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Have we been underestimating the effects of ocean acidification in zooplankton?

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Abstract

Understanding how copepods may respond to ocean acidification (OA) is critical for risk assessments of ocean ecology and biogeochemistry. The perception that copepods are insensitive to OA is largely based on experiments with adult females. Their apparent resilience to increased carbon dioxide (pCO_2) concentrations has supported the view that copepods are 'winners' under OA. Here, we show that this conclusion is not robust, that sensitivity across different life stages is significantly misrepresented by studies solely using adult females. Stage-specific responses to pCO_2 (385–6000 μatm) were studied across different life stages of a calanoid copepod, monitoring for lethal and sublethal responses. Mortality rates varied significantly across the different life stages, with nauplii showing the highest lethal effects; nauplii mortality rates increased threefold when pCO_2 concentrations reached 1000 μatm (year 2100 scenario) with LC_{50} at 1084 μatm pCO_2. In comparison, eggs, early copepodite stages, and adult males and females were not affected lethally until pCO_2 concentrations ≥3000 μatm. Adverse effects on reproduction were found, with >35% decline in nauplii recruitment at 1000 μatm pCO_2. This suppression of reproductive scope, coupled with the decreased survival of early stage progeny at this pCO_2 concentration, has clear potential to damage population growth dynamics in this species. The disparity in responses seen across the different developmental stages emphasizes the need for a holistic life-cycle approach to make species-level projections to climate change. Significant misrepresentation and error propagation can develop from studies which attempt to project outcomes to future OA conditions solely based on single life history stage exposures.

Keywords: copepod, developmental stages, mortality, ocean acidification, recruitment, zooplankton

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Introduction

A significant volume of research has been conducted over the last decade examining the sensitivity of marine organisms to the changes predicted for the ocean's carbonate chemistry as a result of ocean acidification (OA). Responses to OA are much more variable than originally anticipated, with interspecific variation occurring between closely related species, as well as intraspecific variation both between and within populations (Parker *et al*., 2010). Still, little is known of the variation in response to OA at different life history stages within a species' life cycle. While the early developmental stages of many marine species are suspected to be most sensitive to the effects of OA (Dupont & Thorndyke, 2009; Kroeker *et al*., 2010), few studies have directly compared the variation across the different developmental stages of a given life cycle. Knowing the different life-stage-specific effects of OA within a species helps to identify the developmental stage(s) most at most risk, which is essential for projecting outcomes to future CO_2 scenarios. Predictions based only on limited life-stage exposures have clear scope to significantly under- or overestimate the species true overall vulnerabilities.

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Accurate projections of the response of copepods to OA are pivotal to our understanding of future plankton trophic dynamics. Copepods transfer biomass from primary producers to higher trophic levels and in doing so, contribute significantly to the vertical particle flux, influencing global biogeochemical cycles. Previous studies exposing calanoid copepod species have highlighted their apparent resilience to the projected 2100 pCO_2 (Weydmann *et al*., 2012; McConville *et al*., 2013), with lethal and sublethal effects occurring at concentrations that far surpass any climate change scenario (Yamada & Ikeda, 1999; Watanabe *et al*., 2006; Pascal *et al*., 2010). However, these studies have focused largely on the lethal and sublethal effects of acute high pCO_2 on adult females (Kurihara *et al*., 2004a,b; Mayor *et al*., 2007, 2012; Pascal *et al*., 2010; Zervoudaki *et al*., 2011; Zhang *et al*., 2011; Vehmaa *et al*., 2012; McConville et al., 2013). In comparison, few studies (Fitzer et al., 2012b; Lewis et al., 2013) have measured the effects of OA on other life history stages e.g. nauplii. Comparisons between size fractioned stages of mixed copepod assemblages have shown that earlier developmental stages have the greatest sensitivity to elevated pCO_2 (Lewis et al., 2013). Similarly, direct comparisons between two different developmental stages of the species, *Acartia erythera*, found that nauplii were more sensitive to the effects of high pCO_2 (2000 ppm) compared to that of adults (Kurihara et al., 2004a,b). This is indicative that the sole use of adult females to determine effects of high pCO_2 is not a true representation of the species response to OA. Potentially, other life stages (i.e. eggs, copepodites, and adult males) may be even more vulnerable to the effect of OA than that seen in nauplii. Indeed, direct comparisons across all key life stages of a species life cycle are needed to fully appreciate the integrated consequences of high pCO_2.

The aim of this study was to determine the extent of variation in specific responses between different developmental stages in a copepod species. For the first time, several different developmental stages of a calanoid copepod, *Acartia tonsa,* were acutely exposed to five different pCO_2-acidified seawater levels and then monitored for lethal and sublethal responses. While chronic exposure experiments may indicate whether this species could acclimate or adapt to a constant high pCO_2 level over time, the use of multi-stage acute exposure experiments was considered equally realistic in testing the response of this particular species to OA. This is because *Acartia tonsa* populations inhabit a wide range of environments, each with varying levels and fluctuations of pCO_2. Within each population, *A.tonsa* migrate to different depths in relation to their ontogeny (Holliland et al., 2012), resulting in different developmental stages being exposed to different variations in pCO_2. As the projected levels of pCO_2 will be variable over different temporal and spatial scales (Flynn et al., 2012), we argue that exposure of individuals to a range of pCO_2 levels over relatively short periods of time would be similar to gradients that *A.tonsa* may experience in the wild.

Materials and methods

Copepods

The calanoid copepod, *Acartia tonsa*, was obtained originally from Environment & Resource Technology (ERT), Orkney, UK. Stock populations were cultured in the Centre of Sustainable Aquatic Research (CSAR), Swansea, UK. Stock cultures were maintained at 24.4 °C (±0.54) with a 14 : 10 photoperiod (4–9 μmol photons m^{-2} s^{-1}) in aerated (392 ± 27 ppm CO_2) filtered (0.22 μm) seawater. These stock *Acartia tonsa* were fed *ad libitum* on a mixed microalgae diet of *Isochrysis galbana* (Strain CCAP 927/1), *Tetraselmis suecica* (Strain CCAP 66/22C), and *Chaetroceros muelleri* (Strain CCAP 1010/3). The microalgae were grown separately in a seawater-based *f/2* medium (Guillard & Ryther, 1962), maintaining a nutrient-replete status [average (±1SD) mass C : N ratios of *Isochrysis* 5.74 ± 0.41, *Tetraselmis* 7.27 ± 0.84, and *Chaetoceros* 6.22 ± 0.50], and were fed to copepods in a ratio of 1 : 1 : 1 relative to the carbon biomass concentration of the algae (respectively, the initial cell densities at the time of addition to the copepods were 5.0×10^4 cells ml^{-1}, 4.0×10^3 cells ml^{-1}, 2.5×10^4 cells ml^{-1}; total C-biomass added = 1 μg C ml^{-1}). Copepods were reared under these conditions until sufficient numbers of the desired stage [eggs, early nauplii (N_{I-II}), early copepodites (C_{I-II}), male and female adults] were obtained for experimental use.

Treatment levels

The life stages (i.e. eggs, nauplii, copepodite, mature males and females) of *Acartia tonsa* were exposed to five different pCO_2 levels: (i) present-day pCO_2, 385 μatm; (ii) near-future level, 1000 μatm (RCP8.5 2100 pCO_2 projection, Van- Vuuren et al., 2011); (iii) 2000 μatm (ECP8.5 2300 pCO_2 projection, Van- Vuuren et al., 2011), and two extreme pCO_2 levels; (iv) 3000 μatm; and (v) 6000 μatm. The two latter levels were used to determine lethal and sublethal threshold limits, both of which correlate to potential carbon capture and storage (CCS) leakage scenarios (Blackford et al., 2009). These different levels of seawater pCO_2 were obtained through mixing high pCO_2 water with water saturated with ambient CO_2, to attain the desired level (Riebesell et al., 2010). Measurements of pH were made through a three-point decimal place Omega PHB-121 bench top microprocessor pH meter cross-referenced with a WTW 315i portable meter (2A10-101T), both calibrated with pH 7.01 & 10.01 (NBS scale). Total alkalinity (measured by open cell pentiometric titration using an AS-ALK2 Gran Titrator, Apollo SciTech, USA), pH, salinity, and temperature were used to calculate the pCO_2 (μatm) through the programme CO2 SYS (Pierrot et al., 2006), using the K1, K2 constants from Mehrbrach et al. (1973)as refitted by Dickson & Millero (1987).

Experiment design

Four different experiment types were conducted, one for each of the immature developmental stages and a combined experiment for the mature stages, as outlined below. In all instances, two controls were used for each CO_2 level: (i) prey only, with no copepods, to measure phytoplankton prey effects on the seawater chemistry; (ii) no predators or prey, to measure background seawater chemistry variation over the 24 h period.

Eggs. Approximately, 3000 fertilized females of mixed maturity (1–5 days) were split between 5 × 2 l beakers (0.3 individual's ml^{-1}). Each beaker was lined with 150 μm nylon mesh to prevent egg cannibalism. The beaker was filled with ambient aerated seawater with known saturating prey conditions [1 μg C ml^{-1} (prey carbon ratio 1 : 1 : 1 of *I. galbana*, *T.*

suecica and *C. muelleri*)] and females were left for 5 h to produce eggs. Subsequently, all females were filtered out using 150 μm nylon mesh and the eggs collected. Eggs were placed individually into each well of 24-well culture plates with the different pCO_2 treatment (well volume: 3.6 ml; minimum of three replicate plates per treatment). A minimum of 70 eggs were used for each pCO_2 level. All well plates were sealed for the 96 h duration to maintain the pCO_2 level, with pH, temperature, and salinity measured before (t_0) and after (t_{96}) the experiment. Hatching rates were measured every 24 h for the 96 h period; most eggs hatched within 48 h and any not hatching by 96 h were considered nonviable. Mortality rates of the eggs over the 96 h exposure in each of the five different pCO_2 levels were calculated with the following equation.

$$Z = \ln \frac{(N_0/N_t)}{t}. \quad (1)$$

where Z is the mortality rate, N_0 is the initial (t_0) number of eggs, and N_t is the number of hatched eggs after t days. All eggs were considered to have been produced and fertilized under ambient conditions of pCO_2, prior to being exposed to the different pCO_2 levels. This then maintains commonality across the different treatment levels, enabling any mortality of the eggs to be identified as resulting from exposure treatment as opposed to prior maternal or fertilization effects.

Nauplii. For each pCO_2 treatment, 4 × 250 ml tissue culture flasks were each seeded with 25 $N_{I\text{-}II}$ individuals (each <24 h old). An additional 25 $N_{I\text{-}II}$ from the stock culture were fixed with 1% iodine to determine initial (t_0) size data. The nauplii were exposed to the assigned pCO_2 treatment for 96 h, with flasks held on a plankton wheel at 2 rpm, in a constant temperature room (24 °C ± 0.9) with 14 : 10 [light (4–9 μmol photon m^{-2} s^{-1}): dark] photoperiod. Seawater at the appropriate pCO_2 was replenished every 24 h to prevent potential drift in seawater carbonate-pH chemistry.

Copepod survival across all treatments was analysed every 24 h. Mortality of an individual was determined by the lack of movement after physical stimulation with a Pasteur pipette. Dead individuals were removed before replacing the live individuals back into fresh seawater with renewed prey conditions. Mortality rates were determined using Eqn 1. At the end of the 96 h exposure, all treatments were terminated and fixed in 1% Lugols iodine, with size and stage data collected immediately after fixation. Instar developmental stages were identified across all treatments (Ogilvie, 1956; Sabatini, 1990). Total body length (TBL; μm) of the nauplii was measured through Image Analysis (Lecia, LAS 3.8.0) and converted into carbon content (μg C ind^{-1}) using the Berggreen *et al.* (1988) length to carbon conversion; nauplii $\mu gC = 3.18 \times 10^{-6}$ $TBL^{3.31}$. Individuals' carbon-specific growth rates (μ) were determined across all CO_2 treatments post 96 h exposure, using Eqn 2; W_0 & W_t are the initial and end point weights of the individual (μg C), and t is the time period between sample points.

$$\mu = \frac{\mathrm{Ln}(W_t/W_0)}{t} \quad (2)$$

Copepodites. The experimental design and data collection protocol for the copepodite stages were the same as that for the nauplii (see 2.3.2). Twenty copepodite ($C_{I\text{-}II}$) individuals were used for each replicate culture flask (250 ml). The end point growth analysis was determined by measuring the copepodite and adult prosome length (PL, μm), which was converted to carbon using Berggreen *et al.* (1988) length to carbon conversion; copepodite & adult $\mu g\ C = 1.11 \times 10^{-5} PL^{2.92}$.

Adult males and females. For each pCO_2 treatment, 9 × 260 ml tissue culture flasks were used. Six flasks contained adult females [12 (<30 h-old) mature, virgin females without attached spermatophore per replicate], three flasks contained adult males (12 individuals per replicate). Direct lethal effects were measured in the same manner as the nauplii and copepodites. Sublethal effects were measured through fecundity success as follows.

Egg production—Post 72 h exposure, males and females within the same treatment level were combined in a 260 ml tissue culture flask (with four replicate flasks per treatment level). Within each treatment replicate nine females and six males were held for 30 h to copulate, with known saturating prey conditions [>1 μg C ml^{-1} (prey carbon ratio 1 : 1 : 1 of *I. galbana, T. suecica* and *C. muelleri*)]. After 30 h, 10–15 females were randomly selected from each treatment across the four replicates and carefully placed individually into 30 ml vials with their assigned CO_2 treatment. Each vial was prelined with a 150 μm nylon mesh bottom to prevent egg cannibalism. Females were held for 24 h to lay eggs, after which egg production rates were determined for each individual female across the five pCO_2 treatments. Subsequently, the eggs were utilized for egg hatching rates and measurements of egg diameter.

Egg size—The diameter of at least 20 eggs from each pCO_2 treatment was measured from digital images (Lecia LAS 3.8.0). Eggs were assumed to be spherical; volume was calculated with the equation: Egg Volume (μm^3) = $^4/_3 \pi r^3$, and egg volume converted into carbon assuming 0.114 pg C μm^{-3} (Calliari *et al.*, 2006). Using data on carbon, egg size, and egg production per female, C-specific egg production rates were calculated for each pCO_2 treatment.

Egg hatching—same method as described for eggs in Eggs.

Nauplii recruitment success—Daily egg production rates and egg hatching rates were combined to determine the nauplii recruitment success through parental exposure to varying pCO_2 treatment.

Statistics

Within each developmental stage, the mortality rates were compared between pCO_2 treatments. If data failed to fit the normality assumptions of the ANOVA test, a rank-based non-parametric Kruskal–Wallis Test (results reported as; H = test statistic, df_a = degrees of freedom between groups, P = significance value) with Dunn's multiple comparisons and

Mann–Whitney U pairwise comparisons was performed. When data conformed to the normality assumption but failed on homogeneity, the Welch's one-way ANOVA (results reported as; F = test statistic, df_a = degrees of freedom between groups, P = significance value) was performed with Games–Howell *post hoc* analysis between pCO_2 treatments. The concentration of pCO_2 that caused >50% population mortality (LC_{50}) within each developmental stage (post 96 h exposure) was determined through probit regression analysis.

To conduct a Multi-Dimensional Scale (MDS) analysis on the data, the pCO_2 treatments used were first allocated into levels 1–5 (385, 1000, 2000, 3000, and 6000 μatm, respectively) to enable cross-comparisons between the mortality rates of the different developmental stages. All developmental stage mortality data were then normalized and reconstructed into a resemblance matrix using Euclidean Distance, and analysed through a MDS ordinal plot. Observational interpretation of the MDS was confirmed through ANOSIM pairwise comparisons between the mortality rates of the different developmental stages, results report as P (significance value) and R; where R was determined on a scale of 0–1, with 0 representing similar mortality rates between the developmental stages and one representing different mortality rates between the stages.

Sublethal effects across pCO_2 treatments within each developmental stage were analysed using one-way ANOVA's with Tukey's pairwise comparisons and Welch's ANOVA with Games–Howell *post-hoc* analysis. An α-level of $P = <0.05$ was used for assessing statistical significance in all tests. Data were analysed using SPSS (19.0) and PRIMER-e (6.1.15). Data are presented as mean ± 1SD.

Results

Throughout the following text and in the figures, reference is made to the nominal (i.e. target) pCO_2 μatm values (385, 1000, 2000, 3000, and 6000) rather than to the precise values measured, which are reported in Table 1.

Mortality rates across all developmental stages increased significantly upon exposure to increased pCO_2 treatments; males ($H = 11.849$, df_4, $P = 0.019$), females ($F = 19.012$, df_4, $P < 0.001$), copepodites ($H = 12.607$, df_4, $P = 0.013$), nauplii ($H = 17.559$, df_4, $P = 0.002$), and eggs ($F = 15.180$, df_4, $P = 0.002$). Nauplii were the most vulnerable developmental stage to be directly affected by increased levels of pCO_2 (Figs 1b, f and 2), with significantly higher mortality rates compared to all other developmental stages (ANOSIM pairwise comparison, all $P < 0.001$). The greatest deviation in mortality rates from the nauplii stages was the copepodite stages ($R = 0.721$), followed by males ($R = 0.652$), eggs ($R = 0.509$), and females ($R = 0.483$). Upon exposure to the near-future pCO_2 level (1000 μatm), nauplii showed a threefold increase in mortality rates (Mann–Whitney U Test, $P = 0.029$), with 100% mortality found upon exposure to 2000 μatm pCO_2. With 100% nauplii mortality found in two of the pCO_2 treatments, end point growth and development analyses could only be performed on three pCO_2 treatments of the nauplii developmental stages; 385, 1000, and 6000 μatm (albeit with decreased numbers available for analysis at the highest pCO_2 level). Within these treatments, there were significant declines in carbon-specific growth rates of individuals exposed to the highest pCO_2 level (Games–Howell Test, $P = 0.019$). Individuals exposed to the highest pCO_2 level did not develop beyond the nauplii stage (N_V), while a significant proportion (>30%) of individuals exposed to the two lower pCO_2 levels had metamorphosed into early copepodite stages (C_I). No sublethal effects were found in growth or development of the nauplii individuals exposed to the projected pCO_2 values for 2100 (1000 μatm).

The greatest sublethal effect as a result of exposure to elevated pCO_2 was seen in the fecundity of *Acartia tonsa*. Declines in fecundity success were found with males and females exposed to pCO_2 levels projected for the end of this century (Fig. 3a, c, d); significant suppression in egg production rates was seen in individuals exposed to the two highest CO_2 treatments (Games–Howell Test, both $P < 0.001$). Greater impacts were found in the egg hatching rates, with significant declines in hatching success across all pCO_2 treatments (Tukey's Test, 1000 μatm pCO_2 $P = 0.016$, all other treatments $P < 0.001$). Decreases in egg carbon content (Fig. 3b) were found with females exposed to the 3000 and 6000 μatm pCO_2 (Games–Howell Test, $P < 0.001$, $P = 0.009$, respectively). Combining the egg carbon values with daily egg production rates led to >90% decline in daily carbon production $female^{-1}$ day^{-1} in the two highest pCO_2 treatments (Games–Howell Test, for both $P < 0.001$), with significant declines also found at 1000 μatm ($P < 0.001$) and 2000 μatm pCO_2 ($P = 0.008$). Nauplii recruitment negatively correlated with the increasing pCO_2 treatments (Fig. 3d), declining 35% upon exposure to 2100 CO_2 scenarios (Games–Howell Test, for both $P = 0.003$), and further still to <1 nauplii $female^{-1}$ day^{-1} in the two higher CO_2 levels (both $P < 0.001$).

The least affected life stages upon direct exposure to elevated pCO_2 were the copepodites (Figs 1c, f and 2), showing a significantly lower mortality rate across all pCO_2 treatments compared to all other developmental stages (ANOSIM pairwise comparisons, all $P < 0.001$). No pCO_2 treatments attained >50% mortality in copepodites, thus no LC_{50} could be calculated for this life stage. No significant differences were found in C-specific growth rates or development post 96 h exposure across all treatments of *Acartia tonsa* individuals which were initially exposed at early copepodite stages.

Table. 1 Seawater chemistry parameters for all four experiments (mean ± 1SD)

Life stage	Physiochemical water properties	Nominal pCO$_2$ levels (μatm)				
		385	1000	2000	3000	6000
Adults	Male pH* (NBS scale)	8.235 (±0.007)	7.818 (±0.004)	7.610 (±0.004)	7.411 (±0.004)	7.149 (±0.007)
	Male pH† (NBS scale)	8.218 (±0.009)	7.814 (±0.005)	7.608 (±0.007)	7.403 (±0.065)	7.153 (±0.004)
	Female pH* (NBS scale)	8.235 (±0.007)	7.818 (±0.004)	7.610 (±0.004)	7.411 (±0.004)	7.149 (±0.007)
	Female pH† (NBS scale)	8.222 (±0.008)	7.817 (±0.005)	7.614 (±0.005)	7.417 (±0.005)	7.151 (±0.005)
	Egg hatching pH* (NBS scale)	8.235 (±0.007)	7.818 (±0.004)	7.610 (±0.004)	7.411 (±0.004)	7.149 (±0.007)
	Egg hatching pH † (NBS scale)	8.193 (±0.023)	7.832 (±0.011)	7.666 (±0.016)	7.526 (±0.033)	7.295 (±0.050)
	A_T (μmol kg^{-1})	2435.30 (±59.8)	2336.20 (±27.15)	2399.50 (±40.31)	2331.20 (±54.02)	2404.10 (±93.20)
	pCO$_2$ (μatm)‡	399.99 (±10.93)	1141.66 (±14.95)	1972.06 (±30.61)	3071.32 (±59.61)	5924.30 (±194.15)
	Temperature (°C)	23.87 (±0.15)	23.86 (±0.05)	23.93 (±0.05)	23.90 (±0.05)	23.88 (±0.05)
	Salinity (PSU)	27.73 (±0.08)	27.7 (±0.09)	27.63 (±0.05)	27.83 (±0.10)	27.73 (±0.05)
Copepodites	pH* (NBS scale)	8.209 (±0.006)	7.919 (±0.004)	7.619 (±0.004)	7.469 (±0.006)	7.165 (±0.004)
	pH†(NBS scale)	8.202 (±0.009)	7.920 (±0.005)	7.625 (±0.05)	7.472 (±0.007)	7.172 (±0.006)
	A_T (μmol kg^{-1})	2416.50 (±60.1)	2484.00 (±21.21)	2455.30 (±45.13)	2438.2 (±66.19)	2475.2 (±79.4)
	pCO$_2$ (μatm) ‡	427.94 (±10.28)	946.52 (±11.5)	1976.75 (±40.70)	2916.60 (±68.03)	5885.16 (±150.6)
	Temperature (°C)	24.06 (±0.05)	24.13 (±0.05)	24.13 (±0.05)	24.08 (±0.05)	24.05 (±0.09)
	Salinity (PSU)	27.55 (±0.05)	27.80 (±0.09)	27.58 (9 ± 0.05)	27.61 (±0.08)	27.68 (±0.09)
Nauplii	pH* (NBS scale)	8.156 (±0.004)	7.835 (±0.006)	7.610 (±0.007)	7.410 (±0.018)	7.125 (±0.005)
	pH†(NBS scale)	8.160 (±0.005)	7.849 (±0.007)	7.620 (±0.005)	7.418 (±0.006)	7.134 (±0.009)
	A_T (μmol kg^{-1})	2274.60 (±33.23)	2303.40 (±9.34)	2329.0 (±30.98)	2285.5 (±65.7)	2302.0 (±19.79)
	pCO$_2$ (μatm)‡	460.91 (±7.25)	1078.05 ± 15.72	1906.42 (±39.20)	3028.31 (±126.96)	5971.77 (±72.88)
	Temperature (°C)	23.86 (±0.50)	24.03 (±0.10)	23.90 (±0.10)	23.90 (±0.01)	23.88 (±0.05)
	Salinity (PSU)	28.00 (±0.00)	28.06 (±0.05)	28.01 (±0.00)	28.08 (±0.05)	28.08 (±0.05)
Eggs	pH* (NBS scale)	8.255 (±0.005)	7.907 (±0.003)	7.614 (±0.007)	7.424 (±0.013)	7.143 (±0.004)
	pH†(NBS scale)	8.171 (±0.031)	7.926 (±0.125)	7.666 (±0.023)	7.510 (±0.034)	7.313 (±0.038)
	A_T (μmol kg^{-1})	2412.30 (±54.73)	2401.30 (±11.91)	2398.2 (±25.00)	2417.21 (±19.9)	2349.10 (±70.9)
	pCO$_2$ (μatm)‡	375.26 (±8.36)	940.30 (±8.70)	1946.10 (±34.00)	3091.80 (±94.10)	5875.0 (±143.70)
	Temperature (°C)	24.41 (±0.03)	24.13 (±0.19)	24.26 (±0.15)	24.13 (±0.05)	24.36 (±0.05)
	Salinity (PSU)	27.86 (±0.07)	27.86 (±0.05)	28.05 (±0.05)	27.86 (±0.05)	28.08 (±0.05)

*Refers to the averaged initial pH concentrations.
†Refers to the averaged pH concentrations before the 95% water exchange (which occurred every 24 h for adults, copepodites, and nauplii, and after 96 h for eggs).
‡Refers to parameters calculated through CO2 SYS (Pierrot *et al.*, 2006).

Discussion

Significant variations in the mortality rates were found across the different life stages of *Acartia tonsa* within this study. Without using a representative range of different life stages across a species life cycle, the use of acute exposure experiments on just a few stages has clear scope for misrepresenting a species response to OA. Thus, in this present study, exposing just *A.tonsa* nauplii to the different pCO$_2$ treatments would suggest that 100% mortality could potentially be seen by the year 2300 (2000 μatm pCO$_2$, Fig. 1b), with sublethal retardation prior to this (1000–2000 μatm pCO$_2$). In contrast, exposure of just the copepodite stages would indicate the opposite outcome, being that this species has a good resilience to increased pCO$_2$ and will not be affected lethally or sublethally by 2300 (Fig. 1c).

The early developmental stages of many marine species are suspected to be most susceptible to the effects of OA (Dupont & Thorndyke, 2009; Kroeker *et al.*, 2010). In this present study, we have found a greater resilience to increasing levels of pCO$_2$ in *A. tonsa* eggs compared to that of nauplii. Using rates of egg production and hatching (both used as sublethal reproductive

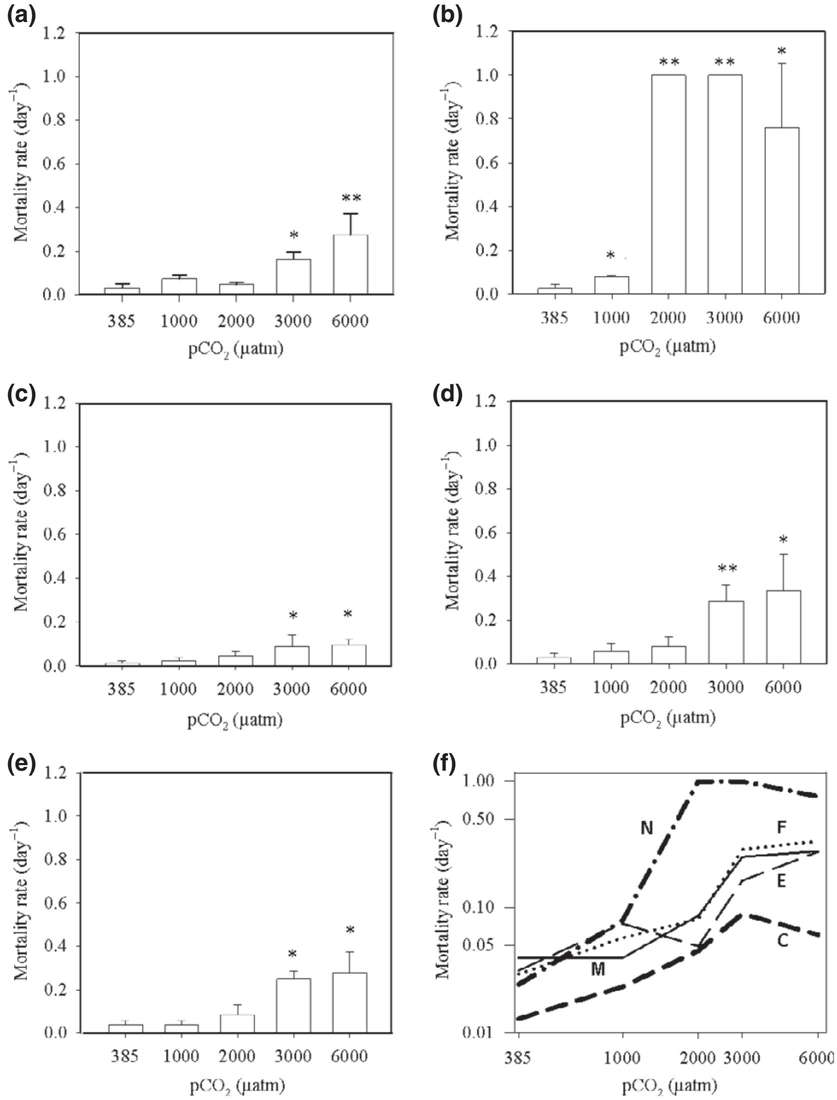

Fig. 1 Individual [panels (a–e); eggs, nauplii, copepodites, females, and males, respectively] and grouped mean [panel (f); eggs = E, nauplii = N, copepodites = C, females = F, males = M] daily mortality rates of the different life stages of *Acartia tonsa* exposed to five pCO_2 treatments (log scale) over a 96 h period. Means ± 1SD. *indicates significant difference of $P \leq 0.05$ from the control treatment. **indicate significant difference of $P \leq 0.01$ from the control treatment. LC_{50} for Eggs = 4291 μatm; Nauplii = 1089 μatm; Copepodites = beyond range of exposure; Males = 4547 μatm; Female = 3888 μatm. Nominal pCO_2 values are indicated; actual pCO_2 values are shown in Table 1.

end points) has the potential to significantly underestimate the damaging effects of OA in copepods. Egg mortality rates across the different pCO_2 treatments were actually similar to that of adult females (ANOSIM, R = 0.003) and adult males (R = 0.189). The observed resilience of *A.tonsa* eggs in comparison to their nauplii stages could be a function of their physiology providing tolerance to environmental change; *Acartia* embryos are surrounded by a restricted permeable double-layered inner plasma membrane that is physically protected by a rigid multilayer chorion shell (Hansen *et al.*, 2012). Investigations into the intracellular pH of copepod diapause eggs have alluded that the thickness of the chorion shell could make it impermeable to larger molecules of CO_2 (Sedlacek, 2008). Thus, the question is whether these eggs are affected under conditions of OA as a result of increased protons (H^+) and/or increased pCO_2, and if this stressor changes with ontogeny. In adult harpacticoid copepods, mortality rates are significantly higher when the seawater carbonate chemistry is manipulated through increased pCO_2, as opposed to HCl addition (Pascal *et al.*, 2010). The diffusion of CO_2

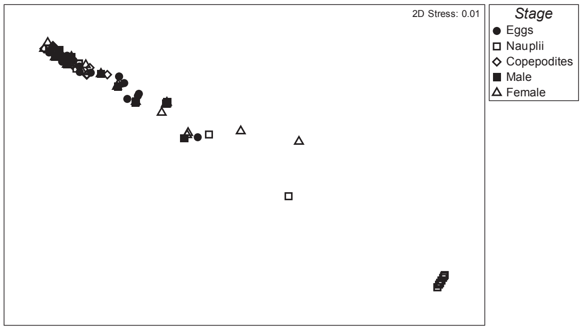

Fig. 2 A Multi-Dimensional Scale (MDS) ordinal plot showing the clustering of overall mortality rates post 96 h exposure of the different *Acartia tonsa* stages exposed to increasing levels of acidity. This shows the differences in sensitivity of the nauplii stages (most sensitive) in comparison to that of the eggs and copepodites (least sensitive).

into the adults' intracellular spaces apparently results in intracellular acidosis causing a more toxic effect on the adults, compared to that of the HCl addition. Determining which stressor, H^+ or CO_2, impacts which life stage of an individual will tease apart the mechanisms of OA that could cause potential adverse effects to the species population.

Within this study, the early developmental nauplii stages of *Acartia tonsa* exhibited the greatest sensitivity to increasing levels of pCO_2. The direct increase in nauplii mortality, coupled with the declines in nauplii recruitment upon parental exposure to 2100 pCO_2 scenario, indicates that these early ontogenetic stages may act as a bottleneck for copepod populations in the near future. These early developmental nauplii (N_{II}–N_{III}) undergo critical physiological changes, switching energy sources from the endogenous yolk to exogenous food available. The additive energetic demand required to maintain metabolic homeostasis under high pCO_2 (Kurihara *et al.*, 2004a,b) may explain why this stage incurs higher mortality rates and sublethal retarded growth compared to other developmental stages within the species life cycle. A critical factor that needs to be considered in future studies is the interaction between survival at high pCO_2 and prey quality during this sensitive early developmental transition. It appears quite likely that under OA the interplay between pH and phytoplankton growth, with knock-on implications for biochemical stoichiometry (Bellerby *et al.*, 2008) and subsequent prey quality (Schoo *et al.*, 2013), will collectively generate the potential for significant changes in the multi-stressor environment for zooplankton populations.

Exposure of adults to high pCO_2 prior to mating has previously shown to influence the outcome of the future progeny in marine animals (Parker *et al.*, 2010;

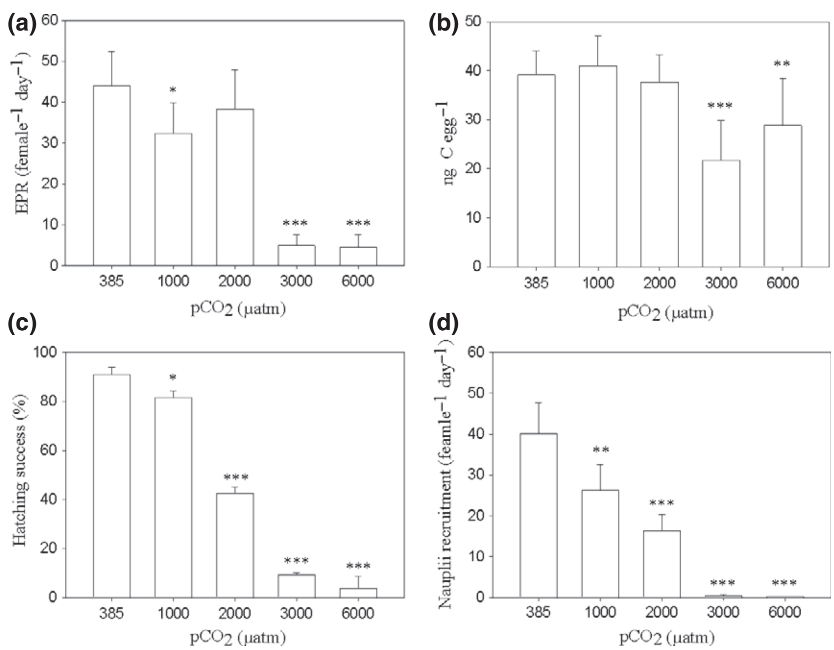

Fig. 3 Fecundity success of *Acartia tonsa* adult females post 96 h exposure to five different pCO_2 treatments. (a) Egg production rate (EPR) per female per day. (b) Carbon content per egg. (c) Hatching success of eggs post 96 h. (d) Nauplii recruitment per female per day. Means ± 1SD. *indicates significant difference of $P \le 0.05$ from the control treatment. **indicate significant difference of $P \le 0.01$ from the control treatment. ***indicate significant difference of $P \le 0.001$ from the control treatment. Nominal pCO_2 values are indicated; actual pCO_2 values are shown in Table 1.

Miller *et al.*, 2012; Allan *et al.*, 2014), including that of copepods (Vehmaa *et al.*, 2012). Within this current study, declines in the fecundity success occurred at a much lower pCO_2 concentration than seen in previous investigations (Mayor *et al.*, 2007; Zhang *et al.*, 2011; Weydmann *et al.*, 2012; McConville *et al.*, 2013), which could be attributed to the combined maternal and paternal exposure to the high pCO_2 within these experiments. The vast majority of previous pCO_2 acute exposure studies have solely utilized copepod females to determine fecundity success (Kurihara *et al.*, 2004a,b; Mayor *et al.*, 2007, 2012; Zervoudaki *et al.*, 2011; Zhang *et al.*, 2011; Weydmann *et al.*, 2012; McConville *et al.*, 2013), and not used males. By not exposing males to the changes in seawater pCO_2, the potential impacts that OA may have on the production and activity of male gametes are discounted, together with the subsequent influence this may have on the fecundity success. While the effect of high pCO_2 on female copepod fecundity success is the subject of active research, there is very limited information on the effects of elevated pCO_2 on the role of the male copepods in reproduction. To the author's knowledge, just one study, Fitzer *et al.* (2012a), has measured the impacts of OA on male copepod gametes, finding significant declines in spermatophore length with increased acidity [pH 7.67; equivalent to ca. 550–647 μatm pCO_2 in their experimental system (Table 1 in Fitzer *et al.*, 2012b)] compared to that of ambient conditions (pH 8.10; equivalent to ca. 204–250 μatm pCO_2 in their system).

Previously, declines in egg production rates have been attributed to the suppression in metabolic activity through decreased protein synthesis consequently decreasing the reproductive output (Kurihara, 2008), which could explain the decline in female carbon production. Increasing levels of pCO_2 have been demonstrated to increase the oxidative stress from the maternal parent in crustaceans, which can subsequently be passed down to the offspring (Rodríguez-Graña *et al.*, 2010). Increased levels of oxidative stress in the eggs of *Acartia biflosia* have found to negatively correlate to the egg viability (Vehmaa *et al.*, 2012). Such an event could account also for the decline in hatching success with increasing pCO_2 levels seen here (Fig. 3c), in addition to the higher hatching success seen in eggs with no prior parental exposure to increased levels >3000 μatm pCO_2 (Fig. 1a) compared to eggs with prior parental pre-exposure to the high pCO_2.

As prior OA studies have found the paternal influence in other marine invertebrates to be a potential limiting factor in reproduction (Havenhand *et al.*, 2008; Morita *et al.*, 2010; Byrne, 2011; Caldwell *et al.*, 2011), it would appear presumptuous to assume that the effect of high pCO_2 solely on copepod females will produce the same reproductive outcome as if both males and females were exposed. The chronic transgenerational exposure (and thus combined parental exposure to pCO_2) of *Acartia tonsa* and *Tisbe battagliai* to 2100 pCO_2 projections (Fitzer *et al.*, 2012b; Rossoll *et al.*, 2012) has illustrated similar decreases (~35%) in fecundity success to that found in this study. The 35% decrease in nauplii recruitment under the 2100 climate change scenario in our study (Fig. 3d), especially when coupled to a decline in the fitness of those nauplii, could significantly alter population dynamics of copepods behaving like *A. tonsa* in the future, with potential impacts for both higher and lower trophic level interactions.

The variation in stage-specific responses seen here highlights the potential for misrepresentation of a species (lethal and sublethal) response to OA when using acute exposure experiments of limited life stages. This has far-reaching implications, beyond that of copepods, for experimental designs projecting species response under elevated pCO_2 scenarios. In using a multi-stage acute exposure study, we have shown that the sole use of mature females to determine the effects of OA has the potential to significantly underestimate the effects of OA in copepods. In addition, using egg hatching and production rates as a reproductive end point measurement could significantly overestimate the species outcome, as other developmental stages are more sensitive to the effects of OA than eggs. The decreased survival and nauplii recruitment of *A. tonsa* upon exposure to 2100 climate change scenarios indicates that copepod species are not as resilient to the effects of OA, and indeed higher CCS levels, as once perceived. Finally, it is worth reflecting that the fecundity results from this study reflect an environment where the copepods had saturating prey quantities (daily replenished prey to maintain ≥1 μg C ml^{-1}), good prey quality (grown under nutrient-replete conditions), prey choice (three prey species), and no predation pressures. The outcome from this study could therefore be perceived as the best-case scenario for this population of *A.tonsa* exposed to high pCO_2 levels, as in the wild these nutritional conditions are most unlikely to be met.

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