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# Thermal fluctuations independently modulate physiological plasticity and the dynamics of the gut microbiome in a tropical rocky shore oyster

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## 31 **Abstract**

32           Extreme high thermal conditions on rocky shores are challenging to the survival of  
33 intertidal ectotherms. Yet, many species are highly successful in these environments in part due  
34 to their ability to regulate intrinsic mechanisms associated with physiological stress and their  
35 metabolic demand. More recently, there has been a growing awareness that other extrinsic  
36 mechanisms, such as animal-associated microbial communities, can also influence the tolerance  
37 and survival of ectotherms under stressful conditions. However, the extent to which the intrinsic  
38 and extrinsic mechanisms are functionally linked as part of the overall adaptive response of  
39 intertidal animals to temperature change and stress is poorly understood. Here, we examined  
40 the dynamics and potential interactions of intrinsic and extrinsic mechanisms in the tropical  
41 high-supratidal oyster, *Isognomon nucleus*. We found that oysters modulate their internal  
42 biochemistry (oxidized PUFA products including 5-F<sub>2t</sub>-IsoP, 10-F<sub>4t</sub>-NeuroP, 13-F<sub>4t</sub>-NeuroP,  
43 and 16-F<sub>1t</sub>-PhytoP) as part of their adaptive regulation to cope with physiological stress during  
44 periods of extreme high temperatures when emersed. While we detected extrinsic microbiome  
45 changes in alpha diversity (ASV richness and Shannon diversity index), the overall taxonomic  
46 and functional structure of the microbiome based on beta-diversity analysis (PERMANOVA)  
47 showed temporal stability and no association with the host biochemical profiles. Our finding  
48 here suggests that the microbiome taxonomic and functional structure is potentially maintained  
49 by a stable host control (not associated to the host biochemistry) and/or that the microbiome  
50 (independent of the host) is resilient to the local thermal fluctuations and extreme high  
51 temperature experienced. This microbiome stability is likely to contribute to the oyster, *I.*  
52 *nucleus* thermal tolerance, in addition to the intrinsic biochemical responses, to survive in the  
53 thermally challenging intertidal environment.

54 **Keywords:** Phenotypic plasticity, ectotherms, microbiome, thermal stress

## 55 **Introduction**

56           The tropical rocky shore is one of the most extreme environments on Earth. This system  
57 is characterised by a marked heterogeneous thermal landscape influenced by tidal dynamics  
58 (Raffaelli and Hawkins, 1999; Helmuth and Hofmann, 2001). Across this vertical gradient  
59 defined by the tides, environmental temperatures shape the physiology, behaviour, and spatial  
60 distribution of tropical intertidal ectotherms (Garrity, 1984; Williams and Morritt, 1995; Chow,  
61 2004; Samakraman *et al.*, 2010; Giomi *et al.*, 2016; Ng *et al.*, 2017). For ectotherms occupying  
62 the high-supratidal zone, thermal and oxidative stress (i.e., excessive production of reactive  
63 oxygen species - ROS) during desiccation periods of extended emersion, are the two of the  
64 major physiological challenges faced (Sokolova and Pörtner, 2001; Freire *et al.*, 2011; Birben  
65 *et al.*, 2012).

66           To survive under these challenging conditions, high-supratidal ectotherms modulate  
67 intrinsic/innate mechanisms associated with their internal physiology (e.g., metabolic rate) and  
68 biochemical activities. This modulation can be observed through, for instance, the removal of  
69 ROS via polyunsaturated fatty acids (PUFA; Sokolova and Pörtner, 2001; Yin *et al.*, 2017;  
70 Monroig *et al.*, 2022), and/or the control of the gene expression associated with heat shock  
71 response (HSR) and oxidative stress response (OSR; Wang *et al.*, 2022). PUFAs such as  
72 Arachidonic acid (ARA) and Docosahexaenoic acid (DHA) are known to be effective  
73 molecules and agent for ROS removal, because they contain unsaturated double bonds making  
74 them susceptible to ROS reaction (particularly to peroxide, HO•) (Sargent, 1976; Leung *et al.*,  
75 2015; Yin *et al.*, 2017; Fadhlouli and Lavoie, 2021). The non-enzymatic peroxidation of these  
76 PUFAs, generates by-products that can be used as biomarkers of the ROS level (Miller *et al.*,  
77 2014; Yonny *et al.*, 2016; Galano *et al.*, 2017). In addition to the biochemical modulation  
78 mechanism, some intertidal organisms (e.g., the oysters, *Isognomon nucleus*) are able to enter  
79 into a hypometabolic state (i.e., metabolic depression, for instance, marked by a drop in heart

80 rate; Marshall and McQuaid, 2011; Hui *et al.*, 2020) to avoid excessive energy consumption  
81 when exposed to high temperatures during low tides. Together, these functional characteristics  
82 are regarded as important adaptive traits for intertidal ectotherms to cope and ameliorate the  
83 extreme stress experienced on tropical rocky shores.

84 Survival under stressful environmental conditions is also hypothesized to be influenced  
85 by extrinsic/acquired mechanisms such as host-associated microbial communities (i.e.,  
86 microbiome; Bang *et al.*, 2018; Ahmed *et al.*, 2019). The structure and functions of these  
87 microbiomes have been suggested to play important roles in shaping tolerances and  
88 biochemical regulation of animals to elevated temperatures (Herrera *et al.*, 2020; Sepulveda  
89 and Moeller, 2020; Jaramillo and Castañeda, 2021). Under such conditions, functional  
90 microbiomes that are resilient/responsive to environmental changes are fundamental to the  
91 host's health and survival (Soen, 2014; Kremer *et al.*, 2018). For instance, Epstein *et al.* (2019)  
92 demonstrated that the resilience of the coral, *Pocillopora acuta*, to a stressful thermal event  
93 may be attributed to the stability of the microbiome composition and structure. In contrast, a  
94 dysbiotic host state is commonly associated with a destabilized microbiome (Fan *et al.*, 2013;  
95 McDevitt-Irwin *et al.*, 2019). This is typically reflected by a marked variation among host-  
96 associated microbiomes, a signal indicating the host losing control of the microbial community  
97 (e.g., increase in opportunistic pathogens) and/or differential sensitivities of microbiomes to  
98 extreme disturbances (e.g., elevated temperature; Carey and Duddleston, 2014; Petersen and  
99 Round, 2014; Lokmer and Wegner, 2015). This pattern is in-line with the “Anna Karenina  
100 Principle”, which states, “all happy families are all alike; each unhappy family is unhappy in  
101 its own way” (Zaneveld *et al.*, 2017; Díaz-Almeyda *et al.*, 2022). In other words, stressed  
102 organisms may host more stochastic microbiomes than healthy organisms. Although  
103 microbiome stability seems important for host homeostasis, some studies have suggested that  
104 flexibility in the microbiome can also provide adaptive capacities to the host, allowing it to

105 cope with rapidly changing environments (Maher *et al.*, 2020; Voolstra and Ziegler, 2020).  
106 Ziegler *et al.* (2017), for example, showed that the coral *Acropora hyacinthus* exhibits changes  
107 in its microbiome composition and structure that are aligned with the local thermal  
108 environmental conditions. Similarly, several studies have also shown seasonal variation in the  
109 host-associated microbiome of many marine organisms such as bivalves and echinoderms, as a  
110 potential plastic/flexible response to environmental change (Pierce and Evan, 2019; Feng *et al.*,  
111 2021). Microbiome stability or flexibility may, therefore, be a sign of host adaptive responses  
112 to environmental changes. However, the influence of these contrasting patterns is context-  
113 dependent and thus, their contribution to the tolerances and survival of animal hosts may differ  
114 in thermally challenging environments such as tropical rocky shores.

115         Previous studies have shown that the capacity for plastic adaptive responses of animal  
116 hosts to environmental stress may be modulated by the interactions between intrinsic  
117 (physiology and biochemical changes of the host) and extrinsic (host's microbiome structure  
118 and function) mechanisms (Muñoz *et al.*, 2019; Marangon *et al.*, 2021; Fontaine and Kohl,  
119 2023). It is unclear, however, to what extent these mechanisms are functionally linked as part  
120 of the whole organism's adaptive response (i.e., holobiont: host and microbiome) to survive in  
121 an extreme environment such as the tropical intertidal rocky shore. In this study, we  
122 investigated the intrinsic and extrinsic mechanisms underpinning tolerances and survival of a  
123 tropical intertidal ectotherm (oyster, *Isognomon nucleus*) by assessing the influence of natural  
124 variation in environmental temperature on i) host physiology (i.e., biochemical dynamics) ii)  
125 the diversity, structure, and function of the host gut-associated microbiome and iii) the interplay  
126 between these mechanisms. The integration of intrinsic and extrinsic mechanisms offers a  
127 holobiont perspective for understanding the stress tolerance regulation of ectotherms in extreme  
128 high temperature and fluctuating environments (Alberdi *et al.*, 2016; Apprill, 2017; Bang *et al.*,  
129 2018).

130

## 131 **Materials and methods**

### 132 *Site and species descriptions*

133 Host-microbiome interactions were investigated in the oyster, *Isognomon nucleus*  
134 (Bivalvia: Isognomonidae), a dominant ectotherm of the high-supratidal zone in Southeast Asia.  
135 Animals were collected from Ko Sichang, Thailand (latitude: 13°08'52''N; longitude:  
136 100°48'11''E). In this area, *I. nucleus* is abundant and forms a distinctive band at the high-  
137 supratidal zone (~2.5 - 3 m above the mean sea level; Samakraman *et al.*, 2010), where rock  
138 temperatures vary daily (emersion period) from ~30°C at dawn to ~50°C at noon (hot season:  
139 March to June; Hui *et al.*, 2020). The oyster exhibits strong variations in physiological and  
140 metabolic adjustments with elevated body temperatures, providing an ideal system to  
141 investigate the functional role of the microbiome to facilitate the host's survival in this  
142 thermally extreme and variable environment (Giomi *et al.*, 2016; Hui *et al.*, 2020).

143

### 144 *Sample collection and processing*

145 A total of 80 individuals of *I. nucleus* were randomly selected at four different  
146 timepoints within an emersion period (Fig. 1A; 20 animals at each time) with contrasting  
147 thermal conditions (09:15 hr, 12:30 hr, 16:12 hr, and 17:40 hr; hereafter, morning, noon, early-  
148 afternoon, and late-afternoon, respectively; Table S1). This cycle represents major changes in  
149 the environmental temperature as a result of the tidal dynamics and air/sun exposure (Hui *et al.*,  
150 2020). Body temperatures were measured from 10 randomly selected individuals at each  
151 timepoint using a digital thermometer ( $\pm 0.1$  °C, TM-947SD, Lutron, Taiwan) where a fine-  
152 tipped type-K thermocouple was inserted into the bodies through the valve opening. At each  
153 time point, the whole tissue of five collected oysters was quickly removed at the site with a  
154 sterile dissecting kit and snap-frozen in liquid nitrogen on shore for oxidative stress  
155 quantification (as a proxy for host physiological and biochemical dynamics). The guts of

156 another five individuals were also dissected at each timepoint on-site, snap frozen in liquid  
157 nitrogen for gut microbiome assessment. All collected samples (20 for oxidative stress and 20  
158 for microbiome analysis across four time points) were stored in a portable liquid nitrogen tank  
159 and transported to The University of Hong Kong within three days, where they were held in a  
160 -80°C freezer until processing and analyses. Because of the weather instability and strong waves  
161 during the fieldwork, temporal sampling was limited to one day to comply with logistic and  
162 safety regulations.

163

### 164 *Rock surface temperature and tidal dynamics*

165 To quantify environmental conditions experienced by *Isognomon nucleus* throughout  
166 the tidal cycle, rock surface temperatures and tidal levels were recorded. To measure and track  
167 surface rock temperature, i-Button loggers (DS1922L, Maxim, US) were firstly embedded in  
168 waterproofing resin (Scotchcast, 3M, US), placed in plastic Falcon tube caps and affixed, using  
169 marine epoxy (A-788, Z-Spar, US; Ng *et al.*, 2021), onto the shore at the height where *I. nucleus*  
170 were collected. Rock surface temperatures were logged by these i-Buttons every 5 minutes at a  
171 resolution of 0.5 °C (n = 5). Additionally, a time-lapse camera (TLC200Pro, Brinno, Taiwan)  
172 was set adjacent to the study site to monitor the immersion state of the *I. nucleus* band during  
173 the day (~ 05:00 hr to 18:00 hr) to assess the tidal states. Images were captured every 10 minutes  
174 and the tidal level was scored according to whether the band was underwater (immersed),  
175 exposed to air (emersed), or being splashed by waves during transitions from  
176 immersion/emersion (awash). During the emersion period, animals showed no intermittent  
177 gaping (Hui, T.Y., unpublished data). Predicted tide height from the lowest low water height  
178 on the sampling date was also obtained from the Hydrographic Department, Royal Thai Navy  
179 Website (<https://www.hydro.navy.mi.th/index1.php#nogo2>): Lowest tidal height - 0.55m  
180 (12:56 hr) and highest tidal height - 3.36m (20:53hr).

181



182 *Determination of polyunsaturated fatty acids-oxidized metabolites*

183

184         The levels of oxidative stress (i.e., associated with ROS production) of the oysters were  
185 determined based on the concentration of non-enzymatic oxidized polyunsaturated fatty acids  
186 (PUFA) products (hereafter, oxidized PUFA products; Durand *et al.*, 2009; Basu, 2010; Leung  
187 *et al.*, 2015). A modified Folch extraction method was used to extract the oxidized PUFA  
188 products from the oysters sampled (n=5) at each timepoint (t=4) (Folch *et al.*, 1957; Dupuy *et*  
189 *al.*, 2016). In brief, 0.05g of oyster flesh was suspended in a 5 ml ice-cold Folch solution  
190 (chloroform: methanol, 2:1, v/v) with 0.01% Butylated HydroxyToluene (BHT; used as anti-  
191 oxidant to prevent lipid peroxidation during sample processing) (w/v) and homogenized at  
192 24,000 rpm in 20 secs bursts on ice using a polytron benchtop homogenizer (T25, Ultra-Turrax,  
193 IKA, Germany). Homogenization was performed twice, and the two parts of the Folch solution  
194 extracts were pooled. The pooled solutions were added with 2 ml of 0.9% NaCl to introduce  
195 phase separation. The mixture was then shaken on ice for 30 min and centrifuged at 2000 × g  
196 for 10 min at room temperature. The lower chloroform phase was transferred into a 30 ml glass  
197 vial. The extracted samples were then added with potassium hydroxide (1M in methanol) and  
198 1X PBS (pH7.4). Samples were then shaken overnight in the dark at room temperature for  
199 alkaline hydrolysis of the extracted samples. This step is required to obtain free forms of fatty  
200 acids by deacylation of phospholipid (most lipids exist in this form) from the extracted samples.  
201 The reaction was terminated by adding 1M of hydrochloric acid, methanol, 20 mM of formic  
202 acid, and 40 mM of formic acid. Deuterated internal standards (5ng prepared in methanol) were  
203 also added after the hydrolysis. Purification of the oxidized PUFA metabolites was performed  
204 with the mixed anion solid phase extraction (MAX SPE, Waters, Milford, MA USA). Before  
205 the start of the solid phase extraction, the SPE column was preconditioned with methanol and  
206 20 mM formic acid. One sample was then loaded into a column and cleaned with 2%  
207 ammonium hydroxide and 20 mM formic acid. The final eluent was collected with

208 hexane/ethanol/acetic acid (70/29.4/0.6, v/v/v). All the samples were then placed on a heating  
209 block and heated at 37°C under a stream of nitrogen gas until they were completely dried. The  
210 dried samples were then re-suspended in 100 µL methanol and cleaned through a 0.45 µm PTFE  
211 filter to remove insoluble impurities, and then immediately analysed by liquid chromatography-  
212 tandem mass spectrometry (LC-MS/MS).

213 In our study, we used the Sciex X500R QTOF LC-MS/MS system (Sciex Applied  
214 Biosystems, MA, USA) which consisted of an Exion LC liquid chromatography with a C18  
215 column (150×2.1 mm, 2.6 µm particle size, Phenomenex, Torrance, CA, USA) which was  
216 maintained at 40°C for LC-MS/MS analysis. A mobile phase consisting of 0.1% aqueous acetic  
217 acid (solvent A) and 0.1% acetic acid/methanol (solvent B) was used. The flow rate was set to  
218 300 µl/min and the injection volume to 10 µl. For each sample analysis, the gradient condition  
219 was maintained at 20% of solvent B for 2 min and between 20% and 98% for 8 min and then  
220 held for 5 min. Then, the percentage of solvent B was reduced to 20% in 1 min and held for an  
221 additional 5 min to equilibrate to the initial conditions. The X500R QTOF system was operated  
222 in negative electrospray ionization (ESI) mode. The spray voltage was set to -4500V and  
223 nitrogen was used as the curtain gas. The ionization chamber temperature was set at 350°C, and  
224 the pressure of the ion source gases 1 and 2 were 35 and 45 psi, respectively. The declustering  
225 potential (DP) was set to -80V and the collision energy (CE) was -10V for the QTOF MS. The  
226 scan mode was set to multiple reaction monitoring (MRM). All data collected by the X500R  
227 QTOF system was analyzed by the Sciex operating system (version 1.2.0.4122). The  
228 quantification of each analyte was determined by correlating the peak area to its corresponding  
229 deuterated internal standard peak. For analytes without corresponding deuterated internal  
230 standards, the following deuterated internal standards, 5(*S*)-HETE-d<sub>8</sub>, 15-F<sub>2t</sub>-IsoP-d<sub>4</sub>, and 4-F<sub>4t</sub>-  
231 NeuroP-d<sub>4</sub> were used for quantification. We identified fifteen oxidized PUFA products that are

232 known to be the products of non-enzymatic lipid peroxidation (Table S2), based on previous  
233 work by Dupuy et al.(2016), Galano et al.(2017) and Lee et al.(2018).

234

### 235 *DNA extraction and 16S rRNA gene sequencing*

236 Total genomic DNA was extracted from the oysters' guts (~0.03g) using the commercial  
237 DNA extraction kit, Dneasy Power Soil Kit (Qiagen GmbH, Hilden, Germany), following the  
238 manufacturers protocol with some modifications (i.e., the solution mixture was incubated at 55°C  
239 in a water bath for 30 minutes after the bead-beating step). The extracted DNA (100µl) was  
240 then collected in a microcentrifuge tube and preserved in a -80°C freezer. All the DNA samples  
241 were then sent to Ramaciotti Centre for Genomics at the University of New South Wales,  
242 Sydney Australia for 16S rRNA amplicon paired-end sequencing (2X 300bp) on an Illumina  
243 Miseq Platform. Before the amplicon sequencing, the V3-V4 variable region of the 16S rRNA  
244 gene from each DNA sample was amplified using the following primers: (1) 341F (Forward  
245 Primer);5'- CCTACGGGNGGCWGCAG-3' (2) 805R (Reverse Primer); 5'-  
246 GACTACHVGGGTATCTAATCC-3'(Herlemann *et al.*, 2011; Klindworth *et al.*, 2013). Raw  
247 reads received were the demultiplexed paired-end sequences in a FASTQ format and they were  
248 deposited in NCBI Sequence Read Archive (SRA) under Bioproject number PRJNA931123.  
249 The data will be made available once published.

250

### 251 *16S rRNA gene sequence processing*

252 Raw demultiplexed paired-end sequences were processed following the Quantitative  
253 Insights into Microbial Ecology (QIIME2 ver. 2021.4) pipeline (Bolyen *et al.*, 2019). Briefly,  
254 the raw data was first visualized with the demux plug-in function for quality-checking and  
255 identifying poor-quality base regions to filter. The sequences were then trimmed, filtered,  
256 denoised, chimeras-removed, and merged using the DADA2 plug-in function in QIIME2  
257 (Callahan *et al.*, 2016). The sequences were identified at a single nucleotide threshold

258 (Amplicon Sequence Variants; ASV; Callahan *et al.*, 2017). Additional steps of quality control  
259 were performed to reduce non-target sequences by aligning (vsearch alignment method) the  
260 ASV sequences to the 99% representative sequences from the greengenes database and only  
261 ASV with a similarity threshold of 0.65% and coverage threshold of 0.5% were retained  
262 following <https://github.com/biocore/deblur/issues/139#issuecomment-282143373>. Singletons  
263 and sequences with less than 349bp were also removed with filter-features and filter-sets  
264 function in QIIME2, respectively. The quality-filtered sequences were then taxonomically  
265 assigned with a naïve-Bayes classifier trained on the V3-V4 region of the 16S gene in the Silva  
266 v138 database (Quast *et al.*, 2013), using the classify-sklearn function in the feature-classifier  
267 plugin (Bolyen *et al.*, 2019). The output from this generated a taxonomy table with taxonomic  
268 naming for each ASV found within our samples. ASV sequences that were identified as  
269 mitochondria, chloroplast, archaea, and eukaryotes were filtered out. The quality-filtered ASV  
270 sequences were then ready to be used for downstream analysis.

271

### 272 *Profiling microbiome functions*

273 The functional microbiome was predicted using the software Phylogenetic Investigation  
274 Communities by Reconstruction of Unobserved States version 2 (PICRUSt2; Douglas *et al.*,  
275 2020). The predicted functions associated with the microbiome (using the quality-filtered  
276 sequences) were inferred based on the MetaCyc pathway abundance, using the PICRUSt plug-  
277 in within QIIME2.

278

### 279 *Data analysis*

280 Unless otherwise specified, the R statistical programming language (ver. 4.2.0, R Core  
281 Team) and R Studio was used to generate graphics and statistical analyses. The oysters' body  
282 temperature data violated the statistical assumption of homogeneity of variances (Levene's test,  
283 p-value < 0.05). Hence, the statistical analysis for this trait across different timepoints was

284 assessed using a generalized linear model (GLM) with a gamma distribution. The likelihood-  
285 ratio chi-square test was used to determine the statistical significance and is indicated if the p-  
286 value was  $< 0.05$  (R package “Car”; Fox and Weisberg, 2019). Post-hoc testing for pairwise  
287 comparison was made using the estimated marginal means with Tukey’s tests from the R  
288 package emmeans (Lenth, 2023)

289 Individual oxidized PUFA products were first evaluated for normality (Shapiro-Wilk’s  
290 Test) and homogeneity of variance (Levene’s test) assumptions. Metabolites that fulfilled both  
291 assumptions were tested with one way Analysis of variance (ANOVA) to investigate the  
292 variation in metabolites’ concentrations across timepoints, followed by *post-hoc* Tukey’s  
293 Honest significant difference (Tukey’s HSD). For metabolites that violated either one of the  
294 assumptions, they were evaluated with GLM (gamma distribution) following a similar  
295 procedure as with the body temperature datasets.

296 Assessment of oxidized PUFA products’ beta diversity (with all the 15 metabolites or  
297 only the 4 significant metabolites) was performed by first generating a Euclidean distance  
298 matrix between samples, using normalized metabolites datasets (i.e., autoscaling; van den Berg  
299 *et al.*, 2006). Euclidean distance metric was selected as it allows for accurate and reliable  
300 analysis for continuous datasets and testing the differences between groups of samples based  
301 on the metabolite data (Qi and Voit, 2017; Mallick *et al.*, 2019; Nguyen *et al.*, 2021). A  
302 principle Coordinate Analysis (PCoA) plot was then created to visualize the oxidized PUFA  
303 products clustering across different timepoints, using the ggplot2 R package (Wickham, 2008).  
304 Permutational multivariate analysis of variance (PERMANOVA) was performed with 999  
305 permutations to test for the differences in oxidized PUFA products profile across timepoints  
306 (Oksanen *et al.*, 2022). We also performed a permutational multivariate dispersion test  
307 (PERMDISP) to assess differences in the dispersion of metabolite profiles between timepoints

308 with 999 permutations followed by pairwise comparisons. All multivariate metabolites analyses  
309 were performed using the package *vegan* in R (Oksanen *et al.*, 2022).

310 Oysters' gut microbiome analysis was conducted by first combining the ASV count data,  
311 taxonomy table, and metadata into a simplified data matrix for ease of downstream processing,  
312 using the *phyloseq()* function from the *phyloseq* R package (McMurdie and Holmes, 2013).  
313 Rarefaction curves (total ASVs captured against sampling depth) were generated for each  
314 sample with the *ggrare()* function from the *ranacapa* R package (Kandlikar *et al.*, 2018). All  
315 samples approached the asymptote level, indicating that the total ASV richness for each sample  
316 has been captured (Fig. S1). Each of the samples was then rarefied to a sampling depth of  
317 21,282 reads (a cut-off that balances the maximum number of sequences retained per sample  
318 and minimizing the number of samples removed) for downstream analysis (Weiss *et al.*, 2017;  
319 i.e., alpha and beta diversity; McKnight *et al.*, 2019). Using the rarefied data, the microbiome  
320 alpha diversity (i.e., the structure of a microbial community with respect to its richness and  
321 evenness; Willis, 2019) was determined based on two indices – ASV richness and Shannon-  
322 Wiener ( $H'$ ) diversity. ASV richness was calculated with the *Base-R* function, while Shannon-  
323 Wiener ( $H'$ ) diversity index was generated using the *diversity()* function from the *Vegan*  
324 package (Oksanen *et al.*, 2022). Differences in the microbiome alpha diversity across the  
325 timepoints were assessed with ANOVA and followed by a *post-hoc* Tukey HSD test. The  
326 ANOVA statistical assumptions of normality (Shapiro-Wilk test) and homogeneity of variance  
327 (Levene's test) for all datasets were tested and fulfilled ( $p$ -value > 0.05). Linear regression  
328 analysis was also conducted to determine the relationship between the oysters' microbiome  
329 alpha diversity (i.e., ASV richness and Shannon-Wiener ( $H'$ ) index) and body temperature (i.e.,  
330 average of the 10 random independent body temperature replicates collected at each timepoint).  
331 The statistical assumptions for the linear regression model were fulfilled based on the normality  
332 test (Shapiro-Wilk test) and residuals plot (variance homogeneity).

333           While alpha diversity focuses on community variation within a community (sample),  
334 beta diversity quantifies (dis-)similarities between communities/samples in terms of species  
335 presence/absence and abundance (Kers and Saccenti, 2022). Metrics of beta diversity are  
336 typically used to measure species turnover or replacement across different locations,  
337 environmental conditions or timepoints (Kers and Saccenti, 2022). Here, we assessed beta  
338 diversity of the oysters' gut microbiome to evaluate temporal differences in taxonomic and  
339 functional structure across all samples simultaneously. Bray-Curtis dissimilarity distance  
340 matrix was first generated between all samples. Then, a PCoA plot was created to visualize  
341 microbiome taxonomic and functional clusters across different timepoints, using the ggplot2 R  
342 package (Wickham, 2008). The chosen dissimilarity distance metric (Bray-Curtis) followed  
343 findings by Mcknight et al. (2019) and Weiss et al. (2017), in which authors showed optimal  
344 and high confidence for community level comparison with rarefied microbiome datasets.  
345 PERMANOVA was performed with 999 permutations to test for the differences in the  
346 microbiome composition and structure between timepoints (Oksanen *et al.*, 2022). PERMDISP  
347 with 999 permutations was performed to test for differences in the taxonomic and functional  
348 microbiome profile dispersion followed by pairwise comparison. All multivariate analyses  
349 were conducted using the package vegan in R.

350           Differential abundance analysis of each microbial taxa was performed with family  
351 taxonomic level data (using unrarefied quality-filtered ASV sequences) and analysed with  
352 Analysis of Composition Microbiome with Bias Correction (ANCOM-BC) from the  
353 ANCOMBC R package (Lin and Peddada, 2020; Nearing *et al.*, 2022). Linear discriminant  
354 analysis (LDA) effect size (LEfSe) was performed to screen for differentially abundant  
355 predicted microbial function (MetaCyc pathways) across the four timepoints, based on an LDA  
356 threshold of 2.0 and a statistical significance cut-off of  $< 0.05$  (All-against-all strategy; Segata  
357 *et al.*, 2011).

358 Mantel test (Spearman correlation method) was employed to determine the correlation  
359 between the *I. nucleus* microbiome taxonomic structure and the oxidized PUFA products using  
360 their dissimilarity matrices across timepoints using the mantel() function in vegan package  
361 (Oksanen *et al.*, 2022), which tested whether higher dissimilarity in microbiome was associated  
362 with higher dissimilarity in metabolite profiles. Since the oysters' microbiome and metabolites  
363 were sampled from independent individuals, we averaged each microbial taxon and each  
364 metabolite concentration across replicates at each timepoints before generating a dissimilarity  
365 distance matrix between samples – Bray-Curtis (microbiome) and Euclidean (metabolites).

366 The GraphPad Prism software (San Diego, California, USA) was used to generate the  
367 figure for oyster's body temperatures, individual oxidized PUFA products and the 10 dominant  
368 microbe's relative abundance across the timepoints.

369

## 370 **Results**

### 371 *Tidal dynamics and temporal changes in environmental and body temperatures*

372 Oysters were regularly exposed to the air as a result of the tidal regime (Fig. 1A) and  
373 this temporal change was aligned to changes in the rock surface temperature across the day,  
374 with the highest temperature recorded at noon (45-50°C, Fig. 1A). The body temperature of the  
375 oysters varied temporally (Fig. 1B; GLM  $\chi^2_{(3)} = 1073.9$ , p-value < 0.05), increasing from 32.11  
376  $\pm 0.12$  °C (mean  $\pm$  SD) in the morning to 39.99  $\pm 0.95$  °C (mean  $\pm$  SD) at noon, after which  
377 body temperatures drop steadily to  $\sim 35$  °C in the afternoon. Besides early-afternoon (35.4°C  $\pm$   
378 0.46) and late-afternoon (35.18 °C  $\pm$  0.46), all pairwise comparisons showed significant  
379 differences in body temperatures between timepoints.

380

### 381 *Temporal assessment of oxidized PUFA products*

382 Individual analyses of each oxidized PUFA products revealed that only four products  
383 (i.e., 5-F<sub>2t</sub>-IsoP, 10-F<sub>4t</sub>-NeuroP, 13-F<sub>4t</sub>-NeuroP, and 16-F<sub>1t</sub>-PhytoP) significantly varied



384 between timepoints (Table 1, Fig. 2). There was a significant difference in the metabolites  
385 structural profile (PERMANOVA) (contributed by fifteen metabolites) between timepoints  
386 ( $pseudo-F = 2.17$ ,  $p\text{-value} < 0.05$ ; Fig. 3A), but PERMDISP indicated no significant difference  
387 in their dispersion ( $F\text{-value} = 0.69$ ,  $p\text{-value} > 0.05$ ; Fig. 3A). An additional PERMANOVA  
388 analysis with the four temporally-varied products showed a significant difference in the clusters  
389 between the morning metabolites profile and all the other timepoints during the emersion period  
390 ( $pseudo-F = 5.62$ ,  $p\text{-value} < 0.05$ ; Fig. 3B). Multivariate dispersion of the four oxidized PUFA  
391 products also differed between timepoints ( $F\text{-value} = 6.10$ ,  $p\text{-value} < 0.05$ ; Fig. 3B). With the  
392 exception of three pairwise comparisons (i.e., noon vs. early-afternoon, noon vs. late-afternoon  
393 and early-afternoon vs. late-afternoon), all the other timepoints showed significant differences  
394 in dispersion ( $p < 0.05$ ).

395

### 396 *Temporal characterization of oyster-microbiomes*

397 The 16S rRNA amplicon sequencing generated a total of 2,032,567 reads from 30  
398 samples which were reduced to 1,126,884 after denoising and quality control. These reads  
399 were then used to characterize the microbial communities of *I. nucleus* across the different  
400 timepoints. Due to the low sequence counts, samples BRM3 (5612 reads) and DRM4 (7519  
401 reads) were removed after each sample was rarefied to a sampling depth of 21,282 reads.

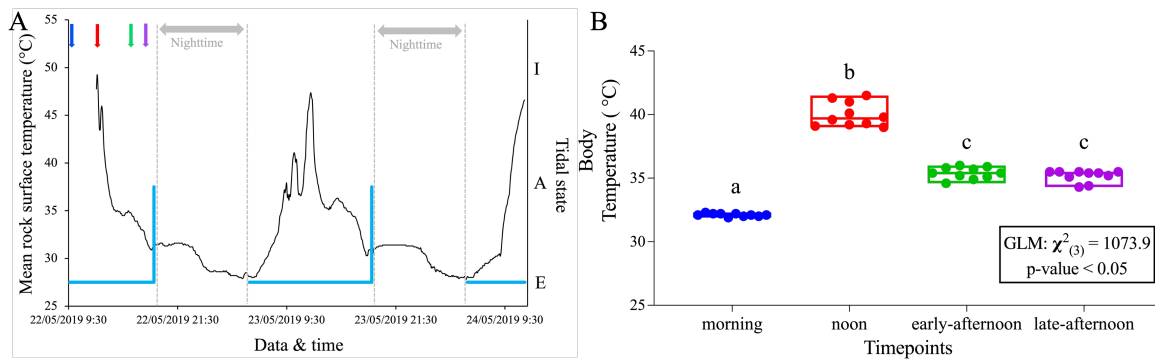
402 The ASV richness and Shannon-Wiener index of *I. nucleus* microbiome were  
403 significantly different between timepoints (ASV richness:  $F_{(3,14)} = 3.67$ ;  $p\text{-value} < 0.05$ ;  
404 Shannon-Wiener index:  $F_{(3,14)} = 3.76$ ;  $p\text{-value} < 0.05$ ; Fig. S2). However, no specific temporal  
405 trends were detected for all pairwise comparisons in ASV richness. Significant differences  
406 were, however, found for the Shannon-Wiener index between morning and noon timepoints.  
407 Oyster body temperature significantly influenced ASV richness and Shannon-Wiener indexes  
408 (Fig. S3). Overall, morning timepoints showed the highest alpha diversity. This diversity then  
409 drastically declined at noon, with partial recovery at the last timepoint – late-afternoon.

410 The top 10 most abundant microbial taxa (at the family level) associated with *I. nucleus*  
411 in a descending order were *Mycoplasmataceae*, *Helicobacteraceae*, *Spirochaetaceae*,  
412 *Xenococcaceae*, *Rhodobacteaceae*, *Rickettsiales* unknown, *Arcobacteriaceae*, *Caldilineaceae*,  
413 *Alphaproteobacteria* unknown and *Saprospiraceae* (Fig. 4). These top 10 families made up on  
414 average 57.09%, 80.88%, 81.42%, and 68.20% of the overall relative abundance in morning,  
415 noon, early-afternoon, and late-afternoon, respectively. Among them, only two families  
416 (*Helicobacteraceae* and *Arcobacteraceae*) showed significant differences in the relative  
417 abundance between timepoints based on the ANCOM-BC analysis (Fig. S4; *Helicobacteraceae*:  
418 morning vs early-afternoon; *Arcobacteraceae*: morning vs noon, and morning vs early-  
419 afternoon).

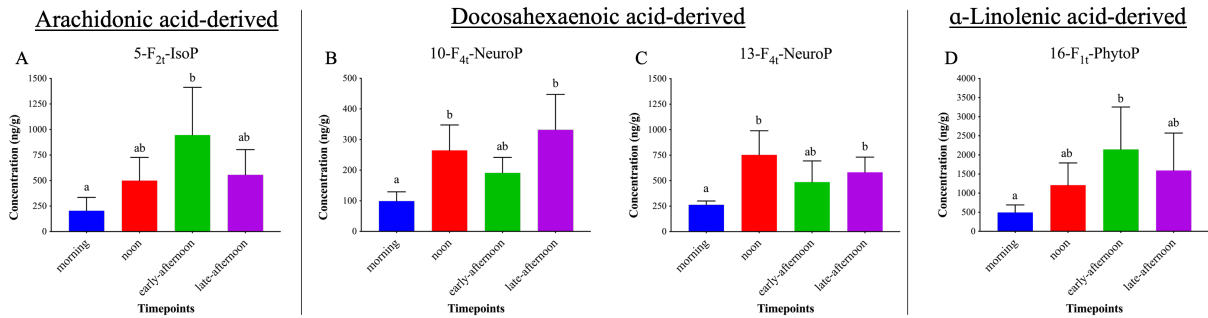
420 LEfSe analysis identified 27 differentially abundant microbial functions (MetaCyc  
421 pathways; LDA threshold of 2.0) that characterises the microbiome at a particular timepoint  
422 (Fig. 5). The most distinctive difference in functions identified were between morning and late-  
423 afternoon timepoints, where the former showed a major enrichment in genes associated with  
424 biosynthetic pathway (and some biomolecules degradation pathways), while the latter had  
425 functions affiliated with degradation, utilization, and assimilation pathways, specifically in  
426 carbon metabolism.

427 Assessing the overall microbiome structure in taxonomy and functions with  
428 PERMANOVA analysis indicates no significant difference between timepoints (taxonomy:  
429  $pseudo-F = 1.18$ ,  $p\text{-value} > 0.05$ ; function:  $pseudo-F = 1.49$ ,  $p\text{-value} > 0.05$  ;Fig. 6), but  
430 significant differences was detected in both of the microbial dispersion profiles (taxonomy: F-  
431 value = 3.55,  $p\text{-value} < 0.05$ ; function: F-value = 3.74,  $p\text{-value} < 0.05$  ; Fig. 6). Major  
432 differences in the taxonomic microbiome dispersion were between morning and early-afternoon,  
433 which corresponds to the pre-and post-noon period, respectively. For the functional  
434 microbiome, the pair early-afternoon-late-afternoon was the cause of the difference in

435 dispersion. We found no Mantel correlation was observed between the microbiome taxonomic  
436 profile and the oxidized PUFA products profile, either with the 15 (all) or 4 metabolite groups  
437 (Table 2).



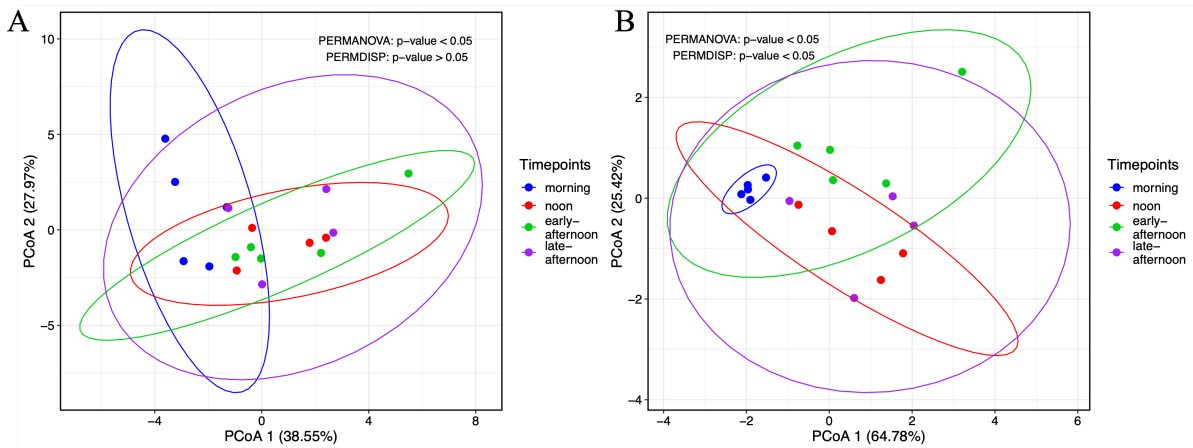
438  
439 **Fig. 1. The body temperature of *Isognomon nucleus* varies across sampling timepoints**  
440 **within a tidal cycle.** (A) Thermal (black line) and tidal conditions (blue line) as experienced  
441 by *I. nucleus* at the study site. Thermal condition is represented by the mean rock surface  
442 temperature (averaged over 3 – 5 i-Button temperature loggers), and tidal condition during the  
443 day is categorized into three states: “E” (exposed), “A” (awash), and “I” (immersed). Coloured  
444 arrows indicate the sampling time points of *I. nucleus* (colours match to boxplot in Fig. 1B) to  
445 assess its physiological responses (i.e., biochemical dynamics) and microbiome  
446 taxonomic/functional profile (B) Boxplots showing *I. nucleus* body temperatures (n=10) across  
447 different timepoints (Centre line represent the median; box limits indicate the first and third  
448 quartiles). Statistical significance (p-value < 0.05) is indicated if the letters above each bar are  
449 different.  
450



451

452 **Fig. 2. Four oxidized PUFA products exhibit variation in concentration across the**  
 453 **emersion period.** Bar graphs showing the four oxidized PUFA products deriving from their  
 454 respective PUFA (labelled at the top of each panel, across different sampling timepoints in *I.*  
 455 *nucleus*. IsoP: isoprostane; NeuroP: neuroprostane; PhytoP: phytoprostanes. Data are presented  
 456 as mean concentration and + 1 standard deviation (error bar) for each metabolite (n=5) at the  
 457 given timepoints. Statistical significance (p-value < 0.05) is indicated if the letters above each  
 458 bar are different.

459



460

461 **Fig. 3. Four oxidized PUFA products exhibit variation in the sample clusters across the**  
 462 **emersion period.** PCoA based on Euclidean distance measured between samples from different  
 463 timepoints based on (A) all oxidized PUFA products (B) four oxidized PUFA products: 5-F<sub>2t</sub>-  
 464 IsoP, 10-F<sub>4t</sub>-NeuroP, 13-F<sub>4t</sub>-NeuroP, and 16-F<sub>1t</sub>-PhytoP. The colours of the datapoints and  
 465 ellipses (95% confidence interval of the true multivariate means) indicate their corresponding  
 466 timepoints.

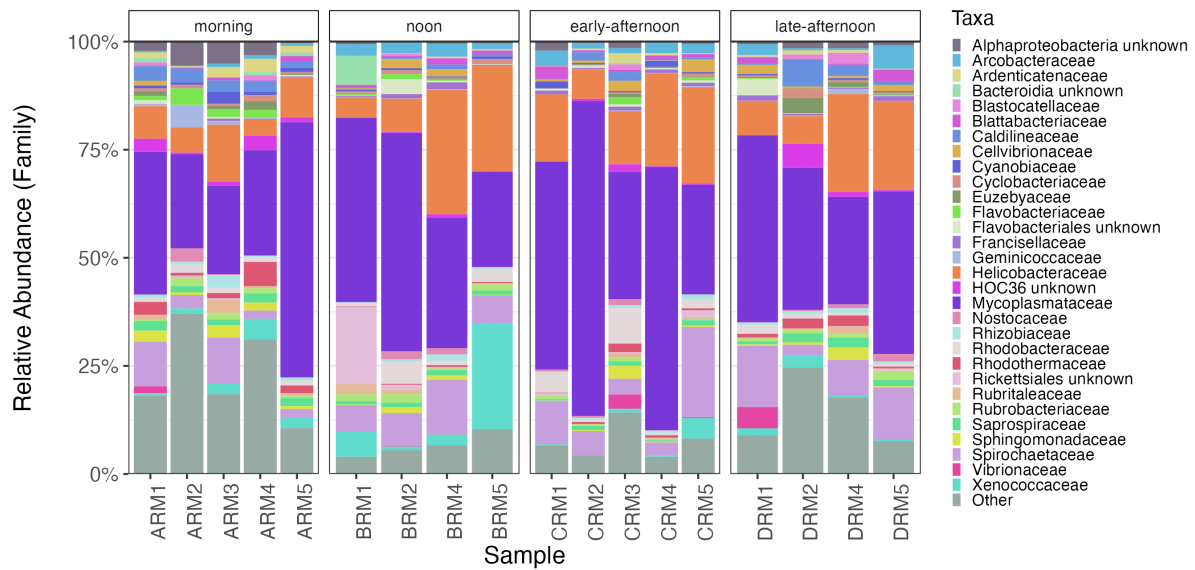
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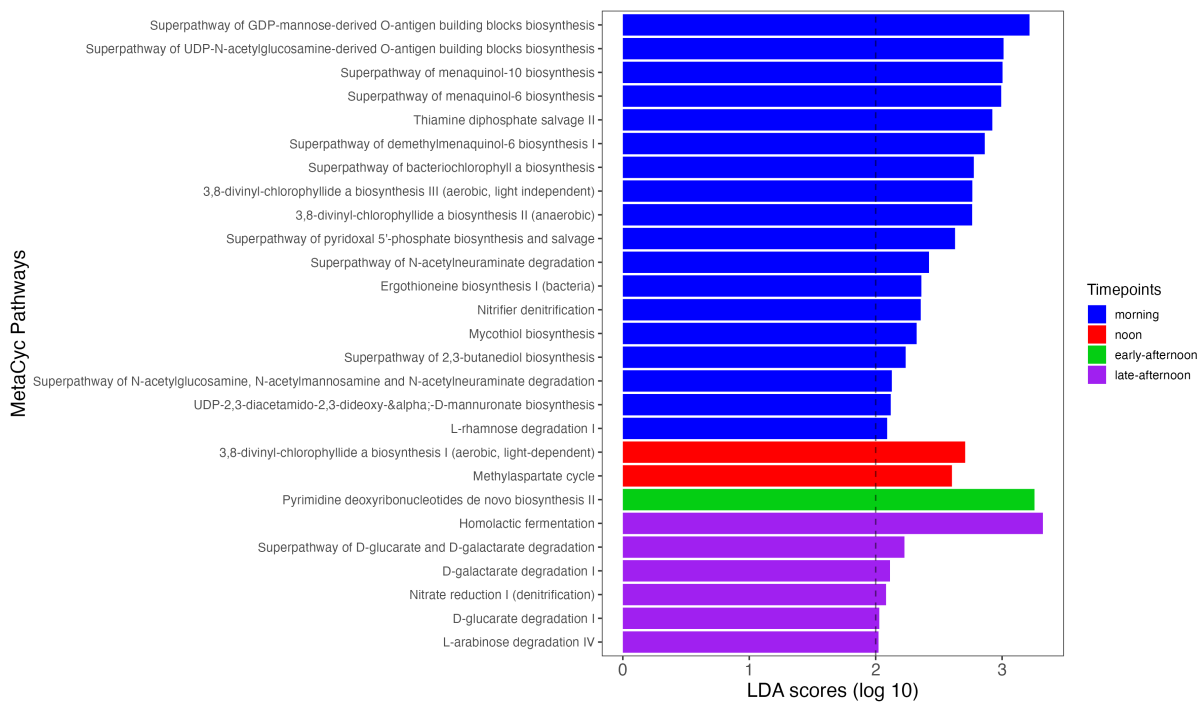
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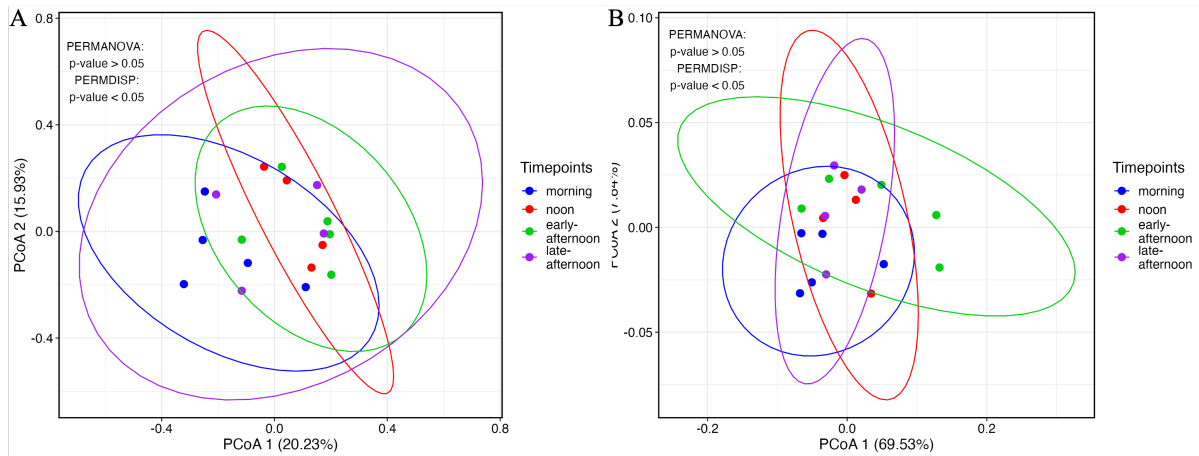
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**Fig. 4. Relative abundance of top 30 most dominant microbial taxa associated with the *I. nucleus*'s gut microbiome at each sampling timepoints.** ASV counts were aggregated into the family taxonomic level. Each bar represents a single individual replicate of oyster gut microbiome.



478  
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**Fig. 5. Differentially abundant microbial functions associated with a particular time point.** LEfSe analysis (LDA scores of > 2 and alpha of 0.05) showing 27 predicted functions (MetaCyc pathways) that was identified as discriminative among the timepoints. Coloured bar represents functions that was derived from a particular timepoint. The LDA scores cut-off is depicted by the dotted lines.



486

487 **Fig. 6. *I. nucleus*-associated microbiome taxonomic and functional structures exhibit**  
 488 **overall stability across the emersion period.** PCoA based on the Bray-Curtis distance metric  
 489 measured between samples from different timepoints, based on (A) taxonomy (ASV level) and  
 490 (B) functions (MetaCyc pathways). The colours of the datapoints and ellipses (95% confidence  
 491 interval of the true multivariate means) indicate their corresponding timepoints.

492

493 **Table 1.** Statistical analysis result (ANOVA or GLM with gamma distribution) testing the effect of  
 494 timepoints on the concentration of each of the oxidized PUFA products.

PUFA	Metabolite	Test Statistic	p-value
Arachidonic acid	5-F <sub>2t</sub> -IsoP	$F_{(3,14)} = 5.08$	<b>0.04</b>
	15-F <sub>2t</sub> -IsoP	$F_{(3,14)} = 1.72$	0.21
Adrenic acid	7-F <sub>2t</sub> -Dihomo-IsoP	$F_{(3,14)} = 3.10$	0.06
	17-F <sub>2t</sub> -Dihomo-IsoP	GLM $\chi^2_{(3)} = 0.66$	0.69
Docosahexaenoic acid	4-F <sub>4t</sub> -NeuroP	$F_{(3,14)} = 3.13$	0.06
	10-F <sub>4t</sub> -NeuroP	$F_{(3,14)} = 8.43$	<b>&lt; 0.01</b>
	13-F <sub>4t</sub> -NeuroP	GLM $\chi^2_{(3)} = 27.46$	<b>&lt; 0.01</b>
$\alpha$ -Linolenic acid	20-F <sub>4t</sub> -NeuroP	$F_{(3,14)} = 1.23$	0.34
	9-D <sub>1t</sub> -PhytoP	$F_{(3,14)} = 0.58$	0.64
	9-F <sub>1t</sub> -PhytoP	GLM $\chi^2_{(3)} = 7.92$	0.05
	16-F <sub>1t</sub> -PhytoP	$F_{(3,14)} = 3.72$	<b>0.04</b>
Eicosapentaenoic acid	16-B <sub>1t</sub> -PhytoP	GLM $\chi^2_{(3)} = 6.66$	0.08
	5-F <sub>3t</sub> -IsoP	$F_{(3,14)} = 2.49$	0.10
	15-F <sub>3t</sub> -IsoP	GLM $\chi^2_{(3)} = 0.32$	0.32
	8-F <sub>3t</sub> -IsoP	$F_{(3,14)} = 0.81$	0.51

495

496

497 **Table 2.** Mantel's spearman's rank correlation (r) and p-value to investigate the association  
 498 between the *I. nucleus* microbiome taxonomic structure (mean abundance of each taxon across  
 499 all timepoints) and oxidized PUFA products profile (mean concentration across timepoints).  
 500 The number of permutations performed was 23(maximum possible).  
 501

Association with microbiome taxonomic structure	Mantel's r	p-value
oxidized PUFA products (all)	0.77	0.21
oxidized PUFA products (Four)*	0.54	0.25

502 \*The four metabolites were: 5-F<sub>2t</sub>-IsoP, 10-F<sub>4t</sub>-NeuroP, 13-F<sub>4t</sub>-NeuroP, and 16-F<sub>1t</sub>-PhytoP

503

## 504 Discussion

505 Organisms living on the intertidal rocky shore regularly experience drastic changes in  
 506 environmental temperature (Somero, 2002; Stillman, 2002; Harley, 2008). For many sessile  
 507 ectotherms, this represents a physiological challenge due to their inability to escape from heat  
 508 stress (e.g. seeking thermal refuge) and so, they rely on various intrinsic (e.g., metabolic and  
 509 physiological plasticity) and extrinsic (e.g., host-associated microbiome; still underexplored)  
 510 mechanisms to cope with temperature fluctuations (Lathlean *et al.*, 2014; Giomi *et al.*, 2016;  
 511 Bang *et al.*, 2018). As with other ectotherms, the body temperature of the high-supratidal zone  
 512 inhabiting oyster, *Isognomon nucleus* was highly influenced by changes in ambient  
 513 temperatures throughout the day. Such changes are known to induce physiological stress and  
 514 metabolic depression (Hui *et al.*, 2020), with the associated oxidative stress (Hermes-Lima *et al.*,  
 515 2015). Here, linked to these physiological conditions, we found adjustments in four major  
 516 non-enzymatic oxidized PUFA products (i.e., 5-F<sub>2t</sub>-IsoP, 10-F<sub>4t</sub>-NeuroP, 13-F<sub>4t</sub>-NeuroP, and  
 517 16-F<sub>1t</sub>-PhytoP) that reflect temporal variation in the oxidative stress experienced by the oysters.  
 518 Contrary, based on beta-diversity analysis that assess all samples simultaneously across  
 519 timepoints, no clear temporal change in the structure and/or function of the gut microbiome  
 520 were detected. This suggests that the oyster gut microbiome is temporally stable and insensitive  
 521 to extreme high thermal conditions (> 40°C in body temperature) at noon that are

522 physiologically stressful to the oyster host. The differences in the host and microbiome  
523 sensitivity to external changes was further supported in the lack of temporal correlation between  
524 the intrinsic (biochemical profile) and extrinsic (microbiome taxonomic structure) mechanisms.  
525 In summary, the microbiome stability may be important for the overall homeostasis of the  
526 oysters, contributing to their thermal tolerance and survival in the challenging tropical intertidal  
527 rocky environment.

528

529 *The intrinsic mechanism underpinning oysters' adaptive response to temporal*  
530 *environmental changes*

531 For high-supratidal zone inhabiting ectotherms, metabolic depression is an adaptive  
532 response that allows them to cope with extreme high and stressful thermal conditions (Sokolova  
533 and Pörtner, 2001; Marshall *et al.*, 2011). In a laboratory setting, Hui *et al.* (2020) demonstrated  
534 that the oyster *I. nucleus* consistently enters into metabolic depression (i.e., measured by heart  
535 rate) when body temperature approaches ~ 37°C (a value that is regularly surpassed on daily  
536 basis at around noon on tropical rocky shores). Our data indicates that the oysters could have  
537 entered a state of metabolic depression when exposed by the tide during noon time and resume  
538 their metabolism when body temperatures lower in late afternoon/evening, or when they are re-  
539 immersed

540 Intertidal bivalves are known to also display intermittent gaping behaviour during  
541 periods of aerial exposure as a way to enhance oxygen intake and evaporative cooling,  
542 particularly when experiencing thermal stress (Lent, 1969; McMahon, 1988; Nicastro *et al.*,  
543 2010). In our study, all individuals shut their valves and no intermittent gaping behaviour was  
544 observed either during the high thermal stress or the recovering period (Hui, T.Y., unpublished  
545 data). Therefore, oxygen depletion in addition to thermal stress in the oysters particularly during  
546 noon time could contribute to the intense oysters' metabolic demand of which, metabolic  
547 depression is likely to be a key strategy for survival during the emersion period.



548           There were four metabolites (i.e., 5-F<sub>2t</sub>-IsoP (Isoprostane), 10-F<sub>4t</sub>-NeuroP  
549 (Neuroprostane), 13-F<sub>4t</sub>-NeuroP (Neuroprostane), and 16-F<sub>1t</sub>-PhytoP (Phytprostane)) that  
550 showed visible temporal patterns that may be associated with their metabolic regulation. Such  
551 patterns may be linked to the sensitivity of the metabolites to ROS changes in the oyster.  
552 Elevation of 5-F<sub>2t</sub>-IsoP, 10-F<sub>4t</sub>-NeuroP, and 13-F<sub>4t</sub>-NeuroP during noon, and 16-F<sub>1t</sub>-PhytoP at  
553 post-noon timepoints, clearly indicates that oysters experienced a rise in the ROS level, a signal  
554 of oxidative stress (Hermes-Lima and Zenteno-Savín, 2002; Storey and Storey, 2004;  
555 Sussarellu *et al.*, 2012; Welker *et al.*, 2013; Galano *et al.*, 2017). This could be caused by an  
556 increase in metabolic demand and/or hypoxia (when individuals shut their valves and show no  
557 gaping behaviour). In our study, the high levels of 16-F<sub>1t</sub>-PhytoP (Phytprostane) metabolites  
558 as compared to other three oxidized PUFA products in the *I. nucleus*, imply that the PUFA  
559 ALA could also be the predominant fatty acid in alleviating oxidative stress in this oyster.

560           Further analysis with the overall structural profile with the four ROS-sensitive oxidized  
561 PUFA products showed a clear temporal variation and inter-individual variation in *I. nucleus*.  
562 The large inter-individual variation in the oxidized PUFA products profile at noon/afternoon  
563 relative to morning timepoint might be related to the thermal and oxidative stress conditions  
564 experienced by the oysters during those periods of elevated temperatures. This variability  
565 suggests inter-individual differences in physiological plasticity, regulation, and sensitivities to  
566 environmental stress. The result here also indicates that the polyunsaturated fatty acids (ARA,  
567 DHA, and ALA) from which these four oxidized PUFA products were derived, may play a key  
568 role as lipid metabolites that help to resolve inflammation and oxidative stress for the oysters  
569 to return to homeostasis (Roy *et al.*, 2017; Lee *et al.*, 2020). It is important to highlight here  
570 that the current understanding regarding the thermal-physiological roles of high PUFA ALA or  
571 the oxidized PUFA products in *I. nucleus* is still limited and that our interpretations are  
572 speculative and idiosyncratic to the specific animals and population studied. Moreover, our

573 assessment was based on the observation and analysis that are known in clinical research and  
574 we remained carefully and strictly conservative in our discussion to just focus on the association  
575 between these products and the host oxidative stress level. Future studies would be dedicated  
576 toward elucidating the role of these molecules that may be essential to their host physiology  
577 and metabolism.

578

579 *The extrinsic mechanism underpinning the oysters' response to temporal*  
580 *environmental changes*

581 Host-associated microbiome diversity, structure, and functions are considered as  
582 important components of animal adaptation and acclimation to environmental change (Foster  
583 *et al.*, 2017; Bang *et al.*, 2018; Voolstra and Ziegler, 2020). Alterations in microbiome diversity  
584 can be associated with the direct influence of the host's physiology and/or with host-  
585 independent environmental effects (Shiu *et al.*, 2017; Li *et al.*, 2018, 2022; Muñoz *et al.*, 2019).  
586 In our study, the composition and diversity (ASV richness and Shannon diversity) of the oyster  
587 gut-associated microbiome varied with time which coincided with variation in the oyster's  
588 body temperature, suggesting that the thermal condition experienced by the host may modulate  
589 the microbial alpha diversity via either direct/indirect physio-biochemical influences (Sorek *et*  
590 *al.*, 2014; Pita *et al.*, 2018; Liu *et al.*, 2023). In the morning, the oyster's gut had a significantly  
591 more diverse microbiome diversity, which may be linked to the less thermally stressed host  
592 (Hui *et al.*, 2020), and/or to the filter-feeding activities before being exposed by the receding  
593 tide (Parris *et al.*, 2019). However, based on the Shannon alpha diversity, at early post-noon  
594 condition, a major decline in diversity was observed, which may be associated with the thermal  
595 physiological stress experienced by the host (body temperature is within the metabolic  
596 depression range; Hui *et al.*, 2020) and oxidative stress (indicated by the rise in the two oxidized  
597 PUFA products at noon and two at early post-noon condition). This decline in diversity is also  
598 a common pattern seen in many marine organisms under physiological stress (Green and Barnes,

599 2010; Li *et al.*, 2018; Hartman *et al.*, 2020). For example, Lokmer & Wegner (2015) showed  
600 that acute thermal stress and/or infection caused a marked decline in the alpha diversity of the  
601 microbiome in the oyster *Crassostrea gigas*. In another study by Li *et al.* (2018) observed an  
602 overall decrease in the gut microbial alpha diversity associated with the mussel, *Mytilus*  
603 *coruscus*, after exposing to higher temperature. Such observation offer support to our result  
604 where the relatively higher temperature at noon may have driven the decline in the oyster-  
605 associated microbial diversity.

606         Whilst the temporal changes exhibited by the *I. nucleus* microbiome and their  
607 association with changes in the oysters' body temperatures suggest that the microbiome  
608 composition may be actively regulated by the host, differential sensitivities of certain bacterial  
609 taxa to external environmental changes may also be an important mechanism driving the  
610 temporal variation in the microbiome composition. *Arcobacteraceae* and *Helicobacteraceae*  
611 are the only two dominant family-level microbial taxa associated with the temporal  
612 thermal/oxidative stress variation of the host. In particular, there was an elevated relative  
613 abundance of these two bacteria groups at noon and/or at the afternoon timepoints, which may  
614 suggest that their abundances during these periods were promoted by specific  
615 host/environmental conditions, facilitating their growth probably by reducing competition  
616 (Garren *et al.*, 2016; Shaver *et al.*, 2017; Sharp and Foster, 2022). *Arcobacteraceae* is a group  
617 of common opportunistic bacteria found in marine bivalves and is known to be associated with  
618 disease-causing bacteria such as *Vibrio* (Lokmer and Wegner, 2015; de Lorgeril *et al.*, 2018).  
619 In many studies, the elevation of the *Arcobacteraceae* has been associated with the thermal  
620 stress condition experienced by the host (Lokmer and Wegner, 2015; Shiu *et al.*, 2017; Neu *et*  
621 *al.*, 2021). Members of *Arcobacteraceae* are also known to be microaerophilic as they tend to  
622 grow better under a low-oxygen environment (Vandamme and De Ley, 1991). This may explain  
623 the dominant presence of *Arcobacteraceae* within the oysters' microbiome due to the prevailing

624 hypoxic and thermally stressful conditions as experienced by the oyster hosts during emersion.  
625 Similarly, the presence and abundance *Helicobacteraceae* can be associated to oxygen levels  
626 in the marine environment. For instance, certain members of this family are capable of oxidising  
627 sulfide, and thus, they might contribute with a detoxification function to the oyster host  
628 (Kodama and Watanabe, 2004; Nakagawa *et al.*, 2017; Neu *et al.*, 2021). The accumulation of  
629 sulfide can be detrimental or lethal to marine bivalves (Le Moullac *et al.*, 2008), and it is  
630 typically associated with hypoxic stress (Le Moullac *et al.*, 2008; Pal *et al.*, 2018). Such stress  
631 is hypothesised to occur in *I. nucleus* during aerial exposure, as no intermittent gaping  
632 behaviour was detected. When oxygen is low, sulfide production is promoted by the activity of  
633 hydrogen sulfide-producing bacteria such as *Desulfobacterota* members (Flannigan *et al.*, 2011;  
634 present in the *I. nucleus*; Dordević *et al.*, 2021; Pimentel *et al.*, 2021). It is unclear, however,  
635 the extent to which the dynamics in the abundance of sulfide-producing bacteria and  
636 *Helicobacteraceae* members (with potential detoxification role), are functionally  
637 interconnected. While this seems likely, we are unaware of any study exploring this functional  
638 link, and it would be important to investigate such species' interaction and its effects on the  
639 physiology and fitness of the host.

640         Variation in the microbiome functional activity is commonly associated with the  
641 functional changes occurring within the host (e.g., dietary changes; Mekuchi *et al.*, 2018;  
642 Masasa *et al.*, 2023) and/or in the external environment (e.g., temperature; Brothers *et al.*, 2018;  
643 Li *et al.*, 2022). Our study is in-line with this, in showing that there were several enriched  
644 functions that vary through time, potentially attributed to either one or both of those two factors.  
645 Specifically, in the morning timepoints, we detected a major proportion of predicted  
646 microbiome function in biosynthetic pathways associated with carbohydrate, cofactor, carrier,  
647 vitamin and secondary metabolites biosynthesis. This suggested that, in the morning, the host  
648 gut had a considerable dietary resources for the building of these organic/inorganic molecules

649 in microbes to support cellular maintenance and growth and/or actively contributing to host  
650 nutritional metabolism (David *et al.*, 2014; Maurice *et al.*, 2015; Dubé *et al.*, 2019). On contrary,  
651 post-noon conditions were significantly dominated by compound/molecular degradation  
652 pathways associated with carbon metabolism. This makes sense since in the post-noon  
653 conditions (post-heat stress), the host are likely in a “recovery” that could incur a high energetic  
654 requirement for various cellular and physiological repair and maintenance purposes (Storey and  
655 Storey, 2004; Sokolova *et al.*, 2011). The breakdown of these carbon compounds by the  
656 microbes can provide as an energy source to meeting the metabolic demand in the host and  
657 microbes (Flint *et al.*, 2012; Tremaroli and Bäckhed, 2012; Den Besten *et al.*, 2013). However,  
658 it is unclear if these predicted microbial functional enrichments are directly contributing to the  
659 host dietary metabolism (e.g., nutrient absorption; English *et al.*, 2023) or simply fulfilment of  
660 need for the microbes themselves (host-independent) to cope and survive to the prevailing  
661 conditions (i.e., host and/or external environmental influence).

662 Host tolerance and resilience to environmental stress are known to be conditioned by  
663 the stability/flexibility of its associated microbiome. Despite the changes in the alpha diversity  
664 and relative abundance of some of the most abundant bacteria (family level) in *I. nucleus*, the  
665 beta analysis that assesses the structural microbiome profiles among samples simultaneously  
666 showed no changes in their taxonomy and function throughout the day (emersion period). This  
667 suggest that despite experiencing high thermal stress at noon (Hui *et al.*, 2020), there is an  
668 overall stability in the microbiome composition and structure. Such stability could be  
669 potentially explained by the ability of the host to control/regulate the microbiome during a  
670 disturbance event (Sharp *et al.*, 2017; Pita *et al.*, 2018; Rocca *et al.*, 2019; Rädercker *et al.*,  
671 2022). Alternatively, the stability of the microbiome may reflect its own resilience (or lower  
672 sensitivity), rather than the host control/regulation, to changing and stressful environments  
673 (Soen, 2014). This characteristic could be essential for host tolerance and acclimation to

674 environmental challenges such as thermal stress. For example, Santoro et al. (2021)  
675 demonstrated that the stability of inoculated “beneficial microorganisms” in the coral  
676 *Mussismilia hispida* provided a thermal protection effect, reducing heat stress and preventing  
677 the associated mortality (40% increase in the survival rate) of the hosts. Contrary, disruptions  
678 in the microbiome structure and function can lead to a weakened thermal tolerance and  
679 performance of the host, and a decrease in survival rate under prolonged heat stress condition,  
680 particularly in early ontogenetic stages such as in the tadpoles of the green frog *Lithobates*  
681 *clamitans* (Fontaine et al., 2022). Regardless whether it is host control or microbiome structural  
682 resilience (independent of the host influence), the temporal stability in the microbiome  
683 structure and function observed suggest that it may contribute to the survival of *I. nucleus* in  
684 the thermally extreme tropical intertidal environment (Bang et al., 2018; Aktipis and Beltran,  
685 2021).

#### 686 *No correlation between microbiome structure and oxidized PUFA products* 687 *profile*

688 Over the decades, many studies have documented the role and action of PUFA (e.g., as  
689 probiotics) in modulating dynamics in the gut microbiome of different animals (Huyben et al.,  
690 2020; Fu et al., 2021; Lau et al., 2022; Ma et al., 2022). Hence, it is expected that stress-driven  
691 changes in these biochemicals and their by-products would influence the oyster gut microbiome.  
692 However, we found no correlation between the oxidized PUFA products (averaged across all  
693 tissues) and the gut microbiome taxonomic structure in *I. nucleus*, despite the temporal and  
694 inter-individual variation in the oxidized PUFA products profile across the emersion period.  
695 The lack of association between the microbial and oxidized PUFA products profiles of *I.*  
696 *nucleus*, suggests two possibilities that are not mutually exclusive. Firstly, as mentioned  
697 previously, physiological/biochemical and microbiome profiles may have different sensitivities  
698 to changes in environmental conditions. Secondly, such microbiome profiles and biochemical  
699 responses may not show strong variation and/or alignment because of local adaptation. Here,

700 the thermal window and oxygen levels experienced by the oysters, although extreme for many  
701 ectotherms, can be considered within the tolerance range of the species. Several studies have  
702 shown that the microbiome-metabolites correlation is detectable only under severe post-  
703 disturbance events and unhealthy host conditions (Nguyen *et al.*, 2021; Pimentel *et al.*, 2021;  
704 Walke *et al.*, 2021; Mallott *et al.*, 2022), which go beyond tolerance levels. Thus, in this study,  
705 oysters may be in a condition where stress is within the tolerance range, and thus no correlation  
706 can be observed between these two components (i.e., host biochemical profile and microbiome  
707 structure). Future studies aiming to explore the functional regulation and interplay between  
708 intrinsic and extrinsic mechanisms in ectotherms living in the thermally extreme and fluctuating  
709 environment like in this study, the intertidal rocky shore, require the assessment of both  
710 components when animals are closer to, or surpass their physiological tolerance limits at  
711 sublethal levels.

712

### 713 *Study limitations*

714

715 While our findings are noteworthy, here we discuss some study limitations and potential  
716 recommendations to address them. First, temperature-modulated dynamics in host-associated  
717 microbial communities and biochemical profiles across the day lacked temporal replication.  
718 Although the selected timepoints are eco-physiologically relevant for the oysters, the  
719 consistency and repeatability of the trends observed can only be assessed by sampling at  
720 different and independent days (i.e., two or more non-consecutive days). Second, the timepoints  
721 selected, although extreme for most ectotherms on Earth, fell within a thermal window that  
722 represents acute low physiological stress to the oysters. Major and divergent differences in  
723 extrinsic and intrinsic responses might potentially be detected under acute high or chronic  
724 physiological stress when body temperatures reach the maximum temperature documented for  
725 this species 50-52°C in the field (Fig. 1). Third, the existence of an interacting drivers that may

726 influence the microbiome altogether, such as, temperature, hypoxic condition (no gaping in the  
727 oyster) and the emersion duration. This makes our study difficult to distinguish the influence  
728 of a sole driver in the field. Fourth, the oyster microbiome can exhibit tissue-specific profiles  
729 associated to functional differences (Dubé *et al.*, 2019). Here, our focus was the gut microbiome  
730 as its captures a large diverse microbial sets associated with their host (King *et al.*, 2012; Pierce  
731 and Ward, 2018; Dubé *et al.*, 2019), and several documented findings showing their strong and  
732 direct signal of their host physiological states (Li *et al.*, 2018; Pierce and Evan, 2019; Fassarella  
733 *et al.*, 2021; Stevick *et al.*, 2021). However, other tissues such as the haemolymph or the gills  
734 of the oysters can provide complementary information regarding the interaction between  
735 microbial communities and their host under environmental stress (Lokmer and Wegner, 2015;  
736 Scanes *et al.*, 2021). Despite these limitations, our study provides for the first-time empirical  
737 evidence of the intrinsic and extrinsic mechanisms underpinning the temperature tolerances and  
738 ecological success of tropical high-supratidal zone inhabiting ectotherms. Future studies can  
739 integrate our recommendations to provide more robust and independent assessments of  
740 temporal dynamics in temperature-modulated functional responses of ectotherms in extreme  
741 environments.

742

## 743 **Conclusion**

744         Intrinsic (e.g., non-enzymatic oxidized PUFA products) and extrinsic (e.g., microbiome  
745 profile) mechanisms linked to the ecophysiology and tolerance/sensitivity of the oyster  
746 *Isognomon nucleus*, showed temporal variation indicative of thermal and oxidative stress. Such  
747 trend showed a temporal association with the environmental and body temperatures during the  
748 tidal cycle where oysters were fully emersed. Although the microbial alpha diversity in the  
749 oyster gut varied along this temporal and environmental cycle, no major changes were detected  
750 in the overall structure and function of the associated microbial communities. This microbiome



751 stability along periods of extreme high temperature and oxygen deprivation, could be explained  
752 by the potential regulatory influence of the host (consistent with the Anna Karenina Principle)  
753 and/or the insensitivity of these microorganisms to the environmental changes experienced in  
754 the tropical rocky shores. When assessing both the intrinsic and extrinsic mechanisms together,  
755 we found no clear correlation between the oxidized PUFA products profile and the microbiome  
756 profile. However, some specific bacteria groups (i.e., *Helicobacteraceae* and *Arcobacteraceae*)  
757 increased during stressful periods, potentially linked to the lower oxygen availability within the  
758 oysters due to the lack of gaping behaviour when emersed. These microorganisms may provide  
759 important functions for the resilience and stability of the overall microbial community, as well  
760 as for the health and survival of the oyster host in the thermally challenging rocky intertidal  
761 environment. The overall microbiome stability can avoid dysbiosis and contribute to the  
762 intrinsic adaptive adjustment of oyster physiology and biochemistry. To our best knowledge,  
763 this is the first study to report the use of non-enzymatic oxidized PUFA products as a proxy for  
764 oxidative stress levels in intertidal animals and profiling short-term dynamics of the host-  
765 associated microbiome in an intertidal environment. Future investigations should aim to explore  
766 the interplay between these biomolecules and microbial patterns when stressful environmental  
767 conditions are unpredictable and beyond tolerance windows of the hosts. Moreover, it is  
768 important to understand whether these functional links between intrinsic and extrinsic  
769 mechanisms are modulated by local adaptation and can be shared among other intertidal  
770 organisms.

771

## 772 **Competing interests**

773 The authors declare no competing or financial interests

774

## 775 **Author Contributions**

776 JDGE and BSA conceived the idea and designed the work. BSA carried out most of the work:  
777 data collection, sample processing, statistical analyses, and data presentation. AWTC, HTY  
778 GAW, and MG were involved in sample and environmental data collection. LKS performed  
779 the oxidized PUFA products extraction and processing. TD supplied the in-house synthesised  
780 oxidized lipids standards for PUFA metabolites quantification. JTY and TD were involved in  
781 analysing the metabolite data. BSA led the manuscript writing with continuous feedback from  
782 JDGE. All authors provided feedback on the final version of this work, and accepted  
783 responsibility for the entire content of this manuscript.

784

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793

## 794 **Data availability**

795 All raw data, and scripts will be made available once published.

796

797 **References**

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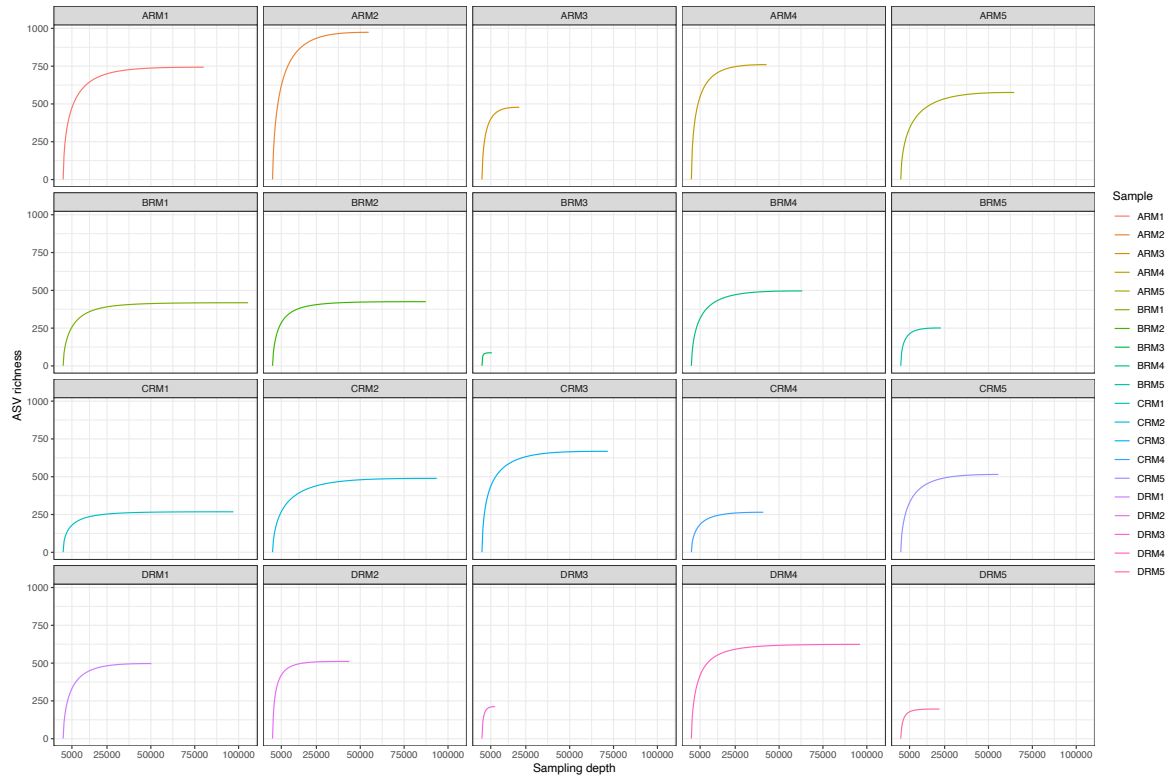
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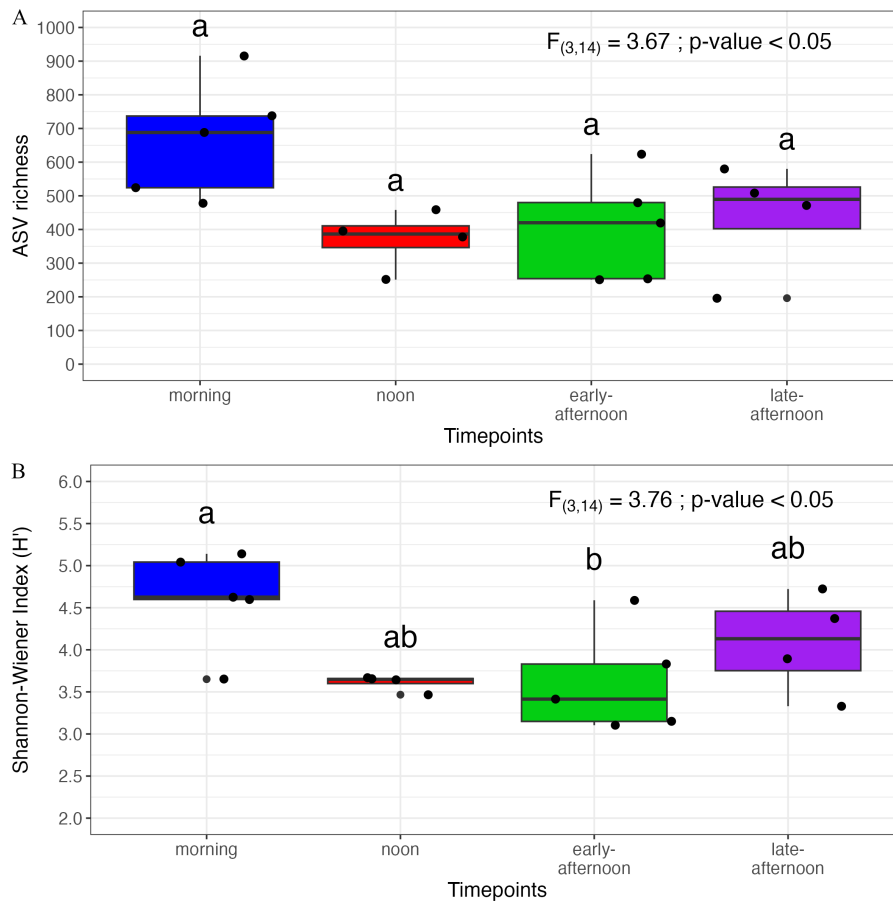
1190 **Supplementary information**



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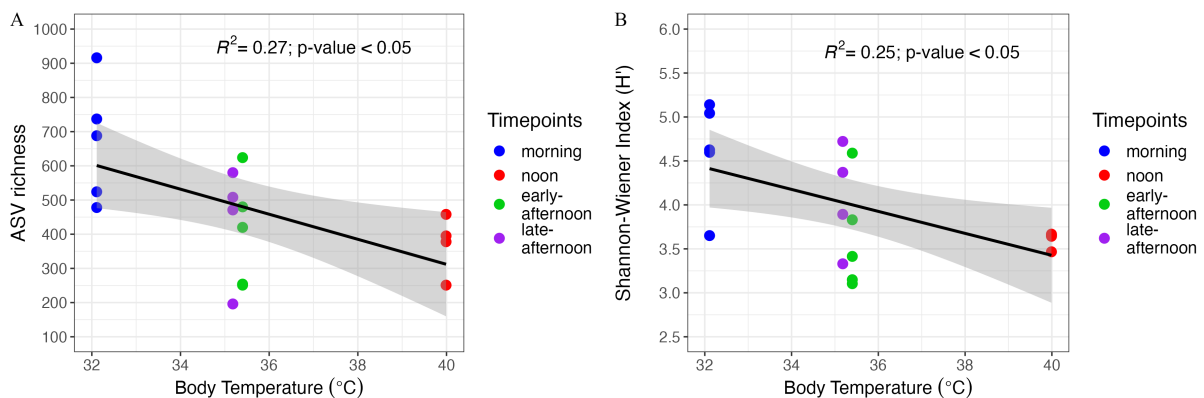
1192 **Fig. S1. Rarefaction curves illustrating the ASVs captured against the sampling depth**  
1193 **which was generated for each sample using their corresponding quality-filtered V3-V4**  
1194 **16SrRNA sequences.**

1195



1196

1197 **Fig. S2. *I. nucleus*-associated microbiome ASV richness and Shannon-Wiener diversity**  
 1198 **across the emersion period.** Boxplot showing the alpha diversity index of (A) ASV richness,  
 1199 and (B) Shannon-Wiener index (H') of the oyster-associated microbiome at different timepoints.  
 1200 (Centre line represents the median; box limits indicate the first and third quartiles; the lower  
 1201 and upper whiskers indicate the smallest and largest values that within 1.5 times of the IQR  
 1202 (Inter-quartile range) from the first and third quartiles, respectively. The data points shown in  
 1203 each timepoint is replicate values. Statistical significance ( $p < 0.05$ ) is indicated if the letters  
 1204 above each boxplot are different between any two timepoints.  
 1205



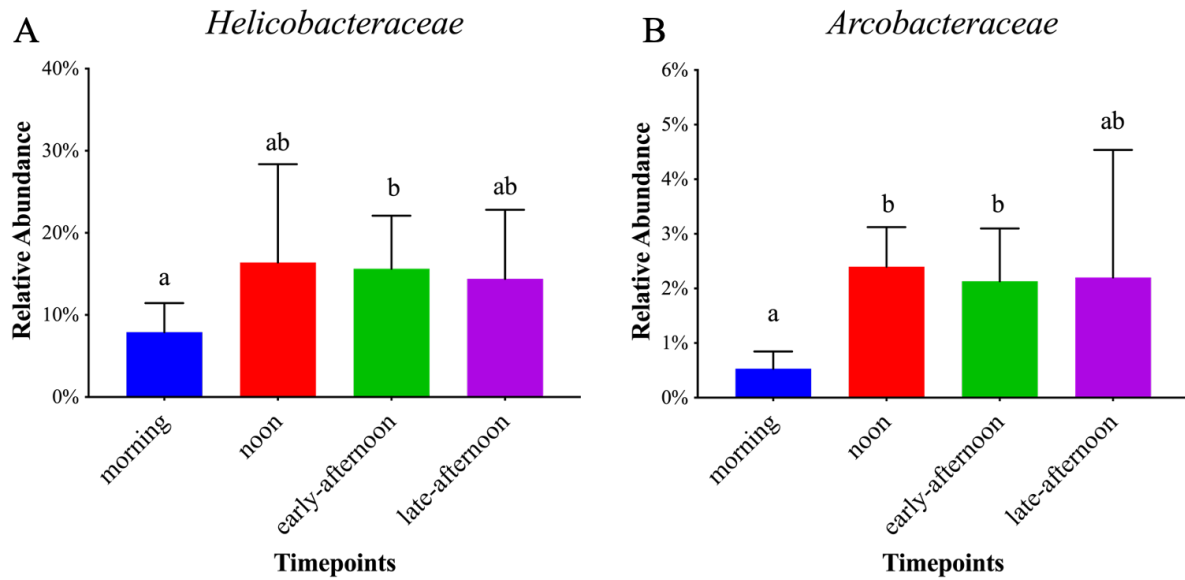
1206

1207 **Fig. S3. Variation in the alpha diversity indices of the *I. nucleus*-associated microbiome**  
 1208 **can be explained by the host body temperature.** Linear regression graph showing the  
 1209 relationship between average body temperature (from 10 random independent replicates  
 1210 collected at each timepoint) and (A) ASV richness (B) Shannon-Wiener index (H'). The

1211 datapoints shown in each panel were the samples' replicates (n= 4-5) from their corresponding  
1212 timepoint. The shaded grey area indicates a 95% confidence interval for the predicted  
1213 regression line. Statistical result (least square regression analysis) is shown in the upper right  
1214 within each panel.

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1219 **Fig. S4. *Helicobacteraceae* and *Arcobacteraceae* mean relative abundance vary across the**  
1220 **emersion period.** Bar graphs showing the two dominant family level taxa in *I. nucleus*'s gut  
1221 microbiome at each timepoint: (A) *Helicobacteraceae* (B) *Arcobacteraceae*. Data are presented  
1222 as mean relative abundance and + 1 standard deviation (error bar) for each taxon at the given  
1223 timepoints. Statistical significance (p-value < 0.05) is indicated if the letters above each bar are  
1224 different

1225

1226

1227

1228 Table S1. Metadata describing the *Isognomon nucleus* samples' collected information

Date	Time	Timepoints label	Replicates	Sample name	Body (mm)
22.05.2019	0915	morning	1	ARM1	12.9
			2	ARM2	13.5
			3	ARM3	15.2
			4	ARM4	12.5
			5	ARM5	13.7
	1230	noon	1	BRM1	11.7
			2	BRM2	11.3
			4	BRM4	11.9
			5	BRM5	12.4
	1612	early-afternoon	1	CRM1	11.7
			2	CRM2	12.9
			3	CRM3	13.0
			4	CRM4	12.0
			5	CRM5	11.7
	1740	late-afternoon	1	DRM1	11.8
2			DRM2	10.9	
4			DRM4	11.1	
5			DRM5	11.9	

1229

1230 Table S2. Description of oxidized poly-unsaturated fatty acids (PUFA) products

PUFA	Oxidized PUFA products
<u>A</u> rachidonic <u>a</u> cid (ARA)	5-F <sub>2t</sub> -IsoP
	15-F <sub>2t</sub> -IsoP
<u>A</u> drenic <u>a</u> cid (AdA)	7-F <sub>2t</sub> -Dihomo-IsoP
	17-F <sub>2t</sub> -Dihomo-IsoP
<u>D</u> ocosah <u>h</u> exaenoic <u>a</u> cid (DHA)	4-F <sub>4t</sub> -NeuroP
	10-F <sub>4t</sub> -NeuroP
	13-F <sub>4t</sub> -NeuroP
	20-F <sub>4t</sub> -NeuroP
<u>α</u> - <u>L</u> inolenic <u>a</u> cid (ALA)	9-D <sub>1t</sub> -PhytoP
	9-F <sub>1t</sub> -PhytoP
	16-F <sub>1t</sub> -PhytoP
	16-B <sub>1t</sub> -PhytoP
<u>E</u> icosap <u>h</u> entaenoic <u>a</u> cid (EPA)	5-F <sub>3t</sub> -IsoP
	15-F <sub>3t</sub> -IsoP
	8-F <sub>3t</sub> -IsoP

1231