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Evaluating bivalve cytoprotective responses and their regulatory pathways in a Climate Change Scenario

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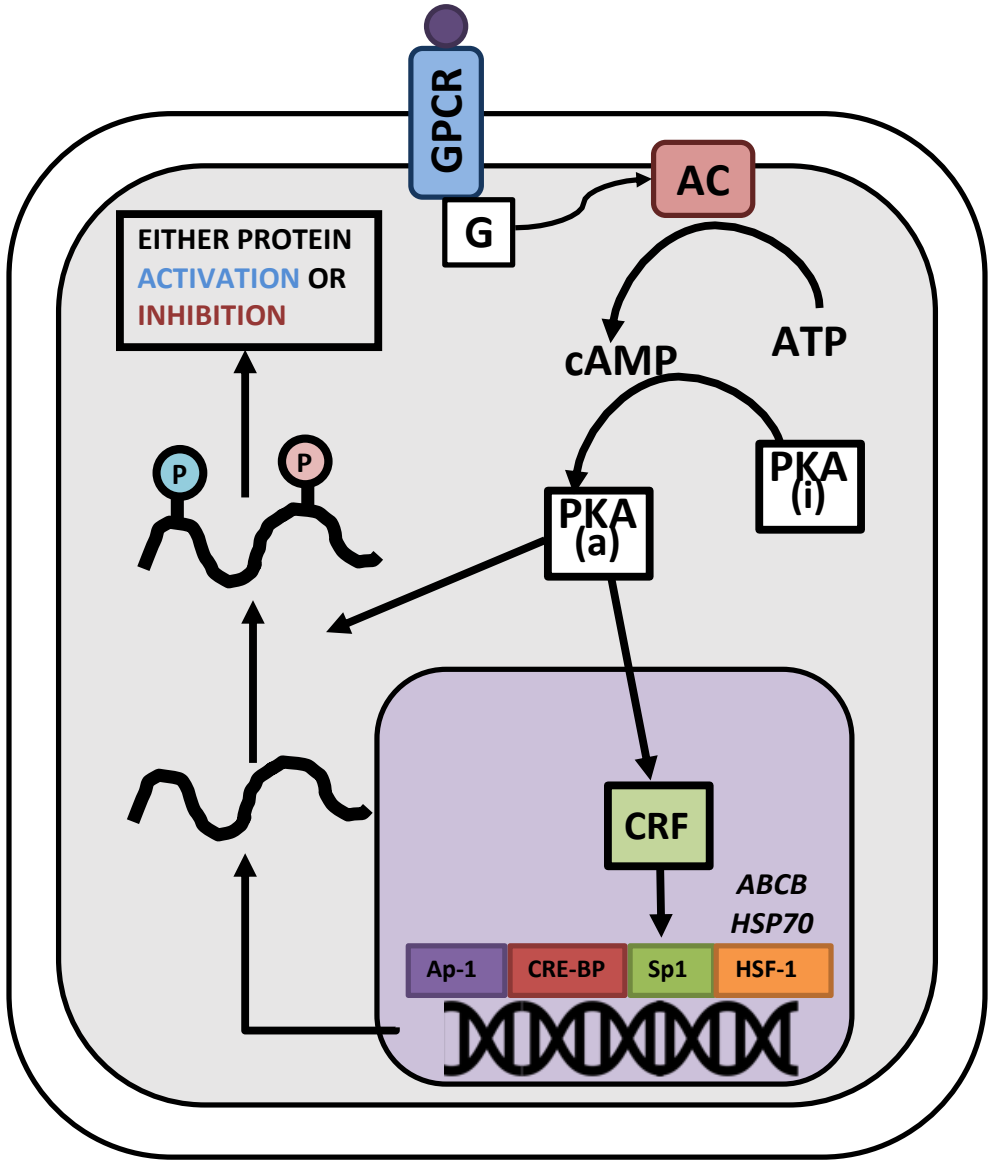
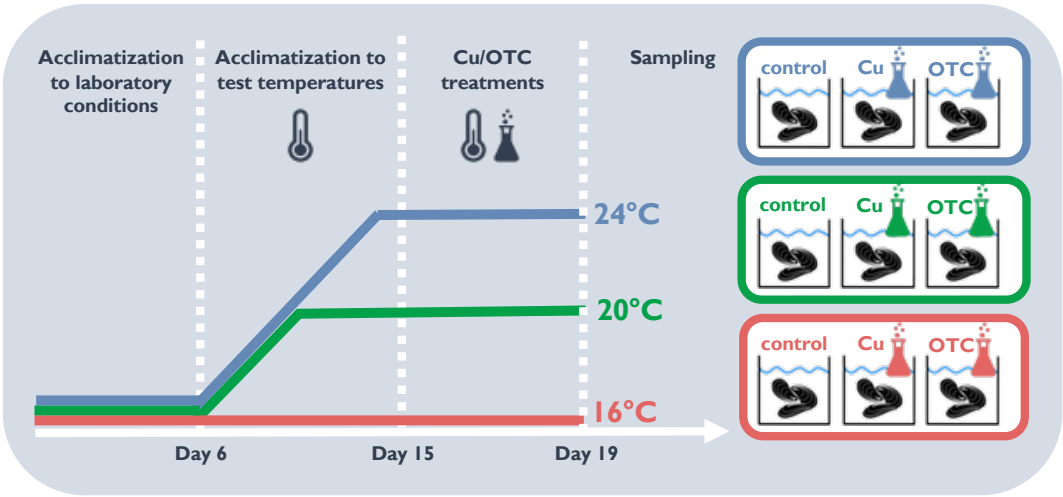
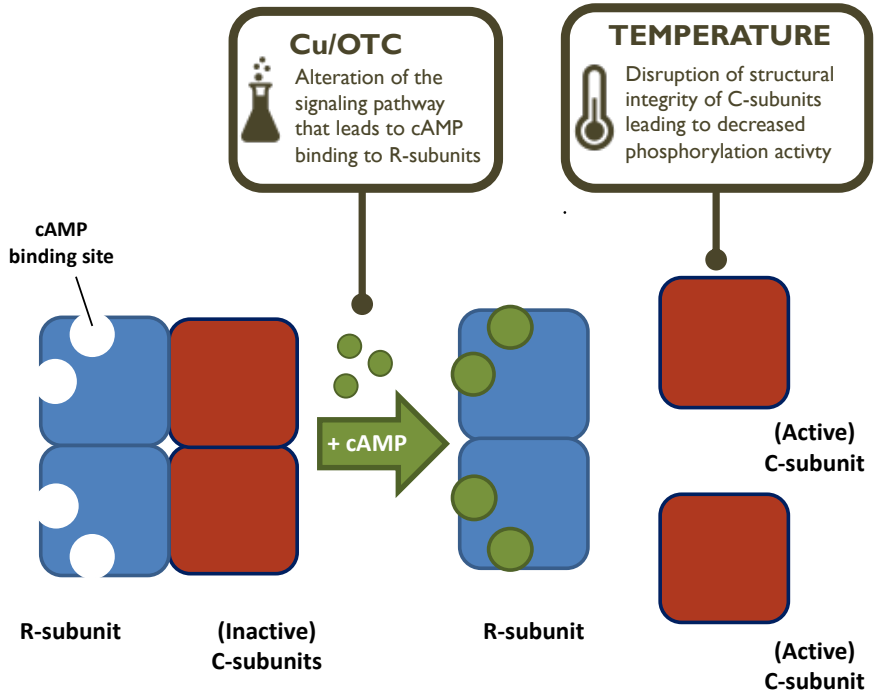
*Graphical Abstract







Abstract

Temperature is a relevant abiotic factor affecting physiological performance and distribution of marine animals in natural environments. The changes in global seawater temperatures make it necessary to understand how molecular mechanisms operate under the cumulative effects of global climate change and chemical pollution to promote/hamper environmental acclimatization. Marine mussels are excellent model organisms to infer the impacts of those anthropogenic threats on coastal ecosystems. In this study, Mediterranean mussels (*Mytilus galloprovincialis*) were exposed to different concentrations of the metal copper (Cu as $CuCl_2$: 2.5, 5, 10, 20, 40 µg/L) or the antibiotic oxytetracycline (OTC: 0.1, 1, 10, 100, 1000 µg/L) at increasing seawater temperatures (16°C, 20°C, 24°C). Transcriptional modulation of a 70-kDa heat shock protein (HSP70) and of the ABC transporter P-glycoprotein (P-gp, encoded by the ABCB gene) was assessed along with the cAMP/PKA signaling pathway regulating both gene expressions. At the physiological temperature of mussels (16°C), Cu and OTC induced bimodal changes of cAMP levels and PKA activities in gills of exposed animals. A correlation between OTC- or Cu- induced changes of PKA activity and expression of *hsp70* and *ABCB* was observed. Temperature increases (up to 24°C) altered *ABCB* and *hsp70* responses to the pollutants and disrupted their relationship with cAMP/PKA modulation, leading to loss of correlation between the biological endpoints. On the whole, the results indicate that temperature may impair the effects of inorganic and organic chemicals on the cAMP/PKA signaling pathway of mussels, in turn altering key molecular mediators of physiological plasticity and cytoprotection.

Keywords: temperature; antibiotic; metal; stress response; transcriptional control; marine mussel.

1. INTRODUCTION

Owing to its impact on biochemical and cellular machinery, temperature is a key abiotic factor affecting organism physiological performances and environmental distribution (Judge et al., 2018; Pörtner and Gutt, 2016). The changes in seawater temperatures associated with global climate change are fostering the research to understand the potential interactive effects of global warming with other sources of physiological stress in marine animals (Freitas et al., 2019; Sokolova and Lannig, 2008). To predict future scenarios, physiological studies attempt to determine the key physiological processes that set the limits of stress tolerance, how these operate in natural conditions where complex exposure scenarios occur, and whether species differ in acclimatization capacities for modifying their stress tolerances (Somero, 2012; Sulmon et al., 2015). In this regard, investigations of the regulatory mechanisms governing acclimatory and stress responses may provide early-warning molecular markers of animal-environment interaction and elucidate ~~on~~ how animal acclimatization is hampered under the cumulative effects of global warming and chemical pollution.

Contamination by metals is a typical anthropogenic footprint in coastal areas (Hatje et al., 2018). Increasing temperatures can influence distribution and fate of metals in sediments and seawater, as well as their bioaccumulation in marine organisms. For example, temperature affects metal bioaccumulation by enhancing bioavailability (Sokolova and Lannig, 2008) or by increasing or decreasing animal uptake through altered ventilation and feeding activity that support the enhanced energy demand (Coppola et al., 2018; Nardi et al., 2018; Negri et al., 2013).

Amongst the emerging pollutants, antibiotics are attracting particular attention since relatively high concentrations are detected in various aquatic ecosystems as a consequence of their worldwide use to treat microbial infections and enhance the growth and feeding efficiency of livestock in aquaculture (Flandroy et al., 2018; Scott et al., 2016),

resulting in the induction and spread of antibiotic resistance genes in natural microbial communities (Dantas et al., 2008; Zhang and Zhang, 2011). However, potential risks to non-target aquatic organisms via mechanisms that are apparently not related to the therapeutic actions of antibiotics are emerging (Stengel et al., 2016; Van Trump et al., 2010). Chemical stability of these compounds is thought to decrease with increasing temperatures, thus modifying their environmental concentrations, bioavailability, and animal accumulation (Chang et al., 2012, 2019).

Marine mussels (*Mytilus* spp.) are sessile organisms and often dominate coastal environments. They live in environments characterized by a wide array of salinities and temperatures, and are extremely tolerant to sudden changes of abiotic and biotic parameters, which makes them ideal model organisms for studying physiological alterations driven by environmental changes (Franzellitti et al., 2010; Viarengo et al., 2007).

This study aims to investigate whether temperature may influence the effects of the metal copper (Cu) or the antibiotic oxytetracycline (OTC), both commonly detected in coastal marine environments (Farajnejad et al., 2017; Scott et al., 2016), on the regulatory pathways that control cytoprotective responses contributing to physiological plasticity of the Mediterranean mussel (*Mytilus galloprovincialis*). Cu is an essential element released in the marine environment through a variety of anthropogenic sources (Wang et al., 2018). At elevated concentrations, Cu can induce redox reactions that generate reactive oxygen species (ROS) capable of direct damage towards cellular proteins, lipids, and DNA (Wang et al., 2018). OTC is extensively used in aquaculture because of its broad-spectrum efficacy in the treatment of infections caused by microorganisms (Limbu et al., 2018). Aquatic toxicity of OTC has been observed on several marine organisms, from algae to crustaceans and fish (Kołodziejska et al., 2013; Limbu et al., 2018; Wu and He, 2019). We assessed mRNA expression changes of a stress-inducible 70 kDa heat shock protein

(*hsp70*) and an *ABCB* transcript encoding the ABC (ATP-binding cassette) transporter P-glycoprotein (P-gp) along with temperature and/or pollutant induced modulations on the cAMP/PKA signaling pathway that may exert both transcriptional and post-transcriptional control on these proteins (Fig 1). Specifically, the cAMP/PKA pathway is involved in the activation of the heat shock transcription factor 1 (HSF1) (Murshid et al., 2010), one of the main mediators inducing *hsp70* gene transcription. It is also involved in *ABCB* transcriptional activation through several transcription factors (Franzellitti and Fabbri, 2013; Yao et al., 2009). P-gp and HSP70 are important players in the core stress response machinery that operates as a broad-spectrum cell protective mechanism allowing marine mussels to tolerate thermal stress (Luedeking and Koehler, 2004), exposure to pollutants (Franzellitti and Fabbri, 2006) or physiologically-demanding environments (Franzellitti et al., 2010). Induction of *hsp70* expression is a common response to temperature developed by mussels in variable thermal regimes (Lockwood et al., 2015; Morris et al., 2013). However, HSP70s are also induced by mussel exposure to metals and organics (Fabbri et al., 2008). The function of HSP70s under stress conditions is to assist in repairing, refolding, and protecting cellular proteins from damages, to minimize protein aggregation, or to facilitate degradation of irreparably damaged proteins, thus contributing to cell homeostasis (Fernández-Fernández et al., 2017). P-gp is the best characterized amongst the bivalve ABC transporters (Franzellitti and Fabbri, 2006). It is a phase 0 membrane transporter mediating the ATP-dependent extrusion of unmetabolized organic compounds, although it may be also involved in the response to further biotic and abiotic stressors (Buratti et al., 2013; Fu et al., 2019; Minier et al., 2000).

Mussels were acclimated to 16°C, 20°C, and 24°C under laboratory conditions and subsequently exposed for 4 days to a wide range of copper (Cu as $CuCl_2$) or oxytetracycline (OTC) nominal concentrations. Transcriptional levels of *ABCB* and *hsp70*, as well as cAMP tissue levels and activity of the cAMP dependent protein kinase A (PKA)

were assessed in gills of exposed mussels. For the purpose of this study, this experimental setup attempts to discriminate the contribution of the chemical and the physical stressors on the observed molecular outcomes and the underlying regulatory impairments, and to drive hypotheses on critical mechanisms that challenge acclimatization of marine organisms to anthropogenically modified environments.

2. METHODS

2.1. Mussel handling and experimental setup

Specimens of *M. galloprovincialis* (5-7 cm in length) were collected from the northwestern Adriatic Sea by professional fishermen of the “Cooperativa Copr.al.mo” (Cesenatico, Italy). They were transferred to the laboratory in seawater tanks with continuous aeration and kept for 6 days in aquaria containing 60 L of aerated 35 psu seawater at 16 °C, under a natural photoperiod (30 animals per aquarium). Mussels were fed once a day with an algal slurry (Koral filtrator, Xaqua, Italy). The duration of the acclimation proved suitable to stabilize the mussel physiological responses at the reference temperature of 16 °C (Banni et al., 2015; Viarengo et al., 2007). Fifteen mussels were sampled at zero time to assess parameters at the onset of each experiment. A scheme of the experimental setup is reported in Fig 2. Following the acclimation period, mussels were randomly selected and divided into groups of 20 animals each and transferred to aquaria containing 20 L of seawater. One liter of seawater per mussel is the suitable volume to avoid overloading and the onset of stress conditions. Four aquaria for each experimental condition were the 4 replicates (N = 4). One group of 44 aquaria was maintained at the reference temperature (16°C) throughout the experimental treatment, while the other 2 groups (each of 44 aquaria) were subjected to a gradual seawater temperature increase up to 20°C or 24°C (1°C per day) and maintained for 24 h at the settled temperature before exposure to the

chemical treatment. The reference temperature (16°C) and the highest exposure temperature (24°C) were derived from the time-series of monthly average SSTs recorded at the sampling area in the North-West Adriatic Sea (retrieved at the Copernicus Marine Service web portal, http://marine.copernicus.eu/), where 16°C matches the average annual temperature, and 24°C approaches the maximum annual recorded values. The 20°C represents a projection of the average annual temperature estimated for the end of the century in the North-West Adriatic Sea (Shaltout and Omstedt, 2014). In each aquarium, water temperature was monitored throughout the acclimation and the experimental periods using FT-800 thermometers (Econorma, Treviso, Italy). Once the selected temperatures in the aquaria were established, mussels were treated for 4 days with nominal 2.5, 5, 10, 20, 40 µg/L Cu (as $CuCl_2$) or 0.1, 1, 10, 100, 1000 µg/L OTC. OTC is found in seawater at the ng/L to µg/L concentrations (max concentration about 15 µg/L) (Chen et al., 2015). Cu concentrations tested in this study encompassed the range of values detected in the Adriatic Sea (from 0.5 µg/L to about 7 µg/L) (Munari and Mistri, 2007; Zago et al., 2000). The selected Cu concentrations were previously shown to decrease lysosomal membrane stability (LMS) in exposed mussels, and to exert further sub-lethal health effects (Negri et al., 2013). OTC effects on LMS were assessed in preliminary experiments in haemocytes of mussels exposed *in vivo* to the antibiotic at 16°C (Supplemental material, Fig S1). LMS was selected as the reference parameter in these preliminary evaluations on chemical concentration ranges to be tested as it is a sensitive and reliable biomarker of general health status in bivalves (Viarengo et al., 2007). All selected OTC concentrations significantly reduced LMS, a sign that mussels were subjected to a physiological stress. Acclimation periods at the selected temperatures and duration of chemical exposures were selected considering the dynamic ranges of the investigated biological endpoints, that constrained our experimental setup. Indeed, cell signaling pathways and transcriptional regulation of stress related genes, such as *ABCB*

and *hsp70*, are early and fast responses to environmental stimuli. Furthermore, according to our previous studies, a 4- to 7-day exposure proved suitable to develop measurable changes in the selected endpoints (Franzellitti et al., 2019, 2014, 2013). A group of unexposed (0 µg/L OTC or 0 µg/L Cu) mussels was maintained in parallel to the treatment groups within each temperature. Mussels exposed to 0 µg/L OTC or Cu at 16°C served as the reference condition for data comparisons and statistics. Seawater was renewed each day and the chemicals added from stock solutions along with mussel feeding. Exposures were conducted under dimmed light to minimize possible photodegradation, in particular of OTC (Jiao et al., 2008).

For all experimental treatments, the gills were dissected from individuals, snap-frozen in liquid nitrogen, and stored at -80°C. Gills were selected as they are the mussel filter-feeding organs and the major barriers between the external environment and internal organs, where physiological conditions are mostly imposed by the external environment (Musella et al., 2020). Therefore, gills are supplied with effective protective mechanisms (Franzellitti et al., 2016; Luckenbach and Epel, 2008). There was no mortality during the exposure period. Mussels at zero time were immediately analyzed for the biological endpoints to assess their initial health status; no significant differences compared to mussels maintained ~~for 4 days~~ under the reference treatment was observed (data not shown).

2.2. Measurements of cyclic AMP (cAMP) levels and PKA activity in mussel gills

For the evaluations of cAMP tissue content, samples (about 200 mg of pooled gills) were homogenized with 6% trichloroacetic acid and further processed as reported by Franzellitti et al. (2014). cAMP contents were assessed in the aqueous extracts through the DetectX^{TM} direct cyclic AMP enzyme immunoassay kit (Arbor Assay, USA) according

to the manufacturer's protocol. Results were finally expressed as pmol cAMP/g fresh tissue.

For the evaluations of PKA activity, samples (about 200 mg of pooled gills) were homogenized in cold extraction buffer (25 mM Tris–HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol and proteinase inhibitor cocktail P8340 from Sigma Aldrich), and further processed according to Franzellitti et al. (2014). Supernatants were assayed for PKA activity using the non-radioactive PepTag PKA assay kit (Promega, Milan, Italy) according to manufacturer's protocol. Results are expressed as nmol/min/mg total protein, with total protein content being estimated with Lowry's method (Lowry et al., 1951).

2.3. Mussel ABCB and hsp70 mRNA expressions

Gills (200 mg tissue) were homogenized in a suitable volume of the TRI Reagent (Sigma Aldrich, Milan, Italy) and total RNA was extracted using the DirectZol kit (Zymo Research, Freiburg, Germany) following the manufacturer's instructions. RNA concentration and quality were confirmed using the Qubit system with the Qubit RNA assay kit (Thermo Scientific, Milan, Italy), electrophoresis using a 1.2% agarose gel under denaturing conditions, and analysis of UV absorbance spectra of the samples (λ = 200 – 340 nm) for the calculation of Absorbance (A) ratio A260/A280 (cut-off values > 1.8 and < 2.0). First strand cDNA for each sample was synthesized from 1 µg total RNA using the iScript supermix (BioRad Laboratories, Milan, Italy) following the manufacturer's instructions.

ABCB and *hsp70* mRNA expressions were assessed by quantitative real-time PCR (qPCR) as reported in previous studies (Balbi et al., 2016; Franzellitti and Fabbri, 2013). Primer sequences and PCR conditions are reported in Supplemental material, Table S1. 18S rRNA and elongation factor 1α were selected as reference gene products for qPCR

data normalization by a preliminary stability analysis of 6 established candidate transcripts (Balbi et al., 2016). Relative expression values of target mRNAs were inferred by a comparative C_T method (Schmittgen and Livak, 2008) using the StepOne and DataAssist softwares (Thermo Fisher, Milan, Italy). Data were reported as relative expression (fold change) with respect to the reference treatment (0 µg/L Cu and 0 µg/L OTC at 16°C).

2.4. Statistical analysis

Statistical analysis of cAMP level and PKA activity data was performed using GraphPad Prism 8 (GraphPad Inc.). Significant differences between treatment groups were determined through the non-parametric one-way ANOVA (Kruskal-Wallis test) followed by the Mann-Whitney U-test, after deviations from parametric ANOVA assumptions being verified (Normality: Shapiro-Wilk's test; equal variance: Bartlett's test). qPCR data were analyzed using the REST software (Pfaffl et al., 2002) to test for statistical differences in mRNA levels of the treatment groups vs the reference condition. Further pairwise comparisons were performed with the Mann-Whitney U-test. Correlation analyses (Spearman's test), data visualization and graphics were obtained with the ggplot2 R package in R (R Development Core Team, 2018). In any case, statistical differences were accepted when $p < 0.05$.

The complete datasets from Cu or OTC treatments were further analyzed by a 2-way permutation multivariate analysis of variance (PERMANOVA) using PRIMER v6 (Anderson et al., 2008) to test for the interactive effects of temperature and Cu or OTC treatments. Log-transformed variations of the target transcripts and log-transformed cAMP levels and PKA activities were used to calculate similarity matrices based on the Euclidean distance (999 permutations; $P\ perm < 0.05$).

The Cu/OTC concentration-dependent trends of the biological endpoints were employed to calculate the Area Under the Curve (AUC) that gives a metric describing the

overall magnitude of cAMP, PKA, *ABCB and hsp70* variations at the different temperatures. Values of AUC were computed by the trapezoidal formula (Fekedulegn et al., 2007) and using GraphPad Prism 8. Details for AUC calculation are reported by Franzellitti et al. (2018).

3. RESULTS

3.1. Variations of cAMP-related signaling parameters in gills of mussels exposed to Cu or OTC at increased seawater temperatures

Results from PERMANOVA analyses demonstrated that temperature and OTC had an overall significant effect on both cAMP levels and PKA activity, while the effects of Cu on cAMP were statistically significant ($P < 0.05$; Table 1). PERMANOVA analysis also showed a significant interaction between each chemical and temperature ($P < 0.05$; Table 1).

Cu treatments at 16°C showed significant increases of cAMP levels and PKA activities up to 5 µg/L Cu, with values decreasing to control levels thereafter (Fig 3A,B). cAMP gill content increased both at 20°C and 24°C in 0 µg/L Cu samples (Fig 3A), while increasing (20°C) and decreasing (24°C) PKA activities compared to the reference condition (0 µg/L Cu at 16°C) were found (Fig 3B). Significant differences of tissue cAMP content between Cu-treated samples and the 0 µg/L Cu samples at the respective temperatures were significant at 2.5, 5.0 and 40 µg/L Cu (20°C), and at 40 µg/L Cu (24°C) ($p < 0.05$; Fig 3A). Significantly different PKA activity values compared to the 0 µg/L Cu samples at the respective temperatures were observed at 2.5 and 5 µg/L Cu ($p < 0.05$; Fig 3B).

OTC treatment at 16°C resulted in a bell-shape trend for both parameters, with values increasing up to 10 µg/L OTC and decreasing thereafter (Fig 4A,B). Mussels

acclimated at 20°C and 24°C showed no significant increase of cAMP levels compared to the 0 µg/L OTC samples at the respective temperatures (Fig 4A). For PKA, significant differences were observed at 1 to 1000 µg/L OTC (20°C) ($p < 0.05$; Fig 4B).

Correlation plots reported in Fig 2C and Fig 3C show that values of PKA activities were significantly correlated with variation of cAMP tissue content across Cu or OTC treatments only at 16°C ($p < 0.05$).

3.2. Variations of ABCB and hsp70 mRNA expressions in gills of mussels exposed to Cu or OTC at increased seawater temperatures

Results from PERMANOVA analyses demonstrated that Cu and OTC had an overall significant effect on both *ABCB* and *hsp70* expression, whereas temperature was effective on *ABCB* in the Cu treatment, while not in the OTC treatment ($P < 0.05$; Table 1). PERMANOVA analysis also showed a significant interaction between both chemicals and temperature ($P < 0.05$; Table 1).

Levels of *ABCB* expression were significantly higher in gills of Cu-exposed mussels at 16°C (5 – 40 µg/L) ($p < 0.05$; Fig 5A and Fig 6A). Expression levels were significantly higher in 0 µg/L Cu samples at 20°C and 24°C (Fig 5A). Significant differences between Cu-treated samples and controls at the respective temperatures were observed at 2.5 and 5 µg/L Cu (24°C) (Fig 4A). At 16°C, the *hsp70* gene product was significantly up-regulated in samples exposed to 2.5, 5, and 40 µg/L Cu, while down-regulated at 20 µg/L Cu ($p < 0.05$; Fig 5B). Expression levels were significantly increased in 0 µg/L Cu samples at 20°C and 24°C (Fig 5B), while significant differences between Cu-exposed samples and controls at the respective temperatures were observed at 40 µg/L Cu (24°C) (Fig 5B).

At 16°C, *ABCB* up-regulation at 1 µg/L OTC and down-regulation at 100 and 1000 µg/L OTC was observed ($p < 0.05$; Fig 6A). Expression levels were significantly increased in 0 µg/L OTC at 20°C and 24°C (Fig 6A), while significant differences between OTC-

treated samples and controls at the respective temperatures were observed at 0.1, 1, 10, 1000 µg/L OTC (20°C), and 1000 µg/L OTC (24°C) (Fig 6A). At 16°C, the *hsp70* gene product was significantly regulated in gills of OTC-exposed mussels, with down-regulation at 0.1 and 1 µg/L OTC, and up-regulation at 10 – 1000 µg/L OTC ($p < 0.05$; Fig 6B). Significantly different *hsp70* expression levels (down-regulation) between OTC-treated samples and the 0 µg/L OTC samples at the respective temperatures were observed at 0.1 – 100 µg/L OTC (20°C), and 1.0 -1000 µg/L OTC (24°C) (Fig 6B). Correlation plots reported in Fig 5C and Fig 6C show that both *ABCB* and *hsp70* expressions were significantly correlated with variation of PKA activity across Cu treatments only at 16°C ($p < 0.05$).

3.3. Analysis of temperature related trends of the biological responses to Cu and OTC

Calculation of AUC was employed to address changes of cAMP, PKA, *ABCB* or *hsp70* response to Cu or OTC at the different temperatures (Fig 7). cAMP and PKA showed a decreased response to both pollutants at increasing temperatures, whereas *ABCB* and *hsp70* showed a decreasing trend towards Cu response and an increasing response to OTC (Fig 7).

4. DISCUSSION

Both Cu and OTC affected the mussel cAMP/PKA pathway with significant changes of cAMP levels and PKA activities in gills of *in vivo* exposed animals. These results are in line with previous reports showing that Cu is a modulator of the cAMP signaling in bivalves (Fabbri and Capuzzo, 2010). Specifically, *in vitro* treatment of mussel gill membranes with Cu^{2+} (10^{-10} – 10^{-5} M) induced a bell-shape modulation of adenylyl cyclase (AC) activity, suggesting a putative direct effect of the metal on the cAMP forming mechanism (Fabbri and Capuzzo, 2006). Although the mechanism of action of OTC is not directly related to

the cAMP pathway, tetracyclines are considered pluripotent drugs in mammals with proved non-antibacterial related effects on inflammation, cell proliferation, cell migration, and apoptosis (Bendeck et al., 2002; Ci et al., 2011). Together with previous data (Banni et al., 2015), results of this study show that OTC in mussels may impair the cAMP signaling, an effect that is likely to broaden the spectrum of the physiological functions potentially impacted by the antibiotic in non-target marine species. Indeed, regulatory pathways mediated by cAMP underpin a variety of vital physiological processes in bivalves as well as in other aquatic species (Fabbri and Capuzzo, 2010; Fabbri and Moon, 2016). A correlation between Cu- or OTC- induced changes of PKA activity and expression of stress-related transcripts *ABCB* and *hsp70* was observed, in agreement with the occurrence of a common cAMP/PKA regulatory pathway (Fig 1) and the finding that *ABCB* (P-gp) and *hsp70* transcripts may be co-regulated as a generalized response to stress (Franzellitti et al., 2010; Luedeking and Koehler, 2004; Minier et al., 2000).

Acclimation to increased seawater temperatures affected the response to Cu and OTC. AUC calculations showed that temperature reduced the magnitude of the cAMP and PKA responses to both pollutants. The response of cAMP is likely linked to the relatively higher tissue levels observed in Cu- or OTC- unexposed samples at the increased temperatures, whereas reduction of PKA activity at 24°C is accompanied by the abolished response to the chemicals; furthermore, a loss of PKA *vs* cAMP correlation is observed at increasing temperatures. cAMP is the direct activator of PKA, and the mechanism by which cAMP regulates PKA activity is conserved from bacteria to humans (Kim et al., 2007). Inactive PKA is a tetrameric holoenzyme composed of two functionally distinct subunits: a dimeric regulatory subunit (R-subunit) and two monomeric catalytic subunits (C-subunits). The main function of the R-subunit is to lock the C-subunits in the inactive state through formation of the holoenzyme inhibitory complex. Binding of cAMP to the R-subunit unleashes the C-subunits, thereby allowing phosphorylation of PKA substrates

(Kim et al., 2007). Several C-subunit isoforms and two distinct isoforms of the R-subunit have been identified in mussels (Bardales et al., 2008, 2007; Díaz-Enrich et al., 2003), showing biochemical properties typical of mammalian type I and type II R-subunits, respectively. Furthermore, the known pharmacological modulators of PKA activity through cAMP in mammals are also effectives on the cAMP/PKA system of mussel haemocytes (Franzellitti and Fabbri, 2013), suggesting the conservation of the activation mechanism. An *in vitro* study showed no effects of temperature increases (up to 40°C) on the cAMP-binding ability of PKA purified from the posterior adductor muscle and the mantle of *M. galloprovincialis*. This suggests that temperature does not affect conformation of R-subunits of mussel PKAs, at least not at the cAMP-binding regions (Bardales et al., 2004). Nevertheless, the same temperature increase significantly modified the protein kinase activity at saturating concentrations of cAMP, when the holoenzyme was completely dissociated, indicating an effect on the conformation of the C-subunits (Bardales et al., 2004). We may hypothesize that while OTC and Cu affected the pathway leading to modulation of PKA activation (i.e. rate of cAMP binding to the R-subunit), temperature may impair the catalytic activity (i.e. altered conformational stability of C-subunits), causing the observed reduced activities at 24°C, the loss of correlation with changes of cAMP levels, and the consequent reduced responsivity to the pollutants. This apparent uncoupling of the PKA activation mechanism from the enzyme catalytic activity may also explain the observed loss of correlation with *ABCB*/*hsp70* expressions in response to the pollutants observed at 20°C and 24°C. AUC calculations also show that temperature reduced (Cu) or increased (OTC) the response of *ABCB* and *hsp70*. On the whole, these results indicate that when an increase of temperature disrupts the cAMP/PKA mediated pathway that normally contributes to *ABCB and hsp70* transcription, Cu and OTC may act through alternative pathways on the onset of P-gp and HSP70 responses. Interestingly, an opposite temperature-related response between Cu and OTC was also observed on

survival, replication rate, and lysosomal membrane stability of the ciliated protozoa *Euplotes crassus* exposed to the chemicals under thermal stress (25-33 °C) (Gomiero and Viarengo, 2014). Those biological endpoints pointed to a reduced toxicity of OTC but an increased toxicity of Cu with temperature increases, likely stemming from decreased stability of the molecule or the production of less toxic metabolites (OTC), or increased accumulation due to temperature-enhanced feeding activity (Cu) (Gomiero and Viarengo, 2014).

A further event that may operate in limiting the induction of *ABCB* and *hsp70* transcription is the so-called constitutive gene frontloading (Barshis et al., 2013). *ABCB* and *hsp70* up-regulation by Cu has been observed under *in vivo* exposure of oysters and mussels (Shi et al., 2015; Xu et al., 2018). Specifically, in gills of surviving oysters (*Crassostrea angulata*) exposed to high concentrations of Cu (30, 100, and 300 µg/L), *abcb1* was continuously over-expressed likely to aid the transport of Cu out of the cell (Shi et al., 2015). Mussels (*M. galloprovincialis*) exposure to low and environmentally relevant Cu concentrations (2 and 8 µg/L) resulted in *hsp70* over-expression which precedes Cu-induced oxidative damage, as evidenced by the induction of antioxidant enzymes activities and increased DNA damage (Xu et al., 2018). These data demonstrate that those proteins are components of the molecular machinery that maintain cellular Cu homeostasis (*ABCB*) and prevent its proteotoxic effects (*hsp70*). Nevertheless, since mussels are used to thrive in extremely variable environments, they are well known to retain a minimal constitutive *hsp70* expression that confers them enhanced physiological resilience by means of faster reaction at the protein level during transient stress events (Franzellitti and Fabbri, 2005). Although *hsp70* gene frontloading is largely acknowledged in marine intertidal invertebrates (Barshis et al., 2013; Fabbri et al., 2008; Morris et al., 2013), our data suggest ~~also~~ that the mussel *ABCB* may also display a frontloading behavior. For both transcripts, mussel acclimation to increased temperatures promoted increased basal

expression that limited the need for further induction due to Cu treatment. Apparently, constitutive gene frontloading was not effective enough to limit the need for further *hsp70/ABCB* mRNAs in OTC-exposed mussels, suggesting that OTC may affect gene transcription through mechanisms that are not biased by or not related to the stress response, which lead to overall independent effects between thermal stress and exposure to the antibiotic.

5. CONCLUSION

Results of this study show that temperature may change the outcome of the mussel cAMP/PKA signaling response to inorganic and organic chemicals, in turn altering the molecular mediators of physiological plasticity and environmental acclimatization, such as HSP70s and P-gp. However, the general consideration that temperature prevails over chemical stressors in eliciting physiological responses in marine organisms (Sokolova and Lannig, 2008) is not fully supported by our results, since a signature for the chemical effects can be observed at hyperthermic conditions. We further hypothesized that the temperature-related loss of correlation between changes in cAMP/PKA signaling and *ABCB/hsp70* transcriptional profiles may be due to the uncoupling of the PKA activation mechanism and the enzyme catalytic activity. Considering the key role of the cAMP/PKA pathway in mussel physiology (Fabbri and Capuzzo, 2010), this finding highlights the importance of considering the regulatory pathways upstream stress response processes when addressing the complex patterns of interactions in multiple stressor scenarios.

Conflict of interest

None

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Table 1. Two-way PERMANOVA results on the effects of oxytetracycline (OTC) or copper (Cu) *in vivo* exposure on cAMP levels, PKA activities, *ABCB* and *HSP70* expressions in gills of mussels at different temperatures (T) (998 permutations).

	df	cAMP		PKA		*ABCB*		*HSP70*	
		Pseudo-F	P(perm)	Pseudo-F	P(perm)	Pseudo-F	P(perm)	Pseudo-F	P(perm)
Cu treatment									
Cu	5	13.85	**0.001**	1.27	0.288	19.12	**0.001**	25.30	**0.001**
T	2	8.02	**0.002**	58.66	**0.001**	23.16	**0.001**	65.17	**0.001**
Cu x T	10	6.50	**0.001**	4.53	**0.001**	7.35	**0.001**	4.31	**0.002**
OTC treatment									
OTC	5	4.02	**0.002**	16.08	**0.001**	24.99	**0.001**	8.46	**0.001**
T	2	17.19	**0.001**	95.54	**0.001**	0.60	0.513	12.02	**0.001**
OTC x T	10	2.18	**0.029**	2.68	**0.006**	17.03	**0.001**	4.40	**0.003**

df: degree of freedom; Pseudo-F: F value by permutation (Anderson et al., 2008); P(perm): probability of pseudo-F.

Figure legends

Fig. 1. Schematic representation of the known cAMP/PKA signaling pathway upstream HSP70 and P-gp expression. (1) *Transcriptional control*: PKA-mediated regulation of several transcription factors (CRF) that initiate HSP70/ABCB (P-gp) transcription (Franzellitti and Fabbri, 2013; Murshid et al., 2010). (2) *Post-transcriptional control*: PKA-mediated phosphorylation of target HSP70/P-gp protein residues resulting in either protein activation (HSP70, P-gp) or inhibition (HSP70) (Arana and Altenberg, 2019; Nitika and Truman, 2017). GCPR, G-protein coupled receptor; G, G-protein; AC, adenylyl cyclase; cAMP, cyclic-AMP; PKA(i), inactive cAMP-dependent protein kinase (PKA; holoenzyme); PKA(a), active PKA (catalytic subunit); CRF, cAMP-responsive factors (amongst others: AP-1, CRE-BP Sp1, HSF1). ***Colored figure is intended only for the online and PDF version***.

Fig. 2. Schematic flowchart of the experimental setup for the impact of thermal stress on the mussel responses to copper (Cu) or oxytetracycline (OTC). The aquaria represent the replicates for each condition (N = 4). ***Colored figure is intended only for the online and PDF version***.

Fig. 3. Changes of cAMP/PKA signaling in gills of Cu-exposed mussels at different temperatures. Bar plots report mean ± SEM values for (A) cAMP tissue levels and (B) PKA activities (N = 4). *$p<0.05$ vs samples at 0 Cu and at 16°C**;** $^{a}p<0.05$ vs sample group at 0 Cu within the 16°C group; $^{b}p<0.05$ vs sample group at 0 Cu within the 20°C treatment groups; $^{c}p<0.05$ vs sample group at 0 Cu within the 24°C treatment groups. (C) Correlation plots show the relationships between cAMP levels and PKA activities at the different temperatures. Correlation analyses are based on data from individual mussels (N = 24

within each temperature). Average values for each data point have been used only for the graphic representation. Only significant Spearman correlations (*p<0.05) are reported in the graphs. Shaded areas show the 95% confidence intervals. ***Colored figure is intended only for the online and PDF version***.

Fig. 4. Changes of cAMP/PKA signaling in gills of OTC- exposed mussels at different temperatures. Bar plots report mean ± SEM values for (A) cAMP tissue levels and (B) PKA activities (N = 4). *p<0.05 vs samples at 0 OTC and at 16°C**;** ^{a}p<0.05 vs sample group at 0 OTC within the 16°C group; ^{b}p<0.05 vs sample group at 0 OTC within the 20°C treatment groups; ^{c}p<0.05 vs sample group at 0 OTC within the 24°C treatment groups. (C) Correlation plots show the relationships between cAMP levels and PKA activities at the different temperatures. Correlation analyses are based on data from individual mussels (N = 24 within each temperature). Average values for each data point have been used only for the graphic representation. Only significant Spearman correlations (*p<0.05) are reported in the graphs. Shaded areas show the 95% confidence intervals. ***Colored figure is intended only for the online and PDF version***.

Fig. 5. *ABCB* (A) and *hsp70 (B)* expressions in gills of Cu-exposed mussels acclimatized at different temperatures. Bar plots report mean ± SEM values of fold change variations. **p<0.05 vs samples at 0 Cu and at 16°C**;** ^{a}p<0.05 vs sample group at 0 Cu within the 16°C group; ^{b}p<0.05 vs sample group at 0 Cu within the 20°C treatment groups; ^{c}p<0.05 vs sample group at 0 Cu within the 24°C treatment groups. (C,D) Correlation plots show the relationships between PKA activity and *ABCB/hsp70* expression at the different temperatures. Correlation analyses are based on data from individual mussels (N = 24 within each temperature). Average values for each data point have been used only for the graphic representation. Only significant Spearman

correlations (*p<0.05) are reported in the graphs. Shaded areas show the 95% confidence intervals. ***Colored figure is intended only for the online and PDF version***.

Fig. 6. *ABCB* (A) and *hsp70* (B) expressions in gills of OTC-exposed mussels acclimatized at different temperatures. Bar plots report mean ± SEM values of fold change variations. **p<0.05 vs samples at 0 OTC and at 16°C**;** [a]p<0.05 vs sample group at 0 OTC within the 16°C group; [b]p<0.05 vs sample group at 0 OTC within the 20°C treatment groups; [c]p<0.05 vs sample group at 0 OTC within the 24°C treatment groups. (C,D) Correlation plots show the relationships between PKA activity and *ABCB/hsp70* expression at the different temperatures. Correlation analyses are based on data from individual mussels (N = 24 within each temperature). Average values for each data point have been used only for the graphic representation. Only significant Spearman correlations (*p<0.05) are reported in the graphs. Shaded areas show the 95% confidence intervals. ***Colored figure is intended only for the online and PDF version***.

Fig. 7. Temperature related trends of AUC (Area Under the Curve) values. For each biological endpoint, Cu or OTC concentration-related variation at each temperature is expressed by the Area Under the Curve (AUC) according to Franzellitti et al. (2018). ***Colored figure is intended only for the online and PDF version***.

754 **Fig 1**

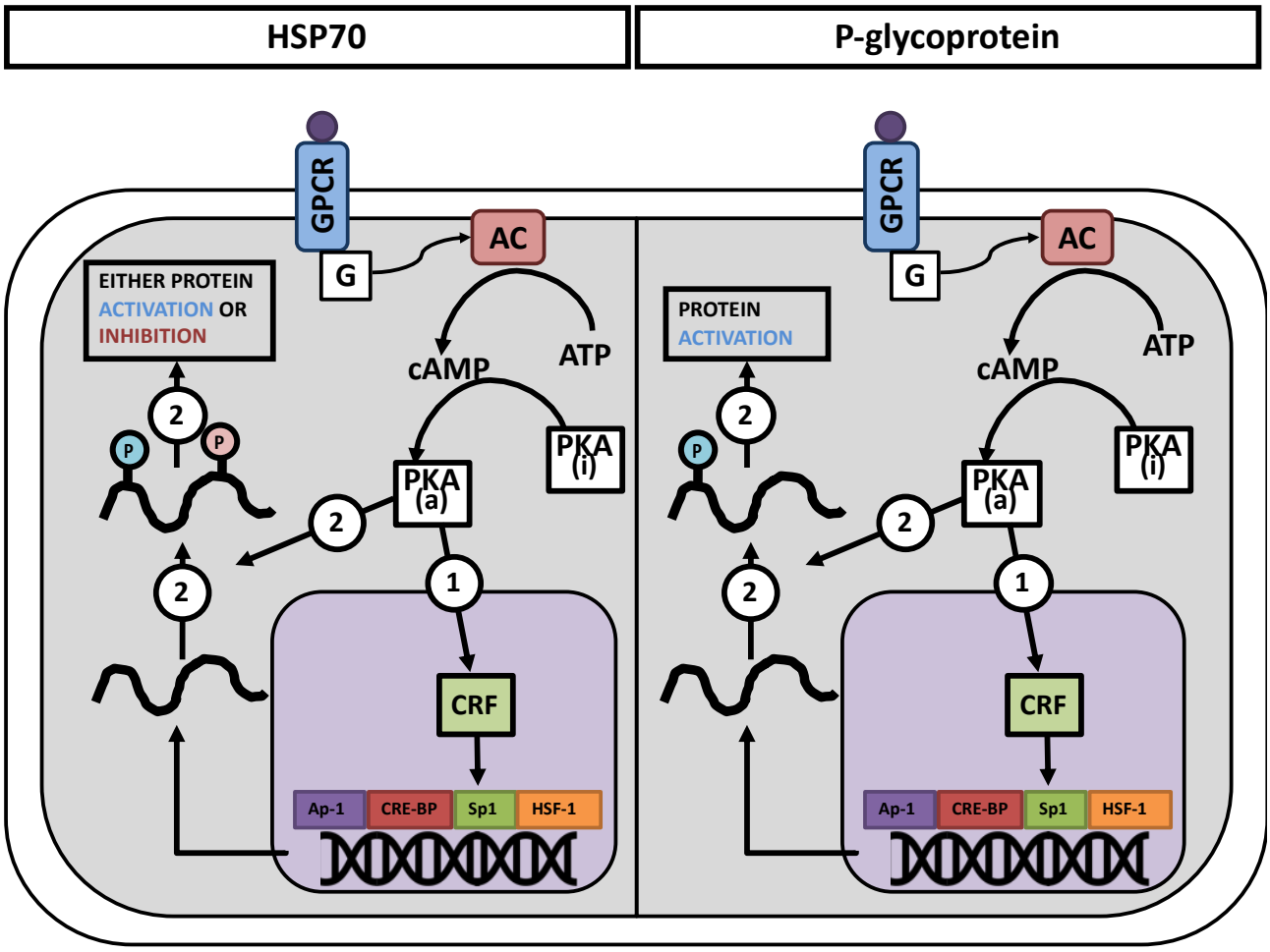

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Fig 2

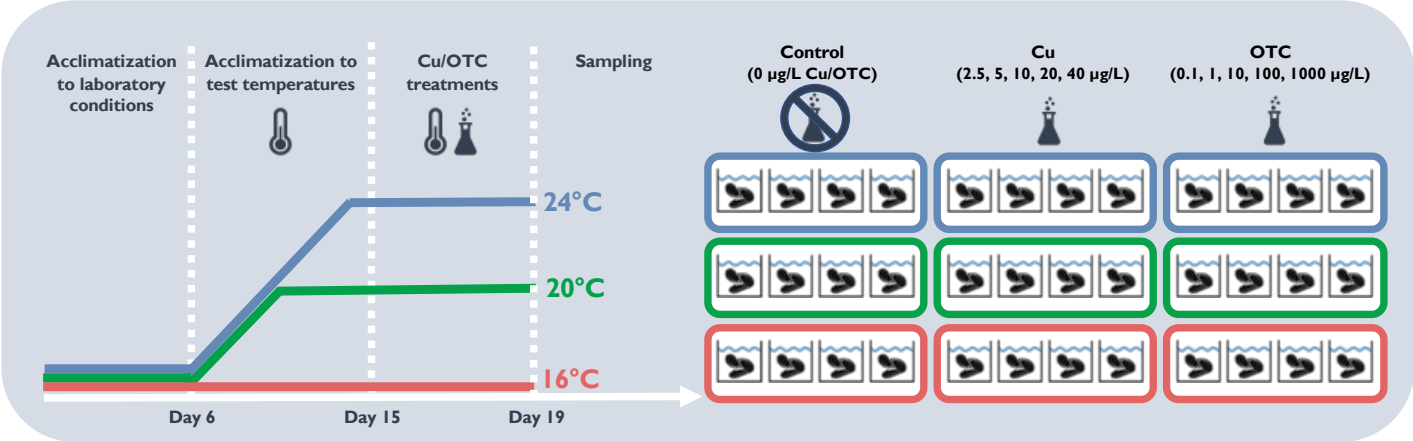

Acclimatization to laboratory conditions
Acclimatization to test temperatures
Cu/OTC treatments
Sampling
24°C
20°C
16°C
Day 6
Day 15
Day 19
Control
(0 µg/L Cu/OTC)
Cu
(2.5, 5, 10, 20, 40 µg/L)
OTC
(0.1, 1, 10, 100, 1000 µg/L)

Fig 3

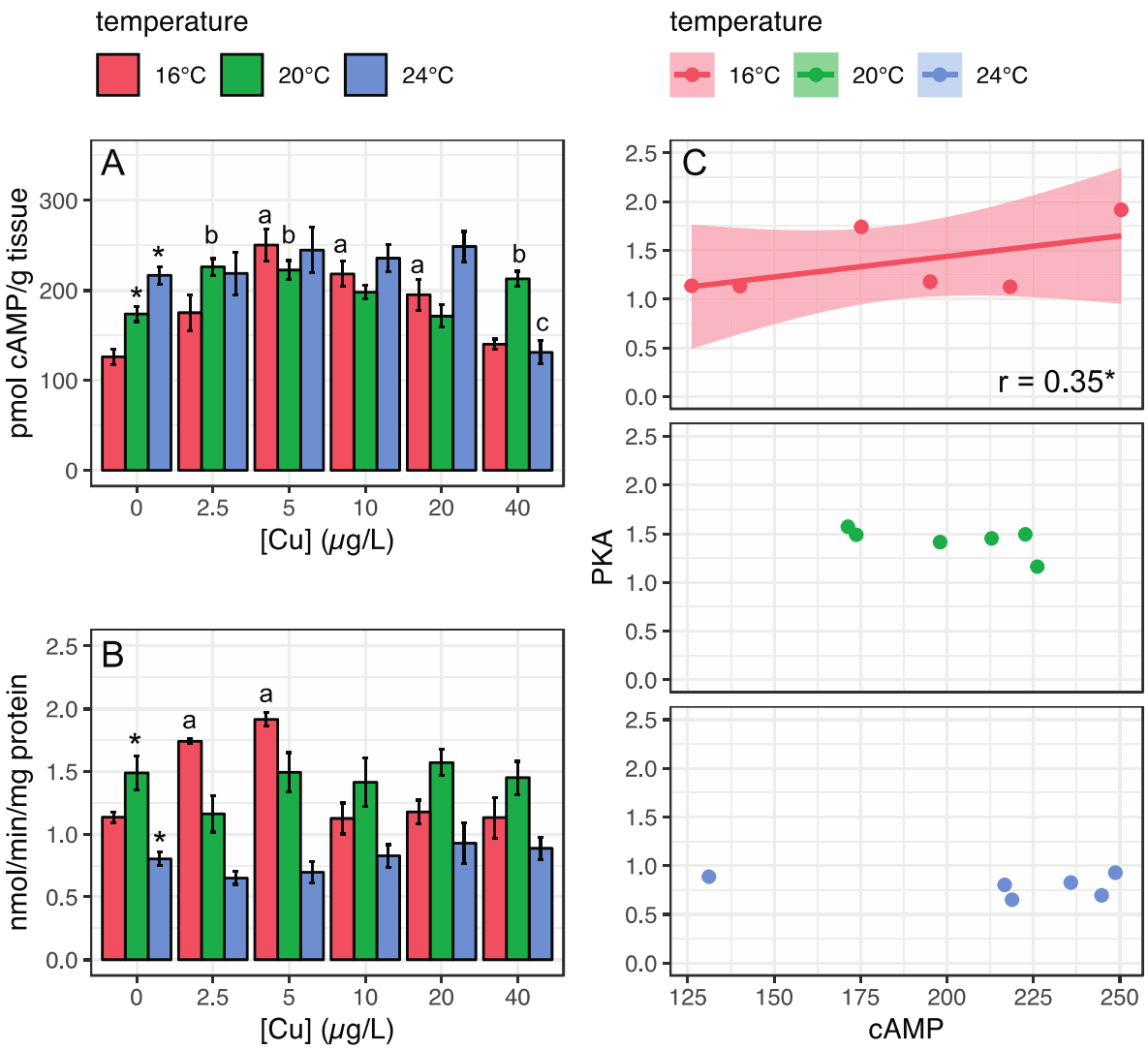

temperature
16°C
20°C
24°C
A
pmol cAMP/g tissue
300
200
100
0
0
2.5
5
10
20
40
[Cu] (μg/L)
B
nmol/min/mg protein
2.5
2.0
1.5
1.0
0.5
0.0
[Cu] (μg/L)
temperature
16°C
20°C
24°C
C
r = 0.35*
PKA
cAMP
125
150
175
200
225
250

Fig 4

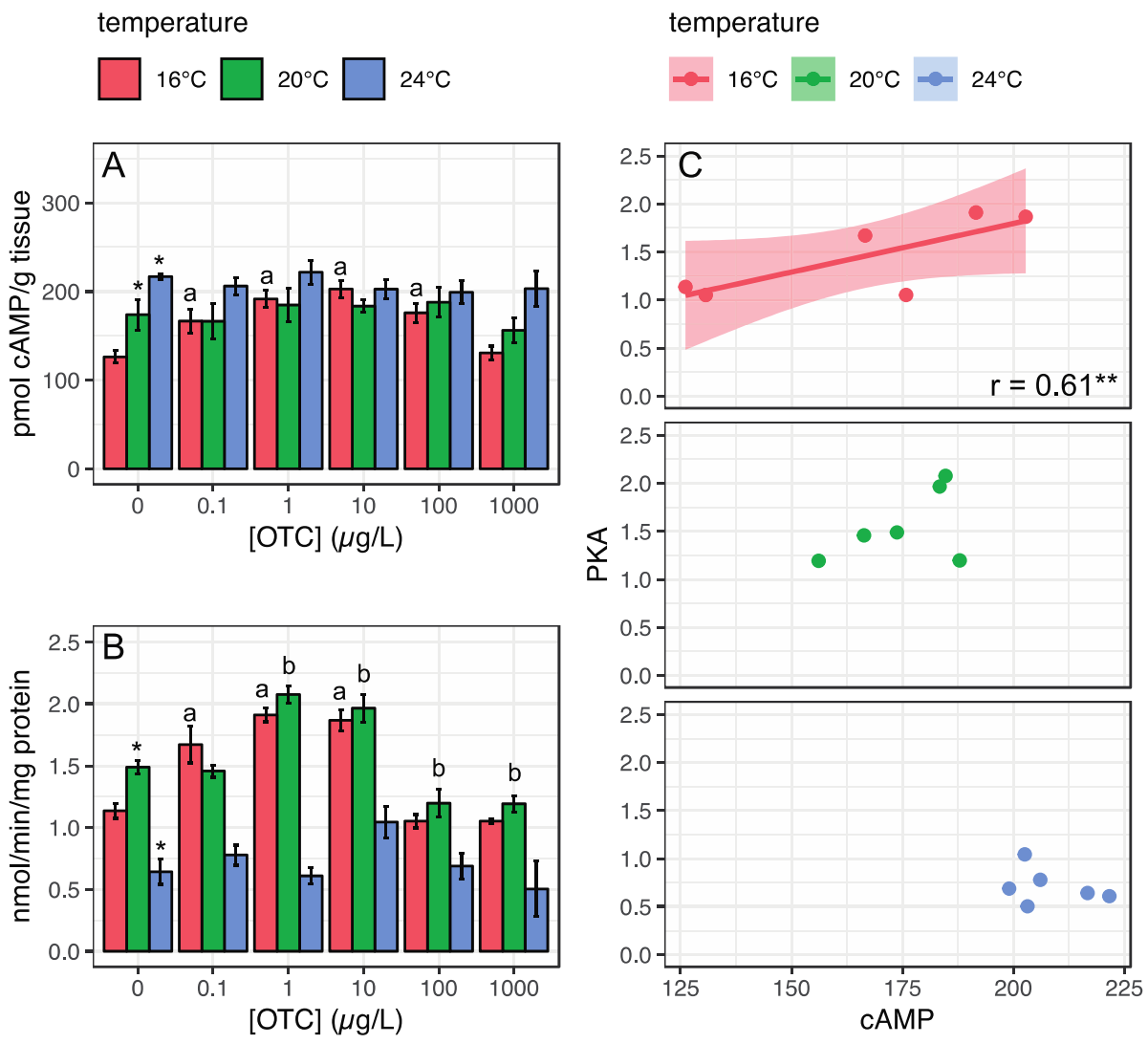

temperature
16°C
20°C
24°C
A
pmol cAMP/g tissue
300
200
100
0
0
0.1
1
10
100
1000
[OTC] (μg/L)
B
nmol/min/mg protein
2.5
2.0
1.5
1.0
0.5
0.0
[OTC] (μg/L)
C
r = 0.61**
PKA
cAMP
125
150
175
200
225

Fig 5

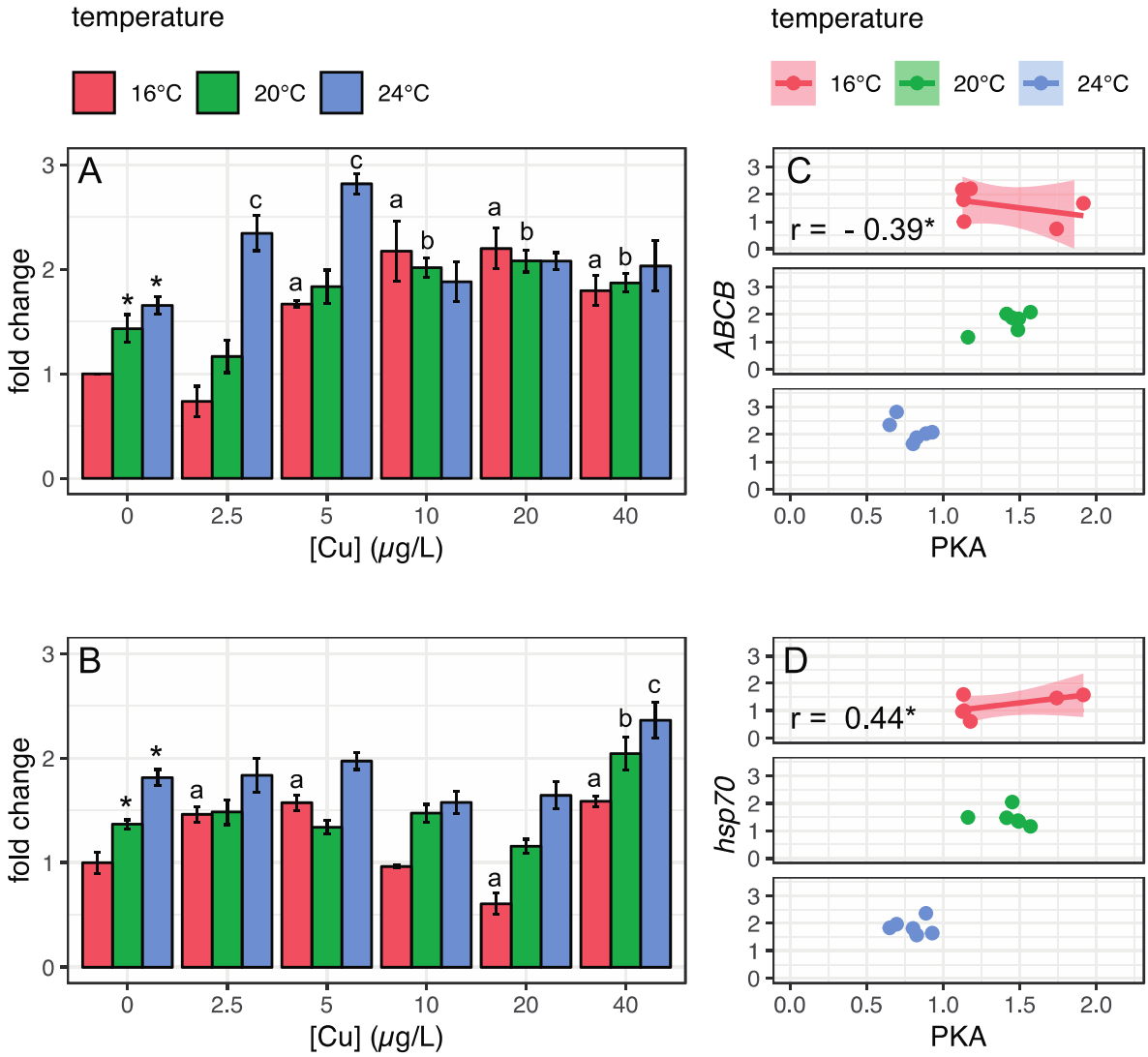

temperature
16°C
20°C
24°C
A
B
C
D
fold change
[Cu] (μg/L)
0
2.5
5
10
20
40
ABCB
hsp70
PKA
r = - 0.39*
r = 0.44*

Fig 6

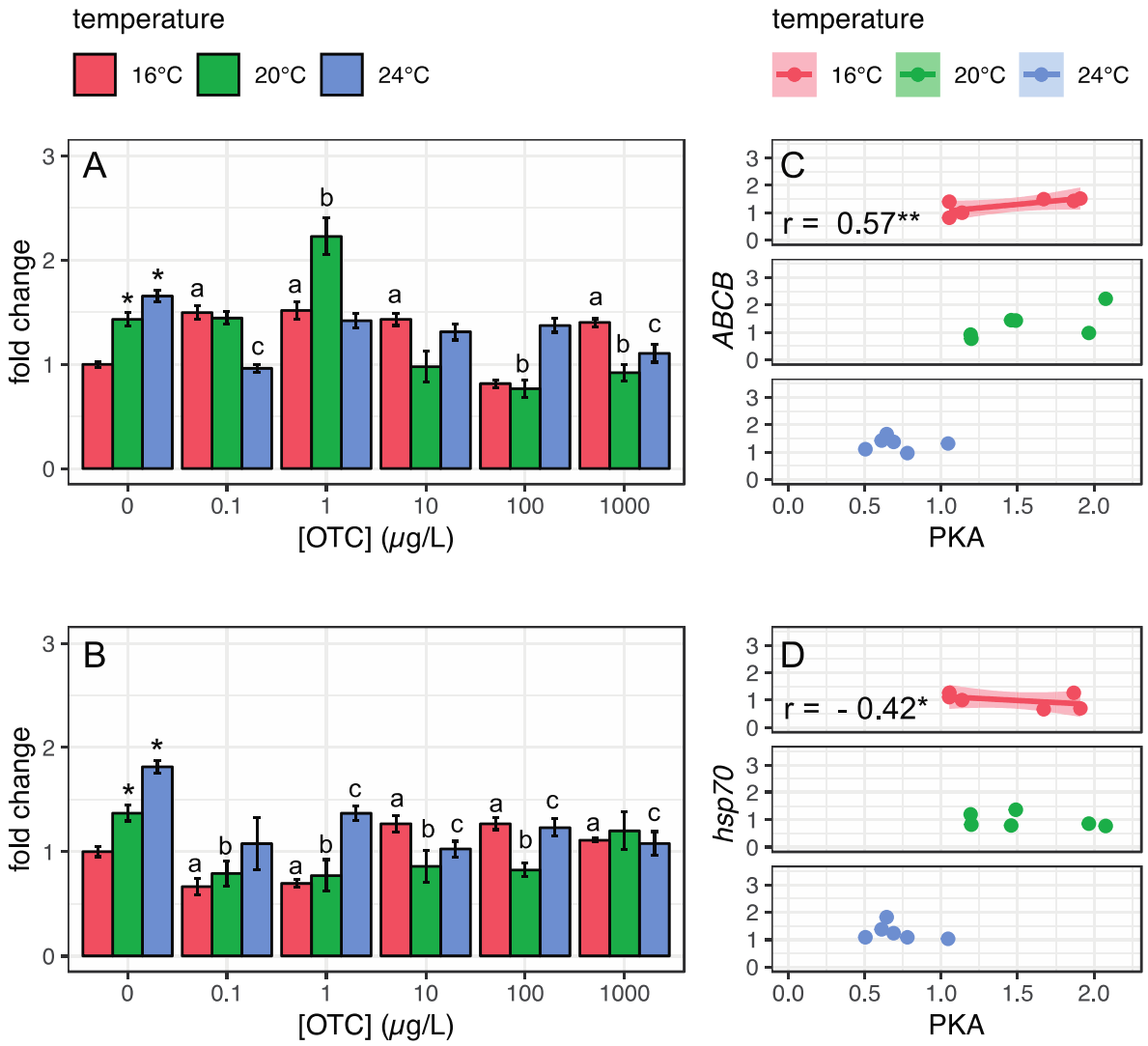
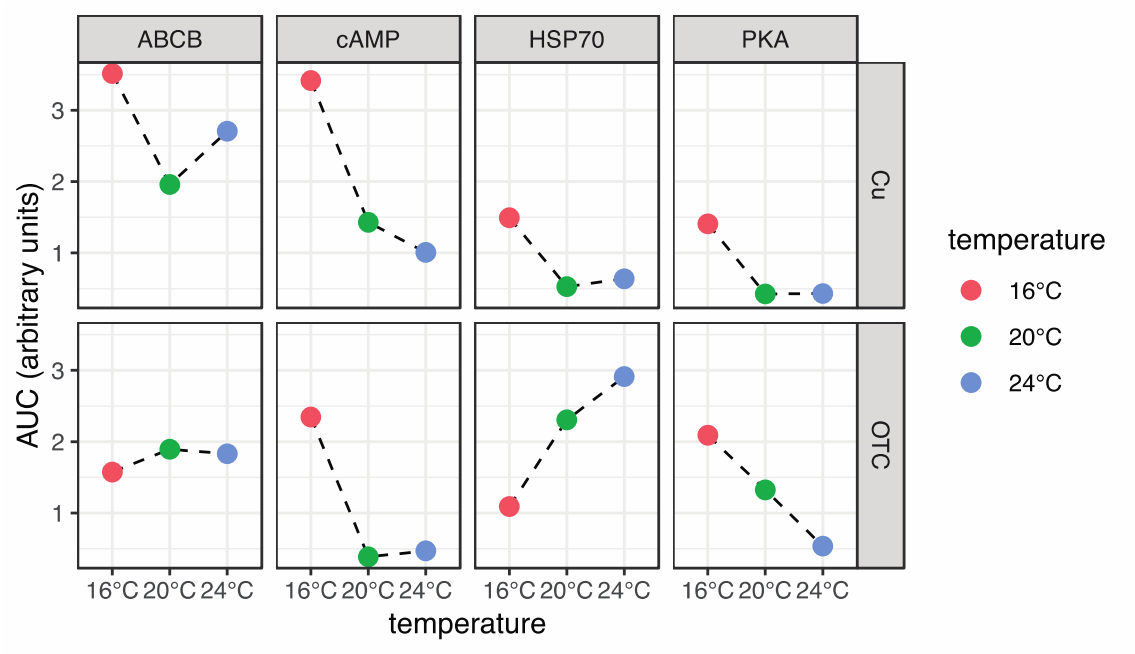

temperature
16°C
20°C
24°C
A
B
C
D
fold change
[OTC] (μg/L)
0
0.1
1
10
100
1000
r = 0.57**
r = - 0.42*
ABCB
hsp70
PKA
0.0
0.5
1.0
1.5
2.0

Fig 7


ABCB
cAMP
HSP70
PKA
Cu
OTC
AUC (arbitrary units)
3
2
1
16°C 20°C 24°C
temperature
temperature
16°C
20°C
24°C