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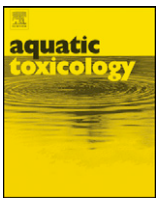

nonylphenol and 17α-ethynylestradiol exposure in the Sydney rock *strea glomerata*: Vitellogenin induction and gonadal development

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A B S T R A C T

Adult *Saccostrea glomerata* were exposed to environmentally relevant concentrations of 4-nonylphenol (1 μg/L and 100 μg/L) and 17α-ethynylestradiol (5 ng/L and 50 ng/L) in seawater over 8 weeks. Exposures were performed to assess effects on vitellogenin induction and gonadal development during reproductive conditioning. Chronic direct estrogenicity within gonadal tissue was assessed via an estrogen receptor-mediated, chemical-activated luciferase reporter gene-expression assay (ER-CALUX®). Estradiol equivalents (EEQ) were greatest in the 100 μg/L 4-nonylphenol exposure (28.7 ± 2.3 ng/g tissue EEQ) while 17α-ethynylestradiol at concentrations of 50 ng/L were 2.2 ± 1.5 ng/g tissue EEQ. Results suggest 4-nonylphenol may be accumulated in tissue and is partly resistant to biotransformation; maintaining its potential for chronic estrogenic action, while 17α-ethynylestradiol, although exhibiting greater estrogenic potency on biological endpoints possibly exerts its estrogenic action before being rapidly metabolised and/or excreted. A novel methodology was developed to assess vitellogenin using high-performance liquid chromatography (HPLC). Exposure to both 17α-ethynylestradiol (50 ng/L) and 4-nonylphenol (100 μg/L) produced increases in vitellogenin for females, whereas males exhibited increases in vitellogenin when exposed to 50 ng/L 17α-ethynylestradiol only. Females exhibited greater vitellogenin responses than males at 50 ng/L 17α-ethynylestradiol only. Histological examination of gonads revealed a number of individuals exhibiting intersex (ovotestis) in 50 ng/L 17α-ethynylestradiol exposures. Male individuals in 1 μg/L and 100 μg/L 4-nonylphenol exposures and 5 ng/L 17α-ethynylestradiol were at earlier stages of spermatogenic development than corresponding controls.

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significant estrogenic compounds in estu-
from a range of anthropogenic sources
discharge from sewage treatment plants,
t works and agricultural/livestock runoff) are

common among the nonylphenol polyethoxylates (Marcomini et al., 1990).

In sewage effluent and open waters 17α-ethynylestradiol is usually reported in the ng/L range, from 0.2 to 42 ng/L in sewage effluent (Desbrow et al., 1998; Ternes et al., 1999) and <0.1 up to 15 ng/L in open waters (Belfroid et al., 1999; Aherne and Briggs,

ted to date withholding the examination
mosquito fish, *Gambusia affinis holbrooki*
usch et al., 2006b). Little work has been con-
marine species, and to our knowledge, no
ate have examined the effects of estrogenic
ic Australian molluscan taxa.
iological relevance of vertebrate-type
vertebrates is debated, considerable evi-
trogens play a functional role. The most
nd Porte, 2007) suggests that selected
le to synthesise sex steroids, including
lysis by cytochrome P450s and steroid
lluscs, compiled evidence to date sug-
nverted to pregnenolone by cytochrome
ge. Pregnenolone may be converted to
5-Δ4-hydroxysteroid dehydrogenase and
umber of cytochrome P450s to cortisol,
xyprogesterone and androstenedione (an
ursor). Androstenedione may also be pro-
ndrostenedione (DHEA), and subsequently
cytochrome P450 aromatase. Seventeen
drogenases (17β-HSDs) facilitate produc-
androstenedione and 17β-estradiol from
006). Thus xenobiotics which mimic the
and exert effects through direct receptor-
on) or contaminants that alter sex steroid
with steroidogenesis, metabolism and/or
ndogenous sex steroids are likely to affect
d to endocrine action in molluscan models

tellin) accumulates in molluscan oocytes
ion, synthesised from its precursor vitel-
s have suggested that vitellogenin is under
tradiol (Suzuki et al., 1992; Li et al., 1998).
-estradiol and 250 nM 4-nonylphenol has
reases in lipophilic alkali-liable proteins
to increased vitellogenin) in both male
iptio complanata, under laboratory condi-
01). Furthermore, exposure of both male
a, in environments contaminated through
icultural runoff waters exhibited increased
gh higher for females than males (Blaise et

ggests estrogenic compounds may also
tion with exposure during early ontogeny
o sex ratios towards females in adult-
Furthermore, estrogenic compounds may
ion of gametes for adult individuals dur-
active gonadal maturation/reproductive

The native Australian Sydney rock oyster, *Saccostrea glomerata*, is thought to exhibit protandric dioecy (Asif, 1979). Male and female sexes are separate; though a subset of the 'male' population are protandric, maturing as males at the juvenile stage, with the ability to change sex to females in later years (Asif, 1979; Guo et al., 1998). Sex reversal usually occurs between spawning seasons when the gonad is undifferentiated, thus the sexes are separate at any sampling point in time, with a shift to greater proportions of females in the population with size/age (Thompson et al., 1996). Hermaphroditism is present but with very low occurrence with reported percentages within populations of 0.7% (Dinamani, 1974) and 0.4% (Cox et al., 1996). Evidence of the sensitivity to estrogens in other bivalve molluscan taxa, coupled with the potential of protandric progression, suggests that exposure to xeno-estrogens may also exert effects on vitellogenesis and the differentiation of gonadal tissue in *S. glomerata*.

S. glomerata forms the basis of the oldest and largest aquaculture industry in NSW with high commercial value ($US28 million) along the East coast of Australia from southern Queensland to southern NSW (White, 2002). Aquaculture initiatives are increasingly occurring in urbanised areas and may be affected (both in terms of productivity and marketability) by wastewater treatment plants where effluent discharges may potentially contain elevated levels of estrogenically active compounds. Indeed *S. glomerata* has been employed as a biomonitor for a range of contaminant classes (metals and organic contaminants) in Australian waters (Scanes, 1997), and may also be an appropriate sentinel species for the detection of biological effects from exposure to estrogenically active compounds.

Our current experiment aimed to determine what effects environmentally relevant concentrations of two xeno-estrogens, 4-nonylphenol and 17α-ethynylestradiol may impart on vitellogenin induction and reproductive condition during a gonadal maturation phase in *S. glomerata*. The estrogen receptor assay (ER-CALUX®) was employed to measure uptake of estrogenic compounds and assess chronic direct estrogenicity within gonadal tissue of xeno-estrogens. It was hypothesised that vitellogenin induction would increase upon exposure to 17α-ethynylestradiol and 4-nonylphenol, particularly in females and at higher exposures. It was further hypothesised that histological examination of individuals exposed to 17α-ethynylestradiol and 4-nonylphenol may reveal significant effects on gonadal development.

2. Materials and methods

2.1. Experimental design

Four hundred and fifty, 18-month-old *S. glomerata*, were used for experimentation. Oysters were sourced from a farm in the Port

igher exposure concentrations were chosen
l values of estrogenically active compounds
ent and lower exposure concentrations based
likely to be typical in estuaries adjacent to
nts and/or found to elicit biological effects in
rne and Briggs, 1989; Desbrow et al., 1998;
, 2005; Langston et al., 2005; Ternes et al.,
posure duration was chosen as the average
adal maturation from resting phase to mature
ning dependant on initial physiological con-
1991).
g/L salinity) within each replicate aquarium
atment exposure regimes maintained thrice
seawater was heated to 22 °C prior to water
nity and temperature were monitored daily.
xed diet daily containing 660 mL of each algal
ing: *Pavlova lutheri*, *Chaetoceros muelleri* and
galbana (1.5×10^9 cells/oyster/day). Temper-
es were chosen in order to facilitate gonadal
g, 2003).
ex and algal consumption were measured to
rential/confounding effects of treatments on
dition due to the presence of estrogenically
r ethanol as a solvent carrier. Wet condi-
sured at the beginning and completion of
was calculated using the formula (Wet tis-
Wet whole weight − wet shell weight) (Lucas
Algal consumption was monitored weekly
riment. Three hours after a water change
f water was sampled from each aquarium
formalin. Algal cell numbers were enumer-
eter from each experimental unit to provide
algae consumption. Neither 4-nonylphenol
diol (at all experimental exposure concen-
ndition (pooled mean of condition index,
index; $F = 0.96$, $p > 0.05$), nor algal concentra-
algal cells per mL, $0.684 \times 10^9 \pm 0.002 \times 10^9$
ng the exposure period.

y of homogenates from oysters exposed to
ubstances were analysed via the BioDetection
ptor-mediated, chemical-activated luciferase
ssion (ER-CALUX®) assay. The ER-CALUX®
man breast cancer cells modified to express
r gene upon exposure to estrogenically active
ailed description of ER-CALUX® refer to Legler
atory analyses for the ER-CALUX® assay were

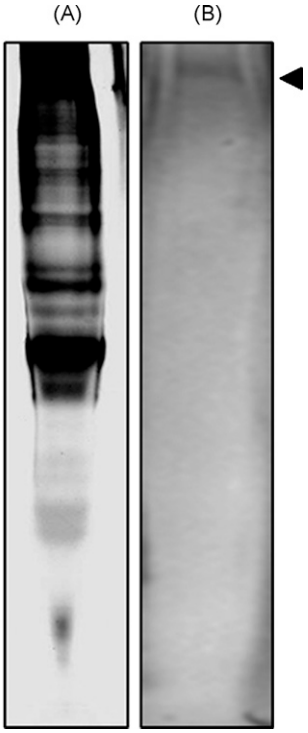
the 48 h incubation period, incubation wells were examined under microscope for any signs of contamination or abnormal cell growth. Cells were then exposed to the oyster test sample(s) and placed back in the CO_2 incubator for a further 24 h. The medium was then removed from the cells, dosed with 50 μL of lysis reagent and incubated at room temperature (<20 °C) for 15 min. The microtiter plates were then shaken for 2 min at room temperature at 300 rpm (2 mm orbit). One microtiter plate was analysed at a time by removing the lid and placing plate into a BMG Lumistar luminometer. Glowmix reagent (luciferin; luciferase substrate) was robotically dispensed into each well immediately prior to reading the sample, and the sample was quenched using 1 M NaOH prior to the next well being read. Analysis of data was undertaken using Biodetection Systems Software (BiodetectionSystems, 2006) and data are reported as EEQ (estrogen equivalents) for ng/g tissue fresh weight (of oysters) (Legler et al., 1999).

2.3. Extraction and HPLC assay for measuring vitellogenin in *S. glomerata*

The method of Gagné and Blaise (1999) was adapted/modified to extract vitellogenin from oyster gonadal tissue for subsequent High Performance Liquid Chromatography (HPLC) analysis. Excised gonad tissue (approximately 100 mg) was stored and homogenised in 0.225 mL citrate buffer (pH 6.5 10 mM and with 16 mg/mL polyethylene glycol) and 25 μl of the protease inhibitor aprotinin (Gagné and Blaise, 1999; Brodeur et al., 2006) and stored at −80 °C.

Gonad samples were later thawed and 500 μL Tris–HCl buffer was added and sample was homogenised using micro pestle. 100 μL of tissue homogenate was transferred to a glass centrifuge tube. Based on the Organic Alkali-liable phosphate method described by (Gagné and Blaise, 1999) 800 μL *t*-butyl methyl ether (Chromsolv grade, Sigma–Aldrich) was used to extract vitellogenin. The mixture was then vortexed and incubated at 4 °C for 30 min. The organic phase was then separated and dried under N_2. This was then resuspended in 1000 μL of PBS. The purification of oyster vitellogenin into the organic phase was also confirmed as 99% (peak area 2256.2 ± 16.7) by also injecting and eluting the discarded aqueous phase; this phase contained less than 1% (peak area 20.5 ± 7.7) vitellogenin but had multiple peaks of other proteins.

Analysis of samples was performed using a Hewlett-Packard 1100 Series HPLC (Waldbronn, Germany). Separation of sample analytes was achieved at room (24 °C) temperature on a Hypersil ODS (C18) (Agilent Technologies, USA) analytical column with dimensions 2.0 mm i.d. × 125 mm, 3 μm particle size and 120 Å pore size. The guard column was a Hypersil ODS (C18) cartridge (Agilent Technologies, USA), with dimensions 4.0 mm i.d. × 4 mm, 5 um par-



rea glomerata vitellogenin. (A) Aqueous extracts were
is with subsequent silver staining. Numerous bands
o >170 kDa (B). Organic extracts were subjected to gel
ent silver staining. The only prominent protein band
d was excised for amino acid sequencing.

ol blue) and heated at 100 °C for 5 min. The
lved on 4–20% gradient Tris glycine poly-
NG31-420) before being stained overnight
lliant blue (G-250, Sigma–Aldrich) then
llowing this treatment, numerous bands
queous phase (Fig. 1A). In comparison, a
was detected in the organic phase cor-
of molecular weight >170 kDa (Fig. 1B).
sensitivity of silver staining (Blum et al.,
ny additional proteins within the sample.
was therefore excised and prepared for

was determined via 1D NanoLC ESI MS/MS
ccess to the Australian Proteome Analy-
under the Australian Government's Major
ities program. The excised gel plug was

```
  1 iesvnptqqqvpenlfrpltgkdadqmikdlsrpinflyvrgnvreikqeaddpewsvnv
 61 kkgllsilevnldkrkqldqsssiprvlrpqssnedsmykvmepsiggecetlykvspht
121 sssddpimyitkvrnydncldrpmyfssmfhgkrcaecvkersd
```

Fig. 2. Alignment of the amino acid sequence of peptide fragments obtained from *Saccostrea glomerata* excised protein band via 1D NanoLC ESI MS/MS analysis with only 'known' sequence of *S. glomerata* vitellogenin; a previously determined vitellogenin amino acid sequence deduced from a partial cDNA fragment (GenBank accession number ABW69671.1). Sequenced peptides with matches to the deduced known vitellogenin sequence are underlined.

charged ions (counts >50) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 1 s (m/z 100–1600). The LC/MS/MS data was used to search *Other Metazoa* entries using Mascot (Matrix Science, London, UK) in the NCBI protein database and seven peptides provided matches with 43% coverage and an alignment score of 63.9 to an oyster vitellogenin amino acid sequence deduced from a partial cDNA fragment of *S. glomerata* origin (Anderson et al. unpublished, GenBank accession number ABW69671.1) (Fig. 2).

2.5. Sex determination

Sexes in *S. glomerata* are separate with low occurrence of hermaphroditism (Dinamani, 1974). Sex assignment was required due to the hypothesised differences in vitellogenin induction between males and females (Blaise et al., 1999, 2003). Approximately 5/54 oysters (9%) in each treatment had insufficient gonadal development to obtain a smear to determine sex. These oysters were thus not included in analysis. The sex of each oyster was determined by sampling a smear of gonadal tissue biopsy via an incision in the gonad using a scalpel no less than 1 h following harvest. This smear was then examined under a compound microscope at 100× magnification with the absence/presence of oocytes used as indicator of sex. Histological analysis was originally performed to assess stage of gametal development which cannot be determined via biopsy and smear techniques. However this also revealed unexpected individuals exhibiting intersex in the 50 ng/L 17α-ethynylestradiol treatment who were originally classed as female via the smear technique. The smear technique is problematic in detecting the individuals exhibiting intersex due to the fact that oocytes are much larger and easier to detect in comparison to spermatozoa. Thus the assignment of sex based on smear testing is reliable for all individuals that were male. Results obtained for females (including EEQ, sex ratio and vitellogenin) in the 50 ng/L EE2 treatment are likely however, to represent values pooled across female and intersex individuals, although it is impossible to separate and quantify these separately, as histological analyses were not performed on all individuals.

ssigned a gonadal developmental stage for oogenesis based on criteria described by described briefly below: (1) follicles contain permatogonia, (2) oocytes (<25 μm) or sper- to mature, (3) maturation of oocytes (>25 μm) togenesis up to spermatids, few spermatozoa, atozoa occupying large proportion of gonad, harged, residual oocytes or spermatozoa, (X) here are residual oocytes or spermatozoa and y. Individuals where an intact gonadal follicle es and spermatids or spermatozoa (ovotestis) rsex.

s

ed using a two-way analysis of variance A (StatSoft, 2005). According to Levene's test riance; vitellogenin units were not homoge- sformed, ln ($x+1$), prior to statistical analysis. nce was observed, Tukey's HSD multiple com- med among groups. A significance level of d for all statistical analyses.

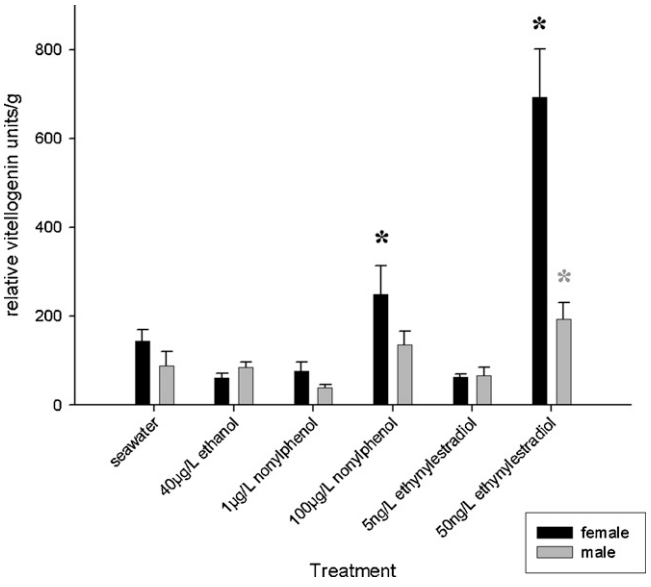


Fig. 4. Vitellogenin units (relative units/g) measured in gonadal tissue of *Saccostrea glomerata,* exposed to 4-nonylphenol (1 μg/L and 100 μg/L) and 17α-ethynylestradiol (5 ng/L and 50 ng/L) over an 8-week exposure period in experimental aquaria (mean ± standard error, $n=14$), $^{*}p<0.05$.

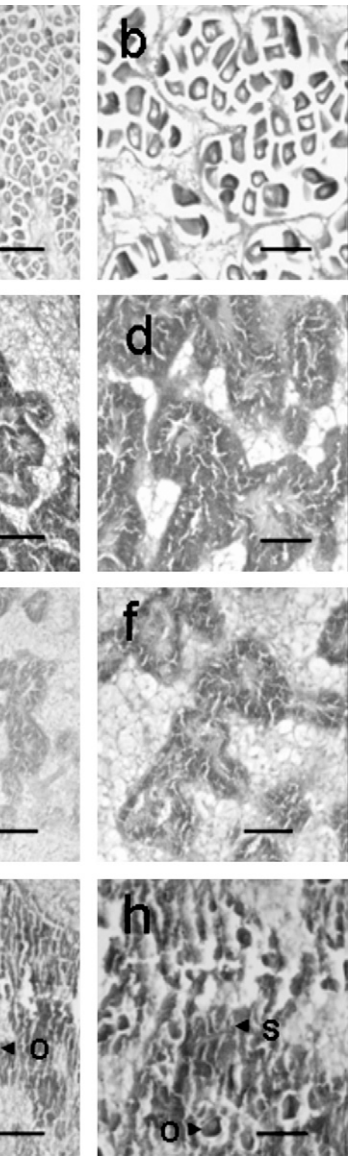
3.2. Vitellogenin analysis

Exposure to both 17α-ethynylestradiol and 4-nonylphenol increased vitellogenin induction. A significant interaction was observed between sex and treatment suggesting differences among treatments differed for each sex ($F=3.5$, $p<0.05$). For females, the high exposure treatments of both 17α-ethynylestradiol (50 ng/L) and 4-nonylphenol (100 μg/L) exhibited significant increases in vitellogenin, up to three-fold and double the controls respectively. For males, significant increases in vitellogenin compared to controls were observed when exposed to 17α-ethynylestradiol (50 ng/L) only. A significant difference between sexes occurred within the 50 ng/L 17α-ethynylestradiol exposure where females exhibited greater vitellogenin responses than males (Fig. 4). No significant differences in vitellogenin were observed between sexes in other treatments. No significant differences in vitellogenin were found between the seawater control and ethanol control.

3.3. Sex ratio

Identification of sex as female or male was determined via the presence or absence of oocytes. The sex ratio of *S. glomerata* was similar among most exposures, controls and oysters at experimen-

ts (EEQ) provide an indication of the relative mulated xeno-estrogen and/or endogenous strogenically active form at time of analysis the T47-D estrogen receptor. Measurement ® analysis revealed that individuals in the nol treatment had significantly higher EEQ ments ($F=15.4$, $p<0.05$). Neither a signifi- en sexes ($F=4.7$, $p>0.05$) nor an interaction and sex were observed ($F=2.1$, $p>0.05$)



dual *Saccostrea glomerata*. (a) Female gonad (control) ol) ×400, (c) male gonad treatment (control) ×200, (d)) ×400, (e) male gonad 17α-ethynylestradiol (50 ng/L) ynylestradiol (50 ng/L) ×400, (g) ovotestis from 17α-00; O, oocyte and S, spermatozoa, (h) ovotestis from) ×400; O, oocyte and S, spermatozoa. Scale bars: in b, d, f and h.

in these individuals was identified through the presence of both spermatozoa and oocytes contained within follicles which were only evident after microscopic examination. In some intersex individuals, large regions of the ovotestis had spermatozoa packed within follicles; however, one small region of the ovotestis had both spermatozoa and oocytes contained within follicles. In the gonadal tissue of all intersex individuals, high hemocytic activity was observed; in some cases within broken follicles (Fig. 5G and H).

4. Discussion

EEQ was highest in the 100 μg/L 4-nonylphenol treatment and thus appears to have a higher potential for accumulation relative to 17α-ethynylestradiol. Individuals in the 100 μg/L 4-nonylphenol treatment accumulated 43% of total EEQ applied over an 8-week period in comparison to individuals in the 50 ng/L 17α-ethynylestradiol where accumulation was <1%. This estimation is based on the assumption that the ER-CALUX® measurement of total EEQ is only due to the presence of 4-nonylphenol or 17α-ethynylestradiol whereas there are likely other endogenous estrogens such as 17β-estradiol present within tissue (as evident in the measurement of low levels of EEQ in the control treatments) which may also increase upon xeno-estrogenic exposure.

Possibly 4-nonylphenol has accumulated in oyster tissue and is not readily metabolised, nor depurated. High accumulation of 4-nonylphenol to tissue may be largely due to the high lipophilic nature of the compound, as reported in previous studies (e.g. Ekeland et al., 1990). McLeese et al. (1980) reported BCFs ranging from 1.4 to 7.9 in *Mytilus edulis* following short-term exposure of nonylphenol. Ekeland et al. (1990) measured the bioaccumulation of 4-nonylphenol in *M. edulis* following a 16-day exposure to 6.2 μg/L and 5.9 μg/L and reported BCF's of 4120 and 2740, respectively. Accumulation of 4-nonylphenol without significant degradation could also potentially imply that its effects may be long-term/chronic. Another explanation may be that endogenous estrogens in oysters are stimulated upon exposure to xeno-estrogens resulting in increased estrogen induction.

Despite greater observed tissue EEQ due to 4-nonylphenol exposure, biological effects observed in individuals exposed to 17α- ethynylestradiol indicated that this compound exerted greater estrogenic potency than 4-nonylphenol. Seventeen α-ethynylestradiol has a comparatively higher estradiol equivalence factor (EEF) than 4-nonylphenol, i.e. an EEF of 2.1 for 17α-ethynylestradiol versus 2.3×10^{-5} for 4-nonylphenol (Legler et al., 2002). Thus at the exposure concentrations employed, 17α-ethynylestradiol (10.2 and 105 EEQ) also had a much higher potential for estrogenic action than 4-nonylphenol (0.023 and 2.3 EEQ). In *M. edulis*, the metabolism of estrogens has been demon-

in a number of molluscan species including
ara et al., 2006), *Octopus vulgaris* (Keay et al.,
Matsumoto et al., 2007). Subsequent studies
e ER in these species is not responsive to, or
en, when recombinantly expressed in a cell-
ected to vertebrate cell lines (reporter gene
Matsumoto et al. (2007) found ER immunore-
d in the nuclei of follicle cells, the site of
s, and they suggest that the ER could possi-
ear receptor regulating the transactivation of
Matsumoto et al. (2003) found 17β-estradiol
s profile with gonadal maturity. Following
adiol *C. gigas* has been demonstrated to induce
., 1998). High levels of vitellogenin mRNA
been demonstrated during the vitellogenic
tsumoto et al., 1997, 2003). Thus estrogens
or vitellogenesis in some molluscan species
enomic action. Alternatively estrogen binding
nay be encoded by genes unrelated to known
y mediate their vitellogenic action through
(Croll and Wang, 2007). Taken together, the
ary significantly among molluscan taxa and
ucidated.

ed however that Sydney rock oysters exposed
unds exhibited elevated vitellogenin induc-
thynylestradiol (50 ng/L) and 4-nonylphenol
significant increases in vitellogenin for
les exhibited significant increases in vitel-
d to 50 ng/L 17α-ethynylestradiol only. The
contribute further confirmation that expo-
ntrations of xeno-estrogenic compounds can
responses in molluscs, especially for females,
ecise mechanism(s) of action remain to be
nin induction in *S. glomerata* appears a sen-
no-estrogenic exposure and effect.

fish have reported vitellogenin as a female
ra et al., 1980; Tyler and Sumpter, 1990;
hang et al., 1994). However male and female
ar in all treatments except for 50 ng/L 17α-
ere the female response was significantly
was found in all male individuals in the cur-
ding those in the seawater control treatment.
hat male molluscs can exhibit sensitivity to
terms of initiation of vitellogenesis. Matazzo
nd that male and female clams (*Tapes philip-*
environmentally relevant concentrations of
g/L and 200 μg/L) exhibited elevated vitel-
ermore, exposure of both male and female
ta, in environments contaminated through

inadvertently overlooked explaining the higher proportion of individuals identified as 'females' in the 50 ng/L 17α-ethynylestradiol treatment. Together, these observations suggest that high 17α-ethynylestradiol exposures may potentially induce oogenesis along with spermatogenesis in individuals that would otherwise develop male gonadal tissue, though larger sample sizes would be required to unequivocally support this hypothesis. For future studies it is recommended that histology is employed for all individuals to provide definitive identification of gametal status and the developmental stage of the gonad.

Based on the histological examination of the ovotestis from intersex individuals in the 50 ng/L 17α-ethynylestradiol treatment, it appears likely that the gonad of these individuals were in a process of transition from male to female. The majority of the gonad was male with small regions containing both male and female gametes. It is unknown whether continued exposure to 50 ng/L 17α-ethynylestradiol would induce a functional hermaphroditic condition, individuals would remain intersex or individuals were in the process of transitioning from male to female at experimental completion. However an earlier study by Mori et al. (1969) suggested that 17β-estradiol exposure resulted in a full sex reversal from male to female gonadal tissue in adult *C. gigas*. Langston et al. (2007) also demonstrated that in vitro exposure of undifferentiated adult clams *S. plana* to sediment spiked with of 17β-estradiol (100 μg/kg), 17α-ethynylestradiol (100 μg/kg), 4-nonylphenol (1000 μg/kg) and octylphenol (1000 μg/kg) (for low exposures and ×10 for higher exposures) caused varying degrees of intersex and enlarged oocytes in the ovotestis of males. Nice et al. (2000) also found that exposure to 1 μg/L and 100 μg/L of 4-nonylphenol at very early life stages in *C. gigas* resulted an altered sex ratio towards females, a higher percentage of functional hermaphrodites in adulthood, and reduced gamete viability in the subsequent generation. A subset of individuals in *S. glomerata* populations does exhibit sequential protandry (i.e. begin as males and may change sex to females later in life), usually occurring when the gonad is undifferentiated (i.e. between reproductive cycles) with hermaphroditism being a rare occurrence (Asif, 1979). Although further experimentation is required, it is possible that that intersex individuals arising from the 50 ng/L 17α-ethynylestradiol exposure in current experiments were protandric individuals capable of undergoing transition from male to female and that exposure to estrogenically active compounds initiated and/or accelerated this transition.

While there is little available evidence to date examining the mechanism of protandric sex reversal in molluscs, findings in fishes suggest a role for estrogens in protandric progression. For the protandrous black porgy, *Acanthopagrus schlegeli*, 17β-estradiol exposure (4 mg/kg in feed, 7-month exposure) induced regression of testicular tissue and development of ovarian tissue (Chang et al., 1994). Furthermore, elevated gonadal aromatase activity

bserved among gonadal development in dividuals in mature male stages while d 4-nonylphenol exposure treatments pro- range of gonadal developmental stages. e low 17α-ethynylestradiol and in both s exhibited earlier stages of gonadal devel- to control treatments including stage 2 enol and 3 for 1 μg/L 4-nonylphenol and diol. In comparison, males in the 50 ng/L ere found to be in a similar gonadal condi- es (stages 4 and 5) where the gonad was and close to spawning or had partially nic compounds may initiate and/or accel- on in a subset of males (protandric males), another subset may not be susceptible to remain male.

ation for the observation of early male posure treatments (compared to mature males) could be that 4-nonylphenol and ay negatively affect the rate of the gonadal Van den Belt et al. (2002) found exposure of 0 ng/L and 25 ng/L) inhibited spermatoge- *rerio* causing earlier spermatogenic stages comparison to control males. It is perhaps evels of estrogens promote the production in but inhibit the development of sperma- versing the mode of gonadal development Conversely, Wang and Croll (2004) found *acopecten magellanicus*, injected with 17β- progesterone or dehydroepiandrosterone l into abductor muscle once per month, ed sexual differentiation of male gonadal ecognisable gametes versus undifferenti- oid exposure groups than in the control of gonadal differentiation are perhaps not

present study suggest *S. glomerata* is an holluscan biomonitor for assessing effects nts in Australian waters, with evidenced strogens, sensitive biochemical biomarker and reproductive endpoint biomarker and intersex) induction upon estrogenic rther the research effort, experiments are se–response relationships between mark- relevant estrogenic exposures, temporal ogenin to changes in estrogenic exposure ning utility of vitellogenin induction to pre- ls of biological organisation with potential . reproductive endpoints). Exploring the 17α-ethynylestradiol). Exposure to both 17α-ethynylestradiol and 4-nonylphenol increased vitellogenin induction. Exposure treatments of both 17α-ethynylestradiol (50 ng/L) and 4-nonylphenol (100 μg/L) exhibited significant increases in vitellogenin for females, whereas males exhibited significant increases in vitellogenin only when exposed to 50 ng/L 17α-ethynylestradiol. Females exhibited greater vitellogenin responses than males in the high exposure of 17α-ethynylestradiol (50 ng/L). Exposure, to 50 ng/L 17α-ethynylestradiol, induced a shift in the sex ratio (i.e. an increase in the proportion of female individuals) with many individuals sexed initially as females via smear testing assigned to an intersex condition upon histological examination, suggesting a protandric progression was induced for subset of males within this treatment.

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