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RESEARCH ARTICLE

Is the chemical composition of biomass the agent by which ocean acidification influences on zooplankton ecology?

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Abstract Climate change impacts prevail on marine pelagic systems and food webs, including zooplankton, the key link between primary producers and fish. Several metabolic, physiological, and ecological responses of zooplankton species and communities to global stressors have recently been tested, with an emerging field in assessing effects of combined climate-related factors. Yet, integrative studies are needed to understand how ocean acidification interacts with global warming, mediating zooplankton body chemistry and ecology. Here, we tested the combined effects of global warming and ocean acidification, predicted for the year 2100, on a community of calanoid copepods, a ubiquitously important mesozooplankton compartment. Warming combined with tested pCO_2 increase affected metabolism, altered stable isotope composition and fatty acid contents, and reduced zooplankton fitness, leading to lower copepodite abundances and decreased body sizes, and ultimately reduced survival. These interactive effects of temperature and acidification indicate that metabolism-driven chemical responses may be the underlying correlates of ecological effects observed in zooplankton communities, and highlight the importance of testing combined stressors with a regression approach when identifying possible effects on higher trophic levels.

Keywords Climate change · *Acartia* sp · Body size · Fatty acids · Fitness · Stable isotopes

Introduction

Global change parameters, such as ocean warming and acidification (OA), are projected to affect marine ecosystems simultaneously, and are considered to be the most important factors for future changes (Anderson et al. 2005; Doney et al. 2012; IPCC 2014; Mackas et al. 2012). The basis of marine food webs is particularly vulnerable for these impacts, i.e., by current and projected global phyto- and zooplankton decreases (Edwards and Richardson 2004; Boyce et al. 2010). In aquatic food webs, zooplankton communities occupy the central, intermediate trophic position, making them key mediators of energy and material fluxes (Sterner 2010).

Determination of in situ physiological state of organisms and communities is among the main challenges to understand changes in composition and structure of aquatic ecosystems. These issues become even more important in the face of climate change impacts on aquatic ecosystems and their multiple levels: populations, communities, and individuals. Climate is changing at an unprecedented rate, with rising pCO_2 and increasing global temperatures (Stocker et al. 2013). These climatic changes may severely affect the performance of aquatic organisms and their trophic interactions [reviewed by e.g., Fabry et al. (2008) and Richardson et al. (2008)], and consequently will have diverse impacts on the structure and functioning of ecosystems. For instance, elevated pCO_2 causes acidification of the oceans with detrimental effects on calcifying organisms (Doney et al. 2012). At the same time, higher CO_2 levels may be beneficial for

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some non-calcifying phytoplankton taxa (Schulz et al. 2013), whereas the effects on non-calcifying zooplankton is less clear. Global temperature increase alters phenology, distribution and size of organisms across ecosystems (Parmesan and Yohe 2003).

The potential interactions of multiple environmental stressors make it difficult to predict impacts based on single-stressor studies (Crain et al. 2008). Recent studies have provided evidence for the interactive effects of warming and other ecological impacts on aquatic ecosystems (Byrne and Przeslawski 2013; Paul et al. 2015, 2016). Consequences for many planktonic systems remain unclear, as only a few studies have experimentally analysed the combined effects of warming and ocean acidification on natural zooplankton communities (Hildebrandt et al. 2014; Zervoudaki et al. 2014; Garzke et al. 2016).

Calanoid copepods play a key role in pelagic marine ecosystems because they contribute up to 80% to the zooplankton biomass (Longhurst 1985), and often represent a crucial trophic link between primary producers and higher trophic levels (Edwards and Richardson 2004). Studies testing extreme OA (≥2000 µatm, pH = 7.56) observed sub-lethal effects on copepods, including compromising egg production, hatching success, survival and development (Kurihara et al. 2004; Kurihara and Ishimatsu 2008; Kurihara 2008; Cripps et al. 2014). However, studies using near future OA predictions in many cases found no effects on copepods (Zhang et al. 2011; Mayor et al. 2009, 2012; Vehmaa et al. 2012; Garzke et al. 2015). The temperature effect on copepod physiology and metabolism is well known (Ikeda et al. 2001), and ranges from responses of increased metabolic rates, and enhanced feeding and respiration activity, to reduced body size, biomass, and compromised survival (Pörtner and Farrell 2008; Daufresne et al. 2009; Whiteley 2011; Kordas et al. 2011; Dell et al. 2011; Garzke et al. 2015, 2016). Although the single effects of OA and warming are thus well studied for exemplary zooplankton species, the copepod community responses to combined OA and warming are less well investigated.

Some studies have tested for responses of combined ocean acidification and warming on organisms but only focused on single ecophysiological performance; i.e., egg production (Vehmaa et al. 2012; Zervoudaki et al. 2014; Thor and Dupont 2015), hatching success (Zervoudaki et al. 2014), excretion rates (Pörtner et al. 2004; Zervoudaki et al. 2014), body size, abundance, and fatty acid composition (Kurihara et al. 2004; Kurihara and Ishimatsu 2008; Garzke et al. 2016), and metabolic activity (Dupont and Thorndyke 2009; Mayor et al. 2015; Przeslawski et al. 2015), or zooplankton body mass, dry weight carbon and nitrogen content (Holste and Peck 2005; Vehmaa et al. 2012, 2015; Hildebrandt et al. 2014; Zervoudaki et al. 2014). However, an integrative assessment of these responses and their physiological implications for marine mesozooplankton is still needed.

In this study, a suite of ecological and physiological responses to warming and pCO_2 (500–3000 µatm) were analysed. We hypothesized that higher temperatures mainly affect copepods by reducing their physiological condition (RNA/DNA ratio), and by lowering their efficiency to use food for biomass build up, which may translate into higher mortality, lower copepod abundance and smaller stage-specific body size. Based on previous findings of minor OA effects compared with warming on zooplankton communities (Holste and Peck 2005; Garzke et al. 2016) it is expected that pCO_2 can play a role within present temperature conditions but its effects on copepod abundance, size, and nutritional composition to be largely over-ridden by warming impacts under a global warming scenario. While investigating the above impacts across a more finely resolved gradient of OA than implemented previously (Garzke et al. 2016), this study asked the key question whether similar orders of effect sizes of warming and OA, and their interactions, also prevail on zooplankton condition, performance in terms of resource use efficiency, trophic position, and ultimately survival, thus taking a course from chemically driven physiological responses, reflected in organism biochemistry, translating to ecological responses.

Materials and methods

Experimental design

We applied a regression design approach, exposing independent plankton communities to one of six pCO_2 levels. This approach has the advantage to enhance predictive power compared to the character state approach (Cottingham et al. 2005; Bolker et al. 2009), which compares among different distinct future climate scenarios (Havenhand et al. 2010).

We conducted a 28-day indoor mesocosm experiment using a natural summer Baltic Sea plankton community (August 14th to September 13th 2013). Each mesocosm had a start volume of ~1400 L. Two temperatures [ambient −3 °C (16.5 °C); and ambient +3 °C (22.5 °C)] were combined with six target pCO_2 levels 500, 1000, 1500, 2000, 2500, 3000 µatm (~50, 100, 150, 200, 250, 300 Pa) in a full-factorial set-up without replicates. The temperature treatments are within the range of the natural average summer sea surface temperature of the coastal western Baltic Sea in August (Lennartz et al. 2014). The lowest pCO_2 target value represents the mean present pCO_2 conditions in the Kiel Fjord in summer, and the highest target value represents the maximum levels (>2300 µatm) that can be temporarily observed in summer at this location

(Thomsen et al. 2010). Intermediate target values of pCO_2 conform to the IPCC predictions for coastal upwelling areas with highly temporal variable pCO_2 values, which exceed the "worst case" scenario predictions for the open ocean (IPCC 2014). Twelve mesocosms were filled simultaneously from 2 m depth of the Kiel Fjord using a rotary pump and spreading the unfiltered water by a distributor to ensure homogenous distribution of species composition and density (Paul et al. 2016). The unfiltered water consisted of the natural composition of bacteria, algae, and protozoa. Due to high losses of mesozooplankton during the pumping process, mesozooplankton was caught from the Kiel Fjord by net catches (10 m depth, 200 µm mesh size), and stored in 10 L buckets. Mesozooplankton individuals acclimated over night to the specific target temperatures in the temperature controlled climate rooms, finally dead organisms were removed from the buckets and bucket-specific copepod densities were estimated. Living individuals were added to each mesocosm in a target density by 20 individuals per liter to mimic the natural density during the experimental start [following Garzke et al. (2015) and Paul et al. (2016)]. Temperature and pCO_2 conditions were manipulated gradually over 2 days, until the target values were reached (Fig. S1, Fig. S2). Each mesocosm was covered with a light permeable PVC cover (polyvinyl-chlorid), which contained a lockable port for sampling. Each experimental mesocosm unit maintained a small headspace above the water surface to reduce outgassing of CO_2. Each unit was equipped with a computer controlled light unit (GHL Groß Hard-und Softwarelösungen, Kaiserslautern, Germany), consisting of 5 HIBay-LED spotlights (100 W each, Lampunit HL3700 and ProfiluxII) was installed (Paul et al. 2016). Light intensity was calculated with the astronomic model of Brock (1981) and adjusted to the natural seasonal patterns and latitude (Paul et al. 2016). Maximum light intensity was on average 382.7 µmol m^{-2} s^{-1} PAR (LICOR Li-250A light meter); 40% of solar irradiation of an approximate cloudless day account for shallow water depths. The light–dark cycle for the present study was set at 14 h:3 min: 9 h:57 min and included a simulated sundown and sunrise period of ~2 h.

Sampling and measurements

Our experimental pCO_2 addition, involving carbon addition to the system, thus provided both an acidity and a carbon availability manipulation. In detail, our experiment exposed mesozooplankters to a DIC gradient of 1830.2–1991.0 µmol L^{-1} and a pH span of 8.01–7.56. PCO_2 manipulation was conducted as in Paul et al. (2016); in short, 0.2 µm filtered seawater from the Fjord (taken at filling day) was enriched with CO_2 and the required volume added to each mesocosm to achieve the target pCO_2 concentration. The needed volume of enriched seawater was calculated using the computer program CO2SYS (Pierrot et al. 2006) for each mesocosm separately on the basis of the measured dissolved inorganic carbon (DIC) and total alkalinity (TA) (see details Figs. S3, S4). CO_2-enriched seawater was added to the mesocosms three times per week after sampling was completed. DIC samples (50 mL) were taken three times a week (Monday, Wednesdays, Fridays) by gently pressure-filtration (0.2 µm, Sarstedt Filtropur) and filled into glass vials with at least 100 mL of overflow directly out of the mesocosms. DIC was analysed by infrared detection of CO_2 by LICOR LI-7000 on an AIRCA system (MARIANDA, Kiel). TA samples were sterile filtered as for DIC but were filled into polyethylene containers (200 mL).

Zooplankton was sampled once a week by three vertical net hauls (hand-held plankton net; 64 µm mesh size, 12 cm diameter; from 150 cm depth). Although, copepod functional traits were measured once a week, this study focuses on measurements on the last day of the experiment, which corresponds to a 1–2 copepod life-cycles under the chosen temperature scenarios (Leandro et al. 2006). Each net haul sampled a volume of 5.1 L For individual measurements of RNA/DNA ratio, fatty acids, and stable isotopes, pooled copepods samples contain only adult female *Acartia* sp. To ensure that only adult *Acartia* sp. individuals were used, additional net catches were performed and only intact adults were selected under a dissecting microscope, rinsed in sterile-filtered seawater, transferred to a tin cup, initially frozen with liquid nitrogen and stored at −80 °C.

Samples for taxonomic identification and abundance estimation were fixed with alkaline Lugols' iodine. The zooplankton samples were divided with a sample splitter (HydroBios), so that ¼ of the each sample volume was counted and identified.

C/N ratios

C/N content analyses were performed on pools of 30 adult female *Acartia* sp. for each treatment. Copepods were selected individually (see above), dried overnight, and then analysed with an organic elemental analyser (FLASH 2000; Thermo Fisher Scientific GmbH, Germany).

Fatty acid content

Fatty acid analyses were performed of pools of 30 adult female *Acartia* sp. for each treatment following the protocol described in Garzke et al. (2015). Briefly, copepods were collected individually (see above) and frozen in liquid nitrogen. Fatty acid methyl esters (FAME) were obtained by lipid extraction in chloroform/dichlormethane / methanol (1:1:1 v/v/v). Prior to extraction

two internal standards, heneicosanoic acid (C21) and FAME mix (C19) were added. After extraction FAME's were analysed with a gas chromatograph (Trace GC Ultra with autosampler AS 3000; Thermo Scientific Fisher GmbH, Germany). Peaks were identified against one external standard (FAME Mix C4-C24 SUPELCO, Sigma–Aldrich, Germany), measured before and after each sample measurement session.

Stable isotope composition ($\delta^{15}N$ and $\delta^{13}C$)

Stable isotopes of C and N are often used to reconstruct food sources to gain a better understanding of trophic interactions in food webs to indicate the trophic position of the consumer. Stable isotope composition of C and N vary between species but also within species due to environmental, physiological and nutritional conditions. Stable isotope analyses were performed on pools of 30 adult *Acartia* for each treatment. Individuals were selected individually (see above), dried overnight, and then analysed with an elemental analyser system (NA 110, Thermo Fisher Scientific GmbH, Germany) connected to an isotope ratio mass spectrometer (DeltaPlus Advantage, Thermo Fisher Scientific GmbH, Germany) as described by Hansen and Sommer (2007). Stable isotope ratios were calculated following Sommer and Sommer (2005).

Resource use efficiency

The copepod resource use efficiency (RUE) was calculated as total copepod biomass (µg C L^{-1}) per unit edible phytoplankton biomass (µg C L^{-1}) [partly following Filstrup et al. (2014)]. Copepod biomass was calculated for each species from average prosome lengths as in Garzke et al. (2015). Phytoplankton bulk samples were fixed with Lugols' iodine. Phytoplankton >5 µm were considered edible for copepods (Paul et al. 2016).

Abundance and body size

Copepods of the copepodite stage 1 to adult were identified to genus and developmental stage level. Prosome lengths (length of carapace) of each genus and developmental stage were measured digitally via photographs and digital software (ZEISS AxioVision 4.8 and AxioCamMRc) with a precision to the nearest µm. The body length constancy between moults enabled clear assignment of size to a given stage. *Acartia* sp. was abundant enough to analyse prosome length changes for all treatments and combinations across all developmental stages.

RNA/DNA ratios

The ratio of ribonucleic acid (RNA) to deoxyribonucleic acid (DNA) of female *Acartia* sp. adults was determined according to Speekmann et al. (Speekmann et al. 2006b) using the cyanine base fluorescent dye RiboGreen® (Thermo Fisher Scientific). Nucleic acids were extracted from pools of five copepods of each treatment, which had been homogenized in an extraction buffer (1× TE 0.1% Triton X-100 and 0.1 mg mL^{-1} protease in RNase/DNase-free water). In short, we used a DNA standard [ranging from ~0.0 (blank) to 500 ng mL^{-1}; type I calf thymus] and RNA standard [ranging from ~0.0 (blank) to 1500 ng mL^{-1}; type III baker's yeast]. RiboGreen® reagent (100 µL) was added to the homogenized copepod samples and incubated in the dark for 5 min; initial fluorescence readings were (FL_1) taken. RNase A (25 µL; type III-A bovine pancreas) were added to the samples and one set of the blanks, which incubated for 30 min at 25 °C. RNase A was added to the RNA standards to control for complete RNA digestion and to DNA standard to ensure no fluorescence disturbance (Speekmann et al. 2006b) The final fluorescence measurements (FL_2) were done. Finally, we calculated the RNA concentration using FL_1–FL_2 (FL_1: measurement of present nucleic acids; FL_2: measurement of present DNA). DNA and RNA concentrations were calculated from standard curves, followed by calculations of RNA:DNA ratios.

Mortality

Average mortality rates were estimated for the predominant copepod taxon throughout the experiment, *Acartia* sp., assuming developmental rates at respective temperature as in Leandro et al. (2006). Mortality rates were calculated from temporal abundances changes of *Acartia* sp. for each developmental stage.

$Z = \frac{[(\ln N_0 - \ln N_t \times 100)]}{t}$, where Z is the mortality rate, N_0 is the initial number of development stage x, and N_t is the number of the developmental stage after t days (Aksnes and Ohman 1996). This approach has several potential flaws, because nauplii abundances included more taxa than *Acartia* sp. which could give a biased view of mortality rates from nauplii stages to the first copepodite stage; nauplii stages and genera could not be distinguished (Garzke et al. 2015, 2016).

Statistical analysis

Temperature and pCO_2 related differences were tested for the following response parameters: C/N ratio, fatty acid content, RUE, trophic position ($\delta^{13}C$ and $\delta^{15}N$), nauplii and copepodite abundance, stage-specific prosome length of *Acartia* sp., RNA/DNA ratio, and

temporal average mortality. All model residuals were firstly tested for normal distribution applying Shapiro–Wilk test for temperature and pCO_2 effects, and, if required, data were ln or ln(x + 1) transformed prior to re-fitting the models. Generalized linear models were used to examine the effects of seawater temperature and pCO_2, and their interactions, on abundances, fatty acids, C/N ratio, RNA/DNA ratio, RUE, and trophic position and temporal average mortality of zooplankton by the last experimental day. Heteroscedacity of the residuals was checked using conditioning plots and accounted for by including a variance component term. The full model was then optimized by stepwise removal of insignificant interactions. For selection of the best-fit model, Akaike's information criterion (AIC) was used as it takes into account both the complexity of the model and the goodness of fit.

Starting zooplankton abundance differences between temperature and pCO_2 treatments were tested with one-way ANOVAs, and stage-specific *Acartia* sp. prosome length responses to temperature and pCO_2 were tested with a three-way ANOVA. Temperature levels and developmental stage were set as categorical, and pCO_2 as a continuous explanatory variable. In cases of non-significant interactions, the ANOVA model was simplified by deleting the interaction term; followed by using Tukey honest significant differences testing to identify where differences occurred. Subsequently, the effect sizes [using Cohen's d for power analysis (Cohen 1992)] of all used model factors were calculated to identify the magnitude of the detected effects. For the interpretation of Cohen's d effect size (d), we distinguished between as small (<0.2), medium ($0.2 \leq d \leq 0.8$) and large effect (>0.8) (Cohen 1992). Statistical analyses were performed using RStudio (version 0.97.551) using the packages compute.es, car and multcomp. All statistical tests were run at significance threshold of $\alpha = 0.05$.

Results

C/N ratios

Only pCO_2 significantly affected the C/N ratio of adult *Acartia* sp. Copepods grown at higher pCO_2 had significantly higher C/N ratios than copepods grown at lower pCO_2 (Table 1; Fig. 1). Partial CO_2 pressure increased C/N ratio in adult copepods, while the increase was differently pronounced between the temperature levels. The increase of C/N with pCO_2 was stronger at lower temperature (Fig. 1).

Table 1 Generalized linear models results of C/N, fatty acids, stable isotopes, resource use efficiency (RUE), copepod abundance, RNA/DNA and mortality

Model	Estimate	Std. error	T value	P
C/N				
Intercept	1.547	0.024	63.771	**<0.001**
Temp	0.017	0.034	0.489	0.642
pCO_2	0.0003	0.0001	2.362	**0.05**
TFA (ln)				
Intercept	−0.996	0.289	−3.443	**0.008**
Temp	−0.507	0.240	−2.113	**0.049**
pCO_2	0.0003	0.0001	2.291	0.051
SFA (ln)				
Intercept	−0.120	0.219	−0.549	0.599
Temp	1.091	0.031	3.535	**0.001**
pCO_2	0.0004	0.0001	3.658	**0.008**
Temp × pCO_2	−0.0007	0.0002	−4.262	**0.004**
PUFA per TFA (ln + 1)				
Intercept	0.266	0.047	5.613	**<0.001**
Temp	−2.266	0.067	−3.387	**0.012**
pCO_2	−0.0004	0.0002	−1.561	0.162
Temp × pCO_2	0.001	0.0003	3.282	**0.013**
DHA per TFA (ln + 1)				
Intercept	0.141	0.035	4.097	**0.005**
Temp	−1.129	0.049	−2.664	**0.003**
pCO_2	−0.0003	0.0002	−1.843	0.108
Temp × pCO_2	0.0007	0.0003	2.969	**0.021**
EPA per TFA				
Intercept	0.089	0.011	8.140	**<0.001**
Temp	−7.773	0.016	−4.978	**0.002**
pCO_2	−0.0002	0.00006	−3.249	**0.015**
Temp × pCO_2	0.0003	0.00008	3.670	**0.008**
δ13 C				
Intercept	−22.530	1.129	−17.426	**<0.001**
Temp	−52.340	1.828	−0.286	0.782
pCO_2	−0.022	0.007	−3.323	**0.011**
Temp × pCO_2	−0.004	0.009	−0.401	0.699
δ15 N				
Intercept	8.213	0.445	18.441	**<0.001**
Temp	−0.663	0.358	−1.854	**0.05**
pCO_2	0.0003	0.0002	1.248	0.244
RUE				
Intercept	3.209	0.523	6.160	**<0.001**
Temp	−0.693	0.418	−1.655	0.132
pCO_2	−0.0006	0.0002	−2.557	**0.031**
Copepodites + adults abundance (ln)				
Intercept	3.958	0.240	16.461	**<0.001**
Temp	−1.210	0.340	−3.559	**0.007**
pCO_2	−0.001	0.0001	−8.482	**<0.001**
Temp × pCO_2	0.0004	0.0002	2.573	**0.033**
Nauplii abundance				
Intercept	171.6	17.11	10.028	**<0.001**

Table 1 (continued)

Model	Estimate	Std. error	T value	P
Temp	−134.4	24.20	−5.552	**<0.001**
pCO_2	−0.046	0.009	−5.185	**<0.001**
Temp×pCO_2	0.042	0.012	3.398	**<0.001**
RNA/DNA (ln+1)				
Intercept	2.977	0.059	49.887	**<0.001**
Temp	−0.692	0.084	−8.195	**<0.001**
pCO_2	−0.002	0.0003	−4.983	**0.001**
Temp×pCO_2	−0.001	0.0004	−2.622	**0.031**
Mortality				
Intercept	3.391	0.209	16.239	**<0.001**
Temp	0.411	0.168	2.450	**0.037**
pCO_2	0.001	0.001	0.938	0.372

Temperature (temp), acidification scenarios (pCO_2), significant values (bold)

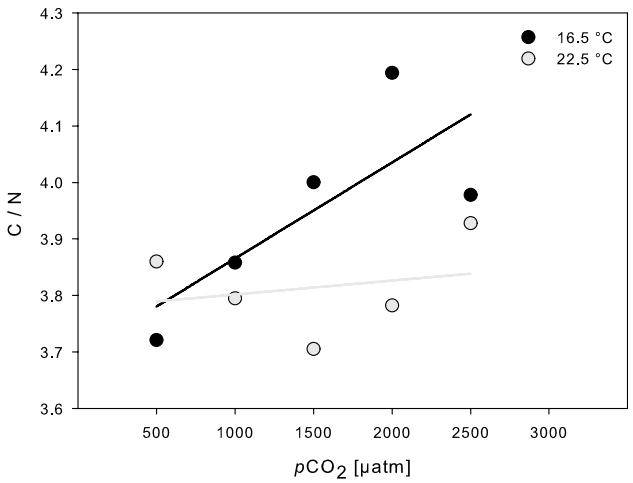

Fig. 1 Average carbon-to-nitrogen ratio (C/N) of female adult *Acartia* sp. individuals at 16.5 °C (*black*) and 22.5 °C

Fatty acid composition

The pCO_2-effect on the total amount of fatty acids (TFA) of adult *Acartia* sp. changed significantly between the temperature levels. Higher pCO_2 affected TFA content negatively under the high temperature scenario, and positively under the low temperature scenario. The regression lines intersected at ca. 1500 µatm (temp×pCO_2: d = 0.89; temp: d = 0.02, pCO_2: d = 0.08, Fig. 2a). At 16.5 °C, copepods had the highest amount of TFA at 2500 µatm, whereas in the warm treatments the lowest amount of TFA was measured at the highest pCO_2 (Fig. 2a).

The amount of saturated fatty acids (SFA) responded significantly to the interaction of temperature and pCO_2 and to each factor alone (Table 1): neither temperature nor pCO_2 drove the interaction term alone. The regression lines intersected at ca. 1500 µatm (Fig. 2b).

Adult *Acartia* polyunsaturated fatty acid (PUFA; in ng) content per ng TFA was significantly affected by the interaction of temperature and pCO_2 and temperature alone (temp×pCO_2: d = 0.12; temp: d = 0.26; pCO_2: d = 0.62, Table 1). Higher temperature decreased the amount of PUFAs, whereas higher seawater pCO_2 concentration increased the amount of PUFA for adult *Acartia* sp. (Fig. 2c). The interaction of temperature and pCO_2 was significantly driven by the impact of pCO_2 on PUFAs and the regression lines intersected at ca 2200 µatm (Fig. 2c). PUFA content increased more strongly with higher pCO_2 levels at higher temperatures, and the differences at the highest pCO_2 concentrations were less pronounced between both temperature treatments than at the lower seawater pCO_2 concentrations (Fig. 2c).

The amount of DHA (docohexaenoic acid, 20:6(n−3); in ng) per ng TFA was significantly affected by the interaction of temperature and pCO_2 and by temperature alone (Table 1), the interaction effect was mostly driven by the temperature impact on DHA (temp×pCO_2: d = 0.05; temp: d < 0.01; pCO_2: d = 0.05). Increasing seawater pCO_2 concentration increased the amount of DHA of adult *Acartia* sp. individuals (Fig. 2d). Higher temperature significantly decreased the relative amount of DHA, under low $pCO2$ (<1000 µatm), but increased it under high (>2100 µatm) pCO_2 (Fig. 2d); increasing pCO_2 concentrations imposed opposite effects on zooplankton DHA content under ambient *versus* under warming-predicted temperatures, and pCO_2 effects were more pronounced for adults *Acartia* when these grew under higher temperatures (Fig. 2d). The regression lines intersected at 2100 µatm; (Fig. 2d).

The amount of EPA (eicosapentaenoic acid; 20:5(n−3); in ng) was significantly affected by the interaction of temperature and pCO_2 and their single effects (Table 1). EPA concentration was significantly lower at higher temperature under pCO_2 concentrations <1500 µatm. EPA also decreased under ambient temperature with increasing pCO_2 and increased in the warming scenario over the lower range of the tested acidification scale. The combined effects of higher temperature and higher pCO_2 concentration on EPA concentration were stronger compared to those on EPA concentrations in copepods grown at lower temperatures (temp×pCO_2: d = 0.02; temp: d = 0.87; pCO_2: d = 0.11, Fig. 2e). The temperature effect on EPA concentration was stronger than the enhancing effect of pCO_2 (Fig. 2e).

Stable isotope ratios

Delta^{13}C was significantly affected by pCO_2 concentration, while neither temperature nor the interaction of temperature and pCO_2 significantly affected the δ^{13}C values

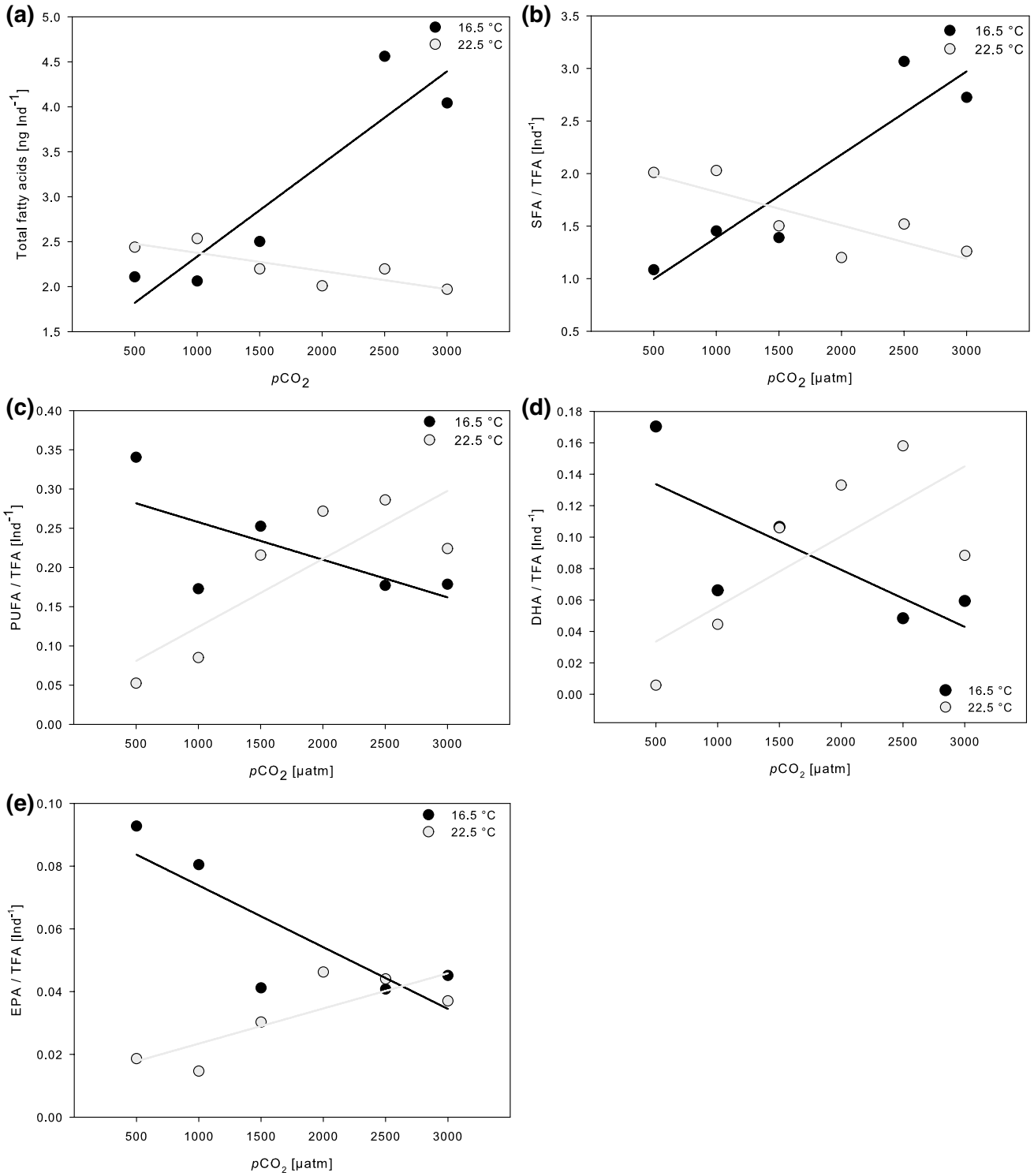

Fig. 2 Fatty acid composition of adult *Acartia* sp. of the last experimental day. **a** Total fatty acids, **b** saturated fatty acids, **c** polyunsaturated fatty acids, **d** DHA and **e** EPA

of adult *Acartia* sp. (Table 1). The single factor analysis showed that pCO_2 mainly affected the $\delta^{13}C$ signature of adult *Acartia* (pCO_2: d = 0.92, temp: d = 0.08). At both temperature treatments, the stable isotope ratio of $\delta^{13}C$ was lower at 2500 and 3000 µatm than at lower pCO_2 concentrations (Fig. 3a). Interestingly, $\delta^{13}C$ values are more variable at the cold temperature compared against the $\delta^{13}C$ values in the warm treatments (Fig. 3a).

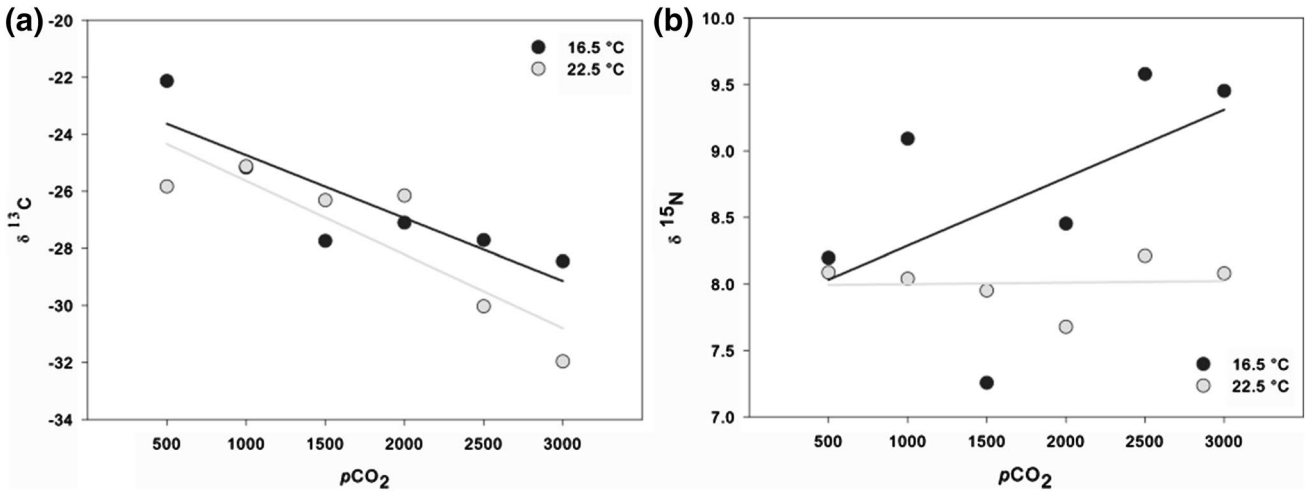

Fig. 3 Stable isotope composition of adult *Acartia* sp. of the last experimental day. **a** $\delta^{13}C$ and **b** $\delta^{15}N$

Delta^{15}N was affected by temperature, but not by pCO_2 nor by the interaction of both (Table 1) (temp: d = 0.54; pCO_2: d = 0.24). We could observe that $\delta^{15}N$ variation was smaller at the warm temperature, whereas in individuals grown at the colder temperature $\delta^{15}N$ was more variable (7.2–9.2‰) (Fig. 3b). Temperature strongly affected values of $\delta^{15}N$, but additionally, we observed that the $\delta^{15}N$ stable isotope signature at the 2500 and 3000 µatm treatments was generally higher compared to the lower pCO_2 (Fig. 3b).

Resource use efficiency

Zooplankton resource use efficiencies were significantly and mainly affected by pCO_2 but not by temperature or the interaction of temperature and pCO_2 (Table 1), while pCO_2 amongst these parameters prevailed a major effect on RUE (temp × pCO_2: d = 0.14, temp: d = 0.24; pCO_2: d = 0.62). After simplifying the model by deleting the interaction, pCO_2 significantly affected the RUE with a medium effect size, while temperature only showed a smaller, and non-significant, effect(temp: d = 0.28; pCO_2: d = 0.72). Increasing pCO_2 concentrations decreased zooplankton RUE significantly (Fig. 4c). The efficiencies of zooplankton biomass that were produced from available edible phytoplankton biomass at the highest pCO_2 concentrations (2500 and 3000 µatm) were similar at both tested temperatures (Fig. 4c).

Abundance

Starting nauplii abundance of the experiment were not significantly different between temperature [16.5 °C: 46.2 (±2.02 SD); 22.5 °C: 43.51 (±4.50 SD)] and pCO_2 treatments [ambient: 42.73 (±5.37 SD); 1000 µatm: 46.80 (±1.32 SD); 1500 µatm: 47.07 (±2.83 SD); 2000 µatm: 45.73 (±2.45 SD); 2500 µatm: 40.40 (±5.09 SD); 3000 µatm: 46.40 (±0.75 SD); Table S1], and starting copepod abundance (stages C1-adult) also did not differ significantly between temperature [16.5 °C: 38.24 (±12.08 SD); 22.5 °C: 59.37 (±22.44 SD)] and pCO_2 treatments [ambient: 33.13 (±6.32 SD); 1000 µatm: 32.13 (±8.48 SD); 1500 µatm: 75.60 (±25.84 SD); 2000 µatm: 48.00 (±1.13 SD); 2500 µatm: 53.87 (±31.38 SD); 3000 µatm: 50.13 (±16.22 SD); Table S1].

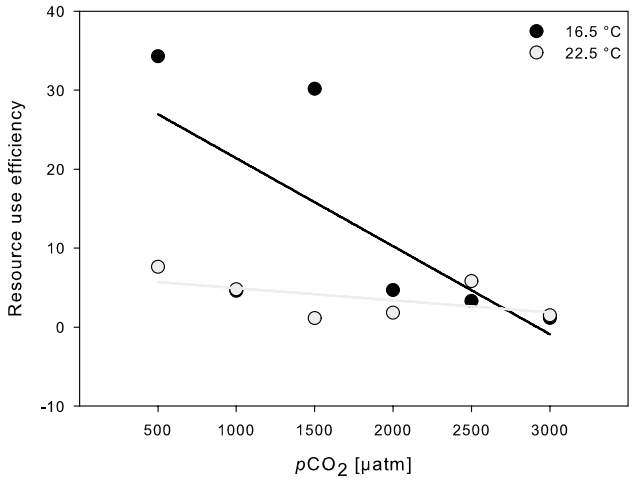

Fig. 4 Resource use efficiency (RUE) of adult *Acartia* sp. of the last experimental day at 16.6 °C (*black*) and 22.5 °C (*grey*)

Nauplii abundance on the last experimental day was affected significantly by the interaction of temperature and pCO_2 (Table 1). Temperature and pCO_2 affected nauplii abundance differently: nauplii abundance in the high temperature decreased with increasing pCO_2 throughout the

tested acidification range, while in the cold treatments, nauplii abundances increased with increasing pCO_2 at medium acidification scenarios, with a peak abundance between 1500 and 2000 µatm, followed by a decrease under stronger acidification (Fig. 5a). Nauplii abundance was significantly higher in the warm treatments and decreased with increasing pCO_2 (Table 1; Fig. 5a).

Final copepodite abundances (including copepodite stages C1 to adult) were significantly affected by the interaction of temperature and pCO_2 and their single effects (Table 1), while pCO_2 amongst these parameters exerted a major effect in copepodite abundance (temp × pCO_2: d = 0.06; temp: d = 0.08; pCO_2: d = 0.86). The highest copepod abundance occurred in the low temperature and ambient pCO_2 treatment, and the lowest abundance under low temperature and highest pCO_2 (Fig. 5a). The regression lines intersected at ca 3000 µatm (Fig. 1a). Higher temperature as well as increasing pCO_2 concentration significantly reduced copepodite abundance, but temperature alone had a small effect compared to the effect of pCO_2 (Fig. 5a). The interaction of temperature and pCO_2 was mainly driven by the strong effect of pCO_2 on copepodite abundance (temp: d = 0.08; pCO_2: d = 0.92). pCO_2 only had an effect under ambient temperature though higher temperature mainly affected copepodite abundance (Fig. 5a).

Body size

Mean stage-specific prosome length responded significantly to temperature, pCO_2, and their interaction (temp: d = 0.21; temp × pCO_2: d = 0.03; temp × stage: d = 0.09; pCO_2 × stage temp × pCO_2 × stage: d = 0.06, Table 2). Generally, higher temperature and higher pCO_2 significantly affected prosome lengths of copepods. Stage dependent effects of warming and acidification showed different effects on prosome lengths (Table 2), only a negative effect on prosome lengths in older copepodite stages (C4-adult) was detected and not for younger stages (Fig. 5a), which shows the significant interaction effect of warming, acidification and developmental stage (Table 2). At older stages, warming and higher pCO_2 significantly decreased the mean prosome length (Table 2; Fig. 5b). The interaction of temperature and pCO_2, without differentiation between the effects on older and younger stages, was mainly driven by the strong negative impact of temperature on stage-specific prosome

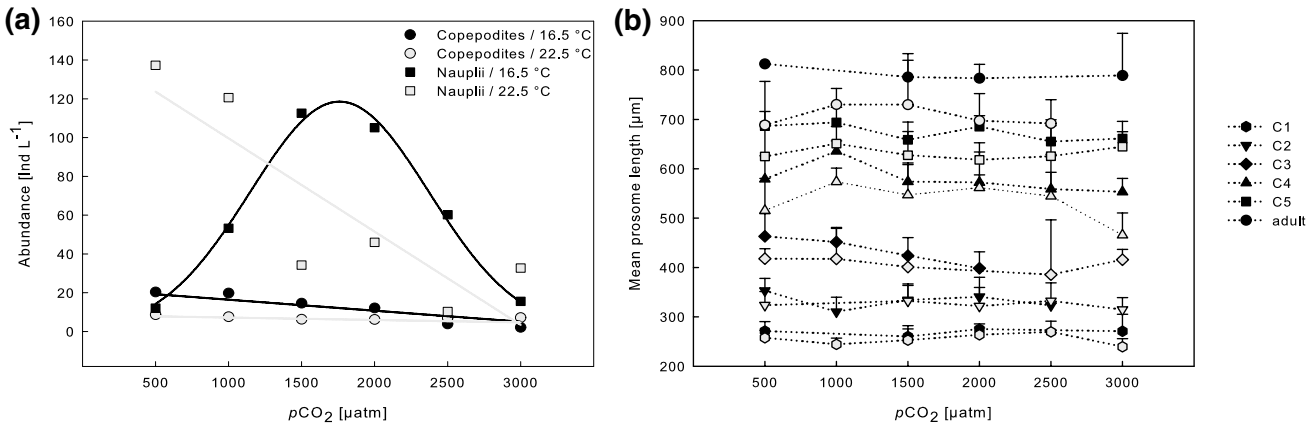

Fig. 5 **a** Abundance of nauplii (*squares*) and copepodites (C1-adult, *circles*) and **b** mean prosome lengths (±SD) of all occurring developmental stages of *Acartia* sp. at 16.5 °C (*black*) and 22.5 °C (*grey*) of the last experimental day

Table 2 Three-way analysis of variance (ANOVA) for *Acartia* sp. prosome length

	df	Sum Sq.	Mean Sq.	F value	P
Temp	1	383,900	383,900	185.802	**<0.001**
pCO_2	1	136,373	136,373	66.003	**<0.001**
Stage	5	1,428,733	2,857,468	1382.970	**<0.001**
Temp × pCO_2	1	18,673	18,673	9.038	**0.003**
Temp × stage	5	57,790	11,558	5.594	**<0.001**
pCO_2 × stage	5	7129	1426	0.690	0.631
Temp × pCO_2 × stage	5	39,745	7949	3.847	**0.002**
Residuals	577	1,192,187	2066		

Temperature (temp), acidification scenarios (pCO_2), significant values (bold)

length (temp: d = 0.74; pCO_2: d = 0.26). Increasing pCO_2 concentrations seem to have a stronger positive effect at higher temperatures than at lower temperatures. Especially prosome lengths at intermediate pCO_2 concentrations are similar between both experimental temperatures within the earlier developmental stages (Fig. 5b).

RNA/DNA ratios

For adult *Acartia* sp., we found clear effects of temperature, pCO_2, and their interaction on the RNA/DNA ratio (Table 1). RNA/DNA ratio differences were majorly driven by the impact of temperature on RNA/DNA ratio (temp × pCO_2: d = 0.06, temp: d = 0.70, pCO_2: d = 0.23, Fig. 6a). Individuals from warm treatments had significantly lower RNA/DNA ratios compared to individuals from cold treatments (Fig. 6a). The RNA/DNA ratio also significantly decreased with higher seawater pCO_2 (Fig. 6a). Higher pCO_2 concentrations had larger impacts on the RNA/DNA ratio of adult *Acartia*, which grew at cold temperatures than on those exposed to warmer temperatures.

Mortality

The calculated temporal average mortality rates were significantly affected by temperature, but neither by pCO_2 nor by the interaction of both factors (Table 1; Fig. 6b). Higher temperature majorly affected the average daily mortality and increased it significantly, while higher pCO_2 concentrations tended to decrease daily mortality at warmer temperature (temp: d = 0.87; pCO_2: d = 0.1, Fig. 6b).

Discussion

These results suggest that ocean acidification and global warming affected zooplankton ecology, in terms of abundance, body size, fitness (RNA/ DNA and mortality) by changed metabolic and physiological traits, in terms of condition and nutritional content, with typically stronger temperature than acidification effects. Particularly under warming conditions, OA effects on many zooplankton traits were largely overridden by temperature effects, while they prevailed in enhancing temperature effects on resource-use-efficiency and trophic position (Fig. 7). Significant responses to ocean acidification were detected particularly at the lower temperatures. Therefore, these experimental results indicate that OA effects on zooplankton communities in temperate marine planktonic ecosystems may be expected to be strongly mediated and in parts overridden by warming (Fig. 7).

The mechanisms behind responses to OA of individuals under warming are not completely understood yet, but can reflect changes in the metabolism and energetic costs to maintain basic functions for survival (Ikeda et al. 2001; Garzke et al. 2016). Organisms have species-specific thermal windows that might be narrowed by the simultaneous occurrence of acidification (Pörtner and Farrell 2008; Whiteley 2011) and may further incur metabolic stress (Vehmaa et al. 2012; Zervoudaki et al. 2014; Thor and Dupont 2015). This may result in increased energetic costs to persist through environmental changes and in altered metabolic allocation to accommodate the increased

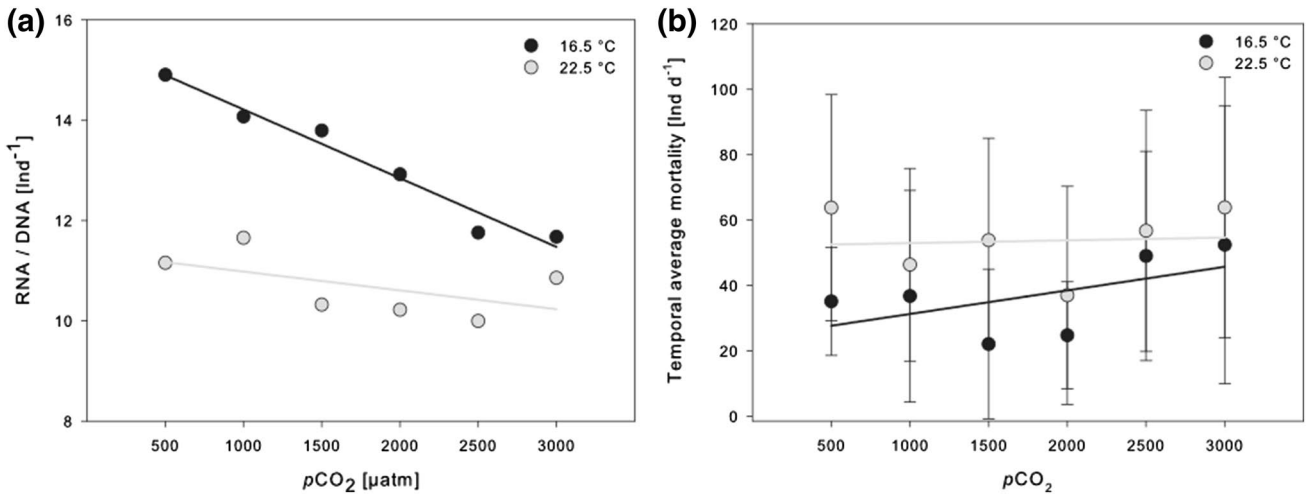

Fig. 6 **a** RNA/DNA per adult *Acartia* sp. of the last experimental day, **b** average temporal mortality of *Acartia* sp. (±SD) at 16.5 °C (*black*) and 22.5 °C (*grey*)

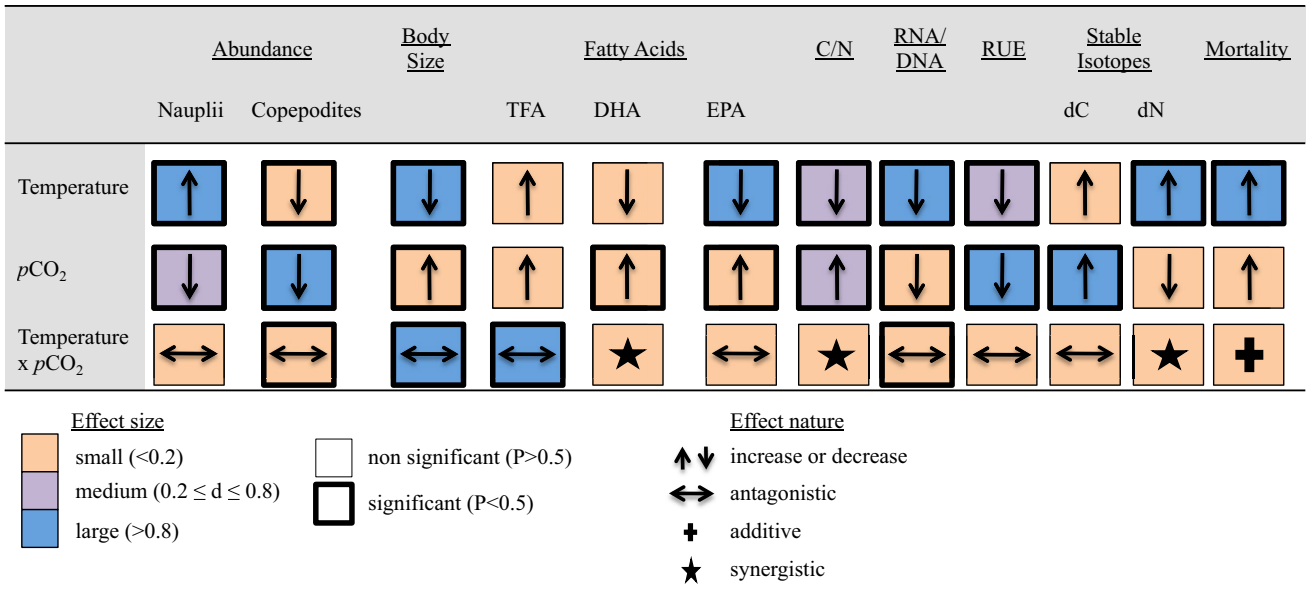

Fig. 7 Summary of temperature, pCO_2 and interaction responses with measured nature of response and effect size [depiction of effects modified after (Bi et al. 2016)]

energetic expenses (Brown et al. 2004; Zervoudaki et al. 2014). Pörtner et al. (2004) and Zervoudaki et al. (2014) stated that such metabolic imbalances might decrease lifetime fitness due to re-allocation of energy. It is discussed that direct effects of ocean acidification on marine organisms can be explained by the decreased external cell pH which leads to an additional energy demands to maintain the homeostasis between cell external and internal pH and by decreased protein synthesis which is a signal for a suppression of metabolic activity (Kurihara et al. 2004; Kurihara and Ishimatsu 2008; Garzke et al. 2016). In line with these patterns, warming and OA can decrease copepodite abundances, with minor interactions between both parameters as suggested from our mesozooplankton study community (Fig. 7). Besides direct effects of OA and warming, indirect effects by food limitation and changed food quality by altered nutritional balance can lead to metabolic imbalances and reduced fitness in copepods (Mayor et al. 2015; Cripps et al. 2016). As our pCO_2 manipulation involved both C addition through DIC pool and direct acidification effects, we recognize the dual nature of direct and indirect pathways by which pCO_2 can impact on zooplankton metabolism and physiology. Especially the indirect effect of food quality is one important factor for copepods and can have a wide range of effects on copepod fitness. Studies have shown that changed C:nutrient ratios in zooplankton prey, caused by ocean acidification (e.g. Bellerby et al. 2008), can lead to altered activity (Plath and Boersma 2001), respiration (Darchambeau et al. 2003; Malzahn et al. 2010; Schoo et al. 2013), digestion efficiency (DeMott and Tessier 2002), growth and development (Boersma 2000; Malzahn et al. 2007), RNA synthesis (Malzahn and Boersma 2012) and fatty acid content (Malzahn et al. 2007, 2010). Contrary to other experiments, e.g., Urabe et al. (2003) and Schoo et al. (2013), the nutritional composition, in terms of C:N, C:P and N:P, was not significantly affected by temperature or pCO_2 over the course of the experiment and phytoplankton stoichiometric ratios were near to Redfield Ratio (Paul et al. 2016). During the present experimental study, temperature-related changes in edible food biomass availability were observed; edible food biomass declined earlier at higher temperature (day 3) compared to the lower temperature treatment (day 9, Fig. S5). Paul et al. (2016) observed differences in edible food biomass availability for copepods between the treatments, generally higher temperature lead to a declined biomass of food sources through enhanced top-down effect by grazing; OA effects were observed only at lower temperatures with bottom-up control.

Mayor et al. (2015) also found that short periods of low food availability failed to meet the metabolic demands of copepods and can affect various aspects of lipid and protein metabolism. Global warming is thought to cause a trophic mismatch between copepods and their prey (Edwards and Richardson 2004; Richardson 2004). Direct and/or indirect effects on copepods by OA and warming during the present study are well in line with predictions and observations in natural ecosystems.

Contradictory to the above predictions, nauplii abundances were primarily temperature-dependent in our experiment, and significantly increased under the warming scenario (Fig. 7). Population success is often measured

in fecundity or reproduction rates. The results thus show opposite effects of warming and OA on the abundance of nauplii and more advanced copepod stages, which is in line with observations of other studies that found that earlier developmental stages are differentially affected by temperature and OA (Dupont and Thorndyke 2009; Mayor et al. 2015; Przeslawski et al. 2015). The observed nauplii responses (see Fig. 7) might result from lower (1) reproduction rates, (2) egg production, (3) hatching success and (4) survival. We measured the abundance of the copepod nauplii stages of the last experimental day, which represents the reproduction success of the f1- and f2-generation after 28 experimental days, respectively (Fig. S6). Even if this study did not investigate reproduction rates, egg production, and hatching success in detail, previous studies have shown nauplii responses to warming and acidification in single and crossed experimental set-ups (Holste and Peck 2005; Vehmaa et al. 2012, 2015; Hildebrandt et al. 2014; Zervoudaki et al. 2014; Cripps et al. 2014). While higher temperatures increase egg production and hatching rates (Holste and Peck 2005; Garzke et al. 2016), elevated pCO_2 decreased nauplii production by a higher number of (1) unfertilized oocytes, (2) non-viable-fecund eggs, and (3) viable fecund eggs in a quiescent state [see Cripps et al. (2014) and references within]. Different responses of nauplii versus copepodites might occur because the early nauplii stages use endogenous yolk and copepodites exogenous food as energy sources, therefore nauplii can be considered as a bottleneck in copepod population dynamics (Eiane and Ohman 2004; Lennartz et al. 2014). The present study further highlights the necessity to analyse functional traits life-stage specifically when assessing the impacts of environmental change.

Growth is often used as a fitness parameter beside fecundity and reproduction success; in this study, we used stage-specific prosome length as growth parameter. Decreasing body size is suggested to be the third universal response to warming (Daufresne et al. 2009) and was observed in experimental (Garzke et al. 2015) and field studies (Rice et al. 2014). The acidification effect on copepod body size is less investigated and only a few studies found that stage-specific prosome length was mainly impacted by temperature and less by acidification (Pedersen et al. 2013, 2014; Garzke et al. 2016). One explanation for lower negative impacts by acidification might be that phytoplankton biomass (as food source for copepods) was positively affected by acidification and resulting in a reduced negative temperature effect on copepods (Garzke et al. 2016). The ability of copepods to invest in their offspring, and develop and grow to maturity depends on their own condition, and on quality and quantity of their food. Nutrient limitation of the food sources can be excluded as a reason for the observed responses to temperature and OA, as nutrients were not limiting throughout the experimental phase (Paul et al. 2016).

Nutritional composition such as C/N ratios and fatty acids are important for individual growth and fitness which is directly translated into population dynamics (Müller-Navarra 2008; Evjemo et al. 2008). C/N ratios give insights into the ratio of lipids (structural lipids i.e., membrane lipids) to protein content (energy storage). Carbon is incorporated into lipids whereas nitrogen is mostly incorporated into proteins (Sterner and Elser 2002). These results indicate that higher temperatures lead to less stored lipid per unit protein as energy source. Decreased C/N indicated that adults relied on internal lipid reserves fueling basic metabolism (Hirche and Niehoff 1996; Hildebrandt et al. 2014). Additionally, single fatty acids species are differently involved in lipid and protein storage, SFAs serve as energy storage, usually seen in cold water species, and PUFAs play an important role in membranes. Copepods at higher temperature and higher pCO_2 had a higher lipid content compared to high temperature and low pCO_2 acclimated copepods, where the stored lipids had the structure of PUFAS. PUFAs, especially the essential fatty acids DHA and EPA (Brett and Müller-Navarra 1997), are important for growth and reproduction to fuel the faster metabolism (Hildebrandt et al. 2014), enhanced growth and higher reproduction rates but also higher mortalities (Hildebrandt et al. 2014).

We used the ratio of RNA and DNA as a proxy of fitness; a lower amount of RNA reflects lower transcriptomic activity, or protein synthesis, and a higher amount of DNA to established growth and survival. OA had a minor effect on copepod body condition in the warmer treatments while the effect was stronger in the cold treatments. In the higher temperature treatment, a constant low RNA/DNA ratio was measured suggesting that organisms need to maintain their basal metabolism for survival and somatic growth. A low RNA/DNA ratio is an indicator of poor nutrition, which is usually transferred into low egg production and fecundity (Speekmann et al. 2006a), as somatic growth has ceased in adult copepods. OA might suppress metabolic activity (RNA transcription) through decreased protein synthesis and consequently reduces the fitness of organisms and suppresses their reproductive output (Kurihara 2008; De Wit et al. 2015). Thus, De Wit et al. (2015) deduced that OA selects copepods according to their RNA synthesis and RNA translation performance. During the early phase of the experiment, copepods exerted a stronger top-down control within the warming scenario causing depleted food sources and starvation, whereas the system was bottom-up controlled within the cold scenario (Paul et al. 2016). Both effects are reflected in the condition measures of copepods in our study, with a predominant warming impact, and a less-than-additive interactive influence of RNA/DNA decrease with rising pCO_2 levels.

The measured resource use efficiency (RUE) was used to approximate trophic transport efficiency from primary producers to consumers (Filstrup et al. 2014). Zooplankton biomass production per unit edible phytoplankton biomass was lower at the higher temperature compared to RUE under low temperature meaning that copepods at higher temperature were not able to build up biomass and subsequently might have used the food sources for maintaining their metabolism for survival. In contrast, more food biomass was efficiently transferred to copepods at colder temperatures.

In additional to the effects on individual fitness and the effects on population dynamics, we were able to observe effects of temperature and OA interaction on the stable isotope composition of copepods within this experimental food web. Even if it was not clearly significant, $\delta^{15}N$ signature increased with pCO_2 and was higher at lower temperature whereas warming generally decreased $\delta^{15}N$ without any OA effect. We speculate that due to more intensified grazing under warming during the beginning of the experiment less edible food biomass was available for copepods in the end of the experiment (Paul et al. 2016), whereas in the lower temperature treatment and with increasing pCO_2 more phyto- and microzooplankton biomass was available during the whole experiment (Paul et al. (2016) and H. G. Horn personal comm.). Consequently, low-temperature acclimated copepods could feed on phyto- and microzooplankton during the end of the experiment (higher enrichment of $\delta^{15}N$), than warm-acclimated copepods that were food-limited with a food source mainly consisting of phytoplankton (Paul et al. 2016 and H. G. Horn personal comm.). Food sources at lower temperature experienced less grazing pressure, while at higher pCO_2 stronger grazing prevailed on microzooplankton or other sources, maintaining and increasing the lipid storage (higher C/N ratio) and essential fatty acid contents in zooplankton. The copepod species *Acartia tons*a, as an omnivore, shows selective feeding behavior between phyto- and microzooplankton species depending on their nutritional needs (Boersma et al. 2016). Our study cannot distinguish if feeding strategy changed between OA and warming treatments, as this would have required high-frequential copepod behavioral sampling and regular taxonomically distinguished prey-abundance sampling, which would necessitate further specifically tailored study. This study therefore cannot distinguish if copepods changed feeding preferences between phyto- and microzooplankton and if this contributed to the observed changes in isotope signature changes. Cripps et al. (2016) assessed chronic effects of direct and indirect OA effects on vital rates and quality (chemical and biochemical stoichiometry) of *Acartia tonsa* and found that OA alters the bottom-up effect by altered phytoplankton quality and led to changes in population structure, a decrease in recruitment by 30% and enhanced respiration rates (>twofold).

We conclude from our results that ecological response parameters of zooplankton communities to warming and acidification are co-occurrent with, and likely mediated by, chemically caused physiological changes to the organisms. Ocean acidification effects seem to affect communities by altered physiological status at lower temperatures whereas warming causes an overall impact on individual physiology and ecology. The potential impacts on marine organism and their ability to adapt or evolve, will determine future marine biodiversity and ecosystem functioning (Fitzer et al. 2012; Cripps et al. 2014; Thor and Dupont 2015). As copepods constitute a major link between lower and higher trophic levels, their reproductive output and the nutritional value can have ecological consequences on the whole ecosystem, including fish species of economic interest (Möllmann et al. 2005). Ocean warming and acidification alone are only one of a suit of anthropogenic perturbations taking place simultaneously in ecological systems (Przeslawski et al. 2015). This uncertainty about the consequences for marine food webs and community structures, and how they are mediated by individual fitness changes, highlights the further need for multi-factorial studies.

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