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Exposure to 17α-ethynylestradiol causes dose and temporally dependent changes in intersex, females and vitellogenin production in the Sydney rock oyster

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Abstract Although mounting evidence suggests exposure to estrogenic contaminants increases vitellogenin production in molluscs, demonstration of dose–response relationships and knowledge of the temporal nature of the vitellogenin response with continual exposure is currently lacking for biomarker utility. To address this knowledge gap, adult Sydney rock oysters, *Saccostrea glomerata*, were exposed to a range of environmentally relevant concentrations of 17α-ethynylestradiol (EE2) (0, 6.25, 12.5, 25 or 50 ng/l) in seawater under laboratory conditions. Vitellogenin induction and gonadal development was assessed following 4, 21 and 49 days exposure to EE2. Vitellogenin was found to increase in a dose dependent manner with EE2 exposure for females (4 and 49 days) and males (4 and 21 days). Histological examination of gonads revealed a number of individuals exhibited intersex (ovotestis) in 50 ng/l EE2 (after 21 days) and in 6.25 and 12.5 ng/l EE2 (after 49 days). Furthermore, a significant shift towards females was observed following 49 days exposure at 50 ng/l EE2 suggesting estrogenic exposure is capable of facilitating a progression for protandric males from male-intersex-female gametal status. Increases in female vitellogenin (4 days) were predictive of later increases in female developmental stages at 21 days and increases in oocyte area following 49 days. Male vitellogenin (4 days) was predictive of decreased male percentages and lower male developmental stages at 49 days. Vitellogenin in *S. glomerata* is a predictive biomarker of estrogenic exposure and effect if sampled soon after exposure and at the commencement of a gonadal development cycle.

Keywords Biomarker · Estrogens · 17α-ethynylestradiol · Intersex · Oyster · Vitellogenin

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Introduction

A range of anthropogenic compounds with structural similarity to estrogen, and its functional moieties, have been implicated as primary causal agents responsible for reproductive perturbations in aquatic wildlife (Langston et al. 2005). One such compound is 17α-ethynylestradiol (EE2); a synthetic estrogen which is widely employed as the active constituent of the female contraceptive pill. Compared to endogenous steroids, such as 17β-estradiol (E2) and its metabolites, EE2 exhibits greater estrogenic potency and is more resistant to metabolism/degradation due to the addition of an ethyl group (Andersen et al. 2003). Consequently, when used as the contraceptive pill in humans, EE2 is only partially metabolised, resulting in excretion and entry to wastewaters. Evidence suggests that during the sewage treatment process EE2, along with a suite of other estrogenic compounds, may only be partially degraded, and/or removed, resulting in entry of EE2 to streams and surface waters (Andersen et al. 2003). Concentrations of EE2 in sewage effluent have been detected between 0.2 and 42 ng/l (Desbrow et al. 1998; Ternes et al. 1999) and in surface waters between 0.1 and 15 ng/l worldwide (Aherne and Briggs 1989; Belfroid et al. 1999). Thus, aquatic organisms, including invertebrates, may be exposed to EE2 in aquatic environments receiving sewage effluent.

Aquatic invertebrates, in particular bivalve molluscs, are ideal candidates for assessing effects of anthropogenic contaminants in aquatic systems. Due to their sessile nature, high filter-feeding capacity and bioaccumulation of both organic and inorganic compounds (Ortiz-Zarragoitia and Cajaraville 2006), molluscs such as oysters and mussels have proved useful in the assessment of contaminant presence, relative contaminant loads among locations and biological responses to contaminant exposure (Scanes 1997). Molluscs may also prove a valuable tool in the assessment of the effects of estrogenic contaminants. Indeed, available literature on both laboratory and field assessment of the effects of estrogenic compounds in molluscan species suggests that exposure to estrogenic compounds can cause reproductive effects including the induction of vitellogenesis in females and males (evidence of endocrine disruption) (Matozzo et al. 2008) and gonadal developmental changes (accelerated oocyte development in females and feminisation of male individuals) (Gagné et al. 2006; Gagnon et al. 2006; Ortiz-Zarragoitia and Cajaraville 2006; Langston et al. 2007).

Molluscan vitellogenins are precursors to egg yolk proteins (vitellins) which accumulate in oocytes during gonadal maturation. Numerous lines of evidence suggest vitellogenesis in molluscs is mediated via endogenous estrogens. In the scallop, *Patinopecten yessoensis*, E2 is synthesised in the gonad and its levels increase during the early phases of oocyte development (Matsumoto et al. 1997). Li et al. (1998) demonstrated that E2 treatment in vivo stimulated vitellogenesis and increases in vitellins in the gonad of the Pacific Oyster, *Crassostrea gigas*. Similarly, Osada et al. (2003) demonstrated E2 induced synthesis of vitellogenin in the gonad of *P. yessoensis*. The site of synthesis is thought to be within ovarian tissue, with in-situ hybridisation demonstrating localisation of *C. gigas* vitellogenin mRNA within follicle (auxiliary) cells (Matsumoto et al. 2003).

With such evidence suggesting that estrogens play a functional role in vitellogenin induction in molluscs, others have hypothesised that exposure to xenoestrogens may elicit similar effects, and have sought to investigate vitellogenin production as a potential biomarker of estrogenically mediated endocrine disruption. Indeed, Gagné et al. (2002) demonstrated that both male and female freshwater mussels, *Elliptio complanata*, deployed 1.5 and 5 km downstream from a sewage treatment plant in the St. Lawrence River, Canada, exhibited increases in alkali-liable phosphates (ALP) (a surrogate vitellogenin estimate) compared to reference locations. Male blue mussels, *Mytilus galloprovincialis*, deployed in Venice canals (receiving raw sewage) have also been shown to exhibit increased ALP levels in hemolymph compared to reference locations (Pampanin et al. 2005). In terms of controlled exposures to specific xenoestrogens, exposure to 4-nonylphenol (NP) (250 nM) for 72 h has been shown to increase ALP levels in both male and female mussels, *E. complanata* (Gagné et al. 2001). Similarly, *M. galloprovincialis*, exposed to NP (0, 25, 50 and 100 μg/l) for 7 days exhibited a dose dependent increase in ALP levels (Ricciardi et al. 2008). Taken together, these findings provide strong evidence of the role of xenoestrogens in induction of vitellogenin-like proteins in molluscs. However, there has been little research to date exploring the potential effects of xenoestrogens with high estrogenic potency, such as EE2, on molluscan vitellogenesis, excluding our own work (see below).

Estrogenic exposure not only induces vitellogenin production in molluscs, but also influences both the rate of gonadal development and sex determination. In *C. gigas*, injections of E2 (50 μg once every 10 days, for a 40 days period) were shown to accelerate female development by increasing oocyte diameter (46.9 ± 0.9 μm in exposed compared to 44.1 ± 2.4 μm in controls) (Li et al. 1998). Langston et al. (2007) demonstrated that exposure of clams, *Scrobicularia plana*, to sediment spiked with a mixture of E2, EE2, NP and octylphenol (OP) caused intersex gonadal status in males and enlarged oocytes in both females and in the ovotestis of intersex individuals. Further, injections of E2 (0.04–0.60 mg estradiol-3-benzoate) during the early stages of seasonal gonadal maturation of the oyster *C. gigas*, have been shown to induce full sex reversal from male to female (Mori et al. 1969). Similarly, exposure to NP (48 h exposure to 1 and 100 μg/l at 7–9 days post-fertilisation) during larval development has resulted in skewed sex ratios towards females during adulthood in *C. gigas* (Nice et al. 2003).

Our own prior research has focused on the development of, *S. glomerata*, as a potential bio-monitoring species for estrogenic contaminants through exploiting vitellogenin induction and gonadal developmental responses following estrogenic exposure. We have previously found that females exposed to EE2 (50 ng/l) and NP (100 μg/l), during a gonadal development cycle, exhibited significant increases in vitellogenin up to three-fold and double controls, respectively. Significant increases in vitellogenin were also found in males exposed to 50 ng/l EE2. Further, exposure to NP (100 μg/l) and EE2 (50 ng/l) induced intersex gametal status for some individuals (Andrew et al. 2008).

As *S. glomerata* exhibits protandric diocey (Asif 1979; Guo et al. 1998), it remains unclear whether intersex individuals observed in our initial experiments were functionally intersex or the result of an opportunistic sampling of individuals that were in the process of undergoing an estrogenically mediated protandric transition from male to female. If the latter is the case, we may expect to observe

shifts in the sex ratio towards females with greater exposure durations to estrogens, at later intervals in the gonadal development cycle and/or with greater sampling frequency during such potential transitions. In the current study, we sought to explore this question by sampling individuals exposed to EE2 at multiple windows temporally during a gonadal development cycle from resting phase to spawning.

Among the criteria required for a successful biomarker is evidence of a dose–response relationship to contaminant exposure (Huggett et al. 1989). Yet a recent review by Ketata et al. (2008) emphasised that clear evidence of dose–response relationships between estrogenic exposure and vitellogenin induction is currently lacking for invertebrate models. In the current study, we sought to redress this knowledge gap by assessing the vitellogenin response upon exposure to an environmentally relevant range of EE2 concentrations. It was anticipated that vitellogenin concentrations would increase with estrogenic dose, particularly in males, who do not usually produce vitellogenin.

Further, for a reliable biomarker, it is desirable that observed dose–response relationships are maintained temporally. Yet there is evidence to suggest that biomarkers may not exhibit static dose–response relationships temporally, but rather may exhibit variable responses to estrogenic exposure dependent on factors such as exposure regime and duration, adaptive response mechanisms and/or developmental status (Wu et al. 2005). For application in environmental monitoring it is critical to understand the temporal nature of biomarker responses to exposure to enable an appropriate sampling design which does not under/over estimate exposure and effect. Thus, we chose to assess the temporal maintenance of the dose–response relationship between EE2 exposure and vitellogenin responses over three sampling periods during a gonadal development cycle.

Finally, as a predictive biomarker, vitellogenin should also possess 'early-warning' predictive ability. It is desirable that early vitellogenin responses correlate with later effects indicative of endocrine disruption at higher organisational levels such as gonadal morphology i.e. gamete maturation, size, expression of intersex and/or total sex reversal.

Thus, the main aims of the present study were to explore dose–response and temporal relationships between EE2 exposure, vitellogenin induction and gametal status during reproductive conditioning in *S. glomerata*. Specifically, we expected that vitellogenin would increase in a concentration dependent manner within days of EE2 exposure (4 days) and that the dose–response relationship would be maintained throughout the exposure duration. Secondly, we predicted that with time (21–49 days) we would observe the presence of intersex individuals in exposure treatments and possibly a shift in the sex ratio towards females. Finally, we hypothesised that early induction of vitellogenin would be predictive of later effects on gametal maturation, expression of intersex and/or total sex reversal.

Materials and methods

Experimental design

Three hundred and sixty, 18 month old *S. glomerata*, were used for experimentation. Oysters were sourced from an oyster farm in the Port Stephens estuary, NSW, Australia; an area with no known history of sources of estrogenic contamination. Oysters were specifically selected to be in 'resting' condition, entering a phase of gonadal development at experimental commencement (Dinamani 1974). Experiments were conducted during Winter (June–August 2007) at the Port Stephens Fisheries Centre, Mollusc Hatchery, Taylor's Beach, NSW according to the protocols of the American Society for Testing and Materials (A.S.T.M.) E 729-96 (1996) for static renewal tests. One hundred and eighty individually aerated aquaria (8 l) were maintained at 22 ± 0.5°C in a temperature controlled room. Each aquarium contained two oysters. Seawater (33 ± 0.5 g kg/l salinity) within each replicate aquarium was completely changed and treatment exposure regimes maintained thrice weekly within a temperature controlled laboratory (maintained at 22°C). Oysters were fed a mixed diet daily containing 660 ml of each algal species culture including: *Pavlova lutheri*, *Chaetoceros muelleri* and Tahitian *Isochysis* aff. *galbana* (1.5×10^9 cells/oyster/day).

Oysters were exposed to one of six nominal treatments in seawater: 6.25, 12.5, 25 and 50 ng/l EE2 (in 1.2 µg/l ethanol), 1.2 µg/l ethanol control and a seawater control ($N = 20$ individuals per treatment/time). Oysters were removed (10 aquaria per treatment/time) from the experiment at three separate sampling occasions (4, 21 and 49 days) in order to assess temporal effects. Sampling times were selected to assess whether vitellogenin was induced following short-term exposure (4 days, 96 h acute exposure) and if vitellogenin responses were likely to be maintained over longer periods of time (chronic effects). The sampling times of 21 and 49 days were considered important time points to measure the shift in sex ratio and sufficient time to allow for gonadal maturation from resting phase to mature gametes prior to spawning (Cox et al. 1996). A group of oysters (from the same population) were maintained in separate aquaria for the purpose of monitoring gonadal development throughout the experiment and to ensure oysters were harvested prior to a spawning event.

Wet condition index was measured to assess the potential confounding effects of estrogenic treatments and/or the solvent carrier on feeding and thus condition. Wet condition index was measured at each harvest. This was calculated using the formula (wet tissue weight × 1,000)/(wet whole weight − wet shell weight) (Lucas and Beninger; 1985). Oyster condition was observed to decline in the 12.5 ng/l, 25 ng/l and 50 ng/l EE2 treatments by 8.5, 7.7 and 12.6%, respectively after 4 days ($F = 2.58$, df = 4, $p < 0.05$), though oysters quickly recovered with no significant differences in condition among treatments at both 21 ($F = 2.095$, df = 4, $p > 0.05$) and 49 days ($F = 2.269$, df = 4, $p > 0.05$).

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

The extraction (modified extraction from Gagné and Blaise 2000) and analysis of oyster gonadal samples were performed using protocols outlined in Andrew et al. (2008). Briefly, 100 mg of oyster gonadal tissue was homogenized in 225 µl citrate buffer (pH 6.5 10 mM and with 16 mg/ml polyethylene glycol) and 25 µl of the protease inhibitor aprotinin then stored at −80°C. Following storage, samples were thawed and 500µL Tris–HCl buffer was added. One hundred microliters of tissue homogenate was transferred to a glass centrifuge tube and ALPs were extracted using 800 µl of *t*-butyl methyl ether (Chromsolv grade, Sigma-Aldrich) with a 30 min extraction at 4°C. The organic phase was then separated and dried under N_2, then re-suspended in 1,000 µl of PBS. Analysis of vitellogenin was performed via HPLC analysis using conditions outlined in Andrew et al. (2008), with the vitellogenin peak initially verified via proteomic sequencing (1DNanoLC ESI MS/MS). It should be acknowledged our use of the term vitellogenin implies the analyte is a vitellogenin-like protein based on genomic/proteomic sequence similarity to known vitellogenin from other taxa (molluscs, fish spp.). Three female and three male individuals from separate aquaria were selected for each treatment/time for vitellogenin analysis.

Histological analysis

Sexes in *S. glomerata* are separate with low occurrence of intersex, i.e. <0.4–0.7% (Dinamani 1974; Cox et al. 1996). Sex assignment was required due to the hypothesised differences in vitellogenin induction between males and females (Blaise et al. 1999, 2003), that vitellogenin induction in males is strong evidence for estrogen mediated endocrine disruption, and finally due to the established effects of estrogenic exposure on sex ratio (Andrew et al. 2008). Each individual oyster was prepared for histological examination resulting in a total of 400 analyses. A cross-section of approximately 5 mm^2 was excised from each oyster between the labial palps and gills. Tissue samples were placed in Davidson's solution (10% glycerine, 20% formalin, 30% alcohol, 30% sodium chloride solution and 10% glacial acetic acid) for 24 h (Cox et al. 1996) with successive dilutions of ethanol (70, 50, 50%) (Howard and Smith 1983). Tissue was embedded into paraffin blocks, sectioned transversely at 5 µm intervals and floated on a water bath heated to 80°C. Sections were placed onto acid washed glass slides and dried overnight at 60°C. Sections were stained with haematoxylin and counterstained with eosin (H&E). Study of the gonadal area was performed under a compound microscope at 200 and 400× magnification.

The sex of each individual was determined as female, male, intersex or indeterminate under microscopic examination (200× magnification) using oocytes or spermatozoa as indicators. Intersex individuals were identified via the presence of both oocytes and spermatozoa within an intact gonadal follicle. Other individuals were found to be indeterminate whereby the gonadal cells were undifferentiated and usually accompanied by an abundance of hemocytes.

It may be possible that estrogen exposure promotes female development resulting in acceleration towards higher proportions of mature female gonad stages in individuals exposed to EE2 compared to controls. Thus, each oyster was assigned a gonadal developmental stage for oogenesis, and also spermatogenesis, based on criteria described by Dinamani (1974). Briefly, stages included: (1) follicles contain primary oogonia or spermatogonia, 2) oocytes (<25 µm) or spermatocytes beginning to mature, (3) maturation of oocytes (>25 µm) or all stages of spermatogenesis up to spermatids, few spermatozoa, (4) oocytes or spermatozoa occupying a large proportion of the gonad, (5) following spawning, many follicles are discharged, residual oocytes or spermatozoa, (6) characteristic of resting, no oocytes or spermatozoa (indeterminate sex) and high hemocyte activity. Eight female individuals per treatment from the 49 days sampling period were randomly selected for oocyte area measurement. For each individual, digitalised images of four randomly selected areas of the gonad were taken with subsequent measurement of 80–100 oocytes (Imagetool 2.0). Only oocytes with a visible nucleus were measured and oogonia were not measured.

Statistical analysis

Linear dose–response relationships between EE2 exposure and vitellogenin concentration in gonadal tissue were assessed to test for biomarker utility via linear regression analyses using STATISTICA (Statsoft 2005). According to Levene's test for homogeneity of variance, vitellogenin units were not homogenous and thus log transformed, ln $(x + 1)$, prior to statistical analysis. Other curve functions

(i.e. sigmoidal, logistic and exponential) were assessed via relative coefficients of variation, though linear fits were found to best represent the data.

A Pearson χ^2 analysis (SPSS version 17, 2009) was used to determine significant differences in sex ratio (female, male, intersex or indeterminate) among time (4, 21 and 49 days exposure) for each exposure to EE2 (0, 6.25, 12.5, 25 or 50 ng/l). Numbers of males, intersex and indeterminate individuals across treatments and time were insufficient for non-parametric analysis and were pooled in order to gain sufficient frequency for comparison to females. The aim was to determine if the proportion of females in the test population increased throughout the duration of the experiment. It was hypothesised that there would be a difference in the sex ratio, specifically a shift towards a greater proportion of females in exposure treatments over time compared to no difference in the controls.

Pearson's correlations were performed between treatments and mean oocyte area (49 days), mean reproductive stages (21 and 49 days) and sex percentages (21 and 49 days) to further determine relationships between exposure and reproductive endpoints. Pearson's correlations were also performed using early mean vitellogenin levels (4 days) with mean oocyte area (49 days), mean reproductive stages (21 and 49 days) and sex percentages (21 and 49 days) in order to test the early warning biomarker predictive utility of vitellogenin. As individuals were sacrificed at each sampling period it was not possible to undertake pair wise correlations between vitellogenin replicates from 4 days with reproductive endpoint replicates from separate individuals at 49 days. Thus, correlation analyses were performed using mean values for each treatment. Due to low N ($N = 3$ per treatment), the power to detect a significant correlation between variables was greatly reduced.

During the experiment an ethanol control was included due to the addition of ethanol as a solvent carrier for EE2 in the exposure treatments (1.2 µg/l). Prior to statistical analyses the seawater and ethanol controls were compared for differences. As no significant differences were observed, both controls were pooled for the purpose of all analyses.

Results

Effects of EE2 on vitellogenin production

Females

While there was greater variability in the vitellogenin response at 4 days compared to later time periods, levels were elevated across all EE2 treatments and increased linearly with EE2 exposure ($R^2 = 0.22, p < 0.05$) (Fig. 1a). Yet the vitellogenin response declined over time, with approximately half of the vitellogenin units found at 49 days compared to the vitellogenin units in corresponding treatments at 4 days. At 49 days, albeit with lower vitellogenin concentrations, a linear relationship with EE2 exposure was found ($R^2 = 0.58, p < 0.05$) while at 21 days the relationship was not significant ($p > 0.05$) (Fig. 1a).

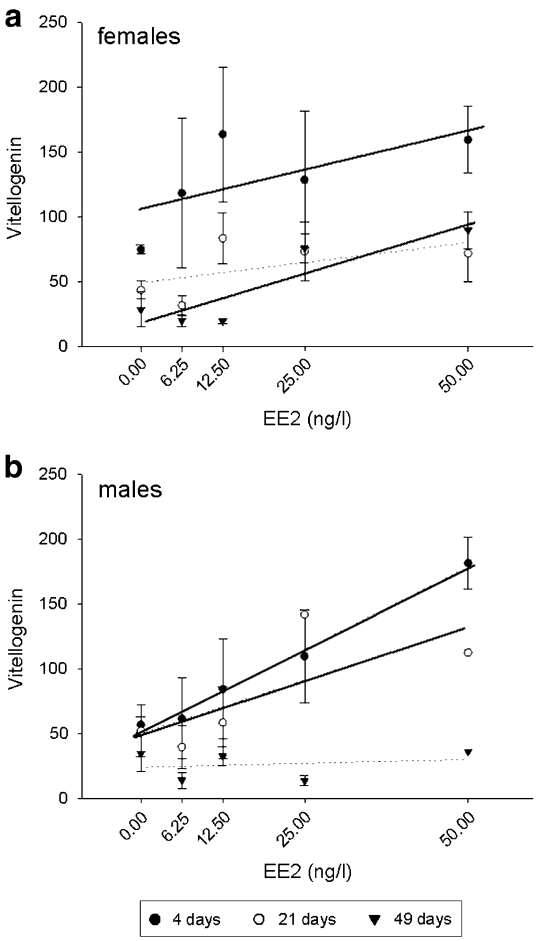

Fig. 1 Comparison of the vitellogenin units measured in Sydney rock oysters, *S. glomerata* following exposure to EE2 (0, 6.25, 12.5, 25 and 50 ng/l) for **a** females, 4 days ($R^2 = 0.22$, $p < 0.05$), 21 and 49 days ($R^2 = 0.58$, $p < 0.05$), **b** males, 4 days ($R^2 = 0.42$, $p < 0.05$), 21 days ($R^2 = 0.31$, $p < 0.05$) and 49 days, $N = 1$–6 per treatment/time (only one male individual was present in 50 ng/l at 21 and 49 days). *Filled lines* indicate a significant relationship $p < 0.05$

Males

Males displayed a similar response with vitellogenin exhibiting a positive linear increase with EE2 exposure at 4 days ($R^2 = 0.42$, $p < 0.05$). A positive linear increase with EE2 was maintained at 21 days ($R^2 = 0.31, p < 0.05$) (Fig. 1b) but, similar to the female response, vitellogenin had declined in comparison to 4 days. At 49 days the

vitellogenin response had declined to basal levels across treatments ($p > 0.05$) (Fig. 1b).

Gonadal sex development

The proportions of sexes (male ~20%, female ~60%, indeterminate ~20%) were similar across EE2 exposure treatments at 4 days (Fig. 2a). This represents what may be expected of sex proportions within a single reproductive season for *S. glomerata*, allowing for sampling variability. A small proportion of individuals were indeterminate at the beginning of the experiment which was expected and reflects individuals that were yet to commence the gonadal maturation process (Fig. 2a). It is also possible these individuals were male due to the difficultly of identification of small proportions of rudimentary spermatogonia within follicles (stage M1) or residual gametes in a male gonad post-spawning (stage M6).

Following 21 days exposure, the proportion of males and indeterminate individuals declined with a concomitant increase in the proportion of intersex individuals in the 50 ng/l EE2 exposure (4/15) (Fig. 2b). No other treatments showed evidence of intersex at this sampling interval. A higher proportion, although not significant, of females were found in the 25 ng/l EE2 treatment compared to all other treatments. After 49 days exposure, proportions of intersex were also evident at lower exposures of 6.25 and 12.5 ng/l EE2 (Fig. 2c). At 50 ng/l EE2, the proportion of intersex individuals declined (relative to 21 days), with a significant shift in the proportion of female individuals at 49 days compared to earlier time periods ($\chi^2 = 6.707$, df = 2, $p < 0.05$) (Fig. 2c). Only one male was observed within this treatment. Taken together, these results suggest a progression from male-intersex-female occurring with time and EE2 exposure. No other significant differences in the female proportions across experimental sampling periods were found in exposure or control treatments.

Oyster gonadal development occurs through the sequential addition of oogonia or spermatogonia from the basal layer of the follicular wall. As gametes mature, they are released to fill the lumen of the follicle. At 21 days, it was observed that intersex individuals from the 50 ng/l exposure all contained male gametes (stage M2) within the lumen along with the development of a layer of oogonia (stage F1) around the inner follicle wall (Fig. 3a). The modal stage of development for males at 4 days was also stage M2, suggesting that the developmental fate of gametes switched soon after exposure to EE2 (Fig. 3b). A similar pattern of development was observed in intersex individuals in 6.25 and 12.5 ng/l at 49 days, whereby male gametes were at stages M2–4 and the new layer of female gametes were consistently at stage F1 (Fig. 3c). It was also observed that for all intersex individuals (particularly where the proportion of oogonia

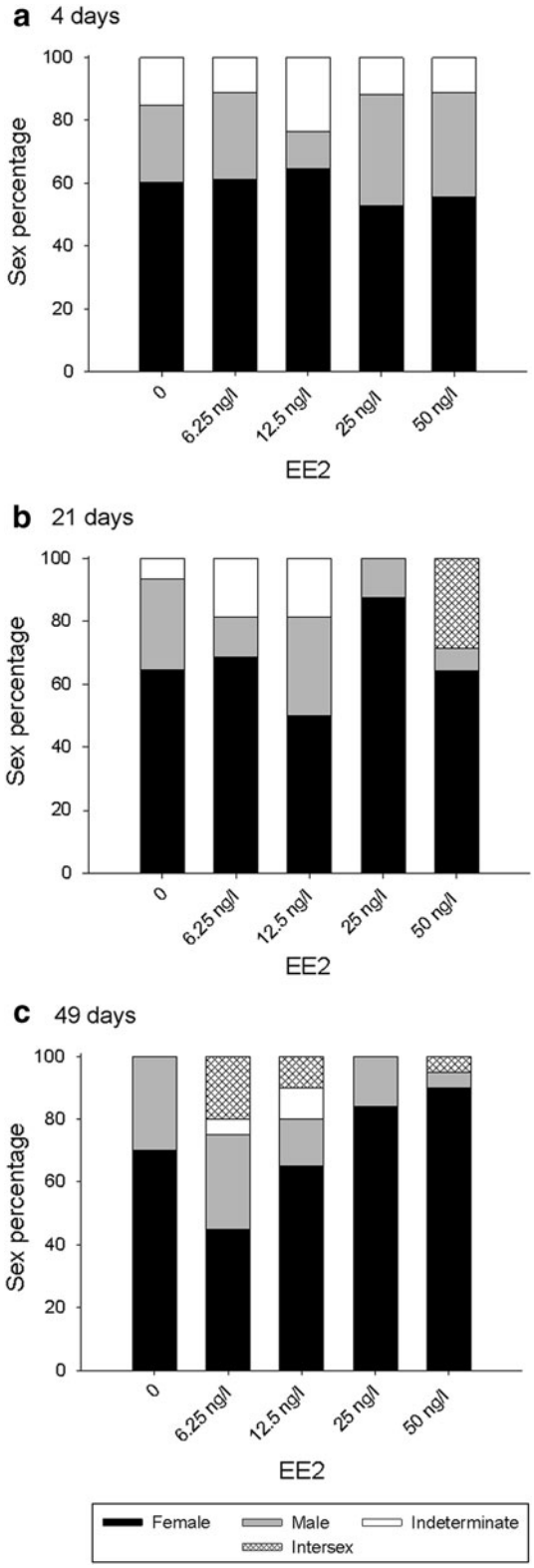

Fig. 2 Comparison of the sex ratio of Sydney rock oysters, *S. glomerata* following exposure to EE2 (0, 6.25, 12.5, 25 and 50 ng/l) in experimental aquaria at **a** 4 days exposure, $n = 17$–18, **b** 21 days exposure, $n = 15$–16 per treatment and **c** 49 days exposure, $n = 18$–20 per treatment. * Indicates a significant shift in the proportion of females via χ^2 analysis

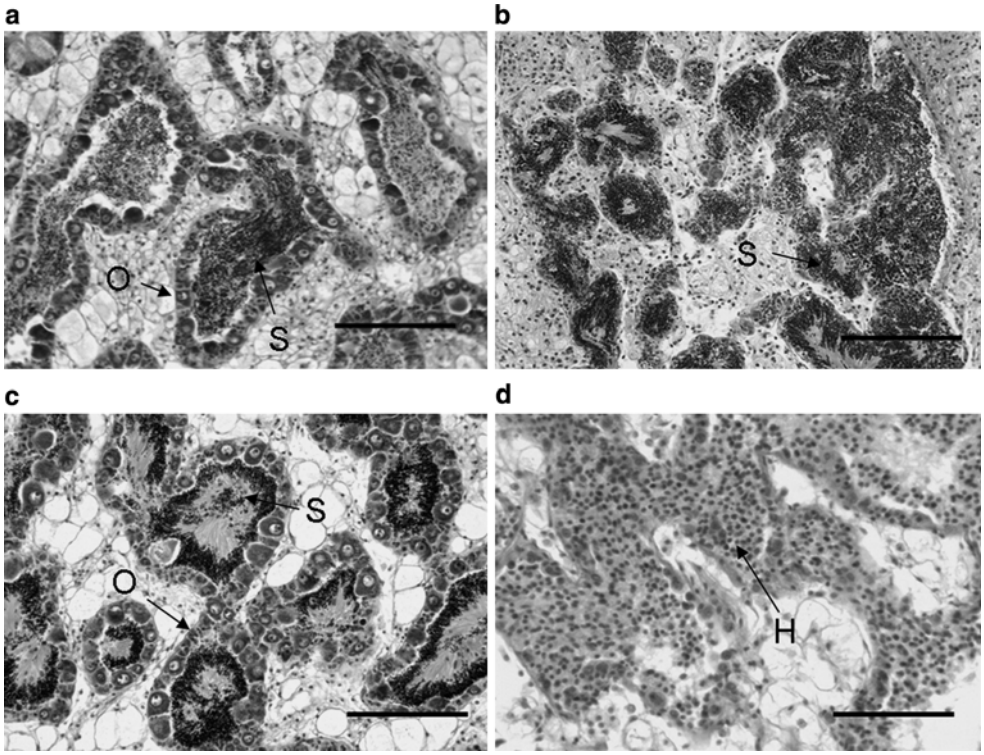

Fig. 3 Gonadal tissue from histological preparation of individual Sydney rock oysters, *S. glomerata*. **a** Intersex follicle with both oogenia and primary oocytes (F1) and spermatozoa (M2) ×200 (21 days, 50 ng/l EE2), **b** male gonad in stage M2 ×200 (4 days, 50 ng/l), **c** intersex follicle with both oogenia and primary oocytes (F1) and spermatozoa (M4) ×200 (49 days, 12.5 ng/l), **d** intersex follicle with proliferation of hemocyte cells ×400 (higher magnification of **c**, 49 days, 12.5 ng/l). O = oocyte cell, S = spermatozoa with the lumen of follicle filled with tails and H = hemocyte. *Scale bars*, A–C = 100 μm, D = 50 μm

was higher than male gametes) there was a proliferation of hemocytic activity within interstitial tissue (Fig. 3d). The combination of maturing male, immature female gametes and enhanced hemocytic activity further suggests that individuals begin gonadal maturation as male, but with exposure to EE2 the developmental pathway of germ cells is redirected from a male to female trajectory. Male gametes are eventually removed via hemocytic clearance and the individual continues gametogenesis along a female developmental pathway.

Female gonadal development stages

At 4 days, the proportions of female gonadal development stages were similar among all treatments. After 21 days exposure, it was observed that all exposure treatments had higher proportions of female development stages F2–F4 (Fig. 4a–c) compared to the controls (where 95% females were within stages F1 or F2) suggesting EE2 exposure accelerated female gametal development. However at 49 days within higher exposures (e.g. 25 and 50 ng/l EE2), the proportions of female stages (F2–F4) were once again similar to the controls (F1–F5) (Fig. 4d). The significant increase in female proportions in 50 ng/l at 49 days together with histological observations of increased proportions of intersex individuals within this treatment at earlier intervals (21 days) suggests that the trend towards higher proportions of earlier female gonadal development stages within these treatments was perhaps the outcome of sampling individuals that have transitioned from a male to female developmental mode. This resulted in a 'mixture' of female developmental stages, with 'new' females in earlier maturation stages such as F2 (Fig. 4d) (that have recently transitioned from intersex or indeterminate gonadal status) and individuals initially female at higher maturation stages (F3 and F4). It is unlikely that EE2 exposure initiated a spawning event in the 25 and 50 ng/l exposure at 49 days and individuals were re-commencing a secondary gonadal development cycle, as spawning (even at low levels) is evident within aquaria.

Does vitellogenin induction and EE2 exposure correlate with later effects on gonadal development?

The effects of EE2 exposure was further explored through examining relationships between EE2 exposure concentration and mean oocyte area, mean reproductive stages and sex percentages from 21 and 49 days. Increasing EE2 exposure

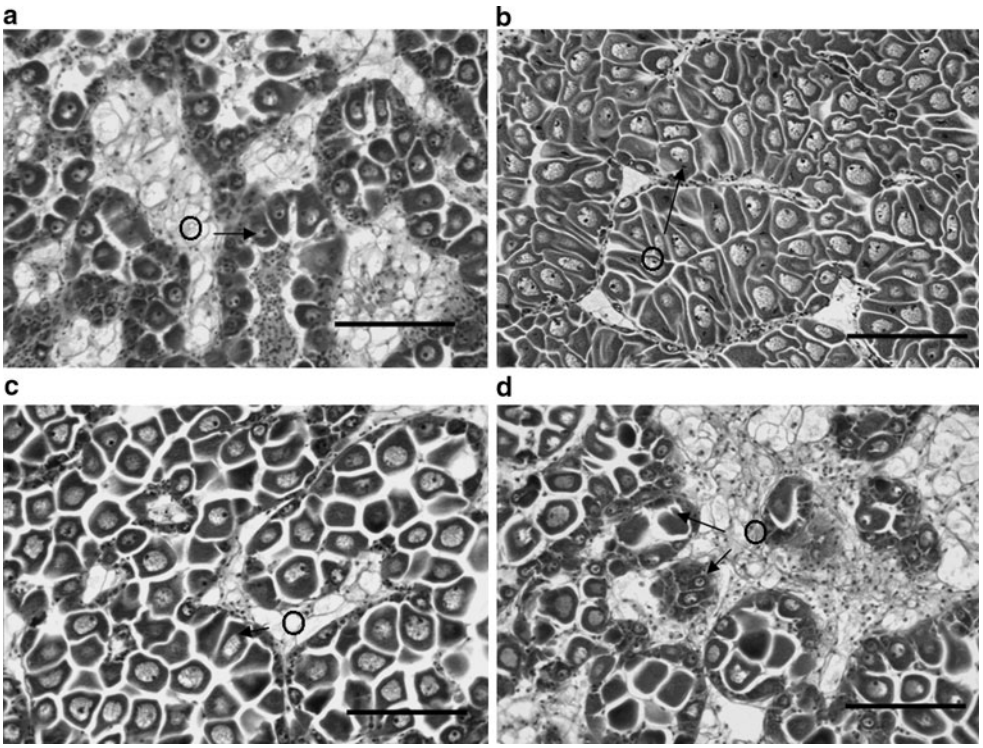

Fig. 4 Gonadal tissue from histological preparation of individual Sydney rock oysters, *S. glomerata*. **a** Female gonad in stage F2 ×200 (21 days, 50 ng/l), **b** female gonad in stage F3 ×200 (21 days, 50 ng/l), **c** female gonad in stage F4 ×200 (21 days, 25 ng/l), **d** female gonad in stage F2 ×200 (49 days, 50 ng/l). O = oocyte cell. *Scale bars* = 100 μm

Table 1 Pearson's correlation coefficient between ethynylestradiol exposure and early mean vitellogenin response from 4 days with sex percentages and mean gonadal development stages from 21 and 49 days and mean oocyte area from 49 days

	Treatment (EE2)		Female vitellogenin (4 days)		Male vitellogenin (4 days)	
	r	*p* Value	*r*	*p* Value	*r*	*p* Value
21 days						
Female percentage	0.57	0.32	0.45	0.45	0.62	0.27
Male percentage	0.01	0.98	0.07	0.91	−0.85	0.89
Intersex percentage	−0.53	0.35	−0.84	0.07	−0.46	0.44
Female development stage	0.72	0.17	**0.96**	**0.01***	0.67	0.21
Male development stage	0.03	0.96	−0.01	0.99	−0.06	0.93
49 days						
Female percentage	0.76	0.14	0.27	0.66	0.78	0.12
Male percentage	**−0.91**	**0.03***	−0.82	0.09	**−0.92**	**0.03***
Intersex percentage	−0.24	0.70	0.24	0.70	−0.28	0.65
Oocyte area	0.71	0.18	**0.93**	**0.02***	0.21	0.22
Female development stage	−0.56	0.33	−0.42	0.49	−0.61	0.27
Male development stage	**−0.95**	**0.01***	−0.52	0.37	**−0.97**	**0.01***

Numbers in bold indicate a significant relationship

negatively influenced male development with a decrease in the percentage of males and decreases in mean male developmental stage following 49 days exposure ($r = -0.91$, $p < 0.05$ and $r = -0.95$, $p < 0.05$, respectively) (Table 1).

Secondly the capability of vitellogenin as a predictive early-warning biomarker of later reproductive effects was examined by performing correlations between mean female vitellogenin at 4 days with mean oocyte area, mean reproductive stages and sex percentages from 21 and

49 days. Increases in female vitellogenin (4 days) were predictive of later increases in female developmental stages following 21 days ($r = 0.96$, $p < 0.05$) and increases in oocyte area following 49 days ($r = 0.93$, $p < 0.05$) (Table 1). Mean male vitellogenin (4 days) may also have useful predictive application of later negative effects on male development with a negative relationship evidenced between early vitellogenin responses and male percentage at 49 days ($r = -0.92$, $p < 0.05$) and between early vitellogenin and mean male development stage at 49 days ($r = -0.97$, $p < 0.05$) (Table 1).

Discussion

Individuals exposed to EE2 exhibited increased vitellogenin in a linear dose dependent fashion, however this was not maintained throughout experimental sampling periods. At 4 days, both sexes exhibited a significant relationship between EE2 exposure and vitellogenin production however, this was not evident at 21 days for females nor at 49 days for males. Thus, the temporal maintenance of the vitellogenin response varies between sexes.

Vitellogenin production, especially for females, is likely to be dependent on stage of gonadal development. Our results indicate that immature females beginning a reproductive cycle were more likely to exhibit increased vitellogenin compared to mature individuals ready to spawn and indicated that developmental stage is an important consideration when assessing estrogenically mediated vitellogenin induction. As a precursor to egg yolk protein (vitellins), vitellogenin may be synthesised most actively during the earlier stages of gonadal development, as oocytes are developing (Li et al. 1998). Our findings for vitellogenin production in control females supported this assertion, with vitellogenin declining with time. Furthermore, female vitellogenin exhibited a linear increase with EE2 at 4 days and subsequently at 49 days only. The relationship found at 49 days was driven by the response in high exposures (25 and 50 ng/l EE2) where a transition from male-intersex-female was likely to have occurred while lower exposures (6.25 and 12.5 ng/l EE2) exhibited similar vitellogenin responses to controls. It may be possible that recently transitioned female individuals within these treatments, at earlier stages of gonadal development, were more responsive to EE2 exposure at 49 days. Matozzo and Marin (2008) have also explored vitellogenin response dynamics at different stages of reproductive development through measurement of ALP in the Manilla clam, *Tapes philippinarum* exposed to E2 (5, 25, 50, 100 and 1,000 ng/l) for 7 and 14 days during both a resting and pre-spawning phase. Females exposed to E2 in a pre-spawning phase for 7 days resulted in lower levels of hemolymph ALP in all exposure treatments compared to controls. Yet, following 14 days, only the 50 ng/l exposure exhibited significantly higher ALP. However, in the resting phase, they found that ALP significantly increased in the hemolymph of females following 7 days exposure to E2 (5, 25, 50, 100 and 1,000 ng/l), but ALP levels in exposure treatments had declined following 14 days and were only significant at the highest exposure (1,000 ng/l) (Matozzo and Marin (2008)). Similarly, Puinean et al. (2006) found that Blue mussels, *Mytilus edulis* exposed to 200 ng/l E2 displayed no significant differences in vitellogenin gene expression compared to control individuals during the mature stages of gametogenesis. Taken together, these observations imply vitellogenin is most actively synthesised, and most responsive to estrogenic stimulation, during earlier phases of gonadal development, suggesting individuals commencing a gonadal development cycle should be targeted when employing vitellogenin as a biomarker of estrogenic exposure in *S. glomerata*. These findings also suggest that future studies of estrogenic effects in *S. glomerata*, and other molluscan taxa, should measure gonadal development alongside vitellogenin.

Despite these temporal response limitations, it appears that vitellogenin is induced in a dose dependent fashion soon after exposure at the commencement of reproductive conditioning for both females and males. The fact that males also exhibit this vitellogenin induction, suggests estrogens exert a response not observed in unexposed males, indicative of endocrine disruption. Indeed, male molluscs (*E. complanata* and *M. edulis*) have been shown to possess a functional, yet silent, gene for vitellogenin which has been shown to be activated upon exposure to exogenous estrogens in *E. complanata* (Gagné et al. 2005) but not in *M. edulis* (Puinean et al. 2006). Further, others have found that male molluscs can exhibit sensitivity to estrogenic exposure in terms of initiation of vitellogenesis (Matozzo and Marin 2005; Blaise et al. 1999, 2003).

In terms of how estrogens may induce vitellogenesis mechanistically, it is well established in vertebrate models, such as fish, that vitellogenin production is mediated through estrogens binding to intracellular estrogen receptors (predominantly ER-β and to a lesser extent ER-α) which function as ligand-modulated transcription factors, binding to estrogen-responsive elements in the promoter region of the vitellogenin gene(s) (Leaños-Castañeda and Van Der Kraak 2007). Despite this well characterized pathway in vertebrate models, little is known on how estrogens may affect vitellogenin production mechanistically in invertebrate models such as molluscs. Primarily, the literature to date is inconclusive on the presence of functional estrogen receptors (ER) in Mollusca. cDNA encoding vertebrate-like estrogen receptors (with high homology in the DNA binding domain to vertebrate ER-α

and ER-β) have been cloned in a number of molluscan species including the rock shell, *Thais clavigera* (Kajiwara et al. 2006), the common octopus *Octopus vulgaris* (Keay et al. 2006) and *C. gigas* (Matsumoto et al. 2007). Matsumoto et al. (2007) suggested that such estrogen receptors may act as nuclear receptors regulating the transactivation of reproductive genes, including the vitellogenin gene, with estrogen receptor immuno-reactivity localised in the nuclei of follicle cells, the site of vitellogenin synthesis (Matsumoto et al. 2007). Yet in vitro observations have found that these estrogen receptors do not bind estrogen both in ligand-binding and cell based gene-reporter assays (Lafont and Mathieu 2007). Although far from clearly characterised, literature documenting vitellogenin induction upon exposure to estrogens, in a variety of molluscan taxa, including our own findings, suggest that estrogens play a functional role in vitellogenesis though the precise mechanism(s) remain to be elucidated.

Along with an acceleration of female gonadal development, EE2 exposure may be capable of initiating a full sex reversal from male to female. The occurrence of intersex individuals present in the 50 ng/l exposure at 21 days together with a significant increase in the proportion of females at 49 days suggested that a number of individuals have undergone a complete sex reversal during this experiment. The histological examinations of intersex individuals suggest that intersex is a transitional event in the switch from male to female gametal status. Within a gonadal follicle of an intersex individual, it appears that the maturing male developmental pathway is interrupted. Subsequent germ cell differentiation is redirected to a female developmental fate. Oogonia are sequentially added and, over time, male gametes may be removed by the proliferation of hemocyte cells (gamete re-absorption). Gamete re-absorption by hemocytes has been described as a process to recycle materials and energy following a spawning event (or in this case, sex transition) or as a survival mechanism in response to stress/diseases (Beninger and Le Pennec 2003; Pipe 1987; Steele and Mulcahy 1999). Without tracking the gametal status of each individual over the entire experimental window it is difficult to unambiguously confirm full sex reversal (as existing sex determination protocols are invasive [smearing] or destructive [histology]). The observation of intersex individuals in lower EE2 exposures (6.25 and 12.5 ng/l) is a significant finding and may indicate that a transition (to higher female proportions) may occur at lower concentrations of EE2 with greater exposure duration. The occurrence of intersex at these lower, environmentally relevant, exposures of EE2 demonstrates that gonad gametal status may be employed as a sensitive biomarker of estrogenic exposure and effect in *S. glomerata*. Although it is likely that these individuals may follow a transitional switch from male to female in a similar fashion to higher exposures (50 ng/l), it may also be possible that lower exposures do not promote full sex reversal and subsequently result in a static intersex condition. Regardless, there are potentially negative effects associated with full sex reversal and/or intersex at both the individual and population level. This could include decreased individual fitness/reproductive capacity and/or altering the sex ratio and thus the overall fitness of a population. It is well established that oysters spawn in synchronization, during the summer months in response to environmental signals such as the full moon, changes in salinity and warmer water temperatures (Roughley 1933). An individual that experiences intersex or a full sex reversal midway during a gonadal cycle is unlikely to develop mature gametes capable of fertilization for a synchronised spawning event compared to individuals that have matured as female only. Thus, estrogenic exposure is perhaps likely to disrupt spawning synchronization among individuals. Further testing is also required to investigate if the gametes from intersex or these (new) female individuals are viable, capable of fertilization and if there is potential for more long term effects in subsequent generations.

Lastly we assessed the utility of vitellogenin as an early warning indicator of later gonadal effects at the individual level. Higher level effects, such as an increase in female development or a full sex reversal may be detrimental in terms of individual fitness or population level related consequences (again via influencing reproductive synchrony). The ability of a biomarker to predict higher level effects is favourable for biological assessment of contaminants under circumstances where a risk of estrogenic exposure can be identified and removed prior to the onset of higher level effects. Our results demonstrated that early increases in female vitellogenin (following 4 days) correlated with an increase in female development (21 days) and increased oocyte area (49 days). Male vitellogenin (following 4 days) exhibited a negative correlation with both male percentages (49 days) and mean male development stages (49 days). Together these results suggest that vitellogenin, if measured during the initial stages of gonadal development, may be useful as an early warning indicator for accelerated female development and decreased male development due to estrogenic exposure. Our results suggest that monitoring of both sexes is useful and perhaps necessary. Females are likely to be more sensitive to effects of estrogenic contaminants, display more pronounced vitellogenin induction and exhibit accelerated oocyte development. Males, in comparison, provide definitive evidence of endocrine disruption via vitellogenin induction, decreased male development and sex reversal.

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