

Transgenerational effects alleviate severe fecundity loss during ocean acidification in a ubiquitous planktonic copepod

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Abstract

Ocean acidification (OA) caused by anthropogenic CO₂ emission is projected for thousands of years to come, and significant effects are predicted for many marine organisms. While significant evolutionary responses are expected during such persistent environmental change, most studies consider only short-term effects. Little is known about the transgenerational effects of parental environments or natural selection on the capacity of populations to counter detrimental OA effects. In this study, six laboratory populations of the calanoid copepod *Pseudocalanus acuspes* were established at three different CO₂ partial pressures ($p\text{CO}_2$ of 400, 900 and 1550 μatm) and grown for two generations at these conditions. Our results show evidence of alleviation of OA effects as a result of transgenerational effects in *P. acuspes*. Second generation adults showed a 29% decrease in fecundity at 900 μatm CO₂ compared to 400 μatm CO₂. This was accompanied by a 10% increase in metabolic rate indicative of metabolic stress. Reciprocal transplant tests demonstrated that this effect was reversible and the expression of phenotypic plasticity. Furthermore, these tests showed that at a $p\text{CO}_2$ exceeding the natural range experienced by *P. acuspes* (1550 μatm), fecundity would have decreased by as much as 67% compared to at 400 μatm CO₂ as a result of this plasticity. However, transgenerational effects partly reduced OA effects so that the loss of fecundity remained at a level comparable to that at 900 μatm CO₂. This also relieved the copepods from metabolic stress, and respiration rates were lower than at 900 μatm CO₂. These results highlight the importance of tests for transgenerational effects to avoid overestimation of the effects of OA.

Keywords: adaptation, copepod, egg production, ocean acidification, phenotypic plasticity, respiration, transgenerational transmission

Received 11 June 2014; revised version received 10 November 2014 and accepted 14 November 2014

Introduction

Marine organisms function in close interplay with the surrounding water. They are adapted to handle challenges visited upon them by predictable changes in water chemistry, and any environmental change is followed by physiological regulation of intracellular chemistry (Pörtner *et al.*, 2004; Zhang *et al.*, 2012). However, while this maintenance of cellular homeostasis allows acclimatization to environmental fluctuations, it comes with an energetic cost. Animals experiencing significant environmental changes must alter their energy distribution to accommodate these changes, and during unfavourable conditions, such alterations may ultimately subtract from the energy available for growth and reproduction (Stumpff *et al.*, 2011; Fitzer *et al.*, 2013).

Distribution of assimilated energy is determined, through natural selection, by a set of heritable allocation 'rules' that maximizes fitness of the individual in a

given environment (Glazier & Calow, 1992; Niewiarowski & Roosenburg, 1993). But for any particular set of rules, the pattern of allocation need not be invariant. During environmental changes, phenotypic shifts in traits governing energy allocation may arise when different patterns of allocation increase lifetime reproductive success (Lardies & Bozinovic, 2006). Such phenotypic shifts are in many cases primarily mediated by plastic physiological reactions to the environment (Gienapp *et al.*, 2008; Sunday *et al.*, 2014), but there is, however, a limit to phenotypic plasticity. Unexpected environmental changes may impose energetic costs of plasticity beyond sustainable levels (DeWitt *et al.*, 1998), or the changes may fall entirely outside the phenotypic plasticity space of the organism. Under these circumstances, organisms with other allocation rule sets may be favoured (Mitton, 1997; DeWitt & Scheiner, 2004). Should such environmental changes persist they may invoke irreversible transgenerational shifts in rates of growth and reproduction in affected populations.

At present, widespread ocean acidification (OA) is occurring. Driven by an increase in global atmospheric

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$p\text{CO}_2$ from 280 μatm at pre-industrial times to the present day 400 μatm (IPCC, 2013), the global ocean mean surface pH has decreased from 8.13 to the present day 8.05. Ocean models predict a continuation of this trend with a further decrease in 0.4 pH units by the year 2100 (Caldeira & Wickett, 2005; Cao *et al.*, 2007). These changes, which are predicted to persist for thousands of years to come, will expose marine organisms to dramatically changed chemical conditions, and significant effects are predicted for many marine animals (Kroeker *et al.*, 2010; Dupont & Pörtner, 2013).

Significant transgenerational responses are expected during such persistent environmental change (Sunday *et al.*, 2011). Nevertheless, most studies have investigated only short-term physiological effects (Kelly & Hofmann, 2013). For copepods, adults have shown little physiological response to OA in some studies (Kurihara, 2008; Mayor *et al.*, 2012; McConville *et al.*, 2013; Zervoudaki *et al.*, 2014), while earlier larval stages and eggs seem more vulnerable (Fitzer *et al.*, 2012; Cripps *et al.*, 2014). Only a few previous studies on effects in offspring and following generations exist for copepods. One showed impairment of larval development beyond the second generation in *Tisbe battagliai* with significantly reduced copepodite size and changes in the chemical composition of the exoskeleton cuticle (Fitzer *et al.*, 2012). Another study showed no effects on egg production and hatching success in *Acartia tsuensis* (Kurihara & Ishimatsu, 2008).

Little is known about the transgenerational effects of parental environments, adaptation by genetic selection, or epigenetic changes in the offspring and the capacity of these processes to alleviate OA effects (Lim & Brunet, 2013; Munday *et al.*, 2013; Sunday *et al.*, 2014). While the studies mentioned above do provide information on changes from one generation to the next, they fail to discover if these changes originated in transgenerational effects (including genetic adaptation) or they were caused by phenotypic plasticity in each generation. Another study tested for parental effects by egg transplantation between environments (Vehmaa *et al.*, 2012). This study showed significant negative maternal effects of pH on egg-hatching success in *Acartia* sp. Thus, effects can be transmitted between generations, but again the study did not test for the origin or possible multigenerational consequences (including adaptation) of these effects. A few studies on other animal groups highlight the key role of adaptation and phenotypic plasticity. Reciprocal transplants of benthic polychaetes inhabiting areas containing underwater CO_2 vents have shown metabolic adaptation to high $p\text{CO}_2$ in *Platyneries dumerilii* but phenotypic plasticity in *Amphiglena mediterranea* (Calosi *et al.*, 2013). This study showed that adaptation and phenotypic plasticity are

equally viable strategies to tackle elevated $p\text{CO}_2$ environments. Another study on the sea urchin *Strongylocentrotus purpuratus* has shown that such adaptation may arise from natural selection for larvae with specific alleles in genes related to membrane composition and ion transport (Pespeni *et al.*, 2013).

In this study, we investigated transgenerational effects of OA in the calanoid copepod *Pseudocalanus acuspes*. Copepods of the *Pseudocalanus* genus are very abundant and widely distributed in temperate, subarctic, and Arctic waters (Aarbakke *et al.*, 2011) and at times they contribute more than one-third of the total zooplankton biomass in the North Atlantic (Head *et al.*, 2003; Thor *et al.*, 2005; Drif *et al.*, 2010). For this study, OA effects on fecundity and metabolism were tested at three different $p\text{CO}_2$ s (400, 900 and 1550 μatm) during two generations. The possibility that transgenerational effects could counter OA effects was tested by measuring egg production rates, egg clutch sizes and respiration rates during reciprocal transplant tests (DeWitt & Scheiner, 2004). While these tests detect transgenerational effects, they cannot distinguish between genetic adaptation and other transgenerational effects. Throughout this study, we therefore use the term 'transgenerational' in its literal sense to describe the transmission of trait changes from one generation to the next. As such, the term covers genetic, epigenetic, somatic and any other detectable trait change transmitted to the next generation.

Materials and methods

Copepods were caught in the Gullmar Fjord, Skagerrak (58°16'N, 11°26'E) by oblique tows of a 200 μm WP-2 plankton net equipped with a closed cod end. The net was towed from approximately 40 m to the surface. On board, the net samples were diluted in 25 L of 0.3 μm filtered sea water produced from water collected at 40 m. Samples were then brought to a cold room at the nearby Sven Lovén Centre for Marine Science, Kristineberg (SLC-K) set to the ambient water temperature during sampling (5 °C). In the laboratory, approximately 1000 adult *Pseudocalanus* spp. were sorted into two 40-L holding tanks until incubation start. The exclusive presence of *P. acuspes* was ensured by pipetting a subsample of approximately 100 individuals into RNAlater for molecular species identification by PCR. DNA was extracted using the DNeasy tissue DNA extraction kit (Qiagen), and PCR was run using primers specific for *P. acuspes*, *P. minutus* and *P. elongatus* (Gudmundsdottir, 2008; Grabbert *et al.*, 2010).

CO_2 incubations

Six unique copepod laboratory populations were established by transferring approximately 200 adults (generation F_0) to 40-L experimental tanks containing 0.3 μm filtered sea water. The water was then adjusted to three different $p\text{CO}_2$ s during

3 days (Table 1). F_0 females were allowed to produce eggs for 2 weeks. At this time, no F_1 copepodites had yet appeared. Tank volumes were then reduced to 10 L by siphoning off approximately 30 L from the inside of a large 50- μm sieve inserted into the tanks, all F_0 adults removed, and the F_1 generation was introduced into new clean tanks. This procedure was repeated for the transition from F_1 to F_2 . The copepods were reared at a 12 h/12 h light/dark cycle at 5 °C until generation F_2 reached maturity (137 days). Satiating food concentrations were achieved by pumping exponentially growing *Rhodomonas baltica* into the tanks (Table 1). Water was changed every second week by siphoning off 30 L as above and reintroducing copepods into new clean tanks.

pH was controlled by pH computers (Aqua Medic, Germany) in populations 3–6. The computers monitored pH and adjusted it to target levels by dynamically controlling bubbling of pure CO_2 into the tanks. CO_2 was administered to the bottom of the tanks at 1 bubble s^{-1} next to the aeration bubbling, which was kept at 4 bubbles s^{-1} to efficiently mix carbonized water into the tanks. The electrodes were placed inside the stream of bubbles to achieve fast responses and prevent undershooting target pHs during bubbling. Once a week, pH_{TS} was measured with a Metrohm 827 pH meter (Metrohm, Switzerland) calibrated against a four-point temperature TRIS standard curve, and total alkalinity was measured by titration of 25 mL samples in a SI Analytics Titroline potentiometric titrator (Riebesell *et al.*, 2010). pCO_2 s were calculated in CO_2 sys version 1.4 (Lewis & Wallace, 1998). Food concentrations were measured every second day on an Elzone 5380 electronic particle counter.

Transgenerational effects were tested by measuring egg production rates, clutch sizes, and respiration rates during reciprocal transplant tests on F_2 adults. Copepods were transplanted from 400 to 900 and 1550 μatm CO_2 and from 900 to 400 μatm CO_2 , and from 1550 to 400 μatm CO_2 (arrows in Figs 1 and 3 show transplant directions). For this purpose, each 400 μatm laboratory population was divided by volume in thirds, and every third was reintroduced to new tanks and adjusted to 400 μatm CO_2 , 900 μatm CO_2 and 1550 μatm CO_2 . Similarly, halves of each of the 900 and 1550 μatm laboratory populations were reintroduced to new tanks and adjusted to 400 μatm CO_2 and their original pCO_2 . Transplanted populations then consisted purely of adults. Incubations of all

original populations and transplants were then continued for 21 days to allow fertilization of unfertilized females and ensure a full egg production cycle before measurements. Egg production rates, egg clutch sizes and respiration rates were measured immediately thereafter.

The importance of phenotypic plasticity and transgenerational transmission of trait changes was estimated from changes in egg production, egg clutch size and respiration caused by transplantation. Phenotypic plasticity was inferred when changes were statistically similar in both transplant directions. Accordingly, transgenerational effects were inferred from statistically dissimilar changes between the two transplant directions (DeWitt & Scheiner, 2004).

Egg production and respiration measurements

Rates of respiration and egg production were measured in 10 or 6 replicate samples from all original populations and their transplants (10 replicates in original populations and six replicates in transplants). In each replicate, five egg-carrying females were transferred to 1.6-mL vials fitted with fluorescent O_2 foil discs (PSt3 spots, PreSens Precision Sensing, Germany) and filled with sea water adjusted to the corresponding pCO_2 . Vials were then sealed with Teflon caps, and O_2 concentrations were measured at 0, 3 and 6 h using a Fibox 3 optode system. Respiration rates ($\text{nmol O}_2 \text{ ind}^{-1} \text{ day}^{-1}$) were calculated by subtracting the average oxygen depletion rate measured in five controls from the oxygen depletion rate in the vials holding copepods, multiplying by vial volume and dividing by number of individuals in each vial. Prior testing of the optode system at 5 °C showed a 2 min 95% reaction time, that is the period of time taken before the output reached within 5% of the final oxygen concentration value (as estimated by exponential regression). Therefore, at every sampling, oxygen concentrations were read for 3 min, and an average of values read during the last minute was used for calculations.

Subsequently, all females were transferred to Petri dishes containing 0.3 μm filtered sea water, photographed under a stereoscope and incubated for 12–18 days for daily counts of carried eggs. One drop of *R. baltica* was administered to each dish every third day to prevent female mortality (final

Table 1 Average experimental conditions during the incubation period

Population	Temperature °C	pCO_2 μatm	pH_{TS}	A_{T} $\mu\text{mol kg}^{-1}$	Food conc. $\mu\text{gC L}^{-1}$
1	$5.68 \pm 0.50^{\text{a}}$	$410 \pm 96^{\text{a}}$	$8.05 \pm 0.07^{\text{a}}$	$2382 \pm 205^{\text{a}}$	$692 \pm 229^{\text{a}}$
2	$5.74 \pm 0.45^{\text{a}}$	$402 \pm 118^{\text{a}}$	$8.07 \pm 0.09^{\text{a}}$	$2454 \pm 100^{\text{a}}$	$761 \pm 173^{\text{a}}$
3	$5.36 \pm 0.76^{\text{a}}$	$890 \pm 107^{\text{b}}$	$7.75 \pm 0.09^{\text{b}}$	$2491 \pm 93^{\text{a}}$	$876 \pm 422^{\text{a}}$
4	$5.67 \pm 0.37^{\text{a}}$	$909 \pm 221^{\text{b}}$	$7.75 \pm 0.10^{\text{b}}$	$2473 \pm 121^{\text{a}}$	$769 \pm 225^{\text{a}}$
5	$5.53 \pm 0.66^{\text{a}}$	$1549 \pm 251^{\text{c}}$	$7.54 \pm 0.08^{\text{c}}$	$2470 \pm 93^{\text{a}}$	$707 \pm 240^{\text{a}}$
6	$5.55 \pm 0.45^{\text{a}}$	$1558 \pm 272^{\text{c}}$	$7.54 \pm 0.14^{\text{c}}$	$2547 \pm 165^{\text{a}}$	$736 \pm 138^{\text{a}}$

pH_{TS} is total scale pH, A_{T} is total alkalinity, and pCO_2 is CO_2 partial pressure. Food concentrations were transformed to $\mu\text{gC L}^{-1}$ according to Montagnes *et al.* (1994). Superscript letters indicate groups of values with no significant difference (1-factor ANOVAs with Holm–Sidak *post hoc* test: $P < 0.05$).

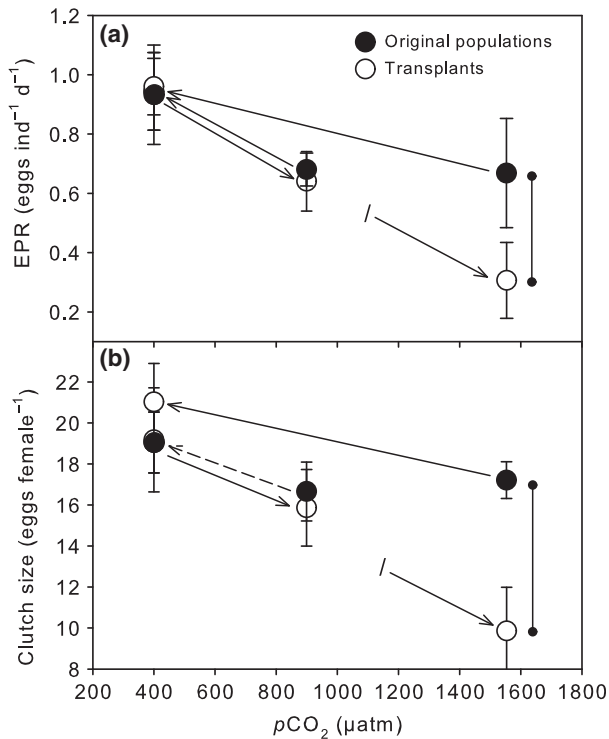


Fig. 1 (a) Egg production rates (EPR) and (b) egg clutch sizes in *Pseudocalanus acuspes* incubated at three $p\text{CO}_2$ s for two generations (137 days). Filled circles show results for second generation females of the originally incubated laboratory populations, and open circles show results for second generation females 3 weeks after transplantation to alternate $p\text{CO}_2$ s ($n = 6$ or 10 for each treatment). Error bars depict 95% confidence intervals. Arrows between filled and open circles show transplant directions. Arrows from $400 \mu\text{atm CO}_2$ to $1550 \mu\text{atm CO}_2$ are truncated to the left for clarity (shown by the dash). Significant and nonsignificant changes (Student–Newman–Keul’s and Holm–Sidak *post hoc* tests) are shown by solid and hatched arrows, respectively. Significant differences between transplant and original target populations are shown by vertical lines with point ends.

concentration ca. $300 \mu\text{gC L}^{-1}$). Egg production rates (eggs $\text{ind}^{-1} \text{day}^{-1}$) were calculated as the slopes of linear regressions on daily egg counts (day^{-1}) multiplied by initial number of eggs counted on the photographs (eggs female^{-1}) assuming egg production rates to equal hatching rates (Runge & Roff, 2000).

Data analysis

Because the design of the reciprocal transplantations included only two environmental states, the phenotypic changes were analysed as character state changes. This is opposed to the reaction norm analysis model, which would otherwise be used to test for phenotypic reactions to continuous scale variables such as $p\text{CO}_2$ /pH. However, it has been shown that the two models are mathematically similar, the character state model

being a special case of the reaction norm model (de Jong, 1995), and to avoid using the implicit regression analysis of the reaction norm model on two-point data, we applied the character state model. Accordingly, differences in means of egg production rates, clutch sizes and respiration rates were tested among populations (both original and transplant populations) by ANOVA. For these tests, the experimental units were the random samples of five females from each population (Hurlbert, 1984). Replication of populations enabled conclusions that observed differences were caused by divergent due to the $p\text{CO}_2$ and not by chance events during the incubations. This was taken into account by applying an ANOVA design where ‘population’ was nested within ‘ $p\text{CO}_2$ ’ population ($p\text{CO}_2$). Levene’s test was used to assure homoscedasticity.

Results

Egg production and respiration in original populations

After two generations, egg production rates were significantly depressed at 900 and $1550 \mu\text{atm CO}_2$ compared to at $400 \mu\text{atm CO}_2$. Rates were 0.945 ± 0.183 eggs $\text{ind}^{-1} \text{day}^{-1}$ (mean \pm SD) at $400 \mu\text{atm CO}_2$, 0.681 ± 0.078 eggs $\text{ind}^{-1} \text{day}^{-1}$ at $900 \mu\text{atm CO}_2$ and 0.668 ± 0.240 eggs $\text{ind}^{-1} \text{day}^{-1}$ at $1550 \mu\text{atm CO}_2$ (Fig. 1a, filled circles) (1-factor nested ANOVA, $p\text{CO}_2$: $F_{6,57} = 16.1$, $P < 0.001$, population($p\text{CO}_2$): $F_{6,57} = 0.99$, $P = 0.44$, Student–Newman–Keul’s *post hoc* test) (Table 2). While there were no significant differences in egg diameter relative to female prosome length among laboratory populations (0.124 ± 0.054 , mean \pm SD) (1-factor nested ANOVA: $F_{2,15} = 0.345$, $P = 0.71$), mean egg clutch sizes were also significantly reduced from 19.0 ± 2.1 eggs ind^{-1} at $400 \mu\text{atm CO}_2$ to 16.7 ± 2.0 eggs ind^{-1} at $900 \mu\text{atm CO}_2$ and 17.2 ± 1.2 eggs ind^{-1} at $1550 \mu\text{atm CO}_2$ (Fig. 1b, filled circles) (1-factor nested ANOVA, $p\text{CO}_2$: $F_{6,57} = 18.3$, $P < 0.001$ and population($p\text{CO}_2$): $F_{6,57} = 0.28$, $P = 0.95$, Student–Newman–Keul’s *post hoc* test) (Table 2).

Egg-hatching rates were constant during the 12–18 days the eggs and females were kept in Petri dishes so that the fraction of eggs remaining in clutches decreased linearly with time in all populations (Fig. 2) (linear regressions: $P < 0.05$). Egg-hatching rates (slopes in Fig. 2) decreased significantly with increasing $p\text{CO}_2$ among original populations (1-factor nested ANOVA, $p\text{CO}_2$: $F_{6,57} = 3.88$, $P = 0.003$, population ($p\text{CO}_2$): $F_{6,57} = 1.10$, $P = 0.38$). All eggs were hatched after 17 days, which indicates that the 21 days acclimation period after the transplantations were sufficient to allow full acclimation of egg production rates. Hence, assuming equal rates of egg production and hatching, an assumption necessary for estimates of egg production in egg-carrying copepods (Runge & Roff, 2000), all

Table 2 Results of Student–Newman–Keul's *post hoc* tests following ANOVA tests on (a) egg production rates, (b) egg clutch sizes and (c) respiration rates

Treatment	<i>n</i>	Subsets			
		1	2	3	4
(a) Egg production rate (eggs female ⁻¹ day ⁻¹)					
400	10			0.945	
900 to 400	6			0.935	
1550 to 400	6			0.962	
900	10		0.681		
400 to 900	10		0.641		
1550	10		0.668		
400 to 1550	6	0.307			
(b) Egg clutch size (eggs female ⁻¹)					
400	10			19.0	19.0
900 to 400	6			19.2	19.2
1550 to 400	6				21.0
900	10		16.7	16.7	
400 to 900	10		15.9		
1550	10		17.2	17.2	
400 to 1550	6	9.9			
(c) Respiration rate (nmol O ₂ ind ⁻¹ day ⁻¹)					
400	10	3.63	3.63		
900 to 400	6		3.89	3.89	
1550 to 400	6	3.37	3.37		
900	10			4.15	
400 to 900	10		3.94	3.94	
1550	10	3.07			
400 to 1550	6		3.91	3.91	

Similar results were obtained with Holm–Sidak *post hoc* tests. *n* is number of replicates. Significantly, equal groups are arranged into subsets and values show average rates. Observed power \geq is 0.99 for all three tests.

eggs were produced after the transplantations. Thus, egg production rates completely reflected the transplant environment.

Oxygen concentrations decreased between 14% and 21% during the 6-h respiration rate incubations, and all individuals appeared unharmed at the end of the incubation period. Respiration rates were significantly higher in the original 900 μ atm populations (1-factor nested ANOVA, $p\text{CO}_2$: $F_{6,53} = 7.49$, $P < 0.001$, population ($p\text{CO}_2$): $F_{6,53} = 0.56$, $P = 0.68$, Student–Newman–Keul's *post hoc* test) (Table 2). They increased from 3.63 ± 0.31 nmol O₂ ind⁻¹ day⁻¹ at 400 μ atm CO₂ to 4.15 ± 0.27 nmol O₂ ind⁻¹ day⁻¹ at 900 μ atm CO₂, but then decreased to 3.07 ± 0.53 nmol O₂ ind⁻¹ day⁻¹ at 1550 μ atm CO₂ (Fig. 3, filled circles).

Effects of reciprocal transplantations

The reciprocal transplant tests showed significant effects in egg production rates and clutch sizes, but not

in respiration rates (Figs 1 and 3). All transplantations induced significant changes in egg production rates (solid arrows in Fig. 1a). Furthermore, egg production rates were fully reversible between 400 and 900 μ atm CO₂. There were no significant differences between rates in the 400 to 900 μ atm transplants and rates in the original 900 μ atm populations. Similarly, there were no differences between rates in the 900 to 400 μ atm transplants and original 400 μ atm populations. Thus, changes in egg production rates were the result of phenotypic plasticity at this level of OA. On the other hand, while there were no significant differences between the 1550 to 400 μ atm transplants and original 400 μ atm populations, we found significant differences between the 400 to 1550 μ atm transplants and original 1550 μ atm populations. Hence, changes in egg production rates were not reversible between 400 to 1550 μ atm CO₂ indicating transgenerational transmission of changes in egg production rates. Clutch sizes showed similar effects. Although clutch sizes did not change significantly in the 900 to 400 μ atm transplants, all other transplantations had significant effects. This indicates that changes in clutch sizes were also induced by phenotypic plasticity between 400 to 900 μ atm CO₂, but the result of transgenerational effects between 400 and 1500 μ atm CO₂. The 400 to 1550 μ atm transplants showed a decrease in egg production rate to 0.31 eggs ind⁻¹ day⁻¹ and clutch size to 9.86 ± 2.03 eggs ind⁻¹. These correspond to decreases of 67% and 43%, respectively. In comparison, the original 1550 μ atm populations showed values for egg production rate and clutch size that were only 29% and 10% lower than in the 400 μ atm populations.

Although the original 400 and 900 μ atm populations showed significantly different respiration rates, there were no significant differences between any of the two transplants and the original populations at the target $p\text{CO}_2$ (Table 2). Hence, the differences in respiration rates between the 400 μ atm and 900 μ atm populations were caused by phenotypic plasticity. On the other hand, the 400 to 1550 μ atm transplants did show rates significantly different than in the original 1550 μ atm populations (Table 2). There was no difference between the reciprocal transplants and the original populations (the 1550 to 400 μ atm transplants and the original 400 μ atm populations), and this indicates a transgenerational development of different reaction norms of respiration at this level of OA.

Discussion

Pseudocalanus acuspes experienced significantly diminished fecundity when subjected to increased $p\text{CO}_2$ during two generations. But most importantly, trans-

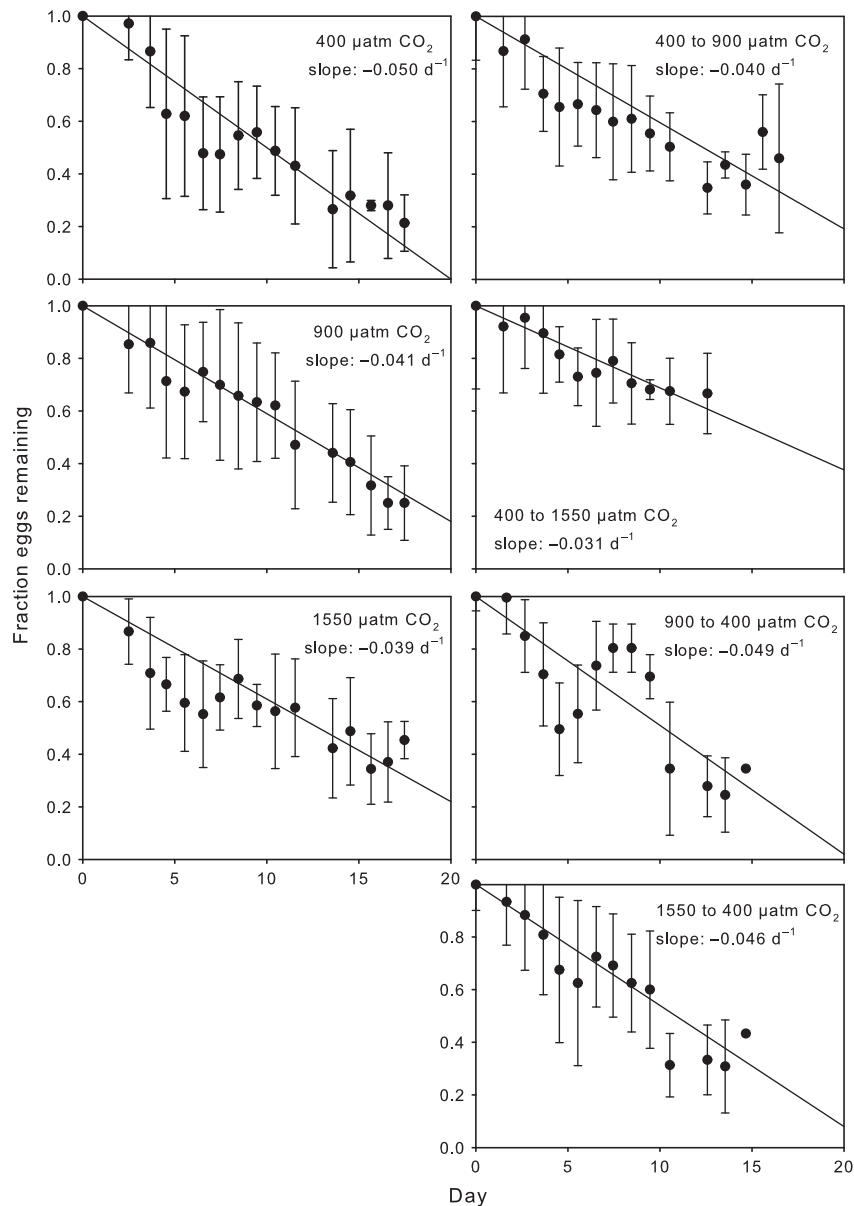


Fig. 2 Decline in mean (\pm SD) number of eggs clutch⁻¹ during the 12- to 18-day incubations for hatching rate estimates. Original laboratory populations (left panels) and transplants (right panels). Lines show mean slopes of all replicates ($n = 6$ or 10 for each replicate). The slopes correspond to mean hatching rates (day^{-1}), which were used to calculate egg production rates.

generational effects partly alleviated this loss at 1550 $\mu\text{atm CO}_2$, and the magnitude of the negative response was only half of what would have resulted from a response caused solely by phenotypic plasticity. All changes between 400 to 900 $\mu\text{atm CO}_2$ were fully reversible and attributable to phenotypic plasticity; hence, our data suggest that transgenerational effects set in at $p\text{CO}_2$ s above the present range of environmental variability (400–900 $\mu\text{atm CO}_2$, Dorey *et al.*, 2013).

The observed depressed egg production rates contrast with some previous short-term effect studies (<1 generation) in pelagic copepods. Studies on different

copepod species have shown no effects on mortality, egg production or larval development up to 2000 $\mu\text{atm CO}_2$ (Kurihara *et al.*, 2004; Kurihara & Ishimatsu, 2008; Mayor *et al.*, 2012; McConville *et al.*, 2013). The copepod exoskeleton contains little CaCO_3 , so no direct impairment by shell dissolution or decreased rates of calcification is expected. However, variations in other physiological processes in affected organisms, calcifiers and non-calcifiers alike, may instigate diminished performance. Recently, reduced egg production and increased naupliar mortality were observed in *A. tonsa* (Cripps *et al.*, 2014), a species otherwise known to toler-

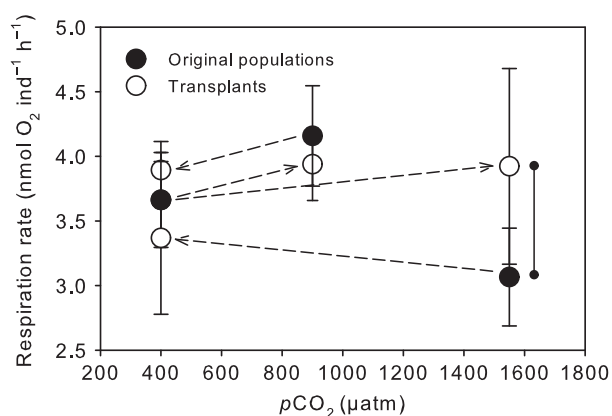


Fig. 3 Respiration rates in *Pseudocalanus acuspes* incubated at three $p\text{CO}_2$ s for two generations (137 days). Filled circles show rates for second generation females of the originally incubated laboratory populations, and open circles show rates for second generation females 3 weeks after transplantation to alternate $p\text{CO}_2$ s ($n = 6$ or 10 for each treatment). Error bars depict 95% confidence intervals. Arrows between filled and open circles show transplant directions. Significant and nonsignificant changes (Student–Newman–Keul’s and Holm–Sidak *post hoc* tests) are shown by solid and hatched arrows, respectively. Significant differences between transplant and original target populations are shown by vertical lines with point ends.

ate wide changes in water chemistry (Calliari *et al.*, 2006). Cripps *et al.* (2014) found significantly increased naupliar mortality and significantly decreased egg-hatching success already at 1000 μatm CO_2 and very severe effects at 2000 μatm CO_2 . Moreover, Lewis *et al.* (2013) found increased mortality in Arctic *Calanus glacialis* nauplii and *Oithona similis* nauplii and adults already at 700 μatm CO_2 . They observed more severe effects in *O. similis* than in *C. glacialis*, and they argue that *O. similis* is adapted to a more narrow range of pH due to the more shallow vertical migration patterns of this species. Both *P. acuspes* and *A. tonsa* show similar shallow migration compared to the *Calanus* species.

One possible explanation for the observed suboptimal performance may be less than optimal energy allocation due to environmental stress. For instance, due to the increased metabolic rates at 1271 μatm CO_2 , sea urchin pluteus larvae were not able to allocate more than 39–45% of the available energy to growth, while larvae growing under present-day conditions could allocate 78–80% of the available energy (Stumpp *et al.*, 2011). Such reallocation has also been hypothesized for the copepod *Tispe battagliai*. By combining data for growth, cuticle composition and nauplii production, Fitzer *et al.* (2012) argued that copepods will preferentially reallocate energy resources to maintain the reproductive output at the expense of larval growth. Our results showed considerable decoupling of respiration

and production so that respiration rates were significantly higher at 900 μatm CO_2 compared to 400 μatm CO_2 despite the decrease in egg production rate. As a result, the ratio between respiration and egg production was twice as high in the 900 μatm populations as in the 400 μatm populations. In unstressed individuals, respiration and production are coupled through specific dynamic action effects (SDA, i.e. increase in metabolic rate due to biosynthesis during food intake) and any change in production is inevitably followed by a similar change in respiration (Thor, 2000). The respiratorial decoupling observed in the 900 μatm populations suggests increased metabolic costs associated with elevated $p\text{CO}_2$. Several previous studies of other environmental stressors in copepods support this notion. For instance, copepods experiencing changes in prey quality exhibit significantly altered SDA (Thor *et al.*, 2002), and the respiration/egg production ratio (another proxy for SDA effects) increases in copepods experiencing stress related to decreasing salinity (Calliari *et al.*, 2006).

Knowledge on the influence of OA on cellular metabolic processes in copepods is scarce, and it is difficult to pinpoint what metabolic changes may be responsible for the decreased performance in *P. acuspes*. One may speculate that increased metabolism curtailed from increased expenses for maintaining homeostasis. Sea urchins, *Strongylocentrotus droebachiensis*, experience increased protein metabolism during prolonged exposure to increased $p\text{CO}_2$, and Stumpp *et al.* (2012) concluded that this is to support homeostasis by enhanced net proton extrusion. In most aquatic crustaceans, decreased cytosolic pH is counteracted primarily by ion exchange over the cell membrane (Henry & Wheatly, 1992). This process demands energy, and in Krill, *Euphausia superba*, elevated $p\text{CO}_2$ induces increased activity of enzymes involved in cycling of ATP and NADH, along with a decrease in body protein content (Saba *et al.*, 2012). We hypothesise that *P. acuspes* may have experienced similar effects at 900 μatm CO_2 and that this caused the observed decrease in egg production and increase in respiration rates.

The reciprocal transplantation tests allowed discrimination of effects into transgenerational effects and effects caused by phenotypic plasticity. These tests showed that the 29% decrease in egg production rate was partly a result of transgenerational effects. Without such effects, egg production rates would have decreased by as much as 67%. No previous studies address the importance of transgenerational effects to counter pH stress in copepods. Previous multigenerational studies have revealed deleterious effects in larval development during consecutive generations in the copepod *Tispe battagliai* (Fitzer *et al.*, 2012), but no

effects on egg production or larval development in another copepod species, *Acartia tsuensis* (Kurihara & Ishimatsu, 2008). Fitzer *et al.* (2012) applied a model to predict changes in larval development through the coming 100 years. Because this study applied a multi-generational approach, it incorporated possible evolutionary or transgenerational effects (although these could not be detected in the applied experimental design). This approach is probably the best we can offer in terms of predictions for the future without knowledge on transgenerational effects. However, as mentioned by the authors themselves, the model assumes an inability of the copepods to adapt. Much emphasis has been put on the importance of adaptation during future global change (Munday *et al.*, 2013; Sunday *et al.*, 2014). We found transgenerational effects already after two generations, which could be a result of adaptation. Consequently, predictions based on models like the one applied by Fitzer *et al.* (2012) will overestimate OA effects. Moreover, the transgenerational effects at 1550 $\mu\text{atm CO}_2$ induced an entirely different reaction to $p\text{CO}_2$ variations, with less steep reaction norms. The implication is that future generations will experience dampened reactions of fecundity also to natural short-term (<1 generation) variations in $\text{pH}/p\text{CO}_2$. The ecological ramifications are that future OA, at least to the level of 1550 $\mu\text{atm CO}_2$, may induce more resilience towards pH variability in *P. acuspes*. But whether or not this will override any other negative effects imposed by, for example, coselection in fitness-related traits responsible for buffering of other environmental variables remains to be seen.

Zooplankton, including copepods, are able to adapt to environmental changes (Dam, 2013). *Acartia hudsonica* has shown transgenerational alleviation of detrimental effects of frequent blooms of toxic dinoflagellates in traits such as ingestion, development and egg production (Colin & Dam, 2002), and subsequent studies indicate that these effects were in fact due to adaptation through evolutionary selection (Avery & Dam, 2007). Moreover, while *Tigriopus californicus* has shown surprisingly little capacity for adaptation to increasing temperature (Kelly *et al.*, 2012), *Scottolana canadensis* shows significant latitudinal transgenerational differentiation in development, adult body size and somatic growth. Unfortunately, this study did not provide any clue to the origin of the transgenerational effects; whether they are a result of natural selection or some nongenetic transgenerational transmission of trait changes (Lonsdale & Levinton, 1985). The observed transgenerational effects in the present study may be a consequence of both. There are indications in previous studies that suggest the possibility of evolutionary adaptation to OA in invertebrates. Allelic shifts

in genes coding for enzymes involved in membrane composition and cellular homeostasis have been observed in sea urchin pluteus larvae during OA perturbation experiments (Pespeni *et al.*, 2013). These shifts allowed physiological compensation to such a degree that normal developmental and morphological progress was upheld at 900 $\mu\text{atm CO}_2$. Adaptation through evolutionary selection can be fast and occur even from one generation to the next in invertebrates. The oligochaete *Tubifex tubifex* showed increased Hg tolerance after exposure for only one generation (Vidal & Horne, 2009). This tolerance was maintained in subsequent outcrosses with nontolerant control groups, a clear indication of natural selection for Hg tolerance. Accordingly, one could hypothesize that the changes observed in the present study were based in natural selection. But, they could also occur as a result of parental effects or nongenetic somatic and epigenetic transmission of trait changes (Lim & Brunet, 2013; Sunday *et al.*, 2014). Apart from the effects found in *Acartia* sp. mentioned above (Vehmaa *et al.*, 2012), such parental effects have been found in the oyster, *Saccostrea glomerata*, where parental exposure to 900 $\mu\text{atm CO}_2$ had positive carry-over effects on larvae. Larvae spawned from adults exposed to 900 $\mu\text{atm CO}_2$ were larger and developed faster than larvae from nonexposed adults (Parker *et al.*, 2012). Moreover, genetic selection and nongenetic transgenerational effects are not mutually exclusive. Nongenetic inheritance can decouple phenotypic change from genetic change over multiple generations, and this may allow adaptation to catch up with fast environmental changes like OA (Whitlock, 1995; Bonduriansky *et al.*, 2012). Finally, while we cannot rule out nongenetic transmission or combined effects, estimated mortalities were sufficiently high (>50% *per* generation) to allow significant selection and hence genetic adaptation in all four original high $p\text{CO}_2$ populations.

It has been argued that individuals selected for increased phenotypic plasticity resulting from changing environments may experience increased energetic costs (DeWitt *et al.*, 1998) and that this may induce detrimental effects in the adapted population. However, the less steep reaction norms of the 1550 μatm populations (i.e. the difference between original 1550 μatm populations and 1550 to 400 μatm transplants) in both egg production and hatching success suggest otherwise. While individuals from the original 400 μatm populations experienced severely reduced fecundity at 1550 $\mu\text{atm CO}_2$ (67% reduction), the 1550 μatm populations upheld an only 29% diminished fecundity compared to the 400 μatm populations. Moreover, the original 1550 μatm populations avoided the increase in respiration rates experienced by the 900 μatm

populations. Rather, respiration followed the decrease in egg production and was more tightly coupled to egg production probably through SDA effects (Thor, 2000). We hypothesize that the transgenerational effects may have altogether relieved the copepods from the metabolic stress they would otherwise have experienced at 1550 $\mu\text{atm CO}_2$.

The three tested scenarios allowed us to cover present day as well as near-future environmental oceanic $p\text{CO}_2$ variability (400–900 $\mu\text{atm CO}_2$) and beyond (1550 $\mu\text{atm CO}_2$). In the Gullmar Fjord, average pH varies between 8.04 and 8.17 pH units. However, pH variation is high, and values as low as 7.57 pH units have been recorded. The shift of all the values by 0.4 pH units, as projected for 2100, predict values ranging from pH 8.28 to 7.17, with a mean annual value of pH 7.71 (Dorey *et al.*, 2013). Our tested scenarios therefore correspond to (i) average pH experienced today (pH 8.05, $p\text{CO}_2$ 400 μatm); (ii) average pH in 2100 and extreme of present natural variability (pH 7.75, $p\text{CO}_2$ 900 μatm); and (iii) extreme pH in 2100 and outside of present natural variability (pH 7.54, $p\text{CO}_2$ 1550 μatm). Thus, our results suggest that *P. acuspes* react with phenotypic plasticity through increased respiration and re-allocation of resources within present day's environment. When exposed to elevated $p\text{CO}_2$ beyond present day's natural variability, transgenerational effects set in so that the copepods are able to avoid further decreases in fecundity.

Pseudocalanus acuspes exists in great numbers, and any serious effects on the genetic diversity of the global standing population are hard to imagine. But, the effects found in the present study may have other ecological implications. While a 29% decrease in fecundity is serious in terms of biomass production and trophic transport of matter, the 67% reduction without transgenerational buffering would have been catastrophic. Calanoid copepods constitute some 80% of the global zooplankton biomass (Mauchline, 1998), they support global fish stocks due to their importance as prey for many species during their larval life (Last, 1980), and *Pseudocalanus* constitute one of the most abundant genera. Stock recruitments of mackerel, *Scomber scombrus*, have been shown to covary with copepod biomass through at least two decades in the Gulf of St. Lawrence, and the emergence of two exceptional strong year classes were directly correlated with high abundances of calanoid copepods (Runge *et al.*, 1999; Castonguay *et al.*, 2008). Such effects have also been observed in the North Sea where survival of larval cod, *Gadus morhua*, has been shown to depend on size and abundance of their copepod prey (Beaugrand *et al.*, 2003). In the Baltic Sea deep basins, increase in rainfall

since the 1980s and lack of intrusion of high saline water from the North Sea have affected reproduction and maturation of a sibling species *P. elongatus* (Mollmann *et al.*, 2003). Investigations of stomach contents showed that herring, *Clupea harengus*, have been forced to revert to less favourable prey than *P. elongatus* during this period, and this has had serious implications for herring growth and development. In view of this, OA effects in copepods will not only affect copepod populations *per se* but have repercussions up through the food web. On the other hand, the transgenerational alleviation of OA effects found here may partly release the constraints of OA on the pelagic ecosystem.

Acknowledgements

We thank the staff at the Sven Lovén Centre for Marine Science at Kristineberg for their skilful help and B. Svenningsen for invaluable logistic support during experiments. We also thank P. Calosi and H. Dam for improvements to the manuscript. P. Thor was financially supported by the Norwegian Ministry of Climate and Environment through the Fram Centre Flagship 'Ocean acidification and ecosystem effects in northern waters', and S. Dupont was supported by the Linnaeus Centre for Marine Evolutionary Biology at the University of Gothenburg and the Swedish Research Councils VR and Formas (Linnaeus grant). Experiments were financially supported by the European Community through ASSEMBLE grant agreement no. 227799.

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