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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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8 146 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 ΔC_T as $C_T(VCL) - C_T(VERL)$ (Anantharaman
35
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.

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_T 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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258 values, and a p-value ≤ 0.05 was considered as statistically significant. DISTLM used the
259 BEST selection procedure and adjusted R² selection criteria.

261 3. RESULTS

262 3.1. Mussel transcriptional profiles in digestive glands

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

269 Significantly different expression levels between males and females are observed
270 for *mt20*, *abcb* and *hex* (P < 0.05). All gene products showed complex transcriptional
271 patterns across season in both males and females (Fig 2), with a tendency to increased
272 (*amil*, *lys*, *mt20*, *abcb*, *cat*, *sod*, *hex*, *ctsl*, *gusb*) or decreased (*pk*, *idp*) expression from
273 winter to summer. DISTLM analyses performed on separate female and male datasets
274 by considering environmental parameters and gonadal maturation level (assessed
275 through *VCL/VERL* expression profiling) showed that in females temperature, salinity,
276 chlorophyll-a, and gonadal maturation explained most of the variation of the observed
277 transcriptional profiles (Fig 3). Among these explaining variables, the BEST/BioEnV
278 analysis showed that temperature and gonad maturation significantly correlated with
279 transcriptional profiles of females, while salinity, surface oxygen and transparency
280 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 ± 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 ($6\% \pm 11.4\%$ 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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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.**

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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 R² 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 ≤ 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.**

Fig 5. Variation of *M. galloprovincialis* DG microbiome composition according to 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 ≤ 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 (σ) and female (♀) 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.**

Fig 6. DISTLM analysis on the gene transcription dataset with superimposed correlation vectors with relative DG microbiome composition. Results from the test
790 of marginality related to the DISTLM analysis is reported in Table S4. DISTLM used the
791 BEST selection procedure and adjusted R^2 selection criteria. **Colored figure is intended**
792 **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	0.001
Sex	1	32.611	0.001
Season x Sex	2	7.2798	0.001

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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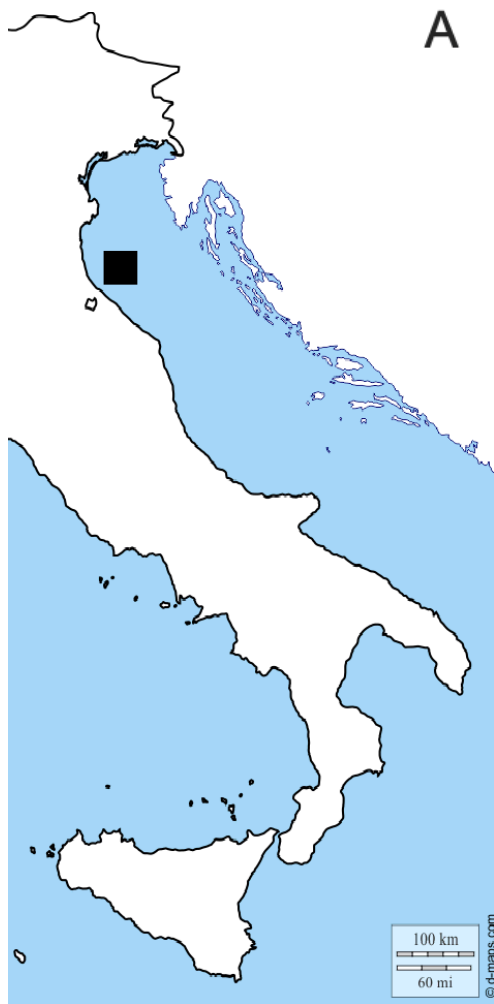
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Season	2	21.897	0.001
Sex	1	32.611	0.001
Season x Sex	2	7.2798	0.001

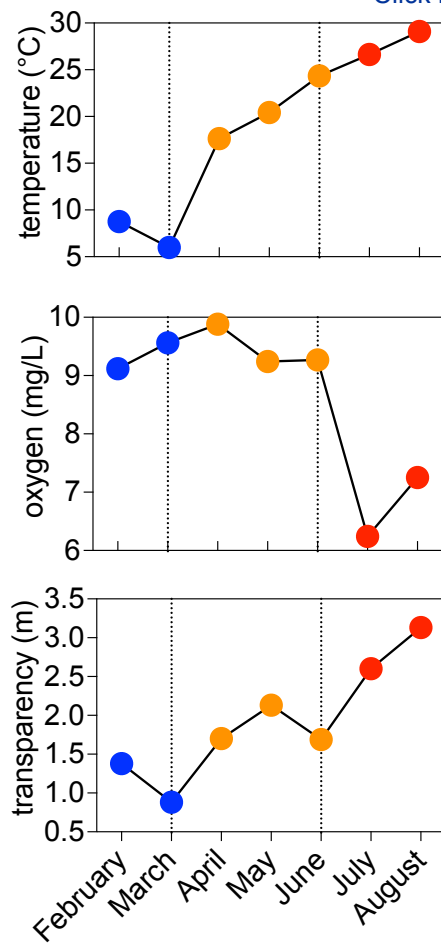
797 df: degree of freedom; Pseudo-F: F value by permutation (Anderson et al., 2008);
798 P(perm): probability of pseudo-F.

Figure 1

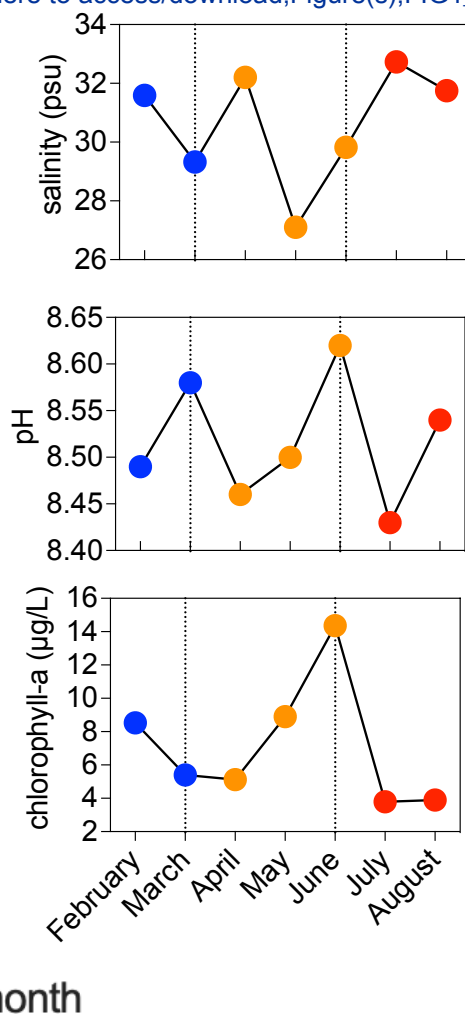
[Click here for access/download;Figure\(s\);FIG1_R2.eps](#)



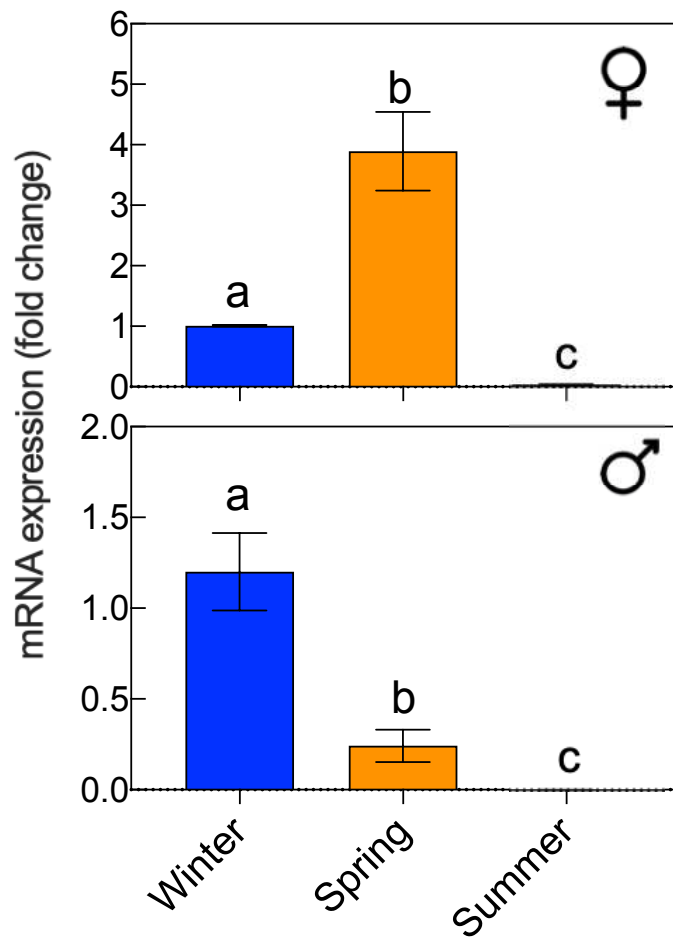
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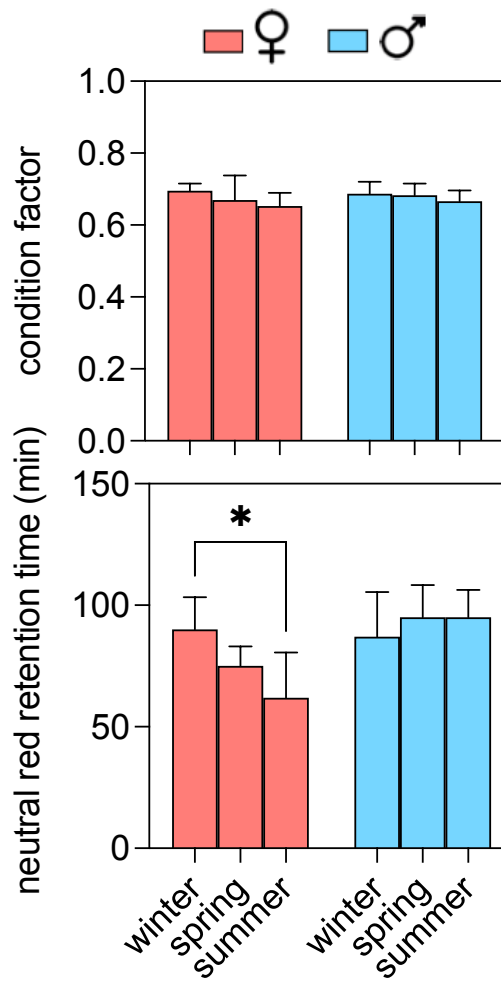
B



C



D



E

Figure 2

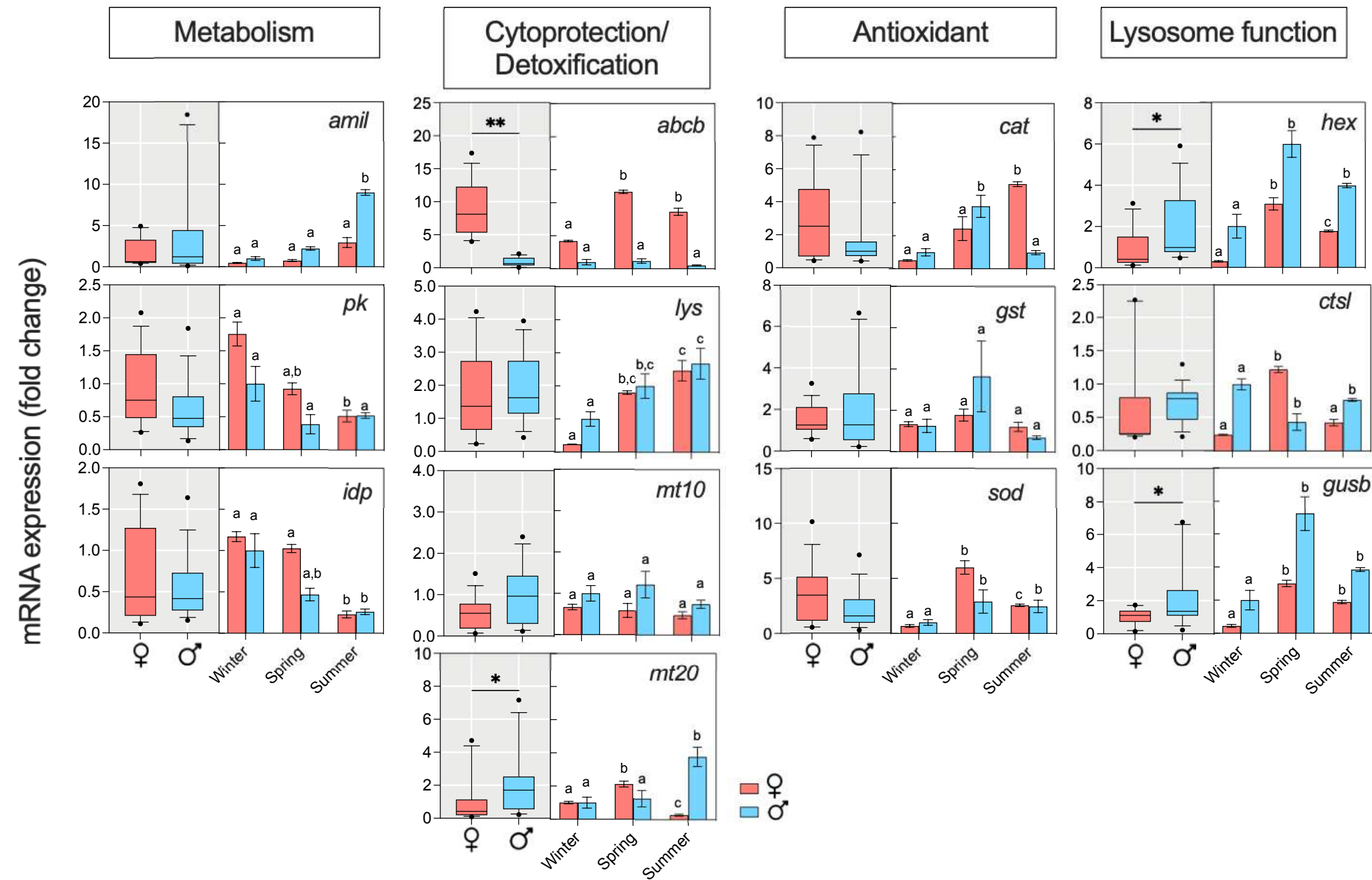


Figure 3

