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RESEARCH ARTICLE

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] and Piero Calosi[2]

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-history traits; (ii) the mechanistic link between adult physiological and fitness-related life-history traits; and (iii) whether the 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, decreases in average reproductive body size and fecundity, and fluctuations in mitochondrial capacity, relative to control conditions. 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.

KEY WORDS: Selection, Acclimation, Phenotypic plasticity, Evolution

INTRODUCTION

An evolutionary race is underway to cope with the ecological impacts of human activity, caused by the release of anthropogenically derived CO_2 into the atmosphere (Palumbi, 2001). The atmospheric partial pressure of CO_2 (P_{CO_2}) has recently surpassed 400 µatm 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). The increase in global CO_2 emissions in such large volumes has had two major effects on the global ocean. Firstly, oceanic surface temperatures have risen by 0.3–0.6°C and, secondly, the ocean pH has dropped by 0.1 pH units 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., 2013; Queirós et al., 2015), which will 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 from the 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., 2016a). 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., 2016) 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, 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 and species (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 organism's fitness and subsequent ecological performance when challenged with high selective pressures (Ghalambor et al., 2007), such as the current rate of global change

[1]Laboratory for Biological Geochemistry, School of Architecture, Civil and Environmental Engineering, École Polytechnique Fédérale de Lausanne (EPFL), Lausanne CH-1015, Switzerland. [2]Département de biologie, chimie et géographie, Université du Québec à Rimouski, 300 Allée des Ursulines, Rimouski, QC, Canada G5L 3A1. [3]College of Science and Engineering, James Cook University, Townsville, QLD4811, Australia.

*Author for correspondence (emma.gibbin@epfl.ch)

E.M.G., 0000-0002-9876-3748

(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 favorable 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 individual's 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; Rodríguez-Romero et al., 2016; 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 P_{CO_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 P_{CO_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 P_{CO_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 *Ophryotroca 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* to a rapid change in P_{CO_2} over six generations (Rodríguez-Romero et al., 2016), 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 and +3°C) for three generations (Munday et al., 2017). 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., 2016; Shama et al., 2016), although epigenetic effects on protein conformation, DNA methylation and chromatin marks may also play a role (Jablonka and Raz, 2009; Bondariansky et al., 2012; Veilleux et al., 2015; Munday et al., 2017; Putnam et al., 2016).

Here, we describe the effects of multi-generational 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., 2016; 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 units) 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.

MATERIALS AND METHODS
Study species
This study was conducted on the benthic marine polychaete *Ophryotrocha labronica* Bacci and La Greca 1961 (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 and Bona, 1993; Paxton and Åkesson, 2007).

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, 12 h light:12 h dark), selected to maximize 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., 2016).

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, 80 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 preventing inbreeding and the accompanying loss of genetic variation (Charlesworth et al., 2003), while ensuring replicates were independent.

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 reach up to 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 fluctuates widely in Porto Empedocle, ranging from 15 to 27°C. We chose to use the summer maximum (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 in Table S1.

Experimental design

Ophryotrocha labronica individuals were exposed to C, W, A and WA for six generations (F1–F6). On the day of hatching, 25 individuals from the F1 parents were transferred to new culture plates and placed in the same treatment as their parents, and 25 individuals from control parents were distributed amongst the experimental treatments. The effects of this acute and transgenerational exposure (F1–F2) are published elsewhere (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 categorized as an 'extinction event' and no further efforts were made to save that replicate.

The 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 25 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 Rodríguez-Romero et al. (2016) 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
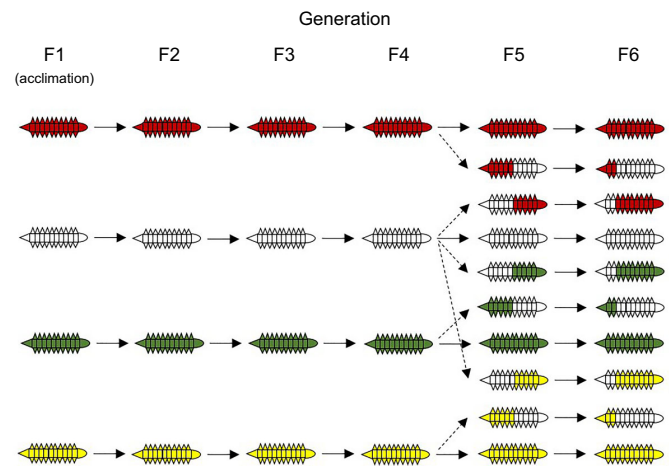
Fig. 1. Schematic diagram of the experimental design. *Ophryotrocha labronica* individuals (*N*=12) were exposed to control (27°C and pH 8, white), ocean warming (30°C and pH 8, red) and ocean acidification (27°C and pH 7.6, green), in isolation and in combination (30°C and pH 7.6, yellow) for six generations (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.

WA-C-C) and vice versa (C-C-W, C-C-A and C-C-WA). Comparing the two generations enabled us to predict whether phenotypic changes were expressions of phenotypic plasticity or new adaptations (Calosi et al., 2013).

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 deemed 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 in which 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, 1 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 by 25 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.

Measurement of physiological traits

Three physiological parameters were measured in female polychaetes: the cellular content of reactive oxygen species (ROS), a marker of oxidative stress, was measured in F2, F3 and F5 using confocal microscopy, whereas the activities of the mitochondrial enzyme citrate synthase (CS) and the mitochondrial electron transport system (ETS) were measured in every generation (N=12 independent replicates per generation, per treatment). CS and ETS 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 focused 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, 2015).

All physiological parameters were measured in sisters of the breeding female. Individuals (N=6) were selected at random and starved for 2 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 µmol l^{-1} CellROX Deep Red and 400 nmol l^{-1} 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) immobilized the sample before the dish was set on 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 standardize ROS to mitochondrial density. A representative confocal image is provided in Fig. S1.

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 mmol l^{-1} phosphate buffer and 2 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA) (pH 8) and the resulting slurry was split in two. Half was transferred to a 100 mmol l^{-1} phosphate buffer containing 2 mmol l^{-1} EDTA (pH 8), 0.1 mmol l^{-1} 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), 0.1 mmol l^{-1} acetyl-CoA and 0.15 mmol l^{-1} oxaloacetate for measurement of ETS

activity and half was placed in a 100 mmol l^{-1} phosphate buffer containing 2 mmol l^{-1} EDTA (pH 8), 0.85 mmol l^{-1} nicotinamide adenine dinucleotide, 2 mmol l^{-1} iodonitrotetrazolium chloride (INT) and 0.03% Triton X-100 for measurement of CS activity. Assays were conducted in triplicate (N=3) on a UV/VIS microplate spectrophotometer (Perkin Elmer Envision, Foster City, CA, USA). CS activity was calculated from the increase in absorbance at 412 nm over 3 min (ε_{412}=13.6 ml cm^{-1} µmol^{-1}), which is caused by the reduction of DTNB (Thibault et al., 1997). ETS 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 4 min (ε_{490}=15.9 ml cm^{-1} µmol^{-1}) (Bergmeyer et al., 1983). Both ETS and CS were standardized to protein content to negate any size-dependent effects, using the bicinchoninic acid method described by Smith and co-workers (1985).

Statistical analyses

The multi-generational response (i.e. F2–F6) was analyzed 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' (five 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 multi-generational exposure to experimental conditions were characterized, we targeted specific traits for correlative analysis to identify potential trade-offs between traits. Only traits significantly affected by multi-generational exposure were used. In these instances, data were 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 analyzed separately. Fixed factors in the F5 analysis were 'parental origin' (C, W, A or WA) and 'offspring exposure' (C, W, A or WA), whereas 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 variance 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).

RESULTS

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

Results for the response to *O. labronica* to increased temperature and acidification are presented in Figs 2–4, whilst statistical outputs are provided in Table 1. Hatching event success is provided in Fig. S2 and the raw data (means±s.d.) are given in Table S2.

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 those exposed to combined conditions (WA) had the lowest success (43%) and the success of those exposed to 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 conditions,
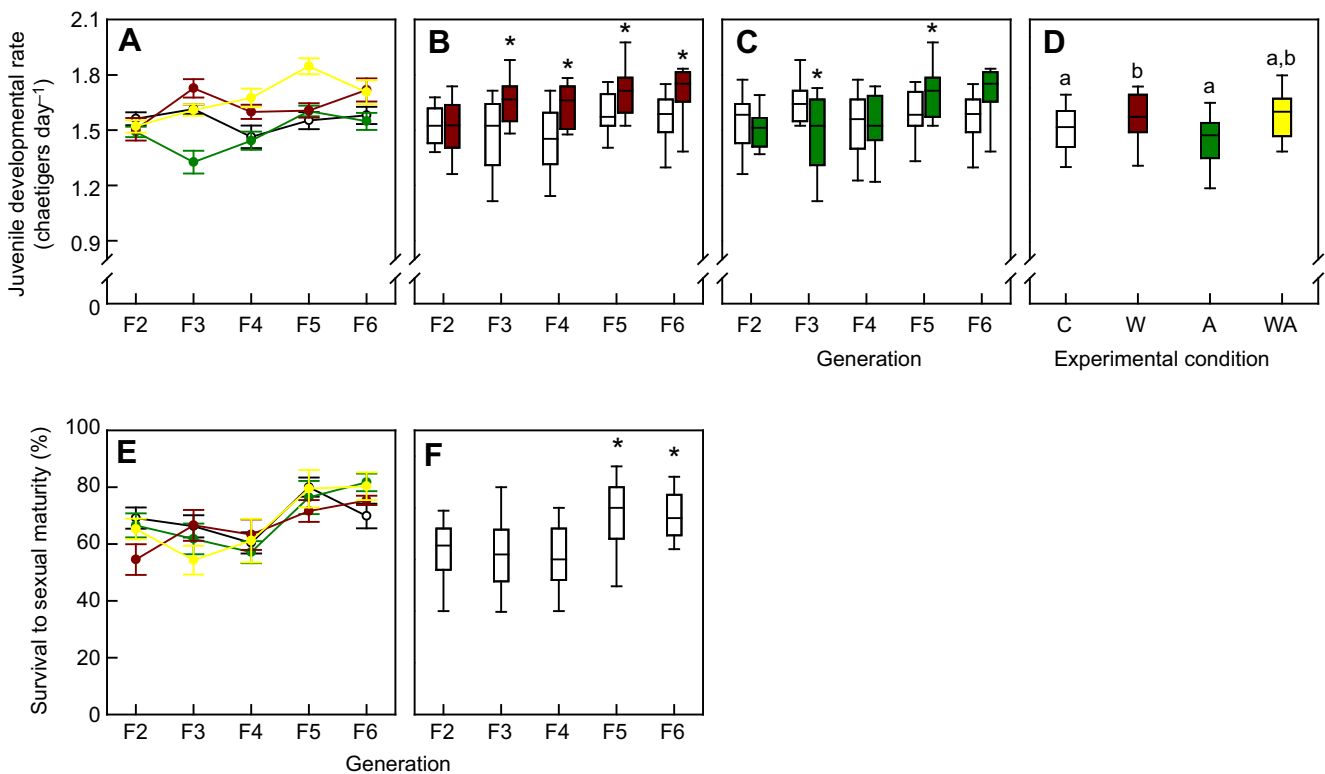
Fig. 2. Effect of multi-generational exposure to global drivers on juvenile life-history traits. *Ophryotrocha labronica* individuals (*N*=12) were exposed to control (C; 27°C, pH 8, white), ocean warming (W; 30°C, pH 8, red) and ocean acidification (A; 27°C, pH 7.6, green), in isolation and in combination (WA; 30°C, pH 7.6, yellow) over six generations (F1–F6). Two juvenile life-history parameters were measured in each generation: juvenile growth rate (A–D) and juvenile survival to sexual maturity (E,F). Raw data are plotted as line graphs where values represent means±s.e.m. (A,E). GLMs were then conducted to determine how traits responded to temperature and pH across multiple generations. Significant interactions are plotted as box plots (B–D,F), 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.

while 24% and 25% were lost in F3 and F5 under WA conditions (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 that 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 in C, but again, this response lasted a single generation (Fig. 2C). In contrast, individuals exposed to W conditions 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 generations 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 conditions (Fig. 3A), with 4% and 2% declines relative to C in F5 and F6, respectively (Fig. 3B), but exposure to neither A nor WA significantly affected the average reproductive body size (Table 1). A similar pattern was observed for fecundity (Fig. 3C), which was reduced by exposure to W and, again, the response differed between generations (Table 1). In F3, individuals exposed to W produced 19% fewer 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).

Multi-generational 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 than those exposed to C conditions (Fig. 4B), as indicated by the presence of a significant generation×temperature interaction (Table 1). CS showed a different trend (Fig. 4C), with levels 38% higher in ocean warming conditions (relative to the control) in F2, before returning to C levels from F3 to F5 and finally increasing again in F6 (Fig. 4D). ETS activity was sensitive to A and W, and their interaction depended on the generation in question (Fig. 4E), as evidenced by the significant three-way interaction between generation, pH and temperature (Table 1). Values were much higher in W than in A conditions in F2 and F6, but this pattern was reversed in F3 and F4 (Fig. 4F). The CS: ETS ratio generally increased over time in both W and C individuals (Fig. 4G); levels were higher in individuals exposed to W in F4 relative to those exposed to C conditions, although this pattern was reversed in F6 (Fig. 4H).

Correlations between traits
Results for relationships between traits are presented in Fig. 5 and statistical outputs are provided in Table 2. We observed significant
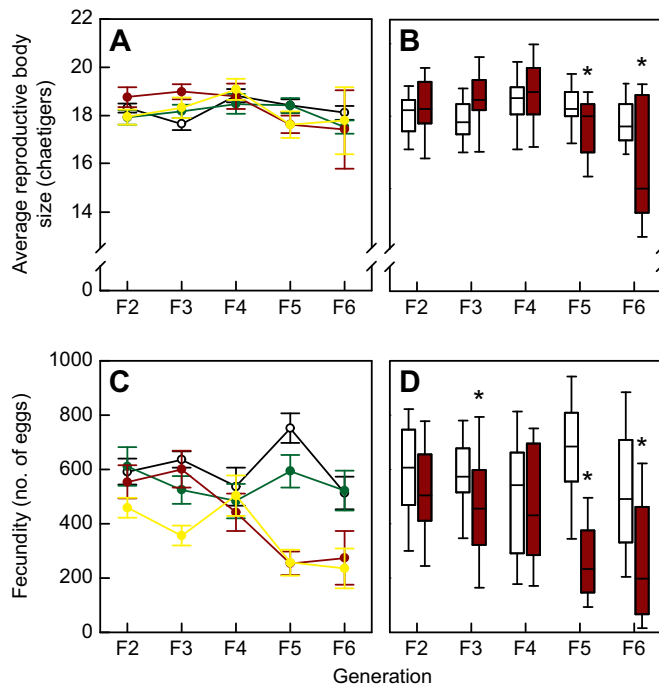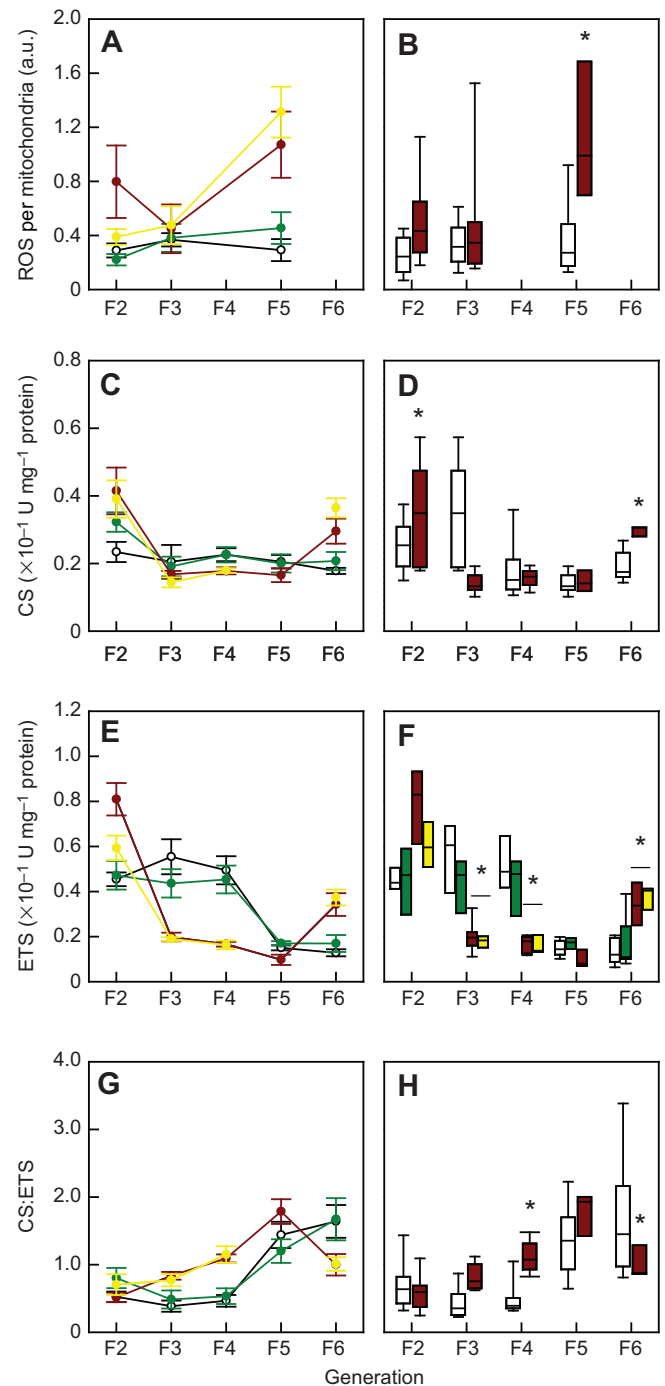
Fig. 3. Effect of multi-generational exposure to global drivers on adult life-history traits. *Ophryotrocha labronica* individuals (*N*=12) were exposed to control (C; 27°C, pH 8, white), ocean warming (W; 30°C, pH 8, red) and ocean acidification (A; 27°C, pH 7.6, green), in isolation and in combination (WA; 30°C, pH 7.6, yellow) over six generations (F1–F6). Two adult life-history parameters were measured in each generation: average reproductive body size (A,B) and fecundity (C,D). Raw data are plotted as line graphs where values represent means±s.e.m. (A,C). GLMs were then conducted to determine how traits responded to temperature and pH across multiple generations. Significant interactions are plotted as box plots (B,D), 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.

positive relationships in response to exposure to W between: (i) survival to sexual maturity and juvenile developmental rate (Fig. 5A); (ii) fecundity and average reproductive body size (Fig. 5B); and (iii) ETS and CS activity (Fig. 5C). Juvenile developmental rate was the only life-history trait significantly affected by exposure to A conditions, but it did not correlate significantly with any other trait (Table 2). There was, however, a significant negative relationship between ETS and juvenile survival to sexual maturity under A conditions (Fig. 5D). 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. 5E).

Transplantation experiment

Results of reciprocal transplantations are presented in Fig. 6, statistical outputs are provided in Table 3 and means (±s.d.) are provided in Tables S3 and S4.

ETS activity was significantly affected by transplantation into the W treatments and so was the CS:ETS ratio (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 ratio 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 the W-C group (Fig. 6A). ETS activity was comparable between individuals

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; 30°C, pH 8, red) and ocean acidification (A; 27°C, pH 7.6, green), in isolation and in combination (WA; 30°C, pH 7.6, yellow) over six generations (F1–F6). Three adult physiological parameters were measured in adults at each generation (with the exception of ROS measurements in generations F4 and F6): reactive oxygen species (ROS) content per mitochondria (A,B), citrate synthase activity (CS; C, D) and electron transport system activity (ETS) (E,F). The ratio of CS to ETS is also given (G,H). Raw data are plotted as line graphs where values represent means±s.e.m. (A,C,E,G). GLMs were then conducted to determine how traits responded to temperature and pH across multiple generations. Significant interactions are plotted as box plots (B,D,F,H), 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.

Table 1. Changes in life history and physiology following multiple generations of exposure to ocean warming and acidification

| Source	Juvenile developmental rate	Juvenile survival to sexual maturity	Average reproductive body size	Fecundity	ROS	CS	ETS	CS:ETS
Generation	$F_{4,199}$=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
Temperature	$F_{1,199}$=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
Generation×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
Generation× temperature	$F_{4,199}$=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×temperature	$F_{1,199}$=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
Generation×pH× temperature	–	–	–	–	–	–	$F_{2,89}$=3.663; **P=0.030**	–

Statistical output from seven general linear models (GLMs). Degrees of freedom (d.f.), F-ratios (F) and probability levels (P) are provided and significant effects (P<0.05) are in bold. Terms that were hierarchically removed from the GLM are indicated by –.

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 into the A treatments (Table 3). Juvenile developmental rate was the only life-history trait significantly affected by transplantation into WA (Fig. 6E), with rates highest in

WA-WA and C-WA, and lowest in WA-C (Fig. 6E). There were no significant differences in juvenile development in F6 (Fig. 6F).

DISCUSSION
Life-history and physiological traits in the marine polychaete *O. 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.
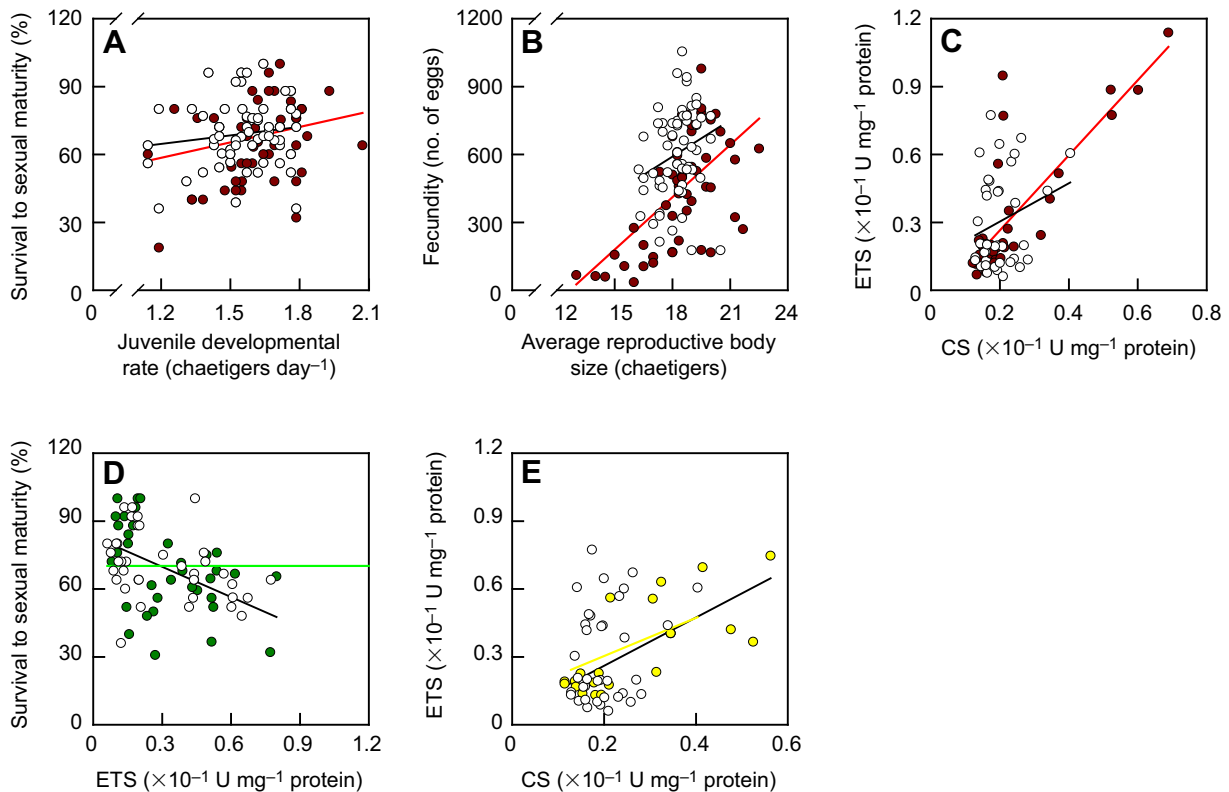
Fig. 5. Energetic trade-offs between life history and physiology. Relationships between traits under ocean warming (30°C, pH 8, red) and acidification (27°C, pH 7.6, green), in isolation and in combination (30°C, pH 7.6, yellow). Parameters measured were: juvenile survival to sexual maturity versus development rate (A), fecundity versus reproductive size (B), ETS versus CS (C,E), and juvenile survival to sexual maturity versus ETS (D). Experimental conditions are plotted relative to the control (27°C, pH 8, white), with each data point representing one individual.

Table 2. The relationship between life-history and physiology traits

Condition	Variable	Juvenile developmental rate	Juvenile survival to sexual maturity	Average reproductive body size	Fecundity	ROS	CS	ETS
Warming	Juvenile developmental rate	–	R^2=0.393; d.f.=53; **P=0.003**	R^2=0.012; d.f.=52; P=0.930	R^2=0.015; d.f.=53; P=0.912	R^2=0.199; d.f.=21; P=0.374	R^2=0.006; d.f.=34; P=0.973	R^2=0.271; d.f.=36; P=0.105
	Juvenile survival to sexual maturity	–	–	R^2=0.198; d.f.=52; P=0.156	R^2=0.169; d.f.=53; P=0.222	R^2=0.171; d.f.=21; P=0.445	R^2=0.030; d.f.=34; P=0.865	R^2=0.256; d.f.=36; P=0.126
	Average reproductive body size	–	–	–	R^2=0.592; d.f.=52; **P=0.002**	R^2=0.171; d.f.=21; P=0.445	R^2=0.030; d.f.=34; P=0.865	R^2=0.256; d.f.=36; P=0.126
	Fecundity	–	–	–	–	R^2=0.160; d.f.=21; P=0.477	R^2=0.085; d.f.=34; P=0.627	R^2=0.159; d.f.=36; P=0.349
	ROS	–	–	–	–	–	R^2=0.285; d.f.=14; P=0.303	R^2=0.234; d.f.=14; P=0.402
	CS	–	–	–	–	–	–	R^2=0.809; d.f.=35; **P<0.001**
Acidification	Juvenile developmental rate	–	R^2=0.082; d.f.=58; P=0.537	R^2=0.020; d.f.=58; P=0.878	R^2=0.550; d.f.=58; P=0.681	R^2=0.118; d.f.=25; P=0.565	R^2=0.058; d.f.=32; P=0.749	R^2=0.187; d.f.=35; P=0.275
	ETS	–	R^2=0.455; d.f.=35; **P=0.005**	R^2=0.197; d.f.=35; P=0.248	R^2=0.094; d.f.=35; P=0.588	R^2=0.229; d.f.=11; P=0.474	R^2=0.324; d.f.=32; P=0.066	–
Combined warming and acidification	Juvenile developmental rate	–	R^2=0.095; d.f.=41; P=0.551	R^2=0.001; d.f.=41; P=0.993	R^2=0.261; d.f.=41; P=0.095	R^2=0.275; d.f.=21; P=0.216	R^2=0.058; d.f.=32; P=0.749	R^2=0.422; d.f.=19; P=0.064
	ETS	–	R^2=0.141; d.f.=19; P=0.553	R^2=0.059; d.f.=19; P=0.804	R^2=0.153; d.f.=19; P=0.521	R^2=0.103; d.f.=10; P=0.764	R^2=0.734; d.f.=21; **P<0.001**	–

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^2, degrees of freedom (d.f.) and probability levels (P) are provided and significant effects (P<0.05) are in bold.

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 did not observe negative fitness impacts of warming until F4 onwards, signifying that at least three generations of exposure to 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 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 multi-generational 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., 2013).

Hatching event success is an important consideration in a multi-generational 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 were lost in ocean acidification conditions, so we conclude that no selection occurs before the juvenile stage. The average hatching success under ocean warming was 57%, although the majority of egg mass loss was confined to the final generation, F5. A similar pattern emerged when ocean warming and acidification were combined, with high losses in F3 producing an average hatching success of just 43%. Interestingly, multiple generations of exposure to ocean warming eliminated 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 (Byrne 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., 2017), so we measured two juvenile life-history traits: developmental rate and survival to sexual maturity. We found 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 observed 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 (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 Wegner, 2014). Parental exposure to elevated temperature (+10°C) doubled juvenile development in the sheepshead minnow,
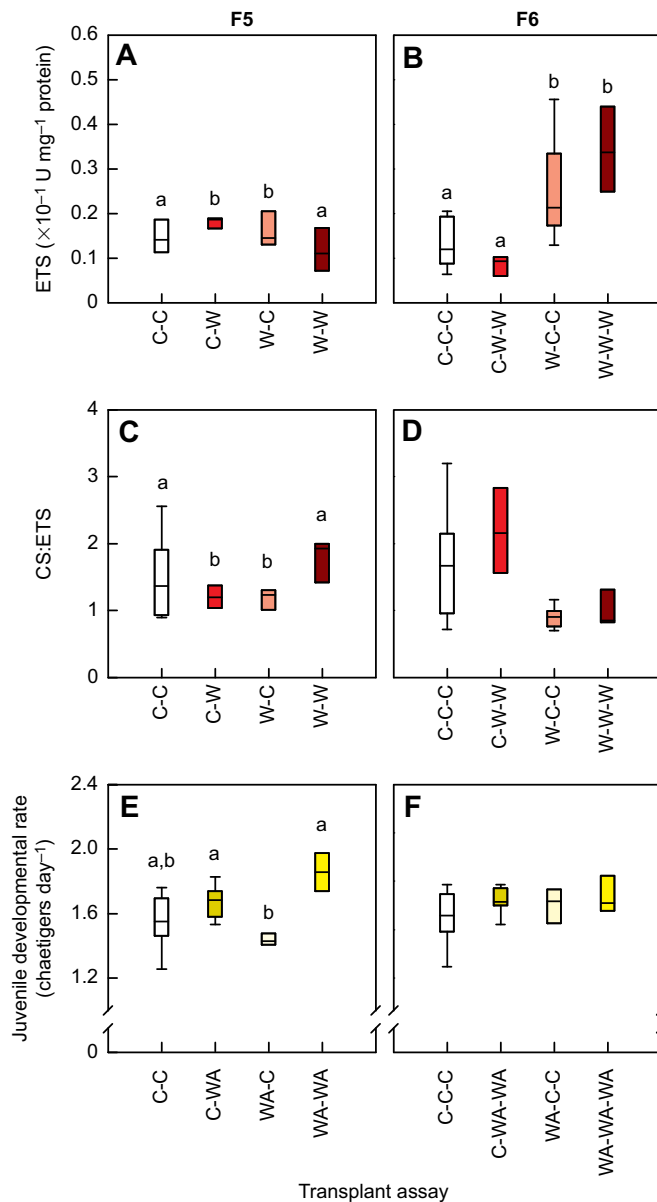
Fig. 6. Plasticity of traits following reciprocal transplantation.
Ophryotrocha labronica individuals (*N*=12) were exposed to control (C; 27°C, pH 8, white), ocean warming (W; 30°C, pH 8, red) and ocean acidification (A; 27°C, pH 7.6, green), in isolation and in combination (WA; 30°C, pH 7.6, yellow) for four generations (F1–F4). Three parameters were measured: ETS (A,B), CS:ETS ratio (C,D) and juvenile development rate (E,F). 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 variance, 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 the changes in life-history and/or physiological traits were. Only significant origin×exposure interactions are plotted and letters identify significant differences between experimental conditions.

Cyprinodon variegatus (Salinas and Munch, 2012), while maternal exposure to a more moderate temperature increase (+4°C) stimulated development in the marine stickleback, *Gasterosteus aculeatus* (Shama et al., 2014). Transgenerational exposure to warming did not result in modifications to juvenile development rates in *O. labronica* (Chakravarti et al., 2016) but after three generations of exposure, we detected increases in the rate of development

(approximately 10%), which persisted over the next three generations. Our findings corroborate those of Shama and Wegner (2014), who also report persistent increases in developmental rate following multi-generational 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 P_{CO_2} in isolation. Changes in juvenile developmental rates between generations exposed to different seawater P_{CO_2} regimes have already been reported in *O. labronica* (Rodríguez-Romero et al., 2016). The values we report are slightly lower, but fall within the same range as those found in the previous study (1.33–1.60 versus 0.92–1.46 chaetigers day^{-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 in the results. 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 the fathers have been exposed to ocean acidification but 25% slower if the mothers experience the same conditions (Lane et al., 2015). As both the males and females were exposed to ocean acidification in this study, it is possible that the neutral response that we observed in F2, F4 and F6 are driven by opposite, additive effects of the parental environment experienced.

The presence of the two drivers in combination results in rates of development that 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. Because 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 by reducing energetic investment in other traits (Stearns, 1992). To determine whether this was the case, we plotted juvenile developmental rate against all of the traits measured in the present study. A positive relationship was evident between the rate of juvenile development and the survival to sexual maturity, with larger individuals more likely to reach sexual maturity, and exposure to ocean warming strengthening the association between the two. Exposure to ocean acidification over multiple generations appears to nullify the negative association between survival to sexual maturity and ETS activity, a marker of mitochondrial capacity suggesting that some metabolic advantage may be gained under high P_{CO_2}.

The majority of changes in adult life-history traits were 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 appeared 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 multi-generational exposure to ocean acidification in the copepod *Pseudocalanus acuspes* (Thor and Dupont, 2016) and in *O. labronica*, respectively

Table 3. Plasticity in life history and physiology following reciprocal transplantation

Condition	Generation	Source	Juvenile developmental rate	Juvenile survival to sexual maturity	Average reproductive body size	Fecundity	CS	ETS	CS:ETS
						Trait			
Warming	F5	O×E	–	–	–	–	–	$F_{1,21}=8.189$; **P=0.009**	$F_{1,21}=5.561$; **P=0.028**
		O	$F_{1,45}=3.304$; 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
		E	$F_{1,45}=9.243$; **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
	F6	O×E	–	–	–	–	–	$F_{1,26}=6.157$; **P=0.020**	–
		O	$F_{1,40}=1.709$; 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**
		E	$F_{1,40}=3.695$; 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
Acidification	F5	O×E	–	–	–	–	–	–	–
		O	$F_{1,44}=1.286$; 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
		E	$F_{1,44}=0.066$; 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
	F6	O×E	–	–	–	–	–	–	–
		O	$F_{1,45}=0.131$; 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
		E	$F_{1,45}=1.441$; 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
Combined warming and acidification	F5	O×E	$F_{1,34}=11.668$; **P=0.002**	–	–	–	–	–	–
		O	$F_{1,34}=0.493$; 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
		E	$F_{1,34}=41.768$; **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
	F6	O×E	–	–	–	–	–	–	–
		O	$F_{1,30}=1.737$; 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
		E	$F_{1,30}=3.393$; 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

Statistical output from two-way analysis of variance (ANOVA), 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. F5 refers to the parental generation; F6 refers to the grandparental generation. Source is given as origin (O) or exposure (E). Degrees of freedom (d.f.), F-ratios (F) and probability levels (P) are provided and significant effects ($P<0.05$) are in bold.

(Rodríguez-Romero et al., 2016) but this has 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 were not evident following transgenerational exposure to ocean warming (Chakravarti et al., 2016); however, after five generations, levels of ROS content were almost twice as high (+190%) as in 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 to 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 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 individual's susceptibility to, and tolerance of, other stressors.

ROS is not the only physiological trait to be affected by ocean warming. ETS activity, a proxy for maximum mitochondrial capacity (Schmidlin et al., 2015), also increased in the ocean warming scenario and, interestingly, this is the only trait in which changes persisted 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) exhibited the same response as those transplanted from ocean warming to control conditions (W-C), with an increase in mitochondrial capacity evident in both. In contrast, in F6, individuals kept in ocean warming conditions for an additional generation (C-W-W) had reduced mitochondrial capacity compared with 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., 2016), 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 multi-generational exposure to ocean warming. As 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 centers 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 they occur via phenotypic plasticity.

In the present study, we posed the question: can multi-generational 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 measured, all but one (maximum mitochondrial capacity) appear to be plastic, rather than adaptive responses. Even under strong selective pressure 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., 2016b). 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., 2016a) and improve the effectiveness of human-assisted parental conditioning projects (van Oppen et al., 2015), ultimately expediting conservation efforts.

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. analyzed the data; and all authors contributed to the writing of the manuscript.

Supplementary information
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Table 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 (pCO_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.

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)
pCO_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)
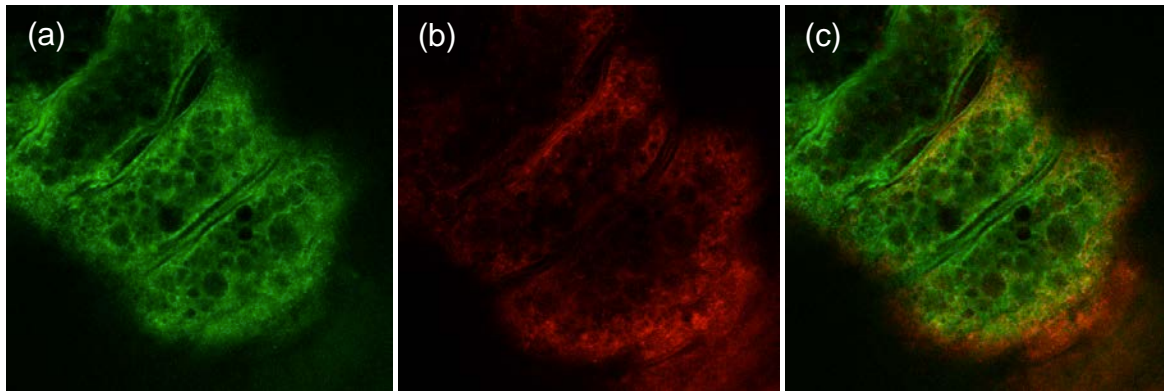
Fig. S1. Confocal image of reactive oxygen species production and mitochondrial density in chætigers 4 and 5 of *Ophryotrocha labronica*. Each individual was incubated with (a) 400 nM of MitoTracker Green FM and (b) 5 µM of CellROX Deep Red for 30 min (c) shows a composite image showing the co-localization 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.
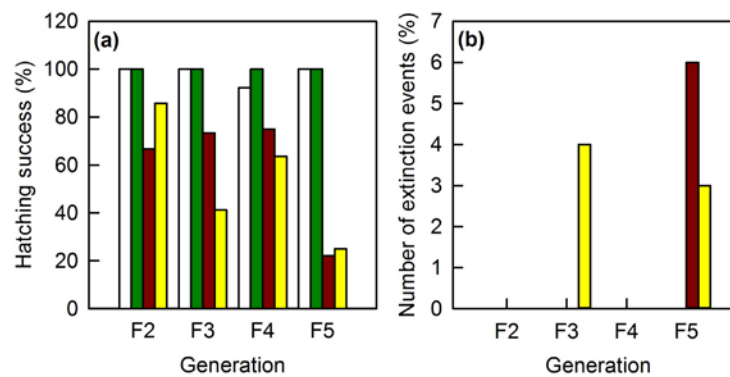
Fig. S2. Selection pressures imposed by multiple generations of exposure to ocean warming and acidification. Histograms show: (A) the percentage of successful hatching events and (B) the number of extinction events imposed by multiple generations of exposure to ocean warming (30 °C, pH 8; red) and ocean acidification (30 °C, pH 7.6; green) in isolation and in combination (30 °C, pH 7.6; yellow) relative to control individuals (white).

Table S2. Mean ± s.d. life-history and physiological traits in *Ophryotrocha labronica* following multiple generations of exposure to ocean warming and acidification, in isolation and combination. Level of replication is given in parentheses.

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
Juvenile developmental 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)
Average 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)
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	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)

Table S3. Mean ± s.d. life-history and physiological traits in *Ophryotrocha labronica* in F5 following reciprocal transplantation of *Ophryotrocha labronica* from control to experimental conditions and *vice-versa*. Level of replication is given in parentheses.

Trait	Transplantation									
	C-C	C-W	W-C	W-W	C-A	A-C	A-A	C-WA	WA-C	WA-WA
Juvenile developmental 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)
Average reproductive 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)
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)
CS activity	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)	
ETS activity	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)	

Table S4. Mean ± s.d. life-history and physiological traits in *Ophryotrocha labronica* in F6 following reciprocal transplantation of *Ophryotrocha labronica* from control to experimental conditions and *vice-versa*. Level of replication is given in parentheses.

Trait	Transplantation									
	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 developmental 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)
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)
Average reproductive body 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)
ROS	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)
CS activity	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)