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Critical Review

Thyroid-like hormone signaling in invertebrates and its potential role in initial screening of thyroid hormone system disrupting chemicals

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Abstract

This review examines the presence and evolution of thyroid-like systems in selected aquatic invertebrates to determine the potential use of these organisms in screens for vertebrate thyroid hormone axis disrupting chemicals (THADCs). Such a screen might support the phasing out of some vertebrate testing. Although arthropods including crustaceans do not contain a functional thyroid signaling system, elements of such a system exist in the aquatic phyla mollusks, echinoderms, tunicates, and cephalochordates. These phyla can synthesize thyroid hormone, which has been demonstrated in some groups to induce the nuclear thyroid hormone receptor (THR). Thyroid hormone may act in these phyla through interaction with a membrane integrin receptor. Thyroid hormone regulates inter alia metamorphosis but, unlike in vertebrates, this does not occur via receptor activation by the ligands triiodothyronine (T3) and thyroxine (T4). Instead, the unliganded nuclear receptor itself controls metamorphosis in mollusks, echinoderms, and tunicates, whereas the T3 derivative tri-iodothyroacetic acid (TRIAC) acts as a THR ligand in cephalochordates. In view of this, it may be possible to develop an invertebrate-based screen that is sensitive to vertebrate THADCs that interfere with thyroid hormone synthesis or metabolism along with interaction with membrane receptors. The review makes some recommendations for the need to develop an appropriate test method. *Integr Environ Assess Manag* 2022;00:1–20. © 2022 The Authors. *Integrated Environmental Assessment and Management* published by Wiley Periodicals LLC on behalf of Society of Environmental Toxicology & Chemistry (SETAC).

KEYWORDS: Endocrine disruption, Invertebrate, Screening, Thyroid

INTRODUCTION

Up to 60 000 different industrial chemicals are estimated to be in commerce globally (International Council of Chemical Associations [ICCA] & the United Nations Environment Programme [UNEP], 2019), but the number could be severely underestimated (Wang et al., 2020). Each year, hundreds of new compounds are introduced to the market. A minority of these are identified as endocrine disrupting chemicals (EDCs) or potential EDCs having adverse effects, but most chemicals have yet to be assessed for their potential effect on human and environmental health (Krimsky, 2017).

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Some EDCs, including certain pesticides, flame retardants, and fluorinated compounds, are thyroid hormone axis disrupting chemicals (THADCs). THADCs can disrupt vertebrate thyroid hormone signaling through multiple molecular targets such as inhibition of enzymes involved in thyroid hormone synthesis, thyroid hormone receptor (THR) agonism, or antagonism (reviewed by Calsolaro et al., 2017; Préau et al., 2015). These molecular-level effects can lead to detrimental increased risk of neurodevelopmental disorders in mammals (reviewed by Moog et al., 2017). Disruption of the well-conserved vertebrate thyroid hormone system is critical because thyroid hormones control development, metabolism, and growth, for example, brain development in humans and metamorphosis in amphibians and fish.

At a European workshop organized by the European Commission in 2017, the expert group agreed that inclusion of thyroid function biomarkers in test guidelines was a major focus for future test development and validation relating to both human and environmental health; research into invertebrate, and especially mollusk endocrinology, was identified as a key knowledge gap (European Commission, 2018).

Currently, there are no internationally validated test guidelines for in vitro assays to screen for thyroid-active chemicals at Level 2 of the Organisation for Economic Cooperation and Development (OECD) Conceptual Framework (OECD, 2018), although some are in development (OECD, 2014). Furthermore, OECD-validated in vivo test guidelines at Levels 3 and 4 for THADCs are based on vertebrates and can raise ethical concerns.

Higher-throughput screening of environmental chemicals is urgently needed, and alternatives to mammalian in vivo assays, including thyroid hormone system-related endpoints that are more sensitive than circulating thyroid hormones and faster than thyroid gland histopathology, are needed to realize this ambition (reviewed by Couderq et al., 2020).

The thyroid gland is the organ of thyroid hormone synthesis in vertebrates and such synthesis had previously been considered a vertebrate-specific trait. Elements of the thyroid hormone signaling pathway, that is, thyroid hormone receptors and enzymes involved in thyroid hormone synthesis, have been identified in some invertebrate species, but knowledge of their basic endocrinology and development is limited (Ford & LeBlanc, 2020). THADCs may nevertheless affect invertebrates and could be a minor contributor to the biodiversity crisis (Albert et al., 2021). In addition, disruption of thyroid hormone signaling in invertebrates may inform on potential effects in vertebrates. Development of new sensitive invertebrate assays that identify chemical interactions with molecular targets of the thyroid hormone system could therefore increase chemical safety screening efficiency and reduce vertebrate animal testing, and the data might be environmentally relevant.

Therefore, the key questions addressed in this review are: (1) do aquatic invertebrates use some form of thyroid hormone signaling as observed in vertebrates and (2) are there invertebrate species, assays, and endpoints that could serve as surrogates for the initial screening of chemicals for thyroid hormone system disrupting activity in vertebrates and, if not, what is the potential for developing such assays or endpoints?

The vertebrate thyroid hormone signaling pathway

The thyroid hormone system regulates development and metabolism in all vertebrates, metamorphosis in fish and amphibians, and tissue regeneration, for example, fins in fish (Bouzaffour et al., 2010; Sekimizu et al., 2007).

To understand the similarities and differences between vertebrate and invertebrate systems, a summary of thyroid hormone signaling in vertebrates is provided below. This is followed by a detailed evaluation of evidence of a functional thyroid hormone signaling pathway in the largest groups of aquatic invertebrates used in standardized toxicity assays and in some deuterostome invertebrate groups that are not commonly used in toxicity testing but may use thyroid hormone signaling in a manner more comparable with vertebrates.

The thyroid hormones L-thyroxine (T4) and 3,3′,5-triiodo-L-thyronine (T3) are synthesized in thyroid follicles in the thyroid tissue of vertebrates. The synthesis and level of thyroid hormones in target tissue is regulated via several mechanisms. Ingested iodine is carried in the circulation as iodide and actively concentrated by the sodium-iodide transporter (NIS) in the basolateral plasma membrane of the thyroid follicular cells. The conjugation of inorganic iodine to thyroglobulin (organification) is performed by thyroid peroxidase (TPO) and the resulting iodine thyroglobulins are building blocks for the thyroid hormones—predominantly T4 but with smaller amounts of T3. Specific cell membrane proteins and binding proteins transfer and transport the hormones to the target tissue where iodothyronine deiodinases (DIO) regulate the availability of the more active T3 by removing an iodine atom from T4. Together, these mechanisms influence thyroid hormone availability to THRs in the cells of the target tissue (Rousset et al., 2015). Both thyroid hormone levels and the expression levels of the multiple THR isoforms change during development (reviewed by Mourouzis et al., 2020), and differential developmental processes, for example, simultaneous growth of legs and tail reabsorption in tadpoles, are finely controlled by tissue-specific expression of different THR isoforms and enzymes (reviewed by Yaoita, 2019). The fetal thyroid hormone signaling profile differs from the adult and may recur in some adult tissues, for example, after tissue injury (Mourouzis et al., 2020). Thyroid hormone regulation is complex and can be disrupted at multiple levels, for example, by chemicals inhibiting thyroid hormone synthesis, interfering with iodide uptake into the thyroid gland, or blocking the transfer of thyroid hormones (Kortenkamp et al., 2020).

The classic vertebrate thyroid hormones T3 and T4 are involved in various signaling cascades and exert their effect primarily via genomic (nuclear) processes involving THR binding. However, nongenomic (nonnuclear) processes have been observed at the plasma membrane, in the cytoplasm, and in organelles. The nongenomic actions of thyroid hormone are less well understood. However, they have emerged as important accessory mechanisms in thyroid hormone actions (Flamant et al., 2017; Heyland & Moroz, 2005). Nonclassical thyroid hormones such as triiodothyroacetic acid (TRIAC), tetraiodothyroacetic acid (TETRAC), tyramines, 3,3′,5′-T3 (reverse T3, rT3), and 3,5-diiodo-L-thyronine (T2) have been shown to exert actions on metabolic parameters and growth (reviewed by Senese et al., 2014).

In the genomic signaling pathway, the thyroid hormones act via binding to and activation of the THR, a member of the nuclear receptor superfamily. Like all nuclear receptors, THRs regulate transcription by binding to the promotor region of a target gene where they activate or repress mRNA synthesis through co-regulators bound to the receptor. THRs bind to hormone responsive elements (HRE) as a monomer, homodimer, or heterodimer with the retinoid X receptor (RXR). In the absence of a ligand, they behave as transcriptional repressors via recruitment of specific co-repressors. Binding of an agonist in the specific ligand-binding pocket, on the other hand, triggers a

conformational change whereby the co-repressors are released and the co-activators are recruited, consequently leading to the activation of target genes (Flamant et al., 2017). Chemicals that bind in the ligand-binding pocket as antagonists prevent occupancy by an agonist and thus block the activation of gene expression by thyroid hormones. However, only a few chemicals with direct ligand effects on THR have been identified, and other potential target sites in the thyroid hormone axis are considered more relevant (Paul-Friedman et al., 2019).

THYROID-LIKE HORMONE SIGNALING IN SELECTED INVERTEBRATE GROUPS

Arthropods, specifically crustaceans, make up the largest group of invertebrates used in internationally standardized ecotoxicity assays, followed by annelids and mollusks, but aquatic tests cover only crustaceans and mollusks (Table 1). Accordingly, we first evaluated arthropods and mollusks for evidence of a functional thyroid-like hormone signaling pathway. Next, we evaluated thyroid-like hormone signaling in some deuterostome invertebrate groups that are not commonly used in toxicity testing but may use thyroid hormone signaling in a manner more comparable with vertebrates.

Arthropoda

Thyroid hormone signaling has been considered by many to be a product of deuterostome evolution (Paris & Laude, 2008). Contributing to this presumption was the absence of the THR gene in the sequenced genome of the protostome invertebrates: the arthropod *Drosophila*, the nematode *Caenorhabditis elegans*, the sponge *Reniera* sp., and the cnidarians *Hydra magnipapillata* and *Nematostella vectensis* (Kostrouchova & Kostrouch, 2014; Wu et al., 2007). A nuclear receptor gene was identified in the genome of the crustacean *Daphnia magna* that was conditionally known as the thyroid hormone receptor (Litoff et al., 2014). However, the low level of similarity between THRs of other species and that of *D. magna* suggests that this receptor is not functionally homologous to the vertebrate THR (Holzer et al., 2017; Sainath et al., 2019). Speculation held that thyroid hormone signaling was lost in crustaceans and insects where the juvenile hormone signaling pathway co-opted many of the regulatory activities associated with thyroid hormone signaling in other species (Davey, 2000; Flatt et al., 2006). However, it should be noted that the regulatory activities of juvenile hormones are mediated through interaction with a receptor of the bHLH-PAS family of proteins and not a member of the nuclear receptor superfamily (LeBlanc et al., 2013; Li et al., 2011). Thus, these two signaling pathways may share functional similarities but are structurally distinct.

Thyroid hormones have not been definitively identified in insects (Tong & Chaikoff, 1961), and genomic analyses failed to identify genes critical to thyroid hormone signaling in *Drosophila* (Flatt et al., 2006). However, fluorescently labeled T3 was shown to be actively taken up by follicle cells of *Locusta migratoria* (Davey, 2000). This observation led the investigator to conclude that thyroid hormones are ingested by locusts. However, no evidence was provided to suggest that thyroid hormones were functional in these insects. Administering thyroid hormone to insects can mimic many of the actions of juvenile hormone, supporting the contention that the molecular actions of thyroid hormones and juvenile hormones are similar (Flatt et al., 2006).

The general lack of evidence of a functional, thyroid-like hormone signaling pathway in arthropods indicates that representatives of this phylum, although commonly used as test species in toxicity assays (Table 1), would not suffice as a model to detect thyroid hormone signaling disruption.

Mollusca

In contrast to the apparent loss of thyroid hormone signaling in arthropods, many components of thyroid-like hormone signaling have been identified in mollusks (Figure 1). The gene for a THR homolog was identified in the gastropods *Haliotis diversicolor*, *Lottia gigantea*, and *Biomphalaria glabrata* (Kaur et al., 2015; Wang et al., 2019; Wu et al., 2007) and in the bivalves *Mytilus coruscus*, *Crassostrea gigas*, *Crassostrea virginica*, and *Mizuhopecten yessoensis* (Huang, Xu, Qu, Zhang, et al., 2015; Li et al., 2020).

The molluscan THR is involved in larval metamorphosis. Suppression of THR levels in *M. coruscus* using siRNA decreased epinephrine-induced metamorphosis of pediveliger larvae (Li et al., 2020). The suppression of THR levels in *H. diversicolor* similarly reduced metamorphosis of larvae (Wang et al., 2019). However, some nonspecific effects cannot be excluded because mortality increased significantly in this treatment group.

Vertebrate THR typically dimerizes with the RXR to function as a ligand-activated transcription factor (Bugge et al., 1992). The RXR has been identified in several mollusks including the gastropods *B. glabrata*, *L. gigantea*, and *Lymnaea stagnalis* (Bouton et al., 2005; Bridgham et al., 2010; Carter et al., 2010) and the bivalve *C. gigas* (Huang, Xu, Qu, Zhang, et al., 2015). In the Pacific oyster *C. gigas*, the RXR has been shown to interact with the THR in a manner indicative of heterodimer formation between the two receptor proteins (Huang, Xu, Qu, Zhang, et al., 2015).

Thyroid hormone-like substances have also been detected in the mollusks *C. gigas* and *Achatina fulica* (Huang, Xu, Qu, Zhang, et al., 2015; Lustrino et al., 2017). Both T3 and T4 were detected during embryogenesis and larval development of *C. gigas* using HPLC and LC/MS (Huang, Xu, Qu, Zhang, et al., 2015). Levels of both hormones were highest during gastrulation, indicating that the hormones were the product of endogenous synthesis and not derived from exogenous sources (Huang, Xu, Qu, Zhang, et al., 2015). The case for endogenous synthesis of thyroid hormones was supported by the identification of two DIO (Huang, Xu, Qu, Li, et al., 2015) and a TPO homolog in this species (Huang, Xu, Qu, Zhang, et al., 2015). An iodothyronine deiodinase was also isolated from the scallop *Chlamys farreri* (Wu et al., 2012), the abalone *H. diversicolor*

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TABLE 1 OECD and US EPA Test Guidelines including invertebrate species representing endocrine sensitive life-stages

Type	Test Guideline number	Invertebrate class	Test Guideline title
Screening (OECD CF level 3)	Draft OECD TG	Arthropod, aqua	Short-term juvenile hormone activity screening assay using *Daphnia magna*
Screening (OECD CF level 3)	OCSPP 850.1025	Mollusk, aqua	Oyster acute toxicity test (shell deposition)
Partial life cycle (OECD CF level 4)	OECD TG 222	Annelid, terr	Earthworm reproduction test
Partial life cycle (OECD CF level 4)	OECD TG 220	Annelid, terr	Enchytraeid reproduction test
Partial life cycle (OECD CF level 4)	OECD TG 225	Annelid, aqua	Sediment-water *Lumbriculus* toxicity test using spiked sediment
Partial life cycle (OECD CF level 4)	OCSPP 850.3100	Annelid, terr	Earthworm subchronic toxicity test
Partial life cycle (OECD CF level 4)	OECD GD 201	Arthropod, aqua	Harpacticoid copepod development and reproduction test with *Amphiascus*
Partial life cycle (OECD CF level 4)	OECD TG 218 & 219	Arthropod, aqua	Chironomid toxicity test
Partial life cycle (OECD CF level 4)	OECD TG 211/OCSPP 850.1300/ISO 10706:2000	Arthropod, aqua	*Daphnia magna* reproduction test (with male induction)
Partial life cycle (OECD CF level 4)	OECD TG 226	Arthropod, terr	Predatory mite reproduction test in soil
Partial life cycle (OECD CF level 4)	OECD TG 232	Arthropod, terr	Collembolan reproduction test in soil
Partial life cycle (OECD CF level 4)	OECD TG 228	Arthropod, terr	Developmental toxicity to dipteran dung flies
Partial life cycle (OECD CF level 4)	OPPTS 850.1350 (draft)	Arthropod, aqua	Mysid chronic toxicity test
Partial life cycle (OECD CF level 4)	OPPTS 850.1790 (draft)	Arthropod, aqua	Chironomid sediment toxicity test
Partial life cycle (OECD CF level 4)	OECD TG 242	Mollusk, aqua	*Potamopyrgus antipodarum* reproduction test
Partial life cycle (OECD CF level 4)	OECD TG 243	Mollusk, aqua	*Lymnaea stagnalis* reproduction test
Full life cycle (OECD CF level 5)	OECD TG 233	Arthropod, aqua	Sediment-water chironomid life cycle toxicity test
Full life cycle (OECD CF level 5)	Draft OECD TG	Arthropod, aqua	*Daphnia* multigeneration test for assessment of EDCs

Abbreviations: aqua, aquatic species; CF, conceptual framework; terr, terrestrial species.

(Wang et al., 2019), and the sea hare *Aplysia californica* (Heyland, Price, et al., 2006a). Reduced expression of this enzyme in the scallop using siRNA increased the T4/T3 ratio in the hemolymph consistent with its role in the conversion of T4 to T3 (Wu et al., 2012).

The putative role of thyroid-like hormone signaling in the metamorphosis of mollusks is strengthened by pharmacologic evidence. Administration of T4 or T3 to juveniles of the sea hare *A. californica* stimulated metamorphosis (Heyland, Price, et al., 2006a).

Although both THR and thyroid hormones appear to regulate aspects of metamorphosis in mollusks, this regulation is apparently not the result of activation of the THR by hormone ligand. T4, T3, and TRIAC failed to activate the transcriptional activity of the THR from *C. gigas* at concentrations as high as 100 nM (Huang, Xu, Qu, Zhang, et al., 2015). Rather, administration of T4 to *C. gigas* or T3 to abalone elevated THR levels (Huang, Xu, Qu, Zhang, et al., 2015; Wang et al., 2019). Taken together, these observations suggest that, in mollusks, endogenously synthesized thyroid hormone induces expression of the THR, which then contributes to the regulation of metamorphosis in an unliganded state. This stands in contrast to vertebrates where thyroid hormone binds to and activates the THR to regulate physiological processes (Harvey & Williams, 2004).

Echinodermata

Echinoderms, particularly sea urchins, are of increasing prominence as toxicity test species (e.g., Aluigi et al., 2010; Barbaglio et al., 2006; Carnevali et al., 2001; Magesky et al., 2016). Echinoderms were evaluated for a thyroid-like hormone signaling pathway because this deuterostome lineage may better model the response of vertebrate species (Figure 2).

Little information is available on the THR of echinoderms other than identification of the THR gene in the sea

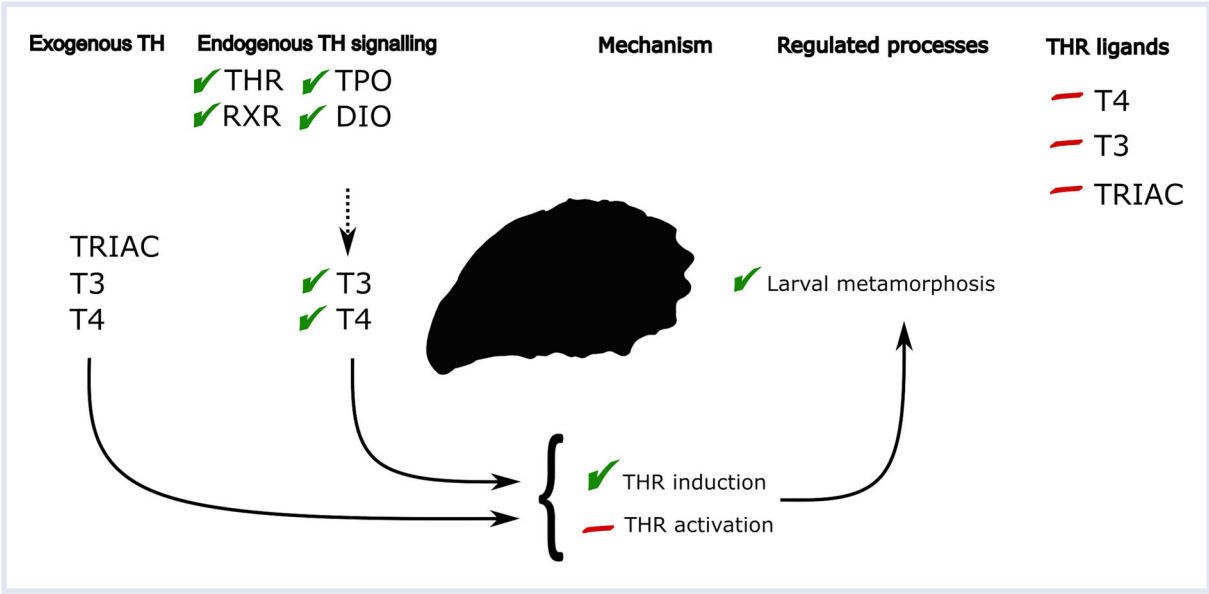

FIGURE 1 Thyroid-like hormone signaling in mollusks. Tick marks denote the presence of the thyroid hormone signaling component or process. Minus signs denote the absence of the thyroid hormone signaling component or process. Black arrows indicate processes regulated by the pathway. Dotted arrow indicates the synthesis pathway is unknown

urchin (Howard-Ashby et al., 2006; Sainath et al., 2019; Wu et al., 2007). The THR partner receptor, RXR, has been cloned in the urchins *Strongylocentrotus purpuratus* and *Paracentrotus lividus* (Capitao et al., 2020; Rafiq et al., 2014). The RXR has also been identified in the transcriptome of the starfish *Patiria pectinifera*. Limited information suggests that the echinoderm THR is incapable of binding the thyroid hormones T4, T3, and TRIAC (Paris et al., 2017).

Echinoderms possess molecules identified as T4 and T3 (Chino et al., 1994; Heyland et al., 2004; Heyland, Price, et al., 2006a; Saito et al., 1998). TPO, the enzyme responsible for the iodination and coupling of iodotyrosine residues in thyroglobulin, has been identified in the sea urchins *S. purpuratus*, *Lytechinus variegatus*, and the sand dollar *Clypeaster rosaceus* (Heyland, Price, et al., 2006a; Miller & Heyland, 2013). TPO activity was detected in *C. rosaceus* when exogenously provided ^{125}I was incorporated into T4, and this ability was blocked when provided with the peroxidase inhibitor thiourea (Heyland, Reitzel, et al., 2006b). The iodothyronine deiodinase gene, whose product is

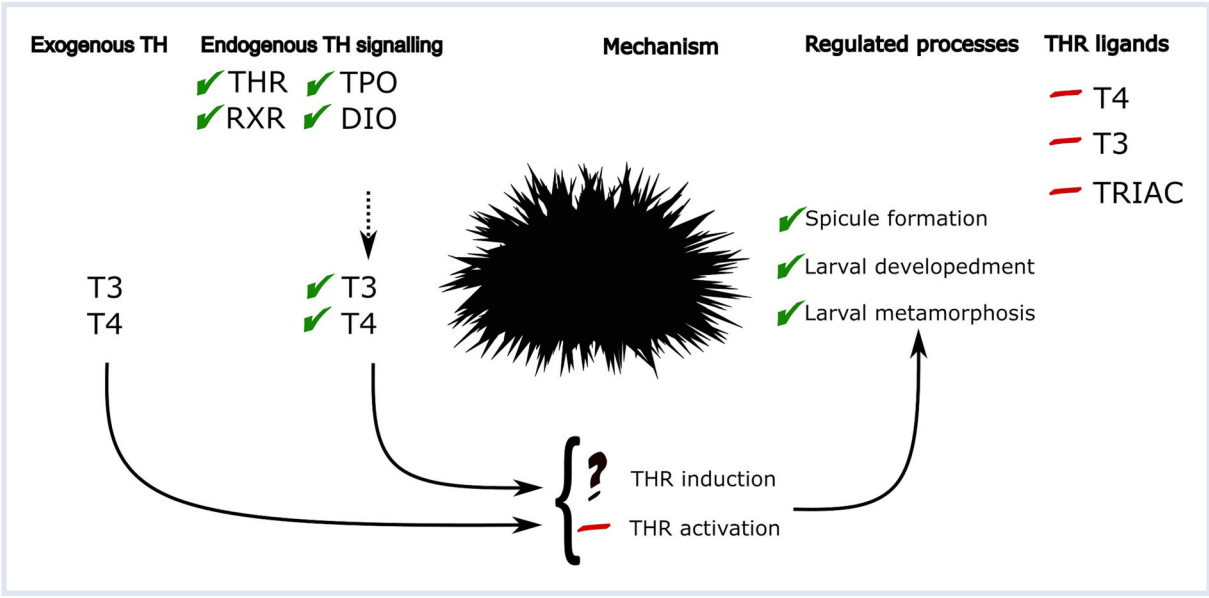

FIGURE 2 Thyroid-like hormone signaling in echinoderms. Tick marks denote the presence of the thyroid hormone signaling component or process. Minus signs denote the absence of the thyroid hormone signaling component/process. Question mark denotes uncertainty about the thyroid hormone signaling component or process. Black arrows indicate processes regulated by the pathway. Dotted arrow indicates the synthesis pathway is unknown

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responsible for the deiodination of thyroid hormones (e.g., the conversion of T4 to T3), was found in the urchin *S. purpuratus* (Paris, Brunet, et al., 2008). All told, echinoderms can synthesize thyroid hormones.

Exogenously provided T4 and T3 accelerated larval development and metamorphosis in several species of sea urchin (Chino et al., 1994; Heyland, Price, et al., 2006a; Johnson, 1998), sand dollar (Heyland & Hodin, 2004; Heyland, Reitzel, et al., 2006b; Saito et al., 1998), and starfish (Johnson & Cartwright, 1996). Further, provision of inhibitors of thyroid hormone synthesis (thiourea and perchlorate) delayed or blocked metamorphosis (Heyland, Reitzel, et al., 2006b; Saito et al., 1998) in *C. rosaceus*. Chino et al. (1994) detected T4- and T3-like substances in both the sea urchin (*Hemicentrotus pulcherrimus*) and algae on which they fed. Thyroid hormone levels increased in developing larvae with maximum levels attained when the adult rudimentary form was attained (eight-armed stage) suggesting that thyroid hormones were instrumental in orchestrating larval development. However, thiourea (a TPO inhibitor) had no effect on the rate of larval development. This led investigators to consider that the algae may have been the source of thyroid hormone in the urchins. Alternatively, the dose of thiourea provided to the urchins may have been insufficient to inhibit endogenous thyroid hormone synthesis.

T4 has been shown to associate with the membrane of sea urchin *S. purpuratus* primary mesenchyme cells by apparent binding to integrin receptors (Taylor & Heyland, 2018). Evidence, generated with the use of various inhibitors of signal transduction, indicated that this binding of T4 activates the transcription factor Ets1 (Taylor & Heyland, 2018), which is responsible for the initiation of skeletogenesis (Koga et al., 2010). Consistent with this observation, T4, as well as T3 and T2, accelerated skeletogenesis as indicated by the premature development of skeletal spicules.

Sand dollar *Leodia sexiesperforata* larvae begin development as nonfeeding individuals, then progress to facultative (optional) feeders, then to obligate feeders prior to metamorphosis (Heyland et al., 2004). Unfed obligate feeding larvae did not undergo metamorphosis. However, unfed obligate feeders given T4 did undergo metamorphosis (Heyland et al., 2004). These authors suggested that exogenous cues (T4 from algae under natural conditions, T4 provision in the laboratory experiments) are responsible for progression to metamorphosis in some echinoderms. Thus, evidence exists for both the endogenous synthesis of thyroid hormones and derivation of thyroid hormone from exogenous sources in echinoderms. Although thyroid hormone clearly orchestrates aspects of larval development and metamorphosis, the source of the hormone may vary among species due to known differences in the timing of embryo and larval development in this phylum.

Tunicata

The tunicates make up a subphylum within the phylum Chordata. These deuterostome invertebrates are more closely related to the vertebrates than those groups discussed above. This group includes the sea squirts, sea tulips, and sea livers. Although tunicates are not among the invertebrate species used in standardized toxicity testing, they have been used in a limited capacity by researchers. Based on their evolutionary relatedness to vertebrates, these organisms were evaluated for evidence of thyroid-like hormone signaling (Figure 3).

The THR has been identified in two species of sea squirt *Styela clava* and *Ciona intestinalis* (Carosa et al., 1998; Wei et al., 2020). The receptor is expressed in both species during embryo and larval development. In particular, the THR of *S. clava* is highly expressed during larval tail regression (i.e., metamorphosis; Wei et al., 2020). Unlike *S. clava*, the tunicate *Oikopleura dioica* retains its tadpole-like appearance throughout its life (Fenaux, 1998). This species lacks the THR gene, providing additional support of a role for the THR in metamorphosis (Wei et al., 2020).

The DNA-binding domain of the *C. intestinalis* THR is highly conserved relative to the vertebrate THRs; however, the ligand-binding domain exhibits poor homology with the THRs of vertebrates (Carosa et al., 1998). Further, the *C. intestinalis* THR was not capable of binding T4, T3, or TRIAC (Carosa et al., 1998; Holzer et al., 2017). Although THR expression in tunicates is associated with larval development and metamorphosis, we are not aware of any direct evidence implicating the THR in these processes. Considering that thyroid hormone levels are also elevated during larval development and metamorphosis, thyroid-like hormone signaling in tunicates may occur through the induction of the THR by thyroid hormone, with the THR acting as a transcription factor in its unliganded state. Unliganded THR functions in the suppression of gene transcription in vertebrates (Fondell et al., 1993; Wen & Shi, 2015), for example, preventing premature metamorphic changes in amphibians (Sachs et al., 2002).

The THR partner receptor RXR has been identified in several tunicates including sea squirts *Polyandrocarpa misakiensis*, *Botrylloides leachii*, and *C. intestinalis* (Blanchoud et al., 2018; Kawamura et al., 2013; Miglioli et al., 2021). Exposure of *S. clava* larvae to UVI3003, an RXR antagonist, completely blocked metamorphosis, indicating a decisive role of this receptor in tunicate development (Wei et al., 2020).

Both T4- and T3-like substances have been measured in several ascidian tunicates including *Phallusia mamillata*, *Ascidia malaca*, *Ascidiella aspersa*, *Halocynthia roretzi*, and *C. intestinalis* (Patricolo & Cammarata, et al., 2001; Roche et al., 1962; D'Agati & Cammarata, 2006; Ogasawara et al., 2002). Ascidians likely synthesize T4 as a homolog of the mammalian TPO gene has been identified in the genome of *C. intestinalis* (NCBI, 2020). Furthermore, ^{125}I binding and peroxidase activity were detected in the endostyle of *O. dioica*, *O. longicauda*, *O. albicans*, *Salpa fusiformis*, *Thalia democratica*, *Dolioletta gegenbauri*, and *Doliolum nationalis* (Fredriksson et al., 1985a, 1988, 1989). The endostyle is considered to be an evolutionary precursor

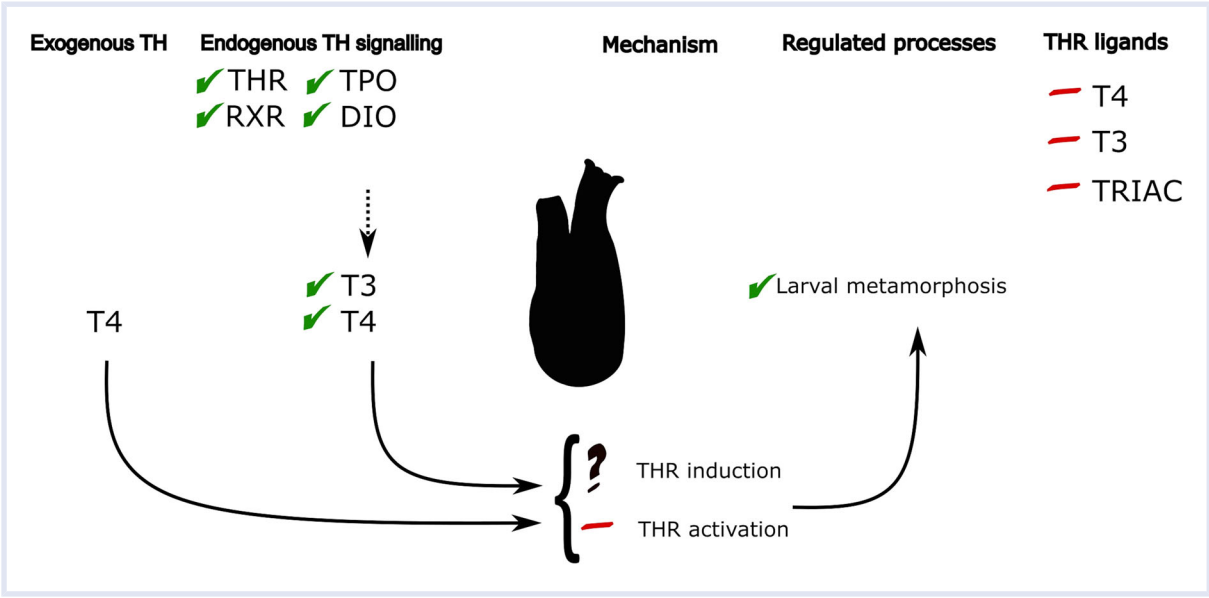

FIGURE 3 Thyroid-like hormone signaling in tunicates. Tick marks denote the presence of thyroid hormone signaling component or process. Minus signs denote the absence of the thyroid hormone signaling component or process. Question mark denotes uncertainty about the thyroid hormone signaling component or process. Black arrows indicate processes regulated by the pathway. Dotted arrow indicates the synthesis pathway is unknown

of the thyroid gland (Canestro et al., 2008). Ascidians can also convert T4 to T3. An iodothyronine deiodinase gene was isolated from *H. roretzi*, and the protein product of this gene exhibited deiodinase activity by converting T4 to T3 (Shepherdley et al., 2004).

As in other invertebrate species discussed, thyroid hormone regulates metamorphosis in ascidian tunicates. Exposure of *A. malaca* larvae to T4 stimulated the onset of metamorphosis and increased the rate at which body reorganization occurred (Patricolo et al., 1982). Exposure of *S. clava* larvae to methimazole, an inhibitor of vertebrate thyroid hormone synthesis via TPO inhibition, reduced the percentage of larvae that underwent metamorphosis (Wei et al., 2020). Interestingly, dissolved copper can cause precocious metamorphosis in ascidian tunicates (Lynch, 1961; Whittaker, 1964). Copper is an essential cofactor in thyroid hormone synthesis in vertebrates (Manisha et al., 2018). Taken together, these observations indicate that endogenously synthesized thyroid hormone regulates metamorphosis in ascidian tunicates.

Cephalochordata

Cephalochordates, commonly known as amphioxus or lancelets, make up another invertebrate subphylum within the phylum Chordata. Cephalochordates have rarely been used as toxicity test organisms; however, as with the tunicates, their evolutionary relatedness to vertebrates suggests that these organisms may be a viable surrogate for detecting the disruption of thyroid-like signaling processes (Figure 4).

The THR was identified in the genome of the amphioxus *Branchiostoma floridae* (Paris, Brunet, et al., 2008), and its cDNA was cloned (Paris, Escriva, et al., 2008). The DNA-binding domain of amphioxus THR shares high homology to that of human THRα (74%), whereas the ligand-binding domain is only 38% identical to the human receptor (Paris, Escriva, et al., 2008). The *B. floridae* THR did not bind T3 but did bind, and was activated by, the T3 derivative TRIAC (Paris, Escriva, et al., 2008). This interaction between TRIAC and the THR was demonstrated by the TRIAC concentration-dependent: (a) activation of a reporter GAL4-THR gene construct, (b) recruitment of the co-activator SRC1 to the THR, and (c) loss of the co-repressor NCOR from the THR. Wang et al. (2009) also observed that TRIAC bound tightly to the *Branchiostoma belcheri* THR. T3 is also bound to this receptor; however, the weak binding of T3 would not likely be of physiological relevance. THR levels gradually increase during larval development and attain maximum expression just prior to metamorphosis (Paris, Escriva, et al., 2008). The THR mRNA levels were elevated significantly by T3 treatment of larvae and adults (Paris, Escriva, et al., 2008). The regulation of THR levels by thyroid hormone is reminiscent of the regulation of the THR in mollusks.

The RXR was identified in the *B. floridae* genome, its cDNA was cloned, and protein expressed (Paris, Brunet, et al., 2008; Paris, Escriva, 2008). Consistent with THR:RXR-DNA interactions observed in vertebrates (Flamant, 2016), the amphioxus THR and RXR can form a heterodimer that binds to a DR4 DNA response element (Laudet, 2011).

Both T4- and T3-like substances have been identified in *Branchiostoma* with highest levels present in the endostyle (Paris, Brunet, et al., 2008; Wang et al., 2009). We are not aware of reports of TRIAC in amphioxus; however, [^{125}I]TRIAC was detected in amphioxus provided [^{125}I]T4 or [^{125}I]T3 (Paris et al., 2010). Thus, amphioxus is capable of

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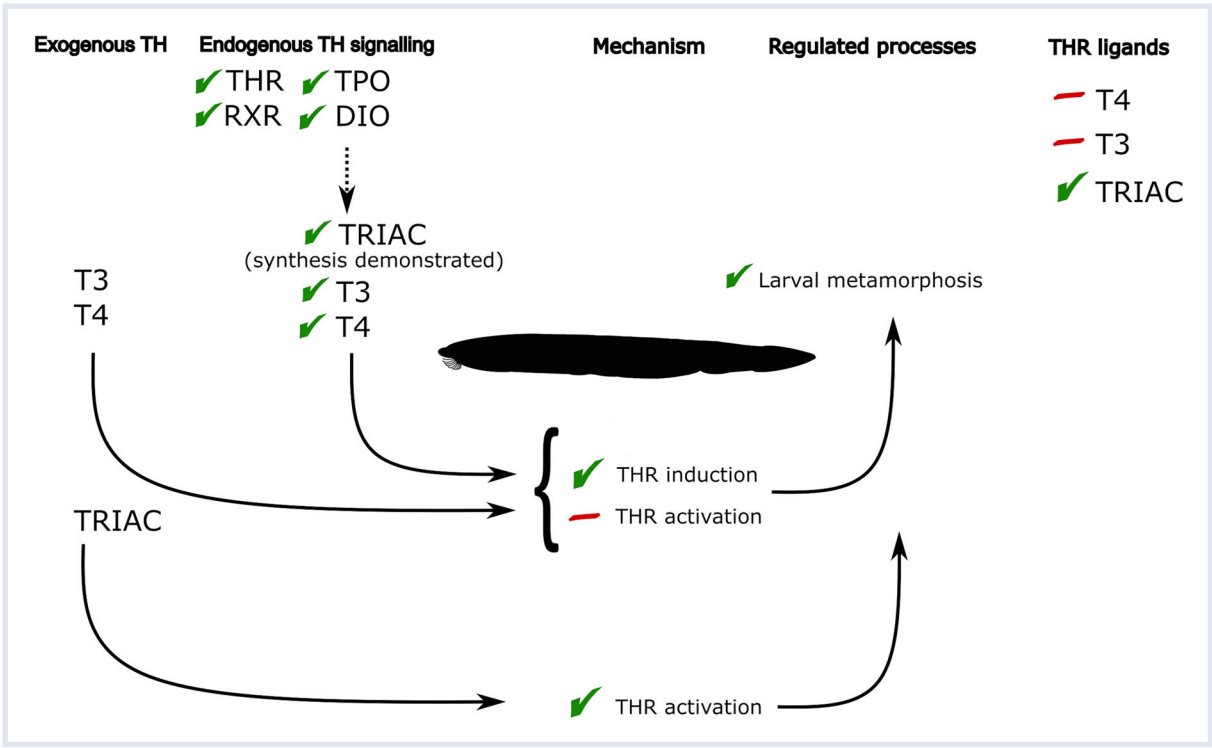

FIGURE 4 Thyroid-like hormone signaling in cephalochordates. Tick marks denote the presence of the thyroid hormone signaling component or process. Minus signs denote the absence of the thyroid hormone signaling component or process. Black arrows indicate processes regulated by the pathway. Dotted arrow indicates the synthesis pathway is unknown

synthesizing TRIAC from thyroid hormone precursors. The lack of success in detecting endogenous TRIAC may be the result of its short half-life (Menegay et al., 1989; Rutgers, et al., 1989).

TPO gene homologs and peroxidase activity have been demonstrated in the cephalochordates *B. belcheri* and *B. floridae*, suggesting the ability to synthesize thyroid hormones (Ogasawara, 2000; Ogasawara et al., 2002; Paris, Brunet, et al., 2008; Tsuneki et al., 1983). TPO activity was measured in the endostyle of *B. lanceolatum* (Fredriksson et al., 1985b), and thyroid hormone synthesis was demonstrated in this organ using a radioiodine tracer (Monaco et al., 1981). As mentioned above, synthesis of TRIAC, the candidate active thyroid hormone in amphioxus, was also demonstrated (Paris et al., 2010). Thus, endogenous synthesis is a likely source of thyroid hormone in amphioxus.

Several deiodinases have been identified in the genome of *B. floridae* (Paris, Brunet, et al., 2008). Iodothyronine deiodinase activity has been measured indirectly in *B. belcheri* through the use of the vertebrate deiodinase inhibitor iopanoic acid (Wang et al., 2009). A unique non-selenoprotein deiodinase was cloned and characterized from *B. floridae* (Klootwijk et al., 2011). This enzyme exhibited deiodinase activity toward the substrate TRIAC but not T4 or T3. The high specificity of an iodothyronine deiodinase for TRIAC provides additional evidence that this compound is hormonally relevant in amphioxus.

Amphioxi represent a transition between the deuterostome invertebrates and the vertebrates. Early life stages of development resemble those of other deuterostome invertebrates. However, the latter stages of larval development are similar to those seen in vertebrates (Bertrand & Escriva, 2011). This developmental transition is mediated, at least in part, by thyroid hormones. Exposure of *B. floridae* larvae to TRIAC significantly advanced the timing of metamorphosis, whereas co-exposure to the THR inhibitor NH3 (Grover et al., 2007) significantly suppressed this advancement (Paris, Escriva, et al., 2008). Furthermore, exposure of pre-metamorphic amphioxus larvae to various inhibitors of thyroid hormone synthesis suppressed the rate of metamorphosis, whereas co-administration of T3 resulted in recovery of the rate of metamorphosis (Paris et al., 2010). Taken together, these results indicate that metamorphosis of *B. floridae* is regulated by TRIAC through its interaction with the THR.

EVOLUTION OF THYROID-LIKE HORMONE SIGNALING IN INVERTEBRATES

The evaluation of thyroid-like hormone signaling processes in the selected invertebrates provides some insight into possible evolution of this signaling pathway. Such insight can assist in the sagacious selection of surrogate species for the screening of chemicals for potential thyroid hormone signaling disruption.

The presence of the THR in both the protostome lophotrochozoans and the deuterostomes (Figure 5) indicates that this receptor existed in metazoa before the split of these two lineages, but after the emergence of the Cnidaria and

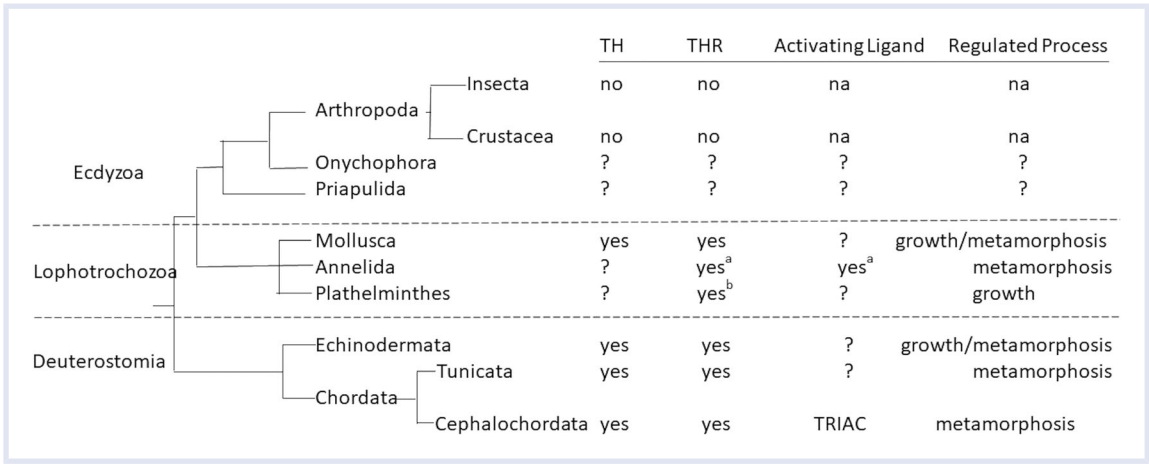

FIGURE 5 Evidence of thyroid hormone signaling among candidate surrogate lineages for the evaluation of endocrine disruption. TH, thyroid hormone; THR, thyroid hormone receptor; na, not applicable; ?, unknown. [a]Annelids are not addressed in this review because no aquatic species are used in standard toxicity tests. [b]THR and its activation were reported in a thesis with T3 (100 nM) and TRIAC (10 nM; Holzer et al., 2016)

Porifera, which do not express the receptor (Taylor & Heyland, 2018). The absence of the THR in the ecdyzoans, but its presence in the lophotrochozoans, suggest that the THR was lost early in ecdyzoan evolution. Alternatively, evolution of the receptor in deuterostomes and lophotrochozoans may have been two unrelated events, which is highly unlikely.

The vertebrate THR is a classic ligand-activated nuclear receptor but there is little evidence of ligand activation of the THR among the lophotrochozoans and deuterostome invertebrates evaluated. Thus, the THR may be an orphan receptor in most invertebrate lineages. Orphan receptors (e.g., estrogen-related receptor [ERR]) do not require ligand activation for functionality and activate target genes in the absence of ligand (Huss et al., 2015). Some nuclear receptors (e.g., constitutive androstane receptor [CAR]) can activate target genes in the unliganded state while suppressing target genes in the liganded state (Forman et al., 1998). Receptors such as ERR and CAR maintain a constitutive conformation that allows for interaction of co-activators. For CAR, ligand binding can place the receptor in a conformation resulting in the loss of co-activators. Unliganded vertebrate THR can bind DNA response elements and act as a repressor of target gene transcription (Sato et al., 2007). Escriva et al. (1997) proposed that the THR originated as an orphan receptor and gained ligand-binding activity later in its evolution. Perhaps the ligand-independent activation of gene transcription among invertebrates reflects its early evolutionary history.

The alternative explanation for the absence of ligand activation among invertebrate THRs is simply that the activating ligand has not yet been discovered. Cephalochordate THR was considered to be an orphan receptor until the T3 metabolite TRIAC was shown to function as a THR agonist (Paris, Escriva, et al., 2008). Perhaps, yet untested metabolites of thyroid hormone activate the receptor in other invertebrate groups.

Thyroid hormones possibly induce THR expression through interaction with a membrane-bound receptor. The THR might then activate gene transcription in a ligand-independent or dependent manner. In vertebrates, thyroid hormone binds to a membrane receptor, integrin αvβ3 (Davis et al., 2011), which stimulates the translocation of THR from the cytoplasm into the nucleus (Cao et al., 2009). Thyroid hormone also upregulates, tissue specifically, the expression of some THR isoforms (Sadow et al., 2003). Integrins are ubiquitous among invertebrate phyla (Burke, 1999), so similar actions may provide a mechanism by which thyroid hormone stimulates THR activity in invertebrates, whether or not the THR is ligand activated.

Taken together, both thyroid hormone and the THR seem to play a role in the development of several protostome and deuterostome groups, but regulation via THR is markedly different from vertebrate regulation because it exerts its action without binding of T3 or T4.

EVALUATION OF EXISTING INVERTEBRATE TEST METHODS FOR THEIR POTENTIAL SENSITIVITY TO SUBSTANCES WITH THYROID ACTIVITY IN VERTEBRATES

This section will evaluate internationally standardized invertebrate toxicity test methods for their potential suitability for modification as thyroid screens. It also examines the scope for developing completely new standardized tests. In line with the argumentation set out above, the animal groups to be considered will be mollusks, echinoderms, cephalochordates, and tunicates. Considering their evolutionary standing among the deuterostomes, the hemichordates may possess many of the thyroid hormone signaling components characteristic of vertebrates, but they were not considered in this review because there is little published information on their endocrinology or their use in toxicology. An important issue will be practicality and potential reproducibility of the methods. Furthermore, in view

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of the close involvement of the vertebrate thyroid system with metamorphosis, potential test methods that include this process will be given particular consideration. It seems unlikely that existing invertebrate test methods will be suitable, as they stand, for screening potential thyroid-active chemicals, but their properties nevertheless require examination. It should also be noted that there are still no internationally standardized in vitro assays for thyroid activity.

Current internationally standardized in vivo invertebrate tests

There are several international organizations involved in the publication of standardized toxicity test methods using invertebrates, but the most important is the Organisation for Economic Cooperation and Development (OECD, 2015; Table 1). The International Organization for Standards (ISO), the International Council for the Exploration of the Sea (ICES), and the Oslo and Paris Commission (OSPAR) have also produced test guidelines, the last two mainly for use as bioassays in monitoring programs.

OECD methods. The two test guidelines (TG) that may be relevant in the present context are TG 242 (a *Potamopyrgus antipodarum* reproduction assay; OECD, 2016a), and TG 243 (a *Lymnaea stagnalis* reproduction assay; OECD, 2016b).

TG 242 *Potamopyrgus antipodarum*. *Potamopyrgus antipodarum* is a freshwater and estuarine prosobranch gastropod mollusk. The TG 242 reproduction test is run with parthenogenetic adult females exposed to solutions of the test substance for 28 days. Embryos develop in the female brood pouch and metamorphose there into juveniles, which are then released to the outside and are immediately able to crawl around on the substrate like adults. In the test, the adult females are dissected at 28 days and the numbers of embryos and metamorphosed individuals counted.

There is little specific information on the possible effects of vertebrate thyroid-active substances on metamorphosis in *P. antipodarum* and, although Geiss et al. (2016) and Alonso and Camargo (2011) have studied the weak thyroid disrupters triclosan and fluoride with this assay, their data did not support a potential use of this species in thyroid testing.

It is easy to measure the ratio of metamorphosed to embryonic individuals in the *P. antipodarum* brood pouch, so it would be straightforward to run TG 242 with a range of thyroid disrupters to investigate whether metamorphosis can be affected at concentrations below systemically toxic levels. However, the fact that metamorphosis occurs within the egg membrane may mean that this process proves to be relatively insensitive to chemical disruption. Another potential disadvantage of TG 242 is the relatively long exposure period, which might be unsuitable for a rapid screening test.

TG 243 *Lymnaea stagnalis*. *Lymnaea stagnalis* is a hermaphroditic freshwater gastropod mollusk that breathes air (a pulmonate). TG 243 exposes adult snails to the dissolved test substance for 28 days and, every few days, measures the numbers of gelatinous egg clutches laid. The guideline also permits measurement of the numbers of eggs per clutch. Under normal circumstances, the veliger embryo would metamorphose in the egg into a young snail approximately 7 days after fertilization, and the snail would then hatch at around 11 days post-fertilization (dpf). However, in TG 243 as currently constituted, the stage of metamorphosis is not reached because the young egg clutches are removed from the test chamber at least twice a week for enumeration of the eggs.

Presumably, it would be possible to modify TG 243 by continuing to expose the egg clutches for longer after they are deposited, thus allowing metamorphosis to occur. However, the fact that the eggs are enclosed in a thick gelatinous envelope may mean, as with *P. antipodarum*, that exposure to the test substance is minimized. On the other hand, it might be possible to develop a new assay that runs only from fertilization to metamorphosis (seven days; or to hatch approximately 14 days) without exposing adult snails, a shorter and more practical timescale for a screening test than the three weeks for *P. antipodarum*. It is possible to separate eggs from the gelatinous cocoon and expose single eggs to solutions of substances for three weeks until hatching (Bandow & Weltje, 2012). In principle, single eggs could therefore be used to investigate effects of thyroid-active chemicals on *L. stagnalis* metamorphosis.

Other nationally and internationally standardized methods. Several invertebrate-based toxicity test methods have been published by ISO, ICES, and OSPAR, plus additional methods recommended by national organizations such as the US Environmental Protection Agency (USEPA). These include methods using bivalve mollusks (ISO, 2015; Leverett & Thain, 2013; USEPA, 2016a, b) and echinoderms (ASTM, 2012; Environment Canada, 2011; OSPAR, 2013; USEPA, 2002).

Although widely recognized as providing considerable acute sensitivity to chemicals, these methods stop well short of metamorphosis, and are thus probably unsuitable for the potential detection of substances with vertebrate thyroid activity.

Nonstandardized invertebrate methods with possible relevance for detecting thyroid-active substances

It will be apparent from the assessment of nationally and internationally standardized invertebrate test methods that there are few that seem to hold much promise as thyroid screens, even after modification. There are, however, several other published methods that should be considered. The evaluation below focuses mainly on procedures that encompass the stage of metamorphosis, although as indicated above, it is not yet clear precisely how thyroid-like signaling is involved in this process in invertebrates.

Mollusks. There is widespread experience with the measurement of metamorphosis and other chronic endpoints in several mollusk species. Much of this has arisen using this group in mariculture industries. Furthermore, the molluscan endocrine system, and its disruption, is probably better understood than that of any other invertebrate phylum except the arthropods (McClellan-Green, 2013).

Gastropods. One possible candidate species is the red abalone *Haliotis rufescens* (Conroy et al., 1996), a gastropod from the North American west coast that grows up to 30 cm long and is widely farmed. Conroy et al. (1996) studied metamorphosis in this species. At 10 dpf, the proportion of metamorphosed larvae (those with a juvenile shell) was recorded. The tested toxicants (zinc and pulp-mill effluent) reduced % metamorphosis, but effects on shell growth after 48 h exposure were of similar sensitivity to the metamorphosis endpoint.

It has been established for some time that metamorphosis in several *Haliotis* species can be induced by treatment with T4 (Fukazawa et al., 2001), and a homolog of the THR is present in *H. diversicolor* (Wang et al., 2019). Furthermore, it has also been demonstrated that several phthalate esters that can disrupt thyroid signaling in vertebrates are able to suppress metamorphosis in *H. diversicolor supertexta* (Zhou, Cai, et al., 2011), and bisphenol A has similar action in this species (Zhou, Zhu, et al., 2011). It should be pointed out, however, that the mechanism(s) of action in these two cases is unknown.

A 10-day *Haliotis* metamorphosis assay would probably be rapid enough for a screening test, and it is reasonably simple. For example, the developing larvae do not need to be fed for the first six days before introduction to their settlement vessels (Conroy et al., 1996). Although *H. rufescens* is restricted to the western American seaboard, it seems likely that other species, such as the green ormer (*H. tuberculata*), found in the northeast Atlantic and Mediterranean, and *H. discus*, *H. gigantea*, and *H. diversicolor* in the western Pacific, would also be suitable. The populations of some *Haliotis* species (e.g., *H. tuberculata*) are under pressure from overexploitation, but others (such as *H. rufescens*) are widely farmed and could relatively easily be brought into laboratories as broodstock.

Bivalves. The recent discovery that knockout of a homolog of the vertebrate THR gene in the bivalve *M. coruscus* leads to a 54% reduction in epinephrine-induced metamorphosis (Li et al., 2020) suggests that bivalves may hold promise for the routine screening of chemicals with suspected thyroid activity. Nevertheless, it should be noted that activation of a molluscan THR by vertebrate thyroid hormones has not been demonstrated in mollusks.

A bivalve metamorphosis test could be very simple, although rearing metamorphosis-ready pediveligers from fertilization can take several months (e.g., Li et al., 2020). For example, it is possible to buy 21-day-old pediveliger larvae of the Pacific oyster *C. gigas* "off-the-shelf" from commercial oyster hatcheries and use them to measure metamorphosis success (e.g., DiPoi et al., 2014; Mottier et al., 2013). This is a practical method that can be conducted in multiwell plates, the larvae requiring no feeding, and metamorphosis is triggered with epinephrine after 24 h exposure to the test substance. The endpoint is the percentage of pediveliger larvae that metamorphose, an easily recorded and unequivocal measure. A similar procedure has been used to study effects of chemicals on larval metamorphosis in the mussel *M. galloprovincialis* (Yang et al., 2011).

There are apparently no published reports of the effects of vertebrate thyroid disrupters on metamorphosis in bivalve larvae. The only relevant study is a 15-day exposure to 1 µg/L of the weak thyroid disrupter 2,2′,4,4′-tetrabromo diphenyl ether, which was reported to reduce T3 and T4 levels in adult Manila clam *Ruditapes philippinarum* (Song et al., 2016), and this concentration also retarded growth in juveniles. However, it is unknown whether these effects represent a form of direct endocrine disruption or simply systemic toxicity.

In summary, relatively simple and practical methods for measuring metamorphosis in both gastropods and bivalves have already been developed, so it would be straightforward to investigate whether vertebrate THADCs at concentrations below those causing systemic toxicity in mollusks are able to interfere with this process. THADCs may also potentially interfere with growth in mollusks, as they do in some vertebrates, and at least one mollusk-based procedure might be adaptable for studying this (USEPA, 2016b).

Echinoderms. As indicated previously, echinoderms contain an ortholog of the vertebrate THR, but typical thyroid hormones do not bind to it. However, metamorphosis can be accelerated by exposure to thyroid hormone (Holzer et al., 2017).

Several researchers have extended the widelyused sea urchin fertilization assay (e.g., Environment Canada, 2011) to investigate various aspects of early development in echinoderms, including growth rate (e.g., Saco-Alvarez et al., 2010), developmental delay, and morphological abnormalities up to the pluteus larval stage (e.g., Bellas et al., 2005; Cesar-Ribeiro et al., 2010; Anselmo et al., 2011; Morroni et al., 2016; Picone et al., 2016). As indicated previously, it has been demonstrated that exposure to T4, T3, or T2 accelerates the initiation of skeletogenesis (spicule formation) in sea urchin *Strongylocentrotus purpuratus* gastrulae (2 dpf) and in six-armed pluteus larvae (10–14 dpf; Taylor and Heyland, 2018). T4 exerted this effect by binding to an integrin membrane receptor and subsequent activation of the mitogen-activated protein kinase [MAPK(ERK1/2]). Taylor and Heyland (2018) exposed the pluteus larvae individually in multiwell plates and, although this involved feeding them with an algal suspension, the procedure does not appear overcomplicated for routine testing. It may be worth investigating this methodology further to determine if THADCs are able to accelerate or retard skeletogenesis, in

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which case this endpoint might form the basis for test method development. It would, of course, also be necessary inter alia to establish the degree to which the skeletogenesis response is thyroid specific.

As described previously, T4 and other thyroid hormones also appear to play an important role in echinoderm metamorphosis. This has been clearly demonstrated in sand dollars, for example, Saito et al. (1998) and Heyland et al. (2004). It seems likely that some other echinoderms are also induced to metamorphose by T4 and other substances such as glycoglycerolipids in their algal food, an adaptation that synchronizes metamorphosis with the presence of food required by growing juveniles (Takahashi et al., 2002). However, this process does not appear to be universal in the phylum. For example, although the progress toward metamorphosis of eight-armed pluteus larvae of the sea urchin *Evechinus chloroticus* was accelerated by treatment with T4, the proportion of larvae which settled and metamorphosed was unchanged (Johnson, 1998).

Several authors have studied the effects of contaminants on echinoderm metamorphosis, although none have apparently investigated thyroid-active substances other than natural thyroid hormones. For example, Magesky et al. (2016) exposed eight-armed pluteus larvae of the sea urchin *Strongylocentrotus droebachiensis* to 100 µg/L nano-silver and revealed that metamorphosis was interrupted. This involved a fairly complex and long, drawn-out procedure—rearing from fertilization to pre-metamorphic pluteus larvae took 5–6 weeks, followed by the exposure phase during metamorphosis lasting two weeks; feeding with biofilm and algal suspensions had to be arranged throughout. In a similar procedure with another urchin (*Paracentrotus lividus*), continuous exposure to chlorpyrifos from 2 to 15 dpf interrupted metamorphosis after this was triggered by exposure to stones (presumably covered in perilithic algae) collected from unpolluted surface waters (Aluigi et al., 2010). Reproduction in many urchin species like *P. lividus* is seasonal, so it will not always be possible to obtain gametes for generation of embryos in vitro.

It appears, therefore, that echinoderm metamorphosis might form the basis of a standardized toxicity testing procedure, and laboratory methods for culturing larvae to metamorphosis are well understood (Hodin et al., 2019). Also, pre-metamorphic pluteus larvae can probably be purchased from the aquaculture industry rather than having to be grown from fertilization in the laboratory. Several species of sea urchins are grown in mariculture systems, especially in the USA and Japan, but the commercial supply of urchin larvae is likely to be patchy and intermittent compared with that of Pacific oyster larvae. As with mollusk larvae, however, considerable research would be required to establish the sensitivity and specificity of echinoderm metamorphosis to vertebrate THADCs.

Yet another aspect of echinoderm biology, which might be useful for testing thyroid-active substances, is arm regeneration in crinoids (Class Crinoidea). In this group, arms can break off by a process known as autotomy and completely regenerate. Interestingly, thyroid hormones are involved in tissue regeneration processes in some vertebrates. Zebrafish (*Danio rerio*) are able to regenerate many tissues and organs, including heart, as their fetal thyroid hormone signaling profile with low T3 levels recurs after tissue injury, and it is well established that T3 inhibits cardiomyocyte proliferation and regeneration (reviewed by Flamant, 2021; Hirose et al., 2019; Mourouzis et al., 2020).

Carnevali et al. (2001) experimentally amputated arms of the feather star *Antedon mediterranea* at autotomy planes and then exposed them for 14 days to a low concentration of polychlorinated biphenyls (14 ng/L ΣPCB). Some of these compounds are known to have disruptive effects on thyroid systems in both humans (e.g., Turyk et al., 2007) and amphibians (e.g., Shirey et al., 2006). Over the 14 days, the amputated arms of PCB-exposed crinoids grew back twice as fast as controls, an effect that was accompanied by a range of histopathological anomalies including hypertrophy of the coelomic canals and proliferation of migratory tissue repair cells. It seems possible that the accelerated regeneration is indeed some type of endocrine disruption rather than systemic toxicity, which might be expected to decrease growth. Similar research with *A. mediterranea* has been conducted by Barbaglio et al. (2006) who found that 14 days of exposures to triphenyltin (225 ng TPT/L) also caused faster regrowth of amputated arms. Triphenyltin activates the THR partner receptor RXR (Hiromori et al., 2015), which might contribute to this effect (Mengeling et al., 2018).

Limb regeneration in crinoids is probably controlled hormonally, but there are currently few data on whether vertebrate thyroid disrupters can affect the process, and no information at all on possible mechanisms. This subject would require further research before any attempt was made to develop standardized test methods. Another important consideration would be the practicality of methods using crinoids because these would probably depend on using wild-caught test organisms.

Tunicates. Tunicate larvae have been used for acute toxicity testing for over 20 years (Zega, Pennati, et al., 2009). Gametes are easily obtained by dissection of the adult gonoduct, and in vitro fertilization results in an embryo that can develop to the point of settlement in <24 h. The larvae of several species (especially *Styela plicata*, *Ciona intestinalis*, and *Phallusia mammillata*) have been used both for drug discovery (e.g., Dumollard et al., 2017) and for testing of environmental chemicals (e.g., Cangialosi et al., 2013; Cima et al., 1996; Eliso et al., 2020; Gallo & Tosti, 2015; Zega, De Bernadi, et al., 2009). For these assays, the endpoints are generally mortality or abnormal development.

Tunicate metamorphosis has also been used as an endpoint in chronic toxicity tests. Patricolo, Mansueto et al. (2001) exposed swimming larvae (24–48 h post-fertilization) of *C. intestinalis* to 10^{-5} or 10^{-7} M tributyltin chloride for 24 h and found that the T4 content of their tissues was decreased by 70% at both concentrations, and T4 synthesis was inhibited. These hormonal changes were accompanied

by blocked tail regression and consequent halting of metamorphosis. It is noteworthy that estuarine tunicate populations exposed to tributyltin (TBT) from shipping antifoulants were severely reduced in the 1980s by this contaminant (Rees et al., 2001). Furthermore, it has been well established that low levels of organotins like TBT and triphenyltin (TPT) can disrupt the thyroid system in a range of vertebrates (e.g., fish: Li & Li, 2020; amphibians: Shi et al., 2014; rodents: Andrade et al., 2018). This suggests the possibility that tunicates could be used to develop an assay for predicting thyroid effects in vertebrates.

More recently, Cahill et al. (2012) studied *Ciona savignyi*, obtaining gametes by dissection and then exposing freshly hatched larvae in Petri dishes to a range of candidate antifoulants. Control larval settlement occurred after approximately 24 h, and the process of metamorphosis was then recorded until its conclusion after seven days. For some chemicals, metamorphosis was inhibited at concentrations close to lethality, whereas in others, metamorphosis was more than two orders of magnitude more sensitive than lethality. A later paper (Cahill et al., 2016) described refined methodology with optimized conditions using multiwell exposure plates, and reported acceptable experimental repeatability (17%) for eight trials run over one year. It was found that metamorphosis was sensitive to inhibition by chlorothalonil (seven days EC50 0.1 µg/ml) and tolylfluanid (seven days EC50 0.3 µg/ml), and it is of interest that the 4-hydroxy metabolite of chlorothalonil, which has been found in fish, exhibits both agonistic and antagonistic activity at THR-β in a reporter gene assay (Zhang et al., 2016).

Larval development and particularly metamorphosis in *Ciona* or *Styela* spp. (or other tunicates) may thus provide a useful model for detecting thyroid disrupters if additional positive reference compounds like methimazole and TBT can be demonstrated to possess activity. However, mechanisms of action need to be better understood, and the possible impracticality of obtaining gametes from adult animals on a routine and universal basis may be a stumbling block for standardization.

Cephalochordates. Like the tunicates, the cephalochordates are another small nonvertebrate chordate group with an endostyle organ that secretes iodo-tyrosines and thyroid hormones. They possess a true notochord that persists throughout life and have several other vertebrate-like features. The separate sexes release their gametes into the surrounding seawater, and the embryos develop rapidly into a ciliated, free-swimming larva, which then metamorphoses into a filter-feeding adult that lives partially buried in sediment. The commonest species are the lancelets or amphioxi (mainly *Branchiostoma* spp.). There is firm evidence that cephalochordates use vertebrate-style thyroid hormone signaling, but the T3 derivative TRIAC acts as the THR ligand and is directly involved in metamorphosis (Holzer et al., 2017).

Cephalochordates have hardly ever been used in traditional ecotoxicology, perhaps because an early acute toxicity study with adult, wild-caught *Branchiostoma caribaeum* (Clark et al., 1987) reported that they are relatively insensitive compared with grass shrimp to three chemicals (fenvalerate, trichlorobenzene, tributyltin oxide) in both water and sediment-water systems. Clark et al. also mentioned that routine collection of wild *Branchiostoma* spp. might present difficulties. The only other published toxicity study appears to be Bhattacharya et al. (2008) who reported on acute toxicity and histopathology in *B. belcheri* adults exposed in sediment to carbon tetrachloride. The acute or chronic effects of chemical exposure on metamorphosis in this group apparently have not been investigated.

Given the close similarity of cephalochordate and vertebrate thyroid systems, it may be considered worthwhile to conduct some research on the effects of THADCs on larval metamorphosis in *Branchiostoma* spp. Raising *B. floridae* (and presumably other *Branchiostoma* species) from hatching through to metamorphosis can be carried out in the laboratory with relative ease, a process that takes 3–4 weeks in this species (Stokes & Holland, 1995). It would therefore be possible to conduct chronic toxicity experiments without the need to collect test organisms from the field.

Conclusions about existing invertebrate methods

It is apparent from this review that, currently, there are no internationally standardized toxicity tests with invertebrates that could be used without major modification for detecting THADCs. However, several species of mollusks, echinoderms, and tunicates exhibit some apical sensitivity to vertebrate thyroid hormones; there are also a few examples in which THADCs demonstrate activity. There are no such examples from the cephalochordates and hemichordates—they are not known to have been experimentally exposed to such substances. The apical effects of chemicals on metamorphosis could probably be easily measured in all these groups because basic methods for rearing metamorphic larvae have been published, and some species of larval mollusks and echinoderms are even commercially available. Furthermore, thyroid-like hormones probably have functions beyond metamorphosis, such as in skeletogenesis and arm regeneration in echinoderms, so these endpoints might also be worth investigating. However, it should also be noted that there are currently no mechanistic assays for endocrine disruption in invertebrates. Both mechanistic and apical endpoints will be needed to identify thyroid-active endocrine disrupters.

There are, of course, many questions that would need to be answered before development and validation of any type of predictive assay could be confidently recommended. A few of these issues are discussed below.

Specificity of response. It will be crucial to understand which possible endpoints such as metamorphosis are sensitive to a range of model vertebrate thyroid disrupters, and the degree to which such responses are specific to thyroid activity. It is obviously important to be able to distinguish

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effects caused directly by anti-thyroid activity from those that merely result indirectly from systemic toxicity. To improve the specificity of a putative apical assay, it would be advantageous to include a biomarker diagnostic of interference with the thyroid system. For example, the Amphibian Metamorphosis Assay (OECD, 2009) has both apical endpoints (e.g., delayed metamorphosis) and thyroid hormone axis specific biomarkers (e.g., thyroid histopathology).

Mechanisms of action. It is clear from this review that the endocrine systems, including thyroid-like systems, of the invertebrates in question are still poorly understood, although useful knowledge is accumulating. This situation needs to be rectified, at least for the endpoints of interest, because that may in turn allow the identification of new and more diagnostic measurements (alluded to in the previous paragraph) to complement or even replace apical endpoints. The development of adverse outcome pathways may be of assistance in this regard.

Choice of test species. A key issue will be the need to confidently extrapolate from invertebrate test results to make predictions about possible thyroid activity in vertebrates, that is, reliable read-across, which could be achieved using the USEPA's SeqAPASS tool (e.g., LaLone et al., 2018). Of course, the responses of various invertebrates to thyroid disrupters would also be of interest in their own right. There may be a temptation to use species that are easily available (such as some mollusks and echinoderms grown in mariculture), but it might be more appropriate to choose species, such as tunicates or cephalochordates, which lie closer in evolutionary terms to vertebrate thyroid hormone signaling (Figure 5). A balance will need to be struck between appropriateness of the test species and ease of use, and of course all species could be relevant if THADCs cause adverse effects on invertebrates in the environment.

Practicality. Screening tests for chemicals need to be relatively quick and simple to perform in contract research laboratories. Those that use organisms from laboratory cultures or from commercial suppliers which do not require complex feeding regimes, do not run for longer than a few days or weeks, do not need excessive laboratory space, and have easily measured endpoints will be preferable. For example, all other things being equal, a test using commercially available pediveliger larvae of *C. gigas* in which metamorphosis success can be measured in a day or two would be preferable, in terms of practicality, to a two-month metamorphosis test with a hard-to-obtain hemichordate species.

Scope for standardization. International standardization of new ecotoxicity tests is a rigorous process. As part of its standardization and validation procedure, the OECD requires that "Intra-test variability, repeatability and reproducibility of the test method within and amongst laboratories should have been demonstrated" (OECD, 2005). This is a challenging objective and must be borne in mind from the beginning of any search for a new THADC screen. In other words, is it likely that a new method is capable of being conducted reliably in laboratories around the world without excessive cost and inconvenience? Another OECD requirement is that the performance of the new method must be evaluated against existing relevant toxicity data—this will be especially important for an assay that seeks to use invertebrate data to predict potential thyroid disruption in vertebrates.

DISCUSSION

Considering the inability of invertebrate THRs to bind conventional (i.e., vertebrate) thyroid hormones (T3 and T4), the assessment of chemicals for THR binding, agonism, or antagonism as a mechanism of endocrine disruption is not appropriate in these organisms. However, there appears to be scope for developing an invertebrate assay sensitive to substances that interfere with thyroid hormone synthesis or metabolism.

In addition to the THR, thyroid hormones have been detected in deuterostome invertebrates and lophotrochozoans but not in arthropods (Figure 5). The co-occurrence of thyroid hormones and the THR suggests that the former organisms use some form of thyroid hormone signaling pathway. Induction of the THR by thyroid hormone has been documented in both lophotrochozoans (mollusks; Huang, Xu, Qu, Zhang, et al., 2015; Wang et al., 2019) and deuterostomes (cephalochordates; Paris, Escriva, et al., 2008).

Both thyroid hormone and the THR appear to be necessary for signaling to occur in lophotrochozoans and deuterostome invertebrates. Thus, analyses of thyroid hormone and THR levels would be viable means of assessing chemicals for mechanistic impacts on this signaling pathway. Radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and chemiluminescent assay (CLA) kits are commercially available for the analysis of T4 and T3 levels (e.g., Invitrogen EIAT3C; Shamsian et al., 2016). These assay kits are designed for the analysis of thyroid hormone levels in blood samples. However, with proper optimization, some may prove useful for analyses of thyroid hormone in invertebrate hemolymph or tissue samples. As THR nucleotide sequences are known for many invertebrate species (Wu et al., 2007), mRNA levels of these receptors could be effectively and rapidly determined using polymerase chain reaction (PCR) methodology.

The regulation of metamorphosis is an evolutionarily conserved function of the thyroid-like signaling pathway among some deuterostome (invertebrate and vertebrate) and protostome lineages (Figure 5). Accordingly, this apical endpoint could prove useful in evaluating potential thyroid signaling disruption by environmental chemicals using surrogate invertebrates. This endpoint, when used alone, may be subject to false positive responses, where metamorphosis is disrupted by the effect of the test chemical on food consumption, energy production, or some other overt

toxicity. However, follow-up mechanistic analyses of thyroid hormone and THR levels could prove to be a powerful means of assessing the potential for a chemical to disrupt thyroid hormone signaling in vertebrates. Such an assay with both mechanistic and apical endpoints would be appropriately placed at Level 3 of the OECD Conceptual Framework (OECD, 2018).

RECOMMENDATIONS FOR RESEARCH

We recommend the following lines of research, which will need to be explored before development and standardization of invertebrate test methods with sensitivity to substances that interfere with thyroid hormone synthesis and metabolism:

1. As the mollusks, echinoderms, tunicates, and cephalochordates appear to possess similar thyroid systems, we suggest that research should focus on the taxonomic group or groups for which widespread ecotoxicological experience already exists. In our view, either the mollusks or echinoderms would be most appropriate, because pre-metamorphic larvae of both groups are freely available commercially or easily produced under laboratory conditions.
2. We recommend that research should investigate the responses of two types of potential test endpoints—alterations in the timing of metamorphosis (an apical endpoint) and alterations in thyroid hormone titres and/or thyroid hormone receptor expression (mechanistic endpoints). If echinoderms are chosen as the focus for research, it may also be desirable to study spicule formation as an alternative apical endpoint.
3. In addition to studying the responses of these endpoints to vertebrate thyroid hormone, it will be necessary to investigate a range of chemicals that are known to interfere with thyroid hormone synthesis and metabolism in vertebrates along with chemicals that have been shown not to interfere with this signaling process. Such lists are developed and will be available via the H2020 EURION cluster projects (www.eurion-cluster.eu).
4. Additional basic research into the mechanisms whereby thyroid disrupters interfere with metamorphosis in the chosen taxa would be desirable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

This review is based entirely on published information.

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