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A mesocosm study investigating the effects of hypoxia and population density on respiration and reproductive biology in the brittlestar *Amphiura filiformis*

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ABSTRACT: Hypoxic events are increasing in frequency and duration, especially in areas susceptible to eutrophication. Such events pose a growing threat to the health and function of marine ecosystems by altering key biological and ecological processes. Of particular concern is how hypoxia impacts upon both the reproductive biology and metabolic activity of marine organisms and what these impacts mean for biodiversity and ecosystem function. Furthermore, information on the effect of population-level processes (e.g. organism density) on individuals' biological responses to hypoxia is currently lacking, hampering predictions on population and species sensitivity to this stressor. Using a mesocosm experiment, we investigated the effects of relatively short-term hypoxia (14 d, 3.59 mg O_2 l^{-1}) and organism density (5, 9, 13, 17, and 21 ind. per aquarium, equating to 500, 900, 1300, 1700, and 2100 ind. m^{-2} respectively) on the aerobic metabolism and reproductive biology of a key infaunal species, the brittlestar *Amphiura filiformis.* While there were no observed effects of organism density, exposure to hypoxia did result in reduced metabolic rates and delayed metabolic recovery rates once normoxic (8.09 mg O_2 l^{-1}) conditions were restored. Additionally, hypoxia resulted in delayed female reproductive cell development, smaller oocyte feret diameter, and a greater number of pre-vitellogenic oocytes present within the ovaries. These disruptions to metabolism and reproductive biology during hypoxia could cause major alterations in the quantity and quality of planktonic propagules supplied by benthic species to the pelagic environment, which in turn could affect benthic community diversity in the long term, as well as bentho-pelagic coupling, if repeated hypoxic events occur.

KEY WORDS: Low oxygen · Aerobic respiration · Benthic invertebrate biology

INTRODUCTION

Coastal hypoxia has been documented in >400 systems worldwide, affecting in excess of 245000 km^2 of the world's oceans (Diaz & Rosenberg 2008), and seems to be a growing problem (Zhang et al. 2010, 2013). As global warming and eutrophication continue to exacerbate hypoxia, there has been an increasing view that reduction in dissolved oxygen is a key environmental stressor within marine ecosystems and that this stress has the potential to define benthic community composition and modify biogeochemical cycles (Rosenberg et al. 2001, Wu & Or 2005, Meire et al. 2012, Zhang et al. 2013). Depending on the severity and duration of a hypoxic event, effects on organisms and communities can vary from short-term behavioural changes, medium-term transitory or lasting physiological alterations, to long-

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term species absences through interruptions to reproductive success, species migrations, or local extinctions (Rosenberg et al. 2001). Consequently, there is growing concern over the long-term impacts of regular or seasonal hypoxia on the sustainability and biodiversity of coastal ecosystems (Diaz & Rosenberg 1995, Wu 2002, Thomas et al. 2007, Zhang et al. 2010, Bijma et al. 2013).

Most studies of the effects of hypoxia in the coastal zone have focused on commercially important species, e.g. larval development in mussels (Wang & Widdows 1991), survival and avoidance behaviour of penaeid shrimp (Wu et al. 2002), settlement and growth in oysters (Baker & Mann 1992, David et al. 2005), and food consumption and growth in fish such as bass, cod and flounder (Chabot & Dutil 1999, Kimura et al. 2004, Brandt et al. 2009, Herbert et al. 2011). A number of studies have examined the effects of hypoxia on benthic community structure and recovery (Diaz & Rosenberg 1995, Lim et al. 2006, Van Colen et al. 2010, Fleddum et al. 2011), but much less information exists on how hypoxia affects reproductive biology, a trait imperative for species survival, community recovery, and biodiversity security. Moreover, the majority of hypoxic events occur during the summer months, often after spring blooms (Diaz & Rosenberg 2008), which is a key reproductive period for many benthic invertebrate species. The paucity of data on the effects of hypoxia on reproductive biology in marine benthic species makes it difficult to predict the ecological consequences of field population resilience, their ability to recover, and ultimately whether these impacts have implications for ecosystem health and function in the long-term (Wu 2002).

The few studies conducted on the effects of hypoxia on reproductive or embryonic development suggest that hypoxia can cause uncoupling of growth and morphogenic processes in mussel larvae (Wang & Widdows 1991), reduced settlement and inhibited growth in oyster larvae and juveniles (Baker & Mann 1992), delayed embryonic development in gastropods (Chan et al. 2008), and delayed spawning in the brittlestar *Amphiura filiformis* (Müller, 1776) (Nilsson & Sköld 1996). The ability of organisms to maintain their metabolic rates and thus energy assimilation for important life-history traits, such as reproductive output, is vital when exposed to environmental stress such as hypoxia, and potentially holds consequences for an organism's distribution and abundance (Wu 2002, Spicer 2014).

The burrowing brittlestar *A. filiformis* is abundant in European waters (O'Reilly et al. 2006) and often aggregates to form population densities ranging from 280 ind. m^{-2} (Sköld et al. 1994) to 2250 ind. m^{-2} (Rosenberg et al. 1997). Geographic, bathymetric, and environmental parameters help explain these vast differences in population density, especially when discrete aggregations coincide with fine sediment characteristics as described by O'Connor et al. (1983). However, it is not known if dense aggregations of *A. filiformis* result in any positive species interactions (i.e. the ecological concept of facilitation) such that survivorship can be positively related to population density (Bruno et al. 2003).

A. filiformis is primarily a suspension feeder that mostly remains buried below the sediment surface and protrudes one or more arms into the water column. *A. filiformis* actively undulates its arms and pumps its disc for respiratory gas exchange, burrow ventilation, collection of food, and the transportation of sediment and waste (Vopel et al. 2003). This species is particularly well studied due to its importance as an ecosystem engineer (Solan & Kennedy 2002) with its capacity to modify the local environment and improve oxygenation within the sediment (Vopel et al. 2003).

Consequently, we hypothesised that *A. filiformis* may benefit from being within dense aggregations, especially when exposed to moderate hypoxia, due to its ability to increase the oxygenation and movement of the surrounding sediment and water, possibly resulting in lower metabolic costs of living (i.e. reduced arm undulations and pumping of the disc), allowing for energy investment into other important traits such as reproductive output. Investigating if there are any potential links between population density, aerobic metabolism and reproductive investment of *A. filiformis* may help define which populations are most at risk when moderate hypoxia occurs.

To test this hypothesis, a 14 d mesocosm experiment was conducted to quantify the impacts of moderate hypoxia on the aerobic metabolism and reproductive biology of the brittlestar *A. filiformis* across a range of organism densities. Rates of O_2 uptake within normoxic and hypoxic treatments were measured as a proxy for aerobic metabolism. In addition, the O_2 uptake of organisms exposed to hypoxia was re-assessed when brittlestars were returned to normoxic conditions. This may provide insight into whether hypoxia can cause longer-lasting disruptions to physiological activity or if *A. filiformis* can exhibit metabolic plasticity when environmental parameters change. Reproductive development was assessed by measuring the mean feret diameter in developed (late-vitellogenic) oocytes and by quantifying the variation in the number of the 3 main oocyte developmental stages.

MATERIALS AND METHODS

Sediment collection

On 25 May 2012, sediment was collected at a water depth of ~10 m from an area of 'very fine sand' with an overlaying surface layer of 'clay/silt' in Cawsand Bay, Plymouth, UK (50° 21.998' N, 4° 07.961' W) using a 0.1 m^2 box core. Once retrieved, the surface layers of sediment (10 to 15 cm) were placed into bags and transported to the Plymouth Marine Laboratory (PML, Plymouth, UK) mesocosm facility where these were sieved (2 mm) in filtered seawater (10 µm diam. Hydrex filters). The sieved sediment was placed into a holding tank and allowed to settle for 48 h to capture the fine fraction. Post-settlement, excess water was carefully drained off and the sediment homogenised by mixing. Fifty experimental glass aquaria (20 cm wide × 5 cm deep × 30 cm high) were filled with sediment up to a depth of 19 cm (±1 cm), leaving 11 cm of overlying water.

Each aquarium was connected to a flow-through seawater system that delivered aerated, twice filtered (10 µm and 1 µm diam. Hydrex filters) seawater from a 450 l header tank (dissolved oxygen or DO = 8.19 ± 0.49 mg O_2 l^{-1}, salinity = 34.50 ± 0.17, temperature = 12.35 ± 0.66°C, pH = 8.08 ± 0.05, mean ± 95% CI) (Table 1) via a peristaltic pump (323E, Watson Marlow, Falmouth, UK) set at a rate of 20 ± 0.5 ml min^{-1}. Water inlet pipes were connected to each aquarium 1.5 ± 0.5 cm above the sediment surface. The average water volume held within each aquarium was 1100 cm^3, resulting in an approximate water renewal rate every 55 min. Aquaria were kept under these conditions for a further 21 d, to allow the sediment to settle and for biogeochemical processes and gradients to re-establish. Aquaria that showed any visual signs of bioturbation during this time were removed from the experimental set up.

Brittlestar collection

Individuals of *Amphiura filiformis* were collected from the same site as the sediment (12 to 14 June 2012). Specimens were handpicked to avoid damage (such as arm loss) and carefully washed with fresh seawater. Only individuals with a disc diameter > 4 mm, based on the size at which adults reach sexual maturity (O'Connor et al. 1983), plus 5 intact arms were placed into containers (vol. = 250 ml, 3 ind. per container) containing freshly collected seawater and transported to PML within 3 h of collection. As large numbers of individuals were required, collection was conducted over 3 consecutive days. After each collection day, individuals were placed in a large (50 l) aerated seawater storage tank (DO = 8.07 ± 0.05 mg O_2 l^{-1}, salinity = 34.20 ± 0.42, temperature = 12.39 ± 0.15°C, pH = 8.08 ± 0.03; mean ± 95% CI) that contained sieved (2 mm) sediment ~7 cm deep. After the final collection day, individuals were maintained unfed in the storage tank for a further 4 d. Following this, brittlestars were carefully extracted from the sediment, and only specimens that appeared undamaged, with 5 intact arms, were used in the experiment.

Before haphazard assignment to the experimental aquaria, individuals were carefully blotted dry and weighed using a Sartorius R200-D balance (±0.01 mg). The wet mass of the brittlestars was used to calculate an appropriate feeding dose per aquaria based on the total biomass within each aquarium. Aquaria were dosed with a marine microalgae shellfish diet (1800 Instant Algae® Marine Microalgae Concentrated Shellfish Diet, Reed Mariculture, CA, USA), a mixture of *Isochrysis* sp., *Pavlova* sp., *Thalossiosira weissflogii*,

Table 1. Summary of seawater treatment conditions throughout the experiment. Values are means ± 95% confidence interval, and the range of values is given in brackets (min–max). Dissolved O_2 measurements are corrected for atmospheric pressure and temperature

	DO (mg O_2 l^{-1})	DO (%)	Temperature (°C)	Salinity (ppt)	pH (NBS)
Normoxic header	8.19 ± 0.49 (6.90–9.42)	98.98 ± 5.92 (83.47–113.93)	15.20 ± 0.66 (12.50–16.50)	34.50 ± 0.17 (34.23–35.90)	8.08 ± 0.05 (8.00–8.21)
Normoxic aquaria	8.09 ± 0.06 (6.92–9.00)	97.77 ± 0.68 (83.71–108.81)	12.30 ± 0.10 (10.90–14.50)	34.32 ± 0.07 (33.80–36.60)	8.13 ± 0.02 (8.07–8.32)
Hypoxic header	3.13 ± 0.21 (2.59–3.87)	37.82 ± 2.54 (31.38–46.79)	14.78 ± 0.89 (12.50–16.90)	34.46 ± 0.16 (34.23–35.90)	8.08 ± 0.08 (7.83–8.22)
Hypoxic aquaria	3.59 ± 0.04 (2.95–4.57)	43.34 ± 0.45 (35.62–55.34)	12.41 ± 0.10 (10.50–14.40)	34.25 ± 0.04 (33.59–35.41)	8.16 ± 0.01 (8.07–8.32)

and *Tetraselmis* sp. (cell count 2 billion ml^{-1}). Dose per aquaria was calculated as 1.5% of the total ash-free dry mass (AFDM) of brittlestars per core, which was estimated using a wet mass (WM) to AFDM conversion factor from Ricciardi & Bourget (1998) (Ophiuroidea: AFDM/WM = 7.4%). Once brittlestars had been added to the experimental aquaria, a 5 d settling period under normoxic conditions commenced. Feeding began during this phase and was repeated daily for the duration of the experiment. The calculated amount of 'Instant Algae mix' for each aquarium was prepared daily and delivered via syringe. Algae were released into the water column approximately 2 cm above the sediment surface and evenly dispersed over the entire sediment area. Water flow to each aquarium was stopped for 1 h after the addition of algae. Ingestion rates were not measured; however, visual recordings of active feeding behaviour were noted. At each time point, aquaria were not fed for 48 h prior to sampling, so that brittlestars' digestion processes would not significantly affect rates of O_2 uptake.

Experimental design and set up

Of the 50 sediment aquaria prepared, 35 were selected for use within the experiment. Aquaria were set up to produce a regression experimental design, in which the effects of individuals' density and time were set as independent variables against which to assess any response. Aquaria were haphazardly assigned to one of two O_2 levels (normoxia = 8.09 ± 0.06 mg l^{-1} or hypoxia = 3.59 ± 0.04 mg l^{-1}) and one of 5 organism density levels (5, 9, 13, 17, and 21 ind. per aquaria, equating to 500, 900, 1300, 1700, and 2100 ind. m^{-2} respectively). Time point T0 marked the start of the experiment where 5 aquaria were haphazardly removed and sampled to create 'pre-exposure' data. After 6, 10 and 14 d (T6, T10, and T14 time intervals), 5 normoxic aquaria and 5 hypoxic aquaria were haphazardly sampled, again including all density levels. Sacrificial sampling of experimental aquaria occurred throughout the experiment.

Seawater manipulations

Hypoxic and normoxic seawater were held in 2 separate header tanks (vol. = 450 l each). To produce hypoxic seawater, DO levels were modified using a computerised control system (Walchem Webmaster Series, MA, USA), which regulated the addition of O_2-free nitrogen gas. This was a modification of the feedback system used by Widdicombe & Needham (2007) to create CO_2 acidified seawater. In the modified system, nitrogen gas was finely bubbled through aquarium air stones, whilst seawater DO concentrations were monitored using a submersible DO sensor (DO6441-T, Sensorex, CA, USA). Once the DO had fallen to the desired level, the supply of nitrogen was halted via an automated feedback relay system. The replenishment of fresh seawater caused the DO in the hypoxic header tank to increase, which triggered the addition of nitrogen until the DO sensor detected the pre-set value. Within the header tank, small plastic spheres floated on top of the hypoxic water to reduce gas exchange with the atmosphere, whilst seawater in the normoxic header tank was kept aerated with a continuous air supply bubbled through air stones. Both header tanks contained submersible pumps to aid water circulation and mixing, and both were shielded with a hard top cover. Each header tank was connected to its respective experimental aquaria via a peristaltic pump, which supplied a constant supply of seawater (20 ± 0.5 ml min^{-1}). As the volume of water within each header tank decreased, seawater was replenished from a large reservoir (vol. = 15000 l), which was periodically restocked with seawater collected ~14 km offshore from Plymouth, UK.

At time point T0, the water supply feeding half of the experimental aquaria was transferred from the normoxic header tank to the hypoxic header tank; it took ~24 h for the water in these aquaria to become fully hypoxic to the desired experimental levels (an approximate decrease of 0.19 mg O_2 l^{-1} h^{-1}). The seawater within both header tanks and the experimental aquaria were monitored daily for DO, temperature, salinity and pH (Table 1) using a multiprobe (9828, Hanna Instruments, RI, USA).

Quantifying metabolic rates

Rates of oxygen uptake (as a proxy for metabolic rate) of *A. filiformis* were measured using a 'closed-respirometry' technique. At T0, 5 aquaria (one from each density treatment) were haphazardly removed for analysis. These aquaria formed the 'pre-exposure data'. On the remaining sampling days (T6, T10, and T14), 5 aquaria receiving hypoxic water (one from each density treatment) and 5 aquaria receiving normoxic water (one from each density treatment) were haphazardly removed from the experiment. Brittlestars were carefully extracted from the sediment

and gently rinsed with filtered seawater over a 2 mm sieve. Four individuals from each aquarium were then haphazardly selected for use in the oxygen uptake trials.

Individuals were carefully blotted dry, weighed using a Sartorius R200-D balance (±0.01 mg) and placed in a numbered mesh basket, containing 11 uniform glass marbles (1.3 cm diam.) which provided an inert structure for the brittlestars to hide and bury within. From then onward, specimens were kept submerged in filtered (0.22 µm diam.) seawater that contained a dissolved O_2 concentration corresponding to their previous experimental water treatment. Whilst submerged, the baskets were placed into individual blackened glass chambers (vol. = 50 ml). Each chamber was left open to the surrounding water in the holding tank for 60 min to allow brittlestars to settle. During this time, DO concentrations in each holding tank were monitored and adjusted accordingly to replicate the water conditions experienced within the experiment. Subsequently, a 10 mm magnetic stirrer was placed within each chamber, separated from the organism by the mesh basket. Chambers were then sealed, ensuring no trapped air bubbles. The sealed chambers were placed within a recirculating chilled (11.5°C) water bath under which lay 3 stirring plates (MIX 15 eco, 2mag, München, Germany) set to 200 rpm. Water velocity within the chambers was not recorded, but preliminary experimentation established that 200 rpm was sufficient to mix the water in the chambers and not cause apparent stress to the brittlestars. Five blank control chambers, containing hypoxic water but no brittlestars, were run simultaneously.

The decline in O_2 within each closed chamber was determined using a non-invasive optical O_2 analyser (5250i, OxySense, TX, USA) as detailed by Calosi et al. (2013). This technique uses a fibre optic reader-pen that contains a blue LED and photodetector to measure the fluorescence characteristics of an O_2 sensitive dot which was previously placed inside the glass chambers. For the first hour, measurements were taken every 15 min, and then every 30 min for the next 4 h. During this time, the reductions in oxygen levels within the chambers were monitored to ensure that the incubation itself did not impose a respiratory stress on the brittlestars. Oxygen levels within the chambers did not fall more than 13.40 ± 1.30% (mean ± 95% CI) of the starting value during the course of the oxygen uptake experiments.

Immediately after the first oxygen uptake trial, all individuals that were within hypoxic water began a second experiment but with refreshed oxygenated seawater (8.09 mg O_2 l^{-1}) being used instead of the hypoxic water. Additionally, 5 new blank chambers were also refreshed with normoxic water. However, due to logistical limitations, all 'control' individuals that were kept within normoxic water during the experimental period and the first oxygen uptake trial were not subjected to a second trial. Consequently, the results from this second trial were interpreted separately and with some caution. In summary, 3 sets of oxygen uptake data were collected: (1) Nt/No (normoxic experimental treatment and normoxic oxygen uptake), (2) Ht/Ho (hypoxic experimental treatment and hypoxic oxygen uptake), and (3) Ht/No (hypoxic experimental treatment and normoxic oxygen uptake), a recovery trial in oxygenated water after exposure to hypoxia.

Small amounts of background O_2 uptake (<10.40% of measured values) were detected in the normoxic and hypoxic blank control chambers. Therefore, background respiration was accounted for when calculating the rates of O_2 uptake by individual brittlestars. All remaining individuals from the experimental cores were preserved in Bakers Formal Calcium solution for subsequent analysis of the gonads.

Measurement of oocyte diameter

Individuals from time points T0 and T14 were removed from Bakers Formal Calcium solution, and their arms were excised close to the disk. Each disk was placed into a small glass vial (vol. = 20 ml) and dehydrated using a sequence of increasing ethanol concentrations (30 min in each 50, 70, and 95% [repeated] and then 95% ethanol/monomer [1:1]). Disks were left in 2-hydroxyethyl methacrylate monomer overnight and then embedded in monomer with activator before being left to set for 24 h. Subsequently, casts were removed from the moulds and air dried for 12 h. A glass knife was used to cut transverse sections through the disk (5 µm thick), which were then mounted on glass slides and left to air dry. Sections were stained using the Periodic Acid Schiff Method (PAS). Due to the unknown distribution of females within each aquaria, and time restraints hindering the sectioning of every individual within the experiment, the above procedures were repeated until a total of 33 female individuals were sectioned, i.e. 11 from T0 (normoxia), 11 from T14 normoxic treatment and 11 from T14 hypoxic treatment. To assess the effects of organism density on the reproductive parameters measured, females from every aquarium covering each density treatment were sectioned. Only one

aquarium (hypoxic treatment, ind. per aquarium = 5, time point = T14), was excluded from this analysis because it contained all male specimens.

The plane of the disk section that intersected the greatest number of ovary sections was chosen for each female, and a series of images were captured under low power (×10) magnification using a microscope (Reichert Polyvar, Leica, Wien, Austria) through a mounted camera system (Coolpix 995, Nikon, Tokyo, Japan) that documented the entire brittlestar section. Photos from each image series were selected at random, and oocyte feret diameter of every late-vitellogenic oocyte displaying a nucleus in each photo was measured until 100 oocytes from each individual had been measured. In total, 3300 late-vitellogenic oocytes were measured. Image analysis was completed using the software Image-Pro Plus (v4.5 Media Cybernetics, MD, USA). Oocyte feret diameter is the greatest diameter measurement through the oocyte, based on the assumption that oocytes are not always spherical. Two basic rules were followed to select oocytes that were measured: (1) oocytes must be in the late-vitellogenic stage, and (2) the nucleoli must be visible (Bowmer 1982, Brogger et al. 2013).

Estimating gonad maturation

Counts of oocyte developmental stages were calculated using the same images taken for oocyte diameter assessment. Using Image-Pro Plus, 20 ovaries were selected randomly from each individual, and the total numbers of late-vitellogenic, mid-vitellogenic, and pre-vitellogenic oocytes within each ovary were counted. Across 33 individuals, a total of 35275 oocytes were examined. The staining method used during slide preparation allowed for distinct colour differences between these 3 major developmental stages, accounting for the different proportions of carbohydrate macromolecules present.

Statistical analyses

Statistical analyses were completed using the software package MINITAB 16 and PRIMER 6. Normality and the homogeneity of variances of samples were examined using Anderson-Darling and Levene's tests respectively. Assumptions for normal distributions of the data and homogeneity of variance were met for all parameters examined, apart from the late-vitellogenic oocyte stage, however after an ln-transformation, these data met assumptions for normality.

Regression analysis was completed on 'oxygen uptake', 'oocyte diameter data', and 'oocyte developmental stage data' to examine the effects of hypoxia against organism density and exposure time. These tests revealed that organism density within aquaria had no effect on any parameters measured, and analyses were re-run, excluding density as a factor. Consequently, 2-sample *t*-tests were used to examine differences between groups of data for oxygen uptake results, oocyte diameter, and developmental stage data. Rates of O_2 uptake data from the Ht/No data set (recovery trial) were examined separately using a paired *t*-test with the corresponding data from the Ht/Ho trial.

RESULTS

Rates of oxygen uptake

Regression analysis revealed that 'organism density' within the aquaria had no significant effects on 'oxygen uptake rates' ($F_{(df=4)}$ range 0.030 to 7.840, p range 0.068 to 0.879). The multivariate test PERMANOVA was also completed to assess the effects of 'density', 'water treatment', and 'exposure time' on 'oxygen uptake rates', and again, density of organisms in aquaria had no effect on the results (Pseudo-$F_{(df=4)}$ = 0.339, p(perm) = 0.849).

Normoxic treatment, normoxic oxygen uptake (Nt/No)

The mean rate of oxygen uptake from specimens of *Amphiura filiformis* pre-exposure (T0) was 0.823 O_2 h^{-1} g^{-1} wet mass. After 6 and 10 d experimental exposure (T6 and T10), the average oxygen uptake rate of organisms kept within normoxic water decreased slightly compared to the rate expressed at T0, but not significantly (Fig. 1, Table 2). After 14 d (T14), the mean rate of oxygen uptake increased significantly compared to all previous readings in the same treatment (Table 2), indicating that there may be an experimental time effect.

Hypoxic treatment, hypoxic oxygen uptake (Ht/Ho)

Organisms subjected to the Ht/Ho trial exhibited a mean oxygen uptake of 0.142 O_2 h^{-1} g^{-1} wet mass at T6, which significantly increased to 0.463 O_2 h^{-1} g^{-1} wet mass at T10 (Fig. 1, Table 2). This increase in

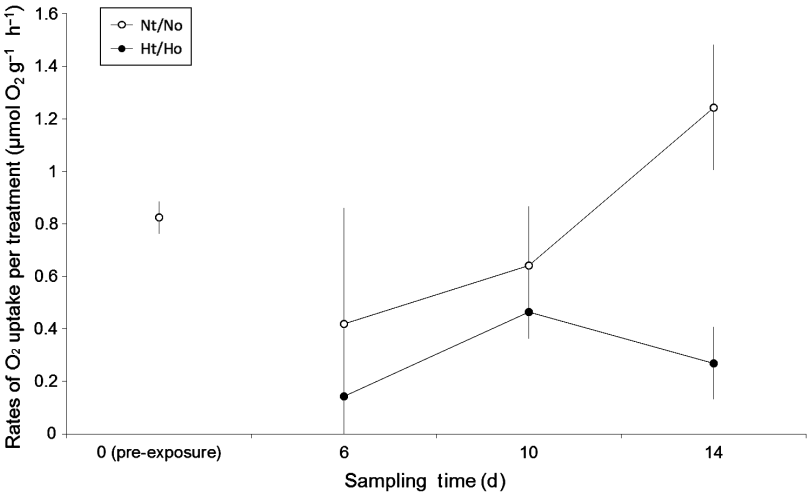

Fig. 1. Rates of oxygen uptake (µmol O_2 g^{-1} wet mass h^{-1}) for *Amphiura filiformis* within normoxic treatment/normoxic oxygen uptake (Nt/No) and hypoxic treatment/hypoxic oxygen uptake (Ht/Ho) trials. Data expressed as means ± 95% confidence intervals, n = 5

Table 2. Two-sample *t*-tests on rates of oxygen uptake data from normoxic set (Nt/No) and hypoxic set (Ht/Ho). *Significant p values (to 95% significance level); n = 5

	Nt/No		Ht/Ho		Nt/No vs. Ht/Ho	
Time	*t*-value	p-value	*t*-value	p-value	*t*-value	p-value
T0 vs. T6	1.780	0.150				
T0 vs. T10	1.540	0.199				
T0 vs. T14	3.340	0.029*				
T6 vs. T10	0.880	0.420	5.250	0.002*		
T6 vs. T14	3.220	0.018*	1.650	0.160		
T10 vs. T14	3.600	0.009*	2.250	0.059		
T6					1.220	0.289
T10					1.420	0.216
T14					6.950	0.001*

oxygen uptake rates from T6 to T10 shadows that of the organisms within the Nt/No trial (Fig. 1). After 14 d (T14) exposure to hypoxic water, oxygen uptake rates were slightly reduced compared to T10 rates, but did not alter significantly. The only significant difference that occurred between Nt/No trial and Ht/Ho trial was at T14, when organisms kept within normoxic water (Nt/No) showed a marked increase in their oxygen uptake rates, but organisms within hypoxia (Ht/Ho) did not (Fig. 1). This may be a reflection on experimental effects and acclimation to laboratory conditions.

Table 3. Paired 2 sample *t*-test on rates of oxygen uptake data from hypoxic set (Ht/Ho) and recovery set (Ht/No). *Significant P values (to 95% significance level); n = 5

	Ht/Ho vs. Ht/No	
Time	*t*-value	p-value
T6	1.220	0.289
T10	2.660	0.057
T14	0.930	0.405

Hypoxic treatment, normoxic oxygen uptake (Ht/No)

Organisms that were exposed to the second oxygen uptake trial, Ht/No (recovery trial), showed no significant differences in their oxygen uptake rates compared to their paired data points collected during the Ht/Ho trial (Table 3), meaning that once returned to oxygenated water, oxygen uptake rates did not immediately increase in a significant manner.

Oocyte feret diameter

Feret diameters of developed (late-vitellogenic) oocytes were measured in individuals at pre-exposure (T0) and at T14 only. Regression analysis showed 'density' of organisms within aquaria had no effect on oocyte feret diameter (F = 0.100, p = 0.754). Initially, at T0, mean (±95% CI) oocyte diameter measured 102.80 ± 2.08 µm (n = 5). After 14 d, brittlestars that had been exposed to normoxic water exhibited a significant increase in mean oocyte diameter (114.00 ± 1.40 µm, mean ± 95% CI, n = 5) compared to individuals measured at T0, indicating growth over the experimental period (t = 8.070, p < 0.001). Specimens exposed to the hypoxic treatment for 14 d (T14) exhibited a mean (±95% CI) oocyte diameter of 105.85 ± 1.36 µm (n = 4), which is a slight increase in diameter compared to individuals at T0, but this difference was not significant (t = 2.320, p = 0.081). Comparing between water treatments, oocyte diameter was significantly smaller in specimens that were exposed to 14 d of hypoxia compared to specimens exposed to 14 d of normoxia, (t = 10.130, p = 0.001), indicating reduced oocyte growth within the hypoxic water treatment over the experimental period.

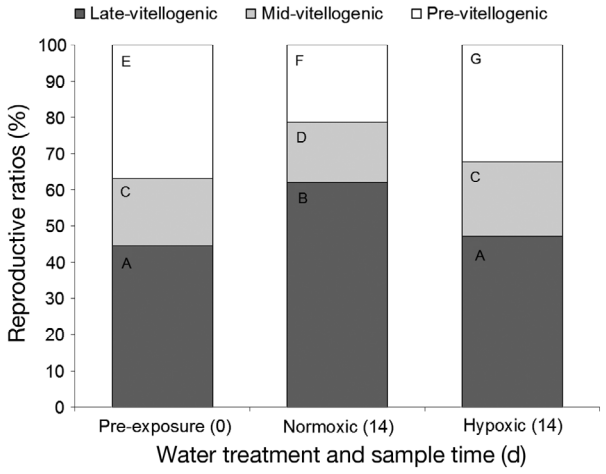

Fig. 2. Reproductive ratios (%) for oocyte development stage within *A. filiformis* pre-exposure (T0) and normoxic and hypoxic treatments at T14. Data are means (95% CI are not shown, but average at 1.82, with a range = 0.91 to 3.97). Different letters on graph represent significant differences within the same oocyte developmental stage, but across the treatment groups (T0 and T14 normoxic n = 5, T14 hypoxic n = 4)

Estimating gonad maturation

Comparing the pre-exposure measurements taken at T0 (normoxia) to the oocyte developmental stages within normoxic conditions 14 d later, there was clear evidence of developmental progression: the number of late-vitellogenic oocytes increased significantly, with a concomitant decrease in pre-vitellogenic oocytes (Fig. 2, Table 4). However, individuals that had been kept in hypoxic water for 14 d showed no significant differences in late-vitellogenic and mid-vitellogenic oocyte numbers compared to the pre-exposure measurements (T0), indicating a significantly reduced amount of developmental progression.

For individuals within the normoxic and hypoxic treatments measured at T14, the amount of time for oocyte development was the same, yet individuals exposed to hypoxia had significantly fewer late-vitellogenic oocytes and a greater number of mid- and pre-vitellogenic oocytes compared to individuals kept in normoxic water at T14 (Figs. 2 & 3, Table 4). Exposure to hypoxia may have disrupted oocyte development, resulting in fewer oocytes maturing and growing into the late-vitellogenic stage.

DISCUSSION

Our findings demonstrate that exposure to 14 d of moderate hypoxia reduces the aerobic metabolism of the brittlestar *Amphiura filiformis* and disrupts its reproductive development to an extent where oocyte diameter was reduced by 7.15% and 14.83% fewer oocytes reached the late-vitellogenic stage. However, these effects ocurred irrespective of population density. Consequently, the fact that the number of individuals per aquarium had no effect on any of the parameters measured here suggests that in the present experiment, individual brittlestars do not appear to gain energetic advantage from being in dense aggregations.

Table 4. Two-sample *t*-test on *Amphiura filiformis* oocyte developmental stage data. Comparisons between T0 (n = 5), T14 N (normoxic treatment) (n = 5) and T14 H (hypoxic treatment) (n = 4). *Significant p values (to 95% significance level)

Time/Treatment	*t*-value	p-value
	Late-vitellogenic	
T0 vs. T14 N	4.970	<0.001*
T0 vs. T14 H	0.990	0.339
T14 N vs. T14 H	5.240	<0.001*
	Mid-vitellogenic	
T0 vs. T14 N	1.180	0.252
T0 vs. T14 H	1.580	0.130
T14 N vs. T14 H	2.730	0.014*
	Pre-vitellogenic	
T0 vs. T14 N	7.010	<0.001*
T0 vs. T14 H	2.600	0.018*
T14 N vs. T14 H	5.360	<0.001*

Effects of density

Organism aggregations are a regular feature of natural ecosystems and occur for many reasons, including social and spawning activities or to provide benefits to individuals such as thermal advantages (Chapperon & Seuront 2012). Here, it was hypothesised that aggregation may benefit *A. filiformis* via increased aeration and movement of the overlying water, aiding burrow ventilation and reducing energetic costs. However, in this study, density of *A. filiformis* did not affect any of the parameters we investigated. One possible explanation for the lack of density-dependent effects could be due to individuals being confined within glass aquaria, where there is reduced laminar flow and potential edge effects. Although brittlestars were collected from an area of low flow and shear stress (Uncles & Torres 2013), previous studies have shown that laminar flow rate has positive effects on *A. filiformis* somatic growth (arm regeneration rates)

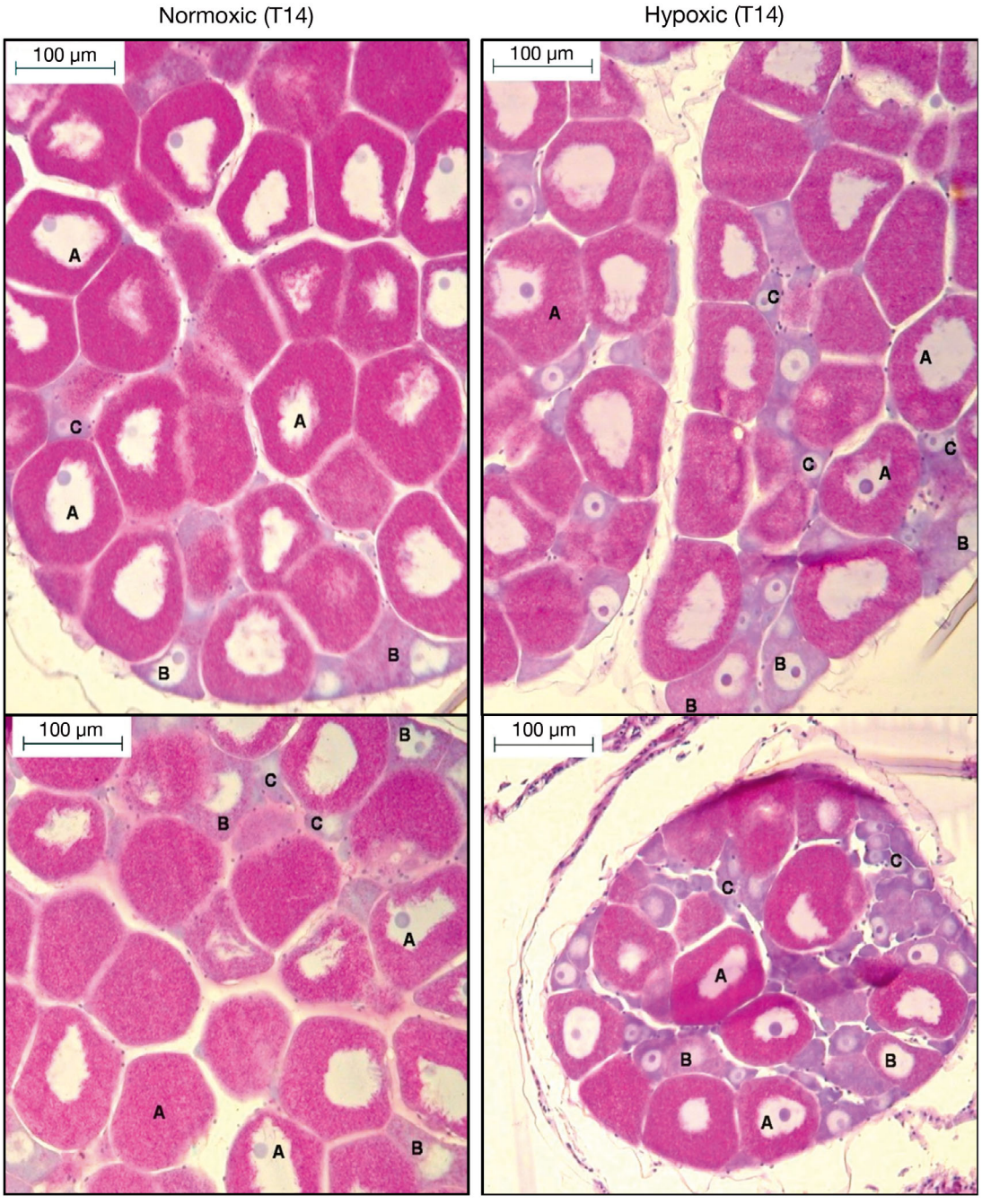

Fig. 3. Examples of *A. filiformis* oocytes from individuals within normoxic (T14) and hypoxic (T14) treatments. Note that the organisms exposed to hypoxia have significantly fewer late-vitellogenic oocytes and more pre-vitellogenic oocytes present compared to the normoxic treatment. A: late-vitellogenic, B: mid-vitellogenic, C: pre-vitellogenic

even when subjected to hypoxia, possibly due to the water pressure gradients created during flow that help to ventilate the burrow (Nilsson 2000). Under low laminar flow (as in this experiment), the capacity to irrigate their burrows requires active pumping using 2 arms. Therefore, irrespective of organism density, active burrow ventilation would still be a requirement to prevent the accumulation of toxic substances, possibly decreasing the energy yield that could be utilised in other important functions such as growth and reproduction. No direct measurements of burrow ventilation were recorded here, although aquaria were checked daily and protruding arms from the sediment were always present, typically indicating burrow ventilation and foraging behaviours.

Although our results suggested no advantages to *A. filiformis* when in dense aggregations, there were also presumably no disadvantages. Vevers (1952) suggested that densely populated patches of the brittlestar *Ophiothrix fragilis* occurred within the Plymouth region as a result of a consistent reliable food source supplied by tidal streams. Therefore, while aggregation benefits, in terms of lowering energetic costs to the organism, may not occur, there are other factors such as food supply which could possibly control distribution patterns for certain populations in their natural habitats. Food supply is an important factor governing growth and energetics within organisms, and may sustain growth rates even in hypoxia (Nilsson 1999). In our experiment, food was supplied daily with the dose of food proportional to the biomass within each aquarium. It may be that a large proportion of the energy gained from food ingestion was invested into maintaining burrow ventilation, especially within the hypoxic treatment. During hypoxic events, there is often an associated increase in ammonia and hydrogen sulphide (Wu 2002); hence, the consequences of allowing toxins to accumulate within the burrow could be more hazardous than exposure to hypoxia itself.

Effects of hypoxia on oxygen uptake

Our results are consistent with the view that *A. filiformis,* in common with a number of other brittlestars, has a tendency to be an 'oxyconformer', as it reduces its metabolic rates with declining pO_2 (partial pressure of O_2) in the surrounding seawater (Binyon 1972, Shick 1983). This was reflected in our results, with brittlestars exposed to hypoxia for 6 to 10 d showing a comparable trend of metabolic rates to that observed for individuals exposed to normoxic water, but showing consistently reduced rates.

Where there is evidence of oxyregulation in echinoderms, it can be a passive consequence of a small amount of biomass in a comparatively large fluid-filled space (Johansen & Vadas 1967, Mangum & Van Winkle 1973, Spicer 1995), as in echinoids, due to active perfusion of respiratory structures (Johansen & Petersen 1971), or due to the presence of an extracellular respiratory pigment like haemoglobin. For example, the burrowing brittlestar *Hemipholis cordifera* was able to maintain its metabolic rate over a broad range of pO_2 (Christensen & Colacino 2000) because of the presence of haemoglobin within red blood cells contained in the water vascular system (Christensen et al. 2003). *A. filiformis* has a relatively small fluid-filled space, does not appear to have a respiratory pigment or targeted mechanisms for perfusing putative gas-exchange surfaces, and shows little evidence of oxyregulation (J. I. Spicer pers. obs.).

Brittlestars exposed to Nt/No exhibited oxygen uptake rates broadly comparable to those recorded by Vopel et al. (2003) but lower (even allowing for temperature differences) than those found by Buchanan (1964) for the same species. The reduced rates of oxygen uptake at T6 and T10, although not a significant change from pre-exposure rates (T0), may be representative of experimental stress and adjustment to mesocosm conditions (Fig. 1). The significant increase in rates of oxygen uptake at T14 could be a sign of acclimation and recovery from the initial stress of collection and laboratory conditions. Although these rates are elevated compared to all other time points recorded for the same water treatment, the values were not dissimilar to those recorded for other brittlestar species (Binyon 1972). Acclimation time was accounted for in the experimental set up, but it is difficult to show exactly what effects are caused to organisms when removed from the field and how long recovery to a 'normal' physiological state would take. If this is the case for organisms exposed to normoxic seawater, our results suggests that exposure to hypoxia has hindered this acclimation process and possibly caused metabolic depression.

Furthermore, results from the Ht/No trial (recovery), although viewed with caution, indicate that *A. filiformis* showed no increase in their rates of oxygen uptake when re-introduced to oxygenated water. This continued reduction of metabolic rate levels even upon return to fully oxygenated water could be interpreted in a number of different ways, none of them mutually exclusive: (1) active metabolic depression triggered by >10 d exposure to moderate hypoxia; a number of marine animals respond to hypoxic exposure by conserving energy through an active down-regulation of certain enzymatic processes (Wu 2002), and thus, it is reasonable to assume that our data here represent metabolic depression due to exposure to hypoxia and the inability to increase rates of oxygen uptake upon re-introduction to oxygenated water; (2) an inadequate detection period; recovery of pre-hypoxic rates of oxygen uptake is a slow process and could have not be detected over the time frame of our experiment (i.e. within 4 h of the respiration experiment); (3) pathological damage; however, this is unlikely as organisms appeared healthy and mortality rate was 0%; and/or (4) an

artefact of the experimental design; our data were possibly affected by brittlestars being exposed to elevated levels of stress due to the undertaking of a second oxygen uptake trial, especially when the data cannot be compared to a second trial for the specimens exposed to normoxia. However, rates of oxygen uptake within the recovery trials remained similar throughout the experiment and at time point T6 almost match the rates of oxygen uptake of the organisms within normoxic water, reducing the possibility that experimental stress may be overriding the effect of the factors of interest.

If the reduction in oxygen uptake by *A. filiformis* exposed to hypoxic conditions is mainly a result of a metabolic reduction (or depression), it could be that assimilated energy may be redirected into other processes. Cheung et al. (2008) found that rates of oxygen uptake by the scavenging gastropod *Nassarius festivus* were reduced as environmental O_2 tension decreased, with fewer egg capsules produced at lower O_2 tensions. At 3.0 mg O_2 l^{-1}, Cheung et al. (2008) documented a 48% reduction in energy allocation to growth and reproduction compared to organisms kept at 6.0 mg O_2 l^{-1}, yet investment into shell growth continued, indicating that under stressful conditions, this species preferentially allocates energy into a trait that would enhance survival. In the case of *A. filiformis* investigated here, energy savings as a result of recourse to metabolic depression could be invested into burrow ventilation (locomotion), to reduce toxins and increase burrow oxygenation. This may be the behaviour/trait that would most enhance survival under experimental hypoxic conditions when laminar flow is reduced and could explain, in part, the delay in reproductive development as observed here.

Effects of hypoxia on reproduction

A. filiformis has a discrete, relatively short breeding period occurring largely in late summer and autumn with the fastest growth often occurring in May to June (Bowmer 1982). This experiment was timed prior to spawning and possibly encapsulated a period of rapid growth. Our results indicate that exposure to 14 d of moderate hypoxia significantly delayed reproductive development, both in terms of oocyte diameter and the number of fully developed oocytes present at the end of the experimental period. Although it is difficult to predict what ecological effect these results may have, they may provide an insight into why spawning was delayed for this species when exposed to hypoxia in the study conducted by Nilsson & Sköld (1996). However, it still remains unclear if this delay in oocyte development could be beneficial to the organism, where gametogenesis would resume normal development when the extrinsic environmental conditions become favourable, or if aerobic energy assimilation whilst exposed to hypoxia was so limited that it restricted energy investment into reproductive output. In previous experiments, *A. filiformis* could allocate a greater fraction of its energy budget to arm regeneration instead of disk growth (Nilsson 2000) in order to rehabilitate its full capacity for capturing food after sub-lethal predation. Therefore, as mentioned above, it is possible that energy was strategically allocated to metabolic processes and locomotory movements, i.e. burrow ventilation, to increase chances of survival during hypoxic stress. Furthermore, our experiment could not take into account the effects of sub-lethal predation occurring in the natural environment of brittlestars, i.e. somatic growth measured as arm regeneration. O'Connor et al. (1986) report estimates of energy flow within *A. filiformis* of 77.4% for respiration, 16.0% for arm regeneration, and 6.6% allocated to gonad output. The number of arms being regenerated affects regeneration rates and energy allocation from the disk, including the gonads (Dobson et al. 1991, Nilsson & Sköld 1996), and therefore, the energy required for arm regeneration during a hypoxic event in the field may impede reproductive development to an even greater extent than measured here. Additionally, studies investigating future scenarios of warmer, more acidic oceans have also shown that these environmental parameters can affect energy allocation in brittlestars. Wood et al. (2010) found that ocean acidification and increased temperature may indirectly affect the fitness and survival of *Ophiura ophiura*, through slower recovery from arm damage. In earlier work, Wood et al. (2008) demonstrated that *A. filiformis* can increase its metabolism and net calcification when exposed to acidified water, but at a substantial cost (arm muscle wastage). The possible interactive effects of hypoxia, acidification, and temperature on brittlestars' energetics and long-term survival are currently unknown.

Conclusions

Exposure to moderate hypoxia for 14 d reduces aerobic metabolism and delays reproductive development in the ecologically important brittlestar *A.*

filiformis. More broadly, there is already evidence to suggest that moderate hypoxia can severely affect the reproductive and endocrine systems in a number of other functionally important marine animals (Wang & Widdows 1991, Baker & Mann 1992, Nilsson & Sköld 1996, Chan et al. 2008). However, research in this area is still in its infancy (Wu 2002). In the long term, recurring hypoxic events may have major implications for benthic and pelagic population dynamics by indirectly affecting metabolic processes and reproduction, possibility resulting in reduced diversity and functionality within these communities. Further investigations of the effects of hypoxia on reproduction and development of other functionally important benthic taxa would provide important insights into the long-term effects to biodiversity in areas where hypoxia is common.

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