

REVIEWER #2

Reviewer wrote: This study integrates laboratory-derived parameters of mussel metabolism and assimilation efficiency to run DEB models testing the effect of pH and hypoxia, using environmental data input (temperature, food) from two sites within the mussel's biogeographic range. I appreciate the approach of introducing hypoxia events (although I have a comment on the design these events) as a means of incorporating environmental variability in the model. This literature is sparse with such perspectives, especially in the context of multiple stressors. However, the paper lacks a perspective of the environmental relevance of the experimental design and modelling.

Author's reply: We thank the Reviewer #2 for helping us improving the readability and the clearness of the ms.

Author's changes: In doing so we applied most of the suggestion for the highlighted points, and we discussed them through the specific comments. A perspective of environmental relevance on modelling outputs has been currently added in the discussion section following what suggested by the reviewer.

Major comments

Methods need much more detail (see detailed comments)

Reviewer wrote: Physiological condition of experimental mussels during the experiment is not quantified. Feeding was ad libitum and it was not assessed if mussels were being fed at conditions of either site used for the modeling. This is problematic as, for example, if the mussels are starving relative to their natural food supply, the derived experimental parameters for the DEB model may be inappropriate. The authors also do not explain the experimental design. Why was the experiment 4 weeks?

Author's reply: We thank the reviewer for highlighting this point.

Author's changes: We estimated the condition index through the biometric available data and compared it through the experiment, resulting in a not significant variation throughout the study period, and this supports that experimental animals were not stressed by starvation. Further, we used the locution "ad libitum" to indicate a food concentration saturating the feeding processes of animals over time. Such an experimental maintenance condition is commonly used throughout the current literature when bivalves are maintained with ad libitum condition of food in bioenergetic experiments (e.g. Sarà et al., 2013; Montalto et al., 2014; Tagliarolo et al., 2016). However, we adopted a four weeks-period to estimate the effect of OA on functional traits of mussels; such a period is judged to be enough to allow mussels to acclimate to new conditions, as showed in many experimental papers across the current literature (references added in the manuscript).

Reviewer wrote: DEB models: As a reader of Biogeosciences, but not an expert on DEB models, it would be helpful if the authors reviewed this approach more clearly (perhaps using a schematic, what program is used to run models, table of input variables etc.). After reading the paper, I am unclear about the exact implementation and conclusions that can be drawn from this simulation based on the following 4 sources of confusion:

1) From what I understand, temperature from 2006-2009 is used as one of the model inputs. However, all the biological parameters taken from the experiments come from 21 degr. C (although this is not stated explicitly for respiration, but I assume it's 21 C). Since environmental data would vary in terms of temperature across the four years, I don't understand how biological performance is scaled across this temperature regime. It would be good to include a figure of the environmental data (means are not great time-series descriptors, especially for biological processes that are seasonal, such as reproduction), as well as a figure on how the biological parameters were scaled for temperature effects over the

years. This same argument applies to food concentration (which I assume varies by time of year as well).

Author's reply: The 2006-2009 thermal series has been used as a forcing variables inside DEB models in current modelling literature (Sarà et al. 2011, 2013b; Montalto et al. 2014). The most important factors driving changes in energy budgets of ectotherms are body temperature, on which every metabolic rates depends via the Arrhenius relationships (Kooijman 2010), and food. Arrhenius temperature, that is species specific, acts as a correction factors inside DEB models to scale all rates to environmental temperature. At the same time DEB models use the 2006-2009 CHL-a series as a second forcing variables to predict LH-traits of our species. Accordingly, including a new figure of environmental data could make the ms. heavier also as the main object is not to contextualise the effect at that period, but to show that stressor's effect is simulated across a long integrated period.

Reviewer wrote: 2) The authors use hypoxia and acidification, two future stressors, with temperature data from a few years ago. This design ignores the fact that warming is currently the dominant stressors for this species in the Mediterranean Sea and is expected to continue in the future. As the environmental relevance of the study design is not discussed, as written, the results do not match any realistic environmental situation. This counters the original intent of using DEB models to better “predict organismal functional traits, capturing variation across species to solve a very wide range of problems in ecology and evolutionary biology” (L58).

Author's reply: We did not test the effect of increasing temperature as we are pretty sure that the thermal effect is not manifested on a period so short (only 4-years); however other companion papers (e.g. Montalto et al. 2017) tested the effect of increasing temperature on mussel's performances throughout the whole Basin. To extrapolate the effect only two stressors, we carried out simulations under two different latitudinal temperature patterns (Trieste, north Adriatic and Palermo, Southern MED). Anyway, we included some discussion lines about this issue.

Reviewer wrote: 3) Given that reproduction of this species can be quite seasonal, how does the DEB model handle this in terms of estimating reproductive output?

Author's reply: DEB manages the seasonal reproduction throughout thresholds based on the temperature-energy relationships.

Reviewer wrote: 4) L188-189: Why is the hypoxia event randomized by month of the year? Hypoxia would most likely occur during summer warming and stratification. It seems that varying the duration of summertime hypoxia is a more environmentally relevant exercise rather than randomizing what month the hypoxia event occurred in. How long was each hypoxia event?

Author's reply: As we said before, we tested the effect of hypoxia as a stochastic event more than testing is in terms of frequency and timing. Thus, here we adopted a very simple scheme but there is a companion paper still under review (G. Sarà submitted PRS B) whose main aim was to test the effect of duration, frequency and timing of disturbance events on three different invertebrate species through the DEB model.

Reviewer wrote: Statistical approach needs to be justified (see detailed comments)

Author's reply: Statistical approach has been justified in the detailed comments.

Reviewer wrote: The choice of using 2500 umol/kg for total alkalinity (TA) based on an oceanographic study for the lab experiments is strange (L106). Especially in static cultures, mussels can alter the TA of a small body of water. I assume the authors did not measure TA

during the experiment. In such a case, it would be best to simulate the experiment again, and measure TA so the authors have some idea of the TA variation in their experimental conditions could have been. Either way, the calculated pCO₂ parameters will be undefinable without the real TA measurement.

Author's reply: We did not find any paper reporting such alteration. We tried at the same time to minimize the number of organism for each tank and to perform a sufficient weekly water change in order to maintain a stable environment for our organisms, even if in mesocosm condition. We are perfectly aware that the suggested one is without any reasonable doubt the most appropriate approach to follow, but as soon as our is not a study focused on the chemistry of the shell but it is to provide a proof to test the predictive potential of DEB model about the effect of two stressors; thus, the use of a value from oceanographic study should be considered a minor approximation due to the metabolic and mechanistic nature of the paper.

Reviewer wrote: In addition to lacking an environmental context, the Discussion lacks comments on the non-DEB model functional traits (shell strength, dissolution patterns), their relevance to the study, and by what mechanism hypoxia and pH would differentially or synergistically impact the periostracum and shell quality.

Author's reply: we didn't analyse the impact on the shell chemistry and ultrastructure in this ms. whose main objective was to predict the effect of two stressors on mussel's LHs.

Author's changes: However, to accomplish the referee's suggestion, we discussed shortly shell fragility related to pH and to both combined stressor.

Detailed comments:

Reviewer #2 specific comment n. 1

Reviewer wrote: The title does not represent the study and reads as if the paper is a literature review. It would behove the authors include more detail in the title (DEB model, hypoxia, OA). Use of "marine bivalves" is inappropriate given that only one species was assessed.

Author's changes: We agreed with Reviewer's #2 point and changed the title.

Reviewer #2 specific comment n. 2

Reviewer wrote: L27-33: sentence is difficult to follow. Consider breaking this up.

Author's changes: Sentence has been spitted up accordingly.

Reviewer #2 specific comment n. 3

Reviewer wrote: L39: this needs clarification (most functional traits? Which ones?) and references

Author's changes: Although references were already present, following Reviewer #2 suggestion we specified the functional traits which we were referring to.

Reviewer #2 specific comment n. 4

Reviewer wrote: L47-48: plenty of labs conduct OA experiments for months up to at least one year

Author's changes: The sentence has been deleted.

Reviewer #2 specific comment n. 5

Reviewer wrote: L48-54: long sentence, CO₂ vents are unrelated to the second half of the sentence. Consider rewriting.

Author's changes: Sentence has been rephrased accordingly.

Reviewer #2 specific comment n. 6

Reviewer wrote: L69: should AE be 'assimilation efficiency'?

Author's changes: Sentence has been rephrased and clarified.

Reviewer #2 specific comment n. 7

Reviewer wrote: Introduction: break text up into paragraphs. Lacks introduction to functional trait-based models; I was expecting this prior to L54.

Author's changes: Introduction was spitted into paragraphs following suggestion and an introduction to functional trait-based models was added.

Reviewer #2 specific comment n. 8

Reviewer wrote: L92: Mussels were fed ad libitum, but this is an energetics study. So how do the authors know the condition of the mussels used to get model parameters?

Author's reply: As in reply to a similar point raised by Rev#1 we used the locution "ad libitum" to indicate a food concentration saturating the feeding processes of animals over time. Such an experimental maintenance condition is commonly used throughout the current literature when bivalves are maintained with ad libitum condition of food in bioenergetic experiments (e.g. Sarà et al., 2013; Montalto et al., 2014; Tagliarolo et al., 2016).

Reviewer #2 specific comment n. 9

Reviewer wrote: L104: dissolution threshold relates to calcium carbonate saturation state, please include the value here, rather than pH.

Author's reply: Unfortunately we do not have such details on calcium carbonate saturation state. We added image details showing the effect of OA on the external shell layer, but a deep analysis on the chemical composition and alteration was out of the purpose of the present investigation.

Reviewer #2 specific comment n. 10

Reviewer wrote: L109: how was CO₂ dissolved? Where their pumps in all the aquaria to ensure mixing?

Author's changes: Details on how CO₂ was dissolved and of water recirculation were added.

Reviewer #2 specific comment n. 11

Section 2.1: Sampling of what (section title)? How often was water replaced in the treatment tanks? Methods need a better description of how carbonate chemistry was calculated. What was the accuracy of the pH measurements? How often was the water sampled for each of the four parameters? How was oxygen maintained and measured in the treatments?

Author's changes: Section title has been changed accordingly. Details on water changes, aeration, water recirculation and accuracy of pH measurements has been added, while details on the calculation of water chemistry were already present in section 2.1. Further details on sampling of water (8 time a day for pH), and on frequency of oxygen and temperature measurement has been added in the same section.

Reviewer #2 specific comment n. 12

Reviewer wrote: Section 2.2 (L112-120): if there are 25 mussels per tank, and there are 3 tanks, why are only five mussels observed for valve opening and closure? Why were observations made 6 times per day every week rather than fewer times per day but more frequently throughout the experiment? Does time of day matter for this behavior? What about time that food was added? I image that flow rates could affect this behavior, but it's unclear if water motion was the same across all tanks.

Author's reply: We thank the referee for his/her interest on the behavioural part of our paper. The restricted number of observation was chosen in order to make the behavioural session during as less as possible in order to not influence with the operator presence mussel's behaviour. Even if it has not proven sometimes mussels suddenly close when moving in front of the tanks. For this reason we decided to observe less individual but more frequently. We didn't notice any difference in the behaviour during the day as mussels were inside a temperature-controlled room, under constant flow through conditions (according to Widdows and Staff 2006 and Sarà et al. 2013) and exposed to automated artificial daylight. The food was added at the end of the day, after all observations were made.

Reviewer #2 specific comment n. 13

Reviewer wrote: L124: define [pm]

Author's changes: [p_M] has been here defined following suggestion.

Reviewer #2 specific comment n. 14

Reviewer wrote: L134: this equation results in units of O₂ concentration x volume per unit time, oxygen units are not defined and there is no explanation as to how this is converted to [pm], which is in J per cubic cm per hour. What level of oxygen undersaturation was reached by the end of the incubation?

Author's reply: Oxygen concentration ($\mu\text{mol l}^{-1} \text{h}^{-1}$) were first converted in J h^{-1} and then in $\text{J cm}^{-3} \text{h}^{-1}$ using a conversion factor (Kooijman, 2010) and following the current literature (Van der Veer et al., 2006; Ren and Schiel 2008). We never reached lethal oxygen concentration due to the short interval of the measurement as the idea was to simulate a sub-lethal effect as that reach at about 1.5-2.0 mg-l DO.

Reviewer #2 specific comment n. 15

Reviewer wrote: L140: this assumption should be justified

Author's changes: As soon as the first two sentences of the section has the same reference, we moved it at the end of the second sentence.

Reviewer #2 specific comment n. 16

Reviewer wrote: Section 2.3: explain that the same individual was used for the respiration rate followed by AE. It's unclear until the end of section 2.4. Given that the respiration methods continue in the end of Section 2.4, merge Section 2.3 and 2.4.

Author's reply: As answered to Reviewer #1 we do not agree in merging both sections because they represent two different part of metabolic stuff (feeding and respiration). However, we now clear specified that specimens were the same between both measure following Reviewer's #2 suggestion.

Reviewer #2 specific comment n. 17

Reviewer wrote: L141-145: Please explain why AE experiment was not done in treatment water, and justify how AE can then be related to experimental treatments.

Author's changes: We now clearly specified that the experiment was conducted with water specifically treated for each treatment.

Reviewer #2 specific comment n. 18

Reviewer wrote: L162: Again, if food availability is important at the field sites, food availability during the experiment should be known. Is it closer to that of Trieste or Palermo?

Author's reply: As in reply to a similar point raised by Rev#1 we used the locution "ad libitum" to indicate a food concentration saturating the feeding processes of animals over

time. Such an experimental maintenance condition is commonly used throughout the current literature when bivalves are maintained with ad libitum condition of food in bioenergetic experiments (e.g. Sarà et al., 2013; Montalto et al., 2014; Tagliarolo et al., 2016).

Reviewer #2 specific comment n. 19

Reviewer wrote: Section 2.7: How are simulations performed (what code or computer program?)?

Author's reply: Our simulation were performed using R routine, and we specified it in the m accordingly.

Reviewer #2 specific comment n. 20

Reviewer wrote: L180: State what DEB parameters these are.

Author's changes:: DEB parameters are now reported in Table 1.

Reviewer #2 specific comment n. 21

Reviewer wrote: L181: Is AE the same as [pM]? AE was already defined in the Introduction

Author's changes: According to Reviewer's #1 point we checked and fixed both assimilation efficiency and the somatic maintenance costs definition.

Reviewer #2 specific comment n. 22

Reviewer wrote: L185-186: Was this data from week 1 or 4? How is a 4-week acclimation period determined sufficient enough to extrapolate to 4 years?

Author's reply: The [pM] parameter used inside our simulations was that calculated at week #4. All the species specific parameters derived from one species and freely available online on the Add my pet collection were previously determined either by the covariation method through data present on literature or experimentally by short experimental sessions. Even without considering the effect of a stressor, a parameter estimated for a well-fed organism is then used inside simulation making predictions up to 4, 10 or even 50 years, as parameters are assumed to be specific for each species (Kooijman, 2010). We did not account any possible evolutionary effect whose effect is still far to be assessed in the DEB theory.

Reviewer #2 specific comment n. 23

Reviewer wrote: Section 2.9: The assumption of normally distributed residuals is not tested for the ANOVA. This needs to be done before moving forward with ANOVAs. A sample size of 16 is not large. The statistical analyses for valve closure does not match the data collection. By using ANOVAs, I assume all the data are pooled across the 4 weeks. This is not appropriate because it does not account for acclimation and it is a repeated measure since there are only 25 mussels in each tank which were observed over 4 weeks. ANOVAs also don't control for the tank replicate per treatment. The authors need to clarify how the data was pooled (and which behavior was analyzed – open or closed). Since this is binary data, reporting both in the bar graph is duplication the data (Section 3.2), report one, or as a stacked bar graph where each bar graph fills 100%.

Author's reply: The assumption of normal distribution has been tested through the Anderson–Darling test using Past® software. We are aware that the sample size is not large, but pooling the six observation per week we obtain a sample of 24, and we believe it is sufficient for the purpose of the present paper.

Author's changes: We repeated the data analysis and accordingly to the suggestion we compared week1 and week4 using two levels of the factor time and 4 levels of the factor treatment. We analysed the open valve behaviour and following suggestion we decided to use

only one category in the graph. The paragraph, the table and the figure has been modified accordingly.

Reviewer #2 specific comment n. 24

Reviewer wrote: Section 3.1: Analysis comparing experimental treatments seems unnecessary, especially given the uncertainty of the calculated parameters using a poor assumption of TA.

Author's changes: Analysis comparing experimental treatments were removed accordingly to both referee's suggestions.

Reviewer #2 specific comment n. 25

Reviewer wrote: L350-352: is this to be expected?

Author's reply: We thank the referee for the question and we have already answered to this point being highlighted by Referee#1. *M. galloprovincialis* in Sicily is observed to be limited by oligo-trophic conditions although it grows in highly trophic-enriched areas such as harbours or under Integrated Multi-Trophic Aquaculture (IMTA) conditions (Sarà et al 2012; 2013b, Giacoletti et al. 2018 in press JEMA) which supports what we gathered in the present ms. through the DEB simulations.

Reviewer #2 specific comment n. 26

Reviewer wrote: Figures: What is the error bar?

Author's reply: The error bar indicated standard errors for means.

Author's changes: Details were added in each figure following Reviewer's suggestions.

Reviewer #2 specific comment n. 27

Reviewer wrote: L259: capital I

Author's changes:: Replaced.

Reviewer #2 specific comment n. 28

Reviewer wrote: L261: replace M&M with Section #

Author's changes:: Replaced accordingly.

Reviewer #2 specific comment n. 29

Reviewer wrote: Table 1: Include temperature

Author's changes: Temperature included inside Table 1.

Reviewer #2 specific comment n. 30

Reviewer wrote: Table 5: include input parameters

Author's changes: DEB parameters were included in Table 1.

1 **Predicting the multiple effects of acidification and hypoxia**
2 **on *Mytilus galloprovincialis* (Bivalvia, Mollusca) life**
3 **history traits**

4 ~~**Functional spatial contextualisation of the effects of**~~
5 ~~**multiple stressors in marine bivalves**~~

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9
10 **Abstract.** Many recent studies have revealed that the majority of environmental stressors experienced by marine
11 organisms (ocean acidification, global warming, hypoxia etc.) occur at the same time and place, and that their
12 interaction may complexly affect a number of ecological processes. Here, we experimentally investigated the
13 effects of pH and hypoxia on the functional and behavioural traits of the mussel *Mytilus galloprovincialis*, we
14 then simulated the potential effects on growth and reproduction dynamics through a Dynamic Energy Budget
15 (DEB) model under a multiple stressor scenario. Our simulations showed that hypercapnia had a remarkable
16 effect by reducing the maximal habitat size and reproductive output differentially as a function of the trophic
17 conditions, where modelling was spatially contextualized. This study showed the major threat represented by the
18 hypercapnia and hypoxia phenomena for the growth, reproduction and fitness of mussels under the current
19 climate change context, and that a mechanistic approach based on DEB modelling can illustrate complex and
20 site-specific effects of environmental change, producing that kind of information useful for management
21 purposes, at larger temporal and spatial scales.

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

24

1 Introduction

25 Since the dawn of research investigating the possible effects of ocean acidification (OA) on aquatic organisms
26 (e.g. Bamber, 1990), most studies have shown that elevated pCO₂ levels, as predicted for the next century, may
27 affect -to some extent the functional traits (Schoener, 1986; Koehl, 1989) of marine organisms (Feely et al.,
28 2004; Navarro et al., 2013). Referring to functional traits, we consider all those specific traits that define each
29 species in terms of their ecological roles (Diaz & Cabido, 2001), and thereby the species' identity. In marine
30 ectotherms such as bivalves, crabs, sea urchins and fish, these traits include tolerance and sensitivity to
31 environmental conditions (e.g. physiological tolerance limits - Kearney & Porter, 2009) defining the ability of
32 each species to support their own metabolic machinery (Sokolova et al., 2012; Sarà et al., 2014). ~~They further~~
33 include the ability to obtain energy from food, the so-called functional response (Holling, 1959) or those
34 behavioural (e.g. swimming behaviour, habitat use, mating system) and morphological (e.g. shape, thickness)
35 traits ~~(Schoener, 1986)~~ which led to optimise the energetic income (Krebs & Davies, 1992) and lastly to reach
36 the ultimate fitness (Roff, 1992).

37 Research performed over the last decade and summarized in the recent IPCC ~~(2014)~~ report ~~(IPCC, 2014)~~ clearly
38 shows that ocean acidification will affect marine organisms and ecosystems (Connell et al., 2017) in the coming
39 decades, and such projections have stimulated new research that aims to understand the impact on calcifying
40 marine organisms. Reductions in growth and calcification rates are just those kinds of the physiological impacts
41 of ocean acidification (Thomsen et al., 2013; Byrne, 2012; Beniash et al., 2010). While much research showed
42 that low pH may impair most functional traits (e.g. respiration), functions connected with energy uptake such as
43 feeding and assimilation seem to be reduced at a larger extent in many species with expected implications for the
44 amount of energy available for growth and reproduction (Kurihara et al., 2008; Appelhans, 2012; Navarro et al.,
45 2013; Zhang et al., 2015). Such information has been obtained through both acute and chronic exposure to OA
46 but no studies are yet available to assess the potential effects of OA on the magnitude of other Life History (LH)
47 traits, such as maximum habitat body size, fecundity, time to reach maturation and the number of spawning
48 events under future conditions of environmental change (*sensu* Kearney and Porter, 2009; Sarà et al., 2011;
49 2013b). ~~To obtain such LH traits, experiments should be long enough to assure a functional effect of lower pH~~
50 ~~for many weeks or months but probably no existing lab mesocosm could currently assure the stability of~~
51 ~~seawater acidification system for such a long time.~~ Thus, apart from long term experiments carried out in few
52 field sites worldwide (e.g. Ischia [Hall-Spencer et al., 2008] and Vulcano [Duquette et al., 2015] islands) in the
53 Southern Mediterranean Sea and in other Seas (Maug Island [Pala, 2009] or ~~CO₂ vents in the SW~~ southwest

54 Pacific [Connell et al., 2017]) where lowered pH seawater is naturally available ~~through CO₂ natural emissions~~
55 ~~from vents~~, the recent introduction of mechanistic functional trait-based (FT) models based on the Dynamic
56 Energy Budget theory (DEB; Kooijman, 2010) can offer a reliable opportunity for disentangling the effect of
57 ~~OA seawater acidification~~ on LH traits.

58 Functional trait-based DEB (FT-DEB) approach (Kearney and Porter 2009; Kearney et al., 2010; Kearney 2012;
59 Sarà et al., 2011, 2012, 2013a, b, 2018) relies on the quantitative prediction of organismal functional traits and
60 fecundity within the fundamental niche limits of one particular species (Hutchinson 1957). Such an approach
61 aim to exploit mechanistic rules to connect environmental human-induced variability to functional traits
62 (Schoener 1986; Diaz & Cabido 2001) and in turn functional traits to species LH (Stearns 1992) traits. The
63 novelty of the FT-DEB approach relies on its intrinsic mechanistic nature deriving from the fact that it is based
64 on flux of energy and mass through an organism which are traceable processes that are subject to conservation
65 laws (according to the new posited concept of ecomechanics; Denny & Helmuth, 2009; Denny & Benedetti-
66 Cecchi, 2012; Carrington et al., 2015). This provides an exceptionally powerful tool to predict organismal
67 functional traits, capturing variation across species to solve a very wide range of problems in ecology and
68 evolutionary biology (Lika et al., 2011; Kearney, 2012; Pouvreau et al., 2006; Pequerie et al., 2010; Sarà et al.,
69 2011; 2012; 2013a; 2013b; 2014). FT-DEB could provide information about the effect of ~~seawater~~ acidification
70 on the fecundity (as expressed by the number of gametes per life span, the so-called Darwinian fitness;
71 Bozinovic et al., 2011) and the degree of reproductive failure of species providing theoretical predictions about
72 LH traits having implications on population dynamics and community structure throughout the species range
73 (Sarà et al., 2013a). Here, we specifically exploited the FT-DEB model spatially and explicitly contextualised
74 along the Italian coasts under subtidal conditions (~~Kearney et al., 2010; Sarà et al., 2011; 2012; 2013a; 2013b~~),
75 using four-year thermal series and satellite ~~Chlorophyll~~ chlorophyll-a (CHL-a) concentrations, to test the multiple
76 effect due to the combination of pH and hypoxia on the physiological and behavioural traits of our target species,
77 the bivalve *Mytilus galloprovincialis* (Lamarck 1819).

78 Recent insights obtained by the experimental research have shown that OA mainly affects feeding rates (FR),
79 assimilation efficiency (AE) and maintenance costs ~~rates of marine organisms~~ (Appelhans, 2012; Navarro et al.
80 2013; Kroeker et al. 2014; Zhang et al., 2015; Jager et al. 2016). Here, we translated the combined effects of
81 hypoxia and hypercapnia on AE-assimilation and oxygen consumption rates as measured under different
82 treatments into effects on assimilation AE and somatic maintenance costs ~~as expressed by the DEB [p_M]~~
83 ~~parameters~~. Somatic maintenance is a crucial suite ~~This latter is a crucial of~~ functional traits used in recent

84 bioenergetics based on the DEB theory that mechanistically can be used to investigate the role played by
85 multiple stressors on LH traits of organisms by using first principles (Sarà et al., 2014). We further documented
86 the effects of those stressors on *M. galloprovincialis* shells through the use of a scanning electron microscope
87 (SEM), and compared the maximum shell breaking load of treated vs. control specimens. A behavioural analysis
88 completed the frame concerning the individual's response to both single and combined stressors. Carried out in a
89 context of OA, this exercise comprises a first step in linking the fields of ecomechanics and climate change
90 ecology, which should yield a more mechanistic understanding of how biodiversity will respond to
91 environmental change (*sensu* Buckley et al., 2012).

92

93

2 Materials and methods

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

100

101 **2.1 ~~Sampling and e~~Experimental set-up.** Specimens of *M. galloprovincialis* (45 - 55 mm) were provided by
102 the Ittica Alimentare Soc. Coop. Arl. (Palermo) and transferred within 30 minutes to the laboratory. Mussels
103 were then carefully cleaned and placed in a 300L tank filled with natural seawater at room temperature (18-
104 20°C), field salinity (37-38 ~~PSU~~), and fed *ad libitum* with cultured *Isochrysis galbana* (Sarà et al., 2011).
105 ~~According to common experimental procedures for studying the bioenergetics of bivalves (Sarà et al., 2008;~~
106 ~~Ezgeta Balic et al., 2011), m~~Mussels were acclimated for two weeks to reduce stress generated by manipulation
107 and transport (Sarà et al., 2013a); ~~and~~ ~~once~~ acclimated, 200 specimens were randomly divided in groups of 25
108 organisms, transferred to 8 independent rectangular glass tanks of 120L capacity (100 cm long, 30 cm deep, 40
109 cm wide) and kept in a conditioned room at 21°C for 4 weeks according to common protocol with bivalves
110 (Braby & Somero 2006; Fields et al., 2012; Kittner and Riisgård 2005). Tanks 1 to 4 were filled with sea water
111 and continuously with aerated through air pumps ~~and recirculating sea water~~, while Tanks 5 to 8 were not aerated
112 and covered with a plastic film disposed on the water surface, in order to avoid gas-exchanges between air and
113 water. Tanks 1-2 were used as a control (CTRL), while hypercapnia was imposed in Tanks 3-4 (Tr1), hypoxia (2

114 ppm) in Tanks 5-6 (Tr2), and both factor (pH 7.5 and hypoxia) in Tanks 7-8 (Tr3) (see Table 2). Mussels were
115 acclimated to two different nominal pH treatments: (i) pH 8.0 in Tanks 1-2 (CTRL) and 5-6 (Tr2), corresponding
116 to present average pH at the sampling site; and (ii) pH 7.5 in Tanks 2-3 (Tr1) and 7-8 (Tr3), deviating from
117 present range of natural variability and relevant for 2100 ocean acidification scenarios. ~~This last point is~~
118 ~~considered the critical dissolution threshold of calcium carbonate in shelled animals as reported in literature~~
119 (Melzner et al., 2011; Gazeau et al., 2013). The carbonate system speciation ($p\text{CO}_2$, HCO_3^- , CO_3^{2-} , ΩCa and
120 ΩAr) was calculated from pH_{NBS} , temperature, salinity and alkalinity ($T_A = 2.5$ mM; Rivaro et al., 2010) using
121 CO2SYS (see Table 2; Lewis and Wallace, 1998) with dissociation constants from Dickson & Millero (1987).
122 The pH was manually controlled 8 times a day by an electronic pH-meter (Cyberscan 510, Eutech Instruments;
123 accuracy = ± 0.01 pH) and gaseous CO_2 was injected directly into the aquarium through a commercial ceramic
124 diffusor, when required. Oxygen concentration and temperature were monitored with the same frequency
125 through the PiroScience FirestingO2 oxygen logger equipped with a dedicated temperature sensor. Water
126 movement and recirculation were assured by water pumps. Tanks were siphoned at the end of each ~~working~~ day,
127 removing all the faecal material in order to avoid the accumulation of waste products, and 20% of water was
128 weekly changed with specific pre-conditioned sea water for each treatment.

129
130 **2.2 Behavioural observations.** The valve gape of mussels was recorded by means of the two simplest
131 behavioural categories reported in Jørgensen et al. (1988): closed valves and opened valves. Each observation
132 was carried out by an operator with the aim to record changes in the behavioural repertoire of bivalves in
133 response to the exposure to a single stressor (pH or hypoxia) and to both pH and hypoxia, compared to
134 individuals kept in normal environmental conditions. All experiments were conducted at environmental (37-38
135 ~~PSU~~) salinity and with well-aerated sea water through a gentle flow (Ameyaw-Akumfi & Naylor, 1987), except
136 for specimens of Tank 5-6 and 7-8, that were not aerated in order to maintain the hypoxia level set through the
137 gaseous nitrogen. Behavioural observations were repeated six times a day at week 1 and 4 of exposure, on day 7,
138 14, 21, 28, and involved involving 5 random specimens for each treatment.

139
140 **2.3 Oxygen consumption.** The rate of oxygen consumption was determined twice (week 1 and week 4) in a
141 respirometric glass chamber (0.3L) inside a temperature-controlled water bath, in order to ~~compare~~ investigate
142 the effects of multiple stressors ~~by converting rates into~~ metabolic somatic maintenance costs. ~~the DEB~~
143 parameter [\dot{p}_M] (expressed as $\text{J cm}^{-3} \text{h}^{-1}$) linked to the energetic cost of maintenance in order and to integrate it in

144 the standard DEB model. Volume-specific somatic maintenance costs, as expressed by the $[\dot{p}_M]$ parameter ($J\ cm^{-3}$
145 h^{-1}), represent the amount of energy needed to fuel basal metabolism (\dot{p}_M) scaled with the organisms' volume,
146 such as $([\dot{p}_M] = \dot{p}_M/V)$. All determinations were performed at 21°C using filtered seawater with the same pH and
147 oxygen content as that of the respective treatment, stirred with a magnetic stirrer bar beneath a perforated glass
148 plate supporting each individual (~~Sarà et al., 2008;~~ Ezgeta-Balic et al., 2011). The decline in oxygen
149 concentration was measured by a PiroScience FirestingO2 respirometer, capable of four sensor connections. We
150 used a total of $n = 64$ mussels per week, 16 for each treatment (8 for each tank) acclimated as above, fed *ad*
151 *libitum* until the day before the experiment. The decline was continuously recorded for at least 1 h, excluding an
152 initial period (~ 10 min) when usually there is a more rapid decline in oxygen caused by a disturbance of the
153 sensor's temperature equilibration. Respiration rate (RR, $\mu mol\ O_2\ h^{-1}$) was calculated according to (~~Ezgeta-Balie~~
154 ~~et al., 2014;~~ Sarà et al., 2008;–2013b): $RR = (C_{t0} - C_{t1}) \times Vol_r \times 60 (t_1 - t_0)^{-1}$, where C_{t0} is oxygen
155 concentration at the beginning of the measurement, C_{t1} is the oxygen concentration at the end of the
156 measurement, and Vol_r is the volume of water in the respirometric chamber. Volume-specific somatic
157 maintenance costs were then calculated by converting oxygen consumption rates expressed in $\mu mol\ h^{-1}$ in $J\ h^{-1}$
158 through a conversion factor (Kooijman 2010) and then in $J\ cm^{-3}$ (van der Veer et al., 2006; Ren and Schiel 2008)
159 (for the calculation of dry weights refer to the end of section 2.4).

160
161 **2.4 Assimilation efficiency.** ~~Assimilation is the final step of food processing and it represents the efficiency with~~
162 ~~which organic material is absorbed from the ingested food (Kooijman, 2010). The assimilation of food is~~
163 ~~assumed to be independent of the feeding rate per se, but proportional to the ingestion rate (Kooijman, 2010).~~
164 Assimilation efficiency (AE) was measured through the Conover ratio (1966) $AE = (F - E) / [(1 - E)F]$, where
165 F is the ratio between ash-free dry weight (AFDW) and dry weight (DW) for food, and E is the same ratio for the
166 faeces; this represents the efficiency with which organic material is absorbed from the ingested food material.
167 Here, after oxygen consumption measurement, the same 16 specimens of *M. galloprovincialis* per treatment
168 were collected twice (week 1 and week 4) and placed into separate beakers containing 1L of filtered seawater
169 (specific for each treatment) and a magnetic stirrer bar. In order to allow the mussels to open their valves and
170 start their filtration activity, they were given 15 minutes before the introduction of food with an initial
171 concentration of ~ 15,000 *Isochrysis galbana* cells ml^{-1} . After a period of 2 h mussels were moved to cleaned 1L
172 glass beakers with filtered seawater for a period of 12 h, after that the water contained in each beaker was filtered
173 on pre-ashed and weighted GF/C fibreglass filters. Once filtered, filters were washed with 0.5 M ammonium

174 formate (purest grade) to remove adventitious salts (~~Widdows & Staff, 2006~~[Sarà et al., 2013a](#)), dried in the oven
175 (95°C for 24 h) and then incinerated in a muffle furnace (450°C for 4 h). After each step, the samples were
176 weighted using a balance (Sartorius BL 120S ± 1µg). For the calculation of AE, together with the faeces
177 collected from the mussels, filters containing algal food were dried and incinerated as above. After respirometric
178 measurement and the collection of faeces each animal was killed by gentle freezing and dissected, and the shells
179 were separated from the body tissue ~~in order~~ to calculate [the condition index according to Davenport & Chen](#)
180 [\(1987\) \(CI = \(body weight/shell weight\) × 100\), and](#) their individual dry weights ~~and to~~ standardize respiration
181 rates ~~to body weights~~.

182
183 **2.5 Water temperature data.** The main forcing driver of shellfish LH inside DEB models is represented by
184 ~~mean~~ seawater temperature (Pouvreau et al., 2006; Kearney et al., 2010; Kooijman, 2010; Sarà et al., 2011;
185 2013). DEB simulations were run under subtidal conditions (body temperature was expressed by the mean
186 seawater temperature; Montalto et al., 2014) with 4 years-hourly data (Jan 2006 - Dec 2009) of seawater
187 temperature measured about 1 m below the surface at the closest meteo-oceanographic station held in Trieste
188 (LAT 45° 38' 57.81"; LONG 13° 45' 28.58") and Palermo (LAT 38° 07' 17.08"; LONG 13° 22' 16.79"). The
189 period of 4 years is consistent with the normal life span of most Mediterranean shellfishes (Sarà et al., 2012;
190 2013b). Both sites were chosen as they represent two opposite temperature and food conditions for mussel
191 growth in Italy, with Trieste as representative of lower temperature (average 16.98 ± 6.19 °C) and higher food
192 levels (average 1.36 ± 0.37 CHL-a), and Palermo of higher temperatures (average 20.19 ± 4.64 °C) and lower
193 food (average 0.19 ± 0.09 CHL-a). Data are available online from the Italian Institute of Environmental Research
194 (ISPRA) web page (<http://www.mareografico.it/>).

195
196 **2.6 CHL-a dataset.** ~~Chlorophyll a (CHL-a) was~~ derived from satellite imageries (µg L⁻¹;
197 <http://emis.jrc.ec.europa.eu/>) ~~was and~~ adopted as a reliable food quantifier for suspension feeders (Kearney et
198 al., 2010; Sarà et al., 2011; 2012) ~~and was downloaded from the EMIS website (<http://emis.jrc.ec.europa.eu/>).~~

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199
200
201 **2.7 Model description.** The Dynamic Energy Budget ([DEB](#)) Theory provides a general framework that allows
202 to describe how physiological mechanisms are driven by temperature and food availability, and influences
203 growth and the reproductive performances in marine organisms ([Sousa et al., 2010](#); [Monaco et al., 2014](#); [Jusup et](#)

204 | [al., 2017](#)). Following the κ -rule (~~DEB theory~~; Kooijman, 2010) a fixed energy fraction (κ) is allocated to growth
205 | and somatic maintenance, while the remaining fraction ($1-\kappa$) is allocated to maturity maintenance plus
206 | maturation or reproduction. If the general environmental conditions deviates from common natural patterns (i.e.
207 | changes in temperature, food availability etc.) reproduction and growth are consequently affected. According to
208 | the DEB theory, a reduction in growth can be caused either by reduced food assimilation (\dot{p}_A), enhanced
209 | maintenance costs \dot{p}_M, or enhanced growth costs (\dot{p}_G). Using this approach, and through the DEB
210 | parameters ~~derived from Sarà et al. (2012)~~ reported in Table 1, except for the variation in the maintenance costs
211 | \dot{p}_M and in the assimilation efficiency of food (AE) which were experimentally estimated throughout this
212 | study, we performed simulations ~~using the standard version of the DEB model~~ (Nisbet et al., 2010) aimed at
213 | investigating the potential variations in growth and fecundity of our model species. To run the DEB simulations,
214 | local thermal series of selected sites were used together with satellite CHL-a concentrations, obtaining a first
215 | model with environmental conditions. A second model was run with the \dot{p}_M calculated from the oxygen
216 | measurements on specimens of *M. galloprovincialis* from Tanks 3-4 (pH 7.5) simulating a chronic hypercapnia
217 | condition for the full cycle (4 years) and the relative estimated AE. Subsequently, further models were run by
218 | simulating one random hypoxia event (duration = 30 days) for each of the four years of the cycle, then
219 | simulating two yearly events, and so on up to six ~~monthly~~ hypoxia events. The starting month of each event was
220 | randomly chosen for every year with the use of a table of random digits. The \dot{p}_M calculated from the oxygen
221 | consumption rate measurements on specimens from Tanks 7-8 (pH 7.5 and hypoxia) was used in substitution to
222 | \dot{p}_M from pH 7.5 tanks 3-4, coupled with the relative estimated AE, when simulating both stressors.
223 | Simulations were performed using the R routine for Standard DEB model developed by M. Kearney (2012), and
224 | further modified (for use in bivalve modelling) by Sarà et al. (2013). Outputs of the DEB models (~~Sarà et al.,~~
225 | ~~2014~~) were: the maximum theoretical total length of shellfish (TL), the maximum total weight (TW), the total
226 | number of eggs (TRO) produced during a life-span of 4 years, the total number of reproductive events (RE) and
227 | the time needed to reach gonadic maturity (TM) for each treatment.

228

229 | **2.7.1 Model limitation.** DEB models are particularly useful to quantitatively assess the effects of multiple
230 | stressors on LH-traits in an integrated manner, leading to test the hypothesis on how OA may affect the
231 | maintenance costs of living organisms (Jager et al., 2016). Maintenance costs, as defined by Dynamic Energy
232 | Budget Theory (Kooijman, 2010), represent the energy requirement of an organism to survive, excluding
233 | investments in growth, reproduction and development. The volume-specific somatic maintenance costs

234 parameter [\dot{p}_M] within the standard DEB model has been up to date estimated only by indirect approaches
235 through changes in energy content by starvation over time (van der Meer, 2006) or measurements of respiration
236 rate of starved organisms (van der Veer et al., 2006; Ren and Schiel 2008). The idea of quantitatively assess the
237 effect of a stressor including it as a modification of a specific parameter was first introduced by Jager et al.
238 (2016) with the *stress factor* “s” applied to assimilation, maintenance and cost of growth. Thus, after estimating
239 the effect induced by a treatment on the oxygen consumption, in our case expressed as percentage variation, we
240 summed/subtracted the energetic amount due to the effect of a stressor to the species-specific [\dot{p}_M] parameter of
241 *M. galloprovincialis* (Sarà et al. 2012) then we run our models. Previous proposed approaches, taking into
242 account starvation for [\dot{p}_M] estimation, wouldn't be realistically applicable for testing and quantifying the effect
243 of a stressor on the energy budget, without adding a further stressor. Jager et al. (2016) was therefore the first to
244 adopt this concept, although using a simplified DEB model (DEBkiss; Jager et al. 2013) that did not involve the
245 concepts of reserve and maturity that play a central role in DEB theory. Although this may not be considered a
246 reliable measure of maintenance costs but a simpler proxy of metabolic effect, negligible costs for growth and
247 gonadic development stand on the assumption of constant protein turnover throughout the experimental range
248 (Hawkins et al. 1989).

249
250 **2.8 Effects on shell: mechanical strength and SEM pictures.** The functional impact of exposure to pH and to
251 validate the pH effect on morphological structure of valves, was tested on mussels exposed to the two nominal
252 pHs for 4 weeks. Twice (week 1 and week 4), 16 mussels for each treatment were collected and dissected, and
253 both valves were cleaned and dried with absorbent paper. The left valve was then sliced transversely using a
254 circular saw (Dremel® 300 series) to section the whole length of the shell. Age was estimated using the analysis
255 of shell rings proposed by Peharda et al. (2011) by counting the number of rings with the use of a stereo
256 microscope (Leica EZ4). The right valves were instead evaluated for their ~~mechanical~~breaking properties ~~at the~~
257 ~~Department of Mechanical Engineering. Experimental through~~ crushing tests, ~~in order to estimate the shell's~~
258 ~~(maximum breaking load (in N) as a further validation step, were realised with as previously done in Martinez et~~
259 ~~al. (2018) a home-made press previously calibrated by an Instron 3367 machine controlled by the Bluehill 2.0~~
260 ~~software.~~ The effects of low pH exposure ~~were~~ was also documented by the use of a scanning electron
261 microscope (SEM; Zeiss LEO 440) that led to a thorough investigation on the integrity of the mussels' external
262 protein layer (*periostracum*) and on the underlying mineral layer, rich in calcite and aragonite.

263

264 **2.9 Statistical analysis.** [The assumption of normal distribution has been tested through the Anderson–Darling](#)
265 [test using Past® software.](#) In order to test for significant differences in respiration rate and the assimilation
266 efficiency, ANOVAs were performed using Treatment (CTRL, Tr1, Tr2, Tr3) and Time (Week 1 and Week 4) as
267 fixed factors, with respectively four and two levels. In order to test for significant differences in behavioural
268 categories, ANOVAs were performed using Treatment (CTRL, Tr1, Tr2, Tr3) as fixed factors, while Breaking
269 load was tested with Treatment (CTRL, Tr1, Tr2, Tr3) and Time (Week 1 and Week 4) as fixed factors. When
270 significant differences were detected, the Student-Newman-Keuls (SNK) post-hoc pair wise comparison of
271 means was used (Underwood, 1997). Cochran’s test was used prior to ANOVA to test the assumption of
272 homogeneity of variance (Underwood, 1997). When no homogeneous variances were rendered with any type of
273 transformation, the significance level was set at 0.01 instead of 0.05, as ANOVA can withstand variance
274 heterogeneity, particularly in large balanced experiments, thereby reducing the possibility of a Type I error
275 (Underwood, 1997).

276

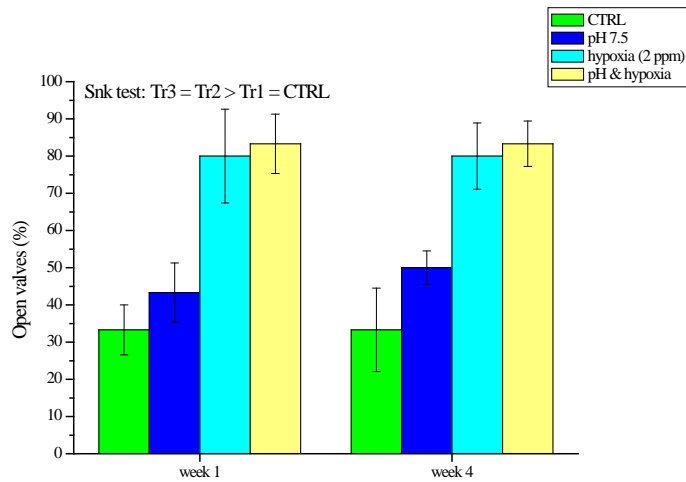
277

3 Results

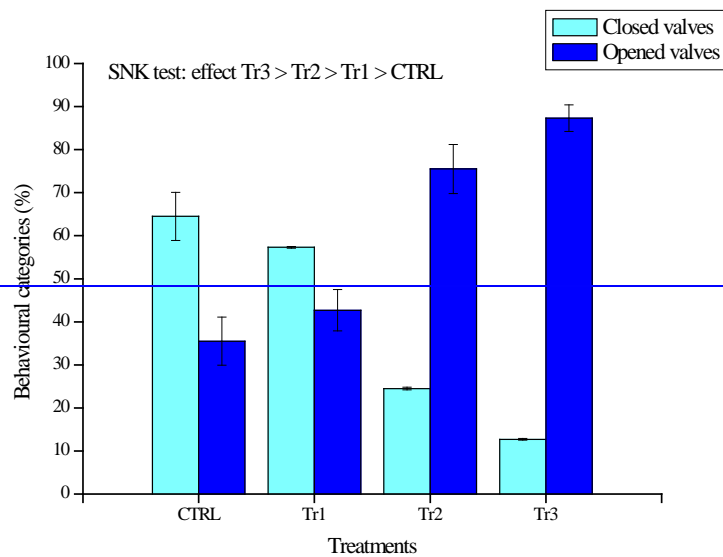
278 **3.1 Water chemistry.** ~~Experimental target pH values were constantly maintained at significantly different levels~~
279 ~~in all tanks (normal pH and 7.5 pH treatments; Table 2; ANOVA, $p < 0.01$; SNK test: CTRL = Tr2, Tr1 = Tr3).~~
280 ~~Oxygen pO_2 was also maintained at significantly different levels between normoxia (7.3 ± 0.02 mg/l) and~~
281 ~~hypoxia (2.4 ± 0.02 mg/l) treatments (Table 2; ANOVA, $p < 0.01$; SNK test: CTRL = Tr1, Tr2 = Tr3). This~~
282 ~~translated into significant different pCO_2 levels in all treatments (Table 2; ANOVA, $p < 0.01$), and in different~~
283 ~~CO_3^{2-} , ΩCa and ΩAr levels in all tanks (Table 2; ANOVA, $p < 0.01$) except between Tr1 and Tr3 (SNK test: Tr1~~
284 ~~= Tr3).~~

285

286 **3.2-1 Valve gaping.** During behavioural observations on *M. galloprovincialis*, specimens showed a significant
287 difference in the behavioural categories, showing respectively ~~64.5 ± 5.6 % (CTRL), 57.3 ± 0.2 (Tr1), 24.5 ± 0.3~~
288 ~~(Tr2) and 12.7 ± 0.2 % (Tr3) of opened valves (Fig. 1; Table 3, ANOVA, $p < 0.001$). The percentage of closed~~
289 ~~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, $p <$~~
290 ~~0.001), 33.3 ± 11.2 (CTRL), 50 ± 4.5 (Tr1), 80 ± 8.9 (Tr2) and 83.3 ± 6.1 of opened valves (Fig. 1; Table 3,~~
291 ~~ANOVA, $p < 0.001$). The percentage of closed valves can be easily calculated as $100 - \text{open valves}$. No~~
292 ~~significant differences resulted between week 1 and 4 (ANOVA, $p > 0.05$), between CTRL and Tr1 and between~~
293 ~~Tr2 and Tr3.~~



294



295

296

297

298

299

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

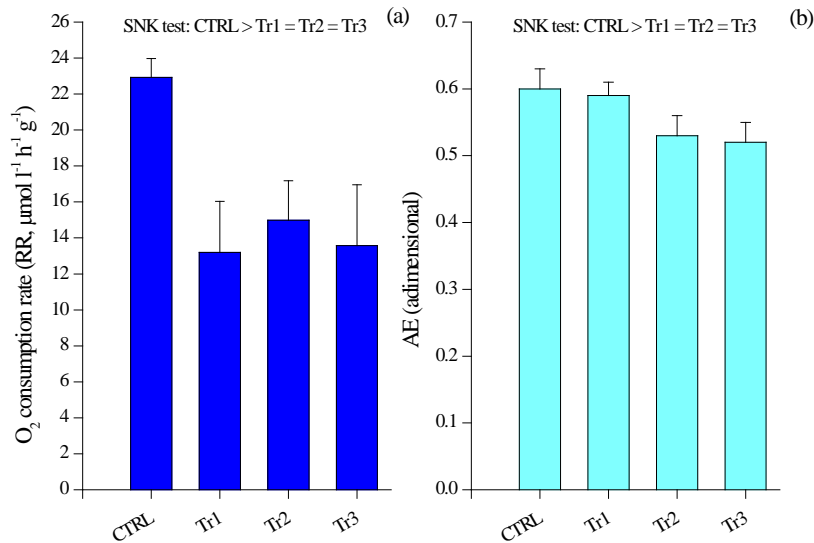
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301

302

3.3-2 Oxygen consumption. Results showed a significant reduction in the oxygen consumption rate by specimens of *M. galloprovincialis* exposed to treatments (Table 4, ANOVA, $p < 0.01$), although the SNK test revealed no significant differences among the various groups (Fig. 2a). No significant effects were highlighted

303 for the time factor (Table 4, ANOVA, $p > 0.05$), so in Fig. 2a we reported only results for week 4. The rate of
 304 oxygen consumption was reduced by up to 42% in Tr1, to 35% in Tr2, and to 41% in Tr3, causing a decrease in
 305 the $[D_M]_{PM}$ by up to 29% in Tr1, to 47% in Tr2, and to 49% in Tr3 across the four weeks of exposure.
 306

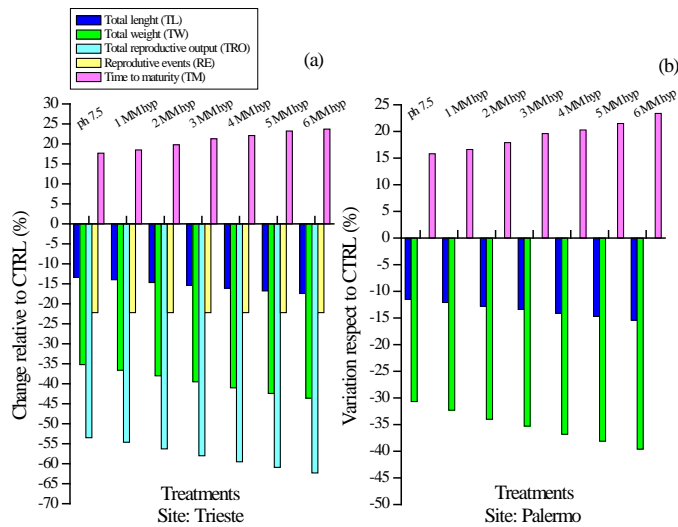


307
 308 Fig. 2 (a) Oxygen consumption rates (RR) and (b) Assimilation efficiency (AE) \pm se of *Mytilus galloprovincialis*
 309 under different treatments of oxygen (normoxia – hypoxia 2ppm) and pH (7.5 – 8.0) at week 4. CTRL = control;
 310 Tr1= pH 7.5; Tr2 = hypoxia 2ppm; Tr3 = pH 7.5 & hypoxia 2ppm.
 311

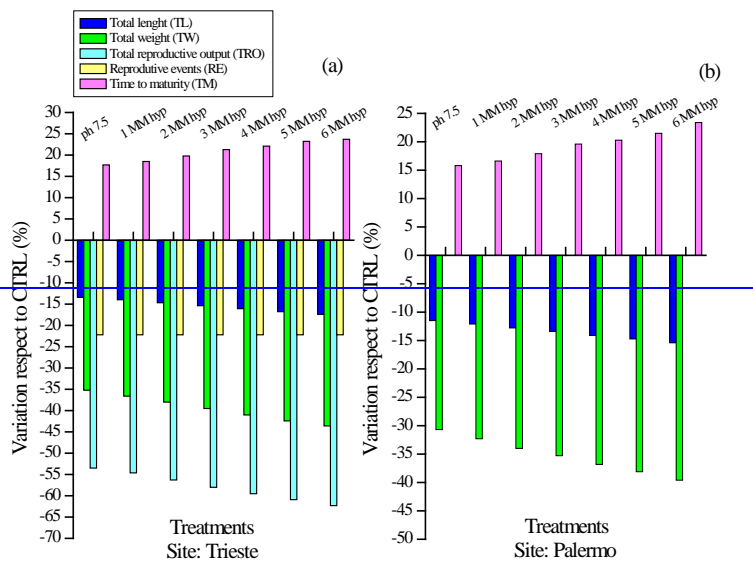
312 **3.4-3 Assimilation efficiency.** Assimilation efficiency of food (AE) ~~resulted was in~~ significantly affected by
 313 treatments (Table 4, ANOVA, $p < 0.001$) after four weeks of exposure. No significant effects were highlighted
 314 for the time factor (Table 4, ANOVA, $p > 0.05$), so in Fig. 2b were reported only results for week 4. In
 315 particular, AE decreased of 2.4% in Tr1, of 12.4% in Tr2, and of 14.4% in Tr3, although the SNK test revealed
 316 no significant differences among ~~the various groups~~ treatments (Fig. 2b). At the end of the 4 weeks exposure, the
 317 BCI resulted comparable ($35.16 \pm 1.12 \%$) to the initial values ($39 \pm 1.9 \%$).
 318

319 **3.5-4 DEB simulation results.** Once $[D_M]_{PM}$ and AE were experimentally estimated under each treatment, we
 320 introduced obtained those parameters were used values under the different treatments to run DEB models and to
 321 obtain the derived in order to predict the effects in on in terms of LH traits. Thus, ~~we performed~~ DEB simulations

322 | were performed under local thermal conditions (as expressed by the thermal series recorded in Trieste and
323 | Palermo; see ~~M&M for details~~Section 2.5) and using satellite CHL-a concentrations (2006-2009) as a proxy of
324 | food. Results showed a remarkable effect exerted by hypercapnia and an increasing ~~addictive effect~~ hypoxia
325 | contribution of hypoxia related to the intensity of disturbance (*i.e.* number ~~of yearly~~ hypoxic events per year) on
326 | LH traits of *M. galloprovincialis* by the end of 4th year (Table 5). Total length (TL) and total weight (TW) in
327 | Trieste and in Palermo were similarly reduced by hypercapnia (Fig. 3), with a progressive additive effect of
328 | hypoxia (Table 5). Total length (TL) resulted unaffected by hypoxia up to a frequency of 2 hypoxia events ($f =$
329 | 0.17; 1/Time), then the Trieste site (representative of eutrophic conditions) reported a larger~~higher~~ effect. On
330 | the opposite the total weight (TW) highlighted a larger~~an higher~~ effect of hypoxia on the oligotrophic site
331 | (Palermo) (Fig. 4). The total number of eggs produced (TRO) and the total number of reproductive events (RE)
332 | in Trieste were strongly reduced by hypercapnia (Fig. 3), with the same progressive ~~addictive effect from~~
333 | hypoxia contribution (Table 5). Maturation time (TM) was affected both in Trieste and Palermo by hypercapnia,
334 | with the same hypoxia contribution previously shown. Palermo showed no reproductive events in the DEB
335 | simulations.



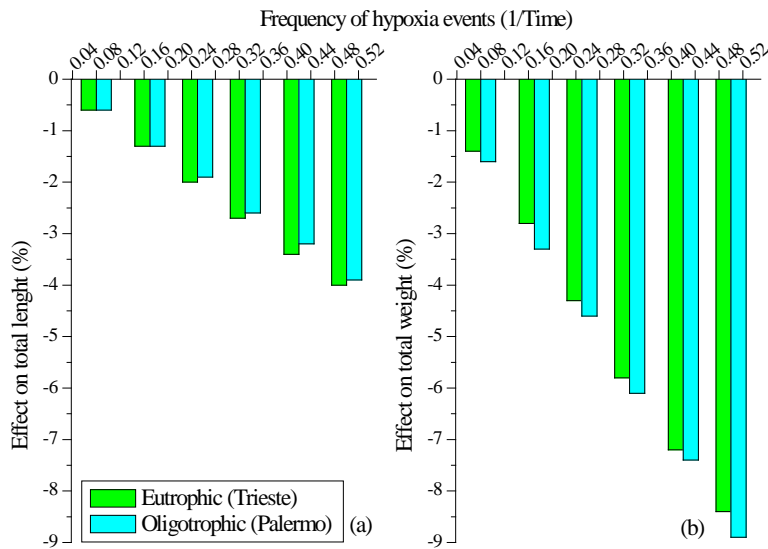
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337

338 Fig. 3 Results from DEB simulation for (a) Trieste and (b) Palermo sites, percentage variation of DEB outputs
 339 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
 340 by hypercapnia, with a progressive additive hypoxia effect up to 8.9%. TRO and RE were reduced by 53.4%
 341 and 66.7% in Trieste by hypercapnia, with a progressive additive hypoxia effect up to 8.8%. TM increased by
 342 17.8% in Trieste and by 15.7% in Palermo with a similar hypoxia effect (up to 7.6%).

343



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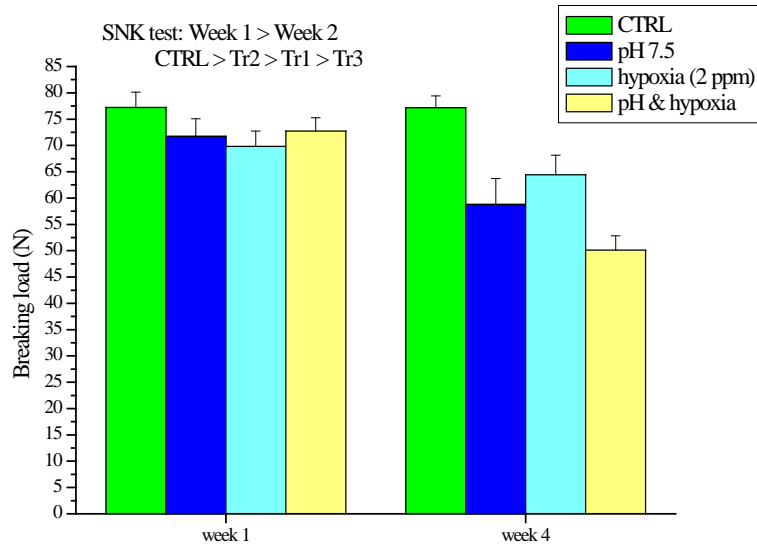
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345 Fig. 4 Percentage effect of Hypoxia from DEB simulations on TL and TW considering the two different trophic
346 conditions represented by Trieste (a) and Palermo (b).

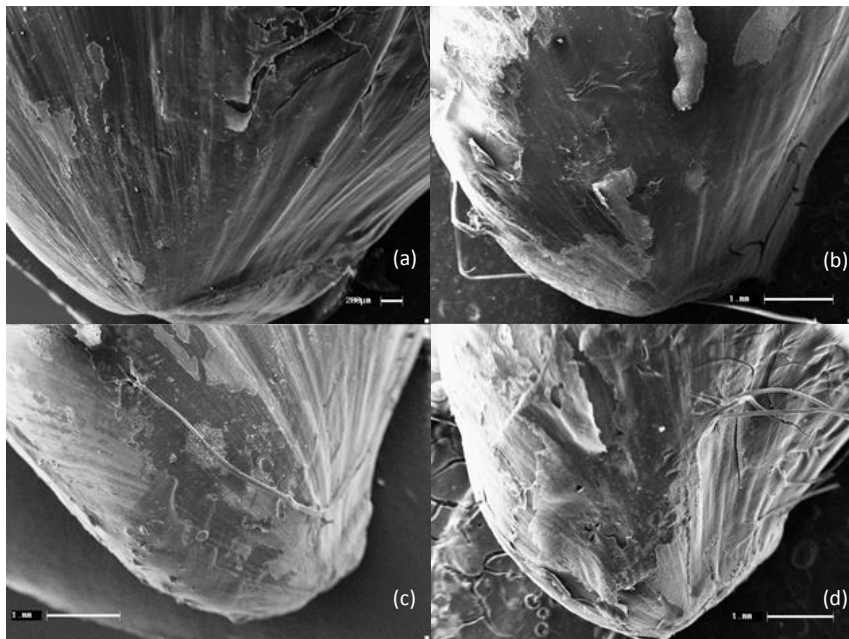
347

348 **3.6 Effects on shell.** Specimens of *M. galloprovincialis* collected ranged in age from 1 to 3 years with a mean
349 age of 1.8 ± 0.04 years ($n = 128$). Overall, 97% of individuals were > 2 years old. Results from the breaking load
350 experiment revealed a significant effect of pH (58.8 ± 5 N) and of combined stressors on the breaking load ($50 \pm$
351 2.7 N), compared to hypoxic (64.4 ± 3.7 N) and CTRL specimens (77.2 ± 2.2 N) (Fig. 45) (Table 3, ANOVA, p
352 < 0.001). In addition, the effect was stronger at week 4 than after one week of exposure (Table 3, ANOVA, $p <$
353 0.01). Deeper investigations through scanning electron microscopy validated an effect by showing an increasing
354 erosion of the shell after exposure to CO_2 -induced acidification. The external dissolution pattern usually started
355 from the umbonal region and progressed toward the margin of the shell, usually associated with some degree of
356 damage to the *periostracum*. The damage was present at differing extensions in all specimens exposed to
357 treatments, except in the control mussels (Fig. 5-6 b, c, d). The alteration of the underlying carbonate layer was
358 instead visible only in Tr1 and Tr3, with details in Fig. 6-7 (b, d). This kind of alteration was never recorded
359 observed under control pH (Fig. 4a).

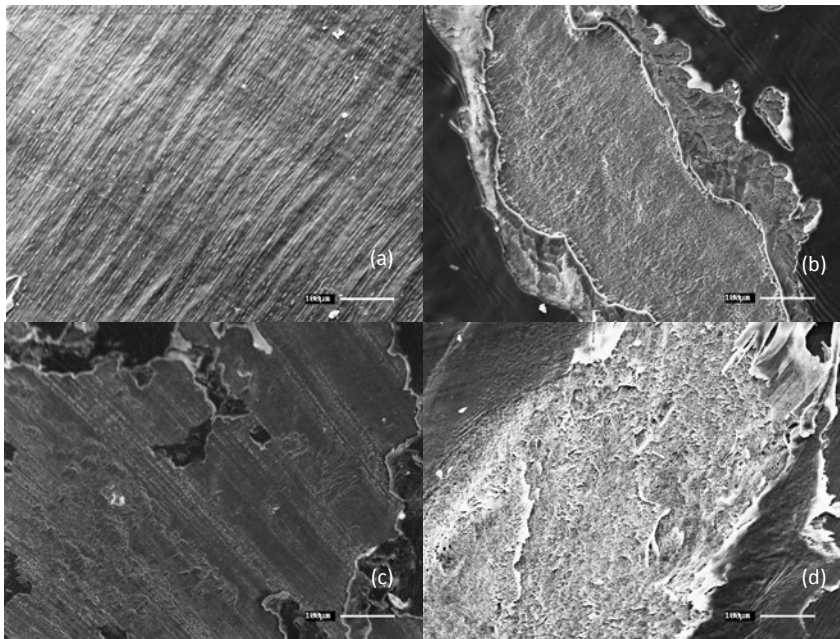
360



361
 362 Fig. 4-5 Breaking load of valves (in Newton, $N \pm se$) exposed to different treatments of oxygen (normoxia –
 363 hypoxia 2ppm) and pH (7.5 – 8.0) at week 1 and 4.



364
 365 Fig. 5-6 SEM pictures of different shells exposed to (a) control condition (CTRL); (b) pH 7.5 and normoxia
 366 condition (Tr1); (c) normal pH and hypoxia condition (Tr2); (d) both pH 7.5 and hypoxia conditions (Tr3).
 367



368
 369 Fig. 6-7 Details of different shells exposed to (a) control condition (CTRL); (b) pH 7.5 and normoxia condition
 370 (Tr1); (c) normal pH and hypoxia condition (Tr2); (d) both pH 7.5 and hypoxia conditions (Tr3).

371
 372

4 Discussion

373 Marine organisms, and in particular intertidal species (Montecinos et al., 2009), have been ~~formally~~-recognized as
 374 being equipped with well-developed and conserved ~~compensatory~~ mechanisms to ~~contrast~~ ~~compensate~~ ocean
 375 acidification such as (i) passive buffering of intra- and extracellular fluids; (ii) transport and exchange of relevant
 376 ions; (iii) transport of CO₂ in the blood in those species that have respiratory pigments; (iv) metabolic suppression
 377 to wait out periods of elevated CO₂ (e.g. Lindinger et al., 1984; Cameron, 1989; Walsh and Milligan, 1989; Hand,
 378 1991; Heisler, 1993; Guppy and Withers, 1999; Pörtner et al., 2004). Several authors recorded ~~suppression~~ ~~of~~
 379 feeding activity and growth, depressed metabolism, increased N excretion and loss of tissue weight for marine
 380 bivalves exposed to reduced seawater pH (Bamber, 1990; Michaelidis et al., 2005; Berge et al., 2006; Gazeau et
 381 al., 2010). Bivalves are in fact capable of maintaining a constant internal pH by decreasing their metabolic rates
 382 and/or dissolving their shell; the shell acting then as a source of CO₃²⁻ (Bamber, 1990; Michaelidis et al., 2005;
 383 Berge et al., 2006) counterbalancing the ~~crossing~~ ~~effect~~ ~~due to lowering of~~ dissolved CO₂ ~~crossing through~~
 384 biological membranes (Fabry et al., 2008). Compensation of low pH, ~~associated with anthropogenic increases in~~
 385 ~~seawater pCO₂~~ (Fabry et al., 2008), through adjustments in ionic composition appears to be a trade-off that is not
 386 likely sustainable ~~on over~~ longer time-scales, ~~such as that associated with anthropogenic increases in seawater~~

387 pCO₂ (Fabry et al., 2008) From our behavioural observations mussels exposed to low pH resulted in a higher, even
388 if not significant, percentage of opened valves respect to CTRL individuals, with the highest significant difference
389 relative to hypoxia exposition. The effect of low pH on the adductor muscle of bivalves has been already
390 documented by Pynnönen & Huebner (1995) and the same effect has been reported after exposition to hypoxia
391 (Sheldon & Walker 1989). In agreement with current literature showing deleterious effects of CO₂-induced
392 acidification on a wide range of invertebrates (Barnhart & McMahon, 1988; Barnhart, 1989; Rees & Hand, 1990),
393 and similarly to other studies by *M. galloprovincialis* (Gestoso et al., 2016; Michaelidis et al., 2005), our results
394 showed how hypercapnia (pH reduced by 0.6 units, relative to the natural pH of the lower Tyrrhenian waters) was
395 able to induce a decline in metabolic rates of mussels. This kind of decline has already been noticed by other
396 authors as an adaptive strategy for survival under transiently stressful conditions (Michaelidis et al., 2005).
397 According to Pörtner et al. (2004), metabolic reduction due to hypercapnia could be a result of acid-base
398 disturbances and therefore be similar to the response of intertidal individuals to anaerobic conditions. Direct
399 effects of hypoxemia have been further proven to cause fatal decrements in an organism's performance in growth,
400 reproduction, feeding, immunity and behaviour (*sensu* Pörtner & Farrell, 2008). ~~Synergistic-Combined effects by~~
401 ~~stressors like such as~~ ocean acidification and hypoxia ~~narrow are capable of narrowing~~ the thermal window of
402 functioning according to species-specific sensitivities, modulating biogeographical ~~distributionses~~, coexistence
403 ranges, community shifts and ~~in general ecological~~ other interactions (Pörtner & Farrell, 2008). The mussel *Mytilus*
404 *edulis* has been proven able to compensate both short- and long-term exposure to hypercapnia by dissolution of ~~its~~
405 shells (Lindinger et al., 1984; Michaelidis et al., 2005), resulting in reduced growth and metabolism. A similar
406 mechanism of release of inorganic molecules into the pallial cavity (as CaCO₃ from valves) has been documented
407 during periods of anaerobic metabolism, to maintain the acid-base balance (Chaparro et al., 2009), determining
408 further physiological and energetic cost such as decreased growth, respiration rate and protein synthesis (Pörtner et
409 al., 2005). During periods of environmental oxygen limitation, many organisms are able to suppress ATP demand,
410 shut down expensive processes, such as protein synthesis (Hand, 1991), but at the same time limiting growth and
411 ~~the~~ reproductive potential. Although suppression of metabolism under short-term experimental conditions is a
412 ~~“sub-lethal”~~ reversible process, reductions in growth and reproductive output will effectively diminish the survival
413 of the species on longer time-scales (Fabry et al., 2008). The contemporary occurrence in our simulations, of
414 monthly hypoxia events, revealed a growing ~~additive~~ contribution to what was already elicited by hypercapnia
415 ~~alone~~ on growth and reproduction. Current literature has not currently explored the combined effects of multiple
416 stressors on long-term experiments by modulating the intensity and duration of disturbance. This would probably

417 translate as a very complex experimental set-up which would be hardly practicable, especially on long-term scales.

418 On the other hand, mechanistic models offer a more ~~sustainable and reliable~~ practical alternative to long-term, in-

419 field research when studying the effects of multiple-stressors-, with the advantage of testing, at the same time, the

420 magnitude and the duration of disturbance on LH_-traits of a model species. Our results highlighted the general

421 hypoxia growing effect following the increasing duration of disturbance, with a particular focus in Trieste on TW

422 and TRO, while in Palermo on TW and TM (~~Table 5~~ Fig. 3). A further important peculiarity of the ~~DEB~~

423 ~~mechanistic modelling simulations~~ deals with the possibility to spatially contextualise the effects of single and

424 multiple stressors on selected outputs by integrating local thermal conditions and food concentrations (Sarà et al.

425 2018c). Comparing the effect of hypoxia across frequencies (Fig. 4), total length (TL) resulted unaffected up to a

426 frequency of 2 hypoxia events in both sites, then the highest effect was recorded in the eutrophic site (Trieste).

427 Trieste, between the two chosen sites, had represented also the one with the lowest temperature. On the contrary a

428 lower-smaller effect of hypoxia was detected resulted considering on the total weight (TW) in the eutrophic

429 site Trieste, suggesting some a sort of food compensation capacity on the effect of environmental stressor

430 (Mackenzie et al., 2014). In particular Also the DEB model easily allowed the estimation of the fecundity potential

431 of ~~cultivated and natural~~ organisms, that is often omitted in other ecological studies, but that represents a crucial

432 quantity for resource (e.g. aquaculture) (Sarà et al. 2018c) and conservation purposes. To verify impacts on

433 shellfish fecundity, we contextualised our simulation by introducing Trieste hourly temperature series after those

434 of Palermo, with the respective local actual CHL-a concentrations, as long as in the first site no reproductive

435 events came out from our simulations, probably due to food limitations and temperature threshold. This is

436 reflected by natural populations in Palermo colonising being represented only in only substrates in highly-trophic-

437 enriched areas sites. A combined effect of the simultaneous stressors, such as those considered across this study,

438 has proven in the present study, through our experimental and mechanistic integrated approach, to affect the

439 organism's performance in growth, reproduction and behaviour. Our results highlighted an effect of pH alone and

440 when combined with hypoxia on the breaking load of shells of our experimental mussels. Through a similar

441 approach, Martinez et al. (2018) showed that temperature was a primary factor driving shell's fragility along a

442 latitudinal gradient. Present findings corroborates that idea that fragility can be affected by both stressors through a

443 combined effect. Multiple stressors Those specific and synergic effects of each stressor seem capable can narrow,

444 especially ~~at when organisms are on the edge of their thermal tolerance range extreme temperatures, of narrowing~~

445 ~~the thermal windows and this has a potential for generating repercussions on ,modulating~~ biogeographical

446 distribution, coexistence ranges, community shifts, food webs and species interactions (*sensu* Pörtner & Farrell,

447 2008). Moreover, an appropriate knowledge of species' biological traits, and a mechanistic understanding of the
448 effect of each stressor, reached through an FT-based approach, will allow the translation of the effects of
449 environmental change into realistic management measures taking into account the optimisation of the species'
450 biological traits (Sarà et al. 2018a,b).

451

452 **6 Conclusions**

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

458

459 **Authors' contributions**

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

463

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Tables

Table 1. DEB parameters for *Mytilus galloprovincialis* (1 = Kooijman, 2010; 2= van der Veer et al., 2006; 3 = Sarà et al., 2011; 4 = Thomas et al., 2006; 5 = Schneider, 2008); Lb, Lp, Ls = length at birth, puberty and seeding respectively; dVw = specific density to convert weight into volume; f = functional response type II $f = X/(XK + X)$; μ_x = chemical potential to convert moles into food energy; SMI is the somatic mass index of both starved and well-fed animals, expressed as somatic ash free dry mass (AFDM, mg); X_K = saturation coefficient expressed as a concentration of chlorophyll a ($\mu\text{g CHL-a l}^{-1}$), where the ingestion rate is half of the maximum.

Symbol	Description	Formulation	Units	<i>Mytilus galloprovincialis</i>	
				Value	Ref
Vb	Structural volume at birth	$V_b = (L_b \times \delta_m)^3$	cm ³	0.0000013	1
Vs	Structural volume at seeding	$V_s = (L_s \times \delta_m)^3$	cm ³	-	-
Vp	Structural volume at puberty	$V_p = (L_p \times \delta_m)^3$	cm ³	0.06	2
δ_m	Shape coefficient	$\delta_m = (Ww \times d_{vw}^{-1}) \times L^{-1}$	-	0.2254	3
{J _{Xm} }	Maximum surface area-specific ingestion rate	{J _{Xm} } = J _X / (f × V ^{2/3})	J cm ⁻² h ⁻¹	8.2	4
ae	Assimilation efficiency	ae = ($\mu_x \times J_x$) / p _A	-	0.88	3
X _K	Saturation coefficient	-	μg l ⁻¹	2.1	3
[E _G]	Volume-specific cost of growth	[E _G] = SMI _{starved} × 23 × (δ_m^3) ⁻¹	J cm ³	5.993	5
[E _M]	Maximum storage density	[E _M] = (SMI _{fed} - SMI _{starved}) × 23 × (δ_m^3) ⁻¹	J cm ³	2.190	2
[p _M]	Volume-specific maintenance cost	[p _M] = p _M / V	J cm ⁻³ h ⁻¹	1	2
κ	Fraction of utilized energy spent on maintenance and growth	-	-	0.7	2
K _R	Fraction of reproductive energy	-	-	0.8	3
T _A	Arrhenius temperature	$T_A = \ln K_{(T_0)} / K_{(T_1)} \times \frac{(T_1 \times T_0)}{(T_0 - T_1)}$	°K	7.022	2
T _L	Lower boundary of tolerance range	-	°K	275	2
T _H	Upper boundary of tolerance range	-	°K	296	2
T _{AL}	Rate of decrease at lower boundary	-	°K	45.430	2
T _{AH}	Rate of decrease at upper boundary	-	°K	31.376	2

Table 42. Seawater carbonate chemistry parameters (mean ± se). Seawater pH on the NBS scale (pHNBS), temperature (T; °C), and salinity were used to calculate CO₂ partial pressure (pCO₂; μatm) as well as aragonite and calcite saturation states (respectively Ω_{ar} and Ω_{ca}), for a total alkalinity of 2500 mmol kg⁻¹.

	Measured	Calculated
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	Temperature (°C)	pH _{NBS}	O ₂ mg/l	Salinity (PSU)	pCO ₂ (µatm)	CO ₃ ⁻	Ω _{ca}	Ω _{ar}
CTRL	20.77 ± 0.01	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	20.77 ± 0.01	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	20.77 ± 0.01	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	20.77 ± 0.01	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				*			*	
	Ω _{ca}				Ω _{ar}			
	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				*			*	

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 17.41	26.03 15.60	***	Treatment (Tr)	3	3838.12	15.18	***	
Time (Ti)	1	1.33	2.08	1.87	ns	Time (Ti)	1	777.19	9.22	**
Tr x Ti	3	0.3056	0.27	ns	Tr x Ti	3	132.92	1.58	ns	
Residuals	40				Residuals	56				
Cochran's C				ns	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

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	Hypoxia events (days)	Frequency (1/Time)	TL (cm)	WW (g)	TRO (n° egg)	RE	TM (days)
Trieste	CTRL	0	0	9.55	11.19	6.74e637889	9	232
Palermo	CTRL	0	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 contributing effect of Hypoxia								
Site	Stressor	Hypoxia events (days)	Frequency	TL (%)	WW (%)	TRO (%)	RE (%)	TM (%)
Trieste	pH+hypoxia	30	0.081-month	-0.6	-1.4	-1.1	0	0.8
Trieste	pH+hypoxia	60	0.172-month	-1.3	-2.8	-2.8	0	2.1
Trieste	pH+hypoxia	90	0.253-month	-2	-4.3	-4.5	0	3.6
Trieste	pH+hypoxia	120	0.334-month	-2.7	-5.8	-6	0	4.4
Trieste	pH+hypoxia	150	0.425-month	-3.4	-7.2	-7.4	0	5.5
Trieste	pH+hypoxia	180	0.506-month	-4	-8.4	-8.8	0	6
Palermo	pH+hypoxia	30	0.081-month	-0.6	-1.6	0	0	0.8
Palermo	pH+hypoxia	60	0.172-month	-1.3	-3.3	0	0	2.1
Palermo	pH+hypoxia	90	0.253-month	-1.9	-4.6	0	0	3.8
Palermo	pH+hypoxia	120	0.334-month	-2.6	-6.1	0	0	4.5
Palermo	pH+hypoxia	150	0.425-month	-3.2	-7.4	0	0	5.7

Palermo	pH+hypoxia	<u>180</u>	month <u>0.506-</u> month	-3.9	-8.9	0	0	7.6
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