Ocean acidification affects the phyto-zoo plankton trophic transfer efficiency

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Abstract

The critical role played by copepods in ocean ecology and biogeochemistry warrants an understanding of how these animals may respond to ocean acidification (OA). Whilst an appreciation of the potential direct effects of OA, due to elevated $pCO_2$, on copepods is improving, little is known about the indirect impacts acting via bottom-up (food quality) effects. We assessed, for the first time, the chronic effects of direct and/or indirect exposures to elevated $pCO_2$ on the behaviour, vital rates, chemical and biochemical stoichiometry of the calanoid copepod Acartia tonsa. Bottom-up effects of elevated $pCO_2$ caused species-specific biochemical changes to the phytoplanktonic feed, which adversely affected copepod population structure and decreased recruitment by 30%. The direct impact of elevated $pCO_2$ caused gender-specific respiratory responses in A.tonsa adults, stimulating an enhanced respiration rate in males (> 2-fold), and a suppressed respiratory response in females when coupled with indirect elevated $pCO_2$ exposures. Under the combined indirect+direct exposure, carbon trophic transfer efficiency from phytoplankton-to-zooplankton declined to < 50% of control populations, with a commensurate decrease in recruitment. For the first
time an explicit role was demonstrated for biochemical stoichiometry in shaping copepod trophic dynamics. The altered biochemical composition of the CO$_2$-exposed prey affected the biochemical stoichiometry of the copepods, which could have ramifications for production of higher tropic levels, notably fisheries. Our work indicates that the control of phytoplankton and the support of higher trophic levels involving copepods have clear potential to be adversely affected under future OA scenarios.

1. Introduction

Mesozooplankton play a crucial role within marine food webs, transferring biomass from primary producers to higher trophic levels, and in doing so significantly contributing to the vertical particle flux. As copepods typically form a significant proportion of the mesozooplankton [1], any influence on their survival, growth or development attributed to ocean acidification (OA) may be expected to have significant implications on trophic dynamics.

To gain an understanding of the potential impacts of OA upon marine organisms, experiments are typically conducted under elevated partial pressures of carbon dioxide ($p$CO$_2$), ideally using $p$CO$_2$ values consistent with predicted future atmospheric CO$_2$ concentrations. In copepods, the direct effects of elevated $p$CO$_2$ have shown to vary between species [2], populations [3], and developmental stages within a species [4,5,6]. The extent of these direct effects appears to be related to the duration of exposure to OA, with recent transgenerational studies demonstrating diminishing effects with prolonged exposure [7]. While our understanding of the direct effects of elevated $p$CO$_2$ on copepods is improving [7,8], little is known of the indirect impacts that OA may cause on copepod populations through indirect, bottom-up, effects mediated through effects of OA on copepod prey [9]. The
increase in \( \text{CO}_2 \text{(aq)} \) in the water column, associated with OA, is suspected to have the potential to increase the carbon-nutrient (e.g., C:N, C:P) ratios of primary producers [10,11,12]. If this was indeed to occur, then the consequential changes in the elemental stoichiometry of the primary producers could translate to poor-quality prey for consumers with decreased trophic transfer efficiency [13] that affects biogeochemistry. Growth under elevated \( p\text{CO}_2 \) also has the potential to alter the biochemical composition of primary produces [14,15,16]. Changes in biochemical content can affect the consumer’s reproduction and development through insufficient supply of critical metabolites [17,11], and thus change the efficiency of energy transfer between the producer and consumer.

In addition to the above mentioned interactions of OA upon trophic transfer, behavioural interactions between predator and prey across marine taxa have also shown to be affected by the projected changes in seawater carbonate chemistry associated with OA [18,19,20]. Although the mode of action remains unclear, copepods have an ability to discriminate between prey types based on size [21] and motility [22], as well as the presence of noxious substances produced by prey [23]. Indeed, copepods have the potential to actively select higher quality prey species with lower C:(N:P) ratios [24,25], when the nutritional variance within the prey is notable [26].

Taken all together, there is scope for OA to affect copepod growth and reproduction and thence interactions to trophic levels below them (their phytoplankton prey) and above (through to fisheries), and associated biogeochemical cycles. A primary driver may be expected to depend on the response of the prey to OA, the number of prey types and quantities available, and if appropriate the predator’s ability to detect the changes in prey quality and choose an alternative prey source.

In this study, we explored the direct (via increased external \( p\text{CO}_2 \) seawater), indirect (via mixed-prey [\textit{Isochrysis galbana}, \textit{Tetraselmis suecica} and \textit{Chaetoceros muelleri}] reared
under increased $pCO_2$) and combined (simultaneous direct and indirect exposure) effects of OA on the ubiquitous calanoid copepod *Acartia tonsa*. To assess if the combined exposure caused a multiplicative effect on the consumer, a cross factorial design of predator and prey reared under elevated (1000 µatm) and low (ambient: 400 µatm) $pCO_2$ levels was utilised to locate sole stressor effects. Vital rates (ingestion, respiration rates and reproduction), behaviour (prey selection) and composition (elemental and biochemical stoichiometry) were measured in copepods after being exposed to $pCO_2$ levels in-line with near-future OA scenarios for one life-cycle. Implications of the different OA pathways on the trophic interactions between phytoplankton and zooplankton were subsequently calculated through elemental and biochemical stoichiometric trophic transfer efficiencies.

2. Method

2.1 Carbonate chemistry

The calanoid copepod *Acartia tonsa* and its phytoplanktonic prey (prymnesiophyte *Isochrysis galbana* [CCAP 927/1], prasinophyte *Tetraselmis suecica* [CCAP 66/22C] and diatom *Chaetoceros muelleri* [CCAP 927/1]) were separately grown under two $pCO_2$ scenarios; (i) low: present-day $pCO_2$ concentrations of 400 µatm, and (ii) elevated: worst-case scenario for the year 2100, 1000 µatm (RCP 8.5 [27]). The details of the method used to achieve these scenarios is outlined in S1, and absolute concentrations for each nominal treatment is detailed in Table S2. These two $pCO_2$ concentrations were combined in a matrix between the two trophic levels to produce 4 treatments: (i) $Z_LP_L$: zooplankton (*A. tonsa*) reared under low $pCO_2$ levels fed mixed phytoplankton (*I. galbana, C. muelleri and T. suecica*) also reared under low $pCO_2$ levels, (ii) $Z_LP_E$: zooplankton reared under low $pCO_2$ levels fed mixed
phytoplankton reared under elevated (RCP 8.5) pCO₂ levels, (iii) ZₐPₐ: zooplankton reared under elevated pCO₂ levels fed mixed phytoplankton reared under low pCO₂ levels, (iv) ZₐPₐ: zooplankton reared under elevated pCO₂ levels fed mixed phytoplankton also reared under elevated pCO₂ level.

2.2 Experimental design

Phytoplankton: phytoplankton prey species were cultured separately in nutrient replete seawater-based medium (88.2 and 5.5 µmol L⁻¹ for NO₃⁻ and PO₄³⁻ respectively; mole N: P ratio 16: 1) in semi-continuous cycles (effective dilution rate: T. suecica 0.30 d⁻¹, I. galbana and C. muelleri: 0.35 d⁻¹) for a minimum of 12 generations. Cultures were grown in a 18:6 hour light: dark cycle (cool-white fluorescent tubes at 50 µmol photons m⁻² s⁻¹) at 22 ± 1.8 °C. Duplicate cultures of each species were used for both pCO₂ treatments (450 mL, total n = 18). I. galbana, C. muelleri and T. suecica cultures (500 mL flasks) were aerated with air at the required pCO₂ concentration (either low or elevated) through a sterilised glass airline via an air-filter (0.2µm) at a flow rate of ca. 52 mL min⁻¹. Cell number (cells mL⁻¹), size (as equivalent spherical diameter, µm) and biovolume (µm³ mL⁻¹) for all replicates were analysed at the end of each light cycle using a Multisizer 4 Coulter Counter (Beckman, USA). Every 48hrs, at the semi-continuous exchange point, cells were collected from each culture for elemental stoichiometry and biochemical analysis. Cellular carbon (µg C mL⁻¹), nitrogen (µg N mL⁻¹) and the C: N of each species grown under both pCO₂ concentrations were analysed using an elemental analyser coupled with an isotope ratio mass spectrometer (SerCon GSL). Relative biochemical stoichiometry (lipids: protein, protein: carbohydrate, and carbohydrate: lipid) of each species cultured at different pCO₂ concentrations was assessed through Fourier Transform Infra-red (FTIR) spectroscopy (PerkinElmer Spectrum
over a wavelength range of 450-4000 cm\(^{-1}\) and at a resolution of 4 cm\(^{-1}\). The methods employed for the FTIR measurements are described in Mayers et al [28], and the quantification of relative biochemical stoichiometry in Stehfast et al [29], and as described further in S1.

**Copepod vital rates:** Copepods were exposed to the four treatments for an entire life cycle, from generation 1\((G_1)\) early nauplii stages \((N_1)\) through to \(G_2\) mid-late nauplii stages \((N_{III-IV})\). Each treatment had four replicate populations \((1L)\), initiated with \(N_1\) at density 890 ind\(^{-1}\) L\(^{-1}\). Fecundity success, respiration rates and ingestion rates of mature adults were measured across the four treatments after a complete life cycle of exposure to the \(p\)CO\(_2\) conditions. For fecundity success, 5-8 females from each replicate population \((n = 20-32\) individuals per treatment) with an attached spermatophore were removed and placed individually into 30 mL vials filled with medium of their assigned treatment and with saturating prey quantities of their allocated mixed-prey \((>1\mu g\) C mL\(^{-1}\)). Each vial was pre-lined with a 150 \(\mu m\) nylon mesh bottom to separate eggs from the female to prevent egg cannibalism. Females were held for 24-30 hours to lay eggs. Egg production rates \((EPR;\) eggs female\(^{-1}\) day\(^{-1}\)), egg hatching success \((EHS [\%])\) and nauplii recruitment \((NR;\) nauplii female\(^{-1}\) day\(^{-1}\)) across the four treatments were calculated as described in Cripps et al [6]. Ingestion rates \((\mu g\) C ind\(^{-1}\) day\(^{-1}\)) of adult males and females were measured separately. A sufficient number of adult copepods \((males: 250\) ind\(^{-1}\) L\(^{-1}\), females:170 ind\(^{-1}\) L\(^{-1}\)) were transferred from the experimental population replicates to 60 mL tissue culture flasks \((6-8\) replicates per life stage, male and female, for each treatment) filled with filtered \((0.2\) \(\mu m\) sterilised seawater of the required \(p\)CO\(_2\) concentration. Prey \((I.\) galbana, \(C.\) muelleri and \(T.\) suecica\), reared under low or elevated \(p\)CO\(_2\), were then added to the corresponding predator tissue culture flasks at the same concentration as used for the stock populations. After 24 hours, ingestion rates were
calculated across the 4 treatments using Frost’s [21] equations. Respiration rates (nL O$_2$ ind$^{-1}$ min$^{-1}$) were calculated over a period of 6-8 hours separately for adult males and females (8-10 replicates per life stage per treatment) using a non-invasive optical fluorescence-based oxygen respirometry (Fibox 3 LCD trace transmitter, PreSens, Germany). The method employed is detailed further in S1.

**Copepod prey selectivity:** Adult male and female prey preference under direct, indirect and combined exposure to elevated $p$CO$_2$, were calculated from the ingestion rates using Chesson’s prey selection index [30].

**Elemental and biochemical stoichiometry of copepods:** Mature males and females (between 1-5 days old) were collected for elemental stoichiometry ($\mu$g C ind$^{-1}$, $\mu$g N ind$^{-1}$ and C: N) and biochemical stoichiometry (lipid: protein, protein: carbohydrate, and carbohydrate: lipid) across the four treatments. The carbon and nitrogen content of the adults were measured separately for males (8-10 replicates per treatment, 15-25 individuals per replicate) and females (8-10 replicates per treatment, 10-15 individuals per replicate).

Individuals were placed into tin cups (6x4 mm; Exeter Analytical, UK), immediately frozen and stored at -80 °C until analysis. The relative difference between the biochemical compositions of *A. tonsa* adults were assessed using FTIR analysis. Individuals were pipetted into 1.5 mL micro-centrifuge tubes, frozen at -80°C, freeze dried (< 24 hours after initial freezing) and then homogenised prior to FTIR analysis. For both elemental and biochemical analyses the same methods were used as described for prey.

**Trophic transfer:** the influence of different $p$CO$_2$ treatments (direct and/or indirect) on the trophic transfer efficiency was calculated using the carbon allocation budgets of adult females in G$_1$. All measured metabolic rates were converted into carbon equivalents; ingestion rates (I, gC gC$^{-1}$ d$^{-1}$), EPR were used as an index for female growth (G, gC gC$^{-1}$ d$^{-1}$), and
respiration rates (nL O$_2$ ind$^{-1}$ min$^{-1}$) were converted into respiratory carbon equivalents (R, gC gC$^{-1}$ d$^{-1}$) using the respiratory quotient of 0.97 [31]. The proportion of carbon ingested (I) that was allocated to growth (G) was calculated as Gross Growth Efficiency (GGE = G/I). The proportion of carbon incorporated into growth in relation to the total carbon assimilated was calculated as Net Growth Efficiency (NGE = G/ G+R). The standard deviation (Xσ) for the calculated transfer efficiencies (NGE and GGE) and weights-specific rates (I, R and G) were calculated to incorporate error propagation. Correlations between the biochemical stoichiometric multivariate responses of the prey (lipid: protein, lipid: carbohydrate and protein: carbohydrate of C. muelleri, I. galbana and T. suecica under both P$_L$ and P$_E$) to that of the predator (lipid: protein, lipid: carbohydrate and protein: carbohydrate of Z$_LP_L$, Z$_EP_E$, Z$_LP_E$ and Z$_EP_E$ populations) were analysed using a Mantel test. Multiple stepwise search analyses were used to determine which biochemical ratio from the prey best matched the multivariate pattern of the predator’s biochemical stoichiometric composition, using the BVSTEP routine.

2.3. Statistical analyses

Phytoplankton: the influence of pCO$_2$ on the growth rates (cells mL$^{-1}$ and BV $\mu$m$^3$ mL$^{-1}$), cell size ($\mu$m), carbon content ($\mu$g C), nitrogen content ($\mu$g N) and C:N ratios of the three phytoplankton species were analysed using permutational multivariate analysis of variance (PERMANOVA). All dependent variables were assembled into a resemblance matrix using Euclidean distance and analysed using a factorial design with two crossed fixed factors; (i) species (I. galbana, T. suecica and C. muelleri), and (ii) treatment (P$_L$ and P$_E$). An additional nested factor of time was incorporated into the ‘treatment’ factor for two of the dependent variables (growth rate and cell size). Main effects and pairwise comparisons of the different
factors were analysed through unrestricted permutations of raw data. If a low number of permutations were generated then the $p$-value was obtained through random sampling of the asymptotic permutation distribution, using Monte Carlo tests. For each dependent variable the dispersion across the factors was first analysed using permutational dispersion. Because cell size had a significantly different dispersion across the different $p$CO$_2$ levels (both, $p = < 0.05$), cell size was transformed ($\log (x+1)$) prior to the PERMANOVA analysis. Fixed factor ($P_L$ and $P_E$) multivariate analysis (PERMANOVA) was used to compared the combined biochemical stoichiometry between the treatments for each species, followed by a one-way fixed factor analysis of variance to compare each stoichiometric ratio between the 2 $p$CO$_2$ treatments ($P_L$ and $P_E$). The lipid: carbohydrate, lipid: protein and carbohydrate: protein ratios in *I. galbana* were transformed prior to analysis, as each ratio had a significantly different dispersion across the different $p$CO$_2$ levels ($p = < 0.05$). An $\alpha$-level of $p = \leq 0.05$ was used for assessing statistical significance. Analyses were carried out in PRIMER-e (version 6.1.15) with the PERMANOVA add-on (version 1.0.3, Plymouth Marine Laboratory, Plymouth, UK) and R-software (version 3.2.1).

**Copepods:** the influence of direct, indirect and combined elevated $p$CO$_2$ exposure on the individual vital rates (fecundity success [EPR: female$^{-1}$ day$^{-1}$, ES: $\mu$m$^3$, EHS: % and NR: female$^{-1}$ day$^{-1}$], ingestion rates [$\mu$g C ind$^{-1}$ day$^{-1}$] and respiration rates [nL O$_2$ ind$^{-1}$ min$^{-1}$]), behaviour ($\alpha$-index) and elemental stoichiometry (C, N and C:N) of *Acartia tonsa* were analysed using PERMANOVA factorial design with two crossed fixed factors; (i) treatment ($Z_LP_L$, $Z_EP_L$, $Z_LP_E$ and $Z_EP_E$) and (ii) life stage (for respiration and ingestion only). Differences in the copepods relative biochemical compositions between the treatments were analysed using the same method employed for the phytoplankton. Means and calculated standard deviations of trophic transfer efficiencies (GGE and NGE) and weights-specific rates (I, R and G) were compared through a fixed-factor analysis of variance design between
the treatments. Correlations between the multivariate biochemical stoichiometric ratios of the
prey and the predators were assessed through a Mantel test, using Spearman’s rank
correlation coefficient (rho). Multiple stepwise search analyses (BVSTEP) determined which
biochemical component across the 3 prey species (lipid: protein, lipid: carbohydrate and
protein: carbohydrate of *C. muelleri, I. galbana* and *T. suecica* under both *P*L and *P*E) had the
greatest influence on the predator’s composition (lipid: protein, lipid: carbohydrate and
protein: carbohydrate of *Z*LP, *Z*EP, *Z*LPE and *Z*EPE populations). The BVSTEP routine
successively adds and removes a variable to obtain the optimum correlation between the
zooplankton and prey’s composition, using spearman’s correlation coefficient. An α-level of
*p* = ≤0.05 was used for assessing statistical significance across main tests, and Bonferroni
corrections were incorporated during multiple testing between the 4 treatments using an α-
level of *p* = ≤0.0125.

### 3. Results

Throughout the following, subscript L and subscript E refer to treatments as low (ambient) or
elevated (OA) *p*CO₂ respectively, as applied to zooplankton (i.e., *Z*L, *Z*E) or phytoplankton
(i.e., *P*L, *P*E). Direct treatments are thus indicated as *Z*EP, indirect as *Z*LPE, and combined as
*Z*EPE, with the control as *Z*LP.

#### 3.1. Phytoplankton

No differences were found in the growth rates, cell size, or elemental content (carbon,
nitrogen, C: N) across the 3 phytoplankton species tested as a result of growth at elevated
*p*CO₂. However, species-specific differences were found in the biochemical composition as
determined by Fourier Transform Infrared Spectroscopy (FTIR). The biochemical stoichiometry of the diatom *C. muelleri* and prymnesiophyte *I. galbana* differed significantly under elevated $p$CO$_2$ (multivariate analysis: $p = 0.014$, $F = 6.25$ and $p = 0.002$, $F = 9.47$, respectively) while no differences were found in the prasinophyte *T. suecica*. The lipid: protein ratio in *C. muelleri* was significantly higher under elevated $p$CO$_2$ levels (1-way analysis of variance: $p = 0.014$, $F = 6.25$, Fig. 1A). Variations between the lipid: carbohydrate ratios and the protein: carbohydrate ratios in *C. muelleri* could not be assessed, as the diatoms silicate peak obstructed the carbohydrate reading on the FTIR. For *I. galbana*, all biochemical stoichiometric ratios varied significantly between the two treatments, Fig. 1B (1-way analysis of variance: lipid: protein: $p = 0.017$, $F = 6.3749$, lipid: carbohydrate: $p = 0.001$, $F = 21.54$ and protein: carbohydrate: $p = 0.005$, $F = 8.65$).

### 3.2. Copepods

**Copepod chemical stoichiometry:** Variations in carbon ($\mu$g C ind$^{-1}$) and nitrogen ($\mu$g N ind$^{-1}$) between the 4 treatments were found in adult males (multivariate analysis of variance: $p = 0.001$, $F = 7.912$), but not in adult females. Both carbon (C) and nitrogen (N) content in males increased in populations exposed to the combined elevated $p$CO$_2$ conditions (pairwise-test: C: $p = 0.007$, $t = 4.46$ and N: $p = 0.001$, $t = 7.12$), though the C: N ratios in males were not found to be different between the 4 treatments. The biochemical composition of copepods varied across the treatments (multivariate analysis of variance: $p= 0.001$, $F = 92.62$), with the greatest stoichiometric similarities found between low $p$CO$_2$ controls and direct OA treatments, where only the copepods were exposed to elevated $p$CO$_2$ ($Z_{LPL}$ vs $Z_{ELP}$ in Fig. 1C). All biochemical stoichiometric ratios significantly differed between the 4 treatments (1-way analysis of variance, lipid: protein: $p = 0.001$, $F = 50.24$, ...
carbohydrate: protein: $p = 0.002$, $F = 142.88$, and lipid: carbohydrate: $p = 0.001$, $F = 27.48$).

Copepod carbohydrate: protein ratios were significantly higher in control populations compared to populations exposed to the indirect and combined OA treatment (pairwise-test: $Z_{LPE}: p = 0.001$, $t = 19.28$, and $Z_{EPL}: p = 0.001$, $t = 21.76$, Fig. 1D). The lipid: carbohydrate ratios significantly declined across the indirect and direct pathways (pairwise-test: $Z_{LPE}: p = 0.001$, $t = 9.00$, and $Z_{EPL}: p = 0.004$, $t = 6.29$, Fig. 1D), but not in the combined OA treatment. The lipid: protein ratios of the copepods declined across all treatments compared to the ambient populations (pairwise-test: $p < 0.0125$ across all treatment ratios [Fig. 1D]), with the greatest declines found across the individual $p$CO$_2$ pathways (direct or indirect pathways).

**Prey selectivity**: *C. muelleri* was preferentially selected by adult copepods across all 4 treatments (Fig. 2A, 2B). The index ($\alpha$-level) of prey selectivity for *C. muelleri* was significantly greater in females exposed to the combined elevated $p$CO$_2$ treatment (>70 %) compared to females preying on phytoplankton reared in ambient $p$CO$_2$ levels (pairwise-test, $Z_{LPL}: p = 0.012$, $t = 3.63$ and $Z_{EPL}: p = 0.003$, $t = 5.50$ [Fig. 2A]). This was also found in male populations, with individuals actively selecting *C. muelleri* to a greater extent (> 65 %) under the combined elevated $p$CO$_2$ treatment compared to ambient conditions (pairwise-test: $p = 0.002$, $t = 4.16$ [Fig. 2B]).

**Prey ingestion**: While the total amount of prey (in terms of phytoplankton-carbon) ingested by adult females did not vary significantly with $p$CO$_2$ exposure, females across the 4 different treatments attained this same total ingestion rate by consuming different prey types (Fig. 2A, C). The combined direct and indirect exposure to elevated $p$CO$_2$ led to a significantly greater consumption of *C. muelleri* (pairwise-test: $p = 0.003$, $t = 4.87$) with lowered ingestion rates of *I. galbana* (pairwise-test, $p = 0.004$, $t = 4.19$). For adult males, the overall ingestion rate...
varied between the treatments (1-way analysis of variance: $p = 0.045, F = 3.06$), and was significantly lower in populations that were exposed to indirect elevated $p$CO$_2$ levels (pairwise-test: $p = 0.008$, $t = 2.96$). Similar to females, males also ingested *C. muelleri* at a greater rate under the combined elevated $p$CO$_2$ treatment (pairwise-test, $p = 0.005$, $t = 3.49$, Fig. 2B, D).

**Respiration:** Respiration rates varied significantly across the 4 treatments in both adult males (1-way analysis of variance: $p = 0.002$, $F = 9.04$), and adult females (1-way analysis of variance: $p = 0.003$, $F = 7.86$, Fig. 3A). Adult males directly exposed to elevated $p$CO$_2$ levels ($Z_{E}P_{E}$ and $Z_{E}P_{L}$) displayed respiration rates 2-2.5 fold higher than males directly exposed to ambient $p$CO$_2$ levels (pairwise-test: $Z_{L}P_{L}$: $p = 0.008$, $t = 3.69$ and $Z_{L}P_{E}$: $p = 0.005$, $t = 3.86$).

In contrast, adult females maintained a significantly suppressed respiration rate under combined elevated $p$CO$_2$ compared to all other treatments (pairwise-test: $Z_{L}P_{L}$: $p = 0.004$, $t = 3.79$, $Z_{E}P_{L}$: $p = 0.007$, $t = 4.22$ and $Z_{L}P_{E}$: $p = 0.006$, $t = 4.74$).

**Phytoplankton to zooplankton trophic transfer efficiency:** Trophic transfer efficiencies declined in populations exposed to the combined elevated $p$CO$_2$ treatment ($Z_{E}P_{E}$) compared to the control ($Z_{L}P_{L}$). The proportion of carbon ingested that was allocated to growth (i.e., gross growth efficiency [GGE]) declined by 78% (pairwise-test: $p = 0.007$, $t = 4.35$), whilst the proportion of carbon incorporated into growth in relation to the total carbon assimilated (i.e., net growth efficiency [NGE]) declined by 52% (pairwise-test: $p = 0.012$, $t = 1.91$, Table S3). Significant correlations were found between the multivariate biochemical stoichiometry of the prey to that of the predators (Mantel test: $p = 0.001$, Rho = 0.68). Multiple stepwise search analyses between two trophic levels indicated that the lipid:protein ratio in *C. muelleri*, the lipid:carbohydrate and protein: carbohydrate ratio in *I.galbana* had the greatest
influence on *Acartia tonsa*'s biochemical composition across the 4 treatments \( p = 0.02, \rho = 0.544 \).

**Population fecundity success:** Different exposure pathways influenced different aspects of the reproductive processes. Egg production rates (EPR) and nauplii recruitment (NR) declined upon indirect elevated \( p\text{CO}_2 \) exposure compared to ambient conditions (pairwise-test: EPR: \( p = 0.009, t = 5.58 \); NR: \( p = 0.011, t = 2.54 \); Fig. 3B). In contrast, egg size decreased through direct elevated \( p\text{CO}_2 \) exposure (pairwise-test: \( p = 0.009, t = 5.91 \)). The combined OA treatment led to an adverse synergistic effect on the EPR and NR, declining both production and recruitment by > 75 \% (pairwise test, both \( p < 0.001 \)).

### 4. Discussion

This study is the first to directly demonstrate the consequences of elevated \( p\text{CO}_2 \) on the trophic transfer between copepods and multiple phytoplankton prey species. The subtlety of the processes that affect prey selection and ingestion, and directly and indirectly then affect growth and reproduction of the consumer are shown to be associated with changes in the biochemical stoichiometry of the prey. While biochemical stoichiometry has been implicated before as an important factor modulating the well-known elemental-level ecological stoichiometry [33,34], here for the first time the event is explicitly demonstrated and also associated with ocean acidification (OA).

The impacts of OA on the elemental stoichiometry of phytoplankton have previously been shown to be species-specific. While some species demonstrate no effects under elevated \( p\text{CO}_2 \) conditions [16,35], other species [10,15,36,37,38] have developed increased C:(N:P) ratios under these conditions. Such deviations have also been seen in mixed-assemblages [12] and phytoplankton communities [39]. In this present study, *C. muelleri, I.galbana* and...
T. suecica displayed an insignificant increase C:N under elevated pCO2. Further, no differences were found in the growth rates or cell size of any of the phytoplankton species between the treatments in our study. The species-specific response to elevated pCO2 only becomes evident in the phytoplankton’s biochemical composition (Fig. 1A, 1B), with C. muelleri and I. galbana expressing relative declines in the lipid: protein (C. muelleri and I. galbana), lipid: carbohydrate and protein: carbohydrate (I. galbana). These differences found across the biochemical composition of C. muelleri and I. galbana highlights the importance of not relying solely upon the use of elemental stoichiometry as an indicator of prey quality. This, then, explains how subtle differences in elemental stoichiometry can have important non-linear effects on predation [33] with serious impacts on predator-prey dynamics ranging from a collapse in growth potential [40] to the rejection of prey consumption and a switch in alternative prey (including cannibalism; [41]).

The influence of direct, indirect or combined elevated pCO2 exposure on the behaviour of copepods is poorly understood. Within this current study, behaviour was assessed through examining prey preference within a mixed prey assemblage that had been reared either under elevated or low CO2 levels (P_E vs P_L). Optimum prey size theories for copepods [42,43,44] indicate that A. tonsa males and females should actively select T. suecica over C. muelleri and I. galbana (calculated using the cell volume of the three prey three species). However, the diatom C. muelleri was ingested and preferentially selected for at a significantly greater rate compared to the other species when it was grown under elevated pCO2 levels (P_E) compared to low pCO2 levels (P_L), irrespective of the predator’s own pCO2 exposure (i.e., Z_EP_E vs Z_LP_E). This active selection of elevated pCO2 reared C. muelleri suggests that the diatom was a more attractive prey type to the predators in comparison to the other prey reared under elevated pCO2. Whilst the exact nature of the link between the prey’s biochemical content and predator preference remains unknown, the potential cause-and-effect
has clear and important tropho-dynamic implications for life under OA. Here, we see that the pivotal significant difference between the growth and reproduction of copepods reared under elevated $p\text{CO}_2$ was attributable to the biochemical stoichiometry of the prey. Potentially, this could suggest that bottom-up indirect impacts of OA on copepod populations are dependent on the species-specific response of the available prey within the predator’s habitat. Such assumptions would also explain the reported declines in copepod reproduction through bottom-up effects of elevated $p\text{CO}_2$ when predators were fed on a sole prey diet [17], whilst the population structure remained unaffected when individuals were fed on a variety of prey from their natural planktonic communities [45].

Recently there has been a significant rise in research exploring the direct acute, chronic and transgenerational effects of elevated $p\text{CO}_2$ on copepod mortality rates [4], vital rates [8], developmental rates [5,46] and elemental composition [47, 48, 49]. However, little is known of the indirect effects of elevated $p\text{CO}_2$ on copepod population dynamics [9], or indeed of the more natural scenario which incorporates the combined interacting effects of direct and indirect exposure to elevated $p\text{CO}_2$. In this current study, the indirect effects of elevated $p\text{CO}_2$ (i.e., $P_E$) predominately influenced the reproduction of $A.\ tonsa$, while the direct exposure (i.e., $Z_E$) primarily affected the male copepods respiratory rates. Combining the two exposures ($Z_EP_E$) resulted in adverse synergistic effects to both the fecundity success and respiratory rates of adult females, and the decline across both net and gross growth efficiencies (NGE, GGE, respectively; Table S3). As the direct effects of elevated $p\text{CO}_2$ on the prey species only affected the biochemical properties (rather than the gross elemental content) of $I.\ galbana$ and $C.\ muelleri$, it seems probable that these subtle alterations were the cause of the indirect effects to the copepods reproduction. This observed sensitivity is consistent with our earlier observation [40] showing that rather minor changes in elemental stoichiometry could have a catastrophic impact upon copepod growth even though ingestion
rates remained high. In that earlier study the quality of the prey was affected by nutrient stress (N-limitation); here the impact was not through N-starvation but through the more ready availability of the substrate for C-fixation (i.e., CO$_2$(aq)).

While the details of the changes in macromolecule functional groups within *C. muelleri* and *I. galbana* in response to growth with elevated $p$CO$_2$ await further investigation, declines in the FTIR absorption spectra implicate significant changes in lipid: protein and lipid: carbohydrate ratios (Fig. 1A, 1B). Both lipid and proteins play critical roles in the somatic growth and reproduction of marine copepods [50]. As *Acartia* lack the biosynthetic capacity for *de novo* synthesis of certain sterols and fatty acids they rely on their dietary intake to meet their metabolic requirements [51]. The different reproductive processes (e.g., gonad development, oogenesis and vitellogenesis) are also energetically expensive and require multiple nutritional components across the different reproductive stages [52]. In *A. tonsa* the concentrations of available sterols, fatty acids (e.g., 20:5n3, 22:6n3 and 18:0) and proteins positively correlate to their egg production rate (EPR [51,52]). In contrast, the nutritional requirements for the success of egg hatching in *Acartia* appear to be less specific, with a wide range of fatty acids and sterols proving adequate for egg viability [52]. Together, these likely explain the declines found in the production rates and size of eggs produced under indirect elevated $p$CO$_2$ exposure, but with no effects found on the hatching success rates in females.

Coupled with the 75% decrease in population recruitment found under the combined elevated $p$CO$_2$ treatment was the 50% decline found in female respiratory rates (Fig. 3). Maintaining internal homeostasis under hypercapnia can cause costly energetic trade-offs, due to less energy being allocated to other physiological activities [53]. If respiratory acidosis cannot be compensated for under elevated $p$CO$_2$ conditions then organisms can undergo metabolic suppression, which acts as a short-term solution to the acid-base imbalance [54].
However, when this metabolic suppression strategy is adopted for a chronic duration it adversely affect organism fitness through the active repression of critical physiological processes (e.g., protein synthesis), which can decrease an individual’s ability to grow and reproduce [55]. Prior to entering metabolic suppression, though, the energetic cost for an individual in maintaining internal homeostasis under hypercapnia can be alleviated through consumption of increased food quality and/or quantity [56]. Within our study, total prey ingestion rates by females did not alter between the four treatments. Thus the variation in prey quality between the treatments (P_E vs P_L) could explain the lack of respiratory and reproductive impacts in females directly exposed to elevated pCO_2 whilst fed prey reared under ambient conditions (i.e., Z_E P_L).

Deviations in an individual’s metabolic rate upon exposure to an environmental perturbation can provide valuable insight into an organism’s ability to preserve internal homeostasis, sustain life history traits and maintain fitness [57]. Research into the metabolic rates of copepods exposed to OA scenarios has emphasised their species-specific response to climate change. Upregulated respiratory rates have been associated with the acute exposure to extreme pCO_2 concentrations (3000 μatm) in adult Centropages tenuiremis [58], in addition to the transgenerational exposure of C_V Calanus finmarchicus (1080-3080 μatm pCO_2, [46]) and Pseudocalanus acuspes (900 μatm pCO_2, [7]). However, no change in respiratory costs were linked to the elevated acute exposure (824 μatm pCO_2) of Acartia clausi [59] or the high chronic exposure (3000 μatm pCO_2) of C_V C. hyperboreus and C_V C. glacialis [49]. One of the novel aspects of this current study is that it demonstrates the contrasting ontogenic respiratory responses to elevated pCO_2. Whilst adult females suppressed their respiratory rates when exposed to the combined treatment, adult males maintained increased oxygen consumption rates under direct elevated pCO_2 exposure, regardless of the status of the prey they ingested (Z_E P_L and Z_E P_E; Fig. 3A).
Further studies are required to investigate how the effects found in this study relate to trophic interactions between wild populations. However, there is every reason to expect the core observations to match, because of the commonality of stoichiometric ecology as a driver in all systems. Thus, trophic dynamics within the plankton food webs are subject to potential feedback loops associated with nutrient regeneration; consumption of good quality prey results in high regeneration rates of nutrients, which maintains the good quality status [34]. Under OA there is scope for additional feedback events. Thus, phytoplankton growth under elevated $p$CO$_2$ generates different scales of basification (increase in pH with C-fixation), which is expected to affect plankton succession [54]. From the current study, we can see scope for an additional level of factors affecting phytoplankton selectivity that may develop through the discriminatory activity of the grazers driven by changes in prey’s biochemical stoichiometry. The totality of these interactions will take some additional effort to fully understand, but for now the combined implications of the results from this study, coupled with that of the phytoplankton-only study of Flynn et al [60] gives us clear cause to suspect that secondary production mediated by copepods has the potential to alter significantly under OA.

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Supporting Information

S1 Text and Table. Detailed methodology

S2 Table. Seawater carbonate chemistry

S3 Table. Trophic transfer efficiencies of adult female Acartia tonsa

Figure legends

Figure 1. Biochemical stoichiometry of phytoplankton prey (A, B) and adult predator copepods (C, D) upon exposure to 4 different OA treatments. A: The lipid: protein ratio of C. muelleri reared at ambient (P_L) and elevated (P_E) pCO_2 levels. B: The lipid: protein, lipid: carbohydrate and protein: carbohydrate ratio of I. galbana reared at ambient and elevated pCO_2 levels. Stars denote significance differences between the 2 treatments: ***, p < 0.001, **, p < 0.01 and *, p < 0.05. C: Multi-dimensional ordinal scale (nMDS) plot representing the ordinal distance between the biochemical stoichiometry of A. tonsa adult populations exposed to 4 different pCO_2 treatments for one-life cycle (Z_LP_L: both plankton prey and copepod predators reared under ambient pCO_2 levels, Z_EP_L: prey reared under ambient pCO_2 levels and predators reared under elevated levels, Z_LP_E: prey reared under elevated pCO_2 levels and predators reared under ambient levels, and Z_EP_E: both prey and predator reared under elevated pCO_2 levels). D: The variation in biochemical ratios across the four pCO_2 treatments in adult Acartia tonsa. Letters denote significant difference between the 4 treatments within each group (biochemical ratio). Columns that do not share the same letter are significantly different from one another. The integrated band ratios assigned for each biochemical group are detailed in Table S1. Corresponding pCO_2 treatment concentrations are detailed in Table S2. Values are average ± 1SE across all graphs.
Figure 2. Prey selection and ingestion rates of adult *Acartia tonsa* exposed to 4 different OA treatments for one-life cycle. **A, C:** Prey selectivity (% of α-index) of adult females and males (respectively). **B, D:** Female and male ingestion rates of *I. galbana* (I), *C. muelleri* (C) and *T. suecica* (T). Letters denote significant difference between the 4 treatments within each group (i.e., male and female). Columns that do not share the same letter are significantly different from one another. Corresponding $pCO_2$ treatment concentrations are detailed in Table S2.

Figure 3. Vital rates of *Acartia tonsa* exposed to 4 different OA treatments after one-life cycle of exposure. **A:** Respiration rates of adult males and females, **B:** Nauplii recruitment per adult female. Letters denote significant difference between the 4 treatments within each group (i.e., male and female). Columns that do not share the same letter are significantly different from one another. Corresponding $pCO_2$ treatment concentrations are detailed in Table S2.
Figure 1
Prey Selection

Figure 2

Prey Ingestion
Figure 3.

A

Respiration rate (nL O$_2$ ind$^{-1}$ min$^{-1}$)

Male  Female

_Acartia tonsa_

B

Nauplii recruitment (female$^{-1}$)

$\rho$CO$_2$ treatments

$Z_{LP_L}$  $Z_{EP_L}$  $Z_{LP_E}$  $Z_{EP_E}$
Supplementary Information (S1)

Detailed Methodology

Method

Throughout the following, subscript L and subscript E refer to treatments as low (ambient) or elevated (OA) $p$CO$_2$ respectively, as applied to zooplankton (i.e., $Z_L$, $Z_E$) or phytoplankton (i.e., $P_L$, $P_E$). Direct treatments are thus indicated as $Z_EP_L$, indirect as $Z_LP_E$, and combined as $Z_EP_E$, with the control as $Z_LP_L$.

1.1. Cultures

The calanoid copepod, Acartia tonsa, was obtained originally from the Environment and Resource Technology (ERT), Orkney, UK. Stock cultures were maintained at 24.5°C ($\pm$ 0.54) with a 14:10 photoperiod (4-9 µmol photons m$^{-2}$ s$^{-1}$) in aerated (392 ± 27 µatm $p$CO$_2$; nominally 400 µatm) filtered (0.22µm) seawater. These stock cultures of A.tonsa were fed a mixed prey diet of the prymnesiophyte Isochrysis galbana (Strain CCAP 927/1), prasinophyte Tetraselmis suecica (Strain CCAP 66/22C) and diatom Chaetoceros muelleri (Strain CCAP 1010/3). The microalgae were grown separately in nutrient replete seawater-based medium (88.2 and 5.5 µmol L$^{-1}$ for NO$_3^-$ and PO$_4^{3-}$ respectively; mole N:P ratio 16:1), at 22 ± 1.8 °C in a 18:6 hour light: dark cycle (cool white fluorescent tubes at 50 µmol photons m$^{-2}$ s$^{-1}$). The algae were fed to the copepods in a ratio of 1:1:1 relative to the carbon biomass concentration of the algae (total carbon biomass added = 1 µg C mL$^{-1}$).
1.2. Carbonate manipulation

OA is a long-term event, integrating over space and time. In experiments, typically attempts are made to mimic the simplest chemico-physico effect of OA by exposing organisms to elevated partial pressures of carbon dioxide ($pCO_2$) in keeping with those projections expected under different emission scenarios. Thus, while often direct reference is made in the literature to the influence of OA, in reality reference should more appropriately be made to the influence of elevated $pCO_2$ that aligns with OA. We henceforth use $pCO_2$ as a proxy to represent OA scenarios. Both trophic levels (*A.tonsa* and the three prey species: *I. galbana*, *T.suecica* and *C.muelleri*) were exposed to two $pCO_2$ scenarios; (i) low: present-day $pCO_2$ concentrations of 400 µatm, and (ii) elevated: worst-case scenario for the year 2100, 1000 µatm (RCP 8.5 [1]). These two $pCO_2$-climate scenarios were achieved through saturation of seawater through the aeration of air:CO$_2$ mixture attained using Mass Flow Controllers (Aalborg GFC17). 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 ($A_T$, measured by open cell pentiometric titration using an AS-ALK2 Gran Titrator, Apollo SciTech, USA), pH, salinity and temperature were used to calculate the seawater $pCO_2$ (µatm) through the programme CO2 SYS [2], using the $K_1$, $K_2$ constants from Mehrbrach *et al* [3], as refitted by Dickson and Millero [4].

1.3. Experimental design

1.3.1. Phytoplankton

All species were cultured separately in semi-continuous cycles (effective dilution rate: *T. suecica* 0.30 d$^{-1}$, *I. galbana* and *C. muelleri*: 0.35 d$^{-1}$). Duplicate cultures of each species
were used for both pCO$_2$ treatments (500 mL, total n = 18). *I. galbana*, *C. muelleri* and *T. suecica* cultures (450 mL) were aerated with air at the required pCO$_2$ concentration (either low or elevated) through a sterilised glass airline via an air-filter (0.2µm) at a rate of ca. 52 mL min$^{-1}$. All phytoplankton cultures were started with low cell densities (*I. galbana* 50,000 cell mL$^{-1}$, *T. suecica* 5,000 cell mL$^{-1}$ and *C. muelleri* 25,000 cells mL$^{-1}$) in order to decrease the influence of algal metabolic processes on the carbonate chemistry of the bulk seawater. The cultures were started in media of the required pCO$_2$, and aeration was initiated 24-hours after the experiment started to promote growth of the low cell density culture [5]. Temperature, salinity and pH were measured daily at the beginning and end of the photoperiod, whilst $A_T$ was measured every 48 hours. All cultures were exposed to the nominal pCO$_2$ treatment for at least 12 generations.

Cell number (cells mL$^{-1}$), size (as equivalent spherical diameter, µm) and biovolume (µm$^3$ mL$^{-1}$) across all replicates were analysed at the end of each light cycle using a Multisizer 4 Coulter Counter (Beckman, USA). Every 48hrs, at the semi-continuous exchange point, cells were collected from each culture for elemental stoichiometry and biochemical analysis. Cellular carbon (µg C mL$^{-1}$), nitrogen (µg N mL$^{-1}$) and the C: N of each species from both pCO$_2$ concentrations were analysed using an elemental analyser coupled with an isotope ratio mass spectrometer (SerCon GSL) using isoleucine as the standard. Biochemical stoichiometry of each species cultured at different pCO$_2$ concentrations was assessed through Fourier Transform Infrared (FTIR) spectroscopy (PerkinElmer Spectrum 2) over a range of 450-4000 cm$^{-1}$ and at a resolution of 4 cm$^{-1}$ (method as described in [6]). The absorbance of infra-red at the assigned spectral wave length is proportional to the concentration of the corresponding bond or molecule within the sample, allowing quantitative analysis of the different functional groups [7,8,9]. Here, a semi-quantitative approach was adopted and integrated FTIR band ratios of specific spectral areas
were used to assess the relative difference between biochemical stoichiometries (outlined in Table S1); (i) lipids: protein, (ii) protein: carbohydrate, and (iii) carbohydrate: lipid. All spectra bands were verified with standards; bovine serum albumin (BSA, grade > 98 %, Sigma-Aldrich) for protein, glucose (grade > 99.5 %, Sigma-Aldrich) for carbohydrates and glycerol tripalmitate (grade > 99%, Sigma-Aldrich) for lipids. FTIR spectra were baseline corrected and normalised to the silicate peak (1074 cm\(^{-1}\)) for \(C. \) muelleri and the amide II bond peak (maximum ordinate values over 1520-1565 cm\(^{-1}\)) for \(I. \) galbana, \(T. \) suecica and \(Acartia \) tonsa to minimise interference between replicate samples.

### 1.3.2. Copepods

The two \(p\)CO\(_2\) concentrations (nominally 400 and 1000 \(\mu\)atm; termed “low” and “elevated” from here on) were combined in a matrix between the two trophic levels to produce 4 treatments: (i) \(Z_L\)P\(_L\): zooplankton (\(A. \) tonsa) reared under low \(p\)CO\(_2\) levels fed mixed phytoplankton (\(I. \) galbana, \(C. \) muelleri and \(T. \) suecica) also reared under low \(p\)CO\(_2\) levels, (ii) \(Z_L\)P\(_E\): zooplankton reared under low \(p\)CO\(_2\) levels fed mixed phytoplankton reared under elevated (RCP 8.5) \(p\)CO\(_2\) levels, (iii) \(Z_E\)P\(_L\): zooplankton reared under elevated \(p\)CO\(_2\) levels fed mixed phytoplankton reared under low \(p\)CO\(_2\) levels, (iv) \(Z_E\)P\(_E\): zooplankton reared under elevated \(p\)CO\(_2\) levels fed mixed phytoplankton also reared under elevated \(p\)CO\(_2\) level. The copepod experiment did not commence until all prey species had been exposed to the appropriate \(p\)CO\(_2\) treatment (\(P_L\) or \(P_E\)) for at least 8 generations.

The copepods were exposed to these four treatments for an entire life-cycle, from generation 1(G\(_1\)) early nauplii stages (N\(_1\)) through to G\(_2\) mid-late nauplii stages (N\(_{III-IV}\)). The N\(_1\) used to initiate the experiment were produced from 3 separate culturing tanks (800L), each with > 20,000 mature adults. N\(_1\) hatchlings (≤ 12 hours from hatch and ≤ 4 hours age difference between individuals) were located under a microscope (Nikon SMZ800) and...
pipetted into autoclaved 1 L Schott flasks (4 replicates per treatment, density 890 ind\(^{-1}\) L\(^{-1}\)) filled with the allocated \(pCO_2\) concentration and prey diet. Each bottle was maintained on a rotating plankton wheel at 2 rpm in a constant temperature room at 21 °C with a 14:10 light: dark photoperiod (4-9 μmol m\(^{-2}\) s\(^{-1}\)). Water exchange (90%) occurred every 48 hours at the end of the copepod light cycle, an event synchronised with the semi-continuous water exchange of the prey species. The frequent copepod water exchange minimised the seawater carbonate drift and ensured saturating prey quantities (>1 μg C mL\(^{-1}\)) for the copepods.

During the water exchange temperature, salinity, pH and \(A_T\) were measured across all replicates, and \(pCO_2\) concentrations calculated as described above. Vital rates, behaviour and trophic transfer efficiencies were calculated in adult copepods after one generation of exposure.

**Vital rates:** When >50% of the *Acartia* population had reached maturity in the preceding water exchange then fecundity success, respiration rates and ingestion rates were measured in adult males and females across the four treatments. For fecundity success, 5-8 females from each replicate population (n = 20-32 individuals per treatment) with an attached spermatophore were removed and placed individually into 30 mL vials filled with their assigned treatment and saturating prey quantities of their allocated prey (>1μg C mL\(^{-1}\)). Each vial was pre-lined with a 150 μm nylon mesh bottom to separate eggs from the female to prevent egg cannibalism. Females were held for 24-30 hours to lay eggs. Egg production rates (EPR [eggs female\(^{-1}\) day\(^{-1}\)], egg hatching success (EHS [%]) and nauplii recruitment (NR [nauplii female\(^{-1}\) day\(^{-1}\)]) across the four treatments were calculated as described in Cripps *et al* [10].

Ingestion rates (μg C ind\(^{-1}\) day\(^{-1}\)) of adult males and females were measured separately. A sufficient number of copepods (males: 250 ind\(^{-1}\) L\(^{-1}\), females:167 ind\(^{-1}\) L\(^{-1}\)) were transferred from the experimental population replicates to 60 mL tissue culture flasks (6-8
replicates per life stage for each treatment) and filled with filtered (0.2 µm) sterilised seawater of the required $p$CO$_2$ concentration. Prey (*I. galbana, C. muelleri* and *T. suecica*), reared under low or elevated $p$CO$_2$, were added to the corresponding predator tissue culture flasks at the same concentration as used for the stock populations; initial ($t_1$) cell counts were taken. Flasks were placed on the plankton wheel (2 rpm) in a constant temperature room (23.9 ± 0.63 °C) in the dark for > 24 hours to capture any diel feeding patterns. At the end of the experiment, each flask was gently inverted for one minute before $t_2$ sub-sample (3 x 5 mL) was measured. Copepods were filtered out onto 60 µm nylon mesh, re-suspended in 10 mL of filtered seawater and enumerated under the microscope and scored as live or dead. Ingestion rates were calculated through Frost’s [11] equations.

Oxygen consumption rates were measured across 2 different stages of *A. tonsa* (G$_1$: male and female) using an optical fluorescence- based oxygen respirometry (Fibox 3 LCD trace transmitter, PreSens, Germany). A planar oxygen sensor spot (diameter of 5mm with optical isolation: type PST3; PreSens, Germany) was secured (silicon glue) to the bottom of each of 50 cylindrical glass vials (2.62 mL). The sensor spots consist of an oxygen-sensitive foil with an immobilised fluorescent dye that undergoes dynamic fluorescence quenching in the presence of oxygen. All sensor spots were calibrated with 2% sodium sulphite and 100% oxygen saturated filtered (0.2µm) seawater (at 20°C and 28.8 salinity) prior to the experiments. The routine respiratory rates of adult males and females were measured separately and at different time intervals. The required number of copepods for each measured life stage (averages of female: 2.31 ind$^{-1}$ mL$^{-1}$ and males: 3.85 ind$^{-1}$ mL$^{-1}$) were transferred into the vials using a sterile glass pipette (8-10 replicates). Each vial was filled to the top with seawater of the appropriate $p$CO$_2$, sealed with silicone rubber bungs and wrapped in parafilm to prevent gaseous exchange during the experiments. For each $p$CO$_2$ treatment 2 duplicate controls (filtered seawater) were used to account for any background
changes due to temperature fluctuation and/or oxygen production by bacteria (any background changes were subtracted from the experimental vials prior to calculating respiratory rates). The vials were placed into a temperature bath that matched the temperature of the constant temperature room. The in-situ temperature was monitored continuously through a temperature sensor probe ($PT_{1000}$, PreSens). Oxygen consumption rates were recorded every hour for a 6-8 hour period. Measurements over the first hour were excluded from the analyses to account for acclimation of the individuals within the vials. At the end of the experimental duration, individuals were enumerated and subsequently fixed with 5% iodine for size analyses (prosome length), which was carried out immediately. The oxygen consumption of the individuals within the vials was calculated as the linear part of the time-dependent oxygen concentration curves through linear regression ($f = y0+a.x$).

**Behaviour:** Adult male and female prey preference under direct, indirect and combined exposure to elevated $p$CO$_2$, was calculated using Chesson’s prey selection index $[12]$. 

$$\hat{\alpha}_i = \frac{\ln((r_i-n_i_0)/n_i_0)}{\sum_{j=1}^{m} \ln \left( \frac{n_{j_0}-r_j}{n_{j_0}} \right)}, \quad i = 1, \ldots, m \quad \text{Eq.1}$$

Here, $\alpha_i$ is the Chesson prey selection index, $n_i_0$ is the number of prey items of prey type $i$ present at the beginning of the experiment, $r_i$ is the number of prey type $i$ (cell number) consumed by each predator (adult males or females), and $j$ is the number of different prey types ($C.\ muelleri$ $j = 1$, $I.\ galbana$ $j = 2$, $T.\ suecica$ $j = 3$). In this study $m = 3$ different prey species available. The Chesson prey selection index ranges from 0 (complete avoidance) to 1 (sole selection of that prey type). Thus here, with 3 prey types, an $\alpha_i$ close to $1/m$ (0.333) represents random feeding, a value greater than $1/m$ (> 0.333) indicates positive selection.
(hereafter referred to as active selection) and less than 1/m (< 0.333) as negative selection (hereafter referred to as avoidance).

**Copepod chemical stoichiometry:** At the end of G₁, adult males and females (between 1-5 days old) were collected for elemental stoichiometry (µg C ind⁻¹, µg N ind⁻¹ and C:N) and biochemical composition across the four treatments. The carbon and nitrogen content of the adults were measured separately for males (8-10 replicates per treatment, 15-25 individuals per replicate) and females (8-10 replicates per treatment, 10-15 individuals per replicate). Individuals were placed into tin cups (6x4 mm; Exeter Analytical, UK), immediately frozen and stored -80 °C until analysis. The relative difference between the biochemical compositions of *A. tonsa* adults were assessed at the same time interval as the elemental stoichiometry. Adults were washed with distilled water on a 200µm mesh to remove the salt. Individuals were transferred into 1.5 mL micro centrifuge tubes, frozen at -80 °C, freeze dried (< 24 hours after being frozen) and then homogenised prior to FTIR analysis. For this, the exact same method and quantification for biochemical stoichiometric analysis as used for the prey was employed for the predators.

**Trophic transfer efficiency:** the influence of different pCO₂ treatments (direct, indirect and combined) on the trophic transfer efficiency was assessed through calculating the carbon allocation budgets of adult females in G₁. All measured metabolic rates were converted into carbon equivalents; ingestion rates (I, gC gC⁻¹ d⁻¹), EPR were used as an index for female growth (G, gC gC⁻¹ d⁻¹); respiration rates (nL O₂ ind⁻¹ min⁻¹) were converted into respiratory carbon equivalents (R, gC gC⁻¹ d⁻¹) using the respiratory quotient of 0.97 [13]. The proportion of carbon and nitrogen ingested (I) that was allocated to growth (G) was calculated as Gross Growth Efficiency (GGE = G/I). The proportion of carbon incorporated
into growth in relation to the total carbon assimilated was calculated as Net Growth Efficiency (NGE = G / G+R). The standard deviation (Xσ) for the calculated transfer efficiencies (NGE and GGE) and weights-specific rates (I, R and G) were calculated through Eq.2 to incorporate error propagation.

\[ X\sigma = ab \sqrt{\frac{\sigma_a^2}{a} + \frac{\sigma_b^2}{b} + \ldots} \] \hspace{1cm} \text{Eq.2}

Here, σ refers to the standard deviation of the individual parameters (a,b: respiration, ingestion, production, growth or body weight) used to calculate the carbon budget or weight specific growth rate.

1.4. Statistical analyses

1.4.1 Phytoplankton

The influence of \( p\text{CO}_2 \) on the growth rates (cells mL\(^{-1}\) and BV \( \mu \text{m}^3 \text{mL}^{-1}\)), cell size (\( \mu \text{m} \)), carbon content (\( \mu \text{g} \text{ C} \)), nitrogen content (\( \mu \text{g} \text{ N} \)) and C:N ratios of the three phytoplankton species were analysed using permutational multivariate analysis of variance (PERMANOVA). All dependent variables were assembled into a resemblance matrix using Euclidean distance and analysed using a factorial design with two crossed fixed factors; (i) species (\( I. \text{galbana}, T. \text{suecica} \) and \( C. \text{muelleri} \)), and (ii) treatment (P\(_L\) and P\(_E\)). An additional nested factor of time was incorporated into the ‘treatment’ factor for two of the dependent variables (growth rate and cell size). Main effects and pairwise comparisons of the different factors were analysed through unrestricted permutations of raw data. If a low number of permutations was generated then the \( p\)-value was obtained through random sampling of the asymptotic permutation distribution, using Monte Carlo tests. For each dependent variable
the dispersion across the factors was first analysed using permutational dispersion. Because cell size had a significantly different dispersion across the different $p$CO$_2$ levels (both, $p = < 0.05$), cell size was transformed ($\log (\chi + 1)$) prior to the PERMANOVA analysis. Fixed factor (P$_L$ and P$_E$) multivariate analysis (PERMANOVA) was used to compared the combined biochemical stoichiometry between the treatments for each species, followed by a one-way fixed factor analysis of variance to compare each stoichiometric ratio between the 2 $p$CO$_2$ treatments (P$_L$ and P$_E$). The lipid: carbohydrate and carbohydrate: protein ratios in *I. galbana* were transformed prior to analysis as each ratio had a significantly different dispersion across the different $p$CO$_2$ levels ($p = < 0.05$). An $\alpha$-level of $p = \leq 0.05$ was used for assessing statistical significance. Analyses were carried out in PRIMER-e (version 6.1.15) with the PERMANOVA add-on (version 1.0.3, Plymouth Marine Laboratory, Plymouth, UK) and R-software (version 3.2.1).

### 1.4.2. Copepods

The influence of direct, indirect and combined elevated $p$CO$_2$ exposure on the individual vital rates (fecundity success [EPR: female$^{-1}$ day$^{-1}$, ES: $\mu$m$^3$, EHS: % and NR: female$^{-1}$ day$^{-1}$], ingestion rates [$\mu$g C ind$^{-1}$ day$^{-1}$] and respiration rates [nL O$_2$ ind$^{-1}$ min$^{-1}$]), behaviour ($\alpha$-index) and elemental stoichiometry (C, N and C:N) of *Acartia tonsa* were analysed using PERMANOVA factorial design with two crossed fixed factors; (i) treatment (Z$_L$P$_L$, Z$_E$P$_L$, Z$_L$P$_E$ and Z$_E$P$_E$) and (ii) life stage (for respiration and ingestion only). Differences in the copepods relative biochemical compositions between the treatments were analysed using the same method employed for the phytoplankton. Means and calculated standard deviations (see Eq. 2) of trophic transfer efficiencies (GGE and NGE) and weights-specific rates (I, R and G) were compared through a fixed-factor analysis of variance design between the treatments [15]. Correlations between the multivariate biochemical stoichiometric ratios of the prey and the predators were assessed through a Mantel test, using Spearman’s rank correlation
coefficient (rho). Multiple stepwise search analyses (BVSTEP) determined which biochemical component across the 3 prey species (lipid: protein, lipid: carbohydrate and protein: carbohydrate of *C. muelleri*, *I. galbana* and *T. suecica* under both P<sub>L</sub> and P<sub>E</sub>)) had the greatest influence on the predator’s composition (lipid: protein, lipid: carbohydrate and protein: carbohydrate of Z<sub>L</sub>P<sub>L</sub>, Z<sub>E</sub>P<sub>L</sub>, Z<sub>L</sub>P<sub>E</sub> and Z<sub>E</sub>P<sub>E</sub> populations). The BVSTEP routine successively adds and removes a variable to obtain the optimum correlation between the zooplankton and prey’s composition, using spearman’s correlation coefficient. An α-level of \( p = \leq 0.05 \) was used for assessing statistical significance across main tests, and Bonferroni corrections were incorporated during multiple testing between the 4 treatments using an α-level of \( p = \leq 0.0125 \).

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**Table legend**

**Table S1.** Specific infrared spectral peaks and assigned functional groups used for microalgae (*Isochrysis galbana*, *Tetraselmis suecica* and *Chaetoceros muelleri*) and copepods (*Acartia tonsa*) during quantification of biochemical of stoichiometry. Assigned functional groups for lipids and proteins were chosen to represent the biochemical groups due to their direct correlations with absolute values [7,9], and the carbohydrate range used to incorporate both polysaccharides and cellulose components [16]. † denotes the carbohydrate wavelength used for the copepods within this study, and represents the carbohydrate backbone within crustaceans [17]. Peak absorbance within each functional group was used to calculate the relative biochemical stoichiometry between the biochemical groups (method as described in [16]). \( v = \) symmetric stretch, \( v_{as} \) asymmetric stretch.
| Biochemical group | Assigned functional group | Wavelength |
|-------------------|---------------------------|------------|
| Carbohydrate      | \( \nu C-O \rightarrow C/\nu as \ P=O, \nu C-O \), polysaccharides and cellulose | 1200-950, (1040-1070)† |
| Lipids            | \( \nu C=O \), membrane lipids and fatty acids | 1750-1730 |
| Protein           | \( \nu C=O \), amide I | 1665-1625 |
Supplementary Information (S2)

Seawater carbonate chemistry

Table legend

Table S2. Seawater carbonate chemistry (average ± 1 SE) of the phytoplankton and copepod cultures during the experiment. Phytoplankton data averaged across the light and dark photoperiod. † denotes that $pCO_2$ was calculated through alkalinity ($A_T$) and pH. P_L: plankton reared under ambient $pCO_2$ levels, P_E: plankton reared under elevated $pCO_2$ levels, Z_LP_L: both plankton prey and copepod predators reared under ambient $pCO_2$ levels, Z_EPL: prey reared under ambient $pCO_2$ levels and predators reared under elevated levels, Z_LP_E: prey reared under elevated $pCO_2$ levels and predators reared under ambient levels, and Z_EP_E: both prey and predator reared under elevated $pCO_2$ levels.
| Species           | Treatment | Temperature (°C) | Salinity  | $A_T$ (µmol kg$^{-1}$) | pH (NBS) | $\rho CO_2^{†}$ (µatm) |
|-------------------|-----------|------------------|-----------|------------------------|----------|-------------------------|
| **Prey**          |           |                  |           |                        |          |                         |
| Chaetoceros muelleri | PL        | 19.8 ± 0.17      | 27.1 ± 0.36 | 2506 ± 55.63           | 8.308 ± 0.015 | 349 ± 39.0              |
|                   | PE        | 20.8 ± 0.14      | 26.3 ± 0.10 | 2529 ± 16.36           | 7.850 ± 0.005 | 1174 ± 51.10            |
| Isochrysis galbana | PL        | 20.4 ± 0.09      | 26.7 ± 0.05 | 2496 ± 22.99           | 8.230 ± 0.006 | 420 ± 18.04             |
|                   | PE        | 20.7 ± 0.14      | 27.0 ± 0.08 | 2499 ± 30.37           | 7.911 ± 0.015 | 1002 ± 97.50            |
| Tetraselminus suecica | PL     | 20.1 ± 0.07      | 27.3 ± 0.10 | 2476 ± 10.39           | 8.331 ± 0.017 | 345 ± 42.25             |
|                   | PE        | 20.2 ± 0.08      | 27.3 ± 0.09 | 2549 ± 14.87           | 7.896 ± 0.016 | 1059 ± 42.25            |
| **Predator**      |           |                  |           |                        |          |                         |
| Acartia tonsa     | ZL        | 20.4 ± 0.13      | 26.7 ± 0.06 | 2403 ± 06.08           | 8.212 ± 0.016 | 429 ± 23.40             |
|                   | ZE        | 20.3 ± 0.18      | 26.9 ± 0.06 | 2431 ± 25.87           | 7.719 ± 0.017 | 1210 ± 68.30            |
|                   | ZL        | 21.0 ± 0.06      | 26.9 ± 0.08 | 2433 ± 10.77           | 8.187 ± 0.014 | 462 ± 20.90             |
|                   | ZE        | 20.7 ± 0.12      | 26.6 ± 0.16 | 2450 ± 25.80           | 7.714 ± 0.017 | 1217 ± 81.20            |
Supplementary Information (S3)

Trophic transfer efficiencies of adult female *Acartia tonsa*

**Table legend**

**Table S3.** Female body weight (F, µg C), and weight-specific (gC gC⁻¹ day⁻¹) ingestion (I), respiration (R) and production (P) rates of female *A.tonsa* exposed to elevated pCO₂ directly (Z₉P₉), indirectly (Z₉P₉) and a combination of both (Z₉P₉). These metabolic rates were used to determine the average growth gross efficiency (GGE) and net growth efficiency (NGE) of the population. Values are averages and calculated standard deviations (± 1SD) using error propagation. Bold values denote significance from ambient populations (Z₉P₉).
Table S3.

| Treatment | F       | I       | R       | P       | GGE     | NGE     |
|-----------|---------|---------|---------|---------|---------|---------|
| Z₁P₁      | 4.129 ± 0.15 | 1.675 ± 0.43 | 0.181 ± 0.04 | 0.198 ± 0.09 | 0.120 ± 0.06 | 0.518 ± 0.27 |
| Z₂P₂      | 4.821 ± 0.33 | 1.424 ± 0.67 | 0.185 ± 0.06 | **0.093 ± 0.08** | 0.065 ± 0.06 | 0.334 ± 0.30 |
| Z₃P₃      | 4.356 ± 0.06 | 1.283 ± 0.47 | 0.197 ± 0.04 | **0.108 ± 0.03** | 0.084 ± 0.04 | 0.353 ± 0.12 |
| Z₄P₄      | 4.591 ± 0.12 | 1.468 ± 0.45 | **0.087 ± 0.03** | 0.038 ± 0.02 | 0.026 ± 0.02 | 0.301 ± 0.21 |