Phenotypical and molecular changes induced by carbamazepine and propranolol on larval stages of *Mytilus galloprovincialis*

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Abstract

The possible impact of carbamazepine (CBZ) and propranolol (PROP), two widespread pharmaceuticals in the aquatic environment, were investigated on morphology and gene transcription of early larvae of *Mytilus galloprovincialis*. Pharmaceuticals were first tested in a wide concentration range (from 0.01 to 1000 µg/L) through the 48-hpf embryotoxicity assay. The results showed that both compounds significantly affected embryo development from environmental concentrations. Although similar EC_{50} were obtained, (≅ 1 µg/L) CBZ induced a progressive increase in embryo malformations, whereas PROP apparently showed greater impacts in terms of arrested development and embryo mortality at higher concentrations (>10 µg/L). Transcriptional analyses of 17 genes involved in different physiological functions in mussels and/or in their response to environmental contaminants, were performed at 24 and 48 h pf at two selected concentrations of CBZ and PROP (0.01 and 1 µg/L). Both compounds induced down-regulation of shell-specific and neuroendocrine related transcripts, while distinct effects were observed on antioxidant, lysosomal, and immune-related transcripts, also depending on the larval stage investigated. The results demonstrate that CBZ and PROP can affect development and gene transcription in mussel early larvae at environmental concentrations.

Keywords: mussel embryo development; pharmaceutical; carbamazepine; propranolol; embryotoxicity; gene transcription.

1 Introduction

Pharmaceuticals are biologically active molecules used in human and veterinary medicine, which are partially excreted as parent compounds or active metabolites and enter wastewater treatment plants (WWTP). Given their increased consumption and low-retention in WWTP, as well as the development of more powerful analytical techniques, pharmaceuticals have been widely detected in the marine environment, mainly in coastal areas (Fabbri and Franzellitti, 2016). Although half-life of these compounds is relatively short, the marine biota is exposed to a continuous input, raising concern on the potential biological impacts (Fent et al., 2006). Research efforts over the last decade also pointed out that pharmaceuticals may affect marine fauna at environmental concentrations when specific responses related to the therapeutic targets or the modes of action known in humans are investigated (Brooks, 2018; Fabbri and Franzellitti, 2016).

Amongst the pharmaceuticals routinely detected in coastal environments, carbamazepine (CBZ) and propranolol (PROP) attracted special attention when it was proved that they induced sublethal effects in marine invertebrates, including mussels (Fabbri and Franzellitti, 2016), and a link between these effects and the therapeutic modes of action in humans was revealed (Franzellitti et al., 2013, 2011; Martin-Diaz et al., 2009).

Carbamazepine (CBZ) is an antiepileptic drug which poorly retained during wastewater treatments (below 10%), thus it is found in all aquatic compartments, including drinking waters, at the low µg/L concentration range (Bahlmann et al., 2012; Calisto et al., 2011; Metcalfe et al., 2014). In seawater, CBZ was documented in harbours (up to 321 ng/L), coastal waters (up to 1400 ng/L) and sediments (up to 89 ng/g), and in river estuaries (up to 178 ng/L) (Fabbri and Franzellitti, 2016). Propranolol (PROP) is administered to counteract hypertension and other cardiovascular pathologies. It is found in surface waters (ng/L range), in transitional waters (up to 142 ng/L), coastal waters (up to 6329 ng/L) and sediments (up to 0.9 ng/g) (Ashton et al., 2004; Fabbri and Franzellitti, 2016; Wille et al., 2011). Data on occurrence of both pharmaceuticals in seawater are summarized in Table S1, Supplemental material.

Recent studies indicated that embryos/larvae from different species can be utilized as promising experimental models to establish the sensitivity of marine species towards pharmaceuticals (Balbi et al., 2016; Di Poi et al., 2017, 2014; Estévez-Calvar et al., 2017; Fabbri et al., 2014; Franzellitti et al., 2017; Minguez et al., 2014). Compounds with widely different therapeutic effects and chemical reactivity have been shown to affect early larval development in the Mediterranean mussel, *Mytilus galloprovincialis* (Balbi et al., 2016; Estévez-Calvar et al., 2017; Fabbri et al., 2014). In particular, 17β-estradiol and diclofenac altered the transcription of genes involved in shell biogenesis and serotonin signaling (Balbi et al., 2018, 2016).

This study is addressed to the potential effects of CBZ and PROP on early larval development of *M. galloprovincialis.* Phenotypical outcomes of the pharmaceuticals were investigated in a wide concentration range (0.01 – 1000 µg/L) through the standardized 48-hpf embryotoxicity assay (Fabbri et al., 2014) which evaluates the impairment of normal D-larvae development. Moreover, transcriptional analyses of 17 gene products at different developmental stages (24 and 48 h post fertilization, pf) (Balbi et al., 2017a, 2016) were carried out after exposure to two selected CBZ or PROP concentrations (0.01 and 1 µg/L). As both compounds may affect physiological pathways in bivalves (Fabbri and Capuzzo, 2010; Franzellitti et al., 2017; Franzellitti and Fabbri, 2013), this latter experimental approach aimed to disclose early signs of CBZ/PROP interactions with developmental processes of mussels.

2 Methods

2.1 Chemicals

Carbamazepine (CBZ), and (±)-propranolol hydrochloride (PROP) were at the molecular biology grade (> 99 % purity) and were purchased from Sigma Aldrich (Milan, Italy). The DirectZol kit was from Zymo Research (Freiburg, Germany). The Qubit RNA assay and the Qubit protein assay were from Thermo Fisher (Milan, Italy). The iScript supermix and iTaq Universal master mix with ROX were from Biorad Laboratories (Milan, Italy). The Tri-Reagent, dimethylsulfoxide (DMSO), and any other reagent was from Sigma Aldrich (Milan, Italy).

2.2 Mussels and gamete collection

Sexually mature mussels (*M. galloprovincialis* Lam.) were obtained from a certified mussel farm (Cooperativa Copr.al.mo, Cesenatico, Italy) and acclimatized in static tanks containing aerated artificial 35-psu seawater (ASTM, 2004) at 16 ± 1 °C (1 L/animal). Procedures for gamete collection, oocyte fertilization and larvae handling were according to Fabbri et al. (2014). Fertilizations were performed with an egg:sperm ratio 1:10 in 96-well (embryotoxicity assay; 200 µL final volume) or 6-well (transcriptional analyses; 10 mL final volume) cell culture plates. After 30 min, fertilization success (n. fertilized eggs / n. total eggs × 100) was verified by microscopical observation (>85%) and carbamazepine (CBZ) or propranolol (PROP) were added to fertilized eggs in the proper wells from concentrated stock solutions prepared in DMSO (CBZ) or sterile milli-Q water (PROP), considering solubility limits of the compounds in the selected medium, and diluted in ASW to reach the final nominal concentrations tested. Concentrations of the stock solutions were verified by LC-MS/MS (Castiglioni et al., 2005). They were within a 8% discrepancy compared to the nominal values. DMSO final concentration never exceeded 0.1% v/v and it did not significantly affect the biological endpoints analysed (data not shown). Control (ASW) and solvent (ASW+DMSO; 0.1% v/v) exposed samples were run in parallel. After pharmaceutical addition, samples were maintained at 16 ± 1 °C for 48 h with a 16 h:8 h light:dark photoperiod according to ASTM (2004).

2.3 Embryotoxicity assay

The 48-h embryotoxicity assay (ASTM, 2004) was performed according to Fabbri et al. (2014) and using a wide range of nominal concentrations from 0.01 to 1000 µg/L. Four independent experiments were performed, each consisting of 6 technical replicates (N = 4). At the end of the incubation time, samples were fixed with buffered formalin (4%). For each well, all larvae were examined by optical and/or phase contrast microscopy using an inverted microscope (Olympus IX53, Olympus, Milan, Italy) at 40X magnification. Observations were carried out by an operator blind to the experimental conditions. Classification of larvae morphotypes was performed according to Balbi et al. (2017a) and Fabbri et al. (2014), with the recorded endpoint being the percentage of normal D-shape larvae in each well over the total. The acceptability of assay results was achieved with controls showing a > 75% normal D-shape larvae (ASTM, 2004).

According to the ASTM guidelines (ASTM, 2004), embryo viability at 48 h pf was assessed as the percentage of live larvae with misshapen or otherwise malformed shells and completely/normally developed shells. Empty shells, larvae with incompletely developed shells or larvae retained at the trocophora stage were considered non-viable because retarded development is likely to reduce survival in the natural environment (ASTM, 2004).

2.4 RNA extraction and qPCR analyses

Treatments for mRNA expression analyses were carried out at 0.01 µg/L and 1 µg/L. Four independent experiments were performed, each consisting of 3 technical replicates (N = 4). Total RNA extraction from embryos at 24 h and 48 h pf was performed according to Balbi et al. (2016). RNA concentration and quality were verified using the Qubit RNA assay through the Qubit 2.0 system (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 using the iScript supermix following manufacturer's instructions. Target transcripts are listed in Table S2, Supplemental material**.** Primer pairs and protocols employed for quantitative real time PCR (qPCR) assays are reported in previous studies (Balbi et al., 2017a, 2016; Capolupo et al., 2018). A preliminary stability analysis performed according to Balbi et al. (2016) selected the pair *helicase/elongation factor 1α* as the reference gene products for qPCR data normalization. A comparative C_T method was used for fold-change calculations (Schmittgen and Livak, 2008) through the StepOne software tool (Thermo Fisher, Milan, Italy). Data are reported as mean ± SEM of log_2-transformed fold changes with respect to controls within each post-fertilization time.

Expressions of transcripts related to antioxidant responses, immune responses and lysosomal functions were also evaluated in unfertilized eggs as well as in 24- and 48-h pf embryos grown under normal physiological conditions, to account for their baseline regulation across early embryo development (Fig. S1, Supplemental material)

2.5 Statistics

Data from embryotoxicity and embryo viability were analysed using the non-parametric 1-way ANOVA (Kruskal-Wallis test) and the Mann-Whitney U-test for pairwise comparisons by the GraphPad Prism 6 software (GraphPad Inc.). Non-accomplishment of parametric ANOVA assumptions was verified (Normality: Shapiro-Wilk's test; equal variance: F-test). EC50 values for the embryotoxicity were calculated using the Hill equation for non-linear regression through the Excel™ macro REGTOX (Vindimian, 2012).

qPCR data were analysed with the REST software (Pfaffl et al., 2002) that employs randomisation tests with pairwise reallocations to assess the significance differently expressed transcripts between each treatment-exposed group and the controls. Further comparisons between pair of treatments were performed using the Mann-Whitney U-test. In all approaches, $p < 0.05$ was set as the threshold level of statistical significance.

Data from qPCR investigations were further submitted to permutation multivariate analysis of variance (PERMANOVA) and distance-based redundancy linear modeling (DISTLM) as detailed by (Balbi et al., 2016). Factors considered were "developmental stage" and "treatment" (CBZ or PROP). DISTLM with a test of marginality was also performed to account for the contributions of the functional groups of transcripts listed in Table S2 in explaining the total variance observed in the CBZ/PROP dataset (Rasika Wathsala et al., 2018).

3 **Results**

3.1 Effects of CBZ and PROP on embryo development

Fertilized eggs were exposed to different concentrations (from 0.01 µg/L to 1000 µg/L) of PROP or CBZ in 96-microwell plates, and the percentage of normal D-larvae was evaluated 48 hpf (Fig. 1). Both compounds induced a significant decrease in percentage of normally developed embryos from 0.1 µg/L (CBZ) and 0.01 µg/L (PROP) ($p < 0.05$), with values decreasing thereafter (Fig. 1A, B).

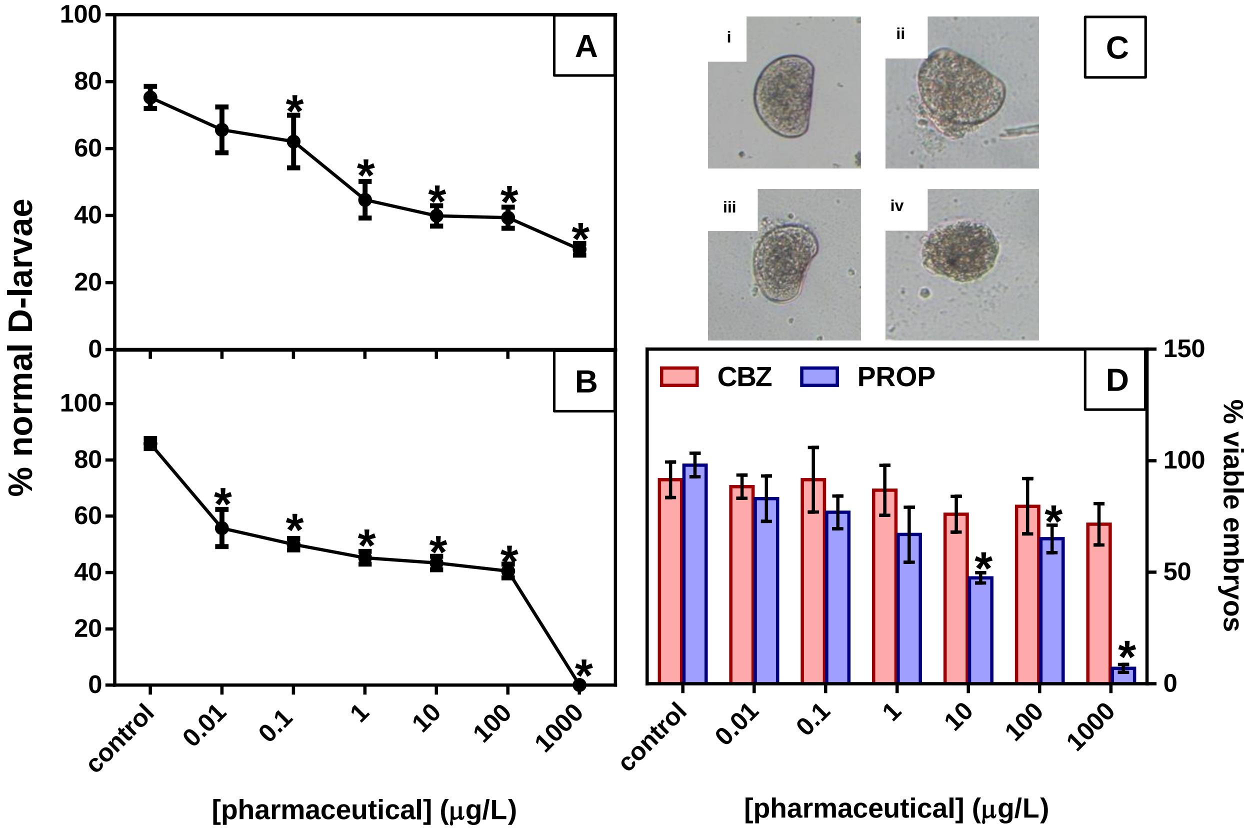

Fig. 1. Effects of different concentrations of CBZ or PROP (0.01 – 1000 μg/L) on *M. galloprovincialis* embryo development. (A, CBZ; B, PROP) Results of the 48-h embryotoxicity assay. (C) Representative light microscope images (32X magnification) showing the main types of abnormalities observed: (i) normal 48 h pf D-shaped veliger; (ii) D-shaped 48 h pf veliger with protruding mantle; (iii) D-shaped veliger with shell hinge malformations; (iv) embryo arrested at the trochophora stage (characteristic of PROP-treated samples). (D) Percentage of embryo viability at 48 h pf. All data are reported as mean ± SEM of 4 experiments carried out in 96-multiwell plates (6 replicate wells for each sample) (N = 4). *p < 0.05 *vs* control (Mann-Whitney U-test). ***Colored figure is intended only for the online and PDF version.***

Although similar EC50 values were obtained for CBZ and PROP (0.82 ± 0.36 and 1.34 ± 0.43 μg/L, respectively), CBZ mainly induced the development of malformed D-veligers at all the concentrations tested, whereas exposure to PROP resulted in a variable proportion of malformed/immature embryos at different concentrations, with all embryos retained at the trocophora stage at the highest concentration tested (1000 μg/L) (Fig. 1C). Furthermore, while no significant mortality due to CBZ was observed, the average proportion of viable embryos at 48 h pf was significantly reduced at 10-1000 µg/L PROP (Fig. 1D).

3.2 Effects of CBZ and PROP on gene transcription

Gene transcription was evaluated in samples exposed to CBZ or PROP at 0.01 and 1 µg/L. The former concentration encompasses the environmental levels of both compounds (10 ng/L, Table S1); the latter is close similar to the EC50 values obtained for both compounds in the embryotoxicity assay, and also represents the highest range of concentrations measured in coastal environments (1000 ng/L, Table S1).

Selected transcripts related to different physiological functions were evaluated: *Antioxidant response*: Catalase, *CAT*; Glutathione transferase-pi, *GST*; Metallotionein 10 and 20, *MT10* and *MT20*; Superoxide dismutase, *SOD*. *Immune response:* Lysozyme, *LYS*; Mytlin B, *MytlB*; Myticin C; *MytcC*. *Lysosomal response* Catheplsin L, *CTSL*; ß-Glucuronidase, *GUSB*; Hexosaminidase, *HEX*. *Putative Neuroendocrine signaling:* Type 1 estrogen receptor, *MeER1*; Type 2 estrogen receptor, *MeER2*; Type 1 serotonin (5-HT) receptor, *5-HT1*. *Shell biogenesis*: Extrapallial protein, *EP*; Carbonic anhydrase, *CA*. (Balbi et al., 2018, 2017a, 2016) (Details are provided in Table S2).

To help comparing overall transcriptional responses among transcripts as well as datasets, fold change variations (log2-transformed) were subjected to similarity analysis which generated heatmaps describing the overall transcriptional responses to CBZ and PROP (Fig. 2). Details of results and statistics are reported in Fig. S2.

Exposure to CBZ for 24h significantly up-regulated a series of transcripts (CA, CTSL, EP, LYS, HEX, MeER2 Mt10, Mt20, MytB, 5HT1 and SOD) at 0.01 µg/L. The effects of 0.01 µg/L CBZ on antioxidant related transcripts were slightly reduced at 48 h pf, except for *GST* which appeared significantly downregulated.

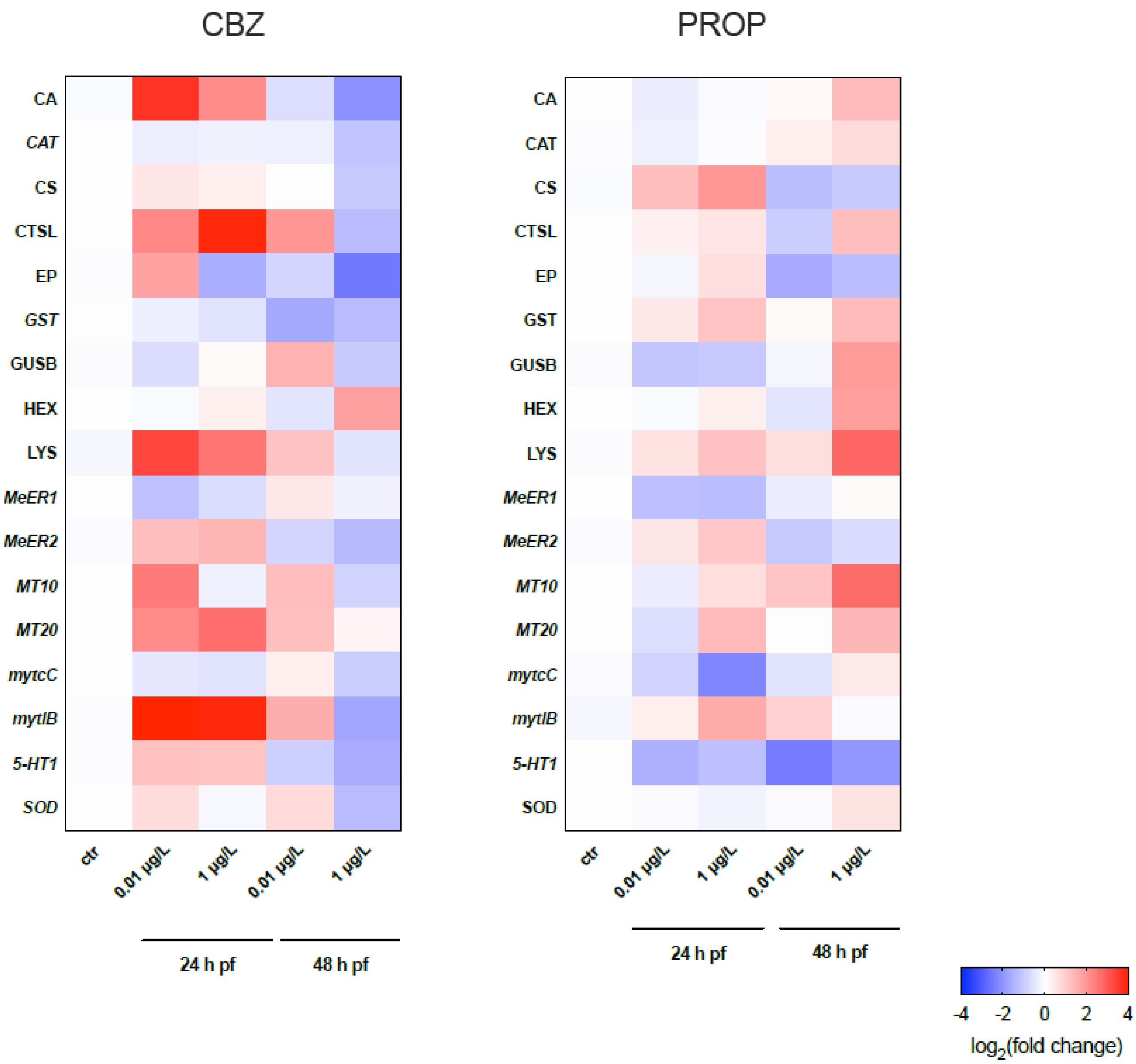

Fig. 2 Heatmaps comparing changes in mRNA levels in early larval stages of *M. galloprovincialis* after exposure to CBZ or PROP. Clustering was performed using fold change variations (log2-transformed) of target transcripts compared to unfertilized eggs (ctr). Colors represent relative expression levels with respect to controls. Transcripts are listed in alphabetical order (see Fig. S2 for detailed data and statistics).

Significant upregulation was observed for CTSL and GUSB, and downregulation for EP, MeER2, and 5HT1 (Fig. 2, Fig. S2). Significant up regulation of CA, CTSL, LYS, MT20 and MytB transcripts was observed after 24 h pf at 1 µg/L CBZ. A general downregulation is shown at 48 h pf, with the exception of HEX (up-regulated) and MeER1 and MT20 (unchanged).

Exposure to PROP for 24h differently affected gene transcription, with significant down regulation of GUSB, MeER1 and 5HT1, and up-regulation of CS transcripts at 0.01 µg/L (Fig. 2, Fig. S2). The effects of 0.01 µg/L PROP were generally reduced at 48 h pf, except for *GST* which appeared significantly down regulated (Fig. 2, Fig. S2). The down regulation of 5HT1 transcript was further enhanced at 48 h pf, while MeER2 and CS became significantly down-regulated (Fig. 2, Fig. S2). After exposure to 1 µg/L PROP, at 24 h pf significant upregulation was observed for CS, EP, GST, LYS, MT10, MT20, MytlB, and MeER2, and down regulation for GUSB, MeER1, MytlC, and 5HT (Fig. 2, Fig. S2). At 48h pf, 1 µg/L PROP caused significant up regulation of all transcripts related to the antioxidant response (CAT, GST, SOD, Mt10 and MT20), lysosomal function (CTSL, GUSB and HEX), and of CA and LYS; while a significant downregulation is shown for 5HT1, CS and EP (Fig. 2, Fig. S2).

PERMANOVA and permutation t-test analyses performed on qPCR data showed that effects of either CBZ or PROP were significant in both embryo stages and at both concentrations tested ($p < 0.05$; Table S3 and Table S4, Supplemental material). A significant interaction between the two factors (developmental stage and CBZ/PROP treatment) was also reported ($p < 0.05$; Table S3, Supplemental material).

Fold change variations ($\log_2$ transformed) of target transcripts were averaged by functional group, as defined in Table S2, and the resulting concentration-related transcriptional profiles at each developmental stage reported in Fig. 3A (CBZ) and Fig. 4A (PROP). CBZ treatments resulted in complex transcriptional profiles, with consistent up-regulations at 24 h pf (lysosomal responses, immune responses, and putative neuroendocrine signaling), down-regulations at 48 h pf (shell biogenesis), and bimodal effects in both embryo stages depending on the tested concentrations (24 h pf: shell biogenesis; 48 h pf: lysosomal responses, immune responses)

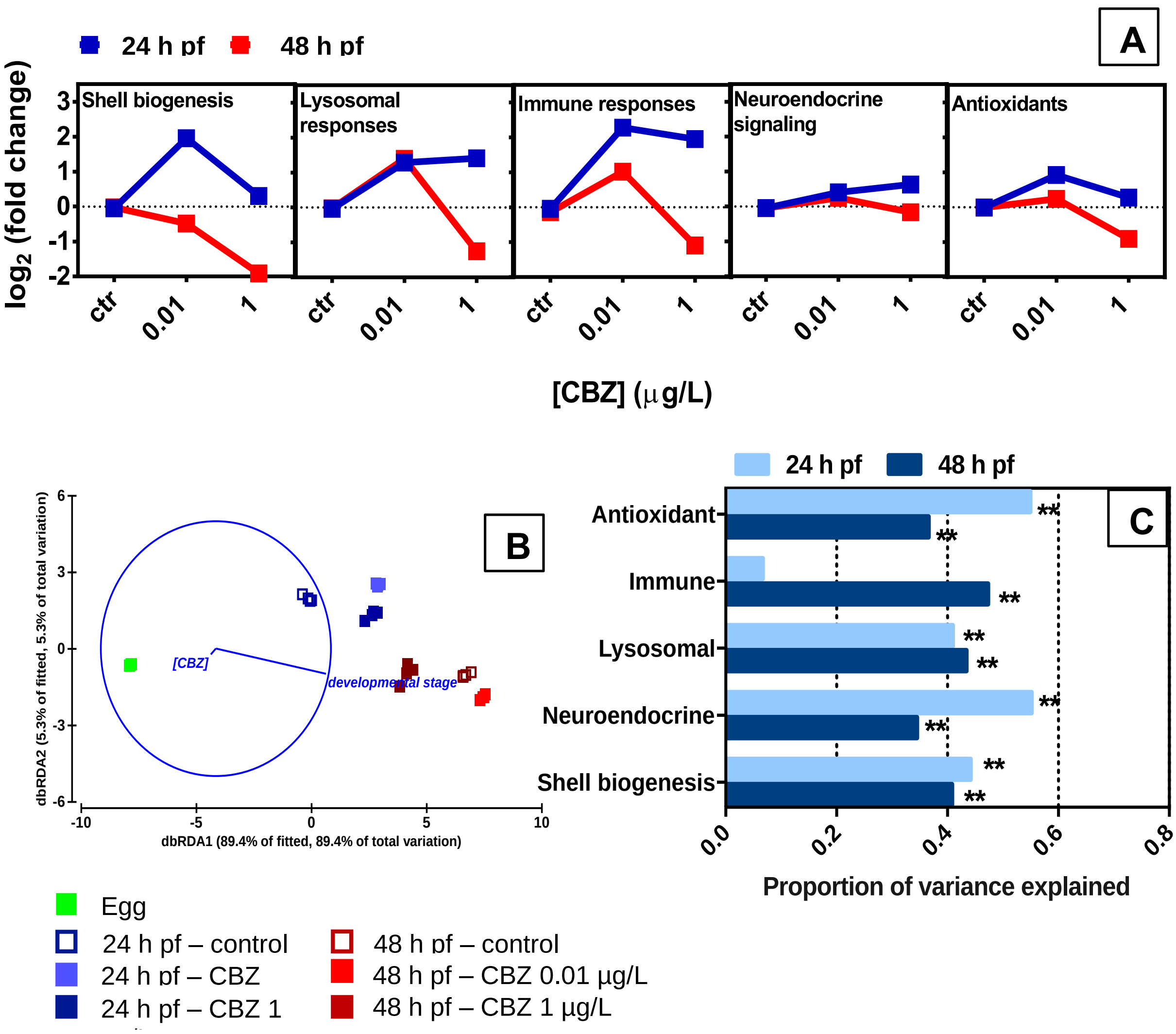

Fig. 3. **Overview of the transcriptional response to CBZ across mussel embryo development**. (A) Graphs report the mRNA expression changes (full squares representing mean values) and the average trend of variations (lines) within each group of transcripts (defined in Table S2). Detailed data for transcript expression changes and related statistics are reported in Fig. S2 (B) Distance-based redundancy (DISTLM) modeling with distance-based redundancy analysis (dbRDA) exploring the amount of the variation in gene transcription to be attributed to CBZ treatment of *M. galloprovincialis* embryos at different developmental stages (Euclidean Distance resemblance matrix, 999 permutations). (C) DISTLM analysis showing contribution of each functional group to the total variance observed in the CBZ dataset. DISTLM used the BEST selection procedure and adjusted R^2 criteria. Asterisks indicate level of statistical significance related to the result (**p < 0.01; *p < 0.05). ***Colored figure is intended only for the online and PDF version***

Distance-based linear model (DISTLM) analyses revealed that expression profiles were strongly dependent on the time post-fertilization that explained about 89.4% total variation (Fig. 3B). Nevertheless, CBZ treatment accounted for about 5% total variation explaining the observed transcriptional changes at the 24 h and 48 h pf (Fig. 3B). DISTLM analysis by functional groups also revealed that expression patterns of transcripts involved in antioxidant responses, lysosomal responses, putative neuroendocrine signaling, and shell biogenesis explained the observed effects of CBZ at 24 h and 48 h pf (Fig. 3C). Responses to CBZ of immune related gene products reached relevance at 48 h pf ($p < 0.05$) (Fig. 3C).

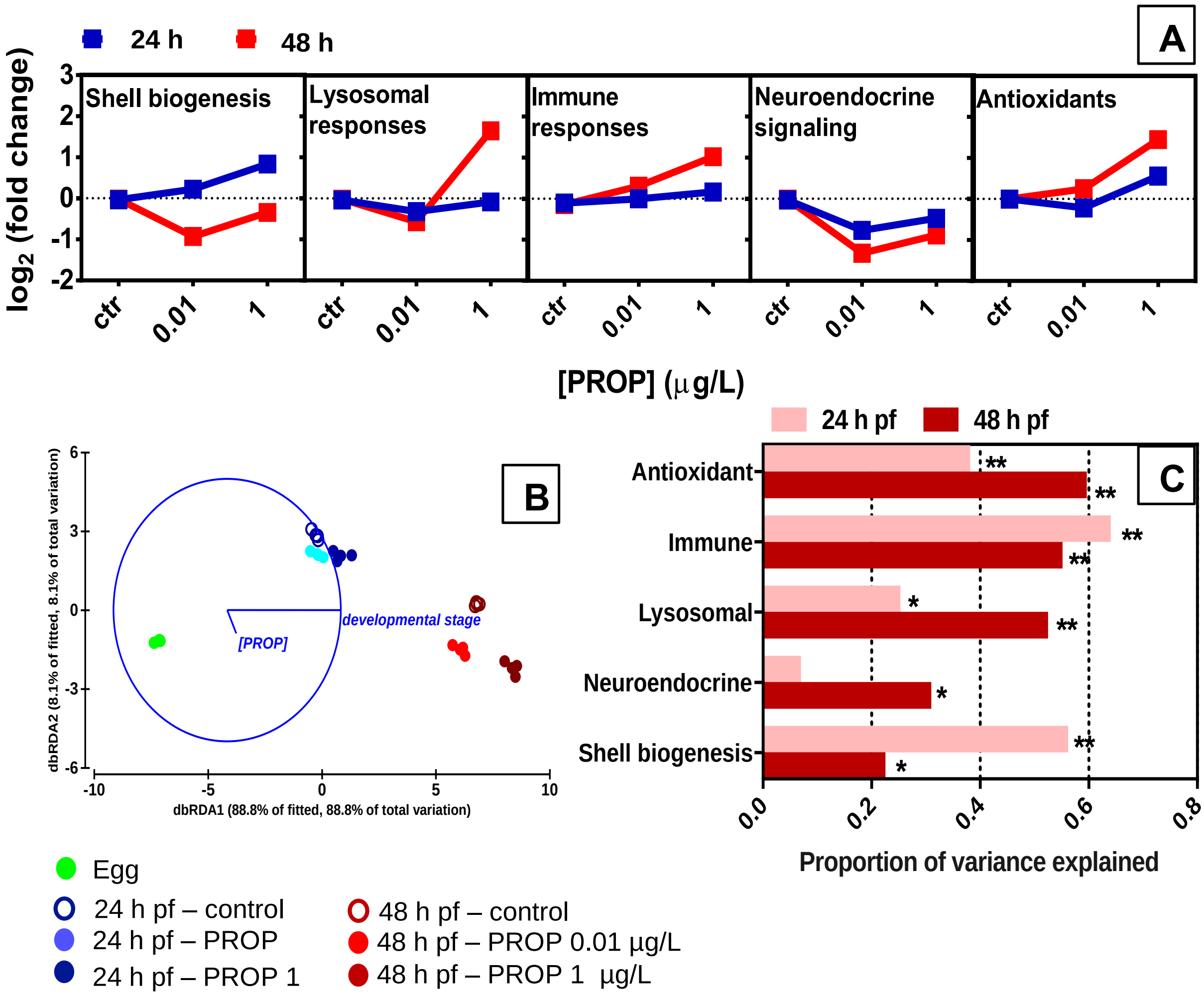

Fig. 4. Overview of the transcriptional response to PROP across mussel embryo development. (A) Graphs report the mRNA expression changes (full squares representing mean values) and the average trend of variations (lines) within each functional group (defined in Table S2). Detailed data for transcript expression changes and related statistics are reported in Fig. S2 (B) Distance-based redundancy (DISTLM) modeling with distance-based redundancy analysis (dbRDA) exploring the amount of the variation in gene transcription to be attributed to PROP treatment of *M. galloprovincialis* embryos at different developmental stages (Euclidean Distance resemblance matrix, 999 permutations). (C) DISTLM analysis showing contribution of each functional group to the total variance observed in the PROP dataset. DISTLM used the BEST selection procedure and adjusted R^2 criteria. Asterisks indicate level of statistical significance related to the result (**$p < 0.01$; *$p < 0.05$). ***Colored figure is intended only for the online and PDF version***

PROP treatments at 24 h pf caused overall up-regulation of transcripts involved in antioxidant responses and shell biogenesis, down-regulation of transcripts involved in putative neuroendocrine signaling, and globally unchanged expression patterns of transcripts encoding lysosomal enzymes and immune-related gene products (Fig. 4A). At 48 h pf, PROP induced down-regulation of transcripts for shell biogenesis and for putative neuroendocrine signaling at 0.01 µg/L, and up-regulation of transcripts for lysosomal and antioxidant responses at 1 µg/L (Fig. 4A). DISTLM showed that expression profiles strongly depend on the time post-fertilization (89% total variation), with PROP treatment accounting for about 8% total variation, which mostly explained the observed transcriptional changes at 48 h pf (Fig. 4B). DISTLM analysis by functional groups showed that expression patterns of transcripts involved in antioxidant response, immune responses, shell biogenesis, and, to a lesser extent, lysosomal responses, explained the observed effects of PROP at 24 h pf (Fig. 4C). The effects of PROP at 48 h pf were mostly explained by expression patterns of transcripts involved in antioxidant and immune responses, while putative neuroendocrine signaling and shell biogenesis were of minor relevance (though statistically significant) (Fig. 4C).

4 Discussion

The 96-microwell embryotoxicity assay has proven as a sensitive methodology for the high throughput screening of emerging contaminants, including anti-inflammatory, blood lipid lowering and antidepressant drugs, on embryo development of *M. galloprovincialis* (Balbi et al., 2018, 2017a, 2016; Estévez-Calvar et al., 2017; Fabbri et al., 2014). The present study showed that the antiepileptic drug CBZ and the β-blocker PROP, two pharmaceuticals widely used in human and veterinary therapies and commonly detected in marine coastal waters, altered embryo phenotypes, with significant effects observed from environmental concentrations (in the ng/L range). Although comparable EC50 values were obtained in embryotoxicity tests (≈ 1 µg/L), CBZ mainly induced shell malformations in D-veligers at 48 h pf at all the concentrations tested, whereas PROP resulted in different percentages of malformed/immature, and progressive developmental arrest/mortality at concentrations much higher than reported environmental levels.

In order to investigate possible impact of CBZ and PROP on gene expression, the effects of both compounds on the transcriptional pattern of 17 genes were evaluated in embryos at both 24 h and 48 h pf. These include both gene sequences corresponding to known biological functions in adult mussels and embryos (Balbi et al., 2017a, 2016), as well as genes whose transcription has been shown to be modulated by exposure to other emerging contaminants in mussel early larval stages (Balbi et al. 2016, 2017a, 2018; Capolupo et al., 2018). Moreover, expression of genes related to lysosomal function was evaluated in this study.

Transcriptional effects were investigated at two selected concentrations: 0.01 µg/L, a value that falls within the range detected in coastal and estuarine waters (see Table S1) and 1 µg/L (a value around the observed EC50 for both compounds in embriotoxicity assays). On the whole, the results show that CBZ and PROP induced significant changes in gene expression at both concentrations and developmental stages.

CBZ and PROP affected transcription of shell-specific transcripts at both post-fertilization times. Previous works indicated that these transcripts are involved in the homeostasis of carbonate chemistry at the site of calcification (*CA, EP*) and in organic matrix synthesis (*CS*), which control calcification rates and morphology of the shell (Chan et al., 2018; Kocot et al., 2016; Ramesh et al., 2017). Accordingly, they are strongly up-regulated in the early stages of mussels along with shell formation (Balbi et al., 2017a, 2016). The down-regulation of both *EP* and *CS* induced by both compounds at 48 h pf suggests that CBZ and PROP may interfere with regulation of shell biogenesis.

Modulation of transcripts encoding for lysosomal enzymes seems a common effect of CBZ and PROP in developing embryos. In embryos grown in physiological conditions, expression of lysosomal enzymes *HEX* and *CTSL*, and, to a lesser extent, of *GUSB* increased dramatically from 24 h to 48 h pf. This is not surprising, since the acquisition of a suitably functional lysosomal system is related to development of the digestive system, where intracellular digestion in hepatopancreatic cells represents the main route of food processing in bivalves (Balbi et al., 2017b), and also endows the animals with an active detoxification system of waterborne chemicals (Balseiro et al., 2013). Lysosomes may accumulate pharmaceuticals (in particular lipophilic compounds with amino groups) by pH partitioning (Kazmi et al., 2013), leading to altered lysosomal functions and drug-drug interactions (Franzellitti et al., 2015, 2013). The capacity of CBZ and PROP to affect lysosomes in marine invertebrates was demonstrated by *in vivo* studies with adult mussels, clams, and crabs, in which the pharmaceuticals decreased lysosomal membrane stability in haemocytes (Aguirre-Martinez et al., 2015; Aguirre-Martínez et al., 2013b, 2013a; Franzellitti et al., 2011; Martin-Diaz et al., 2009), or increased neutral lipid contents in digestive glands, and interactively affected PROP tissue accumulation (Franzellitti et al., 2015). Both CBZ and PROP affected transcription of genes encoding for different lysosomal enzymes, in particular at 48 hpf. Although the effects were not apparently concentration-dependent, the results show that the lysosomal function can be affected by pharmaceuticals also in developing embryos.

CBZ and PROP affected transcription of genes involved in immune and antioxidant responses, causing up- or down-regulations depending on concentration and the stage investigated. In particular, at 24 hpf, immune-related genes were up-regulated in response to low concentrations of CBZ. Both immunomodulatory and pro-oxidant effects of pharmaceuticals have been widely reported in aquatic species (Fabbri and Franzellitti, 2016).

Finally, CBZ and PROP also affected transcription of serotonin and *Mytilus* estrogen receptors (*5-HT1*, *MeER1* and *MeER2*). A putative role for these genes in neuroendocrine signaling during development was suggested by their strong upregulation from fertilization to 48 h pf under basal conditions (Balbi et al., 2016). Moreover, their transcription was affected by exposure to different types of contaminants (i.e. bisphenol A, 17β-estradiol, and styrene) with up and down regulations depending on the chemical and developmental stage (Balbi et al., 2016; Rasika Wathsala et al., 2018). In particular, the observed 5-HT1 downregulation induced by PROP at the different concentrations and times pf tested, indicate an overall impact of this compound on the serotoninergic system.

In an attempt to compare the overall impacts of CBZ and PROP on mussel embryos, a radar plot that summarizes all data on phenotypical and molecular changes is reported in Fig. 5.

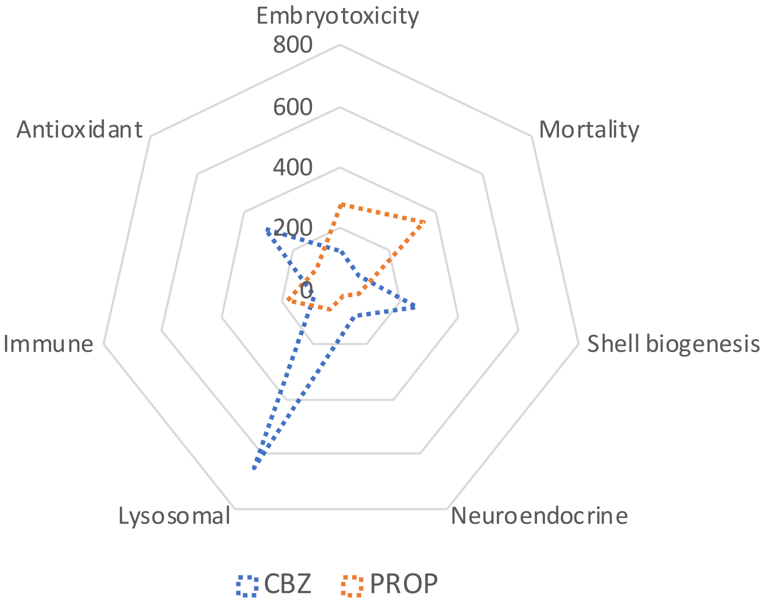

Fig 5. Radar plot summarizing the embryotoxic and molecular effects of CBZ and PROP in *M. galloprovincialis*early larval stages. For each biological endpoint, CBZ or PROP concentration-related variation is expressed by the Area Under the Curve (AUC) according to Franzellitti et al. (2018). Details for AUC calculation are reported in Table S5, Supplemental material. ***Colored figure is intended only for the online and PDF version.***

Although CBZ showed more limited phenotypic outcomes at increasing concentrations, signatures of molecular effects (antioxidant processes, shell biogenesis, lysosomal function) which may forecast upcoming changes at the physiological level are highlighted. For example, the observed changes in lysosomal related gene products in CBZ-exposed embryos may affect not only the development of the digestive system, but also degradative pathways supported by lysosomal enzymes that are crucial in embryo remodeling across development (Dyrynda et al., 1995).

Differently, PROP seems to have a greater impact on development in terms of embryotoxicity and mortality at higher concentrations. Developmental impairments due to PROP were previously reported in oysters; these effects were partly ascribed to the therapeutic action of PROP as an adrenergic antagonist, which may alter the physiological function of catecholamines in mussel embryo development (Yang et al., 2014). Indeed, PROP blocked epinephrine-induced metamorphosis in larvae of *Crassostrea gigas* (Coon and Bonar, 1987), increased mortality rates in zebrafish embryos, and induced developmental arrest in sea urchin embryos (Ribeiro et al., 2015). In embryos of the sea urchin *Arbacia lixula*, reduced cholinergic and serotoninergic signaling was related to impaired skeletogenesis and increased morphological abnormalities (Cappello et al., 2017). In this light, additional investigations are needed to understand the effects of PROP on adrenergic signaling in bivalve development.

5 Conclusions

Overall, the present study supports the use of the 96 microwell embryotoxicity assay to identify those pharmaceuticals that can provoke major types of morphological alterations at environmental concentrations.

With regards to data obtained on gene expression, the results indicate that the main effects on overall transcription (i.e. maximum distance between control and treated samples in the DISTLM analysis) were detected at 24 h pf for CBZ and at 48 h pf for PROP. In particular, as shown by the heatmap analysis, the impact of CBZ was evident at concentrations as low as 0.01 μg/L.

The results as a whole do not reveal a clear pattern in response to different concentrations of CBZ and PROP. In this light, present data do not provide evidence for a major or distinct mechanism of action for either compound, but they may reflect an adaptation response to exposure to these pharmaceuticals.

However, these are the first data demonstrating that both CBZ and PROP, at concentrations encompassing environmental levels, can interfere with multiple processes in mussel early development. Together with recent studies (Balbi et al., 2018, 2016), these observations raise new concerns on the occurrence of pharmaceuticals in coastal environments and their potential impacts on marine fauna. This information can be used to address further investigations on the possible molecular targets and mechanisms of action for either compound, and eventually contribute to establish regulatory priorities to limit the environmental occurrence of these emerging contaminants.

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