Biogeosciences, 13, 1037–1048, 2016
www.biogeosciences.net/13/1037/2016/
doi:10.5194/bg-13-1037-2016

Biogeosciences Open Access EGU

Negligible effects of ocean acidification on *Eurytemora affinis* (Copepoda) offspring production

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Received: 2 October 2015 – Published in Biogeosciences Discuss.: 23 October 2015
Revised: 10 February 2016 – Accepted: 10 February 2016 – Published: 23 February 2016

Abstract. Ocean acidification is caused by increasing amounts of carbon dioxide dissolving in the oceans leading to lower seawater pH. We studied the effects of lowered pH on the calanoid copepod *Eurytemora affinis* during a mesocosm experiment conducted in a coastal area of the Baltic Sea. We measured copepod reproductive success as a function of pH, chlorophyll *a* concentration, diatom and dinoflagellate biomass, carbon to nitrogen (C : N) ratio of suspended particulate organic matter, as well as copepod fatty acid composition. The laboratory-based experiment was repeated four times during 4 consecutive weeks, with water and copepods sampled from pelagic mesocosms enriched with different CO_2 concentrations. In addition, oxygen radical absorbance capacity (ORAC) of animals from the mesocosms was measured weekly to test whether the copepod's defence against oxidative stress was affected by pH. We found no effect of pH on offspring production. Phytoplankton biomass, as indicated by chlorophyll *a* concentration and dinoflagellate biomass, had a positive effect. The concentration of polyunsaturated fatty acids in the females was reflected in the eggs and had a positive effect on offspring production, whereas monounsaturated fatty acids of the females were reflected in their eggs but had no significant effect. ORAC was not affected by pH. From these experiments we conclude that *E. affinis* seems robust against direct exposure to ocean acidification on a physiological level, for the variables covered in the study. *E. affinis* may not have faced acute pH stress in the treatments as the species naturally face large pH fluctuations.

1 Introduction

The concentration of carbon dioxide (CO_2) in the atmosphere is rising at a ten times faster rate than during the past 55 million years. The oceans absorb CO_2 from the atmosphere leading to lower seawater pH and a reduction in carbonate concentration. Since pre-industrial times the ocean acidity has increased by 28 % (IPCC, 2013). The fast increase in CO_2 and change in seawater chemistry will have adverse effects on many marine species and ecosystems (Fabry et al., 2008; Kroeker et al., 2010). Due to lower buffering capacity of brackish water, the Baltic Sea is especially sensitive to elevated CO_2 (Havenhand, 2012). Modelling suggests a decrease of 0.26–0.40 pH units for the Baltic Sea by the year 2100 (BACC II, 2015). In addition, high CO_2 levels interact with other climate change related factors that may have

negative effects on marine organisms (Kroeker et al., 2013; Talmage and Gobler, 2012). Especially the coastal zones are under heavy pressure from anthropogenically driven ocean acidification due to eutrophication and oxygen minimum zones (Fabry et al., 2008; Melzner et al., 2013; Wallace et al., 2014).

Copepods are the most abundant zooplankton in the oceans. They constitute major parts of the diet of juvenile fish, and are hence an important part of the food web. Lowered pH may disturb the acid-base balance, thereby altering the reproduction, hatching, and development (Kurihara et al., 2004; Mayor et al., 2007; Weydmann et al., 2012). Besides the direct effects of acidification, rising CO_2 can adversely affect consumers and food webs due to changed nutritional value of prey (Rossoll et al., 2012). Polyunsaturated fatty acids (PUFA) are essential metabolites for copepods and need to be obtained from the diet. Certain PUFA have specific roles in central processes of copepod reproduction including egg production (20:5ω3 EPA), egg hatching (22:6ω3 DHA), and development (18:3ω3 and 18:5ω3) (Jónasdóttir et al., 2009). Important ω3 fatty acids decreased significantly in the diatom *Thalassiosira pseudonana* grown at high CO_2 with lower levels of PUFA with following decreased egg production in the copepod *Acartia tonsa* (Rossoll et al., 2012). Further, CO_2-related changes in the fatty acid composition and content of several primary producers have been reported (Bermúdez et al., 2016, and references therein). Furthermore, ocean-acidification-induced changes in phytoplankton species composition can have an indirect effect on food quantity and quality for heterotrophic consumers. Elevated CO_2 levels can increase C : N ratios of primary producers, which alter their nutritional value and can adversely affect the growth and reproduction of copepods (Schoo et al., 2013).

Ocean acidification can induce oxidative stress in marine organisms (Tomanek et al., 2011; Kaniewska et al., 2012). Hence, biochemical responses to low pH conditions, such as changed activity of antioxidants and enzymes may show higher sensitivity than, for example, survival and reproduction (Gorokhova et al., 2010; Zhang et al., 2012). An enhanced antioxidant defence in response to increased reactive oxygen species (ROS) concentration may occur at the expense of reduced investment in other metabolic processes, such as growth and reproduction. The defence capacity against oxidative stress can be assessed by measuring the capacity to quench ROS (see review by Monaghan et al., 2009).

E. affinis is a common copepod in the Baltic Sea and dominates the zooplankton community together with *Acartia bifilosa* in the study area during summer. *E. affinis* is an egg-bearing copepod that produces subitaneous eggs during summer and diapause eggs in autumn. The copepods recruit from small overwintering populations, and by hatching from the sediment (Katajisto et al., 1998). Previous studies on the effects of ocean acidification on *A. bifilosa* from the Baltic Sea have shown adverse effects in combination with warming (Vehmaa et al., 2012a, 2013). The increase in egg production with warmer temperature was lower when copepods were simultaneously exposed to warmer temperature and lowered pH (Vehmaa et al., 2012a).

The main objectives of this study were to examine effects of ocean acidification on reproductive success and antioxidant defence of the copepod *E. affinis*, as well as measuring the effects of food quality and quantity on offspring production. We studied how lowered pH, phytoplankton biomass (indicated as chlorophyll *a*), biomass of diatoms and dinoflagellates and the C : N ratio of particulate organic matter (POM) affect the offspring, i.e., nauplii production in *E. affinis*. In addition, we looked at the effect of pH on essential fatty acids of incubated egg-bearing females to reveal indirect effects via the food. We also tested whether the fatty acid levels of the females were reflected in their eggs under a range of fCO_2 values representative for the future ocean (IPCC, 2013).

2 Material and methods

2.1 Experimental set-up

The study was conducted using KOSMOS mesocosms (Riebesell et al., 2013) within the framework of the SOPRAN project (Paul et al., 2015). The mesocosms were located at Storfjärden, an offshore pelagic area in the vicinity of Tvärminne Zoological Station (University of Helsinki) Baltic Sea (59°51′20″ N, 23°15′42″ E) from the beginning of June until the middle of August, 2012. Storfjärden has a maximum depth of 34 m. The water is brackish with a mean salinity of 6. The area receives inflow of freshwater from the river Svartån, and periodical inflows of cold water from the open Baltic Sea with higher salinity (Niemi, 1976). Six mesocosms, consisting of 17 m deep bags made of thermoplastic urethane, each enclosing $\sim 55\,m^3$, were moored on site on June 12. The mesocosms were covered by a net (mesh size 3 mm) at the top and the bottom during filling and left open for 4 days before the net was removed and the top was pulled up 1.5 m above the water surface and closed at the bottom (see Riebesell et al., 2013 and Paul et al., 2015 for details on the experimental design) to enclose the natural plankton community. The water column was mixed at the beginning of the experiment in order to avoid a salinity stratification. Four of the mesocosms were stepwise manipulated with CO_2-enriched seawater, during 3 consecutive days. Two bags were untreated and used as controls. Due to outgassing, CO_2 was also added on day 15 of the experiment to the upper 7 m of the high CO_2 mesocosms to maintain the treatment levels. No nutrients were added. The average fCO_2 levels during the period of our incubation experiments ($t1 - t30$) were 346, 348, 494, 868, 1075, and 1333 µatm (Paul et al., 2015).

2.2 Sampling and incubations

Our copepod experiment was conducted during a 4-week period with weekly incubations. We sampled water and copepods from the mesocosms on days $t3$, $t10$, $t17$ and $t24$ ($t0$ being the day of first addition of CO_2 into the bags). Zooplankton was sampled with a 300 µm net (∅ 17 cm) from 17 m depth to the surface from all mesocosms and transferred to containers pre-filled with 4 L of seawater from a depth of 9 m from the respective mesocosm. On the same day, unfiltered water samples were taken from each mesocosm with depth-integrated water samplers (IWS, HYDRO-BIOS, Kiel) which take equal amount of seawater from every depth (0–17 m). In order to minimize handling of the restricted water available, to keep food conditions as similar to in situ conditions as possible, and to avoid gas exchange, the water was directly transferred into airtight 1.2 L Duran bottles for incubations. Water samples and zooplankton were transported to a light- and temperature-controlled room at Tvärminne Zoological Station. Egg-bearing females of *E. affinis* ($n = 10$ per treatment) were incubated in the 1.2 L Duran glass bottles which contained mesocosm water. Temperature and pH were measured before adding the copepods to the bottles. Bottles were filled up and sealed without airspace, ensuring no air bubbles were present, to prevent CO_2 outgassing. The bottles were slowly inverted after sealing and incubated in a 16:8 h light-dark cycle at in situ temperature, as an attempt to match the natural environment. A light source was installed above the incubation bottles, yielding 7 µmol m^{-2} s^{-1} (LI-COR LI-1000). All pH and temperature measurements were conducted with an Ecosense pH10 pH/temperature Pen directly from the bottles before closing and directly after opening (Table 1). The pen was calibrated with standard buffer solutions (Centipur, Titripac pH 4.00, 7.00 and 10.00) every second day. The bottles were inverted three times a day and their location on the shelf was randomly changed.

Each incubation lasted 4 days. Copepods and nauplii were gently filtered once daily onto a 250 and 30 µm mesh, respectively. The status of the adult copepods was checked under a dissecting microscope by submerging the sieve in a petri dish filled with water from respective mesocosm, before returning the copepods to bottles containing new unfiltered seawater sampled the same day from respective mesocosm. The nauplii were preserved in acid Lugol's solution and counted under a dissecting microscope (Nikon SMZ800, 25 × magnification). As we could not follow individual copepods, we counted the nauplii produced daily, and the number of live females in the incubation bottles (survival > 95 %) when filtering out the nauplii. Only first stage nauplii of *E. affinis* were included in the analyses. The number of nauplii produced per female was calculated from the daily nauplius count divided by the number of females in the bottles. The bottles with new water was temperature-adjusted in the climate chamber before transferring the copepods. When changing the water we checked for oxygen depletion every second day with a hand-held oxygen probe (YSI Environmental ProODO) in the old water used in the incubation bottles.

At the end of each weekly incubation ($t7$, $t14$, $t21$, $t28$) the copepods were counted and checked for eggs and survival. Egg sacs were cut off from incubated egg-bearing females, with a thin needle and transferred to pre-weighted tin cups. The females were then stored separately. The samples were frozen in an ultra-freezer (−80 °C) until fatty acids were measured by gas chromatography as fatty acid methyl esters (FAMEs) following instructions in Klein Breteler et al. (1999). Fatty acids were separated into three groups that were used in the analyses; polyunsaturated (PUFA), monounsaturated (MUFA), and saturated fatty acids (SAFA) and were expressed as ng mg dry $weight^{-1}$.

With each start of the weekly, sub-experiments, female *E. affinis* with egg sacs were picked from the mesocosms for analyses of oxygen radical absorbance capacity (ORAC). The animals ($n = 30 \pm 2$) were carefully moved with tweezers onto a piece of plankton net gauze and stored in Eppendorf tubes in −80 °C until they were homogenised in 150 µL Tris-EDTA buffer containing 1 % sarcosyl. The antioxidative capacity was assayed as ORAC according to Ou et al. (2001). As a source of peroxyl radicals, we used 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH) (152.66 mM) and fluorescein was used as a fluorescent probe (106 nM). We used trolox (218 µM, Sigma-Aldrich) as a standard and the assay was performed on a 96-well microplate and to each well, 20 µL sample, 30 µL AAPH and 150 µL fluorescein were added. ORAC values were normalized to protein concentration and expressed as mg Trolox equivalents mg $protein^{-1}$. Protein concentration was measured with NanoOrange® (Life Technologies).

Phytoplankton was sampled every second day, fixed with acidic Lugol's iodine (2 % final concentration) and counted with the inverted microscope method (Utermöhl, 1958). Samples for chlorophyll *a* (Chl *a*) measurements were collected onto GF/F filters and measured as described by Welschmeyer (1994).

Samples for carbon (C) and nitrogen (N) concentrations were collected as for Chl *a* and stored in glass petri dishes at −20 °C until analyses. For further details on sampling and analyses, please refer to Paul et al. (2015).

2.3 Statistical analyses

2.3.1 Nauplii production

A linear mixed-effects model (LMM) was applied, as we did repeated measures of nauplii production of the same groups of individuals from the same mesocosms, to test if pH or food quantity and quality affected the nauplii production of *E. affinis*. Collinearity between all explanatory variables was checked (Pearson's product-moment correlation). Chl *a* concentration and the abundance of filamen-

Table 1. fCO_2 values ($t1 - t30$), average weekly pH, temperature, and dissolved oxygen (DO) and saturation in incubation bottles.

fCO_2 treatment (µatm)	Mesocosm	Week	pH	temp. (C°)	DO mg L^{-1}	DO%
346	1	1	8.12	11.21	10.61	96.0
	1	2	8.24	14.51	10.30	98.7
	1	3	8.12	15.08	8.71	99.5
	1	4	8.03	15.80	9.42	93.8
348	5	1	8.14	10.00	10.94	96.7
	5	2	8.20	13.37	10.64	98.3
	5	3	8.07	14.99	9.88	99.8
	5	4	8.02	15.10	9.61	98.9
494	7	1	7.93	9.98	10.87	96.2
	7	2	8.02	13.31	10.62	97.7
	7	3	7.90	15.00	9.96	100.6
	7	4	7.91	14.96	9.60	98.7
868	6	1	7.68	10.24	10.83	95.2
	6	2	7.80	13.33	10.56	97.3
	6	3	7.74	15.01	9.85	99.6
	6	4	7.76	15.13	9.65	98.9
1075	3	1	7.59	10.23	10.85	96.4
	3	2	7.72	13.63	10.61	98.3
	3	3	7.67	14.60	10.00	101.4
	3	4	7.71	15.29	9.57	98.5
1333	8	1	7.52	9.96	10.07	96.0
	8	2	7.63	13.35	10.65	98.0
	8	3	7.59	14.76	9.98	100.5
	8	4	7.62	15.14	9.72	99.7

tous cyanobacteria correlated. As these correlating variables explain partly the same thing, the variable that explained the variation in nauplii production the best (Chl *a*) was included in the model. In the model the average number of nauplii produced female^{-1} day^{-1} (log-transformed) for each treatment was set as response variable. Incubation pH (calculated as weekly mean values from daily measurements from incubation bottles), Chl *a* concentration, biomass of diatoms (*Chaetoceros* sp. *Skeletonema marinoi* and pennate diatoms, total µg C L^{-1}), C : N < 55 µm fraction of POM, biomass of mixotrophic dinoflagellates (*Amylax triacantha*, *Dinophysis* spp., *Heterocapsa triquetra* and *Micracanthodinium* spp., size range ∼ 10–100 µm, total µg C L^{-1}) and incubation temperature were used as fixed effects (Table 2). We used only the most abundant diatoms as the other species had a very scarce and inconsistent abundance in the samples. The main groups of diatoms were present in all mesocosms. The smaller fraction of C : N < 55 µm was used instead of total C : N as the total fraction may have included large zooplankton such as copepods which could affect the results. The explanatory variables used included data of each mesocosm of the corresponding day of sampled water used for the incubations. When sampling days were missing, the average values (of total µg C L^{-1} for diatoms and dinoflagellates, and mol : mol of C : N) for the previous and the next day were used. Day nested within week, nested within mesocosm, was used as random intercept as nauplii production of the same animals was measured four times per week and as weekly incubations were dependent on each other, and they were repeatedly sampled from the same mesocosms. The model simplifications were done manually in a backward stepwise manner by removing the non-significant effects and by using Akaike's information criterion (AIC) to achieve the minimum adequate model for the data. We report *t*-statistics of the retained variables for the LMMs (Table 3).

2.3.2 Fatty acids

Linear mixed-effects models were applied to test if pH has a direct effect on the fatty acid content of female copepods. EPA, DHA, and their precursor 18:3ω3 autocorrelated strongly with each other, and with total PUFA (Pearson's product-moment correlation); therefore we decided to use PUFA in the LMM. Separate models were made for each fatty acid group, which was set as a response variable, with pH as fixed effect and mesocosm as random effect. To test the effects of essential fatty acids on weekly nauplii production, we used separate LMMs, as PUFA and MUFA autocorrelated. In the models, PUFA, MUFA, and SAFA were used as fixed effects and mesocosm was tested as a random factor (Table 2).

To test whether female fatty acid content are reflected in the fatty acid content of eggs, each fatty acid group (PUFA, MUFA and SAFA) was tested separately in a LMM. In the

Table 2. Variables that were used in the full LMM models (numbers indicate separate models). Repeated measures were used as random effects in the models, as samples from the same enclosures are dependent on each other.

LMM	Fixed effects	Definition	Response variable
1	pH	The ocean acidification effect	Nauplii production
	Chl *a*	The food quantity effect	
	Diatoms	The food quality effect	
	C : N < 55 µm	The food quality effect	
	Dinoflagellates	The food quality effect	
	Incubation temp.		
			Fatty acids in females:
2	Incubation pH	The ocean acidification effect	PUFA
3	Incubation pH	The ocean acidification effect	MUFA
4	Incubation pH	The ocean acidification effect	SAFA
	Fatty acids in females:		Fatty acids in eggs:
5	PUFA	Relationship between female	PUFA
6	MUFA	fatty acids and their eggs	MUFA
7	SAFA		SAFA
	Fatty acids in females:		
8	PUFA		Nauplii production
9	MUFA		Nauplii production
10	SAFA		Nauplii production
11	pH		ORAC

model, fatty acids of eggs was set as a response variable and female fatty acid content as fixed effect; mesocosm was used as a random factor. Not all females had egg sacs left at the end of weeks 3 and 4 and therefore not enough material (egg sacs) was obtained for all treatments. The variables of corresponding samples that were missing the egg data were therefore removed.

2.3.3 Antioxidative capacity

We tested whether there was an effect of pH on the copepods' antioxidant capacity (ORAC) with a LMM. ORAC was set as response variable, pH (measured the same day from water samples taken for incubations) as fixed factor and mesocosm was set as random factor. In addition, to test for potential correlation between ORAC and nauplii production, a Pearson's product-moment correlation was performed. In the ORAC data, values for mesocosms 5 (control) and 6 (868 µatm) were missing.

For all models, model validation was done by plotting the standardised residuals against the fitted values. All statistical analyses were performed with R 2.15.2 and the nlme-package (Pinheiro et al., 2012) was used for the LMM analyses (R Development Core Team, 2012).

3 Results

The oxygen saturation was continuously high (> 93.8%) in all incubations (Table 1). Temperature in the climate-controlled room followed the in situ temperature except during the fourth weekly incubation ($t24$-$t28$) when the room was not adjusted to the sudden in situ drop in temperature that occurred. Temperature in the treatment bottles increased from around 10 °C in the first week to 15 °C during the fourth week (Table 1). The pH remained stable in the bottles (SD < 0.08 within a week based on daily measurements, (Table 1) and matched the in situ pH and CO_2 treatments. Chl *a* concentration was relatively stable at $\sim 2\,\mu g\,L^{-1}$ in all mesocosms but then decreased to $\sim 1\,\mu g\,L^{-1}$ on $t17$. A significant positive effect of CO_2 on Chl *a* was observed after $t17$ (Paul et al., 2015). Dinoflagellates were on average $4.41 \pm 1.39\,\mu g\,CL^{-1}$ (+SD) (range 0–7.32) and declined rapidly after $t17$. The C : N values included in our analyses (our sampling days) were on average 7.66 ± 0.42 (range 6.13–8.77). A more comprehensive description of C : N is found in Paul et al. (2015). The diatoms included in our analyses were on average 0.06 ± 0.10 (range 0–$0.53\,\mu g\,C\,L^{-1}$). CO_2 treatment did neither effect dinoflagellates, C : N < 55 µm, nor diatoms.

Nauplii production in incubations was highest in water from M3, 1075 µatm (pH 7.6) with on average 12.6 ± 9.6 nauplii produced per female per day during the whole study period. For clarity and easier comparison between studies within this mesocosm project, average fCO_2 lev-

Table 3. T-statistics of the retained fixed effects in the LMM.

LMM	Response variable	Variable	Value	df	t	p
1	Nauplii production*	Chl *a*	1.09 ± 0.20	69	5.440	< 0.001
		Diatoms	-2.79 ± 0.66	69	-4.231	< 0.001
		Dinoflagellates	0.14 ± 0.05	69	2.731	0.008
		Incubation temp.	0.16 ± 0.05	17	3.388	0.004
	Fatty acids in females:					
2	PUFA	Incubation pH	75.99 ± 112.80	16	0.673	0.510
3	MUFA	Incubation pH	-7.70 ± 34.60	16	-0.223	0.827
4	SAFA	Incubation pH	-135.27 ± 325.21	16	-0.416	0.683
	Fatty acids in eggs:	Fatty acids in females:				
5	PUFA	PUFA	1.15 ± 0.40	13	2.864	0.013
6	MUFA	MUFA	1.08 ± 0.37	13	2.922	0.012
7	SAFA	SAFA	-2.51 ± 1.68	13	-1.497	0.158
		Fatty acids in females:				
8	Nauplii production	PUFA	0.09 ± 0.02	17	3.989	0.001
9	Nauplii production	MUFA	0.185 ± 0.09	17	2.031	0.058
10	Nauplii production	SAFA	0.006 ± 0.01	17	0.644	0.528
11	ORAC	Incubation pH	-0.02 ± 0.04	15	0.057	0.580

*log-transformed

els ($t1 - t30$) are included in Fig. 1 to describe the treatments. The effect of pH on nauplii production was not statistically significant. Particulate matter C : N (< 55 µm) had no impact on nauplii production. Chl *a* concentration, as an indicator of total food availability had a positive effect (LMM; $t = 5.440$, $p = < 0.001$, Fig. 2a). Dinoflagellate biomass ($t = 2.731$, $p = 0.008$, Fig. 2c) stimulated nauplii production, whereas diatom biomass (LMM; $t = -4.231$, $p = < 0.001$, Fig. 2b) had an adverse effect. There was a positive relationship between incubation temperature and nauplii production ($t = 3.388$, $p = < 0.004$) (Table 3).

The fatty acid contents (ng mg dry weight^{-1}) of the females were not affected by pH (LMM $p = > 0.5$, see also Supplement). Female MUFA and PUFA content significantly affected the MUFA and PUFA content of the eggs (LMM MUFA; $t = 2.922$, $p = 0.012$, LMM PUFA; $t = 2.864$, $p = 0.013$), whereas female SAFA did not (Fig. 3a–c, LMM; $t = -1.497$, $p = 0.158$). Female PUFA concentration stimulated nauplii production (LMM; $t = 3.989$, $p = 0.001$), whereas MUFA (LMM; $t = 2.031$, $p = 0.058$), and SAFA content had no statistically significant effect (LMM; $t = 0.644$, $p = 0.528$, Fig. 4a–c, Table 3).

ORAC was not affected by pH (LMM; $t = -0.057$, $p = 0.580$) and there was no correlation between female ORAC and nauplii production (rho $= 0.297$, $p = 0.180$) (Fig. 5).

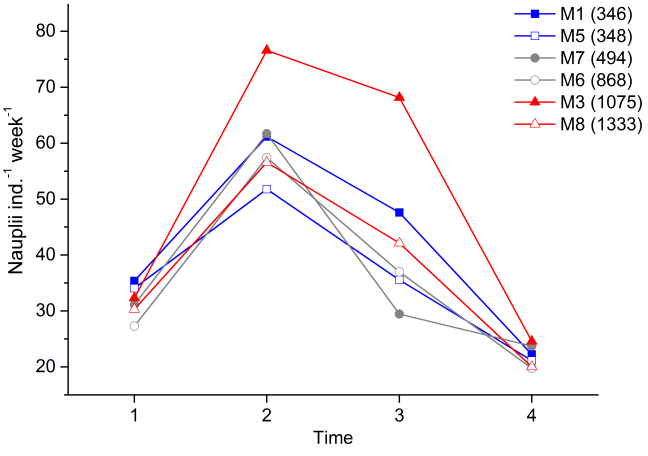

Figure 1. Weekly nauplii production, as averages of 10 females per bottle, for all mesocosms (treatment target $f CO_2$ in brackets, as averages of $t1 - t30$). Time point 1 is the average weekly nauplii production $t3 - t7$, $2 = t10 - t14$, $3 = t17 - t21$, and $4 = t24 - t28$.

4 Discussion

4.1 Effects of lowered pH

Experimental CO_2 concentrations did not affect the nauplii production of *E. affinis* in the current study. However, nauplii production in our incubations corresponded well with patterns of nauplii abundance observed in the mesocosm bags. The total number of copepods in the mesocosms showed no significant relation with CO_2 either (Lischka et al., 2015).

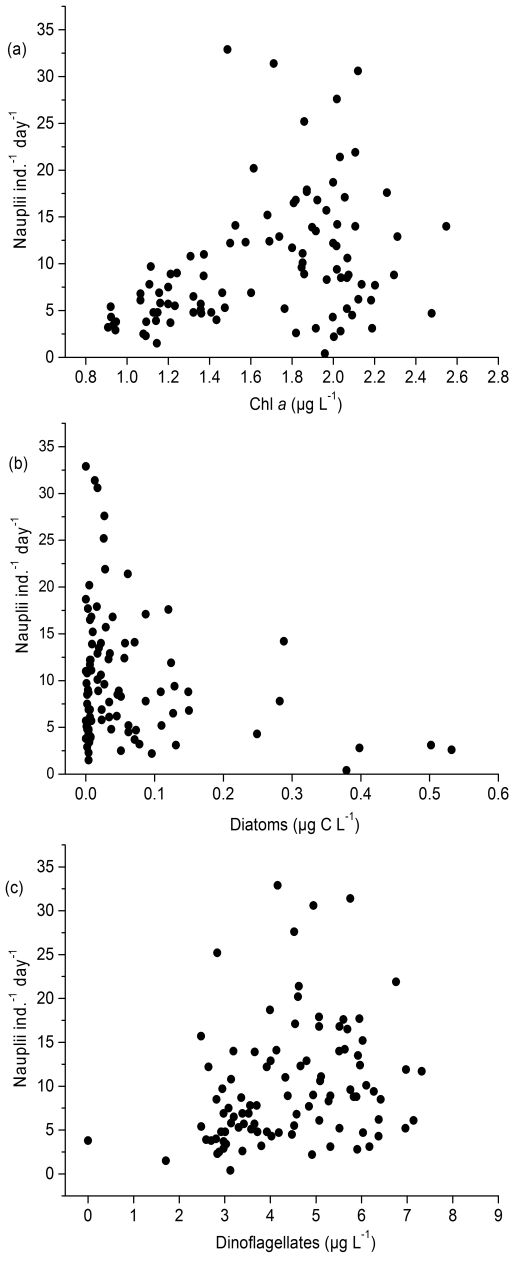

Figure 2. Daily nauplii production of *E. affinis* as a function of **(a)** Chl *a* concentration, **(b)** diatom biomass, and **(c)** dinoflagellate biomass.

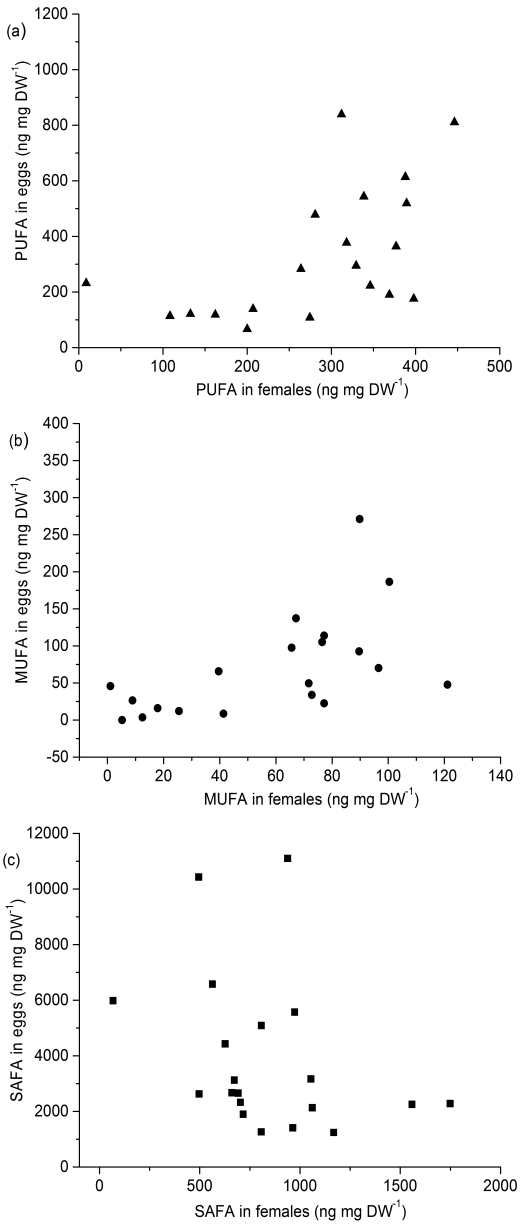

Figure 3. Fatty acids; **(a)** PUFA, **(b)** MUFA, and **(c)** SAFA content of females and eggs.

This is also in line with findings of Niehoff et al. (2013), who found no effect of CO_2 on zooplankton community development or abundance of single taxa in a similar mesocosm study in Kongsfjorden, Svalbard.

The physicochemical conditions in the research area is naturally fluctuating, therefore the plankton community may be adapted to large variability in CO_2 concentration and pH. In addition, organisms such as copepods are exposed to daily variation in pH and there is evidence that species performing vertical migration may be more robust to changes in CO_2 (Lewis et al., 2013). *E. affinis* undertakes diel vertical migration and particularly ovigerous *E. affinis* females stay below 20 m depth and experience > 0.5 units change (7.51–8.1) in pH on a daily basis (Almén et al., 2014), in the area where the current study was conducted. Thus, this could partially explain why *E. affinis* reproduction did not respond to lowered pH. Cripps et al. (2014), on the other hand, found severely reduced nauplii survival for *Acartia tonsa* kept at a pCO_2 of 1000 µatm, while other life stages were less affected. There appears to be a large variation in CO_2 sensitivity between species, even for organisms from the same study area. During this KOSMOS study, Vehmaa et al. (2015) found a nega-

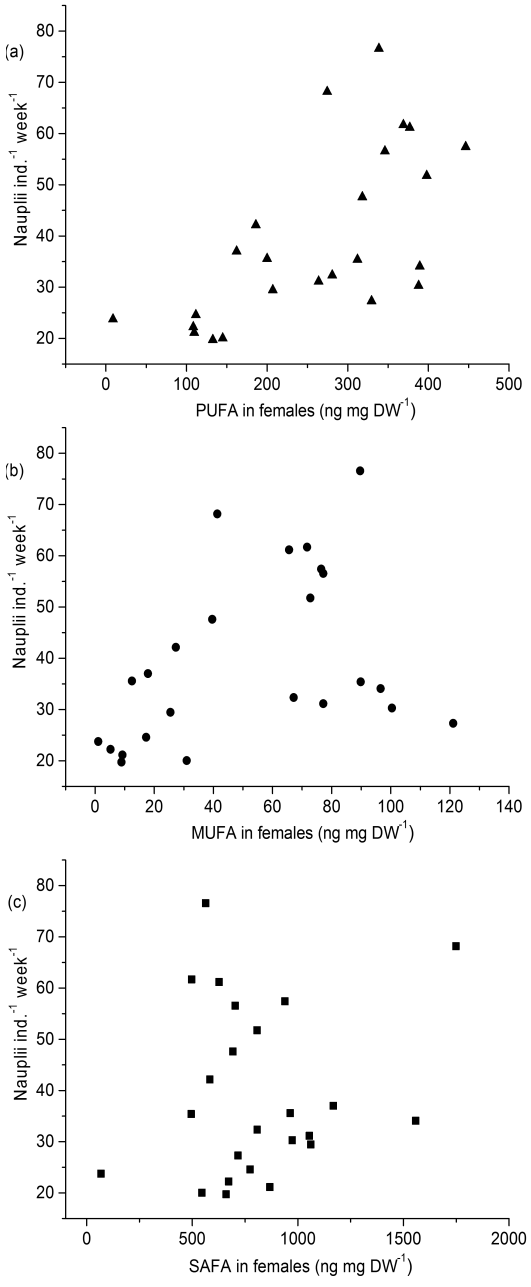

Figure 4. Relationship between nauplii production and female **(a)** PUFA, **(b)** MUFA, and **(c)** SAFA content.

tive effect of increased fCO_2 on body size and development index for *A. bifilosa*, another common copepod in the Baltic Sea. The increasing hatching rate of *E. affinis* with higher temperature reported by Andersen and Nielsen (1997) is also reflected in our results with higher incubation temperatures, affecting the nauplii production positively.

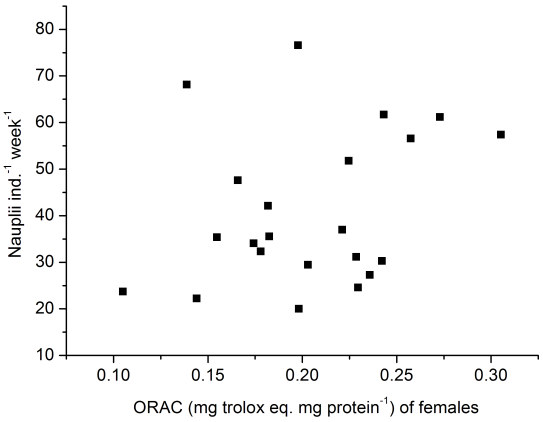

Figure 5. Correlation between weekly ORAC of *E. affinis* females and nauplii production (as averages of 10 females).

4.2 Effects of food

We found that nauplii production was positively affected by food availability (Chl *a* concentration, Fig. 2a). Our results are in agreement with Zervoudaki et al. (2014) who neither found discernible effects of lowered pH, whereas both higher temperature and food concentration (Chl *a*) positively affected egg production in *A. clausi* in a low nutrient Mediterranean system. According to fractionated Chl *a* measurements during the mesocosm campaign (Paul et al., 2015) > 90 % of the Chl *a* consisted of nanophytoplankton (< 20 µm), which possibly constituted an important food source for the filter-feeding *E. affinis* (Motwani and Gorokhova, 2013).

Although nauplii production of *E. affinis* was negatively affected by diatoms, no effect of CO_2 on diatom abundance was found. The abundance of diatoms was high during the first days but then declined rapidly. Low hatching frequency has, however, previously been observed for *E. affinis* during the diatom spring bloom in the same area (Ask et al., 2006). Some diatoms contain inhibitory compounds or lack essential nutrients that may be crucial for copepod reproduction (Lee et al., 1999). In the current study, diatoms consisted of *Chaetoceros* spp., *Skeletonema marinoi* and pennate diatoms. Vehmaa et al. (2012b) reported low egg production for *E. affinis* on a *S. marinoi* dominated diet in the study area. *Skeletonema* can produce potentially harmful aldehydes affecting copepod egg production (Ianora and Miralto, 2010). Significant negative correlation between *Chaetoceros* spp. and *E. affinis* hatching frequency has also been reported (Ask et al., 2006). However, there could potentially be a non-causal relationship between low diatom abundance and high nauplii production. It is possible that the end of the diatom bloom and peak abundance coincided (Ask et al., 2006). Dinoflagellates are in some cases considered superior food source for copepods, as opposed to diatoms (Ianora et al., 2004; cf. Vehmaa et al., 2012b). In this study dinoflag-

ellates positively stimulated nauplii production. Dinoflagellates probably contributed to nutritional quality as they are high in essential fatty acids (Galloway and Winder, 2015). We do not know to which extent the copepods fed on the different species; however, *E. affinis* is able to feed on both *H. triquetra* and *Dinophysis* spp., although the latter has toxic strains (Setälä et al., 2009).

We realize that some copepods and nauplii probably were introduced with the unfiltered water to the incubation bottles. We assume that it did not have a major effect on the results as the copepod nauplii abundance did not vary between the mesocosms (Lischka et al., 2015), and only *E. affinis* nauplii were counted. We observed a lot of epibionts (*Vorticella*) attached to adult copepods during the third week in the mesocosms. This was probably due to ageing (Jamieson and Santer, 2003), or the lack of predators that would otherwise have removed infested individuals which are more visible due to epibionts causing impaired escape ability (Souissi et al., 2013). The age of the *E. affinis* adults incubated in our experiments, was estimated to be 2–3 weeks to > 1 month. The higher age structure of *E. affinis* occurring in the mesocosms, as well as the decreasing Chl *a* levels could partly explain the decreased nauplii production in the third and fourth week of the experiment. Decreasing levels of PUFA in females towards the fourth week (Bermúdez et al., 2016), could also have affected copepod nauplii production. In the current study, the natural phytoplankton composition in the mesocosms did not change significantly due to CO_2 (Bermúdez et al., 2016; Annegret Stuhr, personal communication, 2015). Rossoll et al. (2013) and Bermúdez et al. (2016) suggest that a dampening of CO_2 effects can be expected for coastal communities adapted to strong natural fluctuations (cf. Waldbusser and Salisbury, 2014), as also proposed here. Rossoll et al. (2013) found no changes in phytoplankton community composition and no direct effect of lowered pH or indirect CO_2 effect, via changed food quality on *A. tonsa* reproduction, exposed to similar treatment levels as in the present study.

4.3 Antioxidative capacity and fatty acids

Our results suggest that the oxidative balance was maintained in the copepods in all treatments regardless of pH, as we did not observe any change in ORAC. As noted by Vehmaa et al. (2013), ORAC is affected by lowered pH, rather in combination with warmer temperatures, but not by moderately lowered pH alone. An oxidative imbalance, favouring ROS production can result in oxidative stress, as ROS can attack biomolecules, such as lipids, proteins, and DNA (Monaghan et al., 2009). Developmental stage (Fanjul-Moles and Gonsebatt, 2012), environmental condition (Lushchak, 2011), as well as feeding activity (Furuhagen et al., 2014) can affect levels of oxidative stress, suggesting the importance of measuring several biomarkers (Monaghan et al., 2009). We conclude that *E. affinis* did not face pronounced pH stress and therefore seems fairly robust to future ocean acidification, at least based on results in the present manuscript.

Analyses of fatty acid concentration in *E. affinis* females from our incubations revealed that PUFA in females was transferred to the eggs and stimulated nauplii production significantly, whereas no significant effect of pH on FA content in females was revealed. Despite the fact that Rossoll et al. (2012) found CO_2-induced changes in fatty acid content of phytoplankton in laboratory-based experiments, no CO_2-induced changes on phytoplankton or copepod fatty acid composition were found during the current mesocosm study (Bermúdez et al., 2016). The authors suggest that phosphorus limitation, being homogeneous in all mesocosms as nutrient addition was not practised, may have a stronger influence on community composition and their associated fatty acid profile than CO_2. Isari et al. (2015) found neither direct effects on copepod vital rates, nor indirect effects, via phytoplankton fatty acid composition, in two copepods *Acartia granii* and *Oithona davisae*. However, most PUFA showed a positive correlation with pCO_2 during part of a mesocosm study in Svalbard, which the authors attribute to taxonomical changes due to rising dinoflagellate abundances (Leu et al., 2013). In the present study female MUFA were reflected in their eggs, whereas SAFA were not, and none of them had a significant effect on nauplii production. These fatty acids, at least MUFA, are rather used for metabolism and storage (McMeans et al., 2012).

5 Conclusions

From our results we conclude that *E. affinis* is not sensitive to near-future levels of ocean acidification on a physiological level for the variables measured in the study. Offspring production was not affected after one generation. Food quality, in terms of dinoflagellate biomass and higher PUFA stimulated nauplii production, but we observed no difference in fatty acid composition due to pH. We also did not observe an effect of pH on ORAC. In the study area *E. affinis* is probably adapted to high pH variability due to diel vertical migration and may, therefore, not have faced pronounced pH stress from the treatment levels used in this study. We found that the effects of food quantity had an impact on nauplii production of *E. affinis*. For the time we conducted the laboratory-based experiments, we, however, did not observe an indirect CO_2 effect via phytoplankton biomass. Chl *a* concentration correlated positively with CO_2, but only clearly discernible for picophytoplankton from $t25$ onwards (Paul et al., 2015) and we sampled no longer than $t27$. How the indirect effect of CO_2 (via the food) would affect the copepods on a longer timescale remains unclear. Future studies should focus on copepod adaptation in relation to coastal pH variability and tolerance towards extreme events.

The Supplement related to this article is available online at doi:10.5194/bg-13-1037-2016-supplement.

Author contributions. Anna-Karin Almén, Anu Vehmaa, Andreas Brutemark and Jonna Engström-Öst designed and conducted the laboratory experiment. Anna-Karin Almén counted the nauplii samples, Silke Lischka counted plankton from the mesocosms and Annegret Stuhr counted phytoplankton. Sara Furuhagen analysed ORAC, Allanah Paul analysed C : N samples, J. Rafael Bermúdez analysed fatty acids and Lennart Bach analysed Chl *a*. Anna-Karin Almén and Anu Vehmaa performed the statistical analyses and Anna-Karin Almén wrote the manuscript with contributions from all co-authors. Project coordinator: Ulf Riebesell.

Acknowledgements. We would like to thank the staff of Tvärminne Zoological Station for providing working facilities during the experiment. We also thank the entire KOSMOS team for the joint sampling effort. We thank Michael Sswat for his attribution to the C : N analyses. We also thank the crew of R/V *Alkor* (AL394, A397) for transportation, deployment and recovery of the mesocosms, as well as the diving team. Special thanks go to Andrea Ludwig for organizing logistics and to Bettina Grönlund for assistance with zooplankton sampling and in the lab. The study was funded by Walter and Andrée de Nottbeck Foundation, Victoriastiftelsen, Academy of Finland (project nr. 276947), the Onni Talas foundation, Lennart Bach received funding from the BIOACID project (W. P. 1.3) and A. J. Paul from Excellence Cluster 'The Future Ocean' (Project CP1141). The collaborative project was funded by German Ministry of Education and Research (BMBF) BIOACID II (FKZ 03F06550), SOPRAN II (FKZ 03F0611) and MESOAQUA (228224).

Edited by: C. P. D. Brussaard

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