

Submitted on 05 Oct 2016

Anonymous Referee #3

This mesocosm study considered the response of *Acartia* spp to perturbations of fCO₂ levels. *Acartia* were extracted from the mesocosms at regular intervals over a 45 day period and then incubated in glass bottles for a further 24 h to determine their egg production rates and to harvest eggs for further viability studies. In these, the eggs were incubated in petri dishes for up to 138 hours either in mesocosm water or ambient (Baltic Sea) water and the level of egg hatching success and naupliar development determined. Using the two alternate sources of incubation water was in order to determine transgenerational effects in the response to the perturbation. Selected females were also analysed to establish their antioxidant capacity (ORAC). Feeding conditions in the mesocosms were considered through determining the concentration of total particulate carbon and the C:N ratio of the particulates. Data was analysed through a combination of linear mixed effect models and generalised linear mixed models to determine the effect of acidification, food quantity and food quality on egg production rate, body size, ORAC and hatching success. The study found a significant negative effect of increased fCO₂ on adult female size and some indication that an adaptive maternal effect alleviated the negative effects of acidification on egg hatching and nauplii development. There was no support for either food quantity or quality affecting transgenerational effects.

This was a laudable attempt to determine how exposure of adults and larvae to ocean acidification conditions affects viability, including how conditioning of the females may alleviate OA effects on egg and larval viability. The experiments were repeated over time from regular extractions from mesocosms that were closely monitored for a range of environmental parameters. The cross-transplant of eggs between different sources of water were a valiant attempt to reveal transgenerational effects that had variable results over the course of the experiment. The statistical analyses were thorough and well thought through. I have also considered the comments from previous referees in my assessment of this manuscript. I believe that the authors have modified their manuscript in substantive ways to accommodate criticisms of their work by these reviewers. The system they are studying is complex and variable and many of the criticisms reflect the difficulties encountered in controlling for the numerous variables that could potentially confound results. Although I agree with many of these criticisms, I consider that this work has made an honest attempt to present the variability in their results in a way that allows readership to benefit from their insights and allows them (and others) to repeat these experiments in an improved way. Such experiments are an important part in building up an understanding of the impact of ocean acidification that go alongside bottle experiments that are more tightly controlled and theoretical modelling. Although I am supportive of the publication of this work, I must insist on one substantive change in their conclusions. The extrapolation of the authors to identify 1000 uatm as a potential threshold beyond which adaptive maternal effects cannot alleviate the negative effects of acidification on egg hatching and nauplii development is, I believe, poorly justified by their data. As noted by one referee, it appears that much of the evidence on which this was based on the earlier experiments and was not so apparent in the latter ones. Although I accept certain caveats have been placed around this conclusion, these are not so

apparent in the Abstract, from which false impressions can be made. I accept that the study identified adaptive maternal potentially decrease with increasing fCO₂ levels, but to identify a threshold gives a false level of precision. These statements must be removed from the Abstract and suitably worded within the Discussion so that such false precision is not implied.

Author response:

Thank you very much for the constructive and supportive comments. We have made the suggested changes to the Abstract, Discussion, and Conclusions concerning the possible threshold (page 2, line 6; page 10, line 28; page 11, line 14; page 16, line 20). Hopefully, the text is now better in line with our findings. We have also performed a final careful grammar check.

Ocean acidification challenges copepod phenotypic plasticity

A. Vehmaa^{1,2}, A.-K. Almén^{2,3}, A. Brutemark^{1,2*}, A. Paul⁴, U. Riebesell⁴, S. Furuhausen⁵⁺, J. Engström-Öst²

[1]{Tvärminne Zoological Station, University of Helsinki, J.A. Palménin tie 260, FI-10900 Hanko, Finland}

[2]{Novia University of Applied Sciences, Coastal Zone Research Team, Raseborgsvägen 9, FI-10600 Ekenäs, Finland}

[3]{Environmental and Marine Biology, Faculty of Science and Engineering, Åbo Akademi University, Tykistökatu 6, FI-20500 Turku, Finland}

[4]{GEOMAR Helmholtz Centre for Ocean Research Kiel, Düsternbrooker Weg 20, 24105 Kiel, Germany}

[5]{Department of Environmental Science and Analytical Chemistry, Stockholm University, Svante Arrhenius väg 8, SE-11418, Stockholm, Sweden}

[*]{present address: Calluna AB, Torsgatan 30, SE-11321 Stockholm, Sweden}

[+]{present address: Swedish Chemical Agency, Esplanaden 3A, SE-17267 Sundbyberg, Sweden}

Correspondence to: A. Vehmaa (asvehmaa@gmail.com)

Abstract

Ocean acidification is challenging phenotypic plasticity of individuals and populations. Calanoid copepods (zooplankton) are shown to be fairly plastic against altered pH conditions, and laboratory studies indicate that transgenerational effects are one mechanism behind this plasticity. We studied phenotypic plasticity of the copepod *Acartia* sp. in the course of a pelagic, large-volume mesocosm study that was conducted to investigate ecosystem and biogeochemical responses to ocean acidification. We measured copepod egg production rate,

1 egg hatching success, adult female size and adult female antioxidant capacity (ORAC) as a
2 function of acidification ($f\text{CO}_2 \sim 365\text{--}1231 \mu\text{atm}$), and as a function of quantity and quality of
3 their diet. We used an egg transplant experiment to reveal if transgenerational effects can
4 alleviate the possible negative effects of ocean acidification on offspring development. We
5 found significant negative effects of ocean acidification on adult female size. In addition, we
6 found signs of a possible threshold at high $f\text{CO}_2$ above which adaptive maternal effects
7 cannot alleviate the negative effects of acidification on egg hatching and nauplii development.
8 We did not find support for the hypothesis that insufficient food quantity (total particulate
9 carbon $< 55 \mu\text{m}$) or quality (C:N) weakens the transgenerational effects. However, females
10 with high ORAC produced eggs with high hatching success. Overall, these results indicate
11 that *Acartia* sp. could be affected by projected near future CO_2 levels.

12 Keywords: *Acartia bifilosa*, climate change, maternal effects, total particulate carbon, C:N,
13 oxidative stress

14

15 1 Introduction

16 Increased concentrations of carbon dioxide (CO_2) in the atmosphere is changing the carbon
17 chemistry of the world's oceans. CO_2 dissolves in seawater thereby decreasing ocean pH.
18 Ocean acidification is increasing fast and pH is expected to decrease by a further 0.14–0.43
19 pH units during the coming century (IPCC, 2007). Acidification can cause various problems
20 to biochemical and physiological processes in aquatic organisms. In addition to affecting
21 calcification of calcareous organisms, maintenance of acid-base equilibrium of body fluids
22 may become more difficult and have consequences for example on protein synthesis,
23 metabolism and volume control (Whiteley, 2011).

24 In a changing environment, populations can respond in three main ways: through plastic
25 responses of individuals, through genetic changes across generations, or through escaping in
26 space or time by modification of phenology. During rapid change, phenotypic plasticity, i.e.,
27 the ability of an individual or a population to alter its physiological state, appearance or
28 behaviour in response to the environment, is of major importance (West-Eberhard, 2003).
29 Theory predicts that higher plasticity evolves in extreme environments, and that spatial
30 heterogeneity and dispersal select for higher plasticity (Chevin et al., 2013). One could
31 therefore hypothesise that organisms inhabiting a variable environment, such as the study

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1 area, could be fairly plastic in their response to ocean acidification because they have to cope
2 with both seasonal and sudden changes in pH (Almén et al., 2014; Lewis et al., 2013).

3 Proteomic studies suggest that oxidative stress is a common co-stress of temperature and
4 acidification (Tomanek, 2014). Increased production of reactive oxygen species (ROS) may
5 result in increased antioxidant and/or repair costs, and furthermore in reduced investment in
6 reproduction or other functions, such as immune defence. In addition, increased production of
7 ROS may lead to accumulation of oxidative damage and furthermore to acceleration of
8 senescence (Monaghan et al., 2009). There can also be a connection between maternal
9 oxidative balance and offspring quality. In birds, for example, females allocate diverse
10 antioxidants to the eggs that protect the embryo from oxidative stress. This maternal effect
11 has a positive effect on offspring development and growth (Rubolini et al., 2006).

12 Copepods (zooplankton) are indispensable to the functioning of the whole pelagic ecosystem
13 and contribute significantly to many ecosystem services (Bron et al., 2011). They provide, for
14 example, food for early-life stages as well as some adult fishes of many economically
15 important fish species (Steele, 1974; Cushing, 1990).

16 Previous results suggest that calanoid copepods have high buffering capacity against
17 projected ocean acidification for the year 2100 and beyond (Kurihara and Ishimatsu, 2008;
18 Weydmann et al., 2012; McConville et al., 2013; Vehmaa et al., 2013), meaning that they are
19 able to survive, grow, develop and reproduce in lower pH (Reusch, 2014). However, there are
20 also studies showing negative impacts on moderate CO₂ levels (Fitzer et al. 2012), whereas
21 most of the negative impacts have been discovered for extreme, carbon storage scenarios
22 (Kurihara et al., 2004; Mayor et al., 2007; Weydmann et al., 2012). Many studies have tested
23 only one life-stage, adult females, and have therefore possibly underestimated the effects of
24 ocean acidification on copepods (Cripps et al., 2014a). There are indications that
25 transgenerational effects are one mechanism responsible for the high plasticity of copepod
26 reproduction against altered pH conditions (Vehmaa et al., 2012). This maternal effect is most
27 likely dependent on the condition of the mother and the availability of food and quality of her
28 diet (Vehmaa et al., 2012; Pedersen et al., 2014a). Paternal effects can also influence
29 offspring traits. Exposure of both parents to CO₂ leads to fewer adverse effects on egg
30 production and hatching than exposure of only gravid copepod females (Cripps et al., 2014b).
31 Thor and Dupont (2015) also highlight the importance of testing transgenerational effects.
32 They found significantly lower copepod egg production after two generations when exposed

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1 to 900 and 1500 μatm compared to 400 μatm , but transgenerational effects alleviated the
2 negative CO_2 response in 1500 μatm (Thor and Dupont, 2015).

3 We tested direct and indirect effects of ocean acidification (i.e., via food quantity and quality)
4 on the copepod *Acartia* sp. egg production (EPR), egg hatching success (EH), female body
5 size (measured as prosome length (PL)), as well as antioxidant capacity (ORAC). The study
6 was conducted in association with the KOSMOS (Kiel Off-Shore Mesocosms for Ocean
7 Simulations) project in the Baltic Sea (Paul et al., 2015). The study was intended to cover the
8 low productivity late spring and early summer period, i.e., the post-spring bloom period when
9 $p\text{CO}_2$ concentrations are at the annual minimum. Over the annual cycle, $p\text{CO}_2$ and pH vary
10 substantially at the study site as a result of biological activity and mixing/upwelling of CO_2 -
11 enriched deep water (Niemi, 1975; Omstedt et al., 2014). There are also strong spatial
12 gradients in seawater $p\text{CO}_2$ /pH, most prominently between the surface layer and the CO_2 -rich
13 deeper waters (Almén et al., 2014). Thus, the copepods in the study area are likely to
14 experience strong changes in seawater carbonate chemistry, both seasonally and during their
15 diurnal migration. Total particulate carbon (TPC <55 μm) was used as the measure of food
16 quantity. Food quality was indicated by carbon to nitrogen ratio of the same size fraction of
17 seston (C:N <55 μm) (Elser and Hassett, 1994; Sterner and Hessen, 1994). In addition, in
18 order to separate transgenerational plasticity (i.e., maternal and paternal effects) and the effect
19 of environment on copepod egg hatching and development, we performed an egg-transplant
20 experiment. Half of the produced eggs were allowed to develop in respective mesocosm water
21 and the other half in water collected outside the mesocosm bags.

22 Due to the high buffering capacity of *Acartia* sp., we hypothesised that there are no $f\text{CO}_2$
23 related differences in egg production rate, egg hatching success and prosome length between
24 the mesocosms. In addition, we hypothesised that copepod eggs hatch and develop better in
25 the same environment in which they are produced, because transgenerational effects can
26 alleviate the negative effects of environmental change. Our third hypothesis stated that low
27 food quantity (TPC) and poor quality (high C:N) will weaken the maternal effect by
28 deteriorating the condition of the mother. Finally, we tested if mothers with higher antioxidant
29 capacity (ORAC) produce better quality offspring (EH) by calculating correlation coefficients
30 between the two variables.

2 Materials and Methods

The study was performed in summer 2012 in the vicinity of Tvärminne Zoological Station on the south-western coast of Finland. Six large mesocosms were moored on site in the beginning of June. To enclose the natural plankton community, the mesocosms were left open with only 3 mm mesh size net covering the top and the bottom during filling. After four days, the net was removed and the top was pulled up 1.5 m above the water surface and closed at the bottom (Riebesell et al., 2013; Paul et al., 2015). pH was ~8 and $f\text{CO}_2$ concentrations in the mesocosms prior to adjustment were 237 ± 9 μatm (average \pm std of daily measurements from all bags). Four mesocosms were manipulated with CO_2 enriched seawater, during three consecutive days to reach $f\text{CO}_2$ concentrations of 600-1650 μatm (Paul et al., 2015). Two untreated mesocosms were used as controls. The water column was mixed in the beginning of the experiment to avoid salinity stratification. Due to outgassing, CO_2 was also added on day 15 to the upper 7 m of the high CO_2 mesocosms to maintain the treatment levels. No nutrients were added.

2.1 Sampling

Sampling took place once a week during the first four weeks of the experiment, and once more at the end of the whole experiment (days 3, 10, 17, 24 and 45). Mesozooplankton were sampled [from all mesocosms](#) by taking two hauls with a 300 μm net (17 cm diameter) from 17 m depth. The samples were rinsed into containers with 4 l of seawater from respective mesocosms taken from 9 m depth with a water sampler (Limnos, Hydrobios). On the same day, integrated water samples (0-17 m) were collected from all mesocosms and the Baltic Sea directly into 1.2 l Duran bottles that were closed without head space. Water samples were kept in cool bags and zooplankton samples were protected from light until transported to a temperature and light controlled room at Tvärminne Zoological Station within 4 h. The light: dark cycle in the room was 16:8 h and light intensity was 7 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (LI-COR LI-1000). Temperature followed the *in situ* temperature [9°C (day 3), 11°C (day 10), 15°C (day 17), 16°C (days 24 and 45)].

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2.2 Measurements of egg production, egg hatching success and prosome length

Twenty adult *Acartia* sp. (17 females and 3 males) were picked with pipettes from each sample using stereo microscopes, and gently placed in pre-filled glass bottles with respective mesocosm water. The bottles were closed without head-space, to minimise CO₂-outgassing during the incubation. pH [in the bottles](#) was measured [before closing and right after opening](#) them at the end of the incubation using [an](#) Ecosense pH10 pH/temperature pen (Table S1). The pen was calibrated with standard buffer solutions (Certipur, Titripac pH 4.00, 7.00, and 10.00) every second day. The bottles were incubated in temperature and light controlled room in conditions described above (Materials and Methods 2.1), and mixed three times a day and their place on the shelf was changed randomly. After the incubation (24.3 ± 2.3 h, average \pm std), the copepods and produced eggs were filtered using 250 μ m and 30 μ m sieves, respectively. The copepods were counted and their viability checked before preserving them in RNAlater (Sigma). RNAlater can affect size (Foley et al., 2010), and the effect depends on the number of segments in the animal, i.e., the more segments the larger effect. Shrinkage is ~15% for copepods (Prof. Elena Gorokhova, Stockholm University, pers. comm.). Prosome length of the preserved female copepods was measured using a stereo microscope (Leica MZ12) and ocular micrometer (total magnification 100 \times). As all the measured copepods were adult females, we assume the shrinkage to be in proportion similar for all individuals, which means that our results are quite conservative and comparable between mesocosms.

In the egg transplant experiment, the collected eggs were divided for hatching into two 50 ml petri dishes with different conditions; one dish was filled with respective mesocosm water and the other filled with Baltic water. pH of the water was measured as above before the incubations and right after the petri dishes were opened after the incubation (Table S1). The eggs were counted before the petri dishes were completely filled and sealed without head-space using Parafilm. Egg hatching was followed by counting the number of remaining eggs on the dish through the lid using a stereomicroscope twice a day. When the number of eggs had remained the same on two consecutive counting times, the dishes were opened and the water containing the remaining eggs and hatched nauplii was preserved with acid Lugol's solution. [The hatching incubation time varied between 63.9 and 137.6 h, depending on incubation temperature.](#) *Acartia* sp. nauplii stages were determined and the number of nauplii and remaining copepod eggs counted using a stereo microscope.

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1 Some adults, copepodites, nauplii or eggs could have ended up in the incubation bottles or
 2 petri dishes with the unfiltered incubation water. The possible extra adults and their
 3 contribution to the egg production rate (EPR, eggs copepod⁻¹ d⁻¹) were taken into account as
 4 EPR was calculated using the number of eggs and adult *Acartia* sp. females found in the
 5 incubation bottles after the 24 h incubation. When estimating the egg hatching success (EH,
 6 %), the total number of hatched *Acartia* sp. nauplii and remaining eggs at the end of the
 7 hatching incubation were compared with the number of eggs counted before the hatching
 8 incubation. If the total number exceeded the egg number prior to hatching, the most
 9 developed nauplii (>N4) were considered to be carry-over individuals, and were therefore not
 10 considered in the estimation of EH. For estimation of nauplii development rate, the
 11 development index (DI) was calculated (Knuckey et al., 2005) accordingly,

$$12 \quad DI = \frac{\sum_{i=0}^3 (N_i \times n_i)}{\sum_{i=0}^3 n_i} \quad (1)$$

13 where N_i is the assigned stage value (0 for eggs, 1 for N1, 2 for N2 and 3 for N3 and N4) and
 14 n_i the number of individuals at that stage. We assume all the *Acartia* sp. adults and nauplii to
 15 be species *A. bifilosa*. However, because another *Acartia* species, *A. tonsa* occurs in the area
 16 in late summer too (Katajisto et al., 1998), we cannot be totally sure that we only had one
 17 species in the experiments.

18 2.3 Antioxidant capacity

19 For antioxidant capacity (ORAC) samples ~25 live female *Acartia* sp. were picked from
 20 every zooplankton sample onto a piece of plankton net in the temperature and light controlled
 21 room on days 3, 10, 17 and 31. The net containing the copepods was folded and stored in
 22 Eppendorf tubes at -80°C. The samples were homogenised in 150 µl Tris-EDTA buffer
 23 containing 1% sarcosyl. The antioxidative capacity was assayed as ORAC (Ou et al., 2001).
 24 As a source of peroxy radicals, 2, 2-azobis (2-amidinopropane) dihydrochloride (AAPH)
 25 (152.66 mM) was used and fluorescein was used as a fluorescent probe (106 nM). We used
 26 trolox (218 µM, Sigma-Aldrich) as a standard and the assay was performed on a 96-well
 27 microplate and to each well, 20 µL sample, 30 µL AAPH and 150 µL fluorescein were added.
 28 ORAC values were normalized to protein and expressed as mg Trolox eq. mg protein⁻¹.
 29 Protein concentration was measured with NanoOrange® (Life Technologies).

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2.4 C:N and TPC

Samples for TPC and C:N were collected onto GF/F filters (Whatman, nominal pore size 0.7 µm) using gentle vacuum filtration (<200 mbar) and then stored in glass petri dishes at -20°C. GF/F filters and petri dishes were combusted at 450°C for 6 hours before use. Gauze pre-filters were used to separate the size fraction < 55 µm. Filters were not acidified to remove inorganic carbon, therefore total particulate carbon was used. C and N concentrations were determined on an elemental analyser (EuroEA) following Sharp (1974), coupled by a ConFlo II to a Finnigan Delta^{Plus} mass spectrometer and were used to calculate C:N ratios in mol:mol. For further details on sampling and analyses, please refer to Paul et al. (2015).

2.5 Statistics

The effect of acidification and food quantity and quality on *Acartia* sp. egg production (EPR), prosome length (PL), antioxidant capacity (ORAC) and nauplii development index (DI) was tested using linear mixed effect models (LMM) with restricted likelihood (REML) approximation from the nlme-package (Pinheiro et al., 2014), where EPR, PL or ORAC were used as response variables, *f*CO₂, TPC (<55 µm) and C:N as fixed explanatory variables and repeated measure of the mesocosms over time as a random factor (Table 1). Due to the binomial nature of the data, the effect of *f*CO₂, TPC (<55 µm) and C:N on egg hatching success (EH) was tested with generalized linear mixed model (GLMM) with Laplace likelihood approximation, binomial error structure and logit-link function from the lme4-package (Bates et al., 2014) (Table 1). The average of *f*CO₂, TPC (<55 µm) and C:N measurements from each mesocosm within three days before the zooplankton sampling were used as explanatory variables for EPR, ORAC and EH, because 2–3 days are considered to be an appropriate acclimatisation period for *A. bifilosa* (Yoon et al., 1998; Koski and Kuosa, 1999). For PL, the average of all *f*CO₂, TPC (<55 µm) and C:N measurements from the start of the mesocosm experiment were used since PL reflects the environmental conditions of the whole lifespan of the animal. In addition, Day 3 was excluded in the LMM testing the PL (Table 1), since three days is too short period for detecting differences in copepod size. Egg–adult generation time for *A. bifilosa* at 17°C is approximately 16 days of which ~7.5 d taken by nauplii stages and ~8.5 d by copepodite stages (Yoon et al., 1998). Collinearity between all explanatory variables was checked. Temperature was not considered in the models, because it changed similarly in all the bags (Paul et al., 2015). The model simplifications were done manually in backward stepwise manner by removing the non-

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significant effects and by using Akaike's information criterion (AIC). We report t- or z- statistics (EH) of the retained fixed effects. To separate the effect of hatching environment from maternal environment, EH and DI were divided by the corresponding values measured in the Baltic Sea water. The ratio of Mesocosm EH (or DI) / Baltic EH (or DI) >1 indicates that eggs hatch or develop better in the maternal conditions (Mesocosm water), whereas the ratio <1 indicates that eggs hatch or develop better in the Baltic Sea water. The effect of maternal environment ($f\text{CO}_2$, TPC (<55 μm) and C:N) on the ratio was tested with LMM, where the ratio of Mesocosm EH / Baltic EH and Mesocosm DI / Baltic DI were used as response variables; $f\text{CO}_2$, TPC (<55 μm) and C:N as fixed explanatory variables; and repeated measure of the mesocosms over time as a random factor. The model simplifications were made as above.

To test if maternal antioxidant capacity (ORAC) correlates with egg hatching success, Spearman rank correlation tests were used. Data from Days 3, 10 and 17 were included in the test ($n = 17$, EH result for MC 6 in Day 3 is missing) because those are the days when both ORAC and EH were measured.

All the statistical analyses were performed using software R 3.0.2 (R Core Team, 2013), and the significance level was 0.05.

3 Results

3.1 Egg production, prosome length, antioxidant capacity and egg hatching success

Acartia sp. egg production (EPR) increased in all mesocosms between Day 3 and Day 10, but decreased after that, reaching very low rates (1-2 eggs copepod⁻¹ d⁻¹) on Days 24 and 45 (Fig. 1a). Neither food quantity (TPC, <55 μm), food quality (C:N, <55 μm), nor ocean acidification ($f\text{CO}_2$) had a statistically significant effect on copepod egg production (Table 2), even though there seemed to be variations in those parameters between the mesocosms (Table 3).

Prosome length (PL) of *Acartia* sp. females increased during the first week of the study; however there seemed to be some differences between the mesocosms already on Day 3, which was not included in the analysis (Fig. 1b). From Day 10 onwards, the smallest *A. biflosa* adults were found in the mesocosm with the highest $f\text{CO}_2$ concentration (Fig. 1b).

1 $f\text{CO}_2$, but also TPC ($<55\ \mu\text{m}$) had a statistically significant negative impact on copepod body
2 size (Table 2).

3 Antioxidant capacity (ORAC) of the female copepods increased from Day 3 to Day 10 in all
4 mesocosms (Fig. 1c). Interestingly, on Day 3 ORAC was highest in the three mesocosms with
5 highest $f\text{CO}_2$ treatment, whereas on Day 31 the situation was opposite and ORAC was lowest
6 in the three mesocosms with highest $f\text{CO}_2$ (Fig. 1c). Despite this, only TPC ($<55\ \mu\text{m}$) had a
7 statistically significant effect on ORAC; ORAC decreases with increasing TPC (Table 2).

8
9 The overall egg hatching success (EH) was high throughout the study; over 80 % of the
10 *Acartia* sp. eggs hatched. As seen for EPR, PL, and ORAC, EH also increased from Day 3 to
11 Day 10 in all mesocosms (Fig. 1d). Variance in the EH between the four samplings was
12 highest in the mesocosms with highest $f\text{CO}_2$, whereas EH varied the least and remained >90
13 % in both control mesocosms (MC1, MC5). In spite of this, only TPC ($<55\ \mu\text{m}$) had a
14 statistically significant negative effect on EH (Table 4). Eggs that were produced in MCs 3, 5,
15 6 and 7 had fairly similar hatching success in Baltic water, whereas hatching success of eggs
16 that were produced in MCs 1 (control) and 8 (the highest $f\text{CO}_2$) was alternately either lower or
17 higher than in the other MCs (Fig. 1e).

18 3.2 Egg hatching and nauplii development in mesocosm vs. Baltic Sea 19 conditions

20 Neither the maternal food quantity (TPC) nor the quality (C:N) had a statistically significant
21 effect on offspring quality (EH and DI) in the egg transplant experiment (Table 5). The $f\text{CO}_2$
22 was the only detected variable in the maternal environment that influenced the ratio of EH
23 and DI between mesocosm and Baltic conditions.

24 Egg hatching success for eggs hatching in the mesocosm water differed from eggs hatching in
25 the Baltic water. On Days 3 and 10, hatching success was higher in the mesocosm water for
26 the control (MC1, MC5) and for low $f\text{CO}_2$ -treatment bags (MC7, MC6), whereas eggs
27 produced in high $f\text{CO}_2$ -treatment bags (MC3, MC8) showed higher hatching in the Baltic
28 water (Fig. 2a). Thus, there may be a threshold $f\text{CO}_2$ for hatching success at high $f\text{CO}_2$.
29 However, on Days 17 and 24 the $f\text{CO}_2$ treatment did not have a clear effect on hatching
30 success. Nevertheless, $f\text{CO}_2$ had a statistically significant negative effect on the ratio of EH
31 mesocosm / Baltic, meaning that egg hatching was higher in the Baltic water than in the

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1 maternal environment when the maternal environment had a high $f\text{CO}_2$ (Table 5). When
2 maternal environment had low $f\text{CO}_2$ the situation was vice versa. The level of $f\text{CO}_2$ also had a
3 significant negative effect on the DI mesocosm / Baltic ratio (Fig. 2b; Table 5).

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4 3.3 Correlations between antioxidant capacity and offspring quality

5 Copepod antioxidant capacity (ORAC) was correlated significantly with copepod egg
6 hatching success. The relationship between the two variables is positive and stronger for eggs
7 developing in the mesocosm water ($\rho = 0.75$, $p < 0.001$) than for eggs developing in the
8 Baltic water ($\rho = 0.62$, $p = 0.007$) (Fig. 3).

9 4 Discussion

10 In this study, conducted in semi-natural mesocosm environments, reproduction of the *Acartia*
11 sp. copepod showed high phenotypic buffering against acidification, i.e., the species was able
12 to maintain similar egg production rate and high egg hatching success in all $f\text{CO}_2$ conditions.
13 Nevertheless, we found a significant negative effect of ocean acidification on adult female
14 size. Even more interestingly, we found signs of a possible threshold at high $f\text{CO}_2$ for
15 offspring development, above which adaptive maternal effects cannot alleviate the negative
16 effects of acidification on egg hatching and nauplii development (Fig. 2). However, we did
17 not find support for the third hypothesis that poor food quantity (lower TPC) and quality
18 (higher C:N) would weaken the maternal effect by deteriorating the condition of the mother.
19 Conversely, higher food quantity (TPC $< 55 \mu\text{m}$) correlated negatively with egg hatching
20 success, adult female size and antioxidant capacity, whereas C:N ratio did not correlate with
21 any of the measured variables significantly. Copepods were possibly food limited in all the
22 mesocosms, especially after Day 17 due to a sharp decline in Chl *a* concentrations and in
23 phytoplankton community size structure (Paul et al., 2015). Dominance of picophytoplankton
24 that is too small to be consumed by copepods could be the reason for the observed negative
25 effects of food quantity, and that may also have masked the food quality effect. Also, after
26 Day 17 egg production rate was so low that it was practically impossible to find differences in
27 egg production between the mesocosms. Finally, we found a positive correlation between
28 maternal antioxidant capacity and egg hatching success, suggesting that the female
29 antioxidant defence might also protect the embryo from oxidative stress.

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1 The fact that *Acartia* sp. egg production and egg hatching were unaffected by high $f\text{CO}_2$ but
2 | [the](#) egg transplant experiment revealed that development was slower for nauplii at high CO_2
3 supports the importance of looking beyond egg production and egg hatching, which is also
4 pointed out by Pedersen et al. (2014b). They concluded that the first endogenously feeding
5 nauplii stages of *Calanus finmarchicus* are more sensitive to CO_2 -induced acidification than
6 eggs or later nauplii stages (Pedersen et al. 2014b). Longer developmental times in high
7 CO_2 /low pH have been observed in crustaceans, echinoderms and molluscs (Cripps et al.,
8 2014a and references therein). Weydmann et al. (2012) also reported a significant
9 developmental delay for *Calanus glacialis* eggs when exposed to highly acidified conditions.
10 Pedersen et al. (2014a) observed that development of C4 copepodites of *C. finmarchicus* was
11 | delayed by 8.9 days in high CO_2 treatments in comparison to [the](#) control condition, when also
12 the previous generation had been exposed to the same conditions.

13 We expected maternal effects to be most obvious in a high stress situation (high $f\text{CO}_2$
14 treatments), as seen for three-spined sticklebacks in a study testing the effects of global
15 warming (Shama et al., 2014). Instead, egg hatching was higher and nauplii development
16 faster in the maternal environment than in the Baltic water, when the maternal environment
17 had a low $f\text{CO}_2$ (low stress). In high $f\text{CO}_2$ maternal environment the opposite response was
18 observed, thus indicating that maternal effects are in fact weak and cannot compensate for the
19 higher $f\text{CO}_2$ levels that correspond to near-future levels or that the eggs are damaged by the
20 high $f\text{CO}_2$. This suggests that *Acartia* sp. and its reproduction are after all somewhat sensitive
21 to ocean acidification. However, the effects were not as clear over the following weeks as in
22 the beginning of the study, which may be due to an overall low egg number and large
23 variation in hatching after Day 17, or due to acclimation of the copepods to the treatment
24 conditions. In addition, the maternal effects seemed to weaken over time. This could be due to
25 weakening condition of the mothers. In the absence of fish predators, zooplankton density,
26 | [especially *Bosmina* sp. \(cladocerans\),](#) increased strongly in the mesocosms (Lischka et al.,
27 2015). Senescence and food limitation were thus plausible problems for copepods, and a
28 likely cause of weakening maternal provisioning. In addition, conditions in the Baltic Sea
29 changed after Day 17 due to an upwelling event, which caused an increase in $f\text{CO}_2$ and
30 decrease in pH (Paul et al., 2015). This might have made the Baltic conditions less favourable
31 for copepod egg development and evened out the differences between high $f\text{CO}_2$ mesocosms
32 and the Baltic conditions.

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1 A few studies have highlighted the importance of testing for transgenerational effects to avoid
 2 over- or underestimation of the effects of ocean acidification on copepods. Thor and Dupont
 3 (2015) found decreasing egg hatching of *Pseudocalanus acuspes* with increasing $p\text{CO}_2$. In
 4 addition, transgenerational effects alleviated the negative effects on egg production and
 5 hatching of the second generation when the mothers had been acclimatised to the same
 6 treatment. Also, a reciprocal transplant experiment showed that the effect was reversible and
 7 an expression of phenotypic plasticity (Thor and Dupont, 2015). Contrary to the current study,
 8 Pedersen et al. (2014a) found no effect of the CO_2 environment on egg hatching or
 9 development of pre-feeding nauplii stages N1 and N2 in their multigenerational study using
 10 *C. finmarchicus*. However, the development time of larger nauplii and copepodite stages was
 11 increased by $p\text{CO}_2$, although the development delay was not detected in the following
 12 generation (Pedersen et al., 2014a). Vehmaa et al. (2012) studied combined effects of ocean
 13 acidification and warming, and found indications that negative effects on *Acartia* sp.
 14 reproductive success can partly be combated with maternal effects. The used pH treatments (-
 15 0.4 from ambient) were at the same level as the low $f\text{CO}_2$ -treatments in this study (MC6,
 16 MC7), which makes the results of the two studies consistent.

17 The measurements of female copepod antioxidant capacity were done in order to provide
 18 possible additional information of the maternal provisioning on the offspring. A preferable
 19 practice in oxidative stress studies is to measure several of the four components consisting of
 20 free radical production, antioxidant defences, oxidative damage, and repair mechanisms
 21 (Monaghan et al., 2009). In the current study we only have the estimate for the defences,
 22 antioxidant capacity (ORAC) measurements, which makes our conclusions slightly more
 23 uncertain. However, an earlier study with the same species has indicated that at intermediate
 24 stress levels an upregulation of the antioxidant system enhances protection against oxidative
 25 damage, but at higher stress, the pro-oxidants may exceed the capacity of the antioxidant
 26 system and lead to oxidative damage (Vehmaa et al., 2013). In this study, upregulated
 27 antioxidant defence seemed to have a positive effect on offspring quality, as indicated by the
 28 positive correlation between female ORAC and egg hatching success. Higher ORAC in the
 29 two highest $f\text{CO}_2$ mesocosms in the beginning of the study could be a sign of an upregulated
 30 antioxidant system in a sudden stressful situation, whereas the lowest ORAC in the high $f\text{CO}_2$
 31 treatments at day 31 (Fig. 1c) could be caused by prolonged stress and exhausted antioxidant
 32 defence. The change from positive to negative effect in the course of the study could explain

1 why $f\text{CO}_2$ did not show a significant correlation with ORAC, whereas food quantity (TPC
2 $<55 \mu\text{m}$) did.

3 Ismar et al. (2008) showed that *Acartia* spp. development can be either slow or altered by
4 certain algal groups causing death before the first copepodite or reproductive stage. A non-
5 optimal diet could explain why higher food quantity would cause smaller adult female size,
6 lower egg hatching success or lower antioxidant capacity. *Skeletonema*-diatoms had fairly
7 high abundance in the mesocosms during the first days of the experiment when egg hatching
8 success was lowest in every mesocosm, but then declined rapidly. Diatom-dominated
9 phytoplankton composition has been shown to cause low copepod egg hatching success in the
10 field (Miralto et al., 1999). Another quality aspect is the size and shape of the food, which
11 may make it difficult to ingest or assimilate. From day 16 onwards, over 50% of chlorophyll *a*
12 was in picophytoplankton ($<2 \mu\text{m}$) (Paul et al., 2015), which is too small for *Acartia*
13 consumption (Rollwagen Bollens and Penry, 2003). Since we did not study what the
14 copepods preyed upon we can only speculate on diet quantity and quality. Satiated food
15 conditions can strengthen the maternal or transgenerational effects. The transgenerational
16 effects were of minor importance for hatching success in *C. finmarchicus* when exposed to
17 long term high CO_2 and food limited conditions (Pedersen et al., 2014a). Long term stress and
18 food limitation could thus also be the reason for weakening maternal effects in the current
19 study.

20 We found body size (prosoma length) to be negatively affected by high CO_2 . The result seems
21 to be mostly driven by the mesocosm with the highest $f\text{CO}_2$ (MC 8), where the adult *Acartia*
22 sp. copepods were smallest on all the four sampling times that were included in the analysis
23 (Days 10, 17, 24 and 45) (Fig. 1b). It takes ~ 8.5 days for a sixth stage nauplius of *A. bifilosa*
24 to develop through the five copepodite stages and reach adulthood at 17°C (Yoon et al.,
25 1998). According to the Bělehrádek's temperature function it takes 12–15 days for VI nauplii
26 to reach adulthood at $9\text{--}11^\circ\text{C}$ (Bělehrádek, 1935; McLaren, 1966). The constants used in the
27 equation ($\alpha=1008$, $a=-8.701$) were the same as used in Dzierzbicka-Głowacka et al. (2009) for
28 the Baltic Sea *Acartia* spp. It is thus possible that the copepods could have developed through
29 several stages causing the differences in prosoma length between the treatments on Day 10.
30 Lowered pH may have increased copepods' energy requirements and if energy is reallocated
31 towards maintaining homeostasis, their somatic growth can be reduced. Pedersen et al.
32 (2014a) found *C. finmarchicus* body size to be inversely related to $p\text{CO}_2$. They also found

1 higher respiration rate under more acidified conditions, and claimed that increased energy
2 expenditure via rising respiration and consecutive decreasing growth and reproduction could
3 lower the energy transfer to higher trophic levels and thus hamper the productivity of the
4 whole ecosystem (Pedersen et al., 2014a). This is especially alarming when considering the
5 projected climate warming, since copepod size is negatively correlated with temperature
6 (Foster et al., 2011). In addition to temperature, food quantity and quality can affect the
7 copepod body size (Hart and Bychek, 2011), and create surprising combined effects with
8 acidification. Garzke et al. (2016) reported an indirect positive effect of $p\text{CO}_2$ on copepod
9 body size, which was explained by higher food availability when acidification acted as a
10 fertilizer for phytoplankton. Temperature and food also interact because temperature affects
11 the respiration and metabolism, thus the satisfying diet depends on temperature (Boersma et
12 al., 2016). If high CO_2 treatment (MC 8) caused a developmental delay in maturation, as
13 could be interpreted from the prosome length results (Fig. 1b), the maturation would have
14 occurred at a different temperature than in other mesocosms and possibly in non-optimal food
15 conditions. Anyway, higher food quantity and quality would be expected to increase copepod
16 size, contrary to our results. It is therefore possible that the used food quantity (TPC <55 μm)
17 and quality estimates (C:N <55 μm) do not fully describe the diet that *Acartia* sp. was
18 consuming in the mesocosms.

19 Adult copepods have in general shown robustness against acidification (Mayor et al., 2012,
20 McConville et al., 2013), whereas eggs and nauplii appear to be more sensitive (Cripps et al.,
21 2014b; Fitzer et al., 2012). In addition, there seems to be notable differences in sensitivity
22 between species. Nauplii production, adult female fatty acid content and antioxidant capacity
23 (ORAC) of *Eurytemora affinis* were not affected by $f\text{CO}_2$ in the current mesocosm campaign
24 (Almén et al., 2016). Similarly, Lewis et al. (2013) found differences in ocean acidification
25 sensitivity between the species *Oithona similis* and *Calanus* spp. (*C. glacialis* and *C.*
26 *hyperboreus*). They argued that *O. similis* is more sensitive to future ocean acidification than
27 *Calanus* spp., because *O. similis* remains in the surface waters whereas *Calanus* spp. migrates
28 vertically, and encounters wider $p\text{CO}_2$ ranges daily than *O. similis* (Lewis et al., 2013). The
29 same applies to *Acartia* sp. and *E. affinis* in our study area. Although *Acartia* spp. is exposed
30 to natural variability in pH environment due to daily variations as well as due to staying at
31 greater depths during the day (low pH in deep water), it does not reside as deep as *E. affinis*
32 (Almén et al., 2014) and may therefore show higher sensitivity than *E. affinis* during the
33 current mesocosm campaign (Almén et al., 2016).

The results obtained for *Acartia* sp. reproduction in the current study seem to contradict the results obtained for the *Acartia* sp. abundance determined in the mesocosms. Although our results indicate that *Acartia* sp. reproduction is in fact sensitive to ocean acidification, no $f\text{CO}_2$ effect was found for the abundance of this species (Lischka et al., 2015). It is possible that 45 days was not long enough to detect small negative effects of CO_2 on copepod size, egg hatching and nauplii development, to be reflected in copepod abundance. In addition, especially in the beginning of the study *Acartia* eggs in the mesocosms might have ended up in the sediment trap before hatching due to slow development at low temperature, which might have made it difficult to detect differences in *Acartia* abundance between the mesocosms. On a longer time scale, small acidification induced delays in offspring development could translate into negative effects for the copepod population, and further on energy transfer within the pelagic food web. In addition, warming will probably enhance the sensitivity of the species towards ocean acidification (Vehmaa et al., 2012, 2013).

5 Conclusions

Our results support the idea that it is important to look beyond egg production as hatching and development can be more sensitive to ocean acidification. Parental effects will likely be important in mediating some of the negative effects of ocean acidification. For *Acartia* sp., the transgenerational (maternal) effects may alleviate negative impacts of ocean acidification but **potentially** only under exposure to medium levels of CO_2 . We did not find support for the hypothesis suggesting that poorer food quantity and quality would weaken the maternal effect by deteriorating the condition of the mother, which could be due to the overall food limitation especially during the latter half of the study or the fact that our estimates of food quantity and quality did not describe the diet in a satisfactory manner. Nevertheless, maternal antioxidant defence seems to correlate positively with offspring egg hatching success. Overall, these results indicate that *Acartia* sp. could in fact be affected by CO_2 levels predicted for the year 2100 (IPCC, 2007). However, it is important to remember that this study shows how today's copepods would react to tomorrow's world; thus these results do not take into account the possible effects of evolutionary adaptation. Transgenerational effects can buffer short-term detrimental effects of ocean acidification and thus give time for genetic adaptation and consequently assist persistence of populations under climate change.

Author contributions

A.V. planned the experiment; A.V., A.-K.A., J.E.-Ö., A.B. conducted the laboratory experiment; A.V. performed the statistical analyses; A.P. analysed TPC and C:N; S.F. analysed ORAC; U.R. coordinated the whole project; A.V. and A.-K.A. shared responsibility of writing the manuscript with contributions from all co-authors.

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1 Tables

2 Table 1. The structure of the full LMM or GLMM models that were used to test effects of
3 ocean acidification, food quantity, and food quality on copepod egg production (EPR), egg
4 hatching success (EH), prosome length (PL), antioxidant capacity (ORAC), the ratio of EH
5 mesocosm / EH Baltic, and the ratio of nauplii development index (DI) mesocosm / DI Baltic.
6 The sampling days that were included in each of the models are listed. Repeated measures of
7 same mesocosm bags was used as a random effect in all the models, because copepods that
8 come from the same bags are more alike than copepods from different bags.

9

Response variable	Fixed effects	Effect tested	Days included in the model					
			3	10	17	24	31	45
EPR (LMM)	$f\text{CO}_2$	Ocean acidification	x	x	x	x		x
	TPC (<55 μm)	Food quantity						
	C:N (<55 μm)	Food quality						
EH (GLMM)	$f\text{CO}_2$	Ocean acidification	x	x	x	x		
	TPC (<55 μm)	Food quantity						
	C:N (<55 μm)	Food quality						
PL (LMM)	$f\text{CO}_2$	Ocean acidification		x	x	x		x
	TPC (<55 μm)	Food quantity						
	C:N (<55 μm)	Food quality						
ORAC (LMM)	$f\text{CO}_2$	Ocean acidification	x	x	x		x	
	TPC (<55 μm)	Food quantity						
	C:N (<55 μm)	Food quality						
EH MC/Baltic (LMM)	$f\text{CO}_2$	Ocean acidification	x	x	x	x		
	TPC (<55 μm)	Food quantity						
	C:N (<55 μm)	Food quality						
DI MC/Baltic (LMM)	$f\text{CO}_2$	Ocean acidification	x	x	x	x		
	TPC (<55 μm)	Food quantity						
	C:N (<55 μm)	Food quality						

10

11

1 Table 2. T-statistics of the retained fixed effects in the linear mixed effect models testing the
2 effects of TPC (<55µm), C:N and $f\text{CO}_2$ on egg production rate (EPR), female prosome length
3 (PL) and female antioxidant capacity (ORAC). Repeated measures of same mesocosm bags
4 was used as a random effect in all the models, because copepods that come from the same
5 bags are more alike than copepods from different bags.

6

Response variable	Fixed effect	Estimate	DF	t	p-value
EPR	TPC <55 µm	0.21±0.14	23	1.54	0.137
PL	$f\text{CO}_2$	-0.000027±0.000011	16	-2.39	0.030
	TPC <55 µm	-0.0037±0.0017	16	-2.21	0.042
ORAC	TPC <55 µm	-0.0045±0.0021	22	-2.17	0.041

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8

1 Table 3. Ranges of $f\text{CO}_2$, $\text{TPC} < 55 \mu\text{m}$, and $\text{C:N} < 55 \mu\text{m}$ that were used as explanatory
2 variables in the full LMM and GLMM models. 3-day averages (measured within the latest
3 three days of the sampling day) were used in testing the effects of the explanatory variables
4 on copepod egg production (EPR), antioxidant capacity (ORAC), and egg hatching success
5 (EH), whereas average of all measurements since the start of the experiments until the
6 sampling day were used when testing the effects of the explanatory variables on copepod size
7 (PL). Variations in $f\text{CO}_2$, $\text{TPC} < 55 \mu\text{m}$, and $\text{C:N} < 55 \mu\text{m}$ in the course of the study are
8 presented in Paul et al. (2015).

	$f\text{CO}_2$ (μatm)		$\text{TPC} < 55 \mu\text{m}$		$\text{C:N} < 55 \mu\text{m}$	
	3-d average	Average since Day 1	3-d average	Average since Day 1	3-d average	Average since Day 1
MC 1	267–477	267–365	15.1–31.6	21.4–31.6	5.51–8.43	7.26–8.03
MC 3	745–1201	884–1121	17.4–29.7	20.4–29.7	6.94–8.36	7.79–8.20
MC 5	275–481	274–368	15.8–24.5	19.2–24.8	7.24–8.57	7.24–7.59
MC 6	663–991	683–896	16.5–34.3	21.0–34.3	7.14–8.25	7.60–7.81
MC 7	390–565	390–497	17.5–30.0	21.4–29.9	6.92–8.25	7.43–7.74
MC 8	874–1525	1117–1413	17.4–26.3	21.6–26.3	7.16–8.53	7.59–7.93

9

1 Table 4. Z-statistics of the retained fixed effects in the GLMM testing the effect of $f\text{CO}_2$, TPC
2 ($<55\text{ }\mu\text{m}$) and C:N on egg hatching success (EH). Repeated measures of same mesocosm bags
3 was used as a random effect in the model, because copepods that come from the same bags
4 are more alike than copepods from different bags.

5

Response variable	Fixed effect	Estimate	z	p-value
EH	$f\text{CO}_2$	-0.00062 ± 0.00032	1.94	0.052
	TPC $<55\text{ }\mu\text{m}$	-0.09557 ± 0.02505	3.82	<0.001

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1 Table 5. T-statistics of the retained fixed effects in the LMMs testing the effect of $f\text{CO}_2$, TPC
2 ($<55\ \mu\text{m}$) and C:N on ratio of egg hatching success (EH) mesocosm / EH Baltic and nauplii
3 development index (DI) mesocosm / DI Baltic. Ratio >1 : higher EH or DI in the mesocosm
4 water (maternal environment) than in the Baltic Sea water, ratio <1 : lower EH or DI in the
5 mesocosm water (maternal environment) than in the Baltic Sea water. Repeated measures of
6 same mesocosm bags was used as a random effect in both models, because copepods that
7 come from the same bags are more alike than copepods from different bags.

8

Response variable	Fixed effect	Estimate	DF	t	p-value
EH mesocosm / EH Baltic	$f\text{CO}_2$	-0.000061 ± 0.000028	16	-2.20	0.043
DI mesocosm / DI Baltic	$f\text{CO}_2$	-0.000145 ± 0.000067	16	-2.15	0.047

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1 Figures.

2 Fig. 1. Development of *Acartia bifilosa* a) egg production, b) prosome length (average \pm s.e.),
3 c) antioxidant capacity, and d) egg hatching success in the mesocosms, and e) egg hatching
4 success in Baltic water when eggs are produced in mesocosms in the course of the study. The
5 $f\text{CO}_2$ (μatm) values represent the average in Days 1–43 (Paul et al., 2015).

6

7 Fig. 2. Development of the ratio of a) egg hatching success (EH) mesocosm / EH Baltic and
8 b) nauplii development index (DI) mesocosm / DI Baltic during the study. Ratio >1 : higher
9 EH or DI in the mesocosm water (maternal environment) than in the Baltic Sea water, ratio
10 <1 : lower EH or DI in the mesocosm water (maternal environment) than in the Baltic Sea
11 water. Note that because of different development times, the DI values are not comparable
12 between the days. The $f\text{CO}_2$ (μatm) values represent the average in Days 1–43 (Paul et al.,
13 2015).

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15 Fig. 3. Correlations of copepod egg hatching success (EH) with maternal antioxidant capacity
16 (ORAC).

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