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# Ocean acidification challenges copepod reproductive 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 bifilosa* 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 ( $f\text{CO}_2 \sim 365\text{--}1231 \mu\text{atm}$ ), 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 copepod size and egg hatching success. In addition, we found a threshold of  $f\text{CO}_2$  concentration ( $\sim 1000 \mu\text{atm}$ ), 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  $< 55 \mu\text{m}$ ) or quality (C:N) weakens the transgenerational effects. However, females with high ORAC produced eggs with high hatching success. Overall, these results indicate that *A. bifilosa* could be affected by projected near future  $\text{CO}_2$  levels.

## 1 Introduction

Increased concentrations of carbon dioxide ( $\text{CO}_2$ ) in the atmosphere is changing the carbon chemistry of the world's oceans.  $\text{CO}_2$  dissolves in seawater thereby decreasing ocean pH. Ocean acidity 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/physiological processes in aquatic organisms.

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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 in time by phenology modifications. Under a 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 have to cope with both seasonal and sudden changes in pH (Brutemark et al., 2011; Almén et al., 2014) could be fairly plastic in their response to ocean acidification.

Proteomic studies suggest that oxidative stress is a common co-stress of temperature and acidification stress (Tomanek, 2014). Increased production of reactive oxygen species (ROS) may result in increased antioxidant and/or repair costs and further in reduced investment in reproduction or other functions, such as immune defence. Further, increased production of ROS may lead to accumulation of oxidative damage and further 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). For example, they provide food for early-life stages of many economically important fish species (Beaugrand et al., 2003), as well as some adult fishes such as anchovies and sardines (Alheit and Niquen, 2004). In addition, zooplankton graze phytoplankton,

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and thus participate in controlling harmful algal blooms in the coastal areas suffering from anthropogenic eutrophication (Smayda, 2008).

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, most of the 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<sub>2</sub> 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<sub>2</sub> response in 1500 µatm .

We tested direct and indirect effects of ocean acidification (i.e., via food quantity and quality) on the copepod *Acartia bifilosa* egg production (EPR), egg hatching success (EH), body size (measured as prosome length (PL)), as well as antioxidant capacity (ORAC). 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) (Sterner and Hessen, 1994). In addition, in order to separate transgenerational plasticity 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 the common garden conditions in water collected outside the mesocosm bags.

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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 ( $9^\circ\text{C}$  (day 3),  $11^\circ\text{C}$  (day 10),  $15^\circ\text{C}$  (day 17),  $16^\circ\text{C}$  (days 24 and 45)).

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

Twenty adult *Acartia bifilosa* (17 females and 3 males) were picked with pipettes from every sample using stereomicroscopes, and gently placed in pre-filled glass bottles with respective mesocosm water. The bottles were closed without head-space, to prevent  $\text{CO}_2$ -outgassing during the incubation. The bottles were incubated in the 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 ( $24.3 \pm 2.3$  h, average  $\pm$  SD), the copepods and produced eggs were filtered using 250 and  $30 \mu\text{m}$  sieves, respectively. The copepods were counted and their viability checked before preserving them in RNA/later (Sigma). Prosome length of the preserved copepods was measured using a stereomicroscope (Leica MZ12) and ocular micrometer (total magnification  $100\times$ ).

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 (common garden). 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. Therefore 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 stereomicroscope.





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ments 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  $f\text{CO}_2$ , TPC ( $< 55 \mu\text{m}$ ) 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 to be able to detect differences in copepod size. Egg – adult generation time for *A. bifilosa* at  $17^\circ\text{C}$  is approximately 16 days of which  $\sim 7.5$  d taken by nauplii stages and  $\sim 8.5$  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-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 with the corresponding values measured in the common garden conditions (Baltic Sea water). The ratio of Mesocosm EH (or DI)/Common garden EH (or DI)  $> 1$  means that eggs hatch or develop better in the maternal conditions (Mesocosm water), whereas the ratio  $< 1$  means that eggs hatch or develop better in the common garden conditions (Baltic Sea water). The effect of maternal environment ( $f\text{CO}_2$ , TPC ( $< 55 \mu\text{m}$ ) and C:N) on the ratio was tested with LMM, where the ratio of Mesocosm EH/Common garden EH and Mesocosm DI/Common garden DI were used as response variables;  $f\text{CO}_2$ , TPC ( $< 55 \mu\text{m}$ ) 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



Despite this, only TPC (< 55  $\mu\text{m}$ ) explained variation in ORAC significantly; ORAC decreases with increasing TPC (Table 2).

### 3.2 Egg hatching and nauplii development in mesocosm vs. common garden conditions

Neither the maternal food quantity (TPC) nor the quality (C:N) affected the offspring quality (EH and DI) significantly in the egg transplant experiment (Table 5). The  $f\text{CO}_2$  was the only detected variable in the maternal environment that influenced the ratio of EH and DI between mesocosm and common garden conditions.

Egg hatching success for eggs hatching in the mesocosm water differed from eggs hatching in the common garden environment. On Days 3 and 10, hatching success was higher in the mesocosm water for the control (MC1, MC5) and for low  $f\text{CO}_2$ -treatment bags (MC7, MC6), whereas eggs produced in high  $f\text{CO}_2$ -treatment bags (MC3, MC8) showed higher hatching in the common garden conditions (Fig. 2a). Thus, there seems to be a threshold for  $f\text{CO}_2$  between 821–1007  $\mu\text{atm}$ , above which adaptive maternal effects cannot compensate the negative effects of the environment on offspring development. However, on Days 17 and 24 the  $f\text{CO}_2$  treatment did not have a clear effect on hatching success. Nevertheless,  $f\text{CO}_2$  had a significant negative effect on the ratio of EH MC/CG, meaning that egg hatching was higher in the maternal environment than in the Baltic water when the maternal environment had a low  $f\text{CO}_2$  (Table 5). However, when maternal environment had high  $f\text{CO}_2$  the situation is vice versa. The level of  $f\text{CO}_2$  had also a significant negative effect on the DI MC/CG ratio (Fig. 2b).

### 3.3 Correlations between antioxidant capacity and offspring quality

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

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## 4 Discussion

In this study, conducted in semi-natural mesocosm environment, reproduction of the copepods *Acartia bifilosa* copepod showed high phenotypic buffering against acidification, i.e., the species was able to maintain similar egg production rate and also fairly high egg hatching success in all  $f\text{CO}_2$  conditions. Nevertheless, we found significant negative effect of ocean acidification on egg hatching success and adult female size. Even more interestingly, there seems to be a threshold of  $f\text{CO}_2$  concentration ( $\sim 1000 \mu\text{atm}$ ) 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 second 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 <  $55 \mu\text{m}$ ) 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 (Paul et al., 2015), and that may 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.

The fact that *A. bifilosa* egg production was unaffected by high  $f\text{CO}_2$  but that development was slower in nauplii at high  $\text{CO}_2$  supports the importance of looking beyond egg production and egg hatching, which is also pointed out by Pedersen et al. (2014b). Longer developmental times in high  $\text{CO}_2$ /low pH have been observed in crustaceans, echinoderms and molluscs (Cripps et al., 2014a and references therein). Weydman et al. (2012) also reported a significant developmental delay for *Calanus glacialis* when exposed to highly acidified conditions.

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and quality can affect the copepod body size (Hart and Bychek, 2011); however, the expected effect would be positive, contrary to our results. It is therefore possible that the used food quantity (TPC < 55  $\mu$ m) and quality estimates (C : N < 55  $\mu$ m) do not fully describe the diet that *A. bifilosa* was consuming in the mesocosm bags.

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  $f\text{CO}_2$  in the current mesocosm campaign (Almén et al., 2015). Similarly, Lewis et al. (2013) found differences in ocean acidification sensitivity between the species *Oithona similis* and *Calanus glacialis*. They argued that *O. similis* is less adapted than *C. glacialis* to a narrower range of pH, because of less pronounced vertical migration patterns (Lewis et al., 2013). The same applies to *A. bifilosa* and *E. affinis* in our study area. Although *A. bifilosa* is exposed to natural variability in pH environment due to daily variations as well as staying at greater depths during the day (low pH in deep water), it does not reside as deep down as *E. affinis* (Almén et al., 2014) and may therefore show higher sensitivity than *E. affinis* during the current mesocosm campaign (Almén et al., 2015).

The results obtained for *A. bifilosa* reproduction in the current study seem to contradict the results obtained for the *A. bifilosa* abundance determined in the mesocosm bags. Although our results indicate that *A. bifilosa* 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  $\text{CO}_2$  on copepod size, egg hatching and nauplii development, to be reflected in copepod abundance. On a longer time scale, however, these 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).

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## 5 Conclusions

Our results support the idea that it is important to look beyond egg production as hatching and development can be more sensitive endpoints in the response to ocean acidification. Parental effects will likely be important in mediating some of the negative effects of ocean acidification. For *A. bifilosa*, the transgenerational (maternal) effects may alleviate negative impacts of ocean acidification but only under exposure to medium levels of CO<sub>2</sub>. 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. Nevertheless, maternal antioxidant defence seems to correlate positively with offspring egg hatching success. Overall, these results indicate that *A. bifilosa* could in fact be affected by CO<sub>2</sub> 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. Vehmaa planned the experiment; A. Vehmaa, A.-K. Almén, J. Engström-Öst, A. Brutemark conducted the laboratory experiment; A. Vehmaa performed the statistical analyses; A. Paul analysed TPC and C:N; S. Furuhaugen analysed ORAC; U. Riebesell coordinated the whole project; A. Vehmaa and A.-K. Almén shared responsibility of writing the manuscript with contributions from all co-authors.

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**Table 1.** Variables that were used in the full LMM or GLMM models, their definitions and the sampling days that were included in the models.

Variable type	Variable	Definition	Days	3	10	17	24	31	45
Fixed effects	$f\text{CO}_2$	The ocean acidification effect	EPR (LMM)	x	x	x	x		x
	TPC < 55 $\mu\text{m}$	The food quantity effect	EH (GLMM)	x	x	x	x		
	C : N < 55 $\mu\text{m}$	The food quality effect	PL (LMM)		x	x	x		x
Random effects	Repeated measures of same mesocosm bags	Copepods that come from the same bags are more alike than copepods from different bags	ORAC (LMM)	x	x	x		x	
			EH MC/CG DI MC/CG (LMM)	x	x	x	x		

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**Table 2.** *T* statistics of the retained fixed effects in the LMMs.

	Value	DF	<i>t</i>	<i>p</i> value
EPR				
TPC < 55 μm	0.21 ± 0.14	23	1.54	0.137
PL				
<i>f</i> CO <sub>2</sub>	-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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**Table 3.** Ranges of  $f\text{CO}_2$ ,  $\text{TPC} < 55 \mu\text{m}$ , and  $\text{C} : \text{N} < 55 \mu\text{m}$  that were used as explanatory variables in the full LMM and GLMM models. 3-day averages 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 from the start of the experiments were used when testing the effects of the explanatory variables on copepod size (PL).

	$f\text{CO}_2$ ( $\mu\text{atm}$ )		$\text{TPC} < 55 \mu\text{m}$		$\text{C} : \text{N} < 55 \mu\text{m}$	
	3-d average	since start	3-d average	since start	3-d average	since start
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

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**Table 4.** Z statistics of the retained fixed effects in the GLMM testing egg hatching success (EH).

	Value	<i>z</i>	<i>p</i> value
EH			
$f\text{CO}_2$	$-0.00062 \pm 0.00032$	1.94	0.052
TPC < 55 $\mu\text{m}$	$-0.09557 \pm 0.02505$	3.82	< 0.001

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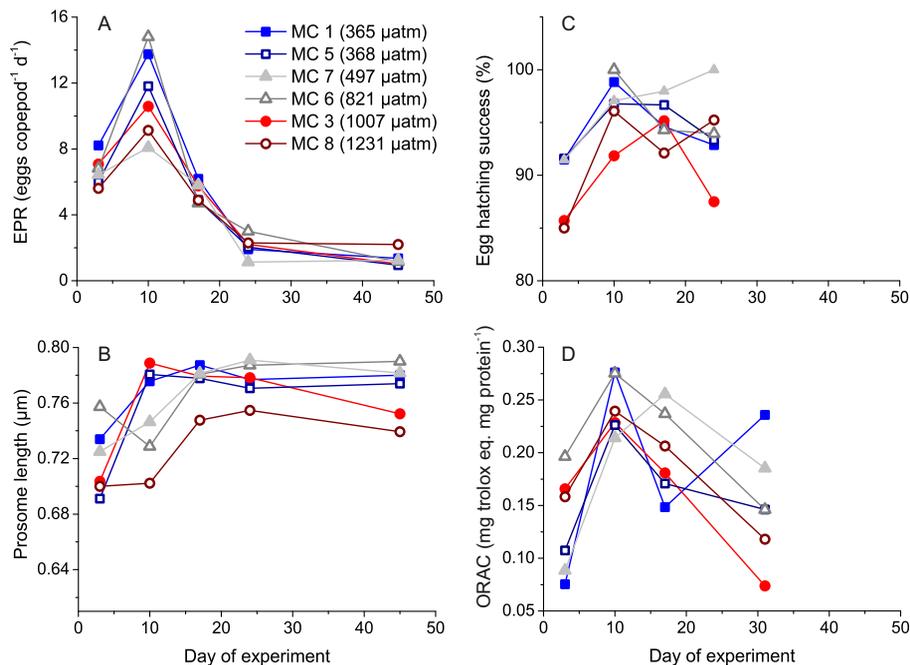
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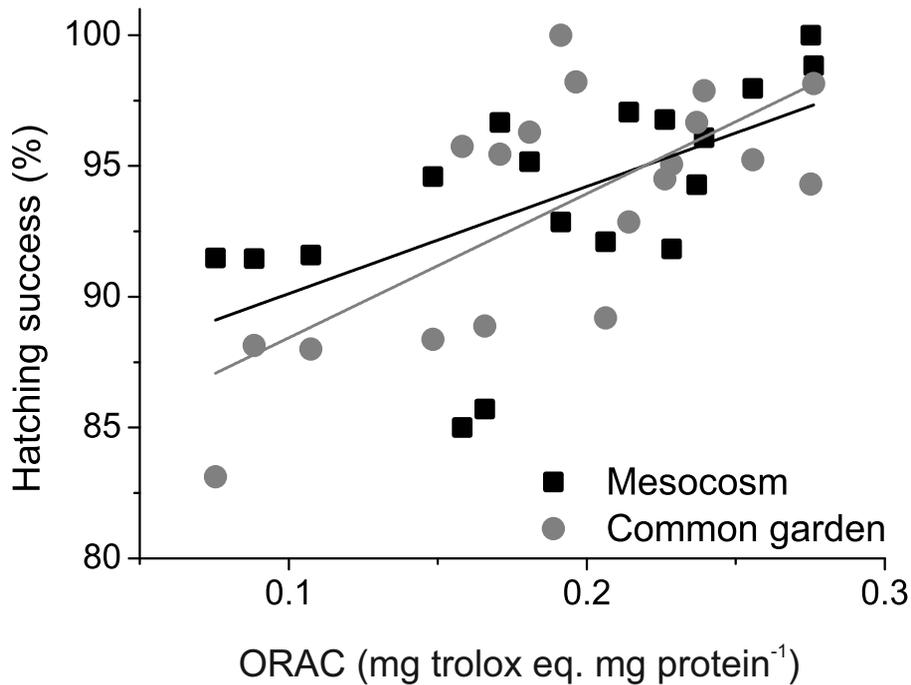
**Table 5.** *T* statistics of the retained fixed effects in the LMMs testing the ratio of egg hatching success (EH) mesocosm/EH common garden and nauplii development index (DI) mesocosm/DI common garden. Ratio > 1: higher EH or DI in the mesocosm water (maternal environment) than in the Baltic Sea water (common garden environment), ratio < 1: lower EH or DI in the mesocosm water (maternal environment) than in the Baltic Sea water (common garden environment).

	Value	DF	<i>t</i>	<i>p</i> value
EH mesocosm/EH common garden <i>f</i> CO <sub>2</sub>	-0.000061 ± 0.000028	16	-2.203	0.043
DI mesocosm/DI common garden <i>f</i> CO <sub>2</sub>	-0.000145 ± 0.000067	16	-2.15	0.047



**Figure 1.** Development of *Acartia bifilosa* (a) egg production, (b) prosome length, (c) egg hatching success, and (d) antioxidant capacity in the mesocosms in the course of the study. The  $f\text{CO}_2$  ( $\mu\text{atm}$ ) values represent the average in Days 1–43 (Paul et al., 2015).





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

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