Can multi-generational exposure to ocean warming and acidification lead to the adaptation of life-history and physiology in a marine metazoan?

Emma M. Gibbin[1,2*], Leela J. Chakravarti[2,3], Michael D. Jarrold[2,3], Felix Christen[2], Vincent Turpin[2], Gloria Massamba N'Siala[2], Pierre U. Blier[2], Piero Calosi[2]

[1] Laboratory for Biological Geochemistry, School of Architecture, Civil and Environmental Engineering, École Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland.

[2] Département de biologie, chimie et géographie, Université du Québec à Rimouski, 300 Allée des Ursulines, Rimouski, Québec, G5L 3A1, Canada

[3] College of Science and Engineering, James Cook University, Townsville, Queensland 4811.
*corresponding author's email: emma.gibbin@epfl.ch ; telephone: +41 2169 380 48

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Summary statement:
We show that a species multigenerational acclimation capacity to ocean warming and acidification is determined by the flexibility of its mitochondrial electron transport system.

Abstract

Ocean warming and acidification are concomitant global drivers that are currently threatening the survival of marine organisms. How species will respond to these changes depends on their capacity for plastic and adaptive responses. Little is known about the mechanisms that govern plasticity and adaptability or how global changes will influence these relationships across multiple generations. Here, we exposed the emerging model marine polychaete *Ophryotrocha labronica* to conditions simulating ocean warming and acidification, in isolation and in combination over five generations to identify: (i) how multiple *versus* single global change drivers alter both juvenile and adult life-traits; (ii) the mechanistic link between adult physiological and fitness-related life-history traits; (iii) whether observed phenotypic changes observed over multiple generations are of plastic and/or adaptive origin. Two juvenile (developmental rate; survival to sexual maturity) and two adult (average reproductive body size; fecundity) life-history traits were measured in each generation, in addition to three physiological (cellular reactive oxygen species content, mitochondrial density; mitochondrial capacity) traits. We found that multi-generational exposure to warming alone caused an increase in: juvenile developmental rate, reactive oxygen species production and mitochondrial density and decreases in: average reproductive body size, fecundity and fluctuations in mitochondrial capacity, relative to control conditions. While exposure to ocean acidification alone, had only minor effects on juvenile developmental rate. Remarkably, when both drivers of global change were present, only mitochondrial capacity was significantly affected, suggesting that ocean warming and acidification act as opposing vectors of stress across multiple generations.

1. Introduction

An evolutionary race is underway to cope with the ecological impacts of human activity, caused primarily by the injection of anthropogenically-derived carbon dioxide (CO_2) into the atmosphere (Palumbi, 2001). The atmospheric partial pressure of CO_2 (pCO_2) has recently surpassed 400 micro atm and continues to rise at a rate and magnitude that is unparalleled in recent geological history (*ca.* 400,000 years; Petit et al., 1999; IPCC 2014). Increasing global CO_2 emissions in such large volumes has had two major effects on the global ocean. Firstly, oceanic surface temperatures have risen by 0.3 to 0.6 °C and secondly, the ocean pH has

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dropped by 0.1 of a unit since the Industrial Revolution (Orr et al., 2005). These two processes, are on-going and are expected to worsen, with current predictions for sea surface warming ranging from +1.7 to +4.8 °C and estimates for seawater pH varying between -0.07 and -0.32 pH units, over this century (IPCC, 2014). Such changes are likely to have profound biological implications for species demography and biogeography (Cheung et al., 2009; Chen et al., 2011; Calosi et al., 2013a; Queirós et al., 2015), which should in turn have severe consequences for the structure, dynamics and function of marine ecosystems (Solan et al., 2004; Hall-Spencer et al., 2008; Christen et al., 2013; Godbold and Solan, 2013).

An individual's ability to respond to periods of rapid environmental change depends on its potential for phenotypic change arising from the plasticity of its genome and those resulting from selection of specific genomes or alleles after many generations (Agrawal, 2001; Ghalambor et al., 2007; Merilä and Hendry, 2014). These processes are usually overlooked in climate change models focusing on biodiversity levels or species biogeographical responses (Cheung et al., 2009; Queirós et al., 2015), potentially resulting in over- or under-estimations of the biological implications of global change (Munday et al., 2013; Sunday et al., 2014; Calosi et al., 2016). In part, this is due to the complexity of plastic and adaptive processes, which require an intimate knowledge of both species' life-history strategy (Byrne and Przeslawski, 2013; Lucey et al., 2015) and the underlying physiological mechanisms, which determine the magnitude of environmental change that a species can withstand (Blier et al., 2014; Seebacher et al., 2010; Tomanek et al., 2015). To confound matters, global change drivers can also interact to modify a species' sensitivity to, or tolerance of a single driver. Ocean warming for example, can either offset the negative effects of ocean acidification, or aggravate them further (Kroeker et al., 2013). Such interactions are responsible for the plethora of responses observed across phyla, functional groups, species and between life-stages (Kroeker et al., 2013; Small et al., 2015).

Phenotypic-plasticity describes the capacity of one genotype to produce a range of phenotypes under different environmental conditions (Fordyce, 2006). Plasticity is pervasive and plays a key role in determining an organisms' fitness and subsequent ecological performance when challenged with high selective pressures (Ghalambor et al., 2007), such as the current rate of global change (Chown et al., 2007; Donelson et al., 2012; Allan et al., 2014). Phenotypic plasticity generates variation, which natural selection can act upon through the differential survival, mortality and reproductive success of individual genotypes (Darwin 1859). Retaining

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favourable genotypes in a population alters the structure of a population by shifting the average phenotype towards a new, optimal fitness peak for the conditions encountered (Sunday et al., 2014). In this respect, the selection pressures experienced by an individuals' parents, grandparents and the procession of generations before that, become crucial for determining the fitness and performance of future generations (Badyaev and Uller, 2009, Shama et al., 2016). Parental conditioning to global change drivers has largely positive effects on an offspring's response to the same stressor (Donelson et al., 2012; Miller et al., 2012; Salinas and Munch, 2012; Massamba N'Siala et al., 2014; Putnam and Gates, 2015; Rodriguez-Romero et al., 2015; Thor and Dupont, 2015). For example, parental exposure to warming (+3 °C; all values are expressed relative to the control conditions) protected offspring from *ca.* 30 % losses in aerobic scope in the damselfish, *Acanthochromis polyacanthus* (Donelson et al., 2012), while parental exposure to warming and acidification (+3 °C and +600 µatm pCO_2) prevented decreases in size, weight and survival in offspring of the anemonefish, *Amphiprion melanopus* (Miller et al., 2012). The effects of parental conditioning are not confined to vertebrates; parental exposure to acidification (+1150 µatm pCO_2), halved the reduction in fecundity caused by acute exposure to the same conditions in offspring of the copepod *Pseudocalanus acuspes* (Thor and Dupont, 2015), while parental exposure to ocean warming and acidification (+2 °C and +400 µatm pCO_2), reduced the negative effects of acute exposure on larval size in the coral, *Pocillopora damicornis* (Putnam and Gates, 2015). However, parental and acute exposure to combined global change drivers does not always affect the progeny, despite the negative effects of single-stressor exposure, as shown recently in the marine polychaete *Ophryothroca labronica* (Chakravarti et al., 2016).

Considerable research effort has been spent understanding the effects of parental conditioning in marine organisms, yet two key questions remain: do the benefits gained *via* parental conditioning persist across multiple generations? And if so, what are the mechanisms responsible for the persistence of these changes? To date, three multi-generational studies (i.e. those lasting longer than the offspring generation) have been conducted on marine metazoans and all have used a single-driver approach. The first, exposed *O. labronica* a rapid change in pCO_2 over six generations (Rodríguez-Romero et al., 2015), the second exposed the marine stickleback, *Gasterosteus aculeatus* to ocean warming (+ 4 °C) for three generations (Shama et al., 2016) and the third exposed *A. polyacanthus* to two ocean warming scenarios (+1.5 °C and +3 °C) for three generations (Munday et al., 2016). In all of these studies, the effects of the initial transgenerational exposure persisted across multiple generations highlighting the

importance of multi-generational acclimation in determining a species response to global change. However, there have been no multi-driver, multi-generational studies in marine organisms, so it is unclear whether a similar pattern will emerge when ocean warming and acidification are combined and this is one avenue of research that must be pursued. Single-driver experiments reveal that benefits are likely to be retained over multiple generations *via* alterations in parental resource partitioning (Marshall and Uller, 2007; Lane et al., 2015) and somatic factors such as antibiotics, antioxidants, hormones and mitochondria, (Hamdoun and Epel, 2007; de Wit et al., 2015; Shama et al., 2016), although epigenetic effects on protein conformation, DNA methylation and chromatin marks may also play a role (Jablonka and Raz, 2009; Bonduriansky et al., 2012; Veilleux et al., 2015; Munday et al., 2016; Putnam at al., 2016).

Here, we describe the effects of multigenerational exposure to ocean warming and acidification on the life-history and physiological response of an emerging model organism for evolutionary studies in the marine realm, O. *labronica* (Massamba N'Siala et al., 2014; Rodríguez-Romero et al., 2015; Chakravarti et al., 2016). This study is an extension of the transgenerational experiment conducted by Chakravarti et al. (2016), who exposed *O. labronica* to control (27 °C, pH 8), ocean warming (+3 °C) and ocean acidification (-0.4 pH unit) scenarios in isolation and in combination over two generations. We followed these individuals for a further five generations, measuring a suite of life-history (juvenile developmental rate, juvenile survival to sexual maturity, average reproductive body size and fecundity) and physiological (cellular reactive oxygen species content, mitochondrial density and capacity) traits in each generation. We then perform reciprocal transplants between control and experimental conditions in F5 and F6 to determine whether any changes are driven by phenotypic plasticity or adaptation.

2. Materials and methods

(a) Study species.

This study was conducted on the benthic marine polychaete *Ophryotrocha labronica* (Polychaeta, Dorvilleidae), a patchily-distributed global occupant of the biofouling community (Cossu et al., 2015). Sexual maturity is reached at 11–12 chaetigers in females and at 7–8 chaetigers in males (Lorenzi and Sella, 2013). Mature adults form breeding pairs and

reproduction is a semi-continuous process, with females producing one cylindrical egg mass a week, which is fertilized externally (Paxton and Åkesson, 2007). Parental care is provided throughout the development of the eggs, with parents continuously swimming through the egg mass ensuring a constant supply of oxygen is available to developing young and preventing parasites growing on the surface of the egg mass (Sella, 1993; Paxton and Åkesson, 2007).

(b) Collection and establishment of the culture.

The initial sample population (*ca.* 100 worms) was collected from a single site in Porto Empedocle harbor (Sicily, Italy - 37°17'N, 13°31'E) in January 2014 (Chakravarti et al., 2016). The culture was transferred to the Marine Evolutionary Physiology (MEP) laboratory at the Université du Québec à Rimouski (Canada), where it was kept for eight generations under control conditions (temperature: 27 °C; pH: 8; salinity: 35, light cycle: 12 h light: 12 h dark), selected to maximise reproductive output (Åkesson, 1970). The polychaetes were fed *ad libitum* with minced spinach (Massamba-N'Siala et al., 2012) and water was partially changed daily to prevent the accumulation of excreta and to remove uneaten spinach (Rodríguez-Romero et al., 2015).

Twelve females and males were selected from the starting population and placed in breeding pairs in six-well plates (Corning, Wiesbaden, Germany). These individuals formed our F0 generation (Chakravarti et al., 2016). The second egg mass spawned by each pair, is generally the largest egg mass so this was left to hatch to form the F1 generation. On the day of hatching, eighty individuals were taken from each pair and transferred to the treatments below (n = 20 hatchlings *per* well, n = 4 plates *per* treatment). Replicates are not referred to as lineages because the genetic identity was not resolved in this study. However, a degree of reproductive isolation was achieved by selecting mothers from the same replicate brood, thus prevented inbreeding and the accompanying loss of genetic variation (Charlesworth, 2003), while ensuring replicates were independent.

(c) Experimental conditions.

We exposed *O. labronica* individuals for six generations (F1-F6) to four experimental conditions representative of present-day, future warming (+3 °C) and acidification (-0.4 pH unit) scenarios. Temperatures vary substantially in the Mediterranean Sea, with values reaching as low as 4 °C in winter and as high as 30.5 °C in summer (Massamba-N'Siala et al., 2011), yet lethal temperatures in *O. labronica* can be as high as 32 °C (Massamba-N'Siala et al., 2012).

The discrepancy between these two values showcases this species' high thermal plasticity. It is possible that this plasticity has evolved in response to abiotic variability in the microenvironment that *O. labronica* inhabits. Benthic temperature fluctuations fluctuate widely in Porto Empedocle ranging from 15 and 27 °C. We chose to use the summer maxima (27 °C) as our control temperature and the average oceanic pH in the region (pH 8) as the control pH. Experimental ocean warming (W; 30 °C and pH 8) and acidification (A; 27 °C and pH 7.6), both in isolation and in combination (WA; 30 °C and pH 7.6), were therefore relative to the designated control. All experiments were performed using the Temperature and CO_2 Manipulation System (Chakravarti et al., 2016). Details of the conditions experienced during the experimental period are provided as Supplementary Information (Table S1).

(d) Experimental design

Ophryotrocha labronica individuals were exposed to C, W, A and WA for six generations (F1-F6). On the day of hatching, twenty five individuals from the F1 parents, were transferred to new culture plates and placed in the same treatment as their parents, and twenty five individuals from control parents were distributed amongst the experimental treatments. The effects of this acute and transgenerational exposure (F1-F2) are published elsewhere by Chakravarti *et al.* (2016). In the *first experiment* of the present study, we followed these 'transgenerational' replicates for a further four generations (F3-F6, Fig. 1). Hatchlings were always derived from the second, largest, egg mass (Massamba-N'Siala et al., 2011). If hatching was unsuccessful in the first instance, a new breeding pair was created using sisters of the female. The original female was retained for life history measurements and the new pair was used for the sole purpose of obtaining the offspring required for the next generation. If the second partnership failed, a third partnership was created. Three failures was categorised as an 'extinction event' and no further efforts were made to save that replicate.

A *second experiment* was designed to run in parallel from F4 onwards with the aim of investigating whether any changes observed in life-history and/or physiology were plastic or adaptive following four generations (F1-F4) of exposure to experimental conditions (Fig. 1). As before, F4 offspring were generated from the second egg mass, except this time 100 H0 hatchlings were removed from each replicate and transplanted back into control conditions. A factorial design was implemented in F5 (Fig. 1) with reciprocal transplants, similar to those used in Rodriguez-Romero et al. (2015) and Chakravarti et al. (2016) performed between

control and experimental conditions (i.e. C-W, C-A, C-WA and W-C, A-C, WA-C). These replicates were retained in the new conditions for two generations, meaning the final generation (F6) contained individuals exposed to three generations of experimental conditions and two generations of control conditions (i.e. W-C-C, A-C-C and WA-C-C) and *vice versa* (C-C-W, C-C-A and C-C-WA). Comparing the two generations enable us to predict whether phenotypic changes were expressions of phenotypic plasticity or new adaptations (Calosi et al., 2013a).

(e) Measurement of life history traits

Hatching event success, two juvenile (developmental rate and survival to sexual maturity) and two adult (average reproductive body size and fecundity) life-history traits were measured in this study. All adult life-history measurements were conducted on females because the maternal environment is more important for determining the offspring response than the paternal environment (Stearns, 1992; Massamba N'Siala et al., 2011; Shama et al., 2014). Here, the traits are explained in the sequence they were measured.

Hatching event success was recorded as the percentage of egg masses that hatched out of the total number of egg masses spawned.

Juvenile developmental rate, expressed as the number of chaetigers added *per* day, represents the number of segments bearing chitinous bristles, one week after hatching. At this stage, juveniles are not sexually mature, so counts were performed on randomly-selected individuals ($n = 6$), using light microscopy (MS5, Leica, St. Gallen, Switzerland). *Ophryotrocha labronica* exhibits high sexual dimorphism and thus, females and males are easily distinguishable at sexual maturity; females are bigger than males, and start to develop oocytes that are clearly visible through the body wall, whereas males grow larger jaws for fighting (Paxton and Åkesson, 2007). The number of individuals alive at this point was counted and divided twenty five to estimate the proportion of juveniles surviving to sexual maturity.

Over the following month, we tracked the progress of the breeding pairs on a daily basis. Whenever a female spawned we counted the number of chaetigers she had, which we subsequently used to calculate average reproductive body size. Fecundity was also quantified for egg masses 1, 3 and 4 on the day of spawning. These egg masses were placed on a light microscope and imaged using a digital camera (14 MP, Omax, Bucheon, South Korea). These

images were later used to determine the total number of eggs produced over the three reproductive events.

(f) Measurement of physiological traits

Three physiological parameters were measured in female polychaetes throughout the experiment; the cellular content of reactive oxygen species (ROS), a marker of oxidative stress, was measured in F2, F3 and F5 using confocal microscopy, while the activities of a mitochondrial enzyme: citrate synthase and a mitochondrial complex: the electron transport system (ETS), were measured every generation (n = 12 independent replicates *per* generation, *per* treatment). These two enzymes were chosen for their function: CS is a proxy for mitochondrial density (Rabøl et al., 2006), while ETS is a marker of maximum mitochondrial capacity (Schmidlin et al., 2015). We focus on mitochondria because it is likely that the mitochondrial response across multiple generations determines the magnitude of temperature change that a species' can withstand (Blier et al., 2014; Seebacher et al., 2010; Tomanek et al., 2015).

All physiological parameters were measured in sisters of the breeding female. Individuals (n = 6), were selected at random and starved for two days to ensure that any chlorophyll auto-fluorescence from the spinach did not interfere with fluorescence from the dyes. One individual was prepared for imaging, while the remaining five were placed in a 1.5 mL microcentrifuge tube and stored at -80 °C for enzyme analysis.

A 30 min incubation in 1 mL of treatment water solution (5 µM CellROX Deep Red and 400 nM of MitoTracker Green FM; both from Life Technologies, Invitrogen, Carlsbad, CA, USA) was used for the confocal microscopy. During incubation, which was conducted at 27 °C, samples were kept in an opaque box and placed on a rotating plate at 60 RPM. Individuals were then washed in fresh treatment water to remove any excess dye and transferred to a 35 mm glass bottom Petri dish (MatTek Corporation, Ashland, MA, USA). A drop of slowing solution (Detain Wards Protist slowing agent, Wards, Rochester, NY, USA) immobilised the sample before the dish was set atop the microscope stage (LSM 700, Carl Zeiss, Oberkochen, Germany). Zen 2009 software captured the fluorescence in two channels; 488 and 640 ± 10 nm, at ×20 magnification. Four chaetae were imaged *per* sample, with images taken at 1.5 µm intervals using the Z-stack function. Analysis was conducted in ImageJ using the 3D Z-project command. Fluorescence at 488 nm was divided by that captured at 640 nm to standardise ROS

to mitochondrial density. A representative confocal image is provided as Supplementary Information (Fig. S2).

Enzyme activity was measured using a protocol adapted from Pichaud and co-workers (2012). The accuracy of the assays was confirmed in preliminary tests conducted before the experiment began by correlating enzyme activity (both ETS and CS) with different volumes of homogenate (1, 2, 5 and 10 individuals). A linear relationship between the two confirmed that enzyme activity was proportional to the quantity of enzyme present in the tissue. These initial tests also revealed that a minimum of five individuals was required to produce enough homogenate for both enzyme assays.

Individuals were homogenized in 120 µL of ice-cold buffer containing 100 mM phosphate buffer and 2 mM EDTA (pH 8) and the resulting slurry was split in two. Half was transferred to a 100 mM phosphate buffer containing 2 mM of EDTA (pH 8), 0.1 mM of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), 0.1 mM of acetyl-CoA, and 0.15 mM of oxaloacetate and half was placed in a 100 mM phosphate buffer containing 2 mM EDTA (pH 8), 0.85 mM of Nicotinamide adenine dinucleotide, 2 mM of Iodonitrotetrazolium chloride (INT) and 0.03% Triton X-100. Electron transport system activity was measured in the former and CS, in the latter. Assays were conducted in triplicate (n = 3) on a UV/VIS microplate spectrophotometer (Perkin Elmer Envision, Foster City, CA, USA). Citrate synthase activity was calculated from the increase in absorbance at 412 nm over 3 mins (ε_{412}=13.6 mL cm^{-1} µmol^{-1}), which is caused by the reduction of DTNB (Thibeault et al., 1997). Electron transport system activity was similarly dependent on an increase in absorbance, which arises from the reduction of INT and so measurements were taken at 490 nm over a 4 min (ε_{490}=15.9 mL cm^{-1} µmol^{-1}) period (Bergmeyer, 1985). Both ETS and CS were standardised to protein content to negate any size-dependent effects, using the bicinchoninic acid method described by Smith and co-workers (1985).

(g) Statistical analyses

The multi-generational response (i.e. F2-F6) was analysed using general linear models (GLMs), with ‘generation’ (five levels: F2, F3, F4, F5 and F6), ‘temperature’ (two levels: 27 and 30 °C) and ‘pH’ (two levels: 8 and 7.6) as fixed factors. Average reproductive body size was included as a co-variate for fecundity because these two variables were strongly correlated. In all cases, ‘tray’ (four levels: 1, 2, 3, 4 and 5) was included as a random factor nested within pH and temperature, while ‘tub’ (four levels: 1, 2, 3 and 4) was designated as a random factor

nested within pH and temperature, and tray. Once the effects of multigenerational exposure to experimental conditions were characterised, we targeted specific traits for correlative analysis to identify potential trade-offs between traits. Only traits significantly affected by multigenerational exposure were used. In these instances, data was pooled by treatment (C, W, A, and WA) and multiple linear regressions were then performed against all other traits.

The aim of the second experiment was to determine whether changes in traits were the expression of phenotypic plasticity or new adaptive characters and thus, the two generations were analysed separately. Fixed factors in the F5 analysis were 'parental origin' (C, W, A or WA) and 'offspring exposure' (C, W, A or WA), whereas the fixed factors in the F6 analysis were 'grandparental origin' (C, W, A or WA) and 'offspring exposure' (C, W, A or WA). The normality of the data and homogeneity of variances were verified using Shapiro-Wilks and Levene's tests respectively. If data did not meet the assumption of normality or homogeneity, the significance of residuals was verified. Residuals were never found to be significant ($P > 0.05$).

3. Results

The multi-generational response of Ophryotrocha labronica *to ocean warming and acidification.*

Results are presented in figures 2 to 4, whilst statistical outputs is provided in table 1. Hatching event success is provided as Supplementary Information (Fig. S3) as is the raw data (mean ± S.D) (Fig. S4).

Out of the 202 partnerships formed over six generations, 150 (74 %) egg masses hatched (Fig. S2A). Individuals exposed to control (C) and ocean acidification (A) in isolation had the highest hatching success (98 and 100 %, respectively), while combined conditions (WA) had the lowest (43 %) and ocean warming (W) was somewhere in between (57 %). Extinction events accounted for 6 % of failed hatching attempts but these events were limited to three generations: 33 % of egg masses were lost in F5 under W, while 24 and 25 % were lost in F3 and F5 under WA (Fig. S2B).

Juvenile developmental rate differed between generations in W and A. Juveniles exposed to A, developed slower than those exposed to W alone, or to WA (Fig. 2A). This was underlined by the presence of a significant 'temperature × pH' interaction which was unchanged across generations (i.e. no significant three-way interaction was evident; Table 1). Developmental rates in these individuals were lowest in F3, where development was 11 % slower compared with those in C conditions (Fig. 2C). This reduction in developmental rate however, was only apparent for one generation. Developmental rates in individuals exposed to A peaked in F5, where rates were 7 % higher than C, but again, this response lasted a single generation (Fig. 2C). In contrast, W showed higher developmental rates relative to C (Fig. 2A), this increase persisting throughout the duration of the experiment (Fig. 2B). Juvenile developmental rates across generations was found to diverge between A and W, whilst rates of development were comparable in individuals exposed to W and WA (Fig. 2D). Juvenile survival to sexual maturity ranged widely among treatments and replicates and was unaffected by A, W or WA (Fig. 2E). Survival was found to increase significantly at generation F5 and F6, this being the only significant term in our analyses (Fig. 2F).

Average reproductive body size differed between generations following exposure to W (Fig. 3A), with 4 % and 2 % declines relative to C in F5 and F6, respectively (Fig. 3B), but neither exposure to A or WA significantly affected the average reproductive body size (Table 1). A similar pattern was observed for fecundity (Fig. 3C). This too was reduced by W and again, the response differed between generations (Table 1). In F3, individuals exposed to W produced 19 % less eggs than their C counterparts (Fig. 3D). By F5 and F6, the difference between the two groups had increased to 62 % and 49 %, respectively (Fig. 3D).

Multigenerational exposure to W and WA resulted in a marked increase in reactive oxygen species content (ROS; Fig. 4A) with values three times higher in W individuals compared to the C (Fig. 4B), as is indicated by the presence of a significant interaction between 'generation × temperature' (Table 1). Citrate synthase (CS) showed a different trend (Fig. 4C), with levels 38 % higher than the control in F2, before returning to C levels from F3 to F5 and finally increasing in F6 (Fig. 4D). Electron transport system (ETS) activity was sensitive to A and W, and their interaction depended on the generation in question (Fig. 4E), as is evidenced by the significant three-way interaction between generation, pH and temperature (Table 1). Values were much higher in the latter than the former in F2 and F6 (Fig. 4F). The ratio of CS: ETS

generally increased over time in both W and C individuals; levels were higher in individuals exposed to W in F4 relative to the C, although this pattern was reversed in F6 (Fig. 4H).

Correlations between traits

Results are presented in figure 5 and statistical outputs are provided in table 2.

We observe significant positive relationships in response to exposure to W between: (i) juvenile developmental rate and survival to sexual maturity (Fig. 5A); (ii) average reproductive body size and fecundity (Fig. 5B) and (iii) ETS and CS activity (Fig. 5D). Juvenile developmental rate was the only life-history trait significantly affected by exposure to A conditions, but it did not correlate significantly to any other trait (Table 2). There was however, a significant negative relationship between ETS and juvenile survival to sexual maturity under A conditions (Fig. 5E). There were no significant correlations between juvenile developmental rate and other traits under WA (Table 2), but a positive relationship between ETS and CS activity was observed under these conditions (Fig. 5F).

Transplantation experiment

Results are presented in figure 6, statistical outputs are provided in table 3 and means (± S.D) are provided as Supplementary Information (S5, S6).

Electron transport system activity was significantly affected by transplantation in the W treatments and so was the ratio of CS: ETS (Table 3). The persistence of these two metrics differed over time: alterations in ETS were evident in both F5 (Fig. 6A) and F6 (Fig. 6B), whereas changes in CS: ETS were only apparent in F5 (Fig. 6C, D). The highest ETS activity in F5 was observed in the C-W exposure, whereas the lowest occurred in individuals belonging to W-C (Fig. 6A). Electron transport system activity was comparable between individuals exposed over two generations to C or W conditions in generation F5 (i.e. C-C and W-W). These patterns were not conserved in F6 (Fig. 6B), with individuals belonging to W-W-W and W-C-C having three times higher ETS activity than those belonging to either C-C-C or C-W-W (Fig. 6B). No traits were significantly affected by transplantation in the A transplants (Table 3). Juvenile developmental rate was the only life-history trait significantly affected by transplantation in WA (Fig. 6E), with rates highest in WA-WA and C-WA, and slowest in WA-C (Fig. 6E). There were no significant differences in juvenile development in F6 (Fig. 6F).

4. Discussion

Life-history and physiological traits in the marine polychaete *Ophryotrocha labronica* differ in their sensitivity to ocean warming and acidification, and their responses greatly depend on the number of generations that they have experienced these conditions for. Exposure to ocean warming in isolation seems to affect *O. labronica* much more than exposure to ocean acidification alone, or the two drivers in combination. However, we do not observe negative fitness impacts to warming until F4 onwards, signifying that at least three generations of exposure this driver is required before benefits gained from parental conditioning (Chakravarti et al., 2016) are lost and harmful (cumulative) effects are observed. This is true for the adult life-history and physiological traits measured in this study. The increase in cellular reactive oxygen species (ROS) following five generations of exposure to ocean warming appears to be particularly important in driving these responses. In the paragraphs below, we integrate life-history changes with physiological responses in order to identify the mechanisms responsible for the multigenerational response to global change. We also examine whether changes persist following transplantation back to control conditions, enabling us to predict whether changes arise *via* phenotypic plasticity or new adaptation (Calosi et al., 2013a).

Hatching event success is an important consideration in a multigenerational experiment because it is indicative of the level of selection that is occurring during either fertilization or early embryonic development (Arnold and Wade, 1984). No egg masses are lost in ocean acidification conditions, so we conclude that no selection occurs before the juvenile stage. The average hatching success under ocean warming is 57 %, although the majority of egg masses lost are confined to the final generation, F5. A similar pattern emerges when ocean warming and acidification are combined, with high losses in F3 producing an average hatching success of just 43 %. Interestingly, multiple generations of exposure to ocean warming eliminates the beneficial effect that ocean acidification has during transgenerational exposure in *O. labronica* (Chakravarti et al., 2016). Several studies report that hatching success is temperature-dependent and most attribute this to the thermal history of the parents (Bryne et al., 2009; Mayor et al., 2012; Zervoudaki et al., 2013), our results support these assertions.

Juvenile life-stages are particularly sensitive to environmental change (Dupont et al., 2010; Byrne, 2012; Munday et al., 2016), so we measure two juvenile life-history traits: developmental rate and survival to sexual maturity. We show that juvenile developmental rates

are modified by exposure to ocean warming and acidification in isolation and in combination, suggesting that this trait is highly plastic in *O. labronica* (Munday et al., 2013; Ghalambor et al., 2015). However, we observe differences in the magnitude, timing and persistence of the responses between drivers.

Enhanced development is a common consequence of warming in marine ectotherms (Angilletta et al., 2004; Forster et al., 2011) and *O. labronica* is no exception (Åkesson 1976; Massamba N'Siala et al., 2012). To date, two studies have investigated how parental exposure to warming influences juvenile developmental rates in aquatic organisms (Salinas and Munch, 2012; Shama et al., 2014), and only one has examined whether these changes persist across multiple generations (Shama and Wagner, 2014). Parental exposure to elevated temperature (+10 °C) doubled juvenile development in the sheepshead minnow, *Cyprinodon variegatus* (Salinas and Munch, 2012), while maternal exposure to a more moderate temperature increase (+4 °C) stimulates development in the marine stickleback, *Gasterosteus aculeatus* (Shama et al., 2014). Transgenerational exposure to warming does not result in modifications to juvenile development rates in *O. labronica* (Chakravarti et al., 2016) but after three generations of exposure we detect increases in the rate of development (approx. 10 %), which persist over the next three generations. Our findings corroborate those of Shama and Wagner (2014), who also report persistent increases in developmental rate following multigenerational exposure to ocean warming.

Ocean acidification similarly impacts developmental rate, but any changes are weaker than those exerted by ocean warming, as is illustrated by the vector of change switching direction between generations (-11 % in F3 and +7 % in F5). Overall, there does not appear to be a specific pattern to the response of this trait to elevated pCO_2 in isolation. Changes in juvenile developmental rates between generations exposed to different seawater pCO_2 regimes have already been reported in *O. labronica* (Rodríguez-Romero et al., 2015). The values we report are slightly lower, but fall within the same range, of this previous study (1.33 to 1.60 chaetigers d^{-1} *versus* 0.92 to 1.46 chaetigers d^{-1}). Several transgenerational studies have measured the effect of ocean acidification on juvenile development (Parker et al., 2012; Schade et al., 2014; Lane et al., 2015), but no pattern is evident. For example, parental exposure to ocean acidification increases the rate of juvenile development in the Sydney rock oyster, *Saccostrea glomerata* (Parker et al., 2012), but decreases developmental rate in *G. aculeatus* (Schade et al., 2014). The relationship is further complicated in the marine tubeworm, *Hydroides elegans*,

whose offspring grow twice as fast if fathers have been exposed to ocean acidification but 25 % slower if mothers experience the same conditions (Lane et al., 2015). Since both the males and females are exposed to ocean acidification in this study, it is possible that the neutral response that we observe in F2, F4 and F6 are driven by opposite, additive effects of the parental environment experienced.

The presence of both drivers in combination, results in rates of development, which fall in between those observed under ocean warming or acidification alone, suggesting that, the two drivers act as opposing vectors of stress across multiple generations (Parmesan, 2006). Indeed, the presence of the two drivers is the only scenario where changes persist after transplantation, with a cost incurred by individuals transferred into control conditions in F5. Since this cost lasts a single generation, we conclude that these changes arise *via* phenotypic plasticity. However, this does not render these changes unimportant. Alterations in juvenile performance can transcend life-history stages either by enhancing or reducing energetic investment into other traits (Stearns, 1992). To see if this was the case, we plotted juvenile developmental rate against all of the traits measured in the present study. A positive relationship is evident between the rate of juvenile development and the survival to sexual maturity, with larger individuals more likely to reach sexual maturity, and this association becomes stronger after exposure to ocean warming. Exposure to ocean acidification over multiple generations appears to nullify the negative association between survival to sexual maturity and electron transport system activity (ETS), a marker of mitochondrial capacity suggesting that some metabolic advantage may be gained under high pCO_2.

The majority of changes in adult life history traits are driven by exposure to ocean warming alone. We report significant decreases in both average reproductive size (-4 % in F5 and -2 % in F6) and fecundity (-19 % in F3, -62 % in F5 and -49 % in F6) that appear after three generations of exposure to ocean warming. The strong, positive correlation between the two traits strongly suggests that this relationship is causative. Reductions in fecundity have been observed following trans- and multigenerational exposure to ocean acidification in the copepod, *Pseudocalanus acuspes* (Thor and Dupont, 2015) and in *O. labronica*, respectively (Rodríguez-Romero et al., 2015) but yet to be shown in response to ocean warming.

So, what are the underlying physiological causes of the changes in life history traits? Increases in ROS are not evident following transgenerational exposure to ocean warming (Chakravarti

et al., 2016), however after five generations, levels of ROS content are almost twice as high (+190 %) as control individuals. This magnitude of stress is not trivial and it is likely that individuals in F5 have lower mitochondrial efficiency (i.e. the amount of ATP produced *per* O_2 is reduced) because a significant number of electrons escape the ETS. It is likely that these individuals are allocating a greater proportion of their energy budget producing antioxidants. Excess ROS has also been linked to reductions in the number of reproductive events *per* breeding pair, the number of eggs produced *per* reproductive event and the proportion of successful hatching events in vertebrates (Bertrand et al., 2006; Dowling and Simmons, 2009; Monaghan et al., 2009), but has not yet been observed in marine metazoans. There were no significant correlations between ROS, average reproductive body size and/or fecundity, suggesting that the increase in ROS alone is not enough to cause the reduction in reproductive performance. The increase in cellular ROS content however is likely to influence an individuals' susceptibility to- and tolerance of, other stressors.

ROS is not the only physiological trait to be affected by ocean warming. Electron transport system activity, a proxy for maximum mitochondrial capacity (Schmidlin et al., 2015), also increases in the ocean warming scenario and interestingly, this is the only trait in which changes persist across both F5 and F6 following transplantation. Closer examination of the responses reveals a change in the direction of the reaction norm between the F5 and F6. In F5, individuals transplanted from control to ocean warming conditions (C-W) exhibit the same response as those transplanted from ocean warming into control conditions (W-C); with an increase in mitochondrial capacity evident in both. Whereas in F6, individuals kept in ocean warming conditions for an additional generation (C-W-W) have reduced mitochondrial capacity compared to those exposed to control conditions for a further generation (W-C-C). The discrepancy between these responses suggests that these are adaptive evolutionary responses, although this would need to be validated using genomic tools (Barshis et al., 2013; Pespeni et al., 2013; De Wit et al., 2015), which are currently unavailable for *O. labronica*.

A strong, positive relationship exists between mitochondrial density and maximum capacity (CS: ETS) and this association is further strengthened by multigenerational exposure to ocean warming. Since exposure to ocean warming increases the potential activity of the ETS relative to CS activity, we suggest that this could be a potential limitation of mitochondrial function at the level of electron transport and/or associated membrane complexes. Numerous factors play a role in determining the response of the ETS to temperature including the distribution of redox

centres within the complex, the efficiency of substrate docking and the thermal sensitivity of metabolic pathways upstream of the ETS (Blier et al. 2014). We cannot pinpoint the exact pathway modified by the ocean warming scenario in this study, but it is likely that such modifications in metabolic organization arise from the differential up-regulation of genes coding for mitochondrial biosynthesis, as has been described in the damselfish, *Acanthochromis polyacanthus* (Veilleux et al., 2015) and *G. aculeatus* (Shama et al., 2016) following multi-generational exposure to ocean warming. It is interesting to note that these changes initially persist following transplantation back to control conditions but only for a single generation (F5), suggesting that these changes occur *via* phenotypic plasticity.

In the present study, we pose the question: can multigenerational exposure to ocean warming and acidification lead to the adaptation of life history and physiology in a marine metazoan? Out of the eight traits we measure, all but one (maximum mitochondrial capacity) appear to be plastic, rather than adaptive responses. Even under strong selective pressures and with high levels of mortality, six generations appears to be too short a time-frame to observe genetic adaptation in *O. labronica*. The differential survival of individuals shows that phenotypic plasticity is sufficient to prevent extinction at a population level and to provide a temporal buffer for genetic variation to respond *via* natural selection (Padilla-Gamiño et al., 2016; Calosi et al., *in press*). Measuring life-history and physiological traits simultaneously, using a multi-factorial experimental design, enables us to dissect how plasticity to one driver influences a species' response to another, from one generation to another. In addition, our results yield several promising avenues for future studies: what controls the accumulation of negative effects over time? Is mitochondrial efficiency a common target of selection? If so, does a species capacity to modify its mitochondrial efficiency control the rate and magnitude of ocean warming that it can withstand? Addressing mechanistic questions with multi-generational experiments such as these, will improve the predictive power of conceptual and mathematical models designed to forecast the response of marine metazoans to global change (Calosi et al., 2016) and improve the effectiveness of human-assisted parental conditioning projects (van Oppen et al., 2015), ultimately expediting conservation efforts.

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Competing interests

The authors declare no competing financial interests.

Author contributions

E.M.G., L.J.C., M.D.J., G.M.N., and P.C. designed the research; E.M.G., L.J.C., M.D.J., F.C. and V.T. performed the research; E.M.G. analysed the data; and all authors contributed to the writing of the manuscript.

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Data availability

Data will be archived and made publically available upon acceptance of the manuscript.

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Figures

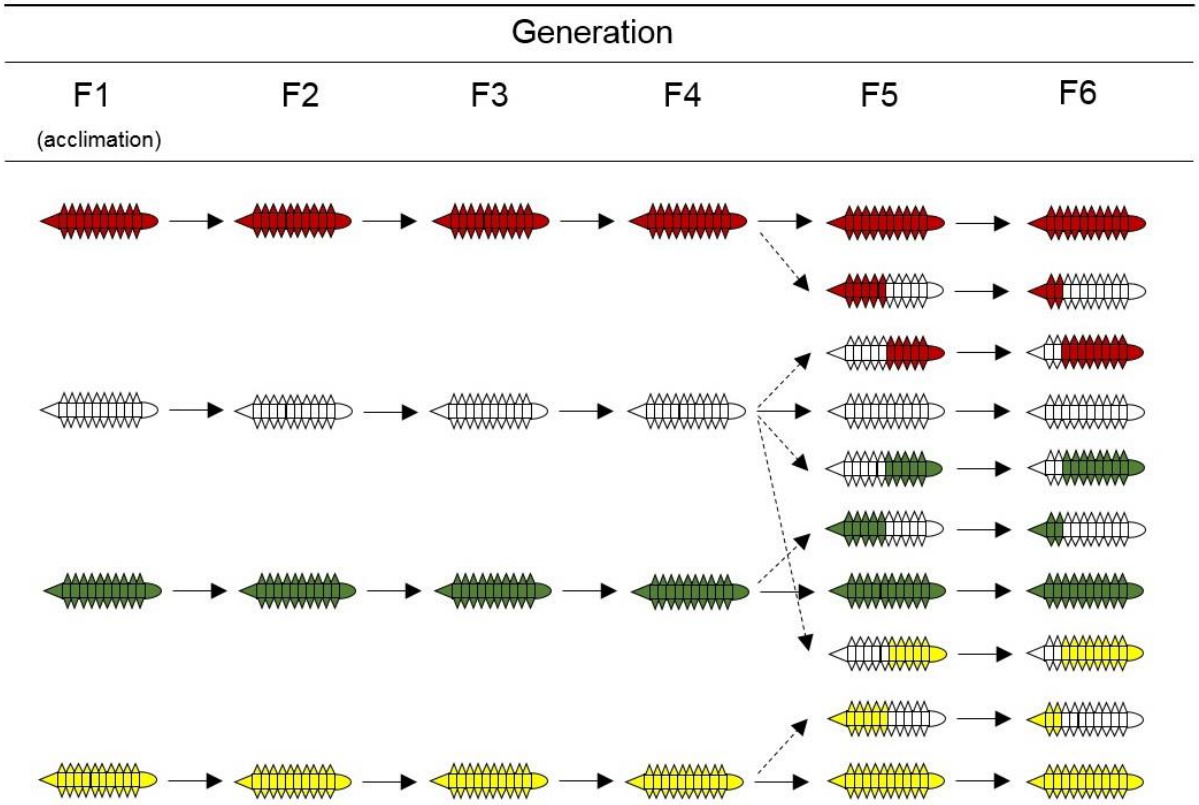

Fig. 1. Schematic of the experimental design.

Ophryotrocha labronica individuals (*n* = 12) were exposed to control (27 °C and pH 8, white), ocean warming (27 °C and pH 7.6, red) and ocean acidification (27 °C and pH 7.6, green), in isolation and in conjunction (30 °C and pH 7.6, yellow) for six generations (F1-F6). Reciprocal transplants were carried out between experimental and control conditions in F5 and these individuals were retained in the same conditions for a further generation (F6). Solid arrows show when parental and offspring conditions match. Dashed arrows indicate reciprocal transplantation.

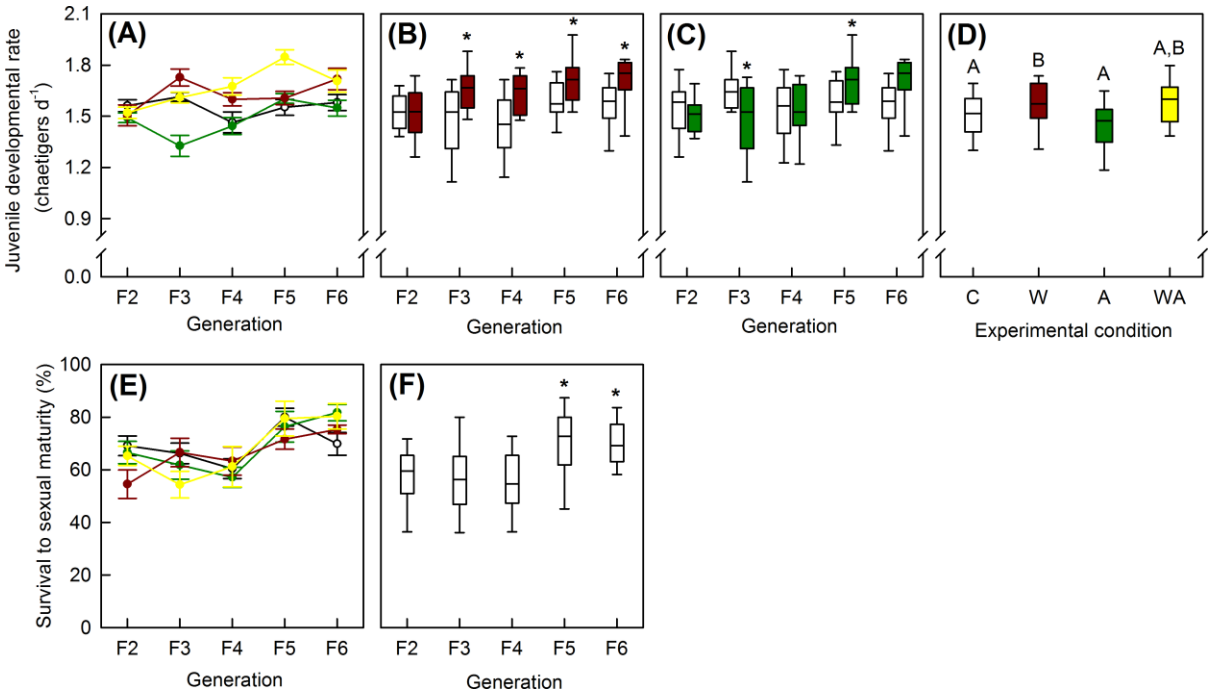

Fig. 2. Effect of multi-generational exposure to global drivers on juvenile life-history traits.

Ophryotrocha labronica individuals (*n* = 12) were exposed to control (C; 27 °C, pH 8, white), ocean warming (W; 27 °C, pH 7.6, red) and ocean acidification (A; 27 °C, pH 7.6, green), in isolation and in conjunction (WA; 30 °C, pH 7.6, yellow) over six generations (F1-F6). Two juvenile life-history parameters: (A-D) juvenile growth rate and (E-F) juvenile survival to sexual maturity were measured in each generation. Raw data are plotted as line graphs where values represent mean ± standard error (A, E). GLM's were then conducted to determine how traits responded to temperature and pH across multiple generations. Significant interactions are plotted as box plots, sized according to the 25th and 75th quartiles, where the line identifies the median and the whiskers indicate the minimum and maximum values. Asterisks (*) denote significant within-generation differences between control and experimental conditions and letters identify significant differences between experimental conditions.

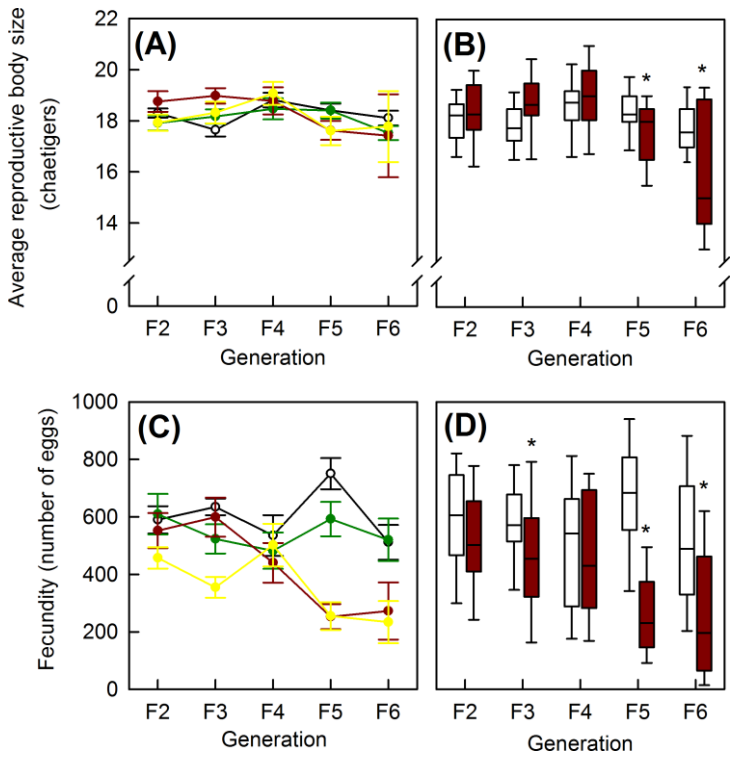

Fig. 3. Effect of multi-generational exposure to global drivers on adult life-history traits. *Ophryotrocha labronica* individuals (*n* = 12) were exposed to control (C; 27 °C, pH 8, white), ocean warming (W; 27 °C, pH 7.6, red) and ocean acidification (A; 27 °C, pH 7.6, green), in isolation and in conjunction (WA; 30 °C, pH 7.6, yellow) over six generations (F1-F6). Two adult life-history parameters were measured in each generation: (A-B) average reproductive body size and (C-D) fecundity. Raw data are plotted as line graphs where values represent mean ± standard error (A, C). GLM's were then conducted to determine how traits responded to temperature and pH across multiple generations. Significant interactions are plotted as box plots, sized according to the 25th and 75th quartiles, where the line identifies the median and the whiskers indicate the minimum and maximum values. Asterisks (*) denote significant within-generation differences between control and experimental conditions.

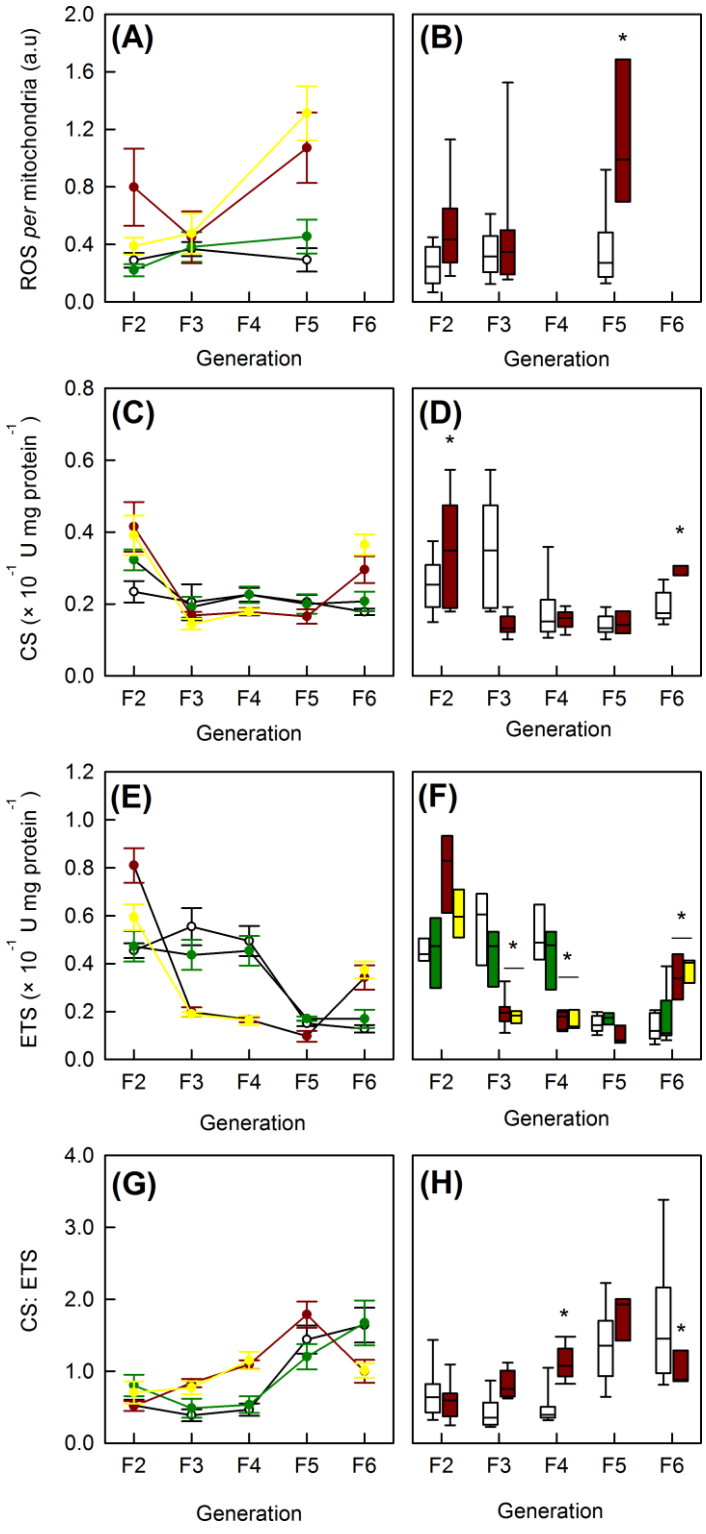

Fig. 4. Effect of multi-generational exposure to global drivers on adult physiological traits.

Ophryotrocha labronica individuals (*n* = 12) were exposed to control (C; 27 °C, pH 8, white), ocean warming (W; 27 °C, pH 7.6, red) and ocean acidification (A; 27 °C, pH 7.6, green), in isolation and in conjunction (WA; 30 °C, pH 7.6, yellow) over six generations (F1-F6). Three

adult physiological parameters were measured: (A, B) reactive oxygen species (ROS) content *per* mitochondria; (C, D) citrate synthase (CS) and (E, F) electron transport system (ETS) activity were measured in adults at each generation (with the exception of ROS measurements in generations F4 and F6). Raw data are plotted as line graphs where values represent mean ± standard error (A, E). GLM's were then conducted to determine how traits responded to temperature and pH across multiple generations. Significant interactions are plotted as box plots, sized according to the 25^{th} and 75^{th} quartiles, where the line identifies the median and the whiskers indicate the minimum and maximum values. Asterisks (*) denote significant within-generation differences between control and experimental conditions.

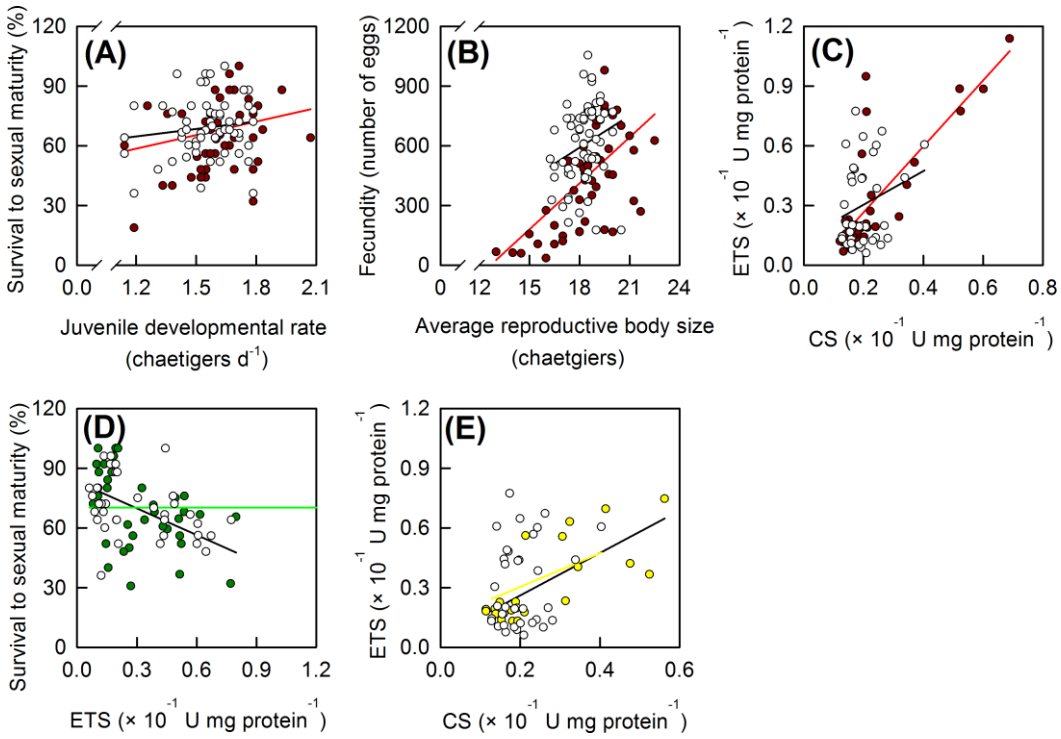

Fig. 5. Energetic trade-offs between life-history and physiology.

Relationships between traits under ocean warming (27 or 30 °C; red) and acidification (pH 7.6 or 8; green), in isolation and in combination (30 °C, pH 7.6; yellow). Experimental conditions are plotted relative to the control (white) with each data point representing one individual.

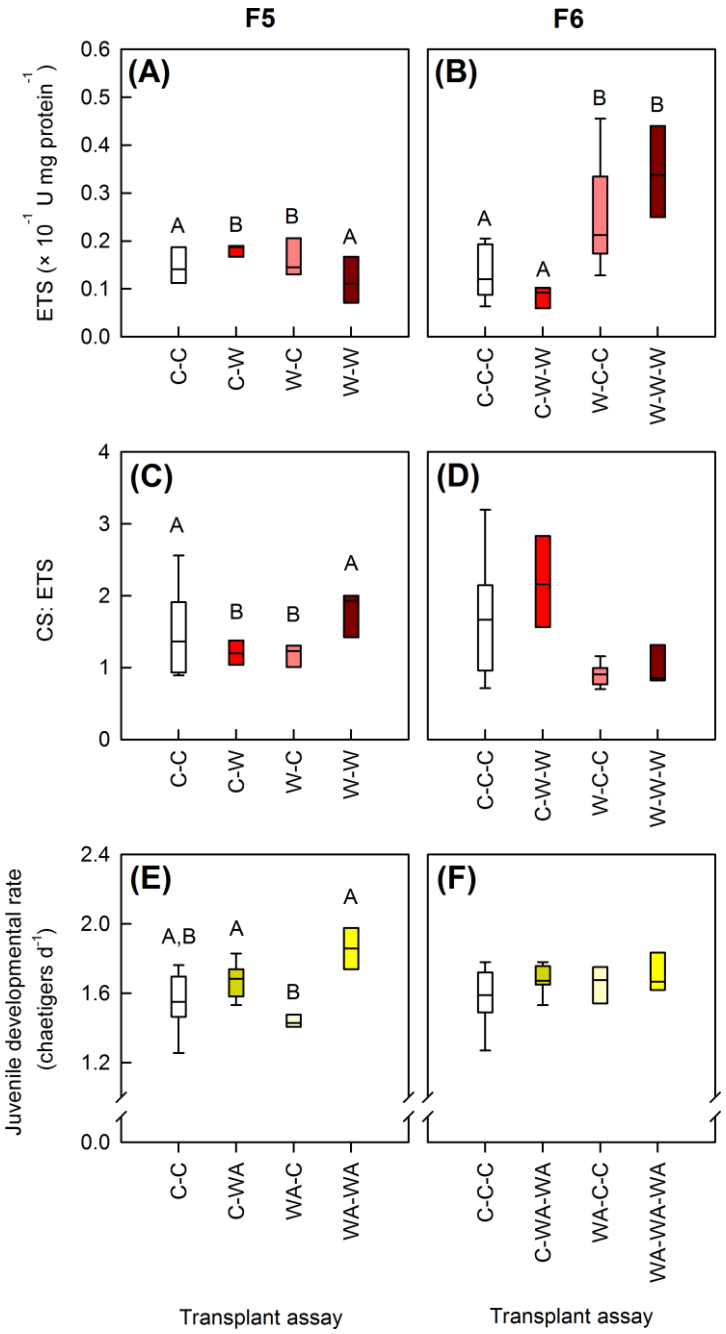

Fig. 6. Plasticity of traits following reciprocal transplantation.

Ophryotrocha labronica individuals (*n* = 12) were exposed to control (C; 27 °C, pH 8, white), ocean warming (W; 27 °C, pH 7.6, red) and ocean acidification (A; 27 °C, pH 7.6, green), in isolation and in conjunction (WA; 30 °C, pH 7.6, yellow) for four generations (F1-F4). Reciprocal transplants were performed in F5 between control and experimental conditions (C-W, C-A, C-WA and W-C, A-C, WA-C). Individuals were maintained in these conditions for a further generation (F6). Two-way analysis of variances with parental or grandparental origin (C, W, A or WA) and offspring exposure (C, W, A or WA) as fixed factors, were used to

determine how plastic changes in life history and or physiological traits were. Only significant origin × exposure interactions are plotted and letters identify significant differences between experimental conditions.

Table 1. Changes in life history and physiology following multiple generations of exposure to ocean warming and acidification. Statistical output from seven general linear models (GLM's) Degrees of freedom (DF), F-ratio's (F) and probability levels (P) are provided and significant effects ($P < 0.05$) are highlighted in bold. Finally, a hyphen (-) denotes terms that were hierarchically removed from the GLM.

Source	Juvenile developmental rate	Juvenile survival to sexual maturity	Average reproductive body size	Fecundity	ROS	CS	ETS	CS: ETS
Gen	$F_{4, 199} = 6.117$; **$P = < 0.001$**	$F_{3, 175} = 8.873$; **$P = < 0.001$**	$F_{4, 192} = 3.585$; **$P = 0.008$**	$F_{4, 167} = 2.949$; **$P = 0.021$**	$F_{2, 86} = 5.225$; **$P = 0.007$**	$F_{4, 116} = 18.864$; **$P = < 0.001$**	$F_{3, 89} = 48.304$; **$P = < 0.001$**	$F_{4,128} = 14.256$ **$P = < 0.001$**
pH	$F_{1, 199} = 0.320$; $P = 0.572$	$F_{1, 175} = 0.432$; $P = 0.512$	$F_{1, 192} = 0.977$; $P = 0.359$	$F_{1, 197} = 4.029$; **$P = 0.046$**	$F_{1, 86} = 0.068$; $P = 0.794$	$F_{1, 116} = 0.188$; $P = 0.666$	$F_{1, 89} = 3.859$; $P = 0.053$	$F_{1,128} = 0.008$; $P = 0.928$
T	$F_{1, 199} = 39.852$; **$P = < 0.001$**	$F_{1, 175} = 1.739$; $P = 0.189$	$F_{1, 192} = 0.083$; $P = 0.774$	$F_{1, 197} = 49.065$; **$P = < 0.001$**	$F_{1, 86} = 20.242$; **$P = < 0.001$**	$F_{1, 116} = 1.864$; $P = 0.175$	$F_{1, 89} = 17.263$; $P = < 0.001$	$F_{1,128} = 1.627$; $P = 0.205$
Gen × pH	$F_{4, 199} = 7.580$; **$P = < 0.001$**	-	$F_{4, 192} = 0.376$; $P = 0.826$	$F_{4, 167} = 2.141$; $P = 0.077$	$F_{2, 86} = 1.786$; $P = 0.174$	$F_{4, 116} = 0.560$; $P = 0.692$	$F_{3, 89} = 0.757$; $P = 0.521$	$F_{4,128} = 0.773$; 0.545
Gen × T	$F_{4, 199} = 3.828$; **$P = 0.005$**	-	$F_{4, 192} = 2.513$; **$P = 0.043$**	$F_{4, 197} = 4.874$; **$P = 0.001$**	$F_{2, 86} = 4.230$; **$P = 0.018$**	$F_{4, 116} = 8.645$; **$P = < 0.001$**	$F_{3, 89} = 30.656$; **$P = < 0.001$**	$F_{4,128} = 7.177$; **$P < 0.001$**
pH × T	$F_{1, 199} = 8.130$; **$P = 0.005$**	-	$F_{1, 192} = 0.421$; $P = 0.517$	$F_{1, 197} = 0.005$; $P = 0.941$	$F_{1, 86} = 0.475$; $P = 0.493$	$F_{1, 116} = 0.592$; $P = 0.443$	$F_{2, 89} = 0.294$; $P = 0.589$	$F_{1,128} = 0.152$; $P = 0.697$
Gen × pH × T	-	-	-	-	-	-	$F_{2, 89} = 3.663$; **$P = 0.030$**	-

Table 2. Plasticity in life-history and physiology following reciprocal transplantation.

Statistical output from two-way analysis of variances (ANOVAs) designed to investigate how reversible any changes in traits were following three generations of exposure to ocean warming and acidification in isolation and in combination. Degrees of freedom (DF), F-ratio's (F) and probability levels (P) are provided and significant effects ($P < 0.05$) are highlighted in bold.

Condition	Generation	Source	Trait						
			Juvenile developmental rate	Juvenile survival to sexual maturity	Average reproductive body size	Fecundity	CS	ETS	CS: ETS
Ocean warming	Parental (F5)	Origin × Exposure	-	-	-	-	-	$F_{1, 21}$ = 8.189; **P = 0.009**	$F_{1, 21}$ = 5.561; **P = 0.028**
		Origin	$F_{1, 45}$ = 3.304; P = 0.076	$F_{1, 45}$ = 5.913; **P = 0.019**	$F_{1, 45}$ = 2.453; P = 0.124	$F_{1, 45}$ = 10.978; **P = 0.002**	$F_{1, 23}$ = 1.269; P = 0.272	$F_{1, 21}$ = 3.930; P = 0.061	$F_{1, 21}$ = 1.273; P = 0.272
		Exposure	$F_{1,45}$ = 9.243; **P = 0.004**	$F_{1, 45}$ = 0.067; P = 0.797	$F_{1, 45}$ = 0.048; P = 0.827	$F_{1, 45}$ = 21.518; **P = < 0.001**	$F_{1, 23}$ = 0.047; P = 0.831	$F_{1, 21}$ = 1.006; P = 0.327	$F_{1, 21}$ = 1.076; P = 0.311
	Grandparental (F6)	Origin × Exposure	-	-	-	-	-	$F_{1, 26}$ = 6.157; **P = 0.020**	-
		Origin	$F_{1, 40}$ = 1.709; P = 0.199	$F_{1, 40}$ = 0.223; P = 0.640	$F_{1, 38}$ = 4.618; P = 0.914	$F_{1, 40}$ = 0.007; P = 0.934	$F_{1, 26}$ = 4.803; **P = 0.038**	$F_{1, 26}$ = 40.989; **P = < 0.001**	**$F_{1, 26}$ = 2.265; P = < 0.001**
		Exposure	$F_{1, 40}$ = 3.695; P = 0.062	$F_{1, 40}$ = 0.818; P = 0.371	$F_{1, 38}$ = 0.004; P = 0.952	$F_{1, 40}$ = 7.764; **P = 0.008**	$F_{1, 26}$ = 1.739; P = 0.199	$F_{1, 26}$ = 0.003; P = 0.957	$F_{1, 26}$ = 0.295; P = 0.136
Ocean acidification	Parental (F5)	Origin × Exposure	-	-	-	-	-	-	-
		Origin	$F_{1, 44}$ = 1.286; P = 0.263	$F_{1, 44}$ = 2.199; P = 0.145	$F_{1, 44}$ = 3.322; P = 0.075	$F_{1, 44}$ = 0.082; P = 0.775	$F_{1, 33}$ = 0.058; P = 0.811	$F_{1, 32}$ = 0.196; P = 0.661	$F_{1, 32}$ = 0.212; P = 0.648
		Exposure	$F_{1, 44}$ = 0.066; P = 0.799	$F_{1, 44}$ = 0.510; P = 0.479	$F_{1, 44}$ = 3.388; P = 0.072	$F_{1, 44}$ = 7.718; **P = 0.008**	$F_{1, 33}$ = 0.014; P = 0.907	$F_{1, 32}$ = 4.844; **P = 0.035**	$F_{1, 32}$ = 0.734; P = 0.398
	Grandparental (F6)	Origin × Exposure	-	-	-	-	-	-	-
		Origin	$F_{1, 45}$ = 0.131; P = 0.719	$F_{1, 45}$ = 5.735; **P = 0.021**	$F_{1, 45}$ = 0.258; P = 0.614	$F_{1, 45}$ = 0.035; P = 0.853	$F_{1, 33}$ = 0.006; P = 0.940	$F_{1, 31}$ = 1.969; P = 0.170	$F_{1, 29}$ = 1.487; P = 0.233
		Exposure	$F_{1, 45}$ = 1.441; P = 0.236	$F_{1, 45}$ = 0.041; P = 0.840	$F_{1, 45}$ = 7.149; **P = 0.010**	$F_{1, 45}$ = 0.481; P = 0.492	$F_{1, 33}$ = 2.413; P = 0.130	$F_{1, 31}$ = 0.000; P = 0.994	$F_{1, 29}$ = 1.260; P = 0.271
Ocean warming and acidification Combined	Parental (F5)	Origin × Exposure	$F_{1, 34}$ = 11.668; **P = 0.002**	-	-	-	-	-	-
		Origin	$F_{1, 34}$ = 0.493; P = 0.487	$F_{1, 35}$ = 0.258; P = 0.615	$F_{1, 35}$ = 1.525; P = 0.225	$F_{1, 35}$ = 12.236; **P = 0.001**	$F_{1, 21}$ = 0.969; P = 0.336	$F_{1, 20}$ = 3.209; P = 0.088	$F_{1, 20}$ = 4.371; P = 0.050
		Exposure	$F_{1, 34}$ = 41.768; **P = < 0.001**	$F_{1, 35}$ = 0.217; P = 0.644	$F_{1, 35}$ = 0.738; P = 0.396	$F_{1, 35}$ = 13.001; **P = 0.001**	$F_{1, 21}$ = 0.249; P = 0.623	$F_{1, 20}$ = 6.263; **P = 0.021**	$F_{1, 20}$ = 1.711; P = 0.206
	Grandparental (F6)	Origin × Exposure	-	-	-	-	-	-	-
		Origin	$F_{1, 30}$ = 1.737; P = 0.197	$F_{1, 29}$ = 1.146; P = 0.293	$F_{1, 30}$ = 4.852; **P = 0.035**	$F_{1, 30}$ = 0.005; P = 0.947	$F_{1, 21}$ = 13.512; **P = 0.001**	$F_{1, 20}$ = 24.479; **P = < 0.001**	$F_{1, 20}$ = 3.889; P = 0.063
		Exposure	$F_{1, 30}$ = 3.393; P = 0.075	$F_{1, 29}$ = 0.085; P = 0.773	$F_{1, 30}$ = 4.557; **P = 0.041**	$F_{1, 30}$ = 33.594; **P = < 0.001**	$F_{1, 21}$ = 18.030; **P = < 0.001**	$F_{1, 20}$ = 9.054; **P = 0.007**	$F_{1, 20}$ = 0.040; P = 0.244

Table 3. The relationship between life-history and physiology traits.

Statistical output from linear regressions designed to investigate energetic trade-offs between life-history and physiology following six generations of exposure to ocean warming and acidification in isolation and in combination. Only traits that were significantly affected by the experimental conditions were targeted. R-squared values (R^2), degrees of freedom (DF), and probability levels (P) are provided and significant effects ($P < 0.05$) are highlighted in bold.

Condition	Variable	Juvenile developmental rate	Juvenile survival to sexual maturity	Average reproductive body size	Fecundity	ROS	CS	ETS
Ocean warming	Juvenile developmental rate	-	R^2 = 0.393; DF = 53; ***P* = 0.003**	R^2 = 0.012; DF = 52; P = 0.930	R^2 = 0.015; DF = 53; P = 0.912	R^2 = 0.199; DF = 21; P = 0.374	R^2 = 0.006; DF = 34; P = 0.973	R^2 = 0.271; DF = 36; P = 0.105
	Juvenile survival to sexual maturity	-	-	R^2 = 0.198; DF = 52; P = 0.156	R^2 = 0.169; DF = 53; P = 0.222	R^2 = 0.171; DF = 21; ***P* = 0.445**	R^2 = 0.030; DF = 34; ***P* = 0.865**	R^2 = 0.256; DF = 36; P = 0.126
	Average reproductive body size	-	-	-	R^2 = 0.592; DF = 52; P = 0.222	R^2 = 0.171; DF = 21; ***P* = 0.445**	R^2 = 0.030; DF = 34; ***P* = 0.865**	R^2 = 0.256; DF = 36; P = 0.126
	Fecundity	-	-	-	-	R^2 = 0.160; DF = 21; P = 0.477	R^2 = 0.085; DF = 34; P = 0.627	R^2 = 0.159; DF = 36; P = 0.349
	ROS	-	-	-	-	-	R^2 = 0.285; DF = 14; ***P* = 0.303**	R^2 = 0.234; DF = 14; ***P* = 0.402**
	CS	-	-	-	-	-	-	R^2 = 0.809; DF = 35; ***P* = < 0.001**
Ocean acidification	Juvenile developmental rate	-	R^2 = 0.082; DF = 58; P = 0.537	R^2 = 0.020; DF = 58; P = 0.878	R^2 = 0.550; DF = 58; P = 0.681	R^2 = 0.118; DF = 25; P = 0.565	R^2 = 0.058; DF = 32; P = 0.749	R^2 = 0.187; DF = 35; P = 0.275
	ETS	-	R^2 = 0.455; DF = 35; ***P* = 0.005**	R^2 = 0.197; DF = 35; P = 0.248	R^2 = 0.094; DF = 35; P = 0.588	R^2 = 0.229; DF = 11; P = 0.474	R^2 = 0.324; DF = 32; ***P* = 0.066**	-
Ocean warming and acidification combined	Juvenile developmental rate	-	R^2 = 0.095; DF = 41; P = 0.551	R^2 = 0.001; DF = 41; P = 0.993	R^2 = 0.261; DF = 41; P = 0.095	R^2 = 0.275; DF = 21; P = 0.216	R^2 = 0.058; DF = 32; P = 0.749	R^2 = 0.422; DF = 19; P = 0.064
	ETS	-	R^2 = 0.141; DF = 19; ***P* = 0.553**	R^2 = 0.059; DF = 19; P = 0.804	R^2 = 0.153; DF = 19; P = 0.521	R^2 = 0.103; DF = 10; P = 0.764	R^2 = 0.734; DF = 21; ***P* = < 0.001**	-

Supplementary Information for Gibbin et al. 2016: Can multi-generational exposure to global change lead to the adaptation of life-history and physiology in a marine metazoan?

Seawater parameter	Experimental condition			
	Control	Ocean warming	Ocean acidification	Ocean warming and ocean acidification combined
Temperature (°C)	27.14 ± 0.80 (921)	29.90 ± 0.69 (667)	27.01 ± 0.92 (606)	29.81 ± 0.59 (640)
Salinity	34.50 ± 0.94 (918)	34.64 ± 0.86 (657)	34.83 ± 1.14 (599)	34.62 ± 0.97 (621)
pH (NBS scale)	8.01 ± 0.14 (921)	8.01 ± 0.16 (661)	7.59 ± 0.13 (601)	7.61 ± 0.16 (631)
DIC (µmol kg$^{-1)}$	3038.68 ± 249.98 (56)	2908.11 ± 377.93 (38)	3141.46 ± 276.34 (38)	3085.47 ± 251.57 (36)
***p*CO$_2$ (µatm)**	673.55 ± 307.78 (56)	728.98 ± 246.15 (38)	1973.86 ± 570.34 (38)	2172.05 ± 536.66 (36)
[HCO$_3^-$] (µmol kg^{-1})	2704.25 ± 249.18 (56)	2598.32 ± 341.89 (38)	2978.43 ± 259.48 (38)	2916.93 ± 234.67 (36)
[CO$_3^{2-}$] (mmol kg^{-1})	321.48 ± 67.86 (56)	297.16 ± 75.89 (38)	121.39 ± 33.81 (38)	111.72 ± 34.97 (36)
Ω $_{cal}$	7.73 ± 1.63 (56)	7.14 ± 1.83 (38)	2.91 ± 0.81 (38)	2.68 ± 0.83 (36)
Ω $_{ara}$	4.85 ± 1.02 (56)	4.48 ± 1.15 (38)	1.83 ± 0.51 (38)	1.69 ± 0.52 (36)

Fig. S1. Mean values (± S.D) for the physico-chemistry seawater parameters. Temperature, salinity and pH were measured daily and dissolved inorganic carbon (DIC) was measured monthly. The remaining parameters: carbon dioxide partial pressure (*p*CO$_2$); bicarbonate (HCO$_3^-$) and carbonate (CO$_3^{2-}$) ion concentration; and calcite (Ω $_{cal}$) and aragonite (Ω $_{ara}$) saturation state, were calculated retrospectively using the programme CO2SYS (Pierrot et al., 2006). Level of replication is provided in parentheses.

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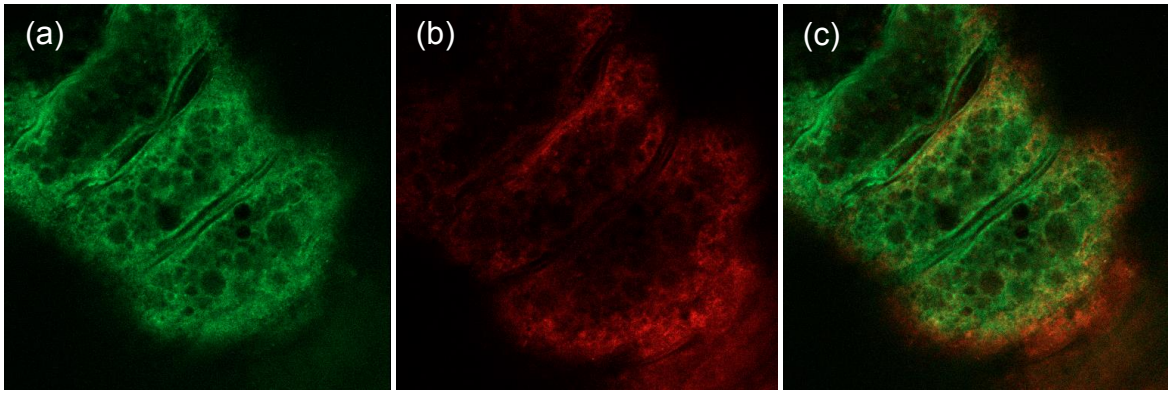

Fig. S2. Confocal image of reactive oxygen species production and mitochondrial density in chætigers 4 and 5 of *Ophryotrocha labronica*. Each individual was incubated with (a) 400 nM of MitoTracker Green FM and (b) 5 µM of CellROX Deep Red for 30 min (c) shows a composite image showing the co-localization between the two dyes. Fluorescence was captured at 488 and 640 ± 10 nm by a confocal laser-scanning microscope (LSM 700, Carl Zeiss, Oberkochen, Germany). All images were taken at ×20 magnification.

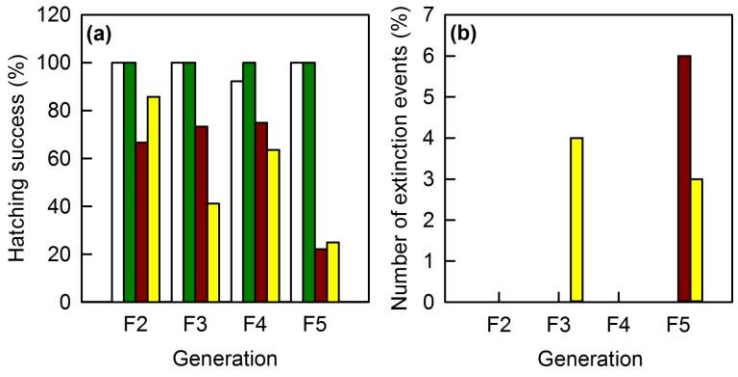

Fig. S3. Selection pressures imposed by multiple generations of exposure to ocean warming and acidification. Histograms show: (A) the percentage of successful hatching events and (B) the number of extinction events imposed by multiple generations of exposure to ocean warming (27 or 30 °C; red) and ocean acidification (pH 7.6 or 8; green) in isolation and in combination (30 °C, pH 7.6; yellow) relative to control individuals (white).

Trait	Control (C)					Ocean warming (W)					Ocean acidification (A)					Ocean warming and acidification (WA)				
	F2	F3	F4	F5	F6	F2	F3	F4	F5	F6	F2	F3	F4	F5	F6	F2	F3	F4	F5	F6
Growth rate	1.56	1.62	1.46	1.55	1.58	1.50	1.73	1.60	1.61	1.72	1.49	1.33	1.44	1.60	1.55	1.52	1.61	1.68	1.85	1.71
	1.22	*0.09*	*0.21*	*0.16*	*0.16*	*0.21*	*0.16*	*0.14*	*0.14*	*0.17*	*0.098*	*0.20*	*0.17*	*0.10*	*0.16*	*0.11*	*0.11*	*0.14*	*0.11*	*0.11*
	(12)	(12)	(12)	(12)	(12)	(12)	(11)	(12)	(12)	(7)	(12)	(11)	(12)	(12)	(12)	(12)	(12)	(8)	(7)	(3)
Survival to sexual maturity	69.10	66.21	60.36	80.00	69.82	54.50	66.55	63.27	71.60	75.33	66.52	61.75	57.09	76.36	81.67	65.24	54.32	61.14	79.43	80.30
	13.03	*13.44*	*12.93*	*11.69*	*15.00*	*18.74*	*18.00*	*18.59*	*13.37*	*4.27*	*14.67*	*17.97*	*13.33*	*20.21*	*10.85*	*12.58*	*17.55*	*21.67*	*17.50*	*8.45*
	(12)	(12)	(12)	(12)	(12)	(12)	(11)	(12)	(12)	(7)	(12)	(11)	(12)	(12)	(12)	(12)	(12)	(8)	(7)	(3)
Mean body size	18.31	17.64	18.82	18.41	18.11	18.76	18.98	18.78	17.63	17.42	17.917	18.17	18.47	18.40	17.52	17.93	18.32	19.07	17.61	17.78
	0.63	*0.88*	*0.99*	*0.90*	*1.00*	*1.40*	*1.00*	*1.83*	*1.29*	*3.98*	*1.03*	*0.93*	*1.43*	*1.09*	*0.94*	*1.11*	*1.49*	*1.27*	*1.46*	*2.41*
	(12)	(12)	(12)	(12)	(12)	(12)	(11)	(12)	(12)	(6)	(12)	(11)	(12)	(12)	(12)	(12)	(12)	(8)	(7)	(3)
Total fecundity	590.00	635.25	535.75	750.76	512.08	552.42	599.73	440.83	253.25	272.86	609.92	523.45	482.50	592.67	520.58	457.34	355.33	502.13	255.43	234.33
	165.07	*100.54*	*244.80*	*188.19*	*208.27*	*211.43*	*226.06*	*238.89*	*150.86*	*262.58*	*242.88*	*167.74*	*218.63*	*207.24*	*254.84*	*127.50*	*124.92*	*210.36*	*128.21*	*127.54*
	(12)	(12)	(11)	(12)	(12)	(12)	(11)	(12)	(12)	(7)	(12)	(11)	(12)	(12)	(12)	(12)	(12)	(8)	(7)	(3)
ROS/Mito	0.29	0.37		0.29		0.80	0.45		1.07		0.22	0.38				0.390	0.48		1.31	
	0.16	*0.15*	-	*0.20*	-	*0.85*	*0.48*	-	*0.60*	-	*0.12*	*0.33*	-	-	-	*0.19*	*0.44*	-	*0.27*	-
	(9)	(9)		(6)		(10)	(7)		(6)		(8)	(10)				(10)	(10)		(2)	
CS activity	0.023	0.02	0.02	0.02	0.02	0.04	0.02	0.02	0.02	0.03	0.032	0.02	0.02	0.02	0.02	0.04	0.01	0.02		0.04
	0.01	*0.01*	*0.01*	*0.01*	*0.00*	*0.02*	*0.00*	*0.00*	*0.00*	*0.01*	*0.01*	*0.01*	*0.01*	*0.01*	*0.01*	*0.01*	*0.00*	*0.00*	-	*0.01*
	(5)	(5)	(11)	(9)	(11)	(8)	(10)	(12)	(3)	(3)	(8)	(4)	(6)	(7)	(8)	(6)	(6)	(5)		(5)
ETS activity	0.045	0.06	0.05	0.02	0.01	0.08	0.02	0.02	0.01	0.03	0.05	0.04	0.05	0.02	0.02	0.06	0.02	0.02		0.04
	0.01	*0.02*	*0.02*	*0.00*	*0.01*	*0.02*	*0.01*	*0.00*	*0.00*	*0.01*	*0.02*	*0.01*	*0.02*	*0.00*	*0.01*	*0.01*	*0.00*	*0.00*	-	*0.01*
	(5)	(5)	(7)	(9)	(11)	(8)	(11)	(12)	(3)	(4)	(8)	(4)	(8)	(7)	(9)	(6)	(6)	(5)		(5)

Fig. S4. Mean values, *standard deviation* (number of replicates) for life-history and physiological traits in *Ophryotrocha labronica*.

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Trait	Reciprocal Transplant									
	C-C	**C-W**	**W-C**	**W-W**	**C-A**	**A-C**	**A-A**	**C-WA**	**WA-C**	**WA-WA**
Growth rate	1.55	1.72	1.54	1.61	1.60	1.64	1.60	1.67	1.44	1.85
	0.16	*0.13*	*0.14*	*0.14*	*0.11*	*0.13*	*0.10*	*0.10*	*0.04*	*0.11*
	(12)	(12)	(12)	(12)	(12)	(11)	(12)	(12)	(7)	(7)
Survival to sexual maturity	80.00	78.40	68.00	71.60	80.00	69.45	76.36	82.91	78.86	79.43
	11.69	*14.19*	*14.47*	*13.37*	*14.97*	*17.18*	*20.21*	*14.28*	*11.71*	*17.50*
	(12)	(12)	(12)	(12)	(12)	(11)	(12)	(12)	(7)	(7)
Mean body size	18.41	18.20	17.43	17.63	17.42	18.47	18.40	17.79	17.52	17.61
	0.90	*1.08*	*2.37*	*1.29*	*1.07*	*0.85*	*1.09*	*1.49*	*1.33*	*1.46*
	(12)	(12)	(12)	(12)	(12)	(11)	(12)	(12)	(7)	(7)
Total fecundity	750.76	526.47	609.39	253.25	515.42	707.27	592.67	532.58	539.95	255.43
	188.19	*214.73*	*288.31*	*150.86*	*250.73*	*220.02*	*207.24*	*225.75*	*272.94*	*128.21*
	(12)	(12)	(12)	(12)	(12)	(11)	(12)	(12)	(7)	(7)
ROS	0.29	0.72	0.86	1.07	0.63	0.73		0.77	0.99	1.31
	0.20	*0.60*	*0.41*	*0.60*	*0.59*	*0.43*	-	*0.55*	*0.46*	*0.27*
	(6)	(8)	(7)	(6)	(12)	(10)		(10)	(5)	(2)
Mitochondrial density	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	
	0.01	*0.00*	*0.01*	*0.00*	*0.01*	*0.01*	*0.01*	*0.00*	*0.00*	-
	(9)	(7)	(7)	(3)	(11)	(9)	(7)	(8)	(7)	
Mitochondrial capacity	0.02	0.02	0.02	0.01	0.02	0.02	0.02	0.02	0.02	
	0.00	*0.00*	*0.01*	*0.00*	*0.00*	*0.00*	*0.00*	*0.01*	*0.01*	-
	(9)	(6)	(7)	(3)	(10)	(9)	(7)	(7)	(7)	

Fig. S5. Mean values, *standard deviation*, (number of replicates) for all life history and physiological traits following reciprocal transplantation of *Ophryotrocha labronica* from control to experimental conditions and *vice-versa*.

Trait	Reciprocal Transplant									
	C-C-C	**C-W-W**	**W-C-C**	**W-W-W**	**C-A-A**	**A-C-C**	**A-A-A**	**C-WA-WA**	**WA-C-C**	**WA-WA-WA**
Juvenile growth rate	1.58	1.68	1.65	1.72	1.55	1.61	1.55	1.68	1.67	1.71
	0.16	*0.11*	*0.14*	*0.17*	*0.10*	*0.12*	*0.16*	*0.08*	*0.12*	*0.11*
	(12)	(12)	(12)	(7)	(12)	(12)	(12)	(12)	(6)	(3)
Juvenile survival to sexual maturity	69.82	75.56	73.63	75.33	68.00	78.00	81.67	69.45	75.63	80.30
	15.00	*13.15*	*18.41*	*4.27*	*21.37*	*14.62*	*10.85*	*13.35*	*25.96*	*8.45*
	(12)	(12)	(12)	(7)	(12)	(12)	(12)	(12)	(6)	(3)
Mean female size	18.11	16.81	18.24	17.42	17.44	18.31	17.52	17.39	19.81	17.78
	1.00	*2.95*	*2.56*	*3.98*	*0.94*	*0.92*	*0.94*	*1.52*	*1.17*	*2.41*
	(12)	(12)	(12)	(6)	(12)	(12)	(12)	(12)	(6)	(3)
Fecundity	512.08	260.92	491.00	272.86	412.17	442.67	520.58	219.08	494.67	234.33
	208.27	*217.36*	*382.13*	*262.58*	*190.67*	*154.90*	*254.84*	*113.34*	*107.02*	*127.54*
	(12)	(12)	(12)	(7)	(12)	(12)	(12)	(12)	(6)	(3)
Mitochondrial density	0.02	0.02	0.02	0.03	0.02	0.02	0.02	0.02	0.02	0.04
	0.00	*0.00*	*0.01*	*0.01*	*0.00*	*0.01*	*0.01*	*0.01*	*0.00*	*0.01*
	(11)	(4)	(11)	(3)	(7)	(10)	(8)	(5)	(4)	(5)
Mitochondrial capacity	0.01	0.01	0.02	0.03	0.02	0.02	0.02	0.02	0.03	0.04
	0.01	*0.00*	*0.01*	*0.01*	*0.01*	*0.01*	*0.01*	*0.01*	*0.01*	*0.01*
	(11)	(4)	(11)	(4)	(7)	(7)	(9)	(3)	(4)	(5)

Fig. S6. Mean values, *standard deviation,* (number of replicates) for all life history and physiological traits following reciprocal transplantation of *Ophryotrocha labronica* from control to experimental conditions and *vice-versa*.