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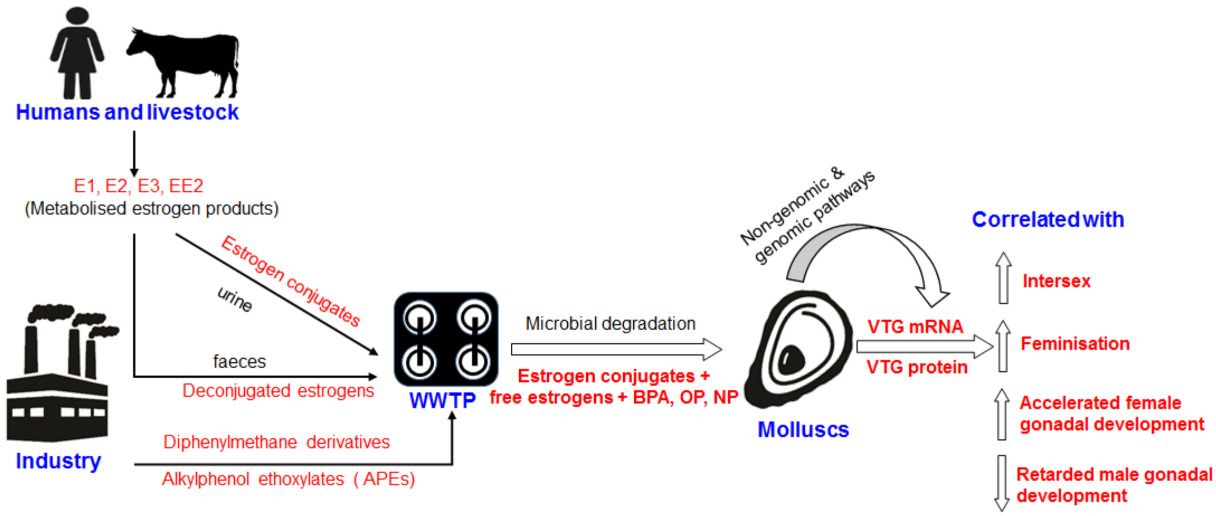

Humans and livestock
E1, E2, E3, EE2
(Metabolised estrogen products)
Estrogen conjugates
urine
faeces
Deconjugated estrogens
Industry
Diphenylmethane derivatives
Alkylphenol ethoxylates (APEs)
WWTP
Microbial degradation
Estrogen conjugates +
free estrogens + BPA, OP, NP
Non-genomic & genomic pathways
Molluscs
VTG mRNA
VTG protein
Correlated with
Intersex
Feminisation
Accelerated female gonadal development
Retarded male gonadal development

The utility of vitellogenin as a biomarker of estrogenic endocrine disrupting chemicals in molluscs

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Abstract

Estrogenic endocrine disrupting chemicals (EDCs) are natural hormones, synthetic compounds or industrial chemicals that mimic estrogens due to their structural similarity with estrogen's functional moieties. They typically enter aquatic environments through wastewater treatment plant effluents or runoff from intensive livestock operations. Globally, most natural and synthetic estrogens in receiving aquatic environments are in the low ng/L range, while industrial chemicals (such as bisphenol A, nonylphenol and octylphenol) are present in the µg to low mg/L range. These environmental concentrations often exceed laboratory-based predicted no effect concentrations (PNECs) and have been evidenced to cause negative reproductive impacts on resident aquatic biota. In vertebrates, such as fish, a well-established indicator of estrogen-mediated endocrine disruption is overexpression of the egg yolk protein precursor vitellogenin (Vtg) in males. Although the vertebrate Vtg has high sensitivity and specificity to estrogens, and the molecular basis of its estrogen inducibility has been well studied, there is growing ethical concern over the use of vertebrate animals for contaminant monitoring. The potential utility of the invertebrate Vtg as a biomonitor for environmental estrogens has therefore gained increasing attention. Here we review evidence providing support that the molluscan Vtg holds promise as an invertebrate biomarker for exposure to estrogens. Unlike vertebrates, estrogen signalling in invertebrates remains largely unclarified

and the classical genomic pathway only partially explains estrogen-mediated activation of Vtg. In light of this, in the latter part of this review, we summarise recent progress towards understanding the molecular mechanisms underlying the activation of the molluscan Vtg gene by estrogens and present a hypothetical model of the interplay between genomic and non-genomic pathways in the transcriptional regulation of the gene.

Capsule

Molluscan vitellogenin holds promise as a biomarker for exposure and effect of estrogenic EDCs, but the molecular mechanisms of estrogen-mediated vitellogenesis require elucidation.

Keywords: biomarker, endocrine disrupting chemicals, estrogen, molluscs, vitellin, vitellogenin.

1. ESTROGENIC EDCs IN AQUATIC ENVIRONMENTS

1.1. *Estrogenic EDCs: Sources, transport and fate*

Endocrine disrupting chemicals (EDCs) are natural hormones or synthetic chemicals (exogenous), or a combination of these (mixture) that interfere with any aspect of the body's endocrine system and produce a critical disruption of hormone-regulated processes of development, reproduction and physiology (Gore et al., 2014). Among the various EDCs, estrogenic EDCs have received the most attention since the discovery of high intersexuality in fish (Jobling et al., 1998) associated with the presence of estrogenic EDCs in wastewater (Desbrow et al., 1998). While it is now clear that other types of EDCs are present in wastewater and that the feminisation of fish may also be due to the presence of anti-androgenic EDCs (Jobling et al., 2009), estrogenicity remains one of the most common and widespread types of endocrine activity detected in (waste) water (Leusch et al., 2018).

Although estrogenic EDCs are a diverse range of chemicals, they can be divided into two broad groups (Williams et al., 2007) including natural hormones (which are required for normal functioning of the endocrine system) and synthetic compounds or xenoestrogens, which are chemicals either intentionally designed to target the endocrine system or man-made chemicals derived from anthropogenic activities. Natural hormones include the steroidal estrogens, such as estrone (E1), 17β-estradiol (E2) and estriol (E3). These are derived from cholesterol and released by the adrenal cortex, ovary, placenta, or testes of human and animals and enter waterways via discharge of biological wastes (D'ascenzo et al., 2003). Synthetic estrogens include 17α-ethinylestradiol (EE2), diethylstilbestrol (DES), and polyestradiol phosphate (PEP), which are mostly found as active pharmaceutical ingredients in contraception practice and hormone replacement therapy and again enter waterways via excretion from humans and livestock. Other synthetic chemicals in wastewater treatment plant (WWTP) effluents, include phthalates, bisphenol A (BPA) and alkyphenol ethoxylates. Phthalates and BPA are plasticizers added to plastics to increase their flexibility, transparency, and durability, and are used for making certain plastics and epoxy resins, water pipelines, and the inner coatings of many food and beverage products (Husain and Qayyum, 2013). Alkylphenol ethoxylates (APEs) are a family of alkylphenols (APs) that are surfactants used in manufacturing antioxidants, lubricating oil additives, laundry and dish detergents, emulsifiers and solubilizers, paints, personal care products, and plastics (Diao et al., 2017). Microbial degradation of APEs during their transit through WWTPs generates persistent shorter-chain APEs and APs such as nonylphenol (NP) and octylphenol (OP) and AP mono- to triethoxylates, all of which possess estrogenic activity (Ying et al., 2008).

Estrogenic EDCs may enter urban aquatic environments as a result of the discharge of both municipal and industrial effluents from WWTPs along with runoff from numerous non-point sources. In contrast, in more rural areas, sources are largely natural estrogens or veterinary pharmaceuticals excreted by livestock and enter waterways via runoff from intensive livestock raising of cattle and poultry (Wang et al., 2017).

Natural and synthetic estrogenic compounds are metabolized in human and livestock to produce water-soluble sulfate or glucuronide conjugates, which are biologically inactive. Both conjugates are excreted in urine (Froehner et al., 2012; Aris et al., 2014). On the other hand, deconjugated estrogens are more persistent in faeces because *Escherichia coli* in the gut deconjugates the estrogen conjugates via β-glucuronidase and sulfatase activities (Aris et al., 2014). In WWTPs, some estrogen conjugates remain unchanged, some are deconjugated and further biodegraded. However, some are deconjugated, but not biodegraded and reform free estrogens with relative stability through the influences of bacterial modification (Liu et al., 2015).

BPA is an industrial chemical belonging to the group of diphenylmethane derivatives and enters aquatic environments via WWTP effluents. Similarly, APEs enter WWTPs and are biodegraded during transit to the estrogenic degradation products of NP and OP (Diao et al., 2017). The main biodegradation pathways of APEs are generally believed under the influences of anaerobic and aerobic microorganisms in WWTPs through the loss of ethoxylate (EO) chains, and then the formation of alkylphenol ethoxy carboxylates as well as alkylphenol polyethoxylates. These degraded products go on to further progressive shortening of the EO chain with the production of alkyl phenols (NP, OP) followed by alkylphenoxyacetic acids and alkylphenol mono ethoxylates (Ying et al., 2002). Most WWTPs are primarily designed to remove solids, nutrients and microbial contamination, but not micropollutants, and thus removal of estrogenic EDCs is often incomplete (Melvin and Leusch, 2016). Therefore, WWTPs can be an important pathway of estrogenic EDCs into receiving water bodies, unless action is taken to equip plants with the means of treating estrogenic EDCs such as membrane filtration, activated carbon and ozonation treatment technologies (Yost et al., 2014).

1.2. Concentrations of estrogenic EDCs internationally

Estrogenic EDCs have been extensively surveyed and are present in appreciable levels in WWTP effluents, marine and coastal waters, freshwater systems (rivers, surface water), and even in groundwater of urban and rural areas (**Supplementary Table 1**). The level of contamination is dependent on a variety of factors, such as how far the sampling point is from the input, the types of wastewater treatment undertaken by WWTPs (e.g., primary, secondary and advanced treatment), and the size of the population/catchment producing the effluent (Zhang et al., 2016). Globally, most natural and synthetic estrogens in WWTPs effluents and receiving aquatic environments are in the low ng/L range, while the industrial chemicals (BPA, NP and OP) are present in higher concentrations, typically in the µg to low mg/L range (Zhang et al., 2016).

In terms of effluent samples, it has been found that most contain some natural estrogens, with E1 concentrations ranging from <0.1 to 180 ng/L (Komori et al., 2004; Allinson et al., 2010) among countries. E2 concentrations are lower with a range of <0.05 to 54 ng/L (Allinson et al., 2010; Schaider et al., 2017), while E3 ranges from 1 to 280 ng/L (Fernandez et al., 2007; Schaider et al., 2017) (**Supplementary Table 1**). The synthetic estrogen EE2 is generally in a lower range of 0.05 to 36.92 ng/L across studied countries (Allinson et al., 2010; Cieck et al., 2007), while xenoestrogens like BPA, NP, and OP typically range from <10 to 3.7×10^4 ng/L (Fukazawa et al., 2001; Barber et al., 2015), 0.6 to 2.5×10^4 ng/L (Fernandez et al., 2007; Ying et al., 2009) and 5.93 to 5.5×10^3 ng/L, respectively (Andrew-Priestley et al., 2012; French et al., 2015).

Natural estrogens in receiving waters are generally lower than effluents due to dilution in the receiving waterways, with ranges of 0.06 to 180 ng/L for E1 (Goonan, 2008; Zhou et al., 2011), 0.6 to 94 ng/L for E3 (Cargouet et al., 2004; Zhou et al., 2011), except E2 ranging from 0.3 to 175 ng/L (Pojana et al., 2007; Goonan, 2008). In comparison, concentrations of synthetic estrogens are generally lower than natural estrogens with EE2 (0.14. to 34 ng/L) (Pojana et al., 2007; Robinson et al., 2009). Xenoestrogens are again lower in receiving waters than effluent with BPA (0.33 to 1.09×10^3 ng/L) (Zhang et al., 2004); Cheng et al., 2018), NP (6.8 to 5.27×10^3 ng/L) (Leusch et al., 2010; Wang et al., 2017), and OP (0.11 to 916 ng/L) (Tremblay and Northcott, 2013; Cheng et al., 2018).

Estrogenic EDCs tend to be accumulated in sediments with concentrations generally in the high ng/kg range (dw) (Supplementary Table 1). For example, E1, E2, E3, EE2, BPA, NP, and OP were reported to be 0.70 to 4.98×10^4 ng/kg (Froehner et al., 2012; Stewart et al., 2014), 0.47 to 3.98×10^4 ng/kg (Froehner et al., 2012; Stewart et al., 2014), <50 to 7.6×10^3 ng/kg (Schmitt et al., 2010; Zhou et al., 2011), <40 to 1.30×10^5 ng/kg (Froehner et al., 2012; Labadie and Hill, 2007), $<1.7\times10^3$ to 4.30×10^5 ng/kg (Gong et al., 2011), 7.55×10^3 to 3.2×10^7 ng/kg (Diao et al., 2017; Stewart et al., 2014), and <170 to 2.10×10^5 ng/kg (Diao et al., 2017; Gong et al., 2011) respectively. Given their hydrophobic properties and high specific octanol-water and sediment-water partition coefficients (logK_{ow} and logK_{oc}), estrogenic EDCs are readily adsorbed onto sediments. For example, logK_{ow} of common estrogenic compounds E2, EE2, BPA, 4-t-OP, and 4-n-NP are 3.94, 4.15, 3.32, 4.12 and 5.76, respectively and logK_{oc} values are 3.56, 3.73, 2.98, 3.86 and 4.89, respectively (Ying and Kookana, 2005). Thus sediment serves as an important sink for estrogenic EDCs in aquatic environments through sorption of these compounds to particulate organic matter (Gong et al., 2011). Since many molluscs are benthic organisms, sediment associated estrogenic EDCs are a potential additional exposure pathway.

1.3. Predicted No Effect Concentrations (PNECS) and the risk of estrogenic EDCs

The concentrations of estrogenic EDCs in effluents and receiving waters are sufficient to cause biological effects on resident taxa and are thus cause for concern. Based on the NOECs (no observed effect concentrations) for chronic reproductive toxicity reported in multiple fish species, Caldwell et al. (2012) developed a species sensitivity distribution (SSD) and PNECs of 0.1 and 2 ng/L were derived for EE2 and E2, respectively. Since insufficient data were available to construct an SSD for E1 or E3, Caldwell et al. (2012) utilised *in vivo* vitellogenin (Vtg) induction studies in fish to establish the relative potencies of these steroid estrogens to E2. Based on the assumption that the relative ability to induce Vtg corresponds with the relative effects on reproductive endpoints, the PNECs of E1 and E3 were derived to be 6 and 60 ng/L, respectively.

A PNEC of 1.6 μg/L for BPA was adopted by the EU (EC, 2010), based on a multi-generation study with fathead minnows (*Pimephales promelas*) (Sohoni et al., 2001). The PNEC was derived by using the 16 μg/L NOEC for egg hatchability in the F_2 generation and dividing by an assessment factor of 10. The same NOEC was also observed for Vtg production in males of the same species (Sohoni et al., 2001) and oviduct formation in male carp (*Cyprius carpino*) (Bowmer and Gimeno, 2001).

Regarding the ecological risk assessment for NP, the EU has adopted an aquatic PNEC of 0.33 μg/L, based on the long-term NOECs for general toxicity from three species representing different trophic levels (for fish a 33-day $NOEC_{survival}$ of 0.0074 mg/L (*Pimephales promelas*); for invertebrates a 21-day $NOEC_{survival\ of\ offspring}$ of 0.024 mg/L (*Daphnia magna*); and for algae a 72-hour $EC_{10\ biomass}$ of 0.0033 mg/L (*Scenedesmus subspicatus*)) and an assessment factor of 10 (EC, 2002). By measuring chronic reproductive effects (including production of testis-ovas and Vtg in males) in a partial life cycle test with the Japanese medaka (*Oryzias latipes*), a PNEC of 0.68 μg/L for NP was determined by the Japanese Ministry of the Environment (MoE, 2001). This verified that the EU's PNEC for NP (0.33 μg/L) is sufficient to cover the reproductive toxicities in vertebrates. Recently, based on a SSD model built using the chronic toxicity data of NP on aquatic organisms covering algae, vertebrates and invertebrates available in the USEPA ECOTOX database, the PNECs for NP in freshwater and seawater were calculated to be 0.48 and 0.28 μg/L, respectively (Gao et al., 2014). These values are close to the EU's PNEC (aquatic) for NP (0.33 μg/L; EC, 2002).

Regarding OP, the EU has adopted an aquatic PNEC of 0.122µg/L, based on the long-term NOECs for general toxicity from vertebrates, invertebrates and algae and an assessment factor of 50 (EC, 2005; Brooke et al., 2005). This value is lower than the lowest most relevant NOEC for endocrine-mediated responses in aquatic organisms (i.e., 12 µg/L from a life-cycle study with the zebrafish *Brachydanio rerio*; Wenzel et al., 2001), and the NOEC for Vtg production (i.e., 1.6 µg/L from rainbow trout after 21 days exposure to OP; Jobling et al., 1996). It was therefore concluded that the conventional endpoint based-PNEC above provides a sufficient safety margin against potential endocrine-mediated effects of OP (EC, 2005).

Overall, the ecological risks posed by the above compounds, from the highest to the lowest, are therefore: EE2 (0.1 ng/L) > E2 (2 ng/L) > E1 (6 ng/L) > E3 (60 ng/L) > OP (0.122 µg/L) > NP (0.33 > µg/L), BPA (1.6 µg/L). This order largely agrees with the relative potencies (estrogenicity) of the same compounds measured with *in vitro* bioassays. For example, using the ERα-CALUX bioassay, Leusch et al. (2014b) determined the relative potency order for the above compounds to be: EE2 (5.4) > E2 (1) > E3 (0.017) ≈ E1 (0.014) > NP (9.1×10^{-5}) > BPA (1.4×10^{-5}) ≈ OP (1.2×10^{-5}).

Concentrations of (xeno)estrogens in effluents and receiving waters often far exceed these PNECs and effects endpoints in many countries (**Supplementary Table 1**), thus we may expect to observe impacts on biota residing in effluent receiving waters, including molluscs. Although previous field studies have not always found evidence of reproductive impacts (Jones at al., 2000) with reasons probably relating to differences in wastewater treatment processes (Jones et al., 2000; Omoruyi and Pohjanvirta, 2015; Leusch et al., 2018), several lines of evidence have shown that field exposure of fish and molluscs in receiving waters of WWTPs with less advanced treatment exhibiting increased Vtg production, intersex production, shifts in the sex ratio towards female-biased populations, aberrant development of male and female reproductive organs and disrupted reproductive cycling (Jobling et al., 1998; Gagne et al., 2004; Matozzo and Marin, 2005; Langston et al., 2007; Jobling et al., 2009; Andrew-Priestley et al., 2012; Ciocan et al., 2012).

2. VITELLOGENIN INDUCTION IN MOLLUSCS

2.1. Vitellogenin and vitellin

Vitellogenin (Vtg) is a high-molecular-mass glycolipophosphoprotein produced in most oviparous and ovoviviparous animals during the process of yolk formation (vitellogenesis). The protein is a precursor of the major egg yolk protein, vitellin (Vn), which serves as the primary nutritional source for embryogenesis. Vtg exists as multiple isoforms encoded by up to seven distinct genes in a number of vertebrate species, including the chicken, frog and fish (Felber et al., 1980; Silva et al., 1989; Wang et al., 2000). In vertebrates, the complete Vtg has a multi-domain structure, consisting of the N-terminal LPD–N domain, the domain of unknown function (DUF1943) and the C-terminal von Willebrand factor type D domain (VWD). From the N- to C-termini, these domains correspond to a signal peptide (Sp), a lipovitellin (Lv), a phosvitin (Pv), a β-component (β′-c) and a C-terminal peptide (C-t) (Hiramatsu et al., 2006; Fin, 2007). Sp is associated with the export of the precursor Vtg protein into the extracellular environment for transport into the vertebrate oocytes. Lv, the largest Vtg-derived yolk product, consists of two polypeptides, a heavy chain (LvH) and a light chain (LvL), which function as a major source of amino acids and lipids for embryogenesis. Pv is rich in phosphorylated serine residues, suggesting its function as a carrier of calcium and phosphate ions required for skeletal development in developing embryos (Hiramatsu et al., 2002). To date, the functions of the β′-c and C-t domains remain unclarified (Hiramatsu et al., 2006).

In vertebrates, such as fish, Vtg in fish is synthesized, lipidated, phosphorylated, and glycosylated in the liver, released into the blood, and finally accumulated by oocytes through receptor-mediated endocytosis (Denslow, 1999). Once inside the oocytes, Vtg is cleaved into its main components (LvH, LvL, Pv and β′-c) by cathepsin D and processed into Vn (Byrne et al., 1989). Therefore, Vtg is known as an extraovarian protein (in the liver or bloodstream), while Vn is an intraovarian protein. In molluscs, both Vtg synthesis and storage occur within the ovary (Matsumoto et al., 2003; Osada et al., 2004; Matsumoto et al., 2008), and to date, neither the protein structure of Vn nor the mechanism of proteolytic processing of Vtg into Vn has been clarified.

2.2. Methods for the determination of vitellogenin in molluscs

Several methods have been developed to detect and quantify the presence of Vtg mRNA and protein(s) in invertebrates including molluscs. Expression of Vtg mRNA is mainly assessed by reverse transcription RT-PCR and quantitative RT-PCR (qRT-PCR). Because of its quantitative nature, qRT-PCR has been extensively used in a number of species such as mussels *Elliptio complanata* (Gagné et al., 2005), *Mytilus edulis* (Puinean and Rotchell, 2006; Ciocan et al., 2010), scallops, *Chlamys farreri* (Zhang et al., 2012) and Sydney rock oysters, *Saccostrea glomerata* (Anderson et al., 2010; Andrew-Priestley et al., 2012; Tran et al., 2016a, b).

With regard to measurement of Vtg proteins, current methods include the enzyme-linked immunological assay (ELISA), ALP and HPLC. ELISA is a highly sensitive and specific method as it relies on the use of a Vtg specific antibody. The limitation of this assay is its species-specificity; antibodies need to be raised within species for accurate quantification despite the fact that Vtg structures among vertebrates and invertebrates are conserved. Although ELISA is a method commonly used for direct quantification of Vtg levels in fish (Jones et al., 2000) and commercial ELISA kits have become available for several fish species (Fazielawanie et al., 2011), antibodies to Vtg or Vtg-like proteins have been only developed for a few molluscan species, e.g., oysters *C. gigas* (Kang et al., 2003), scallops, *Patinopecten yessoensis* (Osada et al., 2003), clams, *R. philippinarum* (Park and Choice, 2004) and freshwater mussels, *Unio tumidus* (Morthorst et al., 2014).

The ALP assay is an indirect method used to estimate Vtg protein levels in molluscs under the assumption that molluscan Vtg is the most abundant phosphorylated protein in the sample (Gagné and Blaise, 1999). The principle of this method is based on the detection of inorganic phosphate liberated from phosphorylated proteins like Vtg after hydrolysis with alkali. To date, ALP has been commonly used to detect Vtg-like protein in molluscs (Gagné and Blaise, 1999; Gagné et al., 2001; Matozzo and Marin, 2007). However, this method has recently been demonstrated unreliable and has thus raised some legitimate concern over it's use for indirect quantification of yolk protein/Vtg levels in molluscs. Indeed, in a study examining biochemical and proteomic characterization of hemolymph serum in mussels (*Mytilus galloprovincialis*), Oliveri et al (2014) demonstrated that the most abundant phosphorylated protein in hemolymph was an extrapallial protein precursor, not yolk protein. Morthorst et al. (2014) exposed the mussel (*U. tumidus*) to 17β-estradiol (at 57, 164 and 512 ng/L) for 7 weeks and then quantified hemolymph yolk protein levels using both ALP and ELISA. Although not responsive to estrogen, the ELISA method detected higher basal yolk protein concentrations in females than males, while the basal ALP levels were similar for both sexes. ALP also provided unreliable information about Vtg levels in marine mussel gonads (Sańchez-Marín et al., 2017). In this study, Vtg proteins were identified and quantified in male and female gonads by a shotgun label-free proteomic approach and the results were then compared with those measured by ALP. The results indicated that the proteomic approach detected Vtg only in females and their levels varied at different stages of gonadal development, whereas the ALP method detected similar amounts of phosphorylated protein regardless of sex or gonadal development stage (Sańchez-Marín et al., 2017).

HPLC is a method previously optimised by our lab for Vtg protein quantification in the Sydney rock oyster following extraction by t-butyl methyl ether (Andrew et al., 2008). This method isolated a sole protein species from the organic phase and subsequently the protein's identity was confirmed to be Vtg by amino acid sequencing. However, since the structural difference between Vtg and Vn is yet to be resolved in molluscs, it is possible that this method may detect a mixture of both Vtg and Vn rather than Vtg alone.

In general, at present there is no standardized method for molluscan Vtg protein quantification and each method has its own limitations. Given the limitations of the ALP method discussed above, it is advisable that ALP should not be used to estimate Vtg protein concentrations in molluscs. Development of specific antibody for ELISA, optimization of conditions for HPLC and employment of proteomic approaches are, therefore, recommended.

2.3. Evidence of vitellogenin induction in molluscs following exposure to estrogenic EDCs

Matozzo et al. (2008) was the first to provide a comprehensive review of evidence of Vtg induction and its use as a biomarker for estrogenic EDCs in invertebrates (including molluscs). Here, we update the discussion with more recently published literature and further examine the mechanistic basis of estrogen-induced Vtg induction in molluscs.

2.3.1. Laboratory-based studies

Induction of Vtg by natural estrogens in molluscs has been extensively investigated under laboratory conditions over the last two decades (**Supplementary Table 2**). Exposure of bivalve mollusc species to E2 has been shown to significantly increase Vtg gene expression as well as Vn protein content. For instance, after female *C. gigas* were injected *in vivo* with E2 at a dose of 50 µg/100 µL every 10 days for 40 days, or their ovary tissues were exposed *in vitro* in a culture medium containing 10^{-7} to 10^{-4} M E2 for 3 days, significant increases in oocyte diameter and Vn content were observed (Li et al., 1998). Similarly, *in vivo* injection of

E2 (50 µg/100 µL every 10 days) into the ovary of scallops, *P. yessoensis* for 90 days or *in vitro* culture of ovarian tissues (during gametogenesis) at 10^{-5} M E2 for 5 days resulted in higher values of Vn compared to controls (Osada et al., 2003).

The increased Vtg induction by E2 was also examined at different gonadal stages in an exposure experiment of blue mussels, *M. edulis* to E2 at 5 and 50 ng/L for 10 days. Vtg gene expression increased in individuals at the early gametogenesis stage, but not in those at the ripe stage (Ciocan et al., 2010). This result suggests that the status of gonadal development may influence the responsiveness of Vtg gene/protein expression to estrogenic EDCs.

In our own recent study, upregulation of Vtg mRNA expression in the ovaries of Sydney rock oysters, *S. glomerata,* was observed following both *in vitro* (explants) and *in vivo* (aquaria) exposure to E2 (Tran et al., 2016a). Particularly, in the explant experiment, sgVtg mRNA expression was significantly increased by ca. 4- to 6-fold after exposure to E2 at nominal concentrations ranging from 10^{-8} to 10^{-5} M for 48 h The results of the aquaria exposure experiments also showed that after 14 days of exposure, the ovarian mRNA levels of sgVtg in oysters exposed to the nominal concentration of 3.67×10^{-10} M (≈100 ng/L) E2 were significantly increased by 8.2-fold, while those exposed to lower concentrations (9.80×10^{-11} M (≈50 ng/L) and 1.84×10^{-10} M (≈25 ng/L) were not significantly different from the controls.

In addition to natural estrogens, several xenoestrogens including EE2, NP, DP, BPA, tetrabromodiphenyl ether (BDE), benzo(*a*)pyrene (B(*a*)P), have been shown to modulate Vtg production in bivalve species (**Supplementary Table 2**). For example, in our own study, *S. glomerata* exposed to EE2 and 4-NP for 8 weeks exhibited an increase in Vtg, with females exhibiting significant induction at 50 ng/L EE2 and 100 µg/L NP, while males responded to 50 ng/L EE2 only (Andrew et al., 2008). In a later study from our lab on *S. glomerata*, Vtg concentrations in the gonads increased in a dose-response manner after exposure to EE2 (6.25, 12.5, 25, 50 ng/L) in females (after 4 and 49 days) and males (after 4 and 21 days) (Andrew et al., 2010).

Concentrations of 4-NP has been shown to increase Vtg or ALP production in clams and cockles, with males being more responsive than females. Exposure of the Manila clams, *Tapes philippinarum*, to nominal 4-NP concentrations of 0.1 and 0.2 mg/L for 7 days produced elevated Vtg-like protein levels in male hemolymph and digestive glands (Matozzo and Marin, 2005). Similarly, exposure of cockles, *Cerastoderma glaucum*, to 4-NP at a series of concentrations (0.0125, 0.025, 0.05, and 0.1 mg/L) for 7 and 14 days resulted in increases in ALP levels in both males and females, although significant induction varied (from 0.0125 to 0.1mg/L) depending on the status of gonadal development and tissue type (Marin et al., 2008).

Induction of Vtg-like proteins by other industrial chemicals has also been observed in marine mussels and scallops. Blue mussels, *M. edulis*, exposed to OP (50 µg/L), BPA (50 µg/L) or BDE (5 µg/L) for 3 weeks showed an elevation of Vtg levels in gonad tissues (Ortiz-Zarragoitia and Cajaraville, 2006). Exposure of scallops, *C. farreri*, to B(*a*)P at 0.4 and 2.0 µg/L and BDE-47 at 0.1 and 1 µg/L for 10 days showed a significant increase in Vtg mRNA expression levels (Zhang et al., 2012).

2.3.2. Field-based studies

Exposure of molluscs to effluents *in situ* has been used to assess estrogenic effects on Vtg induction (**Supplementary Table 3**). For instance, deploying mussels, *E. complanata* at 1.5 km upstream and 5 km downstream of a municipal effluent release station in the St. Lawrence River, Canada for 62 days showed that both males and females exhibited higher ALP concentrations in hemolymph at the downstream location compared to upstream, and females produced greater ALP responses than males (Gagné et al., 2001).

Similarly, Blaise et al. (2003) deployed caged *E. complanata* at an upstream site (~1.5 km) and at two downstream sites (+8 and +10 km) of a municipal effluent outfall of the St. Lawrence River, Canada for one year. Gonadal ALP was significantly elevated at the + 8 km downstream site compared with the upstream site, while gonadal Vtg protein (assessed by gel electrophoresis) was significantly elevated at the +10 km downstream site compared with the upstream site. Interestingly, changes in the sex ratio of mussels were also observed, with downstream sites exhibiting female-skewed sex ratios (8 km downstream, 62% females; 10 km downstream, 66% females) compared to the upstream site (41% females).

Pampanin et al. (2005) collected mussels in two seasons (February and April) at three sites located in the canals of the Venice historic centre, Italy (where raw sewage was discharged) and a reference location external to the city. No differences in hemolymph ALP were observed between canal locations and the reference location during the non-reproductive season. During the reproductive season, however, male hemolymph ALP was significantly higher at all canal locations compared to the reference, whereas no location differences were observed in females. Females, however, generally exhibited higher ALP concentrations than males.

In a more recent study undertaken in Venice, two bivalve species, the clam, *T. philippinarum*, and the cockle, *C. glaucum*, were sampled at six sites when bivalves were at an early-stage of gameteogenesis (January) and at pre-spawning (June). In January, bivalves from the receiving environment of Campalto (sewage treatment plant) and Marghera (contaminated site) had higher (though not significant) ALP levels in hemolymph, than individuals from other sampling sites. In June, when *T. philippinarum* were sexually mature, greater site discrimination was availed with males displaying higher ALP levels within the receiving environment of Campalto (STP) in comparison to individuals from another site influenced by wastewaters from urban areas. In *C. glaucum*, females were more responsive than males, with statistically significant increases in ALP from individuals within the receiving environment of Campalto (STP) compared to other sites (one of which was relatively uncontaminated) (Matozzo and Marin, 2007).

Research from our own group has provided evidence that *S. glomerata* Vtg in gonads was a suitable molluscan biomarker for assessing estrogenic effects *in situ* (Andrew-Priestley et al., 2012). *S. glomerata* were deployed in Newcastle, Australia in the effluent and biosolid receiving marine waters of Burwood Beach WWTP (Burwood Beach "near", <50 m from outfall; Burwood Beach "far", 100–150 m from outfall) and reference locations (Redhead, Fingal Island 1 and Fingal Island 2) at depths of 4, 8 and 12 m for six weeks. Effluent receiving waters of Burwood Beach WWTP were found to be a suitable impact location; demonstrated via measurement of estrogenic compounds and activity throughout the deployment. Female Vtg gene expression was highest at Burwood Beach locations, yet no significant differences were detected among locations for either sex. Vtg protein in females was significantly higher in *S. glomerata* at Burwood Beach Near compared to reference locations at depths of 4 and 12 m. Increased proportions of females were found at Burwood Beach Near, at 4 m depth in comparison to the 8 and 12m depths ($p < 0.05$). Both Burwood Beach locations also had higher proportions of mature female gonadal development stages compared to reference locations.

In summary, induction of Vtg due to exposure to E2 and xenoestrogens has been reported in several molluscan species, although concentrations used in these studies are generally above environmental levels reported in section 1.2. In terms of Vtg responses, EE2 is consistently found to be many orders of magnitude more potent than E2 and phenolic xenoestrogens, such as NP, OP and BPA. For instance, in *S. glomerata*, EE2 is 2.0-8.0 times more potent than E2, while NP is 0.01-0.02 times less potent than E2. In *M. edulis*, the relative potencies of OP, BPA and BDE to E2 are 0.001-0.0001, 0.001-0.0001 and 0.01-0.001 times, respectively. Vtg responses seem to be generally higher in females than males, and on balance, Vtg is more responsive to estrogens at earlier stages of gonadal development. When it comes to cross-species sensitivity, insufficient evidence is available to make a comprehensive comparison (i.e. limited species examined, limited exposure concentrations tested and most studies not designed to establish lowest observed effect concentrations (LOECs) or no observed effect concentrations (NOECs)). Further, it is difficult to reliably compare between species when maturation status, exposure duration and differing endpoints (Vtg protein versus mRNA) are examined among species/studies. However, from available data it seems that Vtg mRNA of *M. edulis* is more sensitive to E2 compared to that of *S. glomerata* (induced at 5 ng/L vs 100 ng/L) and Vtg protein of *C. glaucum* is more responsive to NP compared to *S. glomerata* (0.0125 mg/L vs 0.1 mg/L). In terms of established fish models of Vtg induction, fish are generally more sensitive to (xeno)estrogens than molluscs. The LOEC of E2 for Vtg protein induction in Japanese mekada reported at 5.0 ng/L (Kitamura et al., 2009) is comparable to Vtg mRNA induction in *M. edulis* (Cicoan et al., 2010). However, the LOEC of E2 for Vtg protein induction in rainbow trout (8.8 ng/L; Thorpe et al., 2000), medaka (8.94 ng/L), fathead minnow (28.6 ng/L) and zebrafish (85.9 ng/L); Seki et al., 2006) are much lower than other molluscan species examined to date, e.g. Vtg mRNA induction in *S. glomerata (*100 ng/L; Tran et al., 2016a). The LOEC for EE2 in terms of Vtg protein induction has been reported as low as 1 ng/L in some fish species (see review by Hutchinson et al., 2006), compared to 6.25 ng/L in molluscs, *S. glomerata* (Andrew at al., 2010). Similarly, Vtg protein induction is about 10 times more sensitive to NP (11.4-11.6 μg/L vs 100 μg/L) in Mekada, *Oryzias laptipes* (Seki et al., 2003) compared to molluscs such as *S. glomerata (*Andrew at al., 2010) and fish Vtg protein is about 5 times more sensitive to BPA (10 μg/L for Seabass *Dicentrarchus labrax;* Correia et al., 2007, 11 μg/L for male gold fish *Carassius* *auratus*; Hatsef et al., 2012) than molluscs such as *M. edulis* (50 µg/L; Ortiz-Zarragoitia and Cajaraville, 2006). Differences in estrogenic sensitivity between molluscs and fish are likely attributable to the differing estrogen receptor (ER)-like/ER binding affinities and metabolic rates between taxa.

Despite the fact that Vtg in molluscs is induced only at the higher end of environmentally relevant estrogen concentrations in the lab, evidence from field-based studies exist, with longer exposure windows, confirming the ability of Vtg to respond to lower, environmentally relevant concentrations of estrogens and other xenoestrogens. Despite this, many of these field studies employ ALP as a surrogate measure of Vtg induction and given the recent evidence demonstrating the unreliability of the ALP method (Oliveri et al., 2014; Morthost et al., 2014, 2017) these findings should be viewed cautiously. Our research, and others, who have used appropriate methods such as ELISA, qPCR, and HLPC in the lab and field to measure Vtg mRNA or protein directly, do provide support however that mollucan Vtgs are induced by environmentally-relevant concentrations of estrogenic compounds. Further, it should be noted that although invertebrate hemolymph is considered equivalent to vertebrate blood, bivalve yolk proteins appear to be synthesized directly in the gonad and therefore are less likely to be transported to the hemolymph (Agnese et al., 2013). Therefore, the gonad should be employed as the target tissue in detecting Vtg in molluscs.

3. TOWARDS A MECHANISTIC UNDERSTANDING OF ESTROGEN INDUCED VITELLOGENESIS IN MOLLUSCS

3.1. *The estrogen receptor-dependent pathway of vitellogenin induction in vertebrates*

Estrogen receptors (ERα, ERβ) are ligand-dependent transcription factors that belong to the nuclear receptor (NR) superfamily. In vertebrates it is known that they are divided into six domains, the N-terminal domain (A/B), C domain (DNA binding domain, DBD), hinge region (D), E domain (ligand binding domain, LBD) and C-terminal domain (F) (Kuma et al., 2011). Of these, the DBD and LBD are the most highly conserved among species. The DBD recognises and binds to the estrogen responsive element (ERE) in DNA, while the LBD binds estrogens and mediates hormone-dependent transcription of target genes.

There is ample evidence that exposure of fish to estrogenic EDCs increases Vtg production and the underlying mechanism has been well characterised. These characteristics make fish Vtg induction a reliable biomarker for detecting estrogenic EDCs in aquatic environments (Sumpter and Jobling, 1995). The protein is produced in response to estrogens binding to intracellular estrogen receptors (ERs), which are ligand-activated transcription factors (Arukwe and Goksoyr, 2003; Bouter et al., 2010; Chakraborty et al., 2011). Particularly, in the absence of estrogen ligands, monomeric ER is sequestered in an inactive complex with chaperones. Following ligand binding, ER folds into an active conformation, which in turn triggers a cascade of events, including dissociation from chaperones, dimerization, phosphorylation and translocation to the nucleus. In the nucleus, the activated DNA binding domain binds to specific DNA motifs known as estrogen responsive elements (EREs) in the promoter of target genes (including Vtg), resulting in the recruitment of coactivators and transcriptional activation of the Vtg gene (**Figure 1**).

3.2. Estrogen receptors in molluscs and their responsiveness to estrogens

Although ERs are believed to be unique to vertebrates, their orthologues have been isolated in a number of molluscs, e.g, bivalves (Matsumoto et al., 2007; Zhang et al., 2012; Raingeard et al., 2013; Nagasawa et al., 2015; Tran et al., 2016b), gastropods (Thornton et al., 2003; Kajiwara et al., 2006; Bannister et al., 2007; Stange et al., 2012; Hultin et al., 2014) and the cephalopod *Octopus vulgaris* (Keay et al., 2006). However, unlike vertebrate ERs the molluscan ER appears not to bind estrogens and likely functions as a “constitutively active” transcription factor acting on EREs (Thornton et al., 2003; Kajiwara et al., 2006; Keay et al., 2006; Matsumoto et al., 2007; Tran et al., 2017). Due to the fact that certain molluscan ERs respond to exogenous estrogens with an increase in their mRNA expression (Ciocan et al., 2010; Stange et al., 2012; Völker et al., 2014; Nagasawa et al., 2015, Tran et al., 2016b), and ER mRNA has a close correlation with E2 levels at different stages of ovarian development in the Fujian oyster *C. angulata* (Ni et al., 2013) it is worth investigating whether molluscan ERs play a potential role in estrogen signaling. If increases in ER mRNA expression is reflected at the protein level and the ER-dependent transcription level is dependent on ER availability, ER mRNA upregulation could be one of the factors that activates its target gene expression.

In our recent experiments, activation of *S. glomerata* Vtg by E2 was abolished by ICI 182,780 (a specific ER antagonist), raising the possibility that a novel receptor, which contains a ligand-binding pocket similar to that of the vertebrate ERs, is involved in estrogen signalling in molluscs (Tran et al., 2016a). Because neither of the two steroid receptors (SRs) present in the molluscan genome (i.e., the ER and the orphan nuclear receptor estrogen-related receptor (ERR); Vogeler et al., 2014) exhibits estrogen-binding properties (Kajiwara et al., 2006; Keay et al., 2006; Bannister et al., 2007; Matsumoto et al., 2007; Tran et al., 2017), the hypothetical receptor may regulate E2-dependent gene expression through a non-genomic pathway.

3.3. Vtg promoter and its interaction with the estrogen receptor

It has been well established in vertebrates that estrogen induces Vtg gene transcription primarily through the ERs, which bind to a ERE(s) in the promoter region (Klinge, 2001). Based on the assumption that a similar mechanism exists in molluscs, the Vtg gene and its promoter were first cloned from *S. glomerata* by our research group (Tran et al., 2016a). Sequence analysis revealed that the *S. glomerata* Vtg (*sgVtg*) promoter does not contain a consensus palindromic ERE, but houses three half-EREs (½EREs) which consist two direct repeats and one inverted repeat. Although the consensus EREs have higher affinities for ERs than the non-consensus EREs *in vitro* (Driscoll et al., 1998), evidence exists that the latter components are critical regulators of ER-driven transcription. As reviewed by Klinge (2001), ½EREs or imperfect EREs are much more abundant than consensus EREs in most estrogen responsive gene promoters. It is, therefore, tempting to speculate that the three ½EREs residing in the sgVtg promoter may interact and function in a similar manner.

The interaction between oysters isolated ER (sgER) and sgVtg promoter was then investigated using reporter gene assays in our lab (Tran et al., 2017). Transient transactivation experiments incorporating the Vtg promoter and a luciferase reporter gene indicate that sgER is capable of transactivating sgVtg, however, E2 was not required for the transactivation effect. In order to assess the interaction of sgER with the ½EREs found in the sgVtg gene promoter, mutagenesis experiments of the promoter were performed. The results showed that induction of reporter activity by sgER was decreased by 50% when one of the three ½ERE sites was mutated or was reduced to zero when all three ½ERE sites were deleted or mutated. In addition, the specific binding between the sgER and ½ERE sites in the sgVtg promoter was confirmed by electrophoretic mobility shift assays (EMSA). Altogether, the findings suggest that these ½ERE sites may play a vital role in the regulation of sgVtg gene transcription by sgER, although sgER does not contain an "estrogen-binding site", unlike the vertebrate ERs.

3.4. Towards possible pathways of estrogen- induced vitellogenesis in molluscs

As discussed above, the current evidence supports the presence of a novel E2-dependent receptor(s) in oysters (Tran et al., 2016a,b; Tran, 2017; Tran et al., 2017). Since none of the molluscan nuclear ERs isolated so far have estrogen-binding capacity, the novel receptor could possibly be a membrane-associated receptor and thus the role of estrogens in the regulation of Vtg synthesis could be mediated through a non-genomic pathway. Non-genomic actions of estrogens are usually associated with the activation of second messengers and various protein-kinase cascades that can eventually lead to indirect changes in gene expression due to phosphorylation of transcription factors (Bjornstrom and Sjoberg, 2005). Our prediction is supported by indirect evidence from the literature. For instance, Stefano et al. (2003) showed that treatment of *M. edulis* pedal ganglia with E2-BSA (membrane-impermeable E2 conjugated to BSA) resulted in a rapid increase in nitric oxide release and this stimulation was abolished by tamoxifen, an antiestrogen. In other studies by Canesi et al. (2004, 2006), the extracellular signal-regulated proteins (e.g, mitogen-activated protein kinase-MAPK, extracellular signal-regulated kinase - ERK, protein kinase C-PKC) and CREB (cAMP response element binding protein) in hemocytes were activated within 5 min of E2 treatment and the ERK activation was inhibited by tamoxifen. In human cells, the activation of similar intracellular signalling transducers by non-classical membrane-bound ERs is implicated in increasing gene transcription of the nuclear ERs (Roepke et al., 2008; Sinchak and Wagner, 2012). The Gq-mER, for example, is a novel membrane-bound ER which likely possesses a ligand-binding pocket resembling that of the nuclear ERs; Gq-mER is activated by E2-BSA and antagonised by ICI 182, 780 (Qiu al., 2003, 2006). In addition to estrogen, Gq-mER binds and is activated potently by STX, a diphenylacrylamide compound that is unable to bind either ERα or ERβ (Qiu et al., 2003). This feature of STX allows differentiation of cellular/molecular effects mediated by Gq-mER from those mediated by the classical membrane-bound/cytoplasmic ERs. Previous research found that STX-liganded Gq-mER activates a signal transduction pathway involving phospholipase C (PLC), PKC and protein kinase A (PKA), resulting in increased phosphorylation of CREB and consequently increased expression of CREB-mediated genes, including ERα and ERβ (Roepke et al., 2008). Another possible cross-talk between the Gq-mER mediated-non genomic pathway and the genomic pathway could be due to the PKC-induced activation of MAPK/ERK, which in turn causes phosphorylation of other transcription factors required for target gene expression (Roepke et al., 2008).

Based on the assumption that a similar novel membrane-bound ER is present in molluscs, we propose the following mechanistic model to explain how estrogens regulate transcription of the molluscan ER and Vtg genes via an upstream non-genomic pathway (**Figure 2**): Upon E2 binding, the novel E2-dependent receptor may activate its downstream intracellular targets (e.g., protein kinase cascades). The activation of these targets is expected, in turn, to activate specific transcription factors involved in estrogen-dependent gene expression through increasing their phosphorylation status. This transcriptional activation together with the subsequent increase in ER protein levels may result in the upregulation of ER and ER-responsive genes (e.g., Vtg) in the absence of an estrogen-sensitive nuclear receptor. However, further studies are necessary to test whether the novel receptor is membrane associated and to verify the role of non-genomic mechanisms in the activation of ER and Vtg.

4. DO MOLLUSCS PRODUCE ENDOGENOUS ESTROGENS?

Although the presence of typical sex steroids, testosterone (T), E2 and progesterone (P) have been documented in a number of molluscan species, their endogenous origins have been repeatedly questioned (Scott, 2012, 2013, 2018). Several researchers have expressed views in support of the endogenous origins of molluscan sex steroid hormones. For example, they have argued that most of the crucial enzymes responsible for the biosynthesis and metabolism of steroids have been identified in the main classes of molluscs, including cephalopods (D'Aniello et al., 1996), gastropods (Gottfried and Dorfman, 1970; Di Prisco and Dessi' Fulgheri, 1975; Le Guellec et al., 1987; Oberdörster et al., 1998) and bivalves (De Longcamp et al., 1974; Morcillo et al., 1998; Le Curieux-Belfond et al., 2001). However, there are some who are not in favour of this viewpoint. For instance, Kanazawa (2001) found no evidence of cholesterol synthesis in several of the bivalve and cephalopod species that were examined. In addition, although a cholesterol side-chain cleavage activity (P450scc) (Gottfield and Dorfman, 1970) has been detected in molluscs, it has been reported only in the slug *Ariolimax californicus* and at very low levels (Fernandes et al., 2011). Aromatase CYP19, the key enzyme that converts T to E2 and androstenedione to estrone in vertebrates, has not been identified in molluscs (Fernandes et al., 2011) nor has the gene been found in any of the molluscan genomes (Scott, 2012). Moreover, functional studies have so far failed to demonstrate the responsiveness of any molluscan nuclear receptors to steroid hormones (Tran et al., 2017).

Researchers who have questioned the endogenous origin of molluscan steroids suggest the hypothesis that molluscan steroids are taken up from external sources, i.e. plant or animal steroids (Janeczko and Skoczowski, 2005), from the surrounding waters (Scott, 2012, 2018) or via food intake (Goto et al., 2012). However, a recent study by Zheng et al. (2014) provided evidence that in the absence of external sources of estrogens, sex steroids are synthesised endogenously in molluscs. It was demonstrated in the New Zealand mudsnail that fadrozole, a specific inhibitor of CYP19 aromatase, reduces both E2 levels and female fecundity, an observation that supports the existence of vertebrate-type aromatase in molluscs (Gust et al., 2010).

Although the endogenous origin of mollusc steroids is controversial, their biological functions cannot be ignored. As reviewed by Scott (2013), 85% of the relevant research papers (published between 1970 and 2012) reported that sex steroids in molluscs have at least one biological effect. In fact, many reports have demonstrated the importance of steroids in the reproductive development of molluscs, particularly E2 in sexual differentiation, gonadal maturation, gametogenesis and vitellogenesis (Mori et al., 1969; Reis-Henriques and

Coimbra, 1990; Osada et al., 1992; D'Aniello et al., 1996; Matsumoto et al., 1997; Wang and Croll, 2006; Croll and Wang, 2007).

5. TOWARDS VITELLOGENIN AS A ROBUST BIOMARKER FOR ASSESSING EXPOSURE AND EFFECTS OF ESTROGENIC EDCS

In order for any validated and robust biomarker to be considered useful as a surrogate of exposure to indicate the relative contaminant load, and to provide information on adverse biological effects due to exposure, a number of key criteria have been proposed (e.g. Huggett et al., 1992; Hook et al., 2014).

5.1. Specificity of the biomarker response

It is preferable to understand if the biomarker response is specific to a particular class of contaminant and indicates exposure to that contaminant class only, or alternatively responds to a range of contaminant classes and thus is a more generic biomarker of multiple contaminant stress. In the case of estrogenic EDCs, although the precise mechanism of Vtg induction in molluscs remains to be elucidated, we assume that Vtg is induced through a novel ER-mediated process, thus Vtg should in theory respond only to estrogens, and a range of xenoestrogens, which exhibit some structural similarities to native estrogen. It is well established from a wealth of studies that a variety of estrogens, xenoestrogens and effluents increase Vtg expression (see section 2.3), but further work is required to establish the precise molecular basis for vitellogenesis in molluscs in order to ascertain its estrogenic/xenoestrogenic specificity.

In addition to being involved in yolk protein formation, recent evidence suggests Vtg could also play a novel role in immune function. It has been demonstrated in fish that Vtg is induced after challenge to bacteria (Shi et al., 2006). Further, it appears that fish Vtg acts as a pattern recognition molecule which facilitates macrophage mediated phagocytosis and thus the removal of bacteria (Li et al., 2008, 2009). In molluscs, Bouchard et al. (2009) showed that after exposure of mussels (*E. complanata*) to municipal effluents, Vtg-like production was strongly positively associated with phagocytocis, suggesting Vtg may respond to bacterial challenge in molluscs and may facilitate phagocytosis. If this is the case, one might question the specificity of Vtg to estrogens. Thus, future research effort is required to investigate whether Vtg has immune function in molluscs and whether Vtg is induced upon exposure to pathogens such as bacteria. This is important when employing Vtg as a biomarker in the field, as effluent receiving waters are likely to be elevated not only in estrogens but concomitantly with significant bacterial loads.

5.2. Demonstrated dose-response relationships

If Vtg is employed as a biomarker in the field as a surrogate of estrogenic activity rather than measuring complex mixtures of (xeno)estrogens via direct chemical methods, and if we wish to assess relative estrogenic contaminant loads among locations in field-based scenarios using Vtg, we need to understand the dose-response dynamics between exposure to estrogens and Vtg responses. However, many dose- endocrine biomarker response relationships are not monotonic, making their utility to indicate relative contaminant exposure rather problematic (Vandenberg et al., 2012). Most studies to date have exposed molluscs to limited estrogen exposure concentrations in the laboratory and not examined the nature of the dose-response relationship (see section 2.3). However, a few studies have exposed molluscs in the laboratory to increasing concentrations of environmentally relevant ranges of (xeno)estrogens and have demonstrated linear dose-response relationships, suggesting that Vtg may be useful as a calibrated quantitative indicator of total environmental estrogenicity (Li et al., 1998; Marin et al., 2008; Andrew et al., 2010).

5.3. Biomarkers of exposure versus biomarkers of effect and early warning utility

Biomarkers can be classified into two groups: those indicating exposure, and those indicating adverse effect. Biomarkers of exposure demonstrate that the organism has been exposed to the contaminant class of interest and is eliciting a specific biological response to contaminant exposure. Nonetheless, the biomarker response is not necessarily indicative of an adverse effect in terms of causing a toxic response that will impact the organism's metabolism, physiology or ultimately its fitness (ability to survive and/or reproduce). Conversely, biomarkers of (adverse) effect are biological responses elicited upon exposure to a contaminant that indicate not only exposure has occurred, but that toxic biological effects have occurred with direct impacts on fitness. It is clear from both the lab- and field- based studies that Vtg is a reliable biomarker of exposure to estrogenic chemicals in molluscs, but whether this has any implications in terms of adverse effects on organism functioning and ultimate fitness is debatable. It is clear that Vtg induction in male molluscs is evidence of endocrine disruption, as males do not normally produce Vtg, but links to adverse reproductive effects are thus far limited. Our own study, however, has demonstrated Vtg's early warning utility in molluscs, i.e., early Vtg induction is correlated with later impacts on reproductive physiology. For example, upon exposure to EE2, increases in female Vtg were predictive of later increases in female gonadal developmental stages and increases in oocyte area, and early male Vtg production was predictive of later lower male gonadal developmental stages. Further, Vtg production preceded intersex formation and even protandric progression to female, though the underlying molecular mechanisms are likely not linked (Andrew et al., 2010). Such disrupted gonadal development in both sexes may adversely affect spawning synchronisation in molluscs and could possibly lead to impacts on reproductive success and genetic variability of offspring.

5.4. Temporal dynamics of the biomarker response

In order for Vtg to be employed as a robust biomarker of estrogenic exposure and effect in molluscs, the temporal dynamics of the biomarker response must be understood. We need to ascertain how soon after exposure the response is induced (induction time), how long after cessation of exposure does the response still persist (recovery time) and whether the response may return to basal levels despite continued exposure (adaptation) (Wu et al., 2005). These dynamics are important for utility in the field depending on whether we wish to employ the biomarker to track temporal changes in contaminant exposure (fast induction-fast recovery) or use the biomarker as an archive of past pulse contamination events (slow recovery). Little is known in terms of the temporal dynamics of Vtg induction and recovery in molluscs. It is hypothesised that Vtg mRNA would be more responsive temporally to changes in estrogenic exposure than Vtg protein (as demonstrated in sheepshead minnows, Hemmer et al 2002), though data for invertebrates is lacking. An additional complication is the fact that the site of Vtg synthesis (the gonad) undergoes seasonal developmental cycles where Vtg is upregulated for transport into developing oocytes. The only temporal data available suggests that Vtg induction is most responsive to exogenous estrogens during the early gametogenic phase of gonad development (Andrew et al., 2010; Ciocan et al., 2010).

6. CONCLUSION

In conclusion, estrogenic EDCs in the aquatic environment are of significant environmental concern with most natural and synthetic estrogens in effluents and receiving waters occurring in the ng/L range, while xeno-estrogenic industrial chemicals are present in the µg to low mg/L range. These concentrations exceed laboratory-based predicted no effect concentrations and have been evidenced to impart negative reproductive impacts on resident aquatic biota including elevated Vtg production in females and males, intersex production, shifts in the sex ratio to female-biased populations, and aberrant reproductive development. Although the molecular mechanisms of estrogen-induced vitellogenesis is well established in vertebrates, such as fish, much less is known in terms of estrogen signalling in invertebrates such as molluscs. There is ample laboratory and field-based evidence demonstrating that molluscan Vtg in males and females is induced upon exposure to estrogens, xenoestrogens and effluents, and thus Vtg holds promise as an invertebrate biomarker for exposure to estrogens. In terms of molluscan mechanisms of Vtg induction, exposure experiments in our own lab have shown that Vtg mRNA expression is upregulated by E2 and treatment with an estrogen receptor antagonist abolished the upregulation, suggesting a requirement for an estrogen-dependent receptor for transcriptional activation. We have also found a molluscan ER that can activate Vtg gene expression through increasing luciferase activity driven by a Vtg promoter fragment containing three ERE half sites. However, like other molluscan ERs, a requirement for estrogen is not observed for the transactivation of Vtg by ER and the ER is devoid of estrogen binding. Overall, we hypothesise that the estrogen responsiveness of Vtg is regulated by a novel, yet to be identified, ligand-dependent receptor involved in estrogen signalling. In order for Vtg to be employed as a reliable biomarker for estrogenic exposure and effect, further studies are required to fully understand the molecular mechanisms of Vtg induction and its possible role in immune function so that its estrogenic specificity can be unequivocally established. More evidence linking early Vtg induction to adverse effects at higher levels of biological organisation is needed. Furthermore, the temporal dynamics of Vtg to changes in estrogenic exposure need to be understood if the biomarker is to be employed to track estrogenic loads in the field. Given the limitations of the ALP assay it is recommended that Vtg mRNA/protein is assessed via direct techniques such as qPCR, ELISA and HLPC and that the gonad is the target tissue for analysis. Finally, each of these research goals ideally needs to be addressed on a species by species basis. Overall, molluscan Vtg offers promise as a biomarker for indicating both exposure to, and effect of, estrogenic chemicals in the field, but further focused research effort is required before its utility can be fully realised.

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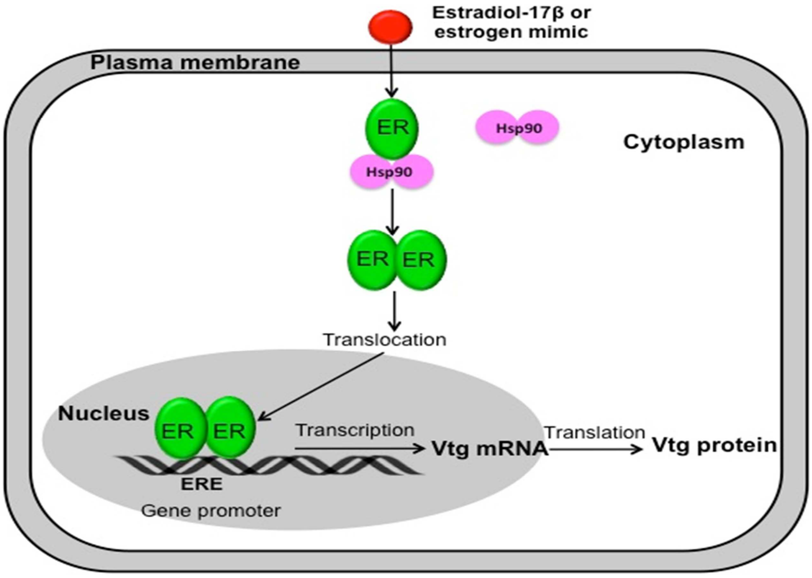
FIGURE CAPTIONS

Figure 1. Estrogen receptor - mediated Vtg induction mechanism in fish. Estrogens or estrogen mimics enter the liver cells and then are retained in the cytoplasm. In the absence of ligands, the ER is found in association with heat shock protein 90 (Hsp90). In the presence of ligands, ER dissociates from Hsp90 and usually dimerises prior to translocation of the complex into the nucleus. The E2-ER complex binds tightly in the nucleus at the EREs located in the Vtg promoter region. This ER-ERE interaction results in the activation of Vtg transcription and a subsequent increase in Vtg mRNA levels.

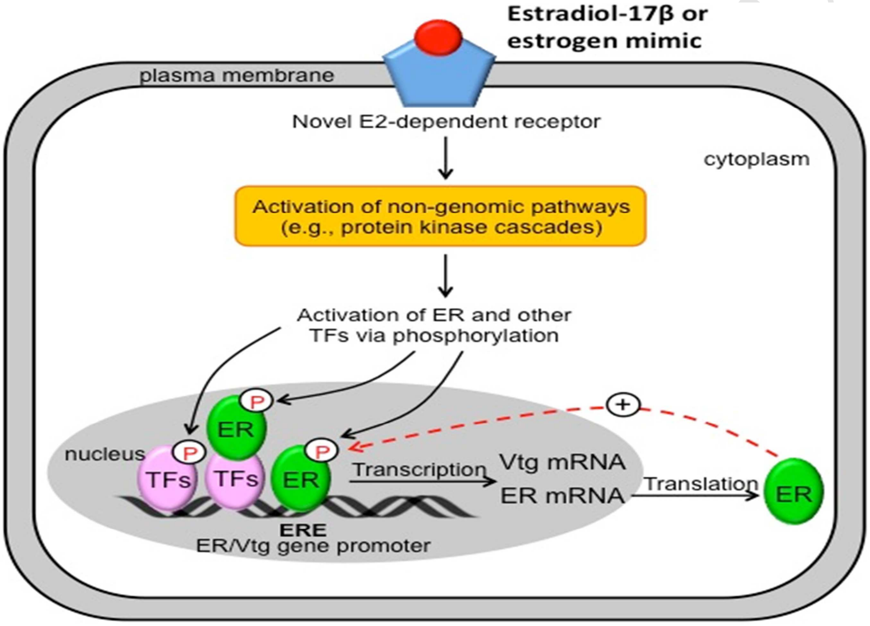
Figure 2. Proposed mechanistic model for the regulation of molluscan Vtg gene expression by estrogens. Estrogens (e.g., E2) induce Vtg gene expression through interplay between nongenomic and genomic pathways. Binding of estrogens to membrane associated receptor(s) triggers the activation of second messengers and protein kinase cascades. These non-genomic effects of E2 result in the activation of ER and other TFs via phosphorylation. Phosphorylated ERs and TFs bind to EREs in the ER and Vtg gene promoters to activate gene transcription. The increased ER transcription subsequently leads to higher levels of the ER protein available for transcription of its own gene (ER) and the Vtg gene**.**

Figure 1:



1302

1303 **Figure 2:**



1304

- Environmental concentrations of estrogenic EDCs exceed lab-based PNECs
- Estrogenic EDCs impart negative reproductive impacts on resident aquatic biota
- Molluscan Vtg is induced upon exposure to estrogens in the lab and field
- Estrogens likely induce Vtg via receptor mediated non-genomic & genomic mechanisms
- More research establishing reliability of the molluscan Vtg biomarker is required