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Ciona spp. and ascidians as bioindicator organisms for evaluating effects of endocrine disrupting chemicals: A discussion paper

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ABSTRACT

In context of testing, screening and monitoring of endocrine-disrupting (ED) type of environmental pollutants, tunicates could possibly represent a particularly interesting group of bioindicator organisms. These primitive chordates are already important model organisms within developmental and genomics research due to their central position in evolution and close relationship to vertebrates. The solitary ascidians, such as the genus *Ciona* spp. (vase tunicates), could possibly be extra feasible as ED bioindicators. They have a free-swimming, tadpole-like larval stage that develops extremely quickly (<20 h under favorable conditions), has a short life cycle (typically 2–3 months), are relatively easy to maintain in laboratory culture, have fully sequenced genomes, and transgenic embryos with 3D course data of the embryo ontogeny are available. In this article, we discuss possible roles of *Ciona* spp. (and other solitary ascidians) as ecotoxicological bioindicator organisms in general but perhaps especially for effect studies of contaminants with presumed endocrine disrupting modes of action.

1. Introduction

The potential of many man-made environmental contaminants to act as Endocrine Disrupting (ED) Chemicals/Contaminants (EDCs), was in July 1991 discussed and conceptualized for the first time by a multi-disciplinary group of experts gathered in Wingspread, Racine, WI, USA (Colborn and Clement 1992; Colborn et al., 1993). At that time, ED relevant research data had been accumulating for many years within the scientific community, possibly earliest documented in the late 1940s when declined sperm counts were found in men involved with aerial application of the insecticide dichloro-diphenyl-trichloroethane (DDT) (Singer 1949). Later, in the early 1960s, Rachel Carson's book 'Silent Spring' (Carson 1962) contributed significantly to raising global awareness about possible ecotoxicological and ecological consequences of pesticide usage and other human activities. Much environmental research and monitoring done ever since has served to broaden and consolidate our understanding of these issues, including about ED phenomena, possibly best exemplified by the discovery of the imposex effect phenomenon in marine gastropods caused by the marine antifoulant tributyltin (TBT) (WHO IPCS 1990; Matthiessen 2019). In our time, ED research represents a broad and vibrant field in environmental science and many major reviews on ED topics have been reported, both for humans, e.g., (McKinlay et al., 2008; Diamanti-Kandarakis et al., 2009; Van den Berg et al., 2012; Gore et al., 2015), and various wildlife, e.g., (Jobling et al., 1998; Santos et al., 2010; Wang et al., 2021; Metcalfe et al., 2022), although many unknowns are still remaining.

An EDC is usually defined as any exogenous substance (or mixture) that alters the function(s) of the endocrine system and thus causes an adverse health effect in an organism, its progeny, or (sub)population (UNEP 2017). Specific endpoints that may indicate that an organism is affected by ED can be changes in hormone concentrations, abnormal expression/function of hormone receptors, or irregularities in developmental events that strictly require hormonal regulation. Endocrine toxicants may induce adverse effects in multiple species, and across taxonomic groups, but because the endocrinology of most non-vertebrates is still largely unknown, ED studies in such taxa are often hampered with misconceptions and over-simplifications (Katsiadaki 2019), and this is a problem especially if the data are used in ecological risk assessment or as a basis for management actions. Most ED studies done so far have dealt with effect issues in vertebrates and mammals (especially humans), but the need for more insight into ED issues in invertebrates is increasingly recognized (Crane et al., 2022),

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both regarding terrestrial, freshwater and marine ecosystems. In context of the latter, the potential for using ascidian tunicates, such as the solitary vase tunicate *Ciona intestinalis* (Fig. 1), as ED bioindicators (i.e., as test organism used in ED toxicity assessments) has gained increasing attention recently, e.g., (Dumollard et al., 2017; Morthorst et al., 2022). The interest is partly due to the unique phylogenetic position of tunicates as ancestral chordates and the closest invertebrate relatives to vertebrates (Delsuc et al., 2006).

The aim of this paper is to discuss the possible role of solitary ascidian tunicates as bioindicators and model test organisms in effect assessment/screening of chemical ecotoxicants in general, and particularly for suspected ED acting pollutants. In our discussion, special attention is given to the solitary vase tunicate *Ciona intestinalis* (Fig. 1), which as discussed herein could be particularly favorable as bioindicator organism in these kinds of studies. For simplicity, the two main congeneric species of vase tunicate, type A: *C. robusta* and type B: *C. intestinalis*, are considered jointly in this study, and referred to as *Ciona*.

2. Modes and mechanisms of actions (MOAs) of EDCs

The degree by which *Ciona*/ascidians are useable as bioindicators in ED assessments relies on the amount of knowledge that is available on their endocrinology, their sensitivity and responsivity to known EDCs, as well as the degree to which their putative ED responses are comparable to other taxonomic groups. From a generalized perspective, the typical modes and mechanisms of actions (MOAs) of ED chemicals regard some disturbance to the regulating effect (stimulatory or inhibitory) of natural hormones. All hormones are produced by specialized cells, and all have target cells that contain specific hormone receptors to which the hormone binds as a ligand with certain (often very high) specificity and affinity, forming the hormone-receptor complex that activates the specific hormone-receptor signaling pathway. The location of a receptor (when initially interacting with the hormone) is either embedded in the cell membrane (G-protein coupled receptors and enzyme-linked receptors), in the cytoplasm (type I nuclear receptors), or in the cell nucleus (type II nuclear receptors). ED induced dysregulation of endocrine systems often involves interaction with ligand-activated nuclear receptor (NR) systems, i.e., the superfamily of gene expression regulators which are key for development, homeostasis and metabolism throughout metazoans. Small lipophilic endogenous ligands such as steroids, retinoids, and phospholipids regulate the actions of most NRs, while "orphan receptors" are NRs for which no ligand has yet been identified. An ED phenomenon may involve: (1) direct binding of the ED chemical to the NRs hydrophobic-binding pocket (the ligand binding domain, LBD), causing stimulatory (agonistic) or inhibitory (antagonistic) regulation of the receptors transcriptional activity, or (2) stimulation or inhibition of biosynthesis and/or degradation of specific endogenous hormone(s), or (3) inhibition or stimulation of hormone binding proteins and thereby increasing or decreasing circulating endogenous hormone availability (Combarnous 2017). Process structuring concepts like the Adverse Outcome Pathway (AOP) approach (Ankley et al., 2010; Perkins et al., 2019) are useful for characterizing ED MOAs, e.g., stating that receptor-mediated ED perturbations are initiated with a Molecular Initiating Event (MIE) when the ED chemical binds to or interferes with a target receptor, erroneously activating or deactivating it, leading subsequently to disruptive irregularities in downstream Key Events (KE), each via different KE functional relationships (KERs), before manifestation of the effect at the apical adverse outcome (AO) level.

In vertebrate organisms, most ED effect studies on NR mediated signaling have focused on estrogen (ER), androgen (AR), thyroid (TR) receptors and steroidogenesis signaling pathways, commonly referred to as the EATS (Estrogen, Androgen, Thyroid and Steroidogenesis) modalities, whereas the numerous non-EATS modalities are much less investigated (OECD 2018). Non-EATS modalities in vertebrates involve NRs such as the retinoic acid receptor (RAR), retinoid X receptors (RXRs), farnesoid X receptors (FXRs), vitamin D receptors (VDRs), pregnane X receptor (PXR), liver X receptors (LXRs), peroxisome proliferator-activated receptors (PPARs), glucocorticoid receptor (GR) and aryl hydrocarbon receptor (AhR) signaling pathways. For invertebrate organisms, both the endocrine systems in general, and their possible modulations by EDCs, are poorly known, possibly except for some systems in insects and crustaceans (Rodriguez et al., 2007; Soin and Smagghe 2007). Currently, no internationally accepted assays for assessing EDC effects in invertebrates are available. Therefore, it is not possible in most cases to evaluate if chemicals of emerging concern are endocrine disruptors in these taxa (OECD 2018).

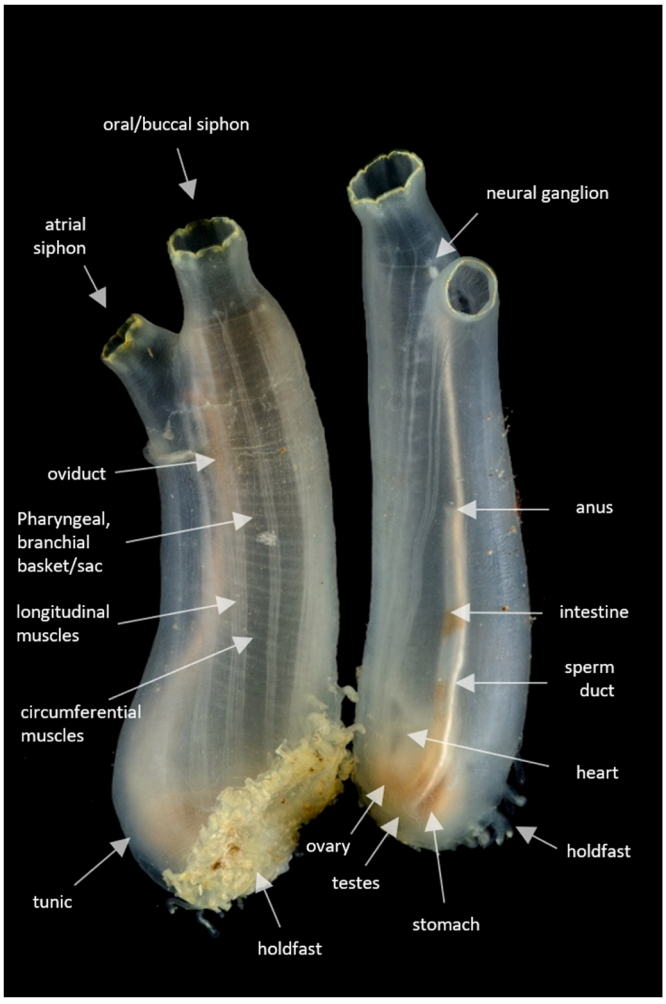

Fig. 1. Adult specimens of *Ciona intestinalis*. The inhalant and exhalant siphons are the upper and lower openings, respectively. The gauze-like pharynx bag and the longitudinal muscle bands on each side of the body are clearly visible. In the right specimen, the whitish sperm duct is clearly visible. Courtesy images: Michael Sars Centre, Bergen, Norway.

3. Why ascidian tunicates could be interesting as ED test models

The three sister groups of tunicates, cephalochordates and vertebrates are believed to have evolved from the same ancestor during the "Cambrian explosion, aka the biological big bang", around 540 million years ago (Fig. 2) (Delsuc et al., 2006). This is when essentially all animal phyla first appear in the fossil record (Marshall 2006). Tunicate larvae have a notochord (Fig. 3, Fig. 4B), a relatively stiff, flexible, cartilage-like, supporting rod, which is the evolutionary forerunner of the vertebra column. Interestingly, tunicates are unique among

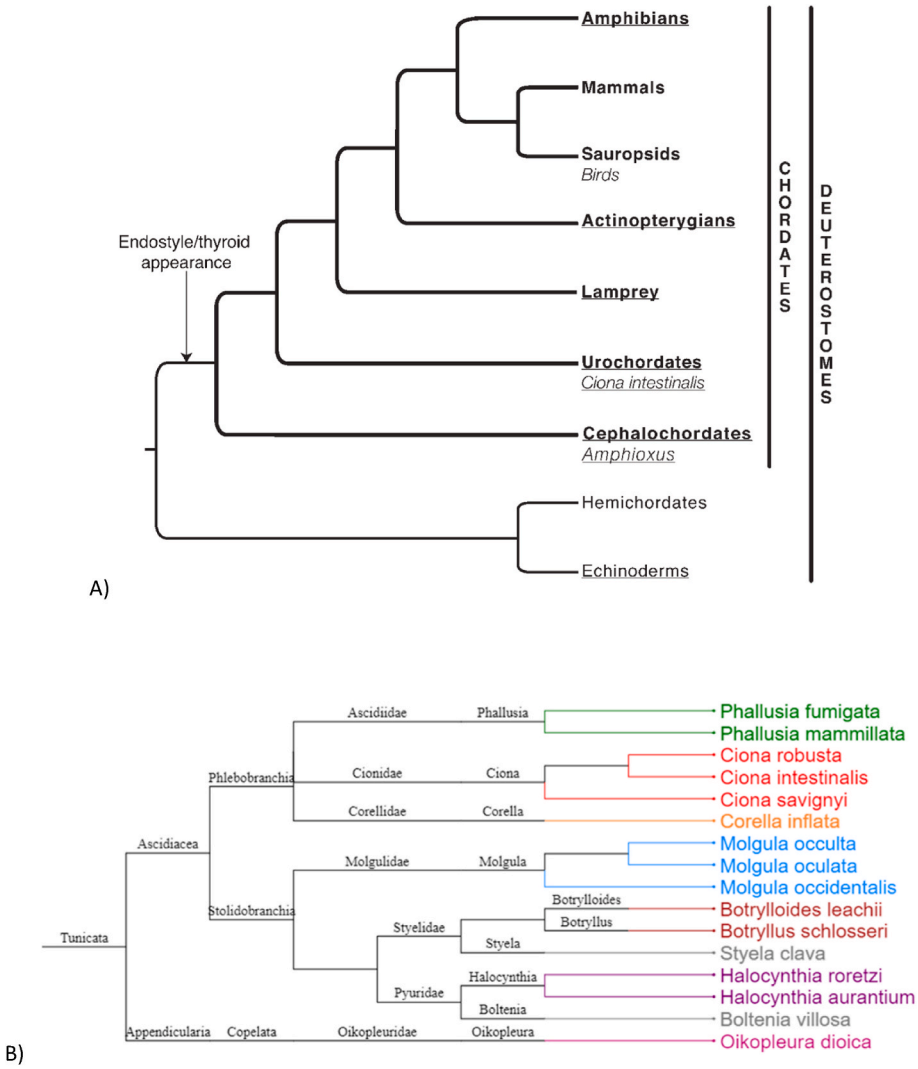

Fig. 2. A) Simplified phylogenetic tree of deuterostomes. Groups where a metamorphosis stage is usually described are underlined. Source of graph A: (Paris and Laudet 2008). B) Taxonomy cladogram showing the ascidian species covered by the ANISEED database, showing the early split of the ascidian and Appendicularia-Oikopleura linages. Source of graph B: ANISEED.

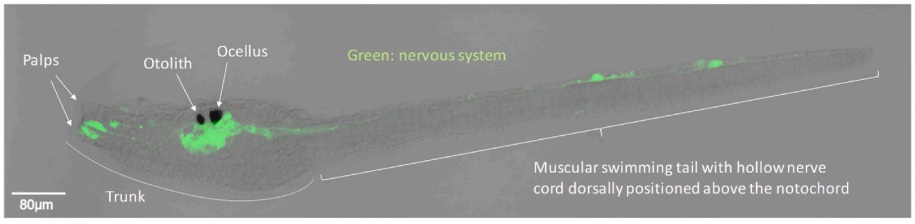

Fig. 3. Transgenic larva of *Ciona intestinalis* (stage 27) with specific staining of the central nervous system which is achieved by expressing green fluorescent protein (GFP) under the control of the proprotein convertase 2 promoter. The two pigmented sensory organs, the anterior geotactic otolith and the posterior photoreceptive ocellus, within the sensory vesicle in the larvae trunk are clearly visible. A fully developed larva consists of only approximately 2600 cells of which 231 are neurons (Christiaen et al., 2009; Kourakis and Smith 2015). Courtesy image: Michael Sars Centre, Bergen, Norway.

metazoans by having an outer coat or "tunica" partly composed of tunicin, which is a variant of cellulose. The tunicates (and the lancelets) are believed to have diverted from the vertebrate lineage prior to the genome duplications and gene diversifications that subsequently occurred within vertebrata (Putnam et al., 2008; Lemaire 2011). They may therefore represent a very basic model for evaluating both functions and perturbations of genetically controlled processes, such as the endocrine system. As invertebrate chordates, the increased application of *Ciona* as bioindicator organisms in toxicity testing could possibly help reducing the need for other chordate (i.e., vertebrate) test organisms, for example in high-throughput EDC testing of the ever-increasing number of man-made chemicals and products. The free-swimming, non-feeding, lecithotrophic ascidian larva is seen as a particularly attractive life stage in context of such toxicity testing, due to its rapid embryological development and many advanced traits, including: an elongated chordate-like body (looking quite like an amphibian tadpole); a central notochord extending within the muscular swimming tail; a hollow nerve cord positioned dorsally above the notochord; a central nervous system with a brain/cerebral vesicle containing a photoreceptive ocellus and a gravity sensing otolith (the two black spots, Fig. 3); and a central

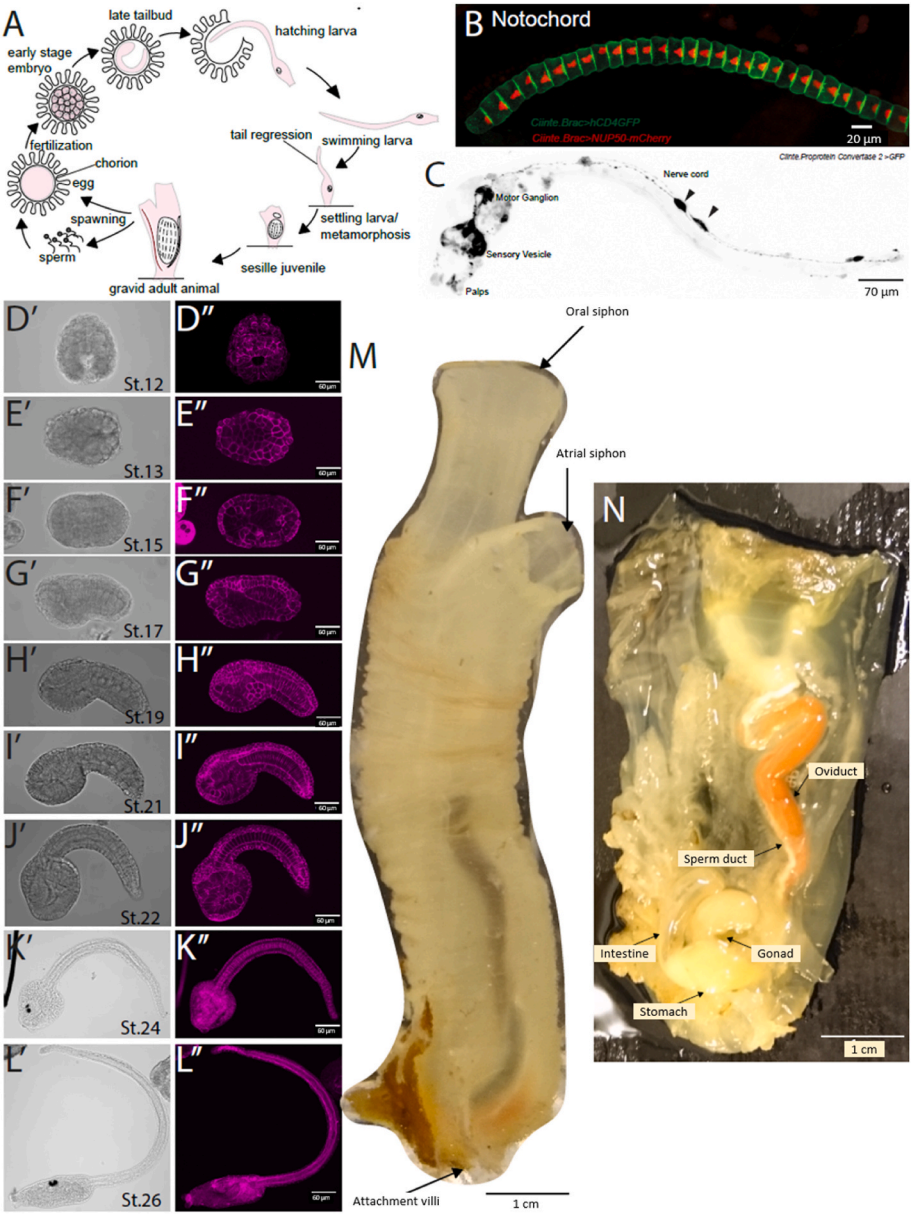

Fig. 4. (A) Main stages of the life cycle of *Ciona intestinalis*. After gametes are released into the marine environment, fertilized oocytes which are covered by a vitelline coat (the chorion) undergo rapid embryogenesis. Hatching from the chorion takes place 18–36 h post fertilization (hpf), depending on the embryo rearing conditions (see references in text). After hatching, the early, non-feeding, free-swimming larvae will disperse a few hours (to several days) until they have developed the three adhesive papillae in their trunk and are able to attach to a suitable, solid substrate where they perform a radical multi-step metamorphosis (up to stage 8), which typically takes about two weeks (after hatching), although the process may take longer depending on temperature and feeding conditions (Chiba et al., 2004). Adult animals reach sexual maturity after approximately 3 months, again depending on the favorability of the environmental conditions. (B) A confocal maximal projection of the notochord of a stage 23 *C. intestinalis* embryo. The embryos were electroporated with Brachyury > hCD4GFP to label the notochord, while the nuclei were marked using Brachyury > NUP50RFP. At the end of convergent extension, the notochord cells become gradually taller in the Anterior- Posterior direction, while they become narrower mediolaterally. Notochord cells become strongly polarized in the AP axis as demonstrated with nuclei localized to the posterior of each cell. (C) Mature swimming larva of *C. intestinalis* with staining of the central nervous system. This stage 26 *C. intestinalis* larva is expressing green fluorescent protein (GFP) under the proprotein convertase 2 promoter, which labels all peptidergic neurons in the larval nervous system (Hotta et al., 2020). Animals were fixed and imaged using a point-scanning confocal microscope. We observed expression of the transgene in the anterior sensory organs called the palps, the sensory vesicle and motor ganglion. The PC2>GFP transgene also labelled a fraction of the peripheral nervous system in the tail including the Bipolar Tail Neurons (BTNs) highlighted with the black arrowheads. (D-L) Pictures of different embryo developmental stages of *C. intestinalis* ranging from stage 12–26, using bright-field microscopy (panels D' - L', left) and maximal projection confocal images (panels D'' - L'', right) after phalloidin staining of actin filaments with Alexa Fluor 647 phalloidin. (M) Adult specimen of *C. intestinalis*. (N) Dissection of *C. intestinalis* showing locations of some key internal organs. Courtesy images: Michael Sars Centre, Bergen, Norway.

nervous system that includes the trunk ganglion and the caudal nerve cord; (Satoh 2003; Hudson 2016; Matsubara et al. 2016, 2019; Satake et al., 2019). Within a few days, the swimming larvae attach permanently to a suitable substrate by means of three anterior adhesive protrusions (papillae or palps) and starts a metamorphosis (Fig. 4A) that involves a complete remodeling of the whole body plan and adaptation to the sessile, filter-feeding life-style, in part by developing two huge siphons, pharyngeal gill slits and the ciliated, mucus-secreting endostyle, the latter organ which also is iodine-absorbing and believed to be an early thyroid (Ogasawara et al., 1999; Chiba et al., 2004; D'Agati and Cammarata 2006; Sasakura et al., 2009; Sasakura et al., 2012b).

The species in the vase tunicate (*Ciona*) genus are invasive and grow opportunistically on literally any substrate submerged in the sea. They can develop high density biofouling on ropes, buoys, shellfish culture gear, fish farm pens and hulls of all kinds of marine vessels (Wilson et al., 2022). They are relatively uncomplicated to maintain in laboratory cultures, and especially due to their rapidly developing embryos, they could be excellent model systems for embryo effect studies (Passamaneck and Di Gregorio 2005; Zega et al., 2009; Dumollard et al., 2017; Eliso et al., 2020b). *Ciona* adults have a transparent, cylindrical, soft, gelatinous body (Figs. 1, Fig. 4M-N), grow up to lengths of 15–20 cm and may produce up to 10–12 thousand eggs per season (Petersen and Svane 1995; Carver et al., 2003; Lemaire 2011). This allows for numerous embryos of the same age to be developed from the same batch. The compact genome of *Ciona* comprises about 16,000 genes and was sequenced in 2002 (Dehal et al., 2002; Shoguchi et al., 2005). Originally, the *Ciona* genus was described by Linnaeus (1767) as one species, but later research distinguished at least two major members, i.e., "type A" *C. robusta* (Hoshino and Tokioka 1967) and "type B" *C. intestinalis* (Linnaeus, 1767) (Caputi et al., 2007; Brunetti et al., 2015), whereas currently the *Ciona* genus is thought to also include at least two other species (type C and D) with more restricted distribution (Malfant et al., 2018; Wilson et al., 2022). *C. robusta*, (or *C. intestinalis* Type A), is native to the Northwest Pacific, has preference to higher temperature and salinity, and is different from *C. intestinalis* Type B by the possession of certain tunic tubercular prominences (Brunetti et al., 2015). Prior to

2015, studies typically made insufficient distinction between *C. intestinal* and *C. robusta*, and numerous studies claiming to use *C. intestinalis* have most likely addressed *C. robusta*, and vice versa. The research literature on *Ciona* is very broad and much additional information is available at high-quality websites such as The Tunicate Web Portal,[1] ANISEED,[2] GHOST[3] and FABA.[4] Therein, access to genome annotations, embryonic development at the level of the genome (cis-regulatory sequences, spatial gene expression, protein annotation), the cell (cell shapes, fate, lineage), and the whole embryo (anatomy, morphogenesis atlas) are available (Dardaillon et al., 2020). The use of ascidians in ecotoxicity testing as alternative/replacement for vertebrates would comply with the European Union legislation on the protection of animals used for scientific purposes (Directive 2010/63/EU) and the so-called 3Rs policy (Refinement, Reduction, Replacement) on experimental animal testing. This is particularly important if the testing may involve large numbers of test organisms, such as in toxicity screening of emerging compounds and chemical mixtures (Lillicrap et al., 2016; Sneddon et al., 2017). Furthermore, using these ancestral chordates could possibly provide ecotoxicity data, including on ED endpoints, that could be relevant for both invertebrate and vertebrate taxa.

4. Endocrine regulation in ascidians

In the past decades, about 40 types of hormones/neuropeptides (some key examples are shown in Table 1) have been identified and characterized in ascidians by purification, cDNA cloning, and peptidomic approaches (Kawada et al., 2011; Matsubara et al., 2016; Satake et al., 2019; Shiraishi et al., 2019). This has contributed to improved understanding of the neuroendocrine control of reproduction and development processes in early chordates. The main ascidian hormones/peptides that have been found can be classified into three categories: (1) vertebrate homologs, such as gonadotropin-releasing hormones (GnRHs), tachykinins (TKs), galanin-like peptides (GALP), calcitonin (CT) and insulin/relaxin paralogs; (2) hormones/peptides that belong to conserved families but with distinct sequences or activities compared to vertebrate homologs, such as GnRH-X and vasopressin (VP); and (3) hormones/peptides that are ascidian-specific, such as Ci-YFV/Ls and Ci-LFs (Satake et al., 2004; Tello et al., 2005; Kawada et al. 2008, 2010, 2011, 2021; Kusakabe et al., 2012; Sekiguchi et al., 2012; Matsubara et al., 2016). Some of these hormones/peptides have been extensively investigated with their functions characterized, but many remain unknown. Studies of the sequenced genome of *Ciona* have identified at least 17 genes that encode for NR transcription factors (Yagi et al., 2003), whereas in humans, for comparison, the number of identified NR genes is 48 or 49 (Robinson-Rechavi et al., 2001; Zhang et al., 2004; Weikum et al., 2018). The NR genes documented to be present in ascidians include, among others, the TR, the peroxisome proliferator-activated receptor (PPAR), the retinoic acid/retinoid X receptors (RAR/RXR), the vitamin D/pregnane X receptors (VDR/PXR), and the estrogen-related receptor (ERR) (Nagatomo et al., 2003; Ishibashi et al., 2005; Kobayashi et al., 2005; Koyano et al., 2009; Pasini et al., 2012; Sasakura et al., 2012a; Pennati et al., 2018). Conversely, NR related genes that are present in humans, but thought NOT to be present in ascidians, include most importantly the steroid hormones and their receptors, i.e., the estrogen receptor (ER), androgen receptor (AR), mineralocorticoid receptor (MR), glucocorticoid receptor (GR), and progesterone receptor (PR) (Dehal et al., 2002; Yagi et al., 2003; Eick and Thornton 2011; Gomes et al., 2019a). NRs positively identified in ascidians represent important focus points for studies into possible ED effect phenomena and MOA of EDCs in ascidian taxa, an example being the study of Gomes et al. (2019b) who addressed the role of ERR in neurodevelopmental toxicity of bisphenol A to embryos of the ascidian *Phallusia mammillata*.

An unknown feature of *Ciona* is whether there are primordial components and functions present in the hypothalamic–pituitary–gonadal (HPG) axis system. This system is essential for regulation of several major processes in vertebrates, including reproduction, immune system, and growth/aging, and is believed to have emerged just prior to (or during) the differentiation of the ancestral agnathans (the jawless vertebrates). A central matter in this context is whether the genes that encode for gonadotropin-releasing hormone (GnRH) show recognizable functionality in *Ciona* compared to vertebrates (Adams et al., 2003). GnRH is the key neuropeptide hormone which is produced in vertebrates in the hypothalamus, acts via the HPG axis to stimulate release of hormones from the anterior part of the pituitary, and are key for regulating the cascade of events that eventually lead to successful reproduction, e.g., (Campbell et al., 2004; Sakai et al., 2020). Both older studies, e.g., (Adams et al., 2003), and more recent omics studies, e.g., (Kawada et al., 2022), have demonstrated the presence and regulatory roles of GnRHs in ovarian follicular development in *Ciona*. Several other studies have demonstrated the presence of a GnRH system in the cerebral ganglion area in the ascidian larvae nervous system, suggesting a neurotransmitter or neuromodulator role of GnRH within central processing and sensory functions (Tsutsui et al., 1998; Kavanaugh et al., 2005; Kusakabe et al., 2012; Okawa et al., 2020). Several *Ciona* GnRHs have been shown to bind to GnRH receptors (GnRHRs) expressed in various tissues within the larval tail (notochord, muscle and nerve cells) and to be involved in organ growth inhibition and the tail absorption process in the settled ascidian larvae (Kusakabe et al., 2012; Okawa et al., 2020), suggesting a prominent role of the GnRH-GnRHR pathway in ascidian metamorphosis (Sakai et al., 2020).

A key question is to what degree ascidians have a functional thyroid hormone (TH) signaling pathway, as this system is pivotal for nervous system developments and many other essential systems and processes in chordates where the regulation acts principally through nuclear TH receptors (TRs), and even mild perturbations by thyroid-disrupting chemicals can produce significant neurological, developmental or motor function defects in developing larvae, such as demonstrated for fish (Macaulay et al., 2015; Sharma et al., 2016; Wei et al., 2018) and amphibians (Dang 2022; Marini et al., 2023). Although ascidians lack a follicular thyroid, a number of studies have shown that the TH pathway and the TH receptor (THR) are present and have regulatory functions in the larval metamorphosis; and that the adult endostyle is site for biosynthesis of thyroid hormones, including thyroxine (T4), triiodothyronine (T3), 3,5,3′-triiodothyroacetic acid (TRIAC) as well as thyroid peroxidase (TPO) and iodothyronine deiodinase (DIO) activities (Eales 1997; Carosa et al., 1998; Patricolo et al., 2001a; D'Agati and Cammarata 2006; Paris and Laudet 2008; Wei et al., 2020; Godefroy et al., 2023). Studies have shown that TH mediated regulation of larval metamorphosis in tunicates can be impaired by known TH synthesis inhibitors (goitrogens) such as methimazole (MMI) (Wei et al., 2020) or thiourea (Patricolo et al., 2001a; Godefroy et al., 2023). Furthermore, treatment of *Ciona* larvae with exogenous T4 has been shown to accelerate tail resorption, suggesting a linkage between this TH and metamorphosis in *Ciona* (Patricolo et al., 2001a). However, there are several issues that remain to be clarified in this field, especially how the action of THs can accelerate ascidian metamorphosis, although their TR have not yet been demonstrated as a functional receptor for ascidian T3, T4 or TRIAC (Holzer et al., 2017b). Hence, the tunicate TR appears to be an orphan receptor and several reviews, such as Morthorst et al. (2022), suggests that thyroid-like hormone signaling in tunicates occur through induction of the THR by thyroid hormone action, and with the THR subsequently acting as a transcription factor in its unliganded state (Fig. 5), as also is the case for mollusks and echinoderms. However, it is still considered an enigma that ascidians synthesize THs which apparently are involved in metamorphosis, and in addition they possess a NR

[1] Tunicate Web Portal (https://tunicate-portal.org/).
[2] https://aniseed.fr/aniseed/?module=aniseed&action=default:index.
[3] http://ghost.zool.kyoto-u.ac.jp/.
[4] https://www.bpni.bio.keio.ac.jp/chordate/faba/1.4/top.html.

Table 1
Ascidian hormones with known roles in major neuroendocrine pathways compared to their vertebrate homologs.

Ascidian hormone/ neuro-peptide	Ascidian receptor	Receptor binding?	Functions in Ascidians	Vertebrate homolog	Vertebrate receptor	Functions in vertebrates
Tachykinin (TK)	TKR	Yes	Oocyte growth (oocyte stage II) (Aoyama et al., 2008)	TK	TKR	Smooth muscle contraction, vasodilation, nociception, secretion, neurogenic inflammation, neurodegeneration and neuroprotection (Satake 2020).
Neurotensin-like peptide (NTLP)	?	?	Follicle growth (oocyte stage II) (Sekiguchi et al., 2012)	NT	NTR	Luteinizing hormone secretion, prolactin release (Vijayan and McCann 1979).
Vasopressin (VP)	VPR	yes	Oocyte maturation (oocyte stage III) (Kawada et al., 2008).	VP	VPR	Regulation of blood pressure, volume and osmolarity (Holmes et al., 2003).
Cionin	CioR	yes	Ovulation (oocyte stage IV) (Sekiguchi et al., 2012).	GAST/CKK	CKKAR	Gastric secretion, blood circulation, muscle contraction (Zeng et al., 2020).
Gonadotropin releasing hormone (GnRH)	GnRHR	yes	Gamete release and metamorphosis (Tello et al., 2005).	GnRH	GnRHR	Secretion of luteinizing hormone (LH), release of follicle-stimulating hormone (FSH) (Millar et al., 2004).
Triiodothyronine (T3), thyroxine (T4)	NR1	no	Embryo and larval development and metamorphosis (e.g., tail regression) (Carosa et al., 1998)	T3, T4	TR	Metabolism, heart functions, brain development, muscle contraction (Mullur et al., 2014).

Abbreviations: CioR, Cionin receptor; GnRH, Gonadotropin releasing hormone; GnRHR, Gonadotropin releasing hormone receptor; NT, neurotensin; NTR, neurotensin receptor; NR1, *Ciona intestinalis* Nuclear Receptor 1; TKR, Tachykinin receptor; TR, Thyroid hormone receptor; VP, Vasopressin; VPR, Vasopressin receptor. GAST/CKK, gastrin/cholecystokinin; CCKAR, cholecystokinin A receptor.

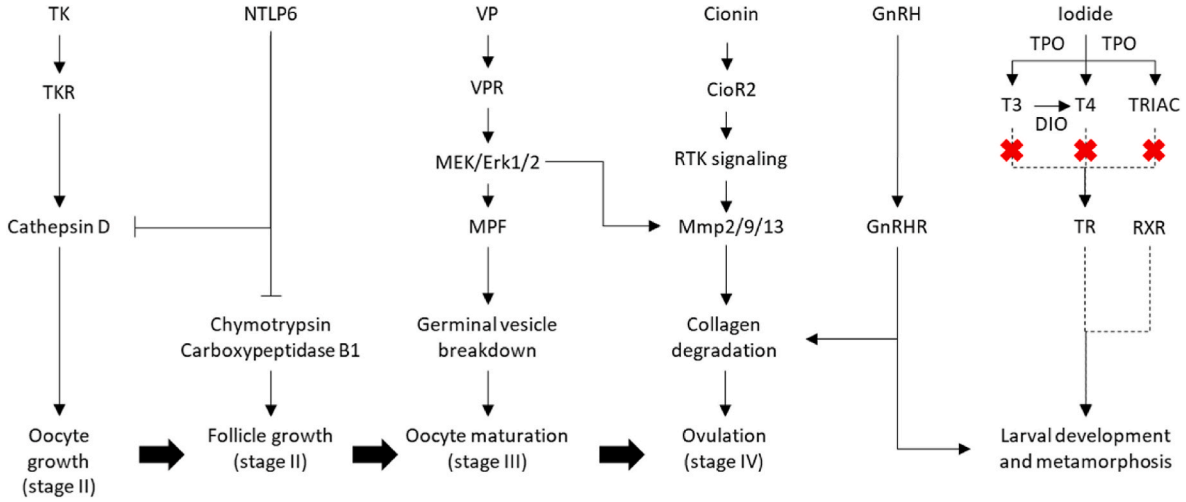

Fig. 5. Regulatory pathways of oogenesis, and development in ascidians. Abbreviations: Tachykinin (TK); tachykinin receptor (TKR); neurotensin-like peptide (NTLP); vasopressin (VP); vasopressin receptor (VPR); Ras/Raf/Mitogen-activated protein kinase/ERK kinase (MEK); extracellular-signal-regulated kinase (ERK); maturation promoting factor (MPF); cionin receptor 2 (CioR2); receptor tyrosine kinase (RTK); matrix metalloproteinase (Mmp); gonadotropin releasing hormone (GnRH); thyroid peroxidase (TPO); triiodothyronine (T3); iodothyronine deiodinase (DIO); thyroxine (T4); 3,5,3′-triiodothyroacetic acid (TRIAC); thyroid hormone receptor (TR); retinoid X receptor (RXR).

which by sequence closely resembles known TRs, yet this receptor can apparently not bind TH (Carosa et al., 1998).

The detailed endocrine regulation of oogenesis and spawning in ascidians has recently been characterized, e.g., (Matsubara et al., 2019; Osugi et al., 2021), and the main features of these pathways are outlined in Fig. 5. The oogenesis is classified into four stages (stage I-IV) based on the diameter, pigmentation, yolk content, mitochondrial distribution, cortical endoplasmic reticulum distribution, DNA condensation level of the egg and the morphology of the follicular cells (Jeffery and Capco 1978; Swalla et al., 1991; Prodon et al., 2006; Mcdougall et al., 2011). In stage I, the previtellogenic oocytes are small and have typical characteristics of preleptotene to pachytene stage oocytes. The stage II oocytes are vitellogenic and increase dramatically in volume. The stage II oogenesis is regulated by the tachykinin (TK) neuropeptides through activation of the TK receptor (TKR). The TKR agonism further upregulates the activities of three enzymes, cathepsin D, chymotrypsin, and carboxypeptidase B1 that are known to be involved in the regulation of oocyte growth and follicle development. In addition, the neurotensin-like peptide (NTLP) is known to suppress TK mediated upregulation of the three enzymes and negatively regulate follicle growth (Aoyama et al. 2008, 2012). In stage III, the post-vitellogenic oocytes have fully grown germinal vesicles (GV) and are ready to mature ((Conklin 1905), see Prodon et al. (2006)). In stage III follicles, VP binds to its receptor VPR to trigger the MEK/Erk1/2 signaling pathway, leading to GV breakdown and oocyte maturation via activation of MPF (maturation promoting factor). The MEK/Erk1/2 pathway can also regulate collagen degradation via activation of MMP2/9/13 and trigger ovulation in stage IV. In addition, another peptide, cionin, can also regulate MMP2/9/3 via receptor (CioR2) agonism and activation of receptor tyrosine kinase (RTK) signaling, leading to collagen degradation and liberation of fertile oocytes from the ovary (Matsubara et al., 2019; Osugi et al., 2021). It has also been suggested that the *Ciona* GnRHR-1/2/4 may be involved in oocyte growth and gamete release through GPCR (G-protein-coupled receptor) heterodimerization (Sakai et al., 2020).

The embryo development of *C. intestinalis* is extremely rapid. The time from fertilization to hatching of the free-swimming larva may take only 18 h at 20 °C, and 22 h at 16 °C (Satoh 2001, 2003; Bellas et al.,

2003; Matsunobu and Sasakura 2015), although more sub-optimal rearing conditions and other factors may delay the hatching significantly. The key steps of the developmental ontology, from fertilized egg, through the larval stages and metamorphosis to the juvenile and adult stages, have been well characterized (Satoh 2003; Chiba et al., 2004; Kourakis and Smith 2015; Hotta et al., 2020) and examples of some of these steps and phases are shown in Fig. 4 (D-L). A web-based collection of high-quality images of embryo and larva development of *Ciona intestinalis* is made available by Hotta et al. (2007), and further details of the 3D anatomy of the *Ciona* tailbud embryo at single-cell resolution level are made available by Nakamura et al. (2012). A few hours to days after the hatching, the free-swimming and non-feeding *Ciona* larva settles permanently at some suitable substrate by means of three adhesive papillae at the anterior-most part of the trunk, and mechanical stimuli from the settlement triggers the onset of the metamorphosis process which subsequently is further controlled by a combination of environmental and endogenous signals (Chiba et al., 2004; Karaiskou et al., 2015). An outline of the endocrine components that are thought to regulate the metamorphosis is shown in Fig. 5, although this process is not yet fully understood. During the metamorphosis, the swimming tail, notochord, musculature, nerve cord, and most other advanced traits of the ascidian larvae degenerate as the whole organism becomes remodeled. The endostyle, will at this stage develop into a longitudinal, ciliated, grooved organ finally localized mid-ventrally in the pharynx wall, and here it will secrete mucoproteins that have a key role in trapping food particles during the filter-feeding behavior. The endostyle organ also exists in cephalochordates as well as in ammocoetes larvae of lampreys, and in the latter it is thought to transform into an adult thyroid gland during metamorphosis (Kluge et al., 2005) (Fig. 2A). The theory of the tunicate endostyle being the precursor to the vertebrate thyroid gland is supported by its iodine-concentrating activity and its ability to biosynthesize thyroid hormones, as commented earlier.

5. Ecotoxicity and ED studies and bioassay with *Ciona* or other ascidians

Despite the large number of research studies on *Ciona*, relatively few have explored their potentials as ecotoxicological bioindicators, and the number of reviews on this topic is still quite small, i.e., (Roberts et al., 2008; Wu et al., 2014; Dumollard et al., 2017; Gomes et al., 2019a; Capela et al., 2020; Morthorst et al., 2022). With most emphasis to studies on teratogenicity and neurodevelopmental toxicity, the chemical stressors that have been addressed in *Ciona*-based effect studies include: heavy metals (Bellas et al. 2001, 2004; Gallo et al., 2011); polycyclic aromatic hydrocarbons (PAHs) (Sekiguchi et al., 2020); TBT or other antifouling biocides (Gianguzza et al., 1996; Patricolo et al., 2001b; Puccia et al. 2001, 2005; Bellas 2005, 2006; Cangialosi et al. 2009, 2010; Mansueto et al. 2011, 2012; Gallo and Tosti 2013, 2015); bisphenol A (BPA) (Mansueto et al., 2011; Cangialosi et al., 2013; Matsushima et al., 2013; Messinetti et al., 2019; Ferrari et al., 2022; Mercurio et al., 2022); flame retardants (Mercurio et al., 2021); polystyrene nanoparticles (Eliso et al., 2020a; Ferrari et al., 2022); oil spill dispersants (Eliso et al., 2020b); and metal nanoparticles (Gallo et al., 2016). In addition, many other relevant ecotoxicological studies with other solitary ascidian species have recently been reported, including: *Phallusia mammillata* (Messinetti et al., 2018; Gomes et al., 2019b; Gazo et al., 2021); *Herdmania momus* (Navon et al., 2020); *Microcosmus exasperates* (Kuplik et al., 2019; Navon et al., 2020); *Polycarpa mytiligera* (Kuplik et al., 2019); and *Styela plicata* (Aydin-Önen 2016; Navon et al., 2020)

The broad knowledge available on the biology of *Ciona* and other ascidians may serve to underpin their potential application as novel ecotoxicological test organisms, both for bioaccumulation and effect studies of CECs in general and for putative EDC studies especially. Data on embryo development and growth of *Ciona* have been reported by virtually hundreds of articles, among which Berrill (1947) is one of the earliest key studies. Standardization of laboratory rearing protocols for *Ciona*, that cover all phases from egg to adult, have previously been provided by Bellas et al. (2003) and by Joly et al. (2007). An updated overview of the ontology of the anatomy and development of *Ciona* was also recently provided by Hotta et al. (2020). The different endpoints that can be addressed in *Ciona*-based *in vivo* biotests may range from unspecific performance parameters, such as mortality, growth, larval swimming behavior, via more specific changes to certain metamorphosis and phenotypic features (much resting on the function of endogenous control systems), and down to ED relevant endpoints such as changes in hormone concentrations, expression/function of hormone receptors or malformations in developmental and phenotypic traits that strictly requires hormonal regulation. Hence, knowing the basic physiology and ontology of *Ciona* and having the capability for establishing and maintaining well-optimized *Ciona* cultures in the lab, is key for virtually any kind of ecotoxicological study that aims to distinguish conditions of stressor-induced abnormalities from conditions that are within the normal range of background variation.

Many studies with *Ciona* or other ascidians have demonstrated various use of high-content analysis of larval phenotypes for evaluating morphological and teratogenic effects of ED acting marine antifouling substances, for example by assessing malformations of swimming tail, pigmented organs and the larval trunk. As noted above, the marine antifouling biocide TBT, which currently is banned from antifouling applications world-wide, is probably the EDC stressor that most prevalently has been addressed in ascidian ED effect studies. Interestingly, many locations worldwide (e.g., close to shipyards and maritime harbors) still are severely TBT contaminated, and these so-called TBT hotspots are interesting locations for field studies of EDC effect issues in *Ciona* and in other invertebrate taxa, as discussed in Beyer et al. (2022). Patricolo et al. (2001b) found teratogenic effects (blocked larval metamorphosis) resulting from TBT exposure and suggested a possible link to impairment of a thyroid hormone mediated regulation. A radioimmunoassay showed the T4 content in the TBT-exposed larvae was decreased by 70% in comparison to the non-exposed controls. Also, new marine antifoulant biocides are relevant to study for their EDC properties in ascidians. For example, Gallo and Tosti (2015) studied the effect of the antifoulant substance chlorothalonil on gamete physiology, fertilization rate, larval malformation and transmissible damage to offspring in *Ciona*. Interestingly, they found that particularly the low chlorothalonil concentrations interfered with embryo development (abnormal curled tails and lack of one of the sensory organs) and led to abnormal larvae, whereas the high exposure concentrations arrested the embryo development at earlier stages.

Beside TBT, bisphenol A (BPA) is probably the individual chemical that has been most addressed in ED effect studies with ascidians. Messinetti et al. (2018, 2019) investigated the effects of BPA on developing larvae of *C. intestinalis* and *Phallusia mammillata* and found effect phenotypes to be characterized by a short and kinked tail, malformations of pigmented organs (otolith and ocellus) and impaired neural development. Interestingly, co-exposure of ascidians with 4-hydroxytamoxifen, which binds and deactivates the estrogen-related receptors (ERRs), resulted in normal pigmented-organ phenotypes. This suggests that BPA exerted its teratogenic effect by binding to and activating ERRs, as also has been observed in vertebrate (zebrafish) models (Tohme et al., 2014), although these observations were not confirmed in the ascidian *Phallusia mammillata* where 2 different inhibitors of ERR (Tamoxifen and DES) phenocopied the effect of BPA, thus suggesting that BPA inhibits ERR activity in *P. mammillata* to exert its phenotype on the pigmented sensory organ (PSO) formation (Gomes et al., 2019b). Moreover, GABAergic and dopaminergic neurons have been found to be target organs of BPA teratogenic actions in *Ciona* (Messinetti et al., 2019), which also is in accordance with BPA effect studies on vertebrate test animals. Mercurio et al. (2021, 2022) compared the potential teratogenic effects of BPA and tris (1-chloro-2-propyl) phosphate (TCPP) exposure on embryo development in both *C. robusta* and *C. intestinalis*. The study suggested a comparable sensitivity to the BPA exposure, whereas TCPP showed very

different teratogenic potential in the two analyzed species, seemingly with *C. robusta* being more sensitive than *C. intestinalis*. Battistoni et al. (2018) developed an Ascidian Embryo Teratogenicity assay (AET) based on *Ciona* larvae morphology for assessing mixture effects of ethanol and the azole fungicide fluconazole. They demonstrated how the combined exposures induced trunk abnormalities, malformed pigmented organs and coiled or flexed tails in the *Ciona* larvae, possibly indicating a need for precaution on combined exposure to alcohol and azoles during human pregnancy. Embryos of *Ciona* (22 hpf) were used by Ferrari et al. (2022) for assessing possible combined effects of BPA and non-functionalized 20 nm polystyrene nanoparticles, used as a proxy for nanoplastics. BPA was confirmed to induce alterations in the pigmented organ, but the combined exposure with nanoparticles did not give any added effect compared to the effect of BPA when administered alone. Sekiguchi et al. (2020) demonstrated teratogenic effects of PAHs (dibenzothiophene, fluorene, and phenanthrene) on early development, metamorphosis and juveniles of *Ciona*, and with a special focus on mechanisms associated with the *Ciona* ortholog of the aryl hydrocarbon receptor (AhR) gene. In the PAH exposed specimens, they found *Ci-AhR* mRNA expression to be localized to the digestive tract, dorsal tubercle, ganglion, and papillae of the branchial sac. This suggests that these organs were associated with PAH metabolism and that *Ci-AhR* mRNA expression could be a candidate biosensor for environmental pollutants. High-content analysis of *Ciona* and ascidian embryo and larval phenotypes for assessing EDC toxicity of CECs will typically involve the use of automated microscopy and image analysis. When such effect phenotype signatures are well characterized in relation to specific EDC structures, concentration requirements, and endocrine perturbation mechanisms, e.g., (Gazo et al., 2021), such analyses can be further adapted into high-content screening, which implies a higher level of analytical throughput than the high-content analyses.

Most ED effect studies on ascidians have focused on endpoints associated with some part of the embryo development process and up to the function of the mature free-swimming larvae. As the mature *Ciona* larva (Figs. 2 and 4C) consists of only approximately 2600 cells, the involvement of advanced techniques such as transgenic embryos, DNA fluorescent tagging and 3D time lapse imaging, can enable *in vivo* EDC effect studies of peptidergic regulation of key organs such as the pharynx, endostyle, alimentary tissues, and heart, even down to the single cell level (Ogura and Sasakura 2013; Osugi et al. 2017, 2020). Cangialosi et al. (2013) assessed dose-response effects of BPA and atrazine in *Ciona* embryos by exposing fertilized eggs at concentrations of 0.1, 1 and 10 μM of each stressor. Both stressors gave concentration-dependent effects with the lowest observed effect concentration in BPA and atrazine treatments being 1 and 0.1 μM, and with the embryos being arrested at morula and at 2–4-cell stages, respectively. Also the functional swimming behaviors of *Ciona* larvae comprise relevant endpoints for possible developments of high-throughput, (semi)automated bioassays that can identify neurotoxic and developmental disruptive properties of ecotoxicants (Zega et al., 2006; Matsushima et al., 2013; Rudolf et al., 2019; Gazo et al., 2021). For example, Matsushima et al. (2013), found altered swimming behavior of *C. intestinalis* larvae when the embryos were exposed to seawater containing 1 μM BPA, whereas adverse effects on embryonic development (measured as decreased hatching rate) was found at BPA concentrations above 3 μM. The most sensitive window of exposure was fertilized eggs exposed to BPA within 7 h post-fertilization (ibid.). Rudolf et al. (2019) used automated image-based tracking combined with machine learning tools to develop an objective ontology of the swimming behaviors for *Ciona* larvae, which subsequently was employed to assess effects of thigmotactic effects of the anxiotropic and nootropic drug modafinil. Thigmotaxis refers to an organism's responses to contact stimuli, which often are key for animal behaviors, whereas nootropic drugs comprise a group of widespread surface water contaminants that could represent an emerging threat to many aquatic animal species, see e.g., (Wilms et al., 2019; Frizzo et al., 2020).

Only relatively few studies on ascidians have assessed the effect of EDCs on juvenile stages, i.e., after the larva have permanently settled at substrates. Mansueto et al. (2011) studied effects of BPA and TBT on organ morphology in post-embryonic (4 d post fertilization) *Ciona* juveniles which were cultured in petri dishes (optimized to 100 juveniles per dish) and exposed for 1 h to 0.1, 1 and 10 μM concentrations of each stressor. Both chemicals caused a dose-responsive effect on the morphology of the tunic, the gonad cells, the nervous system, the digestive system, as well as inhibition of the rhythmic body contractions, with BPA being more toxic than TBT.

Reporter gene bioassays based on ascidian NR genes may comprise a powerful avenue for development of high-throughput CEC test tools. Richter, Fidler and co-workers (2012, 2014, 2015a) developed recombinant yeast strains that expressed ascidian VDR/PXRα LBDs as fusion proteins combined with GAL4-DBD, a generic transcription activation domain (VP16-AD) and the lacZ reporter gene. The bioassay tested ligand binding effects of BPA and three other putative ED contaminants, as well as several marine microalgal biotoxins (okadaic acid, pectenotoxin-11, and portimine). For the contaminants, the bioassays generated EC50 values in the μM range for the EDCs, while for the marine biotoxins the bioassays were found to be activated in the nM range, suggesting a potential for the bioassays in high-throughput screening and effect studies of uncharacterized algae biotoxins as well as pollutants with ED modes of actions. In a follow-up study Richter and Fidler (2015b) characterized transcript structures and sequence variation of three tunicate PXR orthologs: *C. intestinalis* VDR/PXRα and β, and *Botryllus schlosseri* VDR/PXRα, and demonstrated the three predicted proteins to have both DBD and LBD domains typical of nuclear receptors. The studies provide a foundation for studies on the evolution and functioning of tunicate NRs thought to be involved in detection of marine bioactive compounds.

6. Summary and research needs

As ascidians/tunicates are the closest living invertebrate relatives of vertebrates, they have long served as key model organisms in several disciplines of biology. A key question explored herein is whether this central phylogenetic position also make them particularly suitable as bioindicator organisms in effect-oriented testing and monitoring of ED acting pollutants, and whether they (optimally) could act as a replacement of vertebrate bioindicators, such as fish, in such studies. A synthesis of all studies examined herein may suggest that the overall suitability of ascidians as ED bioindicators rests with two major questions, namely (1): how responsive are the ascidian physiological and endocrine systems to exposure of ecotoxicants and EDCs, and (2) to what degree are such responses detected in ascidians representative for other invertebrates and/or vertebrates? The suitability of ascidians as EDC bioindicators also depends on their robustness against influence from common confounding factors such as seasonal variation and near species differences. For example, although the congeneric taxa *C. intestinalis* and *C. robusta* are closely related, species-specific differences in pollutant sensitivity within the *Ciona* genus is so far studied only to a limited degree. As many earlier *Ciona* effect studies don't specify whether it is *C. robusta* or *C. intestinalis* that was used, a proven comparability of sensitivities within the *Ciona* genus would simplify the use of EDC effect data from such older studies.

Although homologs of vertebrate hormones and peptides have been identified in ascidians, the mechanism of actions of the majority of these remain uncharacterized. Why *Ciona* and other urochordates seem to lack the "classical" sex steroid hormones (estrogens, androgens, progestogens, glucocorticoids and mineralocorticoids) and their receptors (ER, AR, PR, GR, MR) is an issue which has attracted much attention. The gene phylogeny review by Eick and Thornton (2011) suggests that the ancestral steroid receptor (SR) gene, AncSR1, the ancestor of chordate ER (α and β), existed even before the split of protostomes (including arthropods, nematodes, mollusks, annelids, etc.) and deuterostomes (including all chordates and echinoderms), and conversely, that a

duplication and divergence of the AncSR1 gene to produce the gene AncSR2, the latter which is thought to be the ancestor gene of AR, PR, GR, and MR, must have occurred prior to the divergence of the chordates (i.e., including early tunicates) linage. And, that the further gene duplication and divergence of the ancient AncSR2 gene, (to produce AR, PR, GR, and MR genes), occurred after the divergence of the vertebrates from the cephalochordate lineage (ibid.). This suggests that modern tunicates have lost the genes required for sex steroid receptor signaling. Furthermore, ascidians seem to lack several of the cytochrome P450 (CYP) enzymes that are needed in steroid hormone biosynthesis pathways, such as CYP17 and CYP19 (P450 aromatase) (Campbell et al., 2004). The absence of these enzymes suggests that ascidians must control their reproductive development without regulatory actions of sex steroids. However, the study of Cangialosi et al. (2010) investigated the presence of several steroid sex hormones and their precursors in *Ciona* ovaries and how these, and ovarian morphology, and the gene expression of steroidogenic pathway enzymes, were modulated by the exposure to TBT, a well-known EDC. The study used a GC-MS assay to identify cholesterol, corticosterone, dehydroepiandrosterone, estrone, estradiol-17 beta, testosterone, pregnenolone, progesterone, and demonstrated their high similarity to vertebrate steroids. In addition, the expression of genes of key enzymes in steroidogenic pathways within the *Ciona* ovary (adrenodoxin, adrenodoxin reductase and 17 beta-hydroxysteroid dehydrogenase) have been studied and demonstrated by means of quantitative (real-time) polymerase chain reaction (qPCR) (Cangialosi et al., 2010). It is also worth mentioning that the steroid ligands themselves might have evolved, and that an ancient steroid (which not yet have been found) could be a ligand for ERs/SRs in *Ciona* or other invertebrates (Holzer et al., 2017a; Markov et al., 2017; Markov and Laudet 2022). One interesting candidate for such an ancestral steroid is paraestrol, such as the paraestrol A compound which has been found in cnidarians (Markov et al., 2017; Khalturin et al., 2018). It has been demonstrated that also ascidians produce sterols, e.g., (Imperatore et al., 2016), and some of these ancient sterols may be mimicked by man-made EDCs (pesticides, pharmaceuticals, etc.), making them able to bind and activate ancestral invertebrate steroid receptors.

The limited knowledge on EDC effects and their mechanisms in marine invertebrate taxa is recognized by chemical regulatory authorities and environmental risk assessors, e.g. (Crane et al., 2022). In this context, a possible increased use of ascidian tunicates, as potential EDC effect models of choice, warrants current and future attention. Using ascidian embryos as a model system in EDC effect studies offers possibilities for sophisticated mechanistic and effect-oriented studies, even down to the single cell level. Ascidians also seem suitable for the development of embryo and larval based high-throughput screening bioassays for contaminant chemicals of emerging concern and chemical mixture assessments. However, far more research is needed to clarify how EDCs, through their interference with NR activities, may influence neurodevelopmental effect phenotypes in ascidians. Although many vertebrate hormone receptor paralogs have been identified in tunicates, the ligand-binding status of many of them (i.e., whether they are just expressed, or they can bind to ligands such as hormones and EDCs) are largely unknown at this time. It may thus be premature to conclude that *Ciona* can indeed serve as an alternative testing target for vertebrate EDCs (even for contaminants interfering with TR regulation mechanisms). The embryogenesis and reproduction pathways of tunicates themselves, however, are relatively better characterized, but the genes/proteins involved are not common to vertebrates (possibly except for GnRH). The development of standardized and validated methods for ED effect assays with ascidians that address non-EATS modalities are particularly needed. *Ciona* based test models for the assessment of EDC effects should also be structured into specific AOPs, and quantitative AOPs when possible. The availability of such AOPs will greatly simplify the targeting of future studies that address EDC effects in *Ciona,* i.e., focusing the design and performance of research and testing to the domains within the respective AOP model where the knowledge still is limited or absent. The new information should also be actively shared at the "Adverse Outcome Pathway Knowledge Base" (AOP-KB) (https://aopkb.oecd.org/index.html) as this website is the centralized portal for EDC toxicologists to share, develop and discuss AOP related findings, and with a goal of facilitating the development of more efficient decision-making tools for risk assessors and regulators of chemicals with putative ED modes of action. Lastly, since ascidians represent the group of animals closest related to vertebrate organisms, whilst not being considered a protected organism according to EU legislation, they can certainly represent a promising surrogate to vertebrate organisms in chemical toxicity testing. It is widely recognized that there is a significant lack of non-vertebrate test methods for marine taxa, particularly related to chronic toxicity assessments and early life stages. Hence, the use of ascidians for early life stage ecotoxicity testing would provide a more ethical alternative than the use of vertebrates such as fish.

CRediT author statement

J. Beyer: Conceptualization, literature collection and review, anatomic interpretation Fig. 1, original writing and editing in all parts of manuscript. Y. Song: Literature collection and review, original writing in neuroendocrine regulation part, original development of Table 1 and Fig. 5, editing all parts. A. Lillicrap: Language optimization and expert editing all parts. S. Rodríguez-Satizábal: Literature collection and review. M. Chatzigeorgiou: Providing visual materials for Figs. 1, fig. 2, & Fig. 4, expert editing and supervision in all parts of manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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