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Ovothiol A synthase transcription levels in different tissues of the mussel *Mytilus galloprovincialis* exposed to copper

Tesi di laurea in Fisiologia molecolare degli animali marini

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ABSTRACT

Marine organisms are continuously exposed to reactive oxygen species (ROS). Although ROS can act as signaling molecules, thus showing a physiological role, their excess may cause oxidative stress resulting in multiple adverse effects such as protein and lipid oxidation and DNA damage. A part of the antioxidant system of marine organisms consists of low molecular weight scavengers, including thiols. Many studies focused on ovothiol, a thiol isolated from marine invertebrate eggs, because of its high antioxidant power. Ovothiol A synthase (Ovo-A) is an iron (II) dependent sulfoxide synthase which catalyzes ovothiol A biosynthesis, and therefore may be a marker of ovothiol response to environmental stress in marine animals. This study investigates transcriptional response of an Ovo-A gene product to oxidative stress in three different tissues of *M. galloprovincialis* (gonads, digestive glands and gills) and considering sex bias. Mussels were in vivo exposed to copper (Cu^{2+}=10 µg/l) and tissues were dissected at 3 and 7 days of exposure. *Ovo-A* expression was assessed by quantitative real time PCR. Gonads were also collected for the histological analyses to determine the development stages. Female gonads showed significant Ovo-A downregulation in stage 5 organisms compared to stage 2 and stage 3. In Cu-treated mussels, down-regulation was observed in male gonads. No significant response occurred in treated digestive glands and gills. Downregulation of *Ovo-A* in relation to female gonads development may be due the conversion to Ovo-A mRNA into the protein which is then accumulated in oocytes as a part of the ‘‘be prepared’’ adaptive strategy allowing fast induction of embryo stress defenses. The lack of *Ovo-A* regulation by Cu in extragonadal tissues and the relevant influence of gonadal maturation stage in *Ovo-A* baseline expression lead to hypothesise that maturation processes may display a major role in *Ovo-A* regulation than exposure oxidative stress generating chemicals.

1 INTRODUCTION

The oxidative stress is the production and accumulation of reduced oxygen species to elevated concentrations that can damage lipids, proteins, and DNA in various organs. Reactive oxygen species (ROS) are naturally produced during several cellular processes including oxidative phosphorylation, electron transport chains in mitochondria and microsomes, the activity of oxidoreductase enzymes producing ROS as intermediates or final products, or even immunological reactions such as active phagocytosis (Regoli and Giuliani, 2014). The univalent reduction of molecular oxygen produces reactive intermediates such as the superoxide radical (O_2^-), singlet oxygen ($1O_2$), hydrogen peroxide (H_2O_2), hydroxyl radical (HO•), and finally water (H_2O). Often biologists address all of the products oxygen reduction as free radicals (Lesser, 2006).

In nature, ROS play an important role in the development, growth, differentiation and proliferation of multicellular organisms. They are kept in cells at a baseline level that supports cellular proliferation and metabolism, but also are used as signal transduction molecules that regulate many important regulatory pathways in cells (Regoli and Giuliani, 2014). Their levels can increase in cells because of the action of multiple abiotic and biotic factors, acting as a trigger for different cell death or autophagy pathways (Zandalinas and Mittler, 2018). Due to the damaging capacity of these reactive species, their metabolism and their biological activity is under strict control inside cells, where their concentration usually does not exceed 10^{-8} M (Halliwell and M.C. Gutteridge, 1992).

Several mechanisms may lead to ROS generation in marine organisms (Regoli and Giuliani, 2014). In most cases, ROS are produced as side products of oxygen metabolism. In eukaryotes, there is a mitochondrial system, while in prokaryotes the production is localized in plasmatic membranes (Bartosz, 2009). Less than 10% of consumed oxygen is reduced via one electron scheme giving rise to ROS. The coenzyme Q and the Complex III are believed to be the main places of mitochondrial electron transport chain where electrons “escape” it and interact with molecular oxygen giving $O_2^{\bullet -}$. The second most important ROS source is electron transport chain of endoplasmic reticulum. The catabolism of cellular and xenobiotic chemicals by cytochromes P450 includes the redox steps and is responsible for ROS

production in endoplasmic reticulum. Certain amounts of ROS are produced in cytosol and peroxisomes by different oxidases. For example, tryptophan di-oxygenase xanthine oxidase, and cytochrome P450 reductase mainly produce $O_2^{\bullet -}$, while such enzymes as oxidases of amino acids and glucose mainly generate H_2O_2 (Lushchak, 2011) Mascio and Murphy 1991).

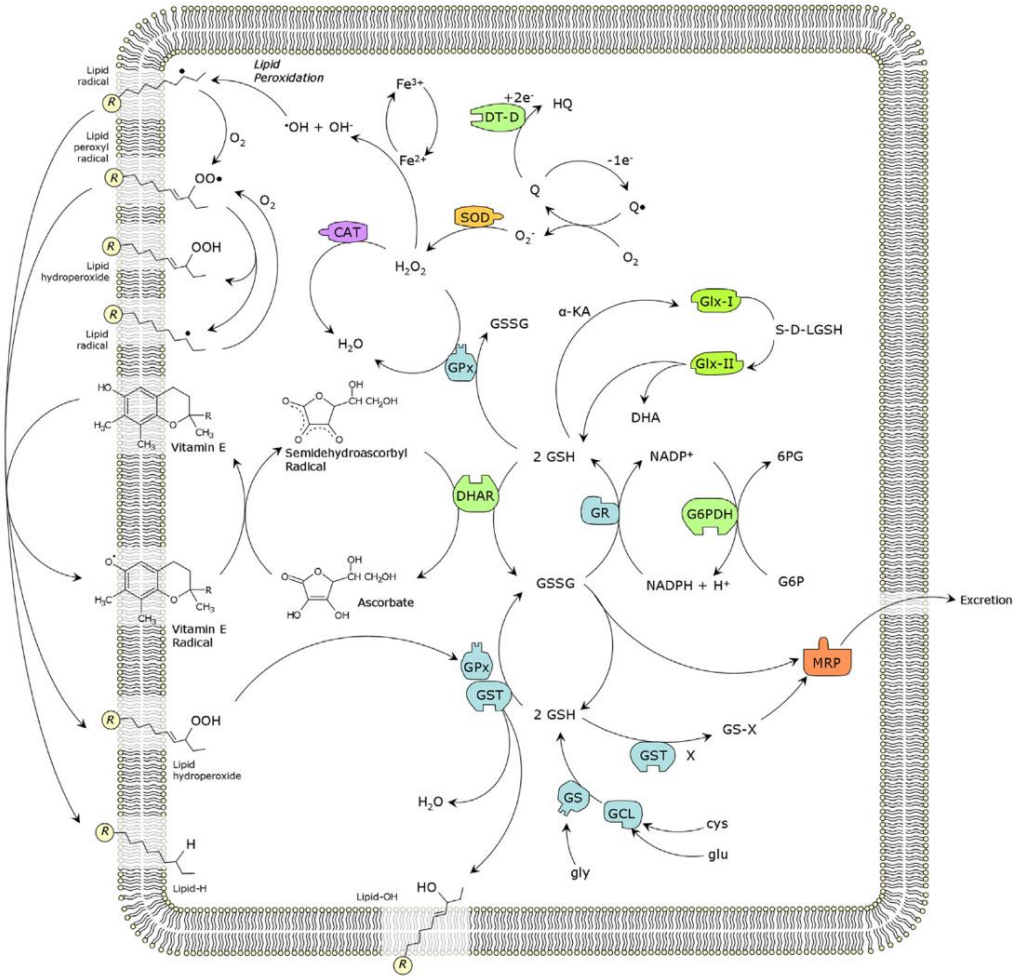

Fig 1: Main cellular antioxidant defences and antioxidant pathways (Regoli and Giuliani, 2014)

The same oxidative pathways could be applied to marine organisms, that are used as a model to understand the mechanism for ROS production and as bioindicators to analyse the environmental quality. The oxidative stress can be a response to the adaptation following a variation of environmental factors as temperature, salinity and oxygen level. Besides, it is the principal response and indicator to environmental pollutant presence. Metal ions as iron, copper and mercury are well known inducers of oxidative stress. They can stimulate ROS production via two different

mechanisms: the first one is related with the interference of metal-related processes; the second one with the generation of free radicals by ions with changeable valence. (Lushchak 2011; Benedetti, Giuliani, and Regoli 2015).

Cells have developed antioxidant defence system to maintain the balance between ROS production and scavenging. Under normal conditions the excess of ROS can be scavenged by antioxidant defence system, that can be divided in two mechanism: the enzymatic system and non-enzymatic low molecular weight compounds acting together to detoxify ROS damage. Several transcriptional factors are also involved in antioxidant defence to regulate transcription of a number of related genes (Meng et al., 2017).

1.1 THE ANTIOXIDANT SYSTEM

Aerobic organisms have a complex antioxidant defence system to regulate cellular redox homeostasis to protect ROS damage. The antioxidant system contains enzymatic and non-enzymatic components. Enzymatic antioxidants comprise superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx), peroxiredoxin and glutathione S-transferase (GST), Nicotinamide adenine dinucleotide phosphate (NADPH), glutathione (GSH) and thioredoxins act together with these enzymes to defend against damages caused by ROS (Meng et al., 2017). Non-enzymatic antioxidants consist of low molecular weight scavengers as glutathione, metallothioneins (MTs), melatonin, vitamins C and E, carotenoids, flavonoids and thiols.

Regarding marine organisms, such as mussels, several metals show an interaction with sulfhydryl groups (-SH). In particular was demonstrated (Benedetti et al., 2015) a reduction on antioxidant defences and induction of synthesis of metallothioneins (MTs). Even if antioxidant activity was observed in all mussel tissues, the organs that shows the highest activity is the digestive gland, because it is the major site of accumulation of xenobiotic and oxyradical generating biotransformation enzymes (Livingstone et al., 1992). Generally, the thiol groups act as intracellular antioxidants by scavenging free radicals and GSH is one of the most important cellular thiol, acting as a substrate for several transferases, peroxidases, and other enzymes that

prevent or mitigate the deleterious effects of oxygen free radicals (Mascio and Murphy, 1991). In these processes, GSH is oxidised to the oxidised form (GSSG). The GSSG can be reduced by glutathione reductase (GR) to regenerate GSH in the presence of NADPH(H^+) as a cofactor. Other great diversity of thiols has been described contributing to these essential cellular processes like trypanothione, with a function parallel to that of GSH (Flohé et al., 1999), and ergothioneine detected in different groups such as bacteria, plants and animals (Hand and Honek, 2005). Ergothineine has been detected in different organs of mammals and at different concentrations but until now there are only evidences of biosynthesis in certain fungi (Melville, 1956) and in actinomycetales, bacteria. Ovothiol is also one of such thiol molecules and plays a very important role in redox regulation.

1.2 OVOTHIOL

Ovothiol or π-N-methyl-5-thiohistidine was first isolated from marine invertebrates and acts as a non-enzymatic glutathione peroxidase system in consuming H_2O_2 and being reduced by glutathione (Palumbo et al., 2018).

It has been isolated from various organism and tissue, including eggs and biological fluids of some marine invertebrates and protists, including protozoa and algae (R. To date three different isoforms have been described, i.e. ovothiol A, B, and C (Fig. 2). They are homologous molecules and their unique difference is their degree of methylation, with ovothiol A being unmethylated, while ovothiols B and C being mono- and di-methylated, respectively (Russo et al., 2014). Ovothiol B was found in the ovaries of the scallop *Chlamys hastata*, while ovothiol C in the eggs ofthe sea urchins *Sphaerechinus granularis* and *Strongylocentrotus purpuratus* (Palumbo et al., 2018).

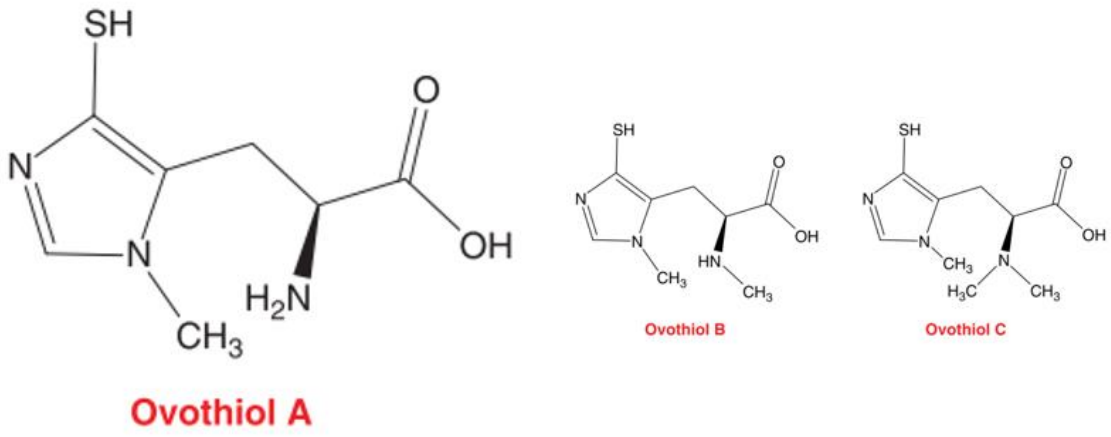

Fig. 2*:* Ovothiol A, B and C structure. (Palumbo et al., 2018)

1.3 BIOLOGICAL FUNCTIONS OF OVOTHIOLS

Since the first studies it was reported that ovothiols played a key role in controlling oxidative stress during fertilization. This was demonstrated in 1980s in the sea urchin *Strongylocentrotus purpuratus* where ovothiol C had a physiological role in the eggs protection from H_2O_2 toxicity (Turner et al., 1986). Recently, ovothiol A has been shown to act also during development, protecting embryos released in seawater from environmental factors (Castellano et al., 2016). In the pathogenic protozoa *Leishmania donovani* and in the non-human infective protist *Crithidia fasciculata* ovothiol A was suggested to be implicated in the protection of parasites from oxidative stress produced by macrophages during infection (Spies and Steenkamp, 1994). The unmethylated histidine derivative of ovothiol A, 5-thiohistidine, is reported in adrenochrome mixture of the branchial heart of *O. vulgaris* (Rossi et al., 1985).On the other hand, the metabolite L-Ovothiol A disulfide could act as a male pheromone during mating in the marine polychaete *Platynereis dumerilii* (Röhl et al., 1999) and a redox regulator in chloroplasts in the microalga *Dunaliella salina,* where ovothiol A disulphide inactivates ATPase by a disulphide exchange reaction (O'Neill et al. 2015; Selman-Reimer et al. 1991). Ovothiol A synthase (Ovo-A) was found to be up regulated in *Nematostella vectnsis* after expose to UV light and PAHs (Tarrant et al., 2018).

Tab.1: List of the main species that shows Ovothiol, with the ovothiol role and form.

SPECIES	REFERENCE	FUNCTION	OVOTHIOL FORMS
Octopus vulgaris	(Rossi, Nardi, and Palumb 1985)	Antioxidant	**Ovothiol A** (π-N-Methyl-5-thiohistidine)
Loligo vulgaris	(Rossi, Nardi, and Palumb 1985)		
Paracentrotus lividus	(Castellano et al. 2016)		
Marthasterias glacialis	(Palumbot et al. 1984)		
Platynereis dumerilii	(Röhl et al. 1999)	Pheromone	
Dunaliella salina	(Selman-Reimer et al. 1991)	Antioxidant	
Euglena gracilis	(O'Neill et al. 2015)	Antioxidant	
Nematostella vectensis	(Tarrant et al. 2018).	Antioxidant	
Leishmania spp., Trypanosoma cruzi, T. brucei, and Crithidia fasciculata	(Spies and Steenkamp 1994).	Antioxidant	
Chlamys hastata	(Eric Turner et al. 1987)	Antioxidant	**Ovothiol B** (π-N-Methyl-N^{α}-methyl-5-thiohistidine)
Strongylocentrotus purpuratus	(Palumbo, Castellano, and Napolitano 2018)	Antioxidant	**Ovothiol C** (π-N-Methyl-Nα, Nα-dimethyl-5-thiohistidine)

Parallel to these studies, a series of ovothiol derivatives was synthesized and their antioxidant properties examined. Among these, 1-methyl-2-(3-trifluoromethylphenyl)-4-mercaptoimidazole (MFP-4MI) was reported to be a potent neuroprotective agent for neuronal cells in the mammalian brain through its activity as ligand for GABAA receptors (agonist), N-methyl-d-aspartate and sodium voltage-gated channels (antagonist) (Vamecq et al., 2003). Therefore, as a consequence of its chemical properties and its potential therapeutic applications in humans, the interest in ovothiol A as a therapeutic agent has increased. Ovothiol A was tested in

human liver carcinoma cell line Hep-G2 and was observed a decrease in cell proliferation and autophagic process (Russo et al., 2014).

1.4 CHEMISTRY OF OVOTHIOL A

Though structurally resembling the 2-thiohistidine derivative ergothioneine, ovothiols exhibit quite different chemical properties because the acidity and the reactivity of the functional thiol is markedly affected by its positioning into the imidazole system. The sulfhydryl group of ovothiol A is characterized by a marked acidic character, as indicated by the low pKa value, estimated in several studies. The ovothiols have four acidic groups.

The thiol group of ovothiol A attached to the 5 position of the imidazole ring provides a pKa much lower ($pKa_{,SH}$ = 1.4) compared to cysteine ($pKa_{,SH}$ = 8.4), glutathione ($pKa_{,SH}$ = 8.7), trypanothione ($pKa_{,SH}$ = 7.4) and coenzyme A ($pKa_{,SH}$ = 9.8). This unique property has led to the belief that ovothiol A may be a particularly efficient scavenger of peroxides. On the other hand, the disulphide of ovothiol A is less stable that the disulphide of glutathione. Unlike most thiols, ovothiols are rapidly oxidized by H_2O_2. They react with H_2O_2 at least twice faster than glutathione to form the sulfenic acid, which then reacts with another molecule of reduced ovothiol to form the disulphide. Therefore, it seems that the more reactive ovothiol A and the more reductive glutathione could cooperate to protect the organism from hydrogen peroxide induced damage (Castellano and Seebeck, 2018).

1.5 OVOTHIOL A SYNTHASE (OVO-A)

The ovothiol A is biosynthesized from L-cysteine, L-histidine, molecular oxygen (O_2) and S-adenosyl methionine (SAM). The first step of the biosynthetic pathway involves the oxidative insertion of a sulphur atom into the C5–H bond on the histidine side chain (Mashabela and Seebeck, 2013). This reaction is catalysed by an iron (II) dependent sulfoxide synthase addressed to as ovothiol A synthase (Ovo-A) (Fig 3).

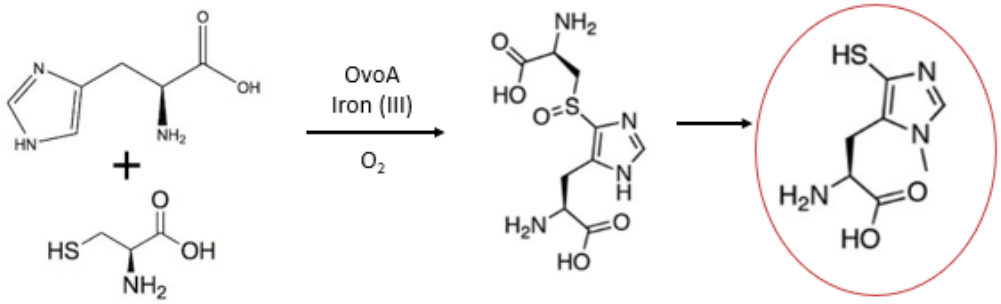

Fig. 3: Ovothiol A Biosynthesis mechanism. The intermediate is a 5-L-histidyl-L- cysteine sulfoxide (Mashabela and Seebeck, 2013)

A recent study reported the characterization of Ovo-A protein primary structure in several species of marine invertebrates (Hernando, 2018) which is schematically reported in Fig.4. By comparing the deduced Ovo-A protein structure with that of ergothioneine biosynthesis protein (EgtB), a sulfoxide synthase that contributes to the biosynthesis of thiol ergothioneine. Similar to EgtB, Ovo-A shows a conserved DNA damage-inducible (DinB) superfamily domain characterized by a four-helix bundle at the N-terminal region; This domain domain contains a putative iron-binding motif (HX3HXE). The DinB superfamily is involved in the SOS response was the first DNA repair system (Cheo et al., 1991). DinB domain is followed by a domain structurally related to that of copper-dependent formyl glycine generating enzymes (FGE sulfatase) that performed the conversion of the cysteine residue of sulfatases (Bojarová and Williams, 2008). Unlike EgtB, Ovo-A contains an additional S-adenosylmethionine methyltransferase (SAM-transferase) domain at the C-terminal region (Fig.4)(Castellano et al., 2016). It is related with different activities as trans-methylation and trans-sulfuration (Cheng and Blumenthal, 1999). In Ovo-A, it could take part in the methylation of ovothiol to generate the different forms, ovothiol A (nonmethylated), ovothiol B (mono-methylated) or ovothiol C (di-methylated) (Hernando, 2018).

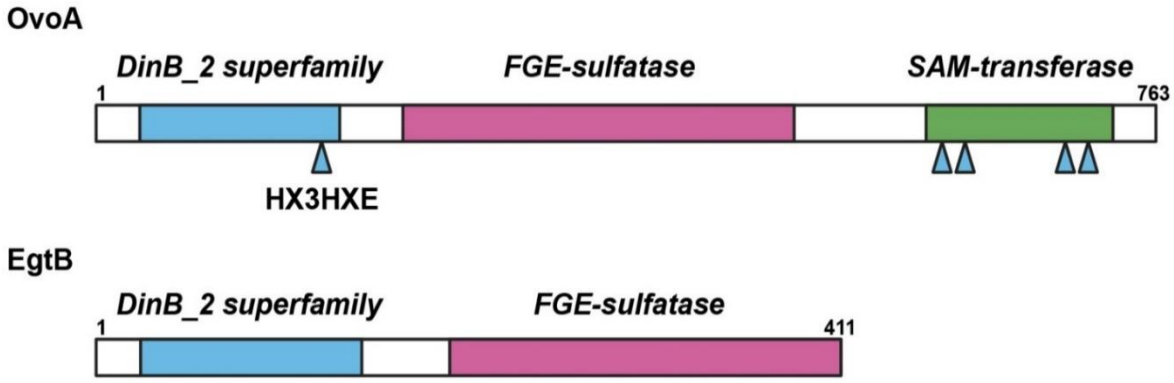

Fig. 4: Representation of Ovo-A and EgtB proteins primary structure (Modified from Castellano et al. (2016)). The DinB-superfamily domain is represented in blue and the FGE sulfatase domain in pink. First arrow indicates the iron-binding site (HX3HXE).

Even if the crystal structures of Ovo-A are not yet available a homology model have been constructed based on the crystal structure of *Mycobacterium thermoresistibile* ergothioneine biosynthesis protein (*Mt*EgtB) (Fig.5). Instead of cysteine and histidine, this enzyme accepts Na-trimethyl histidine and g-glutamyl cysteine as substrates. In analogy to MtEgtB, active site of Ovo-A shows three conserved histidine residues that coordinate the catalytic Fe ion (Castellano et al., 2016). A recent study confirmed that Ovo-A contains a structurally equivalent active site tyrosine (Tyr417) that plays the same pivotal role as Tyr377 in EgtB (Castellano and Seebeck, 2018).

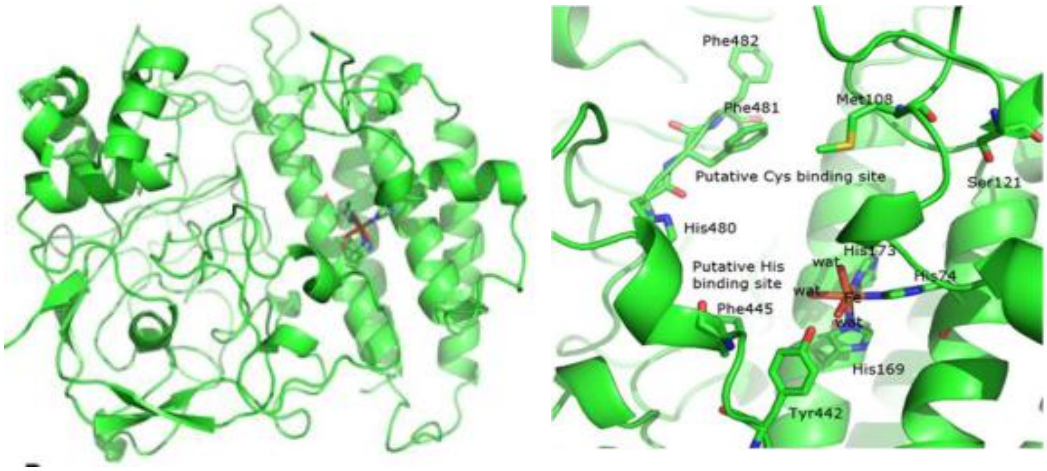

Fig. 5: **Structural model of *P.lividus* Ovothiol A synthetase**. PlOvo-A catalyze C-S bond formation and sulfoxidation between gamma-glutamyl cysteine and N-alpha-trimethyl histidine or between cysteine and histidine as the central steps in the synthesis of ovothiol. (Castellano et al., 2016)

Recently, the promoter region of the sea urchin *Ovo-A* gene has been demonstrate contains to metal responsive elements (MRE), that induces the expression of metallothioneins and other genes involved in metal homeostasis, and stress responsive elements 5 (SRE) that under stress conditions regulate transcription of the gene (Castellano et al., 2016). It is this responsibility which confers adaptability to stressful conditions to the animals and that can constitute an interesting study tool for the environmental toxicologists. Toxins and heavy metals, dispersed in seawater can induce oxidative stress and the resulting ROS production can induced transcription factors, which bind to MRE and/or SRE and activate *Ovo-A* transcription. Finally, Ovo-A enzyme catalyzes the synthesis of ovothiol to counteract redox unbalance (Fig.6).

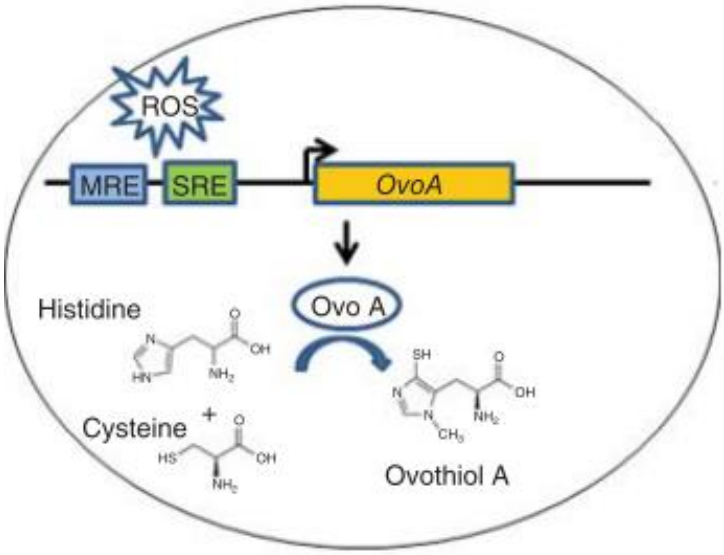

Fig 6: model for *Ovo-A* expression as response to ROS production (Castellano et al., 2016)

1.6 EVOLUTIONARY HISTORY OF THE *OVO-A* GENE

Ovo-A is highly conserved in marine metazoans, from Porifera to Placozoa, Cnidaria, protostomes and deuterostomes (Palumbo et al., 2018). Among deuterostomes, *Ovo-A* is conserved in Echinodermata and Hemichordata, Cephalochordata, Urochordata, and in Chondrichthyes. It seems there has been two independent events that have brought to the loss of *Ovo-A* gene: one in nematodes and arthropods, at least in terrestrial species, and the other one in the ancestor of Teleostei fishes. Has been hypothesized that the loss Ovo-A in Teleostei

may be related to development of the swim bladder and of more efficient systems of respiration and blood circulation with a resulting better control of oxygen exchange (Castellano et al., 2016). Ovothiols was reported also in the eggs of the rainbow trout, *Oncorhynchus mykiss*, as well as from the eggs of the *Oncorhynchus kisutch*. Most likely these higher animals acquire ovothiol through their nutrition, which in the case of salmonidae includes ovothiol producing mollusks (Castellano and Seebeck, 2018).

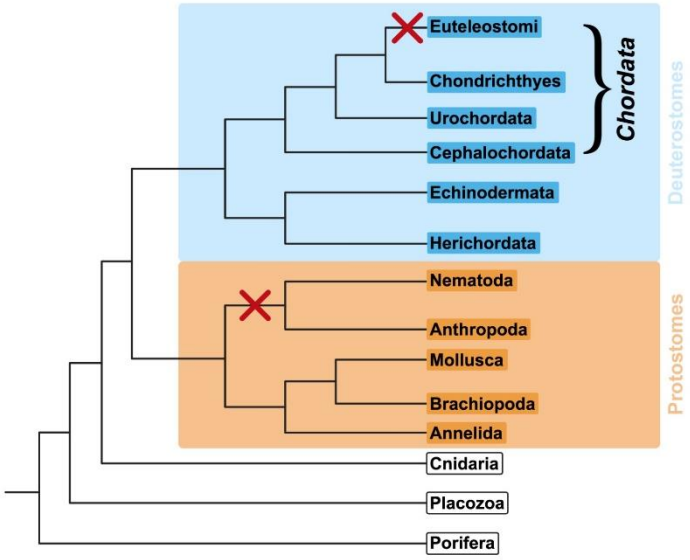

Fig. 7: Phylogenetic tree of *Ovo-A* in metazoans. Modified from (Castellano et al., 2016)

2 RESEARCH OBJECTIVES

In marine organisms, unbalanced ROS production is observed as a response to natural environmental stressors (i.e. temperature and salinity variations, ocean acidification; or to the presence of environmental pollutants (Lushchak 2011)). The non-enzymatic antioxidant defence system includes a series of molecules containing a sulfhydryl (-SH) group, able to accept electrons, called thiols. The most known and best studied is glutathione (GSH), but in the last years the attention has been also set on ovothiols. Ovothiol is a thio-histidine methylated on the imidazole ring. It was isolated from various organisms and tissues, in particular oocytes of some marine invertebrates, and suggested to play a key role in controlling oxidative stress during fertilization and embryo development (Castellano et al. 2016). Ovothiols exist in three different forms, namely ovothiol A, B, and C, which differ for the methylation of the alanine side chain. The thiol group of ovothiol A at the position 5 of the imidazole ring provides a pKa much lower ($pKa_{,SH}$ = 1.4) compared to other antioxidant molecules. This unique property has led to the hypothesise that ovothiol A may be a particularly efficient ROS scavenger. The present study investigates transcriptional expression of an ovothiol synthase (Ovo-A) gene product in the Mediterranean mussel (*Mytilus galloprovincialis*). Ovo-A is an iron (II) dependent sulfoxide synthase which catalyzes the first step in ovothiol A biosynthesis, and therefore can be inferred as a marker of ovothiol response to environmental stress in a marine animal.

Marine mussels are exceptionally well-adapted to live in coastal marine habitats, where they are exposed to fluctuating environmental parameters and elevated levels of natural and anthropogenic pollutants throughout their life-cycle (Franzellitti et al., 2010), and the antioxidant system plays a key role in such a remarkable acclimatory efficiency (Regoli and Giuliani, 2014).

In the present work, mussels were subjected to an in vivo exposure to copper, a known inducer of oxidative stress, and changes of *Ovo-A* expression was investigated in different tissues as well as in males and females at different developmental stages. The aims of the work are:

- to assess the role of Ovo-A in gonadal development, besides its function in fertilization and in early larval stage development (Castellano et al., 2016).

- to assess the involvement of Ovo-A in the mussel antioxidant response.

The experiments were carried out at the laboratories of the Department of Zoology and Animal Cell Biology of the Plentzia Marine Station, University of the Basque Country in Bilbao (Spain).

3 MATERIAL AND METHODS

3.1 ANIMAL HANDLING AND EXPERIMENTAL SETUP

One hundred mussels (*Mytilus galloprovincialis*) were collected at the end of April in the gulf of Plentzia (Basque Country) at the Butroi estuary (43° 24' 32.35'' N - 2° 56' 50.32'' W) (Fig.8)

Fig.8: sampling site. Gulf of Plentzia at the Butroi estuary.

All the mussels were acclimatized for 1 week to laboratory conditions in continuously aerated seawater at 18°C and a light cycle of 12h/12h

. The seawater was filtered naturally pumping the sand of the beach, after that precipitated by decantation and filtered by 0.2 filters and treated by UV. After acclimatization, 50 mussels were taken as control organisms and placed in aquaria with clean seawater (18°C). Further 50 mussels were place in 25-L aquaria containing seawater (at 18°C) contaminated with a solution of copper (II) oxide (CuO) at a final concentration of 10 µg/l.

Samplings from control and Cu-treated mussels were performed at 3 and 7 days of exposure. At each time-point, gonads, digestive gland and gills were dissected from 25 mussels in the control and in the Cu group, respectively, for the molecular

analyses. Tissues were placed in cryovial tubes and immediately frozen in -80ºC after embedding in the RNAlater. Gonads were also collected for the histological analysis, placed into a histology cassette and fixed in 10% Neutral Buffered Formalin (10% NBF) for 24 hours.

3.2 PROCEDURE FOR HISTOLOGICAL ANALYSES OF GONADS

After 24h fixation, samples were transferred to 70% ethanol and then processed for paraffin embedding. Paraffin blocks were cut at a thickness of 5 µm using a Leitz 1512 microtome (Leica, Vienna, Austria).

Sections were then stained with haematoxylin/eosin using a Leica AutostainerXL (Leica) to finally mount the slides with DPX (Sigma-Aldrich, St. Louis, MO, USA) for microscopic analyses. Sex and developmental stage of each animal was determined within any of six gamete developmental stages as described for by Seed (Seed, 1969). Can be classified six development stages.

Tab.2: Gonadal development stages classification in mussels described by Seed.

Stage I	Islands of germinal tissue appearing in the matrix of dense connective tissue. Is difficult to separate into males or females.
Stage II	Ripe gametes appear in the centre of the follicles although these are occupied mainly by early stages of gametogenesis: small, numerous oocytes attached to the germinal epithelium in the female, and spermatogonia and spermatocytes in the male.
Stage III	General increase in the mass of the gonad at the expense of the stored food in the connective tissue. This is a stage of rapid gametogenesis with approximately half of each follicle occupied by ripe gametes, and half with early stages of gametogenesis. The area occupied by genital tissue is about half that of the fully ripe conditions.
Stage IV	Maximum proliferation of genital tissue is almost attained. Preponderance of ripe gametes in each follicle, with a general reduction in the earlier stages of gametogenesis.
Stage V	It differs from developing stage IV only in the greater reduction of early stages of gametogenesis (a few small oocytes in the germinal epithelium of the female, and a narrow band one or two cells deep, of spermatogonia and spermatocytes in the male). Oocytes are compacted into polygonal configuration. The time taken from morphological to physiological ripeness, varies from a few weeks to a few months.
Stage VI	*Spawning Stages*. The follicles are still relatively full of ripe gametes, but active discharge of these is now in progress. This is obvious from the general reduction in density of spermatozoa, and the rounding off of the remaining ova as the pressure within the follicles is reduced following partial emission.

3.3 STUDY OF THE *OVO-A* TRANSCRIPTION PATTERNS

RNA extraction and cDNA synthesis

50-100 mg of tissue samples dissected from each animal were homogenized in 1 mL of TriZol®. reagent (Invitrogen, Carlsbad, California, USA) and RNA extraction performed following the manufacturer's instructions. RNA concentration was assessed by measuring the absorbance at 260 nm with a Biophotometer (Eppenorf, Hamburg, Germany). Moreover, samples were analysed by capillary electrophoresis for testing the RNA quality using the Lab on-a-Chip kit and the Agilent 2100 Bioanalyzer according to the manufacturer´s instructions (Fig.9). RNase degradation of RNA samples is a common reason for failed experiments. The 2100 Bioanalyzer system provides RNA quality control results in both gel-like image as well as electrophoretic data making it easy to detect even small degradation effects (Agilent Technologies, 2017).

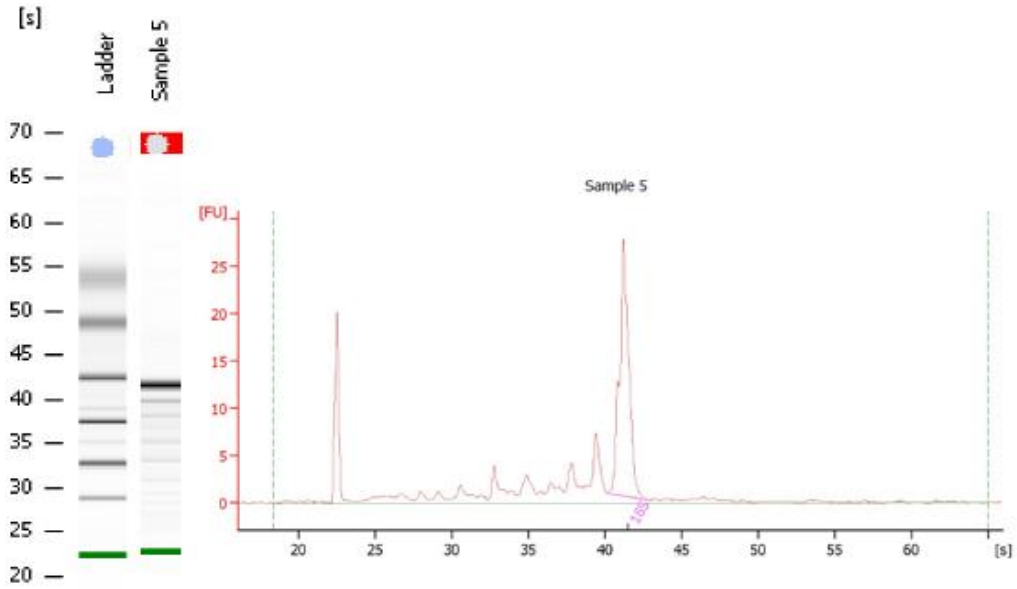

Fig. 9: Result of RNA quality analysis. Are shown the two peaks corresponding to lower marker and 18s portions.

First strand cDNA for each sample was synthesized from 1.3 µg total RNA using the Affinity Script Multiple Temperature cDNA Synthesis Kit (Agilent Technologies). cDNA concentration was further confirmed using the QuantiT ™ OliGreen® Kit (Life Technologies™, Thermo Fisher Scientific, Waltham, Massachusetts, USA), following manufacturer's instructions.

qRT-PCR analysis of *ovo-A* transcription levels

Real-time Polymerase Chain Reaction (PCR) is a method to quantifying levels of gene expression. The difference respect the normal PCR is the ability to detect, at every cycle of the PCR (i.e., in real time), the amount of PCR product (amplicon) using fluorescence that increases in proportion to the accumulation of the products of PCR. A real-time-PCR read-out is given as the number of PCR cycles ("threshold cycle " Ct) necessary to achieve a given level of fluorescence (Ponchel et al., 2003).

qPCR amplifications were performed in triplicate in a final volume of 20 µL containing 2µL diluted cDNA (1:20) and 10µL of FastStart Universal SYBR Green Master Mix (Roche Diagnostics, Mannheim, Germany), and 0.2 µM specific primers (Tab 2). A control lacking cDNA template (no-template) and a minus-reverse transcriptase (no-RT) control were included in the qPCR analysis to determine the specificity of target cDNA amplification. Reactions were carried out in a 7300 Real-Time PCR System (Applied Biosystems). The amplification protocol was programmed to follow an initial denaturation and activation at 50ºC for 2 minutes and 95ºC for 10 minutes, respectively, followed by 40 cycles of denaturation (95ºC, 15 seconds) and annealing/extension (58ºC, 1 minute). Melting curves and agarose gel electrophoresis were utilized to verify the specificity of the amplified products and the absence of artefacts. The amplification efficiency was calculated using a dilution series of cDNA

Tab. 2: Ovo-A primers used for qPCR analysis with the size in base pairs (bp), primer melting temperatures (Tm) and efficiency.

FW (5'-3')	RV (5'-3')	Length (bp)	Tm	Efficiency
GTCCATGGTGGGGAC	GTCCATGGTGGGGAC	179	58	1.522

Efficiency of amplification was estimated by a standard curve, after amplification of serial dilutions of a cDNA pool (dilution factor from 0,2 to 6.25×10^{-3}) and applying following the following equation:

$$\text{Efficiency} = 10^{(-1/\text{slope})}$$

With the obtained value, the Efficiency correction was calculated using the delta *C*t (Δ*C*t) method. Finally, relative quantity (RQ) of all gene transcription levels was calculated. All the results were normalized, using the values obtained from the undetermined phase mussels.

$$\Delta Ct = Ct \text{ sample} - Ct \text{ (indeterminates)}$$

$$\text{Eff. Correction} = \text{Efficiency } \Delta Ct/[\text{cDNA}]$$

$$RQ = \text{Eff correct} / \text{Prom. Eff correct}$$

3.4 STATISTICS ANALYSIS

Data on qPCR quantification of Ovo-A expression were analysed using 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: F-test). These statistical analyses were performed using GraphPad Prism 8. Further statistical inferences were performed using permutation multivariate analysis of variance (PERMANOVA) using the PERMANOVA + add-on in PRIMER v6 (Anderson et al., 2008). Log-transformed Ovo-A fold changes were used to calculate similarity matrices based on the Euclidean distance (999 permutations). Factors considered were “sex” (2 levels), “tissue type” (3 levels), “treatment” (2 levels), “exposure time” (2 levels). Pseudo-F values in the PERMANOVA main tests were evaluated in terms of significance (Anderson et al., 2008), and threshold for statistical differences were set at $P < 0.05$.

4 RESULTS

4.1 HISTOLOGICAL ANALYSIS

All gonads samples were analysed to identified sex and developmental stage.

The female: male ratio was 3:2 in the first day and 1:1 in the second day of dissection. When considering the whole set of organisms, most part of females were at developmental stage III, while most part of part of males were at developmental stage IV (Fig. 10). Examples of histological sections employed in the analyses in Fig. 11 and Fig. 12.

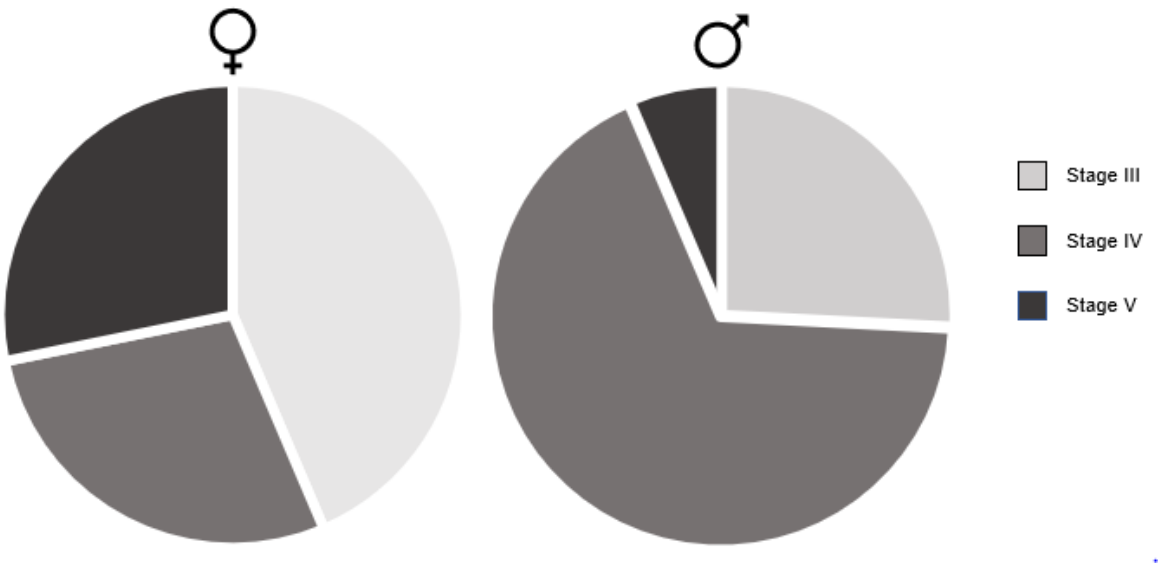

FIG.10: graphic that shows the development stages of gonads determinate using Seed classification (Seed, 1969)

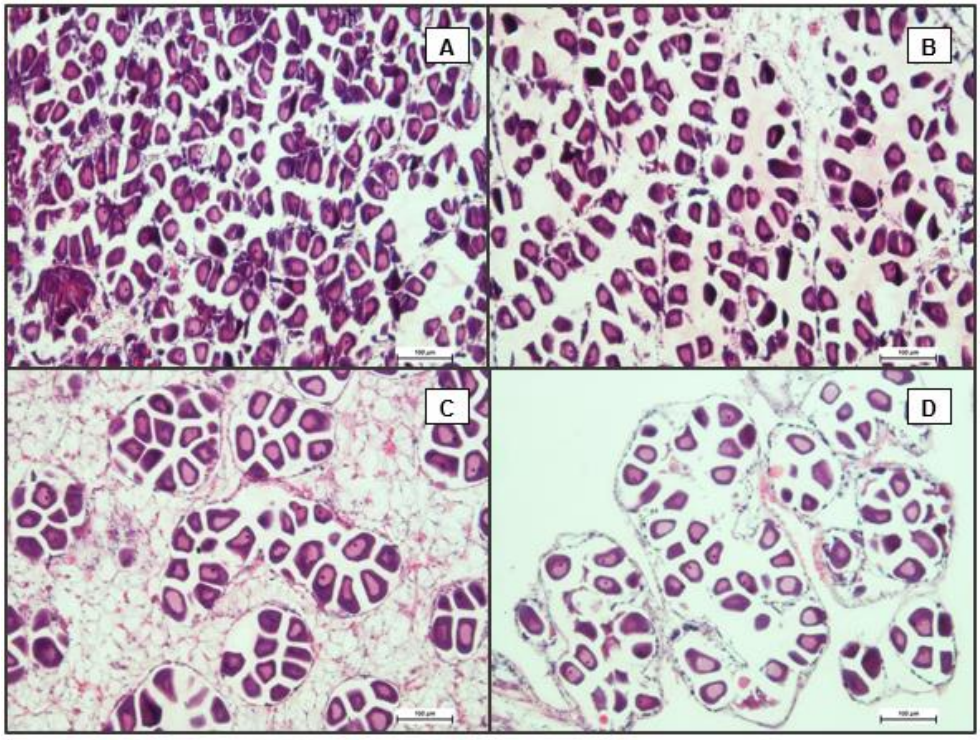

Fig. 11: Histological images of female gonads. (A) Photomicrographs of mussel's ovary in a stage 4**. (B)** Photomicrographs of mussel's ovary in a stage 3. **(C-D)** Photomicrographs of mussel's ovary in a stage 2**.** Scale bars: 100 µm

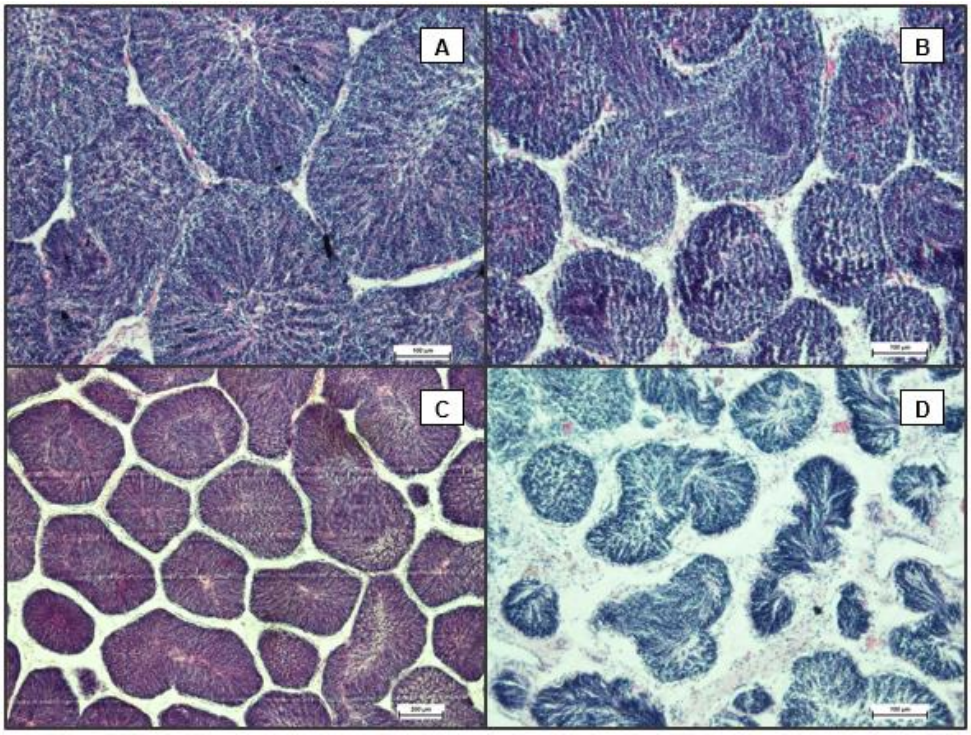

Fig. 12: Histological images of male gonads. (A-B-C) Photomicrographs of mussel's testis stage 4. **(D)** Photomicrographs of mussel's testis stage 2**. (A-B-D)** Scale bars: 100 µm **(C)** Scale bars: 200 µm.

4.2 TISSUE AND SEX RELATED OVO-A TRANSCRIPTION LEVELS

Ovo-A was found to be expressed in all tissue studied (gonads, digestive glands and gills) of both sexes (male and female). Fig.13 shows comparable expression levels in all tissues analysed as well as between males and females.

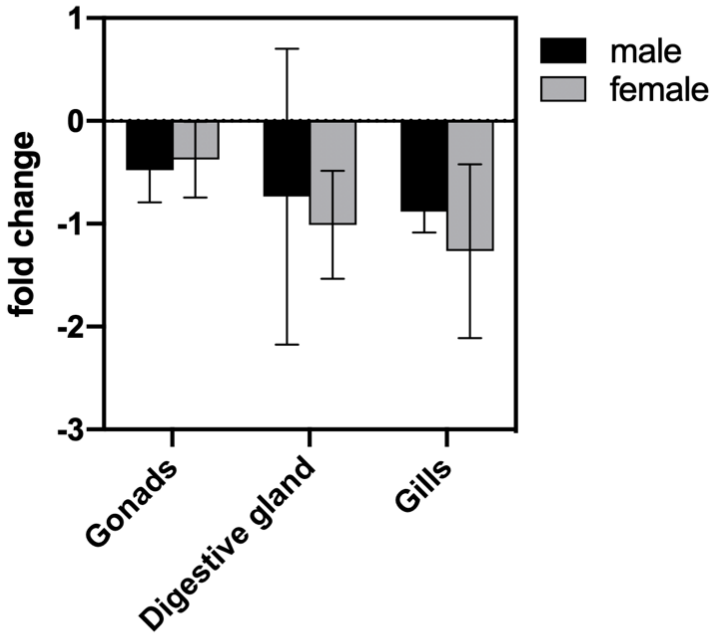

Fig.13: basal levels of *Ovo-A* expression in gonads, digestive glands and gills of mussels at different gonadal stage. Fold change data have been normalized over a reference sample, composed a pool of gonads from different males and females.

Differences in male vs female *Ovo-A* expression was investigated in gonads of mussels at different developmental stages (Fig. 14). Males show no significant expression changes between stage 3 and stage 4, while females show significantly reduced expression levels in stage 5 organisms compared to stage 2 and stage 3. Ovo-A expression in males at stage 5 is not reported due to the low number of organisms.

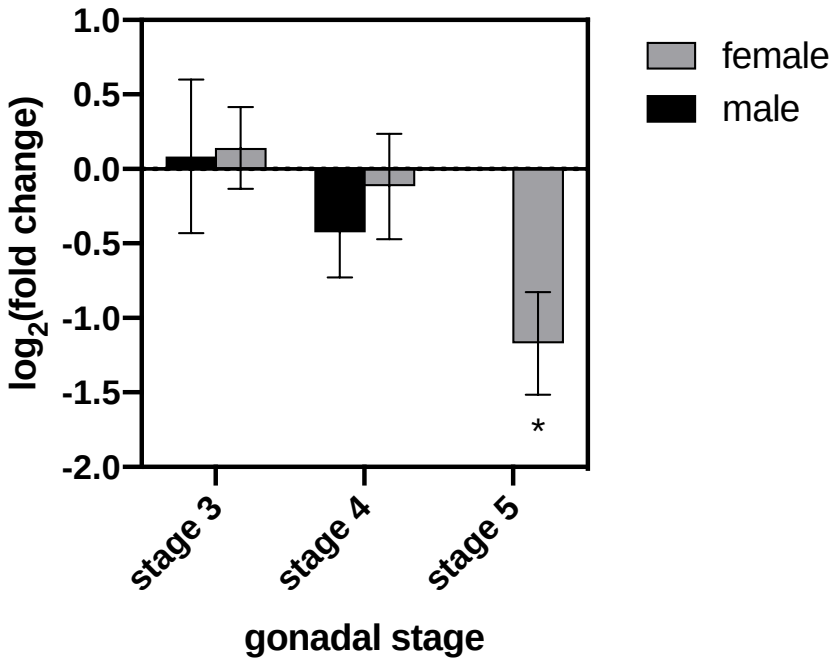

Fig.14: Levels of *Ovo-A* expression in gonads of mussels at different gonadal stage. Asterisks indicate statistical differences compared to samples at stage 3 ($p < 0.05$)

4.3 EFFECTS OF COPPER EXPOSURE ON *OVO-A* TRANSCRIPTION LEVELS IN DIFFERENT TISSUES AND BETWEEN SEXES

Preliminary PERMANOVA analyses was performed to assess whether the effect of Cu treatment could depend on sex of the animals, tissue type, and sampling time-point. Results reported in Table 3, show a significant interaction of Cu-treatment with the factor “tissue” ($P < 0.05$), while the single factor had no significant effect ($P > 0.05$).

Tab.3: PERMANOVA results on ovothiol expression in different tissues of male and female mussels treated with copper (998 permutations). TIM: time; TR: treatment; SE: sex; TIS: tissue. df=degree of freedom; Pseudo-F: F value by permutation (Anderson et al., 2008); P (perm): probability of pseudo-F.

Source	df	Pseudo-F	P(perm)
TIM	1	142.330	**0.001 ***
TR	1	0.014	0.910
SE	1	8.335	**0.004 ***
TIS	3	59.558	**0.001 ***
TIMxTR	1	3.673	0.059
TIMxSE	1	9.940	**0.002 ***
TIMxTIS	3	30.528	**0.001 ***
TRxSE	1	1.093	0.289
TRxTIS	3	3.952	**0.01 ***
SExTIS	3	4.919	**0.006 ***
TIMxTRxSE	1	4.395	0.041
TIMxTRxTIS	3	2.127	0.086
TIMxSExTIS	3	4.231	**0.009 ***
TRxSExTIS	3	0.524	0.656
TIMxTRxSExTIS	3	1.104	0.356

In gonads was observed a general *Ovo-A* down-regulation in Cu-treated mussels was observed at both sampling time-points, although the differences resulted significant only in male gonads ($P < 0.05$) (Fig.15).

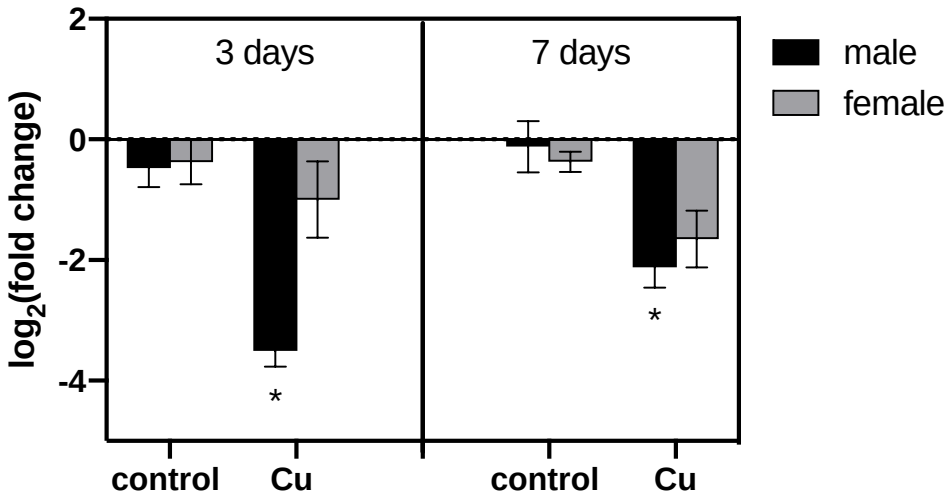

Fig.15: *Ovo-A* expression profile in response to Cu treatment in mussel gonads after 3 and 7 days of exposure. *p < 0.05 vs controls within each time-point.

The digestive glands showed a different trend (Fig.16). In fact, was observed a general up-regulation of transcription levels after 3 days of exposure to copper in both sexes, but the differences resulted significant only in females (*p< 0,05). Moreover, was observed a significant up-regulation of transcription levels (** p< 0,05) between control of day 3 and control of day 7 in both sexes. This difference was also observed in gills (Fig.17) that instead didn't show significant differences between the control and treatment.

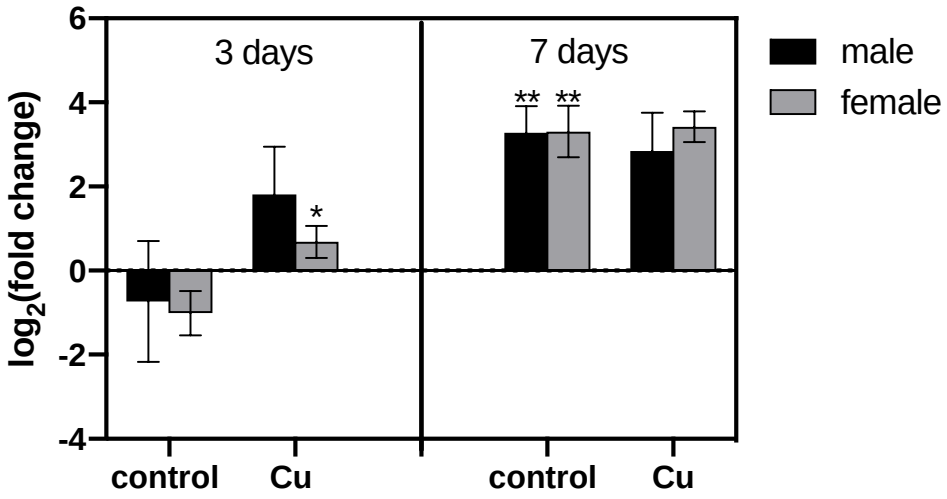

Fig.16: *Ovo-A* expression profile in response to Cu treatment in mussel digestive glands after 3 and 7 days of exposure. * P < 0.05 compared to controls within each time-point. ** P < 0.05 with respect the control at day 3.

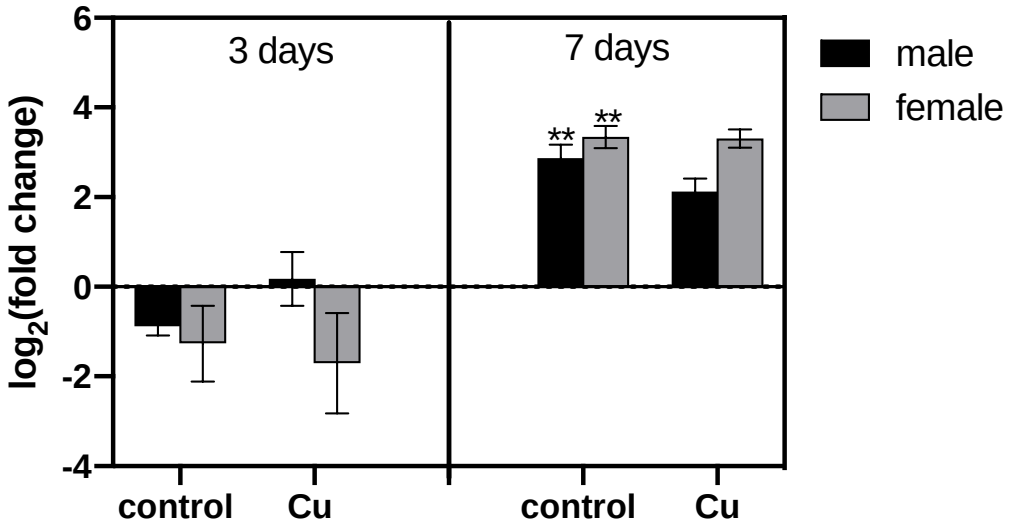

Fig.17: *Ovo-A* expression profile in response to Cu treatment in mussel gills after 3 and 7 days of exposure. ** p<0.05 vs controls at day 3.

5 DISCUSSION

Most of the studies on ovothiols focused on its presence, especially in ovaries and eggs, and on its chemical and structural characteristics (Palumbo et al., 2018b). The pattern of Ovo-A (ovothiol A synthase) expression has been recently studied in sea urchins during embryo development, from unfertilized eggs to the pluteus stage (Castellano et al., 2016). *Ovo-A* expression was reduced soon after fertilization and then further decreased up to the pluteus stage. Nevertheless, a different response to Cd^{2+} exposure was observed across progression through embryo stages, with up-regulation at in blastulae and strong down-regulation in plutei. Such a differential regulation may be related to the involvement of ovothiols in the protective processes the aid in the elimination of primitive tissues and debris during embryo/larvae development (Bassim et al., 2015).

A first investigation on marine mussels (*M. galloprovincialis*) managed to identify and sequencing a partial sequence encoding the ovothiol synthase (Hernando, 2018). The multiple alignment of the partial sequence from *M. galloprovincialis* with the orthologs from *P. lividus, S. purpuratus,* and *C. gigas* confirmed the presence of DNA damage-inducible (DinB) family domain and in Formylglycine generating (FGE) sulfatase domain. Inside the DinB family domain a conserved metal response element (MRE) as iron binding motif (HX3HXE) was also identified. Therefore, the putative mussel Ovo-A retains all the structural features that characterize a functional Ovo-A protein, suggesting a conserved function compared to that in sea urchins (Castellano et al., 2016).

For the first time, this research work investigated the expression patterns of a *Ovo-A* gene product in tissues of adult *Mytilus galloprovincialis* exposed to copper, a metal that is known to induce oxidative stress in exposed animals (Lushchak, 2011). Investigations were performed in three different tissues and taking into account the possible bias given by sex of the animals; therefore, a comparison of the responses between males and females was also performed.

In unexposed mussels, baseline *Ovo-A* expression is not significantly different between the analysed tissues. The three tissues have comparable *Ovo-A* expression levels likely resulting from their specific physiological functions and the

importance of antioxidant responses in supporting such functions. The antioxidant system in the gonads acts especially during gametogenesis in the preparation of the oocytes to the spawning phase and the subsequent fertilization phase. Antioxidant molecules and nutrient reserves are packed into the oocytes to increase the success of reproduction and the survival of free-swimming larval stage (Kautsky, 1982). The mussel digestive gland is a tissue of choice for detecting early signs of exposure to various toxicants, as contaminant bioaccumulation and metabolism of many compounds peak in this organ (Venier et al., 2006). Therefore, the digestive cells of bivalves also possess mechanisms of contaminant accumulation and detoxification (Izagirre and Marigómez, 2009), as those involved in metal homeostasis (Viarengo and Nott, 1993). Gills represent a first site of animal interaction with waterborne pollutants, as such they are endowed with efficient protective mechanisms (Franzellitti et al., 2016).

The down-regulation of *Ovo-A* in relation to female gonads development may be due the conversion to Ovo-A mRNA into the protein which is then accumulated in oocytes, in line with the study on sea urchins (Castellano et al., 2016). The same accumulation process was observed for vitellogenin (Gagné et al., 2005), and, more generally, packaging high levels of cellular defense molecules into the egg prior to release into the environment (thus before exposure to stress) is considered as a ‘‘be prepared’’ adaptive strategy allowing fast induction of these defenses under stress conditions (Hamdoun and Epel, 2007; Franzellitti et al., 2017). As a further support to this hypothesis, a preliminary analysis on antioxidant molecules concentrations in gonads performed by Orbitrap LC-MS showed an increase of Ovothiol-A concentration at increased maturation stage (Fig. 18), which could parallel the increased Ovo-A tissue protein content.

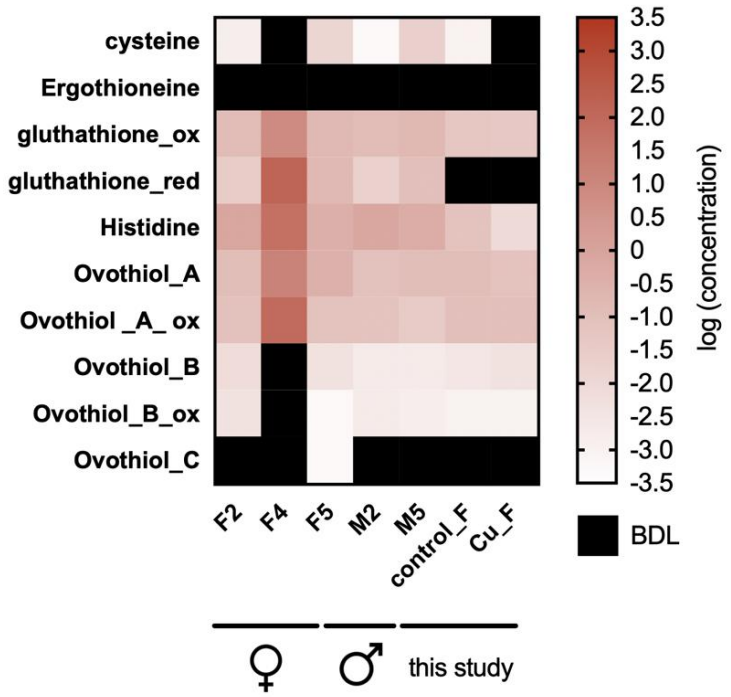

Fig 18. Results of a preliminary chemical analysis by Orbitrap LC-MS showing trends of concentrations for low-molecular weight antioxidant molecules in mussel gonads. Samples assessed in this study (pooled control- and Cu-treated females between 3 and 7 days of exposure) are compared with reference samples at different gonadal maturation stages (females: stage 2, 4, and 5; males: stage 2 and 5). Data are log-transformed chemical concentrations (µg/mg tissue). BDL: below detection limits.

The effects of exposure to copper on *Ovo-A* expression is significantly observed only ion gonads, while digestive glands and gills showed transient (digestive glands in females at 3 days of exposure) or no significant changes in mRNA expression. This finding may suggest that ovothiol in those tissues could display a minor role in the whole antioxidant defence system. Indeed, it is well known that both tissues are targets of investigations addressing the effects of metals on marine organisms (Regoli and Principato, 1995). In particular, the digestive gland is the main tissue of copper accumulation at low concentration, while gills are the target tissue in studies with high copper concentrations (Jing et al., 2006). As such, they are endowed with efficient cytoprotective mechanisms such as the lysosomal system, the expression of metal responsive proteins (i.e. metallohtioneins), and the glutathione system (Benedetti et al., 2015). Therefore, differential involvements of specific antioxidant pathways are likely to occur in response to different stressors. For example, in the clam *Ruditapes decussatus*, copper exposure had an inhibitory effect on the activity of catalase (CAT) and Se-dependent glutathione peroxidase, despite the increased production of oxyradicals (Geret et al., 2002). A similar effect was observed in gills

of the freshwater bivalve *Unio tumidus* (Doyotte et al., 1997). The exposure of mussels to copper was also studied by analyzing expression levels of metallothioneins isoform MT10 and MT20 (Zorita et al., 2007). In particular, MT10s have been reported to be synthesized constitutively and involved in essential–metal regulation, particularly of Cu and Zn. The expression of MT10 was reduced after two days of exposure to copper (40 ppb). Cu is an essential metal and probably Cu-binding MTs are permanently present at given basal levels within the cell before exposure takes place (Langston et al., 1998).

In gonads the response to copper exposure was observed only in males with a down- regulation of *Ovo-A* expression. *Ovo-A* may be expressed at basal levels and may not play a significant role during spermatogenesis process. In females, the low response may be due to interference of packaging processes of Ovo-a with the antioxidant response to copper.

The studies about ovothiol A and in particular on Ovothiol a synthase, are still few. Only recently, as a consequence of the assessment of its chemical properties and of its potential therapeutic applications in humans, the interest has remarkably increased. The present study is the first carried out on extragonadal tissues and the second that uses genus *Mytilus* as a model organism and sex bias is considered. The lack of *Ovo-A* regulation by Cu in extragonadal tissues and the relevant influence of gonadal maturation stage in *Ovo-A* baseline expression lead to hypothesise that maturation processes may display a major role in *Ovo-A* regulation than exposure oxidative stress generating chemicals. Nevertheless, effect between of chemical exposure on antioxidant system in the gonadal tissue are suggested by the significant *Ovo-A* down-regulation observed in males. To get further insights on this last finding it would be useful the employ of a battery of biomarkers along with *Ovo-A* along to get a general view of the antioxidant system in mussel gonads and elucidate the role of Ovo-A and ovothiols.

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