Multi-generational responses of a marine polychaete to a rapid change in seawater $pCO_2$

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Abstract

Little is known of the capacity that marine metazoans have to evolve under rapid $pCO_2$ changes. Consequently, we reared a marine polychaete, Ophryotrocha labronica, previously cultured for approximately 33 generations under a low/variable pH regime, under elevated and low $pCO_2$ for six generations. The strain used was found to be tolerant to elevated $pCO_2$ conditions. In generations F1 and F2 females’ fecundity was significantly lower in the low $pCO_2$ treatment. However, from generation F3 onwards there were no differences between $pCO_2$ treatments, indicating that trans-generational effects enabled the restoration and maintenance of reproductive output. Whilst the initial fitness recovery was likely driven by trans-generational plasticity (TGP), the results from reciprocal transplant assays, performed using F7 individuals, made it difficult to disentangle between whether TGP had persisted across multiple generations, or if evolutionary adaptation had occurred. Nonetheless, both are important mechanisms for persistence under climate change. Overall, our study highlights the importance of multi-generational experiments in more accurately determining marine metazoans’ responses to changes in $pCO_2$, and strengthens the case for exploring their use in conservation, by creating specific $pCO_2$ tolerant strains of keystone ecosystem species.

Introduction

Marine metazoans will face a series of significant changes in oceanic $pCO_2$ levels over the coming centuries. Firstly, increases in oceanic $pCO_2$ levels are predicted to occur at an unprecedented rate, leading to a subsequent reduction in pH; a phenomenon commonly termed ocean acidification (OA) (Doney et al. 2009). Secondly, the successful implementation of low-emissions policies (Frölicher and Joos 2010) and other climate change mitigation measures,
such as carbon capture and storage (Bickle 2009; Szulczewski et al. 2012), should result in a decrease of oceanic pCO2 levels, and thus a rise in seawater pH. Coastal environments will be particularly affected by acidification/‘de-acidification’ processes due to the unstable equilibrium of coastal water carbonate systems, which are affected by multiple human activities operating at various spatial and temporal scales (Strong et al. 2014). Thus, one of the major challenges in marine global change biology, as well as marine coastal conservation, is to accurately predict how marine metazoans will respond to rapid changes in pCO2. Whilst the large body of existing literature documents the potential negative impacts of elevated pCO2 conditions on a wide range of marine species (Kroeker et al. 2013; Wittmann and Pörtner 2013), the majority of studies have focussed on short-term, within-generation, effects of modern-day populations (Kroeker et al. 2013). Consequently, our understanding on the multi-generational responses of marine metazoans to rapid changes in pCO2 is limited (Munday et al. 2013; Reusch 2014; Sunday et al. 2014). This lack of knowledge is restricting our ability to accurately predict whether, and how, marine biodiversity will cope with expected environmental pCO2 changes.

For most marine metazoan species, exposure to changing pCO2 conditions will occur over multiple generations. Future populations may, therefore, be able to maintain current-day performance through the process of evolutionary adaptation. Evolutionary adaptation occurs when selection on existing genetic variation shifts the average phenotype of a population towards the fitness peak that matches its present environment (Sunday et al. 2014). A handful of studies have investigated marine metazoan populations naturally exposed to elevated pCO2 in situ, and show that evolutionary adaptation to OA conditions is possible (Maas et al. 2012; Calosi et al. 2013; Lewis et al. 2013; Pespeni et al. 2013). The rate at which such adaptation occurred, however, is unknown. During rapid climate change, evolutionary adaptation will most likely depend on the extent of existing phenotypic/genotypic variation within populations (Lande and Shannon 1996). Several breeding experiments, focussing on early life stages, have shown that sufficient phenotypic/genotypic variation exists within natural populations of marine metazoans, potentially enabling rapid evolutionary adaptation to changes in pCO2 conditions (e.g. Sunday et al. 2011; Foo et al. 2012; Kelly et al. 2013; Malvezzi et al. 2015). Many marine species, however, have complex life histories, and each life stage can respond to different selection pressures (Miller et al. 2013; Cripps et al. 2014). Therefore, focussing solely on early life stages may over- or under-estimate the potential for any adaptive response to a rapid change in pCO2 (Sunday et al. 2014).

Nongenetic inheritance mechanisms may also influence the potential for evolutionary adaptation to rapid climatic changes (Chevin et al. 2010; Bonduriansky et al. 2012; Klironomos et al. 2012; Gomez-Mestre and Jovani 2013). Trans-generational plasticity (TGP) is the process whereby the environment experienced by parents significantly alters the reactions norms (i.e. phenotypes), and thus fitness, of their offspring (Mousseau and Fox 1998). TGP has the potential to be adaptive, but may also have deleterious effects (Marshall and Uller 2007). Either way, TGP can be an important source of variation in performances between individuals, ultimately influencing short-term selection and the evolutionary trajectories of populations (Mousseau and Fox 1998; Badyaev and Uller 2009; Bonduriansky et al. 2012). TGP has largely been shown to increase offspring fitness of marine metazoans in response to changes in pCO2. For example, Miller et al. (2012) showed that pre-conditioning adult anemone-fish, Amphiprion melanopus, to elevated pCO2 mediated the negative impacts on juvenile growth, survival and metabolic rates (see also Allan et al. 2014; Murray et al. 2014; Pedersen et al. 2014; Parker et al. 2015). However, the extent to which TGP influences the next generation can depend on whether the parental population has experienced within-generation acclimation; the process by which an individual alters its physiological, behavioural or morphological characteristics through phenotypic plasticity to better suit an environment (Munday et al. 2013). Recently, Dupont et al. (2013) showed that the pre-exposure of adult sea urchins to elevated pCO2 for 4 months had a negative effect on larval survival. In contrast, after 16 months of pre-exposure, during which adults had acclimated, the negative effect on larval survival was no longer detected (see also Donelson et al. 2012; Suckling et al. 2014). Furthermore, the majority of trans-generational experiments have been restricted across one, or maximum two, generations leaving it unclear whether or not adaptive TGP can extend into future generations allowing time for evolutionary adaptation to catch up (Chevin et al. 2010), or even facilitate the process (Pigliucci et al. 2006; Crispo 2007; Bonduriansky et al. 2012; Gomez-Mestre and Jovani 2013).

Breeding and trans-generational studies to-date have provided valuable insights into the evolutionary potential of marine metazoans to rapid changes in pCO2 conditions. However, there is still an urgent need for longer term experiments that encompass all life-history stages, across multiple generations, to more accurately predict how populations of marine metazoans might respond to the rapid changes in pCO2 expected to occur (Munday et al. 2013; Sunday et al. 2014). Here, we firstly report the results of a multi-generational exposure experiment in which we reared a laboratory strain of a marine polychaete, Ophryotrocha labronica La Greca and Bacci 1962; for six generations under elevated (1000 μatm) and low (400 μatm) pCO2 conditions. The O. labronica strain used was found to perform better under
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Elevated pCO₂ conditions (generation F1). Secondly, to disentangle whether our observed multi-generational responses were driven by phenotypic plasticity or by evolutionary adaptation (Calosi et al. 2013; Sunday et al. 2014; Thor and Dupont 2015) we report the results of a reciprocal transplant assay experiment between pCO₂ treatments with F7 individuals. Throughout the experiment we measured a range of fitness-related life-history traits, as well as metabolic rates as a proxy for physiological performance. *Ophryotrocha labronica* is a globally distributed benthic polychaete typically found in heterogeneous, organically enriched environments (e.g. fouling communities in harbours, Prevedelli et al. 2005; Simonini et al. 2009). Additionally, it is an excellent species for multi-generational studies as it can be easily cultured under laboratory conditions and has a short generation time (~17 days at 27°C; Åkesson 1976). It was hypothesized that the multi-generational exposure to low pCO₂ conditions would enable worms to initially increase, and then maintain, performance levels similar to those observed under elevated pCO₂ conditions. Although we were specifically investigating the response of an elevated pCO₂ tolerant population to low pCO₂ levels, something that is not an immediate conservation issue as compared with OA studies, the results from this study will give valuable insight into how marine metazoans may respond to rapid pCO₂ changes in general.

**Materials and methods**

**Animal collection and husbandry**

The laboratory strain of *O. labronica* used originated from >40 individuals collected in June 2008 in the harbour of Porto Empedocle (Sicily, Italy; 37°17′4″N, 13°31′3″E). The laboratory strain was housed at the Marine Biology and Ecology laboratory of the University of Modena and Reggio Emilia (Modena, Italy) in culture for approximately 30 generations at relatively constant salinity (mean ± SD: 35 ± 2) (obtained by dissolving an artificial sea salt – Reef Crystals, Instant Ocean – in distilled water) and photoperiod (L:D of 12:12 h), but at variable temperature (min/max = 12/30°C) to mimic natural seasonal variation (Massamba-N’siala et al. 2012). In Nov 2012, 120 individuals from a population of approx. 1200 were transported to the Marine Biology and Ecology Research Centre (MBERC) of Plymouth University (Plymouth, UK) and kept for a further three generations at conditions as close as possible to those experienced by the worms just before collection in the laboratory of Modena (T = 20 ± 1°C, S = 34 ± 1 (0.22 µm filtered natural sea water), L:D of 12:12 h).

**Pre-experimental phase: pH regimes of the worm cultures**

Initially, we did not have details of the pH regime experienced by the worms in culture for the ~33 generations prior to the start of our experiment. However, our discovery that the strain of *O. labronica* had greater fitness under elevated pCO₂ conditions in generation F1 prompted us to monitor the pH of the culture. Daily pH measurements for 3 months at MBERC revealed that the worms in culture experienced on average low, but highly variable, pH conditions (Fig. 1; 7.71 ± 0.20) most likely resulting from the feeding and maintenance protocol employed. Briefly, worm cultures were water changed every 7–10 days (the high peaks) and fed on the same day by adding 0.75–1 mL of spinach minced in sea water (300 g L⁻¹). The fermentation of spinach during the periods between water changes caused recurrent and persistent reductions in pH (Fig. 1). Although pH data were not collected from the cultures of *O. labronica* at the University of Modena and Reggio Emilia, as the feeding and maintenance protocol of these cultures were identical in the two laboratories we are confident that they too experienced a similar pH regime for the 30 generations in which they were in culture there. In fact, pH measurements of the cultures subsequently made at the University of Modena and Reggio Emilia revealed that pH levels varied depending on how frequently water changes were performed, and ranged from 8.2 to 7.4 (G. Massamba-N’siala, personal communication).

**Experimental setup and physico-chemical parameters**

The experimental CO₂ and temperature manipulation system was a modified version of that described in PISTEVOS et al. (2011). Briefly, the system comprised of two trays (60 cm × 30 cm × 15 cm, vol. 13 L), half-filled with deionized water. Each tray housed eight air-tight experimental chambers (four per pCO₂ treatment). Three chambers per pCO₂ treatment contained a six-well culture plate (Corning Ltd, Sunderland, UK), filled with filtered natural sea water (0.22 µm, S = 33), which contained the worms (three broods of offspring and three adult pairs per chamber). The

![Figure 1: Mean pH of the *Ophryotrocha labronica* worm laboratory cultures measured daily for a 3-month period.](https://onlinelibrary.wiley.com/doi/abs/10.1111/eva.12344)
fourth chamber in each treatment contained a glass dish
with filtered natural sea water (0.22 μm, S = 30) at the same
temperature and pH of the wells, which was used for daily
partial water changes of the culturing wells. Culture plates
were covered with a breathable sealing film (Aerasel; Alpha
Laboratories Ltd, Eastleigh, UK), which allowed gas
exchange whilst reducing evaporation, and thus avoiding
large salinity and temperature fluctuations.

Elevated pCO₂ conditions were achieved by mixing ambient air, supplied by an aquarium air pump (Mistral
4000; Aqua Medic, Loveland, CO, USA), with CO₂ gas
using adjustable airline gang vales (Algarde; Armitage Pet
Care, Nottingham, UK) to produce a nominal pCO₂ con-
centration of 1000 μatm (year 2100 predicted levels -
IPCC, 2013). The CO₂ content of the resultant gas mixture
was measured using a CO₂ analyser (LI-840A; LI-Cor,
Lincoln, NE, USA), and then supplied to each chamber
via an airline connected to a de-capped micro centrifuge
tube (Eppendorf) inserted through the top of the chamber.
Low pCO₂ conditions were achieved via supplying air
with an aquarium air pump (Mistral 4000; Aqua Medic)
to each experimental chamber, and represented present-day
levels. The experimental system was maintained at 27°C,
as this temperature produced fast generation times
(Akesson 1976) whilst still being within the thermal range
naturally experienced in the summer months (Massamba-
N’Siala et al. 2012). The temperature in the water in each
tray was controlled by a re-circulating water bath (RS;
Grant Instruments Cambridge Ltd, Herts, UK). Addition-
ally, each tray contained two circulation pumps (Koralia
nano 900; Hydor, Sacramento, CA, USA) to ensure an
even temperature distribution within the tray (max tem-
perature fluctuation recorded during the experiment was
1°C).

Throughout the experiment, all wells were observed daily
using a custom-built bio-imaging system for aquatic ani-
imals (Tills et al. 2013). Polychaetes were fed daily ad libitum
on minced spinach (Massamba-N’Siala et al. 2011, 2012).
Uneaten spinach was removed with daily partial water
changes to maintain good water quality. Additionally, tem-
perature, salinity and pH were measured daily in one ran-
domly chosen well per experimental chamber. Up to twice a
week, seawater samples from each treatment were collected
to determine total alkalinity. This was done by transferring
the water from the glass dish in the fourth experimental
chamber of each treatment to a borosilicate bottles
(vol. = 150 mL), immediately poisoned with mercuric
chloride solution (30 μm, conc. = 0.02%) and kept in the
dark prior to analysis. Total alkalinity was measured using
an automated acid-base alkalinity titrating system (AS-
ALK2; Apollo SciTech Inc, Bogart, GA, USA). Carbonate
system parameters not measured directly were calculated
using CO₂SYS (Pierrot et al. 2006) and the Mehrbach con-
stants (Mehrbach et al. 1973) refitted by Dickson and Mill-
ero (1987). Seawater parameters are presented in Table 1.

### Experimental design

**Experiment one: multi-generational exposure**

To obtain a large enough population of juveniles to
conduct the multi-generational experiment (Fig. 2), 16
breeding pairs were formed from individuals of the
MBERC laboratory culture, acclimated (1°C h⁻¹) to
27°C. Seventy-two hours after hatching, 20 juveniles
from each pair were haphazardly assigned to either the
elevated or low pCO₂ treatment. Juveniles were not
moved on the day of hatching as preliminary observa-
tion showed that handling of juvenile in the first 72 h
from hatching could result in high mortality levels
independent of pCO₂ concentrations. Juvenile growth
rates and survival were determined 7 days posthatching
(see below). When the first reproductive event was
observed new pairs were formed by crossing individuals
from different parents to avoid inbreeding, and placed
into a new well within the same multi-well plate. Spare
worms were kept in their well until offspring from the
pairs made were ready to be transferred to a new well
and then removed. The first egg mass spawned by a
pair was used to produce offspring for the next genera-
tion. The second egg mass was used to determine
fecundity and egg volume. If a male died before the
second egg mass was produced, it was replaced so that
the reproductive performance of females could still be
determined (as in Massamba-N’Siala et al. 2011, 2012).
For this purpose, spare males of the current generation
were kept in the Boveri glass bowl in the fourth exper-
imental chamber of the corresponding treatment. Adult

![Table 1. Physico-chemical parameters of seawater for the elevated and low pCO₂ treatments. Values are means ± 1 SD for pH (NBS scale), salinity, temperature, total alkalinity (TA), carbon dioxide partial pressure (pCO₂), bicarbonate and carbonate ion concentration ([HCO₃⁻] and ([CO₃²⁻]), and calcite and aragonite saturation states (Ω(calc) and Ω(ara)).](image_url)

| Parameter      | Elevated | Low       |
|----------------|----------|-----------|
| pH             | 7.68 ± 0.06  | 7.99 ± 0.06  |
| Salinity       | 34.05 ± 1.04  | 33.76 ± 1.24  |
| Temperature (°C) | 27.11 ± 0.46  | 27.16 ± 0.53  |
| TA (μequiv kg⁻¹) | 2178.77 ± 178.12  | 2201.66 ± 70.05  |
| pCO₂ (μatm)    | 1137.28 ± 193.05  | 461.91 ± 36.21  |
| [HCO₃⁻] (μmol kg⁻¹) | 1955.76 ± 167.23  | 1759.04 ± 58.48  |
| [CO₃²⁻] (μmol kg⁻¹) | 92.58 ± 10.76   | 182.47 ± 14.52  |
| Ω(calc)        | 2.23 ± 0.26    | 4.39 ± 0.35   |
| Ω(ara)         | 1.47 ± 0.17    | 2.89 ± 0.23   |

Superscript capital letters indicate a significant difference between treatments by way of GLM’s (P < 0.05).
size was determined the day that a female produced its second egg mass. The day after a pair had produced their second egg mass, metabolic rates of the female were measured and the male was removed. The above procedure was repeated up until the stage where generation F6 reached maturity. Adult life-history traits, as well as metabolic rates, were only measured in females because their contribution to life-history depiction is more relevant than that of males (Stearns 1992; Masaamba-N’Siala et al. 2011).

**Experiment two: reciprocal transplant assays**

Once F6 juveniles reached maturity, five females per brood were moved into a separate well until they reached a sufficiently large size to ensure enough offspring could be produced in their first brood to perform reciprocal transplants between pCO₂ treatments. Consequently, data were not collected for generation F6 adults. Females were then paired up with males from different parents as in all previous generations. From each pair, 20 juveniles were moved to a different well in the same pCO₂ treatment as their parents (control assay) and 20 juveniles were moved to a well in the other pCO₂ treatment (reciprocal assay) 72 h posthatching (Fig. 2). The experimental procedure followed was identical to that described for generations F2–F5, except that the first three egg masses spawned by a pair were used to determine fecundity, and that metabolic rates of females were measured the day after they spawned their third egg mass and were only carried out haphazardly on half of the females.

**Determination of life-history traits**

Juvenile growth rates were determined by counting the number of chaetigers (i.e. segments bearing bristles) of five randomly selected individuals per brood using the bio-imaging system. Juvenile growth rates were standardized to the number of chaetigers added per day (number of chaetigers day⁻¹).

Juvenile survival per well was measured by counting the number of individuals in a well under low power magnification (×10) (MZ12; Leica, Solms, Germany), and was expressed as the percentage of the total number of individuals at time zero.

Female fecundity was measured as the number of eggs produced per chaetiger (number of eggs chaetiger⁻¹) to account for any effect due to differences in body size (Masaamba-N’Siala et al. 2011, 2012). Counts were made under low-medium magnification (×50) (MZ12; Leica).

Adult size was determined using the bio-imaging system by counting the number of chaetigers a female had on the day it produced its second egg mass.

Egg volume, a proxy for egg quality (Allen and Marshall 2014), was determined by taking a picture of the second egg mass using the bio-imaging system. The longest and shortest axes of 10 eggs were then measured using imageJ.
measurements began. The decline in oxygen (pO2) in sea water within the respirometry chambers was determined using a modified version of the method described by Calosi et al. (2013) for small-size worms. Briefly, polychaetes were placed individually into a respirometer chamber (vol. = 0.12 mL), filled with filtered sea water (0.22 μm, S = 33–34) of the original pCO2 treatment. Once sealed, the chambers were placed inside a water bath to maintain constant temperature (T = 27 ± 0.5°C). All individuals were allowed to settle for 30 min before measurements began. The decline in pO2 within each respirometer was determined using an optical oxygen analyser system (GEN III 5000 series; OxySense, Dallas, TX, USA). Measurements of pO2 were made at regular intervals (20–30 min) for every respirometer for a period of approx. 1.5 h, and the pO2 in the respirometer never fell below 70% O2 saturation to avoid polychaetes experiencing hypoxia. MO2 was calculated as the change in pO2 h−1 from the linear least-squares regression of pO2 (mbar) plotted against time (min). This was multiplied by the solubility coefficient for oxygen, adjusted for salinity and temperature (Green and Carré 1967), and the volume of water within each respirometer. Blanks were also run to correct for any microbial respiration in the sea water. MO2 values were expressed as μmol O2 h−1 STPD.

Determination of metabolic rates

Oxygen uptake was measured as a proxy for metabolic rate (MO2) using closed system respirometry. The partial pressure of oxygen (pO2) in sea water within the respirometry chambers was determined using a modified version of the method described by Calosi et al. (2013) for small-size worms. Briefly, polychaetes were placed individually into a respirometer chamber (vol. = 0.12 mL), filled with filtered sea water (0.22 μm, S = 33–34) of the original pCO2 treatment. Once sealed, the chambers were placed inside a water bath to maintain constant temperature (T = 27 ± 0.5°C). All individuals were allowed to settle for 30 min before measurements began. The decline in pO2 within each respirometer was determined using an optical oxygen analyser system (GEN III 5000 series; OxySense, Dallas, TX, USA). Measurements of pO2 were made at regular intervals (20–30 min) for every respirometer for a period of approx. 1.5 h, and the pO2 in the respirometer never fell below 70% O2 saturation to avoid polychaetes experiencing hypoxia. MO2 was calculated as the change in pO2 h−1 from the linear least-squares regression of pO2 (mbar) plotted against time (min). This was multiplied by the solubility coefficient for oxygen, adjusted for salinity and temperature (Green and Carré 1967), and the volume of water within each respirometer. Blanks were also run to correct for any microbial respiration in the sea water. MO2 values were expressed as μmol O2 h−1 STPD.

Statistical analysis

The effects of pCO2 treatment, ‘generation’ and their interaction (experiment one) on all traits was tested using general linear models (GLM’s), with ‘tray’ as a random factor nested within pCO2 treatment, ‘tub’ as a random factor nested within pCO2 treatment × ‘tray’ and ‘well’ as a random factor nested within pCO2 treatment × ‘tray’ × ‘tub’ (juvenile growth rates and egg volume only). Additionally, body size was used as a covariate for MO2 data. Random, covariate factors and interactions that had no significant effect (P > 0.05) were systematically removed one at a time (highest P value first) from the analysis, until only significant factors and the main effects were left. ‘Well’ had a significant effect on juvenile growth rates (F34, 1093 = 2.71, P < 0.001). ‘Tub’ had a significant effect on juvenile survival, adult size, metabolic rates and egg volume (min F10,170 = 2.09, P = 0.028). However, removing these factors from the analysis did not change the patterns of significance of the main factors, and thus effects were considered marginal. Data from the control transplants of the reciprocal transplant assay experiment in generation F7 were included in the analyses. The effect of reciprocal transplant assays (experiment two) on all traits was analysed using the same design, but with ‘exposure treatment’ and ‘assay treatment’ set as fixed factors.

Except for fecundity and metabolic data (max. KS36 = 0.106, P = 0.200, Kolmogorov–Smirnov’s test), data did not meet the assumptions of normality despite Log10 transformations (min. KS36 = 0.144, P < 0.001). Variances were homogenous for adult size in experiment one and for all data in experiment two (max. F5,67 = 2.36, P = 0.079, Levene’s test), except egg volume (F5,706 = 3.42, P = 0.017). For all other datasets assumptions were not met following Log10 transformations (min. F1,107 = 2.45, P = 0.005). However, given the size of our experiment and replication, we assumed the GLM design employed should be tolerant to deviation from the assumptions of normality and heteroscedasticity (Sokal and Rohlf 1995; Underwood 1997). Nonetheless, we also tested the residuals from each analysis against the factors tested with GLM’s and no significant relationships were detected (P > 0.05). All pair-wise comparisons were conducted using 95% confidence interval levels (95% CI). All analyses were conducted using SPSS 21 (IBM, Armonk, NY, USA).

Results

Experiment one: multi-generational exposure

All data produced from the multi-generational exposure experiment are presented in Table S1. The only trait significantly affected by multi-generational exposure to low pCO2 was fecundity (Fig. 3A; F5,157 = 3.53, P = 0.004). In generation F1, mean fecundity in the elevated pCO2 treatment (6.70 eggs chaetiger−1) was significantly greater than in the low pCO2 treatment (3.55 eggs chaetiger−1; P < 0.05). Similarly, in generation F2 fecundity was significantly greater in the elevated pCO2 treatment (8.12 eggs chaetiger−1) compared to the low pCO2 treatment (6.82 eggs chaetiger−1; P < 0.05). However, this difference did not reappear in subsequent generations (Fig. 3A; P > 0.05).

For all traits, except egg volume (F5,2078 = 0.25, P = 0.942), a significant generation effect was observed (Table 2; min. F5,180 = 2.67, P = 0.023). Mean juvenile growth rates, fecundity and adult size all significantly increased across generations. For all traits, mean values in generation F1 were significantly lower compared to generation F7 (P < 0.05). However, despite a significant generation effect in juvenile survival and metabolic rate data, no
The elevated CO₂ treated worms exhibited significantly greater fitness (i.e. fecundity) under low and variable pH conditions in the laboratory. The worms used in this study exhibited significantly reduced fitness (i.e. fecundity) when initially exposed to low pCO₂ conditions (generations F1 and F2). However, we cannot fully discount that our observed tolerance to elevated pCO₂ conditions was driven by pre-experimental conditions in the laboratory, other than pH, as we did not have access to worms that had been maintained in culture under stable high pH conditions. Nonetheless, long-term
Table 2. Mean generation values ±95% CI for all traits measured for the marine polychaete Ophryotrocha labronica.

| Trait                         | F1   | F2   | F3   | F4   | F5   | F6   | F7   |
|-------------------------------|------|------|------|------|------|------|------|
| Juvenile growth rates         | 0.92 ± 0.04<sup>a</sup> (80) | 1.25 ± 0.02<sup>b</sup> (180) | 1.31 ± 0.03<sup>c</sup> (175) | 1.41 ± 0.03<sup>d</sup> (170) | 1.46 ± 0.03<sup>e</sup> (170) | 1.34 ± 0.03<sup>f</sup> (180) | 1.40 ± 0.03<sup>g</sup> (180) |
| Juvenile survival (%)         | 79.69 ± 10.10<sup>a,b</sup> (16) | 83.33 ± 3.78<sup>a</sup> (36) | 80.29 ± 4.96<sup>b</sup> (35) | 87.22 ± 4.29<sup>a</sup> (36) | 89.71 ± 3.51<sup>b</sup> (34) | 88.61 ± 2.80<sup>b</sup> (36) | 83.47 ± 2.46<sup>a,b</sup> (36) |
| Adult size (number of chaetigers) | 14.21 ± 0.34<sup>a</sup> (34) | 15.37 ± 0.30<sup>b</sup> (35) | 15.44 ± 0.28<sup>b</sup> (34) | 15.89 ± 0.21<sup>c</sup> (35) | 15.75 ± 0.33<sup>b</sup> (36) | 15.40 ± 0.22<sup>a</sup> (35) |
| Fecundity (number of eggs chaetiger<sup>-1</sup>) | 5.13 ± 0.83<sup>a</sup> (34) | 7.45 ± 0.48<sup>b</sup> (35) | 9.85 ± 0.72<sup>c,d</sup> (34) | 10.38 ± 0.81<sup>d</sup> (35) | 9.46 ± 0.82<sup>c,d</sup> (36) | 8.55 ± 0.48<sup>c</sup> (35) |
| Egg volume (x 10<sup>3</sup> mm<sup>3</sup>) | 0.61 ± 0.01 (340) | 0.60 ± 0.01 (350) | 0.62 ± 0.01 (340) | 0.61 ± 0.01 (350) | 0.61 ± 0.01 (360) | 0.61 ± 0.01 (350) | 0.61 ± 0.01 (350) |
| Metabolic rates               | 0.23 ± 0.02<sup>a</sup> (34) | 0.27 ± 0.05<sup>b</sup> (35) | 0.27 ± 0.02<sup>a</sup> (34) | 0.27 ± 0.02<sup>b</sup> (35) | 0.29 ± 0.02<sup>d</sup> (36) | 0.29 ± 0.04<sup>b</sup> (18) |

Capital letters represent significant differences (P < 0.05) between generations. Numbers of replicates are provided in parentheses.
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Table 3. Mean values ± 95% CI for all traits measured in the marine polychaete O. labronica in the reciprocal transplant assay experiment.

| Trait                                    | Transplant assay |
|------------------------------------------|------------------|
|                                          | Elevated-Elevated| Elevated-Low   | Low-Low         | Low-Low-Elevated|
| Juvenile growth rates (number of chaetigers day⁻¹) | 1.40 ± 0.03 (90) | 1.40 ± 0.04 (85) | 1.40 ± 0.04 (90) | 1.39 ± 0.04 (90) |
| Juvenile survival (%)                    | 82.22 ± 4.09 (18) | 78.23 ± 4.59 (17) | 84.72 ± 2.91 (18) | 79.44 ± 5.42 (18) |
| Adult size (number of chaetigers)        | 15.41 ± 0.29 (17) | 15.12 ± 0.33 (17) | 15.39 ± 0.32 (18) | 14.94 ± 0.24 (18) |
| Fecundity (number of eggs chaetiger⁻¹)   | 22.25 ± 1.33 (17) | 19.54 ± 1.21 (17) | 21.44 ± 1.38 (18) | 18.67 ± 1.61 (18) |
| Egg volume (x10⁻³ mm³)                   | 0.62 ± 0.02 (170) | 0.59 ± 0.02 (170) | 0.60 ± 0.02 (180) | 0.60 ± 0.02 (180) |
| Metabolic rates (µmol O₂ h⁻¹ STPD)       | 0.28 ± 0.05 (9)   | 0.31 ± 0.03 (9)   | 0.29 ± 0.06 (9)   | 0.27 ± 0.03 (9)   |

Capital letters represent significant differences (P < 0.05) between treatments. Numbers of replicates are provided in parentheses.

Figure 4 Experiment 2: reciprocal transplant assays. Mean fecundity of O. labronica individuals of the F7 generation exposed to either an elevated or low pCO₂ and then transplanted to same (control assay) or other pCO₂ treatments (reciprocal assay). Capital letters represent significant differences (P < 0.05) between transplant treatments. Bar charts represent mean values ±95% CI. Arrows represent reaction norms between control and reciprocal transplants. Numbers of replicates are reported in Table 3.

Dupont et al. 2013; Suckling et al. 2014), as previously discussed. Within-generation acclimation can refer to both nonpermanent responses (i.e. reversible acclimation), that occur when an organism is exposed to short-term environmental fluctuations, and permanent, irreversible responses that are mainly established during early ontogeny (i.e. developmental acclimation) (West-Eberhard 2003; Angilletta 2009). Ophryotrocha labronica undergoes direct development. In generation F1, offspring were initially moved to low pCO₂ conditions 3 days posthatching. As a consequence, juvenile worms may have experienced limited developmental acclimation. Therefore, it is possible that the acclimation to low pCO₂ conditions we observed in generation F1 was reversible, and consequently not sufficient for TGP to be fully expressed across one generation. Phenotypic plasticity may take at least two generations to be fully expressed, as seen in this study, due to the cumulative effects of different forms of acclimation (e.g. developmental and trans-generational) (Munday et al. 2013).

Potential mechanism for the observed TGP

Trans-generational plasticity can occur through a variety of mechanisms, including the transfer of nutritional (e.g. maternal provisioning) and molecular (e.g. epigenetic) material (Bonduriansky et al. 2012). In this study, there was no difference in egg volume (a proxy for egg quality) between pCO₂ treatments in any generation, indicating no apparent differences in maternal provisioning (see also Miller et al. 2012; Shama et al. 2014). Trans-generational epigenetic effects (Jablonska and Raz 2009; Ho and Burggren 2010) may therefore have been the causative mechanism for restoring and maintaining routine energy allocation, and subsequently fecundity, under low pCO₂ conditions. Trans-generational epigenetic effects were also thought to be responsible for mediating the negative impacts that elevated pCO₂ had on juvenile anemonefish, A. melanopus (Miller et al. 2012). The extent to which epigenetic effects are induced, is suggested to be dependent upon which life-stage experiences the stressor, being greater in earlier life stages (Burton and Metcalfe 2014). Consequently, the fact that offspring were initially exposed to low pCO₂ in generation F1, 3 days posthatching, may have meant that epigenetic effects were not fully induced. This again could potentially explain why it took two generations for our observed TGP to be fully expressed. Irrespective of the mechanism involved, our study adds to the growing body of evidence showing that TGP can be an effective mechanism in buffering populations of marine metazoans against the negative effects of changes in pCO₂ and other climate change stressors (e.g. Jensen et al. 2014; Munday 2014; Pedersen et al. 2014; Parker et al. 2015).

Consequences of multi-generational exposure to low pCO₂ conditions

Reciprocal transplant assay experiment: TGP or evolutionary adaptation?

A reciprocal transplant assay experiment was performed in order to determine whether TGP had persisted across several generations, or if evolutionary adaptation had occurred.
(Calosi et al. 2013; Sunday et al. 2014; Thor and Dupont 2015). Fecundity levels were significantly lower in individuals transplanted from low to elevated pCO2 (low-elevated) compared to those individuals from the elevated pCO2 control line (elevated-elevated). This change in reaction norm of the experimental lines lends support to the idea that evolutionary adaptation to low pCO2 may have occurred after six generations. Indeed, it is now widely recognized that evolutionary adaptation can occur over very short ecological timescales (Reznick and Ghalambor 2001; Stockwell et al. 2003). However, the low CO2 lines showed no significant changes in fecundity when exposed to elevated CO2 conditions (Fig. 4), suggesting that what we have observed could be irreversible effects of TGP persisting across generations. In fact, there is growing evidence to show that trans-generational epigenetic effects can span across multiple (10+) generations (Jablonska and Raz 2009; Ho and Burggren 2010). In conclusion, the ambiguous nature of the results from our reciprocal transplant assay experiment prevents us from us pinpointing the underlying mechanism (i.e. phenotypic plasticity or evolutionary adaptation) which enabled worms to maintain fitness under low pCO2 conditions through to generation F7. Irrespective of the mechanism responsible, worms were able to maintain fitness levels across several generations.

**TGP as a mechanism for facilitating evolutionary adaptation.** If evolutionary adaptation to low pCO2 conditions had occurred, it was likely driven by TGP instead of natural selection. We base this conclusion on the lack of significant differences in juvenile survival between pCO2 treatments in any generation. Furthermore, all pairs produced viable offspring with no evidence of unfertilized eggs or delayed development. Together, these observations demonstrate that the strength of the selection environment was low. We predict that for species which are more resistant to rapid changes in pCO2, evolutionary adaptation may be driven primarily by TGP as opposed to natural selection.

Overall, *O. labronica* had a high capacity to exhibit phenotypic plasticity, both within and across generations. This is not surprising, as *O. labronica* typically occurs in highly heterogeneous environments, where phenotypic plasticity is generally evolutionary favoured (Ghalambor et al. 2007). Thus, it is reasonable to assume that the high levels of plasticity we observed in response to changes in pCO2 had evolved as a result of culturing worms under a highly variable pH regime for ~33 generations. In addition, many coastal environments experience substantial fluctuations in pH on a daily or seasonal basis that can be as large, or even greater, than the decrease in pH projected to occur over the next 50–100 years (Hofmann et al. 2011; Shaw et al. 2012; Melzner et al. 2013). Consequently, many coastal organisms may possess a high capacity to exhibit TGP. Indeed, a recent study showed that natural populations of the Atlantic silverside, *Menidia menidia*, were able to condition their offspring to seasonally acidifying environments (Murray et al. 2014). Such findings are important as TGP could buffer populations against the negative impacts of rapid changes in pCO2 allowing time for evolutionary adaptation to catch up (Chevin et al. 2010), or even facilitate evolutionary adaptation through processes such as the Baldwin effect and/or genetic assimilation (i.e. genetic accommodation) (Pigliucci et al. 2006; Crispo 2007).

**No apparent costs associated with the multi-generational responses.** Plastic and evolutionary responses are often associated with costs and trade-offs to life-history traits and fitness (Hoffmann 1995; Angilletta et al. 2003). However, whilst we observed costs of within-generation plasticity (i.e. reduced fecundity in generation F1 and F2), we detected no apparent costs for our observed multi-generational responses. Although it is still possible that costs exist, potentially affecting traits such as maximum size, longevity and total life-span reproductive output, all of which we were unable to determine with our experimental design. There is urgent need to identify the potential costs of TGP and evolutionary adaptation in response to changes in pCO2 and other climate change stressors (i.e. warming, hypoxia, combined stressors) if we are to more accurately predict whether current populations levels of marine metazoans will be able to persist under rapidly changing conditions. For example, if marine metazoans are able to persist at the cost of reduced body size (Gardner et al. 2011; Sheridan and Bickford 2011; Calosi et al. 2013; Garilli et al. 2015); body size-dependent traits and processes (e.g. fecundity, competitive and predator-prey interactions - Peters 1983; Arendt 2007) and subsequently ecosystem functions may still be negatively impacted (Solan et al. 2004; Sheridan and Bickford 2011). Where feasible, future trans-generational and multi-generational studies should therefore characterize performance over entire life spans to better identify potential costs.

**Summary**

Plastic and evolutionary responses are increasingly recognized as primary rescue mechanisms that could prevent species’ extinctions in the face of rapid climate change (Hoffmann and Sgrò 2011; Godbold and Calosi 2013; Gonzalez et al. 2013; Munday et al. 2013; Salinas et al. 2013; Sunday et al. 2014). Here, we show that a laboratory strain of *O. labronica* was able to rapidly (within two generations) restore its fitness levels, via TGP, and maintain these levels for a further four generations, after experiencing a change in pCO2. Regardless of whether TGP or evolutionary adaptation was the mechanism responsible for the results from
our reciprocal transplant assay experiment, we provide evidence to suggest that marine metazoans may be capable of coping with drastic changes in pH conditions over multiple generations. Our study supports the idea that multi-generational experiments are required to accurately predict how marine organisms will respond to climate change associated stressors (including pCO₂) predicted to occur to the coming centuries (Munday et al. 2013; Sunday et al. 2014). Investigations that span across multiple generations will be valuable for the planning of socio-economic and environmental buffering for the duration of the ‘detrimental’ phase (i.e. whilst fitness is reduced), while planning intervention actions that may preserve and/or speed up the ‘recovery’ phase. Finally, laboratory multi-generational experiments could be used as a valuable conservation tool to select for strains tolerant to specific pCO₂ conditions, with the scope to prevent the extinctions of keystone species in the face of rapid changes in pCO₂ via the process of assisted evolution (Van Oppen et al. 2015).

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Data archiving statement

Life-history, metabolic and water chemistry data have been deposited in the British Oceanographic Data Centre (http://www.bodc.ac.uk, doi:10.5285/22b54764-2448-1318-e053-6c86abc01ae1).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Mean values ±95% CI for all traits measured for the marine polychaete *Ophryotrocha labronica* at elevated (grey rows) and low (white rows) *p*CO$_2$ conditions across generations.

Appendix S1. Acclimation/selection to experimental conditions.