Ocean acidification induces multi-generational decline in copepod naupliar production with possible conflict for reproductive resource allocation.

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ABSTRACT: Climate change, including ocean acidification (OA), present fundamental challenges to marine biodiversity and sustained ecosystem health. We determined

cuticle composition. These responses may drive shifts in life history strategies that favour smaller brood sizes, females and perhaps later maturing females, with the potential to profoundly destabilise marine trophodynamics.

Keywords: Ocean acidification; reproduction; multi-generation; ecological significance; model.

1. Introduction

The burning of fossil fuels over the past 200 years has perturbed the pre-industrial steady-state global cycle of carbon dioxide (CO_2), resulting in increased carbon inventories in the atmosphere, oceans and on land. One consequence of the increased ocean uptake of CO_2 (Sarmiento and Gruber, 2002)

two generations of the calanoid copepod *Acartia tsuensis* were cultured at a single reduced pH (7.32±0.04) with no clear impact on survival, developmental rate, growth, fecundity or hatching success (Kurihara and Ishimatsu, 2008). However, in species with very short life cycles such as *A. tsuensis* (nine days between egg and maturity at 25^{o}C i.e. 225 degree days) two generations may not be sufficient to observe measurable effects.

Concerns over calcium dissolution have favoured the study of calcifying organisms (Anthony et al., 2008; Hofmann et al., 2008; Wood et al., 2008) with comparatively little effort invested in non-calcifiers. However, physiological and metabolic functions common to both groups are also anticipated to be vulnerable to pH perturbations. As such, the responses of both calcifying and non-calcifying organisms must be rigorously evaluated. Physiological responses have been subjected to intense investigations, these concluding that energy-dependent downstream biological processes such as reproduction are likely to be disproportionately affected (Kurihara, 2008; Pörtner, 2008; Pörtner et al., 2004). Initial reproductive studies reported pronounced inhibitory effects on sperm motility, fertilisation and hatching success for several species (Egilsdottir et al., 2009; Ellis et al., 2009; Havenhand et al., 2008; Kurihara et al., 2004a; Kurihara and Shirayam, 2004; Mayor et al., 2007; Morita et al., 2009; Parker et al., 2009). Compromised growth and increased incidences of developmental defects hTf 12 0 044(r)3(e)4(l)(ev)-4(er)-1(al)-6(s)-5(p)-4(e)-10(ci)-6(es)]TJ 0 Tc 0 Tw

determined. *Tisbe battagliai* is a benthic harpacticoid and has a wide geographical distribution, including shallow coastal waters in regions of Europe and off the Atlantic coast of the USA. Our reasons for selecting *T. battagliai* include its ease of culture; its rapid and predictable life cycle (266 degree days between egg and maturity); and its high reproductive output (Cutts, 2003; Gaudy and Guerin, 1982). These properties have enabled us to undertake a three generation investigation. In addition, adopting a multi-generational strategy has enabled us to model trends in naupliar production over a one hundred year period, thereby enhancing our capacity to predict the impacts of OA on copepod population dynamics. Our results are potentially of great value to future modelling studies seeking to gauge the trophodynamic impacts of OA.

2. Methods

2.1. Animal husbandry and experimental incubations

Tisbe battagliai obtained from Guernsey Sea Farms Ltd (Guernsey, UK) were cultured in the laboratory at 19.25±0.36^{o}C with 12:12 photoperiod in aerated 0.22 µm filtered seawater, with weekly water changes. Natural seawater was collected from the Blue Reef Aquarium®, Tynemouth, Tyne and Wear, UK from an inshore sub-sea pipeline. Copepods were fed *ad libitum* on a mixed microalgae diet (2:1 ratios of *Isochrysis galbana* and *Tetraselmis suecica*) ($4.4x10^{5}$cells.ml^{-1} and $2.0x10^{5}$ cells.ml^{-1} respectively), following water changes. Adults and nauplii were separated using a 200 µm sieve. Incubations and recording of life cycle parameters were adapted from a previous approach (Taylor et al., 2007). Briefly, single gravid females were placed into individual wells within modified 12-well plates. The base of the multi-well plates was removed and replaced by 20 µm mesh to allow circulation of

controlled automatically by an Aqua-medic™ pH computer and probes. Carbonate parameters were calculated using CO_2Sys following pH, temperature and salinity measurements made with a Dr DAQ™ data logging system and total alkalinity (TA) titrations (see Table 1 and electronic supplementary data for more details). Oxygen levels were not determined. Experimental pH values were 7.67±0.02, 7.85±0.02, 7.95±0.02 and 8.06±0.06 (control) and naupliar production was recorded daily using light microscopy. Interbrood period for individual gravid females was determined as the time in days between females brooding and hatching nauplii. Due to brood variability, data were collected across three broods (~12 days) also allowing for observation across 72 hours of all nauplii produced per brood (Mayor et al., 2007). Following the initial collection of naupliar production data for three broods per female, the progeny were grown on to adults and the initial gravid females removed; these produced the subsequent generation of gravid females. Only brood one nauplii were used for production of the subsequent gravid females following mixing between wells, taking 14 days from hatching to sexual maturity. On each occasion 36 gravid females were placed into the experimental tank. The process was repeated for four generations; discarding the first generation data to allow sufficient time to acclimatise copepods before data collection.

2.2. Growth

Copepods were sampled at each developmental stage, brood, generation and pH. Samples were all taken from brood one of generation one nauplii. The number sampled varied between two and six depending on stage. Samples were fixed using a 2.5:1 mixture of 40%

the caudal ramus in adults and copepodites and the tip of the caudal armature in nauplii. Measurements were taken using an inverted dissection microscope (Olympus CH x41 at x10 objective) coupled with a digital camera (Sanyo) and Image J software.

2.3. Cuticle composition

Environmental scanning electron microscopy (ESEM) coupled with energy dispersive X-ray (EDX) analysis was used to determine cuticle elemental composition (%) of fixed, air-dried samples (FEI XL30 ESEM-FEG coupled with Quantac EDX system manufactured by Rontec). All specimens sampled for cuticle analysis were gravid females from the same generation. Point analysis was used to produce data from three points along the body for each of three copepods per pH. Samples were analysed at 1 µm below the surface of the exoskeleton at low vacuum at 20 kV and x250 magnification. Despite samples having been rinsed with deionised water prior to air drying, peaks of sodium and chlorine were nevertheless discounted due to the possibility of interference from residual sea salt. Nitrogen was undetected as, using the current system, nitrogen is difficult to detect unless in large quantities and undetectable when using quantitative analysis. Carbon and oxygen content of gravid females were normalised to nauplii production.

2.4. Statistical modelling of multi-generational data

A generalised least squares modelling approach, as described by Pinheiro and Bates (2000), was used to determine potential differences in copepod population growth over the range of OA scenarios. Generalised least squares is a robust regression technique used to offset the effects of pseudo-replication, and/or when the variances within treatment groups are subject to variability (Pinheiro and Bates, 2000). In addition, generalised least squares is also able to estimate relationships between response and explanatory variables in the case of unbalanced experimental designs.

of pH and stage rather than the interactions of pH and stage. Covariance indicating that from a biological perspective changes in pH altered the size of the copepod but this was reliant on the copepods developing through the previous stages.

Elemental composition was analysed using a one-way ANOVA examining differences between copepods with pH. A one-way ANOVA was also used to check for differences between point analyses on each copepod; these were found to be insignificant.

3. Results

3.1. pH and carbonate parameters

production with increasing pH over the next 100 yrs. Differences in the numbers of nauplii produced over each generation were also examined. An increase in naupliar production was observed at generation one from generation zero to 13.99 (*t*-value=3.41, P<0.001), however this reduced to near generation zero levels at generation two (12.25, *t*-value=0.501, P=0.616). The effects of multiplicative interaction between pH and each generation was also examined and significant declines in the mean number of nauplii were observed at pH 7.95 for generation one and two (G1 9.35, *t*-value=-2.73, P-value=0.007 and G2 8.268, *t*-value=-4.13, P-value<0.001). None of the remaining interactions between pH and generation were statistically significant.

3.3. Copepod growth

Growth was significantly reduced compared to the control (mean 362.37±1.07 µm) for pH 7.82 (mean 311.55±1.06 µm, *t*-value=-2.72, P-value=0.008) and 7.67 (mean 271.83±1.05 µm, *t*-value=-5.463, P-value<0.001), however growth at pH 7.95 (mean 398.63±1.06 µm) did not differ significantly from that of the control (Table 3) (Figures 3&4). Copepods cultured at pH 7.82 and 7.67 were observed to reach sexual maturity (C5 stage) (Figure 3) at a considerably smaller body length than copepods cultured at pH 8.06 and 7.95. Reductions in body length shown here at C5 to be 91 µm were observed which equates to an overall reduction in body length of 25% between pH 8.06 and 7.67.

3.4. Copepod cuticle elemental composition

ESEM analyses identified the following elements in the copepod cuticle; carbon, oxygen, sodium, en70 Tcp 0 Td ( )alg4(x)-10(ua)com,551.04 0 0t Td [(0.0i)-2(c)4(l)-2(e)484 Tm [nt he cC ic(e)4

considered. Copepod cuticular oxygen content was significantly lower at pH 7.82 than at pH 8.06 (mean oxygen concentration 48.23, ANOVA; $P<0.02$, df=2, mean carbon concentration 43.28; not significantly different from the control), and copepods at pH 7.67 had significantly lower oxygen concentrations but higher carbon concentrations (mean oxygen concentration 43.40, ANOVA; $P<0.02$, df=2, mean carbon concentration 52.55, ANOVA; $P<0.002$, df=2) (Figure 5a). The carbon to oxygen ratio changed markedly with pH (pH 8.06=0.79; pH 7.95=0.73; pH 7.82=0.90; and pH 7.67=1.21) with the proportion of carbon increasing with decreasing pH. When carbon and oxygen concentrations were normalised to naupliar production, there was a profound impact on female cuticle composition (Figure 5b). When naupliar production was reduced at pH 7.82, carbon and oxygen concentrations increased, suggesting energy reallocation to the cuticle. In the same way, when naupliar production increased at pH 7.95, carbon and oxygen concentrations declined.

4. Discussion

Although previous studies have demonstrated detrimental effects of OA on several reproductive and developmental processes across a broad range of invertebrate taxa (Kurihara, 2008; Kurihara et al., 2004a; Kurihara and Shirayama, 2004; Egilsdottir et al., 2009; Findlay et al., 2009; Talmage and Gobler, 2009), our work is, to our knowledge, the first to establish a direct link between OA and a decline in nauplii production beyond two generations. An interesting and unexpected aspect of our results were the increases in nauplii production observed at pH 7.95 and at pH 7.67, which we attribute to an initial stress response and a hormesis-type response respectively (Calabrese, 2008; Lefcort et al., 2008). Stress may be viewed as a physiological response to a demand (Calabrese, 2008), with initial stress reflecting the first physiological response to a stressor (demand), that may or may not return to homeostasis. Hormesis differs from initial stress, being a bimodal or biphasic response; low stress levels result in a particular response, whereas increased stress may elicit

an opposite response. This may be interpreted as an adaptive biphasic dose response whereby compensatory physiological behaviour follows an initial disruption of homeostasis (Calabrese, 2008; Lefcort et al., 2008), a

7.82 coincided with the significant decrease in nauplii production. The apparent coupling of cuticle elemental composition and nauplii production (Figure 5b) suggests that during periods of prolonged pH stress copepods may selectively reallocate energy between somatic growth and reproductive output. This implies a fine balance between maternal resource (measured as cuticle composition) and reproductive output (naupliar production). Chitin production occurs at the surface of the epidermis at premolt (Adiyodi, 1985). This is a metabolically expensive process. As such, changes in carbon and oxygen concentrations would suggest energy reallocation may be expressed phenotypically as either up or down regulation of chitin synthesis. It was observed that optimisation of carbon resources for growth and gonad maturation were maintained despite low food concentration. The energy allocation strategy was defined in two stages: 1) somatic growth, where assimilated matter is invested to growth; 2) maturation phase, where assimilated matter is invested in gonad maturation (Lombard et al., 2009). C:O ratios in chitin a

maturation is attributable here with significantly reduced copepod length across all life stages at pH 7.82 and pH 7.67. The greatest overall reduction in copepod length was observed at pH 7.67 which coincides with a significant increase in nauplii production compared to pH 7.82. Previous studies identified similar growth reduction with increasing pCO_2 (Berge et al., 2006; Ishimatsu et al., 2008; Kurihara et al, 2008). At pH 7.95, the increase in nauplii production and the subsequent reduction in carbon and oxygen concentrations within the cuticle, normalised to nauplii production, strongly indicate energy allocation in favour of reproduction. This is further evidenced at pH 7.82 and pH 7.67 where investment in somatic growth is sacrificed in preference to reproductive output. By combining data for growth, cuticle composition and nauplii production, we postulate that due to OA-induced stress, copepods will preferentially reallocate energy resources to maintain an overall high level of reproductive output at the expense of somatic growth and cuticle integrity. The overall trend in nauplii production and growth retardation suggests potentially very serious implications for copepod populations under increasing OA. Inevitably a point will be reached whereby growth and cuticle integrity cannot be further compromised without significant impacts on fitness. Assuming an inability to adapt, such a scenario would likely result in a significant down regulation of reproductive output (Figure 2) and may ultimately promote shifts in life history patterns that would favour smaller brood numbers, females and perhaps later maturing females. Such fundamental shifts in reproductive output and life history strategies may potentially destabilise marine trophodynamics. Initial mesocosm and field studies indicate that impacts of temperature and pH change on microzooplankton production are also strongly linked to bottom-up effects due to diminished nutritional quality of phytoplankton food

and Hoegh-Guldberg, 2008). Similar high quality data are essential to accurately model future impacts of OA on marine ecosystems.

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Tables

Table 1. pH parameters maintained within the experimental system. pH, salinity and total alkalinity (TA) all measured to calculate hydrogen carbonate (HCO_3^-), carbonate (CO_3^{2-}), partial pressure of carbon dioxide (pCO_2), calcite (Ω Ca) and aragonite (Ω Ar) using CO2Sys software.

pH	generation	mean pH	mean temperature (^oC)	salinity	mean TA ($CaCO_3$ µmol/kg)	HCO_3^- in (mmol/kgSW)	CO_3^{2-} in (mmol/kgSW)	pCO_2 in (matm)	ΩCa out	ΩAr out
8.06	**0**	**8.02±0.06**	**19.31±0.39**	**36.0**	**1219**	**951**	**92**	**213**	**2.17**	**1.41**
8.06	**1**	**8.04±0.06**	**19.37±0.37**	**36.0**	**1239**	**957**	**97**	**204**	**2.29**	**1.49**
8.06	**2**	**7.98±0.06**	**19.16±0.41**	**36.2**	**1279**	**1021**	**90**	**250**	**2.12**	**1.38**
7.95	0	7.94±0.02	19.42±0.35	38.3	1399	1129	95	302	2.22	1.45
7.95	1	7.93±0.02	19.24±0.39	39.3	1419	1146	96	313	2.22	1.45
7.95	2	7.94±0.02	19.51±0.35	36.0	1299	1055	85	284	2.02	1.31
7.82	**0**	**7.82±0.02**	**19.37±0.34**	**37.7**	**1339**	**1134**	**72**	**401**	**1.68**	**1.09**
7.82	**1**	**7.82±0.02**	**19.37±0.34**	**38.0**	**1319**					

Table 2. Random-effects variance components for the multi-level model.

coefficient	naupliar production number	standard error	*t*-value	*P*-value
pH 8.06	11.97	1.03	76.80	<0.001
pH 7.95	15.05	1.07	3.61	<0.001
pH 7.82	9.67	1.06	-3.89	<0.001
pH 7.67	11.20	1.06	-1.20	0.229
Generation 1	13.99	1.05	3.41	0.001
Generation 2	12.25	1.05	0.50	0.616
pH 7.95:Generation 1	9.35	1.10	-2.73	0.007
pH 7.87:Generation 1	11.05	1.08	-1.03	0.304
pH 7.67:Generation 1	10.92	1.08	-1.17	0.241
pH 7.95:Generation 2	8.27	1.09	-4.13	<0.001
pH 7.87:Generation 2	11.53	1.08	-0.49	0.626
pH 7.67:Generation 2	12.67	1.08	0.72	0.474

Figure Captions

Figure 1. Nauplii production (mean±SE, N=27) for *Tisbe battagliai* cultured under specified OA scenarios. Data are grouped by brood and generation. Symbols; ● = pH 7.67, ■ = pH 7.82, ♦ = pH 7.95 and ▲ = pH 8.06.

Figure 2. Mixed effects model indicating the closeness of fit of the predicted (dashed lines) against measured (solid lines) naupliar production for complete data set across generation with pH changing as a factor for (A) G0, (B) G1 and (C) G2.

Figure 3. Body length (mean±SE) of *Tisbe battagliai* from stage N3 to C5 cultured under specified OA scenarios. All stages were taken from brood one of generation one for each pH, stage C5 copepodites presented were all females. Symbols; ● = pH 7.67, ■ = pH 7.82, ♦ = pH 7.95 and ▲ = pH 8.06.

Figure 4. Mixed effects model indicating closeness of fit of the predicted (dashed lines) against measured (solid lines) copepod length (A) for each pH and (B) for each stage. ( )Tj -0.003 Tc 0c(t)-