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1 **Life-history traits in the Pacific oyster *Crassostrea gigas* are robust to ocean acidification under two**
2 **thermal regimes.**

3

4 **Running head: Oyster life history is unaltered by acidification**

5

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15 **Abstract (191 words):**

16 Ocean acidification and warming (OAW) are pressing contemporary issues affecting marine life and
17 specifically calcifying organisms. Here, we investigated the direct effects of OAW on life-history traits
18 of the Pacific oyster *Crassostrea gigas*, the most cultivated bivalve species worldwide. We also tested
19 whether parental conditioning history shaped the phenotypic characters of their progenies
20 (intergenerational carryover effects). Adult oysters and their offspring were exposed to two
21 temperatures (18°C, +3°C) under ambient pH conditions or under an end-of-century acidification
22 scenario (-0.33 pH units). In adults, we monitored standard biometric and reproductive parameters,
23 stress response by quantifying neuroendocrine metabolites and gamete quality. In larvae, we
24 measured hatching rate, size, biochemical quality and behaviour. We found that reducing pH reduced
25 growth rate and activated the serotonin system, but increasing temperature attenuated these effects.
26 There was no effect of pH on reproduction at either temperature, and no intergenerational carryover
27 effects. Larval characteristics were similar between treatments, regardless of parental conditioning
28 history. Thus, the Pacific oyster seems robust to changes in pH, and increasing temperature is not an
29 aggravating factor. We emphasise that the use of neuroendocrine indicators holds promise for
30 revealing sublethal impacts of environmental changes.

31

32 **Keywords:** Ocean acidification, ocean warming, combined effects, oyster, *Crassostrea gigas*, life
33 stage, intergenerational carryover

34 **Introduction**

35 Over the last 150 years, human activities have led to a massive release of carbon dioxide (CO₂) into the
36 atmosphere, thereby warming the Earth's surface and the oceans (IPCC, 2019). When part of
37 atmospheric CO₂ is absorbed by seawater, it generates an increase in the concentration of hydrogen
38 ions causing a decrease in pH, a process known as ocean acidification (OA) (Caldeira and Wickett, 2005;
39 Orr *et al.*, 2005). Concomitantly, the excess of hydrogen ions leads to a reduction in the bioavailability
40 of carbonate ions (CO₃²⁻) that impacts growth, physiological rates, immune response, behaviour and
41 survival of marine calcifiers such as bivalve molluscs (Orr *et al.*, 2005; Kroeker *et al.*, 2010, 2013; Gazeau
42 *et al.*, 2013; Parker *et al.*, 2013; Figuerola *et al.*, 2021). Bivalvia is the most studied taxonomic class
43 because of the ecological, economic and socio-cultural importance of this group, and they were noted
44 as particularly sensitive to low seawater pH at all life stages (Gazeau *et al.*, 2013; Kroeker *et al.*, 2013;
45 Parker *et al.*, 2013; Gattuso *et al.*, 2015; Waldbusser *et al.*, 2015).

46 Facing the climate emergency, OA research has been one of the fastest growing areas in marine science
47 over the past 15 years (Riebesell and Gattuso, 2015; Browman, 2016). The experimental designs
48 conducted to address this issue have mainly consisted in exposing organisms to stable present and
49 future seawater pH levels as projected for the open ocean (IPCC, 2019). However, for a better
50 assessment of the impacts of OA on bivalves, we must consider the natural pH/pCO₂ fluctuations found
51 in coastal environments where these species thrive (Vargas *et al.*, 2022). Indeed, the diurnal and
52 seasonal variability of pH/pCO₂ in coastal areas is greater than in oceanic waters and can result in
53 present-day situations where seawater pH exceeds the OA projections for the end of the century
54 (Melzner *et al.*, 2013; Gobler and Baumann, 2016; Vargas *et al.*, 2017; Saavedra *et al.*, 2021; Torres *et al.*,
55 2021). The responses of coastal organisms to OA under fluctuating pH regimes are generally
56 different from those observed under stable conditions (Frieder *et al.*, 2014; Mangan *et al.*, 2017). This
57 implies that for studies targeting coastal species, apprehending their OA responses at stable pH
58 conditions may not be reflective of current or future environmental conditions and could lead to
59 misleading extrapolations.

60 In addition, OA occurs concomitantly with other environmental changes such as water warming and
61 deoxygenation (IPCC, 2019). Therefore, OA manipulative experiments should include other interacting
62 factors. Among them, temperature is the most obvious and most studied parameter, as it is the other
63 CO₂-related problem that increases with acidification (Pelejero *et al.*, 2010). Seawater warming can
64 reduce, amplify, or not change the effects of OA depending on the species, population, and life stage
65 (Byrne, 2011; Byrne and Przeslawski, 2013; Kroeker *et al.*, 2013; Przeslawski *et al.*, 2015).

66 Finally, OA experiments should include cross- and multigenerational dimensions to determine whether
67 adaptation can occur rapidly enough that ecosystem functions and services remain unchanged
68 (Riebesell and Gattuso, 2015). These experimental evolutionary studies are generally conducted on

69 species with short generation times, e.g. phytoplankton (Lohbeck *et al.*, 2012; Schlüter *et al.*, 2016).
70 Conversely, for species with longer generation times such as bivalves, we are currently limited in
71 assessing the effects of cross generation exposure to OA, i.e. whether parental conditioning history
72 influences offspring performance traits (Byrne *et al.*, 2020). A pioneer study reveals positive carryover
73 effects on offspring from parents exposed during reproductive conditioning to low pH in the Sydney
74 rock oyster *Saccostrea glomerata* (Parker *et al.*, 2012) that even persist into adulthood and
75 subsequent generations (Parker *et al.*, 2015). However, in the presence of multiple stressors,
76 broodstock exposure to low pH was detrimental on larval survival (Parker *et al.*, 2017). In contrast,
77 parental exposure to low pH made clam *Mercenaria mercenaria* and scallop *Argopecten irradians*
78 larvae more susceptible to OA (Griffith and Gobler, 2017) whereas there was no cross generation effect
79 on the Eastern oyster *Crassostrea virginica* (Clements *et al.*, 2021). Collectively, these results
80 emphasise that the nature of the carryover effects, i.e. beneficial, detrimental or neutral, is species-
81 specific. Thus further research is needed before drawing large-scale conclusions about the adaptability
82 of bivalves to near-future oceanic conditions.

83 Here we aimed to determine whether exposure during reproductive conditioning to naturally
84 fluctuating low pH under two thermal regimes shapes life history traits of the Pacific oyster *Crassostrea*
85 *gigas*, and to test whether parental exposure influences early development of the offspring (from
86 fertilisation to larval hatching) further referred to as intergenerational effects. Adult oysters and their
87 first-generation progeny were exposed to ambient or acidified pH conditions (-0.33 pH units relative
88 to ambient) at a temperature of 18°C, for which reproductive development is optimal, and to +3°C
89 relative to this optimum. These pH and temperature offsets were based on the high greenhouse gas
90 emission scenario (Representative Concentration Pathway, RCP 8.5) predicted for the end of the
91 century (IPCC, 2019), recently redefined as SSP5-8.5 under the CMIP6 Shared Socioeconomic Pathways
92 (Kwiatkowski *et al.*, 2020). Studying the effects of OA in combination with temperature rise will help
93 predicting impacts of climate change in this ecologically and economically relevant species.

94 Specifically, we measured adult growth, food intake, sexual maturity, as well as larval hatching, shell
95 size and calcification. We also investigated new parameters that should shed new light on the potential
96 for oyster adaptation to climate change. Among them, we quantified the concentrations of the
97 components of the neuroendocrine system. Monoaminergic and amino acid neurotransmitters are
98 present in the nervous system throughout the animal kingdom including in bivalves (Walker, 1996;
99 Alavi *et al.*, 2017b). There is accumulating evidence that, in this phylum, the neuroendocrine system
100 plays a critical role in coordinating neurobehavioural systems as well as acts in peripheral organs to
101 regulate physiological functions such as growth, reproduction and development (e.g. for the
102 serotonergic system, reviewed in (Alavi *et al.*, 2017b, 2017a)). It also serves to respond effectively to a

103 wide range of environmental stresses and restores physiological homeostasis (reviewed in (Liu *et al.*,
104 2018)).

105 We also evaluated gamete quality in terms of sperm motility (Boulais *et al.*, 2015a) and egg biochemical
106 contents. Indeed, endogenous lipid reserves invested in eggs fuel energetic needs for embryogenesis
107 in bivalves (Whyte *et al.*, 1990) and maternal reserves are greatly and rapidly mobilised to maintain
108 homeostasis in larvae under acidified and warmer conditions (Talmage and Gobler, 2011; Gibbs *et al.*,
109 2020, 2021). Thus, understanding how biochemical resources are constituted and consumed during
110 early life development may be a promising avenue to further explore the energetic costs to OA and
111 warming. Finally, we characterised swimming behaviour of larvae because of its ecological relevance
112 in survival and dispersal for invertebrate larval stages. Indeed, locomotion is a fundamental
113 behavioural determinant for shellfish larvae that is directly affected by physiological status and shell
114 morphology (Chan and Grünbaum, 2010; Chan, 2012). Swimming behaviour has therefore recently
115 been included in the panel of biological responses of invertebrate larvae that may be altered by OA
116 (Chan *et al.*, 2015, 2016; Meyer-Kaiser *et al.*, 2019; Kavousi *et al.*, 2022).

117

118 **Material and Methods**

119 **Animals**

120 Adult oysters were produced in February 2017 at the Ifremer facilities located at Argenton (Brittany,
121 France, Figure 1A) and grown nearby at Aber-Benoît (see File S1 for details). On 6 February 2018, 412
122 oysters were transferred back to Argenton and kept at 13°C in 500-L flow-through tanks for 6 days
123 prior to the onset of the experiment. The seawater came from a pool which is renewed with each
124 spring tide, filtered at 1 µm and UV-treated (Figure 1A).

125

126 **Experimental set-up**

127 Natural seawater was evenly distributed to 18 independent experimental units consisting of one
128 header tank, where seawater was acidified and/or warmed, connected to one holding tank containing
129 the oysters (Figure 1A). Tanks were 45 L (60 x 40 x 19 cm) and the seawater was renewed every 80
130 min. For larvae, the holding tanks were 5 L cylindrical tube (100 x 9 cm Ø) and the seawater was
131 renewed every 50 min. Seawater in oyster tanks (adult and larvae) was continuously aerated and mixed
132 through air bubbling, the salinity ranged from 34.1 to 34.7 and the photoperiod was fixed at 14L:10D.
133 The acidified conditions were obtained by bubbling pure CO₂ into the seawater of the header tank and
134 the pH-level was controlled using a pH-controller system (JBL, Germany) allowing an accuracy of 0.05
135 pH unit. The controller probes were calibrated on a monthly basis throughout the experiment using
136 NBS buffer solutions at pH 4.00 and 7.00 (HANNA Instruments, France). The pH of the controllers was
137 adjusted relative to the ambient pH twice daily to reach the targeted pH offset in the holding tanks.

138 Each tank was equipped with a thermostat and heating resistor (Biotherm Ecco Hobby and SCHEIGO
139 Titane 300 W, France) to maintain the seawater in the holding tanks at the targeted temperature.
140 There was no tidal regime applied.

141 During the experiment, oysters were fed continuously on a mixed diet of *Chaetoceros muelleri* (CCAP
142 1010/3) and *Tisochrysis lutea* (CCAP 927/14) (1:1 in dry weight). Food concentration was $1500 \mu\text{m}^3 \mu\text{l}^{-1}$
143 of microalgae at the outlet of the tank and controlled twice daily using an electronic particle counter
144 (Multisizer 3, Beckman Coulter, USA) equipped with a 100- μm aperture tube. Oysters did not produce
145 pseudofaeces.

146

147 **Experimental design**

148 At the onset of the experiment on 13 February 2020, the oysters were 12 months-old [55 mm shell
149 length (± 6.1 , SD), 22.5 g wet mass (± 5.3 , SD)] and at an undifferentiated stage of gametogenesis
150 (histological observation; File S2). They were evenly distributed in the holding tanks (N=21 specimens
151 per tank). Each tank was randomly assigned one of four possible conditions: control (C), acidified (OA),
152 warm (W), and acidified and warm (OAW, Figure 1B). There were three replicates for OA and W and
153 six replicates for C and OAW treatments. The control condition corresponded to the ambient seawater
154 pH at the entry of the tanks and a standard seawater temperature for broodstock conditioning (18°C,
155 see (Fabioux *et al.*, 2005). The OA and the warm conditions corresponded to a pH reduction of *ca.* 0.33
156 units compared to the ambient pH and to a temperature increase of *ca.* 3°C respectively, which
157 correspond to the SSP5-8.5 climate projections (Lee *et al.*, 2021). The OAW condition was the
158 combination of the two factors (-0.33 pH units, +3°C relative to the ambient conditions).

159 Under controlled conditions and optimal food availability, gonadal maturation is usually achieved after
160 *ca.* 4.5-6.5 weeks of conditioning at 17°C (Petton *et al.*, 2015). Ripeness was reached after 30 and 35
161 days for the warm ($21.0 \pm 0.1^\circ\text{C}$) and the ambient ($18.0 \pm 0.1^\circ\text{C}$) conditions respectively, corresponding
162 to 630 degree days (Mann, 1979). The gonads were incised and the gametes were recovered with
163 filtered sea water on meshes. For each replicate, the oocytes of three random females from the same
164 tank (*ca.* 250,000 oocytes per female) were fertilised with the sperm pooled from three males from
165 each tank per condition at a ratio of 30 spermatozoa per oocyte (Figure 1C). In total, 18 mating pairs
166 were placed in 2 L jars at the same temperature and pH as the adults.

167 Two hours post-fertilisation, the embryos were transferred to 5 L cylindrical tubes (density of 140
168 larvae mL^{-1}) connected to the header tanks and exposed for 48 h until they reached the D-shaped
169 larvae stage. For OA and W conditions, the progeny were exposed to the same condition as their
170 parents. For C and OAW conditions, the progeny was reallocated in a reciprocal fashion to either the
171 parental condition or to the opposite one, to investigate carry-over effects (Figure 1B). There were six
172 combinations of treatments in three replicate tanks. Seawater temperature for embryos was 1.5°C

173 higher than that of their parents to foster development ($19.5 \pm 0.1^\circ\text{C}$ for control and OA, and $22.5 \pm$
174 0.1°C for W and OAW). Oyster larvae are endotrophic during this period, thus they were not fed.

175

176 **Seawater carbonate chemistry**

177 Seawater pH_{NBS} , temperature, dissolved oxygen and salinity, were monitored twice daily in the holding
178 tanks with a multi-probe reference system (Multi 3630 IDS WT: pH Sentix 940-3 ; oxygen FDO 925 ;
179 salinity TetraCon 925, temperature Checktemp 1 HANNA, Xylem Analytics, Germany). These
180 measurements were used for adjusting the pH-controller settings. The accuracy of the reference pH-
181 probe was checked once a week using Certipur® Merck NBS buffers (pH = 4.00, 7.00, and 9.00) and
182 calibrated three times a week on the total scale (pH_{T}) with a certified Tris/HCl buffer (salinity 35;
183 provided by A. Dickson, Scripps University).

184 Total alkalinity (A_{T}) was assessed on $0.45 \mu\text{m}$ -filtered seawater samples (180 mL) collected three times
185 during the experiment in three randomly chosen header tanks under control, OA and OAW conditions.

186 Seawater samples were poisoned immediately with $75 \mu\text{L}$ saturated mercuric chloride solution. A_{T} was
187 determined potentiometrically using an automatic titrator (888 Titrando Metrohm). Triplicate
188 titrations were performed on 10 mL sub-samples at 20°C and A_{T} was calculated as described by
189 (Dickson, 2007). Titrations of certified reference material provided by A. Dickson (Scripps Institution of
190 Oceanography, USA; batch no. 170) yielded A_{T} values within $12 \mu\text{mol kg}^{-1}$ of the nominal value ($\text{SD} =$
191 $16 \mu\text{mol kg}^{-1}$). The carbonate parameters (partial pressure of CO_2 , aragonite and calcite saturation
192 states, dissolved inorganic carbon, bicarbonate and carbonate concentrations) were determined from
193 pH_{T} , A_{T} , temperature and salinity using the CO2Sys-Excel version 2.1 originally developed by Pierrot *et*
194 *al.* (2011).

195

196 **Biological measurements**

197 Detailed protocols for analytical and behavioural measurements are provided in File S2.

198 **Adults**

199 *Growth* – The shell length (height in mm) and total body weight (shell plus flesh in g) of each oyster
200 were individually measured during the conditioning period after 10, 17, 24 and 30 d for all conditions,
201 and after 35 d for the control and OA conditions. Shell and flesh weights were measured on 12-15
202 individuals per tank after 30 d for W and OAW, and after 35 d for C and OA.

203 *Ingestion rate* – The volume of microalgae, consumed per minute, was measured daily over the course
204 of the experiment in each oyster tank using the following formula:

205 Ingestion rate ($\mu\text{m}^3 \text{ oyster}^{-1} \text{ min}^{-1}$) = $([\text{Cell}_{\text{inlet}}] - [\text{Cell}_{\text{outlet}}]) \times \text{waterflow}$; where the variables were the
206 concentrations of microalgae at the inlet and outlet of the tank ($[\text{Cell}_{\text{inlet}}]$ and $[\text{Cell}_{\text{outlet}}]$ in μm^3 of algae
207 per μL of seawater) and the water flow in the oyster tank (mL min^{-1}).

208 *Reproduction* – A total of 4-5 oysters per tank (out of the 12-15 individuals sampled for shell and flesh
209 weights) were carefully dissected at the end of the conditioning period. A transversal section of the
210 gonads was sampled for histological determination of sex, reproductive stage, and gonadal coverage,
211 which is a proxy of the reproductive effort (Fabioux *et al.*, 2005). The gonadal coverage was calculated
212 as the gonad occupation area divided by the total transverse section area.

213 *Neurochemical profiles* – Within the nervous system, visceral ganglia were sampled, flash-frozen in
214 liquid nitrogen and stored at -80°C for quantification of monoamine neurotransmitters dopamine,
215 noradrenaline, serotonin (5-hydroxytryptamine, 5-HT) and its metabolite 5-hydroxyindoleacetic
216 acid (5-HIAA), as well as amino acid neurotransmitters glutamate and gamma-amino-butyric (GABA)
217 acid, using ultra-high-pressure liquid chromatography coupled with tandem-mass spectrometry
218 (UHPLC-MS/MS). The contents was expressed in pmol mg⁻¹ of tissue, and the ratio of 5-HIAA to 5-HT
219 was used as an index of 5-HT turnover (Winberg and Nilsson, 1993; Winberg and Lepage, 1998).

220 *Gamete quality* – Gametes were sampled from the oysters used for reproduction. Extra males (1-2
221 individuals per tank) were added to increase the sample sizes. The total number of oocytes collected
222 was estimated for each female and the mean diameter was measured using ImageJ (Boulais *et al.*,
223 2015b). Samples of *ca.* 20,000 oocytes per female were collected, centrifuged at 4,000 rpm for 1 min
224 to remove the supernatant seawater, flash-frozen in liquid nitrogen, and stored at -80°C for fatty acid
225 analysis. Total lipids were then extracted and transesterified using methanolic H₂SO₄. Fatty acid methyl
226 esters (FAMES) were analysed in a gas chromatograph equipped with an on-column injector (DB-Wax
227 capillary column) and a flame-ionisation detector, with hydrogen as a carrier gas. The unsaturation
228 index was calculated as the number of double bonds per 100 molecules of fatty acids.

229 Sperm concentration was determined by flow cytometry (EasyCyte Plus, Guava-Marck-Millipore) (Le
230 Goïc *et al.*, 2013). Sperm movement was triggered using a two-step dilution in an activating solution
231 and analysed using the CASA software for ImageJ (Boulais *et al.*, 2015a, 2018). The percentage of
232 motile spermatozoa and their velocity (VAP: Velocity of the Average Path) were assessed on a
233 minimum of 30 spermatozoa. The fertilisation yield is the percentage of fertilised oocytes relative to
234 the total number of oocytes.

235

236 **First-generation progeny**

237 Biological parameters were measured at the D-shaped larvae stage, i.e. 48 h post-fertilisation.

238 *Development* – Hatching rate, i.e. number of D-larvae out of the total, and shell height, i.e. the greatest
239 distance between the umbo and the ventral edge of the shell (minimum of 30 individuals fixed in
240 ethanol 70% per replicate), were measured in each tank by ImageJ software.

241 *Shell calcification* – Samples of larvae were fixed in ethanol 70% and mounted in a glycerol drop. About
242 50 larvae per replicate were photographed under phase contrast and polarised light. The intensity of

243 birefringence was used as a proxy for the evaluation of calcium carbonate (CaCO₃) mineralisation
244 (Wessel *et al.*, 2018). Birefringence was assessed using ImageJ software and quantified as a greyscale
245 score for polarised light images on a scale of 0-255.

246 *Fatty acid composition* – Fatty acid composition of the total lipids of larvae was determined on a pool
247 of 100,000 individuals per replicate. Samples were stored and analysed as previously described for
248 oocytes.

249 *Behaviour* – Larvae were transferred alive in a 96-well micro-plate filled with seawater (8-10 animals
250 per well; 3 wells per replicate) and observed under an inverted microscope connected to a camera
251 (Leica, DMIL, MC 190HD). After a 5-min acclimation period, the mean velocity and the time moving of
252 each animal in each well was recorded for 1 min at 30 frames per second at 1920 x 1080 dpi and
253 quantified with EthoVision XT 13.0 (Noldus Information Technology, The Netherlands).

254

255 **Statistics**

256 General linear mixed-models were performed to assess differences in (1) pH_T among conditions (4
257 levels) and time (38 levels), (2) the total body weight and shell length of adult oysters during
258 conditioning depending on temperature and pH (2 × 2 conditions, main plots), time (4 levels, subplot)
259 and initial parameter values (covariates, subplots), (3) the shell weight and flesh weight of oysters at
260 the end of the conditioning depending on temperature and pH (2 × 2 conditions), (4) the ingestion rate
261 of oysters during the conditioning depending on temperature and pH (2 × 2 conditions, main plots)
262 and time (35 levels, subplots, repeated measurements), (4) the gonadal coverage and neurochemistry
263 at the end of the conditioning depending on temperature and pH (2 × 2 conditions, main plots) and
264 sex (2 levels, subplots), (5) oocyte and sperm quality at the end of the conditioning depending on
265 temperature and pH (2 × 2 conditions), (6) D-larvae quality and behaviour depending on temperature
266 and pH during the conditioning and the larval development (6 conditions). The unit of replication was
267 the tank in which the conditions were applied (random factor). All mutual interactions among the
268 factors were tested, and Tukey's HSD was used as a post hoc test. The normality of residuals and
269 homogeneity of variances were checked. Results are expressed as least square means ± standard error
270 (SE). All statistical analyses were conducted using SAS 9.4 software (SAS institute) and significance was
271 set at α = 0.05. Statistical outputs are summarised in File S3.

272

273 **Results**

274 **Seawater carbonate chemistry**

275 The pH_T of natural seawater ranged from 7.94 to 8.15 throughout the experiment in the non-acidified
276 conditions (C, W) and slightly increased over time (Figure 2, left panel). On average, pH_T ranked as C >
277 W >> OA = OAW (Figure 2, right panel). The average pH_T offset between the acidified (OA and OAW)

278 and control (C) conditions was -0.33 ± 0.04 pH unit. Overall, $p\text{CO}_2$ levels varied over time but were
279 always higher in the acidified conditions than in the control ones (Table 1). Alkalinity was relatively
280 stable regardless of the conditions, varying from 2211 to 2367 $\mu\text{mol kg}^{-1}$. Saturation states of aragonite
281 and calcite (Ω_{ar} , Ω_{ca}) were much lower in the lowered pH conditions compared to the ambient and
282 warmed ones, but seawater remained oversaturated ($\Omega > 1$) in all treatments.

283

284 **Effects of temperature and pH changes on biometrics in adult oysters**

285 During the conditioning period, temperature, pH and time interacted in their effects on shell length
286 and total body weight of adult oysters (Figures 3A, B). On average, shell length increased from 55.8 to
287 66.3 mm, which corresponds to an increase in total body weight from 22.7 to 32.3 g. At the end of the
288 conditioning period, i.e. 30 d in W, OAW conditions and 35 d in C, OA conditions, oysters in acidified
289 water at 18°C (OA) were smaller in terms of both shell length and total body weight than their
290 counterparts reared in acidified and warmed (OAW), warmed only (W), and control conditions (C). The
291 rise in temperature offset the negative effect of acidification alone on growth. There were no statistical
292 differences in final shell and flesh weights between conditions (Figures 3C, D). In contrast,
293 temperature, pH and time interacted in their effects on ingestion rates and there was no significant
294 difference between treatments (Figure 3E).

295

296 **Effects of temperature and pH changes on reproductive attributes in adult oysters**

297 Ripeness was reached after 30 d of conditioning under warmer conditions (21°C in W, OAW) and 35 d
298 under ambient ones (18°C in C, OA). At the end of the conditioning period, sex ratio (*ca.* 60/40
299 females/male), maturity level (majority stage 3) and gonadal coverage were similar for all treatments,
300 while gonadal coverage was consistently higher in males (Figures 4A, B, Table 2).

301

302 **Effects of temperature and pH changes on neurochemistry in adult oysters**

303 The levels of all parent neurotransmitters, i.e. dopamine, glutamate, GABA, noradrenaline and 5-HT,
304 known as neuroendocrine regulators of physiological functions in bivalves, were not significantly
305 affected by temperature and pH changes (Figures 5A-E). There was a significant pH effect on 5-HIAA
306 concentrations, which were higher in the acidified conditions (OA, OAW) than in the other treatments
307 (Figure 5F); while this effect was marginally significant on the 5-HIAA/5-HT ratio (Figure 5G). In
308 addition, there was a significant reduction of this ratio under warmer conditions (W, OAW; Figure 5G).

309

310 **Effects of temperature and pH changes on gamete quality in adult oysters**

311 Oocyte concentration, diameter and fatty acid content, a proxy of total lipid reserves, were similar
312 among treatments (Figures 6A-C). Increasing temperature decreased the unsaturation index of fatty

313 acids and increased levels of both arachidonic (AA, 20:4n-6) and eicosapentaenoic acids (EPA, 20:5n-
314 3, Figures 6D-F). Decreasing pH slightly decreased the level of docosahexaenoic acid (DHA, 22:6n-3)
315 irrespective of temperature (Figure 6G). Temperature rise increased spermatozoa concentrations in
316 oysters but not motility rate and velocity (Figures 6H-J). Fertilisation rates were similar among
317 treatments and were always higher than 90 % under all conditions (data not shown).

318

319 **Effects of temperature and pH changes on life traits of progeny**

320 Hatching rate, an indicator of successful embryogenesis, varied almost twofold within each treatment,
321 but there was no consistent pattern with temperature, pH or parental conditioning history (Figure 7A).
322 Although marginally significant, we found that larvae produced from parents exposed to OA during
323 gametogenesis and then maintained under OA for 48 h were the smallest (Figure 7B). Other indicators
324 such as birefringence level, a proxy of shell calcification, lipid reserves and swimming behaviours (i.e.
325 velocity) were unaffected by lowered pH and increased temperature (Figures 7C-I). Interestingly,
326 offspring facing multi-stress (OAW), either directly during the larval period or via parental exposure,
327 were less mobile, although these results were marginally significant (Figure 7J).

328

329 **Discussion**

330 Here, we investigated the direct effects of OAW on life-history traits of the Pacific oyster *Crassostrea*
331 *gigas*. We also tested whether intergenerational carryover effects occurred, i.e. whether parental
332 conditioning history shaped the phenotypic characters of the first-generation progeny. Adults were
333 exposed to -0.33 pH units relative to ambient seawater conditions at two temperatures during a
334 complete reproductive cycle, and the phenotype of the offspring was evaluated at 48 hours post-
335 fertilisation. Throughout conditioning, we monitored standard parameters in adults such as growth,
336 food intake and sexual maturity, as well as components of the neuroendocrine system, and of gamete
337 and larval quality (e.g. energy reserves, behaviour) that have rarely been appreciated in OA studies.
338 We found that near-future OA exposure during gametogenesis reduced growth rate and activated the
339 serotonergic system in adult oysters, but increasing temperature buffered these effects. Other macro-
340 physiological parameters were however not affected by temperature and pH, and we did not find any
341 evidence of intergenerational linkage. All together, our results suggest that *C. gigas* is robust to near-
342 future ocean changes.

343

344 **Near-future OA reduces adult oyster growth but increasing temperature suppresses this effect**

345 We observed that adult oyster growth, measured as shell length and total body weight, was
346 significantly reduced under OA at a pH offset of -0.33 unit. These results contrast with what we recently

347 observed on juveniles of the same species, i.e. that shell length and growth rate remained unchanged
348 until a tipping point was reached at *ca.* pH 7.10, below which these parameters were strongly affected
349 (Lutier *et al.*, 2022). The overall reduction in oyster growth cannot be attributed to shell dissolution
350 because seawater was not corrosive in our study with respect to the saturation state of calcite ($\Omega_{ca} >$
351 1), the main carbonate mineral composing the adult oyster shell (Stenzel, 1963). Rather, this may
352 reflect a decrease in calcification rate which declined linearly with lowering pH and CO_3^{2-}
353 concentrations in *C. gigas* (Gazeau *et al.*, 2007). However, as previously reported (Clark *et al.*, 2013;
354 Timmins-Schiffman *et al.*, 2014), there was no statistical difference for shell weight. In fact, since
355 growth was limited due to the relatively short experimental period, the newly formed shell was
356 probably negligible relative to the whole shell weight.

357 Adult oyster growth was reduced under near-future OA but food intake was unaltered. The results are
358 consistent with previous data showing that ingestion of juvenile oysters remained unchanged up until
359 reaching pH 6.85 where it decreases thereafter (Lutier *et al.*, 2022). Thus, oysters may not be
360 physiologically responsive to the pH reduction considered in our study because they were fed *ad*
361 *libitum*. Thus the reduced growth rate of oysters under OA was probably not an energetic issue but
362 rather a reflection of a less favourable carbonate environment.

363 Interestingly, we found that increasing temperature suppressed the negative effects of OA on oyster
364 growth. Oysters are ectothermic animals, for which temperature is one of the dominant environmental
365 determinants that controls physiological performances (Bayne, 2017). It is therefore possible that the
366 growth benefit of the temperature increase tested, which was within the optimal thermal window for
367 the species, outweighed the deleterious effects of OA on individual fitness. However, this result
368 contrasts with other studies conducted on clams and scallops that report additive negative effects of
369 these two factors on growth, survival and biomineralization (Talmage and Gobler, 2011; Watson *et al.*,
370 2012; Ivanina *et al.*, 2013). These contradictory results pinpoint the need for conducting more studies
371 combining stressors in OA experiments (Riebesell and Gattuso, 2015) and for increasing knowledge on
372 thermal tolerance of organisms to enhance our prediction capacity of species sensitivity to climate
373 change.

374

375 **Near-future OA and temperature rise have no effect on sex determination and reproductive effort,** 376 **but temperature accelerates gonadal maturity**

377 We did not observe any effect of pH and temperature, alone or in combination, on sex ratio and
378 gonadic coverage of oysters, an indicator of reproductive effort (Fabioux *et al.*, 2005). In contrast, in
379 the eastern oyster *C. virginica*, pH reduction between 7.5-7.7 (-0.2 to -0.4 pH offset) increased gonadic
380 coverage in both sexes (Clements *et al.*, 2021) and gonadal development was delayed at pH \leq 7.1 and
381 inhibited at pH 6.7 (Boulais *et al.*, 2017). In the Sydney rock oyster *Saccostrea glomerata*, pH reduction

382 between 7.4-7.0 (-0.4 pH offset) delayed gonadal development and favoured females (Parker *et al.*,
383 2018). The scientific literature reveals varying and conflicting results within and between species thus
384 preventing generalisations.

385 We note, however, that sexual ripeness was reached earlier at the higher temperature. It is well known
386 that temperature is one major driver of reproduction in *C. gigas* (Fabioux *et al.*, 2005). For instance,
387 we know that a minimum accumulation of 600°C d above ~10°C induces spawning (Mann, 1979).
388 Therefore, increasing seawater temperature will favour accelerated sexual maturity and earlier
389 spawning events with possible impacts on species distribution and phenology (Gourault *et al.*, 2019;
390 King *et al.*, 2021).

391

392 **Temperature rise, but not OA, influences gamete characteristics**

393 Temperature did not influence the quantity and size of the oocytes, but their fatty acid content.
394 Indeed, the unsaturation index of fatty acids, an indicator of lipid fluidity, decreased with increasing
395 temperature, as predicted by homeoviscous adaptation (Hazel, 1995; Pernet *et al.*, 2007). This change
396 in unsaturation index mainly reflected 20:5n-3, as previously reported in other bivalve species (Pernet
397 *et al.*, 2007). Therefore, the fatty acid composition of oocytes changes with temperature consistently
398 with what is generally reported in other tissues. Spermatozoa concentration was enhanced in warmer
399 conditions whereas no change in their motility was observed. It seems that in the case of the oyster,
400 maturation at 21°C may enhance fertilisation success compared to 18°C. It is however noteworthy that
401 the two temperatures tested in our study were within the optimal temperature window for fertilisation
402 in *C. gigas* (20-25°C; Bayne, 2017). While it is admitted that temperature is an important factor
403 influencing sperm quality, an increase in temperature beyond the species optimal is generally
404 unfavourable to sperm movement and energy metabolism. Indeed, prolonged exposure to high
405 temperatures, unsuitable for reproductive activity in the mussel *Mytilus galloprovincialis*, significantly
406 reduced the concentration of spermatozoa and furthermore disrupted their motility and mitochondrial
407 activity (Boni *et al.*, 2016).

408 In addition, we found that OA had no major effect on gamete quality. In most of the published studies,
409 broodstock were maintained under ambient conditions and gametes are generally acutely exposed to
410 low-pH treatments. In such conditions, a pH lowering (-0.3 to -0.4 pH offset) did not induce significant
411 effects on sperm velocity and fertilisation success in oysters and mussels (Kurihara *et al.*, 2007, 2008;
412 Havenhand and Schlegel, 2009; Barros *et al.*, 2013; Falkenberg *et al.*, 2019). In contrast, stronger pH
413 lowering (-0.7 pH offset) substantially reduced spermatozoa motility and fertilisation success in oysters
414 (Kurihara *et al.*, 2007, 2008; Barros *et al.*, 2013; Boulais *et al.*, 2017, 2018). To our knowledge, only one
415 study has examined the effects of acidified conditions throughout the conditioning of *C. gigas* on
416 sperm quality (Omoregie *et al.*, 2019). In contrast to our study, these authors showed that a moderate

417 pH reduction (-0.4 pH offset) reduced sperm motility in a Namibian *C. gigas* population. Such
418 discrepancies in intraspecific responses may reflect effects related to the study populations or
419 experimental conditions other than pH (*e.g.* food regime).

420

421 **Near-future OA enhances serotonin response but increasing temperature counteracts this effect**

422 We did not observe changes in the levels of parent neurotransmitters induced by pH and temperature,
423 even for GABA which is the major neurotransmission system known to be affected by OA in fish
424 (Nilsson *et al.*, 2012; Chivers *et al.*, 2014; Schmidt *et al.*, 2017). However, the content of the 5-HIAA
425 primary metabolite was significantly increased in oysters exposed to OA, as was the 5-HIAA/5-HT ratio,
426 although marginally significant. In bivalves, the serotonergic system modulates reproduction through
427 gametogenesis, spawning induction and sperm motility (Alavi *et al.*, 2017b, 2017a; Canesi *et al.*, 2022).
428 However we observed no effect of OA on oyster reproduction, suggesting that the observed change in
429 5-HIAA under OA may be associated with other biological functions. In particular, the serotonergic
430 system is mobilised in *C. gigas* when exposed to acute and chronic stressors such as air exposure and
431 chemical pollution (Dong *et al.*, 2017; Canesi *et al.*, 2022). Since our study is the first reporting such
432 effects of pH on serotonergic system, it may be premature to state that OA evoked a stress response
433 in oysters.

434 However, we evidenced that OA could affect 5-HT activity by presumably increasing 5-HT catabolism
435 rather than 5-HT synthesis since 5-HT levels remained similar between conditions. In addition, it is
436 worth noting that warming significantly reduced 5-HT turnover for all animals, including those exposed
437 to OA, suggesting an antagonistic interaction between the drivers. To our knowledge, there are no
438 comparable studies on the effects of environmental changes on the neuroendocrine systems in marine
439 molluscs. Interestingly, recent papers showed that near-future changes in pH and thermal regimes
440 affect serotonin metabolism in three fish species (*Salmo salar*, *Gadus morhua*, *Boreogadus saida*)
441 leading to a rise of the 5-HIAA/5-HT ratio (Schmidt *et al.*, 2017; Höglund *et al.*, 2020), with no apparent
442 interaction between the drivers (Schmidt *et al.*, 2017). Unlike these species whose geographical
443 distribution is limited to the North Atlantic, the Pacific oyster is cosmopolitan and has a wide thermal
444 optimum (Bayne, 2017) making it tolerant to a +3°C rise.

445 Overall, our results are of great importance because the serotonergic system is a major
446 neuroendocrine actor mediating various physiological functions in oysters (Alavi *et al.*, 2017b; Canesi
447 *et al.*, 2022). We therefore believe that the use of neuroendocrine indicators is promising in the field
448 of climate change research, particularly the serotonergic system which has been identified as a
449 relevant proxy to reveal sublethal effects of OA in fish (Höglund *et al.*, 2020). Thus, further
450 investigations will be conducive to better understand the role of the 5-HT system in the physiology of
451 *C. gigas* in order to tease the pace of individual acclimation to environmental change.

452

453 **Near-future temperature rise and OA do not affect early development**

454 When parents and offspring were kept under the same conditions, there was no significant effect of
455 pH and temperature on larval quality (hatching rate, shell height, calcification, fatty acid content and
456 composition). We also found that a direct multi-stressor exposure (OAW) during early development,
457 with or without prior parental exposure, had no effect on larval quality. Overall, the lack of a significant
458 effect of near-future OA is consistent with what has already been reported in *C. gigas* larvae when
459 exposed directly during embryogenesis (Kurihara *et al.*, 2007; Barros *et al.*, 2013; Ginger *et al.*, 2013).
460 Collectively, these results contrast with the findings of Harney *et al.* (2016) showing that OA
461 significantly reduces larval size while warming increased both shell size and calcification level. In the
462 latter study, the authors showed a non-additive effect of these two drivers on larval size and proteome
463 (Harney *et al.*, 2016). Furthermore, our exposure conditions did not alter the swimming behaviour of
464 *C. gigas* larvae, which is consistent with previous observations conducted in mollusc larvae exposed to
465 OA in combination or not with warming, such as in the Eastern oyster *C. virginica* at settlement (Meyer-
466 Kaiser *et al.*, 2019) and in the abalone *Haliotis tuberculata* (Kavousi *et al.*, 2022).

467 Moreover, we did not observe any intergenerational carryover effects between adult and offspring at
468 hatching (*i.e.* 48-hour post-fertilisation). The quality of larvae originating from parents matured in the
469 OAW condition and then maintained under control condition, and vice versa, were not different from
470 those kept in the fixed OAW or control conditions throughout the experiment. In several bivalve
471 species, carryover effects under OA are generally positive, *i.e.* parental exposure to OA confers on the
472 offspring, better growth and faster development at decreasing pH from hatching, and this benefit
473 persists throughout the larval life (Parker *et al.*, 2012, 2015; Zhao *et al.*, 2019) and into adulthood
474 (Parker *et al.*, 2015). Although in *C. virginica*, there is no indication of carryover effect, adult OA
475 conditions did not modify early larval development 48 hours after fertilisation (Clements *et al.*, 2021).
476 Interestingly, in several oyster species, when OA is combined with increased temperature, the
477 carryover effect is unnoticeable at hatching (Gibbs *et al.*, 2021) or even negative later in the
478 development (Parker *et al.*, 2017). Our results are consistent with the studies by (Clements *et al.*, 2021;
479 Gibbs *et al.*, 2021) that show no carryover effect in early larval development. Note, however, that the
480 48-hour timeframe was short and we cannot rule out that carryover effects may arise later during
481 development as suggested in *C. gigas* by (Gibbs *et al.*, 2021). So-called "latent carryover" effects
482 (Pechenik, 2006; Hettinger *et al.*, 2013), typically described when the susceptibility of juveniles to
483 environmental stress is influenced by previous larval experience, needs to be further investigated in
484 the context of parental effects. Although intergenerational and developmental plasticity is increasingly
485 being considered as acclimation mechanisms in bivalves to cope with climate change, there may be
486 limitations and differences among species. Therefore, further investigations demonstrating whether

487 or not effects are transferred across life phases, the strength and direction of responses, and their
488 persistence, are needed to provide a more complete understanding of acclimatory capacity of oysters.
489

490 **Apparent robustness of *Crassostrea gigas* to climate change**

491 Molluscs are generally considered as vulnerable to OA because of their dependance to the availability
492 of carbonate ions to produce their shell combined with their low capacity to regulate internal acid-
493 base status (reviewed in Melzner *et al.*, 2009; Gazeau *et al.*, 2013; Parker *et al.*, 2013). Here we suggest
494 that the oyster *C. gigas* is robust to near-future OA and warming during reproduction and early
495 development. This can be explained by the fact that this species is (1) eurythermic and the temperature
496 levels tested here are within the thermal optimum of the species (Bayne, 2017) and (2) intertidal and
497 thus adapted to hypercapnia and internal acidosis (Scanes *et al.*, 2017; Parker *et al.*, 2021). This agrees
498 with the fact that physiological traits such as growth, net calcification and ingestion tipped to much
499 lower pH - between 7.3-6.9 - than those tested here (Lutier *et al.*, 2022).

500 Nevertheless, the experimental conditions may have masked some effects. Oysters were fed *ad libitum*
501 and we know that food availability mitigates the impacts of OA on marine calcifiers (Melzner *et al.*,
502 2011; Thomsen *et al.*, 2013; Ramajo *et al.*, 2016). Also, the pH of the acidified seawater was lowered
503 by 0.3 units but remained non-corrosive regarding calcite saturation level ($\Omega_{ca} > 1$). In fact, it seems
504 that larval shell development and growth are dependent on seawater saturation state, and not on pH
505 (Gazeau *et al.*, 2011; Waldbusser *et al.*, 2015).

506 To go further, we need to consider the natural variability of pH/ pCO_2 experienced by the oysters prior
507 the experiments. A recent study shows that the effect of a given pH/ pCO_2 scenario depends on the
508 deviation from the upper level of pCO_2 experienced by the local populations studied (Vargas *et al.*,
509 2022). This deviation is not known in our study and could explain our results. This requires the
510 implementation of pH/ pCO_2 monitoring along the coastline and in oyster farms to better ground the
511 experiments. We must also consider the importance of local biological adaptation by integrating
512 several populations of oysters across continents and various genetic lines with different life-history
513 traits. These repeating experiments are needed to fully understand and tease apart any differences
514 due to experimental procedures from genetic/phenotypic differences, which may alter conclusions
515 about the impacts of climate change and the capacity for intergenerational plasticity in oysters.

516

517 **Data Availability Statement**

518 The data underlying this article are available in the article and in its online supplementary material (File
519 S4).

520

521 **Supplementary material**

522 The following supplementary material is available at *ICESJMS* online:
523 File S1 describes the production and maintenance of broodstock used in the experiment.
524 File S2 describes in detail the methodology and protocols applied in this study.
525 File S3 provides the full statistical output.
526 File S4 is the data repository.

527

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538 **Author contributions**

539 All authors designed and conducted the experiment. FP performed statistics and figures. CDP and FP
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541

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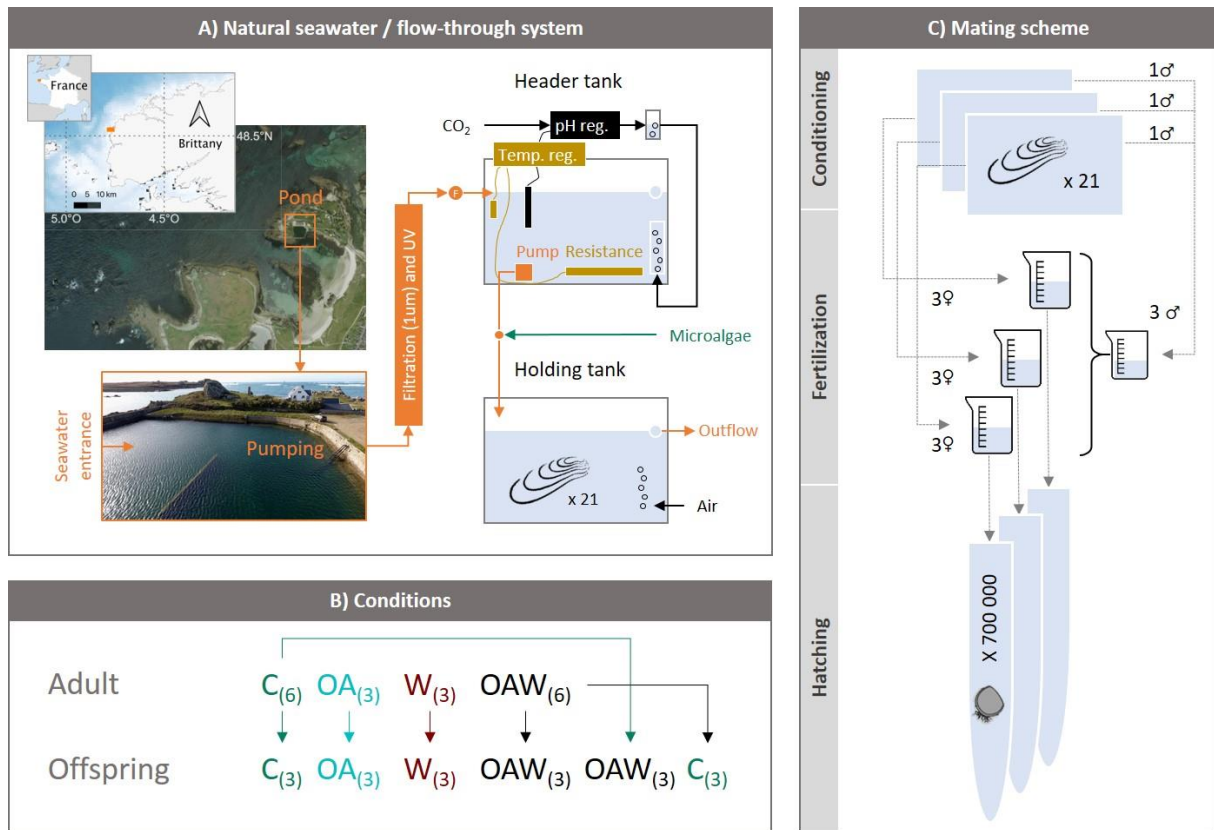
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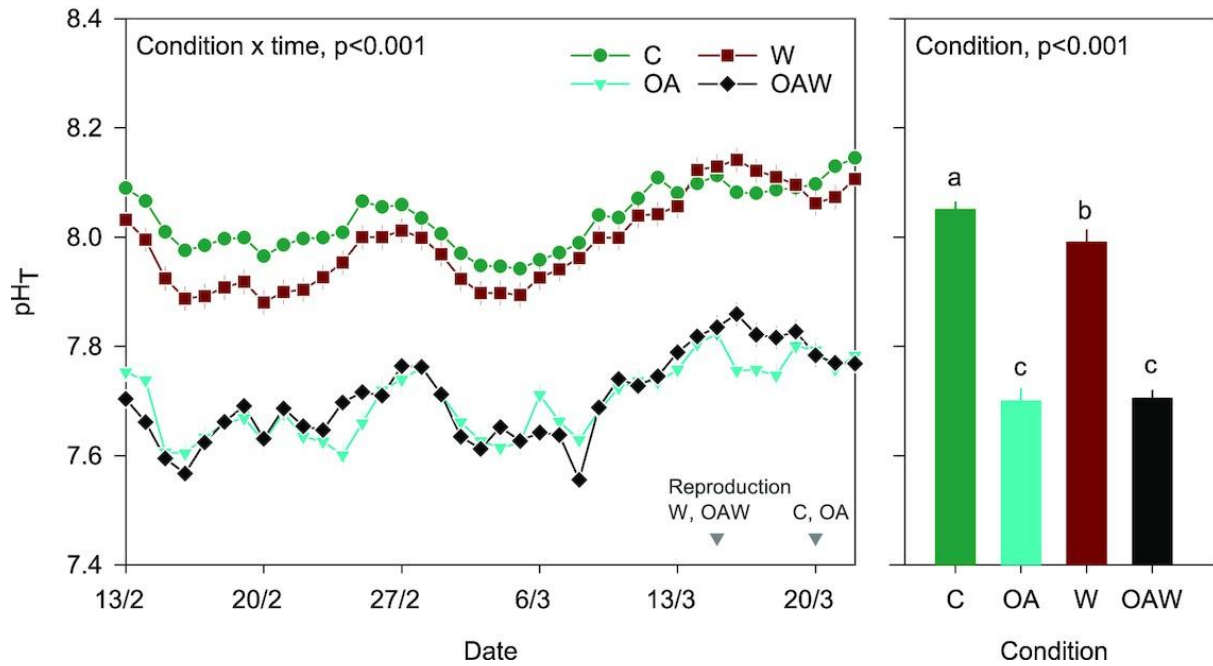
823 **Figure captions**

824 Figure 1. Experimental set-up (A), design (B) and mating scheme (C) during adult conditioning and larval
 825 exposure. Abbreviations: A) reg. = regulation, B) C – control (18°C, ambient pH), OA – ocean
 826 acidification (18°C, -0.33 pH unit), W – warming (21°C, ambient pH), OAW – ocean acidification and
 827 warming (21°C, -0.33 pH unit) and number of replicates is given in parentheses.



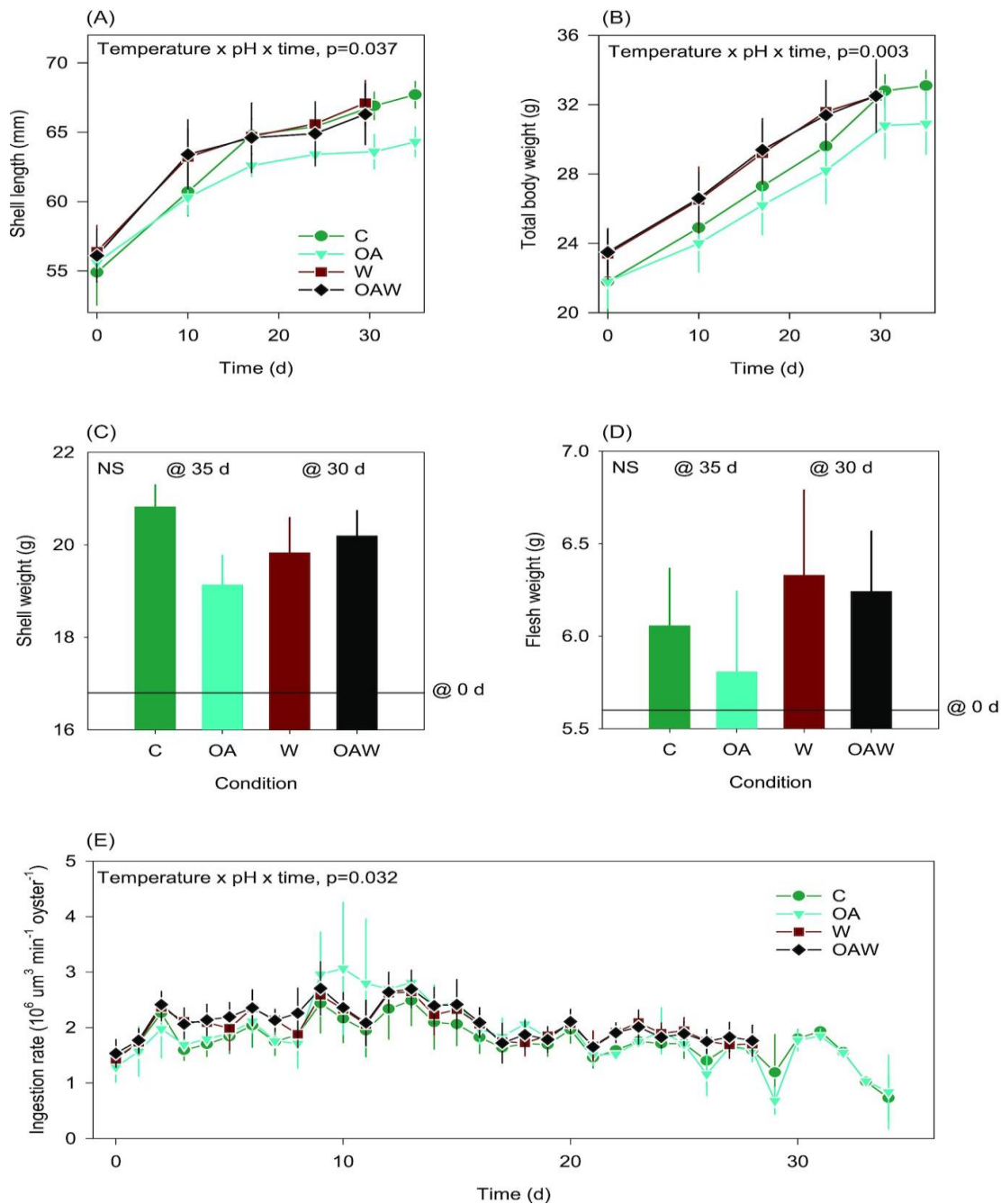
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829 Figure 2. Temporal dynamics of pH_T (on the total scale) in oyster tanks for each condition (left panel)
 830 and averaged values during the course of the experiment (right panel). Data are least square means \pm
 831 SE among tanks ($n=3$ or 6). The dates of reproduction are indicated in grey for each condition in the
 832 left panel. Abbreviations: C – control (18°C , ambient pH), OA – ocean acidification (18°C , -0.33 pH unit),
 833 W – warming (21°C , ambient pH), OAW – ocean acidification and warming (21°C , -0.33 pH unit).



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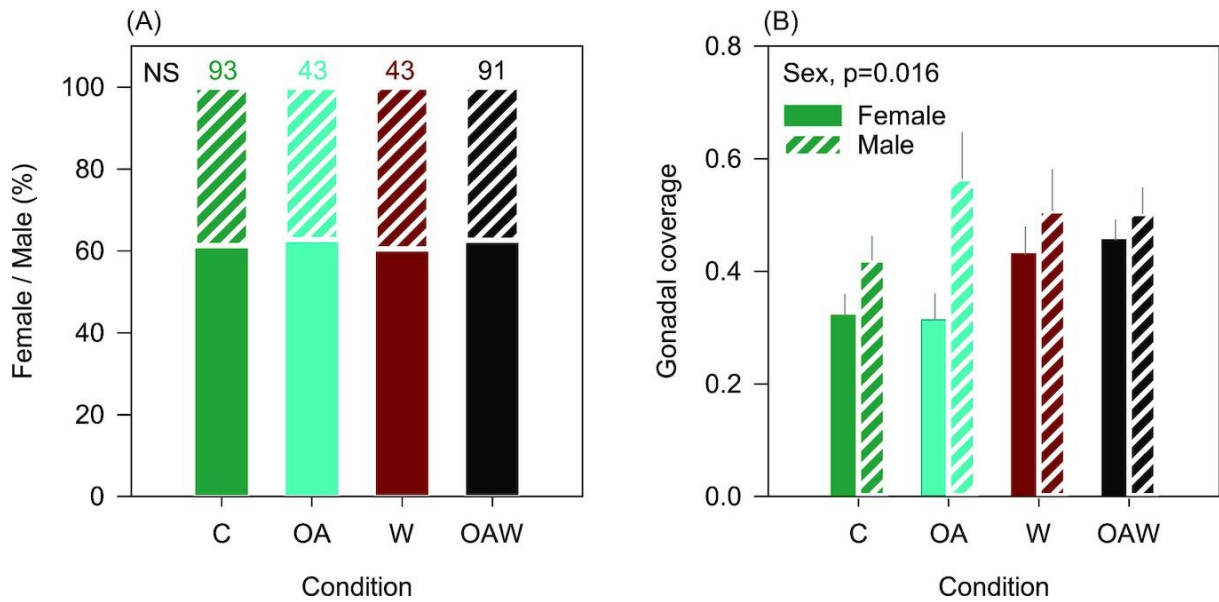
835 Figure 3. Temporal dynamics of oyster shell length (A) and total body weight (B), final shell weight (C)
 836 and flesh weight (D), as well as ingestion rate (E) during the conditioning period. Data are least square
 837 means \pm SE among tanks (n=3 or 6). The black line in (C) and (D) indicates the average weight at the
 838 beginning of the experiment. Effects and *p*-values are indicated otherwise not significant (NS).
 839 Abbreviations: C – control (18°C, ambient pH), OA – ocean acidification (18°C, -0.33 pH unit), W –
 840 warming (21°C, ambient pH), OAW – ocean acidification and warming (21°C, -0.33 pH unit).



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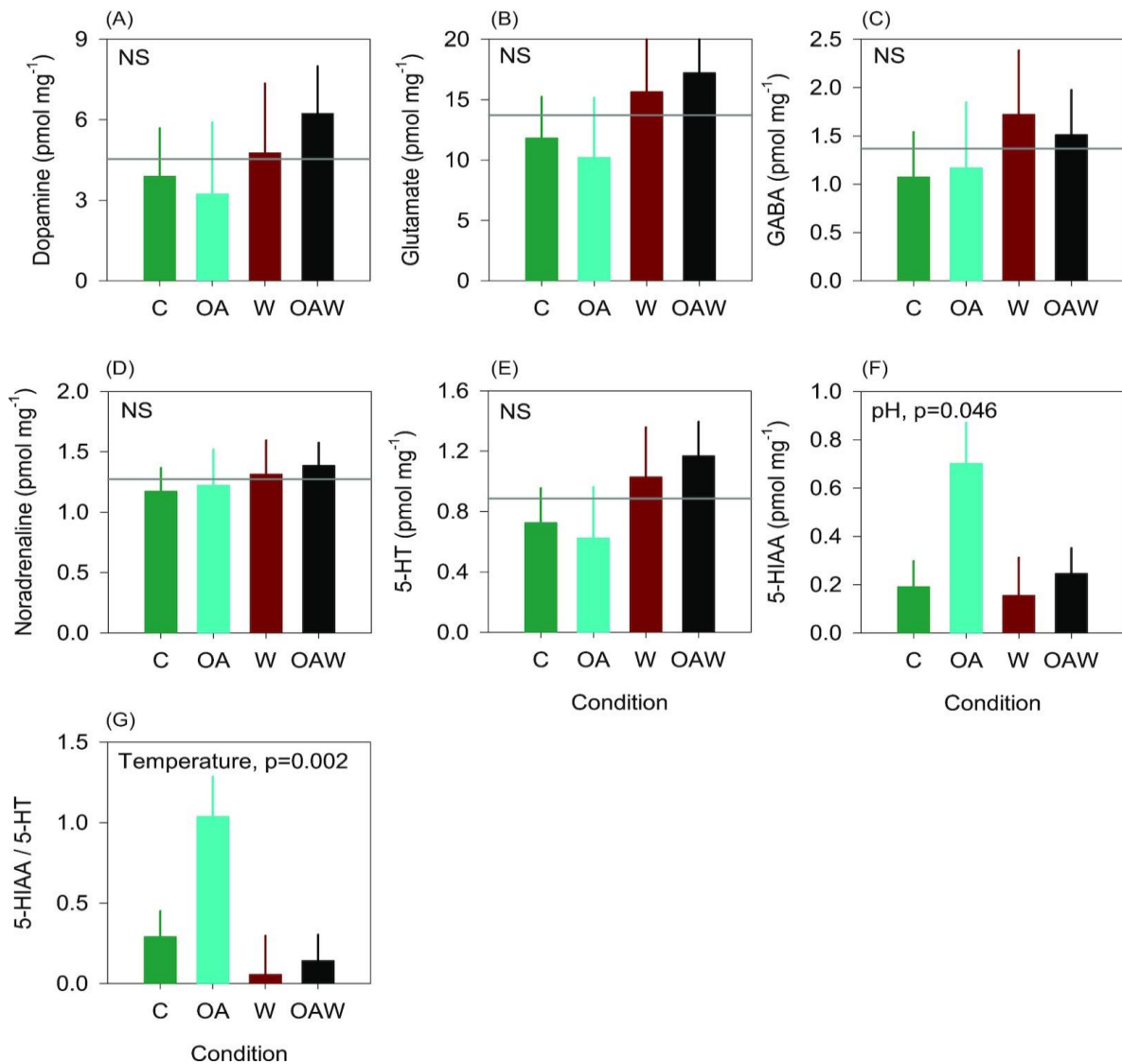
843 Figure 4. Sex ratio with numbers above bars indicating the numbers of adult oysters (A) and gonadal
 844 coverage in both sexes (B). Solid bars represent females and striated bars represent males. Data are
 845 least square means \pm SE among tanks (n=3 or 6). Effects and *p*-values are indicated otherwise not
 846 significant (NS). Abbreviations: C – control (18°C, ambient pH), OA – ocean acidification (18°C, -0.33 pH
 847 unit), W – warming (21°C, ambient pH), OAW – ocean acidification and warming (21°C, -0.33 pH unit).



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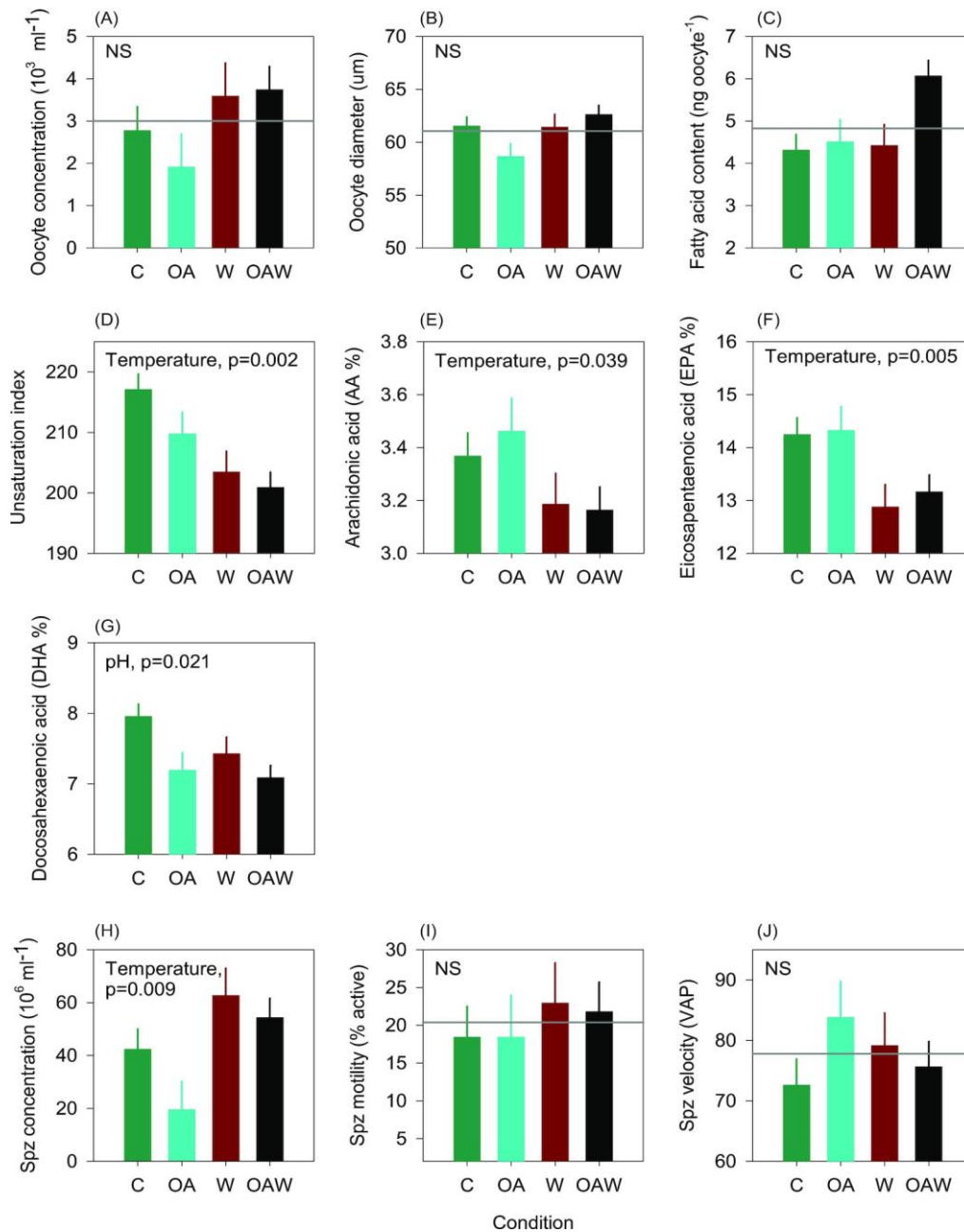
850 Figure 5. Concentration of parent neurotransmitters (A-F) in visceral ganglia of adult oysters, and 5-HT
 851 turnover expressed by the 5-HIAA/5-HT ratio (G). Data are least square means \pm SE among tanks (n=3
 852 or 6). Effects and *p*-values are shown when significant otherwise the mean is shown by the grey line
 853 when non significant (NS). Abbreviations: 5-HT = serotonin or *5-hydroxytryptamine*; 5-HIAA = *5-*
 854 *Hydroxyindoleacetic acid*; C – control (18°C, ambient pH), OA – ocean acidification (18°C, -0.33 pH unit),
 855 W – warming (21°C, ambient pH), OAW – ocean acidification and warming (21°C, -0.33 pH unit).



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858 Figure 6. Quality of gametes produced by conditioned adult oysters. For eggs (A-G): concentration,
 859 diameter, total fatty acid content, unsaturation index and essential fatty acid concentrations (AA, EPA,
 860 DHA). For spermatozoa (H-J): concentration, motility and velocity. Data are least square means \pm SE
 861 among tanks (n=3 or 6). Effects and *p*-values are shown when significant otherwise the mean is shown
 862 by the grey line when non significant (NS). Abbreviations: C – control (18°C, ambient pH), OA – ocean
 863 acidification (18°C, -0.33 pH unit), W – warming (21°C, ambient pH), OAW – ocean acidification and
 864 warming (21°C, -0.33 pH unit).

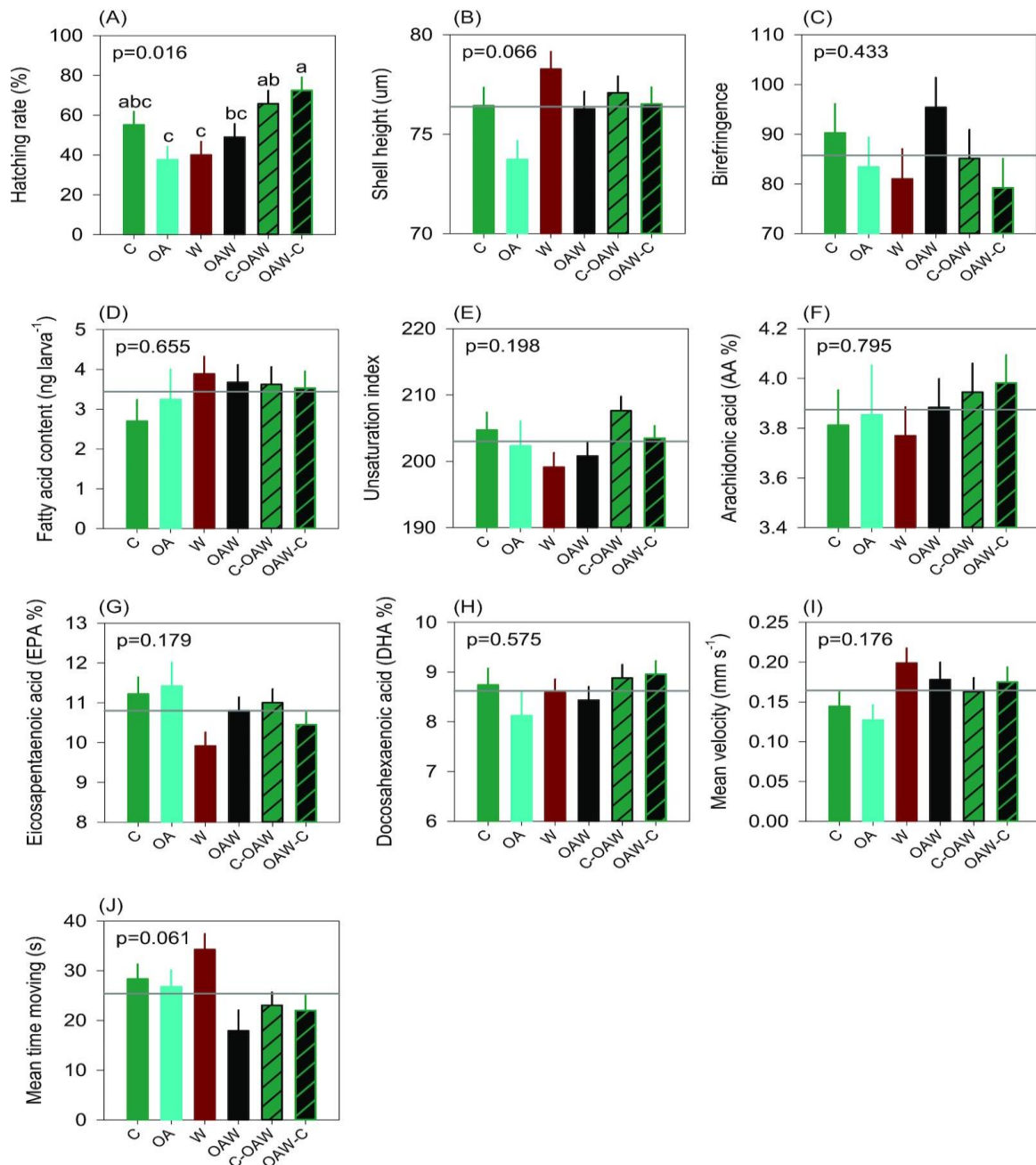


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868 Figure 7. Hatching rate, shell height, birefringence (greyscale score), total fatty acid content and
 869 essential fatty acid concentration (A-H), as well as velocity and motility (I, J). Data are least square
 870 means \pm SE among tanks (n=3 or 6). Effects and *p*-values are shown when significant otherwise the
 871 mean is shown by the grey line when non significant (NS). Abbreviations: C – control (21°C, ambient
 872 pH), OA – ocean acidification (21°C, -0.33 pH unit), W – warming (24°C, ambient pH), OAW – ocean
 873 acidification and warming (24°C, -0.33 pH unit).



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877 **Table 1** Parameters of the seawater carbonate chemistry over time during the adult conditioning and
 878 the larval exposure for each condition: salinity (S), temperature (T, °C), pH (pH_T, total scale), total
 879 alkalinity (A_T, μmol kg⁻¹), partial pressure of carbon dioxide (pCO₂, μatm), dissolved inorganic carbon
 880 concentration (C_T, μmol kg⁻¹), bicarbonate and carbonate concentrations ([HCO₃⁻] and [CO₃²⁻],
 881 respectively, μmol kg⁻¹), saturation state of the seawater with respect to aragonite (Ω_{ar}) and calcite
 882 (Ω_{ca}). Abbreviations: C – control, OA – ocean acidification, W – warming, OAW – ocean acidification
 883 and warming.
 884

Date	Condition	Parameters measured				Parameters estimated					
		S	T	pH _T	A _T	pCO ₂	C _T	[HCO ₃ ⁻]	[CO ₃ ²⁻]	Ω _{ar}	Ω _{ca}
Adult conditioning											
	C	34.3	18.1	7.99	2313.1	490.6	2100.0	1925.0	158.2	2.5	3.8
02/21/18	OA	34.3	18.0	7.72	2279.8	983.8	2182.5	2058.8	89.9	1.4	2.2
	OAW	34.3	21.0	7.68	2255.6	1071.9	2156.5	2031.2	91.4	1.4	2.2
03/02/18	C	34.7	18.0	7.97	2211.1	486.3	2009.8	1845.8	147.4	2.3	3.5
	OA	34.7	18.0	7.63	2288.9	1217.6	2219.2	2101.3	76.3	1.2	1.8
	OAW	34.7	21.0	7.61	2225.0	1272.5	2151.2	2033.3	77.8	1.2	1.9
48-h larval exposure											
	C	34.1	21.1	8.22	2276.9	249.0	1909.6	1643.9	257.8	4.1	6.2
03/26/18	OA	34.1	21.1	7.86	2272.4	684.8	2102.8	1949.4	131.8	2.1	3.2
	OAW	34.1	23.7	7.84	2367.4	736.6	2180.9	2013.1	146.1	2.3	3.5

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887 **Table 2** Number of adult oysters for each gonadal stage determined according to the reproductive
 888 scale of (Steele and Mulcahy 1999) and according to the four conditions. Abbreviations: C – control
 889 (18°C, ambient pH), OA – ocean acidification (18°C, -0.3 pH unit), W – warming (21°C, ambient pH),
 890 OAW – ocean acidification and warming (21°C, -0.3 pH unit).

Conditions	Gonadal stage				Total
	0	1	2	3	
C	0	3	5	19	27
OA	0	3	2	9	14
W	0	0	2	12	14
OAW	0	1	1	26	28

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