SYSTEMATIC, MOLECULAR, AND (ECO)TOXICOLOGY INVESTIGATIONS OF ENDOCRINE MECHANISMS IN MOLLUSCS

A thesis submitted for the degree of Doctor of Philosophy

by

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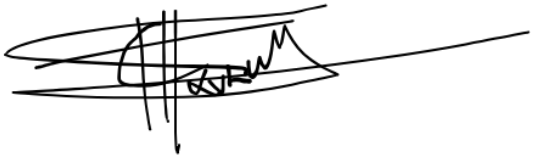
December 2024

Declaration

I, Konstantinos Panagiotidis, confirm that the work presented in this thesis is my own and has not been submitted for any other degree. Where information has been obtained from other sources, I confirm this has been explicitly stated in the thesis.

Signature: 

Date: 21/12/2024

Abstract

Molluscs, integral to ecosystems as habitat engineers and food sources, are extremely sensitive to environmental pollution. While these animals can serve as early indicators for endocrine-disrupting chemicals (EDCs), current testing guidelines lack mechanistic underpinning due to our poor understanding of molluscan endocrinology. Previous molecular investigations have demonstrated that molluscan genomes lack essential enzymes and nuclear steroid receptors to induce vertebrate steroidogenesis. However, some steroidogenic enzymes do appear to exist in molluscs, but their function remains unknown. These include the enzymes 5-alpha-reductases (5αR1, 5αR2) that metabolise testosterone to dihydrotestosterone in vertebrates. Previous work has shown that developmental exposure of *Biomphalaria glabrata* embryos to pharmaceutical 5αR disruptors (dutasteride or finasteride) causes a highly reproducible and dose-dependent disruption to development, resulting in altered shell morphology. However, the impact of dutasteride on adult *B. glabrata* remains unexplored. This doctoral thesis aimed to fill significant knowledge gaps relating to endocrinological pathways in molluscs, investigate the expression of 5αR genes in the embryonic *B. glabrata* and evaluate the effects of dutasteride disruption on *B. glabrata* adults. Using the systematic review guidelines PRISMA, data on the occurrence of hormones, hormone receptors and hormone-metabolising enzymes in Mollusca was identified from 145 eligible studies (published between 2012-2021) and was systematically collected, evaluated and visualised in an openly accessible interactive database. By developing a RT-qPCR assay and validating stable reference genes across embryonic stages of *B. glabrata*, the transcript expression of genes encoding 5αR1 and 5αR2 was assessed. Lastly, the effects of dutasteride on *B. glabrata* adults were evaluated using a 21-day flow-through exposure and an OECD 243 static-renewal test, both with nominal exposure concentrations of 0, solvent control, 1 µg/L, 3.2 µg/L, 10 µg/L, 32 µg/L and 100 µg/L dutasteride. The systematic investigations revealed that most studies assessed were found to be heavily skewed towards vertebrate-type sex steroidogenesis, with over 62% measuring 17β-estradiol in mollusc tissues, despite unconvincing evidence that molluscs can biosynthesise vertebrate-type steroids. However, a limited number of studies are now looking beyond vertebrate-type sex steroids and diverting their focus towards the role of thyroid hormones, phytosterols (plant sterols) and ecdysteroids (insect steroids) in molluscs. The results of the RT-qPCR experiments suggest that 5αR1 and 5αR2 genes are not differentially expressed across day 2 - day 4 post-oviposition embryonic stages. Whereas the minimal variation of the five candidate reference genes *UBI, TUB, EF1a, ACTIN-1, and H2A* across day 2 - day 5 post-oviposition stages, makes them reliable models for normalising gene expression in embryonic *B. glabrata*. Importantly, under static-renewal conditions, dutasteride was observed to cause unexpected mortalities and significant declines

in reproductive output of adults at concentrations above 3.2 µg/L and 32 µg/L, respectively. Together, the findings of this thesis provide novel insights into underexplored endocrinological pathways in molluscs that could be targets of endocrine disruption. Moreover, novel findings on the expression of 5αR in embryonic *B. glabrata* and data on the reproductive toxicity of dutasteride may help enhance our understanding of the role of 5αR in this species.

Acknowledgements

First and foremost, I would like to thank the London NERC Doctoral Training Partnership for funding my PhD project and providing opportunities to develop my skills. I wish to express my sincere gratitude to my supervisor, Dr Alice Baynes, who believed in my abilities and allowed me to join her research group. Apart from being a great supervisor, Alice has been a true mentor, allowing me to experiment, make mistakes and learn without judgment. She has always encouraged me to participate in scientific conferences, science communication competitions and even to take on a policy internship, which have all been instrumental in my training as a researcher. I am beyond thankful for the opportunities that Alice, Brunel University London and NERC have given me which I will forever cherish. I would also like to thank Dr Thomas Miller for being part of my supervisory team and providing technical support and valuable feedback whenever needed. Moreover, I would like to thank Prof Andreas Kortenkamp and Dr Olwenn Martin for encouraging me to conduct and supporting me through the development of the systematic evidence map. I am also grateful to the technical team of the Environmental Sciences Division, including Dr Nicola Beresford, Marta Straszkiewicz and Jolene Jalota for providing training, feedback and assistance, particularly with my chemical exposures. A heartfelt thank you goes to Giang Duong for extensively training me on RT-qPCR and for helping me develop the technical skills needed for those demanding molecular experiments. My gratitude also goes to the entire team at the ecotoxicology laboratory Scymaris, who welcomed me into their facilities to complete part of my PhD work. Specifically, I want to thank Dr Richard Maunder for assisting with my experiments amid unexpected challenges. My sincere appreciation goes to Pete Johnson, who assisted with my chemical analysis and ensured that my study was accurate and successful. My thanks also go to the technical team, including Deana, Sam and Emma for helping with the snail husbandry. A special thank you goes to my friends Maria, Eleni, Vasiliki, Yiannis, Karolina, Andrew, Alvaro, Dimitris and Panos who supported me in times of stress and kept me going with their encouragement, company and humour. I would also like to thank my office buddies, Jasmin, Maurice, Rita and Gideon, for the wonderful times we had on campus, the insightful academic conversations, and for being there when I was feeling low. Finally, I would like to thank my parents and sister for their support throughout my years abroad, especially during my bachelor's and master's degrees. Lastly, I would like to thank my partner, Filip Glatz, for his constant support and encouragement during the most stressful and challenging periods of my PhD.

Dissemination of thesis findings

- **SETAC Europe 34th Annual meeting (Oral presentation)** 05/2024
  A Systematic Evidence Map of Molluscan Endocrinology and an Investigation of the Effects of a Pharmaceutical 5-alpha-Reductases Inhibitor, Dutasteride, on a Freshwater Gastropod Molluscs
- **CHMLS Conference – Brunel University London (Oral presentation)** 01/2024
  Decoding Molluscan Endocrinology: Systematic Mapping and Critical Appraisal of Existing Research
- **Wageningen x Brunel Research Day (Oral presentation & Chair)** 06/2023
  Underlying mechanisms of drug-induced endocrine disruption in gastropod shell to be unravelled by fundamental molecular diagnostics.
- **SETAC Europe 33rd Annual Meeting (Poster presentation)** 05/2023
  Underlying mechanisms of drug-induced endocrine disruption in gastropod shell to be unravelled by fundamental molecular diagnostics.
- **Molluscan Forum (Poster presentation)** 11/2022
  Quantitative gene expression analysis to shed light on the role of 5-alpha-reductase in the development of a freshwater gastropod snail.
- **UKRI/NERC DTP conference (Poster presentation)** 09/2022
  Quantitative gene expression analysis to shed light on the role of 5-alpha-reductase in the development of a freshwater gastropod snail
- **World Congress of Malacology (Poster presentation)** 08/2022
  Quantitative gene expression analysis to shed light on the role of 5-alpha-reductase in the development of a freshwater gastropod snail.
- **CHMLS Conference – Brunel University London (Poster presentation)** 11/2021
  Sex Hormone Biosynthesis in Mollusca: Protocol for a Systematic Evidence Map

Honours and awards

- Conference Prize Award (£750) – Brunel Graduate School (2024)
- Travel grant (£500) – Royal Society of Chemistry (2024)
- 2nd prize winner at 3-Minute-Thesis (3MT) competition – Brunel University London (2023)
- Travel grant (£500) – Malacological Society of London (2022)
- 1st prize winner at CHMLS Conference (Poster presentation) - Brunel University London (2021)

Publications

- **Panagiotidis, K**. et *al.*, ‘Mapping Molluscan Endocrinology: A Systematic and Critical Appraisal’. *Biological Reviews* (under review)
- **Panagiotidis, K.** (2022) ‘Protocol for a systematic evidence map of sex hormone biosynthesis in Mollusca’. doi: 10.5281/zenodo.7061510.

Contents

Chapter 1: General Introduction

1. Overview

Pollution derives from the existence of unfavourable substances in the natural environment which are known to cause adverse, deleterious or damaging effects (Ramakrishnan *et al.*, 2011). Pollutant substances range from man-made chemicals to naturally occurring organic and inorganic compounds. They occur locally and globally, influencing soil, air and water in various ways. Depending on their chemical nature or the rate they spread, pollutants can cause deleterious effects on the elements of an ecosystem (Ramakrishnan *et al.*, 2011).

The first signs of chemical pollution that received public attention were reported by Rachel Carson, whose book "Silent Spring", published in 1962, documented the unfavourable effects of pesticides, such as dichlorodiphenyltrichloroethane (DDT), on bird, fish and human populations (Carson, 1962; Bouwman et al., 2012). The publication of "Silent Spring" helped to differentiate environmental toxicology from classical toxicology, ultimately leading to the establishment of the subdiscipline known as ecotoxicology. Ecotoxicology examines the effects of toxic substances on an ecosystem and, unlike classical toxicology, refers to the ecological impacts of pollutants instead of the poisonous effects on sole organisms (Kendall et al., 2001). Ecotoxicology aims to understand the interaction, transformation and fate of foreign substances, also known as xenobiotics, on organisms, populations and ecosystems. To elucidate those phenomena, ecotoxicology encompasses a range of approaches that stem from various scientific fields including analytical chemistry, molecular biology, physiology, and behavioural ecology (Bard, 2008). To understand the behaviour of a chemical, it is important to estimate the concentration of that chemical in different environmental mediums such as air, water and soil. It is also necessary to evaluate how that chemical is transported within and between those mediums as well as how it is metabolised, stored, degraded or concentrated within them (Kendall *et al.*, 2001).

A fundamental aspect of ecotoxicology is evaluating the effects of chemical pollution on aquatic organisms. Pharmaceuticals are a major source of aquatic pollution and have become prevalent in various water bodies including surface water, groundwater and freshwater habitats (Ortúzar *et al.*, 2022). These compounds enter waterways via different sources, including agricultural runoff, effluents from pharmaceutical factories, or household wastewater (Rzymski, Drewek and Klimaszyk, 2017; Ortúzar *et al.*, 2022). In particular, urban wastewater is considered one of the main sources of pharmaceutical pollution in the aquatic environment (Gracia-Lor *et al.*, 2012; Massima Mouele *et al.*, 2021). Some of the most common types of pharmaceuticals found in wastewater include antibiotics, anti-depressants, antiretroviral drugs, non-steroidal anti-inflammatory drugs and endocrine disruptors (Roberts and Thomas,

2006; Rzymski, Drewek and Klimaszyk, 2017; Gómez-Canela *et al.*, 2021; Ortúzar *et al.*, 2022). The significant presence of pharmaceuticals in wastewater results from their incomplete degradation into mineral components, which often causes their retention in the sewage sludge (Radjenovic, Petrovic and Barceló, 2007; Matesun *et al.*, 2024). As a result, many pharmaceuticals that enter the water environment can remain biologically active for prolonged time periods (Gómez-Canela *et al.*, 2021). Whilst in the water, the active pharmaceutical ingredients (APIs) of these substances can undergo biotransformation and biodegradation processes, resulting in alterations to their physiochemical and pharmacological characteristics (Gómez-Canela *et al.*, 2021). For example, after biotransformation, some APIs may produce products of greater toxicity, higher environmental persistence and higher concentration compared to their starting compounds (Rzymski, Drewek and Klimaszyk, 2017).

2. The endocrine system and EDCs

Endocrine-disrupting compounds (EDCs) include pharmaceuticals and other chemicals that interfere with the normal function of hormones. Hormones are chemical substances that regulate various physiological processes such as growth, development and reproduction (Bertram *et al.*, 2022). Various EDCs have been shown to affect a plethora of hormonal systems in different organisms, including the oestrogen, androgen and thyroid signalling systems (Söffker and Tyler, 2012). Pharmaceuticals with endocrine-disrupting properties, such as synthetic hormones (used in contraceptive and hormone replacement therapies (Darbre, 2015)) or drugs like dutasteride (DUT) and finasteride (FIN) (that are used to treat prostate conditions in humans (Nickel *et al.*, 2011)), are designed to target and interfere with specific hormonal pathways. On the other hand, pesticides such as DDT or antifouling agents such as tributyltin (TBT), also fall under the endocrine-disrupting category of chemicals (Bertram *et al.*, 2022). Many EDCs have also been shown to bioaccumulate and bioconcentrate in various aquatic organisms, including molluscs and fish (Gatidou, Vassalou and Thomaidis, 2010; Álvarez-Muñoz *et al.*, 2015), causing a range of adverse health effects. However, to better understand how these chemicals can interact with organisms, it is important to understand the structure and function of the endocrine system they affect.

The vertebrate endocrine system is made up of specialised cells that produce, store and secrete hormones. Hormones are divided into three major classes based on their chemical structure. These are (1) peptide and protein hormones, (2) steroid hormones and (3) amino acid-related hormones (Norman and Litwack, 1997a). These compounds are produced inside the endocrine glands or in restricted cell groups (e.g. cells of the pancreas) and are transported to other organs through the bloodstream (Brück, 1983). Hormones exert their

action by first interacting with their respective receptors. The type of interaction hormones will have with their receptors depends, to some extent, on their chemical structure (Litwack, 2022). For example, peptide and protein hormones interact with receptors that are membrane-spanning, meaning they do not need to enter the cell to deliver their message. In contrast, steroid hormones enter the cell and bind to receptors located either in the cytoplasm or in the nucleus. Whereas, amino acid-related hormones can vary between each other, as some interact with intracellular receptors and others with membrane-spanning receptors (Litwack, 2022). The synthesis of hormones also depends on their chemical structure. For example, steroids and amino-acid-related hormones are produced from precursor molecules (e.g. cholesterol) through the activity of hormone-metabolising enzymes (Wilkinson and Brown, 2015). Peptide and protein hormones, on the other hand, are produced by the processes of transcription and translation, where genes (composed of DNA) transcribe messenger RNA (mRNA) that is later translated into a protein (Wilkinson and Brown, 2015; Litwack, 2022).

3. Receptor-mediated endocrine disruption

Two of the most well-studied mechanisms by which EDCs exert their effects are receptor-mediated and non-receptor-mediated pathways. Many EDCs can interfere with the action of sex steroid receptors, disrupting an organism's sexual development and reproduction. Sex steroid agonists are chemicals that mimic the action of steroid hormones, bind to their respective receptors and activate them (Fig. 1.1) (Söffker and Tyler, 2012). A well-documented sex steroid agonist is 17α-ethinylestradiol (EE2), a synthetic oestrogen used in oral contraceptive pills and fertility treatments, which binds to the oestrogen receptor (Söffker and Tyler, 2012). EE2 has been demonstrated to cause various detrimental effects on freshwater fish at different concentrations, including decreases in egg production, increased mortalities, and morphological reproductive abnormalities (Scholz and Gutzeit, 2000; Xu *et al.*, 2008; Zha *et al.*, 2008). The synthetic androgen 17α-methyltestosterone, which is used to treat male androgen deficiency, acts as an agonist of the androgen receptor (AR) (Fernández *et al.*, 2018). Despite its beneficial use in humans, research has shown that 17α-methyltestosterone can cause severe developmental abnormalities in zebrafish during their early life stages of (Rivero-Wendt *et al.*, 2016).

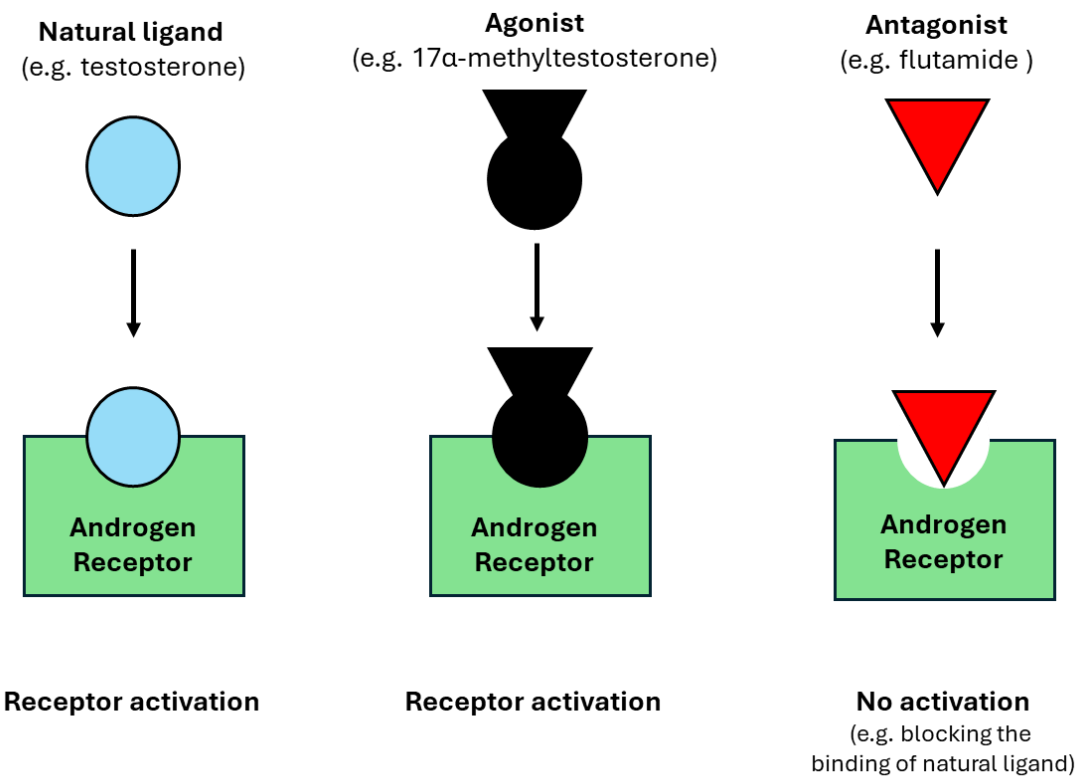


Figure 1.1. Receptor-mediated disruption through the binding of a drug agonist (e.g. 17α-methyltestosterone) or an antagonist (e.g. flutamide), instead of a natural ligand (e.g. testosterone), to the androgen receptor (AR). The figure was adapted from Hackney, (2018).

On the other hand, sex steroid antagonists are compounds that bind to sex steroid receptors but inhibit their activation by blocking other hormones from binding to them (Fig. 1.1) (Wierman, 2007). For many years, anti-androgenic compounds, including AR antagonists, have been reported to exist in both UK (Jobling *et al.*, 2009) and European rivers (Urbatzka *et al.*, 2007). Common AR antagonists include the pharmaceuticals flutamide and bicalutamide which are commonly used to treat prostate cancer in humans. However, research has shown that exposing fathead minnows (*Pimephales promelas*) and Murray River rainbowfish (*Melanotaenia fluviatilis*) to these AR antagonists leads to reproductive impairments and alterations in their sexual characteristics (Panter *et al.*, 2012; Bhatia *et al.*, 2014).

4. Non-receptor-mediated endocrine disruption

Many EDCs can also exert endocrine-disrupting activity in organisms via nonreceptor-mediated mechanisms. For example, EDCs can interfere with proteins that regulate hormone activity. By competing with active hormones and attaching themselves to hormone-binding

proteins, EDCs can indirectly affect the activation of steroid receptors, thus causing changes in hormonal signalling (Rizzo *et al.*, 2023). Moreover, EDCs can interfere with the metabolism of steroid hormones by inhibiting the activity of necessary hormone-metabolising enzymes (Fisher, 2004). For example, the pharmaceutical DUT, which is used to treat benign prostatic hyperplasia by inhibiting the steroidogenic enzymes 5-alpha-reductases (5αR1 and 5αR2) and the conversion of testosterone to the more potent dihydrotestosterone, has been shown to result in reproductive alterations and decreases in fecundity in fathead minnows (*Pimephales promelas*) (Margiotta-Casaluci, Hannah and Sumpter, 2013). Notably, DUT also exhibited different disrupting activities between vertebrate species, as it was shown to increase testosterone (T) concentrations in human males (GSK, 2001), but reduced T levels in the bloodstream of male fish (Margiotta-Casaluci, Hannah and Sumpter, 2013).

5. Ecotoxicity testing for endocrine disruption

Given the range of mechanisms through which EDCs can generate their effects, a robust testing framework is needed to evaluate their impacts on organisms. Standardised ecotoxicity testing methods, such as those developed by the Organisation for Economic Cooperation and Development (OECD), can be used to assess the effects of chemicals on individual organisms. Generally, ecotoxicity tests can be separated into three main categories: (1) those that measure effects at the sub-organismal level; (2) those that measure effects at the whole-organismal level; and (3) those that measure effects at the population and community level (Schuijt *et al.*, 2021).

Sub-organismal ecotoxicity tests can measure biological responses using biomarkers or *in vitro* bioassays. Usually, sub-organismal tests are used to identify early signs of organism sensitivity in response to chemicals. The biomarkers included in these tests, can be used to assess changes at the molecular, cellular, biochemical, histopathological, and physiological levels in response to *in vivo* chemical exposures (Smit *et al.*, 2009). For example, the OECD TG 234 can be used to evaluate the effects of EDCs on the sexual development of fish *in vivo* (OECD, 2011). By chemically exposing organisms for 60 days, sex-specific changes in the vitellogenin concentrations and gonadal histopathology are assessed. Vitellogenin is a protein used by fish for egg development, where changes in its concentration can indicate disruption in the steroidogenic enzyme aromatase. Coupled with observations on sex ratios that are obtained through histopathology, these findings can be used to elucidate the mode of action (MoA) of EDCs in these organisms (OECD, 2011).

On the other hand, *in vitro* bioassays can be used to assess changes at the molecular or cellular levels, by chemically exposing cell cultures or subcellular systems. Although these

assays are limited in ecotoxicology and are usually based on vertebrate models, the biological responses monitored at the *in vitro* level can include changes in receptor or enzyme activity (Schuijt *et al.*, 2021). For example, the OECD TG 249 employs a cell line from the gill of rainbow trout (*Oncorhynchus mykiss*) to predict acute chemical toxicity in fish cells after 24 hours. This test can be used as a pilot screening assay for determining fish toxicity before *in vivo* testing, and to estimate the concentration that causes loss viability in 50% of the exposed cells (EC50 value) (OECD, 2021b). Moreover, although specific to mammalian cells, the OECD TG 458 can be used to determine chemicals that activate (agonists) or inhibit AR (antagonists) using an Androgen Receptor TransActivation (ARTA) assay (OECD, 2023). The mammalian cell lines incorporated in this assay are transfected with a luciferase reporter gene that can respond to AR activity. When the AR is activated by a chemical substrate, the receptor-ligand complex triggers the activation of the receptor gene. In turn, this results in the expression of the luciferase enzyme and the emission of light, allowing the detection of AR activity (OECD, 2023). Consequently, results from the ARTA assay can be used to determine the activity of mammalian AR in response to chemicals which may result in adverse reproductive effects by disrupting the androgen signalling pathway (Belcher *et al.*, 2019; OECD, 2023).

After chemical exposures, alterations seen at the sub-organismal level are often translated at the whole-organismal level. Thus, whole-organismal ecotoxicity tests can be used to assess more traditional endpoints in organisms, such as changes in survival, reproduction, growth, and behaviour in response to chemicals (Schuijt *et al.*, 2021). For example, the OECD TG 203 is an *in vivo* ecotoxicity test that aims to evaluate acute chemical toxicity in fish, by directly exposing the organisms to the test substance for a period of 96 hours. The test employs either a static, semi-static or a flow-through exposure system and its primary objective is to determine the concentration that causes 50% mortality in exposed organisms (LC50 value) (OECD, 2019b). However, a key challenge for ecotoxicity testing is assessing the biological changes following chemical exposures at various levels of biological organisation. This involves linking biochemical and molecular responses (such as changes in gene expression leading to downstream effects of enzyme activity) to the MoA of a chemical and connecting those responses to effects at the whole-organismal level and eventually, at the population and ecosystem level (McCarty and Munkittrick, 1996).

6. Endocrine disruption in molluscs

While standardised ecotoxicity tests provide considerable insights into the effects of EDCs on vertebrate organisms, the impacts of these chemicals on invertebrate animals, such as molluscs, remain less understood (Crane *et al.*, 2022). Molluscs, which display incredible diversity and significant ecological importance (Wang and Wang, 2019), are extremely sensitive to EDCs as demonstrated by the well-documented condition of imposex (Blaber, 1970; Smith, 1971). The poor understanding of endocrine disruption in molluscs stems from a lack of detailed knowledge about their distinct endocrinology, which appears to differ considerably from that of vertebrates (discussed in detail in Chapter 2). As a result, the poor characterisation of different hormonal pathways in molluscs creates significant challenges in understanding the MoA of EDCs and how they interfere with molluscan endocrine processes (Langston, 2020).

In molluscs, two standardised OECD ecotoxicity tests exist. These are the Test Guidelines (TG) 242 (OECD, 2016b) and 243 (OECD, 2016a), which evaluate reproductive and survival effects from prolonged chemical exposures on the gastropod model organisms *Potamopyrgus antipodarum* and *Lymnaea stagnalis*, respectively. Although these endpoints are useful in determining the effects of reproductive toxicants on the whole-organismal level, gastropod ecotoxicity tests lack the mechanistic underpinning needed to address endocrine disruption at the biochemical and molecular levels. For example, there is limited availability of mollusc-specific *in vitro* bioassays to address how EDCs interfere with molluscan enzymes or receptors, and the use of mammalian *in vitro* bioassays for this purpose would be considered unreliable (Schuijt *et al.*, 2021). Hence, the only way to elucidate the MoA of EDCs in molluscs, and protect those organisms from future environmental threats, is by developing a thorough understanding of their endocrinology.

7. The paths to understanding molluscan endocrinology

Steroidogenesis is the biochemical process in which cholesterol acts as a precursor for the synthesis of steroid hormones. In vertebrates, steroidogenesis has been fully addressed and understood over the years. On the other hand, the synthesis of “vertebrate-type” steroid hormones in molluscs has been an area of extensive scientific debate, as it has not been adequately proven to exist. In the past, studies reported the presence of vertebrate-type androgens (male sex hormones) such as testosterone, in molluscan tissues and therefore suggested that molluscs can biosynthesise those hormones *de novo* (Lafont, 1991; Lafont & Mathieu, 2007; Lehoux & Sandor, 1970). Contradictory to these assumptions, genomic

findings demonstrated that molluscs lack an AR as well as essential enzymes that initiate steroid biosynthesis in vertebrates (Kaur *et al.*, 2015; Adema *et al.*, 2017; Markov *et al.*, 2017), yet they appear to have other steroidogenic enzymes whose function remains unknown.

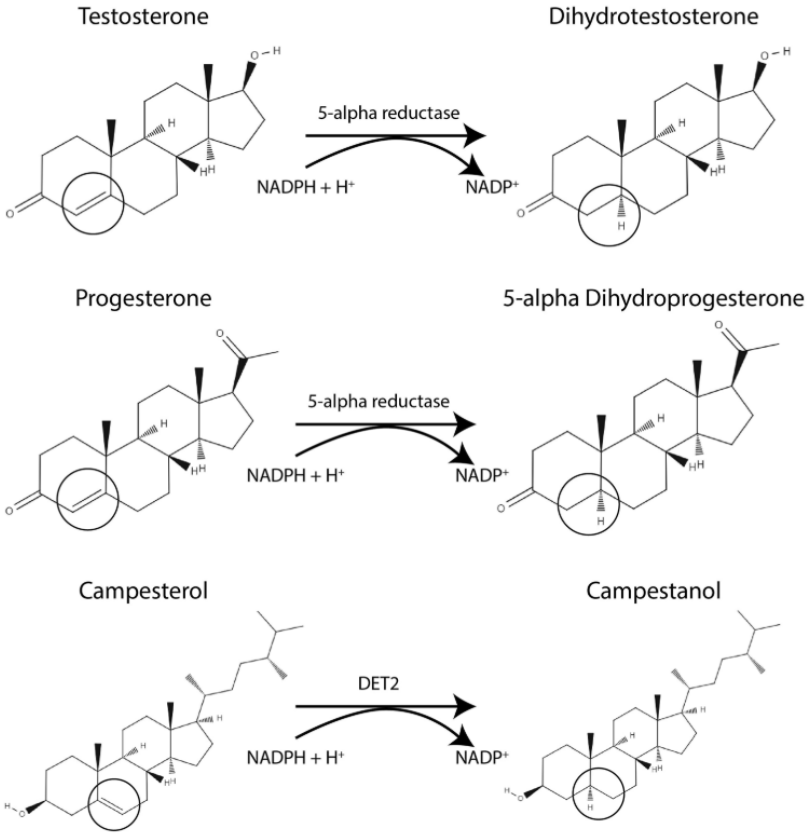


Figure 1.2: The steroidogenic enzyme 5-alpha reductase (5αR) converts testosterone to dihydrotestosterone and progesterone to 5-alpha dihydroprogesterone in vertebrates. Its functional orthologue DET2, converts the brassinosteroid (plant steroid) camplesterol to campestanol. 5αR and DET2 both metabolise steroids by converting the (circled) double-bonded $\Delta^{4,5}$ (testosterone & progesterone) or $\Delta^{5,6}$ (campesterol) rings to single bonds. The figure was obtained and reproduced from Baynes *et al.*, (2019).

A prime example is the presence of the steroidogenic *SRD5A1* and *SRD5A2* gene homologues in the genome of the freshwater gastropod *Biomphalaria glabrata* (Adema *et al.*,

2017), which encode two isoforms of the 5αR enzyme (5αR1 and 5αR2). In vertebrates, 5αR is considered essential for the sexual development and reproductive potential of male organisms. It is responsible for converting testosterone (T) to 5α-dihydrotestosterone (DHT), cortisol to 5α-corticosterone or progesterone into 5α-dihydroprogesterone (Fig. 1.2) (Baynes *et al.*, 2019). To investigate whether 5αR plays a physiological role in the early development of gastropod snails, Baynes *et al.*, (2019) exposed *B. glabrata* embryos to the pharmaceutical 5αR inhibitors, DUT and FIN. Surprisingly, the developing embryos elicited a strong and highly reproducible phenotypic response characterised by an elongated "banana-shaped" shell (Fig. 1.3). Unpublished findings from the same lab group, demonstrated that the phenotypic disruption is strongly initiated during the trochophore stage of embryonic development. Embryonic exposure of another freshwater gastropod, *Physella acuta*, to similar concentrations of DUT, elicited the same response, indicating that phenotypic disruption was not species-specific (Baynes *et al.*, 2019). Interestingly, previous observations have indicated that *SRD5A1* and *SRD5A2* transcripts are expressed in the mantle tissues of *B. glabrata* (Adema *et al.*, 2017), thus raising questions about a potential link of 5αR with molluscan shell formation. Homologues of 5αR have also been identified in plants. The enzyme DET2, which is used by plants for the synthesis of their own steroids (i.e. brassinosteroids), exhibits considerable sequence similarity with mammalian 5αR (Fig. 1.2) (Li and Chory, 1999). Moreover, DET2 was shown to convert T to DHT under experimental conditions, indicating that DET2 is in fact a functional ortholog of mammalian 5αR (Li *et al.*, 1997).

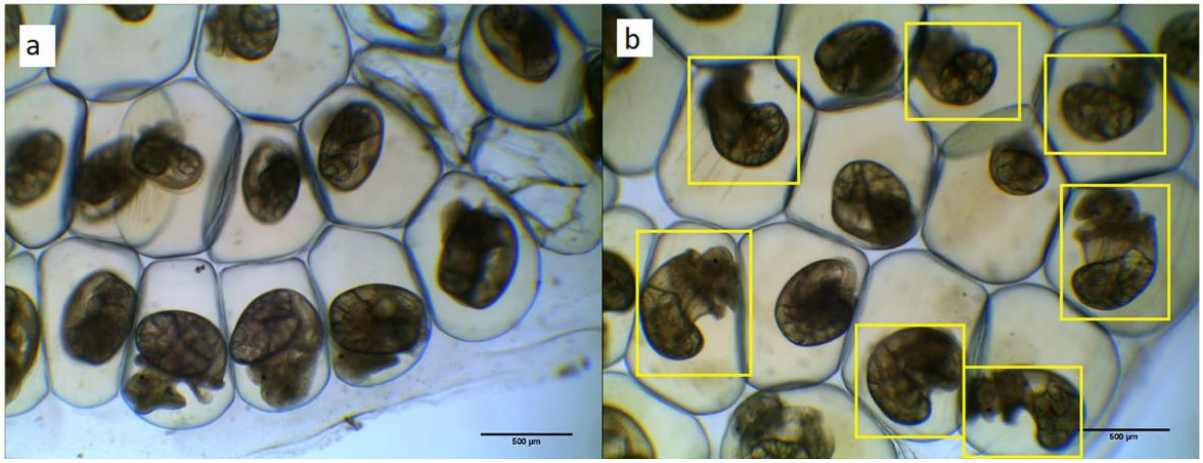


Figure 1.3: Embryonic exposures to pharmaceutical 5αR inhibitor, DUT, were shown to elicit an elongated shell phenotype in *B. glabrata*. *B. glabrata* embryos at (a) solvent control treatment and (b) 100 µg/L DUT treatment, at 4 days post fertilisation. The figure was obtained and reproduced from Baynes *et al.*, (2019).

8. The knowledge gaps in the field

The morphological deformation of gastropod embryos in response to pharmaceutical 5αR inhibitors presents an intriguing area of research that remains poorly understood. In the absence of an AR in molluscan genomes, the function of 5αR in these organisms is likely different from that in vertebrates. However, the limited knowledge about molluscan 5αR highlights broader gaps in our understanding of molluscan endocrinology. To better understand how pharmaceuticals (such as DUT and FIN) or other chemicals interfere with molluscan endocrine systems, it is critical to reach a collective agreement, based on robust scientific evidence, on how molluscs synthesise and regulate hormones.

As of now, 5αR proteins and transcripts of *SRD5A1* and *SRD5A2* were shown to be expressed in *B. glabrata* embryonic tissues (Baynes *et al.*, 2019). However, the temporal expression of *SRD5A1* and *SRD5A2* across different embryonic stages of *B. glabrata* remains a significant knowledge gap. Consequently, it remains unclear whether 5αR is more highly expressed during the sensitive window of pharmaceutical disruption caused by DUT (i.e. the trochophore stage, further investigated in Chapter 3), which could hint at a role in embryo shell formation. We also do not know if the pharmaceuticals DUT and FIN interfere directly with the isoforms of the 5αR enzyme in gastropods, or whether these pharmaceuticals disrupt other substrates that may indirectly affect shell development. Most importantly, given the presence of 5αR in adult tissues (including mantle and reproductive tissues (Adema *et al.*, 2017)), it is unknown if pharmaceutical disruption by DUT and FIN is specific to embryonic development or if adult gastropods are also affected (further investigated in Chapter 4).

9. Aims of the thesis

This PhD thesis aimed to enhance our understanding of molluscan endocrinology, investigate the temporal expression patterns of genes encoding 5αR enzymes in *B. glabrata* embryos, and assess the physiological effects of the pharmaceutical 5αR inhibitor, DUT, on *B. glabrata* adults. Specifically, the objectives of this thesis were to:

- Through systematically reviewing the literature, identify crucial knowledge gaps in our current understanding of molluscan endocrinology, assess biases in this literature, evaluate the reliability of existing evidence in the field, and provide recommendations for future research.
- Identify and validate stable reference genes across embryonic tissues in *B. glabrata*.
- Determine whether *SRD5A1* and *SRD5A2,* the genes encoding 5αR, are more highly expressed during the sensitive window of pharmaceutical disruption in *B. glabrata* embryos.

- Determine if exposure to the pharmaceutical 5αR inhibitor, dutasteride, affects the survival, growth and reproduction of *B. glabrata* adults.

Chapter 2: Systematic evidence mapping and critical appraisal of Molluscan endocrine research

1. Introduction

Molluscs are known for their incredible diversity. The number of known mollusc species (estimated at 81,000-92,000 living species (MolluscaBase eds., 2023)) is second only to arthropods (insects). With a rich fossil record (60,000-100,000 fossil species (MolluscaBase 2023)) dating back to the Cambrian (500 million years ago), molluscs have adapted and radiated into almost every environment from deep ocean trenches and hydrothermal vents, open seas and intertidal shores, freshwater rivers and lakes, to terrestrial locations including deserts and mountains. Molluscs display diversity at many levels beyond species number. Feeding strategies range from giant clams with symbiotic photosynthetic zooxanthellae (Klumpp, Bayne and Hawkins, 1992), to herbivores and detritivores (e.g. typical garden slugs and snails) and carnivores (e.g. squid). Reproductive strategies include gonochorism, hermaphroditism (both sequential and simultaneous) and parthenogenesis, with a range of parental care levels (broadcast spawners and prolific egg layers, to brooders). Molluscs also exhibit a variety of body plans, structures and levels of behavioural complexity. To many, molluscs are either seen as food (e.g. oysters), agricultural pests (terrestrial slugs), parasite vectors (e.g. schistosomiasis), or ornamental (pearls and shells). However, given their ubiquitous and diverse nature, molluscs are vital components of major ecosystems now threatened by climate change, habitat destruction, and pollution (Cuttelod, Seddon and Neubert, 2011; Abreu *et al.*, 2019; Böhm *et al.*, 2021; Thomas *et al.*, 2021).

A clear example of how pollution can disrupt mollusc populations and lead to regional extinctions is the case of the anti-fouling chemical, tributyltin (TBT). In the 1970s two seemingly unrelated disruptions to mollusc populations occurred, namely, reports of normally gonochoristic female marine gastropods developing male sexual structures (penis, vas deferens, prostate) known as “imposex” from American and European harbours (Blaber, 1970; Smith, 1971), and the collapse of the oyster fishery in Arcachon bay, France (Ruiz *et al.*, 1996). However, both were later linked to the widespread use of tributyltin (TBT) anti-foulant on boat hulls (Reviewed in (Santillo, Johnston and Langston, 2001)). The imposex condition, caused by TBT exposure, is known to have driven population declines of marine snails globally (Fernandez, 2019). The link between TBT pollution and imposex has been credited as a clear warning of the risk endocrine-disrupting chemicals could have on wild species (or humans) (Fernandez, 2019). Testing chemicals for possible endocrine-disrupting activity is now being implemented in a number of countries and regions (e.g. EU, USA), with a growing number of

internationally recognised testing methods and protocols available (e.g. OECD, 2018). However, somewhat paradoxically, TBT's mechanism of action leading to imposex in molluscs was misunderstood for decades and has only recently become better resolved ((Nishikawa *et al.*, 2004; Castro *et al.*, 2007; Giulianelli *et al.*, 2020; Lesoway and Henry, 2021; Zhou *et al.*, 2021). Indeed, although molluscs could be viewed as a "canary in the coal mine" for the issues of endocrine-disrupting chemicals, molluscs are yet to be properly integrated into endocrine-disrupting chemical testing guidelines due to a lack of detailed understanding of their endocrinology (hormone systems). Given their economic and environmental value, the paucity of knowledge for mollusc endocrinology is in stark contrast to the wealth of information we have on vertebrate hormone systems (vital for medical and pharmaceutical interventions), as well as our understanding of insect hormones (used to develop insecticides) and plant hormones (used to support agricultural innovations).

Historically, the identification of vertebrate-type sex steroids such as androgens (e.g. testosterone) or oestrogens (e.g. 17β-estradiol) in the tissues of molluscs has led to the assumption that these animals have the ability to biosynthesise or metabolise such steroids *de novo* (Lafont, 1991; Lafont & Mathieu, 2007; Lehoux & Sandor, 1970). Moreover, the presence of Nuclear hormone receptors (NRs) homologs, such as the oestrogen receptor, identified from sequence data, (Ip *et al.*, 2016; Lü *et al.*, 2016), along with the detection of certain vertebrate-type steroidogenic enzymes in molluscan tissues using non-specific techniques (Prisco *et al.*, 2017; Rosati *et al.*, 2019) further supported the hypothesis of endogenous vertebrate-type sex steroid synthesis in this phylum. Contradictory findings, from researchers exposing freshwater gastropods (*Biomphalaria glabrata*, *Lymnaea stagnalis*) to potent vertebrate androgens (testosterone (T), 5α-dihydrotestosterone (DHT) or 17α-methyltestosterone (MT)), showed no effects on the growth, reproductive development or reproductive output (eggs per individual) in the exposed snails (Giusti *et al.*, 2014; Kaur et al., 2016). These "negative" findings have been supported by molecular investigations which demonstrated *B. glabrata* and the marine limpet, *Lottia gigantea,* do not have a nuclear androgen receptor, indeed, the whole 3C group of NRs (including the glucocorticoid receptor, mineralocorticoid receptor and progesterone receptor) were absent in these species (Giusti *et al.*, 2014; Kaur, 2015). Additional genomic and evolutionary searches revealed that molluscs (and other invertebrates) do not contain the cholesterol side-cleavage enzyme (encoded by *CYP11A1* gene) which is essential for the induction of vertebrate steroidogenesis (Adema *et al.*, 2017; Markov *et al.*, 2017). These conflicting observations have given rise to an ongoing debate regarding the ability of molluscs to biosynthesise vertebrate-type steroids *de novo* and what their role in molluscan endocrinology could be (Scott, 2012, 2013). However, our current

understanding of molluscan endocrinology is characterised by significant knowledge gaps that do not reach beyond potential similarities with vertebrate steroidogenesis.

To date, the most thorough discussions on molluscan steroidogenesis have been a series of critical reviews by Scott, 2012, 2013, 2018 and Fodor *et al.*, 2020. While these reviews provide valuable information on the occurrence of vertebrate-type sex steroids in molluscs, the exploration of other hormonal pathways that may exist within the phylum remains a significant knowledge gap. Therefore, the aim of this systematic evidence map is to provide a comprehensive assessment of our current understanding of hormone biosynthesis in molluscs. This is achieved through a critical evaluation of the wider literature bringing together evidence for different hormones, hormone receptors and metabolic pathways present in molluscs. This new evidence base raises important questions and highlights critical knowledge gaps which should guide future research efforts in this area.

2. Methods

The systematic evidence map protocol was drafted according to PRISMA-P (Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols) 2015 guidelines (Shamseer *et al.*, 2015) in consideration with the COSTER (Recommendations for the conduct of systematic reviews in toxicology and environmental health research) checklist (Whaley *et al.* 2020). The second version of the protocol was developed after receiving feedback during open-peer review, and published on the open repository Zenodo (Panagiotidis, 2022) (https://doi.org/10.5281/zenodo.7061510). Additional iterations were made to improve the clarity of the protocol resulting in a third and final version (Appendix S2). The raw data, including data collected from eligible studies, can be found in Appendix S1 which has been published on Zenodo (Panagiotidis, 2024) and can be accessed here: https://doi.org/10.5281/zenodo.14311002.

2.1. Population-Outcome statements and eligibility criteria

In this review, each research question has been defined by a separate PO (Population, Outcome) statement as well as PO-specific inclusion and exclusion criteria; these are summarised in Table S1 (Appendix S1.1, (Panagiotidis, 2024)). The “Mollusca AND Hormones” PO aimed to identify different hormones found in molluscan tissues, whereas the “Mollusca AND Receptors” and “Mollusca AND Enzymes” POs, aimed to identify the presence of hormone receptors and hormone-metabolising enzymes in molluscan tissues, respectively.

2.1.1. Defining 'Population'

Population has been defined as "Mollusca" and consisted of all seven molluscan living classes (Gastropoda, Bivalvia, Polyplacophora, Cephalopoda, Scaphopoda, Aplacophora, Monoplacophora). The seven molluscan classes were included as search terms in addition to several other mollusc-specific terms including "oysters", "mussels", "squids" and "chitons".

2.1.2. Defining Outcome - 'hormones', 'hormone receptors' and 'hormone-metabolising enzymes'

Three sets of keyword strings relevant to our research questions and PO statements were devised to capture all the critical literature. The full details on the database searches and keyword strings used can be seen in the Appendix S1.2 and S1.3, respectively (Panagiotidis, 2024). The keyword string for "Mollusca AND Hormones" PO, comprised of 24 steroid hormone names involved in vertebrate steroidogenesis. These were extracted from the article of Häggström & Richfield (2014) and from the critical evaluation of Fodor et al., (2020). Additionally, 4 identified ecdysteroids (insect steroids) involved in arthropod steroidogenesis were extracted from Niwa & Niwa (2014) and were included as part of the search. To avoid missing important literature, generic terms such as "sterols", "hormones" as well as synonyms for each included steroid were identified and included in the search. Data on retinoids was captured via the "Mollusca AND Receptors" keyword string while data on hormones involved in neurohormonal signalling was outside the scope of this review.

The "Mollusca AND Receptors" PO comprised of general hormone receptor terms, such as "hormone receptors", "nuclear receptors" and "retinoid receptors". The aim of the "Mollusca AND Receptors" PO was to collect data on the occurrence of receptors known to interact with hormones and retinoids, as well as receptors known to be indirectly involved in hormone signalling in molluscs (Appendix S1.1, (Panagiotidis, 2024)). The "Mollusca AND Receptors" PO presents an amended version of the former PO, "Mollusca AND Nuclear receptors" (Panagiotidis, 2022) which aimed to address the occurrence of nuclear receptors in molluscan tissues. However, our pilot systematic searches captured a considerable number of receptors outside the nuclear receptor superfamily, therefore the PO statement was updated accordingly.

Lastly, the "Mollusca AND Enzymes" PO aimed to capture information on the enzymes involved in vertebrate steroidogenesis, insect steroidogenesis and retinoid signalling, in other words defined as "hormone-metabolising enzymes". A list of genes encoding for enzymes involved in vertebrate steroidogenesis were identified and extracted from Wikipedia (Häggström and Richfield, 2014). In addition, the keyword strings comprised of 10 gene names

known to be involved in insect steroidogenesis (Niwa & Niwa (2014)) and names of key transport proteins and enzymes involved in retinoid signalling. The “Mollusca AND Enzymes” PO presents an amended version of the former PO, “Mollusca AND Steroidogenesis-related genes” (Panagiotidis, 2022), which aimed to address the occurrence of steroidogenesis-related genes in molluscs. However, the data captured from the systematic searches revealed a range of hormone-metabolising enzymes outside the scope of vertebrate steroidogenesis. So, the PO statement was updated accordingly (Appendix S1.1 and S1.2, (Panagiotidis, 2024)).

2.2. Search strategy

Eligible studies included in the systematic map were any peer-reviewed publications written in English, that met the eligibility criteria presented in Table S1.1 (Appendix S1.1, (Panagiotidis, 2024)). Grey literature was not included. Data from review studies were excluded. However, relevant review studies were used as supporting material for the discussion. Searches of references from eligible studies were carried out to capture any additional articles not uncovered by the main search.

The date range for inclusion was 1st January 2012 until 10th September 2021 (the date the search was conducted). The 2012 cut-off date was driven by two factors. Firstly, the first draft molluscan whole-genomes were published in 2012 (Takeuchi *et al.*, 2012; Wang *et al.*, 2012), these and subsequent publications, provide high-quality and detailed molecular data not previously available. The second reason is linked to technical advances in measuring hormones. Traditionally, immunological-based assays (e.g. RIA, ELISA) employed to measure hormones in molluscan tissues were widely considered as sensitive and reliable (Warrier, Tirumalai and Subramoniam, 2001; Lavado, Janer and Porte, 2006; Liu, Li and Kong, 2008). However, the reliability and specificity of using antibodies raised for vertebrates in molluscs came into question in the early 2010’s with the emergence of studies that compared immunoassay techniques with analytical chemical methods (Gust *et al.*, 2010; Krasowski *et al.*, 2014), with analytical chemistry (e.g. GC-MS, LC-MS) exhibiting higher precision in detecting low concentrations of steroids and other metabolites than traditional immunoassay methods (Gust *et al.*, 2010).

Date limitations were not applied during the literature search, as this resulted in significant inaccuracies during citation export in trial searches (e.g. the number of exported citations did not match the number of retrieved papers during the original search when a date limit was applied). All retrieved papers were exported from each database individually and were assessed for duplicate removal (using the reference management software Zotero), and then for inclusion (using the screening tool Rayyan), based on the eligibility criteria (Appendix S1.1,

(Panagiotidis, 2024)). The searches for peer-reviewed publications were conducted in PubMed, Web of Science and Scopus.

2.3. Data management and screening

The studies captured by the systematic searches and across all databases were merged and then exported to the reference management software "Zotero" which facilitated the process of duplicate removal. Following duplicate removal, the remaining studies were imported to the online tool Rayyan (Ouzzani *et al.*, 2016) for title and abstract screening. At first, the eligibility criteria from the draft version of the protocol (Panagiotidis, 2021) were applied to 20% (n=1377) of the merged list of eligible studies, by two coders working independently. This pilot screening of retrieved studies allowed us to identify potential limitations with the eligibility criteria of the draft protocol. Disagreements after pilot screening were resolved between the two coders and eligibility criteria were updated accordingly in the second version of the protocol (Panagiotidis, 2022). Following the amendment of eligibility criteria, the remaining 80% of studies were screened by a single evaluator at title and abstract level. Eligible studies were then screened at full-text level, by the same evaluator, and the reason for exclusion was recorded. Studies found eligible for inclusion at full-text screening were included in the data extraction inventory. During full-text screening, some final amendments were made to the eligibility criteria to ensure thoroughness of the present work. For example, the presence of thyroid hormones, thyroid receptors or enzymes that metabolise thyroid hormones (or those involved in thyroid hormone signalling) were not part of the initial inclusion criteria. However, the identification of those biomolecules during full-text screening, provided an opportunity to create a discussion on the occurrence of thyroid hormone pathway in molluscs. Thus, the occurrence of thyroid biomolecules became part of the inclusion criteria, across all PO statements (Appendix S1.1, (Panagiotidis, 2024)). This addition aimed to enhance the comprehensiveness and relevance of our work. The full details on the amendments made to the eligibility criteria are described in the Appendix S1.7 (Panagiotidis, 2024).

2.4. Data extraction

Data on hormones, hormone receptors and hormone-metabolising enzymes were extracted from eligible studies and included in the data extraction inventory entered into the Excel-based data extraction template. The data extraction template was designed to allow the capture of information for all three PO statements and was piloted with 9 eligible studies. The results of the pilot activities are summarised in the draft version of the protocol (Panagiotidis, 2021). Upon completion of the full-text screening and subsequent data extraction, the data extraction template was amended slightly for purposes of clarity and data interpretation. The full details on the data extraction template and inventory, can be seen in Appendix S1.6 (Panagiotidis,

2024), whereas the changes made to the data extraction template are summarised explicitly in the Appendix S1.7 (Panagiotidis, 2024).

2.5. Risk-of-bias assessment

Limitations in the analysis or experimental design of individual studies can result in incorrect assumptions about the origin and synthesis of hormones in molluscan tissues. Critical appraisal tools can be used to assess the internal validity of studies through selection bias, detection bias, performance bias and so forth (Martin *et al.*, 2021). Although attempts have been made to evaluate bias in studies that investigate sex steroid biosynthesis in molluscs (Scott, 2013), to our knowledge, there is no risk-of-bias tool adapted to endocrinological investigations in these animals. For the purposes of this systematic evidence map, a tailor-made Risk-of-Bias tool (Appendix S1.5, (Panagiotidis, 2024)) was developed to critically assess potential flaws or errors in the design, conduct or reporting of eligible studies. The tool consists of a series of criteria that aimed to evaluate the collected data with respect to each PO statement (Appendix S1.1 (Panagiotidis, 2024)). Eligible studies were assessed on both internal validity (e.g. verification of mechanism of action of hormones) and study design criteria (e.g. within or between study repetition) and these are summarised in the accompanied Risk-of-Bias guidelines, alongside detailed information on how studies are coded in different case scenarios (Appendix S1.4, (Panagiotidis, 2024)). Internal validity criteria were PO-specific, whereas study design criteria were applicable to all PO statements. The assessment criteria were crafted according to information provided from peer-reviewed literature and the ARRIVE guidelines 2.0 (Percie du Sert et al., 2020). After the second version of the protocol was published (Panagiotidis, 2022), modifications were made to the Risk-of-Bias tool and Risk-of-Bias guidelines. These changes were introduced to refine the reliability and objectivity of the overall Risk-of-Bias assessment and are addressed in detail in Appendix S1.7 (Panagiotidis, 2024).

The outcomes reported in the included studies varied considerably, so a ‘one-size-fits-all’ approach to Risk-of-Bias was difficult to implement. Therefore, two assessment categories were designed for this purpose. Studies that fell into the “Assessment A” category, were evaluated on internal validity and study design criteria, whereas studies in the “Assessment B” category were evaluated on study design criteria only. Studies were allocated to their respective assessment categories during data extraction. “Assessment A” studies must have focused on the investigation of activity, function and/or mechanism of action (MOA) of hormones, hormone receptors, and/or hormone-metabolising enzymes in molluscs. Consequently, the study objectives should have adhered to a methodology which is considered appropriate for an internal validity assessment. “Assessment B” studies, were any

studies that did not fit within the scope of “Assessment A” study criteria (e.g. toxicological assessments). If the study did not aim to determine (or attempted to comment on) the activity, function and/or MOA of an outcome of interest it was assessed solely on study design criteria (Appendix S1.4, (Panagiotidis, 2024)). Studies assessed for “Assessment B” were included in the systematic map to broaden the range of information captured. However, because these studies primarily focused on objectives different from the investigation of activity, function and/or MOA of hormones, receptors and enzymes of interest, they were evaluated on study design criteria only, to ensure a fairer assessment system. The Risk-of-Bias tool consists of a series of questions that require the evaluator to choose scores between “Definitely low risk of bias (++) ”, “Probably low risk of bias (+) ”, “Probably high risk of bias (-)” and “Definitely high risk of bias (--)”. Every study assessed for Risk-of-Bias received a summary score of Level 1 studies with "Lower risk of bias”, Level 2 studies with “moderate risk of bias” or Level 3 studies with “higher risk of bias” (Fig. 2.1).

Mollusca AND Hormones - Risk of Bias						
		internal validity criteria		study design criteria		
<u>Level 1</u>: All internal validity criteria must be rated as either "**Definitely low risk**" or "**Probably low risk**". The majority of study design criteria (≥2) must be rated as "**Definitely low risk**" or "**Probably low risk**". **<u>Level 2</u>:** Studies that meet neither Level 1 or Level 3 criteria. **<u>Level 3</u>:** All internal validity criteria must be rated as either "**Definitely high risk of bias**" or "**Probably high risk of bias**". The majority of study design criteria (≥2) must be rated as "**Definitely high risk**" or "**Probably high risk**".		Identification of hormones in molluscs:	Verification of MOA of hormones:	Strategy to minimise potential confounders	Within-study or between-study repetition	Statistics
++	Definitely low risk of bias					
+	Probably low risk of bias					
-	Probably high risk of bias					
--	Definitely high risk of bias					
Level 1 (Lower risk of bias)	"Definitely low risk" or "Probably low risk" for internal validity criteria AND "Definitely low risk" or "Probably low risk" for study design criteria (≥2).	**+**	**+**	**--**	**+**	**++**
Level 2 (Moderate risk bias)	Studies that meet neither Level 1 or Level 3 criteria	**--**	**++**	**-**	**+**	**--**
Level 3 (Higher risk of bias)	"Definitely high risk" or "Probably high risk" for internal validity criteria AND "Definitely high risk" or "Probably high risk" for study design criteria (≥2).	**--**	**-**	**-**	**-**	**+**

Figure 2.1: An example of the level system "Assessment A" studies that identified hormones in molluscs ("Mollusca AND Hormones" PO). Individual studies were assessed for Risk-of-Bias based on internal validity and study design criteria prior to summary scoring (Level 1, Level 2, Level 3). Plus (+) and minus (-) signs were used to indicate scoring for the individual questions.

2.6. Data analysis and narrative synthesis

The primary outcome of this systematic evidence map was the development of a narrative synthesis of the data retrieved, based on the three research questions, conducted both individually and in combination. A thorough synthesis of collected evidence was carried out in separate sections (according to each Population and Outcome statement) highlighting key findings for different molluscan classes. The Risk-of-Bias assessment also serves as a primary outcome for this review. The assessment showcases multiple levels of reliability for the collected evidence, based on an extensive list of tailor-made Risk-of-Bias guidelines and case scenarios for how a study was coded. The secondary outcome of this systematic map was to identify the knowledge gaps that exist within our current understanding of molluscan endocrinology. These were highlighted as part of a comparative evidence report which thoroughly evaluated the results obtained from the systematic data mapping.

2.6.1. Mollusca AND Hormones inventory

Hormones were classified in groups based on their chemical structure (steroids, retinoids, biogenic amines, protein hormones) or subgroups (phytosterols, sterols, fungal sterols, zymosterols, secosterols). The structure of hormones was examined manually via online databases (Sud *et al.*, 2007; Kim *et al.*, 2023) or molecule-specific information found in respective studies. Within each (sub)group, hormonal measurements were clustered with respect to species, publication and the tissue they were identified in (Fig. 2.4 – interactive view). Studies included in the "Mollusca AND Hormones" inventory implemented a range of methodological approaches to examine the occurrence of hormones in molluscs. Methodologies were clustered into three main categories: "chemical analysis", "immunoassay", or "other" (Fig. 2.6).

2.6.2. Mollusca AND Receptors

Data for both receptor genes and proteins were collected from eligible studies included in the "Mollusca AND Receptors" inventory. The identified receptors were reported by their nomenclature gene and enzyme name, verified through the nomenclature databases Genenames.org, Flybase.org and WormBase.org (Davis *et al.*, 2022; Gramates *et al.*, 2022;

Seal *et al.*, 2023). For receptors whose nomenclature names could not be identified, they were reported by the names found in respective publications. Receptor genes and proteins were categorised by type (cell surface receptor, G-protein-coupled receptor, intracellular receptor, ligand-gated ion channel, nuclear receptor, unknown), phylogenetic origin (vertebrate or non-vertebrate) and mollusc class (Bivalvia, Cephalopoda, Gastropoda, Polyplachophora) (Fig. 2.7 – interactive view). The classification of receptors by type was achieved through manual searches in the online database UniProt (Consortium, 2023). For clarity, receptors primarily known to be functional in vertebrates (e.g. oestrogen receptors) were defined in this review as "vertebrate-type" receptors, whereas receptors primarily known from invertebrates (e.g. ecdysone receptors) were defined as "non-vertebrate-type" receptors.

The receptors included in the "Mollusca AND Receptors" inventory were identified by a range of molecular assays. To assess the reliability of the collated evidence, the publications in the "Mollusca AND Receptors" inventory were classified into three primary categories based on the methodologies employed: (i) DNA/RNA detection and localisation techniques, (ii) DNA/RNA detection and localisation techniques & other *in vitro* assays, (iii) protein and other *in vitro* assays (Fig. 2.8).

2.6.3. Mollusca AND Enzymes

Information about genes, enzymes and proteins associated with the synthesis of cholesterol, steroids, thyroid hormones, and retinoids were identified and included in the "Mollusca AND Enzymes" inventory. Hormone-metabolising enzymes were classified according to their metabolic or signalling pathways (steroid biosynthesis, cholesterol biosynthesis, retinoic acid signalling, etc) as well as the specific mollusc class in which they were identified (Bivalvia, Cephalopoda, Gastropoda, Polyplacophora). The classification of hormone-metabolising enzymes according to their respective pathways was achieved through manual searches in the online database UniProt (Consortium, 2023). Data from a range of molecules indirectly involved in the main metabolic or signalling pathways of interest was also collected. These include members of the cytochrome P450 superfamily of enzymes known to be involved in xenobiotic metabolism as well as the synthesis of cholesterol, steroids, and other lipids (Fig. 2.9 – interactive view).

Based on the method employed, publications were clustered into three main categories: (i) DNA/RNA detection and localisation techniques, (ii) DNA/RNA detection and localisation techniques & protein assays, and (iii) protein assays. These were further sub-classified according to the specific technique used. Details on method validation were also extracted from studies included in the "Mollusca and Enzymes" inventory (Fig. 2.10 – interactive view).

Studies examining receptor occurrence *in vitro* employed a range of reporter gene assays, transfection and transactivation assays as well and immunostaining diagnostics. As information on method validation can provide important insights into the reliability of the evidence/data reported, details on method validation, such as the implementation and names of reference genes or proteins were also extracted (Fig. 2.10 – interactive view).

2.7. Data visualisation and analysis

In addition to an in-depth evidence report, data collected in the data extraction inventory was visually summarised using the Tableau Public 2023.1 software. For clarity and data interpretation purposes, the data extraction inventory was sub-classified to PO-specific "Mollusca AND Hormones", "Mollusca AND Receptors" and "Molluscan AND Enzymes" inventories. The data was systematically categorised according to several criteria: the tissues where each hormone, receptor, or enzyme was identified; the methods and validation steps taken for their identification; the species in which they were identified; information on the potential activity of hormone, receptor or enzyme; and the observed effect of hormone, receptor or enzyme in response to pharmaceutical interventions. The online database is an interactive, publicly available and fully searchable resource that can be accessed online here.

2.8. Meta-biases

With meta-analysis being absent from this study, statistical methods for detecting meta-biases in the evidence report were not possible to implement. However, to avoid publication bias, any changes made to the final version of the protocol (Panagiotidis, 2022) were explicitly stated in Appendix S1.7 (Panagiotidis, 2024). Of note, are the final amendments made to the eligibility criteria which were not introduced to the keyword strings as part of our search strategy (Appendix S1.3, (Panagiotidis, 2024)). Specifically, keywords for biomolecules involved in the thyroid hormone signalling pathway were not initially part of our search strategy. However, the fortuitous identification of these biomolecules in the papers extracted from our systematic searches, created an opportunity to discuss their occurrence in molluscs. However, the absence of specific keywords in our search strategy could have led to the lack of important information regarding the occurrence of the thyroid hormone signalling pathway in molluscs. Thus, to address this limitation and ensure a comprehensive understanding of thyroid hormone signalling in molluscs, further systematic searches encompassing specific keyword strings are necessary for future research.

3. Results

The systematic literature searches identified 11,656 records from three databases (Fig. 2.2). Following duplicate removal, 6,500 records were screened at the title and abstract level to identify relevance. During this process and based on the initial eligibility criteria of the draft protocol (Panagiotidis, 2021), an additional 6,190 records were excluded. The remaining 310 records were screened at the full-text level to identify eligibility for inclusion in the data extraction inventory. During the full-text screening, 112 studies were found eligible for inclusion in the inventory based on the initial eligibility criteria (draft protocol). Additionally, 33 studies were included in the data extraction inventory based on the modified eligibility criteria (Appendix S1.1, (Panagiotidis, 2024)). Thus, a total of 165 studies were excluded during full-text screening and the remaining 145 studies were included in the data extraction inventory.

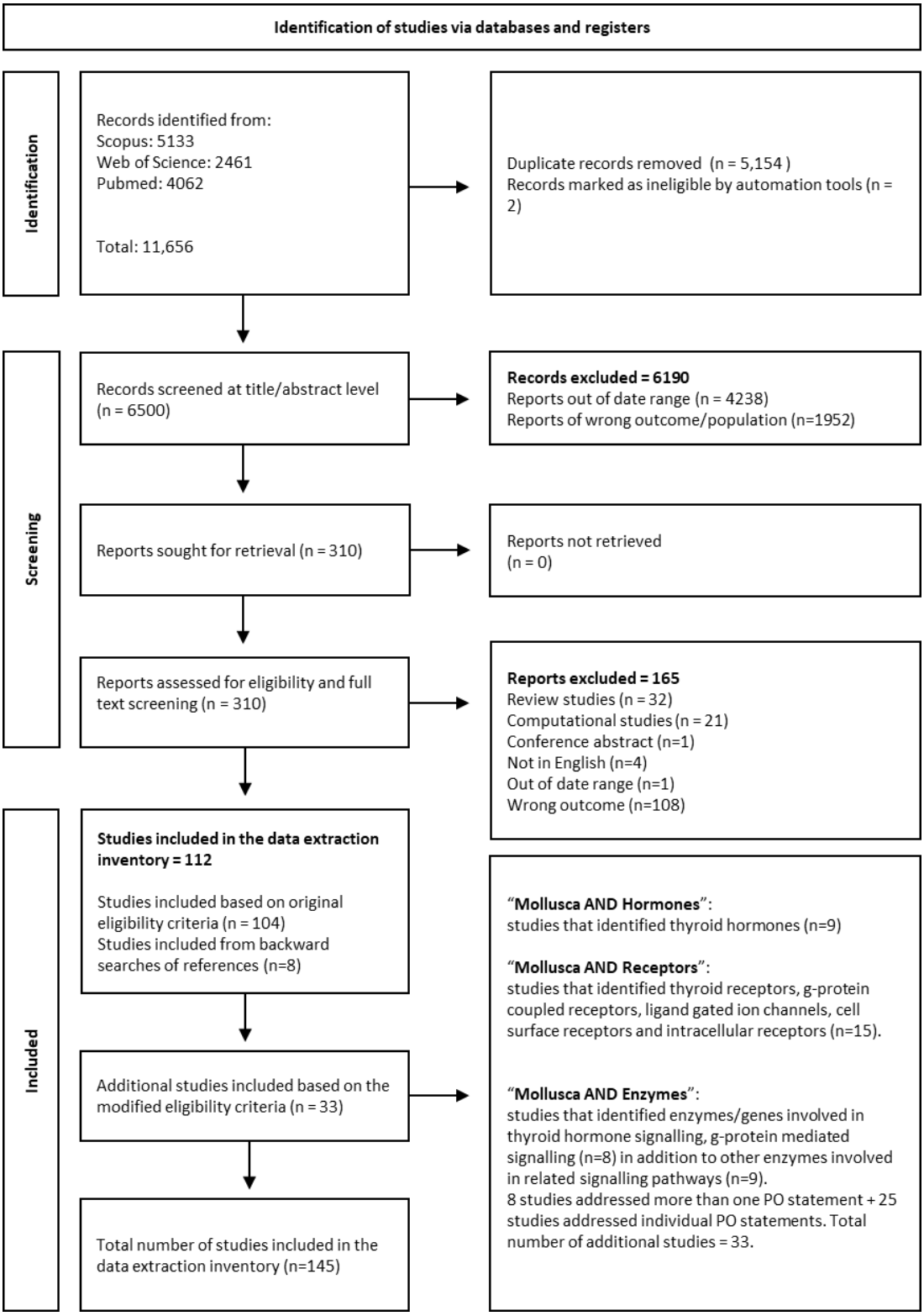


Figure 2.2: Study selection flow diagram, documenting the number of studies identified, removed, screened and included in the data extraction inventory (as per PRISMA 2015 guidelines (Shamseer *et al.*, 2015)). Characteristics of the data extraction inventories, including the number of eligible studies retrieved by year of publication according to Population

and Outcome (PO) statements can be found in Appendix S1.9 (Figure S9.1 and Section 1, (Panagiotidis, 2024)).

3.1. Risk of Bias of included studies

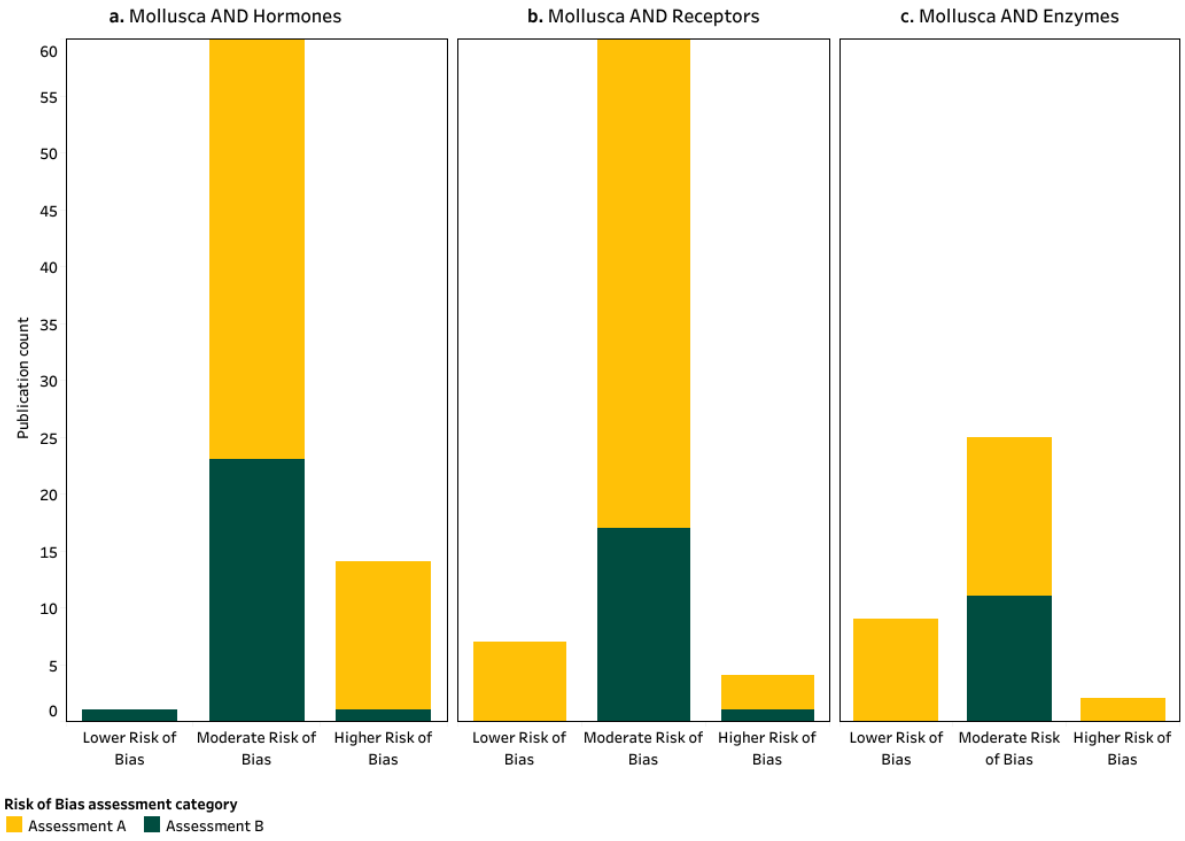


Figure 2.3: Results of the Risk of Bias appraisal for included studies according to assessment category ("Assessment A" shown in yellow and "Assessment B" shown in dark green), for the (a) "Mollusca AND Hormones", (b) "Mollusca AND Receptors", and (c) "Mollusca AND Enzymes" data inventories. Risk-of-Bias assessments were carried out using the Risk-of-Bias tool and guidelines. The results of the Risk-of-Bias assessment can be seen in full via interactive view.

Figure 2.3 and Appendix Material S1.9 section 2 (Panagiotidis, 2024), detail the number of extracted studies allocated to the different Risks of Bias categories for each PO statement (for both Assessment A and Assessment B). Independent of the assessment category, most studies were rated with a moderate risk of bias (Fig. 2.3 and Appendix S1.9 section 2, (Panagiotidis, 2024)). The overwhelming majority of the moderately biased studies were downgraded due to issues related to study design. For example, 83% of studies, included in all three data inventories (Mollusca AND Hormones, Mollusca AND Receptors, Mollusca AND

Enzymes), lacked independent repetition of experiments. Although some studies used biological replicates, these replicates were part of a single study.

Many studies were also downgraded due to issues related to internal validity. For example, a significant factor that contributed to moderate and higher risk of bias scores in the “Mollusca AND Hormones” inventory (Fig. 2.3a), was the absence of ligand binding assays from included studies. Among the studies that focused on steroids, endogenous retinoids, protein hormones, or biogenic amines, a striking 95% did not investigate the ability of these hormones to bind to their respective hormone receptors.

Moreover, 44% of studies that examined nuclear receptors from the “Mollusca AND Receptors” inventory, were considered to have a higher risk of bias due to the lack of a sequence similarity analysis of the receptor’s DNA binding domain (DBD) or ligand binding domain (LBD). DBD and LBD are characteristic regions of nuclear receptor proteins, which in turn recognise and bind to specific DNA fragments or receptor ligands, respectively (Vogeler *et al.*, 2014). Thus, a sequence similarity analysis of DBD and LBD is of particular importance as it can lead to insights regarding nuclear receptor functional ability and can in turn increase the reliability of the reported outcomes. The absence of sequence similarity analyses was also prominent in studies from the “Mollusca AND Enzymes” inventory. In particular, the lack of phylogenetic analyses from 46% of studies aimed to assess the function of hormone-metabolising enzymes in molluscs, contributed to scores of moderate or higher risk of bias. The full details on the Risk-of-Bias assessment criteria and scoring for studies included in the data extraction inventories can be seen in Appendix S1.5 (Panagiotidis, 2024).

3.2. Hormones identified in Mollusca

3.2.1. Inventory characteristics

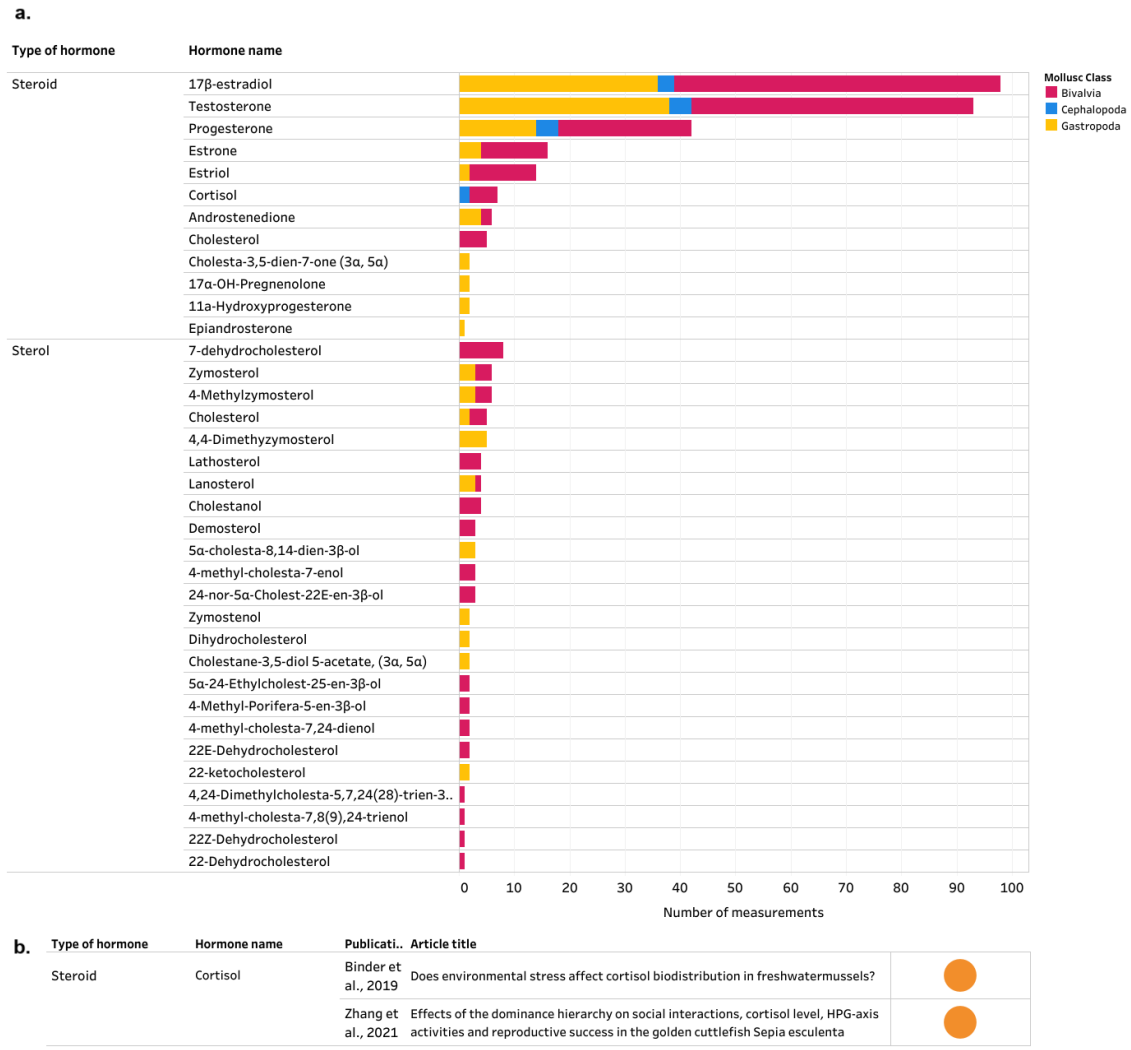


Figure 2.4: (a) A part of the "Mollusca AND Hormones" inventory indicating the steroids and sterols identified in Mollusca. Data on hormones is clustered according to hormone type and name. Different colours represent different Molluscan classes: Bivalvia shown in red, Cephalopoda shown in blue and Gastropoda shown in yellow. The number of measurements in graph (a) represents the times each hormone was measured with regards to the number of species, organism's sex, organism's life stage, tissues examined and methodological approach implemented. Therefore, multiple measurements can come from one publication, as highlighted in 2.4b. As an example, (b) represents the number of studies (n = 2), including article titles, that identified cortisol in Mollusca, where each circle represents a separate study. The number of studies that identified each hormone can be seen using the interactive view. The entire "Mollusca AND Hormones" inventory, including details on all hormones identified,

their reported activity, methodological details of included studies and the list of references can be seen in full in interactive view.

The “Mollusca AND Hormones” inventory was dominated by research on Bivalvia, Cephalopoda, and Gastropoda classes. Bivalvia had the largest number of hits, followed by Gastropoda and lastly, Cephalopoda (Fig. 2.4a – Interactive view). The steroid with the highest number of measurements was 17β-estradiol, followed by testosterone and progesterone in second and third place respectively (Fig. 2.4a). Eighty per cent of studies in the “Mollusca AND Hormones” inventory measured at least one steroid. Sterols (e.g. as 7-dehydrocholesterol) were the second most frequently documented group, reported in 9% of included studies, while biogenic amines (e.g. thyroxine) and retinoids (e.g. 13-cis-Retinoc Acid/13-cis-RA) were the third most frequently documented groups reported in 8% of studies, respectively. Of particular interest is the identification of less commonly recorded hormones, such as phytosterols (e.g. sitosterol), protein hormones (e.g. thyroid-stimulating hormone) or fungal sterols (e.g. ergosterol), found in 7%, 4% and 3% of studies in the “Mollusca AND Hormones” inventory, respectively (Fig. 2.5a). Note, that authors often measured hormones in different sexes within the same species or examined different chemical forms of the same hormone within their investigations (e.g. esterified vs unesterified form of testosterone). This detailed information was recorded in the data extraction template (Appendix S1.6, (Panagiotidis, 2024)) but was not reported explicitly in the figures. Instead, hormone measurements within the same species or of different chemical forms were reported as a total sum with respect to the study they were identified (Fig. 2.4a – Interactive view).

Figure 2.5: (a) A part of the "Mollusca AND Hormones" inventory, indicating the phytosterols and protein hormones identified in Mollusca. Data on hormones is clustered according to hormone type and name. Different colours represent different Molluscan classes: Bivalvia shown in red, Cephalopoda shown in blue and Gastropoda shown in yellow. The number of measurements in graph (a) represents the times each hormone was measured with regards to the number of species, organism's sex, organism's life stage, tissues examined and methodological approach implemented. Therefore, multiple measurements can come from one publication, as highlighted in 2.5b. As an example, (b) represents the number of studies (n = 2), including article titles, that identified luteinizing hormone in Mollusca, where each circle represents a separate study. The number of studies that identified each hormone can be seen using the interactive view. The entire "Mollusca AND Hormones" inventory, including details on all hormones identified, their reported activity, methodological details of included studies and the list of references can be seen in full as interactive graphs.

3.2.2. Methodology characteristics for the “Mollusca AND Hormones” inventory

Figure 2.6: Part of the “Mollusca AND Hormones” inventory indicating methodological characteristics of studies that employed immunoassays to examine the presence of hormones in molluscs. Studies were classified based on the type and method employed and the inclusion of positive quality controls (shown in dark grey) or internal standards (shown in light grey) in their assays. Unspecified information regarding positive controls or internal standards is shown in medium grey. The entire “Mollusca AND Hormones” inventory, including details on

all hormones identified, their reported activity, methodological details of included studies and the list of references can be seen in full as interactive graphs.

The clustering of methodologies revealed that 62% of included studies used at least one type of immunoassay to measure hormones in molluscs (Fig. 2.6), but only 44% of these studies had implemented the use of a positive quality control or internal standard in their methodological procedures. The most common immunoassay method used was enzyme-linked immunosorbent assay (ELISA), implemented by 47% of immunoassay-based studies, followed by radioimmunoassay (RIA) and enzyme immunoassay (EIA) with 33% and 9% respectively. In comparison, chemical analyses were adopted by 37% of studies in the "Mollusca AND Hormones" inventory, out of which 52% included positive controls as part of their method development. Within the studies which employed chemical analysis to measure hormones in molluscs, 33% used High-Performance Liquid Chromatography (HPLC), 30% used Gas Chromatography-Mass Spectrometry (GC-MS) and 30% used Liquid Chromatography-Mass Spectrometry (LC-MS).

3.3. Hormone receptors identified in Mollusca

3.3.1. Inventory characteristics

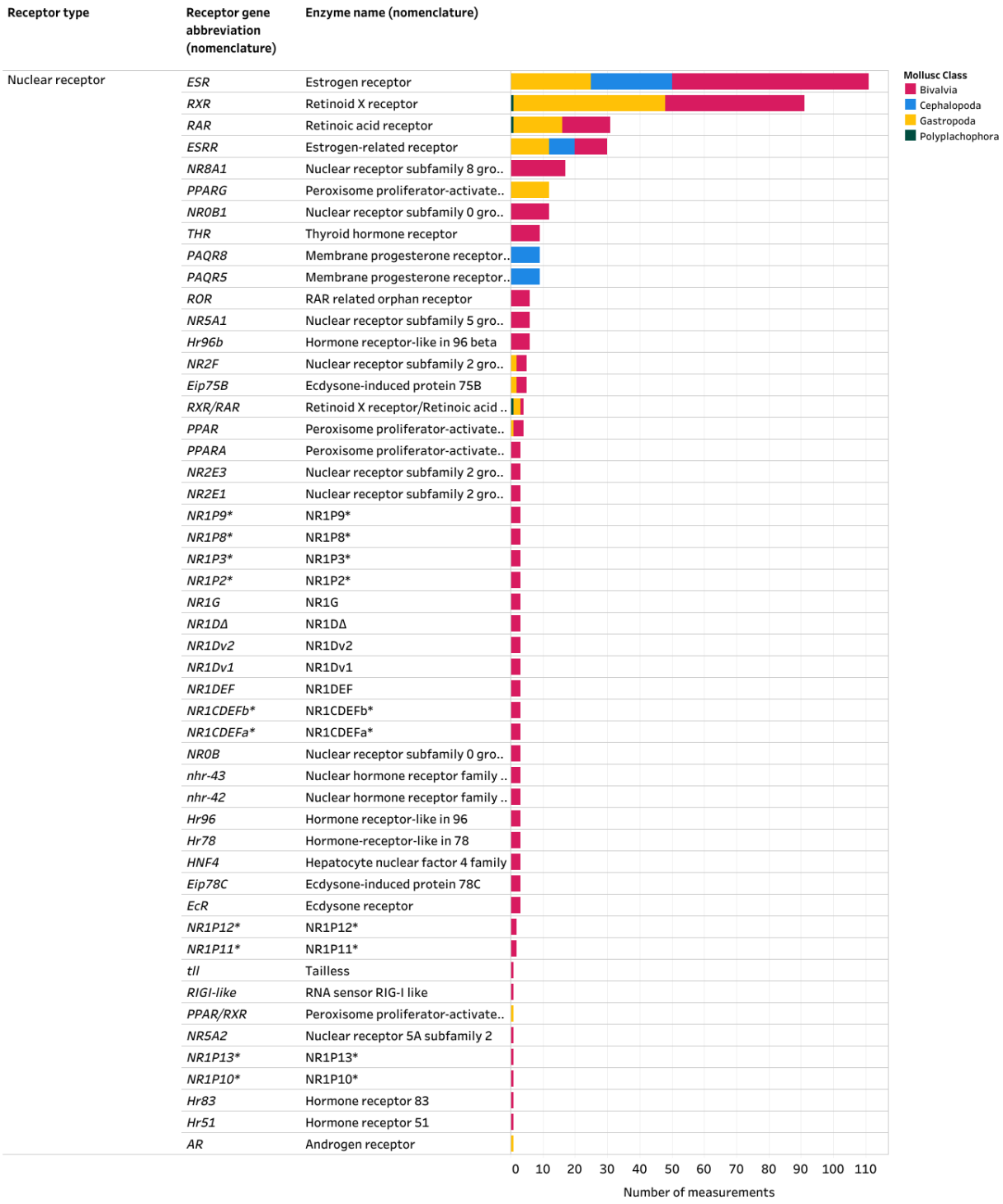


Figure 2.7: A part of the “Mollusca AND Receptors” inventory, indicating the nuclear receptors identified in molluscs. Data in the “Mollusca AND Receptors” inventory is clustered according to receptor type, nomenclature gene abbreviation and enzyme name, as well as molluscan class. Receptors found in Bivalvia are shown in red, Cephalopoda in blue, Gastropoda in yellow and Polyplachophora in dark green. Receptors were reported as the number of

measurements in respect to species, tissues examined, mollusc sex, organism life stage, and methodological approach implemented. Therefore, multiple measurements can come from one publication. Asterisks (*) indicate that a specific nomenclature abbreviation or name for the reported gene (encoding its respective hormone-metabolising enzyme) could not be identified. In these instances, gene abbreviations were reported according to the information found in respective publications. The number of studies that identified each receptor can be seen using the interactive view. The entire "Mollusca AND Receptors" inventory, including details on all receptors identified, their reported activity, methodological details of included studies and the list of references can be seen in full as interactive graphs.

The receptor with the highest number of measurements (hits) in the "Mollusca AND Receptors" inventory (Fig. 2.7), was in the order of *ESR* (oestrogen receptor) > *RXR* (retinoid X receptor) > *RAR* (retinoic acid receptor). As part of the "Mollusca AND Receptors" inventory, data on the occurrence of *ESR* was captured in Bivalvia, Cephalopoda and Gastropoda. Whereas the occurrence of *RXR* and *RAR* were only captured in Bivalvia, Gastropoda and Polyplacophora (Fig. 2.7). Polyplacophora was the least studied molluscan class identified in the "Mollusca AND Receptors" inventory and was only reported in one study (André *et al.*, 2019).

Non-vertebrate type receptors hold particular significance due to their relatively understudied nature and our limited understanding of their mechanisms in relation to molluscs. This distinction was particularly profound in the "Mollusca AND Receptors" inventory, as vertebrate-type receptors were identified in 96% of included studies compared to non-vertebrate-type receptors that were found in 14% of included studies. Some non-vertebrate receptors seem to exclusively occur in molluscs which suggests that distinct receptor signalling mechanisms could exist in this phylum. For instance, the nuclear receptor *NR8A1* found in tissues of the Bivalve *Crassostrea gigas* (as reported in Huang et al., 2015) seems to belong to a novel nuclear receptor subfamily group namely *NR8*. Although phylogenetic analyses have shown that *NR8* originates from eumetazoans (e.g. a sister animal clade to sponges but also more developed), this NR subfamily group seems to have disappeared from vertebrates and ecdysozoans (Huang, Xu, Li, *et al.*, 2015; Simion *et al.*, 2017). There were also other non-vertebrate receptors in molluscs in the catalogue, for example, *Eip78C* (Ecdysone-induced protein 78C) and *EcR* (Ecdysone receptor) were identified in tissues of the bivalve *Crassostrea gigas* (Vogeler, Tim P Bean, *et al.*, 2016). Ecdysone is traditionally considered an arthropod hormone. Its presence in the tissues of bivalve molluscs suggests a possible endocrinological pathway that is not well investigated.

Worth noting is also the identification of a receptor termed gonadotropin-releasing hormone receptor (*GnRHR*) by Zhang *et al.*, (2020), in the "Mollusca AND Receptors" inventory. Whole

genome and transcriptome sequencing data have demonstrated the presence of GnRH-type receptors and peptides that act as ligands for these receptors in molluscs (reviewed in Roch, Busby and Sherwood, 2011). However phylogenetic analyses on the evolutionary origins of GnRH signalling in molluscs revealed they are paralogous with the corazonin (CRZ) signalling system, originating from a gene duplication of the common ancestor of bilaterians (Roch, Tello and Sherwood, 2014; Zandawala, Tian and Elphick, 2018). A duplication of the GnRH signalling system specifically in arthropods has also resulted in the adipokinetic hormone (AKH) and AKH/CRZ-related peptide signalling systems (Hauser and Grimmelikhuijzen, 2014). Additional phylogenetic analyses in molluscs demonstrated that the *Octopus vulgaris GnRHR* is more closely related to arthropod *CRZ* receptors than the vertebrate *GnRHR* (Roch, Tello and Sherwood, 2014). Since studies have shown that CRZ and "AKH-like" signalling exist in molluscs (Li *et al.*, 2016; Dubos, Bernay and Favrel, 2017; Fodor *et al.*, 2024), but true AKH signalling is specific only to arthropods, a change in the nomenclature of these peptides and receptors was proposed. Thus, Zandawala, Tian and Elphick, (2018) recommended that "GnRH-like" peptides and receptors identified in molluscs should be termed as CRZ peptides or receptors, while peptides and receptors previously termed as "AKH" should now be classified as members of the GnRH superfamily. To ensure this is reflected in our "Mollusca AND Receptors" inventory, a note was included for the molluscan "*GnRHR*" nomenclature term (Fig. 7 – interactive view).

3.3.2. Methodology characteristics for “Mollusca AND Receptors” inventory

Figure 2.8: Part of the “Mollusca AND Receptors” inventory indicating methodological characteristics of studies that employed a DNA/RNA detection and localisation technique to measure receptor genes and proteins in molluscs. Studies were classified based on the type and name of the method employed, the implementation of reference genes or proteins, as well

as the experimental validation of those genes or proteins. The names of reference genes or proteins used are shown in different colours. The entire “Mollusca AND Receptors” inventory, including details on all receptors identified, their reported activity, methodological details of included studies and the list of references can be seen in full as interactive graphs.

The majority (84%, n=59) of studies included in the “Mollusca AND Receptor” inventory utilised a DNA/RNA detection and localisation technique (Fig. 2.8). Among these studies, 90% used reference genes in their analyses. However, only 40% of the studies that implemented endogenous controls, validated the expression stability of these genes across experimental samples. Validation of expression stability in mRNA quantification assays is particularly important as it ensures the suitability of reference genes (or proteins in the case of protein quantification assays), which are vital for correct data normalisation and comparative expression (Bustin *et al.*, 2009; Cowan *et al.*, 2017). In contrast, studies that employed protein and other *in vitro* assays accounted for only 16% of the “Mollusca AND Receptors” inventory (Fig. 2.8 – interactive view).

3.4. Hormone-metabolising enzymes identified in Mollusca

3.4.1. Inventory characteristics

a.

b.

Pathway ..	Gene abb..	Enzyme name (nomenclature)	Publication details	Article title
Thyroid hormone signalling	*DIO*	Iodothyronine deiodinase	Huang et al., 2015	Iodothyronine deiodinase gene analysis of the Pacific oyster Crassostrea gigas reveals possible conservation of thyroid ..
			Jiang et al., 2019	Inhibition of growth in juvenile manila clam Ruditapes philippinarum: Potential adverse outcome pathway of TBBP..
			Song et al., 2016	Exposure to2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) alters thyroid hormone levels and thyroid hormone-regulat..

Figure 2.9: (a) A part of the "Mollusca AND Enzymes" inventory indicating the enzymes involved in steroid biosynthesis, retinoic acid signalling, thyroid hormone signalling and thyroid hormone biosynthesis identified in Mollusca. Hormone-metabolising enzymes were reported in the data extraction inventory by their nomenclature gene and enzyme names and were clustered according to the metabolic or signalling pathway they are involved in. Hormone-metabolising enzymes found in different molluscan classes were separated by colour. Bivalvia are shown in red, Gastropoda are shown in yellow and Polyplachophora are shown in dark green. In graph (a) hormone-metabolising enzymes were reported as the number of measurements with respect to species, tissues examined, organism life stage, and methodological approach implemented. Therefore, multiple measurements can come from one publication, as highlighted in 2.9b. Asterisks (*) indicate that a specific nomenclature abbreviation or name for the reported gene (encoding its respective hormone-metabolising enzyme) could not be identified. In these instances, gene abbreviations were reported according to the information found in respective publications. Part (b) represents the number

of studies (n = 3), including article titles, that identified iodothyronine deiodinase in Mollusca, where each circle represents a separate study. The number of studies that identified each hormone can be seen using the interactive view. The entire "Mollusca AND Enzymes" inventory, including details on all enzymes identified, their reported activity, methodological details of included studies and the list of references can be seen in full as interactive graphs.

Some of the identified genes, enzymes or proteins in the "Mollusca AND Enzymes" inventory were found to be involved in more than one metabolic or signalling pathway, such as the *HSD17B8* gene (which encodes for the enzyme 17β-hydroxysteroid dehydrogenase type 8) which is known to participate in cholesterol biosynthesis as well as the metabolism of steroids and other lipids (Fig. 2.9a – Interactive view). Genes, enzymes and proteins involved in the vertebrate-type steroid biosynthesis pathway were the group of molecules with the highest number of hits in the "Mollusca AND Enzymes" inventory, found in 41% of included studies (Fig. 2.9a). *CYP17A1* which encodes the enzyme 17α-hydroxylase, 17,20-lyase, was the most studied biomolecule found in 19% (n=6) of studies included in the inventory (Fig. 2.9a). In humans, 17α-hydroxylase/17,20 lyase adds a hydroxyl group to the 17-carbon position of progesterone or pregnenolone and can further convert these 17-OH products (via lyase activity) to androstenedione or dehydroepiandrosterone (DHEA), respectively. Therefore, in vertebrates, *CYP17A1* facilitates the production of steroid precursors of cortisol, oestrogen and testosterone. Based on the data collected, the steroidogenic genes *CYP17A1, HSD3B1, STAR3, HSD3B,* and *HSD3B2* were only reported in Bivalves. However, sequences of some of these genes (e.g. *STAR3*) in other mollusc classes have been deposited in databases (e.g. ncbi) outside of the scope of this review, possibly suggesting a reporting bias towards Bivalves in the extracted publications. Steroid sulfatase (encoded by *STS*) and Cytochrome P450 family 19 subfamily A member 1 (encoded by *CYP19A1*) were also reported in Bivalve studies, however, these proteins were identified by a western blot and an enzyme-linked immunosorbent assay (ELISA) respectively, and therefore do not confirm that *STS and CYP19A1 genes* are present in Bivalve genomes. Based on the data collected in the "Mollusca AND Enzymes" inventory, the steroidogenic genes *SRD5A1* and *SRD5A2* which encode the enzymes 5αR1 and 5αR2 respectively, were identified in both Bivalves and Gastropods (Fig. 2.9a). In the "Mollusca AND Enzymes" inventory Bivalvia were the most studied molluscan class (73% of studies), Gastropoda (19%) were the second most studied molluscan class, while Cephalopoda and Polyplachophora were observed in 8% and 5% of included studies, respectively.

In addition to several genes involved in retinoic acid and thyroid hormone signalling (discussed in sections 3.5.3 and 3.5.5), of note is the identification of molluscan homologues for the protein Retinochrome in Polyplacophora (Vöcking, Leclère and Hausen, 2021). Retinochrome, which was first identified in Cephalopods, plays an important role in the visual system of some marine molluscs and is known to interact with the vitamin A metabolite, retinal (Vöcking, Leclère and Hausen, 2021). Yet, and to the best of our knowledge, retinochrome has not been identified outside molluscs. Although the role of retinochrome in retinoic acid signalling is currently not well understood, its identification in several molluscan classes hints at the distinct functions of certain non-vertebrate-type proteins in these animals that are known to interact with retinoids.

3.4.2. Methodology characteristics for "Mollusca AND Enzymes" inventory

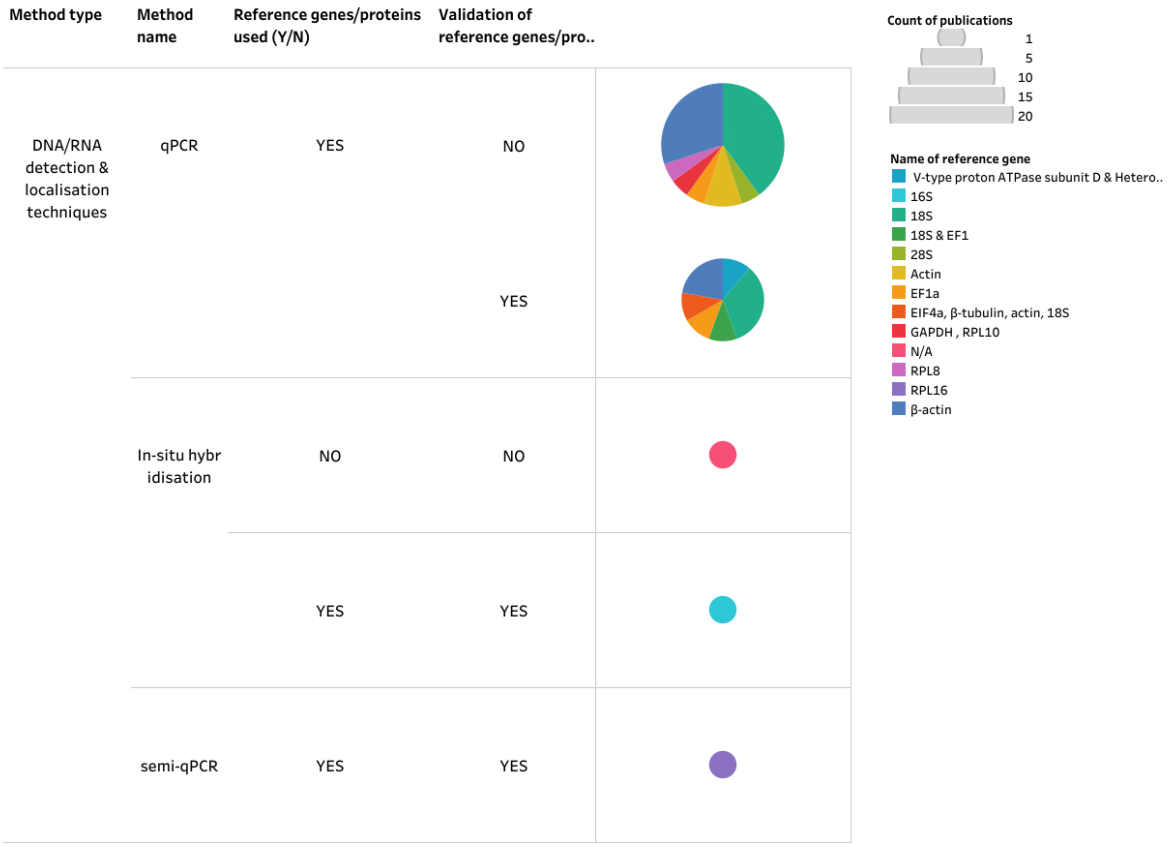


Figure 2.10: Methodological characteristics of studies that measured hormone-metabolising enzymes using a DNA/RNA detection and localisation technique as part of the "Mollusca AND Enzymes" inventory. Studies were classified based on the type and name of the method employed, the implementation of reference genes or proteins, as well as the experimental validation of those genes or proteins. The names of reference genes or proteins used are shown in different colours. The entire "Mollusca AND Enzymes" inventory, including details on

all enzymes identified, their reported activity, methodological details of included studies and the list of references can be seen in full as interactive graphs.

In a similar fashion to the arguments outlined for receptors in section 3.3.2, the identification of mRNA transcripts corresponding to genes involved in metabolic and signalling pathways serves as robust evidence for the existence of their encoded proteins within an organism. However, hormone-metabolising enzymes were identified in eligible studies by DNA/RNA detection and localisation techniques, as well as protein assays. Of all studies included in the "Mollusca AND Enzymes" inventory, 92% (n=32) employed a DNA/RNA detection and localisation technique to investigate the presence of hormone-metabolising enzymes in molluscs (Fig. 2.10). Among these studies, 94% utilised at least one reference gene in their analyses to measure relative gene expression of relevant transcripts. However, only 34% of these studies attempted to validate the expression stability of the endogenous reference genes across experimental samples. Consequently, two-thirds (66%) of studies from the "Mollusca AND Enzymes" inventory, that utilised reference genes or proteins in their DNA/RNA detection and localisation assays, might have reported inaccurate results. Protein quantification assays were employed in 14% of studies included in the "Mollusca AND Enzymes" inventory, none (0%) of these studies utilised reference proteins in their analyses, which raises further concerns regarding the accuracy of reported outcomes.

3.5. Comparative endocrinology across molluscs, vertebrates, and other invertebrates

Given the ongoing debate on the occurrence, and possible role, of vertebrate-type steroids in molluscs (Fodor et al., 2020; Scott, 2012, 2013, 2018) a comprehensive comparison between vertebrate steroidogenesis and the evidence supporting the biosynthesis of these molecules in molluscs is required. Using the findings from our three data inventories (Mollusca AND Hormones, Mollusca AND Receptors, Mollusca AND Enzymes), here we present schematics of the vertebrate cholesterol synthesis pathway (Fig. 2.11) and steroidogenesis pathway (Fig. 2.12) outlining the evidence for the necessary genes and proteins in molluscs and highlighting the gaps in our knowledge. Moreover, a comparative assessment of the retinoic acid signalling pathway between molluscs and vertebrates was also conducted (Fig. 2.14), while the presence of understudied hormonal pathways in molluscs (e.g. ecdysone biosynthesis, thyroid hormone synthesis) is also discussed.

3.6. Evidence on cholesterol biosynthesis in molluscs

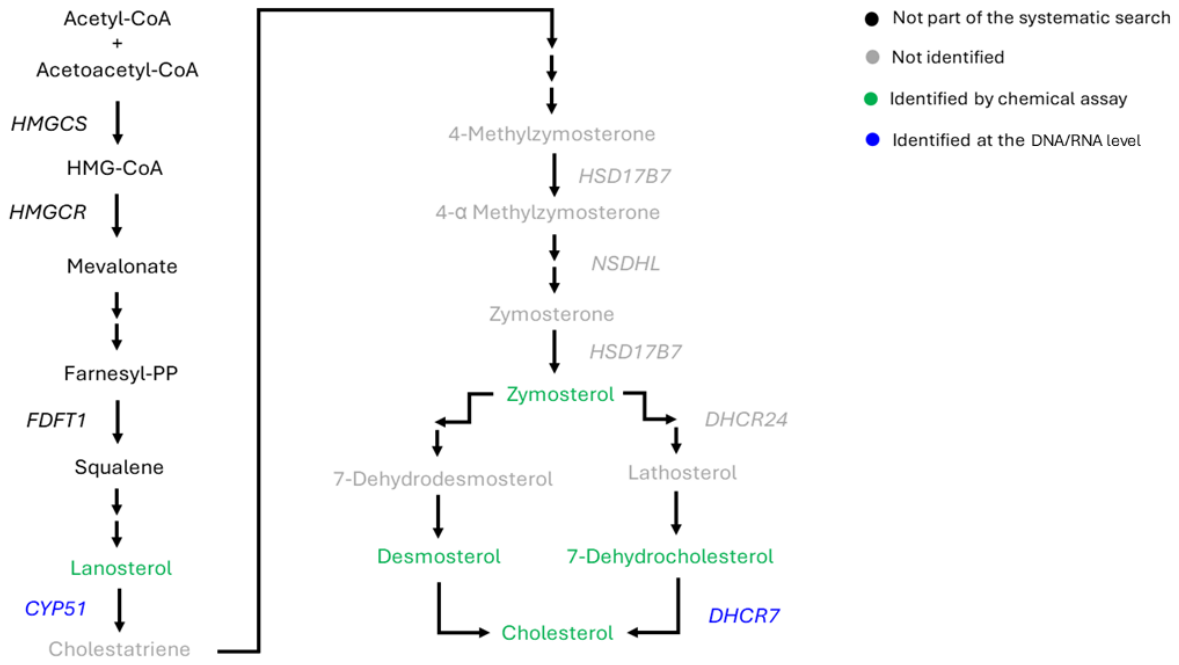


Figure 2.11 Schematic representation of the cholesterol biosynthesis pathway in vertebrates which includes the conversion of sterols (Lanosterol, 4-Methylzymosterone, 4-a Methylzymosterone, Zymosterone, Zymosterol, 7-Dehydrodesmosterol, Lathosterol, Desmosterol, 7-Dehydrocholesterol, Cholesterol) and sterol-related molecules (Acetyl-CoA, Acetoacetyl-CoA, HMG-CoA, Mevalonate, Farnesyl-PP, Squalene, Cholestratriene,) by related enzymes (*HMGCS*: 3-hydroxy-3-methylglutaryl-CoA synthase; *FDFT1:* Farnesyl-diphosphate farnesyltransferase 1, *CYP51:* Cytochrome P450 family 51; *HSD17B7*: Hydroxysteroid 17-beta dehydrogenase 7; *NSDHL:* NAD(P) dependent steroid dehydrogenase-like; *DHCR24*: 24-dehydrocholesterol reductase; *DHCR7:* 7-dehydrocholesterol reductase). Biomolecules included in the "Mollusca AND Hormones", "Mollusca AND Receptors", and "Mollusca AND Enzymes" data inventories are highlighted by colour according to the method used for their identification: black (biomolecules not part of the search strategy), grey (biomolecules that were part of the search strategy but were not identified), green (biomolecules identified in molluscs by a chemical assay), blue (biomolecules identified in molluscs at the DNA/RNA level). The figure was adapted from Saloniemi *et al.*, (2012).

Cholesterol is the most commonly occurring steroid in most living organisms and serves as a precursor for the synthesis of seven classes of steroids in vertebrates (oestrogens, androgens, progestins, glucocorticoids, mineralocorticoids, vitamin D steroids and bile acids) (Norman and Litwack, 1997b). The cholesterol biosynthesis pathway is outlined in Figure 2.11 (Norman and Litwack, 1997b). In molluscs, only a few of the biomolecules known to be involved in cholesterol biosynthesis were identified from our systematic searches (Fig. 2.11). Among them, is the sterol 14-demethylase enzyme which is encoded by the *CYP51* gene and metabolises lanosterol to cholestatriene. Lanosterol was identified in the ovaries, testis and gills of several gastropod species (Kawashima, Ohnishi and Ogawa, 2013; Takishita *et al.*, 2017) while *CYP51* was identified in the gill epithelial bacteriocytes of the bivalve, *Bathymodiolus platifrons* (Takishita *et al.*, 2017). However, neither the activity of *CYP51* nor the identification of the metabolite cholestatriene has been recorded in any of our data inventories (Fig. 2.11). Additionally, several other biomolecules of the cholesterol biosynthesis pathway, including the steroidogenic gene *HSD17B7* which is essential for the conversion of lanosterol to zymosterol, were not captured by our systematic searches (Fig. 2.11). Although the identification of 7-dehydrocholesterol and its metabolising gene *DHCR7* have been identified in the gills of *B. platifrons* (Takishita *et al.*, 2017), functional studies for *DHCR7* have not been reported in any study included in the “Mollusca AND Enzymes” inventory. Currently, the availability of recent evidence in support of a cholesterol biosynthesis pathway in Mollusca is sparse.

3.7. Parallels of vertebrate steroidogenesis identified in mollusc tissues

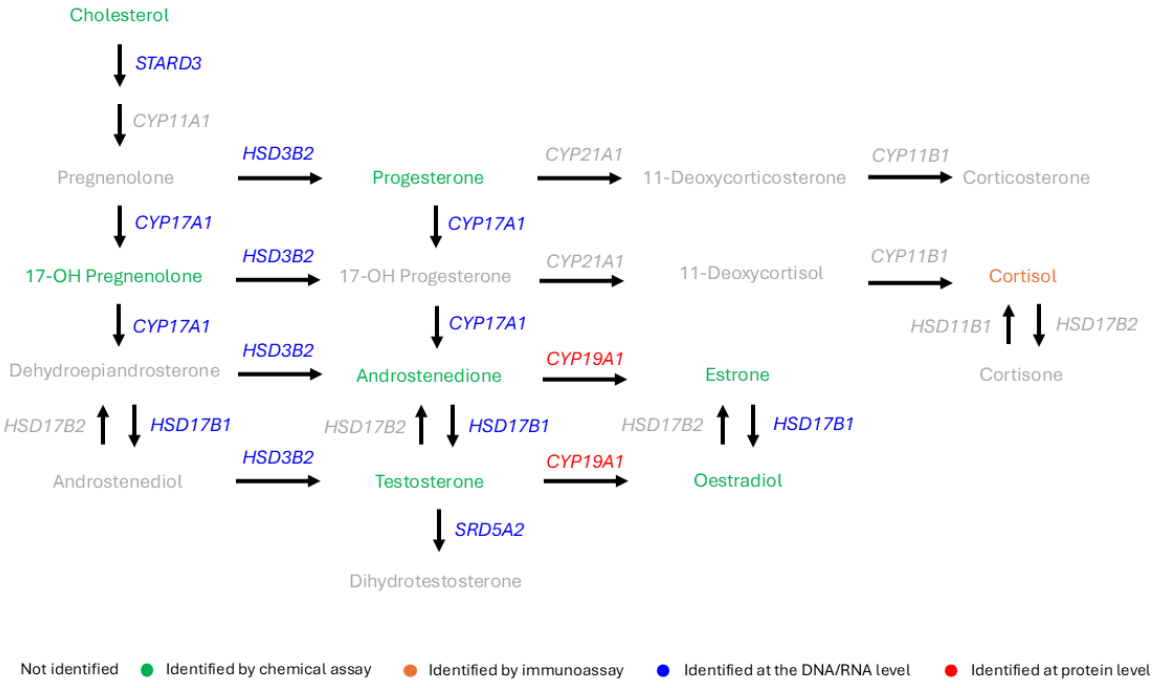


Figure 2.12: Schematic representation of the steroid biosynthesis pathway in vertebrates which includes the conversion of steroids (Cholesterol, Pregnenolone, 17-OH Pregnenolone, Dehydroepiandrosterone, Androstenediol, Progesterone, 17-OH Progesterone, Androstenedione, Testosterone, Dihydrotestosterone, 11-Deoxycorticosterone, 11-Deoxycortisol, Estrone, Oestradiol (17β-estradiol), Corticosterone, Cortisol, Cortisone) by their respective enzymes (*STARD3*: StAR related lipid transfer domain containing 3; *CYP11A1*: cytochrome P450 family 11 subfamily A member 1; *CYP17A1:* Cytochrome P450 family 17 subfamily A member 1; *HSD17B1*: Hydroxysteroid 17-beta dehydrogenase 1; *HSD3B2*: Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2; *CYP17A1*: Cytochrome P450 family 17 subfamily A member 1; *SRD5A2*: Steroid 5 alpha-reductase 2; *CYP21A1:* cytochrome P450 family 21 subfamily A member 1; *CYP19A1:* Cytochrome P450 family 19 subfamily A member 1; *HSD17B2*: Hydroxysteroid 17-beta dehydrogenase 2; *CYP11B1*: cytochrome P450 family 11 subfamily B member 1; *HSD11B1: Hydroxysteroid 11-beta dehydrogenase 1*). Biomolecules included in the "Mollusca AND Hormones", "Mollusca AND Receptors" and "Mollusca AND Enzymes" data inventories are highlighted by colour according to the method use for their identification: grey (biomolecules that were part of the search but were not identified), green (biomolecules identified in molluscs by a chemical assay), orange (biomolecules identified in molluscs by an immunoassay), blue (biomolecules identified in molluscs at the DNA/RNA level), red (biomolecules identified in molluscs at the protein level). The level of robustness is considered higher for biomolecules

identified using chemical assays (green) or at the DNA/RNA level (blue). The figure was adapted from Chakraborty, Pramanik and Mahata, (2021).

The vertebrate steroidogenesis pathway begins with the metabolism of cholesterol to pregnenolone by the P450 cholesterol side chain cleavage enzyme (encoded by the *CYP11A1* gene) (Fig. 2.12, Fig. 2.13). Once pregnenolone is produced it can be converted to a range of steroids, including testosterone, oestradiol (17β-estradiol), cortisol and progesterone by a series of metabolic reactions (Fig. 2.12).

Cortisol pathway: In vertebrate steroidogenesis, pregnenolone is converted to progesterone which in turn is catalysed to corticosterone or cortisol via a series of metabolic reactions, mediated by the enzymes 21-hydroxylase and 11β-hydroxylase, which are encoded by the *CYP21A1* and *CYP11B1* genes, respectively (Fig 2.12). Although cortisol has been reported to be present in molluscan tissues via immunoassays (Binder *et al.*, 2019; Zhang *et al.*, 2021), *CYP21A1* and *CYP11B1* were not reported in any of the studies included in the "Mollusca AND Enzymes" inventory (Fig. 2.12). The lack of molluscan *CYP11B1* in the literature is also supported by phylogenetic analyses that highlight an absence of *CYP11* enzymes from molluscan genomes (Nelson, Goldstone and Stegeman, 2013). Therefore, the only evidence that could be found in the inventory for *de novo* synthesis of cortisol or corticosterone in molluscs relies on the detection of cortisol in tissues via immunoassays, which have known limitations in terms of accuracy and specificity (Gust *et al.*, 2010), suggesting this pathway is not conserved with invertebrates.

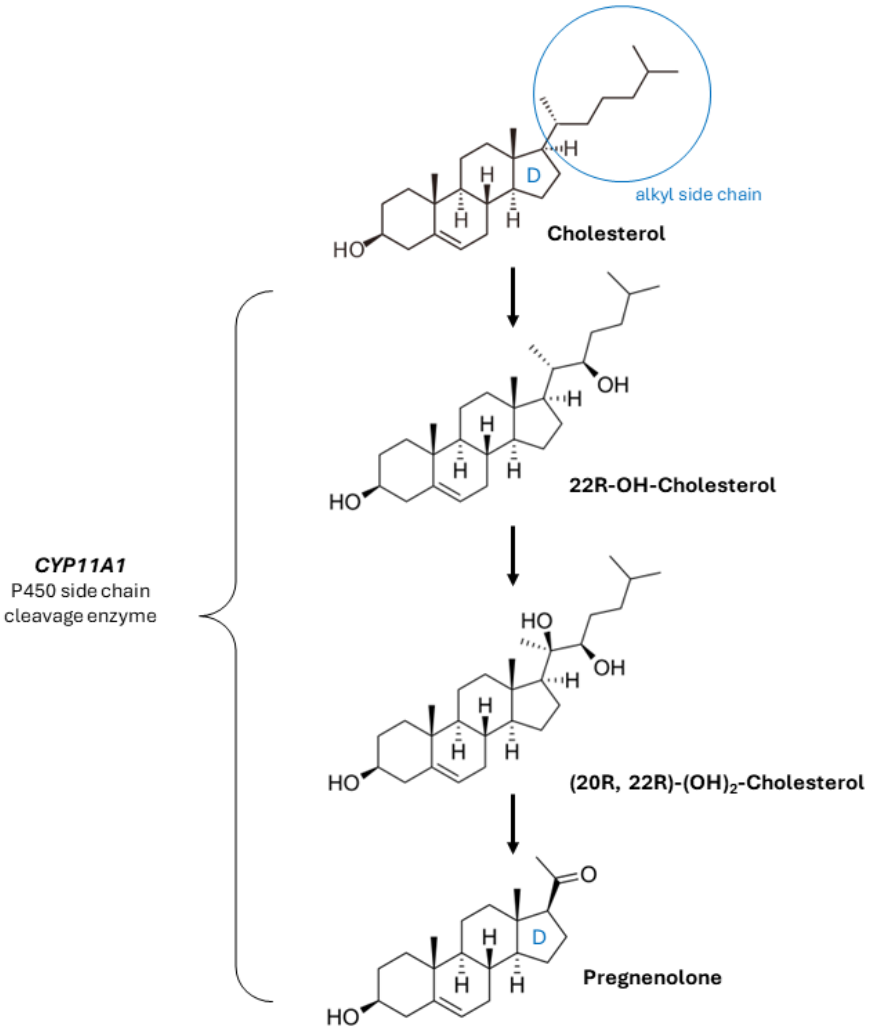


Figure 2.13: Metabolic activity of *CYP11A1* (P450 side chain cleavage enzyme) during vertebrate steroidogenesis, which includes the 22R-hydroxylation and 20R-hydroxylation of cholesterol and its eventual conversion to pregnenolone (via the removal of the alkyl side chain from cholesterol's D carbon ring). The figure was adapted from Norman and Litwack, (1997b).

Sex steroid pathway: In vertebrates, androgens are synthesised via two main metabolic pathways, namely Δ^4 and Δ^5, both of which are derived from pregnenolone (Norman and Litwack, 1997b). Both pathways involve the 17α-hydroxylation of pregnenolone or progesterone via 17α-hydroxylase/17,20 lyase (*CYP17A1*) and the conversion of either dehydroepiandrosterone (DHEA) or androstenedione by 17β-Hydroxysteroid dehydrogenase (*HSD17B1*) to androstenediol or testosterone, respectively. Androstenediol can be converted

to testosterone by 3β-steroid dehydrogenase (*HSD3B3*), which is in turn metabolised to the more potent androgen, dihydrotestosterone, by 5-alpha-reductase (*SRD5A2*). Estrone and oestradiol (i.e. 17β-estradiol), are products of androstenedione and testosterone (respectively) catalysed by the enzyme aromatase, encoded by the *CYP19A1* gene (Fig. 2.12). According to our inventory, a range of steroids (e.g. progesterone, testosterone, 17β-estradiol) in these pathways have been quantified in molluscan tissues via analytical methods (Fig. 2.12), and homologs of many (but not all) of the key steroidogenic enzymes have been identified in molluscs (e.g. *CYP171A*, *HSD3B2*, *HSD17B1*, *SRD5A2*). According to our systematic searches, *CYP19A1* has not been identified in molluscs by a DNA/RNA detection or localisation technique (Fig. 2.12) or in the genomes of molluscs (reviewed by Fodor et al., 2020; Scott, 2012). Aromatase (*CYP19A1*) has been reported in molluscan tissues using vertebrate antibodies (Prisco *et al.*, 2017; Rosati *et al.*, 2019). However, evidence based on vertebrate antibodies in molluscs needs to be considered with caution, as this methodology can be highly inaccurate when it comes to detecting proteins in invertebrates, as extensively reviewed by a recent study (Fodor et al., 2022). This emphasises the necessity for implementing reforms in the way we identify enzymes in molluscs. If we are to ensure reliability in the reported outcomes, future investigations should prioritise the utilisation of molluscan antibodies in protein quantification assays. These analyses should be further accompanied by genomic investigations of the mRNA transcripts that encode for those proteins.

3.8. Evidence on retinoic acid signalling in molluscs

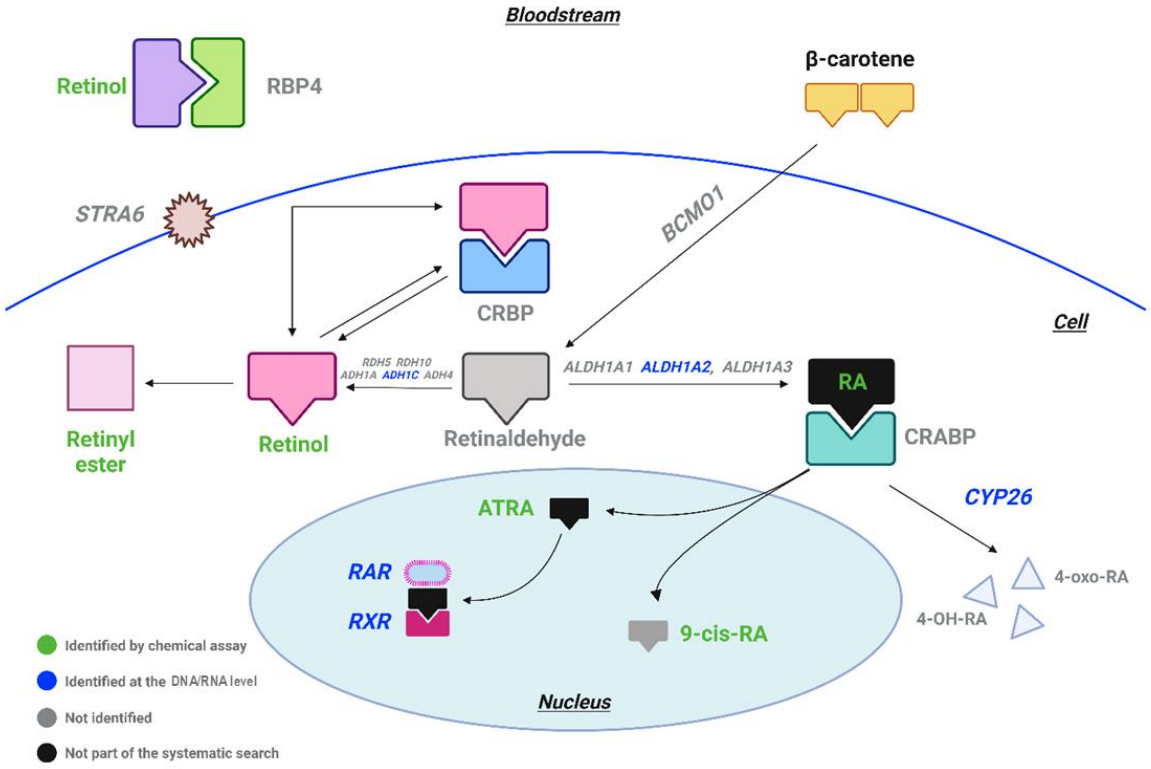


Figure 2.14: The retinoic acid signalling pathway in vertebrates, which includes the enzymes and nuclear receptors involved in retinoid metabolism and retinoid transfer. Biomolecules included in the systematic searches are highlighted by text colour according to the method use for their identification: black (biomolecules not part of the search), grey (biomolecules that were part of the search but not identified), green (biomolecules identified by a chemical assay), blue (biomolecules identified at the DNA/RNA level). The figure was created with the online tool BioRender,com.

Retinoic acid (RA) is a metabolic derivative of retinol (vitamin A). Isomers of RA, such 9-cis-RA, all-trans-RA and 13-cis-RA, exhibit distinct biological activities and play important roles in different biological processes (Ghyselinck and Duester, 2019). In vertebrates, RA synthesis begins with the conversion of retinol to retinaldehyde by two types of enzymes: alcohol dehydrogenases (*ADH1A, ADH1C*, and *ADH4*) or retinol dehydrogenase (*RDH1* and *RDH10*) (Kumar *et al.*, 2012). Retinaldehyde is then metabolised to RA with the help of the enzymes RALDH1, RALDH2 and RALDH3, encoded by the *ALDH1A1*, *ALDH1A2*, and *ALDH1A3* genes respectively (Fig. 2.14). Interestingly, some vertebrate animals, like zebrafish, are known to have lost the *ALDH1A1* ortholog during evolution and thus utilise only *ALDH1A2* and

ALDH1A3 during RA synthesis (Cañestro *et al.*, 2009). Eventually, the metabolism of retinaldehyde to RA will lead to the degradation of RA by *CYP26* enzymes (*CYP26A1*, *CYP26B1*, *CYP26C1*) which thus convert it to inactive metabolites (Ghyselinck and Duester, 2019). Once metabolised, RA isomers act as ligands for the nuclear Retinoic Acid Receptors (RAR). The main RA that binds to RARs is all-trans-RA (ATRA), although other isomers with lesser affinity, such as 9-cis-RA can also bind to RARs (Kumar *et al.*, 2012). Once bound to a ligand, RARs are known to form heterodimers with retinoid X Receptors (RXRs), which in contrast to RARs, can only bind to 9-cis-RA. Once formed, the RAR/RXR complex can then modulate gene transcription by binding to specific DNA sequences known as the RA response elements (RARE) (Ghyselinck and Duester, 2019).

Many of the biomolecules involved in the RA signalling pathway have also been reported to exist in molluscs (Fig. 2.14). Our systematic searches identified the occurrence of retinol, RA and RA isomers in various molluscan species and tissues (Fig. 2.14 and Fig. 2.4a interactive view). Consequently, the presence of *ADH1C* and *ALDH1A2* orthologs in molluscan genomes (Coelho *et al.*, 2012; Rothwell *et al.*, 2014) provide evidence on the potential conservation of retinoid acid signalling in Mollusca. Indeed, exposure of gastropod *N. lapillus* females to retinol was shown to down-regulate the *ADH1C* gene expression levels in the gonads (Coelho *et al.*, 2012). However, whether *ADH1C* and *ALDH1A2* can metabolise their respective retinoids in molluscs, remains to be elucidated. Nonetheless, evidence has previously demonstrated that some retinoid receptors found in molluscs exhibit comparable functions to those found in vertebrates. Particularly, RXR in molluscs was shown to bind to the RA isomer, 9-cis-RA, *in vitro* (Gutierrez-Mazariegos et al., 2014). Conversely, *in vitro* exposure of molluscs to retinoids and retinoic acid isomers including retinal, retinol, all-trans-RA, 9-cis-RA and 13-cis-RA, demonstrated the inability of molluscan RARs to bind to, and thus be activated by, their respective ligands. However, it was shown that RAR can form a heterodimer complex with RXR (Gutierrez-Mazariegos et al., 2014; Urushitani et al., 2013). The evidence suggests that partial similarities exist between the vertebrate and molluscan RA signalling pathways. However, differences exist in the regulatory mechanisms of retinoid receptors between these two groups, which imply that molluscs and vertebrates have followed separate evolutionary paths (Gutierrez-Mazariegos et al., 2014).

3.9. The occurrence of phytosterols and ecdysteroids in molluscs

Interestingly, our systematic searches identified the presence of a wide range of phytosterols (plant sterols) in molluscan tissues. Until recently, phytosterols were thought to exclusively occur in plants and that phytosterols were only able to enter an animal's body through its diet (Özyurt *et al.*, 2013). However, a recent study has demonstrated that some gutless marine annelids can synthesise sitosterol (plant sterol) as well as cholesterol de novo (Michellod *et al.*, 2023). Plant-feeding insects were previously shown to metabolise phytosterols and convert them into cholesterol (Ikekawa, Morisaki and Fujimoto, 1993). This conversion involves the breakdown of alkyl groups that are attached to the 24^{th} carbon atom of the phytosterol molecule (Ikekawa, Morisaki and Fujimoto, 1993). Surprisingly, bivalve molluscs were also found to possess similar metabolic abilities. The northern bay scallop *Argopecten irradians* was shown to metabolise the radiolabelled phytosterols 24-methylenecholesterol, 24-propylidenecholesterol, epioccelasterol, brassicasterol, 4α-methylcholestanol and 4α-methylcholest-8(14)-enol to cholesterol (Giner *et al.*, 2016). An especially interesting observation was the ability of this species to produce $\Delta^{5,7}$ sterols, such as provitamin D, from Δ^{5} (phyto)sterols (Giner *et al.*, 2016). The induction of a double bond at the Δ^{7} position of the sterol molecule is the reverse mechanism from the one that exists in vertebrate sterol biosynthesis. This ability seems to be conserved in nematodes and insects, where the induction of a double bond at the Δ^{7} position of a sterol molecule, induces the biosynthesis of the insect steroid ecdysone in the latter (Chitwood, Lusby and Salt, 1987; Huang, Warren and Gilbert, 2008). Notably, a recent phylogenetic analysis suggests that molluscs do not contain the 24-C sterol methyltransferase (*SMT*) gene in their genomes, which is vital for the synthesis of phytosterols (Brunoir *et al.*, 2023). Together, this evidence suggests that molluscs may uptake Δ^{5} (phyto)sterols from the environment which in turn can convert to $\Delta^{5,7}$ sterols for subsequent steroid biosynthesis. These observations also bring into question whether insect steroid hormones, like ecdysone, are produced in molluscs endogenously.

Although the identification of insect steroids in molluscs was part of our search strategy, 7-dehydrocholesterol was the only ecdysteroid intermediate reported to exist in molluscs, as part of the "Mollusca AND Hormones" inventory (Hurtado *et al.*, 2012; Takishita *et al.*, 2017). Nevertheless, a growing body of evidence has identified many types of receptors in molluscs that were previously thought to exist exclusively in insects (Stange and Oehlmann, 2012; Raingeard *et al.*, 2013; Vogeler, Tim P Bean, *et al.*, 2016; Pes *et al.*, 2021). Among them, gene transcripts of the ecdysone receptor (*EcR*), which binds to and is activated by ecdysone in insects, were found to be expressed in the embryo and whole body of the bivalve *Crassostrea gigas* (Vogeler, Tim P Bean, *et al.*, 2016). Consequently, the relative expression

of *EcR* transcripts was found to vary across developmental stages and was particularly up-regulated 15 days post-fertilisation (Vogeler, Tim P Bean, *et al.*, 2016). A recent study has identified the expression of both membrane and nuclear *EcR* homologs in the genome of *L. stagnalis* (Fodor *et al.*, 2024) whereas another study measured ecdysone concentrations and the relative expression of *EcR* transcripts in tissues and larval developmental stages of the oyster *Pinctada fucata martensii* (Xiong *et al.*, 2022). *EcR* was found to be most highly expressed at the gastrula stage of *P. f. martensii* larvae and the mantle tissue of adults. Most importantly, a shell notching experiment in the same study revealed an increasing ecdysone serum production from 2 - 8 hours post-shell damage which coincided with increasing relative expression levels of *EcR* at the same time points (Xiong *et al.*, 2022). Such findings suggest that molluscs, or at least bivalves, may produce ecdysone endogenously, although care must be taken with these initial studies as ecdysone was measured via an insect 20-hydroxyecdysone ELISA Kit (Xiong *et al.*, 2022). Another hypothesis is that molluscs absorb ecdysone from their diet rather than synthesising it endogenously (Garcia, Griffond and Lafont, 1995), thus leading to its interaction with a functional *EcR* in these animals. Notably, exogenous ecdysone has been demonstrated to interact with an endogenous GPCR-type receptor in mammals, via *in vitro* and *in sicilo* approaches (Lafont *et al.*, 2022). However, it remains unclear whether exogenous ecdysone can activate molluscan *EcR*. Moreover, as highlighted with *RAR* and *ESR* above, molluscan NRs don't always bind to or are activated by their expected ligands. Elucidating possible ecdysteroid biosynthesis pathways, and ecdysone's potential binding affinity to *EcR* will be important to fully understand any role ecdysteroids might play in mollusc endocrinology.

3.10. The occurrence of thyroid hormones in molluscs

Over the past decade, the discovery of thyroid biomolecules in molluscs has generated considerable interest, suggesting the potential existence of a thyroid hormone signalling pathway in these animals. Notably, the two main thyroid hormones thyroxine (T4) and triiodothyronine (T3) have been chemically observed in the haemolymph tissues of the gastropod *Achatina fulica* (Lustrino *et al.*, 2017), as well as in embryos and other tissues of the bivalves *Rutidapes philippinarum* and *Crassostrea gigas* according to our systematic searches (Huang W. et al., 2019; Huang W. et al., 2015; Jiang et al., 2019; Song et al., 2016). Consequently, mRNA transcripts of the thyroid receptor (*THR*) were measured at several larval development stages of another bivalve, *Mytilus unguiculatus* (Li *et al.*, 2020). Interestingly, an RNA interference experiment, which caused the knockdown gene expression of *THR*, revealed significant down-regulation of its relative gene expression levels which in turn led to significant inhibition of larval metamorphosis and a decline in larval viability (Li *et*

al., 2020). Such findings indicate the potential involvement of *THR* in the early development of bivalves, however, the activity of *THR* in molluscs does not seem to be a result of binding to T4 or T3 (Huang, Xu, Qu, Zhang, *et al.*, 2015; Morthorst *et al.*, 2023). Nonetheless, gene transcripts that encode multiple thyroid hormone-metabolising enzymes were also recorded in the "Mollusca AND Enzymes" inventory. These include transcripts of the enzyme thyroid peroxidase (*TPO*) (Song *et al.*, 2016; Jiang *et al.*, 2019) which is involved in thyroid hormone biosynthesis of vertebrates and isoforms of the enzyme deiodinase (*Deio*) known to activate and deactivate thyroid hormones. In fact, the relative mRNA expression of the latter was found to be up-regulated following exposure of *Crassostrea gigas* larvae to exogenous T4 (Huang, Xu, Qu, Li, *et al.*, 2015).

4. Discussion

Gene expression assays such as quantitative PCR (qPCR) and *in situ* hybridisation, can accurately determine the presence of molecules (such as receptors or hormone-metabolising enzymes) at the genomic (DNA) and transcription (RNA) level (Kirby *et al.*, 2007; Nygaard and Hovig, 2009). On the other hand, protein expression assays, like western blots or immunohistochemistry techniques, are only able to detect molecular components at the protein level (Shebl *et al.*, 2010). Consequently, the presence of a protein revealed by such assays in an organism does not necessarily mean that it is encoded by a gene in the organism's genome. Indeed, the concept of exogenous protein uptake in molluscs has been known for decades (Bottke and Sinha, 1979; Bottke, Sinha and Keil, 1982). To confirm the endogenous nature of a receptor or hormone-metabolising enzyme in any organism it is necessary to examine the respective gene at the genome (DNA) and expression (RNA) level (Nygaard and Hovig, 2009; Shebl *et al.*, 2010). Fundamental to the question of vertebrate-type steroidogenesis in molluscs is the *CYP11A1* enzyme, which plays a crucial role during vertebrate steroidogenesis as it presents the very first step of *de novo* steroid biosynthesis from cholesterol (Fig 2.12, Fig 2.13). Positive identification of a molluscan *CYP11A1* was absent from the "Mollusca AND Enzymes" inventory, as well as from the genomes of molluscs studied through previous transcriptomic and whole-genome analysis studies (Adema *et al.*, 2017; Fodor, et al., 2021). This lack of *CYP11A1* implies that molluscs are unable to metabolise cholesterol to produce pregnenolone, and therefore any of the other subsequent vertebrate-type steroid *de novo* (Fig. 2.13). If molluscs are endogenously producing steroids from cholesterol, it is likely their structure(s) will differ from vertebrate ones, as is the case in arthropods. Arthropods use cholesterol to produce ecdysone through a series of metabolic reactions which are catalysed by a distinct set of P450 enzymes involved in insect steroidogenesis (Petryk et al., 2003). Ecdysone's structure varies from that of any vertebrate-

type steroid, such as pregnenolone, as it contains a unique side chain on its D carbon ring (Fig. 2.15).

If molluscs cannot synthesise “vertebrate-type” steroids the considerable body of literature that has measured these steroids, using robust and sensitive analytical chemistry methods, in mollusc tissues needs to be addressed. Why are oestrogens or testosterone detected in molluscan tissues if they are not being produced by these animals? Researchers have investigated this concern and have proposed that molluscs can uptake and accumulate steroids in their tissues from exogenous sources/the environment (Schwarz *et al.*, 2017; Fodor *et al.*, 2022). For example, a study conducted by Schwarz *et al.*, (2017) demonstrated that species within the *Mytilus* genus, possess the ability to absorb radiolabelled testosterone from water, esterify it and store it in their tissues. Similarly, Fodor *et al.*, 2022 confirmed the freshwater gastropod *Lymnaea stangalis* can also absorb and esterify a range of steroids from water, including testosterone, 17β-estradiol and progesterone.

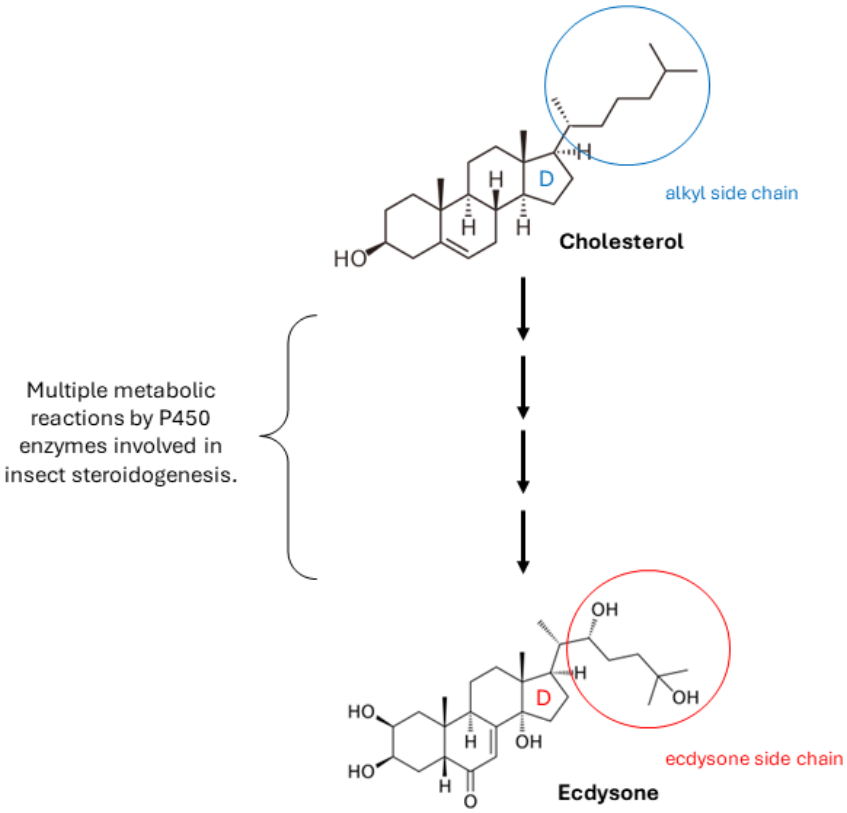


Figure 2.15: The conversion of cholesterol to ecdysone in insect steroidogenesis after a series of metabolic steps catalysed by P450 enzymes specific to this pathway (names of the enzymes not shown). The cholesterol molecule contains an alkyl side chain at its D carbon ring which is converted to an ecdysone-specific side chain attached at the same carbon ring.

Additional lines of evidence also need to be considered, beyond the process of steroid synthesis, to consider steroid signalling pathways. The identification of nuclear oestrogen receptor (*ESR*) in molluscs has been held as supporting evidence for the biological relevance of oestrogens in these animals (Lü *et al.*, 2016). However, although the *ESR* gene in molluscs was initially thought to be homologous to the vertebrate nuclear *ESR*, a recent study suggested that molluscan *ESR* is in fact orthologous to the common ancestor of vertebrate *ESR* and oxosteroid receptors (androgen receptor, glucocorticoid receptor, mineralocorticoid receptor and progesterone receptor) (Hochberg *et al.*, 2020). Moreover, the ligand binding pocket of mollusc ESRs does not bind to oestrogens (or other steroids), and oestrogens do not activate molluscan ESRs (Bridgham *et al.*, 2014). This does not mean mollusc ESRs are redundant. Instead, mollusc ESRs may be constitutively active and regulate gene transcription without a ligand (i.e. steroids). For example, in bivalves, *ESR* expression has been shown to change during key developmental stages (Vogeler *et al.*, 2016)(Fig. 2.16). Additional lines of evidence have indicated the presence of non-genomic oestrogen signalling in bivalves, suggesting exogenous oestrogens may be modulating ESRs through independent receptor pathways (reviewed in Balbi, Ciacci and Canesi, 2019). Such alternative pathways may be associated with membrane or cytosolic adaptor proteins and may take place either on the plasma membrane or in the cytosol, rather than the cell nucleus (Balbi, Ciacci and Canesi, 2019). However, in the absence of evidence for a functional and specific membrane oestrogen receptor homolog in molluscs, capable of mediating non-genomic oestrogen signalling (as it does in vertebrates), these suggestions cannot be confirmed. Notably, a recent study by Fodor *et al.*, (2024) demonstrated the unresponsiveness of the membrane-bound G protein-coupled oestrogen receptor 1 (*GPER1*) when exposed to oestrogens, thus suggesting an absence of this homologue in *Lymnaea stangnalis*. Additionally, the authors demonstrated the inability of a membrane progesterone receptor (*mPR*) homologue to respond to progesterone in the same model organism (Fodor *et al.*, 2024). Together, it was hypothesised that both genomic and non-genomic sex steroid signalling may not be conserved in molluscs. In line with these observations, it has been recommended that genes previously termed molluscan nuclear *ESRs* should be classified as *NR3D* (Markov and Laudet, 2011; Fodor *et al.*, 2024).

Despite poor evidence for vertebrate-type steroid biosynthesis in molluscs, some vertebrate-type steroidogenic enzymes were reported to exist in molluscan genomes. Most notably, the genes *CYP17A1*, *HSD17B1*, *SRD5A1* and *SRD5A2*, whose enzymes in vertebrates metabolise progesterone, androstenedione and testosterone respectively, were all measured in molluscan tissues at the DNA or RNA level. However, the identification of steroidogenic enzymes in molluscan genomes does not confirm the existence of identical enzyme substrates or products (as the ones found in vertebrates). For example, in plants, the *DET2* gene encodes

an enzyme that has a significant sequence similarity with mammalian 5αR1/5αR2 (encoded by *SRD5A1*/*SRD5A2* in vertebrates) (Li and Chory, 1999). In vertebrates, 5αRs convert testosterone to dihydrotestosterone. *DET2* has been proven to be a functional ortholog of the vertebrate 5αR, catalysing testosterone to dihydrotestosterone under experimental conditions (Li and Chory, 1999; Rosati *et al.*, 2003). Although *DET2* has a similar catalytic role as *SRD5A2*, in plants it acts on a different set of substrates, called brassinosteroids, (i.e. plant steroids), metabolising campesterol to campestenol (Li and Chory, 1999). The presence of enzymes like Det2/5αR in broad groups of organisms (e.g. plants, vertebrates, invertebrates) generally suggests they have a long evolutionary history (Markov *et al.*, 2017) and substrates may differ as organisms evolve.

Receptor type	Receptor gene abbreviation (nomenclature)	Enzyme name (nomenclature)	Method_ type (group)	Type of primer/ antibody used	Evidence of Receptor's activity (Y/N)	Type of intervention	Details of activity	Tissue observed	
Nuclear receptor	*ESR*	Estrogen receptor	DNA/RNA Detection and Localisation Techniques	Mollusc-specific primers	NO	Exposure to Testosterone..	No evidence of activity	ovary	●
						No intervention	No evidence of activity	adductor muscle	●●●●
								digestive gland	●●●●●
								gill	●●●●●
								mantle	●●●●
								ovary	●●●
								pedal ganglion	●
								spent gonad	●
								testis	●●●●
					YES	Change of expression following ex..	Up/down-regulated expression	embryo (d-shaped l..	●
								embryo (trochophore..	●
						Exposure to B(a)p at 0.4u..	Up-regulated expression afte..	gonad	●
						Exposure to B[a]P at diffe..	Up/down-regulated expre..	ovary	●
						Exposure to BPA (1ug/L a..	Up/down-regulated expre..	embryo (d-shaped l..	●
						Exposure to different con..	Up/down-regulated expre..	ovary	●
						Exposure to Estradiol (E2..	Up-regulated expression	ovary	●
						No intervention	Up/down regulated expre..	ovary	●
							Up/down-regulated expression acro..	egg	●
								whole body	●●
							Up/down-regulated expression across different tissues.	adductor muscle	●●
								gill	●●
								mantle	●●
								ovary	●
								testis	●
								visceral mass	●●
							Up/down-regulated expression in different tissues.	adductor muscle	●
								digestive gland	●
								gill	●
								haemocytes	●
								heart	●
								mantle	●
								ovary	●
								testis	●
						RNAi (interference)..	Down-regulated expression	haemocytes	●

Mollusc Class
Bivalvia

Figure 2.16. Oestrogen receptor (*ESR*) activity in Bivalves as observed from the studies included in the "Mollusca AND Receptors" inventory. Receptor's activity was clustered

according to receptor type, abbreviation and name, as well as whether evidence of activity has been reported. Details of receptor's activity are reported in respect to the type of intervention used, the tissue observed, the species examined, as well as molluscan class, sex, and life stage. The full details on receptor's activity from the "Mollusca AND Receptors", including details on all receptors identified, their reported activity, methodological details of included studies and the list of references can be seen in full as interactive graphs inventory can be seen in full via interactive view.

For example, Markov *et al.*, (2017) suggested that newly emerged metabolites can act as substrates for already existing enzymes, and this could lead to either an extension or extinction of metabolic pathways. An interesting example taken from their analyses is the late emergence of modern oestrogens (e.g. 17β-estradiol) in basal vertebrates which in turn outcompeted an already existing group of metabolites called 'paraestrols' (Markov *et al.*, 2017). Taken together, the evidence supporting the endogenous synthesis of vertebrate-type steroids in molluscs is unconvincing. Future steroid research in molluscs should be directed towards understanding possible novel substrates, products, and pathways, rather than continuing the vertebrate-centric approach of the last few decades. On the other hand, the evident similarities that exist between molluscan and vertebrate retinoic acid signalling pathways indicate the activity of retinoids and their role in molluscan biology. The retinoid system is known as a key regulator of various biological processes throughout life, from embryo development and body patterning to reproductive and immune function in other organisms (OECD, 2021a). In contrast, the retinoid system in molluscs (beyond the impacts of TBT on marine gastropods) remains largely understudied. Moreover, the evidence on the existence of understudied hormonal signalling pathways in molluscs, including potential biosynthesis of ecdysteroids and thyroid hormones, seems promising. However, the lack of information regarding the activity of some retinoid, thyroid and ecdysteroid biomolecules, calls for further molecular investigations on various components within those pathways, which can potentially unveil insights into the regulatory mechanisms and evolutionary history of molluscan endocrine systems.

5. Summary, Future Research Needs & Perspectives

To the best of our knowledge, this review has provided the most comprehensive and systematic overview of molluscan endocrinology to date. Vitally, it combines the reports of hormones, receptors and enzymes identified in Mollusca with a risk-of-bias assessment to consider the robustness (or weaknesses) in the data presented. Evidently, the majority of research surrounding molluscan endocrinology which has long focused on attempting to discover parallels with the vertebrate steroid hormone signalling pathway still persists in our

three inventories (2012-2021), frequently using approaches or methodologies which are not specific enough or reliable in molluscan tissues. Moreover, the disparity between the number of papers looking at vertebrate-type steroids compared to retinoids is stark, given that retinoids have been known to be involved in key developmental processes in molluscs since the early 2000's (Nishikawa *et al.*, 2004). Vitally, there is evidence indicating endogenous production of other hormones, including insect steroids, retinoids and thyroid hormones in molluscs. This provides opportunities for novel investigations that could unlock a better understanding of molluscan endocrinology.

Given the evidence presented here, it is strongly recommended that the research community begin to explore these less investigated endocrine pathways in molluscs rather than continuing to focus on a vertebrate-centric approach (Goździk *et al.*, 2023).

In summary, our recommendations are:

- Further development and use of non-targeted approaches (e.g. 'omics) to prevent vertebrate-orientated bias in molluscan endocrine research. Combinations of metabolomic, proteomic, lipidomic and genomic are needed to support detailed endocrine pathway analysis.
- Collaboration between experts in the evo-devo, analytical chemistry and experimental biology fields should be encouraged!
- Further investigations of exogenous hormone uptake in molluscs are needed e.g. studying the mechanisms through which molluscs absorb and store hormones from their environment.
- When investigating if certain hormones are involved in an animal's endocrinology, a holistic approach should be taken i.e. by investigating the interactions of hormones confirmed to be present in molluscs (using robust methodologies), with their respective receptors, and hormone-metabolising enzymes known to be expressed in molluscan genomes. Other components of relevant metabolic pathways should also be examined.
- Expand retinoid signalling research in molluscs by examining the presence and function of understudied retinoic acid signalling biomolecules. These investigations should include a comparative assessment of potential similarities and differences between the molluscan and vertebrate retinoic acid signalling pathways.
- Further explore the function of thyroid signalling in molluscs by investigating the role of thyroid hormones T3 and T4, as well as the function of *THR* during molluscan development.

- Explore the presence of ecdysteroid signalling in molluscs by investigating the occurrence and function of related biomolecules in molluscs. Investigations should also focus on the uptake and conversion of Δ^5 (phyto)sterols to $\Delta^{5,7}$ sterols in molluscs and their potential involvement in ecdysteroid biosynthesis.
- For all such investigations, robust analytical methods must be implemented, e.g. investigations performed at the molecular level, must confirm the presence of respective biomolecules at the genome (DNA) and expression (RNA) level using appropriately validated approaches. Protein-level investigations should implement mollusc-specific antibodies to ensure the reliability of findings.

6. Conclusions

This study has developed a fully searchable database that comprises the systematic categorisation and critical appraisal of nine years of evidence on molluscan endocrinology. This resource is available for the scientific community to use and aims to assist in directing future research efforts towards the exploration of understudied hormonal pathways in molluscs using robust methodological design. As of the present, the occurrence of cholesterol biosynthesis in molluscs remains poorly understood as it lacks supporting evidence. Although some key enzymes such as *CYP51* have been identified, evidence of their metabolic activity in molluscs remains sparse. Consequently, the absence of vital steroidogenic genes from molluscan genomes, including *CYP11A1* and *CYP21A1*, suggests the inability of molluscs to synthesise vertebrate-type steroids *de novo*. Existing evidence on retinol metabolism in molluscan tissues suggests a partial similarity between the vertebrate and molluscan retinoic acid signalling pathway. However, more research is needed to elucidate the role of understudied biomolecules in molluscs that are involved in this pathway. The occurrence of thyroid hormones and the reported activity of the thyroid receptor in molluscs, highlights the potential involvement of these biomolecules in developing bivalves but existing evidence on the role of thyroid signalling in these animals remains sparse. Whereas, the presence of a wide range of phytosterols and the production of $\Delta^{5,7}$ sterols in molluscs indicates potential endogenous synthesis of insect steroid hormones (e.g. ecdysteroids), accompanied by evidence of the presence and activity of their respective nuclear receptors. Future investigations should implement robust experimental design and analytical methodologies to study molluscan endocrine systems and should focus on the investigation of understudied or novel hormonal pathways in these animals.

Chapter 3: Development and validation of a RT-qPCR assay to measure *SRD5A1* and *SRD5A2* transcript expression in embryonic *Biomphalaria glabrata*

1. Introduction

1.1. Background

Given that molluscan genomes lack an androgen receptor (AR), as well as important enzymes involved in the synthesis of “vertebrate-type” steroids, the function of 5-alpha reductase (5αR) enzymes in these organisms, remains poorly understood. The systematic investigations conducted in Chapter 2, demonstrated that *SRD5A1* and *SRD5A2* mRNA transcripts, which are homologous to their vertebrate counterparts and encode 5αR1 and 5αR2 respectively, have been identified in at least three molluscan species, including the bivalve *Crassostrea homogenesis* (Tong *et al.*, 2015a) and the gastropod *Biomphalaria glabrata* (Baynes *et al.*, 2019). Toxicological investigations of Baynes *et al.*, (2019) revealed that exposure of gastropod *B. glabrata* and *Physella acuta* embryos to 100 µg/L of the pharmaceutical 5αR inhibitor, dutasteride (DUT), caused the formation of an elongated shell phenotype in 80% and 71.2% of embryos, respectively. This abnormality was termed “banana-shaped shell”, and was first observed at the “hippo stage” of development which occurs 60 hours post-oviposition (when the eggs are laid)(Marxen *et al.*, 2003; Baynes *et al.*, 2019).

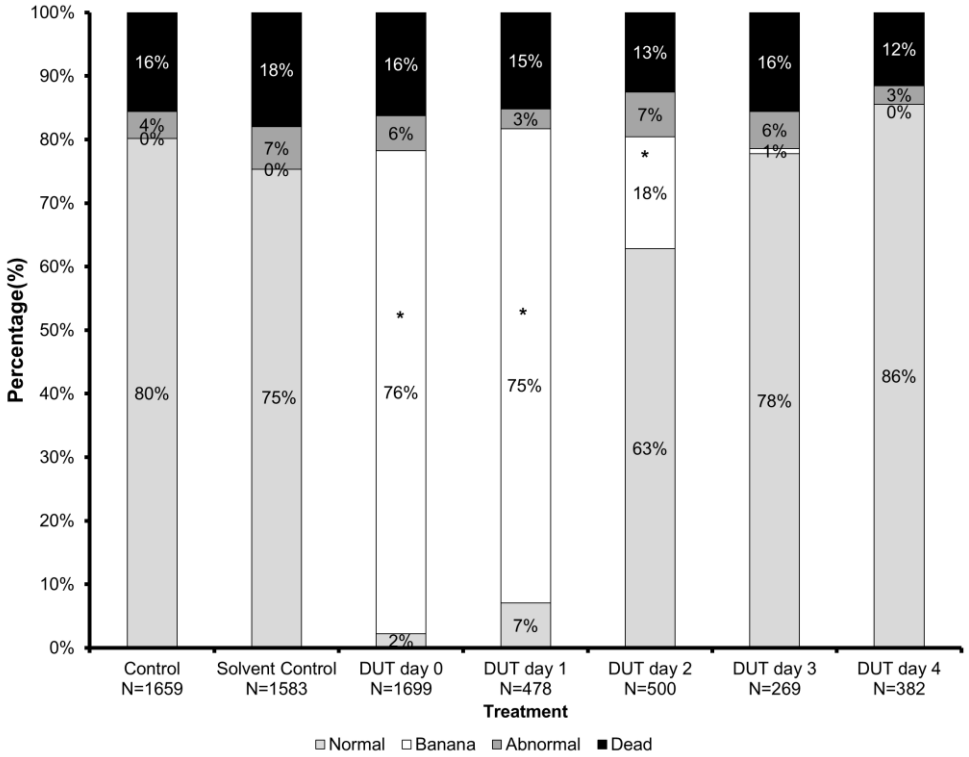


Figure 3.1: Percentages of normal (grey), 'banana-shaped' (white), abnormal (dark grey) and dead (black*) B. glabrata* embryos after being exposed to 100µg/L DUT at different developmental stages (day 0 – day 4 post oviposition). Asterisks represent significant differences ($p < 0.05$). The data was taken from unpublished investigations of Dr Alice Baynes and Hazzel Tabernilla. The total number of embryos exposed to each DUT treatment is presented as "N" underneath each bar. Each experiment included positive (DUT day 0) and negative controls (control, solvent control).

To further investigate the sensitive window of this pharmaceutical disruption, Dr Alice Baynes and Hazzel Tabernilla exposed day 0 – day 4 post-oviposition *B. glabrata* embryos to 100 µg/L DUT and recorded their responses. Their unpublished findings indicated that the "banana-shaped shell" phenotypes were significantly more induced in the day 0 – day 2 post-oviposition embryos (Fig. 3.1) These novel findings suggest that the trochophore stage, which occurs 48 hours post oviposition (Marxen *et al.*, 2003), is likely the sensitive window during which DUT exerts its disruptive effects on *B. glabrata* embryos*.* These observations also indicate a potential correlation between the sensitive period of this pharmaceutical disruption with the timeframe during which gastropod shell formation is induced. Taken together, it is hypothesised that 5αR may play a critical role in the shell formation process in *B. glabrata*.

1.2. Embryonic development of *Biomphalaria glabrata*

To better understand the mechanisms by which DUT exerts its effects and the potential involvement of 5αR in *B. glabrata's* shell formation, a closer look into the early embryonic development of the organism is required. *B. glabrata* eggs develop individually within enclosed egg capsules. An egg mass is formed by approximately 30 encapsulated eggs held together by an outer membrane layer. Developed embryos escape the egg mass by breaking through their egg capsules around the 6th day of development (Marxen *et al.*, 2003). Embryonic development in *B. glabrata* is usually described by specific developmental stages (e.g. trochophore stage) or the hours (or days) after fertilisation. Although not time-specific, another way to distinguish the embryonic developmental stages in *B. glabrata* is by referring to days post-oviposition (Fig. 3.2). Usually, the descriptions of *B. glabrata* embryonic stages are used interchangeably in the literature. For some of them, the consensus seems to be that the blastula stage occurs between 0-15 hours post-oviposition (day 0), the gastrula stage between 24-39 hours post-oviposition (day 1), and the trochophore stage after 48 hours post-oviposition (day 2)(Marxen *et al.*, 2003; Aguiar *et al.*, 2022). However, the timing of the veliger stage varies, with some suggesting it occurs after 60 hours of oviposition (day 3) (Marxen *et al.*, 2003), while others place it between 96-111 hours post-oviposition (days 4-5) (Aguiar *et al.*,

2022). In contrast, the hippo stage is suggested to occur between 144-168 hours post-oviposition (days 6-7)(Aguiar *et al.*, 2022).

Throughout these developmental stages, considerable morphological changes, including changes in the embryonic shell, are observed in *B. glabrata*. In gastropods, shell development is known to occur during the late trochophore stage, where a small thin shell layer starts being formed (Shimizu *et al.*, 2011). The development of the shell is extended during the veliger stages, which coincides with the development of the mantle (Shimizu *et al.*, 2011). In molluscan biology, the mantle is a thin underlying organ which is responsible for regulating the biomineralization of the shell. By secreting proteins and polysaccharides, the mantle helps to form the organic matrix of molluscan shells (McDougall *et al.*, 2011). Notably, the molluscan mantle has a complex structure which is characterised by various cell types, discrete areas and restricted zones where gene expression takes place (McDougall *et al.*, 2011). Moreover, it is believed that its structure varies between species and molluscan classes (McDougall *et al.*, 2011). Despite the research progress made in developmental biology over the years, the mechanisms by which molluscan shells are formed, biomineralized and evolved remain poorly understood. Genetic factors are believed to play a critical role in these processes, where the recent implementation of molecular technologies such as genome sequencing, transcriptomics and proteomics have allowed the identification of important proteins that may help to unveil those underlying mechanisms (Hirota *et al.*, 2023).

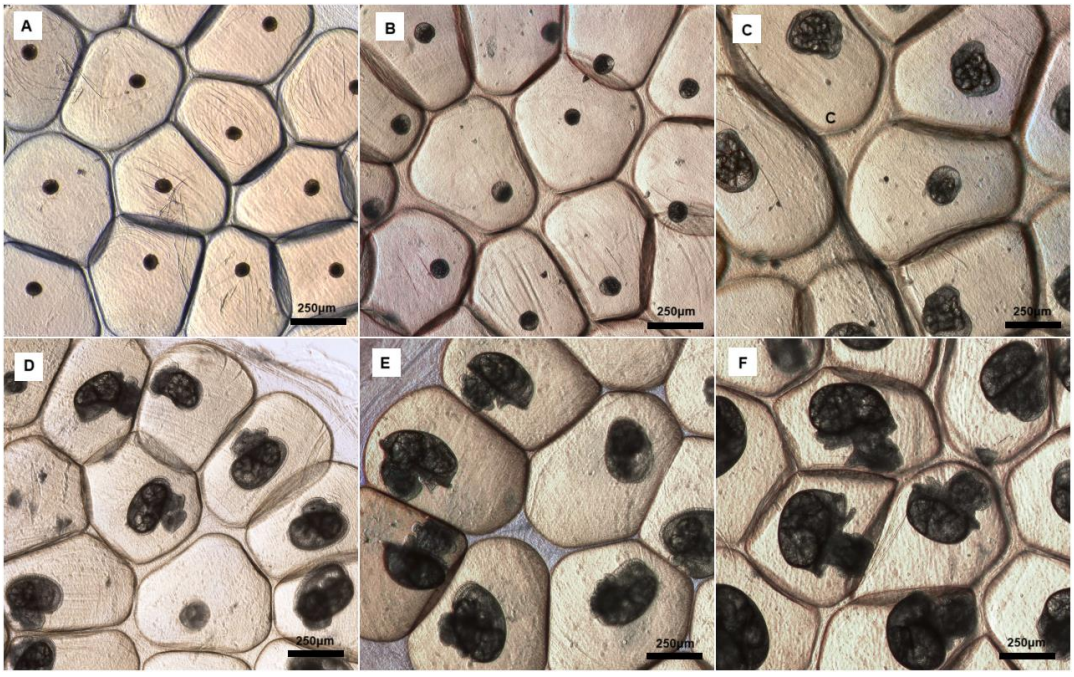


Figure 3.2: Developmental stages of B. glabrata in days post ovispotion: (a) day 0 post-oviposition (blastula stage); (b) day 1 post-oviposition (gastrula stage); (c) day 2 post-

oviposition (trochophore stage); (d) day 3 post-oviposition (early veliger stage); (e) day 4 post-oviposition (veliger stage); (f) day 5 post-oviposition (late veliger stage).

1.3. Gene expression analysis using RT-qPCR

To better understand how proteins function within organisms, it is important to evaluate not only the abundance of these proteins themselves, but also the abundance of mRNA levels that encode those proteins (Brazma and Vilo, 2000; Greenbaum *et al.*, 2003). According to the central dogma of molecular biology, proteins are synthesised through the process of transcription of DNA (within a gene) into messenger RNA (mRNA), followed by their translation. The entire process of transcription and translation is usually referred to as gene expression. Gene expression in an organism's tissue can be assessed using techniques such as quantitative polymerase chain reaction (qPCR) or reverse transcription qPCR (RT-qPCR). Both techniques use fluorescent dyes or probes to amplify and quantify the DNA present in a sample (Adams, 2020). However, the key difference between them lies in their starting material; qPCR uses a DNA template directly, whereas RT-qPCR starts with an RNA template which is converted to complementary DNA (cDNA) prior to amplification (Adams, 2020). Hence, RT-qPCR is considered more appropriate for the study of protein production, as it quantifies the amount of mRNA levels within a sample which are in turn indicative of the genes being transcribed and translated (Brzeszczyńska *et al.*, 2020).

In RT-qPCR, the fluorescent signal is detected and monitored by specialised thermal cyclers. The measured fluorescence demonstrates the amount of amplified cDNA product in each PCR cycle and is visualised by an amplification curve (Fig.3.3) (BioRad, 2006). At the initial phase of the RT-qPCR reaction, the fluorescence remains at background, non-detectable, levels (cycles 0 – 15, Fig. 3.3) until enough DNA product is amplified and starts to accumulate (cycle 16, Fig.3.3). The cycle number that the fluorescence is detected is called the Cq value (BioRad, 2006), and presents the intersection of the amplification curve and the threshold line. The Cq value is dependent on the amount of cDNA template present at the beginning of the amplification reaction. For example, large cDNA template amounts would require fewer amplification cycles for fluorescent detection compared to smaller template amounts (BioRad, 2006). During the exponential phase, the amplified cDNA product approximately doubles with each PCR cycle. However, as the RT-qPCR reaction continues and the components within it (PCR reagents, primers and probes) are utilised, it slows down and enters the plateau phase (30-40 cycles, Fig. 3.3). During the plateau phase, no considerable amplification of cDNA takes place as the PCR components are usually depleted (Joyal, Black and Dassylva, 2007).

To quantify the nucleic acids in the cDNA product that is amplified during a RT-qPCR reaction, two different approaches can be used. These are absolute and relative quantification (Joyal, Black and Dassylva, 2007). In absolute quantification, the amount of target nucleic acids is expressed as a copy number or concentration using external standards. On the other hand, relative quantification determines the amount of the target gene by calculating the ratio between the target nucleic acids and control nucleic acids. The control nucleic acids usually derive from a separate cDNA product, such as an appropriate reference gene that is present in the same sample (Joyal, Black and Dassylva, 2007). Relative quantification is often the method of choice when quantifying gene expression changes through mRNA transcript levels, across multiple samples or different experimental conditions (Fleige *et al.*, 2006; K. Zhang *et al.*, 2020). However, in relative quantification, the expression level of the reference gene must remain stable across different experimental conditions or different tissues. Once this is determined, the quantity of the target gene across tissues or experimental conditions can be calculated by comparing its expression levels with those of the reference gene (Joyal, Black and Dassylva, 2007).

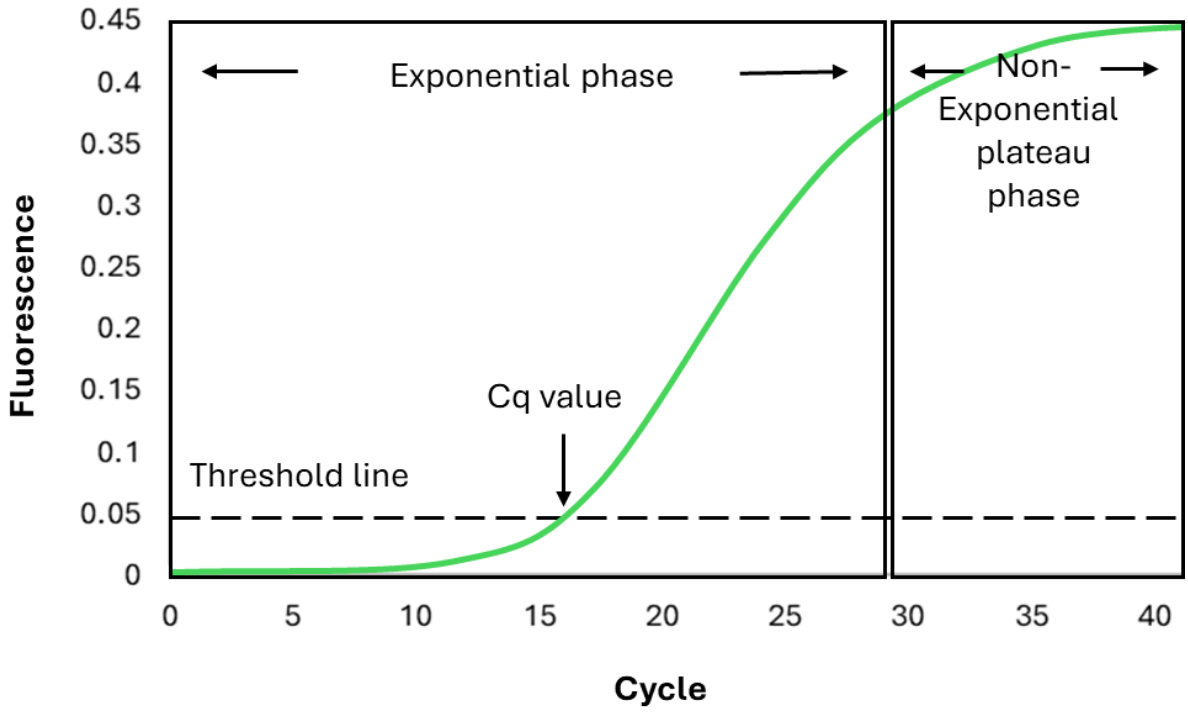


Figure 3.3: Amplification curve (blue line) in a RT-qPCR reaction, which includes the exponential phase, the non-exponential plateau phase, the threshold line and Cq value. The figure was adapted from BioRad's Real-time PCR Applications Guide Report (BioRad, 2006).

1.4. Common RT-qPCR pitfalls

The popularity of qPCR (or RT-qPCR) and its wide applicability has led to several instances where appropriate methodological procedures have not been followed. For example, as observed from the systematic investigations of Chapter 2, many qPCR assays have not employed or validated appropriate reference genes for measuring gene expression levels (Chapter 2, Fig. 2.10). To prevent methodological and reporting bias in qPCR experiments, the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines, published in 2009, provide a series of recommendations that aim to assist researchers in their experimental design (Bustin *et al.*, 2009). Common pitfalls of RT-qPCR assays include poor sample quality, poor amplification efficiency, lack of independent repetitions, and the inappropriate use (or no use) of reference genes for data normalisation. A summary of those steps and their importance is presented.

1.4.1. Sample quality and sample size

In the case of RT-qPCR, isolating RNA from the sample provides the initial template for detecting and quantifying the mRNA transcript. To obtain accurate results, good RNA quality is crucial. During RNA isolation, contamination with DNA often occurs, which in turn can cause amplification and reliability issues as it cannot be distinguished from cDNA in the qPCR reaction. Therefore, when isolating RNA and before proceeding with reverse transcription, it is important to measure the quantity of both RNA and DNA present in the sample, commonly referred to as RNA and DNA yields. In cases where DNA contamination is present in the RNA sample, the use of the DNA degradation treatment, DNase-I, can be used (Adams, 2020). Apart from RNA quality, accurate quantification of mRNA transcripts also requires a high RNA input. Although the suggested quantity of RNA used in RT reactions can vary depending on the kit used, this may range from 100 ng to 5 µg of total RNA. Using high quantities of RNA is usually preferred as it facilitates the detection of low-abundance transcripts and improves the sensitivity of the assay (Romera-Lopez *et al.*, 2012). Moreover, higher RNA input may enable the use of the biological replicate in various validation steps as well as the inclusion of multiple technical replicates in accordance with the MIQE guidelines (Bustin *et al.*, 2009).

1.4.2. Amplification efficiency

An important step in optimising a RT-qPCR assay is to determine the amplification of the primer pairs used. Theoretically, in a RT-qPCR reaction, every copy of the PCR product is perfectly doubled each cycle. However, to determine whether this assumption is met, a standard curve using serial dilutions of a known amount of cDNA template is performed. The

standard curve is plotted according to the log of the starting quantity of the template (e.g. 400 ng cDNA) against the amplified Cq values of each dilution. An R^2 value of the standard curve indicates the linearity of the data whereas an amplification efficiency value (E) that is calculated from the slope of the curve indicates the robustness of the data. For standard curve experiments, a minimum of five dilution points along with an R^2 value > 0.98 and an amplification efficiency value of 90-105% is recommended (BioRad, 2006; Bustin *et al.*, 2009).

1.4.3. Lack of replication

In RT-qPCR experiments, both technical replicates and biological replicates are needed. Technical replicates provide data preservation in cases where amplification does not occur in one well of a PCR plate. In this instance, amplification from wells of other technical replicates may provide the required data. Technical replicates may also used to determine the precision of the PCR amplification, as well as deviations in pipetting. On the other hand, biological replicates provide information on the overall precision and reproducibility of the experiment. A biological replicate usually represents a sample extracted from a single organism within a population. The number of biological replicates in a qPCR experiment can influence variability as fewer replicates within an assay can increase variability in the standard deviation (Applied Biosystems, 2008).

1.4.4. Reference genes

To achieve reliable quantification results that reflect the actual gene expression differences between the individual samples, an appropriate normalisation method is required. Its implementation is necessary to correct the errors that might appear during the sample preparation and processing stages. For example, achieving uniform weight and size of the tissue during sample collection does not ensure a uniform quantity of RNA during isolation. Among others, these variability issues might result from errors during pipetting or from the instability of nucleic acids due to the presence of various degradation enzymes and temperature variations (Kozera and Rapacz, 2013). Normalising the relative expression levels of each target gene against an internal reference gene offers an accurate approach for standardising the initial concentrations of cDNA samples. Reference genes work as internal reaction controls and must be characterised by expression levels that remain unaffected by experimental factors. It is also recommended that reference genes should exhibit a similar Cq value to those of the target gene (Kozera and Rapacz, 2013). Although previously referred to as ‘housekeeping genes’ to indicate the theory that basic metabolism genes can be used as internal calibrators (Kozera and Rapacz, 2013), it is now widely accepted that there are no universal reference genes and that each candidate reference gene must be systematically

validated as stably expressed across all experimental conditions (Gutierrez *et al.*, 2008; Bustin *et al.*, 2013; Zhou, Niu and Quan, 2018).

1.5. The lack of reference genes in embryonic *Biomphalaria glabrata*

Published data on appropriate reference genes in *B. glabrata* (my test species) is sparse. Previous studies using *B. glabrata* were identified in the literature to have utilised reference genes for normalisation in qPCR experiments, namely the ribosomal RNA (rRNA) genes 19S and 28S (Portet *et al.*, 2018; Luviano *et al.*, 2021; Pinaud *et al.*, 2021). However, neither of those studies validated the expression stability of their reference genes during embryonic development, which is the period of interest in this research. Moreover, although rRNAs are commonly employed as reference genes in RT-qPCR assays, they are now generally considered unsuitable for accurately normalising gene expression in various organisms (Tong *et al.*, 2009; Williams and Ghanem, 2022; Shui *et al.*, 2023). This is particularly the case for total RNA samples that have not been specifically enriched for mRNA. Such RNA samples contain an overwhelming amount of rRNA transcripts, making up 80-90% of the total RNA (O'Neil, Glowatz and Schlumpberger, 2013) which makes the detection and quantification of mRNA transcripts difficult to achieve. Although some studies have validated a range of reference genes in other molluscan species including the bivalves *Chlamys farreri* (Tian, Pan and Sun, 2013)*, Crassostrea hongkongensis* (Tong *et al.*, 2015a)*, Crassostrea gigas* (Vogeler, Tim P. Bean, *et al.*, 2016) *and Mytilus galloprovincialis* (Balbi *et al.*, 2016)*,* or the gastropod *Lymnaea stagnalis* (Bouétard *et al.*, 2013; Johnson and Davison, 2019; Young *et al.*, 2019) each species is characterised by distinct genetic and physiological characteristics. Thus, reference genes proven to be stably expressed in one molluscan species are not necessarily stable in another.

1.6. Aims and objectives

Despite previous efforts in elucidating the patterns of 5αR expression in embryonic *B. glabrata*, these were mainly concentrated on detecting 5αR proteins using vertebrate antibodies (Baynes et al., 2019), which while informative, may not fully capture the potential variations that exist in 5αR's function in molluscs. Moreover, although the *SRD5A1* and *SRD5A2* transcripts, which encode 5αR1 and 5αR2 respectively, have been identified in *B. glabrata* embryos, a comprehensive analysis quantifying these transcripts across various developmental stages remains a significant knowledge gap. Such investigations are critical, as understanding the temporal expression patterns of *SRD5A1* and *SRD5A2* could help uncover the potential role of 5αR1 and 5αR2 in molluscan shell development and in relation to the sensitive window of pharmaceutical disruption caused by DUT. Therefore, this chapter

aimed to investigate the expression patterns of *SRD5A1* and *SRD5A2* mRNA transcripts in the embryonic development *of B. glabrata* by developing a robust RT-qPCR assay. Specifically, the objectives of this study were to:

- Identify and validate stable reference genes across embryonic tissues in *B. glabrata*.
- Determine whether *SRD5A1* and *SRD5A2*, the genes encoding 5αR, are differentially expressed during the sensitive window of pharmaceutical disruption in *B. glabrata* embryos.

2. Methodology

2.1. Test species

The freshwater gastropod snail *Biomphalaria glabrata* was chosen as the test species as it has a well-documented physiology and its genome has been recently sequenced (Adema *et al.*, 2017). *B. glabrata* is easily maintained at laboratory conditions, reproduces consistently, and produces many eggs that are easily visible under a microscope. *B. glabrata* embryos were supplied from breeding stocks maintained at Brunel University London (BB02 strain; originally obtained from The Natural History Museum, London). Adult snails were maintained in flow-through glass aquaria and were supplied with de-chlorinated tap water at 27 °C. Snail breeding stocks were fed *ad libitum* three times a week with Tetramin fish flakes.

2.2. Tissue collection and fixation

2.2.1. Collection of egg masses from culture tanks

To initiate egg laying, transparent silicon tubes were placed in the flow-through glass aquaria of the breeding stocks immediately after feeding, three times a week. The silicon tubes with attached egg masses were carefully collected from culture aquariums, after a maximum of 12 hours, using a disposable clean glove and placed in a beaker of tank water. The day the egg masses were collected was the day 0 post-oviposition. Egg masses were carefully scraped off the tubes using the back of a sterile scalpel and placed in a clean petri dish half-filled with dechlorinated tap water. Egg masses were then allocated within a sterile 6-well plate (5 egg masses per well), half-filled with dechlorinated tap water, using a pair of sterile tweezers. They were later kept in an incubator set at 27°C for a maximum of 6 days.

2.2.2. Collection of embryo tissues in RNA-later

For RNA isolation purposes*, B. glabrata* egg masses were either preserved whole (i.e. undissected) or dissected, to allow 'pooling' of embryos in RNA-later. Prior to embryo collection, sterile 2mL Eppendorf tubes were half-filled with RNA-later, labelled, and their

weight was recorded. Tweezers, used for dissecting purposes, were disinfected by being submerged in (1) 1:10 diluted bleach solution with MiliQ H2O; (2) 100% MiliQ water; and (3) 70% Ethanol solution diluted in MiliQ water.

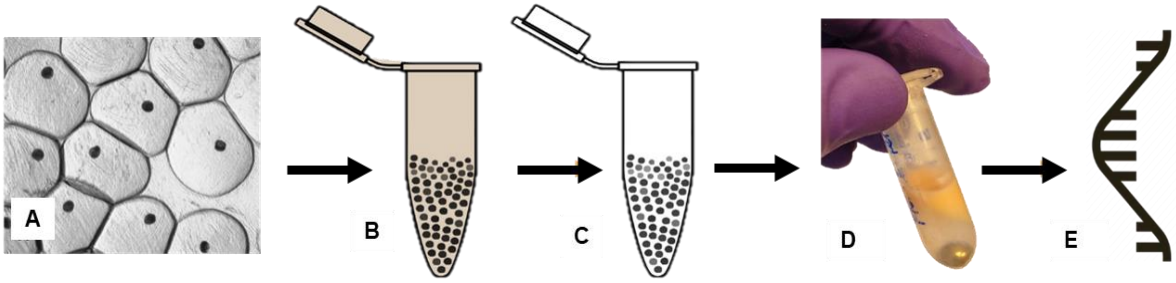


Figure 3.4: Schematic representation of *Biomphalaria glabrata* embryo collection and RNA isolation: (a) Dissection of embryos from the egg capsule; (b) preservation of embryos in RNA-later; (c) removal of RNA-later; (d) addition of lysis buffer and homogenisation of tissue; (e) RNA isolation.

Embryo pooling was achieved either by collecting embryos from dissected egg masses, or by pooling undissected egg masses in RNA-later**.** For embryo pooling from dissected egg masses, egg masses were collected using a pair of sterile tweezers, one at a time, from the 6-well plate and placed inside a clean petri dish. For the rest of the procedure, the 6-well plate was kept inside the incubator at 27 °C. Individual egg capsules were then carefully dissected under a dissecting microscope to release the excess egg mass fluid, using the front edge of a sterile scalpel. With a 2 µl micro-pipette, embryos were carefully collected from the egg capsules and immediately released inside a sterile 2 mL Eppendorf tube half-filled with RNA-later (Fig. 3.4). Following the release of embryos in RNA-later, the number of pooled embryos collected up to that point was recorded. The procedure was repeated until the required number of embryos was collected for each sample (e.g. 200 embryos for each day 2 post-oviposition sample). Finally, the sample was weighed using an analytical balance, and the wet weight of pooled embryo tissue was recorded by subtracting the weight of the 2 mL Eppendorf tube half-filled with RNA-later (without embryos).

For pooling embryos from undissected egg masses, the egg mass was first collected from the 6-well plate as previously mentioned. Under the dissecting microscope, the number of embryos within an egg mass was counted and recorded. This provided an approximation of the total number of embryos pooled in the sample. With the same sterile pair of tweezers, the

whole egg mass was quickly submerged and fixed in RNA-later. The wet weight of the whole egg mass was calculated as previously mentioned.

2.1.1. Collection of albumen gland tissue in RNA-later

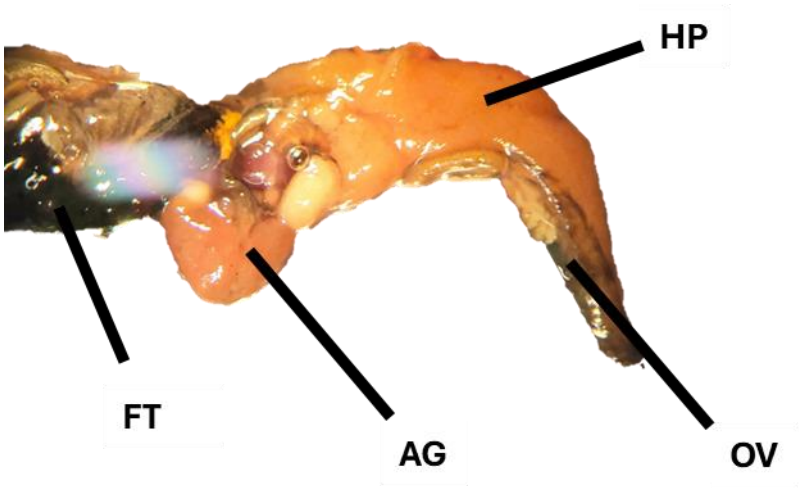


Figure 3.5: Dissected soft body of *B. glabrata* under the microscope (1.25x magnification). Different organs are demonstrated by the abbreviations, where HP: hepatopancreas; OV: ovotestis; AG: albumen gland; FT: foot.

Similarly to the procedures followed during embryo pooling (section 2.2.2), sterile 2 mL Eppendorf tubes half-filled with RNA-later were also used for preserving adult soft body mass tissues (Fig. 3.5) The weight of the half-filled Eppendorf tubes was determined and recorded before the dissections. The dissecting equipment (tweezers, scalpels) were disinfected by dipping them in and out of three individual disinfecting solutions using the exact order: (1) 1:10 Bleach diluted in MiliQ H2O, (2) 100% MiliQ H2O; (3) 70% Ethanol diluted in MiliQ H2O. Adult *B. glabrata* were carefully collected by hand from the culture tanks using a disposable clean glove and placed in a clean petri dish half-filled with dechlorinated tap water. Prior to dissection, every individual was dried using a clean piece of blue roll paper and its shell diameter was measured using electronic callipers (millimetres to two decimal places). To maintain consistency between measurements, the diameter was recorded using the same orientation axis for all individuals (Fig. 3.6). The total weight (including shell) of individuals was determined using an analytical precision balance (grams to three decimal places). Following size determination, *B. glabrata* individuals were killed in a clean petri dish by cutting off their heads instantaneously using a sterile scalpel. Decapitation was used to kill the snails without

narcotisation to avoid the potential degradation of RNA. The shell was carefully removed by compression, to release the snail's soft body mass. The remaining pieces of shell were removed using sterile tweezers. Following removal of the shell, the albumen gland (Fig. 3.5) was dissected from the rest of the organs and was fixated immediately in a 2 mL sterile Eppendorf tube half-filled with RNA-later. The wet weight of the albumen gland was calculated as mentioned in section 2.2.2. and recorded in the lab book.

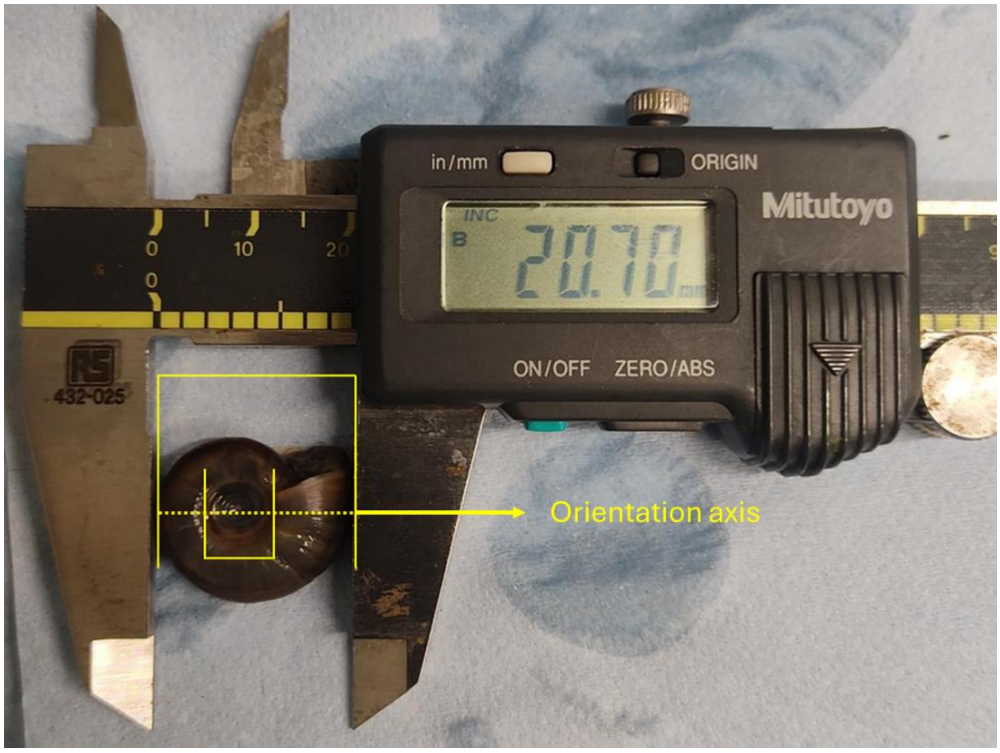


Figure 3.6: Shell diameter measurement of an adult *Biomphalaria glabrata* (shell on) using electronic callipers. The orientation axis (dotted line) facilitated shell diameter measurements for all individuals.

2.3. Total RNA isolation

2.3.1. Optimising RNA yield from embryonic developmental stages

In RT-qPCR the amount of RNA starting material is critical for accurate and reproducible results. In the process of developing a RT-qPCR method, optimisation of different parameters such as primer validation, standard curve quantification and the validation of reference genes is crucial. Most importantly, this series of optimisation steps need to be conducted using the same biological replicate. Consequently, sufficient RNA yield in biological replicates can ensure that an adequate amount of starting material (total RNA) will be transcribed to complementary DNA (cDNA). The amount of cDNA used for subsequent quantification

experiments is also critical as it determines the number of reactions needed during RT-qPCR and ensures the reliability of the results.

In this study, RNA was isolated from embryos of various developmental stages, ranging from day 1 post-oviposition to day 5 post-oviposition. Days post-oviposition were chosen to represent the stages of embryonic development because this timing provided a longer window for collecting the required number of embryos for each sample. To optimise the amount of RNA yield needed for each developmental stage, three RNA isolation kits were used: (1) Qiagen's RNeasy micro kit, (2) Qiagen's Firbous Tissue Mini Kit and (3) Macherey-Nagel's RNA isolation kit. RNA isolation from embryo tissues was performed according to the manufacturer's instructions found in the individual kits. However, due to the small size of the embryos and the inevitable collection of egg mass fluid during fixation, optimising the maximum weight of tissue each kit could process, was found to be particularly challenging. Instead, the optimal number of embryos and their resulting RNA yields were determined for each developmental stage. RNA yields were first determined using the NanoDrop Spectrophotometer, but the Qubit Fluorometer was eventually selected as the method of choice. Removal of genomic DNA contamination was performed by treating the samples with DNase-I.

To obtain a high RNA yield from day 1 post-oviposition embryo samples, a set of techniques was implemented. These included individual collection of 25-150 embryos in RNA-later (as described in section 2.2.2), per sample, and subsequent RNA isolation using either the Macherey-Nagel RNA isolation kit or Qiagen's RNeasy Micro Kit. Attempts to increase the tissue input per sample (200–1567 embryos) were also implemented using Macherey-Nagel's RNA isolation kit. For the latter, lysates obtained from individual samples of 100-200 embryos each, were combined through a single RNA binding column (provided in the kit). Lysates were eluted with a volume ranging from 12 µL–30 µL RNAnase-free H_2O (Appendix S3, Table S3.4).

The optimisation of RNA yield from day 2-5 post-oviposition embryos involved the collection of approximately 80–200 embryos per sample, which were preserved in RNA-later. Embryos of later developmental stages (i.e. day 4-5 post-oviposition) were collected by pooling undissected egg masses, containing 80–100 embryos per sample, and preserved in RNA-later. Subsequently, RNA isolation was performed using the Macherey-Nagel RNA isolation kit for the earlier embryo stages (days 2-3 post-oviposition) and Qiagen's RNeasy Fibrous Tissue kit for later stages (days 4-5 post-oviposition). Lysates were eluted with a volume of 30 µL RNAnase-free H_2O (Table 3.1).

2.3.2. Optimising RNA yield in adult tissue samples

Initial trials were conducted to isolate RNA from the ovotestis, hepatopancreas, mantle, and albumen gland tissues of adult *B. glabrata*. The purpose of using RNA from adult tissues in the RT-qPCR experiments was to serve as control 'calibrators' for data normalisation. However, initial observations revealed that ovotestis, hepatopancreas and mantle tissues contained little RNA content (Appendix S3, Table S3.1) In contrast, the albumen gland was identified as a good tissue candidate due to its high RNA content which was found to be associated with its ability to increase in size prior to snail oviposition (Appendix S3, Table S3.2). Because oviposition is usually influenced by the increased availability of food, albumen gland tissues were dissected from adult individuals before the animals were fed (Lui, 2022). High yield was obtained by isolating RNA from a single albumen gland, derived from an individual snail, thus eliminating the need for tissue pooling. RNA isolation was performed using the Macherey-Nagel RNA isolation kit following the manufacturer's instructions. Due to considerably high RNA yield in albumen gland samples, lysates were eluted with a volume of 60 µL RNAnase-free H_2O. For genomic DNA removal, albumen gland samples were treated with a series of RNAse-I treatments (Table 3.1).

2.3.3. Removal of genomic DNA contamination

Genomic DNA found present in RNA extracts was eliminated by a series of DNase-I treatments, usually found inside the RNA extraction kits, and by following the manufacturer's instructions. In cases where DNAse-I was not present in the kit, subsequent DNase-I treatments were performed after RNA isolation (using the protocol described in Appendix S3 section S3.1). The overall target was to reduce the DNA content as close to 7% as possible (according to suggestions obtained from the technical team at Brunel University London), in relation to RNA yield in all extracts. However, due to the ability of DNase-I to degrade both DNA and RNA material, no embryo samples were treated with DNase-I more than twice because of their lower RNA yields. Due to high RNA yield and high DNA yield in albumen gland samples, DNase-I treatments were performed up to three times (Table 3.1).

Table 3.1: Details on the RNA isolation and reverse transcription (RT, NO-RT) of samples used for quantitative PCR purposes. The table includes details on: the tissue of interest; sample name, RNA isolation method; genomic DNA removal method; RNA and dsDNA yields after genomic DNA removal (ng/µl); percentage of dsDNA yield (%) after genomic DNA removal, RNA template amount used for reverse transcription; and the number of RT and NO-RT reactions performed. Although albumen gland was initially used for reverse transcription and testing reference gene stability, these tissues were not used for quantifying *SRD5A1* and *SRD5A2* transcripts.

Tissue type	Sample name	RNA isolation method	gDNA removal method	RNA yield (ng/µl)	dsDNA yield (ng/µl)	dsDNA yield (%)	RNA template amount (ng)	RT reactions	NO-RT reactions
Day 2 embryos	D2.18	Macherey-Nagel RNA Isolation kit	DNAse-I	135.5	2.0	1.49%	400	2	1
Day 2 embryos	D2.20	Macherey-Nagel RNA Isolation kit	DNAse-I	240	2.0	0.82%	400	2	1
Day 2 embryos	D2.22	Macherey-Nagel RNA Isolation kit	DNAse-I	221	11.9	5.36%	400	2	1
Day 3 embryos	D3.29	Macherey-Nagel RNA Isolation kit	DNAse-I	57	4	7.37%	400	2	1
Day 3 embryos	D3.30	Macherey-Nagel RNA Isolation kit	DNAse-I	209	7.5	7.49%	400	2	1
Day 3 embryos	D3.32	Macherey-Nagel RNA Isolation kit	DNAse-I	262	13.9	5.30%	400	2	1
Day 4 embryos	D4.14	Macherey-Nagel RNA Isolation kit	DNAse-I	137	11	8.05%	400	2	1

Day 4 embryos	D4.15	Macherey-Nagel RNA Isolation kit	DNAse-I	94	1.8	1.96%	400	2	1
Day 4 embryos	D4.27	Qiagen's RNeasy Fibrous Tissue Mini Kit	DNAse-I	145	13.2	9.09%	400	2	1
Day 5 embryos	D5.3	Macherey-Nagel RNA Isolation kit	DNAse-I	133	3	2.29%	400	2	1
Day 5 embryos	D5.9	Macherey-Nagel RNA Isolation kit	DNAse-I	61	1.3	2.15%	400	2	1
Day 5 embryos	D5.27	Qiagen's RNeasy Fibrous Tissue Mini Kit	DNAse-I	179	4.9	2.71%	400	2	1
Albumen gland	S.AG-2	Macherey-Nagel RNA Isolation kit	DNAse-I	315	10.32	3.27%	-	-	-
Albumen gland	S.AG-3	Macherey-Nagel RNA Isolation kit	DNAse-I	500	42.3	8.47%	-	-	-
Albumen gland	S.AG-4	Macherey-Nagel RNA Isolation kit	DNAse-I	650	88.87	13.67%	-	-	-

2.4. Reverse transcription PCR

Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) which contained a mixture of reverse transcriptase (RT) random primers and the MultiScribe™ RT enzyme. Reserve transcription was performed on the total RNA extracts of day 2–5 post-oviposition stages and (initially) albumen gland tissues, to create single-stranded complementary DNA (cDNA) using a reaction size of 20 µL (10 µL Master mix and 10 µL total RNA). A series of two cDNA RT (reverse transcriptase) reactions and one NO-RT reaction were performed for each RNA extract, using 400 ng of total RNA (Tables 3.1, 3.2 & 3.3). Although not an absolute assumption, it was assumed that 400 ng of total RNA would be reverse transcribed to 400 ng of cDNA.

Table 3.2: Preparation of 10 µL total RNA solution, using a day 2 post-oviposition sample, to be used for cDNA synthesis.

Sample name	**Developmental stage**	**RNA yield (ng/µL)**	**Amount of RNA needed (ng)**	**Volume (µL) RNA required in a 10µL reaction**	**Volume (µL) RNAse-free H_2O required in a 10µL reaction**
D2.18	Day 2	135.5	400	2.95	7.1
+ 10% to account for pipette loss	-	-	-	3.2	7.8

As per the manufacturer's instructions, the 20 µL reaction (either RT or NO-RT), comprised 10 µL of the Master mix (Table 3.3) and 10 µL of the total RNA extract (Table 3.2). The volume of the RNA extract needed for each reaction was calculated using the formula:

$$\mathbf{C = m / V}$$

Where, C = RNA yield after genomic DNA removal (ng/µL) , *m* = amount of RNA (ng) needed, and *V* = volume of RNA extract required in a 10µL solution.

In cases where lower volumes of RNA extract were required, total RNA was diluted with RNAse-free water to create a 10 µL solution (Table 3.2). If multiple RT or NO-RT reactions were required from a single RNA extract, an additional 10% volume of RNA sample and RNAse-free water was included in the dilutions. The additional volume was incorporated to account for potential sample loss during pipetting. Reverse transcription was performed using

the PCR thermal cycler (Biometra TProfessional basic Thermocycler 070-701) using the conditions summarised in Table 3.4.

Table 3.3: Preparation of 10 µL Master Mix to be used for cDNA synthesis, as per manufacturer's instructions:

	RT reaction	**NO-RT reaction**
Component	**Volume/Reaction (µL)**	**Volume/Reaction (µL)**
10x RT Buffer	2.0	2.0
25X dNTP Mix (100mM)	0.8	0.8
10X RT Random Primers	2.0	2.0
MultiScribe Reverse Transcriptase	1.0	-
Nuclease-free H2O	4.2	5.2
Total per reaction	10	10

Once reverse transcription was completed, cDNA samples were immediately stored at -20˚C. In cases where pooling of cDNA was required, particularly for the conduct of primer testing and validation**,** samples were combined prior to quantitative PCR. Pooling of cDNA was performed by combining equal volumes of RT cDNA sample of the same tissue together and combining equal volumes of NO-RT sample together, to create pooled cDNA and NO-RT stocks.

Table 3.4: Non-quantitative PCR conditions for performing reverse transcription using the High-Capacity cDNA Reverse Transcription Kit, as per the manufacturer's instructions.

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature (°C)	25	37	85	4
Time (min)	10	120	5	∞

2.5. Validating reference genes for the use in RT-qPCR experiments in *Biomphalaria glabrata*

2.5.1. Primer selection

Since no reference genes had been previously identified for embryonic *B. glabrata*, a set of candidate reference genes was selected from the literature. These candidates were chosen based on studies that tested and validated reference genes in *B. glabrata* or the closely related gastropod, *Lymnaea stagnalis* (Table 3.5). Primers for the target genes *SRD5A1* and *SRD5A2* were selected from Baynes *et al.*, (2019) who had previously designed them and tested them in *B. glabrata* embryos.

2.5.2. Primer testing

Primers of candidate reference genes identified in the literature (Table 3.5) were tested for stability across day 2 – day 5 post-oviposition embryos embryos and albumen gland tissues (Appendix S3, Table S3.3). Following initial observations (based on raw Cq values of RT and NO-RT samples), selected primers were re-designed to be *B. glabrata-specific* (Table 3.6) and were tested again for stability across the same embryo and albumen gland tissues (Fig. 3.10, Table 3.11).

2.5.3. Primer redesigning

The five primer pairs *UBI-3, EF1a, TUB-1, ACTIN-1* and *H2A* were selected and re-designed to be *B.glabrata*-specific using Primer-BLAST (Table 3.6). The *B. glabrata*-specific primers were designed to span exon-exon sequences, to have a length of no more than 200 bp, a melting temperature between 57.14 and 60.32 °C and a GC content between 40% and 60%. The primers of all candidate reference genes (*UBI, EF1a, TUB, ACTIN-1* and *H2A*), as well as primers of target genes (*SRD5A1, SRD5A2*), were first tested using Gradient PCR, to obtain their optimal annealing temperature. For Gradient PCR, pooled stocks of RT cDNA and NO-RT material from embryo samples (day 2 – day 5 post-oviposition embryos) and albumen gland tissues were used. Pooled RT cDNA and NO-RT stocks were prepared to generate representative samples that contained the average quantity of all target and reference genes, as well as the average of all contaminants, present in all individual samples. This approach aimed to ensure the reliable evaluation of gene amplification efficiency by assessing both reference and target genes under the same conditions. To minimise the impact of inhibitors on qPCR efficiency, which could reduce the background signal in the qPCR reaction, the pooled RT cDNA and NO-RT stocks underwent a 5x dilution before incorporating them in the reaction mix. Using the gradient function on the PCR plate a gradient of 55 °C to 65 °C was implemented, where each primer pair consisted of two technical replicates for both RT and NO-RT reactions. Melting curve analysis was employed to evaluate the primer peaks

generated at various temperatures. Optimal results were observed at temperatures of 63.3 °C and 61.4 °C. The annealing temperature chosen for subsequent quantification experiments was 60 °C to align with the optimal annealing temperature of *SRD5A1* and *SRD5A2* primer pairs (Baynes *et al.*, 2019). To validate the reliability of the results, a follow-up gel electrophoresis analysis (2% agarose gel) was conducted for approximately 30 minutes alongside a 12-well 50 bp DNA Ladder. The agarose gels were visualised using a 230V UVP BioDoc-It Imaging System. Two independent agarose gels were visualised using the technical replicates from the PCR products of *UBI, EF1a, TUB, ACTIN-1, SRD5A1*, and *SRD5A2*, from the 63.3 °C and 61.4 °C gradients.

2.5.4. Primer amplification efficiency and standard curve

Pooled RT cDNA stocks, containing volumes from the same day 2 – day 5 post-oviposition embryos embryos and albumen gland samples, were also used for the construction of a standard curve. Due to the limited amount of RNA yields obtained from embryos, the available volumes of cDNA samples were restricted for this study. Thus, primer amplification efficiency tests (for the construction of a standard curve) were conducted for the primer pairs of the two target genes *SRD5A1, SRD5A2* and the two selected reference genes, *UBI* and *TUB*. A six-step serial dilution, using an undiluted pooled cDNA sample (obtained from day 2-5 stages and albumen gland tissues) of 400 ng was tested. Thus, the undiluted sample was tested alongside a series of five dilutions, each with a dilution factor of 1:5 (Table 3.7). The different concentrations were measured in triplicate alongside triplicates for negative template controls (NTC). The standard curve experiments were performed using the same RT-qPCR method implemented during reference gene validation and quantification of target genes (Table 3.9). Standard curves were generated by plotting the Cq values of the different standard dilutions against the logarithmic input amount of the standard cDNA material (Joyal, Black and Dassylva, 2007). Standard curves that produced a value R^2 >0.98 from at least 5 standard concentrations, were considered successful. Quantification values from the last serial dilution were removed from the analysis if they considerably decreased the R^2 value below 0.98. Primer efficiencies for each primer pair were calculated by analysing the template dilution series. The Cq values were plotted against the log template amount, and the slope of the resulting standard curve was determined. Using the slope value (S), the primer efficiency was calculated using the equation below (Joyal, Black and Dassylva, 2007):

$$\textbf{PCR efficiency (\%)} = (10^{(-1/S)} - 1) \times 100$$

Primer efficiencies for each primer pair were estimated using the arithmetic mean of three independent and successful standard curve experiments, using three pooled biological cDNA replicates.

Table 3.5: Primer sequences of candidate reference genes identified in the literature, based on species identified and the percentage similarity with equivalent genes found in the *Biomphalaria glabrata* genome. Primers labelled "F" refer to forward primers and primers labelled "R" refer to reverse primers.

Gene Name	**Encoded protein**	**Primer sequence (5' to 3')**		**Percentage similarity with *B. glabrata***	**Species identified**	**Publication identified**
SRD5A1	5-alpha reductase 1	F	GGCCTGAGTGTATGCGTTC	100% aligned	*Biomphalaria glabrata*	Baynes *et al*., (2019)
		R	CAACACAGCAGGGTAGTTCTTG	100% aligned		
SRD5A2	5-alpha reductase 2	F	CATCATCAACAGATGGGCAGA	100% aligned	*Biomphalaria glabrata*	
		R	CACAAACTCAAACAAGCCTCC	100% aligned		
Bgla-alphaTUB	α-Tubulin	F	CGACATCTGCCGCCGTAACCT	100% aligned	*Biomphalaria glabrata*	Luviano *et al.*, (2021)
		R	GGCGCCATCAAACCTGAGGGA	100% aligned		
Lst-ACTB	Beta-Actin	F	AGGCCAACAGAGAAAAGA	100% aligned	*Lymnaea stagnalis*	Young *et al.*, (2019)
		R	AGATGCGTACAGAGAGAG	100% aligned		
Lst-UBI	Ubiquitin	F	GTATTGTGGTGCTGGTGTTTT	90% aligned	*Lymnaea stagnalis*	
		R	GCTTCCTCCTCTGGTTTGT	90% aligned		
Lst EF1α	Elongation factor 1a	F	ACCACAACTGGCCACTTGATC	Partly aligned	*Lymnaea stagnalis*	
		R	CCATCTCTTGGGCCTCTTTCT	Partly aligned		
Lst-GAPDH	GAPDH	F	CAACAACCGACAAAGCAA	Partly aligned	*Lymnaea stagnalis*	
		R	CATAACAAACATAGGGGCA	Partly aligned		
Lst-TUBB	Beta-Tubulin	F	GGCTAGGGGATGAAGATGA	Partly aligned	*Lymnaea stagnalis*	
		R	AGGATGAGGGTGAATTTGA	Partly aligned		
Lhis 2a	Histone 2A	F	TCAGAGGAGATGAGGAGTTGG	N/A		

		R	CCCCAAGTTATGCTGCCTTC	N/A	*Lymnaea stagnalis*	Johnson and Davison, (2019)
Lube 2	Ubiquitin-conjugating E2	F	CCCCAAGTTATGCTGCCTTC	N/A	*Lymnaea stagnalis*	
		R	TCTGTGGACTGCATATCACTCT	N/A		
Mb	Myoglobin	F	GATGTTCGCCAATGTTCCC	100% aligned	*Biomphalaria glabrata*	Arican-Goktas *et al.*, (2014)
		R	AGCGATCAAGTTTCCCCAG	100% aligned		
Lywhaz	14-3-3 protein zeta	F	GGAGGAGCTGAAGTCAATATGC	N/A	*Lymnaea stagnalis*	Johnson and Davison, (2019)
		R	AGTCACCCTGCATTTTGAGG	N/A		
Lrpl14	60S ribosomal protein L14	F	TAATAAGTCGGTTGCGCGC	N/A	*Lymnaea stagnalis*	
		R	GGGAACAGTCTACTTGGGC	N/A		

Table 3.6: *Biomphalaria glabrata*-specific primer sequences for the candidate reference genes (*UBI, EF1a, TUB, ACTIN-1, H2A*) designed using the online software BLAST. Primers labelled "F" refer to forward primers and primers labelled "R" refer to reverse primers

		Primer sequence		Sequence length (bp)		Melting temperature/Tm (°C)		GC content %		Delta G range (kcal/mol)		
Gene name	**Protein encoded**	**F primer (5' to 3')**	**R primer (5' to 3')**	**F primer**	**R primer**	**Product length (bp)**	**F**	**R**	**F**	**R**	**F primer**	**R primer**	**Accession number**
UBI	ubiquitin-40S ribosomal protein S27a	CCCTCCATCTAGTGCTGAGAC	TTGCCATTCTCATCAACCTTGT	21	22	142	59.31	58.5	57.14%	40.91%	(-) 4.16 (max)	(-) 3.9 (max)	XM_013208032.1
EF1a	elongation factor 1-alpha	GCAGTTCCACGCTCAGGTTA	TTGCCAGAACGACGATCACA	20	20	141	60.32	59.97	55%	50%	(-) 3.61 (max)	(-) 4.62 (max)	XM_013211894.1
TUB	tubulin beta-4B chain-like	CGACTGCTTACAGGGCTTCC	GACTGAGAGGGTTGCGTTGT	20	20	187	60.74	60.25	60%	55%	(-) 3.61 (max)	(-) 3.61 (max)	XM_013241676.1
ACTIN-1	actin, cytoplasmic-like	TCTTGGTGCCTTTTCTTCTCT	ACCAACCATCACACCCTGAT	21	20	197	57.14	58.92	42.86%	50%	(-) 3.14 (max)	(-) 3.14 (max)	XM_013228447.1
H2A	histone H2A.V	CCTGTCGGTCGTATCCATCG	CCAGCTAACTCAAGCACCTCA	20	21	128	60.04	60	60%	52.38%	(-) 3.61 (max)	(-) 6.34 (max)	XM_013205512.1

Table 3.7: Details of the 6-step serial dilution of pooled cDNA stock, containing day 2 -5 post-oviposition embryos and albumen gland tissue, used to assess the primer efficiency of *UBI*, *TUB*, *SDR5A1*, and *SRD5A2* pairs using a standard curve qPCR experiment.

Serial dilutions	**Undiluted cDNA stock**	**1:5**	**1:25**	**1:125**	**1:625**	**1:3125**
cDNA template amount (ng)	400	80	16	3.2	0.64	0.128
Log template amount (ng)	2.602	1.903	1.204	0.505	-0.194	-0.893

2.5.5. Validation of reference gene candidates

The cDNA and NO-RT samples (from day 2–5 post-oviposition embryos and albumen gland tissues) that were previously used in primer testing and amplification tests, were also used for the validation of reference genes and subsequent quantitative PCR experiments. After the preliminary testing of non-specific candidate reference genes (Table 3.5), *B. glabrata*-specific primers for the candidate reference genes *UBI, EF1a, TUB, ACTIN-1* and *H2A* were first validated for stability using one biological cDNA replicate from each embryo developmental stage (day 2-5 post-oviposition) and albumen gland tissue. Due to the limited volumes of cDNA samples, a selection of the two most stable (and most specific) candidate reference genes was performed following the first validation experiment. For this reason, the reference gene stability was initially determined according to the standard deviation (SD) observed in the Cq values for each candidate gene, across tissues tested. A lower SD indicated higher stability, whereas a higher SD indicated lower stability. Subsequent experiments validated the stability of reference genes across the embryo developmental stages only (day 2 – day 5 post-oviposition embryos), using two additional biological cDNA replicates. This is because the candidate reference genes were found to be more stably expressed across all embryo samples but not the albumen gland. Each reference gene validation experiment comprised two technical replicates for all biological replicates as well as a no-RNA-template control (NTC) for each primer pair.

2.6. Quantitative reverse transcription PCR

Relative quantification was the method of choice in determining the expression of the target genes *SRD5A1* and *SRD5A2*. To achieve relative quantification, the ratio between the amplification value of the target gene and a reference gene was established. To investigate

the differential expression of a target gene, this ratio was eventually compared between the embryo tissues examined. The amplification of all reference and target genes was detected using the SYBR Green dye which facilitated the generation of fluorescence signal in the presence of double-stranded DNA. Table 3.8 details the individual components and RT-qPCR reaction setup.

Table 3.8: RT-qPCR reaction setup

Component	**Volume (µL) per 20 µL Reaction**	**Final concentration**
iTaq Universal SYBR Green Supermix (2x)	10 µL	1x
Forward primer	1 µL	500 nM
Reverse primer	1 µL	500 nM
cDNA template	2 µL	400 ng
RNAse-free water	6 µL	-
Total reaction mix volume	20 µL	-

The target gene quantification experiments included three technical replicate controls for each biological replicate, as well as one NO-RT and no-RNA-template control (NTCs) for each embryo sample and primer pair. In total, target gene quantification experiments were performed using three separate biological cDNA replicates for each developmental stage (day 2–5 post-oviposition), whose data was later combined for analysis. The RT-qPCR reactions were performed in a BioRad CFX thermocycler connected to the CFX Maestro software. For all three biological replicates, the target genes (*SRD5A1* and *SRD5A2*) were quantified alongside the two most stable endogenous reference gene candidates determined from the first reference gene validation experiment (*UBI*, *TUB*). Table 3.9 details the thermocycling conditions for the RT-qPCR programme. The conduct of all RT-qPCR experiments adhered to the MIQE guidelines (Bustin *et al.*, 2009).

Table 3.9: Thermocycling conditions

Step	**Temperature (°C)**	**Time (s)**	**Number of cycles**
Initial denaturation	95	30	1
Denaturation	95	5	40
Annealing/extension	55	30	40

Met curve	65 to 95 (increment of 0.5)	5	1

2.7. Statistical analyses

2.7.1. Reference gene stability algorithms

All graphs were constructed using Microsoft Excel and GraphPad Prism (Version 10.2.2). The expression stability of the five candidate reference genes was initially assessed during the first validation experiment (described in section 2.4.3). The arithmetic means and SD values of all candidate genes, from all validation experiments, were calculated using Microsoft Excel. Subsequent expression stability analysis of candidates was tested, using data from all validation experiments, with the help of five statistical algorithms: BestKeeper (Pfaffl *et al.*, 2004), geNorm (Vandesompele *et al.*, 2002), NormFinder (Andersen, Jensen and Ørntoft, 2004), ΔCT (Silver *et al.*, 2006) and RefFinder (Xie *et al.*, 2012). BestKeeper uses the geometric mean of raw Cq values to compute a SD value for each candidate reference gene. The SD values are then corrected via the factor-specific real-time PCR efficiency, calculated by the slope of the linear regression model using log-transformed Cq values (Pfaffl *et al.*, 2004). Any genes exhibiting a SD<1, are considered stable and their raw Cq values are used for determining the BestKeeper Index. The correlation of the Cq data to the BestKeeper Index (r value) is then calculated using a regression model. BestKeeper uses the “r value” and the SD to evaluate gene stability. Consequently, a high “r value” and a low SD value indicate higher expression stability for a given candidate.

geNorm evaluates gene stability by conducting pairwise comparisons between the candidate reference genes, using linearised Cq values, and the generation of the “M value”. The “M value” is a stability metric calculated from the geometric mean of the SD of each pairwise comparison. In geNorm, candidates with a general cut-off “M value”<1.5 are considered stable (Silveira *et al.*, 2009; Yigin *et al.*, 2017), where a lower “M value” indicates higher stability. In the end, geNorm calculates the most stable pair of genes from all candidates tested. In addition, geNorm conducts a pairwise variation analysis to identify the optimal number of reference genes for increasing the normalisation stability of gene expression data. The pairwise-variation analysis is performed according to an assigned “V” value for each candidate reference gene, and by comparing their ratios when using two (V2) versus three (V3), four (V4) or five (V5) reference genes. A ratio value below 0.15 suggests that adding an extra reference gene will not considerably increase normalisation stability (Vandesompele et al., 2002).

NormFinder assigns its stability value using a model-based approach and the intergroup and intragroup variation that exists between the raw Cq values of candidate reference genes. The candidate genes with the lowest stability values are considered to be more stable. The ΔCT algorithm performs pairwise comparisons of candidate reference gene pairs, using their relative gene expression values from all samples. This method assigns its stability ranking based on how big the ΔCT standard deviation is within each pair comparison. Therefore, a smaller ΔCT SD value indicates a more stable gene (Silver *et al.*, 2006). Lastly, RefFinder combines stability values from the previous four algorithms to allocate an overall stability value. Thus, to provide its stability value, RefFinder calculates the geometric mean of BestKeeper's SD values, geNorm's M values, NormFinder's stability values, and ΔCT's SD values.

BestKeeper analysis was run as macros on Microsoft Excel, using raw Cq values. In contrast, geNorm and NormFinder analyses were run on R studio using linearised and raw Cq values, respectively, per the manual instructions. Linearised Cq values for geNorm were calculated according to the formula: $2^{(\text{MIN Cq value}-\text{Cq value})}$. The "MIN Cq value" represents the smallest Cq value obtained from all samples (biological and technical replicates) from each candidate reference gene. This was then subtracted from each Cq value, respectively. The manuals for the R studio analyses were obtained from the manufacturer's websites https://genorm.cmgg.be/ (geNorm) and https://www.moma.dk/software/normfinder (NormFinder). The ΔCT and RefFinder analyses were conducted using RefFinder's online tool accessed at https://www.ciidirsinaloa.com.mx/RefFinder-master/. In addition to its stability ranking, RefFinder provided the results from the BestKeeper, geNorm, and NormFinder analyses. Thus, these were used to cross-validate the results obtained from the Excel and R studio analyses. Notably, RefFinder used raw Cq values, instead of linearised, for all analyses by default. However, no differences in geNorm's stability rankings were observed between RefFinder and R studio analysis.

2.7.2. Relative gene expression analysis

Because the amplification efficiencies of the reference genes (*UBI, TUB*) were not identical to those of the target genes (*SRD5A1, SRD5A2*), the relative gene expression of *SRD5A1* and *SRD5A2* transcripts was calculated using the Pfaffl method (Pfaffl, 2001). To perform this analysis, the primer efficiencies (E) of reference and target genes were first calculated according to the ΔΔCq method criteria (Joyal, Black and Dassylva, 2007). The primer amplification efficiencies of target and reference genes from each quantification experiment were converted from percentages to arithmetic values. According to these criteria, a value of

2 indicated a 100% efficiency, while a value of 1.97 and 2.01 indicated a 97% and 101% efficiency, respectively (Table 3.10).

In each quantification experiment, the average Cq values of all reference and target genes were calculated for each biological replicate based on the raw Cq values of their technical replicates. Then, the ΔCq values were calculated for all reference and target genes in each biological replicate, by subtracting the mean Cq value of the calibrator sample (e.g. day 2 or day 5) from the mean Cq value of each embryo sample. The formula used for this calculation was:

ΔCq = Cq value (calibrator) – Cq value (sample)

The relative quantity (RQ) values were then calculated separately for each target and reference gene using their converted efficiency values (E) according to the equation:

$$RQ = E^{\Delta Cq}$$

The geometric mean for the reference gene RQ values was calculated using the '=GEOMEAN' function in Microsoft Excel. Finally, the relative gene expression values were calculated by dividing the RQ values of the target genes by the geometric mean values of the reference genes, for each biological replicate. The relative gene expressions of each biological replicate (day 2 - day 5 post-oviposition embryos) obtained from three independent quantification experiments were combined, respectively. Statistical differences in gene expression values between samples were determined using a one-way ANOVA ($p \leq 0.05$).

The reference genes *TUB* and *UBI* were selected based on the results of the initial validation experiment (described in section 2.5.5). Because the albumen gland was found unsuitable for inclusion in the analysis due to variations in reference gene stability values (described in sections 2.3.2 and 2.5.5), the day 5 post-oviposition embryo sample was selected as the calibrator. The calibrator sample was used to establish the baseline for relative gene expression analysis across the remaining samples (day 2 – day 4 post-oviposition). To compare potential variations in gene expression between different calibrator samples, the relative gene expression analysis was also run using the day 2 post-oviposition sample as the calibrator.

3. Results:

3.1. Primer specificity and amplification efficiency of candidate reference genes

Agarose gel electrophoresis of cDNA PCR products revealed that all primer pairs were specific to their target DNA sequences and were of the expected size (Fig. 3.7). Moreover, the qPCR melting curves revealed single peaks for all reference gene primer pairs, except for *ACTIN-1*, confirming the amplification of single cDNA products (Fig. 3.8). A smaller second peak was observed in the melt curves of *ACTIN-1*, possibly indicating the amplification of a primer-dimer (Fig. 3.8 b, g, l). The melt curves for the target *SRD5A1* and *SRD5A2* genes were obtained from the three quantification experiments and are shown in Appendix S3 (Fig. S3.1) The amplification efficiencies of the four primer pairs that were used in the final quantification experiment, *UBI, TUB, SRD5A1* and *SRD5A2,* demonstrated values between 1.94 - 2.02 (Table 3.10) while R^2 values exceeded 0.98 (Fig. 3.9).

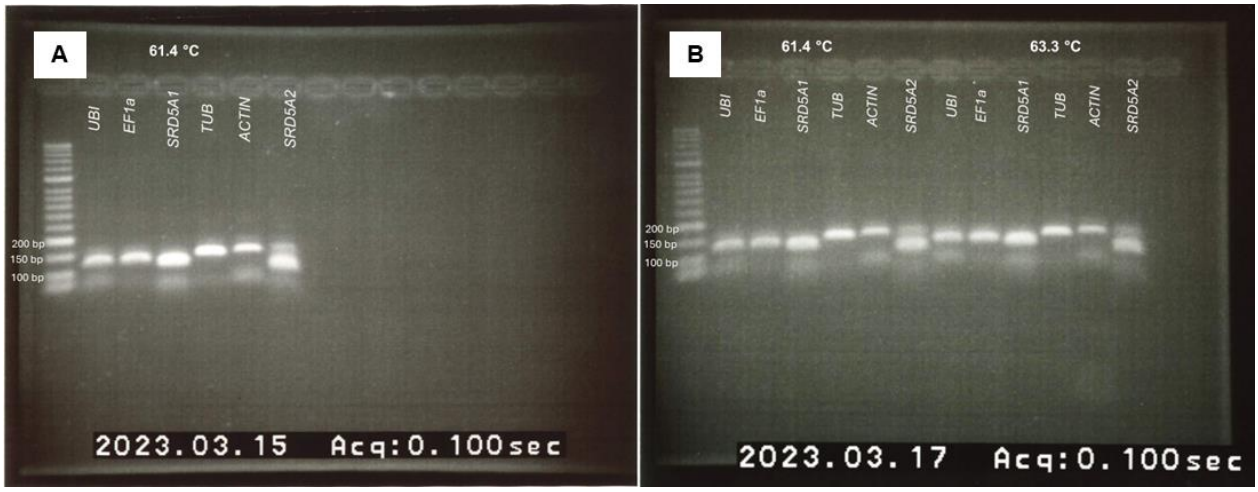


Figure 3.7: Visualised agarose gels of the PCR products from the candidate reference genes *UBI*, *EF1a*, *TUB*, *ACTIN*-1 and the target genes *SRD5A1* and *SRD5A2*. A 15µL sample of each PCR primer product was run on a 2% agarose gel using (a) one technical replicate from 61.4°C wells and (b) a second technical replicate from 61.4°C and 63.3°C wells.

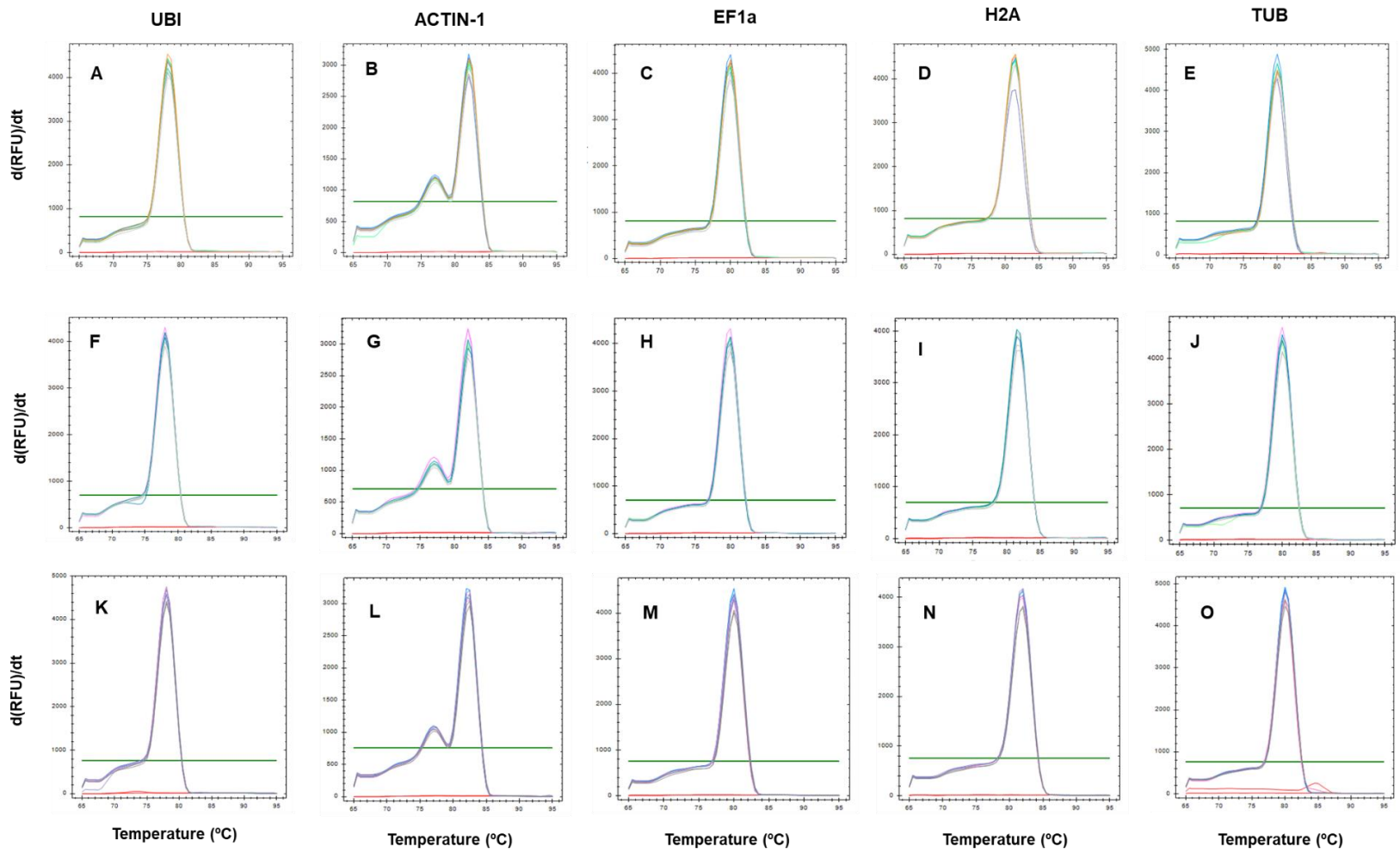


Figure 3.8: Melt curves representing derivatives of the relative fluorescence d(RFU) in respect to temperature (°C) for the candidate reference genes *UBI, ACTIN-1, EF1a, H2A* and *TUB*. Data was obtained from three independent validation experiments, using independent biological replicates. The melt curves (a), (b), (c), (d), (e), were obtained from the first validation experiment; melt curves (f),(g),(h),(I),(j), were obtained from the second validation experiment; and melt curves (k),(l),(m),(n),(o), were obtained from the third validation experiment. Different colours represent different embryo and tissue samples, whereas negative controls (NTC) are shown in red.

Table 3.10: Converted efficiency values of *TUB, UBI, SRD5A1* and *SRD5A2* primer pairs, according to ΔΔCt method criteria, used for the normalisation of target relative gene expression. Primer efficiency values were determined from three independent RT-qPCR experiments.

	Converted efficiency value		
Primer pair	**Exp. 1**	**Exp. 2**	**Exp. 3**
TUB	1.97	1.95	1.99
UBI	2.01	1.96	1.97
SRD5A1	1.94	2.01	2.02
SRD5A2	2.02	1.95	1.98

3.2. Raw Cq values

The raw Cq values for the candidate reference genes (Table 3.11) were obtained from three independent quantification experiments, each using separate biological cDNA replicates for each embryo sample (day 2 – day 5 post-oviposition embryos). Raw Cq values for the candidate reference genes were also obtained using the albumen gland tissue, only in the first validation experiment (Table 3.11). The Cq values represent the cycle threshold at which fluorescence crossed the detection limit during cDNA amplification. In the first validation experiment, Cq values in embryo samples (day 2 – day 5 post-oviposition embryos) and albumen gland samples for *UBI, TUB, EF1a, ACTIN-1*, and *H2A* ranged from 13.07 – 16.10, 12.99 - 20.08, 10.84 – 14.06, 12.02 – 18.02 and 13.54 – 24.12, respectively. Across all three experiments, the Cq values in embryo samples for *UBI, TUB, EF1a, ACTIN-1*, and *H2A* showed smaller variability and ranged from 13.07–14.08, 12.99–15.01, 10.84–13.00, 12.02–14.12, and 13.54–15.59, respectively (Fig. 3.10; Table 3.11). Due to the high variability observed in Cq values, and hence SD, of candidate reference genes across embryo and albumen gland tissues (Table 3.11), subsequent validation and quantification experiments were conducted using solely embryo samples (day 2 – day 5 post-oviposition embryos).

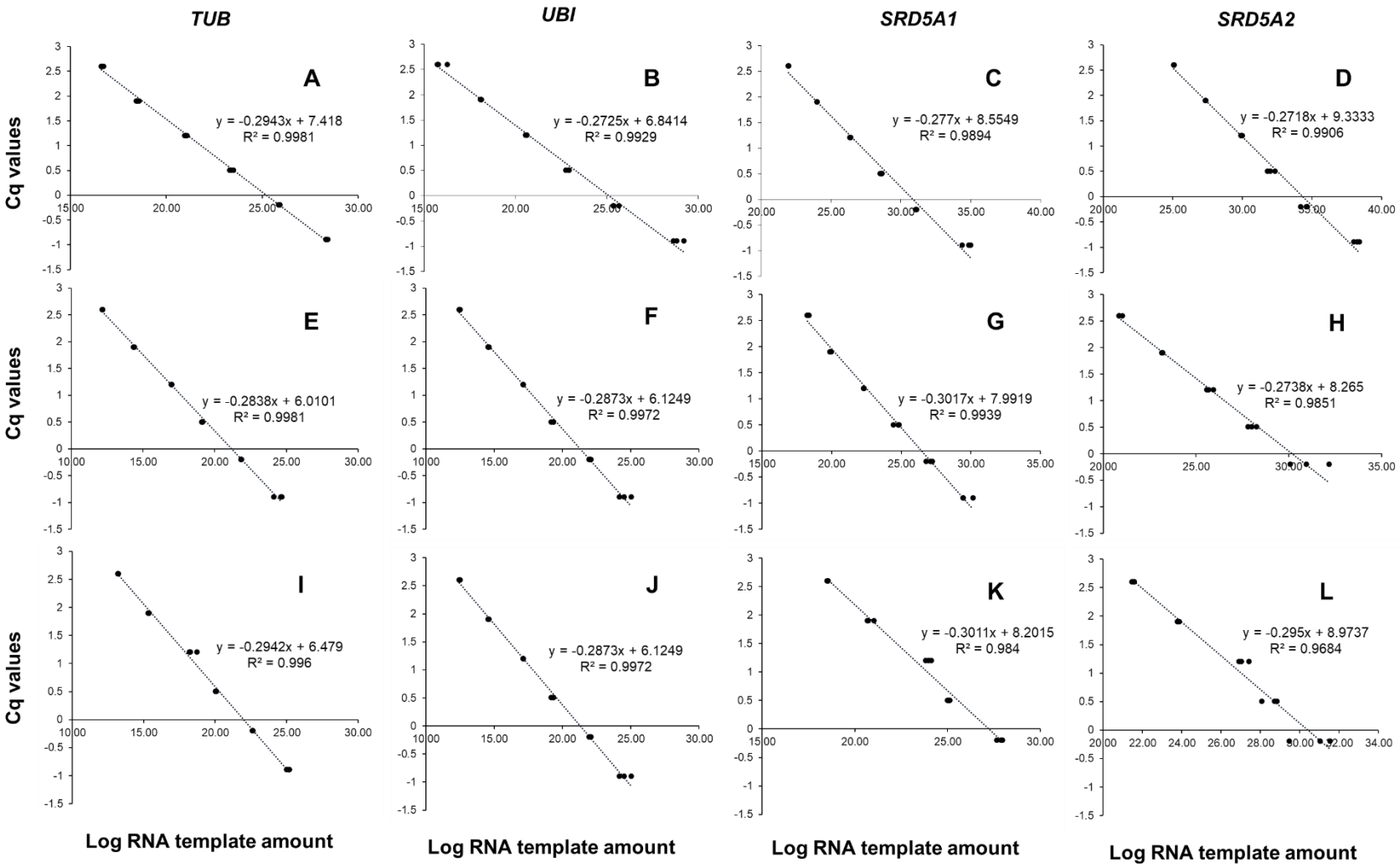


Figure 3.9: Standard curves for the selected reference genes *TUB* (a, e, i), *UBI* (b,f,j) and the target genes *SRD5A1* (c,g,k), *SRD5A2* (d,h,l) based on three independent experiments. Graphs (a),(b),(c),(d) represent data from the first standard curve experiment; (e),(f),(g),(h) represent data from the second standard curve experiment; and graphs (I),(j),(k),(l) represent data from the third standard curve experiment.

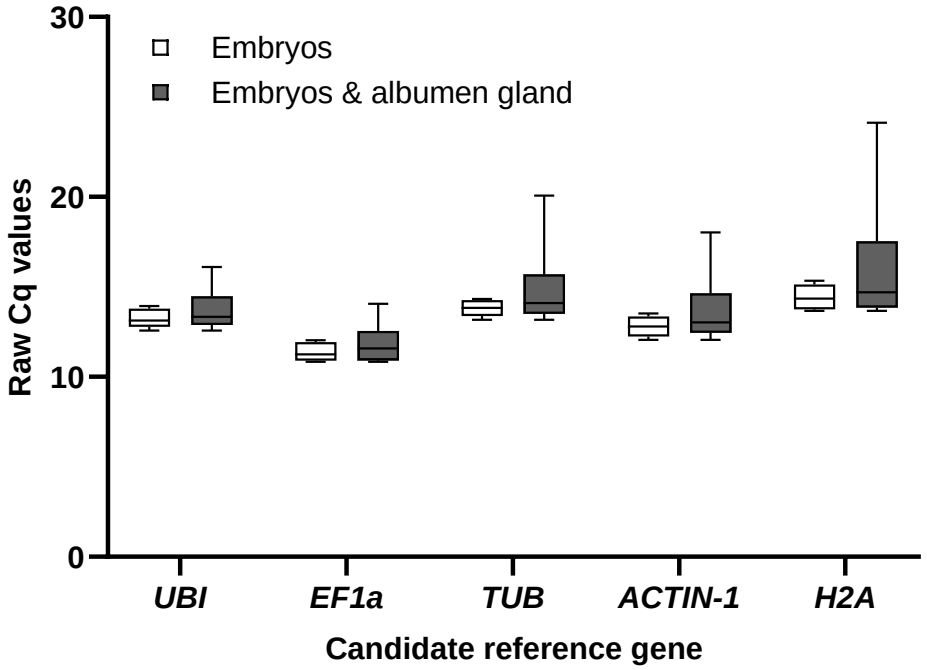


Figure 3.10: Raw Cq values of five candidate reference genes *UBI*, *EF1a*, *TUB, ACTIN-1* and *H2A* demonstrating variability across samples tested. The variability of Cq data is shown between: embryo samples (day 2 – 5 post-oviposition) in white and a combination of embryo samples (day 2 – day 5 post-oviposition) and albumen gland tissue in dark grey. The error bars represent the minimum and maximum Cq values, whereas the middle line in each box represents the median. The data was obtained from the first reference gene validation experiment comprising one biological cDNA replicate for each embryo and albumen gland sample (n=5).

Table 3.11: Raw Cq values from the RT-qPCR validation experiments of candidate reference genes *UBI, TUB, EF1a, ACTIN-1, and H2A* across embryo tissues from four developmental stages (day 2 - 5 post-oviposition) using two technical replicates and three biological replicates (n=12). Raw Cq values from the first validation experiment, which tested the albumen gland tissue are also shown (n = 1). The mean and standard deviation values were calculated for each gene based on the results of three independent experiments, individually and in combination.

Biological replicate	**Tissue**	**Technical replicate**	**Cq values**				
			UBI	***TUB***	***EF1a***	***ACTIN-1***	***H2A***
1	day 2	1	13.07	13.29	11.25	13.17	14.23
		2	13.13	13.55	11.18	13.28	14.26
	day 3	1	14.05	13.74	12.04	13.25	14.46
		2	14.08	13.37	11.79	13.19	14.60
	day 4	1	13.18	12.99	10.98	12.02	13.54
		2	13.13	13.05	11.19	12.03	13.33
	day 5	1	14.07	13.71	11.80	12.65	15.00
		2	14.08	13.66	11.95	12.69	15.05
	Albumen gland	1	16.10	19.77	14.06	17.95	24.04
		2	16.09	20.08	14.05	18.02	24.12
Arithmetic mean	Embryos		**13.60**	**13.42**	**11.52**	**12.79**	**14.31**
	Embryos & Alb. gland		**13.80**	**15.03**	**11.90**	**13.38**	**16.35**
SD	Embryos		**0.47**	**0.27**	**0.39**	**0.49**	**0.58**
	Embryos & Alb. gland		**1.22**	**2.47**	**1.16**	**2.13**	**3.91**
2	day 2	1	13.46	14.08	11.68	13.47	14.68
		2	13.21	14.11	11.50	13.53	14.73
	day 3	1	13.04	13.55	10.84	12.54	13.66

		2	12.94	13.59	10.88	12.57	13.69
	day 4	1	12.72	13.18	10.94	12.15	14.03
		2	12.57	13.32	11.02	12.05	13.90
	day 5	1	13.89	14.32	12.02	13.01	15.28
		2	13.94	14.32	12.04	13.04	15.35
Arithmetic mean			**13.22**	**13.81**	**11.37**	**12.80**	**14.42**
SD			**0.48**	**0.42**	**0.48**	**0.52**	**0.64**
3	day 2	1	13.85	14.65	12.11	14.12	15.10
		2	13.97	14.66	12.13	14.10	15.11
	day 3	1	13.21	13.96	11.17	14.16	14.84
		2	14.58	13.75	11.03	13.47	14.99
	day 4	1	13.62	15.01	12.96	13.22	15.49
		2	13.65	14.89	12.77	13.27	15.49
	day 5	1	14.47	14.47	12.69	12.96	15.59
		2	14.45	13.80	13.00	12.81	14.92
Arithmetic mean			**13.98**	**14.40**	**12.23**	**13.51**	**15.19**
SD			**0.46**	**0.46**	**0.73**	**0.51**	**0.27**
Arithmetic mean (all embryo replicates)			**13.60**	**13.88**	**11.71**	**13.03**	**14.64**
SD (all embryo replicates)			**0.56**	**0.56**	**0.67**	**0.61**	**0.66**

3.3. Reference gene ranking using the BestKeeper, geNorm, NormFinder, ΔCT and RefFinder algorithms

After the selection of two reference genes in the first validation experiment (as described in section 2.5.5), the expression stability of all candidate reference genes was evaluated using the statistical algorithms BestKeeper, geNorm, NormFinder, ΔCT, and RefFinder. For these analyses, raw Cq values of all candidate reference genes were obtained from the embryo samples tested (day 2 – day 5 post-oviposition embryos) and three independent validation experiments. BestKeeper, NormFinder ΔCT and RefFinder methods provided recommendations for the most stable gene, whereas geNorm recommended the most stable pair of genes.

BestKeeper found all candidate genes tested to be stably expressed as they exhibited a SD<1. Solely based on SD values, BestKeeper ranked *TUB* as the most stable gene (SD=0.45), *ACTIN-1* and *UBI* as the second most stable genes (SD=0.49), *H2A* as the third most stable gene (SD=0.54) and *EF1a* as the fourth most stable gene (SD=0.58) (Table 3.12; Fig. 3.12). Based on the "r value", BestKeeper ranked *H2A* as the most stable gene (r value=0.95), TUB as the second most stable gene (r value=0.88), *EF1a* as the third most stable gene (r value= 0.86), *UBI* as the fourth most stable gene (r value=0.77) and *ACTIN-1* as the fifth most stable gene (r value=0.70) (Table 3.12; Fig. 3.12).

Table 3.12: Ranking outcomes of the BestKeeper analysis for each candidate reference gene according to their corrected SD and stability value "r. Data was calculated based on the geometric mean of Cq values from all candidate reference genes tested, across four developmental stages (day 2 - 5 post oviposition), two technical replicates and three biological replicates (n=12).

Reference gene	Ranking	n	Stability value (SD)	Stability value "r"
TUB	1	12	0.45	
	2			0.88
ACTIN-1	2	12	0.49	
	5			0.70
UBI	2	12	0.49	
	4			0.77
H2A	3	12	0.54	

	1			0.95
EF1A	4	12	0.58	
	3			0.86

According to geNorm all of the candidate genes tested were found to be stably expressed as they exhibited an M value < 1.5. Based on this metric, the most stable pair of candidate reference genes were *TUB* and *H2A* with a combined M value of 0.13 (Table 3.13; Fig. 3.12). The second, third and fourth most stable genes were *EF1a, UBI, and ACTIN-1* with M values of 0.16, 0.17, and 0.18, respectively (Table 3.13; Fig. 3.12). Based on geNorm's pairwise variation analysis, the addition of a third reference gene provided a ratio value (V) of 0.14, whereas the addition of a fourth or fifth reference gene demonstrated a ratio value (V) of 0.12 and 0.11, respectively (Fig. 3.11). All "V" values were found to be below 0.15, and thus the data suggested that an addition of a third, fourth or fifth reference gene would not considerably increase normalisation stability.

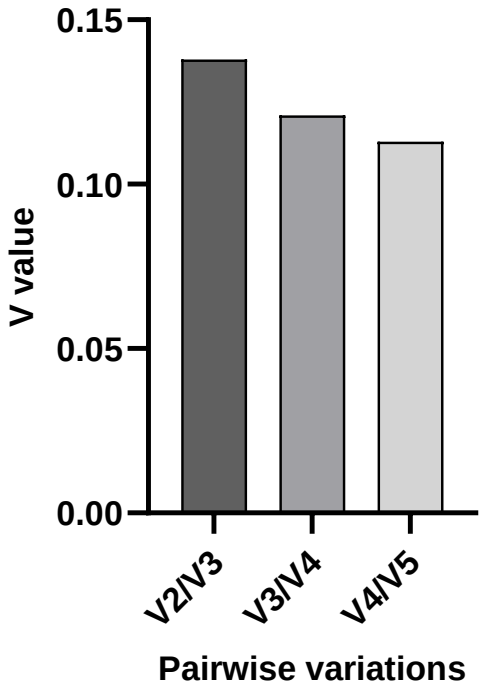


Figure 3.11: Results of geNorm's pairwise variation analysis, indicating the optimal number of reference genes to be used for normalisation of qPCR data. Ratios V2/V3, V3/V4 and V4/V5 compare the pairwise variations when using two (V2) versus three (V3), four (V4) or five (V5) reference genes. A ratio value below 0.15 indicates that adding another reference gene does not significantly increase normalisation stability.

NormFinder calculated the most stable candidate reference gene according to its own stability value, where smaller values indicated greater expression stability. According to NormFinder, *H2A* was found to be the most stable candidate reference gene with a stability value of 0.21 (Table 3.13; Fig. 3.12). The second and third most stable reference genes were found to be *TUB* and *EF1a*, with stability scores of 0.27 and 0.40, respectively (Table 3.13; Fig.2.12). *UBI* and *ACTIN-1* were ranked as the fourth and fifth most stable candidates with a stability value of 0.43 and 0.53, respectively. The ΔCT method ranked the candidate reference genes based on the SD values of different paired comparisons, where a lower SD exhibited greater stability. Based on this method, the ranking of candidate reference genes aligned with the results of the NormFinder analysis (Table 3.13; Fig.2.12). Finally, RefFinder provided a combined result of stability for all candidate reference genes based on the geometric mean of the stability values obtained from the other four algorithms. More stable genes exhibited a lower ranking value. RefFinder suggested *TUB* and *H2A* were the two most stable reference genes, both of which received a ranking value of 1.41 respectively (Fig. 3.12). The second, third and fourth more stable reference genes according to RefFinder were found to be *EF1a*, *UBI*, and *ACTIN-1* with overall ranking values of 3.41, 3.72, and 3.98 respectively (Fig. 3.12).

Table 3.13: Ranking results of the geNorm, NormFinder and ΔCT analyses for each candidate reference gene according to genNorm's M values, NormFinder's stability value and ΔCT's SD values. geNorm identifies the most stable pair of genes, whereas NormFinde and the ΔCT method identify the single most stable gene. Data was calculated based on the Cq values of the embryo tissues from four developmental stages (day 2-5 postoviposition) using two technical replicates and three biological replicates (n=12).

Stability algorithm	Reference gene	Ranking	n	Stability value
geNorm	*TUB / H2A*	1	12	0.13
	EF1A	2	12	0.16
	UBI	3	12	0.17
	ACTIN-1	4	12	0.18
NormFinder	*H2A*	1	12	0.21
	TUB	2	12	0.27
	EF1A	3	12	0.40
	UBI	4	12	0.43
	ACTIN-1	5	12	0.53

ΔCT	*H2A*	1	12	0.47
	TUB	2	12	0.49
	EF1A	3	12	0.55
	UBI	4	12	0.57
	ACTIN-1	5	12	0.63

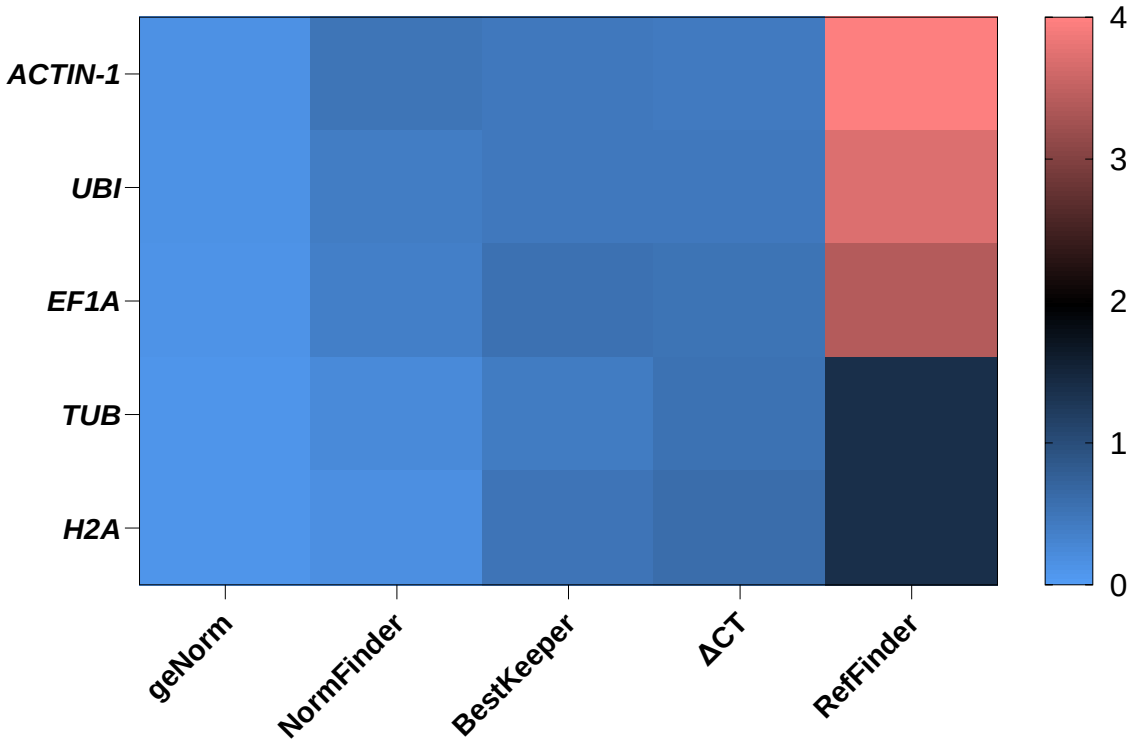


Figure 3.12: Heatmap ranking of candidate reference genes *UBI, TUB, ACTIN-1, EF1A* and *H2A* according to the algorithms geNorm, NormFinder, BestKeeper, ΔCT and RefFinder. geNorm and NormFinder ranked candidates according to their stability values, BestKeeper and ΔCT ranked candidates according to their respective SD values, whereas RefFinder ranked candidates according to the geometric mean of all stability values obtained from the four other methods. BestKeeper's "r values" were not incorporated in the heatmap, as these were not used by the RefFinder algorithm. A smaller value (blue) indicates greater stability, whereas a higher value (red) indicates a smaller stability ranking.

3.4. Quantification of *SRD5A1* and *SRD5A2* transcripts

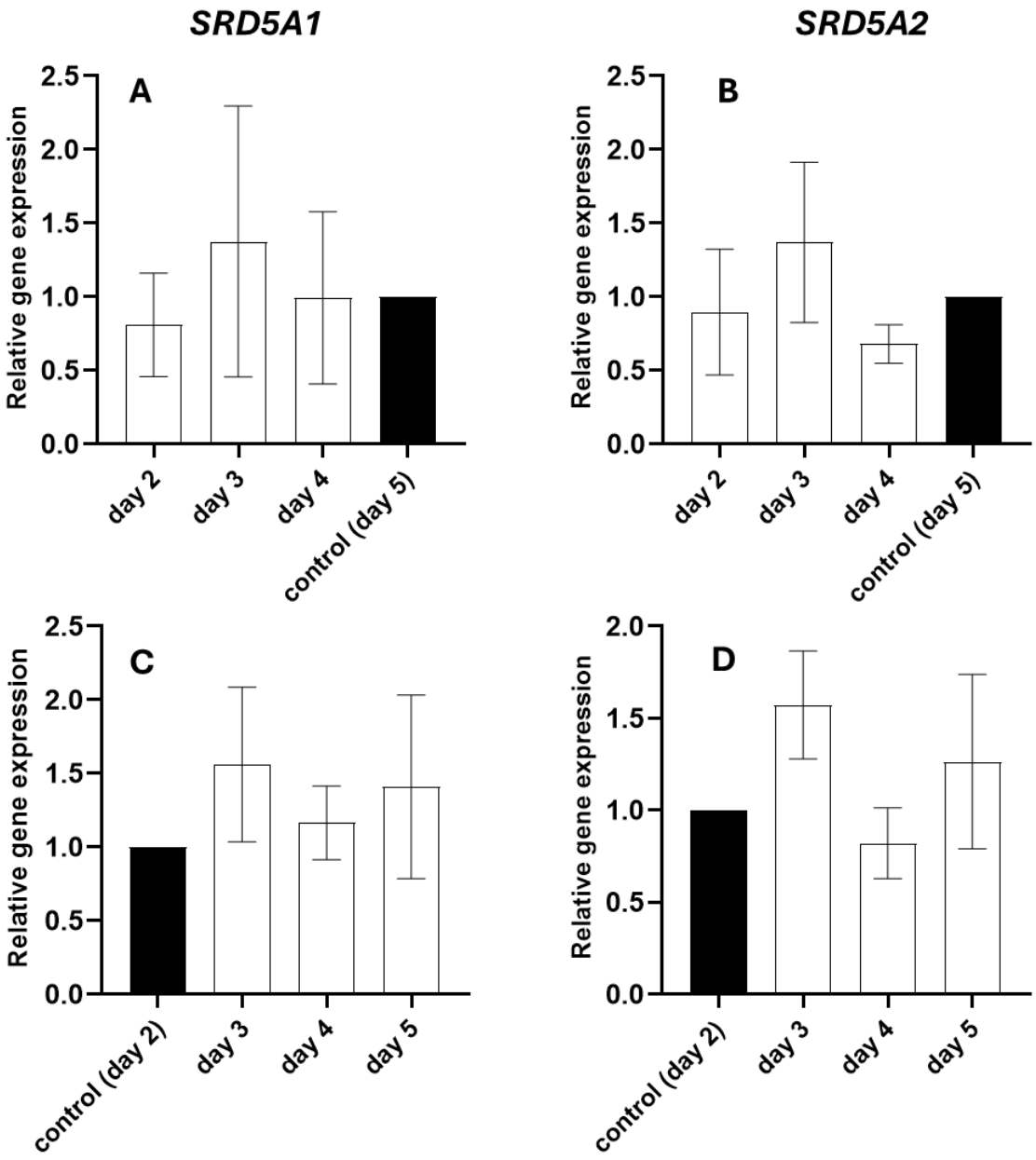


Figure 3.13: Relative gene expression of (a, c) *SRD5A1* and (b, d) *SRD5A2* transcripts across different embryo developmental stages. Graphs (a) and (b) demonstrate relative gene expression across day 2-4 post-oviposition stages with day 5 post-oviposition used as the calibrator sample. Graphs (c) and (d) demonstrate relative gene expression across day 3-5 post-oviposition stages with day 2 post-oviposition used as the calibrator sample. Data of relative gene expression represents the mean of three independent RT-qPCR experiments, comprising three biological replicates for each developmental stage (n=12). Error bars indicate the standard deviation of the mean.

The choice of *UBI* and *TUB* as the most suitable reference genes for quantifying *SRD5A1* and *SRD5A2* was guided by their high primer specificity (Fig. 3.8) and their low standard deviation (SD) observed in the first validation experiment (Table 3.11, Biological replicate 1). The quantification of *SRD5A1* and *SRD5A2* transcripts in embryo samples using *TUB* and *UBI* as reference genes, revealed slight variations across developmental stages, although not statistically significant. On day 2, 3, and 4 post-oviposition stages, the relative gene expression of *SRD5A1* compared to the calibrator (day 5 post-oviposition) was 0.81, 1.38, and 1, respectively (Fig. 3.13a). Similarly, for the same stages, the relative gene expression of *SRD5A2* compared to the calibrator (day 5 post-oviposition) was 0.90, 1.37, and 0.68, respectively (Fig. 3.13b). A second analysis examining relative gene expression across embryo developmental stages, used the day 2 post-oviposition stage as the calibrator sample (Fig. 3.13c, 2.13d). Although not significant, the relative gene expression of *SRD5A1* was slightly higher on day 5 post-oviposition compared to day 4 post-oviposition, with values 1.41 and 1.17, respectively (Fig. 3.13c). With the exception of this result, implementing day 2 post-oviposition as the calibrator sample did not show any other considerable differences in the relative expression of *SRD5A1* and *SRD5A2*, compared to using day 5 post-oviposition as the calibrator. Overall, although not significant, *SRD5A1* and *SRD5A2* were found to be slightly more expressed on day 3 post-oviposition stages compared to other developmental stages. However, the statistical analysis suggests that *SRD5A1* and *SRD5A2* gene expression remains unchanged across the developmental stages tested (Fig. 3.13).

4. Discussion

This study aimed to develop a robust RT-qPCR assay, including the identification of stable reference genes, for normalizing gene expression data across different embryonic stages (days 2 - 5 post oviposition) of *B. glabrata.* Additionally, the study sought to map the mRNA transcript expression of the genes *SRD5A1* and *SRD5A2,* which encode 5αR1 and 5αR2, respectively, and identify whether these are differentially expressed during the sensitive window of DUT disruption in *B. glabrata* embryos.

4.1. Reference gene stability

With the exception of BestKeeper, the statistical algorithms employed in this study consistently ranked *TUB and H2A* as the two most stable reference genes from the five candidates tested (*UBI*, *TUB*, *EF1a*, *ACTIN-1*, and *H2A*) across embryonic developmental stages. Although *TUB* was also identified as stable in the initial SD observations, *H2A* demonstrated the highest SD in the first validation experiment and thus was not selected as a reference gene (Table 3.11). The choice to select only *TUB* and *UBI* as reference genes was due to the small working volume of the biological cDNA replicates (day 2 – day 5 post-oviposition samples), which

limited the inclusion of additional reference genes in the standard curve and target gene quantification experiments. Consequently, the small amount of RNA that was isolated from embryos, restricted these experiments to using no more than four primer pairs—two for the reference genes and two for the target genes. Notably, while the first validation experiment revealed that *ACTIN-1* exhibited an overall greater expression stability than *UBI* and *TUB* (due to its lower SD), the melting curve analysis of *ACTIN-1* revealed a second, smaller peak, indicating non-specific amplification. It is hypothesised that this non-specific amplification, which was consistently observed across all three independent experiments (Fig. 3.8b, g, l), might have been a result of primer-dimer formation. Consequently, *ACTIN-1* was considered an unsuitable reference gene, despite its initially promising stability, as its non-specific amplification could have impacted the accurate quantification of the *SRD5A1* and *SRD5A2* transcripts, leading to normalisation issues and potential inaccuracies in gene expression data. Moreover, although *EF1a* exhibited the smallest SD amongst candidates, it was considered unsuitable for normalising gene expression because its Cq values exhibited the highest divergence from the Cq values of the *SRD5A1* and *SRD5A2* genes (that were obtained from preliminary investigations). To ensure the evaluation of subtle differences in gene expression levels, the expression of reference genes and target genes is recommended to be similar (Silver *et al.*, 2006). Consequently, since *SRD5A1* and *SRD5A2* transcripts were amplified at ~21 and ~24 cycles, respectively (Appendix S3, Table S3.5), *UBI* and *TUB* were chosen as the most appropriate reference genes because they exhibited a combination of low SD, high primer specificity and small Cq divergence from the target genes. The selection of two reference genes in this study aligns with the recommendations of the MIQE guidelines on avoiding the use of a single reference gene (Bustin *et al.*, 2009). This decision was further supported by geNorm's pairwise variation analysis, which indicated that the inclusion of a third reference gene would not considerably affect normalisation stability (Fig. 3.11).

Although each method used different criteria for determining reference gene stability, all candidates tested in this study exhibited small expression variability across the day 2 – day 5 post-oviposition embryos stages and were found potentially suitable for use in RT-qPCR assays according to the standards set by each method. For example, according to geNorm, any candidate reference gene with an M value lower than 1.5 was considered stable enough for inclusion in the ranking analysis (Silveira *et al.*, 2009; Yigin *et al.*, 2017; Renganathan *et al.*, 2023). In our analyses, all candidates demonstrated an M value < 0.6, indicating acceptable levels of stability across the developmental stages tested. Moreover, the BestKeeper analysis showed that every candidate tested exhibited a SD < 0.6, which was lower than its cutoff value of 1, and therefore every candidate tested was considered stable (Pfaffl *et al.*, 2004). (Table 3.13). On the other hand, NormFinder the ΔCT method, and

RefFinder did not have a strict cutoff value but instead relied on the ranking of all candidates tested where a lower value indicated greater expression stability.

4.2. Limitations in reference gene stability, algorithms tested and candidate selection

While all candidate reference genes tested were found stably expressed across the day 2 to day 5 post-oviposition stages, it should be noted that none of these genes maintained stable expression across both embryonic tissues and the albumen gland tissues of *B. glabrata* (Fig. 3.10). Thus, the reference genes identified in this study may be used for normalising gene expression across different embryonic tissues in *B. glabrata* but not a combination of embryo and adult tissues. An adult tissue would have been a more appropriate calibrator sample than embryos as it would not interfere with the evaluation of *SRD5A1* and *SRD5A2* gene expression in the day 2 or day 5 post-oviposition stages. Consequently, this would help to avoid the overlap of comparisons with other embryonic stages, ultimately providing a clearer baseline for analysis. Nonetheless, validating the stability of reference gene expression in earlier developmental stages, such as day 1 post-oviposition, was not possible due to the low RNA yield isolated from those samples (Appendix S3, Table S3.4). Despite efforts to increase the input amount of day 1 embryonic tissue through pooling, the limited RNA quantity each RNA isolation column could process resulted in substantial challenges. These included the loss of a considerable amount of total RNA across day 1 samples, resulting in issues with variability and reproducibility. Isolation of higher RNA amounts from embryo samples (day 2 – day 5 post-oviposition) could have also enabled the quantification of *SRD5A1* and *SRD5A2* transcripts alongside all five candidate reference genes. This more rigorous approach would have enabled a direct comparison of gene expression normalisation with different reference gene pairs, potentially enhancing the reliability of our results. However, given the existing RNA extraction methods used in this study, this process would require the pooling of a greater number of embryos than originally obtained for each sample and thus would be very time-consuming.

Generally, the use of the statistical algorithms employed in this study is considered a robust method for determining reference gene stability in qPCR studies. As of November 2024, geNorm has been cited in 22,166 studies (Vandesompele *et al.*, 2002), BestKeeper in 5,504 studies, (Pfaffl *et al.*, 2004), NormFinder in 7,782 studies (Andersen, Jensen and Ørntoft, 2004), the ΔCT method in 1,772 studies (Silver *et al.*, 2006) and RefFinder in 1,484 studies (Xie *et al.*, 2012). However, it should be noted that each of those algorithms relies on a unique set of assumptions and methods of calculation which in turn determine their stability rankings. Although a comprehensive discussion on the most suitable algorithm for determining reference gene stability is beyond the scope of this chapter, some limitations of these

statistical approaches will be discussed based on previous studies that have attempted to address them. For example, De Spiegelaere *et al.*, (2015) suggested that correcting raw Cq data according to PCR efficiency before feeding them into the algorithms, can considerably affect the outcomes of geNorm NormFinder and RefFinder. With the exception of BestKeeper, which automatically corrected its stability values based on the PCR efficiency, every other algorithm used in this study assumed 100% PCR efficiency. Consequently, the ranking of the candidate reference genes from geNorm, NormFinder, ΔCt and RefFinder could have likely been affected by this limitation. However, considering that *UBI* and *TUB* were selected for normalising *SDRD5A1* and *SRD5A2* gene expression, and were also rated as the two most stable genes in BestKeeper (based on BestKeeper's SD), it is believed this limitation has been minimised. On the other hand, a recent study that evaluated the use of stability algorithms in developmental studies suggested that BestKeeper's SD<1 threshold may be too lenient (Sundaram *et al.*, 2019). Based on their analyses, some reference genes that exhibited SD<1 showed a high coefficient of variation value, indicating greater instability. Thus, an integrated approach which combines the NormFinder algorithm, a coefficient of variation analysis and a one-way Analysis of Variance (ANOVA) (to assess variation amongst different samples) was proposed for accurately determining reference gene stability in such studies (Sundaram *et al.*, 2019). Considering these limitations, it is evident that assuming gene expression stability solely based on uncorrected SD, as done in the present study, may not be the most accurate approach. Nonetheless, it is also important to examine the suitability of different stability algorithms in accordance with the experimental conditions of each study. Although the selection of *TUB* and *UBI* as reference genes aligned with the stability thresholds of BestKeeper and geNorm and may present suitable reference genes for *B. glabrata* embryos, the recommendations of Sundaram *et al.*, (2019) could have been used to increase the robustness of our reference gene validation assay. Finally, it should also emphasised that the identification of candidate reference genes in this study was a targeted approach based on findings previously reported in the literature. In turn, the potential selection bias that may have been introduced in this study should also be addressed. These biases could have been avoided if an agnostic selection of candidate reference genes had been achieved. For example, untargeted transcriptomic investigations of embryo samples across different developmental stages could have identified more stable, and perhaps novel, reference genes. However, given the limited time and resources available for the completion of this project, the validation and use of these methods could not be achieved.

4.3. The potential role of 5αR in molluscs

Even though 5αR's role in vertebrate steroidogenesis is well documented (as discussed in Chapter 2), its function in molluscs remains less understood, despite the identification of 5αR gene homologs (*SRD5A1*, *SRD5A2*) in various species (Chapter 2, Fig. 9). The possible involvement of 5αR enzyme in molluscan development, especially in processes like shell formation, has gained interest due to the phenotypic abnormalities observed in the early development of the freshwater gastropod *B. glabrata* when 5αR activity is disrupted by pharmaceutical inhibitors DUT and FIN (Baynes *et al.*, 2019). This suggests a broader role for 5αR beyond its vertebrate function in the metabolism of testosterone to dihydrotestosterone, potentially influencing critical developmental pathways in molluscs. In *B. glabrata* embryos, shell formation begins during the trochophore stage (around day 2 post-oviposition) (Bielefeld and Becker, 1991; Shimizu *et al.*, 2011). At the veliger stage, which occurs around day 2 – day 3 post-oviposition, the shell of *B. glabrata* starts to coil, marking the early stage of shell development. During the late veliger stage (around day 5 post-oviposition), new shell material begins to attach to the existing shell, allowing for its expansion, as the embryo continues to grow (Bielefeld and Becker, 1991; Shimizu *et al.*, 2011). After 5 days post-oviposition, the embryos are sufficiently developed and may start escaping from their egg capsules (Bielefeld and Becker, 1991; Shimizu *et al.*, 2011).

The period by which *B. glabrata* shell starts to form seems to align with the unpublished findings from Dr Alice Baynes and Hazzel Tabernilla, which demonstrated that the critical period of shell disruption due to DUT occurred around the same timeframe (day 0 – day 2 post-oviposition, Fig. 3.1). This sensitive window of disruption, coinciding with shell-field formation and trochophore stage, could suggest that 5αR may play a role in shell formation, and perhaps be differentially expressed during day 0 – day 2 days post-oviposition compared to later stages of embryonic development (day 3 – day 5 post-oviposition). The results from this study suggest that *SRD5A1* and *SRD5A2,* responsible for encoding 5αR1 and 5αR2 respectively, may not be differentially expressed across the day 2 – day 4 post-oviposition embryonic stages (Fig. 3.13a, 3.13b). This result was consistent with using day 2 post-oviposition stage as a calibrator sample, where no significant differences in expression levels were observed across day 3–day 5 post-oviposition stages (Fig. 3.13c, 3.13d). Although both *SRD5A1* and *SRD5A2* were found to be slightly more expressed during day 3 post-oviposition, this might be a result of the high variability that occurred in the expression levels of the three biological replicates (Fig. 3.13a–3.13d). The variability in the expression levels could be a result of various factors. First, the pooling of embryos for each biological replicate involved collecting tissue from multiple egg masses which could have developed at slightly different rates. This variation in egg development may have occurred due to unknowns in the exact

time the egg mass was laid by the snail. Even though the collection of egg masses from the parent tanks was performed consistently on day 0 post-oviposition, the specific timing of egg-laying was not recorded. Moreover, further variabilities in gene expression could have been introduced from the different approaches used in isolating embryo RNA. For example, embryos from earlier developmental stages (day 2 – 3 post-oviposition) were collected by manually dissecting egg capsules and collecting each embryo individually. Due to the large number of embryos needed in these samples (e.g. 200 embryos per biological replicate), separate collections of 50–100 embryos were preserved in RNA-later over a period of 5 - 6 hours and then pooled together at the RNA isolation stage. In contrast, samples from later developmental stages (day 4 – 5 post oviposition) were collected by pooling entire egg masses in RNA-later within minutes, reducing additional time-related variations. Taken together, the time-related variations in embryo collection could have resulted in the isolation of RNA from embryos with hour-level differences in their development for each sample (day 2 - day 5 post-oviposition). In turn, this could have led to deviations in gene expression between the biological replicates. Notably, the small number of biological cDNA replicates used to obtain the relative gene expression results could have also influenced this high variability. Hence, the inclusion of additional biological replicates from each embryonic developmental stage could have enhanced the robustness of the data.

The overall stable expression of *SRD5A1* and *SRD5A2* transcripts across day 2 – day 4 developmental stages suggests that 5αR1 and 5αR2 expression may remain unchanged during the period that shell formation occurs. However, the high variations observed in gene expression levels between biological replicates make it difficult to draw comprehensive conclusions. Further research employing more time-specific embryo samples is required to accurately determine the patterns of *SRD5A1* and *SRD5A2* transcript expression during the sensitive window of phenotypic disruption caused by DUT. Notably, although not quantified, the presence of *SRD5A1* and *SRD5A2* transcripts in the albumen gland (Appendix S3, Table S3.5) is an important insight which needs further investigation. Despite the non-significant effects observed in the gene expression patterns of *SRD5A1* and *SRD5A2,* the absence of necessary enzymes to initiate testosterone biosynthesis in molluscs (as discussed in Chapter 2) and taking into consideration the morphologic disruption caused by the 5αR inhibitor DUT, may suggest an indirect role of this enzyme in molluscan shell development. For example, 5αR enzymes could be involved in the regulation or synthesis of other signalling molecules that are essential for molluscan shell formation. Although the underlying mechanisms by which molluscan shells are formed are still not fully understood, some signalling pathways that could play a role in molluscan shell morphology as well as the potential involvement of 5αR in these pathways are discussed.

4.4. The role of ecdysone in molluscan shell development

As discussed in Chapter 2, molluscs may possess some endocrinological mechanisms that differ from those of vertebrates. Notably, the insect-originated ecdysone receptor (*EcR*) has been recently identified in the mantle tissues of the pearl oyster *Pinctada fucata* (Xiong *et al.*, 2022). The mantle, crucial for shell secretion and providing a protective layer between the shell and internal organs, plays an important role in molluscan physiology (Clark *et al.*, 2010). Investigations into *P. fucata,* revealed that shell notching (a technique used to induce shell damage) resulted in increased serum levels of the insect steroid ecdysone, as well as an elevated expression of the *EcR* gene (Xiong *et al.*, 2022). Moreover, the insect steroid and *EcR* ligand, ecdysone, has been previously shown to promote shell regeneration in the gastropod mollusc *Helix aspersa* (Whitehead and Saleuddin, 1978). These findings suggest a potential role of ecdysone in molluscan shell regeneration, mediated through the activation of the ecdysone receptor complex (Xiong *et al.*, 2022). It has been previously discussed that ecdysteroids , such as ecdysone, do not act independently during developmental processes in invertebrates but rather through complicated crosstalk between different signalling pathways (Miyakawa *et al.*, 2018). Interestingly, a recent paper examining the effects of another pharmaceutical 5αR inhibitor, finasteride (FIN), in the invertebrate *Daphnia magna*, showed the downregulation of genes involved in ecdysone signalling, such as the ecdysone receptor isoforms *EcR-A* and *EcR-B* and the Retinoid X Receptor (*RXR*) (Cho *et al.*, 2024). Although to the best of our knowledge, a functional sequence of either *SRD5A1* or *SRD5A2* in the genome of *D. magna* has yet to be identified, these findings provide an example of how pharmaceutical 5αR inhibitors can have off-target effects by interacting with various genetic processes. However, whether DUT disrupts ecdysone signalling in molluscs, and its potential effects on shell development, needs to be investigated.

4.5. The role of TGF-β signalling pathway in molluscan shell formation

Molluscan shell formation has been previously demonstrated to be disrupted by the indirect effects of chemicals. For example, short-term exposure of the gastropod *Marisa cornuarietis* embryos to platinum (II) chloride ($PtCl_2$) inhibited the formation of the mantle cavity and prevented the development of external shell in adults (Osterauer *et al.*, 2010). Instead, the formation of a cone-shaped internal shell was observed in platinum-exposed individuals (Osterauer *et al.*, 2010). Exposure of gastropod molluscs to platinum has also been linked with the inhibition of a gastropod-specific developmental process called “torsion”. Torsion occurs during the early embryonic stages of development where the body of gastropods undergo a 180-degree twist, resulting in the repositioning of the animal's internal organs and external structures (Marschner *et al.*, 2013). Further investigations by Marschner *et al.*, (2013)

demonstrated that torsion is the starting point where shell malformations in *M. cornuaerietis* shell occur. The process of torsion, as well as the development of mantle and shell, are characterised by the rapid increase of cells in various tissues, otherwise known as proliferation. Tissue proliferation is in turn regulated by molecules that can affect the growth of cells, known as growth factors, including members of the transforming growth factor-β (TGF-β) superfamily of proteins. Of particular importance are certain cytokines, such as TGF-βs and bone morphogenetic proteins (BMPs), which are involved in various invertebrate ontogenetic developmental processes. It was recently demonstrated that impairment of TGF-β signalling by exposing *M. conruarietis* embryos to a TGF-β pharmaceutical inhibitor, resulted in similar shell malformations to those observed by platinum exposures (Link, Triebskorn and Köhler, 2019). These findings suggest that TGF-β signalling may be involved in shell development and shell positioning in the early development of gastropod molluscs (Link, Triebskorn and Köhler, 2019). In vertebrates, 5αR is indirectly involved in the regulation of the TGF-β cytokine, through the conversion of testosterone (T) to dihydrotestosterone (DHT) and the subsequent binding of DHT to the AR (Lee *et al.*, 2022). Although previous research suggests a potential link of TGF-β with DHT in vertebrate cancer cells (Kim *et al.*, 1996), the effects of 5αR inhibition on TGF-β signalling are not well documented. Molluscan genomes do not possess an AR, and thus the role of 5αR cannot involve the traditional androgenic pathways seen in vertebrates. One theory is that 5αR may be involved in the metabolism of non-androgenic substrates which could still modulate crucial signalling pathways such as TGF-β signalling. Consequently, exposure to pharmaceutical 5αR inhibitors, DUT or FIN, could impair the function of TGF-β in gastropods and may lead to developmental abnormalities in the mantle and shell formation.

4.5. Future perspectives

Although the observations made in this study shed some light on the involvement of 5αR in gastropod embryonic development, further research is needed to fully elucidate the mechanisms by which pharmaceutical 5αR inhibitors result in the surprising open-coil shell phenotype in embryonic *B. glabrata* (as demonstrated by Baynes *et al.*, (2019)). Future investigations should utilise embryonic samples that are more precisely matched to specific developmental stages which would help to eliminate the variations observed in gene expression between biological replicates. Moreover, including earlier developmental stages in the analysis (e.g. day 0 – day 1 post-oviposition) could provide crucial information for the expression of *SRD5A1* and *SRD5A2* transcripts, before gastropod shell formation is initiated. Such time-specific sampling could be facilitated by the adoption of more sensitive quantification methodologies, such as droplet digital PCR (ddPCR). ddPCR can provide even more precise and reproducible data than the RT-qPCR method by using smaller amounts of

RNA starting material, given it does not require the quantification of cDNA using standard curves (Taylor, Laperriere and Germain, 2017).

Nevertheless, the limitations observed in the ranking analysis of candidate reference genes highlight the need for additional validation steps before the statistical analysis is performed. The experimental design of studies should be considered when using the stability algorithms employed in this study. To increase the data robustness of reference gene stability, future analyses using various embryonic samples from *B. glabrata*, should correct the raw Cq values of reference genes according to the recommendations provided in De Spiegelaere *et al.*, (2015). Moreover, the integrated approach described by Sundaram *et al.*, (2019) should also be taken into account.

Future research should also utilise comprehensive omics approaches, including transcriptomics, proteomics and lipidomics to examine changes in the expression of the genome, proteome or lipidome of embryonic *B. glabrata*. These could help the identification of novel candidate reference genes which could be used for future molecular investigations. Moreover, the use of omics approaches could help identify differential expression of *SRD5A1* or *SRD5A2* transcripts in response to DUT, which could provide important insights into the role of 5αR in *B. glabrata* embryos. These could include the exploration of non-androgenic substrates that could be metabolised by 5αR as well as the downstream effects of DUT disruption on various pathways, including TGF-β and ecdysone signalling. Additionally, examining the protein structure of the 5αR in molluscs and its potential affinity in catalysing T to DHT could provide further information into its evolutionary history and the similarities it may possess with its vertebrate counterpart.

5. Conclusions

In conclusion, this study has provided important insights into the role of 5αR1 and 5αR2 in the embryonic development of *B. glabrata*, through novel expression patterns of the *SRD5A1* and *SRD5A2* transcripts. The development of a robust RT-qPCR assay and the identification of stable reference genes, including *TUB* and *UBI*, have been an integral part of this process and present a substantial finding which can assist researchers in future investigations. However, the variations observed in gene expression between biological replicates, as well as the limitations in the statistical methods employed for candidate reference gene ranking, call for further experiments and the use of more rigorous statistical methodologies.

While previous findings indicate a DUT-induced morphological shell disruption during the day 2 post-oviposition embryos of *B. glabrata*, findings from this study suggest that *SRD5A1* and *SRD5A2*, which encode 5αR1 and 5αR2 respectively, exhibit stable expression throughout the developmental stages day 2 – day 4 post-oviposition. However, due to the gene

expression variations that were observed, the potential involvement of 5αR in *B. glabrata* shell formation remains unclear. It is hypothesised that 5αR may have an indirect role in other developmental processes or endocrinological pathways in gastropods, including the TGF-β and ecdysone signalling pathways, through its involvement in the metabolism of non-androgenic substrates. The demonstrated role of ecdysone and its receptor (*EcR*) in molluscan shell development and repair highlights the presence of understudied endocrinological mechanisms in molluscs, whereas the interactions between ecdysone signalling and the pharmaceutical 5αR inhibitor FIN in *D. magna*, demonstrate the complexity of hormonal regulation in invertebrates. Nonetheless, the presence of *SRD5A1 and SRD5A2* transcripts in the albumen gland of *B. glabrata* are important findings that require further investigation. Overall, while significant progress has been made in characterising the stability of reference genes and the effects of the pharmaceutical 5αR inhibitor DUT in embryonic *B. glabrata*, further research is needed to provide a more comprehensive understanding of the shell development in this organism and its deformation by pharmaceutical 5αR inhibitors.

Chapter 4: Effects of a 5-alpha-reductase inhibitor, dutasteride, on the freshwater gastropod *Biomphalaria glabrata*

1. Introduction

1.1. The unknown effects of dutasteride in adult *Biomphalaria glabrata*

The developmental effects observed in *Biomphalaria glabrata* embryos following their exposure to the pharmaceutical 5αR inhibitors, dutasteride (DUT) and finasteride (FIN) (Baynes *et al.*, 2019), raised questions about the mechanisms involved in gastropod shell formation. Further molecular investigations in Chapter 3, revealed that *SRD5A1* and *SRD5A2* genes, which encode the 5αR1 and 5αR2 enzymes respectively, are likely stably expressed during the sensitive window of DUT disruption (day 2 – day 4 post-oviposition stages). Interestingly, preliminary investigations from Chapter 3 confirmed the expression of *SRD5A1* and *SRD5A2* in the albumen gland of *B. glabrata* adults. Additionally, the systematic investigations in Chapter 2, highlighted the expression of the *SRD5A1* gene in multiple adult tissues of another gastropod, *Reisha clavigera*, including the testis, penis, ovary, head ganglia and digestive gland (Ip *et al.*, 2016). Together, these findings suggest that 5αR1 and 5αR2 may be ubiquitously expressed among gastropods and could entail roles that are not specific to early development. Consequently, it is currently unknown whether DUT-induced disruption is only present in the early development of *B. glabrata*, or if it also exerts any physiological effects on adults, given the expression of both enzyme isoforms (5αR1 and 5αR2) in the albumen gland.

Given the low environmental levels of DUT (0.027 µg/L, (Gómez-Canela *et al.*, 2021)), evaluating its downstream effects in *B. glabrata* adults aims to explore the role of 5αR in this species, instead of DUT's broader ecological impacts. Accordingly, physiological complications in response to DUT may indicate a potential involvement of 5αR in the growth, reproduction and overall survival of adult *B. glabrata*, or uncharacterised disruptive effects in later developmental stages. Moreover, assessing the effects of DUT in *B. glabrata* adults provides an opportunity to conduct comparative toxicity assessments with other DUT-affected organisms. These include fish (Margiotta-Casaluci, Hannah and Sumpter, 2013), and crustaceans (Cho *et al.*, 2024), ultimately helping to enhance our understanding of cross-species pharmaceutical disruption caused by 5αR inhibitors. By directly comparing previously tested DUT concentrations in fish, it is also possible to understand the functional discrepancies between vertebrate-type and molluscan 5αR enzymes. For example, the significant declines observed in the reproductive potential of fathead minnows (*Pimephales promelas*) exposed to DUT (Margiotta-Casaluci, Hannah and Sumpter, 2013), reflect the ability of 5αR to metabolise

testosterone and its important role in vertebrate reproduction. However, considering that molluscs lack an androgen receptor (AR) and necessary enzymes needed to synthesise testosterone (discussed in Chapter 2), any DUT-induced interferences with their reproductive mechanisms may indicate a broader role of 5αR that currently remains poorly understood.

1.2. Regulatory ecotoxicity testing in aquatic gastropods

In molluscs, the effects of pharmaceuticals are usually assessed by either full life cycle (FLC) or partial life cycle (PLC) toxicity tests. The former assesses biological responses caused by chemicals across all life stages of an organism, whereas the latter assesses biological responses only during some of its life stages (OECD, 2010). Choosing between a FLC and a PLC is critical when assessing the effects of pharmaceuticals, such as 5αR inhibitors, that are known to interfere with both the embryonic development (Baynes *et al.*, 2019) and the reproductive physiology of adult aquatic organisms (Margiotta-Casaluci, Hannah and Sumpter, 2013). While FLC testing can be highly informative and may demonstrate toxicity effects at even lower concentrations compared to the PLC test (OECD, 2010), it is time-intensive and highly costly. On the other hand, while PLC may restrict any observed effects in the limited life stages tested, it can still provide critical insights about the toxicity of pharmaceuticals especially during molluscan reproduction (OECD, 2010). This is due to the incredible sensitivity that molluscan reproductive mechanisms exhibit in response to chemicals. Specifically, gastropods are known to be particularly sensitive to the reproductive effects of endocrine-disrupting chemicals (EDCs) (Blaber, 1970; Giusti *et al.*, 2013), making the PLC test a suitable framework for assessing the impacts of 5αR inhibitors. A popular PLC-type study in gastropods is the 'reproduction test' which evaluates the effects of chemicals on reproduction based on the number of egg masses or embryos produced per surviving adult (Oehlmann *et al.*, 2006; OECD, 2010; Giusti *et al.*, 2014). An important consideration for any reproductive test is using a test species that has stable yearly reproduction. In the case of molluscs, this can often be challenging due to the reproductive variabilities that exist between different species and across different seasons (Bayne, 1976; Lightfoot, Tyler and Gage, 1979; Wayne, 2001; Santos *et al.*, 2011). Such variabilities often reflect the biological intricacies that exist within the phylum, such as different reproductive strategies or different responses to certain environmental toxicants (Gabbott, 1983; Khabib *et al.*, 2022).

1.3. Assessing pharmaceutical disruption in adult *Biomphalaria glabrata*

Recently, two PLC-type studies for aquatic toxicity testing in gastropods have been developed and validated by the Organisation for Economic Co-operation and Development (OECD). Namely, the Test Guidelines (TG) 242 and 243, can evaluate the effects of chemicals on adult *Potamopyrgus antipodarum* (OECD, 2016b) and *Lymnaea stagnalis* (OECD, 2016a),

respectively. The main objective of both tests is to evaluate the downstream effects of chronic chemical exposure on test species reproduction, by counting the number of egg masses produced per individual snail at the end of a 28-day exposure period. Moreover, the TG 243 enables the assessment of additional endpoints such as the number of embryos produced (within an egg mass), or changes in growth and survival. Although the non-mechanistic nature of TG 242 and TG 243 does not allow the evaluation of underlying molecular mechanisms associated with pharmaceutical disruption (discussed in Chapter 1), they do enable the identification of general physiological toxicity in the test species (Ruppert *et al.*, 2017; OECD, 2018). Additionally, the toxic effects of chemicals on the tested endpoints within TG 242 & 243 can be expressed via standardised risk assessment metrics, including the ECx, NOEC and LOEC. ECx, represents the concentration by which a chemical causes a biological response that is x% of the maximum, whereas NOEC represents the highest concentration by which a chemical does not exhibit any observable effects compared to the controls (Crane and Newman, 2000; Warne and van Dam, 2008). In contrast, LOEC represents the lowest concentration in a test causing a significant effect compared to the controls (Warne and van Dam, 2008). However, using those metrics in ecotoxicity testing necessitates the implementation of an appropriate methodological design, including a range-finding toxicity test that determines an effective concentration range for the tested chemical (OECD, 2010, 2016a).

Therefore, developing a critical understanding of the biological responses and the limitations of standardised ecotoxicity tests is critical for reliably assessing pharmaceutical disruption in molluscs. Consequently, adapting existing regulatory guidelines for testing pharmaceuticals on previously untested organisms, such as DUT on *B. glabrata* adults, can ensure the collection of meaningful results. The adaptation of the OECD TG 243 can provide a suitable framework for assessing the impacts of DUT on adult *B. glabrata* physiology. This is due to the reproductive and phylogenetic similarities that exist between *B. glabrata* and the TG 243-validated organism, *L. stagnalis*. For example, both organisms are simultaneous hermaphrodites as they possess both female and male reproductive organs and can both self- or cross-fertilise (Costa, Grault and Confalonieri, 2004; Kuroda and Abe, 2020). Moreover, reproduction in both species takes place through the release of encapsulated embryos within translucent egg masses, which allows for a simple estimation of their reproductive output under the microscope. Contradictory, populations of the OECD TG-242 organism, *P. antipodarum,* comprise both males and females and have distinct reproductive systems. Females of *P. antipodarum* are usually reproduced parthenogenetically (i.e. without mating and their embryos are developed within a brood pouch inside the oviduct-mantle section (OECD, 2016b). Besides the apparent similarities in assessing reproduction between *B.*

glabrata and *L. stagnalis*, the OECD TG 243 also allows adaptation to a flow-through exposure system as an alternative to the standard static-renewal system. Comparing these two exposure setups can enable the exploration of the sensitivity that *B. glabrata* exhibits in response to DUT, enhancing our understanding of cross-method variability and the influence of DUT across different exposure scenarios.

1.4. Aims and Objectives

This study aimed to evaluate the sensitivity of various DUT concentrations on adult *B. glabrata* under different exposure scenarios, using a flow-through and a static-renewal system, by adapting the OECD TG 243. An overarching aim was to assess the comparability of the two exposure systems in determining the effects of DUT in the test organism. Specifically, the objective of this study was to:

- Determine if exposure to the pharmaceutical 5αR inhibitor, DUT, affects the survival, growth and reproduction of *B. glabrata* adults.

2. Methodology

2.1. Experiment 1 – Flow-through system

The experiment was performed using a continuous flow-through system by adapting the OECD Test Guidelines 243 (OECD, 2016a). The flow-through system was selected due to its general ability to minimise issues found in static toxicity tests, including decreasing oxygen levels and problems associated with the buildup of waste products from organisms (Tišler and Zagorc-končan, 1999). The results obtained from the flow-through study would also enable comparison with those from the static-renewal study (Experiment 2). The exposure period lasted for 21 days, which although a derogation from the OECD TG 243, was considered an appropriate length of for assessing chronic exposure in invertebrates (e.g. the *Daphnia magna* reproduction test lasts for 21 days (OECD, 2012)).

2.1.1. Test species

Biomphalaria glabrata individuals were supplied from breeding stocks as described in Chapter 3, section 2.1. Four-month-old snails were selected for this experiment. On the first day of the acclimation period, snail total weight (g, three decimal places) and shell length (mm, two decimal places) were determined using an analytical balance and an electronic calliper, respectively.

2.1.2. Test conditions

The experiment consisted of five DUT treatments (1 µg/L, 3.2 µg/L, 10 µg/L, 32 µg/L and 100 µg/L), a solvent control (SC) and a dilution water control (DWC). The test substance, dutasteride (DUT, CAS no. 164656-23-9, 99% pure), and the solvent N,N-Dimethylformamide (DMF, CAS no. 68-12-2, ≥99.9% pure), were obtained from Fisher Scientific. The test concentrations were chosen according to the DUT concentrations that were previously tested in fathead minnows (Margiotta-Casaluci, Hannah and Sumpter, 2013) and *B. glabrata* embryos (discussed in Chapter 3 and Baynes *et al.,* (2019)). Each treatment consisted of three replicate tanks, and each tank consisted of 6 randomly allocated *B. glabrata* snail adults (a total of 18 snails per treatment). The tops of the tanks were covered with glass lids. The tanks were gently aerated using an air pump attached to glass pipettes and were maintained in a non-temperature-controlled room with a photoperiod of 12 hours of light: 12 hours of dark.

Five days before the beginning of chemical exposure, tanks were filled with 10.5 L of dechlorinated tap water through a continuous flow-through system at test temperature (27 °C). Adult *B. glabrata* were collected from the stock cultures, where six healthy snails with a shell length between 13.40 mm to 14.21 mm were impartially selected and placed into each tank. The weight of each snail was determined using an analytical balance. The snails were fed *ad libitum* with Tetramin fish flakes. Each replicate tank contained snails of similar shell length to ensure the mean shell length between tanks did not differ considerably. During the 5-day acclimation period, the reproduction of snails under the test conditions was monitored and recorded. Before dosing started a randomized block design was determined using www.Random.org and used to allocate tank replicates with individual snails, to different test concentrations. The dosing of the initial stocks (Table 4.1) started 48 hours before day 0, which was the first day of exposure. On day 7, the finalised dosing stocks (Table 4.2) were introduced.

Thermostatically heated dechlorinated tap water (27±1°C) was supplied from a header tank and flowed through 7 flow meters into 7 mixing chambers (DWC, SC, 1 µg/L, 3.2 µg/L, 10 µg/L, 32 µg/L and 100 µg/L) at a rate of 200 mL/min (Fig. 4.1). The de-chlorinated tap water flowed through the mixing chambers via medical grade silicon tubing (VWR, 9.52 mm). The initial dosing stock test solutions (Table 4.1) flowed through a Watson Marlow multichannel peristaltic pump into their respective mixing chambers at a rate of 0.2 mL/min to achieve the nominal DUT tank concentrations of 1 µg/L, 3.2 µg/L, 10 µg/L, 32 µg/L and 100 µg/L. The percentage of DMF in the replicate tanks was 0.006% for all treatments. The finalised dosing stock test solutions (Table 4.2) flowed through the same peristaltic pump into their respective mixing chambers at a rate of 0.012 mL/min to achieve the same nominal tank concentrations.

The DWC and the SC received water and DMF, respectively, at the same rates. The water from each mixing chamber was distributed at equal flow rates into the replicate tanks through medical-grade silicone tubing (VWR, 2.64 mm). The dosing stocks flowed through the peristaltic pump via manifold tubing (Watson Marlow, Orange/White, 0.8 mm x 0.63 mm) that was in turn connected to medical grade silicone tubing (Watson Marlow Pumpsil Tubing 0.8 mm ID X 1.6 mm) with nipple connectors, facilitating the transfer of dosing stock test solutions from the stock bottle to the mixing chamber (Fig. 4.2). The openings of Winchester bottle dosing stocks were covered in aluminium foil, allowing minimal space for the tubing to be placed.

Figure 4.1: Flow-through system used for the 21-day reproduction study (Experiment 1). From left to right, pictured are the mixing chambers (above) and replicate tanks (below) for the DWC, 1 µg/L, 10 µg/L and 100 µg/L treatments.

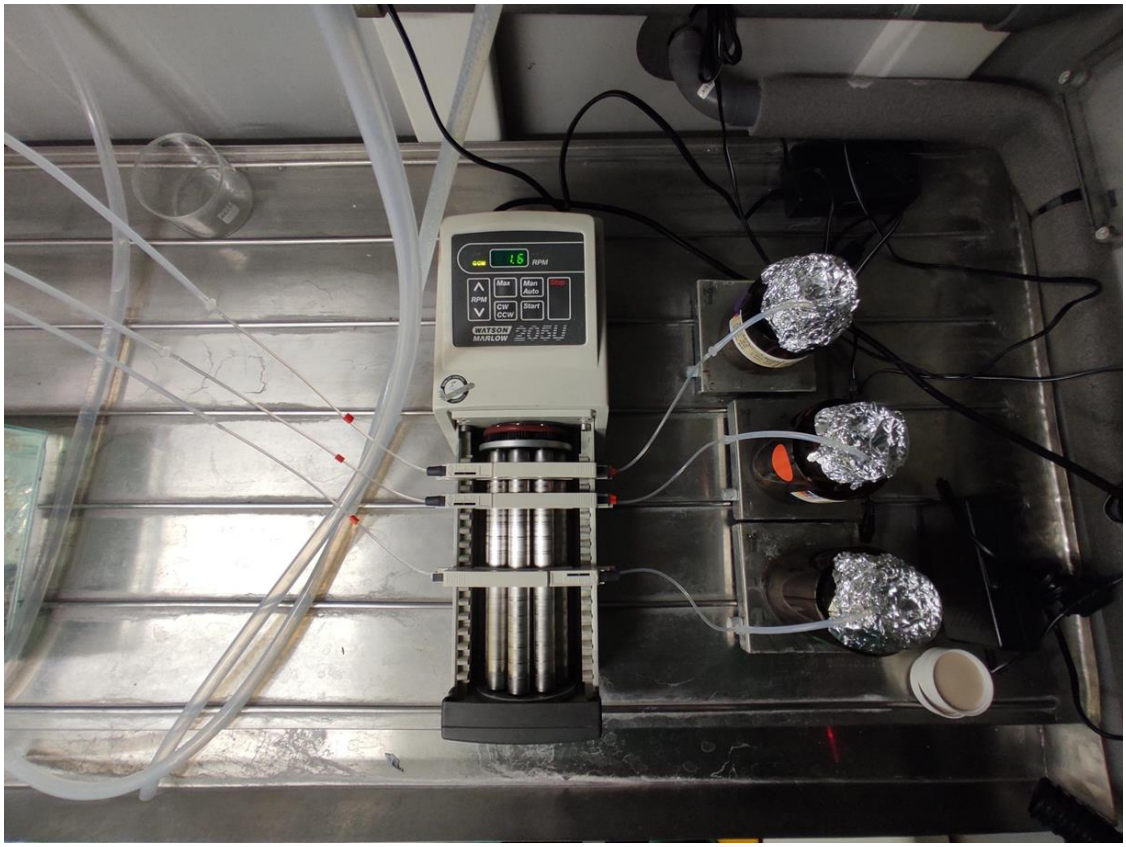


Figure 4.2: The finalised dosing stocks of 32 µg/L DUT, 3.2 µg/L DUT and SC from top to bottom, connected to the peristaltic pump via manifold tubing flowing at a rate of 1.6 rpm.

2.1.3. Test substances, dilution water and preparation of test solutions

The dilution water used for testing and maintaining stock cultures was dechlorinated tap water. Before testing, physical analyses (pH, temperature, water hardness, dissolved oxygen levels) were performed on the dilution water and the data was recorded. One 650 mL master stock concentrated solution of DUT (2 g/L) was prepared in DMF and stored in a Winchester bottle at 4°C. The master stock was initially used to prepare fresh dosing stocks at 1 mg/L, 3.2 mg/L, 10 mg/L, 32 mg/L and 100 mg/L (Table 4.1). Initial dosing stock solutions were prepared by diluting the master stock in dechlorinated tap water (Table 4.1) and were used until the 6th day of exposure. However, observations on exposure day 6 showed that DUT did not dissolve in water-based dosing stocks, and thus finalised dosing stocks were prepared solely in DMF and used from the 7th day of exposure onwards. The same master stock was used to prepare the new dosing stock solutions at 16.67 mg/L, 53.33 mg/L, 166.67 mg/L, 533.33 mg/L and 1666.7 mg/L DUT (Table 4.2). The volume of DMF used for all stock preparations was corrected according to its density (0.948 g/mL) to ensure higher precision.

Table 4.1: Concentrations of the initial DUT dosing stock test solutions, including the volumes used from the master stock and de-chlorinated tap water to achieve the nominal tank concentrations. These dosing stocks were used until the 6th day of exposure.

Dosing stock concentration	Master stock (mL)	100% DMF (mL)	De-chlorinated H_2O (mL)	Total volume of dosing stock (mL)	Percentage of solvent in dosing stock	Dilution Factor in tanks	Nominal tank concentration	Percentage of solvent in the tank
100 mg/L	50	10	940	1000	6%	1000x	100 µg/L	0.006%
32 mg/L	16	44	940	1000	6%	1000x	32 µg/L	0.006%
10 mg/L	5	55	940	1000	6%	1000x	10 µg/L	0.006%
3.2 mg/L	1.6	58.4	940	1000	6%	1000x	3.2 µg/L	0.006%
1 mg/L	0.5	59.5	940	1000	6%	1000x	1 µg/L	0.006%
SC	0	60	940	1000	6%	1000x	Solvent control	0.006%
DWC	0	0	1000	1000	0	1000x	DWC	-

Table 4.2: Concentrations of the finalised DUT dosing stock test solutions, including the volumes used from the master stock to achieve the nominal tank concentrations. These dosing stocks were used from the 7th day of exposure onwards.

Dosing stock concentration	Master stock (mL)	100% DMF (mL)	Total volume of dosing stock (mL)	Dilution Factor in tanks	Nominal tank concentration	Percentage of solvent in the tank
1666.70mg/L	50.0	10.0	60	16666.67x	100 µg/L	0.006%
533.33mg/L	16.0	44.0	60	16666.67x	32 µg/L	0.006%
166.67mg/L	5.0	55.0	60	16666.67x	10 µg/L	0.006%
53.33mg/L	1.6	58.4	60	16666.67x	3.2 µg/L	0.006%
16.67mg/L	0.5	59.5	60	16666.67x	1 µg/L	0.006%
SC	0	60	60	16666.67x	Solvent control	0.006%
DWC	0	0	60	16666.67x	DWC	-

The dosing stocks were prepared fresh in Winchester bottles three times a week (one day before stock renewal) to avoid degradation of the test solution. Immediately after preparation, the dosing stocks were placed in the fridge and maintained at 4°C. During renewal, Winchester bottles with old dosing stocks were removed from the peristaltic pump and were replaced with fresh ones. Seven glass 10 mL pipettes attached to a siphon pump were allocated to each treatment group (one siphon per treatment) and were used to remove snail faeces and food leftovers from the replicate tanks. When bacterial growth was observed on the side of the replicate tanks, it was scraped off with sterile nitrile gloves and removed with the respective siphon. Snails were fed *ad libitum* with Tetramin fish flakes, each renewal day and immediately after cleaning. The replicate tanks of DWC, SC and DUT treatments were monitored every 2-3 days for pH, temperature and dissolved oxygen levels.

2.1.4. Assessment of survival, reproductive output and growth

Throughout the acclimation and 21-day exposure periods, the snails were observed daily for mortality and abnormal behaviour, including avoidance of food, avoidance of water or cannibalism. Dead snails were removed from the test vessel and recorded, and mortalities were considered when calculating the reproductive output. Small pieces of silicone tubing were placed in the treatment tanks to provide a firm surface for adult snails to lay eggs. Before being placed in the tanks, silicone tubing was thoroughly disinfected in a diluted bleach solution and washed with glassware detergent. On collection day, silicone tubing was picked up (one tank at a time) using sterile extra-long nitrile gloves and placed in a clean petri dish. Four 6-well plates were used to collect the egg masses. These were labelled so that each row corresponds to one treatment (e.g. DWC), and each well corresponds to one tank replicate (Fig. 4.3). Egg masses were scraped off the silicone tubes using the back of a sterile scalpel and placed in their allocated well of the 6-well plate half-filled with tank water. The same procedure was repeated for each replicate tank individually. The 6-well plate was stored at 4°C to slow down the development of the encapsulated embryos. Egg masses were collected and counted three times a week. Due to high reproduction rates, the number of encapsulated embryos within each egg mass was counted once a week. Embryos within each egg mass were visualised and counted under a Motic SMZ-171-TLED stereomicroscope with Motic X camera using the Motic Image Plus 3.0 software. The shell length and total weight (including shell) of the snails were measured on the first day of the acclimation period and again at the end of the exposure period. At the end of the 21-day exposure period, the surviving snails from each tank were collected and total weight and shell length measurements were taken. Total weight measurements were taken using an analytical balance and shell length was measured using an electronic calliper, following the methods described in Chapter 3, Figure 3.6. Snails were sacrificed at the end of the exposure period and after the shell length and

total weight measurements were taken. For future histological analyses, soft body tissues from two snails in each tank were fixed in Bouin's solution for 24 hours and then transferred to 70% Ethanol. Soft body tissues from another two snails in each replicate were snap-frozen in liquid nitrogen for future chemical analyses.

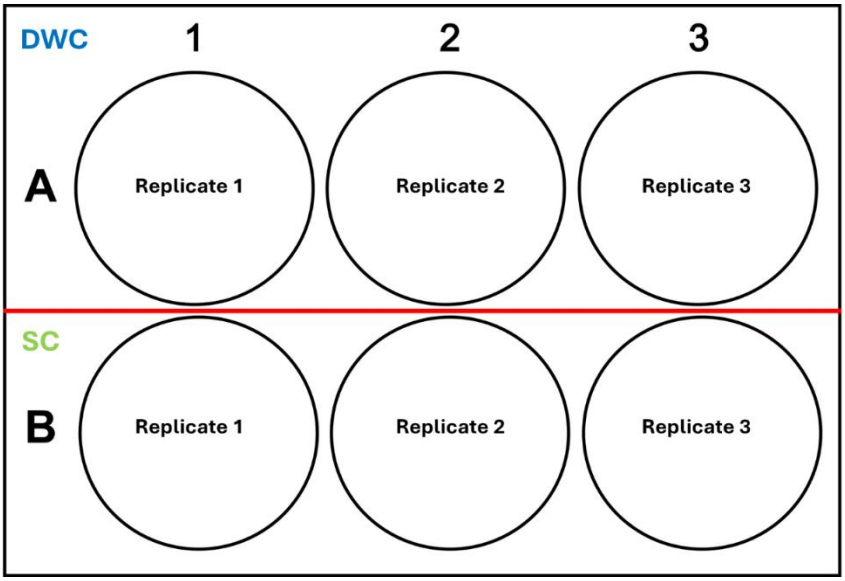


Figure 4.3: Schematic representation of the 6-well plate where egg masses were collected and stored. Each row corresponds to one treatment (e.g. DWC, SC) while each well corresponds to one tank replicate.

2.1.5. Chemical analysis of dutasteride concentrations in water

To measure the actual concentration of DUT in the tanks, water samples were taken from each replicate on day 0, day 1, day 7, day 8, day 12, day 15, day 17 and day 21, and stored in the freezer (-20°C) for future chemical analysis. Due to time constraints and errors encountered during the experimental period, chemistry data was only obtained from water samples of exposure day 1, day 12, day 15, day 17 and day 21. Water samples were analysed using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The test chemical was quantified against standards of DUT spiked with finasteride (FIN) as an internal standard (Table 4.3). FIN was not dosed into the tanks but was selected as the internal standard because it is cost-effective and has chemical and retention time similarities to DUT. An internal standard was used to reliably quantify the analytical recoveries of DUT and correct any potential recovery variations in the replicate tanks (Van De Merbel, Koster and Ohnmacht, 2019).

A dutasteride (DUT, CAS no. 68-12-2, ≥99.9% pure) stock solution (1000 mg/L) was first prepared in 10 mL methanol (MeOH) and subsequently diluted down to 0.1 mg/L, 1 mg/L and 10 mg/L in 10 mL MeOH. Similarly, a 10 mL stock solution containing 1000 mg/L finasteride (FIN, CAS no. 98319-26-7, ≥98% pure) was prepared in MeOH and then diluted to 1 mg/L made in 10mL MeOH. The diluted DUT stocks were first used to prepare calibration standards of 0.5 µg/L, 1 µg/L, 2.5 µg/L, 5 µg/L, 7 µg/L, 10 µg/L, 50 µg/L, 100 µg/L, 200 µg/L in volumetric flasks filled with 10 mL or 5 mL MeOH (Table 4.3). Due to the instrument's sensitivity in detecting DUT at lower concentrations, and thus its limited dynamic range, the calibration standards were separated into two sets: one of a lower calibration curve and one of a higher calibration curve. The lower calibration curve consisted of the standards 0 µg/L, 0.5 µg/L, 1 µg/L, 2.5 µg/L, 5 µg/L, 7 µg/L, and 10 µg/L. The higher calibration curve consisted of the standards 0 µg/L, 5 µg/L, 7 µg/L, 10 µg/L, 50 µg/L, 100 µg/L and 200 µg/L (Table 4.3). Each calibration standard contained 500 µL or 250 µL of the 1 mg/L FIN stock, bringing FIN concentration to 50 µg/L across all flasks.

For chemical analysis, calibration standards were diluted in 50:50 (v/v) dechlorinated tap water: MeOH. This was achieved by transferring 1 mL of each standard to a clean glass vial and mixing it with 1 mL of clean dechlorinated tap water (same matrix as in replicate tanks). This brought the internal standard concentration to 25 µg/L FIN. The resulting 2 mL solution was then filtered in a new glass vial using a syringe attached to a hydrophobic Polytetrafluoroethylene (PTFE)+GMF filter (0.45 µm, 30 mm diameter). 1mL of the resulting supernatant was then transferred to an HPLC vial, which was then used for analysis. A quality control (QC) standard and a blank (0 µg/L) were run after every 5 samples. Fresh sets of calibration standards were prepared and used for each analysis. The limits of detection (LOD) were calculated based on the standard deviation of the response and the slope of each calibration curve (obtained from the calibration standards), according to the ICH guidelines (Harron, 2013). In cases where R^2 values of calibration curves were below 0.98, this was explicitly stated, and the LODs were not considered. Instead, the raw data was reported for exploratory insights. The limits of quantification (LOQ) were not determined due to time constraints.

Replicate tank samples were prepared by first spiking 0.95 mL of water sample with 50 µL of the internal standard FIN (1 mg/L FIN stock) and diluting them with 1 mL of 100% HPLC-grade MeOH. This achieved the same internal standard concentrations as the calibration standards (25 µg/L FIN). The resulting solution was then filtered similarly to the calibration standards, and 1 mL of the resulting supernatant was transferred to an HPLC vial for LC-MS/MS analysis (Method 2, Table 4.4). Following dissolution challenges, initial efforts to quantify DUT concentrations in replicate tanks (using samples from exposure day 1) could not be achieved.

Thus, a series of tests were carried out to evaluate whether filtering in the preparation of water samples was an issue. Five different approaches and two types of syringe filters were used, summarised in Table 4.4.

Table 4.3: Volumes of methanol (MeOH), FIN (1 mg/L) and DUT (0.1 mg/L, 1 mg/L and 10 mg/L) stocks used for preparing calibration standards before being diluted with dechlorinated tap water (50:50 v/v, dechlorinated tap water: MeOH). Calibration standards were separated into two sets, comprising a lower and higher calibration curve.

Calibration standard	Volume of MeOH (mL)	Volume of 1 mg/L FIN stock (µL)	Volume of 0.1 mg/L DUT stock (µL)	Volume of 1 mg/L DUT stock (µL)	Volume of 10 mg/L DUT stock (µL)	Calibration curve
0 µg/L	10	500	-	-	-	Lower
0.5 µg/L	10	500	50	-	-	Lower
1 µg/L	10	500	100	-	-	Lower
2.5 µg/L	5	250	125	-	-	Lower
5 µg/L	5	250	250	-	-	Lower
7 µg/L	5	250	350	-	-	Lower
10 µg/L	5	250	-	50	-	Lower
QC blank	10	500	-	-	-	Lower
QC 10 µg/L	5	250	-	50	-	Lower
0 µg/L	10	500	-	-	-	Higher
5 µg/L	5	250	250	-	-	Higher
7 µg/L	5	250	350	-	-	Higher
10 µg/L	5	250	-	50	-	Higher
50 µg/L	5	250	-	250	-	Higher
100 µg/L	5	250	-	-	50	Higher
200 µg/L	5	250	-	-	100	Higher
QC blank	10	500	-	-	-	Higher
QC 200 µg/L	5	250	-	-	100	Higher

Table 4.4: Methods and filters used for optimising sample preparation for LC-MS/MS.

Method name	Type of filter used	Method description
Method 1	PTFE+GMF filter: 0.45µm, 30mm diameter	0.95mL tank water was first spiked with 50µl internal standard and then filtered. 0.5mL filtered sample was then mixed with 0.5mL MeOH and used for analysis.
Method 2	PTFE+GMF filter: 0.45µm, 30mm diameter	0.95mL tank water first spiked with 50µL internal standard. This was mixed with 1mL MeOH and then filtered. 1mL supernatant was used for analysis.

Method 3	PTFE+GMF filter: 0.45µm, 30mm diameter	0.95mL tank water was filtered and spiked with 50µL internal standard. 1mL MeOH was filtered through the same syringe and filter and mixed with the water sample. 1mL supernatant was used for analysis.
Method 4	GF filter: 0.7 µm, 28m diameter	0.95mL tank water was first spiked with 50µL internal standard and then filtered. 0.5mL of the filtered sample was then mixed with 0.5mL MeOH and was used for analysis.
Method 5	GF filter: 0.7 µm, 28m diameter	0.95mL tank water was filtered (GF filter: 0.7 µm, 28m diameter) and spiked with 50µL internal standard. 1mL MeOH was filtered through the same syringe and filter. Then mixed. 1mL supernatant was used.

The LC-MS/MS instrumentation was comprised of an HPLC Agilent 1260 Infinity series (Agilent Technologies, Waldbronn, Germany), equipped with a G7129A quaternary pump and a G7129A sampler coupled to a Sciex API 5000 triple quadrupole instrument (AB SCIEX Instruments). The main working parameters of the mass spectrometer are summarised in Table 4.5. Liquid chromatography separation was achieved using a ZOBRAX Eclipse XDB-C18 column (4.6mm x 150 mm, 5 µm). The mobile phase was prepared by making a 10 mM ammonium formate buffer in HPLC-grade water and then mixing it with MeOH at a 15/85 (v/v) ratio. The pH was adjusted to 3.0 using formic acid. The mobile phase was pumped at a flow-rate of 0.7 mL/min using an isocratic profile. Data processing was performed on the Analyst 1.7.1 software package (SCIEX).

Table 4.5: Working parameters of the mass spectrometer used in the 21-day reproduction study.

Instrument	**Sciex Triple Quad API 5000**
Scan type	Multiple Reaction Monitoring (MRM)
Source type	Turbo Spray
Polarity	Positive ion
CUR	25 psi
CAD	6.0 psi
Ion source gas 1	10.0 psi
Ion source gas 2	0.0 psi
Dwell time (ms)	150
Spray Voltage	5500 (V)
Source temperature	550 (°C)

2.1.6. Statistical analyses

Statistical analyses and graph generation were performed using the GraphPad PRISM software (Version 10.2.2). Data for reproductive output (number of egg masses, number of

embryos), total weight and shell length were tested for normality using the Shapiro-Wilk test, and for homogeneity of variances using Bartlett's test. When assumptions of normality were met, a one-way analysis of variance (ANOVA) was conducted, followed by Dunnett's multiple comparisons (post hoc) test. When assumptions of normality or homogeneity of variances were not met, the non-parametric Kruskal-Wallis test was used, followed by Dunn's post-hoc test.

For each replicate tank, the reproductive output was calculated by dividing the number of egg masses or embryos for each day, by the number of surviving snails in the tank. This estimated the average number of egg masses or embryos per individual snail (in each replicate tank). Statistical differences were then determined based on the cumulative number of embryos or egg masses on the last day of exposure (day 21). This method of evaluating reproductive output was preferred to the approach recommended in the OECD TG 243. This is because: (1) the biological relevance of DUT in *B. glabrata* was unknown; (2) the tested concentrations did not reflect environmentally relevant DUT concentrations of 0.027 µg/L (Gómez-Canela *et al.*, 2021), but instead previously tested concentrations in fish (Margiotta-Casaluci, Hannah and Sumpter, 2013) and (3) a range-finding test was not conducted to enable the calculation ECx, NOEC or LOEC.

The means of snail shell diameter (mm) and total wet weight (g) were calculated for each treatment, based on the values of respective replicates, at the beginning of the exposure (day 0) and the end of the exposure (day 21). The mean values were used to visualise the data, whereas individual values from each replicate were used for statistical analysis. A statistical analysis was conducted to identify differences in shell length (mm) and total weight (g) between treatments before the exposure (day 0), and a separate analysis was conducted to identify differences between treatments after the exposure (day 21).

Survival data was analysed using the non-parametric Kaplan-Meier survival test, and the overall significance was assessed using the Log-rank (Mantel-Cox) test. Since significant differences between treatments were not observed, pairwise comparisons were not conducted. Statistical differences were identified between the SC group and the rest of the treatment groups, with the significance set at $p<0.05$ for all analyses.

2.2. Experiment 2 – Static-renewal system

This experiment was performed using a static-renewal system by adapting the OECD Test Guidelines 243 (OECD, 2016a). The static-renewal system was selected to provide a direct comparison to the flow-through system (used in Experiment 1), aiming to assess how each testing method may have influenced DUT toxicity in *B. glabrata* adults. To ensure consistency in the test comparison, the static-renewal study implemented the same DUT concentrations

as the flow-through study. The exposure period lasted for 28 days, according to the recommendations provided in the OECD TG 243.

2.2.1. Test species

Biomphalaria glabrata individuals were supplied from breeding stocks maintained at Brunel University London (BB02 strain; originally obtained from The Natural History Museum, London). *B. glabrata* stock cultures were maintained at the ecotoxicology lab Scymaris (Brixham, UK) in static glass aquaria and were supplied with ISO 6341 water medium (OECD, 2016a). The glass aquaria with breeding stocks alongside the ISO 6341 medium were kept in a temperature-controlled room at 27°C. The glass aquaria were cleaned daily and 30% of the tank water was replaced with fresh ISO 6341 medium. Once a week, 60% of the tank water medium was removed and replaced with fresh ISO 6341. *B. glabrata* breeding stocks were fed *ad libitum* daily using organic lettuce leaves. Adult snails aged four months old were selected for this experiment. On the first day of the acclimation period, the total weight (g, three decimal places) and shell length (mm, two decimal places) of snails were determined using an analytical balance and an electronic calliper, respectively. To increase the concentration of calcium carbonate (CaCO3) in the ISO 6341 medium, 0.2g sodium bicarbonate was added to the test solutions, and subsequently to the test vessels, at each test solution renewal (section 2.2.3). The concentrations of CaCO3 were monitored in two ways: first, through an alkalinity test, and second, by determining carbonate hardness (KH) using aquarium test strips (API 5-IN-1). Increased CaCO3 in the ISO 6341 medium was necessary to support shell growth upon initial observations of brittle shells and mortalities in the absence of sodium bicarbonate (Fig. 4.4).

Figure 4.4: *Biomphalaria glabrata* shell condition after two weeks in ISO 6341 medium with no sodium bicarbonate.

2.2.2. Test conditions

Five test concentrations (1 µg/L, 3.2 µg/L, 10 µg/L, 32 µg/L, 100 µg/L), a solvent control (SC) and a dilution water control (DWC) with five replicates of five snails (total 25 snails per treatment) were used. The test substance and solvent were obtained from Fisher Scientific as described in section 2.1.2. The test vessels were 1L tall glass beakers containing 1L of test solution (Fig. 4.5). The tops of the test vessels were covered with a thin net to prevent animals from escaping. The test solutions were gently aerated using glass pipettes, maintained in a temperature-controlled room at 27°C ± 1°C, and a photoperiod of 16 hours light: 8 hours dark, with a 20-minute dawn: dusk transition period. Four days before the beginning of the exposure, test vessels were filled with 1 L of ISO 6341 medium at test temperature. Adult *B. glabrata* were collected from the stock cultures, where five healthy snails with a shell length between 16.35 mm – 19.72 mm were impartially selected and placed into each test vessel containing ISO 6341 medium. The mean shell length of snails in each treatment ranged between 17.62 mm – 17.74 mm. Test vessels contained snails of similar shell length, so the mean shell length between test vessels did not differ considerably. During the 4-day acclimation period, the reproduction of snails under the test conditions was monitored and recorded. On day 0, a randomized block design was determined using www.Random.org and used to allocate replicates to the various test concentrations.

Figure 4.5: Static-renewal system used during the 28-day reproduction study. Pictured are the test vessels of SC (green) and 10µg/L (red) treatments.

2.2.3. Test substances, dilution water and preparation of test solutions

The dilution water used for testing (and maintenance of stock cultures) was ISO 6341 medium prepared according to the OECD TG 243 (OECD, 2016a). Analyses of pH, conductivity, water hardness, alkalinity, oxygen concentration and temperature were performed on the dilution water used for testing and the data was recorded (data records on ISO 6341 medium kept at the ecotoxicology lab Scymaris). The ISO 6341 medium was aerated for a minimum of two hours before use. The study was run with a dilution water control (DWC) and a solvent control (SC) together with nominal concentrations of DUT. Concentrated stock solutions were prepared in solvent (DMF) using glass vials and stirring. One 20mL master stock concentrated solution of DUT (5 g/L) was prepared in DMF and was stored at 4 °C. Four subsequent 10mL stock concentrated solutions (1.6 g/L, 0.5 g/L, 0.16 g/L, 0.05 g/L) were prepared by diluting the master stock concentrated solution in DMF. A sixth 10mL stock solution containing 100% DMF was also prepared (Table 4.6). The volume of DMF used for all stock preparations was corrected according to its density (0.948 g/mL). To achieve nominal DUT test vessel concentrations (SC, 1 µg/L, 3.2 µg/L, 10 µg/L, 32 µg/L and 100 µg/L), 20 µL from the respective concentrated stock solutions were mixed with 0.2 g sodium bicarbonate in 1 L volumetric flask filled with ISO 6341 medium (Table 4.7).

Table 4.6: Concentrations of the DUT stock test solutions, including the master stock (Stock 1) and DMF volumes used to achieve the subsequent concentrated stock solutions.

Preparation of stock solutions	Concentration (g/L)	DUT (g)	Stock solution used	Volume of stock solution used (mL)	Volume of STOCK used (µL)	Volume of DMF added (mL)	Weight of DMF (g)	Final volume (mL)
STOCK 1	5	0.1	-	-	-	20	18.78	20
STOCK 2	1.6	-	STOCK 1	3.2mL	3200	6.8	6.494	10
STOCK 3	0.5	-	STOCK 1	1mL	1000	9	8.496	10
STOCK 4	0.16	-	STOCK 1	0.32mL	320	9.68	9.13792	10
STOCK 5	0.05	-	STOCK 1	0.1mL	100	9.9	9.3456	10
STOCK 6	0	-	-	-	-	10	9.48	10

The test solutions were prepared fresh and renewed three times a week. Renewal days remained consistent throughout the exposure period and there was an equal time between each renewal. During renewal, the snails were first removed and placed in clean test vessels with freshly made test solutions. Snails in test vessels were fed after each renewal with equal

amounts of organic lettuce leaves (2.5 g per test vessel), according to the experimental protocol used at the ecotoxicology lab Scymaris. Old test vessels and food leftovers were checked, and egg masses attached on either the sides of the vessels or on lettuce leaves were carefully removed and collected. Egg masses were carefully removed from the leftover lettuce leaves or the glass walls of the test vessels using a metal spoon. The old test vessels were then thoroughly rinsed with de-chlorinated tap water, wiped off with a blue paper roll and left to dry before the next renewal. The concentration of DMF in the test vessel solutions remained at 0.002% throughout the experiment per the OECD test guidelines 243 (OECD, 2016a). The pH and dissolved oxygen levels for test vessel treatments were monitored and recorded three times a week, while temperature was monitored and recorded daily.

Table 4.7: The DUT nominal concentrations in the test vessels, including the volumes used from concentrated stock solutions (Stock 1-6) and ISO6341 medium, along with the dilution factor and the percentage of solvent present in the test vessels.

Nominal test vessel concentrations (µg/L)	STOCK to be used	Volume of STOCK (µL)	Total volume of DMF solvent (mL)	Volume of ISO6341 in test vessel (mL)	Sodium bicarbonate (g)	Dilution factor in vessels	Percentage of solvent in test vessel (%)
100	STOCK 1	20	0.02	1000	0.2	50000x	0.002%
32	STOCK 2	20	0.02	1000	0.2	50000x	0.002%
10	STOCK 3	20	0.02	1000	0.2	50000x	0.002%
3.2	STOCK 4	20	0.02	1000	0.2	50000x	0.002%
1	STOCK 5	20	0.02	1000	0.2	50000x	0.002%
SC	STOCK 6	20	0.02	1000	0.2	50000x	0.002%
DWC	-	0	0.00	1000	0.2	-	-

2.2.4. Assessment of survival, reproductive output and growth

Throughout the acclimation and 28-day exposure periods, the snails were observed daily for mortality and abnormal behaviour, including avoidance of food, avoidance of water or cannibalism. Dead snails were removed from the test vessel and recorded, where mortalities were considered in calculating the reproductive output. The number of egg masses laid, and the number of embryos within each egg mass were recorded on every renewal day (three times a week) for each replicate. The number of egg masses and embryos were counted under a Motic SMZ-171-TLED stereomicroscope and a tally counter. Embryos that were not fertilised or that experienced atrophied albumen were recorded separately following the OECD TG 243 (OECD, 2016a) to facilitate a comparative analysis between normal and abnormal

embryo production. The shell length (mm, two decimal places) and total weight (g, three decimal places) of snails were measured and recorded on the first day of the acclimation period as well as at the end of the exposure period, using electronic callipers and an analytical balance respectively. At the end of the exposure period and after the shell length and total weight measurements were taken, snails were sacrificed. The wet weight of soft body mass tissue was determined using an analytical balance. One soft body mass tissue per replicate was fixed in liquid nitrogen and another one in 10% Formaldehyde for future chemical and histopathological and analysis, respectively.

2.2.5. Chemical analysis of dutasteride concentrations in water

For this experiment, chemical analysis was conducted by the analytical chemistry team of the ecotoxicology lab Scymaris. To test the concentration of DUT in the test vessels, water samples were taken from one replicate from each treatment on day 0, day 7, day 19, day 21 and day 28, following Scymaris standard practices. Water samples were collected and tested from both freshly made test solutions (ON samples) and old test solutions (OFF samples) on renewal days. The water samples were analysed by LC-MS/MS and were quantified against standards of DUT spiked with FIN as an internal standard. Finasteride (FIN, CAS no. 98319-26-7, ≥98% pure) and DUT (CAS no. 68-12-2, ≥99.9% pure) stock solutions were prepared in MeOH. Calibration standards of DUT were prepared in 50:50 (v/v) ISO 6341 media: MeOH in 20 mL volumetric flasks and covered the range of 0.10 – 100 µg/L (0.1, 0.25, 0.50, 1.0, 2.5, 5.0, 10, 25, 50 and 100 µg/L). 2 mL of 100 µg/L internal standard (FIN) was added to the volumetric flask before being made up to the 20 mL, giving a final concentration of 10 µg/L internal standard. One set of calibration standards was created, as the instrument allowed a greater order of magnitude, and was used for all analyses. Fortified samples at nominal DUT concentrations of 2.5 µg/L and 50 µg/L were prepared to verify the accuracy, precision and recovery of the analytical method. The water samples from fresh test solutions (ON samples) were taken in aliquots of 10 mL and were later mixed with 10 mL of solvent (MeOH) pre-spiked with 2 mL of 100 µg/L internal standard. The concentration of the internal standard in the MeOH was 20 µg/L, giving a final concentration in the diluted sample of 10 µg/L internal standard (to match the standards). To prepare samples from the old test solutions (OFF samples), 30 mL of sample was transferred to a glass centrifuge tube, which was then centrifuged at 1000g for 10 minutes at 20°C. Centrifugation was performed instead of filtering, to separate fecal matter or leftover food from the sample. After centrifugation, a 10 mL aliquot was transferred to a disposable glass vial. The sample was further diluted with 10 mL of MeOH pre-spiked 20 µg/L internal standard, resulting in a final nominal concentration of 10 µg/L internal standard. The LC-MS/MS instrumentation was comprised of a Sciex Exion LC System. Liquid chromatography separation was achieved using a Poroshell EC C18 column (150 x 2.1

mm x 2.7µm). The mobile phase was prepared by making a 10 mM ammonium formate buffer in HPLC-grade water, and then mixing it with acetonitrile at a 15/85 (v/v) ratio. The pH was adjusted to 3.0 using formic acid. The mobile phase was pumped at a flow rate of 400 µL/min. Mass spectrometric detection was performed on a Sciex Triple Quad 7500. The main working parameters of the mass spectrometer are summarised in Table 4.8.

Table 4.8: Working parameters of the mass spectrometer used in the 28-day reproduction study.

Instrument	**Sciex Triple Quad 7500**
Scan type	Multiple Reaction Monitoring (MRM)
Source	E-ANLYT 200+ µL
Polarity	Positive ion
CUR	40 psi
CAD	9.0 psi
Ion source gas 1	60 psi
Ion source gas 2	70 psi
Dwell time (ms)	100
Spray Voltage	5000 (V)
Source temperature	350 (°C)

2.2.6. Statistical analyses

Statistical analyses were performed according to the methods described in section 2.1.6. For determining reproductive output, normal and abnormal numbers of embryos were analysed separately and in combination. To determine the percentage of abnormal embryos to the total number of embryos measured, the number of abnormal embryos (per surviving individual) was calculated for each sampling day. Then, the sum of abnormal embryos (per surviving individual) was determined for the entire experimental period. This value was then divided by the total number of embryos (normal and abnormal) measured throughout the experimental period and multiplied by 100.

3. Results:

3.1. Experiment 1 – Flow-through system

Throughout the experimental period (acclimation and exposure period) the mean pH ranged from 7.6 to 7.7, the mean temperature from 25.7 °C to 29.9 °C and the mean dissolved oxygen from 89.1% - 94.8% across all treatments (Table 4.9).

Table 4.9: Water conditions including oxygen (%), temperature (°C) and pH of DUT treatments across the acclimation period (day -4 to -1) and exposure period (day 2 – day 20). Water conditions were tested every three days starting from the second day of the acclimation period (day -2).

	DWC			**SC**			**1µg/L**			**3.2µg/L**			**10µg/L**			**32µg/L**			**100µg/L**		
Day	Oxy.	Temp.	pH	Oxy.	Temp.	pH	Oxy.	Temp.	pH	Oxy.	Temp.	pH	Oxy.	Temp.	pH	Oxy.	Temp.	pH	Oxy.	Temp.	pH
-4	90.7	26.5	7.5	90.3	26.4	7.5	90.7	26.7	7.5	90.7	27.2	7.5	90.7	26.5	7.5	90.0	26.2	7.5	90.7	26.2	7.5
-1	92.3	26.6	7.5	92.0	25.8	7.5	92.7	26.1	7.5	92.3	26.7	7.5	92.7	26.8	7.5	91.0	26.0	7.5	92.7	25.2	7.5
2	93.3	26.9	7.5	92.8	26.0	7.5	93.7	26.7	7.5	93.0	25.4	7.5	94.1	26.1	7.5	92.3	25.5	7.5	93.7	25.2	7.5
5	94.6	26.9	7.5	92.7	26.2	7.5	90.8	26.6	7.5	92.9	25.1	7.5	92.0	25.6	7.5	91.9	25.3	7.5	92.2	24.7	7.5
8	96.5	27.0	7.5	96.2	26.3	7.5	94.3	26.7	7.5	96.7	26.1	7.5	95.3	26.7	7.5	95.3	26.1	7.5	95.3	26.2	7.5
11	97.2	27.0	7.5	84.2	26.6	7.5	80.1	27.4	7.5	82.1	26.3	7.5	80.2	26.8	7.5	80.5	26.1	7.5	76.6	25.6	7.5
14	97.8	27.1	7.8	84.0	26.8	8.0	81.5	26.3	8.0	83.2	26.6	8.0	81.7	26.8	8.0	82.0	26.3	7.8	77.4	26.1	8.0
17	98.6	27.2	8.0	97.6	27.0	7.8	96.9	26.7	8.0	96.4	26.9	8.0	97.5	27.1	8.0	97.1	26.9	8.0	97.5	26.6	7.7
20	99.0	27.3	8.0	97.3	26.5	8.0	97.7	26.5	8.0	97.3	26.8	8.0	97.3	27.0	8.0	97.0	26.7	8.0	97.0	26.3	8.0
Mean	94.8	26.9	7.6	91.5	26.3	7.6	90.1	26.7	7.7	90.6	26.4	7.7	90.6	26.6	7.7	90.5	26.1	7.6	89.1	25.7	7.6

3.1.1. Chemical analysis of dutasteride concentrations in treatment groups

Following dissolution challenges, LC-MS/MS analyses of water samples from exposure day 1 did not detect DUT in the tank replicates (Appendix S4, Table S4.1 and Fig. S4.1). Although the dosing errors (first observed on exposure day 6) have likely contributed to these limitations, further experimental tests were carried out to understand the influence of the sample preparation method on DUT concentrations. These experiments were conducted using filtered and unfiltered water samples taken from the 32 µg/L treatment at exposure day 12. LC-MS/MS analysis of the unfiltered mixing chamber sample detected 26.2 µg/L of DUT (Table 4.10). The response linearity (R^2 value) of the standards used in this analysis was 0.998 (Appendix S4, Fig. S4.2). In contrast, mixing chamber and tank samples that had undergone filtration, demonstrated no presence of DUT (Table 4.10). Despite these limitations, unfiltered water samples were not used for subsequent LC-MS/MS analyses. This was due to concerns that food remnants and snail faeces from tank water would have caused a column blockage in the LC-MS/MS system. Instead, method alterations were trialled to determine an appropriate filter and sample preparation method (described in section 2.1.5 and Table 4.4). Method 2 (Table 4.4) was found the most appropriate method as it detected the highest DUT concentration at 11.8 µg/L (Table 4.11). For this analysis, the response linearity (R^2 value) of the calibration curve was 0.996 (Appendix S4, Fig. S4.3) which confirmed the precision and accuracy of the calibration method.

Table 4.10: Tank (replicate A) and mixing chamber DUT concentrations from the 32 µg/L treatment using filtered and unfiltered samples, detected by LC-MS/MS. Filtered water samples were prepared using a PTFE+GMF filter and Method 1. Samples were collected on exposure day 12. The LOD for this analysis was 3.46 µg/L.

Nominal concentration of DUT (µg/L)	Replicate A (Method 1)	Mixing chamber (Method 1)	Mixing chamber (unfiltered sample)
	µg/L	µg/L	µg/L
32ug/L	<LOD	<LOD	26.2

Table 4.11: Concentrations of the 32 µg/L mixing chamber sample detected by LC-MS/MS, prepared using different filtering methods. The filters tested were PTFE+GMF (0.45µm, 30mm diameter) and GF (0.7 µm, 28m diameter). Samples were collected on exposure day 15. The LOD for this analysis was 8.11 µg/L.

Nominal concentration of DUT (µg/L)	Method 1	Method 2	Method 3	Method 4	Method 5
	µg/L	µg/L	µg/L	µg/L	µg/L
32 µg/L (mixing chamber sample)	<LOD	11.8	8.8	<LOD	10.4

Subsequent LC-MS/MS analyses were conducted using Method 2 during sample preparation (Table 4.4). To confirm the presence of DUT in the tank replicates during exposure day 1, further chemical analyses were conducted using "replicate A" water samples that were preserved at -20ºC. In those samples, DUT was detected in the "replicate A" of the 10 µg/L and 32 µg/L treatments at concentrations of 0.696 µg/L and 0.626 µg/L, respectively but was not detected in the rest of the treatments (Table 4.12). For this analysis, the lower and higher calibration curves of DUT standards exhibited a response linearity (R^2 value) of 0.996 and 0.972, respectively (Appendix S4, Fig. S4.4 and S4.5). The testing of water samples from exposure day 21 demonstrated the absence of DUT from all replicate tanks and mixing chambers of DWC, SC, 1 µg/L and 3.2 µg/L treatments (Table 4.13). However, DUT was detected in the replicates and mixing chambers of higher concentrations (10 µg/L, 32 µg/L, 100 µg/L). Specifically, DUT concentrations demonstrated an average of 4.87 µg/L in the 10 µg/L treatment, 7.59µg/L in the 32µg/L treatment, and 37.03µg/L in the 100µg/L treatment (Table 4.13). In the mixing chambers, DUT concentrations were observed at 4.13 µg/L, 8.19 µg/L and 79.2 µg/L in the 10 µg/L, 32 µg/L and 100 µg/L treatments, respectively (Table 4.12). The lower and higher calibration curves for this analysis exhibited response linearities (R^2 values) of 0.501 and 0.931, respectively (Appendix S4, Fig. S4.6 and S4.7). It is believed that the lower r^2 values (below 0.98) were a result of improper mixing of calibration standards in their original flasks before their dilution and transfer into HPLC vials.

Table 4.12: Tank replicate A concentrations of DUT, across different treatments, for the samples collected on the first day of the exposure period (day 1) detected by LC-MS/MS. "ND" refers to not detected. The LOD for the lower calibration was 0.73, whereas for the higher calibration, only the raw data was reported due to a low R^2 value. Values marked with an asterisk (*) were reported for exploratory insights. "ND" refers to not detected.

Nominal concentration of DUT (µg/L)	Replicate A
	µg/L
DWC	<LOD
SC	<LOD
1 µg/L	<LOD
3.2 µg/L	<LOD
10 µg/L	0.696 *
32 µg/L	0.626 *
100 µg/L	ND

Table 4.13: Tank replicates and mixing chamber concentrations of DUT, across different treatments, for the samples collected on the final day of the exposure period (day 21) detected by LC-MS/MS. The LODs for the lower and higher calibrations were not reported due to low R^2 values. Instead, the raw data was demonstrated. Values marked with an asterisk (*) were reported for exploratory insights. "ND" refers to not detected.

Nominal concentration of DUT (µg/L)	Replicate A	Replicate B	Replicate C	Arithmetic mean of all replicates	Mixing chamber
	µg/L	µg/L	µg/L	µg/L	µg/L
DWC	ND	ND	ND	N/A	ND
SC	ND	ND	ND	N/A	ND
1 µg/L	ND	ND	ND	ND	ND
3.2 µg/L	ND	ND	ND	ND	ND
10 µg/L	5.16 *	4.16 *	5.28 *	4.87 *	4.13 *

32 µg/L	7.18 *	8.7 *	6.88 *	7.59 *	8.91 *
100 µg/L	45.5 *	29.1 *	36.5 *	37.03 *	79.2 *

3.1.2. Survival, reproductive output and growth

The objective of the first study was to assess the effects of prolonged DUT exposure on the survival, growth and reproduction of *B. glabrata* adults, using a flow-through system over a 21-day exposure period. One mortality was observed at exposure day 19 in the 10 µg/L treatment, where survival rates decreased to 94.4%. For all other treatments survival rates remained at 100% throughout the experimental period. No significant differences were observed in any DUT treatments compared to the SC (Fig. 4.6).

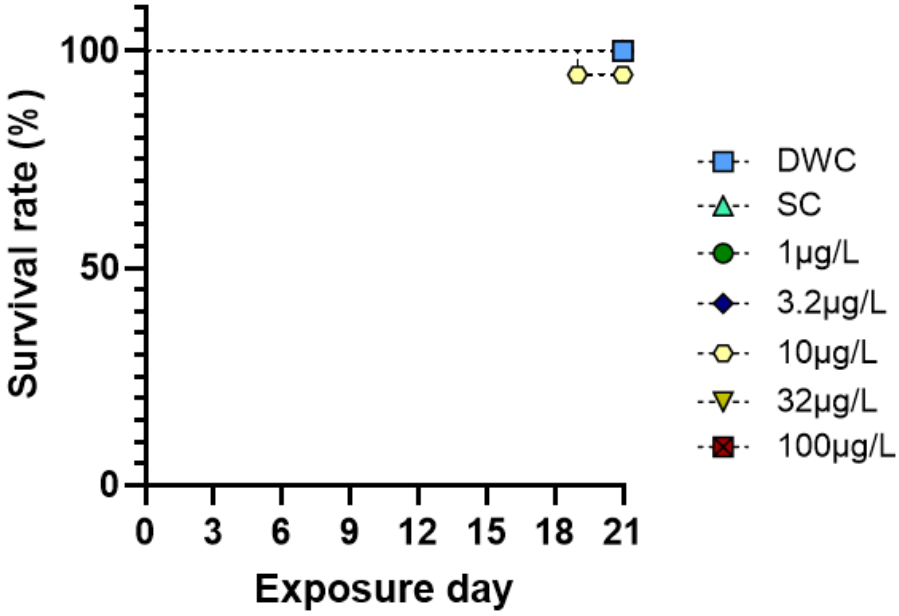


Figure 4.6: Effects of DUT on the survival of *Biomphalaria glabrata* adults over a 21-day exposure period represented as daily survival rates. Data represents the number of survival adults out of the total number of individuals per treatment (n=3 replicates, 6 snails per replicate) converted to percentages (%).

The cumulative embryo and egg mass production for each *B. glabrata* snail at exposure day 21 did not vary significantly between treatment groups compared to the SC (Fig. 4.7). Cumulative embryo production was lower in the DWC but higher in the 10 µg/L, 32 µg/L and

100 µg/L treatments compared to the SC (Fig. 4.7a), throughout the exposure period. Moreover, cumulative egg mass production was found stable throughout the exposure, but slightly higher in the 1 µg/L treatment compared to the SC at exposure day 21 (Fig.4.7b). No significant differences were found in the mean shell length (mm) of snails across treatment groups, before or after the 21-day exposure period (Fig. 4.8a). Before the exposure, the lowest mean shell length (± SD) was 13.49 ± 0.02 mm in the DWC treatment, whereas the highest mean shell length was 13.98 ± 0.23 mm in the 3.2 µg/L treatment. After the exposure, the lowest mean shell length was 17.27 ± 0.51mm in the DWC treatment, whereas the highest was 17.96 ± 0.70 mm in the 32 µg/L treatment (Fig. 4.8a). Significant differences were found in the total weight (g) of snails in the DWC (p=0.03) and 100 µg/L (p=0.02) treatments compared to the SC, before the exposure, but no significant differences were observed between treatments after the exposure (Fig. 4.8b). The mean total weight (± SD) was lowest in the DWC treatment (0.43 ± 0.01 g) and highest in the 100 µg/L treatment (0.53 ± 0.01 g) before the exposure. Whereas, after the exposure the mean total weight (± SD) was lowest in DWC (0.79 ± 0.06 g) and highest in 32 µg/L (0.89 ± 0.07 g) (Fig.4.8ab).

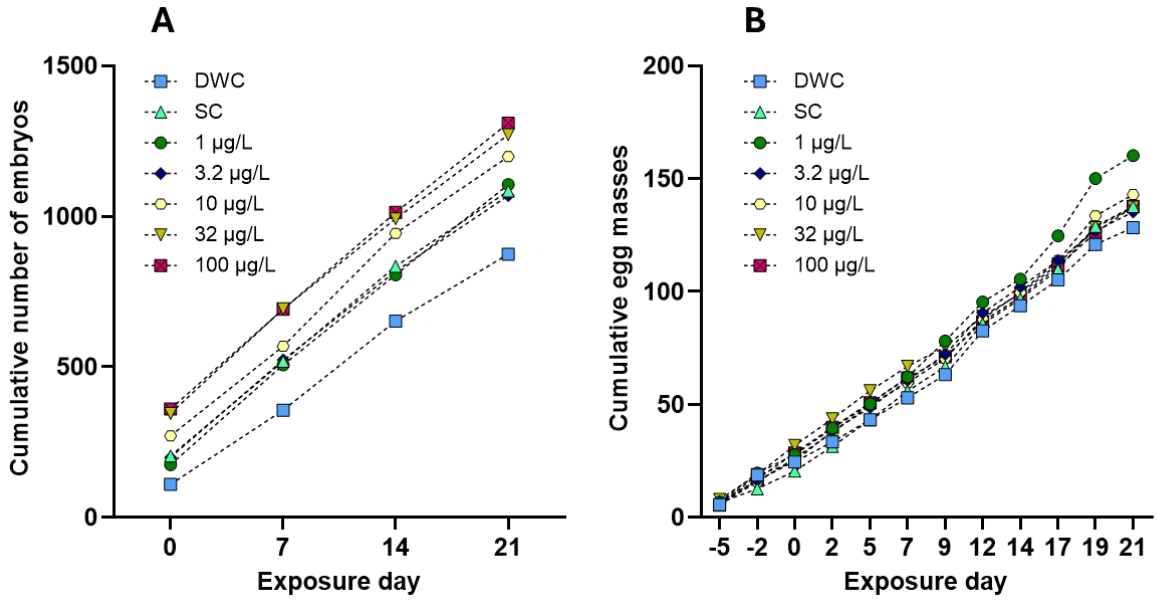


Figure 4.7: Effects of DUT on: (a) the cumulative total embryo production and (b) cumulative total egg mass production, per individual *Biomphalaria glabrata* snail across treatment groups. Data from each treatment represents the sum of the average (a) cumulative embryo or (b) egg mass numbers from three technical replicates (n= 3 replicates, 6 snails per replicate). Concentration-dependent effects on embryo production were demonstrated over the 21-day chemical exposure period.

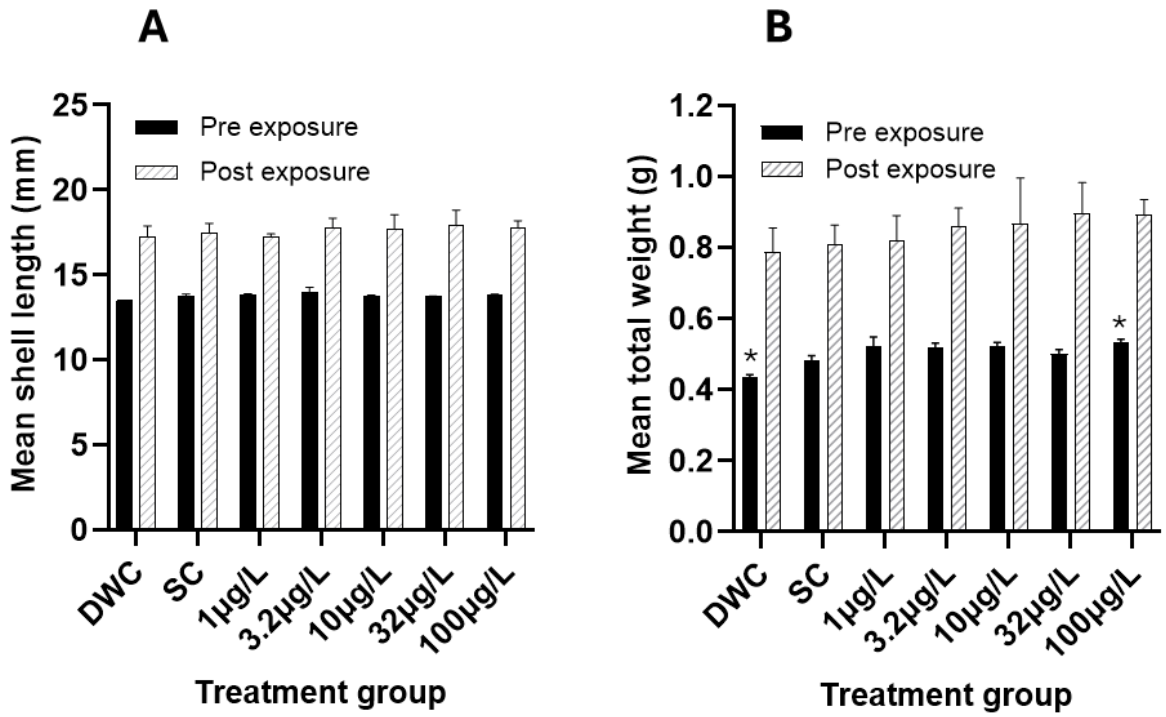


Figure 4.8: Arithmetic mean values (±SD) of (a) shell length (mm) and (b) total weight (g) of *Biomphalaria glabrata* adults pre-exposure (first day of acclimation period) and post-exposure (last day of the 21-day exposure period) to DUT treatments. Data represents the arithmetic mean values taken from the surviving snails across three technical replicates ± SD (n= 3 replicates, 6 snails per replicate). Asterisks indicate statistically significant differences with the SC where *: $p < 0.05$.

3.2. Experiment 2 – Static-renewal system

Throughout the 28-day exposure period, the mean pH and oxygen levels (%) of ON samples across all treatments ranged from 8.1 to 8.2, and from 92.6% to 96.1%, respectively (Table 4.14). For the same period, the mean pH and oxygen levels (%) of OFF samples across treatments ranged from 7.9 to 8.0, and from 79.5% to 85.6%, respectively (Appendix S4, Tables S4.2 and S3). Moreover, throughout the 28-day exposure period, and across different treatments, temperature ranged from 26°C to 27.1°C (Table 4.15).

Table 4.14: Oxygen (%) and pH levels of ON samples during the exposure period for each treatment.

	Day 0		**Day 2**		**Day 5**		**Day 7**		**Dau 9**		**Day 11**		**Day 14**		**Day 16**		**Day 18**		**Day 21**		**Day 23**		**Day 25**		**Mean**	
	Oxy.	pH	Oxy.	pH	Oxy.	pH	Oxy.	pH	Oxy.	pH	Oxy.	pH	Oxy.	pH	Oxy.	pH	Oxy.	pH	Oxy.	pH	Oxy.	pH	Oxy.	pH	Oxy	pH
DWC	86.0	8.0	117.0	8.1	97.2	8.1	98.2	8.1	117.0	8.1	73.5	8.3	102.5	8.0	85.3	8.2	101.2	8.1	78.0	8.1	65.3	8.2	90.0	8.2	92.6	8.1
SC	99.5	8.4	115.0	8.1	104.0	8.2	100.3	8.2	102.0	8.1	83.0	8.2	104.0	8.1	87.8	8.2	106.0	8.2	80.7	8.2	72.1	8.1	80.5	8.3	94.6	8.2
1 µg/L	98.5	8.3	107.0	8.1	100.0	8.2	96.4	8.1	108.0	8.2	90.0	8.2	104.4	8.1	88.0	8.2	105.0	8.2	82.0	8.2	70.3	8.2	87.2	8.3	94.7	8.2
3.2 µg/L	100.0	8.3	112.0	8.1	97.9	8.1	98.1	8.2	109.0	8.2	80.0	8.2	103.0	8.1	90.5	8.2	100.0	8.1	87.0	8.2	68.5	8.2	92.0	8.3	94.8	8.2
10 µg/L	101.0	8.3	107.0	8.0	100.0	8.1	101.2	8.1	101.0	8.1	81.0	8.2	106.0	8.1	95.2	8.1	99.5	8.2	92.5	8.3	73.2	8.2	89.9	8.2	95.6	8.2
32 µg/L	98.0	8.2	110.3	8.0	97.0	8.2	98.8	8.3	110.0	8.1	80.6	8.2	102.0	8.0	89.4	8.1	96.8	8.6	88.5	8.3	72.0	8.2	92.1	8.4	94.6	8.2
100 µg/L	98.5	8.3	114.6	8.2	98.0	8.2	97.5	8.2	112.0	8.2	78.6	8.2	102.0	8.1	94.0	8.1	98.0	8.1	90.0	8.3	83.5	8.1	87.0	8.3	96.1	8.2

Table 4.15: Daily temperature (°C) readings throughout the 28-day exposure period, for alternating replicates of each treatment.

Exposure day	Treatment	Replicate	Temperature (°C)
0	10 µg/L	E	26.3
1	32 µg/L	A	26.4
2	100 µg/L	B	26.5
3	DWC	C	26.5
4	SC	D	26.4
5	1 µg/L	E	26.6
6	3.2 µg/L	A	27
7	10 µg/L	B	26.9
8	32 µg/L	C	26.9
9	100 µg/L	D	26.8
10	DWC	E	26.9
11	SC	A	26.8
12	1 µg/L	B	26.9
13	3.2 µg/L	C	27
14	10 µg/L	D	26.8
15	32 µg/L	E	27.1
16	100 µg/L	A	26.9
17	DWC	B	26.9
18	SC	C	27
19	1 µg/L	D	27.1
20	3.2 µg/L	E	27
21	10 µg/L	A	26.9
22	32 µg/L	B	26.8
23	100 µg/L	C	26.9
24	DWC	D	27
25	SC	E	26.9
26	1 µg/L	A	27
27	3.2 µg/L	B	27.1
28	10 µg/L	C	26.8

3.2.1. Chemical analysis of dutasteride concentrations in treatment groups

Chemical testing using LC-MS/MS demonstrated that DUT concentrations in the test vessel replicates, containing freshly made test solutions, were close to the nominal concentrations indicating high accuracy of the actual exposure concentrations on renewal day (ON samples, Table 4.16). On the other hand, DUT concentrations in the test vessel replicates containing old test solutions varied from the nominal, suggesting a decline of the actual exposure

concentrations over time (OFF samples, Table 4.16). Throughout the experiment, DUT was found absent from the DWC or SC treatment replicates. Throughout the exposure period, ON sample DUT concentrations in the 1 µg/L treatment, ranged from 0.93 µg/L to 1.06 µg/L. In the 3.2 µg/L treatment, DUT concentrations ranged from 2.95 µg/L to 3.71 µg/L, whereas in the 10 µg/L treatment they ranged from 9.96 µg/L to 11.2 µg/L. Finally, in the 32 µg/L treatment, DUT concentrations ranged from 30.9 µg/L to 31.9 µg/L and in the 100µg/L treatment DUT concentrations ranged from 93.4 µg/L to 105 µg/L. In the OFF samples, the concentrations of DUT ranged from 0.35 µg/L to 0.41µg/L in the 1 µg/L treatment and from 1.06 µg/L to 1.53 µg/L in the 3.2 µg/L treatment. In the 10µg/L treatment, DUT concentrations ranged from 3.07 µg/L to 4.52 µg/L, whereas in the 32 µg/L treatment, they ranged from 10.5 µg/L to 13.9 µg/L. Lastly, in the 100 µg/L treatment, concentrations of DUT ranged from 37.4 µg/L to 41.1 µg/L (Table 4.16). The calibration curve of the DUT standards used in this analysis exhibited response linearity (R^2 value) of 0.999 (Appendix S4, Fig. S4.8)

Table 4.16: The concentrations of DUT in replicate samples collected between day 0 – day 28 of the exposure period detected by LC-MS/MS. Fresh test solutions are described as “ON”, whereas old test solutions are described as “OFF”. Samples with an asterisk (*) indicate that centrifugation was performed before dilution with solvent. The limit of detection (LOD) was 0.1 µg/L. As the method was not validated, LOQ remains unknown. Arithmetic mean values were calculated for ON and OFF samples, respectively. One respective replicate from each treatment was used for analysis on tested days.

Nominal concentration of DUT (µg/L)	**Day 0 ON (Rep A)**	**Day 7 OFF* (Rep B)**	**Day 7 ON (Rep B)**	**Day 19 OFF* (Rep C)**	**Day 19 ON (Rep C)**	**Day 28 OFF* (Rep D)**	**Mean (ON)**	**Mean (OFF)**
	(µg/L)	(µg/L)	(µg/L)	(µg/L)	(µg/L)	(µg/L)	(µg/L)	(µg/L)
DWC	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	N/A	N/A
SC	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	N/A	N/A
1.0	0.93	0.35	1.04	0.38	1.06	0.413	1.01	0.38
3.2	2.95	1.21	3.18	1.06	3.71	1.53	3.28	1.27
10	9.96	3.07	10.10	3.15	11.20	4.52	10.42	3.58
32	30.90	10.50	31.10	13.90	31.90	13.90	31	12.77
100	93.40	40.70	95.40	37.40	105	41.10	97.9	39.73

3.2.2. Survival, reproductive output and growth

The survival *B. glabrata* individuals was evaluated across all treatment groups during the 28-day exposure period. Decreases in survival rates were observed across all treatments in a dose-dependent manner. However, the log-rank (Mantel-Cox) test from the Kaplan-Meier survival analysis estimated no significant differences between different treatments (p=0.060) (Fig. 4.9a). Throughout the 28-day exposure period, the total number of mortalities observed in the DWC, 1 µg/L, 3.2 µg/L, 10 µg/L, 32 µg/L, and 100 µg/L treatments were 2 (8%), 1 (4%), 2 (8%), 5 (20%), 6 (24%), 8 (32%), and 7 (28%), respectively (Fig. 4.9b).

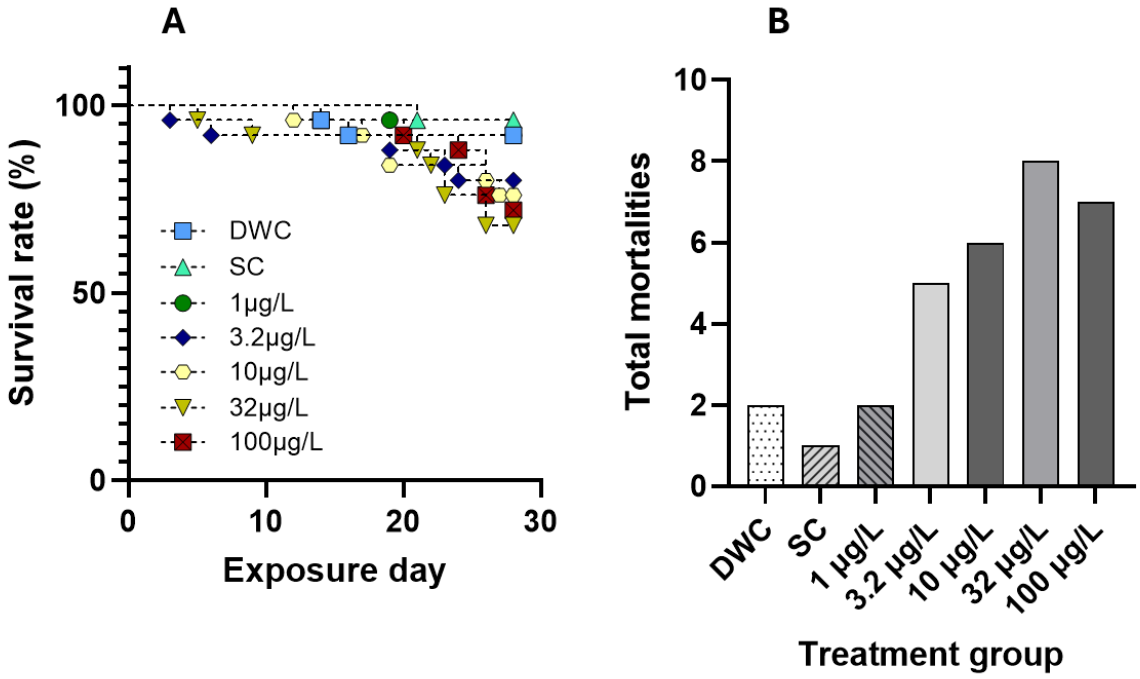


Figure 4.9: (a) Survival rates of *Biomphalaria glabrata* adults over a 28-day exposure period. Data represents the number of surviving individuals from the total number of snails per treatment (n= 5 replicates, 5 snails per replicate) converted to percentages (%). (b) The total number of mortalities observed in each treatment group at the end of the exposure period (day 28). Each treatment group initially consisted of 25 snails.

The cumulative embryo production of individual snails varied significantly across treatments at exposure day 28. The cumulative number of normal and abnormal embryos on the 28th day of exposure was significantly lower in the 32 µg/L (p=0.04) and 100 µg/L (p=0.03) treatments compared to the SC (Fig. 4.10a). Similarly, the cumulative number of normal embryos produced by the end of the exposure was significantly lower in the 100 µg/L (p=0.013) treatment compared to the SC (Fig. 4.10b). Although not significant, on the 28th day of exposure, the cumulative number of abnormal embryos was higher in the 3.2 µg/L treatment

and lower in the DWC and 32 µg/L treatments compared to the SC (Fig. 4.10c). By the end of the exposure period, the highest cumulative number of egg masses was observed in the 3.2 µg/L treatment (Fig. 4.10d). This number also reflects the high levels of abnormal embryos identified in that treatment. Moreover, the cumulative egg mass production was significantly lower in the 32 µg/L ($p=0.005$) treatment compared to the SC (Fig. 4.10d). The percentage of abnormal embryos (from all normal and abnormal embryos counted by the end of the experimental period) was highest in the 100 µg/L treatment reaching 14.1%. The lowest percentage of abnormal embryos was found in the DWC treatment, reaching 5.8%. The second highest percentage of abnormal embryos was observed in the 3.2 µg/L treatment at 11.5%, whereas the percentage of abnormal embryos in the SC treatment was observed at 8% (Fig. 4.11).

No significant differences in mean shell length (mm, ± SD) of individuals were observed across different treatments, before or after the 28-day exposure period (Fig. 4.12a). Before the exposure to DUT, the lowest mean shell length of individuals was 17.72 ± 0.51 mm, observed in the DWC treatment, and the highest mean shell length was 17.77 ± 0.56 mm, which was observed in the 3.2 µg/L treatment. After the 28^{th} day of DUT exposure, the lowest mean shell length was observed in the 3.2 µg/L treatment (18.22 ± 0.64 mm) whereas the highest was found in the SC (18.43 ± 1.02 mm) (Fig. 4.12a). Similarly, no significant differences were observed in the mean total weight (g, ± SD) of individuals before or after the exposure, across different treatments (Fig. 4.12b). Before the DUT exposure, the lowest mean total weight was found in the 100 µg/L treatment (0.83 ± 0.09g) and the highest in the SC treatment (0.88 ± 0.09g). After the exposure, the lowest mean total weight was again observed in the 100 µg/L treatment (0.85 ± 0.07g) and the highest in the SC treatment (0.93 ± 0.16g) (Fig. 4.12b).

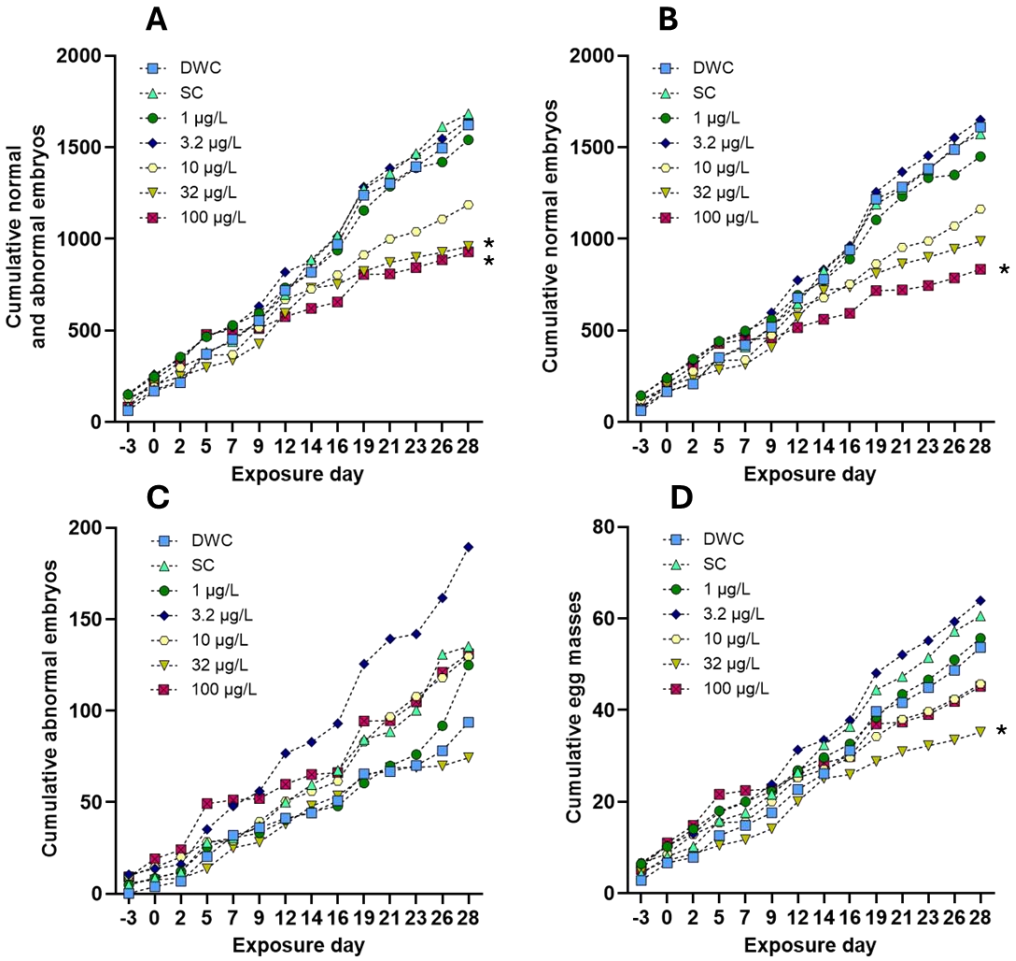


Figure 4.10: Effects of DUT on: (a) the cumulative normal and abnormal number of embryos per snail; (b) the cumulative normal number of embryos per snail; (c) cumulative abnormal number of embryos per snail; and (d) cumulative number of egg masses per snail. Data represents cumulative mean numbers ±SD of embryos (a, b, c) or egg masses (n= 5 replicates, 5 snails per replicate). Concentration-dependent effects on embryo production were demonstrated over the 28-day chemical exposure period. Asterisks indicate statistically significant differences with the SC where *: $p < 0.05$.

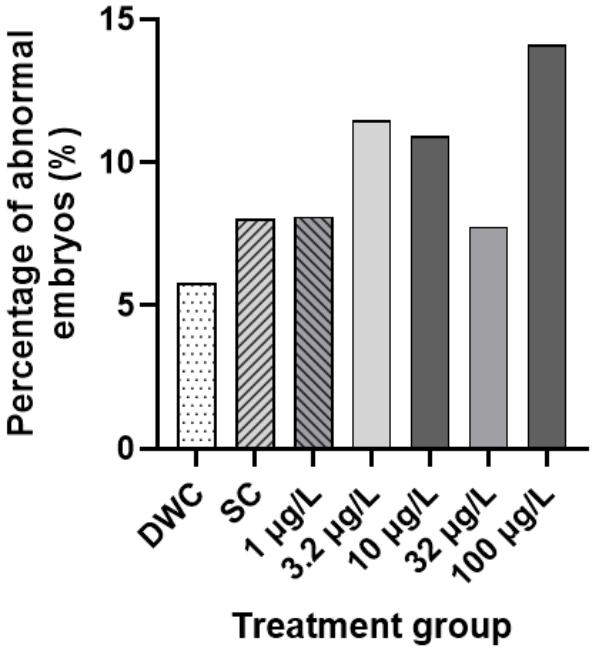


Figure 4.11: Percentage of abnormal embryos (%) at the end of the exposure (day 28), out of the total (normal and abnormal) embryos produced per treatment (n= 5 replicates, 5 snails per replicate). Number of embryos were calculated as the average number of embryos per snail. Percentages were calculated from the sum of average embryo counts from all collection days.

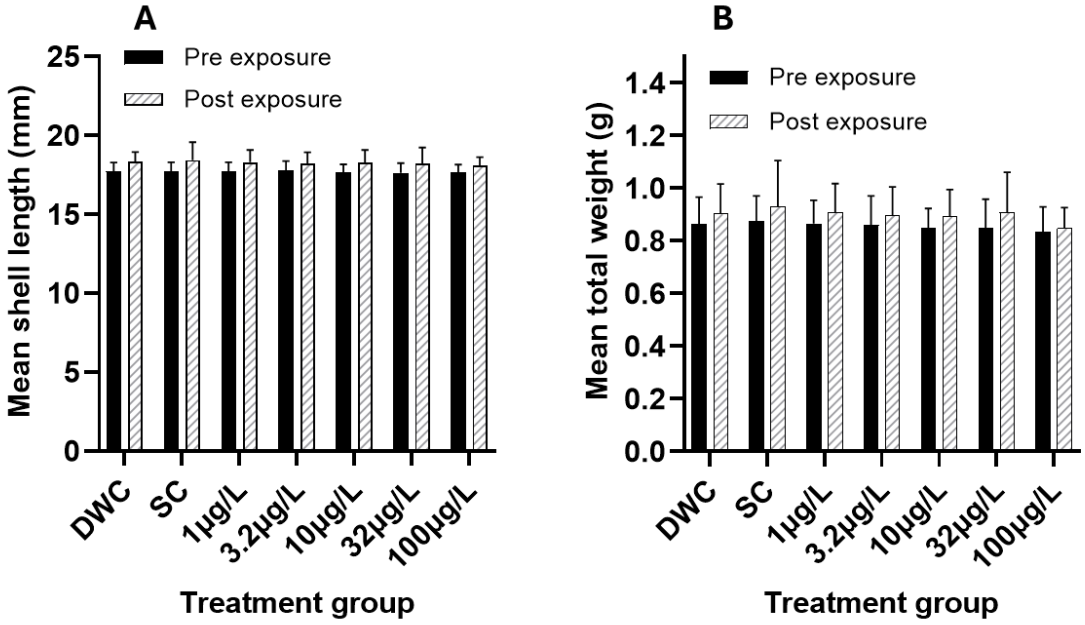


Figure 4.12: Mean values of (a) shell length (mm) and (b) total weight (g) of *Biomphalaria glabrata* adults, before the exposure (first day of acclimation period) and after the exposure

(last day of the 28-day exposure period) to DUT treatments. Data represents the arithmetic mean values (±SD) taken from the surviving snails across five technical replicates (n= 5 replicates, 5 snails per replicate).

4. Discussion

The present study investigated the impacts of DUT on the survival, growth and reproduction of the freshwater gastropod *B. glabrata*, under a flow-through exposure system and a static renewal system. The results revealed considerable differences in the responses of adult gastropods between the two experiments, highlighting the influence of the actual DUT concentrations experienced by individuals.

4.1. Experiment 1 – Flow-through system

Based on the results obtained from the LC-MS/MS analyses, it is evident that the nominal DUT concentrations were not achieved from the beginning of the exposure, while optimisation flaws in the analytical and sample preparation method employed prevented the accurate quantification of DUT concentrations in the tank replicates. Therefore, the results of this experiment should be taken with caution. Explanations for the series of methodological limitations incorporated in the study are discussed below, including factors that may have contributed to the observed results.

The poor dissolution of DUT in the initial dosing stocks had likely prevented the replicate tanks from reaching their nominal concentrations during the early phase of the experiment. Although new and fully dissolved dosing stocks were supplied from the 7th day of exposure onwards, it is important to consider the time needed for these stocks to reach the replicate tanks, fully replace the previous test solutions, and achieve the target nominal concentrations. Thus, flaws in the preparation and supply of initial dosing stocks are very likely to have led to inconsistencies in the DUT concentrations throughout the 21-day exposure period. Moreover, determining the nominal tank concentrations entailed further limitations as the optimisation of the LC-MS/MS method was not completed before starting the experiment. This was due to time constraints that required the timely completion of this study. Consequently, the water samples analysed at the beginning of the experiment might have failed to demonstrate (at least some of) the presence of DUT due to flaws in the sample preparation method employed. Although the presence of DUT from replicate tanks was observed, during subsequent analyses on the first and last day of exposure (Tables 4.12 and 4.13), DUT concentrations were measured at considerably lower levels than the nominal. A possible explanation for this variability between actual and nominal concentrations, especially during the last day of exposure, was the use of PTFE syringe filters during sample preparation. It is suspected that

DUT was partially adsorbed during filtering, resulting in recovery declines in the analyte concentrations. This is indicated by the comparison between filtered and unfiltered mixing chamber samples that reached a non-detectable concentration and a 26.2 µg/L (of the 32 µg/L) concentration, respectively (Table 4.10). Although a direct comparison between filtered and unfiltered water samples from replicate tanks would provide further insights into the limitations of PTFE filters, this was not performed due to concerns of column blockage by particulate matter (e.g. snail faeces or food leftovers).

Thus, a test to understand whether syringe filters interfered with the sample preparation method, was performed (Table 4.4). The results of this experiment (Table 4.11) suggest that the potential adsorption of DUT onto the filter was limited after coming into contact with equal volumes (50:50 v/v) of water: MeOH. This was achieved by either creating a solution containing the water sample, internal standard and MeOH before filtration (Table 4.4, Method 2) or by flushing out the syringe and filter (that were used for the water sample) with MeOH (Table 4.4, Method 3 and 5). Interestingly, a study investigating the effects of different filters (PVDF, Nylon and PTFE) on the recovery of multiple pesticide compounds using LC-MS/MS, demonstrated their adsorption onto the filters during sample preparation (Chamkasem *et al.*, 2009). It was also shown that adding MeOH in water samples prior to filtration can improve the recovery of the analytes, where samples containing over 50% of MeOH demonstrated a recovery above 80% (Chamkasem *et al.*, 2009). Another study that examined the effects of hydrophilic and hydrophobic PTFE syringe filters in the analytical recoveries of various pharmaceuticals and personal care products (PPCPs), demonstrated that the addition of 50% MeOH in water samples during preparation can significantly reduce compound mass loss in both filter types (Dong *et al.*, 2022). These findings align with the observations made in the current study, where water samples mixed with MeOH before filtration demonstrated higher analytical recoveries (Table 4.11). Considering that DUT is a hydrophobic compound (Ali *et al.*, 2014), and thus of a non-polar nature, it is hypothesised that it may have adsorbed onto the hydrophobic, non-polar, PTFE filters during sample preparation. This was likely caused by the strong hydrophobic-hydrophobic interactions between the compound and the filter material (Wang *et al.*, 2018; Al-Jabari and Husein, 2022). It is further hypothesised that the presence of MeOH during sample preparations may have enhanced the dissolution of DUT by disrupting the binding of the substance on the PTFE filter, thereby improving its analytical recovery. An additional factor that may have impacted the analytical recoveries of DUT, is the presence of microbial growth in the mixing chambers and replicate tanks of solvent-based treatments (that is SC, 1µg/L, 3.2 µg/L, 10 µg/L, 32 µg/L and 100 µg/L) where DMF was used. According to the OECD's "Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures", solvent use can likely promote microbial growth in exposure conditions, enhancing

the biodegradability of test substances (OECD, 2019a). This is further supported by evidence that microbial communities can biodegrade a range of pharmaceuticals, including EDCs, in the environment (Blunt *et al.*, 2018; Martins, Sanches and Pereira, 2018). In this study, DMF was consistently present in mixing chambers and replicate tanks at concentrations of 60 µg/L (0.006%) which exceeded the OECD TG 243 suggested limit of 20 µL/L (0.002%) (OECD, 2016a). Although this OECD recommendation mainly addresses the impacts of solvents on the reproductive performance of aquatic species (Hutchinson *et al.*, 2006), it is further suspected that higher concentrations of DMF resulted in increased microbial growth which may have accelerated the biodegradation of DUT. Potential biodegradation effects could likely have been minimised if solvent concentrations adhered to the recommended 20 µL/L limit (OECD, 2016a).

The pharmaceutical dosing limitations in the replicate tanks, and thus the exposure of *B. glabrata* to inconsistent levels of DUT throughout the exposure period, have likely influenced the observed non-significant results in this experiment. It is hypothesised that *B. glabrata* were exposed to lower than nominal levels of DUT until the 6th day of exposure which minimised any significant biological effects at the end of the exposure (day 21). Moreover, the non-significant increases in *B. glabrata* embryo and egg mass production (Fig. 4.7), as well as the total weight differences observed before and after the exposure (Fig. 4.8b), could be a result of the *ad libitum,* unregulated*,* feeding of *B. glabrata* adults. Previous research has shown that restricted food availability resulted in significant declines in embryo production in female apple snails (*Pomacea canaliculate)* (Tamburi and Martín, 2011). Similarly, Ter Maat *et al.* (2007) showed that reduced food availability suppressed egg laying, reduced reproductive output and decreased the dry weight of the freshwater gastropod *Lymnaea stangalis*. Consequently, the similar levels of total weight (g) across treatments observed at day 21, which differ from the significantly different levels before the exposure, may be explained by the variations in food provision which have likely balanced weight levels over time. Nevertheless, it is also believed that variations in food provision across replicate tanks, coupled with inconsistencies in actual DUT exposure concentrations, could have exerted a synergistic effect on the reproductive output of *B. glabrata*.

4.2. Experiment 2 – Static-renewal system

The identification of nominal DUT concentrations in the fresh test solutions (ON samples) across treatments and throughout the exposure period (Table 4.16), indicates the reliability of the collected data, which suggests dose-dependent declines in survival and reproductive output of *B. glabrata*. The centrifugation of water samples during sample preparation for LC-MS/MS analysis proved more effective in retaining DUT concentrations compared to filtering

(used in the flow-through study). This is evident from the mean DUT concentrations observed in the ON samples across different treatments, which were precisely close to the nominal (Tabe 4.16). In contrast, the reduced DUT concentrations in the old test solutions (OFF samples) suggest the potential degradation of the test substance or its adherence to the walls of the test vessel over time. These observations were expected as they reflect well-documented limitations of static-renewal tests (USEPA, 1994). In this experiment, DUT's degradation due to microbial growth is possible, although the frequent renewal of test solutions, coupled with the recommended use of solvent concentrations (0.002%) and the thorough cleaning of test vessels, may have helped to minimise it. On the other hand, the adsorption of DUT onto the surfaces of the test vessels could have also contributed to the observed declines in its concentration. The phenomenon of pharmaceuticals adhering to glass surfaces is well documented in the literature (Geary, Akood and Jensen, 1983; Yahya, McElnay and D'Arcy, 1988), where non-polar compounds (such as DUT) are particularly prone to adsorption onto glass, altering solution concentrations, and reducing analytical recoveries (Dincel *et al.*, 2023). Nonetheless, the accumulation of pharmaceutical compounds in aquatic organisms after long exposure periods is another well-documented phenomenon (Ruhí *et al.*, 2016; Arnnok *et al.*, 2017; Ruan *et al.*, 2020). The ratio between the concentration of a compound in the water and its concentration in the tissues of organisms is often referred to as the bioconcentration factor (BCF) (Fujita *et al.*, 2010). Interestingly, higher compound lipophilicity has been demonstrated to result in higher compound bioconcentration in aquatic organism tissues (Popovi and Perovi, 1999; Świacka *et al.*, 2022). DUT is a highly lipophilic compound and was shown to bioaccumulate in human tissues at concentrations as high as 55% of the administered dose (GSK, 2001; Margiotta-Casaluci, 2011). Although the BCF of DUT in this study is unknown, these findings may indicate a strong ability of this pharmaceutical to bioaccumulate into the soft body tissues of *B. glabrata*. In turn, the potential bioaccumulation of DUT in snail tissues may have also contributed to its declining concentrations in water.

Under the static-renewal system, the effects of DUT on *B. glabrata* adults differed greatly from those observed in the flow-through system. Although not significant, the dose-dependent decreases in survival rates suggest that *B. glabrata* exhibits higher sensitivity to DUT than previously thought (Fig. 4.9). Of particular interest are also the significant, dose-dependent declines in *B. glabrata's* reproductive output, including decreases in the number of normal & abnormal embryos (Fig. 4.10a), solely normal embryos (Fig. 4.10b) and egg mass production (Fig. 4.10d), which indicate DUT-specific disruption at reproductive processes. A noticeable decrease in egg mass size was also observed towards the end of the experiment in the two highest treatments (32 µg/L and 100 µg/L), suggesting a simultaneous effect of DUT on both

the number of eggs released as well as the number of egg capsules contained within the egg mass. The elevated percentages of abnormal embryo production in a dose-dependent manner (3.2 µg/L, 10 µg/L and 100 µg/L) (Fig. 4.11) indicate embryogenic-specific disruption which resulted in the production of either atrophied or unfertilised embryos. In contrast, measurements of shell length and total weight before and after the exposure indicated no disruption in adult growth, which aligns with the findings of the flow-through experiment. Overall, these results suggest that DUT disruption was specific to reproductive processes rather than general somatic growth, where mechanisms involved in growth and development remained unaffected. Moreover, the observed declines in survival may hint at some general physiological toxicity in adults, but this currently remains poorly understood. Notably, the significant reproductive effects on *B. glabrata* adults exposed to DUT were only observed at concentrations much higher than the recently reported DUT concentration of 0.027µg/L in wastewater treatment effluents (Gómez-Canela *et al.*, 2021).

Generally, the effects of 5-alpha-reductase inhibitors, DUT and FIN, on molluscs and other invertebrates remain underexplored. Previous findings from acute early embryonic exposures on *B. glabrata* to DUT and FIN indicated no significant effects in mortalities at concentrations between 0 µg/L to 160 µg/L and 300 µg/L to 1520 µg/L, respectively (Baynes *et al.,* 2019). These findings indicate that exposure of *B. glabrata* to 5αR inhibitors may not be significantly toxic in the early embryonic development of *B. glabrata* (day 0 – day 4 post oviposition, Chapter 3: Fig. 3.1), but exposure to later developmental stages may cause some physiological disruption which could lead to considerable, but not significant, mortalities at concentrations above 3.2 µg/L. Comparatively, the study of Margiotta-Casaluci, (2011) demonstrated that fathead minnow (*Pimephales promelas*) larvae exposed to 100 µg/L DUT exhibited significant survival declines to 65.44% (34.56% mortality) and 55.2% (44.8% mortality) at 14 days and 28 days post-hatching, respectively. However, no survival declines were reported in sexually matured *P. promelas* exposed to the same DUT concentration for 21 days. These findings suggest that fish may be more sensitive to overt toxicity caused by DUT during early development compared to molluscs. Nevertheless, *B. glabrata* and *P. promelas* do seem to exhibit similar reproductive sensitivity to DUT, where significant declines in their reproductive output were observed under the same concentrations (32 µg/L and 100 µg/L) (Fig. 4.10 and Margiotta-Casaluci, Hannah and Sumpter, (2013)). Interestingly, a recent study that chronically exposed zebrafish embryos (*Danio rerio*) to 2.6 µg/L – 1057 µg/L DUT, demonstrated a reduction of dihydrotestosterone (DHT), oestradiol (E2) and vitellogenin (VTG) levels, which coincided with a downregulation of genes involved in fish reproduction, including *SRD5A2* (5αR2), *CYP19A1* (Aromatase), *ESR* (oestrogen receptor) and *VTG* (vitellogenin) (Cho *et al.*, 2025). DUT was also demonstrated to inhibit the *SRD5A1*, *SRD5A2*

and *SRD5A3* genes *in vitro*, but was not observed to have an antagonistic effect on the androgen (AR) or oestrogen receptors (ESR). Together, their findings suggest that DHT plays an important role in zebrafish reproduction and that DUT's mode of action (MoA) may be associated with both androgen and oestrogen signalling in these animals.

On the other hand, a recent study which chronically exposed the invertebrate *Daphnia magna* to FIN, demonstrated notable declines in survival and significant declines in growth and reproductive output at concentrations above 1500 µg/L (Cho *et al.*, 2024). Specifically, *D. magna* was observed to experience a 15% mortality rate at 1500 µg/L and 3000 µg/L FIN, which subsequently rose to 35% and 80% at 4500 µg/L and 6000 µg/L FIN, respectively, after a 23-day exposure period. *B. glabrata* seems to be more sensitive to physiological toxicity caused by pharmaceutical 5αR inhibitors compared to *D. magna*, considering that the former experienced a 20% mortality rate at 5 µg/L DUT. Although the cause of mortality in daphnids exposed to FIN is unclear, molecular analyses revealed that reproduction changes were associated with the downregulation of genes that encode biomolecules involved in crustacean reproduction, such as the ecdysone receptor (*EcR*) and vitellogenin (*VTG*). These findings suggest that FIN is acting through alternative pathways in *D. magna* compared to vertebrates (e.g. androgen or oestrogen signalling pathways) given that *SRD5A1* and *SRD5A2* genes do not seem to be conserved in crustaceans (Rand-Weaver *et al.*, 2013). Additional lipidomic and metabolomic investigations from Cho *et al*. (2024) linked the FIN-induced reproductive and developmental effects in *D. magna* with the downregulation of several lipids, including triacylglycerol, phosphatidylcholine and diacylglycerol. Interestingly, the authors did not observe any increases in oxidative stress levels of FIN-exposed daphnids and thus suggested that toxicity was caused through an endocrine-mediated pathway rather than a cellular one (Cho *et al.*, 2024). While the reproductive and survival observations in *D. magna* may align with the results of this study, as of now, it remains unclear whether pharmaceutical disruption caused by 5αR inhibitors in *B. glabrata* is associated with cellular or endocrine-related toxicity.

According to the standards set by the European Commission in collaboration with the OECD, a substance is considered an endocrine disruptor (ED) if (1) it shows an adverse effect in an intact organism (or its offspring) which may include changes in morphology, physiology, growth, development, reproduction or life span, (2) it has an endocrine MoA and (3) the adverse effect is a result of such endocrine MoA (ECHA/EFSA, 2018). In regulatory testing, the association of an adverse effect in an organism with an endocrine mechanism requires the conduct of a MoA analysis, according to a weight-of-evidence (WoE) approach (Lagadic *et al.*, 2024). However, not all endocrine-related adverse effects are caused by an ED. For instance, changes in reproduction, growth, and behaviour, although sensitive to EDCs, may also be a result of dietary influences or systemic toxicity. In non-mammal organisms, the

concept of maximum tolerated concentration (MTC) can be used to provide insights into the toxicity of a substance (ECHA/EFSA, 2018). When determining endocrine MoA, it is recommended that the top chemical concentration used in a study should not cause significant physiological disorders in the exposed organisms which may prohibit the accurate interpretation of results (ECHA/EFSA, 2018). Specifically in ecotoxicological studies, the MTC threshold is considered the highest concentration of the test chemical which causes less than 10% mortality in exposed organisms (Hutchinson *et al.*, 2009; Wheeler *et al.*, 2013; ECHA/EFSA, 2018). Consequently, adverse effects solely observed above the MTC threshold, should not be used as indicators of endocrine disruption (ECHA/EFSA, 2018). In instances where adverse effects are observed at, or below the MTC threshold, these can be considered related to endocrine toxicity only If they are supported by the MoA analysis (ECHA/EFSA, 2018).

It should be noted, however, that these considerations are based on aquatic animal models (e.g. fish) which have standardised mechanistic assays for assessing endocrine activity of chemicals. In molluscs, the lack of mechanistic testing for EDs prevents from drawing comprehensive conclusions about any potential endocrine MoA of a chemical (OECD, 2018). According to the OECD's guidance on evaluating chemicals for endocrine disruption (OECD, 2018), endocrine-related adverse effects that are only observed at concentrations of clear systemic (non-endocrine) toxicity should be addressed with caution. In this study, the significant effects on reproductive output of *B. glabrata* at the 32 µg/L and 100 µg/L concentrations, coincided with 32% and 28% (non-significant) decreases in survival, respectively. It is therefore likely that those adverse effects might have been a result of systemic toxicity rather than endocrine-specific toxicity. However, considering that DUT has been previously demonstrated to exhibit morphological disruptions on embryonic *B. glabrata* at non-lethal concentrations (Baynes *et al.*, 2019), the possibility of an endocrine MoA of this chemical cannot be completely disproved. Further analyses will need to be conducted to evaluate the disruptive effects of DUT at the histopathological, molecular, lipidomic and proteomic levels, to identify the cause of mortality and determine if this chemical acts through an endocrine MoA. Nonetheless, *in vivo* DUT exposures using the test species *Pomatopyrgus antipodarum* and the OECD TG 242 may also be used to provide insights on the adverse effects of this chemical on species with different (e.g. parthenogenic) reproductive strategies (OECD, 2018).

A possible explanation for the reproductive variations observed in *B. glabrata* could be attributed to the physiological mechanisms involved in the production of eggs. In this species, immature egg cells (i.e. oocytes) are first produced in the hermaphrodite gonads (i.e. ovotestis) and then pass through the hermaphroditic duct, making their way to the carrefour

(Hathaway *et al.*, 2010). Fertilisation occurs inside the carrefour region where eggs are covered in perivitelline fluid, which is produced and released by the albumen gland. Individual eggs are then encapsulated and packed into an egg mass before being released from the snail's vaginal opening (Hathaway *et al.*, 2010). Interestingly, the expression of *SRD5A1* and *SRD5A2* genes in the albumen gland of *B. glabrata* (as discussed in Chapter 3), could suggest that DUT interferes with the activity of 5αR in this tissue, subsequently influencing reductions in egg mass production or increases in the number of abnormal embryos produced by *B. glabrata*. However, without further evidence on the differential expression of *SRD5A1* and *SRD5A2* genes in the albumen gland, particularly before and after DUT exposure, it is difficult to determine any potential interferences.

Besides, reproduction in gastropods is regulated by a complex neuroendocrine system that differs from other molluscan classes (Zając and Kramarz, 2017). Therefore, any evaluation of the effects of DUT disruption in *B. glabrata* must be based on a comprehensive understanding of its distinct hermaphroditic reproductive system. Gastropods tend to have separate reproductive systems which perform male and female functions, but not necessarily at the same time (Zając and Kramarz, 2017). Much of the reproductive research on gastropods has been based on the model organism *L. stagnalis* (Di Cristo and Koene, 2017), which is phylogenetically closely related to the test organism of this study (Kuroda and Abe, 2020). Reproductive behaviour in *L. stagnalis* is mainly mediated by the Caudo-Dorsal Cell cluster (CDC), which functions as the main neuroendocrine centre and is positioned in the cerebral ganglia. CDCs have been shown to induce egg-laying by releasing hormones into the blood through bursting (Geraerts and Algera, 1976; ter Maat *et al.*, 1988). This bursting activity is in turn chemically regulated through the secretion of several peptides, whose genes *CDCH-I* and *CHCH-II* encode at least 11 of those peptides, were shown to be expressed in the CDC of *L. stagnalis* (Van Minnen *et al.*, 1989). The main peptide responsible for the egg-laying process seems to be the CDCH, or as often referred to as egg-laying hormone (ELH), while other peptides are likely involved in organising the egg-laying process after its initiation. Another neuroendocrine peptide that may be involved in the reproduction of gastropods is the gonadotropin-releasing hormone (GnRH), which has been previously shown to regulate reproductive processes in vertebrates (Amoss *et al.*, 1971; Schally *et al.*, 1971). However, in line with the recommendations of Zandawala, Tian and Elphick, (2018) on GnRH nomenclature in molluscs (discussed in Chapter 2), these peptides will be referred to as GnRH/corazonin (CRZ) peptides in this chapter. Although GnRH/CRZ function in molluscs is not fully understood, the presence of a GnRH/CRZ precursor in another gastropod, *Biomphalaria alexandrina*, has been linked with potential involvement in coordinating egg-laying (Rosa-Casillas *et al.*, 2021). Moreover, its presence in the ovotestis, oviduct and

albumen gland of *B. alexandrina* suggests that GnRH/CRZ signalling may participate in several reproductive processes, including the production of oocytes, the fertilisation, nourishment and transport of eggs, or the packaging and release of egg masses (Rosa-Casillas *et al.*, 2021).

Given that molluscs lack an androgen signalling pathway and thereby cannot synthesise testosterone, it is difficult to identify the specific hormonal substrates that might be affected by DUT. Moreover, our incomplete understanding of hormonal pathways involved in gastropod reproduction complicates the elucidation of the indirect effects deriving from the potential 5αR disruption in these animals. However, in line with the hypotheses proposed by Imiuwa *et al.*(2024), DUT could likely influence many of the distinct processes associated with egg-laying in gastropods. These may include the release, maturation or production of oocytes, the availability of fertilised eggs, the number of encapsulated eggs within egg masses, or the frequency at which egg masses are released. Moreover, the identification of *SRD5A1* and *SRD5A2* genes in the albumen gland of *B. glabrata* (Chapter 3) coupled with the observed variations in embryo and egg production in response to DUT in this chapter, provide a further indication for potential disruption in egg-laying mechanisms. Nonetheless, the hypothesis that DUT may have indirectly disrupted the GnRH/CRZ signalling pathway or the release of ELH in *B. glabrata*, is also worth investigating.

5. Conclusions

The present study provides novel insights into the effects of DUT on the physiology and reproduction of the freshwater gastropod *B. glabrata*, highlighting significant reductions in the number of embryos produced in a dose-dependent manner. These reductions coincided with considerable, but non-significant, mortalities at the same concentrations. Given the issues with chemical dosing and food variability in the flow-through study, the findings from the two separate experiments cannot be directly compared. However, DUT concentrations in the static-renewal study were observed close to the nominal, which signifies the reliability of the observed data. Overall, the findings of this study indicate unprecedented sensitivity of *B. glabrata* adults to prolonged exposure to DUT.

The significant, dose-dependent declines in the reproductive output in *B. glabrata* suggest potential disruptions in the mechanisms that mediate reproduction in gastropods. These effects may be due to DUT-derived interferences with the expression of 5αR1 and 5αR2 in the albumen gland, or indirect effects in the release or function of important neuropeptides in gastropods. These may include the release of egg-laying hormone (ELH) or gonadotropin-releasing hormone (GnRH)/corazonin (CRZ) peptides, which were previously demonstrated to coordinate reproductive processes in these animals. However, the considerable declines in

survival which coincide with the adverse reproductive effects at the same concentrations, may indicate non-endocrine specific toxicity from DUT.

The disparities in the findings from the two experiments underscore the influence of actual exposure concentrations on ecotoxicity testing and highlight the need to use standardised methodologies and higher experimental rigour. Repeating the flow-through study using correct dosing, the OECD-recommended solvent concentrations and a validated chemical analysis could allow a more accurate comparison of the efficacy of the two exposure systems. Moreover, given the unique reproductive systems of gastropods, which differ from other molluscs, the findings of this study illustrate the need for further research into the different molluscan hormonal pathways outside steroidogenesis, that remain underexplored. The lack of mechanistic understanding of endocrine disruption in molluscs further underscores the need to conduct more testing to comprehensively determine whether DUT is acting through an endocrine MoA. Future lipidomic, proteomic and histopathological analyses of soft body mass tissues, that were preserved from both experiments but not tested due to time constraints, will help to unravel the disrupted hormonal pathways induced by DUT and identify tissue-specific effects.

Chapter 5: Concluding remarks

1. Overview

This thesis aimed to: (1) develop a greater understanding of molluscan endocrinology by systematically reviewing and assessing findings in the literature; (2) investigate the expression patterns of the genes encoding 5-alpha-reductase 1 (5αR1) and 5-alpha-reductase 2 (5αR2) in embryonic *Biomphalaria glabrata* using a robust RT-qPCR assay; and (3) evaluate the sensitivity of adult *B. glabrata* to the pharmaceutical 5αR inhibitor, dutasteride (DUT), under different exposure systems by adapting the OECD TG 243. As a result, this work successfully created a systematic evidence map and identified key knowledge gaps in the present knowledge of molluscan endocrinology, while highlighting the presence of underexplored endocrinological mechanisms in these animals. Moreover, the identification of stable reference genes in embryonic *B. glabrata* allowed the normalisation of *SRD5A1* and *SRD5A2* mRNA transcripts, whose genes encode 5αR1 and 5αR2 respectively, in the day 2 – day 4 post-oviposition stages. Lastly, the chemical exposure study under a static-renewal system identified unprecedented declines in the survival of adult *B. glabrata* and significant disruption in its reproductive processes. However, limitations in reference gene stability between embryonic and adult tissues, coupled with high expression variability amongst embryonic samples, mean that further investigations might be needed to comprehensively evaluate the expression of *SRD5A1* and *SRD5A2* in *B. glabrata* embryos. Additionally, the challenges encountered during the flow-through exposure prevented a direct comparison of *B. glabrata*'s sensitivity with the results obtained under static-renewal conditions. Overall, the findings of this thesis provide strong evidence that molluscs encompass distinct endocrinological cascades which could be targets of endocrine disruption, but these largely remain underexplored. The expression patterns of 5αR genes in embryonic *B. glabrata,* coupled with the significant reproductive impacts of DUT in adults, suggest a reproductive role of this enzyme in gastropods. Notably, the survival declines observed in response to DUT may indicate systemic toxicity in *B. glabrata,* instead of endocrine-specific toxicity, which requires further investigation.

2. Use of systematic evidence mapping to identify knowledge gaps, assess bias in literature and provide recommendations for future research

The systematic evidence map presented in Chapter 2 offers the most comprehensive review of molluscan endocrinology to date and includes the first-ever critical appraisal (Risk-of-Bias assessment) specifically designed for endocrinological studies in Mollusca. This tailor-made tool surpasses previous efforts in addressing bias in the field, by thoroughly examining the

methods undertaken to investigate the interactions between hormones, their respective receptors, and hormone-metabolising enzymes. The holistic approach taken to evaluate data reliability allowed for a more rigorous assessment of the quality and accuracy of included studies. The key bias identified, comprised a lack of ligand binding assays (in studies examining the presence of hormones), a lack of sequence similarity analysis for receptor ligand binding (LBD) and DNA binding domains (DBD) (in studies identifying receptors), and an absence of phylogenetic analysis (in studies evaluating hormone-metabolising enzymes). Additionally, the lack of reference gene validation experiments in DNA/RNA detection and localisation assays (60% in receptor studies and 66% in hormone-metabolising enzyme studies) is thought to have further biased gene expression findings of included studies. The findings of this assessment suggest that the adoption of analyses which aim to elucidate interactions, structural characteristics and traces of evolutionary relationships between hormones, receptors and hormone-metabolising enzymes could have led to a more comprehensive view of the role of those biomolecules in molluscs.

A key outcome of the systematic evidence map was the development of a fully searchable and publicly available database. This resource offers an exhaustive list of study-specific information that can assist researchers in accessing endocrinological data for future investigations and be continuously refined and updated, ensuring it remains valuable and evolving. The systematic classification of findings in the database enabled the mapping and evaluation of discrepancies between vertebrate and molluscan hormonal pathways (e.g. cholesterol biosynthesis, steroidogenesis and the retinoic acid signalling pathway), which in turn helped to debunk claims on the ability of molluscs to biosynthesise vertebrate sex steroids (e.g. testosterone) *de novo.* Most importantly, by linking scattered data from different publications, this work showcased that molluscs comprise distinct endocrinological processes that remain largely underexplored. The results of the systematic evidence map align with the conclusions drawn by the critical reviews of Scott, (2012), Scott, (2013) and Fodor *et al.*, (2020), which collectively suggest that molluscs lack the essential mechanisms and key molecular pathways required for *de novo* biosynthesis of vertebrate-type steroids. However, this work goes beyond those efforts, filling the gap of a detailed Risk-of-Bias assessment and pointing out new research directions that could shape the field in the next few years. These include the exploration of understudied retinoid, thyroid and ecdysteroid signalling molecules in molluscs, the use of non-targeted approaches (e.g. combinations of metabolomic, proteomic, lipidomic and genomic methodologies) to evaluate the function of endocrinological pathways in these animals, and the adoption of robust analytical methodologies in such studies.

3. Developing a RT-qPCR assay to examine gene expression patterns of *SRD5A1* (5αR1) and *SRD5A2* (5αR2) in the embryonic *Biomphalaria glabarata*

Previous morphological abnormalities observed in the shell development of embryonic *B. glabrata* in response to the 5αR inhibitor, DUT (Baynes *et al.*, 2019), suggest that the few steroidogenic enzymes present in molluscs may serve functions different from those in vertebrates. For this reason, a robust RT-qPCR assay was developed in this study to investigate whether 5αR enzymes are linked with the sensitive window of this pharmaceutical disruption and their potential involvement in shell development. Previous efforts to quantify the genomic expression of 5αRs during the early stages of embryonic development in *B. glabrata* (Baynes *et al.*, 2019) were deemed unsuccessful due to failure to demonstrate stable expression of the reference gene tested (18S rRNA). Moreover, although previous investigations have employed a series of reference genes to study gene expression in *B. glabrata* adults (Portet *et al.*, 2018; Luviano *et al.*, 2021; Pinaud *et al.*, 2021), to the best of my knowledge, no other study has identified stably expressed reference genes during the embryonic development of this species. This study demonstrated that all candidate reference genes tested exhibited minimal expression variability across embryonic development, and thus may be suitable for use in RT-qPCR assay. Notably, the reference gene candidates *TUB* and *H2A* were consistently ranked as the two most stably expressed genes across the four embryonic developmental stages (day 2 – day 5 post-oviposition). In the absence of appropriate reference genes in *B. glabrata* embryos, as well as a record of misuse (or no use) of reference genes in qPCR experiments involving molluscs (as demonstrated in Chapter 2), the identification of these stably expressed genes can allow the conduct of future DNA and RNA detection and quantification investigations in this species. Additionally, findings from this study provide novel insights into the temporal expression of *SRD5A1* and *SRD5A2* transcripts, which appears to remain unchanged during the day 2 – day 4 post-oviposition stages. Preliminary findings of this study also demonstrated the presence of *SRD5A1* and *SRD5A2* genes in the albumen gland of *B. glabrata* adults. To the extent of my knowledge, the expression of *SRD5A2* in the albumen gland of *B. glabrata* has not been previously demonstrated in the literature.

Although the high variations in *SRD5A1* and *SRD5A2* gene expression amongst embryonic replicates make it difficult to draw meaningful conclusions, data from this study provides insight into the role of 5αR in gastropod embryonic development. These findings are consistent with previous observations from Baynes *et al.*, (2019) who detected, but did not quantify, these genes in embryos of the same species. The detection of *SRD5A1* and *SRD5A2* in the albumen gland partially aligns with previous transcriptomic investigations which revealed the

expression of *SRD5A1* in the same tissue (Adema *et al.*, 2017). Interestingly, these transcriptomic investigations have also shown the ubiquitous expression of *SRD5A1* and *SRD5A2* in various other tissues, including the digestive gland, terminal genitalia and mantle (Adema *et al.*, 2017). Such observations are further reflected in literature, where *SRD5A1* has been showcased to be expressed in several tissues of other gastropods and bivalves (Chapter 2, Figure 9) (Tong *et al.*, 2015b; Ip *et al.*, 2016). Together, the expression of 5αR enzymes in the reproductive tissues (e.g. albumen gland, terminal genitalia) and mantle of *B. glabrata*, raises questions about the potential involvement of this enzyme in egg-laying processes and shell development. However, the limited information available on the role of this enzyme in molluscs indicates that more research is needed to gain a thorough understanding of its function.

4. Assessing the effects of DUT on the survival, growth and reproduction of *Biomphalaria glabrata* adults

The morphological disruptions observed in *B. glabrata* embryos following exposure to the 5αR inhibitor, DUT, raised questions about the potential downstream effects of this pharmaceutical in adult organisms. To test this, an ecotoxicity testing framework was adapted according to the OECD TG 243, in which *B. glabrata* adults were chronically exposed to DUT for 21 days (flow-through system) and 28 days (static-renewal system), respectively. While the dosing inconsistencies and methodological flaws in the flow-through study prevented the generation of conclusive findings, the static-renewal study is the first to demonstrate that DUT significantly affects reproductive output in *B. glabrata.* The significant effects of DUT in *B. glabrata* reproduction were dose-dependent and at concentrations above 32 µg/L. Notably, the adverse reproductive effects coincided with dose-dependent mortalities exceeding the 10% maximum tolerated concentration (MTC) threshold. Although not statistically significant, these mortalities may indicate systemic toxicity effects rather than endocrine-specific toxicity in these animals. The survival declines of *B. glabrata* in response to DUT are surprising, as they have not been previously demonstrated to occur in gastropods (Baynes *et al.*, 2019). Nonetheless, it is hypothesised that the adverse reproductive effects of DUT on *B. glabrata* adults may be linked with disruption of 5αR1 and 5αR2 in the albumen gland (observed in Chapter 3), consequently affecting underlying mechanisms associated with egg laying in this species.

Findings in the literature suggest that *B. glabrata* exhibits lower survival sensitivity to pharmaceutical 5αR inhibitors compared to fish (*Pimephales promelas*, Margiotta-Casaluci, (2011)), but higher survival sensitivity compared to crustaceans (*Daphnia magna,* Cho *et al.*, (2024)). Yet, the mechanisms by which 5αR inhibitors, DUT and finasteride (FIN), induce their effects across different species seem to vary. A recent study from Cho *et al.*, (2025)

demonstrated that DUT acts by downregulating the genes encoding 5αR2 (*SRD5A1*), aromatase (*CYP19A1*) and oestrogen receptor (*ESR*) in zebrafish, while FIN was associated with the downregulation of ecdysone receptor (*EcR*) in daphnids (Cho *et al.*, 2024). Notably, both studies demonstrated a downregulation of the *VTG* gene in response to pharmaceutical 5αR inhibitors, suggesting a shared effect on reducing vitellogenin production in fish and daphnids. However, it should not be assumed that DUT acts similarly in molluscs and other vertebrate or invertebrate species. The molluscan *ESR* ligand binding pocket has been previously demonstrated to not bind to oestrogens (Bridgham *et al.*, 2014), while the use of vitellogenin as a potential endocrine disruption biomarker in molluscs has been critically questioned (Katsiadaki, 2019). Besides, *SRD5A1* and *SRD5A2* genes do not seem to be conserved amongst crustaceans (Rand-Weaver *et al.*, 2013), which suggests that DUT disruption could be initiated through a different mode of action (MoA) in *D. magna* compared to *B. glabrata.* Although the environmental concentrations of DUT (0.027 µg/L, Gómez-Canela *et al.*, 2021) remain below the levels that cause significant reproductive effects in *B. glabrata,* the results of this study underscore the need to refine ecotoxicity testing protocols for chemicals that exert endocrine-related effects in molluscs.

5. Limitations of the project and future perspectives

5.1. Systematic evidence map

One limitation of the Risk-of-Bias assessment, within the systematic evidence map, is the potential oversimplification introduced by summarising the data into overall low, moderate and high risk of bias scores. For example, during peer review, a considerable methodological flaw was identified in the study of Chong Sánchez *et al.*, (2019). The authors measured sex hormones in the snail *Lobatus gigas* using HPLC with UV absorption. Amongst other reasons, this approach can be misleading due to the inability of UV detectors to differentiate compounds with overlapping spectra (reviewed in an opinion piece by Scott, (2021)). While this limitation was appropriately flagged as high risk under the “internal validity criteria” of the Risk-of-Bias assessment, the overall score of this study was rated as “Moderate Risk” because of the lower risk of bias scores under the “strategy to minimise confounders” and “statistics” criteria. As irredeemable quantification errors, such as those observed in the study of Chong Sánchez et al., (2019), cannot be corrected by the good use of statistics, the overall “Moderate Risk” score given may not adequately reflect the number of biases in this study. Moreover, it is likely that some confounding factors inevitably remained unaddressed in publications assessed for risk of bias, as this depends on the specific experimental designs adopted in each study. While efforts were taken to ensure the Risk-of-Bias assessment reliably scored each study (through the use of two separate assessments based on the objectives of each study), developing a critical appraisal tool that is extensive enough and accounts for every possible methodological

limitation proved challenging. Therefore, it is recommended that the overall Risk-of-Bias scores addressed in this manuscript should be considered in combination with the individual scores for each “internal validity” and “study design” criterion for each study (Appendix S1).

Notably, the systematic review protocol was published before data extraction and evidence synthesis and aimed to define clear and transparent eligibility criteria (Panagiotidis, 2022). These criteria were designed to be broad enough to capture a wide diversity of studies (e.g. 145 studies were found eligible for data extraction) while ensuring the extracted data could be managed effectively within the time constraints of the project. Therefore, it should be emphasised that some studies which could have provided additional insights were inevitably excluded due to these pre-defined eligibility criteria at the protocol stage. These studies mainly encompassed transcriptomic data and results captured from “non-control” animals (i.e. animals ‘exposed’ to different interventions, results from radioactive precursors or biomolecules identified from chemical exposures). If this type of data were systematically interrogated in a similar way as the included literature, it could reveal additional interactions between hormones, receptors and hormone-metabolising enzymes and possible novel endocrinological pathways in molluscs.

In the future, systematic investigations of such studies are critical to evaluate important data that remains under-investigated. To assess the credibility of their findings, such investigations may incorporate and adapt the Risk-of-Bias assessment presented in this project. Nonetheless, the substantial knowledge gaps this systematic evidence map has underscored should spark a greater interest in advancing research efforts to elucidate the intricate endocrinological processes within this phylum. A shift from the “vertebrate-centric” approach in investigating molluscan endocrine systems should be drastically adopted. The main focus should be the exploration of the thyroid receptor (*THR*) in molluscan larval metamorphosis and the underlying mechanisms that may lead to the biosynthesis of thyroid hormones, T3 and T4, in molluscs. Furthermore, the ability of insects to convert Δ^5 (phyto)sterols to $\Delta^{5,7}$ sterols, which also appear to be conserved in molluscs, should spark further interest in investigating the conservation of ecdysteroid signalling in the latter. The evidence of ecdysone receptor (*EcR*) expression and its potential interaction with ecdysone steroid during shell damage in bivalves, underscores the need to investigate ecdysone’s binding affinity to its respective receptor in molluscs. Crucially, future endocrinological studies should implement rigorous experimental design and robust analytical methodologies, in line with the recommendations set in Chapter 2, to investigate hormone signalling pathways in molluscs.

5.2. Reference gene validation and gene expression analysis

Although the statistical algorithms implemented to study reference gene stability have been extensively used in the wider literature, their use in longitudinal studies (i.e. studies that investigate the same variable over different time periods) has been recently questioned (Sundaram *et al.*, 2019). The drawbacks of algorithms such as geNorm and the ΔCT method in longitudinal studies lie in the influence of (potentially) highly variable candidate reference genes on the stability rankings. While NormFinder is generally considered more robust for longitudinal studies, as it can account for variabilities between different embryonic stages (intergroup variations) and within embryos over time (intragroup variations), its stability ranking can also be affected by highly variable candidate genes. Furthermore, since geNorm produces its ranking results through a pairwise correlation analysis, it can calculate a higher stability value for genes that are co-regulated (Sundaram *et al.*, 2019). This means that candidate reference genes that exhibit similar patterns of expression across different samples, may be considered more stable by geNorm than what they are in biological terms. Instead, Sundaram *et al.* (2019), have proposed an alternative strategy to evaluate candidate reference gene stability. Their approach integrates a revised NormFinder method, a coefficient of variation analysis, and a one-way analysis of variance (ANOVA) between the mRNA fold changes of relative gene expression of each candidate reference gene. Although the raw Cq values of candidate reference genes in this study varied slightly from each other (mean SD from all three validation experiments ranged between 0.56-0.66), the possibility that our ranking analysis could be influenced by the most highly variable candidate cannot be excluded. Further statistical investigations following the considerations reported in Sundaram et *al.,* including an additional coefficient of variation analysis, could help indicate potential ranking discrepancies between the different approaches and algorithms used. Moreover, integrating the model proposed for longitudinal studies could further enhance the reliability of reference gene stability data provided in this study.

The identification and validation of stable reference genes in *B. glabrata* could be further enhanced by the use of untargeted genomic approaches. For example, pilot transcriptomic investigations across embryonic developmental stages could have revealed novel and more stably expressed genes, which may have served as a better reference for data normalisation. For instance, comparative transcriptomic analysis has been previously used in control and drought-stressed plants to identify novel candidate reference genes that showed minimal variation in their expression (Kotrade *et al.*, 2019). Following their identification by the RNA sequencing data, their stability was further validated using gene expression analysis (e.g. RT-qPCR) and the stability algorithms geNorm, NormFinder, and BestKeeper.

Nonetheless, the quantification of *SRD5A1* and *SRD5A2* at earlier embryonic developmental stages (day 0 and day 1 post-oviposition) could have been achieved by adopting more precise quantification methodologies, such as digital PCR (dPCR). The ability of dPCR to work with smaller RNA input amounts (Taylor, Laperriere and Germain, 2017) could allow the collection of embryo samples with smaller developmental variabilities. Potentially, this could have also helped reduce the gene expression variabilities observed in this study, resulting in more comprehensive conclusions about the patterns of expression of 5αR1 and 5αR2 in *B. glabrata* embryos. A phylogenetic analysis of molluscan 5αR1 and 5αR2 protein sequences could further enable the identification of potential structural and functional similarities with their vertebrate homologs as well as the binding affinity of molluscan 5αRs to testosterone, and potentially DUT. For example, phylogenetics has been previously used to evaluate evolutionary similarities between the molluscan and vertebrate oestrogen receptor (*ESR*) (Hultin, Hallgren and Hansson, 2016). The results demonstrated a very high amino sequence similarity of *ESR* between gastropods, heterobranchs and ceanogastropods, but distinct evolutionary differences between molluscan and vertebrate *ESR*. Similarly, a computational investigation of the ligand binding pocket of *ESR* in molluscs identified its binding incompatibility with oestrogens (Bridgham *et al.*, 2014). Furthermore, spatial gene expression analysis, such as whole-mount *in situ* hybridisation (WMISH) could provide additional insights into the function and evolutionary history of *SRD5A1* and *SRD5A2* genes. Given the recent optimisation of a WMISH method on *Lymnaea stagnalis* embryos by Hohagen, Herlitze and Jackson, (2015), this provides an opportunity for adapting it to study the morphological influence of DUT on *B. glabrata* embryonic shell. For instance, WMISH could be used to assess the localisation of *SRD5A1* and *SRD5A2* genes in various developmental stages, tissues and organs within the whole *B. glabrata* embryo, in turn providing important insights into the involvement of 5αR1 and 5αR2 in embryonic developmental processes. A key organ for future research is the mantle, as its formation occurs during the sensitive window of DUT disruption (day 2 post-oviposition) and coincides with the development of the shell.

5.3. Flow-through and static-renewal exposure studies

The errors in the initial dosing stocks of the flow-through study, and subsequently, the inconsistencies in the actual chemical concentrations introduced in the exposure tanks, have very likely influenced the results of this experiment. These significant limitations underline the importance of conducting pilot studies to determine the water solubility of the test substance before the initiation of the exposure (OECD, 2016a). Such considerations are crucially important when aligned with the 3R principles of animal research (Replace, Reduce, Refine). Had a pilot study been conducted, it would have provided more reliable results and refined the methodological approaches implemented while reducing the need for further *in vivo*

explorations using a flow-through system (and thus reducing the need for additional animal testing). Furthermore, the considerable challenges encountered in the LC-MS/MS analysis of the flow-through study have restricted the quantification of the dosed DUT concentrations, consequently adding another barrier to the comprehensive evaluation of the findings. These analytical limitations reinforce the significance of thorough method optimisation before initiating exposure of organisms to the test substance.

The lack of mechanistic underpinning in the regulatory testing of endocrine disruption in molluscs requires further histopathological, molecular, chemical, and potentially, toxicological investigations. Analysis of the preserved *B. glabrata* soft body mass tissues following the completion of the static-renewal study has the potential to provide a more mechanistic understanding of DUT disruption in these animals. Accordingly, lipidomic tests on adult *B. glabrata* tissues exposed to DUT could reveal adversely affected hormonal substrates, ultimately helping to elucidate the MoA of DUT on reproductive processes. Such analyses combined with further molecular investigations, could help confirm whether 5αR is indeed inhibited in molluscs following exposure to DUT. For example, the recent study of Cho *et al.*, (2024) implemented untargeted lipidomic and metabolomic analysis in adult *Daphnia magna* following chronic exposure to the 5αR inhibitor, finasteride (FIN), to reveal the differential expression of 464 lipids and 23 metabolites. These changes were consequently associated with disruption at specific metabolic pathways and reproductive processes in *D. magna*. Another study from the same group, employed molecular (RT-qPCR) and *in vitro* (reporter cell line assay) assays to evaluate the biological mechanisms associated with DUT disruption in zebrafish embryos (*Dario rerio*) (Cho *et al.*, 2025). By demonstrating the downregulation of various reproduction-related genes and insights into androgen (AR) and oestrogen receptor (ESR) interactions, they offered a mechanistic understanding of DUT disruption in these animals. Moreover, histopathological analysis of the preserved *B. glabrata* adult tissues can provide critical information on tissue-specific alterations in response to DUT. Such methodologies were previously applied to evaluate morphological and cellular changes in the albumen gland, ovotestis (i.e. gonads) and glandular complex tissues of *B. glabrata* following exposure to the synthetic androgen methyltestosterone (MT) and the vertebrate steroid dihydrotestosterone (DHT) (Kaur *et al.*, 2016). While histopathological changes in reproductive tissues alone cannot serve as indicators of endocrine disruption in molluscs, they can be valuable biomarkers for detecting cellular toxicity in these animals. Combined with findings from future lipidomic and molecular investigations, this data could also help to assess whether further *in vivo* toxicological investigations, using other mollusc-model organisms, may be required (OECD, 2018a).

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Appendix S1 (Chapter 2): Raw data and supplementary information

All supporting documents for Chapter 2 can be found in Appendix S1 which can be accessed at https://doi.org/10.5281/zenodo.14311002.

Appendix S2 (Chapter 2): Systematic evidence map protocol

1. Introduction

1.1. Molluscs as biomarkers of pollution:

Molluscs represent one of the most diverse phyla of the animal kingdom with seven molluscan classes and more than 130,000 species (Oehlmann *et al.*, 2007). Molluscan species represent a significant part of the biodiversity and are remarkably influential for ecosystem functioning (Oehlmann *et al.*, 2007). Apart from their ecological importance, molluscs also play a key role in ecotoxicological research; they possess a range of characteristics that make them attractive biomarkers for the study of pollution in both aquatic and terrestrial environments. The occurrence of imposex in gastropod snails, following exposure to the organotin tributyltin (TBT), has drawn major scientific attention over the past three decades and represents one of the most widely studied cases of endocrine disruption in aquatic invertebrates (BLABER, 1970; Gibbs et al., 1987). To date, the underlying mechanisms of TBT-induced imposex in molluscs have yet to be fully elucidated. This is due to the lack of our fundamental understanding of molluscan endocrinology which has also led to failure in regulating the distribution of TBT in the past. Although TBT is well regulated now, the biggest challenge for ecotoxicologists is to prevent these detrimental chemicals entering the environment rather than stopping them once they have caused significant effects on wildlife populations. Hence, if an adequate understanding of molluscan endocrinology or appropriate regulatory testing is not achieved, it is expected that mollusc populations might be at risk of similar consequences in the future.

1.2. Molluscan vs vertebrate steroidogenesis:

Over the years, several vertebrate-type steroids and steroidogenesis-related genes have been identified in molluscan tissues. The presence of androgens (i.e. testosterone) in the tissues of some gastropod molluscs had led to the assumption that these species have the ability to biosynthesise or metabolise vertebrate steroids *de novo* (Lafont, 1991; Lafont & Mathieu, 2007; Lehoux & Sandor, 1970). Recently, however, hesitations have arisen on whether molluscs use vertebrate-type steroids in their reproduction and development. Findings of Kaur et al., (2016) demonstrated that exposure of *Biomphalaria glabrata* (Mollusca: Gastropoda) to potent vertebrate androgens (i.e. 5α-dihydrotestosterone (DHT) and 17α-methyltestosterone (MT)) had no effect on neither the growth nor the development of the species' reproductive organs. Such evidence showcases the unlikelihood of vertebrate steroid androgens being involved in the reproductive development of these freshwater gastropods. Additionally, genomic searches have shown that the genetic material of molluscs (and other invertebrates)

does not contain the cholesterol side-cleavage enzymes (CYP11A) which are essential for vertebrate sex hormone steroidogenesis (Markov *et al.*, 2017). Hence, the lack of CYP11A enzymes from the molluscan genome reflects the inability of these animals to process cholesterol and thus biosynthesise vertebrate-type steroids *de novo* (Adema *et al.*, 2017). Therefore, it is fair to assume that the structure of molluscan sex hormones might differ than those of vertebrates, although their identification has yet to be achieved.

1.3. Insect vs molluscan steroidogenesis:

In insects and nematodes, steroid hormones are considered necessary for controlling the development, metamorphosis, and diapause of those organisms. Similar to vertebrates, insects use cholesterol as a precursor for their steroid biosynthesis. Insects can also use plant sterols as precursors for steroidogenesis although these sterols, including cholesterol, must be obtained from their diet (Niwa & Niwa, 2014). Apart from vertebrate-type steroids, arthropod steroids (i.e. ecdysteroids) have also been identified in molluscs (Whitehead, 1977) in rather 'ancient' experiments. Consequently, those studies have failed to provide evidence of ecdysteroid biosynthetic pathways in molluscs as no orthologous genes involved in arthropod steroidogenesis have been identified in their tissues (Adema et al., 2017; Lafont & Mathieu, 2007). Unlike molluscs, a great number of molecules responsible for the regulation of steroidogenesis in Ecdysozoa have been successfully identified and characterised over the past 20 years. A careful investigation of how enzymes that metabolise ecdysteroids are conserved within arthropods, can provide insights into the diversification that exists amongst steroids and between different phyla (Niwa & Niwa, 2014).

Although the synthesis of ecdysteroids is based on cholesterol, insects use a different steroidogenic enzymes than vertebrates. Amongst them, the functions of the enzymes Neverland (Nvd), Phantom (*CYP306A1*), Disembodied (*CYP302A1*), Shadow (*CYP215A1*) and Shade (*CYP314A1*), have been validated both in vivo and in vitro (Niwa & Niwa, 2014). The first step of steroidogenesis in insects is the conversion of cholesterol to 7-dehydrocholesterol (7-dC) which is modulated by Nvd. On the other hand, the conversion of 5β-ketodiol to 20-Hydroxyecdysone (20E) is catalysed by a sequence of cytochrome P450 monooxygenases namely *CYP306A1, CYP302A1, CYP215A1* and *CYP314A1* (Niwa et al., 2005; Niwa et al., 2004; Petryk et al., 2003; Warren et al., 2002, Warren et al., 2004).

1.4. The use of sterols in molluscan steroidogenesis:

Sterols are a subgroup of steroids that can be found on the cell membranes of animals, plants and microorganisms and they possess the ability to bind on and condense the lipid bilayer (Urich and Urich, 1994). Cholesterol is the most important type of sterol in animal tissues and functions as a precursor to steroid hormones and fat-soluble vitamins. However, the potential

endogenous function of sterols in molluscs remains unclear. Notable is the identification of aromatised sterols (i.e. 'paraestrols') in cnidarians and sponges (Markov *et al.*, 2017). Paraestrols are metabolites of cholesterol that have not been subjected to side-chain cleavage, and are thought to be the original ligands for the 'ancestral' steroid oestrogen receptor receptor (ER) in both molluscs and vertebrates. It has been speculated that paraestrols were probably not as efficient in binding to ER as other compounds (i.e. steroids), which resulted from the evolution of side-chain cleavage in vertebrates (Markov *et al.*, 2017). It was also demonstrated that sterols (including testosterone and aldosterone) are substrates of the steroidogenic enzyme 5-alpha-reducatse (5αR) which is present in both molluscan and plant genomes. Thus, further examination of sterol derivatives might shed a light on the elucidation of molluscan endocrinology and the biosynthesis of hormones in molluscs (Fodor *et al.*, 2020).

1.5. The role of nuclear receptors in molluscan endocrinology:

Evaluation of the hormonal function in molluscs does not solely rely on the presence of steroids in their tissues, but also on the occurrence of specific nuclear or membrane bound receptors in their genomes (Ni, Zeng and Ke, 2013). Apart from vertebrate-type and invertebrate-type steroids, several nuclear hormone receptors have also been identified or suggested to exist in molluscs. Nuclear receptors (NRs) belong to a family of ligand-modulated transcription factors specific to metazoans that are known to translate signalling messages of molecules into transcriptional responses (Miglioli *et al.*, 2021). Most signalling pathways, therefore, involve the binding of a signalling molecule (e.g. a ligand) to NRs which in turn trigger events inside the cell. Upon binding, the nuclear receptor goes through a conformational change known as receptor activation. Amongst others, typical ligands for nuclear receptors can include a range steroid hormones such as progesterone and testosterone, as well as retinoids which are derivatives of Vitamin A (Campbell et al., 2014).

In vertebrates, NRs are key players in several embryological and physiological processes including reproduction, metabolism, and development (Miglioli *et al.*, 2021). It is also thought that NR signalling plays an important role in the regulation of several developmental processes in marine invertebrates. Due to their ligand-dependent activity, NRs are susceptible to endocrine disruption. Consequently, a certain set of vertebrate NR orthologs including the retinoic acid receptor (RAR), the retinoid X receptor (RXR) as well as oestrogen receptors (ER, ERR) have now started to be characterised in invertebrates with the aim of developing a greater understanding of their underlying mechanisms (Miglioli *et al.*, 2021). To date, we have developed a good understanding of how NRs are able to regulate transcription though ligand binding, however our knowledge around NR action has been heavily focused on vertebrate

models. Hence, questions still remain about the evolutionary origin of NRs and particularly, around the evolutionary elaboration of certain ligand-receptor pairs (Gutierrez-Mazariegos, E. K. Nadendla, *et al.*, 2014). Lecroisey et al., 2012 demonstrated that certain ligand-receptor pairs might have undergone modifications in their ligand binding processes during animal microevolution which is further evidence that NRs might have not remained unaffected over time. Additionally, in contrast to what was initially thought, orthologs of oestrogen receptors (ER) that have been previously identified in molluscs were shown to be insensitive to oestradiol and thus incapable of binding oestrogens (Iguchi et al., 2007; Thornton & Need, 2003; Tran et al., 2016). Similarly, the presence of retinoic acid receptors (RAR) in the genomes of lophotrochozoa (e.g. molluscs, annelids) does not necessarily mean that RARs possess a functional ability (Gutierrez-Mazariegos et al., 2014). It has been shown that RARs identified in molluscs were unable to bind and thus activated by their respective retinoic acid ligands, 9-cis-RA and all-trans-RA (André *et al.*, 2019). Hence, this is a further indication that certain NRs found in molluscs have possibly lost their functional ability during evolution (Gutierrez-Mazariegos, E. K. Nadendla, *et al.*, 2014).

In contrast, orthologs of the Retinoid X receptor (RXR) identified in molluscan tissues were previously shown to be involved in endocrine disruption processes and are thought to play an important role in molluscan sexual development. In vertebrates, RXR is a key player in cellular endocrine processes with its unique ability to act as a heterodimer or homodimer with other NRs (including RAR and the peroxisome proliferator-activated receptor, PPAR)(Fonseca *et al.*, 2020). Recent findings suggest that TBT-induced imposex in molluscs is a result of the unusual modification of the RXR signalling pathway caused by TBT-exposure (Horiguchi, 2017). The binding of TBT to RXR was shown to initiate the activation of PPARγ, that functions as a heterodimerisation partner of RXR, which in turn induces imposex in female molluscs (Giulianelli *et al.*, 2020). This data suggests that retinoids as well as their interaction with the RXR/ PPARγ complex play a significant role in the development of male reproductive organs in aquatic molluscs (Giulianelli et al., 2020). Although it is still unclear as to what extent the adverse effects of endocrine disrupting chemicals are intervened by NRs in invertebrates, it seems likely that NRs have an active role in the embryonic and post-embryonic development of these animals (Bodofsky et al., 2017; Handberg-Thorsager et al., 2018; Vogeler et al., 2016).

1.6. Steroidogenesis-related genes identified in molluscs:

In most recent years, the advances of genome sequencing technology are used in molluscan models for the discovery of genes and key enzymes that could be involved in molluscan steroidogenesis. Consequently, the identification of vertebrate-type steroid hormones in

molluscs needs to be accompanied by fundamental evidence of their biosynthetic pathways that confirms their involvement in endogenous steroidogenesis. For example, Thitiphuree et al., 2018, has recently confirmed the expression of several steroidogenic genes in molluscs that are known to encode steroid metabolising enzymes. Amongst them, Thitiphuree et al., 2018 revealed an ever-present expression of the steroid metabolizing enzymes star*, cyp17a, hsd17b*, and *hsd3b* in the peripheral and gonadal tissues of the scallop *Mizuhopecten yessoensis*. Additionally, the study demonstrated that *hsd3b* and *hsd17b* genes showed a synchronous pattern related to gonad maturity levels, suggesting their possible involvement in scallop steroidogenesis (Thitiphuree et al., 2018).

Moreover, despite previous indications on the absence of vertebrate-type androgens in the molluscan reproductive development, homologues of the steroidogenic gene 5-alpha-reductase (5αR) have been identified in the genome of the freshwater gastropod *Biomphalaria glabrata* (Adema et al., 2017). In vertebrates 5αR is known to convert testosterone (T) to 5α-dihydrotestosterone (DHT), as well as other steroids such as cortisol to 5α-corticosterone or progesterone into 5α-dihydroprogesterone (Baynes *et al.*, 2019). In *B. glabrata* snails, homologues of 5αR were found to be expressed during embryonic development although the role of this enzyme in gastropods remains unknown. Previous observations of 5αR transcripts in the mantle tissue of *B. glabrata* (Adema *et al.*, 2017) raised questions regarding the potential link of this enzyme with molluscan shell formation. Interestingly, mammalian 5αR has been shown to exhibit significant sequence similarities with DET2, the prime enzyme used for the synthesis of plant steroids (i.e. brassinosteroids) (Li and Chory, 1999). In plants, DET2 catalyses the conversion of campesterol to campestanol. Surprisingly, under experimental conditions it was also shown that DET2 is able to convert T to DHT which suggests that DET2 is a functional ortholog of 5αR in plants (Li *et al.*, 1997).

2. Scientific rationale and methodological approach:

The continuing debate around the presence of vertebrate-type steroids in molluscs as well as their possible involvement in molluscan reproduction and development, reflects the gap of knowledge that exists within molluscan endocrinology. Although previous attempts in addressing the occurrence of vertebrate-type steroids as well as the expression of steroidogenesis-related genes in molluscs (Cuvillier-Hot & Lenoir, 2020; Horiguchi & Ohta, 2020; Scott, 2012) are informative, doubts remain as to whether these steroids possess a functional role. Missing is a systematic collection of evidence on the presence of different hormones, hormone receptors and hormone-metabolising enzymes identified in Mollusca. This systematic evidence map aims to: (1) provide a comprehensive assessment of our current understanding of hormone biosynthesis in molluscs through an appropriate evaluation

of the evidence that exists in the wider literature and (2) highlight gaps in our knowledge for future research. In the hopes of developing a more thorough understanding of molluscan endocrinology this review will thus encompass three research questions:

- What evidence is there for different hormones in molluscan tissues?
- What evidence is there for different hormone receptors in molluscan tissues?
- What evidence is there for different hormone-metabolising enzymes in molluscan tissues?

The protocol was drafted according to the PRISMA-P (Preferred Reporting Items for Systematic review and Meta-Analysis Protocols) 2015 checklist (Shamseer *et al.*, 2015) as well as the "Recommendations for the conduct of systematic reviews in toxicology and environmental health research (COSTER)" (Whaley *et al.*, 2020). A draft version of the protocol was published on Zenodo on 14th July 2021 (Panagiotidis, 2021)(doi: 10.5281/zenodo.4693859) and reviewers were invited to send their comments until 17th of September 2021. No responses were received via the form provided, however, feedback was collected via email communication (Panagiotidis, 2022). Upon receiving feedback from reviewers, the protocol was updated extensively, and the second version was uploaded on Zenodo on 8th September 2022 (Panagiotidis, 2022) (doi: 10.5281/zenodo.7061510). The final amendments made to the protocol are documented in the Supplementary Material S7.

2.1. Eligibility criteria

Each research question has been defined by a separate PO (Population, Outcome) statement as well as PO-specific inclusion & exclusion criteria. The inclusion & exclusion criteria presented in the draft protocol (Panagiotidis, 2021) were revised upon pilot screening activities and are documented in the second version of the protocol (Panagiotidis, 2022). The eligibility criteria were further updated alongside the publication of the systematic map manuscript and presented on Table 1. The changes made to the eligibility criteria are discussed in detail in the Supplementary Material S7.

Table S2.1: PO statements and inclusion & exclusion criteria.

PO Statements		**Inclusion criteria**	**Exclusion criteria**
1. The presence of hormones in molluscan tissues – "Mollusca AND Hormones"			
Population	Mollusca (covering Bivalves, Gastropods, cephalopods, polyplacophores, scaphopods, aplachophora, monoplachophora) of any life stage (embryos, juveniles,		

	adults) and sex (female, male, hermaphrodite).		
Outcome	-Presence of hormones (vertebrate-type and invertebrate-type) in molluscan tissues which include: *Primary outcomes*: -Mollusc class, species, sex -steroid/sterol measured, -their reported concentrations or range of reported concentrations, -tissue observed. *Secondary outcomes:* -concentration variability between seasons if reported (i.e. seasonality), -change of steroid concentrations due to an intervention (e.g. chemical exposure), -method specifications (LOD, method name, extraction procedure, if positive and/or negative controls were used, method validation, etc), - repetition of experiments.	Objectively measured vertebrate-type steroids and invertebrate-type steroids (e.g. ecdysteroids). Objectively measured retinoids involved in the retinoic acid pathway (retinoic acid, retinal, retinol). Objectively measured hormones involved in thyroid signalling pathway. Studies that examined the biological significance of objectively measured sterol concentrations in molluscs and consequently highlight important information about molluscan biology (e.g. how sterols are used in or by the mollusc). Objectively measured hormones from control (i.e. non-exposed) animals, from ecotoxicological studies that have used a chemical intervention (e.g. exposing molluscs to a steroid or a set of steroids).	Data from studies published before 2012. Data from review studies. Neurohormones (e.g. dopamine, serotonin, etc) or anabolic hormones (e.g. insulin). Hormones measured in environmental monitoring studies examining bioaccumulation of contaminants in molluscs (e.g. synthetic steroids including hormones that have derived from pharmaceutical products, such as growth promoters or any other hormones that are considered polluting substances instead of an endogenous synthesis). Hormones derived from studies that have measured sterol/lipid composition in molluscs but do not provide important information about their biological significance in molluscs (e.g. studies investigating the dietary significance of sterols).
2. The presence of hormone receptors in molluscan tissues: "Mollusca AND Receptors"			
Population	Mollusca (covering Bivalves, Gastropods, cephalopods, polyplacophores, scaphopods, aplachophora, monoplachophora) of any life stage (embryos, juveniles, adults) and gender (female, male, hermaphrodite).		
Outcome	Presence of receptors (by examining the expression of receptor genes or proteins) in	Objectively measured hormone receptors, including retinoid receptors and	Data from studies published before 2012.

	molluscs which include: *Primary outcomes:* -Mollusc class, species, gender, -receptor identified, -tissue that is expressed -their relative expression compared to housekeeping gene(s) (if applicable). -evidence of receptor's activity *Secondary outcomes:* -changes in expression due to an intervention (e.g. between seasons, chemical exposure, etc), -Method specifications (method type, method name, validation of housekeeping genes), -data on conservation analyses amongst receptors (e.g. phylogenetic trees and/or sequence conservation analyses showcasing homologies amongst ligand- or DNA-binding domains, between different species/class/phyla, etc). -repetition of experiments.	receptors known to be directly or indirectly involved in hormone signalling pathways, including thyroid hormone signalling.	Data from review studies. Neurohormone receptors. Data from silico/computational studies which did not conduct any molecular work. Data from transcriptomic studies or wide-genome identification studies. Data extraction from studies of such nature is difficult to be achieved with maximum accuracy and efficiency. Thus, data from such studies will only be used as a point of reference for the discussion of results.
3. The presence of hormone-metabolising enzymes in molluscan tissues: "Mollusca AND Enzymes"			
Population	Mollusca (covering Bivalves, Gastropods, cephalopods, polyplacophores, scaphopods, aplachophora, monoplachophora) of any life stage (embryos, juveniles, adults) and gender (female, male, hermaphrodite).		
Outcome	Studies that have measured the expression of hormone-metabolising proteins or genes (vertebrate origin and/or invertebrate origin) in molluscs, which include: *Primary outcomes:* -Mollusc class, species, gender, -hormone-metabolising enzyme/gene identified, -tissue that is expressed,	Objectively measured hormone-metabolising enzymes or genes. These include key enzymes involved in vertebrate and invertebrate steroidogenesis (e.g. insect steroidogenesis), thyroid hormone signalling, as well as transport proteins and enzymes involved in the production and	Data from studies published before 2012. Data from review studies. Observations on enzyme or gene expression after chemical interventions (e.g. gene expression in exposed molluscs).

	-relative expression reported, -evidence of steroidogenesis-related gene activity <u>*Secondary outcomes:*</u> -changes in expression due to an intervention (e.g. between seasons, chemical exposure, etc), -Method specifications (method type, method name, validation of housekeeping genes), -data on conservation analyses amongst hormone-metabolising enzymes/ genes (e.g. phylogenetic trees and/or sequence conservation analyses showcasing homologies amongst gene(s) of interest and between different species/class/phyla, etc). -repetition of experiments.	breakdown of retinoids (e.g. enzymes in the metabolism of Vitamin A/retinol including RBP*, CRBP*, SDR*, CYP26). For chemical intervention studies, data on enzyme or gene expression will be obtained only from control (non-exposed) animals. *RBP: Retinol Binding Protein, CRBP: Cellular retinol binding protein, SDR: short-chain dehydrogenase/reductases (Kin *et al.*, 2012).	Studies that examined neurological processes related to retinoids. Studies that investigated vitellogenin expression. Enzymes involved in metabolising neurohormones. In silico/computational studies which did not conduct any molecular work. Transcriptomic studies or wide-genome identification studies. Data extraction from studies of such nature is difficult to be achieved with maximum accuracy and efficiency. Thus, data from such studies will only be used as a point of reference for the discussion of results. Enzymes of the CYP450 superfamily involved xenobiotic metabolism and detoxification processes (according to the study aims/objectives). Usually members of the CYP1, CYP2 and CYP3 families, these enzymes include: CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. *Note:* Due to the nature of many CYP450 enzymes being involved in the metabolism of both xenobiotic and endogenous compounds, excluded studies will be assessed according to their relevance with the

			objectives of this protocol.

2.1.1. Study design

Eligible studies included in the systematic evidence map were any peer-reviewed publications that have objectively measured hormones, receptors, and/or hormone-metabolising enzymes in any molluscan species. Data from review studies was not extracted, and thus not incorporated in the evidence report. However, review studies identified as relevant to the PO statements were used as supporting material for the discussion of the extracted data. Specific characteristics of eligible studies can be seen on Table 1.

2.1.2. Timing

A date range for inclusion has been applied to studies that were published between 2012 and onwards. This decision was made in consideration to the following criteria:

- Molluscan genome sequencing became more prominent during the past decade (Takeuchi, 2017) by which advances in this technology had revealed an ever-present expression of genes associated with hormone metabolism in molluscs including hormone receptor transcripts. Thereby, the identification of whole-genome sequencing in an organism, and particularly in molluscs, makes gene identification analysis more comprehensive and consequently results in better quality reports. A fully annotated (or at least semi-annotated) genome comprises of important information about the functional elements along the entire genome's sequence, thus providing an explanation to DNA sequences of unknown function (Abril and Castellano, 2019). DNA sequencing technology has increased the quality and completeness of whole genome analyses, which can in turn be used to gain insights into various biological processes (Abril and Castellano, 2019). This criterion is relevant to studies from the POs "Mollusca AND Receptors" and "Mollusca AND Enzymes", respectively.
- During the last decade, the use of chemical analysis (e.g. GC-MS, LC-MS) has been proven to exhibit higher satisfactory precision in detecting low concentrations of steroids and other metabolites than traditional immunoassay methods (Gust *et al.*, 2010; Krasowski *et al.*, 2014). Until the emergence of this evidence, immunoassays used to be a common practice in molluscan endocrinology research and were generally considered reliable. Consequently, this criterion is relevant to studies of the "Mollusca AND Hormones" PO.

The date range was applied in this case, to eliminate bias or outdated information from the data extraction inventory which could in turn affect the quality of the evidence report.

2.1.3. Language

Of interest were articles reported in the English language.

2.1.4. Setting

No restrictions were applied by the type of setting of each study.

2.2. Information sources:

The searches for peer-reviewed papers and articles were conducted in the following bibliographic databases:

- PubMed
- Web of Science
- Scopus

Data on the identification of hormones, hormone receptors and hormone-metabolising enzymes in Mollusca were considered eligible for inclusion only if presented in peer-reviewed literature conducted in an academic or research environment. Grey literature was not searched due fears of including inaccurate or misleading information, that could negatively affect the quality of the present work. Manual searches of the citations and bibliography of eligible studies were carried out in case where highly important data might have been absent or not explicitly addressed in the study of interest. Date limitations on retrieved papers were not applied during literature search, as this was exhibited to result in significant inaccuracies during citation export in trial searches (e.g. the number of exported citations did not match the number of retrieved papers during the original search when a date limit was applied). Alternatively, all retrieved papers were exported from each database individually and were assessed for duplicate removal in combination (using the reference management software Zotero), and thus for inclusion (using the screening tool Rayyan), based on the eligibility criteria mentioned above.

2.3. Search strategy:

2.3.1. Defining ‘Population’

To devise a specific search strategy that is inclusive of all-important literature, we piloted three sets of keyword strings relevant to our research questions and PO statements. Population has been defined as “Mollusca” and consisted of all seven molluscan living classes (Gastropoda, Bivalvia, Polyplacophora, Cephalopoda, Scaphopoda, Aplacophora, Monoplacophora). The

two extinct classes of Mollusca (Rostroconchia and Helcionelloida) were excluded from the search strategy. Additionally, several mollusc-specific terms including “oysters”, “mussels”, “squids” and “chitons” were also defined as key objectives and included in the ‘Population’.

2.3.2. Defining ‘hormones’, ‘hormone receptors’ and ‘hormone-metabolising enzymes’

Throughout the years, the presence of both “vertebrate-type” and “non-vertebrate” steroid hormones have been reported in molluscs, although their origin and synthesis has yet to be elucidated. This systematic map combined all available evidence from 1st January 2012 to 10th September 2021 (the day all papers were extracted) on the identification of different hormones, hormone receptors as well as hormone-metabolising enzymes reported in molluscan tissues. Data on 24 steroid hormones known to be involved in vertebrate steroidogenesis was extracted from the article of Häggström & Richfield (2014) "Diagram of the pathways of human steroidogenesis" and from the critical evaluation of Fodor et al., (2020). Additionally, 4 identified ecdysteroids (insect steroids) involved in arthropod steroidogenesis were extracted from Niwa & Niwa (2014) and were included as part of the “Mollusca AND Hormones” search string. To avoid missing important literature, generic terms such as “sterols”, “hormones” as well as synonyms for each included steroid were identified and included in the search. Data on retinoids was captured via the “Mollusca AND Receptors” keyword string while data on hormones involved in neurohormonal signalling was outside the scope of this review.

In the draft protocol (Panagiotidis, 2021), the search string for “Mollusca AND Receptors” comprised of a set of general receptor terms connected using the Boolean operator ‘AND’ to an additional 147 receptor names found to be expressed in molluscs. These were initially extracted from the papers of Vogeler et al. (2014) and Kaur et al. (2015). However, upon revision of the search strategy, we have noticed that the additional “AND” was narrowing down the searches on the specific receptors: Mollusca AND “general terms for receptors” AND “other specific terms”. To make the search more inclusive, the string was updated as part of the second version of the protocol (Panagiotidis, 2022). There, general terms for hormone receptors were added and the specific receptors names were removed. Consequently, the “Mollusca AND Receptors” PO aimed to collect data on the occurrence of hormone receptors, including retinoid receptors and those known to be directly or indirectly involved in other hormone signalling pathways, such as the thyroid hormone signalling pathway. Data on receptors involved in neurohormonal signalling was outside the scope of this review.

Finally, the keyword string for the “Mollusca AND Enzymes” PO initially aimed to capture information on the enzymes involved in vertebrate steroidogenesis, insect steroidogenesis, and retinoid signalling (Panagiotidis, 2022). However, an interest in thyroid signalling enzymes

was developed after the publication of the second protocol. These were included in the eligibility criteria as part of the systematic map manuscript. A list of genes encoding for enzymes involved in vertebrate steroidogenesis were identified and extracted from Wikipedia. The 'Mollusca AND Enzymes" string comprised of 44 terms identified as the encoding genes for every steroidogenic enzyme previously found in vertebrates. In addition, 10 gene names known to be involved in insect steroidogenesis, were identified from the Niwa & Niwa (2014) paper and included in the search. The search also included names of key transport proteins and enzymes involved in retinoid signalling. To avoid missing important literature, our search strings comprised of gene synonyms and topical vocabulary for steroidogenesis-related processes. The amended search strategy and the updated search strings can be found in Supplementary Material S3.

2.4. Data management:

Initially, the systematic evidence map and literature review process would have been managed with the support of the online tool CADIMA (Kohl *et al.*, 2018). However, trial screening activities found CADIMA to be over-complicated and more time-consuming in managing and screening literature. Thus, the online tool Rayyan (Ouzzani *et al.*, 2016) was chosen as the most appropriate for the conduct of those activities.

2.4.1. Relevance screening

Duplicate records from all exported citations were removed with the help of the reference management software Zotero. After duplicate removal, the remaining records were imported to Rayyan for screening. The list of inclusion & exclusion criteria was applied to 20% (pilot screening) of the merged citation list in duplicate (i.e. by two coders working independently) in two different stages. The first stage involved the pilot screening of title and abstract of obtained studies to identify relevance with the research questions. This had led us to update the eligibility criteria in the second version of the protocol (Panagiotidis, 2022). The online tool Rayyan facilitated the process of consistency check by identifying conflicted decisions between the two coders. Disagreements during pilot screening were resolved through collaboration of the two coders, which has thus allowed the amendment of eligibility criteria and the continuation of the screening process by one of the two evaluators. The results of pilot screening activities are summarised in the supplementary material of the second protocol (Panagiotidis, 2022). The second stage of the screening process involved the full text screening of the selected studies. The eligible studies were then included in the data extraction inventory. The reason for exclusion was recorded during full text screening.

2.4.2. Data extraction

The presence of hormones, hormone receptors as well as hormone-metabolising enzymes in molluscs differed in terms of chemical structure, concentrations or expression levels, tissues they were expressed, methodological approaches taken, and more. The preliminary form of the data extraction template aimed to collect essential information to be used in an evidence synthesis, specific to each research question. Pilot screening revealed an even greater number of factors that are relevant to the scope of this review, and particularly helpful for the Risk-of-Bias assessment of individual studies. To interpret this diversity, the preliminary data extraction template was updated extensively for each PO statement in the second revision of the protocol (Panagiotidis, 2022). To improve clarity, the data extraction template has since been slightly amended and its final version can be viewed alongside the published manuscript. The final version of the data extraction template criteria, for the PO "Mollusca AND Hormones" looks something like this:

- "Mollusca AND Hormones" (PO 1):
    - Hormone abbreviation
    - Type of hormone
    - Extraction method
    - Purification method
    - Intervention used?
    - Type of intervention
    - Effect of intervention
    - Was a ligand binding assay used? *
    - Were tissues spiked with a positive control or Internal standard? *
    - Was a negative control used in the test? *
    - Recovery and reproducibility of the method*
    - Number of measurements between animals (biological replicates), *
    - Number of measurements within animals (technical replicates), *
    - Strategy to minimise potential confounders. If NO strategy was used, was this clearly stated? *
    - Within-study or between-study repetition (Independently verified experiments - separated analyses) *
    - Was the experiment repeated independently? If YES, how many times?*
    - Comments on the activity/function of the hormone.
    - Pathway involved
    - Structure based on/similar to
    - Receptor that interacts
    - Notes

- Other comments

The criteria marked with an asterisk (*) were primarily used for the Risk-of-Bias assessment. Full details on the amendments made to the final version of the data extraction template are summarised in the Supplementary Material S7..

Piloting of the data extraction template was performed independently by two evaluators using a handful of eligible studies. Discrepancies were resolved during in-person meetings between both evaluators, where individual piloting activities of the data extraction template were compared, and conflicts were addressed. Following amendments, further piloting of the data extraction template was carried out by a single evaluator using nine eligible studies (three studies per outcome). The piloting outcomes of the data extraction template can be seen in the supplementary material of the second protocol (Panagiotidis, 2022). Following piloting, data extraction of all eligible studies was conducted by a single evaluator.

2.4.3. Risk-of-bias assessment

Inadequacies in the analysis or experimental design of individual studies can result to incorrect assumptions about the origin and synthesis of sex hormones in molluscan tissues. Critical appraisal tools can be used to assess the internal validity of studies through selection bias, detection bias (reliability of outcome measurements), performance bias and so forth (Martin *et al.*, 2021). To date, many risk-of-bias tools were created for chemical studies involving molluscs, but none of them has ever assessed the internal validity of studies claiming endogenous synthesis of hormones in those organisms. For the purposes of this systematic evidence map, a tailor-made Risk-of-Bias tool has been developed to assess the quality of evidence on the presence of hormones, hormone receptors and hormone-metabolising enzymes in molluscan tissues. The tool consists of a series of criteria that aimed to evaluate the available evidence for each of the three PO statements. Included studies were assessed on both internal validity and study design criteria. Some of the criteria incorporated in the Risk-of-Bias tool were obtained from the ARRIVE guidelines 2.0 (Percie du Sert et al., 2020), which were specifically created to ensure transparency and thorough reporting in studies describing animal research.

The preliminary Risk-of-Bias tool has been amended upon receiving feedback from reviewers, and further amended after uploading the second version of the protocol on Zenodo (Panagiotidis, 2022). The amended tool follows a more thorough approach of assessing individual studies and it is meant to be used in accordance with the updated Risk-of-Bias guidelines. The guidelines consist of detailed information on how a study was coded in different case scenarios. The scoring method was replaced from “Fulfilled”, “Partially fulfilled”,

“Not fulfilled” and “Not determined” in the draft protocol (Panagiotidis, 2021), to “Definitely low risk”, “Probably low risk”, “Probably high risk” and “Definitely high risk” in the second protocol (Panagiotidis, 2022). Individual studies were assessed for risk-of-bias based on internal validity and study design criteria. Internal validity criteria were created individually for each PO statement based on peer-reviewed literature. However, reported outcomes across studies varied significantly and a one-fits-all approach was difficult to implement. Therefore, included studies were assessed for Risk of Bias either fully (based on internal validity & study design criteria) or partly (solely based on study design criteria). Specifically, two types of tailor-made Risk of Bias assessments were designed for this purpose:

- **Risk of Bias Assessment A** (internal validity & study design criteria):

  Eligible studies must provide clear information that ensures an appropriate fit to the research question. Thus, the aims of studies to be assessed with RoB Assessment A must have focused on the investigation of activity, function and/or MOA of hormones/ receptors/steroidogenesis-related genes in molluscs. Consequently, the study objectives should have adhered to a methodology which is considered appropriate for an internal validity assessment. Studies that did not employ appropriate methodology but still attempted to draw conclusions on the activity/function/MOA of the outcomes of interest were marked eligible for a Risk of Bias Assessment A. Studies that used multiple methods to examine a single outcome were assessed for the same outcome independently, based on the methodology implemented. For example, if a study used both a GC-MS and an RIA approach to examine the presence of steroids in a mollusc organism, the study was assessed for RoB twice in respect to each technique.

- **Risk of Bias Assessment B** (study design criteria only):

  Studies that did not fit within the scope of RoB assessment A. For example, ecotoxicological assessments investigating the downstream effects of pharmaceuticals by looking at changes in hormone concentrations or gene expression levels (of receptors or enzymes). If the study did not aim to determine (or attempted to comment on) the activity/function/MOA of an outcome of interest, it was assessed solely on study design criteria.

Studies that provided ill-defined detail about the reported outcomes were marked with “Unclear”, which is equivalent to a “Probably high risk” score. In cases where a single study addressed more than one outcome (e.g. Hormones and hormone receptors), it was assessed independently in respect to the PO statement. Every study assessed for Risk-of Bias received a final summary score, namely Level 1, Level 2 and Level 3. The Level 1 score represents

studies of lower risk of bias, Level 2 score represents studies with moderate risk of bias, whereas Level 3 score represents studies with higher risk of bias studies (Table 2).

Table S2.2: An example of the level scoring system and Risk-of-Bias "Assessment A" for studies that identified hormones in molluscs ("Mollusca AND Hormones" PO). Individual studies were assessed for Risk-of-Bias based on internal validity and study design criteria prior to the final categorical rating (Level 1: Lower risk of bias, Level 2: Moderate risk of bias, Level 3: Higher risk of bias). Plus (+) and minus (-) signs indicate hypothetical scoring of individual studies.

Mollusca AND Hormones - Risk of Bias							
Level 1: All internal validity criteria must be rated as either "**Definitely low risk**" or "**Probably low risk**". The majority of study design criteria (≥2) must be rated as "**Definitely low risk**" or "**Probably low risk**". **Level 2:** Studies that meet neither Level 1 or Level 3 criteria. **Level 3:** All internal validity criteria must be rated as either "**Definitely high risk of bias**" or "**Probably high risk of bias**". The majority of study design criteria (≥2) must be rated as "**Definitely high risk**" or "**Probably high risk**".		internal validity criteria		study design criteria		
		Identification of hormones in molluscs:	Verification of MOA of hormones:	Strategy to minimise potential confounders	Within-study or between-study repetition	Statistics
++	Definitely low risk of bias					
+	Probably low risk of bias					
-	Probably high risk of bias					
--	Definitely high risk of bias					
Level 1 (Lower risk of bias)	"Definitely low risk" or "Probably low risk" for internal validity criteria AND "Definitely low risk" or "Probably low risk" for study design criteria (≥2).	+	+	--	+	++
Level 2 (Moderate risk bias)	Studies that meet neither Level 1 or Level 3 criteria	--	++	-	+	--
Level 3 (Higher risk of bias)	"Definitely high risk" or "Probably high risk" for internal validity criteria AND "Definitely high risk" or "Probably high risk" for study design criteria (≥2).	--	-	-	-	+

Table S2.3: An example of the level scoring system for studies assessed under Risk-of-Bias "Assessment B" criteria. Eligible studies were assessed for Risk-of-Bias solely based on study design criteria prior to the final categorical rating (Level 1, Level 2, Level 3). Plus (+) and minus (-) signs indicate hypothetical scoring of individual studies.

General assessment - Risk of Bias categories				
Level 1: All internal validity criteria must be rated as either "**Definitely low risk**" or "**Probably low risk**". The majority of study design criteria (≥2) must be rated as "**Definitely low risk**" or "**Probably low risk**". **Level 2:** Studies that meet neither Level 1 or Level 3 criteria. **Level 3:** All internal validity criteria must be rated as either "**Definitely high risk of bias**" or "**Probably high risk of bias**". The majority of study design criteria (≥2) must be rated as "**Definitely high risk**" or "**Probably high risk**".		study design criteria		
		Strategy to minimise potential confounders	Within-study or between-study repetition	Statistics
++	Definitely low risk of bias			
+	Probably low risk of bias			
-	Probably high risk of bias			
--	Definitely high risk of bias			
Level 1 (Lower risk of bias)	"Definitely low risk" or "Probably low risk" for internal validity criteria AND "Definitely low risk" or "Probably low risk" for study design criteria (≥2).	--	+	++
Level 2 (Moderate risk bias)	Studies that meet neither Level 1 or Level 3 criteria	-	+	--
Level 3 (Higher risk of bias)	"Definitely high risk" or "Probably high risk" for internal validity criteria AND "Definitely high risk" or "Probably high risk" for study design criteria (≥2).	-	-	+

"Assessment A" studies rated with Level 1, must have received a score of "Definitely low risk" or "Probably low risk" for all internal validity criteria, and a score of "Definitely low risk" or "Probably low risk" for the majority of study design criteria. "Assessment A" rated with Level 2 were the ones that met neither Level 1 nor Level 3 scoring criteria (Table 2). Whereas, studies that rated with Level 3 score must have received "Definitely high risk" or "Probably high risk"

scores for all internal validity criteria and for most of the study design criteria (Table 2). Eligible studies for RoB Assessment B, were assigned a Level 1 score if all their study design criteria were rated as either “Definitely low risk of bias” or “Probably low risk of bias”. Studies that met neither Level 1 nor Level 3 criteria were assigned a Level 2 score, whereas studies rated with a Level 3 scored received a “Definitely high risk” or “Probably high risk” for all study design criteria (Table 3). The idea of the level system was obtained from the OHAT Approach for Systematic Review and Evidence Integration (National Institute of Environmental Health Sciences, 2015), and was adjusted to meet our outcome-specific criteria.

Initial piloting of the Risk-of-Bias tool was performed independently by two evaluators as described in section 2.4.2. Following initial amendments, the Risk-of-Bias tool was piloted using the nine eligible studies that were used in the data extraction template. Both of the Risk of Bias tool and Risk of Bias guidelines were additionally amended after publishing the second protocol (Panagiotidis, 2022). These changes are described in detail in the Supplementary Material S7.

3. Outcomes to be analysed

The primary outcome of this systematic evidence map was the extensive synthesis of an evidence report for collected data, in respect to each PO statement, conducted both individually and in combination. A thorough synthesis of collected evidence was carried out in separated chapters (according to each PO) highlighting key findings for different molluscan classes. The Risk-of-Bias assessment served as a primary outcome for this review. The assessment showcase multiple levels of reliability for the collected evidence, based on an extensive list of tailor-made Risk-of-Bias guidelines and case-scenarios for how a study was coded. The secondary outcome of this systematic map was to identify the knowledge gaps that exist within our current understanding of molluscan endocrinology. These were highlighted as part of a comparative evidence report which thoroughly evaluated the results obtained from the systematic data mapping.

4. Data analysis

In addition to an in-depth evidence report, the data collated for each outcome was visually summarised using the data visualisation software Tableau. Visual comparisons in the form of graphs and summary tables aimed to highlight the relationships amongst the outcomes of interest (e.g. hormones, hormone receptors, hormone-metabolising enzymes) with the rest of criteria included in the data extraction template (e.g. tissue observed, changes in expression due to an intervention, evidence of activity, etc). As it was discussed in the draft protocol (Panagiotidis, 2021), it was expected that experimental designs across studies and PO would vary, and so were the species examined in each study. In the field of environmental sciences,

good quantitative data is often rare and not always abundant and methodological details are usually insufficiently reported (Haddaway and Verhoeven, 2015). Thus, producing a quantitative answer to the research questions using meta-analysis was not be feasible in the present work.

5. Meta-biases

With meta-analysis being absent from this study, statistical methods for detecting meta-biases in the evidence report were not deemed possible to implement. To avoid reporting bias in this study, reviewers were welcomed to access both versions of the protocol on the open-access repository Zenodo and compare the reporting outcomes with those of the final manuscript. Any changes made in the reported outcomes following the submission of this protocol were explicitly stated in the final manuscript.

6. Strength of evidence

The COSTER (Whaley *et al.*, 2020) and PRISMA-P (Shamseer *et al.*, 2015) recommendations for using a strength of evidence assessment are primarily directed towards systematic reviews in the fields of clinical medicine or environmental health. Hence, the GRADE approach (Guyatt *et al.*, 2011) or an interpretation of such approach, was difficult to implement in the context of the present work. Instead, to account for confidentiality in the reported evidence, the collected data was arranged systematically according to a wide range of factors including mollusc class, sex, life stage and species name.

7. Reporting

The systematic evidence map adhered to PRISMA-P (Preferred Reporting Items for Systematic review and Meta-Analysis Protocols) 2015 checklist (Shamseer *et al.*, 2015) in consideration with the COSTER guidelines (Whaley *et al.*, 2020) and was incorporated into Konstantinos Panagiotidis' doctoral thesis. It was also written in the form of a peer-reviewed scientific article with permanent links to the protocol material.

Appendix S3 (Chapter 3): Raw data and supporting information

Table S3.1: RNA yields from preliminary RNA isolation tests of adult tissue samples (ovotestis, hepatopancreas, albumen gland, mantle) and a blank sample, using Macherey-Nagel RNA isolation kit. RNA yields were measured using NanoDrop microvolume spectrometer.

Date collected / fixaded	Date of RNA extraction	Sample name	Tissue name	Eluted volume (µl)	RNA yield (ng/µl - NanoDrop)	A260/280	A260/230	A260	A280
29/07/2021	28/09/2021	1.1	ovotestis	30	7.5	2.56	0.71	0.19	0.07
29/07/2021	28/09/2021	BLK	Blank	30	-0.2	1.23	0.68	0	0
29/07/2021	28/09/2021	1.2	hepatopancreas	30	0.5	3.23	-6	0.01	0
29/07/2021	28/09/2021	1.3	albumen gland	30	122.2	2.14	1.96	3.05	1.43
29/07/2021	28/09/2021	1.4	mantle	30	17.3	2.27	0.59	0.43	0.19
29/07/2021	29/09/2021	2.1	ovotestis	30	6.1	1.99	0.68	0.15	0.08
29/07/2021	29/09/2021	2.2	hepatopancreas	30	11.1	1.96	0.76	0.28	0.14
29/07/2021	29/09/2021	2.3	albumen gland	30	26.7	2.12	1.35	0.67	0.31
29/07/2021	29/09/2021	2.4	mantle	30	5.4	2.16	0.85	0.14	0.06

Table S3.2: RNA yields from albumen gland tissues, obtained the day before snail's oviposition. RNA isolation was performed using the Macherey-Nagel RNA isolation kit. RNA yields and dsDNA yields, before and after DNAse-I treatment, were measured using Qubit Fluorometer.

Date collected / fixaded	Date of RNA extraction	Sample name	Tissue name	Eluted volume (µl)	RNA yield BEFORE DNAse I (ng/µL - Qubit)	dsDNA yield BEFORE DNAse I (ng/µL – Qubit)	RNA yield AFTER DNAse I (ng/µL - Qubit)	dsDNA yield AFTER DNAse I (ng/µL - Qubit)
18/03/2022	22/03/2022	S.AG-1	Albumen gland	60	Too high	565	670.00	113.70
18/03/2022	22/03/2022	S.AG-2	Albumen gland	60	Too high	615	315.00	10.32
18/03/2022	22/03/2022	S.AG-3	Albumen gland	60	Too high	370	220.74	7.01
18/03/2022	22/03/2022	S.AG-4	Albumen gland	60	Too high	390	475.00	15.05

Section S3.1:

DNase I treatment protocol with GlycoBlue

Incubation:

- x ul of sample (x depends on your samples)
- Add x ul of 10x DNase I buffer
- Add 1 ul of DNase I (RNase free) – *You can also adjust it depending on your dsDNA yield*
- Mix well using a pipette
- Incubate at 37^{o}C for 10 min

Add to the mixture:

- 0.1x volume of 5M NaAc
- 1.5 ul of Glyco Blue
- 2.5x volume of ice-cold Ethanol 100% (keep in -20^{o}C before use)

Last part:

- Precool a centrifuge to 4^{o}C
- Centrifuge the samples at 13000rpm, 30min
- Remove the supernatant carefully
- Add 150 ul of ice-cold Ethanol 70% (keep in -20^{o}C before use)
- Centrifuge the samples at 13000rpm, 15min
- Remove the supernatant carefully
- Dry the pellet (should be light blue of GlycoBlue)
- Add the required amount of DEPC treated H_2O (30 µl)

Of note:

- Be quick with the procedure
- Prepare 100% Ethanol and 70% Ethanol and keep them in -20C prior to starting the procedure.
- Pre-cool the centrifuge at 4C and set it to run for 30 minutes before beginning with the procedure.

1. Measure the **dsDNA yield** of each of your samples using NanoDrop. Based on the concentration, calculate the required amount of DNase I enzyme you will need.

Convert your dsDNA yield(s) to µg/µl, and considering that 1µl of DNase I degrades 10µg of DNA (10 units per 1µl – written on the bottle), calculate the amount of enzyme you will need in order to degrade all the DNA you have in your RNA extracts (e.g. If you obtain 53 µg/µl of dsDNA yield, you will need 6 µl of DNase I – round it up to 6 µl) *.

**Note 1: If the DNase I enzyme is known to have less/weaker efficacy, you can increase the volume you use (e.g. if you need 6 µl of DNase I based on your calculations, you can use 7 µl instead).*

***Note 2: Make sure you keep your DNase I enzyme in the ice to thaw. You can keep your DNase I buffer outside of the ice bucket.*

2. During incubation:

Depending on the volume of your RNA extract (often either 30 µl or 60 µl) you need to add 1x of your 10x DNase I buffer (1:10 dilution of you DNase I buffer, so that from 10x concentrated, you will end up with 1x concentrated of buffer in your RNA extract).

Example
If you have a 60 µl RNA extract:
$C_1V_1 = C_2V_2$
(10x) (V_1) *= (1x) (60 µl)*
V_1 = 6 µl of Buffer in 54 µl of RNA.
However, because you want to use the full amount of your RNA extract, you can make a 70 µl solution, using 7 µl of buffer & 60 µl of RNA & 3 µl of H_2O.

Then you can proceed with adding the required amount of DNase I.

3. After incubation, you should add 0.1x of 5M NaAc based on your total RNA volume.

Example: If your RNA mixture is 77 µl, you will add: (0.1) (77 µl) = 7.7 µl of 5M NaAc.
Then you can add 1.5 µl of Glyco Blue.
After adding Glyco Blue, take your Ethanol 100% out of the freezer (it needs to stay ice-cold, because you are working with sensitive RNA) and add 2.5x of ice-cold Ethanol 100% to your mixture based on your total volume.
Example: If your total volume is now 80 µl, you will add (2.5)(80 µl) = 200 µl of 100% Ethanol.

4. When you remove the supernatant, you will notice a small blue dot at the bottom/side of the tube. The blue dot is the Glyco Blue Solution and indicates your RNA material. Make sure to remove the supernatant carefully, by tilting the tube on the side and pipetting out the 100% Ethanol WITHOUT pipetting out the blue dot. You need to be quick with this procedure – 1min.

5. Take your 70% Ethanol out of the freezer and add 150 µl of it into your RNA extracts. Centrifuge your sample(s) and repeat step 4. After removing the supernatant, dry the pellet/tube by placing it to the PCR fume hood (contains clean air to avoid contamination) and leave it for 15 minutes.

Table S3.3: Initial candidate reference genes identified from literature and tested for stability across embryo (day 2 – day post oviposition) and albumen gland tissues. Cq values are shown for the reverse transcriptase (RT) and No reverse transcriptase (No-RT) samples. Standard deviation (SD) was calculated from the Cq values of RT samples.

Candidate reference gene	Tissue	Cq value (RT)	Cq value (No-RT)	SD (RT)
Lrpl14	day2	24.36	3.55	6.24
		15.81	15.17	
		3.79	13.92	
	day3	12.82	15.37	
		3.26	20.74	
		13.76	3.36	
	day4	27.42	4.58	
		19.58	12.83	
		14.86	4.42	
	day5	17.89	3.83	

		20.09	4.02	
		18.99	12.82	
	AG	18.65	13.63	
		17.53	19.46	
		18.77	3.53	
a-TUB	day2	22.04	27.91	4.18
		22.04	27.90	
		22.08	27.85	
	day3	22.94	30.99	
		22.94	31.59	
		23.00	32.41	
	day4	18.28	29.65	
		18.26	29.31	
		18.25	30.27	
	day5	16.07	26.19	
		16.06	26.54	
		16.15	26.81	
	AG	28.21	39.24	
		28.15	32.22	
		28.34	-	
Mb	day 2	17.84	33.43	3.14
		17.78	32.64	
		17.83	32.37	
	day 3	18.26	-	
		18.00	-	
		18.30	39.49	
	day 4	14.12	35.12	
		14.08	31.57	
		14.26	31.58	
	day 5	13.02	30.56	
		12.99	31.17	
		12.82	30.09	
	AG	21.79	36.17	
		21.70	-	
		21.75	33.18	
Lywhaz	day 2	25.23	30.39	1.87
		25.38	30.46	
		25.28	30.51	
	day 3	27.27	30.15	
		26.99	30.38	
		27.14	30.36	
	day 4	23.66	30.03	
		23.40	29.97	
		23.67	30.29	
	day 5	23.11	29.82	
		23.16	30.06	
		23.01	30.03	
	AG	27.37	29.86	
		28.02	29.72	
		27.95	30.15	
Lhis2a	day 2	24.59	29.06	1.92
		24.49	31.42	
		24.52	28.68	
	day 3	26.83	31.81	
		27.23	27.46	
		26.15	28.14	
	day 4	24.07	29.60	

		24.14	31.23	
		24.14	31.91	
	day 5	23.52	27.77	
		23.56	25.70	
		23.70	31.02	
	AG	29.22	28.33	
		27.62	30.60	
		29.02	29.32	
Lst-EF1	day 2	30.44	36.87	1.85
		30.59	-	
		30.18	37.20	
	day 3	32.19	-	
		33.82	-	
		32.64	-	
	day 4	29.22	39.92	
		28.88	39.22	
		29.06	34.51	
	day 5	28.32	36.34	
		29.38	35.80	
		28.57	32.83	
	AG	32.65	39.64	
		33.36	-	
		32.77	-	
Lst-GAPDH	day 2	35.24	-	2.67
		37.15	39.06	
		35.75	-	
	day 3	37.94	-	
		-	-	
		-	-	
	day 4	32.29	-	
		31.84	-	
		32.55	-	
	day 5	31.64	-	
		32.13	-	
		31.54	33.13	
	AG	35.59	-	
		36.01	-	
		39.99	-	

Table S3.4: RNA yields observed from day 1 post oviposition embryos, using different quantities (number of embryos). RNA isolation was extracted using Macherey-Nagel RNA isolation kit. RNA yields were measured using NanoDrop microvolume spectrometer.

Date collected / fixaded	**Date of RNA extraction**	**Sample name**	**Stage**	**Number of embryos**	**Eluted volume (ul)**	**RNA yield (ng/µl - NanoDrop)**	**A260/280**	**A260/230**	**A260**	**A280**
06/10/2021	10/11/2021	**d1.1**	day 1	100	30	3.3	1.59	0.11	0.08	0.05
06/10/2021	10/11/2021	**d1.2**	day 1	100	30	3.8	1.99	0.21	0.09	0.05

20/10/2021	10/11/2021	**d1.3**	day 1	150	30	2.3	1.71	0.14	0.06	0.03
20/10/2021	10/11/2021	**d1.4**	day 1	150	30	2.9	1.93	0.16	0.07	0.04
18/11/2021	30/11/2021	**d1.5**	day 1	1000	30	9.3	1.78	0.72	0.23	0.13
20/01/2022	17/02/2022	**d1.6**	day 1	1000	30	41.4	2.07	1.46	1.03	0.5
02/01/2022	25/02/2022	**d.16**	day 1	1567	30	21.7	1.92	0.74	0.54	0.28

Table S3.5: Raw Cq values from preliminary quantification of *SRD5A1* and *SRD5A2* transcripts across day 1 – 5 post oviposition and albumen gland samples.

Target	Content	Tissue	Stage	Sample ID	Cq
SRD5A1	RT	embryo	day 1	d1.6	21.85
	RT	embryo	day 1	d1.6	21.91
	RT	embryo	day 1	d1.6	21.79
	NO-RT	embryo	day 1	d1.7	22.14
	NO-RT	embryo	day 1	d1.7	22.22
	NO-RT	embryo	day 1	d1.7	22.22
SRD5A2	RT	embryo	day 1	d1.6	24.71
	RT	embryo	day 1	d1.6	24.60
	RT	embryo	day 1	d1.6	24.64
	NO-RT	embryo	day 1	d1.7	24.38
	NO-RT	embryo	day 1	d1.7	24.58
	NO-RT	embryo	day 1	d1.7	24.81
SRD5A1	RT	embryo	day 2	d2.5	21.33
	RT	embryo	day 2	d2.5	21.45
	RT	embryo	day 2	d2.5	21.47
	NO-RT	embryo	day 2	d2.2	21.15
	NO-RT	embryo	day 2	d2.2	21.11
	NO-RT	embryo	day 2	d2.2	20.89
SRD5A2	RT	embryo	day 2	d2.5	25.72
	RT	embryo	day 2	d2.5	25.49
	RT	embryo	day 2	d2.5	25.44
	NO-RT	embryo	day 2	d2.2	24.74
	NO-RT	embryo	day 2	d2.2	24.86
	NO-RT	embryo	day 2	d2.2	24.99
SRD5A1	RT	embryo	day 3	d3.4	20.90
	RT	embryo	day 3	d3.4	20.96
	RT	embryo	day 3	d3.4	21.21
	NO-RT	embryo	day 3	d3.4	20.39
	NO-RT	embryo	day 3	d3.4	20.57
	NO-RT	embryo	day 3	d3.4	20.55
SRD5A2	RT	embryo	day 3	d3.4	25.59

	RT	embryo	day 3	d3.4	25.34
	RT	embryo	day 3	d3.4	25.37
	NO-RT	embryo	day 3	d3.4	24.42
	NO-RT	embryo	day 3	d3.4	24.41
	NO-RT	embryo	day 3	d3.4	24.43
SRD5A1	RT	embryo	day 4	d4.1	20.39
	RT	embryo	day 4	d4.1	20.28
	RT	embryo	day 4	d4.1	20.33
	NO-RT	embryo	day 4	d4.1	20.18
	NO-RT	embryo	day 4	d4.1	20.19
	NO-RT	embryo	day 4	d4.1	20.24
SRD5A2	RT	embryo	day 4	d4.1	25.11
	RT	embryo	day 4	d4.1	25.03
	RT	embryo	day 4	d4.1	25.19
	NO-RT	embryo	day 4	d4.1	24.94
	NO-RT	embryo	day 4	d4.1	25.07
	NO-RT	embryo	day 4	d4.1	25.09
SRD5A1	RT	embryo	day 5	d5.1	20.41
	RT	embryo	day 5	d5.1	20.74
	RT	embryo	day 5	d5.1	20.70
	NO-RT	embryo	day 5	d5.1	20.72
	NO-RT	embryo	day 5	d5.1	21.01
	NO-RT	embryo	day 5	d5.1	21.05
SRD5A2	RT	embryo	day 5	d5.1	25.26
	RT	embryo	day 5	d5.1	24.68
	RT	embryo	day 5	d5.1	24.67
	NO-RT	embryo	day 5	d5.1	25.45
	NO-RT	embryo	day 5	d5.1	25.27
	NO-RT	embryo	day 5	d5.1	25.41
SRD5A1	RT	adult	albumen gland	1.3	25.45
	RT	adult	albumen gland	1.3	25.48
	RT	adult	albumen gland	1.3	25.51
SRD5A2	RT	adult	albumen gland	1.3	26.69
	RT	adult	albumen gland	1.3	26.54
	RT	adult	albumen gland	1.3	26.50

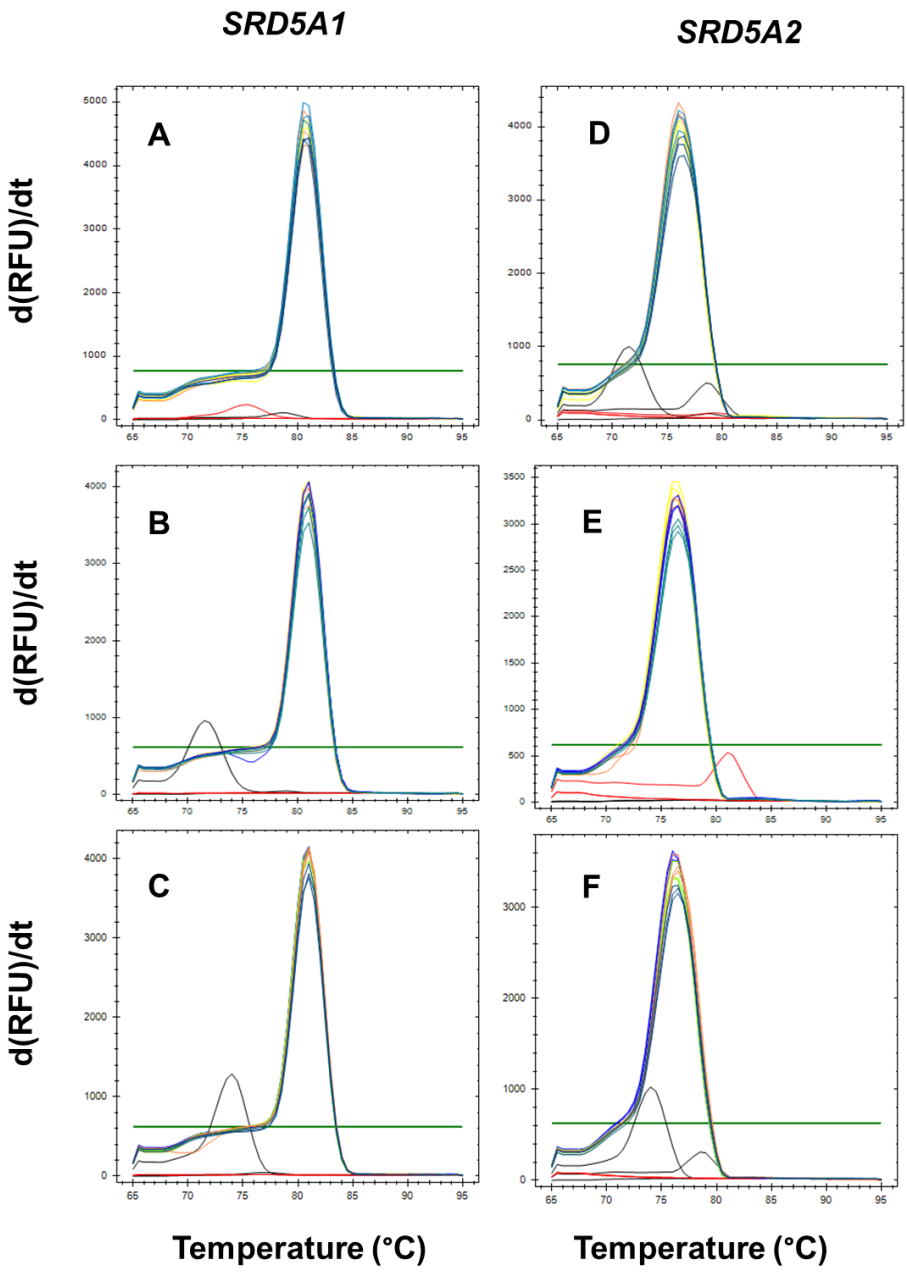

Figure S3.1: Melt curves representing derivatives of the relative fluorescence d(RFU) in respect to temperature (°C) for the target genes *SRD5A1* (a,b,c) and *SRD5A2* (d,e,f). Data was obtained from three separate quantification experiments, using independent cDNA biological replicates. The melt curves (a) and (d), were obtained from the first quantification experiment; melt curves (b) and (e) were obtained from the second quantification experiment; and melt curves (c) and (f) were obtained from the third quantification experiment. Different colours represent different embryo and tissue samples. No-reverse transcriptase controls (NRT) are shown in black whereas negative controls (NTC) are shown in red. The NRT and NTC peaks shown in some graphs were only observed in singular samples which indicates sporadic pipetting errors which were not reflected in the rest of the data

Appndix S4 (Chapter 4): Raw data and supporting information

Table S4.3: Results from the first LC-MS analysis in Experiment 1, using water samples from exposure day 1, and a set of lower calibration standards. Different replicates from the tanks tested are represented by T, where the treatment group tested (DWC, SC, 1 µg/L, 3.2 µg/L, 10 µg/L) is presented in brackets. A separate batch using higher calibration standards was also run but no quantification results were obtained due to methodological errors during sample preparations.

	Sample Name	Sample Type	Analyte Peak Area (counts)	Analyte Concentration (ug/L)	Analyte Signal To Noise	IS Peak Area (counts)	Calculated Concentration (ug/L)
1	Blk	Blank	0.00E+00	N/A	N/A	0.00E+00	-
2	Std 1 0ug/L	Standard	0.00E+00	0	N/A	2.95E+05	ND
3	Std 2 0.5ug/L	Standard	1.12E+03	0.5	3.67E+01	3.10E+05	0.342
4	Std 3 1ug/L	Standard	2.38E+03	1	7.32E+01	3.14E+05	0.864
5	Std 4 2.5ug/L	Standard	6.51E+03	2.5	2.93E+02	3.21E+05	2.53
6	Std 5 5ug/L	Standard	1.54E+04	5	5.25E+02	3.45E+05	5.71
7	Std 6 7ug/L	Standard	1.78E+04	7	7.91E+02	3.24E+05	7.08
8	Std 7 10ug/L	Standard	2.65E+04	10	9.02E+02	3.57E+05	9.6
9	DWC Rep A	Sample	0.00E+00	N/A	N/A	3.31E+05	ND
10	DWC Rep B	Sampe	0.00E+00	N/A	N/A	0.00E+00	-
11	DWC Rep C	Sample	0.00E+00	N/A	N/A	0.00E+00	-
12	SC Rep A	Sample	0.00E+00	N/A	N/A	0.00E+00	-
13	SC Rep B	Sample	0.00E+00	N/A	N/A	0.00E+00	-
14	SC Rep C	Sample	0.00E+00	N/A	N/A	0.00E+00	-
15	QC (std 7)	QC	0.00E+00	0	N/A	0.00E+00	-
16	BLK	Blank	0.00E+00	N/A	N/A	0.00E+00	-
17	1ug/L Rep A	Sample	0.00E+00	N/A	N/A	0.00E+00	-
18	1ug/L Rep B	Sample	0.00E+00	N/A	N/A	0.00E+00	-
19	1ug/L Rep C	Sample	0.00E+00	N/A	N/A	0.00E+00	-
20	3.2ug/L Rep A	Sample	0.00E+00	N/A	N/A	0.00E+00	-
21	3.2ug/L Rep B	Sample	0.00E+00	N/A	N/A	0.00E+00	-
22	3.2ug/L Rep C	Sample	0.00E+00	N/A	N/A	0.00E+00	-
23	QC (Std 7)	QC	0.00E+00	N/A	N/A	0.00E+00	-
24	BLK	Blank	0.00E+00	N/A	N/A	0.00E+00	-
25	10ug/L Rep A	Sample	0.00E+00	N/A	N/A	0.00E+00	-
26	10ug/L Rep B	Sample	0.00E+00	N/A	N/A	0.00E+00	-

27	10ug/L Rep C	Sample	0.00E+00	N/A	N/A	0.00E+00	-
28	QC (std 7)	QC	0.00E+00	N/A	N/A	0.00E+00	-
29	BLK	Blank	0.00E+00	N/A	N/A	0.00E+00	-

Table S4.2: pH levels of OFF samples from Experiment 2 (static-renewal study), across the 28-day exposure period. Measurements were taken every 2-3 days. The mean values of pH across the exposure period are given.

	Day 2	Day 5	Day 7	Dau 9	Day 11	Day 14	Day 16	Day 18	Day 21	Day 23	Day 25	Day 28	Mean
	OFF	OFF	OFF	OFF	OFF	OFF	OFF	OFF	OFF	OFF	OFF	OFF	
DWC	7.8	8.1	7.9	7.9	8.4	7.8	7.9	8.0	7.5	8.0	7.7	7.9	7.9
SC	7.8	8.2	8.0	7.9	8.3	8.0	7.9	7.9	7.8	8.1	8.0	8.0	8.0
1 µg/L	7.9	8.2	8.1	7.9	8.4	8.0	8.1	8.1	7.6	8.2	7.9	8.1	8.0
3.2 µg/L	8.1	8.2	8.1	8.2	8.3	8.1	8.0	7.9	7.6	8.2	7.9	7.8	8.0
10 µg/L	8.0	8.2	8.1	8.0	8.3	6.9	7.7	8.1	7.5	8.1	8.0	7.8	7.9
32 µg/L	8.2	8.0	8.0	8.0	8.4	8.0	7.8	8.0	7.3	8.0	8.0	8.3	8.0
100 µg/L	8.1	8.0	7.6	7.1	8.3	8.0	7.9	7.9	8.5	8.3	7.7	8.0	8.0

Table S4.3: Oxygen levels (%) of OFF samples from Experiment 2 (static-renewal study), across the 28-day exposure period. Measurements were taken every 2-3 days. The mean values of oxygen levels (%) across the exposure period are given.

	Day 2	Day 5	Day 7	Dau 9	Day 11	Day 14	Day 16	Day 18	Day 21	Day 23	Day 25	Day 28	Mean
	OFF	OFF	OFF	OFF	OFF	OFF	OFF	OFF	OFF	OFF	OFF	OFF	
DWC	92.5	92.0	85.3	87.2	68.0	83.0	87.2	80.0	65.3	72.0	70.0	71.0	79.5
SC	83.9	97.5	78.6	91.3	73.0	82.2	68.0	97.0	72.1	75.0	73.0	72.6	80.4
1 µg/L	103.0	100.0	80.2	85.2	80.0	97.0	83.0	88.3	70.3	83.0	78.0	79.0	85.6
3.2 µg/L	105.0	93.5	82.0	88.3	81.0	94.0	91.3	81.0	68.5	81.8	82.0	74.5	85.2
10 µg/L	96.6	97.4	79.4	89.0	80.0	65.0	73.0	92.0	73.2	77.4	81.0	82.3	82.2

32 µg/L	110.5	79.2	83.5	78.3	76.3	82.5	82.2	89.0	72.0	63.4	75.3	81.0	81.1
100 µg/L	83.5	79.5	79.0	75.1	76.0	86.0	85.2	88.5	83.5	77.5	78.4	78.2	80.9

Figure S4.1: Calibration curve of the lower calibration standards used for the first LC-MS/MS analysis (exposure day 1 samples) in Experiment 1 (flow-through system). The calibration curve was run using standards made out of the exposure water medium (Table S4.1).

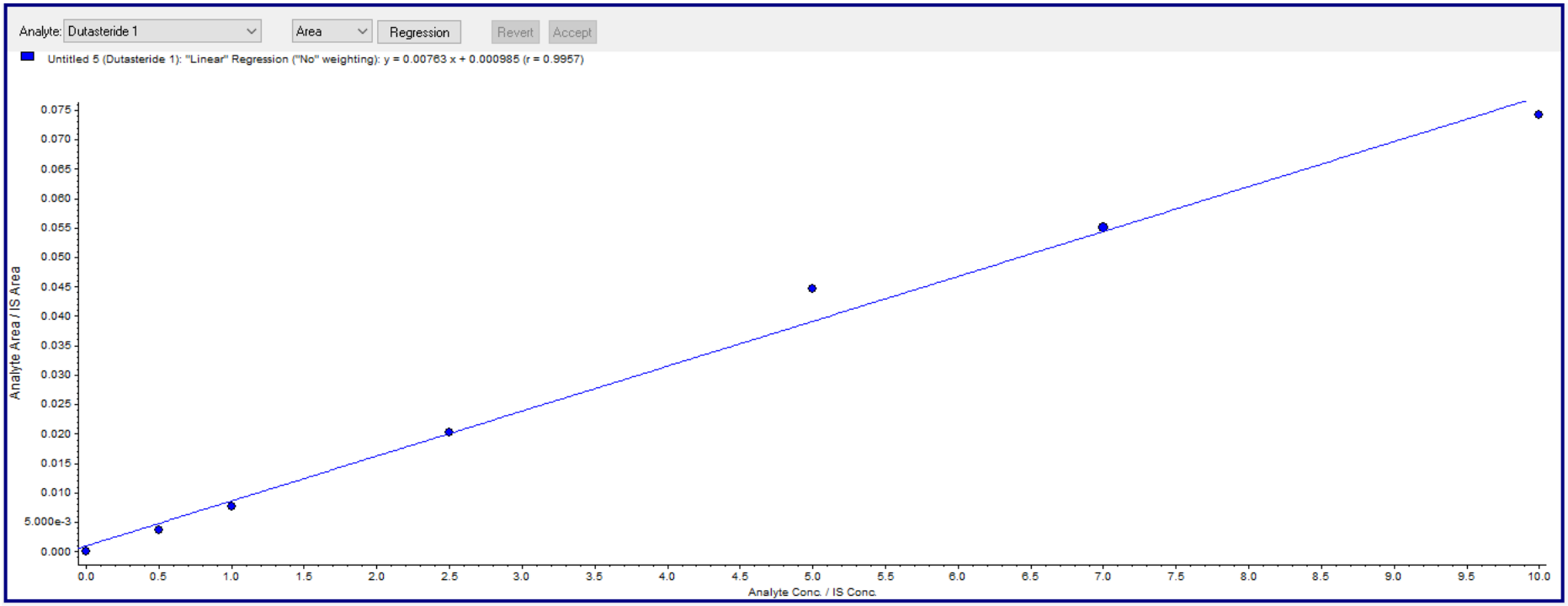

Figure S4.2: Calibration curve of the higher calibration standards that were used during the analysis of filtered and unfiltered samples in Experiment 1. Samples tested were taken from the 32 µg/L DUT mixing chamber and respective tank replicate. R^2 was calculated based on the R value of 0.9995, which equalled to 0.998.

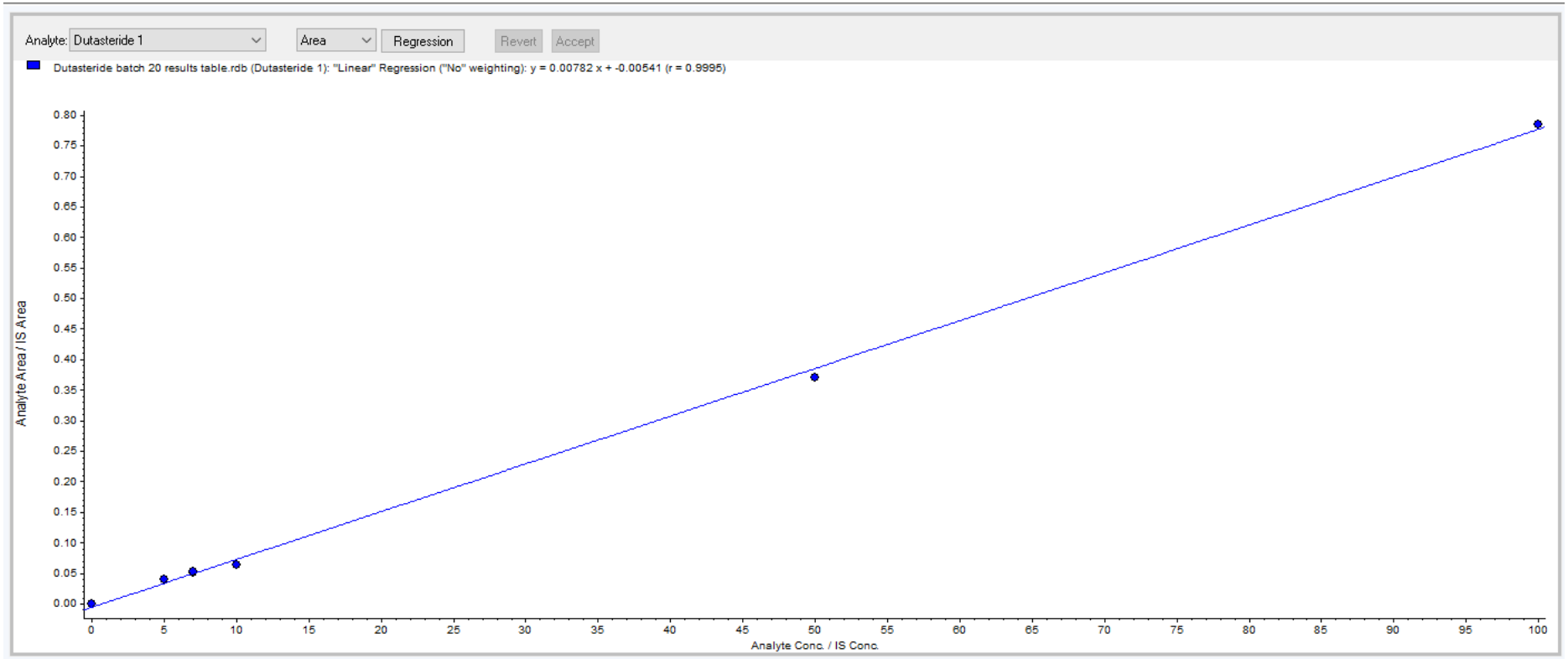

Figure S4.3: Calibration curve of the higher calibration standards that were used during filter optimisation of Experiment 1. Samples tested were taken from the 32 µg/L DUT mixing chamber. R^2 was calculated based on the R value of 0.9978, which equalled to 0.996.

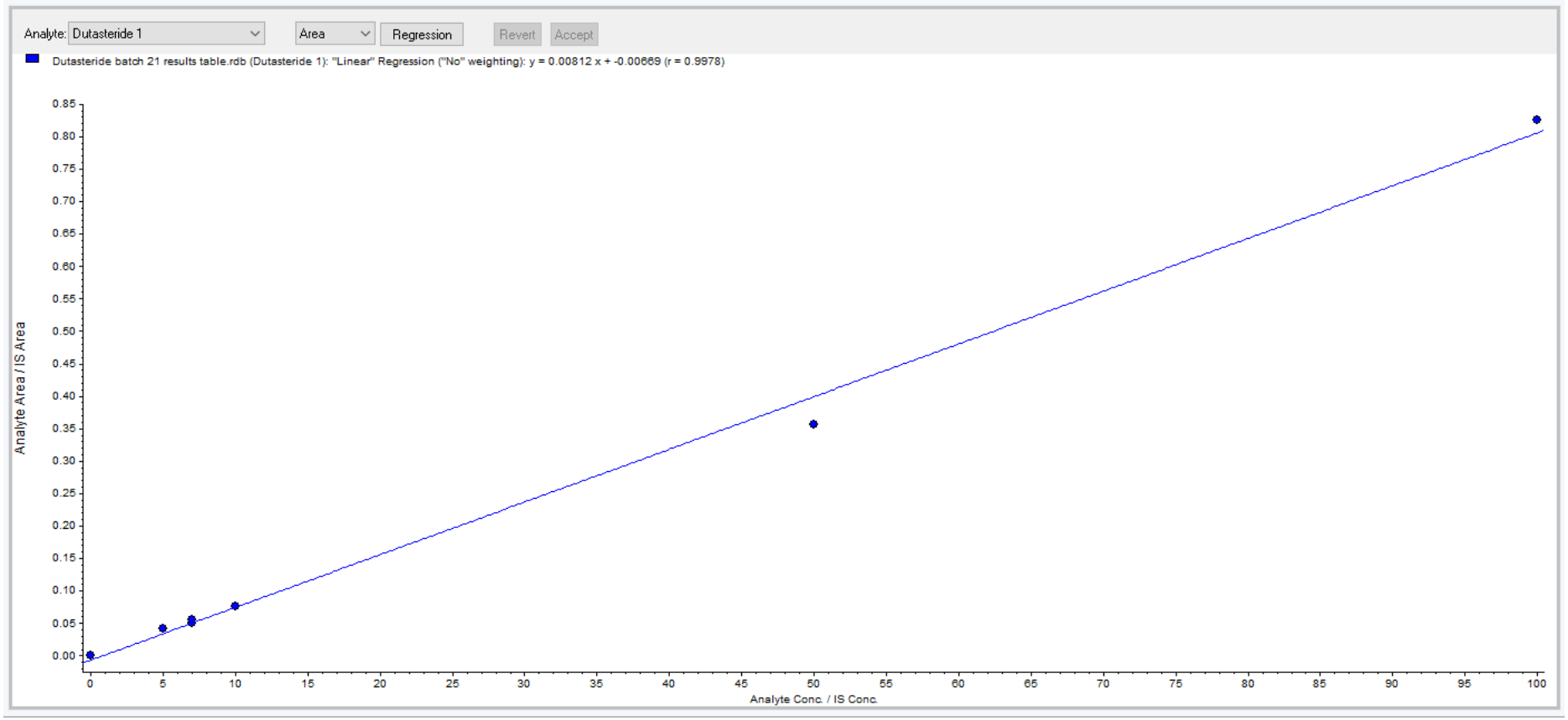

Figure S4.4: Calibration curve of the lower calibration standards that were used during the subsequent LC-MS/MS analysis of day 1 samples, in Experiment 1. Samples tested were taken from the DWC, SC, 1µg/L, 3.2µg/L, 10µg/L treatments. R^2 was calculated based on the R value of 0.9983, which equalled to 0.996.

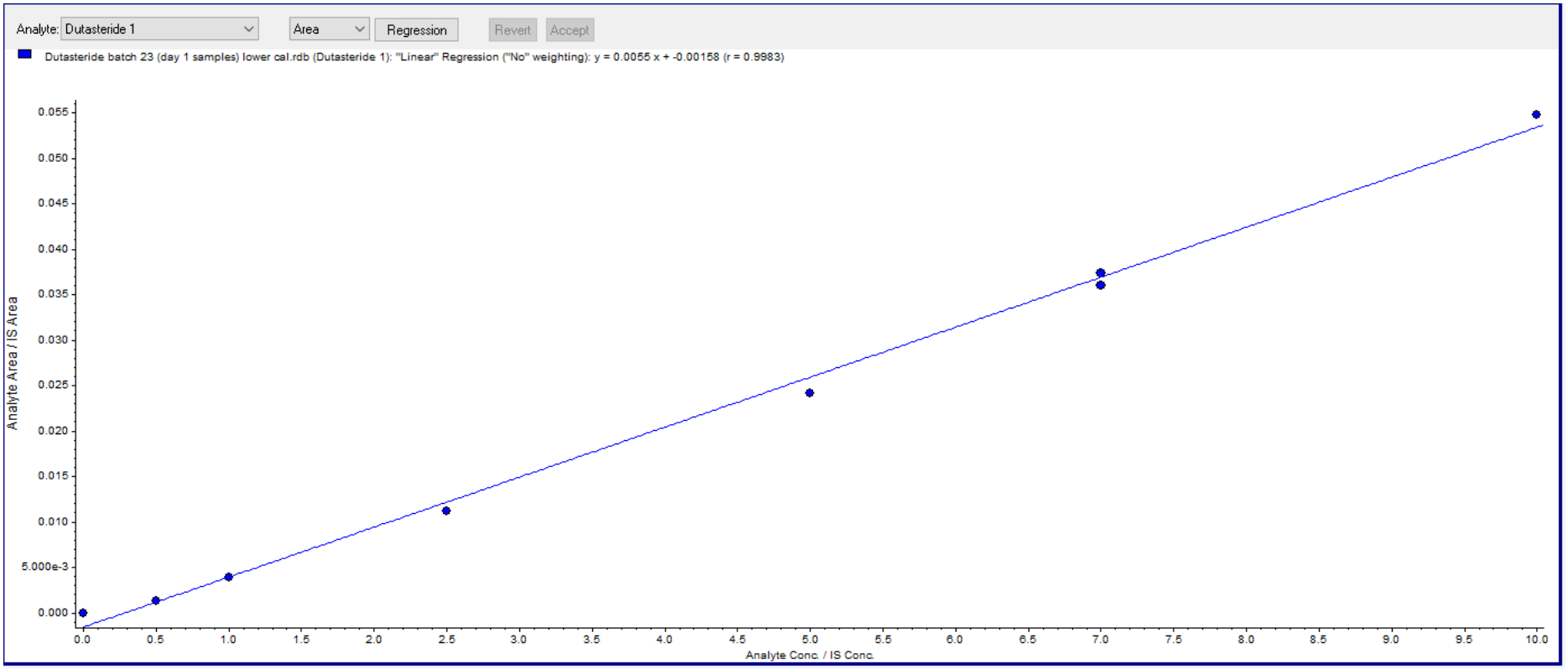

Figure S4.5: Calibration curve of the higher calibration standards that were used during the subsequent LC-MS/MS analysis of day 1 samples, in Experiment 1. Samples tested were taken from the 32 µg/L and 100 µg/L treatments. R^2 was calculated based on the R value of 0.9859, which equalled to 0.972.

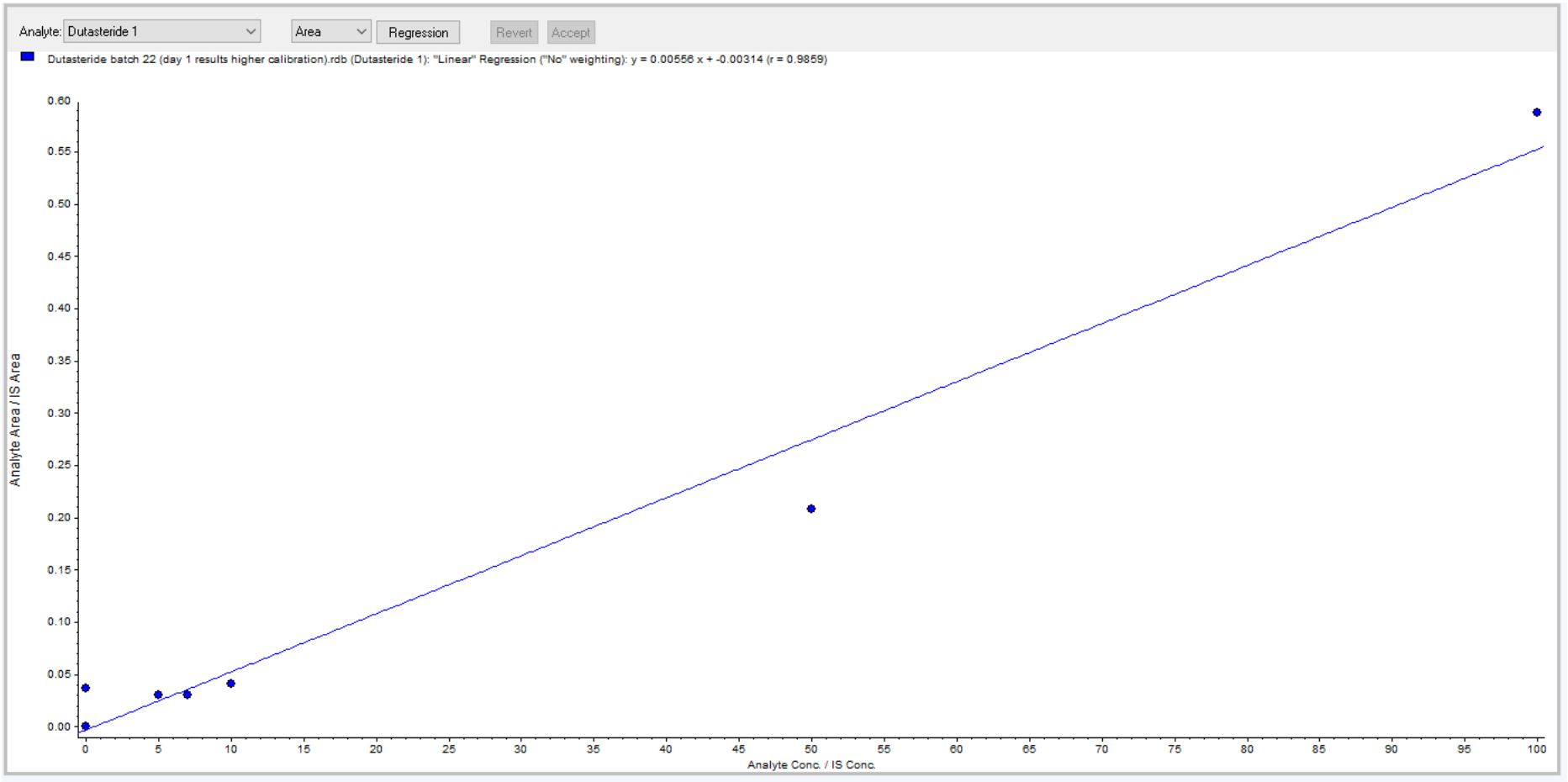

Figure S4.6: Calibration curve of the lower calibration standards that were used during the LC-MS/MS analysis on day 21 samples of Experiment 1. Samples tested were taken from the DWC, SC, 1 µg/L, 3.2 µg/L, 10 µg/L treatments. R^2 was calculated based on the R value of 0.7069, which equalled to 0.501. The two additional calibrants at the high end of the curve represent the quality control (QC) standards.

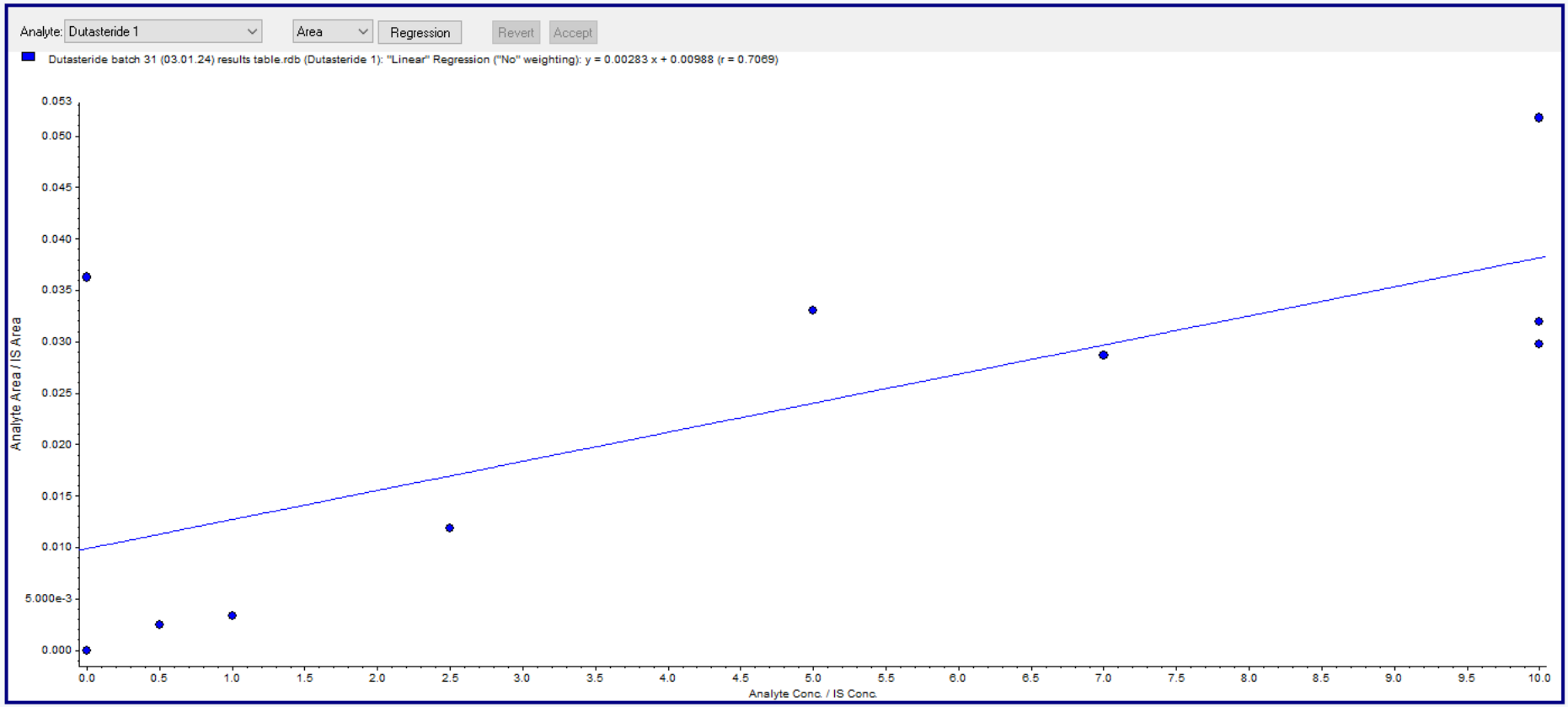

Figure S4.7: Calibration curve of the higher calibration standards that were used during the LC-MS/MS analysis on day 21 samples of Experiment 1. Samples tested were taken from the 32 µg/L and 100 µg/L treatments. R^2 was calculated based on the R value of 0.9648, which equalled to 0.931. The two additional calibrants at the high end of the curve represent the quality control (QC) standards.

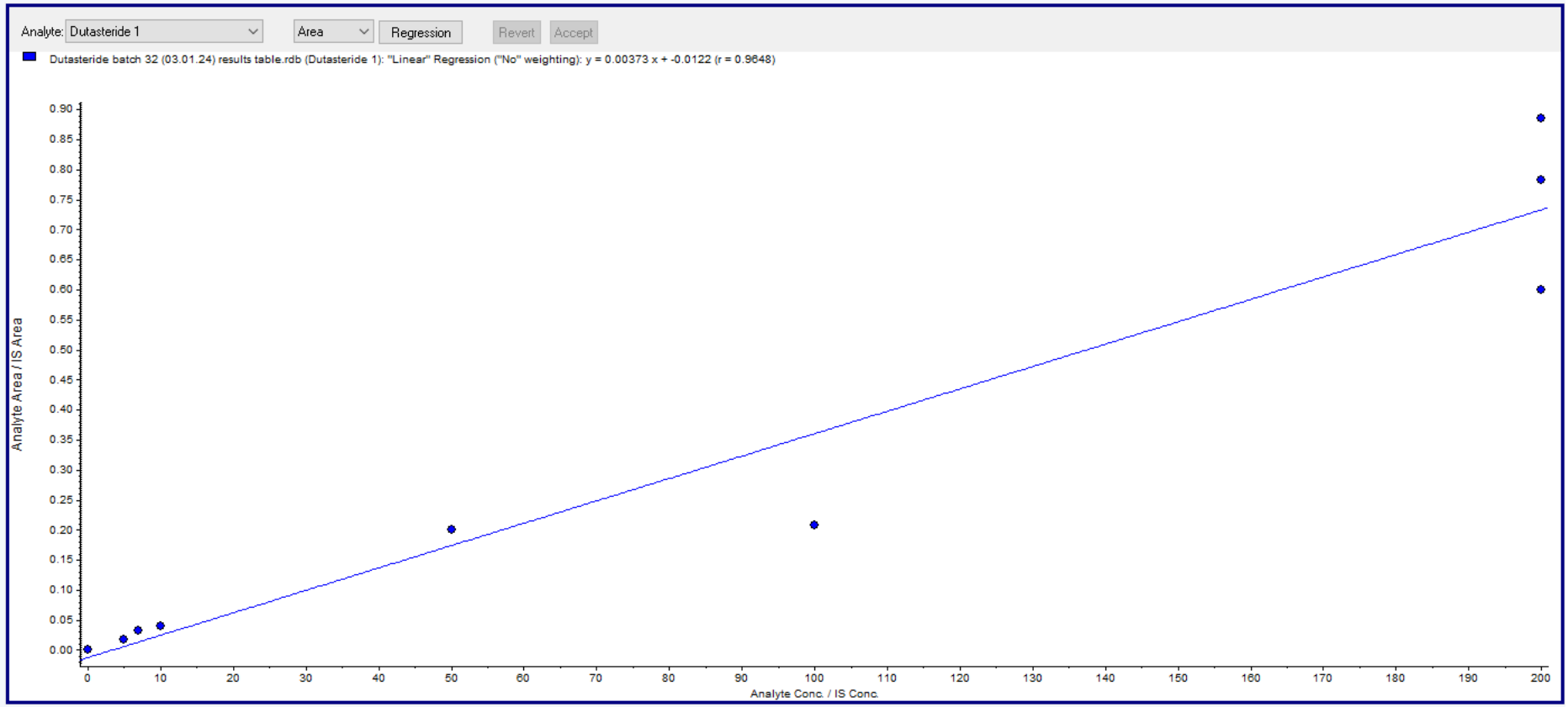

Figure S4.8: Calibration curve of the calibration standards used throughout the LC-MS/MS analyses