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MOLECULAR AND CELLULAR MECHANISMS OF IOXYNIL AND DIETHYLSTILESTROL DISRUPTION OF CARDIAC AND THYROCYTE DEVELOPMENT AND HOMEOSTASIS

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Doutoramento em Ciências Biológicas

Especialidade em Toxicologia

Orientadores:

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Molecular and cellular mechanisms of ioxynil and diethylstilestrol disruption of cardiac and thyrocyte development and homeostasis

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Declaro ser o autor deste trabalho que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Declaration of authorship:

This work has not previously been submitted for a degree in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Yifeng Li

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The heart of the survival is not about knowledge.

It's about embracing failure, making mistakes, and never giving up.

Edward Michael Grylls

Abstract

Endocrine disrupting chemicals (EDCs) are compounds that interfere with endocrine systems, induce alterations in their functionality, and give rise to numerous adverse effects that have been documented in animals and ecosystems. The herbicide ioxynil (IOX) and the synthetic estrogen diethylstilbestrol (DES) are two chemicals still in use, which are environmentally relevant contaminants that act as EDCs. In humans, prenatal exposure to DES is associated with an increased incidence and prevalence of cardiac defects. IOX may disrupt the thyroid system by binding to transthyretin (TTR) and provoke thyroid tumors in rats. The main objective of this thesis was to determine how IOX and DES disrupt the crosstalk between the developing thyroid gland and cardiovascular system in zebrafish. An invertebrate bioindicator species, *Mytilus coruscus*, was included in the study to comprehend the effects of IOX and DES on a bivalve and contribute to a broader understanding of endocrine disruption in both invertebrate and vertebrate organisms. The core achievements were a) characterization of heart function and cardiovascular and thyroid development in IOX- and DES-exposed zebrafish embryos. Transcriptome analysis of vascular endothelial cells of zebrafish embryos that elucidated compound-specific molecular effects associated with endothelial functions; b) identification of the effects of IOX and DES on the physiology of the heart and thyroid in juvenile zebrafish; c) characterization of the effects of IOX and DES on cardiac performance and shell growth of juvenile *M. coruscus*. Transcriptome analysis of juveniles revealed genes related to cardiac function, neuroendocrine regulation, and detoxification were affected. The findings revealed that IOX and DES exposure had a disruptive effect at a molecular and functional level on the cardiovascular system of a vertebrate (zebrafish) and an invertebrate (*M. coruscus*), suggesting that these chemicals function as cardiovascular disruptors in both phyla. Overall, the study highlights for the first time the potential at both a molecular and functional level of adverse outcomes for both fish and bivalves of exposure to IOX and DES in the environment, suggesting they are EDCs with broad impacts across multiple organisms.

Keywords: Diethylstilbestrol, Heart, Ioxynil, *Mytilus coruscus*, Thyroid, Vascular, Zebrafish

Resumo

A desregulação endócrina, uma preocupação ambiental proeminente impulsionada pela industrialização, é significativamente influenciada por produtos químicos como o herbicida IOX e o estrogénio sintético DES. O IOX e o DES continuam a ser utilizados e apresentam riscos ambientais assinaláveis devido ao seu papel como produtos químicos disruptores endócrinos (EDC). Em humanos, a exposição pré-natal ao DES tem sido associada a uma série de efeitos adversos, incluindo o aumento dos riscos de defeitos reprodutivos, de desenvolvimento neurológico e cardiovascular em indivíduos expostos e nos seus descendentes. O IOX foi identificado como um disruptor do sistema tiroideu em modelos de roedores, podendo levar a tumores da tiroide devido à sua interação com a transtirretina (TTR).

Este estudo investiga os efeitos do IOX e do DES nos sistemas cardiovascular e tiroideu em peixe-zebra (*Danio rerio*) e mexilhão (*Mytilus coruscus*). Para avaliar estes impactos, embriões de peixe-zebra foram expostos a concentrações micromolares de IOX e DES na água. Especificamente, doze horas pós-fertilização (hpf), embriões de peixe-zebra Tg(fli1:GFP) e Tg(cmalc2:GFPCaaX) foram expostos a concentrações de 0,1 μM de IOX ou DES por períodos de 36 horas (até 48 hpf) ou 60 horas (até 72 hpf). Os embriões foram utilizados para classificação de células endoteliais vasculares, imunohistoquímica em "whole mount", transcriptómica seletiva de tecidos, análise de expressão génica selecionada por análise quantitativa de reação em cadeia da polimerase em tempo real e determinação da frequência cardíaca por imagens ao *vivo*. A exposição tanto ao IOX como ao DES resultou num aumento da frequência cardíaca, redução do volume ventricular e diminuição do diâmetro da aorta. A análise transcriptómica revelou alterações significativas no perfil de expressão genética das células endoteliais, indicativas de efeitos tóxicos específicos do composto. Estas descobertas indicam que ambos os produtos químicos impactam diretamente o desenvolvimento vascular e cardíaco, com consequentes efeitos indiretos no desenvolvimento da glândula tiroideia. Embora os desfechos finais de toxicidade – como a alteração da morfologia cardiovascular e da tiroide – fossem semelhantes para o IOX e o DES, os químicos pareceram afetar estes sistemas através de vias reguladoras genéticas e mecanismos fisiológicos distintos.

Foram realizados estudos de exposição crónica com peixes-zebra juvenis para avaliar os efeitos a longo prazo do IOX e do DES. Durante um período de 60 dias, peixes-zebra foram expostos a 0,1 μM de IOX ou DES através da dieta. As análises de imunofluorescência e morfométrica 3D do coração juvenil revelaram que a exposição ao IOX levou a uma deformação ventricular

significativa e a um aumento de volume ($p < 0,001$). Em contraste, a exposição ao DES resultou numa alteração da morfologia ventricular sem afetar significativamente o volume ventricular ($p > 0,05$). A exposição ao DES levou também à sobre-regulação de vários genes relacionados com o endotélio, incluindo *angptl1b*, *notch1b*, *mhc1lia*, *mybpc2a*, *ptgir*, *notch1b* e *vwf*, que estão envolvidos na homeostasia vascular. Observou-se que tanto o IOX como o DES alteram a morfologia do folículo tiroideu; o IOX causou hipertrofia folicular, enquanto o DES levou a um aumento do campo tiroideu. Estas descobertas confirmam que ambos os produtos químicos actuam como EDCs com impactos significativos na morfologia do coração e da tiróide, sugerindo que os efeitos cardíacos observados estão provavelmente ligados à alteração da função da tiróide em peixes-zebra juvenis.

Investigações adicionais sobre o bivalve marinho *M. coruscus* revelaram os efeitos do IOX e do DES a um nível diferente de organização biológica. Os mexilhões foram expostos a baixas concentrações de IOX (0,37, 0,037 e 0,0037 mg/L) e DES (0,27, 0,027 e 0,0027 mg/L) na água. Foi observada uma diminuição da frequência cardíaca em ambos os grupos de tratamento após um dia de exposição, com 0,27 mg/L de DES a provocar uma redução significativa da frequência cardíaca ao longo do tempo ($p < 0,05$) e sem sinais de aclimatação. Os efeitos funcionais foram associados à expressão diferencial significativa da sinapse serotoninérgica e de genes relacionados com o coração a 0,027 mg/L de DES, sugerindo interferência na regulação neuroendócrina e efeitos cardíacos diretos. Além disso, a exposição ao DES resultou na regulação positiva de genes relacionados com a desintoxicação e na regulação negativa de genes da função imunitária, indicando processos de desintoxicação melhorados e respostas imunitárias suprimidas. Em contraste, o IOX causou menos perturbações moleculares. Notavelmente, o crescimento da casca foi significativamente suprimido em mexilhões expostos a 0,0027 mg/L de DES, com menor expressão diferencial de genes em relação ao controlo, destacando um impacto direto no desenvolvimento da casca. Estes resultados demonstram que o IOX e o DES atuam como disruptores neuroendócrinos com efeitos alargados no desempenho cardíaco e no crescimento da concha, sendo que o DES apresenta um impacto mais pronunciado em comparação com o IOX nos bivalves marinhos.

Ao analisar estes EDCs em organismos representativos de vertebrados e invertebrados, esta tese oferece um avaliação abrangente dos efeitos adversos do IOX e do DES em diversas espécies. Realça também potenciais efeitos sinérgicos ou antagónicos que com estudos de uma única espécie podem não ser identificados. Os defeitos cardíacos causados pelo DES em humanos, que também são observados no peixe-zebra e nos mexilhões, indicam que a disrupção da vias

conservadas entre estes especies pode levar a resultados adversos para a saúde, sugerindo que tanto o IOX como o DES actuam como disruptores cardiovasculares nestas espécies, apesar das potenciais diferenças na sua mecanismos de ação. As conclusões sublinham a necessidade urgente de medidas regulamentares para mitigar a exposição a estes produtos químicos e proteger tanto o ambiente como a saúde humana. Os significativos efeitos desreguladores endócrinos do IOX e do DES em diversas espécies enfatizam os seus impactos prejudiciais nos sistemas cardiovascular e endócrino, revelando a necessidade de mais investigação sobre as suas consequências ecológicas e de saúde a longo prazo.

Palavras-chave: Coração, Dietilestilbestrol, Ioxinil, *Mytilus coruscus*, Peixe-zebra, Tiroide, Vascular

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List of Abbreviations

18s – 18S ribosomal subunit

Acvr1 - activin receptor type-1-like

Acvr1bb - activin receptor type-1B-like

Acvr2a - actin-related protein 2 precursor

Adra1d - alpha-1A adrenergic receptor-like

Adra2a - alpha-2A adrenergic receptor-like

Adrb1- β1-adrenergic receptor

AFSW - autoclaved filtered seawater

Agtr1a - angiotensin II type I receptor

Angptl1b - angiopoietin-related protein 2

AnpX2 - atrial natriuretic peptide-converting enzyme-like isoform X2

BaP - benzo(a)pyrene

BPA - bisphenol A

Cacna1da - voltage-dependent L-type calcium channel subunit alpha-1D

Cacna1ha - voltage-dependent T-type calcium channel subunit alpha-1H

Cacna1d - muscle calcium channel subunit alpha-1-like isoform X6

Cacna2d1 - voltage-dependent calcium channel subunit alpha-2/delta-2-like isoform X4

Cacna2d2 - voltage-dependent calcium channel subunit alpha-2/delta-1-like

Cacnb2 - voltage-dependent L-type calcium channel subunit beta-2

Cacng2b - calcium channel, voltage dependent, gamma subunit 2b

Cadm1a - cell adhesion molecule 1a

Calcr - calcitonin receptor

Chrnb3a - cholinergic receptor, nicotinic, beta polypeptide 3a

ClO_4^- - perchlorate

CNS - central nervous system

CTR - control

Cxcr4 - C-X-C chemokine receptor type 4

CYP - Cytochrome P450s

Danio rerio - zebrafish

DDT - dichlorodiphenyltrichloroethane

DEHP - Di-(2-Ethylhexyl) Phthalate

DE - differential expression

DEGs - differentially expressed genes

DES - diethylstilbestrol

Dio1/D1 - Deiodinase type 1

Dio2/D2 - Deiodinase type 2

Dio3/D3 - Deiodinase type 3

EDCs - endocrine disrupting chemicals

Edn1- endothelin 1

Egr1- Early growth response protein 1

ER - estrogen receptor

EU - European Union

Fas1 - fasciclin-1-like

Faxc - failed axon connections homolog

FRKM - fragments per kilobase of exon per million mapped reads

GFP - green fluorescent protein

H&E - haematoxylin and eosin

HBM - heartbeats per minute

Hpf - hours post-fertilization

Hif1a - hypoxia-inducible factor 1 alpha

HI-FBS - heat-inactivated fetal bovine serum

HPA - hypothalamic-pituitary-adrenal

HPG - hypothalamic-pituitary-gonad

HPT - hypothalamic-pituitary-thyroid

IF - immunofluorescence

IHC - whole-mount immunohistochemistry

Inpp5a - type I inositol 1,4,5-trisphosphate 5-phosphatase-like isoform X2

IOX - Ioxynil

Itpr1b - inositol 1,4,5-trisphosphate receptor, type 1b

Klf2 - Kruppel-Like Factor 2

Lamb1 - laminin subunit beta-1

MCT8 - monocarboxylate transporter 8

MCT10 - monocarboxylate transporter 10

Mhc1lia - hereditary hemochromatosis

MMI – methimazole

Morpholino – MO

MOA - modes of action

Mybpc2a - myosin binding protein C, fast type a

Mytilus coruscus - Mc

NIS - sodium-iodide symporter

Nk2.1a - thyroid transcription factor 1

NO_3^- - nitrate

Notch1b - notch receptor 1b

Octalpha - alpha-2Db adrenergic receptor-like

PBS - phosphate-buffered saline

PBTr - 0.5 %TritonX-100

PCBs - polychlorinated biphenyls

PFA – paraformaldehyde

Piezo1- piezo-type mechanosensitive ion channel component 1

Pkd2 - Polycystin-2

Plp - perlucin-like protein

PlxnA4 - plexin-A4

Ppiase - peptidyl-prolyl cis-trans isomerase FKBP2-like

Ptgir - prostacyclin receptor-like

PTU - propylthiouracil

qPCR - quantitative real-time polymerase chain reaction

rT_3 - reverse T_3

RXR - retinoid X receptor

S1p1 - Sphingosine-1-phosphate receptor 1

SCN^- - thiocyanate

Slco1a1 - solute carrier organic anion transporter family, member 1a1

Slco1a4 - solute carrier organic anion transporter family, member 1a4

Slco1c1 - solute carrier organic anion transporter family, member 1c1

Stk11 - serine/threonine-protein kinase stk11-like

T_3 – triiodothyronine

T_4 – thyroxine

TBG - thyroxine-binding globulin

TBT - tributyltin

TCB - 3,3′,4,4′-tetrachlorobiphenyl

TCDD - 2,3,7,8-tetrachlorodibenzo-p-dioxin

Tg - thyroglobulin

Tgfbr3 - transforming growth factor beta receptor type 3-like

THBP - Thyroid hormone binding protein

Thrb - thyroid receptor beta

THs - thyroid hormones

Tnni1 - cardiac troponin I

TPM - transcripts per million reads

TPO - thyroid peroxidase

TR/THR - thyroid receptor

TRE - TH response elements

TRH - thyrotropin-releasing hormone

Trpc5 - transient receptor potential canonical 5

Trpm3 - transient receptor potential cation channel subfamily M member 3-like

Trpv5 - transient receptor potential cation channel subfamily V member 5-like isoform X1

TSH - thyroid-stimulating hormone

TTR - transthyretin

Twk7 - TWiK family of potassium channels protein 7

Tyrp1 - tyrosinase-like protein-1

VA - ventral aorta

Vwf - von Willebrand factor

CHAPTER 1

Effects of ioxynil and diethylstilbestrol on cardiovascular development and endocrine function in aquatic organisms: insights from zebrafish and mussels

1.1. General introduction

The present thesis considers the likely impact of disruption by environmental contaminants or endocrine disrupting chemicals (EDCs) on both vertebrates and invertebrates. To fully comprehend the potential mode of action and likely consequences of EDCs, an understanding of the endocrine system and its actions is necessary. Since my study is focused on the thyroid axis, I start this introduction by giving a very brief overview of the main characteristics of the vertebrate endocrine system. In fact, despite the evolutionary distance between fish and mammals, there is generally good conservation of glands, tissues and hormones of the endocrine system. Much less is known about invertebrate endocrine systems and so a general and very brief consideration is provided. A section is dedicated to characterizing the specific characteristics of the thyroid system in fish and chordate species. Notably, I discuss the evidence supporting the existence of thyroid signaling pathway genes in invertebrates, suggesting an early emergence of thyroid system components during evolution. Furthermore, I outline evidence supporting a direct correlation between thyroid morphogenesis and the development and interaction of cardiac and pharyngeal blood vessels. The adverse effects of EDCs on both vertebrates and invertebrates, particularly on the thyroid system, are comprehensively described. Lastly, I underscore the advantages conferred by utilizing vertebrate model organisms, such as zebrafish, alongside invertebrate model organisms, like bivalve molluscs, to elucidate the toxicological impacts of EDCs.

The endocrine system is a vital regulatory network in vertebrates since it is responsible for coordinating physiological processes and maintaining constant internal homeostasis. This complex system in vertebrates is constituted by a diversity of glandular tissues, including the hypothalamus, pituitary gland, thyroid gland, parathyroid glands, reproductive glands, gastrointestinal tract, pancreas, and adrenals (Figure 1). The endocrine glands secrete hormones that control a diversity of physiological functions including growth, development, and metabolism. The release of hormones into the bloodstream means they rapidly reach all parts of the body and act on target organs or cells, where they exert their effects. The effect of hormones is brought about when they interact with specific receptors located on target cells and trigger a cascade of cellular and molecular events that lead to a response that tends to be characteristic of each hormone. The synthesis and secretion of hormones are regulated by negative and positive feedback mechanisms. In negative feedback, when circulating hormone levels reach a specific threshold, they feedback on the producing gland and inhibit further production. In contrast, when concentrations of a hormone in an organism drop, this stimulates

its synthesis and release and, in this way, ensures homeostasis and optimal physiological functioning.

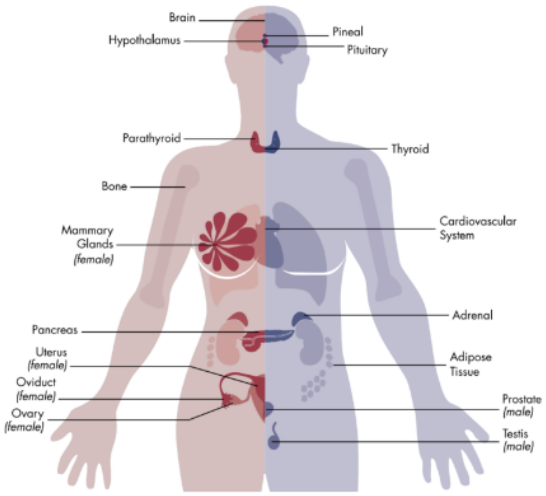

Figure 1.1. Diagram illustrating the anatomical location of the main endocrine glands in humans. Cited from Gore *et al*., Endocrine Reviews, 2015

In vertebrates the hypothalamus-pituitary-organ/tissue interaction regulates a large diversity of actions and connects the central nervous system (CNS) and the endocrine system (Porterfield & Hendrich 1993). The interface between the CNS and endocrine system means animal physiology can be rapidly adjusted to maintain whole organism homeostasis in the face of internal and external change. The hypothalamus contains specialized neurons that play a key role in regulating the pituitary gland. The pituitary gland is composed of the neurohypophysis and adenohypophysis (Amar & Weiss 2003) (Figure 2). The neurohypophysis comprises bundles of neurons originating from the hypothalamus, and it releases various peptide hormones into the bloodstream to act on target organs and maintain homeostasis. In teleost fish no blood portal system exists and instead the bundles of neurons from the hypothalamus project directly to the pituitary gland (Bernier *et al.* 2009; Zohar *et al.* 2010). The adenohypophysis is the glandular part of the pituitary that contains a diversity of cells, corticotrophs, somatotrophs, lactotrophs, gonadotrophs and thyrotrophs that secrete the pituitary hormones (Figure 2). The hypothalamus controls the release of hormones from the adenohypophysis, which releases hormones that can provoke an action on distant tissues or cells and stimulate the secretion of hormones from other endocrine glands of the body. The adenohypophysis controls the adrenal gland, thyroid, and gonads, all of which produce hormones. The regulatory endocrine loops are

designated according to the target tissues modulated and include the hypothalamic-pituitary-thyroid (HPT) axis, the hypothalamic-pituitary-adrenal (HPA) axis, and the hypothalamic-pituitary-gonad (HPG) axis.

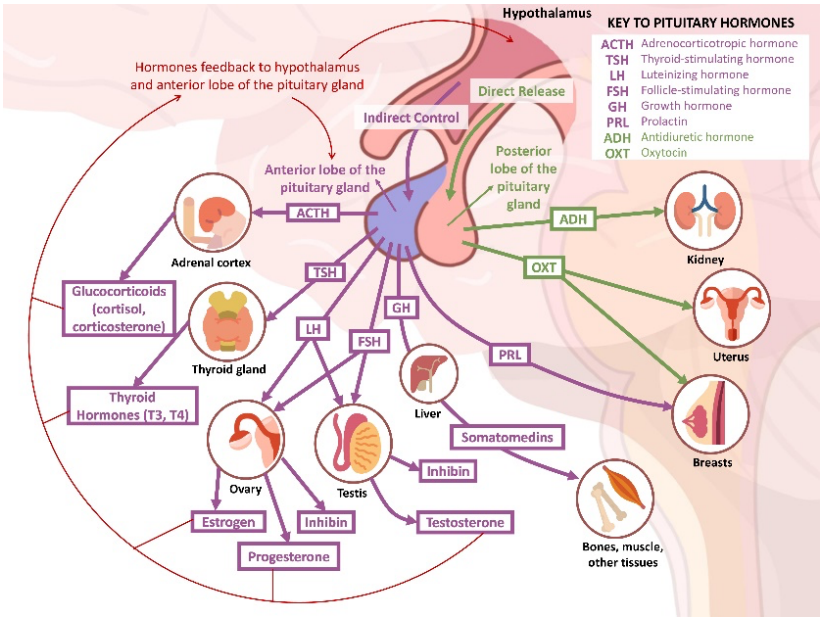

Figure 1.2. The anatomy of the pituitary gland and pituitary hormone regulation in mammals. The hypothalamus directly secretes antidiuretic hormone and oxytocin into the posterior lobe of the pituitary gland (neurohypophysis), which subsequently stimulates target organs. Hypothalamic hormones stimulate pituitary hormone release from the anterior lobe of the pituitary gland (adenohypophysis), which in turn provokes an action in effector organs. (Image modified from Bayram-Weston et al., Nursing Times, 2021)

The invertebrates are a paraphyletic group of animals lacking a vertebral column, they are far more numerous than the vertebrates and include as major phyla, the Cnidaria, Arthropoda and Mollusca (Figure 3). The diversity of organisms within the invertebrates is also reflected in the diversity of their endocrine systems, which are much less studied than in vertebrates. The invertebrate endocrine system differs from that of vertebrates although they do employ hormones and neurohormones to regulate a wide range of biochemical, physiological, and behavioral processes including development, growth, and reproduction (Hartenstein 2006; Holzer *et al.* 2017).

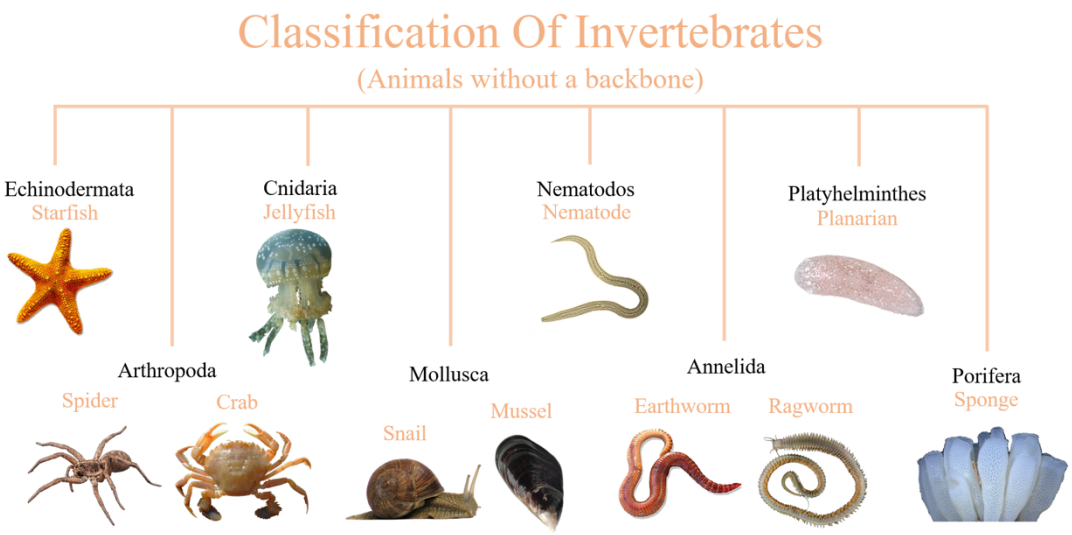

Figure 1.3. Classification of invertebrates illustrated with representative species. (Image modified from https://www.animalwised.com/classification-of-invertebrates-chart-with-definitions-and-examples-3657.html).

For instance, thyroid-like hormone signaling plays a crucial role in the development of invertebrate chordates (including urochordates and cephalochordates) and are synthesized within the endostyle, a homologue structure of the vertebrate thyroid gland (Eales 1997; Heyland & Moroz 2005; Paris *et al.* 2008). Additionally, ecdysozoans, such as arthropods and nematodes, have a distinctive metamorphosis, which originated from the process of molting and is regulated by ecdysteroids (Laudet 2011). In invertebrate phyla such as echinoderms, molluscs, and annelids, their capacity to synthesize THs remains uncertain, despite the presence of key genes associated with TH signaling pathway, such as thyroid receptors (TR) and thyroid peroxidase (TPO) (Bertrand *et al.* 2004; Heyland & Moroz 2005; Heyland & Moroz 2006; Li *et al.* 2020b; Morthorst *et al.* 2023). Invertebrates, like vertebrates, produce both peptide and lipid-based hormones (steroids), typically synthesized within neurosecretory organs or cells, which regulate development, reproduction, and metabolic functions across invertebrate species (Hartenstein 2006; Lafont 2000; Oetken *et al.* 2004; Scott 2012; Scott 2013).

1.2. The hypothalamic-pituitary-thyroid (HPT) axis

The hypothalamic-pituitary-thyroid (HPT) axis serves to maintain optimal levels of thyroid hormone in the circulation, which is vital for the proper functioning of all tissues in vertebrates (Fekete & Lechan 2014). This axis involves a complex interplay between the hypothalamus,

pituitary gland, and thyroid gland (Figure 4). In the hypothalamus, cells of the paraventricular nucleus synthesize and release thyrotropin-releasing hormone (TRH) when stimulated (Fuzesi *et al.* 2009). In mammals to amphibians TRH released from the paraventricular nucleus travels to the adenohypophysis via a specialized portal blood system and stimulates the secretion by pituitary thyrotropes of thyroid-stimulating hormone (TSH), which in turn acts on the thyroid gland to promote the production and release of mainly thyroxine (T_4) and lesser amounts of triiodothyronine (T_3) hormones (Zoeller *et al.* 2007) (Figure 5). The THs are unique as they are the main iodinated proteinaceous material in vertebrates and consist of 2 tyrosine's bound together by an ester bond. THs exert negative regulation on the pituitary gland and hypothalamus by inhibiting the production and release of TSH and TRH, respectively, to maintain plasma thyroid hormones (THs) within the optimal range (Persani 1998).

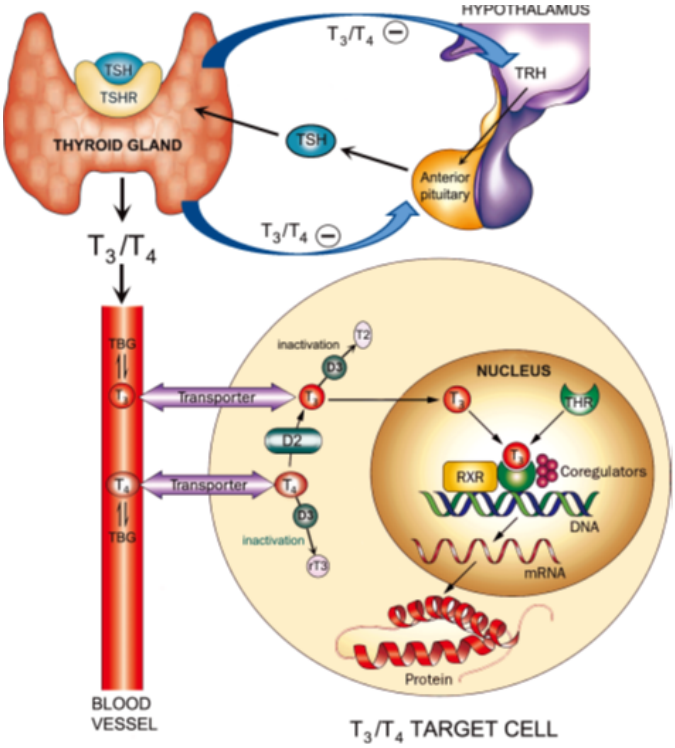

Figure 1.4. A schematic representation of the thyroid axis and thyroid hormone pathway in vertebrates, from regulation to secretion to action. TRH from the hypothalamus stimulates the release of TSH from the anterior pituitary gland, which binds to cell surface receptors in the thyroid gland via membrane thyroid-stimulating hormone receptor (TSHR). In response, the thyroid gland synthesizes and secretes T_4 and T_3. The THs released are transported in the bloodstream bound to thyroxine-binding globulin (TBG) and other binding proteins like transthyretin. Free T_4 and T_3 then enter target cells through specific membrane bound transporter proteins. T_4 undergoes conversion into the active hormone T_3 through the action of deiodinase type 2 by outer ring deiodination (D2). Deiodinase type 3 (D3) catalyzes inner ring deiodination, acting as an inactivating enzyme that converts T_4 into reverse T_3 (rT_3) and T_3 into T_2. Bioactive T_3 binds to THR that form dimers with retinoid X receptor (RXR) prior to binding to target DNA, subsequently activating gene

transcription (mRNA) and facilitating the production of specific proteins. T_3 and T_4 provide negative feedback on both hypothalamic TRH neurons and pituitary thyrotrophs, leading to a decrease in the release of TRH and TSH, respectively. (Image taken from Wilkinson and Imran, Chapter 6, Clinical Neuroendocrinology, 2018).

The thyroid gland in vertebrates produces thyroxine (T_4) and triiodothyronine (T_3) that regulate adult metabolism and growth (Joseph-Bravo *et al.* 2015; Power *et al.* 2001). The present thesis is focused on the HPT axis and, more specifically, the action of THs. The THs are key regulators of many physiological processes but are particularly important in the regulation of morphological development (e.g. metamorphosis) and metabolic function, although their action depends on tissue and developmental stage (Little 2016).

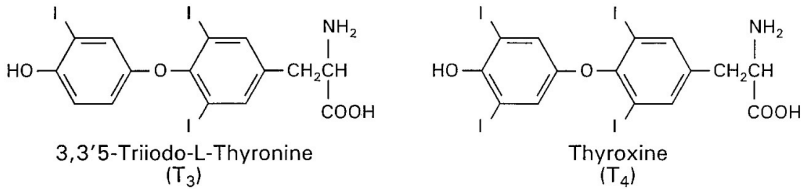

Figure 1.5. Chemical structure of the thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3). T_3 is synthesized from T_4 by removal of an iodine atom from its outer ring by deiodinases. T_4 and T_3 are iodinated, tyrosine-based hormones that are responsible for the regulation of metabolism. The predominant form of the THs is T_4, and total serum T_4 levels are 40 times greater than serum T_3 levels (Yen 2001).

1.2.1. The thyroid system

The vertebrate thyroid gland synthesizes two main hormones, T_4 and T_3, with a relative ratio of approximately 17:1 (Wilkinson & Imran 2018). The biosynthesis of THs in vertebrates involves the uptake by thyroid follicular cells of iodine from the bloodstream. In terrestrial vertebrates the main source of iodine is the diet, and it is absorbed from the gastrointestinal tract into the plasma and is transported to the thyroid (Carvalho & Dupuy 2017). Thyroglobulin in vertebrates is expressed predominantly by thyroid follicular epithelial cells (thyrocytes) and it serves as the primary source of thyroid hormones since it is the main precursor and storage form for thyroid hormones in the follicular lumen (Di Jeso & Arvan 2016). Within the thyroid follicular lumen, thyroglobulin undergoes iodination, which involves the incorporation of iodine atoms into tyrosine residues. This iodination process is catalyzed by the enzyme TPO. Iodinated tyrosine residues on thyroglobulin are coupled together to form either T_4 or T_3 through a reaction that is also mediated by TPO.

When there is a demand for thyroid hormones in the body, thyroglobulin undergoes

endocytosis from the follicular lumen into thyrocytes. Lysosomal enzymes such as cathepsin proteases and plasma glutamate carboxypeptidase (Friedrichs *et al.* 2003; Jordans *et al.* 2009; Suban *et al.* 2012) cleave thyroglobulin, releasing T_4 and T_3 into the circulation where they bind to carrier proteins, circulate in the body and target cell receptors. In humans, thyroid hormones in the bloodstream are transported by specific proteins, including TBG, TTR and albumin (Schussler 2000). Thyroid hormone binding proteins (THBPs) play crucial roles in regulating thyroid hormone distribution and availability within the body (Richardson *et al.* 2005). TBG exhibits a high affinity for T4, while TTR is a carrier of both T_4 and T_3 (Schussler 2000). Albumin also transports thyroid hormones but with lower affinity than TBG and TTR (Schussler 2000). The THBPs ensure efficient delivery of THs to target tissues in vertebrates and facilitates their cellular uptake for appropriate physiological function. TTR has been suggested to play a crucial role in transporting THs across the blood-brain barrier and facilitating their transfer across the placenta into the fetal compartment in mammals (Boas *et al.* 2012).

The intracellular action and metabolism of THs necessitate their transport across the plasma membrane, facilitated by thyroid hormone transporter proteins (Visser *et al.* 2011). Several cellular TH transporters have been identified, such as monocarboxylate transporter 8 (MCT8) and MCT10. MCT8, is expressed in various cell types, and facilitates the transport of THs out of the thyroid gland and their entry into different cells and tissues (Citterio *et al.* 2019). The physiological coordination of upregulated lysosomal thyroglobulin processing and MCT8-mediated TH transport is essential for efficiently releasing and delivering THs to the body (Muller *et al.* 2014; Weber *et al.* 2017).

The activation and inactivation of THs is mediated by the deiodinase family of enzymes, which play an essential role in modulating TH metabolism in the body (Luongo *et al.* 2019). Three deiodinases, type 1 (Dio1/D1), type 2 (Dio2/D2) and type 3 (Dio3/D3), have been identified in vertebrates. T_3 is the most active form of the THs and is produced by the removal of outer-ring (phenolic ring) iodine from T_4 molecules through the catalytic action of D1 and D2 enzymes (Darras & Van Herck 2012). Conversely, D1 and D3 enzymes inactivate T_3 through inner ring (tyrosyl ring) deiodination (Darras & Van Herck 2012). Deiodinases enable tissues to fine-tune and adapt the systemic or local TH activity based on physiological conditions through cell type-specific and developmental expression of deiodinases (Dentice *et al.* 2013; Muller *et al.* 2014). The coordinated enzymatic activity of the three deiodinases exerts regulatory control over the levels of both T_4 and T_3 in both the systemic circulation and tissues

(Bianco & Kim 2006).

1.2.2. Cellular and molecular action of THs (mechanisms of action)

The biological activities of T_3 are predominantly mediated by TRs in vertebrates, which activate downstream gene expression (Ortiga-Carvalho *et al.* 2014). The transcriptional activity of TRs has primarily been studied in mammals and is regulated by multiple factors. In addition to being regulated by T_3, specific TH response elements (TRE) on T_3 target gene promoters, TR isoform expression varies with developmental stage and tissue type, and the involvement of nuclear coregulatory proteins (Cheng *et al.* 2010). Nuclear coregulatory proteins regulate the transcriptional activity of TR in a manner dependent on the presence of T_3. In the absence of T_3, corepressors inhibit the basal transcriptional activity of genes, while in the presence of T_3, coactivators stimulate the transcription of genes (Flamant *et al.* 2007; Cheng *et al.* 2010). Considering the indispensable role of TRs on cellular processes, mutations occurring within TRs may have adverse effects (Cheng 2005; Ortiga-Carvalho *et al.* 2014).

TH actions can also be nongenomic and independent of intranuclear TR ligand binding (Little 2016; Yen 2001). The nongenomic action of TH signaling involves integrin αvβ3, a structural protein located on the plasma membrane, that possesses a binding domain for THs and is an initiation site for complex cellular events such as angiogenesis and cell proliferation (Davis *et al.* 2016). Integrin αvβ3 has a higher binding affinity for T_4 than for T_3 (Liu *et al.* 2019). Integrin αvβ3 binds with both T_4 and T_3 and activates ERK1/2 via PLC and PKCα, promoting phosphorylation of nucleoproteins and modulation of intracellular protein trafficking (Liu *et al.* 2019).

1.2.3. The function of THs in development

The thyroid gland is the first endocrine organ to develop during human embryonic development (Kratzsch & Pulzer 2008). THs play a vital role in organ development and maintaining physiological homeostasis, such as body growth and metabolism, in all vertebrates (Power *et al.* 2001; Yen 2001; Nilsson & Fagman 2017). Thyroid diseases, including Graves' disease and Hashimoto's disease, are frequently observed in humans (Volpe 1994). Moreover, congenital hypothyroidism resulting from thyroid agenesis leads to cretinism which is characterized by irreversible brain dysfunction and dwarfism (Nilsson & Fagman 2017). Thyroid dysfunction is recognized to contribute to aberrations in the heart and cardiovascular system (Klein & Danzi 2007). Both hyperthyroidism and hypothyroidism give rise to

modifications in cardiovascular function such as cardiac contractility, myocardial oxygen consumption, cardiac output, blood pressure, and systemic vascular resistance (Biondi *et al.* 2002; Kahaly & Dillmann 2005). Thyroid dysfunction in early developmental stages results in mental and physical retardation in young mammals (Koibuchi & Chin 2000; Kratzsch & Pulzer 2008) and inhibition of metamorphosis in amphibians and teleosts (Power *et al.* 2001; Laudet 2011; Campinho 2019).

1.2.4. Comparative endocrinology of the thyroid axis

The thyroid gland produces T_4, and the localized expression of deiodinases in target tissues plays a pivotal role in the regulation of TH signaling. Notably, during amphibian metamorphosis, there is a delayed regression of the tadpole tail despite concurrent limb growth induced by thyroid hormone (Brown et al., 2005; Becker et al., 1997). This phenomenon, wherein the tail is protected from thyroid hormone-mediated remodeling, is attributed to the localized activity of deiodinases, which modulate the availability of active T_3 in specific tissues. Such a mechanism of local thyroid hormone regulation via deiodinase expression is not exclusive to amphibians but is also observed in other species, including teleost fishes (Campinho et al., 2012; Isorna et al., 2009). The expression pattern of deiodinases appears to be generally conserved across vertebrates.

The thyroid system in fish shares many similarities with that of other vertebrates, yet it exhibits distinct features. Like mammals and other vertebrates, the fish thyroid gland plays an essential role in regulating metabolism, growth, and development through synthesizing and secreting THs, primarily T_4 and T_3. THs exert their effects by binding to TRs distributed throughout the body. Although the general function of the thyroid system remains conserved across vertebrates, variations exist in the specific molecular mechanisms and regulatory pathways.

In vertebrates, two distinct thyroid hormone receptors (*TRs*) genes, *TRα* and *TRβ*, originated from whole-genome duplication events that occurred at the basis of all vertebrate lineages (Kuraku et al., 2008; Marchand et al., 2001). Teleost fishes, however, underwent an additional genome duplication event, resulting in the emergence of two *TRα* genes, namely *TRα-A* and *TRα-B* (Escriva et al., 2004; Marchand et al., 2001). The differential expression of TR isoforms is evident in teleosts such as the flounder *Paralichthys olivaceus*, where *TRα-A* exhibits earlier and higher expression levels than *TRα-B* and *TRβ* (Yamano and Miwa, 1998). This divergent expression pattern exemplifies how the duplication of TR genes facilitates the divergence of

biological functions between *TRα* and *TRβ*. The greater diversity of TR isoforms observed in teleost fish compared to mammals suggests a more intricate regulation of thyroid hormone signaling in teleost species. In contrast, mammals typically have fewer *TR* isoforms, indicating a more streamlined thyroid hormone response system.

The functional activity of THs has been extensively studied in vertebrates but emerging evidence indicates that they also exhibit functional roles in some invertebrate species (Heyland & Moroz 2005; Laudet 2011). The endostyle, a homolog of the thyroid gland in vertebrates, is a TH-producing gland in invertebrate chordates such as Cyclostomes (lamprey), Urochordates (predominantly ascidians such as *Ciona* or *Halocynthia*) and Cephalochordates (amphioxus) (Tong *et al.* 1962; Monaco *et al.* 1981; Heyland & Moroz 2006; Laudet 2011).

From a functional perspective THs facilitate larval development and metamorphosis in ascidians and amphioxus (Chino *et al.* 1994; Patricolo *et al.* 2001; Heyland & Moroz 2006; Paris & Laudet 2008; Taylor & Heyland 2018). Although THs are found in lamprey, treatment with THs does not induce larval metamorphosis, surprisingly metamorphosis is triggered by goitrogens (Youson & Sower 2001; Manzon *et al.* 2001). Overall, the regulation of metamorphosis in chordates appears to be under the control of the thyroid axis, yet a comprehensive understanding of the molecular mechanisms underlying its contrasting functions remains unknown (Laudet 2011).

In vertebrates, metamorphosis is initiated through binding of THs, T_4 and T_3, to TRs in target cells, while in the cephalochordate amphioxus (*Branchiostoma floridae*), metamorphosis is induced by triiodothyroacetic acid (Paris *et al.* 2008). The increased expression levels of amphioxus TR during metamorphosis and its inhibition by a TR antagonist indicate that TR mediates TH-regulated metamorphosis (Laudet 2011; Paris & Laudet 2008). The deiodinase genes for metabolizing THs were correlated with metamorphosis, which indicates active TH metabolism is present in amphioxus (Paris *et al.* 2008). The basal chordates undergo a remarkable metamorphosis (e.g. the asymmetric larva becomes a relatively symmetric adult in amphioxus and the symmetric flatfish larva transforms into an asymmetric adult), and the presence of shared characteristics among the molecular cascades that govern metamorphosis in sea urchins, amphioxus, teleost fishes, and amphibians is taken as evidence that metamorphosis is an ancestral trait in chordates (Heyland & Moroz 2005; Heyland & Moroz 2006; Laudet 2011; Paris & Laudet 2008).

Many mollusk species undergo metamorphosis when they transform from a pelagic larva to a sessile adult, and this process is characterized by significant physiological and morphological

alterations, including dramatic tissue reorganization and remodeling (Li *et al.* 2020a; Joyce & Vogeler 2018). Only a single *TR* gene has been found in the invertebrates studied to date, and the presence of two isoforms (*TRα* and *TRβ*) in vertebrates is suggested to result from a gene duplication event during the vertebrate radiation (Huang et al. 2015; Li et al. 2020a; Wu et al. 2007). The identification of iodothyronine deiodinase genes in several non-chordate species suggests that TH-like metabolism may exist in invertebrates (Wu *et al.* 2007; Huang *et al.* 2015; Li *et al.* 2021). In the mussel *Mytilus coruscus*, two deiodinase genes (*McDx* and *McDy*) contribute to the development of visceral tissues, the nervous system, mantle and velum (locomotory organ), indicating that *McDx* and *McDy* play an important role during larval development (Shi *et al.* 2022). The knockdown of *McTR*, *McDx* and *McDy* significantly reduced larval metamorphosis in *M. coruscus* and supports the idea that thyroid signaling may be involved in the bivalve metamorphic transition (Shi *et al.* 2022; Li *et al.* 2020b). Nevertheless, despite the identification of multiple TH signaling pathway genes in mollusks and the documented endogenous synthesis of THs in annelids, mollusks, and echinoderms (Eales 1997; Heyland & Moroz 2006; Huang *et al.* 2015; Li *et al.* 2020a; Shi *et al.* 2022; Saito *et al.* 1998), the underlying regulatory mechanisms remain largely unknown.

1.3. Thyroid development and its interaction with the cardiovascular system

The thyroid gland in vertebrates mainly consists of TH synthesizing follicular cells that originate from the endoderm (Noden 1991). Unlike most vertebrates that possess a compact thyroid gland encapsulated in connective tissue, thyroid tissue in fishes is distributed along the ventral midline of the pharyngeal region (Raine *et al.* 2001; Wendl *et al.* 2002). The thyroid primordium develops from the pharyngeal epithelium and migrates downward to the final anatomical location of the thyroid gland both in zebrafish and higher vertebrates (Noden 1991; Shain *et al.* 1972; Wendl *et al.* 2002; Fagman & Nilsson 2011).

In zebrafish and mice, thyroid gland morphogenesis is directly associated with cardiac and pharyngeal blood vessel development and interaction (Alt *et al.* 2006b; Opitz *et al.* 2012; Wendl *et al.* 2002). The heart is the first functional organ to form during vertebrate embryogenesis (Bakkers 2011) and in zebrafish, heart contraction starts approximately 24 hours post-fertilization (hpf) and a regular heartbeat is observed (Chen *et al.* 1996; Vogel & Weinstein 2000). In zebrafish, early thyroid morphogenesis (thyroid bud) occurs between 32 and 55 hpf and is spatially adjacent to the apical pole of the heart in the pharynx (Opitz *et al.* 2012). Pharyngeal vessels, such as the ventral aorta, are in close spatial proximity with the

thyroid gland and play a role in guiding thyroid gland morphogenesis along the pharyngeal region (Opitz *et al.* 2012; Alt *et al.* 2006a). Crosstalk between the thyroid and cardiovascular system was revealed by the zebrafish cloche mutant (Thompson *et al.* 1998), which lacks all pharyngeal vessels and in which the thyroid gland fails to develop properly (Opitz *et al.* 2012). The correlation between the development of the heart and thyroid suggests that heart-angiogenic-thyrocyte interactions are essential for the correct development and function of the thyroid gland, HPT-axis and thyroid homeostasis.

1.4. Endocrine disrupting chemicals

An emerging issue these days is the presence of anthropogenic contaminants in the environment. Anthropogenic chemicals are generated or used by humans, and include chemicals for agriculture, industry, medicine, and the military. Some of these chemicals are classified as EDCs, that is, chemicals, either natural or synthetic, that interfere with or disrupt the endocrine systems by affecting production, secretion, transport, metabolism, binding, action, or elimination of THs in humans and wildlife through environmental exposure (https://www.endocrine.org/patient-engagement/endocrine-library/edcs). Over the past few decades, evidence based on research about EDCs has brought insight into the molecular mechanisms and physiological actions of EDCs and how they affect human health (Skinner 2014; Schug *et al.* 2015; Schug *et al.* 2016). The detrimental impact of EDCs on human health, includes, but is not limited to, reproductive disorders, developmental abnormalities, hormonal imbalances, impaired immune function, and increased susceptibility to certain chronic diseases and cancer in humans, a subject of significant concern over the last few decades (Kar *et al.* 2020; Schug *et al.* 2016; Nowak *et al.* 2019; Lauretta *et al.* 2019). Zebrafish have been widely used in scientific research as an alternative vertebrate model organism due to some conserved genetic features they share with humans and other vertebrates. By studying the effects of various environmental factors on zebrafish, it is possible to foresee how these factors might impact other vertebrates, including humans. The advantages of using zebrafish as a model organism compared to mice or rats are that they are less costly to maintain, have a shorter generation time, and require less space and resources (Lai *et al.* 2021; Roper & Tanguay 2018). This allows experiments to be conducted quickly and efficiently, ultimately leading to a deeper understanding of how environmental factors impact vertebrates and the potential consequences for human health.

Since 1999, the European Union's Framework Programmes for Research and Technological

Development have funded over 50 projects with a budget exceeding €150 million, leading to advancements in understanding endocrine mechanisms, identifying adverse effects on human health and wildlife caused by EDC exposure, and developed tools for their identification and the assessment of their effects (https://commission.europa.eu/strategy-and-policy/policies/endocrine-disruptors_en). The recently adopted EU criteria for identifying EDCs encompass three fundamental elements: chemical-induced adverse effects, chemical-specific endocrine activity, or modes of action (MOA), and a plausible relationship between adverse effects and endocrine activity or MOAs (EC, Commission Regulation EU, 2018. https://eur-lex.europa.eu/eli/reg/2018/605/oj). The proven or suspected EDCs include diverse natural and synthetic substances, with natural EDCs including animal hormones (e.g., estradiol) and phytoestrogens (e.g., genistein), while synthetic EDCs come from plastics (e.g., bisphenol A), personal care products (e.g., perfumes), pharmaceuticals (e.g., diethylstilbestrol), brominated flame retardants, pesticides (e.g., methoxychlor), herbicides (e.g., ioxynil), and other industrial chemicals (Vethaak & Legler 2013; Li *et al.* 2019).

The presence of EDCs in the environment is a topic of significant concern and natural and synthetic EDCs are introduced into the environment including the atmosphere, freshwater, seawater, soils, and marine sediments. EDCs, released from industrial and residential effluents, incineration processes, and/or runoff originating from livestock operations, now have a global distribution. Furthermore, they are transported through the environment and contaminate regions geographically distant from the initial source of contamination.

Persistent synthetic EDCs have been detected in all environmental compartments, albeit with notable declines in concentrations of legacy compounds like polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT), and tributyltin (TBT) probably due to the cessation of their production and use (Vethaak & Legler 2013; Chan & Wong 2013). Such chemicals frequently coexist as complex mixtures, for example, in wastewater effluents, and are mobile in aqueous environments (Rodríguez *et al.* 2007; Vethaak & Legler 2013). Contamination is widespread as persistent chemicals build up in the environment and are transferred to wilderness areas through water runoff and atmospheric deposition (Diamanti-Kandarakis *et al.* 2009; Pironti *et al.* 2021). Aquatic ecosystems can act as a “reservoir” for numerous contaminants and are a significant risk for aquatic organisms when incorporated into the food chain in remote areas (Vethaak & Legler 2013; Annamalai & Namasivayam 2015).

EDCs in the aquatic environment that interfere with the endocrine system of aquatic vertebrates and invertebrates are of public and scientific concern (Gorga *et al.* 2015). Aquatic

organisms can serve as sentinel models for assessing endocrine disruption due to their early exposure to waterborne pollutants compared to other animals (Celino-Brady *et al.* 2020). Aquatic animals such as teleost fish and aquatic invertebrates are directly exposed to EDCs through direct contact or ingestion of contaminated food (Langston 2020; Pironti *et al.* 2021; Ketata *et al.* 2008). The endocrine axis most notably modified and for which evidence of disruption exists both in wildlife (invertebrates and vertebrates) and humans is the reproductive axis due to the abundance of discharged estrogen disrupting chemicals (Kloas *et al.* 2009; Schug *et al.* 2016; Ketata *et al.* 2008). These chemicals include pharmacological estrogens (e.g., DES and 17α-ethinylestradiol) (Prifti *et al.* 2003; Zhang *et al.* 2012b), pesticides (e.g., tributyltin and atrazine) (Eldridge *et al.* 2008; Sharan *et al.* 2013; Albanito *et al.* 2015), food additives (e.g., aurantio-obtusin and semicarbazide) (El-Halawany *et al.* 2007; Maranghi *et al.* 2010), plasticizers (e.g., tricresyl phosphate and benzyl salicylate) (Hashimoto *et al.* 2003; Kojima *et al.* 2013) and industrial pollutants (e.g., dioxins and o, p′-dichlorodiphenylethylene) (Ahmed *et al.* 2009; Bulayeva & Watson 2004).

Several issues in endocrine disruption need to be clarified to understand their mechanism of the action. For example, EDCs may have an effect at very low doses in a tissue or organ and these may cause more severe outcomes than higher doses, and this non-monotonic dose-response increases the complexity of their effects (Vandenberg *et al.* 2012; Schug *et al.* 2016). Early life stages are particularly susceptible to endocrine disruption because of the crucial role of hormones in development (Schug *et al.* 2015). The age at which different development stages are exposed to EDCs can cause divergent outcomes. Indeed, developing fetuses and infants are more vulnerable and susceptible to EDCs than adults, and early exposure to EDCs can cause disease or tissue or organ dysfunction in later life (Diamanti-Kandarakis *et al.* 2009). Accumulating evidence shows that the mechanism of action of EDCs is much broader than initially assumed. For example, exposure to EDCs has been linked to an increased incidence of cardiovascular disease in epidemiological studies (Bae *et al.* 2012; Goncharov *et al.* 2008; Tournaire *et al.* 2016).

The present thesis focuses on both vertebrates and aquatic invertebrates. The disruption of the thyroid axis is considered in both vertebrates and invertebrates since it was recently shown that TRs and DIO genes are present in invertebrates and, more specifically, the mussel, a bivalve (Li *et al.* 2020a; Shi *et al.* 2022). The following section briefly considers thyroid-disrupting effects, mode of action and how EDCs may disrupt organogenesis of the thyroid gland. Related to this, is the interaction of the developing heart and thyroid and for this reason the impact of

thyroid disrupting EDCs on the cardiovascular system is also considered. Indeed, understanding the effects of thyroid disrupting chemicals is important for establishing their likely consequence for humans, wildlife and the environment.

1.5. Effects of EDCs on the thyroid system

1.5.1 The thyroid system

As outlined above the thyroid axis plays a crucial role in the normal development and physiological homeostasis of vertebrates. The maintenance of normal thyroid function is essential for both psychological and physiological well-being and relies on a finely tuned negative feedback mechanism of circulating thyroid hormones at the hypothalamic and pituitary levels (Boas *et al.* 2012). Numerous manufactured chemicals have the potential to interfere with the thyroid system, including various herbicides (IOX), pesticides (DDT), pharmaceutical agents (DES), plasticizers (phthalates), industrial solvents (PCBs), and plastics [bisphenol A (BPA)] (Campinho & Power 2013; Gore *et al.* 2015; Gilbert *et al.* 2020). In zebrafish exposed to IOX and DES, the mRNA expression of *nk2.1a* and thyroglobulin (*Tg*), which are essential for thyroid gland development and function, were decreased suggesting thyroid gland development was impaired (Campinho & Power 2013). Several case studies have shown that environmental chemicals can induce a decrease in serum levels of THs with potentially adverse consequences for public health (Boas *et al.* 2006). Evidence of thyroid hyperplasia in teleost fish from the Great Lakes of Ontario, Michigan, and Erie have been documented and proposed to be the consequence of endocrine disruption (Black & Simpson 1974; Moccia *et al.* 1981; Sonstegard & Leatherland 1976).

Thyroid disruption by EDCs in vertebrates including fish can disrupt thyroid hormone synthesis, release, transport, and metabolism, as well as impair the action of THs on target tissues (Gore *et al.* 2015). Some EDCs exhibit a high degree of structural similarity to THs (T_4 or T_3) and therefore interfere with TH signaling. Accumulated thyroid-disrupting chemicals (e.g., perchlorate, organochlorine pesticides and IOX) can impair the structure and function of the thyroid gland, competitively disrupt the binding of THs to TRs or transthyretin (e.g., DES, bisphenol A and polybrominated diphenyl ethers), and inhibit the enzymes important for TH synthesis and metabolism (e.g., methimazole, iopanoic acid and propylthiouracil) (Brown *et al.* 2004; Campinho & Power 2013; Cai & Brown 2004; Crofton *et al.* 2005; Eguchi *et al.* 2008; Furin *et al.* 2015; Morgado *et al.* 2007; Morgado *et al.* 2009; Miyata & Ose 2012; Tucker *et al.* 2018). Given the physiological feedback loop between TSH and peripheral hormones, the

impacts of EDCs are difficult to predict or detect, particularly since their pervasive distribution means an “exposure-free” state is unlikely. Thyroid disruption by EDCs might not affect circulating levels of THs but may modify other molecules of the axis. Furthermore, peripheral tissues can modulate their sensitivity to thyroid hormones by altering the expression of deiodinases and membrane-bound TH transporters (Kampf-Lassin & Prendergast 2013). Despite the compensatory capabilities of the thyroid gland and axis, the cumulative and prolonged impacts resulting from exposure to EDC mixtures may potentially lead to hypothyroidism or other thyroid disorders (Boas *et al.* 2012).

1.5.2. Cellular and molecular action of THs (mechanisms of action)

EDCs can interfere with almost all processes of TH action, including TH synthesis, transport, metabolism, and action (Predieri *et al.* 2022). TH synthesis involves the active uptake of iodide ions via the sodium-iodide symporter (NIS), the synthesis of thyroglobulin, and the subsequent catalysis of iodine incorporation mediated by the enzyme, TPO (Gore *et al.* 2015). Various environmental chemicals, including perchlorate (ClO_4^-), nitrate (NO_3^-), and thiocyanate (SCN^-), can inhibit NIS function and iodide uptake in a competitive manner (Salazar *et al.* 2021; Tonacchera *et al.* 2004). Perchlorate and nitrate are commonly found in both surface and groundwater sources of drinking water due to industrial production and the use of ammonium perchlorate and nitrate fertilizers (Tonacchera *et al.* 2004). High levels of thiocyanate can arise from diverse origins, including cigarette smoke among other potential sources (Gore *et al.* 2015). Such ions exert inhibitory effects on iodide uptake resulting in inadequate TH levels leading to goiter and, if occurring during development, can cause significant growth abnormalities and mental retardation, a condition called cretinism (Schmutzler *et al.* 2007). In pregnant women with borderline thyroid function perchlorate adversely affected the cognitive function of their offspring (Taylor *et al.* 2014). Other studies showed that TPO enzyme activity can be inhibited by EDCs, such as isoflavones found in soy products and thiocyanates present in tobacco (Kohrle 2008; Gore *et al.* 2015).

TTR and T_4 binding globulin, essential THBPs, are major targets for EDCs, such as PCB and polybrominated diphenyl ethers (Gore *et al.* 2015). Some EDCs, such as IOX and DES, which are by-products of industry, medicine and agriculture, are structurally similar to THs and bind competitively to TTR by displacing T_3 in both humans and fish (Morgado *et al.* 2007; Ogilvie & Ramsden 1988; Ishihara *et al.* 2003). The competitive binding of EDCs to THBPs can diminish the amounts of TH bound and normally distributed across tissues and increase the

bioavailability of free hormones (Boas *et al.* 2006; Gore *et al.* 2015). The outcome of the competition between endogenous THs and EDCs is an elevated free TH concentration and modified tissue uptake and elimination. Chronic exposure to such THBP-competitive EDCs can lead to the excretion of circulating THs through urine and feces and may lead to goiter if not counteracted by increased synthesis and secretion of THs (Kohrle 2008).

Very few studies have investigated whether EDCs influence TH uptake into cells, by TH transporters, such as MCT8 and the solute carrier organic anion transporter family, member 1c1 (Slco1c1) (Kohrle 2008). But for example, fipronil (a broad-spectrum insecticide), that is a thyroid axis disruptor, affects hepatic gene expression of the TH transporters *Slco1a1* and *Slco1a4*, respectively (Roques *et al.* 2013). Furthermore, deiodinase enzymes, which control the activation and inactivation of THs, can be perturbed by EDCs, such as the potent DIO1 inhibitors, (iso-)flavonoids, polyphenols, and aromatic and polycyclic phenolic ring systems (Kohrle 2008). Bisphenol A can disrupt thyroid signaling by altering the metabolism of THs and significantly reduces hepatic DIO1 activity, and increases serum T_4 levels, without affecting brown adipose tissue DIO2 activity, and T_3 levels (da Silva *et al.* 2019). Although the inhibition of TH deiodination by EDCs is highly effective, the administration of these compounds does not necessarily impair T_3 levels (Morgado *et al.* 2009). *In vitro* exposure to some PCBs did not change deiodinase activity in fish, suggesting a compensatory mechanism exists that helps preserve the euthyroid state (Adams *et al.* 2000). Reduced activity of DIO1 can be compensated by the action of DIO2 or by decreased activity of DIO3, and these mechanisms contribute to maintain circulating serum T_3 levels (Kohrle 2008).

EDCs that mimic hormones can have wide-ranging cellular actions through interaction with nuclear hormone receptors such as estrogen and thyroid receptors (Schug *et al.* 2011; Hall & Greco 2019). There are some natural (e.g., genistein) and synthetic chemicals (e.g., diethylstilbestrol, DES) that activate estrogen receptors (ERα and ERβ) (Fuentes & Silveyra 2019). BPA exhibits estrogenic properties by mimicking the effects of estrogen through its binding to ERs, which triggers a cellular signal transduction cascade that is indicative of the activation of the estrogen response (Thomas & Dong 2006; Watson *et al.* 2007). In addition to its estrogen-binding properties, BPA has antagonistic effects on T_3 action at the transcriptional level by displacing TRs (Moriyama *et al.* 2002). DES was the first synthetic estrogen administered for the prevention of miscarriage and other pregnancy-related complications (Hunt *et al.* 2016). DES interacts with ERs and activates the estrogen endocrine pathway (Kiyama & Wada-Kiyama 2015). The available evidence suggests that DES poses risks to

maternal health (Tournaire *et al.* 2016), and is associated with reproductive, cardiac, and urogenital anomalies in offspring (Yamamoto *et al.* 2003; Titus-Ernstoff *et al.* 2010). Studies have demonstrated that hydroxylated PCBs can inhibit the binding of T_3 to the TRs (Miyazaki *et al.* 2004). Moreover, PCBs function as antagonists by inducing partial dissociation of the TR/RXR heterodimer complex from the TH response elements (Kitamura *et al.* 2005). In contrast, DDT and its metabolites, as well as some organochlorine pesticides, do not exhibit competitive binding for the receptor (Cheek *et al.* 1999) and competitive binding of EDCs seems to be receptor- and compound-specific (Boas *et al.* 2006).

1.5.3. Low dose effects

Natural hormones exhibit concentration-response patterns at serum concentrations within the picomolar to the nanomolar range, while EDCs can exert their effect within the nanomolar to the micromolar range, even if some EDCs are active at the picomolar level (Vandenberg *et al.* 2012; Gore *et al.* 2015). The term "low dose" is widely employed in the field of environmental health science and is considered to be a dose falling within the range of typical human exposure or doses below those evaluated in conventional toxicological assessments (Melnick *et al.* 2002).

EDCs can stimulate biphasic dose responses for various endpoints at different levels of organization, as shown by the U-shaped and inverted U-shaped non-monotonic dose-response curves, which suggest that EDCs have an effect even at very low concentrations (Schug *et al.* 2011). For example, DES at 0.02, 0.2, and 2.0 ng per g of body weight per day led to an increase in adult prostate weight in mice, while a dose of 200-ng-per-g resulted in a decrease in adult prostate weight among male offspring, indicating an inverted-U dose-response relationship characterized by an initial increase and subsequent decrease in prostate weight with increasing doses of both estradiol and DES (Saal *et al.* 1997). Similar toxicity effects were observed in rats exposed to a dose of 0.018 ng/g of DES, which resulted in a significant enlargement of preputial glands in males compared to the 0.18 ng/g dose of DES and control groups (Palanza *et al.* 1999). Furthermore, the 18 ng/g dose of o,p'-DDT significantly reduced testes size relative to the 180 ng/g dose of o,p'-DDT and control groups (Palanza *et al.* 1999). A recent study conducted on invertebrates revealed that the anti-thyroid chemicals methimazole and propylthiouracil significantly inhibited larval metamorphosis of the bivalve mussel, *Mytilus coruscus*, with a more severe effect observed at low doses than at higher doses (Li *et al.* 2021). In rats an inverted U-shaped dose-response relationship was observed in Sertoli cells exposed

to varying concentrations of BPA (10, 50, and 100 μM), indicating that only intermediate doses (10 μM-50 μM), rather than high or low doses, induced an elevation in the level of cell-protective glutathione (Gualtieri *et al.* 2011). Although the endpoints commonly investigated in toxicological studies at high doses may differ from those examined in low-dose studies, it cannot be assumed that the effect in a low-dose range will be distinct from a high-dose unless investigated.

1.5.4. Developmental windows of susceptibility

During development, the formation of organs and the differentiation of tissues occurs through the progression of precisely coordinated molecular, biochemical, and cellular processes (Prusinski *et al.* 2016). EDC exposure occurring during pivotal periods of early development (e.g., fetus, birth, and puberty) can influence the subsequent onset of disease and the development of abnormal physiological conditions later in life (Skinner 2014). *In utero* and early childhood are critical windows of exposure due to the heightened vulnerability of developing organisms to disruption caused by chemicals with EDC-like activity (Heindel 2018). The synthetic estrogen, DES, was administered to millions of pregnant women in the world and was subsequently shown to be associated with clear cell adenocarcinoma of the vagina and cervix in their offspring (Herbst *et al.* 1971). In rats, prenatal exposure to DES enhanced thyroid function through the activation of the pituitary-thyroid axis (Yamamoto *et al.* 2003) and increased the probability of pancreatic disorders and pancreatitis in offspring (Troisi *et al.* 2021).

Prenatal exposure to environmental factors can lead to developmental programming, with modified cellular and tissue development and function (Schug *et al.* 2011). The effects of exposures during pivotal perinatal windows may become evident in early life-stages, while fetal development is a critical period of increased sensitivity to chemicals. A study conducted in zebrafish embryos revealed that early exposure to IOX and DES directly disrupted cardiovascular development and disrupted developing thyroid tissue. The exposure of zebrafish embryos to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced significant anomalies in heart morphology by perturbing looping, as well as eliciting defects in heart function, including blood regurgitation and ventricular standstill (Antkiewicz *et al.* 2005). Similarly, clofibrate affected the morphology and contractility of the heart in zebrafish and thyroid gland morphogenesis (Raldúa *et al.* 2008).

Maternal exposure to EDCs has repercussions for the health of their offspring, indicating pregnancy is a sensitive window for environmental exposures (Heindel 2019). This is because

developing organs and systems are much more susceptible to chemical concentrations significantly lower than those deemed harmful in adults (Newbold *et al.* 2004; Newbold *et al.* 2006). Children born to DES-exposed mothers had increased defects in the genital tract, musculoskeletal, and circulatory systems (Titus-Ernstoff *et al.* 2010; Tournaire *et al.* 2016). Although some EDCs are banned from use, the impact of prenatal exposure to those chemicals may be transmitted between generations in the absence of direct exposure (termed epigenetic transgenerational inheritance) (Jirtle & Skinner 2007; Skinner 2014). The process of epigenetic transgenerational inheritance involves alterations in the genome of the germ line, through DNA methylation, noncoding RNAs, histone modifications, and changes in chromatin structure (Skinner 2014). Gestating mice exposed to Di-(2-Ethylhexyl) Phthalate (DEHP), produced offspring that had altered stress hormone levels (corticosterone), pituitary gene expression, and behavioral patterns in both male and female mice of the third generation (Quinnies *et al.* 2015). This suggests that prenatal exposure of a great-grandmother to some EDCs can potentially influence the development of disease in the following generations, even in the absence of direct exposure, thereby leading to the transmission of their effects to grandchildren (Quinnies *et al.* 2015).

1.5.5. Effects of thyroid disruption on the cardiovascular system

The thyroid system exerts direct effects on cardiovascular function, as well as the structure of the heart and the circulatory system (Grais & Sowers 2014). Cardiovascular disorders are associated with thyroid dysfunction in humans (Khan *et al.* 2020). In clinical studies, congenital defects of the cardiovascular system have been previously linked to congenital thyroid abnormalities (Casanova *et al.* 2000; Olivieri *et al.* 2002). Several studies suggest that patients suffering from cardiovascular disorders also have altered thyroid physiology and/or morphology (Duntas & Biondi 2011; Luboshitzky & Herer 2004; Bengtsson *et al.* 2012). A chemical (clofibrate) that affects normal heart development and function in zebrafish also affected thyroid development (Raldúa *et al.* 2008) causing shortening of the ventral aorta and disrupted thyroid tissue morphogenesis in zebrafish larvae (Raldúa *et al.* 2008). Impaired heart development and function in zebrafish were associated with abnormal thyroid development (Campinho & Power 2013). Furthermore, DES and IOX directly affected vascular and heart development and indirectly impaired the development of the thyroid gland and potentially disrupted thyroid homeostasis through their actions (Li *et al.* 2019). The evidence available suggests that DES and IOX have an indirect ED effect on the thyroid system by altering

cardiovascular function and homeostasis (Li *et al.* 2022). The mechanism underpinning the association between ED effects on the cardiovascular system and thyroid axis is explored in the present thesis.

1.6. Animal model in EDC research

1.6.1. Aquatic vertebrate model zebrafish

The zebrafish (*Danio rerio*) is one of the most popular laboratory animal models and is a widely used model organism in developmental and toxicological studies. Its small size, transparent embryos, short developmental cycle, low feeding costs, high reproduction rate, and well-characterized genome make it highly suitable for investigation. Its sequenced genome exhibits homology with the human genome and a large proportion of genes related to human diseases have orthologues in zebrafish, and for this reason, it has been proposed as a model for developmental and toxicological studies (Howe *et al.* 2013). In addition, the short generation time, high fecundity, transparent embryos, and ease of husbandry make zebrafish an interesting model for transgenic research (Roper & Tanguay 2018). Advanced gene manipulation and high-resolution imaging methods have advanced studies of gene function and development (Stewart *et al.* 2014; Varshney *et al.* 2015; Zu *et al.* 2013). The advent of CRISPR-Cas9 gene editing technology has further consolidated the zebrafish as an interesting model for research into cardiovascular and thyroid development (Bowley *et al.* 2021; Ryan *et al.* 2020; Larrivée-Vanier & Deladoëy 2018; Vancamp *et al.* 2019).

The advantages of zebrafish are being increasingly used for studies of EDC toxicity. Exposure of embryonic, larval, and juvenile zebrafish to environmentally relevant doses of pesticides, herbicides, bisphenols, PCBs, phthalates, and flame retardants, induced developmental abnormalities (Dai *et al.* 2014; Jarque & Pina 2014). Phenotypical abnormalities in zebrafish included craniofacial malformations, impaired swim bladder inflation, altered neurogenesis, reduced axonal growth, disruption of the cardiovascular system, and altered thyroid morphology and development (Kinch *et al.* 2015; Lam *et al.* 2011; Macaulay *et al.* 2015; Macaulay *et al.* 2017; Noyes *et al.* 2013; Qiu *et al.* 2016; Tse *et al.* 2013; Li *et al.* 2019; Li *et al.* 2022).

A beneficial characteristics of zebrafish embryos for the present study is that their transparency facilitates observations of the detrimental effects of EDCs on the cardiovascular system, including atrial/ventricle malformations and cardiac rate abnormalities (Campinho & Power 2013; Howe *et al.* 2013; Stewart *et al.* 2014; Li *et al.* 2019). Furthermore, the predictable

timeline of development favours their use as a model (Kimmel *et al.* 1995). In the case of the cardiovascular system, formation of the heart primordia and initiation of contractions occurred at around 22 hpf, followed by the development of the atria and ventricles at 28 hpf (Bakkers 2011). Even though the zebrafish like other teleosts has a single atrium and ventricle, the heart exhibits similar development and function to other vertebrates including humans (Yang *et al.* 2023b). To assess the cardiotoxicity of agrochemicals, cardiac defects such as pericardial edema, abnormal atrial/ventricular morphology, and cardiac rate imbalance are often the first parameters examined (Campinho & Power 2013; Li *et al.* 2019; Li *et al.* 2022; Yang *et al.* 2023a). The identification of changes in biomarkers enables a rapid and more precise evaluation of cardiotoxicity and potential inducers.

Transgenic technology, which involves genetically modifying living organisms, serves as a powerful instrument with diverse applications, including its utilization in environmental monitoring and toxicology (Lai *et al.* 2021). The ability to manipulate the genetic makeup of transgenic fish using reporter genes coupled to green fluorescent protein (GFP) genes or other coloured fluorescent protein genes, has facilitated the induction of visible color change in these organisms (Gong *et al.* 2003; Lawson & Weinstein 2002; Ulrich *et al.* 2011). The use of reporter genes that confer colour can render selected organs or tissues more visible and quantifiable and improve the monitoring of such organisms when exposed to contaminants, especially in embryos and larvae. This approach is advantageous to study the tissue-specific effects of EDCs. For example, the transgenic zebrafish line *Tg*(*fabp10a:DsRed;elaA:egfp*) that continuously expresses red fluorescent protein in the liver and GFP in the exocrine pancreas, was used to evaluate the impact of EDCs on liver morphology and fluorescent intensity in larval stages (Zhang *et al.* 2014). *Tg*(*lysC:DsRed2*) transgenic zebrafish with red fluorescence protein labeled neutrophils revealed treatment with EDCs such as 17β-estradiol and BPA increased their number (Xu *et al.* 2018). The transgenic zebrafish line *Tg*(*fabp10:EGFP-kras*) with a GFP-marked liver was used to evaluate the effects of EDCs [ris(1,3-dichloro-2-propyl) phosphate] on the progression of liver tumors (Chen *et al.* 2020).

1.6.2 Bivalve molluscs

Molluscs exhibit a remarkable level of diversity from an evolutionary standpoint (Figure 6), their origin dates to over 500 MA (millions of years ago), and there are more than 70,000 extant species, that display remarkable adaptability so that they now occupy a vast diversity of ecological niches (Kocot *et al.* 2020; Rosenberg 2014). The phylum *Mollusca* comprises seven

distinct classes, Gastropoda, Bivalvia, Cephalopoda, Scaphopoda, Polyplacophora, Monoplacophora and Aplacophora, and is the second most species-rich phylum after arthropods (Telford & Budd 2011). Bivalves are an important group of the Mollusca and are the second most species-rich molluscan class after Gastropoda (Bieler *et al.* 2014) (Figure 7). Bivalves exhibit a wide range of adaptations and can be found in various aquatic habitats, including freshwater and marine environments. Some well-known examples of bivalves, include oysters, clams, scallops, and mussels, that have a history of aquaculture cultivation and are now being promoted since these lower trophic species, are more sustainable than fish aquaculture (Figure 7). Bivalves have a soft body that is enclosed within two shells that are hinged and are composed primarily of calcium carbonate. Bivalves do not possess a radula for feeding like some other molluscs and instead they are filter-feeding organisms that employ the gills to acquire food particles from the surrounding water. The inhalant siphon is used to take up water and channel it over the gills so that food particles are trapped and conveyed to the mouth by the coordinated action of cilia or other anatomical structures (Ward & Aiello 1973). The mantle is the shell producing organ in bivalves and covers the inner surface of the shell (Malachowicz & Wenne 2019). A muscular foot is used for burrowing in sediment (such as razor clams), producing byssus for attachment to surfaces (such as mussels) among other functions.

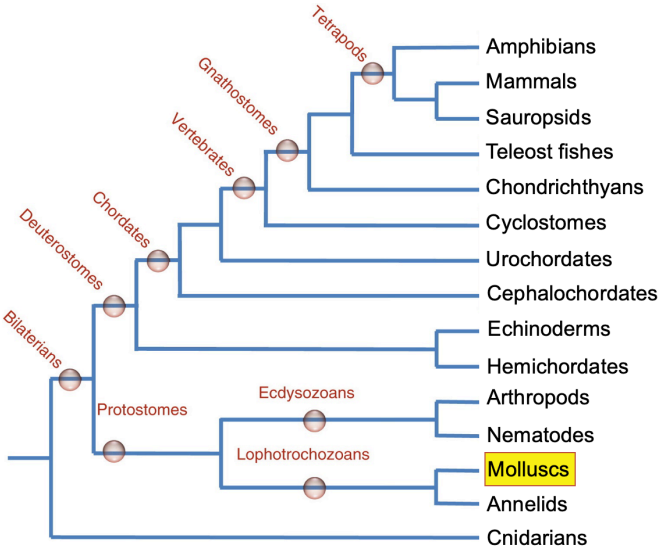

Figure 1.6. A dendrogram revealing the relationships between invertebrates and vertebrates. (Image modified from Laudet, Current Biology, 2011).

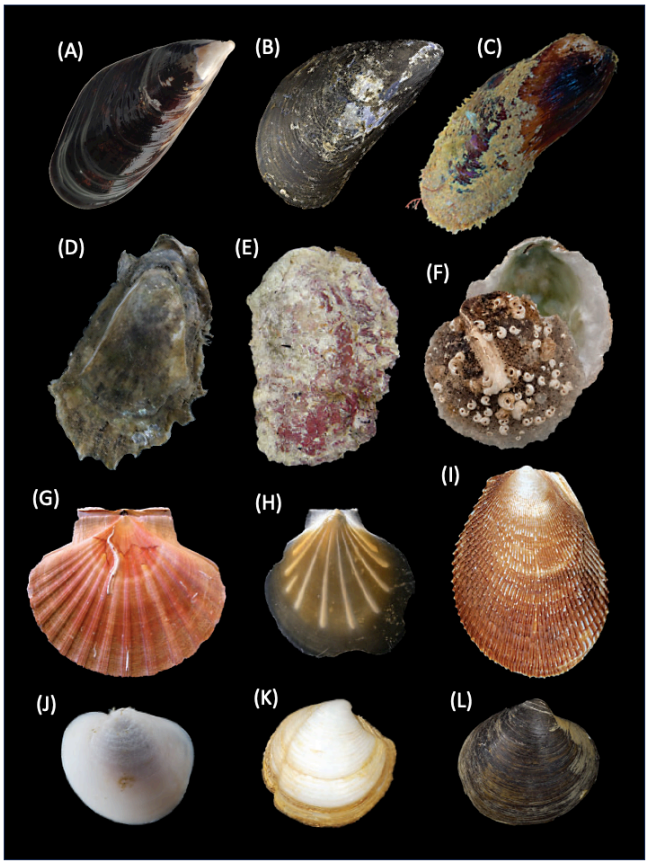

Figure 1.7. Photograph showing the diversity that exists in the shape, form and colour of shells from bivalves. (A) *Mytilus coruscus*. (B) *Mytilus edulis*. (C) *Modiolus rumphii*. (D) *Crassostrea virginica*. (E) *Hyotissa mcgintyi*. (F) *Monia patelliformis*. (G) *Pecten maximus*. (H) *Propeamussium jeffreysii*. (I) *Ctenoides scaber*. (J) *Cavatidens omissa*. (K) *Lucina pensylvanica*. (L) *Arctica islandica*. (Image taken from Bieler et al., Invertebrate Systematics, 2014).

In marine and freshwater ecosystems, bivalve molluscs are abundant and have critical ecological functions (Mondal & Harries 2016). The consumption of phytoplankton by filtering bivalves leads to the depletion of phytoplankton stocks within the pelagic food web. The subsequent release of nutrients through biodeposits and excretion contributes to the dynamics of the benthic food web (Vaughn & Hoellein 2018) and bivalves are a food source for avian and benthic organisms, provide habitats, and are exploited through fisheries and aquaculture (Guo 2009; Vaughn *et al.* 2008).

Bivalves are foundation species in marine and freshwater habitats, offering a hard substrate and three-dimensional structures where a wide variety of other species can thrive (McKindsey *et al.* 2007; Jackson *et al.* 2001; Suchanek 1978). Bivalve assemblages can reach high population densities and form dense aggregations, such as mussel beds and oyster reefs acting as ecological engineers for coastal defence and nature conservation (Fivash *et al.* 2021;

Li *et al.* 2020a; La Peyre *et al.* 2022). Some bivalve species, like the mussels, are ecologically and economically important in coastal marine food webs (Fitzgerald-Dehoog *et al.* 2012).

The fundamental importance of bivalves in ecosystems makes it of primary importance to determine how chemicals might affect them particularly since they are often localized in coastal regions and directly exposed to agricultural run-offs and anthropogenic residues (Azizi *et al.* 2018). Among marine monitoring programs, the "Mussel Watch Program" emerges as a significant initiative (Fernandez 2019; Goldberg 1986). The widespread distribution of mussels in coastal areas for aquaculture, their ease of capture and their resilience and the fact a single medium sized specimen can provide ample tissue for chemical analysis makes them useful for both laboratory-based ecotoxicology studies and *in situ* environmental analysis (Beyer *et al.* 2017). Since mussels are sessile filter feeders and efficiently bioaccumulate environmental pollution from an abiotic phase in water and transfer it to higher trophic levels within the food chain, they are frequently used as environmental bioindicator species (Viarengo & Canesi 1991).

Invertebrate taxa commonly exhibit neurohormonal control systems (Hartenstein 2006), which may possess a similar vulnerability to EDCs as those observed in vertebrates. The high biodiversity, prevalence and niche occupation of invertebrates in natural ecosystems compared to vertebrates make them commonly studied as bioindicators since they give insight into the status of lower trophic levels and allow a more comprehensive understanding of ecosystem dynamics and functioning (Hall *et al.* 2009). Molluscs have been widely employed to investigate the impacts of EDCs on aquatic organisms, primarily due to their prevalence, distribution in many aquatic habitats, and ecological significance (Fong & Ford 2014). EDC exposure of aquatic molluscs can significantly perturb physiological, morphological, and molecular systems within these organisms. A well-documented EDC example in molluscs is tributylin (TBT) that was used as an antifouling biocide to protect ship hulls and aquaculture fish cages (Langston 2020). TBT-induced imposex in some molluscs (Porte *et al.* 2006) and a survey of the gastropod *Nucella lapillus* distribution in the south-west peninsula of England revealed that populations near boating and shipping channels had the highest degrees of imposex and females displayed male characteristics (Bryan *et al.* 1986; Langston 2020). The induction of imposex in *N. lapillus* was triggered by TBT at concentrations as low as 1 ng/l (Bryan *et al.* 1986; Bryan *et al.* 1987). In oysters, nonylphenol increased the incidence of hermaphroditism and reduced gamete viability (Nice *et al.* 2003) and PCB-exposed oysters had reduced egg production and lower numbers of spawning females (Chu *et al.* 2003). Tetrabromobisphenol A, a widely used brominated flame retardant affects shell formation and

the nervous system in the mussel, *M. galloprovincialis* (Miglioli *et al.* 2021).

The lack of knowledge about the endocrine system in invertebrates makes the consequences of exposure to EDCs difficult to predict. The present thesis takes a comparative approach to investigate the effect of two common EDCs, DES and IOX, on vertebrates and mussels to establish their likely endocrine and cardiovascular impact.

1.7. Thesis objectives

The main goal of this PhD thesis was to investigate the effects of IOX and DES on the endocrine system both in a vertebrate species (zebrafish) and in an invertebrate species (hard-shelled mussel *M. coruscus*). The divergence that exists between the endocrine system of vertebrates and invertebrates highlighted above is likely to result in differing responses to EDCs. The present study established how IOX and DES disrupted thyrocyte development and the development of the heart and arterial tissue at a molecular, cellular and systemic level in developing zebrafish. The knowledge produced was used to understand if the effects of IOX and DES on adult HPT-axis physiology in the zebrafish are similar or distinct from those found during development.

Invertebrates, such as molluscs, have less complex and much less studied endocrine systems compared to vertebrates. As outlined above their endocrine systems generally comprise a network of neuroendocrine cells and ganglia that produce and release neurohormones. The inclusion of invertebrates in the study of endocrine disruption is justified since although they are likely to be exposed to EDCs due to their widespread distribution and abundance, they are relatively poorly studied. The important role of invertebrates in ecosystem functioning makes them important environmental indicator species of ecosystem health and integrity. Recent studies have revealed that in some invertebrate species elements of the thyroid axis are present (Li *et al.* 2020b; Li *et al.* 2021; Heyland & Moroz 2005; Shi *et al.* 2022). This discovery challenges existing knowledge about invertebrate endocrine systems and emphasizes the need for more studies of endocrinology in invertebrates. Developing a model for studying endocrine disruption in invertebrates can provide insights into the mechanisms underlying this process and identify potential biomarkers and indicators of EDC exposure. The studies reported in my thesis enhance understanding of the impacts of EDCs on invertebrates and contribute to the broader comprehension of endocrine disruption in both invertebrate and vertebrate organisms. The present thesis elucidated some of the factors and mechanisms that may explain differences in the response of vertebrate and invertebrate species to IOX and DES. The state-of-the art is

significantly advanced since new knowledge is generated about the effects of IOX and DES on a vertebrate and an invertebrate. Enhancing understanding of the effects of IOX and DES on invertebrates, such as mussels, has the potential to contribute fundamental insights into the mechanism of action of these compounds, even in species that lack well-characterised endocrine systems. The knowledge generated broadens comprehension about the implications of EDCs in the environment and highlights the need for a more comprehensive approach to assessing impacts across multiple taxa.

The following three specific objectives were pursued to achieve the aim of this thesis:

1) Determine how IOX and DES disrupt the crosstalk between the developing thyroid gland and the cardio-vascular system in zebrafish embryos. To do this 12 hpf (hours post fertilization) wild type, *Tg(fli1:GFP)* or *Tg(cmalc2:GFPCaaX)* zebrafish embryos were exposed to 0.1 μM IOX or DES for 36 h (up until 48 hpf) or 60 h (up until 72 hpf). Embryos were used for vascular endothelial cell sorting by flow cytometry, whole-mount immunohistochemistry for heart and thyroid visualization, transcriptome analysis of endothelial cells, selected gene expression analysis related to cardiovascular function and calcium homeostasis by quantitative real-time polymerase chain reaction analysis (qPCR) and determination of heart rate by live imaging.
2) Investigate the impact of chronic exposure to IOX and DES on heart and thyroid morphological indexes in juvenile zebrafish. Chronic 60 days exposure of juvenile zebrafish to low doses (0.1 μM) of IOX or DES via food was used to determine the effects of these chemicals on the physiology of the heart and thyroid follicles. The heart ventricle volume was determined in whole hearts dissected from exposed zebrafish juveniles fixed in 4% paraformaldehyde and subject to immunofluorescence. Thyroid morphology was visualized by histology and immunofluorescence. Endothelial and cardiac-specific genes identified in zebrafish embryos exposed to IOX and DES were analysed in juveniles by qPCR.
3) The impact of IOX and DES on cardiac performance and shell growth in the mussel *M. coruscus* was determined. Mussel juveniles were exposed to low doses of IOX (10^{-6}, 10^{-7} and 10^{-8} M) and DES (10^{-6}, 10^{-7} and 10^{-8} M) via the water. The heart rate of juveniles was determined on days 1, 3, 7, 10 and 14 of the treatment under a stereomicroscope and captured in video images. Fifty randomly selected juveniles from each experimental group were used to measure shell length and height on days 0, 7 and 14 using a stereomicroscope fitted with an ocular micrometre. Total RNA was extracted from the IOX and DES treated

juvenile mussels after 14 days of exposure and was used for transcriptome sequencing (RNA-seq). The RNA-seq dataset was validated by qPCR using a series of candidate genes as well as homologues of vertebrate endothelial-related and cardiac-specific genes identified in the transcriptome dataset.

CHAPTER 2

Ioxynil and diethylstilbestrol disrupt vascular and heart development in zebrafish

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2.1. Abstract

Endocrine disruption is one of the consequences of industrialization and chemicals released into the environment have a profound impact on organisms. Waterborne micromolar concentrations of IOX and DES in fish affect the development of the heart, vasculature and thyroid gland. The present study aimed to determine how IOX and DES disrupt the crosstalk between the developing thyroid gland and cardio-vascular system in zebrafish. Twelve hpf wild type, *Tg*(*fli1:GFP*) or *Tg*(*cmalc2:GFPCaaX*) zebrafish embryos were exposed to 0.1 μM IOX or DES for 36 h (up until 48 hpf) or 60 h (up until 72 hpf). Embryos were used for vascular endothelial cell sorting, whole-mount immunohistochemistry, tissue selective transcriptomics, selected gene expression analysis by quantitative real-time polymerase chain reaction analysis and determination of heart rate by live imaging. Exposure of zebrafish embryos to IOX and DES (0.1 μM) increased heartbeat frequency and reduced ventricle volume and aorta diameter. The transcriptome of endothelial cells from blood vessels of hypertrophic, dilated and arrhythmogenic right ventricular cardiomyopathy was significantly changed and compound-specific toxic effects were found in IOX and DES exposed embryos. Both DES and IOX directly affected vascular and heart development and this indirectly impaired thyroid gland development in zebrafish. Even though the toxicity endpoint of the two chemicals was similar, their action seemed to be via different gene regulatory pathways and physiological mechanisms. IOX and DES directly disrupt cardiovascular development and there is an associated disruption of thyroid tissue that most likely has long term consequences for this endocrine axis.

Keywords: Diethylstilbestrol; Endocrine disruption; Heart; Ioxynil; Thyroid; Zebrafish

2.2. Introduction

Endocrine disrupting chemicals are commonly defined as chemicals that can interfere with or disrupt endocrine systems and have a broad negative spectrum of effects on animals, including humans, and ultimately ecosystems (Schug *et al.* 2016; White *et al.* 2017; Patisaul *et al.* 2018). DES was the first synthetic estrogen prescribed for preventing miscarriages and other complications during pregnancy (Hunt *et al.* 2016). Like other synthetic estrogens DES interacts with the estrogen endocrine pathway (reviewed by (Kiyama & Wada-Kiyama 2015) and evidence that DES is an EDC has been accumulating. The evidence indicates that DES is hazardous for maternal health (Tournaire *et al.* 2016) and is associated with reproductive, cardiac and urogenital anomalies in offspring (Yamamoto *et al.* 2003; Titus-Ernstoff *et al.* 2010). A recent study revealed the incidence of heart defects is increased in children of DES exposed women, including ventricular septal disease, tetralogy of fallout, and atrial septal defects (Titus-Ernstoff *et al.* 2010). In rats, prenatal exposure to DES increases thyroid function by stimulating the pituitary-thyroid axis (Yamamoto *et al.* 2003). DES has been banned for human use in the USA since 1971 (Reed & Fenton 2013) but remains a threat to human health due to its widespread use in livestock production. Concentrations as high as 24.9–102 ng L^{-1} and 7.2–16.9 μg L^{-1} are detected in some Chinese rivers and fisheries waters, respectively (Chen *et al.* 2009; Qu *et al.* 2012; Zhang *et al.* 2012a) and in sediments of Mediterranean aquatic environments (Pojana *et al.* 2007).

IOX is an herbicide extensively used in agriculture (Otsuka *et al.* 2014) and in 2015 the use of IOX in Japan was 107.1 tons (METI 2015). IOX may interfere with the human thyroid system by binding to TTR, a thyroid hormone binding protein that transports TH in the blood (Ogilvie & Ramsden 1988) and one of its toxicological effects is to provoke thyroid tumours in rats (European-Commission 2004). In teleost fish, the effect of IOX and DES on the thyroid system has received more attention (Morgado *et al.* 2007; Morgado *et al.* 2009; Campinho & Power 2013). *In vitro* studies reveal that IOX and DES competitively bind to sea bream (*Sparus aurata*) TTR (Morgado *et al.* 2007). Exposure to 1 mg kg^{-1} of IOX or DES for 21-days did not change TH levels or thyroid follicular morpho-histology in adult sea bream but caused down-regulation of some HPT axis genes, suggesting homeostasis was affected (Morgado *et al.* 2009). In zebrafish exposed to IOX and DES, the mRNA expression of the essential thyroid gland developmental gene *nk2.1a* decreased as did that of the thyroglobulin (*Tg*) gene indicating that thyroid gland development was impaired (Campinho & Power 2013). Moreover, exposure of zebrafish embryos to IOX and DES significantly changed heart morphology (Campinho &

Power 2013). Strikingly the effect of IOX and DES on thyroid gland development in zebrafish embryos resembles that of the cardiac troponin I (*tnni1*) gene morpholino knock-down. The similar cardiac phenotype led us to hypothesise that the action of IOX and DES on thyroid gland development might be indirect and a consequence of their effect on the development of endothelial cells or cardiac function or both.

The heart is the first functional organ to form during vertebrate embryogenesis (Bakkers 2011) and in zebrafish heart contraction starts approximately 24 hpf when a regular heartbeat is observed (Chen *et al.* 1996; Vogel & Weinstein 2000). The ventral aorta (VA) of the heart is adjacent to the thyroid gland and it plays a role in guiding thyroid gland morphogenesis along the pharyngeal region (Alt *et al.* 2006b). Crosstalk between the two tissue systems was revealed by the zebrafish cloche mutant (Thompson *et al.* 1998), which lacks all blood vessels and in which the thyroid gland fails to develop properly (Opitz *et al.* 2012). The preceding studies suggest that the heart-angiogenic-thyrocyte interaction may be essential for correct development and function of the thyroid gland, HPT-axis and thyroid homeostasis.

The present study aims to uncover the mode of action of IOX and DES on the heart, angiogenic system and thyrocytes during zebrafish embryogenesis. We analysed the function and morphology of the heart and ventral aorta and demonstrate that the abnormal development of these tissues is directly related to impaired thyrocyte development. The genetic basis of the modified vascular system caused by embryonic exposure to IOX and DES was revealed by the transcriptome of 48 hpf zebrafish embryonic endothelial cells. To the best of our knowledge, this study provides the first multi-level analysis of cardiac, vascular and thyroid system development in IOX and DES exposed animals and extends our previous observations (Campinho & Power 2013) by revealing these chemicals indirectly disrupt the thyroid in zebrafish.

2.3. Materials and methods

2.3.1. Zebrafish maintenance

Adult zebrafish (AB strain) were fed twice a day with dry pellets (Tetramin, Germany) and once a day with live two-day old Red Pepper enriched Artemia. Animals were raised at 28 °C in a recirculating system (Technoplast, Italy) and under a 14 h: 10 h (light: dark) cycle. The embryos were collected and reared at 28.5 °C in an incubator (Sanyo, Japan). All experiments carried out in this work were approved by CCMAR's ethical committee and are in accordance with the regulation of Directive 2010/63/EU and Portuguese National legislation on

experimental animal use.

2.3.2. Zebrafish Tg(cmlc2:GFPCaaX) transgenic line generation

The proximal promoter (−800–1+) of zebrafish *cardiac myosin light chain 2* (*cmlc2*) was isolated by PCR (zfClmc2PrFw: CGATCCGTGACCAAAGCTTAAATCAGT; zfCmlc2PrRv: CTAGCTAGCGTTCACTGTCTGCTTTGCTGT) using zebrafish genomic DNA as the template and Fusion high-fidelity polymerase (Thermo Scientific). The amplified *cmlc2* promoter was isolated from agarose gel using a kit (EZNA) and then cloned into a pCS2+ vector linearized with *Stu*I and the construct confirmed by sequencing. A GFPCaaX sequence isolated from a Tol2kit vector (Kwan *et al.* 2007) by digestion with the restriction enzymes, *Eco*RV and *Eco*RI (Thermo Scientific), was subcloned downstream of the *cmlc2* promoter in pCS2+. After sequence confirmation of the appropriateness of the pCS2+ Zfcmlc2::GFPCaaX construct, it was excised from the pCS2+ vector using the restriction enzymes *Hin*dIII and *Not*I (Thermo Scientific) and subcloned into the pBR322Tol2 vector (Kwan *et al.* 2007). After confirming the correct integration of the cmlc2::GFPCaaX construct into the pBR322Tol2 vector, plasmid DNA was extracted with phenol/chloroform and diluted in water (Kawakami, 2007). For Tol2 mediated transgenesis, 50 pg of the purified pBR322Tol2-Zfcmlc2::GFPCaaX DNA was co-injected (1 nL) with 25 pg of Tol2 mRNA, prepared with an Ambion mESSENGER RNA kit, into the cytoplasm of one cell-stage zebrafish embryos of the AB strain. Embryos with a mosaic cardiac expression of GFP were selected and grown for crossing with WT zebrafish in order to generate heterozygous transgenic *Tg*(*cmlc2::GFPCaaX*) zebrafish lines.

2.3.3. Chemical exposure

IOX and DES exposure was carried out as described in Campinho and Power (2013). Briefly, 12 hpf zebrafish embryos were incubated in 25 mL of E3 medium (control) or to 25 mL of E3 medium containing 0.1 μM IOX or DES for 36 h (up until 48 hpf) or 60 h (up until 72 hpf) at 28.5 °C in an incubator (Sanyo, Germany). For 36 h treatment assays, the E3 medium was not exchanged. In the 60 h exposure experiments 20% of the bathing E3 medium was substituted in each group with a fresh solution of the appropriate composition at 48 hpf. The chemical exposure experiments were carried out using WT, *Tg*(*fli1:GFP*) (Lawson and Weinstein, 2002) or *Tg*(*cmlc2:GFPCaaX*) zebrafish embryos.

Zebrafish embryos at 48 hpf (36 h-exposure to IOX or DES) or larvae at 72 hpf (60 h-exposure) were fixed overnight at 4 °C in 4% PFA/1×PBS, pH 7 and used for whole-mount

immunohistochemistry (IHC) analysis. For relative gene expression analysis by qPCR, WT zebrafish embryos or larvae (n = 5 pools each containing 20–25 embryos) were transferred to a 1.5 mL tube after removing excess water and frozen immediately in liquid nitrogen and stored at −80 °C until analysis.

2.3.4. Heart rate determination

Forty-eight hpf (36 h-exposed to IOX or DES) Zebrafish *Tg*(*cmlc2::GFPCaaX*) embryos (n = 12–15) were placed under a stereoscope (Olympus AZX7) coupled to an Hammamatsu ORCAv2 digital camera and video images of the heart collected for 2 min at 28.5 °C. Heart rate was determined by counting heartbeat recorded in the video images.

2.3.5. Fluorescent immunohistochemistry

Embryos from WT and transgenic lines used for control or IOX or DES treatments were hydrated through a methanol/1× PBS series from 100% to 0% methanol, followed by 3 × 5 min in PBTr (1 × PBS + 0.1% Triton X-100). Hydrated embryos were preincubated with 1 × PBTr/10% sheep serum for 2 h at room temperature to block non-specific binding of antisera. The primary antibodies incubated with the zebrafish embryos were, rabbit anti-GFP (1:1000 dilution; Abcam), Mouse anti-GFP (1:100 dilution; Developmental Studies Hybridoma Bank), Zn8 (1:20 dilution; Developmental Studies Hybridoma Bank) and rabbit anti-thyroglobulin (Tg) (1:1000 dilution; Dako). Fluorescently tagged secondary antibodies were used to detect the primary antisera: goat anti-rabbit 488 (1/400; Jackson Lab), goat anti-mouse 594 (1/400; Anaspec) and goat anti-mouse 488 (1/400; Anaspec). After whole mount staining, zebrafish embryos were stored in 1× PBS at 4 °C and fluorescent microscope images were obtained using a Zeiss Z2 fluorescent microscope coupled to a Zeiss HRm digital camera.

2.3.6. Image analysis

Heart ventricle image volumes were determined using 48 hpf *Tg*(*cmlc2::GFPCaaX*) transgenic embryos. The images were deconvoluted in SVI Huygens software (v2.13, Holland) and heart reconstruction and 3D models were generated. Morphometric analysis to determine aorta diameter was carried out in FIJI (Schindelin *et al.* 2012) using linear measuring tools and the heart ventricle volume was established using the Volumest plug-in (Merzin 2008).

2.3.7. Vascular endothelia cells sorting by flow cytometry

Pools of GFP-positive *Tg*(*fli1:EGFP*) embryos (200−300) were used for cell sorting in each treatment group. The embryo yolk was dissolved in 55 mM NaCl, 1.8 mM KCl and 1.25 mM $NaHCO_3$ by pipetting the eggs up and down in the solution. Subsequently, the embryo and yolk solution were centrifuged at 310g for 1 min at 4 °C. The pellet was washed with 1 mL of 0.5 × Danieau's solution (29 mM NaCl, 0.35 mM KCl, 0.2 mM $MgSO_4 \cdot 7H_2O$, 0.43 mM $Ca(NO_3)_2$, 2.5 mM HEPES, pH 7.6) and then the animals were dissociated using a trypsin-EDTA solution (0.5 mg ml^{-1} trypsin, 0.22 mg mL^{-1} EDTA). Trypsinization was stopped after adding heat-inactivated fetal bovine serum (HI-FBS) to a final concentration of 5%. Embryos were re-suspended in 1 mL FACSmax solution (AMS Biotechnology, Abingdon, UK) and transferred into a 40 μm nylon cell strainer (BD Biosciences, San Jose, CA, USA). A 1-mL syringe plunger was used to force the trypsinized embryos through the cell strainer into a petri dish. Cell suspensions were collected and sorted in a FACSAriaII cell sorter (FACSDiva software v6.1.3, BA Biosystems, Germany) using the GFP fluorescent signal to select endothelial cells. Around 80,000 to 100,000 GFP-positive cells and 260,000 to 370,000 GFP-negative cells in each treatment group were collected from each independent experiment. The experiments were performed in duplicate.

2.3.8. Transcriptome analysis of endothelial cells

RNA was isolated from the sorted cells using a total RNA Extraction Kit (Omega Bio-Tek, Norcross, GA). Extracted RNA was treated with DNAse (DNA-free Kit, Ambion, UK) using a Turbo DNA-free kit (Ambion, UK) and the RNA was precipitated with ethanol and quantified using a Nanodrop (1000 Spectrophotometer, Thermo Fisher Scientific, USA). The total RNA extracted from sorted cells harvested from control, IOX and DES treated embryos was prepared from pools of RNA extracted from 2 independent experiments using the same broodstock (n = 1 transcriptome per treatment).

The sequencing libraries were prepared using a Nextra XT DNA library kit (Illumina) according to the manufacturer's instructions (RNA input was 1 ng). Sequencing was performed using an Illumina MiSeq platform at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) with 150 bp paired-end reads and a total of 189 million sequences were generated. Raw sequences have been submitted to NCBI under the accession number: PRJNA1116337.

2.3.9. Bioinformatics analysis

The raw sequence data was filtered to remove the adapter sequences, low quality reads,

sequences with a high content of N or reads < 20 bp long by using SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle). The filtered data was aligned against the zebrafish reference genome (ftp://ftp.ensembl.org/pub/release-84/fasta/danio_rerio/dna/) using TopHat (V2.0.13) with default parameters (Trapnell *et al.* 2009; Trapnell *et al.* 2012). The aligned read files were entered into the Cufflinks (v2.2.1) software (Trapnell *et al.* 2010) (http://cole-trapnell-lab.github.io/cufflinks/). The gene expression levels were calculated by the method of fragments per kilobase of exon per million mapped reads (FRKM) (Trapnell *et al.* 2010). Differential expression (DE) of transcripts was established using Cuffdiff (http://cole-trapnell-lab.github.io/cufflinks/cuffdiff/index.html) (Trapnell *et al.* 2013). The R package Venn diagram (https://cran.r-project.org/web/packages/VennDiagram/) was used to generate the Venn diagram to obtain an overview of the DE gene transcripts present in one or more of the experimental groups (Chen & Boutros 2011). A heatmap of DE gene transcripts was generated using the function heatmap.2 available in the R package gplots (https://cran.rproject.org/web/packages/gplots/) and the distance between samples and genes were determined by the R function cor with Pearson and dist with the Euclidean distance, respectively. Gene ontology enrichment was analysed by GOatools (https://github.com/tanghaibao/GOatools) and was based on a Fisher's exact test and multiple corrections were adjusted by Bonferroni, Holm, Sidak and false discovery rate (Lu *et al.* 2012). KEGG pathway enrichment of the DE genes was performed using KOBAS (http://kobas.cbi.pku.edu.cn/home.do) (Benjamini & Hochberg 1995; Xie *et al.* 2011).

2.3.10. Real-time quantitative polymerase chain reaction validation

Total RNA from zebrafish WT embryos (n = 5 pools of 20–25 embryos each) was isolated with an Omega Total RNA kit following the manufacturer's instructions and treated with DNase (Ambion Turbo DNase kit). The cDNA was synthesised using 500 ng of DNA free total RNA, random hexamer primers (50 ng/µL) and a RevertAid kit (Thermo Scientific) following the manufacturer's instructions.

The genes selected for qPCR were selected among those with the most significant differential expression levels (log2-fold change; $p < 0.05$, FDR > 95%) in the RNA-seq data obtained for endothelial cells isolated from zebrafish embryos exposed to IOX or DES (Table 2.1). The qPCR primer pairs were designed using the assembled RNA-seq sequence data.

The qPCR reaction was carried out using 1 × FastStart Essential DNA Green Master (Roche

Diagnostics), 300 µM of the specific forward and reverse primers (Table 2.1) and ~200 ng of template cDNA. All samples were analysed in duplicate in a CFX Connect Real-Time instrument (Bio-Rad). The qPCR amplification protocol was as follows: initial denaturation for 3 min at 95 °C followed by 45 cycles of 10 s at 95 °C and 15 s at 60 °C. A melting curve analysis with a temperature gradient of 0.5 °C/s from 65 °C to 95 °C was conducted for each set of primer pairs to confirm that a single amplified product was obtained. Two technical replicates per sample were performed in each qPCR assay. For normalization, 18S was used as the reference gene. The relative mRNA expression was determined using the absolute quantification method using gel isolated amplicons as standards.

Table 2.1. Primers used to qRT-PCR expression analysis of 17 selected genes and *18s*.

Target	Primer	Sequence (5'-3')	Amplicon size (nt)	Efficiency (%)	r^2
Acvr1bb (activin receptor type-1B-like)	FW	ACGCTCTGGGTCTGGTGTACTG	112	101	0.998
	RV	TCTATGGAGGGATCTGACGGCAC			
Adrb1 (β1-adrenergic receptor)	FW	TCGTGGGCATGGGAATCCTCAT	106	93	0.998
	RV	GTGAGCGTCTGGAGCCTCTGAT			
Agtr1a (angiotensin II type I receptor)	FW	CGACTCCAACACGGGACTTGC	137	97	0.999
	RV	AGATGACGGCGACTACCAGACTG			
Angptl1b (angiopoietin-related protein 2)	FW	GACAGTTCACCACGCTGGACAG	122	98	0.998
	RV	TGAGTACCACACGCCGTTCAGA			
Cacna1da (voltage-dependent L-type calcium channel subunit alpha-1D)	FW	CAATGAGCAGCAAGCCGAGGTAA	158	98	0.996
	RV	AGCCTTTGTGGAGGGATATGCCA			
Cacna1ha (voltage-dependent calcium channel subunit alpha-1H-like)	FW	CCTTCAGAGACGCAATGCCTCAG	182	94	0.998
	RV	GCCAACTTGATCGCAGCATCCA			
Cacng2b (calcium channel, voltage-dependent, gamma subunit 2b)	FW	CTCTGGTGGAATGCGGCAGTATG	173	96	0.998
	RV	GCTGGTCTCGTTCTCGTTGGTG			
Cadm1a (cell adhesion molecule 1a isoform 3)	FW	GCCTCAGCAGGTGAACTGGGTA	126	101	0.998
	RV	GACGCCACGCATCGGTAAGTG			
Calcr (calcitonin receptor)	FW	TCGCAGAGGAGCAGCACCTAC	136	98	0.998
	RV	AGCAGATGCGTCTCCACACTCA			
Chrnb3a (cholinergic receptor, nicotinic, beta polypeptide 3a precursor)	FW	CCTTCACGCCAACGACACGATTACT	116	96	0.997
	RV	TCCACTCCTCCCACAACCAGACATT			
HIF1aa (hypoxia inducible factor)	FW	GCTCAGAGAAATGCTGGCACACA	148	99	0.996
	RV	CAGCACCTTCCAGGAGGCAGA			
Mhc1lia (hereditary	FW	TGATGGCGAACCAGGACGAATGA	121	92.6	1

Target	Primer	Sequence (5'-3')	Amplicon size (nt)	Efficiency (%)	r^2
hemochromatosis)	RV	CACATGGGTCGAGACGTTGAAGC			
Mybpc2a (myosin binding protein C, fast type a)	FW	TATCCAAGAACCGCCTGTCACCA	149	105	0.997
	RV	TTCCATCTCGGCTCAGTTCCACA			
Notch1b	FW	TTGACGACTGCACACCGTTCAC	159	94	0.996
	RV	GGTTGGACAGGCACTCGTTGAC			
Ptgir (prostacyclin receptor-like)	FW	AGTGATGGACTCTGCCTGGACAG	169	92	0.998
	RV	GCCTCGATGCTGGTGATGTTCTC			
Itpr1b (inositol 1,4,5-trisphosphate receptor, type 1b)	FW	CGGTGGAGGAGTCGGAGATGTG	160	107	0.997
	RV	GCTTCTGAGGTCTGCGAAGGTATC			
VWF (Von Willebrand Factor)	FW	GCGTGGAGGCATTCGGCAACT	193	97	0.994
	RV	CGGCAGTTCTTCACATACGGCTCAG			
18s	FW	GGAATTGACGGAAGGGCACCAC	135	97	0.995
	RV	GCACCACCACCCACAGAATCG			

2.3.11. Statistical analysis

Data were analysed for statistical significance using Prism software and statistical significance was considered when $p < 0.05$. One-way ANOVA followed by a Bonferroni corrected post-hoc test (significance considered if $p < 0.05$) was used to determine the effect of EDCs on heart function (heart rate), the volume of the ventricle, the ventral aorta diameter, thyroid follicle number, thyroid field and the thyroid follicle area. The relative mRNA expression was analysed using One-Way ANOVA analysis followed by a Turkey post-hoc test (significance considered if $p < 0.05$).

2.4. Results

2.4.1. IOX and DES affect heart function and ventricle morphology

Live image analysis of 48 hpf (36 h-exposed) *Tg*(*cmlc2::GFPCaaX*) embryos revealed the heart rate increased significantly in both 0.1 µM IOX and DES exposed embryos relative to control siblings (Figure 2.1A; $p \leq 0.001$) and that the heart rate of DES exposed embryos was significantly higher than the IOX exposed embryos (Figure 2.1A; $p \leq 0.001$). The change in heart rate was associated with a decreased ventricle volume in the DES and IOX treated zebrafish embryos (Figure 2.1B, Supplementary Movies 2.1-2.3 annex I). IOX and DES treatments significantly affected the ventricle morphology relative to the control groups after 36-h exposure to the chemicals (Figure 2.1B; $p < 0.05$). IOX had the most profound effect and

strongly reduced the volume of the ventricle relative to the control zebrafish embryos ($p < 0.0001$) (Figure 2.1B, Supplementary Movies 2.1-2.3 annex I). Exposure of zebrafish embryos to DES also decreased the volume of the ventricle when compared to control embryos (Figure 2.1B; $p < 0.05$) but less than in IOX-exposed sibling (Figure 2.1B; $p < 0.01$).

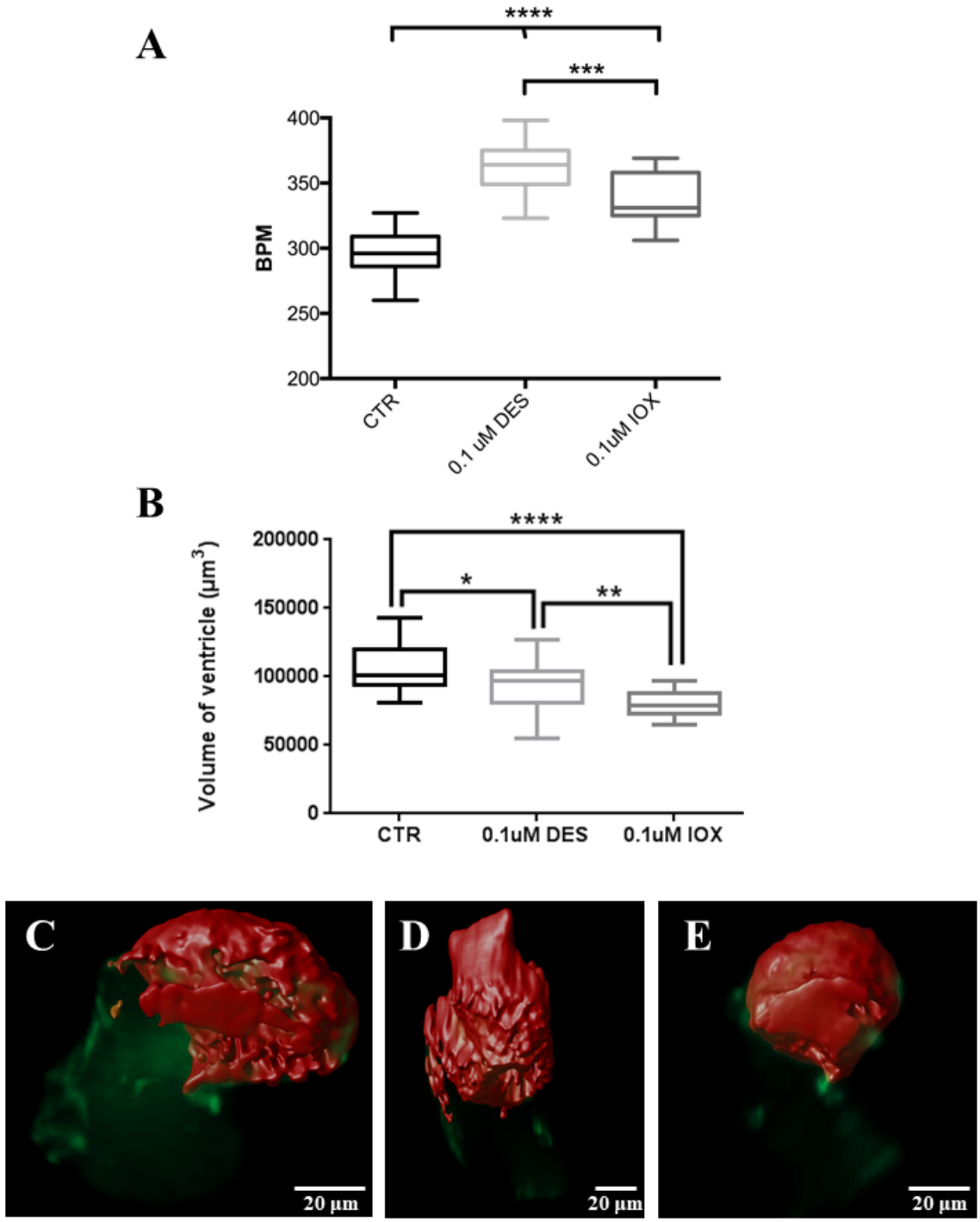

Figure 2.1. Box and whisker plot (minimum and maximum) of (A) heart beats per minute (BPM) and (B) ventricle total volume in 48 hpf zebrafish *Tg(fli1:EGFP)* control (CTR) and 36 h-exposure embryos to DES and IOX. Different letters indicate statistically significant difference ($p < 0.05$). C–E Image of the heart with the ventricle (Red) at the top and atrium (Green) at the bottom, (C) control, (D) DES treated and (E) IOX treated. One-way ANOVA was used to test for statistical significance followed by Bonferroni's

multiple comparisons test *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001, the same as below.

2.4.2. IOX and DES affect aorta and thyrocyte development

In zebrafish, the ventrally localized aorta (VA) is the major artery adjacent to the thyroid and the development of the VA is crucial for thyroid morphogenesis and localization in the pharyngeal region (Alt *et al.* 2006b). To characterize the effect of IOX and DES on VA morphology and thyrocyte development, zebrafish embryos at 72 hpf were analysed by whole-mount immunofluorescence staining (Figure 2.2). Considering that the thyroid field is distributed along the VA, we measured the diameter of the VA in an anterior, middle and posterior position relative to the thyroid tissue (Figure 2.2A). The VA diameter in all three positions measured was significantly decreased in the IOX and DES treated groups compared to the control group (Figure 2.2B; $p < 0.01$). IOX and DES exerted a similar effect on VA diameter (Figure 2.2B, $p > 0.05$).

The effects of IOX and DES on thyroid morphology was analysed by determining the thyroid follicle number, thyroid field and thyroid follicle area (Figure 2.2C). The number of thyroid follicles significantly decreased in IOX treated zebrafish embryos compared to the control groups (Figure 2.2C; $p < 0.05$) but DES did not change thyroid follicle number ($p > 0.05$). The thyroid field length in DES exposure was significantly lower than the control group (Figure 2.2C; $p < 0.05$) but was not significantly different from the IOX treated siblings ($p > 0.05$). The total thyroid follicle area was significantly decreased in both IOX and DES groups relative to the control embryos (Figure 2.2C; $p < 0.05$). DES had a more pronounced effect on total follicle area ($p < 0.01$) than IOX ($p < 0.05$) even though the decrease was not significantly different between treatments (Figure 2.2C; $p > 0.05$). The effect of the IOX and DES on the VA and thyroid development was highly specific, and the growth of zebrafish embryos was unaffected at 72 hpf (Supplementary Figure 2.1 annex I; $p > 0.05$).

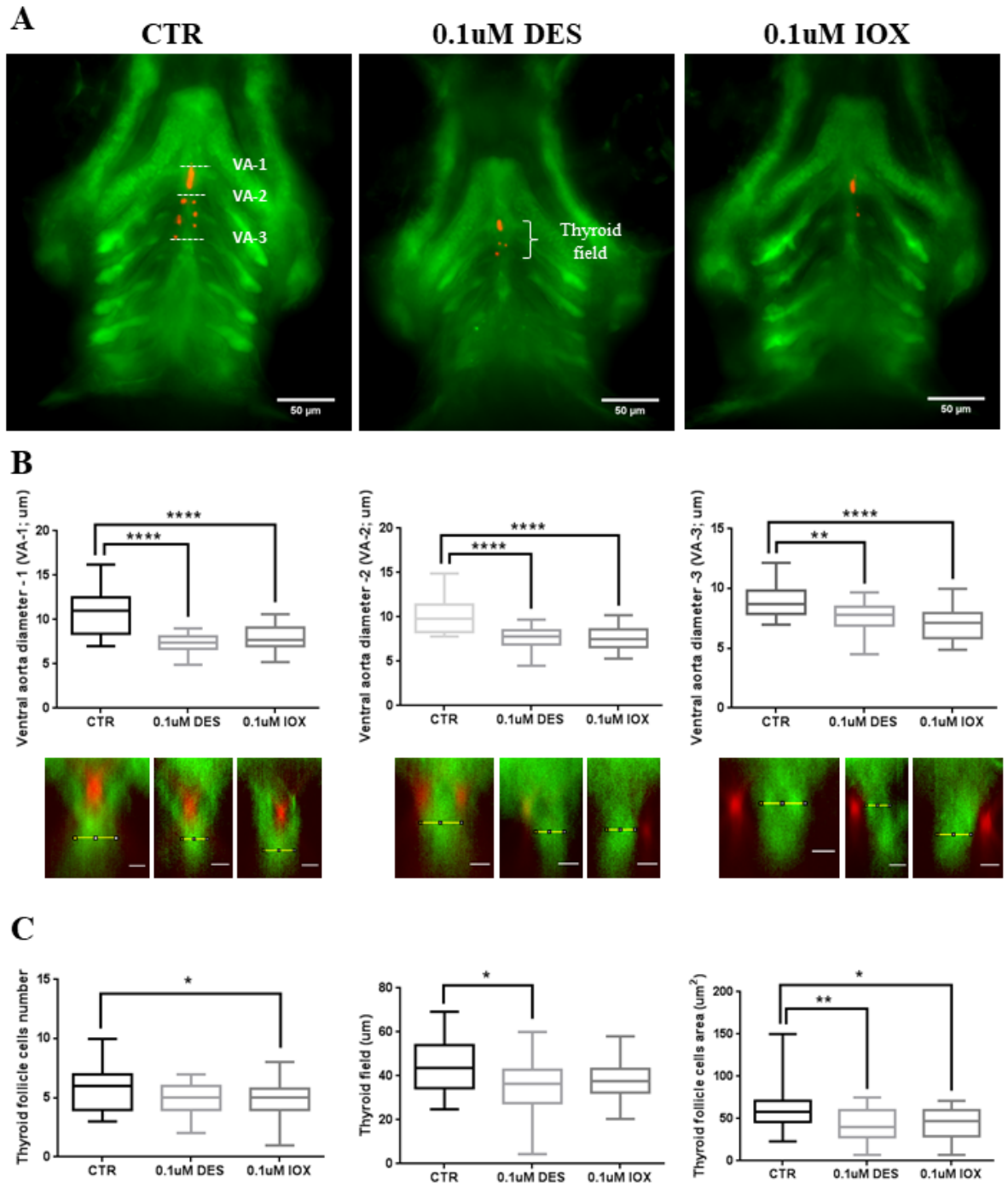

Figure 2.2. Exposure to DES and IOX affects thyroid gland and ventral aorta development. (A) Fluorescent images of the pharyngeal region of *Tg(fli1:EGFP)* zebrafish embryos at 72 hpf. Red and green represent, respectively, thyroglobulin and GFP immunostaining. (B) Determination of aorta diameter in three different regions as depicted in A. From left to right VA-1, VA-2 and VA-3 are represented. The lower panel below the graphs represents examples of Z-slices at each region of the aorta measured. The scale bars in the lower panel below the graphs correspond to 5 µm. (C) Measurements of thyroid follicle number, thyroid field length and the thyroid follicle area.

2.4.3. Transcriptomics of zebrafish endothelial cells exposed to IOX and DES

Analysis of the global gene regulation profiles of endothelial cells in zebrafish embryos

treated with IOX and DES revealed 1688 DE gene transcripts relative to the control embryos. KEGG pathway analysis showed that 28 and 19 pathways respectively were significantly changed in the IOX and DES exposure groups relative to the control embryos (Supplementary Table 2.1 and 2.2 annex I; $p < 0.05$). Several of the pathways significantly changed in DES and IOX treated embryos were related to blood vessel endothelial cell function and three cardiomyopathy pathways were identified including hypertrophic cardiomyopathy, dilated cardiomyopathy and arrhythmogenic right ventricular cardiomyopathy (Table 2.2, $p < 0.05$). IOX and DES also had an effect on the pathways of calcium signalling, cell adhesion molecules, GABAergic synapse (Table 2.2, $p < 0.05$). The type I diabetes mellitus pathway was significantly affected by DES exposure (Table 2.2, $p < 0.05$), while IOX had a significant impact on the complement and coagulation cascades, vascular smooth muscle contraction, amyotrophic lateral sclerosis, platelet activation, retrograde endocannabinoid signalling and ABC transporters (Table 2.2, $p < 0.05$)

The DE gene transcripts identified by RNA-seq were used to generate a heatmap and the pattern of gene expression in the embryos exposed to IOX and DES was compared to the control group (Figure 2.3A). The expression signature of DE gene transcripts in IOX and DES were different from the control but also from each other (Figure 2.3A). DE analysis revealed that some transcripts were affected by both treatments whereas other transcripts were affected by IOX but not DES and vice-versa (in comparison to the control embryos; Figure 2.3B).

Seventeen genes related to the function of endothelial cells, including vascular function, cardiac function, calcium homeostasis and other genes were selected to validate the transcriptome data by qPCR (Figure 2.4). Most of genes such as hypoxia inducible factor (*HIF1aa*), β1-adrenergic receptor (*Adrb1*), von Willebrand factor (*Vwf*), prostacyclin (*Ptgir*) and voltage-dependent T-type calcium channel subunit alpha-1H (*Cacna1ha*) were significantly increased in the IOX treated embryos compared with the control embryos (Figure 2.4, $p < 0.05$). However, no significant differences were found for the gene transcripts analysed between the DES exposed embryos and the control embryos (Figure 2.4, $p > 0.05$).

Table 2.2. Selected enriched KEGG pathways calculated for modulated genes in DES and IOX treated zebrafish embryos at 48 hpf. "—" indicates 'not significantly different'.

	Comparison with control					
	DES			IOX		
	P value	No. of genes		P value	No. of genes	
		Up	Down		Up	Down
Calcium signaling pathway	$2.63E^{-04}$	11	12	$3.09E^{-04}$	14	11
Neuroactive ligand-receptor interaction	0.0166	18	14	$1.03E^{-04}$	27	18
GABAergic synapse	0.0064	8	3	0.0354	6	4
Cell adhesion molecules	0.0276	9	4	0.0348	10	4
Hypertrophic cardiomyopathy	0.0116	4	6	0.0258	4	6
Dilated cardiomyopathy	0.0233	5	5	0.0100	6	6
Arrhythmogenic right ventricular cardiomyopathy	0.0349	4	4	0.0118	3	7
Retrograde endocannabinoid signaling	0.0013	7	7	0.0220	8	4
Type I diabetes mellitus	0.0122	2	4	—	—	—
Vascular smooth muscle contraction	—	—	—	0.0323	7	6
Complement and coagulation cascades	—	—	—	0.0029	9	2
Amyotrophic lateral sclerosis	—	—	—	0.0410	3	7
Platelet activation	—	—	—	0.0476	10	5
ABC transporters	—	—	—	0.0140	3	3

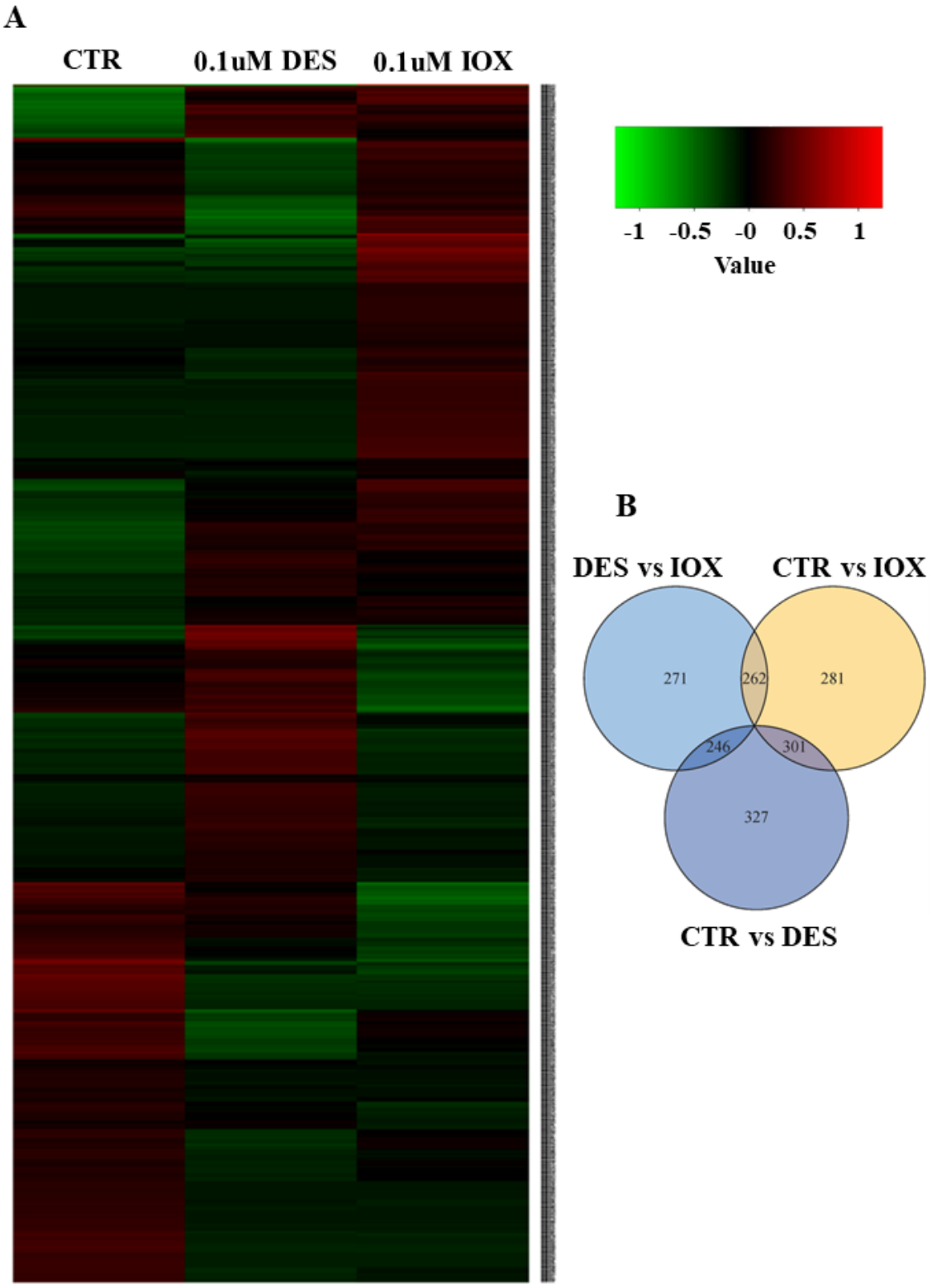

Figure 2.3. Transcriptome analysis of differentially expressed genes in 48 hpf control, DES and IOX treated zebrafish embryos. (A) Heatmap showing the gene expression pattern in control, DES and IOX treated zebrafish; The colour represents the gene expression level (log10 FPKM); Red represents high expression and low expression is indicated in green (B) Venn diagram presenting the differential expressed genes; the number in circles represents the differential expressed transcripts between two groups; the overlapping section of two circles means the common number of differential expressed transcripts.

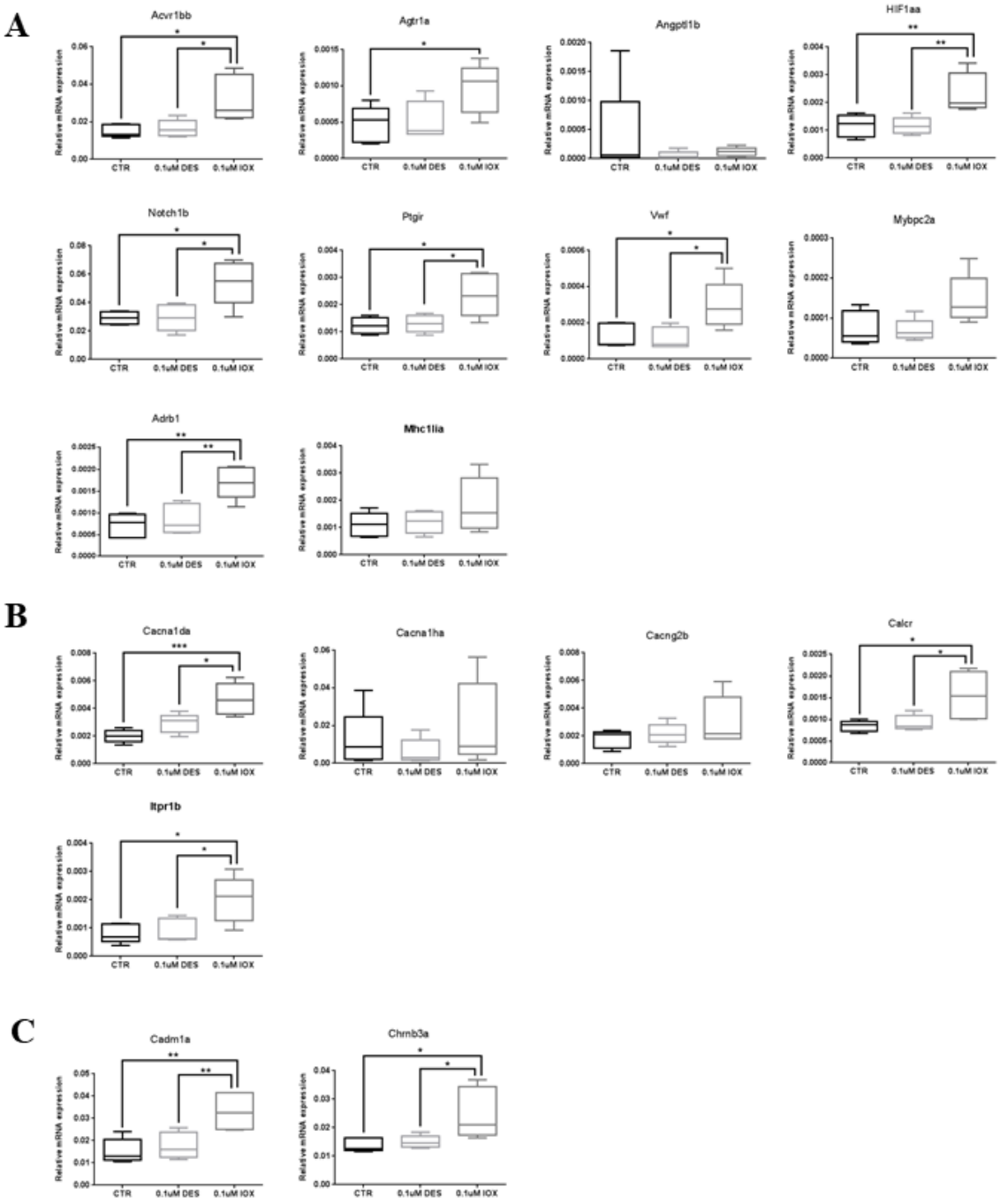

Figure 2.4. qPCR validation of selected RNA-seq differential expressed transcripts between control, DES and IOX-treated zebrafish embryos 48 hpf (36 h-exposure to chemicals). Analysed genes were related to cardiovascular function (A), calcium homeostasis (B) and others (C). *Acvr1bb*: activin receptor type-1B-like; *Agtr1a*: angiotensin II type I receptor; *Angptl1b*: angiopoietin-related protein 2; *HIF1aa*: hypoxia inducible factor (HIF-1); *Ptgir*: prostacyclin receptor-like; *Vwf*: von Willebrand factor; *Adrb1*: β1-adrenergic receptor; *Mhc1lia*: hereditary hemochromatosis; *Mybpc2a*: myosin binding protein C, fast type a; *Cacna1da*: voltage-dependent L-type calcium channel subunit alpha-1D; *Cacna1ha*: voltage-dependent T-type calcium channel subunit alpha-1H-like; *Cacng2b*: calcium channel, voltage dependent, gamma subunit 2b; *Calcr*: calcitonin receptor; Itpr1b: inositol 1,4,5-trisphosphate receptor, type 1b; *Cadm1a*: cell adhesion molecule 1a; *Chrnb3a*: cholinergic receptor, nicotinic, beta polypeptide 3a. Different letters indicate statistically significant difference ($p < 0.05$).

2.5. Discussion

This study provides insight into the mechanisms by which DES and IOX affect zebrafish heart and vascular development and how these changes impinge on thyroid development. The data obtained in the present study, together with our previous results (Campinho & Power 2013), reveal a direct disruptive effect of IOX and DES on the heart and vascular development and an indirect effect on the thyroid. Recently, we found that the action of DES and IOX on zebrafish thyroid development is directly linked to their effect on normal heart development (Campinho & Power 2013) and that this eventually leads to impaired thyroid development in zebrafish embryos. Our data also show that exposure to IOX and DES seems to have a similar morphological and functional endpoint in zebrafish development but that the underlying genetic, cellular and molecular mechanisms differ.

The effects of DES and IOX on the heart are related to increased heartbeat and impaired morphology. The ventricular size and shape are crucial for determining cardiac function (Hu *et al.* 2001). The observed changes in heartbeat in embryos exposed to IOX and DES were further supported by a decrease in ventricle volume when compared to control siblings and this strongly supports the notion that the heart is a target tissue for these two chemicals. It is unclear if the effect of IOX or DES treatments impact more on the definition of heart morphology or heart function. However, it is likely that this is a reciprocal effect given the relationship between form and function in the developing zebrafish heart (Yelon 2001; Torrent-Guasp *et al.* 2005).

It was recently reported that in third generation DES-exposed women there is an increase in the incidence of cardiac defects associated with the atrial and ventricular septal (Titus-Ernstoff *et al.* 2010; Tournaire *et al.* 2016; Parvez *et al.* 2012). This data from human studies is in concordance with the results from our previous (Campinho & Power 2013) and the present study. However, so far no data is available about the thyroid function and physiology in third generation DES-exposed women. Indeed, little is known about the effects of DES on heart development and function in vertebrates in general. This is relevant because our studies in zebrafish (Campinho & Power 2013) showed that alongside the changes in heart function and ventricle volume there were significant changes in thyroid gland development. Here we provide the first evidence on the pernicious effects of low exposure doses of DES in zebrafish, a vertebrate and important biomedical model and thus hint that these observations are conserved in other vertebrates, including humans (Titus-Ernstoff *et al.* 2010).

It was not only heart development that was affected in the IOX and DES treated zebrafish embryos as the ventral aorta diameter was also reduced in both experimental groups. A direct

effect of IOX and DES on the heart is evident from the morphological and functional data (Figure 2.1). The transcriptome data also indicates that developing endothelial cells are differentially affected by these chemicals. The response of endothelial cells to IOX and DES exposure was specific given the restricted subset of genes that responded in both groups when compared to the control embryos. Moreover, the response of endothelial cells to IOX or DES was quite different with different subsets of genes and gene networks being affected in each group.

Three main cardiomyopathy pathways were represented in the DE genes of DES and IOX exposed embryos, including hypertrophic cardiomyopathy, dilated cardiomyopathy and arrhythmogenic right ventricular cardiomyopathy together with other pathways such as the calcium signalling pathway (Table 2.2). qPCR validation of the transcriptome data showed that a cluster of genes such as *Acvr1bb*, *Agtr1a*, *HIF1aa*, *Notch1b*, *Ptgir*, *Adrb1* and *Vwf*, involved in cardiovascular function and form were up regulated in IOX treated groups (Figure 2.4). More specifically changes in *Adrb1* may explain modified heart rate and atrial cardiac muscle contractility (Parvez *et al.* 2012) and changes in *Agtr1a* together with *Ptgir* (a vasodilator effect mediator) may explain the vasoconstriction observed (Whittle *et al.* 2012; Dinh *et al.* 2017). Calcium homeostasis genes (*Cacna1da*, *Calcr* and *Itpr1b*), cell adhesion molecule (*Cadm1a*) and cholinergic receptor gene (*Chrnb3a*) were up regulated by IOX exposure. Although morphological effects on the heart caused by DES exposure were clear, no differences in the genes involved in cardiovascular function and analysed by qPCR were identified. These findings suggest that IOX and DES induce some different genetic pathways, but some genetic responses are similar (Supplementary Table 2.3, 2.4 and 2.5 annex I). This data might also indicate that IOX has a stronger endocrine disruption potential.

The disruption of the heart and VA growth in DES and IOX treated groups may affect the morphology of the vessels or may themselves be affected by the impaired vasculature development. The effect on blood vessel development and homeostasis of the sheer stress of blood cells as they circulate, and the intensity of cardiac function has been extensively demonstrated (Shiojima *et al.* 2005; Lu & Kassab 2011; Boselli *et al.* 2015). Together with increased heartbeat and lower chamber volume (Figure 2.1) it is expected that blood flow will be affected in both IOX and DES-treated embryos when compared to controls. Although not vali dated by qPCR, genes involved in angiogenic mechanosensing (Boselli *et al.* 2015) were DE in the transcriptome (> 2-fold, $p < 0.05$; FDR 0.05) of IOX and DES embryos relative to the control (Supplementary Table 2.3, 2.4 and 2.5 annex I). In both IOX and DES, expression

of early growth response protein 1 (*egr1*) was up-regulated whereas piezo-type mechanosensitive ion channel component 1 (*piezo1*) and endothelin 1 (*edn1*) were down-regulated. In contrast, other genes (e.g. *klf2*, *s1p1*, *pkd2* and *cxcr4*) involved in mechanosensing and response in endothelial cells were either up regulated in IOX (vs control) or downregulated in DES or vice-versa (Supplementary Table 2.3, 2.4 and 2.5 annex I). Taken together, our data seem to suggest that IOX and DES affect blood flow. What is not clear is if the effect is through a direct effect on endothelial cells, cardiomyocytes (and hence heart-function) or both. Of the two chemicals tested IOX presented clearer effects both in relation to endothelial factors (most notably the angiotensin receptor agtr1a) as well as Ca^{2+}-signalling related genes (Figure 2.4A and B). Another important observation was that endothelial cells responded differently to IOX and DES, even though similar morphological endpoints were identified they seem to point out for vessel contraction/stiffness implications.

Blood vessel development is known to affect thyroid gland morphology and modulates thyroid morphogenesis in zebrafish (Alt *et al.* 2006a; Opitz *et al.* 2012). Given that thyroid gland development is highly dependent on the development of the heart and aorta (Opitz *et al.* 2012; Wendl *et al.* 2007; Alt *et al.* 2006a), the data from the present study revealing that IOX and DES disrupt normal cardiovascular development in the zebrafish embryos probably explains the observed modifications in thyroid gland development and homeostasis. We have shown that this is likely mediated by changes in the expression of the thyroid gland master developmental factor, nkx2.1a (Campinho & Power 2013). Taken together our data indicates that thyroid gland development was affected in IOX and DES exposed embryos probably due to disruption of normal cardiovascular development and that this might have repercussions on TH homeostasis in later life stages.

These observations are not entirely unexpected. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been shown to cause severe heart morphology anomalies by altering looping and also provoked defects in heart function such as blood regurgitation and ventricular standstill in zebrafish embryos (Antkiewicz *et al.* 2005). In the other only study in vertebrates where it was studied the effect of an anthropogenic derived chemical exposure on cardiac development and thyroid gland development, it was reported that clofibrate impacts on heart morphology and contractility in zebrafish leading to altered thyroid gland morphogenesis (Raldúa *et al.* 2008). Similarly, a short ventral aorta was observed in clofibrate-exposed zebrafish larvae with disrupted thyroid tissue morphogenesis (Raldúa *et al.* 2008). Taken together the disruption of thyroid gland morphogenesis was due to impairment of ventral aorta development in clofibrate-

exposed zebrafish larvae (Raldúa *et al.* 2008).

While more work is needed to further dissect the effects of DES and IOX on zebrafish thyroid gland development and homeostasis, the present study shows that DES and IOX are thyroid disrupting compounds. We propose that DES and IOX directly affect vascular and heart development and that through this action they indirectly impair the developing thyroid gland and presumably the thyroid axis and disrupt thyroid homeostasis. Notably, the toxicological effects of IOX and DES on zebrafish and humans seem to be remarkably well conserved and highlights the zebrafish as good model for understanding the effect of environmental exposure to IOX and DES in human populations.

2.6. Conclusion

Our findings suggest that low micromolar levels of waterborne IOX and DES can alter normal cardiac and vascular physiology of zebrafish embryos and have the potential to give rise to thyroid endocrine disruption indirectly. Taken together, the subtle but significant morphological, functional and gene expression changes at very low levels of IOX and DES, suggest that what are considered safe limits for these chemicals in the environment are lower than previously thought.

CHAPTER 3

Ioxynil and diethylstilbestrol increase the risks of cardiovascular and thyroid dysfunction in zebrafish

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CRediT statement: YFL processed samples, carried out the molecular analysis, analysed the data and wrote the manuscript; JR processed samples, carry out molecular analysis and analysed the data; MAC designed and carried out the experiments, provided funding, analysed the data and wrote the manuscript. All authors revised the manuscript.

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3.1. Abstract

Endocrine disruption results from exposure to chemicals that alter the function of the endocrine system in animals. Chronic 60 days of exposure to a low dose (0.1 μM) of IOX or DES via food was used to determine the effects of these chemicals on the physiology of the heart and thyroid follicles in juvenile zebrafish. Immunofluorescence analysis and subsequent 3D morphometric analysis of the zebrafish heart revealed that chronic exposure to IOX induced ventricle deformation and significant volume increase ($p < 0.001$). DES exposure caused a change in ventricle morphology, but volume was unaffected. Alongside, it was found that DES exposure upregulated endothelial related genes (*angptl1b*, *mhc1lia*, *mybpc2a*, *ptgir*, *notch1b* and *vwf*) involved in vascular homeostasis. Both IOX and DES exposure caused a change in thyroid follicle morphology. Notably, in IOX-exposed juveniles, thyroid follicle hypertrophy was observed; and in DES-exposed fish, an enlarged thyroid field was present. In summary, chronic exposure of juvenile zebrafish to IOX and DES affected the heart and the thyroid. Given that both chemicals are able to change the morphology of the thyroid it indicates that they behave as EDCs. Heart function dynamically changes thyroid morphology, and function and hence it is likely that the observed cardiac effects of IOX and DES are the source of altered thyroid status in these fish.

Keywords: Diethylstilbestrol; Endocrine disruption; Heart; Ioxynil; Thyroid; Vascular

3.2. Introduction

EDCs are natural or synthetic compounds that interfere with normal endocrine signalling (Santos-Silva *et al.* 2018). These hormone-like chemicals include pharmaceuticals, industrial pollutants, waste products, pesticides, and plastics and have detrimental effects on animal health. Their effects include development, reproduction, metabolism and physiology (Schug *et al.* 2016; Schug *et al.* 2011). A wide variety of EDCs are associated with abnormal physiological conditions that lead to diseases (Skinner 2014).

Epidemiological studies have shown a link between EDC exposure and increased incidence of cardiovascular disease (Bae *et al.* 2012; Goncharov *et al.* 2008; Tournaire *et al.* 2016). In humans, in utero exposure to DES increases the incidence and prevalence of cardiac defects, including heart murmur, ventricular septal disease, tetralogy of fallout, atrial septal defect, and pulmonic stenosis (Titus-Ernstoff *et al.* 2010). Similarly, when zebrafish embryos are exposed to herbicide IOX or synthetic estrogen DES (0.1 μM), heart development, morphology (Campinho & Power 2013) and function are significantly affected (Li *et al.* 2019). Furthermore, IOX- and DES-exposed zebrafish embryos had a higher heartbeat frequency, lower ventricular volume and a reduced ventral aorta diameter, and most likely modified blood flow (Li *et al.* 2019). The observed effects on the cardiovascular system are coherent with a previous study in which short-term exposure to IOX and DES affected heart-vascular-thyrocyte development in zebrafish embryos (Li *et al.* 2019; Campinho & Power 2013).

Vascular endothelial cells in blood vessels play a crucial role in maintaining cardiovascular homeostasis since they mediate vascular tone, regulating vessel-wall permeability and blood flow (Pober & Sessa 2007). Transcriptional analysis of endothelial cells from zebrafish embryos exposed to 0.1 μM IOX or DES revealed both common and compound specific responses and ventricular morphology (Li *et al.* 2019). Several cardiomyopathy pathways were significantly modified in both IOX- and DES-exposed embryos, including hypertrophic, dilated, and arrhythmogenic right ventricular cardiomyopathy, strongly arguing that the cardiac effect of IOX and DES might be due to endothelial specific effects (Li *et al.* 2019). IOX also significantly impacted genes linked to vascular smooth muscle contraction and coagulation related pathways, suggesting it might influence systemic physiological responses via blood vessels (Li *et al.* 2019).

In zebrafish and mice, thyroid gland morphogenesis directly associates with cardiac and pharyngeal blood vessel development and interaction herein (Alt *et al.* 2006b; Opitz *et al.* 2012; Wendl *et al.* 2002). In zebrafish, early thyroid morphogenesis (thyroid bud) occurs between 32 and 55 h post-fertilization (hpf) spatially adjacent to the apical pole of the heart in the pharynx

(Opitz *et al.* 2012). Pharyngeal vessels, such as the ventral aorta (VA), are in close spatial proximity to the thyroid gland and have a role in remodeling thyroid morphogenesis (Alt *et al.* 2006a). Impaired heart development and function are associated with abnormal thyroid development and suggest that normal heart function is essential for thyroid morphogenesis (Campinho & Power 2013). Previous evidence showed that exposure of zebrafish embryos to IOX and DES led to cardiac and vascular development impairment, which induced abnormal zebrafish thyroid development (Campinho & Power 2013; Li *et al.* 2019). Zebrafish embryos revealed more sensitivity to endocrine disruption in a short-term exposure (Campinho & Power 2013; Li *et al.* 2019).

In the USA, human usage of DES has been banned since 1971 (Reed & Fenton 2013). However, given its widespread use in livestock production, it remains a threat to human health. In China, DES concentrations as high as 24.9–102 ng/L and 7.2–16.9 μg/L (approximately 0.1 μM) were detected in Chinese rivers and fisheries waters (Chen *et al.* 2009; Qu *et al.* 2012; Zhang *et al.* 2012a). Therefore, even though some restrictions on using these chemicals have been introduced they are still prevalent in ecosystems and are likely to affect human health.

However, these chemical concentrations are considered low limits in the environment, and "no observed effect concentration" (NOEC) was found when fish were exposed to 3.2 mg/L IOX for 21 days (European-Commission 2004). DES is a human and animal carcinogen that was considered to have no safe exposure level (Rodricks 1988). We have demonstrated that IOX and DES affect zebrafish cardiovascular and thyroid development. However, it is unclear if the same effects are observed in cardiac and thyroid homeostasis and physiology in zebrafish juveniles/adults. To further understand the effects of those chemicals on heart and thyroid physiology under "low dose" exposure and provide evidence for policymakers, we evaluated the impact of chronic (60-days exposure) exposure to IOX and DES on the heart and thyroid morphological indexes in juvenile zebrafish. Given that zebrafish is an outstanding biomedical model, these findings will likely impact IOX and DES action in human health.

3.3 Materials and methods

3.3.1. Zebrafish rearing

Adult transgenic zebrafish *Tg*(*cmlc2:GFPCaaX*) (Li *et al.* 2019) and *Tg*(*kdrl:mCherry*) (Mugoni *et al.* 2013) were crossed and allowed to spawn under natural conditions. Embryos were collected and incubated in E3 medium at 28.5 °C. At 48 hpf, embryos were sorted for the presence of both cardiac GFP and endothelial mCherry signal under a fluorescent stereoscope

(Olympus AZX7, Tokyo, Japan). Double transgenic embryos *Tg*(*cmlc2:GFPCaaX*, *kdrl:mcherry*) were reared under standard zebrafish rearing conditions (Nasiadka & Clark 2012) up until metamorphosis (25–30 dpf) and fed once a day a mix of artemia and Sera Micron dry food (Lisbon, Portugal).

In order to determine the most appropriate IOX and DES dosing, preliminary experiments were performed to determine the growth rate of the juvenile zebrafish for 4 weeks. Immediately after metamorphosis (25–30dpf) 20 juveniles were transferred to 8 L tanks filled with water from the zebrafish rearing facility (pH 7, 700 µS, 28 °C) (Technoplast, Rome, Italy). To this end, every week 5 fish were collected and sacrificed with a lethal dose of phenoxyethanol (>250 mg/L) (Carl Roth, Karlsruhe, Germany). Afterwards, it was determining the wet weight of each juvenile. The weight increment per week was calculated and used to adjust, weekly, the dose of IOX/DES provided via the experimental feed.

All experiments were carried out in accordance with Portuguese and EU law for animal experimentation and welfare.

3.3.2. Experimental feed preparation

Food was prepared on a weekly (7 days) basis using the results of the preliminary growth assay (Section 2.1) to establish the amount of food (g) and quantity of IOX or DES to be added. The experimental feed was given daily at 1.5 % of wet fish weight previously determined and provided a daily exposure dose of 0.1 mM IOX (Merck, Darmstadt, Germany; CAS-1689-83-4) or DES (Merck, Darmstadt, Germany; CAS-56-53-1) (the fish density/tank was ~1 g/L) per individual fish. The feed was prepared by spraying IOX or DES in 70 % ethanol over the food and dried in a fume hood for 24 h at room temperature in the dark, as previously described (Campinho *et al.* 2012). Control food was prepared the same way but only sprayed with the vehicle (70 %).

3.3.3. IOX and DES exposure

Sexually immature (~30 dpf) juvenile double transgenic *Tg*(*cmlc2:GFPCaaX*, *kdrl:mcherry*) zebrafish were used in experiments. Twenty juvenile fish were reared in individually isolated semi-closed system in 8 L tanks with zebrafish system water (pH 7, 700 µS, 28 °C). The water temperature of 28 °C is the optimum temperature for rearing zebrafish (Lawrence 2007; Matthews *et al.* 2002; Kimmel *et al.* 1995; Aleström *et al.* 2020). Fish were fed daily with 1.5 % (g feed/g fish wet weight) of experimental feed prepared as described

above. To ensure all the individuals ate the feed so that each fish was exposed to 0.1 μM IOX or DES, this was done twice daily. The exposure experiment was carried out for 60 days and was terminated before sexual maturation. At the beginning of each experimental day, solid debris was taken out and replenished with zebrafish system water to 8 L. Every two days, one quarter of the tank was renewed. The toxicity exposure procedure is based on modified OECD Test guidelines 215 and our previous experiments with these chemicals. Two identical independent assays were carried out at different times and using different broad stock to mitigate genetic differences.

At the end of the experiments, juveniles were sacrificed with a lethal dose of phenoxyethanol (>250 mg/mL) and samples were collected. For gene expression analysis, 10 fish (5 from each experiment) were snap frozen individually in dry ice and stored at −80 °C until analysis. For histological analysis, 5 fish, from each experiment were fixed separately overnight in 4 % PFA/1 × PBS at 4 °C and then rinsed in sterile water and transferred to 100 % methanol and maintained at −20 °C until use. After transfer to methanol, experimental animals from both independent assays were brought together for storage at −20 °C.

To determine growth curves of experimental fish, daily photos of each tank at a distance of 20 cm were taken to allow all experimental fish to be captured (Supplementary Figure 3.1 and 3.2 annex II). After image analysis, the total length of juvenile fish was determined in FIJI software (Schindelin *et al.* 2012).

It was not possible to determine the actual concentration of IOX and DES in the experimental animals, which is limitation of our study.

3.3.4. Immunofluorescence assays and determination of the heart ventricle volume

The heart ventricle volume was determined on whole hearts dissected from experimental zebrafish juveniles fixed in 4 % paraformaldehyde (PFA) and subject to immunofluorescence (IF). The whole heart was hydrated through a graded methanol series (100 % to water), then rinsed in phosphate-buffered saline (PBS) containing 0.5 %TritonX-100 (PBTr) and incubated with 1/1000 dilution of rabbit anti-GFP antiserum (Abcam, Eugene, Oregon, USA) and 1/200 mouse anti-mCherry serum (St. Johns Lab, London, UK). The detection of the primary antisera was carried out with goat anti-rabbit-CF488 and goat anti-mouse-CF594 secondary antibodies, respectively. After IF, hearts were cleared for 3 days in ScaleA solution (Hama *et al.* 2011) and transferred back to 1 × PBS and imaged using a Zeiss Z1 single plain-microscope (Jena, Germany). Images were fused and reconstructed on Zeiss Zen Blue software. FIJI Volumest

plugin (Merzin 2008) was used to determine the ventricle volume. The images were firstly cropped to select the heart ventricle. The 3D volumes images were transformed to 8-bit. Afterwards, the images were inverted and the area of the heart ventricle in each slice (1 μm Z-step) was measured, and the sum of the total number of slices was used to determine the volume of the ventricle. Five hearts per treatment were randomly selected from experimental animals in the two assays.

3.3.5. Determination of thyroid morphology

Heads of five experimental fish per treatment, randomly selected from the two assays, were dissected PFA-fixed, processed and embedded in paraffin. Serial sagittal histological sections (8 μm) of the whole head were prepared with a rotary microtome and placed on 3-Aminopropyltriethoxysilane (APES; Merck)-coated glass slides. For IF assays, sections were dewaxed in xylene and rehydrated through an ethanol series (100 % to water) and washed in PBTr and IF was carried out using an antiserum specific for thyroglobulin (1/1000, Dako, Copenhagen, Denmark). A 1/30000 dilution of 4′,6-diamidino-2-phenylindole (DAPI, Carl Roth, Karlsruhe, Germany) labeled cell nuclei. After IF, the sections were mounted in glycerol, covered with a glass coverslip and stored at 4 °C until imaging. Imaging was carried out on a Zeiss Z2 up right epifluorescence microscope. Composite images were prepared in FIJI, and thyroid follicle number and diameter were calculated. From this, it was calculated the total area of follicles over the total length of fish, i.e., the sum of the area of all follicles imaged per slide. The extension of the thyroid field over the total length of fish, i.e., the length occupied by thyroid follicles from the most anterior to the most posterior follicle, was also measured in FIJI. All slides used for IF were selected to include the vomer bone (located along the midline of the body) and enabled all measurements to be carried out in similar regions of each experimental fish.

To determine thyroid follicular activity, thyrocyte height was determined as previously described (Campinho *et al.* 2006) using a direct method (Kalisnik *et al.* 1977). To this end, adjacent histological slices to the ones used for immunofluorescence were dewaxed in xylene and rehydrated through an ethanol series (100 % to 50 %) and then washed in water. For haematoxylin and eosin (H&E), staining tissue sections were immersed in Harris haematoxylin (Carl Roth, Karlsruhe, Germany) for 30 s, washed in tap water and then immersed in eosin Y (Carl Roth, Karlsruhe, Germany) for 30 s and washed with distilled water. After the staining procedure, permanent histological preparations were obtained by rapidly dehydrating through

an ethanol series (from 50 % to 100 %), clearing in xylene and mounting in DPX (Carl Roth, Karlsruhe, Germany). Images were taken in a Leica DM2000 bright field microscope coupled to a DFC480 digital camera (Leica, Jena, Germany). Thyrocyte height was measured in FIJI using the line tool. Cell height of four different thyrocytes per follicle, lying 90° from one another, was measured in five to six different follicles per animal (N = 3/treatment) and analysed.

3.3.6. Real-time quantitative polymerase chain reaction analysis

Total RNA from individual juvenile zebrafish (CTR: n = 7; IOX: n = 5; DES: n = 4) was isolated with an Omega Total RNA extraction kit (Omega Bio-Tek, Inc., Norcross, GA, USA) following the manufacturer's instructions. The quality of total RNA was assessed by gel electrophoresis and spectrophotometry. Only the RNA samples that presented clear 18S and 28S bands with a 1:2 ratio were used. The total RNA was treated with a TURBO DNA-free™ kit (Ambion, Foster City, CA, USA) as recommended by the manufacturer and 500 ng DNA free total RNA was used for cDNA synthesis with random hexamer primers (50 ng/µL) and a RevertAid kit (Thermo Scientific, Foster City, CA, USA).

Endothelial and cardiac-specific genes previously affected in zebrafish embryos by IOX and DES (Li *et al.* 2019) were analysed by qPCR using primers previously described (Table 3.1). To further address the specific effect of IOX and DES treatment on thyroid function, thyroglobulin (*tg*) expression was also determined (Table 3.1). The qPCR assay was carried out in a 10 µL reaction volume containing 1 µL template cDNA (around 200 ng), 300 µM of each primer (Table 3.1), 5 µL of 2 × SsoFast EvaGreen supermix (Bio-Rad, Hercules, CA, USA) and 3.4 µL sterile MilliQ water. The qPCR analysis was performed in a CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA) with two technical replicates per sample. Relative mRNA expression was determined using absolute quantification with serial dilutions of the target amplicon as the standard curve. The thermocycling consisted of an initial denaturation step for 3 min at 95 °C followed by 45 cycles of 10 s at 95 °C and 15 s at 60 °C. A melting curve analysis with a temperature gradient of 0.5 °C/s from 65 °C to 95 °C was carried out to confirm that a single amplified product was obtained. *18s* (Li *et al.* 2019) and *ef1a* (Rocha *et al.* 2015) were used as the reference gene for normalization, which did not vary significantly between the treatment's groups analysed. The copy number of target genes was calculated from the calculated standard curve. For normalization of expression of analysed genes, the ratios for the target genes were calculated by dividing by the geometric mean from of copy number of the reference genes (*18s* and *ef1a*).

Table 3.1. Primers used for qRT-PCR expression analysis of 18 selected genes, *18s* and *ef1α*.

Target	Primer	Sequence (5'-3')	Amplicon size (nt)	Efficiency (%)	r^2	Accession number
acvr1bb (activin receptor type-1B-like)	FW RV	ACGCTCTGGGTCTGGTGTACTG TCTATGGAGGGATCTGACGGCAC	112	98.7	0.997	ENSDARG00000052142
adrb1 (β1-adrenergic receptor)	FW RV	TCGTGGGCATGGGAATCCTCAT GTGAGCGTCTGGAGCCTCTGAT	106	96.1	0.999	ENSDARG00000007490
agtr1a (angiotensin II type I receptor)	FW RV	CGACTCCAACACGGGACTTGC AGATGACGGCGACTACCAGACTG	137	104.7	0.995	ENSDARG00000018616
angptl1b (angiopoietin-related protein 2)	FW RV	GACAGTTCACCACGCTGGACAG TGAGTACCACACGCCGTTCAGA	122	93.4	0.995	ENSDARG00000100159
cacna1da (voltage-dependent L-type calcium channel subunit alpha-1D)	FW RV	CAATGAGCAGCAAGCCGAGGTAA AGCCTTTGTGGAGGGATATGCCA	158	100.1	0.998	ENSDARG00000102773
cacna1ha (voltage-dependent calcium channel subunit alpha-1H-like)	FW RV	CCTTCAGAGACGCAATGCCTCAG GCCAACTTGATCGCAGCATCCA	182	97	0.997	ENSDARG00000060496
cacng2b (calcium channel, voltage-dependent, gamma subunit 2b)	FW RV	CTCTGGTGGAATGCGGCAGTATG GCTGGTCTCGTTCTCGTTGGTG	173	97.6	0.997	ENSDARG00000102376
cadm1a (cell adhesion molecule 1a isoform 3)	FW RV	GCCTCAGCAGGTGAACTGGGTA GACGCCACGCATCGGTAAGTG	126	101.4	0.997	ENSDARG00000031075
calcr (calcitonin receptor)	FW RV	TCGCAGAGGAGCAGCACCTAC AGCAGATGCGTCTCCACACTCA	136	93.5	0.999	ENSDARG00000028845
chrnb3a (cholinergic receptor, nicotinic, beta polypeptide 3a precursor)	FW RV	CCTTCACGCCAACGACACGATTACT TCCACTCCTCCCACAACCAGACATT	116	103.6	0.996	ENSDARG00000052764
hif1aa (hypoxia inducible factor)	FW RV	GCTCAGAGAAATGCTGGCACACA CAGCACCTTCCAGGAGGCAGA	148	108.6	0.991	ENSDARG00000006181
mhc1lia (hereditary hemochromatosis)	FW RV	TGATGGCGAACCAGGACGAATGA CACATGGGTCGAGACGTTGAAGC	121	96.9	0.999	ENSDARG00000097766
mybpc2a (myosin binding protein C, fast type a)	FW RV	TATCCAAGAACCGCCTGTCACCA TTCCATCTCGGCTCAGTTCCACA	149	95.3	0.996	ENSDARG00000030157
notch1b	FW RV	TTGACGACTGCACACCGTTCAC GGTTGGACAGGCACTCGTTGAC	159	94.8	0.998	ENSDARG00000052094
ptgir (prostacyclin receptor-like)	FW RV	AGTGATGGACTCTGCCTGGACAG GCCTCGATGCTGGTGATGTTCTC	169	94.5	0.998	ENSDARG00000038278
itpr1b (inositol 1,4,5-trisphosphate receptor, type 1b)	FW RV	CGGTGGAGGAGTCGGAGATGTG GCTTCTGAGGTCTGCGAAGGTATC	160	95.6	0.999	ENSDARG00000074149
vwf (von Willebrand factor)	FW RV	GCGTGGAGGCATTCGGCAACT CGGCAGTTCTTCACATACGGCTCAG	193	96.8	0.999	ENSDARG00000077231
tg (thyroglobulin)	FW RV	GCGAGACCTGCTGTGATGGATTC GATGCGGCTGGAAGAGGAAAGAC	146	88	0.993	XM_689200.4
18s	FW RV	GGAATTGACGGAAGGGCACCAC GCACCACCACCCACAGAATCG	135	103.8	0.995	ENSDARG00000089382
ef1a (Rocha et al, 2015)	FW RV	TTGAGAAGAAAATCGGTGGTGCTG GGAACGGTGTGATTGAGGGAAATTC	99	94.3	1.00	NM_131263.1

3.3.7 Statistical analysis

GraphPad Prism 6 software was used for statistical data analysis. An unpaired Student's t-test was used to infer the statistical significance of differences in the ventricle volume, thyroid follicle number, thyroid field length, thyroid follicle area, thyrocyte cell height and qPCR gene expression data between control fish and IOX or DES siblings. Statistical significance was considered at $p < 0.05$.

3.4. Results

3.4.1. Effect of IOX and DES on the ventricle morphology

Volumetric analysis of the heart assessed after immunostaining against GFP (cardiomyocytes, red) and mCherry (endothelial cells, green) and light-sheet whole heart fluorescent microscopy revealed that exposure of juvenile zebrafish for 60 days to IOX significantly increased the volume of the ventricle relative to the control group (Figure 3.1A; $p < 0.001$). The ventricle of IOX-treated juveniles was deformed compared to the ventricle of control juveniles (Figure 3.1B, Supplementary Movies 3.1 and 3.2 annex II).

DES exposure did not significantly change the volume of the ventricle relative to control juveniles (Figure 3.2A; $p > 0.05$). However, the ventricle of DES-treated animals presented malformations, given that both the external surface of the ventricle and atrium had some obvious irregularities that were not found in control juvenile fish (Figure 3.2B, Supplementary Movies 3.1 and 3.3 annex II).

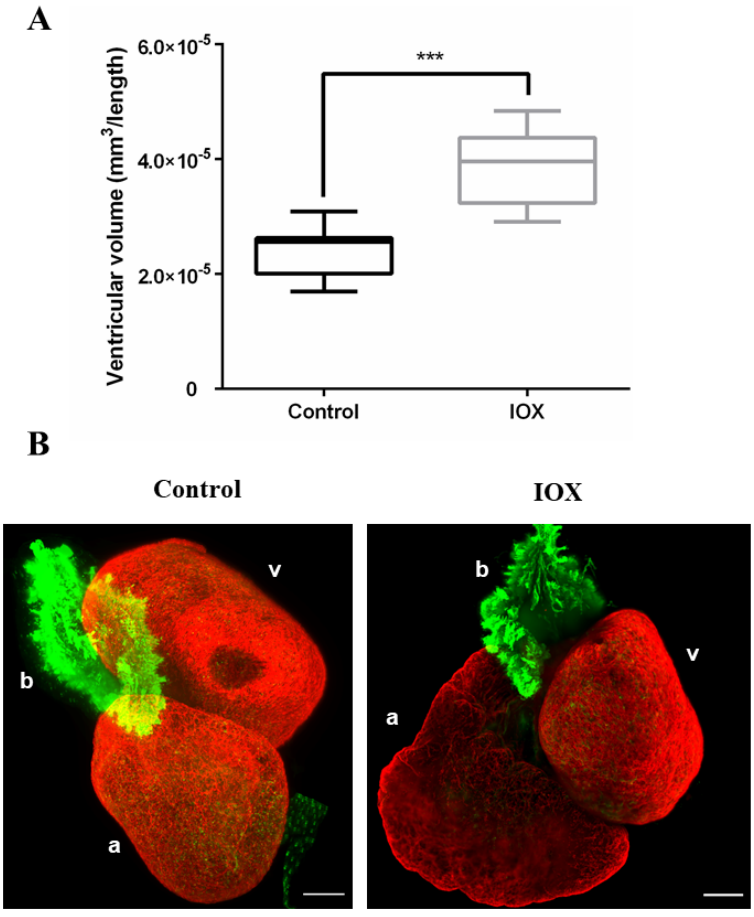

Figure 3.1. Exposure of zebrafish *Tg*(*cmlc2:GFPCaaX*, *kdrl:mcherry*) juveniles (~ 90 dpf) for 60 days to IOX increases ventricle volume and alters heart morphology. (A) Box and whisker plot (minimum and

maximum) of total ventricle volume and (B) maximum projections of whole-heart, after immunostaining for GFP (cardiomyocytes, red) and mCherry (endothelial cells, green) and imaging by light-sheet fluorescent microscopy, for control (CTR, n=5) and IOX (n=5) treated animals. A Student's t-test was used to test for statistical significance, *** $p < 0.001$. Scale bars in figure B correspond to 100 µm. a – atrium, v – ventricle, b – bulbus arteriosus.

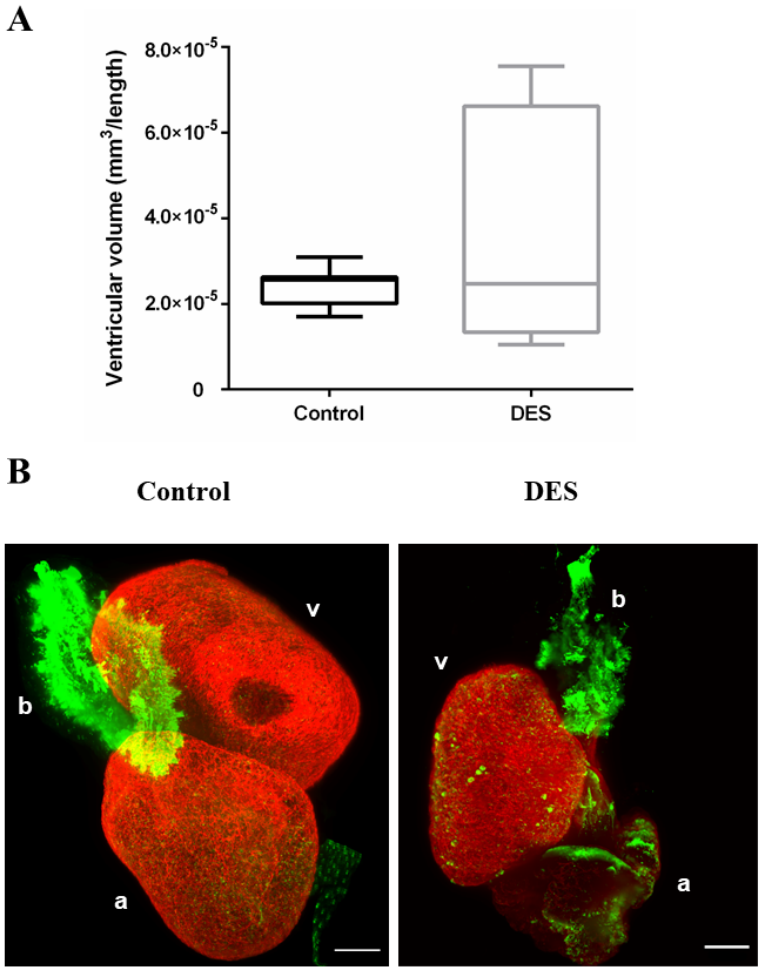

Figure 3.2. Exposure of zebrafish *Tg*(*cmlc2:GFPCaaX*, *kdrl:mcherry*) juveniles (~ 90 dpf) for 60 days to DES does not affect ventricle volume but alters heart morphology. (A) Box and whisker plot (minimum and maximum) of total ventricle volume and (B) maximum projections of whole-heart, after immunostaining for GFP (cardiomyocytes, red) and mCherry (endothelial cells, green) and imaging by light-sheet fluorescent microscopy, for control (CTR, n=5) and DES (n=5) treated animals. A Student's t-test was used to test for statistical significance, $p > 0.05$. Scale bars in B correspond to 100 µm. a – atrium, v – ventricle, b – bulbus arteriosus.

3.4.2. IOX and DES affect the thyroid and gene expression profiles

The effects of IOX and DES on thyroid morphology were analysed after immunostaining for Tg. The staining of Tg with this antiserum only identifies intact protein, indirectly indicative of unprocessed protein. Strong Tg immunofluorescence was observed in the thyroid follicular lumen in control, IOX and DES-treated juvenile zebrafish (Figure 3.3A, Figure 3.4A). In IOX-

treated zebrafish, the thyroid follicles had a modified morphology with enlarged follicles and strong Tg immunofluorescence in the lumen compared to the control group (Figure 3.3A). Follicle Tg staining shows that the follicle lumen is not all occupied by Tg, likely reflecting processed protein into thyroid hormones. The lower stained area in control likely reflects the higher activity of the follicles than in IOX treated animals. Further analysis of thyrocyte follicular activity by cell height analysis further indicated that IOX treated fish presented less active thyrocytes ($p = 0.0048$, Supplementary Figure 3.3A annex II). The thyroid follicle number and field length relative to fish length was not significantly changed in IOX-treated zebrafish compared to the control (Figure 3.3B; $p > 0.05$). However, the follicular area relative to the fish length was significantly increased (Figure 3.3B; $p < 0.05$).

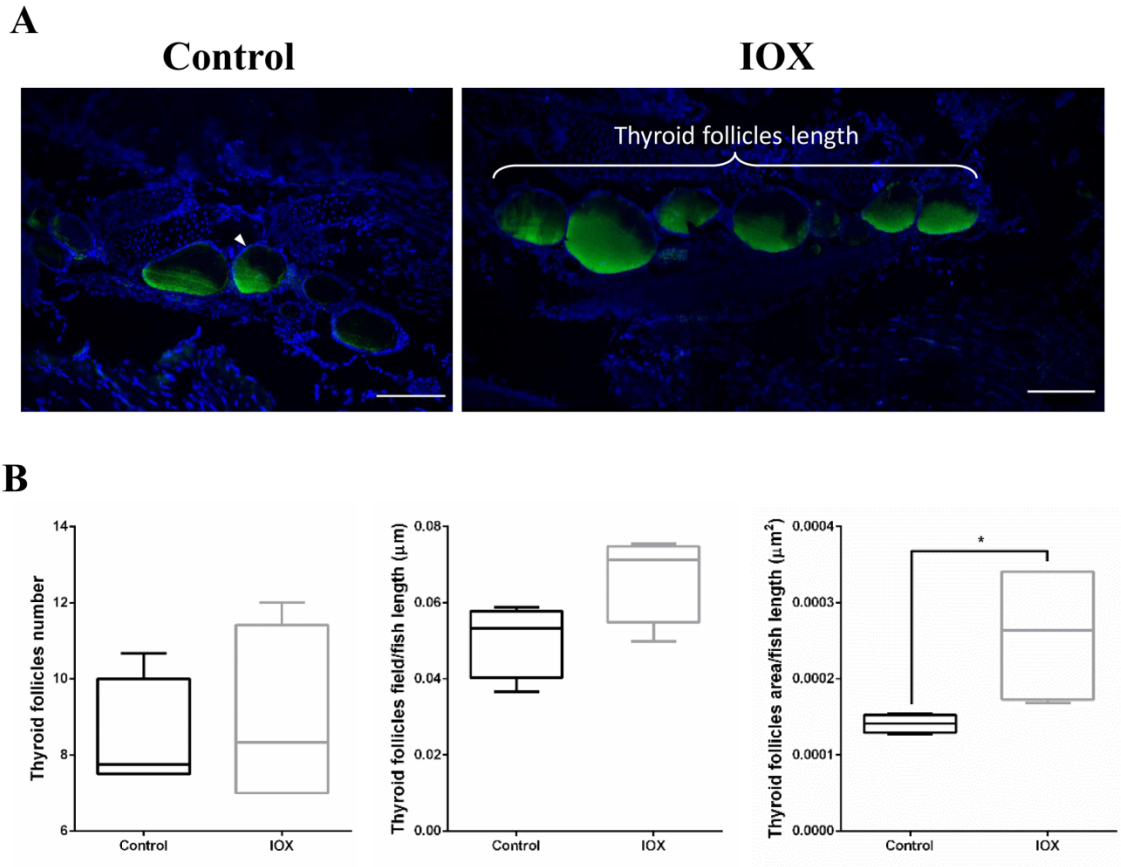

Figure 3.3. Exposure to IOX affects the thyroid follicles' morphology. (A) Tg immunofluorescent detection (green) in the colloid of the thyroid follicle lumen in the pharyngeal region of juvenile zebrafish (*cmlc2:GFPCaaX*, *kdrl:mcherry*) after exposure to IOX for 60 days. Sections were counterstained with DAPI (blue) and the nuclei of the thyrocytes surrounding the follicle lumen are clearly visible. Note that in some follicles of both the control and IOX-treated zebrafish the lumen had little to none Tg immunofluorescent. Enlarged thyroid follicles were consistently found in IOX treated juveniles. (B) Measurement of thyroid follicle number, thyroid follicle field relative to the fish length and thyroid follicle area relative to fish length. Significant differences between the control and IOX-treated zebrafish (n=5/experimental group) were assessed with a Student's t-test with significance at $p < 0.05$ (*). The arrowhead denotes a thyroid follicle. The thyroid follicle field was defined as the distance between the most anterior and the most posterior thyroid

follicles. The thyroid follicle area was defined as the sum area of all thyroid follicles. Scale bars in each figure correspond to 100 μm.

In zebrafish treated with DES, the histological appearance of the thyroid follicle primordium was similar to the control fish, although the thyroid follicle field length relative to fish length increased compared to the control group (Figure 3.4B). The thyroid follicle field relative to fish length in the DES -exposed group was significantly higher than in the control group (Figure 3.4B; $p < 0.05$). DES exposure did not significantly change the thyroid follicles area relative to fish length or the thyroid follicles number (Figure 3.4B; $p > 0.05$), suggesting no differences in thyroid activity. Further analysis of thyrocyte follicular activity by cell height analysis further supports no differences in thyrocytes activity between control, and DES-treated fish ($p > 0.05$, Supplementary Figure 3.3B annex II).

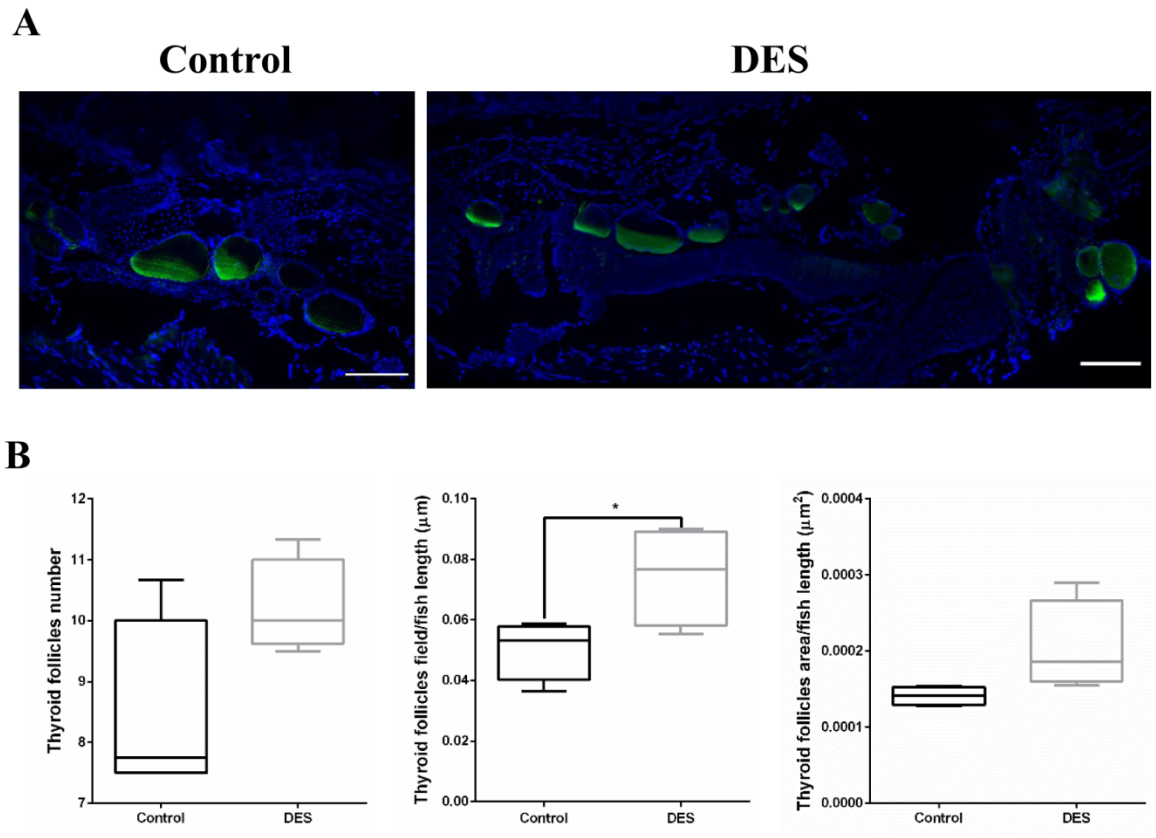

Figure 3.4. Exposure to DES affects thyroid follicles' morphology. (A) TG immunofluorescent detection (green) in the colloid of the thyroid follicle lumen in the pharyngeal region of juvenile zebrafish (*cmlc2:GFPCaaX*, *kdrl:mcherry*) after exposure to DES for 60 days. Sections were counterstained with DAPI (blue) and the nuclei of the thyrocytes surrounding the follicle lumen are clearly visible. Note that in some follicles of both the control and DES-treated zebrafish the lumen did not contain immunofluorescent TG. (B) Measurement of thyroid follicle number, thyroid follicle field relative to the fish length and thyroid

follicle area relative to fish length. Significant differences between the control and DES-treated zebrafish (n=5/experimental group) were assessed with a student's t-test with significance at * $p < 0.05$. The thyroid follicle field was defined as the distance between the most anterior and the most posterior thyroid follicles. The thyroid follicle area was defined as the sum area of all thyroid follicles. Scale bars in each figure correspond to 100 μm.

3.4.3. Gene expression analysis

Eighteen candidate genes related to cardiovascular function and calcium homeostasis were analysed to identify the effect of IOX and DES on gene expression profiles (Figure 3.5). qPCR analysis showed that vascular related angiopoietin-related protein 2 (*angptl1b*), myosin binding protein C fast type a (*mybpc2a*), notch receptor 1b (*notch1b*), prostaglandin I2 receptor (*ptgir*) and von Willebrand factor (*vwf*) gene expression was significantly up-regulated in DES-exposed zebrafish (Figure 3.5B, $p < 0.05$) compared to the control. The expression of these genes was not found to be different from control and IOX-treated fish (Figure 3.5A, $p > 0.05$). However, the expression of the Major Histocompatibility Complex 1lia (*mhc1lia*) gene, related to hereditary hemochromatosis (Dirscherl *et al.* 2014), was significantly up-regulated in IOX-treated fish (Figure 3.5A, $p < 0.05$) but not in DES-treated (Figure 3.5B, $p > 0.05$). No significant differences in expression were found for the other genes analysed between the control and IOX or DES exposed zebrafish ($p > 0.05$; Supplementary Figure 3.4 annex II).

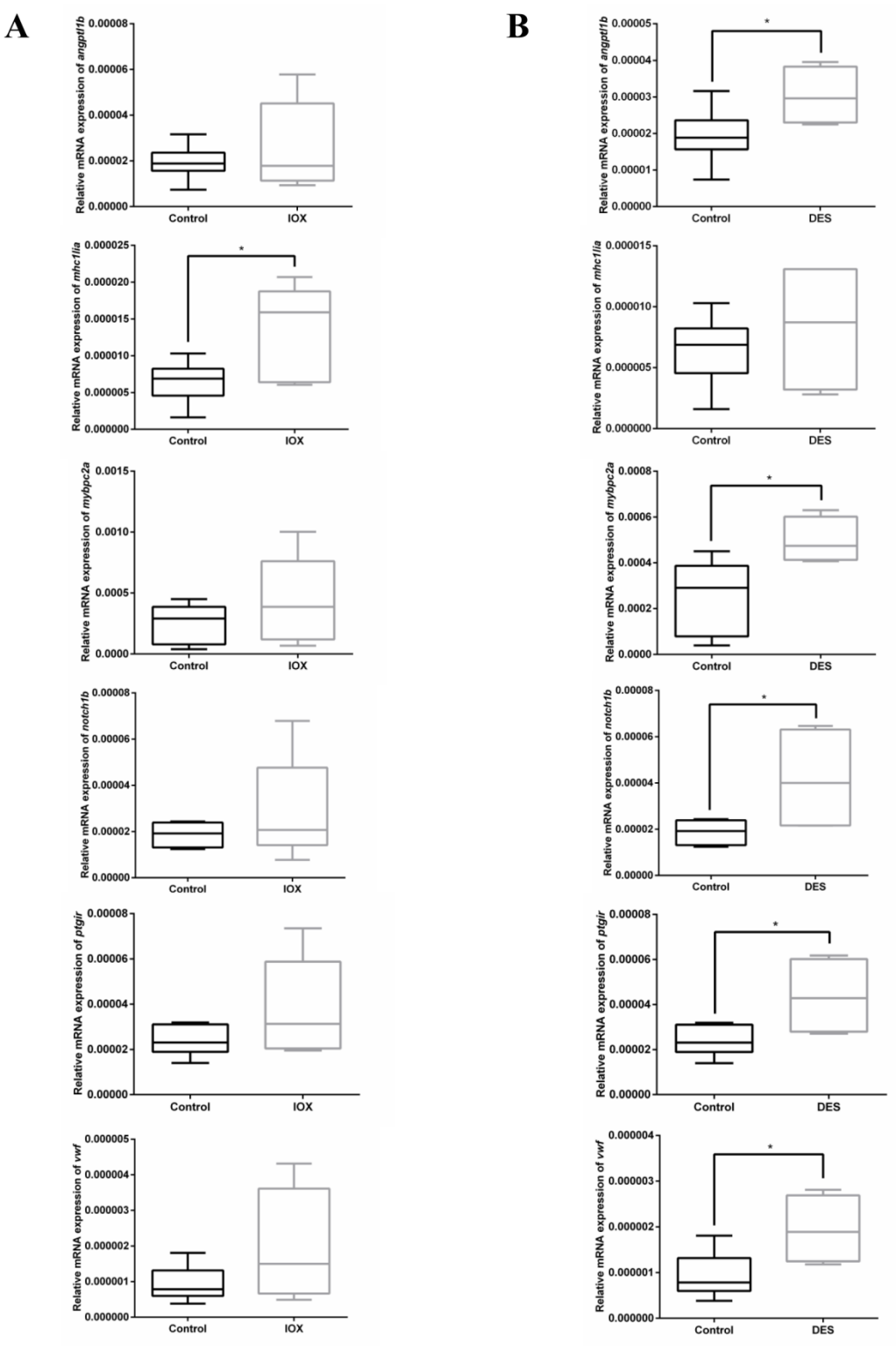

Figure 3.5. qPCR analysis of genes involved in endothelial function (Li *et al.* 2019), *angptl1b*, *mhc1lia*, *mybpc2a*, *notch1b*, *ptgir* and *vwf*, in IOX-treated (A), DES-treated (B) and control juvenile zebrafish. *angptl1b*: angiopoietin-related protein 1b; *mhc1lia*: hereditary hemochromatosis; *mybpc2a*: myosin binding protein C, fast type a; *notch1b*: notch receptor 1b; *ptgir*: prostacyclin receptor-like; *vwf*: von Willebrand factor. A Student's t-test was used to assess if statistically significant difference in gene expression occurred. The biological replicates were as follows: Control: n = 7; IOX: n = 5; DES: n = 4. Significant differences are denoted with * for $p < 0.05$.

3.4. Discussion

Our previous work demonstrated that zebrafish embryos exposed to waterborne 0.1 μM DES and IOX presented impaired cardiovascular system and thyroid development (Campinho & Power 2013; Li *et al.* 2019). Our data revealed that chronic exposure to DES and IOX leads, in a chemical specific-manner, to diverse consequences ranging from cardiac ventricle alterations, up-regulated expression of vascular genes and alterations in thyroid tissue homeostasis in juvenile zebrafish. This work showed that even after the heart and thyroid tissue are fully developed in juvenile zebrafish, exposure to IOX and DES can lead to cardiac and thyroid changes similar to those observed in zebrafish embryos. The evidence presented strongly suggests an indirect endocrine disruption action, via altered cardio-vascular function and homeostasis, of IOX and DES on the thyroid system. It may suggest that cardiac and thyroid are the targets for such a low-level concentration of IOX and DES (0.1 μM) regardless of different life stages.

DES and IOX, like other EDCs, have the potential to interact with the thyroid axis and thyroid cellular signalling and directly disrupt thyroid homeostasis in marine teleosts (Morgado *et al.* 2009; Morgado *et al.* 2007). In zebrafish embryos, exposure to DES and IOX (0.1 μM) impaired zebrafish thyroid and cardiovascular development. Our previous study revealed impaired heart development and function by injection of a morpholino (MO) against zebrafish cardiac troponin I, which stops heart contraction and later cardiac development, and inhibited thyroid morphogenesis, indicating thyroid development is strongly associated with normal heart development and function (Campinho & Power 2013). Notably, our previous studies demonstrate that IOX and DES do not appear to impair thyroid development directly but induce it through altered cardiac and vascular tissue development (Campinho & Power 2013; Li *et al.* 2019). The observations of our previous studies of IOX and DES potential EDC action corroborate studies demonstrating that zebrafish thyroid development is strictly dependent on the correct heart and vascular, most notably ventral aorta, development and function (Alt *et al.* 2006b; Opitz *et al.* 2012; Wendl *et al.* 2002).

The present study aimed to determine in juvenile zebrafish with fully developed cardiovascular and thyroid if 0.1 μM IOX and DES impaired zebrafish heart and thyroid morphology after 60 days of chronic exposure via food. We used previously established morphological indexes to access the heart (Campinho & Power 2013; Li *et al.* 2019) and thyroid function (Pinto *et al.* 2013; Campinho *et al.* 2012; Morgado *et al.* 2009; Campinho *et al.* 2006). The results revealed that as occurred in embryos, DES and IOX affected the heart morphology

and likely function, given that both indexes modulate each other. 3D morphometrics of the juvenile zebrafish heart revealed that the ventricular volume was significantly increased in IOX-treated juveniles, suggesting that IOX had a more pronounced effect on heart morphology than DES. However, endothelial genes differentially expressed between IOX, and control embryos were not significantly different in juvenile zebrafish, suggesting that the effect on cardiac morphology might be a direct action on cardiomyocytes (Figure 3.5). However, this can also reflect the different experimental approaches of the two studies since in embryos, endothelial cells were first isolated and RNA-seq carried only in this cell population (Li *et al.* 2019), whereas here, it was used whole-animals. Nonetheless, the change in ventricle morphology and size observed in IOX treated animals indicates that cardiomyocytes and cardiac endothelial cells were affected. In fact, in cultured human neuroblastoma cells, IOX disrupted gap junction formation by affecting connexin 43 and hence modified cell adhesion (Vicario *et al.* 2017). Therefore, it seems probable that some of the effects of IOX on cardiac morphology and function observed in juvenile zebrafish (present study) and embryos (Campinho & Power 2013; Li *et al.* 2019) may be due to effects of IOX on the cytoskeleton and cell adhesion. Different genes involved in cell adhesion, such as cell adhesion molecule 1b precursor, cell adhesion molecule 1a isoform 3 and platelet endothelial cell adhesion molecule-like isoform X1, were found to be expressed in endothelial cells and significantly up regulated in IOX treated embryos (Li *et al.* 2019). The finding that IOX up regulated *mhc1lia* might also indicate that in juvenile fish IOX might impact the immune system (Dirscherl *et al.* 2014) (Figure 3.5A). That could not be evaluated in embryos since, at this stage of development, the zebrafish does not have a fully developed immune system.

The effect of DES on heart morphology was subtle, and it increased the variability of the ventricular heart volume in exposed juveniles compared to control siblings. Although 0.1 μM of DES did not overtly change ventricle volume, it did change ventricular morphology, and in previous studies of zebrafish embryos exposed to the high concentration of DES (30-fold higher), a significant incidence of heart defects and pericardial oedema occurred (Adam *et al.* 2021), which is in line with our previous observations (Campinho & Power 2013; Li *et al.* 2019). Previous studies have found that the effect of DES on development/physiology may be related to estrogen receptor interactions (Adam *et al.* 2021). Furthermore, high doses of DES (25–100 μM) are reported to have anti-vascular and anti-angiogenic activity by impairing VEGF signaling, promoting a proinflammatory response mediated by cytokines and enhancing YAP/Mst1-FOXO3A signaling (Zhang & Shi 2020). The qPCR results in the present study

reveal differential expression of endothelial-specific genes in DES-treated fish, thus suggesting that endothelial cells are targets for DES during embryonic development (Li *et al.* 2019) and in juveniles. Only five of the candidate genes selected were responsive in juvenile zebrafish exposed to DES for 60 days, *angptl1b*, *mybpc2a*, *notch1b*, *ptgir* and *vwf* (Figure 3.5). This contrasts with the results of embryonic exposure to DES, which did not affect the expression of *angptl1b* in zebrafish embryos (Li *et al.* 2019). Angiopoietins are endothelial cell-specific growth factors involved in the maturation, quiescence, and remodeling of blood vessels by binding to the receptor TIE2 (Tyr kinase with Ig and EGF homology domains) and mediate blood vessel enlargement (Augustin *et al.* 2009; Girling & Rogers 2009; Thurston *et al.* 2005). In mice, angiopoietin-1 expression was down-regulated in neonates after exposure to DES (4 µg injection) *in utero* (Yamashita *et al.* 2013). Angiopoietins are essential for vascular homeostasis (Chislock *et al.* 2013). The up regulation of *angptl1b* after 60 days of exposure to DES in juveniles strongly indicates that DES affects vasculature homeostasis. This conclusion is further supported by the observation that myosin-binding protein C, a component of myosin filaments known to regulate muscle contraction (Merkulov *et al.* 2012; Mun *et al.* 2014) and one of the most common causes of familiar hypertrophic cardiomyopathies (Cheng *et al.* 2013) is also up-regulated. Moreover, *ptgir*, a potent vasodilator (Beaulieu & Freedman 2013) expressed in the zebrafish adult cardiovascular system, was also up regulated, suggesting that DES changes cardiovascular homeostasis. Taken together, the evidence suggests that endothelial cells, after long-term DES exposure, act to balance the effect of the chemical to preserve vascular function and homeostasis. The fact that the *vWF* gene, a multimeric adhesive plasma protein released by endothelial cells in the sites of vascular injury (Zou *et al.* 2019; Favaloro 2020), is also up regulated further strengths our conclusion that endothelial cells are a key target of DES and that the action of the chemical seriously affects vascular function and integrity. Moreover, *notch1b* expression up regulation (Figure 3.5B) further strengthens our conclusion that DES directly targets endothelial cells. In zebrafish, cardiac development notch1b signalling is responsible for luminal endocardium development in response to mechanical cues, and ectopic notch1b signalling leads to impair heart valve formation (Fontana *et al.* 2020). Notably, none of these genes are affected in zebrafish embryos exposed in the short-term to DES (Li *et al.* 2019). The outcomes of the present and previous studies suggest that DES induced cardiovascular defects are associated with prenatal exposure to DES and increased risk of cardiovascular defects such as heart murmur, pulmonic stenosis, atrial and ventricular septal in humans (Titus-Ernstoff *et al.* 2010; Tournaire *et al.* 2016).

The results of the present study, using previously established morphological indexes that indirectly assess thyroid homeostasis (Campinho *et al.* 2006; Campinho *et al.* 2012; Morgado *et al.* 2009; Pinto *et al.* 2013), revealed that thyroid homeostasis responded differently to IOX and DES exposure in juveniles. The enlarged follicles containing more Tg staining in IOX exposed zebrafish likely reflect thyroid hypertrophy after long-term exposure (Figure 3.3), which is in line with previous results in marine teleost exposed to IOX for 21-days (Morgado *et al.* 2009). Moreover, the significant increase in thyroid follicle area in IOX treated fish (Figure 3.3B) correlates with significant decreased thyrocyte height (Fig. S3A), strongly suggesting that Tg processing into thyroid hormones is likely impaired. Thus, the present results seem to point out that even though there are no direct signs of hyperthyroidism (as *tg* expression is maintained, Supplementary Figure 3.4 annex II), this can also signify impaired T_4 secretion due to impaired Tg mobilization from the lumen of the follicle, thus giving rise to increasing follicle area. A similar relationship between thyroid morphology and T_4 levels was previously demonstrated in the marine teleost *Sparus aurata* (Morgado *et al.* 2009; Campinho *et al.* 2012; Campinho *et al.* 2006). A similar situation was found in sea bream, where IOX exposure not only seemed to increase the number of follicles with Tg but also it was observed a slight decrease, in comparison to control, of serum T_4 levels (Morgado *et al.* 2009), arguing that the animals as they are exposed tend to become hypothyroid. However, IOX exposure (10 ppm for 2 years) caused hyperthyroidism and thyroid tumours in rats (Commission 2004; European-Commission 2004). These differences might reflect species specific differences in response to IOX, developmental age and or effects of the different vehicles or methods used for exposure to IOX. In fact, whereas in juvenile zebrafish we observe an increase in heart ventricle volume (present study) the opposite is observed in zebrafish embryos at 48 hpf (Li *et al.* 2019) after exposure to IOX. These observations strongly argue that IOX has different effects on the cardiovascular system and by inheritance on thyroid organogenesis and the HPT-axis homeostasis, dependent on the life-stage of the exposed organisms, as well as the duration of exposure. However, it cannot be discarded that IOX might target some components of the thyroid cellular signaling pathway. In *Xenopus laevis*, XL58 cell line exposure to IOX decreased the expression of thyroid receptor beta (*thrb*) (Otsuka *et al.* 2014). In fact, in the marine teleost sea bream, exposure to IOX for 21 days also decreased *thrb* expression, although this is likely a systemic response (Morgado *et al.* 2009).

A notably extended thyroid field was found in DES-treated juvenile zebrafish, indicating hypertrophy of the thyroid tissue (Figure 3.4B). However, neither *tg* expression (Supplementary

Figure 3.4B annex II) nor thyrocyte height (Supplementary Figure 3.3B annex II) are significantly affected in DES-treated fish compared to control, suggesting that for this level of exposure to DES, the thyroid endocrine system can accommodate the effect of the chemical. This is contrary to our previous study showing a significant decrease in the thyroid field in DES-treated zebrafish embryos (Li *et al.* 2019). Nonetheless, in adult sea bream, it has also been reported that the thyroid tissue of DES-treated fish showed hyperstimulation and hypertrophy as well as reduced colloid content in the follicle lumen (Morgado *et al.* 2009). In other animals, such a situation is generally associated with hypothyroidism, and being human goitre is a common symptom (Ambad 2021). In the sea bream, DES-exposure, although not giving rise to complete hypothyroidism, decreased serum T_4 (Morgado *et al.* 2009). Together, these observations indicate that exposure to DES has different thyroid gland (and hence HPT-axis) endpoints and that the life stage of exposure are important factors in the action of DES in teleosts and likely in other vertebrates. It will be interesting to understand further which thyroid gland developmental/differentiation/physiologic mechanisms are behind different life-stage responsivity to DES. IOX and DES-induced hypertrophy and over-growth of the thyroid tissue may indicate compensatory negative feedback, which might affect the development or function of the thyroid as well as thyroid-stimulating hormone secretion from the pituitary. Taken together, IOX and DES, although indirectly, seem to act as EDCs on the thyroid gland and hence in the HPT-axis.

3.6. Conclusion

Our data demonstrated that long-term exposure to low micromolar levels of IOX and DES can disrupt cardiac, vascular and thyroid homeostasis in juvenile zebrafish. Nonetheless, taken together with our previous studies (Campinho & Power 2013; Li *et al.* 2019), there is no question that the heart, vasculature and thyroid are highly susceptible to the action of IOX and DES even in such low-level concentrations. Notably, the increased risks of cardiovascular defects in the prenatally exposed human offspring bring to our attention that the cardiovascular system is a target of IOX Our findings suggest that low micromolar levels of waterborne IOX and DES are capable and DES. The cellular and molecular mechanisms by which the effects of IOX and DES are not only based on the direct effects on cardiac and vascular tissues but also on the interdependence of different tissues' development and function, as is the case of the thyroid (Li *et al.* 2019; Campinho & Power 2013; Alt *et al.* 2006b). This interdependence is the potential reason behind the effects of these chemicals on the thyroid homeostasis, thus

presenting EDC capacity. Overall, both IOX and DES present life-stage dependent effects. The potential endpoints disrupting the thyroid, and likely HPT-axis function thus translate into the endocrine disruptive potential of these chemicals. Nonetheless, these results do not entirely exclude the possibility that other components of the thyroid endocrine system (deiodinases expression and activity, thyroid receptors, pituitary response) can be affected by IOX and DES, neither that other organ systems are being affected and this should be further explored in the future. None withstanding, our data argue that there are no safe IOX and DES exposure levels.

CHAPTER 4

Ioxynil and diethylstilbestrol impair cardiac performance and shell growth in the mussel *Mytilus coruscus*

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4.1. Abstract

The herbicide IOX and the synthetic estrogen DES are environmentally relevant contaminants that act as EDCs and have recently been shown to be cardiovascular disruptors in vertebrates. Mussels, *Mytilus coruscus*, were exposed to low doses of IOX (0.37, 0.037 and 0.0037 mg/L) and DES (0.27, 0.027 and 0.0027 mg/L) via the water and the effect monitored by generating whole animal transcriptomes and measuring cardiac performance and shell growth. One day after IOX (0.37 and 0.037 mg/L) and DES (0.27 and 0.027 mg/L) exposure heart rate frequency was decreased in both groups and 0.27 mg/L DES significantly reduced heart rate frequency with increasing time of exposure ($p < 0.05$) and no acclimatization occurred. The functional effects were coupled to significant differential expression of genes of the serotonergic synapse pathway and cardiac-related genes at 0.027 mg/L DES, which suggests that impaired heart function may be due to interference with neuroendocrine regulation and direct cardiac effect genes. Multiple genes related to detoxifying xenobiotic substances were up regulated and genes related to immune function were down regulated in the DES group (vs. control), indicating that detoxification processes were enhanced, and the immune response was depressed. In contrast, IOX had a minor disrupting effect at a molecular level. Of note was a significant suppression ($p < 0.05$) by DES of shell growth in juveniles and lower doses (< 0.0027 mg/L) had a more severe effect. The shell growth depression in 0.0027 mg/L DES-treated juveniles was not accompanied by abundant differential gene expression, suggesting that the effect of 0.0027 mg/L DES on shell growth may be direct. The results obtained in the present study reveal for the first time that IOX and DES may act as neuroendocrine disrupters with a broad spectrum of effects on cardiac performance and shell growth, and that DES exposure had a much more pronounced effect than IOX in a marine bivalve.

Keywords: Cardiovascular disruption; Diethylstilbestrol; Growth; Ioxynil; *Mytilus coruscus*; Neuroendocrine disruption

4.2. Introduction

EDCs are compounds that interfere with endocrine systems and alter their function with numerous adverse effects reported for animals and even ecosystems (Schug *et al.* 2016; Patisaul *et al.* 2018). EDCs are present in many manufactured materials with industrial, agricultural, and pharmaceutical applications (Yang *et al.* 2015) and large amounts of EDCs are accumulating in the environment. EDCs are globally ubiquitous contributing to endocrine related disorders and chronic disease risks in terrestrial and aquatic vertebrate species (Patisaul *et al.* 2018; Dang 2016). Even though the use of some chemicals has been banned, they persist in the environment due to their long half-life and remain a hazard to animals since they are in the food chain and air (Chan & Wong 2013; Lauretta *et al.* 2019). Most studies of EDCs have focused on vertebrates to determine how anthropogenic factors are affecting biodiversity and ecosystems and to give insight into the risk of environmental exposure for human health. The launch of the "one health" concept has raised awareness of the need for a more ample understanding of the impact of environmental EDCs on ecosystems including the ubiquitous and numerous invertebrates (Cuvillier-Hot & Lenoir 2020; Ford & LeBlanc 2020; Canesi *et al.* 2022).

Diethylstilbestrol (DES, is a synthetic estrogen) and ioxynil (IOX, an iodine-containing phenolic herbicide) are two chemicals still in use that are persistent in the environment (Otsuka *et al.* 2014; Lei *et al.* 2020). The documented effects of environmental exposure to DES or IOX in vertebrates are through direct endocrine disrupting effects, transgenerational effects and through indirect effects (Kioumourtzoglou *et al.* 2018; Tournaire *et al.* 2016; Perez *et al.* 2005). DES increases the risk of reproductive, neurodevelopmental and cardiovascular defects in humans (Newbold *et al.* 2006; Titus-Ernstoff *et al.* 2010; Schug *et al.* 2011). IOX disrupts the thyroid system in humans by competitively binding to TTR, a thyroxin binding protein (Ogilvie & Ramsden 1988). It induces thyroid tumours in rats (European-Commission, 2004) and has an epigenetic effect on the thyroid hormone receptor-mediated gene regulation cascade in the XL58 *Xenopus laevis* cell line (Otsuka *et al.* 2014). In zebrafish DES and IOX were recently shown to have thyroid disrupting actions and to modify cardiovascular function (Li *et al.* 2019).

Studies of the impact of DES and IOX, on aquatic invertebrates, which have important roles in ecosystem functioning, are urgent (Petersen *et al.* 2003; European-Commission 2004). Moreover, since the regulatory systems in invertebrates are less well studied the effects of vertebrate EDCs are difficult to predict. The likely consequences of environmental anthropogenic contaminants may be exacerbated by the feeding characteristics of invertebrates, and chemical bioaccumulation with associated adverse effects (Cuvillier-Hot & Lenoir 2020).

For example, in Chinese Rivers and oceans environmental concentrations of DES may be as high as 102 ng/L and 16.9 μg/L (approximately 0.1 μM) (Chen *et al.* 2009; Qu *et al.* 2012; Zhang *et al.* 2012a) and in some UK surface water IOX, a common component of pesticides, has been detected at 0.1 μg/L (Croll 1991). Furthermore, due to the low water solubility of IOX and DES and their octanol/water partition coefficient of log Pow >3 (3.4 for IOX and 5.07 for DES) (Linders *et al.* 1994; European-Commission 2004), they tend to bioaccumulate.

The effect of vertebrate EDCs on invertebrates and if DES and IOX have endocrine disrupting effects is poorly documented. An increasingly used approach to assess the ecotoxicological effects of marine contaminants in bivalves is to use cardiac performance tests (Curtis *et al.* 2000; Wedderburn *et al.* 2000; Bakhmet *et al.* 2009; Martinović *et al.* 2015; Bakhmet *et al.* 2021; de Carvalho *et al.* 2022), which give insight into shell opening, respiration rate, filtering activity and growth and health status (Andrewartha *et al.* 2015; Trueman 1967). Since cardiac function in bivalves is under neural regulation and involves various neurotransmitters, changes in cardiac function can give insight into potential neuroendocrine disruption in invertebrates (Paciotti & Higgins 1985; Park *et al.* 2004; Canesi *et al.* 2022). Specific examples of the effects of DES and IOX in invertebrates exist and include the freshwater mussel, *Anodonta cygnea*, where DES exposure altered membrane ion transport and the intracellular pH of the mantle with probable consequences for shell production (Alves & Oliveira 2013). Chronic exposure of *Daphnia magna* to 0.5 mg/L DES for 3 weeks significantly decreased moulting frequency and reduced fecundity of the second-generation (Baldwin *et al.* 1995). IOX repressed larval growth of the invertebrates *Lumbriculus variegatus* and *Chironomus riparius* and was toxic with LC_{50} values of 1.79 and 2.79 mg/L, respectively (Mäenpää *et al.* 2003).

The endocrine system in invertebrates differs substantially from that in humans and endocrine disrupting effects of DES and IOX exposure are poorly documented. Previously, we identified the presence of a thyroid receptor and iodothyronine deiodinase genes in the mussel *Mytilus coruscus* (Li *et al.* 2020b; Li *et al.* 2021; Shi *et al.* 2022). This suggests that the putative thyroid disruptors, DES and IOX, could potentially impact mussel physiology through actions on TRs and DIOs. Considering the essential role of invertebrates in ecosystem functioning, which includes their contribution to nutrient cycling, maintenance of water quality and habitat engineering, changes in their fitness and survival may have devastating environmental consequences (Chen 2021). For this reason, understanding how IOX and DES exert their actions on aquatic invertebrates is important as it can provide insight into the possible

consequences from a broader ecological perspective of these chemicals in the environment.

To increase understanding of the likely impact of environmental contamination by IOX and DES the mussel, *M. coruscus*, an endemic species that is important for aquaculture in the East China and Yellow Sea, was studied. Bivalve wild populations are frequently found in proximity to sewage plants where they have an increased incidence of gonadal regression, atresia, and malformations (Smolarz *et al.* 2017). Although estrogen receptor and thyroid receptor orthologues exist in molluscs their ligands have not been identified (Thornton *et al.* 2003; Matsumoto *et al.* 2007; Raingeard *et al.* 2013; Hultin *et al.* 2014; Li *et al.* 2020a; Li *et al.* 2020b). Furthermore, modifications in the ligand binding pocket of mollusc ERs means estrogen cannot bind (Bridgham *et al.* 2014) and so potential disruption by estrogenic compounds is proposed to be independent of nuclear receptor activation (Canesi & Fabbri 2015). The areas where *M. coruscus* is farmed, around Shengsi Island, China, are susceptible to contamination from pollutants transported by the Yangtze River. The mussel is abundant in many coastal regions, that are impacted by agricultural land run-off and anthropogenic residues and since they are filter feeders and sessile organisms, they are highly exposed to pollutants, which they bioaccumulate and for this reason are frequently used as bioindicator species (Viarengo & Canesi 1991). To assess the potential impacts of IOX and DES in the mussel, *M. coruscus*, in the present study various endpoints were measured, including transcriptome analysis to understand changes at the molecular level, measurement of shell growth to evaluate physiological responses, and assessment of possible cardiovascular effects. By selecting water exposure conditions based on previous literature that reflect realistic exposure levels, we aim to provide valuable insights into the potential effects of IOX and DES on *M. coruscus* and contribute to a better understanding of the ecological implications of these contaminants.

4.3. Materials and methods

4.3.1. Ethics statement

The Animal Ethics Committee at Shanghai Ocean University approved all the protocols for mussel acclimation and experimentation in the present study (registration number SHOU-DW-2018-013).

4.3.2. Juveniles

Juveniles *M. coruscus* were collected from the Jinyi mussel hatchery, Shengsi County, Zhejiang Province, China. The juveniles (shell length: 0.575 ± 0.104 mm, shell height: 0.375 ±

0.085 mm; 50 representative individuals) were washed in clean seawater to remove adhering mud and material and placed in closed circuit 10 L polycarbonate tanks containing filtered seawater (FSW; acetate-fiber filter: 1.2 μm pore size) with a salinity of 30 (Li *et al.* 2020b) at 18 °C in a dark environment. The seawater in the tanks was replaced with new seawater every other day. Mussels were fed once daily with the microalgae *Isochrysis zhanjiangensis* at a density of 5×10^4 cells.

4.3.3. Chemical exposure

The effect on the heart rate and shell growth rate of IOX (Sigma, 36198) and DES (Sigma, D4628) exposure for two weeks at different concentrations (10^{-6} to 10^{-8} M) in juvenile mussels was determined (10^{-6} M IOX = 0.37 mg/L, 10^{-7} M IOX = 0.037 mg/L, 10^{-8} M IOX = 0.0037 mg/L; 10^{-6} M DES = 0.27 mg/L, 10^{-7} M DES = 0.027 mg/L, 10^{-8} M DES = 0.0027 mg/L). For consistency, the chemical concentrations used in this paper are expressed in units of Molarity (M). The chosen concentrations of 10^{-6} M, 10^{-7} M, and 10^{-8} M for IOX and DES in the study on mussels were based on two considerations. Firstly, we aimed to compare our study with previous research on zebrafish (Li *et al.* 2020b; Li *et al.* 2022), where exposure to 0.1 μM (equivalent to 10^{-7} M) of IOX or DES disrupted cardiac, vascular, and thyroid development in both embryos and juveniles. Secondly, the concentrations of 0.1 and 0.01 μM of IOX and DES have been reported in some Chinese rivers and so we considered represented realistic levels that might be found in some environments.

For the control group (CTR) the juveniles were treated with autoclaved filtered seawater (AFSW). For the treatment groups IOX and DES were each dissolved in 0.01 M NaOH to give a stock solution of 10^{-3} M (Campinho & Power 2013) and the desired test concentration (10^{-6} M, 10^{-7} M and 10^{-8} M) was prepared by diluting in AFSW. Exposure of the mussels to the chemicals was carried out in six-well plates for the determination of survival rate and two plates contained 10 juveniles/well (120 juveniles/treatment) in 10 mL of AFSW containing the test chemical at 18 °C were used. A 1 L glass beaker containing AFSW and approximately 1000 juveniles for the control and each treatment group was used for growth and heart rate determination and RNA-seq sample collection. Juveniles were fed daily as described above, and the chemical solutions were renewed every other day when the AFSW in each well or beaker was changed. Mortality was monitored daily, and dead mussels were removed. For transcriptome analysis after 14 days of exposure to IOX or DES, 5 samples/treatment composed of approximately 100 individuals/sample were collected into microcentrifuge tubes, and excess

AFSW was removed before they were snap-frozen in liquid nitrogen.

4.3.4. Heart rate and shell growth determination

The heart rate determination was performed using the visual method, which is commonly employed to determine the heartbeat in various organisms, including vertebrates like zebrafish embryos and invertebrates such as oyster spat (Domnik *et al.* 2016; Li *et al.* 2019; Vereycken & Aldridge 2022). The heart rate of juveniles was determined on days 1, 3, 7, 10 and 14 of the treatment by placing the juveniles in a concave slide with AFSW or AFSW containing IOX or DES under a stereomicroscope (Olympus BX51) and capturing video images for approximately 100 s (n = 10–20) at 18 °C. The observed juveniles were returned to the experimental system after the video recording. Only two biological replicates were determined in the 10^{-6} M DES treated group on day 14 due to the high mortality recorded during the experiment. Heart rate was determined by counting the heart beats per minute (HBM) across 100 s in the recorded video images (Representative Supplementary Movie 4.1-4.7 annex III). Fifty randomly selected juveniles from each experimental group were used to measure shell length and height (Supplementary Figure 4.1 annex III) on days 0, 7 and 14 using a stereomicroscope (Olympus SZX2, Japan) fitted with an ocular micrometre. The measured juveniles were then returned to the experimental system. Dead juveniles were identified during the routine monitoring of the experiment as those specimens without a heartbeat.

4.3.5. Total RNA extraction, cDNA synthesis and Illumina sequencing

Total RNA was extracted from the juvenile samples of IOX, DES and AFSW-treated groups after 14 days of exposure using RNAiso Plus reagent (Takara, Japan) and following the manufacturer's instructions. Genomic DNA was eliminated using gDNA Eraser as recommended by the manufacturer of the PrimeScript™ RT reagent kit (Takara, Japan). Briefly, each reaction contained 1 µL gDNA Eraser, 500 ng total RNA, 2 µL 5× gDNA Eraser buffer and RNase Free dH_2O to give a final volume of 10 µL at 42 °C for 2 min. The extracted total RNA in samples was quantified using a NanoDrop (ND-2000, Thermo Scientific, Wilmington, DE, USA). RNA integrity was confirmed by 1 % agarose gel electrophoresis. The quality of the DNase-treated total RNA samples was determined using an Agilent 2100 Bioanalyser (Agilent Technologies, Inc., Santa Clara CA, USA). Only high-quality RNA samples (RIN ≥ 8.0) were used for library construction. The sequencing libraries were constructed with a TruSeq™ RNA sample preparation Kit from Illumina (San Diego, CA, United States), using 1 µg of total RNA.

The RNA-seq sequencing library was sequenced with an Illumina Novaseq 6000 (2 × 150 bp read length) at Majorbio Bio-Pharm Biotechnology Co., Ltd. (Shanghai, China). Raw sequences have been submitted to NCBI under the accession number: PRJNA855151.

4.3.6. Bioinformatic analysis

Raw reads were cleaned to remove adapter sequences, low quality reads (< 30 bp) and sequences with an N content > 10 %. The clean reads were aligned to the *M. coruscus* genome (NCBI accession number: GCA_017311375.1) using HISAT2 (V2.1.0) (Kim *et al.* 2015) and the default settings. The gene expression levels were calculated by RSEM as transcripts per million reads (TPM) (Li & Dewey 2011). The differentially expressed genes (DEGs) between different groups were identified using DESeq2 (Love *et al.* 2014), and genes with a fold-change ≥ 2 ($\log_2$FC ≥ 1) and a Benjamini-Hochberg correction (p-adjust < 0.05) were considered as DEGs. KOBAS 2.1.1 was applied to identify significantly enriched KEGG pathways using a Fisher's exact test (Mao *et al.* 2005). The p-value was corrected using the Benjamini-Hochberg multiple test correction method, and genes with a corrected p-value ≤ 0.05 were deemed to be significantly different. RSEM software (http://deweylab.github.io/RSEM/) was used to generate a heatmap (mean of four biological replicates/treatment) and Venn diagrams of the DEG transcripts in the treatment and control groups.

4.3.7. Real-time quantitative polymerase chain reaction validation

Twenty-five DEGs related to ion channels, homeostasis, signal transduction, growth and metabolism, as well as homologues of vertebrate endothelial-related and cardiac-specific genes (Table 1) (Li *et al.* 2019) were selected for qPCR. 13 DEGs in the DES treatment from the transcriptome analysis linked to cardiovascular and endocrine disruption were also selected for qPCR (Table 4.1). The *M. coruscus* specific qPCR primer pairs were designed using Primer Premier 5 software and are shown in Table 4.1. Total RNA from treatment groups (AFSW, 10^{-8} M IOX, 10^{-7} M DES and 10^{-8} M DES) after 14 days of exposure (n = 5) was used for cDNA synthesis using 500 ng of DNA free total RNA. Reverse transcription was performed in a 20 µL reaction volume by adding the DNase-treated total RNA (10 µL), 1 µL RT Primer Mix, 1 µL PrimeScript RT Enzyme Mix I, 4 µL 5× PrimeScript Buffer 2 and 4 µL RNase Free dH_2O.

For qPCR the following reaction mix was prepared, 1 µL of template cDNA (2.5 ng), 0.3 µL of the forward and reverse primers (10 µM), 5 µL of 2 × FastStart Essential DNA Green Master (Roche), and sterile MilliQ water to give a final reaction volume of 10 µL. The

thermocycler for qPCR was as follows: initial denaturation at 95 °C for 10 min, followed by 45 cycles of amplification, for 10 s at 95 °C and the optimal annealing temperature for the primers for 10 s. Melting curve analysis confirmed a single reaction product was obtained with all the primers used. Absolute quantification of gene copy number was performed as previously described (Li *et al.* 2020b). A standard curve was prepared for each gene using the target amplicon at a concentration range between 10^7 and 10^1 cDNA copies. The amplified copy number for target genes was determined using their Ct value and the standard curve. qPCR analysis was carried out with two technical replicates and five biological replicates per treatment group in 96 multi-well plates using a Light Cycler 960 (Roche, F. Hoffmann-La Roche Ltd., Basel, Switzerland).

Table 4.1. The primer pairs used in qPCR are presented with the amplicon size, melting temperature, r^2 and efficiency. *Trpm3*: transient receptor potential cation channel subfamily M member 3-like; *Trpv5*: transient receptor potential cation channel subfamily V member 5-like isoform X1; *Twk7*: TWiK family of potassium channels protein 7; *AnpX2*: atrial natriuretic peptide-converting enzyme-like isoform X2; *Fas1*: fasciclin-1-like; *Lamb1*: laminin subunit beta-1; *PlxnA4*: plexin-A4; Faxc: failed axon connections homolog; *Stk11*: serine/threonine-protein kinase stk11-like; *Ppiase*: peptidyl-prolyl cis-trans isomerase FKBP2-like; *Plp*: perlucin-like protein; *Tyrp1*: tyrosinase-like protein-1; *Tgfbr3*: transforming growth factor beta receptor type 3-like. *Acvr2a*: actin-related protein 2 precursor; *Acvr1*: activin receptor type-1-like; *Inpp5a*: type I inositol 1,4,5-trisphosphate 5-phosphatase-like isoform X2; *Hif1a*: hypoxia-inducible factor 1 alpha; *Cacna2d2*: voltage-dependent calcium channel subunit alpha-2/delta-1-like; *Cacna2d1*: voltage-dependent calcium channel subunit alpha-2/delta-2-like isoform X4; *Cacna1d*: muscle calcium channel subunit alpha-1-like isoform X6; *Cacnb2*: voltage-dependent L-type calcium channel subunit beta-2; *Adra2a*: alpha-2A adrenergic receptor-like; *Adra1d*: alpha-1A adrenergic receptor-like; *Octalpha*: alpha-2Db adrenergic receptor-like; *Calcrl*: calcitonin receptor. Gene candidates selected from the DEGs identified in the transcriptome analysis are indicated.

Target	Primer	Sequence (5'-3')	Amplicon size (bp)	Tm (°C)	Efficiency (%)	r^2	DEGs
Trpm3	F	GCTTGGGTTGCTCCGTTAT	187	57	90	1.00	Ion channel
	R	TTGGTGGAGGTAGTAGGGGTC					
Trpv5	F	GGGGAAAGCATTAGTGACAGAC	159	55	95	0.99	
	R	GGGCTAGATTTTGGAGTACCAG					
Twk7	F	TTCCTCTGATGTTGCTCTGCC	171	57	91	0.99	
	R	TATCGCTGTTCCACGCTCC					
AnpX2	F	GCCTCTTTGTTTGCCTGTTC	176	56	90	0.99	Homeostasis
	R	GGTAGGGCGTCACAATCAAGTT					
Fas1	F	TCAGAGGAGACAAGGCAAGC	166	56	91	0.99	
	R	GTTGACCAGGCTGTAGAATGTG					
Lamb1	F	GGATTGAACAGTCCCGAACG	153	58	90	0.99	
	R	TGCTTGCCACCAGCGAGTA					
PlxnA4	F	AGGATGTTCTGAGGCAAAGG	298	55	94	0.99	Signal transduction
	R	GCAGGGAATGCAGATGGT					
Faxc	F	ATACTGGGCCTTGTTGTTGG	130	56	97	0.99	

Target	Primer	Sequence (5'-3')	Amplicon size (bp)	Tm (°C)	Efficiency (%)	r^2	DEGs
	R	ACCGTGACCATACGCATCTC					
Stk11	F	CGTTTATCAACCTCGTCGGA	128	57	90	1.00	Growth & metabolism
	R	CAGCCCTTCTGCATAGAGTGAC					
Ppiase	F	TAGGGCTTGGTGCTGTCATT	188	55	91	0.99	
	R	TGGTTTGGGTCCATCGTG					
Plp	F	TGATGACGGGGAACCAAA	128	56	96	0.99	
	R	GGCAAATGTAACCACCAACC					
Tyrp1	F	GTCGTAGTTCAAGGGGTCG	291	55	90	0.99	
	R	AAGAACGGGTGCGTTCAT					
Tgfbr3	F	AGCCCCAGCCAGAAAGAA	162	56	92	0.99	
	R	GAGGTAACAGCCCCATATCTCA					
Acvr2a	F	TGGCTCACTATGTGACTACC	131	55	110	0.99	Homologues of vertebrate endothelial-related and cardiac-specific genes
	R	TTGCCTCAGAATGACCAG					
Acvr1	F	AGGGATGACAACTGGGGTTC	199	58	99	1.00	
	R	ACGCTATGGCAAGGGAGA					
Inpp5a	F	GAAGACAGTGTTGTTTCCTCCC	173	56	99	0.99	
	R	CCACGCTTGCGAATGCTA					
Hif1a	F	TCACCCAGGCAACCATCA	114	57	110	1.00	
	R	GGAACCCAGCCAGAATCAAA					
Cacna2d2	F	CACTTTGATGAGGTCGGA	165	53	90	1.00	
	R	GGATGGAACACGTAGTCG					
Cacna2d1	F	TGGAGGAACTGATGATGC	186	52	102	0.99	
	R	GCTCTGATTGCTCCCATA					
Cacna1d	F	GCTGGTAGGGAGGATGAT	122	55	91	0.99	
	R	TGCTCTTGACCTTTCTGG					
Cacnb2	F	GATTTAGGGGTGATTGGG	111	54	90	1.00	
	R	AGGTGTTACACTGCTGGACT					
Adra2a	F	GCCATGACTTTTGCCTCG	172	55	105	1.00	
	R	CTCCAATGAACGGTGGAG					
Adra1d	F	TCGGATGGAGGAAAGAGC	173	56	99	0.99	
	R	AGCCATAAGGCAGCGAGT					
Octalpha	F	AAGAACACCAAAGAGGGC	129	55	90	0.99	
	R	CTGAGTAAGCACAGCGGATA					
Calcrl	F	GCGACCTGACGAAAGTGA	158	57	110	1.00	
	R	GTCGGCGAACCACAACAT					

4.3.8. Statistical analysis

Data were analysed using JMP software to detect statistical differences (version 10.0.0). All data were tested for normality (Shapiro–Wilk test) and homogeneity (O'Brien test) before

selecting appropriate statistical tests for analysis. The differences in mussel viability, gene expressions, heart rate, shell length and shell height between each of the treatment groups were analysed using a Wilcoxon/Kruskal-Wallis test. A $p < 0.05$ was considered significantly different.

4.4. Results

4.4.1. Effect of IOX and DES on the heart rate and viability of M. coruscus juvenile

The effects of IOX and DES on heart rate and viability of *M. coruscus* juveniles are shown in Fig. 1 (Representative Supplementary Movie 4.1-4.7 annex III). The heart rate of control mussels exhibited a decrease across the time of the experiment (Figure 4.1), which may be a result of physiological parameters such as neural, humoral, and behavioural changes. The heart rate of juveniles was significantly decreased in the 10^{-6} M and 10^{-7} M IOX-treated groups compared to the control group after one day of exposure (Figure 4.1A; Supplementary Table 4.1 annex III; $p < 0.05$). After seven days of exposure, the heart rate in the 10^{-6} M IOX-treated juveniles was significantly lower than in the control juveniles (Figure 4.1A; $p < 0.05$). No juveniles survived in the 10^{-6} M and 10^{-7} M IOX-treatment groups on day 14 (Figure 4.1B). The viability of juveniles exposed to 10^{-8} M IOX was like the control group (Figure 4.1B; $p > 0.05$).

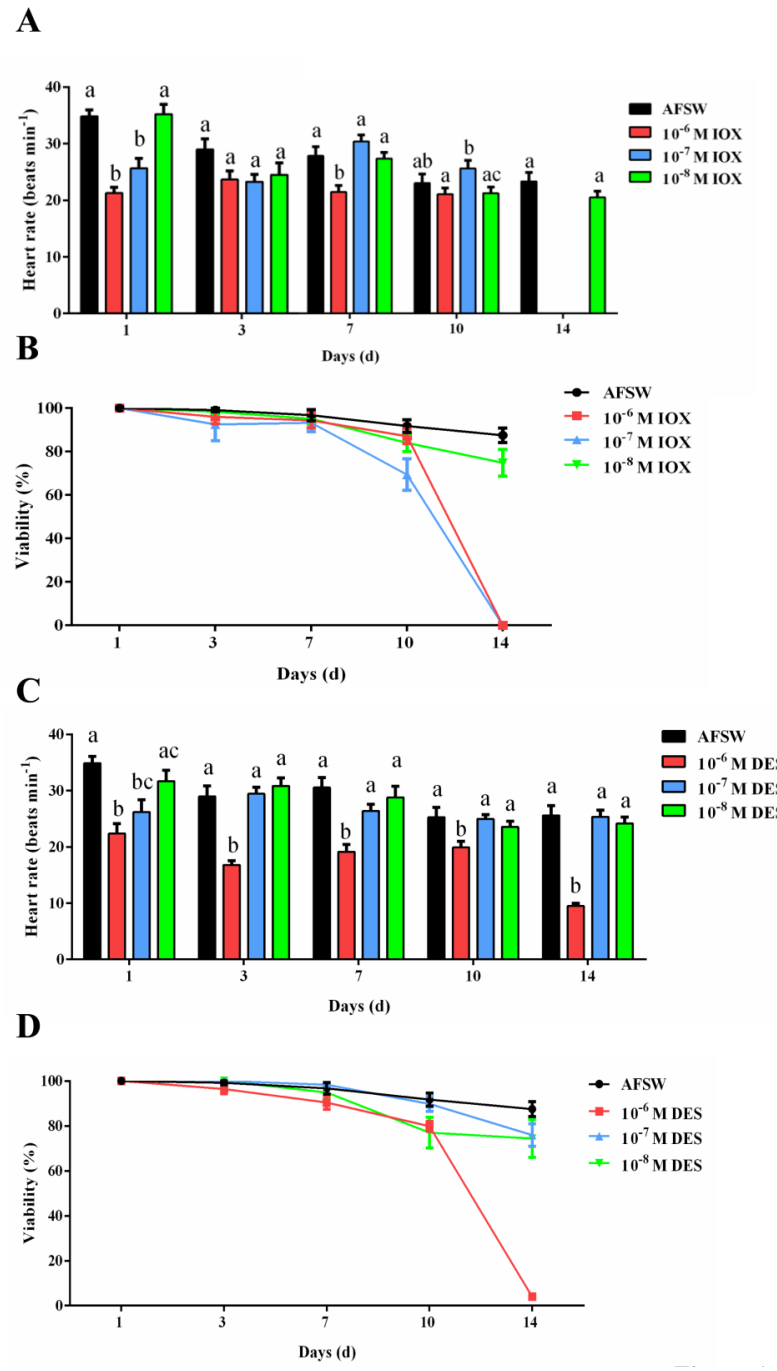

Figure 4.1. Effect of IOX and DES on juvenile *M. coruscus* heart rate (A, C) and their viability when exposed to 10^{-6}, 10^{-7} and 10^{-8} M IOX and DES (B, D) for 14 days. Different letters above bars in graphs A and C indicate groups that are significantly different ($p < 0.05$).

On the first day after DES exposure, a significantly lower heart rate was observed in juveniles exposed to 10^{-6} M and 10^{-7} M DES compared to the control group (Figure 4.1C; Supplementary Table 4.1 annex III; $p < 0.05$). From day 3 to day 14 of DES exposure, a significantly decreased heart rate was only found in the 10^{-6} M DES-treated group compared to other groups (Figure 4.1C; $p < 0.05$). The viability of juvenile mussels was significantly

reduced after 14 days of 10^{-6} M DES exposure compared to the other groups (Figure 4.1D; $p < 0.05$), and in 10^{-7} M and 10^{-8} M DES groups the viability was like the control (Figure 4.1D; $p > 0.05$).

4.4.2. Effect of IOX and DES on the growth of M. coruscus juveniles

The effect of IOX and DES on the shell growth of juveniles was determined (Figure 4.2; Table 4.2). The shell length and height were significantly increased in control mussels on day 14 of the experiment compared to the beginning of the experiment, with a 23.3 % and 32 % increase, respectively (Figure 4.2; Table 4.2). The shell length of *M. coruscus* juveniles was significantly decreased in the 10^{-7} M IOX-treated group compared to the control group after seven days of exposure (Figure 4.2A; $p < 0.05$). IOX had no effect on shell height in the three tested concentrations compared to the control group (Figure 4.2B; $p > 0.05$). 10^{-7} and 10^{-8} M DES exposure for seven days significantly reduced the shell length compared to the control (Figure 4.2C; $p < 0.05$). After 14 days of exposure to 10^{-6} and 10^{-8} M DES there was a significant decrease in the shell length compared to the control (Figure 4.2C; $p < 0.05$). Exposure for seven days to 10^{-6} M DES significantly increased the shell height compared to the control (Figure 4.2D; $p < 0.05$). 10^{-8} M DES exposure for 14 days caused the most pronounced inhibition of shell growth in both length and height compared to the control (Figure 4.2C and D; $p < 0.05$).

Table 4.2. Effects of exposure for 14 days to IOX and DES on the shell growth rate in *M. coruscus* juveniles. "—" indicates "not measured". The growth rate was determined in 50 individuals exposed for 14 days to IOX or DES and normalized by the initial shell length and height. Significant changes were determined by comparing the chemical treatment groups to the control.

Treatment	Shell length (% increase)	Shell height (% increase)
AFSW	23.3%	32.0%
10^{-6} M IOX	—	—
10^{-7} M IOX	—	—
10^{-8} M IOX	20.1%	27.0%
10^{-6} M DES	14.3%	22.8%
10^{-7} M DES	19.3%	27.1%

10^{-8} M DES	0.5%	4.5%

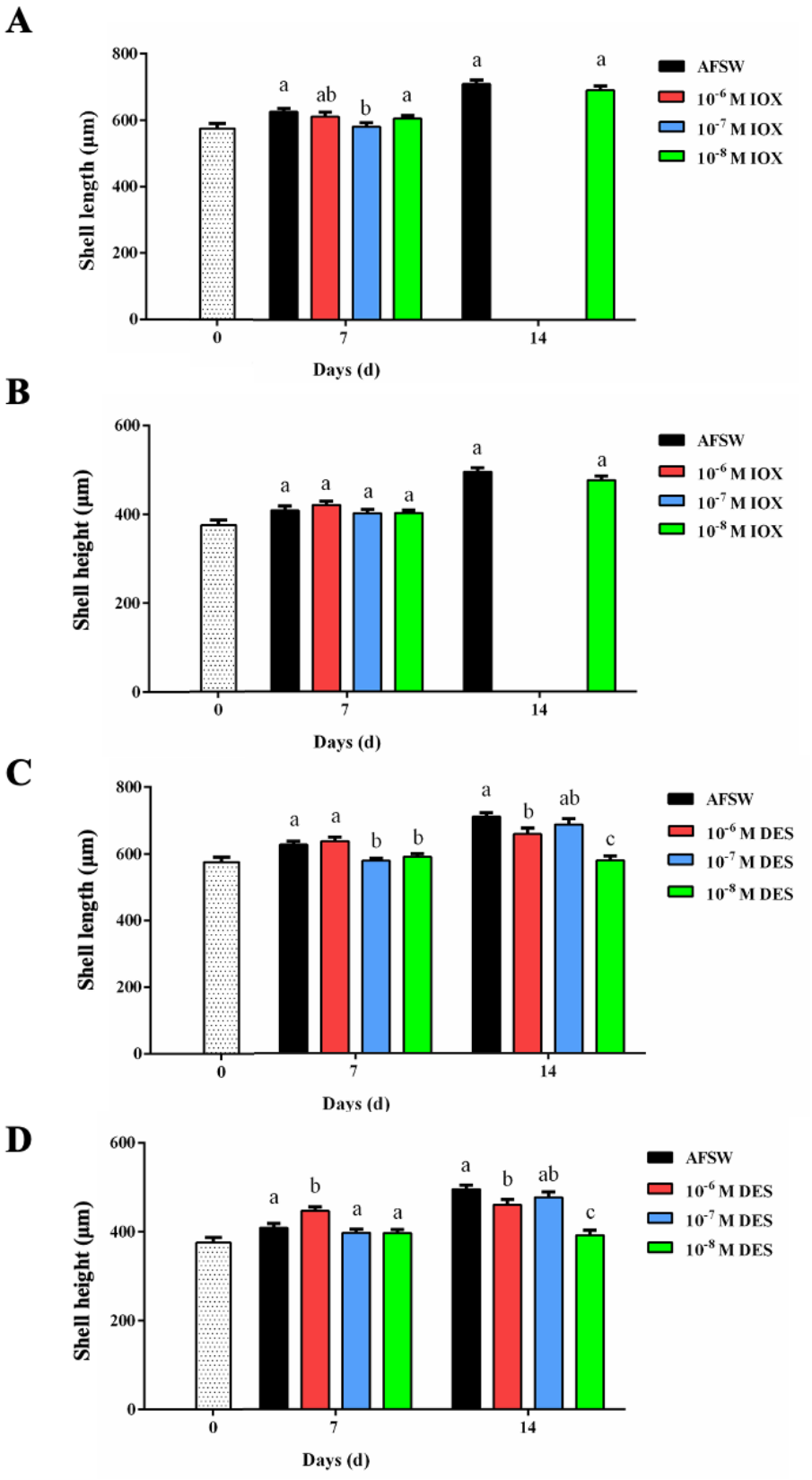

Figure 4.2. The effect of two weeks exposure to IOX and DES (10^{-6}, 10^{-7} and 10^{-8} M) on shell length

(A, C) and shell height (B, D) of juvenile *M. coruscus*. Different letters in bar charts indicate groups that are significantly different ($p < 0.05$).

4.4.3. Transcriptomics of M. coruscus juveniles exposed to IOX and DES

4.4.3.1. Global gene expression

Transcriptome analysis of mussel juveniles after 14 days exposure to DES (10^{-7} M, 10^{-8} M) and IOX (10^{-8} M) yielded a total of 385 DEGs compared to the control group. The 10^{-8} M IOX-treated group contained 19 DEGs, with 3 up regulated and 16 down regulated genes relative to the control group (Supplementary Table 4.2 annex III; $p < 0.05$). The 10^{-7} M DES-exposed mussels had 353 DEGs, with 95 up regulated and 258 down regulated genes (Supplementary Table 4.3 annex III; $p < 0.05$), while the 10^{-8} M DES-treated groups had 13 DEGs, of which 12 were up regulated and 1 was down regulated compared to the control (Supplementary Table 4.4 annex III; $p < 0.05$).

DE analysis revealed that relatively few differentially expressed gene transcripts were common across the DES treatments at different concentrations (Figure 4.3A). The DEGs in DES and IOX were scrutinized to identify genes linked to the functional phenotypes of interest (shell growth, cardiovascular disruption and EDC, Table 4.2). A heatmap was generated based on the average of the differentially expressed gene transcripts per experimental group identified after RNA-seq (Figure 4.3B). The heatmap revealed that the overall gene expression patterns in juveniles exposed to IOX and DES was different from the control group (Figure 4.3B). The 10^{-7} M DES-exposed juveniles had a different expression profile compared to the 10^{-8} M DES-exposed juveniles (Figure 4.3B).

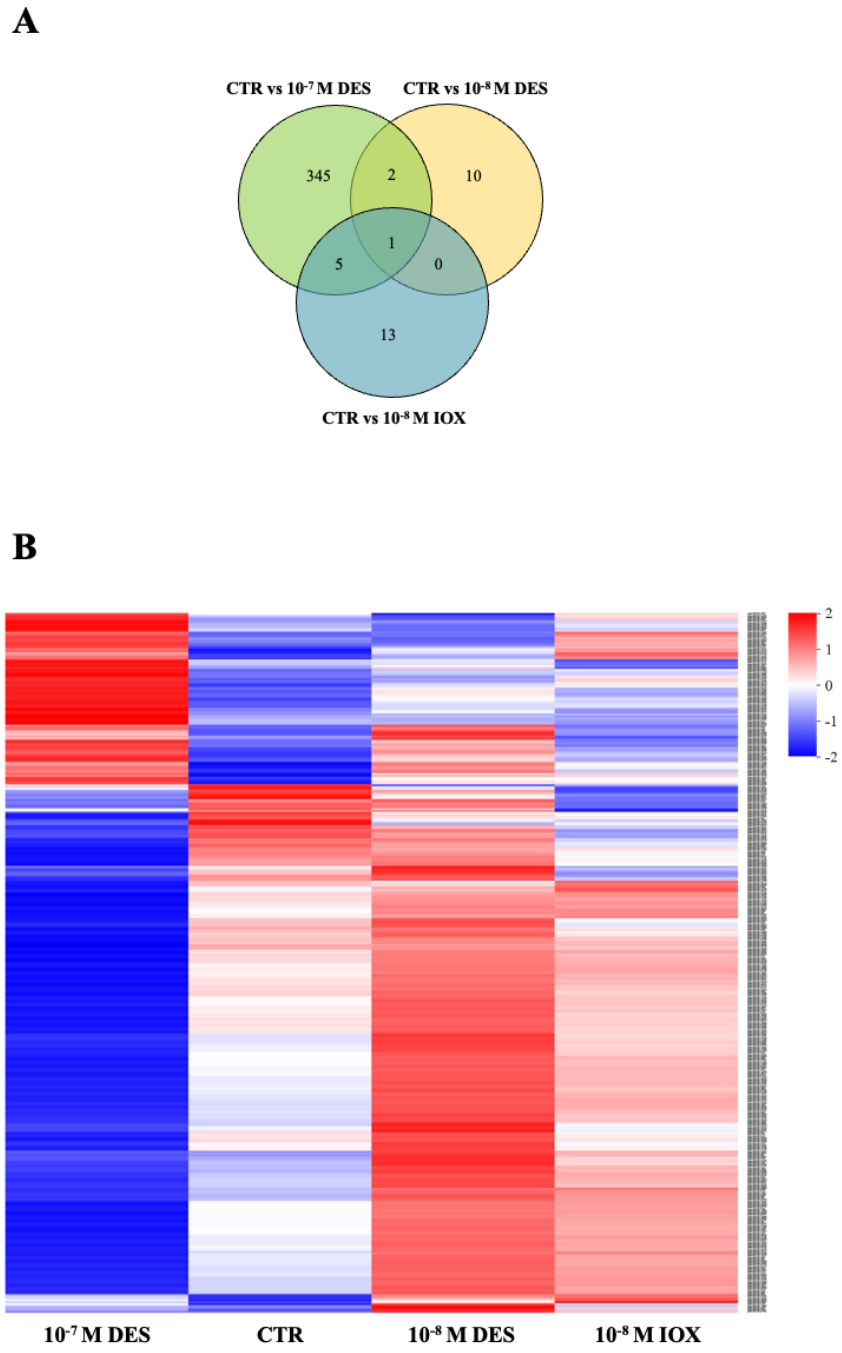

Figure 4.3. Differentially expressed genes in transcriptomes of whole *M. coruscus* juveniles exposed to seawater, DES (10^{-7} and 10^{-8} M) or IOX (10^{-8} M) for 14 days. (A) Venn diagram presenting the differentially expressed genes between the control, 10^{-7} M DES, 10^{-8} M DES and 10^{-8} M IOX groups. The number of differentially expressed gene transcripts in the treatment group and between two groups is reported (Supplementary Table 4.2-4.4 annex III). The overlapping section of two circles identifies the number of common differentially expressed gene transcripts between two groups. (B) Hierarchical cluster analysis of selected differentially expressed genes (DEGs). The heatmap shows the differentially expressed gene transcripts in the control, DES and IOX treated juveniles. Transcriptome data of 10^{-6} M IOX, 10^{-7} M IOX and 10^{-6} M DES are not available due to the death of juveniles on day 14. Each column represents a sample, and each row represents a gene. Red represents high expression, and low expression is indicated in blue. The closer the branches of the two samples, the more similar the expression patterns of all genes between the two samples.

4.4.3.2. Transcripts related to shell growth, cardiovascular disruption and EDC

Several DEGs were identified that were linked to neuroendocrine function, for example, cAMP-responsive element-binding protein-like 2 and peptidylglycine alpha-amidating monooxygenase were significantly changed in juveniles treated with 10^{-8} M IOX compared to the control ($p < 0.05$; Table 4.2, Supplementary Table 4.2 annex III) (Simpson *et al.* 2015; Rao *et al.* 2021; Wang *et al.* 2018). A set of DEGs related to the nervous system, such as arylsulfatase J, teneurin-m, and neuroplastin, were identified in juveniles treated with 10^{-7} M DES compared to the control group ($p < 0.05$; Table 4.2, Supplementary Table 4.3 annex III) (Abzalimov *et al.* 2013; Kenzelmann *et al.* 2007; Beesley *et al.* 2014; Eckhardt 2008).

Table 4.3. DEGs identified in IOX (10^{-8} M) and DES (10^{-7} M and 10^{-8} M DES) compared to the control that were involved in neuroendocrine, cardiac, growth, detoxification, and the immune response.

		Compared with control								
		10^{-8} M IOX			10^{-7} M DES			10^{-8} M DES		
Function	**Swiss-Prot**	*P*-value	up	down	*P*-value	up	down	*P*-value	up	down
Neuroendocrine related effects	cAMP-responsive element-binding protein-like 2	$4.13E^{-06}$	√							
	Peptidylglycine alpha-Amidating Monooxygenase (PAM)	$3.02E^{-09}$		√						
	Arylsulfatase J				$5.77E^{-06}$	√				
	Teneurin-m				$8.19E^{-05}$		√			
	Teneurin-4				$7.86E^{-04}$		√			
	Neuroplastin				$1.38E^{-04}$		√			
Cardiac related effects	Plasma kallikrein				$7.20E^{-04}$	√				
	Low-density lipoprotein receptor				0.0012		√			
	Atrial natriuretic peptide-converting enzyme (Corin)				$3.44E^{-04}$		√			
	P2X purinoceptor 4				0.0012		√			
Growth related effects	Transforming growth factor beta receptor type 3				$1.98E^{-05}$		√			
	POU domain, class 6, transcription factor 2				$2.05E^{-04}$		√			
	Probable G-protein coupled receptor 158				$1.86E^{-04}$		√			
	Annexin A13							$1.86E^{-05}$	√	

		Compared with control								
		10^{-8} M IOX			10^{-7} M DES			10^{-8} M DES		
Function	**Swiss-Prot**	*P*-value	up	down	*P*-value	up	down	*P*-value	up	down
Detoxification related effects	Sulfotransferase 1 family member D1				$2.68E^{-05}$	√				
	Sulfotransferase 1C2A				$1.22E^{-19}$	√				
	Sulfotransferase family cytosolic 1B member 1				$2.82E^{-08}$	√				
	Cytochrome P450 2C29				$1.37E^{-16}$	√				
	Cytochrome P450 2B5				$1.26E^{-06}$	√				
	Cytochrome P450 2U1				$1.49E^{-08}$	√		$1.18E^{-10}$	√	
	Cytochrome P450 2B9				$4.56E^{-22}$	√				
	Nuclear factor erythroid 2-related factor 2				$3.60E^{-04}$		√			
	Solute carrier organic anion transporter family member				$2.33E^{-06}$	√				
Immune related effects	Macrophage mannose receptor 1	$3.76E^{-05}$		√						
	L-rhamnose-binding lectin CSL3	$2.63E^{-11}$		√						
	Scavenger receptor cysteine-rich domain superfamily protein	$4.61E^{-07}$		√	$1.45E^{-09}$		√			

DEGs related to cardiac function were identified in juveniles treated with 10^{-7} M DES and included plasma kallikrein, low-density lipoprotein receptor, atrial natriuretic peptide-converting enzyme and P2X purinoceptor 4 when compared to the control ($p < 0.05$; Table 4.2, Supplementary Table 4.3 annex III) (Bjorkqvist *et al.* 2013; Go & Mani 2012; Yan *et al.* 2000; Ralevic & Burnstock 1991).

Several of the DEGs identified in juveniles treated with 10^{-7} M DES were growth-related and were strongly down regulated compared to the control ($p < 0.05$; Supplementary Table 4.3 annex III) and included transforming growth factor beta receptor type 3, POU domain class 6 transcription factor 2 and probable G protein coupled receptor (Cox 1995; Patel *et al.* 2015; Yang *et al.* 2023a). Annexin A13 was increased significantly in 10^{-8} M DES treated juveniles relative to the control ($p < 0.05$; Table 4.2, Supplementary Table 4.4 annex III) (Moss & Morgan 2004).

An array of DEGs responsible for detoxifying xenobiotic substances or contributing to

antioxidant defence were found in 10^{-7} M DES-treated juveniles, such as *sulfotransferase 1C2A*, *nuclear factor erythroid 2-related factor 2* and *solute carrier organic anion transporter family member 5A1* ($p < 0.05$; Supplementary Table 4.3 annex III) (James & Ambadapadi 2013; Hagenbuch & Gui 2008; Kim *et al.* 2011). Several *cytochrome P450* DEGs were up regulated in both 10^{-7} M DES and 10^{-8} M DES-treated juveniles compared to the control juveniles ($p < 0.05$; Table 4.2, Supplementary Table 4.3, 4.4 annex III) (Galli *et al.* 1988; Livingstone 1988; Snyder 2000; Michel *et al.* 1993). Exposure to IOX and DES also modified the immune response and a variety of immune related DEGs, including *macrophage mannose receptor 1*, *L-rhamnose-binding lectin CSL3* and *scavenger receptor cysteine-rich domain superfamily protein* (Furukawa *et al.* 2012; Gazi & Martínez-Pomares 2009; Watanabe *et al.* 2009) were down regulated in juveniles treated with 10^{-8} M IOX compared to the control ($p < 0.05$; Table 4.2, Supplementary Table 4.2-4.4 annex III).

4.4.3.3. KEGG pathway analysis

KEGG pathway analysis of the DE gene transcripts of the 10^{-8} M IOX, 10^{-7} M DES and 10^{-8} M DES-treated groups retrieved 1, 18 and 4 significantly changed KEGG pathways, respectively (Supplementary Table 4.5-4.7 annex III; $p < 0.05$). Further information is provided about the DE genes allocated to the significantly changed KEGG pathways in Supplementary Table 4.5-4.7 annex III. For example, "protein digestion and absorption" was one of the KEGG pathways that was significantly changed in the mussels exposed to 10^{-8} M IOX (Supplementary Table 4.5 annex III, $p < 0.05$). Both 10^{-7} and 10^{-8} M DES-treated groups had a similar effect on genes linked to the serotonergic synapse pathway of the nervous system (Supplementary Table 4.6, 4.7 annex III, $p < 0.05$). In addition, several of the pathways significantly changed in 10^{-7} M DES-treated juveniles were genes related to cellular functions including endocytosis and adherens junction (Supplementary Table 4.6 annex III, $p < 0.05$). 10^{-7} M DES also had a significant effect on genes of the mTOR signaling pathway, estrogen signaling pathway, arachidonic acid metabolism, lysine degradation, inflammatory mediator regulation of TRP channels, axon guidance, and protein processing in the endoplasmic reticulum (Supplementary Table 4.6 annex III, $p < 0.05$).

4.4.3.4. qPCR analysis of gene expression

Of the twenty-five candidate genes selected due to their link to cardiovascular and endocrine disruption in vertebrate's. qPCR analysis showed that *Ppiase* (peptidyl-prolyl cis-

trans isomerase FKBP2-like) was significantly down regulated in IOX (10^{-8} M) and DES (10^{-7} M and 10^{-8} M) exposed juveniles compared to the control (Figure 4.4, $p < 0.05$). The expression of *Faxc* (failed axon connections), *Plp* (perlucin-like protein) and *Tyrp1* (tyrosinase-like protein-1) was significantly up regulated in 10^{-7} M DES-exposed juveniles compared to the control (Figure 4.4B, $p < 0.05$). *PlxnA4* (plexin-A4) and *Stk11* (serine/threonine protein kinase stk11-like) were significantly decreased in the 10^{-7} M DES-treated juveniles compared to the control (Figure 4.4B, $p < 0.05$). No significant differences were found in transcript abundance of genes related to endothelial and cardiac function in the IOX or DES treatment groups compared to the control (Supplementary Figure 4.2 annex III). Overall, the qPCR results were consistent with the transcriptome data and revealed concordance between the relative abundance of gene transcripts measured in the treatment groups compared to the control group for 10^{-8} M IOX ($R = 0.76$), 10^{-7} M DES ($R = 0.80$) and 10^{-8} M DES ($R = 0.79$) (Supplementary Figure 4.3 annex III).

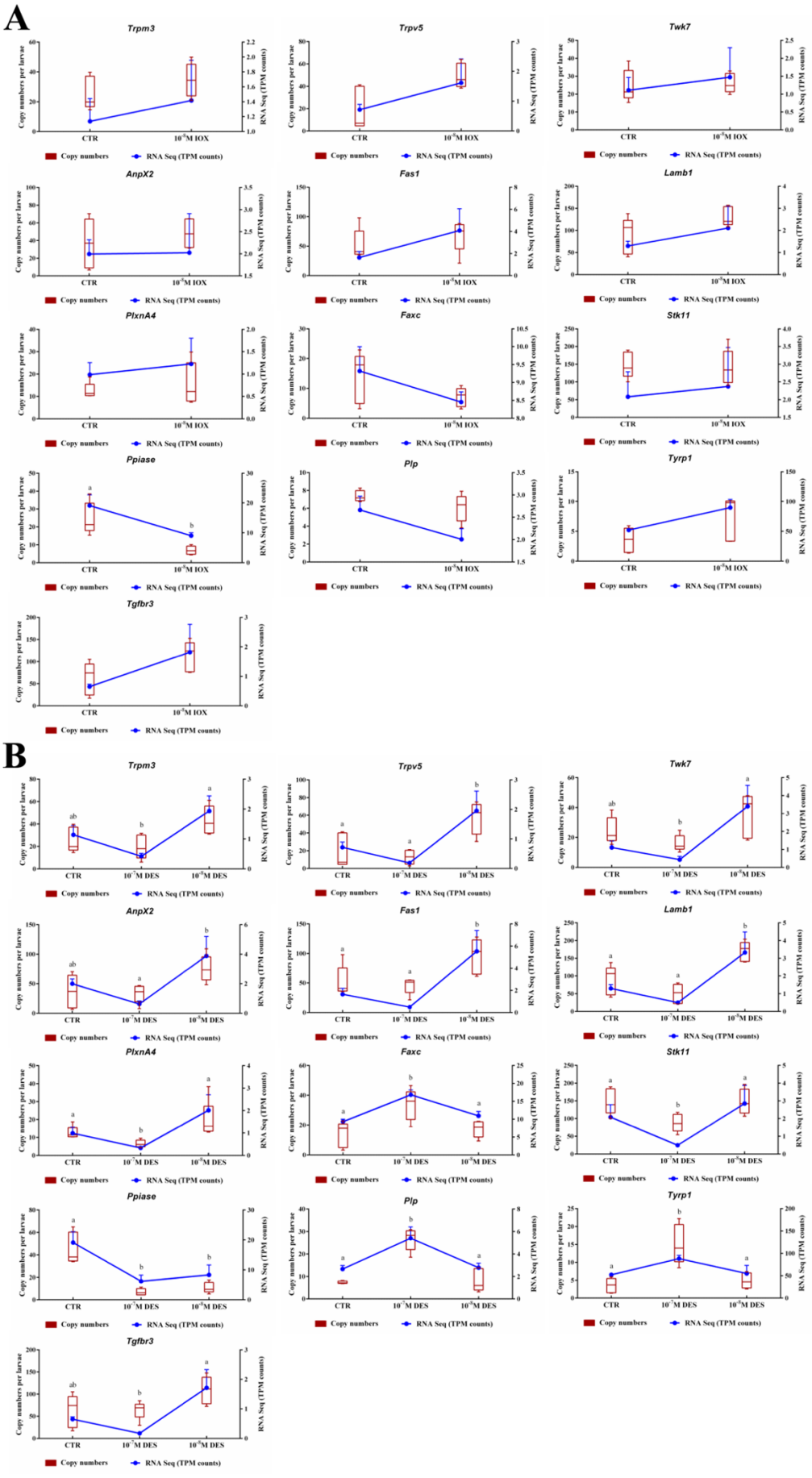
A
Trpm3
Trpv5
Twk7
AnpX2
Fas1
Lamb1
PlxnA4
Faxc
Stk11
Ppiase
Plp
Tyrp1
Tgfbr3
Copy numbers per larvae
RNA Seq (TPM counts)
CTR
10⁻⁵M IOX
Copy numbers
RNA Seq (TPM counts)
B
Trpm3
Trpv5
Twk7
AnpX2
Fas1
Lamb1
PlxnA4
Faxc
Stk11
Ppiase
Plp
Tyrp1
Tgfbr3
Copy numbers per larvae
RNA Seq (TPM counts)
CTR
10⁻⁷M DES
10⁻⁵M DES
Copy numbers
RNA Seq (TPM counts)

Figure 4.4. A graphical comparison of the results of RNA-seq analysis and quantitative PCR (qPCR) analysis of candidate gene expression in IOX-treated (A), DES-treated (B) and Control *M. coruscus* juveniles. The boxplots in the graphs represent the copy number determined by qPCR of cDNA prepared from extracts of whole *M. coruscus* juveniles. The blue line represents the expression levels by TPM counts obtained from RNA-seq. The candidate genes selected for qPCR analysis included genes previously shown in vertebrates to be modified when they were exposed to IOX or DES, namely: *Trpm3*: transient receptor potential cation channel subfamily M member 3-like; *Trpv5*: transient receptor potential cation channel subfamily V member 5-like isoform X1; *Twk7*: TWiK family of potassium channels protein 7; *AnpX2*: atrial natriuretic peptide-converting enzyme-like isoform X2; *Fas1*: fasciclin-1-like; *Lamb1*: laminin subunit beta-1; *PlxnA4*: plexin-A4; *Faxc*: failed axon connections homolog; *Stk11*: serine/threonine-protein kinase stk11-like; *Ppiase*: peptidyl-prolyl cis-trans isomerase FKBP2-like; *Plp*: perlucin-like protein; *Tyrp1*: tyrosinase-like protein-1; *Tgfbr3*: transforming growth factor beta receptor type 3-like. Different letters indicate statistically significantly different of copy numbers of qPCR analysis ($p < 0.05$).

4.5. Discussion

This study revealed a direct disruptive action of IOX and DES on cardiac performance and shell growth in mussel juveniles. Exposure to IOX and DES caused a significant decrease in heart rate, with the most severe effects observed in the 10^{-6} M treatment group. The inhibitory effects of DES on shell growth were more pronounced after 14 days of exposure at higher concentrations (10^{-6} M and 10^{-8} M), indicating a cumulative impact over time. The effects of IOX and DES exposure differed at a molecular level with IOX primarily linked to alterations in neuroendocrine- and immune-related gene transcripts. In contrast, DES exhibited a more substantial effect compared to IOX, influencing gene transcripts associated with cardiac function, growth, neuroendocrine regulation, and detoxification processes. A positive aspect that may explain in part the resilience and wide environmental distribution of bivalves was the observed activation by DES of multiple detoxification related gene transcripts and the acclimation of the mussel heart to low levels of both IOX and DES. However, a negative aspect was the differing targets of IOX and DES as reflected by the transcriptome results that suggests the adverse effects of environmental contaminants may be amplified when they are present as mixtures.

The hazardous potential of IOX and DES and their adverse effects on vertebrates are well documented (Ogilvie & Ramsden 1988; Titus-Ernstoff *et al.* 2010; Otsuka *et al.* 2014; Tournaire *et al.* 2016; Vicario *et al.* 2017; Kioumourtzoglou *et al.* 2018; Hunter *et al.* 2021). In this study, on an invertebrate, the marine mussel, we observed a significant decrease in heart rate after exposure to IOX and DES, which confirmed the potential of these chemicals to disrupt the cardiovascular system in invertebrates as well as in vertebrates (Li *et al.* 2019; Li *et al.* 2022). Heart rate is a dynamic physiological parameter that varies daily and is influenced by a range of factors, including neural, humoral and behavioural changes. Juvenile mussels, as they move

by crawling with their foot, exhibit variations in heart rate that correspond to changes in their behaviour. All these phenomena may contribute to explain the fluctuations normally seen in the mussel heart rate and encountered in our study. Nonetheless, DES and IOX provoked a much more significant reduction in heart rate compared to the control, particularly at higher concentrations (10^{-6} M) and the treatment also elicited a dose dependent effect. The decrease in cardiac performance in response to the 10^{-6} M DES exposure was most likely associated with the observed reduction in activity under stressful conditions (results not presented) although we cannot rule out a role for neural and humoral factors. Interestingly, the pronounced effects of DES on heart rate only occurred on the first day of exposure in the 10^{-7} M DES group, and then a normal heart rate was reestablished from three days exposure onward. This suggests that concentration and duration of exposure to these chemicals determined the effect, and it was dependent on the adaptability of the heart in marine mussels to environmental chemicals. Taking into consideration the dose dependent effects of 10^{-6} M and 10^{-7} M of IOX and DES on the heart rate, the cardiac performance test was a good indicator of exposure to these chemicals in *M. coruscus*. If the disruptive effects of IOX and DES on cardiac performance and DES on cardiac related gene transcripts in mussel are considered together with vertebrate data (Li *et al.* 2022; Li *et al.* 2019), it suggests these chemicals are cardiovascular disruptors in both phyla, although their mode of action seems to be different.

In the present study, analysis of DEGs revealed that the KEGG serotonergic synapse pathway was significantly affected in the 10^{-7} M DES and 10^{-8} M DES-exposed groups after 14 days of exposure (Supplementary Table 4.6, 4.7 annex III), which suggests that the homeostasis of neuronal circuits was likely impaired. Serotonin is an inhibitory neurotransmitter (He *et al.* 2018) and although a prolonged effect on slowing the heart rate was only detected for the 10^{-6} M IOX- and DES-treated juveniles, the up regulation of elements of this system by lower doses of IOX and DES is intriguing and may contribute to explain the reduction in the heart rate observed. The transcriptomics results also showed changes in gene expression related to cardiac function, such as plasma kallikrein, atrial natriuretic peptide-converting enzyme, P2X purinoceptor 4, and low-density lipoprotein receptor (Bjorkqvist *et al.* 2013; Go & Mani 2012; Yan *et al.* 2000; Ralevic & Burnstock 1991), which may explain the modified cardiac function observed in DES-treated groups. The transcriptomics and qPCR results of the mussel taken together with the data on heart function reveal that IOX and DES are potential disruptors of the cardiovascular system in mussels.

Two DGEs linked to neuroendocrine function, *cAMP-response element binding protein* (a

major regulator of neurotrophin responses) (Wang *et al.* 2008) and *peptidylglycine alpha-amidating monooxygenase* (an enzyme important for the biosynthesis of many peptide hormones) (Simpson *et al.* 2015; Rao *et al.* 2021) were significantly affected by 10^{-8} M IOX in treated juveniles relative to the control (Supplementary Table 4.2 annex III). In rats, Trpv5 plays an important role in regulating neural and neuroendocrine pathways in the brain (Kumar *et al.* 2017). 10 μM DES exposure strongly inhibited human transient receptor potential canonical 5 (Trpc5) channels (Naylor *et al.* 2010). Trpv5 and transient receptor potential cation channel subfamily M member 3-like (Trpm3) are essential calcium-permeable ion channels for calcium homeostasis (Dang & Kienzler 2019; Zhou *et al.* 2021). The up regulation of Trpv5 after exposure to 10^{-7} M DES in juveniles of *M. coruscus* indicates that DES affects neuroendocrine regulation (Supplementary Table 4.3 annex III). Plexin-A4 has been implicated in mediating multiple semaphorin signals and regulating axon guidance (Suto *et al.* 2005). Teneurins and neuroplastin have been linked to neural development (Kenzelmann *et al.* 2007; Beesley *et al.* 2014). The down regulation of *PlxnA4*, *teneurin-m*, *teneurin-4* and *neuroplastin* in 10^{-7} M DES-treated juveniles indicates that DES is a potential disruptor of the nervous system in *M. coruscus* (Figure 4.4B, Table 4.2, Supplementary Table 4.3 annex III). Taken together, our data seem to suggest that IOX and DES are disruptors of the nervous and neuroendocrine systems in *M. coruscus*. This seems to be further supported by some overlap in the effects of IOX and DES in bivalves and vertebrates where they are known to disrupt the neuroendocrine system (Morgado *et al.* 2007; Eguchi *et al.* 2008; Morgado *et al.* 2009; Akiyoshi *et al.* 2012).

The presence of DES in the environment is due to its widespread use as a growth promoter in livestock production (Brennan *et al.* 2006; McLachlan *et al.* 1984; Bravo *et al.* 2007) and a similar effect is reported in neonatal mice (0.001 and 0.01 μg/kg) but not at high concentrations (0.01 to 100 μg/kg) (Newbold *et al.* 2004). In contrast, in the invertebrate, *Daphnia magna*, 21 days exposure to 0.5 mg/L DES decreased moulting frequency and hampered growth (Baldwin *et al.* 1995; Brennan *et al.* 2006). The growth inhibition of the mussel shell by DES (10^{-8} M) in the present study may be explained by observations in *Anodonta cygnea* (Bivalvia) that DES causes modified ion transport and intracellular pH, crucial processes for shell production, by the outer mantle epithelium (Alves & Oliveira 2013). The basis of the more severe growth inhibition of the shell by 10^{-8} M DES compared to higher doses, 10^{-6} and 10^{-7} M in *M. coruscus* juveniles remains to be determined. Unlike DES increasing concentrations of IOX reduced *M. coruscus* juvenile survival but lower concentrations (10^{-8} M) did not affect shell growth, and this highlights the differing effects and presumably targets of DES and IOX in bivalves.

The present study revealed that exposure of *M. coruscus* juveniles to DES for 14 days suppressed their growth as has been reported in the teleost, catfish (Król *et al.* 2014; Liu *et al.* 2018). Furthermore, in *M. coruscus* the effect of DES on growth involved changes in gene transcription since it modified the expression of growth-related genes such as those of the cell cycle/proliferation, energy metabolism and protein metabolism (e.g., *Transforming growth factor beta receptor type 3*, *POU domain class 6 transcription factor 2* and *Probable G-protein coupled receptor 158*, *serine/threonine kinase Stk11*) (Supplementary Table 4.3 annex III) (Cox 1995; Patel *et al.* 2015; Yang *et al.* 2023a). The inhibition by 10^{-7} M DES of *Stk11* gene expression further substantiates the role of gene expression in growth impairment in the juveniles of *M. coruscus* (Figure 4.4B). *Peptidyl prolyl isomerase* (*Ppiase*), contributes to efficient protein folding (Young *et al.* 2004), and was up regulated in fast growing larvae of the Pacific oyster *Crassostrea gigas* (Meyer & Manahan 2010); its significant down regulation by IOX and DES (Figure 4.4) may contribute to the general growth inhibition observed in *M. coruscus* juveniles. Several shell growth related genes, such as *perlucin-like protein* (*Plp*) (Wang *et al.* 2008) and *tyrosinase-like protein-1* (*Tyrp1*) (Zhu *et al.* 2021), were also significantly affected by exposure to 10^{-7} M DES (Figure 4.4B) in *M. coruscus* juveniles. A similar inhibitory effect on growth was reported in *M. coruscus* juveniles exposed to an anti-thyroid compound, methimazole (MMI) (Li *et al.* 2021). Moreover, MMI and propylthiouracil significantly reduced larval metamorphosis in *M. coruscus*, suggesting it had a neuroendocrine disrupting effect (Li *et al.* 2021). The disrupted growth caused by low doses of DES is reminiscent of that caused by MMI and suggests it may be an endocrine disruptor.

Cytochrome P450s (CYP) are one of the major enzyme complexes for detoxifying xenobiotic substances (e.g., pesticides, fungicides, hydrocarbons) found in aquatic organisms (Snyder 2000). Cytochrome P450 content was elevated in mussel *M. edulis* and periwinkles *Littorina littorea* when exposure to xenobiotic compounds (Livingstone 1988). Furthermore, benzo(a)pyrene (BaP), 3,3′,4,4′-tetrachlorobiphenyl (TCB) and Na-phenobarbital increased the level of Cytochrome P450 in the mussel *M. galloprovincialis* (Galli *et al.* 1988; Michel *et al.* 1993). Sulfotransferases are crucial molecules for the elimination of xenobiotic molecules such as environmental chemicals and drugs (James & Ambadapadi 2013). In the present study, numerous *cytochrome P450* and *sulfotransferase* genes were significantly up regulated in DES treated juveniles (Table 4.2, Supplementary Table 4.3, 4.4 annex III), indicating exposure to DES activated genes linked to detoxification. Toxic substances are proposed to be detrimental to aquatic organisms, since they weaken their immune system and make them more prone to

disease (Kataoka & Kashiwada 2021). Interestingly the up regulation of detoxification-related genes observed in the present study was associated with down regulation of DEGs related to immune function, such as *scavenger receptor cysteine-rich domain superfamily protein*, *L-rhamnose-binding lectin CSL3* and *nuclear factor erythroid 2-related factor*, in IOX and DES-exposed juveniles (Tables S4, S5, S6) (Furukawa *et al.* 2012; Watanabe *et al.* 2009; Kim *et al.* 2011), suggesting their immune system was affected. It is tempting to speculate that the up regulation of detoxifying-related genes in *M. coruscus* juveniles exposed to 10^{-7} M DES but not to IOX may explain the different survival rates recorded. Further work will be required to consolidate understanding of why *M. coruscus* juveniles had a differing susceptibility to DES and IOX and their mechanism of action.

The present study demonstrated that IOX and DES modified the cardiac performance and shell growth of juvenile mussels and had lethal effects at 10^{-6} and 10^{-7} M IOX. Heart function and shell growth were modified by both IOX and DES in *M. coruscus* juveniles. The alteration by DES of cardiac performance in mussels suggests that IOX and DES may be cardiovascular disruptors in invertebrates a function previously only reported in zebrafish (Li *et al.* 2019). In the present study, we observed that juvenile mussels treated with 10^{-8} M DES exhibited a significant reduction in shell growth, despite no abundant differential gene expression. The results of our experiments do not allow us to establish if the effect of DES was direct or indirect. It is possible that DES directly interfered with processes that regulate shell growth, possibly through impairment of neuroendocrine signaling or of the shell construction machinery. Alternatively, the modified heart function observed with both DES and IOX may have indirectly affected shell growth. An example of direct disruptive actions of DES and IOX comes from studies in zebrafish showing they disrupt normal cardiovascular development (Li *et al.* 2019; Li *et al.* 2022). While the concentration of 10^{-8} M DES employed in this study may be considered relatively elevated compared to the concentrations normally found in seawater, when considering chemical exposure, it is crucial to consider the ecological context of bivalves. *M. coruscus* as a commonly cultured bivalve species is produced in nearshore aquaculture zones within the East China Sea. Specifically, *M. coruscus* aquaculture predominantly occurs in the vicinity of Shengsi Island, located in Zhejiang Province, China. Notably, the aquaculture sites in the Shengsi Sea area are subject to persistent contamination stemming from rivers such as the Yangtze River and the Qiantang River. The continuous influx of pollutants from these rivers may lead to the gradual accumulation of contaminants within the mussels' tissues, posing potential risks to their health. Of note are the recorded concentrations of 16.9 μg/L (~ 0.1 μM)

(Qu *et al.* 2012) and 0.1 µg/L (~ 10^{-9} M) (Croll 1991), respectively of DES and IOX in some Chinese fishery water and in some UK surface water.

Our findings indicate that IOX and particularly DES interfere with a diversity of biological processes, thus rendering mussels more susceptible to adverse environmental conditions. This susceptibility is likely to be further compounded when combined with other stressors such as ocean acidification/warming and highlights the importance of managing and reducing exposure of bivalves to environmental contaminants such as IOX and DES. The effects of IOX and DES on the cardiovascular system, nervous system and neuroendocrine system of the mussel and their resilience, presumably due to the upregulation of detoxifying systems (transcriptome analysis), suggests they may be a good invertebrate model for analysing the effect of vertebrate EDCs. Overall, the results obtained in the mussel, *M. coruscus*, indicate IOX and DES have the potential to be neuroendocrine and cardiovascular disrupters in bivalves and may lead to a decline in mussel populations, which will ultimately threaten the stability and resilience of ocean ecosystems.

CHAPTER 5

General discussion and future perspectives

5.1. General discussion

A properly functioning endocrine system depends on the coordinated action of hormones and is essential for the physiological regulation and homeostasis of all multicellular organisms (Darbre 2019). The body's endocrine system can be negatively impacted by EDCs, resulting in detrimental effects on human development, reproductive function, neurological processes, cardiovascular health, metabolic regulation, and immune responses (Schug *et al.* 2011). A wide variety of EDCs are found in agricultural products, plastics, personal hygiene products, flame retardants and have become widespread contaminants in the environment and a range of disorders have been reported in animal populations living in highly contaminated areas (Tyler *et al.* 1998). DES was the first synthetic estrogen that was utilized to prevent miscarriages and other complications during pregnancy (Hunt *et al.* 2016). Despite being an effective treatment option, DES has harmful side effects, such as the increased risk of cardiac, thyroid, and reproductive abnormalities (Tournaire *et al.* 2016; Blackard *et al.* 1970; de Voogt *et al.* 1986; Palmer *et al.* 2001). Even though use of DES has been prohibited for humans since 1971 in the United States (Reed & Fenton 2013), its detrimental consequences remain a significant concern for public health that extends beyond the maternal population to include their offspring (Yamamoto *et al.* 2003; Titus-Ernstoff *et al.* 2010; Perez *et al.* 2005). IOX, an iodine-containing phenolic herbicide, is still in use and is persistent in the environment (Otsuka *et al.* 2014).

IOX and DES bind to the TH transporter, TTR (Ishihara *et al.* 2003; Morgado *et al.* 2007; Zoeller 2005) and affect the expression of TRs, deiodinases and TSH in the brain, pituitary and liver and in the marine teleost seabream (*Sparus aurata*) they modify the morphology and localization of thyroid follicles and thyrocytes *in vivo* (Morgado *et al.* 2009). In zebrafish embryos exposed to IOX and DES (0.1 μM), the development of the thyroid and heart was impaired and exhibited a strong association between modified heart morphology and altered thyroid development (Campinho & Power 2013). Furthermore, DES exhibited a more pronounced effect on heart morphology at low (0.1 μM) rather than high (1 μM) concentrations, indicating it has nonmonotonic effects (Campinho & Power 2013). The specific effects of IOX and DES on thyrocytes have been demonstrated, but it remains to be determined whether these effects are the result of direct or indirect actions. The potential role of contaminants on other developing tissues, particularly the heart and ventral aorta, which are known to interact extensively with the thyroid gland during development (Alt et al., 2006; Wendl et al., 2007; Opitz et al., 2012), must also be taken into consideration.

Chapter 2 aimed to determine how IOX and DES disrupted the crosstalk between the developing thyroid gland and cardiovascular system in zebrafish. It was found that exposure of zebrafish embryos to IOX and DES (0.1 μM) increased heartbeat frequency and reduced ventricle volume and aorta diameter. The vascular endothelial cells were isolated by flow cytometry to further understand the toxicological effects of IOX and DES on endothelial cells from blood vessels. Transcriptome analysis of endothelial cells from blood vessels exposed to IOX and DES identified the enrichment of three cardiomyopathy pathways, including hypertrophic cardiomyopathy, dilated cardiomyopathy and arrhythmogenic right ventricular cardiomyopathy. This finding reveals that IOX and DES directly affected vascular and heart development, which explains the increased incidence of heart defects observed in children born to women exposed to DES (Titus-Ernstoff et al., 2010). Given that thyroid gland development is highly dependent on the development of the heart and aorta (Alt et al., 2006; Wendl et al., 2007; Opitz et al., 2012). The data from the present study reveal that IOX and DES directly disrupt normal cardiovascular development in zebrafish embryos, probably explaining the observed modifications indirectly in thyroid gland development (Chapter 2).

Chapter 3 described the impact of chronic exposure to IOX and DES (0.1 μM) on thyroid and cardiovascular development in juvenile zebrafish that have a fully developed heart and thyroid tissue. This study contributes to the advancement of knowledge regarding the impact of a low-level concentration (0.1 μM) of IOX and DES on the development of cardiac and thyroid disorders, resembling the observed alterations in zebrafish embryos. The work carried out in early (embryo and juvenile) development in zebrafish suggests that there is no safe limit of IOX and DES exposure levels.

The endocrine system in invertebrates differs substantially from that in vertebrates and endocrine disrupting effects of DES and IOX exposure are poorly documented. Considering invertebrates are key players in maintaining ecological balance and promoting biodiversity within ecosystems, changes in their fitness and survival may have devastating environmental consequences (Chen 2021). The presence of thyroid hormone signalling genes, such as *TRs* and *iodothyronine deiodinases* in mussel species (Li *et al.* 2020b; Li *et al.* 2021; Shi *et al.* 2022) raises the possibility that DES and IOX, may be putative thyroid system disruptors, and may exert physiological effects by acting upon TRs and DIOs. Taking into consideration the cardiovascular effects of IOX and DES on fish, it was decided to study whether these chemicals affect heart function in the invertebrate mussel species *M. coruscus* (Chapter 4). Chapter 4 elucidated the potential effects of IOX and DES on the juvenile mussels to understand how IOX

and DES exert their actions on aquatic invertebrates. IOX and DES impaired heart function and some down-regulated cardiac-related genes were reported and the upregulation of multiple genes related to detoxifying xenobiotic substances suggests that detoxification processes were enhanced in the DES-exposed group. We also observed that juvenile mussels treated with low concentrations of DES exhibited a significant reduction in shell growth. The results suggest that IOX and DES may act as neuroendocrine and cardiovascular disruptors in bivalves, highlighting the possibility that compounds with endocrine-disrupting properties on vertebrates may also affect invertebrate organisms.

5.1.1 IOX and DES impair thyroid development

IOX and DES are known disruptors of thyroid function, affecting crucial developmental processes such as thyroid hormone transport (Ogilvie and Ramsden, 1988) and overall thyroid homeostasis (Morgado et al., 2009). In Chapter 2, thyroid morphology, such as the thyroid follicle number, thyroid field, and thyroid follicle area, was modified in zebrafish embryos exposed to 0.1 μM DES and IOX, indicating impaired thyroid development. These results corroborate a previous study showing that the mRNA expression of thyroid developmental and differentiation marker genes in DES and IOX-exposed zebrafish embryos were reduced (Campinho & Power 2013). Taken together the results suggest that DES and IOX impair thyroid development by directly interfering with the expression of genes associated with thyroid development and differentiation. Altered thyroid gland morphogenesis was also observed in clofibrate-exposed zebrafish larvae (Raldúa *et al.* 2008).

In Chapter 3, we further identified the effects of DES and IOX on thyroid development in zebrafish juveniles after chronic (60-days exposure) exposure. Zebrafish juveniles exposed to 0.1 μM IOX had enlarged thyroid follicles and increased Tg staining, which suggests that 60 days of exposure induced thyroid hypertrophy. Similar observations were reported in juvenile gilthead sea bream exposed to IOX for 21 days indicating this chemical causes thyroid follicle hypertrophy in fish (Morgado *et al.* 2009). The significant increase in thyroid follicle area but not the number of follicles in IOX-treated juvenile zebrafish was correlated with a significantly decreased thyrocyte cell height, suggesting that the follicle area increased but the thyroid follicle activity was lower. This finding implies that the processing of Tg into thyroid hormones is likely impaired. Our results are in general agreement with a similar study on juvenile gilthead sea bream, since IOX exposure led to enlarged thyrocytes although the authors did not identify thyroid hypertrophy, although fish had decreased serum T_4 levels compared to the control group,

implying the fish were hypothyroid (Morgado *et al.* 2009). Additionally, IOX exposure in rats has been associated with liver hypertrophy and thyroid tumours (European-Commission 2004), which indicates the response to IOX may vary between different species. The results presented herein show IOX exposure caused reduced thyrocyte activity and thyroid follicle hypertrophy in zebrafish juveniles.

When comparing embryonic and juvenile zebrafish, our findings revealed distinct and stage-dependent responses to IOX and DES exposure. IOX treatment resulted in a significant reduction in the thyroid follicle area and thyroid follicle number in zebrafish embryos. This finding suggests that IOX may interfere with thyroid organogenesis during early development, leading to a decrease in thyroid follicle size. Conversely, IOX-exposed juveniles exhibited a significant increase in thyroid follicle area, and no significant change was observed in thyroid follicle numbers. These results indicate that IOX may exert different actions on the developing thyroid of embryos compared to the developed thyroid in juveniles. Regarding DES exposure, there was a significant decrease in the thyroid follicle area in zebrafish embryos and this indicates that like IOX it influences thyroid organogenesis during embryonic development. However, no overt differences in the histology of the thyroid were observed in DES-exposed juveniles, although if there were effects on TH production was not assessed. Overall, our findings highlight the stage-dependent effects of IOX and DES on the thyroid and support the notion that the impact of these chemicals on the HPT axis varied with developmental stage and duration of exposure. Further studies are warranted to elucidate the underlying mechanisms and long-term consequences of IOX and DES exposure on thyroid function in zebrafish.

5.1.2 The cardiovascular system is the target of IOX and DES

EDCs are normally defined as chemicals that directly interfere with or disrupt endocrine systems. Previous studies reported that prenatal exposure to DES increased the incidence of cardiovascular defects in their offspring, including ventricular septal disease, tetralogy of fallout, and atrial septal defects (Titus-Ernstoff *et al.* 2010; Tournaire *et al.* 2016). The study on which the present thesis was based revealed that in concordance with the outcome of human studies, IOX and DES exposure caused alterations in the ventricle and heart morphology in zebrafish embryos, as revealed by the altered mRNA expression of *cmlc2*, a marker gene associated with the final differentiation of cardiomyocytes (Campinho & Power 2013). Nevertheless, there is a lack of data about the effects of DES on heart development and function in vertebrates.

To extend understanding of the effects of IOX and DES on heart rate and heart morphology (Chapter 2), zebrafish embryos were exposed to these chemicals (0.1 μM) and were observed to have an increased heart rate, altered heart morphology, reduced ventricle volume, and reduced aorta diameter. The observed reduction in ventricle volume in IOX and DES-exposed embryos suggests that there was a decrease in the amount of blood being ejected from the ventricles, which may be linked to the increased heart rate observed. The changes in cardiac function provide evidence of possible structural abnormalities or developmental alterations induced by IOX and DES exposure, which could explain cardiac dysfunction in zebrafish embryos. Building on the observations in zebrafish embryos we showed that exposure of juveniles for 60 days to IOX and DES had a similar effect on ventricle volume and morphology (Chapter 3). The results of IOX exposure in juveniles suggested it may induce cardiac remodeling, potentially leading to altered ventricular function. In contrast, although DES exposure did not lead to a significant change in ventricle volume compared to control juveniles, it resulted in the presentation of ventricular malformations. The obvious irregularities on the external surface of the ventricle and atrium in DES-treated animals suggest that DES may induce structural alterations possibly through an action on cardiac muscle in the juvenile zebrafish heart. Since cardiac deformities were observed in response to IOX and DES exposure in embryo and juvenile zebrafish this highlights a not previously reported vulnerability of developing cardiovascular tissues. Overall, the results in zebrafish support the notion that even low-dose EDC exposure may have lasting implications for cardiac health.

To assess if IOX and DES have cardiovascular effects in invertebrates, the marine mussel *M. coruscus* was studied (Chapter 4). Based on the findings presented, exposure to IOX and DES resulted in a significant reduction in heart rate, indicating the potential of these chemicals to disrupt the cardiovascular system in both invertebrates and vertebrates. The effects of IOX and DES were influenced by their concentration and the duration of exposure and highlight the importance of understanding the dose-response relationship and the temporal dynamics of EDCs exposure on heart function in marine organisms. Moreover, the reversibility of the heart rate response to prolonged exposure to DES in *M. coruscus* highlights the potential for adaptation or compensatory mechanisms in response to EDCs. The potential mode of action of IOX and DES remains to be established but studies have shown that MMI and propylthiouracil (PTU) anti-thyroid agents commonly employed to investigate the influence of TH synthesis and metabolism in vertebrates influence bivalves (Li et al., 2021). The effects of MMI and PTU in bivalves may be explained by the recent identification of thyroid receptor (*TR*) and

iodothyronine deiodinase (*DIO*) genes in a study on the mussel *M. coruscus* (Li et al., 2020; Li et al., 2021; Shi et al., 2022). The presence of TR and DIO suggests a potential mechanism by which, thyroid disruptors like DES and IOX could influence mussel physiology. In *M. coruscus*, these compounds notably diminish larval metamorphosis, with MMI specifically leading to impaired growth performance (Li et al., 2021). These findings highlight the shared susceptibility of both vertebrates and an invertebrate species to MMI and PTU, emphasizing that although the thyroid axis is not totally conserved there is sufficient conservation of thyroid hormone signaling pathways and actions to provoke disruption of similar physiological processes (metamorphosis and growth).

The transcriptome analysis of endothelial cells of IOX and DES-exposed embryos (Chapter 2) revealed that hypertrophic, dilated and arrhythmogenic right ventricular cardiomyopathy gene pathways were significantly changed and identified a potential link between chemical exposure and adverse cardiac development for future exploration. The response of endothelial cells to IOX or DES diverged, and different subsets of genes and gene networks were affected. Taken together the results indicate that both IOX and DES have adverse impacts on heart development with similar morphological effects observed, although the genetic pathways underpinning the phenotype diverged.

Based on the observed increased heartbeat, lower chamber volume and reduced diameter of the ventral aorta in zebrafish embryos, it is expected that blood flow dynamics may be compromised in both IOX and DES-treated embryos as compared to controls (Chapter 2). Vascular stretching can be induced by both blood flow and pressure through various biological or physical mechanisms, with flow causing vasodilation and pressure resulting in mechanical distension (Lu & Kassab 2011). Blood flow imposes shear stress on the endothelial cells, while cardiac pulsation generates circumferential stretch and imposes mechanical stimulation on both endothelial cells and smooth muscle cells (Boselli *et al.* 2015; Lu & Kassab 2011; Campinho *et al.* 2020). Despite similar heart and ventral aorta morphological endpoints observed in zebrafish embryos, the molecular responses of endothelial cells to IOX and DES differed. The differential effects of IOX and DES exposure on developing endothelial cells emphasize the complexity of the responses elicited by these chemicals within the cardiovascular system. Based on our findings, it appears that both IOX and DES have an impact on blood flow, but it is unclear if this is a direct influence on endothelial cells, affecting their vasodilatory or vasoconstrictive properties, or is a consequence of their influence on cardiomyocytes. Additionally, it is possible that both endothelial cells and cardiomyocytes are affected by IOX

and DES, leading to increased heartbeat and so a modification in blood flow regulation. Further investigation into the underlying cellular and molecular pathways involved in the observed effects of IOX and DES on blood flow is warranted to elucidate the precise mechanisms and potential implications for cardiovascular health.

The development of blood vessels has been shown to influence the morphology of the thyroid gland and to modulate thyroid morphogenesis in zebrafish (Alt *et al.* 2006b; Opitz *et al.* 2012). Thyroid development is highly dependent on the development of the heart and aorta (Opitz *et al.* 2012; Wendl *et al.* 2007; Alt *et al.* 2006b). Moreover, the observed impairment of thyroid development in zebrafish with modified heart function (Campinho & Power 2013), provides compelling support for the interconnected nature of these developmental processes. We suggest that exposure to IOX and DES may disrupt normal cardiovascular development, consequently influencing thyroid gland development in zebrafish embryos. The results reveal the pernicious effects of low-dose exposure to DES in zebrafish, a vertebrate and important biomedical model and highlight the need for further studies in other vertebrates, including humans (Titus-Ernstoff *et al.* 2010). Overall, the thesis highlights the critical role of cardiovascular development in shaping thyroid gland development and indicates the action of EDCs is complex.

5.1.3 DES exposure inhibits growth and activates the detoxification process in mussels

The widespread presence of DES in the environment can be attributed to its use as a growth promoter in livestock production (Brennan *et al.* 2006; McLachlan *et al.* 1984; Bravo *et al.* 2007). DES has been found to have diverse impacts on different organisms within the environment. The effects of DES vary across different species, as evidenced by contrasting observations in neonatal mice and an invertebrate (*Daphnia magna*). While neonatal mice showed an increase in body weight at lower doses, high concentrations did not yield the same response (Newbold *et al.* 2004). Exposure of *Daphnia magna* to DES resulted in decreased moulting frequency and hindered growth after 21 days of exposure, demonstrating the adverse impact of DES on this invertebrate species (Baldwin *et al.* 1995; Brennan *et al.* 2006). In *M. coruscus* juveniles in the present thesis, the severity of shell growth inhibition by DES revealed a complex dose-dependent response (Chapter 4). The effects of DES on ion transport and intracellular pH in the mantle of *Anodonta cygnea* (Bivalvia) (Alves & Oliveira 2013), may explain its effect on shell growth in *M. coruscus*. The different effects of DES and IOX on the survival and shell growth of *M. coruscus* juveniles emphasize the need for more studies into

their mode of action. In our studies, DES exposure led to modifications in gene transcription, particularly affecting the expression of growth-related genes associated with cell cycle/proliferation, energy metabolism, and protein metabolism. Furthermore, the resemblance between the growth inhibition in *M. coruscus* juveniles caused by low doses of DES and that induced by MMI (Li *et al.* 2021) suggests the potential for DES to function as an endocrine disruptor. This emphasizes the need for comprehensive studies of the endocrine system and its disruption by chemicals in aquatic invertebrates like bivalves.

The observed upregulation of detoxification-related genes and concurrent downregulation of immune function-related differentially expressed genes in *M. coruscus* juveniles exposed to 10^{-7} M DES raises intriguing questions regarding the potential impact on their immune system and survival rates. The upregulation of cytochrome P450 and sulfotransferase genes in DES-treated juveniles suggests the activation of detoxification-related molecular pathways in response to xenobiotic exposure. This finding aligns with previous research demonstrating elevated cytochrome P450 content in various aquatic invertebrates upon exposure to xenobiotic compounds, such as pesticides, fungicides, and specific chemicals like benzo(a)pyrene, 3,3′,4,4′-tetrachlorobiphenyl, and Na-phenobarbital (Michel *et al.* 1993; Galli *et al.* 1988; Livingstone 1988). I propose that the induction of cytochrome P450 and sulfotransferase genes in DES-treated mussel juveniles, reflects an adaptive response aimed at enhancing their detoxification capacity. This molecular response may serve as a protective mechanism to counteract the deleterious effects of xenobiotics, which are known to compromise immune function and increase susceptibility to disease in aquatic organisms. Moreover, the absence of similar changes in the IOX and DES-exposed juveniles indicates a specific effect of DES on the regulation of detoxification-related genes.

The differential survival rates recorded between DES-exposed and IOX-exposed juveniles further support the potential link between the observed gene expression patterns and survival outcomes. This led us to speculate that the upregulation of detoxifying genes in DES-exposed juveniles, without a corresponding impact in the IOX-exposed group, explains the disparity in survival rates. It suggests a potential link between the modulation of detoxification-related genes, immune function, and survival outcomes in *M. coruscus* juveniles following DES exposure. While this concentration of 10^{-8} M DES may be considered relatively elevated compared to typical levels found in seawater, it is essential to recognize that marine mussels are filter feeders inhabiting nearshore ecosystems and are continuously exposed to a wide range of environmental contaminants present in the water column. As a result, even at low

environmental concentrations pollutants can accumulate within bivalve tissues over time, potentially reaching levels that elicit a biological response. Understanding the adverse outcomes caused by IOX and DES exposure and other environmental contaminants is crucial for informing effective conservation and management strategies linked to controlling environmental pollutants.

5.2. Conclusions and future perspectives

5.2.1 Conclusions

This thesis work built on previous observations that the commonly used herbicide IOX and the non-steroidal estrogen, DES, have profound effects on thyrocyte development and differentiation and heart function in zebrafish embryos (Campinho & Power 2013). The aim of my thesis was to identify the crosstalk that occurs between thyroid tissue development and the heart and angiogenic systems and how they are modified by IOX and DES in zebrafish. I revealed that exposure to IOX and DES in zebrafish embryos and juveniles significantly altered thyroid and heart development and function.

- Thyroid follicle hypertrophy was observed in IOX and DES-treated zebrafish juveniles and IOX caused ventricle deformation and increased ventricle volume, whereas DES altered ventricle morphology.
- In IOX and DES-treated zebrafish embryos, the heartbeat increased in frequency, and the ventricle volume and ventral aorta diameter were reduced. Evidence was found for the first time using transcriptomics of embryonic endothelial cells of the main molecular changes caused by IOX and DES and revealed genes associated with hypertrophic, dilated, and arrhythmogenic right ventricular cardiomyopathy were significantly changed. The transcriptome data also indicated that developing endothelial cells are differentially affected by IOX and DES exposure and compound-specific toxic effects were found in IOX and DES-treated embryos. We found that although both IOX and DES exerted adverse impacts on heart development with similar morphological effects observed in zebrafish embryos and juveniles, the response of genes involved in cardiovascular function to IOX or DES was quite different, with different subsets of genes and gene networks being affected in each group. We propose that IOX and DES directly affect cardiovascular development and that through this action they indirectly impair the developing thyroid gland and presumably the thyroid axis and disrupt thyroid homeostasis.
- A comparative study was performed showing that exposure of an invertebrate, the mussel,

to IOX and DES also resulted in cardiac-related effects and caused a significant decrease in mussel heart rate. The results indicate that IOX and DES act as EDCs and exert consistent adverse effects on cardiac performance in both vertebrates and invertebrates. Additionally, DES exposure inhibited shell growth and analysis of transcriptomes gave insight into the chemical-specific transcriptional effects caused by DES and IOX but also revealed a capacity of the mussel to combat exposure through the increased expression of detoxification-related genes.

5.1.2 Future perspectives

This thesis comprehensively examined the broad-ranging adverse effects of IOX and DES in vertebrate (zebrafish) and invertebrate (*M. coruscus*) species. Examining these EDCs in different organisms' representative of the main animal divisions gave insight into the common and divergent effects of IOX and DES and uncovered potential synergistic or antagonistic effects that single-species studies might overlook. By using a comparative approach to chemical hazard assessment across multiple species, a more comprehensive and mechanistic understanding can be gained of how toxicity disrupts biological processes that are crucial for health and are shared among various animals, including humans. For instance, vertebrate fish (*Hypseleotris compressa*) and tadpoles (*Limnodynastes peronii*) demonstrated morphological changes when exposed to coal mine wastewater, whereas invertebrate species such as planarian (*Dugesia* sp.) and cladoceran (*Daphnia carinata*) were found to be particularly sensitive to this wastewater (Lanctôt et al., 2016). Similarly, recent efforts in predicting the developmental toxicity of chemicals found in the environment using zebrafish and marine mussels are proving valuable for human health risk assessments (Pinheiro et al., 2023). The use of diverse species in comparative assessments enhances our understanding of shared molecular targets and conserved pathways across species, thereby providing insights that benefit both human and environmental health. The results of my thesis emphasized the environmental risks of EDCs and reinforced the need for further studies to understand their impact at both an individual, species and ecosystem level.

Continued exploration of the endocrine disrupting effects of IOX and DES, requires a) studies to improve the understanding of the endocrine system in marine invertebrates and b) studies targeting the thyroid related axis in vertebrates and invertebrates, particularly THs and TSH. A focused research effort aimed at characterizing the endocrine system in bivalves, both organization and function and an improved understanding of how EDCs work at a molecular

and cellular level and the level in the organisms along with an understanding of dose-response relationships and long-term impacts of EDCs is a priority. Specifically, in zebrafish more detailed mechanistic studies are essential to uncover the pathways associated with endothelial functions, such as hypertrophic, dilated, and arrhythmogenic right ventricular cardiomyopathy through which IOX and DES disrupt cardiovascular development. Advanced molecular techniques, such as the CRISPR-Cas systems, single-cell RNA sequencing, and proteomics, can provide detailed insights into the cellular and molecular alterations induced by these EDCs. A thorough understanding of these mechanisms is critical for developing targeted interventions aimed at mitigating their adverse effects.

In addition to the research focus on vertebrates, it is crucial to consider invertebrates such as the highly abundant and diverse bivalves in the exploration of endocrine-disrupting effects of IOX and DES. To address this, research efforts should be directed toward a comprehensive characterization of the endocrine system in bivalves, encompassing both its organization structure and functional dynamics. It is essential to investigate the effects of EDCs on bivalve physiology, including reproductive, developmental, and growth processes. This includes elucidating how EDCs affect bivalves at a molecular and cellular level, especially in larval developmental stages, as well as understanding dose-response relationships, functional consequences and long-term impacts specific to these organisms. Understanding how EDCs disrupt the bivalve endocrine systems-particularly during sensitive early life stages such as larval development, can reveal broader ecological impacts, including effects on population dynamics, community structure, and ecosystem functioning.

Despite being banned for human use in the USA since 1971 (Reed and Fenton, 2013), DES continues to pose a significant threat to human health due to its extensive utilization in livestock production, with recorded concentrations reaching up to 24.9–102 ng L^{-1} and 7.2–16.9 μg L^{-1} in some Chinese rivers and fisheries waters, respectively (Chen et al., 2009; Qu et al., 2012; Zhang et al., 2012), as well as in sediments of Mediterranean aquatic environments (Pojana et al., 2007). Enhanced environmental monitoring programs covering both vertebrates and invertebrates are needed to track the prevalence and distribution of IOX, DES and other EDCs in aquatic ecosystems. These efforts must include rigorous assessments of their biological effects in exposed organisms, persistence, bioaccumulation dynamics, and potential biomagnification within aquatic food webs. Such data are crucial for informing evidence-based regulatory policies aimed at reducing exposure to EDCs and minimizing their environmental impact. Furthermore, interdisciplinary research collaborations are necessary to address

knowledge gaps regarding the ecological and human health implications of IOX and DES contamination. Integrating ecological risk assessments with epidemiological studies will elucidate the potential health risks for ecosystems and animals associated with chronic exposure to these chemicals. Such holistic approaches will support the development of adaptive management strategies and targeted interventions aimed at preserving aquatic ecosystem health and safeguarding public health.

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