Transcriptomic approach of the response to metals in the hydrothermal mussel Bathymodiolus azoricus

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Transcriptomic approach of the response to metals in the hydrothermal mussel *Bathymodiolus azoricus*

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Para mi hija y esposa,

por todo el tiempo

invertido en esto

y no al estar con ustedes.

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Chapter I
Introduction

In 1962, the marine biologist and conservationist Rachel Carson published her book "A Silent Spring" in which she warned harmful effects of pesticides (DDT) in the environment mainly in birds and humans, establishing a relationship between toxicology and environment. In 1969, René Truhaut introduced the term "ecotoxicology" as the combination of two very different subjects: **ecology** (defined as the scientific study of interactions that determine the distribution and abundance of organisms" ‘Krebs 1985) and **toxicology** (defined as the study of injurious effects of substances on living organisms). If the limit of the toxicological studies is the organisms, ecotoxicology aims to assess the impact of chemicals on a larger scale considering different levels of organization from cell to ecosystems (Truhaut, 1977). At the beginning, the study of environmental toxicology was limited to the detection of toxic residues in the environment or in individual organisms and the use of tests to estimate the toxic effects of chemicals on animals other than human. One main limitation of these approaches was the impossibility to extrapolate those experimental results obtained at an individual level to the understanding of a more complex, multivariate environment. At that point, ecotoxicology was developed in the aim to measure/quantify and predict the impact of pollutants on populations, communities and whole ecosystems rather than on individuals. The exact scope and definition of ecotoxicology still remains on debate. In 1994, Forbes and Forbes defined ecotoxicology as the field of study which integrates the ecological and toxicological effects of chemical pollutants on populations, communities and ecosystems with the fate (transport, transformation and breakdown) of such pollutants in the environment. They also identified three main objectives for ecotoxicology: 1) obtaining data for risk assessment and environmental management, 2) meeting the legal requirements for the development and release of new chemicals into the environment and 3) developing empirical or theoretical principles to improve knowledge of the behavior and effects of chemicals in living systems. To fill these objectives, different steps have to be considered like the identification of the entry, distribution and fate of pollutants within the environment, in living (biota) organisms within an ecosystem and the harmful effects of the chemical pollutants on the constituents (biotic & abiotic) of ecosystems. However, ecotoxicology is different from stress ecology which considers a broader range of natural stressors such as oxygen depletion or the effects of temperature, populations and communities that directly impact on toxicity.

There is further separation between environmental toxicology and ecotoxicology, with a tendency of ecotoxicology to focus on the level of communities and ecosystems and environmental toxicology being more focused on the level of individual organisms or cells. Ecotoxicology aims to understand the mechanisms/mode of action by which environmental contaminants modify normal biological performance in order to develop appropriate measures to prevent adverse outcomes. There are numerous contaminant effects that can compromise the ecological fitness of individuals or populations and the impact of a toxic contaminant or complex contaminant mixture depends on the relative sensitivity of a species, community or ecosystem, but also the intensity and timing of exposure. Ecotoxicology faces the challenge of predicting and assessing the effects of an increasing number of chemical stressors on aquatic species and ecosystems. With increasing ecological relevance the reproducibility, specificity and thus suitability for standardization of methods tends to diminish. A more simple definition has been proposed by Walker et al., (1996) defining ecotoxicology as "the study of the harmful effects of chemicals upon ecosystems". There are 6 main types of toxicants that are studied in a variety of species including **carcinogens** that cause cancer, **mutagens** that cause mutations in DNA, **teratogens** that cause birth defects, a**llergens** that cause unnecessary immune response, **neurotoxins that** damage nervous system and **endocrine disruptors** that interfere with hormones. More, toxicity is affected by many parameters such as the sensitivity to toxicant that vary with sex, age and weight. Exposure to toxicant is defined either as acute (high exposure in short period of time) or chronic (low concentration of toxicants with a long period of time of exposure). In both cases, results of both type of exposure are used in some form of weight of evidence assessment, but without clear guidance as to how to interpret deferential responses and intensities of response.

One of the main objectives of ecotoxicology is the monitoring of environmental pollution, which includes the development of bioassays or single-species tests to obtain a fast and easy access to tools of evaluation of chemicals toxicity and/or effects in both terrestrial and marine ecosystems. However, study of ecotoxicological parameters using only single-species, single toxic and laboratory tests do not represent the complexity of ecosystems and the diversity of responses, so it remains fundamental to understand how populations respond to these environmental changes in their natural habitats. Field studies showed that the ecosystem health mainly depends on three factors: differences in the fate and transport of the toxic substance, the complexity of systems and individual responses of organisms (Burger, 1997).

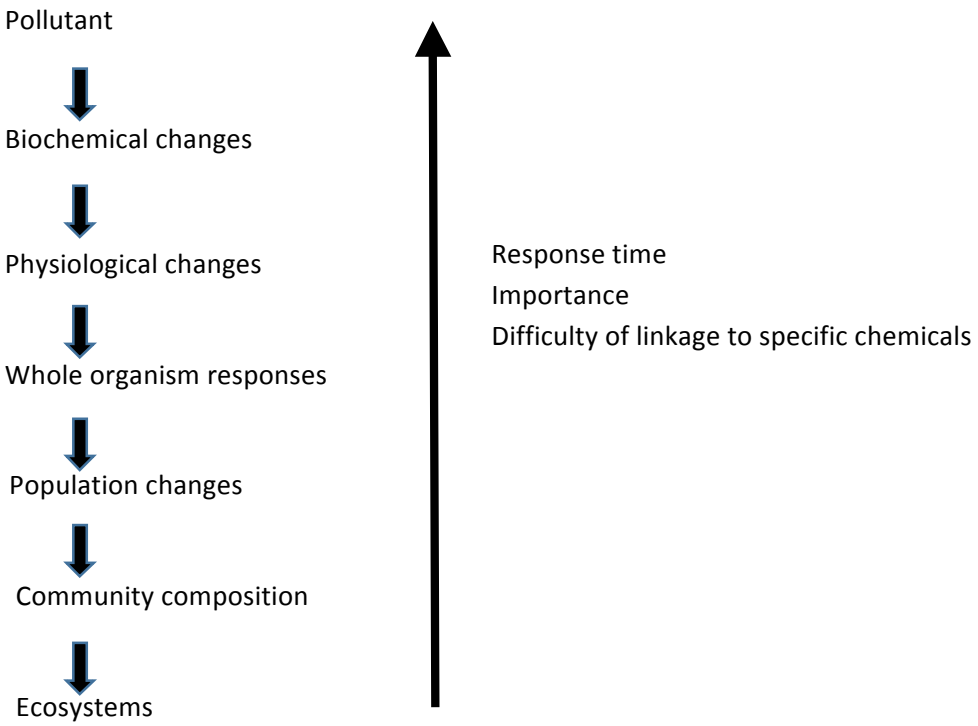

Figure 1: Flow chart for effect of pollutants on ecosystems

1. Biomarkers

During the Nato workshop in 1993, the term biomarker was defined as a biological response that can be related to an exposure to, or toxic effect of, an environmental chemical or chemicals. In the same time, Depledge (1994) defined the term biomarker as a biochemical, cellular, physiological or behavioural variation that can be measured in tissue or body fluid samples at the level of the whole organism (either individuals or populations) that provides evidence of exposure (exposure biomarkers) to and/or effects (health biomarkers) of one or more chemical pollutants. Depledge also gives some indications such as:

* an ideal health biomarker is sensitive to chemical stress and is irrefutably linked to the Darwinian fitness of the organism

* Darwinian fitness is the combined relative probability of survival and rate of reproduction of the individual

* an ideal exposure biomarker is both sensitive and specific to exposure by a single chemical or group of chemicals

* The ideal biomarker in ecotoxicology combines the properties of both types

Several definitions have been given to define what a “biomarker” is. Newman and McIntosh (1991) defines the term biomarkers as a cellular, tissue, body fluid, physiological or biochemical change in extant individuals that is used quantitatively during biomonitoring to either imply the presence of significant pollutant or as an early warming system of imminent effect. Walker et al (1996) defined biomarkers as a change in biological response, ranging from molecular through cellular and physiological responses to behavioral changes, which can be related to exposure to or toxic effects of environmental chemicals. The National Academy of Sciences defines a biomarker or biological marker as a xenobiotically induced alteration in cellular or biochemical components or processes, structures, or functions that is measurable in a biological system or sample (ENTOX/TIWET, 1996). Silbergeld et al (1994) defines biological markers as physiological signals that reflect exposure, early cellular response or inherent or acquired susceptibilities, which provide a new strategy for resolving some toxicological problems. *Sensu stricto*, we define a biomarker as a biological response to a chemical or a group of chemical agents (Walker et al., 1996) but not the presence of the agent or its metabolites within the body (internal dose). However, there is no doubt that the measurement of a xenobiotic in a biological system or sample is a bioindicator of exposure, and thus, it could be considered like a biomarker. The biomarkers have been classified into three main categories based on the effect in the organisms (Silins and Högberg, 2011).

Biomarkers of exposure: these biomarkers measure the internal dose of the toxic compound or metabolite (or any product of the interaction between xenobiotic agent and a target molecule) in one, several body parts compartments of an organism or in the whole body. Bernard and Lauwerys (1986) classified the biomarkers of exposure into two main subgroups according to their selectivity test, which is based on the direct measurement of the unchanged chemicals or their metabolites in biological media. The non-selective tests are used as non-specific indicators of exposure to a group of chemicals. One example is the concentration of 1-hydroxypyrene in the urine of organisms exposed to polycyclic aromatic hydrocarbons (PAHs). PAH are a group of highly carcinogenic elements where the Benzo(a)pyrene is the most studied as a biomarker of pollution, however it is not the only one, has even been reported that other elements are much more carcinogenic, reason by why

many studies preferred report the excretion levels of 1-hydroxypyrene as a biomarker of PAH exposure (Silins and Högberg, 2011).

Biomarkers of susceptibility: they are indicators of a particular sensitivity of individuals to the effect of a xenobiotic or to the effects of a group of such compounds. They correspond to indicator of inherited or acquired trait from an organism in response to exposure substance. Genetic markers are included in this group such as the polymorphisms of specific genes associated with the metabolism of toxic material in the body (Norppa, 2004), alteration in chromosomal structure.

Biomarkers of effect: these biomarkers assess the biochemical, physiological or behavioral disturbance produced in an organism exposed to any toxic substance that can be associated with disease or death. Some examples are the biomarkers of cytotoxicity (Lieggi et al., 2010), levels of necrotic cells (Ward et al., 2008) or unbalance antibody levels (Whitehead and Crawford, 2006) well described in human diseases. The ideal biomarkers of effect should be early detected and be able to show adverse effects before they become irreversible. They represent the most studied biomarkers in the literature and they include different classes of parameters of blood composition, alterations of specific enzyme activities, DNA-adducts appearance, mRNA expression and protein concentrations variations. One of the most emblematic example is the formation of DNA adduct as a consequence of exposure to xenobiotics.

2. Ecotoxicology in aquatic ecosystems

The number of ecotoxicological studies conducted in aquatic ecosystems showed a important growth during the last twenty years principally due to the increasing pollution as a result of an indiscriminate dumping of industrial, urban and agricultural sources into natural aquatic ecosystems. The main contaminants that can be detected in aquatic ecosystems include poly aromatic hydrocarbons (HAPs), pharmaceutical substances, radionuclides and toxic metals. Once contaminants are incorporated into the ecosystem, they can be stored in the sediment, increasing their persistence, be directly incorporated into the organisms and immediately exert their toxic effect like disease, suppression of immune systems, oxidative stress, mutation on DNA or can be bio-accumulated in different tissues and consequently in the food chain.

2.1 Microorganisms

The ability of microorganisms to adapt quickly to environmental changes is one of the main characteristics which has allowed them to colonize different types of ecosystems around the world. Bacteria especially have been associated with a wide variety of functions like antibiotic production, anti-biofilm activity, biodegradation of organic matter and are an important component in the biogeochemical cycle of different elements like carbon and nitrogen. Another characteristic is that they developed a metabolic potential to transform or mineralize organic contaminant into less harmful substance cleaning the contaminated environment, which has allowed the development of bioremediation technology (Dash and Mangwani, 2013).

Petroleum and plastic are one of the major pollutants of anthropogenic origin which are found in large amounts in aquatic ecosystems and some bacteria are able to degrade these pollutants. They can be degraded by oil-eating microbes from different genera that use petroleum as a carbon and energy source (Sakalle and Rajkumar, 2010). Polyethylene, polystyrene and polyvinyl chloride are the main plastics reported in aquatic environments in the last decades and some studies of exposure to plastic in laboratory condition demonstrated the ability of specific bacteria isolated in deep sea ecosystems to degrade those plastics in non toxic forms (Sekiguchi et al, 2010). The biodegradation of Polyaromatic hydrocarbons (PAHs) is also carry out by aerobic and anaerobic bacteria in which the metabolic intermediates in respiratory process like $NADPH^{+}$ or oxidoreductases enzyme activity play a important role (Cerniglia, 1992). High levels of metal bioaccumulation in bacteria has also been reported, like in the bioluminescent bacteria *Vibrio harveyi* that shows a high concentration of cadmium with measured values near to 23.3 mg/g dry cell (Abd-elnaby et al., 2011). A similar tolerance has been reported in other bacteria (*Enterobacter cloaceae*) associated with chelation capacity in different metal as cadmium and copper, removing them from the contaminated environment by the secretion of exo-polysacacharides (Iyer et al., 2005).

The molecular microbiology and genetics have been developed in the last years, which has led to the development of genetically modified microorganisms for the biodegradation of xenobiotic substances, mainly based on the introduction of vectors, which contain specific gene sequences associated to toxic tolerance that are then transformed in the marine bacteria (Dash and Mangwani, 2013) but its applicability has not been tested in field studies.

2.2 Fishes

Fish also have been described as a good model for study in ecotoxicology mainly because they are primary predators in the food chain in aquatic ecosystems and also because many species are commercially exploited for human consumption. Some of the principal commonly species used are zebrafish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*), tilapia (Oreochromis sp.), three-spined stickleback (*Gasterosteus aculeatus*), guppy (*Poecilia reticulata*), killfish (*Fundulus heteroclitus*). The available information on these species concern different aspects of their biology including behavior and evolutionary history, geographic distribution, genomic, transcriptomic and proteomic data, cell lines and mutants and cultivation.

Dispersal is one of its main features of fishes as a response to pollution. However when there are barriers to dispersion, fishes have developed a phenotypic plasticity or evolutionary adaptations to respond to any stressor agent. Numerous studies have been conducted to better understand the molecular mechanisms involved in the response of fishes to different environmental conditions like salinity, hypoxia and temperature. Specific adaptations has been evidenced like the activation of Na^+, K^+ ATPase, shift toward anaerobic metabolism via glycolysis during O2 tension which has is characterized by elevated concentrations of lactate in blood, differential levels of transcription in lactate dehydrogenase (LDH) related to differences in the sequence of promoter region of this gene in populations exposed to different temperature gradient (Cochran and Burnett, 1996; Evans, 2008; Schulte et al., 2000). Fishes can also tolerate high levels of toxic xenobiotic, metals and PAHs with an accumulation principally reported in gill, liver, muscle, kidney, blood. Among the mechanisms of response associated to the exposure of those compounds, case of the activation of the cytochrome P4501A1 (CYP1A1), glutathione S-transferases (GST), superoxide dismutase (SOD), metallothionein (MT), transcription factors (Nrf2) which is involved in the control of a variety of antioxidant defenses, have been reported (Giulio et al., 2015). Pollution in Antarctic area was also examined recently in notothenioids fishes which are well distributed genera in high latitudes and important species in trophic chain. The results obtained suggest different metabolic response between two species (*Notothenia rossii* and *Notothenia coriiceps*) exposed to the same toxic, principally evidenced by variations in glucose levels, differential concentrations of glycogen phosphorylase (GPase) and glucose-6-

phosphatase (G6Pase) enzymes and activities of the enzymatic antioxidant defense (Rodrigues et al., 2015).

2.3 Invertebrates

The invertebrates represent a great majority of the macrospecies living from estuaries to deep sea. In the intertidal environmental, it is possible to find a large diversity of invertebrates from different taxa (crustaceans, mollusks, porifera, annelidas) which are exposed to large changes in environmental parameters that also vary in predictability and intensity, given valuable models system to understand the diversity of adaptive mechanisms developed by these organisms to deal with these fluctuating environments. Bivalves are among the best studied models because they present several characteristic allowing their use in ecotoxicological studies: a diversity of species (mussels, oysters, clams, …), a worldwide distribution, a commercial importance, an easy use in laboratory experiment, and a presence in many contrasted environments.

3. Ecotoxicology and metals in aquatic systems

Ecotoxicology of trace metal in aquatic systems correspond to raise the question « how does toxic and non-toxic metal vary over space and time in and between aquatic habitats and show how they affect living organisms? The term ‘trace metal’ is used to characterize metals that are present at low (trace) concentrations (sometimes defined as 0.01% dry weight) in the environment, in both physical and biotic components. However, some of those metals are detected at high concentration in organisms. Heavy metals term is mainly used for metals that are above a threshold atomic weight, typically incorporating all transition metals of the periodic table. Other chemical characteristics are also considered such as the similar chemical characteristics that make them biologically relevant. These metals become toxic to biota when present in high bioavailability but many are essential to the metabolism of life, consistently across the eukaryotes with sometimes an excellent but not perfect agreement between eukaryotes and prokaryotes. Numerous biochemical pathways underlying life processes are conserved in all organisms and require the same elements to function. Based on another criteria, the term ‘trace metal’ is restricted to essential metals with essential metabolic function then excluding the non-essential metals that have no metabolic function.

For the chemists, a strict definition has been proposed to characterize the term “trace metal”. Nieboer and Richardson (1980) proposed a chemical classification system based on the Lewis acid properties of metal ions. Metals are separated into Class A, Class B or Borderline according to their degree of ‘hardness’ or ‘softness’ as acids and bases. Class A metal ions are Lewis hard acids, readily form cations, and have a ligand affinity order O > N > S. Class B metal ions are Lewis soft acids, ‘more covalent’ and have an affinity order S > N > O. Borderline metal ions present intermediate properties. Metals with Class B or Borderline ions also fit into the category of trace metals. For the non-chemists, the affinity of trace metals for sulphur and nitrogen promotes their binding to molecules in cells especially proteins, and makes some of them essential and all of them toxic link to their ability to bind in the wrong place at the wrong time when available in excess.

There are three major categories of trace metal concentration data that are currently measured in order to compare differences in trace metal pollution in aquatic habitats over space and time: 1) trace metal concentrations in water, 2) trace metal concentrations in sediment and 3) trace metal concentrations in resident biota. Evaluation of dissolved trace metal concentration in water is very important to determine if these concentrations are close from those shown to present a toxic effect on organisms using toxicity tests. Dissolved concentrations usually vary over time, particularly for example in estuaries with differential inputs of river and sea water at different states of the tide, and differential river flow according to recent rainfall in the catchment often varying seasonally. Each measurement represents a single time point that may be very different from the dissolved concentration present at that exact location the day before or the day after

3.1 Biological significance of metals

Living organisms store and transport various transition metals to provide appropriate concentrations for basic metabolism (mainly enzyme cofactors). The normal concentration range varies according to metals and is difficult to determine in biological systems. More, metal deficiencies and excesses both contribute to generate pathological changes. The complexity of cell types in multicellular organisms also participates to the dynamics of metals distribution; the storage and the transport of transition metals being not carried by all cells but specialized ones. The form of the metals remains always ionic but the oxidation state may vary based on cell biological needs. The main heavy metals in environmental monitoring studies are mercury, cadmium, lead and copper.

Mercury (Hg) is a heavy metal present under three chemical forms: elemental (Hg without any additional atoms attached to it), organic, and inorganic that are interconvertible, and can all produce systemic toxicity (Graeme and Pollack, 1998). Origin of Hg in the environment are both natural and from anthropogenic sources like mining, fossil fuels combustion, incineration, emission from smelters, fungicides and catalyst activities. Although mainly present in the atmosphere, a large part of Hg returns into the coastal sea as precipitates. Hg is also present at high concentrations in sediments of aquatic environment since both inorganic and organic Hg are linked to particles, colloids and high molecular weight organic matter (Schiff, 2000). Inorganic Hg can be converted by specific bacteria into methylmercury which represent the most toxic chemical species able to provoke deleterious effects to the central nervous system, deficiencies in the immune system and development [Harada et al., 1998). Dissolved methylmercury is easily bioavailable and can bioaccumulated and biomagnified into the marine food chains to reach very high concentrations in upper levels of the chain. Toxicity of Hg has been studied in several marine invertebrates species such as the clam *Ruditapes philippinarum*, (Liu et al., 2011), the crustacean *Ligia italic*, in which ultrastructural alterations in the hepatopancreas epithelium have been observed (Longo et al., 2013) or *Scylla serrata* in which modifications of several immune related parameters (total haemocyte count, lysosomal membrane stability, phenoloxidase, superoxide generation and phagocytosis) have been reported (Singaran et al., 2013). In embryos of the sea urchin *Strongylocentrotus purpuratus* exposed to mercury, the inhibition of specific molecular transporters increases intracellular accumulation of inorganic Hg but had no effect on accumulation of organic Hg. These results illustrated the existence of a specific elimination of inorganic Hg and a differential accumulation and potency of the two major forms of Hg found in marine environments (Bosnjak et al., 2009). Accumulation of Hg in the soft tissues of the oysters *Saccostrea cucullata* (Shimeshan et al., 2012) and *Crassostrea angulata* illustrated the interest of such organism for monitoring of Hg mercury in the aquatic system. Proteomic analysis conducted on gonads of oysters following food-chain contamination with HgCl evidenced different proteins such as 14 - 3 - 3 protein, GTP binding protein, arginine kinase and 71 kDa heat shock cognate protein) as good candidate biomarkers for environmental Hg contamination (Zhang et al., 2013).

Cadmium (Cd), is a heavy metal released both from natural sources and anthropogenic activities resulting from its large utilization in some industrial and agricultural activities (e.g. pigments, nickel-cadmium batteries, smelting and refining of metals and many

other sources). Cd is a highly toxic environmental pollutant and potent cell poison which induce different types of damage including cell death. Cd toxicity is amplified in organisms as a consequence of the metal's long biological half-life which range from 15 to 30 years according to species and tissues. Cd easily penetrates the cells, via transport mechanisms normally used for other purposes, but is eliminated very slowly (Jarup et al., 1998). Since Cd is a non-essential metal which present no physiological function that is irreversibly accumulated into cells and strongly interact with various cellular components and molecular targets. Cd may enter cells via divalent ion transporters, such as zinc transporters (Kingsley and Frazier, 1979), can cross the plasma membrane as divalent ions, exerting an agonistic role against calcium ionic channels (Foulkes, 2000). Toxicity of Cd is also modulated by various abiotic factors (De Lisle and Roberts, 1988). Among Cd effects, cases of teratogenesis and carcinogenesis, due to cytotoxic concentrations of the ion, have been reported both in invertebrates and in higher organisms. Accumulated evidence has also shown that Cd increased not only cellular ROS levels, but also lipid peroxidation and alteration in glutathione (GSH) levels in various cell types, suggesting that Cd-induced apoptosis may be connected with oxidative stress (Rana, 2008). In invertebrates it up-regulates the expression of antioxidant enzymes, metallothioneins and heat shock proteins (HSPs) and down-regulates the expression of digestive enzymes, esterases and phospholipase A2. Cd also interferes with tissue organization, immune responses and cell cycles by inducing apoptosis (Sokolova et al., 2004). Due to its high level of resilience, Cd is a contaminant which accumulate in the food-chain involving that for many aquatic predators, Cd comes largely from food and the ease with which Cd penetrate tissues mainly depends on the form in which this metal is bound in prey cells (Cd present in the cytosol being more available than Cd associated with insoluble prey components (Dubois and Hare, 2009). High concentrations of Cd has been reported in aqueous organisms including invertebrates, such as sponges, mollusks, crustaceans, echinoderms with very often the existence of significant differences in Cd concentration in tissues in differently contaminated sites. Various effects of Cd have been reported in different species. For example, modifications in cell morphology and cell aggregation in *Scopalina lophyropoda*, by enhancing pseudopodia/filopodia formation which promotes cell movement have been described (Cebrian and Uriz, 2007). High concentrations of Cd are detected in the gills and digestive gland of the mussel *Mytilus galloprovincialis* associated with alteration in the physiology of respiration and feeding processes (Viarengo et al., 1994), in the digestive gland and kidney of mussel *Crenomytilus grayanus* (Podgurskaya and Kavun, 2006), in the renal tissue of Antarctic bivalve *Laternula elliptica* (Rodrigues et al., 2009) or in the

hepatopancreas of *Mytilus edulis* and body wall of echinoderms such as *Asterias rubens.* . Similar observations have been made in few species of Antarctic molluscs highlighting the importance of food in the primary pathway for Cd bio-accumulation (Nigro et al., 1997). Data on the effect of Cd on cellular and molecular defense strategies such as apoptosis, autophagy, metal detoxication and stress proteins have been obtained in *Paracentrotus lividus* embryos (Rochherri and Matranga, 2010).

Lead (Pb) is a heavy metal naturally present in the environment that becomes highly toxic when ingested and cause severe damages to the nervous system and causing different disorders. Pb compounds exist in two main oxidation states, +2 and +4 (Nava-Ruiz et al., 2012).

Pb is a bio-persistent pollutant mainly originating from human activities that accumulates at the top of the food chain. In marine invertebrates, the Pb toxicity varies according to species but also to their life stage in a dose and time dependent manner. A characterization of the cytosolic distribution of Pb was carried out in the digestive gland of *Mytilus galloprovincialis*, Pb is present in molecule with high molecular weight but when Pb concentrations are elevated, Pb is also present in low molecular weight biomolecules, illustrating suitability of the distribution of selected metals among different cytosolic ligands as potential indicator for metal exposure (Strizak et al., 2014). Sea urchin embryos of the sea urchin *Paracentrotus lividus* exposed to Pb exhibit alterations of morphology at gastrula and pluteus stages (Geraci et al., 2004) and a reduction of calcium accumulation is observed in embryos of *Strongylocentrotus purpuratus* (Tellis et al., *2014*). In the mussel *Perna viridis* exposed to environmental Pb concentrations, several enzymes classically used in toxicology (catalase, reduced glutathione, glutathione S-transferase, and lipid peroxides) showed differences in activities (Hariharan et al., 2014).

Copper (Cu) is a metal present in all environments and is present in aqueous solution as copper(II) under the form $[Cu(H_2O)_6]^{2+}$. Copper occurs naturally at low levels in air, soil and water but activities associated to mining and smelting of copper, industrial emissions, municipal wastes, fertilizers, and pesticides have increased copper levels in our biosphere (Eisler 2000). Atmospheric Cu originates primarily from human activities (73%) Precipitation of atmospheric copper is a significant source of Cu to the aquatic environment in mining and industrial areas and deposition patterns vary relative to prevailing winds and intensity of

industrial activity (Nriagu, 1979). Due to its biostatic properties, copper has been used as antifouling to protect against barnacles and mussels in marine paints but also in the aquaculture industry because of its antimicrobial properties. Many proteins need copper as a cofactor. Copper proteins play various roles in biological electron transport and oxygen transportation (that use the interconversion of Cu(I) and Cu(II) (Vest et al., 2013). Copper is also essential in the aerobic respiration of all eukaryotes and is present in mitochondria where the cytochrome c oxidase, (the last protein in oxidative phosphorylation) binds the O_2 between a copper and an iron. Copper is also found in many superoxide dismutases, proteins that catalyze the decomposition of superoxides by converting it to oxygen and hydrogen peroxide. Copper is also present in the major protein involved in the oxygen carrier, hemocyanins, in most mollusks and some arthropods such as the horseshoe crab (*Limulus polyphemus*). Copper enzymes are involved in vital processes such as the control of cellular energetics for synthetic activities, for muscular activity and heat production, for the structural organisation of basement membranes and connective tissues essential for the integrity of both skeletal and soft tissues.

3.2 Metal uptake

The gills of bivalves constitute a key interface for the uptake of dissolved metal ions from water and in a less extent the mantel. To enter the cell metals first must cross the cellular membrane, this process is given by three ways: transport membrane protein of relative wide specificity, transport by intrinsic proteins which metal ions pass selectively or for endocytosis, figure 2 (Khan et al., 2015; Marigómez et al., 2002; Wang and Fisher, 1999).

Transmembrane pumps then play an pivotal role, because they regulate the ionic homeostasis in the cell. Ca^{2+} pump and Na^{+}/H^{+} antiporter exchange are the most studied mechanisms of ion homeostasis, the first regulating the calcium levels which is important in shell formation and is taken actively from the environment principally for transmembrane Ca^{2+} pump, and the second is responsible of intracellular pH and cellular volume. However the presence of toxic metals can disturb the uptake by direct competition with this metal, as the reported for zinc and cadmium that have one of the higher level of assimilation between different invertebrates species and a very slowly removal rate (Ahearn et al., 2001; Machado and Lopes-Lima, 2011; Wang and Fisher, 1999). For example, cadmium may enter the gills

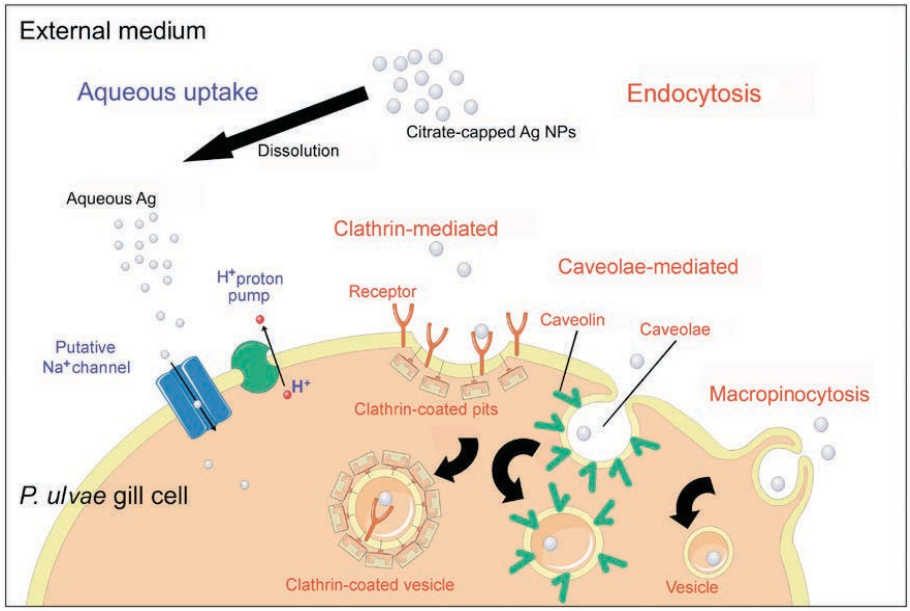

Figure 2. Schematic diagram describing potential uptake pathways during waterborne exposure of Peringia ulvae to Ag NPs. Ag NP dilution in the external media (17 salinity estuarine water) would lead to the presence of aqueous Ag which may enter the cell via ion transport channels, such as the proton-coupled Na+ channels. Intact Ag NPs could be potentially internalised via endocytotic pathways; clathrin-mediated endocytosis, caveolae mediated endocytosis or macropinocytosis. Adapted from Khan et al 2014.

by passive diffusion or across Ca2+-channels probably through a rapid binding to intracellular ligands that ensures removal of cytosolic Cd2+, this uptake process depending on the availability of ATP (Figure 3). Other metal such as iron, when present as insoluble particulate compounds, may be absorbed by pynocytosis. Case of endocytic vesicles in epithelial cells containing lead has been reported in the gills. Similarly, in several freshwater mussels Cd is localized in endocytic vesicles of gill epithelial cells. However metals enter the cytosol either across membrane channels or by endocytosis and can be incorporated into lysosomes or transported in vesicles across the epithelial cells to be later exocytosed basally into the blood and incorporated into circulating hemolymph/blood cells

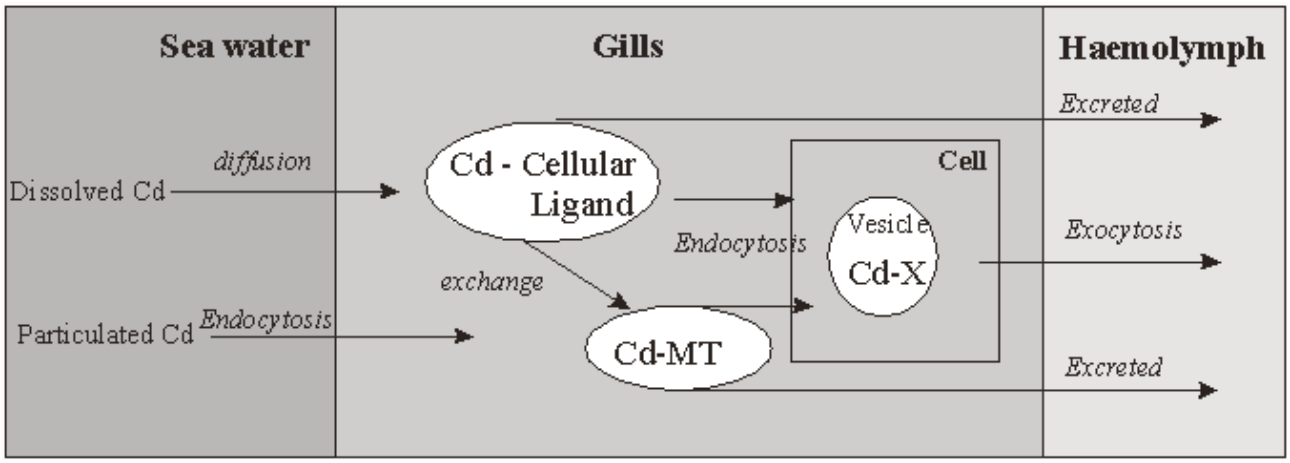

Figure 3. Representation of the ways used by Cd ions to cross the gill cells in bivalves (modified from Carpené and George, 1981).

Some differences exist between filter and grazer species. For example, in grazers, iron is not be endocytosed by gill epithelial cells and the uptake of Zn appears to be directly from water without the use of ion pumps and could be transported across the gill epithelia into the blood. More, mucus layers adhering to the gill may promote epithelial uptake by trapping and concentrating metals and may, thereby, establish a diffusion gradient to favor uptake of the metal. Mucous may also play a role in the fixation of metals ions participating to the decrease of their bio-disponibility. The mantle of molluscs is involved in the metabolism of divalent cations mainly by the active incorporation of Ca2+ and some other elements from the extracellular pallial fluid into the shell. The mantle epithelial cells of bivalves can accumulate Cd, Cu, Fe, Hg and Zn in lysosomes. The shell also serves as a storage matrix for toxic metals even concentrations remain much lower than in soft tissues. Metal ions are replacing calcium in the carbonate complex or are bind by adsorption to the organic component of the shell. Byssus also participate to metal fixation mainly iron since 30% of the Fe from ferric hydroxide can be removed by deposition in the byssal threads of mussels through the fixation of metal on diphenols or semiquinones that are able to form stable ligands with various metals.

3.3 Transport of metals in blood plasma

In molluscs, haemocytes (blood cells) participate to the metals transportation either associated with cytosolic proteins or within lysosomes and blood plasma proteins may bind metals in a non-specific way. In marine mussels and clams, Zn and Cd are transported primarily in blood plasma, associated with high molecular weight proteins (HMWP) and in

the flat oyster *Ostrea edulis*, Cu and Zn are mainly transported within haemocytes. Metals are accumulated in haemocyte lysosomes as non-digested products. Under exposure to Cd, Cu or Pb, a migration of haemocytes can be observed towards gills or digestive tract and other detoxification organs suggesting that blood cells may constitute the most relevant system for metal transport between tissues in molluscs. The presence of lysosomal vesicles of brown cells (cell having an oval or round shape, a presence of membrane fenestrations and yellowish-brown cytoplasmic granules) that can accumulate various metals has been reported in mussels, clams and oysters.

3.4 Metal accumulation

The digestive gland of molluscs is the main center for metabolic regulation but is also involved in immune defense and homeostatic regulation of the internal medium (calcium, haemolymphatic pH, cell volume...), as well as in the mechanisms of xenobiotics detoxification and elimination. When bound to particulate material, metals go through the digestive gland. For example, iron enters the digestive cells via microvilli plasma and pynosomes, and then transferred to lysosomes and further to residual bodies. According to metals, different parts of the digestive gland are involved in ion transport. In the freshwater gastropod, *Lymnaea truncatula*, epithelial cells present in the peritentacular area and the alimentary canal participate to the binding of iron, and copper only enters through cells of the peritentacular area. Cd is heterogeneously distributed along the different regions of the digestive tract in freshwater mussels. Accumulation of metals in lysosomes of the digestive cells occurs by two different pathways: with food or from haemocytes by phagocytosis and directly incorporated in lysosomes or dissolved metals may also be incorporated from either the lumen in the bottom of the cell after having previously immobilized by cytosolic metallothioneins. Endocytic metal-protein complexes can also fuse with primary lysosomes to give heterophagolysosomes in which the biological material is hydrolyzed. At that point, ions are partially available for the cell and partially bound to the undigested material that remains in the endo-lysosomal system. Metal ion can then be later eliminated from the cell via exocytosis of residual bodies.

3.5 Excretion of metals

Elimination of metals may occur by different complementary ways according to metals. Cu can be extruded with mucous secretion or released by exocytosis to the external

medium from frontal cells and Zn may be released by secretory cells in the postlateral zone or by basal exocytosis of lysosomes in the abfrontal epithelial cells. Haemocytes also participate to metal elimination, releasing ions by diapedesis across the epithelia of the mantle and digestive tract. In the freshwater bivalves, the excretory system include the pericardial gland that is involved in ultrafiltration, and under Cd exposure, Cd can be detected in the pericardial gland, bound to small plasma protein-complexes that pass throughout the ultrafiltration barrier to the primary urine. Metal detoxification from digestive gland cells may occur via feces or via basal lamina release towards haemocytes dispersed by the connective tissue of the visceral mass.

4. Response to heavy metals in marine invertebrates.

Exposure to different heavy metals and bioaccumulation in natural population has been reported in many bivalves (Marigómez et al., 2002), showing the high tolerance of these species and given great potential for assess the status of chemical pollutants in aquatic ecosystems (biomarkers of toxicity). Heavy metals have been described as one of the main selective pressure acting in marine invertebrates, affecting embryonic development, stress protein induction, immune system, DNA, RNA and lipid damages with subsequent cellular apoptosis (Chiarelli and Roccheri, 2014). The degree of sensitivity of species depends on the metal type and its concentration exposure, which strongly vary between species. For example, exposure to copper in limpet (*Patella vulgata*), crabs (*Carcinus maenas*) and mussels (*Mytilus edulis*) reveals that at high concentration, metals interfere with different physiological parameters such as survival ability, heart rate, proteins levels in hemolymph, lysosomal stability, neurotoxic effect (acetylcholinesterase activity) and specific biomarkers of metal exposure (metallothionein) and a gradient in sensitivity is observed where limpet is more sensitive than crab and mussel (Brown et al., 2004).

Bivalves species have been chosen as model organisms especially in marine environments to understand the mechanisms involved in the response of metal exposure, because they can accumulate different types of essential and non essential metals in high levels and the major models used are oysters, scallops, clams and mussels (Chandurvelan et al., 2015; Lavradas et al., 2016; Paul-Pont et al., 2012; Sakellari et al., 2013; Varotto et al., 2013; Wang et al., 2009). In bivalves used as metal bioindicators of aquatic contamination different methodologies have been developed to localize and quantify metal concentrations, from histochemistry, spectrophotometric techniques and electronic microscopy, allowing

understanding the uptake pathways from the ambient water (Sakellari et al 2013; Chandurvelan et al, 2015).

Once the metal is acumulated in the organism have the potential to be incorporated in different physiological pathways (normally for the essencial metals, Cu, Zn) but when his concentration exeed the levels of tolerance or are toxic (non essencial metals, Cd, Pb, As, Hg) metals can be stored in compartimentalizated vacuoles or can be begin detoxification mechanism of manner temporaly or permanently, however both process require specific metal binding enzymes or proteins that permit the transport internally to a particular organ for be excreted from the organism (Rainbow, 1997).

At molecular level different specific and non specific biomarkers of metal pollution has benn described, showing a conserved mechanis across different taxas but that vary in the level of efficiency and network complexity.

4.1 Reactive oxygen species (ROS)

Metals increases the production of reactive oxygen species (ROS) including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical ($^{\cdot}OH$), which may interfere in many metal-related processes and generate modification of lipids, proteins and nucleic acids (Lushchak, 2011). The ROS formation occurs through the following reactions:

$$Metal^{ox} + O_2^- \rightarrow Metal^{red} + O_2$$

and

$$Metal^{red} + H_2O_2 \rightarrow OH + OH^- + Metal^{ox}$$

with the net result

$$O_2^- + H_2O_2 \rightarrow OH + OH^- + O_2$$

In response to ROS, an antioxidant machinery as evolved in many species from bacteria to humans, including activation of different low molecular weight scavengers like superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH), ascorbic acid (vitamin C) that interact in a complex network to participate for example to the reduction of the membrane lipid peroxidation (Regoli and Giuliani, 2014; Canesi 2015). ROS are also naturally produced by different cellular compartments including the mitochondria (Figure 4)

as a product of the oxygen metabolism, the endoplasmic reticulum, peroxisomes and phagocytic vesicles that generate oxidative stress as the result of protein folding which requires oxygen as the final oxidant, H_2O_2 production and degradation, transferring electrons across biological membranes through NADPH-oxidases activity (Donaghy et al, 2015).

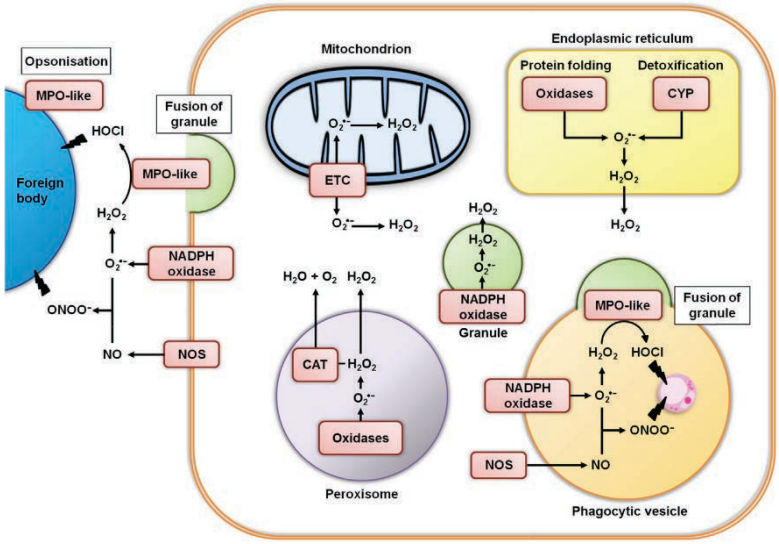

Figure 4. Major sites of reactive oxygen and nitrogen species generation in generalized haemocytes in marine bivalves. Abbreviations: CAT, catalase; CYP, cytochrome P-450; ETC, electron transfer chain; MPO, myeloperoxidase; NOS, nitric oxide synthase. Adapted form Donaghy et al 2015.

4.2 Superoxide dismutase (SOD)

The superoxide dismutase is a dimeric enzyme present in almost all the aerobic organism which principal function is the catalytic conversion of the O_2^- to oxygen (O_2) and hydrogen peroxide (H_2O_2) in presence of H^+ (Fridovich, 1995). SODs are classified in two categories according to their cellular localization, cytosolic and mitochondrial but both types require the presence of specific metals as cofactor for his activation, the specificity been indicated on the active site (figure 5). Manganese SOD (MnSOD) are found in the mitochondria, copper/zinc SODs (Cu/Zn SOD) which is the most abundant form can be found in the cytoplasm such as the iron SOD (FeSOD), usually found in prokaryotes, and the recently described nickel SOD (NiSOD) (Abreu and Cabelli, 2010). The mechanism by SOD activity involves the sequential reduction and oxidation of the metal center, with the

subsequent oxidation and reduction of superoxide radicals. Ecotoxicology studies in marine species such as *Crassostrea gigas, Mytilus edulis, Mytilus galloprovincialis* highlight the activation of SOD as a biomarker of oxidative stress in experimental exposure to xenobiotic agent and both activity (determined at protein level and activity) and gene expression level have been explored (Kim et al., 2015; Boutet et al., 2004; Lewis et al., 2016; Lushchak, 2011). The role of SOD in natural populations has been also studied in a context of naturally anthropogenic contaminated areas to validate the use of this specific biomarker in field context (Orbea et al., 2002; Rodriguez-Ariza et al., 1993).

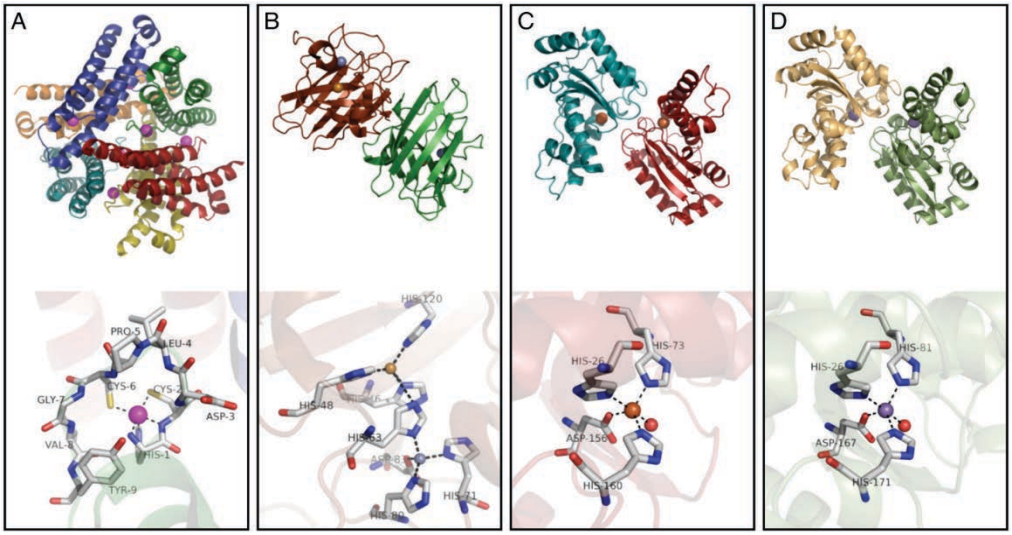

Figure 5. A comparison of the enzyme structures and active sites for the four SODs, (A) *Streptomyces coelicolor* NiSOD, (B) human CuZnSOD, (C) *E. coli* FeSOD and (D) MnSOD Adopted from Abreu and Cabelli, 2010.

4.3 Metallothioneins (MTs)

The metallothioneins (MTs) are one of the best studied stress response associated to metal exposure in invertebrates. Metallothioneins (MTs) are low molecular weight (6 to 7 kDa) cytoplasmic metal-binding proteins with high cysteine content (close to 30% of their amino acid content) that give the ability of the thiol group to capture free metal ions reducing cellular damage) are that bind essential (copper, zinc, iron, manganese) and non essential (cadmium, mercury, lead, silver) metal ions that are sequestered into a metal/sulphur cluster (Amiard et al., 2006; Isani and Carpenè, 2014; Cherian and Goyer, 1978, Kojima and Kägi 1978). Although amino acid composition is variable, the cysteine content is more conserved with characteristic motif like Cys-Cys, Cys-X-Cys and Cys-X-Y-Cys, where X and Y are

amino acids other than cysteine (Paul-Pont et al., 2012). , however not all the function of the Mts as been describes, also free radical scavenger activity, Normally the cysteines are arranged in two metal thiolate cluster (figure 6) which are able to specifically bind metals that later can be exported out of the cellule. Important biological functions of MTs are 1) trace metal homeostasis, 2) protective role against excess reactive heavy metal ions, 3) free radical scavengers, 4) reservoir of essential metals that can be donated to other metalloproteins and 5) cell protection against intracellular oxidative damage (Karin, 1985; Hamer, 1986) and they have been linked to modulations of immune response, genotoxicity and carcinogenicity (Asselman et al., 2012; Nordberg et al., 2009).

Several studies have shown that MTs display a specific tissue expression pattern that is correlated with development and differentiation (Banerjee et al., 1982; Chatterjee and Maiti, 1990; Nemer et al., 1991). A large number of MT isoforms have been isolated from vertebrates and invertebrates, apparently originating from events of gene duplication that may have occurred many times independently (Griffith et al. 1983, Hunziker et al. 1995). Nine MT isoforms have been characterized in the blue mussel *Mytilus edulis* (Barsyte et al. 1999), seven in the sea urchin *Strongylocentrotus purpuratus* (Nemer et al. 1984, Wilkinson & Nemer, 1987), one each in the tropical green mussel *Perna viridis* (Khoo & Patel 1999) and the Eastern oyster *Crassostrea virginica* (Unger et al. 1991), and four in the Pacific oyster *Crassostrea gigas* (Tanguy et al. 2001, Tanguy and Moraga 2001, David et al., 2012) and two in the flat oyster (Boutet et al., 2002).

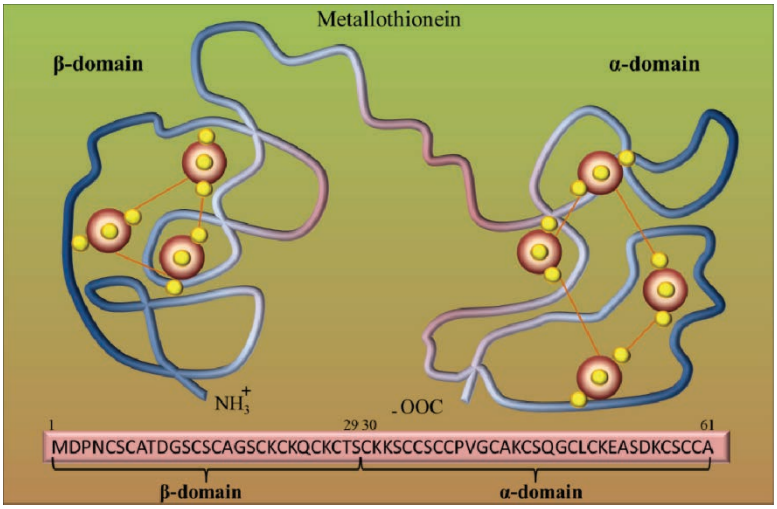

Figure 6. Metallothionein (MT) structure. Model of two binding sites of metallothionein. Red big beads are metal atoms (e.g., Zn), and small yellow beads are sulfur

atoms. Adopted from Ruttkay-Nedecky et al., 2013.

MT transcription is also regulated by a set of binding sites for transcription factors localized in the promoter region as metal responsive elements (MRE), metal transcription factor (MTF) and zinc finger protein (Janssens et al., 2009).

4.4 Ferritin (Fer)

Ferritin which is the principal protein involved in iron storage, is a structurally complex protein formed by 24 subunits of two types of polypeptide chains giving a globular shape that can accommodate large quantity of iron in his interior (Figure 7), and is usually present in the cytosol (Lobreaux et al., 1992). The process by which iron is stored begin in the H (heavy) chain which oxidizes Fe^{2+} in a specific ferroxidase site, and the L (Light) chain that performs the entry of the metal into the protein, while more metals enter the more Fe^{3+} cluster are formed in the cavity of the protein (Strange et al., 1993).

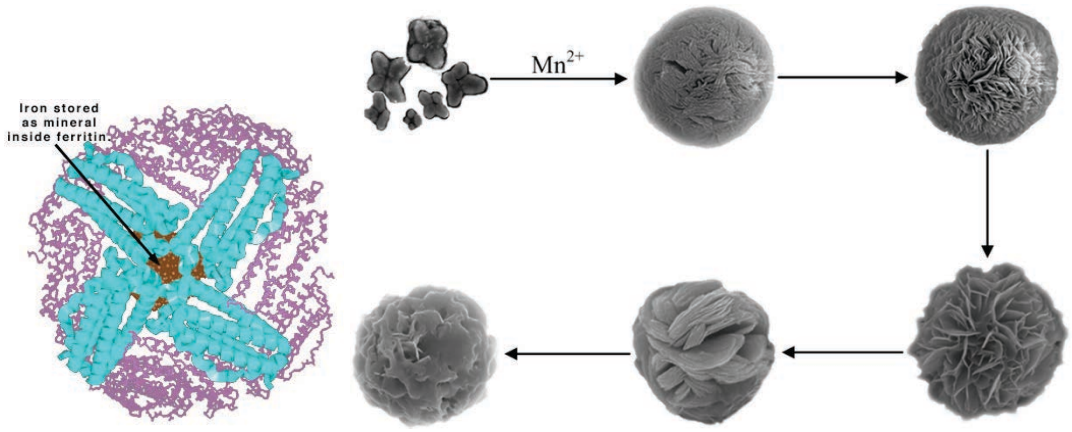

Figure 7. Tridimentional structure of ferritin (left) and manganese ferritin aggregate formation after exposure to manganese (right). Adopded from Chet et al 2015

The principal function of ferritin consists in the storage of free iron when his concentration exceeds the normal levels and its release when needed. In marine invertebrates the mechanism of activation of these proteins is less documented compared to other detoxification proteins but recent works showed that the transcriptionally and translational expression of this protein is positively correlated with the exposure of iron in several bivalves (Zhang et al., 2013; Zhou et al., 2014), but it has also been reported that this protein could store other types of heavy metals (Chen et al., 2015; Zapata et al., 2009).

4.5 Phytochelatin (PCs)

The phytochelatin (PC) is another type of metal binding peptides which is produced from glutathione by the enzyme phytochelatin synthase (PCS) (Figure 8). It has been described primarily in plants and terrestrial invertebrates to present an equivalent function of the MTs in metal detoxification because they also are characterized by the presence of cysteine motif (Cobbett, 2000; Inouhe, 2005; Vatamaniuk et al., 2005). The general structure of PCs consists only in three amino acids Glutamic acid (Glu), Cysteine (Cys) and Glycine (Gly) where the first two are linked through a γ-carboxylamide bond, forming the structure $(\gamma\text{-Glu-Cys})_n$-Gly where n can vary from 2 to 5 repetitions. They are normally found in the cytosol but when they have interacted with a toxic metal, the complex PC-metal are sequestered to the vacuole (Cobbett, 2000; Rodrigo et al., 2013).

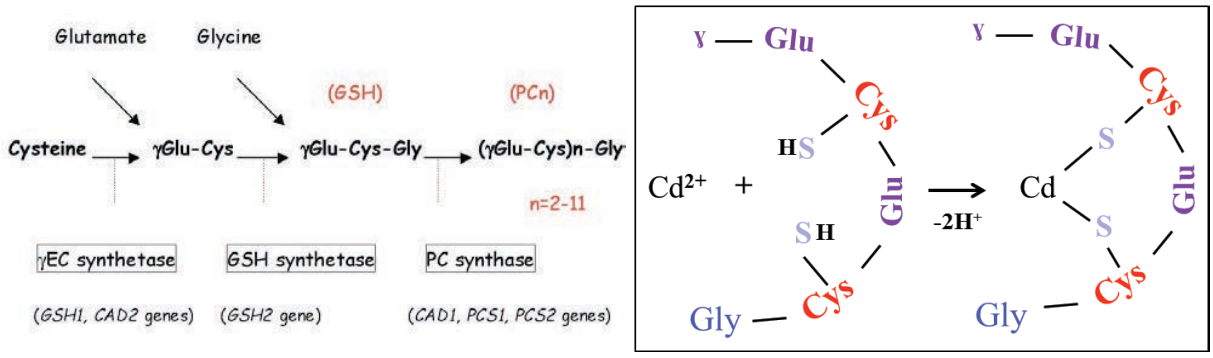

Figure 8. Phytochelatin syntthesis (left) and structural conformation when interact with metals (right). Adopded from Inohue, 2005 and Merlos et al 2013.

In marine invertebrates only a few sequences are reported for the phytochelatin synthase (PCS), particularly in the genome of the pacific oyster *Crassostrea gigas* and recently in the transcriptome of the hydrothermal mussel *Bathymodiolus platifrons* (Wong et al., 2015). The role of PCs could be a potential efficient mechanism in heavy metal sequestration in marine invertebrates which it has so far been less explored.

5. Omics era in pollution response

The Omics term refers to the field of biology that includes genomic, transcriptomic and metabolomics which has considerably increased in the last years due the development of high throughput sequencing techniques as 454, Illumina, Solid and Heliscope which strongly increased the amount of DNA and RNA information, with a low error rate and cost. This opportunity to get a large amount of genomic data represented one of the most important

evolution for the study of non-model species (Pavlopoulos et al., 2013). This amount of genetic information has revolutionized areas of ecology and evolution, greatly expanded the scientific possibilities to assess the mechanisms that govern ecological interactions (Straalen and Feder, 2012). The field of genomics (the study of how an individual's entire genetic make-up, the genome, translates into biological functions) should strongly contribute to provide tools that may assist our understanding of how chemicals can impact on organisms and ecosystem health. Genomic tools can facilitate ecology and evolutionary biology studies, providing useful data to identify ecological performance-regulated gene loci; to perform functional analysis of ecological performance-related traits; evaluate individual, population, community, and ecosystem responses to the environment and examine the degree and significance of genetic variation among ecological performance-related traits (Snape et al., 2004).

The term '*ecotoxicogenomics*' has been proposed to describe the integration of genomics (transcriptomics, proteomics and metabolomics) into ecotoxicology and has been defined as the study of gene and protein expression in non-target organisms that is important in responses to environmental toxicant exposures (Figure 9).

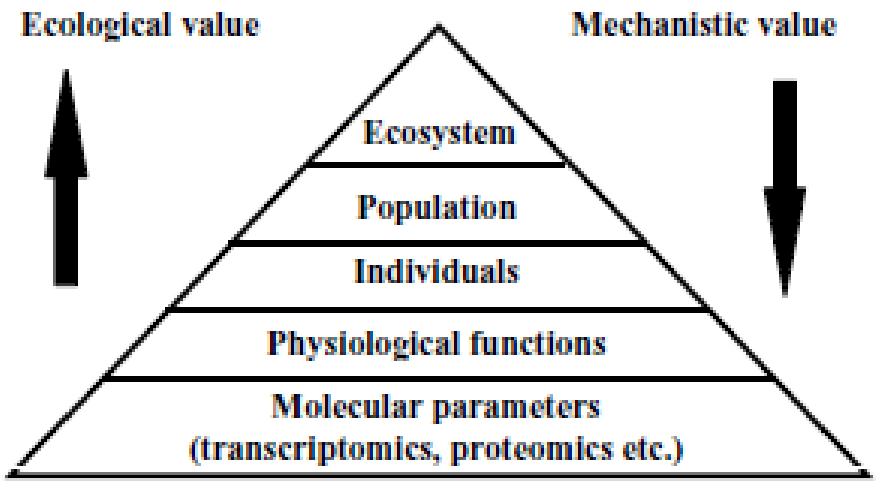

Figure 9: Conceptual framework for ecotoxicogenomics

The way populations can adapt to environmental toxicants also has been explored using omics techniques, where traditionally the response to any toxic agent was studied by a limited number of genes but at present, many genes can be analyzed simultaneously in term of transcript regulation and interaction, protein concentration and activities and how specific mutation can change the protein function or activity. One application of transcriptomics and

proteomics in ecotoxicology that show an increasing interest is the characterization of the mode of action (MOA) profiling of environmental toxicants, in order to identify particularly sensitive subsets of ecological populations, and discover new molecular biomarkers of interest. The use of gene (or protein) expression profiling-based MOA will allow the identification of new toxic substances based on their expression profiling comparison to already known specific toxicants MOAs. The development of this approach is one of the priority of the European program REACH (European Union regulation on Registration Evaluation and Authorization of Chemicals,) that has been established to both optimize resources and to limit the use of animals for testing purposes. The new molecular biomarkers (single gene or complex gene expression pattern) should contribute to provide an early detection of environmental stress, to infer mechanism of action and allow a more relatively efficient monitoring of the environment. But, all this biomarkers have be integrated with phenotypic end points (activities, metabolites concentration, chemical profiles) to establish robust correlations between molecular biomarkers and phenotypic end points which is one of the major criticisms on the application of transcriptomics and proteomics to ecotoxicology.

Three main methods dominate transcriptomic analyses: the single gene expression study using quantitative PCR, the microarray technique that has existed since the mid-1990s and that allow the analysis of the expression of hundreds genes (Schena et al., 1995) and the RNA-seq that utilizes the power of high-throughput sequencing (HTS) for transcriptomics (Wang et al., 2009; Schirmer et al., 2010). Both methods initially use RNA and both result in a data matrix indicating relative expression levels of each transcript in each individual analyzed but both techniques require biological replications to allow robust statistical analysis (Lee et al., 2005). The microarrays use short gene sequences representing part of the DNA that are fixed to a surface and the levels of expression of the genes are estimated by hybridization with complementary DNA (cDNA) transcribed from mRNA transcripts previously labelled with fluorescent dye. After the hybridization, the array is scanned with lasers where the level of fluorescence detected is proportional to the number of cDNA transcript that are hybridizing at particular probe. The RNA-seq use high-throughput sequencing technique to generate hundreds of thousands of fragments of a cDNA library, the length, sequencing depth and quality depending on the sequencing platform used. The main differences with microarrays is that RNA-seq can detect all expressed genes (even new genes) when microarray only provide information on the genes present on the array and that RNAseq

requires a bioinformatics language for assemble and align the huge amount of transcripts generated (Alvarez et al., 2015).

Bivalve genomic resources remain not well annotated or well described, with the exception of some species classically used in biomonitoring studies such as Pacific oyster, *C. gigas*, whose genome has been recently published (Zhang et al., 2012) or the pearl oyster, *Pinctada fucata*, in which genome annotation is still at the draft level (Takeshi et al., 2016). Other bivalve transcriptomes are publicly available for *M. galloprovincialis* (Rosani et al., 2011; Gerdol et al., 2014), *Patinopecten yessoensis* (Hou et al., 2011), *Ruditapes philippinarum* (Milan et al., 2011; Moreira et al., 2012) and *Corbicula fluminea* (Chen et al., 2013). Several reviews papers have been published to provide some examples on how -omic tools have been used in the study of immunity and host–pathogen interactions in mollusc bivalves (Cancela et al., 2010; Gestal et al., 2008; Guo et al., 2008; Romero et al., 2012; Wang et al., 2013; Yue, 2014, Gomez-Chiarri et al., 2015). The use of large scale transcriptomics approach (ie microarray or RNAseq) to monitor pollutant effects is growing and has been mainly developed in the clam *R. philippinarum* in a population context (Milan et al., 2016) or in *M. edulis and M. galloprovincialis* in which correlation between metals concentration in tissues and gene expression profile has been reported (Venier et al., 2006; Dondero et al., 2010, 2011; Poynton et al., 2014, Varotto et al., 2013). These studies reported evidence serves to validate the use of this molecular tools in ecotoxicological studies but the major challenge still remains to get a better understand of how this molecular changes caused by toxic exposure in the organism modulate biological changes in the ecosystem (Schirmer et al., 2010).

6. Hydrothermal ecosystems as natural laboratory for heavy metal adaptation

Deep-sea hydrothermal vents ecosystems are distributed among all the oceans, principally along mid-ocean ridges where new oceanic crust is generated. Vent fluid is formed by infiltration of deep-sea water into fractures of the oceanic plate and chemical transformation at high temperature deep in the crust, under the influence of the underlying magmatic chamber. The vent fluid returns to the ocean through diffuse or focused venting, with typically high temperature (up to 400°C depending on sites), loaded with heavy metals and reduced gases such as carbon dioxide (CO_2), sulfide (H_2S), hydrogen (H_2) and methane (CH_4) (Figure 10). When mixing with cold deep-sea water the hydrothermal flux is diluted, its

high temperature is reduced, the pH becomes more basic, some metals precipitate forming the classical chimney structures and some remain in the water column. However these characteristics are very variable for each vent site, providing a biotope with different environmental conditions (Demina and Galkin, 2016; Von Damm, 1995).

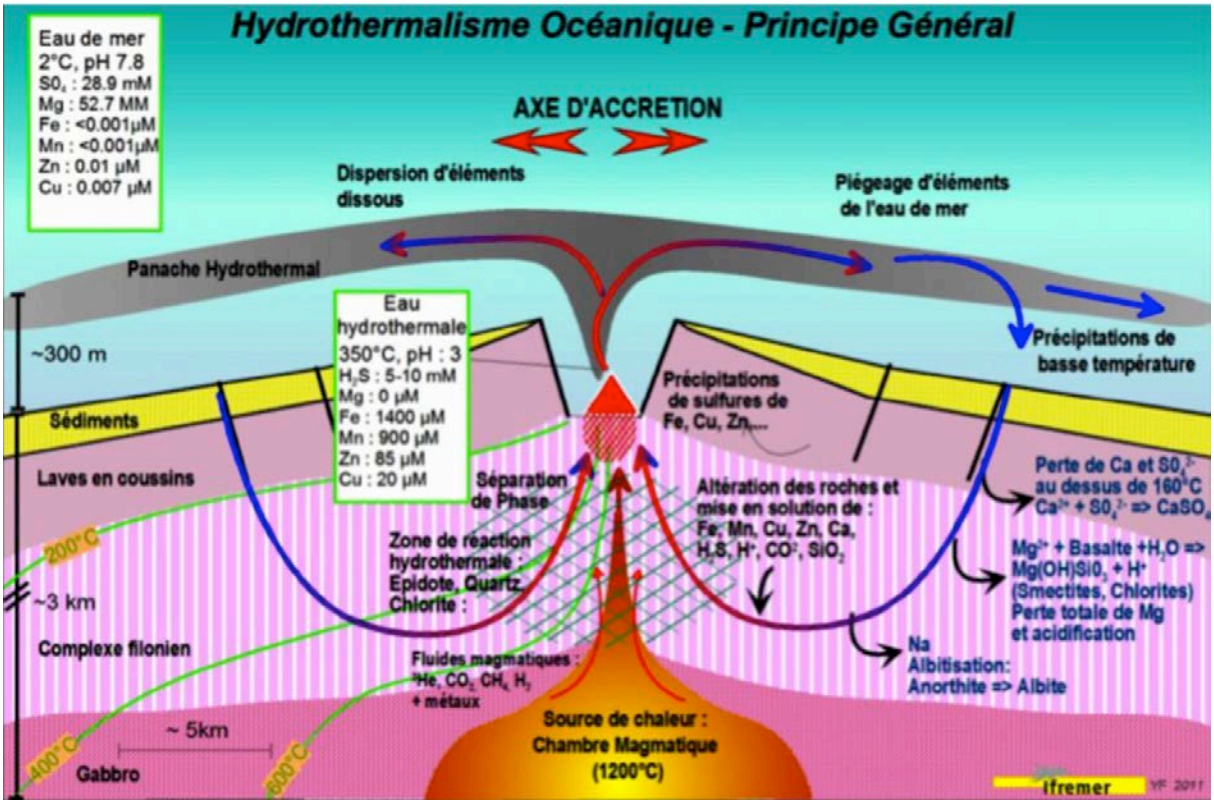

Figure 10. Geological formation of an hydrothermal vent. Adapted from Fouquet and Lacroix, 2012.

The initial discovery of luxuriant vent ecosystems in 1977 around the Galapagos (Corliss et al, 1979) revolutionized the understanding of life in the oceans because until that date the paradigm was that life in the deep ocean was scarce due to the absence of light, and consequently photosynthetic primary production, limiting food availability to what came from the surface layers. In the last forty years the development of new exploratory technology, principally manned submersibles and remotely operated vehicles (ROV), has enabled the discovery of many of these ecosystems across the oceans (Figure 11), revealing the occurrence of local, chemosynthetic primary production supporting very abundant communities of endemic species (Desbruyères et al. 2006), comprising arthropods, molluscs and annelids which have adapted to these ecosystems, and challenging major evolutionary, ecological, physiological hypotheses and paradigms (Danovaro et al., 2014).

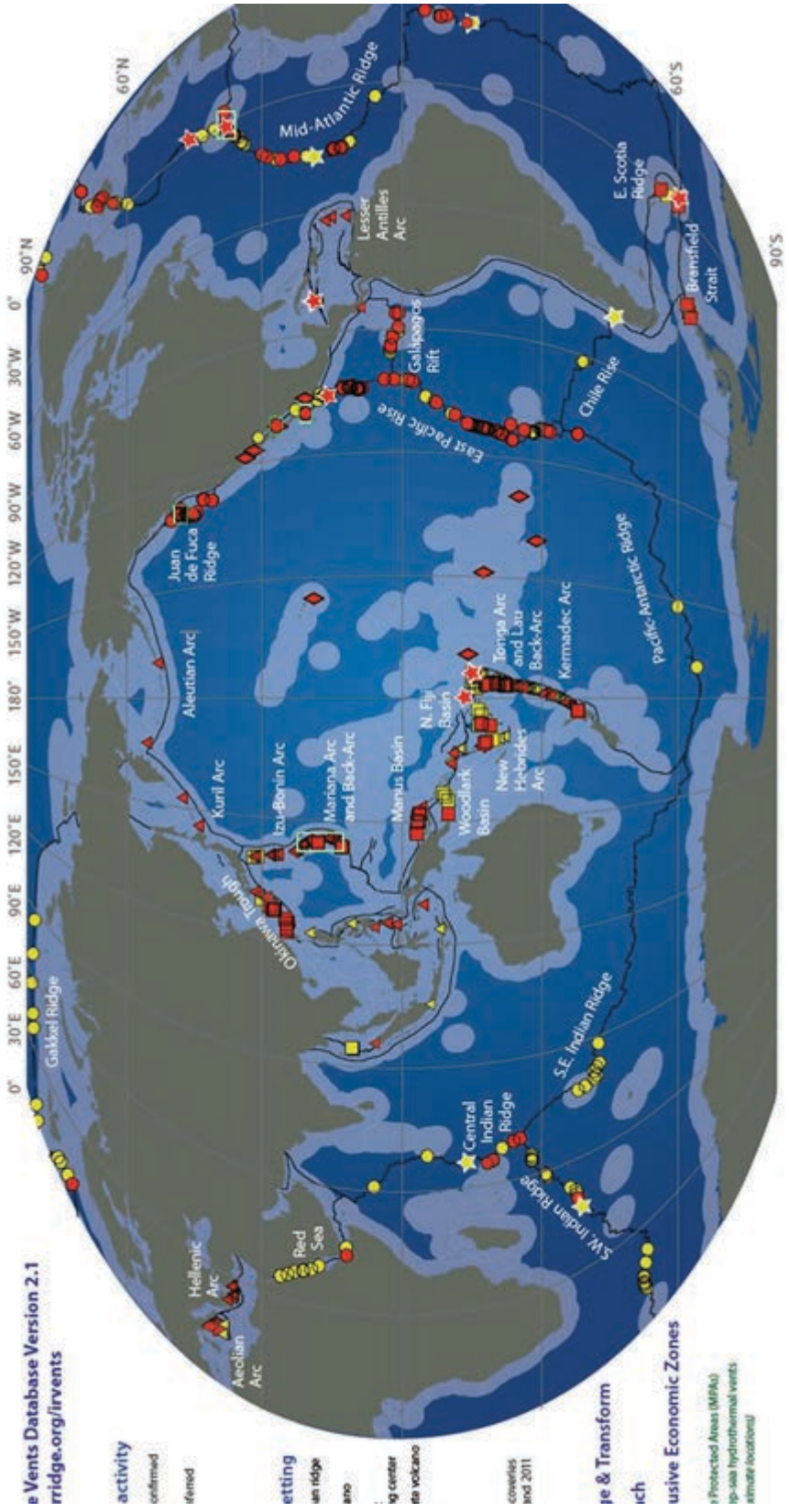

Figure 11. Distribution of hydrothermal vent sites across the oceans. Adopted from InterRidge, version 2.1

6.1 Hydrothermal vents in Mid-Atlantic Ridge (MAR)

Ten years after the discovery of Corliss and colleague's, exploration in the MAR were performed (Rona et al., 1986), evidencing that active hydrothermal vents also exist on slow spreading ridge axes. Until now, many scientific expeditions have been devoted to the MAR where several vent fields have been described between 10º N and 40º N (Figure 12), and some more recently in the south Atlantic. The most studied are the three vent fields located near the Azores Triple Junction (ATJ), characterized by contrasted physicochemical characteristics associated with their depth and the composition of oceanic crust (Charlou et al., 2000; 2002): Menez Gwen (MG, -850m), Lucky Strike (LS; -1700m), and Rainbow (RB; -2300m). An indication of the characteristics of the vent fluid (end-member) is given in Table 1, but one must keep in mind that due to the chaotic nature of hydrothermal venting, large excursions around these values can be measured at a given date.

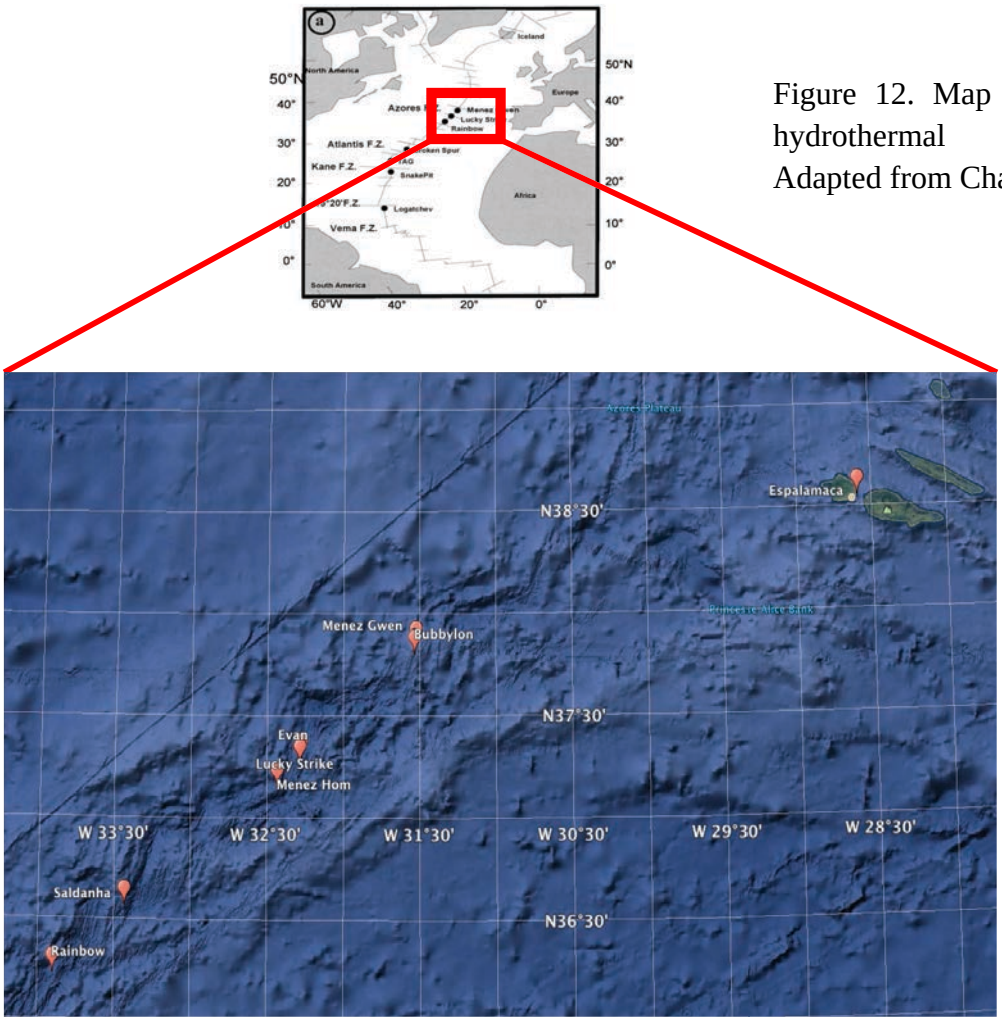

Figure 12. Map of north MAR hydrothermal vent fields. Adapted from Charlou et al 2002.

Table 1. Principal physicochemical characteristics measured in the hydrothermal vent fluid (end-member) at Menez Gwen, Lucky Strike and Rainbow. Adapted from Charlou et al 2002.

	Menez Gwen	Lucky Strike	Rainbow
Depth (m)	850	1700	2300
Temperature (ºC)	275/284	170/364	365
pH	4.2/4.3	3.5/3.7	2.8
Cl (mM)	381	533	750
Br (µM)	710	924	1178
Na (mM)	319	446	553
Ca (mM)	33.1	38.2	66.6
Sr (µM)	111	119	200
Fe (µM)	18	863	24050
Mn (µM)	71	446	2250
Cu (µM)	3	26	162
Zn (µM)	4.3	57	185
H_2S (mM)	1.5	3.0	1.2
CO_2 (mM)	20	28	16
CH_4 (mM)	2.6	0.97	2.5
H_2 (mM)	0.048	0.73	16

Ecological studies at these vent fields started in the nineties and recognized these sites as one biogeographical region separate from the southern sites, principally due to their communities composition and structure (Desbruyères et al., 2001). MG and LS have been the first deep-sea marine protected areas to be established in 2002 and an interdisciplinary deep-sea observatory has been set up at LS since 2010 (http://www.emso-fr.org/fr/EMSO-Azores).

The particular trophic structure described in MAR is conformed by bacteria, arthropods, annelids and molluscs principally. Chemoautotrophic bacteria are the primary producers that can synthetize organic carbon from the energy derived from the oxidation of hydrogen sulfide (SOX bacteria) or methane (MOX bacteria) (Cavanaugh et al., 2006). Consortia of heterotrophic bacteria are typically found within bacterial mats or filamentous aggregates (Crepeau, 2011). Primary consumers are divided in two main groups, mussels and shrimps, that have mixotrophic feeding behavior switching between reliance on symbiotic bacteria and filtering or grazing activities. The next trophic level is represented by detritivorous invertebrates, principally crabs and gastropods that live between mussels beds

and shrimps, and finally the top predator are mostly bathyal species such as fishes that live close to the vent fields and occasionally make incursions into the vent sites for food supply (A. Colaço et al, 2002).

6.2 Heavy metals concentration in MAR and their accumulation in vent fauna

One of the major characteristics of the hydrothermal vent fluid is the high concentration of heavy metals that emerge from the flux, where one part is precipitated to form sulfide structures and the other is mixed with seawater and dispersed with the hydrothermal plume moving hundreds of meters above the vents. However, the availability and concentration of metals depend on the intrinsic characteristics of each vent site such as spreading rate, temperature, depth, rock composition of the ocean floor and hydrothermal circulation (Elderfield 1996; Fouquet and Lacroix, 2012). For MAR fauna, a direct correlation between the concentration measured in the fluid and that in the tissues of different animals (*Bathymodiolus* mussels, *Rimicaris* shrimps and *Segonzaci*a crabs) has been established, showing very high levels of bioaccumulation compared with anthropogenically contaminated ecosystems, where various metals (iron, copper, cadmium, manganese, magnesium, lead, zinc, silver and arsenic) are concentrated in different tissues (gill, mantle, digestive gland, foot and hemolymph fluid) according to the concentration in the environment (Demina and Galkin, 2008). This bioaccumulation represents one of the biggest challenges for species associated with these ecosystems, and endemic species must adopt physiological strategies implying mechanisms of tolerance and/or detoxification which have not been well described for hydrothermal species.

The contrasted metal concentrations reported at MG, LS and RB (Table 1) raises a good environmental laboratory to understand the plasticity of species responses to metal. The concentration of metal in fishes living in surrounding hydrothermal areas has been reported higher with those compared in fishes without the influence of hydrothermal vent (Company et al., 2010; Raimundo et al., 2013), however detoxification in these species is not correlated to metallothionein (MTs) levels in tissues, arguing that this protein is not the major detoxification system for this fishes.

Among endemic species, the crab *Segonzacia mesatlantica* accumulates high concentrations of iron, zinc, copper, manganese and lead principally in gills, gonads, muscles

(Demina and Galkin, 2008) but its detoxification systems are not well studied. Leignel et al 2008 made a characterization of MT genes in this species showing a conserved pattern compared to coastal crabs but a relative expression higher in the hydrothermal crab. High metal bioaccumulation in gill and pylorus is also reported in both shrimps species *Mirocaris fortunata* and *Rimicaris exoculata.* The bioaccumulation of metals is high in *M. fortunata* compared to *R. exoculata* although the latter lives closer to the hydrothermal flux, which would indicate a more efficient detoxification mechanism in this species (Kádár et al., 2006). (Zbinden and Cambon-Bonavita, 2003 report specific physico-chemical conditions in the gut of *R. exoculata* that favor the development of iron reducing Deferribacterales bacteria, suggesting that these bacteria could detoxify iron which would explain the presence of iron oxides in the gut shrimp, a mechanism that has not been well demonstrated in hydrothermal species. *R. exoculata* also possess mechanisms reported in other hydrothermal species and associated to metal exposure, principally the activation of MTs and antioxidant enzymatic activity (CAT, SOD, GST), which suggests that this species could be a good model to study response to metal exposure (Auguste et al., 2016).

Arthropods: crabs and shrimps

The decapod crustaceans are represented by the scavenging crab *Segonzacia mesatlantica* that is the only endemic crab described for the MAR and is well distributed within the three sites (MG, LS and RB) where it is found among mussel beds. The principal adaptation described in this species is the very high affinity of its hemocyanin for oxygen which is present at low concentration around vents (Chausson et al., 2004). A second crab species is *Chaceon affinis* that is not a hydrothermal species but is occasionally found near vents feeding upon mussels beds at Menez Gwen.

The shrimps belonging to the endemic Alvinocarididae family are abundant at MAR vent fields, with three representative species reported: *Rimicaris exoculata, R. (Chorocaris) chacei* and *Mirocaris fortunata*. The first species is dominant at TAG, Logatchev and SnakePit sites but is also abundant at Rainbow (Desbruyères et al., 2000; Zbinden et al., 2004). It forms dense aggregates swimming close to chimneys walls. Symbiosis relationships have been described in these species with filamentous epibionts in the branchial chamber belonging to Epsilonproteobacteria and Gammaproteobacteria. however his relation with the energy supply are not well established (Petersen et al., 2010). Other specific symbiotic bacteria are found in the gut (Durand, 2010). *R. (Chorocaris) chacei* is found at LS where it is

less abundant and consequently less studied than *R. exoculata*, but it carries the same type of gill chamber symbiotic bacteria as *R. exoculata* although less developed (Gebruk, 2000). *Mirocaris fortunata* has been reported at MG and LS where its abundance is positively correlated with hydrothermal fluid flow (Sarrazin et al., 2014) suggesting that its nutritional requirements are obtained by grazing on free living bacteria.

Molluscs: *Bathymodiolus azoricus*

The mussels are represented by Bathymodiolinae species, a family that is well distributed in different hydrothermal systems across different oceans (Miyazaki et al., 2010). The systematic and phylogeny of bathymodiolin mussels have been studied in depth recently (Lorion et al., 2010, Thubaut et al., 2013) and the two species that are found at MAR vents belong to the genus *Bathymodiolus sensu (*Thubaut, 2013). *B. puteoserpentis* lives primarily around the deeper and southern vent sites Logatchev, TAG and SnakePit (Desbruyères et al., 2000). *B. azoricus* is abundant in the northern sites of the Atlantic, RB, LS and MG, forming large communities at the base and walls of vent chimneys (Sarrazin et al., 2014) (Figure 13). Both are mixotrophic species that obtain part of their energy through filtration but mostly rely on their symbiotic MOX and SOX bacteria located in the bacteriocytes of their gill epithelium (Le Bris and Duperron, 2010).

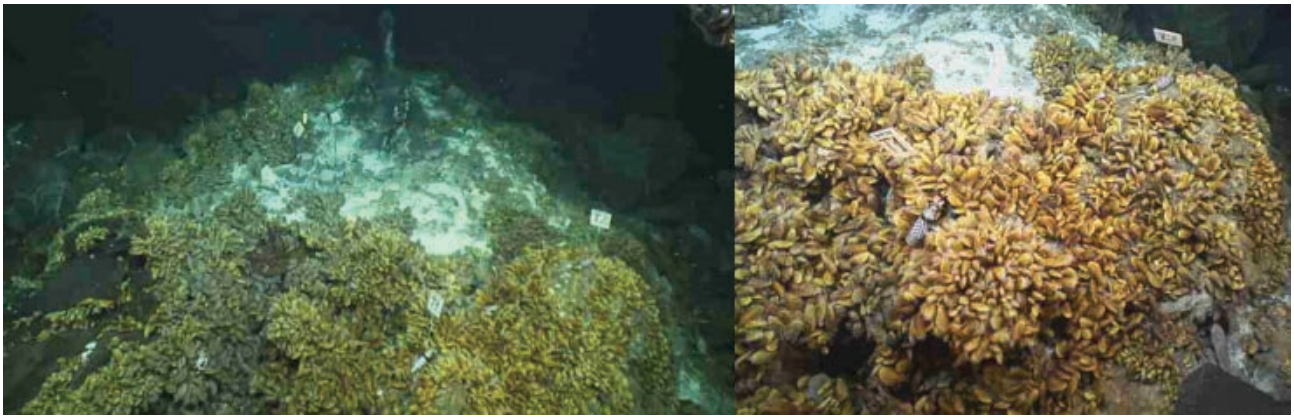

Figure 13. Mussel bed assemblages of *B. azoricus* at Lucky Strike during the BioBaz cruise (Lallier, 2013)

Bathymodiolus species have been the subject of many studies, with about 500 references in the Web of Science, including 200 related to *B. azoricus*. Among these, different aspects of the physiology of *B. azoricus* have been studied, revealing its adaptability to the hydrothermal vent environment and the relevance of gene expression changes affecting its physiological homeostasis. The principal aspects studied are the immune system in response

to bacteria exposure (Bettencourt et al., 2014, 2010; Martins et al., 2014, 2013), effect of post-capture acclimatization on immune genes expression (Barros et al., 2015), inter and intra site variation in symbiotic contents in gills (Guezi et al., 2014), the effect of pressure stress on symbiotic bacteria during capture (Szafranski et al., 2015), transcriptomic regulation in relation to symbiotic bacteria (Boutet et al., 2011), lysozymes activity in relation to symbiont content (Detree et al., 2016) and gill proteome differences between MG, LS and RB (Company et al., 2011). However, the eventual implication of the symbionts in heavy metals resistance or detoxification in *B. azoricus* have not been well studied, even knowing that some free living bacteria can show resistance to metals (Farias et al., 2015).

Obviously, *B. azoricus* is physiologically adapted to high concentrations of different essential and non essential metals such as copper, iron, cadmium, zinc, manganese and lead, that have been found with significant concentrations in gill, mantle and digestive gland, although concentrations vary in relation to each vent site across the MAR (Cosson et al., 2008; Demina and Galkin 2008; Koschinsky et al., 2014).

As has been described in other species, metals can cause oxidative stress and consequently activate the enzymatic machinery reducing this damage. In natural populations of *B. azoricus* differential tissue responses and also intra and inter site variations are reported for SOD and CAT activity. For example, the higher SOD activity is found in gills of Lucky Strike mussels, while CAT levels are higher in Rainbow mussels reflecting the specificity of antioxidative defense to the particular environmental condition (Bebianno et al., 2005). The specific response to certain metals has also been examined in experimental exposure to cadmium, where high levels of antioxidant enzymatic activity are present in gill compared to mantle but SOD and CAT show a significant inhibition in the first days of exposure before gradually increasing, however the levels of both enzymes were similar between control and metal exposed mussels suggesting an inhibitory effect of cadmium (Bebianno et al., 2005), evidencing that the mechanism of response to metal toxicity are not completely understood. Also the MT activity was measured the last two study, where no variation levels were detected across the contrasting environmental and additionally no variation were reported between control and cadmium exposed mussels which puts into question the role of MT in the detoxification mechanism of *B. azoricus*.

At the molecular level the expression of genes involved in different physiological functions in *B. azoricus* mussels exposed to various metal cocktails simulating the gradient

found in MG, LS and RB sites suggest a shared regulation pattern between the three sites and, therefore, a global response to stress and metal exposure. Of course, some genes are regulated only in specific conditions, illustrating the plasticity of the response of this mussel (Bougerol et al., 2015).

The understanding of the mechanisms of tolerance in this and other hydrothermal species remains a challenge in controlled conditions due principally to the difficulty of recreating the hydrothermal conditions in which this organism live, characterized by spatial-temporal variation that change in different orders of magnitude, which makes difficult the comparison of natural and experimental result, so a multidisciplinary approach must be developed with this species to get a better understanding of the physiological and evolutive mechanisms and adaptation to this extreme ecosystem.

7. Objectives

The study of response of marine organisms exposed to different anthropogenic or natural xenobiotic agents has revealed a wide range of mechanisms that vary from molecular to physiological adaptations. Exposure to heavy metals is one of the most selective factors acting in marine aquatic ecosystems but the adaptive response of species in natural, experimental or anthropogenic contaminated areas has not been fully explained.

Hydrothermal vents are widespread around the world and one of their characteristics is the high concentration of heavy metals that naturally emerge from the ocean crust. Endemic species living around vents have developed adaptive strategies to cope with these conditions, making these ecosystems a natural laboratory to study ecotoxicological responses. The Mid-Atlantic ridge (MAR) is one of the best explored areas in which several vents have been described with contrasted physico-chemical characteristics including metal content. Thus, we chose to study the response to metals of one of the most representative species living in three sites of the northern MAR, the mussel *Bathymodiolus azoricus* using a transcriptomic approach. To better understand how *B. azoricus* respond to different environmental metal concentrations, we determined the differential bioaccumulation of the most representative metals in different tissues across different natural *B. azoricus* populations in MAR, but also in a controlled experiment of metal exposure.

We focused on the study of specific molecular markers of response to metal exposure using a candidate gene approach to look at the quantitative expression of well described metal-related responsive genes but also potential new candidates. We conducted this analysis in different tissues and populations of *B. azoricus* with a particular interest for the diversity of genes families. We also perform a global gene expression study using microarrays to identify new physiological pathways involved in metal response, and consequently identify new potential molecular biomarkers that could be implemented in future ecotoxicological research.

This work is divided in five chapters in which each one describes the result related to the objectives described above: Chapter I include a general introduction about ecotoxicology and hydrothermal vent ecosystems. Chapters II include the result of metal bioaccumulation in natural population of *B. azoricus* and the characterization and expression of metallothioneins (MTs) in these populations of MAR. Chapter III describes the metal bioaccumulation in controlled metal exposure experiment and the response of the oxidative stress, based in the expression levels of superoxide dismutase genes (SODs), in this mussels and also natural populations. Chapter IV describe the result from the expression of ferritins genes (Fer) involved in iron homeostasis across exposed metal mussels and natural populations and finally the Chapter V report the result of the gene expression profile in different tissues for natural populations, using microarrays as the molecular tool. The final pages comprise conclusion and future perspectives of this works.

Chapter II

Article I: Metal accumulation and regulation of metal related gene expression in the hydrothermal vent mussel *Bathymodiolus azoricus* as a signature of environmental contamination.

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ABSTRACT

Hydrothermal vent ecosystems are present throughout all the oceans, mainly in active volcanic areas. One of their main feature is the elevated concentration of metals present in the plume emerging from the earth's crust due to infiltration of seawater and sub-seafloor alteration. Despite the toxicity of heavy metals, these ecosystems are biologically highly productive. Bivalves of the genus *Bathymodiolus* are dominant species in most of these environments. *B. azoricus*, an endemic species distributed along the northern mid-Atlantic ridge (MAR), has been described as highly tolerant to heavy metals with a strong accumulation in different tissues. In this work we describe the relative expression pattern of six metal-related genes in three tissues (gill, mantle and digestive gland) in seven mussel populations collected at three different hydrothermal sites on the Medio Atlantic Ridge (Menez Gwen, Lucky Strike and Rainbow), characterized by different concentrations of metals. Three of these genes correspond to classical MT sequences, two genes correspond to sequences related to putative cadmium tolerance protein and one encode a phytochelatin synthase gene. Quantification of eight essential and heavy metals was also conducted in the different tissues in order to establish putative correlation between gene expression and metal accumulation. Our results illustrate the variability of the expression of these genes at both intra- and inter-population level and the potential use of the association of the gene expression pattern with metal concentration in the different tissues as a biomarker for this species. We also evidenced that some metals concentrations are correlated with the abundance of chemosynthetic symbiotic bacteria present in the gills of *B. azoricus.*

Keywords: hydrothermal, metal bioaccumulation, detoxication, gene expression, symbiont bacteria

1. Introduction

Coastal marine mollusc species are permanently exposed to a wide variety of contaminants including pesticides, HAPs and metals that present a large range of concentrations and at both dissolved and particular states. Metal level in bivalve tissues are often considered as a good bio-indicator of the time-integrated response to bioavailable metal in food and water and are widely used in environmental monitoring programs (Langston and Spence, 1995; Boisson et al., 2003). In hydrothermal species, a huge bioaccumulation of metals is detected in different tissues such a in the clam *Calyptogena magnifica* (Roesijadi et al., 1985) or the mussels *Bathymodiolus thermophilus* (Rousse, 1999) and *B. azoricus* (Cosson et al., 2008) but metal

concentrations do not systematically reflect differences in the environmental parameters. It has been suggested that hydrothermal mussels may also exhibit specific adaptations to metal exposure allowing them to limit metal accumulation despite high concentrations in the fluid (Geret et al., 1998; Kádár et al., 2006a, 2006b). *B. azoricus* is a bivalve belonging to the Mytilidae family that is widely distributed in hydrothermal ecosystems, specifically described in Mid-Atlantic Ridge (MAR). The three main MAR vent sites (Menez Gwen, Lucky Strike and Rainbow) present contrasting physicochemical characteristics (Charlou et al., 2002; Desbruyères et al., 2001) and mussels form large communities at the base and walls of vent chimneys (Sarrazin et al., 2014). Previous works evidenced that *B. azoricus* accumulate essential (iron, copper, zinc) and non-essential (cadmium, mercury, lead) metals in different tissues like gills, mantle, digestive gland and foot with variations in metal bioaccumulation observed between sites in relation or not to the metal concentration present in the fluid (Cosson et al., 2008; Martins et al., 2011). *B. azoricus* also harbor dual symbiosis with two distinct sulphur- and methane-oxidizing bacteria located in the bacteriocytes in the gills epithelium (Duperron et al., 2005). Presence of symbionts has been described as an adaptive strategy for energy support in absence of photosynthesis but their putative role in metal content regulation by the host remains poorly documented.

The cellular detoxification of metals is achieved by several molecular mechanisms in which metallothioneins (MTs) are involved. MTs are low molecular weight proteins with a high cysteine content (close to 30%) and are involved in cellular metal homeostasis and heavy metal detoxication (Kojima and Kagi, 1978; Isani and Carpenè, 2014). Although amino acid composition is variable, the cysteine content is always conserved with the presence of characteristic motif like Cys-Cys, Cys-X-Cys and Cys-X-Y-Cys, where X and Y are amino acids other than cysteine (Kagi and Vallee, 1960; Hamer, 1986). These motifs contributes to the formation of the two structural domains α and β (Otvos et al., 1993). Apart from homeostasis of essential and non essential metals, other functions including protection against oxidative stress, free radical scavenger activity, immune response, genotoxicity and carcinogenicity have been reported (Asselman et al., 2012; Nordberg et al., 2009). Quantification of MTs in B. azoricus evidenced a specific tissue response with a higher protein concentration in gills and digestive gland compared to mantle and also revealed a very limited difference between vent sites (Bebianno et al., 2005; Martins et al., 2011) suggesting that MTs may be not the only mechanism involved in heavy metal detoxification. In other species, molecular binding peptides with similar function to MT have been described such as the phytochelatins (PCs). PCs are characterized by three amino acid forming a specific

structure (γ-Glu-Cys)$_n$-Gly where n ranges from 2 to 11 repetition (Cobbett and Goldsbrough, 2002). Originally reported in plants as the major component of Cd-binding complexes (Grill et al., 1987), they have been evidenced in fungal species (Cobbett, 2000), and nematodes (Vatamaniuk et al., 2001). Existence of the PC synthase gene has been evidenced in *A. californica,* in the oyster *C. gigas* genome (Zhang et al., 2012) and in transcriptome of the hydrothermal mussel *Bathymodiolus platifrons* (Wong et al 20015) but his role and regulation in response to metal detoxification is not well documented.

Bivalves have been described as good candidate for pollution biomonitoring mainly due to the high volume of water they filter for feeding and respiratory purposes and because of their ecological and/or commercial value (Paul-Pont et al., 2012). Due to the specificity of the hydrothermal habitat characterized by high metal concentrations, Bathymodiolin mussels could be are relevant models to investigate in more details the relationship between metal related gene expression and heavy metals toxicity in tissues. In this work we used a transcriptomic approach coupled to the quantification of metals in different tissues of several natural populations of *B. azoricus* living in three hydrothermal vent sites of the MAR. Our aim is to determine if both markers can be used as a proxy for metal fluxes in *B. azoricus* and identify specific gene regulation patterns that could be relied to metal contamination. We also addressed the question of the putative influence of symbiont content on metal concentration.

2. Materials and methods

2.1. Biological samples

B. azoricus mussels were collected from Mid-Atlantic Ridge during the BIOBAZ cruise (Lallier, 2013) on the research vessel “Pourquoi Pas? using the Remotely Operated Vehicle (ROV) Victor 6000. The collection was made in three different vents sites Menez Gwen (MG: 37°50’ N, 31°31’ W, 800m depth, three populations called MG2, MG3 and MG4), Lucky Strike (LS: 37°17’ N, 32°17’ W, 1700m depth, two populations called Tour Eiffel and Montsegur) and Rainbow (RB: 36°14’ N, 33°54’ W, 2300m depth, three populations called Termitière and Intermediate, **Appendix A).** The samples were placed in hermetic boxes then brought on board. Pieces of gills, mantle and digestive gland of each individual were immediately dissected in cold room and fixed in liquid nitrogen until further analysis. A total of 25 mussels per population was used for gene expression analysis.

2.2. RNA extraction, cDNA sequencing, qPCR and symbiont content

The relative quantity of symbionts was estimated by real-time PCR on genomic DNA amplification according to the protocol available in Boutet et al., 2011. Total RNA of gills, mantle and gland digestive was extracted using Tri-Reagent (Sigma, St. Louis, MO) according to the manufacturer's instructions. Both quantity and quality of DNA were assessed via UV absorbance (OD260/280/230), using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Delaware, USA). Two µg of total RNA were reverse transcribed using M-MLV reverse transcriptase (Promega, Madison, WI). Partial MTs and PCS sequences used in this study were obtained from Illumina sequences previously generated and deposited in Data Bank of Japan (DDBJ) Sequence Read Archive (DRA, http://trace.ddbj.nig.ac.jp/dra/) with accession number DRA 004082). The MTs and Cd-bp sequences were completed using 5'-RACE and 3'-RACE in SMART^{TM} race cDNA Amplification Kit protocol (Clontech)**.** PCR products were gel-purified using the GeneClean III kit (Bio 101), cloned in pGEM-T vector (Promega, Madison, WI), and sequenced by extension from both ends using T7 and Sp6 universal primers (T7 sequencing kit, Amersham Pharmacia Biotech, Uppsala, Sweden) using an ABI 3130xl automatic capillary sequencer at the "Genomer platform" (Roscoff, France). Sequences were edited using the Geneious software, version 7.1 (Kearse et al., 2012). For gene expression analysis, a volume of 2µl of each diluted reverse transcription product (1:200) was subjected to real-time PCR in a final volume of 5 µl containing 40 nM of each specific primer and 2×Lightcycler®480 SYBR Green I Master mix (Roche Diagnostics, Mannheim Germany). The amplification was carried out as follows: initial enzyme activation at 95 °C for 15 min, then 45 cycles of 95 °C for 10 sec and 60 °C for 30 sec. A dissociation curve was generated and PCR efficiency was estimated for each primer pair. All primer pairs tested generated a single peak in the dissociation curve and a PCR efficiency of 95 to 100%. The ribosomal protein L15 gene (RpL15) was used as the internal PCR control as already used in previous studies (Guezi et al., 2014; Bougerol et al., 2015). All primers sequences used are presented in **Table 1.**

2.3. Metal concentrations in tissues

For each population, three pools of gills, mantle and digestive gland tissues of 5 samples each were lyophilized. Sample digestions were done at 105°C in closed 15-mL Teflon screw-cap vials (Savillex, Minnetonka, USA) with 4 mL Suprapur 65 % nitric acid (Merck, Darmstadt, Germany) and 1 mL Suprapur 30% hydrogen peroxide (Merck). Measurements were then conducted on diluted mixtures (2.5% HNO_3) using different spectrometers operated at the Pole Spectrometry Ocean (PSO, Brest). Cu, Pb, Cd, Mn and Zn were determined using a

High-Resolution ICP (HR-ICP) mass spectrometer (Element 2, Thermo Scientific, Bremen, Germany) with a precision better than 3% (Cd, Cu, Pb) or 5% (Mn, Zn). Ca, Fe and Mg were determined with an inductively coupled plasma (ICP) optical emission spectrometer (Ultima 2, Horiba Jobin Yvon, Longjumeau, France) with a precision better than 1%. The certified reference materials used for assessment of the method accuracy were: DORM-4 (fish protein), DOLT-5 (dogfish liver) and TORT-3 (lobster hepatopancreas) from NRCC, and RM-8414 (bovine muscle) from NIST (**Appendix B)**. All metal concentrations are expressed in μg g^{-1} dry weight of tissue.

2.4. Statistical analyses

The relative expression of each gene was calculated according to the comparative Ct method using the formula RQ = ΔΔCt (with $\Delta Ct_{gene\ X} = Ct_{gene\ X} - Ct_{RpL15}$ and $\Delta\Delta Ct = \Delta Ct_{gene\ X} - \text{mean } Ct_{gene\ X}$). The first analysis was conducted to determine the inter-population effect in the variation of gene expression and the data were normalized by the mean $\Delta Ct_{gene\ X}$ of all populations for one tissue (gill, mantle or digestive gland). The second analysis was conducted to determine the intra-population effect of the expression and the data were normalized by the mean $\Delta Ct_{gene\ X}$ in the three tissues for each population. ANOVA test was used to evaluate differences in metal accumulation and relative expression considering populations and tissues as explanatory factors (R studio software). Canonical redundancy analysis (RDA) was used to evaluate the influence of metal accumulation in the variation of gene expression using the Vegan package (Oksanen et al 2016) in R environment.

3. Results

3.1. Metal concentrations in tissues

Concentrations of the eight metals analyzed in the tissues are presented in **Fig.1;Appen.1**. Metal concentrations varied between populations and tissues but gills and digestive glands exhibited higher concentrations than mantle for several metals. Fe remain the higher enriched element (up to 9500 μg/g) followed by Zn (up to 6000 μg/g), Pb (up to 400 μg/g), Cu (up to 245 mg/ kg), then Cd (up to 30μg/g). Mg remains the most abundant element, followed by Ca and Mn. Results of statistical analysis of metal concentration between populations using ANOVA are presented in **Table 2** and revealed that only calcium shows no significant differences between populations and tissues. Cadmium exhibits the highest concentration in gill especially in the two RB populations (Termitiére and Intermediate) and Tour Eiffel from LS (15,6; 21,4 and 30,5 μg/g respectively). A different picture is observed for the digestive

gland, with the highest concentrations being found in the two populations from MG (MG2 and MG3) and Tour Eiffel from LS (14,5; 16,9 and 19,2 µg/g respectively). No significant differences were found in mantle. A similar pattern is detected for copper with Tour Eiffel, Termitiére and Intermediate that exhibit the higher concentration in gills with 151,2; 187,7 and 104,4 µg/g respectively. Mussels from Termitiére populations also have the lowest levels of copper in both digestive gland and mantle compared to the other sites. For iron, no significant difference is observed in gills between populations. By contrast, in Intermediate and Termitiére populations, highest concentrations are found in digestive gland (8580 µg/g and 4400 ug/g respectively) and mantle (1920 µg/g 3580 µg/g respectively) (**Fig.1**). Lead concentrations vary between tissues with higher values observed in gills from LS and RB populations with a strong accumulation in Termitiére (581 µg/g). In digestive gland and in mantle, a similar pattern is observed even if concentrations remain lower compared to gills. Manganese concentrations present the same pattern for the three tissues with an increase of concentrations from MG to RB. Zinc shows significant differences between populations in gills and digestive gland but no specific hydrothermal site signature is observed. In gills, Intermediate and Tour Eiffel populations present high Zn concentration (5643 µg/g and 2400 µg/g respectively). In digestive gland, the difference observed is mainly due to the high concentration found in Tour Eiffel (1400 µg/g) and mantle do not show any significant differences between populations. Magnesium shows a significant higher concentration in the gills compared to mantle and digestive gland but no significance is found between sites. Correlation analysis made on all samples from the seven populations evidenced a strong positive correlation between some metals illustrating a conserved pattern of metal composition in the three tissues analyzed (**Table 3, 4, 5**). Correlation between iron and calcium and copper and manganese are close to 1.

3.2. Metal related genes characterization

The alignment of the three new MT isoforms (MTa, MTb and MTc) with other bivalves MTs sequences is presented in **Fig.2**. The three MTs shared 50 to 90% of similarity with other bivalve species. The amino acids sequences are 64, 73 and 69 length for MTa, MTb and MTc respectively. All the sequences shared the traditional cysteine motif C-X-C and contain 21 Cys residues except MTc which contains only 20 Cys residues. The MTb isoform corresponds to the most conserved MT in regard with other bivalves MT sequences while MTa and MTc present more deletions. The two putative isoforms that could correspond to cadmium binding protein (called Cd-bp1 and Cd-bp2) according to a previous Blast result

obtained in *E. granulosus* are presented in **Fig.3**. Contrary to classical MTs, we do not observed a high content in Cys residue and a limited presence of C-X-C and C-X-X-C motifs. Although we can not assume that those genes correspond to classical MTs, we decided to keep them in our analysis. The phytochelatin synthase (PCS) gene sequence exhibits 453 amino acids of length and shares between 30% to 45% of similarity with PCS sequences from other species where a the principal similarities are reported in the first two hundreds amino acids (**Fig.4**).

3.3. Metal related genes expression

The analysis of gene expression of MTs, Cd-bp and PCS between populations and ANOVA test results are presented in **Fig.5, Fig.6** and **Table 6**. In the three tissues, MTc showed the highest level of regulation with a low expression in the three tissues in Termitiére population, a high expression in the mantle of mussels from Montsegur and a higer expression in digestive gland of the three MG populations compared to the others populations. At a site scale, we do not identify any conserved pattern of gene regulation. We noticed that in gills, only MTb shows a lower expression in the three MG populations and a higher expression in the two RB populations. In LS, we observed an inverted pattern of expression of MTs when considering the two populations for the three tissues. In MG site, MG3 and MG4 populations present a relatively similar pattern of expression when MG2 shows an opposite regulation especially for gill and digestive gland. Details of the differences in the MTs expression between populations for each tissue are given in **Appendix C, D and E**. PCS expression exhibits a higher expression in gill in mussels from Intermediate population. PCS are also less expressed in the three tissues for MG4, Tour Eiffel and Termitiére populations.

The relative expression of MTs, Cd-bp and PCS between tissues and ANOVA test results are presented in **Fig.7, Fig.8** and **Table 7**. High intra population variations are reported for each population suggesting a tissue specific response of MTs and Cd-bp isoforms, but some patterns can be established. In all populations, the expression of MTa remains higher in gills compared to the two other tissues when expression of MTb remains lower for the mantle. MTb expression is higher in the digestive gland compared to mantle and gills for the three MG populations and is higher or similar in gills for the 4 other populations. For MTc, we noticed a global lower expression in the mantle, with the notable exception of the Montsegur population, and a global higher expression in gills compared to digestive gland. For Cd-bp2, the expression remains higher in the digestive gland for six of the seven populations and for Cd-bp1, we did not identify any clear pattern. A global lower expression of PCS is observed

in mantle for all populations (except MG3). No pattern conservation is detected between gill and digestive gland which exhibit opposite patterns according to populations.

3.4. RDA analysis

The RDA analysis showed that the environmental variable “metal concentration” explained 21%, 14.6% and 17.4% of the variation in the expression of MTs in gills, mantle and digestive gland respectively (**Fig. 9**). RDA analysis showed that the eight metal analyzed have a significant influence on MT expression. In gills, the first two axes explained 15% of the variation in gene expression, mainly attributed to the correlation between MTb, Cd-bp1, Cd-bp2 with cadmium and manganese principally and MTa, and PCS with cadmium. In mantle, the first two axes explained 11% of the variation with a correlation observed between cadmium and PCS, followed by Cd-bp1, while Cd-bp2 is more correlated with the other metals. In the digestive gland, the first two axes explained 13% of the variation in the relative expression with a correlation between Cd-bp1, Cd-bp2, PCS with cadmium, MTb with iron and MTa and MTc with calcium.

3.5. Symbiotic content

The abundance of SOX and MOX bacteria in the gills of the *B. azoricus* for the seven populations studied are shown in **Fig.10**. The SOX content did not show significant differences between populations except for Montsegur population which significantly differs from Eiffel Tower and Termitiére. The MOX content exhibited significant differences between populations characterized by an increased gradient of MOX from MG to RB (**Fig.10**). Correlation analyses between metal concentrations and symbiotic content revealed a number of significant relationships. Negative correlations are found for SOX and manganese (-0,15, p=0.05), copper (-0.20, p=0.01), cadmium (-0.19, p=0.01) and magnesium (-0.15, p=0.05) and positive correlations are detected for SOX and iron (0.21, p=0.01) (**Fig.11**). When considering MOX content, a positive correlation is observed with manganese (0.85, p=0.01), copper (0.62, p=0.01), cadmium (0.41, p=0.01), lead (0.69, p=0.01) and zinc (0.35, p=0.01) and a negative correlation with iron (-0.32, p=0.01) (**Fig.12**).

4. Discussion

4.1 Metal concentrations

Hydrothermal fluids contain variable concentrations of metals that exhibit strong variations according to vent sites due to various parameters including chemical composition of the basement rocks (basaltic and/or ultramafic) and intra-field dynamic characteristics including pressure (Bonatti, 1983; Charlou et al., 2002; Douville et al., 2002)). Mussels that are not living directly in contact with the fluids, are then filtering sea water containing diluted concentrations of metals which bioavailability also depend on the capacities to form precipitates with sulfide (Von Damm, 1995). For example, iron forms preferentially fine grained sulfide particles when Cu and Zn sulfides form large sized grains and more crystalline particles (Feely et al., 1987). Compared to other coastal bivalves, the available portion of soluble metal emerging from the vent and the levels are greater than those found in bivalves inhabiting coastal systems (Sakellari et al., 2013). However, combined with potential differences in storage or detoxification according to metals, the levels of metal bioaccumulation in mussels all the *B. azoricus* populations remain a relative good reflect of the variability of the vent fluid composition and represent the geochemical environmental signature. Differences in metal bioaccumulation between the three tissues reflect the existence of a metal organotropism in *B. azoricus* with a higher accumulation of Cd, Cu, Fe, Pb in gills and digestive gland compared to mantle. This is consistent with previous studies conducted on *B. azoricus* collected in the same area (Colaço et al., 2006; Cosson et al., 2008; Demina, 2008;) and for other hydrothermal vent species such *B. puteoserpentis* (Koschinsky et al., 2014; Smith and Flegal, 1989). Gills are the first permeable respiratory tissues in direct contact with the water and subsequent dissolved elements, the digestive gland is a secretory organ where the metal are transported for subsequent excretion when the mantle is mainly involved in the formation of the shell (Rainbow, 1997). Usually, in marine invertebrates, the digestive gland plays a role in the elimination of metabolic products, but in *B. azoricus*, the primary source of energy comes from the symbiosis with SOX and MOX symbionts (Duperron et al., 2005; Guezi et al., 2014), suggesting that the digestive gland could be mainly involved in storage and excretion of heavy metals. In gills, the mean concentrations of Cd, Cu, Pb, Mn showed an global increasing gradient from MG to RB populations (except Montsegur population at LS) in coherence with metal concentrations measured in the fluids by Charlou et al. (2002). Ca and Mg show no specific variation between populations as often observed probably due to the strong regulation in the numerous metabolic cellular processes in which they are involved (Bara et al., 1993). The low levels of iron observed in gills from RB mussels compared to mantle and digestive gland suggest the existence of a translocation process through the gills towards the other tissues and/or a subsequent excretion from gills. A

similar mechanism of organotropism has been described in other species of marine invertebrates for various heavy metals involving the presence of ligands (mainly proteins) which bind and transport the metallic ions through hemolymph to others organs (Al-Sid-Cheikh et al., 2013; Chalkiadak et al., 2014). This phenomenon is not observed in MG and LS sites, which present similar and lower iron signatures in their fluid composition.

Calcium and magnesium have the highest levels of accumulation across the sites, but are less representative in the hydrothermal fluid (Charlou et al., 2002; Douville et al., 2002) so their high concentrations can be explained by their high concentration in sea water, with $1x10^4$ µM and $1x10^5$ µM respectively and their important physiological role in living organisms and particularly in bivalves. Magnesium is a cofactor for several enzymes whereas calcium is involved in many cellular transmembrane exchange processes (through calcium channels and Ca-ATPase pumps) (Toyoshima, 2009) and, in the case of bivalves, in the formation of the calcium carbonate shell (Li et al., 2004; Rousseau et al., 2003).

Regarding lead, our results showed an increasing accumulation in gills samples from MG to RB, which is coherent with the lead concentrations in fluids. In previous studies, relatively low concentrations of lead was evidenced in samples from MG and RB (Demina, 2008). In our populations from RB (particularly Termitière), the lead concentration measured is close from those detected in gills of *B. puteoserpentis* collected at Snake Pit site by the same authors and at four hydrothermal vent sites on the same species (Koschinsky et al., 2014). Lead presents a clear organotropism, being mainly accumulated in gills with an increasing gradient between populations in accordance with Pb concentration in the fluids. This suggests a limited ability of mussels to regulate this metal in gills. In other bivalves species such as *Dreissena polymorpha* (Kraak et al., 1994), accumulation of lead has also been detected in gills. Lead and other metals are also known to be sequestrated and immobilized in lysozymes which could constitute the main storage site of metals before excretion (Nott, 1991).

4.2 Characterization and expression of MT, Cd-bp and PCS

In highly variable and extreme habitats such as hydrothermal vents, the metal concentrations are higher than those found in most coastal ecosystems suggesting the existence of efficient metal detoxification or sequestration mechanisms in species inhabiting these ecosystems. In bivalves the characterization and induction of MTs after metal exposure has been widely investigated under controlled and natural conditions. In most studies, MTs expression levels increased in response to exposure (Tanguy and Moraga, 2001; Boutet et al., 2002; Dondero et al., 2005; Bourdineaud et al., 2006; Park et al., 2007; Aceto et al., 2011) but, in some cases,

no correlation is observed (Le et al., 2016). The two new specific sequences that could correspond to cadmium binding protein (Cd-bp1 and Cd-bp2) differ from classical MT structure mainly because of the limited number of cysteine motifs in the sequence. Although the specific function of this gene regarding metal sequestration has not been elucidated in marine invertebrates, our redundancy analysis showed a correlation of both genes expression and cadmium and manganese in gills, between Cd-bp1 and cadmium in mantle and between both genes and cadmium in digestive gland. Those data do not allow to conclude that these two genes are effective MTs or Cadmium binding proteins but at least, we evidenced a correlation between their expression and the metal content in some tissue. Additional analysis (i.e over-expression and functional tests) are needed to confirm or invalidate the ability of these two proteins to bind cadmium or other metals. The percentage of the total variance found in gene expression that can be explained by metal concentration range from 14 to 21% according to tissues; the remaining variance being attributed to other environmental variables such as temperature, salinity, which also affect the levels of MTs in bivalves (Le et al., 2016) and must be considered in future studies to get a better explanation about the pattern of MT expression in natural populations of *B. azoricus.*

The diversity of MTs expression patterns observed in the three tissues at the Mid-Atlantic ridge scale strongly illustrates the effect of environmental parameters. Differences observed between populations and even inside the same vent site can reflect the different responses of *B. azoricus* to specific environmental variations at local scale. Few studies have measured the expression or levels of MTs in natural populations (Roesijadi, 1994; Mourgaud et al., 2002; Lavradas et al., 2016). Our RDA analysis suggests a relationship between MTs expression and heavy metal content in *B. azoricus* at the MAR scale with a high correlation of cadmium and manganese with MTb, Cd-bp1, Cd-bp2. Some of the gene expression patterns may be used to characterize populations such as the strong down-regulation of MTc in the three tissues of mussels from Termitiére or the global high expression levels of all MTs gene in gills samples from MG2 compared to MG3 and MG4.

Involvment of PCS in mollusc species in response to metal detoxification is poorly illustrated. In a recent study, Wong et al 2016 identified a PCS gene in the hydrothermal mussel *B. platifrons* but do no detect any difference in expression level between gills, mantle and foot. In *B. azoricus*, the PCS expression shows differences between tissues according to the population suggesting environmental effect on the PCS regulation. Interestingly, difference in expression ratio between tissues remain relatively low suggesting a relative constitutive expression of this enzyme. We notice that PCS expression remains lower in

mantle for all the populations and that cadmium concentration in mantle is also lower. RDA analysis revealed a positive correlation between cadmium with PCS expression in the three tissues with different degree of correlation, which makes us confirm the implication of PCS in cadmium binding activity as demonstrated other species (Franchi et al., 2014; Vatamaniuk et al., 2005). Those results suggest that variations in MTs, Cd-bp and PCS levels and heavy metal bioaccumulation found across populations could reflect local environmental variations within a hydrothermal vent site.

4.3 Symbiont bacteria and heavy metal content

The SOX and MOX symbiotic bacteria content strongly depends on the availability of substrates (i.e, sulfide and methane) and comparisons over years could reflect relative temporal fluctuations (Halary et al., 2008; Szafranski et al., 2015; Guezi et al., 2014). The low differences in SOX content observed in our populations could reflect a similar sulfide concentrations in the three vent sites and the slightly higher SOX content observed in MG populations compared to RB populations could be partly explained but the interaction between sulfides and iron at RB that may lead to a decrease of available sulfide for organisms (Le Bris and Duperron, 2010). We observed an increasing gradient of the MOX content from MG to RB as previously reported by Szafranski et al 2015 and this result is in accordance with the higher level of methane available at RB (Charlou et al, 2002). In this study, we looked for correlations between metal accumulation and symbiont content. Indeed, previous studies showed that both SOX and MOX bacteria exhibited high levels of iron, copper and zinc (Kadar et al 2006b). The ability of tolerate high concentrations of metals has also been previously evidenced for hydrothermal free-living bacteria (Silver and Phung, 1996; Wang et al., 1997). In the sponge *Spongia officinalis* and the hydrothermal polychaetes *Alvinella pompejana* and *A. caudata*, the high heavy metal tolerance of their isolated bacteria exposed to different metals suggested a possible contribution of these bacteria to the mechanism of metal resistance (Bauvais et al., 2015; Jeanthon and Prieur, 1990). Our results indicate that some significant correlations are observed between metal and bacteria content in gills suggesting that symbiotic bacteria may strongly contribute to metal uptake and sequestration in this species limiting the toxic effect of those metals for *B. azoricus* and then contributing to the accumulation of metals at least in gills. The positive correlation of MOX levels with Mn, Cu, Cd, Pb, Zn, and SOX levels with Fe, and the negative correlation of MOX with Fe and SOX with Mn, Cu, Cd and Mg, also suggest that both bacteria may have a different role in heavy metal homeostasis in the gills of *B. azoricus.*

In conclusion, this study make appear *B. azoricus* as a useful bioindicator species for hydrothermal habitats subject to metal enrichments. Indeed, both tissue metal signature and metal detoxication related gene expression pattern provided insight into the biological stress responses of mussels and allowed to distinguish vent sites. Additional analysis mainly based on MT properties have to be conducted through over-expression of all MTs isoforms and more specially the two potential Cd-MTs.

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7. Figures and tables

Table 1. Primers used in qPCR, RACE amplification of metal related genes and SOX and MOX bacteria content quantification.

Gene name	Primers sequence (5´-3´)	Method
MTa	F: CTAGCGTATGCATCTGTGGAACTG	qPCR - RACE
	R: CAAGAAGAAGTACATCCGACACCG	
MTb	F: ATGTAGCGGAGAAGGTTGCC	qPCR - RACE
	R: GGTCCAGTACATCCGACACC	
MTc	F: AACTGCGCCGACATCTGCGGCACCGG	qPCR
	R: CAAGAAGATGGTCCAGTACATCCG	
Cd-bp1	F: GCAGACCACTTTTCTAAGGCAG	qPCR
	R: GGTACTTGCTTGTCCTGCTCT	
Cd-bp1	F: GTTTACAATTGTTGTTTTCAT	RACE
	R: ACATTGTTGTATTTGTAGGACAA	
Cd-bp2	F: TGGAAGGCCGACAAGAAGTC	qPCR
	R: ACATTGCATCATGGACACACC	
Cd-bp2	F: TGTGTCGTAAAGTAGTTCAA	RACE
	R: GCACAGTCCTCCTGCATGTA	
PCS	F: CGATTTATCTGTCGCTTTCATGGC	qPCR
	R: AGTTCTTGGTCAAACTGGAGATGG	
MDH	F: ATGGAGGAAAGAGATATGGCACTGAGCGT	qPCR
	R: TAACATTAAACATAGCCTAGGAACCTAATG	
SOX (16S)	F: GAGTAACGCGTAGGAATCTGC	qPCR
	R: CGAAGGTCCTCCACTTTACTCCATAGAG	
MOX (16S)	F: GTGCCAGCMGCCGCGGTAA	qPCR
	R: GCTCCGCCACTAAGCCTATAAATAGACC	
RbL15	F: TATGGTAAACCTAAGACACAAGGAGT	qPCR
	R: TGGAATGGATCAATCAAAATGATTTC	

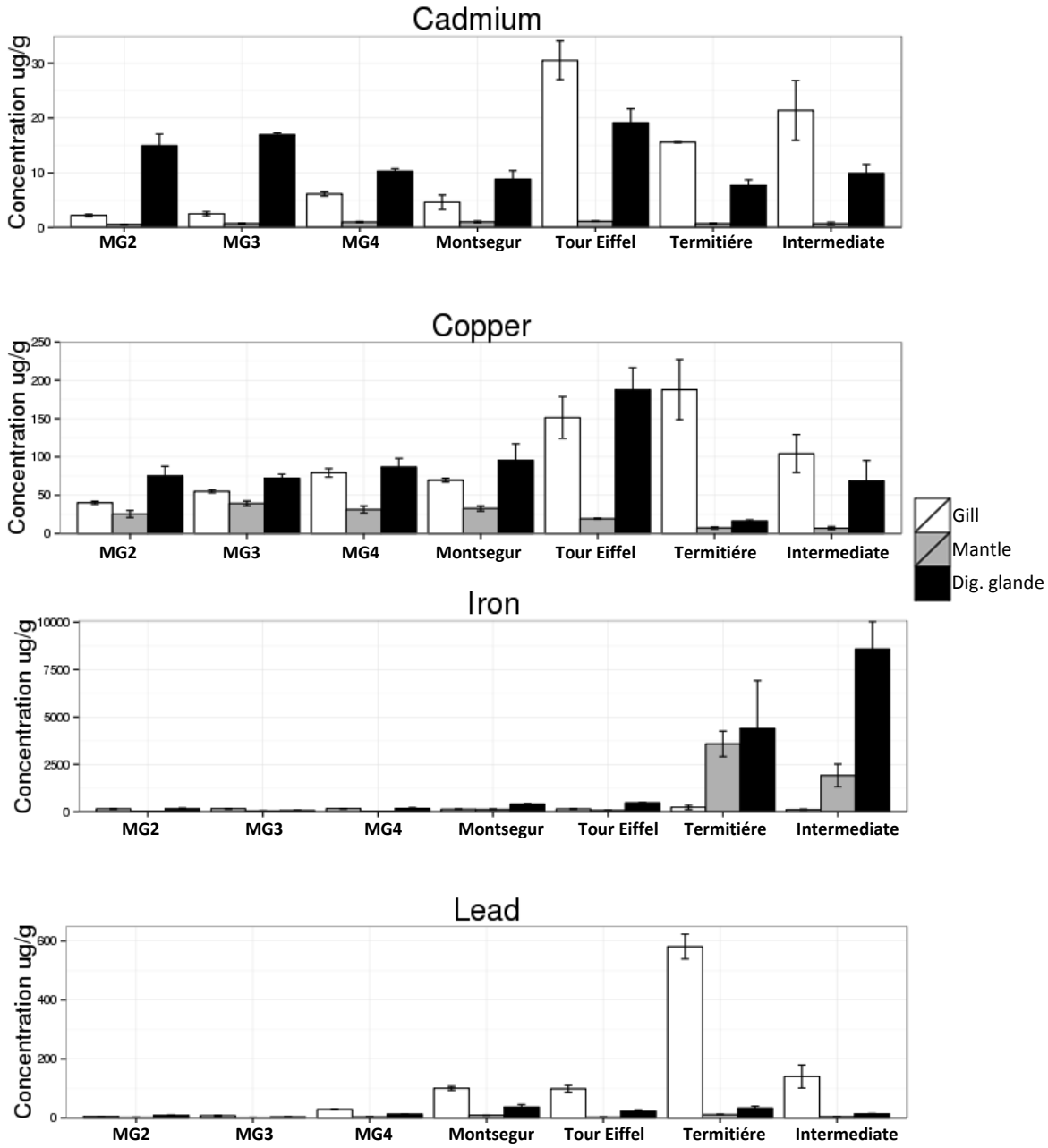


Fig 1. Average concentration and standard deviation of each metal analyzed in the three tissues gill, mantle and digestivegland in the seven populations.

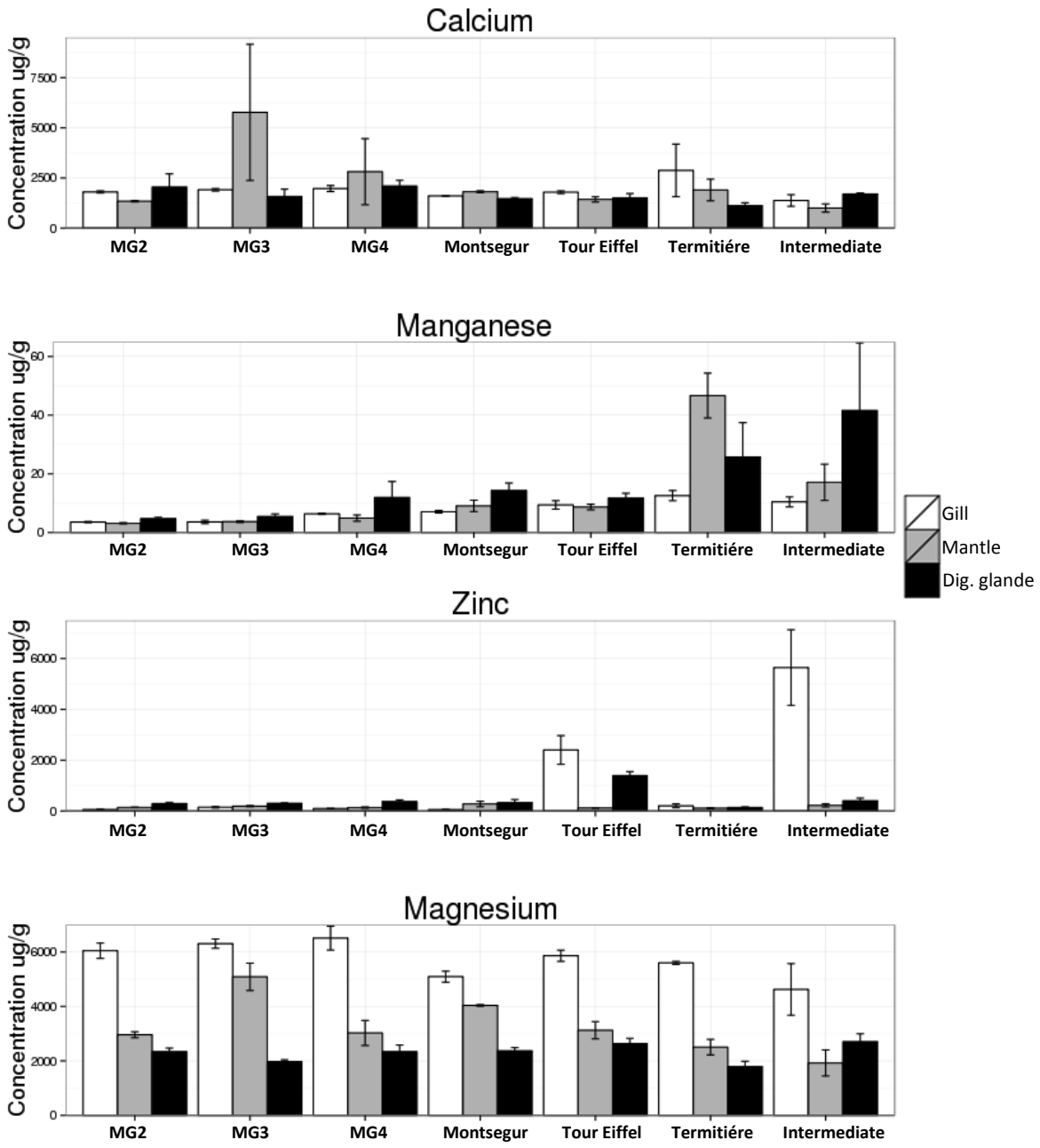


Fig 1. Continued.

Table 2. Analysis of variance (Anova) between populations for each metal measured in the three tissues.

Metal	Tissue	Df	MS	F	p-value
Cadmium	Gill	6	359.1	18.81	5.85e-06
	Mantle	6	0.13	2.18	0.107
	Digestive gland	6	355.4	7.98	7.00e-04
Copper	Gill	6	8630	6.82	0.0015
	Mantle	6	474.3	15.61	1.78e-05
	Digestive gland	6	7919	7.97	0.0007
Iron	Gill	6	4846	0.85	0.55
	Mantle	6	5850	16.92	1.10e-05
	Digestive gland	6	3214	8.87	0.0004
Lead	Gill	6	1229	82.33	4.14e-10
	Mantle	6	47.74	101.2	1.02e-10
	Digestive gland	6	469.3	7.65	8.60e-04
Calcium	Gill	6	6728	0.857	0.5
	Mantle	6	8028	1.28	0.32
	Digestive gland	6	3490	1.14	0.38
Manganese	Gill	6	34.08	9.52	0.00028
	Mantle	6	716.5	16.34	1.36e-05
	Digestive gland	6	511.3	1.68	0.19
Zinc	Gill	6	1356	12.47	6.48e-05
	Mantle	6	10652	1.47	0.25
	Digestive gland	6	52762	21.86	2.34e-06
Magnesium	Gill	6	13508	2.45	0.078
	Mantle	6	32276	8.5	5.10e-04
	Digestive gland	6	32278	2.96	0.043

Table 3. Correlation analysis (r^2) between metals in gill.

	Manganese	Copper	Cadmium	Lead	Magnesium	Calcium	Iron	Zinc
Manganese	-	0.91	0.74	0.81	-0.56	0.37	0.37	0.47
Copper		-	0.76	0.83	-0.23	0.60	0.60	0.25
Cadmium			-	0.33	-0.37	-0.02	-0.02	0.69
Lead				-	-0.30	0.79	0.78	-0.01
Magnesium					-	0.35	0.35	-0.66
Calcium						-	1.00	-0.50
Iron							-	-0.50
Zinc								-

Table 4. Correlation analysis (r^2) between metals in mantle.

	Manganese	Copper	Cadmium	Lead	Magnesium	Calcium	Iron	Zinc
Manganese	-	0.92	0.73	0.82	-0.54	0.42	0.42	0.45
Copper		-	0.76	0.83	-0.23	0.62	0.62	0.25
Cadmium			-	0.32	-0.34	0.01	0.01	0.69
Lead				-	-0.30	0.81	0.81	-0.02
Magnesium					-	0.31	0.31	-0.62
Calcium						-	1.00	-0.47
Iron							-	-0.47
Zinc								-

Table 5. Correlation analysis (r^2) between metals in digestive gland.

	Manganese	Copper	Cadmium	Lead	Magnesium	Calcium	Iron	Zinc
Manganese	-	0.92	0.73	0.82	-0.54	0.43	0.43	0.45
Copper		-	0.76	0.83	-0.23	0.62	0.62	0.26
Cadmium			-	0.33	-0.34	0.02	0.01	0.69
Lead				-	-0.31	0.81	0.81	-0.02
Magnesium					-	0.30	0.30	-0.61
Calcium						-	1.00	-0.47
Iron							-	-0.47
Zinc								-

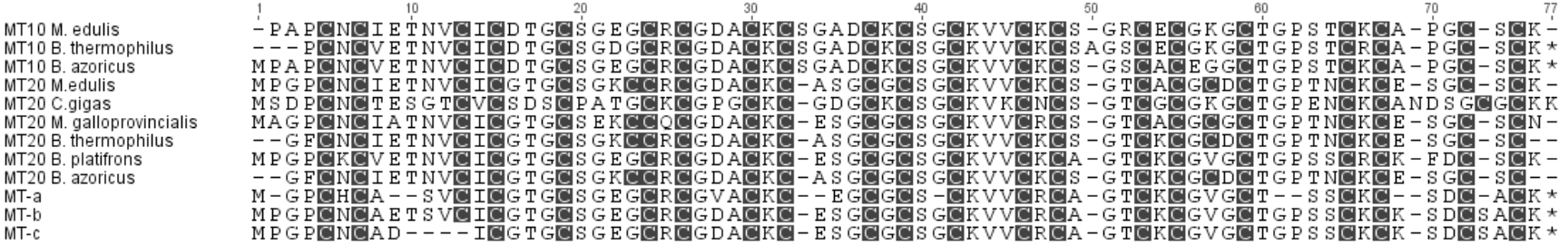


Fig.2 Alignment of MTs sequences. The cysteine motifs are highlighted in grey. Accession number *M. edulis* (MT10: AAB29061.1; MT20: P80252.2), *B. thermophilus*(MT10: CAE11860.1; MT20: Hardivillier et al 2004), *B. azoricus* (MT10: CAF34421.1; MT20: CAF34424.1), *C. gigas* (CAB64869.1), *M. galloprovincialis* (AAT72935.1) and *B. platifrons* (from Wong et al 2016).

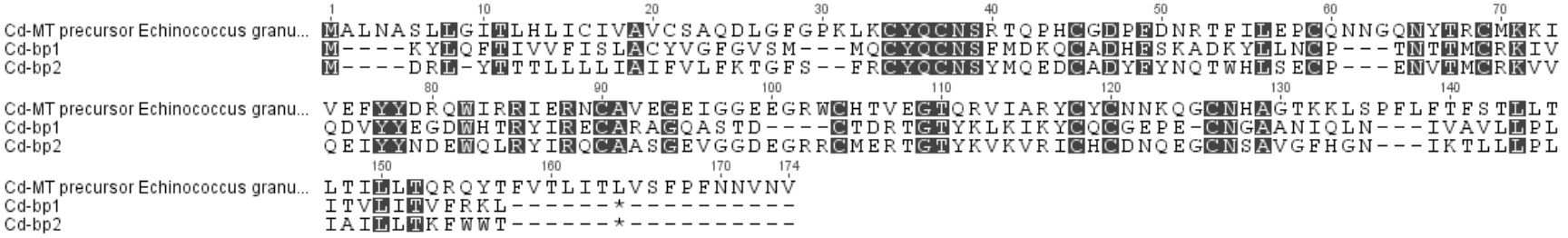
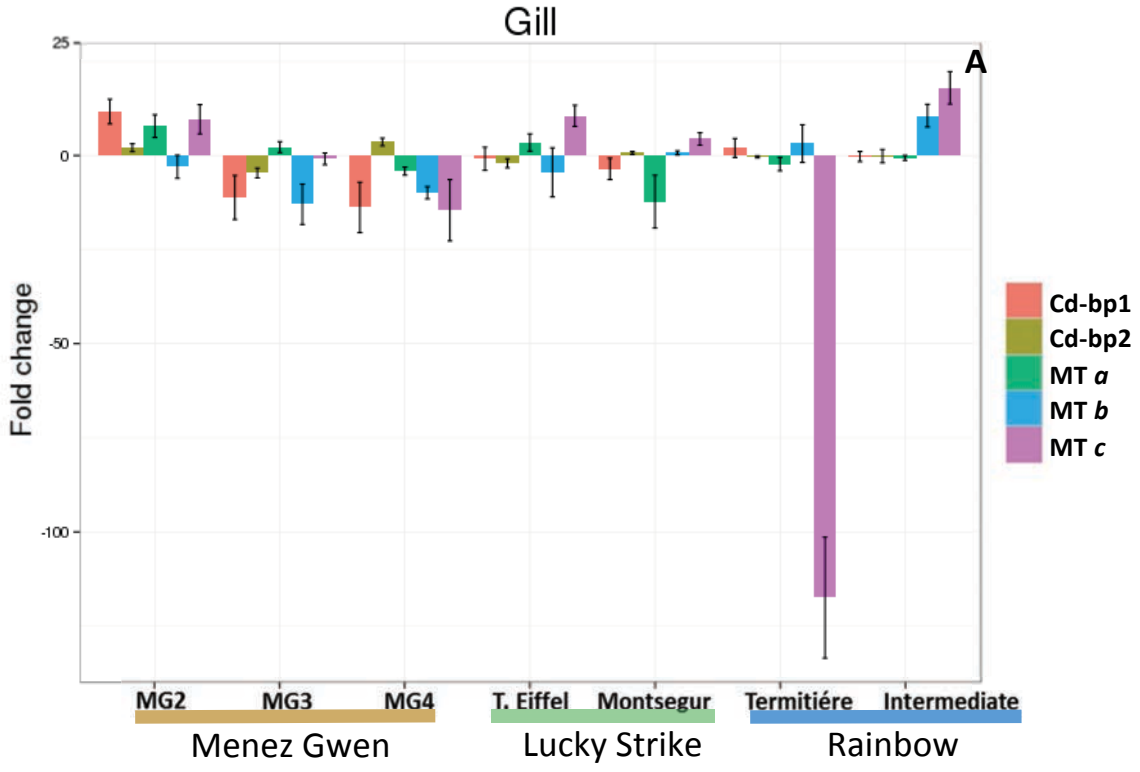
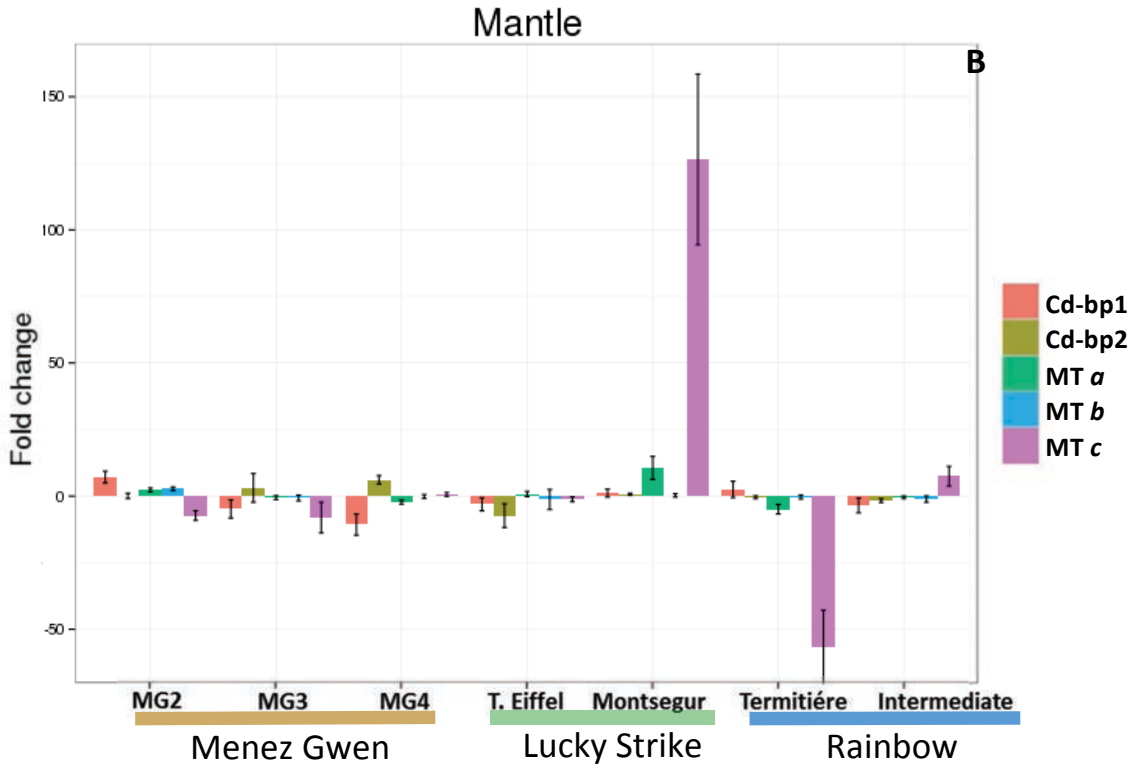


Fig.3 Alignment of the two putative *B. azoricus* cadmium binding protein sequences (*E. granulosus* accession number: EUB55305.1).Conserved amino acids are highlighted in grey.

Fig.4 Alignment of the PCS sequences. Conserved amino acids are highlighted in grey. Accession numbers: *A. thaliana* (Q9ZWB7), *O. bimaculoides* (XP_014771294), *H. vulgaris* (XP_002156317), *E. fetida* (ABR13683), *A. californica* (XP_005110845.1) and *C. gigas* (EKC27807.1).


Gill
A
25
0
-50
-100
Fold change
Cd-bp1
Cd-bp2
MT a
MT b
MT c
MG2
MG3
MG4
T. Eiffel
Montsegur
Termitiére
Intermediate
Menez Gwen
Lucky Strike
Rainbow


Mantle
B
150
100
50
0
-50
Fold change
Cd-bp1
Cd-bp2
MT a
MT b
MT c
MG2
MG3
MG4
T. Eiffel
Montsegur
Termitiére
Intermediate
Menez Gwen
Lucky Strike
Rainbow

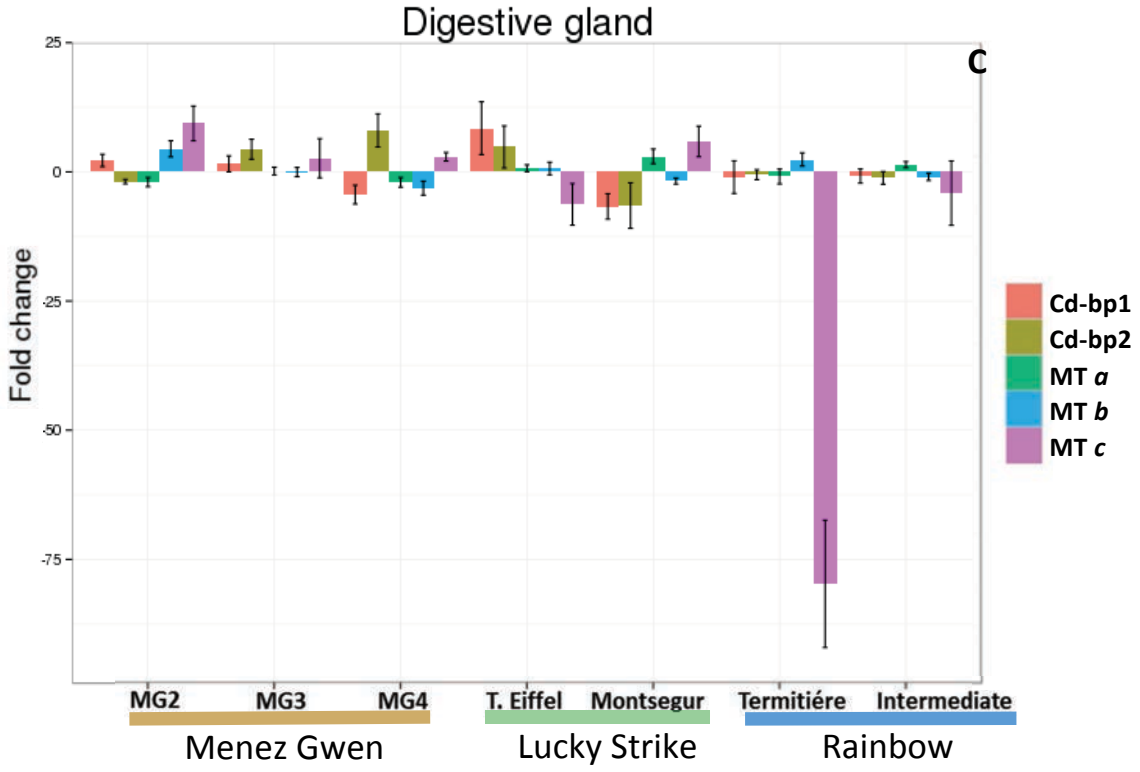


Fig 5. Relative expression (fold change) of MTs and putative cadmium binding proteins genes between the 7 populations in A) gill, B) mantle and C) digestive gland. Gene expression is normalised by the mean deltaCt value of genes in all samples for each tissue.

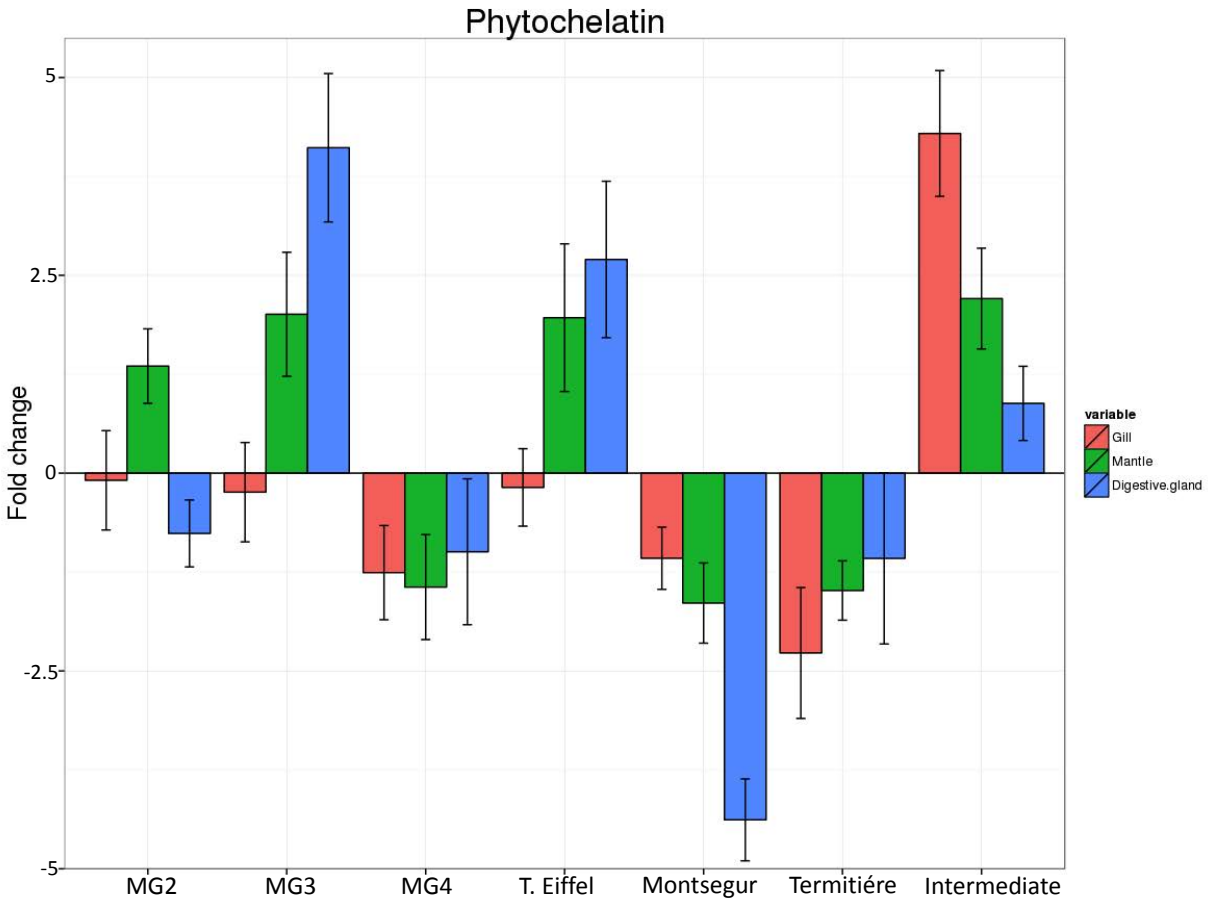


Fig 6. Relative expression (fold change) of PCS gene between the 7 populations in gill, mantle and digestive gland. Gene expression is normalised by the mean deltaCt value of genes in all samples for each tissue.

Table 6. Results of the Global ANOVAanalysis between populations for MTs, putative cadmium

binding proteins and PCS relative expression in the seven populations for each tissue.

Tissue	Response	Df	MS	F	p-value
Gill	Cd-bp1	6	2171.5	4.51	0.00026
	Cd-bp2	6	216.8	5.89	1.08e-05
	MT-*a*	6	1286.3	3.89	0.00106
	MT-*b*	6	1903.0	3.79	0.00132
	MT-*c*	6	6714.9	42.96	2.00e-16
	PCS	6	131.71	10.73	2.28e-10
Mantle	Cd-bp1	6	967.9	4.19	0.00055
	Cd-bp2	6	502.8	2.28	0.0371
	MT-*a*	6	728.9	6.37	3.78e-06
	MT-*b*	6	54.0	0.67	0.676
	MT-*c*	6	9654.4	13.16	4.84e-15
	PCS	6	96.95	7.132	6.96e-7
Dig. gland	Cd-bp1	6	723.0	3.20	0.005
	Cd-bp2	6	720.4	3.10	0.0063
	MT-*a*	6	99.0	3.21	0.0049
	MT-*b*	6	193.7	5.03	8.02e-05
	MT-*c*	6	2921.5	28.87	2.00e-16
	PCS	6	228.38	11.01	1.53e-10

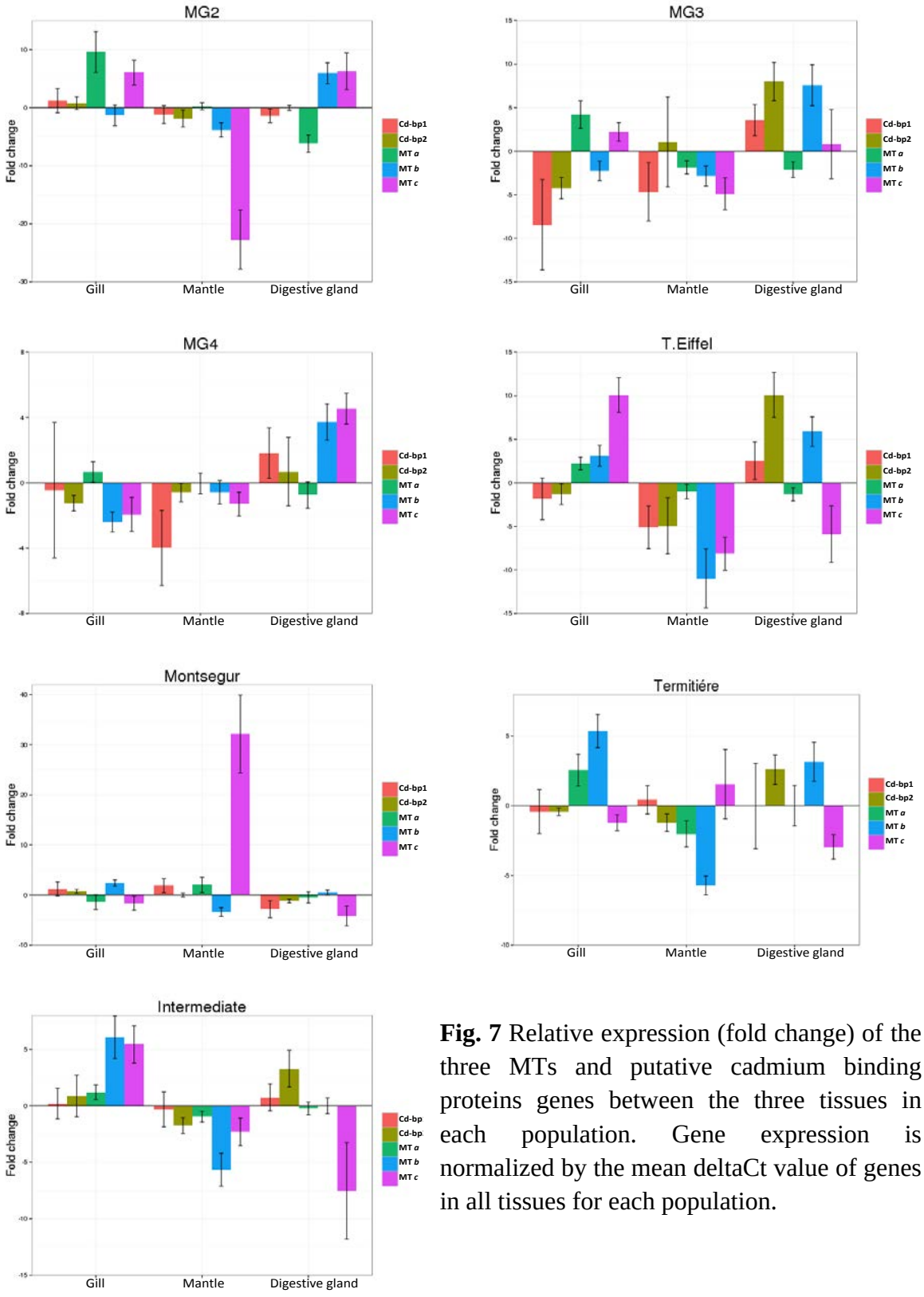


Fig. 7 Relative expression (fold change) of the three MTs and putative cadmium binding proteins genes between the three tissues in each population. Gene expression is normalized by the mean deltaCt value of genes in all tissues for each population.

Table 7. Results of the Global Anova analysis conducted on the relative expression of MTs, putative cadmium binding proteins and PCS between the three tissues for each population.

Population	Response	Df	MS	F	p-value
MG-2	Cd-bp1	2	65.42	0.788	0.458
	Cd-bp2	2	56.1	1.536	0.221
	MT-*a*	2	1898.3	12.03	2.43e-05
	MT-*b*	2	753.3	9.427	0.000197
	MT-*c*	2	8337	20.79	4.18e-08
	PCS	2	12.30	1 087	0.341
MG-3	Cd-bp1	2	1109	2.7	0.0729
	Cd-bp2	2	1107.7	3.64	0.0305
	MT-*a*	2	374.6	9.581	0.000179
	MT-*b*	2	992.8	12.7	1.52e-05
	MT-*c*	2	406.6	2.097	0.129
	PCS	2	192.29	8 302	0.000501
MG-4	Cd-bp1	2	240.3	0.995	0.374
	Cd-bp2	2	27.46	0.598	0.552
	MT-*a*	2	14.14	1.02	0.365
	MT-*b*	2	280.21	14.17	5.17e-06
	MT-*c*	2	359.3	15.28	2.29e-06
	PCS	2	52.87	5.073	0.00832
Tour Eiffel	Cd-bp1	2	453.1	2.755	0.0691
	Cd-bp2	2	1888.9	10.03	0.000119
	MT-*a*	2	116.74	6.496	0.00234
	MT-*b*	2	2473.9	15.71	1.47e-06
	MT-*c*	2	2976.6	15.99	1.19e-06
	PCS	2	249.92	13.38	8.21e-6
Montsegur	Cd-bp1	2	190.59	2.691	0.0733
	Cd-bp2	2	27.69	6.273	0.00283
	MT-*a*	2	96.56	1.613	0.205
	MT-*b*	2	270.93	18.79	1.55e-07
	MT-*c*	2	126.12	18.33	2.16e-07
	PCS	2	47.39	11.53	3.23e-5
Termitiére	Cd-bp1	2	5.45	0.042	0.959
	Cd-bp2	2	120.88	7.646	0.00087
	MT-*a*	2	158.07	3.648	0.0301
	MT-*b*	2	1032.1	26.52	1.02e-09
	MT-*c*	2	156.05	2.143	1.23e-01
	PCS	2	83.82	8.11	0.00057
Intermediate	Cd-bp1	2	6.84	0.138	0.871
	Cd-bp2	2	152.86	2.437	0.0943
	MT-*a*	2	32.99	3.766	0.0276
	MT-*b*	2	921.5	15.17	2.94e-06
	MT-*c*	2	1154.9	6.504	2.48e-03
	PCS	2	89.94	8 753	0.000329

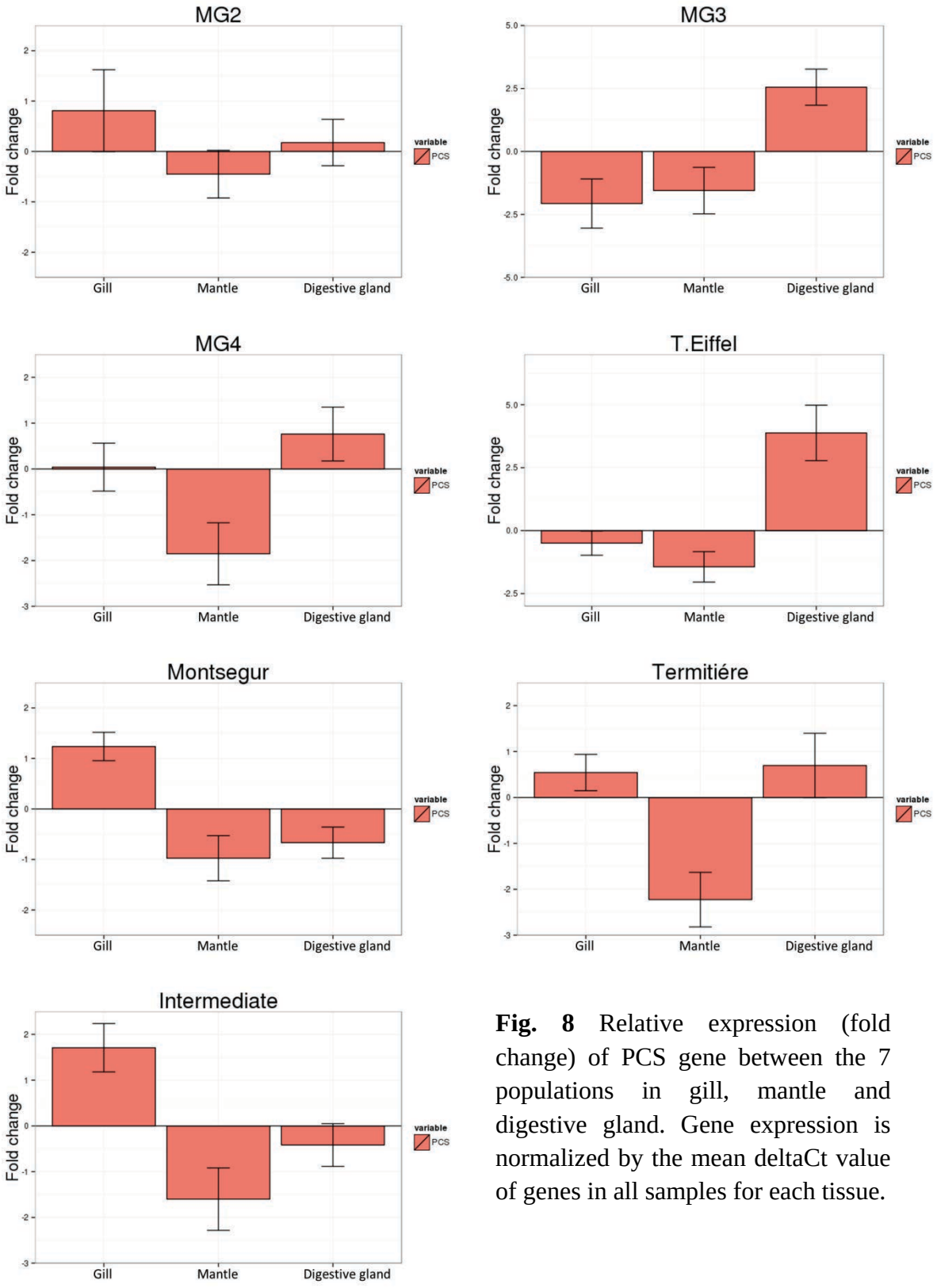
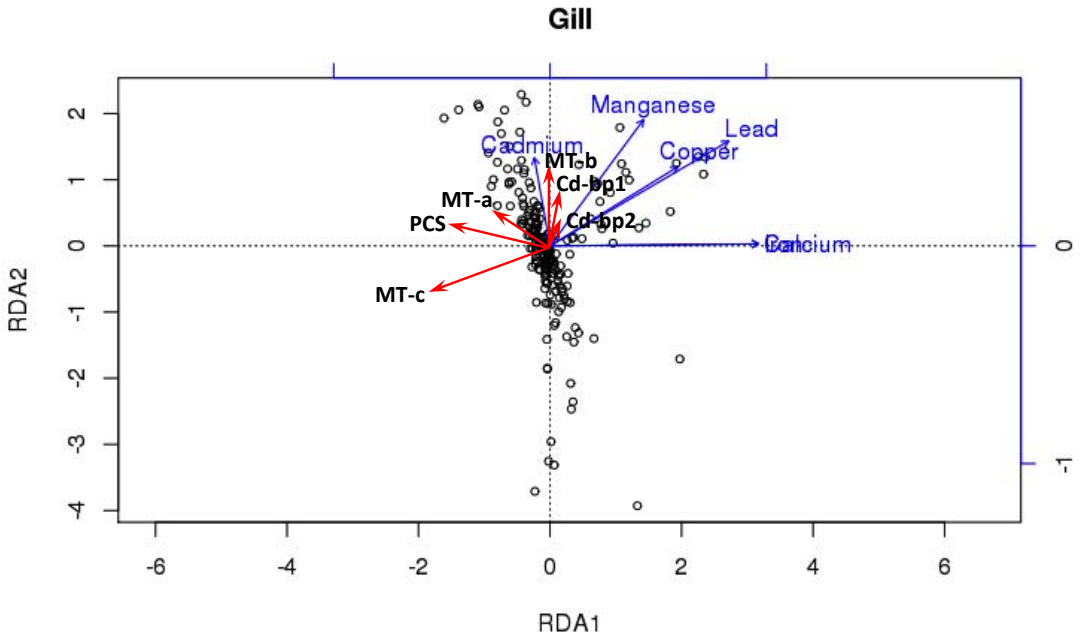


Fig. 8 Relative expression (fold change) of PCS gene between the 7 populations in gill, mantle and digestive gland. Gene expression is normalized by the mean deltaCt value of genes in all samples for each tissue.


Gill
Manganese
Lead
Cadmium
Copper
MT-b
Cd-bp1
MT-a
PCS
Cd-bp2
Calcium
MT-c
RDA2
RDA1
-6
-4
-2
0
2
4
6
-4
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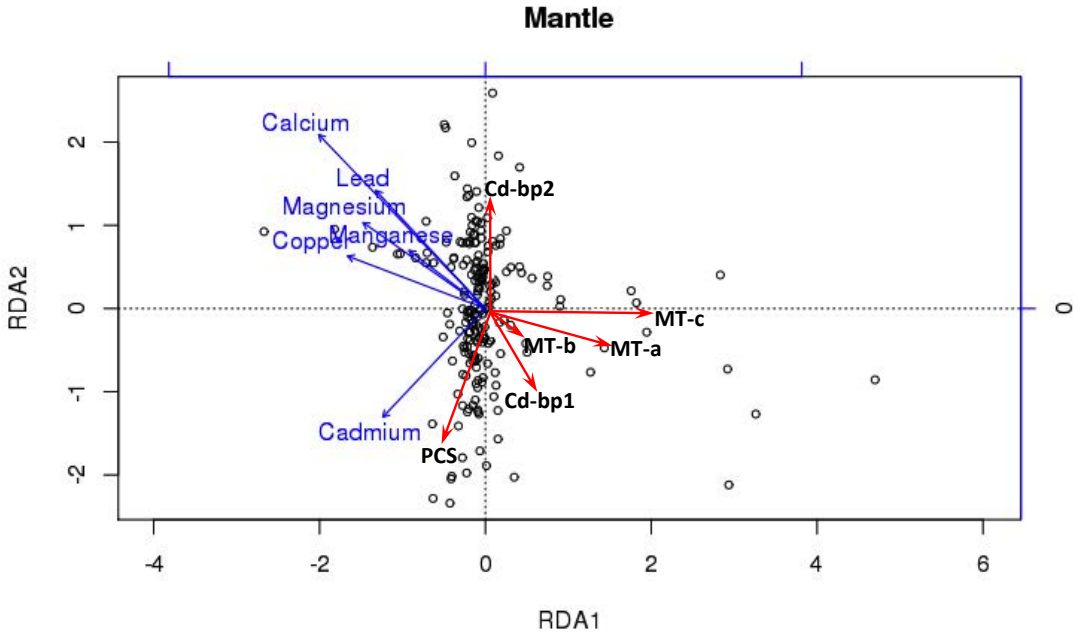
-2
-1
0
1
2


Mantle
Calcium
Lead
Magnesium
Copper
Manganese
Cd-bp2
MT-c
MT-b
MT-a
Cd-bp1
Cadmium
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4
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-1
0
1
2

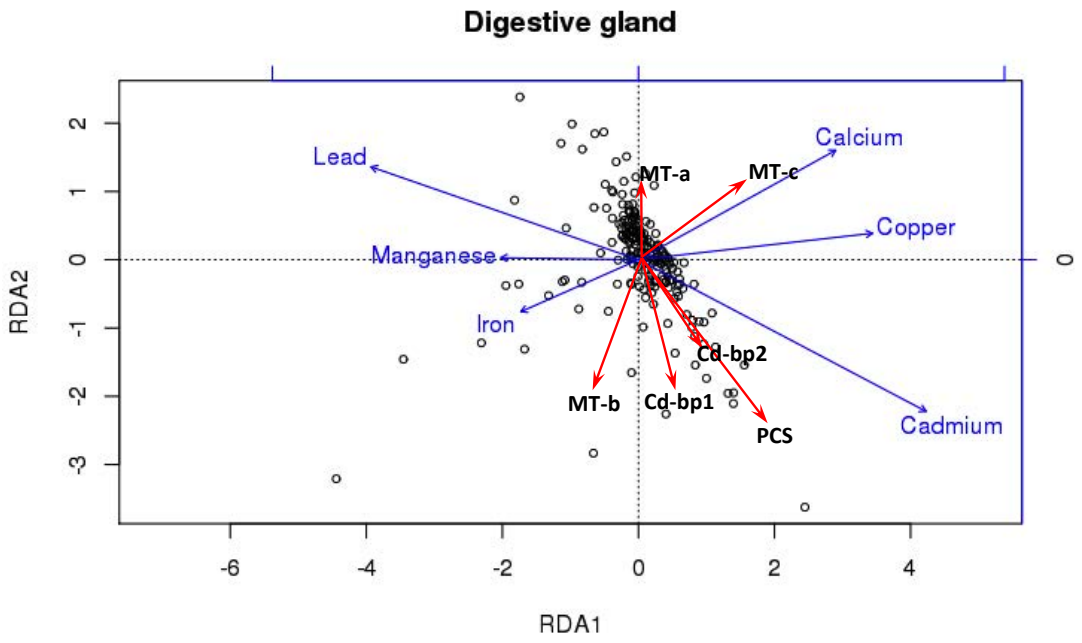


Fig 9. Global redundancy analysis (RDA) for metal concentration and relative gene expression in each tissue for the seven populations. The angle between arrows represents the degree of correlation between variables.

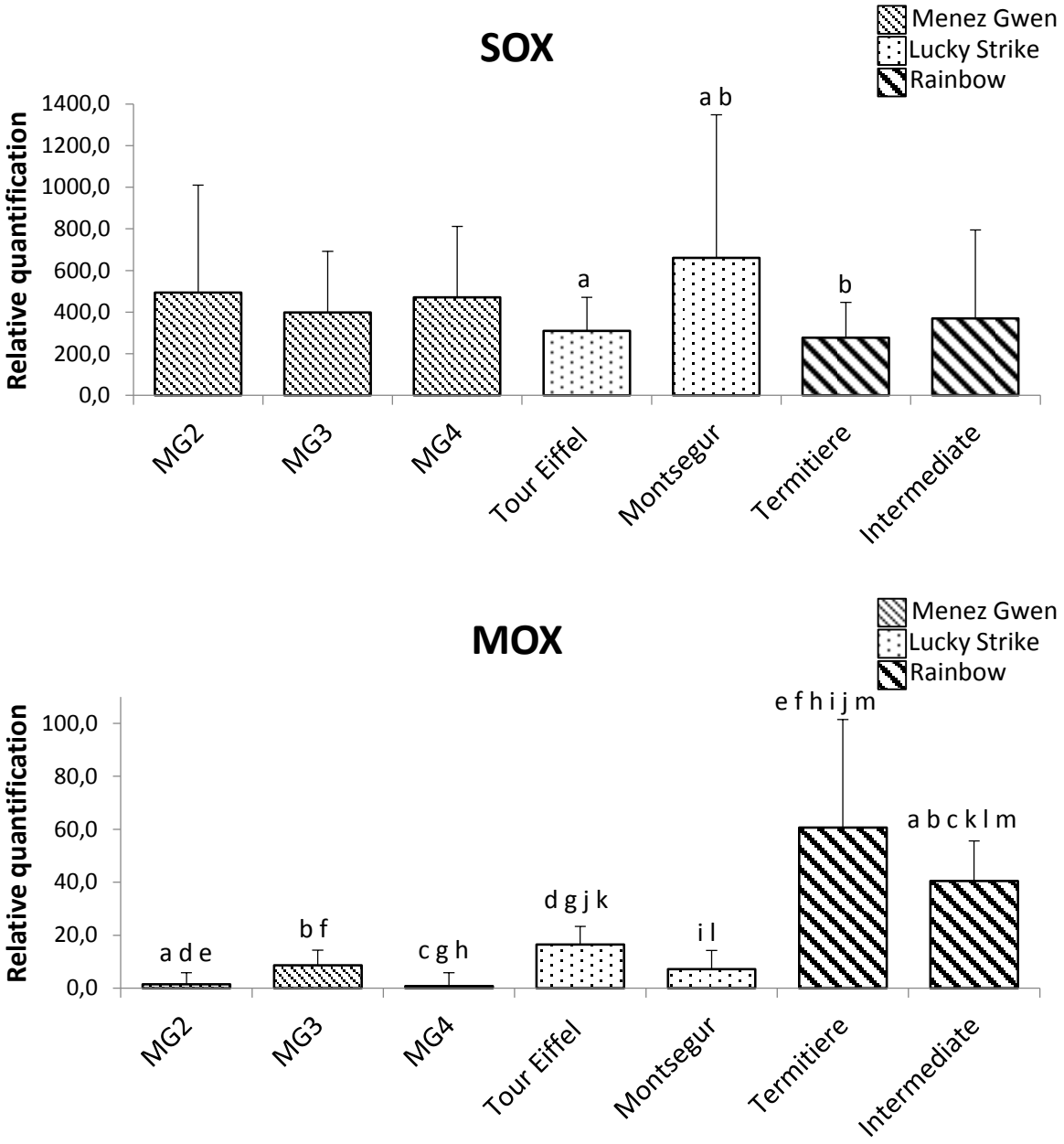


Fig 10 Thiotrophic (SOX) and methanotrophic (MOX) symbiont content in the seven populations studied. Significant differences are represented by letters. For SOX: **a**=Tour Eiffel/Montsegur and **b**=Montsegur/Termitiére. For MOX: **a**=MG2/Intermediate, **b**=MG3/Intermediate, **c**=MG4/Intermediate, **d**=MG2/Tour Eiffel, **e**=MG2/Termitiére, **f**=MG3/Termitiére, **g**=MG4/Tour Eiffel, **h**=MG4/Termitiére, **i**=Montsegur/Termitiére, **j**=Tour Eiffel/Termitiére), **k**=Tour Eiffel/Intermediate, **l**=Montsegur/Intermediate and **m**=Termitiére/Intermediate.

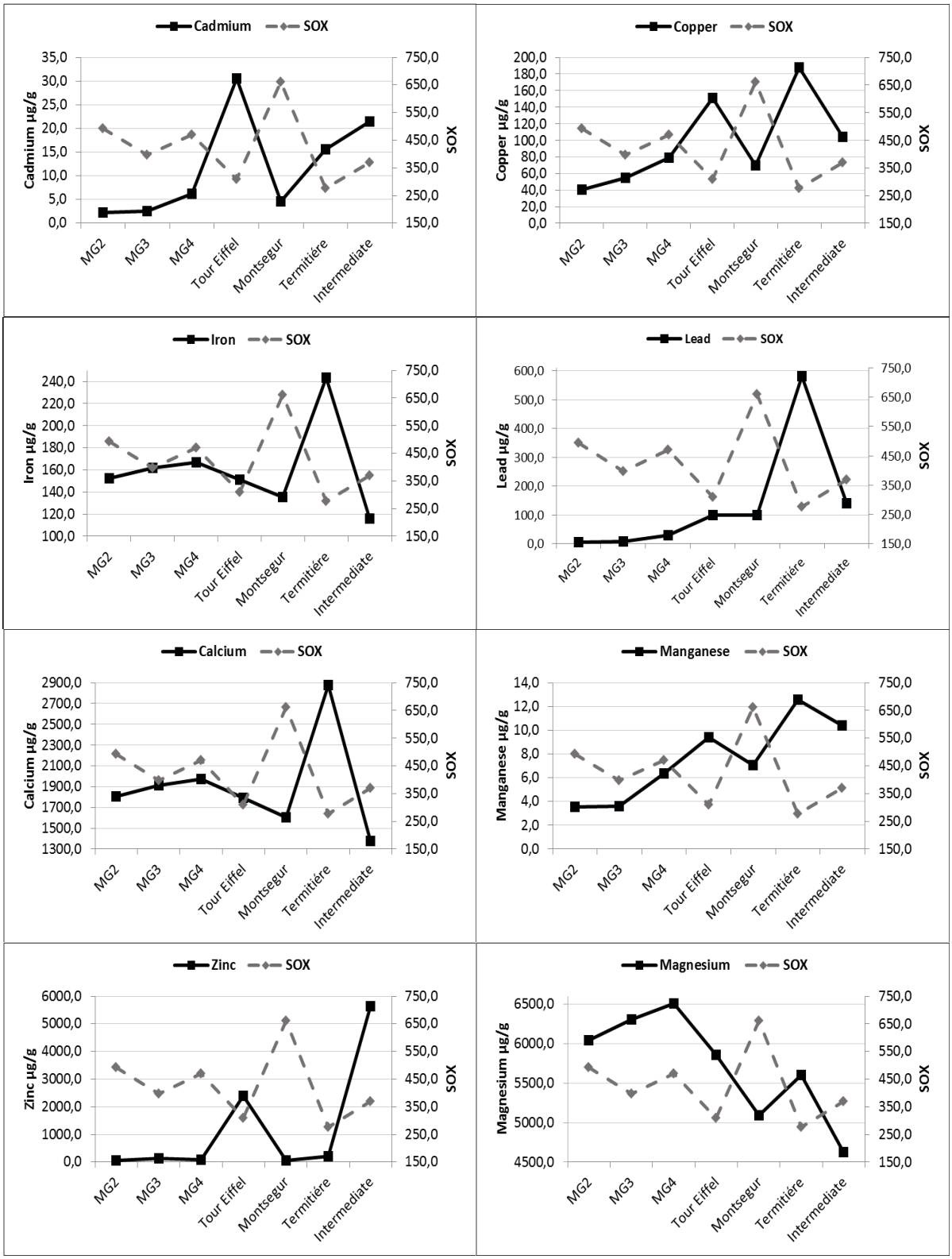


Fig 11. Mean concentration of thiotrophic (SOX) symbiont and mean concentration of heavy metal measured in gill for all the populations. Standard deviations is omitted for better interpretation.

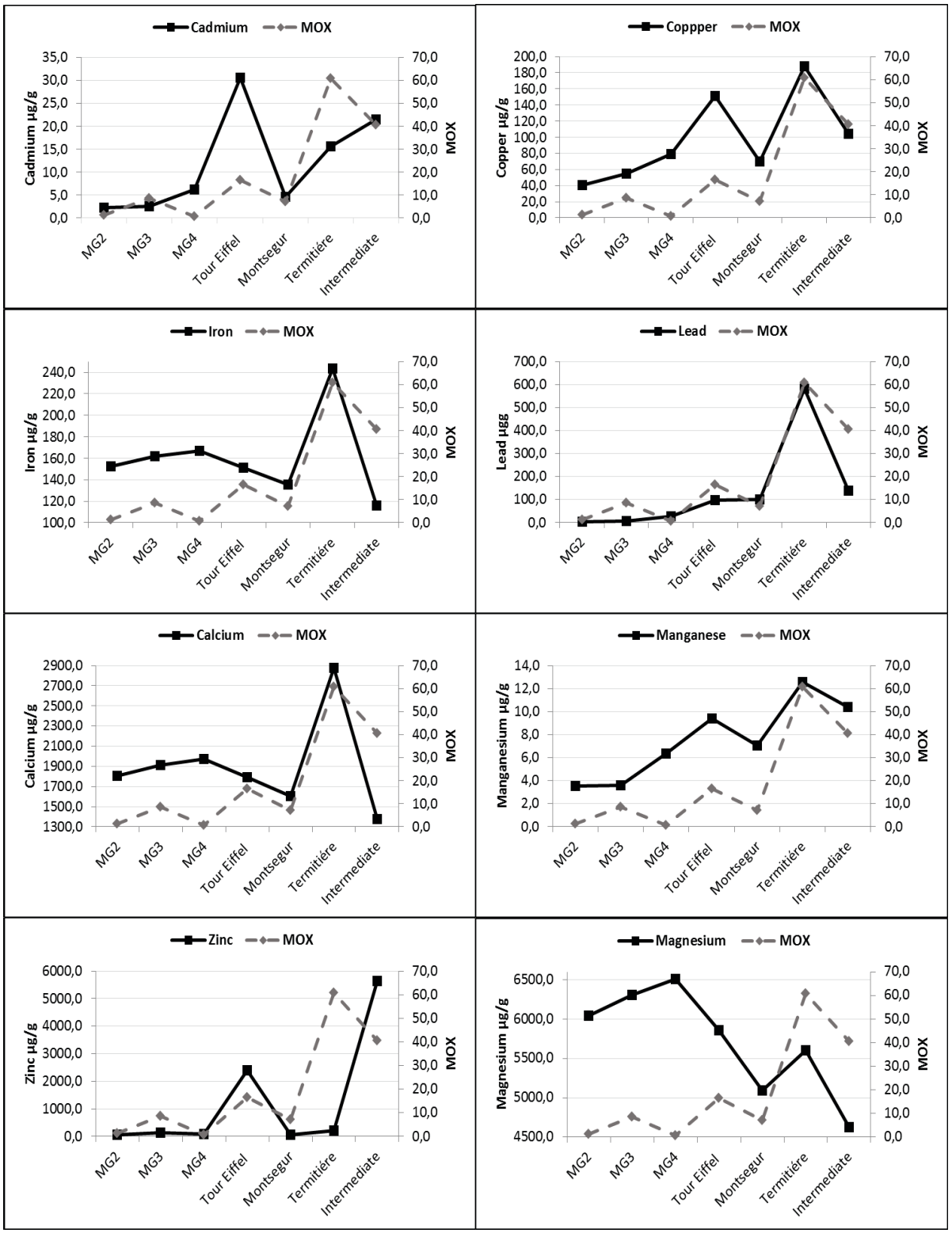
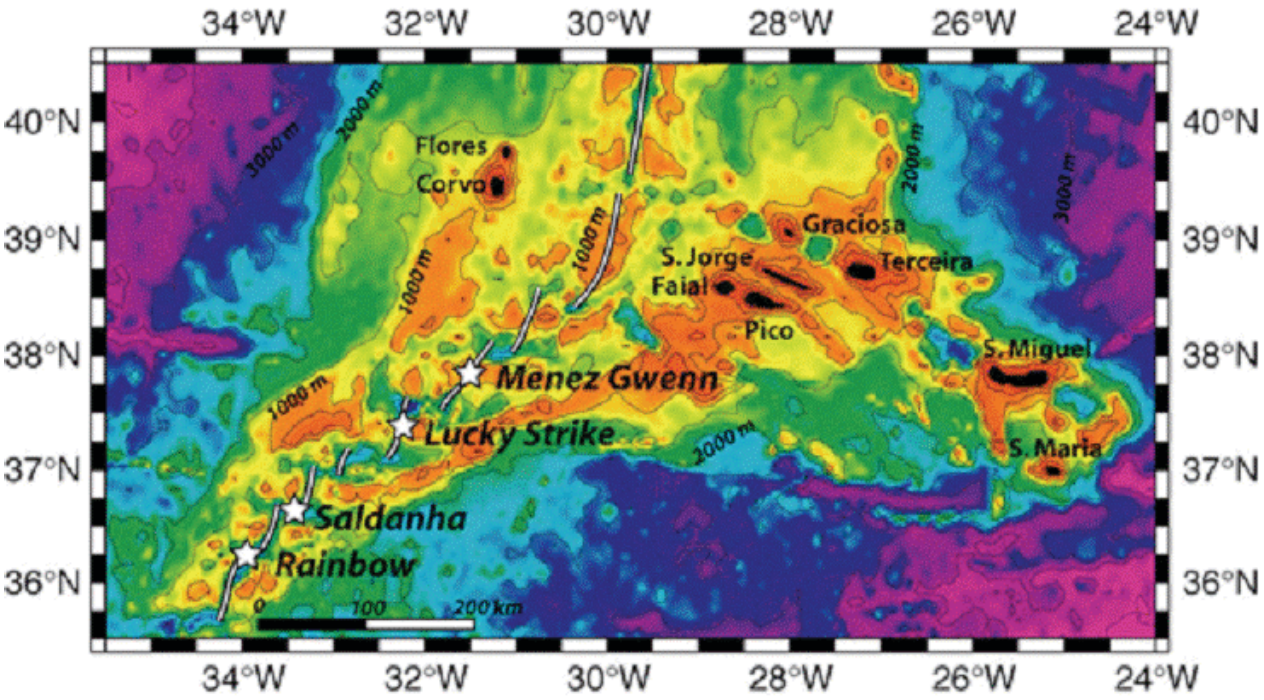


Fig 12. Mean concentration of methanotrophic (MOX) symbiont and mean concentration of heavy metal measured in gill for all the populations. Standard deviations is omitted for better interpretation

Appendices

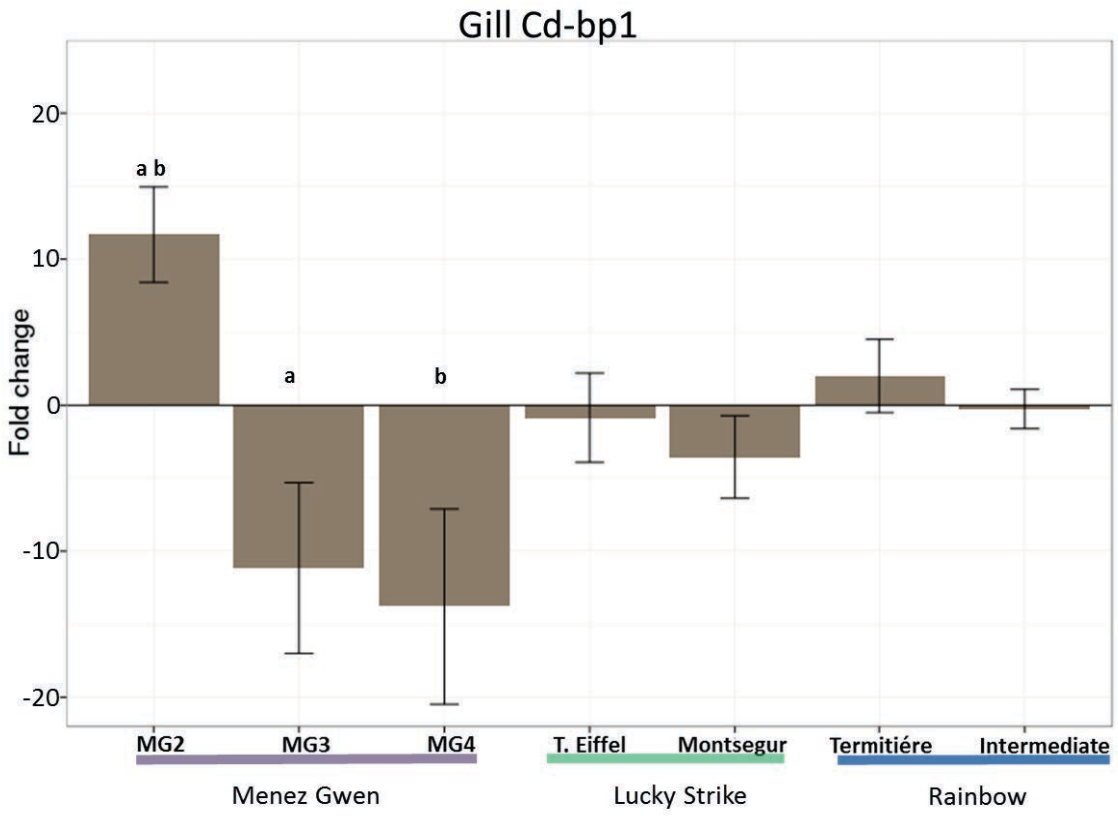


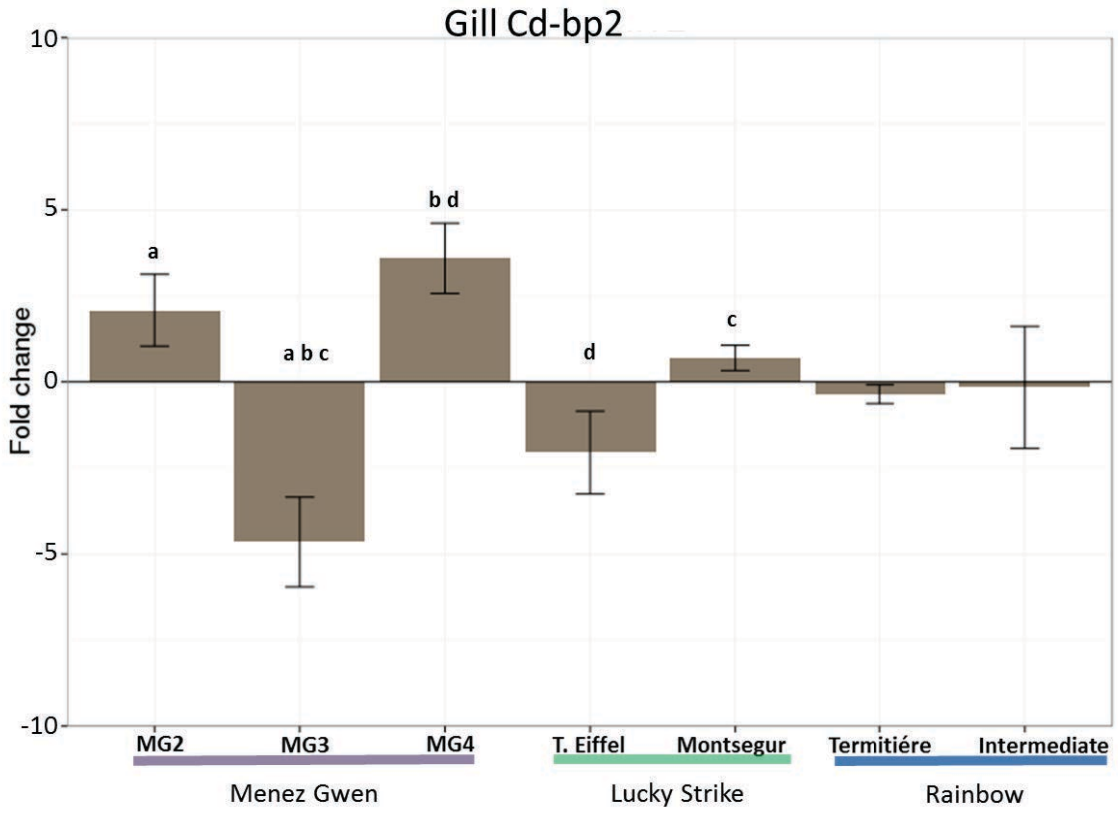
	MG	LS	RB
Depth (m)	850	1700	2300
Tem (° C)	265-284	152-333	360-365
pH	4.2-4.8	3.5-4.9	2.8-3.1
Fe (mg l^{-1})	**1.3-1.6**	**1.7-48**	**1339**
Mn (mg l^{-1})	3.2-3.7	4.2-24.7	123
Cu (mg l^{-1})	**40-180**	**60-1650**	**8900**
Zn (mg l^{-1})	0.16-0.33	0.33-3.79	10.5
Cd (mg l^{-1})	**1.01-1.34**	**2.02-8.85**	**14.6**
Pb (mg l^{-1})	4.4-11.6	7.2-26.9	30.6
H_2S (mM)	1.3-1.82	0.6-3.3	1-2.52
CH_4 (mM)	1.7	0.52	2.5

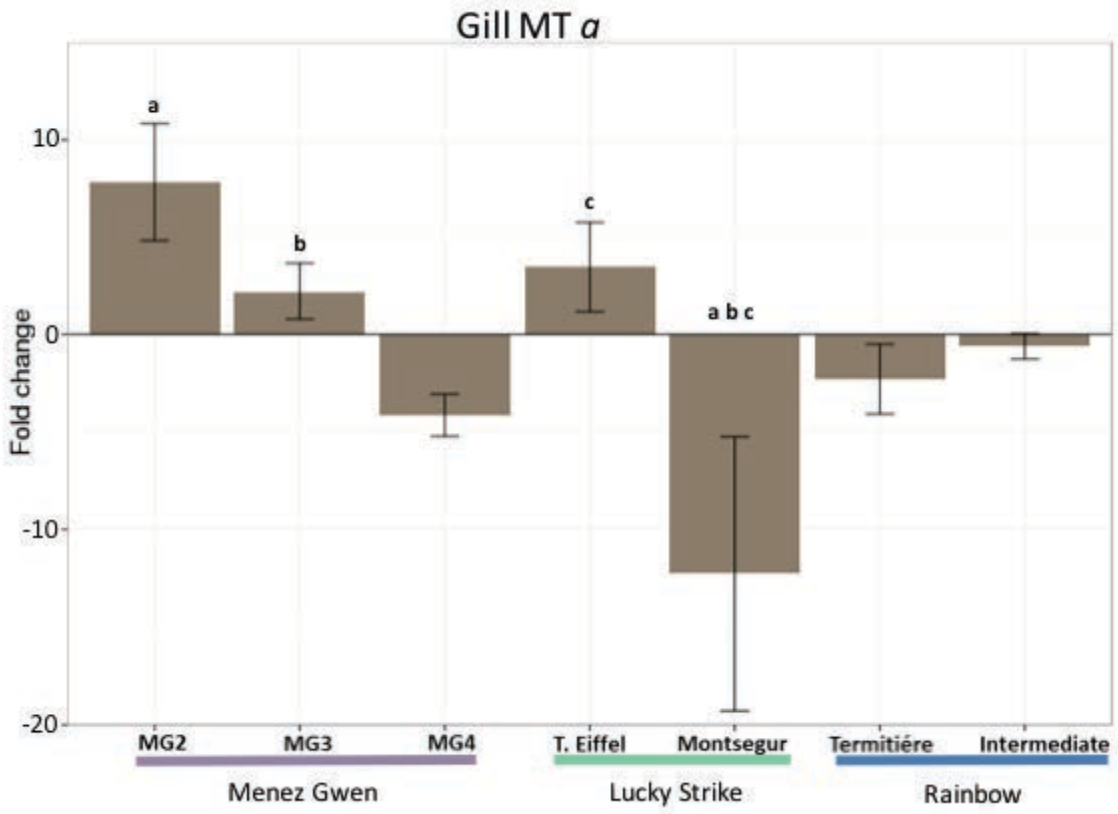
A. Sampling area and physico-chemical conditions at the three hydrothermal vent sites of the Mid-Atlantic Ridge : Menez Gwen (MG). Lucky Strike (LS) and Rainbow (RB). Adapted from Charlou et al 2002.

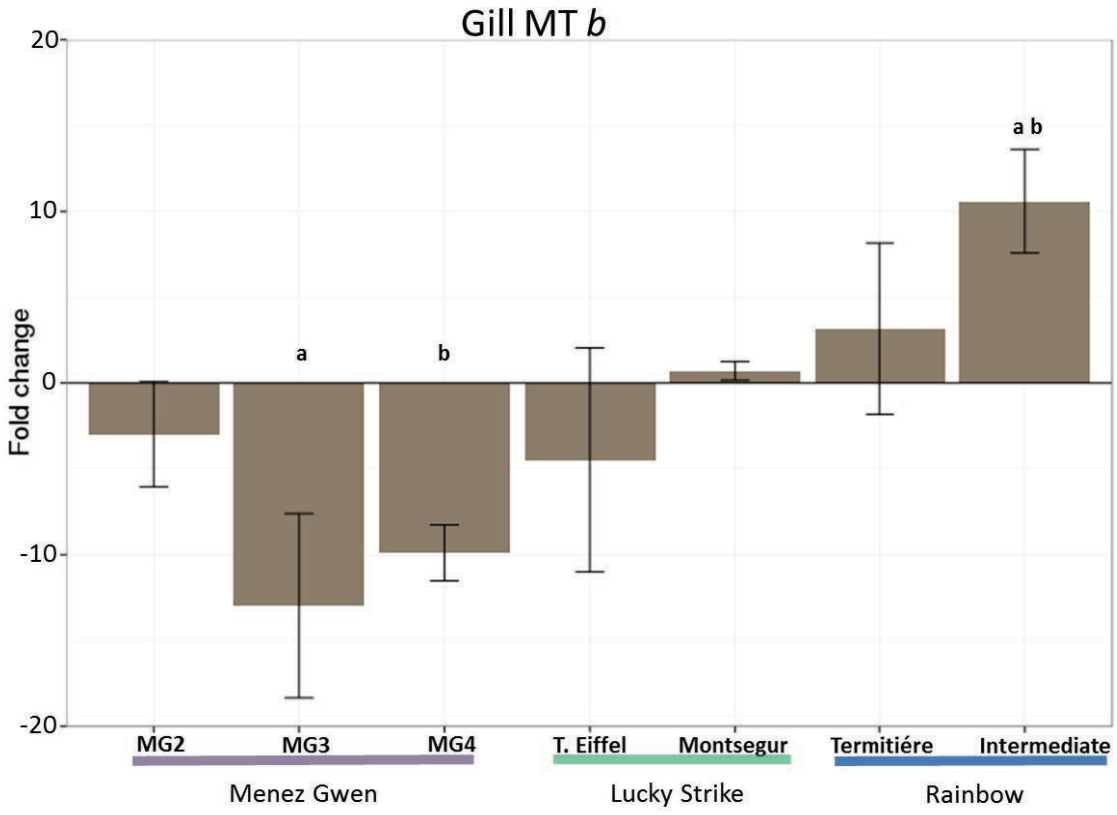
		Ca	Cd	Cu	Fe	Mg	Mn	Pb	Zn
DORM-4	measured	2070±90	0.42±0.06	25±4	310±30	690±40	3.0±0.4	0.38±0.07	68±7
	certified	2360±140	0.30±0.02	15.7±0.5	340±30	910±80	3.2±0.3	0.40±0.07	52±3
DOLT-5	measured	480±30	17±2	48±7	1010±60	710±50	9.2±1.0	0.35±0.13	130±17
	certified	550±80	14.5±0.6	35±3	1070±80	940±100	8.9±0.7	0.16±0.04	105±6
TORT-3	measured	2280±150	55±7	670±80	153±10	900±50	18±2	0.30±0.06	170±20
	certified		42±2	500±30	179±8		15.6±1.0	0.22±0.02	136±6
RM8414	measured	137±20	0.017±0.002	3.8±0.2	64±3	760±30	0.43±0.08	0.65±0.12	162±8
	certified	145±20	0.013±0.011	2.8±0.5	72±10	960±100	0.37±0.09	0.38±0.24	142±14

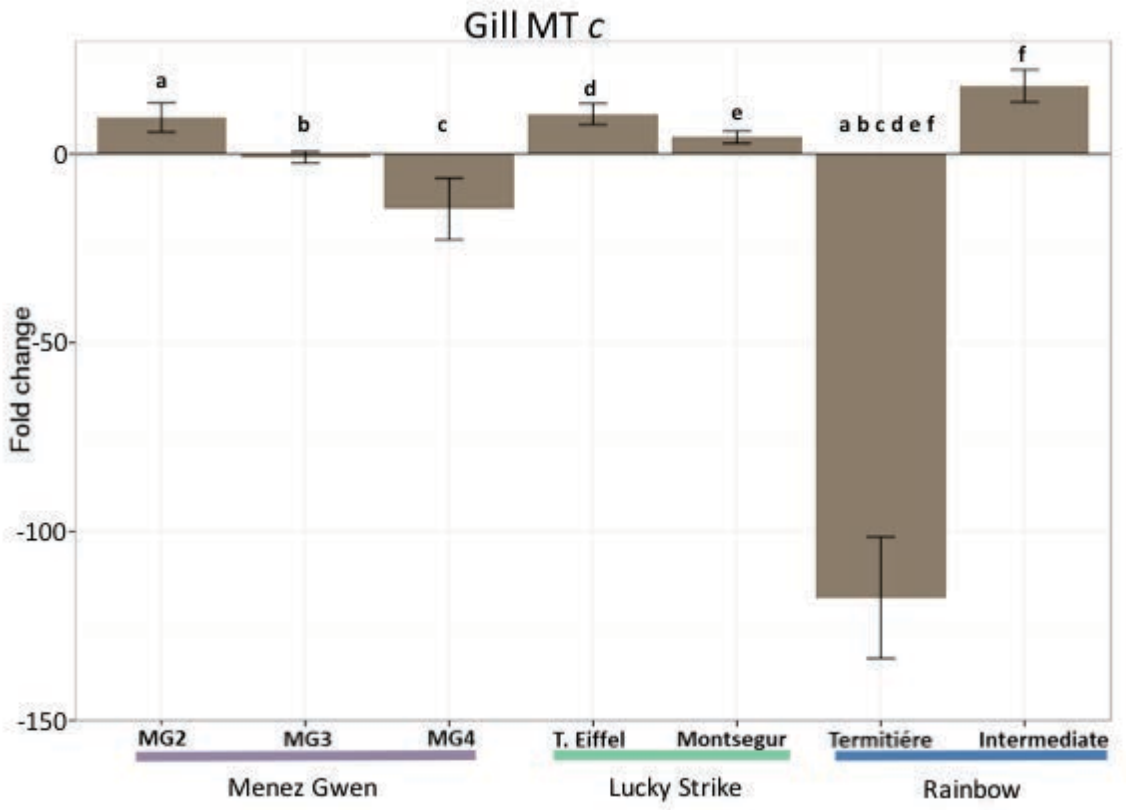
B. Determined elemental concentrations (in μg g^{-1}) as compared to certified values for several certified reference materials: DORM-4 (fish protein). DOLT-5 (dogfish liver) and TORT-3 (lobster hepatopancreas) and RM-8414 (bovine muscle).


Gill Cd-bp1
Fold change
20
10
0
-10
-20
a b
a
b
MG2
MG3
MG4
T. Eiffel
Montsegur
Termitiére
Intermediate
Menez Gwen
Lucky Strike
Rainbow

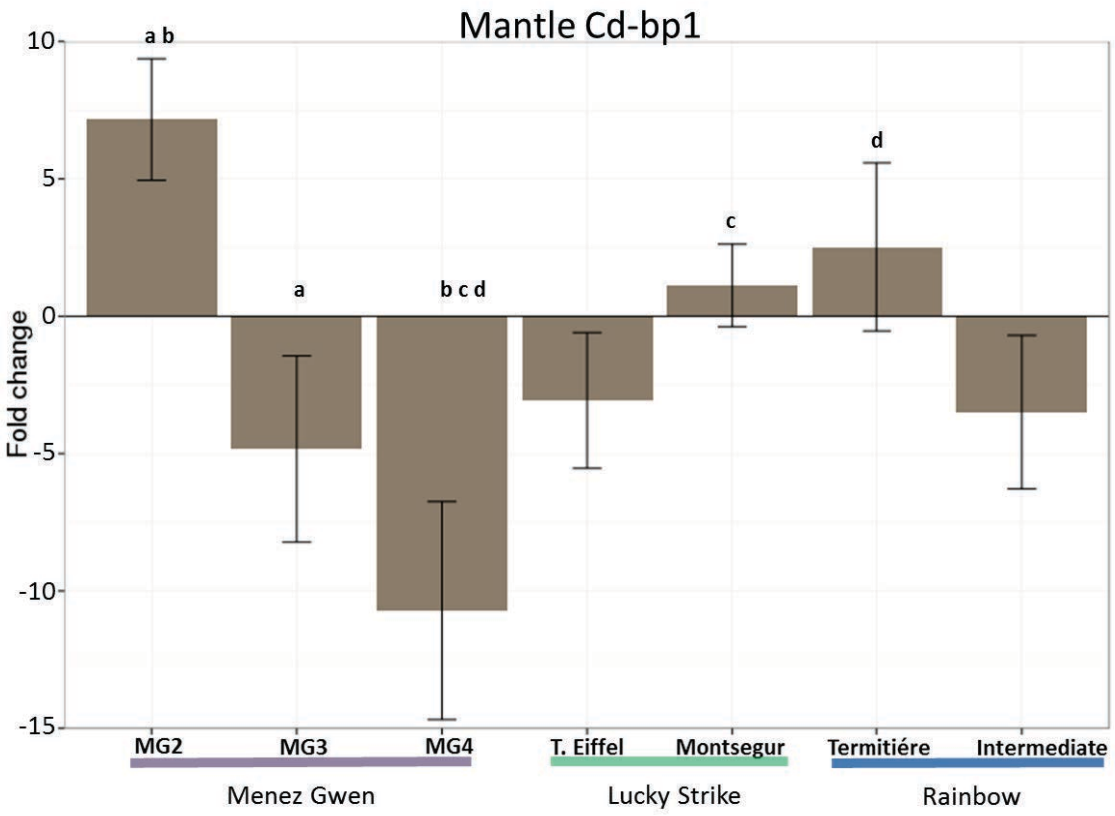

Gill Cd-bp2
Fold change
10
5
0
-5
-10
a
a b c
b d
d
c
MG2
MG3
MG4
T. Eiffel
Montsegur
Termitiére
Intermediate
Menez Gwen
Lucky Strike
Rainbow

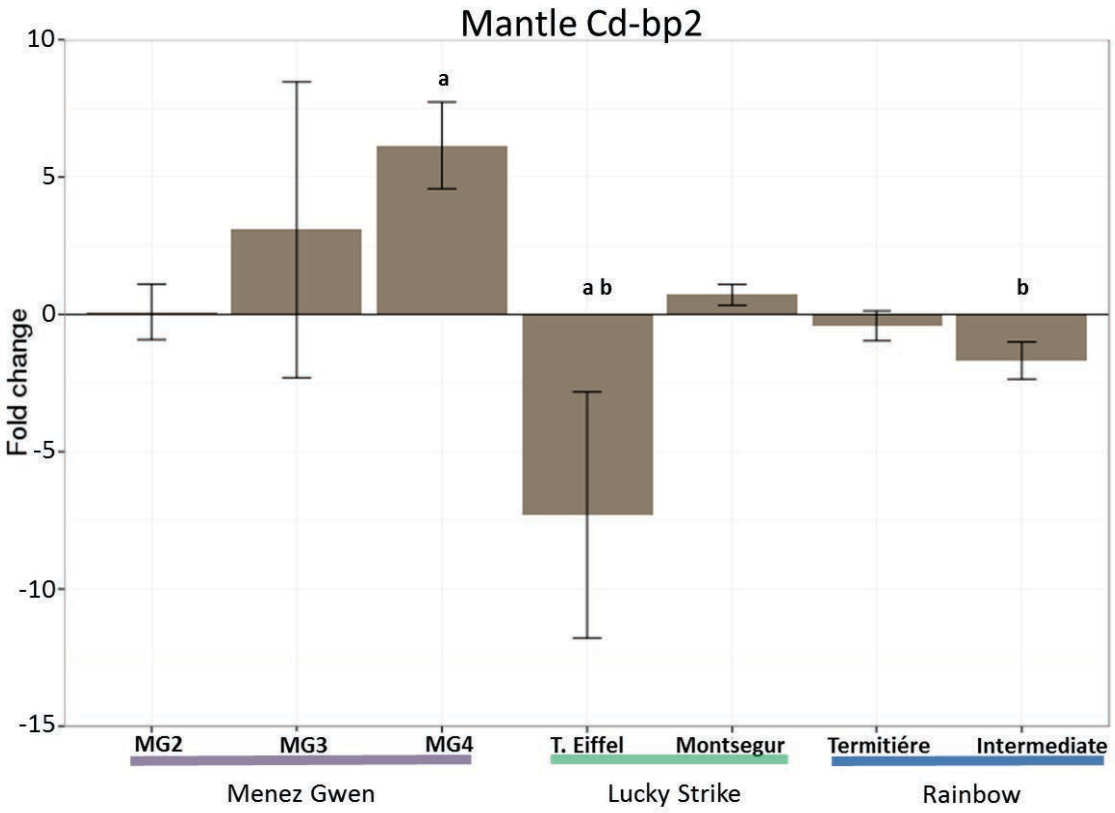

Gill MT *a*
Fold change
10
0
-10
-20
a
b
c
a b c
MG2
MG3
MG4
T. Eiffel
Montsegur
Termitiére
Intermediate
Menez Gwen
Lucky Strike
Rainbow

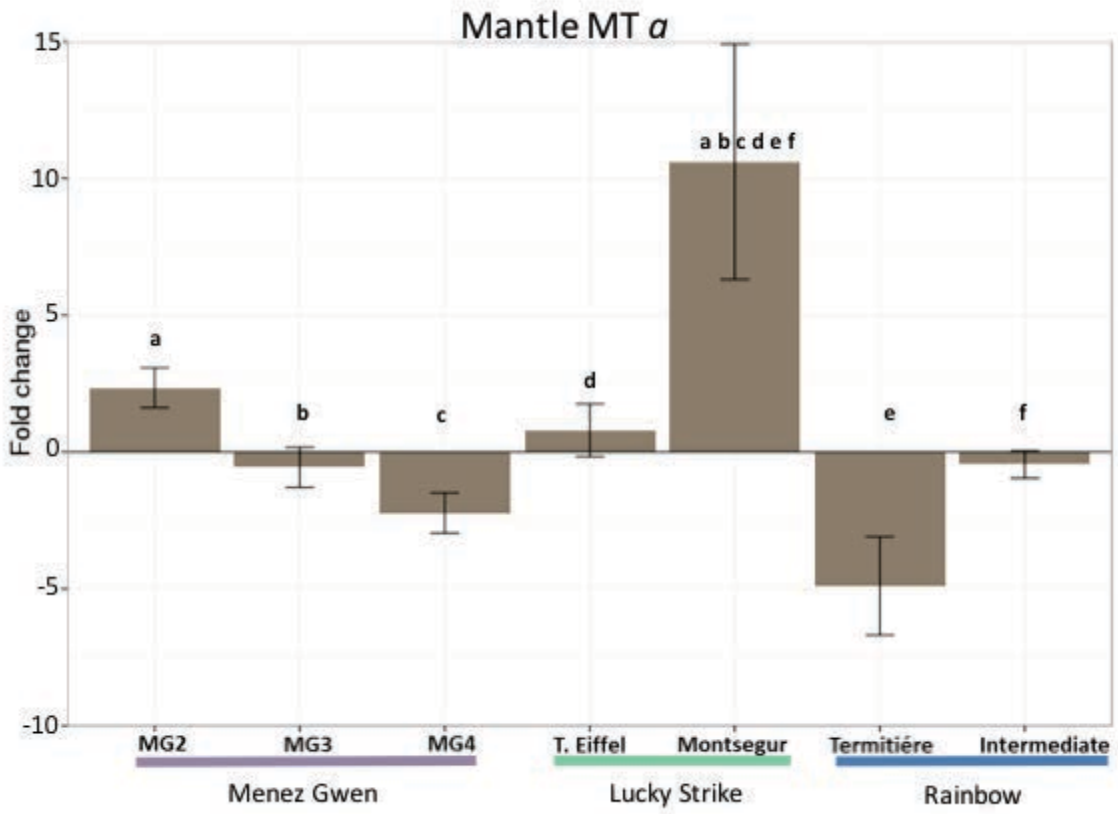

Gill MT *b*
Fold change
20
10
0
-10
-20
a
b
a b
MG2
MG3
MG4
T. Eiffel
Montsegur
Termitiére
Intermediate
Menez Gwen
Lucky Strike
Rainbow

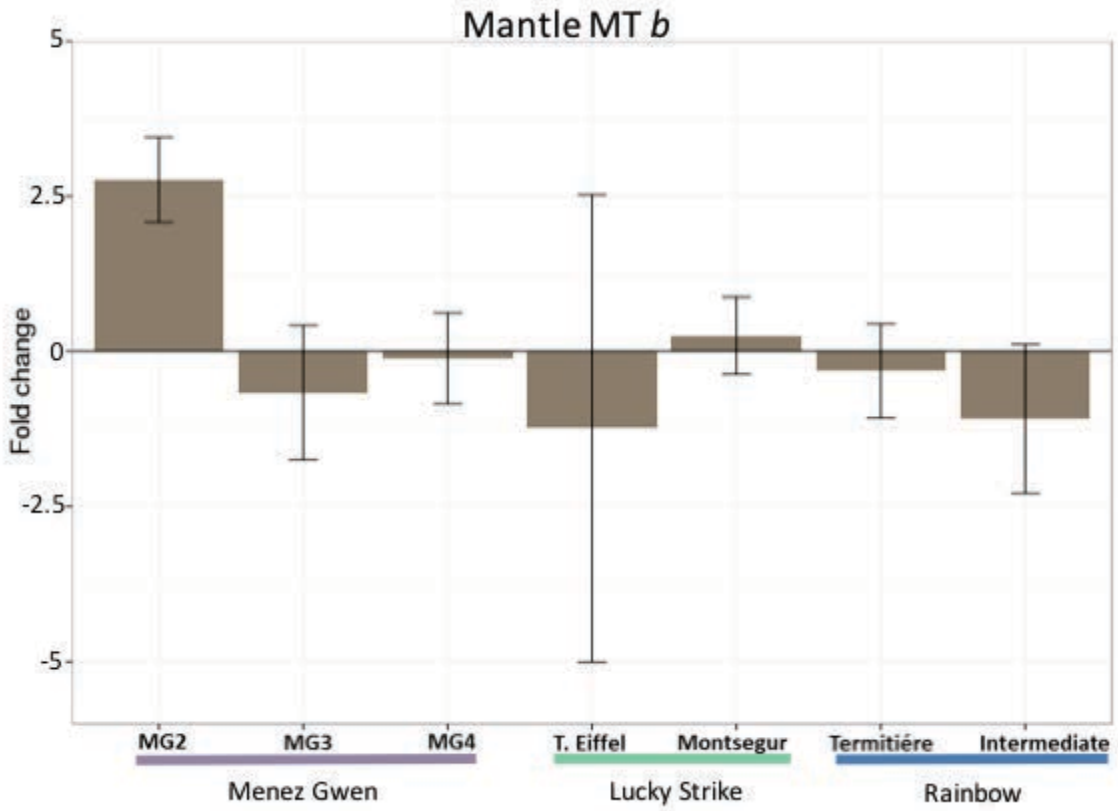


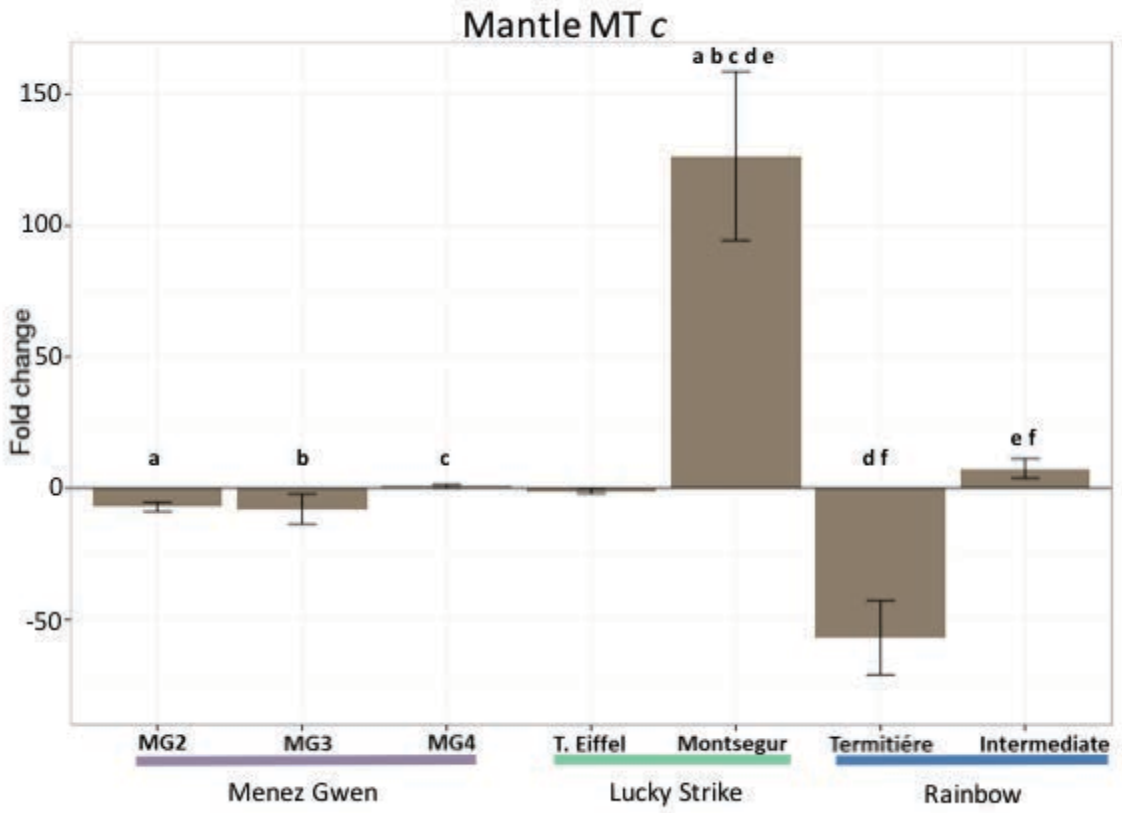
C. Relative expression of the two putative cadmium binding proteins and the three MTs in gill in the seven populations analyzed. The data are normalized by the mean deltaCt value of each MT isoform. Shared letters represent significant differences between populations. For Cd-bp1: **a**=MG2/MG3 and **b**=MG2/MG4. For Cd-bp2 **a**=MG2/MG3, **b**=MG3/MG4, **c**=MG3/Montsegur and **d**=MG4/Tour Eiffel. For MT*a:* **a**=MG2/Montsegur, **b**=MG3/Montsegur and **c**=Tour Eiffel/Montsegur. For MT*b:* **a**=MG3/Intermediate, **b**=MG4/Intermediate. For MT*c*: **a**=MG2/Termitiére, **b**=MG3/Termitiére, **c**=MG4/Termitiére, **d**=Tour Eiffel/Termitiére, **e**=Montsegur/Termitiére and **f**=Termitiére/Intermediate.


Mantle Cd-bp1
Fold change
10
5
0
-5
-10
-15
a b
a
b c d
c
d
MG2
MG3
MG4
T. Eiffel
Montsegur
Termitiére
Intermediate
Menez Gwen
Lucky Strike
Rainbow

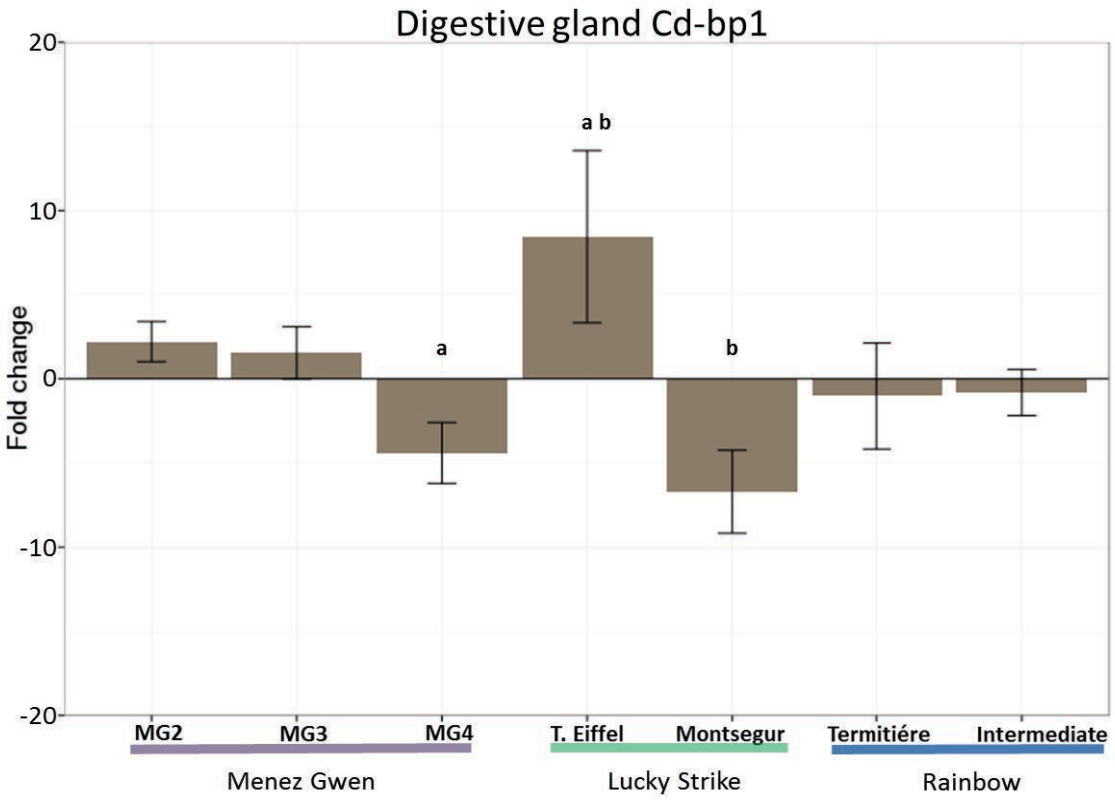

Mantle Cd-bp2
Fold change
10
5
0
-5
-10
-15
a
a b
b
MG2
MG3
MG4
T. Eiffel
Montsegur
Termitiére
Intermediate
Menez Gwen
Lucky Strike
Rainbow

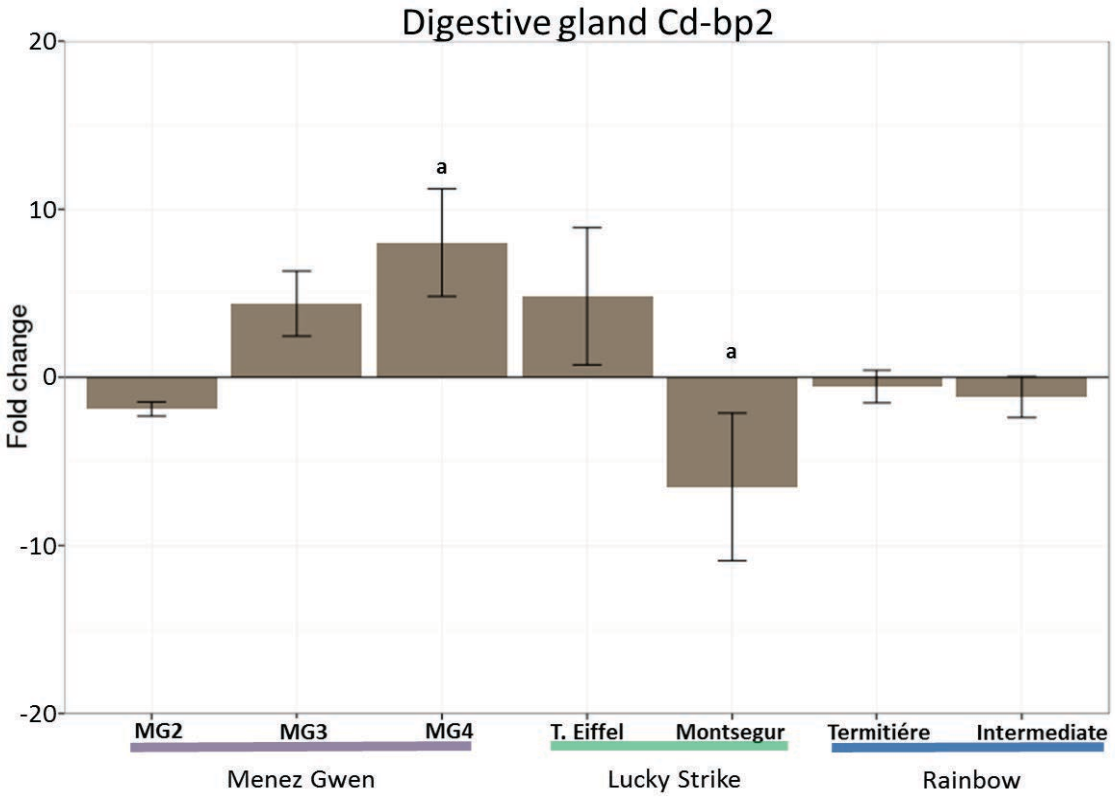

Mantle MT a
Fold change
15
10
5
0
-5
-10
a
b
c
d
a b c d e f
e
f
MG2
MG3
MG4
T. Eiffel
Montsegur
Termitiére
Intermediate
Menez Gwen
Lucky Strike
Rainbow

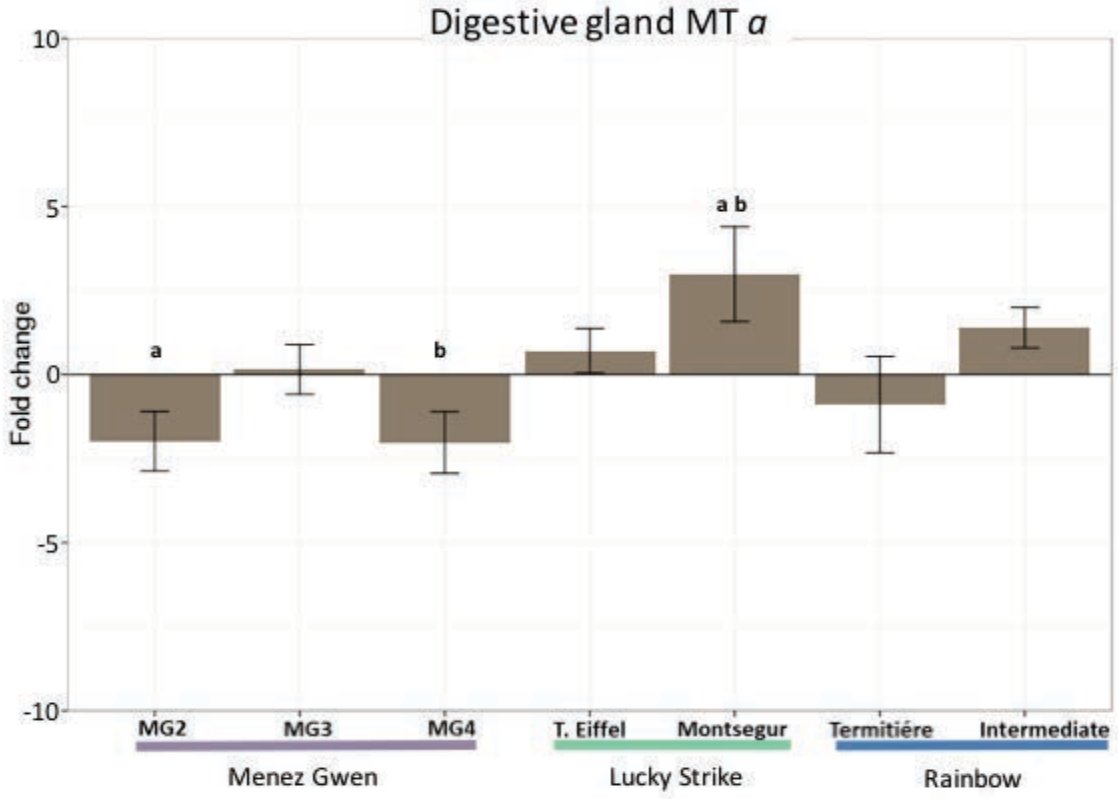

Mantle MT b
Fold change
5
2.5
0
-2.5
-5
MG2
MG3
MG4
T. Eiffel
Montsegur
Termitiére
Intermediate
Menez Gwen
Lucky Strike
Rainbow

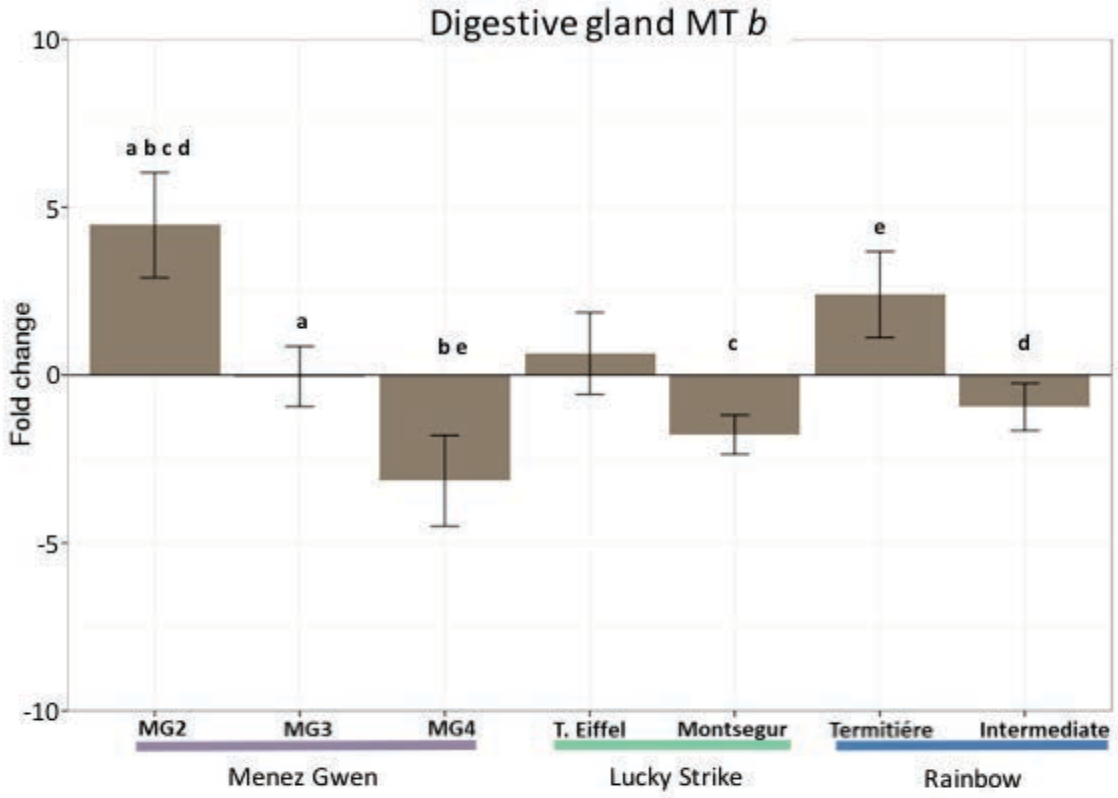


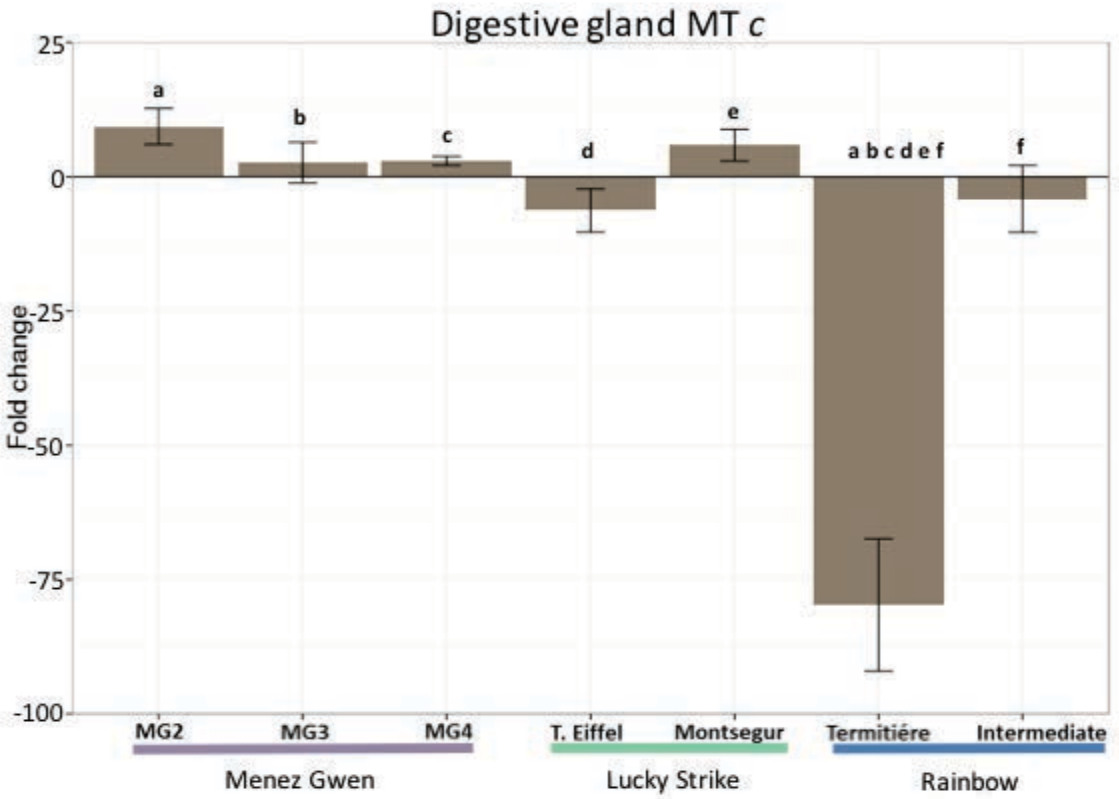
D. Relative expression of the two putative cadmium binding proteins and the three MTs in mantle in the seven populations analyzed. The data are normalized by the mean deltaCt value of each MT isoform. Shared letters represent significant differences between populations. For Cd-bp1: **a**=MG2/MG3, **b**=MG2/MG4, **c**=MG4/Montsegur, **d**=MG4/Termitiére. For Cd-bp2: **a**=MG4/Tour Eiffel, **b**=Tour Eiffel/Intermediate. For MT*a:* **a**=MG2/Montsegur, **b**=MG3/Montsegur, **c**=MG4/Montsegur, **d**=Tour Eiffel/Montsegur), **e**=Termitiére/Montsegur and **f**=Intermediate/Montsegur. For MT*c:* **a**=MG2/Montsegur, **b**=MG3/Montsegur, **c**=MG4/Montsegur, **d**=Termitiére/Montsegur), **e**=Intermediate/Montsegur and **f**=Termitiére/Intermediate.


Digestive gland Cd-bp1
Fold change
20
10
0
-10
-20
a b
a
b
MG2
MG3
MG4
T. Eiffel
Montsegur
Termitiére
Intermediate
Menez Gwen
Lucky Strike
Rainbow


Digestive gland Cd-bp2
Fold change
20
10
0
-10
-20
a
a
MG2
MG3
MG4
T. Eiffel
Montsegur
Termitiére
Intermediate
Menez Gwen
Lucky Strike
Rainbow


Digestive gland MT a
Fold change
10
5
0
-5
-10
a
b
a b
MG2
MG3
MG4
T. Eiffel
Montsegur
Termitiére
Intermediate
Menez Gwen
Lucky Strike
Rainbow


Digestive gland MT b
Fold change
10
5
0
-5
-10
a b c d
a
b e
c
e
d
MG2
MG3
MG4
T. Eiffel
Montsegur
Termitiére
Intermediate
Menez Gwen
Lucky Strike
Rainbow



E. Relative expression of the two putative cadmium binding proteins and the three MTs in digestive gland in the seven populations analyzed. The data are normalized by the mean deltaCt value of each MT isoform. Shared letters represent significant differences between populations. For Cd-bp1: **a**=MG4/Tour Eiffel, **b**=Tour Eiffel/Montsegur. For Cd-bp2: **a**=MG4/Montsegur. Fpr MT*a:* **a**=MG2/Montsegur, **b**=MG4/Montsegur. For MT*b:* **a**=MG2/MG3, **b**=MG2/MG4, **c**=MG2/Montsegur, **d**=MG2/Intermediate, **e**=MG4/Termitiére. For MT*c:* **a:**MG2/Termitiére, **b**=MG3/Termitiére, **c**=MG4/Termitiére, **d**=Tour Eiffel/Termitiére, **e**=Montsegur/Termitiére, **f**=Termitiére/Intermediate.

Appendix 1. Measured metal concentration in gill for each population (mean ± SD) expressed in µg g^{-1} dry weight.

Population	Cadmium	Copper	Iron	Lead	Calcium	Manganese	Zinc	Magnesium
MG-2	2,23 ± 0,3	40,24 ± 3,4	152,22 ± 7,7	4,67 ± 0,8	1802,88 ± 91,3	3,55 ± 0,3	56,02 ± 18,3	6045,79 ± 489,2
MG-3	2,52 ± 0,6	54,98 ± 3,1	161,728 ± 8,5	7,36 ± 3,3	1911,87 ± 101,0	3,55 ± 0,9	147,35 ± 44,2	6305,62 ± 293,7
MG-4	6,14 ± 0,6	79,27 ± 9,7	166,67 ± 21,9	28,9 ± 3,0	1973,38 ± 258,9	6,33 ± 0,3	96,39 ± 13,2	6508,15 ± 760,4
LS-Montsegur	4,63 ± 2,2	69,63 ± 4,6	135,50 ± 1,84	100,48 ± 11,2	1605,8 ± 21,16	7,07 ± 0,7	54,17 ± 20,4	5091,95 ± 349,3
LS-Eiffel Tower	30,53 ± 6,1	151,19 ± 47,1	151,12 ± 9,60	98,75 ± 20,5	1791,64 ± 112,6	9,38 ± 2,5	2403,69 ± 980,9	5860,37 ± 354,7
Rb-Termitiére	15,59 ± 0,1	187,71 ± 68,1	243,63 ± 92,6	580,91 ±72,7	2880,15 ± 2269,7	12,56 ± 2,9	206,07 ± 127,9	5599,02 ± 92,9
Rb-Intermediate	21,39 ± 9,4	104,35 ± 42,9	116,20 ± 42,0	140,37 ± 67,7	1378,4 ± 497,7	10,42 ± 2,9	5643,62 ± 2575,4	4623,39 ± 1641,6

Measured metal concentration in mantle for each population (mean ± SD) expressed in µg g^{-1} dry weight.

Population	Cadmium	Copper	Iron	Lead	Calcium	Manganese	Zinc	Magnesium
MG-2	0,57 ± 0,08	25,47 ± 7,9	26,04 ± 12,5	1,32 ± 0,05	1339,96 ± 61,02	3,13 ± 0,3	142,83 ± 14,2	2960,8 ± 190,1
MG-3	0,76 ± 0,2	39,37 ± 5,6	54,56 ± 10,3	1,11 ± 0,06	5769,8 ± 5882,8	3,7 ± 0,6	192,8 ± 35,9	5085,9 ±867,4
MG-4	1,01 ± 0,2	31,31 ± 8,2	28,46 ± 12,7	2,90 ± 0,78	2812,08 ± 2853,1	4,9 ± 1,8	136,8 ± 38,2	3026,6 ± 795,3
LS-Montsegur	1,03 ± 0,3	32,72 ± 5,7	126,14 ± 37,7	9,20 ± 0,9	1813,09 ± 84,05	9,06 ± 3,3	276,8 ± 189,9	4036,75 ± 48,6
LS-Eiffel Tower	1,14 ± 0,1	19,40 ± 1,3	81,25 ± 7,02	1,94 ± 0,3	1433,3 ± 225,2	8,69 ± 1,6	124,6 ± 4,6	3125,31 ± 548,1
Rb-Termitiére	0,73 ± 0,2	7,43 ± 2,3	3580,5 ± 1165,4	11 ± 0,8	1900,8 ± 934,8	46,66 ± 13,2	115,8 ± 31,8	2504,9 ± 496,5
Rb-Intermediate	0,72 ± 0,4	6,97 ± 3,6	1921,15 ± 1029,5	3,83 ±1,02	1001,2 ± 353,8	17,11 ± 10,6	220,9 ± 102,8	1922,3 ± 829,12

Measured metal concentration in digestive gland for each population (mean ± SD) expressed in µg g^{-1} dry weight.

Population	Cadmium	Copper	Iron	Lead	Calcium	Manganese	Zinc	Magnesium
MG-2	14,96 ± 3,7	75,22 ± 21,7	161,42 ± 76,5	8,76 ± 2,0	2052,4 ± 1128,0	4,74 ± 0,7	290,8 ± 89,7	2340,8 ± 221,2
MG-3	16,94 ± 0,5	72,20 ± 9,2	77,39 ± 21,6	3,02 ± 0,70	1576,08 ± 634,9	5,44 ± 1,51	301,03 ± 34,9	1976,3 ± 112,0
MG-4	10,3 ± 0,8	86,70 ± 19,6	180,9 ± 71,7	12,9 ± 0,94	2098,8 ± 488,54	11,93 ± 9,4	375,08 ± 113,5	2342,72 ± 412,8
LS-Montsegur	8,84 ± 2,7	95,61 ± 36,9	405,49 ± 71,7	36,48 ± 14,9	1469,05 ± 90,4	14,30 ± 4,4	337,13 ± 200,1	2369,8 ± 212,9
LS-Eiffel Tower	19,16 ± 4,4	187,5 ± 50,1	483,5 ± 18,0	22,67 ± 8,2	1511,8 ± 372,65	11,74 ± 2,8	1395,5 ± 268,4	2633,8 ± 340,3
Rb-Termitiére	7,66 ± 1,8	16,44 ± 2,4	4397,0 ± 4368,02	32,99 ± 11,4	1125,8 ± 240,8	25,73 ± 20,3	142,7 ± 33,3	1793,2 ± 334,4
Rb-Intermediate	9,91 ± 2,8	68,67 ± 46,2	8581,4 ± 2501,6	13,77 ± 1,6	1701,46 ± 94,43	41,57 ± 39,9	403,5 ± 183,3	2705,9 ± 505,9

Chapter III

Article II: Differential expression of Superoxide Dismutase isoforms as indicator of oxidative stress in response to metals in the hydrothermal mussel *Bathymodiolus azoricus*: application to field and experimental populations.

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AQUATIC TOXICOLOGY.

ABSTRACT

Hydrothermal vent ecosystems are characterized by the presence of high concentration of metals that are known to generate the production of ROS. The hydrothermal mussel *Bathymodiolus azoricus*, is known to present a strong tolerance to metals that are differentially accumulated in the soft tissues. The superoxide dismutase (SOD) is one of the key enzymes involved in the ROS scavenging and several isoforms are present in *B. azoricus*. This study aims to evaluate the responses of molecular biomarkers in the mussel providing data for potential use in environmental monitoring in the MAR. Mussels were collected at different vent sites characterized by contrasted environmental parameters to compare populations and used in laboratory experiments of metal exposure using several concentrations and sampling time. Determination of metal accumulation was also made to determine potential correlation between metal content and SOD gene expression pattern. Contrasted profiles of SOD expression were detected between the different tissues analyzed and within population without highlighting a specific vent site signature or a specific metal response after exposure to copper, cadmium or iron. However, we identified three SOD isoforms that showed a strong regulation in response to metal exposure particularly in digestive gland. Some correlations have also been detected between this SOD expression and the metal content in tissues. These results indicate possible oxidative stress effects on organisms in response to metal and the involvement of SODs in the cellular response to metal exposure.

Keywords: Mid Atlantic Ridge, Metal exposure, SOD, Gene expression, hydrothermal mussel

1. Introduction

Marine ecosystems are exposed to constant environmental fluctuations that vary in amplitude, duration and predictability which conduct species to elaborate different strategies to respond to these fluctuations. Marine invertebrates are characterized by a strong ability to accumulate different types of metals in different tissues at high concentrations but the physiological consequences of this are not well elucidated (Rainbow, 1997). Metals can increase the production of reactive oxygen species (ROS) including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical (OH), which are the regular products of oxygen metabolism. However, the presence of excess of metals generate an imbalance in the ROS concentration which

subsequently cause lipid peroxidation, protein modifications and DNA strand cleavage (Lushchak, 2011). To respond to ROS excess, organisms activate an antioxidant machinery that include different enzymes like superoxide dismutase (SOD), catalase (CAT) and Glutathione-S-Transferase (Canesi, 2015) and non-enzymatic antioxidants, such as glutathione, vitamin E (de Zwart et al., 1999). The main function of SOD is the conversion of O_2^- to oxygen (O_2) and hydrogen peroxide (H_2O_2) in presence of H^+ (Fridovich, 1995). SODs are classified into three main groups: the cytosolic copper/zinc SOD (Cu/Zn-SOD) which is the most abundant and contain a extracellular and an intracellular form, an iron SOD (Fe-SOD) usually found in prokaryotes and a manganese SOD (Mn-SOD) which can be found in the mitochondria (Abreu and Cabelli, 2010). In many marine species, SODs are used as biomarker of susceptibility to oxidative stress in response to environmental parameters (Boutet et al., 2004; Sheader et al., 2006; Lewis et al., 2016; Lushchak, 2011). Transcriptional expression of SODs exhibits differential regulation in response to metals very often associated to an increase of SOD expression in response to metal exposure (Taylor et al., 2013; Kim et al., 2013; Nicosia et al., 2015). SODs are also used as biomarkers in natural populations to characterize the effect of anthropogenic pollutants on ecosystems (Orbea et al., 2002; Rodriguez-Ariza et al., 1993).

In hydrothermal vents, the physico-chemical variables such as hydrostatic pressure, temperature, low oxygen levels and pH, high concentrations of gases (methane and sulfides) and metals concentration exhibit a large range of variation (German and Von Damm, 2004; Le Bris et al., 2006). These ecosystems are biologically productive and are characterized by an endemic fauna mainly composed by bivalves, arthropods and annelids which have evolved using different adaptive strategies like symbiosis with methane and sulphur oxidizing bacteria in bacteriocytes in gills of for energy support (Kiers and West, 2015; Le Bris and Duperron, 2010), anatomic modifications (Jones, 1981) and physiological strategies (Childress et al., 1992). The high metal concentration is one of the shared characteristics between vents around the oceans that represent a physiological challenge for the organism living here. The diversity and concentration of metals that emerge from the hydrothermal flux vary in each vent according to the physicochemical condition as temperature, rock composition of the ocean crust and oceanic circulation around the vent which moderate the availability of particulate and dissolved metal that can be transported hundreds of meters by the hydrothermal plume (Von Damm, 1995) and be available for the absorption in the fauna associated to these ecosystems. In the Mid-Atlantic ridge, bivalves from the Mytilidae family are widely distributed, where *Bathymodiolus azoricus* is a model specie used for understand the adaptation to this extreme environmental, principally because forms large communities in the base and walls of vent chimneys, with high abundance (Sarrazin et al., 2014). Previous works showed that *B. azoricus*

can accumulate essential (iron, copper, zinc) and non-essential (cadmium, mercury, lead) metals in different tissues like gills, mantle, digestive gland, foot and this bioaccumulation can vary between site related or not to the concentration found in the fluid (Colaço et al., 2006; Kadár, 2007; Kádár et al., 2007; Cosson et al., 2008; Martins et al., 2011; Koschinsky et al., 2014). However the biochemical and physiological adaptation to metals have not been well described in this species. The characterization of SODs in hydrothermal species from different vent systems has been reported such as in *Alvinella pompejana (Bruneaux et al., 2013)*, Riftia pachyptila *and* Calyptogena magnifica (*Blum and Fridovich, 1984),* Brachyura (*Marchand et al., 2009) or* Paralvinella grasslei *(Shin et al., 2010). In B. azoricus,* experimental exposure to cadmium, copper and mercury induced an inhibition in SOD activity in the gills of *B. azoricus* from Menez Gwen but over long periods of exposure to cadmium, the activity increases compared to the initial levels (Company et al., 2004) and a higher SOD activity is observed in response to cadmium exposure by Company et al. (2006).

The use of gene expression profile in an ecotoxicological assessment as a tool of response to stress see an increasing interest due to its high sensitivity (Huggett et al. 2005; Hellemans et al. 2007). In the case of SODs, most studies conducted in marine mollusc species only analyzed the expression of single SOD isoform and in parallel, studies based on the SOD activity are not able to distinguish the specific activity of the different isoforms with the exception of the mitochondrial versus cytosolic compartment. The goal of the present study is to investigate the pattern expression variations of seven SOD isoforms in response to metal exposure under controlled conditions and compare their regulation profiles to those obtained in natural populations.

2. Materials and methods

2.1. Biological samples, Natural population

Samples of *B. azoricus* were collected from Mid-Atlantic Ridge in August 2013, during the BIOBAZ cruise on the research vessel “Pourquoi Pas?, using the Remote Operating Vehicle (ROV) Victor 6000. The collection was made in three different vents sites Menez Gwen (37°50’ N, 31°31’ W, 800mdepth, 3 populations called MG2, MG3 and MG4), Lucky Strike (37°17’ N, 32°17’ W, 1700m depth, 2 populations collected at Tour Eiffel and Montsegur) and Rainbow (36°14’ N, 33°54’ W, 2300m depth, 2 populations collected at Termitière and Intermediate) **(Appendix 1).** Mussels were collected in hermetic boxes then brought on board. Gills, mantle and digestive gland of each individual were immediately dissected and fixed in liquid nitrogen until further analysis.

2.2. Experimental exposure to heavy metals

For this experiment, mussels were collected at Menez Gwen site (MG2) because of their ability to survive for a long time at atmospheric pressure (Kadar et al., 2005).Once on board, ten groups of 150 mussels from various size (from 12 to 80 mm) were placed in tanks containing 30 liters of filtered sea water. Then, in each tank, mussels are exposed to 0,2µM; 1µM; 4µM; 20µM of cadmium, 4µM; 25µM and 60µM of copper, 1µM, 5µM and 20µM of iron. Concentrations have been determined according to concentrations determined in the fluids composition by Charlou et al. (2002) and represent concentration detected in the fluid for copper and iron and the 0.2 and 1µM for cadmium, the 4 and 20µM of cadmium corresponding to sub-lethal levels. An additional set of 200 mussels was placed in a control tank without metal added to follow the effect of experimental design (use of surface sea water and absence of pressure and gases). After 5, 10, 15, 20 and 25 days, 10 individuals were sampled in each tank and pieces of gills and digestive gland were dissected and fixed in liquid nitrogen for further analysis.

2.3. Metal concentrations in tissues

For each metal concentration and sampling time, we make pools of 10 individuals for gills, mantle and digestive gland then tissues were lyophilized. Sample digestions were done at 105°C in closed 15-mL Teflon screw-cap vials (Savillex, Minnetonka, USA) with 4 mL suprapur 65 % nitric acid (Merck, Darmstadt, Germany) and 1 mL suprapur 30% hydrogen peroxide (Merck). Measurements were then conducted on diluted mixtures (2.5% HNO_3) using different spectrometers operated at the Pole Spectrometry Ocean Brest (PSO, Brest). Cu, Pb, Cd, Mn and Zn were determined a High-Resolution ICP (HR-ICP) mass spectrometer (Element 2, Thermo Scientific, Bremen, Germany) with a precision better than 3% (Cd, Cu, Pb) or 5% (Mn, Zn). Ca, Fe and Mg were determined with an inductively coupled plasma (ICP) optical emission spectrometer (Ultima 2, Horiba Jobin Yvon, Longjumeau, France) with a precision better than 1%. The certified reference materials used for assessment of the method accuracy were: DORM-4 (fish protein), DOLT-5 (dogfish liver) and TORT-3 (lobster hepatopancreas) from NRCC and RM-8414 (bovine muscle) from NIST. All metal concentrations are expressed in µg g^{-1} dry weight.

2.4. RNA Extraction, cDNA sequencing and qPCR

For natural population samples, total RNA was extracted from gills, mantle and gland digestive and for experimental samples, total RNA from gills and digestive gland only was extracted using Tri-Reagent (Sigma, St. Louis, MO) according to the manufacturer's instructions. Two µg of total

RNA were reverse transcribed using M-MLV reverse transcriptase (Promega, Madison, WI). For gene expression, a volume of 2 µl of each diluted reverse transcription product (1:200) was subjected to real-time PCR in a final volume of 5µl containing 40 nM of each specific primer and 2×Lightcycler®480 SYBR Green I Master mix (Roche Diagnostics, Mannheim Germany). The amplification was carried out as follows: initial enzyme activation at 95°C for 15 min, then 45 cycles of 95 °C for 10 sec and 60 °C for 30 sec. A dissociation curve was generated and PCR efficiency was estimated for each primer pair. All primer pairs tested (**Table 1**) generated a single peak in the dissociation curve and a PCR efficiency of 95 to 100%. The ribosomal protein L15 gene (RpL15) was used as the internal PCR control as already used in previous studies (Bougerol et al., 2015; Boutet et al., 2011; Guezi et al., 2014).

2.5. Statistical analyses

The relative expression of each SOD isoform was calculated according the comparative Ct method using the formula RQ = ΔΔCt (with $\Delta Ct_{gene\ X} = Ct_{gen\ X} - Ct_{RiboL15}$; $\Delta\Delta Ct = \Delta Ct_{gene\ X} - \text{mean } Ct_{gene\ X}$). The first analysis was conducted to determine the inter-population effect in the variation of MTs expression and we normalized the data by the mean $deltaCt_{gene\ X}$ of all populations for one tissue. The second analysis was conducted to determine the intra-population effect of the MTs expression and we normalize the data by the mean $deltaCt_{gene\ X}$ in the three tissues in each population. For the mussels exposed to metal we normalize the data by the mean in of each metal across the 25 days of exposure. ANOVA test was used to evaluate differences in metal accumulation and SODs expression considering population and tissues as explanatory factors (R studio software). Canonical redundancy analysis (RDA) was used to evaluate the influence of metal accumulation in the SODs expression using the Vegan package (Oksanen et al., 2016) in R environment. Test of significativity between SODs in each experimental condition in both tissues were performed using additional Student T-tests with 100 permutations and a p-value= 0.05.

3. Results

3.1. *SODs characterization.*

The seven SODs studied correspond to 5 cytosolic Cu/ZnSOD (SOD1, SOD2, SOD3, SOD4 and SOD7; A**ppendix2**), one mitochondrial MnSOD (SOD6; **Appendix3**) and one copper chaperone for superoxide dismutase (SOD5) (**Appendix4)**. The alignment of the cytosolic *B. azoricus* SODs sequences and other SODs described in bivalve species and in a hydrothermal annelid *A. pompejana*, shows a moderate percentage of similitude indicating a relative low conservation of these proteins (**Table 2**). The higher similarity is reported for *B.*

azoricus SOD2 with the *A. pompejana* and *M. chilensis* SOD while the lowest value is found between SOD3 and SOD4 within *B. azoricus* sequences. The percentage of identity of the mitochondrial SOD-6 with other marine invertebrates does not exceed the 51% compared with sea urchin *S. pupuratus* and the brachiopod *L. anatine* (**Table 3**) and similar value are found for the copper chaperone SOD-5 with sequences of other coastal invertebrates (**Table 4**).

3.2 SODs expression between populations

The relative expression of the seven SODs genes reveals contrasted patterns between populations (**Figure 1** and **Table 5**) but no clear vent site effect is detected. In gills, all the SODs gene except SOD6 are significantly regulated. MG2 and MG4 populations exhibit a similar expression pattern characterized by a high expression of the cytosolic SOD1 and a low expression of SOD2. Both populations of Lucky Strike show a higher expression ratio of all isoforms except SOD1. The two populations of Rainbow exhibit an opposite expression pattern for all SODs. Surprisingly, SODs expression pattern of Termitiére and MG3 populations are very similar. In mantle, all the SODs gene except SOD1 are significantly regulated. MG2 exhibits a higher expression of all SODs except SOD5 compared to other populations. SOD2 appears more expressed in the three MG populations and SOD5 in both Rainbow populations. In the digestive gland, all the SODs are significantly regulated and no clear expression pattern can be detected between the three vent sites. MG3 and Tour Eiffel exhibit a higher expression for all SODs when Montsegur and Termitiére show the lower expression ratio. .

3.3 Relative expression of SODs between tissues

The regulation of the seven SOD genes is characterized by a global down regulation in mantle compared to other tissues across all the populations except MG2 and MontSegur (**Figure 2** and **Table 6**). A higher expression is observed in the digestive gland compare to gills for three populations (MG3, Tour Eiffel and Intermediate) and a similar ratio is observed between both tissues for the other populations with the exception of MG2 that shows a general up-regulation in gills. At MG2 for SOD5 and at Montsegur for SOD6 only, a significant higher expression is observed in gills (**Table 6**). For MG3, MG4 and Tour Eiffel populations, almost all SOD isoforms are significantly regulated ($p<0,01$), MG3 and Tour Eiffel exhibiting the same regulation pattern. In Intermediate population, SOD1 and SOD7

when in Termitière population, SOD6 and SOD7 are significantly up-regulated in the gills and SOD4 in the digestive gland.

3.4 Redundancy analysis (RDA)

The redundancy analysis showed that 16.6% of the variation in relative expression of SODs is explained by the variable "metal concentration" in gills, 13.5% in the mantle and 19.2% in the digestive gland (**Figure 3**). In gills, the SOD1 gene does not show any correlation with metals while SOD3 and SOD4 are correlated to lead and copper, SOD2 and SOD5 are with manganese and SOD6 and SOD7 with cadmium. In mantle, SOD5 shows a strong correlation with iron and manganese and in lesser extent with lead, SOD1 appears correlated to copper and the other SODs are not correlated to metals. In the digestive gland, SOD3, SOD5 and SOD6 show a weak correlation metals but SOD7 exhibit a strong correlation with cadmium, and SOD1, SOD2 and SOD4 with copper.

3.5 Mortality in metal exposure experiment

After 25 days of metal exposure the highest mortalities rate are reported in cadmium exposure with a similar rate for the 1, 4 and 20µM concentrations. In iron exposure, mortality is limited and increase from 1 to 20 µM when in copper exposure, mortality rate remain comparable between concentrations (**Figure 4**).

3.6 Metal concentration in tissues during metal exposure

The concentration of the eight metals are reported for gills (**Figure 5**) and digestive gland (**Figure 6**). The specific measurement of each metal in all metal exposure conditions are reported in the **Appendix 5 and 6.** In gills, cadmium concentration do not increase with time whatever the concentration of exposure. For copper, the concentration increased after 5 days and remain high and constant up to 20 days (3 to 5 times compared to control) in mussels exposed to copper with no clear effect of concentration exposure effect. We also noticed that copper is accumulated in gills from mussels exposed to cadmium at a similar level than the one observed in copper exposure. Copper levels are similar to control in mussels exposed to iron and lead. For iron, a higher and constant concentration is detected only in mussel exposed to iron from day 5 (1.5 times higher than in control and other metal exposure conditions). No change are observed for the four metal calcium, magnesium, manganese and zinc. In the digestive gland, the cadmium concentration shows a slight decrease in control with time and remain similar in all other metal conditions. For copper, the

concentration increases from day 5 to day 20 (2 to 4 times) compared to control and the other metal exposure conditions in mussels exposed to copper with no specific time exposure pattern. For iron, we only notice a global higher concentration in mussels exposed to iron after day 20 compared to control. No change is observed for the three metal magnesium, manganese and zinc. We notice a relative decrease of calcium concentration from day 10 in control mussels compared to those exposed to metal conditions.

3.7 Relative expression of SODs in metal exposure experiments

In gills, the general expression pattern of SODs during the metal exposure shows strong similarities for the three metal experiments. Regarding the regulation of SODs in the control which represent a condition of depuration, we notice that some SODs show a significant regulation according to time especially SOD2 and SOD3 with a significant down-regulation of SOD2 and 3 at day 5 and 10 and an up-regulation at day 15 and 20 (**Table 9**). In cadmium exposure (**Figure 7**), ANOVA analysis reveals that all SODs except SOD5 and SOD7 are significantly regulated during the experiment (**Table 7**). At the 0.2 µM concentration, SODs expression differ from controls at day 10 with a significant increase of 4 SODs isoforms, and at day 20 with a significant decrease of SOD2,3 and 6. At the 1 µM concentration, SODs expression increase at day 5 compared to control especially SOD2 and 4 then remain similar at day 10 and 15 and significantly decrease at day 20 for 4 SODs. At the 4 µM concentration, SODs expression does not differ from control at day 5 and 10 except SOD2 and SOD1 and 4 decrease at day 15. At the 20 µM concentration, a global lower SODs expression is observed but very few SODs show a significant variation in expression compared to control (**Table 9**). In copper experiment, all SODs except SOD3 and SOD6 are significantly regulated (**Table 7**). At day 5, no difference in SODs regulation is observed at any concentration compared to control except for SOD2 which is up-regulated at day 5 and 10 and down-regulated at the 60µM concentration. At day 10, an induction of SOD 1 and 4 at 4 µM, SOD3 at 25µM and SOD2 at 60µM is detected. At day 15, an induction of SOD 1 at 4 µM, SOD2 at 60µM and a decrease of SOD4 at 4µM is detected when at day 20, a significant decrease of SOD2 expression is observed at the three concentrations. In iron experiment, all SODs except SOD7 are significantly regulated. At day 5, 10, 15 and 25, we mainly observed a significant expression at the three concentrations for SOD2 compared to the control and a decrease at day 20. Other SODs showed some significant difference at different sampling time but with contrasted pattern.

In the digestive gland, the global expression pattern during the metal exposure shows major differences according to metals. In the control, we notice that SOD2, 3 and 6 present a down-regulation at day 15 and 20 compared to the initial time T0 (**Table 9 and Figure 8**). In cadmium

exposure, all SODs are significantly regulated (**Table 8**). At day 5, we observe a contrasted regulation of SODs with a up-regulation at 4µM and a down-regulation at 1 and 20µM for 4 to 6 SODs. At day 10, SOD2 and 4 are significantly down-regulated in all cadmium concentrations. At day 15, a contrasted response is observed with a significant increase of SOD1, 2, 3 and 6 at 1µM and SOD 6 at 20µM when SOD2 is down-regulated at 20µM. At day 20, SOD2 is up-regulated at the 1, 4 and 20µM concentrations. In copper exposure, all SODs are significantly regulated except SOD6. A global down regulation of all SODs is observed at day 5 for the 4 and 25µM concentrations. At day 10, SODs expression increase in mussels exposed to 25 µM for SOD 2, 3 and 7 and at 60µM for SOD1, 4 and 5 and SOD5 is down-regulated at 4µM. At day 15, an increase of SOD1, 3, 6 and 7 expression is observed at the 4µM concentration and at day 20, a major up-regulation is detected for the SOD1, 2, 3, 4 and 7 in the three concentrations compared to the control. In iron exposure, SOD1, ANOVA showed that SOD2, SOD3, SOD4 and SOD6 are significantly regulated .At day 5, mussels exposed to 20µM show a down-regulation for SOD 3, 4, 5 and 6 compared to the control when at day 10, all mussels exposed to the three concentrations exhibit a significant down-regulation of SOD3 and 4. At day 15, few variations are observed except for SOD1 and 3 which are down-regulated SOD7 that is up-regulated at 20µM. At day 20, expression of SOD1, 2 and 3 increases in all iron concentrations and at day 25, levels of SOD expression are similar in both experimental and control mussels.

4. Discussion

4.1 SODs characterization and relative expression in natural populations

The antioxidant defense has been studied in several invertebrates species from hydrothermal vent ecosystems, particularly enzymes involved in the reduction of the cellular content of superoxide and hydrogen peroxide (Blum and Fridovich, 1984; Bruneaux et al., 2013; Company et al., 2004; Genard et al., 2013). Indeed, ROS production can result from the oxidation of H_2S (Tapley et al., 1997), radioactivity (Cherry et al., 1992), ferrous iron and other metallic compounds present in the fluid (Sung and Morgan, 1980). SOD activities can increase in H_2S-rich environments (Morill et al., 1988; Abele-Oeschger, 1996) and during hypoxic events in order to prevent the oxidative stress due to normoxic recovery (Abele et al., 1998). In the three populations collected at the three vent sites Menez Gwen, Lucky Strike and Rainbow, Bebianno et al. (2005) evidenced a higher SOD activity in gills compared to mantle with a higher cytosolic SOD activity compared to mitochondrial SOD activity. The authors also detected a specific site pattern, the cytosolic SOD having a higher activity in LS followed by MG and RB while the mitochondrial form showed a decreasing activity from MG to LS

and RB but only for gills. A similar trend has been reported in transplanted mussels from RB to LS, however this study represent the contribution of both symbiotic and host SOD levels and a limited sample number (Company et al., 2007). None of the previous studies evidenced the existence of several isoforms that could modulate the global SOD activity. The present study intends to characterize the oxidative status in the hydrothermal vent mussel *B. azoricus* through the relative expression of several genes encoding SODs in both natural and experimental populations. We report the existence of several SOD isoforms in *B. azoricus* including new cytosolic and mitochondrial sequences. We also report for first time the existence of a specific copper SOD chaperone (SOD5) which has a similar structure to the classical SOD but which function as a metallochaperone is the incorporation of copper inside the cytosolic SOD when this metal is in low concentrations (Schmidt et al., 1999). Their relative expression revealed both specific tissues and population pattern. In all populations, a global higher level of expression is detected in gills compared to mantle which is in agreement with the higher level of SOD activity evidenced in gills (Company et al 2007). SODs expression patterns between gills and digestive gland vary according to populations highlighting the role of organotropism in ROS scavenging. In *Mytilus edulis*, a comparison of polluted sites versus control sites showed an induction of SOD gene expression in gills but not associated with an increase of SOD activity (Rola et al., 2012). The comparison of SODs expression in the three tissues between the seven populations exhibits significant differences between populations but no specific hydrothermal vent is detected. The strongest differences are observed in the digestive gland where a clear higher expression of all SODs is observed in MG3 (MG site) and Tour Eiffel (LS site) and a lower expression in Montsegur (LS site) and Termitiér (RB site). When considering the expression pattern of SODs in the three tissues, each population show a specific signature which could be used as a specific population signature. The three vent sites strongly differ in terms of depth, temperature, pH and metal concentrations but none of these environmental differences seems to influence the global SODs expression pattern. All those results reflect the phenotypic plasticity of *B. azoricus* related to different susceptibilities to oxidative stress according to each vent condition. Our RDA analysis also showed that tissue metal content only explained a part of the SODs expression pattern (range from 13 to 19% according to tissues) illustrating the fact that metal accumulation is not directly correlated with ROS level. However in gills, we noticed that SOD expression increases with metal content (except SOD1) which could be linked to the direct contact of gills with water and metals and their subsequent role in respiration and osmo-regulation (Regoli and Principato, 1995). In mantle, SODs expression globally decreases with increasing metal content and in digestive gland, SOD expression is related to copper and cadmium content for SOD1, SOD2, SOD4 and SOD7. Regarding the copper SOD chaperone, RDA analysis shows that SOD5 present a lower expression when copper concentration increase regulation in mantle and digestive gland. Part of the difference in the SODs expression

regulation between the population could also be partly explained by the fact that chronic exposure to some pollutants may affect the response to other pollutants illustrating the effect of a strong pressure selection and potential genetic adaptation in allele frequencies at specific genes (Weis et al., 1999; Bélanger-Déchênes et al., 2013) or epigenetic modifications that can affect gene expression (Aluru et al., 2011; Timme-Laragyet al., 2005) or altered gene expression pattern (Whitehead and Crawford, 2006; Whitehead et al., 2012).

4.2 Metal bioaccumulation in metal exposure condition

Bathymodiolus azoricus is one of the species with higher biomasses in the MAR and as in other bivalves species has shown ability to accumulate different metals at higher concentrations principally in gills, mantle, foot and digestive gland (Kádár et al., 2007; Koschinsky et al., 2014). The differences in mortality detected after 25 days of exposure to copper, cadmium, iron and lead suggest a metal sensibility in *B. azoricus*, characterized by higher susceptibility to cadmium followed by copper and iron. The metal accumulation reveals a tissue specific bioaccumulation pattern. Cadmium do not accumulate with time in gills contrary to the digestive gland after 20 days but surprisingly, in presence of copper (without additional cadmium), we observed a strong co-accumulation of both copper and cadmium in gills and digestive gland suggesting the existence of a similar pathway of chelation for the two metals but the mechanism of metal regulation in many mollusk species remains not clear. We may assume that cadmium concentrations used in this study represent an acute stress and that mussel, as a rule, first isolate themselves from the environment by closing their valves and increasing mucus production on the gills, then limiting the entry of cadmium in organs. In case of iron, we also observed an accumulation of iron in both tissues and especially in gills but with no time effect since level of iron remains similar at all sampling time. Accumulation of iron into soft tissues of mollusc species has been reported in many species (Depledge et al., 1994, Brown and Depledge, 1998). In the bivalve *Modiolus modiolus* exposed to iron, an increase of iron is observed after only 10 days of exposure but not in digestive gland suggesting an active excretion of this metal (Podgurskaya and Kavun, 2012). Calcium and magnesium ion are found at high concentration in sea water so is possible that they can interact with biological membranes and facilitate other metals absorption. Calcium has been reported as a modulator in filtering behavior and also in uptake of others metals as cadmium and zinc in experimental condition in *Daphnia magna* (Tan and Wang, 2008). In our experiment, calcium and magnesium which are principal components in the biological pumps involved in ion homeostasis (Toyoshima, 2009) have similar trends in the two tissues and in all experiments and showed no specific pattern of regulation. The concentrations of manganese and zinc reported in our study do not evidence strong variations during

the experiment, the major levels measured in manganese being reported in the digestive gland of mussels exposed to copper. Global higher levels of zinc are reported in control mussels in both tissues especially at the end of the experiment and a slow decrease of zinc concentration is observed in gills after 20 days, as previously reported in *B. azoricus* (Kadar, 2007) or in oysters in natural transplanted experiment (Wallner-Kersanach et al., 2000). Manganese and zinc are essential metals that act as cofactor or activator of many enzymatic reactions but at high concentration or prolonged exposure time, they can be toxicity and cause important physiological changes (Bini et al., 2015; Oweson and Hernroth, 2009; Oweson et al., 2010).

4.3 *SODs regulation in mussels exposed to metals*

The antioxidant protection of the superoxide dismutase has been widely studied in marine invertebrates exposed to different environmental conditions as sulphide exposure (Abele-Oeschger, 1996), hydrocarbon exposure (Boutet et al., 2004), industrial chemical and biocides (Kim et al., 2011; Kim et al., 2015) demonstrating the physiological importance of this enzymes in response to different environmental conditions. The hydrothermal vent is one of the most extreme ecosystems found in the oceans, characterized by strong environmental variation in temperature, pH, toxic gases and heavy metal, thus the role of the SOD in the maintenance of oxidative stress is of vital importance as an adaptive response in the hydrothermal species to these environmental fluctuations. In this work we report a robust experiment of metal exposure during 25 days, with a large samples size, different metals in different concentrations which shows that the exposure of metal can significantly trigger the expression of SOD in gills and digestive gland. We try to better understand the regulation of the different SODs in response to various metals at different concentrations in order to identify specific pattern of regulation and subsequently determine if SODs gene expression may be used as a tool to evaluate the effects of metal exposure. Due to the specificity of the hydrothermal mussel model, both heavy metals and laboratory conditions participate to the SODs regulation. Indeed, in hydrothermal vent environments metal-sulfide complexes buffer the concentration of bioavailable metals before physiological detoxification reactions occur (Edgecomb et al., 2004). Because of the absence of any sulfur or methane in our tanks, we suggest that metals are more bioavailable than those naturally encountered by mussels because no interaction with sulfurs is possible. Experiments were also conducted at atmospheric pressure and with seawater containing an oxygen level close to 20% versus an average concentration of 10% in situ. These differences in the laboratory environment could contribute to alter the physiological conditions of the mussel mainly by generating a global

oxidative stress. The pattern of SODs expression observed in the control condition indicates that the mussels are affected by the experimental condition metal concentrations used and the increase of SOD regulation observed after. The up-regulation observed after 15 days suggests an increase of ROS probably generated by the higher level of oxygen in the sea water compared to in situ conditions. However, the comparison of SOD expression in experimental conditions along the exposure time with the respective control showed that some SODs present strong regulations with in particular SOD2, SOD3 and SOD4. Interestingly, these three isoform showed the stronger relation between their expression and the metal concentration in tissues when considering field populations ‘as shown in Fig.3. We also noticed that SOD regulation was more significant in the digestive gland than in the gills for the three metals tested illustrating the tissue specificity in the oxidative stress response. These results suggest that gills may rapidly eliminate or chelate metals to limit the ROS production. In bivalves, cadmium may enter the gills by passive diffusion or across Ca2+-channels then be a rapidly bind to intracellular ligands and specific proteins such as metallothioneins that ensures removal of cadmium (Carpene and Georges, 1981, Frazier and George, 1983). In *Mytilus galloprovincialis* exposed to nanoparticules containing metals, the gills appeared to be more susceptible to dissolved metal toxicity, while digestive gland is more sensitive to nanoparticules illustrating the differential tissue response in the uptake, accumulation and detoxification processes (McCarthy et al., 2013; Gomes et al., 2012). An absence of regulation in the transcription of a SOD gene has been observed in gills of *M. gallorpincialis* in response to nanoparticules exposure (Bebianno et al., 2015) and in gills and digestive gland of *C. gigas* exposed to silver nanoparticules or dissolved silver (Mc Carthy et al., 2013). At a proteomic level, an extra cellular Cu/Zn SOD from the digestive gland of *C. gigas* was also found to be down-regulated after Cu exposure associated with an elevation of the copper contain in the protein suggesting a competition between zinc and copper (Xu et al., 2014). The active site of the SOD is characterized by the presence of metal binding site, normally copper, zinc or manganese. However, due to the differences in metal affinity for the binding site, a competition between metals can cause the replacement of regular metals by others with higher affinity such as cadmium or lead, but this replacement also depends of the metal concentration (Abreu et al 2010). .

Our analysis also revealed that SODs regulation is not linear and show a complex pattern according to metal and concentrations. In the hydrothermal vent mussel *B. azoricus,* the SOD activity has been studied in natural populations in the MAR evidencing specific population patterns together

with temporal variations (Bebianno et al., 2005; Company et al., 2010). Company et al (2006) also reported a positive increment between cadmium exposure and cytosolic SOD activities in gills after 6 days but not for the mitochondrial SOD where the activities decrease after the first day. In our study, the mitochondrial MnSOD present a stronger regulation in response to iron in both tissues and a limited regulation in response to cadmium. One interesting result of this study is that the difference in ratio of expression in experimental conditions is never very high suggesting either a strong regulation of SOD expression or a limited involvement of SODs in the metal response due to the limited ROS production associated with a rapid metal detoxification. We cannot exclude that other mechanisms or proteins than SODs could participate to counteract oxidative stress in the cells. The activities of total glutathione peroxidases and cytosolic catalases have been quantified in the two hydrothermal shrimps, *Mirocaris fortunate* and *Rimicaris exoculata* and are higher than in their costal homologs (Gonzalez-Rey et al.,2008). We may suggest that SOD expression is naturally high in *B. azoricus* as a response to hydrothermal environment and that the range of up-regulation of SODs genes is then limited. In the hydrothermal crabs *Segonzacia mesatlantica*, higher cMnSOD and mMnSOD mRNA expressions have been detected in individuals from Rainbow compared to crabs collected at LS suggesting an impact of environmental parameters (Marchand et al., 2009). At the contrary, based on SOD activities, Morril et al (1988) suggested that thiobiotic meiofauna which avoid oxic habitats should have much lower catalase and SOD levels than their oxybiotic counterparts.

5. Conclusion

These experiments of metal exposure have been generated to investigate the validate role of metals in the generation of oxidative stress in *B. azoricus* with the idea that SOD expression levels reported in gills and digestive gland could bring reflect the effect of metal exposure. Three SOD isoforms, SOD2, SOD3 and SOD4 could be considered as putative good biomarkers of metal exposure in *B. azoricus*. Additional experiments could also be conducted especially in response to hypoxia, temperature but also in presence of gases to better estimate the relative importance of the main environmental factors in the SODs regulation in this species. As a perspective, a purification of the different SOD isoforms of *B. azoricus* associated with the determination of their metal content could also bring useful information and the analysis of their specific activity will also contribute to understand their relative contribution in SOD activity so as the link between gene expression and protein activity.

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8. Figures and tables

Table 1. Primers used in qPCR for SOD relative expression.

Gene name	Sequence	GC	Tm	Pb Size
SOD1	F: GGATCACCTACTGATAGTAAACGGC	48	60.8	160
	R:GTCTAGCATGTACCACTATGGACCT	48	61.4	
SOD2	F: CTCGTCAGTAAATGTAACAGGGGAG	48	60.9	159
	R: CTCACCTCATCAGTTGGACTACCAT	48	61.9	
SOD3	F: GACAGAGCACTACAGCTACCATAGA	48	61.4	160
	R: GGACTACACTGAGAAGGGTAATTTCC	46.2	60.9	
SOD4	F: CTGATGGTCCAGTTAATCTCGACATC	46.2	61.2	154
	R: GTTGTTCCATCTGGGTTATAGTGAGG	46.2	61.1	
SOD5	F: ACAGAGAGCAGTGTTACAGGGATAC	48	61.8	162
	R: GGAGATAAGCCATCCAATGTTCCATC	46.2	61.7	
SOD6	F: GATAGGGAAACAGTTACAGTGCATC	44	59.7	160
	R: ATTTTTCTGGAATGCTCTGTACTGC	40	60.1	
SOD7	F: GCCCACATAAGACATATCGGTGATC	48	61.4	160
	R: CTAATCCCATATCGTCTGGTCCAAAG	46.2	61	
RbL15	F: TATGGTAAACCTAAGACACAAGGAGT			
	R: TGGAATGGATCAATCAAAATGATTTC			

Table 2. Percentage of similitude between the cytosolic SOD sequences of *B. azoricus* with other marine invertebrates, *M. galloprovincialis* (AFQ32467.1), *C. gigas* (EKC41617.1), *A.pompejana* (ABY50192.1) and *M. chilensis* (AEE60900.1).

	SOD-1 Cu/Zn	SOD-2 Cu/Zn	SOD-3 Cu/Zn	SOD-4 Cu/Zn	SOD-7 Cu/Zn
SOD-1 Cu/Zn					
SOD-2 Cu/Zn	44.8				
SOD-3 Cu/Zn	5.0	5.9			
SOD-4 Cu/Zn	19.5	19.6	3.4		
SOD-7 Cu/Zn	26.8	27.3	5.1	36.7	
Cu/Zn SOD *M. galloprovincialis*	27.2	29.2	5.0	35.6	56.0
Cu/Zn SOD *C. gigas*	5.1	5.6	37.6	3.3	5.0
Cu/Zn SOD *A.pompejana*	42.6	73.6	5.2	21.3	27.1
SOD *M. chilensis*	46.0	77.4	6.0	21.2	28.4

Table 3. Percentage of similitude between the mitochondrial SOD sequence of *B. azoricus* with other marine invertebrates, *S. purpuratus* (XP_791826.1), *L.anatina* (XP_01342 0018.1) and *E. pallida* (KXJ16556.1).

	SOD-6 Mn	Mn SOD *S. purpuratus*	Mn/Fe SOD *L. anatina*
SOD-6 Mn			
Mn SOD *S. purpuratus*	50.4		
Mn/Fe SOD *L. anatina*	50.4	43.7	
Fe SOD *E. pallida*	45.5	41.9	46.1

Table 4. Percentage of similitude between the Cu-chaperone SOD sequence of *B. azoricus* with other marine invertebrates, S. purpuratus (XP_011674044.1), L. anatina (XP_013404594.1), C. gigas (XP_011446778.1), C. intestinalis (XP_002122009.1).

	SOD-5 Cu chaperone SOD	SOD Cu chaperone *S. purpuratus*	SOD Cu chaperone *L. anatina*	SOD Cu chaperone *C. gigas*
SOD-5 Cu chaperone SOD				
Cu chaperone SOD *S. purpuratus*	41.7			
Cu chaperone SOD *L. anatina*	42.1	47.0		
Cu chaperone SOD *C. gigas*	48.0	44.4	43.4	
Cu chaperone SOD *C. intestinalis*	40.7	49.4	43.2	42.5

Table 5. Global Anova table between population for SOD relative expression in the seven populations for each tissue.

Population	Response	Df	MS	F	p-value
Gill	SOD1	6	963.7	7.92	1.08e-7
	SOD2	6	1266.6	10.24	6.52e-10
	SOD3	6	140.47	6.924	1.03e-6
	SOD4	6	201.52	7.234	5.11e-7
	SOD5	6	227.57	6.9	1.09e-6
	SOD6	6	184.51	3.469	0.0027
	SOD7	6	285.87	65.80	1.33e-5
Mantle	SOD1	6	49.81	0.686	0.661
	SOD2	6	213.3	8.05	8.93e-8
	SOD3	6	116.80	6.782	1.52e-6
	SOD4	6	168.35	6.71	1.79e-6
	SOD5	6	208.46	3.03	0.00734
	SOD6	6	87.03	5.71	1.72e-5
	SOD7	6	223.4	5.45	3.11e-5
D. Gland	SOD1	6	124.77	4.237	0.00049
	SOD2	6	304.64	6.272	4.86e-6
	SOD3	6	147.82	10.03	1.23e-9
	SOD4	6	160.34	13.7	6.13e-13
	SOD5	6	138.4	6.49	2.91e-6
	SOD6	6	171.17	9.462	4.15e-9
	SOD7	6	193.4	5.04	7.81e-5

Table 6. Anova table for relative expression of SODs between the three tissues for each population.

Population	Response	Df	MS	F	p-value
MG-2	**SOD1**	2	38.04	0.496	0.61
	SOD2	2	253.19	3.279	0.0424
	SOD3	2	5 934	0.369	0.692
	SOD4	2	6.36	0.141	0.869
	SOD5	2	1563.2	11.96	0.0000258
	SOD6	2	85.41	2.042	0.136
	SOD7	2	208.5	1.855	0.163
MG-3	**SOD1**	2	336.8	7.66	0.000879
	SOD2	2	109.51	1.306	0.279
	SOD3	2	356.3	7.827	0.000763
	SOD4	2	234.4	6.841	0.00177
	SOD5	2	499.9	11.58	0.0000362
	SOD6	2	346.7	6.309	0.0028
	SOD7	2	378.0	3.454	0.0362
MG-4	**SOD1**	2	421.9	3.498	0.0349
	SOD2	2	250.15	5.58	0.0053
	SOD3	2	219.43	7.661	0.000891
	SOD4	2	334.7	9.0	0.000293
	SOD5	2	246.05	6.099	0.00339
	SOD6	2	236.1	7.331	0.00118
	SOD7	2	63.44	0.625	0.538
Tour Eiffel	**SOD1**	2	887.4	7.558	0.000935
	SOD2	2	623.1	5.726	0.00459
	SOD3	2	191.2	3.438	0.0365
	SOD4	2	510.2	17.32	0.000000455

	SOD5	2	741.5	9.456	0.000191
	SOD6	2	968.8	10.82	0.0000631
	SOD7	2	416.9	5.22	0.0072
Montsegur	**SOD1**	2	26.63	2.95	0.0574
	SOD2	2	4.14	0.605	0.548
	SOD3	2	17.02	2.484	0.0891
	SOD4	2	17.41	2.973	0.0562
	SOD5	2	6.55	0.209	0.811
	SOD6	2	148.03	6.955	0.00155
	SOD7	2	12.59	0.708	0.495
Termitiére	**SOD1**	2	3 284	0.561	0.573
	SOD2	2	42.57	2.078	0.131
	SOD3	2	45.73	2.237	0.113
	SOD4	2	57.09	5.664	0.00487
	SOD5	2	31.04	0.797	0.454
	SOD6	2	462.0	18.91	0.000000152
	SOD7	2	617.6	13.83	0.0000061
Intermediate	**SOD1**	2	144.1	6.11	0.00347
	SOD2	2	113.68	4.44	0.015
	SOD3	2	67.64	3.474	0.0361
	SOD4	2	100.6	3.751	0.028
	SOD5	2	79.57	4.65	0.012
	SOD6	2	71.18	4.22	0.018
	SOD7	2	84.74	4.975	0.00936

Table 7. Anova table for relative expression of SOD in gill in mussels exposed to cadmium, copper and iron.

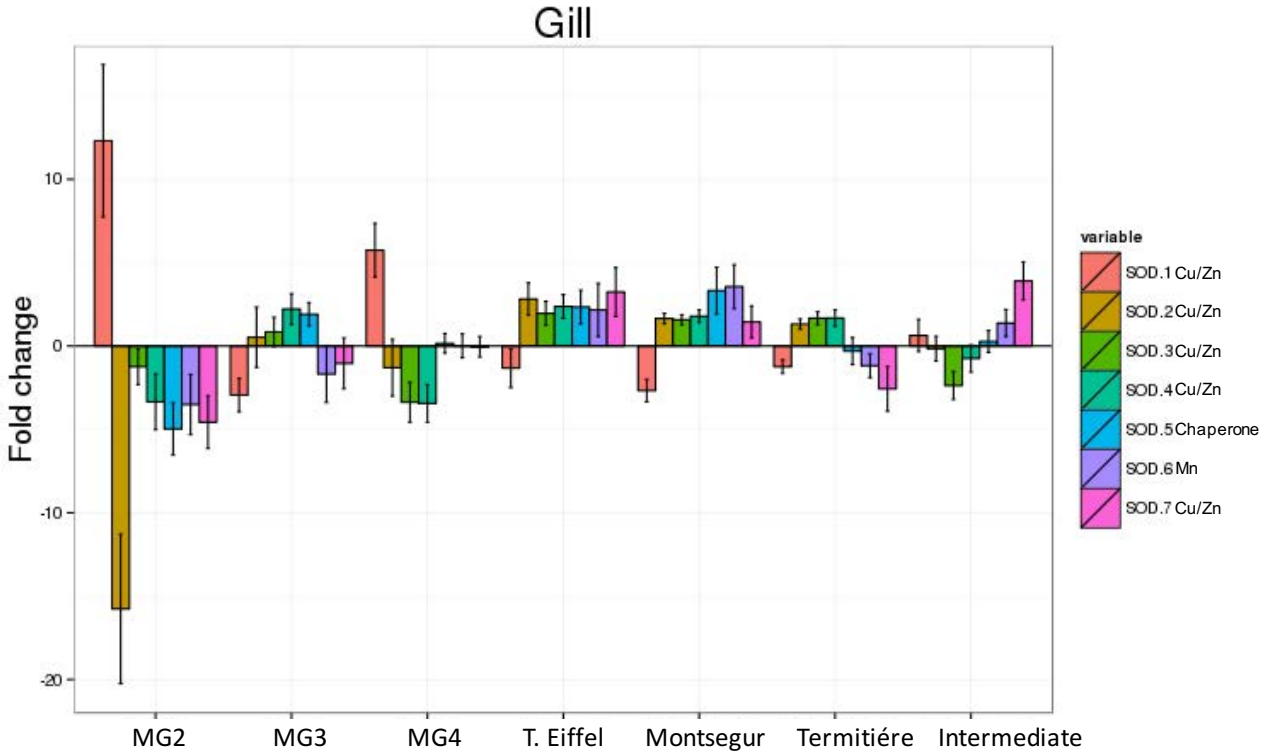
Metal	Response	Df	MS	F	p-value
Cadmium	SOD1	18	294.42	8.183	1.39e-14
	SOD2	18	156.99	9.131	3.53e-16
	SOD3	18	97.72	4.33	2.49e-7
	SOD4	18	36.81	2.726	0.000495
	SOD5	18	10.94	0.999	0.465
	SOD6	18	30.42	2.473	0.0016
	SOD7	18	25.02	1.413	0.134
Copper	SOD1	16	138.84	2.862	0.000616
	SOD2	16	358.5	10.53	7.91e-16
	SOD3	16	113.5	1.075	0.388
	SOD4	16	39.38	2.809	0.00076
	SOD5	16	28.62	1.644	0.0689
	SOD6	16	15.22	1.477	0.121
	SOD7	16	30.70	2.609	0.0017
Iron	SOD1	19	32.05	2.246	0.00342
	SOD2	19	288.37	17.28	2.0e-16
	SOD3	19	84.08	8.157	1.15e-15
	SOD4	19	34.92	2.468	0.0011
	SOD5	19	35.92	2.482	0.0010
	SOD6	19	23 282	3.074	5.58e-5
	SOD7	19	11 118	1.684	0.0434

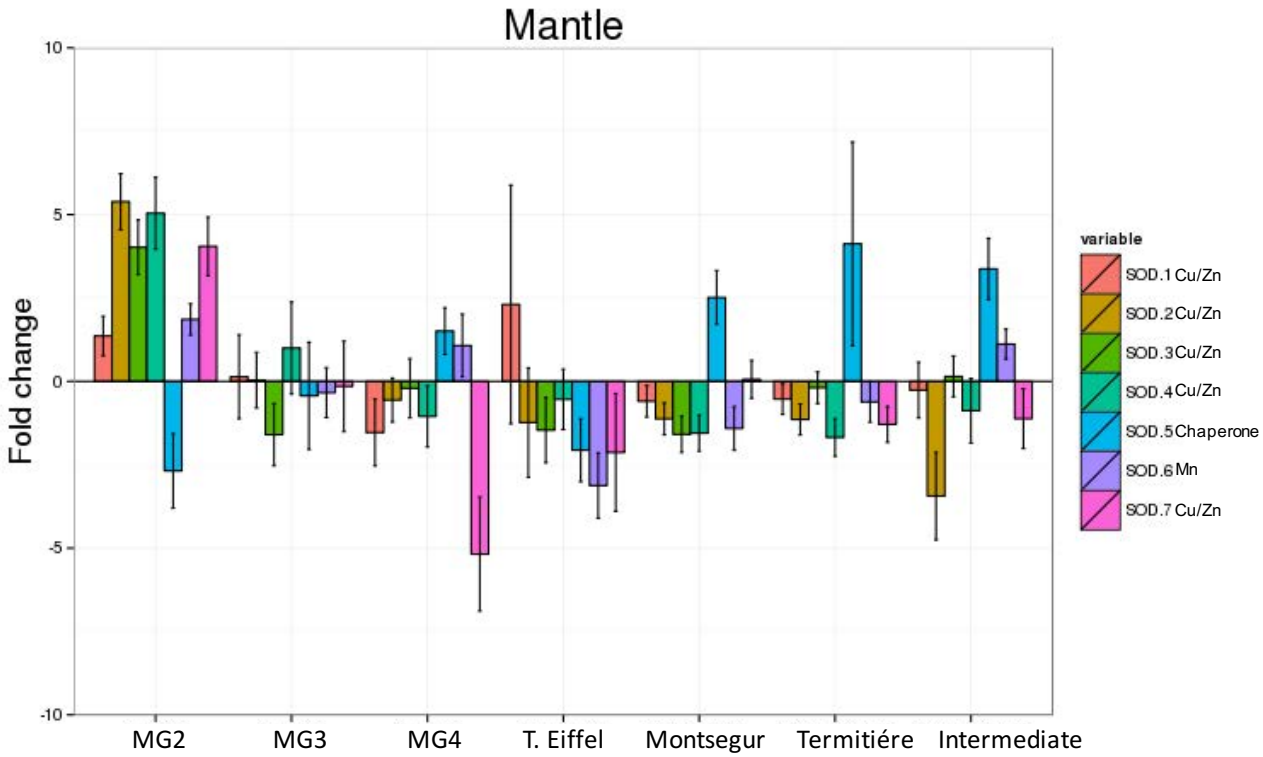
Table 8. Anova table for relative expression of SOD in digestive gland in mussels exposed to cadmium, copper and iron.

Metal	Response	Df	MS	F	p-value
Cadmium	SOD1	18	34.30	3.716	3.38e-6
	SOD2	18	53.81	6.447	7.44e-12
	SOD3	18	49.93	4.544	5.86e-8
	SOD4	18	40.02	4.203	3.08e-7
	SOD5	18	20.92	3.601	5.94e-6
	SOD6	18	42.37	5.574	4.23e-10
	SOD7	18	29.155	3.288	2.77e-5
Copper	SOD1	16	29.899	5.35	1.12e-8
	SOD2	16	47.44	6.219	3.21e-10
	SOD3	16	53.34	4.297	9.56e-07
	SOD4	16	54.97	5.595	4.05e-9
	SOD5	16	401.0	6.454	1.26e-10
	SOD6	16	18.469	2.521	0.00203
	SOD7	16	26.67	3.065	0.00019
Iron	SOD1	19	12.445	2.162	0.0048
	SOD2	19	28.075	7.333	1.94e-14
	SOD3	19	32.11	6.602	6.5e-13
	SOD4	19	26.62	3.544	4.09e-6
	SOD5	19	13527	2.019	0.00948
	SOD6	19	12.43	2.769	0.000234
	SOD7	19	15.51	1.399	0.132

Table 9: List of SOD significantly regulated in the different metal exposure experimental conditions and in the two tissues at the different sampling times. Significant values have been obtained by the comparison between each sampling time and their respective control using a Student's t-test after 100 permutations and a p-value < 0.05.

Experimentation	Comparison	T5	T10	T15	T20	T25
T0 –gill		2, 3	2, 3	1, 2, 3, 4	2, 3, 6	
T0- dig gland		2	5	2, 3, 6	2, 3, 4, 6	7
Cd-gill	0.2 µM/CTR	1	1, 2, 3, 4		2, 3, 6	
	1µM/CTR	2, 4	2		1, 2, 3, 6	
	4 µM/CTR	2	2, 3	1, 4		
	20 µM/CTR	2, 7	3	1, 4		
Cd-dig gland	0.2 µM/CTR		5	2	1	
	1µM/CTR	1, 2, 3, 4	2, 4	1, 2, 3, 6	2, 4	
	4 µM/CTR	4, 5, 6, 7	1, 2, 4		2	
	20 µM/CTR	1, 2, 4, 5, 6, 7	2, 4	2, 5, 7	2	
Cu-gill	4 µM/CTR	2	1, 4	1, 4	2, 3	
	25 µM/CTR	2	3		2	
	60 µM/CTR	2, 7	2	2	2	
Cu-dig gland	4 µM/CTR	1, 2, 3, 4, 6	5	1, 3, 5, 6, 7	1, 2, 3, 4	
	25 µM/CTR	2, 3, 4, 6, 7	2, 3, 7	1, 2	1, 2, 3, 4, 7	
	60 µM/CTR	2	1, 4, 5	7	1, 2, 3, 4, 7	
Fe-gill	1 µM/CTR	2	2	2	2, 6, 7	1, 2
	5 µM/CTR	2, 4,	4	2, 4, 7	2, 3	2, 6
	20 µM/CTR	2, 6	2	5	2, 6	
Fe-dig gland	1 µM/CTR		3, 4	5	1, 2, 3	1
	5 µM/CTR	2, 7	3	4	1, 2, 3, 5, 6	1
	20 µM/CTR	3, 4, 5, 6	3	1, 3, 7	1, 2, 3, 4	


Gill
Fold change
10
0
-10
-20
MG2
MG3
MG4
T. Eiffel
Montsegur
Termitiére
Intermediate
variable
SOD.1 Cu/Zn
SOD.2 Cu/Zn
SOD.3 Cu/Zn
SOD.4 Cu/Zn
SOD.5 Chaperone
SOD.6 Mn
SOD.7 Cu/Zn


Mantle
Fold change
10
5
0
-5
-10
MG2
MG3
MG4
T. Eiffel
Montsegur
Termitiére
Intermediate
variable
SOD.1 Cu/Zn
SOD.2 Cu/Zn
SOD.3 Cu/Zn
SOD.4 Cu/Zn
SOD.5 Chaperone
SOD.6 Mn
SOD.7 Cu/Zn

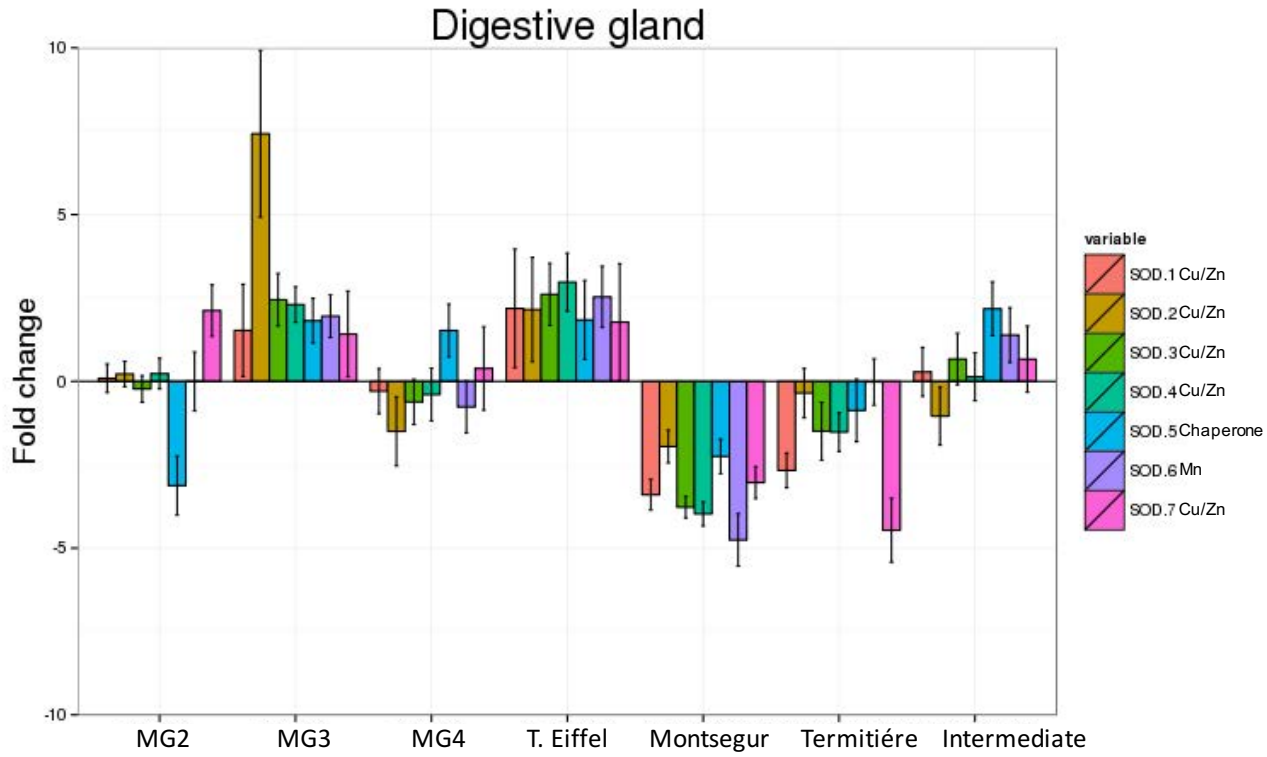


Figure 1. Relative expression (fold change) of the seven SODs between the seven populations analysed in the three tissues gill, mantle and digestive gland. Data are normalized using the mean expression of each SODs isoform in the same tissues in all populations.

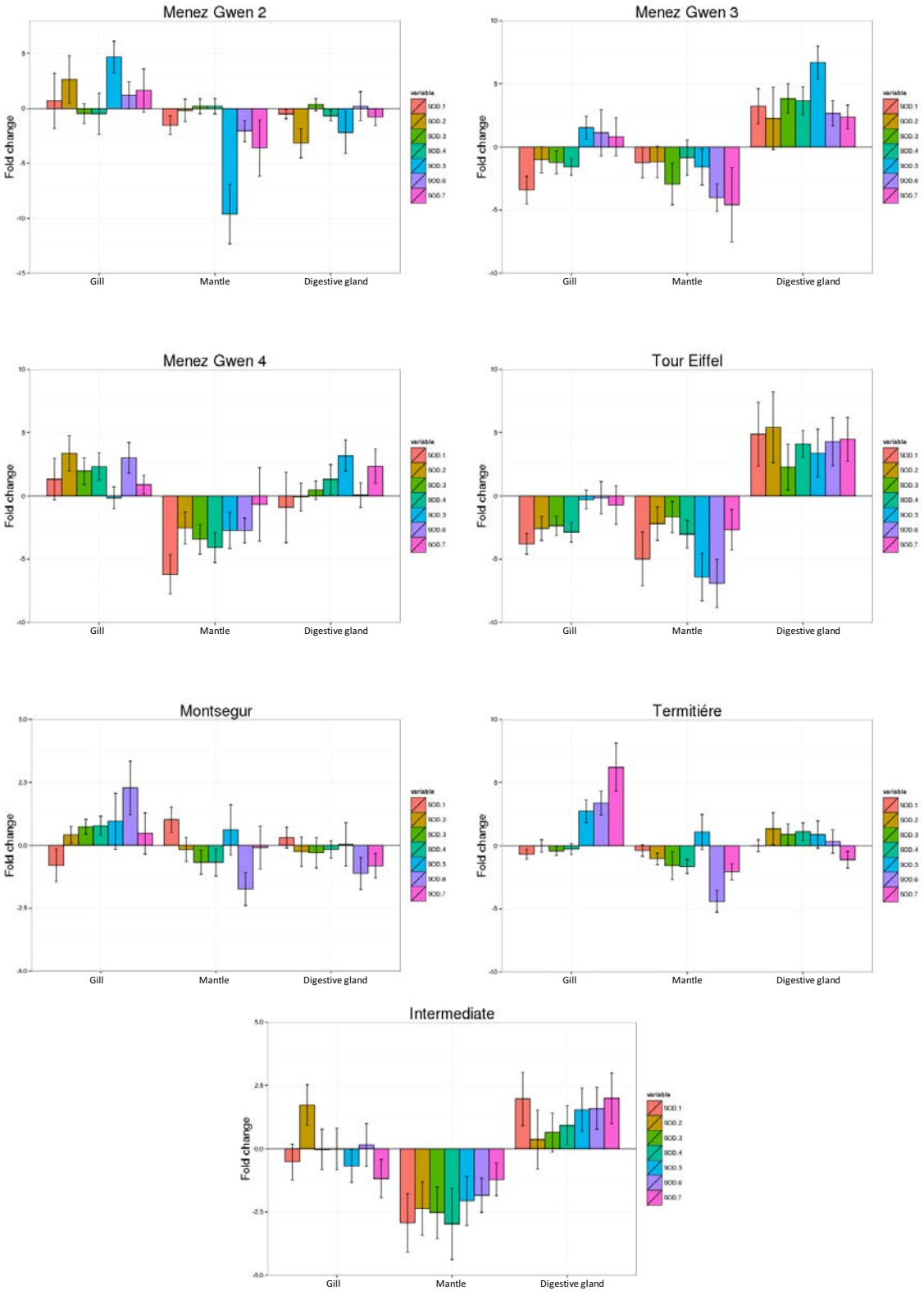
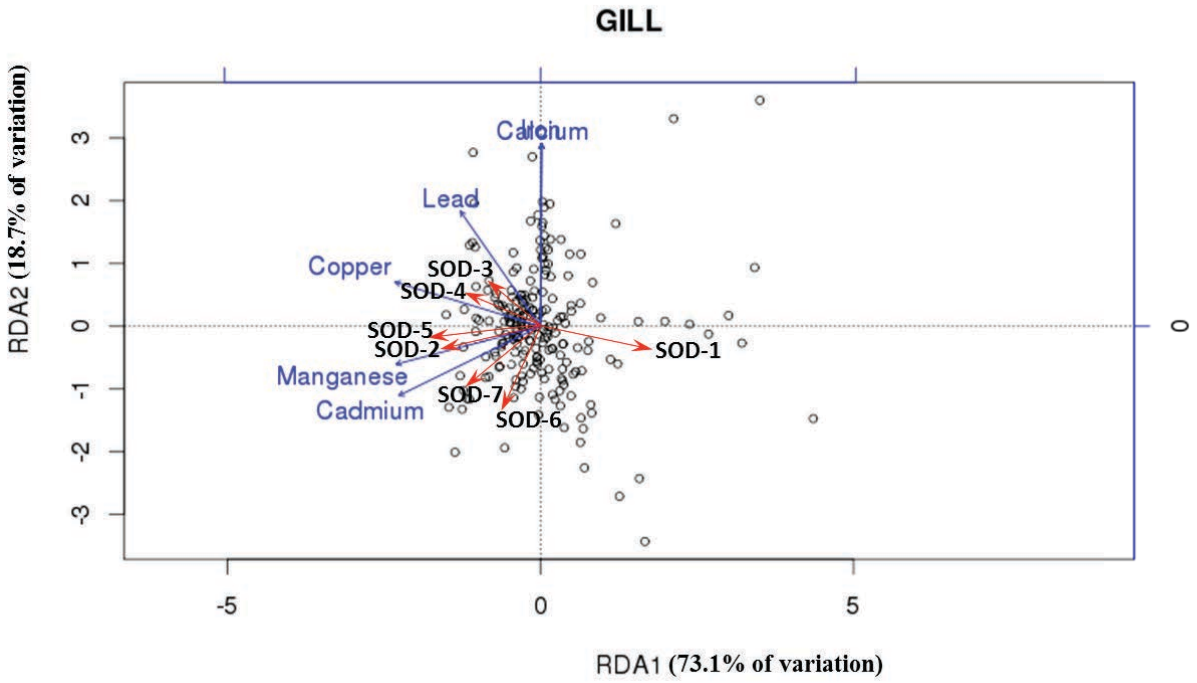
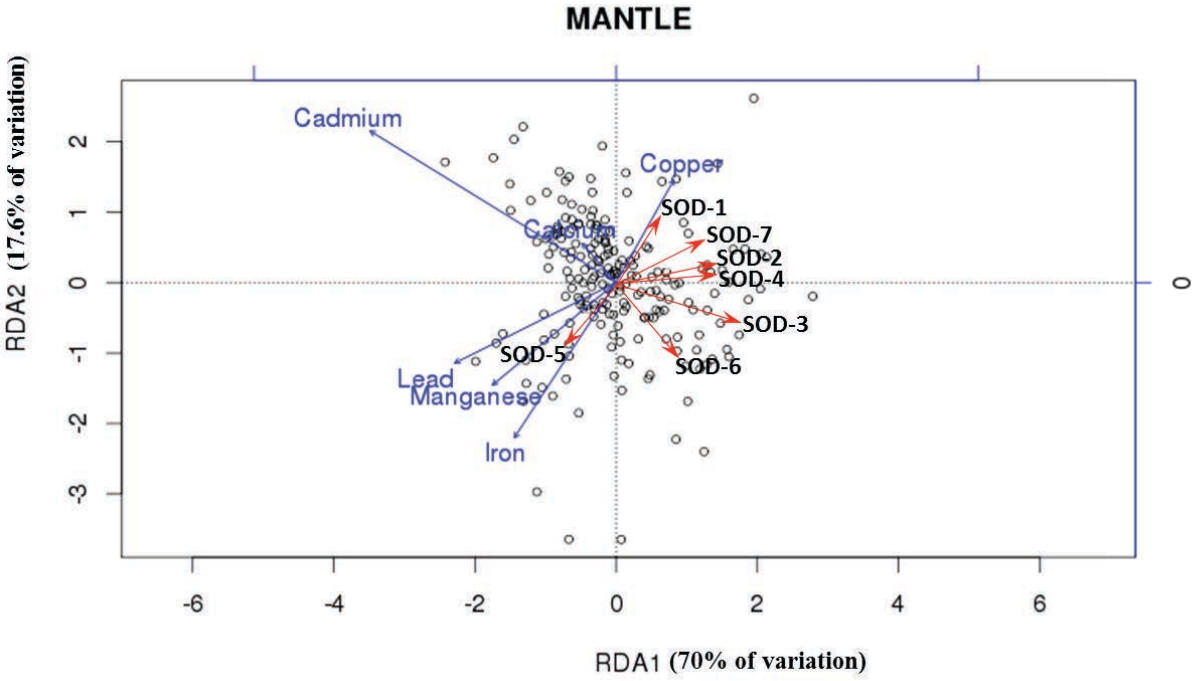


Figure 2. Relative expression (fold change) of the seven SODs in the three tissues gill, mantle and digestive gland analysed in the seven populations. Data are normalized using the mean expression of each SODs isoform in the three tissues within each population.


GILL
RDA2 (18.7% of variation)
RDA1 (73.1% of variation)
Calcium
Lead
Copper
SOD-3
SOD-4
SOD-5
SOD-2
SOD-1
Manganese
Cadmium
SOD-7
SOD-6


MANTLE
RDA2 (17.6% of variation)
RDA1 (70% of variation)
Cadmium
Copper
SOD-1
Calcium
SOD-7
SOD-2
SOD-4
SOD-3
SOD-5
SOD-6
Lead
Manganese
Iron

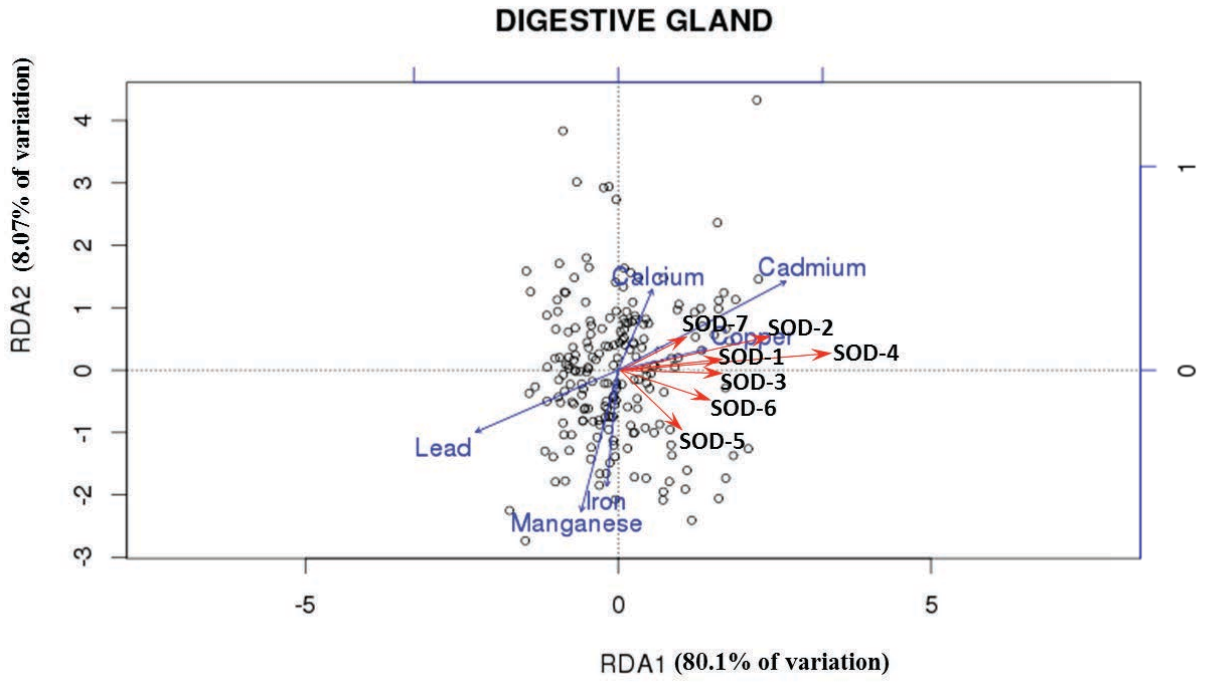


Figure 3. Global redundancy analysis (RDA) for metal concentration and relative SODs gene expression in the three tissues, gill, mantle and digestive gland. The angle between arrows represents the degree of correlation between variables.

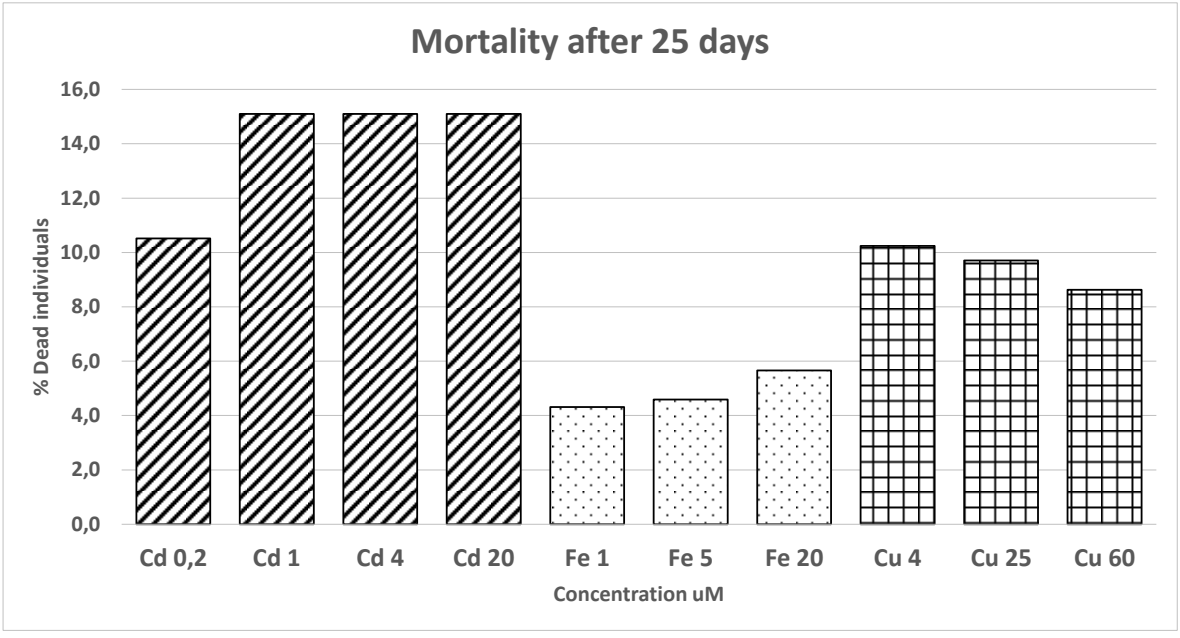


Figure 4. Percentage of mortality after 25 days of exposition to the different metals and concentrations.

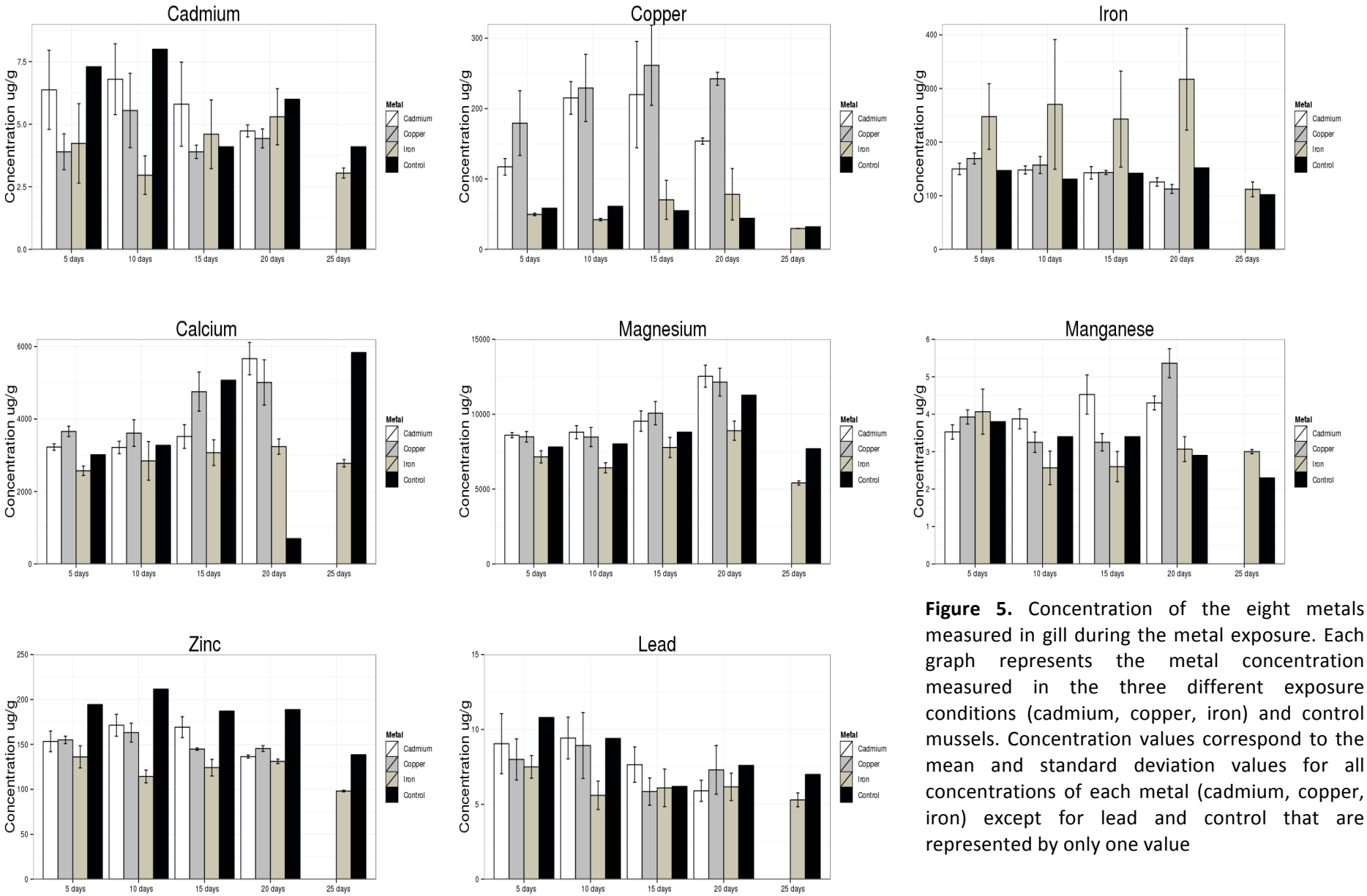


Figure 5. Concentration of the eight metals measured in gill during the metal exposure. Each graph represents the metal concentration measured in the three different exposure conditions (cadmium, copper, iron) and control mussels. Concentration values correspond to the mean and standard deviation values for all concentrations of each metal (cadmium, copper, iron) except for lead and control that are represented by only one value

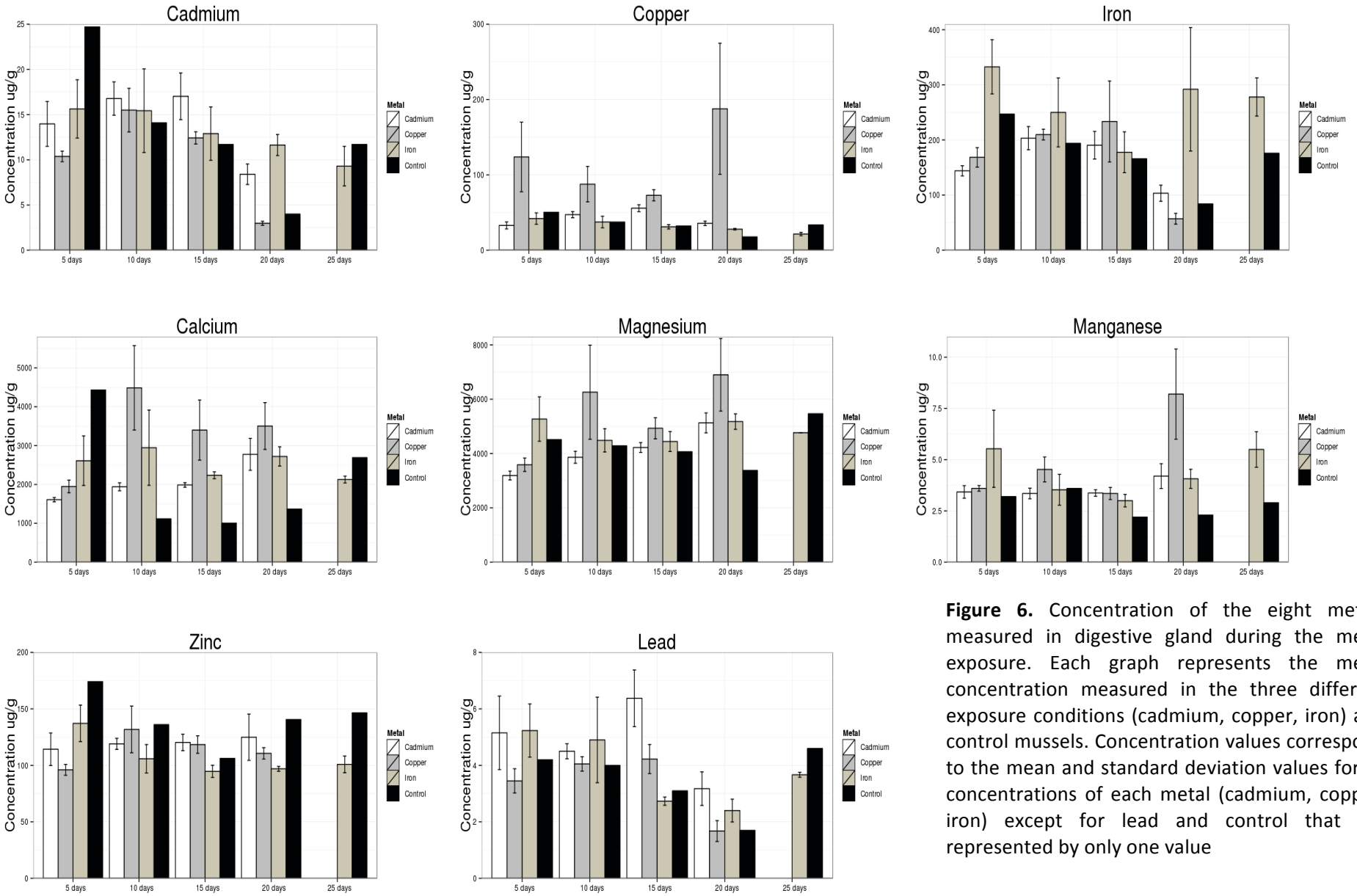
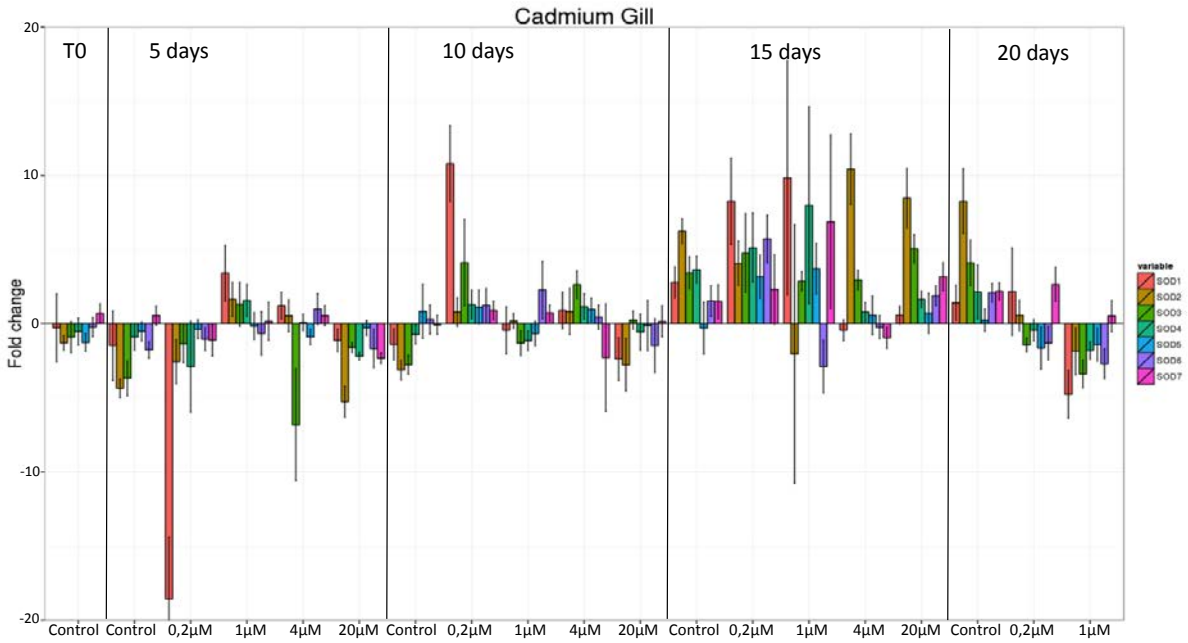
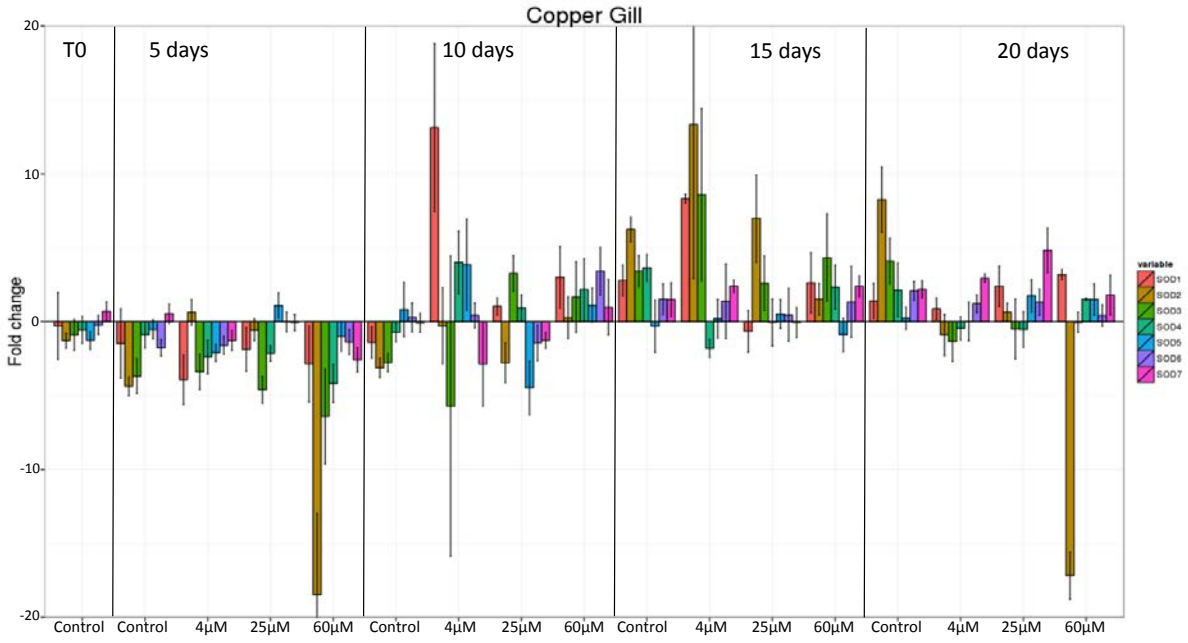


Figure 6. Concentration of the eight metals measured in digestive gland during the metal exposure. Each graph represents the metal concentration measured in the three different exposure conditions (cadmium, copper, iron) and control mussels. Concentration values correspond to the mean and standard deviation values for all concentrations of each metal (cadmium, copper, iron) except for lead and control that are represented by only one value


Cadmium Gill
T0
5 days
10 days
15 days
20 days
Fold change
20
10
0
-10
-20
Control Control 0,2µM 1µM 4µM 20µM Control 0,2µM 1µM 4µM 20µM Control 0,2µM 1µM 4µM 20µM Control 0,2µM 1µM
variable
SOD1
SOD2
SOD3
SOD4
SOD5
SOD6
SOD7


Copper Gill
T0
5 days
10 days
15 days
20 days
Fold change
20
10
0
-10
-20
Control Control 4µM 25µM 60µM Control 4µM 25µM 60µM Control 4µM 25µM 60µM Control 4µM 25µM 60µM
variable
SOD1
SOD2
SOD3
SOD4
SOD5
SOD6
SOD7

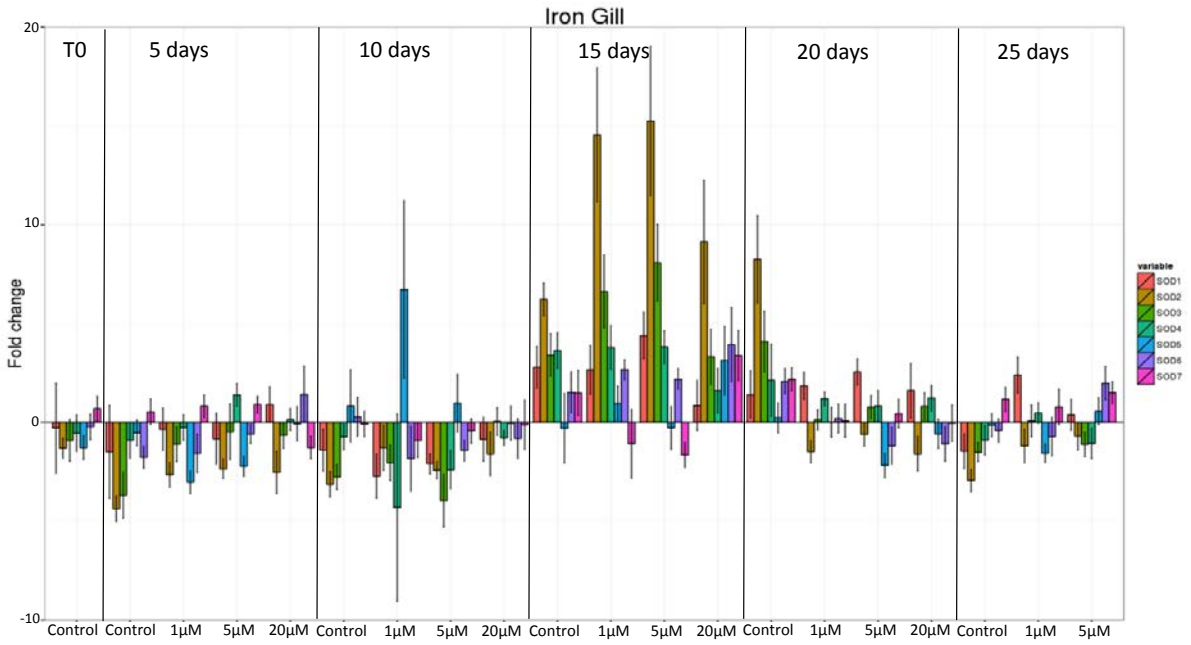
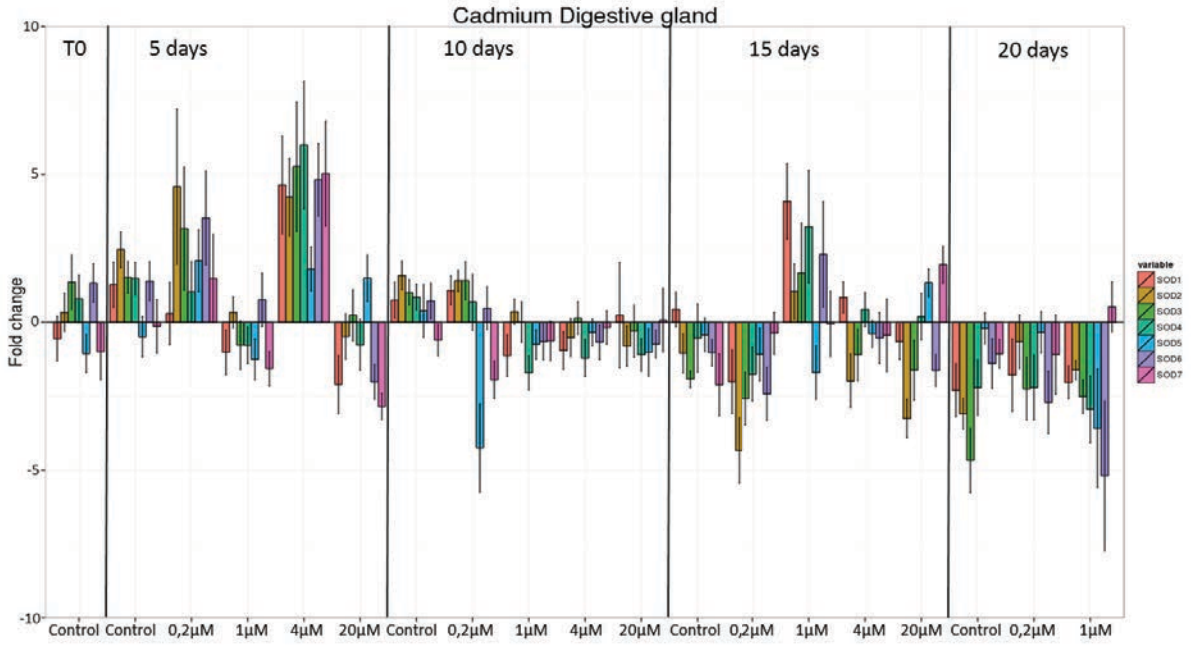


Figure 7. Relative expression levels in gills for the seven SODs genes during 20-25 days of exposure to cadmium, copper and iron in different concentrations.



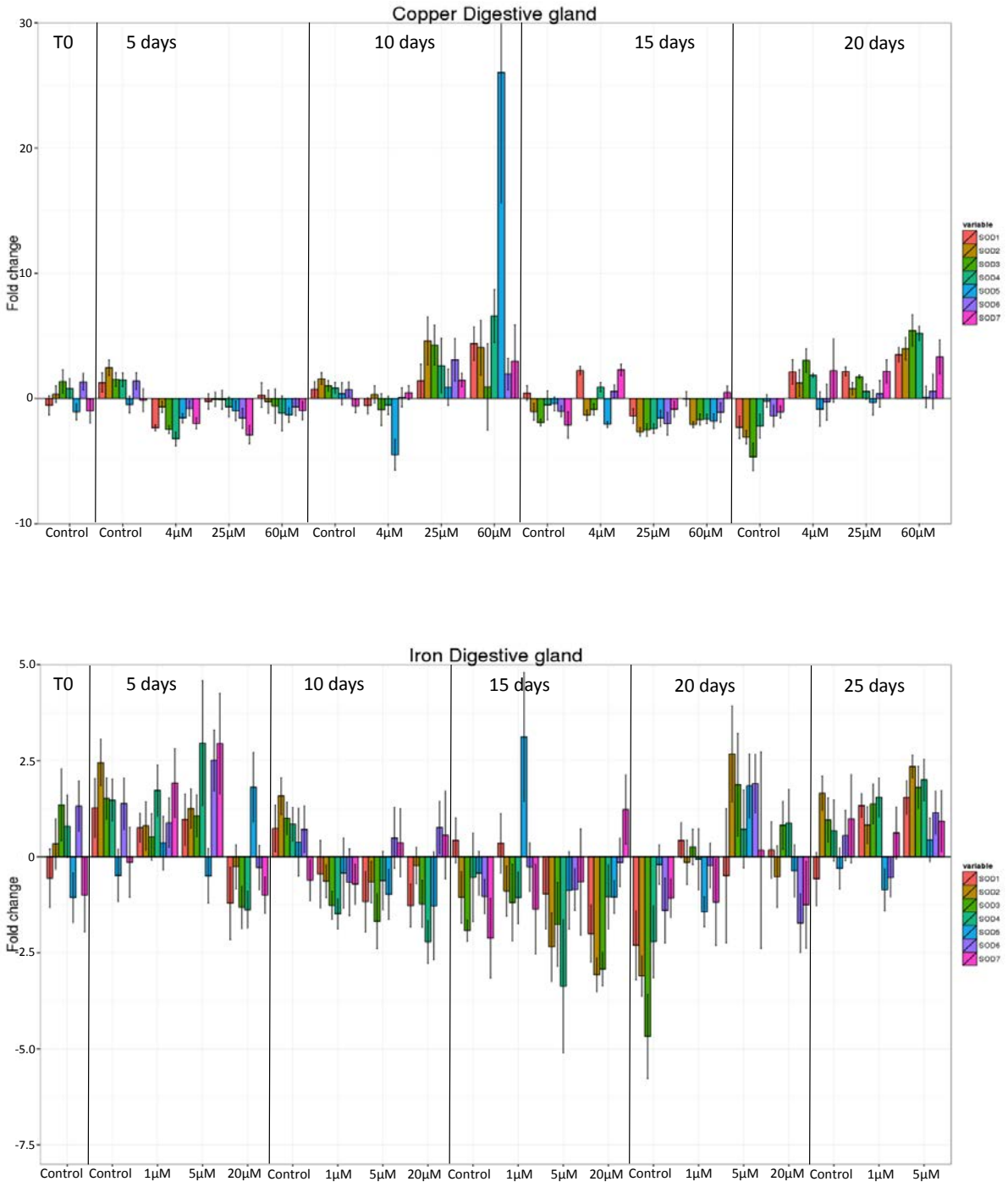
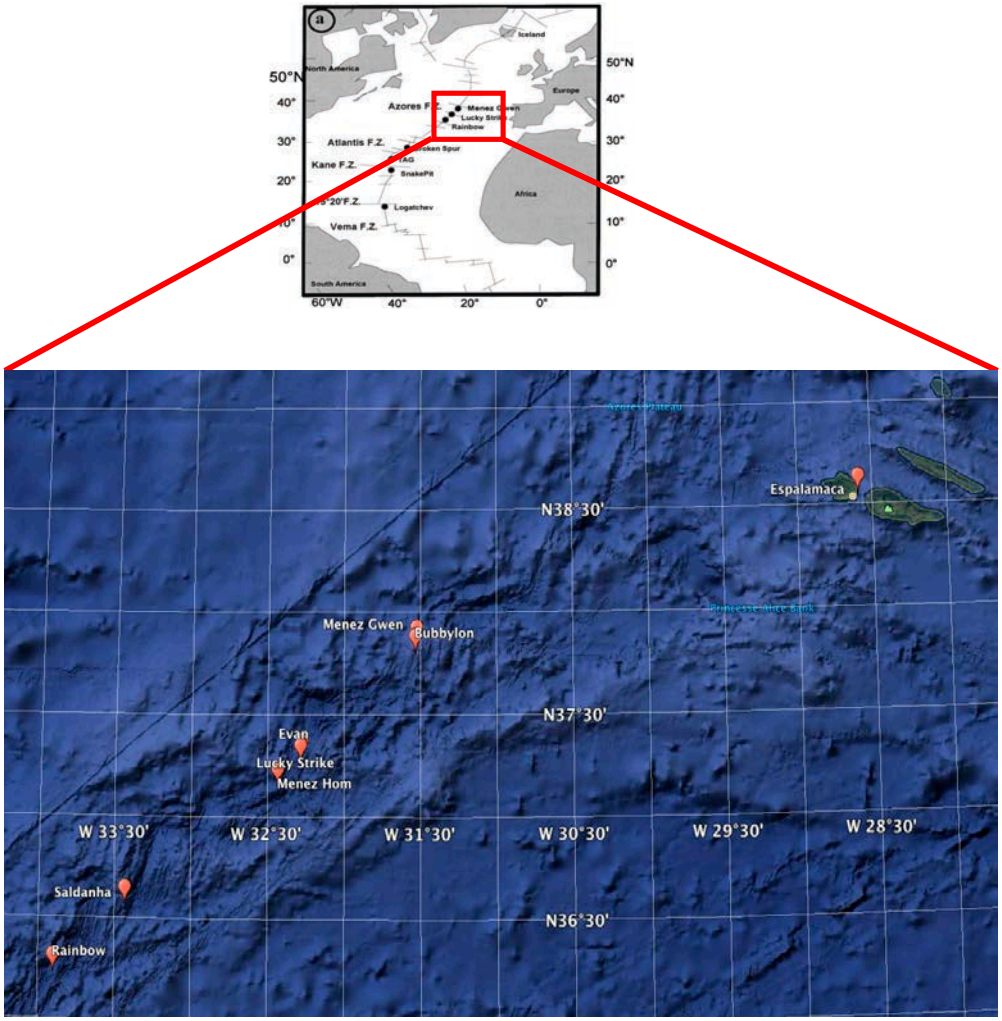


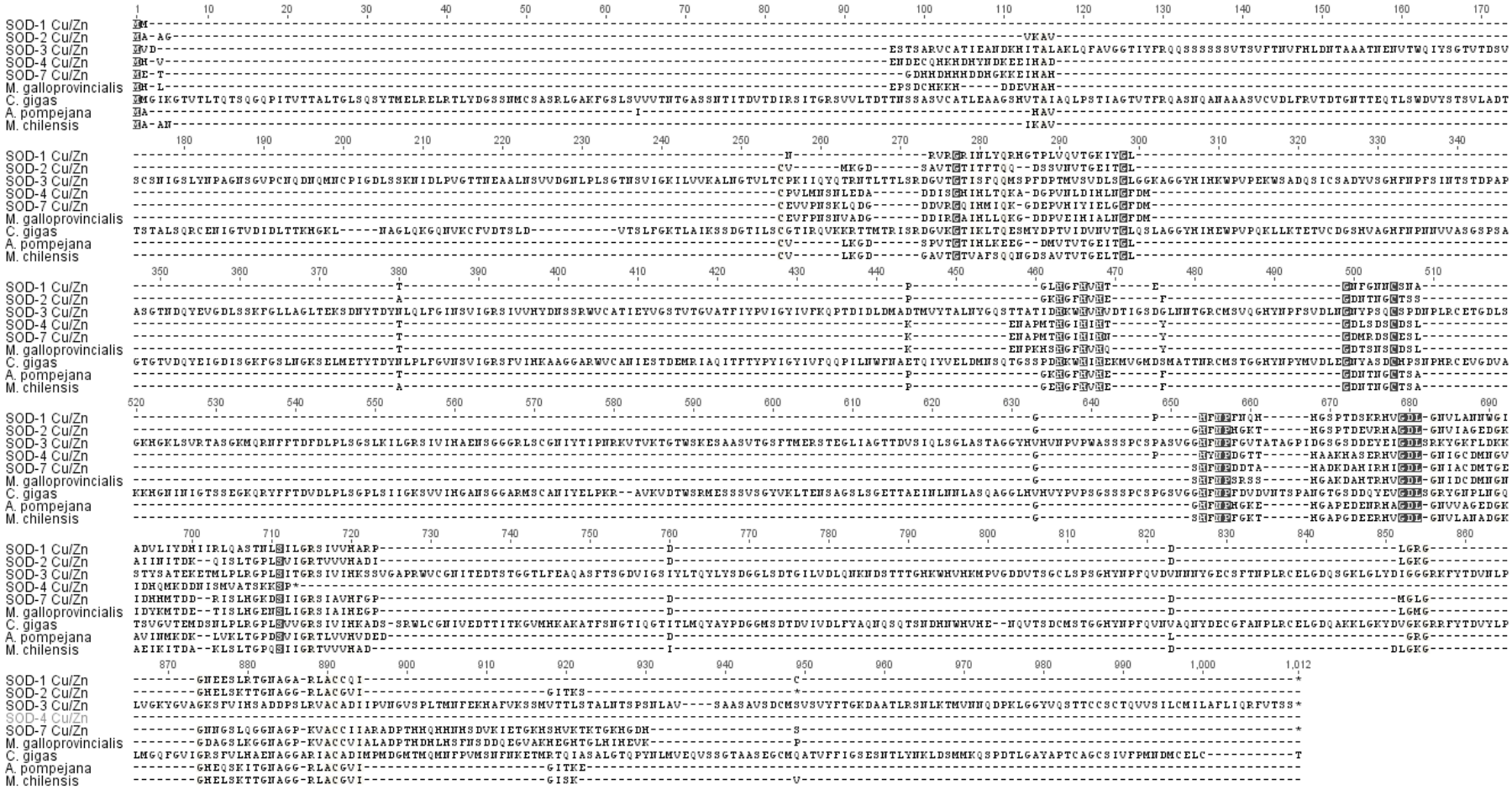
Figure 8. Relative expression levels in digestive gland for the seven SODs genes during 20-25 days of exposure to cadmium, copper and iron in different concentrations.

Appendices

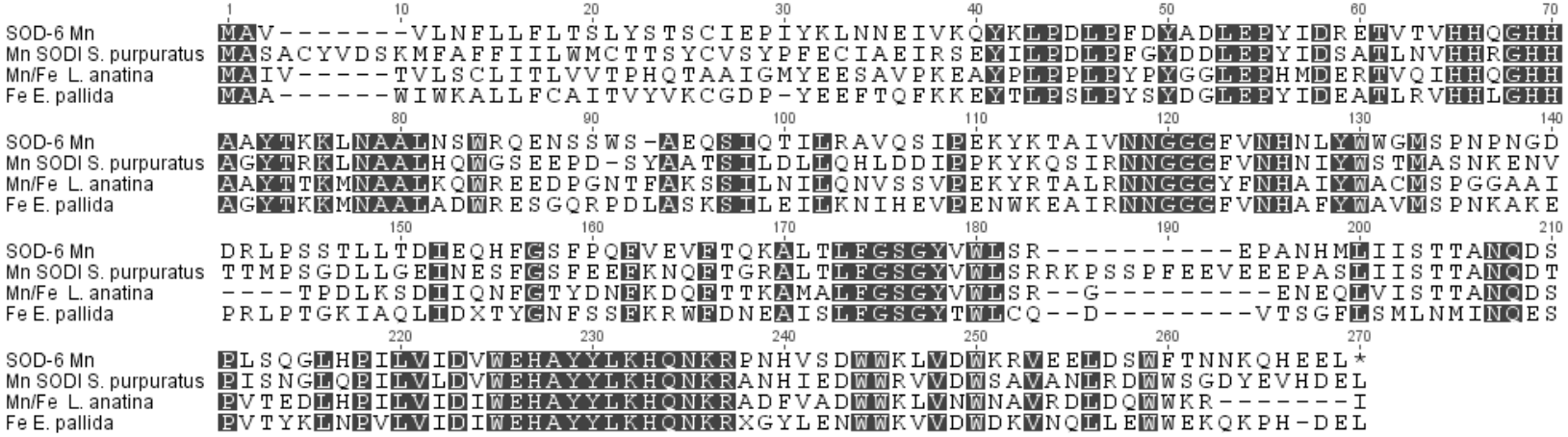
Apendix 1. Sampling area and physico-chemical conditions at the three hydrothermal vent sites of the Mid-Atlantic Ridge : Menez Gwen (MG). Lucky Strike (LS) and Rainbow (RB). Adapted from Charlou et al 2002.



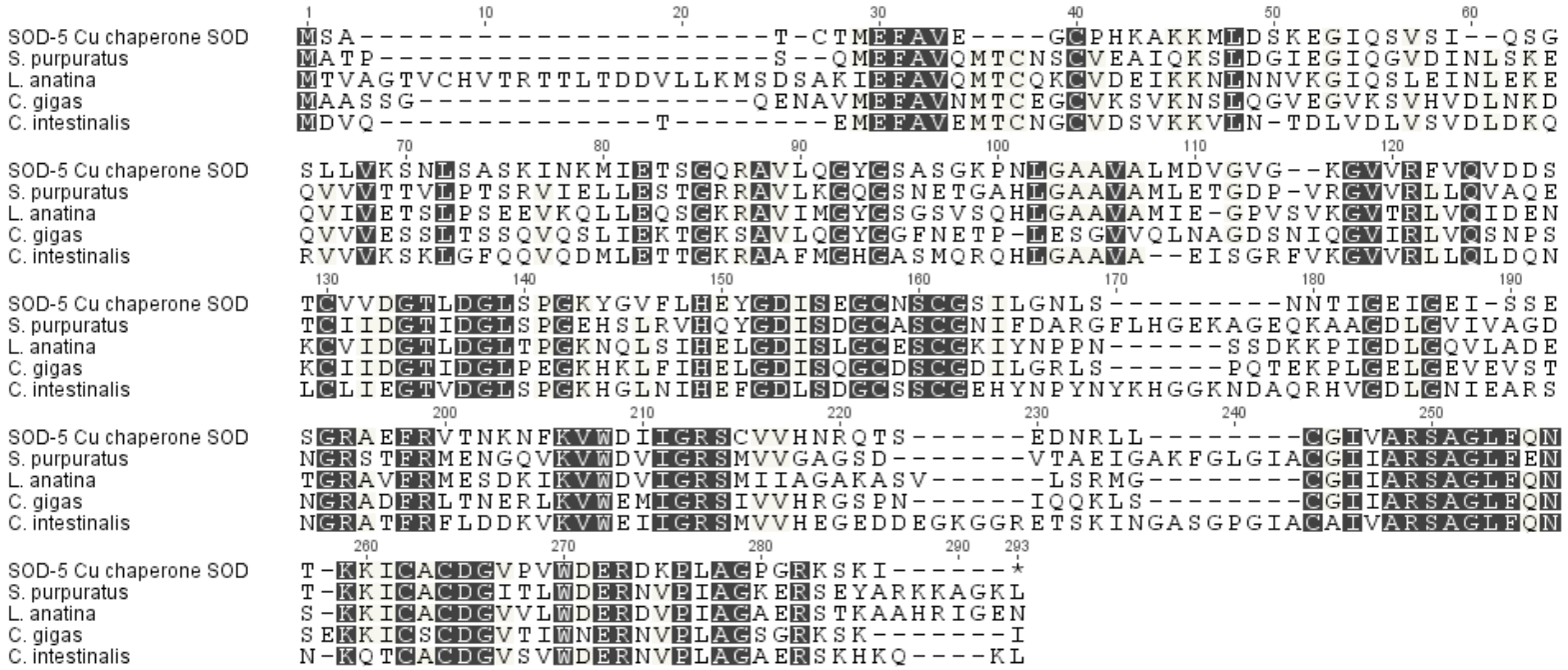
Apendix 2. Alignment of the five cytosolic superoxide dismutase with the sequences of *M. galloprovincialis* (AFQ32467.1), *C. gigas* (EKC41617.1), *A.pompejana* (ABY50192.1) and *M. chilensis* (AEE60900.1).



Apendix 3. Alignment of the mitochondrial superoxide dismutase (SOD6)with the sequences of *S. purpuratus* (XP_791826.1), *L.anatina* (XP_013420018.1) and *E. pallida* (KXJ16556.1).



Apendix 4. Alignment of the chaperone superoxide dismutase (SOD5)with the sequences of S. purpuratus (XP_011674044.1), L. anatina (XP_013404594.1), C. gigas (XP_011446778.1), C. intestinalis (XP_002122009.1).



Apendix 5. Measured concentrations in gills and digestive gland of cadmium, copper, iron and lead across 25 exposure days for each metal concentration and control mussels, (x) represent not measured concentration due mortality.

µg/g of dry weight	GILLS																			
	After 5 days				After 10 days				After 15 days				After 20 days				After 25 days			
Metal	**Cd**	**Cu**	**Fe**	**Pb**	**Cd**	**Cu**	**Fe**	**Pb**	**Cd**	**Cu**	**Fe**	**Pb**	**Cd**	**Cu**	**Fe**	**Pb**	**Cd**	**Cu**	**Fe**	**Pb**
Cd 0,2 µM	3,1	93,5	167	5,5	10,6	209,2	140	13,1	3,6	141,6	174	6,2	4,5	156,5	148	7,9	x	x	x	x
Cd 1 µM	6,0	100,7	162	7,1	4,8	152,6	170	9,5	10,8	153,9	147	10,5	5,4	141,9	113	5,0	x	x	x	x
Cd 4 µM	10,7	137,8	151	14,7	4,5	241,6	136	6,4	4,1	137,8	123	5,3	4,3	163,3	116	4,8	x	x	x	x
Cd 20 µM	5,7	137,3	120	8,9	7,3	257,4	146	8,7	4,7	446,6	127	8,6	x	x	x	x	x	x	x	x
Cu 4 µM	5,9	98,9	139	8,5	3,8	231,2	143	7,3	4,5	242,1	136	8,3	4,0	225,8	89	11,9	x	x	x	x
Cu 25 µM	2,6	107,6	177	4,0	2,8	130,5	198	5,0	3,5	113,1	151	3,9	3,8	267,9	127	4,9	x	x	x	x
Cu 60 µM	3,9	222,0	181	9,5	9,5	358,2	164	15,2	4,2	311,3	149	5,7	5,5	233,6	122	5,1	x	x	x	x
Fe 1 µM	2,0	53,1	190	6,0	4,5	45,7	130	7,5	2,8	125,6	148	4,7	7,5	41,3	143	7,9	3,4	29,6	88	6,1
Fe 5 µM	3,4	48,6	183	8,1	2,3	40,3	170	4,6	7,3	38,0	159	8,6	4,6	42,2	340	4,8	2,7	29,7	136	4,5
Fe 20 µM	7,3	47,3	370	8,4	2,1	40,9	511	4,7	3,7	47,6	422	5,0	3,8	151,3	469	5,8	x	x	x	x
Controles	7,3	58,5	147	10,8	8,0	61,2	131	9,4	4,1	55,0	142	6,2	6,0	44,2	152	7,6	4,1	32,2	102	7,0
µg/g of dry weight	DIGESTIVE GLAND																			
	After 5 days				After 10 days				After 15 days				After 20 days				After 25 days			
Metal	**Cd**	**Cu**	**Fe**	**Pb**	**Cd**	**Cu**	**Fe**	**Pb**	**Cd**	**Cu**	**Fe**	**Pb**	**Cd**	**Cu**	**Fe**	**Pb**	**Cd**	**Cu**	**Fe**	**Pb**
Cd 0,2 µM	11,0	29,4	153	3,6	20,3	48,6	191	4,6	12,7	44,4	262	7,0	8,1	33,8	122	2,7	x	x	x	x
Cd 1 µM	11,6	22,6	122	3,2	17,4	35,2	171	5,1	22,7	60,6	152	8,5	11,3	43,4	126	4,8	x	x	x	x
Cd 4 µM	21,4	44,5	164	8,9	11,6	52,4	265	3,8	20,1	52,7	188	6,3	5,8	29,6	62	2,0	x	x	x	x
Cd 20 µM	11,9	35,0	137	4,9	17,8	52,5	186	4,5	12,6	65,2	160	3,7	x	x	x	x	x	x	x	x
Cu 4 µM	11,2	26,1	149	4,0	12,4	61,0	189	4,3	10,4	76,8	150	5,3	2,6	46,4	55	1,0	x	x	x	x
Cu 25 µM	11,3	195,6	204	2,3	13,8	156,6	231	4,4	12,9	54,1	145	3,2	3,6	84,0	82	2,7	x	x	x	x
Cu 60 µM	8,8	64,0	129	3,3	22,7	55,0	221	4,2	13,2	70,6	187	3,5	2,7	432,8	34	1,3	x	x	x	x
Fe 1 µM	18,4	57,1	431	7,1	24,1	51,2	202	7,8	8,0	31,3	110	2,5	13,5	25,9	172	2,4	5,5	17,7	218	3,8
Fe 5 µM	9,2	33,7	284	4,5	8,3	24,8	174	2,7	12,5	35,8	185	3,0	9,5	28,4	188	1,7	13,1	25,3	338	3,5
Fe 20 µM	19,3	35,1	283	4,1	13,9	36,2	374	4,2	18,2	26,0	238	2,7	11,9	29,3	516	3,1	x	x	x	x
Controles	24,7	50,4	247	4,2	14,1	37,4	194	4,0	11,7	32,2	166	3,1	4,0	17,7	84	1,7	11,7	33,6	176	4,6

Apendix 6. Measured concentrations in gills and digestive gland of zinc, calcium, magnesium and manganese across 25 exposure days for each metal concentration and control mussels, (x) represent not measured concentration due mortality.

µg/g of	GILLS																			
dry weight	After 5 days				After 10 days				After 15 days				After 20 days				After 25 days			
Metal	**Zn**	**Ca**	**Mg**	**Mn**	**Zn**	**Ca**	**Mg**	**Mn**	**Zn**	**Ca**	**Mg**	**Mn**	**Zn**	**Ca**	**Mg**	**Mn**	**Zn**	**Ca**	**Mg**	**Mn**
Cd 0,2 µM	152,7	2994	8376	4,1	200,5	3017	8434	4,1	181,6	3416	9603	5,7	140,7	5898	14367	3,9	x	x	x	x
Cd 1 µM	130,8	3426	8889	3,4	182,1	3721	9971	4,3	187,2	4311	10783	5,1	136,1	6628	12477	4,8	x	x	x	x
Cd 4 µM	184,9	3263	8892	3,3	156,0	3149	8883	4,0	135,6	3617	10129	3,5	132,6	4473	10756	4,2	x	x	x	x
Cd 20 µM	145,0	3222	8224	3,3	146,8	2974	7895	3,1	172,3	2726	7624	3,8	x	x	x	x	x	x	x	x
Cu 4 µM	149,6	3241	8185	3,6	151,3	2917	8219	3,1	144,1	3333	9799	2,8	142,9	4114	9836	5,4	x	x	x	x
Cu 25 µM	152,8	3748	9241	4,3	162,8	4387	9186	3,9	141,5	5940	11890	3,9	154,1	6777	14391	4,4	x	x	x	x
Cu 60 µM	167,3	3886	8875	3,6	192,8	4089	9720	3,4	147,1	4732	10458	3,2	139,2	4135	12192	6,3	x	x	x	x
Fe 1 µM	117,7	2311	6343	4,9	128,6	2072	6038	3,2	128,5	3743	8700	3,3	132,3	3599	9838	3,4	96,6	2604	5166	3,1
Fe 5 µM	159,4	2705	7656	4,4	107,8	3872	6120	2,8	106,3	2545	6475	2,6	134,8	3248	9184	3,4	99,6	2954	5652	2,9
Fe 20 µM	131,3	2699	7470	2,9	106,6	2589	7101	1,7	137,6	2927	8168	1,9	126,4	2869	7674	2,4	x	x	x	x
Controles	194,4	3015	7812	3,8	211,5	3276	8018	3,4	187,1	5072	8802	3,4	188,8	700	11270	2,9	138,5	5837	7698	2,3
µg/g of	DIGESTIVE GLAND																			
dry weight	After 5 days				After 10 days				After 15 days				After 20 days				After 25 days			
Metal	**Zn**	**Ca**	**Mg**	**Mn**	**Zn**	**Ca**	**Mg**	**Mn**	**Zn**	**Ca**	**Mg**	**Mn**	**Zn**	**Ca**	**Mg**	**Mn**	**Zn**	**Ca**	**Mg**	**Mn**
Cd 0,2 µM	98,8	1496	3125	3,4	113,9	1786	3441	4,1	110,6	2000	4197	3,6	87,0	2483	4965	3,5	x	x	x	x
Cd 1 µM	94,8	1589	3037	2,7	108,6	2000	4313	3,3	141,3	1816	3902	3,7	181,8	3895	6095	5,9	x	x	x	x
Cd 4 µM	156,8	1761	3661	4,2	130,8	1766	3532	3,0	118,6	2073	4051	3,1	106,0	1945	4325	3,2	x	x	x	x
Cd 20 µM	106,7	1583	2934	3,4	122,9	2203	4159	3,0	110,6	2056	4735	3,1	x	x	x	x	x	x	x	x
Cu 4 µM	93,9	1492	3018	3,7	108,6	6534	5189	3,7	139,7	2512	5649	3,3	96,6	2270	3987	6,2	x	x	x	x
Cu 25 µM	93,4	2135	3632	3,2	192,5	6142	11399	5,3	116,1	2509	3886	3,0	115,8	3098	6268	4,1	x	x	x	x
Cu 60 µM	87,2	1947	3480	3,8	123,3	2208	3960	5,8	102,3	2868	4810	2,9	119,5	5139	10443	14,3	x	x	x	x
Fe 1 µM	162,5	3883	6903	9,3	131,0	2083	4045	5,0	83,9	2060	3711	3,4	93,3	2893	5421	4,2	88,1	1972	4773	4,0
Fe 5 µM	141,9	2011	4376	3,7	92,9	1875	4067	2,5	101,3	2301	4886	3,2	100,8	2231	5499	4,8	113,6	2279	4760	7,0
Fe 20 µM	107,1	1933	4532	3,6	93,6	4878	5342	3,1	98,8	2346	4727	2,4	96,7	3036	4609	3,2	x	x	x	x
Controles	174,1	4430	4514	3,2	136,1	1116	4287	3,6	106,2	1004	4067	2,2	140,6	1367	3380	2,3	146,5	2690	5467	2,9

Chapter IV

Article III: Identification and regulation of ferritins the hydrothermal vent mussel *Bathymodiolus azoricus* in natural and experimental populations.

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In this chapter, we present the draft of a paper in preparation dedicated to the characterization of seven isoforms of ferritins identifed in *B. azoricus*. We addressed the question of the regulation of these isoforms in different tissues in both natural and experimental populations and try to identify correlation between transcriptomic profile and iron content in tissues.

1. Introduction

In oceans, iron is an essential metal for many forms of life since it represents an micronutrient that control plankton blooms and consequently have an strong impact on biogeochemical cycles of carbon and nitrogen (Boyd et al., 2007). Iron is physiologically involved in several cellular process like electron transport, oxidative phosphorylation and DNA biosynthesis (Andrews, 2008). Depletion in iron may generate a cellular malfunction when excess can be toxic by triggering the generation of oxidative stress (Lushchak, 2011). Therefore the mechanisms involved in iron storage, metabolism and transport have critical importance for living organism. Ferritin, a conserved protein found from bacteria to humans, is the principal protein involved in iron storage (Aisen et al., 2001; Theil, 1987). Ferritins are structurally complex proteins usually present in the cytosol formed by 24 subunits of two types of polypeptide chains giving a globular shape that can accommodate large quantity of iron in his interior (Lobreaux et al., 1992). The process by which iron is stored begin in the H (heavy) chain which oxidizes Fe^{2+} in a specific ferroxidase site, and the L (Light) chain perform the entry of the metal into the protein, while more metals enter the Fe^{3+} cluster formed in the protein cavity(Strange et al., 1993). In marine ecosystems, iron is originated from natural source or by anthropogenic contamination and is mainly present under ferrous Fe^{2+} and ferric Fe^{3+} forms with great redox potential. The role of ferritins in marine invertebrates is less documented compared to other detoxification proteins. Ferritins have also been shown to play a role in development (Chen et al., 2003; Levenson and Fitch, 2000), or immunity (Li et al., 2008; Zhang et al., 2006). In the clam *Meretrix meretrix*, ferritin is reported to be involved in larval shell development (Wang et al., 2009). The molecular characterization of ferritins genes has been reported in several species (Beck et al., 2002; Durand et al., 2004; Ren et al., 2014), but in marine species, the physiological role of ferritins has been only partly described in few organisms. Ferritins have been suspected to play a role in the immune defense in response to bacteria challenge (Chávez-Mardones et al., 2013; Núñez-Acuña et al., 2013), in the response to hypoxia/anoxia periods (Larade and Storey, 2004) or thermal stress (Salinas-Clarot et al., 2011) and it also has been reported that they could participate to the storage of other types of heavy metals acting as a detoxification mechanisms in cells and tissues (Chen et al., 2015; Zapata et al., 2009). However, the principal function of ferritins remains iron homeostasis and several studies showed that both gene transcription and protein expression are positively correlated with the exposure of iron in several bivalves species (Zhang et al., 2013; Zhou et al., 2014).

The hydrothermal vent are ecosystems characterized by high concentrations of metals that emerge from the hydrothermal chimney, however the concentration and quantity of

metals vary between vents associated to the characteristics of the sea floor (Von Damm, 1995). The vents from the Mid Atlantic Ridge (MAR) and particularly the Azores triple junction, are characterized by different physico-chemical condition specially the site of Menez Gwen (MG), Lucky Strike (LS) and Rainbow (RB) where the concentration of metals widely vary between these sites and especially iron which show very high concentrations at RB (Charlou et al., 2000, 2002). In this vent one of the most abundant species is is the bivalve *Bathymodiolus azoricus* which form dense bed mussels with high density (Desbruyere et al., 2000; Sarrazin et al., 2015). Previous work evidence the plasticity of B. azoricus to the accumulation of essential (iron, copper, zinc) and non-essential (cadmium, mercury, lead) metals in different tissues like gills, mantle, digestive gland, foot with different concentrations related or not to the concentration found in the fluid (Demina, L.L, 2008; Kádár, 2007; Koschinsky et al., 2014). The role of ferritin is not well reported in hydrothermal species, in the case of *B. azoricus*, Martins et al (2015) revealed a higher relative expression in the population of Menez Gwen compared to Lucky Strike that was associated with variations in immune system. In a second study the expression of four ferritin genes was analysed in *B. azoricus* exposed to several cocktail of metals and strong variations in gene expression was detected for those genes according to metals cocktails, revealing a complex pattern of iron homeostasis in *B. azoricus* (Bougerol et al., 2015). In this work we analyzed the differential expression of seven genes encoding ferritins in natural and experimental populations in different tissues in the aim to better characterize the role of ferritins regulation in the response of *B. azoricus* in a naturally iron rich ecosystem.

2. Materials and methods

2.1. Biological samples

Samples of *B. azoricus* were collected from Mid-Atlantic Ridge in August 2013, during the BIOBAZ cruise on the research vessel “Pourquoi Pas?, using the Remote Operating Vehicle (ROV) Victor 6000. The collection was made in three different vents sites Menez Gwen (37°50’ N, 31°31’ W, 800mdepth, 3 populations called MG2, MG3 and MG4), Lucky Strike (37°17’ N, 32°17’ W, 1700m depth, 2 populations collected at Tour Eiffel and Montsegur) and Rainbow (36°14’ N, 33°54’ W, 2300m depth, 2 populations collected at Termitière and Intermediate. The samples were collected

and placed in hermetic boxes then brought on board. Gills, mantle and digestive gland of each individual were immediately dissected and fixed in liquid nitrogen until further analysis.

An additional population of mussels was collected at Menez Gwen site (MG2) and used in an experimental exposure to iron because of their ability to survive for a long time at atmospheric pressure (Kadar et al., 2005). Once on board, 4 groups of 150 mussels from various size (from 12 to 80 mm) were placed in tanks containing 30 liters of filtered sea water and kept at 7°C. Then, in three tanks, mussels were exposed to 1µM, 5µM and 20µM of iron. The control tank was not exposed to iron to follow the effect of experimental design (use of surface sea water and absence of pressure and gases). Every five days, 10 individuals were sampled in each tank and pieces of gills and digestive gland were dissected and fixed in liquid nitrogen for further analysis.

2.2. RNA Extraction and qPCR

Total RNA of gill, mantle and gland digestive was extracted using Tri-Reagent (Sigma, St. Louis, MO) according to the manufacturer's instructions. Two µg of total RNA were reverse transcripted using M-MLV reverse transcriptase (Promega, Madison, WI). For gene expression, a volume of 2 µl of each diluted reverse transcription product (1:200) was subjected to real-time PCR in a final volume of 5 µl containing 40 nM of each specific primer and 2×Lightcycler®480 SYBR Green I Master mix (Roche Diagnostics, Mannheim Germany). The amplification was carried out as follows: initial enzyme activation at 95 °C for 15 min, then 45 cycles of 95 °C for 10 sec and 60 °C for 30 sec. A dissociation curve was generated and PCR efficiency was estimated for each primer pair. All primer pairs tested (Table 1) generated a single peak in the dissociation curve and a PCR efficiency of 95 to 100%. The ribosomal protein L15 gene (RpL15) was used as the internal PCR control as already used in previous studies (Bougerol et al., 2015; Boutet et al., 2011; Guezi et al., 2014). All ferrtins sequences were obtained from the seqeuncing of *B. azoricus* transcriptome (Sequence Read Archive (DRA, http://trace.ddbj.nig.ac.jp/dra/; accession number DRA 004082)

2.3. Statistical analyses

The relative expression of each gene was calculated according to the comparative Ct method using the formula RQ = ΔΔCt (with $\Delta Ct_{gene\ X} = Ct_{gen\ X} - Ct_{RpL15}$; $\Delta\Delta Ct = \Delta Ct_{gene\ X} - \text{mean } Ct_{gene\ X}$). The first analysis was conducted to determine the inter-population effect in the variation of gene expression and we normalized the data by the mean $\Delta Ct_{gene\ X}$ of all populations for one tissue (gills, mantle or digestive gland). The second analysis was conducted to determine the intra-population effect of the expression and we normalized the data by the mean $\Delta Ct_{gene\ X}$ in the three tissues for each population. ANOVA test was used to evaluate differences in metal accumulation and relative expression considering population and tissues as explanatory factors (R studio software). For the metal exposure experiment the data was normalized by the mean $\Delta Ct_{gene\ X}$ in each gene for each iron concentration. Canonical redundancy analysis (RDA) was used to evaluate the influence of metal accumulation in the variation of gene expression using the Vegan package (Oksanen et al 2016) in R environment. Test of significativity between ferritins in each experimental condition in both tissues were performed using additional Student T-tests with 100 permutations and a p-value= 0.05.

3. Results

3.1 Ferritins characterization

We report the sequences of seven ferritin in *B. azoricus.* Five ferritins isoforms (Fer1 to Fer5) show high levels of similarity between them and with other marine bivalves with values ranging from between 68 to 94% of identity (Fig.1). The two other ferritin sequences encoding a Soma ferritin (Sfer) and a Yolk ferritin (Yfer) have lower levels of similitude that not exceed the 30% (Fig.2) and 45% respectively (Fig.3).

3.*2 Relative expression of ferritins between populations*

In gills, the relative expression of ferritin in the seven populations is presented in Figure 4. ANOVA analysis shows that all ferritins except S-fer are significantly regulated between populations (Table 2). (Fig. 4). No clear pattern of regulation is observed at a vent site scale and but specific population signature can be identified. MG2 population show a global higher expression for all ferritins with the exception of Sfer compared to all populations except Intermediate. MG3 and Tour Eiffel present the lowest level of expression for all ferritins and the three other populations present a relative similar pattern of regulation.

In mantle, ANOVA analysis shows that all ferritins except Y-fer and Fer4 are significantly regulated between populations (Table 2). The pattern of expression observed in mantle is similar to gills with a higher expression in MG2 and Intermediate populations when MG3, MG4 and T. Eiffel show a global down regulation for all ferritins (Fig. 4). In the digestive gland, ANOVA analysis show that ferritins Y-fer, Fer1, Fer4 and Fer45 are significantly regulated between populations (Table 2, at $p<0.01$). (Fig. 4), The ferritin regulation pattern in digestive gland is characterized by a global higher expression of ferritins in MG3, Tour Eiffel and Intermediate populations and a lower expression in Termitière and Montsegur.

3.*3 Tissues differential expression of ferritins within the populations*

The results of the relative expression of ferritins between tissues in the seven populations are presented in Figure 5 and ANOVA analysis results are presented in Table 3. In all the populations, a global down regulation pattern is observed in the mantle for all the ferritins genes compared to the other tissues, while the high levels of expression are detected in the digestive gland especially for Fer3, Fer4, Sfer and YFer (the highest values being observed in both populations of Lucky Strike (Montsegur and Tour Eiffel). ANOVA analysis shows that most of the ferritins exhibit a significant difference in expression between tissues within a population. However, we notice that Fer 2 is only significantly regulated in the three MG populations.

3.*4 Redundancy analysis (RDA)*

We use RDA analysis to test the significance of the metal concentration in the relative expression of ferritins genes in gill, mantle and digestive gland (Figure 6). In gills, metal concentration explains 17.6% of the variation and the first axis of the RDA1 explain 83.5% of the 17.6% and versus only 9.4% for the RDA2. RDA show an opposite relationship between ferritins expression and metal content except for Soma ferritin (Sfer) which appears mainly correlated with iron and calcium followed by lead and manganese and in less extend with copper. In mantle, the RDA analysis explains 14.3% of the variation with the first axis explaining 71.5% of this variation. A relationship is mainly observed for Fer2 and Fer5 with iron and manganese. In the digestive gland, the RDA analysis explains 10.3% of the total variation with the first axis explaining 75.2%. A relationship between Fer3, Fer5 and Sfer is observed mainly with calcium and cadmium and Fer1, Fer2, Fer4 and Yfer with copper and cadmium.

3.*5 Relative expression of ferritins in mussels exposed to iron.*

We report the levels of relative expression of the seven genes in gill and digestive gland of individuals exposed for 25 days to three iron concentrations, 1µM, 5µM and 20µM concentration (Figure 7). In gills, the relative expression of almost all the ferritin genes have significant differences during the course of the experiment with the exception of Yfer (Table 4). In this tissue, the expression in control mussels do not strongly differ from T0 except at day 20 where a significant increase of Fer2, Fer3, Fer4, Fer5 (T-test, $p<0.05$) is observed. At day 5, no variation in ferritin expression is observed except a significant increase of Fer1 expression (T-test, $p<0.05$) detected at the 1 and 5µm concentrations. At day 10, a significant down-regulation is observed for Yfer at both 1 and 5µm and an up-regulation of Fer2 at 20 µM and Fer3 at 5 µM. At day 15, only Fer1 is significantly up-regulated at 1µM and a higher expression in observed for Sfer at 5 and 20µM. At day 20, a strong significant down-regulation is observed at 5µM except for Fer1 and Yfer and for Fer2 at 1 and 20 µM compared to the control. At day 25, a significant up-regulation is only observed at 1µM for Fer1 and Fer2. In the digestive gland, ANOVA results showed that a significant regulation is observed for all ferritins except Fer3 and Yfer (Table 3). The expression in control mussels no not differ from T0 except at day 25 for Fer2 (T-test, $p<0.05$). At day 5, no variation in ferritin expression is detected between exposed and control mussels. At day 10, Fer4 and Yfer expression is significantly decreased at 5µM. At day 15, no significant change are detected. At day 20, a significant increase of all ferritins except Fer3 is detected (T-test, $p<0.05$) at 20 µM, and for Fer3 Fer4, Sfer and Yfer at 1µM and Fer3 at 5µM. At day 25, only Fer3 and Fer4 are significantly up-regulated at 5µM.

4. Discussion

The understanding of the mechanism of metal tolerance and detoxification in hydrothermal species remain limited compared to data reported in many other bivalve species living in coastal ecosystems despite the fact that hydrothermal vent has been described as a natural laboratory for pollution studies due to the high concentration of metals. In *B. azoricus, t*he tolerance to heavy metals has been evidenced (Kádár, 2007; Kádár et al., 2006) and several molecular markers have been suggested to play a role in metal tolerance and detoxification (Bebianno et al., 2005; Bougerol et al., 2015; Company et al., 2010; Hardivillier et al., 2004). However, the role of ferritins is not well documented in this species neither in other hydrothermal species.

4.1 Ferritin characterization

In our work, we described seven ferritin genes in the bivalve *B. azoricus* where Fer1, Fer2, Fer3, Fer4 and Fer5 show the higher degree of identity with other marine mussel species ferritins as *M. edulis* and *M. chilensis*. The amino acid residues of ferroxidase center and nucleation center are conserved in *B. azoricus* sequences compared with the other species, those centers being strongly involved in the iron loading into the ferritin protein (Hilton et al., 2012). Stronger differences are observed in the Soma ferritin (Sfer) and Yolk ferritin (Yfer), where in the Sfer, amino acid change is observed in the nucleation center at the position 62 of *B. azoricus* where a Serine (S) residue replace the Glutamic acid (E) present the other bivalve sequence, and another replacement is detected at the position 140 where a Lysine (K) is found in B. azoricus instead of a Glutamine (Q) in the other mussel species. In Yfer, amino acid changes are reported for the ferroxidase center in the position 75, 78 and 82. The two sequences are structurally different to the other five ferritins genes, so their main function may not be directly related to iron detoxification processes.

4.2 Ferritin regulation in natural populations

One of the characteristics of the vent from the Azores triple junction is the elevated concentration of iron that emerge from the chimney associated with large spatio-temporal fluctuations (Sarrazin et al., 2014). Based on known iron concentrations in fluids (Charlou et al., 2002), we may expect that regulation of ferritins in natural populations could be a relevant biomarkers of iron exposure. Biochemical changes in an organism are highly labile but can also be related to environmental factors or genetic differences within a population (Romeo et al., 2003) and the metal distribution among organs represent a total index which can be considered as an accurate assessment of the organism's response to increased heavy metal levels in the environment (Kavun and Shul'kin, 2005). In gill, the higher levels of expression reported in MG2, MG4 and Intermediate cannot be directly associated to the iron concentrations in this tissue, the highest iron concentration being detected the Termitiere population that exhibit a lower expression of all ferritins. The same trend is observed for the mantle. In the digestive gland, the population of Intermediate also exhibits a higher gene expression but for all the six other populations, the expression pattern of ferritins is inverted compare to gill and mantle. Recently works in bivalves exposed to metals reveal that both metal uptake and the expression of methallothioneins and ferritins can be modulated by the levels of pH and CO_2 concentration (Ivanina et al., 2015). Both parameters are fluctuating in hydrothermal vent with different spatio-temporal scales, so the variation in ferritin expression reported in this work may reflect the interaction between iron present in the surrounding water and those factors.

The comparison of ferritin expression between tissues reveal a conserved pattern of expression in all the populations, the mantle showing the lower level of expression for all the genes and the digestive gland globally exhibiting the higher levels of expression. This high expression in digestive gland is in agreement with the major iron concentration measured in this tissue. Similar differences between gills and mantle has been reported for the surf clam *Mesodesma donacium* in natural conditions (Maldonado-Aguayo et al., 2014), however the fold change differences are lower compared to those reported in the present study work with ratio ranging from 10 to 70 times more in digestive gland that in mantle. Similar trend is reported for oysters exposed to pesticides evidencing a organ and site specific ferritin response possibly related to acclimation to pollution (Collin et al., 2010). To test the role of metal concentration in ferritin expression we chose the redundancy analysis to detect possible specificity for certain metals previously measured in tissues form the same populations and our result suggest a tissue specific response in ferritins genes but not directly related to iron concentration. Sfer remain the only gene related to metals, principally calcium, iron, lead and manganese and in mantle only Fer2 and Fer5 are related to iron and no correlation is reported in digestive gland.

4.3 Ferritin regulation in iron exposure experiment

In marine bivalves the expression of ferritins in experimental metal exposure conditions is restricted to a low number of species and no long term exposure has been described in the literature leading to a difficult comparison of our results. Ferritin up-regulation has been described in response to situations of anoxia/ischemia such as in snail (Larade and Storey, 2004), and frog (Storey, 2004). In the mollusc *Tegillarca granosa*, a ferritin subunit similar to GF1 was found to be up-regulated after 24h of exposure to iron then down-regulated after 48h, suggesting a complex pattern of expression for ferritin (Jin *et al*., 2011). Typical yolk protein precursors are developmentally regulated proteins and show a tissue- and sex-specific expression (Wahli, 1988). Yolk ferritins are also able to store iron together with some phosphate (Bottke *et al*., 1982) and the presence of the ferroxidase center in the yolk ferritin suggests a possible ability of this protein to accelerate iron oxidation. In snails, it has been evidenced a putative role of these proteins in ion transport during the vitellogenesis production, specifically inside gametocytes for yolk ferritin (Bottke et al., 1988) and in somatic tissues for the soma ferritin (Von Darl et al., 1994). Gills constitute an interface for dissolved metal uptake, feeding and gas exchange while the digestive gland is responsible for the absorption of nutrient and is also a major site for metal accumulation (Marigómez et al., 2002) and in natural population of *B. azoricus* , digestive gland exhibit the higher iron concentrations. During the exposure, the relative

expression of ferritins in gills show an increase after 20 days which is partly maintained at day 25 in control mussels. In the digestive gland, less variations are observed in the ferritin regulation except for Fer3, Fer4 and Yfer especially after 20 days of exposure suggesting that this tissue may be not affected by iron in the beginning of the experiment. The iron quantification made on samples used in this experiment only shows an accumulation of iron after 20 days in the digestive gland and not in gills (Figure 8 and 9). The control can be considered as a depuration experiment in which no metal was added so the activation of ferritins expression could be interpreted as a response to the capture of the released iron stored in lysosomes and cellular ligands but also as a global response to experimental conditions. We detect a regulation of the Sfer gene expression but not in the Yfer according to time exposure Inhibition of Yfer was reported in experimental cocktail metal exposure recreating natural conditions in *B. azoricus* (Bougerol et al., 2015). Sfer and Yfer have been implicated in processes of vitellogenesis even this specific role remain unclear, and iron supply for embryo or protection of embryo and larva against bacterial growth have been proposed as possible functions (Schussler et al., 1995). We did not detect particular traces of reproduction in our samples so we suggest that Sfer could be involved in other iron metabolism.

The use of ferritins as a proxy for transcriptomic analysis revealed stronger differences in the natural population analysis and allowed us to distinguish all our populations. The results obtained during the experimental exposure do not strongly evidence the involvement of specific ferritins in the response to iron leading to a more complex picture. Functional analysis of these isoforms could bring interesting informations about the ferritins properties in term of iron binding but also influence of other metals on the iron chelation.

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6. Figures and tables

Table 1. Primers used in qPCR Ferritins relative expression.

Gene	Sequence
Fer1	F: CATGAAGTACCAGAACAGAAGAGGTG
	R: CTTTGTGAAGATCCAAGAGAGACTGG
Fer2	F: GTCAGATCAACATGGAACTCTACGC
	R: CACCTCTTCTGTTCTGGTACTTCATG
Fer3	F: GTATGCCAGCTATGTTTACCAGTCC
	R: GATTCGTCCTCCTCTCTTGTTCTGA
Fer4	F: CATGTCCTACTACTTCGATAGGGATG
	R: GGTCAGCTTTCTTGATGTCCTGAAG
Fer5	F: GTCAGATCAACATGGAACTCTACGC
	R: CACCTCTTCTGTTCTGGTACTTCATG
Sfer	F: GATAGCACTTCCTGGATTCCACAAG
	R: CCCTTGGTTCCTTTATCCCTGTAAG
Yfer	F: TATCTGAATCAGAGAGGAGGAGAGG
	R: CAGATTCCATGACTGATCTTTGGCC

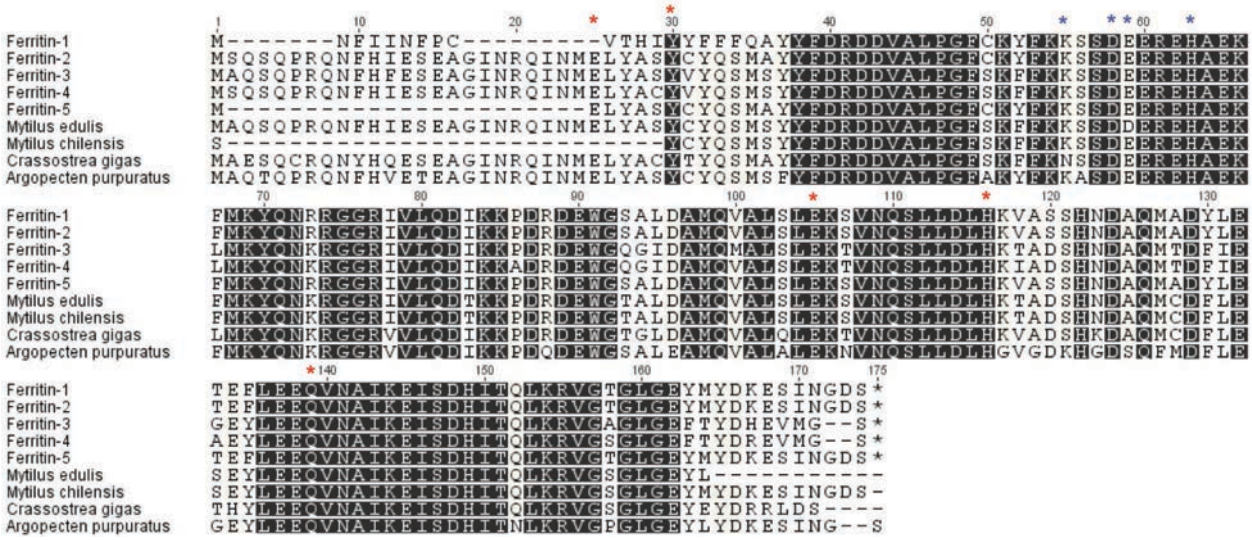


Figure 1. Alignment of the five Ferrtitin with sequences of other marine bivalves, *M. edulis* (AEM36072.1), *M. chilensis* (AEE60904.1), *C. gigas* (AAP83793.1) and *A. purpuratus* (ALV83427.1). Red asterisks represent residues that comprise the ferroxidase center and blue asterisks residues from the nucleation center.

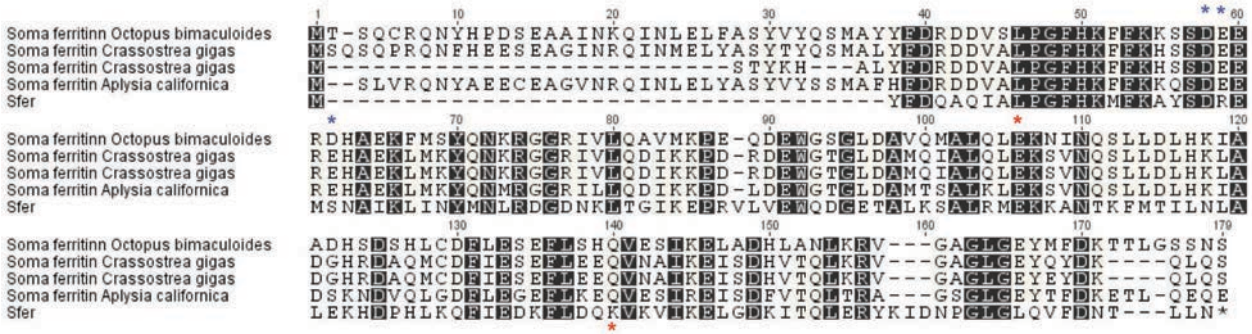


Figure 2. Alignment of the Soma ferritin with sequences of other marine invertebrates, *O. bimaculoides* (XP_014779712.1), *C. gigas* (XP_011432947.1 and XP_011448525.1) and *A. californica* (XP_005096044.1). Red asterisks represent residues that comprise the ferroxidase center and blue asterisks residues from the nucleation center.

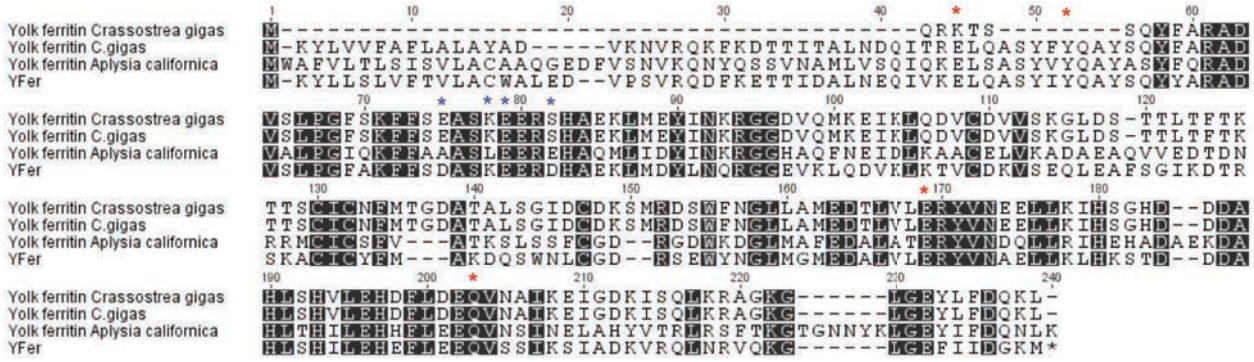
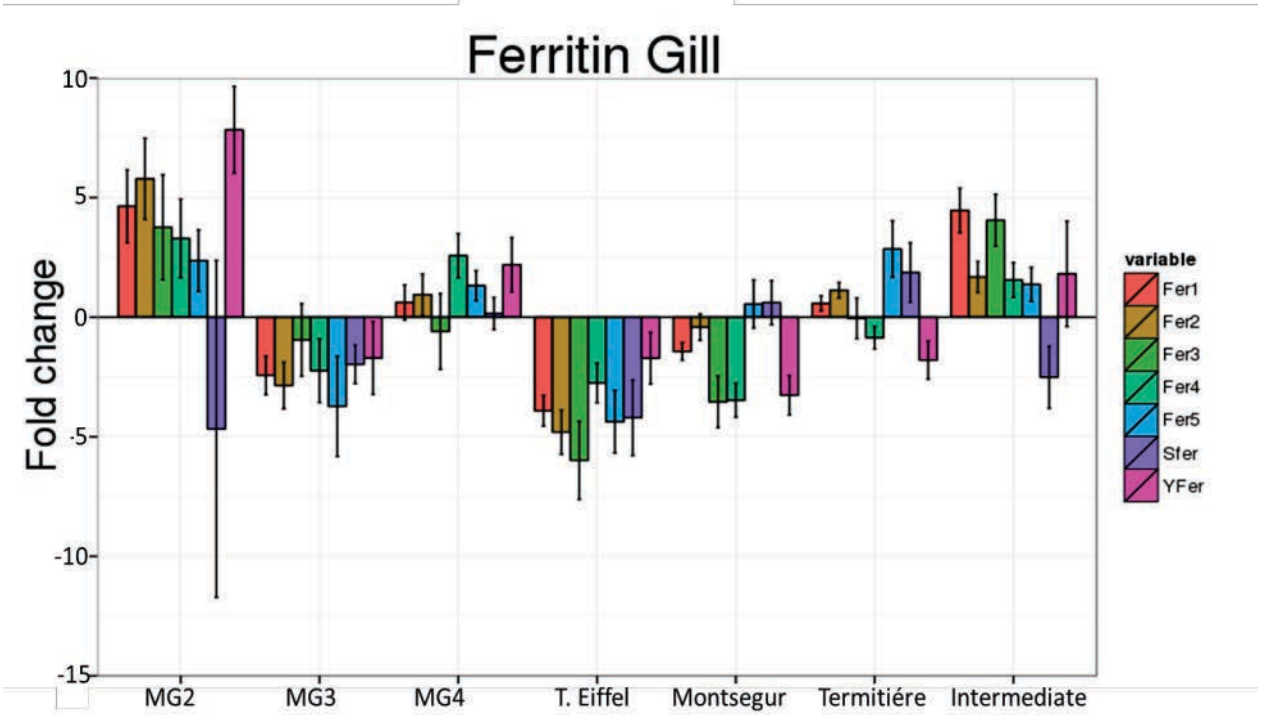


Figure 3. Alignment of the Yolk Ferrtitin with sequences of other marine invertebrates, *C. gigas* (EKC42967.1 and XP_011438862.1) and *A. californica* (XP_005089125.2). Red asterisks represent residues that comprise the ferroxidase center and blue asterisks residues from the nucleation center.



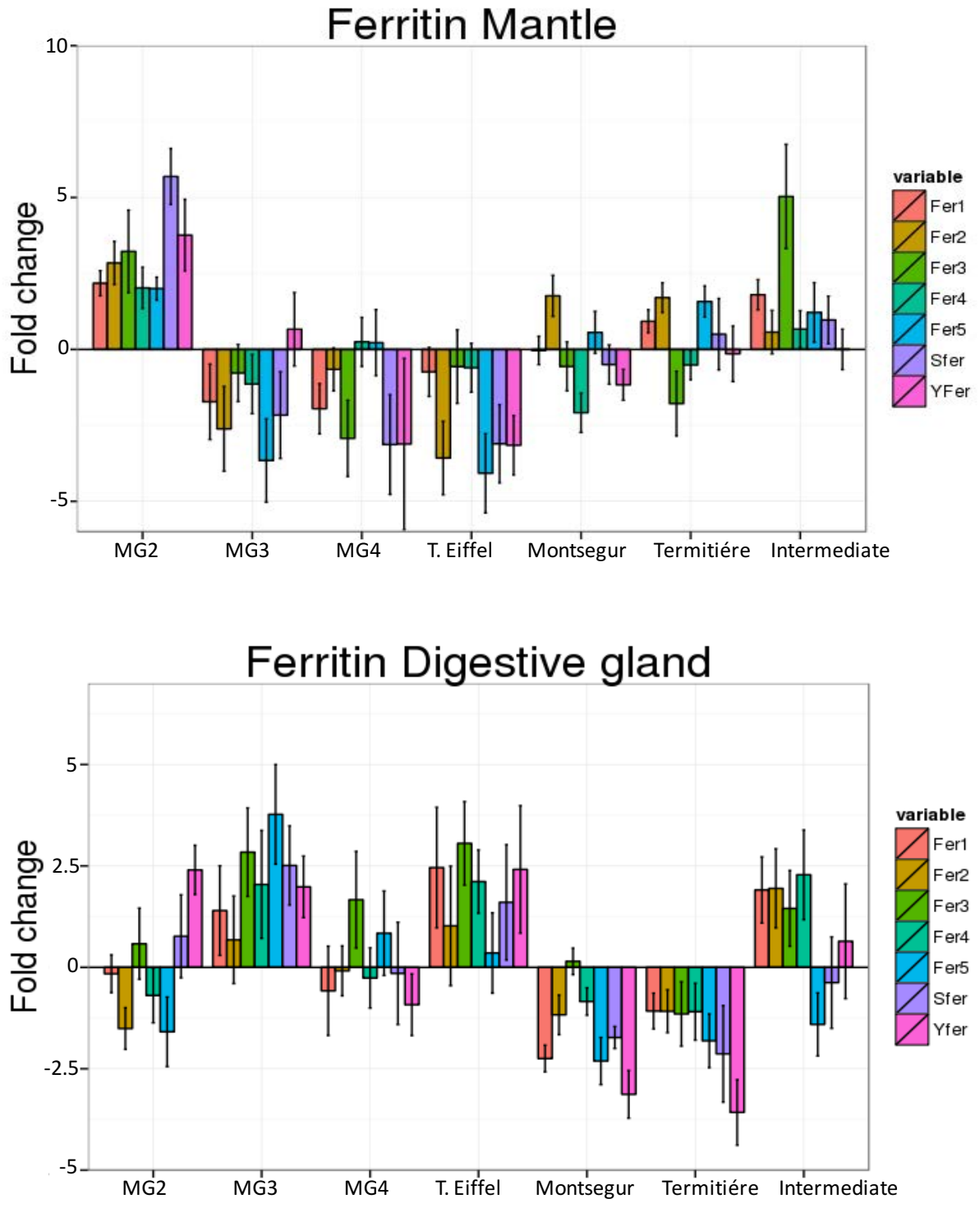


Figure 4. Inter-population effect of relative expression (fold change) of ferritins in gills mantle and digestive gland for the seven populations analyzed. Gene expression is normalized by the mean deltaCt value of genes in all samples for each tissue. Positive values represent up regulation and negative values represent inhibition.

Table 2. Results of the Global Anova analysis conducted on the relative expression of the seven ferritins genes between the seven populations for each tissue.

Population	Response	Df	MS	F	p-value
Gill	Fer1	6	327.1	14.92	3.89e-14
	Fer2	6	353.3	12.91	2.33e-12
	Fer3	6	399.4	6.00	8.41e-6
	Fer4	6	228.01	7.23	5.16e-7
	Fer5	6	254.09	5.31	4.05e-5
	Sfer	6	188.4	0.73	0.626
	Yfer	6	451.3	7.35	3.89e-7
Mantle	Fer1	6	75.33	5.11	6.68e-5
	Fer2	6	170.1	7.38	3.99e-7
	Fer3	6	214.72	5.24	4.97e-5
	Fer4	6	52.46	3.42	0.0031
	Fer5	6	182.29	6.91	1.13e-6
	Sfer	6	282.24	7.13	6.99e-7
	Yfer	6	168.04	3.151	0.00569
D. gland	Fer1	6	85.55	3.47	0.0028
	Fer2	6	46.15	2.01	0.065
	Fer3	6	66.85	2.70	0.015
	Fer4	6	68.65	3.33	0.0038
	Fer5	6	132.33	5.63	2.08e-5
	Sfer	6	84.25	2.42	0.0276
	Yfer	6	193.96	6.87	1.26e-6

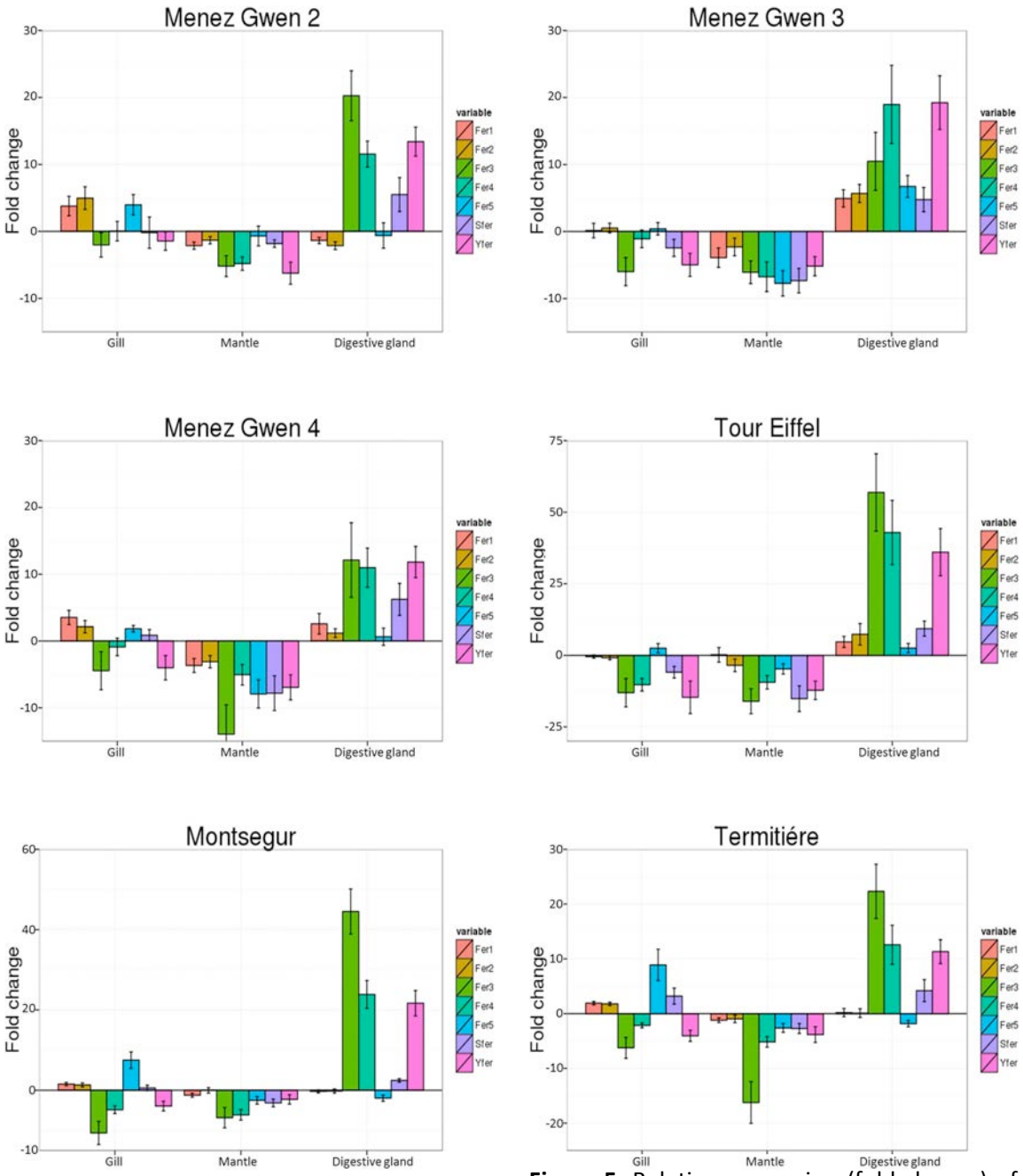
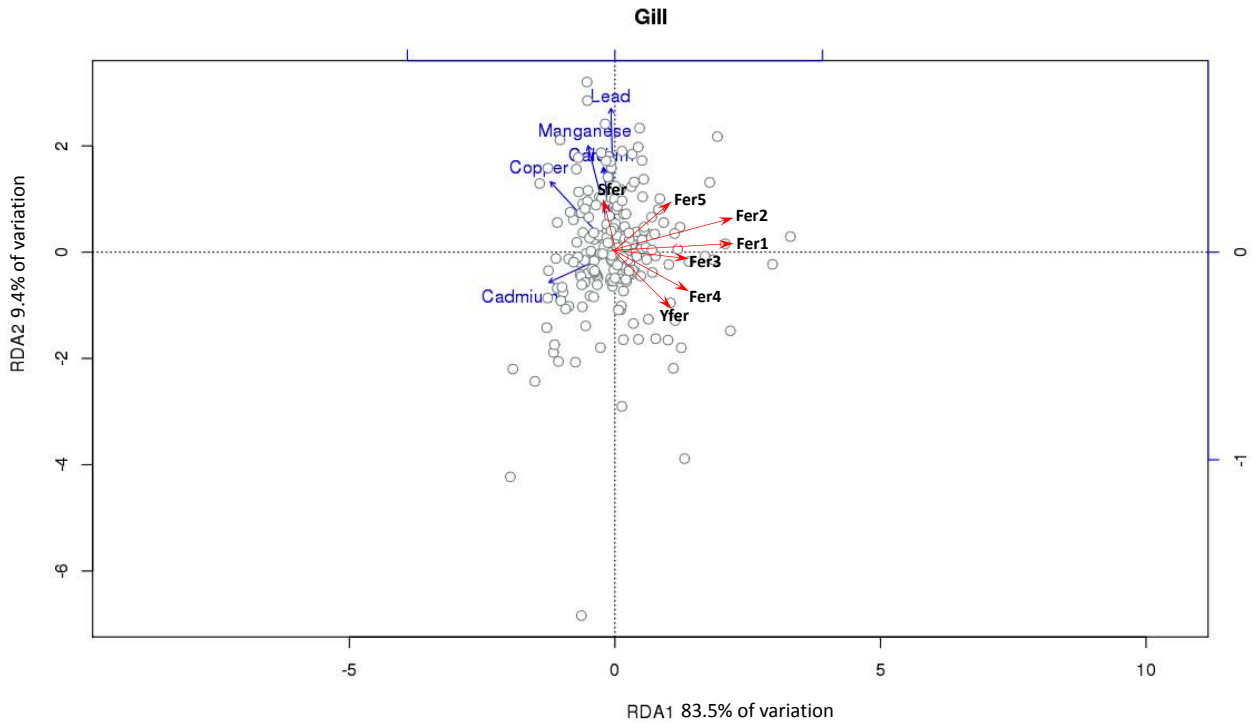


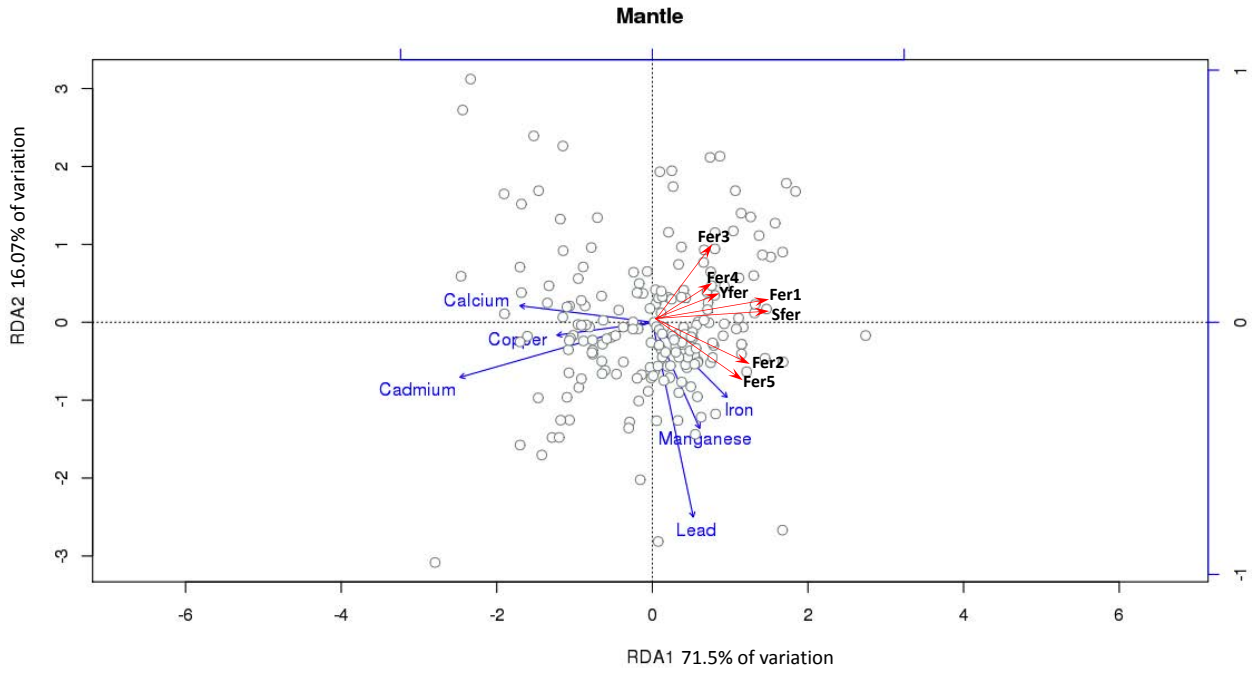
Figure 5. Relative expression (fold change) of the ferritins genes between the three tissues in each population. Gene expression is normalized by the mean deltaCt value of genes in all tissues for each population. Positive values represent up regulation and negative values represent inhibition

Table 3. Results of the Global Anova analysis conducted on the relative expression of the seven ferritins genes in the seven populations between tissues.

Population	Response	Df	MS	F	p-value
MG-2	Fer1	2	316.05	11.41	3.96e-5
	Fer2	2	459.6	12.48	1.71e-5
	Fer3	2	5.658	29.79	1.4e-10
	Fer4	2	2074.2	30.66	8.35e-11
	Fer5	2	217.07	2.70	0.0726
	Sfer	2	434.7	3.67	0.032
	Yfer	2	3083.8	33.94	1.27e-11
MG-3	Fer1	2	558.8	11.91	2.8e-5
	Fer2	2	468.4	12.2	2.23e-5
	Fer3	2	2632.8	10.44	8.96e-5
	Fer4	2	5251	13.44	8.63e-6
	Fer5	2	1494.8	22.25	1.76e-8
	Sfer	2	1056.5	13.41	8.85e-6
	Yfer	2	5711	27.97	4.9e-10
MG-4	Fer1	2	431.4	9.98	0.000132
	Fer2	2	220.86	11.02	5.77e-5
	Fer3	2	4876	8.96	0.000301
	Fer4	2	1942.3	16.45	9.83e-7
	Fer5	2	800.7	13.6	7.92e-6
	Sfer	2	1409	11.53	3.87e-5
	Yfer	2	2864.3	24.81	3.75e-9
Tour Eiffel	Fer1	2	235.8	2.24	0.112
	Fer2	2	957.9	4.95	0.0091
	Fer3	2	5114	22.63	1.22e-8
	Fer4	2	2785	20.38	5.52e-8
	Fer5	2	527.9	6.27	0.00285
	Sfer	2	4579	14.69	3.19e-6
	Yfer	2	2453	22.19	1.63e-8

Montsegur	Fer1	2	63.43	10.69	6.82e-5
	Fer2	2	22.07	2.20	0.116
	Fer3	2	2839	63.79	2,00E-16
	Fer4	2	8604	62.96	2,00E-16
	Fer5	2	999.6	16.18	9.94e-7
	Sfer	2	6152	50.58	1.89e-15
	Yfer	2	251.78	15.05	2.3e-6
Termitiére	Fer1	2	72.99	8.81	0.000328
	Fer2	2	57.86	4.94	0.0092
	Fer3	2	1201	28.14	3.76e-10
	Fer4	2	2703.7	19.68	8.87e-8
	Fer5	2	1240.7	13.57	7.4e-6
	Sfer	2	418.9	5.96	0.0037
	Yfer	2	2333.1	29.69	1.48e-10
Intermediate	Fer1	2	240.88	15.29	2.45e-6
	Fer2	2	403.9	5.15	0.0079
	Fer3	2	1184	20.1	9.85e-5
	Fer4	2	3134	23.03	1.51e-8
	Fer5	2	280.18	18.13	3.66e-7
	Sfer	2	598.4	12.61	1.87e-5
	Yfer	2	2288.7	22.19	2.57e-8


Gill
Lead
Manganese
Copper
Sfer
Fer5
Fer2
Fer1
Fer3
Fer4
Yfer
Cadmium
RDA2 9.4% of variation
RDA1 83.5% of variation


Mantle
Fer3
Fer4
Yfer
Fer1
Sfer
Calcium
Copper
Fer2
Fer5
Cadmium
Iron
Manganese
Lead
RDA2 16.07% of variation
RDA1 71.5% of variation

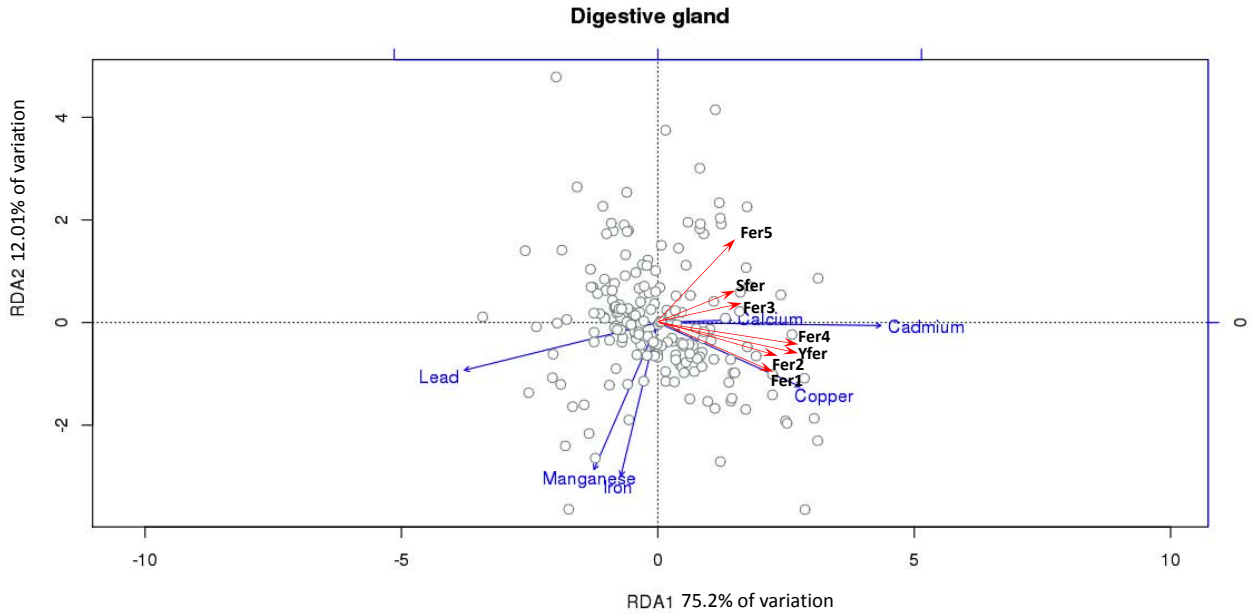
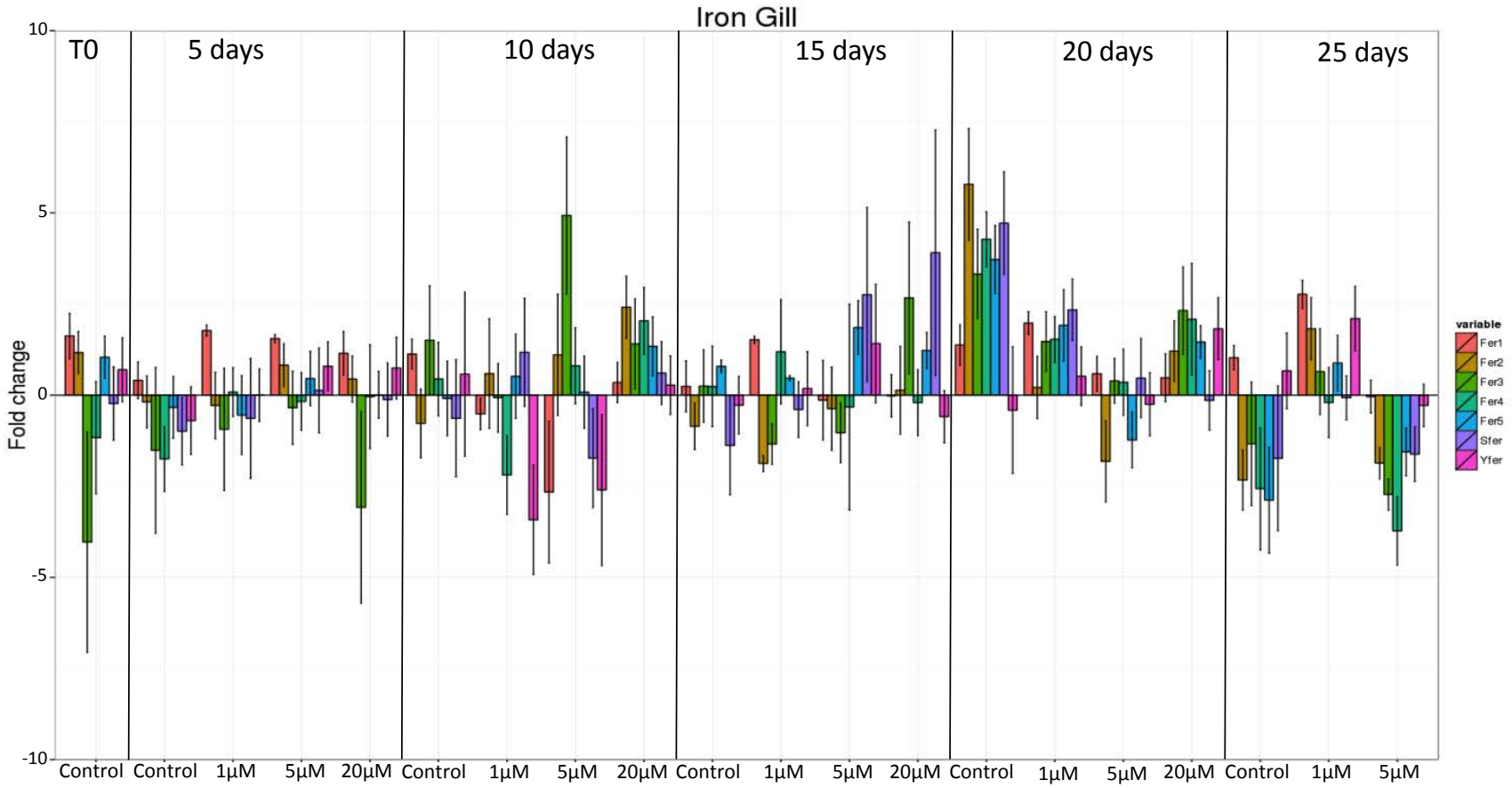


Figure 6. Global redundancy analysis (RDA) representing the relationship between the response variable (relative expression) and the explicative variable (metal concentration) in the gills, mantle and digestive gland, The angle between arrows represents the degree of correlation between variables


Iron Gill
T0
5 days
10 days
15 days
20 days
25 days
Fold change
10
5
0
-5
-10
Control
Control
1µM
5µM
20µM
Control
1µM
5µM
20µM
Control
1µM
5µM
20µM
Control
1µM
5µM
20µM
Control
1µM
5µM
variable
Fer1
Fer2
Fer3
Fer4
Fer5
Sfer
Yfer

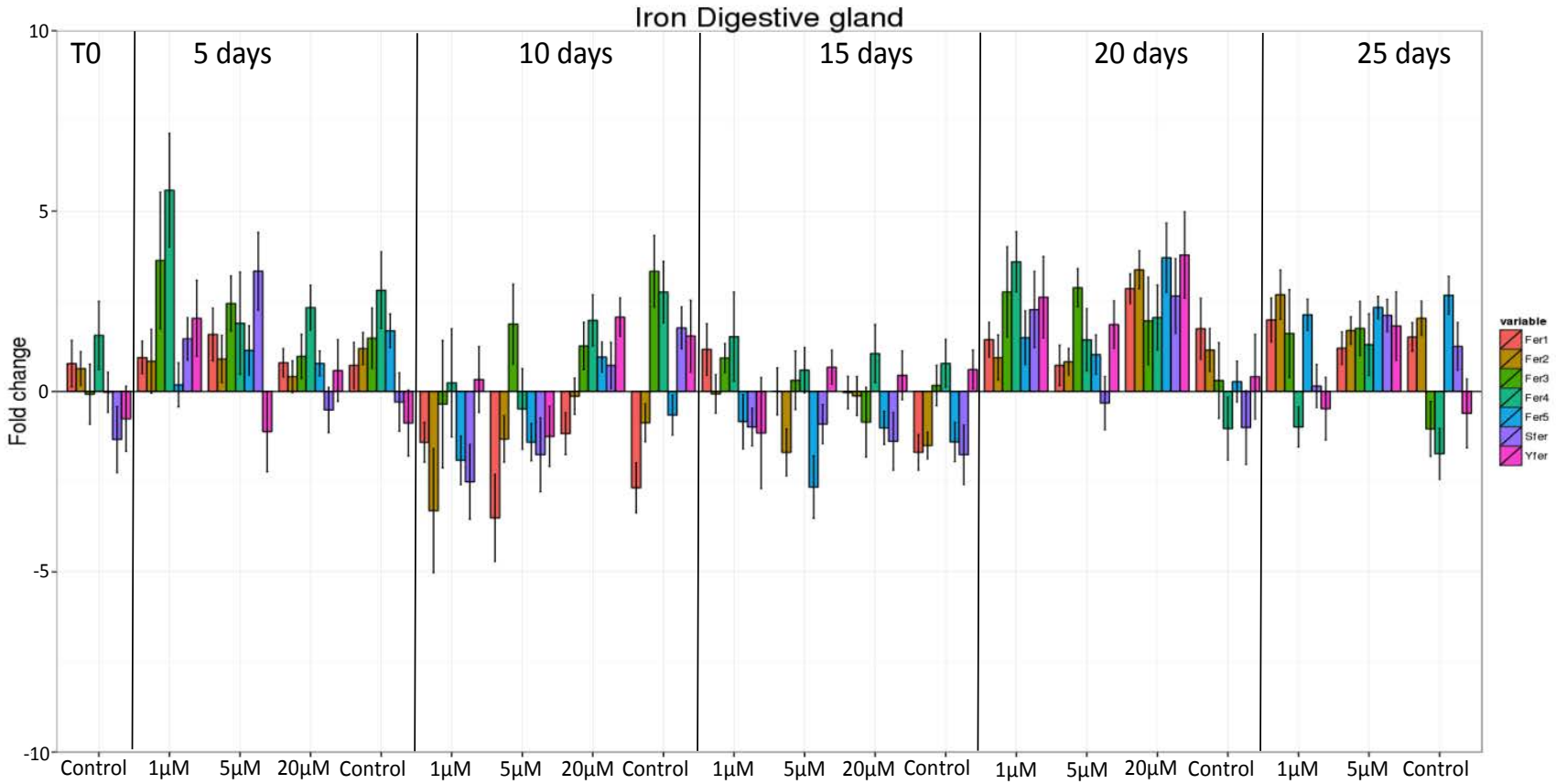


Figure 7. Relative expression levels in gill and digestive gland for the seven ferritins genes during 25 days of iron exposure.

Table 4. Results of the Global Anova analysis conducted on the relative expression of the seven ferritins genes in gills and digestive gland of mussels exposed to iron.

Tissue	Response	Df	MS	F	p-value
Gills	Fer1	18	12.57	3.32	2.61e-5
	Fer2	18	31.39	3.48	1.2e-5
	Fer3	18	38.67	1.93	0.0167
	Fer4	18	31.90	2.79	0.00033
	Fer5	18	19.40	2.65	0.000635
	Sfer	18	28.87	1.75	0.036
	Yfer	18	17.05	1.39	0.139
Digestive gland	Fer1	18	28.10	7.08	2.64e-13
	Fer2	18	25.49	5.66	1.98e-10
	Fer3	18	18.56	1.78	0.029
	Fer4	18	30.67	3.23	3.22e-5
	Fer5	18	28.34	7.93	5.71e-15
	Sfer	18	28.07	4.38	1.03e-7
	Yfer	18	19.20	2.11	0.0073

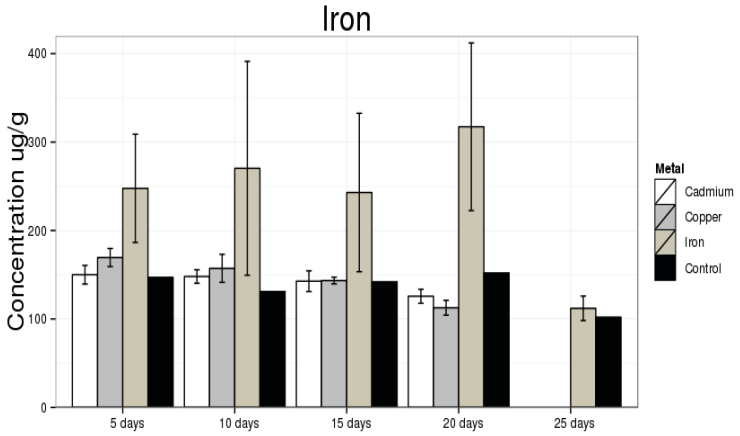


Figure 8. Concentration of iron measured in gill during the metal exposure. Each graph represents the metal concentration measured in the three different exposure conditions (cadmium, copper, iron) and control mussels. Concentration values correspond to the mean and standard deviation values for all concentrations of each metal (cadmium, copper, iron) except for control that are represented by only one value

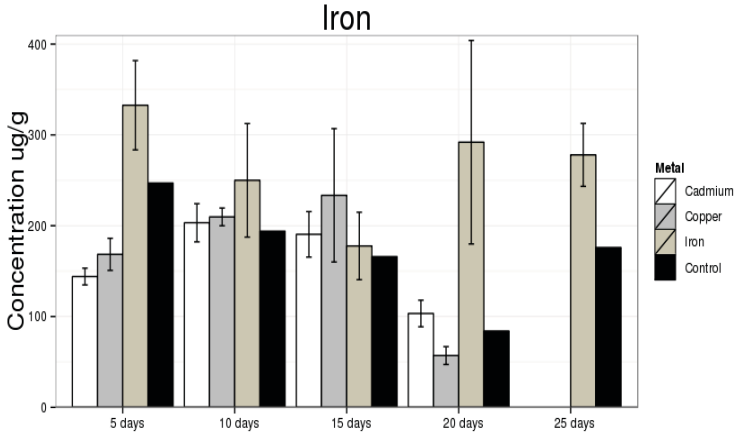


Figure 9. Concentration of iron measured in digestive gland during the metal exposure. Each graph represents the metal concentration measured in the three different exposure conditions (cadmium, copper, iron) and control mussels. Concentration values correspond to the mean and standard deviation values for all concentrations of each metal (cadmium, copper, iron) except for control that are represented by only one value

Chapter V

Large scale transcriptomic analysis of *Bathymodiolus azoricus*

In this chapter, we present the first results of the analysis of the transcriptomic variations in six populations sampled in the three vent sites Menez Gwen, Lucky Strike and Rainbow. This chapter contains data related to a tissue comparison within vent sites

1. Introduction

In ecosystems where abiotic fluctuations are highly variable, the ability of the species to acclimatize (short term response) and evolutionary adapt (long term response) is of vital importance for determine the survival, where tolerance limits are one of the central topics to understand the maintenance of cellular homeostasis and consequently in organism and populations, but for this, biochemical systems biology and genomic studies are required to explain these responses to external perturbation (Bozinovic and Pörtner, 2015; Doney, 2010). New approaches has been then developed in the past years using "omics" technologies (genomics, transcriptomics and proteomics) which generate a high quantity of genetic information that has revolutionized areas as ecology and evolution by greatly expanding the scientific possibilities to asses the mechanism that govern ecological interaction principally in environmental genomics, who study the functioning of a genome of an organism and the relationship with biotic and abiotic environment (Straalen and Feder, 2012).

In marine ecosystems the environmental conditions vary at a spatio-temporal scale, where temperature, oxygen concentration, reactive oxygen species (ROS), salinity and pH have been described as being the main factors that moderate stress changes that can be detected through gene and protein expression which also vary according the intensity of the abiotic factors. In this context different transcriptomic tools has enable the investigation of gene profile of thousand of genes in natural or experimental conditions (Somero, 2012) such as microarrays. The use of transcriptomic profiling has been developed in many species but the molluscs and particularly in bivalves which represent pioneer species in marine ecosystems, which are important for aquaculture resource and are described as biomonitoring species of marine pollutions with mainly *Mytilus* sp and *Crassostrea* species (Suárez-Ulloa et al., 2013; Tanguy et al., 2008). In the bivalve *Mytilus californianus*, a complex pattern of transcriptional changes are related to the vertical distribution in the intertidal zones, reflecting the physiological strategy used by this specie to accommodate with their fluctuating environment (Gracey et al., 2008). The exposure to pollutants of anthropogenic origin (pesticides, polycyclic aromatic compounds, heavy metals) is recognized as being one abiotic factors able to generate strong physiological response in marine invertebrates which are detected through alteration in mRNA levels after exposure for genes that encode proteins involved in xenobiotic detoxification, immune system, antioxidant stress systems and specific metal detoxification pathways (Dondero et al., 2006; Ki et al., 2009; Tanguy et al., 2005). The expression of these genes remains however more difficult to interpreted in natural exposed populations due the complex environmental spatio-temporal variations.

Bivalves from the Mytilidae family are widely distributed around the oceans and particularly in the hydrothermal vent of Mid-Atlantic Ridge (MAR) where the environmental fluctuations in temperature, pH, oxygen gas and metal concentration reach extreme levels. However, the mussel

Bathymodiolus azoricus dominates this area (specifically Azores triple junction, ATJ) and has been described as potential model specie to understand the adaptation to this extreme environmental. *B. azoricus* forms large communities in the base and walls of vent chimneys, with high abundance (Sarrazin et al., 2014) and its physiological plasticity has been characterized by the presence of symbiosis with methane and sulphur oxidizing bacteria in bacteriocytes in gill (Kiers and West, 2015; Le Bris and Duperron, 2010) as their ability to accumulate different metals in different tissues and at higher levels compared to congeners species from littoral ecosystems (Colaço et al., 2006; Enik Kádár et al., 2006; Kádár et al., 2007; Koschinsky et al., 2014a). Additionally transcriptomic and genomic data in this specie has been previously reported for this specie (Bougerol et al., 2015; Boutet et al., 2011; Tanguy et al., 2008). However, those studies are limited to the analysis of a limited number of genes. For this purpose we developed a microarrays analysis using all sequencing data available in *B. azoricus*, with the goal to identify differentially expressed genes in different sites of ATJ, which are characterized by a gradient in depth, concentration of toxic gases and heavy metal concentrations: Menez Gwen, Lucky Strike and Rainbow).

2. Materials and methods

2.1 Biological samples

Samples of *B. azoricus* were collected from Mid-Atlantic Ridge in August 2013, during the BIOBAZ cruise on the research vessel “Pourquoi Pas?, using the Remote Operating Vehicle (ROV) Victor 6000. The collection was made in three different vents sites Menez Gwen (37°50’ N, 31°31’ W, 800mdepth, 2 populations called MG2 and MG4), Lucky Strike (37°17’ N, 32°17’ W, 1700m depth, 2 populations collected at Tour Eiffel and Montsegur) and Rainbow (36°14’ N, 33°54’ W, 2300m depth, 2 populations collected at Termitière and Intermediate. The samples were collected and placed in hermetic boxes then brought on board. Gills, mantle and digestive gland of each individual were immediately dissected and fixed in liquid nitrogen until further analysis.

2.2 Array construction

We use a oligoarray format of 4x180K formed by sequences of 60pb length obtained from transcriptomic and genomic data in collaboration with the laboratory of Nori Satoh (Okinawa Institute of Science and Technology, OIST). Probes were synthetized with positive and negative controls using 4x180K- feature Agilent slide format.

2.2 RNA Extraction and labelling

Total RNA of gill, mantle and gland digestive was extracted using Tri-Reagent (Sigma, St. Louis, MO) according to the manufacturer's instructions. For each of the six populations, three pools containing an equal amount of total RNA extracted from 5 individuals for each tissue was pooled.. Labeled Cy3 and Cy5 probes were synthetized from 200ng of RNA from each pool using Agilent Low Imput Quick Amp labeling kit following manufacturer protocol. Labeled probes were then purified using Kit Illustra CyScribe GFx purification (GE HealthCare) and both quantity and quality were determined using NanoDrop spectrophotometer. For microarray hybridization 800ng of probes were firstly fragmented at 60ºC for 30 min. using fragmentation buffer (Agilent Technologies, USA) and then added to 25ul of 2X hybridization buffer (Agilent Technologies, USA). Arrays were hybridized in chambers at 65ºC for 17 h. Following hybridization the arrays were washed, twice, with wash buffer 1 containing 500ml Agilent Technologies, IncWilnington, USA and 250ml Triton 10X for 1 min, once with wash buffer 2 containing Agilent Technologies, IncWilnington, USA and 250ml Triton 10X for 1 minute to 37ºC and finally one with acetonitrile (200ml) for 10 second. Hybridized microarrays were scanned with an Agilent scanner using the Feature Extraction Software (version 9.5.3.1, Agilent Technologies, Inc). This process was repeated for all the hybridized slides with a 3um resolution mode. Data from each probe was then extracted and analyzed.

2.3 Statistical analysis

The microarray data were analyzed using the statistical language R studio (www.rstudio.com), BioConductor (Gentleman et al., 2004) and Limma library (Smyth et al., 2005). The Background correction of the probe intensity was carried out using the normexp method (Ritchie et al., 2007). Technical artifacts were removed using quantile normalization and to remove intensity dependent trends, intra and inter slide lowess normalization were applied. Only probes showing an intensity signal three times superior to the background signal in more than 20% of the samples were kept for the analysis. Intensities data were then log2-transformed and replicated values of each gene were averaged. The ratio of expression of each gene was then calculated by dividing log2 signal intensity of the gene for one given sample by the mean log2 intensity of the gene in all samples in order to standardize gene expression to a mean of zero.

Hierarchical clustering was performed using Tmev (Saeed et al., 2003), http//www.m4.org/mev.html) using the Euclidean distance metric and the complete linkage clustering parameters were used to identify clusters of genes that have most similarity in all samples after the number of cluster generated was determined using the FOM (Figure of merit) function to determine the optimal number of clusters and the KMC. Differential expression patterns between mussels were identified by SAM (Significant Microarray Analysis) program. Briefly, SAM computes a statistic for each gene measuring the strength of the relationship between gene expression and the response variable. We applied a permutation test (100 permutations) to better estimate false discovery rate

(FDR) which was used to estimate the proportion of false positive. Only genes with an FDR lower or equal to 5% were considered for further analysis.

Among the genes showing a significant regulation, those having a functional annotation were considered in a functional enrichment analysis for Gene Ontology term using DAVID software (Database for Annotation Visualization and Integrated Discovery) (Huang et al., 2009a, 2009b). Analysis were performed for the six comparisons, MG-LS; MG-RB; LS-MG; LS-RB; RB-LS; RB-MG, for gill, mantle and digestive gland. In each comparison, we obtained a list of the most relevant (overrepresented) biological terms. In the way to simplify the long GO term list obtained, we chose only those which were significantly enriched (p-value < 0.05) after DAVID analysis and we constructed a networks of similarities between GO terms using an algorithm similar to the neighbor joining approach implemented in a web server REVIGO (Reduce Visualize Gene Ontology) (Supek et al., 2011) which reduces the functional redundancies within list of GO terms and generate a interactive graph-based visualization keeping joined similar GO terms (represented by nodes) through edges that represent the degree of similarity.

3. Results and discussion

The number of significant regulated genes varies between 3800 to 13000 genes (Fig.1) across each site comparison. Gills and digestive gland record the major number of regulated genes with the exception of mantle in the comparison MG>LS, where the numbers of reported genes is higher for this tissue compared to gills an digestive gland. The comparison between RB>MG revealed the higher number of regulated genes. High number of regulated genes is reported in MG>RB where particularly the digestive gland has similar values to what is reported in RB>MG. Both hydrothermal sites has been reported to have the more contrasting environmental conditions, principally hydrostatic pressure due to the depth (-800m and -2300m respectively) and the composition of the seafloor that explain the differences in metal and gas concentration that emerge from the vent (Charlou et al., 2002). The second higher level of regulated genes is reported in LS compared to MG (LS>MG) and particularly in the digestive gland in MG site compared to RB (MG>RB), while the lowest and similar levels are reported in LS>RB and RB>LS. This difference in number of regulated genes between sites may reflect differences in physiological conditions.

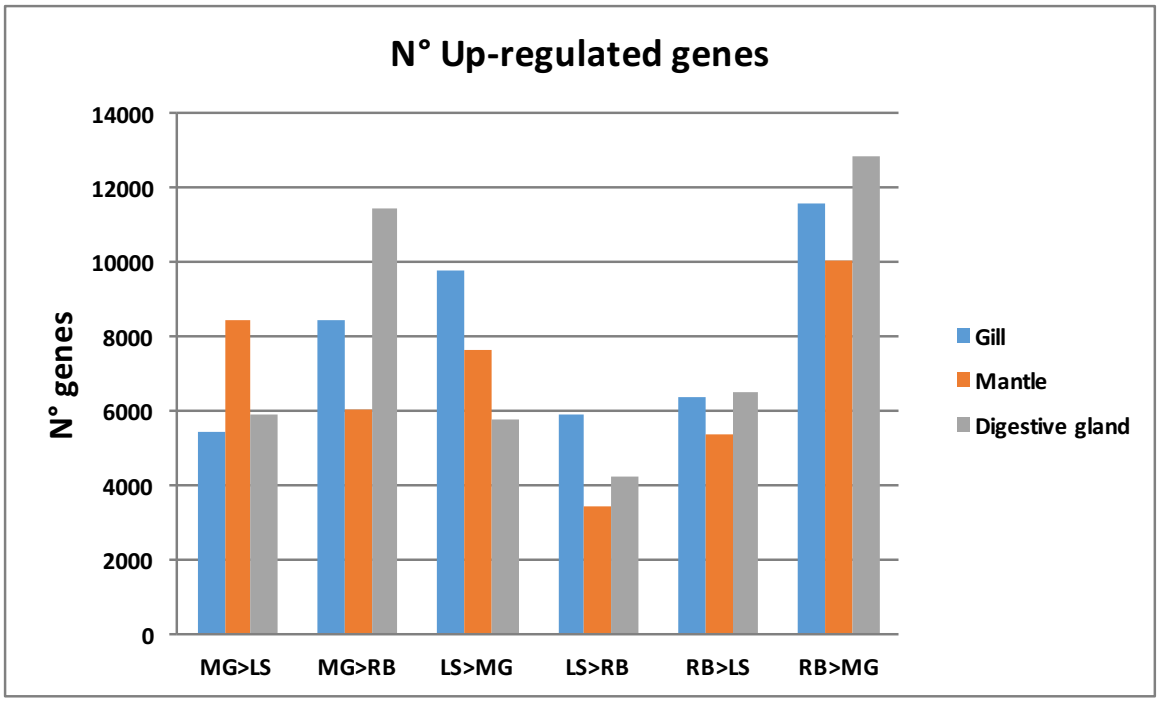
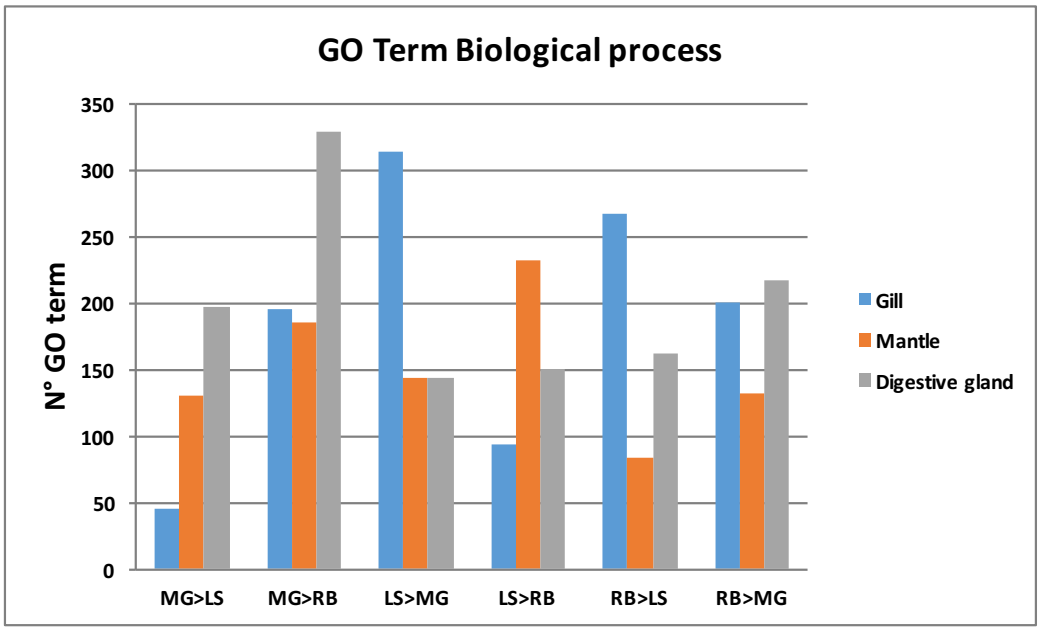
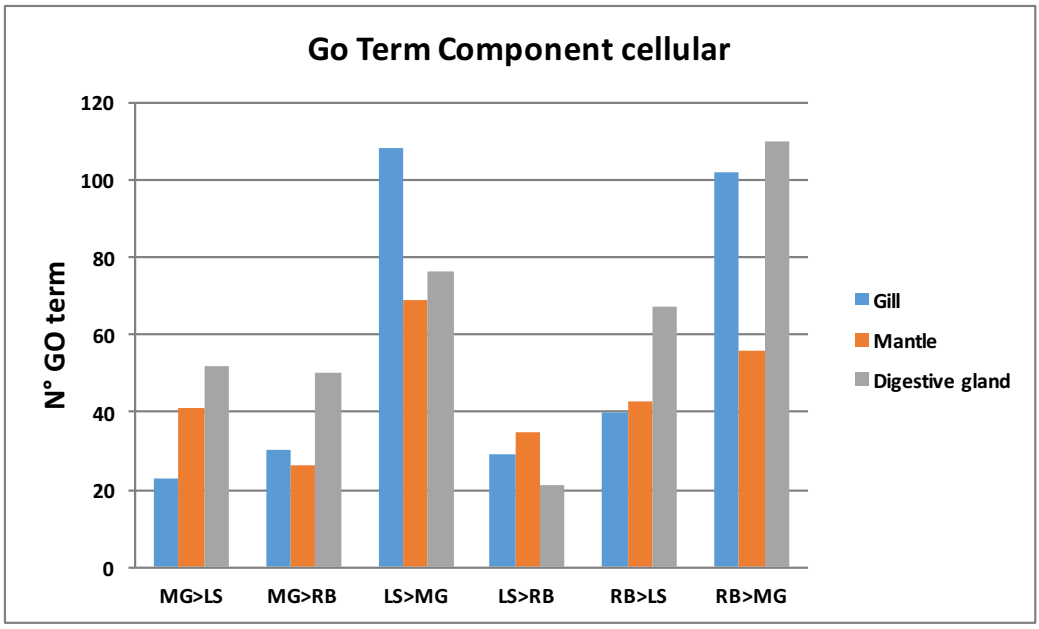


Figure 1. Total number of significantly regulated genes between each site for gills, mantle and digestive gland.

The physiological difference raised above is more evident when we observed the numbers of significant enriched GO term when the sites are compared between them, differences that vary at biological function, component cellular and molecular function and which could reflect the plasticity of response in natural populations of *B. azoricus.*





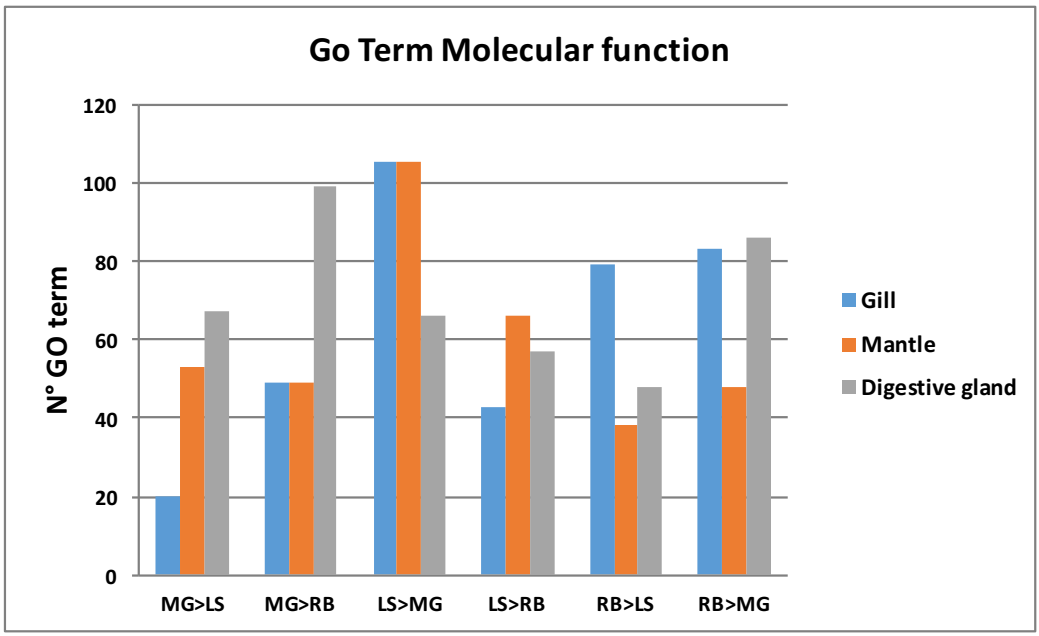


Figure 2. Number of significant enriched GO terms for each tissue and site comparison for Biological process, Component cellular and Molecular function.

For each site comparison, we made a GO term network that regroups terms based on their functional similarity in the different GO ontology terms, with the objective to identify the principal pathways in which the genes significantly regulated and enriched are involved. This information give us a global overview of the principal physiological status of each sites for the three different tissues, revealing interesting specific site and tissue pathways. Each one of the network described represents

the similarity between different GO terms, and each node represents a GO term, his color the levels of significance after enrichment analysis and width line of each edge indicate the degree of similarity.

Up-regulated genes in Menez Gwen compared to Lucky Strike.

This comparison (Fig.3, 4 and 5) revealed at the Biological process level a few number of regulated process in gills (Fig.3a) with essentially the reactive oxygen specie response and drug response together with ion transport. In mantle (Fig.4a), the mains process regulated include morphological and tissue formations as angiogenesis, skeletal muscle development and contraction, cell adhesion and cAMP signaling that are grouped in a cohesive network and additional fragmented networks with less similarity between them are represented for metabolic functions as vitamin metabolism, energy reserve and proteins transport and interestingly a node that regulate calcium ion transmembrane is represented in this tissue. In digestive gland (Fig. 5a) a very connected network bring together function related to response to stress, response to reactive oxygen species, regulation of protein kinase activity and cellular metabolism and a second network join nodes with ion regulation and transport principally for sodium, calcium. Protein and glucose metabolism are represented by singles nodes.

At the Cellular component level (Fig. 3b, 4b and 5b) few nodes are represented in the three tissues, mainly plasma membrane component and component for mitosis process (Kinesin complex), integral component of membranes and transport membrane receptor. At the Molecular function levels, the copper and calcium ion binding are strongly regulated in gills (Fig. 3c) and high significant networks of nodes related to protein activity as acetyl-CoA carboxylase, glutamic acid and UDP ligase activity are detected for mantle (Fig. 4c). Proteins binding activity, peptidic receptors and calcium ion binding function are also regulated in the digestive gland (Fig. 5c). The complete list of GO term used in this analysis can be found in the appendix 1.

Up-regulated genes in Menez Gwen compared to Rainbow.

At the Biological process level, a high activity, represented for the elevated number enriched GO term, is detected in gills (Fig. 6a), mantle (Fig. 7a) and digestive gland (Fig. 8a) when MG and RB are compared. In this three tissues, a shared response is recorded and represented by term such as response in the immune system, regulation to viral response, cellular response to exogenous RNA, Toll signaling pathways, response to gram positive bacterium, development of central nervous systems. A specific response in gills is related to DNA methylation, thermosensory regulation and hypoxia response, in mantle to morphological formation and lipid storage in digestive gland.

At the cellular component level (Fig. 6b, 7b and 8b), several pathways are shared in the three tissues such as plasma integral components, extracellular matrix and Golgi lumen. At the Molecular function level, GO term enriched in gill (Fig. 6c) include actinin, fibrinogen and collagen binding activity and calcium ion binding. In mantle (Fig. 7c), binding activity is also reported related to calcium and zinc binding, protein binding, collagen binding, receptor activity and peptidase activities. In the digestive gland (Fig.8c), a different patter is observed whit an enrichment in terms related to reproduction process as spermatogenesis, mitotic nuclear division and DNA replication.

Up-regulated genes in Lucky Strike compared to Menez Gwen.

Different tissue regulation process are reported at the Biological level for this site comparison where gill (Fig. 9a) is characterized by GO term linked to response immune, viral process, Toll-like receptor, necroptotic process, cell death process, protein phosphorylation, endocytosis, autophagosome, lipoprotein and lysosome transport. In mantle, different pathways are represented, principally mitochondrial translation, RNA processing, translation and process related to energy generation as Tricalboxilic acid cycle and ATP synthesis. In the digestive gland, process to energy generation are present with TCA cycle and ATP metabolism together with process related to cellular division as mitotic nuclear division, DNA replication and repair and RNA processing.

In the Cellular component level, the most representative organelles are the cytoplasm, cytoskeleton, golgi apparatus, mitochondrial, nucleolus and ribosomal subunit in the three tissues (Fig. 9b, 10b, 11b). In the Molecular function level, gills are characterized by the presence of high binding proteins domains, ligase activity, and ATP binding with a second network formed by enzymatic activities as glycine ligase activity, coenzyme gamma-glutamyl ligase and carboxylase activity (Fig. 9c). In mantle (Fig. 10c, 10d), Go term enriched are dominated by transferases activity, while in digestive gland the dominant pattern is ATPase and binding activity where interestingly a sulfur cluster binding is detected as a single node (Fig. 11c).

Up-regulated genes in Lucky Strike compared to Rainbow

The differences in this site are represented by a low number of GO term in gill (Fig. 12a) but with higher levels of significance and the contrary is observed in mantle (Fig. 13a) with a high number of GO terms linked in a big network where the more significant node is represented by the innate immune system followed by Kappa kinase/NF-kappaB signaling, and other interesting pathways are reported such as hydrogen peroxide biosynthesis, DNA methylation, regulation of calcium ion concentration, mineralization process, detection of bacterium, cell surface recognition receptor and necroptotic and apoptotic process. In digestive gland, the response in immune system and hydrogen peroxide catabolism are also present together with response to oxidative stress, regulation of

inflammatory process and DNA methylation mechanism and apoptotic process (Fig. 14a). At the Cellular component level, lower differences between tissues is reported (Fig. 12b, 13b and 14b) with principally the cytoplasm and cellular membranes being represented. For the Molecular function, a low numbers of GO terms is reported for gills (Fig. 12c) with principally the binding activity linked with ATPase activity. For mantle, the binding activity is represented by more GO terms, in a similar manner to the ligase activity terms (Fig. 13c). For digestive gland (Fig. 14c), the ligase activity is over represented like the receptor signals and peroxidase activity, calcium and zinc ion binding and NAD(P)H oxidase activity.

Up-regulated genes in Rainbow compared to Menez Gwen.

At the Biological process level, the three tissues present different numbers of physiological pathways regulated. In gills (Fig.15a), the most represented are related to tissue and organ development, post-translation protein modifications, intracellular protein transport, apoptotic signaling pathways, TOR signaling, detection of mechanical stimulus, response to organic substances, apoptotic signaling and DNA repair. Mantle (Fig. 16a) is characterized by the process of fatty acid oxidation, glutamate metabolism, viral response process, bicarbonate transport, RNA processing, translational and elongation process, and organ differentiation process. Digestive gland (Fig. 17a) is overrepresented in DNA repair process, RNA splicing, mitotic cellular process, response to heat, ATP synthesis related to proton transport, mitochondrial NADH electron transport. At the Cellular component level, lower differences between tissues are detected but some component are over represented as lysosomal and endosome membrane and nuclear membrane in gills (Fig. 15b), mitochondrial respiratory chain complexI, ribosome, nucleoplasma in mantle (Fig. 16b) and nucleus, nucleoplasm, methylosome, and mitochondrial membrane and respiratory chain complexI in the digestive gland (Fig. 17b). At the Molecular function level, in gill (Fig. 15c) binding and transporter activities are overrepresented with protein binding and ATP binding. In mantle, ATPase, anion transporter activity, acyl-CoA dehydrogenase activity, sulfur cluster binding and gamma-glutamyltransferase activity are some of the most interesting function overrepresented. In the digestive gland (Fig. 17c), RNA and DNA binding activities, ATPase activity, NADH dehydrogenase activity, 4 sulfur cluster binding are the principal pathways represented.

Up-regulated genes in Rainbow compared to Lucky Strike.

The principal Biological process recorded in the gills (Fig. 18a) show a major regulation in process related to innate immune response, regulation of inflammatory response, Toll like receptor signaling, ROS metabolism and hydrogen peroxide catabolism. In the mantle (Fig. 19a), the DNA repair process is related to cellular heat response, detection of temperature stimulus and ionizing radiation and also hydrogen transmembrane transport related to calcium ion transport, and a

significant representation is detected for cilium movement and Golgi vesicle transport. In the digestive gland (Fig. 20a), the more significant GO term are related to mitotic division, spermatogenesis, cilium assembly and movement, collagen organization, DNA repair, protein phosphorylation and organ formation. Like in the others site comparison, the lower representation of GO term is also reported for the Cellular component, where no major differences between tissues is reported characterized by the shared representation of membrane extracellular region, Golgi apparatus, microtubule and cytoskeleton formation (Fig. 18b, 19b and 20b). For the Molecular function, the GO term protein ligase activity, matrix protein component, sulfate transmembrane, oxalate transmembrane, succinate transmembrane, peroxidase activity, NAD(P)H oxidase and calcium, zinc binding proteins are highly represented in gills (Fig. 18c), microtubule motor activity, ATP binding, sulfotransferase, calcium ion binding and metallopeptidase activity, for mantle (Fig. 19c) and ATP binding, ATPase activity, kinase activity, calcium ion binding, sodium independent anion transmembrane activity and hemoglobin binding in digestive gland (Fig. 20c).

The development of the "omics" technology in the study of environmental interaction and gene expression profiling in marine species has increased notably in the last decades where environmental changes has been reported to alter metabolic pathways, developmental process, reproductive conditions, behavior and disease susceptibility. Those profiles can be used as prospective biomarkers of environmental change and have been proposed as an important tool in the context global change (Somero, 2012).

Our microarrays analysis developed in contrasting hydrothermal site allow us to illustrate the high physiological plasticity in the hydrothermal mussel *Bathymodiolus azoricus*, which is reflected principally by the differences in biological process that are over represented when site comparison is performed. This response vary between tissues. However, we can set up the specific response to some of the most characteristic environmental factors described such as the activation of reactive oxygen species response, that has been explored previously in *B. azoricus* in response to bacteria and metal exposure (Bebianno et al., 2005; Company et al., 2006; Martins et al., 2015, 2016). The bacterial response can also be attributed to the assimilation of the chemosynthetic thiotrophic (SOX) and methanotrophic (MOX) bacteria located in gills of *B. azoricus*, that use CO_2 and methane as carbon energy source. The presence of symbionts suggests the activation of physiological mechanisms involved in immune response such as Toll like receptor, response to exogenous RNA, lysozyme regulation as found in our analysis and that has explored recently in this mussels (Detree et al., 2016; Guezi et al., 2014). Bicarbonate transport was also over-represented in our study as being highly expressed in mantle when Rainbow is compared to Menez Gwen which is in agreement with the physiological importance of bicarbonate in the regulation of pH and in the formation of the shells of bivalves with also the regulation of mitochondrial activity (Haider et al., 2016). Also we report the

significant overexpression of biological process regulated to mechanical stimulus in the more deeper site which can be related to the effect of the pressure in this site or to the sampling effect when the mussels where removed from the vent.

Considering the response of *B. azoricus* related to abiotic factor as metal concentration, we can suggest the activation of specific biological responses based in the over representation found in our analysis as metal binding proteins, iron sulfur cluster binding, hydrogen peroxide catabolism, calcium-zinc ion transmembrane transport, glutathione biosynthesis. In marine bivalves, the exposure to toxic agent, particularly metal, can cause DNA damage (Emmanouil et al., 2007; Michel and Vincent-Hubert, 2015) which has a hereditable potential to increase the susceptibility of organisms to diseases or alter their capacity of stress response, implying that organisms have to develop an effective mechanism of DNA repair. In our analyses, we detected in different sites the over representation of these mechanism so we can suggest that this response is linked to the extreme condition of hydrothermal vent that may cause DNA damage. Response to hypoxia mechanism were also represented in our study which can be attributed to the low levels of dissolved oxygen present in the hydrothermal vent. However, the mechanism of oxygen exchange has not well elucidated for hydrothermal vent even some data reported in the literature suggest morphological change in decapods gills (Decelle et al., 2010), difference in protein regulation in annelid linked to changes in oxygen concentration (Mary et al., 2010) and specificity of hemoglobin structure (Hourdez and Weber, 2005). However, in natural populations, this specific and the others physiological pathways involved in adaptation to hydrothermal vent are not well identified in *B. azoicus*.

This work has generated a considerable amount of information about the different physiological pathways in hydrothermal vent characterized by different environmental conditions. This comparative site analysis revealed interesting differences at site and tissue level that can be interpreted as the strategy of *B. azoricus* in his adaptive process to the hydrothermal vent. However this information and particularly the high numbers of regulated genes challenge represent a challenge for his interpretation whereby we only focus in the significant GO terms obtained after the enrichment analysis. Additionnal analysis have to be performed especially in link with other physiological index.

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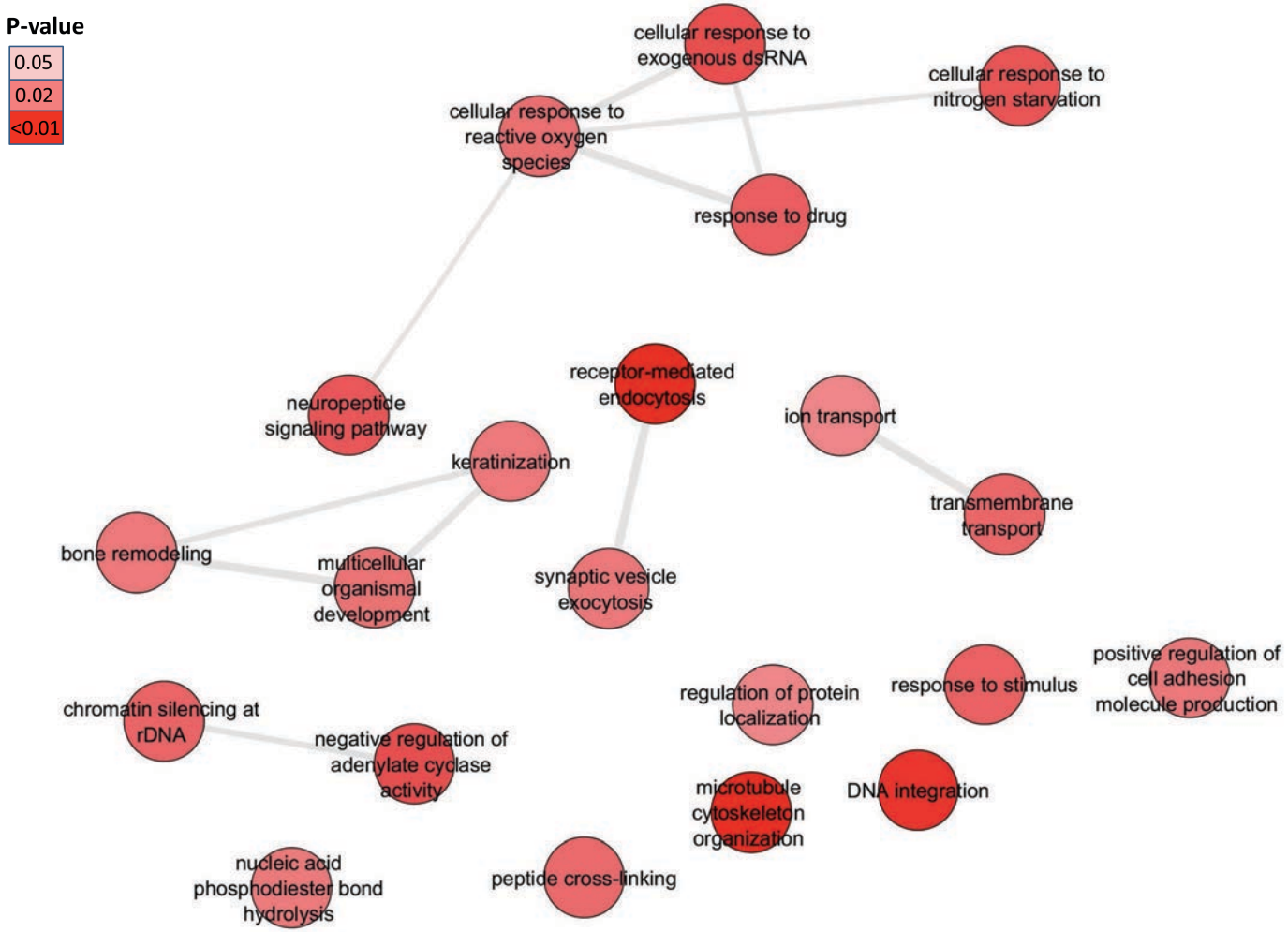

Figure 3a. MG genes higher expressed than LS for gill in biological process. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

CELLULAR COMPONENT

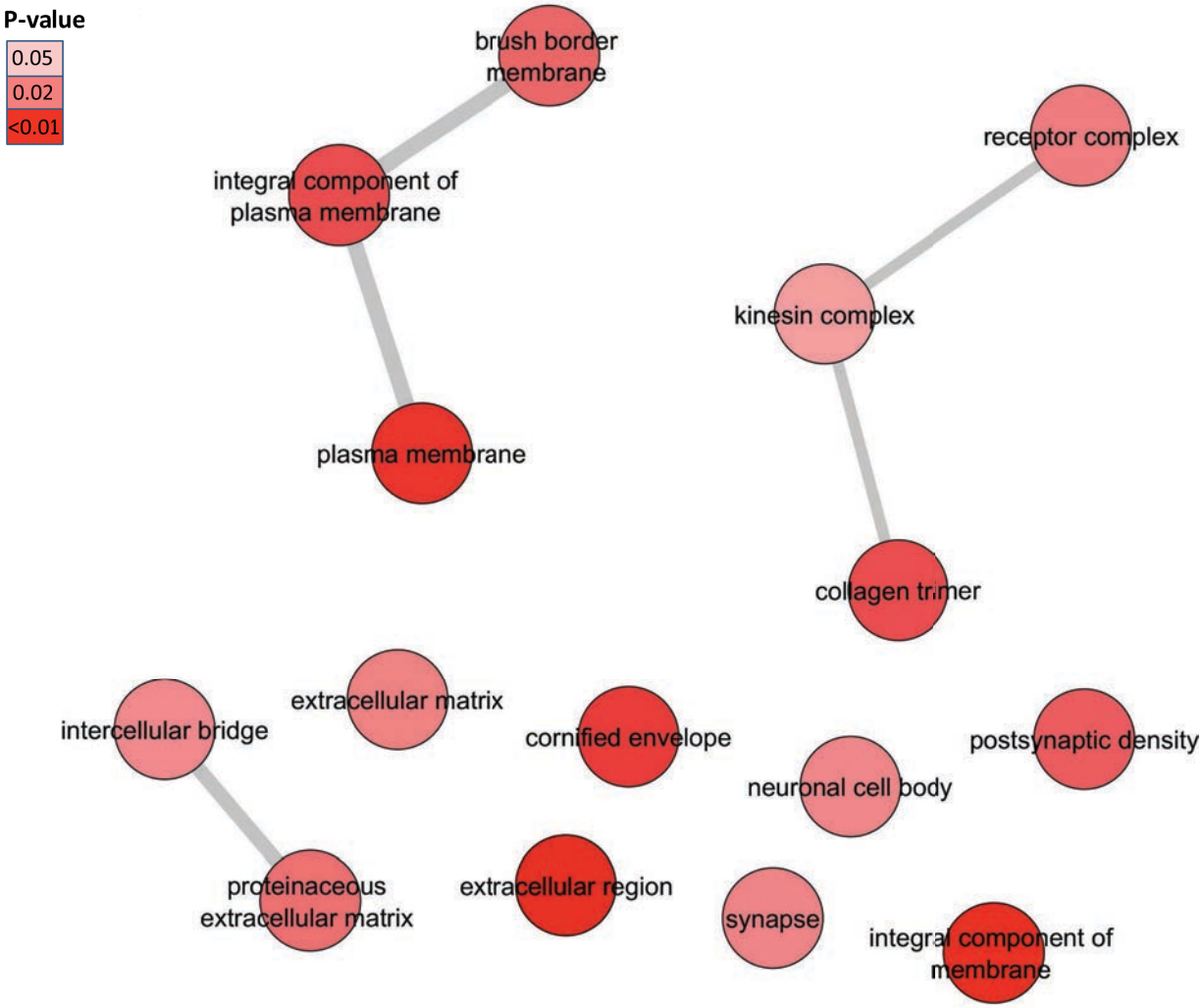

Figure 3b. MG genes higher expressed than LS for gill cellular component. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represent the degree of significance.

MOLECULAR FUNCTION

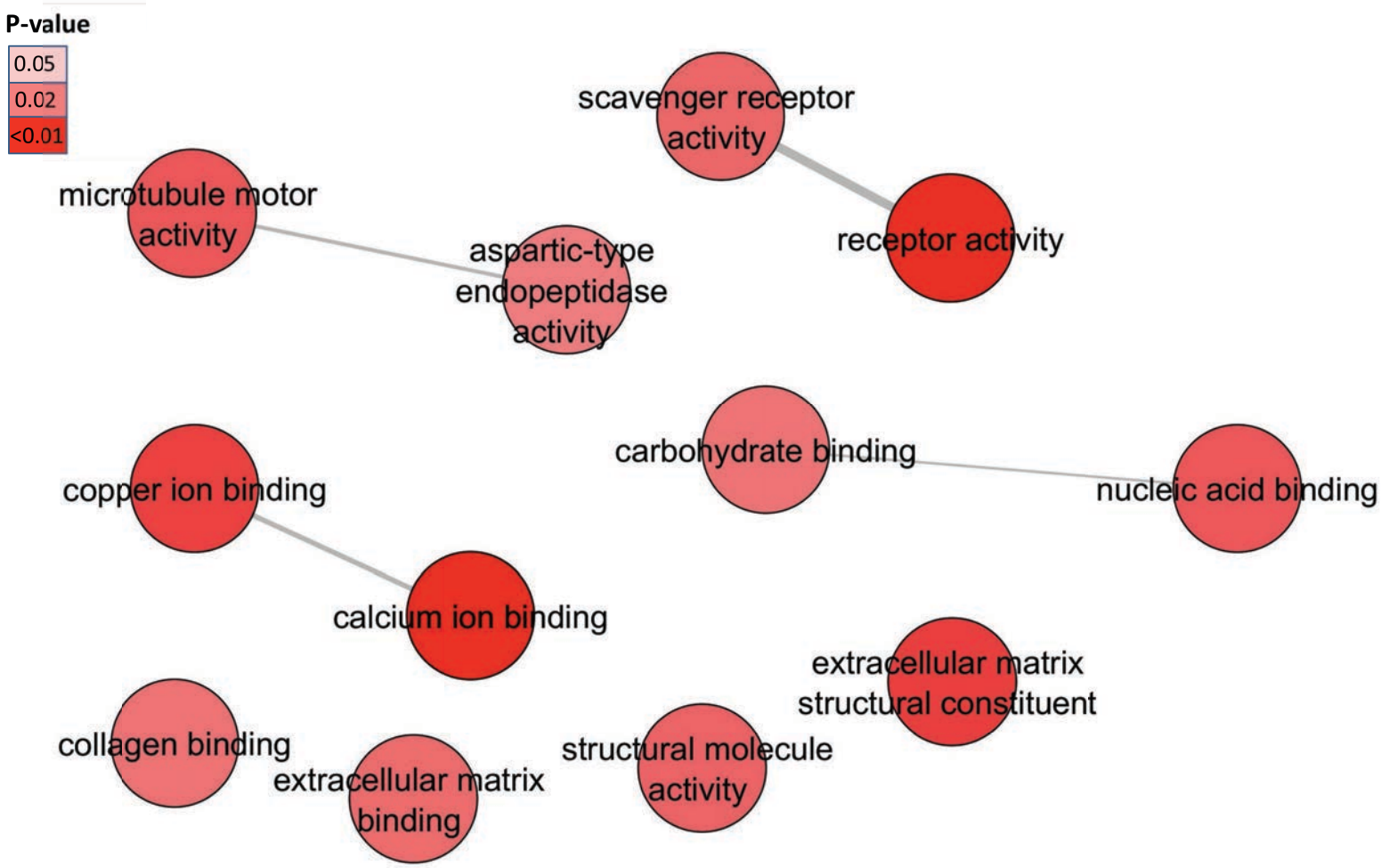

Figure 3c. MG genes higher expressed than LS for gill in molecular function.. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represent the degree of significance.

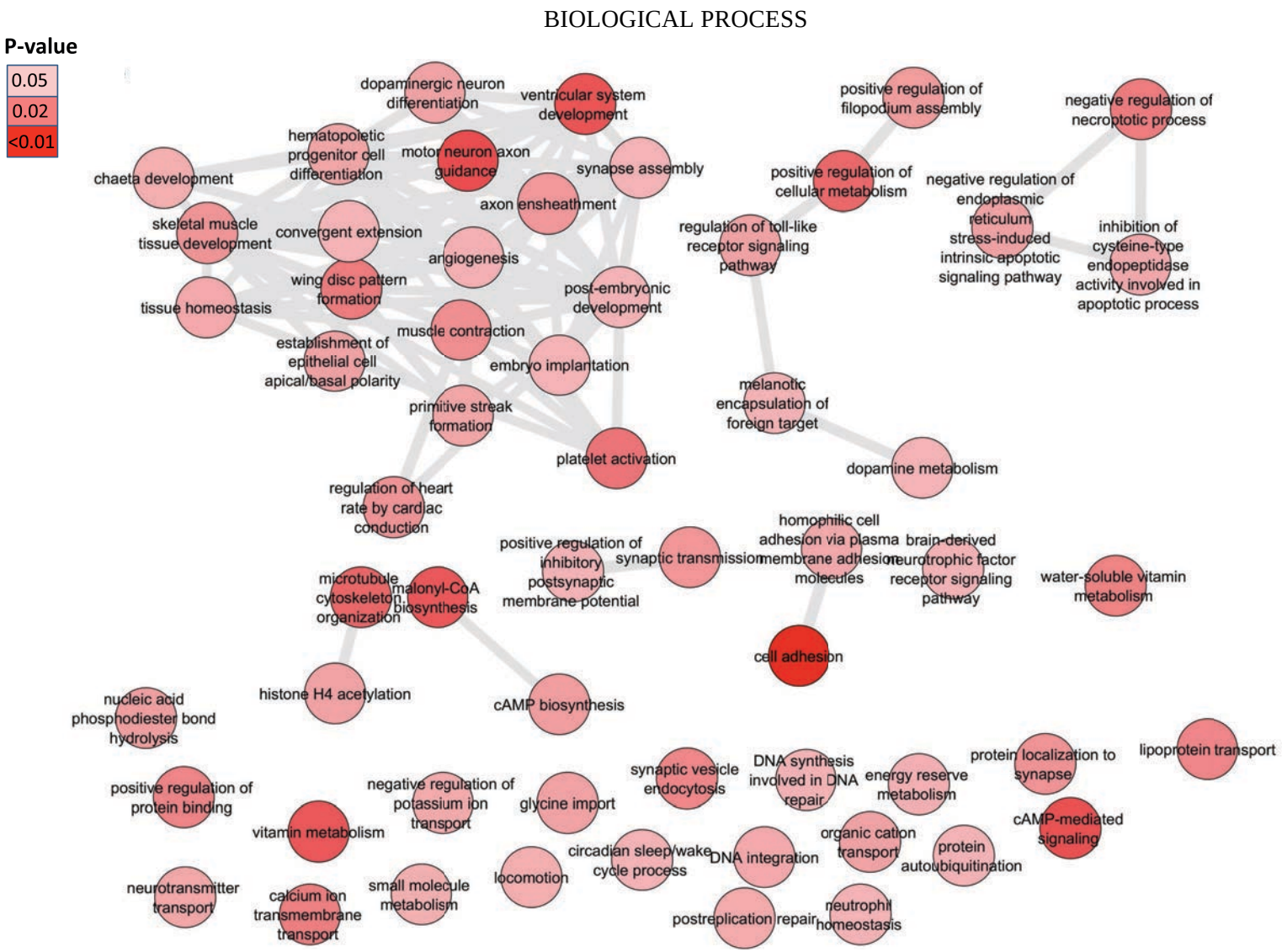

Figure 4a. MG genes higher expressed than LS for mantle in biological process. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

CELLULAR COMPONENT

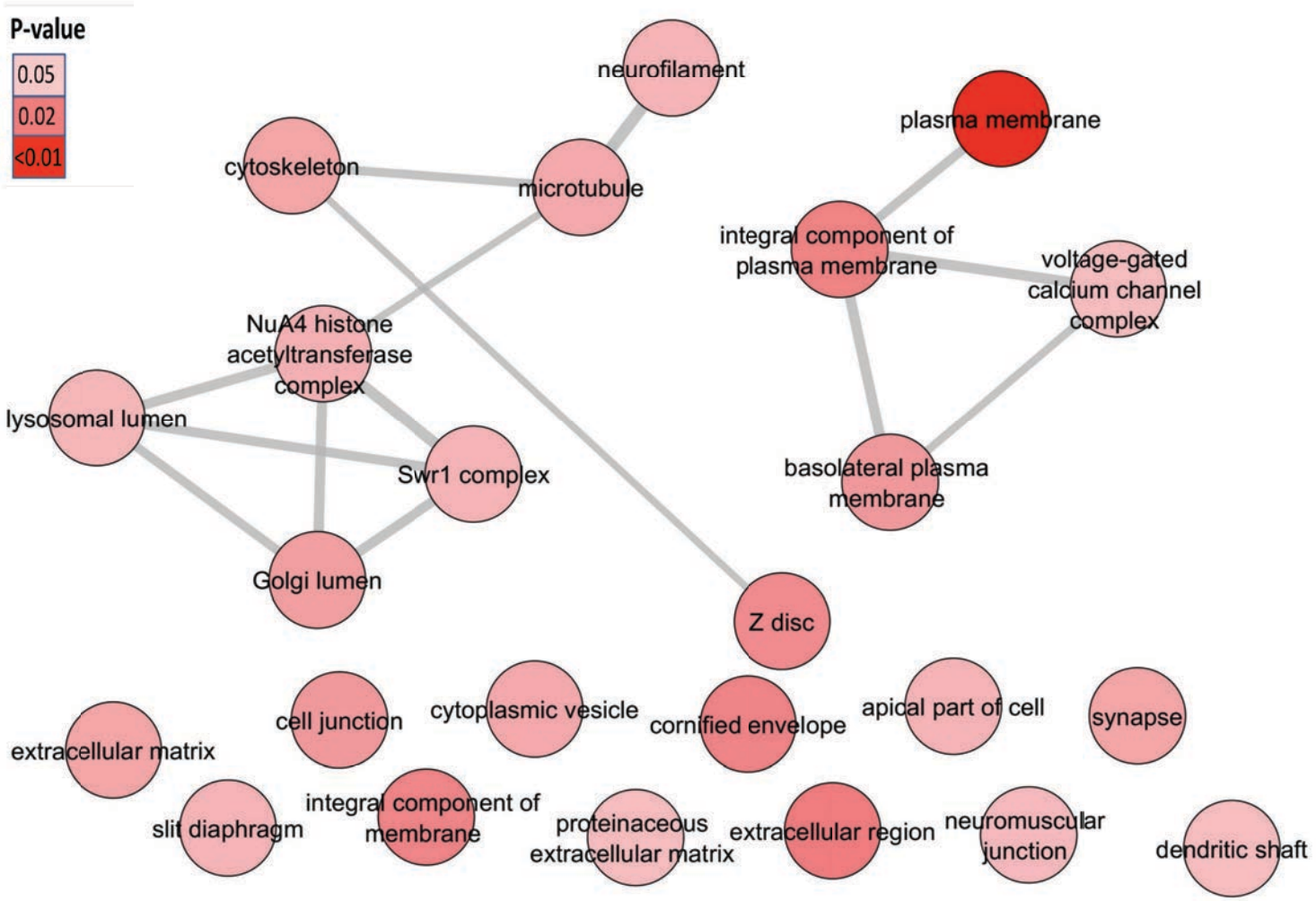

Figure 4b. MG genes higher expressed than LS for mantle in cellular component. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

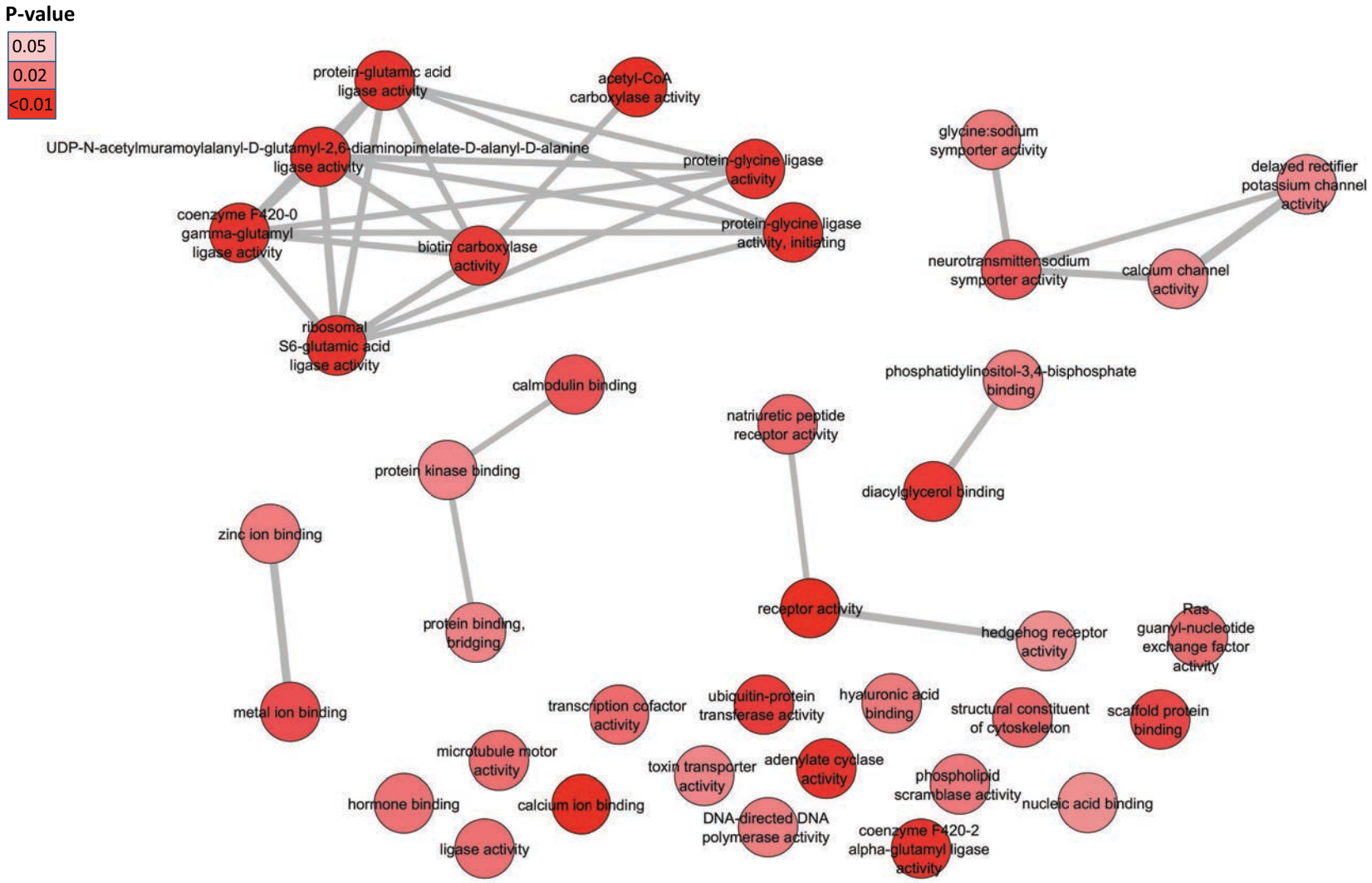

Figure 4c. MG genes higher expressed than LS for mantle in molecular function. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

BIOLOGICAL PROCESS

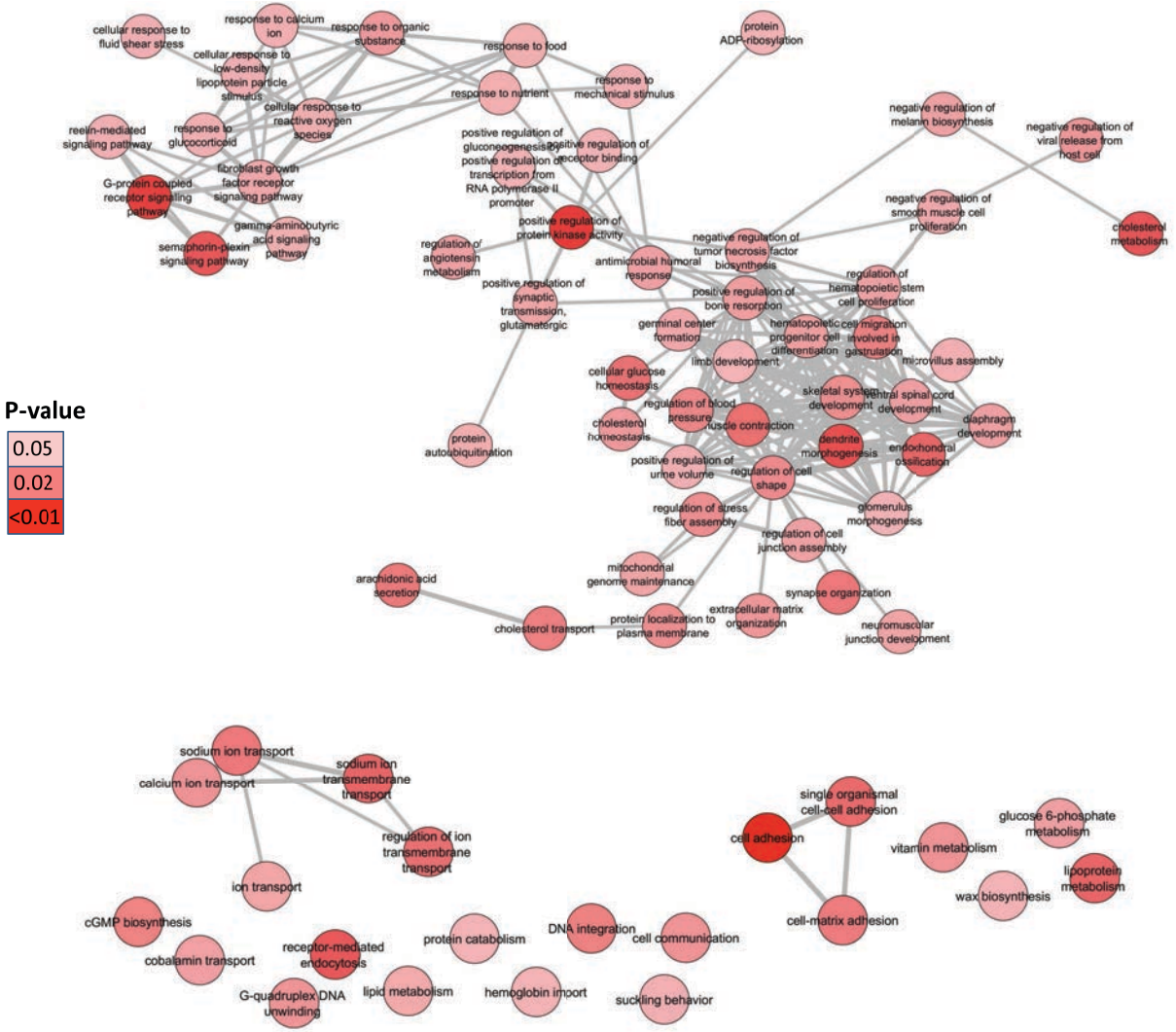

Figure 5a. MG genes higher expressed than LS for digestive gland in biological process. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

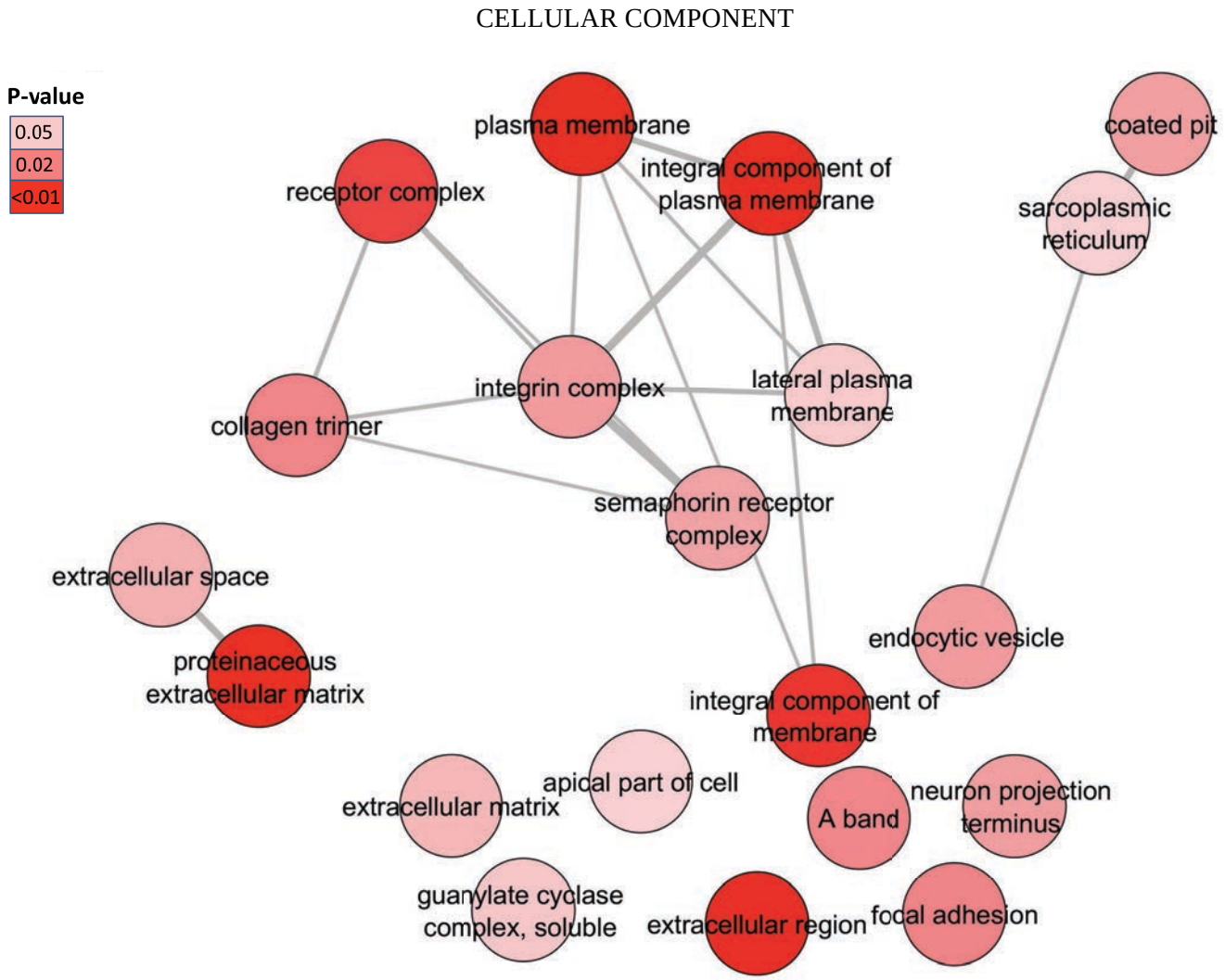

Figure 5b. MG genes higher expressed than LS for digestive gland in cellular component. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

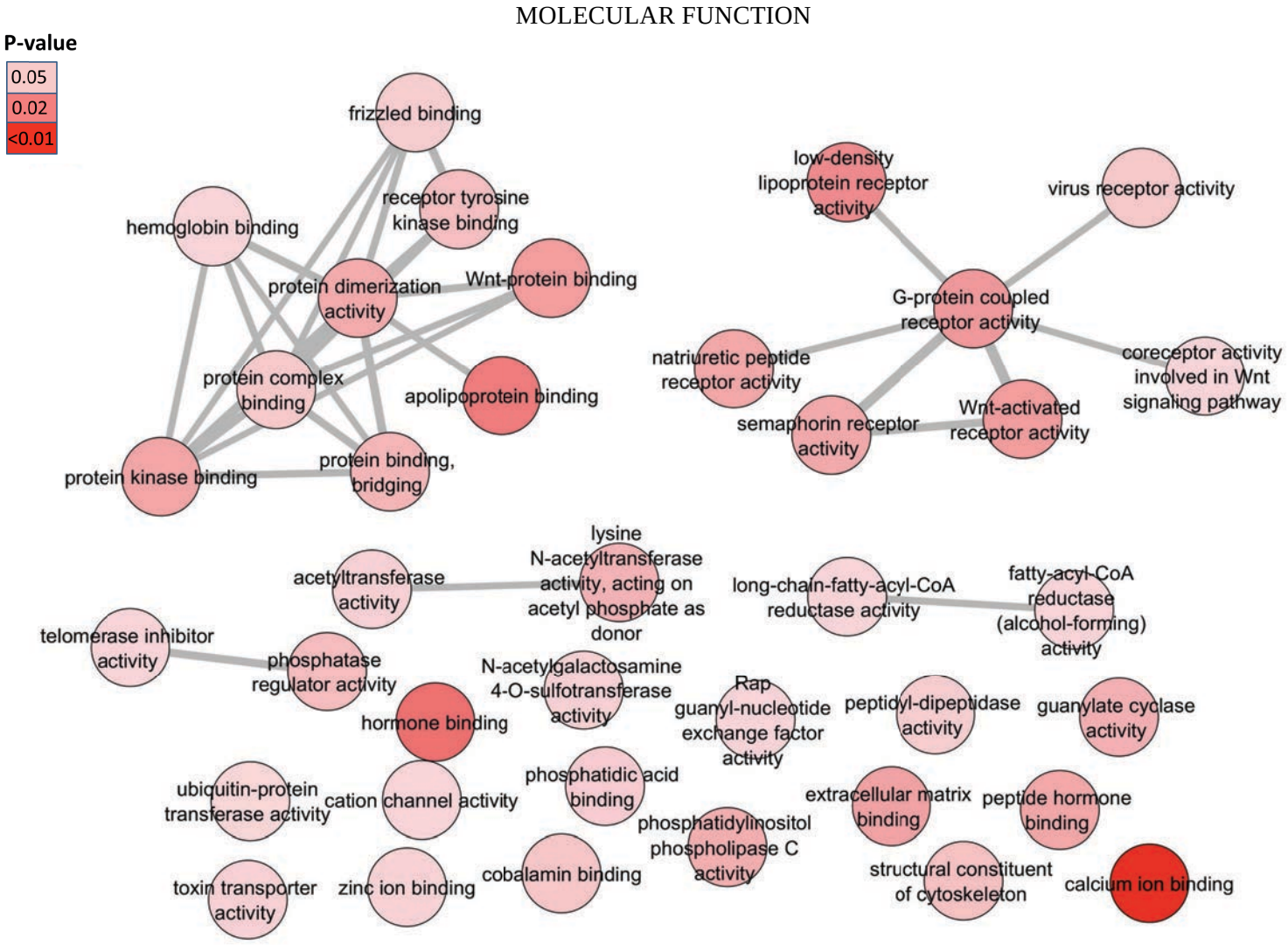

Figure 5c. MG genes higher expressed than LS for digestive gland in molecular function. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

BIOLOGICAL PROCESS

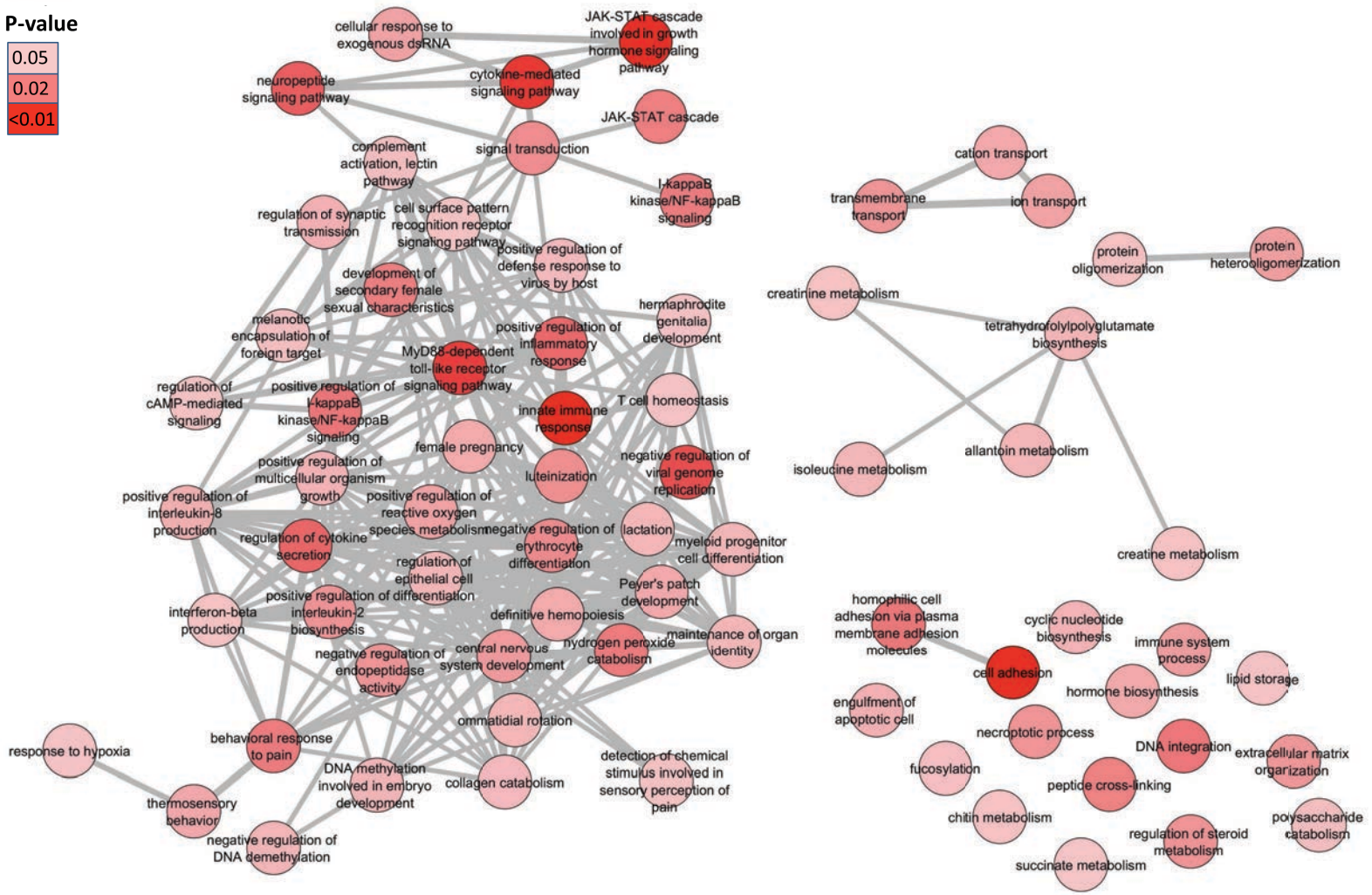

Figure 6a. MG genes higher expressed than RB for gill in biological process. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

CELLULAR COMPONENT

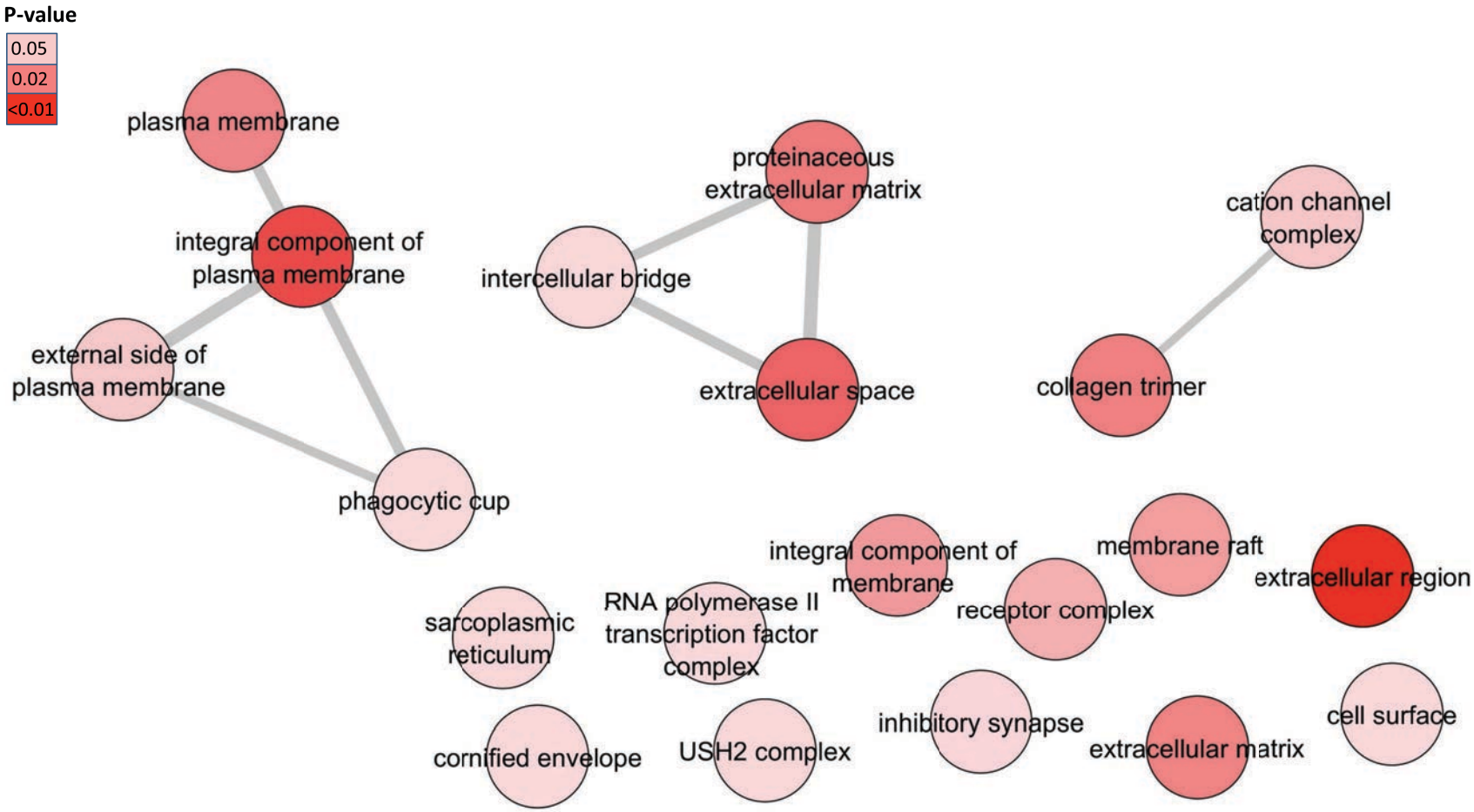

Figure 6b. MG genes higher expressed than RB for gill cellular component. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

MOLECULAR FUNCTION

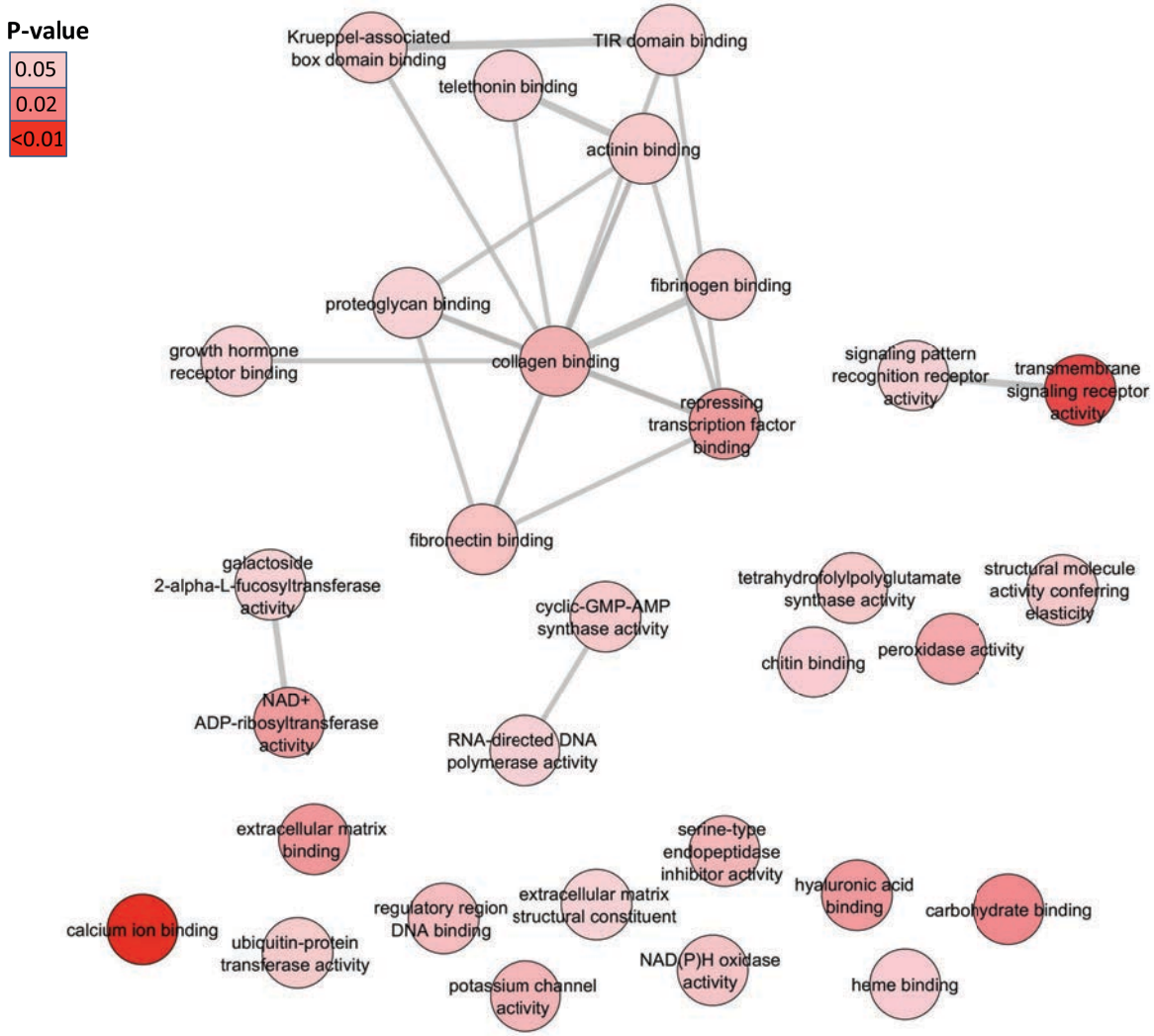

Figure 6c. MG genes higher expressed than RB for gill in molecular function.. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represent the degree of significance.

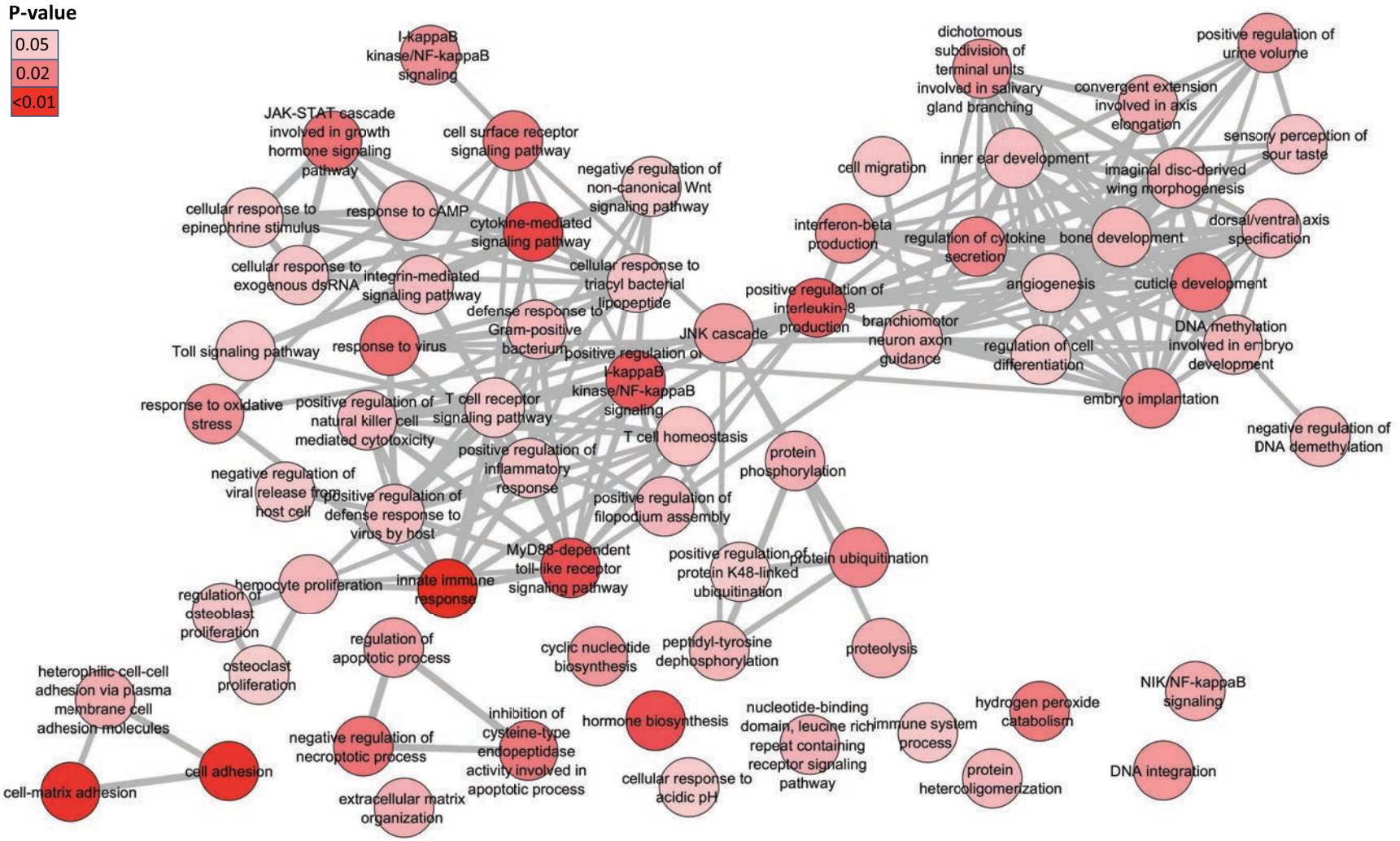

Figure 7a. MG genes higher expressed than RB for mantle in biological process. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

CELLULAR COMPONENT

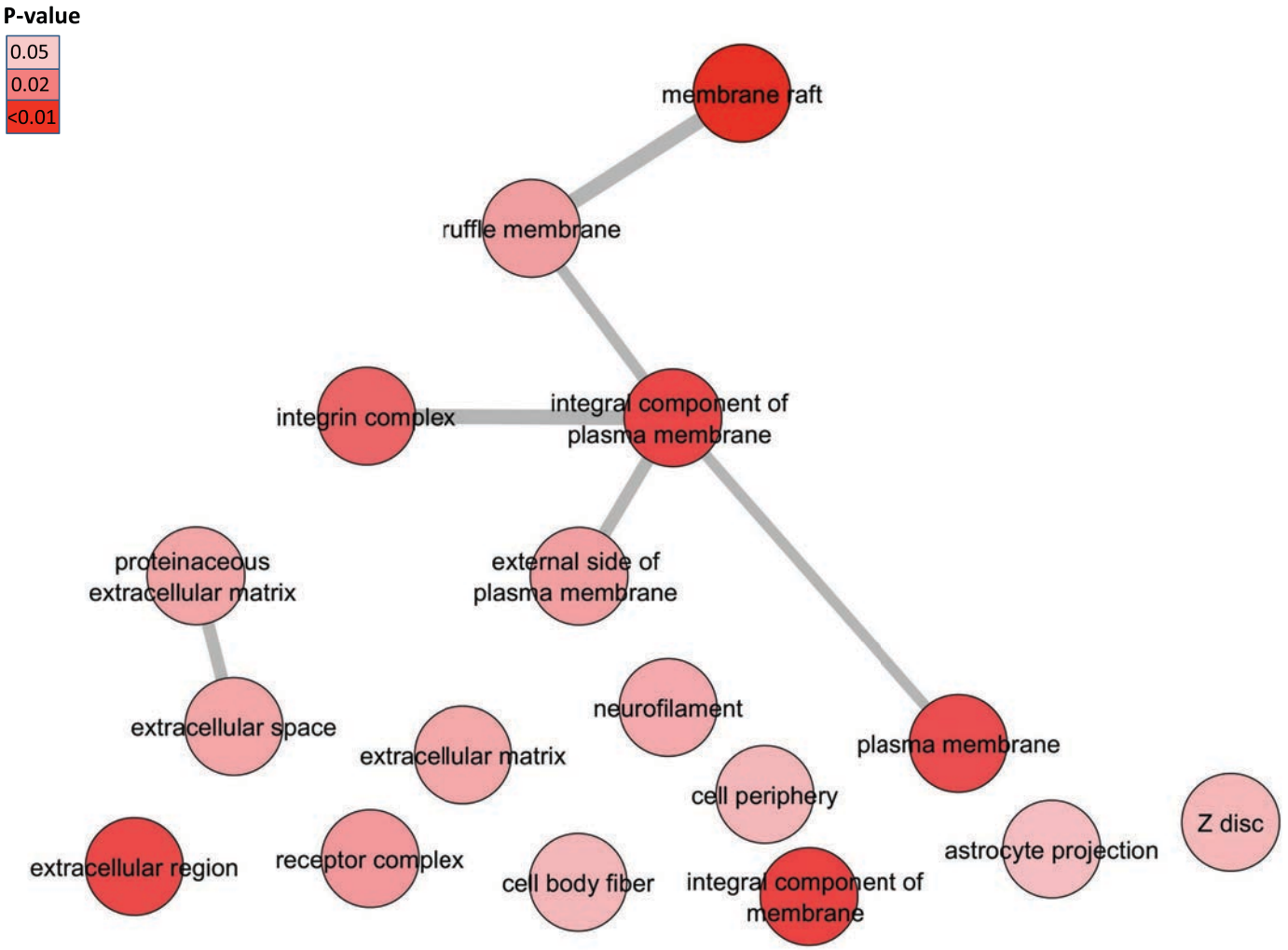

Figure 7b. MG genes higher expressed than RB for mantle in cellular component. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

MOLECULAR FUNCTION

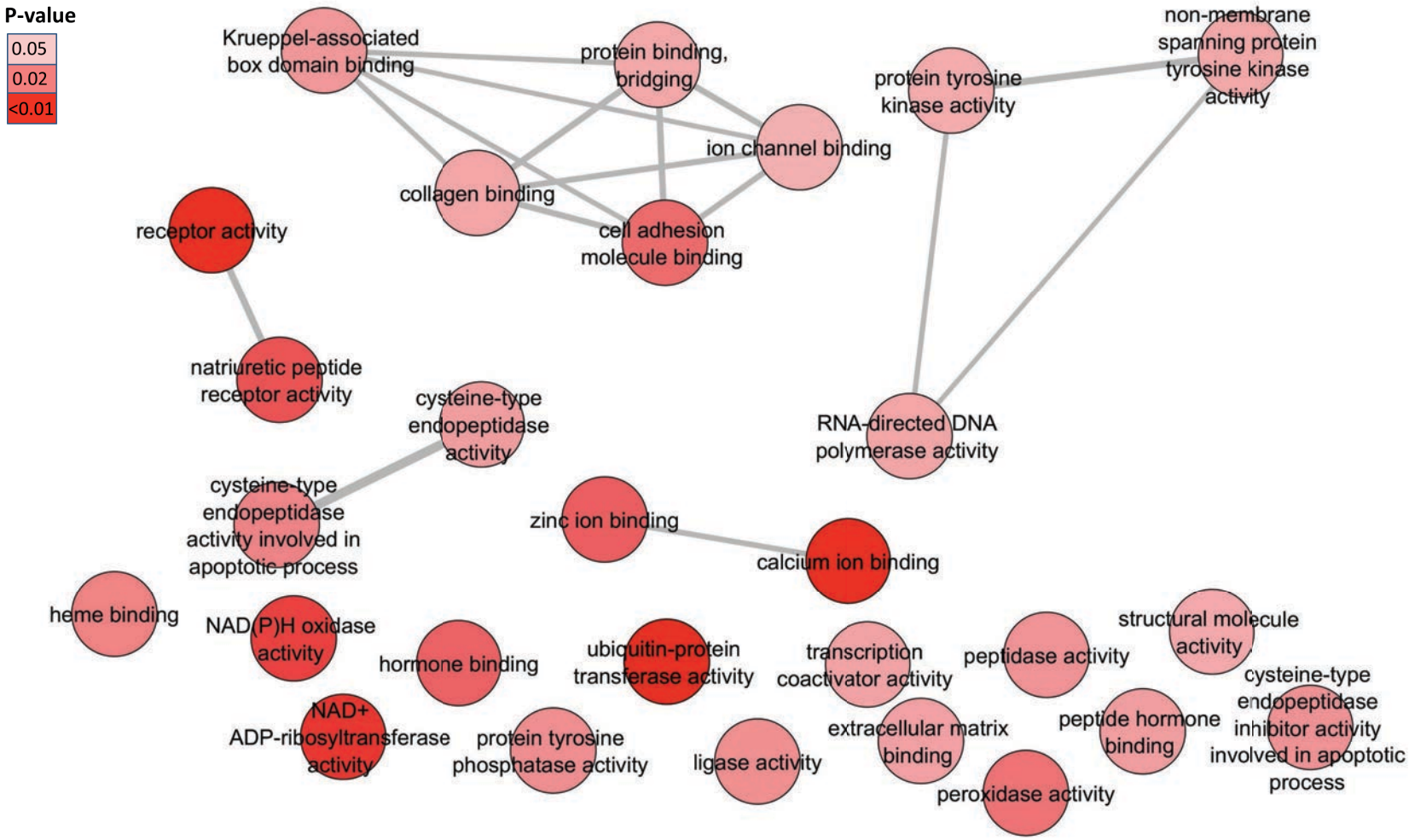

Figure 7c. MG genes higher expressed than RB for mantle in molecular function. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

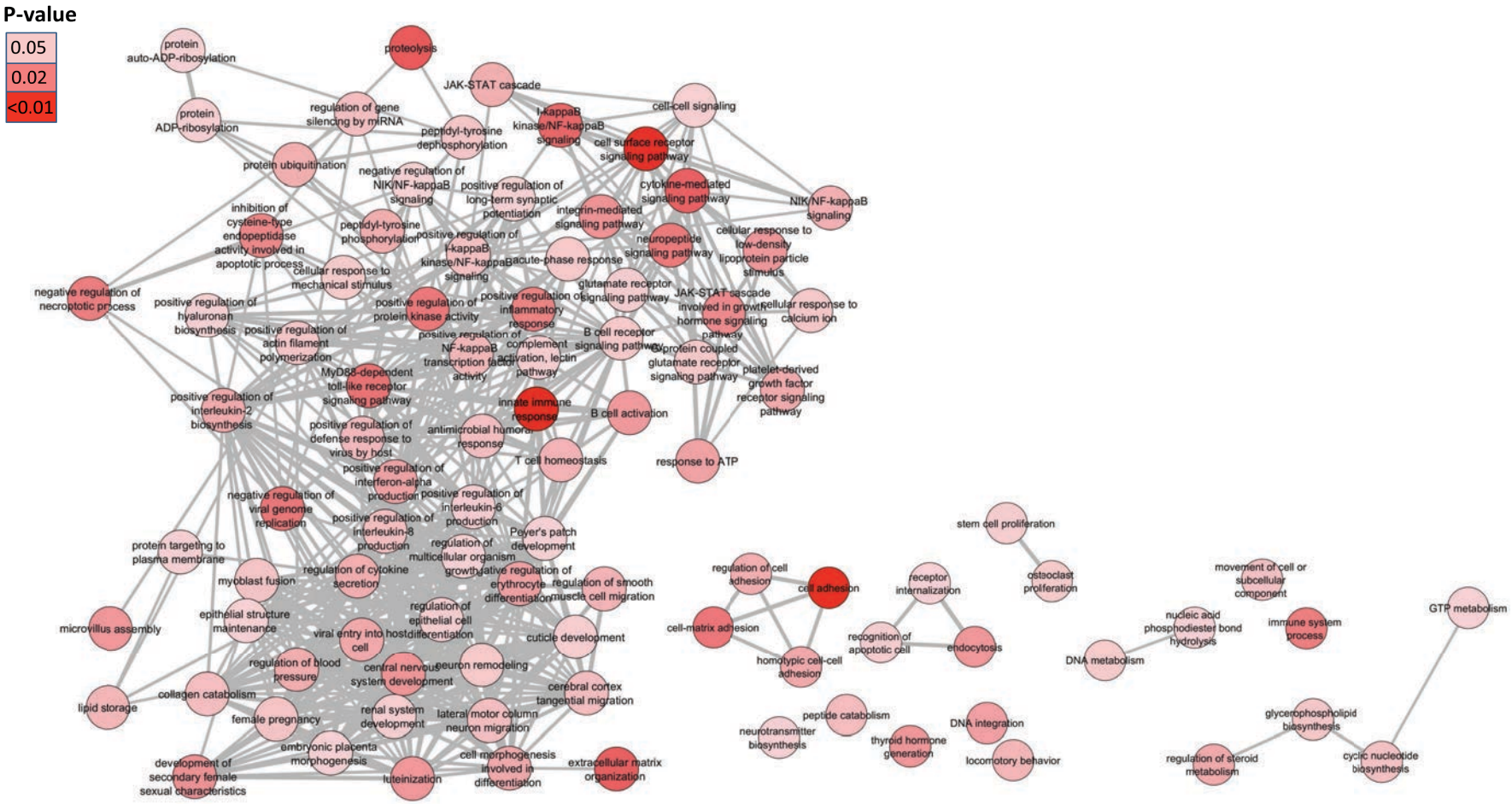

Figure 8a. MG genes higher expressed than RB for digestive gland in biological process. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

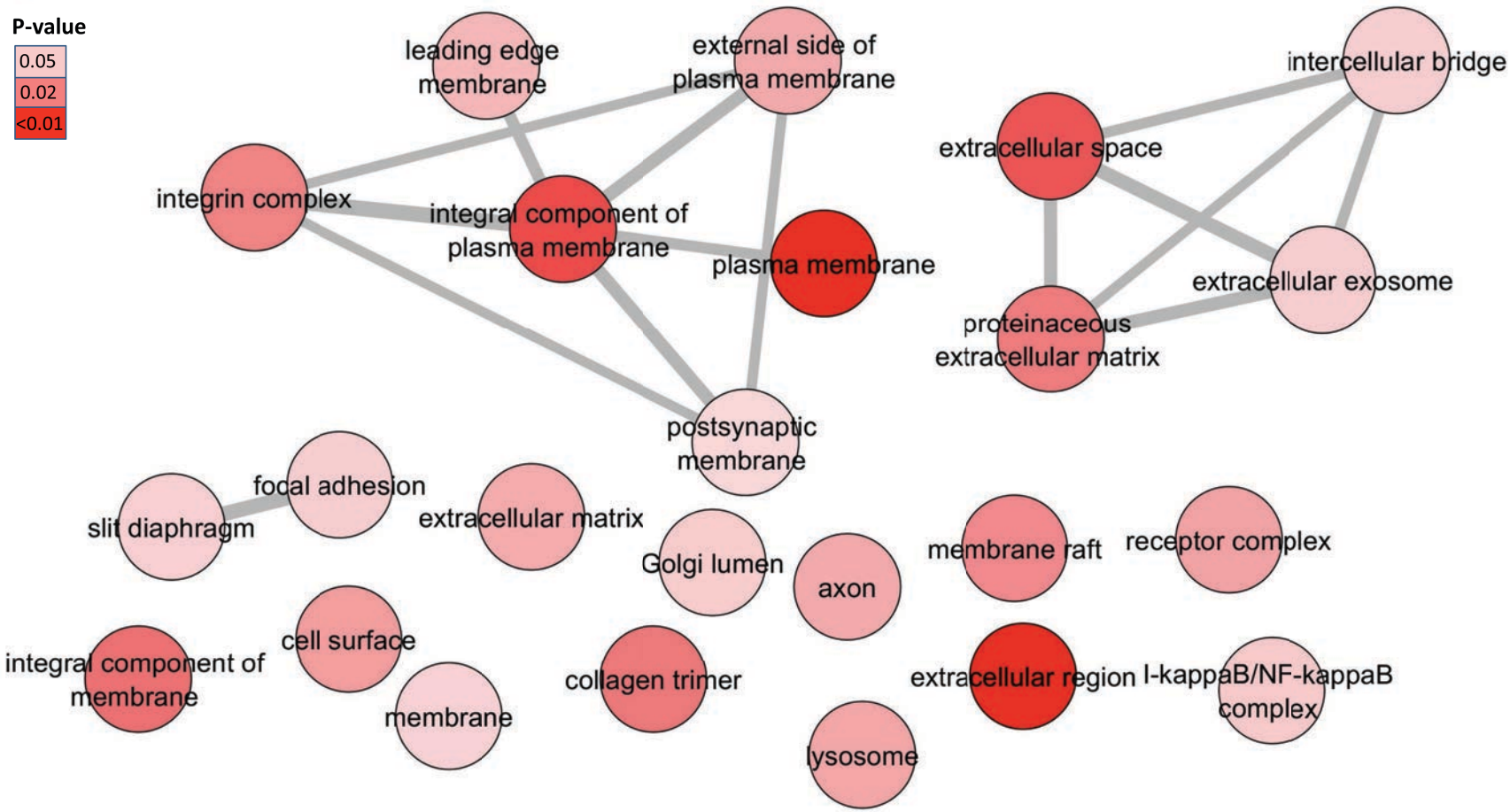

Figure 8b. MG genes higher expressed than RB for digestive gland in cellular component. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

MOLECULAR FUNCTION

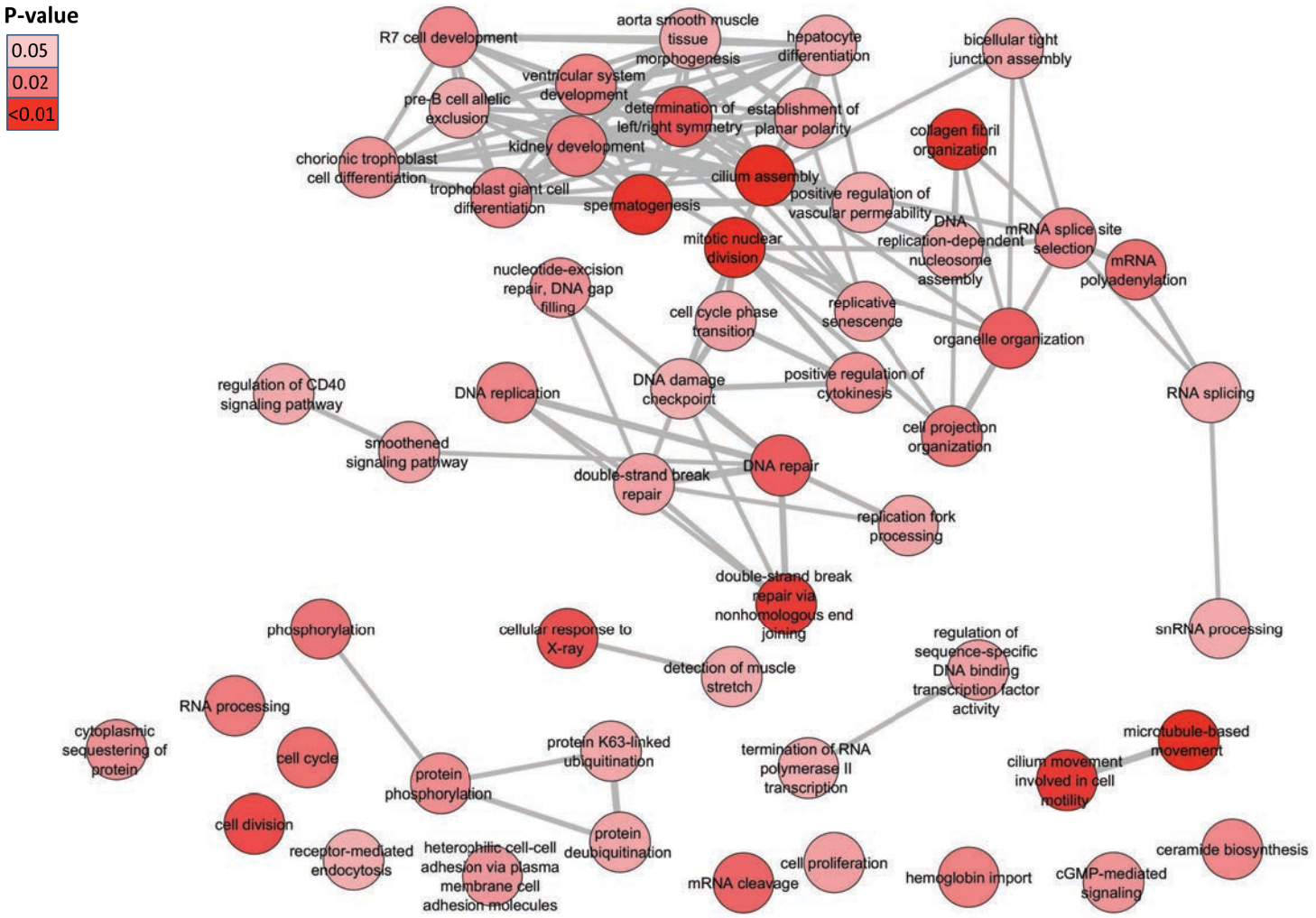

Figure 8c. MG genes higher expressed than RB for digestive gland in molecular function. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

BIOLOGICAL PROCESS

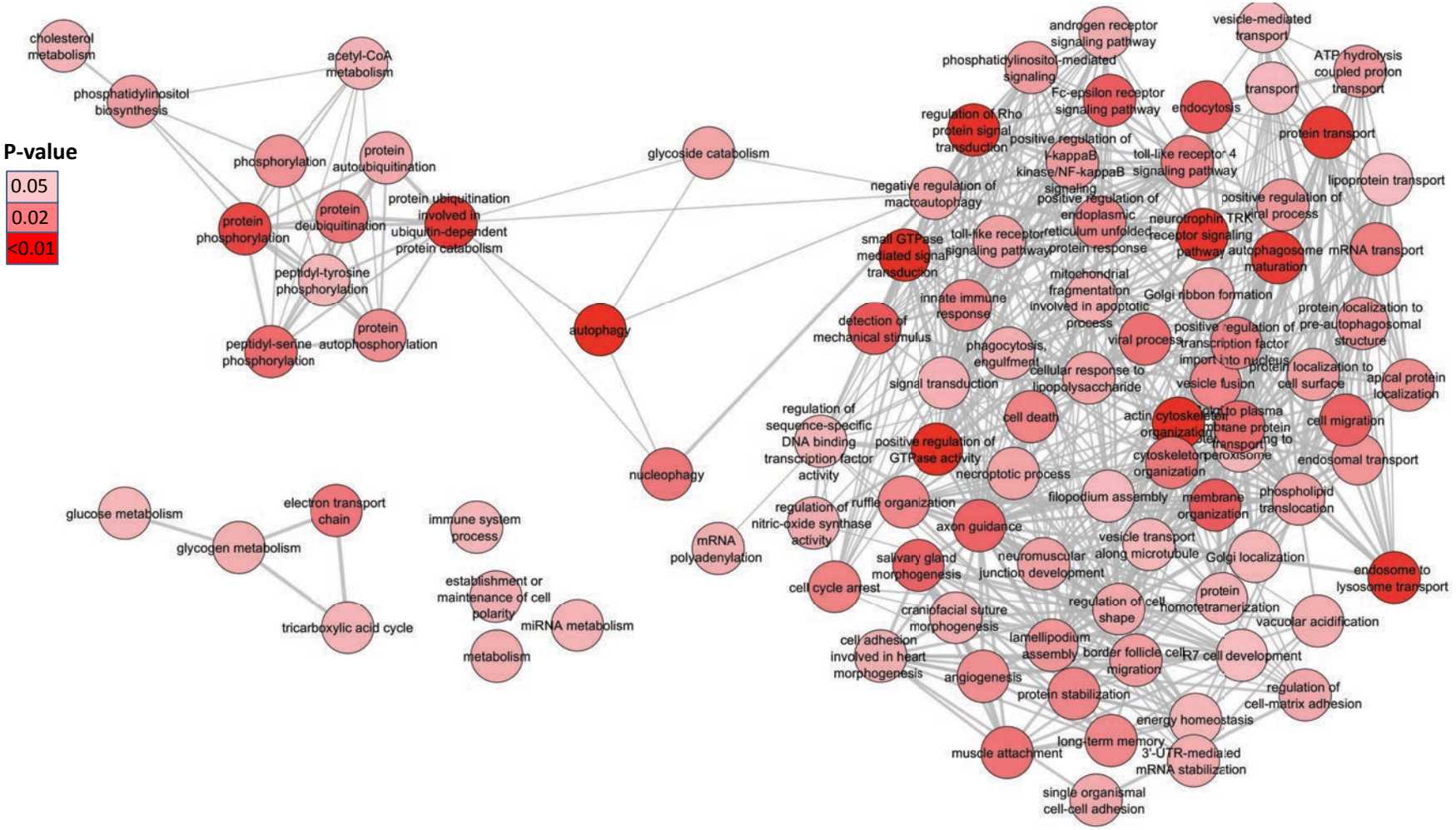

Figure 9a. LS genes higher expressed than MG for gill in biological process. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

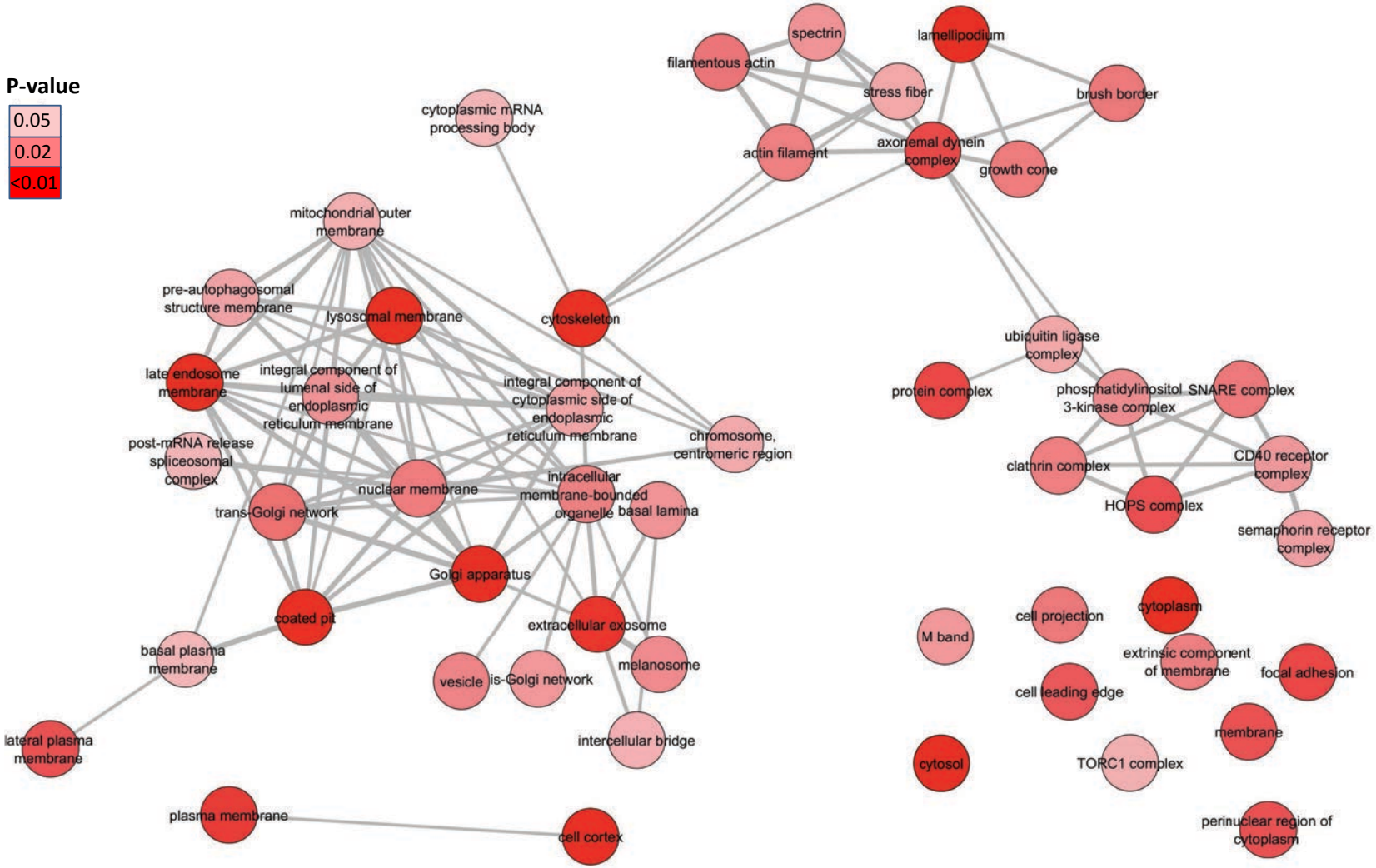

Figure 9b. LS genes higher expressed than MG for gill cellular component. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

MOLECULAR FUNCTION

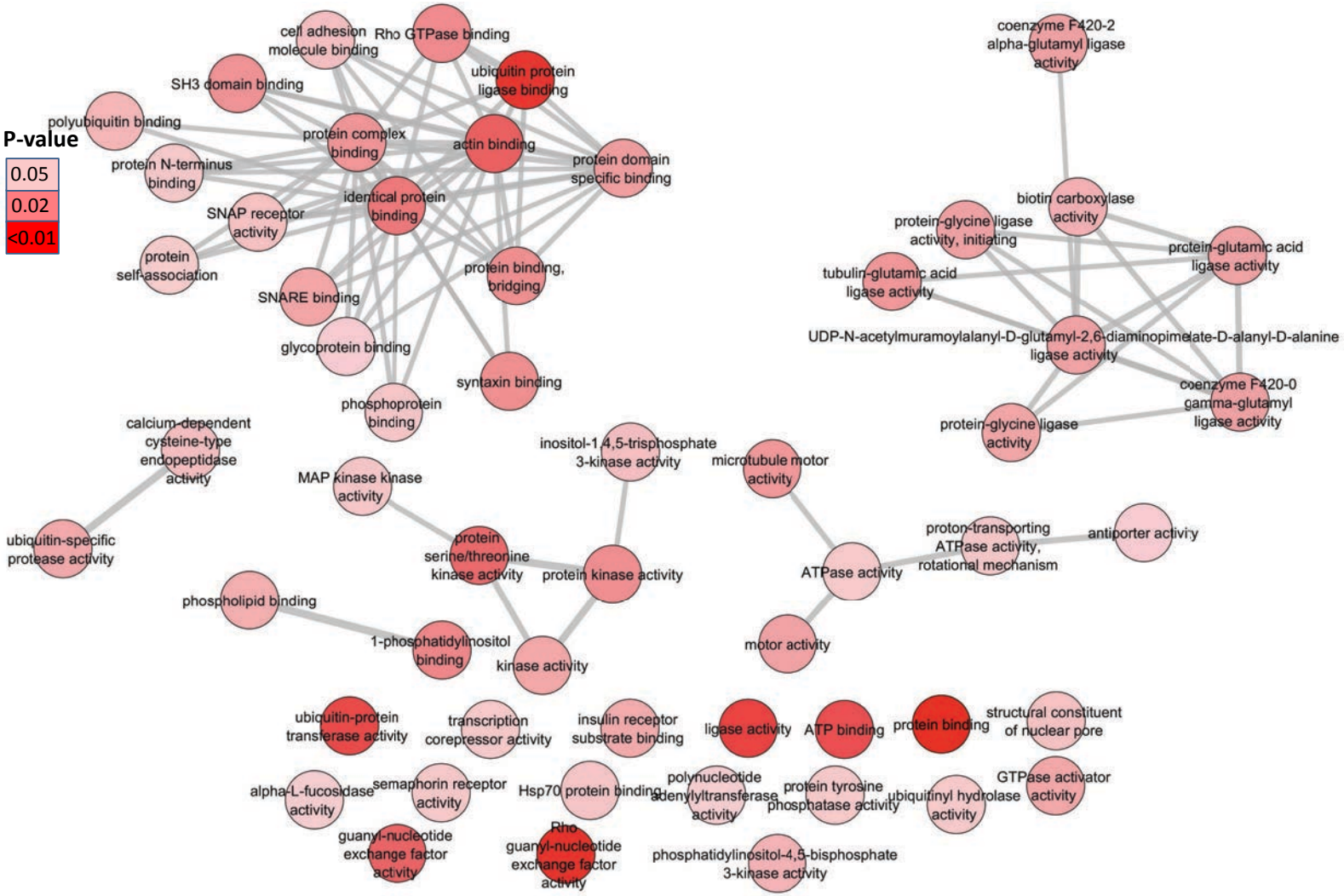

Figure 9c. LS genes higher expressed than MG for gill in molecular function. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

BIOLOGICAL PROCESS

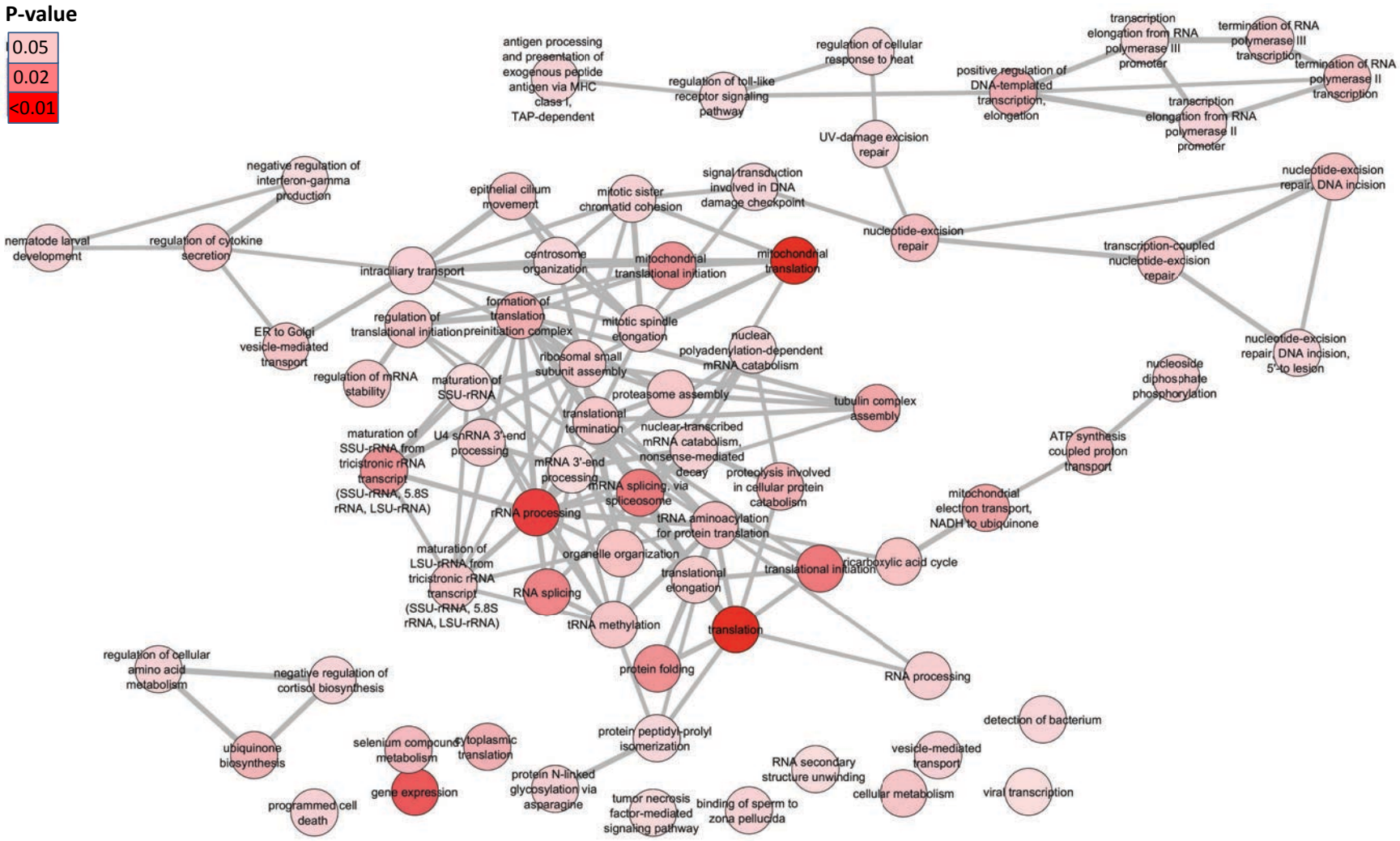

Figure 10a. LS genes higher expressed than MG for mantle in biological process. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

COMPONENT CELLULAR

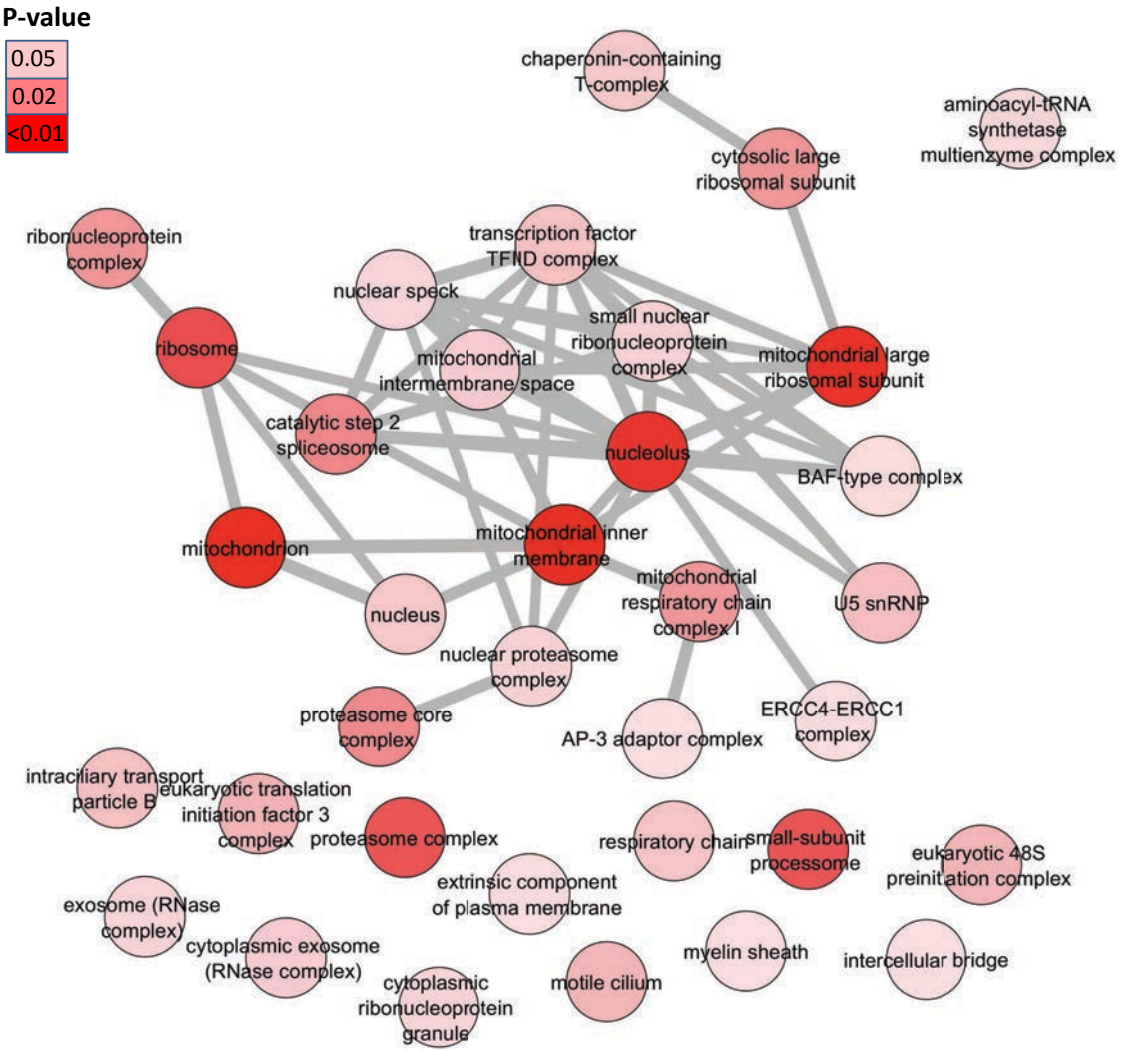

Figure 10b. LS genes higher expressed than MG for mantle in cellular component. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

MOLECULAR FUNCTION

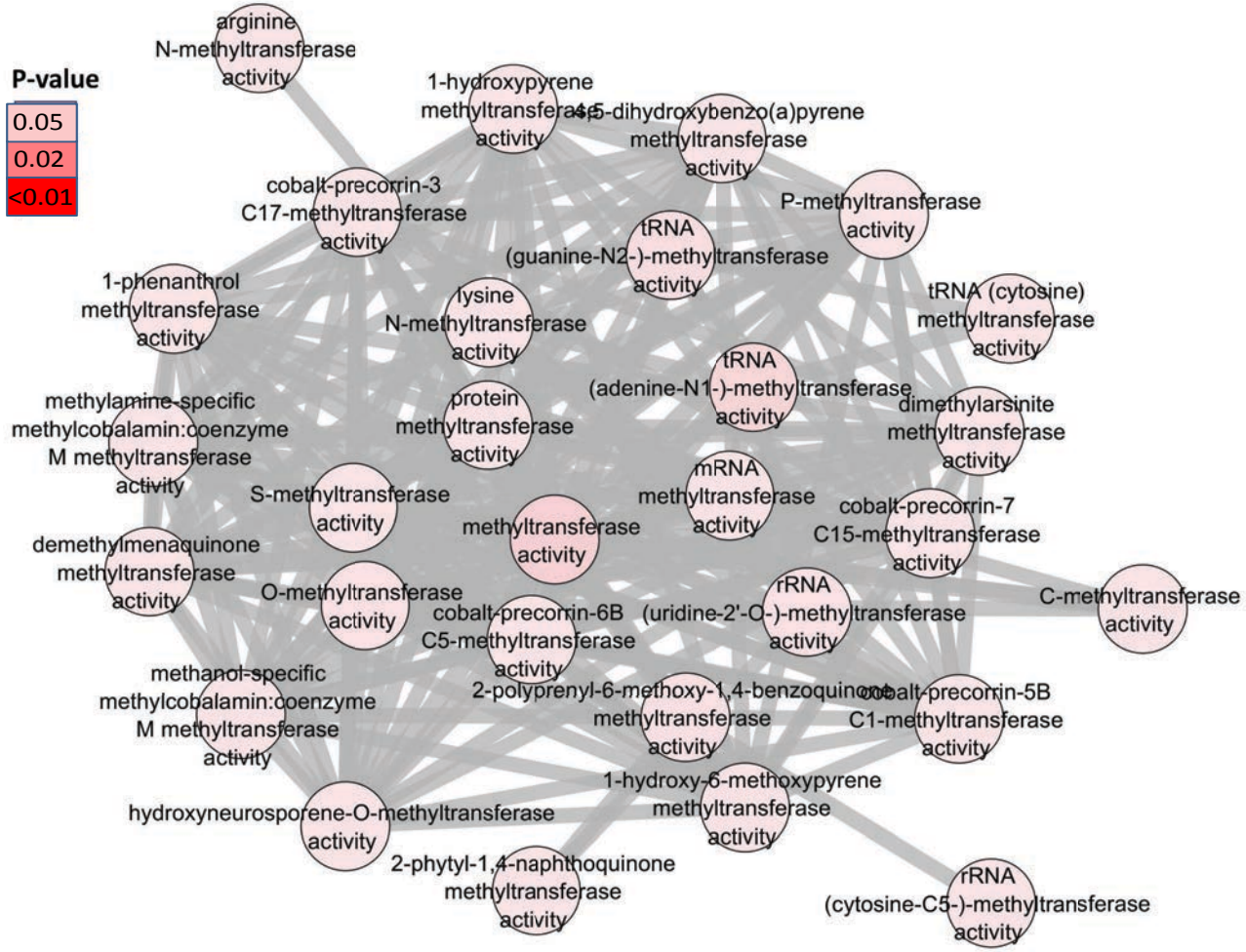

Figure 10c. LS genes higher expressed than MG for mantle in molecular function. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

MOLECULAR FUNCTION

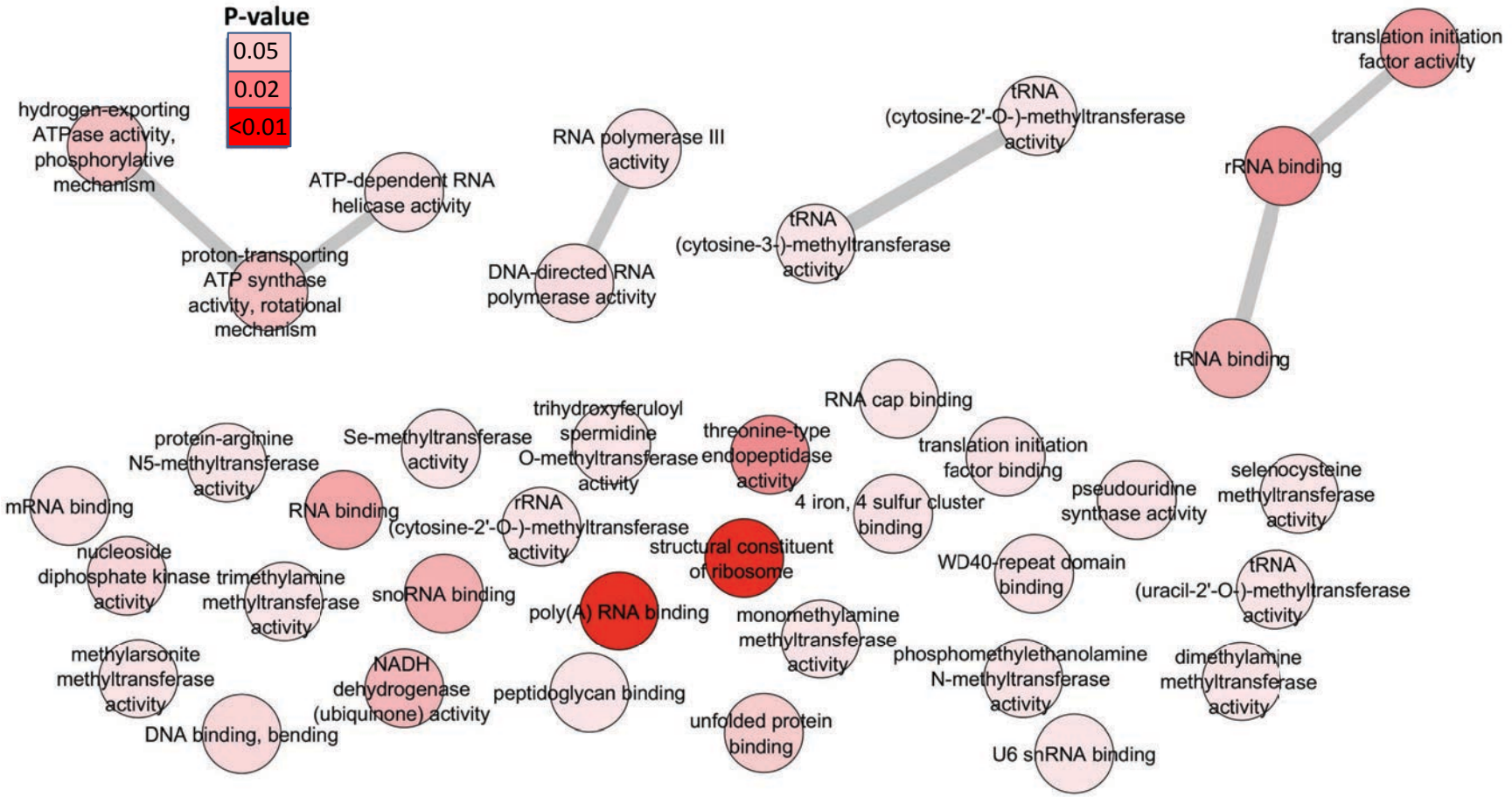

Figure 10d. LS genes higher expressed than MG for mantle in molecular function. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

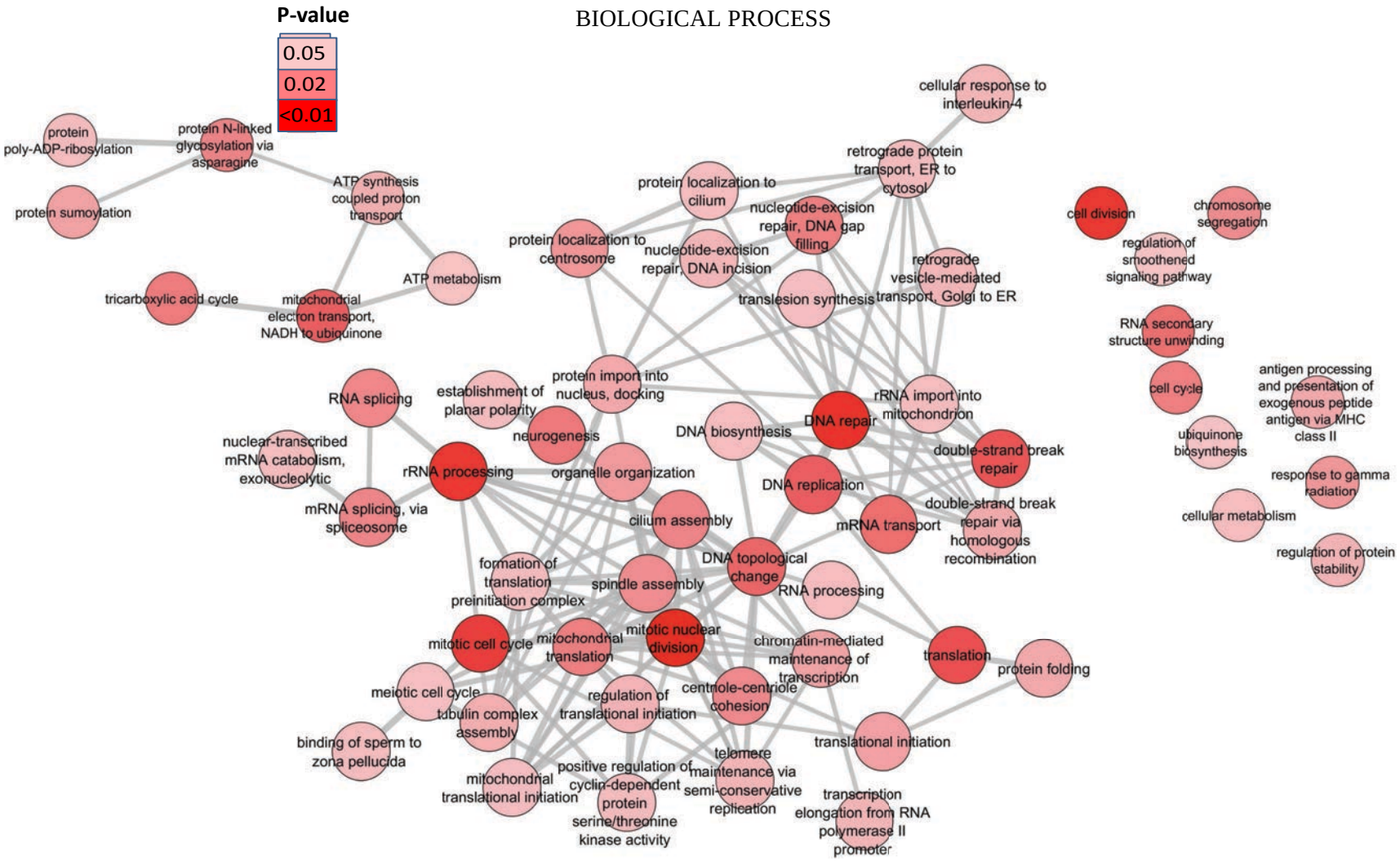

Figure 11a. LS genes higher expressed than MG for digestive gland in biological process. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

CELLULAR COMPONENT

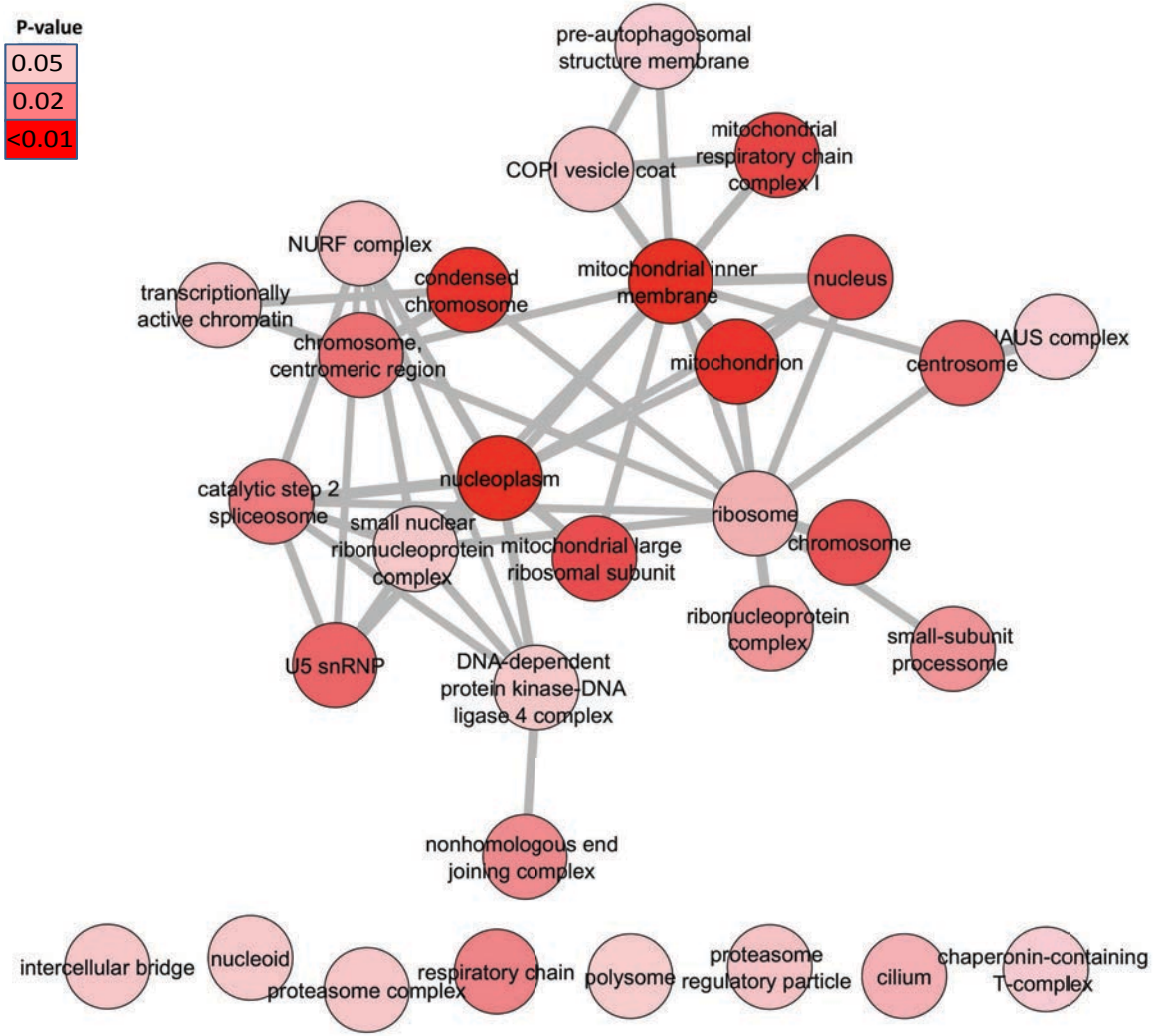

Figure 11b. LS genes higher expressed than MG for digestive gland in cellular component. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

MOLECULAR FUNCTION

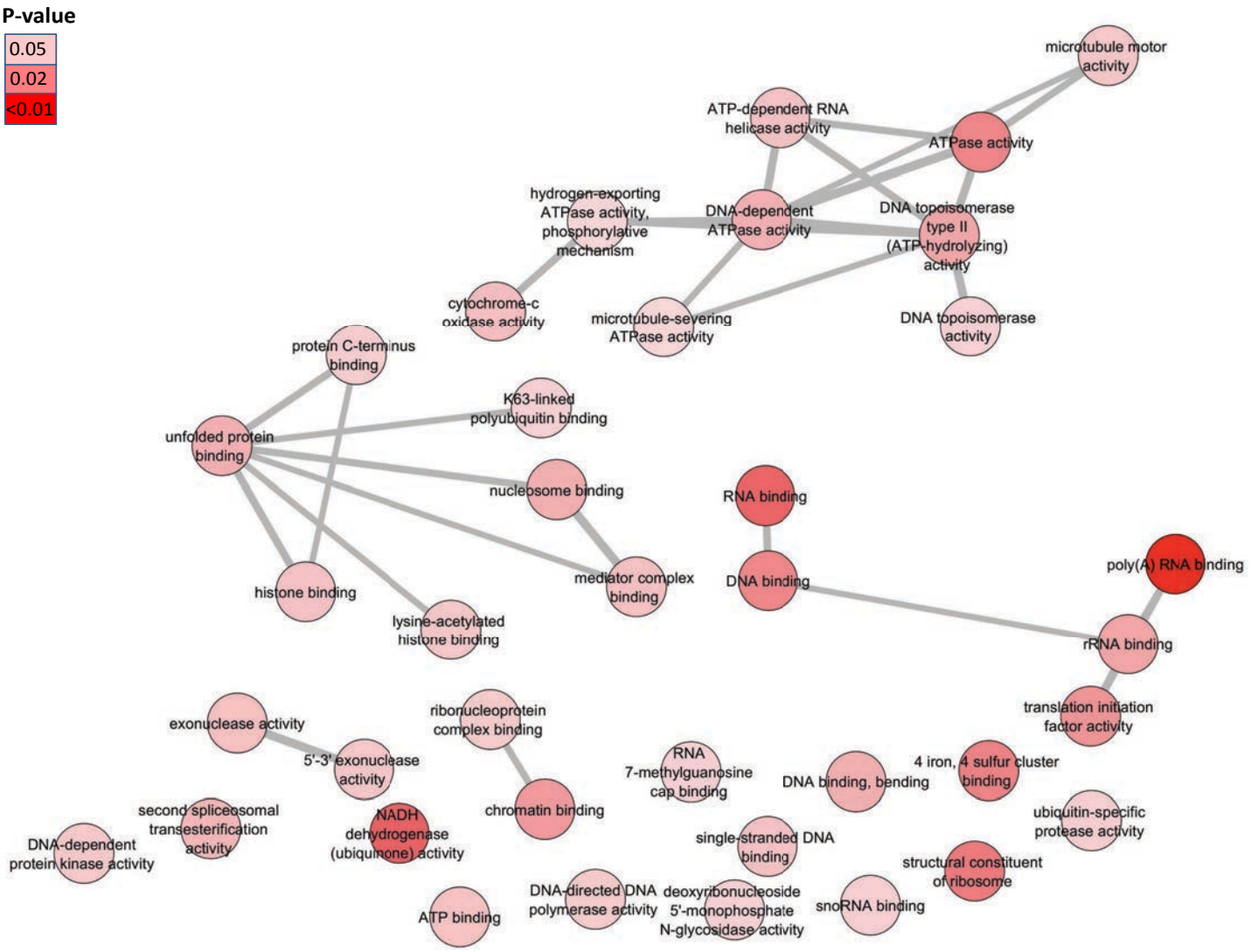

Figure 11c. LS genes higher expressed than MG for digestive gland in molecular function. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

BIOLOGICAL PROCESS

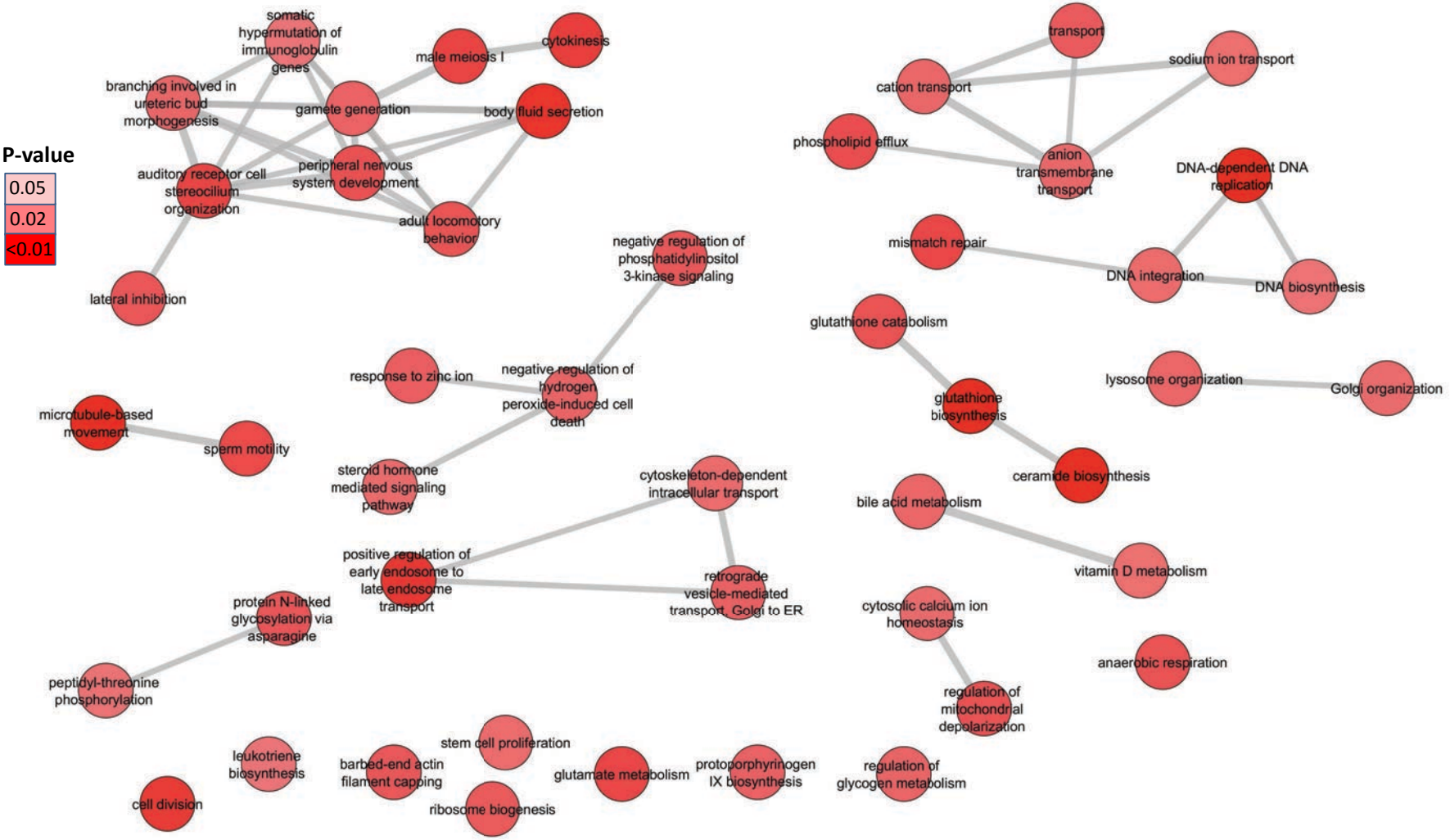

Figure 12a. LS genes higher expressed than RB for gill in biological process. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

CELLULAR COMPONENT

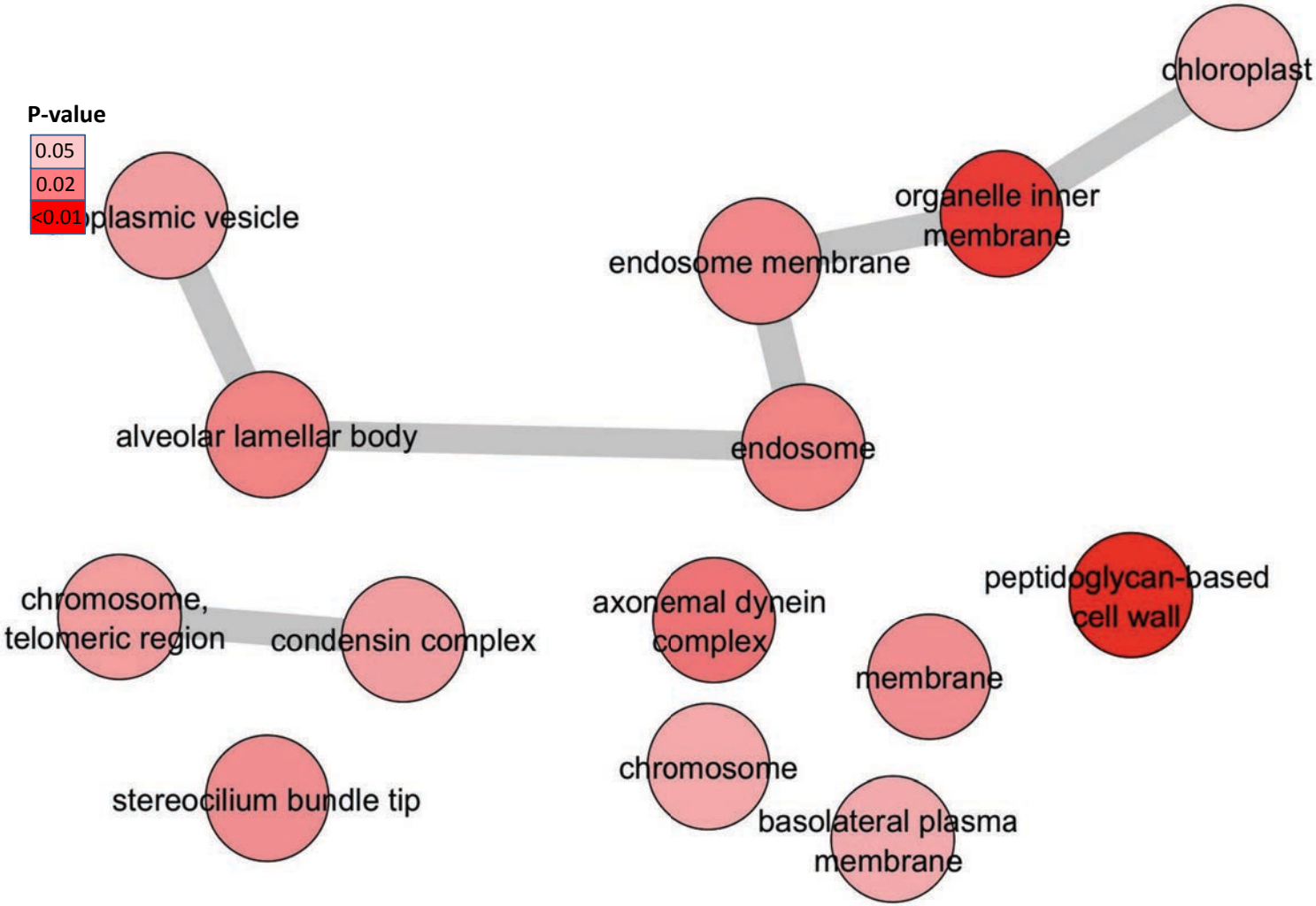

Figure 12b. LS genes higher expressed than RB for gill cellular component. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represent the degree of significance.

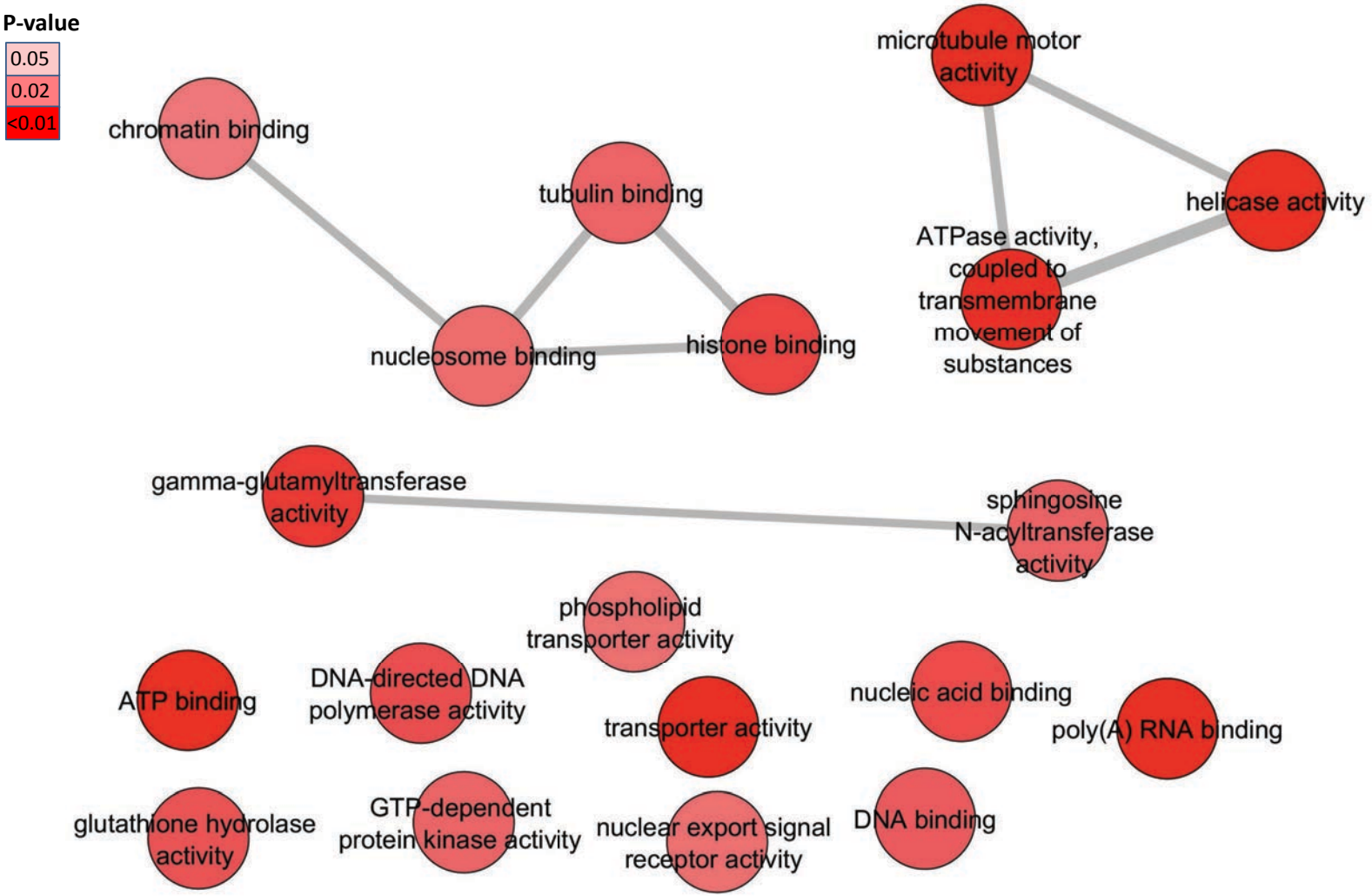

Figure 12c. LS genes higher expressed than RB for gill in molecular function. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represent the degree of significance.

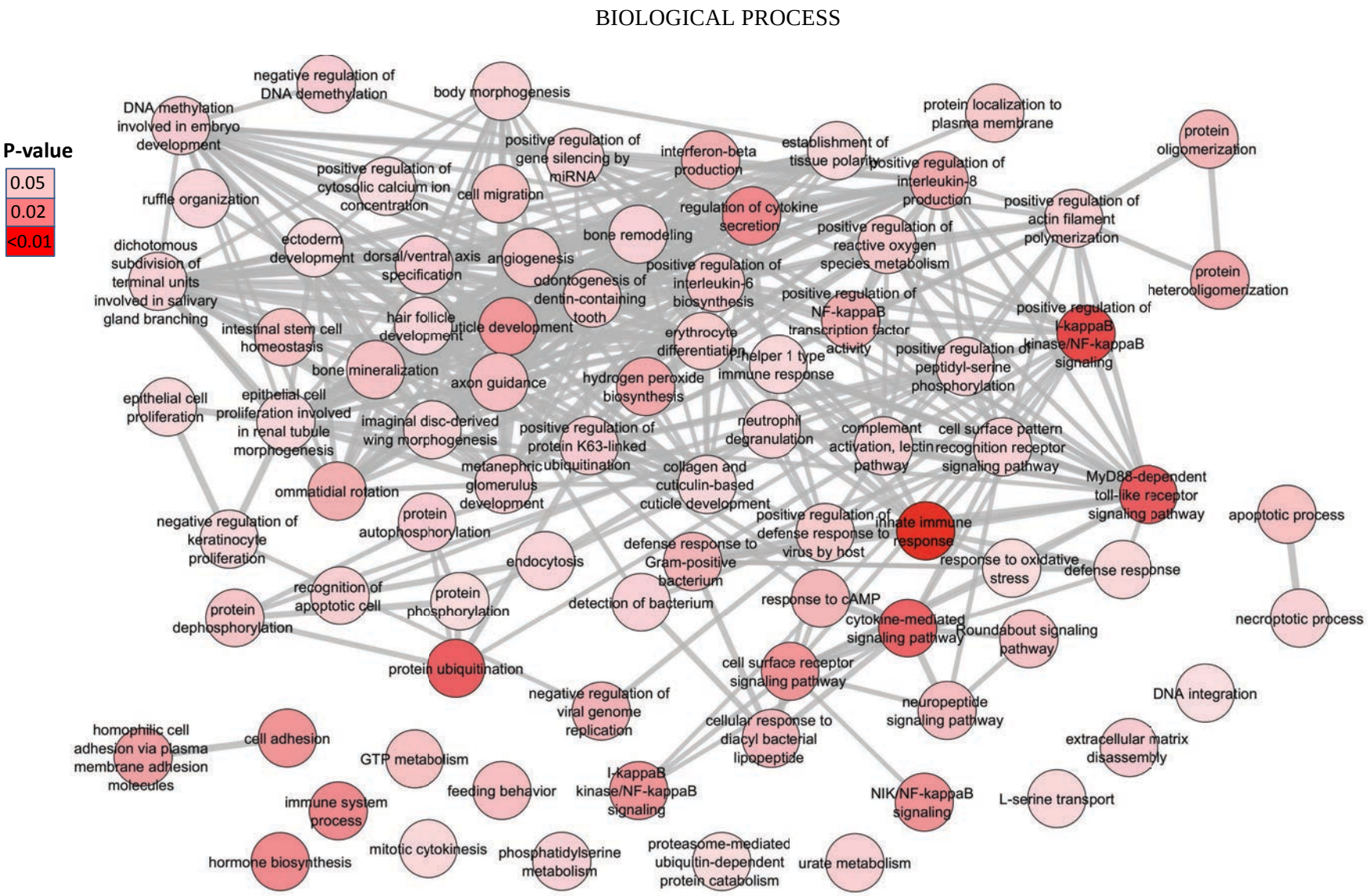

Figure 13a. LS genes higher expressed than RB for mantle in biological process. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

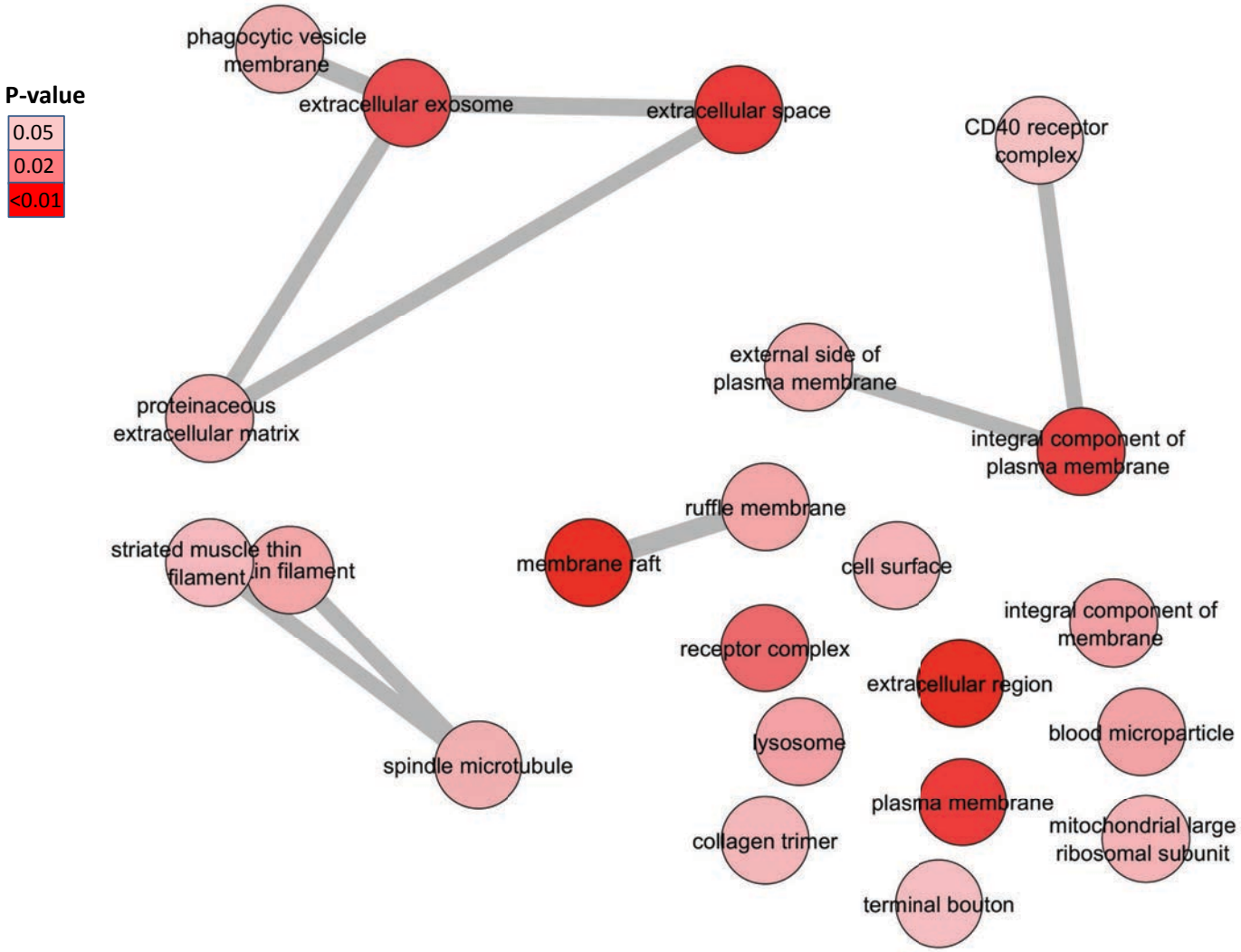

Figure 13b. LS genes higher expressed than RB for mantle in cellular component. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

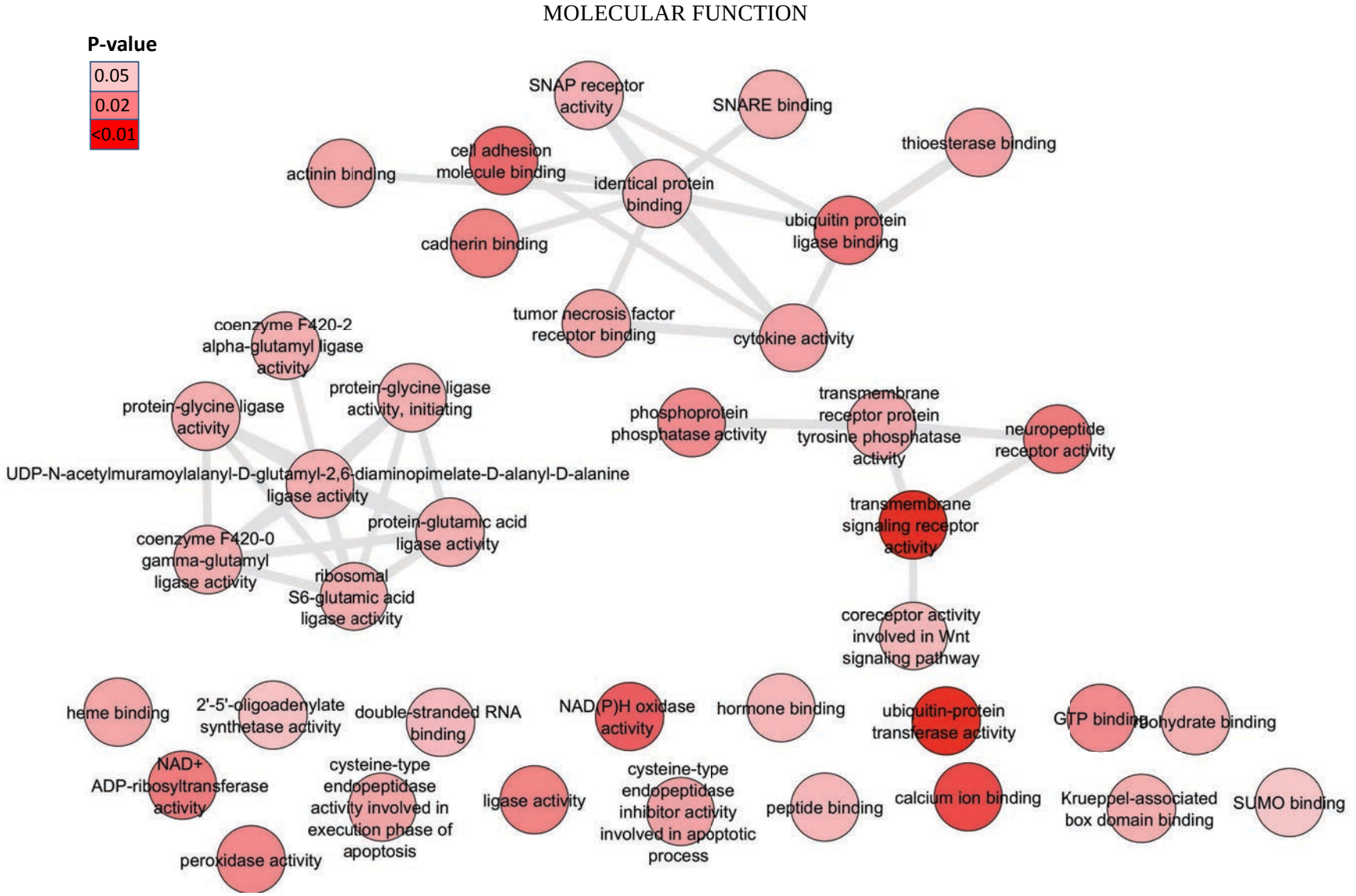

Figure 13c. LS genes higher expressed than RB for mantle in molecular function. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

BIOLOGICAL PROCESS

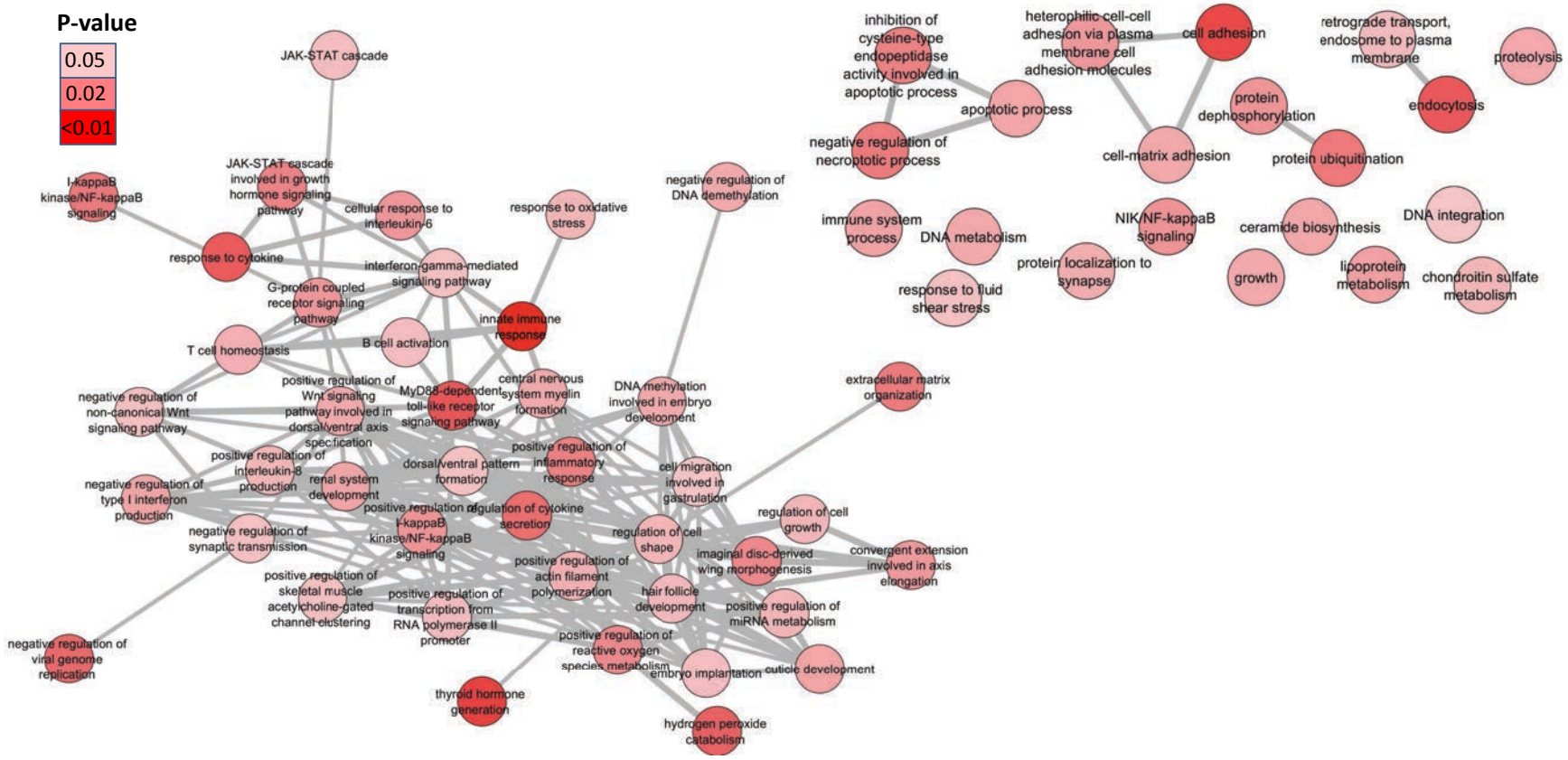

Figure 14a. LS genes higher expressed than RB for digestive gland in biological process. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

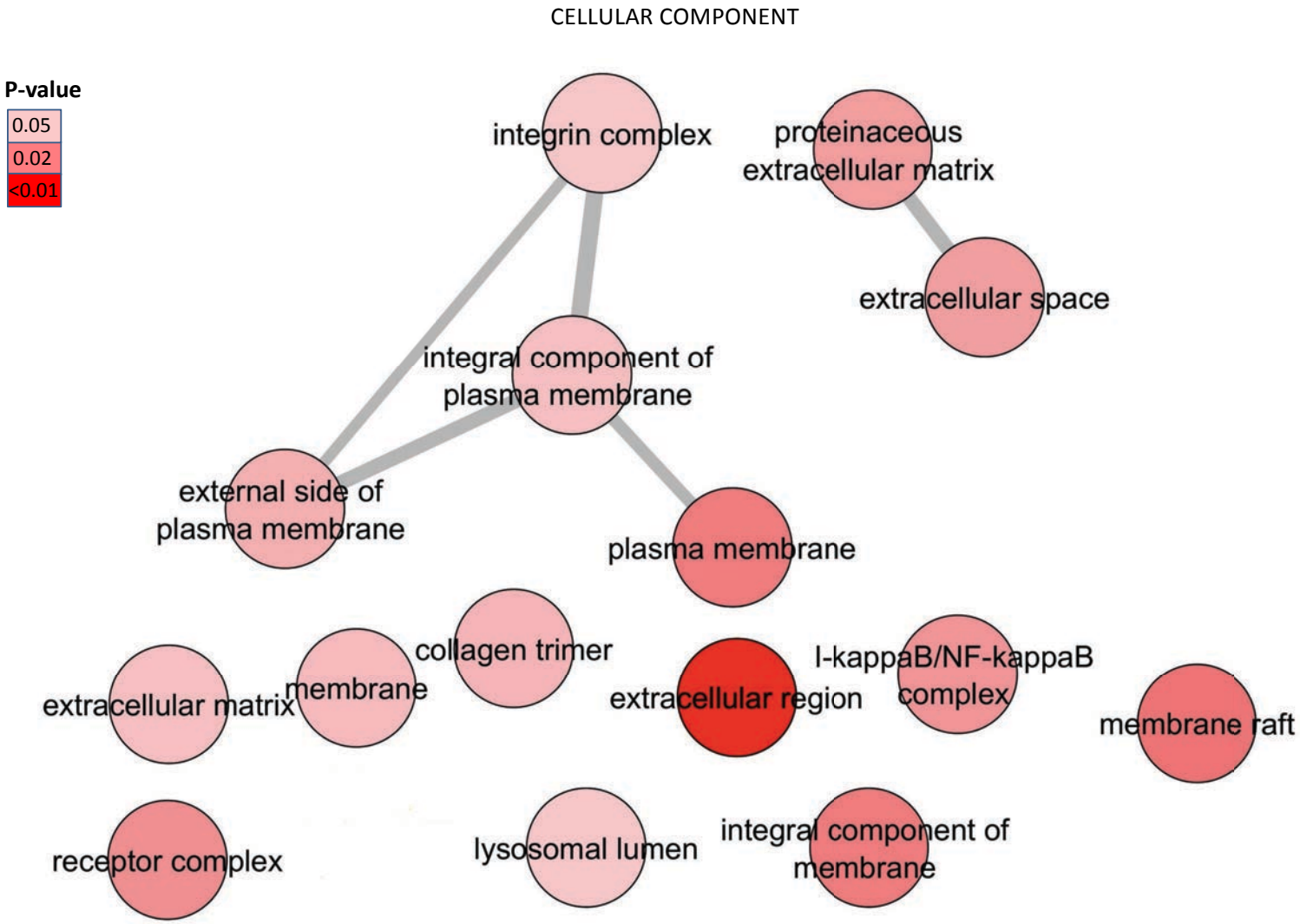

Figure 14b. LS genes higher expressed than RB for digestive gland in cellular component. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

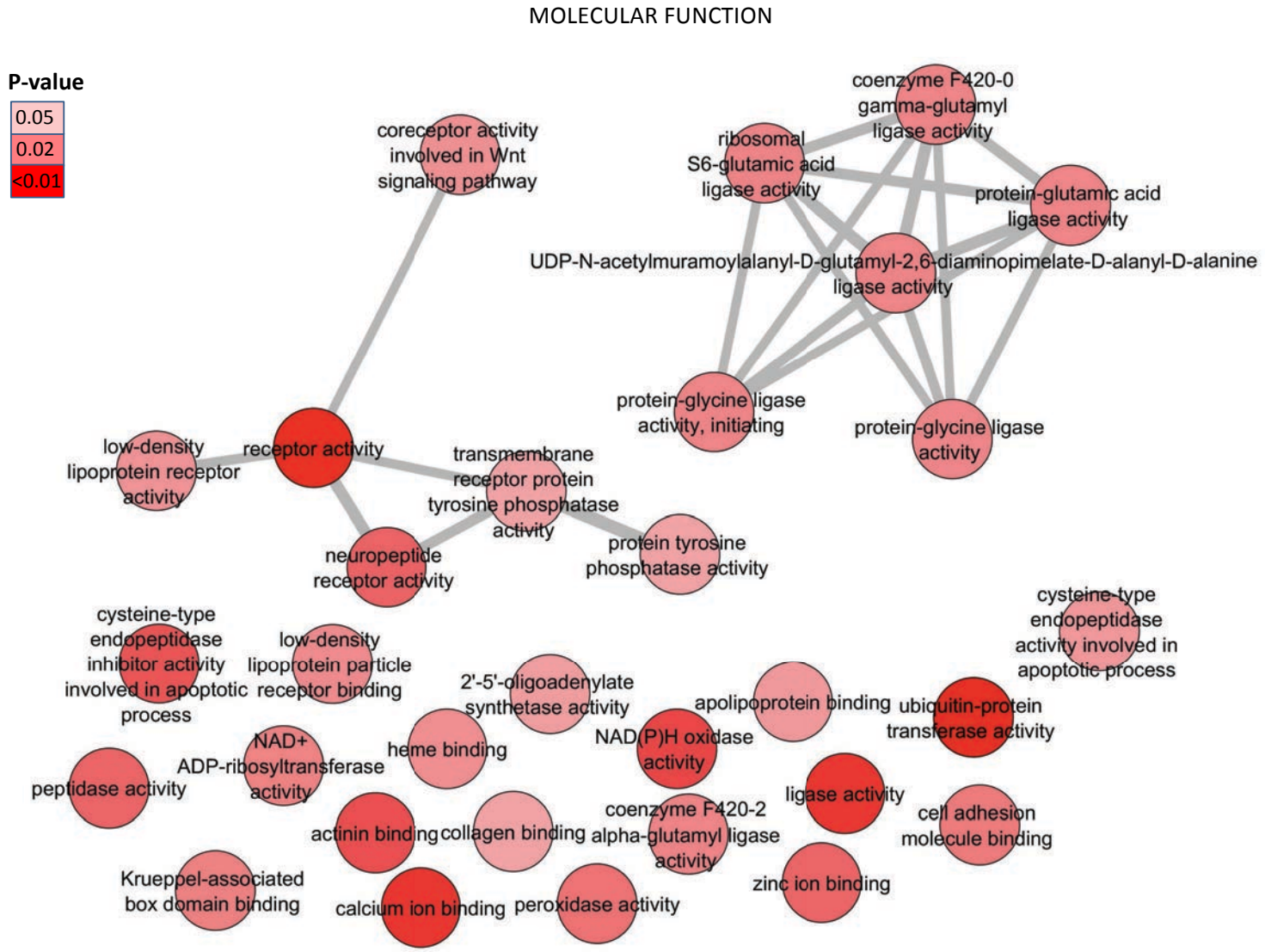

Figure 14c. LS genes higher expressed than RB for digestive gland in molecular function. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

BIOLOGICAL PROCESS

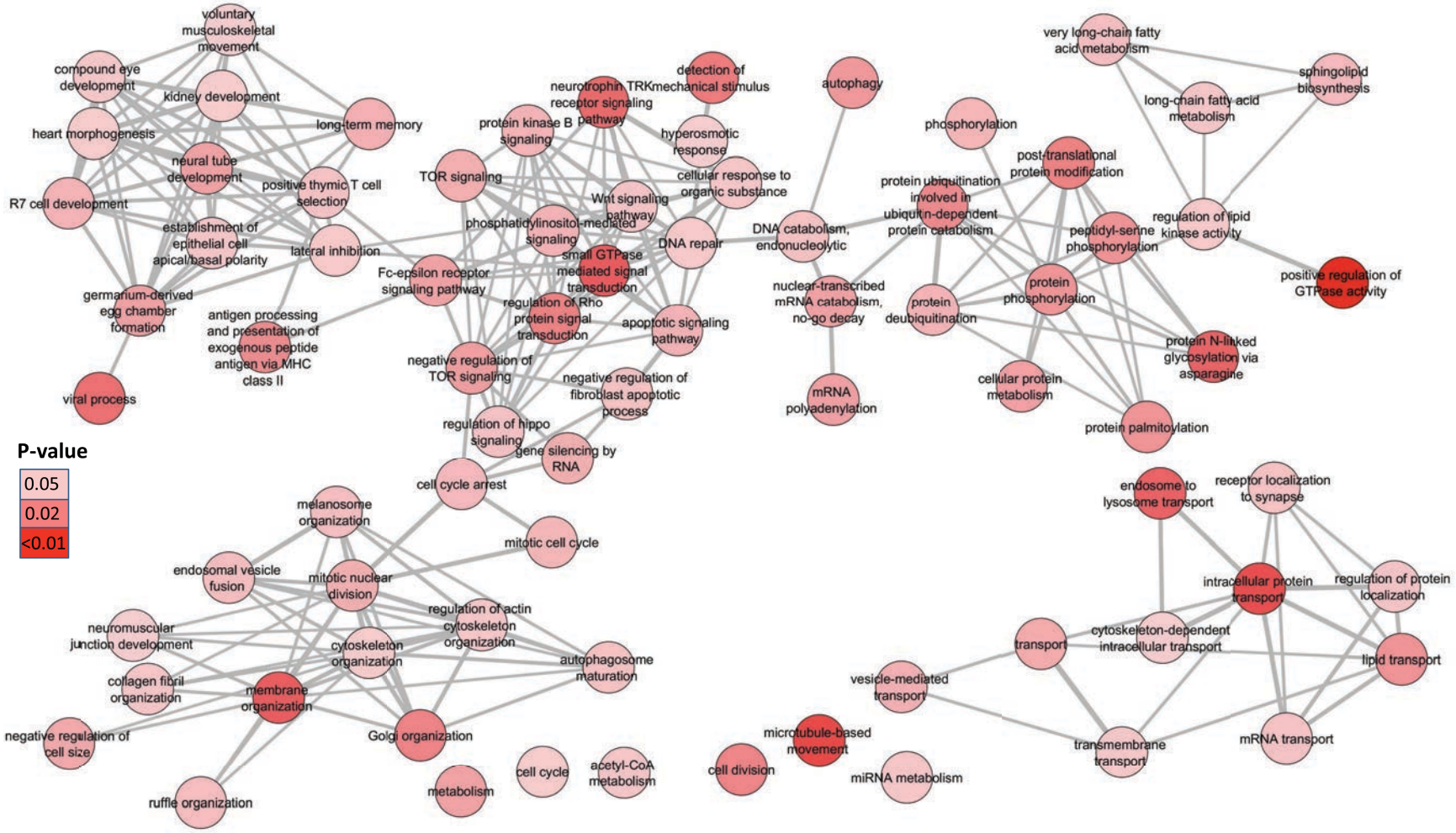

Figure 15a. RB genes higher expressed than MG for gill in biological process. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

CELLULAR COMPONENT

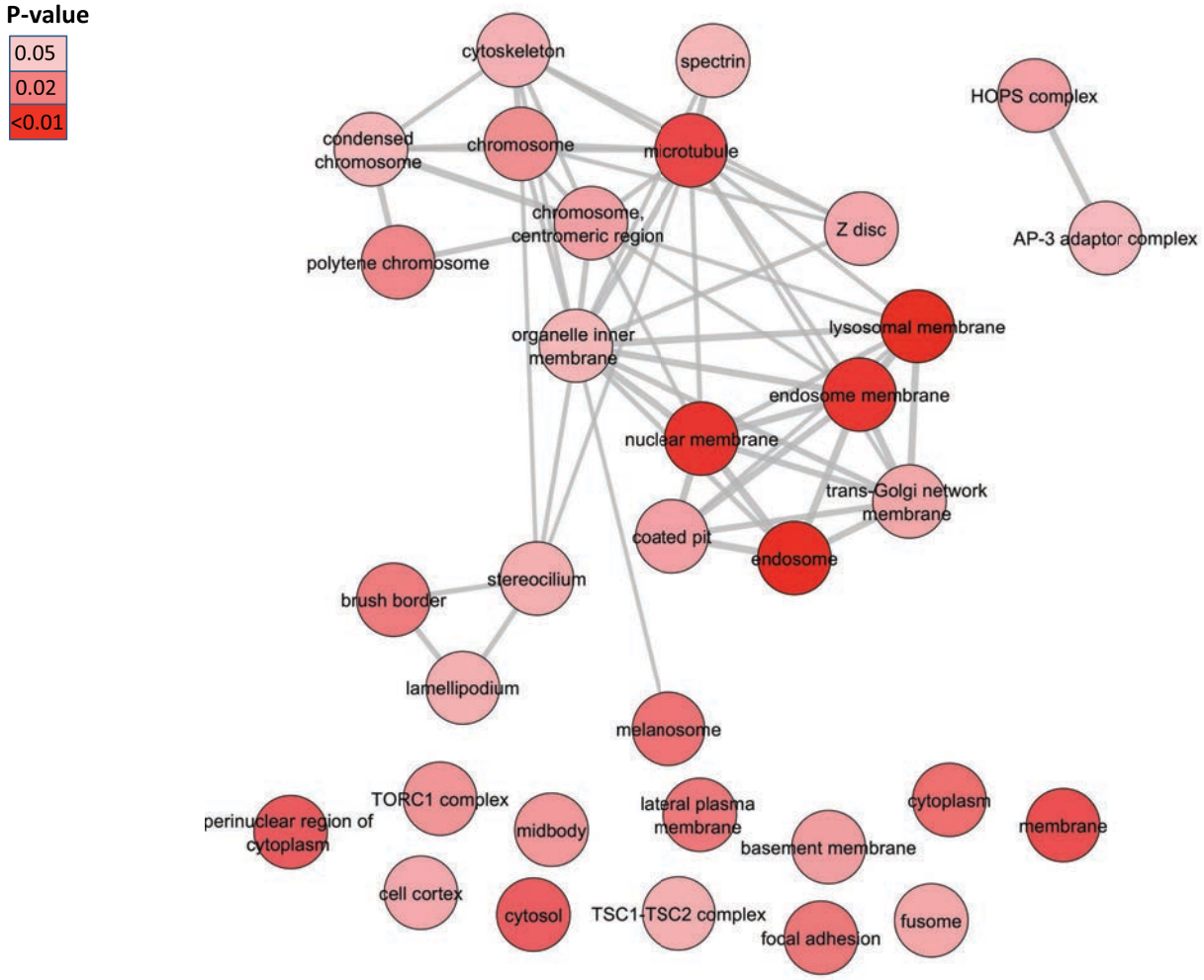

Figure 15b. RB genes higher expressed than MG for gill cellular component. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represent the degree of significance.

MOLECULAR FUNCTION

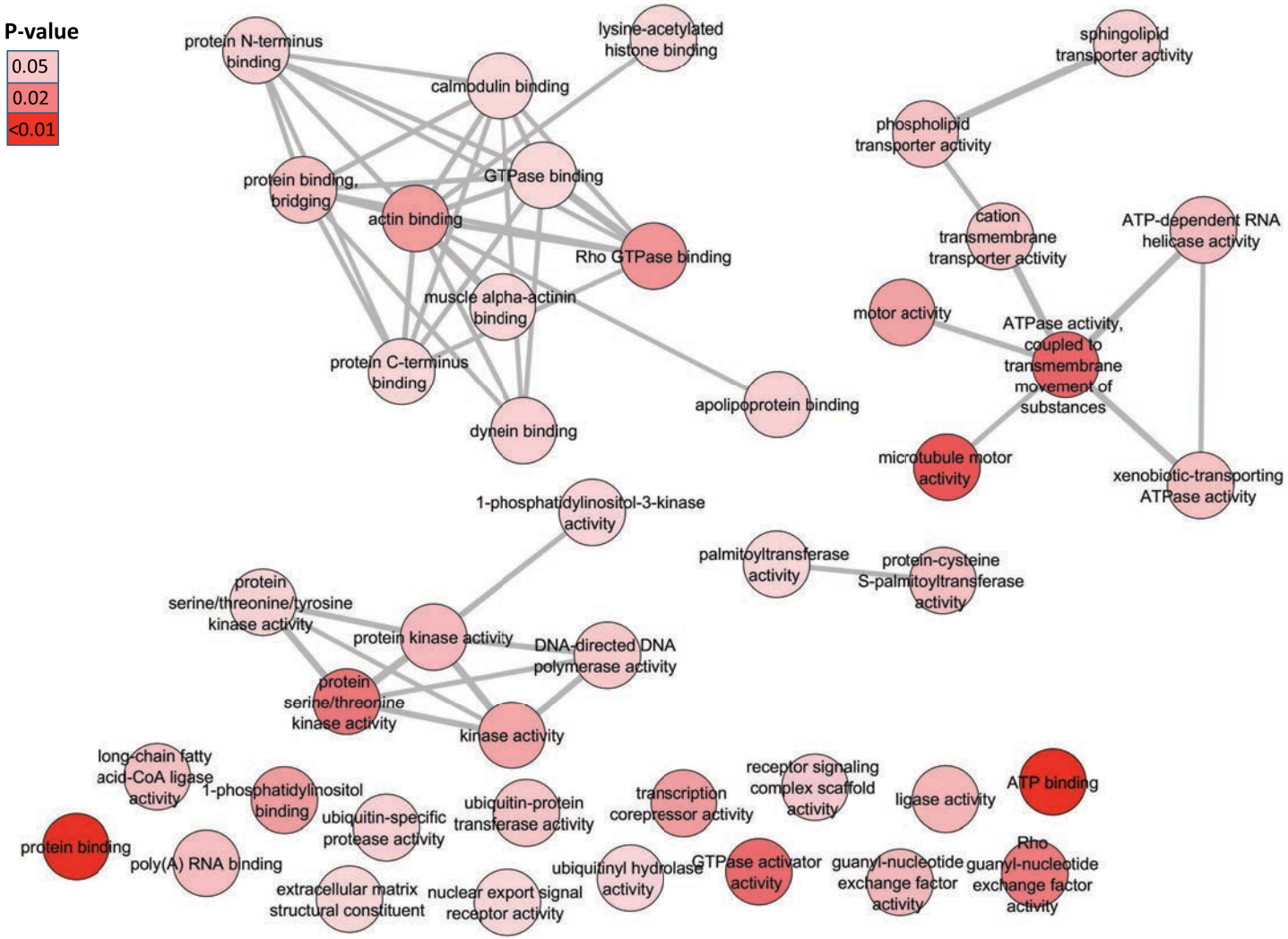

Figure 15c. RB genes higher expressed than MG for gill in molecular function. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represent the degree of significance.

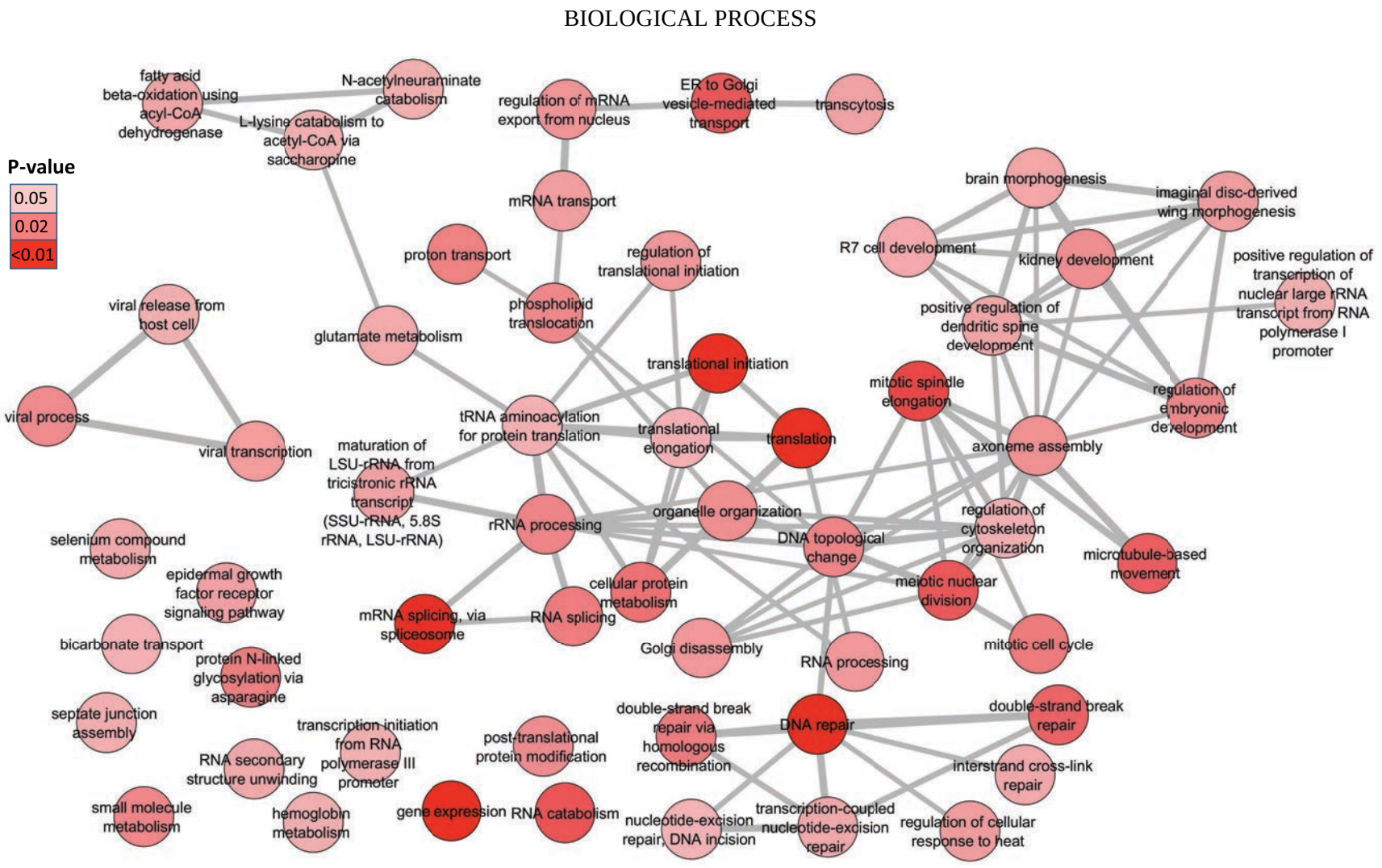

Figure 16a. RB genes higher expressed than MG for mantle in biological process. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

CELLULAR COMPONENT

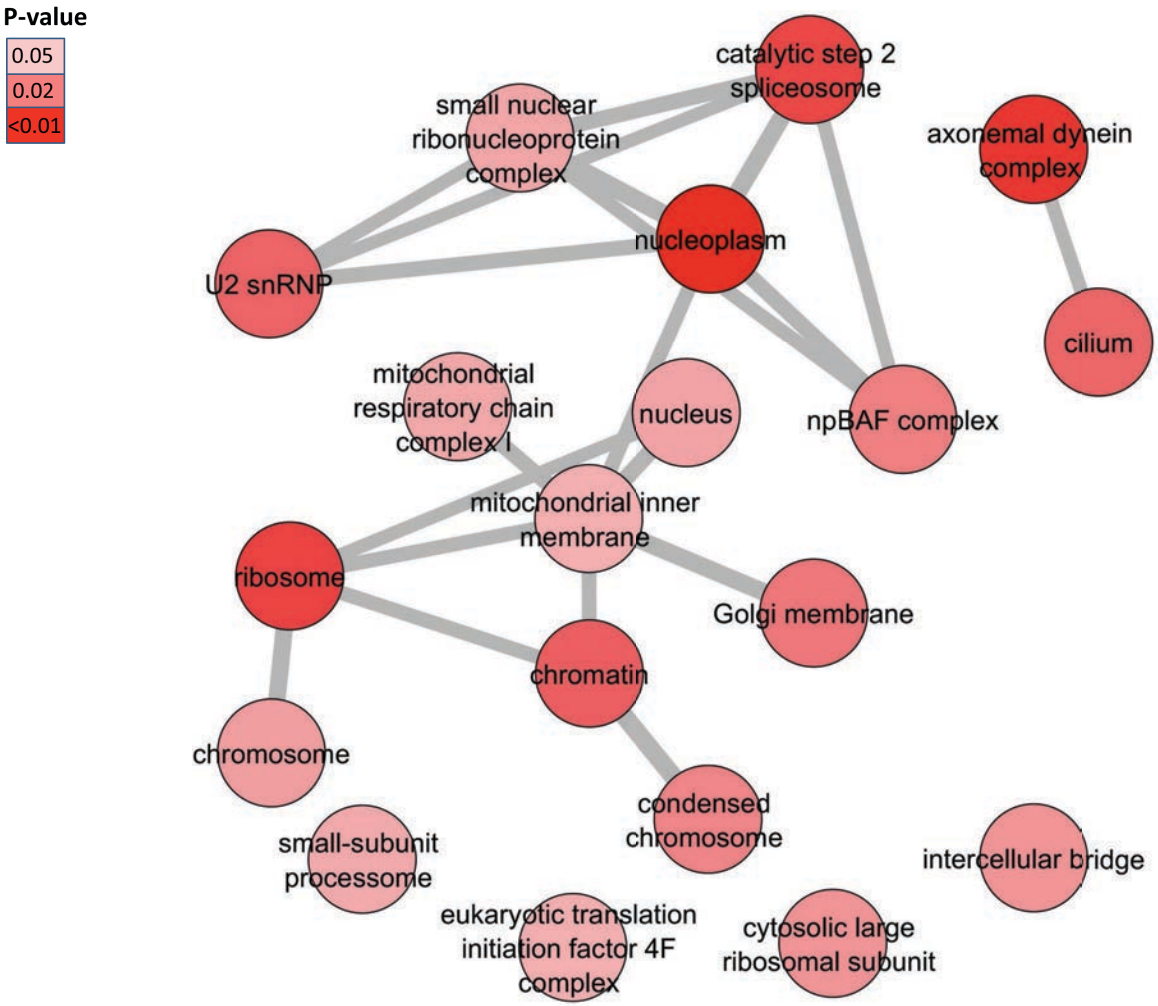

Figure 16b. RB genes higher expressed than MG for mantle in cellular component. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

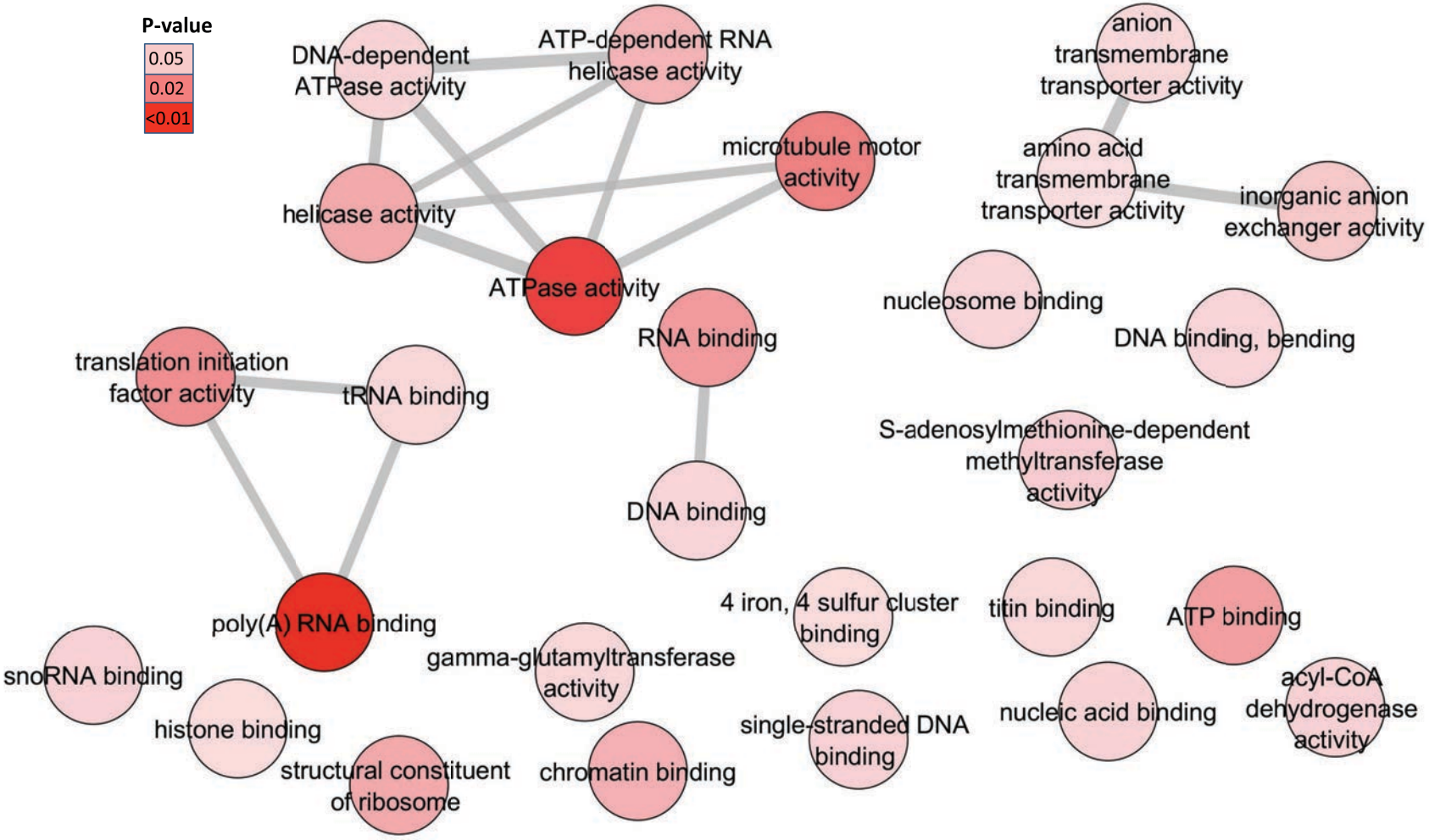

Figure 16c. RB genes higher expressed than MG for mantle in molecular function. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

BIOLOGICAL PROCESS

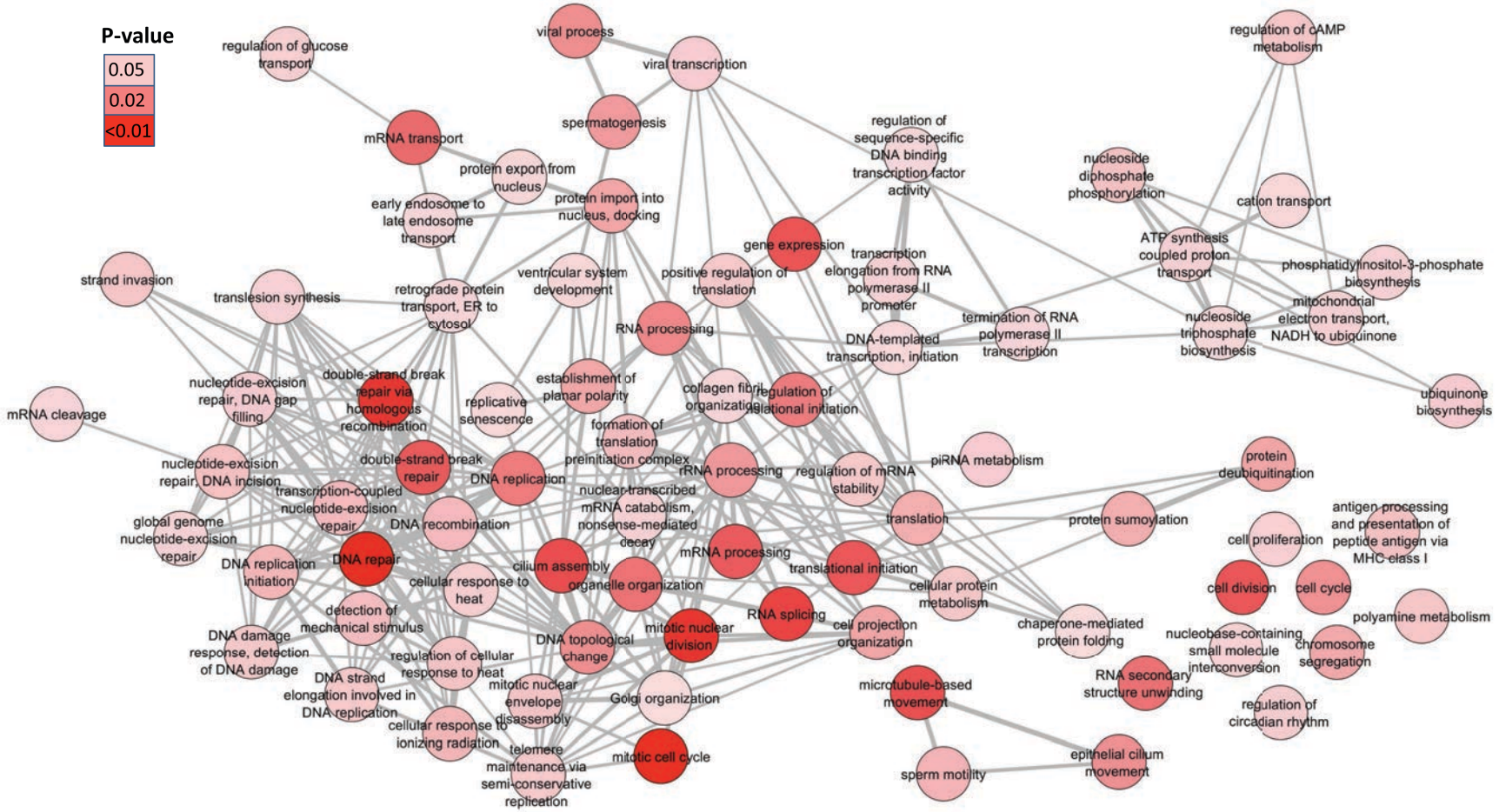

Figure 17a. RB genes higher expressed than MG for digestive gland in biological process. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

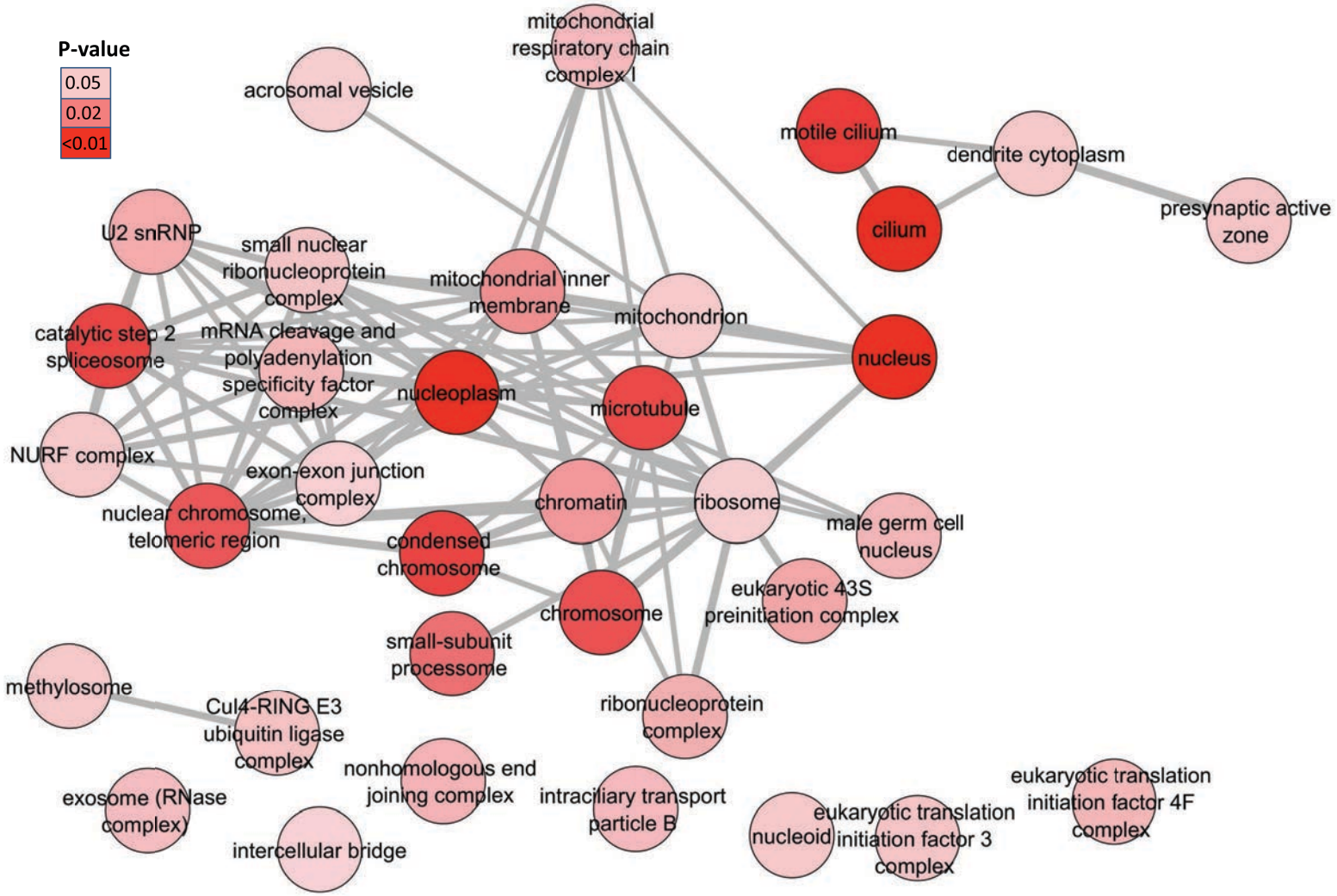

Figure 17b. RB genes higher expressed than MG for digestive gland in cellular component. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

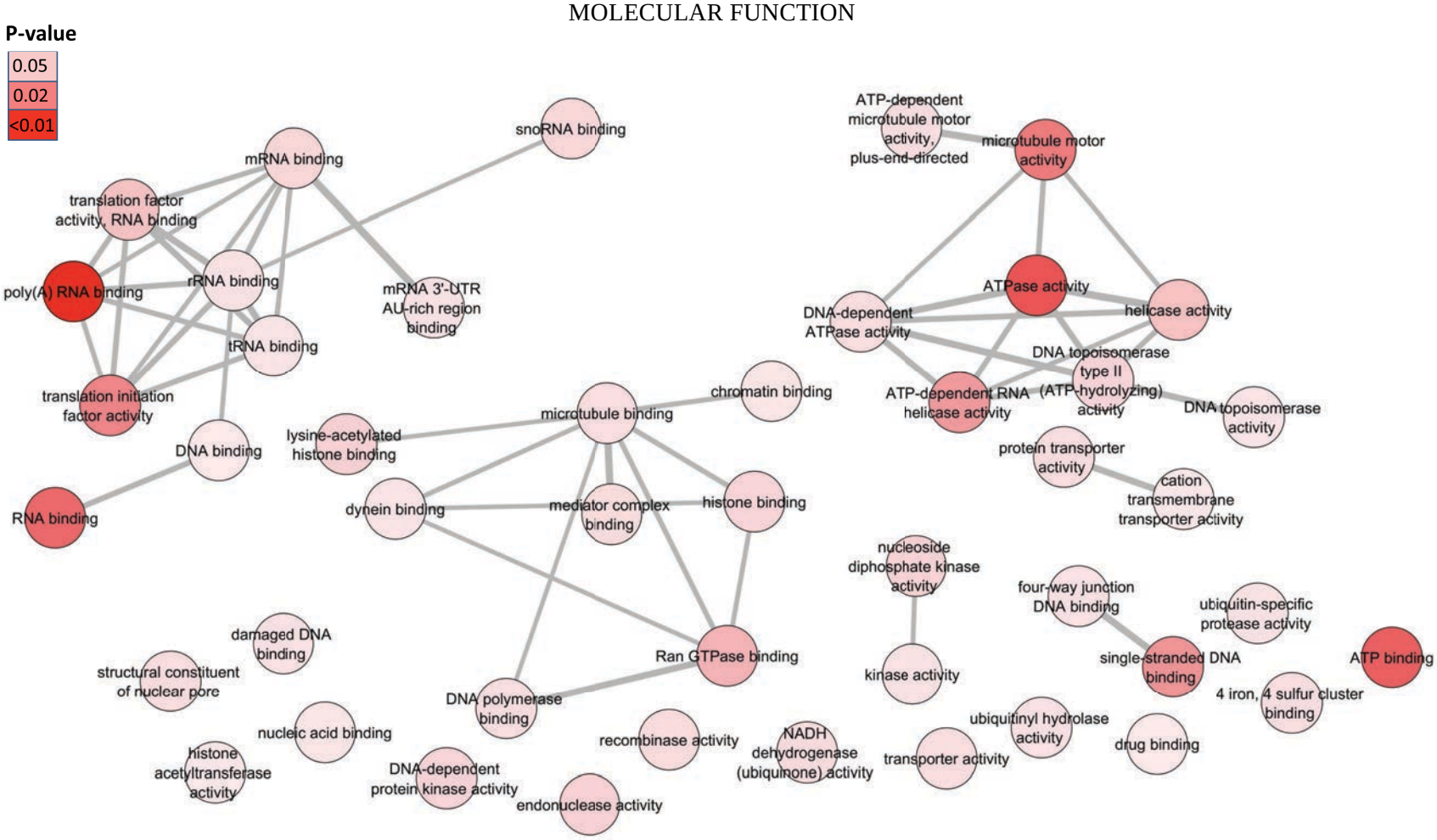

Figure 17c. RB genes higher expressed than MG for digestive gland in molecular function. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

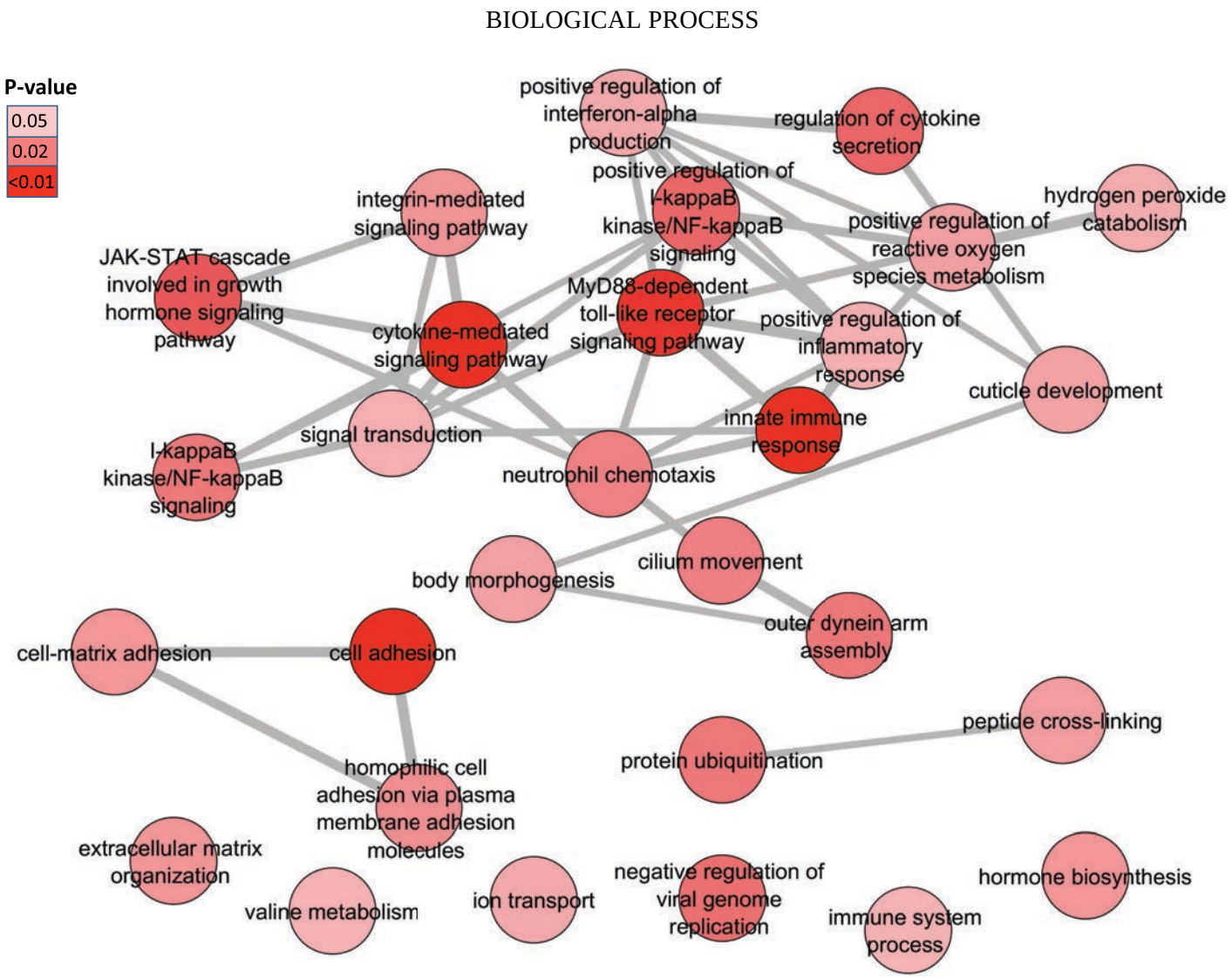

Figure 18a. RB genes higher expressed than LS for gill in biological process. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

CELLULAR COMPONENT

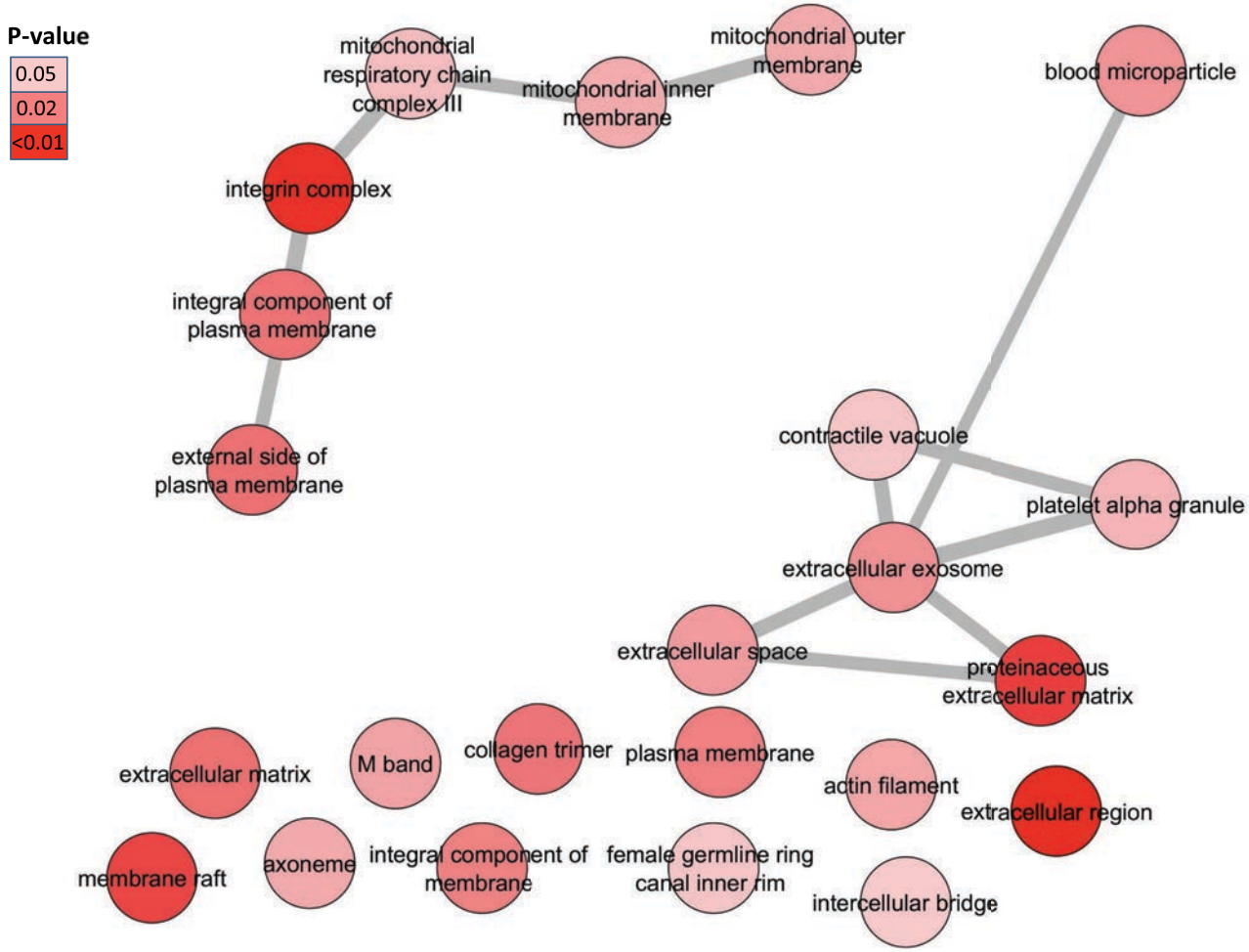

Figure 18b. RB genes higher expressed than LS for gill cellular component. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represent the degree of significance.

MOLECULAR FUNCTION

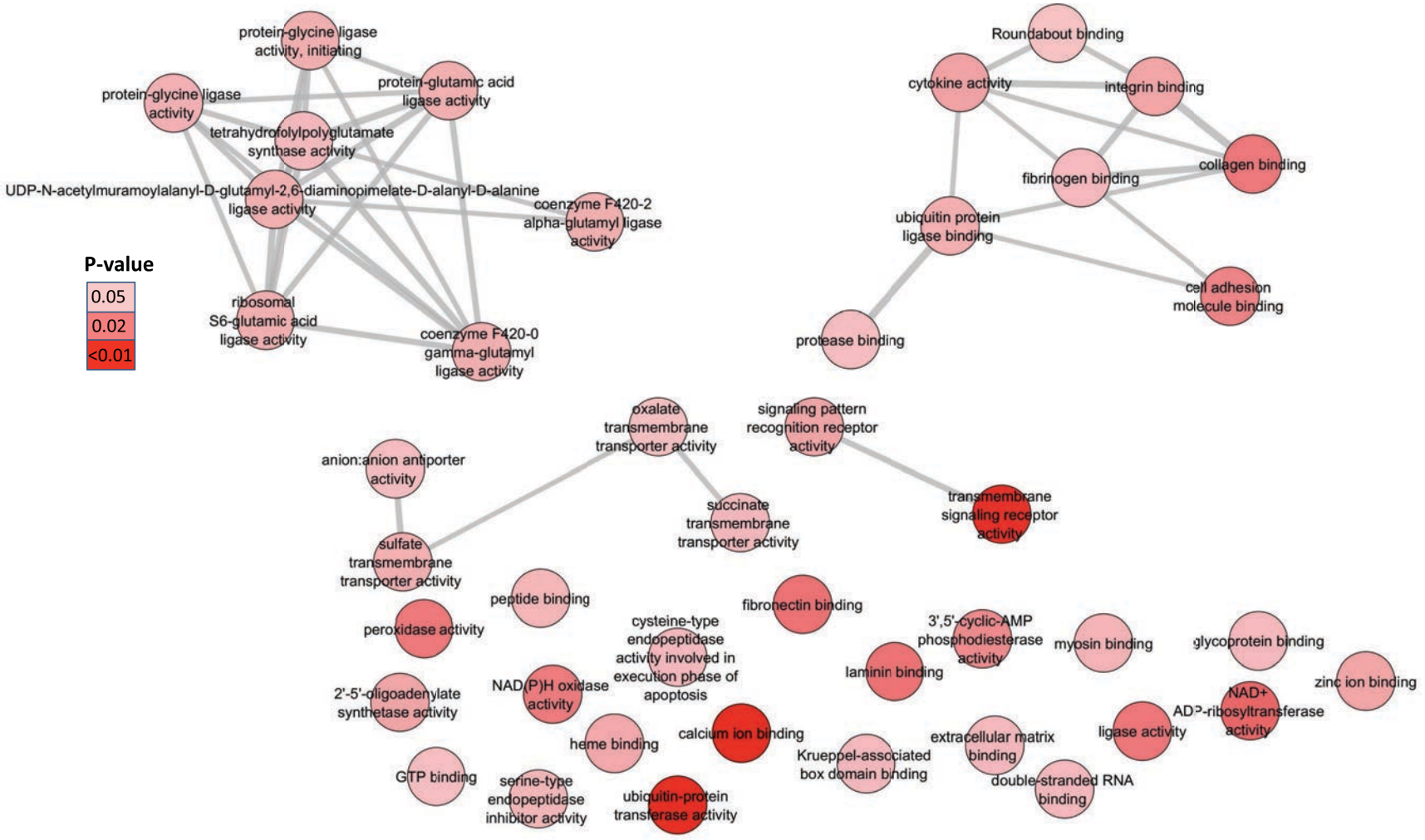

Figure 18c. RB genes higher expressed than LS for gill in molecular function. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represent the degree of significance.

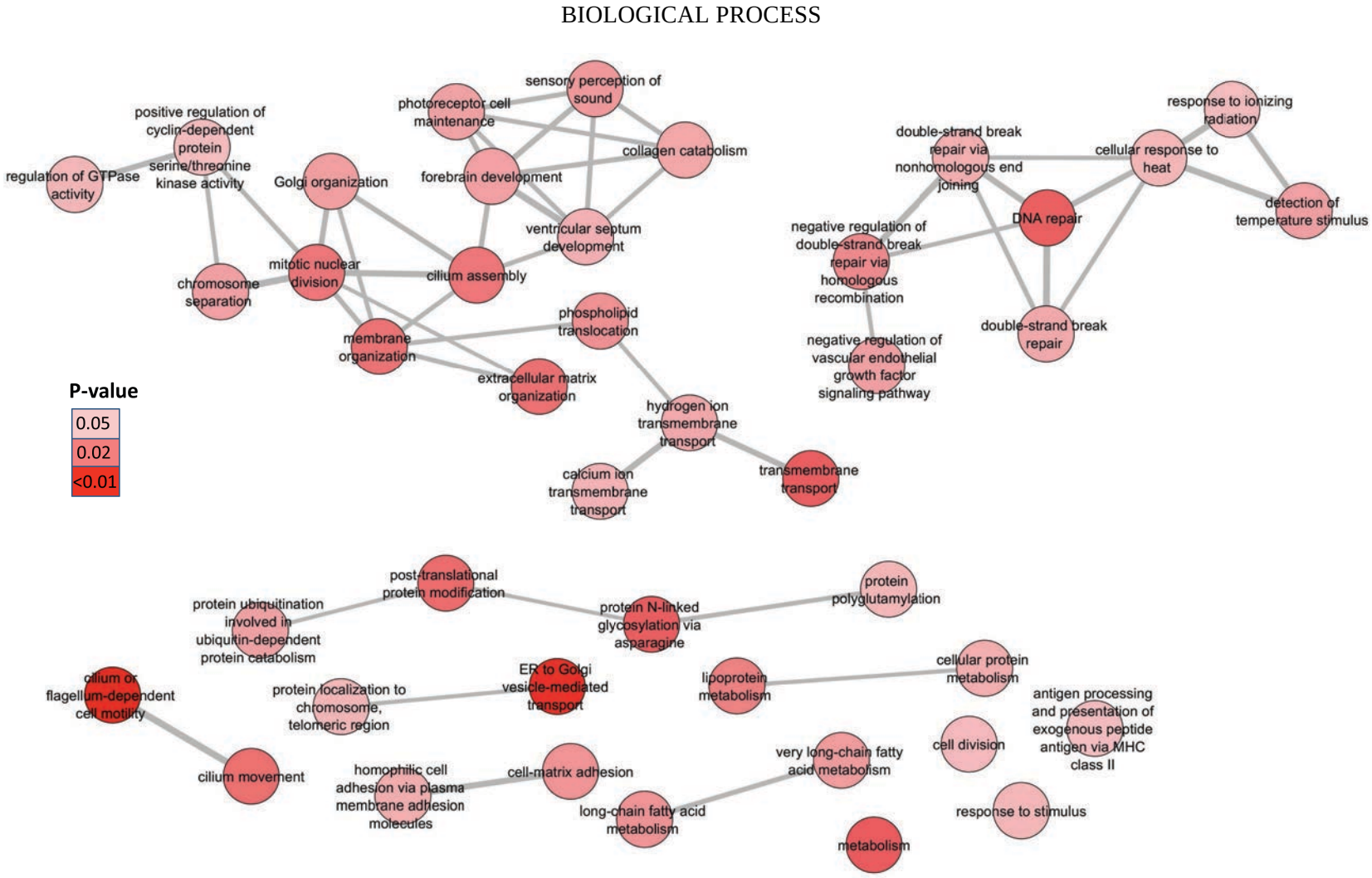

Figure 19a. RB genes higher expressed than LS for mantle in biological process. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

CELLULAR COMPONENT

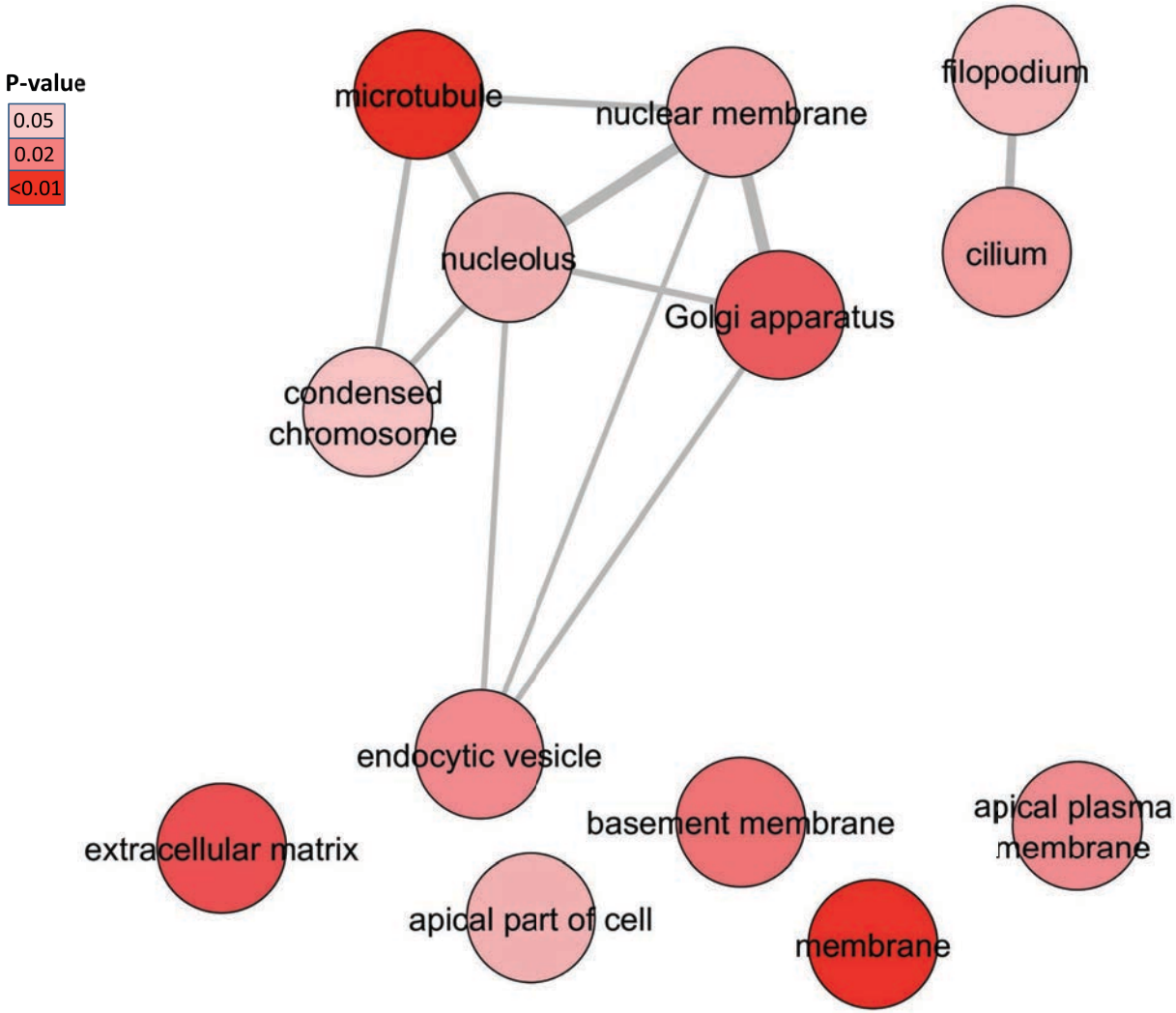

Figure 19b. RB genes higher expressed than LS for mantle in cellular component. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

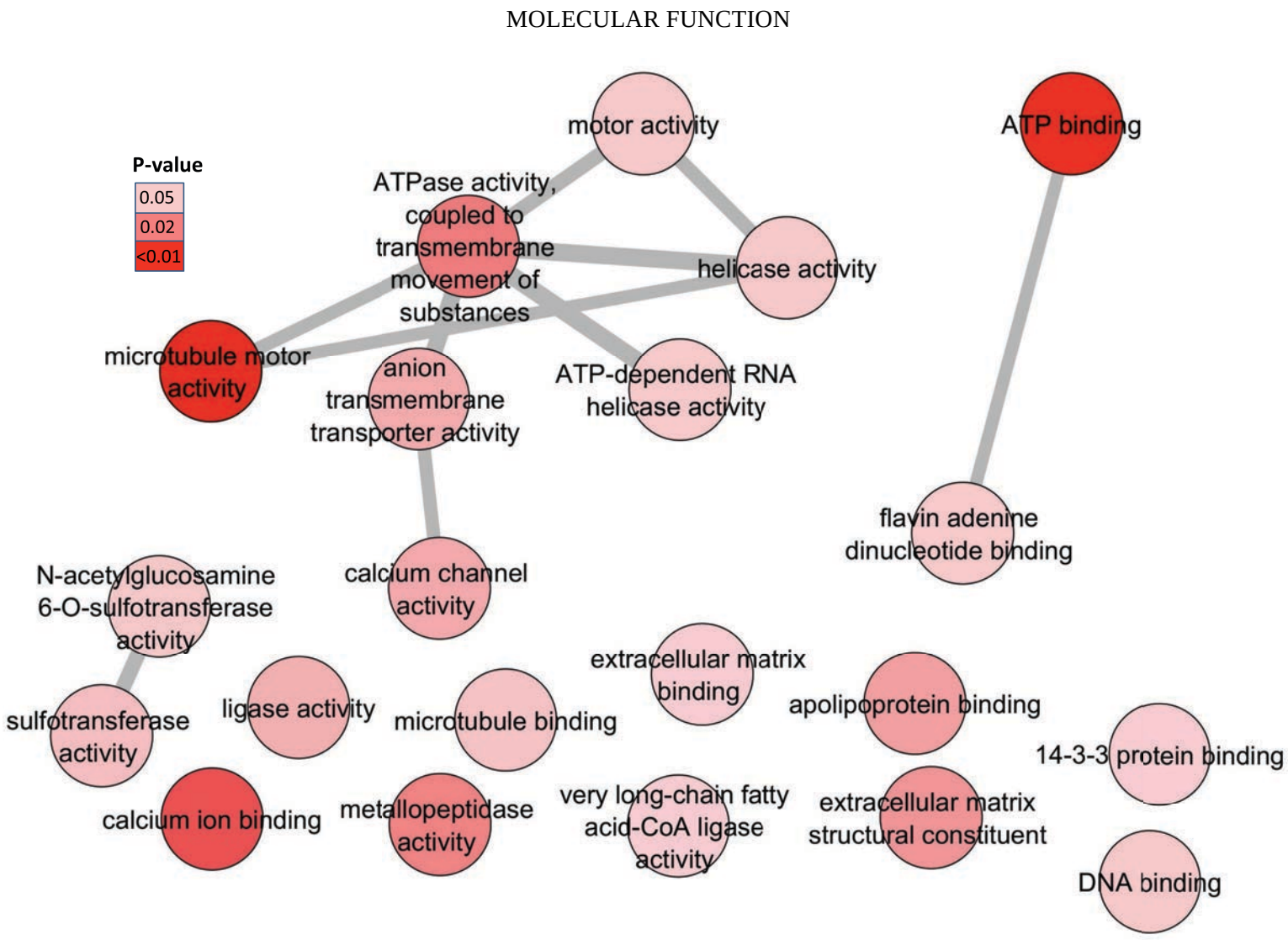

Figure 19c. RB genes higher expressed than LS for mantle in molecular function. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

Figure 20a. RB genes higher expressed than LS for digestive gland in biological process. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

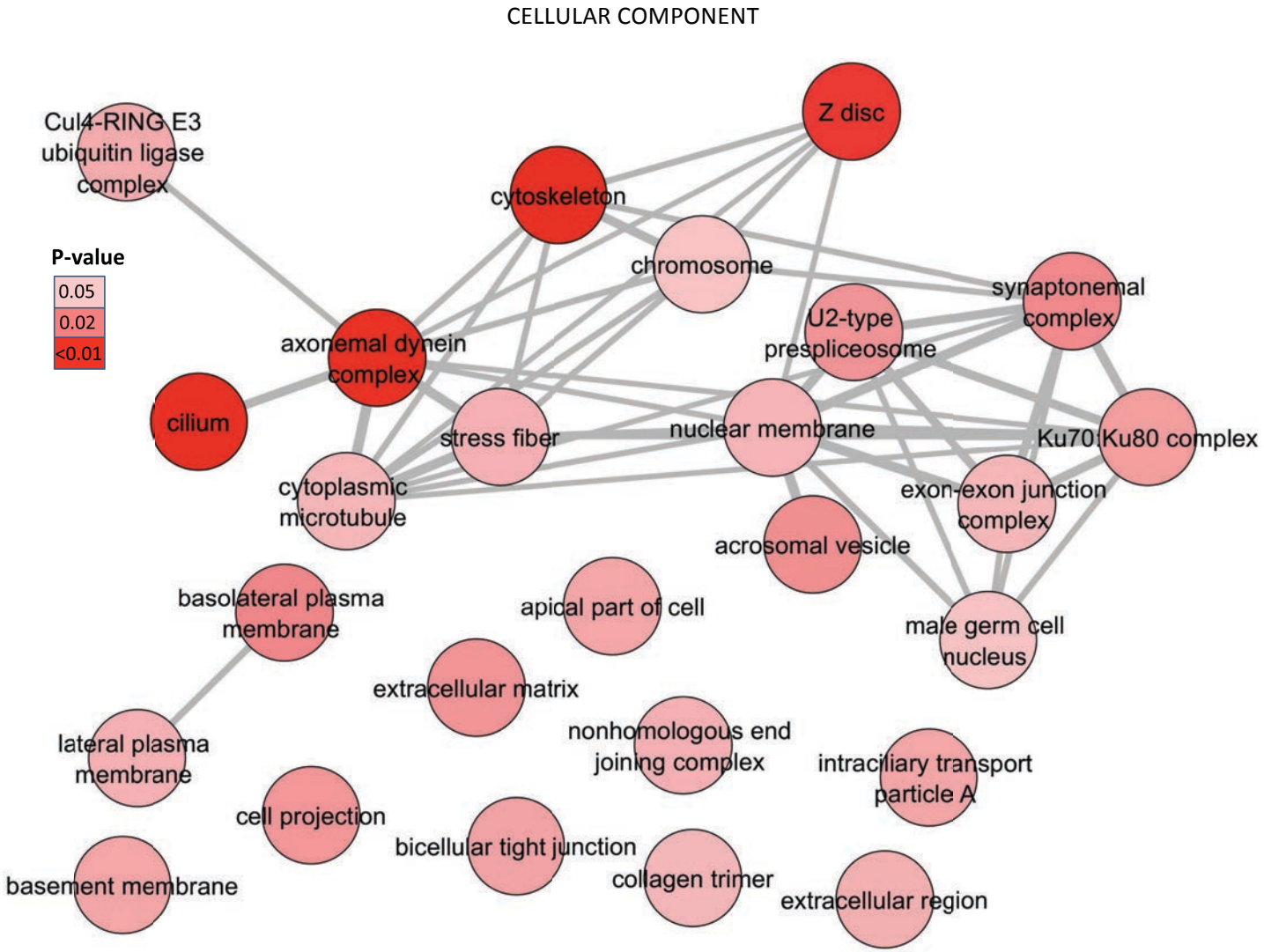

Figure 20b. RB genes higher expressed than LS for digestive gland in cellular component. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance.

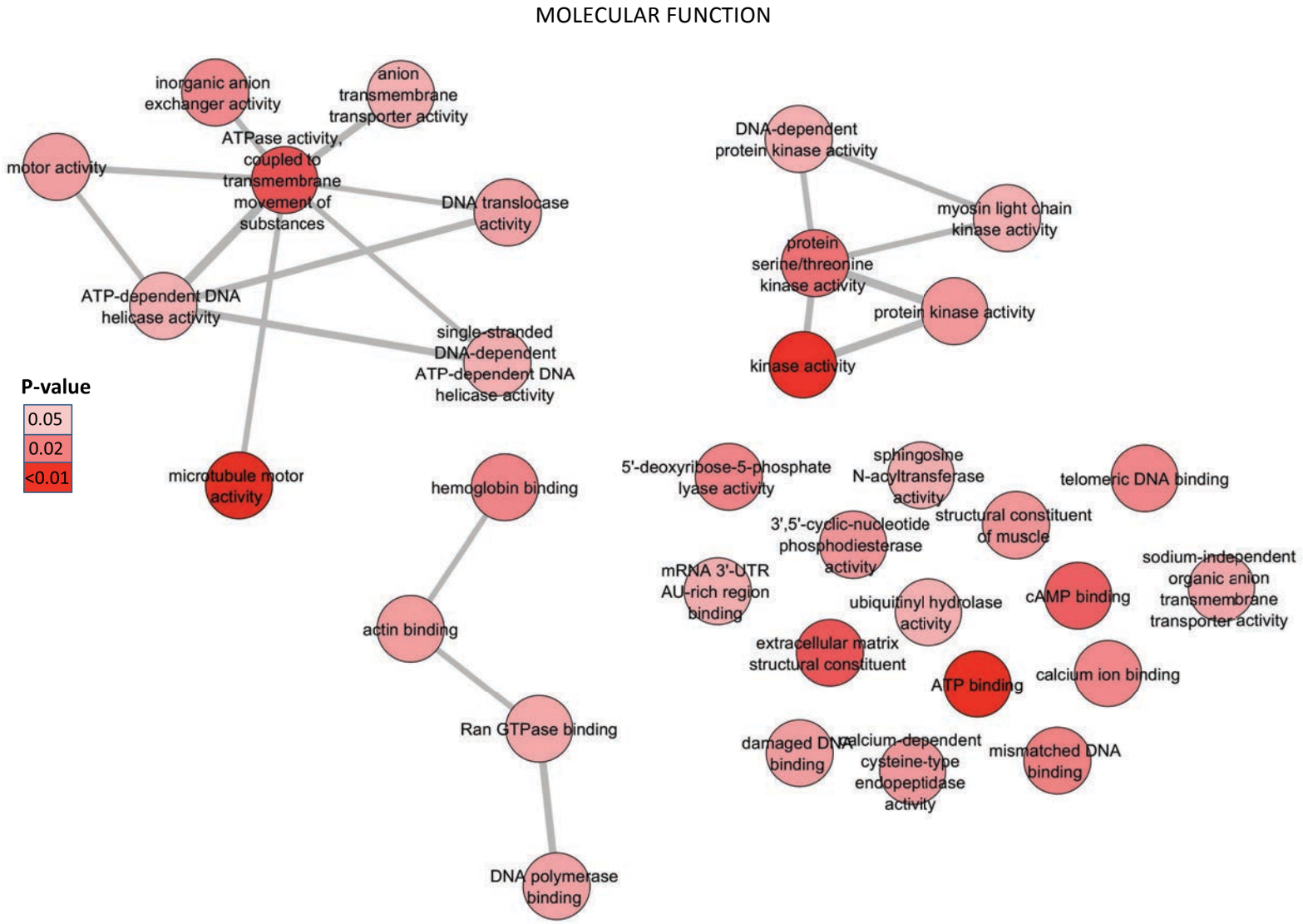

Figure 20c. RB genes higher expressed than LS for digestive gland in molecular function. Each of the GO term is represented for a node and the degree of similarity is represented for the number and width of the edge. The color represents the degree of significance

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Discussion/Conclusion and perspectives

The metal concentrations that emerge from the hydrothermal vent is one of the main characteristic of this ecosystems, where *in situ* concentrations in fluids and in different species in the MAR has been reported for many sites, particularly for Menez Gwen, Lucky Strike and Rainbow and strong significant differences have been evidenced for some metals such as iron, copper, cadmium, manganese and lead (Charlou et al., 2000, 2002; Desbruyeres et al., 2000). *Bathymodiolus azoricus* is one of the most abundant species in these sites and represent an emblematic species, which present a high tolerance and bioaccumulation capacities of essential and non-essential metals (Company et al., 2004; Kádár, 2007; Kádár et al., 2006; Koschinsky et al., 2014). At present, many questions remain unanswered about the kinetic of metal accumulation in natural environmental and experimental conditions and the identification of good biomarkers able to characterize the populations according to their chemical environmental signature has not been done. In a general context of the use of *B. azoricus* as a sentinel species for the ecological monitoring of vent sites (ie MOMARSAT program), the identification of reliable biomarkers represent an important challenge. The use of ecotoxicological biomarkers in hydrothermal ecosystem is a not well studied area but recently those ecosystems have attracted the attention of the mining industries that are interested in the massive sulphide and manganese nodules deposits. However the knowledge of the functioning of the hydrothermal vent ecosystems and the associated fauna is not fully understood, therefore it is urgent to increase those knowledge to provide usefull data in the aim to create a correct management protocols of deep sea exploitation to minimize the impact for the hydrothermal fauna (Mestre et al., 2014; Van Dover et al., 2011).

In this context, the work conducted in this thesis try to describe the metal composition of soft tissues (gill, mantle and digestive gland) for eight metals (cadmium, copper, iron, lead, calcium, manganese, zinc and magnesium) that represent the main metals present in the fluids in different populations collected on three vent sites previously known to strongly differ in term of metal composition: Menez Gwen, Lucky Strike and Rainbow. We collected seven natural populations in these three hydrothermal vents in order to test our biomarkers with the main idea that if fluid composition represents a specific signature of each site, transcriptomic profiles should be relatively similar in all populations from a same vent site and, at least two populations were sampled to get biological replicates of each vent site. We hypothesize that if transcriptomic pattern are similar between populations within a site and different from the other sites, metals could potentially be one explanatory variable. In this case, we also could not forget the possibility that pressure may be another explanatory variable. In the case of the existence of higher

variations in gene expression between populations within sites than between sites, we will have to consider that vent site are not homogenous but the sum of micro-habitats exhibiting small scale variations.

Whatever the biomarker analyzed, our results (obtained in natural and experimental populations) suggest that the characterization of biomarkers of metal exposure is not trivial. Based on data available and our assumptions, one of our first surprising result is the fact that the metals quantified in the tissues do not necessarily represent the gradient of concentrations (RB>LS>MG) previously described for these sites (Charlou et al., 2002). We cannot exclude that according to the activity of the vent site, fluid composition may change in time scale between young and old chimneys. Our results indicate a probable stronger heterogeneity in fluid composition within each vent site but because we do not have fluid water sampling available for metal quantification for the populations sampled, this hypothesis cannot be confirmed by chemical analysis. However, if we consider that at a same site, mussels cannot have a different behavior in term of metal accumulation, the difference of metal concentrations in their soft tissues should reflect real differences in the metal composition in the fluid they are filtering. The tissue specific response observed in *B. azoricus* in which gill and digestive gland show the higher concentration levels compared to mantle may either reflect the environmental variations related to hydrothermal activity or the existence of a organotropism in *B. azoricus* as one strategy for metal tolerance. More, we identified high levels of correlations for some metals which conduct to the identification of conserved metal profiles in each tissues and may reflect a possible shared mechanism of tolerance, uptake or detoxification for the correlated metals. It also mean that each population can be characterized by a specific tissue metal signature when considering the metal concentration in the three tissues together.

B. azoricus live in symbiosis with two types of bacteria that strongly depends on the availability of sulfide and methane as substrate for carbon source but the role of this in metal tolerance or detoxification mechanism is not described. The study of correlation between bacteria and metal concentration in gills revealed significant correlations pattern particularly with iron, one of the most representative metals of hydrothermal vent. This result could indicate a role for bacteria in some metal regulation in *B. azoricus* but this relationship need to be studied more in depth. The recent genomic data obtained on the SOX symbiont genome need to be explored to identify putative genes involved in metal homeostasis or detoxication in bacteria to test some hypothesis of co-regulation of gene expression in both partners.

The model species chosen for this work has proven to be a good species for the development of experiment in controlled laboratory conditions, particularly individuals collected from Menez Gwen site, because they can survive for a long time under hydrostatic pressure. This provided an advantage which allows the development of a long time experiment of metal exposure. In our experiment of metal exposure

using different metals and concentrations, the metal quantification operated in the different tissues revealed a high plasticity in *B. azoricus* related to metal accumulation. In our experiments, we focused our attention in the kinetic of metal accumulation of copper, iron and cadmium because are the most representative elements of the hydrothermal vent and are representing both categories: essential and non essential metals. The results obtained (presented in the SOD regulation study) showed that accumulation of cadmium do not operate even after 25 days when copper is accumulated much faster. This represent the existence of different strategies in metal accumulation and probably fixation/detoxication of heavy metals (ie cadmium) and essentials metals (ie copper). However, the surprising co-accumulation of cadmium and copper in the copper exposure suggest that some molecule involved in the copper fixation could also be involved in cadmium sequestration. This question has to be further addressed. A complementary approach based on the isolation of proteins containing copper could be envisaged in order to identify these actors that could be new candidate to study metal detoxication in *B. azoricus*.

The use of relative gene expression has been used extensively in marine invertebrates exposed to different biotic and abiotic factors, providing valuable information about how the species respond to this factors. In *B. azoricus* this approach has been used to measure the response in different aspects, symbiont quantification (used also in this study), response to immune systems and response to metal exposure principally, but the response in natural population has been poorly reported. Therefore, another objective of this study was to describe genes involved in metal tolerance and detoxification process that can be used in the future as biomarkers related to metal exposure in this extreme environmental. The candidate genes selected for this work where chose because they have been previously reported as playing a potential role in metal detoxification process (Bebianno et al., 2005; Bougerol et al., 2015; Hardivillier et al., 2004; Wong et al., 2015). In natural populations, the study of these biomarkers are restricted to low sample number, total protein quantification and/or activity (including the bacteria proteins) or limited to a specific hydrothermal site (one population).

We selected our candidate genes on their physiological importance in the metal tolerance and detoxification but also on the fact that they are used in other marine invertebrates as biomarkers of metal response. In this context, metallothioneins and superoxide dismutase are among the best studied mechanisms related to metal detoxification and oxidative stress respectively. However the previously reported works in *B. azoricus* were focused to controlled experimental situations in which the response of these genes were related to only one variable, one of the principal limitations in ecotoxicological studies. Indeed, the extrapolation of the results obtained on the effect of one parameter to the response in natural conditions where multiple variables can influence the physiological status of individuals and consequently population responses is not accurate. On the other hand, the study of phytochelatin (a cadmium binding

protein well described in plant and terrestrial worms principally) and ferritins (related to iron homeostasis) are poorly studied in marine invertebrates, even their important role in metal detoxification process has been evidenced. Those markers presented a strong interest because cadmium and iron are the most representative metals in the hydrothermal vent ecosystems.

We report new sequences in *B. azoricus* especially for MTs and two new potential cadmium-related proteins that showed some interesting correlation between their expression and tissue cadmium concentrations. We did not characterize a strong correlation between MTs expression and metal content in natural populations especially when regarding the vent site effect but we evidenced a specific signature for each population which still can reflect the signature of environment. To go further, a quantification of MTs at a protein level can be envisaged even such approach will not allow to characterize the level of each isoform except if monoclonal antibodies are developed. Another issue will be the production of recombinant proteins for each of the MTs and cadmium-related proteins to investigate their functional properties (metal binding specificity, thermostability, pH effect,…). This approach is going on with the three MTs and the two cadmium-related proteins. Another level of analysis has to be investigated which concerns the potential effect of polymorphism in gene promoters (particularly in Metal responsive Elements), the polymorphism of gene copy number (between isoforms, between populations), and the role of SNPs in link to the expression.

We also evidenced a high diversity of SODs and ferritins isoforms (no similar study has been conducted in other mollusk species) that could participate to the understanding of the resistance of hydrothermal mussel: the involvement of a higher number of genes to improve the production of proteins then better afford the stress. For all biomarkers tested, the relative expression pattern reported did not clearly represent the metal concentration of the sites but reflect strong differences at inter population levels and specific tissue response. We may also suggest that the limited response could also reflect the fact that the expression ratio for these genes is already high in mussels and that there is no specific need or no capacity to strongly improve this expression in response to stress. In the case of the metal exposure, concentrations used for copper and iron (that mimics the conditions observed at the three vent sites), the stress intensity was maybe not sufficient to generate a strong stress response or at the contrary, the observed response, even limited in term of fold, was sufficient to deal with the consequences of the stress. As for MTs, protein quantification and activity (especially for SODs) can be envisaged with the same problem link to the fact that those parameters reflect a global response and do not allow to characterize the importance of each isofom. Again, all the genetic approach has to be considered.

For all markers analyzed, MTs, PCs and SODs and ferritins, high variation in expression levels have been reported at population levels and no global pattern can be detected making more difficult to catalog this genes as real good biomarkers of metal exposure in natural populations of *B. azoricus.* The use of microarrays allowed the comparison of thousands of genes simultaneously in different situations generating an enormous amount of information even a significant amounts of them do not have a functional annotation. The result of the gene profiling performed in this study are under complementary analysis, but the preliminary data presented in the last chapter of this manuscript revealed different population pattern and also differences at tissue levels which are in agreement with the variability reported in the candidate gene expression, that can be interpreted as the differences in the physiological status of each population and also in agreement with the hypothesis of tissue specific response of *B. azoricus*. Additionally some pathways related to metal response are described, however the detected differences between populations does not allow us to establish clearly that these differences are essentially due to metal concentrations measured in populations. The integration of additional environmental variables must be considered in the future as the concentrations of methane and sulphide gases, variations in temperature, pressure under natural conditions.

To go further on this work, we will explore the response of other molecular markers on the same samples (field populations and experimental individuals) and add other kind of genetic markers to go deeply in the understanding of the adaptation developed by *B. azoricus* to scope with such environments. Among the molecular markers identified, we will address a specific focus on the HSP family, especially the HSP70. The heat shock proteins (HSP) and especially HSP70s is probably the most studied proteins in response to different environmental conditions, is a conserved protein in many taxa and which principal function is to act as chaperone molecules that re-fold proteins denatured for different factor, principally temperature. Induction of HSP70 in response to heavy metals has been reported for some marine invertebrates (Tedengren et al 1999; Haap et al., 2016). For example, Tedengren et al. (1999) reported differential expression of HSP70 proteins in two populations of the mussel *Mytilus edulis* exposed to cadmium, with a rapid activation of HSP at low levels of cadmium exposure associated with low levels of mortality in one population (North sea),and in the other population (Baltic sea), low levels of HSP activation but high levels of cadmium and elevated mortality, those differences reflecting a putative differential efficiency in metal detoxification between populations. From the transcriptomic and genomic data available on this species, we identified 9 isoforms of HSP70 on which the expression is actually analyzed in the same populations and tissues.

Another metabolism will be investigated: the energetic pathway. The response of the organism to any environmental variation inevitably brings change in metabolic pathways that should provide the

energy (ATP production) needed to address these changes, so metabolic genes and enzymes can be considered as important hotspot for natural selection. The enzymes of glycolysis and TCA cycle have emerged has a target to understand how the organism respond physiologically to different environmental conditions and glycolysis and TCA cycle related genes have been shown to be regulated with respect to others genes and also his intermediary metabolites can have a potential effect in the metabolism (Marden, 2013).

In this work, we also characterize a gene profile using microarrays in natural populations in ways to found new metabolic pathways that can be associated to metal response, however for a better identification of this pathways will be interesting to make the same analysis in the mussels exposed to metals, which would allow to found specific response to each metal at different expression concentration, determining the mechanism that could explain the high metal tolerance reported for *B. azoricus*. Complementary analysis have to be perform to identify potential markers associated with pressure effect (represented by the depth of the populations) but also between populations from the same vent site to distinguish local effect.

This study gives abundant information about the biochemical and molecular response of *B. azoricus* related to metal tolerance mechanism (candidate genes) and global gene expression profile (microarrays analysis). However it does not provide information about molecular ecology aspects as genetic diversity and gene flow in natural populations of this species, which is one of the key issues in evolutionary biology to understand how the species respond to environmental changes. To bring this information, we developed a SNP library using a RAD sequencing approach using individuals from six populations from the three vent sites. RADseq is a molecular technique that has enabled the generation of the identification of thousands of SNPs (single nucleotide polymorphism) that optimize the number of genetic markers for a specific biological system allowing a better picture of historical process (phylogenetic relationships, population structure) and functional mechanism (genotype-phenotype), which could help to understand in better way the variability in gene expression profile described in this work.. Pattern of genetic adaptation to environmental change has been well documented in several marine species where several loci associated to different physiology process, including energy metabolism are differentially expressed and are under selection demonstrating the potential role of this evolutive force to allow population to adapt in different environmental changes (Debes et al., 2012; De Wit and Palumbi, 2013; Schoville et al., 2012; Tovin et al., 2012; Whitehead and Crawford, 2006; Xie et al., 2016)

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Approche transcriptomique de la réponse aux métaux chez la moule hydrothermale *Bathymodiolus azoricus*

Résumé:

Bathymodiolus azoricus est un bivalve endémique des sources hydrothermales de la dorsale Médio-Atlantique qui présente une forte capacité à accumuler différents métaux dans ses tissus, la qualifiant comme espèce modèle en écotoxicologie. L'objectif de ce travail a consisté à décrire les mécanismes moléculaires de tolérance et de détoxication des métaux, que ce soit au sein des populations naturelles ou durant des expositions expérimentales, par le biais d'approches chimiques (quantification des métaux) et transcriptomiques (PCR quantitative et puces à ADN), afin de comprendre comment les facteurs environnementaux pouvaient influencer l'expression des gènes de *B. azoricus* et d'identifier des biomarqueurs potentiels utiles pour des études écotoxicologiques en environnement extrême.
Les mesures de bioaccumulation de différents métaux (Cd, Cu, Fe, Pb,…) sur des individus provenant de sites aux caractéristiques contrastées ont révélé des patrons spécifiques aux populations et aux tissus examinés chez cette espèce, suggérant des phénomènes d'organotropisme. Nos résultats suggèrent également que les bactéries symbiotiques des branchies pourraient participer à la tolérance aux métaux et aux processus de détoxication.
Des tendances comparables sont observées pour l'expression relative de certains gènes candidats impliqués dans la réponse au stress métallique, par exemple les métallothionéines, les superoxyde dismutases, les ferritines et les phytochélatines. Les réponses distinctes observées pour certaines populations ou certains tissus traduisent des différences d'état physiologique qui ne seraient pas directement reliées avec l'accumulation de métaux.
En outre, l'analyse des puces à ADN à une échelle globale nous a permis d'identifier les réseaux métaboliques principaux pour chaque population et chaque tissu.

Mots clés : *Bathymodiolus azoricus*, Sources hydrothermales, Ecotoxicologie, Stress métallique, Transcriptome

Transcriptomic approach of the response to metals in the hydrothermal mussel *Bathymodiolus azoricus*

Abstract:

Bathymodiolus azoricus is an endemic bivalve from hydrothermal vents in the Mid-Atlantic ridge, which is known to accumulate different types of metals in various tissues at high concentrations, and has therefore been granted model species for exotoxicology. The objective of this work is to describe the mechanisms of metal tolerance and detoxification as they occur in natural populations and during experimental exposures, using both chemical (metal quantification) and transcriptomic (qPCR and microarrays) approaches to understand how environmental factors influence gene expression response in *B. azoricus* and to identify potential biomarkers useful for ecotoxicological studies in extreme environments.
Bioaccumulation of different metals (Cd, Cu, Fe, Pb,…) was measured on individuals from contrasted vent sites, revealing specific population patterns and tissue differences for this species that could be related to processes of organotropism. Our results also suggest that the symbiotic bacteria in gills may be involved in metal tolerance and detoxification.
Similar variation trends are observed in the relative expression of candidate genes involved in response to metal stress, e.g. metallothioneins, superoxide dismutase, ferritin, and phytochelatin, revealing contrasted responses at population and tissue level, and reflecting differences in physiological status not directly correlated with the accumulation of metals. In addition, global scale microarray analysis allowed us to identify the principal biological pathways representative of each population and tissues.

Keywords: *Bathymodiolus azoricus*, Hydrothermal vents, Ecotoxicology, Metallic stress, Transcriptome