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Thermal priming mitigates the effects of lethal marine heatwaves on the Manila clam *Ruditapes philippinarum*

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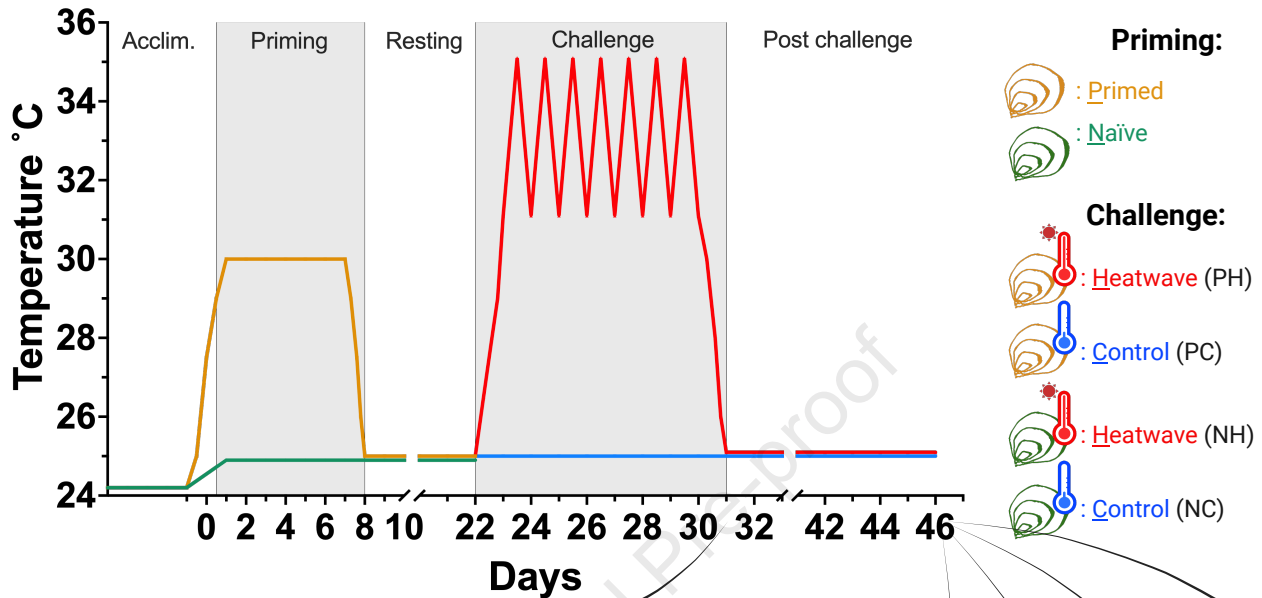
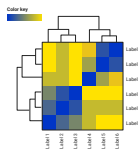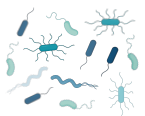Day 31: PH show better burying behaviour than NH
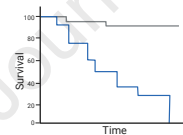
Day 46: PH show greater survival than NH

Day 46: Higher antioxidant activity in Primed clams vs Naïve

Day 46: Gene expression showed up-reg. of HSP genes and metabolic pathways in PH vs NH and evidence of stress memory in PC vs NC.

Day 46: 16S metabarcoding revealed higher abundance of beneficial and lower abundance of detrimental taxa in PH vs NH.

1 ## Title

2 **Thermal priming mitigates the effects of lethal marine heatwaves on the Manila clam *Ruditapes***

3 ***philippinarum***

4

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20 ## Keywords

23

Summary

Climate extreme events like heatwaves (HWs) increasingly threaten marine ecosystems. Using the Manila clam Ruditapes philippinarum as a model organism, this study assessed the effects and costs of thermal priming in a realistic scenario. Results showed increased resilience to lethal HWs in primed clams, with significantly higher survival and better defensive behavior compared to naïve clams. Priming costs were evidenced by a reduced condition index, but hardened animals showed higher antioxidant capacity, upregulation of heat shock response genes, and shifts in microbial composition favouring beneficial taxa. Gene expression analysis revealed environmental memory via upregulation of respiratory chain complex genes, detectable 38 days after priming—the longest reported in molluscs in a controlled setting. Evidence suggests that thermal priming can be an effective mitigation strategy not just for Manila clams but, potentially, for other farmed shellfish species (e.g. oysters, mussels) and for ecological conservation projects against climate extreme events.

Introduction

In a world with a fast-growing population, the need to bolster food production is more and more compelling. In this context, bivalve aquaculture provides an excellent source of animal proteins with a very limited carbon footprint[1]. Bivalves also provide numerous ecosystem services that range from the regulation of water turbidity to nutrient cycling, habitat provision, and carbon sequestration[2]. Unfortunately, bivalve aquaculture will be heavily impacted by climate change (CC) as it is predicted that, by 2090, coastal areas suitable for this activity will globally decrease by about 10%[3]. In addition to habitat reduction, ocean acidification and higher prevalence of pathogens, are expected to slowly increase in parallel with ramping average temperatures. While such phenomena will certainly have a negative impact on shellfish, CC-related extreme events such as marine heatwaves (MHWs) have rapidly emerged as a much more immediate and dramatic threat to sedentary and sessile species as demonstrated, for instance, by the mass mortality of benthic communities registered in 2003 in the North Adriatic[4-6]. Frequency and intensity of MHWs will further increase in the future, multiplying the risks for the benthic fauna[7].

52   A possible strategy for mitigating the negative effects of MHWs is constituted by heat-
53   hardening or priming, defined by Bowler[8] as a transient 'adaptation' to a lethal thermal stress
54   following prior exposure to a sub-lethal temperature. Hardening has been extensively studied in
55   plants and other species without a nervous system[9] as well as in other metazoans (e.g. [10-13]) and
56   it represents a form of stress memory that prepares (i.e. "primes") an organism to future stress
57   by improving its response to adverse environmental conditions. Hardening is a form of phenotypic
58   plasticity as it does not entail any change in the organism genotype and it constitutes a fast
59   "adaptation" response against environmental challenges[14]. While priming improves the response
60   to future stress conditions, it is generally expected to be associated with some costs[13]; in fact,
61   maintaining stress memory must require changes in regulatory networks to ensure relevant
62   biological systems to be in a "vigilant" state, ready to cope with stressful conditions[9]. While
63   hardening may represent a general phenomenon naturally occurring in living organisms to better
64   cope with recurrent environmental stress, the physiological and molecular mechanisms of heat
65   hardening are not well understood[15] and, most importantly, its efficacy in granting higher
66   resilience against MHW that resemble (in terms of daily thermal profile, intensity and duration)
67   those that occur in nature is, so far, still limited to a handful studies[11,12,16].

68   Hitherto, great efforts have been devoted towards the understanding of the response to
69   MHWs in marine animals. A plethora of adverse consequences across different animal taxa
70   (e.g.[17,18]) beyond mass mortality has been reported, ranging from altered ecosystem services[19,20]
71   to impaired reproduction[21]. In addition, several authors have reported substantial changes in the
72   microbial communities living in several species that generally lead to dysbiosis[22-26]. In many
73   studies, MHWs are simulated as acute thermal stress in a high-temperature challenge of relatively
74   short duration that has limited resemblance with environmental conditions in the field (e.g. [11,27]).
75   However, as demonstrated by Isotalo et al.[28] and He et al.[29], the consequences of MHWs are
76   largely dependent on their duration and intensity. Hence, it is essential that experiments
77   simulating extreme temperatures closely mirror the temporal and thermal profile of current or
78   predicted MHWs as they occur in the natural environment, in order to draw accurate conclusions
79   on their negative impacts and to test mitigation strategies.

80   In this framework, the present paper aimed at filling those gaps by exploring the efficacy

81   of heat-hardening in conferring greater resilience against a simulated MHWs that mimicked, in

82   terms of intensity and duration, a natural event occurred in 2015 in the Venice lagoon. As model

83   organism we employed the Manila clam *Ruditapes philippinarum* because of its key role in the

84   ecology of transitional habitats (e.g. lagoons, river deltas) worldwide and its equal importance as

85   highly sustainable food source. Four million metric tons of Manila clam per year are farmed from

86   coastal lagoons and deltas worldwide, supporting a relevant economic sector. Unfortunately, this

87   benthic species is severely threatened by extreme events such as MHW, which are particularly

88   intense in coastal habitats where the majority of clam farming takes place.

89   To test our hypothesis, we employed an experimental design with four groups: naïve and

90   primed animals triggered with potentially lethal thermal conditions, and naïve and primed ones

91   without triggering. The priming stimulus was represented by a seven days period at 30°C, a

92   thermal condition that is frequently recorded during summer in the environment where the

93   species is usually grown or found (i.e. lagoons and river deltas). The triggering stimulus closely

94   simulated a real MHW event that occurred in 2015 in the Venice lagoon (one of the major farming

95   sites for this species) with temperatures rising during the day to a maximum of 34 ˚C and then

96   decreasing gradually during the night to a minimum of 31 ˚C. By using an integrative approach,

97   we assessed the effects of priming on fitness-related traits (i.e. behavior, oxidative stress damage,

98   survival), we measured hardening-associated costs (via changes in condition index), and we

99   elucidated the biological mechanisms underlying priming and stress memory, through the

100   analysis of behavioral, metabolic, biochemical alterations induced by hardening as well as of

101   changes at the transcriptome and microbiome level in the digestive gland, an organ that was

102   reported to be highly relevant in the response of Manila clam to thermal stress[26].

103

Results

Behaviour

Because of its non-invasive nature, the burrowing behavioural test could be applied before the end of the experiment, which was not the case for all the other read-outs. The first test was performed immediately after priming and showed that a similarly high percentage of animals completed the burrowing both in the primed and naïve clams, although the burrowing speed was significantly slower in primed individuals (Figure 1A). A second round of tests was carried out at day 32 since the beginning of the experiment. When comparing primed and naïve animals maintained constantly at 25°C, a significantly different behaviour was again observed. In this case with a lower percentage of primed clams having completed the burrowing, while at a similar speed (Figure 1C). The pattern was completely reversed in the comparison between primed and naïve clams that had experienced the MHW triggering event (Figure 1B). As before, there was no difference in the burrowing speed, but the percentage of animals having completed the process was much lower in naïve clams after the simulated MHW. Notably, the percentage of HW-triggered primed clams that buried was similar to the percentage of naïve animals not exposed to the triggering event (Figure 1B and C).

Survival, condition index and oxidative stress biomarkers

Survival was surveyed for the entire duration of the experiment, including a 15-days post thermal challenge recovery period at 25°C since in preliminary trials carried out to optimize experimental settings, it was observed that mortality often occurred after the end of HW challenge. In fact, in naïve clams exposed to HW significant mortality started one week into the recovery period, while it was negligible in primed animals (Figure 2A) and by the end of the monitoring period, a higher significant mortality rate (~25%) was found in naïve animals in comparison to primed ones (~4%). Control groups showed no mortality at all (Figure 2B).

Condition index (CI), which indicates the overall physiological status of clams, showed that priming entails a cost, as primed clams showed a significantly lower CI both with and without triggering event (Figure 3A), although the difference was significantly smaller after experiencing HW. In fact, the overall pattern showed that CI dramatically decreased in naïve clams after being

132 exposed to thermal stress, while it did not decrease significantly in primed animals after the

133 triggering event.

134   Concerning oxidative stress response, SOD activity was higher albeit not significantly in

135 primed clams in the absence of HW, while the difference became significant with a decrease in

136 the activity of SOD in naïve clams exposed to HW (NH) compared to naïve controls (Fig. 3B). The

137 activity of GPx was significantly higher in both primed groups (i.e. PC and PH) in contrast with

138 both naïve groups (i.e. NC and NH), suggesting that priming induces a stable and trigger-

139 independent expression of this antioxidant enzyme (Fig. 3C). Cellular damage as a consequence

140 of oxidative stress was examined in the four groups by assessing the extent of lipid peroxidation.

141 Comparison of primed clams with naïve ones under non-triggering conditions confirmed that

142 priming comes at a cost, as a significantly higher amount of peroxidation in primed versus naïve

143 clams was observed (Fig. 3D). In naïve animals the triggering event had a limited effect, with a

144 non-significant increase in lipid peroxidation between primed and naïve animals. When

145 comparing lipid peroxidation between the two primed groups, animals that were exposed to the

146 triggering stress paradoxically showed a significant decrease in comparison to non-triggered

147 animals.

148

149 Transcriptome analysis

150   Transcriptional profiles were analysed in the four experimental groups at the end of the

151 final recovery phase, depicting the medium-term effects on gene expression of priming and/or

152 the triggering event. The digestive gland was analysed here as previous studies showed that this

153 organ is significantly involved in the clam response to HWs. A discriminant analysis of principal

154 components (DAPC) was used to explore overall patterns, with a clear separation of samples into

155 four clusters, overlapping the experimental groups (Fig. 4A). Evidence from the DAPC suggested

156 that naïve animals experiencing the triggering event showed the most relevant transcriptional

157 changes. Such evidence was confirmed when significant differentially expressed genes (DEGs)

158 were identified in all possible pairwise comparisons, as the number of DEGs was substantially

159 higher when examining the effects of HW in naïve clams (Figure 4B). Full lists of DEGs for each

160 comparison are reported in Table S1 A, B, C and D.

161　　　　The comparison between PC and NC groups revealed genes and gene networks potentially
162　　involved in thermal stress memory as several significant DEGs and gene sets were observed over
163　　a month (38 days) after the end of the priming event. Noteworthy, among DEGs were two
164　　chaperones, *HSPA12A* (fold change (FC) 4.5 FDR<0.01) and *TRAP1* (FC 0.4 FDR<0.05). The former
165　　belongs to the hsp70 family, the latter to the hsp90 one. Remarkably, there was no overlap
166　　between DEGs identified in the comparison between primed and naïve clams after the triggering
167　　event and those differentially regulated just by the priming. As external and internal stimuli often
168　　affect biological processes through modest, but coordinated expression changes in the set of
169　　genes that underlie those processes, a Gene Set Enrichment Analysis (GSEA) was also
170　　implemented here (Table S1 E, F, G and H). Gene network analysis via GSEA showed different sets
171　　of genes that were significantly regulated. Negative regulation of protein translation (GO:0002181
172　　*cytoplasmic translation*, normalized enrichment score (NES) -1.9, FDR 0.01) and positive
173　　regulation of ATP synthesis (GO:0015986 *proton motive force-driven ATP synthesis*, NES 2.1, FDR
174　　0.03; GO:0042776 *proton motive force-driven mitochondrial ATP synthesis* NES 2.2, FDR 0.03)
175　　were observed. Two larger and partially overlapping gene networks underlying cell response to
176　　stimuli (*GO:0032870 cellular response to hormone stimulus*, NES -1.7, FDR 0.03; GO:0071495
177　　*cellular response to endogenous stimulus*, NES -1.5, FDR 0.04) appeared both to be down-
178　　regulated (Fig.4).

179　　　　In the comparison between primed and naïve animals that both experienced the
180　　triggering event (i.e. PH and NH), several genes were differentially regulated (Figure 4B) over two
181　　weeks after the HW challenge ended, including the up-regulation of genes potentially implicated
182　　in thermal tolerance (Fig. 4C): *HSPA12B* (FC 1.8, FDR 0.04)*, a heat shock protein; NPHS1* (FC 2.1;
183　　FDR 0.01), a molecule involved in the regulation of epithelial barriers; *ADGRL3* (FC 3.5; FDR 0.04)*,
184　　which plays a role in cell-cell adhesion; *FASN* (FC 9.8; FDR 0.02)*, which is involved in fatty acid
185　　synthesis and endothelial permeability. Broad gene sets were found to be significantly enriched
186　　at FDR<0.05 (Figure 4E), involving lipid and amino acid metabolism (GO:0006629 *lipid metabolic
187　　process*, NES 1.5, FDR 0.02; GO:1901605 *alpha-amino acid metabolic process*, NES 3.8, FDR 0.04).
188　　Both gene networks were up-regulated.

189   In the comparison between the two naïve groups (i.e. NH and NC), we found many down-

190 regulated genes in NH linked to heat shock response (e.g. *HSPA12B*, *HSPA12A*, *HSPA9, DNAJB9*)

191 and to epithelial barrier permeability (e.g. *SEMA5B*, *SEMA5A*, Fig. 4D). In NH, GSEA highlighted a

192 significant down-regulation of pathways associated to protein folding and protection

193 (GO:0034620 *cellular response to unfolded protein*, NES -1.9, FDR 0.01; GO:0006986 *response to*

194 *unfolded protein*, NES -1.7, FDR 0.05, Fig. 4E) and to pathways linked with cellular adhesion and

195 structure (GO:0030036 *actin cytoskeleton organization*, NES -1.9, FDR <0.0001; GO:0034330 *cell*

196 *junction organization*, NES -1.7, FDR 0.001; GO:0120193 *tight junction organization*, NES -1.8, FDR

197 0.03) such as "cell-cell junction organization", "tight junction organization" "actin cytoskeleton

198 organisation" and "cellular response to unfolded protein" (Fig. 4E).

199

200 Clam associated microbiota:

201   The microbiota associated with the clam digestive gland was characterized in all four

202 experimental groups. Shannon alpha diversity showed a significant increase in naïve clams after

203 exposure to HW (Table S2 A, B, C and D, Fig. 5A). The beta diversity was calculated using the Bray-

204 Curtis index at the genus level and visualized with a PCoA (Fig. 5B). Naïve and primed clams

205 appeared to be separated along the second axis, irrespective to the presence or absence of the

206 triggering event, although PERMANOVA revealed significant differences in all pairwise

207 comparisons (Fig. 5B insert).

208   Analysis of taxonomic composition showed that the microbial community of NH animals

209 was characterized by a conspicuous relative abundance of bacteria of the orders *Flavobacteriales*

210 and *Rhodobacterales* (Fig. 5C), while *Clostridiales* were highly abundant in PH clams. At the genus

211 level, pairwise comparisons by means of multiple linear regression with covariate adjustment

212 between PH and NH highlighted the presence of 32 differentially abundant genera. Several of

213 these genera, which are included in the putatively detrimental orders of the *Flavobacteriales* and

214 *Rhodobacterales,* were indeed more abundant in naïve clams after the triggering event (Table S2

215 A), while several taxa belonging to the potentially beneficial order *Clostridiales* were more

216 abundant in primed and triggered clams. The comparison between PC and NC showed long lasting

217   effects of priming on clam-associated microbiome, with two over-represented taxa in primed

218   clams (PC) and one more abundant in NC.

219

220 Discussion

221   Nowadays it is widely recognised that extreme climate events such as MHWs will strike at

222 increasing frequency, duration, and intensity worldwide[19,30], with great impact on marine biota.

223 In this context, priming and environmental stress memory represent a biological mechanism to

224 potentially cope with such extreme events. Priming is known to the scientific community since

225 the '70s, although the majority of research in multicellular organisms has mostly focussed on

226 plants[9]. In metazoans, possibly the best studied case is that of corals[31]. In this taxonomic group it

227 emerged that priming and environmental stress memory might represent a more rapid strategy

228 than genetic adaptation for sedentary and sessile animals to counteract recurrent stressful

229 episodes without relocation. It could be envisaged as an operational tool to obtain more resilient

230 animals before they are seeded in the natural environment either in the context of restoration

231 programmes or for aquaculture purposes.

232   Analysis of thermal priming in corals also highlighted the potential importance of the host-

233 associated microbial communities in stress memory[32], although the relevance and cause-effect

234 links remain to be fully elucidated. Studies on more complex marine animals are unfortunately

235 still scarce and the available ones often follow an experimental design where short (i.e. hours)

236 sub-lethal exposure times at fairly high temperatures are used to induce priming. Likewise,

237 triggering stimuli generally consist of acute heat shock events often at non-realistic temperatures

238 rather than conditions resembling those in a natural HW event (e.g. [27,33-35]). This approach limits

239 the understanding of the potential of priming in counteracting natural extreme events both as an

240 operational tool and a natural mechanism of acclimatization. In this work, as already mentioned,

241 priming conditions were implemented resembling in length and intensity the average sublethal

242 MHWs currently recorded in transitional zones along the coast of the Adriatic Sea and, in parallel,

243 the triggering stimulus closely simulated a lethal MHW that occurred in 2015 in the same area.

244   All three fitness-related read-outs measured in this work (i.e. behaviour, mortality and

245 oxidative stress) showed a clear beneficial effect of priming after the triggering stimulus. As

246 reported by Luo et al.[36], "behaviour is the most direct response of an organism and reflects the

247 prospect of surviving" and, in clams, is particularly relevant as it allows them to evade predators

248 and helps mitigating the effects of high-water temperatures. Recent studies showed behavioural

249   responses of naïve animals were significantly impaired following MHW exposure[26,35-38]. The

250   potential fitness costs of priming are clearly appreciated as burrowing speed slowed down

251   immediately after priming, while in the longer term it drastically reduced the percentage of

252   burrowed clams (Figure 1A, C) compared to untriggered naïve animals. However, as already

253   noted, the pattern was entirely reversed in the presence of the triggering event, with the

254   percentage of completed burrowing events significantly higher in primed clams (a percentage

255   that closely mirrored that recorded in untriggered naïve animals, Figure 1B). Survival, the trait

256   most directly affecting animal fitness, was significantly greater in comparison to naïve clams.

257   Mortality was observed only after the triggering event, therefore there seemed to be no priming-

258   associated costs in terms of survival (Figure 2). The third fitness-related read-out, oxidative stress

259   damage measured through lipid peroxidation, showed a pattern similar to that recorded for

260   burrowing behaviour: at the end of the experiment, hardened clams displayed a significantly

261   higher cell membrane damage in the absence of a triggering event. However, hardened and

262   triggered animals had a lower level of damage, comparable to naïve ones (Figure 3D).

263   The fact that priming entails biological costs, as expected based on existing literature[9],

264   was clearly evident from the analysis of condition index, which was significantly lower in primed

265   clams compared to naïve ones when priming was not followed by a triggering stimulus (Figure

266   3A). The ratio between soft body weight and shell weight, the condition index (CI) used here, is

267   an indicator of energy storage in bivalves[39]. Lower CI in primed clams suggested higher energetic

268   demand, perfectly fitting the energy budget hypothesis, where changes necessary to withstand

269   harsher environmental conditions require an energy investment of the organism, which

270   reallocates energies from other processes[40,41]. However, it is interesting to note that this energetic

271   cost was not paid again when primed animals were exposed to the triggering stimulus, as there

272   was no difference in CI between PH and PC (Fig. 3A). The metabolic costs of priming and stress

273   memory maintenance paid off as a potentially lethal HW appeared to entail no extra energetic

274   cost. On the other hand, naïve clams spent a significant amount of energy after exposure to the

275   triggering event even if, such energetic costs, were not sufficient to prevent a significantly higher

276   mortality in these animals. The other fitness-related measures, burrowing behaviour and

277   oxidative stress damage, showed a similar, even more pronounced pattern in naïve clams.

278   The energetic investment was likely utilised by primed animals to face both the priming

279   stimulus, which is in itself a stressful condition that induces a response in the animal, and to set

280   up the regulatory changes to maintain stress memory. The comparison at the molecular and

281   enzymatic level between naïve and primed clams in the absence of triggering stimulus more than

282   a month after priming revealed at least part of such changes. Enzymatic activity of SOD was

283   higher, but not significant, in primed animals without triggering stimulus, although SOD activity

284   significantly decreased in naïve clams as a long-term consequence of HW exposure, while it was

285   unaffected in primed ones. The activity of GPx was significantly higher in primed clams

286   irrespective to the occurrence of HW (Fig. 3). Thermal stress is known to increase reactive oxygen

287   species (ROS), which are detrimental for cells because they cause oxidative damage to

288   membranes and organelles[42]. In fact, many authors have reported an increased activity in the

289   components of the antioxidant pathway (e.g. SOD, GPx, Cat) in the acute response to MHWs

290   (e.g.[15,26,43-45]). Primed clams appeared therefore to be better equipped for oxidative stress

291   response than naïve ones, with priming being able to confer higher protection against ROS, as

292   reported by others[15,46]. When searching for differential mRNA expression specifically for GPX- and

293   SOD encoding genes, five isoforms of GPX (*GPX1, GPX2, GPX3, GPX5, GPX6*) were found in the

294   annotated clam genome. Only *GPX5* showed marginally significant differential expression with

295   FC=1.6 (uncorrected p=0.055). Several SOD isoforms were also identified, although none

296   appeared to be differentially regulated, mirroring the results of SOD enzymatic activity.

297   While antioxidant response did not show a specific profile at the gene expression level,

298   RNA-seq analysis revealed a vast transcriptional program induced by priming, which likely

299   underlies environmental stress memory. At the single gene level, the involvement of one protein

300   chaperone confirmed the importance of maintaining protein quality during thermal stress. The

301   observed negative regulation of cytoplasmic protein translation is fully in agreement with the

302   known phenomenon of general protein synthesis reduction as a response to most types of cellular

303   stress. In general, such phenomenon is mediated by post-translational changes, e.g. through

304   phosphorylation of EIF2a, which ensure a faster response. Stress-induced translation repression

305   represents a protective mechanism to ensure protein quality, while allowing reallocation of

306   cellular resources toward the synthesis of specific stress proteins[47]. In the case of stress memory,

307 evidence from the present work suggested that the same goal might be achieved on the longer

308 term through the coordinated down-regulation of several genes involved in protein translation,

309 including key translation initiation factors (e.g. *EIF4G*, different components of the EIF2 complex),

310 ribosomal proteins, and proteins with an important regulatory role in translation control such as

311 *RACK1*, a scaffold protein that transduces upstream signals to translation, and mTOR[48].

312 Two other broad sets of genes were significantly involved in clam environmental memory.

313 These gene sets are largely overlapping and include several genes encoding protein subunits of

314 respiratory chain complex I and complex V (ATP synthase), which were all up-regulated in primed

315 clams. Expression of genes encoding respiratory complexes subunits have been shown to be

316 regulated by a broad array of non-coding RNAs (i.e. miRNAs, lncRNAs, ciRNAs[49]), which are

317 considered to play a key role in plant stress memory[50]. Complexes I and V represent respectively

318 the first and the last component of the respiratory chain, the core of the mitochondrial machinery

319 for energy production in eukaryotic cells. A possible interpretation of this finding is that

320 coordinated upregulation of several components of these two complexes of the electron

321 transport chain might help facing the increased energy demand during heat stress and reduce the

322 associated production of reactive oxygen species that is known to occur under such condition. In

323 murine cardiomyocytes upon anoxia/reoxygenation treatment, which induces oxidative stress

324 like thermal stress, the cellular response is mediated by *miR-762* and leads to decrease complex

325 I activity, lowering intracellular ATP, increasing ROS levels, and promoting apoptotic cell death[51].

326 Evidence that repressing cell response might improve stress tolerance has often been

327 reported and has provided the basis for the hypothesis of hormesis. It might also explain the

328 observed down-regulation of two other complex gene sets, which both include a large number of

329 genes that participate in the response to hormonal/internal stimuli. Of note is the repression of

330 *SIRT1* in primed clams. Sirtuin1 belongs to the class III histone deacetylase family of proteins and

331 is crucial for various biochemical processes within cells, including oxidative stress, inflammation,

332 lipid metabolism, and autophagy as recently reviewed by Wang et al.[52] for hepatic cells, which

333 functionally recapitulate the role of clam hepatopancreas. Since *SIRT1* functions both indirectly

334 through epigenetic modifications of regulatory regions in several target genes and directly

335   through deacetylation of key transcription factors, its effects on transcriptional programs are

336   potentially very broad.

337   The comparison of primed and naïve clams after the end of the triggering event also

338   showed a significant divergence in their transcriptome profiles, suggesting that priming and stress

339   memory translate into a modified response to thermal stress, targeting key cell biological

340   processes. As exposed by Moseley[53], when the body is not able to deal with the excessive heat,

341   then this stressful condition can cause tissue injury. Some tissues, in particular the gut, must

342   preserve their integrity to fulfil their barrier function between the external and the internal

343   environments. For instance, excessive heat can damage the intestinal epithelial barrier and

344   induce a release of endotoxins in the body[53,54]. Animals that are acclimatised to their environment

345   have a greater ability to effectively disperse heat excess, thus avoiding damage to epithelial

346   barriers, mostly thanks to heat shock proteins (HSPs), which prevent denaturation of proteins and

347   lower barrier permeability[53]. In primed clams exposed to heat stress, the observed up-regulation

348   of *HSPXX* and *NPHS1* and *ADGRL3,* two regulators of barrier permeability suggested a more

349   efficient acclimatory response. Recent studies reported upregulation of the tight junction

350   pathway, cell adhesion molecules, and of several HSP genes in heat-hardened *Mytilus coruscus*[55],

351   *Argopecten irradians irradians*[56], *Crassostrea gigas*[57], and *Pinctada maxima*[58]. Our hypothesis is

352   further supported by the fact that an opposite response (i.e. the down-regulation of six heat

353   shock proteins and many molecular chaperones and the down-regulation of processes such as

354   "cell junction" and "response to unfolded protein") was observed in naïve clams (NH) after the

355   triggering event, reinforcing the idea that these changes (i.e. preventing protein denaturation

356   with HSPs and maintaining epithelial barrier integrity) at molecular level were indeed acclimatory

357   responses that play an important role in dealing with heat damage.

358   In order to maintain the internal homeostasis and preserve normal functions, the body

359   requires energy to enable stress adaptation[40,59], hence metabolic adjustments are foreseen when

360   animals are exposed to stress. In the present study, up-regulation of two large sets of genes

361   involved respectively in lipid and amino acid metabolism suggested that primed clams exposed

362   to lethal HW might be able to mount more quickly than naïve animals a metabolic response in

363   the digestive gland, which is fundamental in food digestion and in storing energy-rich compounds,

364 using non-sugar compounds such as lipid and amino acids, putatively to sustain the production

365 of ATP for the entire body. Recently, Georgoulis *et al.*[15] and [60] showed that priming *Mytilus*

366 *galloprovincialis* resulted in metabolic adjustments to increase ATP production, by acting on the

367 Krebs cycle and on the electron transport system with responses that were either stronger, earlier,

368 faster or more sensitive than naïve animals. Furthermore, as reported in Gurr *et al.*[10], clams

369 primed to acidified conditions or thermal stress, respectively, were able to respond more quickly

370 to a second encounter with lethal stress by triggering genes enriched for mitochondrial recycling

371 and immune defences. In contraposition to priming with sub-lethal thermal stress, He et al.[61]

372 showed that repeated exposures to extreme thermal stress in the pearl oyster induce a general

373 down-regulation of energy metabolism and argued that this may be linked with a disfunction of

374 mitochondria. This latter observation highlights the difference between repeated exposure to

375 extreme (and potentially lethal) heat stress and priming via non-lethal stress. In the non-lethal

376 treatment animals can trigger transcriptomic changes and frontload genes for important

377 pathways such as metabolic pathways (e.g. [10]), while repeated lethal treatments damage to key

378 cellular systems for energy production and regulation of metabolic processes might be too

379 extensive for priming being beneficial. In fact, in corals it has been observed that excessive stress

380 exposure during priming have detrimental effects in the recovery phase and after a triggering

381 event[31].

382   As already observed mainly in corals, priming and triggering event induced significant

383 changes in clam-associated microbiota. The role of host-associated microbiota is increasingly

384 recognised as fundamental in animal health and disease[62,63]. Despite the digestive gland

385 microbiome shows a remarkable resilience to change[64], it was recently proved that sub-lethal,

386 prolonged HWs can induce a substantial shift in its composition, resulting in increased relative

387 abundance of detrimental and concomitant decrease of beneficial taxa[26]. In this study taxa

388 belonging to the families *Flavobacteriaceae, Cryomorphaceae* and *Rhodobacteraceae* (Fig. 5)*,*

389 identified as detrimental by Vompe *et al.*[22] in corals, were more abundant in naïve clams after

390 exposure to HW, suggesting in line with previous studies[26,65], that HW can induce dysbiosis by

391 favouring the onset of detrimental taxa. As reported by Scanes *et al.*[25] on the Sydney rock oyster,

392 the ability of the host in contrasting the onset of pathogenic bacteria and the parallel decrease of

393 beneficial one is an essential aspect to predict the host ability to survive thermal stress. Primed

394 clams showed no such increase in relative abundance of detrimental taxa after the triggering

395 stimulus, whereas a higher abundance of beneficial bacteria belonging to the order *Clostridiales*

396 was observed. This is in agreement with results from Liu *et al.*[65], who reported that oysters

397 resistant to heat stress were enriched for bacteria of the orders *Clostridiales* and

398 *Verrucomicrobiales*. In the absence of triggering stimulus, significantly higher abundance of

399 *Clostridiales* was detected in primed clams compared to naïve ones. It should be noted, however,

400 that the presence of putatively beneficial taxa in the digestive gland microbiome of primed clams

401 does not prove that the microbial community has a positive causal effect on the host fitness traits.

402 Proving or disproving such effect would require the creation of naïve gnotobiotic clams,

403 harbouring either a "primed" microbiome or a "naïve" one.

404   As already mentioned, in the present study the triggering stimulus closely mirrored

405 naturally occurring HWs. The gap of 15 days between priming and triggering recapitulates

406 consecutive MHW events observed in the wild (Fig. 6A, B). As reported by Moyen *et al.*[11], the

407 majority of MHW occurring in the same year in the Bay of Monterey had a gap interval between

408 consecutive events (in term of days) of less than 16 days. In the Mediterranean Sea, a similar

409 pattern was observed: summer MHWs had a median value for the time gap of 10-12 days (Fig.

410 6B). A similar pattern was found in the Venice lagoon, which together with the Po River delta is

411 the most productive site in Europe for Manila clam. Based on the evidence obtained here it could

412 therefore be hypothesised that "artificial" priming, induced before seeding clams in on-growing

413 areas by artificially raising water temperatures in pre-fattening structures (e.g. flupsies), might

414 represent a highly effective operational tool to mitigate the effects on climate change on clam

415 aquaculture.

Limitations and conclusion

417   In this study it was shown under realistic conditions that priming might have a great

418 potential for mitigating the effects of climate extreme events. In fact, we report increased

419 resilience to a lethal HW that resembled (in terms of intensity and duration) an event occurred in

420 the Venice lagoon in 2015. A complex interplay of adjustments at behavioural, physiological, and

421   molecular levels underpins this increased tolerance, which could be exploited to increase the

422   thermal performance of clams and also to support clam aquaculture. However, in this framework,

423   much remains to be done especially in relation to its optimisation under different priming

424   conditions (i.e. intensity, duration). Finally, since clams reproduce during summer when HWs

425   occur, it should be tested whether at the end of the reproductive season, when energy reserves

426   have been depleted by the reproduction, priming costs for maintaining stress memory and

427   ensuring an improved response to triggering stimuli might be excessive for the animal's energy

428   budget. However, overall priming holds great promise as a mitigation strategy that can be applied

429   also to other important farmed shellfish species (e.g. oysters, mussels) to help sustain the

430   aquaculture sector against climate change induced threats.

431   RESOURCE AVAILABILITY

432   Lead Contact

433   Further information and requests for resources and reagents should be directed to and

434   will be fulfilled by the lead contact, Luca Peruzza (luca.peruzza@unipd.it).

435   Materials Availability

436   This study did not generate new unique reagents.

437   Data and code availability:

438   • All Next-generation sequencing data have been deposited at the NCBI data repository and

439   are publicly available as of the date of publication. Accession numbers are listed in the key

440   resources table. All remaining data can be shared by the lead contact following request.

441   • This study did not generate any original code.

442   • Any additional information required to reanalyse the data reported in this paper is

443   available from the lead contact upon request.

Author contributions

445   LP and LB conceived the project; CFT, MM, GDR, IB, SF, RF, MB, MP and LP performed the

446   experiments and collected samples; CFT analysed behavioural data; CFT, RF and GDR performed

447   biomarker and molecular analyses; CFT and LP performed RNAseq and 16S microbiota analyses;

448   LP wrote the first draft of the manuscript with input from CFT, MM and LB; all authors commented

449   and critically revised the manuscript.

450   ## Acknowledgments

466

467   ## Declaration of interest

468   The authors declare no competing interests.

469

470   ## Figure legends

471

472   **Figure 1:** Impact of priming on clam behavior. The upper panels show the proportion of primed

473   and naïve clams that successfully buried in the sand A) after priming and B) after HW challenge

474   and C) without HW challenge. The lower panels show the burying speed (i.e. the amount of time

475   that individual clams took to hide in the sand) of primed and naïve clams A) after priming and B)

476   after HW challenge and C) without HW challenge. In upper panels A) B) and C) Pearson's chi-

477   squared test with significance, confidence interval (CI) and number of observations is reported in

478   the subtitle. Results of one-sample proportion test are displayed on top of each bar. In lower

479   panels A) B) and C) box and violin plots with individual observations are depicted. Student t-test

480   with significance, CI and number of observations is reported in the subtitle. Statistical tests are

481   reported in the caption. Different letters indicate significant differences between groups after

482   post-hoc multiple comparisons (significance level: adjusted p-value <0.05).

483

484   **Figure 2:** Impact of priming on clam's survival following MHW conditions. Kaplan-Meier survival

485   estimates on primed (yellow dashed line) and naïve (green dotted line) clams exposed to A) MHW

486   (grey area) and then kept at 25 ˚C or B) kept at control conditions (25 ˚C) for the entire time.

487   Differences in survival were computed using Log-Rank (Mantel-Cox) test. Statistical details (i.e.

488   significance, degree of freedom and number of observations) are reported in the plot. The grey

489   shaded area in A) indicates the onset and duration of the MHW (refer to Figure 1 for details on

490   MHW conditions). Note that the start time (i.e. day 0) on the x-axis of both graphs corresponds

491   to day 23 in Fig. 1A.

492

493   **Figure 3:** Impact of priming on clam's overall status and antioxidant defenses. A) Condition index

494   of primed and naïve clams after challenge. Enzymatic activity of B) SOD and C) GPx and D) lipid

495   peroxidation in primed and naïve clams after challenge. For each plot, individual observations are

496   plotted and graphical details (i.e. mean ± CI or median ± IQ range) and statistical details are

497   reported in the caption. Different letters indicate significant differences between groups after

498   post-hoc multiple comparisons (significance level: adjusted p-value <0.05).

499

500   **Figure 4:** Impact of priming on clam's gene expression. A) Discriminant analysis of principal

501   components (DAPC) showing the distribution, along the first two component of variation, of each

502   biological sample (individual points). Points are shape-coded according to their treatment (circles:

503   naïve; triangles: primed) and are color-coded according to the challenge (red: exposure to MHW;

504   blue: no MHW). B) Numbers of differentially expressed genes of selected pairwise comparisons,

505   split by up-regulated (purple) and down-regulated (light blue) genes. Heatmaps of differentially

506   expressed genes in the C) "PH vs NH" and D) "NH vs NC" comparisons. Gene names on the right

507   side of the heatmap are added to label genes involved with molecular chaperones (red),

508   metabolism (grey) and cell-junction (yellow). Annotation on top of the heatmap illustrates the

509   membership of individual samples to each treatment. E) Functional analysis (i.e. GSEA) associated

510   with the different pairwise comparisons (reported in the grey, left column). A positive enrichment

511   score indicates up-regulation, while a negative enrichment score indicates down-regulation.

512   Significant hits are colour coded according to their FDR value and their size is proportional to the

513   percentage of genes contributing to the observed enrichment score for the pathway/process.

514

515 **Figure 5:** Impact of priming on clam's microbiota. Effects on A) alpha and B) beta diversity of

516 primed (triangles) and naïve (circles) clams. C) Taxonomic composition of the average microbiome

517 abundance at order level. Bar plot of significant genera (genera with relative abundance <5% are

518 not shown). In A) mean ± CI and individual points are plotted. Statistical details are reported in

519 the caption. Different letters indicate significant differences between groups after post-hoc

520 multiple comparisons. In B) points are shape-coded according to their treatment (circles: naïve;

521 triangles: primed) and are color-coded according to the challenge (red: exposure to MHW; blue:

522 no MHW).

523

524 **Figure 6:** Heatwave characteristics across the Mediterranean basin. A) Map of the Mediterranean

525 basin where 13 random coastal areas (red dots) and the Venice lagoon (grey dot labelled "VL")

526 have been investigated for the occurrence of MHW over the period June 1st 2000 to June 1st

527 2024. MHW data for each location were retrieved from

528 https://www.marineheatwaves.org/tracker.html#. B) Boxplot showing the interval gap (in days)

529 between the end of one MHW and the beginning of the following one for the Venice lagoon and

530 for all coastal areas indicated in A. Each red triangle represents an interval between two MHW

531 event. Only MHW occurring during summer were considered.

532

533

534 ## STAR Methods

535 EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

536   Adult individuals of Manila clam (*R. philippinarum*), with an average length of 26.01 ± 1.78

537 mm and an average weight of 3.75 ± 0.81 g, were purchased from SATMAR (France) in February

538 2022 and brought to the marine station of the University of Padua, located in Chioggia where all

539 experiments were carried out. It was not possible to sex experimental animals used in the

540 experiment. Experimental activities were carried out in 25L tanks with 30 clams per tank, ensuring

541 at least 0.5 liters for each clam, and were filled with natural sea water from the Venice lagoon.

542 For the entire duration of the experimental activities (Suppl. Figure 1), about 50% of the water

543 was replaced every two days, maintaining an average salinity of 30 PSU. Each tank was equipped

544 with a pump, air stones, an iButton temperature logger (Maxim Integrated, USA, model:

545 DS1921H-F5), and a 100W heater connected to a precision thermoregulator (MPT91 Mect, Turin

546 Italy) programmed to maintain the desired temperatures (i.e. either constant or fluctuating

547 throughout the day). Clams were fed daily with the commercial mixture of microalgae New Coral

548 Fito Concentrate (A.G.P., Italy), composed of *Isochrysis* (T-Iso) (33.3%), *Nannochloropsis* (31%),

549 *Tetraselmis* (18%) and *Phaeodactylum* (18%), at a final concentration of ~40 * 10^6 cell L^{-1}.

550

551 METHOD DETAILS

552 Experimental design

553   A 2x2 full factorial design was performed with "Priming" (levels: priming "P" and naïve

554 "N") and "Marine HW" (levels: heatwave "H" and control "C") as fixed factors. The experimental

555 design is depicted in Suppl. Figure 1.

556   Acclimation in experimental tanks lasted 15 days. During this period water temperature

557 was gradually increased from 18 to 25°C (at the rate of 1 °C day^{-1}, until the target temperature

558 was reached). Following acclimation, animals were tagged, weighted, and measured, then

559 randomly divided into two groups: primed (P) and naïve (N, i.e. non-primed, as defined by

560 Hackerott *et al.*[31]). The temperature of the primed group was increased to 30°C (at the rate of 2.5

561 °C day^{-1}) and then kept constant at 30 °C for 7 days, while the control group was maintained at

562 25°C, which represents the average summer water temperature recorded in the Venice lagoon.

563   We chose a priming temperature of 30 ℃ because it represents a sublethal stressful condition, as

564   demonstrated previously[26], and a duration of 7 days to test the effectiveness of a long priming

565   treatment on eliciting long-lasting memory effects. Subsequently, a resting period of 15 days

566   (chosen because it represents the average gap between consecutive heatwaves in the

567   Mediterranean Sea) at 25 °C was carried for both groups to allow recovery from the priming

568   conditions. Then each of the two treatments was randomly split in two groups: one was triggered

569   with a simulated MHW (see below for additional details) while the other group was kept at 25 °C

570   as control. This experimental design resulted in four different conditions in total, namely PH:

571   primed triggered with MHW; PC: primed non-triggered with MHW; NH: naïve triggered with

572   MHW; NC: naïve non-triggered with MHW. Each of these four conditions was tested in duplicate,

573   in 25 L aquaria with 30 clams per tank.

574   The simulated MHW lasted for 7 days, and water temperatures gradually oscillated each

575   day from 31 to 35°C (Fig. 1B). These oscillations mimicked a MHW recorded in the Venice lagoon

576   in 2015 (https://www.marineheatwaves.org/tracker.html), except for the fact that temperatures

577   were shifted upward +1 ℃ to account for near future predictions of increase in water temperature

578   due to CC[66]. Daily thermal oscillations were obtained via programming the precision

579   thermoregulators (MPT91 Mect, Turin Italy) equipped in each tank. Groups that were not exposed

580   to the MHW were kept at 25°C. At the end of the MHW, temperatures were brought back to 25 ˚

581   C and kept constant for additional 15 days.

582   Animal survival was monitored daily throughout the duration of the experiment and for

583   the 15 days following the end of the simulated MHW. The total duration of the experiment was

584   46 days.

585

586   Sample analysis

587   *Behavioral Tests*

588   To assess the behavioral response of clams following the exposure to priming and HW,

589   tests were conducted on a random subset of 25 clams for each group. The test was carried out

590   twice: at the end of the priming treatment (i.e. day 8, Fig. 1A) and at the end of the HW (i.e. day

591   32, Fig. 1A). In accordance with Peruzza *et al.*[26], for each behavioral test an 80L tank was prepared

592 by adding four centimeters of sand at the bottom of the tank and topped with 10 centimeters of

593 seawater and equipped with a small pump and aerator. A Logitech C920 PRO HD camera

594 (Logitech, Switzerland) was positioned above each tank, and once the clams were placed on the

595 sand, they were filmed for 2 hours in agreement with [67]. During the first behavioral test, water

596 temperature was maintained at 30°C for primed animals and at 25°C for naïve animals. The

597 second behavioral test, performed at the end of the MHW, was carried at the respective

598 temperature of each experimental group to avoid that abrupt changes in temperature could

599 interfere with animal's behavior (i.e. water temperature was 25 °C for groups PC and NC while it

600 was 31 °C for PH and NH groups).

601   Behavioral videos were double-blind analyzed, recording for each clam the time it was

602 placed on the sand, the time of the first recorded movement, and the time the clam had

603 successfully completed burrowing in the sand, in accordance with Peruzza et al.[26]. This allowed

604 to calculate the percentage of clams that successfully completed the burrowing and the time

605 taken for this process.

606

607 *Physiological and biochemical assays*

608   The physiological status of clams was assessed by looking at their condition index. This

609 index was calculated at the end of the experiment (i.e. day 46, Figure 1) on all surviving clams

610 from the four groups. With the help of a scalpel, the shell was separated from the soft body tissue,

611 which was then blotted on paper and weighed on a precision scale (Sartorius, Fisher Scientific).

612 Similarly, the shell was blotted on paper and weighed. The condition index, defined as the weight

613 of the soft tissue over the weight of the shell, is a measure of the general well-being of the clam.

614   At the end of the experiment, the mantle of 40 clams, 10 per group, was cut into two

615 pieces, snap frozen on dry ice and stored at -80 °C. These samples were later used to evaluate the

616 extent of lipid degradation and the activity of two enzymes produced in response to oxidative

617 stress: glutathione peroxidase (GPx) and superoxide dismutase (SOD). The assays used for these

618 analyses are based on spectrophotometric techniques: the absorbance of a reaction product or

619 of an intermediate is measured to derive its concentration through the construction of a

620 calibration curve with known concentration standards. All samples were run in duplicate using

621 transparent 96-well plates (SARSTEDT, model 82.1582.001) and a microplate spectrophotometer

622 (Multiskan Go1510, Thermo Fisher).

623 First, tissue homogenate was prepared from the mantle tissue. About 10mg of sample was

624 immersed in 300 μL of homogenization buffer (containing NaCl 150 mM, EGTA, 1 mM EDTA 1 mM

625 pH 8, TrisHCl 10 mM pH 7.4, TritonX100 0.2%, Phosphatase Inhibition Cocktail, Nonidet 5%, PIC

626 1x, and H$_2$O) with metal beads using the TissueLyser II (QIAGEN, Germany). The samples were

627 then centrifuged at 4°C for 20 minutes at 10000 rcf and the supernatant was taken. Total protein

628 content was determined via Bradford method (Bradford Reagent, B6916, Sigma). The remaining

629 homogenate was aliquoted and stored at -80°C for subsequent processing.

630 To measure SOD activity, the Dismutase Activity Assay Kit (CS0009, Sigma) was used

631 following the manufacturer's instructions.

632 The glutathione peroxidase activity was determined indirectly by assessing the amount of

633 NADPH consumed in the reducing reaction of oxidized glutathione (GSSG) to reduced glutathione

634 (GSH). GSH is the substrate for glutathione peroxidase (GPx) and gets oxidized to GSSG. Six

635 standards of known NADPH concentrations were obtained by diluting 1 mM NAPDH with a

636 diluting solution composed of PBS 62.5 mM, EDTA 6.25 mM, H2O. GPx 2 U/ml (G6137-100UN,

637 Sigma Aldrich) were used as positive control and the diluting solution was used as reagent control.

638 All samples, standards, and controls were run in duplicate. 25 μL of each sample were put in the

639 sample wells and 80 μL of the assay solution containing all the necessary reagents to recreate the

640 GSSG reduction and GSH oxidation reaction (GSH 130 mM, GR 2.5 U/ml, NADPH 40 mM (481973-

641 25MG, Sigma Aldrich) suspended in the diluting solution) were added to the samples and controls

642 wells. The plate was shaken for 15 minutes at room temperature, and to ensure an excess of

643 NADPH in the samples, if the optical density in the wells was less than 1.0, 1 μL of 40 nM NADPH

644 was added. The GSH oxidation reaction by GPx was initiated by adding 20 μL of 1.875 mM cumene

645 hydroperoxide (247502-5G, Sigma Aldrich). Finally, absorbance at 340 nm was read for 10

646 minutes to determine how much NADPH was consumed in that time interval. A calibration curve

647 was obtained by the standard absorbances to get the concentration of NADPH in the samples and

648 to derive GPx activity.

649   Lipid peroxidation was estimated by measuring the concentration of the final product of

650   a polyunsaturated lipid peroxidation, malondialdehyde (MDA), in accordance with the protocol

651   of a commercial kit (MAK085, Sigma Aldrich).

652

653   *RNAseq analyses*

654   At the end of the experiment, from the same animals from which the mantle was taken

655   for lipid and enzymatic assays, the digestive gland was collected and stored at -20°C in RNAlater®

656   (ThermoFisher, USA). RNA was extracted using the RNeasy Mini Kit (Qiagen, Germany), quantified

657   with Qubit Fluorometer (Invitrogen, USA) and qualitatively assessed with the Bioanalyzer

658   (Agilent). RNA samples with concentration >20 ng/µl and RIN > 8 were sent to CRIBI (University

659   of Padua, Italy) for the preparation of 3' Tag Lexogen (Lexogen GmbH, Austria) library and

660   sequencing. Sequencing was performed on Illumina Novaseq 6000 platform with 75 SE

661   sequencing. The sequences obtained are available in NCBI Sequence Read Archive (BioProject ID:

662   PRJNA1132140).

663   The quality of the raw reads was assessed using FastQC/v0.11.9

664   (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and BBduk of the suite BBTools

665   was used for trimming. STAR/v2.7.11a[68] was employed to map the trimmed reads onto the Manil

666   clam genome from Xu et al.[69] with the option "--outFilterMismatchNoverLmax=0.1". Gene counts

667   were obtained using the program featureCounts/v2.0.0[70] by counting multimapping reads with

668   options "-M" and "--fractional" and the count table was analyzed with R/v4.0[71] to determine

669   differentially expressed genes between four pairs: NH vs NC, PH vs PC, PH vs NH, PC vs NC. Before

670   conducting the pairwise differential expression analysis, transcriptome data was filtered to

671   remove genes with low expression values that could contribute to background noise in

672   accordance with Bernardini et al.[72], data were then normalized using the 'RUVs' function of the

673   RUVSeq/v1.36 package[73], selecting respectively 1, 2, 6 and 3 factors of unwanted variation for

674   each pairwise comparison.

675   Pairwise differential expression analysis was performed using the R package edgeR/v4.0[74]

676   running a likelihood ratio test (LRT) to identify differentially expressed genes. From the differential

677 expression result file containing all genes, only those with an adjusted FDR < 0.05 and a log2FC

678 threshold > |0.58| were deemed significant.

679   To determine if there were overrepresented categories among the differentially expressed

680 genes, a Gene-Set Enrichment Analysis (GSEA) was performed to test for enriched functions using

681 the software clusterProfiler/v4.6.2[75] and the databases: Gene Ontology, KEGG, and Reactome.

682 The functional databases were downloaded from gProfiler's webpage

683 (https://biit.cs.ut.ee/gprofiler/gost) using human as reference species. Identity between human

684 genes and Manila clam genes was assessed by running Blastx search as specified in Peruzza *et*

685 *al.*[26]. For these analyses, the FDR threshold was set at 0.2.

686

687 *Clam-associated microbiota:*

688   Microbiota analyses were carried on the same RNA used for transcriptomic analysis.

689 Preparation for the biological material followed steps described in Peruzza *et al.*[26]. The 16S

690 amplicon sequencing were performed at Biomarker Technologies facility (BMK GmbH, Germany)

691 targeting V3-V4 region of 16S gene, all samples were then sequenced on Illumina Novaseq 6000

692 with 250 pair-end approach. The sequences obtained are available in NCBI Sequence Read

693 Archive (BioProject ID: PRJNA1132140).

694   Sequences were analyzed using the pipeline of QIIME2[76], which constated in filtering raw

695 reads based on quality score, dereplication, merging and chimera removal by using

696 dada2/v1.14.1[77], as reported in detail in Peruzza *et al.*[26]. ASVs were taxonomically classified up to

697 genus level using the SILVA 16S Database (NR98 version 138.1). The ASV table was then imported

698 in microbiomeanalyst (https://www.microbiomeanalyst.ca) where it was further processed. ASV

699 table was filtered to remove low count ASV with less than 4 counts in 10% of the samples. ASV

700 table was not rarefied, in accordance with Willis[78]. Alpha and beta diversity metrices were

701 calculated using default values. Pairwise comparisons were performed using the "Multiple Linear

702 Regression with Covariate Adjustment" tool and ASV with FDR < 0.05 were deemed significant.

703

704 QUANTIFICATION AND STATISTICAL ANALYSIS

705   Differences in survival between treatments were assessed and plotted using the
706 survminer/v0.4 library in R.

707   For each quantified parameter, normality and homogeneity of variances were assessed
708 using the "shapiro.test" and "var.test" functions of base R. When normality and/or homogeneity
709 of variances were not met, an attempt to normalise them was performed using the
710 bestNormalize/v1.8 library.

711   Behavioural analyses in relation to the proportion of clams that completed burying were
712 analysed and plotted using the "ggbarstats" function from the ggstatsplot/v0.8[79] library.
713 Behavioural analyses relating the burying speed were analysed and plotted either using the
714 "ggbetweenstats" function, or by using a custom 2-Way ANOVA code in R followed by plots with
715 ggplot2[80].

716   Physiological and biochemical data were analyzed using a 2-way ANOVA: when normality
717 and homogeneity of variances were granted, a classical 2-way ANOVA was performed with post-
718 hoc tests in R; if data were not normally distributed, pairwise comparisons using Wilcoxon rank
719 sum exact test were performed. An adjusted p-value <0.05 was deemed significant. Detailed
720 results of the 2-way ANOVA analyses are reported in Data S1 (see Supplemental Item). Post-hoc
721 pairwise comparisons results are integrated within each plot by means of the compact letter
722 display method where groups sharing the same letter are not significantly different from each
723 other, while groups with different letters have statistically significant differences.

724 ## Excel-format supplemental table legends:

725 **Table S1:** Transcriptomic analyses. Pairwise differential expression analysis results for each gene
726 tested for differential expression with edgeR in A) NV vs NC; B) PH vs PC; C) PH vs NH; D) PC vs
727 NC. Each table contains the ids of the clam genome on column A, the log-transformed fold change
728 on column I and the adjusted p-value (FRD) on column M. Gene set enrichment analysis results
729 for each pairwise comparison: A) NV vs NC; B) PH vs PC; C) PH vs NH; D) PC vs NC. Each table
730 contains the pathway id on column A, the normalised enrichment score (NES) on column E and
731 the adjusted p-value on column G.

732

733 **Table S2:** 16S metabarcoding analyses. Pairwise differential abundance analysis results for each

734 ASV at genus level in A) PH vs NH; B) PC vs NC; C) NH vs NC; D) PH vs PC. Each table contains the

735 genus of each ASV on column A, the log-transformed fold change value on column B and the

736 adjusted p-value on column E.

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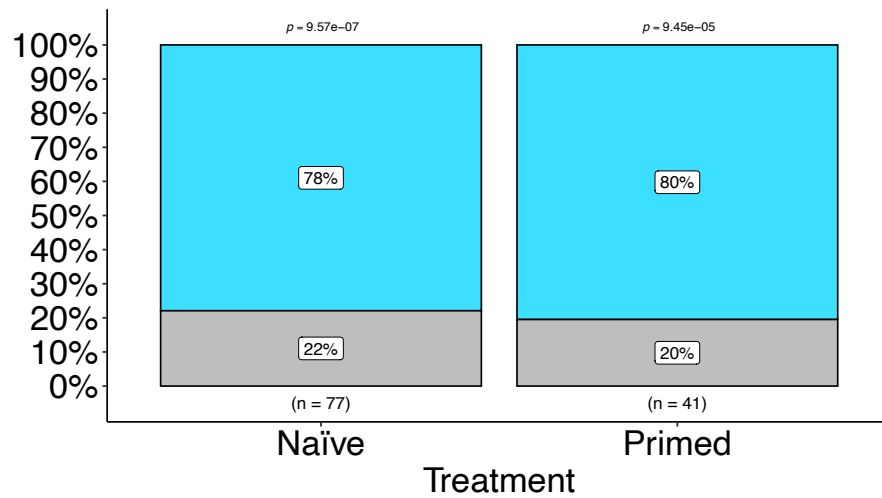951   2864-2880. 10.1111/eva.13319.

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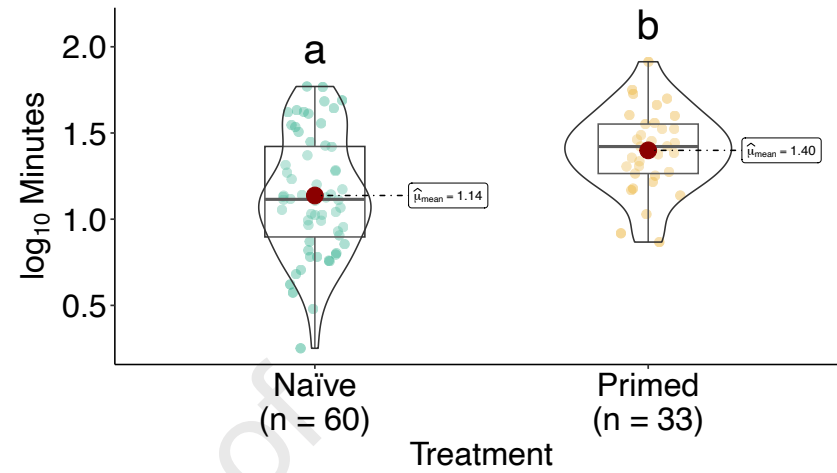994   Open Source Software *6*, 3167.

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A

After priming

$\chi^2_{\text{Pearson}}(1) = 0.11$, $p = 0.75$, $\widehat{V}_{\text{Cramer}} = 0.00$, $\text{CI}_{95\%}$ [0.00, 0.18], $n_{\text{obs}} = 118$
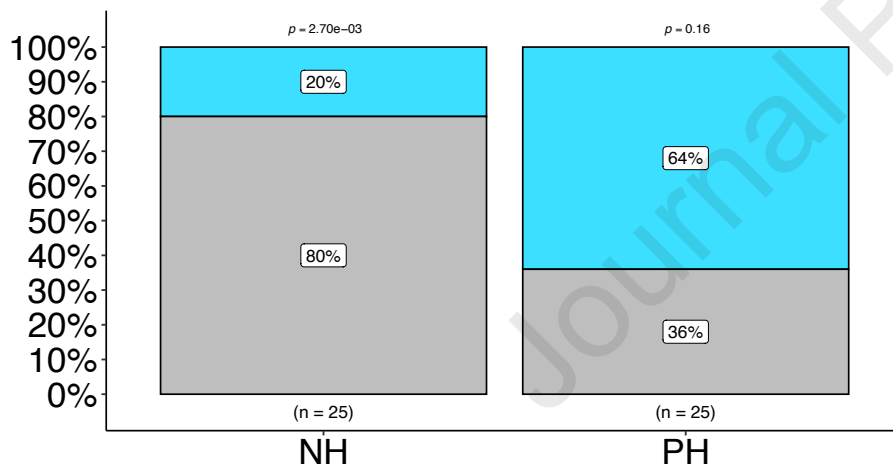
Completed ■ Yes ■ No

$p = 9.57e{-}07$ $p = 9.45e{-}05$

78% 80%

22% 20%

(n = 77) (n = 41)

Naïve Primed

Treatment

$t_{\text{Welch}}(87) = -4.31$, $p = 4.28e{-}05$, $\widehat{g}_{\text{Hedges}} = -0.87$, $\text{CI}_{95\%}$ [-1.29, -0.45], $n_{\text{obs}} = 93$

Treatment ● Naïve ● Primed

a b

$\widehat{\mu}_{\text{mean}} = 1.14$ $\widehat{\mu}_{\text{mean}} = 1.40$

Naïve Primed
(n = 60) (n = 33)

Treatment

$\log_{10}$ Minutes

B

Priming + triggering

$\chi^2_{\text{Pearson}}(1) = 9.93$, $p = 1.62e{-}03$, $\widehat{V}_{\text{Cramer}} = 0.43$, $\text{CI}_{95\%}$ [0.09, 0.72], $n_{\text{obs}} = 50$

Completed ■ Yes ■ No

$p = 2.70e{-}03$ $p = 0.16$

20% 64%

80% 36%

(n = 25) (n = 25)

NH PH

$t_{\text{Welch}}(6.14) = -1.03$, $p = 0.34$, $\widehat{g}_{\text{Hedges}} = -0.47$, $\text{CI}_{95\%}$ [-1.39, 0.48], $n_{\text{obs}} = 21$

Treatment ● Naïve + Heatwave ● Primed + Heatwave

a a

$\widehat{\mu}_{\text{mean}} = 1.51$ $\widehat{\mu}_{\text{mean}} = 1.64$

NH PH
(n = 5) (n = 16)

$\log_{10}$ Minutes
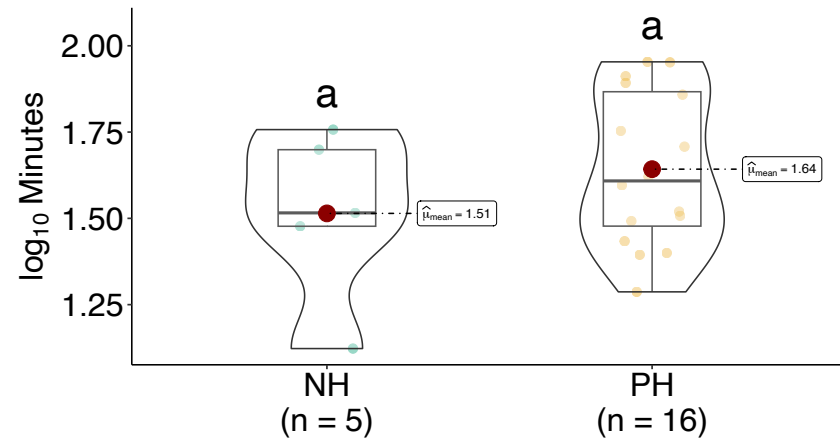
C

Stress memory

$\chi^2_{\text{Pearson}}(1) = 6.65$, $p = 9.91e{-}03$, $\widehat{V}_{\text{Cramer}} = 0.34$, $\text{CI}_{95\%}$ [0.00, 0.63], $n_{\text{obs}} = 50$

Completed ■ Yes ■ No

$p = 0.32$ $p = 9.32e{-}03$

60% 24%

40% 76%

(n = 25) (n = 25)

NC PC
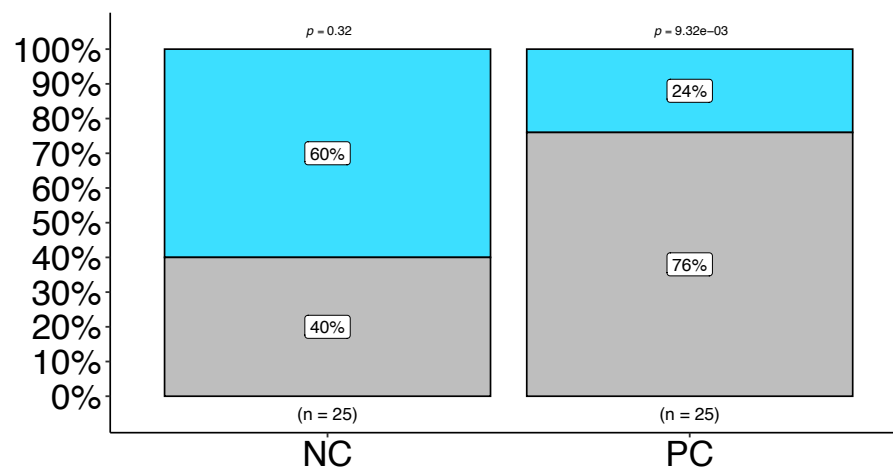
$t_{\text{Welch}}(9.2) = -1.57$, $p = 0.15$, $\widehat{g}_{\text{Hedges}} = -0.70$, $\text{CI}_{95\%}$ [-1.60, 0.24], $n_{\text{obs}} = 21$
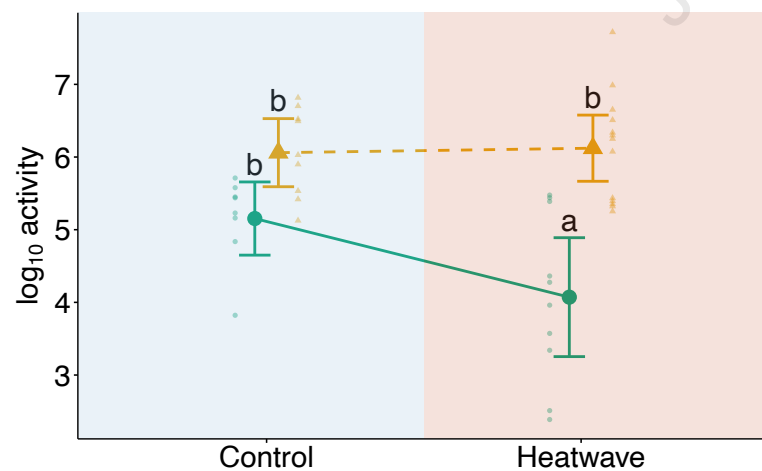
**Treatment** ● Naïve + Control ● Primed + Control

a a

$\widehat{\mu}_{\text{mean}} = 1.39$ $\widehat{\mu}_{\text{mean}} = 1.54$

NC PC
(n = 15) (n = 6)

$\log_{10}$ Minutes

A Triggered   **B** Control

Survival probability

Heatwave $\chi^2_{Log-rank}(1) = 5.1$, $p = 0.024$, $n_{obs} = 59$

$\chi^2_{Log-rank}(1) = 0$, $p = 1$, $n_{obs} = 60$

Time (d)   Time (da

Treatment   ⋯+⋯ Naïve   – + – Primed

A

Condition Index

Plot: mean ±CI.
Analysis: 2−way ANOVA with scheffe post−hoc comparisons

B

SOD Activity

Plot: mean ±CI.
Analysis: 2−way ANOVA with hsd post−hoc comparisons

C

GPx Activity

Plot: median with IQ range.
Analysis: Kruskal−Wallis rank test with pairwise Wilcoxon post−hoc comparisons

D

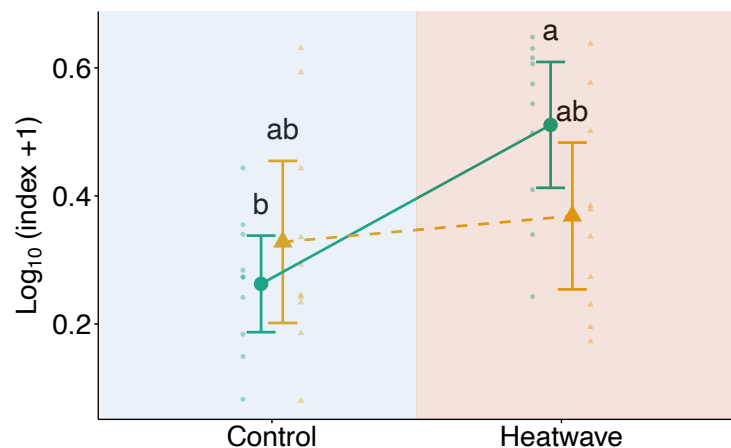Lipid peroxidation
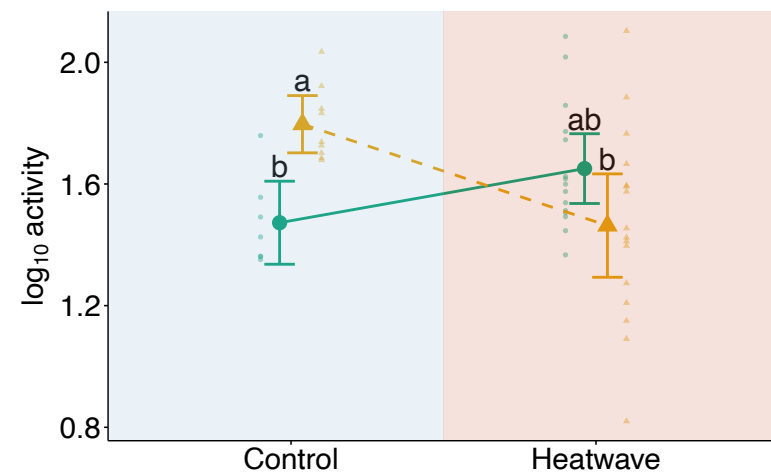
Plot: mean ±CI.
Analysis: 2−way ANOVA with hsd post−hoc comparisons
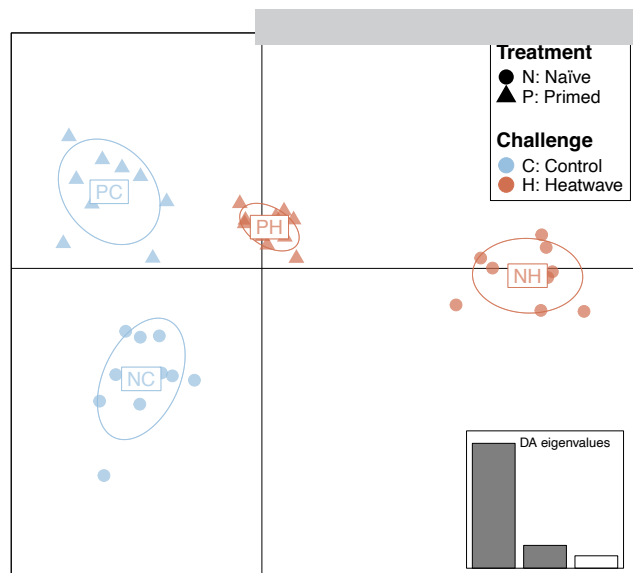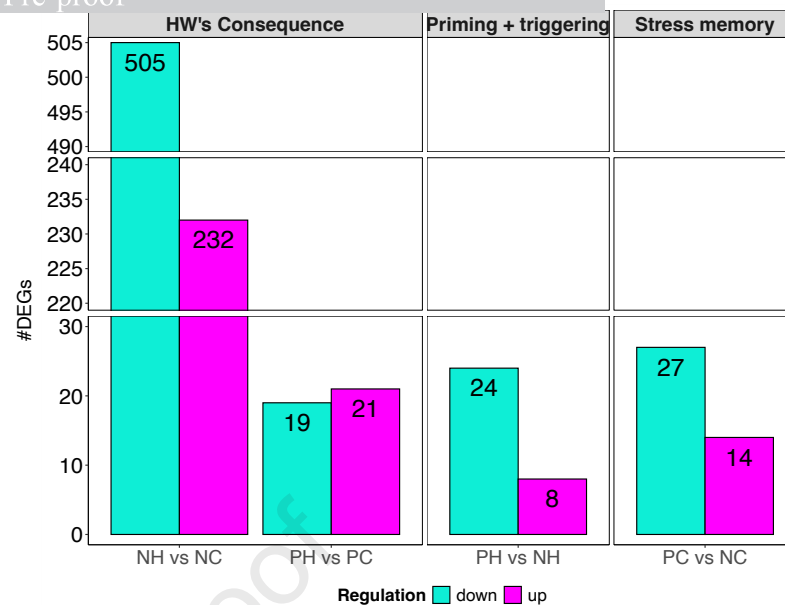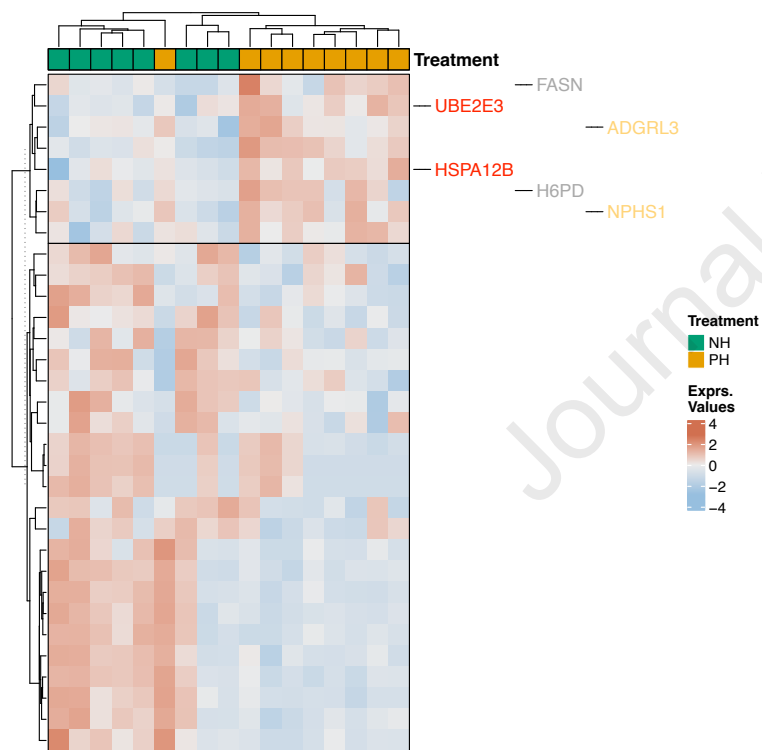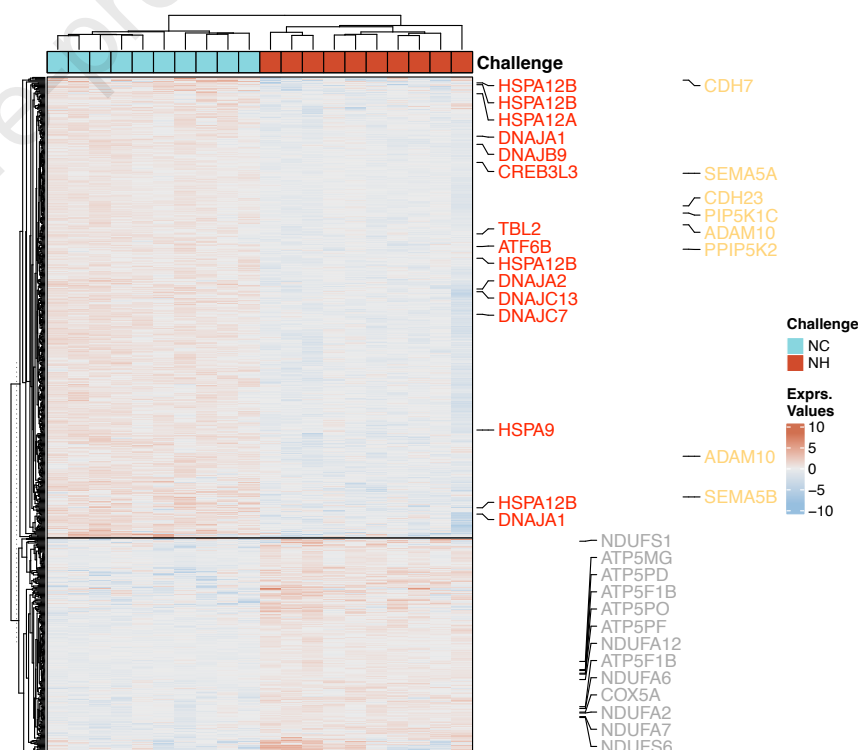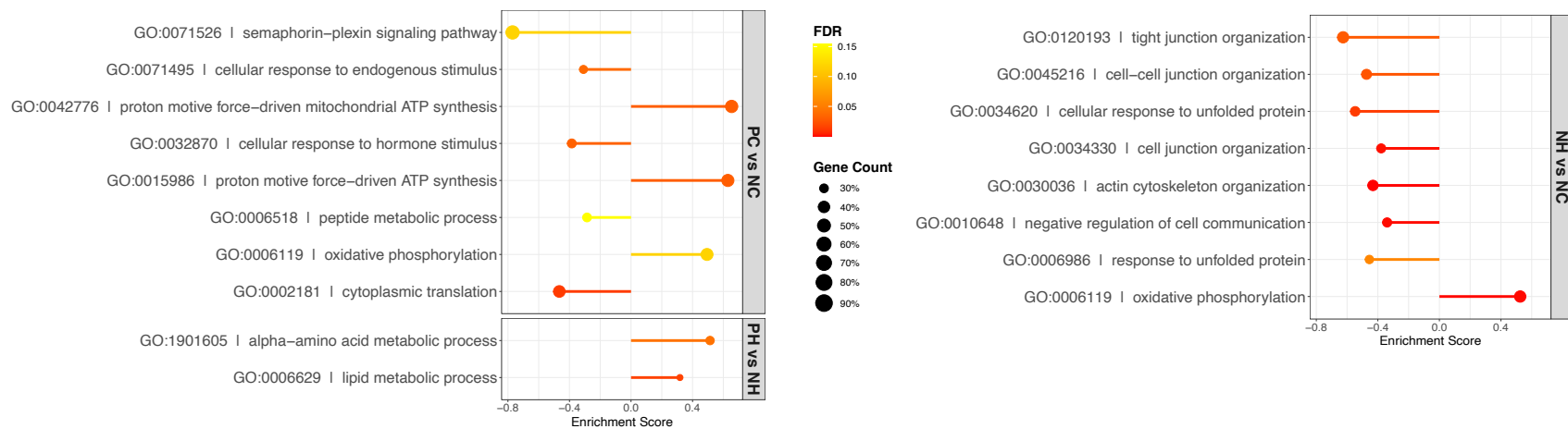
A Shannon alpha diversity

Treatment ● Naïve ▲ Primed

Plot: mean ±CI.
Analysis: 2-way ANOVA with scheffe post-hoc comparisons

B Beta div. – Bray Curtis

Pair	F-value	FDR
NH - PH	3.91	0.0015
NH - PC	2.87	0.0015
PH - NC	4.51	0.0015
NC - PC	2.9	0.0015
PH - PC	2.2	0.0096
NH - NC	1.85	0.045

Treatment
● N: Naïve
▲ P: Primed

Challenge
● C: Control
● H: Heatwave

C Most abundant orders
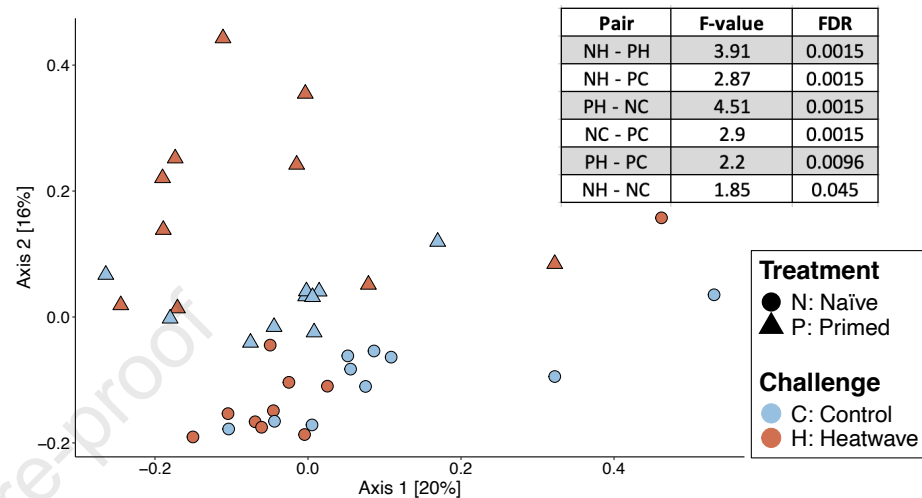
Order
■ Bacteroidales ■ Clostridiales ■ Not_Assigned ■ Rhodobacterales ■ Rickettsiales
■ Campylobacterales ■ Flavobacteriales ■ Others ■ Rhodospirillales ■ Spirochaetales
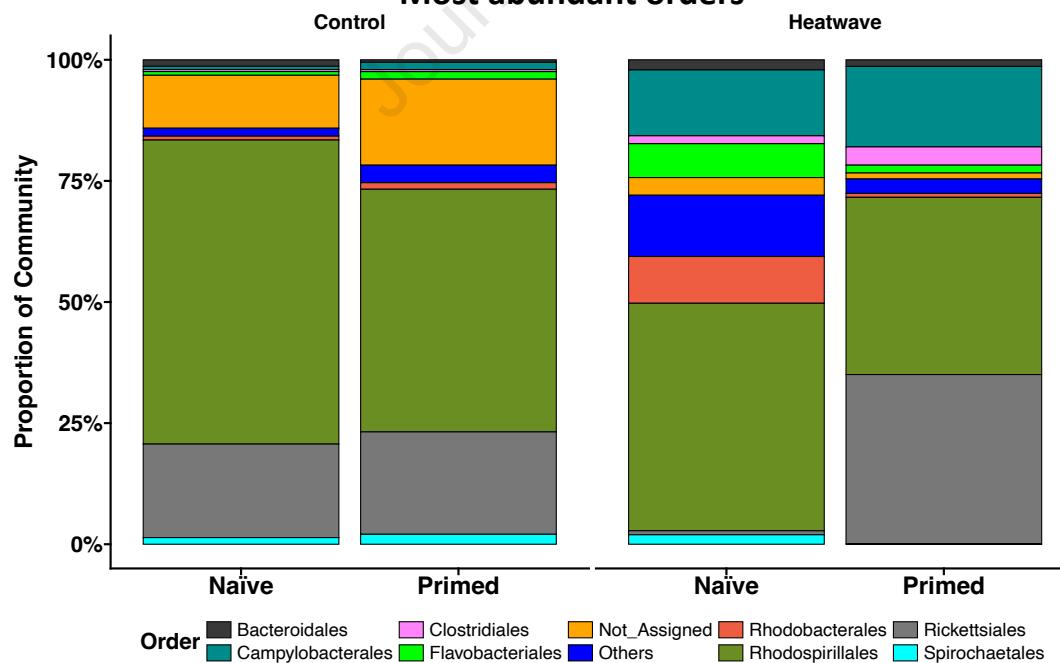
A
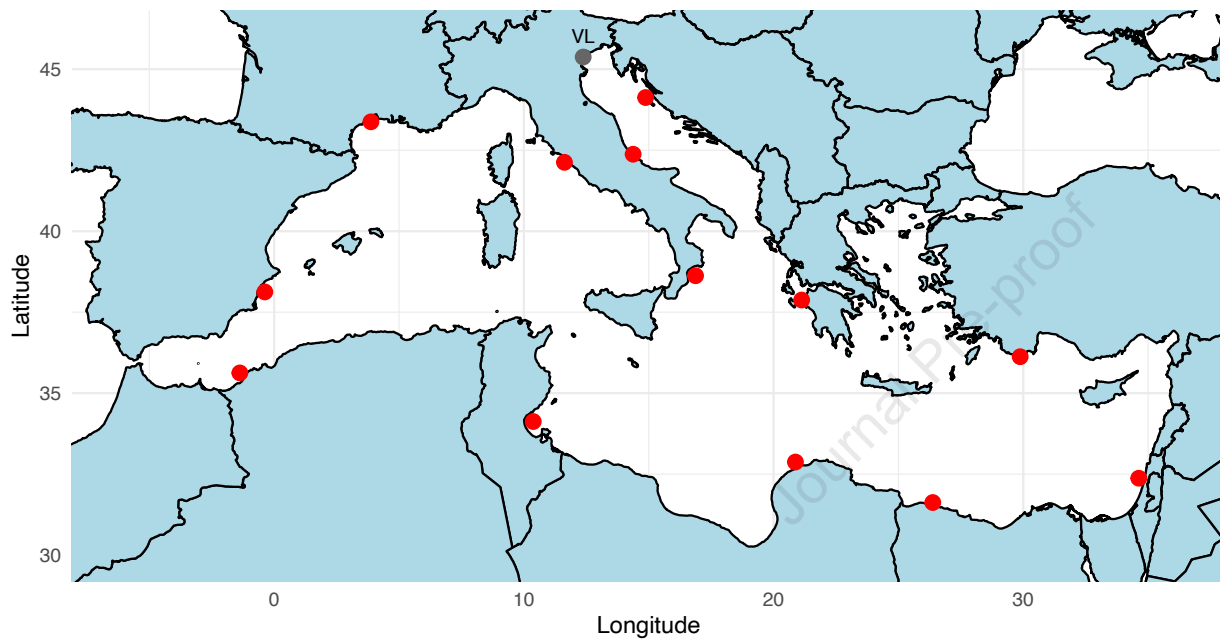
B
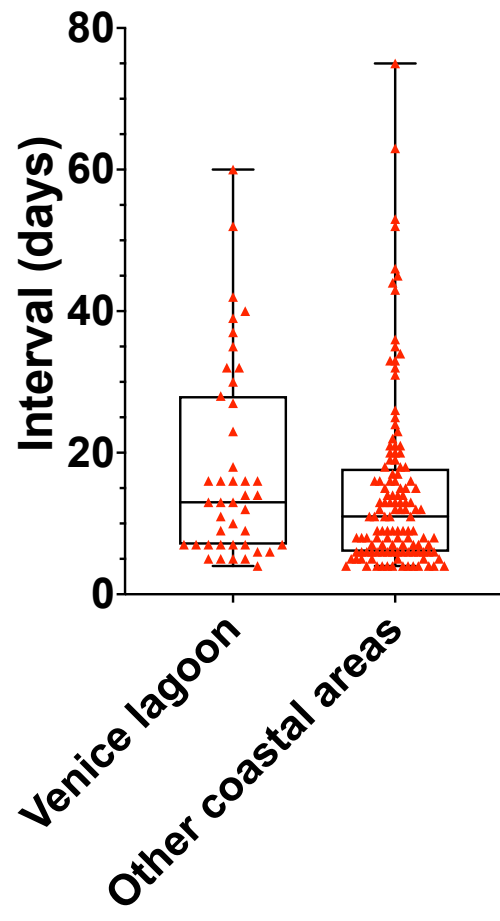
Highlights:

- Priming reduced clam death following heatwave that mimicked a realistic event
- Priming had a cost, but triggered behavioural and biochemical adjustments in primed clams
- Primed clams showed metabolic upregulation following heatwave and exhibited stress memory
- Naïve clams had more detrimental bacteria while primed clams had more beneficial taxa

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
SOD Assay test: Dismutase Activity Assay Kit	Sigma	CS0009
Lipid peroxidation Assay	Sigma	MAK085
Deposited data		
RNA-seq and 16S metabarcoding data:	BioProject ID: PRJNA1132140	NCBI - SRA Archive
Experimental models: Organisms/strains		
Manila clam. Adult organisms.	Animals bred in a commercial hatchery were used.	SATMAR hatchery (France)
Software and algorithms		
STAR Mapper	https://github.com/alexdobin/STAR	
FeatureCounts	https://subread.sourceforge.net/featureCounts.html	
edgeR	https://bioconductor.org/packages/release/bioc/html/edgeR.html	
RUVseq	https://bioconductor.org/packages/release/bioc/html/RUVSeq.html	
clusterProfiler	https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html	
microbiomeanalyst	https://www.microbiomeanalyst.ca	