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REVIEW

What can an ecophysiological approach tell us about the physiological responses of marine invertebrates to hypoxia?

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ABSTRACT

Hypoxia (low O_2) is a common and natural feature of many marine environments. However, human-induced hypoxia has been on the rise over the past half century and is now recognised as a major problem in the world's seas and oceans. Whilst we have information on how marine invertebrates respond physiologically to hypoxia in the laboratory, we still lack understanding of how they respond to such stress in the wild (now and in the future). Consequently, here the question 'what can an ecophysiological approach tell us about physiological responses of marine invertebrates to hypoxia' is addressed. How marine invertebrates work in the wild when challenged with hypoxia is explored using four case studies centred on different hypoxic environments. The recent integration of the various -omics into ecophysiology is discussed, and a number of advantages of, and challenges to, successful integration are suggested. The case studies and -omic/physiology integration data are used to inform the concluding part of the review, where it is suggested that physiological responses to hypoxia in the wild are not always the same as those predicted from laboratory experiments. This is due to behaviour in the wild modifying responses, and therefore more than one type of 'experimental' approach is essential to reliably determine the actual response. It is also suggested that assuming it is known what a measured response is 'for' can be misleading and that taking parodies of ecophysiology seriously may impede research progress. This review finishes with the suggestion that an -omics approach is, and is becoming, a powerful method of understanding the response of marine invertebrates to environmental hypoxia and may be an ideal way of studying hypoxic responses in the wild. Despite centring on physiological responses to hypoxia, the review hopefully serves as a contribution to the discussion of what (animal) ecophysiology looks like (or should look like) in the 21st century.

KEY WORDS: Comparative animal physiology, Ecophysiology, Field experiments, Hemocyanin, Natural experiments, -omics approaches, Oxygen, Respiration biology

Introduction

Hypoxia occurs in marine systems when substantial biological O_2 demand and restricted water movement co-occur. It is a natural feature of enclosed (e.g. rock pools, sediments), semi-enclosed (e.g. poorly mixed fjords) or seemingly open (e.g. the oxygen minimum zone, deep ocean basins, hydrothermal vents, cold seeps) systems (Taylor, 1988; Burnett, 1997; Rosenberg et al., 2002; Levin, 2003; Garcia et al., 2010; Decelle et al., 2010). However, this natural feature can be exacerbated by anthropogenic stress. Coastal eutrophication results in accumulation of particulate organic matter, which initially enhances microbial activity and O_2 consumption. This, in turn, leads to coastal waters becoming periodically hypoxic, producing so-called 'dead zones' (Garcia et al., 2010). Both the number and extent of dead zones have increased dramatically (Diaz and Rosenberg, 2008; Stramma et al., 2010; Diaz et al., 2011) since Rosenberg (Rosenberg, 1985) suggested that eutrophication/hypoxia was the 'future coastal nuisance'. Furthermore, significant de-oxygenation has occurred since the 1960s in the north Pacific and tropical oceans, which Keeling et al. (Keeling et al., 2010) suggested means that even larger changes are looming. There is also lively discussion on how global warming will directly affect the amount of dissolved O_2 in the seas and oceans (Shaffer et al., 2009; Keeling et al., 2010). Chan et al. (Chan et al., 2008) reported the first record of the rise of water-column shelf anoxia off the northern California, which, they claim, highlights the potential for rapid and discontinuous ecosystem change in coastal waters, waters that support most of the world's fisheries. There has never been a time that our understanding of how hypoxia affects marine life has been so important.

Good information is available on the effect of hypoxia (and anoxia) on vertebrates, partly because of its medical importance (e.g. Hochachka, 1990; Lutz and Storey, 1997; Boutilier, 2001; Richards et al., 2009; Gorr et al., 2010; Roach et al., 2013). However, comparatively less is known about how hypoxia affects the physiology of marine invertebrates (Vernberg, 1972; Mangum and van Winkle, 1973; De Zwaan, 1977; Herreid, 1980; Pamatmat, 1980; Ellington, 1983; McMahon, 1988; McMahon, 2001; Gnaiger, 1991; Taylor and Atkinson, 1991; Pörtner and Grieshaber, 1993; Grieshaber et al., 1994; Lutz and Storey, 1997; Mill, 1997; Burnett, 1997; Mangum, 1997; Larade and Storey, 2002; Wu, 2002; Hourdez and Lallier, 2007; Seibel, 2011). Whilst many of the molecular, cellular and tissue responses of marine invertebrates to hypoxia may have some commonality with each other, with vertebrates and even humans (e.g. Haddad, 2006), and we have access to 70+ years of excellent studies carried out by comparative physiologists on some of these groups (references above), we still need to construct an understanding of how environmental hypoxia affects the physiology of 'wild' marine invertebrates from the molecular level up to the whole organism. It will be suggested that such an understanding may come from taking what will be termed an ecophysiological approach to our understanding of how hypoxia affects (and could affect) the physiology of marine invertebrates.

What is ecophysiology?

Interestingly, there still seems little firm agreement on what (animal) physiological ecology or ecophysiology actually is (Tracy and Turner, 1982; Bartholemew, 1987; Bennett, 1987; McNab,

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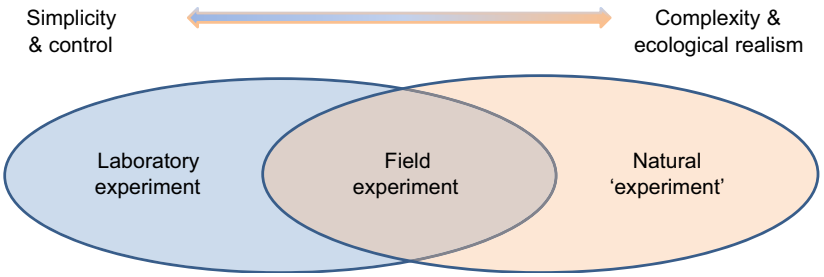

Strengths		
Can draw causal conclusions	Can make some causal conclusions	Ecologically realistic
Extraneous variables minimised	More ecological realism	
Easily replicated	Little experiment effect	
Weaknesses		
Not realistic – contrived	Less control than laboratory	Cannot draw causal conclusions
Experiment effects	More ecological realism	Many extraneous factors
	Little experiment effect	'Flawed' data

Fig. 1. Diagram illustrating the relationship between the three different but related experimental approaches used by ecophysiologists, and the strengths and weaknesses of each. In laboratory experiments, investigations are carried out in a specially designed environment where all variables apart from the one of interest can be controlled. In field experiments, the investigator still attempts to carry out laboratory-type manipulations but the experiments are carried out in the field. The natural 'experiment' is actually a quasi-experiment that takes advantage of the fact that the environmental variable of interest varies naturally, so its effect on a physiological mechanism or response can be recorded. This final approach is often serendipitous.

2002), and there is even the inference that the term has been superceded (Feder and Mitchell-Olds, 2003). A strange situation perhaps for a research area that 'has remained an orphan, healthy and growing, but at home neither in comparative physiology nor in ecology and evolution' (Brown in McNab, 2002). And yet is this surprising? To understand 'how animals work' in the wild demands that the ecophysiologist draw on the strengths and capabilities of a number of different disciplines encouraging collaboration. Ecophysiology is no orphan. Its 'home', it is suggested, is in the 'how does this work' question. Addressing this question requires careful integration of laboratory, field and 'natural' experiments (Fig. 1). Its home is not in any (or all) of the disciplines on which it draws. Comparative animal physiology (CAP) has its context firmly in chemistry and physics. The techniques of ecophysiology have largely been those of the modern comparative animal physiologist, but the context of ecophysiology, it is suggested, is ecology [in agreement with McNab (McNab, 2002)]. As indicated above, the disciplines it can and does draw on are numerous. Bartholemew (Bartholemew, 1987) believed that ecophysiology had its origin in natural history, and McNab (McNab, 2002) (quoting Heinrich) refers to ecophysiology as 'modern natural history, a science rooted in mechanisms'.

Bennett (Bennett, 1987) suggested that ecophysiology was wider than just 'how animals work in the wild' and should encompass how they adapt to their environment, i.e. how they could work or why they work the way they do in the wild. Incorporating evolutionary biology, as Bennett (Bennett, 1987) (amongst others) suggested, brings us close to McNab's (McNab, 2002) modification to the statement quoted above: the techniques of ecophysiology are largely those of the modern comparative animal physiologist, but its context is ecology and evolution.

While there continues to be much discussion about what ecophysiology actually is, I suggest that a good starting place to define the ecophysiological approach adopted here is in a phrase from Speakman's review of McNab's *Physiological Ecology* (McNab, 2002): 'This is a book about physiology in wild animals that contains no molecular biology' (Speakman, 2002).

Aims and objectives

This present work tackles the question 'what can an ecophysiological approach tell us about the physiological responses of marine invertebrates to hypoxia?' This will be addressed using two parts of the above quote from Speakman's (Speakman, 2002) review to centre our discussion around.

1. '…physiology in wild animals…'. We will explore the extent to which laboratory experiments tell us about how marine invertebrates work in the wild, and also address the related question: does taking physiological measurements or performing experiments 'in the wild' bring us closer to the 'real world' of understanding what physiological responses actually occur in wild animals? This is done by examining four case studies that combine laboratory and field investigations of the effects of environmental hypoxia on marine invertebrates. These are drawn largely from my own work, allowing a more critical approach than would be charitable with other people's studies, but also as a reminder (to myself as much as anyone else) that work done in the past should not be forgotten or ignored; those who do not know their historical literature are destined to repeat it.

2. '...that contains no molecular biology.' The fourth case study takes an -omics approach to understanding how hypoxic marine invertebrates work in the wild. However, this discussion must be prefaced by the following. Ten (plus) years on from Speakman's (Speakman, 2002) review, it is worth asking (1) to what extent have inroads been made by ecophysiology, not just in accommodating but also integrating the -omics, into this disciplinary convergence, and (2) will (should) such integration change our understanding of the effect of environmental hypoxia on marine invertebrates, firstly in laboratory experiments and secondly in the wild?

To conclude, the final section of this review summarises (1) what an ecophysiological approach can and does tell us about the physiological responses of marine invertebrates to hypoxia and (2) how the integration of the -omics has modified and will continue to modify this understanding. While primarily about physiological responses to hypoxia of marine invertebrates, this review also contributes to the discussion of what ecophysiology more generally looks like (or should look like) in the 21st century.

‘Physiology in wild animals...’

Case study 1. Response of crustaceans to nocturnal hypoxia in rockpools (enclosed)

High intertidal rock pools experience diel and periodic changes in environmental factors including O_2 (Taylor, 1988). While hyperoxia may occur due to algal photosynthesis during the day (particularly if sunny), during the night animal and algal respiration can reduce dissolved O_2. How O_2 uptake and transport by high intertidal species responds to acutely declining O_2 tensions is relatively well known from laboratory studies (Taylor, 1988; McMahon, 1988; McMahon, 2001). Crabs and shrimp, for example, show good powers of oxy-regulation, as a result of a combination of hypoxia-sensitive hyperventilation, increased cardiac output, recourse to anaerobic metabolism and possession of a lot of high-O_2-affinity respiratory pigment, haemocyanin (Hc).

Taylor and Spicer (Taylor and Spicer, 1987; Taylor and Spicer, 1989; Taylor and Spicer, 1991) investigated some of the physiological differences in the hypoxic responses of two closely related prawns, the high intertidal *Palaemon elegans* and the low intertidal/subtidal *P. serratus*. Respiratory and metabolic responses of *P. elegans* were better than those of *P. serratus* during hypoxic exposure, i.e. they possessed a lower critical O_2 tension for O_2 uptake (P_{crit}, or the point at which oxy-regulation breaks down), lower ventilation and cardiac activity, higher Hc O_2 affinity, better developed anaerobic capacity, less extracellular acid-base disturbance and faster recovery from that disturbance. These species differences are consistent with what is known of their distribution on the shore, and seem to provide a physiological explanation of their differential distribution. However, during a study of physico-chemical parameters of rock pool water *in situ*, it was noted that as an uncovered high shore pool became markedly hypoxic during the night, *P. elegans* came to the surface and lay on its side in the shallows, with one of its gill chambers partially emersed (J.I.S., personal observations). Its swimming appendages beat furiously, disturbing the water surface, and within the gill chamber the scaphognathite agitated the air–water interface. Occasionally prawns violently tail-flicked, which launched them clear of the pool and they lay totally emersed on the rocks. Previous to this observation prawns had been caged in hypoxic pools to gauge their ‘realistic’ metabolic response to hypoxia (Taylor and Spicer, 1987), but this caging prevented individuals from emersing themselves. Hypoxia-related emersion is a common response of intertidal fish (Richards, 2011) and crabs (Wheatley and Taylor, 1979). However, the hypoxia-related partial emersion response shown by *P. elegans* seemed to allow an intertidal animal with no specific physiological or morphological adaptations to emerse itself to survive brief periods of aquatic hypoxia, but without drying out. A subsequent laboratory experiment inspired by this observation found that haemolymph O_2 was higher and haemolymph L-lactate (end-product of anaerobic metabolism) was lower in partially emersed individuals compared with individuals either immersed in hypoxic water or fully emersed in humidified air (Taylor and Spicer, 1988). *Palaemon serratus* did not display this partial emersion response. Thus while the physiological differences between the species could be correlated with their ecology, and *P. elegans* seemed well suited physiologically to deal with hypoxic water, behavioural change and difference were crucial factors in correctly interpreting the physiological findings. For *P. elegans*, behaviour partially negated the physiological effects (lactate accumulation, reduced O_2 in arterialised haemolymph) of being in a pool that was becoming progressively more hypoxic.

Only comparatively recently have these studies been followed up and the crucial experiment performed, investigating the physiological consequences of the partial-emersion response of *P. elegans* to hypoxia *in situ* (J.I.S., unpublished). A combined field and natural ‘experiment’ was carried out. *In situ* L-lactate accumulation in partially emersed *P. elegans* was found to be significantly less than either that of immersed or emersed individuals (Fig. 2), and was also considerably less than predicted by the laboratory experiments alone (Taylor and Spicer, 1988). However, the logistic and technical difficulties in carrying out natural ‘experiments’ (where the investigator has very little control over what is happening, and controlling for critical features such as exposure time is not really possible) were considerable. Although the design of the natural ‘experiment’ is ‘flawed’ (and so publishing it on its own would be difficult), the results are still invaluable for interpreting the laboratory study. To conclude, laboratory experiments on their own were not enough to elucidate the actual physiological response to hypoxia of these prawns in the wild – they also required field experiments and natural ‘experiments’ even with their, often severe, limitations. In this instance, potential physiological responses were modified by behavioural change.

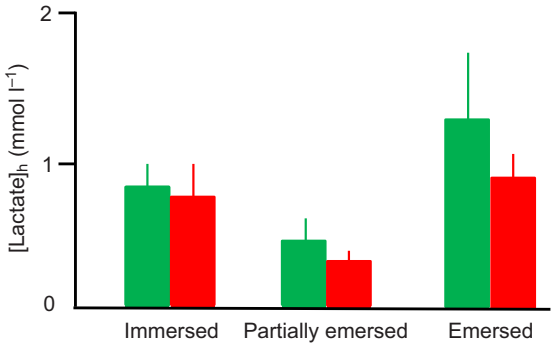

Fig. 2. Concentrations of L-lactate in the haemolymph of caged and free-living *Palaemon elegans* exposed to natural progressive hypoxia (lowest P_{O_2}=3.8–2.1 kPa) *in situ* (22 August 2010) in a high intertidal pool, Wembury Bay, Devon, UK, in the middle of the night (J.I.S., unpublished). Values are means ± 1 s.d. Numbers in parentheses are sample sizes. Caged individuals (green bars, *N*=5 in each case) were placed in mesh containers and left for 1 h before they were removed and their haemolymph sampled. The cages containing immersed individuals were placed in a damp location (on top of seaweed), protected from the wind. The cages containing emersed individuals were placed in the deepest part of the pool in a manner that prevented prawns from initiating their partial emersion response. The cages containing partially emersed individuals were placed in the shallows of the pool where prawns had the choice of exhibiting the partial emersion response. Free-living individuals (red bars) that inhabited this pool were sampled whenever possible and designated as partially emersed or emersed, and the duration of the behaviour was recorded. There are few samples of emersed individuals as those that did tail flick out of the pool either tail flicked and found their way back into the water or died within 30 min of emersion. Consequently, the emersed individuals sampled were only emersed for a maximum of 15–20 min. Partially emersed individuals had been partially emersed for between 24 and 46 min. Immersed individuals were collected from beneath rocks in the deep part of the pool after the prawns from the caged experiment were harvested. There was no way of knowing whether these individuals had exhibited a partial or full emersion response earlier. All haemolymph samples were kept on ice and analysed for L-lactate, using a well-established method, within 4 h of sampling.

Case study 2. Diel migration of northern krill into hypoxic water (semi-enclosed)

Northern krill, *Meganyctiphanes norvegica*, are a predominantly open-water species occurring in regions that rarely experience environmental hypoxia (Spicer et al., 1999). However, they also occur in deep-water fjords, which, depending on their bathymetry,

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may become seasonally hypoxic, e.g. the Gullmarsfjord, southwest Sweden. Early studies from the Gullmarsfjord characterised northern krill as a species with some ability to oxy-regulate under acutely declining O_2 tensions (van den Thillart et al., 1999). However, they seemed to alter their diel vertical migration (DVM) if the deeper water was hypoxic. They migrated down to the pycnocline but did not traverse it (Bergström and Strömberg, 1997). Occasionally the annual flushing of the Gullmarsfjord, which restores normoxic conditions, fails and the deep water in the fjord remains severely hypoxic. This happened in 1997. As a result, the benthic fauna were decimated at 80–100 m depth and disappeared beyond 100 m depth (Rosenberg et al., 2002). This 'fortuitous' event allowed a natural 'experiment' and a field experiment on the effect of hypoxia on the DVM and physiology of northern krill (Spicer et al., 1999) to be conducted concurrently. Laboratory studies confirmed that northern krill had a modest oxy-regulatory ability under acutely declining O_2 conditions, but the critical O_2 tension was much greater than the environmental O_2 tensions it would encounter if it carried out its 'normal' DVM (Strömberg and Spicer, 2000). The capacity for anaerobic metabolism was also poor, and anoxia tolerance was <1 h (Spicer et al., 1999). And yet twice a day, northern krill migrated into deep hypoxic water, and experienced O_2 tensions *in situ* lower than the laboratory-determined, critical O_2 tension for oxy-regulation (Spicer et al., 1999; Strömberg and Spicer, 2000). Manipulating krill DVM by caging individuals at different depths showed that if they had migrated as deep as they usually did (90 m), they would die (Fig. 3). Even at the depth they occur at during the day (60–70 m), L-lactate accumulation was pronounced and close to the maximum generated by 1 h anoxia. Indeed, if caged krill were retrieved any later than they would have naturally ascended, mass mortality was noted (J.I.S., unpublished observations). These data were not published because, at the time, it was felt that the replication was insufficient. Trawling krill from different depths throughout the day and sampling them immediately post harvest (i.e. a natural 'experiment') confirmed the results of the field experiment. Northern krill seem very poorly equipped to deal with hypoxia and yet in the Gullmarsfjord, during our investigation, they migrated into hypoxic water twice a day, before returning to the surface just before their physiology was overwhelmed.

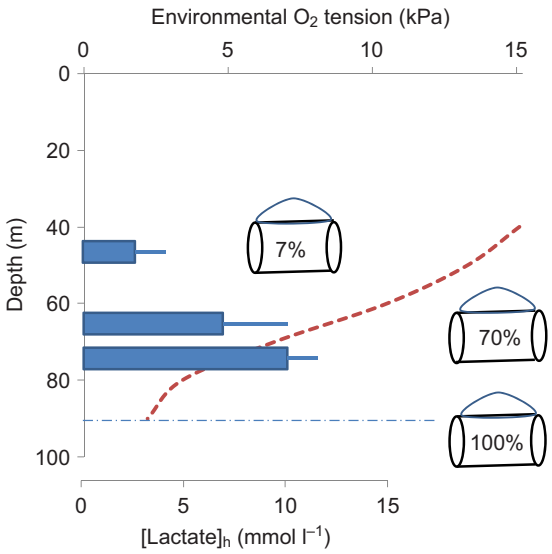

Fig. 3. Concentration of L-lactate in haemolymph from krill caged at different depths, retrieved just before they would normally ascend into shallower water as part of their diel vertical migration, and the environmental O_2 tension at those depths. Values within the cage cartoons are percentage mortality in the cages noted upon retrieval. Environmental O_2 tensions are represented by the dashed line. The dot-dashed line represents the depth at which the cage where 100% mortality was noted (hence no lactate values) was suspended (data from Spicer et al., 1999). Horizontal bars are means ± 1 s.d.

Case study 3. Benthic hypoxia and Hc in Norway lobster (seemingly open)

Changes in the respiratory pigment Hc can be induced by environmental challenges (Mangum, 1997). Hypoxia tends to increase the Hc concentration in the haemolymph ($[Hc]_h$), but the response in laboratory experiments is variable, with sometimes no significant difference or even a hypoxia-related decrease recorded (Senkbiel and Wriston, 1980; Hagerman and Uglow, 1985; Baden et al., 1990; Mangum, 1997).

In situ $[Hc]_h$ of decapods is extremely variable, particularly when we investigate locations that historically have seen some of the worst incidents of coastal hypoxia over the last 50 years, e.g. the Kattegat at the mouth of the Baltic Sea (Diaz and Rosenberg, 2008). A study of frequency distributions of $[Hc]_h$ from three different crustacean species each from two different populations [Gullmarsfjord (normoxic) and Kattegat (hypoxic), southwest Sweden] found that only in one population was the $[Hc]_h$ distribution normal, and only in two (different) populations was the distribution symmetrical (Spicer and Baden, 2000). This is perhaps surprising given that it might be expected that respiratory pigment concentration should be under strong stabilising selection. Median values for $[Hc]_h$ also varied inter- and intra-specifically. A number of studies have investigated the effects of natural hypoxic events on $[Hc]_h$ from decapods (mainly, though not exclusively, the commercially important Norway lobster, *Nephrops norvegicus*) (Hagerman and Baden, 1988; Pihl et al., 1991; Engel et al., 1993). The results can appear contradictory and such discrepancies are accounted for (not always successfully) by highlighting the different levels of environmental hypoxia *in situ*, both within and between studies.

Field and laboratory studies of $[Hc]_h$ from *N. norvegicus* show that both the concentration (median and frequency distribution) and its response to hypoxia are variable. This prompted Spicer and Baden (Spicer and Baden, 2001) to test the idea that individuals with different $[Hc]_h$ might respond differently to exposure to environmental hypoxia (Fig. 4). Individuals with initially low $[Hc]_h$ showed a substantial increase (red lines), individuals with some of the highest $[Hc]_h$ showed a decrease (green lines), and for some individuals their $[Hc]_h$ remained unchanged (blue line) (Fig. 4A). In other words, a paired experimental design showed that individuals respond differently to hypoxia, but that this response is predictable. Using a non-paired experimental design, the response to hypoxia observed was shown to be merely a function of the frequency of different initial $[Hc]_h$ in the experimental population. As can be seen in Fig. 4B, a left-skewed distribution frequency curve for $[Hc]_h$ under normoxic conditions (dotted line) became considerably less variable and tightly symmetrical under hypoxia (solid line), and the median for $[Hc]_h$ did increase with increasingly severe hypoxic exposure.

To test whether this happened *in situ*, a natural 'experiment' was carried out where *N. norvegicus* were collected from three well-oxygenated sites (Skagerrak) and from two sites that were currently experiencing chronic hypoxia (Kattegat). $[Hc]_h$ of individuals from Skagerrak displayed a very wide range of values and the frequency

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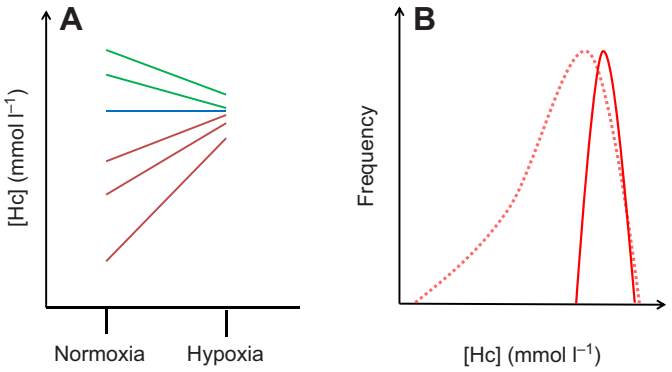

Fig. 4. Changes in haemocyanin concentration ([Hc]) in the Norway lobster, *Nephrops norvegicus*, in response to hypoxia. (A) Reaction norms depicting the different responses of individuals in Spicer and Baden's (Spicer and Baden, 2001) study. Those with comparatively low initial [Hc] showed an increase (red lines), while those with comparatively high initial [Hc] showed either no change (blue line) or a decrease (green line). (B) Frequency distributions for [Hc] from populations of a normoxic (broken line) and a hypoxic (solid line) population (based on Spicer and Baden, 2000; Spicer and Baden, 2001).

curve was left-skewed (as in the dotted line in Fig. 4B). By contrast, the frequency curve of $[Hc]_h$ from individuals from Kattegat was symmetrical and tightly leptokurtic (solid line in Fig. 4B), and the median $[Hc]_h$ was greater than that for the Skagerrak individuals, supporting the conclusions of our individual-based experimental design in the laboratory. However, checking the water chemistry of the hypoxic water revealed that the hypoxic condition that had persisted in this area for many months had been replaced by normoxic water a day before sampling. The predictions fitted the O_2 levels recorded right up until 24 h before sampling, but the measurements were considerably higher.

Thus, as a product of the dialectic interaction of field and laboratory studies, we now suggest that hypoxia does indeed increase median $[Hc]_h$ but that the increase is a product of individual responses determined by the initial $[Hc]_h$ of the individuals exposed to the challenge. Neither normoxic nor hypoxic individuals showed a normal $[Hc]_h$ distribution. The effect of hypoxia on $[Hc]_h$ in *N. norvegicus* is predictable and nowhere near as variable as has been portrayed in the literature to date, even with the uncertainty cast by our failed successful field 'experiment'.

Before finishing with the fourth case study, which takes an -omics approach to how marine invertebrates respond to hypoxia in the wild, we first consider the integration of -omics into ecophysiology generally and into laboratory studies on the effects of hypoxia specifically. Having done so we will then be better placed to examine the lessons we can learn from all four case studies.

'...that contains no molecular biology'

Integration of -omics and CAP

A key aim of '-omic' approaches (genomics, transcriptomics, proteomics and metabolomics) is to link the phenotype to specific aspects of biomolecular organisation. The push to integrate the various -omics and CAP is well underway (Feder and Mitchell-Olds, 2003; Gracey and Cossins, 2003; Cossins and Somero, 2007), even using traditional non-model marine invertebrates, such as molluscs (e.g. Zhang et al., 2012) and crustaceans (e.g. Terwilliger et al., 2006; Towle and Smith, 2006; Stillman et al., 2008). Easier access to high-throughput sequencing technologies has increased the number of genetic non-model species and is generating its own CAP community alongside the more well-established communities in biomedical and agricultural functional genomics (Mitchell-Olds et al., 2008). This community is particularly visible in investigating the effects of global climate change, specifically warming and ocean acidification, on animal function (Hofmann et al., 2005; Johnson and Browman, 2007; Stillman et al., 2008; Evans and Hofmann, 2012), and is currently much better developed than the community studying how marine invertebrates (though not necessarily vertebrates) respond to hypoxia. So Speakman's (Speakman, 2002) claim that books or reviews of ecophysiology written now (he says 'in 10–15 years time') would be unpublishable without those -omics seems credible.

-omics and physiological responses of marine invertebrates to hypoxia in the laboratory

Although not appearing as quickly as some general proponents of -omics approaches predicted, over the past 10 years a number of studies have been published that would have to be included in any review of ecophysiological approaches to environmental hypoxia in the marine environment.

The study of Zhang et al. (Zhang et al., 2012) on the Pacific oyster, *Crassostrea gigas*, is, to date, one of the most comprehensive investigations of genome-wide responses to stress. Having sequenced 61 transcriptomes from oysters subjected to numerous environmental challenges (temperature, salinity, air exposure and different trace metals), a total of 5844 genes were differentially expressed for each challenge. While different genes did respond to different challenges, there was also significant overlap. Although they did not explicitly test environmental hypoxia, Zhang et al. (Zhang et al., 2012) noted that functional hypoxia is a likely consequence of shell closure while emersed during the low tide period. Therefore, it is interesting that air exposure induced a response from the largest number of genes, 4420. However, the effect of environmental hypoxia on the *C. gigas* transcriptome was published 2 years previously (Sussarellu et al., 2010). The authors recorded a hypoxia-induced upregulation of genes associated with antioxidant defence and the respiratory chain compartment. Sussarellu et al. (Sussarellu et al., 2010) attributed these upregulations to either hypoxia-induced oxidative stress or an 'anticipatory response for normoxic recovery' (see also Le Moullac et al., 2007). They found that another 1694 genes varied independent of hypoxia. It would be interesting to formally compare these oyster studies, looking for common expression patterns for emersion and hypoxia, but this is currently a formidable task and beyond the scope of this review.

There are a few transcriptomic studies of the response to hypoxia of crustaceans in the laboratory. Exposure of the black tiger shrimp, *Penaeus monodon*, to hypoxia resulted in a complex gene expression pattern through time with approximately the same number of genes being either upregulated or downregulated (de la Vega et al., 2007). In common with other crustaceans, discussed below, genes related to Hc production were downregulated on exposure to the level of hypoxia used.

Cells of many organisms have a system for switching on a suite of genes that confer hypoxia tolerance. This 'master switch' involves a family of related proteins, transcription factors termed hypoxia-inducible factors (HIFs). HIFs are considered essential in the maintenance of O_2 homeostasis in metazoans. However, HIF involvement in regulating gene expression is much wider than just responding to hypoxia: HIF-1 controls the expression of hundreds of genes, and because these genes vary from one cell type to another, it is probable that the HIF-1 transcriptome includes literally

thousands of genes with roles in development, physiology and disease. HIF-1 is formed from two subunits (α and β) expressed constitutively, with the α-subunit post-translationally modified in response to changes in O_2. It has been studied in a number of marine invertebrates (Flűck et al., 2007). Soñanez-Organis et al. (Soñanez-Organis et al., 2009) characterised the *hif-1* gene and investigated its hypoxia-induced upregulation in different tissues of the white shrimp, *Litopenaeus vannamei*. To silence the *hif-1* gene they then injected dsRNA directly into different tissues (Soñanez-Organis et al., 2010). In normoxic controls, dsRNA injection reduced the concentration of the corresponding transcripts in gill, but not muscle, tissue. Under hypoxia, however, silencing of *hif-1* affected the corresponding transcripts, and as a result, affected the concentrations of L-lactate and glucose (fermentation substrate) in the haemolymph. They also found that HIF-1 affected the expression of L-lactate dehydrogenase (an enzyme that catalyses the transformation of pyruvate into L-lactate) during hypoxia (Soñanez-Organis et al., 2012).

Clark et al. (Clark et al., 2013) found an upregulation of transcripts with putative functions associated with combatting reactive O_2 species, the unfolded protein response and activation of the immune system in hypoxic Antarctic clam, *Laternula elliptica*, with the immune system activities more heavily impacted in older (19 years) than younger (3 years) individuals.

Jiang et al. (Jiang et al., 2009) investigated how hypoxia affected the proteome of the shrimp *Fenneropenaeus chinensis*. There was a decrease in the levels of a large number of proteins, such as those related to energy production, metabolism, immune response, antioxidant defence, chaperones and cytoskeletal activity. Though from a completely different phylum and habitat, when exposed to hypoxia, the proteome of the deep-sea hydrothermal vent worm *Alvinella pompejana* showed broadly similar increases in three groups of proteins associated with cytoskeletal activity, enzymes involved in energy metabolism and chaperones (Mary et al., 2010).

-omics, ecophysiology and hypoxia

Certainly the -omics approach to studying physiological responses of marine invertebrates to hypoxia is part of an ecophysiological approach. However, it is incomplete without field studies and experiments. So what is known of -omics data from hypoxic marine invertebrates *in situ* from the point of view of how those animals work? Currently there are few studies, but what exists is tantalising. For our fourth case study, the identification of hypoxia-responsive genes and proteins in the blue crab, *Callinectes sapidus*, in the laboratory and *in situ* is a promising, if complicated, first step towards integrating -omics with ecophysiology in understanding the responses of marine invertebrates to hypoxia.

Case study 4. Gene expression in hypoxic blue crabs in the laboratory and *in situ*

Brouwer and co-workers (Brouwer et al., 2005; Brown-Peterson et al., 2005) set out to identify hypoxia-responsive genes and proteins in the blue crab, *C. sapidus*, to develop a sensitive molecular tool for detecting sublethal effects of hypoxia in this estuarine-resident species *in situ*. They cloned 23 potential hypoxia-responsive genes from blue crabs and used them to construct microarrays. Five days of hypoxic exposure (2.5 ppm O_2) resulted in reductions in gene expression of heat shock protein 70 (*Hsp70*), cytosolic MnSOD (*cyt-MnSOD*) and ribosomal proteins S15 and L23 but not copper metallothionein (*CuMt3*). While Hc mRNA levels decreased over this period, the actual $[Hc]_h$ increased. After 15 days of hypoxia the expression of target genes had reverted to pre-exposure levels. However, interpreting these changes was complicated by the fact that expression of *Hsp70*, *CuMt3* and *cyt-MnSOD* increased in normoxic controls over the 15 days of the experiment. Exposing crabs to intermittent hypoxia (2.5 to 8 ppm O_2 over a 24 h cycle) for 10 days resulted in upregulation of *cyt-MnSOD* and cytochrome *c* oxidase subunit 1 (*ccox1*), but no change in $[Hc]_h$ (even though 15 days in chronic hypoxia altered $[Hc]_h$). So under laboratory conditions, intermittent and chronic hypoxia affected gene regulation differently. To see whether the findings of the laboratory studies were comparable to the field, crabs were sampled from a diurnally hypoxic marsh site on Pensacola Bay, Florida. They showed hypoxia-related downregulation of *ccox1* and *cyt-MnSOD* gene expression and low $[Hc]_h$.

There are another two examples of -omics applied to field-collected marine invertebrates. Short-term exposure of the grass shrimp *Palaemonetes pugio* to hypoxia highlighted a possible 74 differentially regulated genes, with some related to ATP metabolism (7), O_2 transport (6), protein synthesis (9) and protein degradation (6), but expression patterns were different between intermittent and chronic hypoxia (Brouwer et al., 2007; Brown-Peterson et al., 2008). In another study, gene expression in field-collected mussels (*Mytilus californianus*) was examined using a cDNA library prepared from different tissues of adult mussels exposed to a number of stressors including hypoxia (Place et al., 2008). Although the patterns observed were complex, and difficult to interpret (the main aim of the study was to investigate latitude/temperature differences), interestingly some of the responses were similar to those found for the intertidal fish *Gillichthys mirabilis* exposed to environmental hypoxia (Gracey et al., 2001).

Overall, we are beginning to produce data on molecular and physiological responses of marine invertebrates (mainly selected molluscs and crustaceans) to hypoxia, which needs careful consideration if we are to begin to link the two different types of response. Making this truly comparative is not a trivial task. As far as field tests of this link are concerned, the few data we have are difficult to interpret. Arguably we may be behind those ecophysiologists interested in temperature effects (see above), as well as ecologists (Travers et al., 2007) and ecotoxicologists (Veldhoen et al., 2012), but a start has been made in our understanding of -omics reponses to hypoxia by marine invertebrates.

Lessons from the ecophysiological approach

So given the four case studies above, and the brief review of -omics studies and ecophysiology, what can an ecophysiological approach tell us about the physiological responses of marine invertebrates to hypoxia? What follows are four suggestions.

1. Hypoxic responses in the wild are not always predictable from laboratory experiments because…

…Responses can be modified by behaviour. When investigating the effect of hypoxia on prawns, a study using physiological and biochemical techniques ended up having to take cognisance of natural history (cart before the horse?) and incorporate behaviour into the experimental design. The behaviour and natural history of krill and their DVM was the context for the laboratory and field experiments and measurements investigating their metabolism when migrating into hypoxic waters. An ecophysiological approach is ideal because ecophysiology is more of a disciplinary convergence than a discipline of its own.

…The starting point of investigations is often different. It is rarely possible to ‘standardise’ at the beginning of a ‘natural’ (or even

field) experiment. While this makes it difficult to interpret the data, working with natural variability may throw light on physiological mechanisms, as was the case with Norway lobster, where the pattern of the response of the respiratory pigment to hypoxia was to a large extent determined by investigating initial respiratory pigment concentration *in situ*.

…Not just one, but three experimental approaches are necessary. Working out how animals actually work in the wild, in all four case studies, did not (and could not) emerge solely from the laboratory experiments. The ecophysiological approach to understanding physiological responses to hypoxia requires not just a mixture, but an interaction, a dialectic, between laboratory experiments, field experiments and what is referred to throughout the text as natural 'experiments'. But as seen in the first and third case studies, the data from natural 'experiments' are difficult to obtain, almost impossible to standardise, and nearly as impossible to publish (particularly on their own as is the case with the prawns where the laboratory experiments were performed many years ago). And yet such data are still invaluable in interpreting field and laboratory experiments.

Laboratory and field experiments are rarely the same animal (Calisi and Bentley, 2009). Natural 'experiments' are even more different. In all four case studies, the laboratory experiments were not entirely successful in predicting what happened in the field, although there were degrees of success. This means that although all three approaches may not always be possible, those interested in physiological responses to hypoxia should always seek opportunities to explore as many of these experimental approaches as possible, accepting the often severe limitations of each approach.

2. Assuming we know what a physiological response is 'for' can be misleading

Whether it is explaining the physiological responses in an ecological or an evolutionary context, the reasoning must be robust and warranted. We must avoid the danger of assuming we know what the physiological responses we measure 'in response to' hypoxia are 'for'. *Palaemon elegans* was clearly more developed in its respiratory and metabolic response than its congenitor, *P. serratus*, which seems to fit with the high shore distribution of the former. But it was the behaviour of *P. elegans* that actually allowed it to survive nocturnal hypoxia *in situ*. Conversely, the physiology of the krill *M. norvegica* is not as well developed in its response to hypoxia as similar species that migrate into permanent O_2 minimum zones (Seibel, 2011), and so the idea that it would ever enter severely hypoxic water (which it does) seems implausible. This implausibility is only strengthened by the knowledge that *M. norvegica* has one of the highest $[Hc]_h$ within the Crustacea, but its O_2 affinity is extremely low and there is even some evidence that this species may actually catabolise its Hc when it migrates into hypoxic water (Spicer and Strömberg, 2002). If anything, *M. norvegica* could actually appear maladapted for diurnal excursions into severely hypoxic water, from which, *in situ*, it eventually emerges literally exhausted. And it seems unlikely that a physiological trait such as $[Hc]_h$ that is seen as critical for respiratory gas transport might not be normally distributed, as was the case for *N. norgegicus*, but becomes so under hypoxic conditions, and yet that is precisely what happens.

Finally, many of the interpretations of what the various -omics patterns mean for the physiological phenotype appear reasonable, but being reasonable does not make it so. It is possible to cannibalise some electronics from a 'broken' advanced washing machine and use them to help build a radio. However, it is wrong to draw the inference that inside every such washing machine is a little radio! Avoiding similar reasoning is particularly important when assigning putative functions to genes or gene products. We must interpret our molecular patterns with due care and design our experiments more carefully, with an eye to comparability with other similar studies, if we are to reliably attribute cause and effect, or function, to changes in gene or protein expression.

3. Taking parodies of ecophysiology seriously closes off interesting questions in our understanding of how marine invertebrates respond to hypoxia

Brown (in McNab, 2002) identified three parodies of ecophysiology. While each certainly contains some element of truth, they do not have to – and none of them do – apply to the case studies presented here. Indeed, taking any of these parodies seriously arguably could have weakened research directions in understanding the effect of hypoxia in the wild. Ecophysiology has been parodied as:

(i) Endlessly cataloguing weird and wonderful attributes of 'oh my' organisms that live in far-off stressful places. Leaving aside the observation that all organisms have the inherent capacity to be 'oh my' organisms, and that what is one organism's 'stressful place' is another's 'couldn't-do-better-anywhere-else environment', the most important thing to note is this – the context of ecophysiology is ecology, so the model species chosen must be ecologically important. In CAP, species are chosen not just for their suitability for the question being asked (the Krogh principle), but because they are common and laboratory hardy. Most species on Earth are not common, and are not particularly laboratory hardy, and often these are, or will be, the species we need for our ecophysiological studies. While the charge of endlessly cataloguing, if true, is a damning one, so too is the assumption that because we have good information on the physiology of one particular species (e.g. the 'lab rat' of marine ecophysiology, the green crab *Carcinus maenas*) we can generalise to other species (i.e. say we know how crabs, or crustaceans work). Northern krill across most of their geographical range never experience hypoxia; would it have been considered an interesting question to investigate their responses to hypoxia if the interesting 'natural experiment' in the Gullmarsfjord had escaped our attention?

(ii) Interpreting every trait as adaptation. To some extent this has already been covered above. Ecological or evolutionary importance cannot be assumed and should be tested formally. In the past, biologists were generally guilty of assuming adaptation and constructing 'just-so' stories to account for observed physiological responses to hypoxia.

(iii) Trivially proving that an organism can actually live where it lives. Admittedly there are examples of this in the literature. However, this statement ignores two key points. First, some animals, such as the krill in the second case study, live where it would be thought unlikely that they would live. This krill species has a physiology seemingly unsuited for exposure to hypoxia, but they migrate into hypoxic water twice a day. Second, 'where an animal lives' is not static. The issue is that 'in the wild', environmental hypoxia is not often a passive or constant environment, on a number of different temporal and spatial scales. This is perfectly illustrated in all four case studies. So while what an animal does do is important (its realised physiological niche if you like), knowing what an animal can do (its fundamental physiological niche) is crucial if we are to predict the influence of physiology on the abundance, distribution and diversity of species exposed to environmental hypoxia. This is particularly important for species such as the northern krill, which does not normally experience

environmental hypoxia, but may well do so as the number and extent of dead zones increase with time. Also to predict the evolvability of a particular physiological function, when acclimatisation is not a sufficient response to ensure population persistence, fits in well with Bennett's (Bennett, 1987) view, expressed earlier, that ecophysiology has evolution as well as ecology as its context. So although not as snappy as Schmidt Nielsen's 'how animals work', we could define ecophysiology as a question: 'how do (or might) ecologically important animals work in the constantly changing "wild"… and why?'

It is in understanding what animals can do that ecophysiology is, perhaps, closest to CAP. Given all of this, it may not be surprising that McNab (McNab, 2002) thought that ecophysiology was central to conservation, and that recently we have seen the emergence of conservation physiology, defined as the application of physiological theory, approaches and tools to elucidate and address conservation problems with an aim to provide a mechanistic understanding of new environmental disturbances and threatening processes that impact physiological responses and thereby ecological function, population persistence and species survival – or applied ecophysiology (Seebacher and Franklin, 2012).

4. An -omics approach is, and is becoming, a powerful approach to understanding hypoxic responses

In his book review, Speakman (Speakman, 2002) noted the absence of any molecular biology in McNab's *Physiological Ecology*, and 'because looming on the horizon is a mid-life crisis for [ecophysiology]', he wondered 'how physiological ecology will accommodate the genomics revolution'. Based on what was discussed above, it is suggested that the -omics are slowly but successfully being integrated into our understanding of how marine invertebrates respond to hypoxia, albeit not as quickly as ecophysiologists interested in thermal responses or the ecologists generally appear to be managing (references above). Below are listed three opportunities and three challenges for our continued advancement with this integration.

Opportunities

(i) Genomic tools such as microarrays and next-generation RNA sequencing allow analysis of expression of thousands of genes, enabling us, by ever increasing degrees, map out the genetic architecture underpinning physiological responses to the environment. The technological advances are remarkable (Buckley, 2007; Gracey, 2007). Large-scale mapping, expressed sequence tag and genome sequencing projects, and construction of a bioinformatics infrastructure for handling these data are becoming less expensive and (comparatively) less time consuming. New technologies, such as transcriptome shotgun sequencing and bioinformatics, are constantly improving. This has increased the desire to, and capability of, increasing the number of non-model species genomes, transcriptomes, etc., allowing us to use ecologically relevant species for our ecophysiological work, marine invertebrates that either do or do not experience hypoxia in their natural environment.

(ii) Use of -omics tools can lead to unexpected (and therefore unlooked for?) findings of biological significance. For example, the hypoxic induction of myoglobin in unexpected tissues of intertidal fish came out of a study of the effect of hypoxia on its transcriptome (Fraser et al., 2006; Gracey, 2007).

(iii) Measurement of the hypoxia-induced products of -omics studies may be more easily studied *in situ* than some physiological characters. In the study of the response of the blue crab transcriptome to hypoxia in the fourth case study, it was possible to quantify gene products related to respiration, whereas the direct quantification of aerobic and anaerobic respiration in the field is, at best, difficult.

Challenges

(i) Interpreting -omics data is, and will continue to be, 'no walk in the park'. As in the -omics/physiology experiments summarised above, linking transcriptional changes to what is happening at the protein level is far from routine. For example, in some studies quoted above, transcriptional Hc expression decreased while at the same time the amount of Hc increased. And Sussarellu et al. (Sussarellu et al., 2010) could not infer a global metabolic depression at the transcriptional level in the Pacific oyster. A number of investigators, while strong advocators of -omics approaches, have highlighted the major challenges of knowing what we can take from the data they generate and, in particular, making sense of the transcriptome (Feder and Walser, 2005; Rupert, 2008; Vijay et al., 2013). The simplistic 'one gene, one RNA, one protein' model that held sway until recently cannot account for the transcriptional complexity now emerging, and there has recently been a seismic shift in our thinking. Genes can produce many different transcripts as a result of variable start and termination sites and alternate splicing. Furthermore, much of the extragenic ('outside genes') genome is also transcribed, producing a perplexing assortment of noncoding RNAs. These include antisense transcripts and microRNAs believed to be involved in post-transcriptional regulation of gene expression and controlling transcription during development.

Add to this the high sensitivity of gene expression to even subtle alterations in environmental conditions (and not just the experimental treatment either) noted in the -omics/physiology projects examining the effects of hypoxia on molluscs and crustaceans summarised above, and there is a real possibility, even despite the advent of next generation sequencing, that we may not always be able to detect relevant changes in field measures of transcription profiles [see Travers et al. (Travers et al., 2007) for the equivalent situation in more ecological studies]. Finally, making our different studies truly comparable is both essential and fraught with difficulties in logistics and identification of function.

(ii) Emerging and unforeseen technical and logistic problems will retard (but not prevent) progress. To take one example, Taris et al. (Taris et al., 2008) reported that sequence polymorphism in Pacific oysters, one of the few non-model marine invertebrates available to ecophysiologists (see above), may produce serious artefacts in real-time PCR assays, holding back, but not preventing, the use of oysters in our studies.

(iii) Data generation could easily substitute for thinking. Suarez and Moyes (Suarez and Moyes, 2012) point out that certain -omics approaches are used to draw unwarranted, questionable or even simplistic conclusions about physiological processes (e.g. metabolism). They plead that we never allow large-scale data-gathering -omics techniques to become a substitute for thinking. Such a possibility is made more likely by the fact that the greatest challenge we face is the ability to produce more data than we can analyse at the expense of the scientific questions we can address. Feder and Mitchell-Olds (Feder and Mitchell-Olds, 2003) remind us that -omics approaches "require mechanistic biology (biochemistry, physiology and so on), ultimately under realistic cellular and environmental conditions – not just '-omics', but 'functional -omics'." However, it is conceivable that a comprehensive data set of the realistic environmental context and the phenotypes of animals

responding and/or adapting to novel environments itself could, paradoxically, become the main limiting factor in our understanding of physiological responses to hypoxia and even ecophysiology more generally.

Overall, it could be that the genomic responses to hypoxia we observe not just in marine invertebrates, but in marine animals more generally may well have some common themes, e.g. despite tissue-specific patterns of gene regulation predominantly there is: (1) upregulation of genes associated with anaerobic ATP production, gluconeogenesis and reactive oxygen species effects, and (2) downregulation of genes associated with protein synthesis and cell growth. Each new laboratory study seems to add some complication, and interpreting what is happening in the field seems to compound this. That said, even with the little knowledge and understanding we have at present, the -omics technologies look to be ideal for analysing and assessing field data in ways that could be more accessible than current traditional physiological approaches. They will fundamentally alter how we investigate and how we think about ecophysiological responses of marine invertebrates to hypoxia, as they continue to alter the way we think about ecophysiology more generally.

Perspectives

The past 10 years have seen a renaissance in ecophysiology. The fact of global climate change, which includes the reduction of O_2 in our seas and oceans, is indisputable. Uncertainty about how organic life will respond to this change has risen to the top of the scientific agenda. As a result, ecophysiology has found its niche, linking mechanistic understanding to ecological change, in an ecological and evolutionary context, either under its own title or in its applied form, conservation physiology. Physiological responses of marine invertebrates to hypoxia is just one area of active interest amongst many, but given what we now see of spatial and temporal variations in our seas and oceans, it is increasingly seen as an important one. It is one that urgently requires an ecophysiological approach, much like the one advocated here, if we are to both understand and predict how marine invertebrates in the wild will respond to these environmental changes. Sadly, it has taken the growing recognition that our world is a ‘world of wounds’ (Ehrlich, 1997) to rejuvenate interest and funding in this fascinating and intellectually challenging disciplinary convergence we find so difficult to pin down.

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Competing interests

The author declares no competing financial interests.

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