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Article

Induction of vitellogenin-like lipoproteins in the mussel *Aulacomya ater* under exposure to 17β-estradiol

Inducción de lipoproteínas tipo vitelogenina en el mejillón *Aulacomya ater* expuesto a 17β-estradiol

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Resumen.- El mejillón *Aulacomya ater* fue expuesto a diferentes concentraciones de 17β-estradiol (E2) (0, 1, 100 µg L^{-1}) por 21 días y durante 4 periodos de muestreo, con el objeto de evaluar como reaccionan estos organismos ante la exposición a compuestos estrogénicos externos. Además, se inyectaron 2 concentraciones de 17β-estradiol (0,5 y 1 mmol) en el músculo abductor de 5 individuos. El efecto del E2 se determinó a través de la medición de los niveles de vitelogenina (Vg) en gónadas, utilizando el ensayo del fosfato alcalino lábil (ALP) y mediante el análisis histológico de las gónadas. Los resultados demuestran un incremento en los niveles de ALP para los machos expuestos a los menores niveles de E2, principalmente durante los primeros 4 días de exposición, indicando que la respuesta de la Vg al E2 es dependiente del tiempo. Se observaron algunas diferencias en los parámetros reproductivos (Índice gonadosomático y madurez) de machos y hembras expuestas y no expuestas a E2. Además, se encontraron claras diferencias en los niveles de ALP entre los sexos, siendo estos más elevados en las gónadas de los machos. No se detectaron respuestas en los niveles de Vg en hembras expuestas a las concentraciones experimentales de E2. Nuestros resultados sugieren que el ciclo reproductivo de *A. ater* podría ser alterado por la exposición a E2, lo que resulta relevante al considerar los recientes informes de altos niveles de E2 en los sedimentos de Chile centro-sur.

Palabras clave: Disrupción endocrina, estrógenos, ensayo del fosfato alcalino-lábil, bivalvos

Abstract.- The mussel *Aulacomya ater* was exposed to 17β-estradiol (E2) (0, 1, 100 µg L^{-1}) for 21 days with 4 sampling periods to evaluate how these organisms deal with exogenous estrogenic compounds. In addition, 2 concentrations of 17β-estradiol (0.5, 1 mmol) were injected into the abductor muscle of 5 individuals. The effects of E2 were determined by measuring the vitellogenin (Vg) levels with an alkaline labile phosphate (ALP) assay for gonads and using histological analyses. The results show an increment in ALP levels for males exposed to the lowest E2 levels, principally during the first 4 days of exposure, indicating that the response of Vg to E2 is time-dependent. Some differences were observed between the reproductive parameters (gonadosomatic index and maturity) of males and females exposed and unexposed to E2. Clear differences were also found in ALP levels between sexes, with the highest value occurring in male gonads. No response in Vg levels was detected in females after exposure to E2. Our results suggest that the reproductive cycle *A. ater* may be altered by E2 exposure, which is relevant considering recent reports of high levels of E2 in the sediments off south-central Chile.

Key words: Endocrine disruption, estrogens, Alkali-labile phosphate assay, bivalves

Introduction

Estrogens are hormones that play a major role in sexual differentiation, sex-gland development, and oocyte growth of most oviparous species (Wang *et al*. 2008); they also play an important role in bone maintenance, the cardiovascular system and the central nervous system (Janer 2005). These compounds may be of natural [17β-estradiol (E2), estrone (E1), estriol (E3)] or synthetic [17β-ethynylestradiol (EE2), and diethylstilbestrol (DES)] origin; natural estrogens are principally synthesized and excreted by the ovary. At present, both types of estrogens are released into the environment due mainly to the use of estrogen-replacement therapy and via livestock manure (Chen *et al*. 2010). This has become an important area of research, given that estrogens are currently recognized as the most potent endocrine disrupting compounds (Caliman & Gaverilescu 2009). Estrogens have the potential to interact with the estrogen-signaling pathway, leading to the increased production of the egg-yolk protein precursor vitellogenin (Vg), to the feminization of the reproductive duct, and to intersex in fish (Thorpe *et al*. 2003, Wester *et al*. 2004) and bivalves (Gagné *et al*. 2001).

Estrogen-disrupting compounds include E2, the most potent natural estrogenic hormone naturally present in the environment. This compound is of concern since it can reach aquatic environments through domestic effluents (Gagné *et al.* 2005, 2010), livestock waste (Chen *et al*. 2010), and agricultural runoff (Lee *et al*. 2007). Different studies have shown that E2 can induce Vg at very low concentrations (1 ng L^{-1}) in male trout (Hansen *et al*. 1998, Tremblay & Van der Kraak 1998). Moreover, due to its high estrogenic potential E2 has often been used as 'a model estrogenic compound' in studies that assess the effects of xenoestrogenic compounds on aquatic organisms (Janer *et al*. 2005).

The presence of E2 and other estrogens in aquatic environments has led to increasing research on the impact of these compounds on bivalves, which filter large quantities of water and increase their exposure to contaminants (Gagné *et al.* 2004). Furthermore, these organisms are relevant for analysis given their abundance in nature, their ecological importance, and their use as bioindicators in monitoring water pollution (Ketata *et al*. 2008).

Though little is known about invertebrate endocrinology, several studies have shown that the main effect of E2 on the reproductive system of bivalve mollusks is the increase of Vg levels (Li *et al*. 1998, Blaise *et al*. 1999, Matozzo & Marin 2008, Gagné *et al.* 2010, 2011, Moura-Costa *et al*. 2010). The production of Vg, the major precursor of the egg-yolk proteins (vitellins), is the main indicator used to determine endocrine disruption in oviparous organisms (Wallace 1985). It is the major energy-rich glycolipophosphoprotein for developing embryos in vertebrates and invertebrates. Vg levels generally increase in sexually mature females, but are lower or undetectable in juveniles and males, probably due to low levels of estrogens in plasma (Wallace 1985). However, males have a functional, silent Vg gene that may be activated by (xeno)-estrogens (Flouriot *et al.* 1995). In bivalves, Vg is synthesized in the vesicular connective tissue of the female gonads, transported to the oocytes by hemolymph and then incorporated in developing oocytes by pinocytosis (Matozzo & Marin 2005). This process is induced and regulated by estrogens (Li *et al.* 1998) and appears to be susceptible to endocrine-disrupting compounds (EDCs), since bivalves respond to exposure to estrogen and xeno-estrogens by increasing levels of Vg. An antibody for mussel Vg is not commercially available at present, however Vg levels can be measured through an indirect assay termed alkali-labile phosphate assay (ALP), based on the determination of labile phosphates released by Vg after hydrolysis with alkali (Blaise *et al*. 1999). This method offers a simple cost-effective biomarker of endocrine disruption in mussels (Porte *et al.* 2006). Some studies of bivalves have used this approach, finding that Vg seems to be relatively sensitive to estrogen exposure (Gagné *et al*. 2001, Aarab *et al.* 2006, Matozzo *et al*. 2008) and that endocrine disruption occurs after exposure to estrogenic compounds and urban effluents (Gagné *et al.* 2001, Quinn *et al.* 2004, Ortiz-Zaragoitia & Cajaraville 2006).

Aulacomya ater (Molina, 1782) is an economically important mussel inhabiting the shallow waters of Chile's south-central coast, forming extensive beds on rocky substrates. It is a gonochoric species found in South Africa (Griffiths 1977) and South America, from Callao (Peru) to the Strait of Magellan (Chile) in the Pacific, and from southern Brazil, along Argentina, to the Falkland Islands in the Atlantic (Jaramillo & Navarro 1995). Little is known about the life cycle of *A. ater* in south-central Chile, but Jaramillo & Navarro (1995) showed a continuous gamete release over several months during the year for individuals from southern Chile. They observed clear peaks of pre-spawning stages in April, August, November and February. The spawning stage in females peaked in July and December, but males released gametes from May to January. Also continuous spawning was found from June to January, related to fluctuations in phytoplankton levels.

The coast of south-central Chile, where shoals of this species are frequently found, is strongly influenced by domestic and industrial waste coming from coastal cities and river runoff (Rudolph *et al.* 2002). This may lead to significant sediment contamination by steroid estrogens, principally released from human sewage, with levels of E2 and 17β-ethinylestradiol in south-central Chile between 0.06 and 4.61 ng g^{-1} dry weight (dw) and 0.06 to 16.81 ng g^{-1} dw, respectively (Bertin *et al.* 2011). Therefore, the main goal of this study was to investigate the estrogenic effect of the natural estrogen E2 on the bivalve *A. ater* through the induction of Vg-like proteins and histological observations.

Materials and methods

Mussel collection and maintenance

Specimens of *Aulacomya ater* (5.5-6.5 cm shell length) were collected from Coliumo Bay (Chile; 36º31'S; 72º55'W)

a small and shallow bay commonly used as a control site in environmental studies due to its low anthropogenic activity (Palma *et al*. 2007, Aguirre- Martinez *et al*. 2009, Srain & Rudolph 2010). The samples were obtained during December 2009, and acclimatized in the laboratory for 7 days before exposure to E2. Mussels were maintained in 30 L polystyrene aquaria provided with aerated seawater at a salinity of 35 ± 1 and temperature of 11 ± 0.3ºC, under a 12 h light/12 h dark cycle. The water was changed every day, at which point the mussels were fed a mixture of the microalgae (*Isochrysis galbana* and *Chaetoceros muelleri*).

Exposure experiments and sampling

Exposure to E2 dissolved in water

During a 21 day exposure period, mussels (n=27) were exposed to the following concentrations of E2 (Sigma-Aldrich): E2-1= 1 µg L^{-1}, E2-100= 100 µg L^{-1}. The lower concentration was chosen on the basis of data available in the literature concerning E2 estrogenicity (Janer *et al*. 2004, Matozzo & Marin 2008), while the higher was selected as a saturated concentration. A stock solution of E2 was prepared in acetone (Matozzo & Marin 2008) and stored at room temperature for the duration of the experiments. Working solutions were prepared by diluting the stock solution into the microalgae feed mixture. For the control, acetone was added at the same concentration (20 µL L^{-1}) used in both treatments. The acetone solvent was diluted 20,000 times in the tanks. Of the 27 mussels allocated per treatment, 6 were sampled from each tank at 0, 90, 282 and 474 h.

Six replicates were sampled per treatment group and the following biometric data were recorded: weight (g), total shell length (cm), whole soft and gonad tissue weights (g), condition index (CI = wet weight of soft tissues/ wet weight of shell) and gonadosomatic index (GSI = gonad wet weight/ total tissue wet weight). The sex was first determined visually, since females have black colored gonads while in males they are yellowish, and then with histological analysis. The gonads of each individual were sectioned in two parts: one for the Vg analysis described below and the other for histology.

Exposure to E2 via injection

Five *A. ater* specimens were injected in the anterior abductor muscle with 0.5 µmole of E2 and 5 specimens with 1 µmole of E2; both concentrations were chosen according to Gagné *et al*. (2001). The solvent dimethyl sulfoxide (DMSO, 100%) was selected to dissolve E2 (Blaise *et al*. 1999, Gagné *et al*. 2001). In the experiment, 25 µl of the substance per 40 g of total body plus shell weight were injected into each mussel. Control mussels received only the solvent (n= 5; 25 µl of DMSO per 40 g of total body plus shell weight). After injection, the mussels were incubated for 96 h at 11 ± 0.3ºC in aerated seawater. At the end of the incubation period the mussels were sampled as described above.

Vitellogenin determination

An indirect quantitative method was used to establish Vg levels in the gonads of *A. ater,* through ALP.

Gonad tissues were homogenized in 25 mM Hepes–NaOH, pH 7.4, containing 125 mM NaCl, 1 mM dithiothreitol and 1 mM EDTA at 4ºC. The homogenate was centrifuged at 12000 x *g* for 20 min at 4ºC. The supernatant (S12) was carefully removed from the pellet and kept at -80ºC until analysis.

Vg levels in gonad homogenate extracts were determined using the indirect alkali labile phosphate method (ALP) (Blaise *et al*. 1999) modified by Gagné *et al*. (2003) and Ortiz-Zaragoitia & Cajaraville (2006). A subsample of the supernatant was adjusted to 35% acetone and centrifuged at 10,000 x *g* for 5 min. The pellet was dissolved in 200 ml of NaOH 1M at 60ºC for 30 min. Inorganic phosphate levels were determined using the phosphomolybdenum method (Stanton 1968). A subsample of 100 µl was mixed with 25 µl trichloroacetic acid, 600 µl ultrapure water, 125 µl molybdenum reagent (0.02 M ammonium molybdate tetrahydrate and 5.25 M H_2SO_4 solution), and 125 µl ascorbate (Sigma-Aldrich) (Francois Gagné *pers. comm.*). After incubating for 20 min at 37ºC, the absorbance was measured at 660 nm using a Shimadzu UV-1603 spectrophotometer. A series of KH_2PO_4 concentrations were used for the inorganic phosphate standard curve. ALP levels in gonads are given as µg phosphate mg^{-1} protein. Total protein in the supernatants was determined according to Bradford (1976).

Gonad histology

Gonads were fixed in 10% neutral buffered formalin, dehydrated through increasing concentrations of ethanol and embedded in paraffin. Serial sections (5 µm thickness) were cut and stained with hematoxylin and eosin. Histological alterations (hemocytic aggregates,

macrophage aggregates (GMA), hemocytes, fibrosis and atresic oocytes) and developmental stages were determined for each animal. Five developmental stages were determined in mussels according to Gauthier-Clerc *et al.* (2006): phase I= undifferentiated (pre-vitellogenesis/ spermatogenesis); phase II = development (early vitellogenesis/spermatogenesis); phase III = mature gonad (late vitellogenesis/spermatogenesis); phase IV = spawning gonad; and phase V = post-spawning gonad (Fig. 1). The prevalence of pathologies was examined with an optical microscope (4x, 10x, 40x).

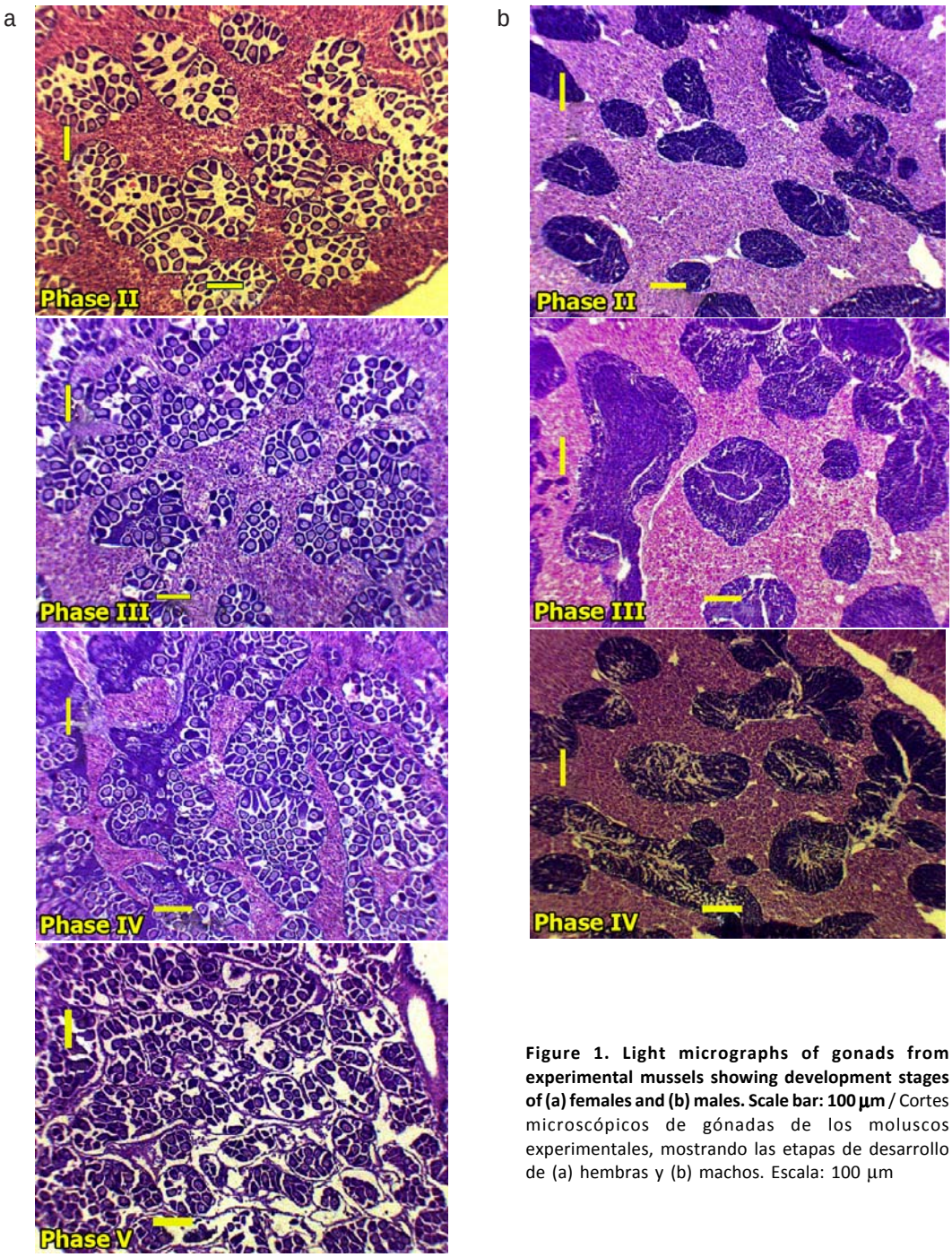

Figure 1. Light micrographs of gonads from experimental mussels showing development stages of (a) females and (b) males. Scale bar: 100 µm / Cortes microscópicos de gónadas de los moluscos experimentales, mostrando las etapas de desarrollo de (a) hembras y (b) machos. Escala: 100 µm

Statistical analysis

Statistical analyses were performed with the software GraphPad Prism® (Version 4). Data were checked for normality using the Kolmogorov-Smirnov (KS) test. A factorial two-factor analysis of variance, with time and treatments as major factors, followed by a Tukey post hoc test was used to test for significant differences in ALP concentration. This analysis was done separately for males and females. Furthermore, to test for significant differences in the ALP concentration between sexes in each treatment a two-factor analysis of variance was used with time and sex as major factors. Significance was established at $P < 0.05$. The results are expressed as mean ± standard error.

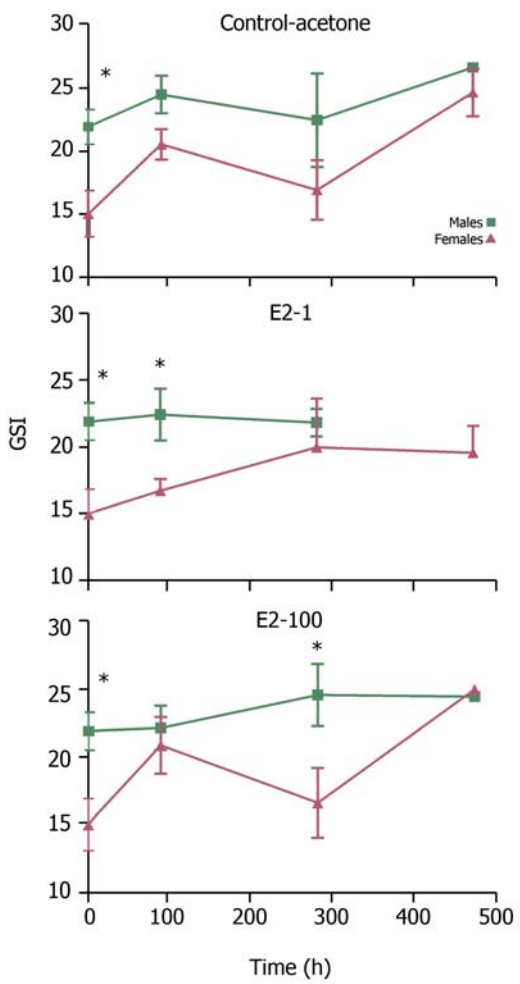

Figure 2. Gonadosomatic index (GSI) variation with time, for males and females of (a) control-acetone, (b) E2-1 (1 µg L^{-1}) and (c) E2-100 (100 µg L^{-1}) treatments. * significant differences between sexes ($P < 0.05$) / Variación del índice gonadosomático (IGS) en el tiempo, para los machos y hembras del (a) control-acetona, (b) tratamiento con E2-1 (1 µg L^{-1}) y (c) tratamiento con E2-100 (100 µg L^{-1}). * Estadísticamente significativo ($P < 0,05$)

Results

Organisms exposed to the 2 treatments showed a similar gonadosomatic index (GSI), without significant changes with time of exposure. However, the GSI of males and females differed, especially in the acetone control and during the first days of exposure (Fig. 2). According to the histological analyses, most individuals were in developmental stage II, III, and IV (Figs. 1 and 3). Males presented a less advanced development stage than females, which was confirmed by histological analyses (Fig. 3).

Vg concentrations obtained by ALP showed significant differences between males and females (Fig. 4), especially for the group subjected to the treatment with the lower E2 concentration (1 µg L^{-1}) (Fig. 4b). Males had higher ALP levels, especially during the first sampling period (90 h). In the treatment with the higher E2 concentration (E2-100), males and females showed similar ALP levels.

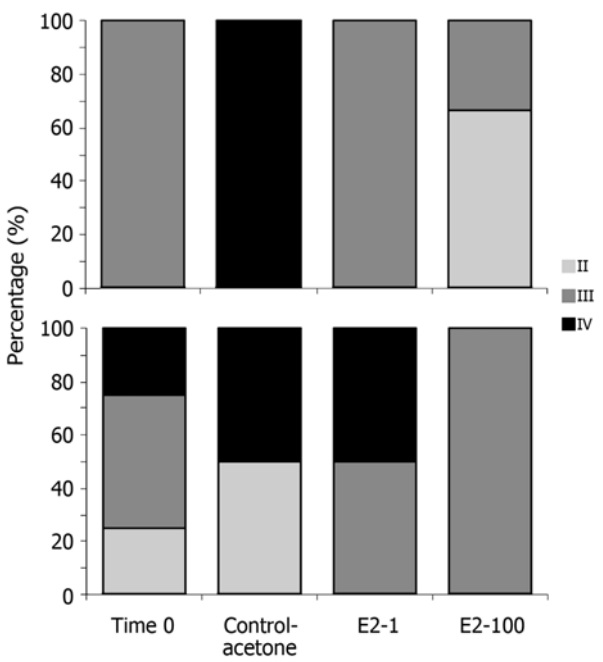

Figure 3. Percentages of mussels at each gonad developmental stage: (a) females and (b) males. There was only one female in stage V and it was not included in this figure / Porcentaje de moluscos en cada etapa de desarrollo: (a) hembras y (b) machos. Sólo se encontró una hembra en estado V por lo que no se incluyó en esta figura

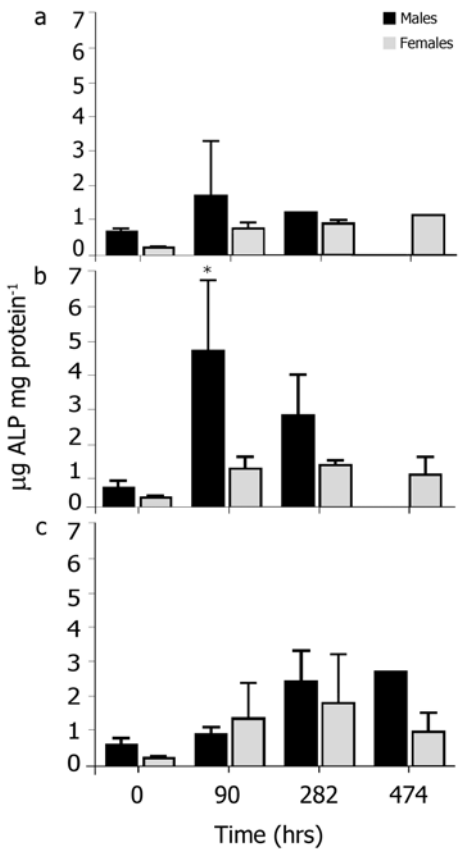

Figure 4. Differences in gonad ALP levels between males and females of *A. ater* exposed to (a) control acetone, (b) 1 µg L^{-1} (E2-1), (c) 100 µg L^{-1} (E2-100). *significant differences between sexes (P < 0.05) / Diferencias entre los niveles de ALP de gónadas de machos y hembras de *A. ater* expuestos a (a) control-acetona, (b) 1 µg L^{-1} (E2-1), (c) 100 µg L^{-1} (E2-100). * diferencias significativas entre sexos (P < 0,05)

Due to the different concentrations of ALP between sexes, we compared the different treatments for males and females separately (Fig. 5). In the case of the females, the time is the main factor explaining the observed differences in ALP concentration, and no significant differences were found between treatments exposed to the 2 experimental concentrations of E2. In males, time was also the main factor but the post hoc test showed, for the second sampling interval (90 h), a significant higher ALP level in the treatment with lower E2 concentration (P < 0.05) (Fig. 5b). After this initial period, ALP levels dropped to concentrations similar to the control. In addition, a trend to increase ALP concentration over time was observed in the treatment with high concentration of E2 (100 µg L^{-1}) (Fig. 5b).

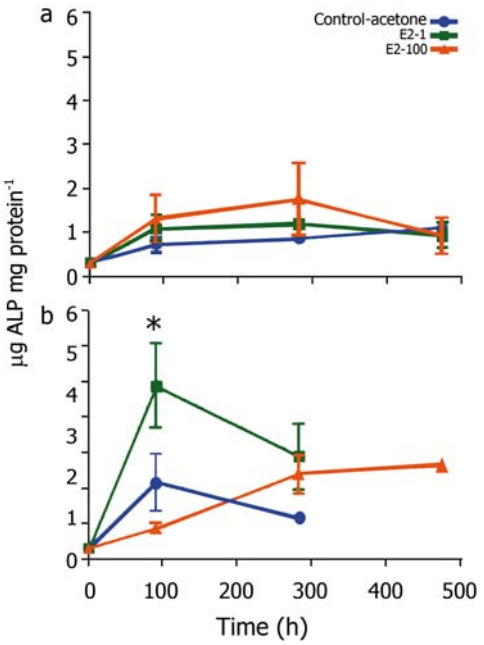

Figure 5. Relation between ALP levels and time, showing differences between treatments and control for (a) females and (b) males. *: significant differences between treatments (P < 0.05) / Relación entre los niveles de ALP y el tiempo, mostrando las diferencias entre los tratamientos y el control para (a) hembras y (b) machos. *: diferencias significativas entre tratamientos (P < 0,05)

The induction assay using the direct injection of 2 E2 concentrations was impaired due to the fact that it was not possible to know in advance the sex of the specimen, and accordingly, the sex proportion in the experiment was not adequate (Table 1). Nevertheless, we have reported the results in Table 1 because they also show differences in ALP concentration between males and females (Fig. 6). On the average, the concentration of Vg found was higher in males than in females (Fig. 6), particularly in males exposed to the lower E2 concentration.

Table 1. Gender segregated average ALP concentrations (µg ALP mg protein^{-1}) for induction experiments / Promedio de las concentraciones de ALP (µg ALP mg protein^{-1}) separadas por sexos, para los experimentos de inducción

	Females		Males	
	n	Mean	n	Mean
Control DMSO	3	0.055 ± 0.005	0	-
E2-0.5	0	-	3	5.769 ± 5.080
E2-1	3	0.299 ± 0.074	1	2.085
Total Mean	6	0.177 ± 0.142	4	4.541 ± 4.200

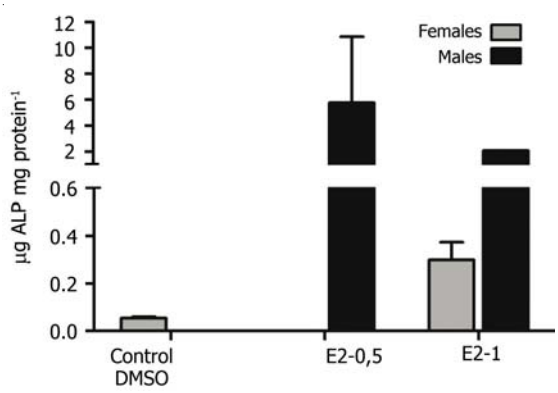

Figure 6. ALP levels in gonads of directly induced (injected) organisms with 0.5 and 1 µmol E2 and DMSO as control, showing differences between the sexes / Niveles de ALP en gónadas de organismos inducidos directamente (inyectados) con 0,5 y 1 µmol E2 y DMSO como control, mostrando las diferencias entre los sexos

Figure 7 shows the most common pathologies found in this experiment, principally hemocytic aggregates, macrophage aggregates (GMA), hemocytes, fibrosis and atresic oocytes. Again there was a clear difference between sexes, with more pathology found in males. However, the presence of atresic oocytes in all females is remarkable. No significant differences were found between the treatments and the control for either sex.

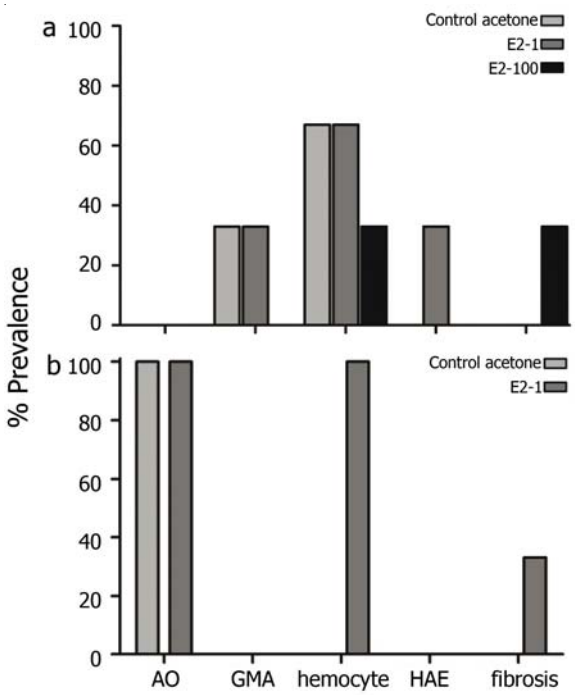

Figure 7. Prevalence of pathologies in gonads of *A. ater* exposed to 17β-estradiol: (a) males and (b) females. Values are averages over sampling periods. HAE: Hemocytic aggregates, GMA: Macrophage aggregates / Prevalencia de patologías encontradas en gónadas de *A. ater* expuesta a 17β-estradiol: (a) machos y (b) hembras. Los valores son promedios de los periodos de muestreo. HAE: agregación hemolítica, GMA: agregación macrófaga

Discussion

With regard to the general condition of the organisms, both the condition and the gonadosomatic indices failed to show important differences between the treatments. Nonetheless, we noted a much higher GSI in males in comparison to females in all treatments as well as in the control, which is consistent with the high levels of atresic oocytes observed in females. A high percentage of females with atresic gonad follicles was also reported in *Mytilus galloprovincialis* by Suárez Alonso *et al*. (2007), indicating the reabsorption of oocytes, either due to lack of favorable environmental conditions (temperature, food) or to the end of the gonadal cycle (summer). In the sampling period (December) the development stages were different for both sexes, with males presenting a less advanced development stage than females. This is coherent with the reproductive cycle of *A. ater* described by Jaramillo & Navarro (1995), with females having a peak of gamete release between July and December whereas males release gametes continuously between May and January.

In this study we used the indirect method for determining Vg-like proteins (ALP) due to the lack of antibodies against specific Vg for this species and for mollusks in general (Blaise *et al*. 1999). Using this method we were able to detect important differences in ALP level between males and females. We noted a significant increase of ALP in males exposed to the lower E2 concentration, mainly during the first 4 days of exposure (90 h). This suggests that the response of Vg to E2 is time-dependent, and thus it is a factor that should be taken into account in bivalve endocrine disruption studies. Most studies of Vg induction by E2 in mollusks have only considered one incubation period (Blaise *et al.* 1999, Gagné *et al.* 2001, Janer *et al.* 2004), thereby omitting the time-dependent response to the endocrine disruptor. One exception is the study of Matozzo & Marin (2008), which included sampling during 2 different periods of the induction experiment (at 7 and 14 days), finding differences in the responses of Vg to the EDCs. These temporal variations may be due to mechanisms of

regulation of free steroid-levels (*e.g.*, conjugation rates and esterification), which could influence the decline of Vg over time. These mechanisms play an important role in the endocrine homeostasis of mollusks (Janer 2005, Lavado *et al.* 2006).

On the other hand, Vg only increased significantly with the lower E2 concentration (1 µg L^{-1}), in agreement with recent studies that propose that estrogens generate more effects at low concentrations (non-monotonic dose response) in mammals and various other species (Welshons *et al.* 2003, 2006, Weltje *et al.* 2005). This same effect was observed by Matozzo & Marin (2008) in clams (*Tapes phillipinarum*) exposed to different E2 concentrations and by Ciocan *et al*. (2010) concerning the expression of Vg mRNA in *Mytilus edulis*. The response to low E2 concentrations may be due to the fact that this compound behaves like an endogenous steroid and can regulate physiological functions, whereas high concentrations of E2 significantly increase the activity of inactivation mechanisms for excessive E2 (palmitoyl-CoA; estradiol transferases) (Janer *et al.* 2005). However, it is important to note that the lower E2 experimental concentration used in the present study is very high compared to ambient levels (about 0.02 µg L^{-1} in sediments), and therefore we cannot discard induction of Vg when *A. ater* is exposed to E2 at environmental concentrations lower than 1 µg L^{-1}.

The significant difference found between the ALP levels of males and females, mainly in the control and in the treatment with the lower E2 concentration, leads us to suggest the following hypotheses: (i) the higher level of ALP in males is explained by the lower levels of proteins in their gonads because they are in an earlier stage of gamete development, and (ii) males were induced by endocrine disruptors present in the natural environment. Other studies also reported the same situation: Matozzo & Marin (2005) for the clam *Tapes philippinarum*, Hashimoto *et al*. (2000) for male flounder *Pleuronectes yokohamae*, and Pampanin *et al*. (2005) for *Mytilus galloprovincialis*. In the last study the authors suggested that the presence of chemicals with estrogenic effect was probably able to induce the expression of the Vg-like proteins in males, whereas in females, where this protein is normally expressed during gametogenesis, the parallel process could be masked. Our hypothesis of a natural environmental vitellogenin induction in males of *A. ater* is supported by the presence of high concentrations of E2 and ethinyl-estradiol (EE2) in the coastal sediments off south-central Chile (Bertin *et al*. 2011). The presence of EDCs in the coastal environment such as polyaromatic hydrocarbons (PAHs) and alkylphenols are known to produce in mussels high prevalence of oocyte athresia and low ALP levels in females, and high ALP levels in males (Ortiz-Zaragoitia & Cajaraville 2003[1]). In our experiments we also found high levels of oocyte athresia including those animals that were not exposed to E2. Unfortunately, to the best of our knowledge, there is no information available about the natural level of this pathology in *A. ater* populations.

In conclusion, the results of this study show that males and females of *A. ater* respond differently to E2 exposure. Vg levels increased in *A. ater* males exposed to low E2 concentrations, but only in the first days of exposure, thereby suggesting that the mechanisms regulating the levels of sexual steroids are activated by 90 h of exposure. ALP values on the average were lower in females than males and no response in Vg levels was detected in females after the exposure to E2 experimental concentrations. The results of this study do not answer the question of which processes are involved in the responses of *A. ater* to estrogen exposure, mainly due to the lack of information on the endocrine system of this bivalve. Nonetheless, this study stands out as a first approach to understanding how the endocrine system of this species may be affected by estrogen exposure. Our results suggest that the reproductive cycle *A. ater* may be altered by E2 exposure, which is relevant considering recent reports of high levels of E2 in the sediments off south-central Chile (Bertin *et al.* 2011).

Acknowledgments

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