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Functional spatial contextualisation of the effects of multiple stressors in marine bivalves

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Abstract. Many recent studies have revealed that the majority of environmental stressors experienced by marine organisms (ocean acidification, global warming, hypoxia etc.) occur at the same time and place, and that their interaction may complexly affect a number of ecological processes. Here, we experimentally investigated the effects of pH and hypoxia on the functional and behavioural traits of the mussel *Mytilus galloprovincialis*, we then simulated the potential effects on growth and reproduction dynamics trough a Dynamic Energy Budget (DEB) model under a multiple stressor scenario. Our simulations showed that hypercapnia had a remarkable effect by reducing the maximal habitat size and reproductive output differentially as a function of the trophic conditions, where modelling was spatially contextualized. This study showed the major threat represented by the hypercapnia and hypoxia phenomena for the growth, reproduction and fitness of mussels under the current climate change context, and that a mechanistic approach based on DEB modelling can illustrate complex and site-specific effects of environmental change, producing that kind of information useful for management purposes, at larger temporal and spatial scales.

Key-words: Acidification; Climate change; DEB Model; Hypoxia; *Mytilus galloprovincialis*; Multiple-Stressor; Mussel.

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1 Introduction

Since the dawn of research investigating the possible effects of ocean acidification (OA) on aquatic organisms (e.g. Bamber, 1990), most studies have shown that elevated pCO_2 levels, as predicted for the next century, may affect to some extent the functional traits (Schoener, 1986; Koehl, 1989) of marine organisms (Feely et al., 2004; Navarro et al., 2013). Referring to functional traits, we consider all those specific traits that define each species in terms of their ecological roles (Diaz & Cabido, 2001), and thereby the species' identity. In marine ectotherms such as bivalves, crabs, sea urchins and fish, these traits include tolerance and sensitivity to environmental conditions (e.g. physiological tolerance limits - Kearney & Porter, 2009) defining the ability of each species to support their own metabolic machinery (Sokolova et al., 2012; Sarà et al., 2014), the ability to obtain energy from food, the so-called functional response (Holling, 1959) or those behavioural (e.g. swimming behaviour, habitat use, mating system) and morphological (e.g. shape, thickness) traits (Schoener, 1986) which led to optimise the energetic income (Krebs & Davies, 1992) and lastly to reach the ultimate fitness (Roff, 1992). Research performed over the last decade and summarized in the recent IPCC report (IPCC, 2014) clearly shows that ocean acidification will affect marine organisms and ecosystems (Connell et al., 2017) in the coming decades, and such projections have stimulated new research that aims to understand the impact on calcifying marine organisms. Reductions in growth and calcification rates are just those kinds of the physiological impacts of ocean acidification (Thomsen et al., 2013; Byrne, 2012; Beniash et al., 2010). While much research showed that low pH may impair most functional traits, functions connected with energy uptake such as feeding and assimilation seem to be reduced at a lager extent in many species with expected implications for the amount of energy available for growth and reproduction (Kurihara et al., 2008; Appelhans, 2012; Navarro et al., 2013; Zhang et al., 2015). Such information has been obtained through both acute and chronic exposure to OA but no studies are yet available to assess the potential effects of OA on the magnitude of other Life History (LH) traits, such as maximum habitat body size, fecundity, time to reach maturation and the number of spawning events under future conditions of environmental change (*sensu* Kearney and Porter, 2009; Sarà et al., 2011; 2013b). To obtain such LH traits, experiments should be long enough to assure a functional effect of lower pH for many weeks or months but probably no existing lab mesocosm could currently assure the stability of seawater acidification system for such a long time. Thus, apart from long term experiments carried out in few field sites worldwide (e.g. Ischia [Hall-Spencer et al., 2008] and Vulcano [Duquette et al., 2015] islands) in the Southern Mediterranean Sea and in other Seas (Maug Island [Pala, 2009] or CO2 vents in the southwest Pacific [Connell et al., 2017]) where lowered pH seawater is naturally available through CO_2 natural emissions from vents, the

recent introduction of mechanistic functional trait-based (FT) models based on the Dynamic Energy Budget theory (DEB; Kooijman, 2010) can offer a reliable opportunity for disentangling the effect of seawater acidification on LH traits. The novelty of the FT-DEB approach relies on its intrinsic mechanistic nature deriving from the fact that it is based on flux of energy and mass through an organism which are traceable processes that are subject to conservation laws (according to the new posited concept of ecomechanics; Denny & Helmuth, 2009; Denny & Benedetti-Cecchi, 2012; Carrington et al., 2015). This provides an exceptionally powerful tool to predict organismal functional traits, capturing variation across species to solve a very wide range of problems in ecology and evolutionary biology (Lika et al., 2011; Kearney, 2012; Pouvreau et al., 2006; Pequerie et al., 2010; Sarà et al., 2011; 2012; 2013a; 2013b; 2014). FT-DEB could provide information about the effect of seawater acidification on the fecundity (as expressed by the number of gametes per life span, the so-called Darwinian fitness; Bozinovic et al., 2011) and the degree of reproductive failure of species providing theoretical predictions about LH traits having implications on population dynamics and community structure throughout the species range (Sarà et al., 2013a). Here, we specifically exploited the FT-DEB model spatially and explicitly contextualised along the Italian coasts under subtidal conditions (Kearney et al., 2010; Sarà et al., 2011; 2012; 2013a; 2013b), using four-year thermal series and satellite Chlorophyll-a (CHL-a) concentrations, to test the multiple effect due to the combination of pH and hypoxia on the physiological and behavioural traits of our target species, the bivalve *Mytilus galloprovincialis* (Lamarck 1819). Recent insights obtained by the experimental research have shown that OA mainly affects feeding (FR), assimilation (AE) and maintenance cost rates. Here, we translated the combined effects of hypoxia and hypercapnia on AE and oxygen consumption rates as measured under different treatments into effects on assimilation and somatic maintenance costs as expressed by the DEB [$\dot{p}_M$] parameter. This latter is a crucial functional trait used in recent bioenergetics based on the DEB theory that mechanistically can be used to investigate the role played by multiple stressors on LH traits of organisms by using first principles (Sarà et al., 2014). We further documented the effects of those stressors on *M. galloprovincialis* shells through the use of a scanning electron microscope (SEM), and compared the maximum breaking load of treated *vs.* control specimens. A behavioural analysis completed the frame concerning the individual's response to both single and combined stressors. Carried out in a context of OA, this exercise comprises a first step in linking the fields of ecomechanics and climate change ecology, which should yield a more mechanistic understanding of how biodiversity will respond to environmental change (*sensu* Buckley et al., 2012).

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2 Materials and methods

This study articulated three steps: 1) laboratory investigation on the effects of pH and hypoxia on functional and behavioural traits of *Mytilus galloprovincialis*; 2) collection of water temperature data, and Chlorophyll-a (CHL-a) data from two Mediterranean sites (Trieste and Palermo), as a further forcing variable in the DEB model and lastly 3) model running to simulate growth and fitness of *M. galloprovincialis* under stressful conditions by using estimated DEB parameters arising from the activities in the first step.

2.1 Sampling and experimental set-up. Specimens of *M. galloprovincialis* (45 - 55 mm) were provided by the Ittica Alimentare Soc. Coop. Arl. (Palermo) and transferred within 30 minutes to the laboratory. Mussels were then carefully cleaned and placed in a 300L tank filled with natural seawater at room temperature (18-20°C), field salinity (37-38 PSU), and fed *ad libitum* with cultured *Isochrysis galbana* (Sarà et al., 2011). According to common experimental procedures for studying the bioenergetics of bivalves (Sarà et al., 2008; Ezgeta-Balic et al., 2011), mussels were acclimated for two weeks to reduce stress generated by manipulation and transport (Sarà et al., 2013a). Once acclimated, 200 specimens were randomly divided in groups of 25 organisms, transferred to 8 independent rectangular glass tanks of 120L capacity (100 cm long, 30 cm deep, 40 cm wide) and kept in a conditioned room at 21°C. Tanks 1 to 4 were filled with aerated and recirculating sea water, while Tanks 5 to 8 were not aerated and covered with a plastic film disposed on the water surface, in order to avoid gas-exchanges between air and water. Tanks 1-2 were used as a control (CTRL), while hypercapnia was imposed in Tanks 3-4 (Tr1), hypoxia (2 ppm) in Tanks 5-6 (Tr2), and both factor (pH 7.5 and hypoxia) in Tanks 7-8 (Tr3). Mussels were acclimated to two different nominal pH treatments: (i) pH 8.0 in Tanks 1-2 (CTRL) and 5-6 (Tr2), corresponding to present average pH at the sampling site; and (ii) pH 7.5 in Tanks 2-3 (Tr1) and 7-8 (Tr3), deviating from present range of natural variability and relevant for 2100 ocean acidification scenarios. This last point is considered the critical dissolution threshold of calcium carbonate in shelled animals as reported in literature (Melzner et al., 2011; Gazeau et al., 2013). The carbonate system speciation ($pCO2$, HCO_3^-, CO_3^{2-}, ΩCa and ΩAr) was calculated from pH_{NBS}, temperature, salinity and alkalinity (T_A = 2.5 mM; Rivaro et al., 2010) using CO2SYS (Lewis and Wallace, 1998) with dissociation constants from Dickson & Millero (1987). The pH was manually controlled 8 times a day by an electronic pH-meter (Cyberscan 510, Eutech Instruments) and gaseous CO_2 was injected directly into the aquarium when required. Tanks were siphoned at the end of each working day, removing all the faecal material in order to avoid the accumulation of waste products.

112 **2.2 Behavioural observations.** The valve gape of mussels was recorded by means of the two simplest
113 behavioural categories reported in Jørgensen et al. (1988): closed valves and opened valves. Each observation
114 was carried out by an operator with the aim to record changes in the behavioural repertoire of bivalves in
115 response to the exposure to a single stressor (pH or hypoxia) and to both pH and hypoxia, compared to
116 individuals kept in normal environmental conditions. All experiments were conducted at environmental (37-38
117 PSU) salinity and with well-aerated sea water through a gentle flow (Ameyaw-Akumfi & Naylor, 1987), except
118 for specimens of Tank 5-6 and 7-8, that were not aerated in order to maintain the hypoxia level set through the
119 gaseous nitrogen. Behavioural observations were repeated six times a day, on day 7, 14, 21, 28, and involved 5
120 random specimens for each treatment.
121
122 **2.3 Oxygen consumption.** The rate of oxygen consumption was determined twice (week 1 and week 4) in a
123 respirometric glass chamber (0.3L) in a temperature-controlled water bath, in order to compare the effects of
124 multiple stressors by converting rates into the DEB parameter [$\dot{p}_M$] (expressed as J cm^{-3} h^{-1}) linked to the
125 energetic cost of maintenance in order to integrate it in the standard DEB model. All determinations were
126 performed using filtered seawater with the same pH and oxygen content as that of the respective treatment,
127 stirred with a magnetic stirrer bar beneath a perforated glass plate supporting each individual (Sarà et al., 2008;
128 Ezgeta-Balic et al., 2011). The decline in oxygen concentration was measured by a PiroScience FirestingO2
129 respirometer, capable of four sensor connections. We used a total of n = 64 mussels per week, 16 for each
130 treatment (8 for each tank) acclimated as above, fed *ad libitum* until the day before the experiment. The decline
131 was continuously recorded for at least 1 h, excluding an initial period (~ 10 min) when usually there is a more
132 rapid decline in oxygen caused by a disturbance of the sensor's temperature equilibration. Respiration rate (RR,
133 µmol O_2 h^{-1}) was calculated according to (Ezgeta-Balic et al., 2011; Sarà et al., 2008; 2013b): $RR = (C_{t0} - C_{t1}) x\, Vol_r x 60 (t_1 - t_0)^{-1}$, where C_{t0} is oxygen concentration at the beginning of the measurement, C_{t1} is
135 the oxygen concentration at the end of the measurement, and Vol_r is the volume of water in the respirometric
136 chamber.
137
138 **2.4 Assimilation efficiency.** Assimilation is the final step of food processing and it represents the efficiency with
139 which organic material is absorbed from the ingested food (Kooijman, 2010). The assimilation of food is
140 assumed to be independent of the feeding rate *per se*, but proportional to the ingestion rate. Here, 16 specimens
141 of *M. galloprovincialis* per treatment were collected twice (week 1 and week 4) and placed into separate beakers

containing 1L of filtered seawater and a magnetic stirrer bar. In order to allow the mussels to open their valves and start their filtration activity, they were given 15 minutes before the introduction of food with an initial concentration of ~ 15,000 *Isochrysis galbana* cells ml^{-1}. After a period of 2 h mussels were moved to cleaned 1L glass beakers with filtered seawater for a period of 12 h, after that the water contained in each beaker was filtered on pre-ashed and weighted GF/C fibreglass filters. Once filtered, filters were washed with 0.5 M ammonium formate (purest grade) to remove adventitious salts (Widdows & Staff, 2006), dried in the oven (95°C for 24 h) and then incinerated in a muffle furnace (450°C for 4 h). After each step, the samples were weighted using a balance (Sartorius BL 120S ± 1µg). For the calculation of AE, together with the faeces collected from the mussels, filters containing algal food were dried and incinerated as above. After respirometric measurement and the collection of faeces each animal was killed by gentle freezing and dissected, and the shells were separated from the body tissue in order to calculate their individual dry weights and standardize respiration rates to body weights.

2.5 Water temperature data. The main forcing driver of shellfish LH inside DEB models is represented by mean seawater temperature (Pouvreau et al., 2006; Kearney et al., 2010; Kooijman, 2010; Sarà et al., 2011; 2013). DEB simulations were run under subtidal conditions (body temperature was expressed by the mean seawater temperature; Montalto et al., 2014) with 4 years-hourly data (Jan 2006 - Dec 2009) of seawater temperature measured about 1 m below the surface at the closest meteo-oceanographic station held in Trieste (LAT 45° 38′ 57.81″; LONG 13° 45′ 28.58″) and Palermo (LAT 38° 07′ 17.08″; LONG 13° 22′ 16.79″). The period of 4 years is consistent with the normal life span of most Mediterranean shellfishes (Sarà et al., 2012; 2013). Both sites were chosen as they represent two opposite temperature and food conditions for mussel growth in Italy, with Trieste as representative of lower temperature (average 16.98 ± 6.19 °C) and higher food levels (average 1.36 ± 0.37 CHL-a), and Palermo of higher temperatures (average 20.19 ± 4.64 °C) and lower food (average 0.19 ± 0.09 CHL-a). Data are available online from the Italian Institute of Environmental Research (ISPRA) web page (http://www.mareografico.it/).

2.6 CHL-a dataset. Chlorophyll-a (CHL-a) derived from satellite imageries (µg L^{-1}) was adopted as a reliable food quantifier for suspension feeders (Kearney et al., 2010; Sarà et al., 2011; 2012) and was downloaded from the EMIS website (http://emis.jrc.ec.europa.eu/).

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2.7 Model description. The Dynamic Energy Budget Theory provides a general framework that allows to describe how physiological mechanisms are driven by temperature and food availability, and influences growth and the reproductive performances in marine organisms (Monaco et al., 2014). Following the κ-rule (DEB theory; Kooijman, 2010) a fixed energy fraction (κ) is allocated to growth and somatic maintenance, while the remaining fraction (1-κ) is allocated to maturity maintenance plus maturation or reproduction. If the general environmental condition deviates from common natural patterns (i.e. changes in temperature, food availability etc.) reproduction and growth are consequently affected. According to DEB theory, a reduction in growth can be caused either by reduced food assimilation ($\dot{p}_A$), enhanced maintenance costs ($\dot{p}_M$), or enhanced growth costs ($\dot{p}_G$). Using this approach, and through the DEB parameters derived from Sarà et al. (2012), except for the variation in the maintenance costs ($\dot{p}_M$) and in the assimilation efficiency of food (AE) which were experimentally estimated throughout this study, we performed simulations aimed at investigating the potential variations in growth and fecundity of our model species. To run the DEB simulations, local thermal series of selected sites were used together with satellite CHL-a concentrations, obtaining a first model with environmental conditions. A second model was run with the $\dot{p}_M$ calculated from the oxygen measurements on specimens of *M. galloprovincialis* from Tanks 3-4 (pH 7.5) simulating a chronic hypercapnia condition for the full cycle (4 years) and the relative estimated AE. Subsequently, further models were run by simulating one random hypoxia event for each of the four years of the cycle, then simulating two yearly events, and so on up to six monthly hypoxia events. The month of each event was randomly chosen for every year with the use of a table of random digits. The $\dot{p}_M$ calculated from the oxygen consumption rate measurements on specimens from Tanks 7-8 (pH 7.5 and hypoxia) was used in substitution to $\dot{p}_M$ from pH 7.5 tanks 3-4, coupled with the relative estimated AE, when simulating both stressors. Outputs of the DEB models (Sarà et al., 2014) were: the maximum theoretical total length of shellfish (TL), the maximum total weight (TW), the total number of eggs (TRO) produced during a life-span of 4 years, the total number of reproductive events (RE) and the time needed to reach gonadic maturity (TM) for each treatment.

2.8 Effects on shell: mechanical strength and SEM pictures. The functional impact of exposure to pH and to validate the pH effect on morphological structure of valves, was tested on mussels exposed to the two nominal pHs for 4 weeks. Twice (week 1 and week 4), 16 mussels for each treatment were collected and dissected, and both valves were cleaned and dried with absorbent paper. The left valve was then sliced transversely using a circular saw (Dremel® 300 series) to section the whole length of the shell. Age was estimated using the analysis

of shell rings proposed by Peharda et al. (2011) by counting the number of rings with the use of a stereo microscope (Leica EZ4). The right valves were instead evaluated for their mechanical properties at the Department of Mechanical Engineering. Experimental crushing tests, in order to estimate the shell's maximum breaking load (in N) as a further validation step, were realised with a home-made press previously calibrated by an Instron 3367 machine controlled by the Bluehill 2.0 software. The effects of low pH exposure were documented by the use of a scanning electron microscope (SEM; Zeiss LEO 440) that led to a thorough investigation on the integrity of the mussels' external protein layer (*periostracum*) and on the underlying mineral layer, rich in calcite and aragonite.

2.9 Statistical analysis. In order to test for significant differences in respiration rate and the assimilation efficiency, ANOVAs were performed using Treatment (CTRL, Tr1, Tr2, Tr3) and Time (Week 1 and Week 4) as fixed factors, with respectively four and two levels. In order to test for significant differences in behavioural categories ANOVAs were performed using Treatment (CTRL, Tr1, Tr2, Tr3) as fixed factors, while Breaking load was tested with Treatment (CTRL, Tr1, Tr2, Tr3) and Time (Week 1 and Week 4) as fixed factors. When significant differences were detected, the Student-Newman-Keuls (SNK) post-hoc pair wise comparison of means was used (Underwood, 1997). Cochran's test was used prior to ANOVA to test the assumption of homogeneity of variance (Underwood, 1997). When no homogeneous variances were rendered with any type of transformation, the significance level was set at 0.01 instead of 0.05, as ANOVA can withstand variance heterogeneity, particularly in large balanced experiments, thereby reducing the possibility of a Type I error (Underwood, 1997).

3 Results

3.1 Water chemistry. Experimental target pH values were constantly maintained at significantly different levels in all tanks (normal pH and 7.5 pH treatments; Table 2; ANOVA, $p < 0.01$; SNK test: CTRL = Tr2, Tr1 = Tr3). Oxygen pO_2 was also maintained at significantly different levels between normoxia (7.3 ± 0.02 mg/l) and hypoxia (2.4 ± 0.02 mg/l) treatments (Table 2; ANOVA, $p < 0.01$; SNK test: CTRL = Tr1, Tr2 = Tr3). This translated into significant different pCO2 levels in all treatments (Table 2; ANOVA, $p < 0.01$), and in different CO_3^-, ΩCa and ΩAr levels in all tanks (Table 2; ANOVA, $p < 0.01$) except between Tr1 and Tr3 (SNK test: Tr1 = Tr3).

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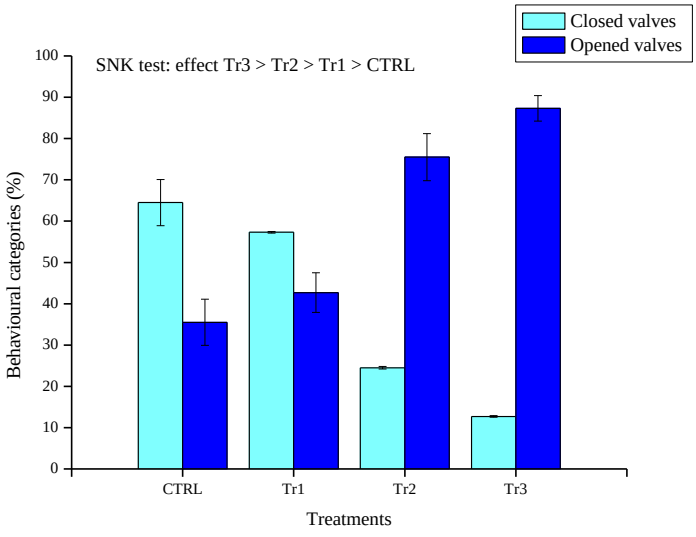
232 **3.2 Valve gaping.** During behavioural observations on *M. galloprovincialis*, specimens showed a significant
233 difference in the behavioural categories, showing respectively 64.5 ± 5.6 % (CTRL), 57.3 ± 0.2 (Tr1), 24.5 ± 0.3
234 (Tr2) and 12.7 ± 0.2 % (Tr3) of opened valves (Fig. 1; Table 3, ANOVA, $p < 0.001$). The percentage of closed
235 valves was instead 35.5 ± 5.6 % (CTRL), 42.7 ± 4.8 (Tr1), 75.5 ± 5.7 (Tr2) and 87.3 ± 3.1 % (Tr3) (ANOVA, p
236 < 0.001).



237

238 Fig. 1 Behavioural observations of *Mytilus galloprovincialis* under different treatments of oxygen (normoxia –
239 hypoxia 2ppm) and pH (7.5 – 8.0). The two behavioural categories represented were: closed and opened valves.

240

241 **3.3 Oxygen consumption.** Results showed a significant reduction in the oxygen consumption rate by specimens
242 of *M. galloprovincialis* exposed to treatments (Table 4, ANOVA, $p < 0.01$), although the SNK test revealed no
243 significant differences among the various groups (Fig. 2a). No significant effects were highlighted for the time
244 factor (Table 4, ANOVA, $p > 0.05$), so in Fig. 2a we reported only results for week 4. The rate of oxygen
245 consumption was reduced by up to 42% in Tr1, to 35% in Tr2, and to 41% in Tr3, causing a decrease in the $\dot{p}_M$
246 by up to 29% in Tr1, to 47% in Tr2, and to 49% in Tr3 across the four weeks of exposure.

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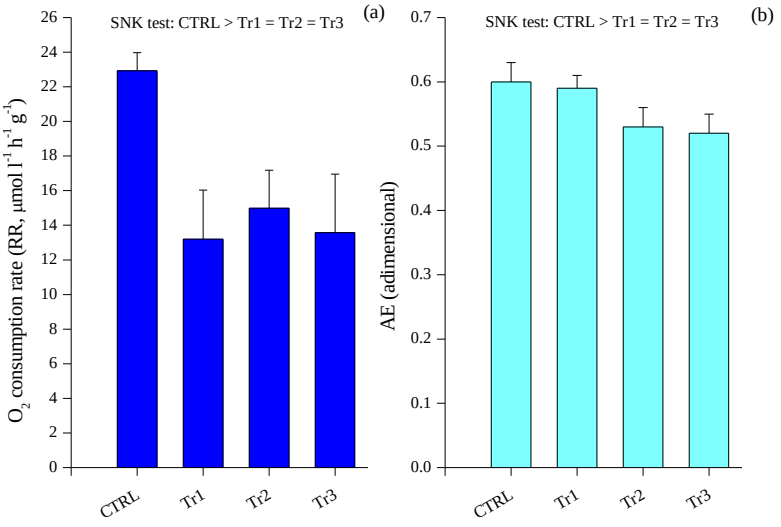

Fig. 2 (a) Oxygen consumption rates (RR) and (b) Assimilation efficiency (AE) of *Mytilus galloprovincialis* under different treatments of oxygen (normoxia – hypoxia 2ppm) and pH (7.5 – 8.0) at week 4.

3.4 Assimilation efficiency. Assimilation efficiency of food (AE) resulted in significantly affected treatments (Table 4, ANOVA, $p < 0.001$) after four weeks of exposure. No significant effects were highlighted for the time factor (Table 4, ANOVA, $p > 0.05$), so in Fig. 2b were reported only results for week 4. In particular, AE decreased of 2.4% in Tr1, of 12.4% in Tr2, and of 14.4% in Tr3, although the SNK test revealed no significant differences among the various groups (Fig. 2b).

3.5 DEB simulation results. Once $\dot{p}_M$ and AE were experimentally estimated, we introduced obtained values under the different treatments to run DEB models and to obtain the derived effects In terms of LH traits. Thus, we performed DEB simulations under local thermal conditions (as expressed by the thermal series recorded in Trieste and Palermo; see M&M for details) and using satellite CHL-a concentrations (2006-2009) as a proxy of food. Results showed a remarkable effect exerted by hypercapnia and an increasing addictive effect of hypoxia related to the intensity of disturbance (*i.e.* number of yearly hypoxic events) on LH traits of *M. galloprovincialis* by the end of 4th year (Table 5). Total length (TL) and total weight (TW) in Trieste and in Palermo were similarly reduced by hypercapnia (Fig. 3), with a progressive addictive effect of hypoxia (Table 5). The total number of eggs produced (TRO) and the total number of reproductive events (RE) in Trieste were strongly

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reduced by hypercapnia (Fig. 3), with the same progressive addictive effect from hypoxia (Table 5). Maturation time (TM) was affected both in Trieste and Palermo by hypercapnia, with the same hypoxia contribution previously shown. Palermo showed no reproductive events in the DEB simulations.

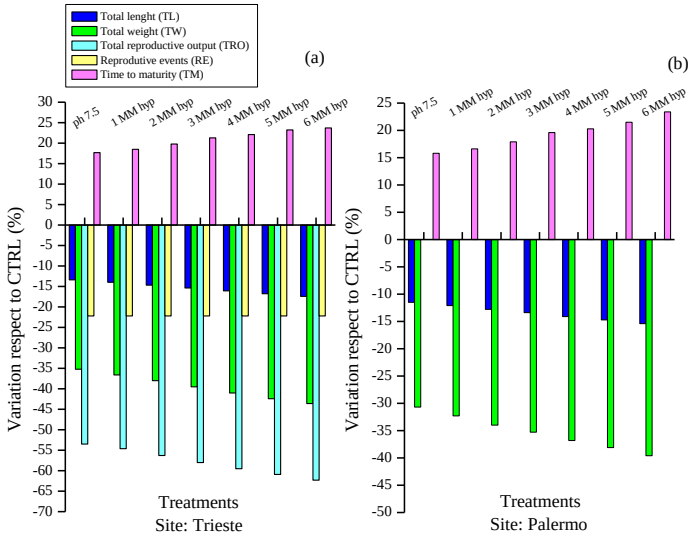

Fig. 3 Results from DEB simulation for (a) Trieste and (b) Palermo sites, percentage variation of DEB outputs respect to CTRL. TL and TW were reduced by 13.4% and 35.2% in Trieste, and by 11.5% and 30.7% in Palermo by hypercapnia, with a progressive addictive hypoxia effect up to 8.9%. TRO and RE were reduced by 53.4% and 66.7% in Trieste by hypercapnia, with a progressive addictive hypoxia effect up to 8.8%. TM increased by 17.8% in Trieste and by 15.7% in Palermo with a similar hypoxia effect (up to 7.6%).

3.6 Effects on shell. Specimens of *M. galloprovincialis* collected ranged in age from 1 to 3 years with a mean age of 1.8 ± 0.04 years (n = 128). Overall, 97% of individuals were > 2 years old. Results from the breaking load experiment revealed a significant effect of pH (58.8 ± 5 N) and of combined stressors on the breaking load (50 ± 2.7 N), compared to hypoxic (64.4 ± 3.7 N) and CTRL specimens (77.2 ± 2.2 N) (Fig. 4) (Table 3, ANOVA, $p < 0.001$). In addition, the effect was stronger at week 4 than after one week of exposure (Table 3, ANOVA, $p < 0.01$). Deeper investigations through scanning electron microscopy validated an effect by showing an increasing erosion of the shell after exposure to CO_2-induced acidification. The external dissolution pattern usually started from the umbonal region and progressed toward the margin of the shell, usually associated with some degree of damage to the *periostracum*. The damage was present at differing extensions in all specimens exposed to

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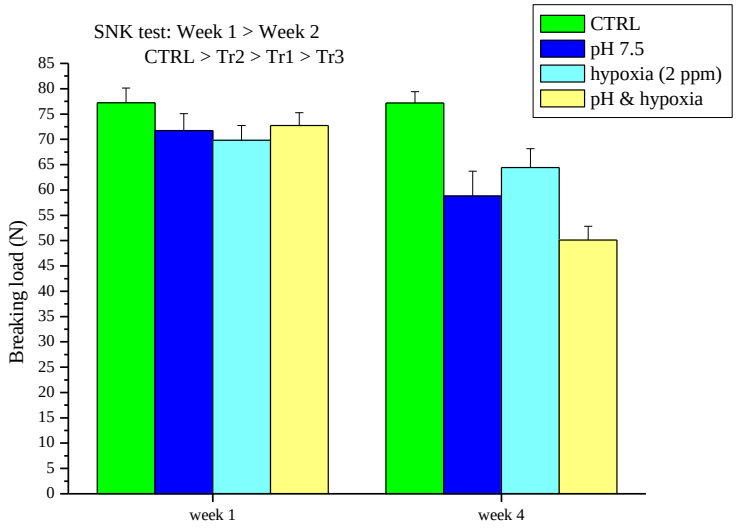
286 treatments, except in the control mussels (Fig. 5 b, c, d). The alteration of the underlying carbonate layer was
287 instead visible only in Tr1 and Tr3, with details in Fig. 6 (b, d). This kind of alteration was never recorded under
288 control pH (Fig. 4a).
289



290
291 Fig. 4 Breaking load of valves (in Newton, N) exposed to different treatments of oxygen (normoxia – hypoxia
292 2ppm) and pH (7.5 – 8.0) at week 1 and 4.

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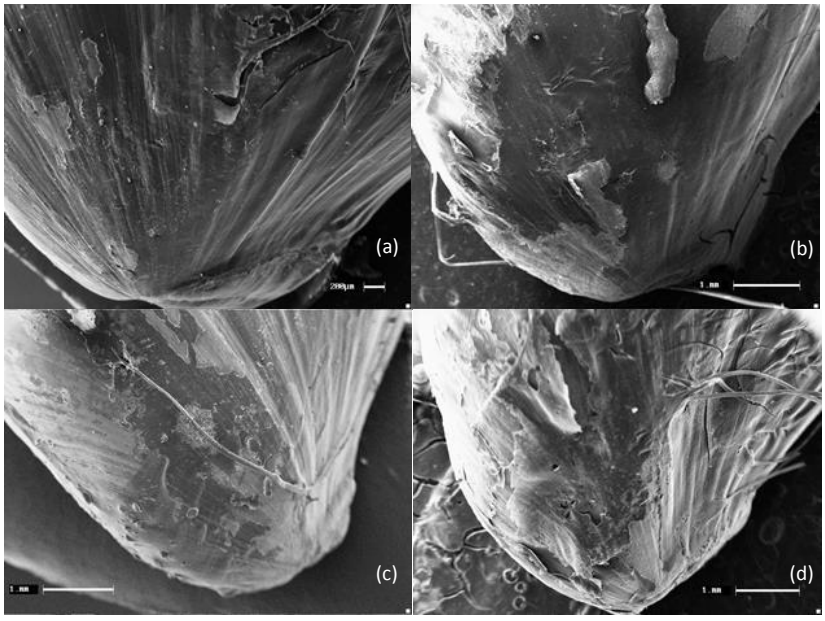
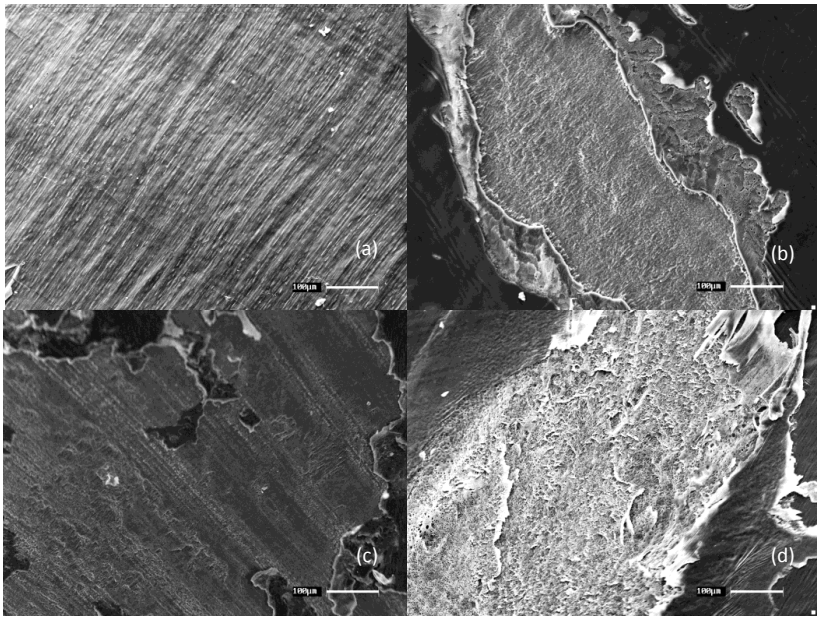


293

294 Fig. 5 SEM pictures of different shells exposed to (a) control condition (CTRL); (b) pH 7.5 and normoxia
295 condition (Tr1); (c) normal pH and hypoxia condition (Tr2); (d) both pH 7.5 and hypoxia conditions (Tr3).

296



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298 Fig. 6 Details of different shells exposed to (a) control condition (CTRL); (b) pH 7.5 and normoxia condition
299 (Tr1); (c) normal pH and hypoxia condition (Tr2); (d) both pH 7.5 and hypoxia conditions (Tr3).

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4 Discussion

Marine organisms, and in particular intertidal species (Montecinos et al., 2009), have been formally recognized as being equipped with well-developed and conserved compensatory mechanisms to contrast ocean acidification such as (i) passive buffering of intra- and extracellular fluids; (ii) transport and exchange of relevant ions; (iii) transport of CO_2 in the blood in those species that have respiratory pigments; (iv) metabolic suppression to wait out periods of elevated CO_2 (e.g. Lindinger et al., 1984; Cameron, 1989; Walsh and Milligan, 1989; Hand, 1991; Heisler, 1993; Guppy and Withers, 1999; Pörtner et al., 2004). Several authors recorded suppression of feeding activity and growth, depressed metabolism, increased N excretion and loss of tissue weight for marine bivalves exposed to reduced seawater pH (Bamber, 1990; Michaelidis et al., 2005; Berge et al., 2006; Gazeau et al., 2010). Bivalves are in fact capable of maintaining a constant internal pH by decreasing their metabolic rates and/or dissolving their shell; the shell acting then as a source of CO_3^{2-} (Bamber, 1990; Michaelidis et al., 2005; Berge et al., 2006) counterbalancing the crossing effect due to lowering dissolved CO_2 through biological membranes (Fabry et al., 2008). Compensation of low pH through adjustments in ionic composition appears to be a trade-off that is not likely sustainable on longer time-scales, such as that associated with anthropogenic increases in seawater pCO_2 (Fabry et al., 2008). In agreement with current literature showing deleterious effects of CO_2-induced acidification on a wide range of invertebrates (Barnhart & McMahon, 1988; Barnhart, 1989; Rees & Hand, 1990), and similarly to other studies by *M. galloprovincialis* (Gestoso et al., 2016; Michaelidis et al., 2005), our results showed how hypercapnia (pH reduced by 0.6 units, relative to the natural pH of the lower Tyrrhenian waters) was able to induce a decline in metabolic rates of mussels. This kind of decline has already been noticed by other authors as an adaptive strategy for survival under transiently stressful conditions (Michaelidis et al., 2005). According to Pörtner et al. (2004), metabolic reduction due to hypercapnia could be a result of acid-base disturbances and therefore be similar to the response of intertidal individuals to anaerobic conditions. Direct effects of hypoxemia have been further proven to cause fatal decrements in an organism's performance in growth, reproduction, feeding, immunity and behaviour (*sensu* Pörtner & Farrell, 2008). Synergistic stressors like ocean acidification and hypoxia are capable of narrowing the thermal window of functioning according to species-specific sensitivities, modulating biogeographies, coexistence ranges, community shifts and other interactions (Pörtner & Farrell, 2008). The mussel *Mytilus edulis* has been proven able to compensate both short- and long-term exposure to hypercapnia by dissolution of its shell (Lindinger et al.,1984; Michaelidis et al., 2005), resulting in reduced growth and metabolism. A similar mechanism of release of inorganic molecules into the pallial cavity (as $CaCO_3$ from valves) has been documented during periods of anaerobic metabolism, to maintain the acid-base balance (Chaparro et al.,

2009), determining further physiological and energetic cost such as decreased growth, respiration rate and protein synthesis (Pörtner et al., 2005). During periods of environmental oxygen limitation, many organisms are able to suppress ATP demand, shut down expensive processes, such as protein synthesis (Hand, 1991), but at the same time limiting growth and reproductive potential. Although suppression of metabolism under short-term experimental conditions is a "sublethal" reversible process, reductions in growth and reproductive output will effectively diminish the survival of the species on longer time-scales (Fabry et al., 2008). The contemporary occurrence in our simulations, of monthly hypoxia events, revealed a growing additive contribution to what was already elicited by hypercapnia on growth and reproduction. Current literature has not currently explored the combined effects of multiple stressors on long-term experiments by modulating the intensity and duration of disturbance. This would probably translate as a very complex experimental set-up which would be hardly practicable, especially on long-term scales. On the other hand, mechanistic models offer a more sustainable and reliable alternative to long-term, in-field research when studying the effects of multiple-stressors , with the advantage of testing, at the same time, the magnitude and duration of disturbance on LH-traits of a model species. Our results highlighted the general hypoxia growing effect following the increasing duration of disturbance, with a particular focus in Trieste on TW and TRO, while in Palermo on TW and TM (Table 5). A further important peculiarity of the DEB simulations deals with the possibility to spatially contextualise the effects of single and multiple stressors on selected outputs by integrating local thermal conditions and food concentrations. In particular the DEB model easily allowed the estimation of the fecundity potential of cultivated and natural organisms, that is often omitted in other ecological studies, but that represents a crucial quantity for resource (e.g. aquaculture) and conservation purposes. To verify impacts on shellfish fecundity, we contextualised our simulation by introducing Trieste hourly temperature series after those of Palermo, with the respective local actual CHL-a concentrations, as long as in the first site no reproductive events came out from our simulations, probably due to food limitations and temperature threshold. A combined effect of the simultaneous stressors, such as those considered across this study, has proven, through our experimental and mechanistic integrated approach, to affect the organism's performance in growth, reproduction and behaviour. Those specific and synergic effects of each stressor seem capable, especially at extreme temperatures, of narrowing thermal windows, modulating biogeographical distribution, coexistence ranges, community shifts, food webs and species interactions (*sensu* Pörtner & Farrell, 2008).

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6 Conclusions

Additional research is still required to improve our knowledge of organismal response to multiple stressors, in particular, of many marine ectotherms with indeterminate growth amongst invertebrates (e.g. crustaceans, molluscs). Nevertheless, modelling the growth and reproductive potential (and failure) of species vulnerable to those stressors with predictive tools, such as bioenergetic models is a useful approach for management and protection purposes, but also for shellfish culture in general.

Authors' contributions

AG and GS conceived the idea and led the writing. AG carried out all experiments in mesocosms, performed modelling work and analysed data. GS provided lab facilities and research funds. All authors contributed critically to the drafts and gave final approval for publication.

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Tables

Table 1. Seawater carbonate chemistry parameters (mean ± se). Seawater pH on the NBS scale (pH_{NBS}), temperature (T; °C), and salinity were used to calculate CO_2 partial pressure (pCO_2; µatm) as well as aragonite and calcite saturation states (respectively Ωar and Ωca), for a total alkalinity of 2500 mmol kg^{-1}.

	Measured			Calculated			
	pH_{NBS}	O_2 mg/l	Salinity (PSU)	pCO_2 (µatm)	CO_3^-	Ωca	Ωar
CTRL	8.01±0.001	7.29±0.02	37.18±0.11	624.31±4.9	167.93±0.95	3.95±0.02	2.58±0.01
Tr1	7.53±0.002	7.30±0.02	37.12±0.05	2151.17±22.02	62.05±0.73	1.46±0.02	0.95±0.01
Tr2	8.01±0.001	2.44±0.02	37.07±0.04	729.88±18.24	152.53±1.51	3.59±0.04	2.34±0.02
Tr3	7.53±0.002	2.44±0.02	37.21±0.17	2238.83±20.72	59.59±0.42	1.40±0.01	0.91±0.01

Table 2. ANOVA on seawater chemistry parameters. Comparison between CTRL (normal pH) and TREAT (low pH and hypoxia) (* = p < 0.05; ** = p < 0.01; *** = p < 0.001; ns = not significant).

	pH_{NBS}				pO_2		
	df	MS	F	p	MS	F	p
TREAT	3	10.73	41450.84	**	1083.21	18798.36	**
Residuals	548	0.0003			0.06		
Cochran's Snk				*			*

	pCO_2				CO_3^-		
	df	MS	F	p	MS	F	p
TREAT	3	1.06e08	2426.84	**	460157.7	3433.17	**
Residuals	548	43851.09			134.03		
Cochran's Snk				*			*

	Ωca				Ωar		
	df	MS	F	p	MS	F	p
TREAT	3	254.09	3432.44	**	108.26	3426.14	**
Residuals	548	0.07			0.03		
Cochran's Snk				*			*

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Table 3 ANOVA table of results. Effect on valve gape and breaking load of *Mytilus galloprovincialis* (* = p < 0.05; ** = p < 0.01; *** = p < 0.001; ns = not significant).

Source	df	Valve gape			Source	df	Breaking load		
		MS	F	P			MS	F	P
Treatment (Tr)	3	34.53	26.03	***	Treatment (Tr)	3	3838.12	15.18	***
Residuals	84	1.33			Time (Ti)	1	777.19	9.22	**
Cochran's C				ns	Tr x Ti	3	132.92	1.58	Ns
					Residuals	56			
					Cochran's C				ns

Table 4 ANOVA table of results. Respiration rate (RR) and assimilation efficiency (AE) of *Mytilus galloprovincialis* (* = p < 0.05; ** = p < 0.01; *** = p < 0.001; ns = not significant).

Source	df	RR st			AE		
		MS	F	P	MS	F	P
Treatment (Tr)	3	312.9183	6.95	***	0.2783	12.21	***
Time (Ti)	1	205.1325	4.56	*	0.0424	1.86	ns
Tr x Ti	3	40.7752	0.91	ns	0.0198	0.87	ns
Residuals	120	45.0271			0.0228		
Cochran's C				*			ns

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Table 5 DEB simulation outputs. Percentage variation of treatments from CTRL: Total length (TL), Total weight (WW), Total reproductive output (TRO), Total reproductive events (RE), Time to maturity (TM).

DEB outputs (CTRL) after 4 years							
Site	Stressor	Frequency	TL (cm)	WW (g)	TRO (n° egg)	RE	TM (days)
Trieste	CTRL	0	9.55	11.19	6737889	9	232
Palermo	CTRL	0	3.08	0.31	0	0	739

DEB outputs: percentage variation respect to CTRL after 4 years							
Site	Stressor	Frequency	TL (%)	WW (%)	TRO (%)	RE (%)	TM (%)
Trieste	pH 7.5	baseline	-13.44	-35.20	-53.49	-22.22	18
Trieste	pH+hypoxia	1-month	-14.04	-36.58	-54.61	-22.22	18
Trieste	pH+hypoxia	2-month	-14.71	-38.04	-56.34	-22.22	20
Trieste	pH+hypoxia	3-month	-15.40	-39.52	-58.01	-22.22	21
Trieste	pH+hypoxia	4-month	-16.09	-40.97	-59.52	-22.22	22
Trieste	pH+hypoxia	5-month	-16.77	-42.37	-60.95	-22.22	23
Trieste	pH+hypoxia	6-month	-17.38	-43.62	-62.26	-22.22	24
Palermo	pH 7.5	baseline	-11.47	-30.69	0	0	16
Palermo	pH+hypoxia	1-month	-12.09	-32.26	0	0	17
Palermo	pH+hypoxia	2-month	-12.84	-33.97	0	0	18
Palermo	pH+hypoxia	3-month	-13.42	-35.26	0	0	20
Palermo	pH+hypoxia	4-month	-14.10	-36.78	0	0	20
Palermo	pH+hypoxia	5-month	-14.70	-38.08	0	0	22
Palermo	pH+hypoxia	6-month	-15.42	-39.63	0	0	23

Percentage additive effect of Hypoxia							
Trieste	pH+hypoxia	1-month	-0.6	-1.4	-1.1	0	0.8
Trieste	pH+hypoxia	2-month	-1.3	-2.8	-2.8	0	2.1
Trieste	pH+hypoxia	3-month	-2	-4.3	-4.5	0	3.6
Trieste	pH+hypoxia	4-month	-2.7	-5.8	-6	0	4.4
Trieste	pH+hypoxia	5-month	-3.4	-7.2	-7.4	0	5.5
Trieste	pH+hypoxia	6-month	-4	-8.4	-8.8	0	6
Palermo	pH+hypoxia	1-month	-0.6	-1.6	0	0	0.8
Palermo	pH+hypoxia	2-month	-1.3	-3.3	0	0	2.1
Palermo	pH+hypoxia	3-month	-1.9	-4.6	0	0	3.8
Palermo	pH+hypoxia	4-month	-2.6	-6.1	0	0	4.5
Palermo	pH+hypoxia	5-month	-3.2	-7.4	0	0	5.7
Palermo	pH+hypoxia	6-month	-3.9	-8.9	0	0	7.6