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 (pCO2) 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 pCO2 (385–6000 μatm) were studied across different life stages of a calanoid copepod, monitoring for lethal and sublethal responses. Mortality rates varied significantly across different life stages, with nauplii showing the highest lethal effects; nauplii mortality rates increased threefold when pCO2 concentrations reached 1000 μatm (year 2100 scenario) with LC50 at 1084 μatm pCO2. In comparison, eggs, early copepodite stages, and adult males and females were not affected lethally until pCO2 concentrations ≥3000 μatm. Adverse effects on reproduction were found, with >35% decline in nauplii recruitment at 1000 μatm pCO2. This suppression of reproductive scope, coupled with the decreased survival of early stage progeny at this pCO2 concentration, has clear potential to damage population growth dynamics in this species. The disparity in responses seen across 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 risk, which is essential for projecting outcomes to future CO2 scenarios. Predictions based only on limited life-stage exposures have clear scope to significantly under- or overestimate the species true overall vulnerabilities.

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 pCO2 (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 pCO2 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 pCO2 (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 pCO2 (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 pCO2 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 pCO2.

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 ctenophora copepod, Acartia tonsa, were acutely exposed to five different pCO2-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 pCO2 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 pCO2. Within each population, A. tonsa migrate to different depths in relation to their ontogeny (Holliday et al., 2012), resulting in different developmental stages being exposed to different variations in pCO2. As the projected levels of pCO2 will be variable over different temporal and spatial scales (Flynn et al., 2012), we argue that exposure of individuals to a range of pCO2 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 ctenophora 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 CO2) 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 Chaetoceros 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 × 10⁵ cells ml⁻¹, 4.0 × 10⁶ cells ml⁻¹, 2.5 × 10⁷ 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 (N1), early copepodites (C1), 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 pCO2 levels: (i) present-day pCO2 385 μatm; (ii) near-future level, 1000 μatm (RCP8.5 2100 pCO2 projection, Van-Vuuren et al., 2011); (iii) 2000 μatm (ECP8.5 2300 pCO2 projection, Van-Vuuren et al., 2011), and two extreme pCO2 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 pCO2 were obtained through mixing high pCO2 water with water saturated with ambient CO2 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 Titration, Apollo SciTech, USA), pH, salinity, and temperature were used to calculate the pCO2 (μatm) through the programme CO2 SYS (Pierrot et al., 2006), using the K1, K2 constants from Mehrbruch et al. (1973) as refigited 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 CO2 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. muellera)] 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 pCO₂ treatment (well volume: 3.6 ml, minimum of three replicate plates per treatment). A minimum of 70 eggs were used for each pCO₂ level. All well plates were sealed for the 96 h duration to maintain the pCO₂ level, with pH, temperature, and salinity measured before (t₀) and after (tₙa) 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 pCO₂ levels were calculated with the following equation.

$$Z = \ln\left(\frac{N_n}{N_0}\right)$$  

(1)

where Z is the mortality rate, N₀ is the initial (t₀) number of eggs, and Nₙ is the number of hatched eggs after t days. All eggs were considered to have been produced and fertilized under ambient conditions of pCO₂ prior to being exposed to the different pCO₂ levels. Then, 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 pCO₂ treatment, 4 × 250 ml tissue culture flasks were each seeded with 25 Nₙ₉,ₚ individuals (each <24 h old). An additional 25 Nₚ₉,ₚ from the stock culture were fixed with 1% iodine to determine initial (t₀) size data. The nauplii were exposed to the assigned pCO₂ 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 pCO₂ was replenished every 24 h to prevent potential drift in seawater carbonate-pH chemistry.

Copepod survival across all treatments was assessed 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 µg C = 3.18 × 10⁻⁶ TBL³.¹. Individuals’ carbon-specific growth rates (µ) were determined across all CO₂ treatments post 96 h exposure, using Eqn 2; W₀ & Wₙ are the initial and end point weights of the individual (µg C), and t is the time period between sample points.

$$\mu = \frac{\ln(W_t/W_0)}{t}$$  

(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 (Cₙ,ₚ) 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 µg C = 1.11 × 10⁻⁸PL².⁹².

Adult males and females. For each pCO₂ 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 CO₂ 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 pCO₂ 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 pCO₂ 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 (µm³) = 4/3πr³, and egg volume converted into carbon assuming 0.114 pg C µm⁻³ (Calliairi et al., 2006). Using data on carbon, egg size, and egg production per female, C-specific egg production rates were calculated for each pCO₂ 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 pCO₂ treatment.

Statistics

Within each developmental stage, the mortality rates were compared between pCO₂ 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 = 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 = degrees of freedom between groups, P = significance value) was performed with Games–Howell post hoc analysis between pCO2 treatments. The concentration of pCO2 that caused >50% population mortality (LC50) 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 pCO2 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 pCO2 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 x-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) pCO2 μatm values (385, 1000, 2000, 3000, and 6000 μatm) 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 pCO2 treatments; males (H = 11.849, df6, P = 0.019), females (F = 19.012, df6, P < 0.001), copepodites (H = 12.607, df6, P = 0.013), nauplii (H = 17.559, df6, P = 0.002), and eggs (F = 15.180, df6, P = 0.002). Nauplii were the most vulnerable developmental stage to be directly affected by increased levels of pCO2 (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 pCO2 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 pCO2. With 100% nauplii mortality found in two of the pCO2 treatments, end point growth and development analyses could only be performed on three pCO2 treatments of the nauplii developmental stages; 385, 1000, and 6000 μatm (albeit with decreased numbers available for analysis at the highest pCO2 level). Within these treatments, there were significant declines in carbon-specific growth rates of individuals exposed to the highest pCO2 level (Games–Howell Test, P = 0.019). Individuals exposed to the highest pCO2 level did not develop beyond the nauplii stage (NV), while a significant proportion (>30%) of individuals exposed to the two lower pCO2 levels had metamorphosed into early copepodite stages (CI). No sublethal effects were found in growth or development of the nauplii individuals exposed to the projected pCO2 values for 2100 (1000 μatm).

The greatest sublethal effect as a result of exposure to elevated pCO2 was seen in the fecundity of Acartia tonsa. Declines in fecundity success were found with males and females exposed to pCO2 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 CO2 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 pCO2 treatments (Tukey’s Test, 1000 μatm pCO2 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 pCO2 (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 pCO2 treatments (Games–Howell Test, for both P < 0.001), with significant declines also found at 1000 μatm (P < 0.001) and 2000 μatm pCO2 (P = 0.008). Nauplii recruitment negatively correlated with the increasing pCO2 treatments (Fig. 3d), declining 35% upon exposure to 2100 CO2 scenarios (Games–Howell Test, for both P = 0.003), and further still to <1 nauplii female−1 day−1 in the two higher CO2 levels (both P < 0.001).

The least affected life stages upon direct exposure to elevated pCO2 were the copepodites (Figs 1c, f and 2), showing a significantly lower mortality rate across all pCO2 treatments compared to all other developmental stages (ANOSIM pairwise comparisons, all P < 0.001). No pCO2 treatments attained >50% mortality in copepodites, thus no LC50 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 pCO2 levels (μatm) |
|------------|---------------------------------|---------------------------|
|            |                                 | 385                       |
|            |                                 | 1000                      |
|            |                                 | 2000                      |
|            |                                 | 3000                      |
|            |                                 | 6000                      |
| Adults     |                                 |                           |
| Male       | \(\text{pH}^*\) (NBS scale)     | 8.235 (±0.007)            |
| pCO2       |                                 | 7.818 (±0.004)            |
|            | \(\text{pH}^*\) (NBS scale)     | 7.610 (±0.004)            |
|            |                                 | 7.411 (±0.004)            |
|            |                                 | 7.149 (±0.007)            |
| Female     | \(\text{pH}^*\) (NBS scale)     | 8.235 (±0.007)            |
| pCO2       |                                 | 7.818 (±0.004)            |
|            | \(\text{pH}^*\) (NBS scale)     | 7.610 (±0.004)            |
|            |                                 | 7.411 (±0.004)            |
|            |                                 | 7.149 (±0.007)            |
| 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)            |
| Copepodes  | \(\text{pH}^*\) (NBS scale)     | 8.209 (±0.006)            |
|            |                                 | 7.919 (±0.004)            |
|            | \(\text{pH}^*\) (NBS scale)     | 7.619 (±0.004)            |
|            |                                 | 7.469 (±0.006)            |
|            |                                 | 7.165 (±0.004)            |
| Nauplii    | \(\text{pH}^*\) (NBS scale)     | 8.156 (±0.004)            |
|            |                                 | 7.835 (±0.006)            |
|            | \(\text{pH}^*\) (NBS scale)     | 7.610 (±0.007)            |
|            |                                 | 7.411 (±0.007)            |
|            |                                 | 7.134 (±0.009)            |
| Eggs       | \(\text{pH}^*\) (NBS scale)     | 8.255 (±0.005)            |
|            |                                 | 7.907 (±0.003)            |
|            | \(\text{pH}^*\) (NBS scale)     | 7.614 (±0.007)            |
|            |                                 | 7.424 (±0.013)            |
|            |                                 | 7.143 (±0.004)            |
|            | A_T (μmol kg\(^{-1}\))      | 2461.50 (±60.1)           |
|            | pCO2 (μatm)†                   | 2484.00 (±21.21)          |
|            |                                 | 2455.30 (±45.13)          |
|            |                                 | 2438.2 (±66.19)           |
|            |                                 | 2475.2 (±79.4)            |
|            | Температура (°C)               | 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 (±0.05)             |
|            |                                 | 27.61 (±0.08)             |
|            |                                 | 27.68 (±0.09)             |
|            | A_T (μmol kg\(^{-1}\))      | 2461.50 (±60.1)           |
|            | pCO2 (μatm)†                   | 2484.00 (±21.21)          |
|            |                                 | 2455.30 (±45.13)          |
|            |                                 | 2438.2 (±66.19)           |
|            |                                 | 2475.2 (±79.4)            |
|            | Температура (°C)               | 24.06 (±0.05)             |
|            |                                 | 24.13 (±0.05)             |
|            |                                 | 24.13 (±0.05)             |
|            |                                 | 24.08 (±0.05)             |
|            |                                 | 24.05 (±0.09)             |
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|            |                                 | 2438.2 (±66.19)           |
|            |                                 | 2475.2 (±79.4)            |
|            | Температура (°C)               | 24.06 (±0.05)             |
|            |                                 | 24.13 (±0.05)             |
|            |                                 | 24.13 (±0.05)             |
|            |                                 | 24.08 (±0.05)             |
|            |                                 | 24.05 (±0.09)             |

*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, copepodes, 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 pCO2 treatments would suggest that 100% mortality could potentially be seen by the year 2300 (2000 μatm pCO2, Fig. 1b), with sublethal retardation prior to this (1000–2000 μatm pCO2). In contrast, exposure of just the copepodite stages would indicate the opposite outcome, being that this species has a good resilience to increased pCO2 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 pCO2 in *A. tonsa* eggs compared to that of nauplii. Using rates of egg production and hatching (both used as sublethal reproductive
end points) has the potential to significantly underestimate the damaging effects of OA in copepods. Egg mortality rates across the different pCO2 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 CO2 (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 pCO2, 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 pCO2, as opposed to HCl addition (Pascal et al., 2010). The diffusion of CO2...
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, \( \text{H}^+ \) or \( \text{CO}_2 \), 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 \( \text{Acartia tonsa} \) exhibited the greatest sensitivity to increasing levels of \( \text{pCO}_2 \). The direct increase in nauplii mortality, coupled with the declines in nauplii recruitment upon parental exposure to 2100 \( \text{pCO}_2 \) scenario, indicates that these early ontogenetic stages may act as a bottleneck for copepod populations in the near future. These early developmental nauplii (NII–NIII) 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 \( \text{pCO}_2 \) (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 \( \text{pCO}_2 \) 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 \( \text{pCO}_2 \) prior to mating has previously shown to influence the outcome of the future progeny in marine animals (Parker et al., 2010;
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 pCO2 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 pCO2 within these experiments. The vast majority of previous pCO2 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 pCO2, 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 pCO2 on female copepod fecundity success is the subject of active research, there is very limited information on the effects of elevated pCO2 on the role of the male copepods in reproduction. To the author’s knowledge, just one study, Fitz 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 pCO2 in their experimental system (Table 1 in Fitz et al., 2012b)] compared to that of ambient conditions (pH 8.10; equivalent to ca. 204–250 µatm pCO2 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 pCO2 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 pCO2 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 pCO2 (Fig. 1a) compared to eggs with prior parental pre-exposure to the high pCO2.

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 pCO2 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 pCO2) of Acartia tonsa and Tisbe battaglai to 2100 pCO2 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 pCO2 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 pCO2 levels, as in the wild these nutritional conditions are most unlikely to be met.

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