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Acclimation and adaptation of the coastal calanoid copepod *Acartia tonsa* to ocean acidification: a long-term laboratory investigation

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ABSTRACT: Ocean acidification impacts many marine biota. Although evolutionary responses should occur during persisting environmental change, little is known about the adaptability of copepods. Therefore, we set up a 3½ yr long selection experiment, maintaining *Acartia tonsa* populations in seawater treated with 200 and 800 µatm CO_2, and feeding them with algae cultured under corresponding CO_2 conditions. In 3 reciprocal transplant experiments, roughly 1 yr apart, we measured developmental rates, C:N and C:P ratios, egg production and hatching rates of the different lines. In the transplant experiments, we observed significantly lower developmental rates in the high CO_2 treatment independent of the selective history. Egg production and hatching success were unaffected by the experimental conditions, but we observed an earlier hatching of eggs from females with a high CO_2 selective history. Over the experimental period, beneficial adaptations of the copepods cultured under high CO_2 conditions of elevated seawater pCO_2 and associated food quality were not detected. However, towards the end of the experiment, copepods cultured under elevated pCO_2 and fed with high CO_2 algae showed increased body mass and decreased prosome length.

KEY WORDS: Ocean acidification · CO_2 · *Acartia tonsa* · Adaptation · Body stoichiometry

1. INTRODUCTION

Since the industrial revolution, oceanic uptake of CO_2 from the atmosphere has resulted in an average ocean surface water pH decrease of more than 0.1 units, the most rapid change in carbonate chemistry experienced in the past 20 million yr (Caldeira & Wickett 2003). Above all, these changes affect calcifying organisms, such as coccolithophores, foraminifera, echinoderms, molluscs and corals, as seawater carbonate chemistry directly impinges on their ability to build up calcium structures (Riebesell et al. 2000, Fabry et al. 2008). Direct effects on non-calcifying zooplankton taxa such as cnidaria (Lesniowski et al. 2015) and crustaceans (Wittmann & Pörtner 2013) are rarer. Most experiments on adult copepods, for example, have indicated low sensitivities to increased pCO_2 (decreased seawater pH) conditions (Kurihara & Ishimatsu 2008, Mayor et al. 2012, Thor et al. 2018). Only under pCO_2 pressures above 5000 µatm were developmental rates, egg production, hatching rates and survival of copepods negatively affected (Kurihara et al. 2004, Mayor et al. 2007, Niehoff et al. 2013). However, younger devel-

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opmental stages seem to be more vulnerable to direct effects of increased seawater pCO_2 than older ones. Fitzer et al. (2012) observed a negative effect of increased pCO_2 (~600 µatm) on nauplii production in *Tispe battagliai*. Furthermore, Cripps et al. (2014b) detected lethal effects of increased CO_2 on *Acartia tonsa* nauplii at concentrations lower than 1000 µatm pCO_2. In contrast, others observed no effects of increased pCO_2 (~800 µatm) on developmental rates of juveniles and females' egg production, even in the same species (Meunier et al. 2016).

Since primary producers reflect the nutrient composition of their surrounding medium, they show higher carbon (C)-to-nutrient element ratios as pCO_2 availability increases (Burkhardt et al. 1999, Urabe et al. 2003). Therefore, increased pCO_2 levels can also have indirect effects on herbivores through stoichiometry changes (Rossoll et al. 2012, Verschoor et al. 2013). Those stoichiometry changes may be particularly pronounced under nutrient limitation during phytoplankton bloom periods (Bach et al. 2016). Since heterotrophs are more restricted in their elemental composition than autotrophs, stoichiometric discrepancies between predator and prey can reduce consumer growth, fecundity and survival (Sterner & Elser 2002, Bukovinszky et al. 2012). Indeed, in previous studies, the copepod *A. tonsa* showed significant decreases in development and reproduction when fed algae cultured under high pCO_2 (~800 µatm) conditions (Schoo et al. 2013, Meunier et al. 2016).

Confronted with a changing environment, organisms can react with geographic range shifts, physiological acclimation or genetic adaptation (Kelly & Hofmann 2013). Experimental evidence of evolutionary adaptations towards ocean acidification (OA) is still rare, and essentially only available for species with short generation times such as microalgae. Indeed, studies on species with short generation times, for example the coccolithophore *Emiliania huxleyi* (Lohbeck et al. 2012) and the microalga *Chlamydomonas* (Collins & Bell 2004), revealed evolutionary adaptations to OA after 500 and 1000 generations, respectively, although contrasting results with no evolutionary response have also been reported (e.g. Low-Décarie et al. 2013). Most temperate copepod species have generation times of approximately 15 to 30 d (Peterson 2001) and their cultivation is laborious and time-consuming. Therefore, investigations on acclimation and adaptation to environmental change are scarce. In a laboratory experiment, Colin & Dam (2004) exposed the copepod *Acartia hudsonica* to a diet containing the toxic dinoflagellate *Alexandrium fundyense*, leading to reductions in survival, growth and fecundity of the copepod. After 3 generations, copepods acclimated or adapted to the food conditions, reflected by improved ingestion and egg production. Thus, although there is some evidence that some species can adapt to highly selective environmental conditions within a short time scale, organisms with longer generation periods such as copepods often show phenotypic plasticity to deal with changes in their environment (Dam 2013, Thor & Dupont 2015). This kind of flexibility allows species to persist in the face of climate change and gives populations time to adapt to new environmental settings (Chevin et al. 2010). Additionally, as an extension of phenotypic plasticity, transgenerational effects such as epigenetic inheritance have been observed. Through, for example, the transmission of nutritional, somatic, cytoplasmatic or epigenetic material, parents can significantly affect the performance and thereby the fitness and survival of following generations (Bonduriansky et al. 2012). Transgenerational carry-over effects of marine organisms exposed to OA have been found in fish (Munday 2014), oysters (Parker et al. 2012), sea urchins (Dupont et al. 2013) and copepods (Vehmaa et al. 2012, Cripps et al. 2014a). In the study of Thor & Dupont (2015), for example, populations of the copepod *Pseudocalanus acuspes* were cultured for 2 generations under 400, 900 and 1550 µatm pCO_2. Reciprocal transplant experiments showed fecundity decreases under higher pCO_2 conditions, which were reversible and thus an expression of phenotypic plasticity. Furthermore, due to transgenerational effects, the reduction in fecundity of offspring generations was lower for parents cultured in a high pCO_2 environment.

In this study, we investigated the effects of elevated pCO_2 conditions, with associated low food quality, on laboratory populations of *A. tonsa* during a 3½ yr experiment. We hypothesised that the high CO_2 selection line (SL) populations should adapt to the high CO_2 conditions of elevated seawater pCO_2 and associated food quality reduction, leading to higher fitness under high CO_2 conditions compared to the low CO_2 SL copepods. We compared several fitness-relevant parameters (developmental rate, egg production and hatching rate) and elemental body stoichiometry with the help of 2 reciprocal transplant experiments, repeated yearly. Further, we measured the prosome length of adult *A. tonsa* females at regular intervals. To investigate genetic changes, the neutral gene region cytochrome-*c*-oxidase I (COI) was analysed.

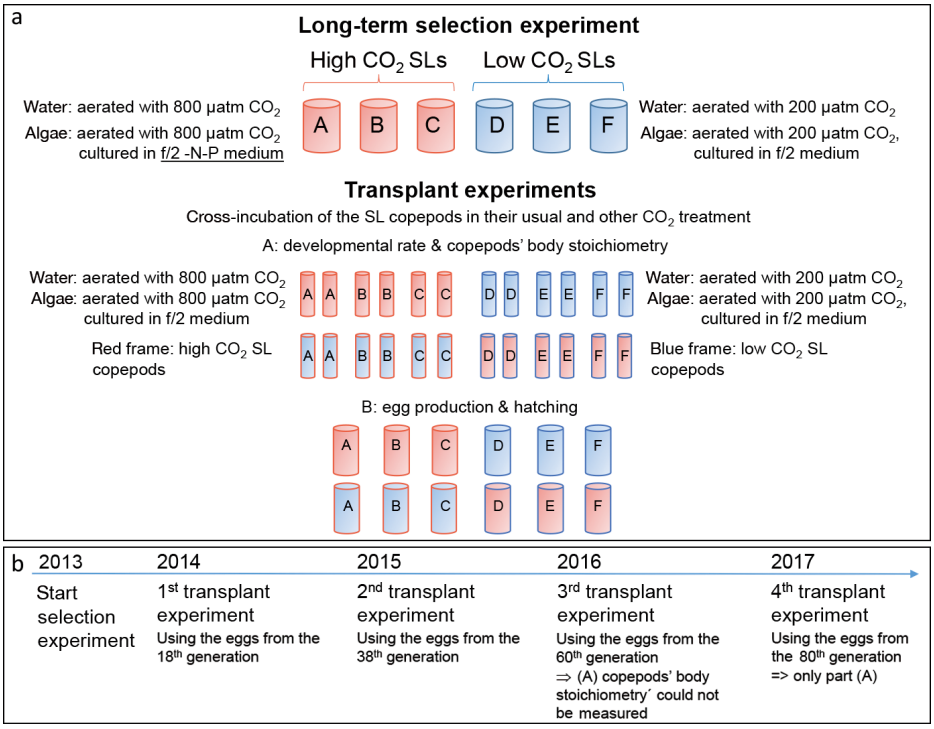

Fig. 1. Schematic illustration of (a) the experimental setup and (b) the overall timeline of the long-term *Acartia tonsa* selection study. SL: selection line

2. MATERIALS AND METHODS

2.1. Long-term CO_2 selection experiment

In September 2013, we started the long-term CO_2 SLs of *Acartia tonsa* using a random selection of eggs from our copepod cultures already present in the laboratory. The SLs ran for 41 mo, which represents around 80 generations based on the investigations of Peterson (2001). Fig. 1 shows a schematic illustration of the experimental setup and time line.

2.1.1. Long-term CO_2 selection experiment: *A. tonsa* SLs

The copepods were cultured in 100 l plastic tanks containing artificial sterile and nutrient-free seawater (ASW; Aquamarin; Söll) at a salinity of 32 and temperature of 18°C. Copepod cultures were kept in the dark and covered with a plastic lid to avoid outgassing. When necessary, new ASW was added to the tanks, and a complete water exchange was conducted every second month. We did not manage copepod densities; natural fluctuations resulted in densities between ~50 and 100 ind. l^{-1}. The experiment consisted of 2 pCO_2 SLs with 3 replicates each. CO_2 values used to constantly aerate the water in the tanks were 800 µatm (tanks A, B, C) and 200 µatm (tanks D, E, F). We chose 200 µatm as a pre-industrial control rather than today's pCO_2 level (400 µatm) to maximise the difference between the experimental CO_2 treatments without exceeding the pCO_2 concentrations predicted for the end of the century. The CO_2 content and flow rate of the gas–air mixture was continuously monitored by a sensor (HTK Hamburg) and automatically adjusted. The pH of the tanks was checked after cleaning activities or maintenance work on the gas mixing system, using a ProLab 3000 pH meter with an IoLine pH combination electrode with temperature sensor (type IL-pHT-A170 MFDIN-N). From 2015 on, we documented pH values on a weekly basis (see Fig. 2). At the start of the long-term selection experiment we also measured the total alkalinity (TA) of the water from each SL tank. The SL tanks D, E and F aerated with 200 µatm CO_2 had a TA (and pH) of 3102 µmol kg^{-1} ASW (8.09), 2962 µmol kg^{-1} ASW (8.06) and 3060 µmol kg^{-1} ASW (8.12), respectively. For the SL tanks A, B and C aerated with 800 µatm CO_2 a TA (and pH) of 3041 µmol kg^{-1} ASW (7.87), 3106 µmol kg^{-1} ASW (7.86) and 3082 µmol kg^{-1} ASW (7.49) was measured, respectively. TA was determined by open-cell duplicate potentiometric titration and calculation

with modified Gran plots (Bradshaw et al. 1981), using a TitroLine alpha plus titrator and an IoLine pH combination electrode with temperature sensor (type IL-pHT-A120MF-DIN-N). Measured values were corrected using certified reference material (CRM; Batch No. 104, Scripps Institution of Oceanography). For the final pCO_2 calculation, the online program CO2calc v.1.3.0 (Robbins et al. 2010) was used, utilising the dissociation constants of carbonic acid of Mehrbach et al. (1973) and Hansson (1973), refitted by Dickson & Millero (1987), and the dissociation constants for H_2SO_4 from Dickson (1990). The back-calculated pCO_2 concentrations of the low CO_2 SL tanks D, E and F were 495, 512 and 450 µatm pCO_2; the high CO_2 SL tanks A, B and C were 871, 913 and 838 µatm pCO_2, respectively. Additional alkalinity and pH control measurements of the water from one low CO_2 SL tank D and one high CO_2 SL tank A in 2016 resulted in a TA (and pH) of 2826 µmol kg^{-1} ASW (8.13) and 3093 µmol kg^{-1} ASW (7.86), respectively. The back-calculated pCO_2 concentrations were 403 µatm for the low and 909 µatm for the high CO_2 treatment. As the CO_2 content and flow rate of the gas–air mixture introduced in the SL tanks was continuously monitored by a sensor and automatically adjusted, there was no reason for additional TA measurements. Additionally, a change in the CO_2 inflow would have led to a change in the water pH, which was measured regularly.

We took samples from each tank every 2nd wk, by pulling a small sieve through the water column (collecting ~50 ind.). For subsequent genetic analyses and prosome length measurements, individuals were preserved in 96% ethanol and sodium tetraborate buffered formaldehyde (4%), respectively. Prosome length was measured from every 4th sample, yielding 19 sampling points in total; ~20 adult females were taken from formaldehyde samples, rinsed with tap water (2–3 min) and measured under a stereo microscope (SZX16) using the CellSens Dimension software version 1.6 (Olympus).

To analyse the genetic variability of the *A. tonsa* start population and to observe changes in the haplotype frequencies over the experimental period, we analysed the marker region COI. The DNA of 5 ind. SL^{-1} tank (A–F) from 5 time points (t_1: 11 Oct 2013 A–F; t_2: 06 Dec 2013 D–F, 08 Jan 2014 A–C; t_3: 17 Mar 2014 A–F; t_4: 15 Apr 2015 A–F; t_5: 01 Jun 16 A–F) were isolated (ISOLATE II Genomic DNA Kit; Bioline) and amplified. The master mix consisted of 2.5 mM $MgCl_2$, 1 mM 10× PCR buffer, 0.2 mM deoxynucleotide triphosphates (dNTPs), 0.2 mM each primer (HCO2198_t1 [5′-CAG GAA ACA GCT ATG ACT AAA CTT CAG GGT GAC CAA A-3′], LCO1490_t1 [5′-TGT AAA ACG ACG GCC AGT GGT CAA CAA ATC ATA AAG A-3′]; Messing 1983, Folmer et al. 1994) and 0.5 mM *Taq* polymerase in a total volume of 20 µl with 1 µl template DNA. The amplification conditions for the PCR reaction were 2 min at 95°C followed by 35 cycles of 1 min at 95°C, 1 min at 45°C and 1 min 30 s at 72°C and a final elongation for 7 min at 72°C. Amplification success was verified via agarose gel electrophoresis. Positive PCR products of the predicted length and purity were sequenced. Single sequences were edited and aligned in Geneious v.7.0.4 and used to calculate a haplotype network (median joining) in PopART v.1.7 using default settings. Additionally, we calculated the frequency of each haplotype for the ambient and high CO_2 treatment at the different time points throughout the experimental period.

Eggs were collected every month and stored at 4°C to ensure the persistence of the long-term experiment. Over the whole experiment, there was only one case of extinction of a SL population, when we needed to restart the low CO_2 SL tanks from the collected eggs after 1 yr.

2.1.2. Long-term CO_2 selection experiment: food cultures

Two 5 l chemostat cultures of the algae *Pyrenomonas salina* from the Experimental Phycology and Culture Collection of Algae (EPSAG) at the University of Göttingen were grown under a 16 h light:8 h dark regime (light intensity: 185 µmol m^{-2} s^{-1}) and stirred continuously. Chemostat cultures were continuously supplied with f/2 medium (Guillard & Ryther 1962), at a rate of 500 ml d^{-1}, resulting in a dilution rate of 10% d^{-1}. Chemostats were cleaned monthly. For the preparation of the f/2 medium, we used sterile filtered sea water (CA, pore size 0.2 µm; Sartorius). The chemostat cultures were aerated with the same 200 or 800 µatm CO_2 gas–air mixture as the copepod tanks. To prepare the food for the low CO_2 SL copepods, we mixed 500 ml of the 200 µatm pCO_2 chemostat culture with 2.5 l of the f/2 medium. For the high CO_2 SL copepods, 500 ml of the 800 µatm pCO_2 algae culture were mixed with 2.5 l of the f/2 –N–P medium (f/2 recipe without NaH_2PO_4 and 20% of the original $NaNO_3$). These algae mixtures were prepared daily, and grown for a further 3 d, aerated with the appropriate CO_2–air mixture of 200 or 800 µatm. Copepods were fed 1 l of the corresponding algae culture, daily, which we computed to be ad libitum.

We specifically chose to manipulate the medium of the high CO_2 SL food algae, thereby accelerating their stoichiometric change, for several reasons. As algae take up nutrients from the surrounding water, a less intense stoichiometric change would have made it necessary to clean the tanks daily to assure constant food qualities (algae stoichiometry), which was not feasible. Therefore, it was necessary to foster the stoichiometry change (higher C-to-nutrient element ratio) of the high CO_2 food algae to ensure that the differences in food qualities between the CO_2 SL tanks persisted. Furthermore, as indirect effects of OA are more important than direct ones (Rossoll et al. 2012, Verschoor et al. 2013, Meunier et al. 2017), especially under realistic OA scenarios, it was essential to ascertain that food quality changes were included in the long-term selection experiment. We specifically chose this approach as we were not interested in a distinction between direct and indirect effects of increased pCO_2 on copepods, but rather in the combined, ecologically relevant, effect.

2.2. Transplant experiments

We conducted 2 different short-term reciprocal transplant experiments, each repeated 3 to 4 times at an interval of at least 1 yr, to investigate adaptations of the SLs to the CO_2 treatments. With transplant Expt A, we investigated the developmental rate and elemental body composition of the copepods (C:N; C:P). Egg production and hatching rate were measured in transplant Expt B. The transplant experiments were conducted using eggs produced by the copepods of the long-term selection experiment of roughly the 18th generation in May 2014, the 38th generation in April 2015 and the 60th generation in April 2016. Due to high nauplii mortality during the transplant experiments in 2016, we repeated transplant Expt A in January 2017 using the eggs of the 80th copepod generation. All transplant experiments consisted of 2 treatments (high CO_2, low CO_2) with 3 replicate tanks and 2 replicates within each tank. Thereby the 2 CO_2 SLs were cross-incubated under their usual and other CO_2 condition and fed with the corresponding CO_2 manipulated algae (Fig. 1).

2.2.1. Transplant experiments: food cultures

For the transplant experiments, we set up *P. salina* stock cultures grown in f/2 medium under ambient aeration. To ensure that the copepods were fed with equal algal stoichiometric qualities every day, pre-cultures were prepared daily from the stock culture at a start concentration of 10×10^5 cells ml^{-1}. After 1 d, the pre-cultures were used to prepare the food cultures started at concentrations of 7.5×10^5 cell ml^{-1} and aerated with either 200 or 800 µatm CO_2. After 5 d, these cultures were harvested and used as food. During the transplant experiments we took samples of the food algae (at least every 2nd day), which were analysed after the experiment to investigate the (molar) C:N and C:P ratios. For the C and N measurements, 4×10^6 cells of *P. salina* were filtered onto pre-combusted GF/F filters (Whatman) and analysed using a vario MICRO cube CHN analyzer (Elementar). The P content of the algae was measured photometrically (Hansen & Koroleff 2007). The cell concentrations were determined with a CASY cell counter (Schärfe System CASY Cell Counter and Analyzer System).

2.2.2. Transplant Expt A: *A. tonsa* cultures

In transplant Expt A, we investigated the developmental rate of the nauplii. Eggs of the long-term SLs were collected at 1 d and incubated for 2 d in ASW at 18°C for hatching. Afterwards, ~1500 nauplii were transferred into 1 l Schott incubation bottles, filled with ASW and connected to the CO_2 aeration system. One-half of each SL was gently aerated with 200 µatm and the other half with 800 µatm CO_2 (24 bottles in total). In correspondence with recommendations from the literature (Marcus & Wilcox 2007), copepods were fed 20 000 cells ind.$^{-1}$ d^{-1}. To minimise changes in the nutrient composition of the algae by animal waste products, we exchanged the water with CO_2 pre-conditioned (overnight) ASW every day before feeding. For the transplant experiment in 2017, we used ~3000 nauplii and 2 l incubation beakers. Furthermore, the incubation beakers were not directly aerated with CO_2. Instead, to minimise the disturbance and mortality of the copepods, we aerated the headspace of the incubation beakers with the appropriate CO_2 concentrations.

We checked the pCO_2 of the pre-conditioned ASW by measuring TA and pH during the first transplant experiment in 2014. The ASW aerated with 200 µatm CO_2 had a TA of 2958 µmol kg^{-1} ASW (and a pH of 8.43); for the ASW pre-conditioned with 800 µatm CO_2, a TA of 2951 µmol kg^{-1} ASW (and a pH of 8.00) was measured. The back-calculated pCO_2 concentration of the low (200 µatm) and high (800 µatm) CO_2 treatments were 180.98 and 615.23 µatm pCO_2,

respectively. The mean pH of the ASW within the incubation bottles during the transplant experiments in 2015, 2016 and 2017 did not differ from the ones in 2014, and were 8.59 (±0.16) for the low and 8.17 (±0.14) for the high CO_2 treatment (data pooled from n = 18 measurements; 4 per CO_2 treatment and year). Further TA measurements in 2015, 2016 or 2017 were not performed as pH values did not differ.

After 10 d of incubation, we investigated the stage distribution of the copepods. We fixed individuals from the incubation bottles in acidic Lugol's solution and determined the developmental stage of at least 60 ind. bottle^{-1}. The arithmetic mean of the counted stages of each incubation bottle was calculated, averaged over the replicates and divided by the number of incubation days to obtain the developmental rate (the numerical values of the developmental stages to calculate the arithmetic mean were defined as follows: nauplii stages 1–6, copepodite stages 7–12). Furthermore, in 2014, 2015 and 2017, we measured the C, N, P content of the copepods from each incubation bottle, and calculated the C-to-nutrient element ratios. In 2016, a measurement of the copepods' body stoichiometry was not possible due to the low numbers of individuals remaining after the transplant experiment.

2.2.3. Transplant Expt B: *A. tonsa* cultures

With the second transplant experiment, conducted in 2014, 2015 and 2016, we investigated the reactions in egg production and hatching. Eggs from the different CO_2 SLs, taken on the same day as for transplant Expt A, were placed in 6 separate ASW-filled 5 l beakers, where they hatched and developed. We kept the copepods in those beakers for 14 d, at 18°C, without light and with gentle aeration until they reached adulthood. During this time, copepods were fed ad libitum with *P. salina* and cultured in f/2 medium. Afterwards, the transplant experiment was conducted for 4 d. Each culture was gently homogenised and split roughly in half. Each half was poured into a 5 l beaker with ASW and covered with a light-diminishing foil (12 beakers in total). The subsequent CO_2 treatment, water exchange and feeding procedures were identical to transplant Expt A. After the incubation, we chose 18–34 females from each beaker placed them individually into 6-well plates containing ASW and ~20 000 cells of *P. salina*. After 24 h, we counted the number of produced eggs and removed the females from the wells to avoid cannibalism (Boersma et al. 2014). The number of nauplii hatched from these eggs was counted after 24 and 48 h.

2.3. Statistical analyses

We conducted all statistical analyses using the program STATISTICA v.9.1 (StatSoft). Data homogeneity was investigated using Shapiro-Wilk tests. For the evaluation of the C, N, P and prosome length measurements, we calculated whisker plots to identify outliers and extreme values. Values outside the 1.5 upper and lower interquartile range, the calibration range or negative values were not considered. For the investigation of differences in the haplotype frequencies between the low and high CO_2 SLs over the experimental period, we performed a 2-way ANOVA using haplotype as the dependent and date and SL as independent factors. We evaluated prosome length measurements of the adult SL females with a repeated measures ANOVA using prosome length as a dependent, and SL and time as independent factors. The significance of the differences in the food algae stoichiometry (C-to-nutrient element ratio and C, N, P cell content) between the low and high CO_2 treatments during the transplant experiments was investigated using Student's *t*-tests. To investigate the stoichiometry (C-to-nutrient element ratios and C, N, P body content) of the high and low CO_2 SL copepods in the 2 CO_2 treatments during the transplant experiments, we performed separate 2-way ANOVAs using SL and treatment as independent factors and C, N, P concentrations and C-to-nutrient element ratios as dependent factors. To compare copepods' C:N ratios between the transplant experiments, we conducted a Student's *t*-test using the C:N ratio as the independent variable. We determined the significance of the differences in developmental rate d^{-1} between the high and low CO_2 SLs under high and low CO_2 conditions during the transplant experiments using 2-way ANOVAs with developmental rate d^{-1} as the dependent and SL and treatment as independent factors. Differences between the transplant experiments (different time points) were investigated by a 1-way ANOVA using developmental rate d^{-1} as the dependent and year as the independent factor.

For the evaluation of the egg production of the high and low CO_2 SL females cultured under high and low CO_2 conditions during the 3 transplant experiments (2014, 2015, 2016) and the corresponding evaluation of the hatching success of the nauplii, observations of the single females within replicates were pooled. We then performed 2-way ANOVAs with eggs female^{-1} d^{-1} or percentage of hatched nauplii after 24 or 48 h as dependent and SL and treatment as independent factors. Means are presented ± SD; for all statistical analyses, $p < 0.05$ was considered significant.

3. RESULTS

3.1. Long-term CO_2 selection experiment: *Acartia tonsa* SLs

Over the experimental period, the mean pH was 8.14 ± 0.11 in the low CO_2 SLs and 7.91 ± 0.09 in the high CO_2 SLs (Fig. 2). The pH together with the alkalinity measurements implied that the intended pCO_2 of 200 µatm was not reached, but was instead around 400 µatm pCO_2 during the experiment.

The prosome length of the SL copepods changed significantly over the experimental period (repeated measures ANOVA; SL: $F_{1,4}$ = 81.3, p < 0.001, SL × time: $F_{16,64}$ = 4.9, p < 0.001, time: $F_{16,64}$ = 1.2, p = 0.279). Until August 2016, prosome length of the different SLs was similar, with a mean length of 867.44 ± 17.95 and 855.73 ± 17.95 µm) for the low and high SL copepods, respectively (Fig. 3). However, during the last half year of the experiment, prosome length of the high CO_2 SL females started to decrease (low CO_2 SLs: 887.37 ± 10.29 µm, high CO_2 SLs: 812.23 ± 14.03 µm; data pooled from the last 3 time points; Fig. 3).

The sequencing of the COI gene region of DNA from a total of 150 individuals resulted in an alignment including 129 high-quality sequences of 442 bp length. Genetic analyses identified 4 polymorphic segregating sites resulting in 2 main and 2 less frequent substitution haplotypes (Fig. 4). Over 3 yr experiencing different CO_2 conditions we observed no significant differences or changes in the frequency of the haplotypes among the CO_2 SLs (2-way ANOVA; date: $F_{4,119}$ = 0.04, p = 0.995, SL: $F_{1,119}$ = 0.05, p = 0.818, date × SL: $F_{4,119}$ = 0.51, p = 0.512).

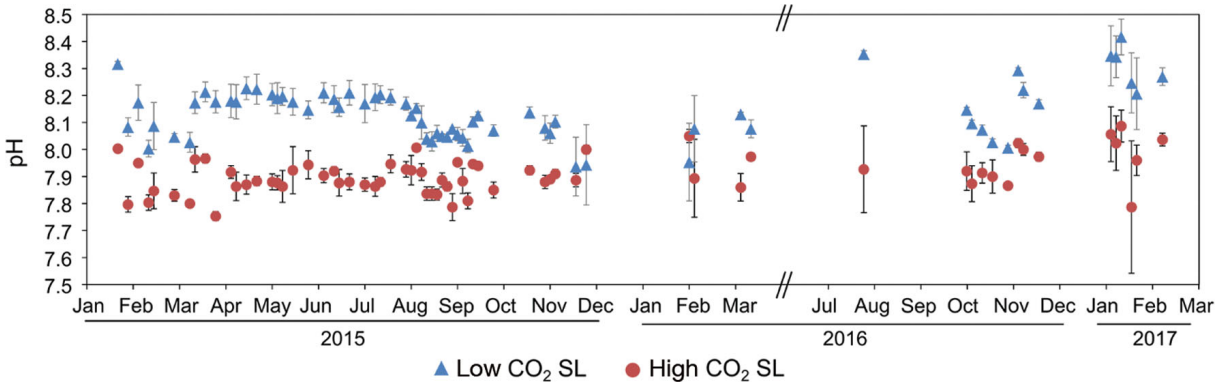

Fig. 2. Mean (±SD) pH of the water in the *Acartia tonsa* selection line (SL) tanks. CO_2 treatments of the SLs: high CO_2: food and water manipulated with 800 µatm CO_2; low CO_2: food and water manipulated with 200 µatm. Error bars: SD; data are pooled from the SL replicates (n = 3)

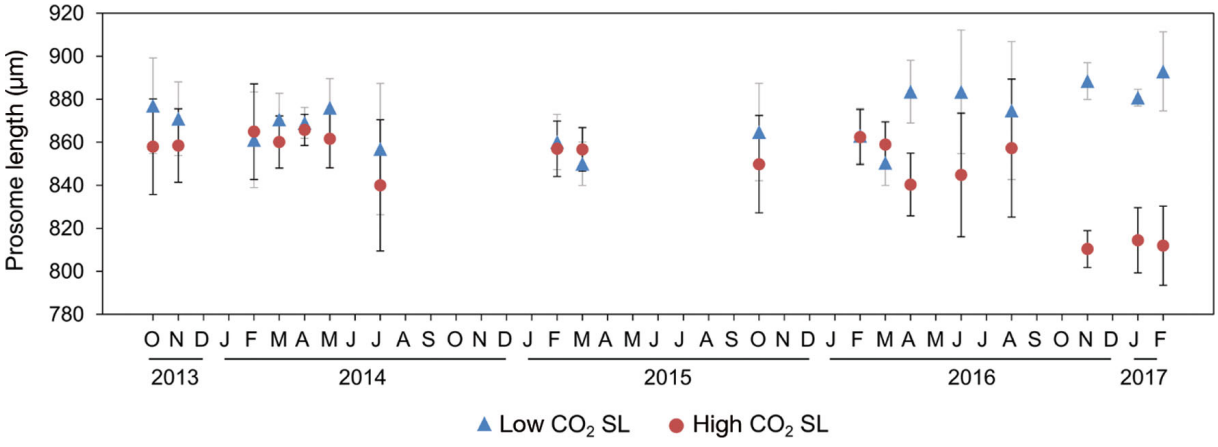

Fig. 3. Mean (±SD) prosome length differences of *Acartia tonsa* between the selection lines (SLs) during the long-term experiment. *x*-axis: letters indicate the month; *y*-axis: mean prosome length of 6 to 49 adult females (µm). CO_2 treatments of the SLs: high CO_2: food and water manipulated with 800 µatm CO_2; low CO_2: food and water manipulated with 200 µatm. Data pooled from the SL replicates (n = 3) with n = 30 (±14) measurements SL^{-1}

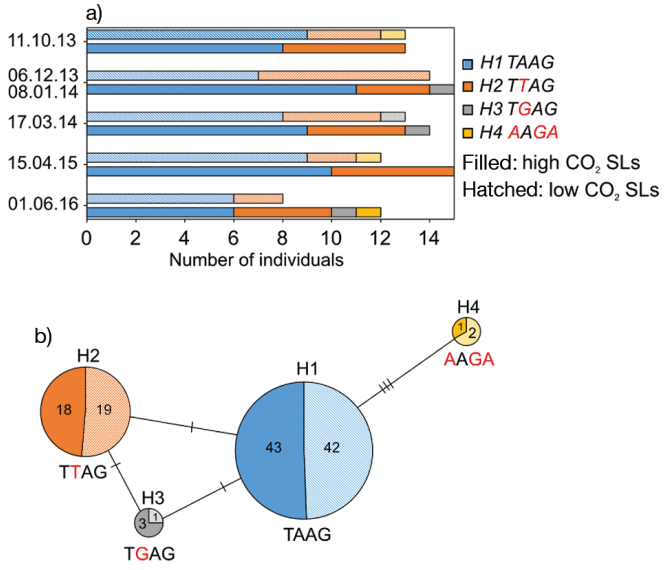

Fig. 4. (a) Haplotype frequencies and (b) network of *Acartia tonsa* individuals from the CO_2 selection line (SL) tanks based on COI sequences. (a) *x*-axis: no. ind. haplotype^{-1} (haplotype frequencies); *y*-axis: time points. Haplotypes H1, H2, H3, H4 (bases varying between the substitution sites among the haplotypes are written in red); filled bars or network parts represent the high CO_2 SLs (food and water aerated with 800 µatm CO_2); hatched bars or network parts represent the low CO_2 SLs (food and water aerated with 200 µatm). Number of dashes between the pies in (b) represent the number of substitutions between haplotypes; numbers on pies represent the number of associated individuals

3.2. Transplant experiments

3.2.1. Developmental rate of *A. tonsa*

In all transplant experiments except the one conducted in 2016, the developmental rate of the copepods in the high CO_2 treatment was significantly reduced. Moreover, the reduction appeared independent of the prior experienced CO_2 SL conditions (Figs. 5 & 6, Table 1).

After 10 d of incubation, 60–75% of the copepods kept under low CO_2 conditions reached the late copepodite stages C4–C6, whereas under high CO_2 conditions only 5–7% (2014), 41–49% (2015), 44–55% (2016) and 44–48% (2017) of the copepods reached those stages (Fig. 6). Furthermore, from 2015 on, copepods of both SLs developed significantly faster under high CO_2 conditions compared to 2014 (1-way ANOVA; $F_{3,44} = 4.92$, p = 0.005; Figs. 5 & 6). Over the years, we did not observe differences in the developmental rate between the high and low CO_2 SL individuals, nor did we observe any significant interactions between the CO_2 treatment and CO_2 SL conditions.

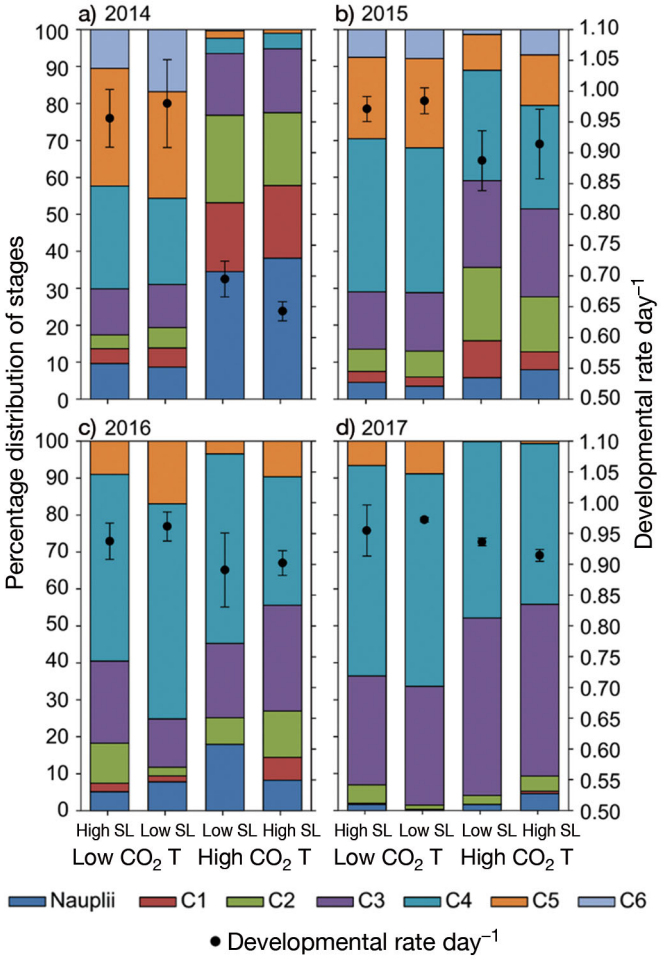

Fig. 5. Stage distribution of *Acartia tonsa* measured after the transplant experiments in (a) 2014, (b) 2015, (c) 2016 and (d) 2017. *x*-axis: transplant combinations: SL: selection line; T: treatment; high CO_2: food and water manipulated with 800 µatm CO_2; low CO_2: food and water manipulated with 200 µatm CO_2. Left *y*-axis: percentage of the respective stages. Key: Nauplii: nauplii stages N1–N6; C1, C2, C3, C4, C5, C6: copepodite stages; right *y*-axis: developmental rate d^{-1}. Error bars: SD; data pooled from the SL replicates (n = 3)

3.2.2. Cell and body stoichiometry of *Pyrenomonas salina* and *A. tonsa*

The C content, and consequently the C:N and C:P ratios of the algae, were significantly higher under high CO_2 conditions compared to the low CO_2 conditions (Table 2). Furthermore, the high CO_2 algae contained significantly less N compared to the low CO_2 cultures. In the transplant experiment in 2014, the C:N and C:P ratios as well as the C content of the copepods from the high CO_2 treatment were significantly higher than the values from the low CO_2 treatment, independent of the CO_2 SL conditions experienced before

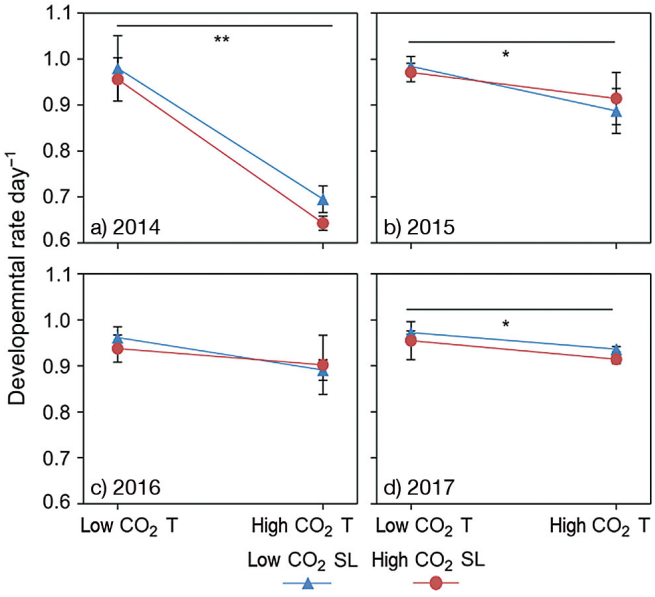

Fig. 6. Developmental rate of *Acartia tonsa* during the transplant experiments in (a) 2014, (b) 2015, (c) 2016 and (d) 2017. *x*-axis: CO_2 treatments (T) of the transplant experiments: high CO_2 (food and water manipulated with 800 µatm CO_2), low CO_2 (food and water manipulated with 200 µatm CO_2); *y*-axis: developmental rate d^{-1}. Key: CO_2 treatments of the selection lines (SLs). Asterisks indicate significant differences (2-way ANOVA using developmental rate d^{-1} as dependent and SL and T as independent factors; *p < 0.05; **p < 0.001). Error bars: SD; data pooled from the SL replicates (n = 3)

Table 2. Stoichiometric differences of the algae food cultures between the CO_2 treatments in 2014, 2015 and 2016. Degrees of freedom (df) and p-values are given for treatment (T) effects (Student's *t*-test using C, N, P concentrations and C-to-nutrient ratios [molar] as independent variables). SD is given in parentheses; significant differences at p < 0.05 are shown in **bold**

Year	200 µatm	800 µatm	df	p T
C-to-nutrient ratios (molar)				
C:N 2014	9.16 (1.39)	12.61 (1.09)	26	**<0.001**
C:N 2015	6.41 (0.89)	14.69 (3.84)	42	**<0.001**
C:N 2016	6.84 (0.44)	14.94 (0.67)	6	**<0.001**
C:N 2017	9.85 (0.69)	15.46 (1.02)	12	**<0.001**
C:P 2014	215.25 (54,64)	267.48 (51.65)	25	**0.021**
C:P 2015	206.62 (66.82)	282.83 (102.56)	38	**0.009**
C:P 2016	317.71 (53.54)	476.06 (127.23)	7	0.231
C:P 2017	246.97 (47.26)	414.63 (59.37)	12	**<0.001**
C, N, P [pg cell^{-1}]				
C 2014	67.25 (9.61)	84.62 (8.02)	25	**<0.001**
C 2015	55.53 (7.61)	88.77 (8.85)	41	**<0.001**
C 2016	41.26 (2.83)	67.2 (5.47)	7	**<0.001**
C 2017	69.31 (7.32)	99.51 (10.18)	12	**<0.001**
N 2014	8.61 (0.92)	7.85 (0.72)	25	**0.03**
N 2015	10.1 (1.94)	7.41 (1.52)	42	**<0.001**
N 2016	6.05 (0.53)	5.09 (0.77)	7	0.647
N 2017	8.21 (0.63)	7.49 (0.41)	12	**0.039**
P 2014	0.79 (0.06)	0.80 (0.14)	24	0.736
P 2015	0.72 (0.26)	0.78 (0.30)	42	0.963
P 2016	0.30 (0.01)	0.34 (0.13)	7	0.647
P 2017	0.74 (0.10)	0.63 (0.10)	12	0.089

(Table 3, Fig. 7). In 2015, copepods from the high CO_2 treatment again had a significantly higher C:N ratio compared to the copepods from the low CO_2 treatment. The C:P ratio and C content of the copepods were, however, similar between CO_2 treatments. Furthermore, as was the case in 2014, we observed no differences between the SLs. In 2017, the C and N content of the copepods as well as the resulting ratio were significantly different between the CO_2 treatments, with a higher C and N content of the copepods (both SLs) from the high CO_2 treatment (Table 3, Fig. 8). Thereby, the C and N content was higher still in those individuals having a high CO_2 selective history (Table 3, Figs. 7 & 8). The increased C content of the high CO_2 SL individuals also affected their C:P ratios, which were significantly higher in both CO_2 treatments compared to the control SLs (Table 3, Fig. 7). Additionally, in 2015 (5.66 ± 0.55) and 2017 (6.94 ± 0.33) the C:N ratios of the copepods (both SLs) from the high CO_2 treatment were significantly lower compared to 2014 (8.91 ± 1.02) (Student's *t*-tests; 2014 vs. 2015: df = 38, p < 0.001, 2014 vs. 2017: df = 21, p < 0.001; Table 3, Fig. 7).

In 2016, we could not measure the body stoichiometry of the copepods because there were not enough individuals left after the transplant experiment.

Table 1. Two-way ANOVA results for investigating the effects of elevated pCO_2 and associated food quality reduction on the developmental rate of the *Acartia tonsa* selection lines during the transplant experiments in 2014, 2015, 2016 and 2017. Results of CO_2 treatment during the transplant experiment (T), of the selection line (SL) and the combined effect (T × SL) are shown. Significant differences at p < 0.05 are shown in **bold**

Year	T	SL	T × SL
2014	$F_{1,8}$ = 85,14, **p < 0.001**	$F_{1,8}$ = 1.39, p = 0.272	$F_{1,8}$ = 0.19, p = 0.673
2015	$F_{1,8}$ = 6.12, **p = 0.038**	$F_{1,8}$ = 0.04, p = 0.831	$F_{1,8}$ = 0.42, p = 0.535
2016	$F_{1,8}$ = 3.72, p = 0.089	$F_{1,8}$ = 0.05, p = 0.822	$F_{1,8}$ = 0.41, p = 0.539
2017	$F_{1,8}$ = 9.37, **p = 0.015**	$F_{1,8}$ = 2.51, p = 0.151	$F_{1,8}$ = 0.03, p = 0.866

3.2.3. Egg production and hatching success of *A. tonsa* females and eggs

The number of eggs produced in 2014 (14 ± 1 eggs female^{-1} d^{-1}) and 2015 (6 ± 1 eggs female^{-1} d^{-1}) did not

Table 3. Body stoichiometry of *Acartia tonsa* measured after the transplant experiments in 2014, 2015 and 2017. SL: selection line; T: transplant experiment; 800 and 200: food and water manipulated with 200 or 800 µatm CO_2. *F* and p-values are given for T, SL and combined (SL × T) effects (2-way ANOVA using SL and T as independent factors and C, N, P concentrations and C-to-nutrient ratios as dependent factors). Standard deviations are given in brackets; significant differences at $p < 0.05$ are shown in **bold**

Year	200 SL 800 T	800 SL 800 T	200 SL 200 T	800 SL 200 T	SL	T	SL × T	SL p-value	T p-value	SL × T p-value
C:N (molar)										
2014	9.14 (0.79)	8.69 (1.09)	5.57 (0.36)	5.99 (0.74)	$F_{1,20} < 0.00$	$F_{1,20} = 78.60$	$F_{1,20} = 1.50$	0.970	**<0.001**	0.235
2015	5.66 (0.61)	5.65 (0.44)	4.84 (0.38)	5.05 (0.37)	$F_{1,53} = 0.59$	$F_{1,53} = 31.38$	$F_{1,53} = 0.68$	0.447	**<0.001**	0.412
2017	6.87 (0.34)	7.02 (0.25)	6.29 (0.41)	6.19 (0.42)	$F_{1,19} = 0.02$	$F_{1,19} = 17.78$	$F_{1,19} = 0.53$	0.885	**<0.001**	0.477
C:P (molar)										
2014	195.26 (52.22)	212.57 (80.74)	153.64 (43.93)	117.20 (54.77)	$F_{1,19} = 0.12$	$F_{1,19} = 6.19$	$F_{1,19} = 0.95$	0.887	**0.022**	0.152
2015	143.02 (59.93)	144.89 (57.48)	147.60 (62.14)	116.47 (63.62)	$F_{1,52} = 0.89$	$F_{1,52} = 0.59$	$F_{1,52} = 1.13$	0.350	0.446	0.292
2017	169.93 (43.90)	279.40 (55.07)	127.76 (46.81)	153.66 (43.28)	$F_{1,18} = 9.00$	$F_{1,18} = 13.84$	$F_{1,18} = 3.43$	**0.008**	**<0.001**	0.081
C [µg ind.$^{-1}$]										
2014	3.47 (0.86)	4.10 (0.72)	2.43 (0.71)	2.04 (0.46)	$F_{1,20} = 0.14$	$F_{1,20} = 24.21$	$F_{1,20} = 2.65$	0.714	**<0.001**	0.119
2015	2.01 (1.34)	1.85 (1.09)	1.67 (1.02)	1.51 (0.83)	$F_{1,51} = 0.30$	$F_{1,51} = 1.26$	$F_{1,51} < 0.00$	0.960	0.493	0.578
2017	3.46 (0.34)	4.86 (0.67)	1.96 (0.22)	2.89 (0.88)	$F_{1,19} = 18.76$	$F_{1,19} = 41.18$	$F_{1,19} = 0.74$	**<0.001**	**<0.001**	0.402
N [µg ind.$^{-1}$]										
2014	0.44 (0.11)	0.55 (0.07)	0.51 (0.14)	0.40 (0.09)	$F_{1,20} < 0.00$	$F_{1,20} = 0.82$	$F_{1,20} = 5.25$	0.965	0.375	**0.033**
2015	0.42 (0.27)	0.37 (0.21)	0.46 (0.30)	0.39 (0.25)	$F_{1,53} = 0.66$	$F_{1,53} = 0.17$	$F_{1,53} < 0.00$	0.421	0.680	0.946
2017	0.59 (0.04)	0.81 (0.09)	0.37 (0.06)	0.54 (0.14)	$F_{1,19} = 21.27$	$F_{1,19} = 31.97$	$F_{1,19} = 0.26$	**<0.001**	**<0.001**	0.613
P [µg ind.$^{-1}$]										
2014	0.04 (0.11)	0.05 (0.01)	0.04 (0.01)	0.05 (0.02)	$F_{1,20} = 1.77$	$F_{1,20} = 0.13$	$F_{1,20} = 0.18$	0.397	0.754	0.804
2015	0.03 (0.02)	0.02 (0.01)	0.02 (0.01)	0.03 (0.01)	$F_{1,45} = 0.15$	$F_{1,45} = 2.29$	$F_{1,45} = 4.48$	0.705	0.137	**0.041**
2017	0.06 (0.02)	0.05 (0.01)	0.04 (0.02)	0.05 (0.02)	$F_{1,19} < 0.00$	$F_{1,19} = 0.68$	$F_{1,19} = 1.82$	0.978	0.420	0.194

significantly differ between the 2 CO_2 SLs and treatments (2-way ANOVA; treatment: [2014] $F_{1,8} = 0.38$, $p = 0.555$, [2015] $F_{1,8} = 0.45$, $p = 0.519$; SL: [2014] $F_{1,8} = 0.17$, $p = 0.693$, [2015] $F_{1,8} = 0.76$, $p = 0.408$; treatment × SL: [2014] $F_{1,8} = 0.13$, $p = 0.731$, [2015] $F_{1,8} = 0.86$, $p = 0.381$).

In 2016, we observed similar egg production in animals from the high (tanks B and C: 7 ± 1 eggs female^{-1} d^{-1}) and low (tanks D, E and F: 8 ± 1 eggs female^{-1} d^{-1}) SL tanks under high CO_2 conditions (Student's *t*-test, df = 3, $p = 0.456$). Since females from SL tank A did not produce any eggs, those measurements were not considered for the calculation of the egg production in the high CO_2 treatment. In the low CO_2 treatment, egg production was low in all SL tanks (2 ± 1 eggs female^{-1} d^{-1}).

In 2014 and 2015, the percentage of hatched nauplii in the first 24 h was significantly different between the CO_2 SLs independent of the CO_2 treatment (2-way ANOVA; treatment: [2014] $F_{1,8} = 0.08$, $p = 0.778$, [2015] $F_{1,8} = 1.25$, $p = 0.297$; SL: [2014] $F_{1,8} = 31.00$, $p < 0.001$, [2015] $F_{1,8} = 24.53$, $p < 0.001$; treatment × SL: [2014] $F_{1,8} = 0.73$, $p = 0.417$, [2015] $F_{1,8} = 0.56$, $p = 0.476$; Fig. 9), with a higher hatching rate of eggs from females with a high CO_2 selective history. In 2014, 76 ± 27% of the eggs from females with a high CO_2 selective history and 55 ± 25% of the eggs from low CO_2 SL females hatched during the first 24 h. After 48 h, 90 ± 1% of the eggs from both SLs hatched. In 2015, the percentage of hatched nauplii after the first 24 h differed only by 8% between the eggs from high and low CO_2 SL females, but this difference was still significant. In 2016, we observed no significant differences between the SLs and CO_2 treatments (2-way ANOVA; treatment: $F_{1,6} = 0.01$, $p = 0.912$; SL: $F_{1,6} = 0.3$, $p = 0.60$; treatment × SL: $F_{1,6} = 0.15$, $p = 0.713$; Fig. 9).

4. DISCUSSION

Within the 3½ yr of this long-term selection study, we did not observe any clear adaptive advantages of the high CO_2 SL copepods under high CO_2 conditions (e.g. an increased developmental rate or egg production under high CO_2 conditions compared to the control SLs). Nevertheless, increased seawater pCO_2 in combination with food quality reduction significantly altered copepods' stoichiometry, and in the long-term also affected physical characteristics such as body length and mass (indicated by a higher C and N content of the copepods in relation to body length).

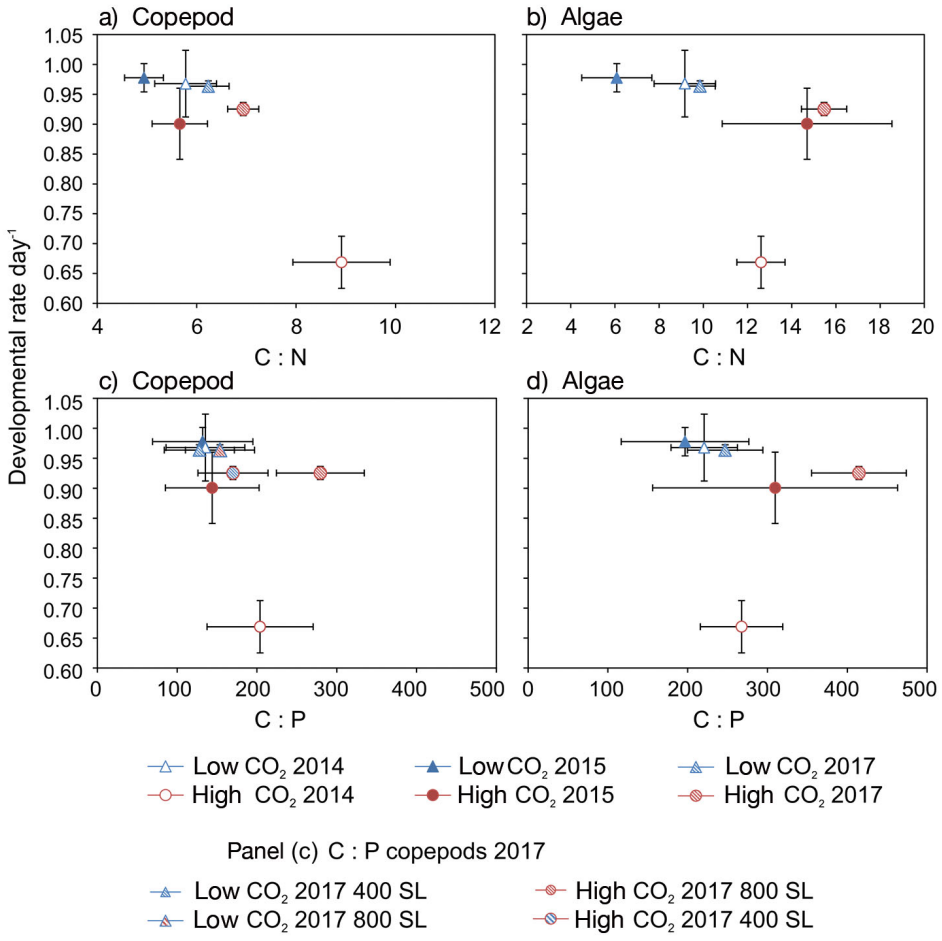

Fig. 7. Developmental rate of *Acartia tonsa* during the transplant experiments in 2014, 2015 and 2017 in dependency of the C:N and C:P ratios of (a,c) the copepods and (b,d) the food algae. *x*-axis: C:N and C:P ratios; *y*-axis: developmental rate d^{-1}. Key: CO_2 treatments: high CO_2 (food and water manipulated with 800 µatm CO_2), low CO_2 (food and water manipulated with 200 µatm CO_2); open, filled and hatched signs represent values from 2014, 2015 and 2017, respectively. Note additional differentiation between the 400 and 800 pCO_2 SL copepods in 2017 due to significant different C:P ratios in the high CO_2 treatment. Error bars: SD; developmental rate: data pooled from the SL replicates (n = 3)

4.1. Experimental setup

In our experiment, we chose to study realistic end-of-the-century OA scenarios, implying the use of a relatively small range of pCO_2 concentrations. We aimed to lower our control treatment from today's pCO_2 level (400 µatm) to pre-industrial conditions (200 µatm) in order to maximise the difference between experimental CO_2 treatments without exceeding these realistic pCO_2 concentrations. However, we did not reach a pCO_2 concentration of 200 µatm in the low CO_2 SL tanks. The amount of the gas–air mixture introduced was probably not high enough to compensate for the respiratory activities in the tank. The pCO_2 concentration of 200 µatm were only reached during the transplant experiments. Modest fluctuations in experimental parameters are inevitable when conducting an experiment such as this over several years. However, the seawater pH in the high and low CO_2 SL tanks remained different over the complete experimental period. As we aimed to study long-term effects of simulated OA (combining direct and indirect effects) on *Acartia tonsa*, short-term fluctuations in the experimental parameters were irrelevant.

Increasing pCO_2 concentrations in the world's oceans will change both the pH as well as the availability of carbon for primary producers, and hence the nutrient stoichiometry of the food for herbivores (Verschoor et al. 2013). The purpose of this study was to investigate these joint effects, as they will never occur separately in a future ocean. Hence, we reduced the nutrient amount in the growth medium of the food algae supplied to the high CO_2 SL tanks,

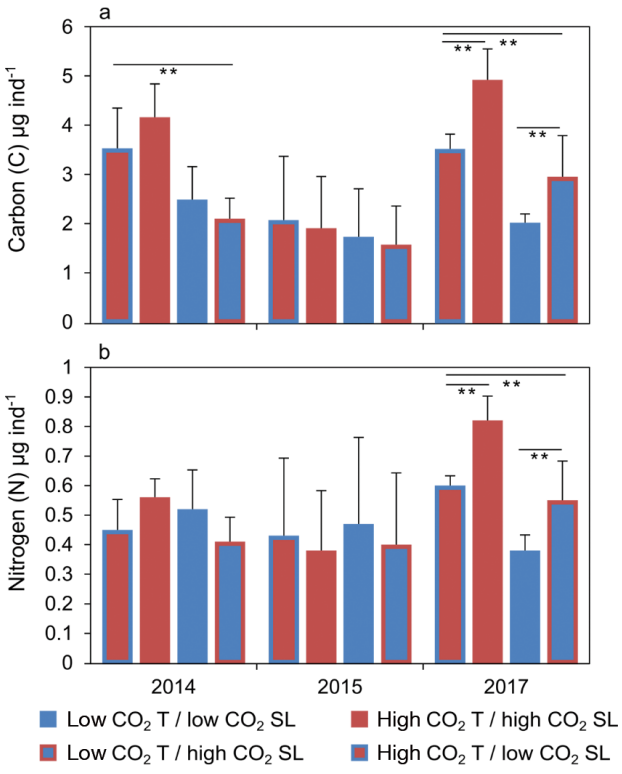

Fig. 8. (a) Carbon (C) and (b) nitrogen (N) content of *Acartia tonsa* measured after the transplant experiments in 2014, 2015 and 2017. *x*-axis: years; (a) *y*-axis: C content [µg ind.$^{-1}$]; (b) *y*-axis: N content [µg ind.$^{-1}$]. Key: red bar = high CO_2 treatment (T) (food and water manipulated with 800 µatm CO_2), blue bar = low CO_2 treatment (food and water manipulated with 200 µatm CO_2), red frame = high CO_2 selection lines (SL), blue frame = low CO_2 SLs. Asterisks: significant differences between the SLs and treatments (2-way ANOVA using C and N concentrations as dependent factors and SL and treatment as independent factors; **$p < 0.001$). Error bars: SD; data pooled from the SL replicates (n = 3)

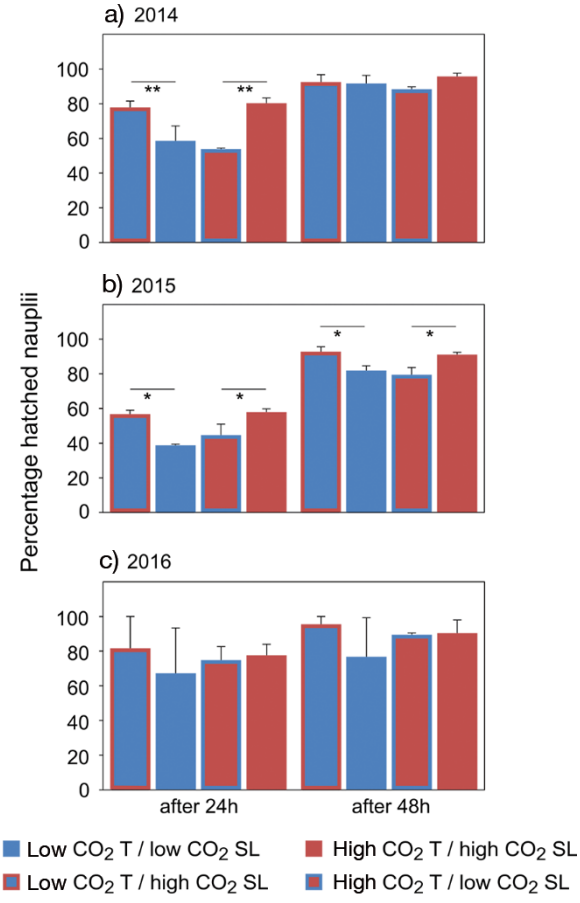

Fig. 9. Percentage of hatched nauplii measured after the transplant experiments in (a) 2014, (b) 2015 and (c) 2016. *x*-axis: counting times of hatched nauplii, (after 24 h) all nauplii hatched after 24 h, (after 48 h) all nauplii hatched after 48 h; *y*-axis: percentage of hatched nauplii. Key: red bar = high CO_2 treatment (T) (food and water manipulated with 800 µatm CO_2), blue bar = low CO_2 treatment (food and water manipulated with 200 µatm CO_2), red frame = high CO_2 selection lines (SL), blue frame = low CO_2 SLs. Asterisks indicate significant differences between the SLs (2-way ANOVA using percentage of hatched nauplii as dependent and SL and treatment as independent factors; *$p < 0.05$; **$p < 0.001$). Error bars: SD, data pooled from n = 3 replicates in 2014 and 2015, n = 2 replicates in 2016 (high CO_2 T / high CO_2 SL, low CO_2 T / high CO_2 SL)

which was necessary to ascertain stable (high C-to-nutrient element ratio) food quality in the high CO_2 SL tanks. This circumstance obviously implies that we cannot attribute changes in animal characteristics solely to different pCO_2 concentrations, but as stated above, we never aimed to do so. Manipulating only the pCO_2 concentrations in the SL tanks would have underestimated the response of *A. tonsa* to future OA conditions, as it would have focused only on changes in seawater pH.

4.2. Genetic diversity of the *A. tonsa* populations

Based on the COI gene region, the genetic diversity of our laboratory population was comparable to natural *A. tonsa* populations investigated in other studies (Caudill & Bucklin 2004, Costa et al. 2014), with less than 2 % variation among haplotypes of one lineage and an average of 4 substitution haplotypes per lineage. Although haplotype frequencies fluctuated, there was no indication of directional change. More sensitive analytical methods, such as microsatellite analyses, could have revealed directional changes in the genetic diversity, so we cannot completely rule out changes in the genetic composition of the SLs.

4.3. Effects of elevated CO_2 and nutrient-deficient food on the stoichiometry and development of *A. tonsa*

During the long-term experiment, we observed an increased cellular C content of the primary producer, *Pyrenomonas salina*, under elevated pCO_2 conditions, supporting the results of previous studies (e.g. Riebesell et al. 2007, Schoo et al. 2013). Concomitantly, the molar C:N and C:P ratios of high CO_2 algae were also higher. As grazers have defined metabolic requirements, stoichiometric discrepancies between prey and consumer needs can impact predator fitness (Sterner & Elser 2002, Meunier et al. 2017). In each of our transplant experiments, we observed a significantly reduced developmental rate of the nauplii reared under high CO_2 conditions feeding on high CO_2 algae. However, developmental rates of the nauplii were always similar between the SLs and thus independent from the selective history of the copepods.

Many grazers, including copepods, are fairly homeostatic with respect to their elemental body composition, thus adjusting their body composition by, for example, the selective retention of scarce nutrients or the excretion of excess elements (Hall 2009). This homeostatic capacity is, however, not perfect, and as a result we observed higher C:N and C:P ratios of *A. tonsa* individuals reared under high CO_2 conditions and fed with high CO_2 algae, and we did not detect any difference between the SLs until 2017. In 2014 and 2017, the significant differences in the C:N and C:P ratios between the CO_2 treatments were due to a significantly higher C content of the copepods (both SLs). As in 2015, the C contents of both SLs were not different between the CO_2 treatments; the significantly different C:N ratio was merely the effect of a slightly lower N and a slightly higher C content of the copepods under high CO_2 conditions.

Unexpectedly, we observed enhanced regulation of the elemental body composition of both SL populations from 2015 on—they both became more homeostatic throughout the long-term experiment. In 2015 after 38 generations, copepod C:N ratio (both SLs) increased by just 15% and after 82 generations in 2017 by just 11% when reared under high pCO_2 conditions and fed high CO_2 algae. Concurrent to this apparent homeostasis regulation, we observed significantly faster juvenile development (both SLs) under high CO_2 conditions (Fig. 7), suggesting a correlation between the elemental body composition of *A. tonsa* and its developmental rate. During the transplant experiments in 2015, 2016 and 2017, the high and low CO_2 SL individuals developed significantly faster in the high CO_2 treatment compared to 2014. In a previous experiment, conducted with eggs from the same laboratory cultures also used to start the long-term experiment, and with equal algal food quality, Meunier et al. (2017) observed similar developmental rates as in our study in 2014. Therefore, it is highly unlikely that the observed developmental rates in the high CO_2 treatment in 2014 were just artefacts. Nevertheless, we do not have an explanation for the change in homeostasis regulation of both SL populations, although it is conceivable that it was caused by the constant experimental conditions. However, regulation of the elemental body composition of *A. tonsa* is rather flexible and independent of the formerly experienced pCO_2 concentration and ingested food qualities. Although low CO_2 SL individuals were not exposed to high CO_2 conditions before the transplant experiments, low CO_2 SL copepods could reach a similar level of homeostasis as copepods with a high CO_2 selective history, which suggest a high plasticity level of *A. tonsa* in the regulation of its elemental body composition.

4.4. Long-term effects of elevated CO_2 and nutrient-deficient food on the phenotype of *A. tonsa*

There are 3 main physiological ways for herbivores to deal with excess C: (1) reduced assimilation across gut walls; (2) stored as C-rich compounds such as lipids; and (3) disposed of by respiration or extracellular release of organic substances (Sterner & Hessen 1994). Our results indicate that *A. tonsa* can use different strategies to deal with excess C. In 2015, the C content of the copepods (both SLs) measured after the transplant experiment was not elevated when they were reared under high CO_2 conditions and fed high CO_2 algae, suggesting an increased excretion of C (see also Schoo et al. 2013). As observed after the first transplant experiment in 2014, in 2017, the C content of both SLs was significantly higher under high CO_2 conditions compared to the low CO_2 treatment (Table 3, Fig. 8). However, different from 2014, the N content of the copepods also significantly increased in the high CO_2 treatment (Table 3, Fig. 8). Therefore, we pose that *A. tonsa* individuals retained more N to compensate for their elevated C content, which is also not an atypical reaction. Likewise, investigations by Miller & Glibert (1998) of the N excretion of *A. tonsa* during a mesocosm study showed a high plasticity level of the copepod to regulate the amount of retained and released N, depending on its physiological and nutritional status.

Although in our study *A. tonsa* showed a high phenotypic buffering capacity to deal with the impaired food quality under high CO_2 conditions, the detrimental elemental composition of the food probably had extensive long-term physical consequences for the copepod. Over the period of the long-term experiment, mean female prosome lengths slightly fluctuated, although not exceeding the range observed in other studies at 18°C (Fiedler et al. 2004, Holste & Peck 2006). Approximately 3 yr after the start of the experiment, we observed that *A. tonsa* individuals cultured under high CO_2 conditions became significantly smaller compared to the ones from the low CO_2 SL tanks (Fig. 3). Furthermore, measurements performed after the transplant experiment in 2017 showed a significantly increased C and N content of the high CO_2 SL individuals compared to the low CO_2 SL copepods independent of the CO_2 treatment (Table 3, Fig. 8). The higher C and N content implies that the body mass of the high CO_2 SL individuals that grazed on high C (energy) algae increased, even though they became smaller. Although there is usually a positive correlation between body length and body mass (C and N ind.$^{-1}$), especially in the growth phase of copepods, this relationship is weak among adults (Kankaala & Johansson 1986, Durbin et al. 1992). Therefore, it is likely that the adult copepods gained body mass while simultaneously becoming smaller. In our study, *A. tonsa* individuals probably stored excess C partly in the form of lipids as adipose tissue, resulting in the increased body mass. Generally, when food is sufficiently available herbivores store lipids as an energy reserve for times of low food supply and for reproduction (Lee et al. 2006). However, a large lipid load can also result from an unbalanced diet (Sterner & Hessen 1994), as was the case for the high CO_2 SLs (excess C and N, P in deficiency).

4.5. Effects of elevated CO_2 and nutrient-deficient food on the reproduction of *A. tonsa*

Although extensive research has been conducted on the effects of food concentration on the egg production of *A. tonsa* (Roman 1991, Zhang et al. 2015 and many others), surprisingly little is known about the effects of food quality (other than using different food species) on egg production. The few experimental studies dealing with the indirect effects of OA (changed nutritional quality of the food) on *A. tonsa* reproduction indicate a connection between the fatty acid composition of the prey and the egg production of the copepod, with a lower production rate under a high CO_2 nutrition (Kleppel et al. 1998, Hazzard & Kleppel 2003). Since those studies comprised only one copepod generation, so far nothing is known about long-term dietary effects on the reproductive output of *A. tonsa*. During our experiment, copepod egg production (number of eggs female^{-1}) was not impacted by the combination of increased seawater pCO_2 and changed algal food quality. Measurements performed after the transplant experiments in 2014 and 2015 showed no substantial differences between the CO_2 SLs and treatments according to the number of eggs female^{-1}. Yet, our results indicated that the nutrition of the high CO_2 SLs affected females' egg production in terms of egg size or nutrient content. In 2014 and 2015 we observed that eggs from high CO_2 SL females hatched significantly earlier than the eggs from low CO_2 SL females. Guisande & Harris (1995) found a positive correlation between the size, protein, carbohydrate and lipid content of eggs from *Calanus helgolandicus* females and the amount of available food. Thus, the nutritional status of a female can directly affect egg size. The availability of N (protein) especially is known to affect egg production in copepods (Checkley 1980, Kiørboe 1989). Steele & Steele (1975) previously showed that the time for embryonic development is positively correlated with egg size. Turning this interdependency around would imply that earlier hatching indicates smaller eggs. Under nutrient depleted conditions, caused by the changed stoichiometry of the food algae (high C-to-nutrient element ratio), high CO_2 SL females opted for reproductive quantity rather than quality, and invested less resources into the production of each egg. In addition, the earlier hatching of eggs from high CO_2 SL females occurred independently of the CO_2 treatment experienced during the transplant experiment. So even under optimal environmental conditions, females with a high CO_2 selective history invested less in the production of each egg. This pattern suggests epigenetic inheritance, whereby the quality of produced eggs is determined by the environmental conditions of the previous generations. It is known that transgenerational effects can actually decouple phenotypic from genetic change over multiple generations and allow populations to respond to environmental changes even in the absence of genetic variation (Bonduriansky et al. 2012). However, whether the expression of the phenotype observed here is solely due to transgenerational effects or has already been genetically assimilated remains unclear.

5. IMPLICATIONS

Our results show that *Acartia tonsa* can be substantially affected by OA, both immediately and in the long term. In the first place, most likely due to the dietary conditions in the high CO_2 treatment, copepods' elemental body composition changed, and their developmental rate decreased. Although *A. tonsa* showed high plasticity in the regulation of its body stoichiometry, we found evidence that the ingestion of high CO_2 algae with a high C-to-nutrient element ratio for several years can result in a reduced body length accompanied by an increase in lipid storage, as indicated by the increased C and N content of the high CO_2 SL copepods in both CO_2 treatments in 2017. However, we do not know whether these changes were phenotypic or genotypic in nature, as we did not test the reversibility of these processes. Besides corresponding implications for the copepod itself, such changes in the phenotype and nutritional composition can have consequences for trophic interactions in the food web. Several studies on various species (oysters, sea urchins, mussels and phytoplankton) predict that complex adaptations to OA are likely over longer time scales (Sunday et al. 2011, Parker et al. 2012, Reusch & Boyd 2013). Nevertheless, we did not detect any SL-specific adaptations to the CO_2 conditions in the observed traits (body stoichiometry, developmental rate, egg production and hatching rate) during our long-term CO_2 selection study. Phenotypic buffering and transgenerational effects, as observed in *A. tonsa* in the regulation of its elemental body composition and egg production, probably reduced the selective pressure on the high CO_2 SL populations.

Results from laboratory experiments on single species or artificial predator–prey combinations cannot simply be translated to the community level, as species richness and complex trophic interactions might mitigate or accelerate the potential direct and indirect effects of OA. Furthermore, in natural field populations many traits are under selection and an effective response to one stressor may limit the response to another, which hampers adaptations when multiple environmental changes occur (Hofmann & Todgham 2010, Gunderson et al. 2016). Besides, there is evidence for inter-population differences in the response to OA due to local adaptations (Vargas et al. 2017).

To be able to make fundamental predictions on how OA and the potentially associated stoichiometry changes of primary producers will influence food web interactions, further investigations under natural field conditions are needed, including multiple producer and consumer species as well as fluctuations in abiotic and biotic factors.

Data archive. The data sets generated in this study are available in the PANGAEA data repository (https://doi.org/10.1594/PANGAEA.879370).

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