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ORIGINAL PAPER

Effects of multiple climate change stressors: ocean acidification interacts with warming, hyposalinity, and low food supply on the larvae of the brooding flat oyster *Ostrea angasi*

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Abstract Ocean acidification, rising temperatures, and increased intensity of rain events are occurring due to climate change. Individually, each of these stressors has the potential to influence the growth and survival of many marine organisms, particularly during early development. Together the interactive and multiple impacts of elevated pCO_2, temperature, and salinity may be exacerbated by a lack of food. Life history traits are important in determining the response of organisms to climate change. Larvae that develop within a brood chamber, such as the flat oyster, *Ostrea angasi*, may be pre-exposed to living a higher CO_2 environment. This study determined the pH of the fluid surrounding the gills of adult oysters where larvae are brooded and investigated the interactive effects of the multiple climate-related stressors: ocean acidification, warming, hyposalinity, and reduced food availability, on development of *O. angasi* larvae. The fluid surrounding the larvae was of pH 7.88 ± 0.04, lower than that of surrounding sea water, and was significantly reduced (to pH 7.46 ± 0.05) when oysters remained closed as occurs in nature during periods of stress caused by low salinity. Elevated pCO_2 [853–1194 µatm (pH_{NBS} 7.79)] resulted in larvae being 3 % smaller, but it had no effect on the timing of progression through developmental stages, percentage of abnormalities, or survival of larvae. Exposure to elevated pCO_2 together with increased temperature (+4 °C) or reduced salinity (20) had a negative effect on the time to the eyed larval stage and with an increase in the percentage of abnormal larvae. Unexpectedly, larvae did not meet their higher metabolic requirements to survive under elevated pCO_2 by eating more. In a sublethal effect of elevated pCO_2, larval feeding was impaired. We found that *O. angasi* larva were relatively resilient to elevated pCO_2, a trait that may be due to the acclimatisation of hypercapnic conditions in the brood cavity or because they are released from the brood cavity at an older, possibly less sensitive stage. This result contrasts with the larvae of broadcast spawning oysters which are extremely sensitive to elevated pCO_2.

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Introduction

As a result of anthropogenic activities, oceanic conditions are changing placing stress on marine species and marine ecosystems (IPCC 2013, 2014). Increases in carbon dioxide levels in the atmosphere are exacerbating the multistressor world of marine organisms (Byrne and Przeslawski 2013). Increases in the atmospheric partial pressure of CO_2 (pCO_2) and its absorption by the ocean have resulted in a decrease in the saturation state of calcium carbonate and a decrease in the pH of the ocean (Feely et al. 2004; Orr et al. 2005; Doney et al. 2009; Mackenzie et al. 2014). This

changing ocean chemistry, known as ocean acidification, is driving a decrease in calcification of marine species as a direct effect of the three co-varying stressors (pH, the saturation state of calcium carbonate, and pCO_2), each which can impact biomineralisation (Byrne et al. 2013), interfering with the shell formation of calcifying molluscs (Gazeau et al. 2007; Wood et al. 2008; Thomsen et al. 2010). Independently, the three stressors associated with ocean acidification (pH, pCO_2, and saturation state) can impact different aspects of the physiological processes of molluscs (Waldbusser et al. 2015a, b).

Ocean acidification has been shown to have mainly negative effects on a wide range of marine biota with calcifying species being negatively impacted, and molluscs ranking amongst the most vulnerable (Ries et al. 2009; Kroeker et al. 2010, 2013; Ross et al. 2011; Gazeau et al. 2013; Parker et al. 2013). The negative impacts of ocean acidification include decreased growth and calcification of adults (Ries et al. 2009), and reduced development and survival of larvae, including scallops (White et al. 2013; Scanes et al. 2014), clams (Talmage and Gobler 2011; Van Colen et al. 2012; Jansson et al. 2013; Gobler et al. 2014), mussels (His et al. 1989; Kurihara et al. 2009), and oysters (Parker et al. 2012; Barros et al. 2013).

Due to climate change, marine biota are also experiencing increased sea surface and aerial temperatures. Increasing atmospheric CO_2 is predicted to cause a global increase in temperature of up to 4 °C by the 2100 (IPCC 2013). The effects of climate warming can exceed those of ocean acidification (Byrne et al. 2009; Talmage and Gobler 2011; Nguyen and Byrne 2014). Environmental temperature is a key determinant of the distribution of marine species due to the physiology of organisms operating within defined thermal windows (Helmuth and Hofmann 2001; Pörtner Pörtner 2008). Typically, growth and rates of development increase with warming (Zimmerman and Pechenik 1991; Byrne et al. 2009), and synergistic interactions are more evident with acidification (Parker et al. 2010; Ross et al. 2011; Hiebenthal et al. 2013; Matoo et al. 2013) than additive or antagonistic interactions with a broader suite of anthropogenic stressors (Przeslawski et al. 2015). For example, elevated pCO_2 has been shown to reduce the growth of the blue mussel *Mytilus galloprovincialis*, but under moderate warming the effect was minimised (Kroeker et al. 2014) as also found for other calcifiers (Sheppard-Brennard et al. 2010). Furthermore, studies on metamorphosis of the Pacific oyster, *Crassostrea gigas*, found elevated temperature removed any negative effects of elevated pCO_2 (Ko et al. 2014).

Broadscale climatic change will increase the frequency of storm events and subsequent coastal run-off and flooding in the catchment (IPCC 2013). Changes to salinity and coastal run-off due to rainfall will exacerbate other anthropogenic stressors such as nutrients, sedimentation, and metal contamination (Przeslawski et al. 2015), which are known to have negative impacts on marine invertebrates (Pedersen and Perkins 1986; Schone et al. 2003). Studies have also shown interactive effects of salinity with pCO_2 on the development and physiology of oysters, but the results have varied. For example, for juvenile oysters, *Crassostrea virginica*, combined exposure to elevated pCO_2 and reduced salinity weakened the shells and increased the consumption of energy (Dickinson et al. 2012). Furthermore, Ko et al. (2014) found interactive effects of decreased pH, elevated temperature, and reduced salinity slowed growth of the Pacific oyster, *C. gigas*. Conversely, Waldbusser et al. (2011) found that although calcification declined with elevated pCO_2 in juvenile *C. virginica*, these negative effects were partially ameliorated by elevated salinity and temperature. For bivalves, the combined negative effects of increased temperature and decreased salinity on development, growth, and mortality are long recognised (e.g. Kinne 1971; Robert et al. 1988; His et al. 1989).

Increased food availability has the potential to ameliorate the energetic stress experienced by marine organisms due to acidification (Hettinger et al. 2013). Under stress, bivalve species have higher metabolic requirements to maintain homeostasis (Pörtner and Farrell 2008; Melzner et al. 2009, 2011). Increased food supply is reported to reduce the negative impacts of increased pCO_2 on juvenile scallops and mussels (Sanders et al. 2013; Thomsen et al. 2013), and oyster larvae (Hettinger et al. 2013). Hettinger et al. (2013) found that the combination of elevated pCO_2 and low food availability led to a large reduction in larval performance of *Ostrea lurida*. Thomsen et al. (2013) found that the *Mytilus edulis* was able to tolerate high (>3000 µatm) pCO_2 when food supply was abundant. Thus, it appears that abundant food resources may partially ameliorate the negative impacts of ocean acidification on bivalves (Hettinger et al. 2013).

To be able to make predictions about which molluscs species will be most vulnerable in a future climate-changed ocean, it is essential to understand the additive, synergistic, or antagonistic effect of the multiple stressors associated with climate change (Byrne 2011). In a recent meta-analysis of multiple stressors on marine larvae and embryos, it was found that the majority were synergistic interactions (65 %), and that responses were taxon-specific (Przeslawski et al. 2015). Within bivalves, additive effects of pCO_2 and temperature impact growth in the clam *Mercenaria mercenaria* and the oyster *C. virginica* (Talmage and Gobler 2011). Specifically, increases in temperature and pCO_2 had negative and additive effects on the survival, development, and growth of larvae of these species (Talmage and Gobler 2011). For the oysters, *Saccostrea*

glomerata and *C. gigas*, there were synergistic negative effects of pCO_2 and temperature (Parker et al. 2009; Dickinson et al. 2012; Ko et al. 2014). Under the combination of elevated pCO_2 and temperature, Parker et al. (2010) found reductions in size and development and an increase in the number of abnormally shaped larvae for both species of oysters.

For many marine molluscs with biphasic life histories, the early life stages have been shown to be the stage that is the most sensitive (Parker et al. 2010, 2011), which has led to concerns of early-stage bottlenecks and issues of persistence of populations (Byrne 2011). Some molluscs may, however, be relatively robust to ocean acidification due to pre-exposure to elevated pCO_2 conditions. Parker et al. (2012) found that pre-exposure of adult oysters to ocean acidification during reproductive conditioning had positive "carry-over" effects and increased the resilience of their offspring. These carry-over effects persisted into the second generation, depending on their environmental exposure to pCO_2 (Parker et al. 2015). Thomsen et al. (2010) also found that calcifying benthic species, including the mussel *M. edulis*, thrived in naturally CO_2-enriched waters of the Western Baltic Sea. When placed under artificially elevated pCO_2 in the laboratory, this long-term exposure to naturally elevated pCO_2 increased the ability of *M. edulis* to calcify (Thomsen et al. 2010).

Some invertebrate species such as seastars that live in variable environments in tide pools are physiologically acclimated to withstand elevated pCO_2 (Nguyen and Byrne 2014). The embryos and larvae of molluscs that rear their young within a brood chamber experience respiration-driven hypercapnic conditions during periods that the chamber is closed (Chaparro et al. 2008, 2009; Noisette et al. 2014). During stressful conditions, e.g. low salinity, females close off their brood chambers to protect the larvae, and the pH of the brood chamber can decrease to below pH 6.5 (Chaparro et al. 2009). Thus, the larvae of brooding bivalves maybe more tolerant of elevated pCO_2 than larvae of broadcast spawning bivalves which are not naturally exposure to periods of hypercapnia. The larvae of the flat oyster, *Ostrea angasi*, are not within a brood chamber but initiate development on the gill filaments of the female and are likely to experience hypercapnic conditions as the respiratory pCO_2 of water in the mantle cavity increases when the oyster is closed due to environmental conditions (Dix 1976). Larvae of species adapted to living in a brood, such as *O. angasi*, may have the phenotypic plastic characteristics that increase their acclimation capacity to respond to a high CO_2 environment. The conditions experienced by larvae while brooded may therefore provide preconditioning to ocean acidification conditions. Gradual acclimation to elevated pCO_2 has shown to allow preconditioning and provide less physiological and behavioural stress (e.g. intertidal isopods, *Paradella dianae*, Munguia and Alenius 2013). Thus, the progeny of brooding oysters may be more resilient to ocean acidification.

This study investigated the effect of multiple stressors: elevated pCO_2, elevated temperature, hyposalinity, and reduced food availability on the larvae of the flat oyster, *O. angasi*. Flat oysters are a brooding species which retain developing larvae directly on the gill filaments and then release their young as planktotrophic larvae (Dix 1976; O'Sullivan 1978). In Australia and elsewhere, flat oysters are important aquaculture species (O'Connor and Dove 2009). *Ostrea angasi* occurs subtidally in coastal areas of relatively stable seawater salinities (Thomson 1954; Nell and Gibbs 1986), although heavy rainfall in south-eastern Australia during the summer spawning season often results in acute episodes of reduced salinity (hours to days). We predicted that there would be a reduction in the pH of the branchial area where the larvae are reared, during closure of adult shells. In ocean acidification experiments, we tested the hypothesis that the negative effects of elevated pCO_2 on larval development, as widely reported for broadcast spawning molluscs, would be mitigated, in *O. angasi* due to the acclimation of brooded larvae to low pH conditions. In combination with warming, hyposalinity, and reduced food availability, it was predicted that there would be synergistic effects on the size, progression of development to the eyed larval stage, survival, and in the percentage of abnormally developed larvae. Additionally, due to the higher metabolic requirements of responding to stress, it was hypothesised that acidification, warming, and hyposalinity would increase food clearance rates by *O. angasi* larvae.

Methods

Determination of the pH of the pallial cavity of adult oysters

Adult *O. angasi* of the same age (3 years) were collected from farmed oyster leases at Port Stephens (32°42′38″S, 152°4′5″E) on the mid-coast of New South Wales. The oysters were maintained in the Port Stephens Fisheries Institute. To determine the pH of the pallial cavity, adult oysters were either forced to remain closed, simulating a stressful event such as hyposalinity, or left unmanipulated (e.g. Chaparro et al. 2009). The acute exposure reflects what occurs in nature when the salinity changes rapidly with heavy rain during February where these oysters are grown (Roy et al. 2001). Individual oysters were placed in 10-L buckets with 3 L of 1-µm filtered sea water (salinity 35.5; pH_{NBS} 8.16 ± 0.02). They were left for 20 min until they were no longer disturbed and visibly open. Water was gently pumped into each bucket, so the oysters were not

Table 1 Four orthogonal factors were tested, each with two levels

Factor	Ambient/optimum	Stressed
pCO_2[a]	297–394 µatm (pH_{NBS} 8.2)	853–1194 µatm (pH_{NBS} 7.79)
Salinity[b]	32	20
Temperature[b]	26 °C	30 °C
Food[c]	50,000 cells mL^{-1}	25,000 cells mL^{-1}

Conditions were defined according to the literature and represented the ambient or optimum conditions and corresponding stressed (elevated or reduced) conditions

[a] IPCC (2013); [b] O'Connor et al. (2012); [c] O'Connor et al. (2014)

disturbed, with either sea water or freshwater to a volume of 7 L. For buckets of low salinity, salinity was dropped to 20 as monitored by a WTW 3420 metre with SenTix 940 probe.

The larvae of *Ostrea* spp. are free to move around inside the pallial cavity and are closely associated with the gills of their mothers (Mardones-Toledo et al. 2015). The pH of the fluid surrounding the gills where the larvae are held was measured by carefully drilling a 1-mm hole into the shell (Crenshaw 1972) and carefully extracting the fluid with a needle. The needle was inserted through the hole, avoiding the main body of the oyster, to the approximate mid-point between the inner surfaces of the two valves, to minimise the risk of the collection of either haemolymph or extrapallial fluid. As the larvae of *O. angasi* are directly brooded on the gills, the fluid that was sampled is that of which is surrounding the larvae whist within the adult. Approximately 0.5 mL of the fluid was extracted from each oyster and placed into a 2-mL microcentrifuge tube, ensuring a minimum of 5 mm of the pH microprobe was submerged in the fluid. The pH of the fluid was determined using a pH metre and microprobe (Metrohm 826 pH mobile with Metrohm 6.0224.100 microprobe). This was done for five oysters from each of the treatments immediately (<5 min) after the salinity had dropped (time 0), and after 1 h (time 1). During this time, oysters were observed to ensure they either remained open in the ambient sea water or closed in the reduced salinity treatment.

Untransformed data were analysed with a two-factor analysis of variance (ANOVA) in GMAV 5 (Underwood and Chapman 2002), with time (two levels) and salinity (two levels) as fixed and orthogonal factors, and $n = 5$ replicate oysters. Prior to analysis, the data were tested for homogeneity of variances, Cochran's test. Based on the hypothesis of interest, Student–Newman–Keuls (SNK) tests were used to determine the direction of difference relative to the interaction of the factors of time and salinity.

Interactive effects of pCO_2, temperature, salinity, and food on larvae

Collection of larvae

Female *O. angasi* ($n = 27$) obtained from a farm in from Wagonga Inlet (36°13′18″S, 150°7′15″E) were relaxed with MgCl (50 g L^{-1}) in 25 % sea water with 75 % freshwater and then rinsed with 1-µm filtered sea water, and the larvae were gently rinsed from the brachial chamber (Butt et al. 2008). Prior to the experiments, all larvae were maintained in Port Stephens Fisheries Institute hatchery using standard larval rearing protocols (O'Connor et al. 2012). Late-stage larvae (shell length 257 ± 2 µm) were selected for the experiment to maximise the amount of time the larvae were within the adults and were at the umbonate stage that are released into the water column and start feeding (Dix 1976; O'Sullivan 1978). Larvae of this stage have developed on the gill filaments for 15–18 days (O'Sullivan 1978; Hickman and O'Meley 1988), and metamorphose 10–13 days after release (O'Connor et al. 2014). These larvae were used in either the multistressor experiment or the experiment to determine clearance rates.

Experimental design

To determine the effects of multiple stressors on the larvae of *O. angasi*, the four factors pCO_2, temperature, salinity, and food were tested (Table 1). Each treatment had two levels: ambient pCO_2 (388–417 µatm, equivalent to pH_{NBS} 8.2, Table 2) or elevated pCO_2 (1158–1206 µatm, equivalent to pH_{NBS} 7.79, Table 2), optimum (26 °C) or elevated temperature (30 °C), ambient (32) or low salinity (20) (Table 2), and full (50,000 cells mL^{-1}) or half diet (25,000 cells mL^{-1}). The elevated pCO_2 level was based on predictions under a medium-level scenario corresponding to the mean global surface pH predicted under scenario RCP8.5 for 2100 (IPCC 2013, 2014). The levels for each treatment of salinity, temperature, and food were based on the previous studies (O'Connor et al. 2012, 2014). Temperatures were based on the optimum temperature for rearing larvae of *O. angasi* under hatchery conditions (O'Connor et al. 2014), or a 4 °C increase in temperature under a high scenario for 2100 (IPCC 2014). The salinity was either ambient or reduced to a level experienced in the estuaries where *O. angasi* are grown and previously shown to decrease growth (O'Connor et al. 2014). The larvae were either fed a full food diet for maximum growth under hatchery conditions or a diet of half that amount of food known to results in decreased growth within the sublethal range for the larvae of *O. angasi* (O'Connor et al. 2012).

Sea water was trucked in from the Port Stephens coast and filtered to 1 µm. The elevated pCO_2 level was obtained

through adjustment of the seawater pH by direct bubbling of pure carbon dioxide (CO_2) into sea water (et al. 2012). The total alkalinity (TA) was quantified in triplicate by Gran titration prior to each water change (mean TA = 2285 ± 36 μmol kg^{-1}; Gran 1952; Butler 1982). Salinity, temperature, and pH_{NBS} were measured with each daily water change and remained constant throughout the experiment (Table 2). Following the titrations, the TA, salinity, temperature, and pH_{NBS} were entered into a CO_2 system calculation program (CO2 SYS, Lewis and Wallace 1998), pCO_2 using the dissociation constants of Mehrbach et al. (1973).

The filtered sea water had a salinity of 32 throughout the study and is referred to hereafter as ambient salinity. The salinity of the water was decreased from ambient to 20 by adding deionised freshwater and then monitoring salinity changes with a WTW 3420 meter with SenTix 940 probe. Larvae were not acclimated to salinity over time to reflect the natural conditions that they would experience (Roy et al. 2001). The change in pH with decreasing salinity was also measured with a WTW 3420 metre with SenTix 940 probe (Table 2).

Larval rearing temperature was set to the optimal (26 °C) or elevated (30 °C) temperature (O'Connor et al. 2014) using thermostatically controlled water baths. Each 120-mL polypropylene container with larvae was randomly allocated to one of the three 500-L tanks set at 26 or 30 °C. With each daily water change, pH, salinity, and temperature were monitored to insure these experimental conditions remained consistent throughout the experiment (Table 2).

Larvae were fed either the full food diet (50,000 algal cells mL^{-1}) or half food diet (25,000 algal cells mL^{-1}) of the microalga, *Isochrysis* aff. *galbana* (T. Iso CS-177) daily. Microalgae used in the trial were cultured semi-continuously in 10-L polycarbonate vessels (Aquatek, SA), under cool-white fluorescent light exposed to a 16:8 h, light–dark photoperiod at 23 ± 1 °C using f/2 growth media (Guillard 1975). Microalgal cell concentrations in the algal cultures were determined every second day by triplicate cell counts using a Neubauer haemocytometer. Experiments were conducted in the dark to limit the potential impacts of algal photosynthesis on pCO_2, but no differences in the pH of the water within each of the containers were detected after each 24-h period between water changes (Table 2).

At the start of the experiment, the mean size of larvae was determined from measurement of 30 randomly chosen individuals (shell length 257 ± 2 μm) using an eyepiece micrometer at 40× magnification under a compound light microscope. Larvae were stocked in tightly sealed 120-mL polypropylene containers at a concentration of 2 mL^{-1}. After 4 days, 10 mL of 5 % formalin buffered with sea water was added to each container. The effects of temperature, pCO_2, salinity, and diet on the final size, percentage of abnormalities (larvae exhibiting abnormal shell growth), percentage of larvae developing an eyespot, and larval mortality were determined from the assessment of 30 randomly encountered individuals per replicate container. There were $n = 3$ replicate containers for each combination of treatments.

Table 2 Physicochemical conditions of the sea water for each of the treatments

Condition	Salinity	Temp (°C)	pH_{NBS}	TA (μmol kg^{-1})	pCO_2 (μatm)	$\Omega_{calcite}$	$\Omega_{aragonite}$
Ambient salinity, optimum temperature, ambient pCO_2	32	26	8.20	2300.72 ± 33.46	388.42 ± 6.70	5.82 ± 0.09	3.82 ± 0.05
Ambient salinity, optimum temperature, elevated pCO_2	32	26	7.79	2300.72 ± 33.46	1158.07 ± 19.57	2.62 ± 0.04	1.72 ± 0.03
Ambient salinity, elevated temperature, ambient pCO_2	32	30	8.20	2300.72 ± 33.46	394.03 ± 6.85	6.46 ± 0.10	4.30 ± 0.06
Ambient salinity, elevated temperature, elevated pCO_2	32	30	7.79	2300.72 ± 33.46	1193.5 ± 20.20	2.95 ± 0.04	1.96 ± 0.03
Reduced salinity, optimum temperature, ambient pCO_2	20	26	8.20	1446.26 ± 40.69	296.9 ± 8.6	2.72 ± 0.08	1.70 ± 0.05
Reduced salinity, optimum temperature, elevated pCO_2	20	26	7.79	1446.26 ± 40.69	853.10 ± 24.40	1.18 ± 0.03	0.74 ± 0.02
Reduced salinity, elevated temperature, ambient pCO_2	20	30	8.20	1446.26 ± 40.69	302.1 ± 8.80	3.08 ± 0.09	1.94 ± 0.06
Reduced salinity, elevated temperature, elevated pCO_2	20	30	7.79	1446.26 ± 40.69	879.3 ± 25.1	1.36 ± 0.04	0.85 ± 0.02

Values for pCO_2, $\Omega_{calcite}$, and $\Omega_{aragonite}$ calculated from salinity, temperature, TA, pH_{NBS} using CO_2 SYS

Salinity, temperature, and pH_{NBS} were measured with each daily water change and remained constant throughout the experiment

Values are mean ± SE ($n = 3$)

Data analysis

The interactive effects of pCO_2, temperature, salinity, and availability of food were tested. Data were analysed with a four-factor analysis of variance using GMAV 5 (Underwood and Chapman 2002). Each factor was orthogonal, fixed, and had two levels: ambient (297–394 µatm) or elevated pCO_2 (853–1194 µatm), optimum (26 °C) or elevated temperature (30 °C), ambient (32) or low salinity (20), and full (50,000 cells mL^{-1}) or half food diet (25,000 cells mL^{-1}), $n = 3$ replicates of each treatment. Prior to analyses, the data were tested for homogeneity of variances with Cochran's *C* test. To satisfy the assumption of homogeneity of variances, and to make comparisons among the different variables, the data for percentages of abnormalities, mortality, and eye larvae were ArcSin-transformed. Data that did not satisfy the assumption of homogeneity of variances were still analysed and interpreted with caution ($\alpha = 0.01$), as large balanced ANOVAs such as these are robust to such violations (Underwood 1997). SNK tests were done for significant sources of variation to determine the patterns of difference relative to hypotheses of interest (Underwood 1997; Underwood and Chapman 2002).

Clearance rates of algae

The effects of pCO_2, temperature, and salinity on clearance rates of food by larvae were determined over a 12-h period on larvae that were the same size as those used for the multistressor experiment. Experimental treatments comprised the orthogonal treatments of ambient or elevated pCO_2, optimal or elevated temperature, and ambient or low salinity (Table 1). Following the previously described procedure, 120-mL polypropylene container was stocked with two larvae mL^{-1}. Experimental controls consisted of the same treatments without the addition of larvae, also with $n = 3$ replicate containers per treatment. In each jar, 50,000 cells mL^{-1} of *Isochrysis* aff. *galbana* was added, and after 12 h, the experiment was stopped with the addition of formalin. The number of algal cells remaining in each container was determined, and larval clearance rates (cells $larvae^{-1}$ h^{-1}) were calculated (Coughlan 1969).

For the clearance rate experiment, untransformed data were analysed with a three-factor ANOVA (Underwood and Chapman 2002), testing for fixed and orthogonal treatments of pCO_2, salinity, and temperature, $n = 3$ replicates of each treatment. Prior to the analysis, data were tested for homogeneity of variances. *Post hoc* SNK tests were done to determine the patterns of difference (Underwood 1997).

Results

Determination of the pH of the pallial cavity of adult oysters

At the beginning of the experiment, the mean ± SE pH of the fluid surrounding the gills of adult oysters was 7.88 ± 0.04, considerably lower than ambient sea water (pH 8.16 ± 0.02). The pH of the fluid surrounding the gills of adult *O. angasi* that remained opened did not change. For oysters that were closed for 1 h, the pH of the fluid surrounding the gills was lower, dropping to a mean ± SE pH of 7.46 ± 0.05 ($n = 5$; range 7.39–7.64 pH; Table 3).

Interactive effects of pCO_2, temperature, salinity, and food on larvae

Effects on size

The effect of food on the size of larvae was contingent on pCO_2 conditions (Table 4a). Larvae reared in ambient conditions were 3 % larger when fed the full diet (full food: 280 ± 4 µm, half food: 271 ± 2 µm, Fig. 1; Table 4a). Under elevated pCO_2 conditions, larvae fed a full or half food diet were similar in size (Fig. 2; Table 4a). Although larvae under elevated pCO_2 and a full food diet were significantly smaller than under ambient conditions, there was only a 3 % reduction in size (Fig. 2). Although larvae under elevated pCO_2 and a full food diet were significantly smaller than under ambient conditions, there was only a 3 % reduction in size.

Salinity and temperature interacted with each other to influence the size of larvae (Table 4a). For both the optimum and elevated temperatures, larvae were significantly smaller under reduced salinity conditions (Fig. 1; Table 4a). The difference between salinity treatments was greater under elevated temperature (Fig. 1). In ambient salinity, elevated temperature significantly increased the size of larvae (Fig. 1; Table 4a). The pattern was reversed for larvae in reduced salinity conditions such that larvae were significantly larger in the optimum temperature than in the elevated temperature (Fig. 1; Table 4a).

Effects on development

All four factors, pCO_2, temperature, salinity, and food, interacted with each other to influence the percentage of larvae that reached the eyed stage of development (Table 4b). Specifically, there was no effect of pCO_2 on development in the absence of other stressors, i.e. comparing treatments of ambient pCO_2 with elevated pCO_2

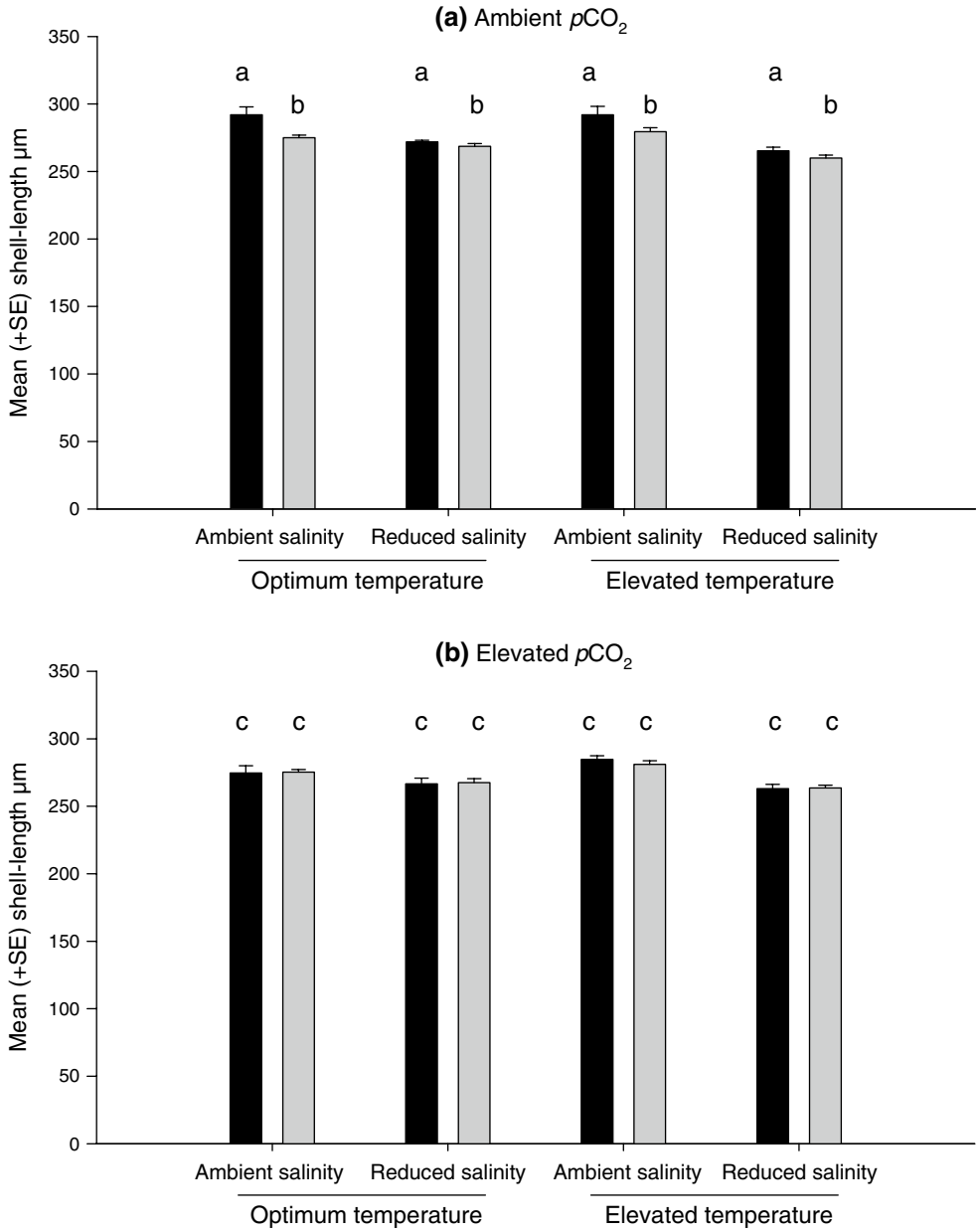

Fig. 1 Mean (+SE, $n = 3$) shell length (µm) of larvae under **a** ambient pCO_2 (390 µatm), or **b** elevated pCO_2 (856 µatm), from optimum (26 °C) or elevated (30 °C) temperature, and ambient (32) or reduced (20) salinity, and fed either the full diet (50,000 cells mL^{-1}; *black bars*) or half diet (25,000 cells mL^{-1}; *grey bars*). Analyses were based on the mean shell length of 30 randomly chosen individuals from each replicate container. *Letters* represent significant differences (SNK $P < 0.05$) for the significant interaction between pCO_2 and food (ANOVA $P < 0.05$, Table 4a)

in combination with ambient salinity, optimum temperature, and full food diet (Fig. 2; Table 4b). Elevated pCO_2 did, however, have a negative effect on development to the eyed larval stage when they were reared in ambient salinity and optimum temperature and were fed the half food diet (Fig. 2; Table 4b). Exposure to elevated pCO_2 combined with hyposalinity (Fig. 2; Table 4b), or elevated temperature resulted in a decrease in the percentage of larvae that developed to the eyed larval stage.

Under the combined stress of reduced salinity and elevated temperature, the percentage of eyed larvae was also very low. There was no difference between ambient and elevated pCO_2 conditions on the percentage of eyed larvae (Fig. 2; Table 4b). Furthermore, these differences were even greater when larvae were fed the half food diet (Fig. 2).

Table 3 Analysis of variance (ANOVA) comparing the pH of the fluid of the pallial cavity of oysters at the beginning of the experiment (T0) and after 1 h (T1), under ambient salinity (AS) and reduced salinity (RS) conditions

Source	*d.f.*	M.S.	*F*	*P*
Salinity (Sa)	1	0.1797	19.15	0.0005
Time (Ti)	1	0.2536	27.02	0.0001
Sa × Ti	1	0.1627	17.34	**0.0007**
Residual	16	0.0094		
Total	19			
SNK		Sa × Ti		
		AS: T0 = T1		
		RS: T1 > T2		
		T0: AS = RS		
		T1: AS > RS		

Variances were homogenous, Cochran's *C* test, $C = 0.324$, $P > 0.05$. *Post hoc* SNK tests were done for significant sources of variation, shown in bold, relative to the hypotheses of interest to determine the direction of differences, $P < 0.05$

Effects on abnormalities

All four factors combined influenced the percentage of abnormally developed larvae (Table 4c). There was no effect of elevated pCO_2 on the percentage of abnormal larvae when all other stressors were absent, i.e. ambient salinity, optimum temperature, and full food diet (Fig. 4; Table 4c). With the addition of any of the other stressors (reduced salinity, elevated temperature, or the half food diet), the percentage of abnormalities was greater under elevated than the ambient pCO_2 (Fig. 3; Table 4c). This pattern was not statistically significant (Table 4c) for the treatment with reduced salinity, elevated temperature, and the full food diet, but the trend was in the same direction such that there was a greater percentage of abnormalities under elevated pCO_2 (Fig. 3).

Effects on mortality

Hyposalinity and warming increased mortality of larvae, but this was not affected by the level of pCO_2 or amount of food (Fig. 4; Table 4d). Overall, mortality was greater under the combined effects of reduced salinity and elevated temperature (Fig. 4). Furthermore, the effect of salinity on mortality of larvae was dependent on the temperature. Under the optimum temperature, there was no effect of salinity on mortality of larvae (Fig. 4; Table 4d). Under elevated temperature, mortality was significantly greater in the reduced salinity treatments (Fig. 4; Table 4d).

The effect of temperature on mortality of larvae also depended on salinity. Under ambient salinity, temperature did not affect mortality of larvae (Fig. 4; Table 4d). Under reduced salinity, mortality was significantly greater in the elevated temperature treatments (Fig. 4; Table 4d).

Clearance rates of algae

Under elevated pCO_2, larvae had reduced clearance rates (Fig. 5). Specifically, larvae cleared fewer algal cells within the 12-h period in the elevated pCO_2 compared to the ambient pCO_2 (Fig. 5; Table 5). Clearance rates were also reduced with elevated temperature (Fig. 5; Table 5) and reduced salinity (Fig. 5; Table 5).

Discussion

This study found that when adult *O. angasi* close, even for short periods of time, the reduced pH conditions experienced by their brooded larvae are well in excess of the conditions expected under future models of ocean acidification for 2100. The pH of the fluid within the pallial cavity that larvae encounter was much lower than that of the ambient sea water (7.88 pH units).

Overall, the negative impacts of elevated pCO_2 (853–1194 µatm) as a single stressor were relatively minor, with no effects on the timing of development, abnormality, and mortality of *O. angasi* larvae. There was an effect of elevated pCO_2 on the size of larvae, with larvae being 3 % smaller compared to those reared under ambient pCO_2. Effects on larval size were exacerbated by additional stressors including reduced food (that was half the diet required for optimum hatchery conditions), reduced salinity (reduced down from 32 to 20 which is encountered by *O. angasi* during heavy rain), and elevated temperature (4 °C increase predicted for 2100). There was delayed development, reduced size, and an increase in the prevalence of abnormal larvae reared in combined stressor treatments. Regardless of the presence of other stressors, there was no effect of elevated pCO_2 on mortality of larvae.

Larvae were significantly smaller when reared under elevated pCO_2, exhibiting a 3 % reduction in final size. Under similar conditions, however, the impact of elevated pCO_2 on the growth of broadcast spawning bivalves was much greater. For example, elevated pCO_2 resulted in up to a 65 % decrease in growth of larvae of *S. glomerata* (et al. 2011), and decreases of 18 and 8 % for the clams, *Argopecten irradians* and *M. mercenaria*, respectively (Gobler and Talmage 2013). In contrast to the robust response of the larvae of *O. angasi* to elevated pCO_2, larvae of broadcast spawning bivalves reared in the same conditions used here are extremely sensitive to acidification (Kurihara et al. 2007; Kurihara 2008; et al. 2010; Talmage and Gobler 2011). Elevated pCO_2 as a single stressor did not result in negative impacts on the timing of development

Table 4 Analysis of variance (ANOVA) of (a) shell length of larvae, (b) percentage eyed larvae, (c) percentage abnormalities, and (d) percentage mortality

Source		(a) Shell length			(b) Eyed larvae			(c) Abnormalities			(d) Mortality		
		Untransformed $C = 0.189$ ($P > 0.05$)			ArcSin (%)-transformed $C = 0.211$ ($P > 0.05$)			ArcSin (%)-transformed $C = 0.400$ ($P < 0.05$)			ArcSin (%)-transformed $C = 0.376$ ($P < 0.05$)		
	d.f.	M.S.	*F*	*P*	M.S.	*F*	*P*	M.S.	*F*	*P*	M.S.	*F*	*P*
pCO_2=CO	1	141.80	3.96	0.055	2952.77	77.47	0.000	2323.97	128.5	0.000	41.59	0.18	0.674
Salinity (Sa)	1	3048.05	85.02	0.000	3866.83	101.45	0.000	450.89	24.92	0.000	4787.17	20.74	0.000
Temperature (Te)	1	1.17	0.03	0.858	891.92	23.40	0.000	246.11	13.6	0.001	6315.15	27.36	0.000
Food (Fo)	1	295.85	8.25	0.007	796.01	20.89	0.000	80.56	4.45	0.043	20.34	0.09	0.769
CO × Sa	1	57.42	1.60	0.215	340.26	8.93	0.005	47.87	2.65	0.114	0.036	0.00	0.990
CO × Te	1	68.88	1.92	0.175	0.43	0.01	0.916	33.69	1.86	0.182	114.46	0.50	0.486
CO × Fo	1	248.28	6.93	**0.013**	10.33	0.27	0.601	86.12	4.76	0.037	9.34	0.04	0.842
Sa × Te	1	347.58	9.70	**0.004**	78.38	2.06	0.161	1.47	0.08	0.777	3420.94	14.82	**0.001**
Sa × Fo	1	119.81	3.34	0.077	199.29	5.23	0.029	7.72	0.43	0.518	18.69	0.08	0.778
Te × Fo	1	0.71	0.02	0.889	3.87	0.10	0.752	163.06	9.01	0.005	410.11	1.78	0.192
CO × Sa × Te	1	2.45	0.07	0.796	1241.81	32.58	0.000	185.44	10.25	0.003	4.98	0.02	0.884
CO × Sa × Fo	1	50.36	1.40	0.245	405.42	10.64	0.003	83.44	4.61	0.039	15.94	0.07	0.794
CO × Te × Fo	1	9.04	0.25	0.619	1.47	0.04	0.845	0.73	0.04	0.842	1.55	0.01	0.935
Sa × Te × Fo	1	1.17	0.03	0.858	147.12	3.86	0.058	89.63	4.95	0.033	3.74	0.02	0.900
CO × Sa × Te × Fo	1	19.81	0.55	0.463	246.74	6.47	**0.016**	270.04	14.93	**0.001**	2.74	0.01	0.914
Residual	32	35.85			38.11			18.09			230.8		
Total	47												
SNK		Sa × Te			CO × Sal × Te × Fo			CO × Sal × Te × Fo			Sa × Te		
		OT: AS > RS			AS/OT/FF: AC = EC			AS/OT/FF: AC = EC			OT: AS = RS		
		ET: AS > RS			AS/OT/HF: AC > EC			AS/OT/HF: AC > EC			ET: AS < RS		
		AS: OT < ET			AS/ET/FF: AC > EC			AS/ET/FF: AC > EC			A S: OT = ET		
		RS: OT > ET			AS/ET/HF: AC > EC			AS/ET/HF: AC > EC			RS: OT < ET		
					RS/OT/FF: AC > EC			RS/OT/FF: AC > EC					
		CO × Fo			RS/OT/HF: AC > EC			RS/OT/FH: AC > EC					
		FF: AC > EC			RS/ET/FF: AC = EC			RS/ET/FF: AC = EC					
		HF: AC > EC			RS/ET/HF: AC = EC			RS/ET/HF: AC < EC					
		AC: FF > HF											
		EC: FF = HF											

Treatments were orthogonal combinations of the fixed factors: pCO_2 (*AC* ambient or *EC* elevated), salinity (*AS* ambient salinity or *RS* reduced salinity), temperature (*OT* optimum temperature or *OT* elevated temperature), food (*FF* full food or *HF* half food), $n = 3$

Prior to analyses, data were tested for homogeneity of variances, Cochran's *C* test

Post hoc SNK tests were done for significant sources of variation, shown in bold, relative to the hypotheses of interest to determine the direction of differences ($P > 0.05$)

Treatments were orthogonal combinations of the fixed factors: pCO_2 (ambient or elevated), salinity (ambient or reduced salinity), temperature (optimum or elevated temperature), food (full or half food), $n = 3$

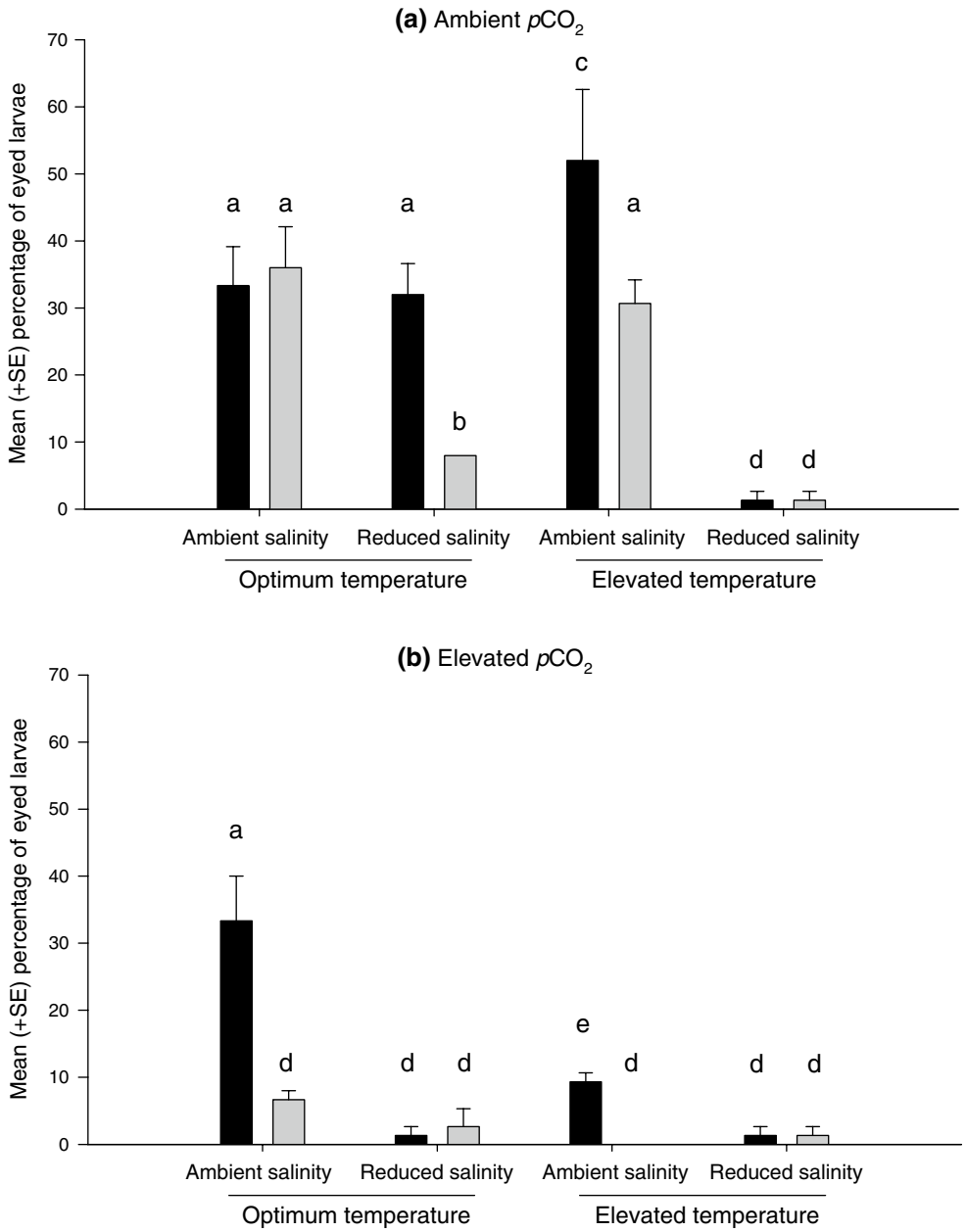

Fig. 2 Mean (+SE, $n = 3$) percentage of larvae that had reached the eyed stage of development jar^{-1} under **a** ambient pCO_2 (390 µatm), or **b** elevated pCO_2 (856 µatm), from optimum (26 °C) or elevated (30 °C) temperature, and ambient (32) or reduced (20) salinity, and fed either the full diet (50,000 cells mL^{-1}; *black bars*) or half diet (25,000 cells mL^{-1}; *grey bars*). Analyses were based on the percentage of 30 randomly chosen individuals from each replicate container. *Letters* represent significant differences (SNK $P < 0.05$) for the significant interaction amongst all factors (ANOVA $P < 0.05$, Table 4b)

of *O. angasi*, abnormality or on mortality of larvae. The robust nature of the larvae of *O. angasi* to elevated pCO_2 may be due to their acclimation to episodes of hypercapnia or because they are released at an older, possibly less sensitive stage. We used these later-stage larvae which are typically more robust than early-stage larvae (Gibson et al. 2011). Parker et al. (2010) did, however, use this umbonate larval stage and noted a reduction in size and increase in abnormal morphology of larvae under elevated pCO_2 at the levels used here in *S. glomerata* and *C. gigas*.

Under ambient conditions, the fluid surrounding the larvae of *O. angasi* attached to the gills remained lower than the surrounding sea water (7.88 compared with 8.20 pH units). While it may be possible sample contamination by small amounts of extrapallial fluid and haemolymph occurred when pallial fluid was extracted from drilled

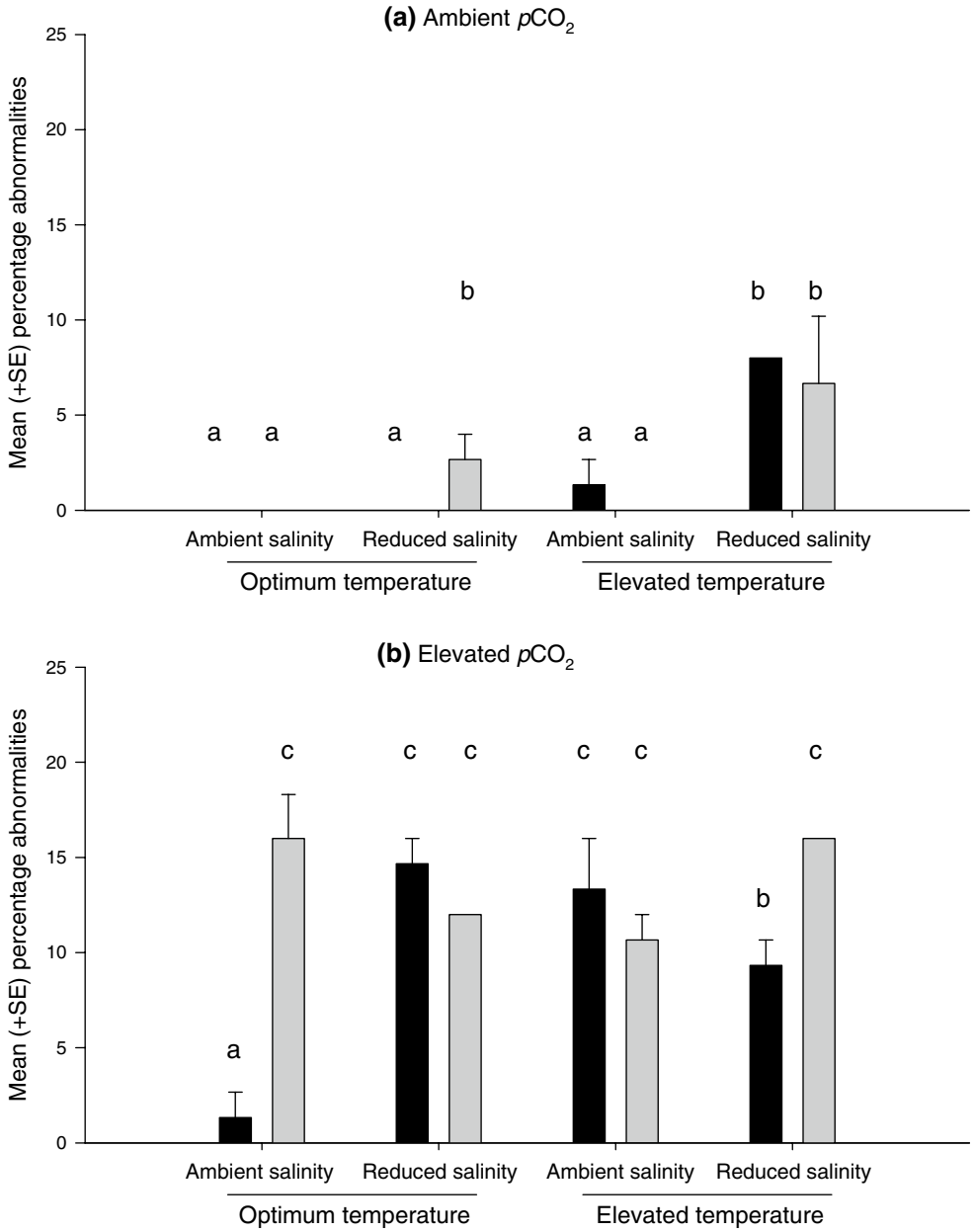

Fig. 3 Mean (+SE, $n = 3$) percentage of abnormally developed larvae jar^{-1} under **a** ambient pCO_2 (390 µatm), or **b** elevated pCO_2 (856 µatm), from optimum (26 °C) or elevated (30 °C) temperature, and ambient (32) or reduced (20) salinity, and fed either the full diet (50,000 cells mL^{-1}; *black bars*) or half diet (25,000 cells mL^{-1}; *grey bars*). Analyses were based on the percentage of 30 randomly chosen individuals from each replicate container. *Letters* represent significant differences (SNK $P < 0.05$) for the significant interaction amongst all factors (ANOVA $P < 0.01$, Table 4c)

oysters, it may also be influenced by respiratory CO_2. When the individuals were closed for at least an hour, as occurs in response to heavy rain where *O. angasi* is farmed, the pH larvae experienced was lower (to pH 7.46) than that expected under extreme scenarios of elevated pCO_2 for 2100 (IPCC 2014). While closure of adults for long periods may result in hypercapnic conditions which are detrimental to the survival of the larvae (Chaparro et al. 2009; Noisette et al. 2014), acute episodic exposure of larvae to reduced pH prior to liberation from the pallial cavity may be beneficial to larvae as our oceans continue to decrease on pH over this century. Similar resilience of larvae to elevated pCO_2 occurs for broadcast spawners following laboratory exposure of their parents to elevated CO_2 prior to spawning. For example, larvae of the oyster, *S. glomerata*, exhibited lower impacts of elevated CO_2 on their growth, development, and abnormality (but not survival) if their parents were also reared at elevated compared to ambient pCO_2 (Parker

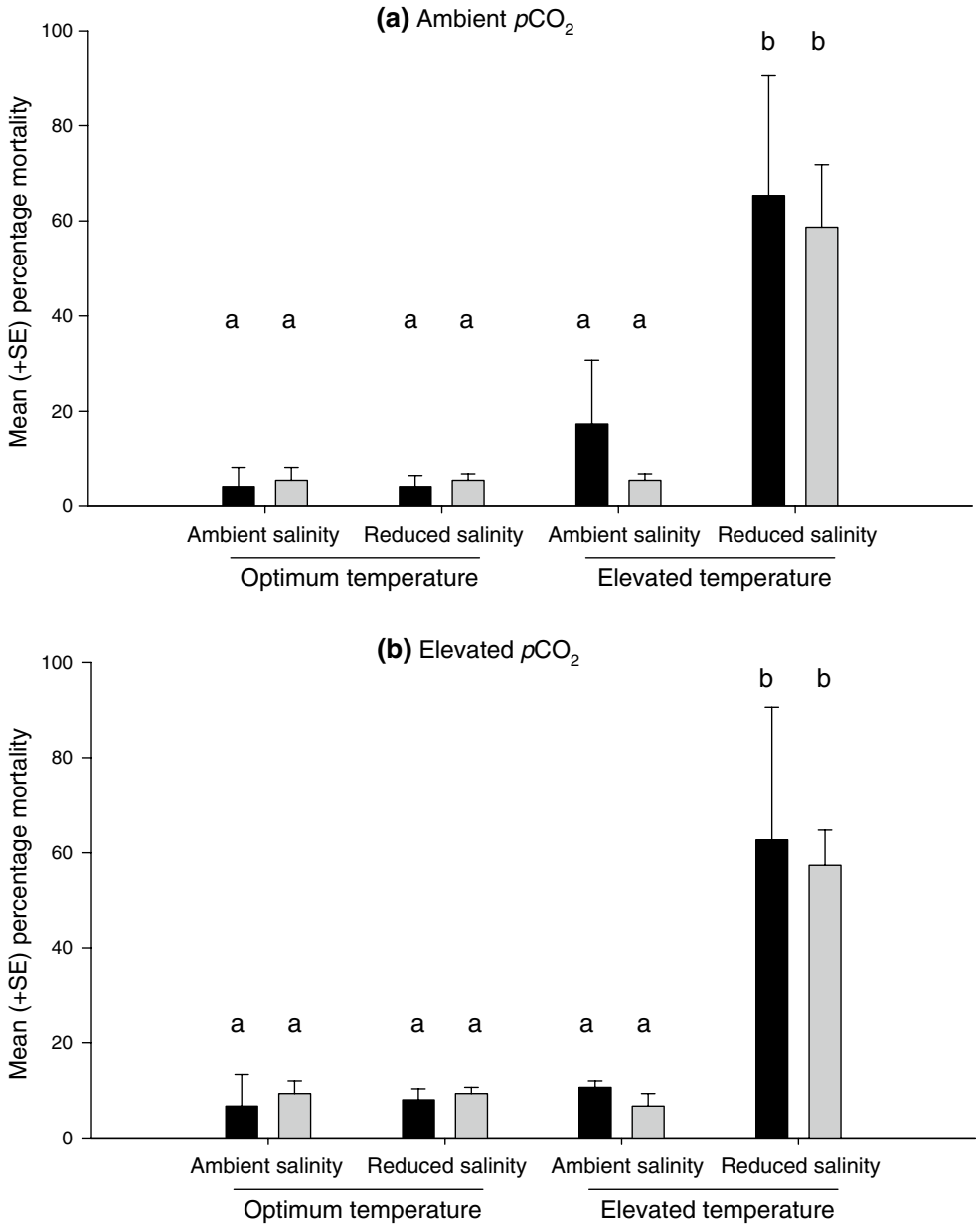

Fig. 4 Mean (+SE, $n = 3$) percentage of larvae that died jar^{-1} under **a** ambient pCO_2 (390 µatm), or **b** elevated pCO_2 (856 µatm), from optimum (26 °C) or elevated (30 °C) temperature, and ambient (32) or reduced (20) salinity, and fed either the full diet (50,000 cells mL^{-1}; *black bars*) or half diet (25,000 cells mL^{-1}; *grey bars*). Analyses were based on the percentage of 30 randomly chosen individuals from each replicate container. *Letters* represent significant differences (SNK $P < 0.05$) for the significant interaction between salinity and temperature (ANOVA $P < 0.01$, Table 4d)

et al. 2012). Similar results were found in larvae of the sea urchin, *Strongylocentrotus droebachiensis*, which displayed no reduction in survival if their parents were reared at elevated compared to ambient pCO_2 for 16 months prior to spawning (Dupont et al. 2013). It is unknown whether similar exposure of *O. angasi* parents to elevated pCO_2 during reproductive conditioning would further increase the resilience of their larvae to elevated pCO_2 or have negative consequences.

While early development of *O. angasi* larvae on the gills of adults (O'Sullivan 1978; Hickman and O'Meley 1988) in elevated pCO_2 conditions relative to surrounding water may have provided resilience to detrimental effects of acidification, the presence of additional stressors (temperature, hyposalinity, and reduced food availability, which will occur in the real world, will likely minimise any advantage of *O. angasi* larvae to elevated pCO_2. When fed the half food diet, development was slower and the percentage

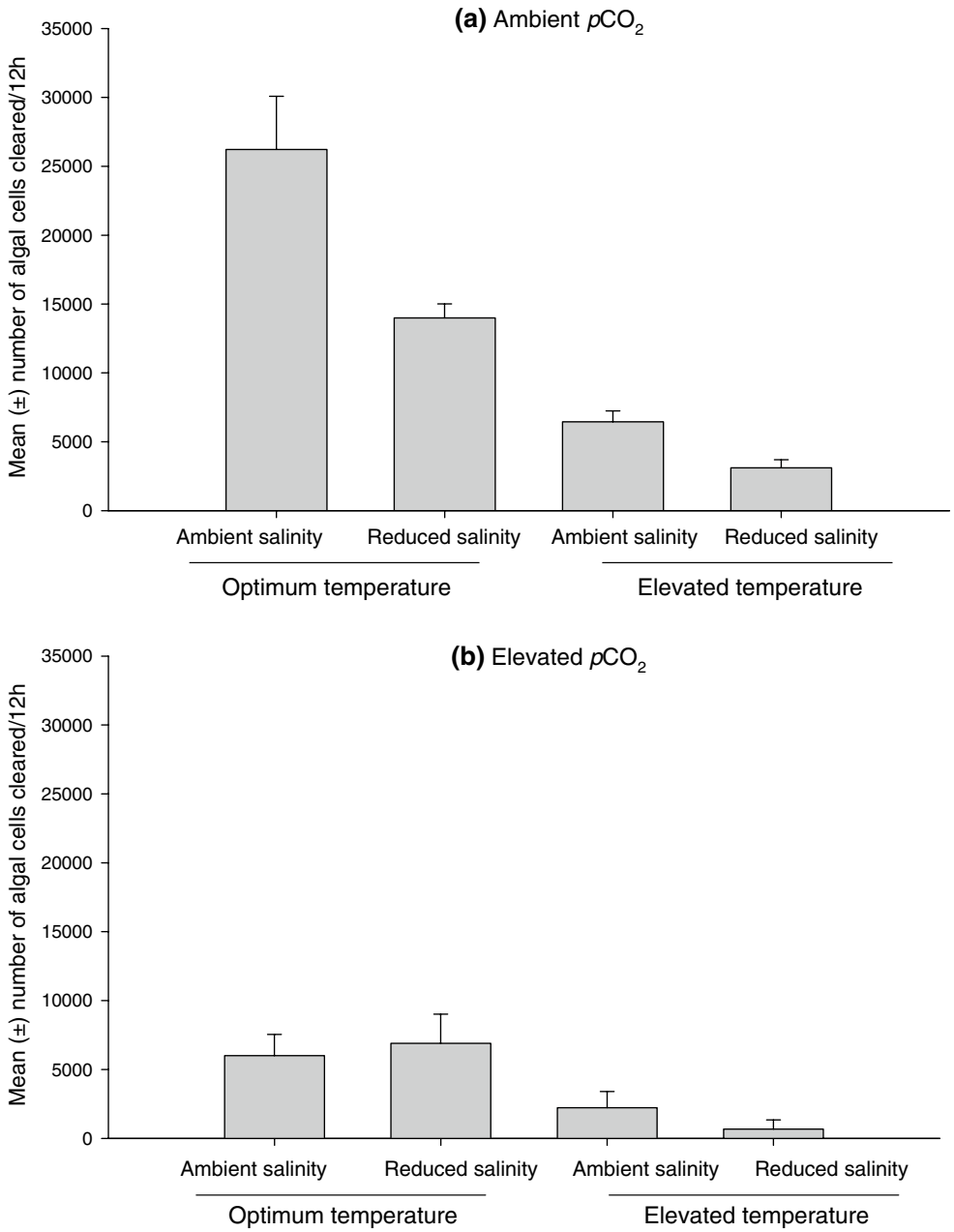

Fig. 5 Mean (+SE, $n = 3$) number of algal cells mL^{-1} cleared after 12 h from under **a** ambient pCO_2 or **b** elevated pCO_2, and ambient (32) or reduced (20) salinity, and optimum (26 °C) or elevated (30 °C) temperature

of abnormally shaped larvae was greater under elevated pCO_2. The presence of other stressors exacerbated the effects of elevated pCO_2 on decreased larval size. Moreover, elevated pCO_2 as a single stressor negatively impacted clearance rates of larvae. Ocean acidification is believed to cause a higher energetic cost of living (Pörtner and Farrell 2008; Melzner et al. 2009). Many mollusc species have higher metabolic needs under elevated pCO_2, with more energy being diverted to sustain processes such as acid–base balance and calcification (Melzner et al. 2009). It is hypothesised that at higher food concentrations, these higher metabolic costs can be met, thereby minimising the effects of elevated pCO_2 (Pörtner and Farrell 2008; Melzner et al. 2011; Thomsen et al. 2013). At lower food concentrations, the higher costs cannot be met, exacerbating the impacts of elevated pCO_2 (Pörtner and Farrell 2008). In the present study, we found that larvae of *O. angasi* decreased their feeding rate under elevated pCO_2. Under high food concentrations, larvae reared under elevated pCO_2 did not consume the food that they encountered as successfully

Table 5 Analysis of variance (ANOVA) of clearance rates in terms of the number of algal cells mL^{-1} cleared after 12 h

Source	*d.f.*	M.S.	*F*	*P*
pCO_2=CO	1	15,547.46	37.14	**0.000**
Salinity (Sa)	1	22,745.09	54.33	**0.000**
Temperature (Te)	1	2790.24	6.67	**0.020**
CO × Sa	1	648.55	1.55	0.231
CO × Te	1	894.81	2.14	0.163
Sa × Te	1	32.68	0.08	0.784
CO × Sa × Te	1	775.70	1.85	0.192
Residual	16	418.64		
Total	23			
SNK		CO: AC > EC		
		Sa: AS > RS		
		Te: OT > ET		

Treatments were orthogonal combinations of the fixed factors: pCO_2 (*AC* ambient or *EC* elevated), salinity (*AS* ambient salinity or *RS* reduced salinity), temperature (*OT* optimum temperature or *OT* elevated temperature), food (*FF* full food or *HF* half food), $n = 3$

After data were Sqrt $(x + 1)$-transformed, variances were homogeneous Cochran's *C* test, $C = 0.3272$ ($P > 0.05$)

Post hoc SNK tests were done for significant sources of variation, shown in bold, relative to the hypotheses of interest to determine the direction of differences, $P < 0.05$

as larvae reared in ambient conditions. Similarly, Vargas et al. (2013) found a decrease in feeding by larvae of the gastropod, *Concholepas concholepas*, with elevated pCO_2 and highlighted the susceptibility of larval feeding ability to ocean acidification. Whether the larvae of *O. angasi* were unable to feed or had slowed metabolism as a defence mechanism is unknown. It is expected that the observed clearance rates by *O. angasi* larvae would be lower under low food concentrations because larvae encounter food less frequently (e.g. Tenore and Dunstan 1973; Widdows et al. 1979; Bayne 1993). Despite reduced feeding coupled with higher energetic requirements to deal with stress, over the longer term, larvae may ultimately survive if there is enough food available. These reduced clearance rates seen here as a sublethal effect are likely to ultimately compromise development and survival. Moreover, metabolic costs, not met by food intake, can lead to elevated stress which will ultimately lead to mortality (Pörtner 2008; Pörtner and Farrell 2008). It should also be noted that the positive impacts of food in oysters are limited with respect to minimising the negative impacts of ocean acidification (Hettinger et al. 2013).

Elevated pCO_2 in combination with elevated temperature (30 °C) or decreased salinity (20) resulted in was a greater negative effect of acidification on larval development, abnormalities, and clearance rates. Bivalve development is well known to be sensitive to warming and hyposalinity (Przeslawski et al. 2015). As found here, juvenile clams (*M. mercenaria*) showed the greatest negative effects on biomineralisation and energy metabolism in conditions of low salinity and high pCO_2 (700–800 µatm), while these stressors in isolation had no effects (Dickinson et al. 2013). Similarly, Ko et al. (2014) found that elevated pCO_2, elevated temperature, and reduced salinity reduced pre-settlement growth of *C. gigas*, while these stressors used in isolation had no significant effects. Negative interactive effects of multiple climate stressors have implications for the larvae of *O. angasi* and other marine species as climate change is likely to result in exposure to many stressors with synergistic impacts leading to widespread changes to marine systems (Fabry et al. 2008; Przeslawski et al. 2015).

The interaction between temperature and salinity had a negative effect on the size and mortality of larvae and, independently, these stressors affected clearance rates. In ambient salinity, growth was faster at 30 °C than at 26 °C. In reduced salinity, the pattern was reversed. On average, over 50 % of larvae died under elevated temperature and reduced salinity, regardless of the level of pCO_2 or food availability. The ability of larvae to clear food from the water column was limited when reared under elevated temperature or reduced salinity. Salinity is an important determinant of the distribution of oysters and their larvae (Dekshenieks et al. 2000). As a coastal species, *O. angasi* may be capable of withstanding some variation in salinity but are considered to inhabit a stenohaline environment (Thomson 1954; Nell and Gibbs 1986). The salinity of tidal estuaries where the larvae occur can drop to 20 for a number of days (Roy et al. 2001), which resulted only small effects on growth of *O. angasi* larvae level that reduces (O'Connor et al. 2014). Another estuarine oyster, *S. glomerata*, also shows low rates of growth and development at salinities of 20 or less (Dove and O'Connor 2007). Although the larvae were able to survive with reduced salinity at the optimal temperature of 26 °C, when temperature increased to 30 °C, fewer survived.

This study reared the larvae to the eyed stage. Taking the larvae to metamorphosis might have revealed differential mortality because stress in early life stages can influence the extent and magnitude of latent effects at later stages of development (Pechenik 2006. Stresses experienced by oysters in an early life stage can have negative "carry-over" to subsequent stages Hettinger et al. (2012). Larvae of *O. lurida* reared under ocean acidification conditions displayed decreased growth as juveniles regardless of their present environment (Hettinger et al. 2012). Naturally elevated levels of pCO_2 had delayed effects on larval development of *C. gigas*, such that conditions affected mid-stage growth but not the early D-stage (Barton et al. 2012). In addition to carry-over effects within the life of individuals,

oysters such as *S. glomerata* may carry responses to elevated pCO_2 to their offspring and future generations (Parker et al. 2012, 2015).

In summary, *O. angasi* was affected by multiple climate change-related stressors, indicating that the impacts of climate change on *O. angasi* will be complex. Based on the findings of the present study, we predict that survival of *O. angasi* will continue in a changing climate, facilitated by its brooding life history. Further research is required to investigate climate change impacts on larval ontogeny and the effects on future generations in the long term. The results from this study suggest a complex interaction of pCO_2, temperature, salinity, and availability of food on the growth, shape, development, and mortality of larvae. This study fills a vital gap in our understanding of the response of marine invertebrates to a changing climate, particularly when organisms are faced with multiple stressors (Harley et al. 2006). Due to preconditioning of larvae while brooded, the larvae of *O. angasi* may potentially have an improved capacity to compensate for changes to their acid–base status (Pörtner 2008). Without investigating the physiological mechanisms, it is not known whether larval preconditioning within the maternal shells, and ability to withstand experimentally created ocean acidification, will enable *O. angasi* to adapt to a high CO_2 world in the future. It is essential for future studies to address multiple stressors associated with climate change (Byrne 2011; Przeslawski et al. 2015).

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