Manuscript Details

Manuscript number	CBPC_2019_131
Title	Estrogenic compounds as exogenous modulators of physiological functions in molluscs: signaling pathways and biological responses
Short title	estrogen in molluscsignaling pathways and effects
Article type	Invited review article

Abstract

Molluscs have been widely utilized to evaluate the effects of estrogenic compounds, one the most widespread class of Endocrine Disrupting Chemicals-EDCs. However, knowledge on steroid signaling and metabolism in molluscs has considerably increased in the last decade: from these studies, a considerable debate emerged on the role of 'natural' steroids in physiology, in particular in reproduction, of this invertebrate group. In this work, available information on the effects and mechanisms of action of estrogens in molluscs will be reviewed, with particular emphasis on bivalves that, widespread in aquatic ecosystems, are most likely affected by exposure to estrogenic EDCs. Recent advances in steroid uptake and metabolism, and estrogen receptors-ERs in molluscs, as well as in estrogen signaling in vertebrates, will be considered. The results so far obtained with 17β-estradiol and different estrogenic compounds in the model bivalve Mytilus spp, demonstrate specific effects on immune function, development and metabolism. Transcriptomic data reveal alternative estrogen signaling pathways in mussel tissues that are supported by new observations at the cellular level. In vitro and in vivo data show, through independent lines of evidence, that estrogens act through alternative, non-genomic signaling pathways in bivalves. In this light, regardless of whether molluscs synthesize estrogens de novo or not, and despite their ERs are not directly activated by ligand binding, estrogens can interact with multiple signaling components, leading to modulation of different physiological functions. Increasing knowledge in endocrine physiology of molluscs will provide a framework for a better evaluation and interpretation of data on the impact of estrogenic EDCs in this invertebrate group.

Keywords	alternative signaling pathways; bivalves; development; estrogens; estrogen receptors; immunity; steroid metabolism; transcriptomics
Taxonomy	Endocrinology, Physiology
Manuscript category	Endocrine disruptors

Corresponding Author	Laura Canesi
Corresponding Author's Institution	Dept. of Eart, Environmental and Life Sciences, University of Genoa
Order of Authors	teresa balbi, CATERINA CIACCI, Laura Canesi

Suggested reviewers	Elena Fabbri, Cinta Porte, Alexander Scott, Helen Poynton, Maria Marino, Miren Cajaraville

Submission Files Included in this PDF

File Name [File Type]

CoverLetter.docx [Cover Letter]

Highlights.docx [Highlights]

GraphicalAbstract.tif [Graphical Abstract]

MsRevCBPC.docx [Manuscript File]

Fig1.tif [Figure]

Fig2.tif [Figure]

Fig3.tif [Figure]

Fig4.tif [Figure]

Fig5.tif [Figure]

Fig6.tif [Figure]

Declarations of interest.docx [Conflict of Interest]

Supplementary information.docx [Supplementary Material]

To view all the submission files, including those not included in the PDF, click on the manuscript title on your EVISE Homepage, then click 'Download zip file'.

Research Data Related to this Submission

There are no linked research data sets for this submission. The following reason is given:
No data was used for the research described in the article

Dear Prof. Grosell,

As suggested by Prof. Elena Fabbri, member of the Editorial board of CBP, I send you the review 'Estrogenic compounds as exogenous modulators of physiological functions in mollusks: signaling pathways and biological responses' to be considered for publication in CBP part C.

The ms. is intended to provide the state of the art on the effects and mechanisms of action of estrogens in mollusks, bivalves in particular. Recent advances in sex steroid uptake and metabolism, and estrogen receptors in mollusks, as well as in estrogen signaling in vertebrates, are summarized. The pro- and cons- on the role of natural vertebrate estrogens as 'hormones' in regulation of physiological processes in mollusks is also considered.

Literature data obtained with different estrogenic compounds in the model bivalve Mytilus spp, from cellular to organism level, demonstrate specific effects on immune function, development and metabolism. Few original data are also reported on the effects of estrogenic compounds in *Mytilus* hemocytes and embryos. Overall, in vitro and in vivo data show, through independent lines of evidence, that estrogens present in the environment modulate different physiological functions acting through alternative, non-genomic signaling pathways.

Available information will provide a framework for a better evaluation and interpretation of the effects of estrogen exposure, that could be utilized in understanding the potential impact of estrogenic EDCs as ubiquitous contaminants of aquatic ecosystems.

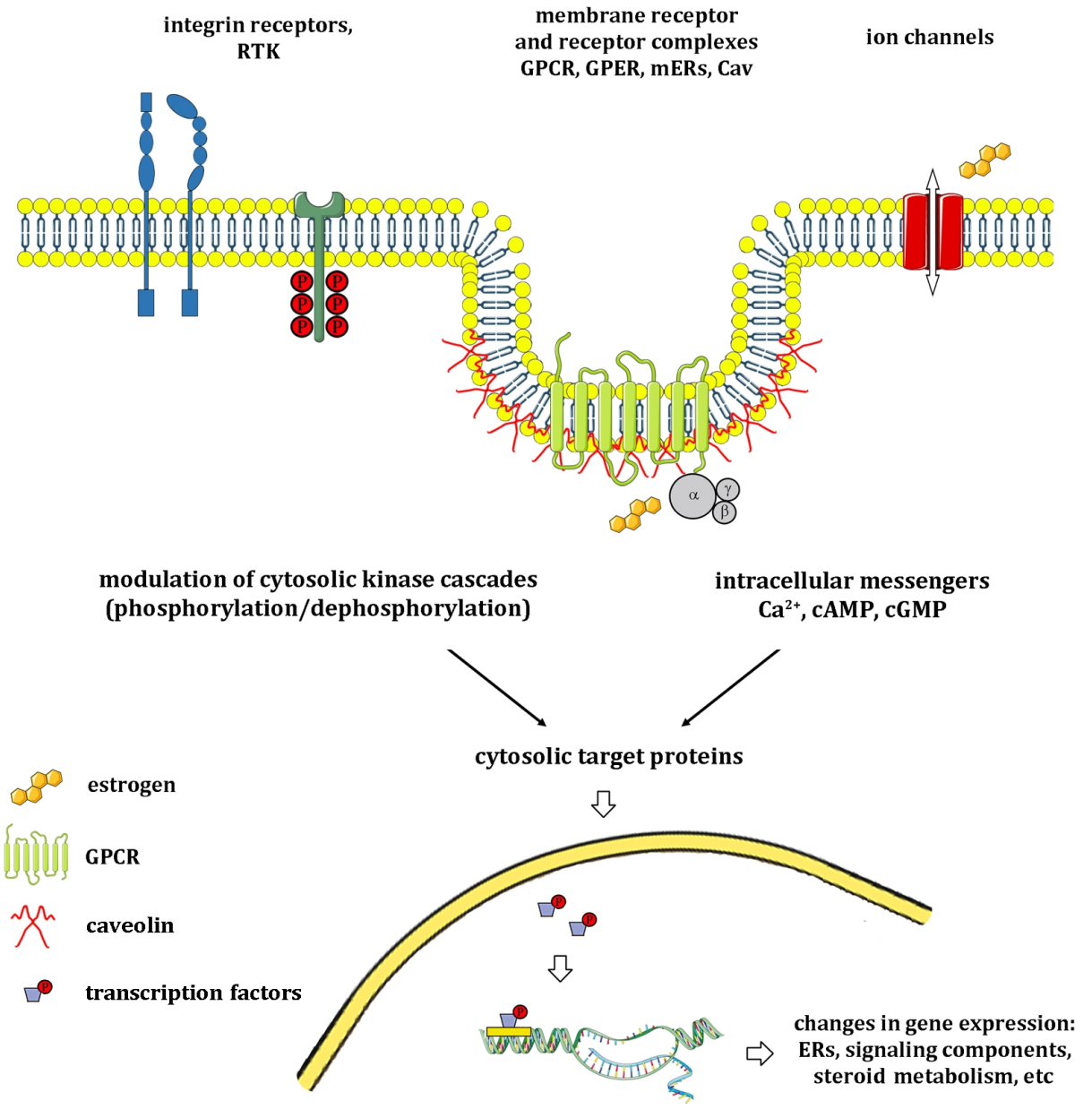
I thank you for your kind attention and look forward to hearing from you

Sincerely yours

Laura Canesi

Highlights

- Review on the effects/mechanisms of action of estrogenic compounds-EC in molluscs
- Their sources, uptake and metabolism, receptors/ signaling pathways are considered
- In Mytilus, different ECs affect immune function, development and metabolism.
- Independent lines of evidence that ECs act through alternative signaling pathways
- ECs can act as exogenous modulators of physiological functions


integrin receptors,
RTK
membrane receptor
and receptor complexes
GPCR, GPER, mERs, Cav
ion channels
P
α
γ
β
modulation of cytosolic kinase cascades
(phosphorylation/dephosphorylation)
intracellular messengers
Ca2+, cAMP, cGMP
cytosolic target proteins
estrogen
GPCR
caveolin
transcription factors
changes in gene expression:
ERs, signaling components,
steroid metabolism, etc

Invited Review article

Estrogenic compounds as exogenous modulators of physiological functions in molluscs: signaling pathways and biological responses

Teresa Balbi[1], Caterina Ciacci[2], Laura Canesi[1]

[1]*Dept. of Earth, Environment and Life Sciences (DISTAV), University of Genoa, Genoa, Italy*

[2]*Dept. of Biomolecular Sciences (DIBS), University 'Carlo Bo' of Urbino, Urbino, Italy*

Corresponding Author

Laura Canesi

Laura.Canesi@unige.it

Abstract

Molluscs have been widely utilized to evaluate the effects of estrogenic compounds, one the most widespread class of Endocrine Disrupting Chemicals-EDCs. However, knowledge on steroid signaling and metabolism in molluscs has considerably increased in the last decade: from these studies, a considerable debate emerged on the role of 'natural' steroids in physiology, in particular in reproduction, of this invertebrate group.

In this work, available information on the effects and mechanisms of action of estrogens in molluscs will be reviewed, with particular emphasis on bivalves that, widespread in aquatic ecosystems, are most likely affected by exposure to estrogenic EDCs. Recent advances in steroid uptake and metabolism, and estrogen receptors-ERs in molluscs, as well as in estrogen signaling in vertebrates, will be considered.

The results so far obtained with 17β-estradiol and different estrogenic compounds in the model bivalve *Mytilus* spp, demonstrate specific effects on immune function, development and metabolism. Transcriptomic data reveal alternative estrogen signaling pathways in mussel tissues that are supported by new observations at the cellular level. *In vitro* and *in vivo* data show, through independent lines of evidence, that estrogens act through alternative, non-genomic signaling pathways in bivalves. In this light, regardless of whether molluscs synthesize estrogens *de novo* or not, and despite their ERs are not directly activated by ligand binding, estrogens can interact with multiple signaling components, leading to modulation of different physiological functions. Increasing knowledge in endocrine physiology of molluscs will provide a framework for a better evaluation and interpretation of data on the impact of estrogenic EDCs in this invertebrate group.

Key words: *alternative signaling pathways, bivalves, development, estrogens, estrogen receptors, immunity, steroid metabolism, transcriptomics*

1. Introduction

Invertebrates, that represent about 95% of animal species and important components of aquatic ecosystems, are at considerable risk of exposure to Endocrine Disrupting Chemicals (EDCs), in particular to estrogenic compounds. For EDC assessment, assays with aquatic invertebrates would offer some practical advantages over vertebrates' (fewer ethical considerations, easier dose delivery, cheaper cultural requirements) that allow larger datasets to be collected. In contrast, available information on the impact of EDCs is still scattered, due to the large number of species, diversity of endocrine systems, scarcity of data on metabolism/mechanisms of action of natural hormones.

Among invertebrates, Molluscs, the 2nd largest animal Phylum, have been widely utilized to evaluate the biological effects of EDCs. Although validated tests are available in aquatic species based on reproduction (i.e. OECD TG 242/243), they are not specific for EDCs, and data from these tests can be used only for classification and hazard. However, knowledge on endocrine physiology in molluscs has considerably increased in the last decade, also thanks to the development of –omics technologies and molecular modelling, allowing for the identification of receptors/signaling pathways/effectors involved in steroid signaling and metabolism. From these studies, a considerable debate has emerged on the role of 'natural' or vertebrate steroids in mollusc physiology. This represents a key question to be answered in order to understand not only the basic endocrine mechanisms, but also the possible impact of EDCs, and in particular of estrogenic chemicals, in this invertebrate group.

In this work, we will provide an update on the effects and mechanisms of action of estrogenic compounds in molluscs, also reporting pros- and cons- on the role of natural vertebrate estrogens as 'hormones' in regulation of their physiological processes. We will mainly focus on bivalves that, due to their widespread occurrence in different aquatic environments, including estuarine and

coastal areas, are most likely affected by exposure to estrogenic EDCs, and therefore have been the subject of most extensive research (Canesi and Fabbri, 2015).

In particular, data on the effects of 17β-estradiol (E_2) in the marine bivalve *Mytilus* spp, in comparison with estrogenic EDCs, will be reviewed. The results obtained *in vitro*, utilizing the immune cells, the hemocytes, and *in vivo*, during early embryo development, will be integrated with those obtained from transcriptomics data at the tissue level, in adult mussels exposed to estrogenic compounds. Available knowledge on the possible mechanisms of action of estrogenic compounds, from molecular to organism level, will provide a framework for a better evaluation and interpretation of data on the effects of estrogen exposure in molluscs, that could help understanding the potential impact of estrogenic EDCs as ubiquitous contaminants of aquatic ecosystems.

2. Estrogens and molluscs

A PubMed search on estrogen literature (estrogen & molluscs) gave 326 hits, with the majority of publications in the last two decades (Fig. 1). Bivalves are the most represented group (52%), followed by gastropods (38%) and cephalopods (5%), with data reporting both effects and tissue levels not only of the natural estrogen 17β-estradiol (E_2), but also of synthetic estrogens and estrogenic chemicals (Fig. 1A). With regards to those papers specifically referring to 'estradiol & molluscs' (E_2 or 17α-ethynyl estradiol-EE), about 207 hits were found. Among these, if we look at the biological processes, 154 matches were found for 'estrogen & metabolism', 70 for 'reproduction', 60 for 'development', 15 for 'immune function'; if we consider the mechanisms of action, 56 hits were obtained, 41 for 'estrogen receptors', and 15 for 'signaling' (Fig. 1B). It is therefore evident that most research has been focused on metabolism, reproduction and development, and the underlying signaling mechanisms related to the occurrence of Estrogen Receptors-ERs, in analogy with those of vertebrates, including aquatic species. For example, several studies investigated the effects of estrogen exposure on induction of vitellogenin, a marker of endocrine disruption in fish. Actually, data indicating a correlation between fluctuations in sex

steroid tissue concentrations and the stage of gonad maturity in molluscs, previously suggested analogies with the main effects and mechanisms of action of natural estrogens in vertebrate systems (Scott, 2013 and references therein). A similar approach has been also used in other invertebrate groups (Porte et al., 2006; Crain et al., 2007; Scott, 2012, 2013; Canesi and Fabbri, 2015). However, from these studies also stemmed the still ongoing debate on the presence and physiological role of 'vertebrate' estrogens as endogenous modulators of physiological functions in invertebrates.

A first accurate analysis on the literature on the effects of steroids and EDCs concluded that there is no indisputable bioassay evidence that vertebrate sex steroids (or their agonist/antagonists) have endocrine or reproductive roles in molluscs (Scott, 2013). This conclusion was essentially based on 1) methodological criteria, which may lead to publication bias (e.g. lack of statistics, single experiments only, no concentration- response curves, confounding factors not corrected for, not independently validated, and emphasis on positive results only); 2) scientific criteria: molluscs have neither the enzymes necessary to synthesize vertebrate steroids nor nuclear receptors with which to respond to them (Scott, 2012).

Despite the relevant objections raised by these two review papers, other studies have been subsequently published in an attempt to demonstrate a relationship between sex steroid levels and reproduction in molluscs. However, more recent data obtained in bivalves seem to support Scott's arguments. In *Mytilus trossolus*, the content of sex steroids, including estrogens, was analyzed during gametogenesis (Zabrzańska et al., 2015; Smolarz et al., 2018). The Authors concluded that the observed fluctuations are unlikely to be caused by the reproductive cycle. but are rather of unknown nature, probably linked with environmental conditions, also indicated by the higher levels of steroids found in gills with respect to gonads. In the clam *Ruditapes decussatus*, prolonged exposure to high concentrations of E_2 (400 ng/L) increased both free and esterified E_2 concentration in tissues and led to two distinct effects. During exposure, gametogenesis was clearly activated in both males and females. However, in the long term, after transplantation of the clams in the field,

only females formerly exposed to E_2 showed lower health status, and ovaries with atretic oocytes. Overall, the results indicate a sex-specific effect of exposure to high E_2 and suggest either a direct or indirect role for E2 in *R. decussatus* reproduction (Mezghani-Chaari et al., 2017). Although these data appear to argue against evidence for estrogens acting as natural hormones in the control of gametogenesis, they further support their possible role as exogenous modulators of reproduction.

3. Steroid metabolism in molluscs

More recent information on steroid metabolism in invertebrates, including molluscs, has been provided by two recent key papers from independent research groups, addressing two main questions.

1) *do mollusc synthetize sex steroids?* The evolution of steroid metabolism was investigated by cladistic analysis (Markov et al., 2017). Estradiol synthesis pathway in vertebrates and distribution across metazoans of related biochemical features were thoroughly analyzed. Vertebrate estradiol synthesis from cholesterol is a multistep pathway involving side-chain cleavage of cholesterol to pregnenolone by the CYP11A enzyme, a complex suite of reactions from pregnenolone to testosterone, and aromatization by the CYP19A enzyme. CYP19A is already present in chordates, whereas CYP11A is vertebrate-specific. Aromatization has been described in cnidarians, molluscs, and cephalochordates. In cephalochordates, CYP19A may catalyze the aromatization reaction, but in molluscs and cnidarians the reaction would involve a different enzyme, possibly a paralogous CYP. Because there is no consistent evidence for side-chain cleavage activities in these organisms, the exact endogenous substrate of those aromatizing enzymes remains elusive. Despite this, with the advance of –omics technologies and genome sequencing, other studies have focused on steroidogenesis-related molecules in molluscs. The steroidogenesis pathway previously proposed by Janer and Porte (2007) in bivalves was re-examined by a study where the *Mytilus edulis* transcriptome was explored for gene homologues to steroidogenesis genes in vertebrates; the analysis successfully identified the majority of genes in this pathway, except for aromatase (Blalock

et al., 2018). In a recent study on steroidogenesis-related genes in gonadal tissues of the scallop *Mizuhopecten yessoensis,* it was postulated that, if P450 aromatase is not a member of the aromatizing gene family in invertebrate phyla, then invertebrates could possess other ancestral types of cyp genes for use in aromatization; the cyp1 and cyp3 genes are thought to represent likely candidates (Thitiphuree et al., 2019). However, at present, in the absence of identification of the enzyme(s) responsible for the conversion of testosterone to 17β-estradiol, the endogenous source for vertebrate-like estrogens,cannot be demonstrated in molluscs.

2) *if molluscs do not have the enzymes of sex steroid synthesis, what is the origin of estrogens and other steroids found in their tissues?* It has been established that molluscs metabolize steroids into more apolar products by esterification with fatty acids, which leads to their storage in lipid rich tissues. This process likely acts as a homeostatic regulation of the free, potentially bioactive form of steroids (Janer et al., 2005; Janer and Porte, 2007; Labadie et al., 2007; Giusti and Joaquim-Justo, 2013). For estrogens, this question was addressed by a paper from Scott and co-workers, on uptake, metabolism and depuration of 17β-estradiol in the model marine bivalve *Mytilus* spp. (Schwarz et al., 2017a). It was demonstrated that mussels have a large capacity for uptake of E_2 from water, and that E_2 is esterified and retained in soft tissues. In the light of these results, the fact that vertebrate steroids can be measured in invertebrates is still used as evidence of their endogenous biosynthesis, and that their observed variations are consistent with their perceived role in the endocrine control of reproduction, were debated. The Authors concluded that the source of tissue E_2 is exogenous based on two main observations: 1) the ability to absorb E_2 from water (with the rate of uptake being hardly affected by the actual exposure concentration) is so great that it is not possible to use the presence of E_2 as evidence for their endogenous synthesis; 2) the E_2 absorbed by the mussels mostly remains intact (i.e. it is not heavily metabolized). Moreover, a fast decrease of labelled E_2 in exposure medium (from 4 h) was observed at very low concentrations (2 ng/L), demonstrating the high capacity of molluscs to take up the E_2 present in medium at environmental levels. Mussels also have a very high capacity for uptake and retention of waterborne Testosterone and Progesterone;

however, both compounds undergo more extensive metabolism *in vivo,* and their concentrations in the aquatic environment are likely to be much lower than those of E_2 (Schwarz et al., 2017b, 2017c). In the light of these data, the value of measuring vertebrate steroids, including estrogens, in invertebrates, was recently discussed (Scott, 2018). On the basis of available evidence, it was concluded that 'if invertebrates, and molluscs in particular, can readily absorb vertebrate steroids from the environment, are able to conjugate them to fatty acids with great efficiency, and subsequently retain them for a very long time, this, together with the lack of biosynthetic enzymes, cast strong doubts on those data claiming that steroid concentrations are functionally linked to reproductive cycles' (Scott, 2018).

4. Estrogen receptors in molluscs

With regards to the presence of steroid receptors, a gene that is the orthologue of the vertebrate estrogen nuclear receptor (ER) is present in mollusc genomes; however, it has been shown in several species that ERs have constitutive transcriptional activity, and are not activated by ligand binding. The ERs of the sea slug *Aplysia californica*, the cephalopod *Octopus vulgaris*, the clam *Thais clavigera*, the oyster *Crassostrea gigas*, the mussel *Mytilus edulis*, have all been shown experimentally to activate transcription at high levels in the absence of any added ligands; furthermore, they do not bind estrogens, and their transcriptional activity is unaffected by estrogen addition (Thornton, 2001; Keay et al., 2006; Bannister et al., 2013; Nagasawa et al., 2015; Tran et al., 2016). The mechanisms by which the mollusc ERs constitutive transcriptional activity evolved were thoroughly investigated in *C. gigas*, combining structural and genetic analysis (Bridgham et al., 2014). The results indicate that two ancient mutations conferred full constitutive activity, shifting the equilibrium towards the active conformation even in the absence of ligand. Additional mutations further filled the ligand cavity and further stabilized the activation conformation. The structural characteristics required for a hormone-sensitive activator (allosteric modulation) were thus lost, with no apparent functional effect on the receptor's functional output. Overall, the results

supported the conclusion that ligand-independence evolved in molluscs from an estrogen-sensitive ancestral state (Thornton, 2001; Keay et al., 2006; Bridgham et al., 2014; Markov et al., 2017).

In *C. gigas*, other nuclear receptors have been recently identified, in particular two isoforms of the retinoid X receptor, CgRXR-1 and CgRXR-2, and a retinoic acid receptor ortholog CgRAR (Vogeler et al., 2017). Computer modelling of each receptor based on 3D crystal structures of human proteins was used to predict their ability to bind to different ligands in silico. All three *C. gigas* receptors possess a LBD, including ligand binding sites, and sequence consensus between oyster, other molluscs, and human RXRs are very high for the LBD; however, six residues in the LBD of the oyster RAR prevent binding of retinoids in the required conformation. Similarly, neither two gastropod receptors, RcRAR or NlRAR, respond to retinoids *in vitro* (Gutierrez-Mazariegos et al., 2014). Overall, these data support the theory of ligand binding loss not only for molluscan ERs, but also for RARs.

However, in spite of its ligand-independence, a potential role for the ER in estrogen signaling cannot be fully dismissed (Tran et al., 2016, 2017 and refs. quoted therein). In different molluscan species, ERs respond to exogenous estrogens with an increase in their mRNA expression at tissue level in adults (Canesi et al., 2007a,b; Ciocan et al., 2010; Stange et al., 2012; Völker et al., 2014; Nagasawa et al., 2015), as well as in early developing embryos (Balbi et al., 2016) (see below). Moreover, correlations between E_2 levels and ER mRNA and have been observed in the ovary of oysters during gametogenesis (Ni et al., 2013), although, as discussed above, this cannot be considered as a proof of the hormonal action for estrogen in reproduction. According to Tran et al. (2016), assuming that the upregulation of ER mRNA is reflected at the protein level and that the rate of ER-dependent transcription is limited by ER availability, these findings suggest that ER mRNA upregulation could contribute, at least in part, to the activation of expression of its target genes. But how can exogenous estrogens modulate ER expression?

Since none of the NRs in the molluscan genome has been found to bind estrogens (Vogeler et al., 2014, 2017), such an effect may be part of a receptor independent pathway and/or may involve

signaling components or ER forms possibly located on the plasma membrane and/or in cytosol. In *Mytilus*, the possible involvement of ERs was first suggested by *in vitro* data showing that in different experimental systems (isolated ganglia, hemocytes) classical antagonist of mammalian nuclear ERs (Tamoxifen, ICI 182780) reduced or abolished the effects of E_2 (Stefano et al., 2003; Canesi et al., 2004a). These observations, made before the identification and characterization of *M. edulis* ERs (Puinean et al., 2006; Nagasawa et al., 2015), were recently confirmed in cultured ovarian explants (Tran et. al., 2016). However, also in mammals, the tissue specificity ER modulators can depend on differential expression of ER subtypes (i.e., ERα and ERβ), of co-regulatory proteins (i.e., co-activators and co-repressors), and varying ER conformational changes induced by ligand binding (Martinkovich et al., 2014). Whatever the source of estrogens in molluscs, knowledge on their mechanisms of action leading to biological responses has been hampered by the lack of suitable *in vitro* models for investigating estrogen signaling at the cellular level.

Overall, the most recent evidence seems to confirm that the definition of estrogens as 'hormones' cannot be applied in molluscs as in vertebrate systems. However, natural estrogens, estrone (E1), 17β-estradiol (E2) and estriol (E3) are discharged consistently and directly into surface waters with wastewater treatment plants (WWPTs) effluents, disposal sludge and in storm-water runoff (Nazari and Suja, 2016). Estrogens are therefore ubiquitous in the aquatic environment at ng/L concentrations (Tiedeken et al., 2017) and their impact on humans, animals and plants is well documented (Adeel et al., 2017). In particular, estrone-E_1; 17β-estradiol-E_2; and 17-α-ethinylestradiol-EE, have been included in a Watch List of contaminants of emerging concern (CECs) for European Union monitoring of surface waters (Decision 2015/495).

In this light, information on whether and how estrogenic compounds can act in molluscs, the mechanisms of action and pathways involved, and the resulting effects on different target cells and tissues, will help understanding their possible impact as environmental or hexogenous modulators of physiological processes in this relevant invertebrate group.

5. Alternative pathways of Estrogen signaling in vertebrate systems

Mammalian studies have widely demonstrated that ligand binding to nuclear receptors, including ERs, through the 'classical' genomic mechanism, is not the only way to activate steroid signaling. Estrogens can also trigger rapid (within seconds to minutes) effects through non-genomic or ''alternative pathways'' (for most recent reviews see Saczko et al., 2017; Levin, 2018; Stefkovich et al., 2018). These may be initiated at either membrane or cytosolic locations and include lipid modification of the ER (palmitoylation), and interactions with membrane and cytoplasmic adaptor proteins and other signaling molecules (kinases and phosphatases). These complex interactions enable crosstalk among different signaling cascades that can result in both direct local effects (such as modification of ion fluxes) and regulation of gene transcription secondary to activation of kinase pathways (involving cAMP, MAPKs, PKC and PKA, PI-3K, etc.). Elicited responses depend upon the cell type studied and the conditions used; however, rapid changes in phosphorylation state of mitogen activated protein kinases (MAPKs) and in cytosolic [Ca^{2+}] are among the most common events observed in non-genomic effects of E_2. A number of studies on the mechanisms of estrogen action provided evidence that non-genomic and genomic effects converge on modulation of gene transcription (Saczko et al., 2017). However, in contrast to nuclear or genomic estrogen signaling, the events initiating the non genomic signaling pathways of E_2 are not yet completely understood even in mammalian cells. It was soon apparent that both mammalian ERs (ERα and ERβ) or novel receptors may also be responsible for the rapid, membrane-associated effects of estrogens. Due to the multiple and critical roles of estrogens in physiology and pathophysiology, the role, location, and molecular nature of these receptors in different cell types has been the focus of intensive research in the two last decades (Levin, 2018).

5.1 Role of GPER in estrogen signaling

In the mid-1990s, several laboratories independently reported the cloning of an orphan G protein-coupled receptor from different mammalian cells that was named GPR30 (reviewed in Barton, 2012, Barton et al., 2018). Research published between 2000 and 2005 provided evidence that GPR30 binds and signals via estrogen, indicating that this intracellular receptor is involved in rapid, non-genomic estrogen signaling. In particular, GPR30 was shown to act independently of estrogen receptors, ERα and ERβ, and probably functions as a heptahelical ER that activates cell signaling cascades initiated by estrogens and convey intracellular signals via EGF receptors (Filardo and Thomas, 2005). Thus, through GPR30 estrogens can signal by the same mechanism as various other hormones. This field of research was greatly stimulated by studies that described the binding of estrogen to GPR30-expressing cell membranes, followed by the identification of a GPR30-selective agonist (that lacked binding and activity towards ERα and ERβ) (Prossniz and Arterburn, 2015; Barton et al., 2018). Renamed GPER (G protein-coupled estrogen receptor) in 2007, there are now over 1000 publications in PubMED on this receptor (Barton et al., 2018).

GPER mediates the rapid activation of multiple signaling pathways, including MAPK, PI3K, Ca^{2+} and cAMP, that can in turn regulate gene expression (Barton, 2012, Barton et al., 2018). In the last decade, evidence has emerged towards the role of GPER in the pleiotropic effects of estrogens on mammalian physiology (Sharma et al., 2018). GPER is capable of coordinating the activation of plasma membrane receptors that regulate adhesion to provisional extracellular matrices (integrin) and responsiveness to epidermal growth factor (EGF), critical events for cell survival (Gaudet et al., 2015). However, also in mammalian systems, GPER-mediated E2 signaling and effects depend on the type of tissue, the abundance of receptor types and cross talk between different receptor types (Sharma et al., 2018). Although GPER is presumed to be located on the plasma membrane as most GPCRs are, many reports observed that in diverse cells and tissues, GPER is predominantly, though not exclusively, localized to intracellular membranes, particularly those of the endoplasmic reticulum and Golgi apparatus (Sharma et al., 2018).

With regards to non mammalian systems, conserved estrogen binding and signaling functions of the GPER was first demonstrated in fish (Thomas et al., 2010). Subsequent studies showed the role of GPER in mediating multiple effects of estrogens in different fish cells and tissues, with up to 36 publications to date in PubMed. Comparison of the partial sequence of goldfish GPR30 predicted protein with full-length zebrafish, Atlantic croaker, zebra finch, rat, and human proteins showed that GPR30 is highly conserved among vertebrates (Mangiamele et al., 2017).

However, also when performing experiments with E_2 in vertebrate models, it is difficult to ascribe the effects to a particular receptor, since E_2 binds to all the three estrogen receptors (ERα, ERβ and GPER). Moreover, it is now clear that the combined rapid and genomic effects of estrogen are critical to its overall function, and that even in the absence of estrogen (i.e. in the unliganded state), "classical" ERs and GPER exert some constitutive (i.e. ligand-independent) functions on physiology and disease (Barton et al., 2018).

Given the complexity of estrogen signaling in vertebrate systems, unraveling the pathways of estrogen action in invertebrates, including molluscs, has proven a difficult task. Most available data are from the marine bivalve, the mussel *Mytilus* spp. Due to their widespread occurrence in coastal areas, characterized by rapidly fluctuating environmental conditions, they have long been utilized for evaluating the responses, from molecular to organism level, to a variety of chemicals (Beyer et al., 2017 and refs. quoted therein).

6. Rapid Estrogen signaling in *Mytilus* hemocytes

In bivalves, hemocytes are responsible for the innate immune response, together with soluble hemolymph factors, that operate in a co-ordinated way to provide protection from invading micro-organisms and non-self materials (Canesi et al., 2002; Canesi and Pruzzo, 2016). The mechanisms involved in activation of immune signaling have been thoroughly investigated (reviewed in Canesi et al., 2006a; Canesi and Pruzzo, 2016). The first evidence of estrogen action in these cells was provided in the mid 2000's, showing that E_2, in a narrow concentration range (5-50 nM) induced a

dose-dependent destabilization of lysosomal membranes. E_2 also induced significant morphological changes in hemocytes, indicating activation, and stimulated oxidative burst, intracellular ROS production, lysozyme release and NO production (Canesi et al., 2004a, 2006b, 2007a). In the same experimental conditions, it was later shown that E_2 affected hemocyte adhesion to different substrates, and increased a2 integrin subunit levels (Koutsogiannaki and Kaloyianni, 2011). All the effects of E_2 occurred very rapidly, from 5 to 30-60 min post addition, and were mediated by non genomic mechanisms of action similar to those identified in mammalian cells; these involved increase of cytosolic Ca^{2+}, activation of MAPKs (ERK, p38 and JNK) and PKC, and phosphorylation of the transcription factors STAT3, STAT5 and CREB (Canesi et al., 2004a, 2006b, 2007b; Porte et al., 2006).

The *in vitro* effects of estrogens on mussel hemocytes were further investigated testing E_2 in comparison with different estrogenic compounds, 17α-ethynyl estradiol (EE), mestranol (MES), nonylphenol (NP), nonylphenol monoethoxylate carboxylate (NP1EC), bisphenol A (BPA), benzophenone (BP), in a wide concentration range, on lysosomal membrane stability-LMS (Canesi et al., 2007a). For LMS, a complete dose-response curve was observed, allowing to calculate the NOEC, LOEC and EC_{50} for all compounds. For E_2, values of 1, 5, and 13.34 nM were obtained, respectively; much higher EC_{50} values (at μMolar concentrations) were recorded for both synthetic estrogens and estrogenic chemicals. Interestingly, unexpected values were obtained with respect to the known affinity of different estrogens for mammalian ERs; for example, for the synthetic estrogens EE and MES, the EC_{50} values were 1000 times higher than those of E_2, and comparable to those of weak estrogenic chemicals such as for example, BPA. LMS is a functional endpoint characterized by a well-known monotonic response, that decreases at increasing concentration of chemicals or stress conditions (Martínez-Gómez et al., 2015). This is accordance with the conclusions of Scott (2013), that considered the effects on LMS the only independent observation of estrogen specific effect validated in molluscs. Although this is not as a proof that estradiol acts as a hormone in mussel hemocytes, E_2 also affected other functional parameters of mussel hemocytes,

indicating multiple immunomodulatory effects of estrogens similar to those observed in mammalian systems (Kovats, 2015). Indeed, E_2 also showed a higher potency on phagocytosis, oxidative burst or lysozyme release, with respect to other estrogenic compounds, with effects observed at nM and μM concentrations, respectively (Canesi et al., 2004a,b, 2006b, 2007a). However, these immune related endpoints typically show non-monotonic responses to a wide range of stimulators/inhibitors in mussel hemocytes (Canesi and Pruzzo, 2016; Canesi et al., 2016) and EC_{50} values could not be therefore calculated. In this light, these data could be not considered as fulfilling the correct methodological requirements for demonstrating a specific estrogen-dependent effect (Scott, 2013).

Interestingly, as summarized in Fig. 2, all functional parameters rapidly affected by E_2 exposure were prevented by cell pre-treatment with the anti-estrogen Tamoxifen (TAM) at concentrations comparable with those utilized in mammalian cells (Canesi et al., 2004a, 2006b). The results suggested a possible role for ER-related sequences responsive to TAM in mediating the rapid effects of E_2. Immunoreactive ERα and ERβ bands were identified by Western blot analysis of soluble hemocyte protein extracts using mammalian anti-ERα and anti-ERβ antibodies directed against a specific region in the NH_2-terminal domain of either form (Canesi et al., 2004a). When their intracellular localization was investigated, ER-α immunoreactivity was diffused in the nuclei and cytoplasm, whereas that of ER-β was exclusively extranuclear, showing a granular or punctate pattern that suggested association with plasma membrane patches and intracellular vesicles, these latter mainly concentrated in the perinuclear region (Canesi et al., 2006b).

The immunomodulatory effects of E_2 were confirmed *in vivo*, indicating lysosomal destabilization, changes in phagocytic activity and lysozyme activity; overall, the effects were observed in a narrow concentration range, with stimulation of immune responses at low concentrations (5-25 nM) and inhibition at concentrations higher than 50 nM. The *in vivo* effects of E_2 were evaluated at 6 and 24 h post injection, probably bypassing the *in vivo* homeostatic control of E_2 levels by esterification in the tissues (Janer et al., 2005; Schwarz et al., 2017a).

When the *in vitro* effects of E_2 on kinase mediated transduction pathways were compared with those of synthetic and environmental estrogens, different components involved in immune signaling were identified as common targets for all compounds; however, whereas E_2 induced activation (phosphorylation) of different cytosolic kinases and transcription factors, exposure to estrogenic chemicals resulted in general downregulation of immune signaling (reviewed in Porte et al., 2006). These effects were also observed *in vivo*; in particular, large decreases in p38 MAPK phosphorylation were observed in mussels injected with both BPA and nonylphenol-NP (Canesi et al., 2005, 2007b).

6. Transcriptomics reveal alternative estrogen signaling pathways in mussel tissues that address further studies at the cellular level

In molluscs, the hepatopancreas or digestive gland plays a key role in metabolism and nutrient distribution to the gonad during gametogenesis. In *M. galloprovincialis* digestive gland, E_2 was first shown to modulate expression of ERs, as well as the lysosomal function, and lipid and glucose metabolism (Canesi et al., 2007c). Similar results were obtained with BPA (Canesi et al., 2007d). Microarray data later showed that E_2 administration resulted in the modulation of 44 genes (about 2.5% of total 1700 sequences present in the array) (Canesi et al., 2011). Functional genomics indicated that about 50% of the annotated DEGs found in response to E_2 were involved in primary metabolic processes, lipid catabolism in particular, and underlined the occurrence of virtual biological processes and molecular functions typical of a hormone-induced response. Indeed, specific GO terms such as ‘‘hormone response’’, ‘‘receptor activity’’, ‘‘vasculogenesis’’ and ‘‘heart development’’ were over-represented in the E_2 DEG list (Canesi et al., 2011). As for the *in vivo* effects on immune function, the results were obtained after 24 h injection with E_2. However, these represent the only data available to date on the effects of E_2 exposure on transcriptional changes at the tissue level in molluscs.

More recently, a much more extensive and thorough transcriptomic approach was utilized to evaluate the effects of chronic exposure (32 and 39 days) to different concentrations of the estrogenic chemicals EE and NP in the digestive gland of *M. edulis* (Blalock et al., 2018). The results show 1,735 total DEGs, indicating that exposed samples grouped together across different treatment conditions and sex. Network and targeted analyses identified the steroidogenesis pathway and non genomic estrogen signaling pathway as the likely mechanisms of action for a putative Adverse Outcome Pathway-AOP for estrogenic compounds in mussels. Network biology was also utilized to examine pathways and genes that may be influencing reproduction and development. In both females and males networks identified down-regulated genes with GO terms connected to steroid metabolic process, cell cycle, response to stress, development and up-regulated genes involved in response to steroid hormone stimulus, steroid metabolic process, regulation of intracellular protein kinase cascade, cell cycle, and phosphorylation. The results also showed that StAR (steroidogenic acute regulatory protein), that controls the rate-limiting step of steroidogenesis, was among the most highly DEGs, clearly indicating interference with steroid metabolism. Interestingly, when different biomarker genes were selected to validate the microarray results by RT-qPCR, Cav-1 was the most highly differentially expressed gene in non genomic signaling pathways in both EE and NP exposure groups. Cav-1 was also identified as a hub protein in both male and female up-regulated subnetworks.

Moreover, the observed DEGs seemed to span several functions of stress response including production of protective/stress proteins, DNA repair mechanisms, activation of cell cycle checkpoints, biotransformation, and apoptosis. Therefore, players involved in the non genomic estrogen signaling pathway may cross-talk with other pathways such as stress response (Blalock et al., 2018). These results are in line with those previously obtained in the digestive gland with E_2 and BPA, indicating lysosomal membrane destabilization and accumulation of neutral lipids, that are considered as general stress biomarkers (Canesi et al., 2007c,d). A recent *in vitro* study in isolated digestive cells showed that BPA can rapidly affect lipid metabolism (Balbi et al., 2017). Exposure

to BPA from 3 h induced accumulation of intracellular Triglycerides (TAGs) and of both neutral and polar lipids, paralleled by a net decrease in TAG secretion. The effects were prevented by cell pretreatment by specific inhibitors of PI-3K and ERK MAPKs, indicating that activation of kinase pathways is involved also in mediating the rapid metabolic effects of BPA (Balbi et al., 2017).

Overall, the results obtained so far in *Mytilus spp*. underline the key role of rapid signaling pathways in the multiple effects of estrogenic compounds in bivalves. Together with the increasing information on the rapid non genomic mechanisms of action of estrogenic compounds in vertebrates, these data address further studies in bivalve cells. For example, the results obtained by Blalock et al. (2018), underlined the role of Caveolin in the response to EDCs in mussel digestive gland. Caveolin-1 is the main structural component of caveolae, functional microdomains that cluster related signaling molecules in the plasma membrane, including ERs, G protein–coupled receptors-GPCRs, Src tyrosine kinases; interactions between caveolins and ERs are critical in activation of a multitude of intracellular signaling pathways activated by estrogenic compounds (Kiss et al., 2005; Luoma et al., 2008; Patel et al., 2008; Watson et al., 2012). In this light, we investigated the possible presence of caveolin in mussel hemocytes using heterologous anti-Cav-1 antibodies. As shown in representative images reported in Fig. S1, a distinct distribution of total and phospho-Cav immunoreactivity was observed, with the latter only localized outside the nucleus (Fig S1, a and b). Moreover, p-Cav-1 showed partial colocalization with ERβ immunoreactivity in punctated patches and perinuclear vesicles (Fig S1 d). These observations suggest that Caveolin may participate in rapid E_2 signaling also in mussel hemocytes; preliminary data obtained by Western Blotting indicate that E_2 exposure rapidly increases the level of p-Cav (not shown).

The results of microarray analysis in mussel digestive gland (Canesi et al., 2011; Blalock et al., 2018) also indicated that exposure to estrogenic compounds resulted in several DEGs related to hormone response and receptor activity, including GPCR. Screening of genomic and transcriptomic data indicate the presence of multiple GPCR sequences in molluscs, with similarities for receptors for a large number of peptides, whose function has been related to reproduction, development and

phototransduction (reviewed in Cardoso et al., 2016). Unfortunately, no GPER-like sequences have been identified so far. However, the molluscan ER is an example of how the presence of homologous receptor sequences does not imply a homologous mechanism of action in vertebrate and invertebrate systems. Moreover, all components involved in membrane activated E_2 signaling (MAPKs, PI3K, PKC, Ca^{2+}, GPRC, cAMP) (reviewed in Canesi et al. 2007b; Fabbri and Capuzzo, 2010) as well as EGF-mediated pathways (Canesi et al., 2000) and integrin receptors (Koutsogiannaki and Kaloyianni, 2011; Maiorova and Odintsova, 2015) are present in mussel hemocytes. In this light, the involvement of putative GPER may further contribute to explain the rapid effects of E_2 in these cells. In both mammalian and non mammalian systems, the identification and characterization of selective agonists (G1) and antagonists (G15 and G36) of GPER greatly helped in the assessment of GPER function (Prossniz and Arterburn, 2015). In fish granulocytes, the use of G1 allowed to demonstrate, for the first time, that estrogens are able to modulate vertebrate granulocyte functions through a GPER/cAMP/protein kinase A/CREB signaling pathway (Cabas et al., 2013).

The effects of GPER modulators were therefore tested in mussel hemocytes. Cells were pre-treated with G15 (100 nM) before exposure to E_2, in the same conditions previously utilized (25 nM, 30 min) (Canesi et al., 2004a, 2006b) and LMS was evaluated (see SI for Methods). As shown in Fig. 3, G15 fully prevented the E_2-induced lysosomal membrane destabilization. The results were confirmed by CLSM using Lysotracker Green, a fluorescent dye that stains acidic compartments in live cells. E_2 induced lysosome enlargement and fusion events, and the effects were prevented by G15. Moreover, the GPER agonist G1 alone induced effects similar to those of E_2 (Fig. 4, upper panel). E_2 also induced cytoskeletal alterations, as shown by actin green immunostaining (Fig. 4, lower panel); in particular, E_2 caused disappearance of actin filaments in pseudopodia and actin condensation, in line with the previously observed changes in cell shape and adhesion (Canesi et al., 2004a), also involving integrin signaling (Koutsogiannaki and Kaloyianni, 2011). The effect of E_2 was fully prevented by G15; in contrast, the effect of G1 was similar to that of E_2. These data

represent the first indication of a role of putative GPERs in mediating the response to E_2 in invertebrate cells.

7. Effects of Estrogenic compounds on mussel early embryo development

The effects of estrogenic compounds at the whole organism level were investigated on *Mytilus* early embryo development, utilizing the adaptation of the standard embryotoxicity assay, that evaluates the development of normal D-veligers at 48 h post fertilization, for the screening of emerging contaminants (Fabbri et al., 2014). The phenotypical changes induced by E_2 were evaluated in a wide concentration range: E_2 induced a dose-dependent developmental delay, with an increasing percentage of immature embryos (trocophorae and pre-veligers) and an EC_{50} of 6.9 µg/L, corresponding to 25 nM (Balbi et al., 2016). The results demonstrate that E_2 affects embryo development in *Mytilus*, with data fulfilling all the methodological criteria indicated by Scott (2013). Interestingly, the *Mytilus* ER isoforms MeER1 and MeER2 were among the most upregulated genes across early development, from fertilized eggs to 48 hpf (Fig. 5A), indicating a physiological role for these receptors in early stages. Exposure to E_2 affected gene transcription at different times post-fertilization, with downregulation of both MeER isoforms (Fig. 5B), of the serotonin receptor (5-HTR) and immune-related genes (Balbi et al., 2016). DISTLM analysis revealed that E_2 treatment accounted for 7.5% of total variation, and affected transcription at both times pf. For the xenoestrogen BPA, similar EC_{50} values were obtained in the embryotoxicity assay (3.7 µg/L, corresponding to 16 nM); the same applies to changes in gene expression, in particular downregulation of MeER isoforms (Balbi et al., 2016). Recent data also indicate that the phenotypical effects of both E_2 and BPA on early embryo development are associated with changes in expression of tyrosinase, a key enzyme in shell matrix deposition in the first steps of shell formation (Miglioli et al., 2018, ms. in preparation).

The effects of E_2 and BPA on embryo development (Balbi et al., 2016) were compared with those of their respective analogs, the synthetic estrogen 17α-ethynyl estradiol (EE), and the BPA

substitute Bisphenol F (BPF). Interestingly, neither compound was effective at concentrations lower than 100 µg/L (Fig. 6). It is worth noting that the results obtained on embryo development, at the whole organism level, indicate different relative potencies of different estrogenic chemicals with respect to those observed at the cellular level measuring LMS in the hemocytes (Canesi et al., 2007a). Overall, these data clearly underline that in mussels the effects and relative potencies of different estrogenic compounds (natural vertebrate steroids, synthetic or environmental estrogens) cannot be predicted on the basis of specific molecular events as in vertebrate systems (i.e. their affinity for ERs). Different outcomes can depend on the exposure conditions (concentrations and times of exposure) and, most of all, on different uptake and metabolism of individual compounds at different levels of biological organization (cell, tissue, organism). In this light, data on uptake and metabolism of different estrogenic compounds may contribute to explain the outcome of exposure in different experimental conditions.

8. Conclusions and perspectives

Overall, the results obtained so far demonstrate through independent lines of evidence that, regardless of whether bivalves synthesize estrogens *de novo,* or they rely on uptake of exogenous estrogens from their environment, they are clearly bioactive compounds in bivalves that can modulate different physiological functions at environmental concentrations.

In mussels, the natural vertebrate estrogen 17β-estradiol seems to represent to date the most powerful estrogen: this could be due to the high uptake and specific metabolism of exogenous E_2 (Schwarz et al. 2017a and refs. therein), as well as to the capacity of E_2 to interact at the cellular level through rapid signaling pathways, thus activating different biological responses (Canesi et al., 2006b, 2007b; Porte et al., 2006 and refs. therein). In this regard, the results of transcriptomics obtained *in vivo* (Blalock et al., 2018) and additional *in vitro* data obtained in hemocytes, although preliminary (this work), further support the role of multiple signaling components in mediating the alternative pathways of estrogen action in mussels. In this light, together with the knowledge that

molluscan ERs are not activated by estrogen binding, ruling out direct nuclear effects on transcription, the possible signaling pathways involved in the cellular action of natural and environmental estrogens previously proposed (Porte et al., 2006) should be revisited. Whatever the initiating event, and/or the molecular components involved in triggering the action of estrogens at the plasma membrane or at intracellular membrane sites, they can interact with multiple regulatory pathways in mussel cells, leading to modulation of their physiological functions.

Further research on identification of estrogen signaling in molluscan cells is needed: despite the obvious limitations of utilizing heterologous antibodies for receptors, signaling components, and effectors, as well as of agonist/antagonist of mammalian receptors, their use can still pave the way for understanding the conservation of these pathways in invertebrate cells. Actually, several components of cell signaling, including membrane receptors, kinase components, transcription factors, whose putative role had been indicated more than 10 years ago by functional assays or indirect methods have been subsequently identified by –omic approaches. Next-Generation Sequencing (NGS) technologies have significantly increased our basic knowledge on the genomes of several species of bivalves (Murgarella et al., 2016). Their application in different molluscs will provide further information on the mechanisms of action of estrogenic compounds in this invertebrate group.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

Adeel, M., Song, X., Wang, Y., Francis, D., Yang, T., 2017. Environmental impact of estrogens on human, animal and plant life: A critical review. Environ. Int. 99, 107-119.

Balbi, T., Franzellitti, S., Fabbri, R., Montagna, M., Fabbri, E., Canesi, L., 2016. Impact of bisphenol A (BPA) on early embryo development in the marine bivalve *Mytilus*: effects on gene transcription. Environ. Pollut. 218, 996-1004.

Balbi, T., Ciacci, C., Grasselli, E., Smerilli, A., Voci, A., Canesi, L., 2017. Utilization of *Mytilus* digestive gland cells for the *in vitro* screening of potential metabolic disruptors in aquatic invertebrates. Comp. Biochem. Physiol. C 191, 26-35.

Bannister, R., Beresford, N., Grangerc, D.W., Pounds, N.A., Rand-Weaver, M., White, R., Jobling, S., Routledge, E.J., 2013. No substantial changes in estrogen receptor and estrogen-related receptor orthologue gene transcription in *Marisa cornuarietis* exposed to estrogenic chemicals, Aquat. Toxicol. 140-141, 19-26.

Barton, M., 2012. Position paper: The membrane estrogen receptor GPER – Clues and questions. Steroids 77, 935-942.

Barton, M., Filardo, E.J., Lolait, S.J., Thomas, P., Maggiolini, M., Prossnitz, E.R., 2018. Twenty years of the G protein-coupled estrogen receptor GPER: Historical and personal perspectives. J. Steroid Biochem. Mol. Biol. 176, 4-15.

Beyer, J., Green, N.W., Brooks, S., Allan, I.J., Ruus, A., Gomes, T., Bråte, I.L.N, Schoyen, M., 2017. Blue mussels (Mytilus edulis spp.) as sentinel organisms in coastal pollution monitoring: A review. Mar. Environ. Res. 130, 338-365.

Blalock, B.J., Robinson, W.E., Loguinov, A., Vulpe, C.D., Krick, K.S., Poynton, H.C., 2018. Transcriptomic and network analyses reveal mechanistic-based biomarkers of endocrine disruption in the marine mussel, *Mytilus edulis*. Environ. Sci. Technol. 52, 9419-9430.

Bridgham, J.T., Keay, J., Ortlund, E.A., Thornton, J.W., 2014. Vestigialization of an allosteric switch: genetic and structural mechanisms for the evolution of constitutive activity in a steroid hormone receptor. PLoS Genet. 10, e1004058.

Cabas, I., Rodenas, M.C., Abellán, E., Meseguer, J., Mulero, V., García-Ayala, A., 2013. Estrogen signaling through the G protein-coupled estrogen receptor regulates granulocyte activation in fish. J. Immunol. 191, 4628-4639.

Canesi, L., Malatesta, M., Battistelli, S., Ciacci, C., Gallo, G., Gazzanelli, G., 2000. Immunoelectron microscope analysis of epidermal growth factor receptor (EGFR) in isolated *Mytilus* digestive gland cells: evidence for ligand-induced changes in EGFR intracellular distribution. J. Exp. Zool. 286, 690-698.

Canesi, L., Gavioli, M., Pruzzo, C., Gallo, G., 2002. Bacteria-hemocyte interactions and phagocytosis in marine bivalves. Microsc. Res. Tech. 57, 469-476.

Canesi, L., Ciacci, C., Betti, M., Lorusso, L.C., Marchi, B., Burattini, S., Falcieri, E., Gallo, G., 2004a. Rapid effect of 17β-estradiol on cell signalling and function of *Mytilus* hemocytes. Gen. Comp. Endocrinol. 136, 58-71.

Canesi, L., Lorusso, L.C., Ciacci, C., Betti, M., Zampini, M., Gallo, G., 2004b. Environmental estrogens can affect the function of mussel hemocytes through rapid modulation of kinase pathways. Gen. Comp. Endocrinol. 138, 58-69.

Canesi, L., Betti, M., Lorusso, L.C., Ciacci, C., Gallo, G., 2005. "*In vivo*" effects of Bisphenol A in *Mytilus* hemocytes: modulation of kinase-mediated signalling pathways. Aquat. Toxicol. 71, 73-84.

Canesi, L., Betti, M., Ciacci, C., Lorusso, L.C:, Pruzzo, C., Gallo, G., 2006a. Cell signaling in the immune response of mussel hemocytes. Inv. Surv. J. 3, 40-49.

Canesi, L., Ciacci, C., Lorusso, L.C., Betti, M., Guarnieri, T., Tavolari, S., Gallo, G., 2006b. Immunomodulation by 17β-Estradiol in bivalve hemocytes. Am. J. Physiol. Integr. Resp. Comp. Physiol. 291, R664-R673.

Canesi, L., Lorusso, L.C., Ciacci, C., Betti, M., Rocchi, M., Pojana, G., Marcomini, A., 2007a. Immunomodulation of *Mytilus* hemocytes by individual estrogenic chemicals and

environmentally relevant mixtures of estrogens: *In vitro* and *in vivo* studies. Aquat. Toxicol. 81, 36-44.

Canesi, L., Ciacci, C., Lorusso, L.C., Betti, M., 2007b. Effects of endocrine disrupting chemicals on the immune system of the edible bivalve mollusc *Mytilus*. In: Marino, M., Mita, D.G. (Eds.), The Endocrine Disruptors, Trivandrum, pp. 1-12.

Canesi, L., Borghi, C., Fabbri, R., Ciacci, C., Lorusso, L.C., Gallo, G., Vergani, L., 2007c. Effects of 17β-estradiol on mussel digestive gland. Gen. Comp. Endocrinol. 153, 40-46.

Canesi, L., Borghi, C., Ciacci, C., Fabbri, R., Vergani, L., Gallo, G., 2007d. Bisphenol-A alters gene expression and functional parameters in molluscan hepatopancreas. Cell. Mol. Endocrinol. 276, 36-44.

Canesi, L., Negri, A., Barmo, C., Banni, M., Gallo, G., Viarengo, A., Dondero, F., 2011. The organophosphate Chlorpyrifos interferes with the responses to 17β-Estradiol in the digestive gland of the marine mussel Mytilus galloprovincialis. PlosONE 6, e19803.

Canesi, L., Fabbri, E., 2015. Environmental effects of BPA: Focus on aquatic species. Dose-Response 13, 1-14.

Canesi, C., Ciacci, C., Balbi, T., 2016. Invertebrate model for investigating the impact of nanomaterials on innate immunity: the example of the marine mussel *Mytilus* spp. Current Bionanotechnol. 2, 71-76.

Canesi, L., Pruzzo, C., 2016. Specificity of innate immunity in bivalves: a lesson from bacteria. In: Ballarin, L., Cammarata, M., (Eds.), Lessons in Immunity: from single-cell organisms to mammals, Elsevier Inc, pp. 79-91

Cardoso, J.C.R., Félix, R.C., Bjärnmark, N., Powe, D.M., 2016. Allatostatin-type A, kisspeptin and galanin GPCRs and putative ligands as candidate regulatory factors of mantle function. Mar. Genom. 27, 25-35.

Ciocan, C.M., Cubero-Leon, E., Puinean, A.M., Hill, E.M., Minier, C., Osada, M., Fenlon, K., Rotchell, J.M., 2010. Effects of estrogen exposure in mussels, *Mytilus edulis*, at different stages of gametogenesis. Environ. Pollut. 158, 2977-2984.

Crain, D.A., Eriksen, M., Iguchi, T., Jobling, S., Laufer, H., LeBlanc, G.A., Guillette, L.J. Jr, 2007. An ecological assessment of bisphenol-A: evidence from comparative biology. Reprod. Toxicol. 24, 225-239.

Fabbri, E., Capuzzo, A., 2010. Cyclic AMP signaling in bivalve molluscs: an overview. J. Exp. Zool. A Ecol. Genet. Physiol. 313, 179-200.

Fabbri, R., Montagna, M., Balbi, T., Raffo, E., Palumbo, F., Canesi, L., 2014. Adaptation of the bivalve embryotoxicity assay for the high throughput screening of emerging contaminants in *Mytilus galloprovincialis*. Mar. Environ. Res. 99, 1-8.

Filardo, E.J., Thomas, P., 2005. GPR30: a seven-transmembrane-spanning estrogen receptor that triggers EGF release. Trends Endocrinol. Metab. 16, 362-367.

Gaudet, H.M., Cheng, S.B., Christensen, E.M., Filardo, E.J., 2015. The G-protein coupled estrogen receptor, GPER: The inside and inside-out story. Mol. Cell. Endocrinol. 418, 207-219.

Giusti, A., Joaquim-Justo, C., 2013. Esterification of vertebrate like steroids in molluscs: a target of endocrine disruptors? Comp. Biochem. Physiol. B 158, 187-198.

Gutierrez-Mazariegos, J., Nadendla, E.K., Lima, D., Pierzchalski, K., Jones, J.W., Kane, M., Nishikawa, J., Hiromori, Y., Nakanishi, T., Santos, M.M., Castro, L.F., Bourquet, W., Schubert, M., Laudet, V., 2014. A mollusc retinoic acid receptor (RAR) ortholog sheds light on the evolution of ligand binding. Endocrinology 155, 4275-4286.

Janer, G., Lavado, R., Thibaut, R., Porte, C., 2005. Effects of 17β-estradiol exposure in the mussel *Mytilus galloprovincialis*: a possible regulating role for acyltransferases. Aquat. Toxicol. 75, 32-42

Janer, G., Porte, C., 2007. Sex steroids and potential mechanisms of non-genomic endocrine disruption in invertebrates. Ecotoxicology 16, 145-160.

Keay, J., Bridgham, J.T., Thornton, J.W., 2006. The *Octopus vulgaris* estrogen receptor is a constitutive transcriptional activator: evolutionary and functional implications. Endocrinology 147, 3861-3869.

Kiss, A.L., Turi, A., Müllner, N., Kovács, E., Botos, E., Greger, A., 2005. Oestrogen-mediated tyrosine phosphorylation of caveolin-1 and its effect on the oestrogen receptor localisation: an *in vivo* study. Mol. Cell. Endocrinol. 245, 128-137.

Koutsogiannaki, S., Kaloyianni, M., 2011. Effect of 17β-estradiol on adhesion of *Mytilus galloprovincialis* hemocytes to selected substrates. Role of alpha2 integrin subunit. Fish Shellfish Immunol. 31, 73-80.

Kovats, S., 2015. Estrogen receptors regulate innate immune cells and signaling pathways. Cell. Immunol. 294, 63-69.

Labadie, P., Peck, M.R., Minier, C., Hill, E.M., 2007. Identification of the steroid fatty acid ester conjugates formed *in vivo* in *Mytilus edulis* as a result of exposure to estrogens. Steroids 72, 41-49.

Levin, E.R., 2018. Membrane estrogen receptors signal to determine transcription factor function. Steroids 132, 1-4.

Luoma, J.I., Boulware, M.I., Mermelstein, P.G., 2008. Caveolin proteins and estrogen signaling in the brain. Mol. Cell. Endocrinol. 290, 8-13.

Maiorova, M.A., Odinstova, N.A., 2015. β integrin-like protein-mediated adhesion and its disturbances during cell cultivation of the mussel *Mytilus trossulus*. Cell. Tissue Res. 361, 581-592.

Mangiamele, L.A., Gomez, J.R., Curtis, N.J., Thompson, R.R., 2017. GPER/GPR30, a membrane estrogen receptor, is expressed in the brain and retina of a social fish (*Carassius auratus*) and colocalizes with isotocin. J. Comp. Neurol. 525, 252-270.

Markov, G.V., Gutierrez-Mazariegos, J., Pitrat, D., Billas, I.M.L., Bonneton, F., Moras, D., Hasserodt, J., Lecointre, G., Laudet, V., 2017. Origin of an ancient hormone/receptor couple revealed by resurrection of an ancestral estrogen. Sci. Adv. 3, e1601778.

Martínez-Gómez, C., Bignell, J., Lowe, D., 2015. Lysosomal membrane stability in mussels. In: ICES Techniques in Marine Environmental Sciences No. 56, 41 pp.

Martinkovich, S., Shah, D., Planey, S.L., Arnott, J.A., 2014. Selective estrogen receptor modulators: tissue specificity and clinical utility. Clin. Interv. Aging 9, 1437-1452.

Mezghani-Chaari, S., Machreki-Ajimi, M., Hamza-Chaffai, A., Minier, C., 2017. High estradiol exposure disrupts the reproductive cycle of the clam *Ruditapes decussatus* in a sex-specific way. Environ. Sci. Pollut. Res. 24, 26670-26680.

Miglioli, A., Balbi, T., Dumollard, R., Canesi, L., 2018. Role of tyrosinase in early shell formation of *Mytilus galloprovincialis*: a possible marker of endocrine disruption in marine bivalves? Abstract presented at the 31st European Society for Comparative Physiology and Biochemistry Congress-ESCPB (Porto, Portugal), ms. in preparation.

Murgarella, M., Puiu, D., Novoa, B., Figueras, A., Posada, D., Canchava, C., 2016. A first insight into the genome of the filter-feeder mussel Mytilus galloprovincialis. PlosONE 11, e0151561.

Nagasawa, K., Treen, N., Kondo, R., Otoki, Y., Itoh, N., Rotchell, J.M., Osada, M., 2015. Molecular characterization of an estrogen receptor and estrogen-related receptor and their autoregulatory capabilities in two *Mytilus* species. Gene 564, 153-159.

Nazari, E., Suja, F., 2016. Effects of 17β-estradiol (E2) on aqueous organisms and its treatment problem: a review. Rev. Environ. Health 31, 465-491.

Ni, J., Zeng, Z., Ke, C., 2013. Sex steroid levels and expression patterns of estrogen receptor gene in the oyster *Crassostrea angulata* during reproductive cycle. Aquaculture 376-379, 105-116.

Patel, H.H., Murray, F., Insel, P.A., 2008. Caveolae as organizers of pharmacologically relevant signal transduction molecules. Annu. Rev. Pharmacol. Toxicol. 48, 359-391.

Porte, C., Janer, G., Lorusso, L.C., Ortiz-Zarragoitia, M., Cajaraville, M.P., Fossi, M.C., Canesi, L., 2006. Endocrine disruptors in marine organisms: approaches and perspectives. Comp. Biochem. Physiol. C 143, 303-315.

Prossniz, E.R., Arteburn, J.B., 2015. International union of basic and clinical pharmacology. XCVII. G protein-coupled estrogen receptor and its pharmacologic modulators. Pharmacol. Rev. 67, 505-540.

Puinean, A.M., Labadie, P., Hill, E.M., Osada, M., Kishida, M., Nakao, R., Novillo, A., Callard, I.P., Rotchell, J.M., 2006. Laboratory exposure to 17beta-estradiol fails to induce vitellogenin and estrogen receptor gene expression in the marine invertebrate *Mytilus edulis*. Aquat. Toxicol. 79, 376-383.

Saczko, J., Michel, O., Chwiłkowska, A., Sawicka, E., Mączyńska, J., Kulbacka, J., 2017. Estrogen receptors in cell membranes: regulation and signaling. Adv. Anat. Embryol. Cell. Biol. 227, 93-105.

Schwarz, T.I., Katsiadaki, I., Maskrey, B.H., Scott, A.P., 2017a. Mussel (Mytilus spp.) display an ability for rapid and high capacity uptake of the vertebrate steroid, estradiol-17β from water. J. Steroid Biochem. Mol. Biol. 165, 407-420.

Schwarz, T.I., Katsiadaki, I., Maskrey, B.H., Scott, A.P., 2017b. Data on the uptake and metabolism of testosterone by the common mussel, Mytilus spp. Data Brief 12, 164-168.

Schwarz, T.I., Katsiadaki, I., Maskrey, B.H., Scott, A.P., 2017c. Uptake and metabolism of water-borne progesterone by the mussel, *Mytilus* spp. (Mollusca). J. Steroid Biochem. Mol. Biol. 178, 13-21.

Scott, A.P., 2012. Do molluscs use vertebrate sex steroids as reproductive hormones? Part I: Critical appraisal of the evidence for the presence, biosynthesis and uptake of steroids. Steroids 77, 1450-1468.

Scott, A.P., 2013. Do molluscs use vertebrate sex steroids as reproductive hormones? II. Critical review of the evidence that steroids have biological effects. Steroids 78, 268-281.

Scott, A.P., 2018. Is there any value in measuring vertebrate steroids in invertebrates? Gen. Comp. Endocrinol. 265, 77-82.

Sharma, G., Mauvais-Jarvis, F., Prossnitz, E.R., 2018. Roles of G protein-coupled estrogen receptor GPER in metabolic regulation. J. Steroid Biochem. Mol. Biol. 176, 31-37.

Smolarz, K., Zabrzańska, S., Konieczna, L., Hallmann, A., 2018. Changes in steroid profiles of the blue mussel *Mytilus trossulus* as a function of season, stage of gametogenesis, sex, tissue and mussel bed depth. Gen. Comp. Endocrinol. 259, 231-239.

Stange, D., Sieratowicz, A., Horres, R., Oehlmann, J., 2012. Freshwater mudsnail (Potamopyrgus antipodarum) estrogen receptor: identification and expression analysis under exposure to (xeno)hormones. Ecotoxicol. Environ. Saf. 75, 94-101.

Stefano, G.B., Zhu, W., Mantione, K., Jones, D., Salamon, E., Cho, J.J., Cadet, P., 2003. 17β-estradiol downregulates ganglionic microglial cells via nitric oxide release: presence of an estrogen receptor β transcript. Neuroendocrinol. Lett. 24, 130-136.

Stefkovich, M.L., Arao, Y., Hamilton, K.J., Korach, K.S., 2018. Experimental models for evaluating non-genomic estrogen signaling. Steroids 133, 34-37.

Thitiphuree, T., Nagasawa, K., Osada, M., 2019. Molecular identification of steroidogenesis-related genes in scallops and their potential roles in gametogenesis J. Steroid Biochem. Mol. Biol. 186, 22-33.

Thomas, P., Alyea, R., Pang, Y., Peyton, C., Dong, J., Berg, A.H., 2010. Conserved estrogen binding and signaling functions of the G protein-coupled estrogen receptor 1 (GPER) in mammals and fish. Steroids 75, 595-602.

Thornton, J.W., 2001. Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions. Proceedings of the National Academy of Sciences 98, 5671-5676.

Tiedeken, E.J., Tahar, A., McHugh, B., Rowan, N.J., 2017. Monitoring, sources, receptors, and control measures for three European Union watch list substances of emerging concern in receiving waters - A 20 year systematic review. Sci. Total Environ. 574, 1140-1163.

Tran, T.K.A., MacFarlane, G.R., Kong, R.Y.C., O'Connor, W.A., Yu, R.M.K., 2016. Potential mechanisms underlying estrogen-induced expression of the molluscan estrogen receptor (ER) gene. Aquat. Toxicol. 179, 82-94.

Tran, T.K.A., MacFarlane, G.R., Kong, R.Y.C., O'Connor, W.A., Yu, R.M.K., 2017. The constitutively active estrogen receptor (ER) binds and activates the promoter of the vitellogenin (Vtg) gene in the Sidney rock oyster, *Saccostrea glomerata*. Mar. Pollut. Bull. 118, 397-402.

Vogeler, S., Galloway, T.S., Lyons, B.P., Bean, T.P., 2014. The nuclear receptor gene family in the Pacific oyster, Crassostrea gigas, contains a novel subfamily group. BMC Genomics 15, 369.

Vogeler, S., Galloway, T.S., Isupov, M., Bean, T.P., 2017. Cloning retinoid and peroxisome proliferator-activated nuclear receptors of the Pacific oyster and in silico binding to environmental chemicals. PlosONE 12, e0176024.

Völker, C., Gräf, T., Schneider, I., Oetken, M., Oehlmann, J., 2014. Combined effects of silver nanoparticles and 17 α-ethinylestradiol on the freshwater mudsnail *Potamopyrgus antipodarum*. Environ. Sci. Pollut. Res. Int. 21, 10661-10670.

Watson, C.S., Jeng, Y.J., Hu, G., Wozniak, A., Bulayeva, N., Guptarak, J., 2012. Estrogen- and xenoestrogen-induced ERK signaling in pituitary tumor cells involves estrogen receptor-α interactions with G protein-αi and caveolin I. Steroids 77, 424-432.

Zabrzańska, S., Smolarz, K., Hallmann, A., Konieczna, L., Bączek, T., Wołowicz, M., 2015. Sex-related differences in steroid concentrations in the blue mussel (*Mytilus edulis trossulus*) from the southern Baltic Sea. Comp. Biochem. Physiol. A 183, 14-19.

Figure 1

Studies on the effects of estrogen in mollusks. A) Percentage of publications on major molluskan groups with respect to total publications; B) Number of publications referring to different functions and signaling pathways (source http://www.ncbi.nlm.nih.gov/pubmed at 25/03/2019)

Figure 2

Summary on the effects of pre-treatment with the ER modulator Tamoxifen (Tam) on the changes in functional immune parameters induced by 17β-estradiol (E_2) (25 nM, 30 min) in mussel hemocytes (% of control). Data from Canesi et al., 2004a, 2006b.

Figure 3

Effect of pre-treatment with the GPER antagonist G15 on the lysosomal membrane destabilization induced by E_2 in mussel hemocytes (C = control; E_2 = E_2 (25 nM, 30 min); G15 = G15 (100 nM, 10 min); G15/E_2 = G15 10 min/E_2 30 min. Data, expressed as Neutral Red Retention Time (min) (see Supplementary Information) are the mean ± SD of 4 experiments. * = $p<0.05$

Figure 4

Effects of GPER modulators in the presence and absence of E_2 on lysosomal and cytoskeletal alterations in mussel hemocytes, evaluated by Confocal Laser Scanning Microscopy (CLSM) using LysoTracker Green and ActinGreen488 (see Supplementary Information). Representative images are reported of C = control cells; E_2 = E_2 25 nM, 30 min; G15 = cells pretreated with G15 and then exposed to E_2 as in Fig. 3; G1 = cells exposed to G1 alone (100 nM, 30 min).

Upper panel: hemocytes stained with LysoTracker Green

Lower panel: hemocytes stained with ActinGreen488

Figure 5

Expression of *Mytilus* Estrogen Receptors isoforms MeER1 and MeER2 in mussel early embryo development, evaluated by RTqPCR.

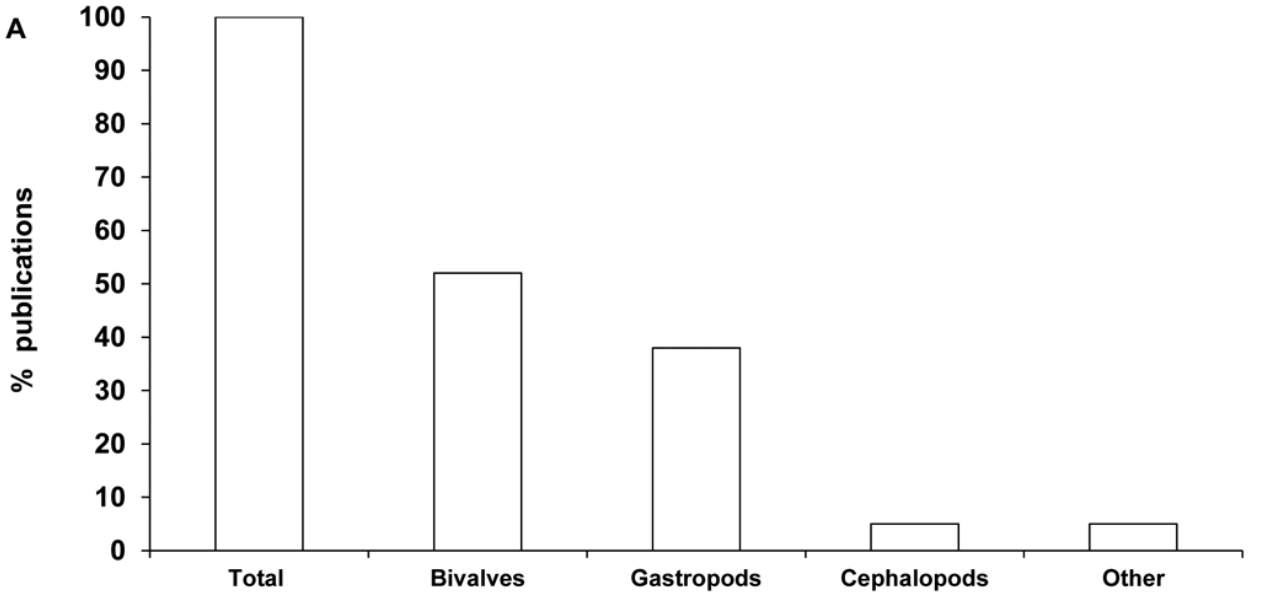
(A) Transcriptional profiles of MeERs gene products evaluated in embryos of *M. galloprovincialis* grown under physiological conditions at 24 and 48 hpf. Data, reported as log_2-transformed relative expressions with respect to unfertilized eggs, represent the mean ± SD (N = 12). Expression of all transcripts was significantly changed at both 24 and 48 hpf with respect to unfertilized eggs (1-way non-parametric ANOVA followed by the Mann-Whitney *U* test; $p<0.05$).

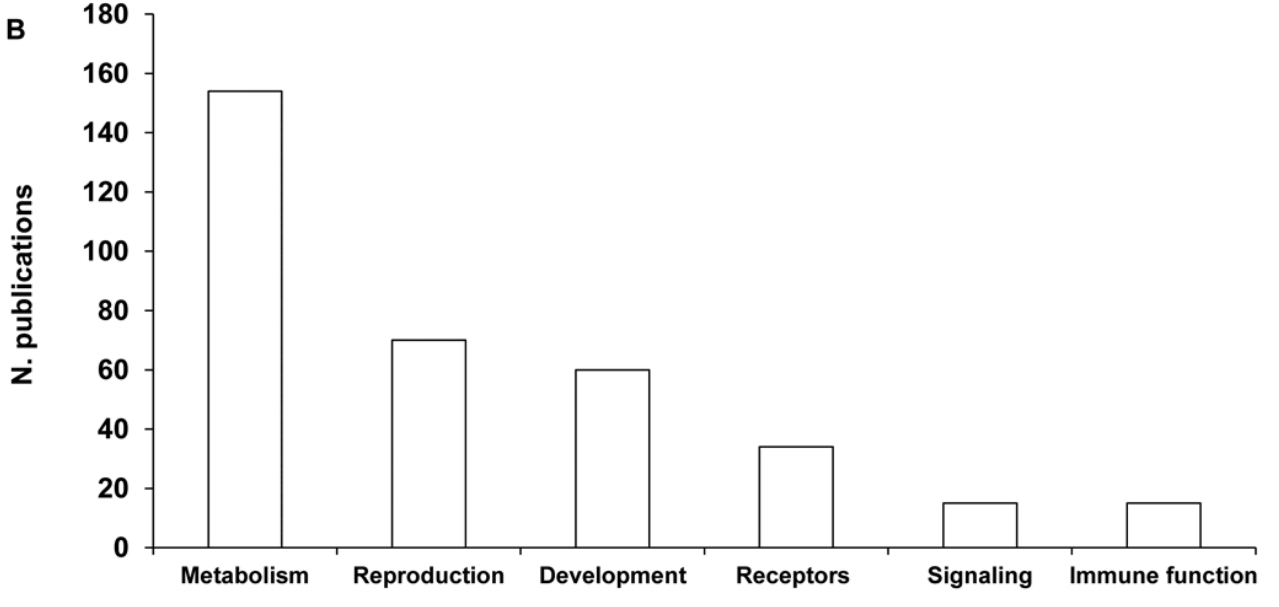
(B) Effects of exposure to E_2 (10 μg/L) on gene transcription in *Mytilus* embryos at 24 h and 48 hpf. Data are reported as mean ± SD of the relative expression with respect to untreated samples within each life stage (N = 4) (all * = $p<0.05$ vs control, according to the Mann-Whitney *U* test).

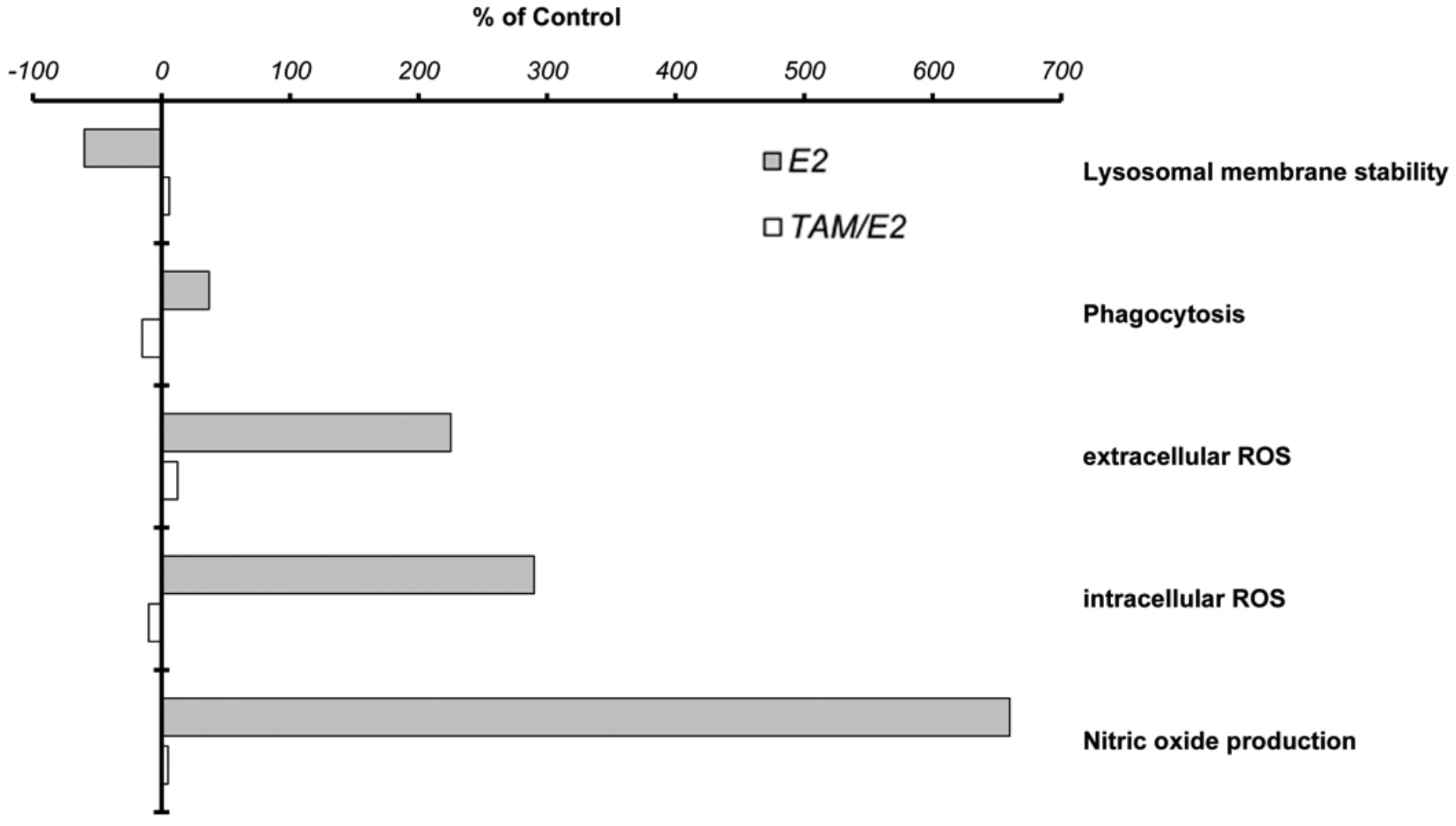
Data from Balbi et al., 2016.

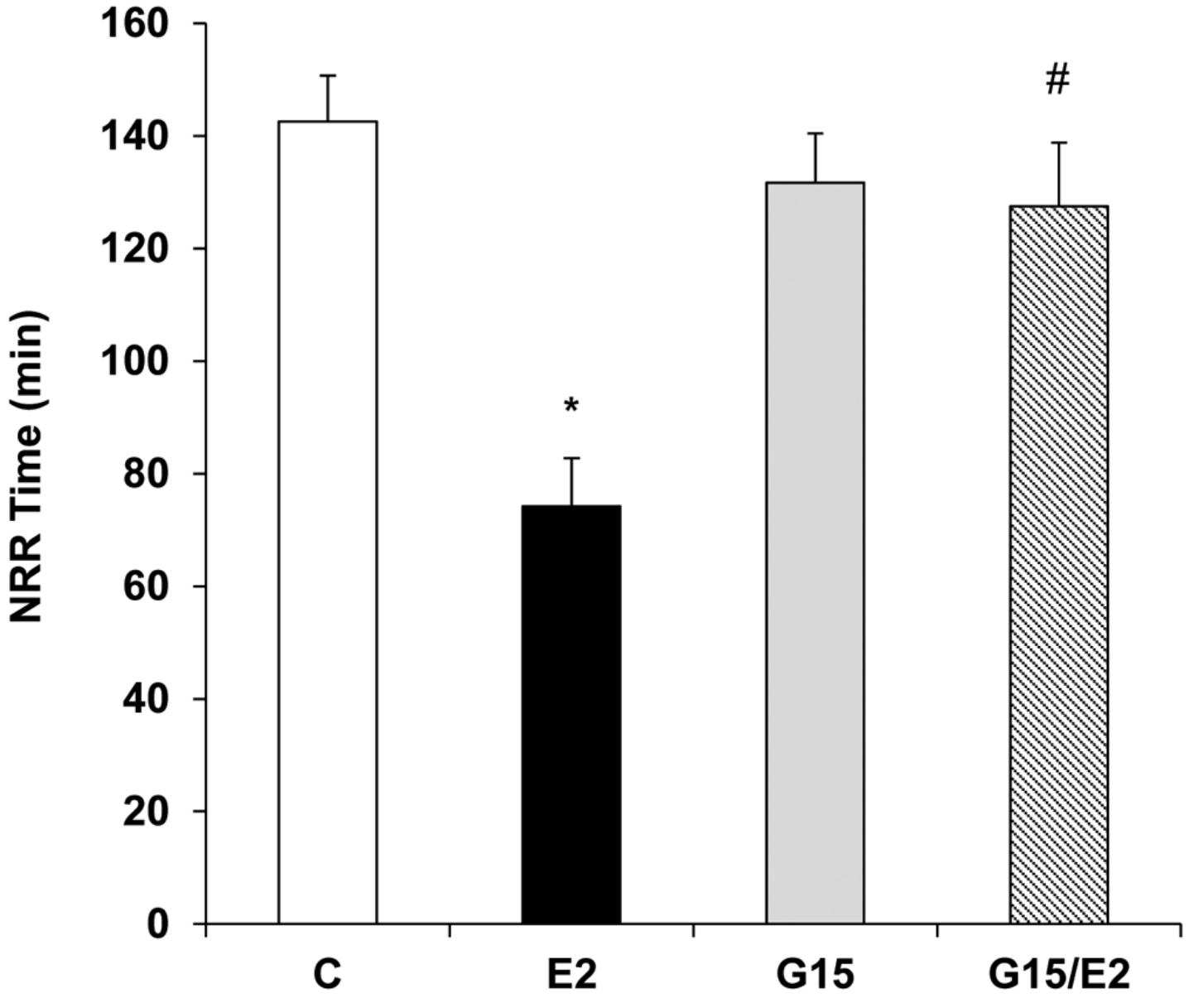
Figure 6

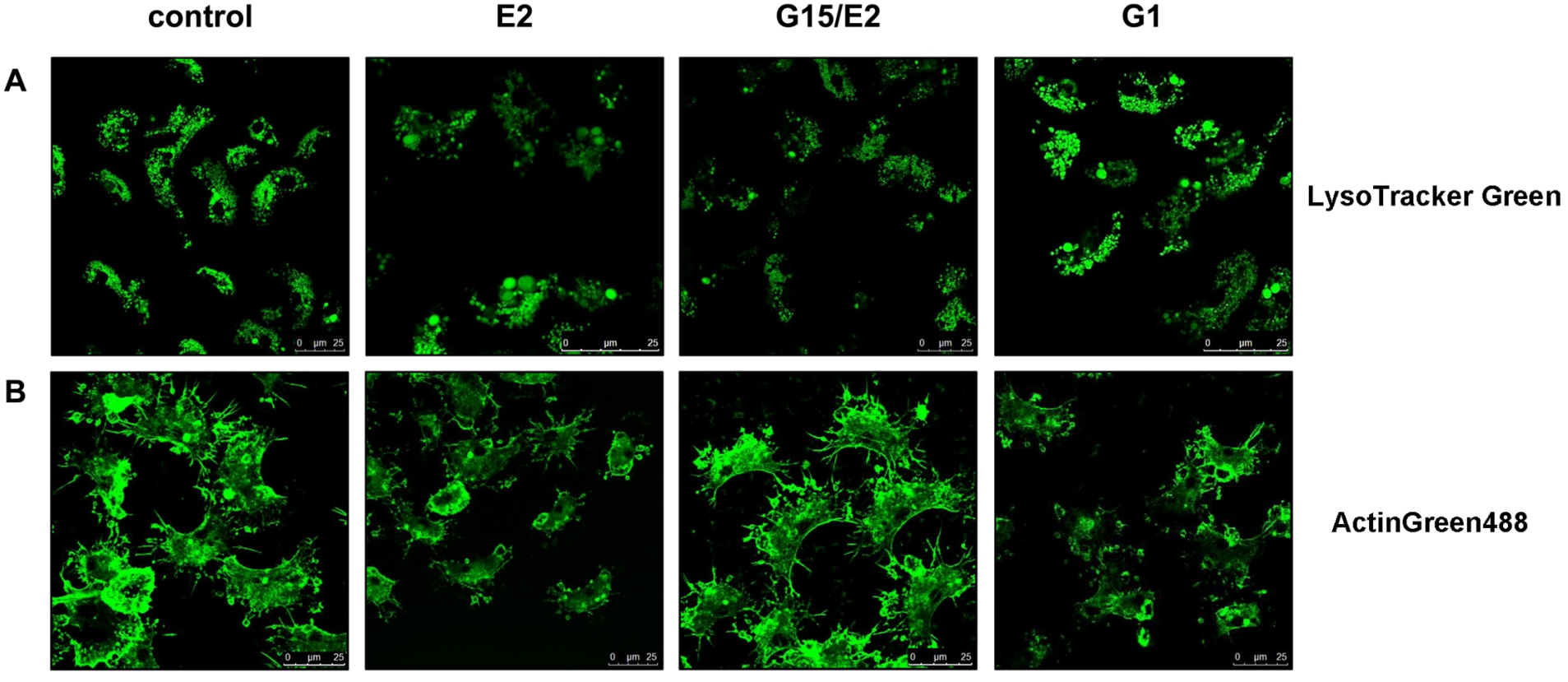
Comparison of the effects of E_2, BPA (Balbi et al., 2016), EE and BPF (this work) on *M. galloprovincialis* larval development evaluated by the 48-h embryotoxicity assay.

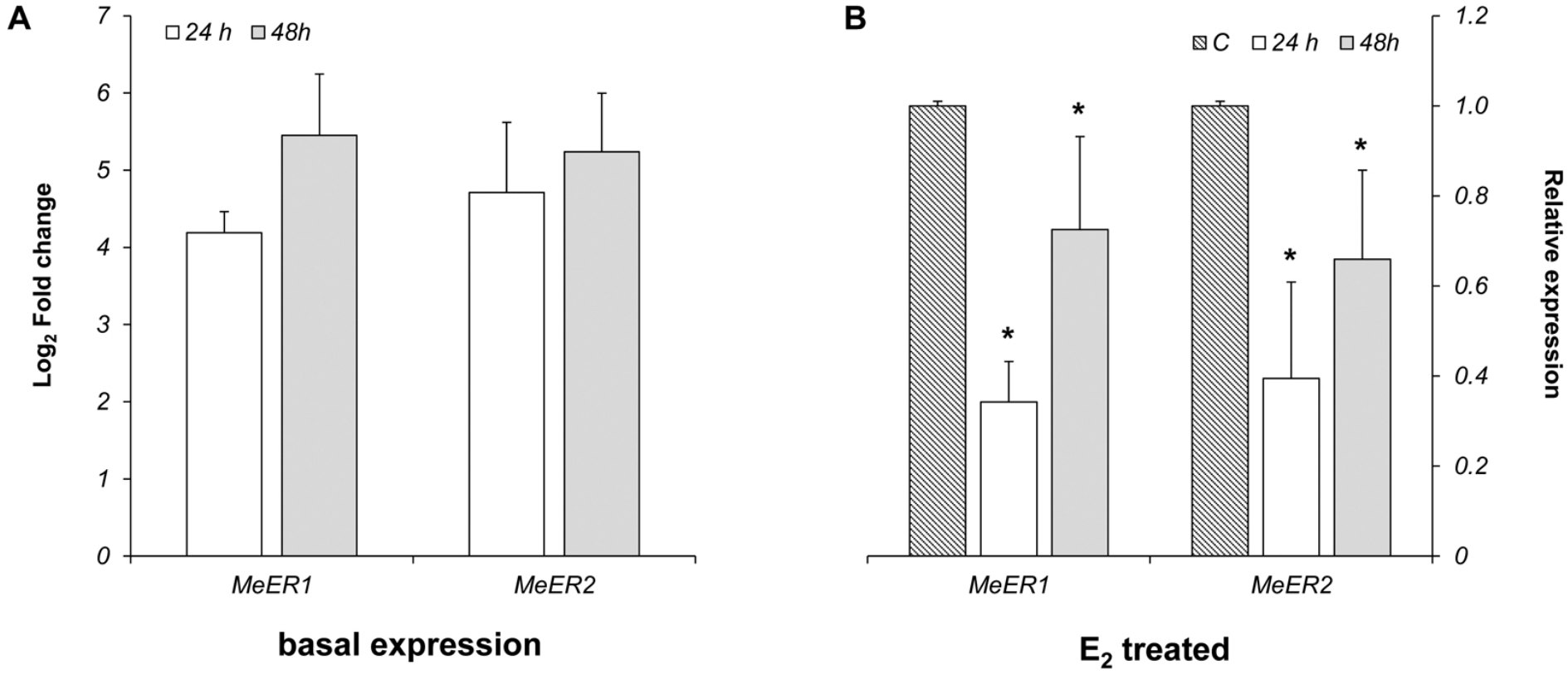

A
% publications
100
90
80
70
60
50
40
30
20
10
0
Total
Bivalves
Gastropods
Cephalopods
Other

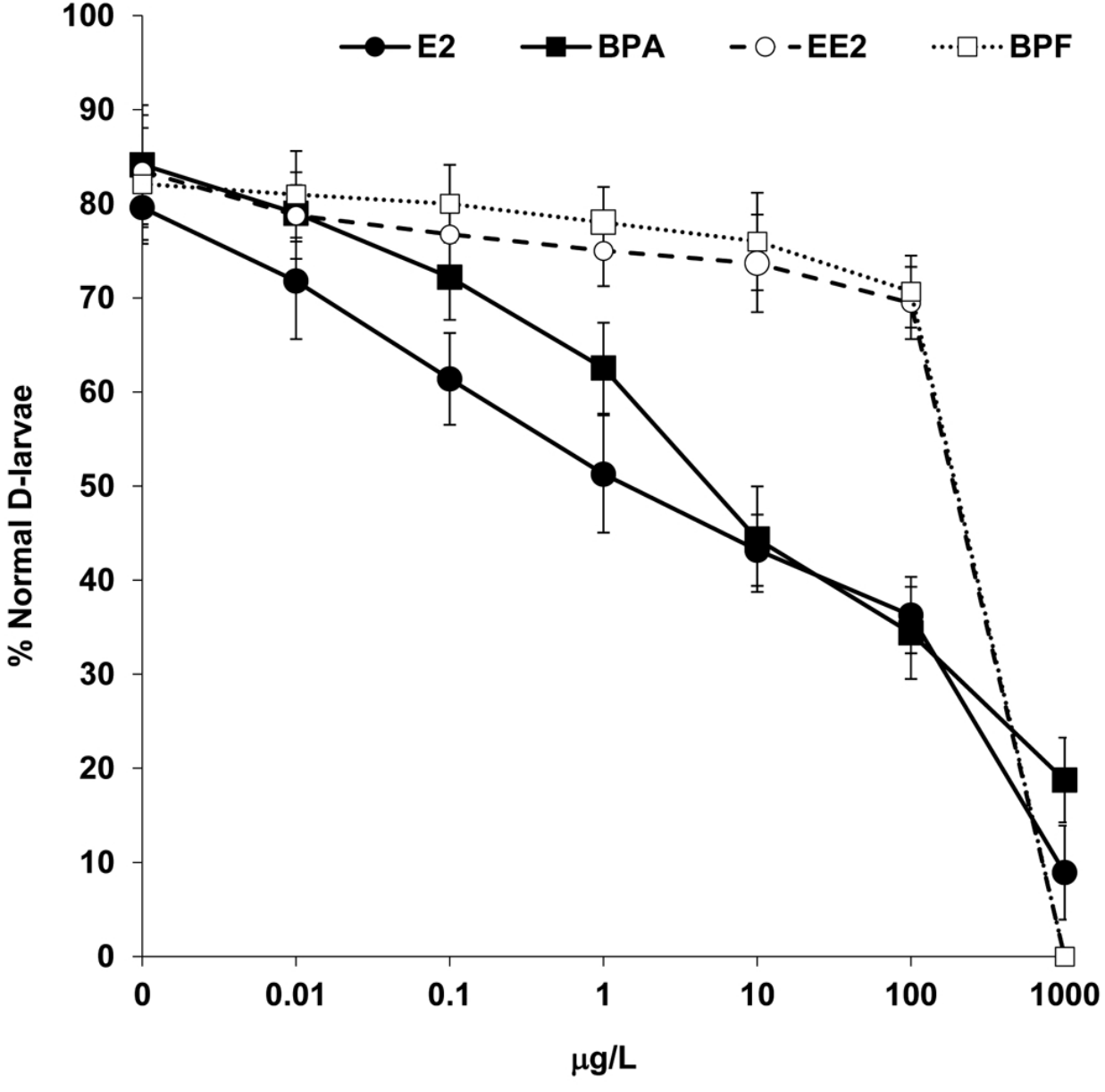

B
N. publications
180
160
140
120
100
80
60
40
20
0
Metabolism
Reproduction
Development
Receptors
Signaling
Immune function


% of Control
-100
0
100
200
300
400
500
600
700
E2
TAM/E2
Lysosomal membrane stability
Phagocytosis
extracellular ROS
intracellular ROS
Nitric oxide production


160
140
120
100
80
60
40
20
0
NRR Time (min)
*
#
C
E2
G15
G15/E2


control
E2
G15/E2
G1
A
B
LysoTracker Green
ActinGreen488
0 µm 25


A
24 h
48h
Log2 Fold change
0
1
2
3
4
5
6
7
MeER1
MeER2
basal expression
B
C
24 h
48h
Relative expression
0
0.2
0.4
0.6
0.8
1.0
1.2
*
*
*
*
MeER1
MeER2
E2 treated


E2
BPA
EE2
BPF
% Normal D-larvae
100
90
80
70
60
50
40
30
20
10
0
0
0.01
0.1
1
10
100
1000
µg/L

'Declarations of interest: none'.

Supplementary information

Methods

Immunolocalization of Caveolin- and ERβ-like proteins in mussel hemocytes

Immunoreactivity of Total Caveolin, Phosphorylated caveolin (P-Cav), and ERβ receptors in hemocytes was evaluated by confocal laser scanning microscopy (CLSM) using heterologous antibodies as previously described (Canesi et al., 2006). Hemocyte monolayers were fixed for 30 min at room temperature in 4% paraformaldheyde in 5 mM PBS, pH 7.4, with 2% NaCl. All subsequent steps were carried out at room temperature in the presence of PBS-NaCl. The cells were washed three times with PBS, permeabilized for 10 min with 0.2% Triton X-100 in PBS, and treated for 30 min with 2% BSA in PBS (PBS-BSA) to minimize possible unspecific binding of the antisera. Hemocytes were then incubated with primary antibody polyclonal rabbit anti-caveolin-1 (1:100; 610059, BD Biosciences, San Diego, CA1) or rabbit Phospho-Caveolin-1 (Tyr14) (1:100; sc-373837, Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min. The cells were washed three times in PBS-BSA and incubated for 60 min with secondary antibody mouse anti-rabbit IgG-FITC-labeled (1:250; Santa Cruz Biotechnology) following the manufacturer's instructions. For the colocalization evaluation hemocytes were also incubated with monoclonal mouse anti-ERβ antibody (1:50, sc-373853, Santa Cruz Biotechnology, Santa Cruz, CA; 1:50) for 60 min and visualized with secondary antibody goat anti-mouse IgG-CY3- labeled (1:50, Sigma-Aldrich, St. Louis, MO). The cells were washed three times in PBS, and coverslips were mounted on glass slides using 5% glycerol-1,4- diazabicyclo[2.2.2]octane in a 90% glycerol-10% PBS (10X) antifading mixture. Fluorescence was detected using a Leica TCS SP5 confocal setup mounted on a Leica DMI 6000 CS inverted microscope (Leica Microsystems, Heidelberg, Germany) using 63x 1.4 oil objective (HCX PL APO 63-1.40 OIL UV) with 488 nm and 545 nm laser excitations, for FITC and Cy3, respectively. Images were analyzed by the Leica Application Suite Advanced Fluorescence (LASAF) and ImageJ Software (Wayne Rasband, Bethesda, MA).

Embryotoxicity assay

The 48-h embryotoxicity assay (ASTM, 2004) was carried out in 96-microwell plates as previously described (Fabbri et al., 2014; Balbi et al., 2016). When mussels begun to spontaneously spawn, each individual was immediately placed in a 250 mL beaker containing 200 mL of aerated ASW until complete gamete emission. After spawning, mussels were removed from beakers and sperms

and eggs were sieved through 50 µm and 100 µm meshes, respectively, to remove impurities. Egg quality (shape, size) and sperm motility were checked using an inverted microscope. Eggs were fertilized with an egg:sperm ratio 1:10 in polystyrene 96-microwell plates (Costar, Corning Incorporate, NY, USA). After 30 min fertilization success (n. fertilized eggs / n. total eggs × 100) was verified by microscopical observation (>85%). Aliquots of 20 µl of a 10x solution of E_2, BPA, EE, BPF (obtained from a 1 mg/L stock solution prepared in DMSO) suitably diluted in ASW were added to fertilized eggs in each microwell to reach the nominal final concentrations (0.01-0.1-1-10-100-1000 µg/L) in a 200 µl volume. Microplates were gently stirred for 1 min, and then incubated at 18 ± 1°C for 48 h, with a 16h:18h light:dark photoperiod. All the following procedures were carried out following ASTM (2004). At the end of the incubation time, samples were fixed with buffered formalin (4%). All larvae in each well were examined by optical and/or phase contrast microscopy using an inverted Olympus IX53 microscope (Olympus, Milano, Italy) at 40X, equipped with a CCD UC30 camera and a digital image acquisition software (cellSens Entry). Observations were carried out by an operator blind to the experimental conditions. A larva was considered normal when the shell was D-shaped (straight hinge) and the mantle did not protrude out of the shell, and malformed if had not reached the stage typical for 48 h (trocophore or earlier stages) or when some developmental defects were observed (concave, malformed or damaged shell, protruding mantle). The recorded endpoint was the percentage of normal D-larvae in each well respect to the total, including malformed larvae and pre-D stages. The acceptability of test results was based on controls for a percentage of normal D-shell stage larvae >75% (ASTM, 2004). Data were finally expressed as means ± SDs of 4 experiments carried out in 6 replicate-wells (N = 4). The EC_{50} was defined as the concentration of chemical causing 50% reduction in the embryogenesis success, and 95% confidence intervals (CI) were calculated by PRISM 5 software (GraphPad Prism 5 software package, GraphPad Inc.).

Effects of the GPER modulators on the lysosomal system and active cytoskeleton

Hemocytes were pre-treated for 20 min with the GPER antagonist G15 (100 nM) before exposure to E_2 (25 nM, 30 min) and LMS was evaluated by the Neutral Red Retention time assay as previously described (Canesi et al., 2004, 2006, 2007).

In the same experimental conditions, lysosomal compartments and actin cytoskeleton were evaluated by Confocal microscopy. The effect of the GPER agonist G1 alone (100 nM, 30 min), were also evaluated.

LysoTracker® Green DND-26 (from Thermo Fisher Scientific, Massachusetts, USA) is a green fluorescent dye that stains acidic compartments in live cells. Acidification of lysosomal compartments was evaluated in hemocytes loaded with LysoTracker Green (250 nM, 30 min).

Actin cytoskeleton structure was evaluated in hemocytes loaded with ActinGreen™488 ReadyProbes® Reagent for 30 min to reveal F actin as previously described (Auguste et al., 2018).

Fluorescence of Lysotracker (excitation 504, emission 511 nm), and ActinGreen™488 (excitation 495 nm, emission 518 nm) was detected using a Leica TCS SP5 confocal setup as described above.

References

ASTM, 2004. International standard guide for conducting static acute toxicity tests starting with embryos of four species of salt water bivalve mollusks. E 724-798.

Auguste, M., Ciacci, C., Balbi, T., Brunelli, A., Caratto, V., Marcomini, A., Cuppini, R., Canesi, L., 2018. Effects of nanosilver on *Mytilus galloprovincialis* hemocytes and early embryo development. Aquat. Toxicol. 203, 107-116.

Balbi, T., Franzellitti, S., Fabbri, R., Montagna, M., Fabbri, E., Canesi, L., 2016. Impact of bisphenol A (BPA) on early embryo development in the marine bivalve *Mytilus*: effects on gene transcription. Environ. Pollut. 218, 996-1004.

Canesi, L., Lorusso, L.C., Ciacci, C., Betti, M., Zampini, M., Gallo, G., 2004. Environmental estrogens can affect the function of mussel hemocytes through rapid modulation of kinase pathways. Gen. Comp. Endocrinol. 138, 58-69.

Canesi, L., Ciacci, C., Lorusso, L.C., Betti, M., Guarnieri, T., Tavolari, S., Gallo, G., 2006. Immunomodulation by 17β-Estradiol in bivalve hemocytes. Am. J. Physiol. Integr. Resp. Comp. Physiol. 291, R664-R673.

Canesi, L., Lorusso, L.C., Ciacci, C., Betti, M., Rocchi, M., Pojana, G., Marcomini, A., 2007. Immunomodulation of *Mytilus* hemocytes by individual estrogenic chemicals and environmentally relevant mixtures of estrogens: *In vitro* and *in vivo* studies. Aquat. Toxicol. 81, 36-44.

Fabbri, R., Montagna, M., Balbi, T., Raffo, E., Palumbo, F., Canesi, L., 2014. Adaptation of the bivalve embryotoxicity assay for the high throughput screening of emerging contaminants in *Mytilus galloprovincialis*. Mar. Environ. Res. 99, 1-8.

Supplementary Results

Supplementary Figure 1

CLSM images of Total Caveolin (a), P-Cav (b) (green), and of ERβ (c) (red) immunoreactivity in mussel hemocytes. Note the diffuse cytoplasmic and nuclear immunoreactivity of Total Caveolin (a), whereas that of P-Cav is exclusively extra-nuclear and mainly associated with vesicles of different sizes, the largest of which are localized in the perinuclear region (b).

c) ERβ-like immunoreactivity is also extra-nuclear, and associated with cytoplasmic vesicles of different sizes as previously described (Canesi et al., 2006).

d) Merged image showing Colocalization of P-cav- and ERβ-like immunoreactivity (yellow) that is apparently concentrated in larger perinuclear vescicles.

Scale bars: 10 µm