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Impaired reproduction, energy reserves and dysbiosis: The overlooked consequences of heatwaves in a bivalve mollusc

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ABSTRACT

Extreme events like Marine Heatwaves (MHWs) are becoming more intense, severe, and frequent, threatening benthic communities, specifically bivalves. However, the consequences of non-lethal MHWs on animals are still poorly understood. Here, we exposed the Manila clam *Ruditapes philippinarum* to non-lethal MHW for 30 days and provided an integrative view of its effects. Our result indicated that albeit non-lethal, MHW reduced clam's energy reserves (by reducing their hepato-somatic index), triggered antioxidant defenses (particularly in males), impaired reproduction (via the production of smaller oocytes in females), triggered dysbiosis in the digestive gland microbiota and altered animals' behaviour (by impacting their burying capacity) and filtration rate. Such effects were seen also at RNA-seq (i.e. many down-regulated genes belonged to reproduction) and metabolome level. Interestingly, negative effects were more pronounced in males than in females. Our results show that MHWs influence animal physiology at multiple levels, likely impacting its fitness and its ecosystem services.

1. Introduction

In a world where human population is constantly growing, the best choice to meet the increasing protein demand of our species is centred around aquaculture. Currently, aquaculture produces more seafood than wild capture fisheries, and a great deal of aquaculture farming (~20 %) is based on bivalves (Hough, 2022), since their production is highly sustainable, and they have highly beneficial nutritional properties (Gentry et al., 2017). The majority (89 %) of bivalve production comes from aquaculture and the economic value of this sector has been estimated at 20.6 billion US$ per year (Wijsman et al., 2019). At present, mollusc aquaculture is mainly practiced in coastal ecosystems, such as lagoon, river deltas or coastal waters. These are among the most ecologically and socio-economically vital ecosystems on the planet, but are also threatened by Climate Change (CC). In fact, it is predicted that by 2090 the total area suitable for bivalve aquaculture will globally decrease by 10 % due to CC (Froehlich et al., 2018).

However, most recent models assessing CC impact on aquaculture and fisheries use predictions of mortality based on species' thermal tolerance ranges and the projected mean water temperature increase, e.g. (Bertolini and Pastres, 2021; Froehlich et al., 2018; Gentry et al., 2017; Lam et al., 2020). The vast majority of research on CC has focussed on the predicted average increase in water temperature (Smale et al., 2019), while climate extreme events such as marine heatwaves (MHWs), which are defined as daily sea surface temperatures exceeding the 90th percentile of climatological observations for >5 days (Hobday

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et al., 2016), have received much less attention. Unfortunately oceans are increasingly subject to MHWs, whose intensity duration and frequency are rising (Oliver et al., 2018; Oliver et al., 2021), and as stated by Oliver (2019) "much of the oceans will possibly reach a permanent MHW state by late twenty-first century". Only after 2003, the effects of MHWs have gained attention mostly because of the dramatic mass mortality events with which they have been associated. In example the mass mortality of molluscs leading to a shift in the benthic community of a lagoon (Munari, 2011); the mass mortality of benthic organisms along the coast of Australia (Wernberg et al., 2013), across the Mediterranean basin (Garrabou et al., 2009; Garrabou et al., 2022) and coral bleaching events (Smale et al., 2019).

While mortality is probably the most astonishing outcome of exposure to MHWs, hereby we advocate that MHWs constitute a present (and likely more severe) threat that is still largely underestimated. As stated by Minuti et al. (2022), "the impact of increased temperature on marine ectotherms depends on their physiological capacity to adjust to change". Even when MHWs do not trigger mass mortalities, they might cause non-lethal effects by impacting energy reserves with consequences on organismal fitness. Such effects include for example impacting the reproductive success of a marine copepod (Siegle et al., 2022) or altering stickleback's reproductive behaviour (Isotalo et al., 2022). In addition to this, it has been shown that MHWs can elicit sex-specific effects (i.e. when the response differs between males and females) in animals, such as changing the metabolic profile of mussels (Ellis et al., 2014) or significantly affecting the survival of gravid females in guppies (Breedveld et al., 2023).

Our current knowledge of the organismal response to MHWs at different levels of biological organisation is still fragmented (He et al., 2022). The use of next generation sequencing technologies has greatly advanced our knowledge in helping characterise the gene expression responses (Hu et al., 2022; Jahan et al., 2022; Xu et al., 2021) and in assessing changes in the microbiota (Li et al., 2023; Scanes et al., 2021a; Scanes et al., 2021b) of animals during MHWs. In general molecular and transcriptomics studies have evidenced how exposure to MHWs triggers upregulation of the stress response and the transcription of heat-shock proteins (e.g. (De Marco et al., 2023)) while a recent meta-analysis on the effects of temperature on the microbiota found a decrease in microbiome alpha diversity. However most of these studies does not include evidence from multiple sources (e.g. transcriptomics with physiological data) and, as stated by De Leij et al. (2022), a whole organism approach is necessary to understand how MHWs trigger the deterioration of organismal functioning. Such a knowledge is pivotal and should be integrated in prediction models to correctly assess the impact of CC on marine resources and habitats.

Additionally, most controlled experiments investigating the effects of MHWs have focussed on relatively short MHWs (i.e. from 3 to 10 days, e.g. (Dominguez et al., 2020; He et al., 2022; Isotalo et al., 2022; Jahan et al., 2022; Scanes et al., 2020; Xu et al., 2022b)), thus ignoring longer MHWs, despite the fact that, according to recent models, their average duration will increase from ~5 days in 2001 to ~30 by 2050 under RCP4.5 (Darmaraki et al., 2019; Galli et al., 2017). Recently, Isotalo et al. (2022) demonstrated the importance of considering the duration of HWs when investigating their impact. To gain a deeper understanding of the different biological processes that are affected by MHWs, we exposed adult Manila clams *Ruditapes philippinarum* to a non-lethal HW of 30 days. Manila clam is among the most widely farmed mollusc species worldwide (its aquaculture sector was worth 162.1 million € in 2016 (STECF, 2018)) and is a key ecological species in its native habitats as well as where it has been introduced (i.e. Europe, North America). Interestingly, in certain areas which have long been associated with high clam production, such as the Venice lagoon, a sharp decline in clam stock has been reported since 2003 and a potential link to MHWs has been suspected (Ponti et al., 2017).

We hypothesized that, when exposed to 30 days long MHW at temperatures within the species thermal tolerance range, animals might not be able to fully adapt to the prolonged thermal stress despite a significant energetic investment which would reduce energy reserves; this would in turn impact energy expensive processes like reproduction with consequences that potentially hinder the species fitness.

2. Materials and methods

2.1. Clam maintenance

A population of 240 Manila clam *Ruditapes philippinarum* (average shell length 25.7 ± 1.9 mm, average weight 4.11 ± 1.0 g) were purchased from the hatchery SATMAR (France) in February 2021 and kept in eight 25 L aquaria with artificial sea water (ASW, Aquaforest Sea Salt) at a salinity of 32 PSU and 20 °C for 15 days, with air stones providing constant water oxygenation. Manila clam is known to be a sequential hermaphrodite species (Lee et al., 2013) in which a small proportion of individuals (about 19 %) can change sex from one year to another. However according to Lee et al. (2013) the sex reversal process takes place following the spawning season, which finishes in September/October, and it is fully completed before the next reproductive season, which starts in February/March (Drummond et al., 2006; Meneghetti et al., 2004), and thus any sex reversal process was completed by the time our experiment took place (i.e. March).

Animals were fed once per day with New Coral Fito Concentrate (A. G.P., Italy), a commercial mixture of microalgae composed by *Isochrysis* (T-Iso) (33.3 %) + *Nannochloropsis* (31 %) + *Tetraselmis* (18 %) + *Phaeodactylum* (18 %), at a final concentration of ~40 * 10^6 cell L^{-1}. About 40 % of the water from each tank was replaced three times per week with new ASW at the appropriate temperature. At the beginning of the experiment, all clams were measured (shell length, height, and width by a calliper with precision ±0.05 mm and wet weight by a precision scale (Sartorius, Fisher Scientific) with precision ±0.001 g) and were subsequently randomly allocated to one of the two treatment groups (controls – CTRL or heatwave – HW) by using the R library randomizr/v0.24 with the function "complete_ra".

2.2. Overview of the experimental design

The overall aim was to look at the effects of a heatwave on behavioural, physiological and molecular traits. Specifically we wanted to test heatwave effects on burying behaviour, filtration rate, haemocyte count and viability, condition index, hepato-somatic index, fertility, enzymatic activity of Superoxide dismutase (SOD), Catalase (Cat), Glutathione peroxidase (GPx) and Phenoloxidase (PO), transcriptomics, metabolomics and microbiota of clams. To do so, we randomly assigned clams to either the control (CTRL) or heatwave (HW) treatment (HW duration and intensity are detailed in "Experimental Set-up"). Each treatment was replicated on 4 independent tanks. A total of 240 clams were used (30 clams for each tank; 120 clams for each treatment). After the experiment 5 clams from each tank were randomly collected and used to measure all listed traits (except for burying behaviour and filtration rate where a different set of individuals was used). For burying behaviour 20 clams from each tank were randomly collected while for filtration rate 3 clams from each tank were collected. When possible, results were presented by sex (the species does not have sexual dimorphism, hence the determination of sex was performed a posteriori after the collection of tissue samples) to disentangle the effect of the heatwave on the two sexes.

2.3. Experimental set-up

The experimental set-up consisted of two conditions: HW clams were exposed to a simulated Marine Heatwave (MHW) of 30 °C for 30 days whereas CTRL clams were kept at 20 °C for 30 days. Each condition was run in quadruplicate and each experimental replicate consisted of a 25 L experimental tank with 30 randomly allocated clams. After random clam allocation to the treatment, the temperature was gradually

increased over the course of two days from 20 °C to 30 °C in the HW aquaria only. Once temperature reached the desired value, the experiment was started. Temperature was controlled with 100-W water-heaters AQL AquaT (Italy) set at 20 or 30 °C. Temperature loggers iButton (Maxim Integrated, USA, model: DS1921H-F5) were placed in each tank to ensure constant monitoring of the water temperature. Throughout the experiment, animal maintenance (i.e. water changes and feeding regime) followed the procedures detailed in the previous section and clam mortality was assessed daily. Differences in survival between treatments were assessed and plotted using the survminer/v0.4 library in R/v4.0 (R Core Team, 2014).

2.4. Statistical analyses

For each quantified parameter, normality and homogeneity of variances were assessed using the "shapiro.test" and "var.test" functions of base R. If variables did not meet normality and/or homogeneity of variances, an attempt to normalise them was performed using the bestNormalize/v1.8 library. Statistical differences between treatments were then assessed and plotted using the "ggbetweenstats" function from the ggstatsplot/v0.8 library: if normality and homogeneity of variances were granted, a Student *t*-test was performed; if data were normally distributed but had unequal variances, a Welch two-sample t-test was performed; if data were not normally distributed, a non-parametric Mann-Whitney test was performed. Within each plot, the figure subtitle contains all the statistical details concerning the statistical analysis that was performed.

2.5. Behavioural measurements

At the end of the experiment, a subset of clams was used for behavioural measurements. For each treatment, two tanks with ~4 cm of sand (filtered and autoclaved) at the bottom and ~5 cm of ASW overlay were prepared at appropriate temperature (i.e., 20 °C for CTRL, 30 °C for HW). Twenty clams from each of the technical replicate tanks were gently located at the top of the sand. Animal behaviour was recorded using a Logitech C920 PRO HD camera (Logitech, Switzerland) for 2 h. Subsequently, each video was analysed (in a double-blind set-up) in order to test for differences among groups in: *i)* the percentage of clams that successfully buried (i.e. each clam for which only the two siphons were visible was considered as "successfully buried"); *ii)* the burrowing speed (i.e. the amount of time elapsed between the beginning and the completion of the burrowing movements); *iii)* the reactivity (i.e. the time elapsed between the start of the observation and the first burrowing movements). Differences between treatments in the proportion of clams that buried were assessed with a Pearson's χ^2 test within the "ggbarstats" function of the ggstatplot package.

2.6. Clam filtration rate

At the end of the experiment, three clams from each of the technical replicate tanks were used to quantify the filtration rates, with slight modifications from Carneiro et al. (2020) and Munari and Mistri (2007). Briefly, individual clams were placed in clean glass beakers with 90 mL of ASW at the respective treatment temperature. Beakers were kept in a water bath to ensure the desired temperature. Each clam was fed with 775 μL of the commercial solution of algae (as specified above) at the initial concentration of $8.1 * 10^5$ cells L^{-1}. Immediately after the administration of food and subsequently every 30 min, a 10 μL sample of water was collected and placed on a counting chamber Fast-Read 102® (Biosigma) and the number of cells was counted on an inverted microscope (Olympus IX50) according to manufacturer's recommendations. The experiment was terminated 2 h after the initial administration of food. For each treatment, an empty beaker with ASW and algae (but without clams) was run as negative control, to check that algal concentration was not decreasing in the absence of clams. For each individual clam, the decrease in algal concentration with time was plotted and the slope of the linear regression was calculated with R/v3.6 (R Core Team, 2014) using the "lm" function. Since the algal concentration was deemed to decrease with time, only slopes smaller than 0 were retained. Then the filtration rate was obtained by dividing the absolute value of the calculated slope by the wet weight of each animal, measured with the precision scale mentioned above.

The temperature coefficient (Q_{10}) for the filtration rate was calculated, in accordance with Han et al. (2008):

$$Q_{10} = (R_{HW}/R_{CTRL})^{10/(tHW-tCTRL)}$$

where R_{HW} and R_{CTRL} are the average filtration rates in HW and CTRL treatments, respectively and t_{HW} and t_{CTRL} are the treatment temperatures (30 °C and 20 °C, respectively).

Values of Q_{10} calculated at different temperatures in *R. philippinarum* were retrieved from the literature, plotted using ggplot2/v3.4. The linear dependence of the filtration rate on temperature was tested using Prism/v9 (https://www.graphpad.com/scientific-software/prism/) with the module "non-linear fit" where a linear regression model (i.e. representing the linear dependence) was compared with a second order polynomial (quadratic) model by performing an Extra sum-of-squares F Test with a significance threshold value of 0.05. The best fitting model was plotted using the "geom_smooth" function of ggplot2 package and its equation and R^2 value were added on the plot.

2.7. Analysis of haemocyte parameters by flow cytometry

All haemocyte parameters were analysed with a CyFlow Space (Sysmex-Partec, GmbH, Sysmex Europe, Germany). The flow cytometer was equipped with two specific lasers (blue with an emission length of 488 nm and red with an emission length of 638 nm) and a True Volumetric Absolut Counting system to obtain the absolute count of events. Haemolymph extraction, processing and flow cytometry analyses were performed in accordance to Flye-Sainte-Marie et al. (2009); Lopez et al. (1997); Rolton and Ragg (2020), with slight adjustments.

At the end of the experiment, 100 μL of haemolymph were taken by carefully inserting a 27G needle with an insulin syringe in the abductor muscle of each clam ($n = 8$ clams from each treatment). Haemolymph was immediately mixed with 325 μL of an Anti-aggregant solution (AAS) from Lopez et al. (1997) (AAS: 20.80 g/L glucose, 8.00 g/L sodium citrate, 3.36 g/L EDTA, 22.50 g/L sodium chloride in distilled water) and 325 μL of ASW (salinity 30 PSU). The mixture was filtered through a 80 μm mesh to remove large debris, as suggested by Flye-Sainte-Marie et al. (2009) and immediately processed as suggested by Rolton and Ragg (2020).

Haemocyte counts and viability were assessed by adding 10 μL of Propidium Iodide (PI) (ab14083, Abcam) at the initial concentration of 1 mg/mL and 50 μL SYBR Green 10× (Sigma) to the haemolymph solution and left to incubate in the dark for 20 min at room temperature. SYBR Green can stain live and dead cells containing DNA; whereas Propidium Iodide stains only dead cells. SYBR Green fluorescence is detected by the FL1 channel, and PI fluorescence is detected by the FL3 channel. Total haemocyte count was obtained gating haemocytes stained by SYBR Green to exclude other particles in the haemolymph, in accordance with Flye-Sainte-Marie et al. (2009). The percentage of dead cells in each sample was estimated in a of FL1 vs FL3 density plot.

2.8. Physiological measurements

At the end of the experiment, physiological and molecular parameters have been assessed in 5 clams from each of the technical replicate tanks for each treatment (in total 20 HW clams and 20 CTRL clams). Initially, each clam was individually measured as detailed in the previous section, then the entire soft tissue was dissected, blotted on paper, and weighted on a precision scale. In particular, the digestive gland was

dissected, blotted on paper, and weighted on a precision scale. The weight of the empty shell was measured as well and used, together with the weight of the soft tissue and digestive gland, to calculate the Hepato-Somatic Index – HSI – and the Condition Index – CI. HSI and CI (following Gvozdenović et al. (2020)) were defined as follows:

$$HSI = weight_{digestive\ gland} / weight_{soft\ tissue}{}^{*}\ 1000$$

$$CI = weight_{soft\ tissue} / weight_{empty\ shell}$$

The digestive gland was then stored in RNAlater® (ThermoFisher, USA) for transcriptomics and 16S DNA barcoding analyses. Mantle and foot samples were dissected from the same clams for enzymatic assays and metabolomic analyses and stored in accordance with the specific requirements for each analysis (see details in the following sections).

Due to the relatively small size of the animals and the numerous samples that were collected from each of them for the different assays, the sex of these animals was identified using a Machine Learning (ML) approach as detailed below in a specific section.

2.9. Enzymatic assays

Enzymatic activity of Superoxide dismutase (SOD), Catalase (Cat), Glutathione peroxidase (GPx) and Phenoloxidase (PO) and total protein content were assessed on a portion of the mantle tissue dissected from clams (as detailed in "Physiological measurements"), and immediately stored at −80 °C until processing (in accordance with Richard et al. (2016)). Each sample was homogenised with an IKA T8.01 ULTRA-TURRAX in 300 μL of cold homogenisation buffer composed of NaCl (150 mM), EDTA (1 mM), EGTA (1 mM), Phosphatase Inhibitor cocktail (1 %), Triton X100 (1 %) Tris HCl (10 mM) and centrifuged at 10000*g* for 55 min at 4 °C. The resulting supernatant was aliquoted and stored at −80 °C until further processing.

Each sample was analysed in duplicate on 310 μL transparent 96-well plates (SARSTEDT, model 82.1582.001) and assayed on a Multiskan Go 1510 Microplate Spectrophotometer (Thermo Fisher). In each assay, negative controls (blanks) were run in duplicate, and the average absorbance value of the blank was subtracted from the absorbance values of each sample. For total protein content, SOD, Cat and GPx assays, a standard curve was produced via the addition of a positive control and a linear regression (by considering at least 4 different dilutions of the standard) was fitted. The results of each assay were not considered valid if the R^2 of the linear regression was below 0.8.

Total protein content in each sample was assessed using the Bradford method (Bradford Reagent, B6916, Sigma) using a protein standard (P0914, Sigma) to produce a standard curve, following manufacturer's instructions. For each sample, the enzymatic activity was normalised by the total protein content of the corresponding sample.

SOD activity was assessed by using the commercial kit Superoxide Dismutase Activity Assay Kit (CS0009, Sigma), as recommended by the manufacturer.

Cat, GPx and PO were quantified in accordance with Richard et al. (2016), with slight modifications. Cat was assessed aliquoting in a 96-well plate 3 μL of each sample with 260 μL of a 50 mM pH 7.0 potassium phosphate buffer solution with 10 mM H_2O_2. After 2 min at room temperature, the absorbance was read at 240 nm for 120 s. A Catalase standard (C9322, Sigma) was used to produce a standard curve.

Total GPx was measured by using 25 μL of each sample and mixing it with 100 μL of an assay solution (62.5 mM PBS, pH 7.4; 6.25 mM EDTA; 0.5 mM NADPH; 2.5 mM Reduced glutathione (GSH); 2.5 U/mL Glutathione reductase (GR); 1.875 mM Cumene hydroperoxide). The absorbance of the solution was immediately read at 340 nm and continuously logged for 3 min. A Glutathione peroxidase standard (G6137, Sigma) was used to make a standard curve.

PO activity was measured by incubating 50 μL of each sample with 50 μL of Tris-HCl buffer (0.1 M, pH 8.0) for 10 min at 25 °C. Then, 100 μL of 0.04 M L-DOPA were added and the increase in absorbance was monitored for 30 min at 492 nm.

2.10. RNA-seq analyses

RNA-seq analyses were performed on the dissected digestive gland tissues of clams (as detailed in "Physiological measurements"). Digestive gland is mostly composed of glandular tissue, but it also contains some reproductive tissue, since the gonads in *R. philippinarum* form a sheath around the digestive gland, as reported by Çolakoğlu and Palaz (2014). After dissection, samples were stored in RNAlater at −20 °C. RNA was extracted using RNeasy Mini Kit (Qiagen, Germany) and its purity, concentration and integrity were assessed on a Qubit Fluorometer (Invitrogen, USA) and a Tape Station (Agilent, Germany). The extracted RNA was used for both gene expression and microbiota analyses (16S). QuantSeq 3′ mRNA-Seq Library Prep Kit FWD for Illumina (Lex- ogen GmbH, Vienna, Austria) was used to prepare all RNAseq libraries, which were then sequenced on an Illumina Novaseq 6000, with a single-end 75 bp sequencing setup. The sequences obtained are available in NCBI (BioProject ID: PRJNA906528).

Sequencing reads were quality-checked using FastQC/v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/); low-quality reads and adaptors were removed with the program BBDuk (program specific options were taken from Lexogen's website at https://www.lexogen.com/quantseq-data-analysis/) of the suite BBTools (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/). High-quality trimmed reads were then mapped on the clam genome (Xu et al., 2022a) using STAR /v2.7.3a (Dobin et al., 2013) (program specific options were taken from Lexogen's website). Gene counts were produced using the program featureCounts/v2.0.0, with the option "–primary".

The generated count table was imported in R where it was split according to the sex of the animals (see next section for relevant details) and gene expression data was analysed separately per each sex. Then, sex-specific counts comprising HW and CTRL clams were filtered to retain gene transcripts that had >5 reads across 70 % of all analysed samples, thus removing genes with low coverage that would increase background noise (Peruzza et al., 2021; Verma et al., 2021). A total of 15,698 and 14,392 genes were retained for female and male clams, respectively. Counts were then normalised using the "RUVs" function built in the RUVseq/v.1.30 package, with a number of unwanted factors "k = 1" for both count tables. Differential expression (DE) analysis was performed with the package edgeR/v3.38 by using a likelihood ratio test with the functions "glmFit" and "glmLRT" on the comparison "HW vs CTRL". Genes with FDR < 0.05 were deemed significant.

Functional analysis on the Differentially Expressed Genes (DEGs) was carried out with the package clusterProfiler/v4.4.4. Briefly, gene transcripts were initially annotated on the base of sequence similarities between the clam's predicted protein set and the Ensembl human proteome by means of protein BLAST (BLASTp – Basic Local Alignment Search Tool (Altschul et al., 1990)) with a stringent *E*-value cut-off $<10^{-6}$ (instead of the commonly used threshold of 10^{-3} for BLASTp). BLASTp was preferred over a homology-based approach (e.g. OrthoFinder (Emms and Kelly, 2019)) due to the taxonomic distance between mollusc and human, which would have left most of the clam's genes without an orthologous and hence with no functional annotation. The lists of DEGs were then separately subjected to over-representation analysis with the function "enricher" of the clusterProfiler package that implements a hypergeometric test. As suggested by Wijesooriya et al. (2022), all genes that passed the filtration criteria during DE analysis (i.e. 15,698 and 14,392 genes for the female and male datasets, respectively) were used as background in the analysis and a FDR threshold <0.15 was set to deem significant pathways. Functional databases (e.g. Gene Ontology, Reactome, KEGG) were downloaded from the gProfiler's web page (https://biit.cs.ut.ee/gprofiler/gost) and used in the analysis.

Heatmaps of DEGs were generated with the ComplexHeatmap/v2.14 package while Principal Component Analysis plots, bar plots and dot-plots of the over-representation analysis were all generated using ggplot2/v3.4.

2.11. Determination of sex

In relation to the physiological measurements, enzymatic assays and -omics analyses, the sex of clams was identified using a Machine Learning (ML) approach, because of the relatively small size of the animals and the numerous tissue samples that were collected for the different assays, which prevented the application of histological approaches for the determination of sex.

The approach used to sex animals was based on the application of ML algorithms that were trained to recognise the transcriptome of the digestive gland of known male and female clam samples from a reference population which was of comparable size, origin and was sampled in the same period of the year of our study population. To achieve this, the digestive glands of 6 reference males and 6 reference females (identified by observing gametes under the microscope) were dissected as described above and stored in RNAlater®. RNA was extracted, processed and RNA-seq libraries were sequenced and processed to obtain count tables as detailed above. RNAseq libraries for such reference population have been deposited on NCBI (BioProject ID PRJNA906977).

The count table was imported in R and analysed using the package DaMiRseq/v2.8, following the author's recommendations. Briefly, genes with <5 counts in at least 40 % of the libraries were removed, together with genes whose coefficient of variation was >3. Genes were then normalised using the "vst" transformation before removing the effect of surrogate variable via the "DaMiR.SVadjust" function. Then, we trained our ML model on 8 out of 12 samples using Cross-fold validation by: *i)* identifying a subset of robust features to discriminate classes with the functions "DaMiR.FSelect", "DaMiR.FReduct" and "DaMiR.FSort"; *ii)* building an Ensembl prediction model with "DaMiR.EnsL_Train". Finally, the trained model was tested on the 4 samples that were not used during the training phase; we assessed that the trained model was able to correctly discriminate male and females with 100 % accuracy.

Once the training of the ML algorithm was complete, we then uploaded the count table of the clam samples whose sex was unknown and, following the protocol designed by Chiesa et al. (2018), we predicted the sex of the unknown samples by running the "DaMiR.EnsL_Predict" function.

2.12. ^{1}H NMR metabolomics

The metabolic profiles of clams were determined on a portion of the foot, dissected as described above, and immediately stored at −80 °C until processing. Metabolites were extracted using the methanol-chloroform extraction technique described in Eymann et al. (2020) with slight modifications. Frozen samples were homogenised in cold methanol (8 mL g^{-1} tissue) using an IKA T8.01 ULTRA-TURRAX, then MilliQ water was added (2.5 mL g^{-1} tissue) and the mixture was shaken for 10 min at room temperature. Subsequently, chloroform (8 mL g^{-1} tissue) and MilliQ water (4 mL g^{-1} tissue) were added, the solution was vortexed for 1 min and left on ice for 10 min; samples were then centrifuged for 15 min at 6000*g* and 4 °C. Finally, the upper water-methanol layer containing polar metabolites was separated from the lower chloroform layer, containing apolar metabolites. Both fractions were freeze-dried overnight (−56 °C, <0.100 mbar; Alpha 1–2 LDplus, Martin Christ) and stored at −28 °C until analysis. The polar fraction was resuspended in 600 μL of deuterated water (D_2O) containing a K_2HPO_4/KH_2PO_4 buffer solution at pH 7.1 as suggested by Bertini et al. (2014), while the apolar fraction was resuspended in 600 μL of deuterated chloroform ($CDCl_3$). Both fractions were then transferred into a 5-mm NMR tube and analysed. NMR spectra were acquired with a Bruker Avance Neo 600 MHz spectrometer (Bruker BioSpin, Karlsruhe, Germany) equipped with a Prodigy cryoprobe and using Topspin 4.1 software (Bruker).

Polar extracts were acquired with a "noesypr1d" pulse sequence with spectral width (SWH) 11,904 Hz, acquisition time (AQ) 2.75 s, relaxation delay (D1) 10 s, receiver gain (RG) 101, 32 scans (NS), and 4 dummy scans (DS). Apolar extracts were acquired with a "zg" pulse sequence with SWH 11904 Hz, AQ 2.75 s, D1 10 s, RG 39, NS 32, and DS 4.

Acquired data was pre-processed in Topspin 4.1: free induction decays were multiplied by an exponential function equivalent to a 0.3 Hz line-broadening before applying Fourier transform. Transformed spectra were phase-corrected automatically. 1D-NMR spectra were then processed in R 4.1.2 using the tidyverse/v1.3.1, ASICS/v2.8.0, AlpsNMR/v3.2.3, PepsNMR/v1.10.1, speaq/v2.6.1 packages, and custom scripts. Spectra from polar and apolar extracts were interpolated into two separated datasets, baseline-corrected, and referenced to the *N*-trimethyl- resonance from Betaine (3.27 ppm, polar exacts) and Chloroform (7.26 ppm, apolar extracts). Signal intensity from water (4.7–5 ppm, polar extracts) and chloroform regions (7.20–7.30 ppm, apolar extracts) were set to 0, then spectra were normalised by total peak area and aligned to compensate for minor sample-specific variations in chemical shifts. Data binning was performed by bucketing and peak detection. Statistical analysis was performed using the metaboanalystR/v3.0.0 package: statistical significance was assessed using two-sample *t*-test, setting the FDR threshold to 0.1; significant signals were filtered by Fold Change, setting a minimum threshold of 1.5. Principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA) were performed to discover the features involved in group-specific variations. Relevant signals were assigned by submitting their chemical shifts to public databases and tools such as GISSMO (https://doi.org/10.1021/acs.analchem.7b02884), the Human Metabolome Database (https://pubmed.ncbi.nlm.nih.gov/34986597/) (HMDB), the Biological Magnetic Resonance Database (https://doi.org/10.1093/nar/gkm957) (BMRB).

Relative intensity, multiplicity, and J coupling for each assigned signal of each identified compound. Putative assignments were further characterised and confirmed with targeted 1H—2D TOCSY and 1H—1D TOCSY experiments.

2.13. Clam-associated microbiota

The RNA extracted from the digestive gland of clams and used for both transcriptomic and microbiota analysis. Briefly, Superscript IV kit (Invitrogen, Life Technologies, Monza, Italy) was used to reverse-transcribe 1 μg of RNA to cDNA. Diluted cDNA 0.2 ng/μL with specific primers (at the concentration of 10 μM) for the V3-V4 region of the bacterial 16S rRNA were used for library preparation as described by Bernardini et al. (2021). Libraries and sequencing were performed by BMR Genomics (Padova, Italy). The final libraries were then sequenced with MiSeq Illumina 300 PE. The sequences obtained are available in NCBI Sequence Read Archive (BioProject ID: PRJNA906528).

Sequences were analysed according to the pipeline developed by Callahan et al. (2016). Reads were filtered based on their quality using the package dada2/v1.14.1 on R/v3.6.3. Then, reads were de-replicated, obtaining a unique representative sequence, and finally, they were merged. The sequences obtained were then clustered using the Amplicon Sequence Variants approach (ASV), and chimera sequences were removed at the end of the process. ASVs were taxonomically classified up to genus level using the SILVA 16S Database (NR98 version 138.1). To remove low-abundance and rare taxa, a prevalence threshold was set at 0.05 % (out of 1) for the entire dataset, resulting in the exclusion of all unique sequences (singletons). Taxon raw counts were then transformed into relative abundance levels. The taxonomic table, ASV counts, and sample metadata were finally merged in a phyloseq object (using the package phyloseq/v1.30.0), and a set of explorative analyses was performed to investigate microbiome composition:

Faith's Phylogenetic Diversity index was estimated for each sample using picante/v1.6.1; Beta diversity was calculated basing on Unweighted UniFrac distance and validated through Adonis algorithm from the vegan/v2.4.2 package, while dispersion was calculated using "betadisper" function from the same package. Taxonomic significant differences between groups were assessed with the DESeq2 algorithm from DESeq2/v1.12.3 package.

A Canonical Correlation Analysis (CCA) was performed to associate microbiome taxonomic variables with sample metadata. A permutation analysis to assess the significance of enzymatic variables that fitted with the ordination was applied through the "envifit" function from the vegan package. The correlation between enzymatic activity and the relative abundance of *Vibrio* spp. Was performed with the "ggcorplot" function of the ggstastplot package.

2.14. Histological analysis of the gonads

To study the impact of MHWs on gametogenesis, a population of clams (of similar size as the one of the population used in the other experiment) was collected from the Venice lagoon in April, at the beginning of the gametogenic cycle, according to Meneghetti et al. (2004). Clams followed the same acclimation protocol and were exposed to the same experimental procedure as detailed in "Experimental set-up". At the end of the MHW, gonad tissues ($n = 15$ for each treatment) were dissected following Drummond et al. (2006) and fixed in Dietrich's Fixative for at least 24 h. Fixed samples were then dehydrated and embedded in paraffin according to Meneghetti et al. (2004). Semi-thin sections (7 μm) were taken using a Leica microtome (RM2125 RTS, Leica Biosystems, Germany), stained in Harris's haematoxylin and counter-stained in eosin, examined using a Leica DM1000 (Leica, Germany) optical microscope and pictures were taken using a Leica MC190 HD camera.

Clam reproductive cycle was characterised following criteria proposed by Drummond et al. (2006) and Vázquez et al. (2021) and analysed using the software ImageJ (Schneider et al., 2012). Females were initially staged, and only "Ripe" and "Spawning" females were considered; then for each of the selected female, the oocyte area of 50 different oocytes (for which the nucleus was visible) was measured and compared between treatments. For males, the size of at least 20 different follicles was measured and compared between treatments in accordance with

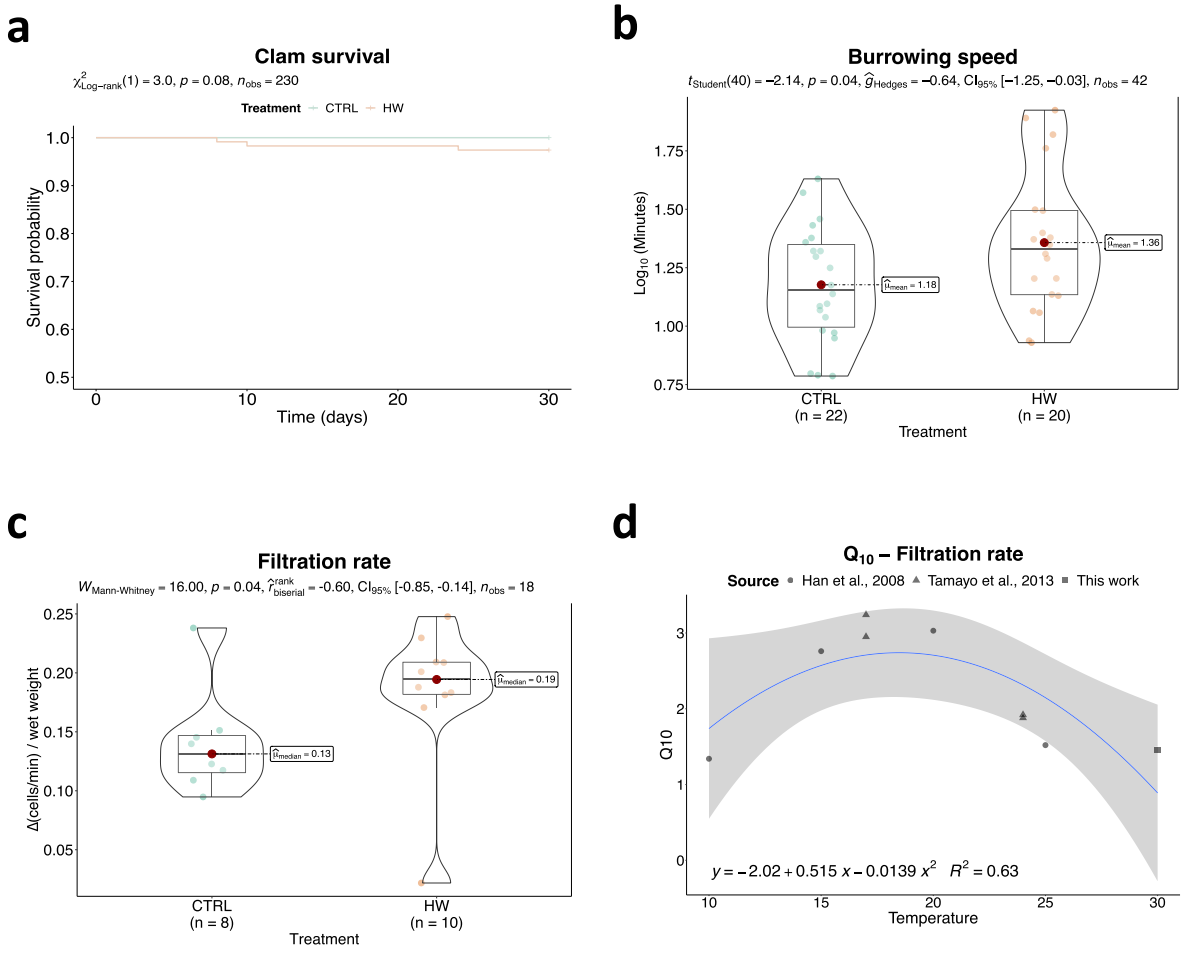

Fig. 1. Impact of a 30-days long HW on whole-organism physiological traits. a. Kaplan-Meier survival estimates on clam during exposure to HW: Differences in survival were computed using Log-Rank (Mantel-Cox) test. Statistical details (i.e. significance, degree of freedom and number of observations) are reported in the subtitle. HW exposed clams in red, CTRL clams in blue. b,c. The effect of HW on clam behaviour quantified as the amount of time that individual clams took to hide in the sand (b) and on clam filtration rate quantified by comparing the decrease in algal concentration with time (c). Scatterplot of all independent behavioural observations with superimposed box and violin plots. Red dots represent the mean/median value for each treatment; the number of independent observations for each category are shown in parentheses. Statistical details (i.e. statistical test, significance, effect size, confidence intervals and number of observations) are reported in the subtitle. d. Temperature dependence of clam filtration rate quantified at different temperatures. Q_{10} values were obtained from Han et al. (2008) (circles), Tamayo et al. (2013) (triangles) and this work (square). Data follow a negative quadratic response [$y = -2.02 + 0.515x - 0.0139x^2$ $R^2 = 0.63$ $p = 0.052$] typical of performance curves where the optimal temperature has been exceeded. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Piazza et al. (2015).

3. Results

3.1. Alterations at whole-organism level

Based on the working hypothesis that MHWs significantly affect fitness even without causing massive mortality, we carried out a pilot test to assess clam survival exposing animals for 30 days at three different temperatures (28 °C, 30 °C, 32 °C). An important decrease in clam survival was observed at the highest temperature (Log-rank χ^2, p-val <0.0001), while no significant change in mortality was recorded at 28 °C and 30 °C (Log-rank χ^2, p-val > 0.05, Suppl. Fig. 1a). All subsequent experiments were therefore run at 30 °C. A prolonged MHW at 30 °C represents a realistic scenario (Darmaraki et al., 2019). For instance, in the Venice Lagoon, the frequency of MHWs has increased since the early 90s and their maximum intensity has reached, in the last years, the temperature of 30 °C, with an average MHW duration of 10.2 ± 8.3 days (Suppl. Fig. 1b,c). Experimental exposure to this temperature confirmed its limited effect on survival (Fig. 1a), revealing at the same

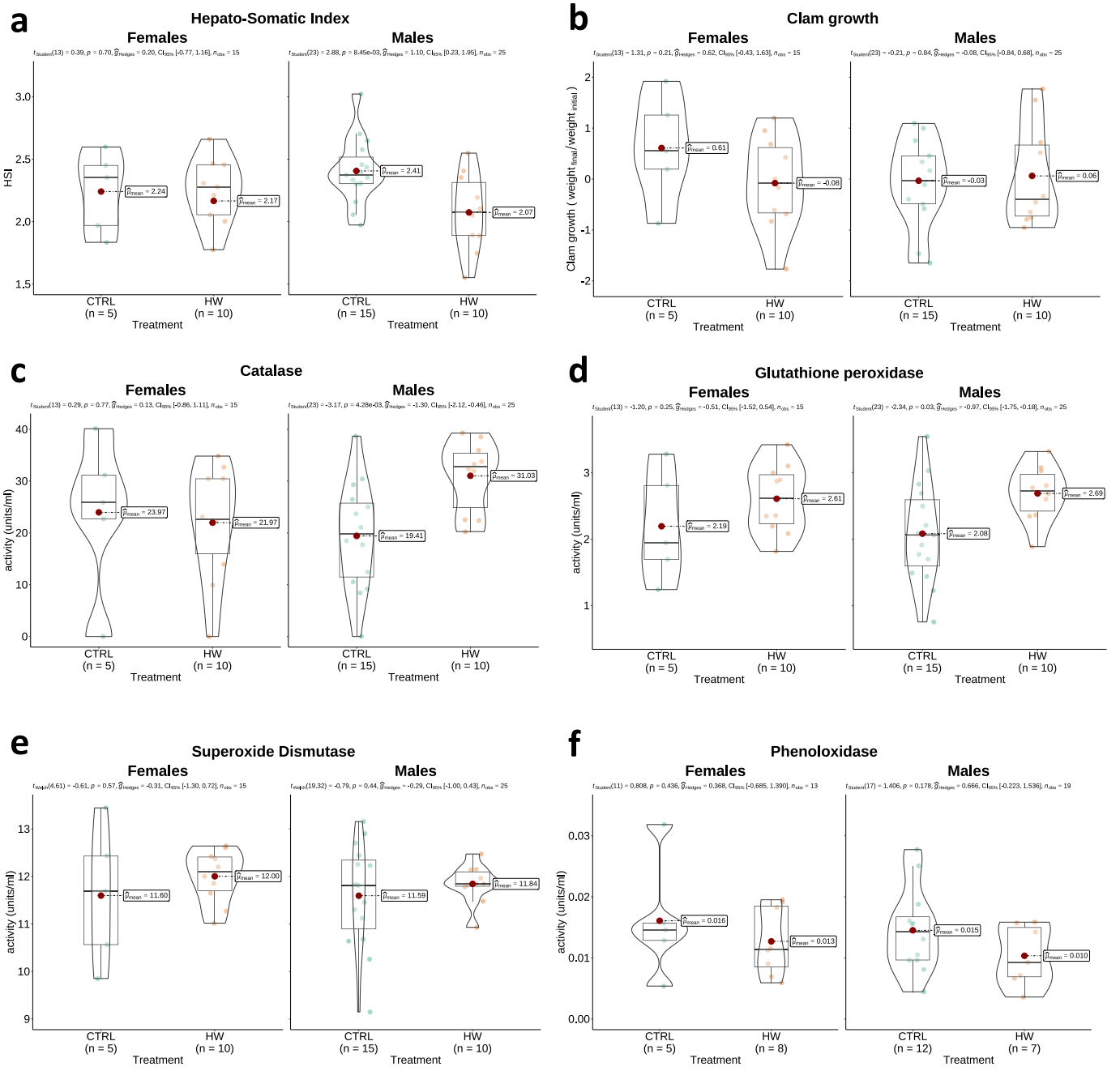

Fig. 2. Impact of HW on physiological traits and biochemical mechanisms of clams grouped by sex. a,b. The effect of HW on the hepato-somatic index (i.e. amount of energy reserves in the body, (a)) and on clam growth (b) defined as the weight at the end of the experiment divided by the weight of the same animal at the beginning of the experiment. c,d,e,f. The effect of HW on the activity of the major components of the antioxidant pathway (Catalase – Cat (c), Glutathione peroxidase – GPx (d), Superoxide dismutase – SOD (e)) and on the immune-related enzyme Phenoloxidase – PO (f). Scatterplot of all independent physiological and biochemical observations with superimposed box and violin plots. Red dots represent the mean/median value for each treatment, the number of independent observations for each category are shown in parentheses. Statistical details (i.e. statistical test, significance, effect size, confidence intervals and number of observations) are reported in the subtitle. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

time multiple critical consequences on clam biology. It affected burrowing behaviour, which is considered a key evasion response from predators: while neither the proportion of clams that burrowed nor their reactivity changed (Suppl. Fig. 2a,b), clam's burying time was significantly higher in HW clams than controls (Fig. 1b). In addition, HW clams showed a significantly higher filtration rate in comparison to CTRL clams (Fig. 1c), but an analysis of the Q_{10} temperature dependence of clam filtration rate revealed a negative quadratic response (Fig. 1d), typical, as suggested by Macho et al. (2016), of performance curves where the upper limit of a process has been exceeded. At the immune

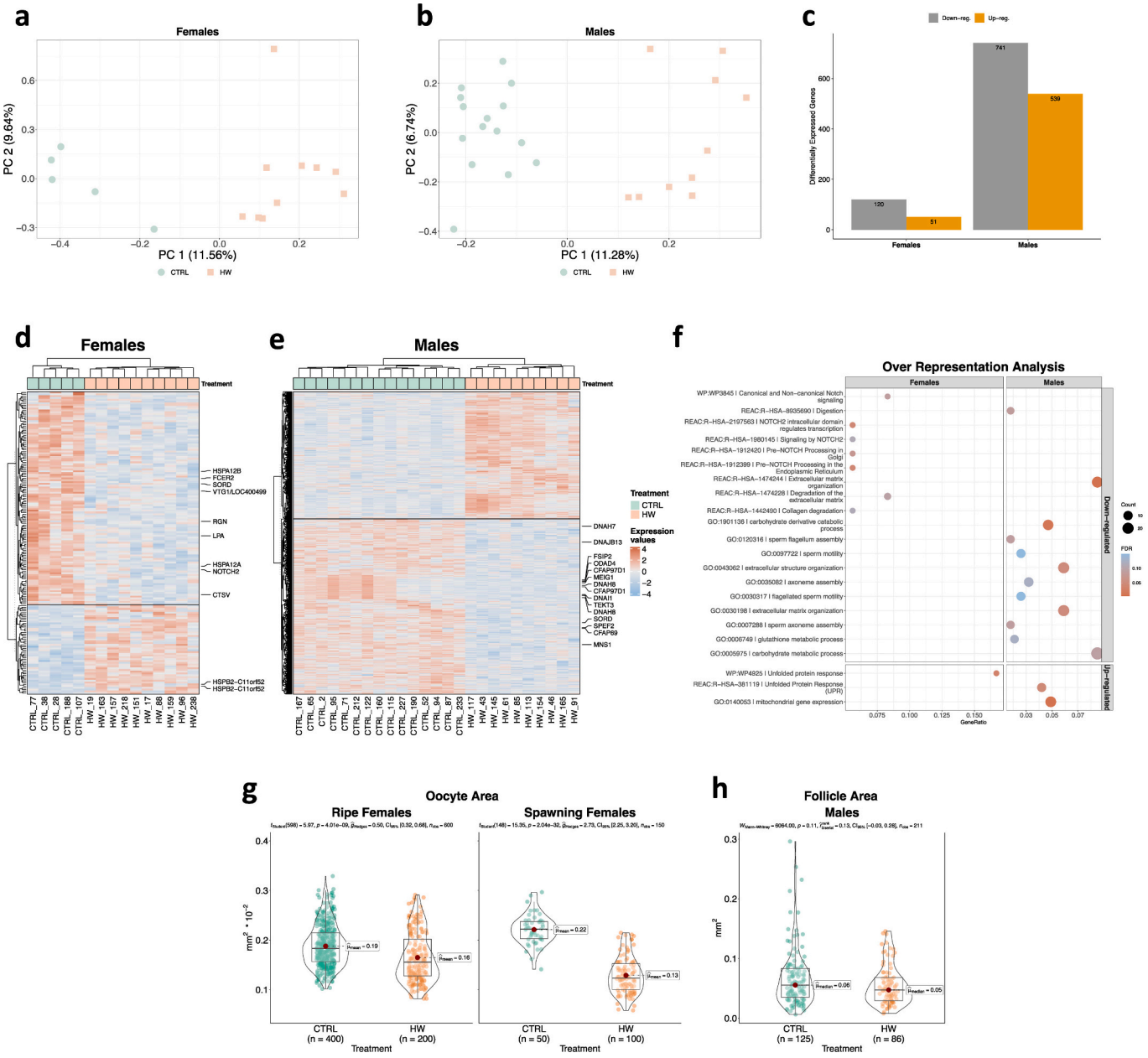

Fig. 3. Impact of MHW on the transcriptome of the digestive gland of clams and on their reproductive performances. a,b. Principal component analysis (PCA) showing the relative distribution of female (a) and male (b) RNA-seq samples along the first (PC1) and second (PC2) components. Numbers in parentheses indicate the percentage of variance explained by each component. Circles denote control samples, squares denote MHW-exposed samples. c. Number of differentially expressed genes – DEGs – (FDR < 0.05) in males and females split by the direction of regulation (up-regulated in yellow, down-regulated in grey). Numbers in the bar indicate the number of DEGs. d,e. Heatmaps of DEGs (FDR < 0.05) in female (d) and male (e) clams. Gene names on the right side of the heatmap are added to label genes involved with the reproduction. Annotation on top of the heatmap illustrates the membership of individual samples to each treatment. f. Functional analysis (i.e. over-representation analysis) associated with the different lists of DEGs: significantly enriched pathways/processes within the lists of down-regulated DEGs in females (top-left panel) and males (top-right panel) and within the lists of up-regulated DEGs in females (bottom-left panel) and males (bottom-right panel). Significant hits are colour coded according to their FDR value and their size is proportional to the number of DEGs that are found within the pathway/process. g,f. Histological analysis on the reproductive performances of females (g) and males (h) after exposure to HW. The effect of HW on the oocyte area (a proxy for female's energetic investment) developed by females in both ripe and spawning clams (g). The effect of HW on the size of sperm follicles in males. Scatterplot of all independent histological observations with superimposed box and violin plots. Red dots represent the mean/median value for each treatment, the number of independent observations for each category are shown in parentheses. Statistical details (i.e. statistical test, significance, effect size, confidence intervals and number of observations) are reported in the subtitle. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

level, no significant change was observed in haemocyte count, but a trend was observed in the percentage of dead haemocytes, which was increased in HW clams (Suppl. Fig. 2c).

3.2. Impact at physiological and biochemical level

Further physiological measures showed that the hepato-somatic index (HSI) was significantly reduced in HW exposed males (compared to CTRL males), while no difference could be observed in females (Fig. 2a). Condition index (CI) (Suppl. Fig. 2d) and weight gain during the experimental period for both males and females (Fig. 2b) was comparable in HW animals and controls. Males (but not females) were impacted by MHW also at the biochemical level: two components of the antioxidant defence system, i.e. Catalase (Cat) and Glutathione peroxidase (GPx), showed a higher activity in HW exposed males (compared to CTRL exposed males, Fig. 2c,d), while Superoxide Dismutase (SOD) and Phenoloxidase (PO) showed no difference in activity (Fig. 2e,f).

3.3. Impact on the reproduction

To gain an integrated view of the impacts of MHWs at multiple levels of biological organisation, the animals used for the physiological assessments presented in Fig. 2 were also investigated at the transcriptome, microbiome and metabolome level. RNA-seq analysis was performed on tissue samples of digestive gland (which also included a thin layer of gonadic tissue that envelops the digestive gland). In both sexes, MHW induced a substantial change in the overall gene expression profiles (Suppl. Table 0) between HW and CTRL animals, as shown by the clear separation of the two treatments along the first component of the PCA (Fig. 3a,b). Males had a higher number of differentially expressed genes (DEGs) in comparison to females, suggesting that MHWs had a greater impact on males (Fig. 3c, Suppl. Tables 1–6). For both sexes, up-regulated DEGs were significantly enriched for genes that belonged to the "Unfolded protein response (UPR)" such as *HSPA5*, *HSP90B1* and the transcription factor *ATF4*. In females, the down-regulated DEGs were enriched for genes that were included in the Notch signalling pathway (Fig. 3f), a key regulator during oogenesis in Drosophila (Xu and Gridley, 2012), while in males DEGs were enriched for GOs linked with male gametogenesis (e.g. "sperm axoneme assembly" and "sperm motility") and carbohydrate metabolism. Key genes involved in the maturation of gametes were down-regulated in both sexes (Fig. 3d,e). Among them, vitellogenin (*VTG1*) and apolipoprotein (*LPA*) and Notch (*NOTCH2*) were under-expressed in females. The observed changes were mirrored by findings at the histological level (Fig. 3g). In both ripe and spawning HW females, the size of the oocytes (a proxy for yolk content) was smaller in comparison to CTRL females, while no change in follicle size was found in males (Fig. 3h).

3.4. Metabolic adjustments

The different metabolome profiles in the foot of HW and CTRL clams were investigated via 1D—^{1}H NMR. The separation between CTRL and HW clams was less evident in females than in males (Fig. 4a,b) and such weaker separation was reflected in the number of differentially abundant metabolites between the two sexes (Fig. 4c). Within polar metabolites, Glutamine and Arginine were more abundant in CTRL clams for both sexes (Fig. 4d,e, Suppl. Tables 7–9), while Glutamic acid, Alanine, Proline, and Aspartic acid were more abundant only in HW male clams. Results from the enrichment analysis on males revealed the potential involvement of the glucose-alanine cycle (i.e. Cahill cycle, Fig. 4f), in which amino groups and carbons are transported from muscle to the digestive gland to replenish glucose reserves. Within apolar metabolites, several classes of compounds, mainly involved in regulating the fluidity of the cellular membrane (CM) were found to be different between HW and CTRL clams (Fig. 4g,h, Suppl. Tables 10–11). In general, in both sexes the amount of unsaturated and poly-unsaturated fatty acids (FA, which increase CM fluidity) significantly decreased in HW clams. In HW females, sterols (which decrease CM fluidity) were significantly lower, while they increased in HW males. Additional evidence for adjustments of membrane fluidity were found in males: the amount of saturated FA (which decrease CM fluidity) and the SFA:UFA and SFA:PUFA ratios were all higher in HW clams compared to CTRL, suggesting homeoviscous adaptation in the foot.

3.5. Alterations on the microbiota

MHW also impacted the microbiota of the digestive gland. MHW caused an overall reduction in microbial diversity (Suppl. Fig. 3a) and an increase in beta dispersion (Suppl. Fig. 3b,c), which were both significant in females, despite the low sample size of the CTRL group (Fig. 5a, b). Such changes resulted in a sex-specific shift in microbiome composition (i.e. males and females are characterised by differently abundant microbes, Fig. 5c, Suppl. Fig. 3d) and were characterised by the disappearance of potentially beneficial strains (e.g. *Phaeobacter*) and the concomitant abundance of potentially pathogenic species (e.g. *Vibrio* spp.) (Suppl. Fig. 3e). This shift was more evident in males (Fig. 5c,d), where the relative abundance of *Vibrio* was positively correlated with the activity of catalase (Fig. 5e, Suppl. Fig. 3f).

4. Discussion

We provide here an integrative view at multiple levels of biological organisation of the effects of MHW by showing that prolonged MHWs induce a global response in clams to counteract the effects of high temperatures. Well-known adjustments to heat stress were observed such as the Unfolded Protein Response to ensure protein quality, expression of antioxidant enzymes to reduce the damage of temperature-associated oxidative stress (Yu et al., 2022), and homeoviscous adaptation to preserve cell membrane stability. However, these adaptive responses come at a high cost. It is likely that clams exposed to prolonged MHWs reallocate energy toward maintenance, decreasing investment in reproduction and evasive behaviour. Such a trade-off most likely leads to lower reproductive success and higher vulnerability to predation, decreasing the species fitness. In males, the cost appeared to be higher, significantly decreasing energy reserves despite the mobilization of energy-rich compounds from muscle to digestive gland through the Cahill cycle.

While it has been traditionally sustained that producing eggs entails higher energetic costs, Lewis and Ford (2012) argued that sperm cells have little or no capacity to protect from DNA damage or from oxidative stress and, consequently, males may need to invest extra energy to provide a suitable environment for such cells (e.g. by increasing antioxidant defence). This might be the case of male clams, where such an investment, however, might not be sufficient to fully protect sperm development as suggested by the significant down-regulation of genes involved in gametogenesis. Based on evidence from transcriptome analysis, sperm motility might be the most affected as numerous down-regulated genes belong to the pathways "flagellated sperm motility" and "sperm axoneme assembly". As reported by Lehti and Sironen (2017) "the axoneme is the core structure of the sperm tail and contains dynein arms, which function as motors for the motility". A significant impact of MHWs on the production of sperm cells and on male fecundity has been recently observed in a terrestrial insect, the flour beetle *Triboleum castanum* (Sales et al., 2018), and in the mussel *Mytilus galloprovincialis* (Boni et al., 2016).

A significant impact of MHWs on the reproductive biology of clams was also observed in females. This was suggested by the down-regulation of two major proteins involved in egg yolk formation (i.e. VTG1 and LPA) and confirmed at the histological level by the reduction in oocyte area of HW females. Overall, these changes suggest a reduction of per-offspring investment (i.e. the quantity of resources allocated to offspring) in females and this can, in turn, negatively impact larval

development resulting in a reduced offspring fitness (Oliphant et al., 2013).

Host-associated microbiota are increasingly recognized as a key player in animal health and disease. Analysis of Manila clam in natural populations already demonstrated that its digestive gland microbiome is affected by environmental factors (e.g. temperature, salinity, pollutants (Milan et al., 2018)), but shows a remarkable resilience to change (Iannello et al., 2021). Here, in partial agreement with Li et al. (2023), after 30 days exposure to 30 °C, we observed decreased richness, a shift in composition, and greater variability of the microbiota following

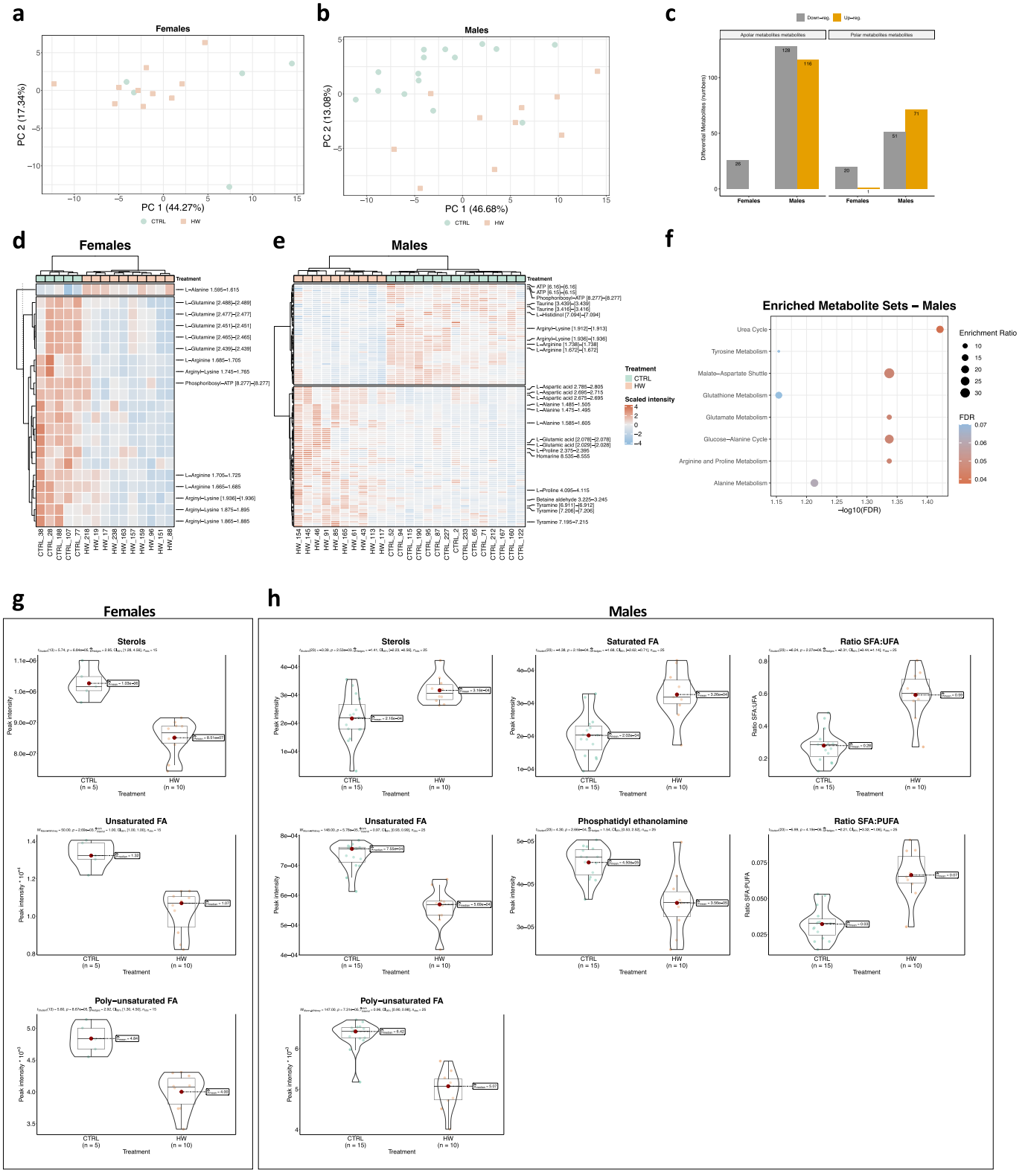

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Fig. 4. Impact of MHW on the metabolomic profile of the foot of clams. a,b. Principal component analysis (PCA) of metabolomic samples (polar and apolar compounds together) showing the relative distribution of female (a) and male (b) samples along the first (PC1) and second (PC2) components. Numbers in parentheses indicate the percentage of variance explained by each component. Circles denote control samples, squares denote MHW-exposed samples. c. Number of differential metabolites (FDR < 0.1) in males and females grouped into polar (i.e. metabolites, right panel) and apolar (i.e. lipids, left panel) compounds and split by the direction of regulation (up-regulated in yellow, down-regulated in grey). Numbers in the bar indicate the number of differential metabolites. d,e. Heatmaps of differential polar metabolites (FDR < 0.1) in female (d) and male (e) clams. Metabolites with known identification are labelled on the side of the heatmap. Annotation on top of the heatmap illustrates the membership of individual samples to each treatment. f. Functional analysis (i.e. over-representation analysis) associated with metabolites that had higher abundance in MHW-males. Significant hits are colour coded according to their FDR. g,h. Effects of MHW on the differently abundant apolar compounds (grouped by chemical classes) in females (g) and males (h). Abbreviations: FA Fatty acids; SFA Saturated Fatty acids; UFA Unsaturated Fatty acids; PUFA Poly-unsaturated Fatty acids. Scatterplot of all independent observations with superimposed box and violin plots. Red dots represent the mean/median value for each treatment; the number of independent observations for each category are shown in parentheses. Statistical details (i.e. statistical test, significance, effect size, confidence intervals and number of observations) are reported in the subtitle. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MHW, all of which might signal the onset of dysbiosis, a condition increasingly associated with pathogenesis of intestinal and extra-intestinal disorders (Carding et al., 2015). Hence, it is not surprising that the relative abundance of *Vibrio* spp. increased ~18-fold in HW clams. Altogether, these data lead us to speculate that changes in microbiome composition driven by MHWs in males affect nutrient availability and metabolic pathways that are required for the replicative success of certain species (e.g. *Vibrio* spp.), which outcompete symbiotic host species (e.g. *Phaeobacter*) and might concur to host stress response and disease, and may trigger episodes of mass mortality, as reported by Green et al. (2019), with important ecological, economic, and societal implications.

The duration of the simulated HW challenge most likely allowed us to observe the integrated biological response to prolonged heat stress as well as the associated costs, which might have long-term effects on clam fitness. So far, most studies have focussed on the short-term effects of MHW on bivalve molluscs (e.g. (Dominguez et al., 2020; He et al., 2022; Jahan et al., 2022; Scanes et al., 2020; Xu et al., 2022b)). However, considering that molluscs from transitional systems (i.e. lagoons or river deltas) are more vulnerable to MHW than other taxa (Munari, 2011) and that the average MHW duration in the Mediterranean basin will be ~30 days by 2050 under RCP4.5 according to model predictions (Darmaraki et al., 2019; Galli et al., 2017), we believe that the real consequences of MHWs on marine bivalves have been substantially underestimated. Hoppit and Schmidt (2022) recently performed a meta-analysis on the responses of benthic organisms to climate change in controlled experiments (although they did not specifically focus on extreme events like MHWs) and found that stress duration generally amplified responses on benthic organisms. A similar response has recently been documented in the Pacific oyster by De Marco et al. (2023) who showed that oysters can activate metabolic responses against stress upon short MHW exposures, but that longer exposures lead to extensive impairment in the oyster's physiology. In fact, Isotalo et al. (2022) proposed the hypothesis that HW duration is "an important factor to take into consideration when studying responses to CC".

Another important aspect that should be taken into consideration is the frequency of MHWs. In fact, we report here the effects of a single MHW, albeit a long one, but it should be noted that MHWs might strike multiple times during the same summer, which also represents the reproductive season for Manila clam. Especially in males, the extra energetic investment may render clams vulnerable on the long term because they may have insufficient energy reserves left to counteract consecutive MHWs, a scenario that is increasingly likely according to future climate predictions.

The negative effects on clam reproduction reported here might have occurred already in the Venice lagoon after the 2003 MHW, for which Ponti et al. (2017) documented a reduction in larval recruitment with an important impact on clam stock and, similarly, in a population of Pacific oyster *Crassostrea gigas* in the Thau lagoon, France (Correia-Martins et al., 2022) and in the Coos Bay estuary, USA (Shanks et al., 2019). Many invertebrate species show a higher vulnerability to environmental stressors during development and/or settlement than during adulthood, as documented by Collin et al. (2021). At present, while there are many studies documenting the impact of larval development in relation to CC (e.g. Armstrong et al. (2019); Byrne (2011); Collin et al. (2021)) our knowledge on the effects of MHWs on development is still very limited (e.g. Minuti et al. (2022)) and requires deeper investigation.

Finally, we found a significant impact on the filtration rate of clams. More specifically, we found that the filtration rate upper limit is exceeded in clams exposed to MHWs, indicating that such temperature has a deleterious effect on this trait. An impact on filtration rate has been observed after short-term MHW in other bivalves (e.g. oysters (De Marco et al., 2023; Giomi et al., 2016), marine (Dominguez et al., 2020) and freshwater (Ferreira-Rodríguez et al., 2018) clams) revealing how this trait is very "sensible" to both short- and long-term MHWs. This may in turn affect an important ecosystem function provided by the species, which, according to Naylor et al. (2021), provides a key role in mitigating coastal eutrophication via removal of excess nutrients with its filtration activity.

5. Conclusions

We have provided here an integrative view of the organismal response to MHW at multiple levels of biological organisation and have shown that even if MHWs do not reach lethal temperatures, thereby causing massive mortalities, the consequences on marine bivalves can result in a reduction of the species' fitness via alteration of the reproduction, filtration and avoidance behaviour, decline of the energy reserves and onset of dysbiosis. We believe that all the experimental evidence reported in the present study clearly stands for a substantial change in the way we predict the impact of CC on marine animals. To avoid significant underestimation of such impact, prediction models should be updated to account for non-lethal, yet significant, impacts due to these extreme events. In fact, it is likely that MHWs have already manifested their negative effects: if one looks at production data, an important decline in clam production can be observed in the past 20 years, especially in concomitance with important MHW events (Hough, 2022; Ponti et al., 2017).

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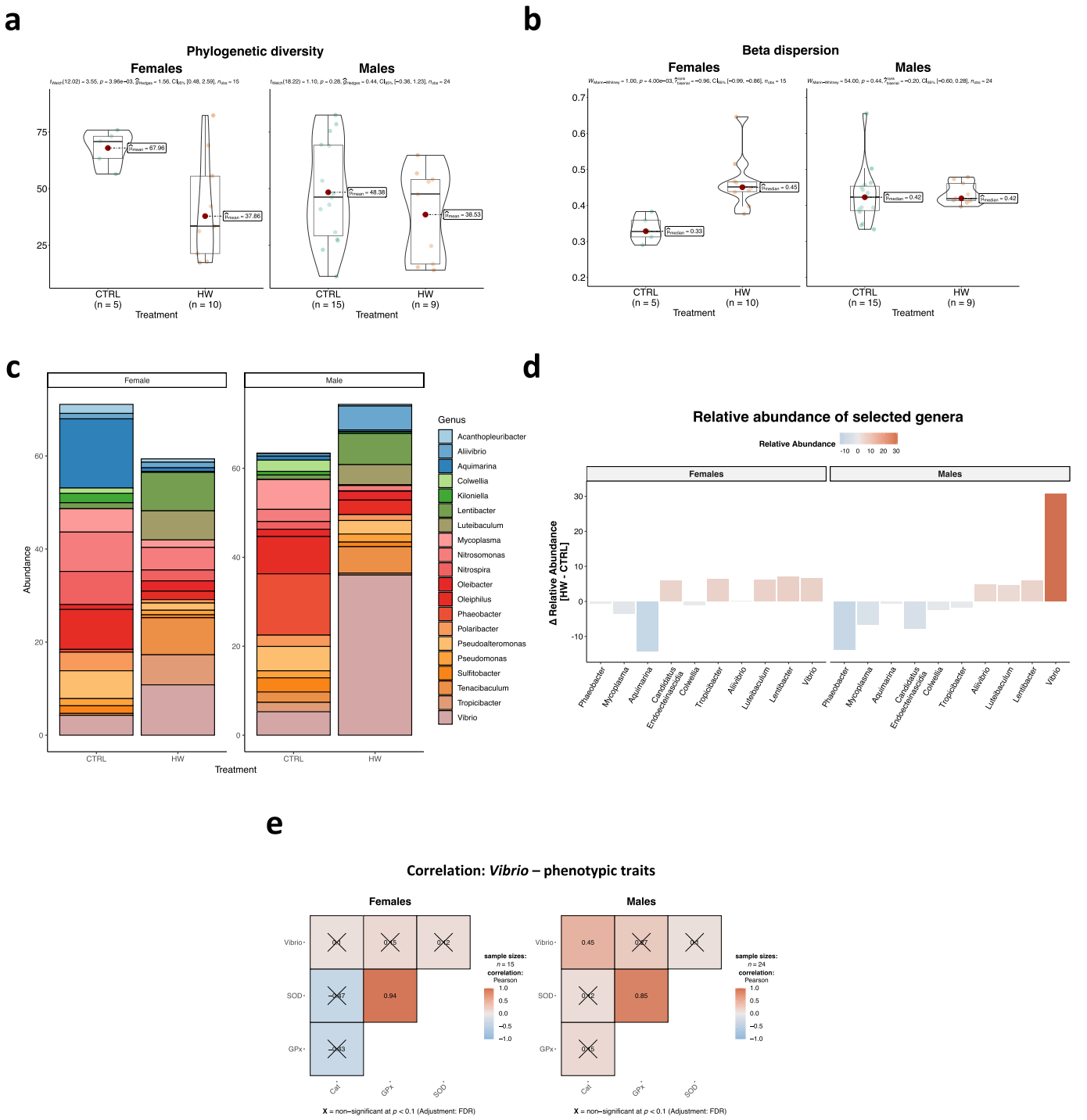

Fig. 5. Impact of MHW on the microbiome of the digestive gland. a,b. Effects of MHW on the alpha diversity (a) and beta dispersion (b) of clams. Scatterplot of all independent observations with superimposed box and violin plots. Red dots represent the mean/median value for each treatment; the number of independent observations for each category are shown in parentheses. Statistical details (i.e. statistical test, significance, effect size, confidence intervals and number of observations) are reported in the subtitle. c. Taxonomic composition of the average microbiome abundance at genus level. Barplot of significant genera (genera with relative abundance <5 % are not shown) in female (left panel) and male (right panel) clams. d. Relative abundance of genera known for their probiotic or pathogenic role in clams, according to their sex. Bars show the average relative abundance across all CTRL samples minus the average relative abundance across all HW samples. Negative bars indicate higher relative abundance in CTRL clams, positive bars indicate higher abundance in HW clams. e. Pearson's correlations between the relative abundance of *Vibrio* spp. and the activity of antioxidant enzymes Catalase (Cat), Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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CRediT authorship contribution statement

PL, BL and HC conceived the project. PL, BO, CF, PM, DRG, MA, TC performed the experiments and collected samples. PL, TC, BF, GME

performed flow cytometry. PL, FR, RT, MaS performed metabolome analysis. PL, DRG, QA, MME performed microbiota characterization. PL performed transcriptomic analysis. CF, TC performed histological analyses. PL, TC performed data analysis. PL wrote the first draft of the manuscript with inputs from BL, MM. All authors commented and critically revised the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The datasets used and/or analysed during the current study are available upon request. The -omics datasets have been deposited on NCBI under accession (BioProject ID: PRJNA906528 and PRJNA906977).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2023.115192.

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