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Title: Seasonal variability in different biomarkers in mussels (Mytilus galloprovincialis) farmed at different sites of the Gulf of La Spezia, Ligurian sea, Italy

Article Type: Research Paper

Keywords: Mytilus; biomarkers; seasonal; monitoring; health status

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Abstract: Mussels (Mytilus spp), are worldwide utilized in marine biomonitoring by a multi-biomarker approach. However, for a correct interpretation of different biomarker responses, information is needed on their natural seasonal variability due to environmental/physiological factors.
In this work, the seasonal variations of different biomarkers were investigated in M. galloprovincialis from 4 different sites from the gulf of La Spezia (Ligurian sea, Italy), an intensive rearing area in the north-western Mediterranean near La Spezia harbor, an important commercial and touristic port. Lysosomal membrane stability-LMS, stress on stress-SoS, phagocytosis, tissue metallothionein-MT content, oxidative stress related enzyme activities (GST, catalase), and nitric oxide (NO) production were evaluated. The results underline the importance of LMS and SoS as core descriptors of the mussel health status in relation to seasonal variations in temperature and reproduction. These data represent the baseline information for ongoing biomonitoring studies related to dredging activities in this area.

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Dear Editor,

I send you the revised manuscript 'Seasonal variations of different biomarkers in mussels (*Mytilus galloprovincialis*) farmed at different sites of the Gulf of La Spezia, Italy'.

We thank the Reviewer for the interesting and helpful comments. Response to all queries are enclosed on a point by point basis. In particular, Figures 2-8 and their legends have been revised adding the requested statistical information and the text has been accordingly modified following all Referee's indications.

We hope that the changes made will meet the Reviewer requirements.

Thank you very much for your attention

Sincerely yours

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Reviewer #1: Notes:
Overall this is a well written manuscript with no typos and excellent grammar throughout. There is some very useful data presented in this paper regarding biomarker expression in Mytilus galloprovincialis over a 12 month period, but I don't feel that it has been analysed to its full potential.

The data has been analysed for differences between sites for each month, but also needs to be analysed for each site over the 12 month sampling period.

This would provide very valuable background information and as far as I can tell has not been undertaken on all the data.

As detailed below the results section needs to be made clearer to the reader and the significant results visible on the graphs. The discussion is well written putting the results into context. This paper makes a significant contribution to research in the area and with the inclusion of the suggestions below is suitable of publication in MPB.

We thank the Reviewer very much for the helpful comments.

With regards to statistical data analysis, data were analyzed for monthly differences in each site, as well as for site-related differences at each month. In an attempt to make data presentation simpler, we missed showing relevant information on significant seasonal variations at each site.

Since for most biomarkers seasonal differences largely prevailed over site-related differences, the former have been indicated by symbols in all graphs of the revised ms, and the appropriate explanation in Figure legends. Site-related differences, when observed, are only reported within the text of the results section.

The results have been revised describing, for each biomarker, in the following order:
1) general seasonal trend (if any, with average maxima and minima)
2) significant monthly differences for each site (also reported in graphs)
3) differences observed among sites (only reported within the text)

General observations:
* What does graphical abstract tell us? I like the idea of it but in its current form it is not very descriptive. Possible to add in the key changes in biomarker expression or spawning?

The graphical abstract has been modified in order to better give the idea of the relationship between T, gametogenesis, LMS and SoS biomarker data.

* Why was n=4 chosen? Seems a little low for a biomarker study? Do they think more significance could have been observed with a higher sampling number?

In this work a total of 10 different determinations (7 different biomarkers, 3 of which in two tissues) were performed for 11 months in 4 different mussel samples. Due to the large amount of analyses to be performed, the number of replicates was carefully chosen on the basis of previous experience for each biomarker.
N=4 was utilized for SOS response (4 groups of 10 mussels each), as in the original method and its subsequent applications.
With n=4 small SDs are generally obtained for many of the other biomarkers (LMS, phagocytosis, MT and GST), and this was confirmed by the present work. For other parameters, such as Catalase

activity (measured as the sum of activities in both crude cytosolic fraction and lysosomal pellet containing peroxisomes) and tissue nitrite accumulation (which is an indirect measurement of NO production), larger SDs are often observed, also in relation to the type of assay. Actually, for these two biomarkers, the number of replicates could be increased (up to 6 or 8), in some samples at certain months, where higher variability was observed. However, increasing the number of samples did not decrease the resulting SDs, and therefore, statistical significance, reflecting the inner variability of the results.
The number of replicates for each biomarker has been specified in each Figure legend of the revised ms.

* Good to equate the baseline data to dredging impact. Although this is mentioned in the introduction and briefly in the discussion, it could do with a bit more detail. Have these biomarkers been used to assess the impact of dredging previously?
* Good idea to use as a tool to monitor the health of mussels in aquaculture.
* Introduction is relatively short, but contains relevant information. More info on the use of these biomarkers in aquaculture would be good, buy may be provided in the discussion.

We thank the Reviewer for these comments. Actually, available data on the use of different biomarkers in relation to dredging operations, in addition to lysosomal biomarkers, Metallothioneins, biomarkers of genotoxicity, mainly utilized multiple enzymatic biomarkers mainly related to oxidative stress, GSH metabolism and biotransformation, all biomarkers that show a bell-shaped trend with increasing stress conditions (see discussion of the original ms., page, line).
SoS response, immune biomarkers and NO production have not been previously utilized to evaluate the impact of dredging.
Moreover, Biomarkers have not been previously utilized to monitor aquacultured mussels.
A short para on these two points has been added in the Introduction (two more references have been added (Martins et al., 2012; Bebianno et al., 2015) and related comments in the Discussion of the revised ms.

M&M
* Why keep the mussels in the lab for 24h before observation? Practical restrictions? Would they recommend this approach to others? Issue with spawning and how that impacts on biomarker expression.
The referee's guess is right, but maintaining mussels in water before observation was only partly due to practical reasons. This monitoring program was carried out in collaboration with local environmental and health institutions (for contaminant burden and microbiological analyses). This implies that after sampling and before distribution to different groups and the journey to the lab mussels were be kept for few hours in air, although in controlled conditions (due to the large number of animals it was impossible to keep them in water). This lapse of time was as short at possible (2-5 hr max) but not always the same in 11 different samplings, introducing a further variable or an additional source of stress to the mussels, which may affect the determination of the parameters measured.

This attempt to minimize stressful conditions was already mentioned in Methods, 2.2, line 121 of the original ms. However, it particularly applies to hemolymph sampling and hemocyte analyses: in our experience, even few hours in air can affect the amount of hemolymph that can be withdrawn from the animals, as well as the number of circulating hemocytes and the values of NRR time, since LMS is the most sensitive biomarker of stress. Rapid recovery can be observed after 24 of re-immersion. In addition, this period allows to easily monitor spawning of ripe mussels, as well as possible mortalities.

Therefore, we would recommend this approach in particular when measuring hemocyte biomarkers, but also in general for standardization of the procedures between sampling and analyses, as well as for the observation of spawning and mortality.

Results:

* Fig 2, why is site 4 so markedly different from the others regarding stage 0-II?

The referee is right, Site 4 showed the more marked seasonality in the gametogenic period. However, since no differences in physico-chemical parameters (T, salinity, ph, O2, etc) were detected in comparison with other sites, we could only guess that this may depend on its location, which is at the inner mouth of the port, and on the local changes in water currents, that may reduce the food availability, or on other unknown disturbances related to the passage of ships at the entrance of the port.

Interestingly, in samples form Site 4, that showed a shorter gametogenic period, smaller fluctuations in LMS were also observed. A comment on this point has been added in the revised ms. in both results and discussion.

* Fig 3: why is there no indication of statistical significance on the graph? Do they think that the small sample number (n=4) could have impacted on the levels of significance?

The only reason why statistical significance was not shown in the graph is an attempt to make it as simpler as possible. In the revised figure, for each site statistical differences over the 12 month sampling period have been added.

* Line 236: is this the average maximum as Sept has higher levels for for site 1, 2 and 3?

The sentence has been corrected. For all samples average values in both July and September were > 200 min.

* Although there was a significant difference below 120 min at site 1, was there a significant difference between sites for each time period? This has not been mentioned and is very relevant information. I feel the data presented in Fig 3A needs clearer description and interpretation.

The description of Fig. 3A has been revised. With regards to differences among sites, only the differences between Site 1 and the other sites have a biological importance , since the extent of these different is indicative of stressful conditions. Occasional differences among the other sites, although statistically significant, were always observed among LMS values > 120 min, indicating stable lysosomes and healthy conditions (see discussion).

* Line 244, do you mean between sites here? Was there no Signiant difference between each site for each time point? Needs to be clearer. Similarly in line 235 do you mean among sites (all sites for a specific time period) or between sites (different sites for a specific month)? This needs to be clearerâ€¦

See the response to the general comment on statistical significance.

* Fig 3 has no error bars. These should be included

Error bars and Statistical differences have been added.

* For Fig 3 legend, please include the sample number.

All figure legends have been modified indicating the sample number.

* Table 1. has been presented twice. Where they positive or negative correlations? Please indicate on the table.

Sorry for the mistake in uploading the ms. In Tab. 1 all correlations are positive. This has been specified in the text of the revised ms.

* Line 255, was that tested for each site over the 12 month period or between sites for each month? Please make this clearer. I would be surprised if there were not significant differences found anywhere.

With regards to phagocytosis, no seasonal differences were observed, and values always > 60% were observed, indicating full immunocompetence.

However, some site-related differences were recorded, and they have been reported within the text in the results section of the revised ms..

Fig 5a. Please indicate significance on the graph. Was significance for each site between months tested? this would be important information that could be taken from the data.

Significant monthly differences for each biomarker at each site have been added (see general comments).

* Line 272: can you please make this clearer as I am not sure what you mean. Jan site 1 significantly higher than other site 1s, May site 3 significantly lower than all other site 3s? what about each site over the 12 month period?

See response to general comments to the results section.

* Fig 6 legend. What is the N number then for 4 experiments in triplicateâ€¦ please make this clearer.

For spectrophotometric evaluation of enzyme activities (GST and CAT) each sample was read three times.

* Fig 6. Please indicate significance on graphs.

Significant monthly differences have been added(see general comments).

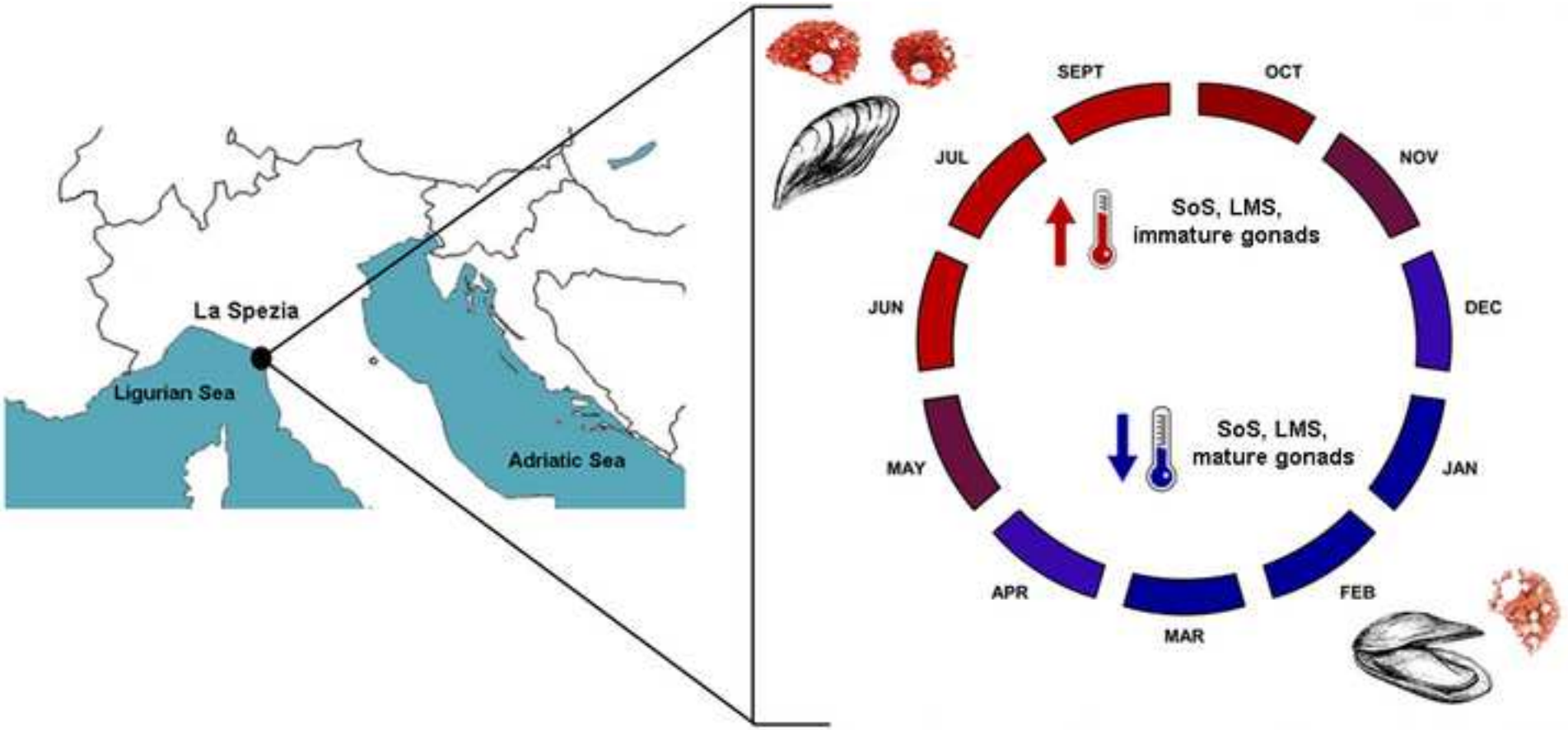
* Line 359: please replace in different samples, with samples taken from different sites.

Corrected

* Line 413 mentions data on seasonal changes in different biomarkers. However I feel this has not been fully explored in the paper. It would be good to have a greater analysis of biomarker expression for each site over the 12 month period.

Significant monthly differences for each biomarker at each site have been added(see general comments).

Graphical Abstract



Highlights

- Biomarker seasonal variations in mussels farmed at 4 sites of the La Spezia gulf
- Seasonal changes in LMS and SoS correlated with temperature and reproduction
- No differences in the health status of mussels from different sites
- First data of seasonal variability of mussel biomarkers in western coast of Italy
- Basal information for future biomonitoring studies related to dredging activities

Seasonal variability of different biomarkers in mussels (*Mytilus galloprovincialis*) farmed at different sites of the Gulf of La Spezia, Ligurian sea, Italy

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Abstract

Mussels (*Mytilus spp*) are worldwide utilized in marine biomonitoring by a multi-biomarker approach. However, for a correct interpretation of different biomarker responses, information is needed on their natural seasonal variability due to environmental/physiological factors.

In this work, the seasonal variations of different biomarkers were investigated in *M. galloprovincialis* from 4 different sites from the gulf of La Spezia (Ligurian sea, Italy), an intensive rearing area in the north-western Mediterranean near La Spezia harbor, an important commercial and touristic port. Lysosomal membrane stability-LMS, stress on stress-SoS, phagocytosis, tissue metallothionein-MT content, oxidative stress related enzyme activities (GST, catalase), and nitric oxide (NO) production were evaluated. The results underline the importance of LMS and SoS as core descriptors of the mussel health status in relation to seasonal variations in temperature and reproduction. These data represent the baseline information for ongoing biomonitoring studies related to dredging activities in this area.

Key words: Mytilus, biomarkers, health status, seasonal, monitoring, aquaculture

1. Introduction

Mussels of the genus *Mytilus* are among the commonest marine mollusks in coastal waters, with *Mytilus galloprovincialis* representing the warm water species present in the Mediterranean. Apart from their ecological importance and economic value, mussels have long been utilized in biomonitoring of marine pollution by a multi-biomarker approach (UNEP-Ramoge, 1999; Viarengo et al., 2007; Cravo et al., 2009; Davies and Vethaak, 2012; Vethaak et al., 2015; Martínez-Gómez et al., 2015). However, it is widely accepted that different biological parameters utilized as biomarkers of the mussel health status can show seasonal fluctuations driven by the complex interactions between natural environmental factors (temperature, salinity, food availability, etc.) and endogenous factors (sex, age, reproductive status) (Bayne, 1976; Hagger et al., 2010; Schmidt et al., 2013; Dallas and Jha, 2015). These interactions complicate the interpretation of biomarker responses observed in environmental monitoring studies (Davies and Vethaak, 2012). In this light, a thorough knowledge on the natural variability of different biomarkers in different mussel populations is needed for a better interpretation of biomarker results in biomonitoring of coastal environments. Moreover, also in the absence of potential pollutant exposure, application of the biomarker approach to cultivated mussels can represent a powerful, early warning tool for mussel farmers to monitor the health status of the organisms, and to identify their susceptibility to different environmental stressors in different farming areas at different times of the year (Schmidt et al., 2013).

Despite the number of studies published on seasonal variations of biomarkers in *M. galloprovincialis*, only a few were carried out on populations of this species living along the Italian coast, and all of them in the eastern coast (Adriatic sea) (Nesto et al., 2004; Bocchetti and Regoli, 2006; Bocchetti et al., 2008; Moschino et al., 2011). The gulf of La Spezia (Ligurian Sea, Italy) has long been an intensive rearing area for *M. galloprovincialis* in the north-western Mediterranean; mussel aquaculture is carried out at different sites near the La Spezia harbor, an important

commercial and touristic port. Mussels commercialized from this area, after depuration and treatment according to the EC and FAO Regulations, have been utilized in the last decades for the development and application of different biomarkers in laboratory (es: Viarengo et al., 1995, 1997) and in transplantation studies (es: Viarengo et al., 2007; Dagnino et al., 2007). However, no information is available on the natural seasonal variability of biomarkers in mussels reared within the gulf of La Spezia before depuration treatment. These data are important not only to gain basal information on the health status of mussels in relation to abiotic and biotic factors, but also in relation to activities carried out within the near La Spezia harbor, such as periodical dredging operations necessary to maintain accessibility and navigational depths, that may have possible impacts on aquaculture.

Biomarkers have been utilized in mussels to evaluate the impact of dredging in harbor areas, with oxidative stress, genotoxicity and metallothionein representing the most common biomarker responses (Bocchetti and Regoli, 2006; Martins at al., 2012; Bebianno et. al. 2015). However, the biomarker approach has not been specifically applied so far to aquacultured bivalve species. In the present study, seasonal variations of different biomarkers were investigated in mussels farmed at 4 different sites of the Gulf of La Spezia over a 11 month period. A battery of biomarkers at different levels of biological organization were evaluated: lysosomal membrane stability (LMS), stress on stress (SoS) response, immunocompetence (hemocyte phagocytic activity), gill and digestive gland metallothionein (MT) content and biotransformation and antioxidant enzyme activities (Glutathione transferase and catalase). Nitric oxide (NO) accumulation in the gills was also determined as a possible marker of tissue inflammation.

2. Methods

2.1 Sampling area

Waters for mussel cultivation in the Gulf of La Spezia are classified as Type B (max 4.600 *E. coli* /100 g), according to EU Reg. 854/2004. Before commercialization, mussels are treated at the

depuration center of S. Teresa (Lerici) (EU I 11C.D.M and I 11C.S.M.). Analyses of water physico-chemical parameters, chemical contamination and microbiological analyses in water and mussel samples are routinely carried out at different sites within the activities co-ordinated by the La Spezia Port Authority (APSP) (www.porto.laspezia.it) in collaboration with ARPAL (Agenzia Regionale per la protezione dell'Ambiente Ligure), IZS-PV (Istituto Zooprofilattico Sperimentale Piemonte-Liguria Veneto), ISS (Istituto Superiore di Sanità), ICRAM (Istituto Centrale per la Ricerca Scientifica e Tecnologica applicata al Mare) (see Marine Coastal Information System (http://apsp.macisteweb.com/).

Mussels were sampled monthly, from October 2015 to September 2016, at 4 different sites internal and external to the seawall of the Gulf of La Spezia, Italy, chosen among those included in the APSP monitoring program: Portovenere (Site 1), Diga Levante Interno (Site 2), Diga Ponente Esterno (Site 3) and Diga Ponente Interno (Site 4) (Fig. 1) (ARPAL, 2008). Water physico-chemical parameters (salinity, O_2, pH) measured monthly at different sites were within the range of annual values for the Gulf of La Spezia, and showed little seasonal variations, with average salinities of 36.5 ± 1.9 g/L, dissolved O_2 7.66 ± 0.64 mg/L, pH 8.29 ± 0.08. Only temperature showed large seasonal changes (see below).

2.2 Mussel sampling

Mussels (at least 120 individuals per each site) were collected in collaboration with Cooperativa Mitilicoltori Spezzini and rapidly transferred in humidified containers at 15°C to the laboratory, where individual of approximately the same size (4-5 cm long) were selected. Groups of mussels were exposed to air for SoS evaluation. For subsequent determination of all other biomarkers, about 60 mussels from each site were acclimatized in aquarium for 24 h in static tanks containing aerated artificial seawater (ASW), salinity 36 ppt (1 L/mussel) at 16-20 °C, depending on the monthly water temperature. This standard protocol was utilized to minimize stressful conditions that may affect hemolymph sampling and hemocyte stress in the intercourse between sampling and analyses, as

well as to allow for the observation of gamete emission and possible mortalities. After 24 h acclimation, the reproductive stage of mussels from different sites was evaluated by microscopical observations of gonad smears during cell and tissue sampling. Gonads were classified as 0-II = early stage, III = developing/ripe, III-IV = ripe/spawning, IV = spawning. Since the proportion of males and females showed a great variability in animals sampled at different times of the year and at different sites, determinations of all biomarkers at cellular and tissue level were performed on pooled samples of both sexes. No mortality was observed in mussels sampled from different sites during acclimation in the laboratory.In order to assess hemocyte functional parameters, hemolymph from 8-10 mussels was extracted from the posterior adductor muscle using a sterile 1 ml syringe with an 18 G1/2" needle. With the needle removed, hemolymph was filtered through sterile gauze and pooled in 50 ml Falcon tubes at 16°C. To evaluate biomarkers at tissue levels, gills and digestive glands from 4 groups of 8-10 mussels were rapidly dissected, pooled, frozen in liquid nitrogen and maintained at -80°C for further analysis.

2.3 Lysosomal membrane stability

LMS was evaluated by the NRR (Neutral Red Retention time) assay as previously described (Ciacci et al., 2009; Martínez-Gómez et al., 2015). Hemocyte monolayers on glass slides were incubated with 20 µl of a neutral red (NR) solution (final concentration 40 mg/ml from a stock solution of NR 40 µg/ml in DMSO); after 15 min excess dye was washed out, 20 µl of filtered artificial sea water (ASW) was added and slides were sealed with a coverslip. Every 15 min, slides were examined under an optical microscope and the percentage of cells showing loss of the dye from lysosomes in each field was evaluated. For each time point 10 fields were randomly observed, each containing 8-10 cells. The end point of the assay was defined as the time at which 50 % of the cells showed sign of lysosomal leaking (the cytosol becoming red and the cells rounded). All incubations were carried out at 16°C.

2.4. Stress on stress (SoS) response

SoS was determined according to Viarengo et al. (1995). Upon arrival to the laboratory, mussels (4 groups of 10 animals per each Site, n=4) were placed onto plastic containers over a paper dampened with water (to achieve a continuous humidity of approximately 100 %) and kept at a constant temperature of 18°C. Survival was inspected every 24 h until 100 % mortality was reached. Mussels were considered alive when they resisted forcible valve separation and dead when the valves gaped and external stimulus (squeezing of valves) did not produce any response. Results are expressed by the Lethal Threshold for 50 % mortality (LT_{50}), median of the survival time or the day in which 50 % of mussels from a site were dead.

2.5 Phagocytic activity

Phagocytic activity was evaluated as uptake of Neutral Red-conjugated zymosan particles in hemocyte monolayers as previously described (Ciacci et al., 2009). Neutral Red-stained zymosan in 0.05 M Tris-HCl buffer (TBS), pH 7.6, containing 2.5 % NaCl was added to each monolayer at a concentration of about 1:50 hemocytes:zymosan diluted in filtered ASW, and allowed to incubate for 60 min at 16°C. Monolayers were then washed three times with ASW, fixed with Baker's formol calcium (4 % v/v formaldehyde, 2 % NaCl, 1 % calcium acetate) for 30 min and mounted in Kaiser's medium for microscopical examination with an inverted Olympus IX53 microscope (Olympus, Milano, Italy). For each slide, the percentage of phagocytic hemocytes was calculated from a minimum of 200 cells. Data are expressed as % of phagocytizing cells.

2.6 Tissue metallothionenin (MT) content

The metallothionein tissue content was analyzed as described by Viarengo et al. (1997). Both gills and digestive glands were homogenized in 3 volumes of 0.5 M sucrose, 20 mM Tris-HCl, pH 8.6, containing 6 μM leupeptine, 1 mM phenylmethylsulpfonyl-fluoride (PMSF), and 0.01 % β-mercaptoethanol. The homogenate was treated to obtain a partially purified metallothionein fraction

by ethanol/chloroform precipitation. Metallothionein concentration in the samples was quantified by spectrophotometric titration of the sulphydrylic residues using the Ellman's reagent, 5,5-dithiobis-2-nitrobenzoic acid. Spectrophotometric analyses were carried out at 25°C using a Varian Cary 50 spectrophotometer (Varian, Torino Italy). Metallothionein content (μg prot/g w.w. sample) was calculated against a standard curve of glutathione (GSH).

2.7 Biotransformation and antioxidant enzyme activities

Gills and digestive glands were homogenized respectively in 4 and 6 volumes of homogenization buffer (20 mM Tris buffer, 0.5 M sucrose, 0.15 M NaCl, pH 7.6) and centrifuged at 500 x *g* for 15 min at 4°C. The supernatants were then centrifuged at 14,000 x *g* for 30 min. Both supernatant and pellet (containing mitochondria and peroxisomes) were utilized for the spectrophotometric evaluation of catalase activity following the decomposition of H_2O_2 at pH 7, 25°C, at 240 nm (Viarengo et al., 1991). GSH transferase (GST) activity was evaluated on the 14,000 x *g* supernatants with CDNB (1-chloro-2,4-dinitrobenzene) as a substrate. The formation of S-2,4-dinitro phenyl glutathione conjugate was evaluated by monitoring the increase in absorbance at 340 nm (Canesi and Viarengo, 1997). Enzyme activities were measured in triplicate readings for each sample. All data are reported as μmol/min/mg prot.

2.8 NO production

NO production in mussel gills was evaluated by Griess reaction, a simple spectrophotometric method for the quantification of nitrite content (Tafalla et al., 2002). Gills were homogenized in 4 volumes of 0.05 M Tris buffer solution 2 % NaCl, pH 7.6 and centrifuged at 1,000 x *g* for 15 min at 4°C. The supernatants were then centrifuged at 16,900 x *g* for 30 min and incubated for 2 hours with the enzyme nitrate reductase in order to allow the reduction of nitrates to nitrites. After incubation, Griess reagent (1 % Sulfanilamide, 0.1 % N-(1-Naphthyl)ethylenediamine dihydrochloride, 5 % H_3PO_4) was added to each sample. After 10 min incubation in the dark,

absorbance at 540 nm was evaluated by a Varian Cary 50 spectrophotometer (Varian, Torino Italy) equipped with a microplate reader. Values were corrected for sample blanks readings. Nitrite accumulation (nmol/mg prot/ml) was determined by a standard curve of known concentrations of sodium nitrite ($NaNO_2$). Protein content was determined according to the bicinchoninic (BCA) method using bovine serum albumin (BSA) as a standard.

2.9 Statistics

Data, representing the mean ± SD of at least 4 samples, were analyzed by ANOVA followed by Tukey's post-test ($P \leq 0.05$). Significant monthly changes for each site are shown in the graphs, whereas significant differences among sites at each month are reported in the text (see Results). LT_{50} values were calculated with a 95 % confidence interval. Correlation was calculated by the Spearman non parametric test ($P<0.05$). All statistic calculations were performed by the PRISM GraphPad software.

3. Results

3.1 Water temperature and gametogenetic stage

Seasonal changes in water temperature recorded at different sites showed a minimum in Jan-Mar (12-13°C) and a maximum in July (24-25°C) (mean values between 0.2-5 m depth), in line with previous data on average temperatures at the Gulf of La Spezia (Fig. 2A). No significant differences were observed among different sites at different times of the year. Accordingly, mussels collected at different sites showed an extended reproductive period: although during acclimation in the laboratory a major gamete emission was observed in January-March, when lowest T values were recorded, spawnings were also recorded at different times of the year, when the water temperature never exceeded 18-19°C, as previously observed for mussels commercialized form this farming site (Fabbri et al., 2014). The reproductive stage of mussels sampled at different sites was evaluated by microscopical observations of gonad smears and data are reported in Fig. 2B. Mussels sampled at

Site 1 showed ripe/spawning gonads from October to June. In samples from Site 2 and 3 gonads at final stages of gametogenesis and spawning (stage III-IV) could be observed from November to May, with slight shifts in spawning among sites. Mussels from Site 4 showed a more definite and shorter gametogenic period, with immature mature gonads form May to October.

3.2 Lysosomal membrane stability and Survival in air (SoS response)

LMS and SoS were first determined in mussels sampled at different sites as core stress biomarkers at the cellular and organism level, respectively, and the results are reported in Fig. 3. LMS was evaluated by the NR Retention Time assay in hemocytes: as shown in Fig. 3A, seasonal changes were clearly observed in samples from all sites. As a general trend, high and stable LMS average values (>120 min) were observed in Oct-Dec at all sites, followed by a decreasing trend, with strong oscillations, in late winter-early spring, with a minimum in February, indicating stressful conditions in relation to the gametogenic period. Higher LMS values were recorded at all sites in summer, with maximal average values > 200 min between July and September. Samples from Site 1 showed the greatest oscillations in LMS, with significant monthly changes from January to July ($P \leq 0.05$). In the same period, significant, although smaller changes were observed in samples from Site 2 and 3. Samples from Site 4 showed the smallest oscillations, with significant decreases only in Jan-Feb, followed by an increase in Mar-Apr ($P \leq 0.05$).

The different extent of these seasonal changes was reflected by significant differences among sites at certain times of the year: Site 1 LMS was significantly lower than in samples from the other sites from January to April, as well as in June ($P \leq 0.05$). In particular, LMS <50 min were observed in both February and April, indicating strong stressful conditions. Differences were also occasionally observed among Sites 2-4 in April, May and September: however, LMS values were always high (> 120 min), indicating healthy lysosomes.

SoS also showed seasonal differences at all sites (Fig. 3B): lower values were recorded in late winter-early spring, with a significant decrease from January to February (average $LT_{50} \cong 5$ days;

P≤0.05). Significant increases were observed in May, followed by high, stable values throughout late summer (average LT_{50} for all sites in May-Sept of 7 days). No significant differences were observed among sites. Seasonal changes in LT_{50} were also reflected by 100 % mortality data, that showed a minimum in February (6 days) and were highest (>9 days) from May to September (not shown).

LMS and SoS data were analyzed by Spearman rank correlation test (P≤0.05) (Tab. 1). Seasonal changes in LMS showed a significant positive correlation with SoS for all sites, except for Site 4. Both LMS and SoS also showed significant positive correlations with Temperature at all sites at all times of the year.

3.3 Phagocytic activity

Phagocytic activity of circulating hemocytes was evaluated as a proxy of immunocompetence (Ellis et al., 2011). High phagocytic activity was recorded in mussels from all sites throughout the year (≥60 %), without significant seasonal variations, this indicating a good immune capacity (Fig. 4). However, phagocytosis could not be determined in hemocytes of mussels from Site 1 in February and in April, due to the high number of spermatozoa affecting the microscopical examination. Some differences between Site 1 and the other sites could be observed: phagocytosis was significantly higher with respect to Sites 2-4 in October, to Site 2 in March and in May-July, to Site 3 in July, and to Site 4 in January (P≤0.05). No differences among the other sites were recorded.

3.4 Tissue Metallothionein (MT) content

MT content was evaluated in mussel digestive gland and gills and the results are reported in Fig. 5. In the digestive gland, average annual values in all samples were about 300 µg/g tissue, with a maximum in January (395.45 ± 13.48) and a minimum in June (231.70 ± 26.61)(Fig.5A).

Significant monthly increases were observed for all sites in January, for Sites 1 and 4 in March, and for Sites 1-3 in July (P≤0.05). However, these oscillations did not show a clear seasonal trend at

different sites. Site related differences were also observed: Site 1 showed a higher MT content with respect to the other sites in November, and to Site 3 and 4 in May. The MT content was lower in samples from Site 4 than in those of all the other sites in July ($P \leq 0.05$).

Lower concentrations of MTs were measured in the gills, with average annual values of 140 µg/g tissue (Fig. 5B). In this tissue, a clear seasonal trend was observed, with a significant increase from December to January (217.96 ± 7.33, representing highest annual values; $P \leq 0.05$), followed by a progressive decrease in spring that was significant from February to March at all sites ($P \leq 0.05$). Lowest annual values were observed in May (average 71.09 ± 4.88). A subsequent significant rise in MT concentration was observed in the summer, with a second peak in July at all sites. No significant differences were observed among different sites throughout the year.

3.5 Tissue GST and Catalase activities

GST and Catalase activities were evaluated in mussel digestive gland and gills and the results are reported in Fig. 6 and Fig. 7. In the digestive gland, GST activity showed clear seasonal variations at all sites, with highest average values in winter (≥0.07 µmoles/min/mg prot in January) and lowest in summer (0.029 ± 0.003 µmoles/min/mg prot in June) (Fig. 6A). In particular, significant decreases were observed at all sites in October, February and April; increases were recorded at all sites in December and at Site 1-3 in March ($P \leq 0.05$). With regards to differences among sites, significantly lower values were observed at Site 1 with respect to all sites in December and to Site 4 in September; higher values were recorded in February at Site 4 and in March at Site 2 compared to all the other sites ($P \leq 0.05$).

Higher GST activities were recorded in the gills (Fig. 6B); although significant differences in different months could be observed at different sites, no clear seasonal trends were observed. Highest average values for all sites were recorded in March (0.167± 0.015 µmoles/min/mg prot), showing a significant increase with respect to February at all sites ($P \leq 0.05$), and lowest values in June (0.100 ± 0.015µmoles/min/mg prot). Site-related differences were also recorded: Site 4

showed significantly higher gill GST activity in October, and Site 3 in May and June with respect to the other sites; significantly lower values were also observed at Site 2 in December, February and April and at Site 4 in April ($P<0.05$).

In the digestive gland, catalase activity showed large oscillations, without clear seasonal trend or site-related differences (Fig. 7A). Lowest average values were recorded in October (≤50 μmoles/min/mg prot) and highest values in March (≥200 μmoles/min/mg prot), followed by a significant decrease in April for all sites ($P\leq0.05$). In contrast, seasonal changes were observed in the gills (Fig. 7B): from a significant decrease in December ($P\leq0.05$), low activities (≤100 μmoles/min/mg prot) were recorded until May, when a large increase, reaching maximal annual values (>200 μmoles/min/mg prot) was observed at all sites ($P\leq0.05$). A significant decrease was subsequently observed in July ($P\leq0.05$). No significant site-related changes in gill catalase activity were recorded at different times of the year.

3.6 Nitric oxide (NO) production in gills

NO production in gills, determined as tissue nitrite accumulation, was evaluated as a possible biomarker of inflammation as recently described (Della Torre et al., 2015). The results (Fig. 8) show large seasonal variations in NO production at Sites 2, 3 and 4; a first peak was observed in November (up to 600 nmoles/mg protein) followed by a progressive and steady decline in winter to lowest average values in February-March (≅150 nmoles/mg protein). A second peak was recorded in April, with a significant increase with respect to March, followed by a rapid decrease in May ($P\leq0.05$). Low and stable levels were detected until September. Interestingly, between November and April, a significant positive correlation between NO production and temperature was observed ($r = 0.9429$; $P<0.05$). Less marked seasonal changes were observed in the gills of mussels collected at Site 1; however, nitrite accumulation was significantly higher with respect to the other sites in March, May and June ($P\leq0.05$).

4. Discussion

The results obtained in this work represent the first data on seasonal variations of different biomarkers in specimens of *M. galloprovincialis* cultured at 4 sites of the gulf of La Spezia, the main mussel farming area in the Ligurian sea, Italy.

The gametogenic cycle was first evaluated in mussels sampled monthly from October 2015 to September 2016. In mussels, gametogenesis is influenced by different environmental factors, and it is likely subject to a year to year variability, mainly dependent on temperature and food availability. In particular, warmer temperatures reduce rates of gametogenesis in *M. galloprovincialis* (Fearman and Moltschaniwskyj, 2010). In the present study, water temperatures at sampling sites were always between 14 and 18 °C from November to May, this allowing for almost continuous maturation/spawning of gametes throughout this period. Differences in the gonad maturation stage at different sites from June to October are probably related to different food availability, since no clear seasonal changes in other physico-chemical parameters were observed. This in particular may apply to mussels from Site 4, located at the inner mouth of the port, where local currents or disturbances caused by ship traffic may affect gametogenesis, this resulting in a more clearly defined gametogenic period, with immature gonads from May to October.

A battery of seven different biomarkers, at cellular, tissue and organism level, was evaluated in mussels collected at different sites. LMS was first determined in hemocytes as the main biomarker of stress at the cellular level, and, in the absence of mortality, SoS as the main biomarker at the whole organism level (Viarengo et al., 2007). Both LMS and SoS have a monotonic decreasing trend with increasing stress conditions, and have been shown to represent core biomarkers of mussel health status (Viarengo et al., 2007; Dagnino et al., 2007). Measurements of LMS in animals from climatically and physically diverse ecosystems indicate that it is potentially a universal indicator of health status (OSPAR, 2013). In mussels, LMS is directly correlated with physiological scope for growth (SFG) and protein turnover. When LMS is evaluated by the NRR assay, animals are considered to be healthy if NRR is ≥120 min; stressed but compensating if <120 but ≥ 50 min,

and severely stressed and probably exhibiting pathology if <50 min (OSPAR, 2013). LMS can be affected by non-contaminant factors such as severe nutritional deprivation and hyperthermia and prolonged hypoxia; however, the main confounding factor is the adverse effect of the final stage of gametogenesis and spawning, which is a naturally stressful process. In the present work, seasonal changes in LMS showed a similar trend at all sites: generally healthy or mild stress conditions were observed for Sites 2-4 throughout the year with a minimum in February, during the main spawning period. In this month, only at Site 1 lowest LMS indicated severe stress conditions. Although some differences in LMS could be detected at different times of the year among sites, these were much smaller than those observed in relation to season; moreover, except for those observed for Site 1, the observed site-related differences were always among high LMS values, indicating generally stable and healthy lysosomes.

The reduction of survival in air, or stress on stress (SoS), is a simple, low–cost, whole-organism response and can show pollutant-induced alterations in an organism's physiology that render the animal more sensitive to further environmental changes (Viarengo et al., 1995; OSPAR, 2013). SoS has been adopted as a general stress biomarker in the UNEP-RAMOGE Mediterranean Biomonitoring Programme (1999). The results obtained in this work show a similar seasonal trend in SoS of mussels sampled from all sites of the Gulf of La Spezia: in samples from all sites, maximal values were recorded at the end of the summer, whereas lower values were observed in winter, with minima in February. These data confirm previous observations made during the development of the original SoS method, where mussels from the same area were utilized (Viarengo et al., 1995, Canesi and Viarengo, unpublished data). No significant site-related differences could be detected. Taken together, the results indicate that in mussels from La Spezia average SoS values are in line with EAC (Environmental Assessment criteria, $LT_{50} \geq 5$) but lower than BAC (Background Assessment Criteria, $LT_{50} \geq 10$) (OSPAR, 2013). Lower values of SoS could be partly ascribed to the extended spawning period observed in mussels from all sites, since spawning mussels with a low Condition Index tend to be weak and will die quickly when measured

for SoS (OSPAR, 2013). Moreover, the tide range in the Gulf of La Spezia is negligible (few cms), and mussels grown on ropes are not naturally subjected to aerial exposure. These data further support the need to continuously update and review boundaries such as BAC and EAC in the light of the physiological fluctuations of each biomarker for each mussel population in different coastal environments.

Overall, the results indicate clear seasonal variations in LMS and SoS, with lowest values detected in winter, during the reproductive period, with minima in February. The gametogenic stage is the main confounding factor affecting determination of these biomarkers: reduction in membrane stability and survival in air has been reported as a result of the reproductive effort associated with spawning (Davies and Vethaak, D., 2012; OSPAR, 2013). Seasonal changes in LMS positively correlated with SoS data, and both parameters showed positive correlations with water temperature. Overall, the results obtained for LMS and SoS data indicate similar health conditions in mussels cultivated at different sites.

Phagocytosis is considered as a proxy of immunocompetence of the whole organism, since it represents the main parameter of cell-mediated immunity in invertebrates (Ellis et al., 2011). Although this parameter is not routinely determined in biomonitoring programs, we also evaluated phagocytic activity of mussel hemocytes. High phagocytic activities were recorded in the hemocytes sampled from different sites throughout the year. Average annual values were higher (about 80%) than those previously reported in mussels sampled in the North Adriatic sea (about 60%) (Ciacci et al., 2009). Phagocytosis did not show seasonal changes in mussels sampled at different sites, indicating that despite the stressful conditions associated with gametogenesis observed in winter months, no decrease in mussel immunocompetence was observed. As for LMS, although some site-related differences were detected, phagocytic activity was always high, indicating no impairment of the overall immune defence. The results underline the importance of immune-related biomarkers in the evaluation of the overall health status of mussels.

Biomarkers related to heavy metal, antioxidant and biotransformation response (MTs, catalase, GST) are widely utilized in mussel biomonitoring. Seasonal changes in MT content and antioxidant enzyme activities have been documented in mussel tissues from different areas (Viarengo et al., 1991; Bocchetti et al., 2008; Schmidt et al., 2013; Jarque et al., 2014). The results of the present work indicate that MT content and catalase activity showed seasonal oscillations in the digestive gland, and a clearer seasonal trend in the gills. Seasonal oscillation were also observed in digestive gland GST activity; in contrast, high level were detected in the gills throughout the year, in line with the first role of this tissue in biotransformation of xenobiotics. Gill MT content negatively correlated with catalase activity ($r = -0.4273$) at all sites, but the relationship was not significant ($P<0.18$). No other significant correlation was observed among these three biomarkers. Although both MT and antioxidant enzymes participate in antioxidant processes, the observed seasonal changes may reflect natural fluctuations in tissue content of essential metals (Zn, Cu or Fe), as well as in oxyradical production related to metabolic activities (Viarengo et al., 1997; Vlahogianni et al., 2007; Benali et al., 2015). Moreover, since all these protein-related biomarkers show a bell-shaped curve with increasing stress conditions, their response should be interpreted in relation to changes in other biomarkers with a clear monotonic trend (increasing or decreasing) (Dagnino et al., 2007). However, in the present study no significant correlation was observed between tissue MT content and antioxidant enzyme activities and changes in LMS or SoS in mussel sampled at different sites; the results indicate little contribution of these protein related biomarkers in describing the overall health status of mussels in the absence of contamination. Although also for MT and antioxidant enzymes some site-related differences could be observed at certain months of the year, their extent and occurrence do not seem to reflect significant changes in the physiological status of the mussels at different sites.

Tissue inflammation represents a consequence of the response of the host to pathogen exposure, but also to the presence of contaminants. In bivalves, tissue hemocytic infiltration is generally considered to be indicative of inflammatory stress conditions (OSPAR, 2013). In this work, nitric

oxide (NO) production was evaluated in the gills as a possible biomarker of inflammation. In molluscs, NO, an ubiquitous intracellular signaling molecule, is mainly involved in immune response, neuromodulation and metamorphosis (Torreilles, 2001; Mantione et al., 2006). Increased NO accumulation has been previously observed in the gills of mussels exposed to heavy metals (cadmium) and TiO_2 nanoparticles (Della Torre et al., 2015). Increased NO production in the tissues can be partly ascribed to infiltrating hemocytes. However, in mussel gills, physiological NO production is also due to dopaminergic neurons that control activity of lateral cilia, generating the water currents that regulate gas exchange, food intake and waste removal (Cadet et al., 2004; Carrol and Catapane, 2007). This activity may vary in response to both environmental factors (such temperature and food availability) and endogenous neuromodulators related to reproduction. The results here obtained show large seasonal changes in NO production at Sites 2, 3 and 4 between November and April, when mussels were at the final stages of gametogenesis and spawning; interestingly, in this period, a significant positive correlation between NO production and temperature was observed. Much smaller seasonal changes were observed in the gills of mussels sampled at Site 1, that showed a more extended reproductive period. Analysis of tissue-specific mussel transcriptome recently revealed expression of genes related to nitric oxide metabolism in the gills (Moreira et al., 2015). The results reinforce the hypothesis that determination of NO production in the gills may represent a significant biomarker of the physiological status of mussels.

Overall, the results represent the first data on seasonal changes in different biomarkers in mussels cultivated in at different sites of the gulf of La Spezia, the main site of intensive mussel farming in the north western coast of Italy. Although no major differences were observed among different sites, the results confirm how seasonal variations in the reproductive status mainly related to changes in temperature are a key event in mussel physiology during their annual cycle (Lemaire et al., 2006; Jarque et al., 2014). These data clearly identify the higher susceptibility of mussels to additional stressful conditions at certain times of the year, and support the utilization of the biomarker approach as a tool to monitor the health of mussels in aquaculture. This information is

important for mussel farmers to follow-up, and most of all, for the correct interpretation of responses under stress conditions, as those that may occur during dredging operations.

The results further highlight the importance of assessing baseline levels of different biomarkers in mussel populations in different areas of the Mediterranean prior to their use in biomonitoring programms. Finally, within the application of the multi-biomarker approach, the results obtained in this work confirm the importance of LMS and SoS as core descriptors of the mussel health status (Viarengo et al., 2007). As demonstrated by the ICON experience (Martínez-Gómez et al., 2016), determination of these two general and non-expensive stress biomarkers in mussels can provide a cost-effective and harmonised approach within the framework of wide-scale biomonitoring programmes, such as that proposed by the European Union, i.e. the Marine Strategy Framework Directive.

Author contributions.

TB, RF, MM, GC performed all experiments and data analyses and drafted parts of the manuscript. LC conceived the study. LC and TB wrote the ms. All authors read and approved the manuscript.

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Figure legends

Fig. 1 - Location of the four sampling sites in the Gulf of La Spezia, Italy. Portovenere (Site 1), Diga Levante Interno (Site 2), Diga Ponente Esterno (Site 3) and Diga Ponente Interno (Site 4).

Fig. 2 - Seasonal changes in water temperature in the Gulf of La Spezia and mussel reproductive stage from October 2015 to September 2016.

A) Mean water temperatures recorded at 4 different Sites in 2015/2016 (solid line) and average water temperature recorded in the Gulf of La Spezia in 2006/2007 (dotted line).

B) Temporal changes of gonad development across an annual cycle in mussel *Mytilus galloprovincialis* (Lam.). Gonads were classified as 0-II = early stage, III = developing/ripe, III-IV = ripe/spawning, IV = spawning.

Fig. 3 - Seasonal changes in LMS and SoS in mussels sampled in the Gulf of La Spezia.

A) LMS was determined in hemocytes and data are expressed as NRRT (min).

B) SoS (Stress on Stress response or anoxic survival time) expressed by the Lethal Threshold for 50 % mortality (LT_{50}).

Data, represent the mean ± SD of 4 samples. For each site, significant differences observed at each month with respect to the previous month are indicated * = ≤ 0.05.

Fig. 4 - Seasonal changes in phagocytic activity of hemocytes from mussels sampled at different sites of the Gulf of La Spezia. Data, representing the mean ± SD of 4 samples, are expressed as % of phagocytizing cells.

Fig. 5 - Seasonal changes in metallothionein (MT) content in the tissues of mussels sampled at different sites of the Gulf of La Spezia. Data, representing the mean ± SD of 4 samples, are expressed as μg prot/g w.w. sample.

A) digestive gland; B) gills

For each site, significant differences observed at each month with respect to the previous month are indicated * = ≤ 0.05.

Fig. 6 - Seasonal changes in Glutahione Transferase (GST) activity in the tissues of mussels sampled at different sites of the Gulf of La Spezia. Data, representing the mean ± SD of 4 experiments in triplicate, are expressed as μmol/min/mg prot.

A) digestive gland; B) gills.

For each site, significant differences observed at each month with respect to the previous month are indicated * = ≤ 0.05.

Fig. 7 - Seasonal changes in Catalase activity in the tissues of mussels sampled at different sites of the Gulf of La Spezia. Data, representing the mean ± SD of at least 4 experiments in triplicate, are expressed as μmol/min/mg prot.

A) digestive gland; B) gills.

For each site, significant differences observed at each month with respect to the previous month are indicated * = ≤ 0.05.

Fig. 8 - Seasonal changes in NO production in the gills of mussels sampled at different sites of the Gulf of La Spezia. Data, representing the mean ± SD of at least 4 samples, are expressed as nitrite accumulation (nmol/mg prot). For each site, significant differences observed at each month with respect to the previous month are indicated * = ≤ 0.05.

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Table 1

Spearman rank correlation analysis between biomarkers in mussels and temperature (T) at different sites. LMS = Lysosomal membrane stability; SoS = Stress on Stress response. * = P≤ 0.05

Site 1	**LMS**	**SOS**	**T**
LMS			0.0039*
SOS	0.0018*		
T		0.0306*	
Site 2	**LMS**	**SOS**	**T**
LMS			0.0306*
SOS	0.0162*		
T		0.0480	
Site 3	**LMS**	**SOS**	**T**
LMS			0.0060*
SOS	0.0182*		
T		0.0278*	
Site 4	**LMS**	**SOS**	**T**
LMS			0.0007*
SOS	0.0708		
T		0.0162*	

Figure 1

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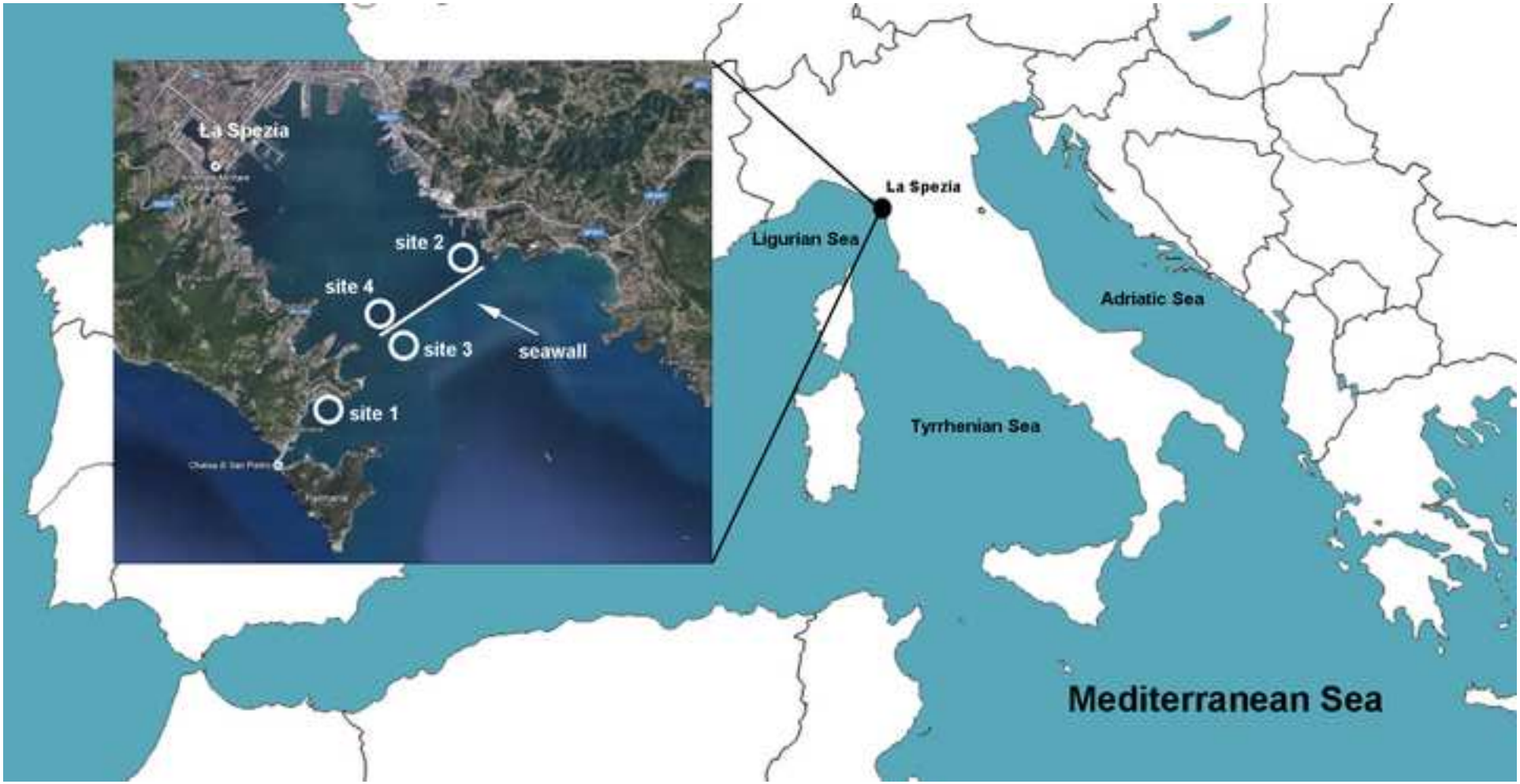

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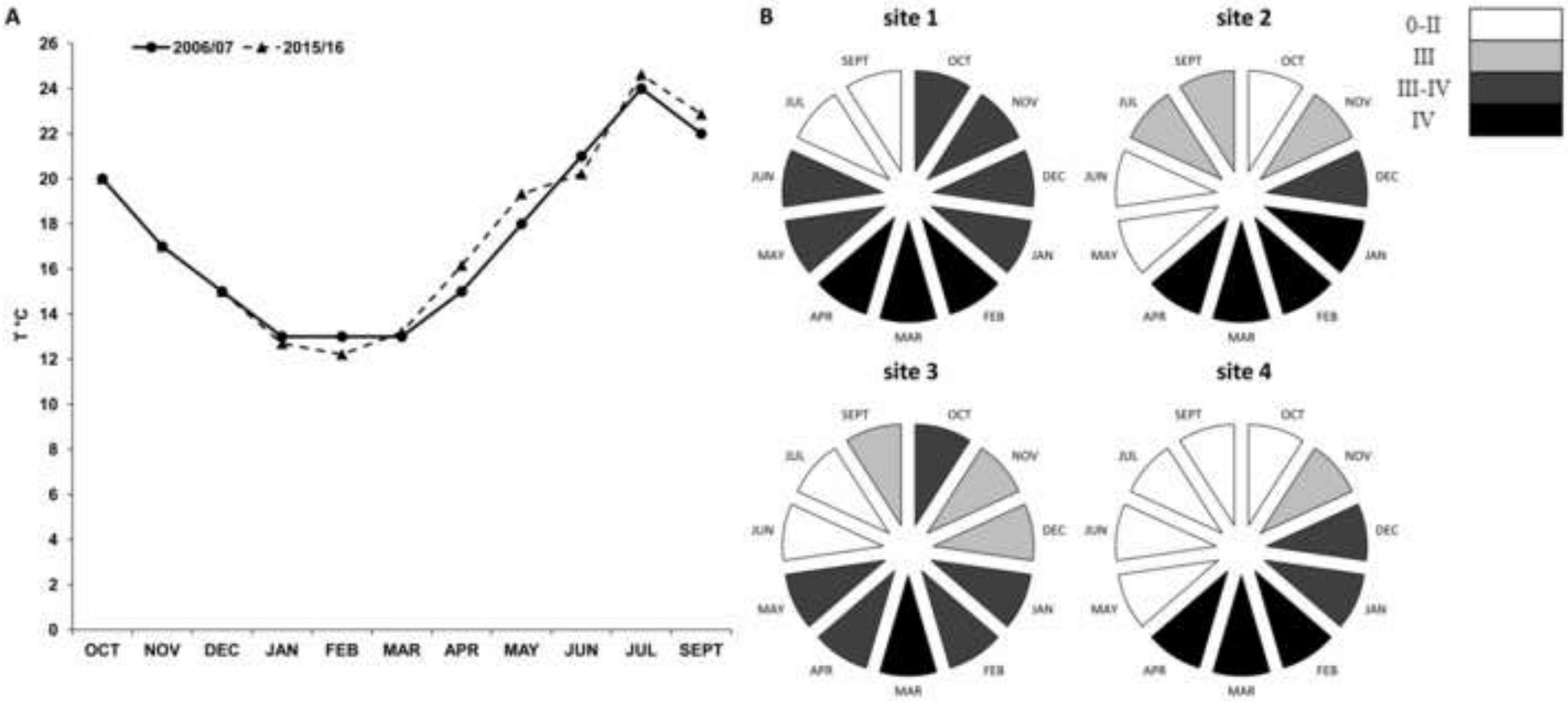

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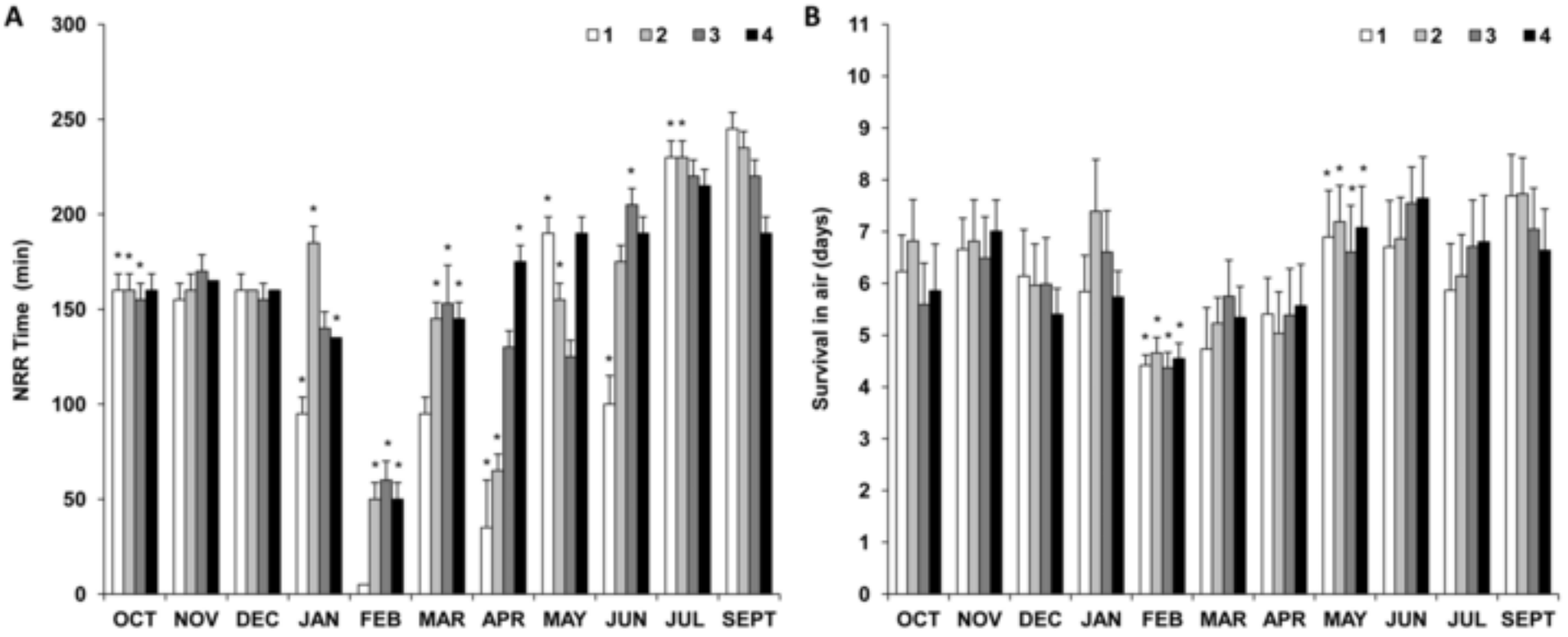

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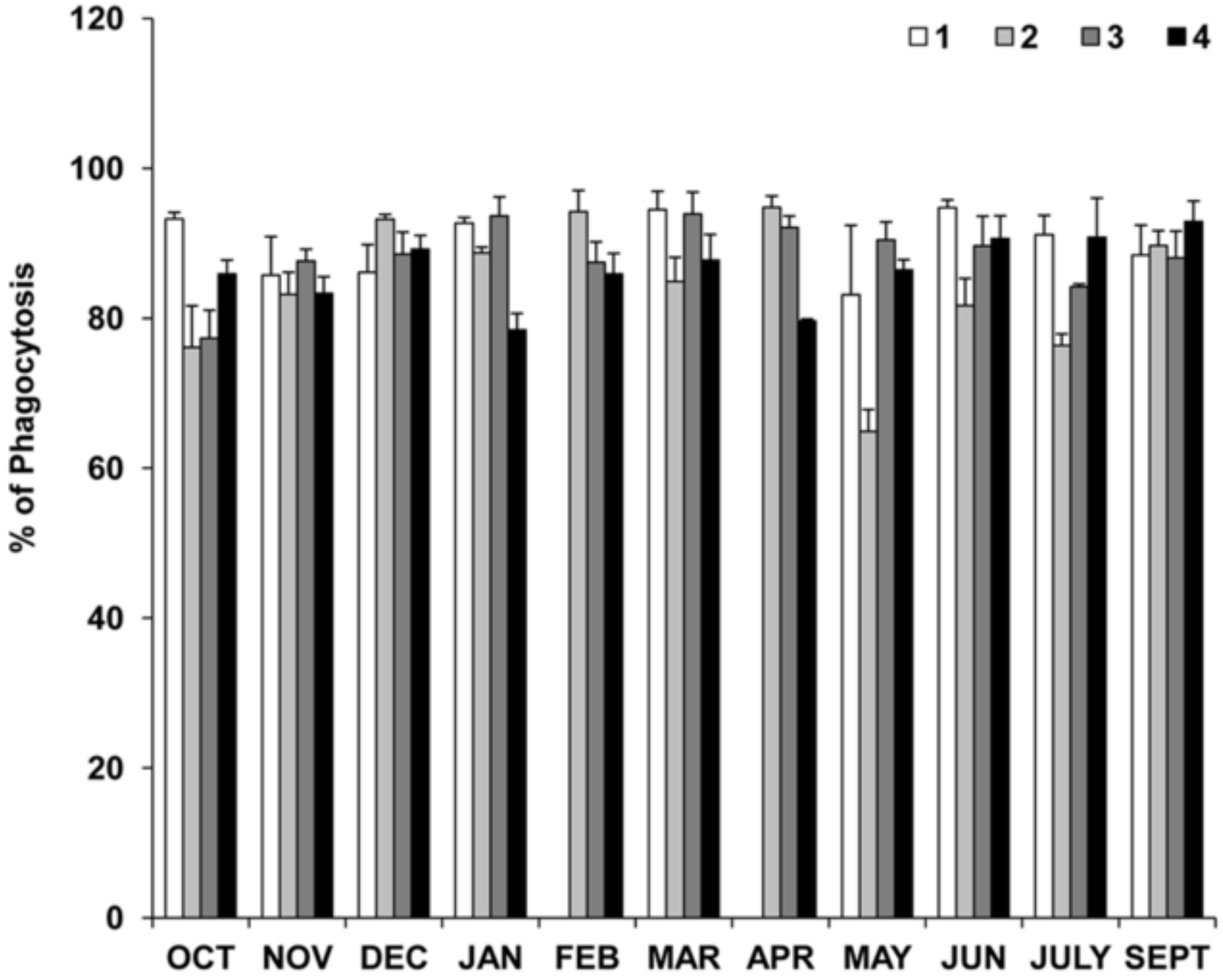

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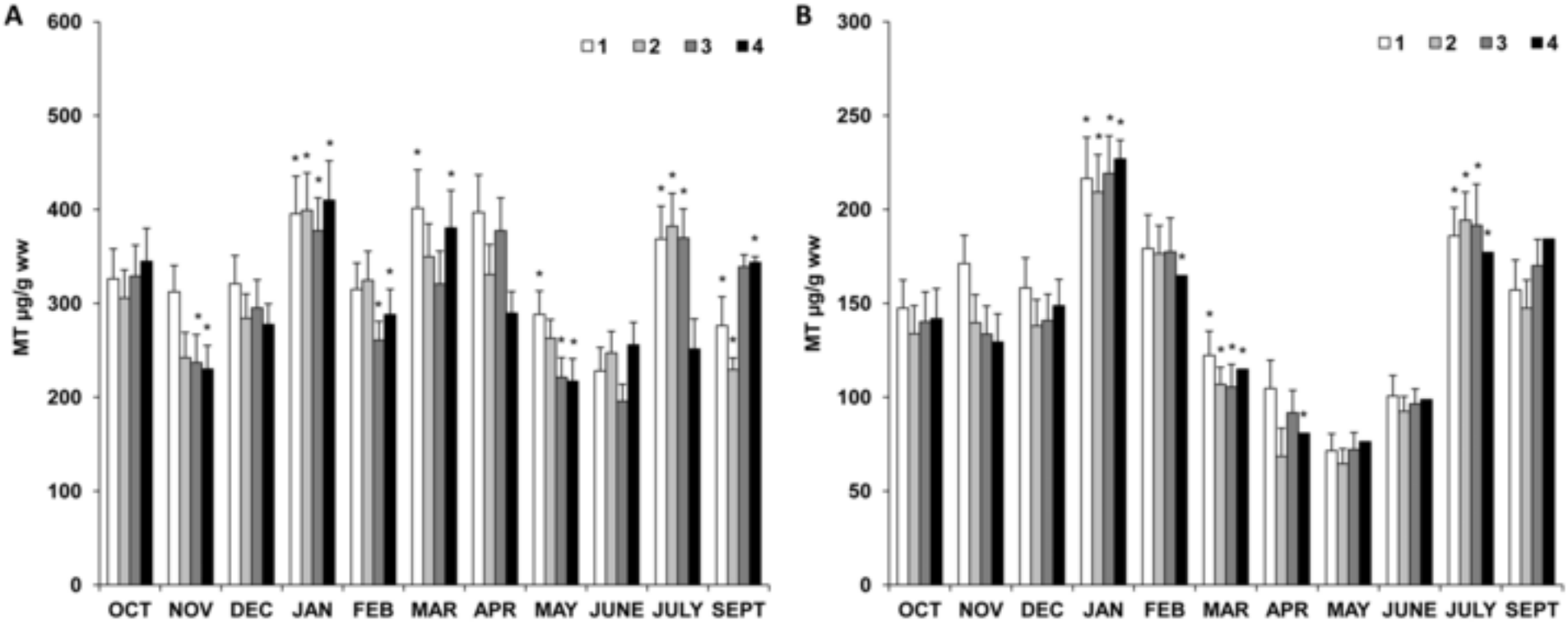

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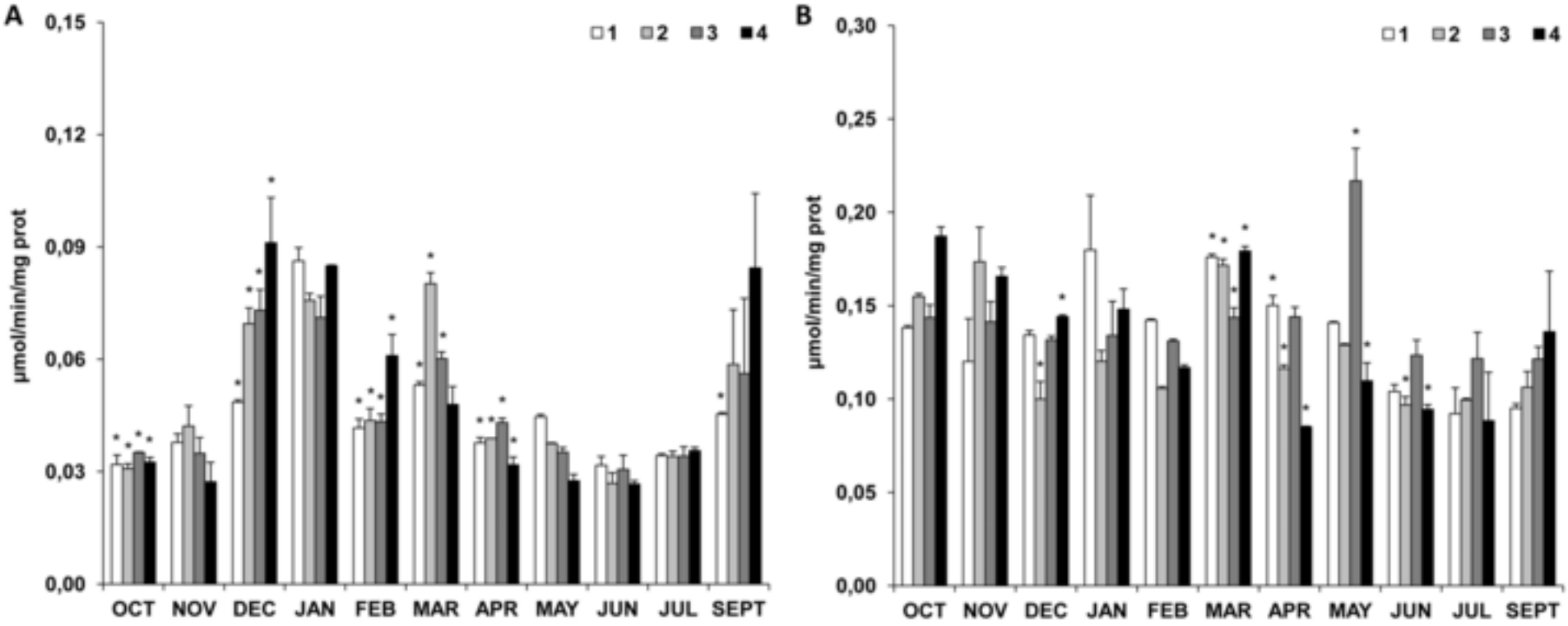

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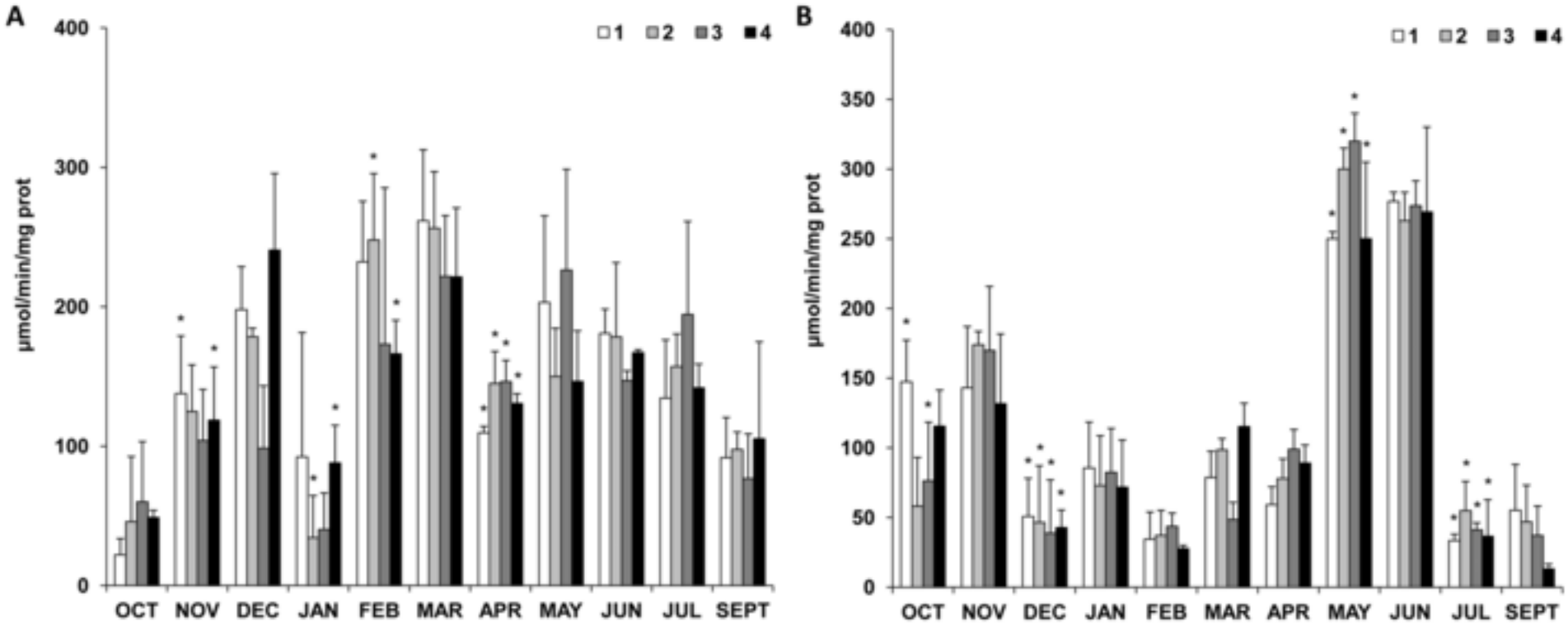

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