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 and Discussion concerning the possible threshold (page 2, line 6; page 10, line 28; page 11, line 14). Hopefully, the Abstract and the Discussion are now better in line with our findings.
We have also performed a final careful grammar check.

Ocean acidification challenges copepod phenotypic plasticity

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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, egg hatching success, adult female size and adult female antioxidant capacity (ORAC) as a function of acidification (fCO_2 ~365−1231 µatm), and as a function of quantity and quality of their diet. We used an egg transplant experiment to reveal if transgenerational effects can alleviate the possible negative effects of ocean acidification on offspring development. We found significant negative effects of ocean acidification on adult female size. In addition, we found signs of a possible threshold at high fCO_2, above which adaptive maternal effects cannot alleviate the negative effects of acidification on egg hatching and nauplii development. We did not find support for the hypothesis that insufficient food quantity (total particulate carbon < 55 µm) or quality (C:N) weakens the transgenerational effects. However, females with high ORAC produced eggs with high hatching success. Overall, these results indicate that *Acartia* sp. could be affected by projected near future CO_2 levels.

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

1 Introduction

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

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

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

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

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

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Previous results suggest that calanoid copepods have high buffering capacity against projected ocean acidification for the year 2100 and beyond (Kurihara and Ishimatsu, 2008; Weydmann et al., 2012; McConville et al., 2013; Vehmaa et al., 2013), meaning that they are able to survive, grow, develop and reproduce in lower pH (Reusch, 2014). However, there are also studies showing negative impacts on moderate CO_2 levels (Fitzer et al. 2012), whereas most of the negative impacts have been discovered for extreme, carbon storage scenarios (Kurihara et al., 2004; Mayor et al., 2007; Weydmann et al., 2012). Many studies have tested only one life-stage, adult females, and have therefore possibly underestimated the effects of ocean acidification on copepods (Cripps et al., 2014a). There are indications that transgenerational effects are one mechanism responsible for the high plasticity of copepod reproduction against altered pH conditions (Vehmaa et al., 2012). This maternal effect is most likely dependent on the condition of the mother and the availability of food and quality of her diet (Vehmaa et al., 2012; Pedersen et al., 2014a). Paternal effects can also influence offspring traits. Exposure of both parents to CO_2 leads to fewer adverse effects on egg production and hatching than exposure of only gravid copepod females (Cripps et al., 2014b). Thor and Dupont (2015) also highlight the importance of testing transgenerational effects. They found significantly lower copepod egg production after two generations when exposed to 900 and 1500 µatm compared to 400 µatm, but transgenerational effects alleviated the negative CO_2 response in 1500 µatm (Thor and Dupont, 2015).

We tested direct and indirect effects of ocean acidification (i.e., via food quantity and quality) on the copepod *Acartia* sp. egg production (EPR), egg hatching success (EH), female body size (measured as prosome length (PL)), as well as antioxidant capacity (ORAC). The study was conducted in association with the KOSMOS (Kiel Off-Shore Mesocosms for Ocean Simulations) project in the Baltic Sea (Paul et al., 2015). The study was intended to cover the low productivity late spring and early summer period, i.e., the post-spring bloom period when pCO_2 concentrations are at the annual minimum. Over the annual cycle, pCO_2 and pH vary substantially at the study site as a result of biological activity and mixing/upwelling of CO_2-enriched deep water (Niemi, 1975; Omstedt et al., 2014). There are also strong spatial gradients in seawater pCO_2/pH, most prominently between the surface layer and the CO_2-rich deeper waters (Almén et al., 2014). Thus, the copepods in the study area are likely to experience strong changes in seawater carbonate chemistry, both seasonally and during their diurnal migration. Total particulate carbon (TPC <55 µm) was used as the measure of food quantity. Food quality was indicated by carbon to nitrogen ratio of the same size fraction of seston (C:N <55 µm) (Elser and Hasset, 1994; Sterner and Hessen, 1994). In addition, in order to separate transgenerational plasticity (i.e., maternal and paternal effects) and the effect of environment on copepod egg hatching and development, we performed an egg-transplant experiment. Half of the produced eggs were allowed to develop in respective mesocosm water and the other half in water collected outside the mesocosm bags.

Due to the high buffering capacity of *Acartia* sp., we hypothesised that there are no fCO_2 related differences in egg production rate, egg hatching success and prosome length between the mesocosms. In addition, we hypothesised that copepod eggs hatch and develop better in the same environment in which they are produced, because transgenerational effects can alleviate the negative effects of environmental change. Our third hypothesis stated that low food quantity (TPC) and poor quality (high C:N) will weaken the maternal effect by deteriorating the condition of the mother. Finally, we tested if mothers with higher antioxidant capacity (ORAC) produce better quality offspring (EH) by calculating correlation coefficients 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 $f\mathrm{CO}_2$ concentrations in the mesocosms prior to adjustment were 237±9 µatm (average±std of daily measurements from all bags). Four mesocosm were manipulated with CO_2 enriched seawater, during three consecutive days to reach $f\mathrm{CO}_2$ 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 mesocosm, 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 µmol photons m^{-2} 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 CO_2-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 ± std), the copepods and produced eggs were filtered using 250 µm and 30 µm sieves, respectively. The copepods were counted and their viability checked before preserving them in RNA*later* (Sigma). RNA*later* 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 ×). 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.

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

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$$DI = \frac{\sum_{i=0}^{3}(Ni \times ni)}{\sum_{i=0}^{3} ni} \qquad (1)$$

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

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2.3 Antioxidant capacity

For antioxidant capacity (ORAC) samples ~25 live female *Acartia* sp. were picked from every zooplankton sample onto a piece of plankton net in the temperature and light controlled room on days 3, 10, 17 and 31. The net containing the copepods was folded and stored in Eppendorf tubes at -80°C. The samples were homogenised in 150 µl Tris-EDTA buffer containing 1% sarcosyl. The antioxidative capacity was assayed as ORAC (Ou et al., 2001). As a source of peroxyl radicals, 2, 2-azobis (2-amidinopropane) dihydrochloride (AAPH) (152.66 mM) was used 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 and expressed as mg Trolox eq. mg protein^{-1}. Protein concentration was measured with NanoOrange® (Life Technologies).

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$^{\mathrm{Plus}}$ 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).

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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, $f\mathrm{CO}_2$, 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 $f\mathrm{CO}_2$, 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 $f\mathrm{CO}_2$, 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 $f\mathrm{CO}_2$, 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 nonsignificant 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 ($f\mathrm{CO}_2$, 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; $f\mathrm{CO}_2$, 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.

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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 ($f\mathrm{CO}_2$) had a statistically significant effect on copepod egg production (Table 2), even though there seemed to be variations in those parameters between the mesocoms (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. bifilosa* adults were found in the mesocosm with the highest $f\mathrm{CO}_2$ concentration (Fig. 1b).

fCO$_2$, but also TPC (<55 µm) had a statistically significant negative impact on copepod body size (Table 2).

Antioxidant capacity (ORAC) of the female copepods increased from Day 3 to Day 10 in all mesocosms (Fig. 1c). Interestingly, on Day 3 ORAC was highest in the three mesocosms with highest fCO$_2$ treatment, whereas on Day 31 the situation was opposite and ORAC was lowest in the three mesocosms with highest fCO$_2$ (Fig. 1c). Despite this, only TPC (<55 µm) had a statistically significantl effect on ORAC; ORAC decreases with increasing TPC (Table 2).

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

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

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

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

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

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3.3 Correlations between antioxidant capacity and offspring quality

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

4 Discussion

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

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

We expected maternal effects to be most obvious in a high stress situation (high $f\mathrm{CO_2}$ treatments), as seen for three-spined sticklebacks in a study testing the effects of global warming (Shama et al., 2014). Instead, egg hatching was higher and nauplii development faster in the maternal environment than in the Baltic water, when the maternal environment had a low $f\mathrm{CO_2}$ (low stress). In high $f\mathrm{CO_2}$ maternal environment the opposite response was observed, thus indicating that maternal effects are in fact weak and cannot compensate for the higher $f\mathrm{CO_2}$ levels that correspond to near-future levels or that the eggs are damaged by the high $f\mathrm{CO_2}$. This suggests that *Acartia* sp. and its reproduction are after all somewhat sensitive to ocean acidification. However, the effects were not as clear over the following weeks as in the beginning of the study, which may be due to an overall low egg number and large variation in hatching after Day 17, or due to acclimation of the copepods to the treatment conditions. In addition, the maternal effects seemed to weaken over time. This could be due to weakening condition of the mothers. In the absence of fish predators, zooplankton density, especially *Bosmina* sp. (cladocerans), increased strongly in the mesocosms (Lischka et al., 2015). Senescence and food limitation were thus plausible problems for copepods, and a likely cause of weakening maternal provisioning. In addition, conditions in the Baltic Sea changed after Day 17 due to an upwelling event, which caused an increase in $f\mathrm{CO_2}$ and decrease in pH (Paul et al., 2015). This might have made the Baltic conditions less favourable for copepod egg development and evened out the differences between high $f\mathrm{CO_2}$ mesocosms and the Baltic conditions.

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

The measurements of female copepod antioxidant capacity were done in order to provide possible additional information of the maternal provisioning on the offspring. A preferable practice in oxidative stress studies is to measure several of the four components consisting of free radical production, antioxidant defences, oxidative damage, and repair mechanisms (Monaghan et al., 2009). In the current study we only have the estimate for the defences, antioxidant capacity (ORAC) measurements, which makes our conclusions slightly more uncertain. However, an earlier study with the same species has indicated that at intermediate stress levels an upregulation of the antioxidant system enhances protection against oxidative damage, but at higher stress, the pro-oxidants may exceed the capacity of the antioxidant system and lead to oxidative damage (Vehmaa et al., 2013). In this study, upregulated antioxidant defence seemed to have a positive effect on offspring quality, as indicated by the positive correlation between female ORAC and egg hatching success. Higher ORAC in the two highest $f\mathrm{CO}_2$ mesocosms in the beginning of the study could be a sign of an upregulated antioxidant system in a sudden stressful situation, whereas the lowest ORAC in the high $f\mathrm{CO}_2$ treatments at day 31 (Fig. 1c) could be caused by prolonged stress and exhausted antioxidant defence. The change from positive to negative effect in the course of the study could explain why $f\mathrm{CO}_2$ did not show a significant correlation with ORAC, whereas food quantity (TPC <55 µm) did.

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

We found body size (prosome length) to be negatively affected by high CO_2. The result seems to be mostly driven by the mesocosm with the highest $f\mathrm{CO}_2$ (MC 8), where the adult *Acartia* sp. copepods were smallest on all the four sampling times that were included in the analysis (Days 10, 17, 24 and 45) (Fig. 1b). It takes ~8.5 days for a sixth stage nauplius of *A. bifilosa* to develop through the five copepodite stages and reach adulthood at 17°C (Yoon et al., 1998). According to the Bělehrádek's temperature function it takes 12−15 days for VI nauplii to reach adulthood at 9−11°C (Bělehrádek, 1935; McLaren, 1966). The constants used in the equation (α=1008, a=-8.701) were the same as used in Dzierzbicka-Glowacka et al. (2009) for the Baltic Sea *Acartia* spp. It is thus possible that the copepods could have developed through several stages causing the differences in prosome length between the treatments on Day 10. Lowered pH may have increased copepods´ energy requirements and if energy is reallocated towards maintaining homeostasis, their somatic growth can be reduced. Pedersen et al. (2014a) found *C. finmarchicus* body size to be inversely related to $p\mathrm{CO}_2$. They also found higher respiration rate under more acidified conditions, and claimed that increased energy expenditure via rising respiration and consecutive decreasing growth and reproduction could lower the energy transfer to higher trophic levels and thus hamper the productivity of the whole ecosystem (Pedersen et al., 2014a). This is especially alarming when considering the projected climate warming, since copepod size is negatively correlated with temperature (Foster et al., 2011). In addition to temperature, food quantity and quality can affect the copepod body size (Hart and Bychek, 2011), and create surprising combined effects with acidification. Garzke et al. (2016) reported an indirect positive effect of $p\mathrm{CO}_2$ on copepod body size, which was explained by higher food availability when acidification acted as a fertilizer for phytoplankton. Temperature and food also interact because temperature affects the respiration and metabolism, thus the satisfying diet depends on temperature (Boersma et al., 2016). If high CO_2 treatment (MC 8) caused a developmental delay in maturation, as could be interpreted from the prosome length results (Fig. 1b), the maturation would have occurred at a different temperature than in other mesocosms and possibly in non-optimal food conditions. Anyway, higher food quantity and quality would be expected to increase copepod size, contrary to our results. It is therefore possible that the used food quantity (TPC <55 µm) and quality estimates (C:N <55 µm) do not fully describe the diet that *Acartia* sp. was consuming in the mesocosms.

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

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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*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 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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Tables

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

Response variable	**Fixed effects**	**Effect tested**	**Days included in the model**					
			3	**10**	**17**	**24**	**31**	**45**
EPR (LMM)	fCO_2	Ocean acidification	x	x	x	x		x
	TPC (<55 µm)	Food quantity						
	C:N (<55 µm)	Food quality						
EH (GLMM)	fCO_2	Ocean acidification	x	x	x	x		
	TPC (<55 µm)	Food quantity						
	C:N (<55 µm)	Food quality						
PL (LMM)	fCO_2	Ocean acidification		x	x	x		x
	TPC (<55 µm)	Food quantity						
	C:N (<55 µm)	Food quality						
ORAC (LMM)	fCO_2	Ocean acidification	x	x	x		x	
	TPC (<55 µm)	Food quantity						
	C:N (<55 µm)	Food quality						
EH MC/Baltic (LMM)	fCO_2	Ocean acidification	x	x	x	x		
	TPC (<55 µm)	Food quantity						
	C:N (<55 µm)	Food quality						
DI MC/Baltic (LMM)	fCO_2	Ocean acidification	x	x	x	x		
	TPC (<55 µm)	Food quantity						
	C:N (<55 µm)	Food quality						

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

Response variable	Fixed effect	Estimate	DF	t	p-value
EPR	TPC <55 µm	0.21±0.14	23	1.54	0.137
PL	$f\mathrm{CO}_2$	-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

Table 3. Ranges of fCO_2, TPC<55 µm, and C:N< 55 µm that were used as explanatory variables in the full LMM and GLMM models. 3-day averages (measured within the latest three days of the sampling day) were used in testing the effects of the explanatory variables on copepod egg production (EPR), antioxidant capacity (ORAC), and egg hatching success (EH), whereas average of all measurements since the start of the experiments until the sampling day were used when testing the effects of the explanatory variables on copepod size (PL). Variations in fCO_2, TPC <55 µm, and C: <55 µm in the course of the study are presented in Paul et al. (2015).

	fCO_2 (µatm)		**TPC<55 µm**		**C:N <55 µm**	
	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

Table 4. Z-statistics of the retained fixed effects in the GLMM testing the effect of *f*CO_2, TPC (<55 µm) and C:N on egg hatching success (EH). Repeated measures of same mesocosm bags was used as a random effect in the model, because copepods that come from the same bags are more alike than copepods from different bags.

Response variable	Fixed effect	Estimate	z	p-value
EH	*f*CO_2	-0.00062±0.00032	1.94	0.052
	TPC <55 µm	-0.09557±0.02505	3.82	<0.001

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

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

Figures.

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

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

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