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European Lobster Larvae Development and Fitness Under a Temperature Gradient and Ocean Acidification

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13 Abstract

14 Climate change combined with anthropogenic stressors (e.g. overfishing, habitat destruction) may have particularly strong effects on threatened populations of coastal invertebrates. The collapse of the 15 16 population of European lobster (Homarus gammarus) around Helgoland constitutes a good example 17 and prompted a large-scale restocking program. The question remains if recruitment of remaining 18 natural individuals and program-released specimens could be stunted by ongoing climate change. We 19 examined the joint effect of ocean warming and acidification on survival, development, morphology, 20 energy metabolism and enzymatic antioxidant activity of the larval stages of the European lobster. 21 Larvae from four independent hatches were reared from stage I to III under a gradient of ten seawater temperatures (13-24°C) combined with moderate (~470 µatm) and elevated (~1160 µatm) seawater 22 23 pCO₂ treatments. Those treatments correspond to the shared socio-economic pathways (SSP), SSP1-24 2.6 and SSP5-8.5 (i.e. the low and the very high greenhouse gas emissions respectively) projected for 25 2100 by the Intergovernmental Panel on Climate Change. Larvae in CO₂ – acidified seawater had not only lower survival rates, but also significantly smaller rostrum length. However, temperature was 26 27 the main driver of energy demands with increased oxygen consumption rates and elemental C:N ratio 28 towards warmer temperatures, with a reducing effect on development time. Using this large 29 temperature gradient, we provide a more precise insight on the aerobic thermal window trade-offs of lobster larvae and whether exposure to the worst hypercapnia scenario may narrow it. This may have 30 31 repercussions on the recruitment of the remaining natural and program-released specimens and thus, 32 in the enhancement success of future lobster stocks.

33 1 Introduction

- 34 Since the industrial age, the burning of fossil fuels has led to an exponential increase in CO₂
- emissions and temperature. To understand the impact of climate change and potential risks, future 35
- scenarios have been developed by the Intergovernmental Panel of Climate Change (IPCC), which are 36
- 37 based on diverse degrees of mitigation efforts to decrease CO₂ emissions. The low mitigation
- 38 scenario SSP5-8.5, predicts that by the end of this century, sea surface temperature (SST) will have
- 39 increased by 2.58 °C, seawater surface pH will have decreased to 7.7; and atmospheric CO₂ could
- 40 have reached 1000 µatm. In contrast, the high mitigation scenario SSP1-2.6 corresponding mean 41 changes are +0.73 °C SST, reduction to pH 8.0 for surface seawater, and approximately 430-480
- 42 µatm CO₂ concentrations (IPCC, 2014; Pörtner et al., 2019; IPCC, 2021).
- 43 The combined effects of ocean acidification (OA) and warming on marine life have been studied for
- 44 at least two decades, but they are still challenging to interpret and predict. A growing number of
- 45 experiments using ecologically and economically important species, such as Pacific herring
- 46 (Villalobos et al., 2020), Pacific oysters (Lemasson et al., 2018), gilthead seabream, meagre
- (Pimentel et al., 2016), American and European lobster (Small et al., 2015; Waller et al., 2017) have 47
- shown an exacerbated impact of OA on survival, physiology, and growth when it was combined with 48
- 49 elevated temperatures. Synergistic impacts (the result of stressors interacting and producing a greater
- 50 effect than the cumulative or individual effects) of climate change vary across life stages with the
- 51 tendency that early life stages are more sensitive and less tolerant to environmental stressors than
- 52 adults (Kikkawa et al., 2003; Ishimatsu et al., 2004; Kurihara, 2008). Understanding the synergistic
- 53 effects of OA and warming on larval development is critical to predict how climate change will
- 54 influence species abundance, distribution and community structure (Cowen and Sponaugle, 2009; 55
- Giménez et al., 2020). This is particularly important for the future of commercially important and 56 vulnerable species, like crustaceans, which have complex life cycles and undergo distinct
- 57 ontogenetical changes. As in the majority of marine species with planktonic larvae, the transition
- 58 between the larval pelagic stage to the benthic post-larval stage larvae has been described as a
- 59 population bottleneck (Marshall and Morgan, 2011).
- 60 European lobster (Homarus gammarus) develops through three pelagic larval stages (stages I, II and
- III), a postlarval stage (stage IV) and then reaches the juvenile stage which marks the complete 61
- transition to a benthic lifestyle (Charmantier et al., 1991). The International Union for Conservation 62
- 63 of Nature has listed the European lobster as 'least concern' as the examination concluded that this
- 64 species has a broad geographic range, despite commercial fisheries. This is perhaps true on a global
- 65 level, but not for the lobsters of the German Bight, North Sea, that inhabit the rocky shores of the
- 66 island of Helgoland. This population experienced a dramatic decline in the 1950s and 1960s from a 67
- combination of overfishing, pollution and extensive habitat destruction (Franke and Gutow, 2004). 68 The decline of this population prompted a large-scale restocking program on Helgoland, presently
- 69 carried out by the lobster conservation company, Reefauna. After ten years (1999 – 2009) of
- 70 releasing hatchery-reared juveniles into the wild, the success of the restocking program was
- 71 evaluated; the results showed that re-stocked lobsters could be re-caught; survival rates averaged
- 72 40% and the proportion of caught cultured lobsters to wild lobsters was 3-8% between the years
- 73 2007-2009 (Schmalenbach et al., 2011). Nevertheless, despite recapture of marked lobsters, it is
- 74 unknown whether recruitment is successful. Most of the catches were older individuals and lobster
- 75 larvae are rarely caught in long-term plankton net monitoring around the island (Greve et al., 2004).
- 76 Therefore, the question remains if recruitment of remaining natural individuals and program-released 77
- specimens is currently successful, or could be affected by ongoing climate change (i.e. ocean
- 78 warming and acidification).

- 79 Only two studies have assessed the joint effects of OA and ocean warming on larvae of lobsters of
- 80 the genus *Homarus*. They provide the first insight on how lobsters may respond to the synergistic
- 81 effects of environmental changes predicted for the end of the 21st century (Small et al., 2015; Waller
- 82 et al., 2017). These studies have in common an experimental design based on only two temperatures
- and two pCO_2 regimes, comparing (in a factorial design) ambient temperature and pCO_2 conditions with increased temperature and pCO_2 . Both studies demonstrated that elevated temperature has a
- stronger effect on life history (survival and development) and physiological responses (oxygen
- 86 consumption rates) of lobster larvae than elevated pCO_2 . Nevertheless, it remains unknown how
- 87 lobsters will react to a broader range of temperatures under ocean acidification. Regional differences
- 88 from the global mean SST and CO₂ uptake trends can result in a "temperature buffering" effect.
- 89 possibly mitigating some of the negative impacts of OA. Therefore, as suggested by Humphreys
- 90 (2017), OA experimental setups should be combined with a thermal gradient to reflect regional
- 91 variation from the global mean SST more realistically.

92 How marine crustaceans will perform under future high CO₂ can be interpreted by their physiological 93 capacities to adjust to environmental change. Crustaceans are water breathers and are directly 94 exposed to ocean acidification through their gills which are specialized for respiratory gas and ion 95 exchange (Taylor and Taylor, 1992). An acute rise in seawater pCO_2 reduces (or reverses) the pCO_2 96 diffusion gradient across the gills, causing additional CO₂ to accumulate in the haemolymph 97 (extracellular compartment) until an excretory gradient is restored (Whiteley, 2011). Regulating 98 haemolymph pH is necessary to maintain proper oxygen supply, when the concentration of CO₂ in 99 the haemolymph increases and pH decreases it causes hemocyanin (oxygen transporting proteins) to 100 release their load of oxygen molecules as explained by the Bohr effect (Hirota et al., 2008; Strobel et 101 al., 2012). Nonetheless, crustaceans are equipped to buffer changes in haemolymph pH to some 102 extent through iono-regulation (Whiteley et al., 1999; Whiteley, 2011). However, acute and long-103 term exposure to OA could interrupt this acid-base equilibrium and alter metabolism and growth 104 (Whiteley and Taylor, 1992; Whiteley et al., 1999). Thus, routine metabolic rate (RMR) is 105 traditionally investigated in studies on multiple environmental stressors as an approach to assess if 106 elevated CO₂ concentrations affect the sensitivity of organisms to thermal extremes (Storch et al., 107 2011; Waller et al., 2017; Laubenstein et al., 2019). At optimal temperatures, organisms have 108 maximal aerobic capacity and proper functioning. (Pörtner, 2001). While at suboptimal temperatures 109 aerobic capacity is limited and failure to sustain a balance between metabolism, development and 110 growth can result in reduced body mass at critical life history stages (Anger, 2001; Pörtner, 2008; 111 Torres and Giménez, 2020). The suboptimal temperatures can be divided into the *pejus* range, where 112 an organism performance starts to decrease (Frederich and Pörtner, 2000; Frederich et al., 2009); and 113 the *pessimus* limit, when an organism switches from aerobic into anaerobic metabolism (Jost et al., 114 2012). Additionally, the energetic costs of maintaining proper functioning under acidic conditions 115 can further interrupt defense mechanisms against reactive oxygen species (ROS), leading to oxidative 116 stress and lipid peroxidation (Rato et al., 2017). Therefore, the decrease in antioxidant response and 117 consequent lipids' oxidative damage can serve as a proxy to identify when an organism's defense mechanism has been compromised or suppressed by environmental stressors (Beliaeff and Burgeot, 118 119 2002; Rato et al., 2017; Tremblay et al., 2020).

120 The purpose of our study was to provide a more complete picture on how European lobster larvae

121 will perform in future CO_2 conditions by including a wider range of temperature treatments. Our

- study investigated the ability of European lobster larvae to survive and develop successfully as well
- as their aerobic metabolic capacity when exposed to the projected SPP1- 2.6 scenario OA conditions
- and a temperature range that covers cold and warm suboptimal temperatures. Larvae from four

- 125 independent hatches were reared from stage I to III under a gradient of ten different seawater
- temperatures (13-24°C) combined with moderate (average ~470 μatm) and elevated (average ~1160
- 127 μ atm) seawater *p*CO₂ treatments (corresponding to the very stringent [SSP1-2.6] and worst-case
- 128 emission scenario [SSP5-8.5] projected for 2100 by IPCC).

129 **2** Materials and Methods

130 2.1 Animal Collection and Maintenance

131 The study was carried out at AWI Helmholtz-Zentrum für Polar- und Meeresforschung (Helgoland, 132 Germany). The experiment was repeated four times under the same temperature and light regimes 133 (12:12h light/dark), each experimental run was carried out with larvae from different females or 134 hatches, hereafter referred to hatches. Hatches typically vary due to maternal and environmental effects 135 prior to the hatching of larvae. Thus, pooling larvae together from different females could potentially 136 mask responses to the treatments. This is the reason why we chose to repeat the experiment with four 137 independent hatches to increase the robustness of the results. European lobster larvae (Homarus 138 gammarus) hatched during summer from four ovigerous female lobsters captured by local fishermen 139 in the rocky subtidal zone around the island of Helgoland (German Bight, North Sea, 54°11:3'N, 140 7°54.0'E). Females were fed by the lobster conservation company Reefauna and kept in separate tanks 141 (29 x 79 cm), filled with running seawater from the North Sea under a natural light cycle until hatching 142 occurred. Freshly hatched larvae were transferred to 60 X 800 mL glass beakers and were distributed evenly into four sections to minimize cannibalism. Two 100 cm² plastic meshes (mesh size: 500 µm) 143 144 were sewn in the middle and placed in the beaker to delimit these areas. The number of larvae was 15 145 per beaker for the first hatch and was adjusted to 12 afterwards for better survival. In total 2,880 lobster 146 larvae were used. The research presented in this paper complies with the guidelines from the directives 147 2010/63/ EU of the European parliament and of the Council of 22nd September 2010 and the German 148 law on the protection of animals used for scientific purposes.

149 2.2 Experimental Design and Seawater Parameters

150 A thermal gradient incubator similar to the one used by Thomas et al., (1963) was used for the 151 experimental setup. The table was built to hold 60 beakers (10 columns X 6 rows) and connected to 152 two cooling bath thermostats with engine coolant flowing through a closed system (HUBER 153 Compatible Control K6, Offenburg, Germany) that were set at 11.8°C and 27.8°C. A gradient of ten 154 temperatures was obtained in the horizontal axis (Supplementary Table 1). On the vertical axis, two 155 CO2 concentrations were set at target 450 µatm (SSP1-2.6 scenario) and 1150 µatm (SSP5-8.5 156 scenario) and supplied with gentle bubbling in three rows for a total of 30 beakers per CO₂ 157 concentration. Each beaker had a plastic hose with a glass tube extremity connected to a CO₂ 158 distributor. The targeted CO₂ levels were reached using a system that removes CO₂ from ambient air 159 with a soda lime filter. The CO₂-free air (< 1 μ atm CO₂) was mixed with pure CO₂ (Air Liquide 160 Deutschland ltd., Düsseldorf, Germany), and the pCO_2 of the mixture was continuously monitored with 161 a gas detection unit (GDZ 401, Umsitec, Denkendorf, Germany) that automatically adjusts the CO₂ 162 concentration and flow rates to maintain the target values. All beakers were covered by a clear plastic bag to limit CO₂ outgassing throughout the experiments. The setup resulted in a triplicate per 163 164 temperature and CO₂ concentration (see Supplementary Table 1). Lobster larvae were raised from 165 stage I to stage III under the temperature and pCO_2 conditions related to their position in the gradient table. Larvae were fed ad libitum (ca. 200-300 Artemia salina nauplii) after the daily water change at 166 167 9:00.

Seawater parameters were measured daily (n=1 for each combination of temperature CO2 168 169 concentration, using a pH meter (WTW pH315i, Wilheim, Germany) and pH electrode (WTW SenTix 21 Basis pH-combined electrode, Wilheim, Germany), salinometer (WTW Cond 3110 SET 1, 170 171 Wilheim, Germany) and salinity sensor (WTW, Conductivity Cells TetraCon), and thermometer 172 (VOLTCRAFT DET2R, Wernberg-Köblitz, Germany) (see Supplementary Table 1). Total alkalinity 173 (TA) was measured at the beginning and end of all experimental runs (n=2 for each combination of 174 temperature CO₂ concentration). For TA, water was sampled airtight in 100 mL bottles and stored at 175 4°C until later measurements with a TitroLine α plus titrator (SI Analytics GmbH [Xylem], Weilheim, 176 Germany) in technical duplicates with Dickson Batch 104 (NOAA, Reference material for oceanic CO₂ 177 measurements, 2010) as a standard. The seawater carbonate system was calculated based on measured 178 TA, temperature, pH, salinity and pressure using the CO₂SYS Excel Macro software (Pierrot et al., 179 2006). The following calculations were used, Mehrbach et al., (1973) refitted by Dickson and Millero 180 (1987) for the CO₂ constant, total scale (mol/kg-SW) for pH scale, Uppström (1974) for total boron 181 and Dickson (1990) for KHSO₄ to calculate the carbonate system. The obtained values are summarized 182 in Supplementary Table. 2, the mean values of pCO_2 treatments among all temperatures were 467

183 ± 19 for the moderate pCO₂ treatment and 1156 ± 27 for the high pCO₂ treatment.

184

185 2.3 Survival and Development Time

186 Lobster larvae in each beaker were monitored during the daily water change to record mortality and 187 dead larvae were removed immediately. Cumulative survival was expressed as the percentage of the 188 number of larvae introduced into each beaker at the start of the experimental run. To monitor 189 development, beakers were checked daily at 9:00 h for evidence of stage change (e.g molting). Larvae 190 were individually observed for stage characteristics, such as the formation of pleopods for stage II and 191 the formation of uropods for stage III. When larvae molted to stage III, they were removed from the 192 beaker for further measurements (see next sections). Sampling was divided into three groups once 193 larvae reached stage III: (1) three larvae from each beaker were sampled for RMR and afterwards 194 frozen for biomass, carbon and nitrogen measurements; (2) three larvae per beaker were photographed 195 for size and morphology analysis and (3) three larvae from each beaker were immediately frozen for 196 enzymatic antioxidants analysis. Each experimental run lasted approximately 28 days, to allow all 197 larvae in different temperatures treatments to reach stage III.

198 2.4 RMR Measurements

199 RMR was used as a proxy to investigate the effect of elevated pCO_2 and temperature on stage III larvae 200 metabolism. RMR measurements were done under the corresponding experimental temperature and 201 freshly prepared pCO_2 conditioned seawater. To make sure larvae were in a post-absorptive state, 202 larvae were starved for 2 h to allow gastric processing (Kurmaly et al., 1990; McGaw and Curtis, 2013) 203 in 20 mL glass vials implemented with an optically isolated oxygen sensor type PSt5 at its bottom 204 (PreSens, Regensburg, Germany). During this 2 h, vials were covered with a mesh to avoid larvae 205 escape and permit oxygen diffusion in the conditioned seawater. This period also allowed larvae to 206 recover from handling stress. After 2 h, vials were tightly closed with a plastic lid, while submerged in 207 the corresponding conditioned seawater in order to avoid air bubbles and placed on a SDR 208 SensorDish® Reader (PreSens, Regensburg, Germany). This system consists of a 24-channel reader of 209 oxygen luminescence quenching and provides a high-quality measurement without oxygen 210 consumption or gas exchange between the environment and the vial functioning as the incubation 211 chamber. The system was calibrated at each temperature with seawater at 100% and 0% air saturation

212 following the manufacturer's protocol. A 12-well microplate was adapted to the system to measure 213 simultaneously 12 glass vials (20 mL). Vials without larvae (n=2) were used as a control to account 214 for microbial oxygen consumption. Vials and channel readers were placed on a rocking platform shaker 215 (IKA Rocker 2D digital, Staufen, Germany) at 80 revolutions per minute (rpm) to avoid oxygen 216 stratification within the vials during measurement. The vials were incubated in the dark with an opaque 217 black plastic box. The oxygen concentration was recorded every 15 s during 4 h. Oxygen levels during 218 measurement were monitored closely to avoid suboptimal levels ($<4 \text{ mg} \cdot L^{-1}$) inside the chambers. 219 Oxygen consumption was determined by a linear regression of the change in O₂ concentration data plotted against time. After RMR was measured larvae were frozen for further biomass, carbon and 220 nitrogen measurements (see next section) to express RMR in O₂ mg•h⁻¹•mg DM⁻¹. RMR was measured 221 222 in postmolt larvae to allow comparison at all temperature treatments, as the intermolt period of larvae 223 is greatly dependent on temperature and is thus highly variable. Past studies measuring RMR in lobster 224 larvae show respiration rates are fairly consistent between intermolt and postmolt stage III larvae 225 (Sasaki et al., 2011).

225 (Sasaki et al., 2011).

226 2.5 Biomass, Carbon and Nitrogen Content

227 Freshly molted stage III larvae used for RMR were sampled for dry body mass, and carbon/nitrogen 228 content measurements. Carbon was measured as a proxy for reserves (lipid content) and nitrogen as a 229 proxy for protein content. The same parameters were measured in freshly hatched larvae (8-15 230 replicates per hatch) (see Supplementary Table 3). Larvae were rinsed gently with distilled water, 231 blotted dry to remove salts and excess water and stored in 1.5 mL microcentrifuge tubes at -20°C for 232 later analysis. For the analysis, larvae were placed in pre-weighed zinc cartridges (8 x 11 mm, 233 LabNeed, Germany), then freeze-dried for 48 h (Christ Alpha 1-4 freeze dryer, Germany) and 234 afterwards weighed to the nearest 0.0001-mg using a microbalance [Sartorius SC2, Germany]). Carbon 235 and nitrogen contents were then measured using an element analyzer (vario MICRO cube CHNS 236 analyzer, Elementar Analysensysteme, Germany).

Dry mass (DM) was measured in freshly hatched larvae (8-15 replicates per hatch) to calculate
 instantaneous growth. Instantaneous growth rate was calculated as:

239
$$g = \frac{\log \left(\frac{DM_f}{DM_0}\right)}{T}$$

In this formula DM_f is the corresponding dry mass value at stage III, DM_0 is the dry mass value at hatching, and *T* is the development time from hatching to stage III. Total production was calculated as an additional parameter to investigate fitness of larvae and was calculated as the number of survivors to stage III in each treatment multiplied by the corresponding dry mass.

244 2.6 Morphological Measurements

As a proxy to assess possible malformation under high pCO_2 , as seen in a study on the effect of OA on lobsters (Agnalt et al., 2013), we measured eight morphological traits. Stage III larvae were placed laterally in a Petri dish and photographed using an Olympus SZX16 stereo microscope. Pictures were then analyzed using ImageJ Software (ImageJ 1.45s, National Institute of Health, Madison, WI, USA). Eight morphological characteristics were measured following the protocol of a similar study in American lobsters (Homarus americanus) (Menu-Courey et al., 2019): (1) rostrum length (RL), (2)

251 carapace length (CL), (3) total length (TL), (4) telson length, (5) the dominant claw pollex, (6) the

252 dactylus, (7) the eye diameter, which consisted of measuring the dark area. The abdomen length was calculated as the difference between TL and the sum of RL and CL. 253

254 2.7 **Antioxidant Enzyme Activity**

255 Stage III larvae were sampled and immediately snap-frozen in liquid N₂ and kept at -80°C until 256 analyzed. To determine the level of cellular stress larvae experienced under experimental conditions 257 and the mechanisms involved in the response, three antioxidant enzymes were analyzed in technical 258 triplicates: superoxide dismutase (SOD), glutathione S-transferase (GST), glutathione peroxidase 259 (GPx) and catalase (CAT). Each individual was cut into two pieces below the carapace and ground in 260 liquid N₂ using a ceramic pestle. The front part (carapace) was used for antioxidant enzymes. We aimed to quantify lipid damage using the abdomen part of the larva via malondialdehyde (MDA) 261 262 formation, but these data were discarded as they were mostly under the detection level. For the 263 enzymes' analysis, the samples were transferred to microcentrifuge tubes with 125 µl of phosphate 264 buffer solution [50 mM potassium phosphate dibasic and monobasic mixture (K₂HPO₄/KH₂PO₄, 265 30.5% and 19.5% respectively), 50 mM Ethylenediaminetetraacetic acid (EDTA), 1 mM 266 phenylmethanesulphonyl fluoride, pH 7.5], homogenised using a laboratory ball mill (MIXER MILL 267 MM 400, Retsch, Haan, Germany) and centrifuged at 23,897 g for 3 min at 4 °C to obtain the 268 supernatant used for the assays. SOD catalyses the conversion of O_2^{-1} to H_2O_2 and was measured using xanthine-xanthine oxidase as a superoxide radical generating system and nitroblue tetrazolium 269 270 as a detector (Suzuki et al., 2000). GST modifies xenobiotics into other conjugates using reduced 271 glutathione (GSH) as substrate, and was estimated by detecting the formation of the thioether product from the reaction between GSH and 1-chloro,2,4-dinitrobenzene (Habig and Jakoby, 1981). GPx 272 273 removes H₂O₂ using nicotinamide adenine dinucleotide phosphate (NADPH) as substrate and was 274 measured by monitoring the decrease in the concentration of NADPH at 340 nm upon addition of 275 H₂O₂ to the assay mixture (Ahmad and Pardini, 1988). CAT eliminates H₂O₂ too and prevents its accumulation in cells and tissues. The decrease of the H₂O₂ concentration catalyzed by CAT was 276 277 measured at 240 nm according to Aebi, (1984). Soluble protein was also measured as per Bradford 278 (1976) in all supernatants to obtain enzyme activities expressed in activity units (U)•mg protein⁻¹. All 279

- spectrophotometric measurements were done at room temperature (20 °C) using a spectrophotometer
- 280 (THERMO Multiskan Spectrum, Waltham, USA).

281 2.8 **Data Analysis**

282 After data visualization, statistical analyses of the defined variables were performed in RStudio

- 283 (2021). Generalized additive models (GAM) with random effects using the package mgcv (Wood,
- 284 2017) were done with temperature and CO_2 concentration as fixed factors, plus the addition of the
- 285 hatch as a random factor (specified as: *s(hatch, bs= "re")*) for all measured variables: survival,
- 286 development time, morphological measurements, RMR, biomass, and antioxidant enzyme activities.
- 287 The best model (interactive, additive, temperature and CO₂ only, null model) was then chosen based
- 288 on the Akaike information criterion (AIC) score and simplicity of the model (Supplementary Table
- 289 4). Line graphs were plotted using the smoothing command from the package *mgcv* (Wood, 2017)
- 290 with the predicted regression line in ggplot2 (Wickham, 2016). The lines are the predicted regression
- 291 lines: solid black lines were plotted when there was a temperature effect, but no effects of OA. Red
- 292 and blue lines were plotted when there was a temperature and CO₂ additive effect; the red and blue
- 293 dots represent each sampled larva under high or moderate pCO₂ conditions respectively.
- 294 Additionally, a multivariate analysis using a principal component analysis (PCoA) was used to

- visualize morphological measurements by CO₂ and temperature treatments and permutational
- 296 multivariate analysis of variance (PERMANOVA) to test significance.

297 **3 Results**

We did not observe any evidence of a synergistic effect of high temperature and high pCO_2 in any of the studied variables. Statistically this means that in no case the model including interactions between

- the studied variables. Statistically this means that in no case the model including interactions between temperature and pCO_2 concentration provided a better fit to the data than the models with the two main
- 301 factors alone. In general, most variables were affected by temperature and effects of pCO_2 (when
- 302 present) were additive with respect to temperature. We present our results as the average response of
- 303 larvae under each experimental condition (pCO_2 and temperature) and hatch for better visualization.
- 304 For results separated by hatch see **Supplementary Figures 1 to 5**.

305 3.1 Survival and Development Time

We observed evidence of a negative effect of high *p*CO₂ on larval survival (**Supplementary Table 4**)

- 307 but not on development time to reach stage III. At higher temperatures the differences in average 308 survival between CO₂ treatments were small compared to lower temperatures. However, the best model
- did not retain a term indicating that smooths are conditional on the CO_2 level. Overall, mean survival
- for all temperatures under moderate pCO_2 was 33.3% in comparison to 27.9% for high pCO_2 .
- 311 Moreover, survival increased with temperature while duration of development time to reach stage III
- decreased(Figure 1).

313 **3.2** Biomass and Carbon and Nitrogen Content

Best models retained temperature but not pCO_2 as predictors (Figure 2; Supplementary Table 4). Dry mass, carbon and nitrogen content, and C:N ratio increased with temperature. Instantaneous growth also increased with temperature but there was no evidence of an effect of pCO_2 (Supplementary Figure 6). Temperature and pCO_2 had an additive effect on total production (Figure

- 318 7). This result matches the trend and significance seen in the survival results.
- 319

320 3.3 Morphological Measurements

321 Best models retained both pCO_2 and temperature for predictors of rostrum length (Figure 3; 322 Supplementary Table 4). Lobster larvae in high pCO_2 treatment had, on average, shorter rostrum 323 length than those in moderate pCO_2 . Rostrum length (RL) increased with increasing temperature in 324 both CO₂ treatments. For the remaining variables, only temperature was retained in the best model 325 (Supplementary Table 4). Carapace length (CL), abdomen length (AL), total length (TL) and claw 326 size increased with temperature (Figure 3; Supplementary Table 4). By contrast, the CL:AL ratio 327 decreased with temperature (Figure 3; Supplementary Table 3). Neither temperature nor pCO2 were 328 retained as predictors for eye diameter size and telson length. Multivariate analysis using measured 329 morphological characteristics (RL, CL, AL, TL and telson) did not give any significant morphological 330 difference between larvae under moderate and high pCO_2 . Temperature had a significant effect on 331 larval morphology in the colder temperatures (Figure 4; PERMANOVA test: F_{1, 165}= 7.37, p=0.003).

332 3.4 Routine Metabolic Rate Measurements and Antioxidant Enzyme Activity

333 The routine metabolic rate (RMR) increased with temperature (Figure 5; Supplementary Table 4).

However, we did not find any evidence of an effect of OA (i.e. the best model contained only

temperature as predictor). Best models did not retain temperature nor CO₂ as predictors for variation in

antioxidant activity of the enzymes SOD, GST, GPx and CAT(Figure 6).

337 4 Discussion

338 Contrary to our expectations, we did not find any evidence of synergistic or interactive effects of 339 temperature and pCO_2 on any of the studied response variables. Ocean warming and acidification 340 impacts can range from the highest level of sensitivity seen in the whole organism functioning, down 341 to the cellular and molecular levels (Pörtner, 2008). We accordingly discuss our results from whole 342 body functioning (i.e. survival, growth), to energy metabolism and finally to antioxidant responses. 343 Our findings demonstrate that future high CO₂ concentrations could have an impact on survival and 344 morphology of lobster larvae. However, at the organizational levels analysed here, we did not detect physiological responses in lobster larvae. The latter will be discussed below. 345

346 4.1 Survival

347 Our results suggest that near-future pCO_2 conditions have a negative effect on lobster larvae survival to stage III. In lobster larvae, a bottleneck is expected in the transition between the last larval pelagic 348 349 stage (stage III) and the benthic stage IV; the stage III of *H. gammarus* is the one that accumulates 350 most of the body mass leading to the stage IV (Torres et al., 2021). Such bottlenecks are important as 351 life history transitions (Giménez, 2004; Pechenik, 2006; Marshall and Morgan, 2011). Our study 352 clearly showed OA has the potential to increase mortality before reaching the transitional metamorphic 353 stage (stage III to IV). In the wild, this could translate to an additional obstacle for successful 354 recruitment. Similarly, reduced survival due to elevated pCO_2 has been observed in early life stages in 355 the European lobster (Small et al., 2016) and in the congeneric American lobster (Menu-Courey et al., 356 2019; Noisette et al., 2021). Moreover, this increase in mortality in early life stages of crustaceans 357 exposed to ocean acidification has been noted in several other species: e.g. red king crab, Paralithodes 358 camtschaticus (Long et al., 2013), edible crab, Cancer pagurus (Metzger et al., 2007) and the porcelain 359 crab, *Pethrolisthes cinctipes* (Ceballos-Osuna et al., 2013). Previous studies on crustaceans and thermal 360 stress revealed elevated pCO_2 can narrow the thermal tolerance of the edible crab, C. pagurus and the spider crab, H. araneus (Metzger et al., 2007; Walther et al., 2009; Whiteley, 2011). Our survival 361 362 results provide no evidence of larvae reaching a temperature threshold or *pessimus* range on the warm side of our gradient with a maximum temperature (24 °C) under high pCO_2 . However, on the cold side, 363 364 the low number of lobster larvae that reached stage III at 13 and 14 °C in both moderate and high pCO₂ 365 treatments suggest the *pessimus* survival limit is below 15 °C. The lack of an interaction shows though 366 that the limits were not affected by OA, in contrast to previous observations (stated above) and 367 Pörtner's (2008) predictions. The other significant driver for survival was temperature: higher 368 temperatures resulted in higher survival in both moderate and high pCO_2 treatments. We observed 369 similarities with a study carried out in the same region (Helgoland) on the effect of climate warming on European lobster larvae (Schmalenbach and Franke 2010). Their results showed that optimal larval 370 survival occurred within 16 to 22 °C which largely matches our results; we also tested warmer 371 372 temperatures (23 °C and 24 °C) where survival was even higher.

373 4.2 Growth: Development Time, Biomass, Carbon and Nitrogen Content

There was no evidence of an effect of elevated pCO_2 on development time from hatching to stage III. Our results are consistent with studies focusing on temperature only (Schmalenbach and Franke, 2010)

376 as well as pCO₂ and temperature (Arnold et al., 2009; Small et al., 2015; Waller et al., 2017) where 377 pCO_2 had no effect on lobster larval development rate. This led us to further enquire if there was 378 possibly a trade-off between slower development rate under pCO_2 . For instance, at moderately low 379 salinities, larval development of *H. gammarus* is extended, possibly as a way to minimize the negative 380 effects on lipid and protein levels (Torres et al. 2021). Studies on the combined effect of food limitation 381 and increased temperatures have analyzed the integrated response of dry mass and development under 382 different temperatures (Torres and Giménez, 2020; Griffith et al., 2021) to provide insight if delayed 383 development time could be a compensatory response to maintain body mass (and reserves) at stage. 384 We investigated this integrated response, and our results show there was no trade-off between 385 developing slower under elevated pCO_2 conditions as larvae were reaching similar biomass when 386 molting to stage III (Figure 8). Temperature alone was the principal driver in development rate, lobster 387 larvae in warmer temperatures molted to stage III faster independently of pCO_2 treatment. We did not 388 find any evidence of effects of pCO2 on dry mass and elemental carbon (C) and (N) content, either; 389 thus larvae grew to the (thermal dependent) maximum body mass without any need of extending 390 development. If present, the compensatory responses to increased pCO2 levels operated at a different level of organization, potentially at the intracellular level through acid-base balance mechanisms 391 392 (Whiteley et al.2011, 2018).

393 There was an increase in biomass and C and N content with increasing temperature, consistent with

the increased survival. In crustacean larvae, carbon content (approximately >35% of biomass) is

395 correlated with lipid content (a proxy for accumulation of reserves), while nitrogen (approximately 8-

11% of biomass) reflects the protein content (Anger and Harms, 1990; Anger, 2001; Torres and

397 Giménez, 2020). Biomass can also be divided into composition of proteins, lipids, chitins and free

carbohydrates, making up >30%, <20%, <15%, and <5% respectively (Anger and Harms, 1990;
Anger, 1998). However, chitin is mainly associated with cuticle formation and plays a minor role in

400 the accumulation and utilization of energy reserves. Likewise, carbohydrates are stored as glycogen

401 and for the synthesis of non-essential amino acids and thus have no significant influence on the C:N

402 mass quotient (Anger and Harms, 1990).

403 The effect of temperature on biochemical composition in crustaceans has been studied in both field 404 and controlled laboratory conditions. Field experiments showed that temperature changes, related to 405 seasonal variation, can influence biochemical composition of decapod crustaceans (Buckup et al., 406 2008; Urzúa and Anger, 2013). Whereas laboratory experiments demonstrated that an increase in 407 temperature led to an augmentation in lipid content in adult male whiteleg shrimps (Litopenaeus 408 vannamei) (Perez-Velazquez et al., 2003) and a decrease in protein content in the northern shrimp 409 larvae (Pandalus borealis) (Brillon et al., 2005). Our results are in line with previous results 410 reporting higher lipid content in cherry shrimp (Neocaridina heteropoda heteropoda) at 24 °C 411 (Tropea et al., 2015). However, the same authors noted a decrease in lipid concentrations at 28 °C 412 and 32 °C, which we did not see with our experimental design. We did not explore extremely high 413 temperature where consumption rates of lipids (reflected in a decrease in carbon content) could have 414 increased due to increased energy demands. More specifically, studies on American and European 415 lobsters have shown varying results that are challenging to compare due to the difference in 416 temperature treatments and life stages. A study done by Small et al., (2016) on juvenile European 417 lobster acclimated at 10 °C and 13°C showed carbon content decreased and nitrogen increased in the 418 warmer temperature treatment. Another experiment done on stage I to stage IV European lobster 419 larvae reared at 17 °C and 21 °C found no significant effects of elevated temperature on organic 420 content (Small et al., 2015). Additionally, an experiment done on OA and warming on American 421 lobster stage III larvae (Waller et al., 2017) showed higher C:N ratio at 19 °C compared to 16 °C.

422 However, this difference was not seen in stage I, II or IV larvae. Our experiment similarly saw an

423 increase in C:N ratio in stage III larvae at higher temperatures.

424 The high dry mass, and C and N content matches the energetic demands for rapid growth, accelerated

- 425 cellular mechanisms and bigger size in warm temperatures. Because C:N ratios were lower towards
- 426 the lower end of the temperature range, we hypothesize that such low temperatures limited the rate of 427 accumulation of lipids more than that of proteins. Lower changes in proteins than in lipids has also
- 428 been found when larvae are exposed to low salinity (Torres et al., 2002, 2021). In contrast, on the
- 429 warmer side an increase in C:N ratio can be indicative of protein degradation due to high metabolism
- 430 (Weiss et al., 2009). Thus, the decrease seen in C could be related to a degradation of lipids due to
- 431 extra energetic requirements and a decrease in N could translate to a shortage in protein (Anger and
- 432 Harms, 1990).
- 433

434 **4.3 Size and Morphology**

435 Evidence of an effect of high pCO_2 on size and morphology was found only for rostrum size, larvae 436 exposed to high pCO_2 showing shorter rostrum length than those in the high pCO_2 treatment. The 437 rostrum is the region which protects the eyes (Ingle, 1997), a reduced rostrum could potentially lead to 438 eve damage; and proper eye-functioning is necessary for localizing prev and predators (Wahle, 1992). 439 Consequently, we measured the eye diameter, as a proxy to assess if there was any correlation with 440 nervous system underdevelopment (Letourneau, 1976; Laverack, 1988; Beltz and Sandeman, 2003) in 441 elevated pCO_2 . Nevertheless, despite larvae having smaller rostrum in the high pCO_2 there is no 442 evidence that the eye size was influenced by high pCO2 or high temperatures. Additionally, we 443 checked for "puffy" carapace, bent rostrum, and telson deformities, as seen in past studies of juvenile 444 H. gammarus exposed to increased pCO₂ at cold (12 °C) and optimal temperatures (18 °C) (Agnalt et 445 al., 2013). Our results show only one case of a puffy carapace in larva under high pCO_2 at 24 °C. 446 Lobster larvae reduced size under high pCO_2 has been observed before (e.g. in carapace length (Keppel 447 et al., 2012; Rato et al., 2017). Conversely, a study on H. americanus found a positive correlation 448 between elevated pCO_2 and carapace and abdomen length (Menu-Courey et al., 2019). We believe the 449 effects on size observed in our study are not as strong as the study by Menu-Courey et al., (2019) and 450 Noisette et al., (2021) possibly because the pCO_2 gradient treatments used in those studies reached 451 higher concentrations (1200, 2000 and 3000 µatm) than ours. Furthermore, our study only focused on 452 larval stages and decapod larvae exoskeletons are unmineralized while those of benthic juveniles are 453 partially calcified (Anger, 2001). This lack of calcification may be the reason why elevated pCO_2 did 454 not have a stronger impact on the size of the larvae's different body parts.

455 4.4 Routine Metabolic Rate and Antioxidant Enzyme Activity

Temperature is one of the most important factors influencing routine metabolic rates (RMR) in lobsters and other decapods (McLeese, 1964). Our results show that lobster larvae RMR increased linearly with increasing temperature, independent of pCO_2 treatment. Our findings do not show an exponential relationship between RMR and temperature because this pattern is typically seen in standard and maximal metabolic rate measurements. Moreover, the results are highly dependent on the acclimation of animals, the larva in our experiments were acclimated to a certain temperature and then respiration rate was measured at the same temperature. Exponential increase of oxygen consumption with temperature is usually seen and obtained with a different methodology (Schulte et

463 consumption with temperature is usually seen and obtained with a different methodology (Schulte et 464 al., 2011). A different approach where RMR is measured at acute temperatures independent of the

465 temperature treatment larvae were acclimated to, could have help identify bottlenecks in cell

- 466 functionality derived from the compensation costs provoked by combined higher temperature and
- 467 pCO₂ treatments. However, the design of our experiment and sample size did not allow for this kind
- 468 of approach. The "Temperature Induced Metabolic Rate" method could be tested on lobster larvae in
- the future, as it is suitable for studying the effects of temperature on the metabolic capacities of non-
- 470 constantly swimming organisms (Paschke et al., 2018). For this standardized method, the researcher
 471 is required to evaluate critical thermal maximum (CT max) and critical thermal minimum (CT min)
- 471 is required to evaluate critical inermal maximum (C1 max) and critical inermal minimum (C1 min) 472 to set the measurement temperature for high and low metabolic rates at each acclimation temperature
- 472 to set the measurement temperature for high and low metabolic r 473 to calculate an aerobic budget.
 - 474

475 The higher RMR at warmer temperatures can be associated with faster development rate and larger 476 size. In physiology, the cost of growing faster comes at the expense of an increase in feeding rates and 477 thus swimming to catch food. These activities have been suggested to be energetically expensive in 478 planktonic crustaceans (Morris et al., 1985). Our results are in line with previous studies on early life 479 stages of Homarus sp. (Small et al., 2015; Waller et al., 2017; Menu-Courey et al., 2019), northern shrimps, Pandalus borealis (Arnberg et al., 2013), and juvenile porcelain crabs, P. cinctipides (Carter 480 481 et al., 2013), where pCO_2 did not significantly affect respiration rates. Significant oxidative stress 482 responses would allow us to infer with more certainty on the optimal, pejus and pessimus ranges of 483 lobster larvae. However, without evidence of significant antioxidant response fluctuations, it is difficult to separate *pejus* and *pessimus* ranges. From the oxygen consumption point of view and survival alone, 484 the optimal range would be temperatures between 17-24 °C as lobster were able to use their energy 485 486 supply to maintain maximal physiological functions. Helgoland's European lobster larvae appear to be 487 quite tolerant to temperatures above those found in the German Bight (Schmalenbach and Franke, 488 2010).

489 On the cold side of our temperature gradients, our results show suboptimal temperatures under 15 °C,

490 expressed in low survival rates and low RMR. These temperatures are unusual for summertime in
491 Helgoland when lobster larvae hatch (Schmalenbach and Franke, 2010). Nevertheless, temperatures

492 recorded at the Helgoland long-term sampling indicate temperature increases are most noticeable

- 493 during winter (Franke et al., 1999; Wiltshire et al., 2008). Experimental evidence shows winter
- 494 warming (+3 °C) can alter larval recruitment and result in lobster larvae hatching earlier, mid-April
- instead of mid-June. In the wild, lobster larvae could be faced with suboptimal temperatures thatcould cause the lengthening of development time in the pelagic stage, thus increasing the danger of
- 497 mortality through predation (Schmalenbach and Franke, 2010).

498 Enzymatic antioxidant responses were measured for the first time in lobster larvae exposed to 499 multiple environmental stressors. To date there is only one study by Rato et al., (2017) that analyzed 500 the biochemical responses of H. gammarus under acidification alone and highlights the occurrence of 501 oxidative stress. They found out lobster larvae under high pCO₂ (710 µatm) had reduced SOD and 502 higher DNA damage. Our study included variables not measured before under OA and thermal stress, 503 such as the enzyme activity of GST, GPx and CAT. However, there was no evidence that OA and 504 temperature had a negative impact on the antioxidant enzyme activity. We recognize further deeper 505 investigation at the molecular level (proteomic or transcriptomic) could reveal further information on 506 the processes lobster larvae go through to cope with elevated pCO_2 (Noisette et al., 2021). For 507 instance, Noisette et al. (2021) findings show elevated pCO_2 (up to 3000 µatm) did not have an effect 508 on larvae at a physiological level, however, there is evidence they underwent intensive metabolic

509 reprogramming.

- 510 In conclusion, European lobster larvae demonstrated to be resilient to near future pCO_2
- 511 concentrations at temperatures beyond 17–18 °C, including higher temperatures than those
- 512 experienced by the local population. Our results show larvae do not appear to have reached the
- 513 critical temperatures or pejus range under the elevated temperatures tested (23-24 °C). Raising the 514 temperature even further and reaching the thermal limit of lobster larvae would have been interesting
- 514 temperature even further and reaching the thermal limit of lobster larvae would have been interesting 515 from a physiological point (e.g. higher antioxidant responses and compromised respiration).
- 516 However, for the purposes or our research question, we wanted to understand how lobster larvae will
- 517 cope with the predicted SSP5-8.5 scenario for 2100 in which SST will increase by 2-3 °C. We
- 518 observed no interactive effect of temperature and pCO_2 on the measured variables; temperature was
- the greatest driver and there was an additive effect of CO_2 and temperature on survival and size.
- 520 Examining the results from the perspective of different levels of biological organization, even though
- 521 pCO_2 did not elicit a response at the cellular level (i.e. enzyme activity) or physiological level (i.e.
- development time to reach stage III); at the population level (survival) there were significant negative
 effects. We used total production (survival times biomass) as a way to integrate physiological and
- 524 population responses, and it was evident there was an increase in mortality in larvae exposed to high
- pCO_2 accompanied with lower biomass in the suboptimal temperatures (<15 °C). Integrating
- 526 physiological responses to environmental stressors and life history traits is key for species
- 527 conservation strategies and stock enhancement management. As worst-case climate change scenarios
- 528 could potentially have repercussion on ongoing restock efforts of endangered populations under
- 529 recovery, like the European lobster population of Helgoland.

530 5 Conflict of Interest

531 This study received funding from the Business Development and Technology Transfer Corporation

532 of Schleswig Holstein (WT.SH). All authors declare no other competing interests.

533 6 Author Contributions

534 MB acquired funding for the experiment. RK provided resources. All authors were involved in

experiment design, LL and NT carried out the experiment. LL, NT and LG analyzed the data. NT, MB,
GT and LG supervised the project. LL wrote the first draft of the manuscript; all authors revised the

537 manuscript.

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- 817 **10 Figures**

818 FIGURE 1 | Effect of temperature and ocean acidification on the survival and development time 819 of *Homarus gammarus* larvae to stage III. (A) survival was influenced by CO₂ and temperature, (B) 820 development time was influenced by temperature. Each point represents the average response 821 quantified in larvae originated from the same female. Curves correspond to smooths fitted with the best 822 (general additive) model, obtained after backwards model selection.

FIGURE 2 | Effect of temperature on biomass of stage III *Homarus gammarus* larvae. (A) dry
mass, (B) carbon content, (C) nitrogen content and (D) C:N was positively correlated with temperature.
Each point represents the average response quantified in larvae originated from the same female.
Curves correspond to smooths fitted with the best (general additive) model, obtained after backwards
model selection.

828 FIGURE 3 | Effect of temperature and ocean acidification on size and morphology of stage III

829 Homarus gammarus larvae. (A) ocean acidification and temperature effect on rostrum length; and

temperature effect on (B) carapace length [CL], (C) abdomen length [AL], (D) total length, (E) CL:
AL ratio and (F) claw length. Each point represents the average response quantified in larvae originated

from the same female. Curves correspond to smooths fitted with the best (general additive) model,

833 obtained after backwards model selection.

FIGURE 4 | Results of principal coordinates analyses (PCoA) for morphological traits of stage III *Homarus gammarus* larvae. Plots were made using rostrum length, carapace length, abdomen length, total length and telson size. (A) comparison by temperatures (13-24°C), significant differences among temperatures. (PERMANOVA test: $F_{1, 165}=7.37$, p=0.003). (B) comparison by pCO₂ concentrations, no significance differences among pCO₂ concentrations.

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840 FIGURE 5 | Effect of temperature on the routine metabolic rate of stage III *Homarus gammarus*

841 larvae. Each point represents the average response quantified in larvae originated from the same

842 female. Curves correspond to smooths fitted with the best (general additive) model, obtained after

- 843 backwards model selection.
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845 FIGURE 6 | Effect of temperature and ocean acidification on antioxidant enzyme activities of

846 stage III Homarus gammarus larvae. There was no effect of experimental conditions (temperature

gradient and pCO₂ levels) on antioxidant response: (A) SOD, (B) GST, (C) GPx and (D) CAT. Each

- point represents the average response quantified in larvae originated from the same female. Curves
- 849 correspond to smooths fitted with the best (general additive) model, obtained after backwards model
- 850 selection.

- 851 FIGURE 7 | Additive effect of temperature and CO₂ on total production of stage III *Homarus*
- 852 gammarus larvae for the four hatches analyzed. Panels 1 to 4 respectively.
- 853 FIGURE 8 | Integrated response between development time and drymass. The triangle and (-)
- 854 sign represent the moderate pCO_2 treatment and the circle and (+) sign the high pCO_2 treatment.
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