Elsevier Editorial System(tm) for Environmental Pollution

Manuscript Draft

Manuscript Number:

Title: Impact of bisphenol A (BPA) on early embryo development in the marine bivalve Mytilus galloprovincialis: effects on gene transcription

Article Type: Research Paper

Keywords: endocrine disrupters; bisphenol; marine mussel; larvae; gene transcription; shell formation

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Abstract: Bisphenol A (BPA), a monomer used in plastic manufacturing, is weakly estrogenic and a potential endocrine disruptor in mammals. Although it degrades quickly, it is pseudo-persistent in the environment because of continual inputs, with reported concentrations in aquatic environments between 0.0005 µg/L and 12 µg/L. BPA represents a potential concern for aquatic ecosystems, as shown by its reproductive and developmental effects in aquatic vertebrates.
In invertebrates, endocrine-related effects of BPA were observed in different species and experimental conditions, with often conflicting results, indicating that the sensitivity to this compound can vary considerably among related taxa. In the marine bivalve Mytilus galloprovincialis BPA was recently shown to affect early development at environmental concentrations. In this work, the possible effects of BPA on mussel embryos were investigated at the molecular level by evaluating transcription of 13 genes, selected on the basis of their biological functions in adult mussels. Gene expression was first evaluated in trocophorae and D-veligers (24 and 48 h post fertilization) grown in physiological conditions, in comparison with unfertilized eggs. Basal expressions showed a general up-regulation during development, with distinct transcript levels in trocophorae and D-veligers. Exposure of fertilized eggs to BPA (10 µg/L) induced a general upregulation at 24 h pf, followed by down regulation at 48 h pf. Mytilus Estrogen Receptors serotonin receptor and genes involved in biomineralization (Carbonic Anydrase and Extrapallial Protein) were the most affected by BPA exposure. At 48 h pf, changes in gene expression were associated with irregularities in shell formation, as shown by scanning electron microscopy (SEM), indicating that the formation of the first shelled embryo, a key step in bivalve development, represents a sensitive target for BPA. Similar results were obtained with the natural estrogen 17β-estradiol. The results demonstrate that estrogenic chemicals can affect Mytilus early development through dysregulation of gene transcription.

DEPARTMENT OF BIOLOGICAL, GEOLOGICAL,
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July 4th, 2016

Dear Editor,

I send you the manuscript "*Impact of bisphenol A (BPA) on early embryo development in the marine bivalve Mytilus galloprovincialis: effects on gene transcription*" by Teresa Balbi, Silvia Franzellitti, Rita Fabbri, Michele Montagna, Elena Fabbri, Laura Canesi, to be considered for publication in Environmental Pollution.

This work deals with the potential concern for endocrine disrupting chemicals (EDCs), and in particular for the estrogenic chemical Bisphenol A (BPA), in aquatic ecosystems. Although BPA is considered as one of the most common EDC that can affect reproduction and development in vertebrates, little information is available on the developmental effects of this compound in aquatic invertebrate species The results here reported demonstrate that in the model marine invertebrate, the bivalve *Mytilus*, environmental concentrations of BPA significantly affect early embryo development through dysregulation of gene transcription. Moreover, the formation of the first calcified embryo, a key step in bivalve development, is showed as a sensitive target for BPA as well as for the natural estrogen 17β-estradiol (E_2).

To the best of our knowledge, this study represents the first investigation on transcriptional changes occurring during early development of *M. galloprovincialis*, as well as the first indication on the molecular mechanisms of estrogenic compounds impacts in embryos of marine bivalves.

The results underline how exposure during early life stages can help identifying potential effects and modes of action of potential EDCs in aquatic invertebrates. Moreover, since calcifying larvae of marine species are particularly vulnerable to abiotic stressors, studies on the effects of potential EDCs on early life stages of sensitive species may contribute to the development of future multiple stressor studies in a global change scenario.

I do hope the manuscript meets the requirements for publication on Environmental Pollution.

Looking forward to hearing from you,

Sincerely yours

Prof. Elena Fabbri

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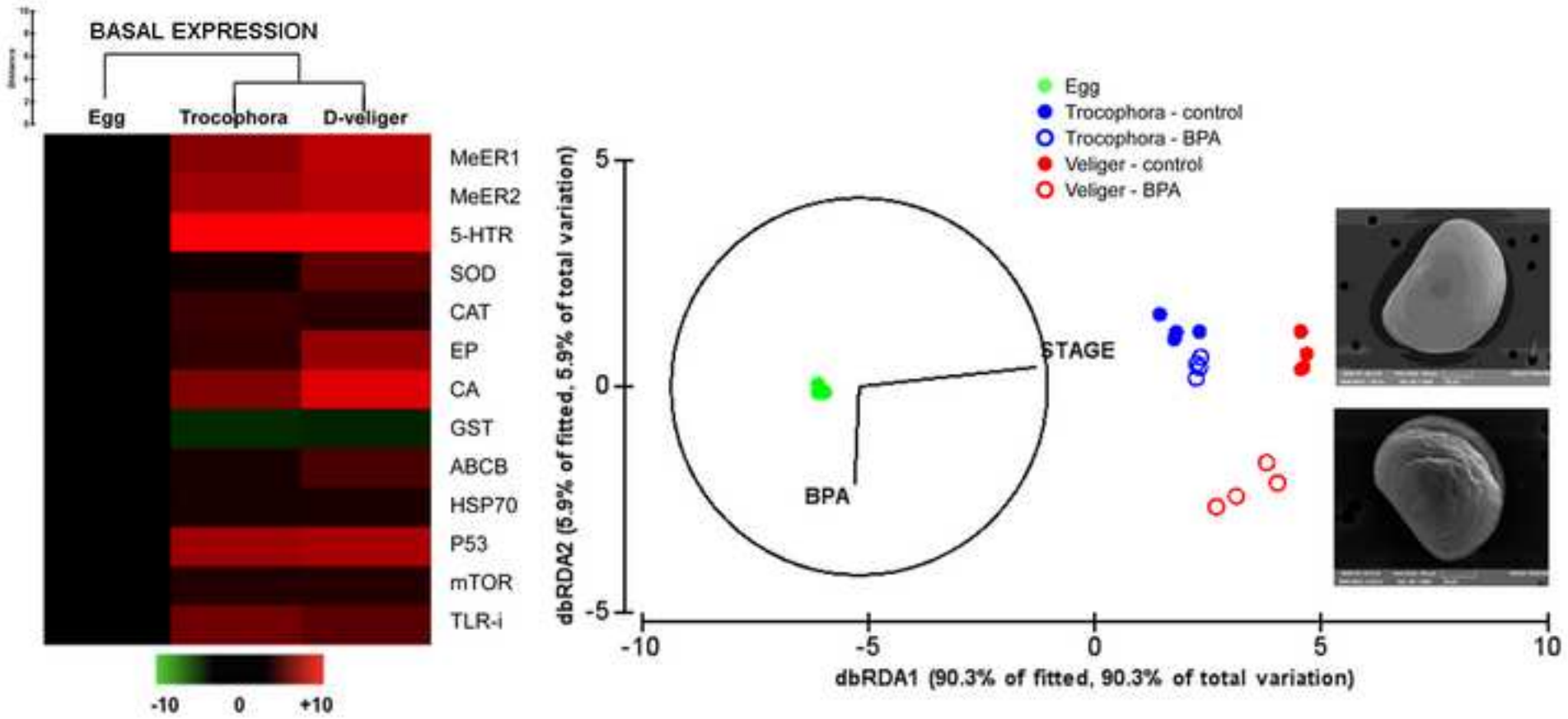
*Graphical Abstract



Highlights

- The effects of BPA on Mytilus early embryo development were investigated
- Changes in transcription of 13 genes were observed at 24 and 48 h pf
- At 48 h pf, gene downregulation was associated with alterations in shell formation
- Similar results were obtained with the natural estrogen 17β-estradiol
- Estrogenic chemicals affect bivalve embryos by dysregulation of gene transcription

Impact of bisphenol A (BPA) on early embryo development in the marine bivalve *Mytilus galloprovincialis*: effects on gene transcription

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Abstract.

Bisphenol A (BPA), a monomer used in plastic manufacturing, is weakly estrogenic and a potential endocrine disruptor in mammals. Although it degrades quickly, it is pseudo-persistent in the environment because of continual inputs, with reported concentrations in aquatic environments between 0.0005 μg/L and 12 μg/L. BPA represents a potential concern for aquatic ecosystems, as shown by its reproductive and developmental effects in aquatic vertebrates.

In invertebrates, endocrine-related effects of BPA were observed in different species and experimental conditions, with often conflicting results, indicating that the sensitivity to this compound can vary considerably among related taxa. In the marine bivalve *Mytilus galloprovincialis* BPA was recently shown to affect early development at environmental concentrations. In this work, the possible effects of BPA on mussel embryos were investigated at the molecular level by evaluating transcription of 13 genes, selected on the basis of their biological functions in adult mussels. Gene expression was first evaluated in trocophorae and D-veligers (24 and 48 h post fertilization) grown in physiological conditions, in comparison with unfertilized eggs. Basal expressions showed a general up-regulation during development, with distinct transcript levels in trocophorae and D-veligers. Exposure of fertilized eggs to BPA (10 μg/L) induced a general upregulation at 24 h pf, followed by down regulation at 48 h pf. *Mytilus* Estrogen Receptors serotonin receptor and genes involved in biomineralization (Carbonic Anydrase and Extrapallial Protein) were the most affected by BPA exposure. At 48 h pf, changes in gene expression were associated with irregularities in shell formation, as shown by scanning electron microscopy (SEM), indicating that the formation of the first shelled embryo, a key step in bivalve development, represents a sensitive target for BPA. Similar results were obtained with the natural estrogen 17β-estradiol. The results demonstrate that estrogenic chemicals can affect *Mytilus* early development through dysregulation of gene transcription.

Keywords: endocrine disrupters; bisphenol; marine mussel; larvae; gene transcription; shell formation

Capsule: *Bisphenol A effects on larval stages of Mytilus galloprovincialis*

1. Introduction

Aquatic environments are the ultimate reservoirs for many environmental anthropogenic chemicals, including those that can interfere with the functions of natural hormones. These compounds, defined as endocrine disrupting chemicals (EDCs) can adversely impact reproduction and development in humans and wildlife (Zoeller et al., 2012). Fish and other aquatic organisms often have the greatest exposures to such chemicals during critical periods of development or even their entire life cycles.

Bisphenol A (BPA), generally considered one of the most common EDC (Rubin, 2011; Vandenberg et al., 2013), is a monomer used in the manufacture of polycarbonate plastics and in a variety of plastic products, including food and beverage packaging, flame retardants, adhesives, building materials, electronic components, and paper coatings (EFSA FIP, 2015). Due to its widespread applications, BPA has become one of the highest production-volume chemicals (5.5 million tons per year) in the last decades (Alonso-Magdalena et al., 2015; Flint et al., 2012). Although it degrades quickly, it is pseudo-persistent in the environment because of continual inputs, leading to reported concentrations in aquatic environments between 0.0005 μg/L and 12 μg/L (Flint et al., 2012). Available data underline that environmental concentrations of BPA may represent a potential concern for aquatic ecosystems (Tišler et al., 2016). In aquatic vertebrates, BPA causes developmental and reproductive effects, as well disturbances in immune function and metabolism (Canesi and Fabbri, 2015). In invertebrates, endocrine-related effects of BPA have been observed in different species and experimental conditions, with often conflicting results. Available data underline how some invertebrates appear to be quite sensitive to BPA (freshwater and marine mollusks, insect larvae, marine copepods in particular), with effects documented at environmentally relevant concentrations (reviewed in Canesi and Fabbri, 2015; Flint et al., 2012).

Research on the impact of BPA in model aquatic organisms has major regulatory implications towards the ecosystem health. In this light, studies involving exposure during early life

stages in invertebrates, which can be highly sensitive to environmental perturbations, would greatly help identifying potential adverse effects of BPA as an EDC, in analogy with what reported in vertebrates (Hamlin and Guillette, 2011; Santangeli et al., 2016; Tse et al., 2013).

We have recently shown that in the marine bivalve *Mytilus galloprovincialis* exposure of fertilized eggs to BPA at environmentally relevant concentrations affects the formation of fully developed D-larvae at 48 h post fertilization (pf) (Fabbri et al., 2014). Larval development in mussels chronologically unfolds following a mechanical remodeling of the form and behavior, which requires controlled mechanisms engaged in tissue distribution and organ system differentiation (Ackerman et al., 1994). In *Mytilus*, after external oocyte fertilization embryos undergo fast cell divisions that lead to their segmentation, and differentiate into motile forms (trocophorae, 24 h pf), with successive development of various shelled forms (D-veligers) from 48 h pf (Marin et al., 2008). Although a number of genetic regulators during late-stages of larval development have been recently identified (Bassim et al., 2014), no information on the molecular mechanisms underlying the physiological processes involved in the early critical stages, such as the formation of the first shelled embryo, is available.

In this work, the effects of BPA on gene transcription in early embryo development of *M. galloprovincialis* were investigated. Fertilized eggs were exposed to BPA 1 µg/L (< EC_{50}, LOEC 10x) and 10 µg/L (> EC_{50}, LOEC 100x) as previously described (Fabbri et al., 2014), and the differential expression of 13 gene products related to known biological functions in adult mussels was assessed. These include neuroendocrine signaling, antioxidant defense, biomineralization, stress response, apoptosis, autophagy, and immune response. Transcriptional profiles were evaluated in trocophorae and D-veligers grown under physiological conditions or in the presence of BPA at environmentally realistic concentrations (Corrales et al., 2015). Morphology of control and BPA-exposed embryos was evaluated by scanning electron microscopy (SEM). The effects of BPA on embryo development, gene expression and morphology were compared with those of the natural estrogen 17β-estradiol (E_2).

2. Methods

2.1. Animal holding and larval rearing

Sexually mature mussels (*M. galloprovincialis* Lam.), purchased from an aquaculture farm in the Ligurian Sea (La Spezia, Italy) between November 2014 and March 2014, were transferred to the laboratory and acclimatized in static tanks containing aerated artificial sea water (ASTM, 2004), pH 7.9 - 8.1, 36 ppt salinity (1 L/animal), at 16 ± 1°C.

Gamete collection and fertilization were performed following the procedure described by Fabbri et al. (2014). Details are reported in Fig S1 (Supporting Information Appendix 1) with the description of the 96-microwell embriotoxicity assay performed to assess the effects of E_2. Unfertilized eggs (about 24,000 eggs/mL) obtained from at least 6 female individuals were collected by centrifugation at 400 xg for 10 min at 4°C, and the resulting pellet was frozen in liquid nitrogen. Eggs were fertilized with an egg:sperm ratio 1:10 in polystyrene 6-well plates and a final 6 mL volume. After 30 min, fertilization success (n. fertilized eggs / n. total eggs × 100) was verified by microscopical observation (>85%). At 30 min pf, BPA or E_2 were added to fertilized eggs in each well from 1 mg/L concentrated stock solutions prepared in ethanol, and suitably diluted to reach the final nominal concentrations of 1 and 10 μg/L for BPA, and 10 μg/L for E_2. Control wells (negative controls) contained only ASW. Four replicates for each experimental condition were made. In all cases, ethanol final concentration did not significantly affect the biological endpoints analysed (data not shown). Concentrations of BPA and E_2 in test solutions were checked by LC/MS (Table S1, Supporting Information Appendix 2).

2.2. RNA extraction and cDNA preparation

At 24 and 48 h pf larvae were collected by a nylon mesh (40 μm pore-filter) and washed with ASW. Three wells for each condition were pooled in order to obtain approximately 7000

embryos/replicate. The larval suspension was centrifuged at 800 xg for 10 min at 4°C. Larval pellets and unfertilized eggs were lysed in 1 mL of the TRI Reagent (Sigma Aldrich, Milan, Italy) and total RNA was further extracted using the DirectZol kit (Zymo Research, Freiburg, Germany) following manufacturer's instructions. RNA concentration and quality were verified using the Qubit RNA assay (Thermo Fisher, Milan, Italy) and electrophoresis using a 1.2% agarose gel under denaturing conditions. First strand cDNA for each sample was synthesized from 1 µg total RNA according to Balbi et *al.*(Balbi et al., 2014).

2.3. qPCR

Transcript expressions were assessed by real-time PCR (qPCR). Primers pairs were as reported in previous studies or were designed with Primer Express (Thermo Fisher, Milan, Italy) using nucleotide sequences retrieved from the GeneBank database (https://www.ncbi.nlm.nih.gov/genbank/) for *Mytilus galloprovincialis* (Table S2, Supporting Information Appendix 2). qPCR reactions were performed in duplicate in a final volume of 10 µl containing 5 µl iTaq universal master mix with ROX (BioRad Laboratories, Milan, Italy), 2 µl diluted cDNA, and 0.2 µM specific primers (Table S2, Supporting Information Appendix 2). A control lacking cDNA template (no-template) and a minus-reverse transcriptase (no-RT) control were included in the qPCR analysis to determine the specificity of target cDNA amplification. Amplifications were performed in a StepOne real time PCR system apparatus (Thermo Fisher, Milan, Italy) using a standard ''fast mode'' thermal protocol. For each target mRNA, melting curves and agarose gel electrophoresis were utilized to verify the specificity of the amplified products and the absence of artifacts. The amplification efficiency of each primer pair was calculated using a dilution series of cDNA (Table S2, Supporting Information Appendix 2). A preliminary stability analysis of 6 established candidate reference transcripts (Cubero-Leon et al., 2012; Franzellitti and Fabbri, 2013) was performed to achieve a robust normalization of qPCR data in mussel larval stages. Detailed methods and results are reported in Supporting Information

Appendix 3. *HEL* and *EF-α1* were selected as the best performing combination of reference gene products for data normalization. Calculations of relative expression of target mRNAs was performed by a comparative C_T method (Schmittgen and Livak, 2008) using the StepOne software tool (Thermo Fisher, Milan, Italy). Data were reported as relative expression (fold change or log_2-transformed fold changes according to the data ranges) with respect to unfertilized eggs (basal gene expression across larval development) or control samples within each life stage (BPA or E_2 treatments).

2.4. Scanning Electron Microscopy (SEM)

At different times post-fertilization, depending on the experiment, control and treated embryos grown in 96 microwells were fixed in 3% glutaraldehyde in ASW. After fixation, samples from 6 microwells were pooled, placed onto Whatman 22 µm filters, dehydrated in an ascending series of ethanol washes (50% - 80% - 90% - 100%) and air-dried. Then samples were sputter-coated with gold, and observed at 20 kV with a Vega3 - Tescan scanning electron microscope.

2.5. Statistics

Statistical comparisons between unfertilized eggs *vs* trococophora/D-veligers, or controls *vs* BPA/E_2 treated larvae (within each developmental stage) were performed using the non-parametric one-way ANOVA (Kruskal-Wallis test) followed by the Mann-Whitney U-test ($P < 0.05$), after deviations from parametric ANOVA assumptions being verified (Normality: Shapiro-Wilk's test; equal variance: F-test). Data analyses were performed using GraphPad Prism 5 software (GraphPad Inc.).

Additional statistical approaches were utilized for analysis of qPCR data to evaluate:

i) Basal gene expression across larval development. Ordination analysis (carried out by Principal Coordinates, PCO) and clustering of basal transcript expressions across the larval stages were

performed using the PRIMER v6 software (PRIMER-E Ltd, UK), which employed log_2-transformed fold change variations of the target transcripts to calculate similarity matrices based on the Euclidean distance (999 permutations). The similarity matrices were also used to perform a canonical analysis of principal coordinates ordination (CAP) to attribute the specific transcripts responsible for condition differences (i.e. differences between larval stages). A heatmap describing the overall transcriptional profile was generated using the Gene Cluster software v2.0 (Eisen et al., 1998) and the TreeView software for heatmap visualization. Similarity was measured by Euclidean distance.

ii) Effects of BPA or E_2 treatments on target transcript expressions in different larval stages. Relative expression data from BPA or E_2 treatments were submitted to permutation multivariate analysis of variance (PERMANOVA) using the PERMANOVA+ add-on in PRIMER v6 (Anderson et al., 2008). Log-transformed copy number variations of the target transcripts were used to calculate similarity matrices based on the Euclidean distance (999 permutations). Factors considered were "developmental stage" and "treatment" (BPA or E_2). Pseudo-F values in the PERMANOVA main tests were evaluated in terms of significance (Anderson et al., 2008). When the main test revealed statistical differences ($P < 0.05$), PERMANOVA pairwise comparisons were carried out (using the Euclidean distance matrix and 999 permutations). Distance-based redundancy linear modeling (DISTLM) followed by a redundancy analysis (dbRDA) in PRIMER with PERMANOVA+ extension was also performed on BPA or E_2 treatments to examine the relationship between the multivariate dataset (i.e. the suite of transcripts assayed and their expression levels) and the predictor variables (life stage and BPA/E_2 treatment). Numerical metric for life stage progression was indicated by the post fertilization time (0: unfertilized egg; 24 h: trocophora; 48 h: D-veliger). Treatment was indicated by the nominal concentrations of exposure (BPA/E_2: 0 and 10 µg/L). DISTLM used the BEST selection procedure and adjusted R^2 selection criteria.

3. Results

3.1. Transcriptional profiles during early larval development

Transcription of selected genes related to neuroendocrine signaling (estrogen receptors *MeER1*, *MeER2*, serotonin receptor, *5-HTR*), antioxidant defense (superoxide dismutase, *SOD*, catalase, *CAT*), biomineralization (extrapallial protein, *EP*, carbonic anhydrase, *CA*), detoxification response (glutathione-s-transferase, *GST-π*, ABC transporter p-glycoprotein, *ABCB*), stress-response (70kDa heat shock proteins, *HSP70*), apoptosis (*p53*), autophagy (serine/threonine-protein kinase mTOR, *mTor*), immune response (Toll-like receptor, *TLR-i*), was evaluated at both 24 and 48 h pf in embryos grown under normal physiological conditions. Data on relative expression of each transcript across larval development (fold changes with respect to unfertilized eggs) are reported in Fig S2 (Supporting Information Appendix 1**).** To help comparing overall transcript levels among datasets, fold change variations (log_2-transformed) of target transcripts were subjected to a cluster analysis and to an ordination analysis using a Principal Coordinate Ordination (PCO) extraction procedure (Fig 1). Basal transcript expressions showed substantial time-course variations across developmental stages with general and significant up-regulation (Fig 1A; Fig S2, Supporting Information Appendix 1). The highest mRNA levels were reported for *MeERs*, *5-HTR*, *CA* and *p53* (Fig S2, Supporting Information Appendix 1). On the contrary, down-regulation was observed for *GST-π* in both larval stages (Fig1A; Fig S2, Supporting Information Appendix 1). The PCO analysis (Fig 1B) detected two principal coordinates (PCO1 and PCO2) explaining about 97% of total variation (89.8% and 5.8%, respectively). Samples from unfertilized eggs formed one group with evident separation from those belonging to trocophora and D-veliger stages, which also formed distinct clusters. The canonical analysis of principal coordinates ordination (CAP analysis) attributed much of the differences between these latter two stages to *5-HTR, EP, CA, MeER1, p53, CAT, SOD, ABCB* profiles (Fig. 1C).

3.2. Effects of BPA on gene transcription

Fertilized eggs were exposed to BPA 10 µg/L (> EC_{50}, LOEC 100x), as previously described (Fabbri et al., 2014) and gene transcription was evaluated at different times pf (24 h and 48 h) in comparison with untreated samples (Fig 2). Distinct changes in gene transcription at different stages were observed. In trocophorae, all transcripts were significantly up-regulated, with the exception of *HSP70*, which showed down-regulation (Fig. 2A). A maximum 3.5-fold increase was observed for the *EP* transcript. In contrast, in D-veligers 10 out of 13 transcripts were significantly down-regulated, with mRNA levels ranging from -30% to -54% with respect to controls (Fig.2A). Only *SOD* and *CA* were significantly up-regulated, with *CA* showing up to 4.2-fold increase with respect to controls (Fig. 2A). To obtain further details on concentration-dependent effects of BPA on both larvae developmental stages, parallel experiments were performed exposing fertilized eggs to BPA 1 µg/L (< EC_{50}, LOEC 10x) (Fabbri et al., 2014). The results, reported in Fig S3 (Supporting Information Appendix 1), showed a small though significant up-regulation of most transcripts (from + 35 % to + 55 %, depending on the transcript and the developmental stage). Significant down-regulation was observed for *SOD* and *EP* in trocophorae, and for *SOD* and *CA* in D-veligers (from -30 % to - 60 %).

Distance-based linear model (DISTLM) analysis revealed that expression profiles were strongly dependent on embryo development (time from pf) that explained about 90 % total variation. Nevertheless, BPA treatment accounted for 6% of total variation, which mostly explained the observed changes in transcript expressions at 48 h pf (Fig. 2B). Results from PERMANOVA and permutation t-test analyses demonstrated that the effects of BPA were statistically significant in both larval stages ($P < 0.05$; Table S3, Supporting Information Appendix 2). PERMANOVA analysis also showed a significant interaction between the two factors (developmental stage and BPA treatment; $P < 0.05$; Table S3, Supporting Information Appendix 2).

3.3. SEM analysis

Larval development was observed by Scanning electron microscopy (SEM) first in larvae grown under physiological conditions, and representative images are reported in **Fig 3**a-f. Fig. 3a and Fig. 3b show a trocophora at 24 h pf, and the detail of the ciliated epithelium, respectively. During the trocophora stage, the onset of shell mineralization occurs (generally at 20 h pf). The process is initiated by a specialized group of ectodermal cells, the shell field, that are responsible for production of the first outer organic shell layer, the periostracum (Weiss et al., 2002). The saddle-shaped shell field is well visible in Fig. 3c. From about 32 h pf, first shelled embryos could be observed (Fig. 3d); at this stage, most shells were extremely fragile, and damaged by the sample preparation procedure for SEM observations. Micrometric spherules of amorphous calcium carbonate (ACC) could be observed both at the surface and margins of the valvae (Fig. 3e) typical of initial shell formation (Cartwright et al., 2012; Weiss et al., 2002). Finally, at 48 h pf the D-Veliger stage is reached (Fig. 3f), with shells showing straight hinge, symmetric valvae, and uniform surface, indicating that formation of the calcified shell occurred simultaneously in the whole shell field. In samples exposed to BPA (10 µg/L), no appreciable morphological changes were observed at 24 h pf (Trocophora) (not shown). On the other hand, clear changes were observed in the morphology of D-larvae at 48 h pf (Fig. 3g-i). Common shell alterations were rough surface, thinner valvae, convex hinge, asymmetry and/or gap between valvae; moreover, many samples showed the externalized velum (ciliated swimming and feeding organ), a feature typical of the pre-veliger stage (Fig. 3i and insert to Fig. 3i).

3.4. Effects of 17β-estradiol on mussel embryo development and gene transcription

The effects of BPA were compared with those of the natural estrogen 17β-estradiol (E_2). Fig S1a (Supporting Information Appendix 1) shows results from the 48-h embryotoxicity assay performed utilizing E_2 in the same concentration range as that previously utilized for BPA (Fabbri et al., 2014).

E_2 induced a dose-dependent decrease in normal larval development that was significant from 0.1 µg/L, with a calculated EC_{50} value of 6.904 µg/L (95% confidence interval of 3.284 - 14.511 µg/L). Optical microscopy observations indicated that, as previously observed with BPA (Fabbri et al., 2014), E_2 mainly induced a delay in development, with a prevalent increase in the percentage of trocophorae/not fully developed D-veligers, and a variable percentage of malformed embryos at different concentrations. At the highest concentration tested (1,000 µg/L) more than 90% embryos were at the pre-veliger stage or showed gross shell malformations (not shown). At 10 µg/L E_2, SEM observations showed that most common shell alterations induced by E_2 were hinge malformations, asymmetric and thinned valvae and rough surface (Fig. S1b-d, Supporting Information Appendix 1).

The effects of E_2 (10 µg/L) on gene transcription are illustrated in Fig. 4. The results show that most transcripts were significantly down-regulated both at 24 h and 48 h pf, to a different extent depending on the type of gene and developmental stage. In particular, at 24 h pf E_2 induced a general decrease in gene transcription, except for *5-HTR*, *ABCB*, *EP* and *CA*. Dramatic down-regulation (≤ 70% with respect to controls) was observed for *MeER1*, *CAT*, *p53*, and *mTor*, and almost complete inhibition resulted for *TLR-i* transcription. At 48 h pf, a significant down-regulation was observed for all transcripts except for *HSP70* and *mTor*, whose mRNA levels were unchanged, and for *ABCB*, that was up-regulated.

DISTLM analysis revealed that E_2 treatment accounted for 7.5 % of total variation, and affected both trocophora and D-veliger stages (Fig. 4B). Results from PERMANOVA and permutation t-test analyses demonstrated that the effects of E_2 were significant in both larvae stages ($P < 0.05$; Table S4, Supporting Information Appendix 2). PERMANOVA analysis also showed a significant interaction between the two factors (developmental stage and E_2 treatment; $P < 0.05$; Table S4, Supporting Information Appendix 2).

4. Discussion

We have previously shown that BPA significantly affected 48 h embryo development in *M. galloprovincialis*, with a LOEC and EC_{50} of 0.1 µg/L and 3.68 µg/L, respectively (Fabbri et al., 2014). Since these values fall within the concentration range of BPA found in coastal environments (Flint et al., 2012), these data underlined the sensitivity of mussel embryos towards exposure to environmental concentrations of BPA, and supported the hypothesis that this compound could act as an EDC in marine bivalves. The results here obtained demonstrated that BPA affects gene transcriptions in early mussel embryos, with effects depending on the BPA concentration, the target transcript and the developmental stage. These data represent the first indication of molecular mechanisms for BPA impacts on developmental stages in a marine bivalve.

Basal transcription of 13 genes involved in different biological processes was first evaluated in larvae grown under physiological conditions at both 24 and 48 h pf, and relative expression was quantified with respect to unfertilized eggs. Genes were selected on the basis of their recognized physiological functions in *Mytilus spp*. and/or of their sensitivity to different environmental chemicals, including BPA (i.e. neuroendocrine signaling, antioxidant defenses, biomineralization, detoxification processes, stress response, apoptosis, autophagy, immunity). These pathways are reported to provide potent defenses in embryos against common stressors, including changes in temperature, hypoxia, and pollutants, to permit optimal development despite relevant environmental challenges (Hamdoun and Epel, 2007). To the best of our knowledge, the results represent the first data on basal mRNA expressions in early development of *M. galloprovincialis*. All transcripts were significantly up-regulated in both trocophora and D-veliger stages with respect to unfertilized eggs, except for *GST-π*, which resulted significantly down-regulated in both trocophore and D-veligers compared to eggs, thus suggesting that early embryos rely on maternal GST, if any, for Phase II biotransformation pathways. PCO and cluster analyses disclosed a further distinct clustering between trocophore and D-veliger samples, consistent with the progressive differentiation of

different processes. Main contribution to the observed data ordination was provided by the increased expressions of *5-HTR, EP, CA, MeER1, p53, CAT, SOD, ABCB* in D-veligers compared to trocophorae, as suggested by CAP analysis. If on one hand the results obtained for *5-HTR* confirm the early and progressive development of serotonergic transmission in *Mytilus spp.* reported by previous immuno-histochemical investigations (Voronezhskaya et al., 2008), on the other hand increased expressions of *SOD*, and to a lesser extent, of other transcripts with cytoprotective functions (i.e. *ABCB, CAT, p53*), indicate a reinforcement of defenses in the first D-shelled embryo stage.

Although a number of genes involved in biomineralization are being described in different bivalve species (Bassim et al., 2015; Vendrami et al., 2016), those involved in the initial formation of a first calcified shell (prodissoconch I), a key step in early development, have not been identified yet. Carbonic anhydrase (CA) is known to regulate matrix mineralization by generating an acidic environment (Clark et al., 2010). *Mytilus* EP (Extrapallial Protein), an acidic calcium binding protein, regulates the production of different polymorphs of calcium carbonate (Yin et al., 2009). Both *EP* and *CA* showed the highest expression in D-veligers, supporting the results from SEM analyses, showing that the formation of a complete normal shell occurred only at 48 h pf, and confirming the key physiological role of both transcripts in initial biomineralization (Medakovic, 2000; Yin et al., 2009, 2005).

Exposure of fertilized eggs to 1 µg/L BPA resulted in small but significant up-regulation of most transcripts. However, down-regulation was observed for *SOD*, and for *EP* and *CA* in trocophorae and D-veligers, respectively. More evident changes were observed at 10 µg/L. In these conditions, distinct trends in gene transcription were observed at different post-fertilization periods, with a general up-regulation in the trocophorae and down-regulation in D-veligers for all genes tested, except for *CA* and *SOD*, whose mRNA levels were further increased at 48 h pf. Interestingly, transcription of both *EP* and *CA* was strongly affected by BPA exposure at both developmental stages.

The effects of BPA on mussel embryos were compared with those of the natural estrogen 17β-estradiol (E_2). E_2 significantly affected larval development in the 48 h embryotoxicity tests (EC_{50} = 6.904 µg/L), with effects similar to those of BPA (Fabbri et al., 2014). Therefore, gene transcription was evaluated after exposure to 10 µg/L E_2. At 24 h pf, E_2 induced a general decrease in gene transcription, in particular of genes involved in apoptosis, autophagy and immunity, showing a distinct effect with respect to BPA. At 48 h pf, E_2, like BPA, induced down-regulation of most genes, including those involved in biomineralization. An exception to this trend was represented by *ABCB*, whose transcription was unaffected in trocophorae, and significantly increased in D-veligers.

At the concentration utilized for gene expression studies (10 µg/L) the main morphological effects induced by BPA (Fabbri et al., 2014) and E_2 (this work) were represented by the presence of immature, not fully developed D-shell larvae at 48 h pf. Similar results were obtained when BPA or E_2 were added at 24 h pf, instead of at 30 min post oocyte fertilization (not shown), indicating that the transition from the trocophora stage to the first D-shelled larva represents a critical developmental step affected by both compounds. Accordingly, a common effect of BPA and E_2 observed at 48 h pf was the general down-regulation of gene expression and alterations in shell formation, as indicated by SEM analysis.

Overall, the results demonstrate that both the estrogenic chemical BPA and the natural estrogen E_2 affect *Mytilus* early development through dysregulation of gene transcription. In the marine gastropod *Haliotis diversicolor*, BPA affected different stages of larval development, with an EC_{50} of 1.02 µg/L at 96 h (completion of metamorphosis) (Liu et al., 2011). Furthermore, proteomic analysis indicated that both chemicals interfered with different physiological pathways, including energy and substance metabolism, cell signaling, formation of cytoskeleton and cilium, immune and stress responses at the same time, leading to the failure of metamorphosis (Liu et al., 2011).

The results of the present work underline that in *Mytilus MeERs* and *5-HTR* represent significant targets for both BPA and E_2 during early embryo development. Similar results on *MeER* expression were observed in the hepatopancreas of adult mussels exposed to BPA (Canesi et al., 2011, 2007, 2006). Taken together the results support a common neuroendocrine-related effect of these compounds in mussels. However, from an accurate analysis on the literature on the effects of steroids and EDCs Scott (2013) concluded that there is no indisputable bioassay evidence that vertebrate sex steroids (or their agonist/antagonists) have endocrine or reproductive roles in mollusks. Estrogen receptors (ERs) generally show constitutive transcriptional expressions in mollusks, and are not activated by either natural estrogens or estrogenic compounds, this rising a debate on their physiological function (Bridgham et al., 2014). In this respect, molluscan ERs seem to resemble mammalian orphan estrogen-related receptors (ERRs), that play multiple and overlapping roles in cell growth, differentiation and metabolism (Huss et al., 2015). However, cloning and phylogenetic analysis of estrogen–like receptors in *Mytilus edulis* and *M. galloprovincialis* revealed the Me/MgER2 is an ER, whereas the Me/MgER1 is an ERR (Nagasawa et al., 2015). Our results show that in physiological conditions both transcripts encoding the MeER1 and MeER2 isoforms were among the most up-regulated across different developmental stages, while these receptors were down-regulated by exposure to both BPA and E_2 in D-veligers. These data indicate that *Mytilus* ERs may play a role in early development and represent significant targets for estrogenic chemicals. Similar results were obtained for the 5-*HTR* transcript, encoding a putative type 1 serotonin receptor (Cubero-Leon et al., 2010; Franzellitti and Fabbri, 2013), indicating that BPA and E_2 have a significant impact on early development of the serotoninergic signaling in mussels (Kreiling et al., 2001; Voronezhskaya et al., 2008).

In addition, the results underline how estrogenic compounds significantly affected transcription of *EP* and *CA* involved in mussel biomineralization, with down-regulation of both transcripts observed at 48 h pf. These data were supported by SEM observations, which clearly showed similar irregularities in shell formation in both BPA and E_2 -treated embryos at 48 h pf.

These effects are in line with the effects of estrogenic chemicals on bone metabolism observed in vertebrate systems (Agas et al., 2013). Overall, these represent the first data on the effects of EDCs on early biomineralization processes in marine invertebrates. The effects of BPA on the shell structure are under investigation. Calcifying larvae of marine species are particularly vulnerable to abiotic stressors (Przeslawski et al., 2015). Interestingly, the shell malformations induced by BPA and E_2 were similar to those observed in *M. galloprovincialis* embryos by inhibiting of the synthesis of chitin, a key biological component that forms the framework for other macromolecules that guide initial shell deposition (Schönitzer and Weiss, 2007; Weiss and Schönitzer, 2006), as well as by exposure to high pCO_2 or low pH (Kadar et al., 2010; Kurihara et al., 2008). In this light, chitin synthesis may represent an important target for the effects of both chemical pollutants and altered environmental parameters in the developing embryo. Studies on the effects of individual chemicals that represent potential EDCs on early life stages of sensitive marine species may contribute to the development of future multiple stressor studies in a global change scenario.

5. Conclusions

The results of the present study show that environmental levels of estrogenic compounds may adversely affect physiological processes involved in early embryo development of marine mussels through dysregulation of transcription for genes involved in different key regulatory pathways

In particular, the results address the transition from the trocophora stage to the first D-shelled larva as a very sensitive target for estrogenic compounds. The results indicate that, although physiological processes in early embryos are well buffered for environmental challenges (Hamdoun and Epel, 2007), anthropogenic stressors can overwhelm this intrinsic robustness and compromise developmental processes.

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Acknowledgement

The research has been funded by the Italian Ministry of University and Research (RFO2014) to E.F. and to L.C.

581 **Figure legends**

582 **Fig. 1 – Transcriptional profiles of selected gene products during early larval development in**
583 **mussels. (A)** Treatment ranking according to the qPCR data describing transcript levels at different
584 developmental stages. Hierarchical clustering was performed using fold change variations (log_2-
585 transformed) of target transcripts at each developmental stage (In-detail data are reported in Fig. S2,
586 Supporting Information Appendix 1). Colors represent relative expression levels with respect to
587 unfertilized eggs. **(B)** Principal coordinates ordination (PCO) bi-plot of mRNA expression levels
588 with super-imposed cluster analysis by condition (Euclidean distance matrix; 999 permutations).
589 **(C)** Canonical analysis of principal coordinates ordination (CAP analysis) illustrating transcripts
590 whose expression patterns are likely to discriminate the differences between life stages. Transcripts
591 to be displayed in the CAP bi-plot were filtered according to their Spearman correlation's
592 coefficients ($r > 0.6$ with at least CAP1 or CAP2).

593

594 **Fig. 2 - Effects of BPA (10 µg/L) on gene transcription in *Mytilus* embryos at 24 h and 48 h**
595 **post fertilization times. (A)** Expression levels of target transcripts evaluated by qPCR. Data are
596 reported as mean ± SD of the relative expression with respect to untreated samples within each life
597 stage (N = 4). *P < 0.05, **P < 0.01 according to the Mann-Whitney U test. **(B)** DISTLM modeling
598 with distance-based redundancy analysis (dbRDA) to explore the amount of the variation in gene
599 transcription to be attributed to BPA treatment (Euclidean Distance resemblance matrix, 999
600 permutations).

601

602 **Fig. 3 - Scanning electron microscopy (SEM) of *Mytilus* embryos.** a-f: representative images of
603 different stages of larvae grown under physiological conditions: a) trocophora at 24 h pf, and b)
604 detail of the ciliated epithelium; c) trocophora with super-imposed margins of the saddle-shaped
605 shell field; d) early D-veliger at 36 h pf, showing the presence of amorphous calcium carbonate
606 (ACC) spherules (e); f) normal D-veliger at 48 h pf, with straight hinge, symmetric valvae and

607 uniform surface. g-i: representative images of the effects of BPA (10 μg/L) of shell morphology in
608 D-veligers at 48 h pf, showing asymmetric valvae and rough surface (g), convex hinge and thinner
609 valvae (h), externalized velum (i).
610
611 **Fig. 4 – Effects of E_2 (10 μg/L) on gene transcription in *Mytilus* embryos at 24 h and 48 h post**
612 **fertilization times. (A)** Expression levels of target transcripts evaluated by qPCR. Data are reported
613 as the mean ± SD of the relative expression with respect to untreated samples within each life stage
614 (N = 4). *P < 0.05, **P < 0.01 according to the Mann-Whitney U test. **(B)** DISTLM modeling with
615 distance-based redundancy analysis (dbRDA) to explore the amount of the variation in gene
616 transcription is attributable to E_2 treatment **(**Euclidean Distance resemblance matrix, 999
617 permutations).
618

Figure 1

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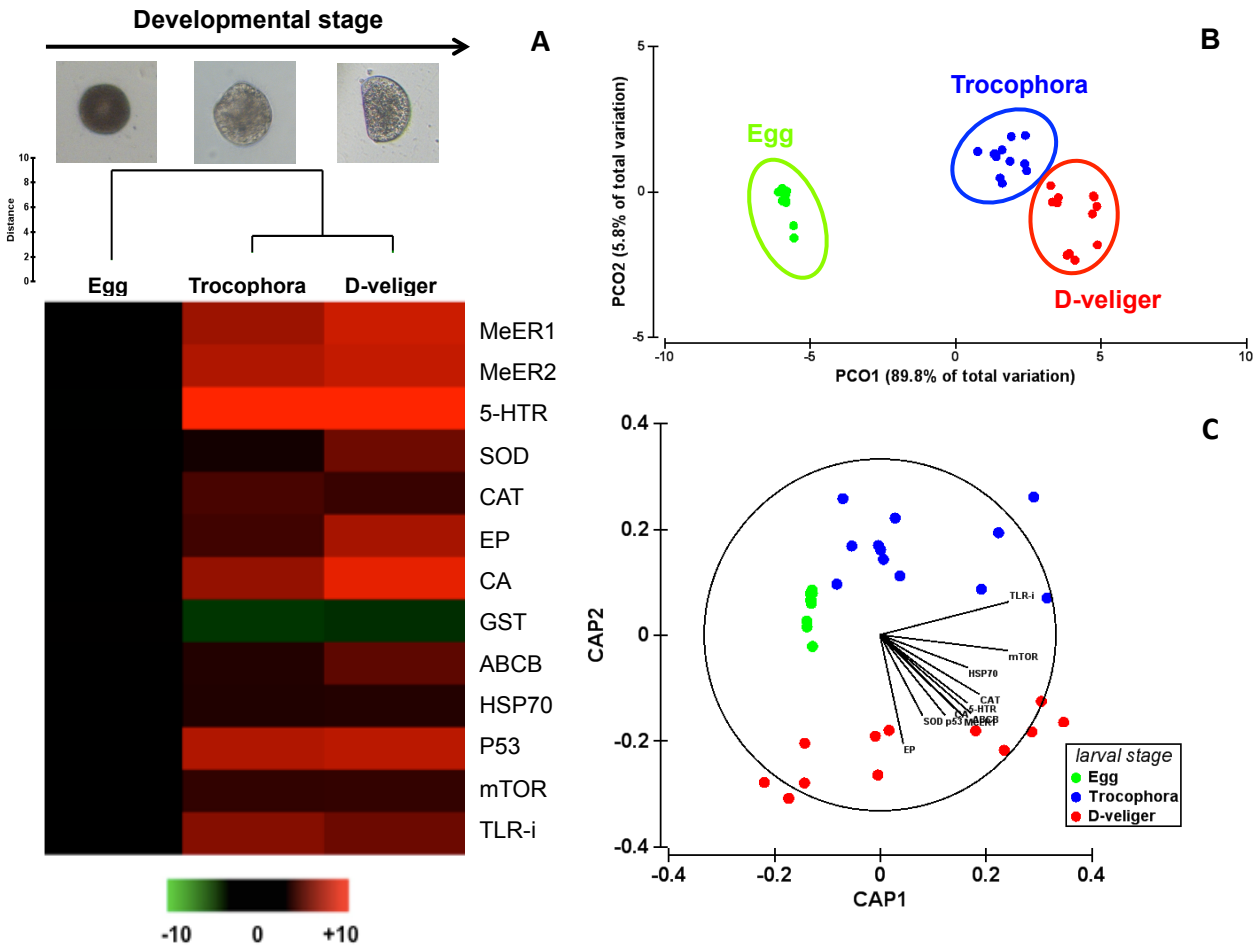

Figure 2

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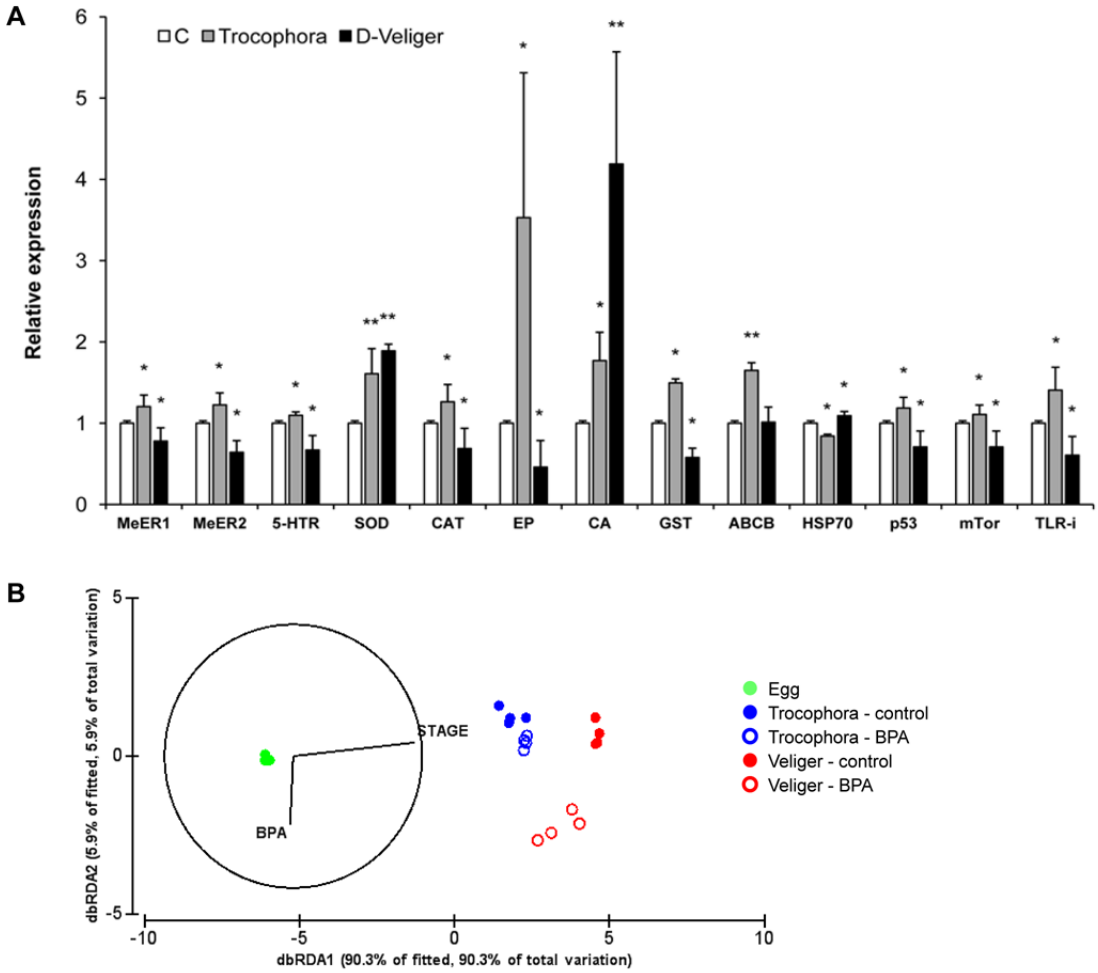

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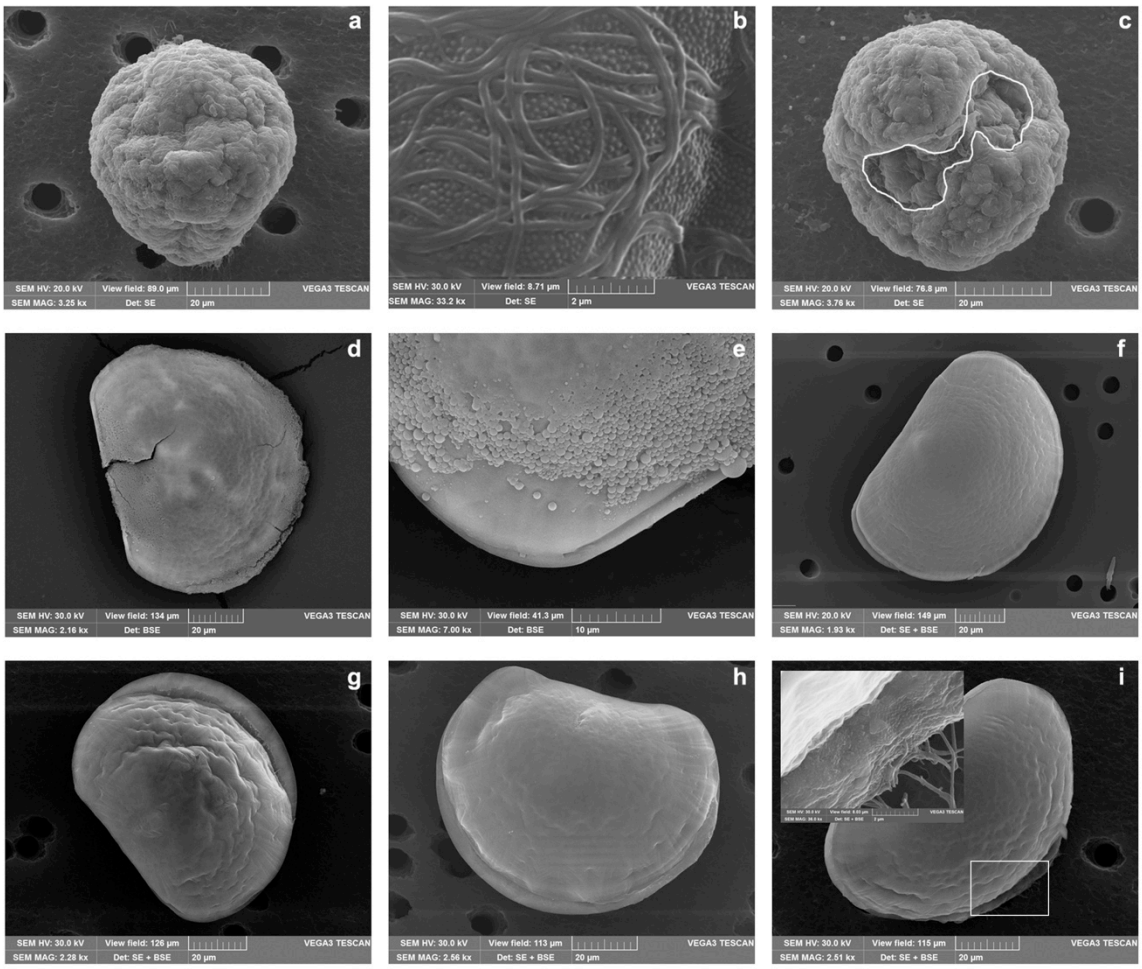

Figure 4

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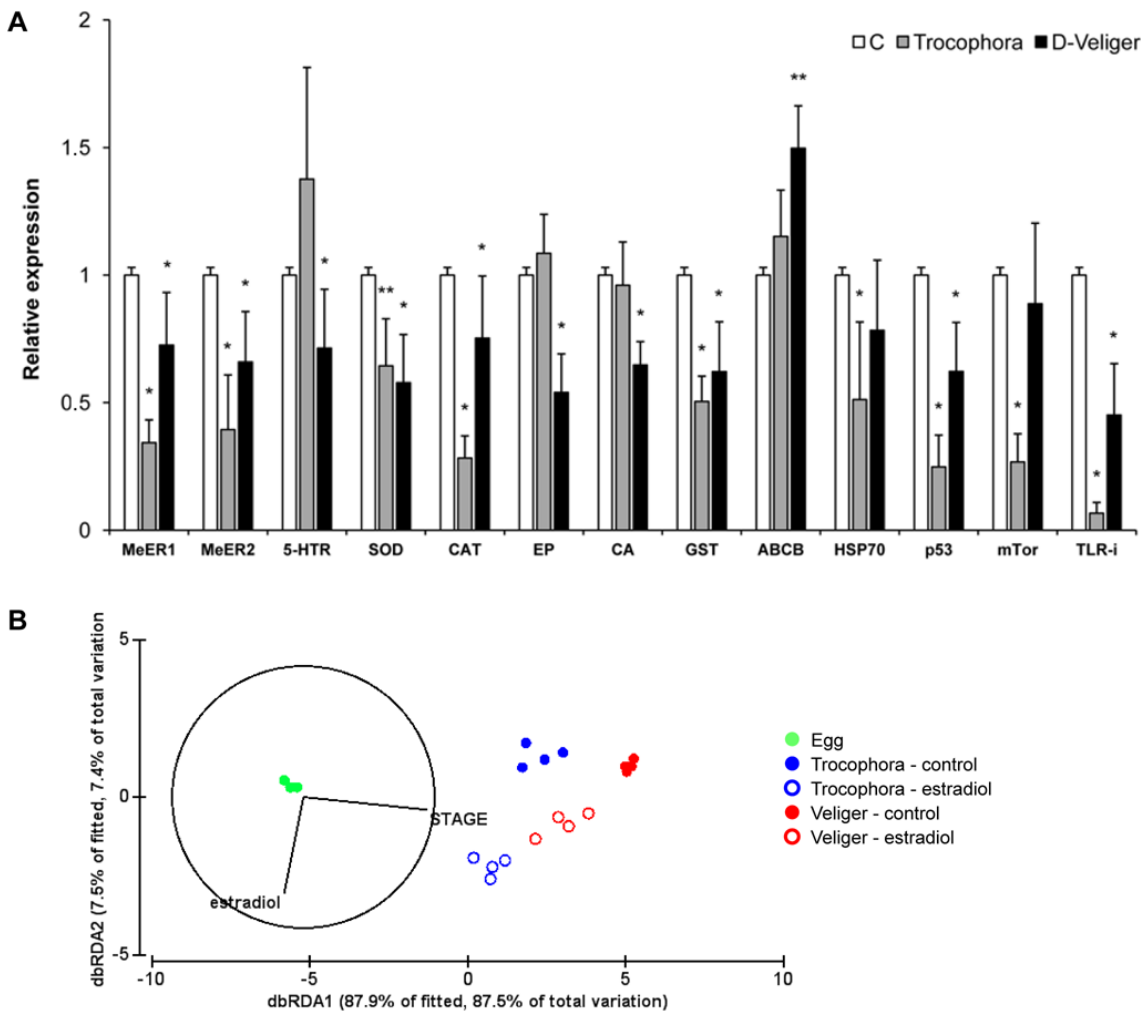

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