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Adult exposure to ocean acidification and warming remains beneficial for oyster larvae following starvation

Mitchell C. Gibbs[1,2], Laura M. Parker[1,3], Elliot Scanes [1,2], Maria Byrne [1,2], Wayne A. O'Connor[4], and Pauline M. Ross [1,2,*]

[1]*The University of Sydney, School of Life and Environmental Sciences, Camperdown, NSW 2006, Australia*
[2]*The Sydney Institute of Marine Science, Mosman, NSW 2088, Australia*
[3]*The University of New South Wales, School of Biological, Earth and Environmental Sciences, Kensington, NSW 2052, Australia*
[4]*New South Wales Department of Primary Industries, Port Stephens Fisheries Institute, Taylors Beach, NSW 2316, Australia*

**Corresponding author: tel: +61 2 9351 5026; e-mail: pauline.ross@sydney.edu.au.*

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Climate change is expected to warm and acidify oceans and alter the phenology of phytoplankton, creating a mismatch between larvae and their food. Transgenerational plasticity (TGP) may allow marine species to acclimate to climate change; however, it is expected that this may come with elevated energetic demands. This study used the oysters, *Saccostrea glomerata* and *Crassostrea gigas*, to test the effects of adult parental exposure to elevated pCO_2 and temperature on larvae during starvation and recovery. It was anticipated that beneficial effects of TGP will be limited when larvae oyster are starved. Transgenerational responses and lipid reserves of larvae were measured for 2 weeks. Larvae of *C. gigas* and *S. glomerata* from parents exposed to elevated pCO_2 had greater survival when exposed to elevated CO_2, but this differed between species and temperature. For *S. glomerata*, survival of larvae was greatest when the conditions experienced by larvae matched the condition of their parents. For *C. gigas*, survival of larvae was greater when parents and larvae were exposed to elevated pCO_2. Larvae of both species used lipids when starved. The total lipid content was dependent on parental exposure and temperature. Against expectations, the beneficial TGP responses of larvae remained, despite starvation.

Keywords: climate change, ocean acidification, oyster, starvation, transgenerational plasticity

Introduction

Climate change is occurring over multiple generations of marine species, potentially allowing for responses to improve across generations (Donelson *et al.*, 2012; Parker *et al.*, 2012, 2015, 2017; Ross *et al.*, 2016; Byrne *et al.*, 2020). Transgenerational plasticity (TGP) is a mechanism for rapid acclimation of marine organisms, a form of phenotypic adjustment which results in climate change resilient offspring when adult parents are exposed to climate change conditions (Donelson *et al.*, 2012; Parker *et al.*, 2012, 2015, 2017; Ross *et al.*, 2016; Byrne *et al.*, 2020). For example, Parker *et al.* (2012) found that exposure of adult parents of the oyster *Saccostrea glomerata* to elevated CO_2 improved the response of their offspring to elevated CO_2. This positive TGP effect was found to persist into adulthood and the next generation (Parker *et al.*, 2015), but these advantages were extinguished when larvae experienced multiple stressors (Parker *et al.*, 2017). Putnam *et al.* (2020) found that exposure of parents of the coral, *Pocillopora acuta* to elevated CO_2 during reproductive conditioning improved the survival and settlement rate of the larval offspring and increased the growth rate of settled spat. Furthermore, Vehmaa *et al.* (2012) found that parental exposure of the copepod, *Acartia bifilosa* to elevated temperature of +3°C, improved nauplii production (Vehmaa *et al.*, 2012). While TGP has been shown to improve responses of marine organisms to climate change, improved responses of offspring have been shown for some species to be accompanied by increased oxygen

consumption and metabolic rates (Parker *et al.*, 2012, 2015). Increased metabolic rates infer a greater energy requirement to support homeostasis (Pörtner, 2008) which for planktotrophic species needs to be fuelled largely by energy from food (Thomsen *et al.*, 2013). Not all studies report beneficial effects of TGP, some studies report detrimental or neutral (no change) when adults are exposed (Uthicke *et al.*, 2013; Kong *et al.*, 2019; Venkataraman *et al.*, 2019).

While positive effects of TGP have been observed in marine species, these are typically observed under ideal feeding conditions (Donelson *et al.*, 2012; Hettinger *et al.*, 2012, 2013; Parker *et al.*, 2012, 2015, 2017; Ross *et al.*, 2016). It is anticipated that over this century, climate-mediated alterations in currents and flow will shift the phenology of phytoplankton and zooplankton, creating temporal mismatches between marine species and the availability of their plankton food sources, further constraining larval energy budgets (Edwards and Richardson, 2004; Poloczanska *et al.*, 2013). The potential for such mismatches has already been observed, with spring phenology in the ocean advancing at different rates across taxonomic and functional groups (Poloczanska *et al.*, 2013). In the only study to our knowledge to date to assess the impacts of reduced food supply following transgenerational exposure, larvae of the oyster *S. glomerata* from parents exposed to elevated CO_2 had greater mortality at elevated CO_2 when fed a reduced food diet, compared to larvae from non-exposed parents (Parker *et al.*, 2017). It remains untested whether parental conditioning to climate change stressors can alter the use of energy in offspring via TGP when offspring are starved.

Marine calcifying organisms such as molluscs display particular vulnerability to climate change. In coastal and estuarine environments where molluscs are commonly found, warming and acidification are manifesting more rapidly (Scanes *et al.*, 2020; Cai *et al.*, in press). Here we test whether transgenerational responses native Sydney rock oyster *S. glomerata* and the invasive Pacific oyster *Crassostrea gigas* (alternatively named *Magallana gigas*; Salvi *et al.*, 2014) are evident following periods of starvation and delayed access to food in larval offspring. Further, we test whether TGP will reduce the survival of larvae during starvation due to a faster depletion of their energy reserves. It was anticipated that larval performance improved by parental exposure to elevated pCO_2 and temperature will not be sustained when larvae experience starvation, due to an increase in metabolic rate (see Parker *et al.*, 2017) and faster depletion of their endogenous energy reserves.

Material and methods

To determine whether TGP responses will be still evident following periods of starvation and delayed access to food in larval offspring, adult *S. glomerata* and *C. gigas* were conditioned in ambient and elevated CO_2 (340 µatm and 856 µatm, respectively) at ambient and elevated temperature (24°C and 28°C, respectively) during gamete development. Larvae were then reared at ambient and elevated CO_2 (340 µatm and 856 µatm, respectively) and ambient and elevated temperature (24°C and 28°C, respectively) and food was made available after 5-, 9-, or 11-day starvation. Larval size, mortality, metabolic rate, and lipid dynamics were measured following the different intervals of starvation and to assess the response of larvae during starvation and subsequent recovery following provision of food.

Experimental organisms, adult treatments, and acclimation

Six hundred oysters, three hundred of each species, *S. glomerata* and *C. gigas*, were collected from Holbert's Oyster Supplies (152°4′0.69″E, 32°43′19.69″S) and Diemer's Oysters Supplies (152°3′58.91″E, 32°43′27.49″S) respectively. Both suppliers grow oysters in Cromarty Bay, Port Stephens, NSW, Australia. Inspection of the gonad both macroscopically and microscopically with gonad smears (Leica 400×) confirmed that the oysters were in a regressive-early ripening reproductive condition (Dinamani, 1974). The oysters were transported to the Department of Primary Industries Port Stephens Fisheries Institute, Taylors Beach, NSW, Australia (152°3′20.16″E, 32°44′42.29″S). On arrival, the oysters were scrubbed and cleaned from mud and any potentially fouling organisms then transferred into 40-l tubs filled with 1 µm nominal filtered sea water (FSW, 16°C, 34.5 psu) supplied from a 750-l recirculating header tank where they were maintained for two weeks to acclimate to laboratory conditions.

During acclimation and subsequent experiments, adult oysters received water changes every second day using pre-equilibrated FSW. Throughout adult conditioning, oysters were fed an algal diet of 25% *Chaetoceros muelleri*, 25% *Dicrateria lutheri*, 25% *Tisochrysis lutea*, and 25% *Tetraselmis chuii* at a concentration of 1×10^9 cells oyster^{-1} day^{-1}.

To assess the effects of parental conditioning on larval responses, adult *S. glomerata* and *C. gigas* were exposed to orthogonal treatments of ambient (400 µatm) and elevated (856 µatm) pCO_2 selected to represent the CO_2 concentrations of Earth's atmosphere in the year 2100 based on multi-model averages (Collins *et al.*, 2013). Adult oysters were also conditioned at two temperatures; 24 and 28°C selected to represent the current and predicted temperatures for eastern Australia (Collins *et al.*, 2013; Hobday and Pecl, 2014; Scanes *et al.*, 2020). The mean (± SE) summer temperature of Port Stephens has been measured at 24 ± 0.05°C (calculated from data within Scanes *et al.*, 2020), this temperature was selected to represent the current summer ambient temperature in Port Stephens (24°C). The elevated temperature treatment of 28°C was selected to represent the current upper temperature experienced in Port Stephens during summer, and a temperature that is predicted to become more common in the coming decades [Collins *et al.*, 2013; Hobday and Pecl, 2014; Commonwealth Science and Industry Research Organisation (CSIRO), 2020; Scanes *et al.*, 2020]. Both species of oysters were split into 12 groups of 25 individuals (2 × pCO_2 treatments, 2 × temperature treatments, 3 × replicates), each group of 25 was placed into a 40-l tub of recirculating FSW. One 40-l tub containing *S. glomerata* and one tub containing *C. gigas* were fed FSW from a single 750-l header tank.

Six groups of oysters of each species were randomly assigned to an elevated pCO_2 treatment, and six were assigned to an ambient pCO_2 treatment. The CO_2 was slowly added to the tank to reduce pH to the desired level of 7.7 (856 µatm), over 8 days. From each pCO_2 level, three groups of 25 oysters of each species were assigned to the ambient temperature treatment (24°C) and three groups were assigned to the elevated temperature (28°C) treatment (Figure 1a). Both ambient and elevated temperature treatments were warmed to the experimental temperature of 24 and 28°C over a period of 8 days using an individual 300 W aquarium heater (Seabillion model: HL-388, HaiYi Electrical Appliance

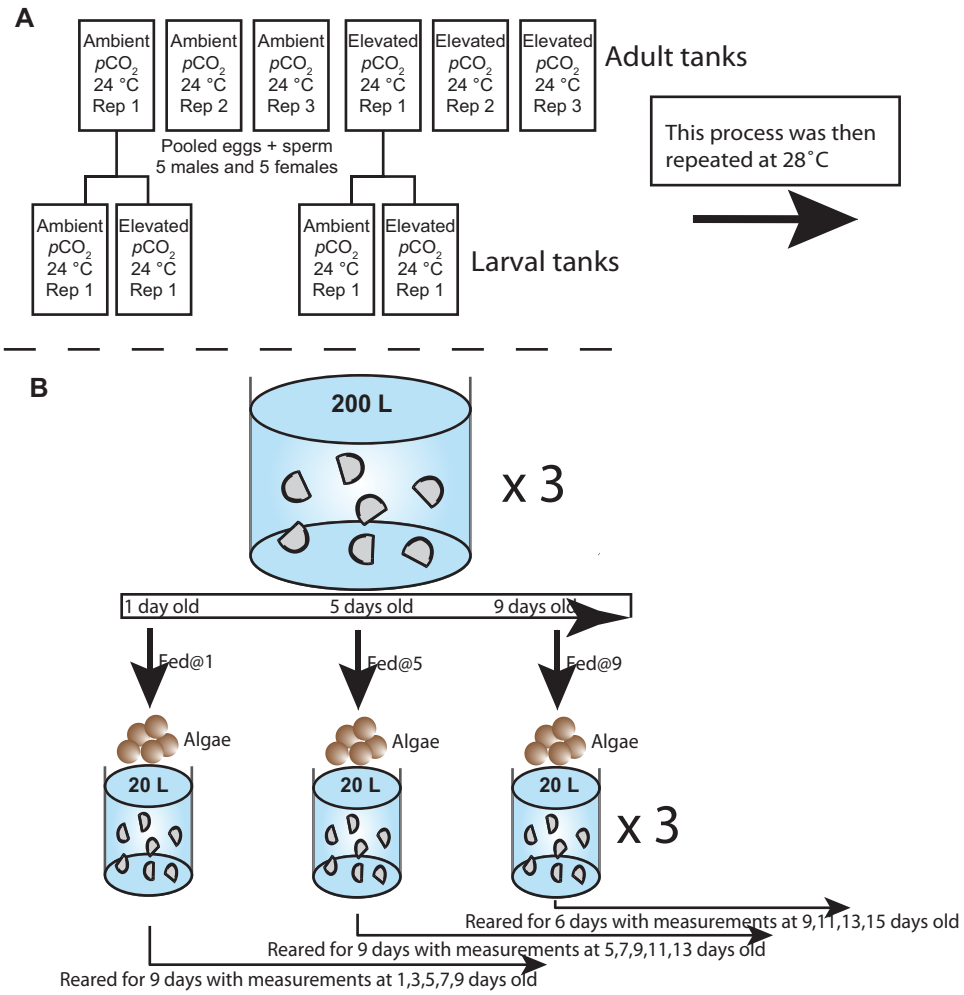

Figure 1. (a) Experimental design used for larval experiments. The first row is tanks representing the adult exposure treatment. Pooled eggs and sperm from five male and five female oysters in each replicate tank were then stocked into two 200-l tanks set at ambient or elevated pCO_2. (b) Conceptual diagram of the feeding delay experimental design.

Table 1. Mean (± SE) physiochemical variables of seawater, from each pCO_2 treatment level over the 8-week parental exposure period ($n = 3$ for each treatment).

Nominal pCO_2 treatment	400 µatm, 24°C		856 µatm, 24°C		400 µatm, 28°C		856 µatm, 28°C	
Total alkalinity	2 449.1	± 64.0	2 482.3	± 58.0	2 471.7	± 60.0	2 467.5	± 50.6
Temperature	24.0	± 1	24.0	± 1	28.0	± 1	28.0	± 1
pH total	8.1	± 0.01	7.8	± 0.01	8.1	± 0.01	7.8	± 0.01
Salinity (ppm)	34.1	± 0.2	34.1	± 0.2	34.1	± 0.2	34.1	± 0.2
pCO_2 (µatm)	367.2	± 1.6598	842.8	± 3.7	369.0	± 10.1	850.7	± 9.7
DIC	2 096.6	± 50.5	2 313.7	± 51.8	2 070.0	± 49.9	2 270.0	± 43.9
$\Omega_{calcite}$	6.1	± 0.3	3.3	± 0.1	6.6	± 0.3	3.8	± 0.1
$\Omega_{aragonite}$	4.0	± 0.2	2.6	± 0.1	4.4	± 0.2	2.5	± 0.1

Measurements were taken weekly.

Factory, Zhongshan, Guangdong, China; accuracy ±1°C) for each 750-l tank.pCO_2 was manipulated in adult conditioning tanks by adding gaseous CO_2 to header tanks using a negative feedback system as described in detail by (Parker *et al.*, 2012). The pH values of each tank (Table 1) were monitored daily, and the pH electrode of each controlling system was checked daily against another calibrated pH probe (Tris buffers, WTW 3400*i*).

Spawning and larval populations

Adult oysters remained in their treatments for eight weeks to build reproductive condition. After eight weeks of conditioning, oysters were gravid (Dinamani, 1974), confirmed by inspecting gonad smears of three oysters from each treatment using a light microscope (Leica 200×). All oysters were then spawned by

Table 2. Mean (± SE) physiochemical variables of seawater, from each pCO_2 treatment level over the 2-week larval exposure experiment ($n = 12$ for each treatment).

Nominal pCO_2 and temperature treatment	400 µatm, 24°C		856 µatm, 24°C		400 µatm, 28°C		856 µatm, 28°C	
Total alkalinity	2 470.0	± 25.7	2 456.9	± 33.4	2 443.5	± 45.2	2 433.1	± 39.4
Temperature	24.0	± 1	24.0	± 1	28.0	± 1	28.0	± 1
pH total	8.1	± 0.01	7.8	± 0.01	8.1	± 0.02	7.8	± 0.01
Salinity (ppm)	34.1	± 0.2	34.1	± 0.2	34.1	± 0.2	34.1	± 0.2
pCO_2 (µatm)	379.0	± 12.8	851.6	± 18.5	371.8	± 25.0	846.0	± 24.0
DIC	2 138.6	± 21.3	2 288.5	± 29.9	2 075.9	± 46.8	2 239.1	± 35.0

lightly scoring the gonad with a scalpel blade and rinsing the gametes out with seawater.

To create larval cultures, a population of larvae from each species was produced from each adult conditioning tank (12 total per species). Eggs of five females per replicate tank, and per species were collected and pooled into a 1-l container and allowed to rest for 2 h. A 0.05-ml subsample of concentrated egg mixture was taken, and eggs were counted on a Sedgewick-Rafter slide. Another subsample was then taken from the mixture at the correct volume to sample 10,000 eggs. These were placed in a 1-ml tube, centrifuged (ca. 5 min at 500*g*) to remove water and stored at −80°C for lipid analysis.

The sperm from each male was collected in a 1-l container and a subsample was checked for motility under a light microscope (Leica 400×). Males with non-motile sperm were excluded. After 2 h, the sperm of five males from the same replicate tank was pooled and then added to the pooled eggs of females from the same replicate tank. Sperm was added incrementally until 5–10 sperm were visible around each egg under a light microscope (Leica 400×). This created a 5 × 5 cross per conditioning tank. Fertilization was confirmed as the presence of polar bodies in >80% of eggs under a light microscope (Leica 200×). Embryos from each conditioning tank were divided into ambient or elevated pCO_2 treatments similar to the design of Scanes *et al.* (2018) (Figure 1b). This was done for each replicate tank ($n = 3$) for each temperature ($n = 2$), pCO_2 ($n = 2$) treatments, and species ($n = 2$), resulting in 48 embryo populations.

Five million embryos from each of 48 embryo populations were transferred to a 200-l (25 larvae ml^{-1}) polyethylene larval rearing tank (48 × 200-l tanks). Each tank was drained after 24 h through a 45 µm mesh to catch D-veliger larvae. The number of D-Veliger larvae in a subsample of known volume was determined for each tank on a Sedgewick-Rafter slide under a light microscope (Leica 200×). New 200-l tanks were restocked with one million D-veliger larvae giving a 5 ml^{-1} stocking density.

To determine the impact of elevated pCO_2 and temperature on the size, mortality and lipid content of larvae, embryos from each parental tank were reared in orthogonal combinations of two pCO_2 treatments (400 and 856µatm). Larvae were also reared at the same temperature as their parents were conditioned (24 and 28°C) for each species (Figure 1b). The temperatures were maintained using individual 300-W aquarium heaters (±1°C) in each tank.

The pCO_2 of tanks containing larvae were manipulated via the constant delivery of premixed CO_2 in air at 856 µatm (BOC Gas Co., Tighes Hill, NSW) to give a pH_{NIST} of 7.8 (checked by calibrated pH probe; WTW 3400*i*). Three individual tanks of premixed gas supplied each of three replicate tanks per treatment. Treatments that were exposed to ambient pCO_2 were continuously bubbled with air at the same rate as the elevated pCO_2 tanks. Water samples were collected for total alkalinity 12 times through experiment and analysed by potentiometric titration and the data were input into CO_2SYS with other parameters to determine the carbonate chemistry (Table 2).

Feeding treatments

The feeding treatments were designed to determine how larvae respond to periods of starvation (see Moran and Manahan, 2004; Gibbs *et al.*, 2020; Figure 1b). Larvae of *S. glomerata* and *C. gigas* were maintained without food from fertilization to the end of starvation treatments. On days 5, 9, and 11 post-fertilization, 100 000 larvae were removed from each 200-l tank and transferred to a 20-l tank where they were fed a diet of 25% *C. muelleri*, 25% *D. lutheri*, 25% *T. lutea*, and 25% *C. calcitrans* at a rate at concentration of 1×10^4–10^5 cells ml^{-1}. These feeding delays were determined by a pilot study that indicated 3 days of starvation did not elicit an effect in larvae; therefore, a minimum of 5 days starvation was selected. Starvation treatments were then selected to occur every second day (except day 7) to coincide with water changes. These feeding delays also matched closely with those used by Moran and Manahan (2004). This created the feeding treatments Fed@5 days, Fed@9 days, and Fed@11 days. In the treatments Fed@5 days, Fed@9 days, and Fed@11 days, the larvae were starved for 4, 8, and 10 days after fertilization, respectively (depending on survival; Figure 1b). After feeding commenced, Fed@5 larvae were reared for 9 days with measurements at 1, 5, 7, 9, 11, and 13 days and Fed@9day larvae were reared for 9 days with measurements at 1, 5, 9, 11, 13, 15, and 17 days or until death. Fed@11 day larvae were reared for 7 days with measurements at 1, 5, 9, 11, 13, 15, and 17 days or until death (Figure 1b; Gibbs *et al.*, 2020).

Larval sampling and morphology

To determine the size, survival, and energetics of larvae, following transfer to the 20-l tank larvae were sampled every second day. Survival was determined by counting the number of live larvae in a subsample of the first 30 encountered in a sub sample under a light microscope (Leica 200×). The size of 30 larvae was measured along their posterior anterior axis using an ocular micrometer under a light microscope (Parker *et al.*, 2010) (Leica 200×). For lipid analysis, 5000 larvae from each replicate were transferred to a 1.5-ml centrifuge tube, centrifuged to allow removal of the sea water (ca. 5 min at 500*g*) and immediately frozen at −80°C.

Larval metabolic rate

Larval metabolic rate (LMR) was measured when larvae were transferred from the 200–20-l tanks. This occurred at 1 day (24 h), 5 days, 9 days, and 11 days (*C. gigas* only). This meant that LMR was always measured on starved larvae. Larvae from each 200-l tank were placed into 5-ml oxygen monitoring sensor vials (PreSens SV-PSt5) at a concentration of 25 000 larvae vial^{-1} including a control vial containing no larvae, to allow larval respiration rates to be corrected for background bacterial respiration. Oxygen concentrations were monitored and LMR was calculated as per Parker *et al.* (2017).

Lipid extraction

To determine the impact of elevated pCO_2 and temperature on total lipid levels, egg and larval samples were freeze dried (Christ Alpha 1-4 LSC). Lipids were extracted using a modified Bligh and Dyer (1959) and quantified using an Iatroscan MK VI TH10 thin-layer chromatography-flame ionization detector analyser (Tokyo, Japan) following the methods described in Gibbs *et al.* (2020). Total lipid was determined as the sum of all lipid classes.

Data analyses

Size, mortality, and total lipid of larvae *C. gigas* and *S. glomerata* were analysed separately for each species and time point to avoid co-dependence and allow for meaningful interpretation of results (Green, 1979). Data were analysed at two meaningful time points: the end of starvation, being 5 days for Fed@5, 9 days for Fed@9, and 11 days for Fed@11 and for valid comparisons at 13 days of age for all treatments. A three-way analysis of variance (ANOVA) was used with "Temperature" (2 levels; 24°C and 28°C), "CO_2 treatment (parents; 2 levels, ambient and elevated)", and "CO_2 treatment (larvae; 2 levels, ambient and elevated)" as fixed

Table 3. Fed@5; results of three-way ANOVAs on the size and % mortality of *S. glomerata* and *C. gigas* exposed to elevated pCO_2 as parents and larvae, and elevated temperature as both larvae and parents from the Fed@5 day treatment.

	Mortality (%)					**Size**			
		Fed@5, 5 days old		**Fed@5, 13 days old**		**Fed@5, 5 days old**		**Fed@5, 13 days old**	
	DF	***F***	***p***	***F***	***P***	***F***	***p***	***F***	***p***
S. glomerata									
CO_2 (larvae)	1,16	0.39	0.54	10.3	<0.01**	58.3	<0.001***	9.37	<0.01*
Temperature	1,16	101.6	<0.001***	42.9	<0.001***	4.3	0.1	8.82	<0.05*
CO_2 (parent)	1,16	1.68	0.21	0.1	0.7	16.1	<0.01**	3.81	0.08
CO_2 (larvae) × temperature	1,16	19.58	<0.001***	6.9	<0.05*	35.7	<0.001***	12.72	<0.01*
CO_2 (larvae) × CO_2 (parent)	1,16	6.48	<0.05*	0.2	0.7	37.5	<0.001***	0.56	0.47
Temperature × CO_2 (parent)	1,16	0.25	0.62			0.3	0.6		
CO_2 (larvae) × temperature × CO_2 (parent)	1,16	0.07	0.8			1.5	0.2		
C. gigas									
CO_2 (larvae)	1,16	0.01	0.94	6.58	<0.05*	0.04	0.85	9.90	<0.01**
Temperature	1,16	7.11	<0.05*	4.19	0.06	14.07	<0.01**	2.31	0.15
CO_2 (parent)	1,16	3.06	0.10	6.95	<0.05*	14.07	<0.01**	0.15	0.70
CO_2 (larvae) × temperature	1,16	1.03	0.33			0.21	0.65		
CO_2 (larvae) × CO_2 (parent)	1,16	0.79	0.39	1.95	0.19	0.62	0.44	0.15	0.71
Temperature × CO_2 (parent)	1,16	0.00	0.98	1.62	0.23	1.70	0.21	5.98	<0.05*
CO_2 (larvae) × temperature × CO_2 (parent)	1,16	0.36	0.56			1.77	0.20		

Measurements were taken at 5 and 13 days of age. Significance levels: <0.05*, <0.01**, <0.001***. Not all comparisons could be made due to insufficient sample sizes.

Table 4. Fed@9; results of three-way ANOVAs on the size and % mortality of *C. gigas* exposed to elevated pCO_2 as parents and larvae, and elevated temperature as both larvae and parents in the Fed@9 day treatment.

	Mortality (%)					**Size**			
		Fed@9, 9 days old		**Fed@9, 13 days old**		**Fed@9, 9 days old**		**Fed@9, 13 days old**	
	DF	***F***	***p***	***F***	***p***	***F***	***p***	***F***	***p***
C. gigas									
CO_2 (larvae)	1,16	0.01	0.93	0.63	0.44	0.45	0.51	10.18	<0.01**
Temperature	1,16	16.25	<0.001***	5.94	<0.05*	2.99	0.1	0.52	0.48
CO_2 (parent)	1,16	1.72	0.21	0.01	0.93	2.53	0.13	7.36	<0.05*
CO_2 (larvae) × temperature	1,16	1.2	0.29	0.17	0.68	0.05	0.82	5.16	<0.05*
CO_2 (larvae) × CO_2 (parent)	1,16	0.77	0.39	0.02	0.9	0.08	0.79	5.22	<0.05*
Temperature × CO_2 (parent)	1,16	0	1	0.21	0.66	0.36	0.56	2.19	0.16
CO_2 (larvae) × temperature × CO_2 (parent)	1,16	0.03	0.88	0	0.99	2.83	0.11	0.3	0.59

Measurements were taken at 9 and 13 days of age. Significance levels: <0.05*, <0.01**, <0.001***.

factors. *Post hoc* Tukey tests were used to determine significant (adjusted p-value <0.05) differences among treatment levels for significant factors of interest. Residual normality for all analyses was checked using Pearson's-residual Plots. All analyses were done using R V3.5.4 statistical software (R core team) and significance was set as $\alpha < 0.05$. Cochran's test was used to detect any heterogeneity of variances. If necessary, the data were log transformed and re-checked with Cochran's test (Underwood, 1997).

Results

Lipid content in eggs

The total lipid content of the eggs was generally greater in *C. gigas* compared to *S. glomerata* in all treatments (Supplementary Figure S1 and Supplementary Table S1). Lipid content of eggs of *S. glomerata* was significantly greater at elevated CO_2 at 28°C (Supplementary Table S1).

Mortality and size of larvae

Saccostrea glomerata

Due to *S. glomerata* larval mortality, a complete set of statistical analysis of data on larval size and mortality is only available up to the Fed@5 day treatment. Larval mortality of *S. glomerata* was dependent on larval CO_2 exposure and temperature (Tables 3 and 4). There was less mortality when parent and larval treatments were the same, either both ambient or elevated CO_2 (Figure 2a and Tables 3 and 4). Temperature also affected mortality. Mortality of larvae of *S. glomerata* was greater at 28 compared to 24°C with 100% mortality observed after 9 days of starvation at 28°C (Figure 2a, Tables 3 and 4, and Supplementary Table S2). The maximum size of *S. glomerata* was at 28°C (Figure 2b). At 28°C, larvae of *S. glomerata* were significantly larger when reared in ambient conditions from parents exposed to elevated pCO_2 (Figure 2b, Tables 3 and 4, and Supplementary Table S2). At 24°C, the size of larvae was not dependent on parental exposure to elevated CO_2.

Crassostrea gigas

Larvae of *C. gigas* were present in most treatments for the duration of the experiments. Larval mortality of *C. gigas* was generally lower than that of *S. glomerata*. Mortality of larvae from parents exposed to elevated CO_2 was less and survival greater than larvae from ambient parents (compare Fed @9 days and Fed@11 days Figure 3a and Tables 3 and 4). Mortality of larvae was generally greater at the elevated temperature of 28°C compared to 24°C except when larvae had been starved for 11 days, when the only survivors where larvae from elevated parents at 28°C (Figure 3a, Tables 3 and 4, and Supplementary Table S2).

Starvation affected the size of larvae of *C. gigas*. For *C. gigas*, parental and larval CO_2 exposure and temperature affected the size of larvae (Figure 3b and Tables 3 and 4). When starved for 5-day larvae were greater in size when reared in ambient CO_2 from parents exposed to ambient conditions compared to larvae of these parents reared in elevated CO_2. This pattern persisted at 28°C, although on day 13, larvae of *C. gigas* were larger in size from parents exposed to elevated CO_2 (Fed@5, Figure 3b and Tables 3 and 4). When larvae were starved for 9 and 11 days, larvae of *C. gigas* from parents exposed to elevated CO_2 were generally smaller than larvae from parents exposed to ambient CO_2 (Fed@9 days and Fed@11 days, Figure 3 and Supplementary Table S2).

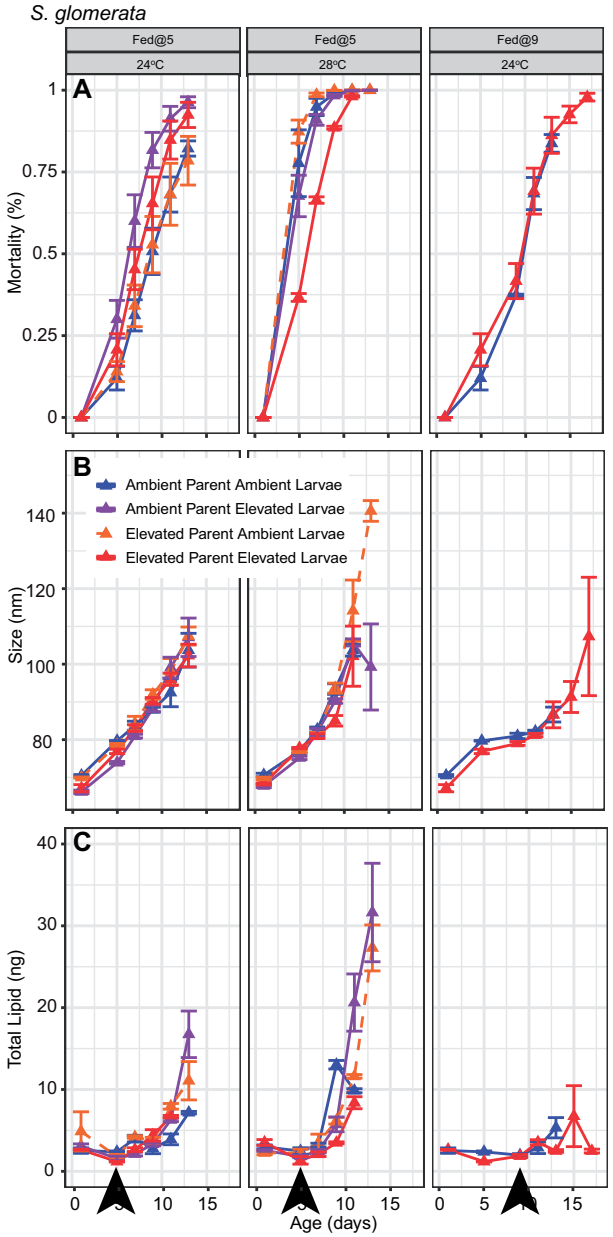

Figure 2. Mean (± SE, $n = 3$) (a) larval mortality (%), (b) larval size (shell length µm), and (c) total lipid content (ng larva^{-1}) of the larvae of *S. glomerata* exposed to experimental treatments of ambient (400 µatm) and elevated (856 µatm) pCO_2at 24 and 28°C. Larvae were measured every second day from day 5 and for a maximum of 9 days following the introduction food, larvae in some treatments died before 9 days. Arrows on the *x*-axis indicate the day at which food was introduced following the feeding delay.

Lipid content in larvae

Saccostrea glomerata

Larvae of *S. glomerata* used lipids when starved (Figure 2c). When larvae of *S. glomerata* were starved for 5 days, their total lipid content was significantly greater when they were exposed to ambient compared to elevated CO_2 (Fed@5 days: 5 days old). Following the commencement of feeding, this pattern was reversed, with larvae exposed to elevated CO_2 having greater total

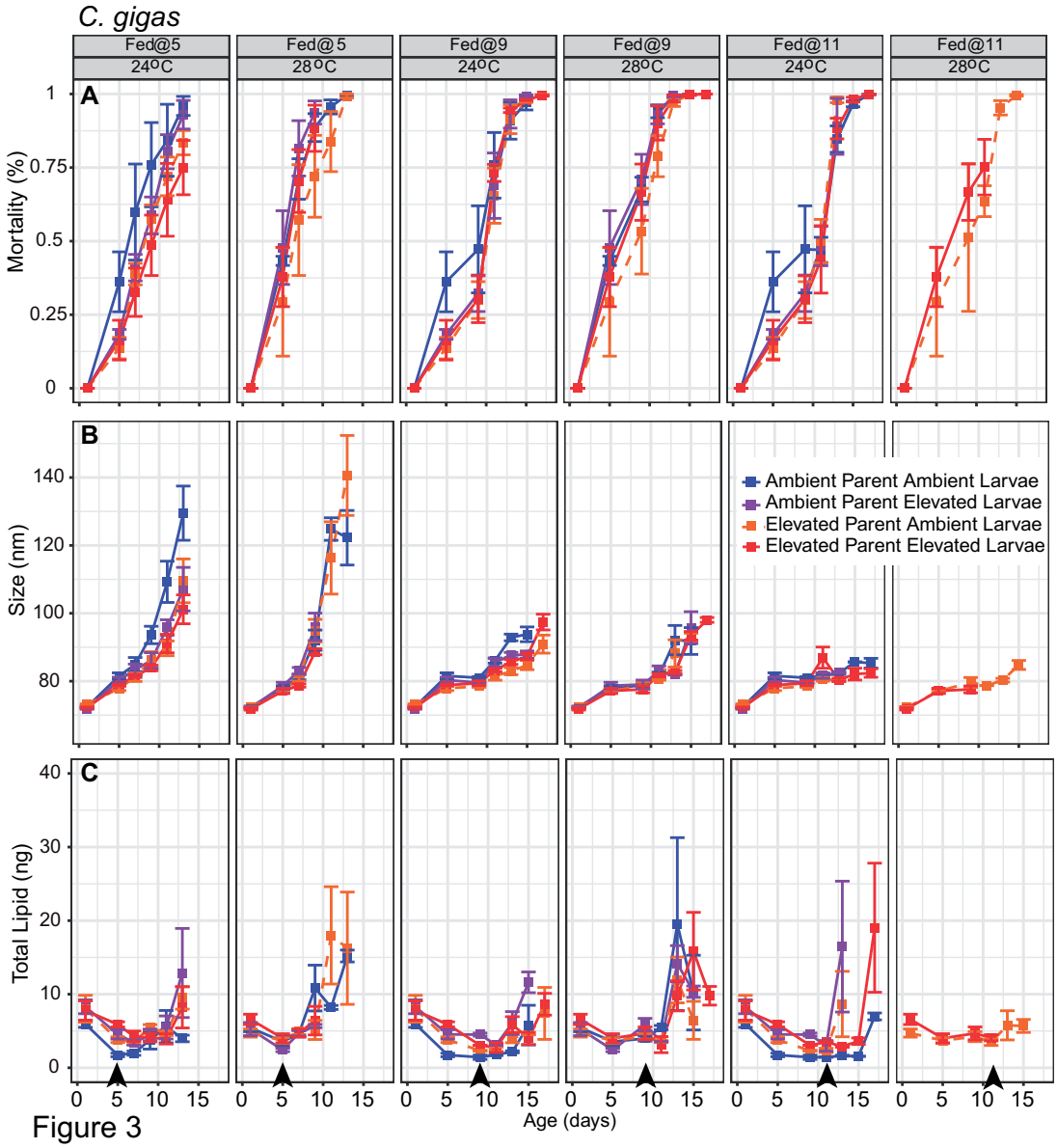

Figure 3. Mean (± SE, $n = 3$) (a) larval mortality (%), (b) larval size (shell length µm), and (c) total lipid content (ng larva^{-1}) of the larvae of *C. gigas* exposed to experimental treatments of ambient (400 µatm) and elevated (856 µatm) pCO_2at 24 and 28°C. Larvae were measured every second day from day 5 and for a maximum of 9 days following the introduction food, larvae in some treatments died before 9 days. Arrows on the *x*-axis indicate the day at which food was introduced following the feeding delay.

lipid content (Fed@5 days: 13 days old; Figure 2c, Table 5, and Supplementary Table S3). By 13 days, the larvae reared at 28°C had a greater lipid content than those reared at 24°C as shown by the significant effect of temperature (Table 5).

Crassostrea gigas

Like *S. glomerata*, larvae of *C. gigas* used lipids when starved (Figure 3c). Larvae of *C. gigas* had greater total lipid content when they were from parents exposed to elevated CO_2 when starved for 5, 9, and 11 days at 24°C (Figure 3c and Table 5) compared to larvae at ambient CO_2 (5 days old, Fed@5 days; 9 days old, Fed@9 days; and 11 days old, Fed@11 days; $p < 0.01$, Figure 3c, Table 5, and Supplementary Table S2). When feeding commenced, there was no effect of larval or parental CO_2 exposure by day 13 for larvae Fed@5 days, Fed@9 days, or Fed@11 days (Figure 3c and Table 5). There was an effect of temperature. The longer larvae were starved, the greater the depletion of total lipids. This depletion was generally greater at 24 compared to 28°C (Fed@9 days and Fed@11 days, Figure 3 and Table 5). Larvae of *C. gigas* had greater total lipid content at 28 compared to 24°C at 13 days old when starved for 9 days (Fed@9 days) (Table 5).

LMR

At 24 h, the LMR of *S. glomerata* was greater when larvae were reared in the opposite CO_2 level to their parents (i.e. ambient parents: elevated larvae; elevated parents: ambient larvae; Figure 4a, Table 6, and Supplementary Table S3). After 5 days of

Table 5. Fed@5; results of three-way ANOVAs on the total lipid content of *S. glomerata* and *C. gigas* exposed to elevated pCO_2 as parents and larvae, and elevated temperature as both larvae and parents from the Fed@5 day, Fed@9 day, and Fed@11 day treatments.

		Fed@5				Fed@9				Fed@11			
		5 days old		13 days old									
	DF	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
S. glomerata													
CO_2 (larvae)	1,16	11.26	<0.01**	8.44	<0.05*								
Temperature	1,16	1.09	0.31	28.44	<0.001***								
CO_2 (parent)	1,16	3.72	0.07	0.89	0.37								
CO_2 (larvae) × temperature	1,16	0.02	0.89	0.04	0.85								
CO_2 (larvae) × CO_2 (parent)	1,16	0.01	0.94										
Temperature × CO_2 (parent)	1,16	0.07	0.79										
CO_2 (larvae) × temperature × CO_2 (parent)	1,16	0.23	0.64										
C. gigas													
CO_2 (larvae)	1,16	9.60	<0.01**	0.53	0.48	18.89	<0.001***	2.847	0.112	11.17	<0.01**	0.98	0.35
Temperature	1,16	3.10	0.10	3.27	0.10	27.82	<0.001***	27.64	<0.001*	9.13	<0.05*	0.01	0.93
CO_2 (parent)	1,16	15.59	<0.01**	1.02	0.33	1.13	0.30	0.001	0.981	3.20	0.10	0.48	0.51
CO_2 (larvae) × temperature	1,16	19.03	<0.001***			1.10	0.31	3.701	0.074	8.42	0.38		
CO_2 (larvae) × CO_2 (parent)	1,16	0.32	0.58	1.00	0.34	7.19	<0.05*	1.248	0.282	0.24	0.63	4.73	0.06
Temperature × CO_2 (parent)	1,16	1.68	0.21	1.02	0.33	0.07	0.80	2.023	0.175				
CO_2 (larvae) × temperature × CO_2 (parent)	1,16	2.73	0.12			0.42	0.53	0.277	0.606				

Measurements were taken at 9 and 13 days of age. Significance levels: <0.05*, <0.01**, <0.001***. Not all comparisons could be made due to mortality of *S. glomerata*.

starvation, this pattern was still observed at 24 but not at 28°C. Instead, at 28°C, LMR was reduced when larvae were reared at elevated CO_2 irrespective of the parental exposure. LMR of *S. glomerata* larvae was greater at 28°C compared to 24°C at 24 h but not after 5 days of starvation, where LMR at 28°C became depressed (Figure 4a and Table 6). By day 9, only larvae in the 24°C survived and there was no difference in LMR between offspring of adults from ambient or elevated CO_2 treatments (Figure 4a).

At 24 h, larvae of *C. gigas* reared at ambient CO_2 had a greater LMR if their parents were reared at elevated CO_2. When larvae were exposed to elevated CO_2, LMR increased in larvae from parents exposed to ambient CO_2 but not in larvae from parents exposed to elevated CO_2. Following starvation, the LMR of larvae of *C. gigas* was low regardless of parental source (Figure 4b). While there was no effect of temperature on the LMR of *C. gigas*, there was a significant interaction effect of temperature × CO_2 (parent) × CO_2 (larvae) (Figure 4b, Table 6, and Supplementary Table S3).

Discussion

The majority of studies to date that have assessed the impacts of elevated CO_2 and temperature on oyster larvae, both acute and transgenerational, have assessed these impacts under optimal food concentrations (Gazeau *et al.*, 2013; Parker *et al.*, 2013). While these experiments provide essential information on how vulnerable marine species will respond over this century, they do not fully account for the natural environment, where food concentration is not always optimal. This study explored a knowledge gap by assessing the impacts of elevated CO_2 and temperature on the transgenerational response of larvae *S. glomerata* and *C. gigas*, when larvae were subjected to different levels of starvation due to delayed access to food. While it is unlikely that marine larvae will experience periods of no food in their natural environment, the incremental starvation and delayed provision of food in this study provided an opportunity to determine whether there were beneficial effects of TGP and tease apart the impacts of endogenous and exogenous energy supply on the TGP developmental responses and lipid dynamics of *S. glomerata* and *C. gigas* exposed to climate change.

Overall, the results of this study indicate that beneficial TGP in response to elevated pCO_2 can occur despite larval starvation. We found a clear positive effect of TGP on the survival of larvae despite starvation. For *C. gigas*, the only larvae remaining alive after 11 days of starvation were those from parents exposed to elevated CO_2. For *S. glomerata*, larval survival was greatest when the conditions experienced by larvae matched the condition of their parents. Parental exposure to elevated CO_2 did not improve the growth rate of larvae for either species when exposed to elevated CO_2. This result contrasts with previous transgenerational studies done on *S. glomerata* under optimal feeding conditions (Parker *et al.*, 2012, 2015, 2017), however, for this study is likely due to the slow grow of larvae during starvation across all treatments.

It was hypothesized that the beneficial transgenerational effects passed from parents to their larval offspring during exposure to elevated CO_2 and temperature would become maladaptive during periods of starvation, as transgenerational larvae would have an inherently increased LMR and therefore deplete their energy reserves sooner (Parker *et al.*, 2017). Instead, across nearly all treatments, starved larvae of *S. glomerata* and *C. gigas* had a reduced LMR, presumably conserving energy and prolonging survival until food became available (Pörtner, 2012; Sokolova, 2013). Such reductions in metabolic rate during episodes of starvation have previously been observed in other animals (Storey and Storey, 1990, 2004; Hahn and Denlinger, 2007; McCue *et al.*, 2017). Interestingly, in the few treatments where LMR was not reduced during starvation (i.e. Pacific oyster larvae from ambient

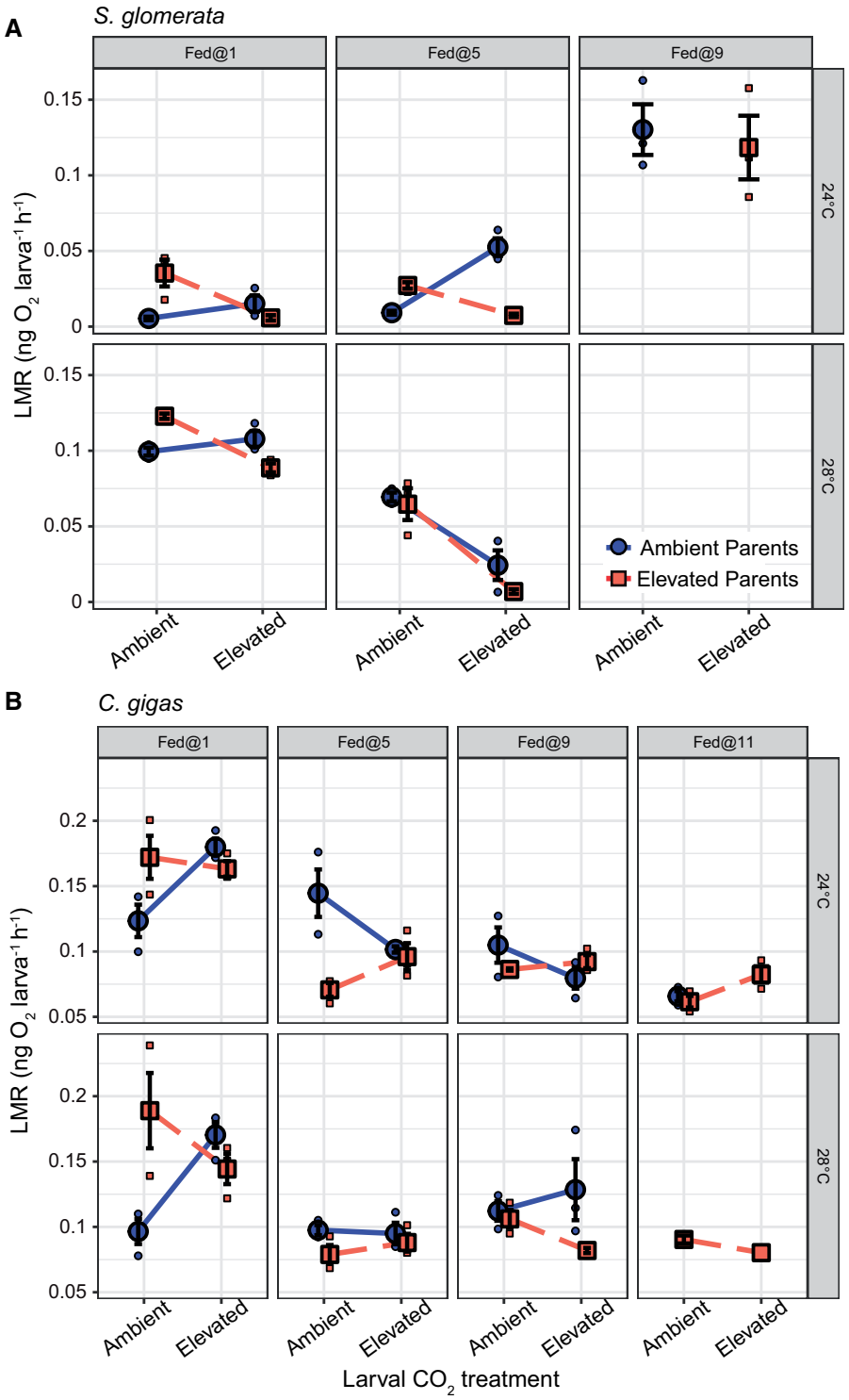

Figure 4. Mean (large symbols, ± SE, $n = 3$) LMR (ng O_2 $larva^{-1}$ h^{-1}) of the larvae of oysters (a) *S. glomerata* and (b) *C. gigas* exposed to orthogonal combinations of ambient (400 µatm) and elevated (856 µatm) pCO_2 as both parents (ambient = blue circles, elevated = red squares) and larvae, as well as 24 and 28°C temperature treatments and feeding delay treatments of 5-day starvation (Fed@5), 9-day starvation (Fed@9), and 11-day starvation (Fed@11). LMR was measured on starved larvae at 1, 5, 9, and 11 days following metamorphosis. The raw data for each replicate are also shown as smaller symbols.

parents, exposed to ambient CO_2 in the Fed@5 day treatment at 24°C, or Sydney rock oyster larvae from ambient parents, exposed to elevated CO_2 in the Fed@5 day treatment at 24°C), larvae experienced a greater depletion of total lipids, greater mortality but maintained a faster rate of growth, compared to larvae from treatments which experienced a reduction in LMR. This further

Table 6. Results of three-way ANOVAs on the LMR of *S. glomerata* and *C. gigas* exposed to elevated pCO_2 as parents and larvae, and elevated temperature as both larvae and parents from the Fed@5 day, Fed@9 day, and Fed@11 day treatments.

		Fed@1				Fed@5				Fed@9				Fed@11	
		S. glomerata		*C. gigas*		*S. glomerata*		*C. gigas*		*S. glomerata*		*C. gigas*		*C. gigas*	
	DF	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
CO_2 (larvae)	1,16	13.1	<0.01*	3.6	0.1	24.4	<0.001***	0.0	0.9	0.2	0.7	0.8	0.4	4.7	0.4
Temperature	1,16	807.1	<0.001***	0.9	0.4	18.5	<0.001***	3.3	0.1			4.7	<0.05*	3.7	<0.05*
CO_2 (parent)	1,16	3.9	0.1	5.8	<0.05*	9.4	<0.01**	20.3	<0.001***			3.7	0.1	0.8	0.1
CO_2 (larvae) × temperature	1,16	0.2	0.6	0.2	0.7	62.0	<0.001***	0.2	0.6			0.1	0.7	2.3	0.7
CO_2 (larvae) × CO_2 (parent)	1,16	43.0	<0.001***	20.4	<0.001***	22.3	<0.001***	11.0	<0.01**			0.1	0.8	0.1	0.8
Temperature × CO_2 (parent)	1,16	1.7	0.2	0.7	0.4	0.1	0.8	4.1	0.1			2.3	0.1	0.1	0.1
CO_2 (larvae) × temperature × CO_2 (parent)	1,16	0.1	0.8	1.7	0.2	9.9	<0.01**	4.4	0.1			5.6	<0.05*	5.6	<0.05*

Significance levels: <0.05*, <0.01**, <0.001***. Not all comparisons could be made due to insufficient sample sizes.

supports the notion that larvae reduce their LMR during periods of starvation to conserve energy and prolong survival.

The total lipid content of larvae of *C. gigas* during starvation was greatest in larvae from parents exposed to elevated CO_2—a result which coincided with greater survival in these larvae compared to those from parents exposed to ambient CO_2. Previous analysis of the total lipid content of eggs of *C. gigas* has shown that mothers exposed to elevated CO_2 do not increase the total lipids which they invest per egg (Supplementary Figure S1). This suggests that the increased total lipid content of larvae from CO_2-exposed parents during starvation occurred due to differences in the usage of endogenous lipid reserves between larvae. Following the commencement of feeding the effect of parental exposure on total lipid content was no longer present, however, lasting effects on survival were still observed.

In contrast to *C. gigas*, mothers of *S. glomerata* have previously been found to increase total lipid investment per egg following exposure to elevated CO_2 (Gibbs *et al.*, 2020). Yet here, we found that this increase in maternal investment did not lead to an increase in the total lipid content in the larvae. Following starvation, the total lipid content of larvae of *S. glomerata* was greater when larvae were reared at ambient compared to elevated CO_2. This result was like that found by Talmage and Gobler (2011) who showed that the lipid index of larvae of the clam, *Mercenaria mercenaria* and scallop, *Argopecten irradians* decreased during exposure to elevated CO_2. But, when starved larvae of *S. glomerata* were given food, the pattern of total lipid content was reversed, with larvae exposed to elevated CO_2 having greater total lipid content than those exposed to ambient CO_2. This suggests differences in the mechanisms involved in the accrual or conversion of lipid in the two groups of larvae. However, in contrast to the larvae of *C. gigas*, an increase in total lipid did not appear to be closely linked to survival in *S. glomerata*. For *S. glomerata*, larval survival was greatest when the conditions experienced by larvae matched the conditions that their parents experienced. For both species, the total lipid content of larvae was greater at the elevated temperature of 28 compared to 24°C. This result was surprising given that the LMR of larvae was similar at 24 and 28°C during periods of starvation.

In summary, TGP may be a beneficial mechanism for mollusc larvae to survive climate change despite potential food shortages. The capacity for TGP may prove decisive for marine mollusc populations living in oceans that are rapidly changing with little sign of slowing in the coming decades. From this study, it appears that the invasive *C. gigas* may have a competitive advantage over the native *S. glomerata* as our oceans continue to acidify and warm, as they survived considerably longer through periods of starvation. *Crassostrea gigas* has proven to be a major invasive species across the globe (Ruesink *et al.*, 2005), yet despite its faster rates of growth and filtration and greater metabolic efficiency, has failed to widely displace the native *S. glomerata* in Australia (Scanes *et al.*, 2016). Climate change may provide *C. gigas* with an advantage that could displace *S. glomerata* from Australian estuaries over this century.

Supplementary data

Supplementary material is available at the *ICESJMS* online version of the manuscript.

Data availability statement

The data underlying this article will be shared on reasonable request to the corresponding author.

Author contributions

The study was conceived and designed by MG, PMR, LMP, and WAO. Data analysis and presentation was done by MG, ES, and PMR. All authors were involved in the writing, editing, and revising of the manuscript.

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