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Pathways of endocrine disruption in the larval development of the mediterranean mussel *Mytilus galloprovincialis*

Angelica Miglioli

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PhD thesis of the Università degli studi di Genova
and Sorbonne Université

Doctoral Schools of Marine Sciences (BIO/09-8702)
and Complexité du Vivant (ED515)

**Pathways of endocrine disruption in the larval development
of the mediterranean mussel *Mytilus galloprovincialis***

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Questa tesi è dedicata a mamma e papà

*« Dum taxat, rerum magnarum parva potest res
exemplare dare et vestigia notitiae »*

Tito Lucrezio Caro. De Rerum Natura, Liber II. I century a.C.

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Preface

This PhD project has been developed in the context of a collaboration between Prof. Laura Canesi, Team leader of the Environmental Physiology team, from the Università degli studi di Genova (IT) and PhD. Remi Dumollard, Team Leader of Ascidian BioCell Team in the Laboratoire de Biologie du Développement, based at the Institut de la mer de Villefranche (IMEV). Accordingly, the PhD project has been carried out in the form of a co-tutelle between the two institutions: Sorbonne Université and Università degli studi di Genova. Prof. Canesi's team is a pioneer in the study of invertebrate physiology and developmental biology by using the mediterranean mussel *M. galloprovincialis* as a model organism for the understanding of the physiological mechanisms of action of environmental pollutants on marine invertebrate populations. The Ascidian BioCell Team historically studies ascidian and invertebrate embryology and cell cycle although in recent years this main research line was added with a novel project aimed at understanding the impact of environmental contaminants on marine invertebrates by exploiting developmental biology and cell biology approaches. The collaboration was due to the common interest of the two teams in understanding and shedding more light on the issue of endocrine disruption in marine invertebrate organisms.

The current manuscript will develop in the following order. A summary of the PhD work will be initially reported. The introductory section will follow, and it is divided in three parts: the first addresses the topic of Endocrine Disrupting Chemicals, the second reports a literature review on the function of Nuclear Receptors in the development of marine invertebrates, and the third will introduce the mediterranean mussel *Mytilus galloprovincialis*, the model organism on which the study relied. Based on the problematics presented in the introduction, the aims and scopes of the thesis will be explained. The results of this PhD work are organised in 3 chapters that include research articles performed and published during the PhD. In the first Chapter, the process of *M. galloprovincialis* larval shell development is explained. The second chapter groups the research articles addressing the neuroendocrine effects of the model EDCs Bisphenol-A and Tetrabromobisphenol-A on the larval development of *M. galloprovincialis*. In the third chapter, the neuroendocrine aspects of endocrine disruption through Nuclear Receptors in *M. galloprovincialis* early larval development are investigated by exploiting the model EDC TBT and the Retinoid X Nuclear Receptor. After, the main results and their contribution to the current understanding of invertebrate endocrine disruption will be discussed and consequent future work perspectives proposed.

Abstract

Endocrine Disrupting Chemicals (EDCs) are a class of environmental contaminants of anthropogenic origin that, through the interference with any aspect of hormone action, affect organisms by disrupting their endocrine systems. The effects of this class of chemicals, and consequent definition, were in the first place characterised in vertebrate organisms as their endocrine as well as hormonal systems are historically well understood. Eventually, it was discovered that the most common mechanism of action of EDCs in vertebrates is to mimic endogenous hormones and compete with their Nuclear Receptors (NRs) as agonists or antagonists. However, the vertebrates represent only the ~5% of all the known species. Conversely, invertebrates consist of the remaining ~95% of all known animals and possess endocrine systems that can significantly vary across clades and phyla and differ from the one of vertebrates. Moreover, large groups of these species are aquatic and marine thus inhabiting the environment which represents the primary sink for any chemicals released by human activities, including EDCs. Aquatic and marine invertebrates are thus at high risk of exposure to EDCs. Nevertheless, the current knowledge regarding invertebrate endocrine disruption has not significantly advanced in the past two decades with respect to initial expectations. In fact, although there is key and accumulating evidence that EDCs impact on almost all groups of invertebrates, the lack of detailed description of their endocrine signalling pathways and Nuclear Receptors function prevents the understanding of endocrine disruption in these organisms. In this light, the research work presented in this thesis is meant to address the issue of endocrine disruption in marine invertebrates by exploiting the early larval development of the mediterranean mussel *M. galloprovincialis* as a model organism. The present work is, by applying an Adverse Outcome Pathway-AOP investigation rationale, an attempt to characterise the mechanism of action of selected model EDCs in mussel larvae and identify plausible pathways that could be linked to adverse biological outcomes. We first characterised the neuroendocrine elements regulating larval shell biogenesis and established the morphogenetic process as susceptible to neuroendocrine disruption. Consequently, the mechanisms of action of the model EDCs BPA and TBBPA in mussel larvae were investigated and the plausible neuro-/endocrine AOPs that could be linked to their adverse biological outcomes were identified. In addition, by studying and characterising the possible AOP of TBT in *M. galloprovincialis* larvae through the modulation of RXR activity, a plausible relationship between NR signalling and the neuroendocrine system in *M. galloprovincialis* larvae was established. The results of this thesis represent pivotal evidence that, put into a comparative approach, will help and push forward the current understanding of the mechanisms of endocrine disruption in invertebrate organisms.

Key words: *Mytilus galloprovincialis*, Early Larval Physiology, Invertebrate Endocrine Disruption, Neuroendocrine signalling, Nuclear Receptors

Résumé

Les Perturbateurs Endocriniens (PE) sont une classe de contaminants environnementaux d'origine anthropique qui, par interférence avec n'importe quel aspect de l'action hormonale, affectent les organismes en perturbant leur système endocrinien. Les effets de cette classe de produits chimiques, et la définition qui en découle, ont été en premier lieu compris et caractérisés chez les organismes vertébrés, car leurs systèmes endocriniens et hormonaux sont historiquement bien compris. Notamment, il a été découvert que le mécanisme d'action le plus courant des PEs chez les vertébrés consiste à imiter les hormones endogènes et à entrer en compétition avec leurs récepteurs nucléaires (NR) en tant qu'agonistes ou antagonistes. Cependant, les vertébrés ne représentent que ~5% de toutes les espèces connues. À l'inverse, les invertébrés représentent les 95 % restants de toute la biodiversité et possèdent des systèmes endocriniens qui peuvent varier considérablement selon les clades et les *phyla* et aussi être différent de celui des vertébrés. De plus, de grands groupes de ces espèces sont aquatiques et marines et habitent ainsi l'environnement qui représente le principal réservoir pour tous les produits chimiques libérés par les activités humaines, y compris les PEs. Les invertébrés aquatiques et marins courent donc un risque élevé d'exposition aux perturbateurs endocriniens. Néanmoins, les connaissances actuelles concernant la perturbation endocrinienne des invertébrés n'ont pas progressé de manière significative au cours des deux dernières décennies par rapport aux attentes initiales. En fait, bien qu'il existe des preuves clés et de plus en plus nombreuses que les perturbateurs endocriniens ont un impact sur presque tous les groupes d'invertébrés, le manque de compréhension détaillée de leurs voies de signalisation endocrinienne et de la fonction des récepteurs nucléaires empêche la compréhension de la perturbation endocrinienne dans ces organismes. Dans cette optique, les travaux de recherche présentés dans cette thèse visent à aborder la question de la perturbation endocrinienne chez les invertébrés marins en exploitant le développement larvaire précoce de la moule méditerranéenne *Mytilus galloprovincialis* comme organisme modèle. Le présent travail, en appliquant une logique d'investigation Adverse Outcome Pathway-AOP, a tenté de caractériser le mécanisme d'action de modèles sélectionnés d'EDC chez les larves de moules et d'identifier des voies plausibles qui pourraient être liées à des effets biologiques délétères. Nous avons d'abord caractérisé les éléments neuroendocriniens régulant la biogenèse de la coquille larvaire et établi le processus morphogénétique comme étant sensible à la perturbation neuroendocrinienne. Par conséquent, les mécanismes d'action des EDC modèles BPA et TBBPA chez les larves de moules ont été étudiés et les AOP neuro-/endocrines plausibles qui pourraient être liées à leurs effets biologiques indésirables ont été identifiées. De plus, en étudiant et en caractérisant l'AOP possible du TBT chez les larves de *M. galloprovincialis* via la modulation de l'activité RXR, une relation plausible entre la signalisation des NRs et le système neuroendocrinien chez les larves de *M. galloprovincialis* a été établie. Les résultats de cette thèse représentent des preuves essentielles qui, mises dans une approche comparative, aideront et feront avancer la compréhension actuelle des mécanismes de perturbation endocrinienne chez les organismes invertébrés.

Mots clés : *Mytilus galloprovincialis*, Physiologie larvaire précoce, Perturbation endocrinienne des invertébrés, Signalisation neuroendocrinienne, Récepteurs nucléaires

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Introduction

Part 1

Potential impacts of EDCs on marine invertebrates: facts and controversies.

This first part of the chapter aims at summarizing the current concepts of endocrine disruption with particular regards to aquatic invertebrate species and marine molluscs

1. Endocrine Disrupting Chemicals (EDCs): summary of consensus statements

Endocrine Disrupting Chemicals (EDCs) are man-made compounds originally designed and employed for pharmaceutical, agriculture and industrial purposes that nowadays represent a global-scale issue for both environmental and human health (Noguera-Oviedo & Aga, 2016). The potential risk related to this new class of pollutants has been complex to decipher and isolate. Their effects on biological systems were unprecedented so that the definition for these chemicals has been updated several times in the past decades in line with research advances and it is likely to be modified again (Yilmaz et al., 2020; Kassotis et al., 2020; Demeneix et al., 2020; Heindel and Blumberg, 2018; Zoeller et al., 2012). The term “endocrine disruptor” was first issued with the Wingspread Statement in the early 1990s relatively to the effects of EDCs on human and environmental health, but it was the Environmental Protection Agency (EPA) in 1996 that published the first detailed definition of what is EDC: *an exogenous agents that interfere with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behaviour* (Yilmaz et al., 2020). The Endocrine Society later reconsidered such description and released the definition of EDCs currently in use today: *“exogenous chemicals, or mixture of chemicals, that can interfere with any aspect of hormone action”* (Kassotis et al., 2020; Demeneix et al., 2020; Yilmaz et al., 2020). The class of EDCs is heterogeneous, diverse and estimated to count up to 1000 chemicals (Yilmaz et al., 2020). Compounds with ED properties can be inorganic and organic and are found among metals, industrial chemicals, plasticizers, nonylphenols, pharmaceuticals, pesticides, fungicides, phytoestrogens, flame retardants, paints, and cosmetics (Schug et al. 2016; Kabir et al. 2015; Bergman et al., 2012). Most of the organic EDCs are extremely persistent or pseudo-persistent, so that they are also considered among another class of environmental pollutants, the Persistent Organic Pollutants (POPs) (Bergman et al., 2012). Their persistence can be strictly due to their chemical properties or to the fact that they are also lipophilic and bioaccumulate in living organisms, thus having a very long half-life (Yilmaz et al., 2020). Due to the variety of EDC chemical structures, it has been especially problematic for States and Unions to establish specialized regulations and governmental investigation organs (Kassotis et al., 2020). For instance, only in the European Union, up to 3 distinct legislations (REACH/European Chemical Agency-ECHA for industrial chemical and biocidal products, the European Food Safety Authority-EFSA for pesticides and the Scientific Committee on Consumer Safety-SCCS for cosmetics) are in charge of screening and reporting potential EDCs. So far, the EU endocrine disruptors list includes 99 chemicals divided in substances identified as endocrine disruptors at EU level (List I, 18 chemicals), substances under evaluation for endocrine disruption

under an EU legislation (List II, 81 chemicals) and substances considered, by the evaluating National Authority, to have endocrine disrupting properties (List III, 8 chemicals also included in list I and II) (<https://edlists.org/the-ed-lists>). The substances identified as endocrine disruptors at EU level (List I) are presented in table 1.

Substances identified as endocrine disruptors at EU level						
Name and abbreviation	CAS no.	EC / List no.	Health Effects	Environmental Effects	Status	Regulatory Field
Benzyl butyl phthalate (BBP)	85-68-7	201-622-7	Yes		Health candidate list year: 2017	REACH
p-(1,1-dimethylpropyl)phenol	80-46-6	201-280-9		Yes	Environmental candidate list year: 2017 CoRAP list year: 2014	REACH
3-benzylidene camphor	15087-24-8	252-946-0		Yes	Environmental candidate list year: 2019	REACH
Cholecalciferol	67-97-0	200-673-2	Yes	Yes		Biocides
Bisphenol A	80-05-7	201-245-8	Yes	Yes	Health candidate list year: 2017 Environmental candidate list year: 2018	REACH
Reaction products of 1,3,4-thiadiazolidine-2,5-dithione, formaldehyde and 4-heptylphenol, branched and linear (RP-HP)	N/A	N/A		Yes	Environmental candidate list year: 2018	REACH
4-(1,1,3,3-tetramethylbutyl)phenol	140-66-9	205-426-2		Yes	Environmental candidate list year: 2011	REACH
Tris(4-nonylphenyl, branched and linear) phosphite (TNPP) with $\geq 0.1\%$ w/w of 4-nonylphenol, branched and linear (4-NP)	N/A	N/A		Yes	Environmental candidate list year: 2019	REACH
Bis(2-ethylhexyl) phthalate (DEHP)	117-81-7	204-211-0	Yes	Yes	Health candidate list year: 2014 Environmental candidate list year: 2017	REACH
4-(1,1,3,3-tetramethylbutyl)phenol, ethoxylated (group)	N/A	N/A		Yes	Environmental candidate list year: 2012 Environmental authorisation list year: 2019	REACH
Butylparaben	94-26-8	202-318-7	Yes		Health candidate list year: 2020 Commission EDC list year*: 2019	REACH, Cosmetics
Diisobutyl phthalate (DIBP)	84-69-5	201-553-2	Yes		Health candidate list year: 2017	REACH
4-heptylphenol, branched and linear (group)	N/A	N/A		Yes	Environmental candidate list year: 2017	REACH
Dicyclohexyl phthalate (DCHP)	84-61-7	201-545-9	Yes		Health candidate list year: 2018 CoRAP list year: 2017	REACH
4-Nonylphenol, branched and linear (group)	N/A	N/A		Yes	Environmental candidate list year: 2012	REACH
Dibutyl phthalate (DBP)	84-74-2	201-557-4	Yes		Health candidate list year: 2017	REACH
4-Nonylphenol, branched and linear, ethoxylated (group)	N/A	N/A		Yes	Environmental candidate list year: 2013 Environmental authorisation list year: 2019	REACH
4-tert-butylphenol	98-54-4	202-679-0		Yes	Environmental candidate list year: 2019 CoRAP list year: 2014	REACH

Table 1: List of the substances identified as endocrine disruptors at EU level (List I, <https://edlists.org/the-ed-lists>)

2. Endocrine disruption: what is known

2.1. The definition of “endocrine disruption” is largely vertebrate-centric

The first evidence of endocrine disruption was found in aquatic vertebrates such as mammals, amphibians and reptiles, for which a link between environmental pollution and population declines had been hypothesised already in 1988; but it was only with the publication of the book *Our Stolen Future* by Myers, Dumanoski and Colborn in 1996 that widespread attention was finally paid to the discovery of a new class of contaminants that was able to interfere with hormonal signalling at extremely low doses (Gercken, and Sordyl, 2002; Houlihan et al., 2000; Guillette et al., 2000; Willingham and Crews, 1999; Key, 1997; Bergeron et al., 1994; Woodward et al., 1993; Jennings et al., 1988; Brown and Scott, 1988). The book unleashed the interest and concern of the scientific community as it claimed that this new class of chemicals could threaten the survival of our species by proving that such chemicals were able to alter foetal development through this mechanism (Key, 1997). It follows that EDCs have been primarily characterised and studied with respect to their possible impact on human health and on aquatic vertebrates and have been consequently named after the primary target of their disruption: the endocrine system. Consequently, the concept of endocrine disruption is historically based on humans, vertebrates, and their endocrine systems.

2.2. The basics of Endocrine Disruption: lessons from vertebrates

In vertebrate-human biology, the term “endocrine” defines any internal secretion of endogenous signalling molecules into the systemic circulation of an organism (Bahadoran, Mirmiran, Azizi and Ghasemi., 2019). Such signalling molecules are made and released by the endocrine glands and tissues directly into the circulatory system so that they can exert their physiological effects by binding to specific receptors associated with their target cells (Bahadoran et al., 2019). In synthesis, the endocrine system comprises the ensemble of endocrine tissues and ductless glands, their secretions, and respective receptors in target cells that regulates animal physiology. Endocrine secretions release messenger bioactive molecules responsible for the endocrine signalling throughout the body and are conventionally, although simplistically, referred to as hormones (Bahadoran et al., 2019). In fact, the etymology of the word “hormone” derives from the Greek word for “setting in motion” (*ὁρμῶν*) and thus defines any member of a class of signalling molecules that is transported to distant organs and acts distant from its site of production to regulate physiology and behaviour (Bahadoran et al., 2019). For simplicity, from now on the word hormone will be used to define any endocrine tissue/gland borne messenger molecule. The human body includes five strictly endocrine glands (pituitary, thyroid, parathyroid, thymus, and adrenal glands) and other glands that contain endocrine tissue (i.e. adipose tissue, the pancreas, ovaries, and testes) that secrete specific hormones controlling a variety

of vital biological functions from development and growth to reproduction and behaviour (Garcia-Reyero, 2018; La Perle and Dintzis, 2018) (Fig. 1). As hormones are defined by their function, they can have very diverse chemical structures. Structurally, hormones are thus classified in Peptide (made of a chain of amino acids; i.e. insulin, thyroid hormones), Amino Acid (derived from amino acids such as tyrosine; i.e. melatonin, dopamine, noradrenaline and adrenaline), Steroid (derived from cortisol; i.e. the sex hormones Estradiol and Testosterone, Glucocorticoids) and Eicosanoid (derived from lipids; i.e. prostaglandins) hormones (La Perle and Dintzis, 2018; Heyland, Hodin and Reitzel, 2005). Moreover, some hormones, in particular the amino acid hormones, are further defined as neuro-hormones as they can also have a role of neurotransmitters and are made by neurosecretory cells which are part of, so-called, neuroendocrine glands, such as the Hypothalamic and Adrenal glands, and compose the neuroendocrine system (Toni, 2004). In fact, the endocrine system does not regulate the body's internal activities alone, but in association with the nervous system as well as the immune system (Galoyan, 2000; Toni, 2004). Finally, hormones transmit and operate the biological functions of the endocrine system thus being key regulator of almost every aspect of the biology of an organism via their action in target tissues. Accordingly, hormonal balance (the physiological equilibrium between synthesis, degradation, transport and circulating levels of hormones) is essential to animal life and its deregulation leads to detrimental repercussions on the endocrine, nervous and immune system leading to endocrine gland dysfunctions, life-threatening pathologies, infertility, developmental and neuronal disorders, immunodepression, as well as transgenerational effects (Ruggeri et al., 2018; Asa and Mete, 2018).

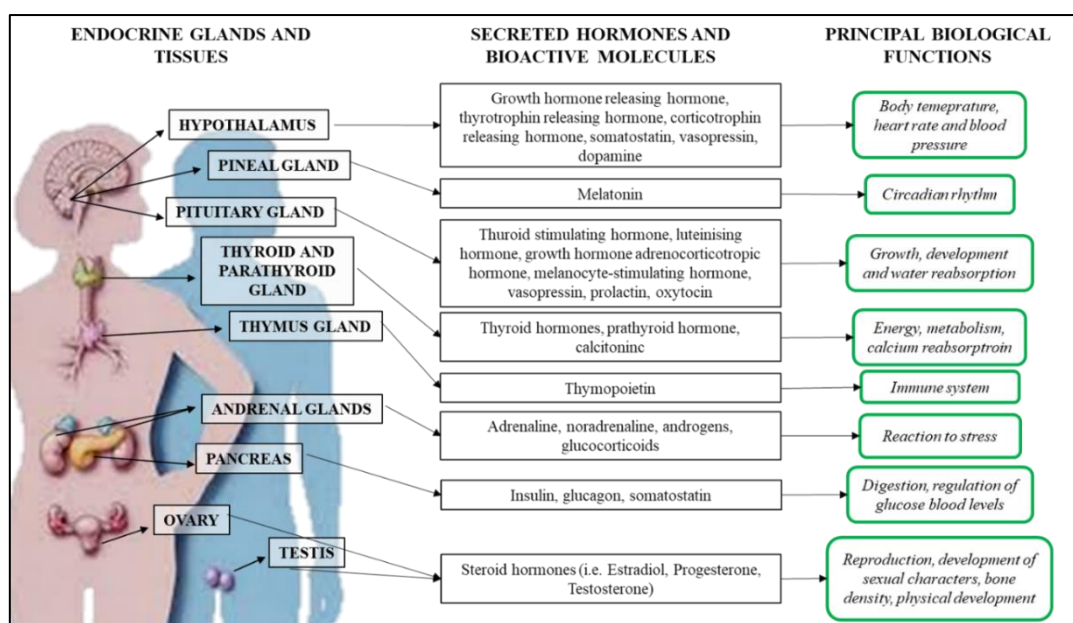


Figure 1: Schematic representation of the principal endocrine glands, hormones, and their functions in the human body.

On that account, ten key characteristics of EDCs have been recently identified and published in a *consensus* statement; each one is relative to aspects of hormones action (Fig. 2, from La Merrill et al., 2020). Endocrine Disrupting activity of a chemical is thus narrowed to a specific form of toxicity which is triggering adverse health effects by disrupting the endogenous hormone system (Solecki et al., 2017). Finally, EDCs operate by affecting hormone metabolism and circulating level, as well as by jeopardizing their specific activity in target-tissues by acting as agonists and antagonists of their receptors (Yilmaz et al., 2020) (Fig. 2).



Fig. 2: The ten specific key characteristics (KCs) of endocrine-disrupting chemicals (EDCs).

KC1: an EDC can interact with or activate hormone receptors.

KC2: an EDC can antagonize hormone receptors.

KC3: an EDC can alter hormone receptor expression.

KC4: an EDC can alter signal transduction in hormone-responsive cells.

KC5: an EDC can induce epigenetic modifications in hormone-producing or hormone-responsive cells.

KC6: an EDC can alter hormone synthesis.

KC7: an EDC can alter hormone transport across cell membranes.

KC8: an EDC can alter hormone distribution or circulating hormone levels.

KC9: an EDC can alter hormone metabolism or clearance.

KC10: an EDC can alter the fate of hormone-producing or hormone-responsive cells. Taken from La Merrill et al., 2020.

However, due to the presence of the neuroendocrine system, whose hormones are neuropeptides and neurotransmitters, a novel subclass of EDCs has been also defined: the neuroendocrine disruptors (NEDCs) (León-Olea et al., 2014; Trudeau, Kah and Bourguignon, 2011). NEDCs were at first described as environmental pollutants that are capable of acting as agonists/antagonists or altering the synthesis and/or metabolism of neuropeptides, neurotransmitters, or neurohormones, which subsequently affect diverse physiological, behavioural, or hormonal processes to affect an animal's capacity to reproduce, develop and grow, or deal with stress and other challenges (Trudeau, Kah and Bourguignon, 2011). However, the definition of NEDCs was narrowed to any pollutant exerting specific effect on well-defined neuroendocrine cells, thus diverging from the *consensus* general definition of EDCs which is hormones based (Olea et al., 2014).

2.3. EDCs mimic hormones and compete for the binding to Nuclear Receptors

Among the ten key characteristics, the most common mechanism of action of EDCs is to mimic endogenous hormones and compete with their receptors as agonists or antagonists (Toporova and Balaguer, 2020; La Merrill et al., 2020). The main hormone receptors targeted by EDCs are Nuclear Receptors (NRs), a superfamily of phylogenetically related transcription factors present in all metazoans that regulate gene transcription either constitutively or upon the binding of a ligand, which, for some of them, is a hormone (Escriva et al., 2004). Hormone activated NRs (HNRs) have thus the unique ability to control the endocrine signalling at the gene expression level, so that they are named after their hormone ligands. In a similar fashion, HNR-acting EDCs can be qualitatively catalogued depending on the hormones whose signalling they disrupt, or the biological effect induced. For instance, the EDCs affecting Estradiol and Estrogen NR (ER) activity are called xeno-estrogens and include compounds such as the plasticizers Bisphenol-A (BPA) and Phthalates, the synthetic estrogen ethinylestradiol employed in birth control pharmaceuticals, and Polybrominated Biphenyls-PCBs once employed in coolant fluids. Exposure to xenoestrogens has been linked to reproduction failure, male infertility, cancer and transgenerational defects (Dickerson and Gore, 2007). EDCs affecting testosterone and the androgen NR (AR) signalling such as Tributyltin (TBT), Triphenyltin (TPT) are called xeno-androgens; these chemicals were linked to female masculinisation and reproduction failure throughout vertebrates and invertebrates (Micael et al., 2007). EDCs affecting the thyroid system, hormones and NR (THR) are called thyroid disrupting chemicals (TDC) or thyromimetics, and include PCBs, BPA, Tetrabromobisphenol-A (TBBPA), Phthalates, Polybrominated Diphenyl

Ethers (PBDEs) and pesticides such as the Dichlorodiphenyltrichloroethane (DDT); this class of EDCs is known to affect amphibian metamorphosis and thyroid function in vertebrates (Calsolaro et al., 2017). On the other hand, the novel EDC class of Obesogens groups all the xenobiotics able to disrupt lipid homeostasis and metabolism, generally via the Peroxisome proliferator-activated NR (PPAR) and Retinoid X NR (RXR); obesogens have been linked to metabolic syndromes, diabetes and obesity (Heindel and Blumberg, 2019). However, this type of classification can create ambiguities as some EDCs such as BPA, PCBs, organotins belong to more than one category. In fact, another characteristic of EDCs in vertebrate organisms is their pleiotropic effect on endocrine systems (Li and Gramatica, 2010).

3. EDC pollution and Marine Invertebrates

3.1. EDCs in the aquatic and marine environment

Endocrine-disrupting compounds are involved in the water compartment contamination of both rivers and lakes as well as marine waters (Pironti et al., 2021). Similarly to any environmental pollutant, EDCs enter the aquatic environment from land anthropogenic activities in the form of sewage effluents, industrial waste and land run off and concentrate in rivers and lakes which represent the primary sink for any chemicals released by human activities (Katsiadaki, 2019; Ahmed et al. 2017; Bergman et al., 2013). EDCs can thus reach high concentrations in rivers and lakes and ultimately reach the marine coastal waters, their final reservoir, in which they dilute (Pironti et al., 2021; Bergman et al., 2013). The marine environment is also additionally impacted by the potential EDCs input from harbours, fisheries, aquaculture, tourism, and excavations practices (Pironti et al., 2021; Katsiadaki, 2019; Bergman et al., 2013). It follows that aquatic species are more exposed to EDCs than the terrestrial ones and at a considerably higher risk (Pironti et al., 2021; Ahmed et al. 2017; Bergman et al., 2013). It is thus not surprising that, as described in previous sections, some of the very first evidence of the effects of EDCs was found in aquatic species (Cuvillier-Hot and Lenoir, 2020; Katsiadaki, 2019; Fernandez et al., 2019; Gercken, and Sordyl, 2002; Houlahan et al., 2000; Guillette et al., 2000; Willingham and Crews, 1999; Key, 1997; Bergeron et al., 1994; Woodward et al., 1993; Jennings et al., 1988; Brown and Scott, 1988). Although research efforts and investigations on the effect of EDCs on aquatic species initially focused on vertebrate organism, it was soon clear that these chemicals represent a threat also to aquatic invertebrates, among which molluscs provided the best documented examples of endocrine disruption (Cuvillier-Hot and Lenoir, 2020; Katsiadaki, 2019; Fernandez et al., 2019).

3.2. Effects of EDCs on marine invertebrates

Invertebrates represent more than 95% of the known species in the animal kingdom fulfilling many crucial ecological roles in every ecosystem, and large groups of these species are marine (Wilson, 2006). Moreover, the marine environment is the final reservoir of any kind environmental pollutant, including EDCs (Pironti et al., 2021). It follows that studying the issue of endocrine disruption in marine invertebrate species is meaningful and requires to be handled urgently (Cuvillier-Hot and Lenoir, 2020). Due to their worldwide economic relevance in aquaculture and coastal distribution, the relationships between EDCs and population declines of marine invertebrates were first observed in molluscs following the contamination of the organotin TBT and TPT, historically employed as biocides in the antifouling paints of boats (Horiguchi et al., 2005; Ellis et al., 1990). The effects of these EDCs were especially tremendous in the case of the basin of Arcachon, where together with molluscs also many other invertebrate populations collapsed (Ruiz et al., 1996; Alzieu et al., 2000). The most evident biological effect of organotin in molluscs is female masculinisation in gastropods which occurs in the forms of the imposex phenotype (superimposition of male organs on females), intersex (the shift of the pallial organs towards male morphological structures) and ovo-testis (suppressed oogenesis and/or occurrence of spermatogenesis), accompanied by infertility and consequent lower rates of reproduction (Fernandez et al., 2019; Titley-O'Neal et al., 2011). With respect to intersex and ovo-testis, the imposex has received a lot of attention since it is an external, evident phenotype, that does not require animal sacrifice, and was described for over 268 species of gastropods (Titley O'Neal et al., 2011) (Fig 3). Although with some reserves, the occurrence of the imposex phenotype in gastropods is now considered a marker of organotin pollution (Fernandez et al., 2019; Titley O'Neal et al., 2011). However, this biomarker is strictly limited to adult specimens of a specific class of molluscs and a single type of EDC whose production is now banned almost worldwide. Apart from gastropod molluscs, Organotins were shown to affect several other big groups of invertebrates in marine waters including echinoderms, annelids, arthropods, bivalve molluscs and crustaceans (Fernandez, 2019). In addition, a long series of other EDCs such as herbicides (i.e. atrazine, diuron), metals (i.e. mercury, cadmium), insecticides (i.e. DDT, Endrin), PCBs and natural/synthetic vertebrate steroids (i.e. diethylstilbestrol, testosterone) were also documented to be capable of affecting natural populations of a variety of marine invertebrates (Cuvillier-Hot and Lenoir, 2020; Fernandez, 2019).



Figure 3: Morphology of the gastropod mollusc *Buccinanops globulosus*. 1) *B. globulosus* male; 2) normal female; 3) imposex-affected female. p: penis, dg: digestive gland, ag: albumen gland, cg: capsule gland, h: head, f: foot. Adapted from Giulianelli et al., 2020.

In addition, EDCs can affect multiple developmental and life stages other than adulthood as well as causing distinct effects across species of the same *phylum*. Using again Organotin and molluscs as example, the bivalve population of Arcachon bay collapsed because TBT and TPT affected the development and shell growth of oyster larvae while adult female oyster did not show any sign of masculinisation or non-fecundity (Alzieu, 2000). It follows that the actual impact of EDCs on marine invertebrates could be largely underestimated, especially when their biological effects are not shown in the form of evident phenotypes or at distinct developmental stages.

3.3. Canonical Endocrine Disruption in marine invertebrates: facts and controversies

In line with the information presented in previous paragraphs and the most recent *consensus* publications regarding EDCs, it is cautious to state that the interference with the hormonal system is the *conditio sine qua non* for Endocrine Disrupting Chemicals and Endocrine Disruption to be defined as such (La Merrill et al., 2020). This vertebrate hormones-centric conceptualisation of EDCs makes it particularly difficult to consistently apply the *consensus* guidelines to the observed effects of these chemicals in invertebrate organisms (Cuvillier-Hot and Lenoir, 2020; Ford and Le Blanc, 2020; Fernandez, 2019; Katsiadaki, 2019). In fact, although almost all known EDCs have proved to affect a variety of invertebrates it is still not understood to which level the observed adverse effects are due to interference with neuro-/endocrine or hormonal pathways (Katsiadaki, 2019). Eventually, the knowledge regarding invertebrate hormones and endocrine systems is, to some level, present for arthropods and chordates but almost non-existent for all the rest of invertebrates which represent the majority of the animal kingdom and would thus require copious dedicated research (Ford and Le Blanc, 2020; Fernandez et al., 2019; Castro and Santos, 2014). Substantially, the lack of detailed comprehension of the endocrine signalling pathways in disregarded groups of invertebrates is the

main gap of knowledge regarding endocrine disruption in these organisms (Cuvillier-Hot and Lenoir, 2020; Castro and Santos, 2014; Hutchinson, 2007). Invertebrates are eventually not considered to possess a canonical endocrine system, but only the neuroendocrine system that exerts a variety of regulatory and developmental functions through the secretion of neuropeptides and neurotransmitters (Joyce and Vogeler, 2018; Malagoli and Ottaviani, 2017). A neurosecretory/neuroendocrine system appeared very early in metazoan history so that all invertebrates possess neuroendocrine cells releasing neuropeptides in the haemolymph or coelomic fluid; moreover, in many invertebrates such as polychaetes and molluscs, many neurons, although not organised in a gland-like structure, were proved to be neurosecretory cells (Hartenstein, 2006; Tessmar-Raible, 2007) (Fig. 4). However, it has been pointed out by distinct authors in invertebrates neuroendocrinology that, despite general similarities, it is difficult to compare possibly corresponding neurosecretory systems between the adult nervous systems of invertebrates, as neurons can undergo long migrations during development, which makes a comparison based on their adult position hardly meaningful, suggesting that the study of such system should be done by comparing species at early developmental stages (Hartenstein, 2006; Tessmar-Raible, 2007).



Fig. 4: Position of neurosecretory cells and neurosecretory regions in the «forebrain» of adult gastropod molluscs and polychaete annelids. The nervous system is marked in yellow; neurosecretory regions in green; selected neurosecretory neurons in blue, blood vessels in red. Adapted from Tessmar-Raibler, 2007.

Accordingly, it could be argued that endocrine disruption in invertebrates is largely neuroendocrine. Although Neuroendocrine Disruption started to be discussed only in recent years, most EDCs have already been found to be also NEDCs in vertebrate models. However, the current definition of NEDCs, together with the scarce knowledge and difficulty related to invertebrate neuroendocrinology, makes it difficult to theorize and define what could be evidence of neuro-/endocrine disruption in these organisms. Consequently, the study of developmental invertebrate neuroendocrinology, together with the characterisation of NEDC actions could be essential for the understanding of the true “endocrine” impact of EDCs on marine invertebrates (León-Olea et al., 2014; Trudeau, Kah and Bourguignon, 2011). Nevertheless, the scientific community eventually searched for alternative elements of investigation that could serve the purpose of studying the effect and assessing the risks related to EDC pollution in invertebrates and initiated a new discipline based on shared molecular pathways susceptible to EDCs regardless of their possible involvement in the neuroendocrine system of invertebrates, this new discipline takes the name of Invertebrate Molecular Disruption (IMD) (Cuvillier-Hot and Lenoir, 2020).

3.4. Invertebrate Molecular Disruption (IMD): Invertebrate Nuclear Receptors respond to EDCs

IMD takes advantage of our best knowledge regarding modes of action of EDCs in vertebrate physiology and transposes it to invertebrates, focusing on the shared molecular pathways (Cuvillier-Hot and Lenoir, 2020). However, as already explained in the previous paragraph, invertebrate neuro-/endocrinology is poorly described; moreover, most vertebrate hormones are unique to the subphylum and when detected in invertebrate animals they are likely to be of exogenous origin, thus not fitting within the definition of hormone (Katsiadaki, 2019). On the contrary, NRs, the main mode of action of EDCs in vertebrate organisms, are present in all metazoan clades, including invertebrates (Toporova and Balaguer, 2020; Castro and Santos, 2014; Escriva et al., 2004). Consequently, IMD firstly investigated the possible modes of EDCs in invertebrates through these transcription factors. Invertebrate NRs have consequently received increasing attention and turned out to be efficient tools to screen potential EDCs, at least, for a few classes of invertebrates and of NRs (Cuvillier-Hot and Lenoir, 2020) (Fig. 5).



Fig 5: From Cuvillier-Hot and Lenoir, 2020 (Fig. 1): NRs susceptible to EDCs in invertebrates. In blue the ones shared with vertebrates, in light blue those specific to arthropods. Outer coloured rings indicate invertebrate groups. Hatched rings mean that the NR does not respond to ligands, the question marks highlight that the NR is present in the group, but the activity needs to be confirmed. Under each NR are listed the respective EDCs suspected to act the same in the invertebrate groups mentioned (IGD, insect growth disruptors; BPA, bisphenol A; BPS, bisphenol S; NP, nonylphenol; DEHP, di-ethylhexyl phthalate; BBP, benzyl butyl phthalate; E2, 17 β -estradiol; EE2, 17 α -ethinylestradiol; TBT, tributyltin; TPT, triphenyltin).”

Emblematic, once again, is the case of Organotin. Organotin are obesogenic EDCs in vertebrates as they disrupt the NRs PPAR and RXR, which regulate lipid metabolism (Heindel and Blumberg, 2019). The same NRs have been proved to mediate, at least in part, the effect of Organotin in gastropods, thus being involved in the insurgence of the imposex phenotype and female infertility. However, this remains, for now, the only IMD case in which an invertebrate NR disruption leads to evident biological effects of physiological relevance through a conserved NR pathway with vertebrates (Katsiadaki, 2019). The remaining evidence of IMD in invertebrate organisms based on vertebrate studies has been mostly assessed with *in vitro* tests for which the biological relevance is not obvious (Knigge, Le Blanc and Ford, 2021). This is because, apart from arthropods, NRs pathways and functions are not known and necessarily shared in invertebrates and vertebrates. It remains though out of any doubt that this is the approach that permitted to better identify EDCs and refine our knowledge on their mode of action in invertebrate species, as well as set up molecular *in vitro* tests for EDCs in invertebrates that can influence decision makers and environmental law (Cuvillier-Hot and Lenoir, 2020; Castro and Santos, 2014). Still, remains the question of which are the biological and endocrine implications of molecular endocrine disruption *via* NRs in marine invertebrates.

3.5. The Adverse Outcome Pathway approach in invertebrate EDC research: linking molecular pathways of disruption to biological effects

A survey of the research progress regarding the issue of endocrine disruption in invertebrates has been recently published and, sadly, the results indicate that in the last 20 years the research has not

advanced as vigorously as expected (Ford and Le Blanc, 2020). However, the survey also pointed out novel directions and research approaches that could nowadays be promising, one of which is the application of the Adverse Outcome Pathway (AOP) concept (Ford and Le Blanc, 2020; Allen et al., 2014). The AOP is defined as the sequence of events that from the exposure of an individual to a chemical, lead to its adverse effect at the individual or population level (OECD, 2012). Each AOP begins with a molecular initiating event (MIE) in which a chemical interacts with a biological target leading to a first cellular response which induces a sequential series of higher order effects at the organ and the organism level to produce an adverse biological outcome (Fig. 6 from Ankley et al., 2010). The first three steps described in Fig. 6 denote the parameters that define a toxicity pathway, while the last two steps are indicative of the effects on individuals that could lead to loss of population sustainability (Ankley et al., 2010; NRC, 2007). Accordingly, the application of the AOP approach could unravel plausible mechanisms of disruption by invertebrate endocrine-disrupting chemicals along with their effects on individuals that could lead to declines of natural populations (Ford and Le Blanc, 2020).



Fig 6: Conceptual diagram of key features of an adverse outcome pathway (AOP) from Ankley et al., (2010).

However, AOPs need a molecular initiating event (MIE) for which the knowledge regarding the chemistry of the molecule is essential in order to search for its possible interacting receptor/process in the test organism. Accordingly, the survey from Ford and Le Blanc also points at a biological target discovery (BTD) approach to identify and understand the targets of endocrine disruption (i.e. NRs signalling) in various invertebrate phyla by comparative analysis of their ecological-evolutionary and developmental biology (Ford and Le Blanc, 2020). In the context of marine invertebrates, it has been pointed out in a recent review on the topic that the applicability of the current AOPs should be

expanded with the priority to cover marine diversity in line with the discovery and characterisation of the pathways involved in neuro-endocrine signalling in these organisms (Katsiadaki, 2019). So far, the few elements that were identified as plausible MIE are to be found with the molecular approach and are thus the Nuclear Receptors (Cuvillier-Hot and Lenoir, 2020). It is thus essential to characterize the activity and possible neuro-endocrine function of these transcription factors in invertebrates as well as the neuro-endocrine system in its globality in order to describe and be able to recognise signs of Endocrine Disruption in invertebrates.

3.6. Marine invertebrate larvae can shed light on invertebrate endocrine disruption

Marine invertebrate larvae and early life stages have long been used as experimental models in various disciplines and not exclusively related to environmental issues (Ortega and Olivares-Bañuelos, 2020; Wilson-Sanders, 2011). Nowadays, larval development of a number of marine invertebrates from distinct *phyla* is also exploited by standard embryotoxicity tests designed by the International Organization for Standardization - ISO (www.iso.org) and ASTM international (www.astm.org). The main reason is that marine invertebrate larvae meet almost all the characteristics of a good experimental model organisms: they are unexpensive, easy to obtain from adult specimens, they develop fast, they are easy to grow in laboratory conditions, their exploitation for research purposes is not affected by ethical restrictions and the collection of ripening adult specimens from the environment does not generally risks to depauperate natural populations (Lewis et al., 2012; Love, 2009; Ségalat, 2007). Moreover, marine invertebrate larvae are proportionally more sensitive to environmental pollutants and stressors, including EDCs, with respect to their adult stages (Przeslawski, Byrne and Mellin, 2015). Regarding the particular context of endocrine disruption research, marine invertebrate larvae have already been pointed out in previous paragraphs to be the most suitable model for the characterisation of the invertebrate neuro-endocrine system, due to the variety of body plans and morphologies characterising adult stages (Tessmar-Raible, 2007). In addition to that, embryonic and post-embryonic development are the most dynamic periods of NR activity and signalling in vertebrates, carrying out a variety of distinct morphogenetic and developmental processes that are also required, at least in part, in the development of all metazoans, including marine invertebrates (i.e., neurogenesis, lipid metabolism, growth) (Chung and Cooney, 2003; Erwin, 1993). Finally, the fast larval morphogenesis and early developmental transitions of marine invertebrates could serve as potent endpoints of adverse effects of EDCs in the context of the AOP application and establish neuro-endocrine related biomarkers specific to invertebrates (Ford and Le Blanc, 2020; Carrier, Reitzel and Heyland, 2018).

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Part 2

Nuclear Receptors and development of Marine Invertebrates

Miglioli A, Canesi L, Gomes I. D. L., Schubert M., Dumollard R.

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Based on the information presented in the first section of the introduction, this following part is aimed at summarizing what is known regarding the role of Nuclear Receptors in the development of marine invertebrates and the possible limitations relative to the use of these transcription factors as molecular tools for the study of invertebrate endocrine disruption.



Review

Nuclear Receptors and Development of Marine Invertebrates

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Abstract: Nuclear Receptors (NRs) are a superfamily of transcription factors specific to metazoans that have the unique ability to directly translate the message of a signaling molecule into a transcriptional response. In vertebrates, NRs are pivotal players in countless processes of both embryonic and adult physiology, with embryonic development being one of the most dynamic periods of NR activity. Accumulating evidence suggests that NR signaling is also a major regulator of development in marine invertebrates, although ligands and transactivation dynamics are not necessarily conserved with respect to vertebrates. The explosion of genome sequencing projects and the interpretation of the resulting data in a phylogenetic context allowed significant progress toward an understanding of NR superfamily evolution, both in terms of molecular activities and developmental functions. In this context, marine invertebrates have been crucial for characterizing the ancestral states of NR-ligand interactions, further strengthening the importance of these organisms in the field of evolutionary developmental biology.

Keywords: nuclear receptors; development; marine invertebrates; evolutionary developmental biology



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1. Introduction

Nuclear receptors (NRs) are a superfamily of phylogenetically related transcriptional regulators and act as activators or repressors of gene transcription, either constitutively or depending on the binding of a ligand [1,2]. NRs are characterized by a conserved structural organization, comprising a variable N-terminal region (A/B domain), a DNA-binding domain (DBD, C domain), a hinge region (D domain), a ligand-binding domain (LBD, E domain) and a C-terminal domain (Figure 1) [3].

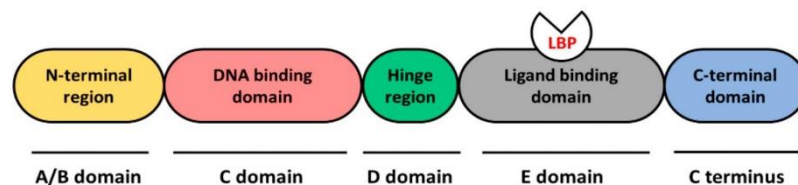


Figure 1. General structure of nuclear receptor (NR) proteins. Different domains of the NR protein are shown in different colors. A schematic representation of the ligand-binding pocket (LBP) is included in the ligand binding domain.

The LBD permits ligand binding, mostly through Van der Waals interactions and hydrogen bonds of specific amino acid residues in the ligand-binding pocket (LBP) [4]. The DBD is the region of the NR that mediates interactions with DNA and hence controls the specificity of the transcriptional response. NRs bind DNA at so-called response elements

(REs), specific sequences located in the *cis*-regulatory regions of target genes [5]. REs consist of direct, inverted or palindromic repetitions of a core consensus motif separated by a variable number of nucleotides that are bound by NRs as monomers, homodimers or heterodimers [6]. Efficient dimeric complexes are formed in vivo through cooperative protein-protein and protein-DNA interactions [7]. While protein-protein dimerization interfaces are found in the DBD, the LBD and the hinge region of NRs, REs serve as NR dimerization sites on DNA [8–10].

NRs are divided into two main classes: NRs with known ligands and NRs, for which ligands do not exist or have yet to be identified, the so-called orphan receptors [11,12]. Recent phylogenetic analyses of DBD and LBD sequences defined nine NR subfamilies (Table 1), which have probably originated from a single ancestral NR [2,12–16].

Table 1. Nuclear receptor (NR) complements characterized in metazoan genomes.

Subfamily	Group	Name	NRNC Symbol	Abbreviation	Physiological Ligand
# *	A	NRs with two DBDs	NR#A1	2DBD-NR α	
	B		NR#B1	2DBD-NR β	
	C		NR#C1	2DBD-NR γ	
	D		NR#D1	2DBD-NR δ	
0	A *	Zygotin gap protein	NR0A1	KNI	x
		Zygotin gap protein- related	NR0A2	KNRL	x
		Egon	NR0A3	EG	x
		ODR-7	NR0A4	ODR-7	x
		Trithorax	NR0A5	TRX	x
	B	Dosage-sensitive sex reversal-adrenal hypoplasia congenital critical region on the X chromosome, gene 1	NR0B1	DAX1	x
		Small heterodimer partner	NR0B2	SHP	x
	A	Thyroid hormone receptor	NR1A1,2	THR α,β	T3
	B	Retinoic acid receptor	NR1B1-3	RAR $\alpha-\gamma$	All- <i>trans</i> -RA
	C	Peroxisome proliferator-activated receptor	NR1C1-3	PPAR $\alpha-\gamma$	Fatty acids, Prostaglandins
D	Rev-ErbA	NR1D1,2	Rev-ErbA α,β	x	
E *	Ecdysone-regulated E78 gene	NR1E	E78		
F	RAR-related orphan receptor	NR1F1-3	ROR $\alpha-\gamma$	x	
		NR1F4	HR3		
G *	CNR14-like	NR1G1	Sex-1	x	
1	H	Liver X receptor-like	NR1H1 *	EcR	Ecdysteroids
			NR1H2,3	LXR α,β	Oxysterols
			NR1H4,5	FXR α,β	x
	I	Vitamin D receptor-like	NR1I1	VDR	Vitamin D
			NR1I2	PXR	Xenobiotics
			NR1I3	CAR	Androstane
	J *	NHR96	NR1J1	DHR96	
	K *	VDR/PXR α,β	NR1K1,2	VDR α,β -like	
	L *	HNR-like 97	NR1L	HR97	
	M *	HNR-like 19	NR1M1	HR10	
N *	HNR-like 11	NR1N1	HR11		
O *		NR1O			
P *		NR1P1-11			
2	A	Hepatocyte nuclear factor 4	NR2A1-3	HNF4 α,γ	Fatty acids
			NR2A4 *	HNF4	

Table 1. Cont.

Subfamily	Group	Name	NRNC Symbol	Abbreviation	Physiological Ligand	
	B	Retinoid X receptor	NR2B1-3	RXR α - γ	x	
			NR2B4	USP	x	
	C	Testicular receptor	NR2C1	TR2	x	
			NR2C2	TR4	x	
D *		DHR78	NR2D1	HR78		
E	Tailless / Photoreceptor cell-specific nuclear receptor		NR2E1	TLX	x	
			NR2E2 *	TLL	x	
			NR2E3	PNR/HR51 *	x	
		Dissatisfaction nuclear receptor *	NR2E4	DSF		
		Nuclear hormone receptor FAX-1 *	NR2E5	FAX1		
F	Chicken ovalbumin upstream promoter transcription factor		NR2F1,2	COUP-TF1,II	x	
		Seven-up *	NR2F3	SVP		
		Chicken ovalbumin upstream promoter transcription factor III *	NR2F4	COUP-TFIII		
		Seven-up related protein 46 *	NR2F5	SVP-46		
			NR2F6	EAR-2	x	
			NR2F6	EAR-2	x	
A		Estrogen receptor	NR3A1,2	ER α , β	Estradiol	
B	Estrogen-related receptor		NR3B1-3	ERR α - γ	x	
			NR3B4 *	ERR	x	
3	C	Steroid receptor / Ketosteroid receptors	NR3C1	Glucocorticoid receptor, GR	Cortisol	
			NR3C2	Mineralocorticoid receptor, MR	Aldosterone	
			NR3C3	Progesterone receptor, PR	Progesterone	
			NR3C4	Androgen receptor, AR	Testosterone	
D *		Estrogen receptor-like in Protostomia	NR3D	ER-like		
E *		Estrogen receptor-like in Cnidaria	NR3E	ER-like		
F *		Estrogen receptor-like in Placozoa	NR3F	ER-like		
4	A	Nerve growth factor 1B	NR4A1	NGF1B	x	
			Nuclear receptor related 1	NR4A2	NURR1	x
			Neuron-derived orphan receptor 1	NR4A3	NOR1	x
		DHR38 *	NR4A4	HR38		
5	A	Steroidogenic factor 1	NR5A1	SF1	Phosphatidylinositols	
			Liver receptor homolog-1	NR5A2	LRH1	Phosphatidylinositols
			NHR FTZ1- α *	NR5A3	FTZ1- α	
B *		NHR39/FTZ1- β	NR5B1	HR39		
6	A	Germ cell nuclear factor	NR6A1	GCNF	x	
			HR4 *	NR6A2	HR4	
7/8 *	A		NR7/8A1			

Non-vertebrate NR subfamilies and receptors are indicated by (*). Physiological ligands refer to in vivo conditions in vertebrates and the (x) highlights orphan NRs [12–26]. NRs with two DBDs are listed as (#), because they do not yet have an unequivocal subfamily status. Like NR0s, NRs with two DBDs do not have the conventional NR structure as shown in Figure 1. The NR7/8 subfamily has been called either NR7 or NR8 [14,22,24]. When information is absent or disputed, cells are left empty.

NR genes have been found in all extant metazoan taxa, but not in fungi, plants or unicellular eukaryotes, suggesting that NRs originated at the base of the metazoans [1,2]. NR phylogenies have indicated that the ancestral receptor might have been a ligand-activated receptor, with fatty acids as possible ligands [15,16]. In the course of evolution, the NR superfamily has experienced a complex pattern of gene expansions that led to the current diversity of metazoan NRs (Figure 3) [17–24,27].

Genomes of placozoans contain members of NR subfamilies 2 and 3, suggesting that a first wave of NR gene expansion occurred relatively early in metazoan evolution [20,21,29,30]. The NR complement of cnidarians indicates that the diversification of the NR2 and the appearance of the NR6 and NR7/8 subfamilies predate the split of the cnidarian and bilaterian lineages (Figure 3) [20,27,30]. Another NR expansion occurred very early in bilaterian evolution, leading to the appearance of all extant NR subfamilies and an estimated complement of about 25 NR genes (Figure 3) [1,15,27]. The NR superfamily subsequently experienced lineage-specific expansions. The NR3C receptors, for example, arose at the base of the chordates and whole genome duplications further expanded the NR complement in the vertebrate lineage (Table 1, Figure 2) [31–33]. A specific lineage-specific duplication has further been reported in cephalochordates, whose genomes encode 10 NR1H receptors (Figure 2) [14]. Similarly, in nematodes, the orphan NR HNF4 experienced a lineage-specific burst of duplications (Figure 2) [34].

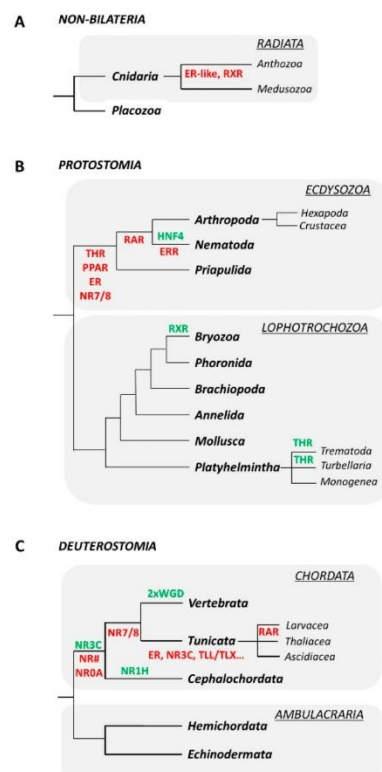


Figure 2. Examples for nuclear receptor (NR) duplications and losses during metazoan evolution. (A) Non-bilaterians. (B) Protostomes. (C) Deuterostomes. Gene expansions are shown in green and gene losses are shown in red. WGD: Whole genome duplications. Animal phylogenies are based on Laumer et al. (2019) [28]. NR nomenclature is as defined in Table 1.

to pathophysiological and pathological conditions, such as neurodevelopmental disorders and developmental dysfunction/arrest in all vertebrate taxa [42,43]. It was initially thought that invertebrates are not affected by EDCs as they were said to lack an endocrine system similar to that of vertebrates [44,45]. However, it is now known that invertebrates are extremely sensitive to EDC exposure, in particular during their embryonic and post-embryonic development [44–46]. For this reason, a specific set of NRs, including thyroid hormone (THR), retinoic acid (RAR), retinoid X (RXR) and estrogen (ER, ERR) receptors, is starting to be characterized in invertebrates in an effort to understand their functional traits in relation to those of their vertebrate orthologs. Even though it is still not clear to what extent the teratogenic effects of EDCs in invertebrates are mediated by NRs, it has been shown that NRs are involved in embryonic and post-embryonic development in these animals [47–53]. Yet, the mechanisms at play are not necessarily conserved with vertebrates, revealing lineage-specific adaptations in both invertebrates and vertebrates [1,10,17,47–57]. The aim of this review is to describe and discuss the developmental functions of NRs in invertebrates, with a special focus on marine organisms, highlighting the particular importance of comparative approaches using emerging marine invertebrate models for the field of evolutionary developmental biology.

2. Development of Marine Invertebrates and Associated NR Cohorts

In this section, we will correlate NR expression with the development of marine invertebrates, focusing on representatives from three phyla: cnidarians, mollusks and chordates. Marine invertebrate development can generally be classified into four stages: embryonic development, embryo to larva transition (EtL), larval development and metamorphosis. Embryonic development is characterized by cell proliferation and germ layer specification [47]. EtL consists of a series of morphogenetic processes resulting in the formation of a primitive larva. These processes include axial and body patterning, initiation of neurogenesis and organogenesis [58]. During larval development, additional structures for feeding, light sensing and swimming/crawling are formed [59]. Most of these structures will subsequently be lost during metamorphosis, which results in the emergence of the adult body plan [60]. This typical life cycle can be completed by an asexual reproduction phase. Juvenile polyps of Medusozoan cnidarians, for example, can undergo strobilation, leading to segmentation of the polyp into so-called ephyrae, which will subsequently grow into adults [61]. Furthermore, several tunicate lineages independently gained the capacity for asexual reproduction using different budding strategies [62,63].

As detailed above, NR complements can vary greatly between different marine invertebrate phyla. Cnidarians, for instance, possess only a limited number of NRs [20,30,61,64]. The genome of the cnidarian *Nematostella vectensis*, an anthozoan, was thus estimated to encode 17 NRs, including orthologs of vertebrate COUP-TF, TLX/PNR, HNF4, TR2/4 and GCNF [30]. The genomes of medusozoan cnidarians, such as *Aurelia aurita*, include additional NRs, such as orthologs of vertebrate RXR and an ER-like NR, called NR3E (Figure 3; Figure 2) (Table 1) [20,64,65]. In comparison, mollusks have larger NR complements with representatives of each NR subfamily. In the genomes of *Crassostrea gigas*, *Biomphalaria glabrata* and *Lottia gigantea*, for example, 43, 39 and 33 genes encoding NRs were respectively identified, with most of them being orthologous to vertebrate NRs [19,23,24]. While the stereotypical NR complement of chordates is similar to that of mollusks, tunicates are characterized by significantly reduced NR complements. The ascidian tunicate *Ciona intestinalis*, for example, only has 17 NR genes [36].

It is intriguing to speculate that differences in NR complements are correlated with the diversity of life cycles, developmental strategies and reproductive adaptations observed in marine invertebrates [66]. NRs are essential regulators of vertebrate development [67,68]. Given that a certain number of developmental processes are conserved in metazoans (or at least bilaterians), it is conceivable that NRs are also pivotal regulators of embryonic and post-embryonic development in invertebrates [48,69]. However, developmental expression

of NRs has only been established in a limited number of marine invertebrates, and their developmental functions in marine invertebrates are chiefly unknown [30,47,48,53,65].

In *N. vectensis* (Figure 4), 13 of the 17 NRs are dynamically expressed during development, with distinctive temporal patterns during embryogenesis, EtL, larval development and metamorphosis. While homologs of COUP-TF and TLX/PNR are highly expressed during EtL, HNF4, TR2/4, COUP-TF and NR7/8 expression is characteristic for larval development. Intriguingly, although GCNF is detectable throughout development, its expression increases during late development and marks the metamorphic stage [30]. Furthermore, in *A. aurita*, RXR expression peaks during strobilation (Figure 4) [64,65]. In the bivalve mollusk *C. gigas*, 34 of the 43 NRs are characterized by dynamic expression patterns during development (Figure 4). Orthologs of several NRs, including 2DBD-NR γ , HNF4 and NR7/8, are strongly expressed during embryonic development, with transcripts being almost exclusively of maternal origin [24,47]. While EtL is characterized by a number of NR transcripts including THR, RAR, PPAR, RXR, Rev-Erba, TLL/TLX and PNR, larval development is marked by a general downregulation of NR expression [24,47,70]. Metamorphosis, in turn, is characterized by NR0B, 2DBD-NR δ , EcR, RXR, COUP-TF/SVP, TLL/TLX, ER and ERR expression [47]. Intriguingly, various members of the *C. gigas*-specific NR1P subgroup are dynamically expressed during development [17,47]. In the ascidian tunicates *C. intestinalis* and *Phallusia mammillata*, a principal component analysis of NR transcripts has not yet been performed. However, the expression profiles of ascidian NRs available in the Aniseed database (<https://www.aniseed.cnrs.fr/>) allow at least some level of developmental clustering (Figure 4). Embryonic development is thus characterized by expression of TR2/4, RXR, PPAR and LXR, all of which are of maternal origin. EtL is associated with RAR, HNF4, GCNF and ROR expression, while peaks in the expression of THR, Rev-Erb, ERR, COUP-TF and PPAR occur during larval development [53]. Altogether, these results demonstrate that the development of marine invertebrates is characterized by the stage-specific expression of NR subsets, suggesting that NR activity is correlated with distinctive developmental processes at different stages of the life cycle.

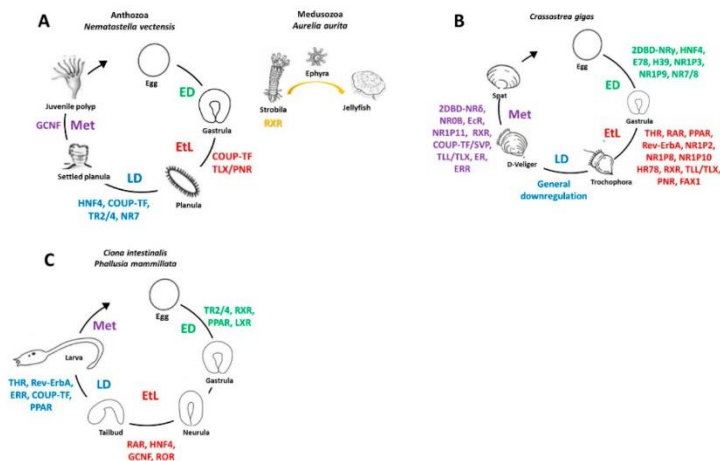


Figure 4. Nuclear receptor (NR) expression during development of marine invertebrates. (A) Cnidarians. (B) Bivalve mollusks. (C) Ascidian tunicates. NRs dynamically expressed during embryonic development (ED) (green), embryo to larva transition (EtL) (red), larval development (LD) (blue), metamorphosis (Met) (violet) and during cnidarian strobilation (orange). NR nomenclature is as defined in Table 1. Data for the cnidarians *Nematostella vectensis* and *Aurelia aurita* from Reitzel et al. (2009), Fuchs et al. (2014) and Brekhman et al. (2015) [30,64,65], for the bivalve mollusk *Crassostrea gigas* from Vogeler et al. (2016) and Huang et al. (2015; 2020) [19,24,70]. Raw data for the ascidian tunicates *Ciona intestinalis* and *Phallusia mammillata* are from the Aniseed database (<https://www.aniseed.cnrs.fr/>) and from Gomes et al. (2019) [53].

3. Development of Marine Invertebrates and NR Diversification

Despite the gene duplications and losses that accompanied NR diversification, a basic set of NRs is implicated in the development of distantly related metazoans. This basic set of NRs comprises COUP-TF/SVP, TLX/PNR, HNF4 and RXR (Figure 5, Table 1). Information regarding their developmental functions in marine invertebrates is still extremely scarce and is largely derived from the biological activity of their vertebrate orthologs.

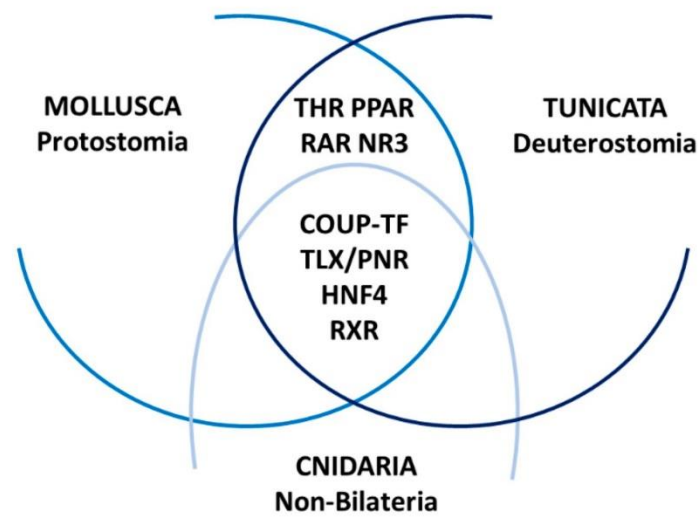


Figure 5. Ancestral sets of nuclear receptors (NRs) define the development of marine invertebrates. Members of the NR2 subfamily (COUP-TF, TLX/PNR, HNF4, RXR) are dynamically expressed during development of cnidarians, mollusks and tunicates. Albeit their presence in the genomes of at least medusozoan cnidarians, NR3 receptors only contribute to the developmental NR complements of the two bilaterian taxa, mollusks and tunicates. Members of the NR1 subfamily (THR, PPAR, RAR) are dynamically expressed during development of both protostomes and deuterostomes, further expanding the bilaterian set of NRs with developmental functions.

3.1. Chicken Ovalbumin Upstream Promoter Transcription Factor, COUP-TF

In vertebrates, COUP-TF generally acts as a transcriptional repressor and regulates the development of muscles and heart as well as the differentiation of hindbrain and photoreceptors [71,72]. COUP-TF orthologs are expressed in early and late larval stages of *N. vectensis* and in late pre-metamorphic stages of both *C. gigas* and *C. intestinalis* (Figure 4). As this receptor is expressed in nematoblasts and subsets of neural cells in the cnidarian *Hydra vulgaris* as well as in the posterior photoreceptive ocellus of *P. mammillata*, COUP-TF is considered as a conserved neural marker involved in the formation of photoreceptive organs [53,73].

3.2. Tailless/Photoreceptor Cell-Specific Nuclear Receptor, TLX/PNR

In vertebrates and fruit flies, TLL/TLX is an orphan receptor involved in eye and forebrain development as well as in anteroposterior patterning of the embryo, suggesting some level of functional conservation between vertebrates and invertebrates [48,74]. In both *N. vectensis* and *C. gigas*, TLX/PNR orthologs are highly expressed in EtL stages (Figure 4). Their predominant expression in neural tissues is indicative of functions in neurogenesis and in the development of photoreceptive organs [30,47]. While TLX/PNR receptors were secondarily lost in tunicates, the TLL/TLX ortholog of the cephalochordate amphioxus is dynamically expressed in EtL stages. In neurulae, the amphioxus TLL/TLX

gene marks developing sensory neurons, and in larvae, the gene is expressed in the central nervous system and anterior notochord [75]. It is thus likely that TLX/PNR genes are also involved in neurogenic processes in developing invertebrate chordates.

3.3. Hepatocyte Nuclear Factor 4, HNF4

In humans, HNF4 binds endogenous fatty acids as ligands and regulates hepatocyte differentiation, energy metabolism, xenobiotic detoxification and stem cell maintenance in the germ line [76,77]. HNF4 also participates in primary endoderm development in frogs, regulates expression of transcription factors necessary for endoderm specification in mice and is required for gut formation in insects [78–80]. HNF4 is highly expressed during larval development in *N. vectensis*, is a maternal transcript in *C. gigas* early embryos and expressed at EtL stages in *C. intestinalis* (Figure 4) [30,47]. In *P. mammillata*, HNF4 is expressed in endoderm cells of the trunk [53]. Developmental expression of HNF4 could thus play a role in endoderm specification and the formation of endodermal organs [30,47,53].

3.4. Retinoid X Receptor, RXR

In vertebrates, RXR is commonly the silent heterodimeric partner of NR1 and NR4 subfamily members and is involved in a variety of developmental processes in subordination to its heterodimeric binding partners. In the medusozoan cnidarian *A. aurita*, RXR is dynamically expressed during early strobilation, suggesting a potential role for RXR in this asexual reproduction process (Figure 4) [65]. RXR is highly expressed in *C. gigas* EtL stages, together with the NR1 subfamily members THR, RAR and PPAR [47,70]. In addition, there is a second peak of RXR expression prior to metamorphosis, which is paralleled by the NR1 subfamily member EcR [47]. Conversely, RXR in *C. Intestinalis* is expressed mainly during embryonic development, together with PPAR, and less strongly during EtL and larval stages, which are, respectively, characterized by the expression of the NR1 subfamily members RAR and ROR and THR, Rev-erb and PPAR [53]. While the developmental clustering of RXR does not strictly follow that of the NR1 subfamily members, there is nonetheless a tendency for regrouping RXR expression with that of the representatives of the NR1 subfamily. The notable absence of NR4 subfamily members from the developmental clusters in cnidarians, mollusk and tunicates suggests that heterodimers of RXR and NR1 receptors might play much more important roles during invertebrate development than heterodimers of RXR and NR4 receptors.

The receptors defining the basic set of NRs acting during development of marine invertebrates are all members of the NR2 subfamily. This subfamily has appeared very early in the metazoan lineage and has diversified before the cnidarian-bilaterian split [27,30]. Of all NRs, the NR2 subfamily member HNF4 is actually considered the extant NR that most closely resembles the ancestral NR that originated in the last common ancestor of all metazoans [15,16]. Based on the observations detailed above, it thus seems likely that the early diversification of the NR2 subfamily was accompanied by the elaboration of a NR2-dependent gene regulatory network involved in different aspects of animal development [81]. If this hypothesis is correct, at least some elements of this core gene regulatory network should still exist in extant animals and control conserved developmental functions in different metazoans. The basic set of NRs identified here and their potential involvement in invertebrate development could serve as a starting point for future studies aimed at identifying these ancestral NR-dependent features of animal development.

4. Thyroid Hormone Receptor (THR) Signaling Regulates Developmental Transitions in Marine Invertebrates

In vertebrates, THR is a ligand-activated transcription factor and its ligands are generally referred to as thyroid hormones (THs), which are either synthesized endogenously or taken up from the environment (Table 1) [82,83]. The main THs of vertebrates are triiodothyronine (T3) and tetraiodothyronine (T4), with T3 being the biologically active TH. THs are key regulators of vertebrate development and homeostasis and are involved, for example, in animal growth and metabolism as well as in the regulation of metamor-

phosis [84]. THR is dynamically expressed during development of both mollusks and tunicates, which is suggestive of a possibly conserved function in bilaterian development (Figure 5). THR genes originated at the base of bilaterians and have already been identified in the genomes of a wide variety of protostomes and deuterostomes [10,52,85]. However, the THR gene was lost in ecdysozoans and lineage-specific THR duplications occurred, for example, in platyhelminths, likely by independent duplication events in trematodes and turbellarians (Figure 2) (Table 2) [27,85].

Table 2. Summary of thyroid hormone receptor (THR) functions in marine invertebrates.

Taxon	Clade	Phylum	Receptor Activity	Developmental Function
Deuterostomia	Chordata	Tunicata	Unknown	Suspected role in metamorphosis
		Cephalochordata	Activated by TRIAC	Pivotal regulator of metamorphosis
	Ambulacraria	Echinodermata	Presumably ligand-activated and/or controlled by alternative signaling pathways	Suspected role in growth, metamorphosis, skeletogenesis
Protostomia	Lophotrochozoa	Annelida	Ligand-activated by T3 or TRIAC	Regulator of developmental transition from trochophore to crawling larva
		Mollusca	Presumably ligand-activated and/or controlled by alternative signaling pathways	Suspected role in growth and developmental transitions
		Platyhelminthes	Presumably ligand-activated and/or controlled by alternative signaling pathways	Suspected role in growth
Non-Bilateria	Radiata	Cnidaria	Absent from the genome	THs with a role in metamorphosis, strobilation, skeletogenesis

Ascidian tunicates possess a THR ortholog and can endogenously produce T4 [86–88]. T4 is present in pre-metamorphic stages, and experimental evidence suggests that it could be a regulator of metamorphosis [87,88]. However, a direct involvement of THR in this process remains elusive [86,87]. In the cephalochordate amphioxus, several elements of the THR signaling system are shared with vertebrates, but the biologically active TH is triiodothyroacetic acid (TRIAC), rather than T3 [89,90]. TRIAC binds and strongly activates the amphioxus THR, and TH-dependent signaling plays a pivotal role in the regulation of metamorphosis [89,90]. In echinoderms, a THR gene has been cloned from sea urchins, but it has been shown that this receptor is not activated by THs or their metabolites [52,83]. Yet, T3 and T4 are actively accumulated during echinoderm development, and an exogenous supply of T4 can accelerate development, skeletogenesis and metamorphosis in a variety of echinoderm species [52,91].

Ligand-controlled THR signaling has further been suggested to regulate development and metamorphosis in different protostomes. However, in the absence of convincing evidence of endogenous TH synthesis in protostomes, it is currently believed that protostomes have to take up THs, or its precursors, from external sources [52,54,92]. In annelids, the THR of *Platynereis dumerilii* is activated in the presence of T3 or TRIAC [10,54]. Treatments with exogenous T3 or TRIAC induce an acceleration of the morphological switch from the trochophore to the crawling larva. This morphological switch is further characterized by a peak of THR expression [10,54]. The THR of the annelid *P. dumerilii* might thus be a ligand-activated receptor with endogenous ligands similar to T3 or TRIAC that mediates developmental transitions between larval stages [54,93].

In mollusks, experimental evidence also points to a morphogenetic function of THR and THs. While the transcriptional activity of *C. gigas* THR is not stimulated at relevant physiological concentrations of T4, T3 or TRIAC in vitro, both T4 and T3 are present in vivo in embryos and larvae, with their concentrations increasing significantly between the gastrula and the feeding larva [94]. In addition, the THR protein is detectable from blastula to trochophore stages in *C. gigas*, suggesting that the THR and the TH signaling system might be involved in the regulation of the embryo to larva transition [94]. Similarly, although platyhelminth THR is not activated by THs, exogenous THs accelerate the development of parasitic platyhelminth lineages [85,95,96]. Taken together, it seems likely that THs have an ancestral role in lophotrochozoan development and that THR is involved in mediating these roles. However, the endogenous ligands of lophotrochozoan THR seem to be different from those of vertebrate THR.

THs were further shown to function in developmental transitions in animals that lack a THR, such as cnidarians where THs regulate metamorphosis and strobilation [52,83]. A role for TH signaling in the control of developmental transitions might thus predate the evolutionary origin of THR [52,54,83]. It has been proposed that the first TH signaling systems to evolve used TH precursors obtained from algal sources and that the ancestral THR evolved as a sensor for iodinated tyrosine and indicator of food availability [10,52,83,92]. The appearance of THR in bilaterian animals thus allowed for the elaboration of a ligand-dependent control mechanism for development and growth, whose activity is directly coupled to environmental cues favoring larval survival [10,52,83,92,97].

5. Retinoic Acid Receptor (RAR)-Dependent Signaling Is Required for Neurogenesis in Marine Invertebrates

As in the case of THR, RAR is also dynamically expressed during larval morphogenesis in invertebrates (Figure 5). It is thus reasonable to assume that this NR is also involved in the regulation of developmental functions in marine invertebrates. Vertebrate RARs are ligand-activated transcription factors that act as constitutive repressors in the absence of a ligand [98]. RARs bind different isomers of the small, lipophilic molecule retinoic acid (RA), such as all-*trans*-RA, 9-*cis*-RA or 13-*cis*-RA, with all-*trans*-RA being the main biologically active RA isomer [99]. RA acts as a morphogen, whose functions are mediated by RARs. During vertebrate development, RA signaling is, for example, required for axial patterning, nervous system development and organogenesis, with HOX genes being amongst the major targets of this signaling pathway [99,100]. Most bilaterian genomes contain a single RAR gene [17,27,36,49,50,101–103]. However, RARs have been lost in most ecdysozoans as well as in appendicularian (larvacean) tunicates (Figure 2) (Table 3) [27,101,103].

Many elements of a vertebrate-like RA signaling system are present in invertebrate deuterostomes, including the genes encoding the receptors and the enzymes required for the synthesis and degradation of endogenous RA [99]. In tunicates that have not secondarily lost the RAR gene, RAR-dependent RA signaling is implicated in neurogenesis as well as in tissue regeneration and bud development of budding tunicates [104,105]. The cephalochordate amphioxus is characterized by the most vertebrate-like RA signaling system of all invertebrates [99,106]. It has been demonstrated that the amphioxus RAR/RXR heterodimer can be activated by all-*trans*-RA, that HOX genes are directly regulated by RAR/RXR and that HOX-mediated RA signaling is essential for neurogenesis and axial patterning [107]. Conversely, little is known about RAR and RA signaling in ambulacrarians. In echinoderms, the sea urchin RAR can bind RA in vitro, although with low affinity, and, while RA treatments might either disrupt or delay sea urchin development, the molecular mechanisms underlying these effects remain to be established [49,99].

In lophotrochozoans, extensive functional characterizations of RAR orthologs and RA signaling have been carried out in both annelids and mollusks [49–51,55,102]. The annelid *P. dumerilii* possesses a vertebrate-like RA signaling machinery composed of a ligand-activated RAR as well as enzymes for RA synthesis and degradation [49,101]. *P. dumerilii* RAR is activated by RA binding and regulates transcription in a heterodimer with RXR [49]. However, the *P. dumerilii* RAR has a lower affinity for RA than its vertebrate orthologs, and

the conformation of the ligand within the LBP is not conserved between the annelid and vertebrate receptors (Figure 1) [49]. When exposed to all-*trans*-RA or 13-*cis*-RA, *P. dumerilii* embryos experience neuroblast depletion leading to reduced numbers of differentiating motor neurons and suggesting a direct effect of RA on dividing neural stem cells [49]. Moreover, knockdown of *P. dumerilii* RAR or RXR causes severe malformations of the developing larval nervous system [49]. RAR-dependent RA signaling is thus required for neurogenesis in annelids [49].

Table 3. Summary of retinoic acid receptor (RAR) functions in marine invertebrates.

Taxon	Clade	Phylum	Receptor Activity	Developmental Function
Deuterostomia	Chordata	<i>Tunicata</i>	Ligand-activated by retinoic acid	Neurogenesis, budding
		<i>Cephalochordata</i>	Ligand-activated by retinoic acid	Neurogenesis, axial patterning
	<i>Ambulacraria</i>	<i>Echinodermata</i>	Ligand-activated by high concentrations of retinoic acid	Presumably involved in developmental growth
Protostomia	Ecdysozoa	<i>Priapulida</i>	Ligand-activated by high concentrations of retinoic acid	Unknown
		<i>Hexapoda</i>	Absent from the genome	RA with role in nervous system regeneration, tissue repair
	<i>Lophotrochozoa</i>	<i>Annelida</i>	Ligand-activated by high concentrations of retinoic acid	Neurogenesis
		<i>Mollusca</i>	Ligand-binding pocket occluded and potential activation by liganded RXR	Neurogenesis
Non-Bilateria	<i>Radiata</i>	<i>Cnidaria</i>	Absent from the genome	RA with role in neurogenesis, metamorphosis, strobilation

Contrasting the situation in annelids, mollusk RARs have lost the ability to bind RA. It has been suggested that mollusk RARs secondarily lost the capacity to bind RA by accumulating independent single mutations in different mollusk lineages [51,56]. Accordingly, mollusk RARs likely function as constitutive transcriptional repressors [50,51,102]. It has recently been proposed that this repressive activity of RAR within the RAR/RXR heterodimer could be modified by RXR-dependent ligand binding [50,102]. It thus remains elusive how RA controls development and neurogenesis in mollusks [50,55,56,99,106]. Similarly, although the genomes of most ecdysozoans do not encode a RAR gene, there are indications for active roles of RA signaling in ecdysozoans, for example, during nervous system regeneration and in tissue repair of insects [108,109]. As a matter of fact, RAR has been lost very early during ecdysozoan evolution, after the split of the priapulid lineage [103]. Notably, the RAR of the priapulid *Priapulid caudatus* binds all-*trans*-RA and 9-*cis*-RA with affinities similar to those of the *P. dumerilii* RAR [49,103]. In cnidarians, whose genomes do not encode RARs, RA has been implicated in the regulation of metamorphosis and neural development [55,64,99,110]. Independent of the presence of RAR, RA can thus be considered a potent morphogen involved in metazoan neurogenesis. Future work will have to address how the RA signal is mediated in the absence of RAR, and several scenarios, including the involvement of a liganded RXR, have already been proposed for the evolutionary origin of RA signaling at the base of metazoans [50,99]. RARs only arose later, at the base of bilaterians, as ligand-activated RA sensors that significantly facilitated the regulatory control of RA signals [49].

6. Retinoid X Receptor (RXR) Functions during Marine Invertebrate Development

In vertebrates, RXRs act as heterodimeric binding partners of NR1 and NR4 subfamily receptors. Their activity is thus subordinated to those of the heterodimeric binding partners, which participate in a wide variety of developmental processes [39,111]. However, under

very specific circumstances, RXRs may also function as ligand-activated receptors. In vitro, RXRs are activated by a number of different compounds, including 9-*cis*-RA and fatty acids, such as docosahexaenoic acids (DHAs) [112,113]. Orthologs of vertebrate RXRs have been identified in most metazoan taxa, including sponges, placozoans and cnidarians, suggesting that RXRs originated at the base of the animal tree of life [57,114]. Lineage-specific duplications of RXR genes are rare and have so far only been reported in two lophotrochozoans: bryozoans and platyhelminths (Figure 2) [57]. RXR is widely expressed during development of most marine invertebrate taxa, including cnidarians, mollusks and tunicates. Compared to other NRs, RXR does not show a clear pattern of developmental clustering. Its expression profiles tend to follow the ones of its heterodimeric binding partners (Figure 4).

Marine invertebrates provide excellent examples for the importance of RXRs as regulators of development and point to a possible ligand-activated function of these receptors (Table 3). In the sea urchin *Strongylocentrotus nudus*, for example, an ambulacrarian deuterostome, knockdown of RXR induces abnormal early embryonic development and leads to a complete arrest of embryonic development at the early gastrula stage, suggesting that RXR is crucially required for sea urchin development [115]. Furthermore, a study of the RXR from the sea urchin *Paracentrotus lividus* suggests that exposure to an endogenous gonadal fatty acid mixture stimulates the activity of the PPAR/RXR heterodimer *in cellulo* and that this stimulation is mediated by ligand binding to RXR. In this particular context, the transcriptional activity of the PPAR/RXR heterodimer might thus be regulated by RXR [116]. In ecdysozoan and lophotrochozoans protostomes, RXR mainly acts as a heterodimeric partner of other NRs, in accordance with the process of RXR subordination [117,118]. However, a ligand-activated function of RXRs has been proposed in mollusks, where it was shown, in *C. gigas*, *Nucella lapillus* and *Acanthochitona crinita*, that RXR-specific ligands abrogate the repressive state of RAR/RXR heterodimers [50]. Given that mollusk RARs do not seem to bind RA, the activity of RAR/RXR during mollusk development might thus be regulated by ligand binding to RXR. Further studies need to address this hypothesis *in vivo* and evaluate the biological relevance of RXR ligands during development.

While the genomes of non-bilaterians encode RXR genes, their heterodimeric binding partners, i.e., members of the NR1 and NR4 subfamilies, are absent. The RXR of medusozoan cnidarians binds 9-*cis*-RA with high affinity and is required for strobilation [64]. Cnidarian RXRs might further be involved in the RA-dependent regulation of nervous system patterning and neurogenesis [65,99,110]. It is thus tempting to speculate that RXR is mediating the developmental roles of RA in cnidarians, potentially as a homodimer [119].

7. Estrogen Receptor (ER), Estrogen-Related Receptor (ERR) and the Development of Marine Invertebrates

In vertebrates, ERs are activated by estradiol (E2) and function during the formation of the nervous system, during development of secondary sexual characteristics, in the regulation of the immune system, in the maintenance of bone density and in the control of social behavior [120–122]. ERRs form a group of orphan receptors that are closely related to ERs and that are critical for the regulation of neurogenesis and metabolism as well as for cell proliferation and cell movements [123–125]. While the endogenous ligand of ERRs still remains elusive, their activity can be modulated by synthetic ligands, some of which also activate or inhibit ERs, such as, respectively, diethylstilbestrol (DES) or 4-hydroxytamoxifen (4-HT) [126]. ER and ERR are members of the NR3 subfamily, which originated early in metazoan evolution, likely after the split of the sponge lineage, and experienced diversification in early bilaterians and subsequently also in early chordates (Figure 3; Figure 2) [20,21,127]. Phylogenetically, non-chordate ERs thus group at the base of chordate ERs and SRs [20,127,128]. Single orthologs of ER and ERR have been identified in most protostomes, with ecdysozoans having lost ER and the nematodes, which are ecdysozoans, having additionally lost ERR (Figure 2) [27]. The genomes of invertebrate deuterostomes also encode single copies of ER and ERR and those of cephalochordates additionally a single SR [14,31]. Within chordates, the tunicates have secondarily lost both

ER and SR (Figure 2) [27,36,127]. The lineage-specific loss of an ER-like gene has further been reported in anthozoan cnidarians (Figure 2) [20,30]. Even though their expression profiles are indicative of a function during invertebrate development (Figure 5), the role played by these NRs outside vertebrates remains virtually unknown.

7.1. ERR Might Play a Role during Development of Marine Invertebrates

Ascidian tunicates possess a single ERR gene [36,53], whose expression, albeit dynamically modulated during late larval development, is exclusively localized in the brain (Figure 4) (Table 4) [53,129]. DES and 4-HT both affect brain formation and trunk elongation in developing *P. mammilata* larvae, suggesting that ERR acts during nervous system development and body extension of tunicates [53,129]. In the cephalochordate amphioxus, ERR is expressed in the hindbrain homolog and in the developing musculature [130]. The segmented patterns of ERR expression in hindbrain and muscles suggest that this NR is involved in establishing neuromuscular contacts in developing amphioxus [130]. In protostomes, ERR functions have been established in ecdysozoans, where it regulates metabolic processes supporting larval growth and cell proliferation [131,132]. ERR is further suspected to be a pivotal player during metamorphosis [131]. In contrast, there is only limited information available on ERR expression and functions in lophotrochozoans. In *C. gigas*, ERR is dynamically expressed during development with a peak at pre-metamorphic and metamorphic stages [47]. Accordingly, it is parsimonious to assume that ERR plays specific functional roles during development of mollusks, and more generally, of lophotrochozoans.

Table 4. Summary of estrogen receptor (ER) and estrogen-related receptor (ERR) functions in marine invertebrates.

Taxon	Clade	Phylum	Receptor Activity	Developmental Function
Deuterostomia	Chordata	Tunicata	ERR: Orphan receptor ER: Lost	ERR: Suggested role in sensory cell differentiation in the larval brain
		Cephalochordata	ERR: Orphan receptor ER: Unknown	ERR: Suspected role in establishment of neuromuscular contacts ER: Unknown
		Ecdysozoa	Arthropoda	ERR: Orphan receptor ER: Lost
Protostomia	Lophotrochozoa	Annelida	ERR: Unknown ER: Ligand-activated receptor binding estrogens	ER: Regulation of formation and proliferation of primordial germ cells
		Mollusca	ERR: Orphan receptor ER: Occluded ligand binding pocket, but constitutive transcriptional activity	ERR: Unknown ER: Unknown
		Rotifera	ERR: Unknown ER: Ligand-activated receptor binding estrogens	ERR: Unknown ER: Unknown
Non-Bilateria	Radiata	Cnidaria	ER-like: Ligand-activated receptor binding paraestrol A, an ancestral estrogen	ER-like: Unknown

7.2. Developmental Functions of ER in Marine Invertebrates Remain Largely Elusive

In the cephalochordate amphioxus, estrogens have been proposed to play important roles in reproductive functions, such as spawning [133]. However, it is currently unknown if ER and SR are required to mediate these functions (Table 4). In contrast, experimental evidence from marine protostomes, such as annelids and rotifers, supports the hypothesis that ERs are involved in reproductive processes of at least some invertebrates [134–137]. However, studies

addressing the developmental roles of ER-like receptors in protostomes remain extremely rare, with the only description of developmental functions of ER-like receptors in protostomes coming from annelids. In fact, estrogens are endogenously synthesized in annelids and are required for the formation and proliferation of primordial germ cells [136–138]. This regulation is dependent on ER, which binds the endogenous estrogens and hence directly controls this process [136,138]. Estrogen also induces proliferation of primitive germ cells in vertebrates, suggesting that germ cell regulation represents an ancestral trait of ER signaling in bilaterians [138]. In contrast, estrogen binding and ligand-dependent activation were lost in mollusk ERs [128,139]. Mollusk ERs are constitutively active and retained the ability to regulate their own gene transcription, but do not bind estrogens or other steroids, as their LBPs underwent vestigialization [128,139,140]. Nevertheless, in the bivalve mollusks *C. gigas* and *Mytilus galloprovincialis*, dynamic ER expression was detected at larval stages, which is suggestive of a role for ER in larval development [47,141]. Outside bilaterians, ER-like receptors have been identified in placozoans and cnidarians [20,21]. While their expression and function have yet to be assessed, it has been shown that the *H. vulgaris* ER-like receptor can bind paraestrol A, an ancestral estrogen [20,134]. Notably, a compound isolated from the cnidarian *Dendronephthya studeri* is structurally similar to paraestrol A, suggesting that ER-like receptors might act as ligand-activated transcription factors, at least in cnidarians [134,142].

8. Are NRs a Primary Target of EDCs in Marine Invertebrates?

Invertebrate protostomes and deuterostomes were initially thought not to be affected by EDCs, as they supposedly lack an elaborate endocrine system [44]. This assumption was shown to be incorrect, following the collapse of gastropod and bivalve mollusk populations in the Arcachon Bay in France. It was later shown that the mollusks were affected by EDCs in marine antifouling paints, already known to modulate the activity of PPAR/RXR heterodimers in vertebrates [45]. Today, it is well established that marine invertebrates are sensitive to EDC pollution and clearly manifest their adverse effects, with embryonic stages being particularly vulnerable. The scientific community has thus invested greatly in the study of EDC exposure and endocrine systems of marine invertebrates, turning their embryos into valuable alternative models for EDC and chemical testing [143]. However, with the exception of the RXR-dependent imposex phenotype in gastropod mollusks [144], evidence for NRs as primary targets of EDCs in marine invertebrates is still extremely circumstantial and mainly based on *in vitro* studies that do not necessarily reflect the molecular mechanisms *in vivo* [44–46,145,146]. This does not mean that NR-mediated endocrine disruption does not occur on a large scale in marine invertebrates. In fact, the chemicals currently classified as EDCs have chiefly been identified and characterized in vertebrates, and most studies into marine invertebrates have simply used these compounds to assess whether they also affect their endocrine system [46]. This vertebrate-centric view has thus heavily biased our current understanding of invertebrate endocrine systems.

Based on the work reviewed here, it is not surprising that high affinity EDCs of vertebrate NRs do not necessarily affect NRs of marine invertebrates (and vice versa) [44,45,145]. In the course of their evolution and diversification, NRs have been subjected to significant alterations of their sequences and structures, which explains why orthologous NRs are not necessarily characterized by conserved ligand binding affinities, downstream targets and biological functions [1,2,13,15–17,25]. Accordingly, there are probably numerous substances that can act as NR-mediated EDCs in marine invertebrates, and their modes of action are likely to be very different from those known in vertebrates [46,146]. Furthermore, it is currently impossible to estimate to what extent the endocrine systems of marine invertebrates are comparable to those of vertebrates, including the involvement of NRs [147–149]. There are thus a number of different points that need to be addressed in order to establish the endocrine disrupting potential of a particular set of chemicals in marine invertebrate taxa. These include the characterization of the roles of NRs in marine invertebrate physiology and endocrine systems, the assessment of their functions in embryonic and post-embryonic

development and the detailed definition of the physiological and developmental outcomes of NR-dependent endocrine disruption.

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Part 3

The larval development of *Mytilus galloprovincialis*: an experimental model for invertebrate endocrine disruption studies

This last part of the introduction aims to introduce the experimental model on which this research study relies. The current section will describe *M. galloprovincialis* larval development as well as the main morphogenetic processes characterising early life stages in bivalves in the context of the exploitation of this model for invertebrate endocrine disruption studies in marine species.

1. *Mytilus galloprovincialis* (Lamarck, 1819)

1.1 Characteristics and distribution

Mytilus galloprovincialis is a bivalve, marine mollusc belonging to the order of *Mytilida*, mostly distributed in the mediterranean region (Fig. 1) (FAO, 2021). Especially in the mediterranean region, *M. galloprovincialis* is an important species as it carries out essential ecosystem services as a bio-remediator capable of removing and segregating in its tissue pollutants and excess of organic material from the water column due to its filter feeding *habitus* (Brzozowska et al., 2012). The mediterranean mussel is also one of the major species in the yearly shellfish aquaculture production in the southern European region thus assuming a relevant importance in the socioeconomic context of the mediterranean territory (FAO, 2020). In this context, research aimed at identifying the effects and mechanisms of action of environmental contaminants are particularly solicited for both environmental monitoring and aquaculture practices.

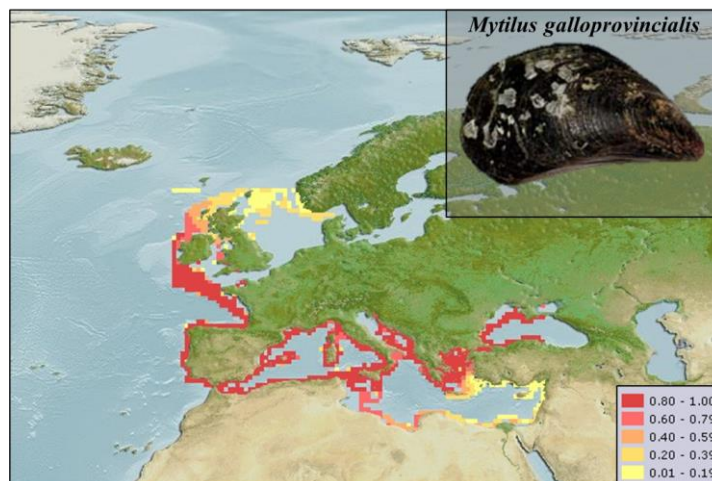


Figure 1: *Mytilus galloprovincialis* distribution in the mediterranean region. Shades of colours represent the probabilities of occurrence (www.aquamaps.org).

1.2 The life cycle of *Mytilus galloprovincialis*

M. galloprovincialis specimens are gonochoric and seasonal broadcast spawners (Gosling, 2015). Fertilisation happens externally upon gamete release in the seawater columns at given times of the year that normally coincide with winter (Gosling, 2015). The life cycle of *M. galloprovincialis* is analogous to any other bivalve molluscs and characterised by the succession of a planktonic, free swimming and benthic sessile phase (Fig. 2) (Carrier, Reitzel and Heyland, 2018; Gosling, 2015). The first life stages of the developing embryos and larvae of *M. galloprovincialis* are planktonic. The embryonic development follows the stages of spiralia metazoans with the progressive transitions to morula, blastula, and gastrula stage (Carrier, Reitzel and Heyland, 2018). The gastrula stage signs the end of the embryonic planktonic phase and the transition to the larval free swimming planktonic phase

(Carrier, Reitzel and Heyland, 2018). Molluscs are Lophotrochozoans, a group of different animal *phyla* within protostome bilaterians characterised by specific larval form called trochophore (Giribet et al., 2020). *M. galloprovincialis* early larval forms are thus called trochophores. During the trochophore stage, *M. galloprovincialis* larvae start to grow the larval shell and develop a swimming ciliated tissue called velum that, extruding from the shell, permits free swimming; the larvae at this point are referred to as pre-Veligers (Giribet et al., 2020; Gosling, 2015). The larval shell will progressively grow and take on a D-shape; by the time the larval shell becomes big enough to enclose the whole larval body, *Mytilus* larvae are referred to as D-Veligers (Giribet et al., 2020; Carrier, Reitzel and Heyland, 2018). The D-Veliger stage eventually signs the start of the transition to the juvenile stage. The velum, which is strictly a larval organ, is progressively transformed into the mantle, the tissue responsible for environmental sensing and exchange as well as shell biogenesis already in advanced larval stages and finally in adults (Carrier, Reitzel and Heyland, 2018, Gosling, 2015). The larvae develop the foot, the organ responsible for the adhesion to the substrate that signs the larval pediveliger stage (Giribet et al., 2020; Carrier, Reitzel and Heyland, 2018; Gosling, 2015). The transformation of the velum, morphogenesis of the foot as well as the global morphological and physiological remodelling required for the transition from the planktonic to the benthic phase is referred to as metamorphosis. After metamorphosis, *Mytilus galloprovincialis* larvae reach the final life stage that signs the start of the sessile benthic adult phase (Carrier, Reitzel and Heyland, 2018; Gosling, 2015).

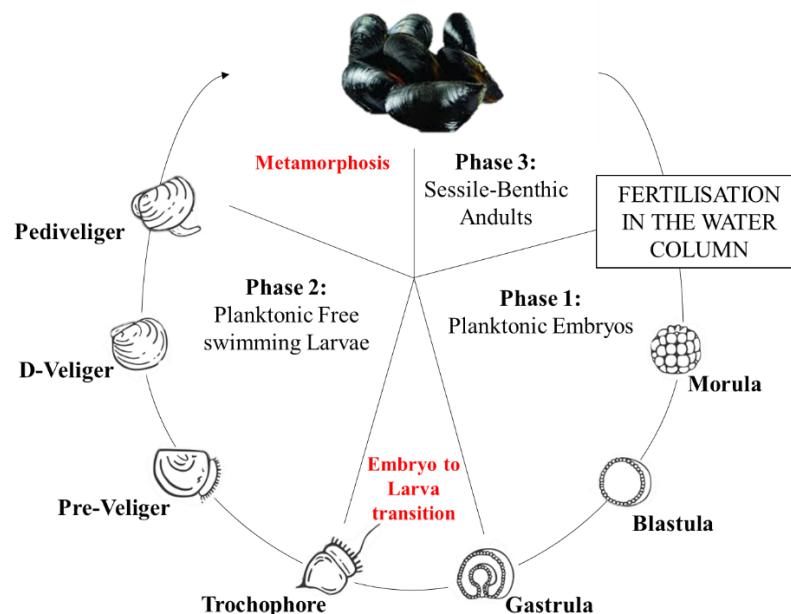


Figure 2: The life cycle of *Mytilus galloprovincialis*. Figure adapted from Vogeler et al., (2016).

2. *M. galloprovincialis* larval development as a model in invertebrate endocrine disruption research

2.1 The larval development of *Mytilus galloprovincialis*

Throughout invertebrates, the morphogenetic processes occurring during larval development are mainly dedicated to equipping the larvae with structures and systems able to provide protection from external stressors as well as converting external stimuli from the environment into appropriate responses (Carrier, Reitzel and Heyland, 2018, Gosling, 2015). In *M. galloprovincialis*, the larval development spans over all the developmental stages from the trochophore to the D-Veliger stage and is characterised by two main morphogenetic processes: shell biogenesis and neurogenesis (Giribet et al., 2020; Gosling, 2015). The process of shell biogenesis enables larvae to protect themselves from external insults while the formation of the nervous system permits to convert external stimuli from the environment in appropriate responses for the regulation of larval behaviour and development (Yurchenko et al., 2019; Marin, 2012).

2.1.1 Larval shell biogenesis in *M. galloprovincialis*

The growing of a shell is the distinctive character of all conchiferan molluscs, including *M. galloprovincialis*, on which they rely for protection from external stressors. In addition, the different stages of shell development in molluscs constitute the main diagnostic element of the out-course of their larval development which depends on the growth and shape of the larval shell as well as its ability to enclose the velum (Fig. 2). The larval shell is composed of an organic and inorganic layer (Wanninger and Wollesen, 2018). The organic layer is called organic matrix while the inorganic layer is constituted by the deposition of calcium carbonate (CaCO₃). Surprisingly, the ontogeny of the larval shell formation has been described first in *M. galloprovincialis* (Kniprath, 1980). Shell biogenesis starts with the secretion of the organic matrix operated by a group of ectodermic cells that take the name of shell field (Fig. 3 A) (Kniprath, 1981; Kniprath, 1980).

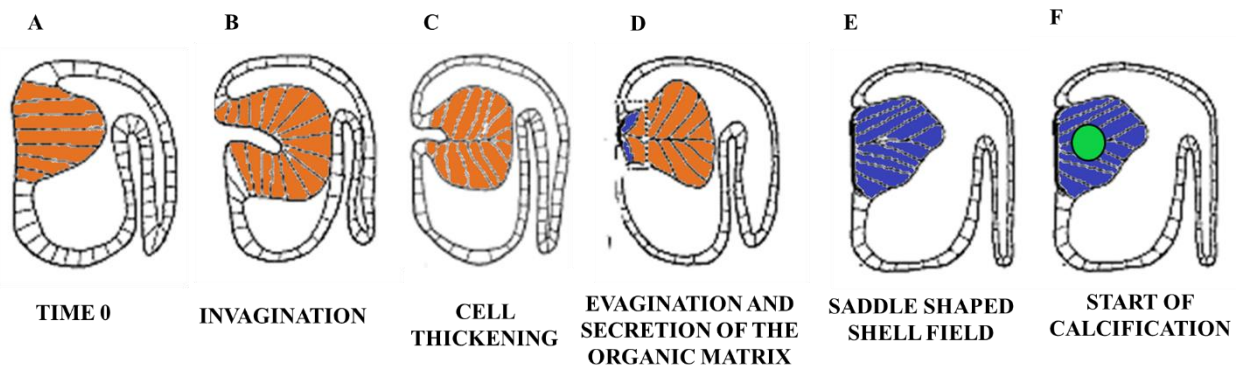


Figure 3: Schematic representation of the steps leading to the larval shell formation in *M. galloprovincialis* adapted from Kniprath (1980). In orange are highlighted the cells of the shell field on the dorsal side of the larva, blue indicates the expansion of the organic matrix, green CaCO_3 deposition.

The cells composing the shell field tissue are already present at gastrulation, during which they also undergo a complex invagination and successive cell thickening process (Fig. 3 B) (Kurita et al., 2009). The columnar thickening of the shell field cells leads to an apparent progressive evagination of the shell field when the embryos reach the trochophore stage and is followed by the start of the secretion of the organic matrix (Fig. 3 C, D) (Kniprath, 1981). Early in development, the exclusively organic shell takes on the shape of a saddle and the whole complex of shell and shell field takes the name of saddle shaped shell field (Fig. 3 E) (Marin, 2012). The organic matrix is mainly composed of chitin and acidic polysaccharides, proteins and glycoproteins, which have essential roles in different aspects of shell formation, such as CaCO_3 nucleation, growth and choice of polymorphs (Falini and Fermani, 2004). The inorganic layer soon starts to be deposited over the organic matrix which provides a scaffold for mineral deposition (Fig. 3 F) (Ramesh et al., 2018). The two components start growing together in line with global larval development in the form of two symmetric valves that progressively cover and enclose the whole larval body in a D-shaped shell by the D-Veliger stage (Ramesh et al., 2018). The shell field is at this point differentiated into the mantle, the shell forming tissue in adults (Marin, 2012).

2.1.2 Larval neurogenesis

The larval neurogenesis of *M. galloprovincialis* is yet to be documented; however, the process has been studied and described in other bivalve molluscs such as the pacific oyster *Crassostrea gigas* and *Mytilus trossulus* (Yurchenko et al., 2019; Voronezhskaya et al., 2008). The *consensus* tells that the first neurons to appear in larvae are sensory cells, responsible for converting external stimuli from the environment and appropriately regulating larval behaviour and development (Yurchenko et al., 2019). In *C. gigas*, starting by the trochophore stage, early peripheral neurons appear on both the dorsal and ventral side of the larvae which are followed by the formation of the larval Apical Sensory

Organ (ASO) and every neuron of the ASO possess unique neurotransmitter identity (Yurchenko et al., 2019). The ASO is a sensory organ typical of trochophore stages localised in the region of the apical tuft (Fig. 4). During early trochophore development the first neurotransmitters to appear are Serotonin and FRMFamide with Serotonin being exclusively localised in the ASO and FRMFamide in both the ASO and peripheral neurons (Yurchenko et al., 2019). In *Mytilus sp.* the first serotonergic and FRMFamidergic neurons are detected in the ASO starting by the trochophore stage together with two peripheral unidentified neurons (Fig. 4) (Yurchenko et al., 2019; Voronezhskaya et al., 2008). FRMFamidergic neurons increase in number in the ASO as the larvae develop and by the D-veliger stage two other groups appear on the ventral side of the larvae (Fig. 4) (Yurchenko et al., 2019; Voronezhskaya et al., 2008). What is more, many of the larval neurons disappear after metamorphosis, as do the other larval structures suggesting that the crosstalk between larval morphogenesis and nervous system is specific to this developmental phase (Croll and Dickinson, 2004).

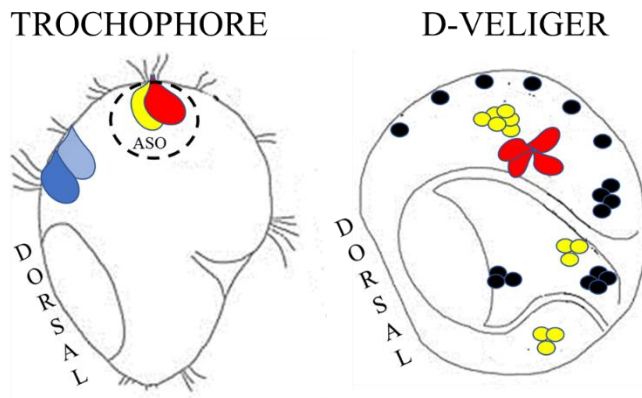


Figure 4: Schematic representation of the neuronal cell populations of *Mytilus sp.* larvae at the trochophore and D-Veliger stage. Blue indicates sensory peripheral neurons, serotonergic neurons are marked in red, yellow indicate FRMFamidergic neurons and catecholamine containing cells are shown in black. The region of the ASO is indicated by a dotted circle.

Serotonergic neurons also increase in number, but they remain localised in a single ganglion-like structure that by the D-Veliger stage has migrated towards the dorsal side of the larva (Fig. 5) (Yurchenko et al., 2019; Voronezhskaya et al., 2008). Starting by the Veliger stage, catecholamine containing cells are also found. Catecholamines are a group of neurotransmitters as well as neurohormones that includes dopamine, adrenaline, and noradrenaline. Catecholamine containing cells start to be detected at the pre-Veliger stage and mostly localise in the extruding velum and later in the D-border of D-Veliger shell and in a small dorsal group (Fig. 4) (Yurchenko et al., 2019; Voronezhskaya et al., 2008). In bivalves, the early neurons and their neurotransmitters have been linked to a series of developmental and regulatory functions during larval development including locomotion, the patterning of the nervous system itself as well as larval settling and metamorphosis in later stages (Yurchenko et al., 2019; Voronezhskaya et al., 2008). Additionally, Serotonin and

dopamine action were recently linked to the process of larval shell biogenesis in *C. gigas* (Liu et al., 2020; 2018).

2.2 *Mytilus galloprovincialis* larvae as model organism

M. galloprovincialis fulfils many criteria for a suitable model organism. As a research species the mediterranean mussel is relatively inexpensive, easy to handle in the laboratory and, as an intertidal organism, can tolerate wide ranges of salinity and temperature (Gosling, 2015). Additionally, specimens can easily be acquired from mussel farms as well as collected from the shores. Moreover, embryo and larval development can easily be monitored and sampled using the well-established *Mytilus sp.* embryo-larval bioassay protocols (Fabbri et al., 2014). In fact, in laboratory conditions, *Mytilus sp.* embryos reach the trochophore stage in 24 hours post fertilisation (hpf) and the D-Veliger stage by 48 hpf. The neat and characteristic shape of the shell of D-Veligers permits to efficiently employ *Mytilus* larvae for standard embryo-toxicity tests as the presence of toxic substances or stressors will prevent the larvae to reach the D-Veliger stage by 48 hpf with the presence of hinge malformations or arrested development at the trochophore or pre-Veliger stage (Fig. 3) (Fabbri et al., 2014).

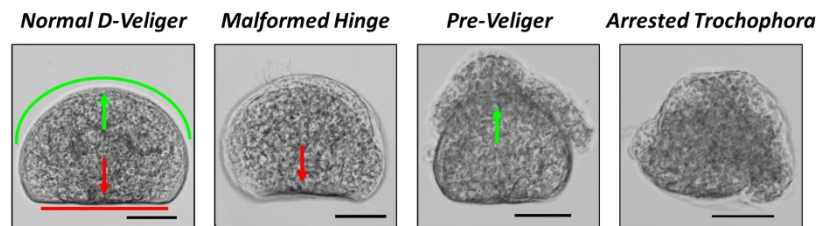


Figure 5: Representative image of normal D-Veliger larvae of *M. galloprovincialis* at 48 hpf and most common developmental defects induced by toxic substances at this stage. The hinge and D-border regions are indicated in red and green respectively. Scale bar: 20 μ m.

2.3 The larval development of *M. galloprovincialis* and invertebrate endocrine disruption

In the context of invertebrate endocrine disruption research, the larval development of the mediterranean mussel could serve as a potent model. In addition to the powerful tool that shell biogenesis in itself already constitutes in screening the potential toxicity of environmental pollutants, evidence suggests that the morphogenetic process is at least in part controlled by the larval neuroendocrine signalling operated by the larval neurons and neurotransmitters that arise during early trochophore development (Liu et al., 2020, 2018). What is more, NR signalling supposedly plays a role in regulating these morphogenetic processes in molluscs (Miglioli et al., 2021). It follows that

the main morphogenetic processes happening during larval development and their regulation in *M. galloprovincialis* are potential endocrine disruption targets that could serve as diagnostic endpoints to assess the endocrine effect of environmental pollutants in invertebrates as well as their specific mechanisms and essential information to unravel the functioning and regulatory elements of invertebrate neuro-endocrine systems and of Nuclear Receptors.

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Aim of the thesis

Following the problematics and information exposed in the introductory section, the research work presented in this thesis is meant to address the issue of endocrine disruption in marine invertebrates by exploiting the early larval development of the mediterranean mussel *M. galloprovincialis* as experimental model. The present work is, by applying an AOP investigation rationale, an attempt to characterise the mechanism of action of selected model EDCs in mussel larvae and identify plausible pathways that could be linked to adverse biological outcomes. In particular, an attempt has been made to identify a possible endocrine disrupting activity initiated at the level of the neuroendocrine system and of Nuclear Receptors signalling.

To reach this goal, the research work successively addressed the following questions:

- 1) What are the key elements regulating shell biogenesis and neurogenesis in *M. galloprovincialis* early larval development?
- 2) How do different EDCs affect shell biogenesis and neurogenesis in developing larvae of *M. galloprovincialis*?
- 3) What are the relationships between endocrine disruption, shell biogenesis and neurogenesis?
- 4) Could NRs be involved in the neurodevelopmental defects induced by EDCs in developing *M. galloprovincialis* larvae?

The questions were sequentially investigated starting by the detailed characterisation of the process of shell biogenesis and its regulation in *Mytilus* larvae. Next, the xenoestrogen Bisphenol-A and the thyroid disrupting chemical Tetrabromobisphenol-A were employed to evaluate if key components of both shell biogenesis and neurogenesis could represent sensitive targets of EDCs. Finally, Tributyltin- TBT was employed as a model EDC to investigate the possible relationship between disruption of NR signalling and neurodevelopmental effects.

Chapter 1.

The process of larval shell biogenesis in *M.*
galloprovincialis

Context of the study:

As mentioned in previous sections, larval neurogenesis and shell biogenesis could serve as sensitive and potent endpoints of investigation to determine endocrine disrupting effects of chemicals in developing larvae of *M. galloprovincialis*. While the protocols and methods to study larval neurogenesis as well as information regarding its regulation are already available in the literature (Yurchenko et al., 2019; Voronezhskaya et al., 2008), the same information was lacking for larval shell biogenesis, in particular regarding the development and growth of the organic matrix. Accordingly, this study was performed to:

- 1) develop experimental methods to follow the process of shell biogenesis throughout larval development;
- 2) establish a quantitative index to evaluate the developmental progress of the process;
- 3) characterise, at least partially, the key regulatory elements of early larval shell biogenesis;
- 4) identify molecular markers for early larval shell growth.

- ❖ **Voronezhskaya** EE, Nezhlin LP, Odintsova NA, Plummer JT, Croll RP. Neuronal development in larval mussel *Mytilus trossulus* (Mollusca: Bivalvia). *Zoomorphology* 2008;127:97–110. <https://doi.org/10.1007/s00435-007-0055-z>.
- ❖ **Yurchenko** OV, Savelieva AV, Kolotuchina NK, Voronezhskaya EE, Dyachuk VA. Peripheral sensory neurons govern development of the nervous system in bivalve larvae. *EvoDevo* 2019;10. <https://doi.org/10.1186/s13227-019-0133-6>.

Supplementary Material

Final Remarks:

Based on the results of this first study it is possible to conclude that larval shell biogenesis is driven by the development and growth of the organic matrix. The main achievement of this study is the design of a reliable method that enables to follow every step of larval shell biogenesis, analyse it quantitatively and summarise the information in a ratio capable to synthetically evaluate the out-course of the whole process. In addition to that, the study identified a novel pivotal player in the process of *M. galloprovincialis* larval shell biogenesis, Tyrosinase, which by governing organic matrix development determines the subsequent calcification as well as the global success of the morphogenetic process. Of note, for the purpose of this thesis, the results of the experiments carried out with the tyrosinase inhibitor PTU whose effects were not limited to altered shell development but also induced global larval development arrest. In fact, Tyr-3, on which this study focused, is only one member of the very diverse and copious family of tyrosinase enzymes (Joyce and Vogeler, 2018). The tyrosinases family includes in fact all the enzymes responsible for the conversion of the amino acid tyrosine into L-DOPA which is a crucial step not just for the construction of the organic matrix but also to produce neurotransmitters and neurohormones such as the catecholamines dopamine, noradrenaline and adrenaline (Joyce and Vogeler, 2018). What is more, these neurotransmitters and neurohormones are active actors in the neuroendocrine and neuroendocrine-immune signalling of molluscs with dopamine being already suggested to be a regulator of early larval shell biogenesis in oysters (Liu et al., 2020; Joyce and Vogeler, 2018;).

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Chapter 2.

Neuroendocrine disrupting effects of EDCs in
developing larvae of *Mytilus*
galloprovincialis: case studies with
Bisphenol-A and its brominated derivative
Tetrabromobisphenol-A

Context of the study:

This chapter addresses and attempts to shed light on two questions previously reported in the aims of the thesis: How do EDCs affect shell biogenesis and neurogenesis in developing larvae of *M. galloprovincialis*? What are the relationships between endocrine disruption, shell biogenesis and neurogenesis in developing *M. galloprovincialis* larvae?

In line with the aims, the current chapter reports the characterisation of the neuro-developmental and neuroendocrine disrupting effects of two model EDCs, Bisphenol-A (BPA) and its derivative Tetrabromobisphenol-A (TBBPA), in developing *M. galloprovincialis* larvae in the form of two case studies. The first case study is focused on BPA. BPA is a well-known xenoestrogen in vertebrates but not in molluscs, whose Estrogen and Estrogen Related NRs are so far considered to be devoid of a liganded function (Introduction, Part 2). The effects of BPA have been avidly investigated on aquatic species although its endocrine disrupting activity in molluscs is still debated. Accordingly, we investigated more in detail its effect on *M. galloprovincialis* larvae focusing on the process of shell biogenesis. In addition, the potential neuroendocrine action of BPA was tested on the onset of the serotonergic system and signalling that, as previously described, is one of the first neuronal as well as neurotransmitter identities appearing in bivalve early development (Yurchenko et al., 2019). The second case study addresses TBBPA, an EDC affecting, in vertebrates, the metabolism of thyroid hormones (THs) as well as the activity of the thyroid hormone receptor (THR). Since the functioning and physiological relevance of THs and THR in molluscs is still unclear (Introduction, Part 2), we first compared the effects of TBBPA on *M. galloprovincialis* larvae with the effects of pharmacological modulators of TH metabolism and THR activity. Consequently, we evaluated the effects of TBBPA on larval shell biogenesis and serotonergic system as previously described for BPA. In addition, during this case study we also investigated the onset and elements of the dopaminergic signalling, previously suggested to play a role in bivalve shell biogenesis (Liu et al., 2018), in order to provide solid evidence linking neuroendocrine signalling to larval shell formation and thereby confirm that adverse outcomes of larval shell formation in response to an environmental pollutant could be indicative of neuroendocrine disruption.

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Case study n°1:
Bisphenol A interferes with first shell formation
and development of the
serotonergic system in early larval stages of
Mytilus galloprovincialis.

Miglioli (a) A, Balbi T, Besnardeau L, Dumollard R, Canesi L.

Science of The Total Environment 2021;758:144003.

<https://doi.org/10.1016/j.scitotenv.2020.144003>.

Supplementary Material

Case study n°2:
Tetrabromobisphenol-A acts a
neurodevelopmental disruptor in early larval
stages of *Mytilus galloprovincialis*.

Miglioli (b) A, Balbi T, Montagna M, Dumollard R, Canesi L. Tetrabromobisphenol A acts a neurodevelopmental disruptor in early larval stages of *Mytilus galloprovincialis*. Science of The

Total Environment 2021;793:148596.

Supplementary Material

Final remarks:

Finally, with the present work it was possible to investigate into detail the endocrine disrupting pathway of known EDCs on the larval development of *M. galloprovincialis* that is initiated by the altered metabolism of monoamine neurotransmitters and neurohormones (i.e., serotonin and dopamine) as well as the interference with early regulators of larval neurogenesis (i.e., GABA). The results of these studies also identify the monoaminergic system as a sensitive neuroendocrine target of EDCs disruption in *M. galloprovincialis* larvae. In addition, by formally establishing the link between the process of shell biogenesis and the onset monoaminergic signalling, the present work also demonstrates that the process of shell biogenesis and neurogenesis are susceptible to neuroendocrine disruption in the larvae of *M. galloprovincialis*.

Chapter 3.

(MANUSCRIPT IN PREPARATION)

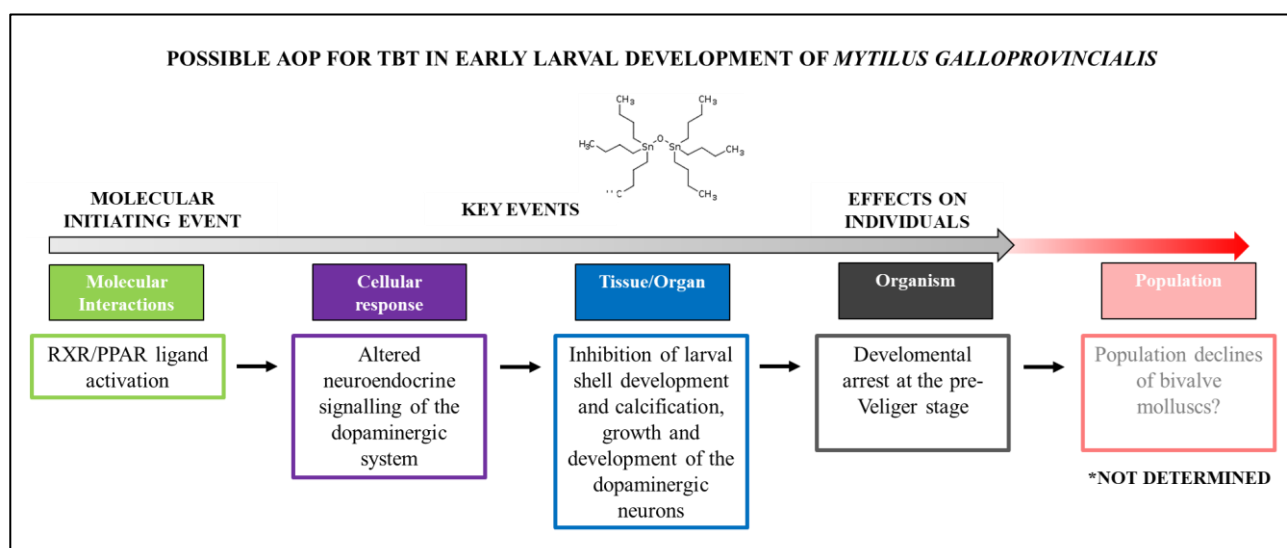
Implications of NR disruption in developing larvae of *M. galloprovincialis*: a case study with RXR

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Abstract:

Endocrine Disrupting Chemicals are a global threat to populations of marine invertebrates; however, the search for plausible physiological pathways through which such chemicals affect invertebrate neuroendocrine systems has been largely based, to the best of our knowledge, on endocrine disruption in vertebrate systems, that predominantly takes place through modulation of Nuclear Receptors (NR) activity. Organotin compounds, such as Tributyltin and Triphenyltin, have been shown *ex vivo* to bind the nuclear receptor Retinoic X Receptor (RXR) in a variety of invertebrate clades. Disruption of RXR signalling has been thus proposed as the main mode of action through which Organotins have historically affected populations of marine invertebrates, molluscs in particular. However, the *in vivo* demonstration of RXR disruption by these pollutants leading to the adverse biological effects is still lacking. In this work, the possible role of RXR in TBT-induced endocrine disruption in marine molluscs was investigated using the model of early larval development in the mediterranean mussel *Mytilus galloprovincialis*. The results demonstrate *in vivo* that RXR activation is a predominant mode of action of TBT in mussel developing larvae. Furthermore, the results show that exposure to TBT, as well as to the natural RXR ligand 9-*cis*-Retinoic Acid, leads to altered larval development through different pathways, ultimately interfering with the neuroendocrine signalling regulating larval shell biogenesis.



1. Introduction

Endocrine disrupting chemicals (EDCs), exogenous chemicals that can interfere with any aspect of hormone action, are pervasive and persistent pollutants in the aquatic environment, thus representing a major threat to marine invertebrate populations (La Merrill et al., 2020; Cuvillier-Hot and Lenoir, 2020; Lu et al., 2020; Katsiadaki, 2019; Fernandez, 2019). However, the limited knowledge of the elements of invertebrate neuro-endocrine systems prevents a global understanding of the effects of endocrine disruption at the individual or population level in these organisms (Ford and Le Blanc, 2020; Hartenstein et al., 2006). Such a gap of knowledge strongly encouraged transposing to invertebrates the main mode of action of EDCs in vertebrate organisms to hypothesise binding and modulation of invertebrate Nuclear Receptors (NR) by EDCs acting as agonists and antagonists (Cuvillier-Hot and Lenoir, 2020; Ford and Le Blanc, 2020; Toporova and Balaguer, 2020; Balaguer et al., 2019; Santos and Castro, 2014). NRs are superfamily of phylogenetically related transcription factors characterised by the presence of a DNA binding domain and Ligand binding domain (LBD) that regulate gene transcription constitutively or upon binding to a ligand in the form of monomers, dimers, and heterodimers (Germain et al., 2013; Markov et al., 2008; Bain et al., 2007; Gronemeyer et al., 2004; Escriva et al., 2000; Mangelsdorf et al., 1995). NR proteins are specific to metazoans and can show high levels of conservation in function and ligand-binding affinity across distinct and evolutionary distant phyla (Bodofsky et al., 2017; Bridgham et al., 2010; Escriva et al., 2004). Invertebrate NRs have consequently received increasing attention and turned out to be efficient tools to screen potential EDCs; however, the *ex vivo* and *in vitro* approaches applied to invertebrates provided limited information regarding the biological functions of these transcription factors as well as the possible adverse effects of their exogenous modulation by xenobiotics at the organism level (Knigge et al., 2021; Cuvillier-Hot and Lenoir, 2020; Ford and Le Blanc, 2020; Santos and Castro, 2014). Moreover, the NR superfamily varies in members and functions depending on the taxonomic clade, so that such approach has been applicable to exiguous classes of invertebrates and of NRs (Miglioli et al., 2021; Cuvillier-Hot and Lenoir, 2020; Bridgham et al., 2010; Bertrand et al., 2004). Only one NR shows conserved ligand binding capabilities throughout metazoans: the Retinoic X Receptor (RXR) (Fonseca et al., 2020). Despite RXR is normally considered as an orphan receptor, whose activity is subordinated to one of its heterodimeric partners, it was shown to bind to and transactivate in response to the *bona fide* ligand 9-*cis*-Retinoic Acid, as well as Organotin EDCs in most invertebrate clades (Miglioli et al., 2021; Fonseca et al., 2020; Evans and Mangelsdorf, 2014; Benoit et al., 2006). In the EDC context, the binding of RXR to the Organotin Tributyltin (TBT) and Triphenyltin (TPT) was linked to the insurgence of female masculinisation and infertility in gastropod molluscs, as well as developmental teratogenicity and shell biogenesis defects in bivalves (Castro et

al., 2007; Alzieu et al., 2000). However, even for this emblematic case, the formal *in vivo* demonstration of the relationships between the biological effects of Organotin and the modulation of RXR activity is still lacking (Ford and Le Blanc, 2020; Fernandez et al., 2019). Moreover, the physiological role of this receptor in molluscan neuro-endocrine regulation is largely unexplored. In this light, in the present work the role of RXR and the possible *in vivo* mechanisms of action of the organotin TBT were investigated in the model of early development of the mediterranean mussel *Mytilus galloprovincialis*. To this aim, RXR (*MgRXR*) was first searched in *M. galloprovincialis* NR complement and compared with orthologs from other metazoan, to assess its ligand binding capabilities to TBT, the *bona fide* ligand 9-*cis*-Retinoic Acid (RA) and its full antagonist UVI3003 (Nahoum et al., 2007; Egea et al., 2002).

The effects TBT were investigated on the process of larval shell biogenesis and onset of the neuroendocrine signalling by immunohistochemistry and *In situ* Hybridisation-ISH, and compared with those of of the RXR *bona fide* ligand 9-*cis*-RA. The relationships between the observed adverse outcomes of TBT, 9-*cis*-RA and RXR activation were tested by performing rescue experiments by co-incubation of the developing larvae to either 9-*cis*-RA and TBT alone and the RXR full antagonist UVI3003 (Nahoum et al., 2007; Egea et al., 2002). To shed some light on the possible relationships between the observed adverse outcomes and *MgRXR* developmental functions, its expression pattern was further investigated by ISH. In addition, given the functioning of RXR in heterodimeric complexes, the expression pattern of *MgRXR* was compared with those of some of its known heterodimeric partners suspected to carry out neuro-developmental functions in marine invertebrates and the molluscan *phylum*, *i.e.* Thyroid Hormone Receptor (THR), Retinoic Acid Receptor (RAR), Peroxisome Proliferator-Activated Receptor (PPAR) and Hormone Receptor 38 (NR4A/HR38) (Miglioli et al., 2021). The results of the present work provide the very first description of the plausible physiological pathways by which RXR disruption upon binding to TBT affects larval development in a marine mollusc. This represents a step forward in the understanding of the mechanisms of endocrine disruption in invertebrate organisms.

2. Materials and Methods

2.1. Identification of putative nuclear receptors in the genome of *Mytilus galloprovincialis*

Putative *M. galloprovincialis* NR sequences were searched in the published genome of the mediterranean mussel (Gerdol et al., 2020) through a combination of Blastx, tBlastn and Blastp. Searches were performed using the protein sequences of the isolated DBDs and LBDs of 48 *H. sapiens*, 21 *D. melanogaster* and 38 *C. gigas* canonic NR as templates (Vogeler et al., 2014). The template sequences were retrieved from GenBank and the IDs are reported in Table 1. The putative NR sequences isolated from *Mytilus* genome (*MgNRs*) were verified by the identification of a single DBD (NUCLEAR_REC_DBD, zf C4-type, pfam00105) and LBD (NR_LBD, pfam00104) using Expasy-Prosit (<https://prosite.expasy.org/>) and NCBI conserved domain database (Lu et al., 2020) that also served for a first annotation of the NRs. In order to preliminary establish the phylogenetic relationships of *MgNRs* with respect to other metazoans, the amino acid sequences of the DBDs and LBDs of the 34 *MgNRs* isolated were aligned with the ones of the template sequences with Muscle (v.3.8.31). The alignment was then used to perform maximum likelihood (ML) phylogenetic analysis. The tree was constructed using MEGAX with a JTT+I+G substitution matrix (determined as best model) and nodes were assessed with 1,000 bootstraps (BS).

Nuclear receptor subfamily group	Nuclear receptors	<i>H. sapiens</i>	<i>D. melanogaster</i>	<i>C. gigas</i>	<i>M. galloprovincialis</i>
1A	THRa THRb	P10827 P10828		XP_011443300	VDI39449
1B	RARa RARb RARg	P10276 P10826 P13631		XP_019927427	VDI75840
1C	PPARa PPARb PPARg	Q07869 Q03181 P37231		XP_011412920	VDI52408 VDI49937 VDH90301
1D	Rev-Erb a Rev-Erb b E75 CgNR1CDEFa CgNR1CDEFb	P20393 Q14995	P17672	XP_034303845 XP_011445715 XP_011446959	ABU89808 VDI35675 VDI76186
1E	E78		P45447	XP_011419905	VDI25772
1F	RORa RORb RORg DHR3	P35398 Q92753 P51449	P31396	XP_034321282	VDI83019
1H	Ecr LXRb LXRa FXR	P55055 Q13133 Q96R11	P34021	XP_011416716	VDI41128
1I	VDR PXR CAR	P11473 O75469 Q14994			
1J	DHR96 NRja NRjb NRjc		Q24143	XP_011436006 XP_011428273 XP_011454352	VDI41434 VDI17683 VDI45893 VDI43945
1P	NR1P NR1P1/7 NR1P1 NR1P2 NR1P3 NR1P4 NR1P5 NR1P6 NR1P7 NR1P8 NR1P9 NR1P10 NR1P11 NR1P11a NR1P11b NR1P12 NR1P13			XP_034307552 XP_011449920 XP_011431575 XP_011439771 XP_011431105 XP_011423680 XP_011431570 XP_011431576 XP_034307585 XP_011431624 XP_011431632	VDI41594 VDI43370 VDI43372 VDH95509 VDI77872 VDI27984 VDI24803 VDI63409
2A	HNF4a HNF4b HNF4	P41235 Q14541	P49866	XP_011451244	VDI60739
2B	RXRa RXRb RXRg USP	P19793 P28702 P48443	P20153	XP_011434498	VDH93983
2C	TR2 TR4	P13056 P49116			
2D	DHR78-TR2/4		Q24142	XP_011438114	VDI70513
2E	TLX ILL PNR/DHR51 DSF	Q9V466 Q9V5X4	P18102 AAM68536 AAD05225	XP_011438551 XP_011433241 XP_011416690	VDI42436 VDI48231 VDI68522
2F	COUP-TF1 COUP-TF2 SVP EAR2	P10589 P24468 P10588	P16375	XP_011456035	VDI02832
3A	ER a ER b	P03372 Q92731		XP_011424805	VDI74939
3B	ERRa ERRb ERRg DERR	P11474 Q95718 P62508	AAF50473	XP_011414542	VDI7225
3C	GR MR PR AR	P04150 P08235 P06401 P10275			
4A	NGFIB NURR1 NOR1 DHR38	P22736 P43354 Q92570	P49869	XP_034301982	VDI18106
5A	SF1 LRH FTZ-F1	Q13285 NP_995582	P33244	XP_0114387919	VDI56451
5B	DHR39		Q05192	XP_011413193	
6A	GCNF DHR4	Q15406	Q9W539		VDI62239
7/8A	NR7/8			XP_011436094	VDI47149

Table 1: Nuclear receptor amino acid sequence GenBank accession numbers for *Homo sapiens*, *Drosophila melanogaster*, *Crassostrea gigas* and *Mytilus galloprovincialis*

2.2. *MgRXR* Sequence Analysis

Once the clear RXR ortholog was identified among the *MgNRs*, the LBD was isolated and subjected to further analysis to identify the residues identified in previous studies to interact with its *bona fide* agonist 9-*cis*-Retinoic Acid (9-*cis*-RA), the EDC Tributyltin (TBT) and its full antagonist UVI3003 (Nahoum et al., 2007; Fonseca et al., 2020). The LBD of *MgRXR* was aligned to the LBDs of other molluscs and metazoans whose sequences were retrieved from Genbank database: *Homo sapiens* (NP_002948.1), *Priapulid caudatus* (QFQ33540.1), *Bonellia viridis* (MT264999.1), *Platynereis dumerilii* (AVR59237.1), *Lottia gigantea* (ESO92876.1), *Patella vulgata* (ALQ43971.1), *Nucella lapillus* (ABS70715.1), *Reisha clavigera* (AAU12572.1), *Mytilus galloprovincialis* (VDH93983.1), *Crassostrea gigas* (XP_011434492.1), *Phoronopsis californica* (MT264997.1), *Bugula neritina* (MT265000.1), *Aurelia aurita* (AGT42223.1), *Trichoplax adherens* (ATD53319.1). The sequences were aligned using Muscle (v.3.8.31) and the alignment was analysed and edited in Geneious® (v7.1.7).

2.3. *Mussels, gamete collection and fertilization*

All procedures were carried out as previously described (Miglioli et al., 2019, 2021a, 2021b). Sexually mature specimens of *M. galloprovincialis* were collected from the natural population in the Bay of Villefranche-sur-mer (43.682°N, 7.319°E - France) during the spawning season of 2020/21 (December–March). Animals were maintained at the Institut de la Mer de Villefranche (IMEV) by the Centre de Ressources Biologiques Marines of the institute (CRBM), transferred to the laboratory and acclimatized in flow-through vessels containing Millipore filtered natural seawater-MFSW (pH 7.9–8.0, 38 ppt salinity, 15°C). Gametes were obtained by heat-shock induced spawning in MFSW (Miglioli et al., 2019). At the moment of emission, sperms and eggs were filtered to remove eventual contaminations with 55µm and 100µm nylon mesh filters, respectively, and suspended in MFSW. Fertilisation was then performed following the guidelines described in standard embryo-toxicity protocols (ISO 17244:2015). After checking quality of the gametes (eggs shape and size; sperm motility), eggs and sperms were mixed in culture vessels filled with MFSW (pH7.9-8.1, salinity 36 ppt and temperature 16°C) identified by the presence of the polar body, a globule forming at the animal pole of the egg cell. After 30 min, fertilization success (n. fertilized eggs/n. total eggs x 100) was verified by microscopic observation (usually >90%). Larval cultures were prepared in MFSW with a maximum concentration of 200 embryos/mL (Fabbri et al., 2014).

2.4. Experimental conditions of exposure

Fertilized eggs were exposed to increasing concentrations of Tributyltin-TBT (Sigma-Aldrich, France), a model EDC interacting with RXR, the RXR *bona fide* ligand 9-*cis*-Retinoic Acid (R 4643, Sigma-Aldrich, France) and the RXR full antagonist UVI3003 (B6688, Sigma-Aldrich, France). The range of concentrations for the test substances was chosen in order to build a dose-response curve of 9-*cis*-Retinoic Acid, TBT and identify the highest non-toxic concentration of UVI3003 (final concentrations 0.1, 1, 10, 100 nM of 9-*cis*-Retinoic Acid; 0.1, 1, 10 and 100 nM TBT; 0.5 and 5 μ M UVI3003). Concentrations within the range were then selectively tested depending on the endpoint measured. Culture vessels were chosen depending on the type of analysis: 50 mL cell culture Flasks for situ Hybridisation (ISH) and Immunostaining practices (Miglioli et al., 2021 a, b), 24-wells and 48-wells plates for the study of shell biogenesis and larval development (Miglioli et al., 2019). The desired final concentrations of chemicals were obtained from stock solutions of 9-*cis*-Retinoic Acid (10mM in Dimethyl-sulfoxide/DMSO) TBT (10 mM in MFSW) and UVI3003 (50mM in Dimethyl-sulfoxide/DMSO) suitably 10X serially diluted in MFSW. Parallel control samples were run in MFSW added with the vehicle at the final concentration utilised (0.01% DMSO).

2.5. Effect of 9-*cis*-Retinoic Acid, TBT and UVI3003 on larval growth at 48 hpf

To test the potential toxic effects of the chemicals on *M. galloprovincialis* larval development, fertilized eggs were exposed to the chemicals (0.1, 1, 10, 100 nM 9-*cis*-Retinoic Acid; 0.1, 1 and 10 nM TBT; 0.5 and 5 μ M UVI3003) and vehicle (0.01% DMSO) in 50mL flasks for suspended cell cultures at 30 mins pf. Their effect was firstly evaluated at 48 hours post fertilisation (hpf) by scoring the percentage of normally developed D-veliger larvae according to the bivalve larval toxicity assay (ISO 17244:2015) and measuring shell lengths in larvae obtained from, minimum, 3 independent parental pairs and in at least 50 larvae for each parental pair. In each flask, 5 mL of the 10X solutions were added to 45 mL of fertilized eggs suspension (0.01% DMSO). Larvae were sampled and concentrated at 48 hpf with a 60 μ m nylon filter, fixed overnight in 4% Paraformaldehyde in Phosphate Buffer Saline-PBS (137 mM NaCl, KCl 2.7 mM, 10 mM Na₂HPO₄, KH₂PO₄ 1.8 mM, pH 7.4) and imaged with a Zeiss Axio Imager A2 (Zeiss, France). The frequency of shell malformations (% values) and mean shell lengths (μ m) were measured. Shell lengths were obtained by measuring the anterior-posterior dimension of the shell parallel to the hinge line and the results are presented normalized as % of controls. A larva was considered normal when the shell was enclosed and D-shaped (straight hinge), malformed when developmental arrest prevented the larva to reach the stage typical for 48 hpf (trochophore or earlier stages) or when some developmental defects were observed (concave, malformed or damaged shell, protruding mantle) (Fabbri et al., 2014).

Accordingly, the larval phenotypes were grouped into four main categories: Normal D-Veligers (D-shaped shell and straight hinge), Pre-Veligers (immature Veligers with mantle protrusions), Malformed hinge (D-Veligers showing an unregular hinge) and Arrested trochophorae (larvae that did not reach the Veliger stage). Representative images of the listed phenotypes are shown in Fig. 1. The acceptability of test results was based on control samples for a percentage of normal D-shell stage larvae >75% (ISO 17244:2015).

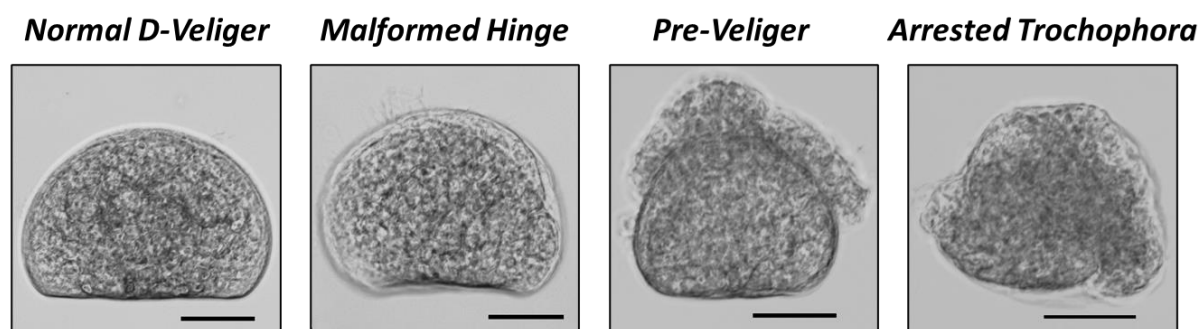


Figure 1: Representative images of 48 hpf D-Veliger larvae of *Mytilus galloprovincialis* showing normal morphology, malformations and arrested development. In physiologic conditions, larvae of *M. galloprovincialis* reach the D-Veliger stage by 48 hpf. The D-Veliger is characterised by a D-shaped shell with a straight hinge that encloses the whole larval body. When exposed to stressors, the larvae can manifest abnormal shell morphologies or developmental arrest which are referred to as phenotypes. The malformed hinge phenotype is applied when the hinge is not straight, the Pre-Veliger is assigned when the larval body is not entirely enclosed in the D-shaped shell, when the larvae do not reach the D-Veliger stage and their development is arrested to the trochophore stage they are referred to as arrested trochophorae. Scale bar: 20 μ m

2.6. Effect of 9-cis-Retinoic Acid, TBT and UVI3003 on shell formation: organic matrix deposition and calcification.

To test the potential adverse effect of the chemicals on shell formation, fertilized eggs were exposed to 1, 10 and 100 nM 9-cis-Retinoic Acid, 1 and 10 nM TBT, 5 μ M UVI3003 and the vehicle (0.01% DMSO) in 24-wells plates at 30 mins pf. The time-course of shell formation was investigated at different hours post fertilization (hpf) between the trochophora and the D-veliger stage (24, 28, 32 and 48 hpf). Larval shell components were visualized by Calcofluor White Fluorescent Brightener 28 (Sigma Aldrich, Lyon, France) for organic matrix, and Calcein (Sigma Aldrich, Lyon, France) for CaCO₃ deposition, respectively (Miglioli et al., 2019, 2021a, 2021b). Stock solutions of Calcein (4 M in Dimethyl-sulfoxide/DMSO) were diluted in MFSW at 4 mM (4x final concentration) and directly added to the culture medium. Calcofluor (UV channel, Exc: 408 nm/Em: 450–490 nm) was added to each single well at the time of sampling (final concentration 0.02 mM in 0.01% DMSO), as

previously described (Miglioli et al., 2019). In each well, aliquots of 0.5 mL of 4x Calcein (FITC channel, Exc: 488 nm/Em: 520–560 nm) solution and 200 μ L of 10x solutions were added to 0.5 mL of fertilized egg suspension (0.01% DMSO in MFSW) to reach the final nominal concentrations in a 2 mL volume. Calcofluor was added to the wells 5 mins prior to sampling, the excess was abundantly rinsed with MFSW prior to larvae immobilisation in 4% PFA-MFSW and the imaging was performed immediately after (Miglioli et al., 2019; 2021a, b). Larvae were imaged with a Leica SP8 Confocal Laser Scanning Microscope (CLSM - Leica, France) scanning sequentially Brightfield, Calcofluor, and Calcein signals with a 0.5 μ m Z-stack interval. Channels were merged, 3D rendered and rotated to measure the area (in μ m²) of each shell component in a single valve of each larva by manual drawing using IMAGEJ software (Kapsenberg et al., 2018; Miglioli et al., 2019, 2021a, 2021b). Controls cultures were considered acceptable when the mean values of organic matrix and calcified areas or the larvae at the distinct developmental stages fitted within the range of values reported in Table 2 (Miglioli et al., 2019). Analyses were performed on larvae from 3 independent parental pairs (at least 15 individuals for each parental pair and experimental condition). Data are presented normalized as % of controls.

<i>Hours post fertilisation</i>					
24		28		32	
<i>Organic Matrix</i> (μ m ²)	<i>Calcified Shell</i> (μ m ²)	<i>Organic Matrix</i> (μ m ²)	<i>Calcified Shell</i> (μ m ²)	<i>Organic Matrix</i> (μ m ²)	<i>Calcified Shell</i> (μ m ²)
≥180	0	≥1000	≥ 500	≥2000	≥1500

Table 2: Minimum area (μ m²) of organic matrix and calcified shell at the different hours post fertilisation determining the acceptability of the control cultures.

2.7. Prevention of the effects of 9-cis-Retinoic Acid and TBT on shell formation by co-incubation with UVI3003

In order to assess if the possible effects induced by 9-cis-Retinoic Acid and TBT on larval development might be due to *Mg*RXR ligand-activation, we tested if pharmacological blockage of RXR activity by the full-antagonist UVI3003 could prevent the responses to the *bona fide* ligand and to TBT alone. The rescue experiment was thus performed by co-exposing fertilized eggs to the highest non-effective concentration of UVI3003 (5 μ M) and either 9-cis-Retinoic Acid or TBT at concentrations able to induce shell malformations (10nM) chosen on the basis of experiments described in previous paragraphs. Experiments were carried out in 48-wells plates. The negative control added with the vehicle (0.02% and 0.01% DMSO for 9-cis-Retinoic Acid and TBT rescues

respectively) and the three positive controls added with 5 μ M UVI3003 and 10 nM of 9-*cis*-Retinoic Acid or TBT, were run in parallel. At 48 hpf, larvae were fixed in 4% PFA and imaged with a Zeiss Axio Imager A2 (Zeiss, France). The effect of the treatments and success of the rescue on global larval development were evaluated by scoring shell malformations and measuring shell lengths in larvae from 3 independent parental pairs and in at least 50 larvae for each parental pair as described in previous paragraphs.

2.8. *In situ* Hybridisation

In situ hybridisation protocols were carried out as previously described (Miglioli et al., 2019; 2021 a,b). In brief, larvae were grown in 50 mL flasks for suspended cell cultures in MFSW added with 10 nM 9-*cis*-Retinoic Acid, TBT and the vehicle. Larvae were then sampled at 32 and 48 hpf, fixed in 4% PFA and preserved in 100% Methanol. The ISH protocol developed for *M. galloprovincialis* larvae (Miglioli et al., 2019; 2021a, b) served in the first place to investigate the expression pattern of *MgRXR* in physiologic conditions and after exposure to 9-*cis*-Retinoic Acid and TBT. In order to understand the tissues and/or cell populations in which *MgRXR* might be localised, *MgRXR* expression pattern was compared with the ones of Tyrosinase (Tyr), a key enzyme for larval shell morphogenesis and growth, and the elements of the dopaminergic signalling pathway (Dopamine Receptor 1 - DR1; Dopamine Tyrosine Hydroxylase – TH, Dopamine- β -Hydroxylase – DBH) which were proved to play pivotal roles in larval shell formation and physiology in bivalve molluscs as described in previous work (Miglioli et al., 2019, 2021 a, b). In addition, the expression pattern of *MgRXR* was as well confronted with the immunoreactivity signals of the neurotransmitter serotonin (5-HT) and γ -aminobutyric acid (GABA), also described in previous work (Miglioli et al., 2021 a, b). Consequently, the protocol was further employed to investigate the effect of 9-*cis*-RA and TBT on the expression pattern of Tyrosinase (Tyr) and the elements of the dopaminergic signalling pathway (Dopamine Receptor 1 - DR1, Tyrosine Hydroxylase – TH, Dopamine- β -Hydroxylase – DBH). Finally, since RXR transcriptional activity is normally considered to be subordinated to the activity of its heterodimeric partners, we compared the expression pattern of *MgRXR* with the ones of its heterodimeric partners *MgTHR*, *MgRAR*, *MgPPARa* and *MgNR4A/HR38* to derive which heterodimers could be at work in *M. galloprovincialis* development. The ISH was carried out on larvae from three independent parental pairs and the primers employed for ISH-probe synthesis and template sequence IDs are shown in Table 3. Analyses were performed in larvae obtained from three independent parental pairs.

	Gene	Primers	Sequence ID
Nuclear Receptors	<i>MgRXR</i>	Fw: ATGTCATCAGGGGGCATGG	MGAL_10B092503T1
		Rev: GGTATTAGTTGGGCTTTCTA	
	<i>MgTHR</i>	Fw: CGCGTCGACAATGGACAACAGAATATATTCAAA	MGAL_10B092997T1
		Rev: CGCGCGGCCGCTTACAGGAAGTCAAATAAATTCAAC	
	<i>MgRAR</i>	Fw: CGCCCCGGAATGGAAGCATGAAGAGAA Rev: CGCGCGGCCGCTTAGGGCATTTCAGCCGTAA	MGAL_10B093840T1
<i>MgNR4A</i>	Fw: ATGTTACTTTTACAGCCACAGG Rev: AAATGGTAGTGCACCTTGATAAAT	MGAL_10B093974T1	
<i>MgPPARa</i>	Fw: ATGCGACATGATTTTGACCA Rev: TATATTAACATTTCCTTTGATTAATGGT	MGAL_10B094094T1	
Shell Formation	Tyrosinase-3	Fw: ATGCGATTCTTTATAACATGAAA Rev: TTGGTGGTTTTGGTACATGT	KV583276.1
Dopaminergic Markers	Dopamine Receptor 1	Fw: ATGATAACAAATTTTCTATATTGG Rev: ACAAAAGCAGTTACCTTGTCTT	Unigene27870_All
	Tyrosine Hydroxylase	Fw: ATGATTACCCGACACCTCG Rev: ACACCTCAGTTCTTTTACTG	GGUW01014444.1
	Dopamine - β - Hydroxylase	Fw: CCTTCTGAAGTTTGCCTTTTC Rev: CTGGCTCCCCGGGCACT	GHIK01112718.1

Table 3: Primer pairs and IDs of the template sequences employed for synthesis of *In situ* Hybridisation probes.

2.9. Immunocytochemistry

In order to evaluate the possible neurodevelopmental effects of 9-*cis*-RA and TBT, antibodies against the neurotransmitter serotonin (5-HT) were employed to visualize and estimate the population of serotonergic neurons in 48 hpf in larvae grown in control conditions and exposed to 10 nM of each chemical. The immunostaining protocols was carried out as previously described (Miglioli et al., 2021 a, b). Briefly, subsamples of the same larval pools used for ISH were rehydrated from 100% Methanol, decalcified with 0.1 M Ethylenediaminetetraacetic acid (EDTA) in PBS. Non-specific binding sites were minimized by overnight incubation in Blocking Solution [10% normal goat serum, 0.25% bovine serum albumin (BSA), 0.03% sodium azide (NaN₃), PBS-Tx 1% (Triton X-100)] at 4° C. D-Veligers were then washed in PBS-Tx 0.1%, soaked for 10 min at RT in the primary Antibody Buffer (1:10 Blocking Buffer in PBS) and added with 1: 5000 polyclonal primary antibody against Rabbit 5-HT (Immunostar, Hudson, WI, USA) with which they were incubated for 5 days at 4° C. After rinsing in PBS-Tx 0.1%, larvae were incubated for 3 days at 4°C with 1:500 Goat Anti-Rabbit Rhodamine Red™-X (RRX) IgG secondary antibody (Ex/Em: 570/590 nm - Jackson laboratory, Bar Harbor, Maine, USA) in PBS-Tx 0.1%. Non-specific binding was evaluated in subsamples incubated only in the presence of the secondary antibody (not shown). The nuclei were stained with 1 µg/mL Hoechst in PBS (UV, Ex/Em: 352/461nm - Hoechst 33342, Invitrogen) and mounted in CitiFluor™

AF1 (pH 10, Agar scientific). Images were acquired with a Leica Sp8 Confocal microscope with a 0.5 μm interval between each stack along the z axis. To assess the number of 5-HT-*ir* cells, subsequent Z-stacks were assembled using ImageJ following the steps previously described (Miglioli et al., 2021b).

2.10. Statistical Analyses

All data obtained from at least three independent parental pairs were initially analysed with Shapiro-Wilk normality test, statistical differences were assessed accordingly with either nonparametric ANOVA (Kruskal-Wallis test) followed by the Dunn's test ($p < 0.05$) or One-Way ANOVA followed by Tukey's test ($p < 0.05$). The test used for each analysis is indicated in the corresponding result section. All statistical calculations were performed using GraphPad Prism 5 software (GraphPad Inc.).

3. **Results**

3.1. ***Isolation and Characterisation of Mytilus galloprovincialis RXR***

3.1.1. ***The Nuclear Receptors of M. galloprovincialis***

Thirty-four canonical NR proteins were identified in *M. galloprovincialis* genome. Putative NRs were verified upon the presence of a single conserved DNA and ligand binding domains. The protein IDs for each *MgNR* are given in Table 1. Phylogenetic analysis was performed using the amino acid sequences of the DBD and LBD of the 34 *MgNRs* and the maximum likelihood tree is shown in Fig. 2. *M. galloprovincialis* NRs include representatives of all the seven NR subfamilies investigated and the preliminary phylogenetic analysis indicated that most of the canonical *MgNRs* fit in the current nomenclature and classification, with the only exception of the representatives of the bivalve specific NR1P subfamily, previously described in the pacific oyster *C. gigas*, and the subfamily NR1J described in molluscs, insects, and nematodes (Vogeler et al., 2014; Kaur et al., 2015). The preliminary phylogeny of *MgNRs* segregated NR1s in a monophyletic group and the subfamilies NR2-NR7/8 in a second major clade, similarly to what was previously described for the NR complements of *C. gigas*, *B. glabrata* and *L. gigantea* (Vogeler et al., 2014; Kaur et al., 2015). Twenty-three of the 34 *MgNRs* are members of subfamily NR1, with 4 of them belonging to the protostome NR1J group, 2 to the mollusc NR1CDEF group and 8 of them clustering with the bivalve NR1P group (Vogeler et al., 2014; Kaur et al., 2015). Leaving the invertebrate NR1J and molluscan NR1P subgroups aside, *M. galloprovincialis* possesses single orthologs for all the *MgNR1s* traditional subgroups, confirming and corroborating data previously obtained in the pacific oyster, apart from the NR1C (PPAR) sub-group, for which three *MgNRs* were found. Seven *MgNRs* belong to subfamily 2 (NR2), with a single NR in each subgroup except for NR2E, for which three *MgNRs* clustering with *D. melanogaster* TLL, *H. sapiens* TLX and *H. sapiens* PNR / *D. melanogaster* DHR51 were found. Two *MgNRs* fitted in subfamily 3 (NR3) with high support values with respect to human ER (NR3A) and human/fruit fly ERR (NR3B), whereas no *MgNRs* clustered in NR3C. Only one *MgNR* clustered with subfamilies NR4 and NR5. With regards to NR5, the only *MgNR5* belonged to the subgroup NR5B, while none clustered with NR5A. Surprisingly, *M. galloprovincialis* NR complement includes a representative of sub-group NR6A previously believed to be lost in bivalve molluscs, which showed high support values to both the fruit fly and human orthologous sequences (Vogeler et al., 2014; Kaur et al., 2015). *M. galloprovincialis* NR complement also includes a representative of the newly discovered subfamily NR7/8 (Huang et al., 2018).

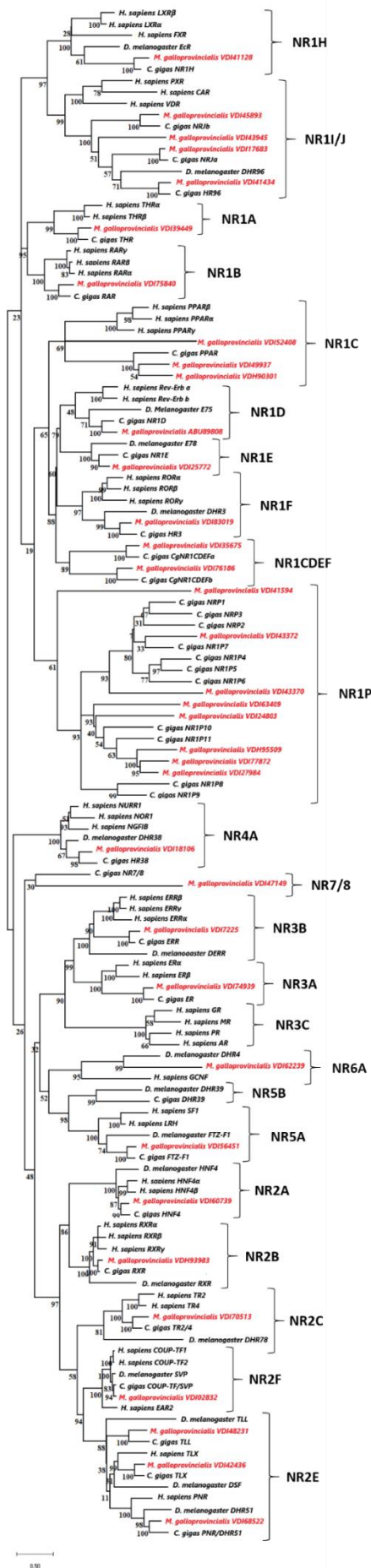


Figure 2: Phylogenetic relationships of nuclear receptors in *Mytilus galloprovincialis*, *Crassostrea gigas*, *Homo sapiens* and *Drosophila melanogaster*. The alignment was constructed using the DBD plus LBD and phylogenetic relationship was conducted by a Maximum likelihood (ML) analysis. ML support values (percentage of 1000 Bootstraps) are provided above the nodes. *MgNRs* are reported in red, the subfamily groups are reported next to the correspondent NRs.

3.1.2. Sequence analysis of *MgRXR*

Among the 7 *MgNR2s*, only one protein (Genbank ID: VDH93983.1) showed high bootstrap values with *H. sapiens*, *C. gigas* and *D. melanogaster* RXR/USP. Accordingly, *Mytilus galloprovincialis* has a single RXR. The LBD of *MgRXR* was further investigated to understand its affinity to *bona fide* ligand 9-*cis*-Retinoic Acid (9-*cis*-RA), the endocrine disruptor Tributyltin (TBT), previously shown to bind and activate RXR throughout metazoans (Fonseca et al., 2020), and the full antagonist of vertebrate RXR UVI3003. Alignment of the LBD amino acid sequence of *MgRXR* with those from *H. sapiens* RXR α and the newly isolated RXRs of other invertebrates from different levels of the metazoan tree of life, including bivalve and gastropod molluscs (Fonseca et al., 2020), is shown in Fig. 3. The 18 amino acids forming the ligand binding pocket in human RXR α were identified. The affinity of *MgRXR* for 9-*cis*-RA was assessed by searching for the arginine residue in Helix 5, that permits binding to and transactivation of the human RXR α (Egea et al., 2002). The affinity for TBT and organotin was assessed by individuating the cysteine in Helix 11, the essential residue for the binding and transactivation in human RXR α (Hiromori et al., 2015). The affinity to UVI3003 was evaluated by searching for the Leucine 451 situated in the Helix 12 of the human RXR α (Nahoum et al., 2007). This residue showed to be conserved throughout the investigated invertebrates, suggesting that UVI3003 could be a universal inhibitor of RXR activity in metazoans. The presence in *MgRXR* LBD of all the elements required for interaction with the three chemicals suggested that the activity of the receptor could be susceptible to these modulators *in vivo*.

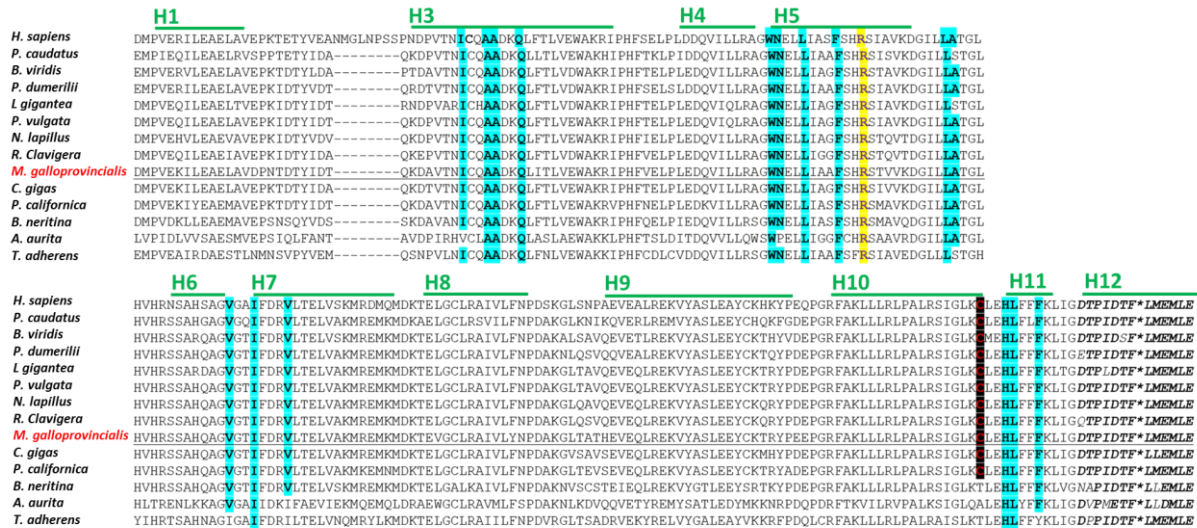


Figure 3: Alignment of RXR ligand binding domains (LBD). Sequences correspond to *Homo sapiens* RXR α (NP_002948.1), the Ecdysozoan *Priapulus caudatus* (QFQ33540.1), the annelids *Bonellia viridis* (MT264999.1) and *Platynereis dumerilii* (AVR59237.1), the gastropod mollusc *Lottia gigantea* (ESO92876.1), *Patella vulgata* (ALQ43971.1) and *Nucella lapillus* (ABS70715.1), the bivalve mollusc *Reisha clavigera* (AAU12572.1), *Mytilus galloprovincialis* (VDH93983.1) and *Crassostrea gigas* (XP_011434492.1), the Phoronida *Phoronopsis californica* (MT264997.1), the Bryozoan *Bugula neritina* (MT265000.1), the cnidarian *Aurelia aurita* (AGT42223.1), the placozoan *Trichoplax adherens* (ATD53319.1). The α -helices from human RXR α are indicated in green. The residues forming the ligand binding pocket in human RXR α are indicated in cyan, the arginine residue determining the ionic interaction with 9-cis-retinoic acid is indicated in yellow, the cysteine residue forming the covalent bond with TBT is red and highlighted in black, the leucine 451 in H 12 determining the interaction with UVI3003 is indicated with an asterisk.

3.2. Effect of 9-*cis*-RA on larval shell biogenesis.

3.2.1. Effect of 9-*cis*-RA on development of D-Veligers at 48 hpf.

The phenotypic effects of different concentrations of 9-*cis*-RA (0.1, 1, 10 and 100 nM) were first evaluated on the formation of normal D-larvae at 48 hpf and the results are reported in Fig. 4. 9-*cis*-RA induced a concentration-dependent decrease in the percentage of normally developed D-Veligers (Fig. 4 A), as well as a significant decrease in mean shell length (Fig. 4 B). From 1 nM 9-*cis*-RA induced the formation of up to 40% of immature larvae, characterized by mantle protrusion (Fig. 4 A). The occurrence of this phenotype was also predominant at increasing concentrations of 9-*cis*-RA (up to 90% at 100 nM), while other phenotypes such as arrested trochophores remained negligible (Fig. 4 A). Interestingly, no significant teratogenicity, i.e. developmental arrest at the trochophora or earlier stages, was observed, irrespective of the concentration. In line with the phenotypic evidence, also the mean shell size of the immature D-Veligers was significantly decreased by 9-*cis*-RA in a dose dependent manner with 1, 10 and 100 nM concentrations inducing, respectively, a 5, 10 and 20% of shell length reduction with respect to that of controls (Fig. 4 C).

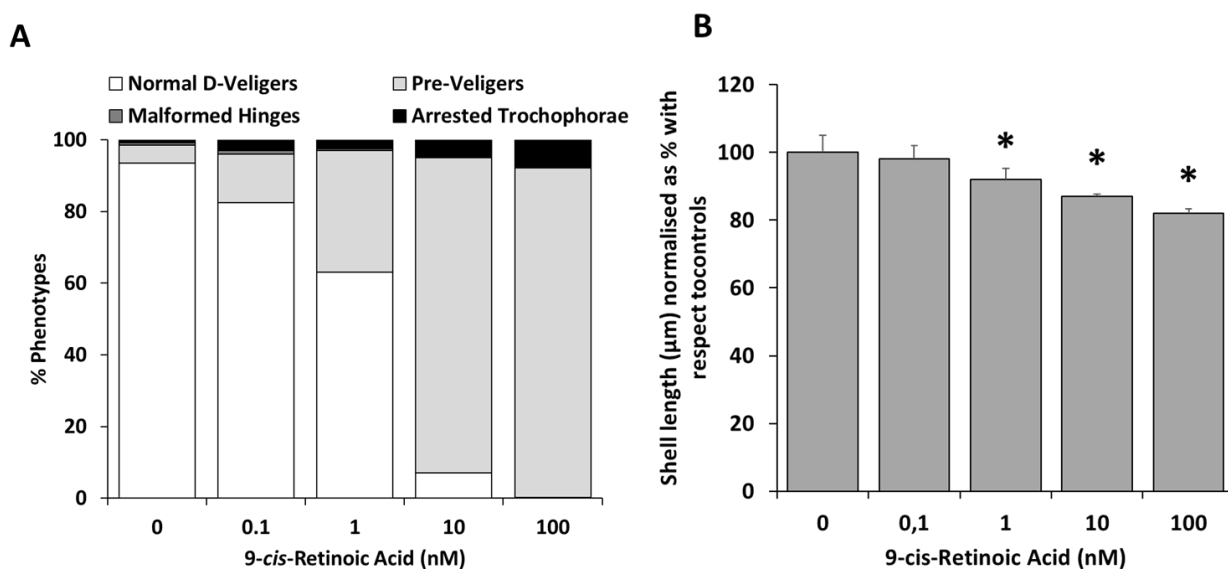


Figure 4: Effect of increasing concentrations of 9-*cis*-RA on the larval development of *Mytilus galloprovincialis* at 48 hpf. A) Percentage of different phenotypes: normally developed D-Veligers (white), Pre-Veligers with protruding mantle (light gray), larvae with hinge malformations (dark gray) and arrested at the trochophora stage (black); B) Shell length of D-Veligers, i.e. the anterior-posterior dimension of the shell parallel to the hinge line normalized as % with respect to controls. N=3. One-way ANOVA followed by Tukey's test (* $p \leq 0.05$).

3.2.2. *Effect of increasing concentrations of 9-cis-RA on larval shell formation from 24 to 48 hpf*

The effect of 9-*cis*-RA on shell formation was evaluated by Calcofluor/Calcein double staining at close times pf between 24 and 48 hpf in order to follow the growth of the organic and calcified components of the larval shell as previously described (Miglioli et al., 2019, 2021a, 2021b). Figure 5 shows the time-course of shell formation at 24, 28, 32 and 48 hpf, with representative z-projections of the merged signals of the organic matrix (blue) and the calcified layer (green) of the growing shell in larvae from each experimental condition. In control samples, shell formation followed the steps previously described (Miglioli et al., 2019): at 24 hpf the larval shell is entirely composed of a saddle shaped organic matrix (blue), by 28 hpf CaCO₃ deposition starts in the centre of each valve, the calcified shell (green) reaches the external borders of the organic matrix by 32 hpf and the whole larval shell is entirely calcified when the larvae reach the D-Veliger stage at 48 hpf. 9-*cis*-RA did not induce evident alterations of shell formation from 24 to 32 hpf with respect to controls. At 48 hpf, when control larvae reached the normal D-veliger stage, larvae exposed to all concentrations of 9-*cis*-RA presented extensive mantle protrusion and a weaker calcein signal, thus having the typical appearance of immature stages. The developmental delay was enhanced in a dose dependent manner with both mantle protrusion and uncomplete calcification being more and more evident in line with increasing concentrations of 9-*cis*-RA. The effect of 9-*cis*-RA was further evaluated quantitatively by analysing the areas occupied by the organic matrix and calcified shell throughout development and the results are presented in Fig. 6. The lowest concentration of 9-*cis*-RA tested, 1nM, induced a significant decrease in the area of the organic matrix only at 24 hpf and of the calcified shell only at 28 hpf, while concentrations of 10 and 100 nM always induced a significant decrease of both larval shell components at all times pf (Fig. 6 A, B, C). The results are summarized as organic matrix:calcified shell ratios at 28 and 32 hpf in Fig. 6 D. At 28 hpf, 1 and 10 nM 9-*cis*-RA induced a stronger decrease in the area occupied by the calcified shell with respect to that of the organic matrix, thus resulting in a higher ratio than in controls. In contrast, 100 nM caused a significant increase in the ratios at both 28 and 32 hpf. In summary, 9-*cis*-RA affects the growth of both organic and inorganic components of the larval shell although calcification is apparently more affected than the growth of the organic matrix.

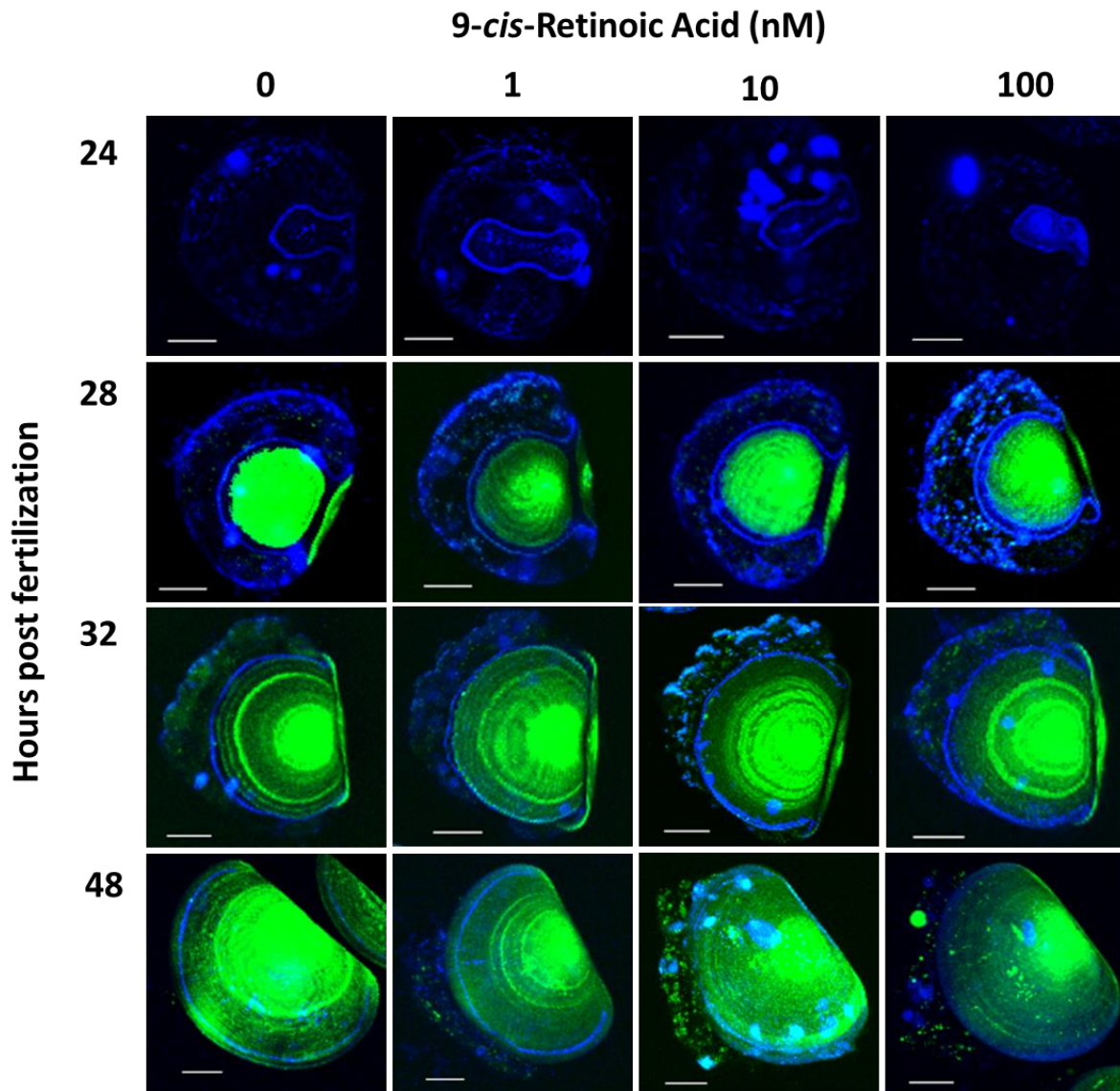


Figure 5: Effect of increasing concentrations of 9-*cis*-RA on the formation of the larval shell in *Mytilus galloprovincialis* larvae at 24, 28, 32 and 48 hpf. Columns correspond to experimental conditions (from left to right: Control, 1, 10 and 100 nM 9-*cis*-Retinoic Acid). Images (lateral view) show merged fluorescent signals of calcofluor (blue) and calcein (green) staining, respectively, the organic matrix and CaCO₃ deposition (scale bars: 20 μm).

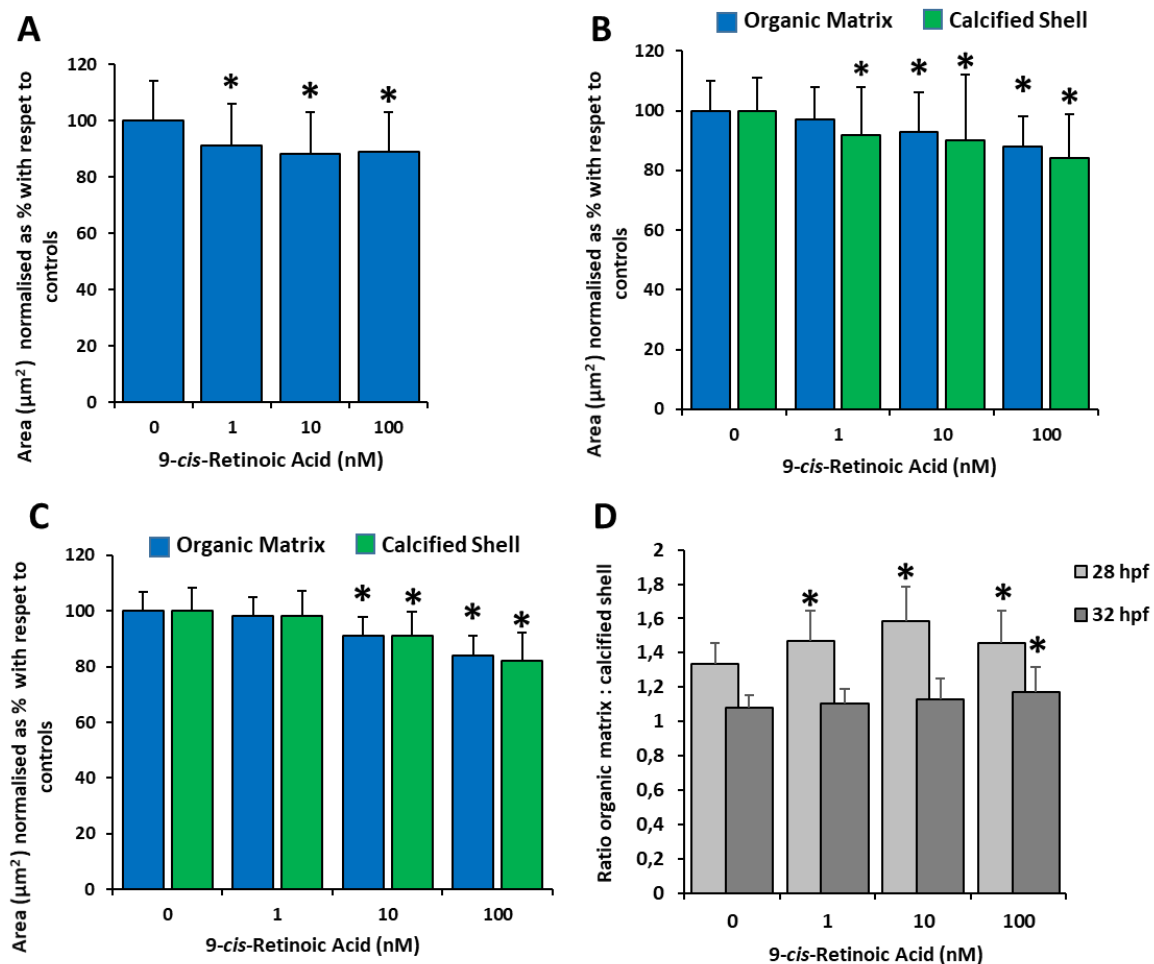


Figure 6: Effect of increasing concentrations of 9-cis-RA on the growth of the organic matrix (blue) and calcified shell (green) in *Mytilus galloprovincialis* larvae from 24 to 32 hpf. Areas of each shell component (μm^2) measured in a single valva of 9-cis-Retinoic Acid-exposed larvae are reported as % values with respect to controls (unexposed) larvae at 24 (A), 28 (B) and 32 hpf (C). In D) ratios of areas matrix/calcified shell in different experimental conditions are reported. Measurements were made on at least 50 larvae for each condition obtained from 3 parental pairs (N=3). Non-parametric Anova (Kruskal Wallis) followed by Dunn's test (* $p \leq 0.05$).

3.3. Effect of TBT on larval shell biogenesis

3.3.1. Effect of TBT on development of D-Veligers at 48 hpf

The phenotypic effect of TBT (0.1, 1, 10 and 100 nM) was first evaluated in 48 hpf larvae and the results are reported in Fig. 7. Similarly to 9-*cis*-RA, TBT induced a concentration-dependent decrease in the percentage of normally developed D-Veligers (Fig. 7 A). Starting by a 1 nM concentration, TBT induced the formation of immature D-larvae characterized by mantle protrusion and, accordingly, a significant decrease in mean shell length of 10 and 40% at 1 and 10 nM TBT respectively (Fig. 7 B). Overall, the effects of TBT were even stronger than those of 9-*cis*-RA, with proportions of immature larvae from 13 to 79 % from 0.1 to 10 nM. (Fig. 7B). At 100 nM, TBT completely prevented larval development from the first hrs post-fertilization (not shown). The percentage of other phenotypes remained negligible with the exception of arrested trochophores that reached the 20% at 10 nM TBT (Fig. 7A).

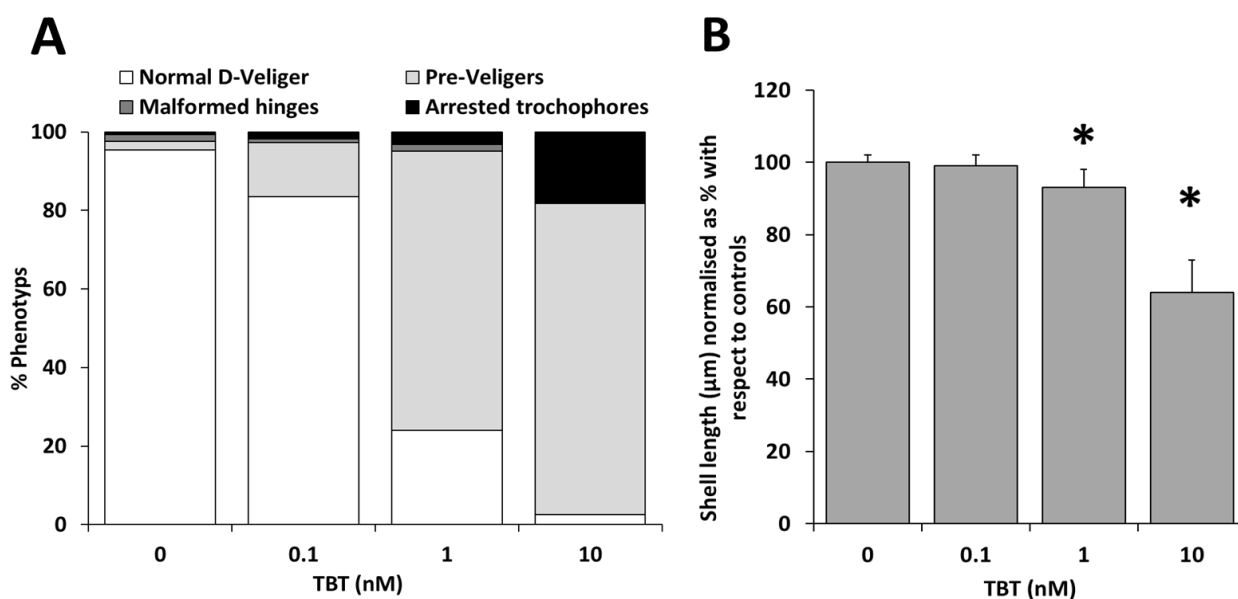


Figure 7: Effect of increasing concentrations of TBT on the larval development of *Mytilus galloprovincialis* at 48 hpf A) Percentage of different phenotypes: normally developed D-Veligers (white), Pre-Veligers with protruding mantle (light grey), larvae with hinge malformations (dark grey) and arrested at the trochophora stage (black); B) Shell length of D-Veligers, i.e. the anterior-posterior dimension of the shell parallel to the hinge line normalized as % with respect to controls. N=3. One-way ANOVA followed by Tukey's test (* $p \leq 0.05$).

3.3.2. *Effect of TBT on larval shell formation from 24 to 48 hpf*

Deposition of the organic matrix and shell calcification were evaluated in larvae exposed to 1 and 10 nM TBT and the results are shown in Fig. 8 in the form of z-projections of the merged channels for the organic matrix (blue) and calcified shell (green). Both concentrations of TBT caused an appreciable delay of the expansion of the organic matrix as well as of the calcified portion of the shell, which was visibly undersized. The effect TBT on shell formation was particularly evident by 48 hpf: larvae exposed to 1 nM TBT showed a weaker calcein signal and mantle protrusions and those exposed to 10 nM TBT also presented incomplete shell calcification in the D-border (Fig.8). These observations are supported by quantitative data (Fig. 9). The mean area of the organic matrix was significantly affected only by 10 nM TBT at 24 hpf, while both concentrations induced a significant decrease at 32 hpf. Conversely, the area of the calcified shell was significantly affected by both 1 and 10 nM TBT at 28 and 32 hpf (Fig. 9 A, B, C). The effect of TBT on the growth of the larval shell resulted in altered organic matrix:calcified shell ratios (Fig. 9 D).

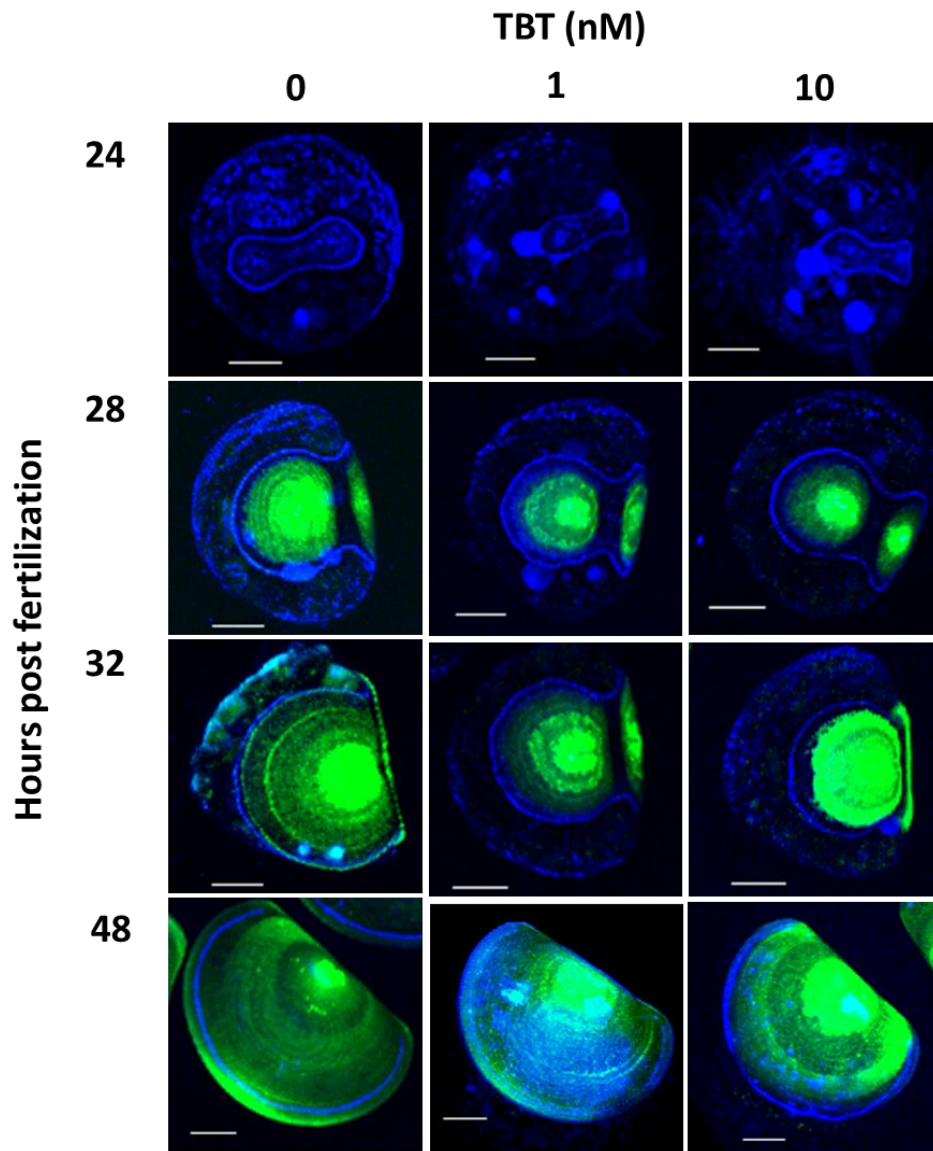


Figure 8: Effect of increasing concentrations of TBT on the formation of the larval shell in *Mytilus galloprovincialis* larvae at 24, 28, 32 and 48 hpf. Columns correspond to experimental conditions (from left to right: Control, 1 and 10 nM TBT). Representative images (lateral view) show merged fluorescent signals of calcofluor (blue) and calcein (green) staining, respectively, the organic matrix and CaCO₃ deposition (scale bars: 20 μ m).

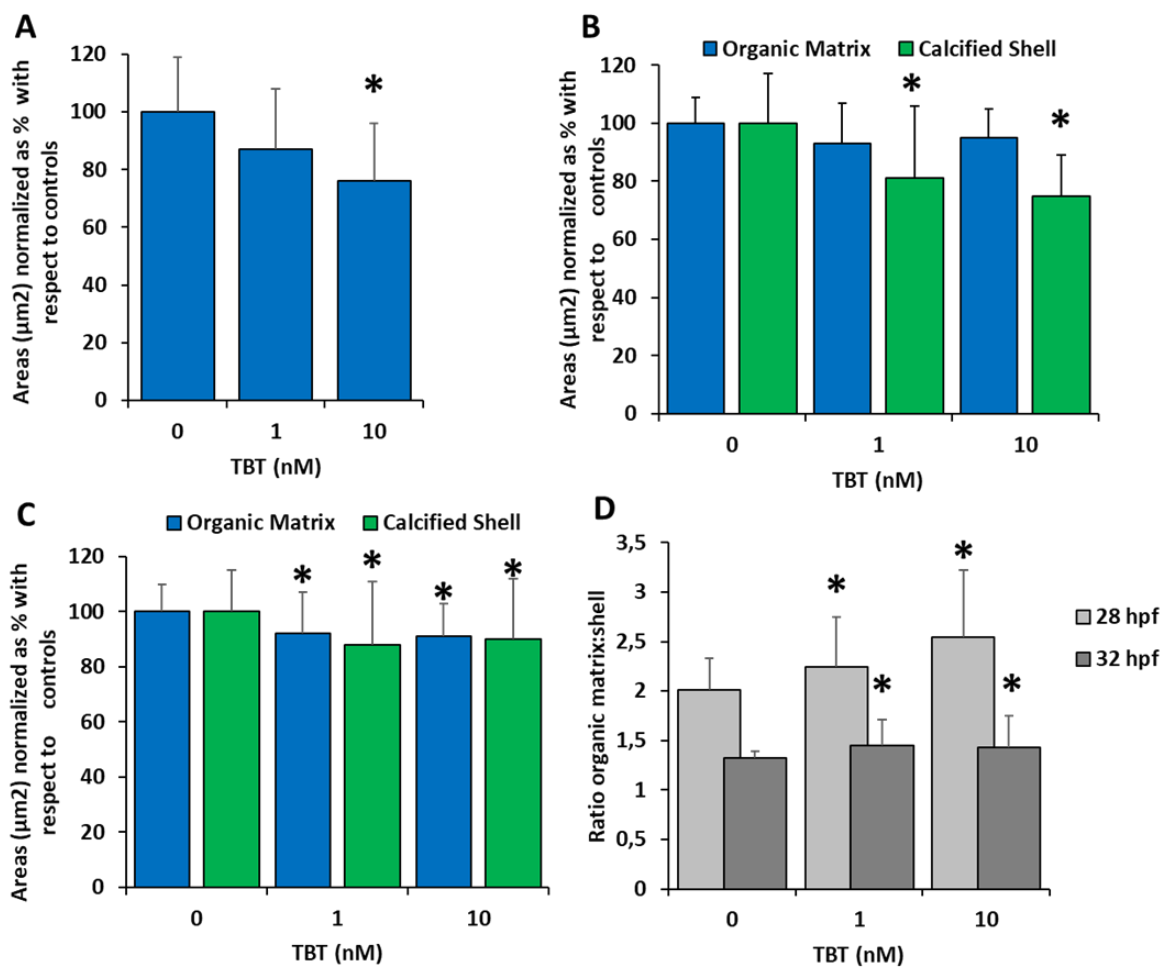


Figure 9: Effect of increasing concentrations of TBT on the growth of the organic matrix (blue) and calcified shell (green) in *Mytilus galloprovincialis* larvae from 24 to 32 hpf. Areas of each shell component (μm²) measured in a single valva of 9-cis-Retinoic Acid-exposed larvae are reported as normalised values with respect to controls (unexposed) larvae at 24 (A), 28 (B) and 32 hpf (C). In D) ratios of areas matrix/calcified shell in different experimental conditions are reported. Measurements were made on at least 50 larvae for each condition obtained from 3 parental pairs (N=3). Non-parametric ANOVA (Kruskal Wallis) followed by Dunn's test (* p ≤ 0.05).

3.4. Effect of UVI3003 on larval development of *Mytilus galloprovincialis*

As for the other chemicals, the effect of the full RXR antagonist UVI3003 was first evaluated at 48 hpf and the results are showed in Fig. 10. Despite the high concentrations tested (μMolar), UVI3003 showed little effects on larval phenotypes, (maximum 10-15% of malformed D-Veligers or Pre-Veligers, depending on the concentration) and did not affect the mean shell size (Fig. 10 A, B). When shell morphogenesis was investigated at the highest concentration tested (5 μM) UVI3003 did not induce any significant decrease of the area of the organic matrix and calcified shell from 24 to 32 hpf (Fig. 11). UVI3003 was therefore utilized in co-exposure experiments with either 10 nM 9-*cis*-RA or TBT to investigate the possibility that the effects of these compounds on larval phenotypes at 48 hpf were mediated by RXR (Phenotype Rescue experiments).

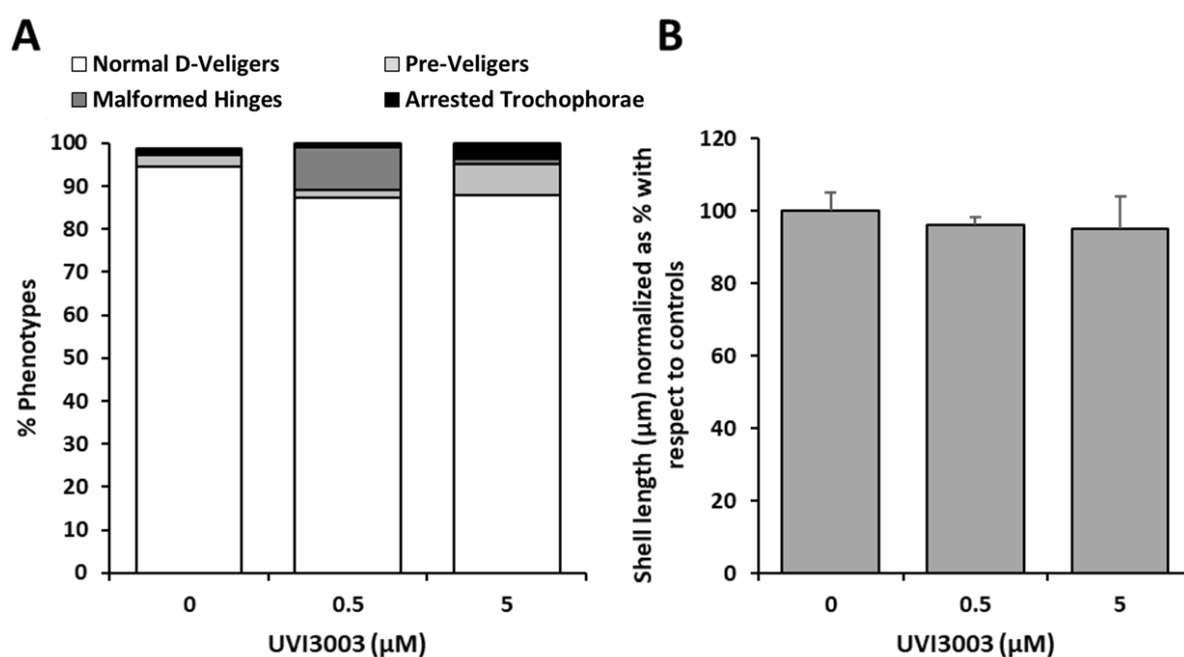


Figure 10: Effect of increasing concentrations of UVI3003 on the larval development of *Mytilus galloprovincialis* at 48 hpf. A) Percentage of different phenotypes: normally developed D-Veligers (white), Pre-Veligers with protruding mantle (light gray), larvae with hinge malformations (dark gray) and arrested at the trochophora stage (black); B) Shell length of D-Veligers, i.e. the anterior-posterior dimension of the shell parallel to the hinge line normalized as % with respect to controls. N=3. One-way ANOVA followed by Tukey's test (* $p \leq 0.05$).

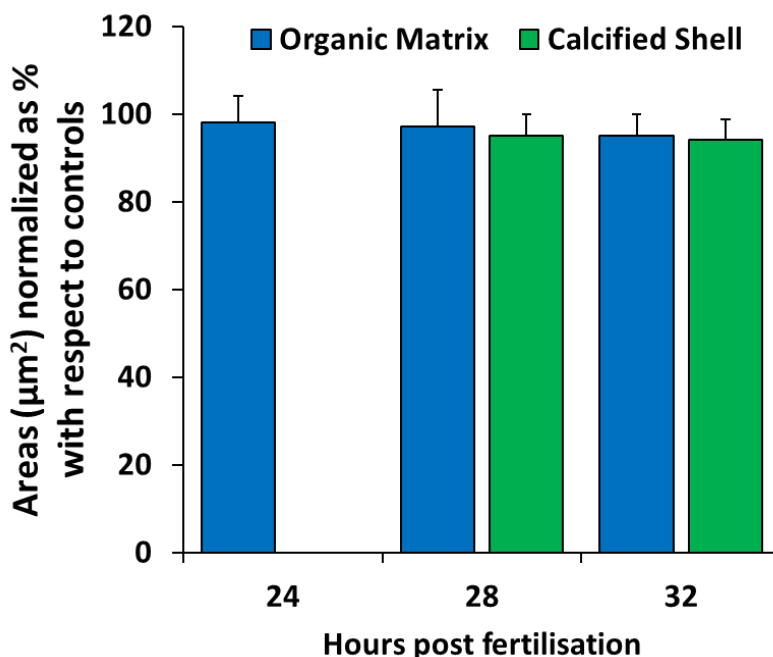


Figure 11: Effect of 5 µM UVI3003 on the growth of the organic matrix (blue) and calcified shell (green) in *Mytilus galloprovincialis* larvae from 24 to 32 hpf. Areas of each shell component (µm²) measured in a single valva of 9-*cis*-Retinoic Acid-exposed larvae are reported as normalised values with respect to controls (unexposed) larvae at 24 (A), 28 (B) and 32 hpf (C). In D) ratios of areas matrix/calcified shell in different experimental conditions are reported. Measurements were made on at least 50 larvae for each condition obtained from 3 parental pairs (N=3). Non-parametric ANOVA (Kruskal Wallis) followed by Dunn's test (* p ≤ 0.05).

3.5. 9-*cis*-RA and TBT phenotype rescue experiment at 48 hpf by co-incubation with UVI3003

Larvae exposed to 10 nM 9-*cis*-RA and TBT and 5 µM UVI3003 showed the same phenotypic response reported above (Fig. 12 A, B and Fig. 4, 7, 10). Both 9-*cis*-RA and TBT induced the formation of Pre-Veligers as the predominant phenotype, while UVI3003 was ineffective (Fig. 12 A, B). Co-exposure to either 10 nM 9-*cis*-RA or TBT alone plus 5 µM UVI3003 prevented the occurrence of the pre-Veliger phenotype in over 80% of the samples, that thus presented a % of normally developed D-Veligers similar to those of controls. However, co-exposure with UVI3003 did not fully rescue the decrease in shell length induced by both 9-*cis*-RA or TBT alone, as shown in Fig. 12 C.

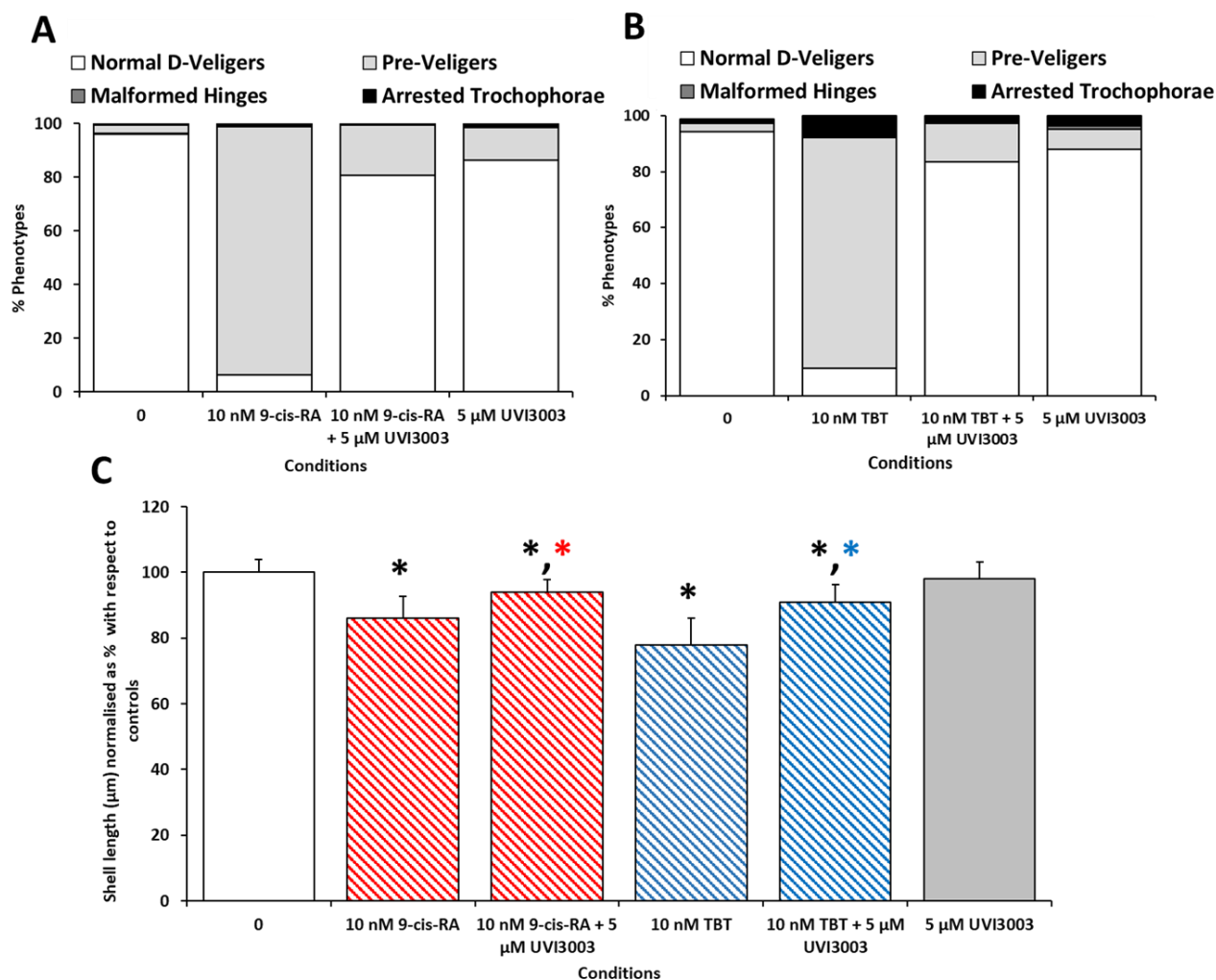


Figure 12: Outcomes of the rescue experiments by the RXR inhibitor UVI3003 on larval phenotypes induced by 9-cis-RA or TBT at 48 hpf.

A) **9-cis-Retinoic Acid phenotype rescue.** Percentage of different phenotypes: normally developed D-Veligers (white), Pre-Veligers with protruding mantle (light gray), larvae with hinge malformations (dark gray) and arrested at the trochophora stage (black); B) **TBT phenotype rescue.** Percentage of different phenotypes: normally developed D-Veligers (white), Pre-Veligers with protruding mantle (light gray), larvae with hinge malformations (dark gray) and arrested at the trochophora stage (black); C) Shell length of D-Veligers, i.e. the anterior-posterior dimension of the shell parallel to the hinge line normalized as % with respect to controls. N=3. One-way ANOVA followed by Tukey's test (* $p \leq 0.05$). The colour of asterisks indicates significant difference with respect to negative controls (black) and correspondent positive controls (9-cis-RA: red; TBT: blue).

3.6. Effect of 9-cis-RA and TBT on the expression pattern of MgRXR

Since both 9-cis-RA and TBT largely affected the transition from Pre-Veliger to the fully developed first D-Veliger stage, and this specific effect was prevented by the inhibitor of RXR activation, the possible effects of either compound on the expression of *MgRXR* was investigated by ISH at, 32 and 48 hpf, when the shell phenotypes plausibly arise, and the results are reported in Fig. 13. Neither 9-cis-RA and TBT visibly affected the expression pattern of *MgRXR* at either 32 or 48 hpf with respect to larvae grown in physiologic conditions. However, in control samples, *MgRXR* showed a distinct localization at 32 and 48 hpf. Therefore, *MgRXR* expression pattern was compared with those of other components of neuroendocrine signalling involved in neurogenesis and shell biogenesis previously identified in mussel larvae (Miglioli et al., 2019; 2021a, b), namely those involved in dopaminergic, serotonergic and GABAergic signalling (DR1, TH and DBH, 5-HT and GABA) and tyrosinase-Tyr, the key enzyme in shell matrix deposition. At 32 hpf *MgRXR* is strongly expressed in areas corresponding with those of 5-HT- and GABA-*ir* cells, as well as in sites of expression of TH (tyrosine hydroxylase, the marker of dopaminergic neurons), DR1 and Tyr (Fig. 13). At 48 hpf, *MgRXR* showed a similar pattern to that of the dopamine receptors DR1 and of Tyr in the border of the D-Veliger shell, together with distinct expression sites in hinge areas were also 5-HT-*ir* cells and DBH expression are detected (Fig. 13). Therefore, the possible effects of 9-cis-RA and TBT (10 nM) on the expression pattern of these genes were investigated.

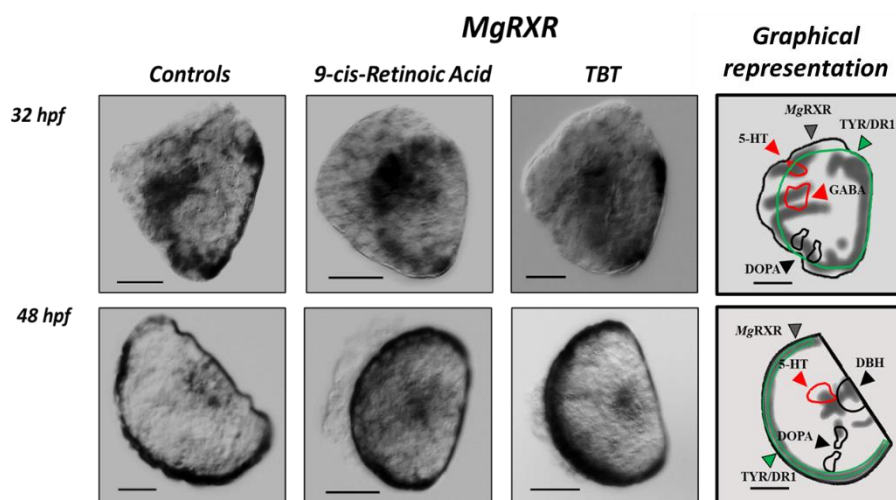


Figure 13: Localisation of RXR transcripts in *Mytilus galloprovincialis* larvae grown in control conditions and exposed to 10 nM 9-cis-RA and TBT at 32 and 48 hpf. The expression pattern of *MgRXR* is shown in larvae of *M. galloprovincialis* grown in physiologic conditions and exposed to 10 nM 9-cis-RA and TBT. Columns correspond to the experimental conditions, rows to the developmental timepoint. In the last column is shown the expression pattern of RXR (grey) in control larvae at 32 and 48 hpf with those of the Dopamine Receptor 1 (DR1) of Tyrosinase (Tyr) and in green, Tyrosine Hydroxylase (DOPA) and Dopamine- β -Hydroxylase (DBH) in black, with the immunoreactivity (*ir*) signals of Serotonin (5-HT) and γ -aminobutyric acid (GABA) in red. Scalebar: 20 μ m.

3.7 Effect of 9-*cis*-RA and TBT on the expression pattern of Tyrosinase as a marker of shell development.

The mechanisms of action of 9-*cis*-RA and TBT (10 nM) were further investigated focusing on the expression pattern of Tyrosinase (Tyr), a marker of larval shell development, in late developmental stages leading to the D-veliger phenotype (i.e., at 32 and 48 hpf). As shown in Fig. 14, in physiologic conditions, Tyr expression strongly localizes along the borders of the growing shell at 32 hpf and by 48 hpf the signal is limited to a thin layer of cells along the D border of the shell (Miglioli et al., 2019). Both 9-*cis*-RA and TBT altered the localisation of Tyr transcripts, although inducing distinct expression patterns. At 32 hpf, in samples exposed to 9-*cis*-RA the signal was apparently more concentrated in few distinct areas on the lateral margins of the hinge, whereas in TBT-exposed samples, Tyr expression was more homogeneous. Such effects were also evident at 48 hpf with 9-*cis*-RA inducing a scattered expression pattern and TBT inducing Tyr expression in the whole shell area.

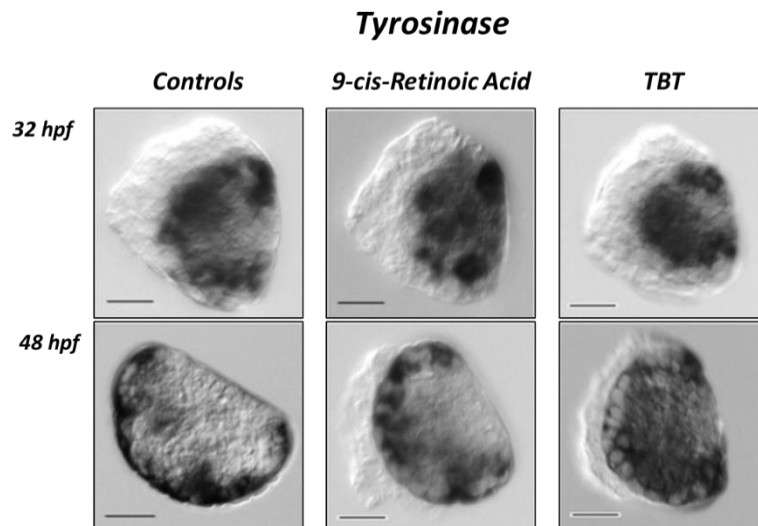


Figure 14: Effect of 10 nM 9-*cis*-RA and TBT on Tyrosinase (Tyr) expression pattern at 32 and 48 hpf. The expression pattern of Tyr is shown in larvae of *M. galloprovincialis* grown in physiologic conditions and exposed to 10nM 9-*cis*-Retinoic Acid and TBT. Columns correspond to the experimental conditions, rows to the developmental timepoint. Scalebar: 20µm.

3.8 Effect of 9-*cis*-RA and TBT on the dopaminergic system.

The effect of 9-*cis*-RA and TBT was additionally investigated at 32 and 48 hpf on the expression patterns of dopaminergic components, that play a key role in bivalves larva shell formation and neurogenesis (Liu et al., 2020; Miglioli et al., 2021b). Expression patterns of Dopamine Receptor 1 (DR1), Tyrosine Hydroxylase (TH) and Dopamine- β -Hydroxylase (DBH) were evaluated by ISH and results are presented in Fig. 15. In physiologic conditions, DR1 localises along the growing margins of the larval shell throughout development (Miglioli et al., 2021b). 9-*cis*-RA did not affect DR1 expression pattern at 32 hpf while TBT strongly decreased the intensity of the signal (Fig. 15 A). By 48 hpf, the localisation of DR1 in 9-*cis*-RA and TBT exposed larvae did not differ from those of control conditions: putative ectopic sites of expression were present within the shell area, but the signal mostly concentrated along the shell margins. TH (Fig. 15 B) is a rate limiting enzyme for dopamine synthesis and is considered to be a marker for dopaminergic neurons in *M. galloprovincialis* larvae (Miglioli et al., 2021b). As previously described, in control conditions TH is expressed in a group of symmetric cells that at 32 hpf are positioned in the area of the oral cavity in the ventral side of the larvae and by 48 hpf are located more dorsally and enclosed in the D-Veliger shell (Miglioli et al., 2021b) (Fig. 14 B). 9-*cis*-RA did not significantly affect TH expression patterns at both 32 and 48 hpf; however, a decrease in the expression signal was observed, especially at 48 hpf (Fig. 15 B). TBT exposure induced an even stronger decrease in TH expression at both times pf; moreover, at 48 hpf the group of dorsal TH-neurons was absent (Fig. 15 B). Dopamine- β -Hydroxylase (DBH) is the rate limiting enzyme for the catabolism of dopamine and its conversion to noradrenaline, thus being also defined as a molecular marker for adrenergic neurons. In control larvae, DBH has a clear and distinctly localised expression pattern only at 48 hpf, in a group of cells in the centre of the hinge region. (Miglioli et al., 2021b) (Fig. 15 C). Neither 9-*cis*-RA and TBT induced any clear alteration of the expression pattern of DBH (Fig. 15 C).

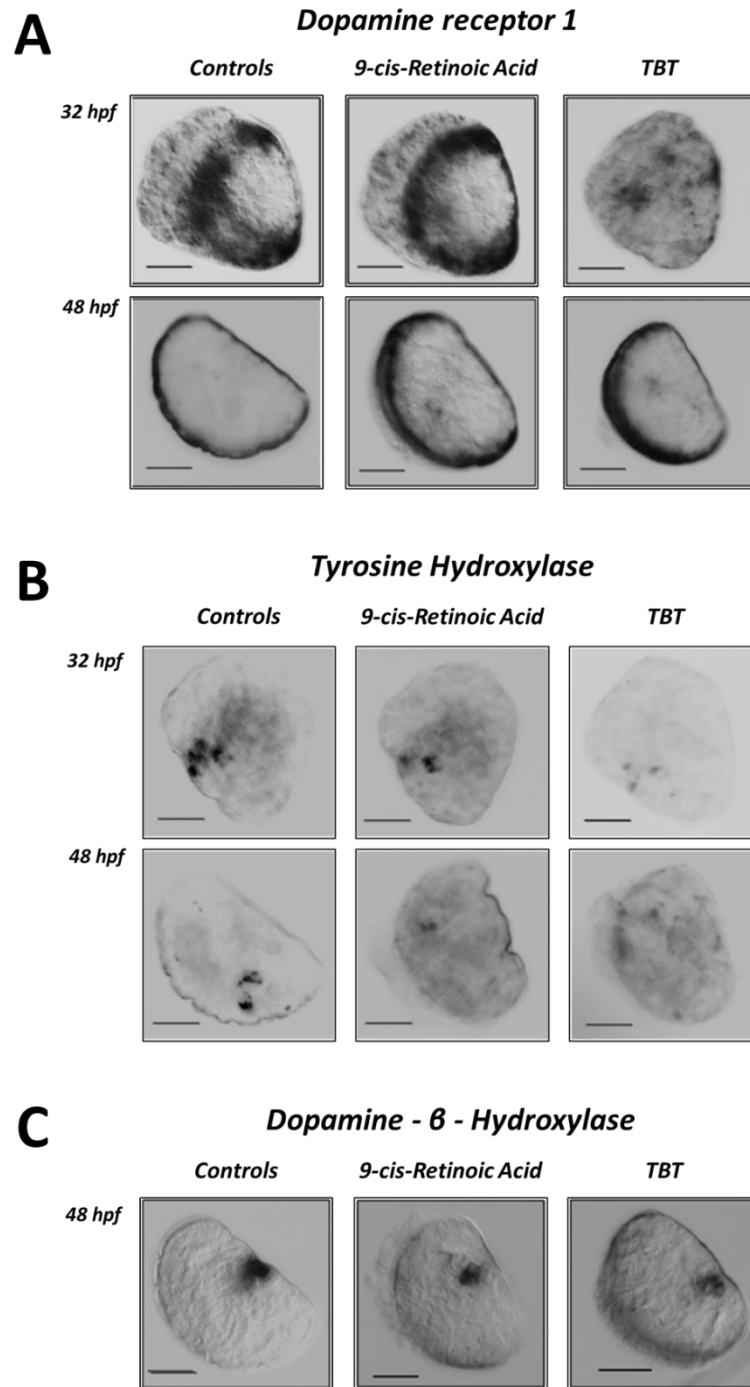


Figure 15: Effect of 10 nM 9-cis-RA and TBT on the expression patterns of the elements of the dopaminergic system. A) Expression pattern of Dopamine Receptor 1 at 32 and 48 hpf; B) Expression pattern of Tyrosine Hydroxylase at 32 and 48 hpf; C) Expression pattern of Dopamine - β - Hydroxylase at 48 hpf. Columns correspond to experimental conditions and rows to hpf. Scalebar: 20 μ m.

3.9 Effect of 9-cis-RA and TBT on the number of 5-HTir cells at 48 hpf.

The effect of 10 nM 9-cis-RA and TBT was evaluated on the population of serotonergic (5-HTir) neurons in 48 hpf D-Veligers, as previously described (Miglioli et al., 2021a, 2021b), and the result are presented in Fig. 16. In physiologic conditions, 48 hpf D-Veligers possess from five up to eight 5-HTir cells (mean \pm SD: 6 ± 1) organized in a ganglion-like structure further characterized by two neuritic emergencies. Exposure to 9-cis-RA significantly decreased the number of 5-HTir neurons to 4 ± 1 (mean \pm SD). TBT also induced a slight, but not significant reduction in the number of 5-HTir cells, (5 ± 0.84) and more than 2 emerging neurites were often observed.

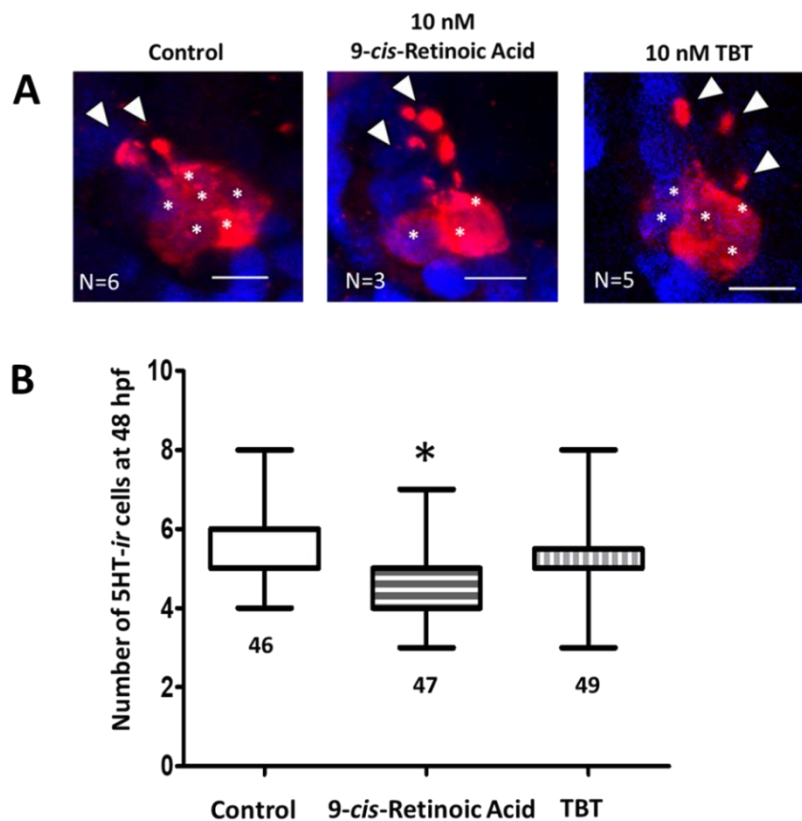


Figure 16: Effect of 10 nM 9-cis-RA and TBT on the number of 5-HTir cells at the D-Veliger stage.

A) Representative images of the serotonergic ganglion in the different experimental conditions showing the 5-HT (red) and Hoechst (blue) merged channels. Asterisks indicate the number of 5-HTir cells (see materials and methods) and the number of 5HT-ir cells is reported in the bottom left of the images. White arrowheads indicate the emerging neurites. Scalebar: 5 μ m. B) Histogram showing the # 5-HTir cell counts in each condition. The n numbers of embryos analysed for each condition are reported above each bar. N=3. Nonparametric ANOVA (Kruskal-Wallis) followed by Dunn's test (*p < 0.05).

3.10 In search of possible *MgRXR* heterodimers involved in *M. galloprovincialis* larval development

RXR activity as an orphan NR is generally considered to be subordinated to the one of its heterodimeric partners. In molluscs and protostomes, NR heterodimerisation has been confirmed for few NRs: THR, RAR, PPAR in molluscs and NR4A/HR38 in insects (Miglioli et al., 2021; Shen et al., 2020; Evans and Mangelsdorf, 2014). Since the functioning of the heterodimers implies the colocalization of RXR and its partners (Evans and Mangelsdorf, 2014), we investigated the expression patterns of the orthologous NR proteins identified in *M. galloprovincialis* genome in control 32 hpf larvae by ISH in comparison with that of *MgRXR*, and the results are shown in Fig. 17. The results show that *MgRXR* and its heterodimeric partners are show several co-localization sites; moreover, they appear generally co-expressed in either shell-related or both shell and neuronal-related domains (Fig. 17). Furthermore, the co-expression suggests that all the heterodimers plausibly play a role in *M. galloprovincialis* larval development, and their activity could be affected by RXR exogenous modulation (Miglioli et al., 2021).

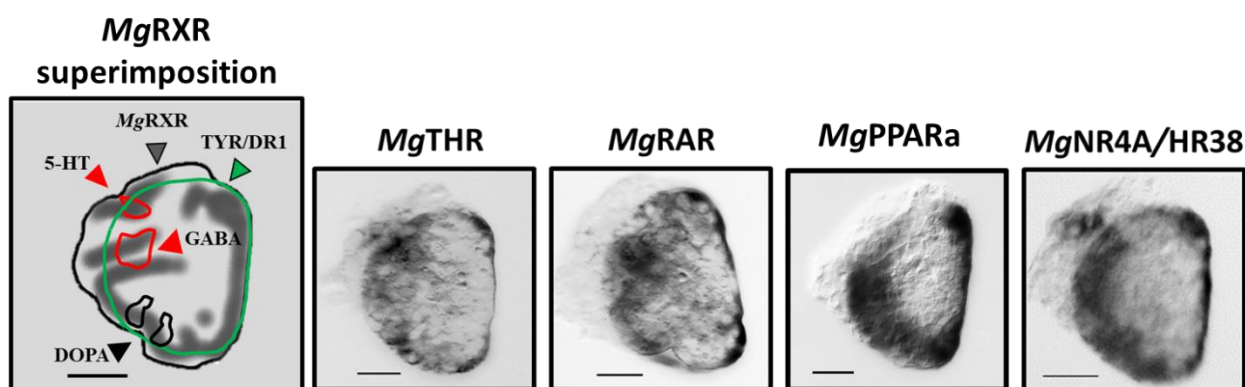


Figure 17: Expression patterns of *MgTHR*, *MgRAR*, *MgPPARa* and *MgNR4A/HR38* in 32 hpf *M. galloprovincialis* larvae with respect to the one of *MgRXR*. The illustration of *MgRXR* expression pattern is taken from Fig. 13. Scale bar: 20 μ m.

4. Discussion

The results obtained in this work provide information on the potential role of RXR in early larval development of *M. galloprovincialis*. We first isolated the protein sequence of *MgRXR* from the NR complement of *M. galloprovincialis* and identified the conserved residues responsible for ligand binding of the *bona fide* vertebrate RXR agonist 9-*cis*-Retinoic Acid, the EDC TBT, a well-known endocrine disruptor in molluscs acting through RXR *in vitro* (Fonseca et al., 2020; André et al., 2019; Castro et al., 2007), and the RXR antagonist UVI3003. Our results show that exposure to both 9-*cis*-RA, as well as to TBT, significantly affected larval development, both in terms of altered phenotypes and of processes involved in deposition of shell components. Both compounds impaired the full development of D-Veligers at 48 hpf, resulting in a predominance of Pre-Veligers: these effects were related to a reduced deposition of the shell organic matrix at shorter times pf (from 24 h), followed by a reduction in calcification at later times pf (28 and 32 hpf). The developmental effects of both 9-*cis*-RA and TBT were concentration dependent in the low nMolar range, with TBT acting at concentration 10 times lower than 9-*cis*-RA and inducing full developmental arrest at 100 nM. These data extend previous observations and knowledge on the adverse effects of TBT as endocrine disruptor in marine molluscs (Cuvillier-Hot and Lenoir, 2020; Fernandez et al., 2019; Alzieu et al., 2000). In particular, the results support previous observations in natural populations of marine bivalves, in which developmental teratogenicity and shell formation defects were observed (Cuvillier-Hot and Lenoir, 2020; Fernandez et al., 2019; Alzieu et al., 2000). In order to investigate the possible role of RXR activation in mediating the effects of TBT, *in vivo*, rescue experiments were performed, consisting in co-exposure of developing larvae to either 9-*cis*-RA or TBT in the presence of UVI3003. The results demonstrate that pharmacological blockage of *MgRXR* almost fully prevented the phenotypic changes induced by both 9-*cis*-RA and TBT (i.e., developments delay at the Pre-Veliger stage). These data provide first *in vivo* evidence that RXR activation disruption is a predominant mode of action of TBT in bivalve larval development. Effects such as altered shell formation and developmental delay/arrest have been already shown to occur in *M. galloprovincialis* early larvae in response to a variety of xenobiotics and EDCs (Miglioli et al., 2021 a., b; Franzellitti et al., 2019; Balbi et al., 2019; 2018; 2017; 2016; Fabbri et al., 2014). However, the developmental outcome of both TBT and 9-*cis*-RA resulted peculiar. In fact, different contaminants have been shown to induce variety of altered shell phenotypes that in a dose dependent manner can progressively lead to developmental arrest or larval death. Conversely, exposure to both TBT and 9-*cis*-RA almost exclusively induced the pre-Veliger phenotype, whose occurrence increased a concentration dependent manner and was accompanied by a decrease in mean shell length. The relative absence of other phenotypic defects, together with the similarities in the effects of TBT and 9-*cis*-RA reinforces

the notion that RXR might represent a unique target for the two chemicals in developing *M. galloprovincialis* larvae. This evidence was also supported by the results of the rescue experiment in which co-incubation of TBT and 9-*cis*-RA with UVI3003 fully prevented the formation of pre-Veliger larvae and significantly prevented the reduction in mean shell-length. It follows that the biological *in vivo* effects of TBT in *M. galloprovincialis* larvae could be primarily induced by RXR activation (Castro et al., 2007). In order to shed more light on the physiological pathways that from RXR activation lead to the observed adverse developmental outcomes, we characterised the expression pattern of *MgRXR* at 32 and 48 hpf, when the late developmental arrest presumably takes place, and further investigated the effects of TBT and 9-*cis*-RA on key elements of *M. galloprovincialis* larval physiology and neuroendocrine signalling previously shown to be sensitive to other EDCs (Miglioli et al., 2021 a, b). *MgRXR* localised in distinct regions of the larval body plausibly corresponding to neurogenic and shell biogenic tissues but neither 9-*cis*-RA or TBT induced appreciable effects on the global expression pattern of *MgRXR*.

However, TBT (10 nM) affected the expression patterns of different components of dopaminergic signalling, in particular DR1 and TH, at both 32 and 48 hpf. Smaller effects were observed by equimolar concentrations of 9-*cis*-RA. In contrast, 9-*cis*-RA but not TBT affected the number of the 5-HT_{ir} cells at the D-Veliger stage. Both compounds, in particular TBT, altered the expression pattern of the shell building enzyme Tyrosinase. A link between dopaminergic and serotonergic systems and tyrosinase has been previously shown in early oyster and mussel larvae ultimately showing that they all participate to the neuroendocrine signalling network governing the process of shell formation in bivalve larvae (Miglioli et al., 2021 a, b; Liu et al., 2020; Miglioli et al., 2019). Overall, it could be argued that RXR disruption in bivalve larvae can lead to altered shell biogenesis and development through the interference with distinct elements of the larval neuroendocrine signalling including the dopaminergic and serotonergic system, and Tyrosinase. In the present work, the differences observed between the effects of equimolar concentrations of TBT and 9-*cis*-RA might not indicate a different mechanism of action of the two RXR modulators but could simply reflect the different potency of the two compounds towards each of the neuroendocrine components investigated. Furthermore, it must be considered that RXR is physiological active in the form of heterodimeric complexes with other NRs; it is thus likely that the distinct effects of TBT and 9-*cis*-RA could also derive from the modulation of the activity of distinct heterodimers upon RXR ligand activation (Miglioli et al., 2021 and references quoted therein; Evans and Mangelsdorf, 2014). For instance, TBT is classified within the Obesogenic EDCs due to its disruption of the RXR/PPAR heterodimer, a key regulator of lipid homeostasis (Capitão et al., 2021; 2018). In molluscs it was initially thought that the activation of the heterodimer was strictly due to RXR ligand activation, until a recent

publication showed that TBT could transactivate the heterodimer also *via* the binding to PPAR (Capitão et al., 2021). Moreover, in molluscs and protostomes, RXR was shown to dimerise also with THR, RAR and NR4A/HR38 (Miglioli et al., 2021; Medzikovic et al., 2019; Brunet Avalos et al., 2019; Shen et al., 2020). Interestingly, such heterodimers are suggested to play important role in molluscan and protostome development. THR/RXR is supposed to play a role in growth and developmental transitions (Miglioli et al., 2021). RAR/RXR in neurogenesis (Miglioli et al., 2021). PPAR/RXR in growth and lipid metabolism (Capitão et al., 2021; 2018). NR4A-HR38/RXR has received little attention in molluscs; however, it was shown to play a role in chitin sclerotization in insects and onset of the monoaminergic system in vertebrates (Shen et al., 2020; Brunet Avalos et al., 2019; Medzikovic et al., 2019). To shed some light on the possibility that these mechanisms may occur in mussel larvae, the expression patterns of these NRs were compared with that of *MgRXR*. While *M. galloprovincialis* presented a single copy of THR, RAR and NR4A/HR38, the phylogenetic analysis identified three NRs clustering with the PPAR subgroup. The PPAR (*MgPPARa*) with highest support values to the human orthologs was selected for the study (Capitão et al., 2021; 2018). The results show that *MgRXR* and its heterodimeric partners are all expressed in *M. galloprovincialis* larvae and colocalise almost entirely. *MgPPARa* was strictly expressed in the tissue surrounding the growing shell thus suggesting that the effects of TBT, and possibly also 9-*cis*-RA, on larval shell biogenesis could be symptomatic of the disruption of the PPAR/RXR heterodimer (Capitão et al., 2021; 2018). In addition, THR, RAR and NR4A-HR38 localised in both shell biogenic and neurogenic regions. It follows that a variety of heterodimers and their respective physiologic pathways could potentially contribute to *M. galloprovincialis* development and thus be susceptible to disruption upon RXR ligand activation (Miglioli et al., 2021). What is more, the expression pattern of the analysed NRs is in line with their potential role in molluscan development and neurogenesis supported by previous studies (Miglioli et al., 2021; Vogeler et al., 2017; Vogeler et al., 2016). For instance, the heterodimer RAR/RXR has long been speculated to play a role in molluscan larval neurogenesis in response to retinoic acid (Miglioli et al., 2021; Schubert and Gilbert, 2020). Our results indicate that RXR exogenous modulation by 9-*cis*-RA but not TBT led to a decrease in the number of serotonergic neurons. A similar effect has been described in developing annelids due to premature neuronal differentiation following the activation of the RAR/RXR heterodimer by exogenous retinoic acids (RA) (Handberg-Thorsager et al., 2018). Although in annelids such effect was due to RAR ligand activation, molluscan RAR is incapable of binding ligands so that the activation of the heterodimer in response to RA has been recently attributed to RXR (André et al., 2019; Gutierrez-Mazariegos et al., 2014). Accordingly, the effect of 9-*cis*-RA on the number of serotonergic neurons in *M. galloprovincialis* larvae could be due to the modulation of RAR/RXR

activity upon RXR ligand activation thus supporting the concept that the heterodimer could be involved in molluscan larval neurogenesis (Miglioli et al., 2021). Of note, *MgNR4A/HR38* surprisingly showed a very neat expression pattern that covered almost all the border of the growing shell border as well as the neuronal regions of the monoamines dopamine and serotonin which could be suggestive of similar developmental functions with respect to the ones described for insects and vertebrates (Shen et al., 2020; Brunet Avalos et al., 2019; Medzikovic et al., 2019). In addition, the consistent localisation of NRs transcripts in proximity of the shell forming tissue is suggestive of a role of RXR and its heterodimers in the process of shell formation thus further reinforcing the link between RXR disruption and altered shell development in the larvae of bivalve molluscs as well as a plausible role of RXR in regulating the process in physiologic conditions though the activity of its heterodimers.

5. Conclusions

The present study is the first to formally demonstrate *in vivo* that disruption of RXR is a major mode of action of TBT in developing larvae of marine bivalves. In order to enhance our current understanding of invertebrate endocrine disruption pathways by linking plausible initiating molecular events, such as Nuclear Receptor modulation, to adverse biological outcomes at the organism level, the Adverse Outcome Pathway (AOP) has been proposed (Ford and Le Blanc, 2020; Ankley et al., 2010). Following an AOP rationale, the study demonstrated that RXR disruption in bivalve larvae can lead to altered shell development through different pathways, and plausible modulation of the activity of distinct heterodimers, ultimately interfering with the neuroendocrine signalling at the base of the regulation of shell formation. In the context of the AOP framework, it is accepted that the same molecular initiating event of a given xenobiotic can lead to a plurality of adverse effects and, vice versa, that homologous adverse effects can be induced by multiple molecular initiating events (Allen et al., 2014). In addition, the distinct effects of 9-*cis*-RA and TBT on diverse elements of *M. galloprovincialis* larval physiology corroborated previous claims on the role RXR and its heterodimers in regulating molluscan development and thus expanding our current knowledge regarding the possible morphogenetic and developmental processes susceptible to RXR disruption in bivalve larvae which now include shell biogenesis as well as neurogenesis.

6. References

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General Discussion

The work presented in this PhD manuscript addressed the issue of endocrine disruption in marine invertebrates by exploiting the early larval development of the mediterranean mussel *M. galloprovincialis* as experimental model. We first characterised the neuroendocrine elements regulating larval shell biogenesis and established the morphogenetic process susceptible to neuroendocrine disruption. Consequently, the mechanisms of action of the model EDCs BPA and TBBPA in mussel larvae were investigated and the plausible neuro-/endocrine AOPs that could be linked to their adverse biological outcomes were identified. In addition, an attempt has been made to identify a possible endocrine disrupting activity initiated at the level of the neuroendocrine system and of Nuclear Receptors signalling. The NR complement of *M. galloprovincialis* was isolated and put in a phylogenetic and developmental context. In addition, by studying and characterising the possible AOP of TBT in *M. galloprovincialis* larvae through the modulation of RXR activity, a plausible relationship between NR signalling and the neuroendocrine system in *M. galloprovincialis* larvae was established.

1. Shell biogenesis in *M. galloprovincialis* larvae is regulated by the neuroendocrine system

As previously discussed in the introductory section, the functions and elements of the neuro-/endocrine system in invertebrates represent the main gap of knowledge that prevents the understanding of the consequences and biological impact of endocrine disruption in these organisms (Ford and Le Blanc, 2020; Cuvillier-Hot and Lenoir, 2020; Katsiadaki, 2019). In fact, recent reviews on the topic highlighted the need to discover and characterise neuro-/endocrine processes in invertebrate organisms in order to employ them as endpoint of analysis to screen potential endocrine disrupting activities of environmental pollutants (Ford and Le Blanc, 2020).

In previous work conducted in the pacific oyster *Crassostrea gigas*, it was shown that the process of larval shell biogenesis in bivalve larvae might be subject to neuroendocrine regulation. In fact, *C. gigas* larval shell biogenesis is initiated by the signalling of the monoamine neurotransmitter serotonin and neurohormone dopamine that, through the TGF- β smad pathway, trigger the expression of tyrosinase and chitinase, key enzymes in the formation of the initial shell (Liu et al., 2020). In the present work, we investigated and characterised the initial steps of larval shell formation in the model of *M. galloprovincialis* larval development and searched if a similar neuroendocrine network could regulate the process in mussels.

At first, tyrosinase was shown to be involved in organic matrix remodelling and to be expressed in the tissues responsible for shell biogenesis in developing larvae (Miglioli et al., 2019). In addition, upon inhibition of Tyrosinase enzymatic activity, the whole process of shell biogenesis was inhibited (Miglioli et al., 2019). Secondly, the onset the serotonergic and dopaminergic systems was

investigated and shown to occur during *M. galloprovincialis* early larval development, leading to the specific neuronal populations at different time post-fertilization (Miglioli et al., 2021 a). The dopaminergic signalling proved to be a pivotal regulator of *M. galloprovincialis* larval shell biogenesis, with the Dopamine Receptor 1 (DR1) being also localised in the tissues responsible for shell biogenesis. Moreover, pharmacological blockage of DR1 was capable to arrest the whole process of larval shell biogenesis and development (Miglioli et al., 2021 b). In addition, the rate limiting enzymes of dopamine metabolism were also investigated and showed to be consistently expressed throughout larval development. The results confirm that dopamine is a potent morphogenetic agent in *M. galloprovincialis* larval shell biogenesis (Miglioli et al., 2021 b). Together with Tyrosinase, Serotonin and Dopamine, Chitinase was shown to be also expressed in *M. galloprovincialis* early larvae and localised in shell biogenic tissues.

Although the role and functions of the TGF- β smad pathway were not investigated with respect to the process of shell biogenesis, the presence and demonstrated roles of all the other elements suggest that a neuroendocrine network similar to that of the pacific oyster could be at the base of the process of shell biogenesis also in *M. galloprovincialis* larvae. In addition to mussels and oysters, serotonin and dopamine have been shown to play a role in the early larval development in multiple species and families of molluscs (Joyce and Vogeler, 2018).

It follows that the process of larval shell biogenesis in bivalve molluscs is regulated by the neuroendocrine signalling of the monoamines serotonin and dopamine, thus possibly representing a potential investigation endpoint for EDCs with neuroendocrine disrupting activity.

2. BPA and TBBPA are neuroendocrine disruptors in *M. galloprovincialis* larvae

Following the hypothesis that larval shell biogenesis could be a sensitive target of neuroendocrine disruption, we investigated the effects of the well-known model EDCs, BPA and TBBPA, on early larval development of *M. galloprovincialis* and enquired if the adverse developmental outcomes observed in previous studies could be due to the disruption of neuroendocrine signalling pathways. BPA was already shown to affect *M. galloprovincialis* larval development in the 48 h embryotoxicity assay in a wide concentration range, including environmental concentrations (Fabbri et al., 2014). In addition, BPA significantly affected the transcription of several genes, including the serotonin receptor 5-HT_{1A} as well as those involved in biomineralization (Balbi et al., 2016). Moreover, BPA was already indicated to interfere with the enzymatic activity of Tyrosinase enzymes (Wu et al., 2012). The results presented in this thesis show that BPA could significantly impair the process of shell biogenesis at very low (nMolar) concentrations and could affect the expression of Tyrosinase (Miglioli et al., 2021 a). BPA could also induce ectopic localisation and a decrease in number of 5-

HT-*ir* neurons, accompanied by a significant effect on the expression levels of the serotonin receptor 5-HTR1 (Miglioli et al., 2021 a). Such results suggest that BPA adverse effects on *M. galloprovincialis* larvae could be due neuroendocrine disruption, affecting the onset of the serotonergic system as well as tyrosinase activity which altogether lead to impaired larval shell growth (Miglioli et al., 2021 a).

TBBPA was also demonstrated to affect *M. galloprovincialis* early larval development with standard embryotoxicity assay and the effects were comparable with those observed with the related compound BPA (Balbi et al., 2016; Fabbri et al., 2014). Consequently, we further investigated the effect of this EDC on different elements of the neuroendocrine signalling regulating *M. galloprovincialis* development. The results showed that TBBPA impaired the whole process of larval shell biogenesis and affected the development of dopaminergic, serotonergic as well as GABAergic systems, thus corroborating the notion that the neuroendocrine system is a sensitive target of EDC action and by which the adverse developmental outcomes on shell biogenesis and larval development are induced (Miglioli et al., 2021 b). The data obtained with TBBPA permitted to draw a plausible adverse outcome pathway-AOP of the action of both EDCs in *M. galloprovincialis* early larval stages, where the molecular initiating event is represented by the interference with the synthesis of neurotransmitters serotonin and dopamine involved in neurodevelopment and shell biogenesis, that ultimately results in delayed or arrested development in individual *Mytilus* larvae (Miglioli et al., 2021 b). Moreover, the range of concentrations utilised for the studies is close to the amount of both chemicals found in the environment thus suggesting that the identified AOP might potentially lead to detrimental consequences on *Mytilus* and bivalve populations (Miglioli et al., 2021 b).

Finally, both EDCs are capable of affecting the development of the larval shell of *M. galloprovincialis* through the neuroendocrine disruption of the monoaminergic system with TBBPA additionally interfering with the signalling of the GABAergic one. Accordingly, it is parsimonious to theorise that the neuroendocrine signalling carried out by the monoaminergic system could be a sensitive target of neuro-/endocrine disrupting chemicals in developing bivalve molluscs, and the first diagnostic evidence of such mechanism is the adverse development of the larval shell.

What is more, the action of BPA and TBBPA on *M. galloprovincialis* larvae could fit into the definition of Neuroendocrine Disrupting Chemicals, as their mode of action involves the interference with the action of neurotransmitters (i.e., serotonin and GABA) and neurohormones (i.e., dopamine) as well as a direct effect on their neuronal populations with plausible neurosecretory functions (León-Olea et al., 2014; Trudeau, Kah and Bourguignon et al., 2011). In addition, such neuroendocrine disrupting pathway could potentially occur in diverse invertebrate clades because neurotransmitters and neurohormones, and especially monoamines, are the main signalling molecules of the

neuroendocrine system of invertebrate organisms (Joyce and Vogeler, 2018; Malagoli and Ottaviani, 2017). However, neuro-/endocrine diagnostic elements, such as shell development in bivalve molluscs, should be first discovered and characterised for each major invertebrate clade (Ford and Le Blanc, 2020).

3. Nuclear Receptors as possible target for EDCs in early development of *M. galloprovincialis*

Nuclear receptors are the main target of the action of EDCs in vertebrates and have been linked to a variety of developmental processes in invertebrates, including molluscs (Miglioli et al., 2021). In particular, a subset of NRs has been shown to be involved in embryonic and post-embryonic development throughout vertebrate and invertebrate phyla which include TR2/4, HNF-4, COUP-TF, TLL/TLX and RXR (Miglioli et al., 2021). With respect to bilaterians alone, the heterodimeric complexes of RAR, THR and PPAR with RXR were shown to be involved in precise morphogenetic processes in the development of invertebrates, and molluscs, which include, respectively, neurogenesis, developmental transitions and lipid metabolism (Miglioli et al., 2021; Capitão et al., 2021). In addition, these heterodimers are also major targets of EDCs in vertebrate organisms (Miglioli et al., 2021). In this thesis, the NR complement of *M. galloprovincialis* was isolated from the published genome (Gerdol et al., 2020). A preliminary phylogenetic analysis was performed and the possible relationships between the site of expression of the heterodimeric complexes and their potential biological functions was investigated during the stage of mussel development that leads to the formation of the first shelled larva (D-veliger). The results show that the subset of NRs ancestrally involved in metazoan, as well as bilaterian embryonic and post-embryonic development, is present in *M. galloprovincialis* NR complement (Fig. 2, Chapter 3). Single orthologs were identified for each NR, with the exception of PPAR for which three *MgNRs* were identified (Fig. 2, Chapter 3). *MgPPARa* showed the highest support values with respect to the human orthologs, while *MgPPAR b-c* clustered with the ortholog of the pacific oyster *C. gigas* (Fig. 2, Chapter 3). PPAR duplication in molluscs has been already documented for the gastropod molluscs *B. glabrata* and *L. gigantea* and showed similar phylogenetic relationships with one of the PPARs clustering with the vertebrate orthologs and the second with the one of *C. gigas* (Capitão et al., 2021). The occurrence of three PPARs in *M. galloprovincialis* is thus plausible, although further phylogenetic analysis as well as functional investigations are required to confirm this evidence. The expression patterns of *MgTHR*, RAR and *PPARa* were investigated and showed to be conspicuously expressed in tissues related to larval shell growth and neuronal populations of the monoaminergic system, i.e., serotonin and dopamine.

The results of this study support the hypothesis that the RAR/RXR heterodimer could be involved in larval neurogenesis in bivalve molluscs upon RXR ligand activation, since exogenous modulation of *MgRXR* by RA was able to induce a decrease in the number of serotonergic neurons as previously described in annelids (Miglioli et al., 2021; André et al., 2019; Handberg-Thorsager et al., 2018). In addition, the results support the evidence that the PPAR/RXR heterodimer is involved in growth, since disruption of *MgRXR* by TBT was able to arrest larval development and growth in *M. galloprovincialis* larvae (Chapter 3). In addition, the results indicate that the PPAR/RXR and THR/RXR heterodimers could also participate to the regulation of larval shell biogenesis in *M. galloprovincialis*, as their expression was consistently localised in the growing margins of the larval shell (Fig. 17, Chapter 3).

The present study also investigated the NR4A/HR38. The functions and characteristics of this NR are completely unknown in the molluscan *phylum*, although its activity is required for insect cuticle sclerotization as well as the monoaminergic signalling in vertebrates (Shen et al., 2020; Medzikovic et al., 2019). Given the evidence that NRs could be involved in shell biogenesis and that the process is regulated by monoamine neurotransmitters and neurohormones, the expression pattern of *MgNR4A/HR38* was investigated and showed to localise in all shell biogenetic, as well as monoaminergic neuronal domains (Fig. 17, Chapter 3). It follows that the heterodimer NR4A/HR38 might be involved in the neuroendocrine network at the base of the onset of monoaminergic system and thus participate to the process of larval shell biogenesis. Finally, NRs might be involved in neurogenesis, shell biogenesis as well as growth during early larval development of *M. galloprovincialis*. All these processes were shown in this thesis to be especially sensitive to EDCs of different sort. It follows that NRs could be involved in the AOPs of diverse xenobiotics and EDCs. Nevertheless, the limited knowledge regarding NR functions in invertebrate organisms makes it hard to elaborate the direct link between EDCs adverse outcomes at the organism level and a possible disruption of NR activity. Accordingly, further study and characterisation of NRs biological functions in *M. galloprovincialis* and, more in general, invertebrates, is highly needed to fill this gap of knowledge and push forward the current understanding of the involvement of NRs in the adverse outcomes of EDCs at the organism level in invertebrate species.

4. RXR initiates the neuroendocrine AOP of TBT in *M. galloprovincialis* larval development

In order to test the possible relationships between NR disruption and the neuroendocrine system, the thesis attempted to characterise a plausible AOP of the model EDC TBT on larval development of *M. galloprovincialis*. Through IMD (Invertebrate Molecular Disruption) approaches, TBT has been shown to disrupt the activity of the nuclear receptor RXR throughout metazoans and, in multiple

invertebrate clades including molluscs, to disrupt the activity of the PPAR/RXR heterodimer (Capitão et al., 2021; Fonseca et al., 2020; Capitão et al., 2018). The results show that in *M. galloprovincialis* larvae, TBT affects multiple elements of the neuroendocrine system, in particular the dopaminergic signalling, thus leading to developmental arrest and strong adverse outcomes at the level of shell biogenesis. Moreover, the results show that such effects were in large part, but not exclusively, due to the disruption of *MgRXR*. In fact, recent molecular evidence showed that TBT in molluscs acts through the disruption of the PPAR/RXR heterodimer by binding to both receptors and that upon pharmacological blockage of RXR, TBT was still capable of inducing a transcriptional response of the heterodimer through binding to PPAR alone (Capitão et al., 2021). Such mechanism could explain the partial success of the rescue experiment performed with the RXR inhibitor, as well as the distinct effects of TBT with respect to 9-*cis*-RA in *M. galloprovincialis* larval development. Nevertheless, the *in vivo* evidence provided by the study show that disruption of NRs can potentially lead to a plurality of adverse developmental outcomes through the interference with the neuroendocrine system. These data shed more light of the potential involvement of NRs in invertebrate endocrine disruption, as well as in the neuro-/endocrine system of bivalve molluscs. In addition, the present study has validated at the organism level some of the notions and findings obtained *ex vivo* through the IMD approach. The *in vivo* results not only corroborated part of the molecular pathway of action of TBT, but also connected the disruption of RXR to a series of biological adverse outcomes specific to *M. galloprovincialis* early larvae. Such effects, when observed in response to xenobiotics and environmental pollutants, could then potentially be indicative of neuro-/endocrine AOPs involving the disruption of RXR and/or PPAR/RXR activity in bivalve molluscs. It follows that the functional *in vivo* validation of IMD findings through an AOP approach in invertebrate model organisms could be a resolute experimental rationale to relate the adverse effects at the organism level to plausible molecular target of disruption, including NRs, specific to invertebrate species.

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General Discussion

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Concluding Remarks and Future Perspectives

In this thesis the issue of invertebrate endocrine disruption was investigated in the model of *M. galloprovincialis* larval development. The work added up to current knowledge on invertebrate neuroendocrine system and its function in development, by investigating the neuroendocrine networks and element regulating the process of larval shell biogenesis. Through an AOP approach the results help deciphering the neuroendocrine mode of action of EDCs of different sorts, showing that the onset and functioning of the monoaminergic system is a sensitive target and thus informative endpoint of investigation to assess neuro-/endocrine activities of environmental pollutants. In addition, this work formally established a link between NRs and the neuroendocrine system of *M. galloprovincialis* and, potentially, bivalve molluscs in general. Altogether, the results of this thesis represent pivotal evidence and novel experimental pipelines that, applied to distinct invertebrate clades by a comparative approach, might greatly help and push forward the current understanding of the mechanisms of endocrine disruption in invertebrate organisms.

- The characterisation of the neuroendocrine function in invertebrate organisms is essential to the understanding of the issue of invertebrate endocrine disruption.

The results of this thesis underlined the importance of the monoaminergic system in the neuroendocrine signalling of *M. galloprovincialis* and bivalve molluscs. Consequently, the neuroendocrine disrupting activity of model EDCs BPA, TBBPA and TBT, could be assessed through the characterisation of their effects on larval shell biogenesis and on the newly discovered neuroendocrine elements regulating *M. galloprovincialis* larval development. Accordingly, the characterisation of the neuroendocrine system as well as the biological functions in diverse groups of invertebrates with a comparative approach could greatly help in the identification of plausible target and pathways of disruption specific to invertebrate organisms. Even with respect to *M. galloprovincialis*, the present work has only partially characterised a single neuroendocrine pathway involved in one process of larval development that could be sensitive to endocrine disruption. It follows that further studies addressing diverse aspects and neuronal population of *M. galloprovincialis* larval neuroendocrine system should be investigated and their susceptibility to endocrine disrupting pathways tested. Consequently, the topic will be investigated by a new project based at the Università degli studi di Genova predominantly focused on the study of the onset and biological functions of the monoaminergic system in *M. galloprovincialis* early larval stages and the susceptibility of its signalling to diverse xenobiotics, EDCs and pharmaceuticals with neuroendocrine activity.

- The characterisation of the biological functions of Nuclear Receptors in invertebrates is essential to the understanding of their potential involvement in the endocrine disruption pathways of environmental pollutants

In this thesis, a selected group of NRs were investigated in *M. galloprovincialis* larval development and linked to a possible developmental function on the base of their expression pattern in the context of the available literature. The findings helped in deciphering the AOP of TBT and linking the disruption of *MgRXR* to adverse biological effects at the organism level. Moreover, the findings also permitted to establish plausible relationships between NRs activity and the process of shell biogenesis thus adding up to the already known developmental functions of NRs in invertebrate development. Still, the evidence is circumstantial. Accordingly, the developmental functions of NRs in *M. galloprovincialis*, as well as their potential role in mediating the effects of EDCs and xenobiotics will be investigated in the near future with the ANR project “Nuclear receptor signalling and endocrine disruption in a mollusc: the power of functional experiments”. Using the model of *M. galloprovincialis* larval development, the project will develop state of the art protocols and resources in embryology, molecular biology and genomics for invertebrate organisms and make the mediterranean mussel a new marine model system for developmental studies. Taking full advantage of the model, the molecular basis of NR signalling and endocrine disruption during *Mytilus* development will be defined and interpreted at the organism level with functional studies. The results of this project will provide an experimental pipeline able to merge the field of Invertebrate Molecular Disruption (IMD) with the Adverse Outcome Pathways (AOP) of emerging pollutants and EDCs that will permit to shed light on the issue of invertebrate endocrine disruption not only in *M. galloprovincialis* but potentially in a variety of invertebrate organisms.

**List of oral communications
and published articles**

Congresses

- ❖ 69° Congress of the Italian Society of Physiology-SIF, 19-21 September 2018, Firenze (Italy). Unraveling the physiological mechanisms involved in early development of marine bivalves. Miglioli A, Dumollard R, Franzellitti S, Fabbri E, Canesi L
- ❖ 31st European Society for Comparative Physiology and Biochemistry Congress-ESCPB, 9-12 September 2018, Porto (Portugal). Role of tyrosinase in early shell formation of *Mytilus galloprovincialis*: a possible marker of endocrine disruption in marine bivalves? Miglioli A, T Balbi, R Dumollard, L Canesi
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Publications

- ❖ Kapsenberg L, **Miglioli A**, Bitter MC, Tambutté E, Dumollard R, Gattuso J-P, 2018. Ocean pH fluctuations affect mussel larvae at key developmental transitions. Proc. Royal Soc. B: Biological Sciences 2018;285:20182381. (doi:10.1098/rspb.2018.2381).
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- ❖ **Miglioli A.**, Boulifard N., Besnardeau L., Schubert M., Canesi L. and Dumollard R. 2022. Implications of NR disruption in developing larvae of *M. galloprovincialis*: a case study with RXR. **Manuscript in preparation. Submission planned to Frontiers in Endocrinology.**

Annex 1

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Ocean pH fluctuations affect mussel larvae at key developmental transitions

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Coastal marine ecosystems experience dynamic fluctuations in seawater carbonate chemistry. The importance of this variation in the context of ocean acidification requires knowing what aspect of variability biological processes respond to. We conducted four experiments (ranging from 3 to 22 days) with different variability regimes (pH_T 7.4–8.1) assessing the impact of diel fluctuations in carbonate chemistry on the early development of the mussel *Mytilus galloprovincialis*. Larval shell growth was consistently correlated to mean exposures, regardless of variability regimes, indicating that calcification responds instantaneously to seawater chemistry. Larval development was impacted by timing of exposure, revealing sensitivity of two developmental processes: development of the shell field, and transition from the first to the second larval shell. Fluorescent staining revealed developmental delay of the shell field at low pH, and abnormal development thereof was correlated with hinge defects in D-veligers. This study shows, for the first time, that ocean acidification affects larval soft-tissue development, independent from calcification. Multiple developmental processes additively underpin the teratogenic effect of ocean acidification on bivalve larvae. These results explain why trochophores are the most sensitive life-history stage in marine bivalves and suggest that short-term variability in carbonate chemistry can impact early larval development.

1. Introduction

Coastal marine ecosystems experience dynamic spatio-temporal variability in seawater inorganic carbonate chemistry [1]. Short-term variability occurs on top of baseline changes associated with ocean acidification. Ocean acidification causes a decrease in mean seawater pH and aragonite saturation state (Ω_a) via absorption of anthropogenic carbon dioxide (CO₂) emissions [2]. As ocean acidification progresses, the acidity of naturally occurring low pH events is enhanced [3]. How the interplay of acidification and coastal pH variability will impact marine organisms requires understanding what aspect of pH variability influences biological processes that occur over similar time frames [4,5].

Fluctuations in coastal carbonate chemistry often occur on a diel period. Diel fluctuations result from daytime photosynthetic removal of CO₂ by phytoplankton and benthic photoautotrophs followed by night-time respiration of the whole community [6]. Depending on habitat characteristics, seagrasses, macroalgae and kelp forests can induce diel pH fluctuations that span a few

tenths to a full pH unit [5–8]. These short-term pH fluctuations are of similar magnitude as anthropogenic ocean acidification (–0.4 units pH by end of the century) [9], which is known to affect a myriad of biological processes [10]. In addition to baseline changes in carbonate chemistry, ocean acidification is expected to increase the magnitude of diel fluctuations [5].

The biological impact of fluctuating carbonate chemistry in the context of ocean acidification is understudied [11]. A few recent studies that address this issue show non-generalizable responses across calcifying taxa and species [11,12]. For molluscs, one the most studied taxa, growth appears largely indifferent to variability [13–16] (but see [17]) despite the fact that exposure to fluctuating conditions may be energetically costly [18]. Understanding impacts of variability requires knowing which parameter of carbonate chemistry drives a biological response and the reaction norm thereof. Given recent advances in these criteria for molluscs, here we focus on the early development of bivalve larvae.

The early development of bivalves is completed within 2–3 days after fertilization and is extremely sensitive to carbonate chemistry [19–22]. Specifically, abnormal development and reduced growth of D-veliger larvae occur under conditions of low Ω_a or low substrate inhibitor ratio ($SIR = [\text{HCO}_3^-]/[\text{H}^+]$) [23,24], which are tightly coupled in manipulations using CO_2 . Previous work suggests that the first 24 h of development are insensitive to CO_2 acidification and exposure thereafter drives abnormal development [21]. Subsequent shell growth is highly dependent on Ω_a or SIR [23,24], due to the fact that larvae have limited control over carbonate chemistry at the site of calcification [25]. These findings suggest that early development of bivalves is comprised of process-specific sensitivities, and short-term fluctuations in carbonate chemistry typical of shallow coastal waters may be important to larval development. Despite its environmental relevance, only a few studies have investigated the impact of variable carbonate chemistry on early development of marine bivalves [13,14], with inconsistent outcomes across species [17]. For example, Frieder *et al.* [17] found that semi-diel variability enhanced larval growth in *Mytilus galloprovincialis*, but not in *M. californianus*. As the aforementioned studies do not control for timing of fluctuations, it remains unclear how fluctuating carbonate chemistry affects specific developmental processes in bivalve larvae.

The aim of this study was to identify what aspects of diel fluctuations in carbonate chemistry (e.g. timing, magnitude) are important to the early growth and development of the mussel *M. galloprovincialis*. Global aquaculture of *M. galloprovincialis* largely depends on natural recruitment for seed supply [26], so natural variability in seawater chemistry in the context of ocean acidification is highly relevant to the persistence of this industry. The first experiment (Exp. 1) tests the hypothesis that the magnitude of variability influences larval growth and development. Results from Exp. 1 informed the design of Exp. 2 and Exp. 3, which test the hypothesis that development is influenced by the timing of variable exposures. A fourth experiment (Exp. 4) was conducted to specifically test the hypothesis that CO_2 -acidified seawater alters development of the trochophore shell field. For two of the four experiments, larvae from unique parental crosses were cultured in isolation to explore the biological variation of the response across parental pairs (Exp. 2 and Exp. 4).

2. Material and methods

For reading simplicity and based on methods of manipulation, we describe treatments in terms of pH, whereby low pH represents the full suite of chemical changes brought on by CO_2 -acidification under stable temperature and salinity (electronic supplementary material, table S1). Low pH treatments were chosen to reach levels of aragonite undersaturation known to induce abnormal development and reduced larval growth [24].

(a) Experimental design

In the context of ocean acidification in dynamic coastal zones, a range of variable pH treatments was used (pH_T 7.4–8.1). Exp. 1 assessed the impact of stable compared to variable pH treatments with the same mean pH (figure 1; electronic supplementary material, table S1). Four treatments were set up: control treatment of stable pH_T 7.8 (pH 7.8—), and three variability treatments with a mean pH_T 7.8 and a diel range of either 0.4 (pH 7.8 ± 0.2), 0.8 (pH 7.8 ± 0.4), or 0.8 offset by 12 h (pH 7.8 ± 0.4), in order to control for the timing of variability exposures. Embryos from three unique families, using three males and nine females (1 male \times 3 females, replicated three times with unique individuals), were cultured separately in the four treatments (12 cultures total, $N = 3$ biological replicates). Larvae were sampled on day 3 (size and morphology), 9 (size) and 22 (size). Treatment pH 7.8 ± 0.2 was discontinued after day 9 for technical reasons.

Variable pH treatments in Exp. 2 and Exp. 3 (figure 2a; electronic supplementary material, table S1) were designed to assess the effect of pH exposure at the start of shell morphogenesis, using a single diel pH fluctuation down from pH_T 8.1 (pH 8.1^-) or up from pH_T 7.4 (pH 7.4^+) centred around the start of calcification, approximately 30 hours post-fertilization (hpf). Stable pH_T exposures were used as controls (pH 8.1— and pH 7.4—) and larvae were sampled on day 3 (size and morphology). In Exp. 2, embryos from three unique pairs were cultured separately (12 cultures in total, $n = 3$ biological replicates per treatment). In Exp. 3, embryos from nine unique pairs were pooled and then distributed across three replicate cultures (total of 12 cultures, $n = 3$ technical replicates). In Exp. 4 (figure 2a), embryos from five unique pairs were cultured separately in pH_T 8.1 and pH_T 7.4 (10 cultures in total, $n = 5$ biological replicates per treatment) and sampled at two time points: 35 hpf (fluorescent staining) and 68 hpf on day 3 (size, morphology, scanning electron microscopy [SEM]).

(b) Larval cultures

Gravid *M. galloprovincialis* were collected from two sites during local spawning seasons. The first group was collected from a dock in Thau Lagoon (43.415°N, 3.688°E), a shallow semi-enclosed bay in Sète, France on 11 October 2016 (Exp. 1). The second group was collected from shallow buoy lines in the Bay of Villefranche-sur-Mer (43.682°N, 7.319°E), France on 9 February 2017 (Exp. 2–4). During the spawning season, pH variability in Thau Lagoon ranged from pH_T 7.80 to 8.10 (L.K., 2016, unpublished data) and from pH_T 8.10 to 8.15 in the Bay of Villefranche [27]. Adult mussels were kept in a single temperature-controlled flow-through sea table (approx. 16°C) and fed three times per week a mixture of Instant Algae (Reed Mariculture, Iso 1800 and Shellfish Diet 1800) until spawning. Experiments were conducted from January through March 2017 (see electronic supplementary material). Spawning was induced by cleaning the mussels of epibionts and warming seawater to 28°C. Sperm was kept on ice and eggs were kept at approximately 16°C until fertilization. Test fertilizations were performed to ensure gamete compatibility prior to batch fertilization. Fertilization was performed simultaneously across biological replicates, except for Exp. 4, which required 30 min

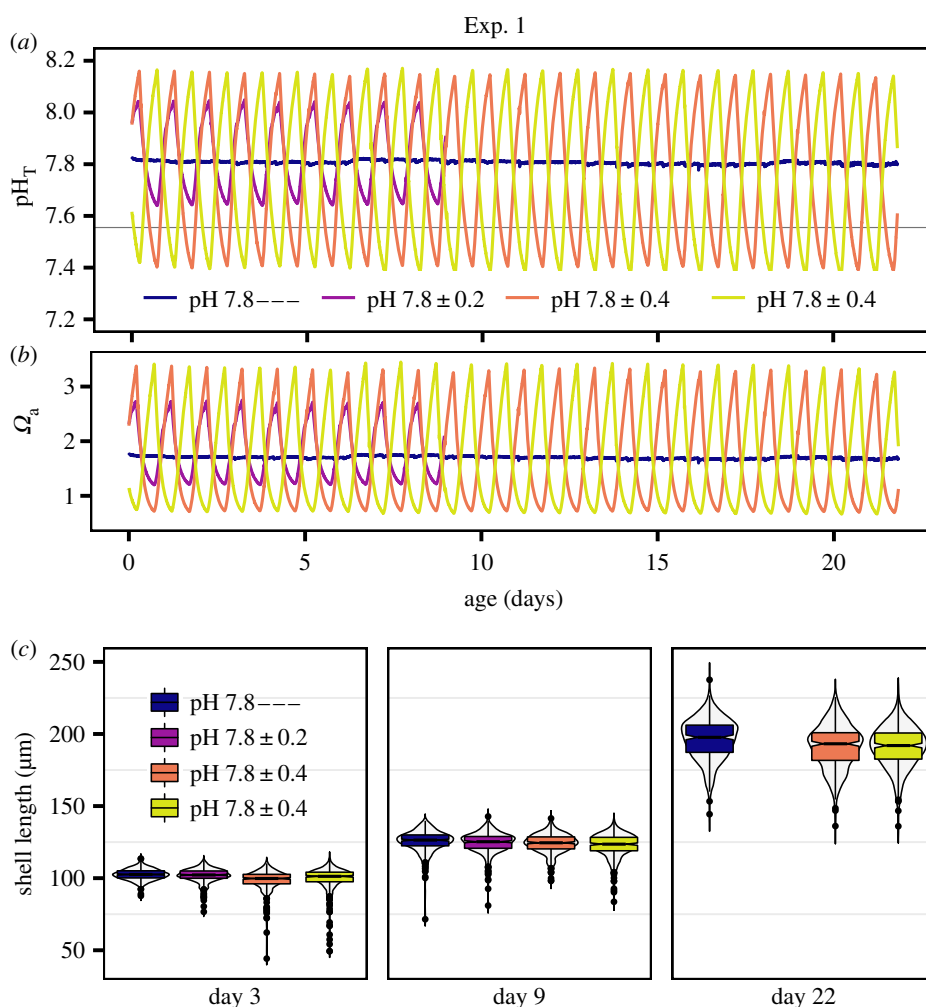


Figure 1. D-veliger shell size correlates to mean exposures: Exp. 1. (a) pH time series of Exp. 1, grey references $\Omega_a = 1$, hpf is hours post-fertilization. (b) Ω_a time series of Exp. 1. (c) Shell size of larvae in Exp. 1 on days 3, 9 and 22. Box plots denote median, median confidence interval (notch width), quartiles and outliers (dots). Violin plots show the size distribution of the observations ($n \approx 300$).

staggered fertilization by pair to facilitate sampling at precisely 35 hpf. Successful fertilization was determined by greater than 90% presence of polar bodies, after which embryos were transferred to culture vessels at a density of 14 embryos ml^{-1} . All experiments were run at 14.3°C (near February habitat conditions [27]) to maintain equal developmental rates. In Exp. 1, larvae were fed 1×10^8 live cells of *Tisochrysis lutea* (CCAP 92714) once or twice per day after day 3.

(c) Experimental system and seawater chemistry

Larvae were cultured in a temperature-controlled pH variability system described in Kapsenberg *et al.* [28]. Four header tanks (35 l) were supplied with seawater pumped from 5 m depth in the Bay Villefranche, filtered to 0.35 μm and UV-sterilized (FSW). Header tank pH was manipulated via the addition of pure CO_2 gas using a glass pH electrode feedback system (IKS Aquastar) and constant aeration with CO_2 -free air. Treatment water was pumped from header tanks to culture buckets ($n = 3$ per header tank, see electronic supplementary material for control of header tank effects) using irrigation drippers (21 h^{-1}). Variable pH treatments switched header tank pH every 12 h, resulting in smooth pH oscillations in cultures as documented by Honeywell Durafet III pH sensors (for performance quality see electronic supplementary material). Exp. 4 consisted of static cultures (sourced from two independent header tanks of pH_T 8.1 and pH_T 7.4) due to the use of calcein dye. Water samples were collected from each treatment header tank for salinity (Mettler Toledo SevenEasy Conductivity) and total alkalinity (A_T) every

2–3 days for Exp. 1, every 2 days for Exp. 2, every day during Exp. 3, and once at the start of Exp. 4. Samples for A_T were run in duplicate using open cell titration (Metrohm Titrando 888) [29]. Accuracy of A_T measurements ranged between -7 and $+5 \mu\text{mol kg}^{-1}$ as compared to certified reference material (A. Dickson, Scripps Institution of Oceanography). Precision of A_T measurements was $2 \mu\text{mol kg}^{-1}$ (mean standard deviation of all duplicate measurements, $n = 68$). Ω_a and $p\text{CO}_2$ were calculated from pH_T using mean temperature, and header tank salinity and A_T per treatment, per experiment (electronic supplementary material, table S1). Carbonate system calculations were performed in RStudio (version 1.0.143) using the seacarb R package [30], with constants K_1 and K_2 , K_f , and K_s from Lueker *et al.* [31], Perez *et al.* [32] and Dickson [33], respectively.

(d) Shell size, morphology and SEM

Shell size was determined as the maximum shell length parallel to the hinge, using bright field microscopy and IMAGEJ software ($n \geq 100$ culture $^{-1}$). On day 3, larval morphology was scored ($n \geq 100$ culture $^{-1}$) according to His *et al.* [34]: normal (D-shaped shell with a straight hinge), trochophore (undeveloped larvae, often shell-less), abnormal hinge (concave D-shaped shell), protruding mantle (D-shaped shell with protruding tissue or velum), and combined abnormal hinge and protruding mantle (D-shaped shell with both abnormalities). For SEM imaging, D-veligers were preserved in 100% EtOH. Prior to imaging (JEOL 6010LV), shells were cleaned (rinses of 2 min tap water, 5 min R.O. H_2O with 1% bleach, 2 min tap water, 5 s

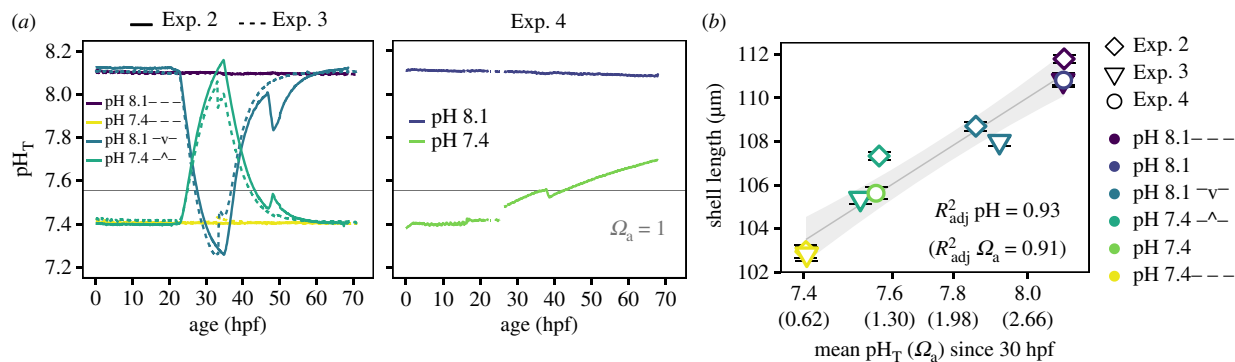


Figure 2. D-veliger shell size correlates to mean exposures: Exp. 2–4. (a) pH time series of Exp. 2–4, grey references $\Omega_a = 1$, hpf is hours post-fertilization. (b) Mean shell size ($n \approx 300$, \pm s.e.) of D-veligers from Exp. 2–4 (symbols) on day 3 plotted against the treatment-respective mean pH exposure since the start of calcification (30 hpf) to sampling time (68–72 hpf). Mechanistically, as calcification is driven by Ω_a (or SIR) and not pH, the corresponding Ω_a data is shown in parentheses (x -axis, R^2_{adj}). Colours indicate pH treatments in (a). Linear regression for pH is in grey (\pm s.e.).

ddH₂O, temporary storage in 100% EtOH), dried at 42°C, and sputter coated with gold.

(e) Fluorescent staining

To visualize the first CaCO₃ precipitation, calcein (0.001 M, final concentration) was added to static cultures of Pair 2 and 4 at 25 hpf in Exp. 4 [35]. Pilot experiments using calcein dye showed that the first calcification occurred during the early trochophore stage at 30 hpf in 14.3°C. Immediately after calcein addition, motorized paddles were turned on in all culture vessels, resulting in slight CO₂ off-gassing in pH_T 7.4. Calcein culture pH was checked daily using a glass pH electrode which was compared against a calibrated Durafet (addition of calcein caused a 0.03 unit pH_T increase in the high pH treatment and 0.10 unit pH_T decrease in the low pH treatment).

To visualize the organic matrix of trochophores in Exp. 4, larvae from all pairs were sampled and stained live with calcofluor (Calcofluor White M2R, #F3543 from Sigma-Aldrich) at 35 hpf. Calcofluor is a fluorochrome that binds to chitin (and cellulose) and has been used to study chitin in adult abalone [36]. Its precision for identifying chitin from other matrix molecules has been debated [37] and the results presented here are restricted to assessing the shape and extent of the organic matrix in general. Concentrated larvae were stained by calcofluor in FSW for 5 min (final exposure of 1:50000 w/v by diluting a stock solution of 1:100 w/v in DMSO, stored at -20°C), washed three times with FSW, fixed with a drop of 4% paraformaldehyde, and immediately imaged on a confocal microscope (Leica SP8, electronic supplementary material). Images were 3D rendered and composite images were rotated for each larva to measure the area of one valve stained by calcein and calcofluor via manual drawing using IMAGEJ software ($n = 13$ –42 culture⁻¹).

(f) Statistical analyses

Data analysis was performed in RSTUDIO (v. 1.0.143) [38]. Residuals of larval shell size data were not normally distributed, violating assumptions required for ANOVAs. For Exp. 1, a two-factor permutation analysis of variance was used with 1000 permutations in RVAideMemoire R package [39]. Treatment and age were fixed factors (interaction was not significant and removed from the final model) and family was used as a blocking factor. Size data from the first 100 larval measurements were used and data from treatment pH 7.8 \pm 0.2, stopped after day 9, was excluded. For Exp. 2–4, a linear regression was used to assess the impact of mean pH exposure on mean larval size during the shell growing period (30–68 hpf). Exp. 2–4 had different biological design (isolating versus pooling larvae from unique parental pairs), so a linear regression was performed on

overall mean larval size ($n \approx 300$ treatment⁻¹ experiment⁻¹). Cultures with calcein dye in Exp. 4 were excluded as these cultures did not contain a Durafet by which to calculate the mean pH exposure. For Exp. 1 and Exp. 2 (family and pair replication), proportions of larvae with normal development was analysed using a generalized linear mixed effects model with treatment as a fixed effect and family or pair as a random effect, using the lmer R package [40]. Significance of the fixed effect was tested against the null model using a likelihood ratio test. For Exp. 3 (bucket replication), treatment effect was assessed using a one-way ANOVA. Analyses were repeated for Exp. 2 and Exp. 3 for proportion of larvae with an abnormal hinge (irrespective of a protruding mantle). All model residuals exhibited a normal distribution (Shapiro–Wilk normality test) and equal variance (Levene’s test). Least-squares means were used for *post hoc* pairwise contrasts of treatment effects for Exp. 2 and Exp. 3, using a Bonferroni correction for six comparisons in the lsmeans R package [41].

3. Results

(a) Shell size responds to mean conditions

The impact of variable carbonate chemistry on shell growth was analysed by comparing shell length of larvae reared under treatments of either constant pH_T 7.8 or mean pH_T 7.8 with a total diel range of 0.4 or 0.8 units pH_T in Exp. 1 (figure 1). Shell length was not significantly affected by a 0.8 unit pH_T diel variation over the course of a three-week exposure (figure 1c; treatment effect: $F_{1,2} 3.33$, $p = 0.093$; shell size increased over time, $F_{1,2} 1712.68$, $p < 0.001$), which suggests that shell length is a function of mean exposures. This was empirically tested and verified in Exp. 2–4, using a combination of stable and variable treatments spanning a range of mean exposures (figure 2). Shell length was significantly correlated to mean exposures during the shell growing period, 30 hpf until the sampling time on day 3 (for pH: $F_{1,8} 119.6$, $p < 0.0001$; for Ω_a : $F_{1,8} 92.57$, $p < 0.0001$). Mean pH_T (Ω_a) explained 93% (91%) of the variance in average shell size of larvae across treatments, respectively (figure 2b). This correlation was independent of developmental effects (abnormal larvae are smaller than normal D-veligers in low pH treatments; electronic supplementary material, figure S1 and table S2) as the correlation was maintained for larvae in the top 10th percentile size class (for Ω_a , $F_{1,8} 32.27$, $p < 0.001$, $R^2_{adj} = 0.78$).

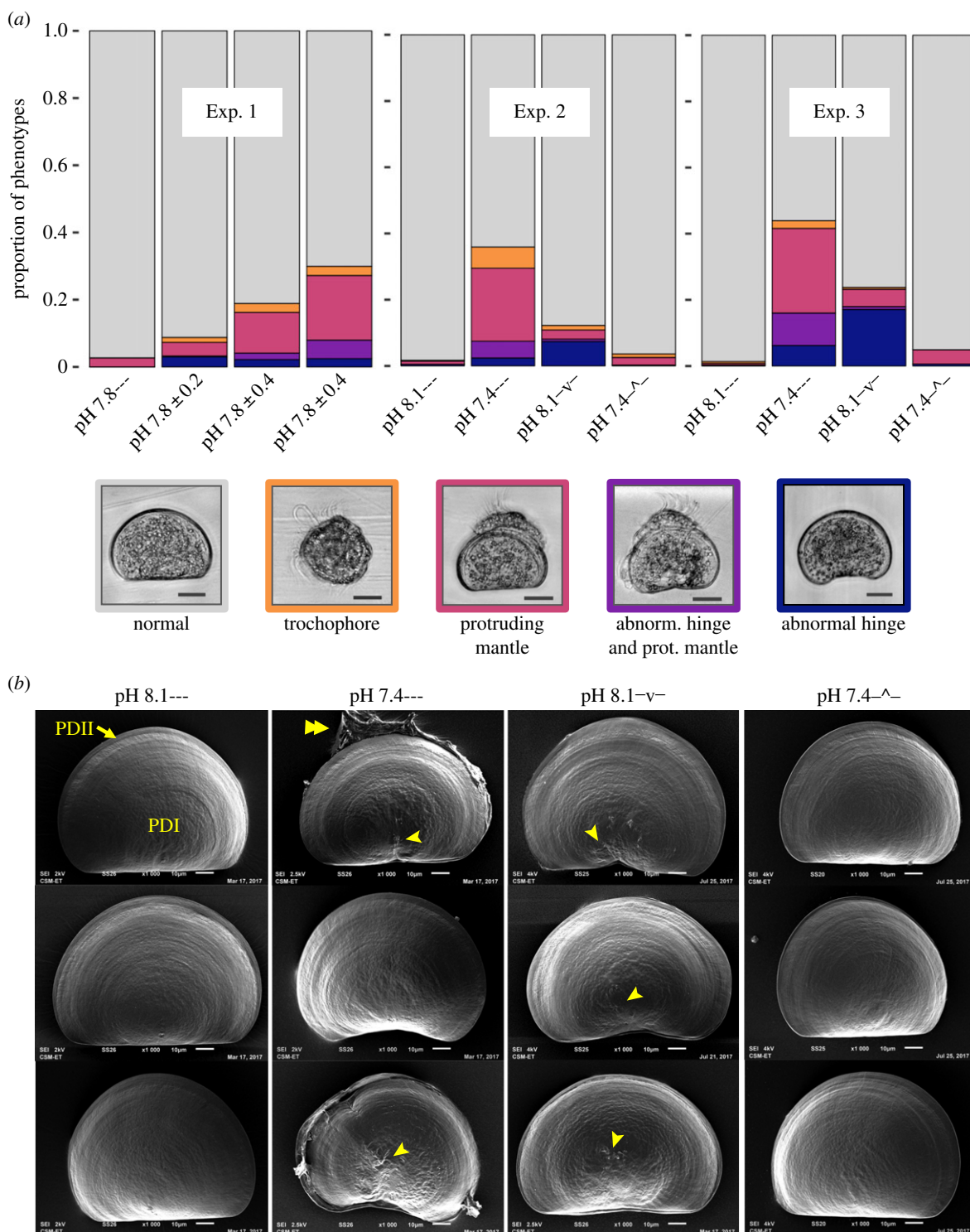


Figure 3. Variable pH alters development of mussel larvae. (a) Proportion of larval phenotypes on day 3 in Exp. 1, Exp. 2 and Exp. 3. Treatment labels match those in figures 1a and 2a. Colour coding follows the border colour of representative phenotype images (scale bar = 30 μm). Per experiment, proportions of specific phenotypes were calculated from summed observations across three cultures per treatment ($n \approx 300$; see text for design of biological replication of cultures). (b) SEM images of D-veliger shells observed on day 3 in Exp. 3 (scale bar = 10 μm , brightness and contrast was adjusted per image). Examples of three larvae were chosen to highlight phenotypes associated with treatment effects. Prodissoconch I (PDI) and II (PDIi) is indicated for a larva from pH 8.1— (top). Examples from pH 7.4— and pH 8.1^{-v-} show larvae with abnormal hinges and scarring at the centre of PDI (arrowhead). Larva with a protruding mantle is indicated by a double arrowhead.

(b) Larval development depends on timing of exposure to acidified seawater

In Exp. 1, abnormal development increased with the magnitude of the diel pH range (figure 3a; p -value < 0.007 for all pairwise comparisons, electronic supplementary material, table S3). The increase in abnormal development across treatments with diel cycles offset by 12 h (pH 7.8 \pm 0.4 versus pH

7.8 \pm 0.4; $p = 0.0013$) suggests that the timing of low pH conditions was an important factor for developmental outcomes. This was empirically tested and verified in Exp. 2 and Exp. 3 (electronic supplementary material, tables S4 and S5). In both Exp. 2 and Exp. 3, the most striking result was that $\geq 95\%$ of larvae developed normally in both pH 8.1— and pH 7.4—^{-^} (figure 3a), whereas larvae in pH 8.1^{-v-} exhibited 76–88% normal development, mostly due to the presence of abnormal

hinge phenotypes. In pH 7.4—, only 56–64% of larvae exhibited normal development due to frequent observations of larvae with protruding mantles and abnormal hinges. Larvae with both an abnormal hinge and protruding mantle were most prevalent in pH 7.4— (5.1% in Exp. 2, and 9.8% in Exp. 3), wherein 60–70% of larvae with a hinge abnormality also exhibited a protruding mantle. The combination of an abnormal hinge and a protruding mantle was observed in less than 1% of larvae in pH 8.1^{-v-} (due to few protruding mantles), and never in pH 8.1— and pH 7.4-^— (due to absence of abnormal hinges). The temporal partitioning of unfavourable seawater chemistry exposure and associated abnormal phenotypes suggests that abnormalities in the hinge and mantle are additive. This additive effect was also apparent in the shell size of these phenotypes (electronic supplementary material, figure S1): larvae with an abnormal hinge, protruding mantle or both are 1, 4 and 6% smaller, respectively, than normal D-veligers from the same low pH treatment (electronic supplementary material, table S2).

(c) Abnormal development of the shell field is correlated to hinge abnormalities

In both Exp. 2 and Exp. 3, the proportion of larvae with an abnormal hinge (irrespective of a protruding mantle) was significantly greater in treatments that experienced low pH conditions around 30 hpf (7–8% in Exp. 2, 16–18% in Exp. 3, in pH 7.4— and 8.1^{-v-}) compared to those that experienced high pH at this time (less than 1% in pH 8.1— and pH_T 7.4-^—; pairwise comparisons p -values ≤ 0.004 ; electronic supplementary material, tables S6 and S7) and equal between treatments which experienced the same pH at that time (p -values = 1.0; electronic supplementary material, tables S6 and S7). Therefore, hinge abnormalities were induced by exposure to unfavourable low pH conditions around 30 hpf (27–35 hpf), independent of prior or later exposures. Larvae exposed to unfavourable carbonate chemistry during this period frequently exhibited irregular texture and scarring in the centre of the first larval shell (PDI, prodissoconch I) (e.g. figure 3*b*; in pH 7.4— and pH 8.1^{-v-} in Exp. 3). In contrast, D-veliger shells of larvae reared in pH 8.1— and pH 7.4-^— were indistinguishable (figure 3*b*).

The developmental process underpinning the abnormal shell development was investigated in Exp. 4. In pH_T 7.4 at 35 hpf, the organic matrix of some larvae exhibited an indentation along the hinge line (figure 4*a*). In contrast, in pH_T 8.1, this chitinous isthmus was straight and well developed. The proportion of larvae with an indented matrix at 35 hpf was highly correlated to the proportion of D-veligers with an abnormal hinge at 68 hpf (figure 4*b*; $F_{1,8} = 47.89$, $p = 0.0001$, $R^2_{\text{adj}} = 0.84$). Larvae from Pair 1 exhibited greater than 99% normal development in pH_T 7.4 at both trochophore (35 hpf) and D-veliger stage (68 hpf), with normal flat shell matrices in both pH_T 8.1 and pH_T 7.4 at 35 hpf (figure 4*c*). In contrast, 55% of larvae from Pair 2 exhibited matrix indentations in pH_T 7.4 ($n = 12$, of 22 larvae), and 35% of D-veligers exhibited hinge abnormalities by day 3 ($n = 42$, of 121 larvae). While some variation in the correlation between phenotypes at 35 and 68 hpf may be related to sample size, these data, along with results from Exp. 2 and 3, provide strong evidence that matrix

indentations persists throughout development and cause hinge abnormalities in D-veligers.

Matrix indentations occurred in the presence and absence of CaCO₃ (electronic supplementary material, figure S2), suggesting that abnormal shell field development is a phenotype that is fixed prior to calcification. At 35 hpf, only 87% of pH_T 7.4 larvae had started calcification ($n = 40$ out of 46). Shell abnormalities in pH_T 7.4 were visible as keyhole-shaped indentations near the hinge, indicating no calcification in that region by 35 hpf (figure 4*a*). In pH_T 8.1, this abnormality was not observed and all larvae exhibited substantial shell growth ($n = 58$). Both the organic matrix and larval shell were larger for larvae reared in pH_T 8.1 compared with those in pH_T 7.4 at 35 hpf (electronic supplementary material, figure S3), regardless of the normal or abnormal developmental trajectory of the larval cohort (visually apparent in figure 4*a,c*).

(d) Protruding mantle indicates sensitivity of the PDI-II transition

The protruding mantle phenotype occurred during the early D-veliger stage around 40 hpf. In Exp. 2 and 3, larvae with protruding mantles (irrespective of an abnormal hinge) were associated with low pH exposure, with approximately 30% observed in pH 7.4— and $\leq 1\%$ in pH 8.1— (figure 3*a*). In contrast, treatments pH 7.4-^— and pH 8.1^{-v-} induced a low occurrence of protruding mantle (respectively, 2.3 versus 3.5% in Exp. 2; 4.3 versus 6.1% in Exp. 3) indicating that this phenotype was induced by low pH at a time when pH in both treatments were similar. There are only two periods where this occurs, near 27 and 40 hpf. The most relevant time-point is 40 hpf, as embryos are resistant to CO₂ acidification during the first day of development [21], and developmental processes around 40 hpf relate to the shell–mantle interface at the transition from the first (PDI) to the second larval shell (PDII).

4. Discussion

The aim of this study was to identify which aspect of mussel larval development is influenced by temporal variability of carbonate chemistry. By using a range of variability treatments, we show that shell growth responds to mean exposures while development depends on time-sensitive exposures (Exp. 1–4), and these processes additively contribute to overall D-veliger phenotypes. Based on timing of unfavourable conditions, sensitive developmental processes were identified and linked to specific abnormal phenotypes (Exp. 2–3), with mechanistic evidence at the tissue layer (Exp. 4). Electronic supplementary material, figure S4 details the developmental timeline and windows of sensitivity.

(a) Mussel larval growth and development

Larval shell growth was driven by mean exposures regardless of variability regimes (figures 1*c* and 2*b*). This reflects the instantaneous nature of Ω_a -dependent precipitation kinetics in bivalve larvae [42], whereby the benefit of high Ω_a is neutralized by the negative effect of low Ω_a . This has also been observed in other mollusc larvae, including abalone, hard clams, oysters and scallops [13–15], although oddly not for *M. galloprovincialis* from California [17]. Frieder

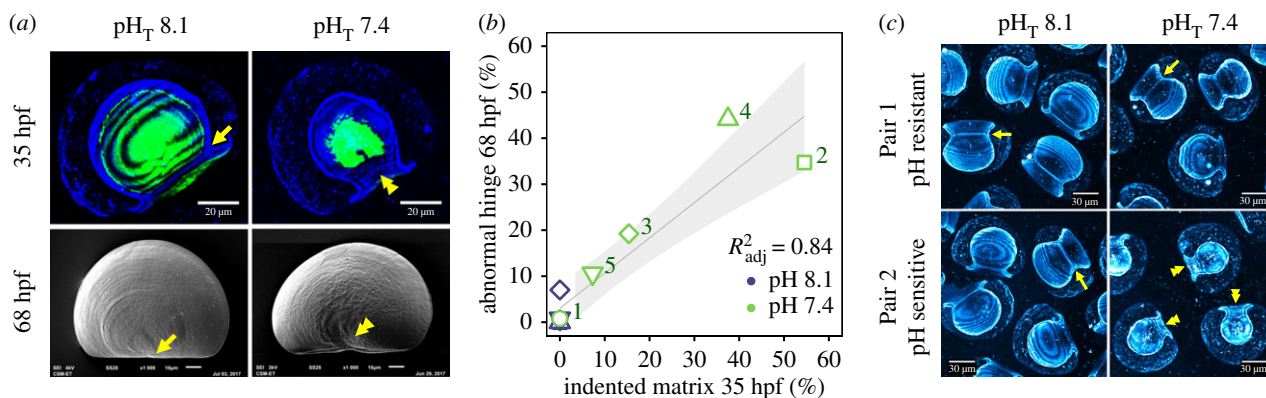


Figure 4. Relationship between organic matrix morphology and D-veliger shell phenotype in Exp. 4. (a) Images of larvae in pH_T 8.1 (straight hinge, arrow) and pH_T 7.4 (indented matrix and hinge, double arrowhead). Composite confocal images show larvae at 35 hours post-fertilization (hpf; organic matrix stained in blue, CaCO₃ stained in green, scale bar = 20 μm) and SEM show larvae at 68 hpf (scale bar = 10 μm). Larvae are of unique individuals from Pair 2 and were selected to best visualize the correlation shown in (b). (b) Percentage of larvae with an indented organic matrix at 35 hpf ($n = 13–42$) correlated with percentage of D-veliger larvae exhibiting an abnormal hinge at 68 hpf ($n = 109–155$), across five unique male–female pairs (symbol, numbered for pH_T 7.4). Linear regression line is grey (\pm s.e.). (c) Trochophore larvae (Pair 1 and Pair 2) reared in pH_T 8.1 and pH_T 7.4 and stained with calcofluor (cyan) at 35 hpf. Colour was adjusted equally across images for clarity of the hinge line (scale bar = 30 μm). Arrows denote normal organic matrices and hinge lines (arrow) and indented matrix (double arrowhead). Ring-like pattern in staining is an artefact of confocal imaging larvae through multiple slices.

et al. [17] found that *M. galloprovincialis* larvae increased growth in low pH treatments when semi-diurnal fluctuations were included. Culture temperatures and larval growth rates in control treatments in their study and ours are comparable (approx. 125 μm; day 8 in Ω_a 1.9 at 15.7°C versus day 9 in Ω_a 1.7 at 14.4°C in our study). As Frieder *et al.* [17] conducted a single experiment with few replicate measurements, further experiments will be necessary to identify the source of these contrasting observations.

In terms of development, we identified two processes that, when they occur during conditions of low pH (low Ω_a and SIR), give rise to abnormal D-veligers: (i) formation of the shell field prior to calcification around 30 hpf, and (ii) velum retraction around 40 hpf prior to the transition from PDI to PDII.

Exposure to low pH conditions around 30 hpf produced D-veligers with an abnormal hinge (figure 3, Exp. 2 and 3). The major developmental process occurring at this time is the formation of the shell field during the mid-trochophore stage (electronic supplementary material, figure S4). This process is initiated in the early trochophore stage by the invagination of a group of ectoderm cells, which create a pore. This brings together a rosette of outer surface cells that secrete what appears to eventually become the periostracum [43]. The pore closes and deeper invaginated cells then evaginate back to the surface epithelium where, under control conditions, they create a flat region that expands via mitotic division, under the expanding periostracum [43–45]. During this process, shell field cells exude a chitin-based organic matrix wherein calcification takes place [43,46,47]. In low pH treatments, the organic matrix often exhibited a central indentation at the site of the shell field invagination (figure 4). At 35 hpf, the lack of calcification in this area and scarring in the centre of PDI on D-veligers suggests a treatment effect on cells associated with the shell field evagination (figure 3b). Larvae in pH 7.4– \wedge – calcified smooth shells, despite being in unfavourable carbonate chemistry from approximately 40 hpf onward. This indicates that the effect of unfavourable carbonate chemistry at the start of calcification (approx. 30 hpf), which generated abnormal

shell texture, is different from the effect of low Ω_a that drives the rate of linear, but smooth, extension of the shells after this period [24]. Similar distortions in PDI have previously been observed in oyster larvae of *Ostrea edulis* and linked to the process of the shell field evagination, although this was not in the context of environmental conditions [48]. Hinge abnormalities may result from abnormal or incomplete restructuring of the ectoderm during development of the shell field, probably prior to calcification. The resulting abnormal trochophore body shape alters the calcification blueprint, whereby the shell simply takes on the shape of the cellular landscape over which the organic matrix is exuded, thereby producing D-veligers with an indented hinge.

Within low pH treatments, the abnormal hinge phenotype is only 1% smaller than normal D-veligers (electronic supplementary material, figure S1). In Exp. 1, abnormal hinge larvae were still present on day 9 (L.K. 2017, personal observation). By day 22, however, curvature of the shell masked the angle of the shell hinge, so there is no evidence to infer the effect of the abnormal hinge phenotype on larval fitness.

Regardless of normal or abnormal shell field development, the organic matrix and calcified area at 35 hpf was consistently smaller in low pH treatments, which we interpret as developmental delay. Developmental delay likely occurs from exposures during the early trochophore stage (electronic supplementary material, figure S4). The energetic cost of building the protein-rich organic matrix is much greater than the cost of external calcification [49,50]. In oyster larvae, low pH causes a decrease in the protein deposition efficiency [51]. Developmental delay of the shell field may thus stem from protein production issues associated with building the organic matrix. Consequently, this could also delay the onset of calcification.

Exposure to low pH conditions near 40 hpf was linked to increased frequency of D-veligers with protruding mantle tissue or velum (irrespective of an abnormal hinge). At this time, the extension of the calcified PDI shell has caught up with the leading edge of the expanding organic matrix, which

then both fully cover the larval body (L.K. 2017, personal observation). Around 47 hpf, larvae gain the ability to retract their velum and close their shell (a behavioural response to ethanol exposure; L.K. 2016, personal observation). Shell closure marks the transition from PDI growth to concentric growth lines distinctive of PDII (electronic supplementary material, figure S5) [48]. Protruding tissue is evidence of either abnormal tissue development or inability to retract the velum. It is likely that these larvae cannot progress in development to PDII growth. Such a developmental arrest is evident in shell size. D-veligers with protruding tissue tend to be 4–6% smaller than normal D-veligers (electronic supplementary material, figure S1). Previous experiments showed that protruding mantle phenotypes of *M. galloprovincialis* lacked PDII growth, even after a 5-day exposure to Ω_a 0.49 [21], which suggests that this phenotype is terminal.

From this study, it is clear that CO₂-acidified seawater impacts development of the larval body in multiple ways, independent from impacts on calcification (electronic supplementary material, figure S4). This study raises the question as to how seawater CO₂-acidification disrupts tissue development in mussel larvae and highlights the need to identify mechanisms of ocean acidification impacts at a cellular level.

(b) Environmental and global change context

Our results suggest that developmental success of mussel larvae will be unpredictable in habitats with high variability in carbonate chemistry. Cues that determine the moment of a spawning event in the field are not well understood. The mussel population in Villefranche appears to spawn out during storms, and mussels have spawned in a bucket on the boat, which suggests that the final trigger may be physical and unrelated to time of day. Temperature-dependent developmental rates could also influence when a given larval cohort will be sensitive to carbonate chemistry. As shell growth depends on mean conditions, areas with high mean Ω_a , regardless of variability regimes, may significantly benefit normally-developed D-veliger larvae, if larvae are retained in this body of water throughout their pelagic phase.

Normal larval development in pH_T 7.4 ranged from 30% to 99% across unique parental pairs (electronic supplementary material, figures S6 and S7). Such biological variation is common among bivalves and may facilitate adaptation to global change [52,53]. Consequently, as ocean acidification progresses, dormant genotypes previously unselected for in natural populations may be favoured via new natural selection pressures operating on early development [54].

Although we did not test for this, evidence for adaptation can be found via population comparisons between sites with different pH regimes [55]. Overall, future recruitment of *M. galloprovincialis* is likely to increase in variance as emerging factors such as local carbonate chemistry variability, timing of spawning, parental effects, and other co-occurring global change stressors gain importance in determining successful development of a larval cohort.

5. Conclusion

By controlling the timing of low pH events, this study partitions the effect of ocean acidification on mussel larval growth from that on development. Larval growth is a function of mean exposures. This extends previous research on the Ω_a -dependency of calcification in bivalve larvae [23,24] by demonstrating that calcification responds instantaneously to changes in seawater chemistry. Independent from calcification, abnormal development was driven by sensitivity to low pH conditions during specific soft-tissue developmental processes: (i) formation of the shell field and (ii) transition from PDI to PDII. This is the first study documenting ocean acidification sensitivity in soft tissues of bivalve larvae and how formation thereof determines D-veliger morphology. These additive and short-lived processes explain why trochophores are the most sensitive life-history stage in marine bivalves.

Ethics. Number of adult mussels used and duration of stress was minimized.

Data accessibility. Data are archived on BCO-DMO, <https://www.bco-dmo.org/project/720349>.

Authors' contributions. L.K. designed the research with input from A.M., M.C.B. and J.-P.G. L.K., A.M., M.C.B. and E.T. performed research. R.D. and J.-P.G. contributed resources. L.K. analysed the data and wrote the paper with contributions from all authors.

Competing interests. The authors declare they have no competing interests.

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Annex 2

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Molecular basis of ocean acidification sensitivity and adaptation in *Mytilus galloprovincialis*.

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