© 2015. Published by The Company of Biologists Ltd | The Journal of Experimental Biology (2015) 218, 2148-2151 doi:10.1242/jeb.117705

SHORT COMMUNICATION

Energy metabolism and cellular homeostasis trade-offs provide the basis for a new type of sensitivity to ocean acidification in a marine polychaete at a high-CO_2 vent: adenylate and phosphagen energy pools versus carbonic anhydrase

Lucy M. Turner[1,2,*], Elena Ricevuto[3], Alexia Massa-Gallucci[3], Maria-Cristina Gambi[3] and Piero Calosi[1,4]

ABSTRACT

Species distributions and ecology can often be explained by their physiological sensitivity to environmental conditions. Whilst we have a relatively good understanding of how these are shaped by temperature, for other emerging drivers, such as P_{CO_2} we know relatively little. The marine polychaete *Sabella spallanzanii* increases its metabolic rate when exposed to high P_{CO_2} conditions and remains absent from the CO_2 vent of Ischia. To understand new possible pathways of sensitivity to CO_2 in marine ectotherms, we examined the metabolic plasticity of *S. spallanzanii* exposed *in situ* to elevated P_{CO_2} by measuring fundamental metabolite and carbonic anhydrase concentrations. We show that whilst this species can survive elevated P_{CO_2} conditions in the short term, and exhibits an increase in energy metabolism, this is accompanied by a significant decrease in carbonic anhydrase concentration. These homeostatic changes are unlikely to be sustainable in the longer term, indicating *S. spallanzanii* may struggle with future high P_{CO_2} conditions.

KEY WORDS: Individual approach, P_{CO_2}, Climate change, Homeostatic capacity, Annelid, Mediterranean Sea

INTRODUCTION

An understanding of a species' ecophysiological limits helps us identify the reasons underpinning species and population processes over different spatial scales. Whilst we have a relatively good understanding of how species distributions are shaped by temperature (see Bozinovic et al., 2011), for other emerging environmental drivers, such as increasing seawater partial pressure of CO_2 (P_{CO_2}), which is causing the acidification of the oceans, we know relatively little (see Calosi et al., 2013; Kroeker et al., 2011; Maas et al., 2012). Shallow water, high CO_2 vents have been used as analogues to investigate the potential ecological and evolutionary implications of ocean acidification. In particular, at the CO_2 vent of Ischia the polychaete fauna have been characterised in relation to the venting activity (e.g. Kroeker et al., 2011). Based on their distribution patterns, species found inside and outside these naturally acidified areas can be considered to be either 'tolerant' (abundant both inside and outside the low pH/high P_{CO_2} areas) or 'sensitive' (found outside the vents in similar habitat). Tolerant species include those that are able to maintain their metabolic rate levels unchanged during acute exposure to elevated P_{CO_2}, thus maintaining their energy metabolism and metabolic scope levels. In comparison, sensitive species show extreme decreases or increases in metabolic rates, corresponding to an extreme decrease of aerobic metabolism and an increase in metabolic costs, respectively, with both responses probably leading to a substantial decrease in metabolic scope (see Calosi et al., 2013 and references therein). In general, species that are poor regulators of metabolic rate under high P_{CO_2} conditions have been shown to have lesser homeostatic control, with some undergoing metabolic depression (Melzner et al., 2009). The fan worm *Sabella spallanzanii* (Gmelin, 1791) (Sabellidae) is present in the waters around Ischia (including those near the vents; M.-C.G. and P.C., personal observation), being especially abundant in areas with high nutrient levels, e.g. harbours (Bocchetti et al., 2004). It is absent from the high venting areas, despite showing the ability to increase its metabolic rates when exposed *in situ* to high P_{CO_2} conditions (e.g. Calosi et al., 2013), and thus possibly maintain its metabolic scope, unless it undergoes a physiological trade-off between energy metabolism and other important functions (e.g. enzymatic activities, osmo-ionic or pH intracellular regulation). Thus, *S. spallanzanii* allows us to investigate the biochemical mechanism underpinning a type of sensitivity to high P_{CO_2} not connected to metabolic depression, which may contribute towards explaining the distribution of this polychaete around the CO_2 vent of Ischia.

To determine the extent to which the cellular physiological condition explains the sensitivity of *S. spallanzanii* to ocean acidification, we conducted *in situ* transplant experiments (e.g. Calosi et al., 2013), transferring specimens to either control pH/P_{CO_2} or low pH/high P_{CO_2} conditions, and examined the concentration of fundamental aerobic and anaerobic metabolites and of carbonic anhydrase. Carbonic anhydrase is an essential enzyme involved in an organism's acid–base and respiratory function (see Fehsenfeld et al., 2011), which are key to defining its tolerance to high P_{CO_2}. This approach allowed us to test for the effect of high P_{CO_2} on this species' biochemical metabolic responses, enabling us to unravel possible functional trade-offs among different traits, which may help explain its sensitivity. Our use of an 'individual approach' allowed us to examine the significance of inter-individual variation in the metabolic versus enzymatic responses, which may otherwise remain masked by using an independent samples analysis (see discussion on 'the golden mean' in Bennett, 1987). Furthermore, our study is the first to provide mechanistic evidence for an alternative metabolic pathway of sensitivity to high P_{CO_2} conditions, characterised by a significant reduction in metabolic rates and energy metabolism (e.g. Ivanina et al., 2013). This mechanism seems to be

[1]Marine Biology and Ecology Research Centre, Plymouth University, Drake Circus, Plymouth PL4 8AA, UK. [2]Department of Biological and Environmental Sciences, University of Gothenburg, Box 461, Gothenburg 405 30, Sweden. [3]Stazione Zoologica Anton Dohrn, Villa Dohrn, Ischia 80121, Napoli, Italy. [4]Département de Biologie, Chimie et Géographie, Université du Québec à Rimouski, Rimouski, Quebec, Canada G5L 3A1.

*Author for correspondence (lucy.turner@gu.se)

Received 1 December 2014; Accepted 11 May 2015

The Journal of Experimental Biology

The Journal of Experimental Biology (2015) 218, 2148-2151 doi:10.1242/jeb.117705

underlined by a regulatory trade-off between a fundamental enzyme and the maintenance of this species' metabolic homeostatic machinery upon exposure to high P_{CO_2} conditions. This provides a possible explanation for the distribution of *S. spallanzanii* around the CO_2 vent of Ischia, and further assists our understanding of the variety of mechanisms through which climate change may pose a threat to extant marine biodiversity.

RESULTS AND DISCUSSION

We provide the first evidence for the biochemical sensitivity to high P_{CO_2} of a marine polychaete capable of metabolic up-regulation. The fan worm *S. spallanzanii* is able to sustain its energy metabolism under high P_{CO_2} conditions, increasing both the production of ATP and the dephosphorylation of arginine phosphate, but also shows a reduction in carbonic anhydrase (Fig. 1A). Together, these changes may lead to the overall homeostatic ability of *S. spallanzanii* being compromised under high P_{CO_2} conditions. This may in part explain *S. spallanzanii*'s inability to colonise the high CO_2 vents of Ischia, although this may be coupled with other external ecological factors (e.g. low food supply; Bocchetti et al., 2004). More broadly, our findings further support the idea that differences in marine invertebrate distribution in relation to high P_{CO_2} conditions will, at least in part, depend upon each species' homeostatic capacity and ultimately on their ability to acclimatise and adapt to new environmental conditions (Calosi et al., 2013; Maas et al., 2012).

Our results demonstrate that ATP levels increase significantly upon exposure to elevated P_{CO_2}, as might be expected for a species that is known to increase its metabolic rate in response to exposure to high P_{CO_2} conditions (Calosi et al., 2013). Because the seawater temperature of the acidified site was 1°C lower (supplementary material Table S1), this is likely to have masked what would have been an even more pronounced increase in these metabolic metrics had the two sites been the same temperature. The increase in ATP concentration under high P_{CO_2} conditions was accompanied by a significant increase in arginine concentration (Fig. 1A), which is indicative of the dephosphorylation of arginine phosphate to arginine and therefore evidence of the contribution of high energy phosphagens to the annelid energy pool (Urich, 1994). The concomitant decrease in succinate and D-lactate concentration, which have previously been shown to be the main products of anaerobiosis in annelids (Urich, 1994), indicates that this species had to significantly increase its aerobic capacity to sustain this energy release (Fig. 1A). However, our analyses show this occurred at the expense of carbonic anhydrase, as mean carbonic anhydrase levels decreased with increasing environmental P_{CO_2}, and carbonic anhydrase concentration is negatively correlated with arginine and AMP concentration (minimum r=0.452, P=0.05; Fig. 1B,C). Not only do our results add further to our knowledge of the complex physiological phenotypic responses to ocean acidification in annelids, a currently understudied group within this context, but also they enable us to document a new type of physiological sensitivity to high P_{CO_2} in a marine invertebrate species, that of a trade-off between increasing aerobic respiration and carbonic anhydrase concentration. Finally, our work highlights the benefits of using an individual approach, further demonstrating the powerful insights that we can gain in organismal physiology if we investigate individual variation in single-trait phenotypes and complex phenotypes (e.g. relationships among multiple single-trait phenotypes). In the current study, we specifically explored the contribution of an individual's physiological performance to observed differences in metabolic state (Fig. 1B,C), rather than

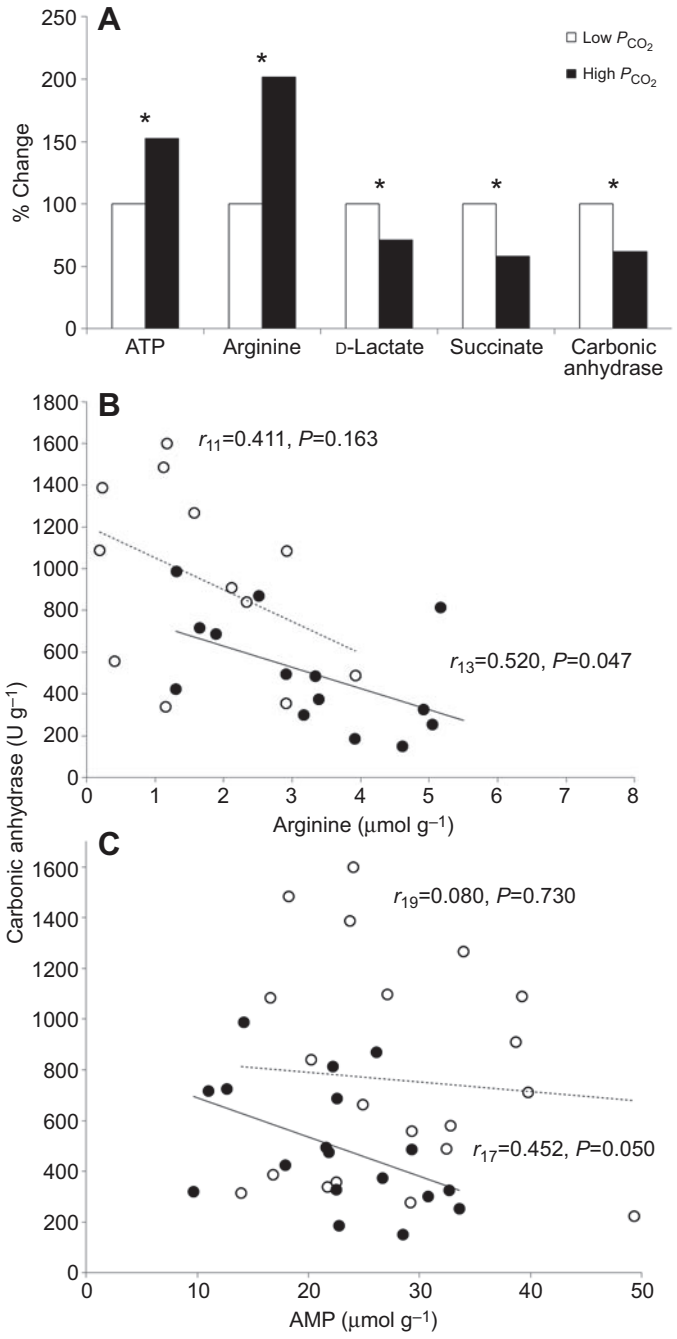

Fig. 1. Metabolite and carbonic anhydrase levels in *Sabella spallanzanii* in response to high P_{CO_2} conditions. (A) Histograms representing the percentage change of mean ATP, arginine, D-lactate, succinate and carbonic anhydrase of the polychaete *S. spallanzanii* collected from low P_{CO_2}/high pH conditions outside CO_2 vents and exposed to (i) low P_{CO_2} conditions outside the vents (control) and (ii) high P_{CO_2} conditions within the vents (acidified). The mean biochemical parameter measured under control conditions was set as 100% and the mean biochemical parameter measured under acidified conditions was recalculated accordingly. *Significant difference between the mean biochemical parameter measured under control conditions and acidified conditions according to the generalised linear model (GLM) test (P<0.05). (B,C) The relationship between individuals' levels of the enzyme carbonic anhydrase and arginine (B) and AMP (C) in *S. spallanzanii* when exposed to high P_{CO_2} (filled circles) or low P_{CO_2} (open circles) conditions. Data points represent individual measurements. The Pearson correlation coefficient and degrees of freedom, together with the probability values (P) are provided. Regression lines are shown as solid or dashed lines denoting significant and non-significant relationships, respectively.

The Journal of Experimental Biology (2015) 218, 2148-2151 doi:10.1242/jeb.117705

Table 1. Mean±s.e.m. metabolite and carbonic anhydrase concentrations in *Sabella spallanzanii* after exposure to control or acidified conditions

Metabolite	Control	*N*	Acidified	*N*	*F*	d.f.	*P*
ATP (μmol g^{-1})	28.56±3.21	19/21	43.56±6.27	19/20	5.920	1	**0.020**
ADP (μmol g^{-1})	17.32±1.70	21/21	14.21±1.65	19/20	1.703	1	0.200
AMP (μmol g^{-1})	27.46±1.99	21/21	22.75±1.63	19/20	3.277	1	0.078
TAN (μmol g^{-1})	70.56±6.37	18/21	82.13±7.47	19/20	1.375	1	0.249
Arginine (μmol g^{-1})	1.67±0.34	12/21	3.38±0.37	15/20	10.718	1	**0.003**
Arginine phosphate (μmol g^{-1})	92.73±17.48	13/21	81.50±10.39	16/20	0.332	1	0.569
Glycogen (μmol g^{-1})	12.89±0.78	21/21	11.88±0.95	20/20	0.687	1	0.412
Glucose (μmol g^{-1})	0.09±0.02	17/21	0.07±0.02	9/20	0.536	1	0.471
L-Lactate (μmol g^{-1})	0.02±0.003	18/21	0.02±0.004	12/20	0.100	1	0.755
D-Lactate (μmol g^{-1})	0.02±0.001	16/21	0.01±0.001	19/20	8.481	1	**0.006**
Succinate (μmol g^{-1})	0.07±0.01	21/21	0.07±0.01	17/20	9.687	1	**0.004**
Malate (μmol g^{-1})	not detected	0/21	not detected	0/20	N/A	N/A	N/A
Carbonic anhydrase (U g^{-1} protein)	761.41±94.92	21/21	472.61±59.72	19/20	6.318	1	**0.016**

Results for the generalised linear model (GLM) tests are reported, with number of specimens investigated (*N*), G-ratio (*F*), degrees of freedom (d.f.) and probability (*P*). Significant *P*-values are given in bold.

analysing average responses of a population, as occurs when we rely solely upon 'golden mean'-type analyses (e.g. Bennett, 1987).

A high P_{CO_2}-induced down-regulation of branchial carbonic anhydrase has previously been shown in other marine invertebrate species, such as the crab *Carcinus maenas*, suggesting that respiratory function and acid–base processes would ultimately be compromised (Fehsenfeld et al., 2011). However, in marine calcifiers such as the bivalves *Crassostrea virginica* and *Mercenaria mercenaria*, exposure to high P_{CO_2} has been shown to generally increase carbonic anhydrase levels to maintain $CaCO_3$ deposition (Ivanina et al., 2013). Whilst the role of carbonic anhydrase in shell calcification is fundamental for calcifying species, in the case of both calcifying and non-calcifying marine invertebrates this increase in carbonic anhydrase is needed to ultimately maintain cellular homeostasis through this enzyme's role in facilitating gas exchange and CO_2 excretion. It therefore appears that when these species are faced with high P_{CO_2} conditions they utilise the up- or down-regulation of carbonic anhydrase as a physiological trade-off to extend their metabolic capacity. Clearly, these types of physiological trade-offs are not without cost. In the current study, despite the fact that *S. spallanzanii* possesses the metabolic plasticity to maintain an elevated aerobic scope when exposed to high P_{CO_2}, at least in the short-term in our *in situ* test, the loss of homeostatic capacity this species undergoes is unlikely to be sustainable in the long term, whilst it is also undertaking (energy-demanding) processes such as growth and reproductive investment. Although other factors may influence to different extents the distribution and/or absence of *S. spallanzanii* from the studied area (e.g. oligothrophic conditions, habitat features, poor competitive ability), the results of this study do lend further support to the idea that it will be those species with greater homeostatic capacity that will be better able to cope with future ocean acidification scenarios (Melzner et al., 2009), as well as helping to explain and predict future adjustments in species' geographic distribution in relation to changes in seawater pH (Bozinovic et al., 2011; Calosi et al., 2013; Maas et al., 2012).

MATERIALS AND METHODS

Animal collection and preparation for *in situ* transplant

Adult individuals of *S. spallanzanii* (*N*=42, mean 9.87 g wet mass) were collected from pontoons in Ischia (40°44′41″N, 13°56′32″E) and Casamicciola (40°44′51″N, 13°54′46″E) harbours, Ischia (control pH, pH 8.13±0.01, mean±s.e.m.), where they are found in large numbers, and transferred to the Benthic Ecology Laboratory (Villa Dohrn, Ischia) within 30 min of collection using cool boxes (~10 l) to minimise thermal stress. Once in the laboratory, worms were maintained for 2 days prior to the experiment in a tank (60 l, ~1.2 individuals l^{-1}) supplied with a flow of natural seawater (*T*=19°C, salinity 38, pH 8.15), and maintained on a dark:light regime of 9 h:15 h. Worms were observed feeding with their branchial crown fully open and responded readily to dark/light stimulus, indicating they were healthy.

Study area, experimental design and procedure

To investigate the potential physiological mechanism underpinning the sensitivity of *S. spallanzanii* to CO_2, an *in situ* transplant experiment utilising the natural CO_2 vents of Ischia was conducted. The specimens collected from control pH/P_{CO_2} areas were transplanted to both control pH/P_{CO_2} and low pH/high P_{CO_2}, mean pH 7.29±0.04 (three stations per treatment). For detailed descriptions of the study areas and deployment areas used, see Calosi et al. (2013). In brief, two experimental areas were selected, an area with acidified conditions with high CO_2 venting activities (>10 vents m^{-2}, mean pH 7.29), which was adjacent to the Castello Aragonese d'Ischia (40°43′53″N, 13°57′47″E), and a control area at San Pietro point (40°44′48″N, 13°56′39″E; mean pH 8.13) directly adjacent to the Benthic Ecology Laboratory (~4 km from the acidified area). For each area, three stations were identified, ~50 m from each other, to allow for spatial replication, and consisted of a weighted line (~2.5 m depth) with a buoy to which the experimental containers or 'transplantation chambers' (TCs) could be attached (~1 m from the bottom). These consisted of cylindrical cages constructed from plastic mesh (15×30 cm, 1 cm mesh), whose size allowed for the continual flow through of seawater, but at the same time prevented the worms from being washed away or predated upon.

On the day of deployment, *S. spallanzanii* specimens were transferred to tanks (~10 l each, seven specimens per tank) and transported to the experimental area (in the case of the acidified area, this was via boat). Immediately before deployment, the worms were carefully introduced to the TCs underwater (*N*=7 per transplantation chamber) and then secured to the main structure using cable ties whilst minimising handling. TCs (*N*=1 transplantation chamber per station) were then immediately deployed by SCUBA to each station in both the control and acidified areas, where they remained for 5 days under natural conditions.

Seawater temperature, salinity and pH were measured at each station daily during the 5 day experimental period and seawater samples were also taken for total alkalinity analysis. Results for the carbonate system (supplementary material Table S1) confirmed that pH and P_{CO_2} differed significantly outside and inside the CO_2 vent areas, consistent with previous studies (Kroeker et al., 2011; Calosi et al., 2013).

After exposure, TCs were recovered by SCUBA, placed underwater in 10 l tanks and immediately transferred to tanks containing fresh seawater of the appropriate pH collected from the respective experimental areas, avoiding any exposure to air. In addition, to avoid thermal shock, *S. spallanzanii* were transported to the laboratory within 30 min. Upon arrival in the laboratory, worms were removed from the TCs, the tube of each worm was gently removed with small scissors, and excess water was

removed via blotting with laboratory roll paper. Worms were then immediately weighed and rapidly snap frozen in liquid nitrogen inside individual Falcon tubes before being shipped to the Marine Biology and Ecology Research Centre (MBERC) in Plymouth, UK, on dry ice. At the MBERC, specimens' ATP, ADP, AMP, L-lactate, D-lactate, malate, succinate, arginine, arginine phosphate, glucose, glycogen and carbonic anhydrase concentrations were determined (see 'Biochemistry' below).

Environmental monitoring and profiles

Seawater temperature, salinity and pH were measured at each station daily during the 5 day experimental period as described in Calosi et al. (2013). To determine seawater total alkalinity (TA), 100 ml samples of seawater were also collected at each station daily [see Calosi et al. (2013) for details] and shipped to the laboratory and poisoned upon arrival with $HgCl_2$ within ∼1 h of collection. Samples were subsequently shipped to the MBERC laboratory (Plymouth, UK), where TA was determined using an alkalinity titrator (AS-ALK2, Apollo SciTech, Bogart, GA, USA).

Dissolved inorganic carbon (DIC), P_{CO_2}, calcite and aragonite saturation (Ω_{calc} and Ω_{ara}), and bicarbonate and carbonate ion concentration ($[HCO_3^-]$ and $[CO_3^{2-}]$, respectively) were calculated from pH and TA measurements as described in Calosi et al. (2013).

Biochemistry

At the MBERC laboratory, specimen levels of ATP, ADP, AMP, L-lactate, D-lactate, malate, succinate, arginine, arginine phosphate, glucose, glycogen and carbonic anhydrase were determined spectrophotometrically in a microplate format. Beforehand, tissue extracts were prepared using a $HClO_4$ extraction protocol.

Tissue ATP, ADP and AMP, glucose, glycogen, arginine and arginine phosphate concentrations were determined based on the methods of Bergmeyer (Bergmeyer, 1985a, 1985b, 1985c). L-Lactate, D-lactate, malate and succinate concentrations were assayed using commercial kits (L-lactate 735, Trinity Biotech Wicklow, Ireland; D-lactate, K-DATE 12/12; malate, K-LMALR/K-LMALL 12/12; succinate, K-SUCC 12/12, Megazyme International Ireland, Wicklow, Ireland). Finally, carbonic anhydrase concentrations were measured using the methods detailed in Ivanina et al. (2013) but adapted to a microplate format.

Statistics

Generalised linear model (GLM) tests were used to investigate the effect of high P_{CO_2} on the mean cellular physiological traits investigated separately, with the term 'station' as a random factor nested within P_{CO_2} treatment and individual body mass as covariate (Table 1). All data met assumptions for normality and for homogeneity of variances (Kolmogorov–Smirnov/Levene's test, $P>0.05$). In a preliminary analysis, the terms 'station' and 'body mass' (within the range tested) were shown not to have a significant effect on the parameters investigated, and were therefore removed. To understand in more depth the biochemical sensitivity of *S. spallanzanii* to high P_{CO_2}, and further validate results from mean analyses, whilst at the same time avoiding any limitation surrounding the use of the 'golden mean', individual approach analyses were also utilised (Bennett, 1987), correlating individual values for traits investigated using the Pearson correlation test. All analyses were conducted in SPSS v. 21.

Acknowledgements

We thank the staff of the Benthic Ecology Centre (Stazione Zoologica at Villa Dorhn, Ischia), particularly Captain V. Rando for help with boat operations.

Competing interests

The authors declare no competing or financial interests.

Author contributions

P.C., L.M.T. and M.-C.G. conceived the study. All authors carried out the fieldwork. L.M.T. was responsible for the biochemical determinations. L.M.T. and P.C. carried out the statistical analyses. L.M.T. wrote the first draft of this manuscript with input from the other authors.

Funding

We acknowledge the support of the following funding: UKOARP NERC (NE/H017127/1) and Plymouth University to P.C., ASSEMBLE (MetabolAdapt) to P.C. and L.M.T., Stazione Zoologica PhD fellowship to E.R., and Campania Region (PON-MODO Project) to A.M.G.

Supplementary material

Supplementary material available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.117705/-/DC1

References

Bennett, A. F. (1987). Chapter 7 – Interindividual variability: an underutilized resource. In *New Directions in Ecological Physiology* (ed. M. E. Feder, A. F. Bennett, W. W. Burggren and R. B. Huey), pp. 147-169. New York: Cambridge University Press.

Bergmeyer, H. (1985a). *Methods of Enzymatic Analysis*, Vol. 6, *Metabolites 1: Carbohydrates*. Weinheim: VCH Verlagsgesellschaft.

Bergmeyer, H. (1985b). *Methods of Enzymatic Analysis*, Vol. 7, *Metabolites 2: Tri and dicarboxylic acids, purine, pyrimidines, inorganic compounds.* Weinheim: VCH Verlagsgesellschaft.

Bergmeyer, H. (1985c). *Methods of Enzymatic Analysis*, Vol. 8, *Metabolites 3: lipids, amino acids and related compounds*. Weinheim: VCH Verlagsgesellschaft.

Bocchetti, R., Fattorini, D., Gambi, M. C. and Regoli, F. (2004). Trace metal concentrations and susceptibility to oxidative stress in the polychaete *Sabella spallanzanii* (Gmelin) (Sabeliidae): potential role of antioxidants in revealing stressful environmental conditions in the Mediterranean. *Arch. Environ. Contam. Toxicol.* **46**, 353-361.

Bozinovic, F., Calosi, P. and Spicer, J. I. (2011). Physiological correlates of geographic range in animals. *Annu. Rev. Ecol. Evol. Syst.* **42**, 155-179.

Calosi, P., Rastrick, S. P. S., Lombardi, C., de Guzman, H. J., Davidson, L., Jahnke, M., Giangrande, A., Hardege, J. D., Schulze, A., Spicer, J. I. et al. (2013). Adaptation and acclimatization to ocean acidification in marine ectotherms: an in situ transplant experiment with polychaetes at a shallow CO_2 vent system. *Philos. Trans. R. Soc. B Biol. Sci.* **368**, 20120444.

Fehsenfeld, S., Kiko, R., Appelhans, Y., Towle, D. W., Zimmer, M. and Melzner, F. (2011). Effects of elevated seawater pCO_2 on gene expression patterns in the gills of the green crab, *Carcinus maenas*. *BMC Genomics* **12**, 488.

Ivanina, A. V., Dickinson, G. H., Matoo, O. B., Bagwe, R., Dickinson, A., Beniash, E. and Sokolova, I. M. (2013). Interactive effects of elevated temperature and CO_2 levels on energy metabolism and biomineralization of marine bivalves *Crassostrea virginica* and *Mercenaria mercenaria*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **166**, 101-111.

Kroeker, K. J., Micheli, F., Gambi, M. C. and Martz, T. R. (2011). Divergent ecosystem responses within a benthic marine community to ocean acidification. *Proc. Natl. Acad. Sci. USA* **108**, 14515-14520.

Maas, A. E., Wishner, K. F. and Seibel, B. A. (2012). The metabolic response of pteropods to acidification reflects natural CO_2-exposure in oxygen minimum zones. *Biogeosciences* **9**, 747-757.

Melzner, F., Gutowska, M. A., Langenbuch, M., Dupont, S., Lucassen, M., Thorndyke, M. C., Bleich, M. and Pörtner, H. O. (2009). Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? *Biogeosciences* **6**, 2313-2331.

Urich, K. (1994). *Comparative Animal Biochemistry*. Berlin: Springer-Verlag.