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Variability of metabolic, protective, antioxidant, and lysosomal gene transcriptional profiles and microbiota composition of *Mytilus galloprovincialis* farmed in the North Adriatic Sea (Italy)

Rajapaksha Haddokara Gedara Rasika Wathsala[a], Margherita Musella[b,c], Paola Valbonesi[a], Marco Candela[b,c,*], Silvia Franzellitti[a,c,*]

[a]Animal and Environmental Physiology Laboratory, Department of Biological, Geological and Environmental Sciences (BiGeA), University of Bologna, Ravenna, Italy

[b]Unit of Microbiome Science and Biotechnology, Department of Pharmacy and Biotechnology, University of Bologna, Bologna, Italy.

[c]Fano Marine Center, The Inter-Institute Center for Research on Marine Biodiversity, Resources and Biotechnologies, Fano, Italy

*corresponding authors: via S. Alberto 163, 48123 Ravenna (Italy), phone: +39 0544937311, e-mail: silvia.franzellitti@unibo.it (S. Franzellitti); Via Belmeloro 6, 40126 Bologna (Italy), phone: +39 0512099727, e-mail: marco.candela@unibo.it (M. Candela).

Abstract

This study evaluates the transcriptional profiles of genes related to physiological responses in digestive glands (DG) of *Mytilus galloprovincialis* under the influence of seasonal changes of environmental variables, gender bias, and gonadal development. Composition of the DG microbiome was also explored. Mussels were collected across 7 months encompassing 3 seasons from a farm in the Northwestern Adriatic Sea. All gene products showed complex transcriptional patterns across seasons. Salinity, surface oxygen and transparency significantly correlate with transcriptional profiles of males, whereas in females temperature and gonadal maturation mostly explained the observed transcriptional changes. Seasonal variations and gender-specific differences were observed in DG microbiome composition, with variations resembling metabolic accommodations likely facing season progression and reproductive cycle. Results provide baseline information to improve actual monitoring strategies of mussel farming conditions and forecast potential detrimental impacts of climatological/environmental changes in the study area.

Keywords: Mediterranean mussel; gene transcription; microbiome; season; gender; gonadal cycle

Highlights

- Natural seasonality, gender bias, and gonadal cycle effects on mussel digestive gland (DG) gene transcriptions were evaluated
- Composition of the DG microbiome was assessed.
- temperature and gonadal maturation mostly explained the transcriptional changes of females
- salinity, oxygen, transparency affected transcriptional profiles of males.
- Microbiome composition resembled metabolic accommodations to face season progression and reproductive cycle

1. INTRODUCTION

The marine ecosystem is interconnected with the terrestrial ecosystem, hence changing any component in each system automatically affects another's functioning. In particular, coastal habitats are severely impacted by the contaminants receiving from the terrestrial surroundings that affect organism health, biodiversity, and consequently, ecosystem functioning (Islam and Tanaka, 2004; Lacroix et al., 2017). Besides chemical pollution, changes may also occur due to climate changes such as seawater warming, salinity variations, and ocean acidification (Landrigan et al., 2020). In this context, investigations of the regulatory mechanisms governing stress responses of marine organisms may elucidate the critical pathways setting the limits of animal acclimatization to anthropogenically modified marine environments.

Marine mussels (*Mytilus* spp.) dominate sessile fauna of many coastal areas and estuaries. These environments are characterized by wide fluctuations of abiotic and biotic parameters, which make mussels ideal model organisms for studying physiological alterations driven by environmental changes (Figueras et al., 2019; Franzellitti et al., 2020).

Environmental studies with mussels highlighted seasonal fluctuation of microbial indices, contaminant bioaccumulation, cellular biomarkers, and key physiological functions (Azizi et al., 2018; Caricato et al., 2010; Ivanković et al., 2005; Roméo et al., 2003; Sheehan and Power, 1999; Shen et al., 2020; Vernocchi et al., 2007), and suggest the influence of abiotic factors (temperature, pH, salinity, food availability), and endogenous factors (i.e., gender bias and reproductive stage) for those biological responses (Blanco-Rayón et al., 2020; Grbin et al., 2019; Grenier et al., 2020).

Molecular biomarkers based on expression analysis of stress responsive genes are pointing out crucial insights into molecular mechanisms regulating animal ability to survive and thrive in dynamic and changing marine environments (Evans and Hofmann, 2012; Gracey, 2007). Indeed, in environmentally relevant species as marine mussels, the modulation of mRNA levels is the earliest signal of an ongoing physiological alteration that can potentially forecast changes at higher levels of the biological organization (Gracey, 2007). There are several studies employing mussels as model organisms in field experiments to infer transcriptomic changes with environmental quality (Blalock et al., 2020; Franzellitti et al., 2010; Kerambrun et al., 2016; Rossi et al., 2016; Sforzini et al., 2018; Venier et al., 2006). Conversely, few studies emphasized that mussel gene transcription may be modulated by natural environmental parameters or by endogenous factors (Banni et al., 2011; Counihan et al., 2019; Schmidt et al., 2013b).

This study evaluates the transcriptional profiles of genes related to metabolic, detoxification, antioxidant, and lysosomal responses in Mediterranean mussels (*Mytilus galloprovincialis*) under the influence of natural seasonal variations of environmental variables, gender bias, and gonadal cycle. We purposely addressed those environmental parameters and/or endogenous factors that may modulate some functional categories of stress-responsive gene transcripts, likely affecting the capability of the animals to cope with further environmental changes or the occurrence of natural and anthropogenic toxins. Furthermore, a recent literature review (Lindsay et al., 2020) shows that the composition of gut microbial community of a species can vary seasonally with host diet, metabolic demands, and life stage. These changes in microbial community composition seem to comprehensively contribute to the host flexibility to cope with environmental

changes, enabling the host to live within different environments, adapt to seasonal changes and maintain its physiological performance. Therefore, considering this vital role of microbial communities in the maintenance of the host health (Rausch et al., 2019; Simon et al., 2019) also in response to environmental conditions (Vanwonterghem and Webster, 2020), this study explores basal responses of the mussel digestive gland (DG) microbiome to seasonal changes, allowing to figure out microbiome variations occurring concomitantly with host physiological changes across seasonality.

2. METHODS

2.1. Mussel sampling

Seven sampling campaigns were performed from a mussel farm located in the Northwestern Adriatic Sea by professional fishermen of the “Cooperativa Pro.mo.ittica” (Cesenatico, Italy) (Fig 1A). This area is characterized by a combination of shallow waters and high riverine inputs (dominated by the Po river outflow) (Marini et al., 2008), that makes its coastal environments as one of the most eutrophic and most productive in the Mediterranean, promoting an intense mussel farming activity (Brigolin et al., 2017). The study area is generally characterized by sudden and anomalous rise/drop of temperature, salinities, or eutrophic level, mainly related to climatological events and riverine inputs from the Italian border (https://www.arpae.it). Furthermore, the area is characterized by the periodical rise of algal blooms and the occurrence of algal toxins which are accumulated by mussels (Buratti et al., 2013). These phenomena may elicit transitory stress conditions in mussels.

The selected sampling site is routinely monitored by the Regional Agency for Prevention, Environment and Energy of Emilia-Romagna, Italy (ARPA-ER) to evaluate seawater parameters, algal biomass and the occurrence of algal toxins (https://www.arpae.it). During the sampling period, no relevant events of algal blooms were recorded, although in June a peak of chlorophyll-a > 10 µg/L indicates the onset of transitory eutrophic conditions (Fig 1B). No hypoxic conditions (dissolved oxygen < 3 mg/L) were recorded; however, a relevant reduction was recorded in July and August. Sea surface temperatures followed the monthly profile and overall range of variability typical of a shallow-water ecosystem as the study area, with winter temperatures < 10°C, summer values > 27°C (Fig 1B). In March and in May, two events of relatively low salinity were recorded (Fig 1B). In March, climatological records reported in the ARPA-ER database indicates the occurrence of heavy rains and snow melting as well as high riverine inputs. The low salinity was paralleled by a reduction of transparency, likely related to the input of sediments and debris along with freshwater inflow, while the low chlorophyll-a values indicate a low algal biomass (Fig 1B). In May, high riverine inputs were recorded. These inputs determined an increase of eutrophic level with rise of algal biomass (mainly diatoms of the genus *Chaetoceros* sp.).

Mussel samples were collected once a month, from February to August 2018. At each sampling time point, 60 randomly selected mussels were collected directly in the field, immediately stored in coolers (+4°C) and transferred to the laboratory, where they were cleaned and washed and immediately processed for tissue (mantle/gonad complexes and digestive glands) dissection under sterile conditions. Tissues were snap-frozen in liquid nitrogen and then stored at -80°C.

Sex was determined in individual mussels using the sex-specific gene method (Fraser et al., 2016). Specifically, the method consists in the quantification through real-time PCR (qPCR) of expression of the mussel vitelline envelope receptor for lysine (*VERL*), and vitelline coat lysine (*VCL*) mRNAs in the mantle/gonad complex. The transcripts are specifically expressed in females and males, respectively, and serve as a proxy of gonadal cycle (Hines et al., 2007). This method proved suitable differentiating males from females both during gametogenesis and sexual resting stage, when histology does not allow the observation of gametes (Anantharaman and Craft, 2012; Fraser et al., 2016). RNA extraction and cDNA preparation from mussel mantle/gonad complexes was as reported below. qPCR reactions were performed in duplicate for each sample using primer pairs and protocols reported previously (Anantharaman and Craft, 2012) (Table S2). Threshold cycle (C_T) values were determined by setting a constant baseline. Sex was determined calculating the intra animal ΔC_T as C_T(VCL) – C_T(VERL) (Anantharaman and Craft, 2012). Negative values indicate males and positive values indicate females. Relative *VCL* or *VERL* expression values across season (Fig 1C) were inferred by a comparative C_T method (Schmittgen and Livak, 2008) using the normalization and statistical strategy reported below. As reported previously (Anantharaman and Craft, 2012), both VERL and VCL expression levels significantly decreased from winter to summer (Fig 1C). Based on visual microscopic inspection of gonads (Hines et al., 2007), transcript levels of both sex specific genes was found to be associated to the presence and abundance of gametes.

Mussel biometric parameters are reported in Table S1. A production metric, the condition factor, was calculated, with values being unchanged across seasons and similar between

females and males (Fig 1D; Table S1). The lysosomal membrane stability (LMS) was assessed in mussel living haemocytes through the neutral red retention assay according to (Buratti et al., 2013). LMS is a well-consolidated general stress biomarker and a prognostic indicator for putative pathologies. As such, it addressed to as an integrated pathophysiological indicator of general health status (Martínez-Gómez et al., 2015). According to Martínez-Gómez et al. (2015), neutral red retention time (NRRT) values recorded in this study fall within the range representing stressed but compensating organisms (Fig 1E; Table S1). Furthermore, while males show almost constant NRRT values across season, females show a significant reduction of NRRT values from winter to summer, which indicate increased stress levels.

2.2. RNA extraction, cDNA preparation, and qPCR analyses

For each animal, 200 mg of mantle/gonad complex (sex identification and gonadal cycle) or of digestive glands were independently homogenized in a suitable volume of the TRI Reagent (Sigma Aldrich, Milan, Italy) and total RNA was extracted using the DirectZol kit (Zymo Research, Freiburg, Germany) following the manufacturer's instructions. RNA concentration and quality were confirmed using the Qubit system with the Qubit RNA assay kit (Thermo Scientific, Milan, Italy) and electrophoresis using a 1.2% agarose gel under denaturing conditions. The analysis of UV absorbance spectra of the samples (λ = 200 – 340 nm) allowed the calculation of Absorbance (A) ratio A260/A280 addressing the occurrence of protein contaminations (cut-off values > 1.8 and < 2.0), and the ratio A260/A230 addressing the occurrence of contaminants that may be present in the samples, such as guanidine thiocyanate, which is a component of the TRI Reagent (cut-

off value > 1.7). First strand cDNA for each sample was synthesized from 1 µg total RNA using the iScript supermix (BioRad Laboratories, Milan, Italy) following the manufacturer's instructions.

Expression profiles of selected transcripts in digestive glands were assessed by qPCR using primer pairs listed in Table S2 and protocols reported in previous studies (see references in Table S2). 18S and 28S were selected as reference gene products for qPCR data normalization by a preliminary stability analysis of 6 established candidate transcripts (Balbi et al., 2016). Relative expression values of target mRNAs were inferred by a comparative C_T method (Schmittgen and Livak, 2008) using the StepOne and DataAssist softwares (Thermo Fisher, Milan, Italy). Data were reported as relative expression (fold change) with respect to a reference sample (Winter male).

2.5. Microbial DNA extraction and sequencing

Total microbial DNA was extracted from approximately 20 – 30 mg of digestive gland tissue using the DNeasy PowerSoil kit (Qiagen, Hilden, Germany) according to (Musella et al., 2020). The V3–V4 hypervariable region of the 16S rRNA gene was amplified using the 341F and 785R primers with added Illumina adapter overhang sequences, as previously described (Barone et al., 2019). The thermal cycle consisted of initial denaturation at 95°C for 3 minutes, 30 cycles at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds and 5 minutes at 72°C for final extension. PCR reactions were then cleaned up with Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA). Indexed libraries were prepared by limited-cycle PCR, using the Nextera technology and then pooled after a further clean up step as

described above and normalized to 4 nM. The sample pool was denatured with 0.2 N NaOH and diluted to a final concentration of 6 pM with a 20% PhiX control. Sequencing was performed on Illumina MiSeq platform using a 2 × 250 bp paired end protocol, according to the manufacturer's instructions (Illumina, San Diego, CA). Sequencing reads were deposited in SRA-NCBI (*SRA Accession Numbers will be available upon manuscript acceptance*).

2.6. Statistical and bioinformatic analyses

qPCR data were analyzed using the REST software (Pfaffl et al., 2002) to test for statistical differences in mRNA levels of the treatment groups vs the reference condition. Further pairwise comparisons were performed with the Mann-Whitney U test (GraphPad Prism v9). Data visualization, and graphics were obtained with the ggplot2 R package in R (R Development Core Team, 2018). In any case, statistical differences were accepted when $P < 0.05$.

The complete dataset was further analyzed by a 2-way permutation multivariate analysis of variance (PERMANOVA) using PRIMER v6 (Anderson et al., 2008) to test for variations of transcriptional profiles amongst sex and season groups. Log-transformed variations of the target transcripts were used to calculate similarity matrices based on the Euclidean distance (999 permutations). When the main tests revealed statistical differences ($P < 0.05$), PERMANOVA pairwise comparisons were carried out. Distance-based redundancy linear modeling (DISTLM) with a test of marginality in PRIMER was also performed to account for the contribution of environmental parameters and gonad cycle in explaining the total observed variance in the transcriptional profiles. DISTLM used

the BEST selection procedure and adjusted R^2 selection criteria. BEST/BioEnV analysis in PRIMER 6 was also carried out using a Spearman rank correlation to identify the best correlated environmental variables that explained the observed patterns of gene transcriptions (999 permutations).

For DG microbiome analyses, raw sequences were processed using a pipeline combining PANDAseq (Masella et al., 2012) and QIIME 2 (https://qiime2.org) (Bolyen et al., 2019). High-quality reads were clustered into amplicon sequence variants (ASVs) using DADA2 (Callahan et al., 2016). A normalized ASV table have been used, so that for all samples the same number of reads have been considered. Taxonomy was assigned using the SILVA database as a reference (Quast et al., 2013). Unassigned sequences and those assigned to eukaryotes (i.e. chloroplasts and mitochondrial ones) were discarded. Beta diversity was estimated by computing unweighted UniFrac distance. All statistical analyses was performed using R software version (R Development Core Team, 2018). ASVs were filtered for prevalence, retaining only ASV showing a relative abundance >1% in at least 10% of samples. UniFrac distances were plotted using the vegan package, and permutation test pseudo-F ratios (function adonis in the vegan package) was computed to test the significance of data separation in the principal coordinate's analysis (PCoA).

Kendall correlation test and a DISTLM analysis with a test of marginality was used to determine associations between the PCoA coordinates (Kendall correlation) or relative abundances of microbic phyla (DISTLM) and expression profiles of selected transcripts. False discovery rate (FDR) (function p.adjust in the stats package) was used to adjust p-

values, and a p-value ≤ 0.05 was considered as statistically significant. DISTLM used the BEST selection procedure and adjusted R^2 selection criteria.

3. RESULTS

3.1. Mussel transcriptional profiles in digestive glands

Variations of gene transcriptional profiles between sexes or across season are reported in Fig 2. Results from PERMANOVA analyses demonstrated that the single factors “Season” and “Sex” had a significant effect on the whole dataset ($P < 0.05$; Table 2). Furthermore, PERMANOVA analysis showed a significant interaction ($P < 0.05$; Table 2) between the factors. The BEST/BioEnV analysis showed the environmental variables that best correlated with the overall transcriptional dataset (Table S3).

Significantly different expression levels between males and females are observed for *mt20*, *abcb* and *hex* ($P < 0.05$). All gene products showed complex transcriptional patterns across season in both males and females (Fig 2), with a tendency to increased (*amil, lys, mt20, abcb, cat, sod, hex, ctsl, gusb*) or decreased (*pk, idp*) expression from winter to summer. DISTLM analyses performed on separate female and male datasets by considering environmental parameters and gonadal maturation level (assessed through *VCL/VERL* expression profiling) showed that in females temperature, salinity, chlorophyll-a, and gonadal maturation explained most of the variation of the observed transcriptional profiles (Fig 3). Among these explaining variables, the BEST/BioEnV analysis showed that temperature and gonad maturation significantly correlated with transcriptional profiles of females, while salinity, surface oxygen and transparency significantly correlated with transcriptional profiles of males (Fig 3; Table S3).

3.2. Microbiome analysis

The compositional structure of the DG microbiome from 41 mussels collected across the sampling period was obtained by NGS sequencing of the V3–V4 hypervariable region of the 16S rRNA gene. A total number of 623000 high quality reads were obtained (mean per sample ± SD, 15195 ± 11581) and clustered in 614 ASVs at 97% identity.

To explore overall differences in the DG microbiome composition between samples, an unweighted Unifrac-based PCoA of the correspondent compositional profiles was carried out. According to our findings, mussel samples clustered in 3 groups which correspond to the collection seasons (permutation test with pseudo-F ratios, P-value ≤ 0.02) (Fig 4A). From the compositional point of view, Firmicutes characterized winter samples, while Tenericutes were most abundant in the summer. Conversely, Proteobacteria appeared to be constant throughout the year (Fig 4B). Besides seasonal variation, Fig 5A shows a tendency of microbiome composition segregation according to mussel sex, though not statistically significant (permutation test with pseudo-F ratios, P-value = 0.12). Particularly, as shown in Fig 5B, males are most abundant in Cyanobacteria (6% ± 11.4% in male, 2.1% ± 7.6% in female), Planctomycetes (5.3% ± 7.7% in male 0.6% ± 1.5% in female) and Chlamydiae (2% ± 4.7% in male, 0.6% ± 1.5% in female), while females show an increase in Firmicutes (16.1% ± 22.5% in male, 19.7% ± 26.9% in female), Bacteroidetes (2.7% ± 3.4% in male, 8.9% ± 16.2% in female) and Actinobacteria (4.7% ± 6.6% in male, 5.9% ± 12.7% in female). To detect possible associations between mussel transcriptional profiles and the observed seasonal pattern DG microbiome segregation, we performed an indirect gradient analysis using Kendall correlation test. No

significant correlation (P > 0.05) was observed between the samples PCoA coordinates and the correspondent expression profiles of the genes analyzed in Fig 2. Nevertheless, the DSTLM analysis (Fig 6; Table S4) shows that sample grouping based on transcriptional changes correlate with vectors describing trends of relative abundance of some microbial phyla disclosed in the DG microbiome. In particular, Chlamydiae and Planctomycetes appear correlated with transcriptional changes between males and females, while Actinobacteria, Bacteroidetes, Firmicutes, and Tenericutes seem correlated with transcriptional changes between winter to sprimg/summer samples (Fg 6, Table S4).

4. DISCUSSION

Data reported in this study show the influence of both seasonality and gender bias on transcriptional profiles and microbiota composition of *M. galloprovincialis* from the Northwestern Adriatic Sea.

Season related fluctuations of molecular and biochemical biomarkers in mussels can be expected, as reported by a relevant amount of scientific evidence on this topic (Balbi et al., 2017; Benito et al., 2019; Leiniö and Lehtonen, 2005), and suggested to mainly depend on seawater temperature and salinity variations, which are considered amongst the main drivers of physiological regulation for mussels and other intertidal marine invertebrates (Lockwood et al., 2015). Indeed, the BEST/BioEnV analysis performed on the whole transcriptional dataset showed that temperature and salinity are the best correlated environmental variables with the observed biological outcomes, together with pH and chlorophyll-a variations. This finding suggests a more complex

interaction with the environmental conditions provided by the sampling area in the Northwestern Adriatic Sea, which are characterized by a large river runoff from the Italian border and by highly variable meteorological conditions (Alvisi and Cozzi, 2016). DG microbiome composition also followed a seasonal pattern, with Firmicutes and Tenericutes characterizing winter and summer samples, respectively.

The overall seasonal pattern of gene transcription shows a general increasing expression from winter to summer, except for transcripts encoding metabolic enzymes, that show both increasing (*amyl*) and decreasing (*pk*, *idp*) expressions across season. Amylase is a key enzyme in carbohydrate metabolism; pyruvate kinases and isocitrate dehydrogenases are engaged in channeling glycolytic substrates towards aerobic metabolic pathways (Canesi et al., 1999; Liu et al., 2017). On the whole, the relative expression patterns of these gene products suggest a lower aerobic capacity of the mussels in summer, or, alternatively, an enhanced occurrence of substrates for anaerobic metabolism. Interestingly, the DISTLM analysis show the (significant) correlation between gender and season sample groupings based on the overall mussel gene transcriptional profiles and vectors describing trends of relative abundance of some microbial phyla disclosed in the DG microbiome that may be related to the host metabolic layout. At low (winter) temperatures, the mussel DG microbiome enriches fiber fermenting anaerobes belonging to Firmicutes, which generally populate digestive tract of terrestrial and marine animals (Musella et al., 2020; Rausch et al., 2019), and can take advantage of the oxidative propensity of the host overall metabolic layout. Conversely, with a raised temperature (summer), the DG microbiome becomes characterized by Tenericutes, a microbiome taxon that includes non-peptogenic parasites living in close association (and

dependence) with host cells (Lee et al., 2018), which do not suffer the host shift toward an anaerobic metabolic layout. Further investigations integrating transcriptomic, proteomic, and metabolomic profiles could probably disclose the crosstalk interactions occurring between host physiology and microbiome composition (Balbi et al., 2021; Fernández Robledo et al., 2019; Utermann et al., 2018). At any rate, measured values condition factor, an indicator of the physiological state and growth of mussels (Andral et al., 2004), and LMS, a well-consolidated general stress biomarker, within the range representing stressed but compensating organisms, likely indicating that the overall host transcriptional and microbiome composition layout is suitable to support such a physiological condition of the animals.

Results of this study further demonstrate sex related expression of some gene transcripts and of DG microbiota composition. Generally speaking, females and males differ for their expression profiles across seasons. Both DISTLM and BEST/BioEnV analyses indicated that transcriptional profiles of males seem related only to environmental variables, mainly to salinity, surface oxygen, and transparency, whereas in females seawater surface temperature and gonad maturation are the best correlated factors and explained most of the variance of the transcriptional dataset. Furthermore, while males show almost constant LMS levels values across season, females show a significant reduction of NRRT values (i.e. decreased LMS) from winter to summer, which indicate an increase of stress levels. Besides environmental conditions, LMS is known be affected by endogenous factors as reproduction and dietary budget (Moore, 2004; Múgica et al., 2015) Taken together, results of this study agree with previous findings assessing that season-related differences in biomarker responses of mussels between females and

males may reflect the progression of the reproductive cycle (Blanco-Rayón et al., 2020). From the DG microbiome side, males resulted enriched in environmental aerobes from the water column, such as Cyanobacteria and Planctomycetes, supporting a closer connection with the surrounding environment. Being characterized by a higher abundance of Firmicutes, Bacteroidetes and Actinobacterial, females showed DG microbiome enriched in host-associated taxa with a clear functional propensity toward carbohydrate fermentation.

Some gene products displayed significantly different overall expression levels between sexes. The most remarkable difference is observed for the *abcb* transcript encoding the mussel P-glycoprotein (P-gp), whose expression is significantly higher in females than in males. P-gp is a member of the ATP-binding cassette (ABC) membrane transporters. ABC transporters are ATP-dependent active transporters pumping out from cells both endogenous chemicals and xenobiotics, thus preventing their accumulation and toxic effects (Bard, 2000). These proteins are generally considered to build up a first-tier defense against chemical toxicities. Besides this, their role in mammalian oocyte maturation has been postulated (Bloise et al., 2016). It is worth noting that well detectable levels of *abcb* mRNA were observed in unfertilized (after spawning) and fertilized mussel oocytes (Franzellitti et al., 2017), suggesting a similar function in mussels. The maternal origin is not restricted to P-gp or ABC transporters, but it is a general strategy to package high levels of cellular defenses into the egg prior its release into the environment to achieve a fast induction under stress conditions (Hamdoun and Epel, 2007). For instance, it has been also suggested for antioxidant, immune, and lysosomal related gene products (Balbi et al., 2016; Franzellitti et al., 2019). Egg production requires for females to invest

a large proportion of the available energy in gametogenesis (Bedulina et al., 2020). It has been suggested that in females such sex-specific processes may impair the induction of cytoprotective mechanisms, altering their capacities to cope with environmental stressors (Bedulina et al., 2020; Meistertzheim et al., 2009). Together with LMS results, which indicate the season related onset of stress conditions for females but not for males, this observed differential expression and season regulation of cytoprotective mechanisms corroborates previous investigations showing sex related differences in pollutant bioaccumulation and in biological responses to pollutants (Blanco-Rayón et al., 2020; Schmidt et al., 2013a).

5. CONCLUSIONS

Results of this study integrate previous investigations on season- and sex- related differences in mussel responsiveness to environmental stressors by showing that the differential regulation of gene transcripts that may underpin such physiological responses may be affected by natural environmental variables and by endogenous factors, such as gender bias, gonadal cycle, and, likely, microbiome composition. Indeed, putative physiological variations occur with compositional changes in microbiome of digestive gland, the organ in which digestive and detoxification processes allow animal to tolerate and accumulate xenobiotics of natural and anthropogenic origin (Faggio et al., 2018). Widespread contamination by different classes of chemicals have been largely documented in the Northwestern Adriatic Sea, including metals, polyaromatic hydrocarbon (PAHs), pesticides, and, more recently, microplastics and pharmaceuticals (Bajt et al., 2019; Combi et al., 2016; Frapiccini et al., 2018; Mezzelani et al., 2020; Strafella et al., 2019). As showed for fish and bivalves sampled in the same area (Elia et

al., 2020; Frapiccini et al., 2020), the combination of reproductive cycle progression and seasonality may affect the pattern of pollutant accumulations, animal detoxification, and putative health outcomes.

Data reported in this study may provide baseline information on the seasonal progression of *M. galloprovincialis* physiological traits in the study area, which may improve the actual monitoring strategies of physiological performances of farmed mussels and forecast potential detrimental impacts of climatological/environmental changes. Therefore, future approaches may be improved to establish business plans that project mussel farm annual production more realistically.

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Figure legends

Fig 1. Study area, variations of environmental parameters, gonadal cycle, condition factor and mussel stress levels (lysosomal membrane stability, LMS) across the sampling period. (A) Location of the mussel farm assessed in this study in the North-West Adriatic Sea (Italy). Map was generated using the Dmaps database (https://d-maps.com). (B) Temporal trends of sea water parameters at the sampling location. Data are retrieved from the web portal of the Regional Agency of Environmental Protection, ARPA-ER (https://www.arpae.it). Blu dots: winter; orange dots: spring; red dots: summer. (C) Transcriptional profiles of male-specific vitelline coat lysin (*VCL*) and female-specific vitelline envelope receptor for lysin (*VERL*) mRNAs in mantle/gonads of farmed Mediterranean mussels addressing trends of gonad maturation across seasons. Different letters indicate statistical differences among pair of sample groups ($P < 0.05$). (D) Calculated condition factor (i.e. the ratio of the soft tissue weight to dry shell weight; mean ± SD, N = 7). (E) LMS (mean ± SD; N = 7) in hemocytes of female and male mussels collected at the different seasons (Buratti et al., 2013). The graph reports the neutral red retention time (NRRT), i.e. the time at which about the 50% of the lysosomes retained the nutral red dye (Martínez-Gómez et al., 2015). Biometric parameters employed for the calculations and statistics are reported in Table S1. *P<0.05 between pairs of sample groups. ***Colored figure is intended only for the online and PDF version***.

Fig 2. Transcriptional profiles of metabolic (*amil, pk, idp*), cytoprotective/detoxification (*lys, mt10, mt20, abcb*), antioxidant (*cat, gst, sod*), and lysosomal (*hex, ctsl, gusb*) mRNAs in females (♀) **and males** (♂) **farmed**

Mediterranean mussels from the North Adriatic Sea. For each target transcript box plots (grey area) report overall expression levels in females *vs* males (median, upper and lower quartiles; N = 21), while bar plots (white area) show transcriptional profiles across the sampling seasons and for the different genders (mean ± sem; N = 7). In box plots: *P<0.05 male *vs* female. In bar plots: different letters indicate statistical differences between samples within male or female sample groups (P < 0.05). Full transcript names are reported in Table S2. ***Colored figure is intended only for the online and PDF version***.

Fig 3. DISTLM analysis to explore trends of biological parameters with environmental variables in females (♀) **and males** (♂) **sample groups.** Results from the test of marginality related to the distance-based redundancy (DISTLM) analysis showing contribution of each environmental variable to the total variance observed in female and male datasets of gene transcription profiles. *VERL/VCL* expression levels (which are proxies of Gonadal cycles in females/males) reported in Fig 1C have been included as a predictor variable. DISTLM used the BEST selection procedure and adjusted R^2 selection criteria. Dark red (females) and dark cyan (males) bars indicate the best correlated environmental variables according to the BEST/BioEnV analysis reported in Table S2. ***Colored figure is intended only for the online and PDF version***.

Fig 4. Variation of *M. galloprovincialis* DG microbiome according to seasonality. (A) Principal Coordinates Analysis (PCoA) based on unweighted UniFrac distances between samples compositional profiles. Samples are significantly separated (permutation test

with pseudo-F ratios, P-value ≤ 0.02). The percentage of variance in the dataset explained by each axis, first and second principal component (PCo1 and PCo2), is 21% and 12%, respectively. (B) Boxplot showing relative abundance of dominant phyla in winter, spring and summer. The color legend is depicted at the top-right of the plot in panel A. ***Colored figure is intended only for the online and PDF version***.

Fig 5. Variation of *M. galloprovincialis* DG microbiome composition according to sex. (A) Principal Coordinates Analysis (PCoA) based on unweighted UniFrac distances between samples compositional profiles. Samples, color coded according to sex, showed a tendency to separate (permutation test with pseudo-F ratios, P-value ≤ 0.2). The percentage of variance in the dataset explained by each axis, first and second principal component (PCo1 and PCo2), is 21% and 12%, respectively. (B) Bar plot showing phylum-level mean relative abundance in male (♂) and female (♀) samples. Only phyla with relative abundance >1% in at least 10% of samples are represented. ***Colored figure is intended only for the online and PDF version***.

Fig 6. DISTLM analysis on the gene transcription dataset with superimposed correlation vectors with relative DG microbiome composition. Results from the test of marginality related to the DISTLM analysis is reported in Table S4. DISTLM used the BEST selection procedure and adjusted R^2 selection criteria. ***Colored figure is intended only for the online and PDF version***.

Table 1. PERMANOVA results on transcriptional profiles (998 permutations).

Source	df	Pseudo-F	P(perm)
Season	2	21.897	**0.001**
Sex	1	32.611	**0.001**
Season x Sex	2	7.2798	**0.001**

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

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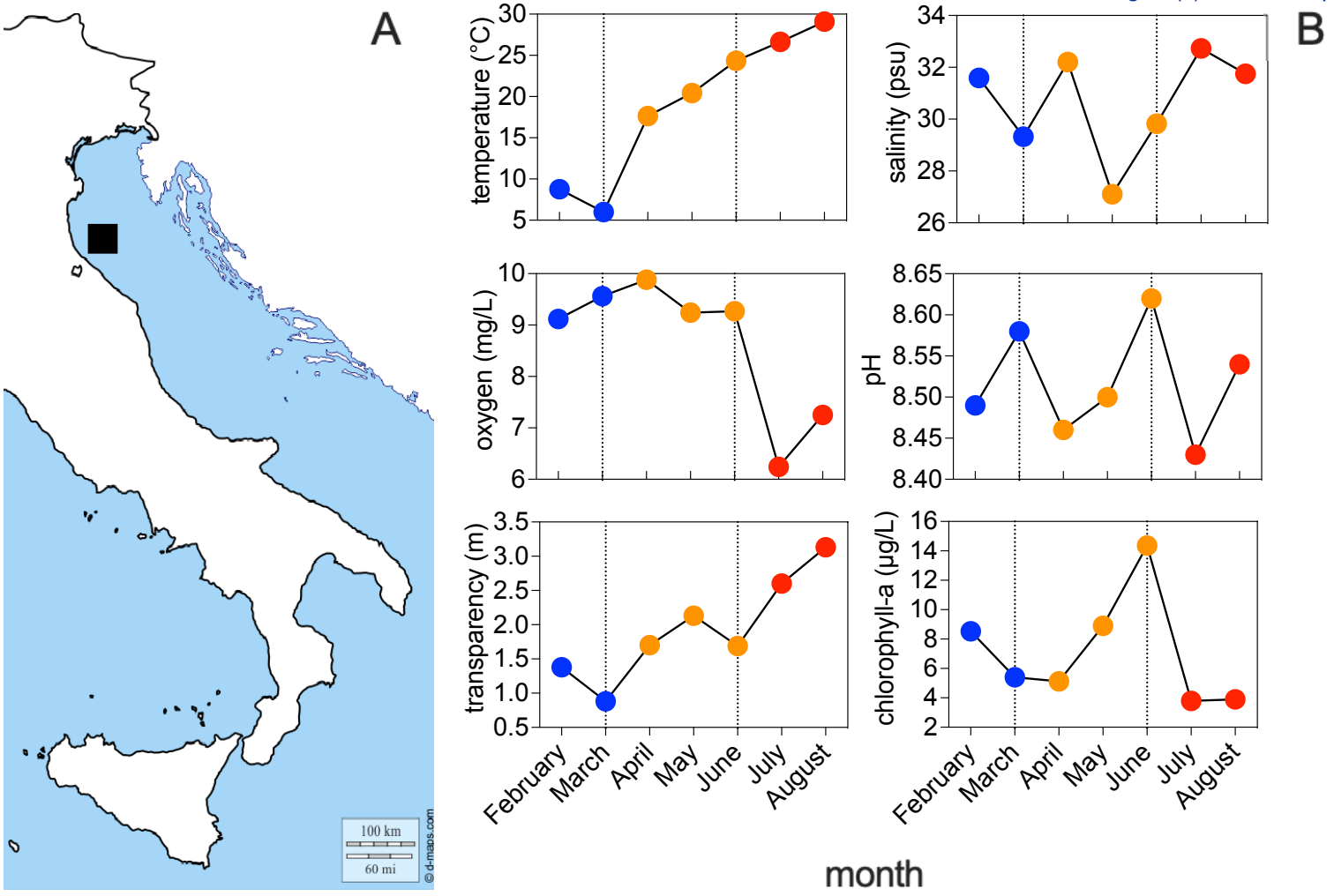
Figure 1



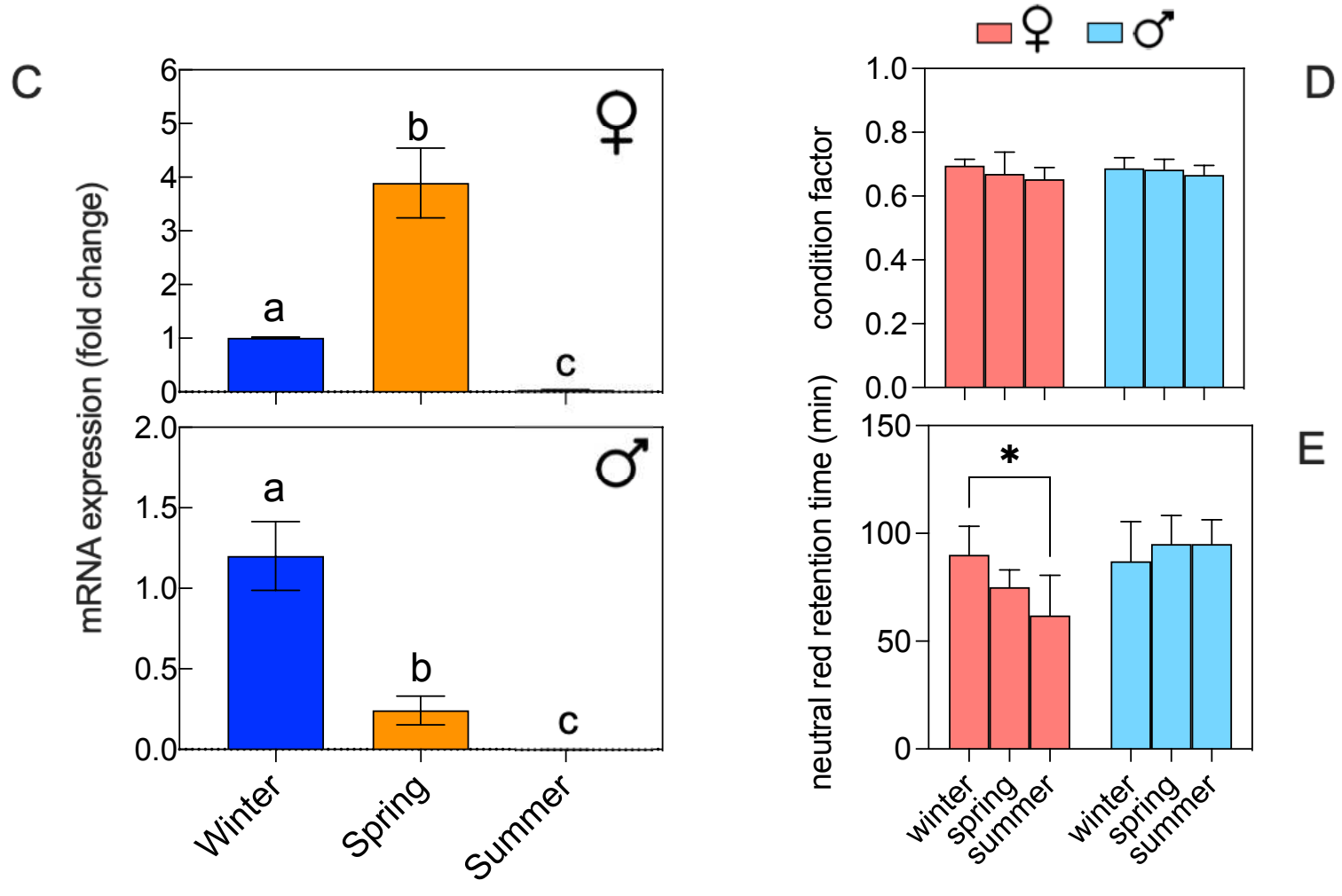


Figure 2

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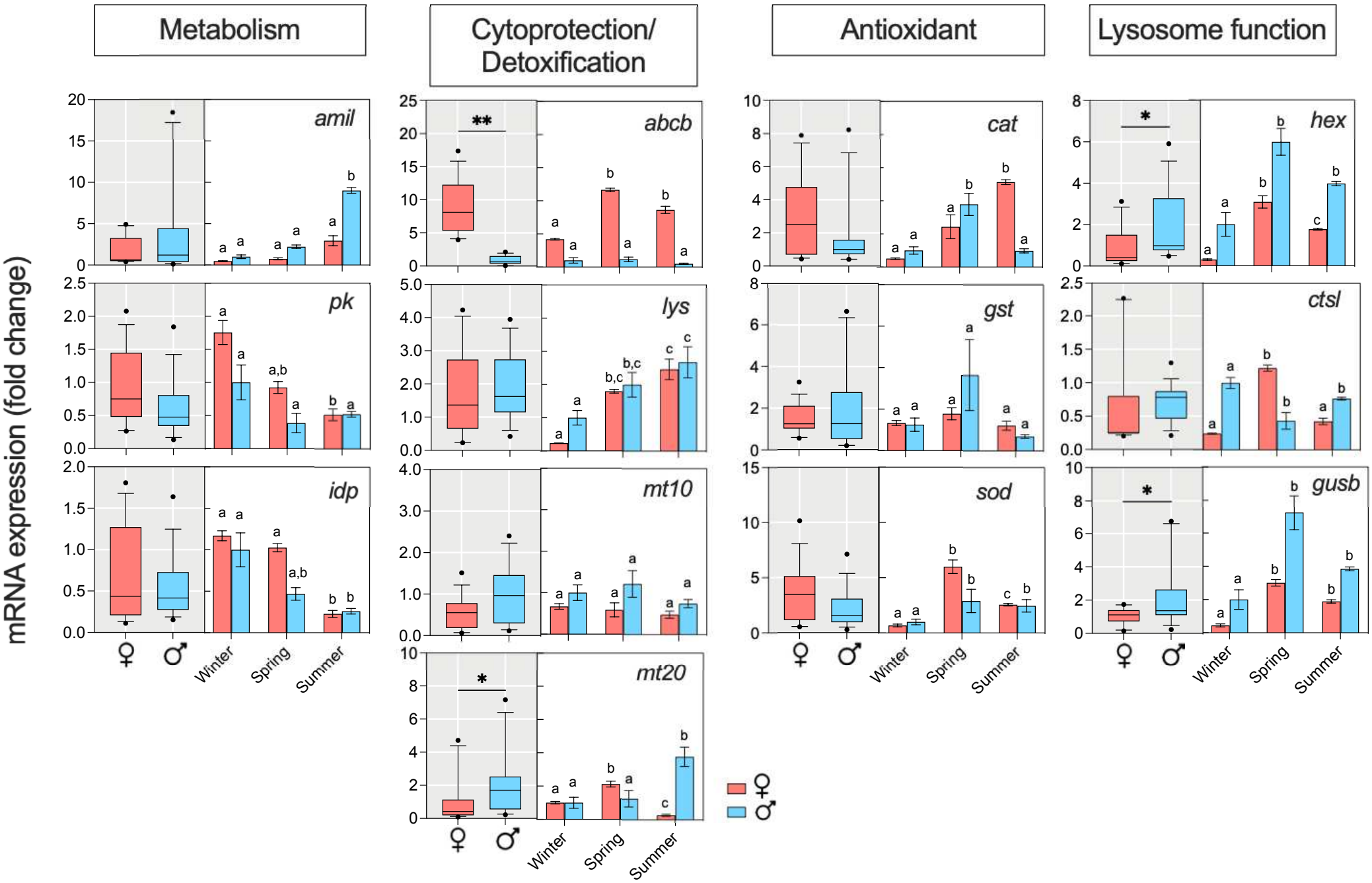

Figure 3

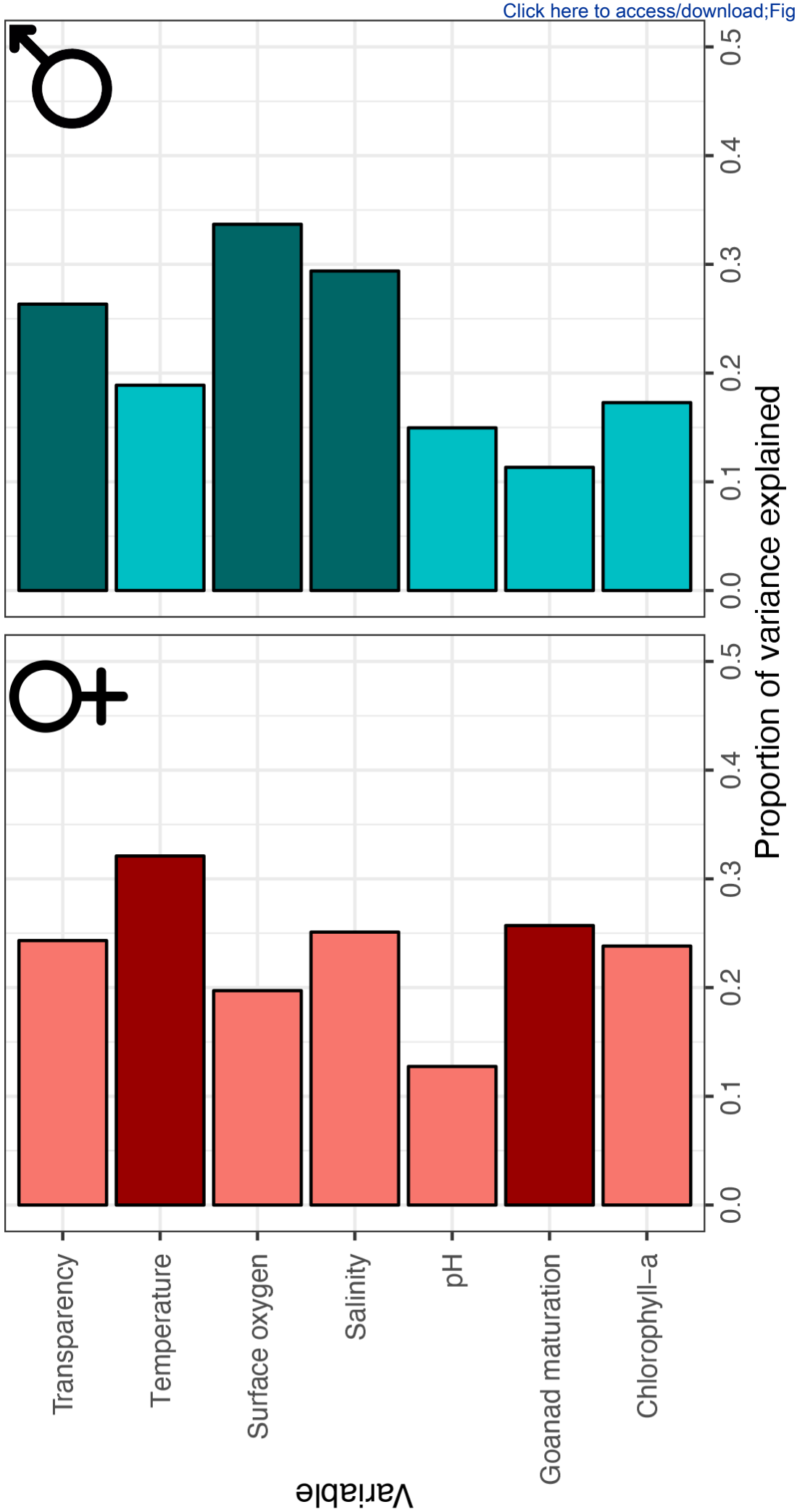

Figure 4

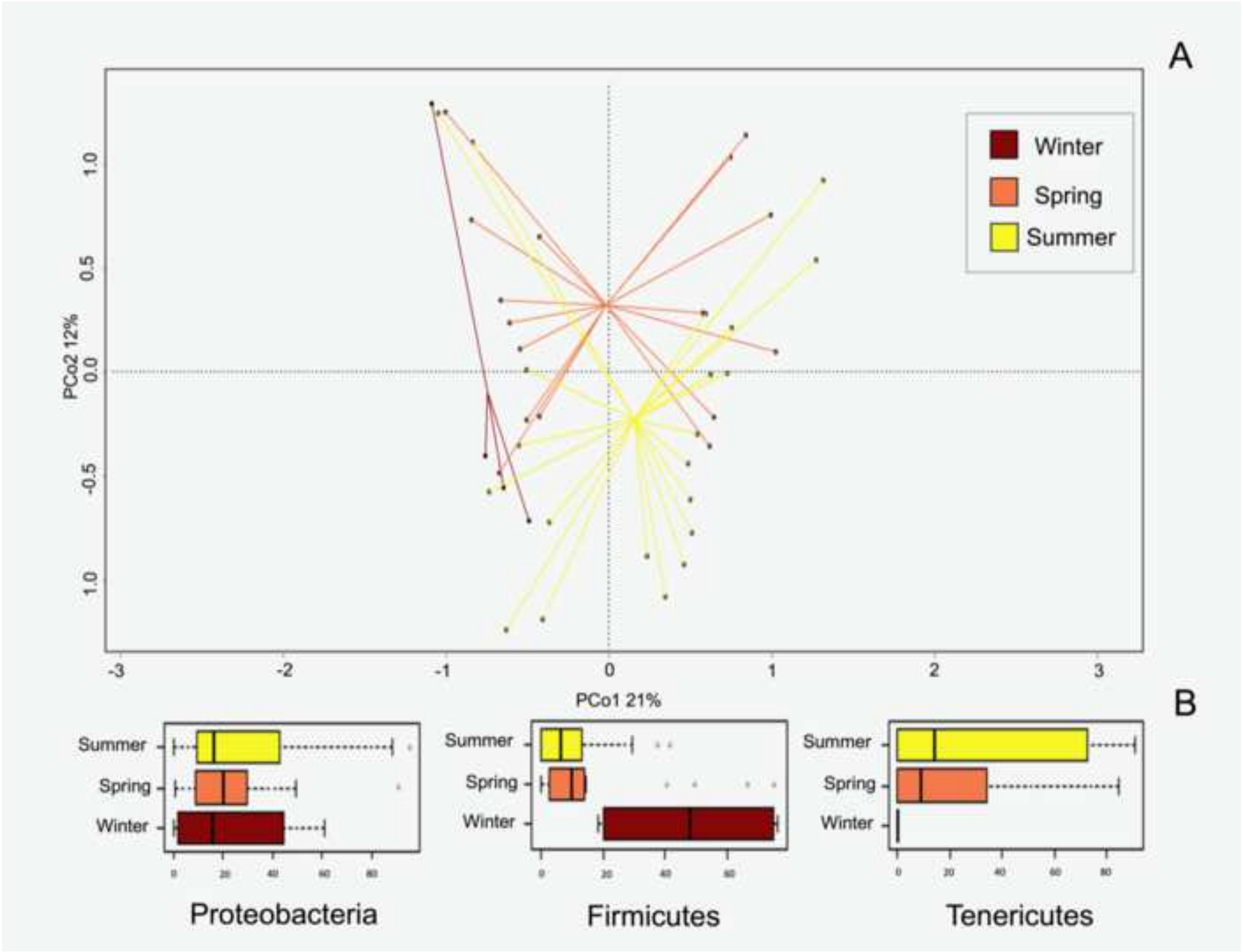

Figure 5

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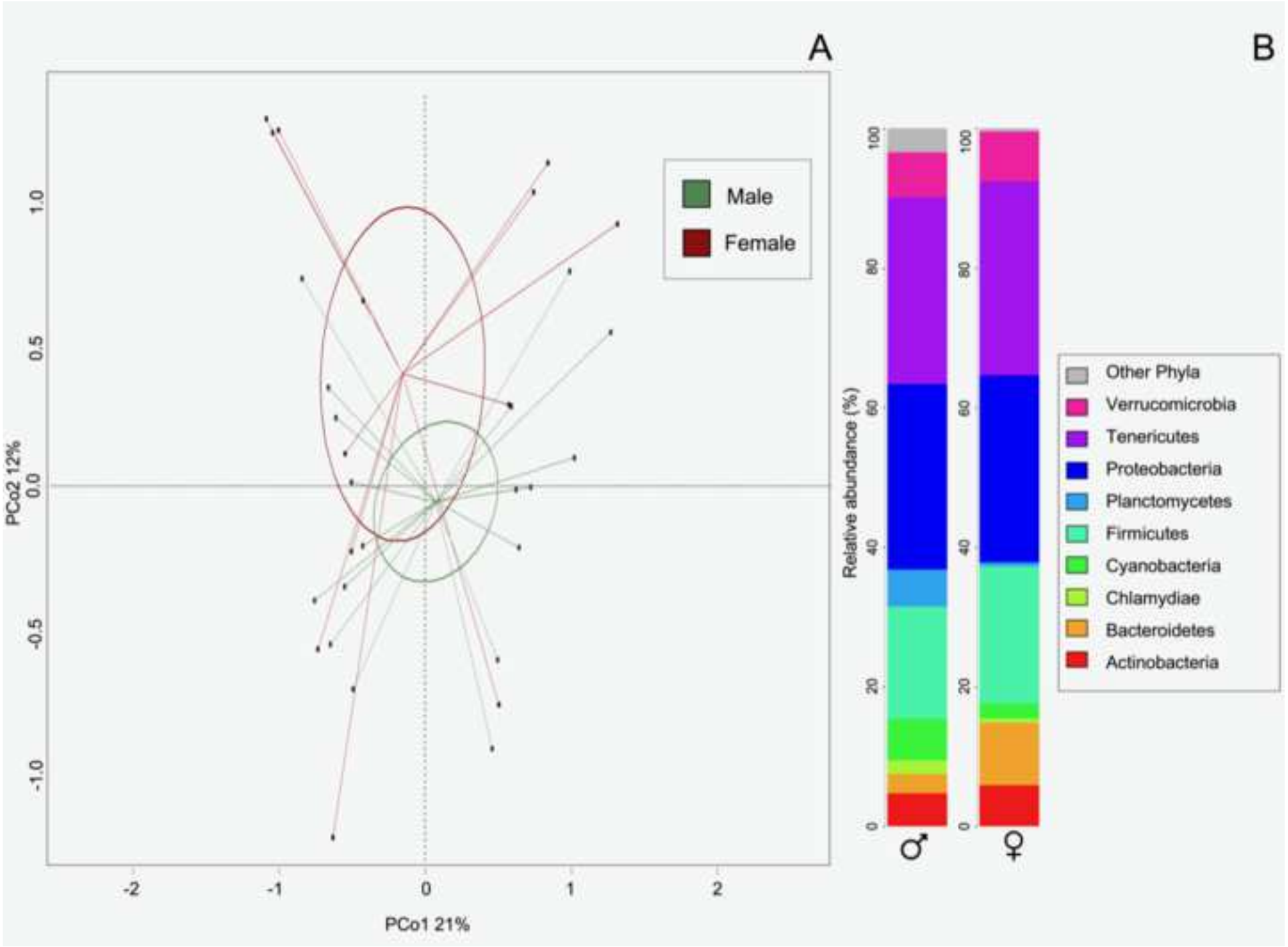

Figure 6

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