Long-term environmental tolerance of the non-indigenous Pacific oyster to expected contemporary climate change conditions

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Highlights:

- Long-term effects of climate change on non-indigenous species are rarely studied
- Pacific oysters were exposed to warming, ocean acidification and reduced salinity
- Warming and ocean acidification predicted for the year 2100 did not affect fitness
- Low salinity reduced clearance rates and increased oxygen consumption rates
- Long-term observations highlighted potential seasonal trends in physiological rates

Abstract: The current global redistribution of biota is often attributed to two main drivers: contemporary climate change (CCC) and non-indigenous species (NIS). Despite evidence of synergetic effects, however, studies assessing long-term effects of CCC conditions on NIS fitness remain rare. We examined the interactive effects of warming, ocean acidification and reduced salinity on the globally distributed marine NIS *Magallana gigas* (Pacific oyster) over a ten-month period. Growth, clearance and oxygen consumption rates were measured monthly to assess individual fitness. Lower salinity had a significant, permanent effect on *M. gigas*, reducing and increasing clearance and oxygen consumption rates, respectively. Neither predicted increases in seawater temperature nor reduced pH had a long-term physiological effect, indicating conditions predicted for 2100 will not affect adult physiology and survival. These results suggest that *M. gigas* will remain a globally successful NIS and predicted CCC will continue to facilitate their competitive dominance in the near future.

Key words: Climate change, invasive species, *Magallana gigas*, mesocosm, multiple stresses, seasonality

1. Introduction

Non-indigenous species (NIS) and contemporary climate change (CCC) are two of the most significant threats to global biodiversity. From their main points of introduction (e.g. agricultural exploitations, harbours and aquaculture sites), NIS can spread to surrounding areas and establish new populations, becoming invasive (Eno *et al.*, 1997; Manchester & Bullock, 2000; Richardson *et al.*, 2000; Jensen *et al.*, 2004; Hellmann *et al.*, 2008). In addition, NIS can negatively impact native biodiversity and alter the structure and functioning of ecosystems (Nichols *et al.*, 1990; Carlton & Geller, 1993; Travis, 1993; Grosholz Edwin *et al.*, 2000). Knowledge of the potential impacts of CCC on marine NIS has advanced rapidly in recent years through the use of laboratory and field experiments (for example Lemasson *et al*., 2018; Miranda *et al*., 2019; Nguyen *et al.*, 2020), habitat suitability modelling (for example Sarà *et al*., 2018; D’Amen & Azzurro, 2019; Zhang *et al*., 2020) and mechanistic modelling (Thomas *et al*., 2016). Mounting evidence suggests that CCC is likely to facilitate NIS spread and alter the current global distribution of species (Dukes & Mooney, 1999; Carlton, 2000; Occhipinti-Ambrogi, 2007; Hellmann *et al.*, 2008; Rahel & Olden, 2008; Occhipinti-Ambrogi & Galil, 2010; Smith *et al.*, 2012).

Anthropogenic activities have led to both an increase in global temperatures and ocean acidification over the last 150 years (Caldeira & Wickett, 2003; Caldeira & Wickett, 2005; Gattuso *et al.*, 2015; IPCC, 2019). It is expected that by the end of the century, global surface temperatures and ocean pH will rise by 2 - 4°C and decrease by 0.3 - 0.4 units, respectively, relative to 1986 – 2005 (Caldeira & Wickett, 2003; Sabine *et al.*, 2004; Caldeira & Wickett, 2005; IPCC, 2019). In response to these environmental changes, species distributions are shifting to higher latitudes and altitudes, or deeper depths in the oceans (Parmesan & Yohe, 2003; Parmesan *et al.*, 2005; Helmuth *et al.*, 2006; Hickling *et al.*, 2006; Mieszkowska *et al.*, 2006; Cheung *et al.*, 2009; Rijnsdorp *et al.*, 2009; Chen *et al.*, 2011; Pecl *et al.*, 2017). Range expansions and increases in abundance have already been reported on NIS that have experienced a 50-year period of warming (Rius *et al.*, 2014). Consequently, range expansions of NIS are expected throughout this century in both marine and terrestrial ecosystems (Occhipinti-Ambrogi, 2007; Rahel & Olden, 2008; Pecl *et al.*, 2017). A meta-analysis of NIS performance under CCC conditions showed that NIS displayed more positive responses to increased ocean acidification and warming than native species (Sorte *et al.*, 2013), probably due to successful NIS being able to survive across a wider range of these parameters. Overall, the potential positive effects of CCC on NIS may ultimately facilitate NIS dominance in the near future.

The environmental factors linked to CCC rarely act in isolation and interactions between multiple environmental stressors are known to exacerbate the effects of CCC on species performance (Przeslawski *et al.*, 2005; Staudt *et al.*, 2013; Delorme & Sewell, 2014; Przeslawski *et al.*, 2015). It is, therefore, crucial to study the combined effects of multiple abiotic stressors on species physiology to fully understand how CCC will affect the success of

NIS. However, assessments of the effects of multiple stressors, particularly those using more than two stressors, are uncommon with the majority of CCC experiments focusing on short-term exposures (Kroeker *et al.*, 2013). Although these experiments provide key insights into how CCC affects the physiology of both native and NIS, short-term experiments may not account for the acclimation of the species to experimental conditions and only provide limited evidence of chronic stress responses (Hollister *et al.*, 2005; Leuzinger *et al.*, 2011; Dupont *et al.*, 2013; Smith & Dukes, 2013; Stewart *et al.*, 2013; Suckling *et al.*, 2015). Data from long and short-term experiments have revealed contrasting results (Form & Riebesell, 2011; Dupont *et al.*, 2013; Munday *et al.*, 2013; Suckling *et al.*, 2015), with species showing acclimation and resilience only in long-term and/or trans-generational exposure to abiotic stressors (Donelson *et al.*, 2011; Munday, 2014; Palumbi *et al.*, 2014; Suckling *et al.*, 2015; Ross *et al.*, 2016). Long-term studies are, therefore, crucial for accurate predictions of the effects of CCC on species fitness. Another advantage of long-term experiments is that they can encompass seasonal changes in environmental conditions, which are often overlooked in short-term experiments. By including maximum and minimum temperatures, an assessment can be made of the effects of stressors across phenological cycles.

In this study we assessed how changes in key environmental conditions, as predicted under CCC at the end of the 21st century, interact and affect the long-term performance of the highly successful marine NIS the Pacific oyster, *Magallana gigas* (Thunberg, 1793). From its native range in northeast Asia, *M. gigas* has been introduced into over 65 countries for use in aquaculture worth approximately $1.2 – $1.4 billion per year (Fig. S1a). *M. gigas* has already established wild populations in more than 17 countries (Fig. S1b), making it a globally successful NIS (Herbert *et al.*, 2016). *M. gigas* is classified as an invasive species due to its ability to spread and form extensive reefs, modifying the habitats and threatening indigenous biodiversity (Richardson *et al.*, 2000; Lejart & Hily, 2005; Smaal *et al.*, 2008; Troost, 2010; Holm *et al.*, 2016).

There are a wealth of studies investigating environmental change on *M. gigas* fitness, however, contrasting results have shown both neutral/positive (Havenhand & Schlegel, 2009; Falkenberg *et al.*, 2019) and negative (Kurihara *et al.*, 2007; Lannig *et al.*, 2010; Barros *et al.*, 2013; Lemasson *et al.*, 2018) effects of warming and ocean acidification on adult and larval stages. Whilst the majority of these experiments have shown the short-term sensitivity of *M. gigas* to CCC, *M. gigas* may acclimate and express resilience to these conditions in the long-term. Further investigation is, therefore, needed to assess the effects of prolonged, seasonal exposure of adult *M. gigas* to predicted CCC conditions and how the long-term interactive effects between altered abiotic factors will contribute to their continued success.

Here, *M. gigas* were exposed to varying levels of three abiotic stressors, temperature, pH and salinity, over a ten-month period. We hypothesized that: (1) long-term exposure to expected CCC conditions and reduced salinity would negatively affect *M. gigas* fitness, and (2) the manipulated environmental conditions will have synergistic interactions, ultimately affecting the performance of *M. gigas*.

2. Methods

2.1. Mesocosm set up and conditions

We created a mesocosm system with 12 independent experimental treatments (Fig. 1). Each treatment consisted of a closed system with a header and footer tank circulating approximately 200 litres of natural, untreated, filtered seawater from Plymouth Sound through six, nine litre experimental tanks. The 12-level (2 temperature x 3 pH treatments x 2 salinity) factorial experiment included different temperature (ambient and +4 °C), pH (ambient at 8.1, 7.7 and 7.5), and salinities (34 and 20) levels (Fig. 1). Temperature and pH were chosen to reflect ambient and forecasted projections from both IPCC and UKCP09 reports (Jenkins *et al.*, 2009; Lowe *et al.*, 2009; Hughes *et al.*, 2017; IPCC, 2019). Reduced salinity is a key range limiting factor for marine species, often having a negative impact on species physiology and survival (Cognetti & Maltagliati, 2000; Paavola *et al.*, 2005; Braby & Somero, 2006; Pourmozaffar *et* *al.*, 2020), and thus salinity was manipulated to reflect the tolerance range of *M. gigas* with conditions experienced in northwest Europe as intertidal coastal and estuarine organisms. Salinities above 20 are regarded as important for the establishment of Pacific oysters and adult oysters having been observed in salinities ranging from 20 (Scandinavian coast (Wrange *et al.*, 2010)) to more than 35. *M. gigas* are also common in estuaries where salinity fluctuates on a semi-diurnal basis. Each of the 12 treatments was run for 10 months between June 2018 to March 2019.

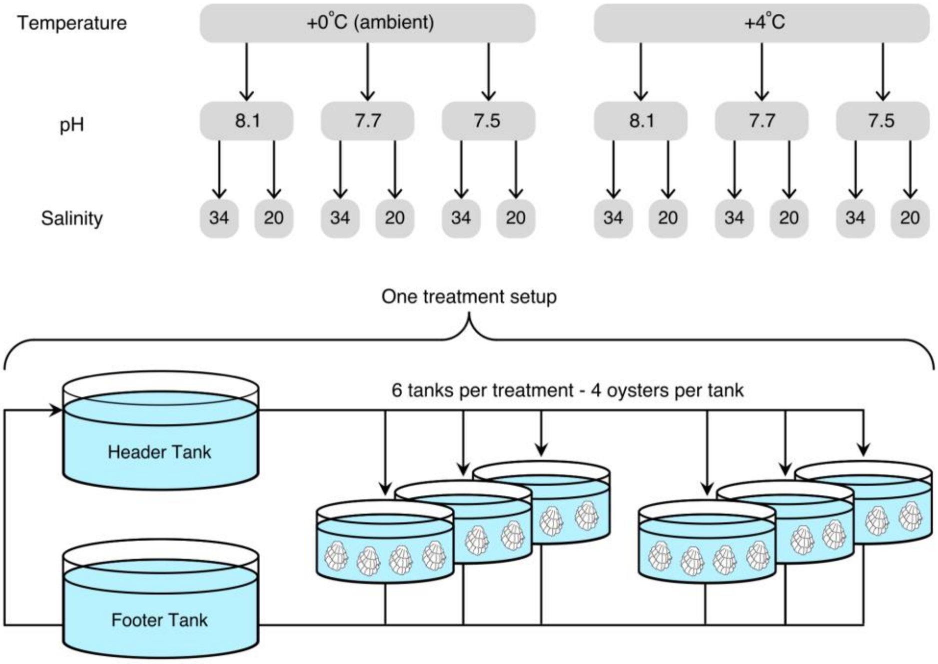

Figure 1. Experimental design used for the 12-level factorial experiment and the layout of each closed treatment system. Treatment refers to each of the 12 independent combinations of the environmental parameters. The six, nine litre tanks per treatment acted as the units of replication.

The pH was regulated using Aqua Medic pH computers, which administered CO_2 when the pH deviated by 0.02 above the treatment value. To reflect seasonality in natural systems, daylength and temperature were adjusted at the start of every month, with seawater temperature reflecting the average sea surface temperature at L4 station in the Western English Channel off the coast of Plymouth (www.westernchannelobservatory.org.uk) (Fig. 2). Air temperature was used to control ambient seawater temperature and was adjusted to 2°C below the monthly average at L4 to achieve the desired seawater temperatures. Seawater temperature in the +4°C treatments were achieved by placing heaters in the reservoir tanks. Seawater temperatures varied by ±0.5°C across the two temperature levels. Total alkalinity for each treatment was measured every other week using an Alkalinity Titrator (Model AS-ALK2, Apollo SciTech, Bogart). The water chemistry recorded for each treatment is shown in Table S1.

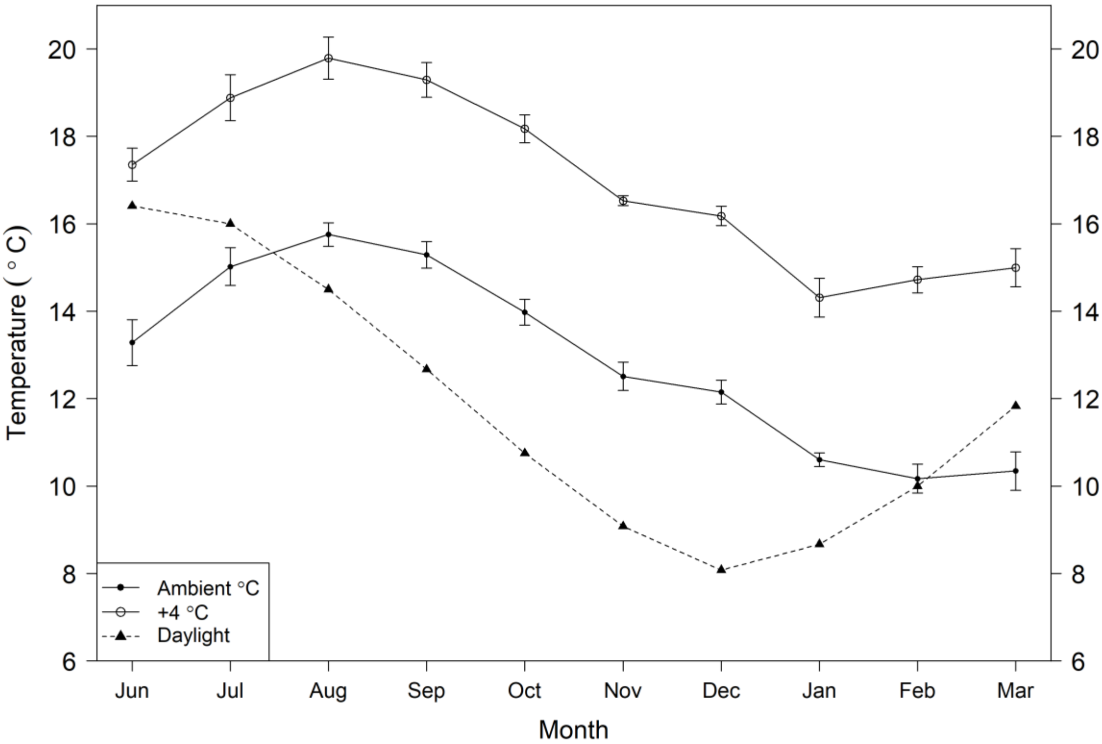

Figure 2. Monthly mean seawater temperature (ambient and +4ºC) and daylight hours in the mesocosm system between June 2018 and March 2019.

Adult *M. gigas* individuals (60 - 113 mm maximum length) were collected at the end of March 2018 from the low intertidal zone of the River Yealm estuary (50.3098°N, 4.0537°W), which is 15 km away from the laboratories of the Marine Biological Association of the United Kingdom (50.3641°N, 4.1391°W) where the experiments were conducted. The collected individuals were placed in insulated containers that were transported to the laboratory within one hour. Once cleaned of epibionts, a total of 288 oysters were randomly allocated to tanks, with 24 *M. gigas* per treatment across six replicate tanks, i.e. four oysters per tank (based off Utting & Spencer, 1991) (Fig. 1). *M. gigas* were initially acclimated to their temperature and salinity treatments for four weeks prior to introducing CO_2 to the systems, then acclimated for a further four weeks before measuring physiological parameters. Oysters were fed with a diet of *Isochrysis galbana* and *Tetraselmis sp.* (Instant Algae® by Reed Mariculture) five days a week at an amount equivalent to 2% of the mean oyster dry weight (dry weights based on destructively sampling a representative sample of oysters from the Yealm Estuary) (Helm & Bourne, 2004). The concentration of food in the tanks at each feed was in the order of $x10^8$ cells per litre. Tanks were checked prior to feeding and dead oysters removed; only 11 oysters died over the duration of the experiment. A 10% water change with filtered seawater occurred twice a week; freshwater was added to reduce salinity to 20 in half of the treatments.

2.2. Growth

Initially, all 288 oysters were weighed to the nearest 0.01g prior to treatment exposure to obtain a baseline weight. Weight of the oysters was then assessed at the start of each month (with the exception of January and February). Every oyster was removed from their tank, carefully dried to remove excess water from the shell and total live wet weight (shell and wet tissue) of each individual oyster was measured. Weight change was then determined as the difference in the monthly weight compared to baseline weight.

2.3. Physiological measurements

Physiological measurements were taken during the fourth week of each month, after *M. gigas* were exposed to the monthly environmental conditions for three weeks. Daily feeding was stopped two days prior to measuring physiological parameters to obtain values at a routine metabolic rate. Oysters were returned to their respective tanks after physiological measurements were taken.

2.3.1. Clearance rate

The clearance rates for *M. gigas* (i.e. the volume of water cleared of algal cells per hour) were measured to assess their efficiency to remove seston within each treatment. Clearance rates were measured on six individual oysters from each treatment (one randomly selected from the replicate tanks). Oysters were placed in an enclosed chamber with 1 litre of filtered seawater with temperature, pH and salinity levels matching their respective treatments. Once the oyster was visibly feeding (valves open) a concentration of 2 x 10^8 cells per litre (below pseudofaeces production threshold) of *Isochrysis* sp. was added to the water and the oyster left to feed for 30 minutes. Strong, continuous aeration was used to keep the water constantly mixed and prevent the settlement of algae cells. Water samples at the beginning and end of each 30-minute period were collected and the cell concentration of each sample analyzed using a Beckman Coulter Counter Z Series. Clearance rate (*CR*) was calculated from the exponential decrease in cells using the following equation described in Coughlan (1969):

$$CR = V \times \ln(C0) - \ln\,(C1)/t$$

where *V* is the volume of water in the enclosed chamber, *C1* and *C2* are the concentration of cells before and after 30 minutes, respectively, and *t* is the time in hours. Clearance rates were then standardized to 1 g total wet weight of the oyster. Total wet weight was used to standardize the rates due to oyster size and condition having an effect on physiological parameters. As a control, tanks were run without oysters to assess potential algal settlement during clearance rate measurements.

Due to unforeseen circumstances, the clearance rates in August from all four treatments with a pH of 8.1 were removed from the analysis due to an equipment failure leading to measurements being unreliable.

2.3.2. Oxygen consumption

Oxygen removal from the water by *M. gigas*, a proxy for metabolic demand (Treberg *et al.*, 2016), was measured monthly (last week of every month) using a fibre-optic oxygen logger (Piccolo2; PyroScience). Oxygen consumption rates were obtained from four oysters per treatment (one randomly selected from four tanks per treatment). Individuals were isolated in a closed chamber with 1 litre of seawater with temperature, pH and salinity levels matching their respective treatments. Oysters were left to acclimatize to the chamber for at least 15 minutes before measuring oxygen concentration. Once the oysters began to uptake oxygen (valves opened), after the acclimatizing period, oxygen concentration was recorded every second for a minimum of 30 minutes. If the oyster closed its values during the recording, the timer was restarted when valves were opened to ensure 30 minutes of continuous oxygen decline in the water. Seawater was continuously stirred during each trial with a magnetic stirrer to ensure oxygen was equally mixed throughout the water. If the individual remained shut for more than 60 minutes, and therefore was not taking up oxygen, the individual was replaced with another from within the same tank. Oxygen in the water did not fall below 70% saturation during any of the trials and therefore did not limit oxygen consumption (Ren *et al.*, 2000). Oxygen removal in control tanks, without oysters, was measured for each treatment each month to determine the potential rate of decline from bacterial respiration.

Oxygen consumption was estimated as the rate of oxygen decrease per second in the chamber. Linear regression was used to obtain the average rate of oxygen consumption. The rate was taken after the first ten minutes of the oyster consuming oxygen to allow time for the oyster to reach a steady state of respiration. The rate of change was then scaled up to obtain a rate of uptake per hour and standardized to 1 g total wet weight of the oyster by dividing the rate by the total wet weight (including shell) of the oyster.

2.4. Data analysis

Weight change from baseline, clearance rates and oxygen consumption rates were analysed separately using linear, mixed effect, repeated measures analysis of variance (RM-ANOVA) models with an autocorrelated error structure (Quinn & Keough, 2002). The models included a term for month and the 12 treatments (see details in Fig. 2) were fitted as a 2x2x3 factorial with main effects, 2-factor interactions, and the 3-factor interaction (Quinn & Keough, 2002). Month-by-treatment interactions were also fitted. For weight change, individual oyster was used in the model as the repeated measure, and for clearance and oxygen consumption rates, tank was used as the repeated measure.

Assumptions of parametric tests were checked by analyzing: normality, which was assessed by plotting theoretical quantiles with versus standardized residuals (Q-Q plots) and using Shapiro-Wilk test; and homogeneity of variances, which was tested by plotting residuals versus fitted values and Levene test. All assumptions were met, except for clearance rate where there was evidence of non-normality. A non-parametric ANOVA (randomization test) was performed and the results were consistent with the parametric test, therefore, given the conclusions are the same, we have reported the values from the parametric model. All models were fitted in R (R Core Team, 2019).

3. Results

3.1. Growth

Oysters lost an average of approximately 0.5g in weight in the first month after establishment in the mesocosm. Weight increased at the start of the experiment before decreasing for all treatments to a minimum in September (Fig. 3a). There were statistically significant interactions between month and the three treatment factors however, as no clear or consistent treatment-related trends were observed (Fig. 3b, c, d).

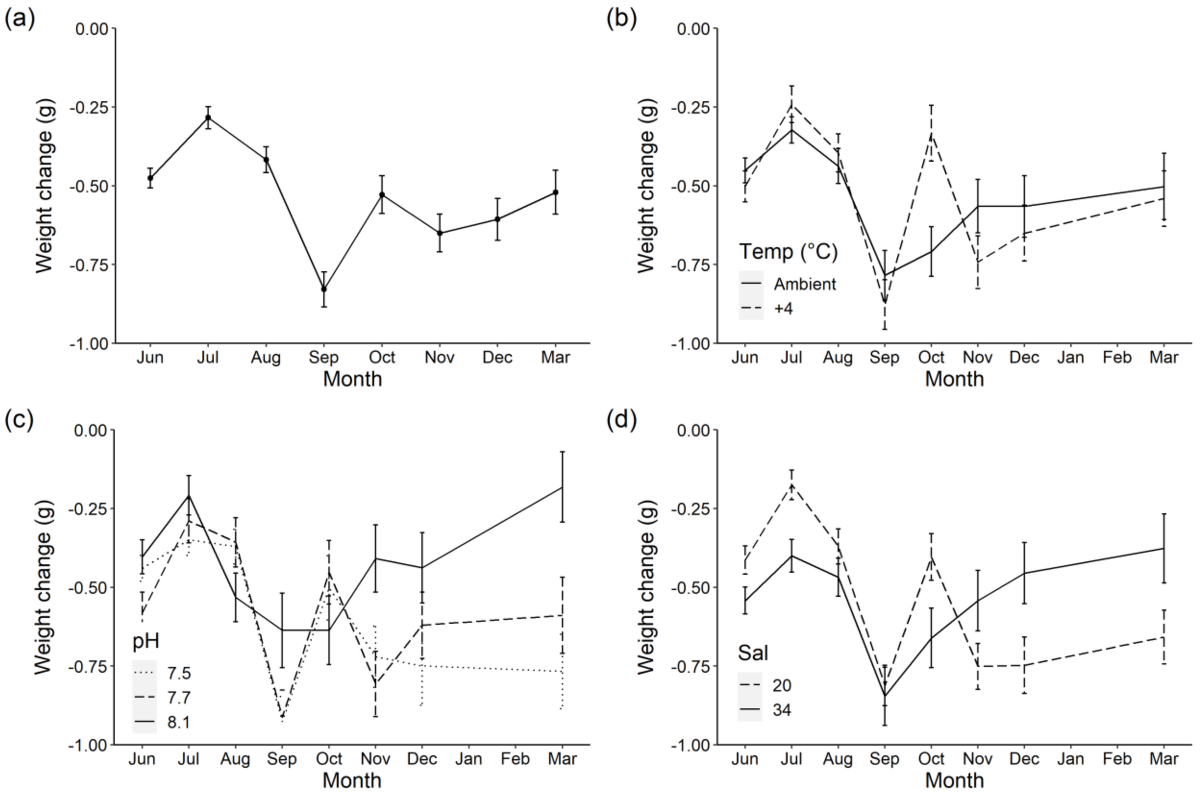

Figure 3. Mean weight change (l hr^{-1} ± standard error) from their baseline weight at collection between June 2018 and March 2019 (a) across all treatments, (b) averaged for treatments at ambient or +4 °C to investigate temperature effect (c) averaged for treatments at a pH of 8.1, 7.7 or 7.5 to investigate pH effects and (d) averaged for treatments at a salinity of 20 or 34 to investigate salinity effect.

3.2. Clearance rate

Overall, mean clearance rates were highest at the beginning of the experiment in June and July 2018 at a maximum of 0.024 (±0.001) l hr^{-1}, standardized to 1g total weight. Clearance rate then decreased by more than half in August at 0.011 (±0.001) l hr^{-1} and reached a minimum in January 2019 at 0.005 (±0.0004) l hr^{-1}. Clearance rate was also relatively stable between November 2018 and March 2019 (Fig. 4a).

The estimate of the autocorrelation parameter for both clearance rate models was 0.04 indicating almost complete independence of the measurements over time. There was no significant three-factor or two-factor interaction between any of the factors nor was there a significant long-term effect of temperature or pH (Fig. 4b, c). There was a statistically significant difference between the two salinity levels, with a larger volume of water cleared in a salinity of 34 compared a salinity of 20 (Table 1, Fig. 4d). There was no significant interaction between month and salinity, therefore the difference between the salinity levels was constant over time. Oysters in a salinity of 20 cleared an estimated 0.0016 (±0.0004) l hr^{-1} less per month, approximately 20% less than those in a salinity of 34.

Overall, mean clearance rate across all treatments did not change with actual temperature of the water (Weighted linear regression: $R^2 = 0.38$, $F_{1, 8} = 4.78$, $p = 0.06$). Further, the salinity effect (difference between the two salinity levels per month) did not correlate actual temperature (Weighted linear regression: $R^2 = 0.39$, $F_{1, 8} = 5.02$, $p = 0.06$).

Table 1. Repeated measures ANOVA output stating the degrees of freedom, F-statistic and p-values for each individual factor and their interactions. Significant effects are denoted with an asterisk.

	Clearance rate			Oxygen consumption rate		
	df	*F* (*df*, 94)	*P*	*df*	*F* (*df*, 101)	*P*
Month	9	112.1	<0.0001*	9	21.8	<0.0001*
Temperature	1	0.04	0.83	1	11.29	0.001*
pH	2	0.11	0.90	2	1.44	0.24
Salinity	1	10.1	<0.002*	1	7.37	0.009*
Temp X pH	2	0.95	0.39	2	0.71	0.50
Temp X Salinity	1	0.72	0.40	1	0.06	0.81
pH X Salinity	2	0.85	0.43	2	1.17	0.32
Temp X pH X Salinity	2	1.68	0.19	2	0.35	0.71
Month X Temp	9	0.21	0.99	9	2.05	0.03*
Month X pH	18	0.72	0.78	18	0.89	0.59

Month X Salinity	9	1.63	0.11	9	0.77	0.65

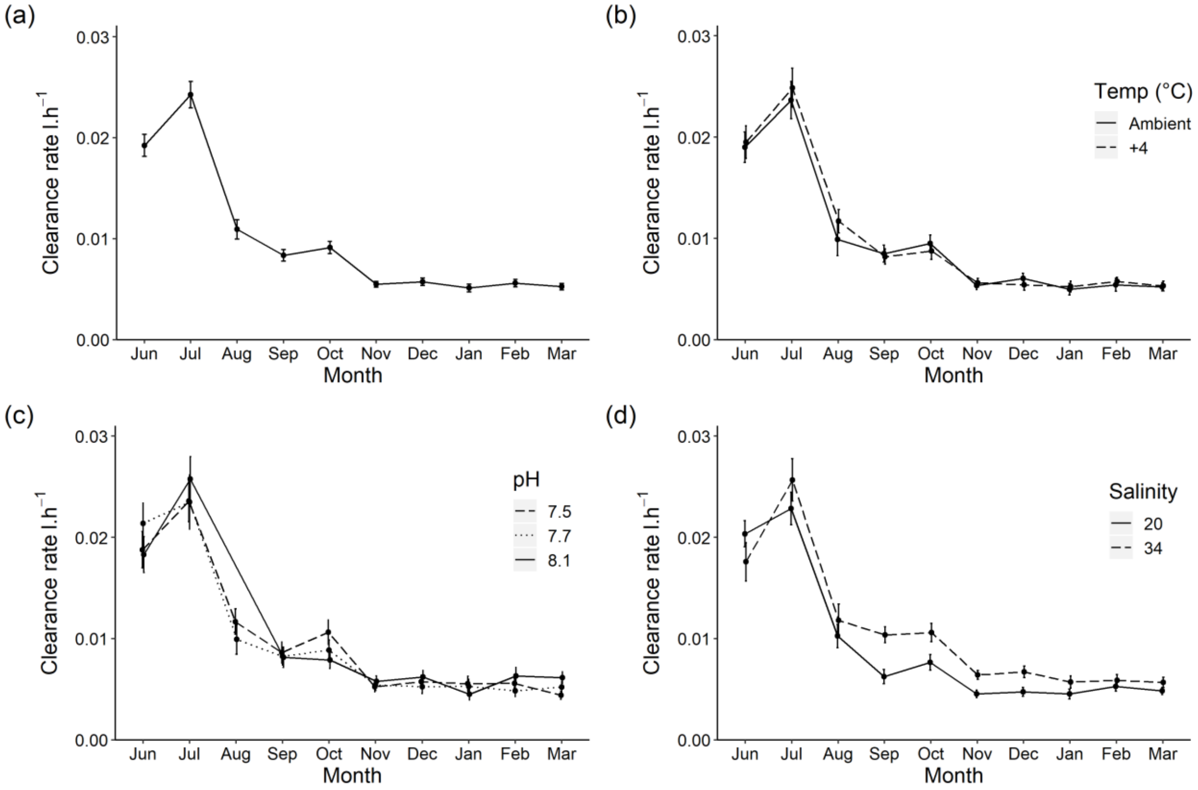

Figure 4. Mean clearance rates (l hr^{-1} ± standard error) between June 2018 and March 2019 (a) across all treatments, (b) averaged for treatments at ambient or +4 °C to investigate temperature effect, (c) averaged for treatments at a pH of 8.1, 7.7 or 7.5 to investigate pH effects and (d) averaged for treatments at a salinity of 20 or 34 to investigate salinity effect. All rates are standardized to 0.01g total weight.

3.3. Oxygen consumption

Mean oxygen consumption rate across all treatments was highest between June and September 2018 before decreasing between September 2018 at 0.0077 (±0.0024) mg hr^{-1}, standardized to 1g total weight, to March 2019 at 0.0038 (±0.0019) mg hr^{-1} (Fig. 5a). This decrease coincided with a decrease of 6°C between the warmest and coldest months of the experiment. Consumption was high at the beginning of the experiment although temperatures in June and July 2018 are similar to those in October and November 2018 where rates were lower.

The estimate of the autocorrelation parameter for oxygen consumption rate models was 0.04 indicating almost complete independence of the measurements over time. There was no significant interaction between any of the three experimental factors. There was a significant effect of temperature (Table 1, Fig. 5b). This was observed as those at +4°C had either equal to or higher oxygen consumption rates than the ambient treatments. There was a significant interaction between month and temperature, therefore, the difference in oxygen consumption rates between the temperature levels was not constant over time. There was, however, a large difference between factors during the first couple of months and minimal difference in oxygen consumption rates between August and January (Fig. 5b).

Oxygen consumption rate had no long-term trend with pH (Fig. 5c) and overall, the effect of pH was not statistically significant. There was a statistically significant effect of salinity (Table 1, Fig. 5d). There was no significant interaction between month and salinity, therefore the observed differences between the two salinity levels was constant over time. On average, a salinity of 20 led to an estimated 0.00056 (±0.0002) mg hr^{-1} more oxygen being consumed, approximately 10%, compared to those kept at a salinity of 34.

Oxygen consumption increased significantly with increasing actual temperature (Fig. S2) (Weighted linear regression: $R^2 = 0.89$, $F_{1, 8} = 66.42$, $p < 0.001$) at 0.0004 mg hr^{-1} every 1°C increase. The salinity effect was not significantly affected by actual temperature.

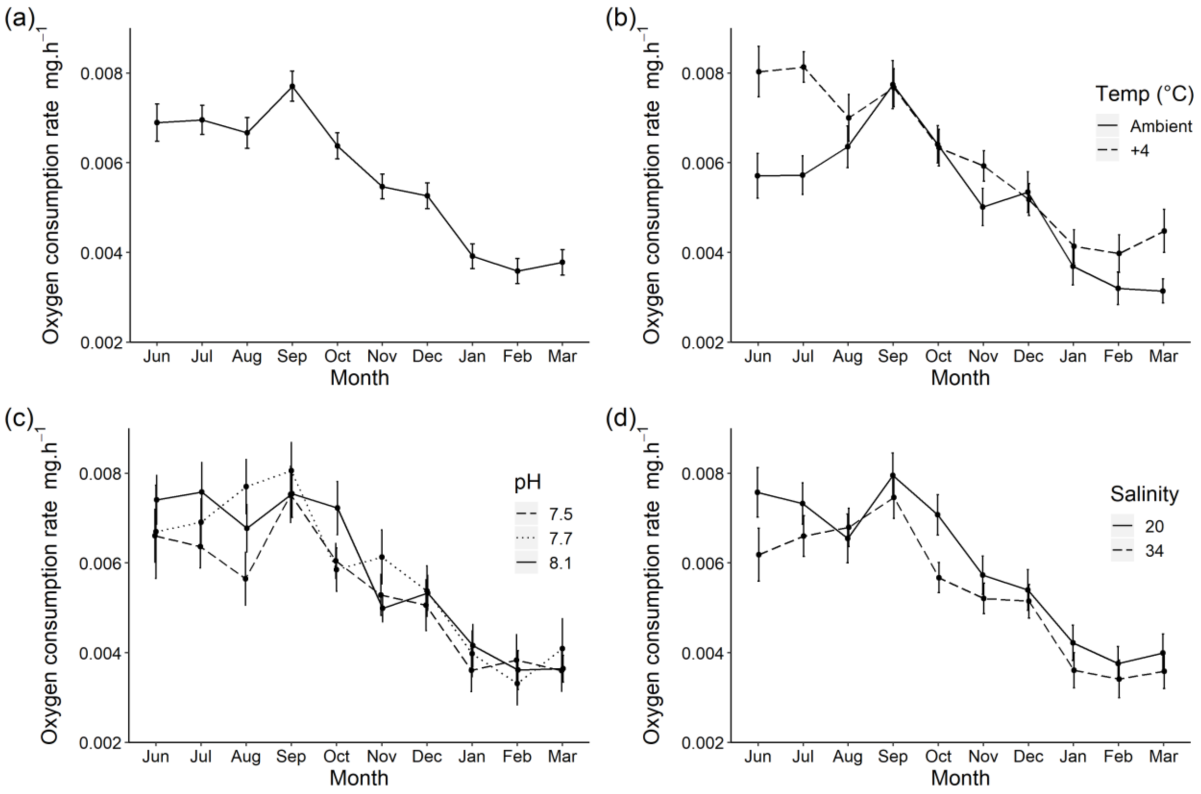

Figure 5. Mean oxygen consumption rates (mg hr^{-1} ± standard error) between June 2018 and March 2019 (a) averaged across all treatments (b) averaged across treatments at ambient or +4 °C to investigate temperature effect, (c) averaged across treatments at a pH of 8.1, 7.7 or 7.5 and (d) averaged across treatments at a salinity of 20 or 34. All rates are standardized to 0.01 g total weight.

4. Discussion

Our long-term mesocosm experiment showed that reduced salinity conditions had a statistically significant, persistent impact on the physiology of *M. gigas*. In turn, water temperature and pH had little to no impact on clearance and oxygen consumption rates of *M. gigas*. This suggests that key environmental conditions as predicted by the end of the century will not negatively affect adult physiology and survival of this highly successful NIS. Our study highlights the importance of conducting long-term, seasonal experiments to fully understand the tolerance of NIS to abiotic stressors.

Broad environmental tolerance and phenotypic plasticity to environmental change are common traits of NIS compared to native species in marine and terrestrial environments (Daehler, 2003; Funk, 2008; Davidson *et al.*, 2011; Tepolt & Somero, 2014). The ability of a species to alter its physiology and morphology to novel environmental conditions, such as CCC, may give them a competitive advantage over other species and, in the case of NIS, enable them to spread to a wide range of habitats (Stachowicz *et al.*, 2002; Davidson *et al.*, 2011). Our results support growing evidence that successful NIS have high environmental tolerance and will tolerate predicted future environmental conditions.

Salinity had a constant effect on *M. gigas* physiology over the duration of the experiment. It may, therefore, be physiologically necessary for *M. gigas* individuals to increase oxygen consumption rates and decrease clearance rates to ensure survival in areas of freshening. Other physiological studies have shown that *M. gigas* have faster growth and gonadal development at a salinity of 30 and reduced growth and larval mortality at lower salinities (Shumway & Koehn, 1982; Muranaka & Lannan, 1984; Brown & Hartwick, 1988; His *et al.*, 1989). Environmental stressors such as low salinity result in changes in water chemistry (Dickinson *et al.*, 2012; Casas *et al.*, 2018b) and osmotic pressure (Maoxiao *et al.*, 2019), which in turn increases energy expenditure (increase metabolic rate) or reduced energy storage capacity leading to reduced fitness, survival and growth (Lannig *et al.*, 2006; Dickinson *et al.*, 2012; Maoxiao *et al.*, 2019). Our results showed that a salinity of 20 led to an increase in energy demand of *M. gigas*, as shown by an increased oxygen uptake (Hawkins & Hilbish, 1992; Lannig *et al.*, 2006; Sokolova *et al.*, 2012), therefore, may have had a negative impact on performance. This negative effect of salinity has also been observed in European flat oysters (*Ostrea edulis*) and eastern oysters (*Crassostrea virginica*) (Hutchinson & Hawkins, 1992; Dickinson *et al.*, 2012).

Decreased clearance rates with salinity have already been reported as a stress response in other bivalves (Wang *et al.*, 2011). Our results are consistent with the results found in Gray & Langdon (2018) with *M. gigas* which showed a decrease in clearance rates under reduced salinity conditions. Sarà *et al.* (2008) described how clearance rate decreased with lower salinities in the non-indigenous bivalve *Brachidontes pharaonis* but salinity followed the normal-shaped tolerance curve (decline in rates towards the extremes) and concluded this species can tolerate a wide range of salinities. Although lower salinities may be less favourable, *M. gigas* may be able to tolerate and survive these conditions better over the long-term compared to indigenous species. Whilst lower salinities may reduce the fitness of *M. gigas*, areas with low salinity such as estuaries have fewer competing species and predators, therefore, settlement will likely occur in these locations if conditions are tolerable to larval stages (Gunter, 1955).

It has been proposed that organisms inhabiting coastal areas and exposed to a range of temperatures and carbonate chemistry are more likely tolerant of future warming and ocean acidification (Range *et al.*, 2012). *M. gigas* individuals used in our experiment were collected from a low-intertidal, estuarine environment that is classed as highly stressful for marine organisms as temperature, salinities and pH fluctuate on a daily basis (Lannig *et al.*, 2006; Range *et al.*, 2012). Local adaptation to this type of environment may explain why our manipulation of temperature and pH showed little to no long-term effect on *M. gigas* physiology. Although ocean acidification has been shown in many cases to suppress the growth and important physiological processes such as feeding and respiration (Range *et al.*, 2012; Barros *et al.*, 2013; Gazeau *et al.*, 2013; Ivanina *et al.*, 2013; Bressan *et al.*, 2014; Zhao *et al.*, 2017), some species show neutral or positive effects to near-future pH levels (Ries *et al.*, 2009; Matoo *et al.*, 2013; Gazeau *et al.*, 2014). Zhang *et al.* (2012) found that *M. gigas* have an extensive set of genes that leads to unique adaptation to the stressful conditions of intertidal and estuarine environments. Further, both adult and early life-history stages of *M. gigas* have shown to have a high degree of plasticity in their physiology, such as thermal stress (Hamdoun *et al.*, 2003), resource allocation and feeding (Honkoop *et al.*, 2003; Ernande *et al.*, 2004; Dutertre *et al.*, 2007), reproductive timing (Fabioux *et al.*, 2005), growth (Bayne, 2002; Taris *et al.*, 2006), and circadian rhythms (Mat *et al.*, 2012).

No clear temperature effect was observed in clearance rates whereby the overall rates were relatively similar between the highest and lowest temperatures. A similar trend has been recorded in *Crassostrea virginica* with no difference in clearance rate between individuals in the winter (17 °C) and summer (27 °C) (Casas *et al.*, 2018b). Seasonality can have an important influence on physiological energetics. Studies have shown that clearance rates in bivalves are not always temperature dependent as seasonal cycles in life history traits (reproduction and growth) can lead to variation in filtration rates in bivalves, typically with reduced feeding in the winter months and an increase in spring and summer (Hornbach *et al.*, 1984; Viergutz *et al.*, 2012; Rahman *et al.*, 2020). For example, Viergutz *et al.* (2012) showed that at peaks in clearance rate of the invasive bivalve *Corbicula fluminea* likely corresponded to spawning patterns increasing food (energy) demand. In our study, *M. gigas* clearance rates were not directly influence by seawater temperature, however, high rates were measured in the spring/early summer (at the start of the experiment). These rates were followed with a decrease in oyster weight in all treatments. *M. gigas* typically spawn between July and October in Europe which leads to a substantial decrease in wet meat weight (up to 50%) (Enríquez-Díaz *et al.*, 2009; Bernard *et al.*, 2016; Reise *et al.*, 2017; Ubertini *et al.*, 2017; Balić *et al.*, 2020). It is, therefore, likely that an increase in energy demand (and, therefore, clearance rate) occurred prior to spawning.

Whilst a seasonal pattern in clearance rate is likely to have been observed, acclimation to new environmental conditions at the start of the experiment may have also led to increased clearance rates through an increased stress response. Acclimation can be characterized by an initial stress response (for example, increased metabolic rate) followed by a stabilization of the organism’s response (Suckling *et al.*, 2015). The acclimation of species to new environmental conditions can take several months (Dupont *et al.*, 2013; Suckling *et al.*, 2015; Pintor *et al.*, 2016) and, as clearance rates were high at the very start of the experiment, the effects of species acclimation on *M. gigas* cannot be fully ruled out.

A seasonal effect on oxygen consumption was observed across all treatments in line with changes in SST. Similar trends in oxygen consumption rates have been reported *in situ* for *M.*

gigas where the highest and lowest oxygen consumption rates occurred in the summer and winter, respectively (Mao *et al.*, 2006; Casas *et al.*, 2018b). Increasing oxygen consumption with actual temperature agrees with several studies on marine bivalves (Bougrier *et al.*, 1995; Ren *et al.*, 2000; Sarà *et al.*, 2008; Casas *et al.*, 2018b), reflecting an increase in physiological and biochemical reactions with warming. Seasonality in metabolic demand may also reflect life history cycles, which are often linked to seawater temperature (Clarke, 1993; Casas *et al.*, 2018a). Oxygen consumption rates also showed a significant effect of temperature with month, however, the effect of +4°C was variable. Rates were similar to that of the ambient temperature treatment with the exception of June and July and, to a lesser extent, February and March where +4°C is associated with higher oxygen consumption rate (Fig. 4b). The effects of end of the century temperatures on *M. gigas* may, therefore, be dependent on time of the year.

Although the adult oysters used in this study exhibited no negative effect with ocean acidification, studies on early life-history stages have shown vastly different results. Ocean acidification has been shown to have a negative effect on the development and calcification of early life stage *M. gigas* under pH values predicted for 2100 and 2300 (Kurihara *et al.*, 2007; Parker *et al.*, 2010) and varying effects on fertilization due to intraspecific differences between populations (Havenhand & Schlegel, 2009; Parker *et al.*, 2010; Barros *et al.*, 2013). Studies over multiple generations are rare but give an important insight on the potential carry-over effects between different life stages. These studies would increase understanding regarding trans-generational acclimation which has been shown in a variety of species to reduce the effects of CCC (Donelson *et al.*, 2011; Munday, 2014; Parker *et al.*, 2015; Ross *et al.*, 2016). For a more complete understanding of the long-term effects of CCC on individual species, it is crucial for future work to incorporate individuals from multiple populations and multiple generations into their experimental designs to investigate both intraspecific differences and the potential acclimation of a species through trans-generational carry-over effects to environmental change.

5. Conclusion

This long-term multiple stressor study on *M. gigas* demonstrated that a globally distributed NIS is tolerant to key CCC stressors, as predicted for the end of the century. Our results imply that NIS with broad environmental tolerances like *M. gigas* may thrive under expected CCC conditions, potentially leading to an increase in NIS populations and further impacting native biodiversity (Dubois *et al.*, 2006; Kelly *et al.*, 2008; Lejart & Hily, 2011; Anglès d'Auriac *et al.*, 2017). Although previous studies have shown negative synergistic effects of CCC factors on organism fitness, we found no interactive effects between any of the three stressors over the long-term. However, further long-term, trans-generational studies are required to investigate the presence of possible synergistic effects across multiple life-history stages (Rius *et al.*, 2010). Our results also demonstrated how seasonality may influence physiological rates and the importance of allowing the studied organism to reach a physiologically stable state (Suckling *et al.*, 2015), which is key for interpreting laboratory experiments of differing temporal lengths. Ultimately, the long-term effects of CCC on NIS are still relatively unknown and continued research in this area will advance our understanding on the physiological resilience of NIS to environmental change.

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Supplementary Figures

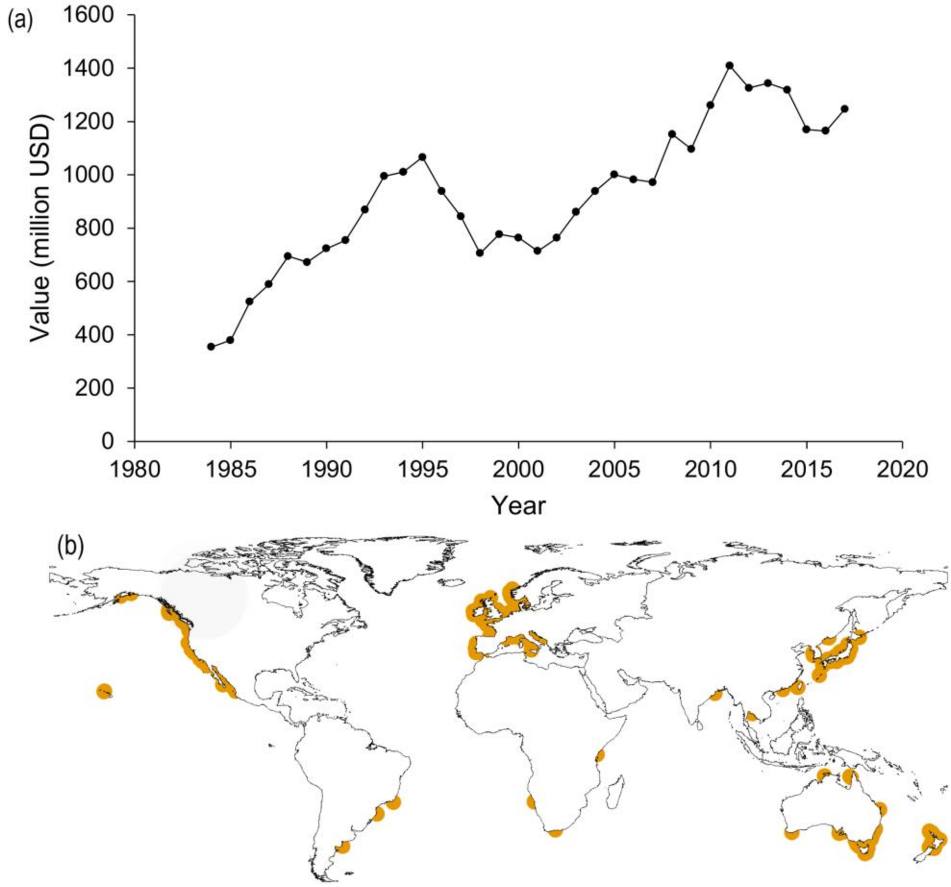

Figure S1. (a) The global aquaculture value of *M. gigas* between 1984 and 2017 (FAO, 2019) and (b) the global distribution of wild *Magallana gigas* from Global Biodiversity Information Facility (GBIF, accessed: 7th October 2019).

Table S1. Mean (± standard deviation) salinity, pH and total alkalinity for each of the 12 treatments over 10 months. Salinity and pH were monitored every 1-2 days, and total alkalinity measured every 2 weeks for the duration of the experiment.

Treatment temperature (°C) - salinity - pH	Salinity	pH	Total alkalinity (umol kg-1)
Ambient - 20 - 7.5	19.9 ± 0.9	7.54 ± 0.05	2302.6 ± 95.3
Ambient - 20 - 7.7	20.2 ± 1.1	7.70 ± 0.04	1807.4 ± 98.4
Ambient - 20 - 8.1	21.2 ± 1.6	8.05 ± 0.04	2293.9 ± 15.4
Ambient - 34 - 7.5	33.5 ± 1.8	7.54 ± 0.04	2334.6 ± 95.2
Ambient - 34 - 7.7	33.0 ± 1.4	7.72 ± 0.03	1838.5 ± 88.2
Ambient - 34 - 8.1	33.9 ± 1.2	8.01 ± 0.03	2304.6 ± 92.0
+4 - 20 - 7.5	20.4 ± 1.1	7.51 ± 0.06	2317.0 ± 93.1
+4 - 20 - 7.7	21.3 ± 1.1	7.72 ± 0.04	1828.0 ± 81.5
+4 - 20 - 8.1	20.5 ± 1.3	8.10 ± 0.06	1835.4 ± 81.4
+4 - 34 - 7.5	34.3 ± 0.9	7.53 ± 0.06	2332.7 ± 92.1
+4 - 34 - 7.7	34.1 ± 1.9	7.72 ± 0.03	1833.4 ± 102.9
+4 - 34 - 8.1	34.0 ± 1.8	8.07 ± 0.05	1810 ± 105.1

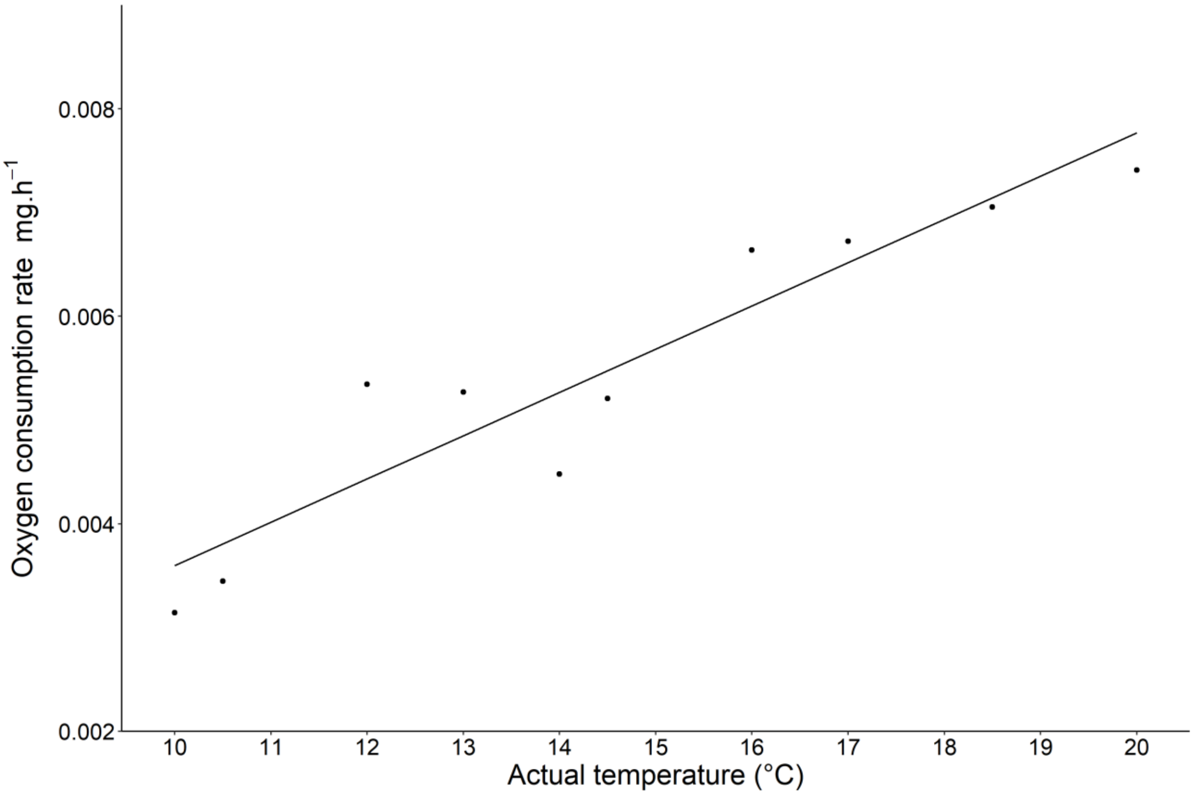

Figure S2. Mean oxygen consumption rates (mg hr^{-1}) across all treatments against average monthly temperature of the water. Temperature was based on mean monthly SST from the Western Channel Observatory L4 station data. Data were weighted by sample size and standardized to 1g oyster ($R^2 = 0.87$).