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

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21 **Abstract**

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

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

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42 **Highlights**

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

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## 52 1. INTRODUCTION

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

63 Marine mussels (*Mytilus* spp.) dominate sessile fauna of many coastal areas and  
64 estuaries. These environments are characterized by wide fluctuations of abiotic and biotic  
65 parameters, which make mussels ideal model organisms for studying physiological  
66 alterations driven by environmental changes (Figueras et al., 2019; Franzellitti et al.,  
67 2020).

68 Environmental studies with mussels highlighted seasonal fluctuation of microbial  
69 indices, contaminant bioaccumulation, cellular biomarkers, and key physiological  
70 functions (Azizi et al., 2018; Caricato et al., 2010; Ivanković et al., 2005; Roméo et al.,  
71 2003; Sheehan and Power, 1999; Shen et al., 2020; Vernocchi et al., 2007), and suggest  
72 the influence of abiotic factors (temperature, pH, salinity, food availability), and  
73 endogenous factors (i.e., gender bias and reproductive stage) for those biological  
74 responses (Blanco-Rayón et al., 2020; Grbin et al., 2019; Grenier et al., 2020).

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4 75 Molecular biomarkers based on expression analysis of stress responsive genes  
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6 76 are pointing out crucial insights into molecular mechanisms regulating animal ability to  
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9 77 survive and thrive in dynamic and changing marine environments (Evans and Hofmann,  
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11 78 2012; Gracey, 2007). Indeed, in environmentally relevant species as marine mussels, the  
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14 79 modulation of mRNA levels is the earliest signal of an ongoing physiological alteration  
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16 80 that can potentially forecast changes at higher levels of the biological organization  
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19 81 (Gracey, 2007). There are several studies employing mussels as model organisms in field  
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21 82 experiments to infer transcriptomic changes with environmental quality (Blalock et al.,  
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23 83 2020; Franzellitti et al., 2010; Kerambrun et al., 2016; Rossi et al., 2016; Sforzini et al.,  
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25 84 2018; Venier et al., 2006). Conversely, few studies emphasized that mussel gene  
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28 85 transcription may be modulated by natural environmental parameters or by endogenous  
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30 86 factors (Banni et al., 2011; Counihan et al., 2019; Schmidt et al., 2013b).

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33 87 This study evaluates the transcriptional profiles of genes related to metabolic,  
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35 88 detoxification, antioxidant, and lysosomal responses in Mediterranean mussels (*Mytilus*  
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37 89 *galloprovincialis*) under the influence of natural seasonal variations of environmental  
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39 90 variables, gender bias, and gonadal cycle. We purposely addressed those environmental  
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41 91 parameters and/or endogenous factors that may modulate some functional categories of  
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43 92 stress-responsive gene transcripts, likely affecting the capability of the animals to cope  
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45 93 with further environmental changes or the occurrence of natural and anthropogenic  
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47 94 toxins. Furthermore, a recent literature review (Lindsay et al., 2020) shows that the  
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49 95 composition of gut microbial community of a species can vary seasonally with host diet,  
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51 96 metabolic demands, and life stage. These changes in microbial community composition  
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53 97 seem to comprehensively contribute to the host flexibility to cope with environmental  
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98 changes, enabling the host to live within different environments, adapt to seasonal  
99 changes and maintain its physiological performance. Therefore, considering this vital role  
100 of microbial communities in the maintenance of the host health (Rausch et al., 2019;  
101 Simon et al., 2019) also in response to environmental conditions (Vanwonderghem and  
102 Webster, 2020), this study explores basal responses of the mussel digestive gland (DG)  
103 microbiome to seasonal changes, allowing to figure out microbiome variations occurring  
104 concomitantly with host physiological changes across seasonality.

## 106 2. METHODS

### 108 2.1. Mussel sampling

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

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

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



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4 144 Sex was determined in individual mussels using the sex-specific gene method  
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6 145 (Fraser et al., 2016). Specifically, the method consists in the quantification through real-  
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9 146 time PCR (qPCR) of expression of the mussel vitelline envelope receptor for lysine  
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11 147 (*VERL*), and vitelline coat lysine (*VCL*) mRNAs in the mantle/gonad complex. The  
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14 148 transcripts are specifically expressed in females and males, respectively, and serve as a  
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16 149 proxy of gonadal cycle (Hines et al., 2007). This method proved suitable differentiating  
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19 150 males from females both during gametogenesis and sexual resting stage, when histology  
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21 151 does not allow the observation of gametes (Anantharaman and Craft, 2012; Fraser et al.,  
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24 152 2016). RNA extraction and cDNA preparation from mussel mantle/gonad complexes was  
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26 153 as reported below. qPCR reactions were performed in duplicate for each sample using  
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29 154 primer pairs and protocols reported previously (Anantharaman and Craft, 2012) (Table  
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31 155 S2). Threshold cycle ( $C_T$ ) values were determined by setting a constant baseline. Sex  
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34 156 was determined calculating the intra animal  $\Delta C_T$  as  $C_T(VCL) - C_T(VERL)$  (Anantharaman  
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36 157 and Craft, 2012). Negative values indicate males and positive values indicate females.  
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39 158 Relative *VCL* or *VERL* expression values across season (Fig 1C) were inferred by a  
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41 159 comparative  $C_T$  method (Schmittgen and Livak, 2008) using the normalization and  
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43 160 statistical strategy reported below. As reported previously (Anantharaman and Craft,  
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46 161 2012), both *VERL* and *VCL* expression levels significantly decreased from winter to  
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48 162 summer (Fig 1C). Based on visual microscopic inspection of gonads (Hines et al., 2007),  
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51 163 transcript levels of both sex specific genes was found to be associated to the presence  
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53 164 and abundance of gametes.  
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56 165 Mussel biometric parameters are reported in Table S1. A production metric, the condition  
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58 166 factor, was calculated, with values being unchanged across seasons and similar between  
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167 females and males (Fig 1D; Table S1). The lysosomal membrane stability (LMS) was  
168 assessed in mussel living haemocytes through the neutral red retention assay according  
169 to (Buratti et al., 2013). LMS is a well-consolidated general stress biomarker and a  
170 prognostic indicator for putative pathologies. As such, it addressed to as an integrated  
171 pathophysiological indicator of general health status (Martínez-Gómez et al., 2015).  
172 According to Martínez-Gómez et al. (2015), neutral red retention time (NRRT) values  
173 recorded in this study fall within the range representing stressed but compensating  
174 organisms (Fig 1E; Table S1). Furthermore, while males show almost constant NRRT  
175 values across season, females show a significant reduction of NRRT values from winter  
176 to summer, which indicate increased stress levels.

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178 *2.2. RNA extraction, cDNA preparation, and qPCR analyses*

179 For each animal, 200 mg of mantle/gonad complex (sex identification and gonadal  
180 cycle) or of digestive glands were independently homogenized in a suitable volume of the  
181 TRI Reagent (Sigma Aldrich, Milan, Italy) and total RNA was extracted using the DirectZol  
182 kit (Zymo Research, Freiburg, Germany) following the manufacturer's instructions. RNA  
183 concentration and quality were confirmed using the Qubit system with the Qubit RNA  
184 assay kit (Thermo Scientific, Milan, Italy) and electrophoresis using a 1.2% agarose gel  
185 under denaturing conditions. The analysis of UV absorbance spectra of the samples ( $\lambda =$   
186 200 – 340 nm) allowed the calculation of Absorbance (A) ratio A260/A280 addressing the  
187 occurrence of protein contaminations (cut-off values > 1.8 and < 2.0), and the ratio  
188 A260/A230 addressing the occurrence of contaminants that may be present in the  
189 samples, such as guanidine thiocyanate, which is a component of the TRI Reagent (cut-

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

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

201  
202 *2.5. Microbial DNA extraction and sequencing*

203 Total microbial DNA was extracted from approximately 20 – 30 mg of digestive  
204 gland tissue using the DNeasy PowerSoil kit (Qiagen, Hilden, Germany) according to  
205 (Musella et al., 2020). The V3–V4 hypervariable region of the 16S rRNA gene was  
206 amplified using the 341F and 785R primers with added Illumina adapter overhang  
207 sequences, as previously described (Barone et al., 2019). The thermal cycle consisted of  
208 initial denaturation at 95°C for 3 minutes, 30 cycles at 95°C for 30 seconds, annealing at  
209 55°C for 30 seconds, extension at 72°C for 30 seconds and 5 minutes at 72°C for final  
210 extension. PCR reactions were then cleaned up with Agencourt AMPure XP magnetic  
211 beads (Beckman Coulter, Brea, CA). Indexed libraries were prepared by limited-cycle  
212 PCR, using the Nextera technology and then pooled after a further clean up step as

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

## 220 2.6. Statistical and bioinformatic analyses

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

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

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

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

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

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values, and a p-value  $\leq 0.05$  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).

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### 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  $\pm$  SD,  $15195 \pm 11581$ ) 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  $\leq 0.02$ ) (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 ( $6\% \pm 11.4\%$  in male,  $2.1\% \pm 7.6\%$  in female), Planctomycetes ( $5.3\% \pm 7.7\%$  in male  $0.6\% \pm 1.5\%$  in female) and Chlamydiae ( $2\% \pm 4.7\%$  in male,  $0.6\% \pm 1.5\%$  in female), while females show an increase in Firmicutes ( $16.1\% \pm 22.5\%$  in male,  $19.7\% \pm 26.9\%$  in female), Bacteroidetes ( $2.7\% \pm 3.4\%$  in male,  $8.9\% \pm 16.2\%$  in female) and Actinobacteria ( $4.7\% \pm 6.6\%$  in male,  $5.9\% \pm 12.7\%$  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

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

#### 314 4. DISCUSSION

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

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



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

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

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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

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

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

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396 a large proportion of the available energy in gametogenesis (Bedulina et al., 2020). It has  
397 been suggested that in females such sex-specific processes may impair the induction of  
398 cytoprotective mechanisms, altering their capacities to cope with environmental stressors  
399 (Bedulina et al., 2020; Meistertzheim et al., 2009). Together with LMS results, which  
400 indicate the season related onset of stress conditions for females but not for males, this  
401 observed differential expression and season regulation of cytoprotective mechanisms  
402 corroborates previous investigations showing sex related differences in pollutant  
403 bioaccumulation and in biological responses to pollutants (Blanco-Rayón et al., 2020;  
404 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

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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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### References

Alvisi, F., Cozzi, S., 2016. Seasonal dynamics and long-term trend of hypoxia in the coastal zone of Emilia Romagna (NW Adriatic Sea, Italy). *Sci. Total Environ.* 541, 1448–1462. <https://doi.org/10.1016/j.scitotenv.2015.10.011>

Anantharaman, S., Craft, J.A., 2012. Annual Variation in the Levels of Transcripts of Sex-Specific Genes in the Mantle of the Common Mussel, *Mytilus edulis*. *PLoS One* 7. <https://doi.org/10.1371/journal.pone.0050861>

Anderson, M., Gorley, R., Clarke, K., 2008. PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods.

Andral, B., Stanisiere, J.Y., Sauzade, D., Damier, E., Thebault, H., Galgani, F., Boissery, P., 2004. Monitoring chemical contamination levels in the Mediterranean based on the use of mussel caging. *Mar. Pollut. Bull.* 49, 704–712.

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<https://doi.org/10.1016/j.marpolbul.2004.05.008>

Azizi, G., Layachi, M., Akodad, M., Yáñez-Ruiz, D.R., Martín-García, A.I., Baghour, M., Mesfioui, A., Skalli, A., Moumen, A., 2018. Seasonal variations of heavy metals content in mussels (*Mytilus galloprovincialis*) from Cala Iris offshore (Northern Morocco). *Mar. Pollut. Bull.* 137, 688–694. <https://doi.org/10.1016/j.marpolbul.2018.06.052>

Bajt, O., Ramšak, A., Milun, V., Andral, B., Romanelli, G., Scarpato, A., Mitrić, M., Kupusović, T., Kljajić, Z., Angelidis, M., Çullaj, A., Galgani, F., 2019. Assessing chemical contamination in the coastal waters of the Adriatic Sea using active mussel biomonitoring with *Mytilus galloprovincialis*. *Mar. Pollut. Bull.* 141, 283–298. <https://doi.org/10.1016/j.marpolbul.2019.02.007>

Balbi, T., Auguste, M., Ciacci, C., Canesi, L., 2021. Immunological responses of marine bivalves to contaminant exposure: contribution of the -omics approach. *Front. Immunol.* 12. <https://doi.org/10.3389/fimmu.2021.618726>

Balbi, T., Fabbri, R., Montagna, M., Camisassi, G., Canesi, L., 2017. Seasonal variability of different biomarkers in mussels (*Mytilus galloprovincialis*) farmed at different sites of the Gulf of La Spezia, Ligurian sea, Italy. *Mar. Pollut. Bull.* 116, 348–356. <https://doi.org/10.1016/j.marpolbul.2017.01.035>

Balbi, T., Franzellitti, S., Fabbri, R., Montagna, M., Fabbri, E., Canesi, L., 2016. Impact of bisphenol A (BPA) on early embryo development in the marine mussel *Mytilus galloprovincialis*: effects on gene transcription. *Environ. Pollut.* 218, 996–1004. <https://doi.org/10.1016/j.envpol.2016.08.050>

Banni, M., Negri, A., Mignone, F., Boussetta, H., Viarengo, A., Dondero, F., 2011. Gene expression rhythms in the mussel *Mytilus galloprovincialis* (Lam.) across an annual cycle. *PLoS One* 6, e18904. <https://doi.org/10.1371/journal.pone.0018904>

Bard, S.M., 2000. Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms. *Aquat. Toxicol.* [https://doi.org/10.1016/S0166-445X\(00\)00088-6](https://doi.org/10.1016/S0166-445X(00)00088-6)

Barone, M., Turrone, S., Rampelli, S., Soverini, M., D'Amico, F., Biagi, E., Brigidi, P., Troiani, E., Candela, M., 2019. Gut microbiome response to a modern Paleolithic diet in a Western lifestyle context. *PLoS One* 14, e0220619. <https://doi.org/10.1371/journal.pone.0220619>

Bedulina, D., Drozdova, P., Gurkov, A., von Bergen, M., Stadler, P.F., Luckenbach, T., Timofeyev, M., Kalkhof, S., 2020. Proteomics reveals sex-specific heat shock response of Baikal amphipod *Eulimnogammarus cyaneus*. *Sci. Total Environ.* 143008. <https://doi.org/10.1016/j.scitotenv.2020.143008>

Benito, D., Ahvo, A., Nuutinen, J., Bilbao, D., Saenz, J., Etxebarria, N., Lekube, X., Izagirre, U., Lehtonen, K.K., Marigómez, I., Zaldibar, B., Soto, M., 2019. Influence of season-dependending ecological variables on biomarker baseline levels in mussels (*Mytilus trossulus*) from two Baltic Sea subregions. *Sci. Total Environ.* 689, 1087–1103. <https://doi.org/10.1016/j.scitotenv.2019.06.412>

Blalock, B.J., Robinson, W.E., Poynton, H.C., 2020. Assessing legacy and endocrine disrupting pollutants in Boston Harbor with transcriptomic biomarkers. *Aquat. Toxicol.* 220, 105397. <https://doi.org/10.1016/j.aquatox.2019.105397>

Blanco-Rayón, E., Ivanina, A. V., Sokolova, I.M., Marigómez, I., Izagirre, U., 2020. Sex and sex-related differences in gamete development progression impinge on biomarker

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2  
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492       responsiveness in sentinel mussels. *Sci. Total Environ.* 740, 140178.  
493       <https://doi.org/10.1016/j.scitotenv.2020.140178>

494 Bloise, E., Ortiga-Carvalho, T.M., Reis, F.M., Lye, S.J., Gibb, W., Matthews, S.G., 2016. ATP-  
495 binding cassette transporters in reproduction: A new frontier. *Hum. Reprod. Update* 22,  
496 164–181. <https://doi.org/10.1093/humupd/dmv049>

497 Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander,  
498 H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A.,  
499 Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope,  
500 E.K., Da Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvall, C.,  
501 Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson,  
502 D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower,  
503 C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K. Bin,  
504 Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciulek, T., Kreps, J.,  
505 Langille, M.G.I., Lee, J., Ley, R., Liu, Y.-X., Lofffield, E., Lozupone, C., Maher, M., Marotz,  
506 C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A. V., Metcalf, J.L., Morgan, S.C.,  
507 Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson,  
508 T., Peoples, S.L., Petras, D., Preuss, M.L., Pruesse, E., Rasmussen, L.B., Rivers, A.,  
509 Robeson, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J.,  
510 Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A.,  
511 Turnbaugh, P.J., Ul-Hasan, S., van der Hooft, J.J.J., Vargas, F., Vázquez-Baeza, Y.,  
512 Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K.C.,  
513 Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R.,  
514 Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data  
515 science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. <https://doi.org/10.1038/s41587-019-0209-9>

516

517 Brigolin, D., Porporato, E.M.D., Prioli, G., Pastres, R., 2017. Making space for shellfish farming  
518 along the Adriatic coast. *ICES J. Mar. Sci.* 74, 1540–1551.  
519 <https://doi.org/10.1093/icesjms/fsx018>

520 Buratti, S., Franzellitti, S., Poletti, R., Ceredi, A., Montanari, G., Capuzzo, A., Fabbri, E., 2013.  
521 Bioaccumulation of algal toxins and changes in physiological parameters in Mediterranean  
522 mussels from the north Adriatic Sea (Italy). *Environ. Toxicol.* 28, 451–470.  
523 <https://doi.org/10.1002/tox.20739>

524 Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016.  
525 DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13,  
526 581–583. <https://doi.org/10.1038/nmeth.3869>

527 Canesi, L., Ciacci, C., Betti, M., Malatesta, M., Gazzanelli, G., Gallo, G., 1999. Growth Factors  
528 Stimulate the Activity of Key Glycolytic Enzymes in Isolated Digestive Gland Cells from  
529 Mussels (*Mytilus galloprovincialis* Lam.) through Tyrosine Kinase Mediated Signal  
530 Transduction. *Gen. Comp. Endocrinol.* 116, 241–248.  
531 <https://doi.org/10.1006/gcen.1999.7366>

532 Caricato, R., Lionetto, M.G., Schettino, T., 2010. Seasonal variation of biomarkers in *Mytilus*  
533 *galloprovincialis* sampled inside and outside Mar Piccolo of taranto (Italy). *Chem. Ecol.* 26,  
534 143–153. <https://doi.org/10.1080/02757541003627639>

535 Combi, T., Pintado-Herrera, M.G., Lara-Martin, P.A., Misericocchi, S., Langone, L., Guerra, R.,  
536 2016. Distribution and fate of legacy and emerging contaminants along the Adriatic Sea: A

1  
2  
3  
4 537 comparative study. Environ. Pollut. 218, 1055–1064.  
5 538 <https://doi.org/10.1016/j.envpol.2016.08.057>  
6  
7 539 Counihan, K.L., Bowen, L., Ballachey, B., Coletti, H., Hollmen, T., Pister, B., Wilson, T.L., 2019.  
8 540 Physiological and gene transcription assays to assess responses of mussels to  
9 541 environmental changes. PeerJ 2019, 1–33. <https://doi.org/10.7717/peerj.7800>  
10  
11 542 Elia, A.C., Burioli, E., Magara, G., Pastorino, P., Caldaroni, B., Menconi, V., Dörr, A.J.M.,  
12 543 Colombero, G., Abete, M.C., Prearo, M., 2020. Oxidative stress ecology on Pacific oyster  
13 544 *Crassostrea gigas* from lagoon and offshore Italian sites. Sci. Total Environ. 739, 139886.  
14 545 <https://doi.org/10.1016/j.scitotenv.2020.139886>  
15  
16 546 Evans, T.G., Hofmann, G.E., 2012. Defining the limits of physiological plasticity: how gene  
17 547 expression can assess and predict the consequences of ocean change. Philos. Trans. R.  
18 548 Soc. B Biol. Sci. 367, 1733–1745. <https://doi.org/10.1098/rstb.2012.0019>  
19  
20 549 Faggio, C., Tsarpali, V., Dailianis, S., 2018. Mussel digestive gland as a model tissue for  
21 550 assessing xenobiotics: An overview. Sci. Total Environ. 636, 220–229.  
22 551 <https://doi.org/10.1016/j.scitotenv.2018.04.264>  
23  
24 552 Fernández Robledo, J.A., Yadavalli, R., Allam, B., Pales Espinosa, E., Gerdol, M., Greco, S.,  
25 553 Stevick, R.J., Gómez-Chiarri, M., Zhang, Y., Heil, C.A., Tracy, A.N., Bishop-Bailey, D.,  
26 554 Metzger, M.J., 2019. From the raw bar to the bench: Bivalves as models for human health.  
27 555 Dev. Comp. Immunol. 92, 260–282. <https://doi.org/10.1016/j.dci.2018.11.020>  
28  
29 556 Figueras, A., Moreira, R., Sendra, M., Novoa, B., 2019. Genomics and immunity of the  
30 557 Mediterranean mussel *Mytilus galloprovincialis* in a changing environment. Fish Shellfish  
31 558 Immunol. 90, 440–445. <https://doi.org/10.1016/j.fsi.2019.04.064>  
32  
33 559 Franzellitti, S., Balbi, T., Montagna, M., Fabbri, R., Valbonesi, P., Fabbri, E., Canesi, L., 2019.  
34 560 Phenotypical and molecular changes induced by carbamazepine and propranolol on larval  
35 561 stages of *Mytilus galloprovincialis*. Chemosphere 234, 962–970.  
36 562 <https://doi.org/10.1016/j.chemosphere.2019.06.045>  
37  
38 563 Franzellitti, S., Buratti, S., Donnini, F., Fabbri, E., 2010. Exposure of mussels to a polluted  
39 564 environment: insights into the stress syndrome development. Comp. Biochem. Physiol.  
40 565 Part C 152, 24–33. <https://doi.org/10.1016/j.cbpc.2010.02.010>  
41  
42 566 Franzellitti, S., Prada, F., Viarengo, A., Fabbri, E., 2020. Evaluating bivalve cytoprotective  
43 567 responses and their regulatory pathways in a climate change scenario. Sci. Total Environ.  
44 568 720, 137733. <https://doi.org/10.1016/j.scitotenv.2020.137733>  
45  
46 569 Franzellitti, S., Striano, T., Pretolani, F., Fabbri, E., 2017. Investigating appearance and  
47 570 regulation of the MXR phenotype in early embryo stages of the Mediterranean mussel  
48 571 (*Mytilus galloprovincialis*). Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol. 199, 1–  
49 572 10. <https://doi.org/10.1016/j.cbpc.2016.11.004>  
50  
51 573 Frapiccini, E., Annibaldi, A., Betti, M., Polidori, P., Truzzi, C., Marini, M., 2018. Polycyclic  
52 574 aromatic hydrocarbon (PAH) accumulation in different common sole (*Solea solea*) tissues  
53 575 from the North Adriatic Sea peculiar impacted area. Mar. Pollut. Bull. 137, 61–68.  
54 576 <https://doi.org/10.1016/j.marpolbul.2018.10.002>  
55  
56 577 Frapiccini, E., Panfili, M., Guicciardi, S., Santojanni, A., Marini, M., Truzzi, C., Annibaldi, A.,  
57 578 2020. Effects of biological factors and seasonality on the level of polycyclic aromatic  
58 579 hydrocarbons in red mullet (*Mullus barbatus*). Environ. Pollut. 258, 113742.



- 1  
2  
3  
4 580 <https://doi.org/10.1016/j.envpol.2019.113742>  
5  
6 581 Fraser, M., Fortier, M., Roumier, P.H., Parent, L., Brousseau, P., Fournier, M., Surette, C.,  
7 582 Vaillancourt, C., 2016. Sex determination in blue mussels: Which method to choose? *Mar.*  
8 583 *Environ. Res.* 120, 78–85. <https://doi.org/10.1016/j.marenvres.2016.07.008>  
9  
10 584 Gracey, A.Y., 2007. Interpreting physiological responses to environmental change through gene  
11 585 expression profiling. *J. Exp. Biol.* <https://doi.org/10.1242/jeb.004333>  
12  
13 586 Grbin, D., Sabolić, I., Klobučar, G., Dennis, S.R., Šrut, M., Bakarić, R., Baković, V., Brkanac,  
14 587 S.R., Nosil, P., Štambuk, A., 2019. Biomarker response of Mediterranean mussels *Mytilus*  
15 588 *galloprovincialis* regarding environmental conditions, pollution impact and seasonal effects.  
16 589 *Sci. Total Environ.* 694, 1–10. <https://doi.org/10.1016/j.scitotenv.2019.07.276>  
17  
18 590 Grenier, C., Román, R., Duarte, C., Navarro, J.M., Rodriguez-Navarro, A.B., Ramajo, L., 2020.  
19 591 The combined effects of salinity and pH on shell biomineralization of the edible mussel  
20 592 *Mytilus chilensis*. *Environ. Pollut.* 263, 114555.  
21 593 <https://doi.org/10.1016/j.envpol.2020.114555>  
22  
23 594 Hamdoun, A., Epel, D., 2007. Embryo stability and vulnerability in an always changing world.  
24 595 *Proc. Natl. Acad. Sci. U. S. A.* 104, 1745–50. <https://doi.org/10.1073/pnas.0610108104>  
25  
26 596 Hines, A., Yeung, W.H., Craft, J., Brown, M., Kennedy, J., Bignell, J., Stentiford, G.D., Viant,  
27 597 M.R., 2007. Comparison of histological, genetic, metabolomics, and lipid-based methods  
28 598 for sex determination in marine mussels. *Anal. Biochem.* 369, 175–186.  
29 599 <https://doi.org/10.1016/j.ab.2007.06.008>  
30  
31 600 Islam, M.S., Tanaka, M., 2004. Impacts of pollution on coastal and marine ecosystems including  
32 601 coastal and marine fisheries and approach for management: A review and synthesis. *Mar.*  
33 602 *Pollut. Bull.* 48, 624–649. <https://doi.org/10.1016/j.marpolbul.2003.12.004>  
34  
35 603 Ivanković, D., Pavičić, J., Erk, M., Filipović-Marijić, V., Raspor, B., 2005. Evaluation of the  
36 604 *Mytilus galloprovincialis* Lam. digestive gland metallothionein as a biomarker in a long-term  
37 605 field study: Seasonal and spatial variability. *Mar. Pollut. Bull.* 50, 1303–1313.  
38 606 <https://doi.org/10.1016/j.marpolbul.2005.04.039>  
39  
40 607 Kerambrun, E., Rioult, D., Delahaut, L., Evariste, L., Pain-Devin, S., Auffret, M., Geffard, A.,  
41 608 David, E., 2016. Variations in gene expression levels in four European zebra mussel,  
42 609 *Dreissena polymorpha*, populations in relation to metal bioaccumulation: A field study.  
43 610 *Ecotoxicol. Environ. Saf.* 134, 53–63. <https://doi.org/10.1016/j.ecoenv.2016.08.018>  
44  
45 611 Lacroix, C., Duvieilbourg, E., Guillou, N., Guyomarch, J., Bassoulet, C., Moraga, D., Chapalain,  
46 612 G., Auffret, M., 2017. Seasonal monitoring of blue mussel (*Mytilus* spp.) populations in a  
47 613 harbor area: A focus on responses to environmental factors and chronic contamination.  
48 614 *Mar. Environ. Res.* 129, 24–35. <https://doi.org/10.1016/j.marenvres.2017.04.008>  
49  
50 615 Landrigan, P.J., Stegeman, J.J., Fleming, L.E., Allemand, D., Anderson, D.M., Backer, L.C.,  
51 616 Brucker-Davis, F., Chevalier, N., Corra, L., Czerucka, D., Bottein, M.-Y.D., Demeneix, B.,  
52 617 Depledge, M., Deheyn, D.D., Dorman, C.J., Fénichel, P., Fisher, S., Gaill, F., Galgani, F.,  
53 618 Gaze, W.H., Giuliano, L., Grandjean, P., Hahn, M.E., Hamdoun, A., Hess, P., Judson, B.,  
54 619 Laborde, A., McGlade, J., Mu, J., Mustapha, A., Neira, M., Noble, R.T., Pedrotti, M.L.,  
55 620 Reddy, C., Rocklöv, J., Scharler, U.M., Shanmugam, H., Taghian, G., Van de Water,  
56 621 J.A.J.M., Vezzulli, L., Weihe, P., Zeka, A., Raps, H., Rampal, P., 2020. Human Health and  
57 622 Ocean Pollution. *Ann. Glob. Heal.* 86, 151. <https://doi.org/10.5334/aogh.2831>  
58  
59  
60  
61  
62  
63  
64  
65

- 1  
2  
3  
4 623 Lee, M.D., Kling, J.D., Araya, R., Ceh, J., 2018. Jellyfish life stages shape associated microbial  
5 624 communities, while a core microbiome is maintained across all. *Front. Microbiol.* 9, 1534.  
6 625 <https://doi.org/10.3389/fmicb.2018.01534>
- 8 626 Leiniö, S., Lehtonen, K.K., 2005. Seasonal variability in biomarkers in the bivalves *Mytilus edulis*  
9 627 and *Macoma balthica* from the northern Baltic Sea. *Comp. Biochem. Physiol. - C Toxicol.*  
10 628 *Pharmacol.* 140, 408–421. <https://doi.org/10.1016/j.cca.2005.04.005>
- 12 629 Lindsay, E.C., Metcalfe, N.B., Llewellyn, M.S., 2020. The potential role of the gut microbiota in  
13 630 shaping host energetics and metabolic rate. *J. Anim. Ecol.* 89, 2415–2426.  
14 631 <https://doi.org/10.1111/1365-2656.13327>
- 16 632 Liu, C., Lin, D., Dong, Y., Xue, Q., Yao, H., Lin, Z., 2017. Association of  $\alpha$ -amylase gene with  
17 633 growth traits in the razor clam *Sinonovacula constricta*. *Invertebr. Surviv. J.* 14, 494–504.  
18 634 <https://doi.org/10.25431/1824-307x/isj.v14i1.494-504>
- 20 635 Lockwood, B.L., Connor, K.M., Gracey, A.Y., 2015. The environmentally tuned transcriptomes  
21 636 of *Mytilus* mussels. *J. Exp. Biol.* 218, 1822–1833. <https://doi.org/10.1242/jeb.118190>
- 23 637 Marini, M., Jones, B.H., Campanelli, A., Grilli, F., Lee, C.M., 2008. Seasonal variability and Po  
24 638 River plume influence on biochemical properties along western Adriatic coast. *J. Geophys.*  
25 639 *Res.* 113, C05S90. <https://doi.org/10.1029/2007JC004370>
- 27 640 Martínez-Gómez, C., Bignell, J., Lowe, D., 2015. Lysosomal membrane stability in mussels.  
28 641 ICES Techniques in Marine Environmental Sciences No. 56.  
29 642 <https://doi.org/10.17895/ices.pub.5084>
- 31 643 Masella, A.P., Bartram, A.K., Truszkowski, J.M., Brown, D.G., Neufeld, J.D., 2012. PANDAseq:  
32 644 paired-end assembler for illumina sequences. *BMC Bioinformatics* 13, 31.  
33 645 <https://doi.org/10.1186/1471-2105-13-31>
- 35 646 Meistertzheim, A.-L., Lejart, M., Le Goïc, N., Thébault, M.-T., 2009. Sex-, gametogenesis, and  
36 647 tidal height-related differences in levels of HSP70 and metallothioneins in the Pacific oyster  
37 648 *Crassostrea gigas*. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 152, 234–239.  
38 649 <https://doi.org/10.1016/j.cbpa.2008.10.004>
- 40 650 Mezzelani, M., Fattorini, D., Gorbi, S., Nigro, M., Regoli, F., 2020. Human pharmaceuticals in  
41 651 marine mussels: Evidence of sneaky environmental hazard along Italian coasts. *Mar.*  
42 652 *Environ. Res.* 162, 105137. <https://doi.org/10.1016/j.marenvres.2020.105137>
- 44 653 Moore, M.N., 2004. Diet restriction induced autophagy: A lysosomal protective system against  
45 654 oxidative- and pollutant-stress and cell injury. *Mar. Environ. Res.* 58, 603–607.  
46 655 <https://doi.org/10.1016/j.marenvres.2004.03.049>
- 48 656 Múgica, M., Sokolova, I.M., Izagirre, U., Marigómez, I., 2015. Season-dependent effects of  
49 657 elevated temperature on stress biomarkers, energy metabolism and gamete development  
50 658 in mussels. *Mar. Environ. Res.* 103, 1–10. <https://doi.org/10.1016/j.marenvres.2014.10.005>
- 52 659 Musella, M., Wathsala, R., Tavella, T., Rampelli, S., Barone, M., Palladino, G., Biagi, E., Brigidi,  
53 660 P., Turrone, S., Franzellitti, S., Candela, M., 2020. Tissue-scale microbiota of the  
54 661 Mediterranean mussel (*Mytilus galloprovincialis*) and its relationship with the environment.  
55 662 *Sci. Total Environ.* 717, 137209. <https://doi.org/10.1016/j.scitotenv.2020.137209>
- 57 663 Quast, C., Priesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner,  
58 664 F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing

1  
2  
3  
4 665 and web-based tools. *Nucleic Acids Res.* 41, D590–D596.  
5 666 <https://doi.org/10.1093/nar/gks1219>  
6  
7 667 R Development Core Team, 2018. R: A language and environment for statistical computing.  
8 668 Vienna, Austria. <https://doi.org/R> Foundation for Statistical Computing, Vienna, Austria.  
9 669 ISBN 3-900051-07-0, URL <http://www.R-project.org>.  
10  
11 670 Rausch, P., Rühlemann, M., Hermes, B.M., Doms, S., Dagan, T., Dierking, K., Domin, H.,  
12 671 Fraune, S., von Frieling, J., Hentschel, U., Heinsen, F.-A., Höppner, M., Jahn, M.T.,  
13 672 Jaspers, C., Kissoyan, K.A.B., Langfeldt, D., Rehman, A., Reusch, T.B.H., Roeder, T.,  
14 673 Schmitz, R.A., Schulenburg, H., Soluch, R., Sommer, F., Stukenbrock, E., Weiland-Bräuer,  
15 674 N., Rosenstiel, P., Franke, A., Bosch, T., Baines, J.F., 2019. Comparative analysis of  
16 675 amplicon and metagenomic sequencing methods reveals key features in the evolution of  
17 676 animal metaorganisms. *Microbiome* 7, 133. <https://doi.org/10.1186/s40168-019-0743-1>  
18  
19 677 Roméo, M., Hoarau, P., Garello, G., Gnassia-Barelli, M., Girard, J.P., 2003. Mussel  
20 678 transplantation and biomarkers as useful tools for assessing water quality in the NW  
21 679 Mediterranean. *Environ. Pollut.* 122, 369–378. [https://doi.org/10.1016/S0269-](https://doi.org/10.1016/S0269-7491(02)00303-2)  
22 680 [7491\(02\)00303-2](https://doi.org/10.1016/S0269-7491(02)00303-2)  
23  
24 681 Rossi, F., Palombella, S., Pirrone, C., Mancini, G., Bernardini, G., Gornati, R., 2016. Evaluation  
25 682 of tissue morphology and gene expression as biomarkers of pollution in mussel *Mytilus*  
26 683 *galloprovincialis* caging experiment. *Aquat. Toxicol.* 181, 57–66.  
27 684 <https://doi.org/10.1016/j.aquatox.2016.10.018>  
28  
29 685 Schmidt, W., Power, E., Quinn, B., 2013a. Seasonal variations of biomarker responses in the  
30 686 marine blue mussel (*Mytilus* spp.). *Mar. Pollut. Bull.* 74, 50–55.  
31 687 <https://doi.org/10.1016/j.marpolbul.2013.07.033>  
32  
33 688 Schmidt, W., Rainville, L.C., Mceneff, G., Sheehan, D., Quinn, B., 2013b. A proteomic  
34 689 evaluation of the effects of the pharmaceuticals diclofenac and gemfibrozil on marine  
35 690 mussels (*Mytilus* spp.): Evidence for chronic sublethal effects on stress-response proteins.  
36 691 *Drug Test. Anal.* 6, 210–219. <https://doi.org/10.1002/dta.1463>  
37  
38 692 Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C<sub>T</sub>  
39 693 method. *Nat. Protoc.* 3, 1101–1108. <https://doi.org/10.1038/nprot.2008.73>  
40  
41 694 Sforzini, S., Oliveri, C., Orrù, A., Chessa, G., Pacchioni, B., Millino, C., Jha, A.N., Viarengo, A.,  
42 695 Banni, M., 2018. Application of a new targeted low density microarray and conventional  
43 696 biomarkers to evaluate the health status of marine mussels: A field study in Sardinian  
44 697 coast, Italy. *Sci. Total Environ.* 628–629. <https://doi.org/10.1016/j.scitotenv.2018.01.293>  
45  
46 698 Sheehan, D., Power, A., 1999. Effects of seasonality on xenobiotic and antioxidant defence  
47 699 mechanisms of bivalve molluscs. *Comp. Biochem. Physiol. - C Pharmacol. Toxicol.*  
48 700 *Endocrinol.* 123, 193–199. [https://doi.org/10.1016/S0742-8413\(99\)00033-X](https://doi.org/10.1016/S0742-8413(99)00033-X)  
49  
50 701 Shen, H., Kibria, G., Wu, R.S.S., Morrison, P., Nuggeoda, D., 2020. Spatial and temporal  
51 702 variations of trace metal body burdens of live mussels *Mytilus galloprovincialis* and field  
52 703 validation of the Artificial Mussels in Australian inshore marine environment. *Chemosphere*  
53 704 248, 126004. <https://doi.org/10.1016/j.chemosphere.2020.126004>  
54  
55 705 Simon, J.C., Marchesi, J.R., Mougel, C., Selosse, M.A., 2019. Host-microbiota interactions:  
56 706 From holobiont theory to analysis. *Microbiome* 7, 1–5. [https://doi.org/10.1186/s40168-019-](https://doi.org/10.1186/s40168-019-0619-4)  
57 707 [0619-4](https://doi.org/10.1186/s40168-019-0619-4)  
58  
59  
60  
61  
62  
63  
64  
65

1  
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54  
55  
56  
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58  
59  
60  
61  
62  
63  
64  
65

708 Strafella, P., Fabi, G., Despalatovic, M., Cvitković, I., Fortibuoni, T., Gomiero, A., Guicciardi, S.,  
709 Marceta, B., Raicevich, S., Tasseti, A.N., Spagnolo, A., Scarcella, G., 2019. Assessment  
710 of seabed litter in the Northern and Central Adriatic Sea (Mediterranean) over six years.  
711 Mar. Pollut. Bull. 141, 24–35. <https://doi.org/10.1016/J.MARPOLBUL.2018.12.054>

712 Utermann, C., Parrot, D., Breusing, C., Stuckas, H., Staufenberg, T., Blümel, M., Labes, A.,  
713 Tasdemir, D., 2018. Combined genotyping, microbial diversity and metabolite profiling  
714 studies on farmed *Mytilus* spp. from Kiel Fjord. Sci. Rep. 8, 1–13.  
715 <https://doi.org/10.1038/s41598-018-26177-y>

716 Vanwonterghem, I., Webster, N.S., 2020. Coral Reef Microorganisms in a Changing Climate.  
717 iScience 23, 100972. <https://doi.org/10.1016/j.isci.2020.100972>

718 Venier, P., De Pittà, C., Pallavicini, A., Marsano, F., Varotto, L., Romualdi, C., Dondero, F.,  
719 Viarengo, A., Lanfranchi, G., 2006. Development of mussel mRNA profiling: Can gene  
720 expression trends reveal coastal water pollution? Mutat. Res. - Fundam. Mol. Mech.  
721 Mutagen. 602. <https://doi.org/10.1016/j.mrfmmm.2006.08.007>

722 Vernocchi, P., Maffei, M., Lanciotti, R., Suzzi, G., Gardini, F., 2007. Characterization of  
723 Mediterranean mussels (*Mytilus galloprovincialis*) harvested in Adriatic Sea (Italy). Food  
724 Control 18, 1575–1583. <https://doi.org/10.1016/j.foodcont.2006.12.009>

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727 **Figure legends**

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

746  
747 **Fig 2. Transcriptional profiles of metabolic (*amil*, *pk*, *idp*),**  
748 **cytoprotective/detoxification (*lys*, *mt10*, *mt20*, *abcb*), antioxidant (*cat*, *gst*, *sod*), and**  
749 **lysosomal (*hex*, *ctsl*, *gusb*) mRNAs in females (♀) and males (♂) farmed**

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**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  $\pm$  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<sup>2</sup> 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

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

778  
779 **Fig 5. Variation of *M. galloprovincialis* DG microbiome composition according to**  
780 **sex.** (A) Principal Coordinates Analysis (PCoA) based on unweighted UniFrac distances  
781 between samples compositional profiles. Samples, color coded according to sex, showed  
782 a tendency to separate (permutation test with pseudo-F ratios, P-value  $\leq 0.2$ ). The  
783 percentage of variance in the dataset explained by each axis, first and second principal  
784 component (PCo1 and PCo2), is 21% and 12%, respectively. (B) Bar plot showing  
785 phylum-level mean relative abundance in male ( $\sigma$ ) and female ( $\text{♀}$ ) samples. Only phyla  
786 with relative abundance  $>1\%$  in at least 10% of samples are represented. **Colored figure**  
787 **is intended only for the online and PDF version.**

788  
789 **Fig 6. DISTLM analysis on the gene transcription dataset with superimposed**  
790 **correlation vectors with relative DG microbiome composition.** Results from the test  
791 of marginality related to the DISTLM analysis is reported in Table S4. DISTLM used the  
792 BEST selection procedure and adjusted  $R^2$  selection criteria. **Colored figure is intended**  
793 **only for the online and PDF version.**

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

Source	df	Pseudo-F	P(perm)
Season	2	21.897	<b>0.001</b>
Sex	1	32.611	<b>0.001</b>
Season x Sex	2	7.2798	<b>0.001</b>

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



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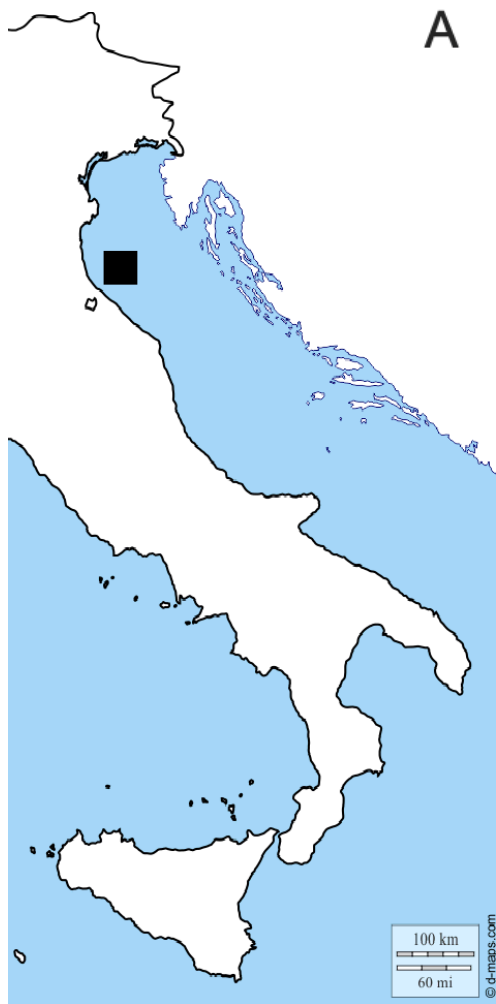
796 **Table 1. PERMANOVA results on transcriptional profiles (998 permutations).**

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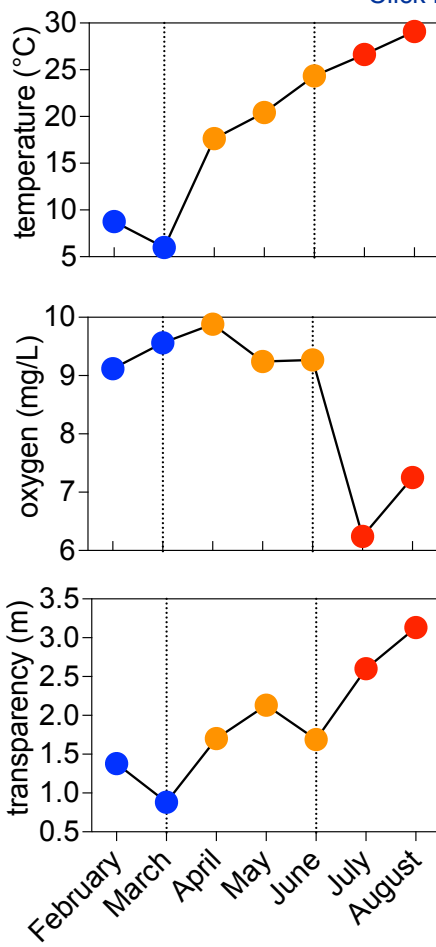
797 df: degree of freedom; Pseudo-F: F value by permutation (Anderson et al., 2008);  
798 P(perm): probability of pseudo-F.

Figure 1

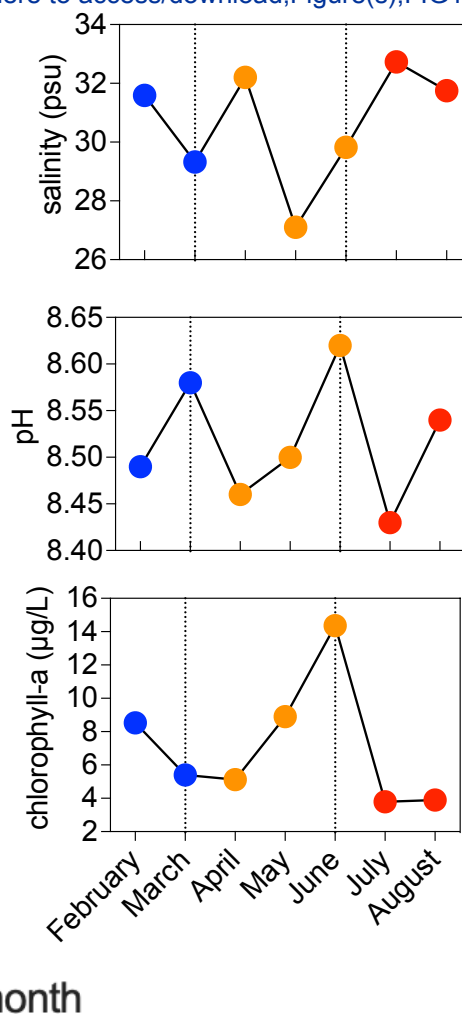
[Click here for access/download;Figure\(s\);FIG1\\_R2.eps](#)



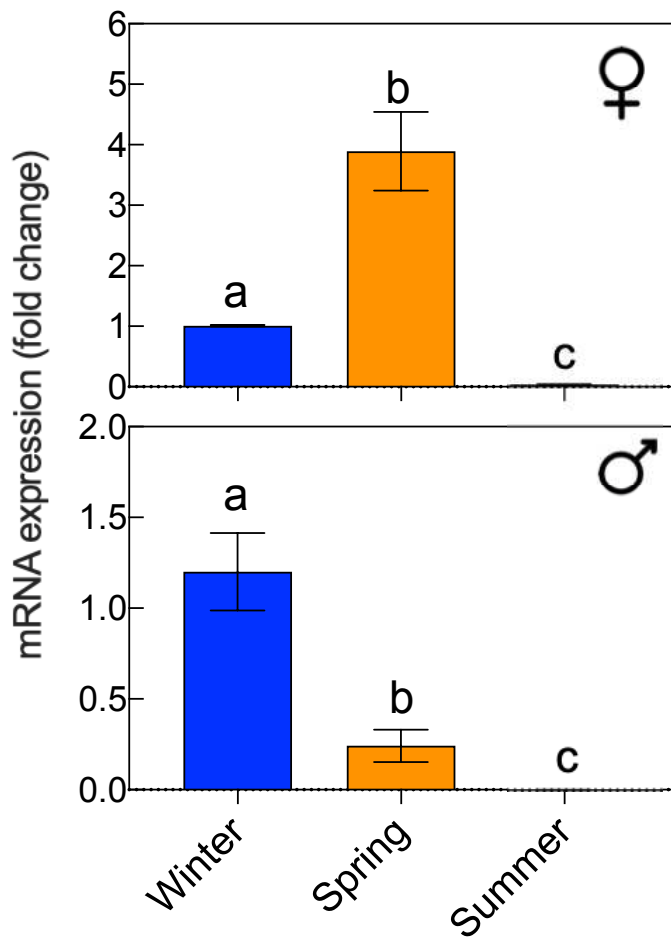
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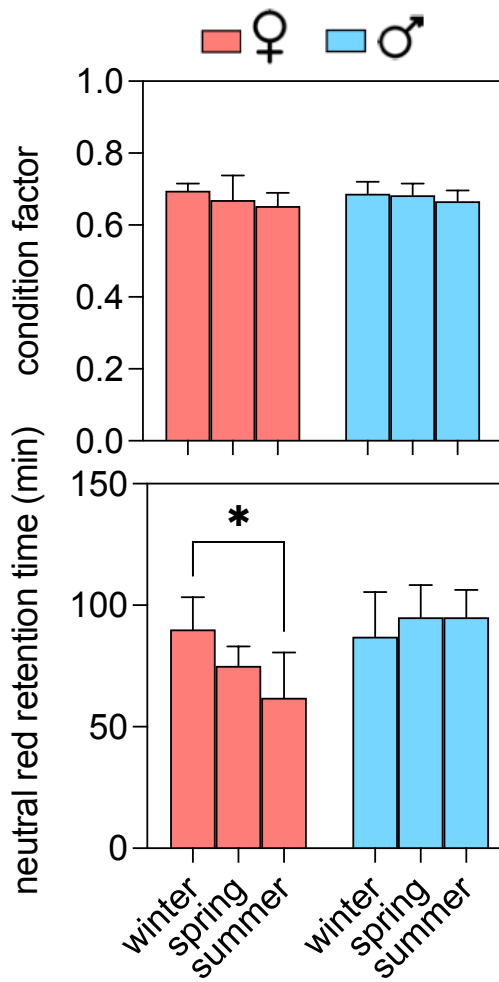
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Figure 2

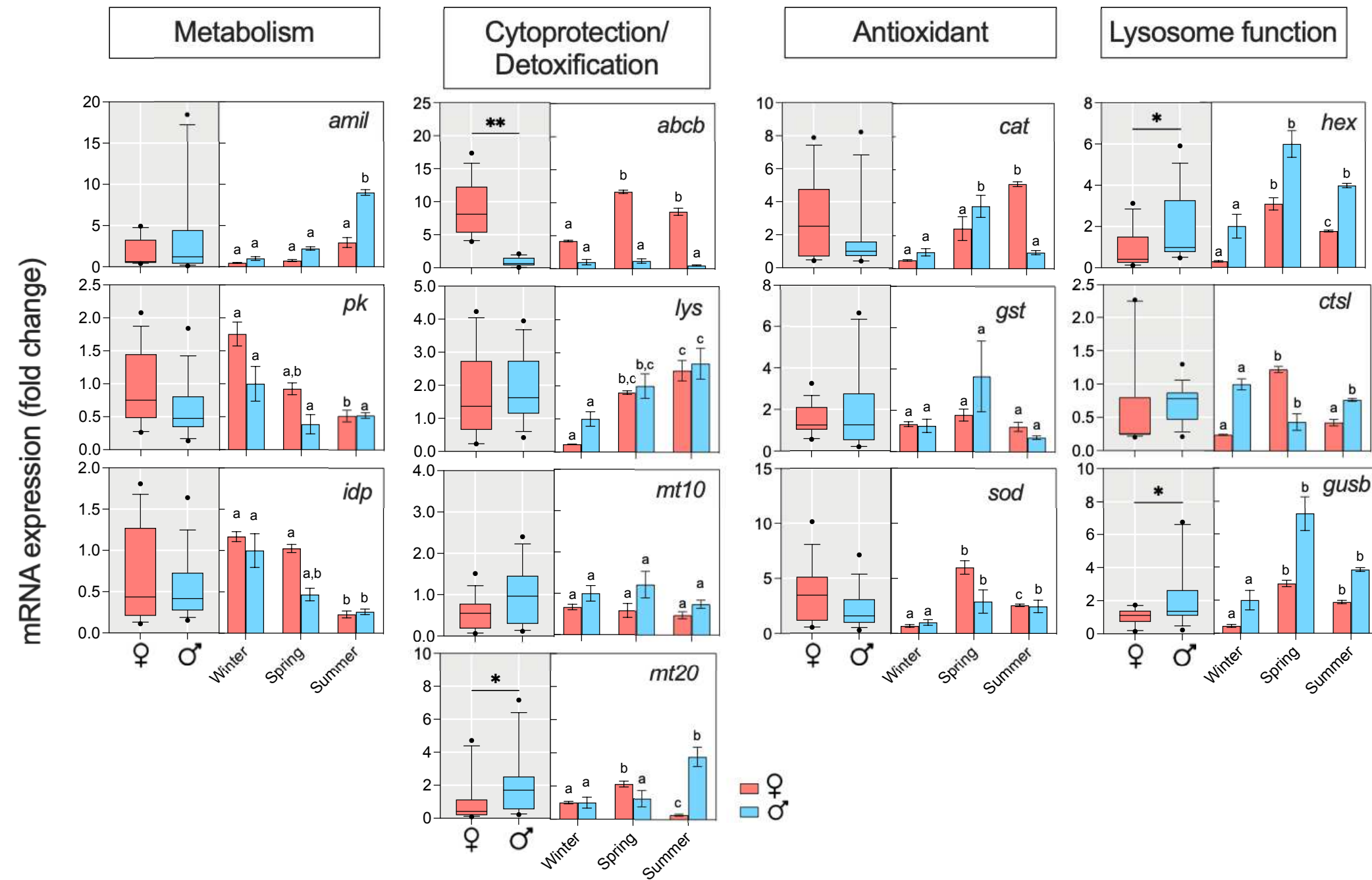


Figure 3

