



Sub-lethal and lethal effects of chronic and extreme multiple stressors on a critical New Zealand bivalve under hypoxia

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ABSTRACT: Warming of coastal regions due to climate change is exerting chronic thermal stress on intertidal organisms, many of which are already living at or near their limits for thermal tolerance. In addition, increasing frequency of marine heat waves and algal blooms leading to hypoxic events in estuaries exert acute stresses on aerobic organisms. Hypoxia and warming rarely occur in isolation, and when combined, likely have synergistic detrimental effects on bivalves. Further, midday low tides have been identified as important drivers of mass mortality events for bivalves as a consequence of the combined effects of thermal stress and desiccation. Accordingly, the current study focuses on the sublethal and lethal effects of these combined stressors on the critical estuarine bivalve *Austrovenus stutchburyi*. Experiments were used to test the effects of simulated midday low tides combined with chronic warming and oxygen depletion on activity, respiration and survival of *A. stutchburyi*. A lethal emersion thermal threshold was surpassed at 33°C for treatments exposed to both hypoxic and normoxic treatments, suggesting extreme thermal events such as heatwaves during midday low tides could have substantial detrimental effects on bivalve populations and their vital ecosystem functions. Further, the ability of bivalves to survive heat and desiccation of midday low tide events was much reduced when they were exposed to even minor heat and hypoxia stress when immersed at high tide. The findings of the current study highlight important biological consequences of coastal warming combined with extreme temperature and hypoxic events observed in estuaries.

KEY WORDS: Hypoxia · Bivalve · Marine heatwave · Multiple stressors · Thermal stress · Ecosystem engineer

1. INTRODUCTION

Anthropogenically driven deoxygenation of coastal marine environments has occurred since at least the early 20th century (Diaz & Rosenberg 2008, Keeling et al. 2010), and it is projected to accelerate with climate-induced warming (Bopp et al. 2013), enhanced ocean stratification (Ummenhofer & Meehl 2017), and increased eutrophication. Accordingly, deoxygenation in the coastal ocean has gained attention with increased reporting and research into the consequences of low oxygen events since the 1960s

(Barlow et al. 1963). A 54% increase in the occurrence of annual marine heat wave (MHW) days globally and a 34 and 17% rise in MHW frequency and duration, respectively, with associated declines in the oxygen saturation point, have been observed since 1925 (Oliver et al. 2018). Dissolved oxygen (DO) concentrations are declining faster, and are becoming more variable, in coastal zones than in open oceans where a 2% decline in oceanic oxygen content has been observed in recent decades (Rabalais et al. 2014, Schmidtko et al. 2017). Fluctuations and declines in the coastal oxygen saturation baseline result

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in increases of both the frequency and intensity of lethal hypoxia events in coastal bays and inlets (Schmidtko et al. 2017, Breitburg et al. 2018, Altieri & Diaz 2019). Accordingly, the frequency of acute coastal hypoxia events has been reported to be growing at an exponential rate of 5.5% per year as coastal seas warm and become more eutrophic (Vaquer-Sunyer & Duarte 2008).

Below a species-specific DO threshold, DO deficiency and hypoxic stress affect metabolic rates, behaviour, growth, reproduction and survival (Vaquer-Sunyer & Duarte 2008). The critical hypoxia threshold of 2.0 ml O₂ l⁻¹ defined in Diaz & Rosenberg (1995) is used as a general threshold for metazoan survival below which large-scale mortality events of critical species can occur. However, taxon-specific and intra-specific thresholds exist (Vaquer-Sunyer & Duarte 2008) and can covary with population size structure and/or reproductive state (e.g. Taylor & Brand 1975). Hypoxia-induced loss or decline of critical species such as bivalves have resulted in changes in ecosystem functioning, particularly in fluxes of organic matter and nutrient cycling within coastal food webs and in ecosystem services such as fisheries production and formation of biogenic habitats (Sumaila et al. 2011, Rabalais et al. 2014, Pecl et al. 2017).

Localised and mass mortality events (MMEs) in the marine environment have been documented for at least 5 decades (Johnson 1966, Tsuchiya 1983). However, the magnitude of MMEs and the quantity of events published in the scientific literature has increased in the past 10 yr; while many others are anecdotally reported (Fey et al. 2015, Soon & Zheng 2020). Many of the observed MMEs have been attributed to disease outbreaks and contributing synergistic effects of multiple stressors on disease susceptibility (Fey et al. 2015). For example, a meta-analysis by Fey et al. (2015) indicated that the likely causes of multiple stressor MMEs included sensitivity to toxicity in conjunction with thermal and hypoxic stress. Multiple stressor-induced events are especially prevalent in intertidal and estuarine areas where environmental changes on land and sea combine (Petes et al. 2007).

Intertidal species are naturally exposed to dynamic physical conditions, however the combined thermal and hypoxic stresses associated with climate change and MHWs can exert lethal additive or synergistic combinations of chronic and acute stress onto organisms (Bible et al. 2017). Chronic, lower intensity, longer-term stress of rising temperatures has the capacity to divert energy allocation away from feeding, respiration, growth and reproduction (Kooijman 2010), rendering an organism more susceptible to

acute stressors (i.e. 'extreme' thermal or hypoxic events). Extreme thermal events are intensifying in coastal and estuarine systems, exerting severe stress when hot, dry aerial temperatures are coincident with midday low tides, resulting in repeated distinct physiological challenges (e.g. Helmuth et al. 2006, Jones et al. 2017). If bivalves are already stressed due to increased water temperatures and the concomitant lowered oxygen conditions, the result can be large-scale MMEs of intertidal bivalve populations. Accordingly, observations of MMEs as a combined result of coastal warming and aerial heat stress and desiccation have been increasing in frequency in marine environments in New Zealand (e.g. Tricklebank et al. 2021) and globally (e.g. Harley 2008, Seuront et al. 2019), causing concern for persistence of important ecosystem engineers such as bivalves.

Austrovenus stutchburyi (Bivalvia: Veneridae) (Wood, 1828), the endemic cockle or Tuaki (hereafter *Austrovenus*), is a critical infaunal suspension feeding bivalve and ecosystem engineer in coastal bays and inlets throughout New Zealand and subject to both prehistoric and modern day changes in environmental conditions (Tallis et al. 2004, Wells et al. 2019, O'Connell-Milne et al. 2020). *Austrovenus* is an important benthic–pelagic coupler (e.g. Marsden 2004), influencing biogeochemical cycling in estuarine environments and trophic pathways (Thrush et al. 2006, Karlson et al. 2021) as well as supporting customary, recreational and commercial harvest (O'Connell-Milne et al. 2020). Behavioural responses by *Austrovenus* to low oxygen include reduced burrowing depths and extending siphons above the sediment surface (Marsden & Bressington 2009). More general physiological responses of bivalves to hypoxia include lowered metabolic rates, induced short-term anaerobic metabolism and production of alanine, succinate and propionate (Carroll & Wells 1995, Haider et al. 2020). Each of these ecosystem functions, services and physiological responses are sensitive to increased heat and hypoxic stresses with warming coastal seas.

New Zealand's longest sea surface temperature (SST) time series (1953–present) collected at the Portobello Marine Laboratory, Otago Harbour, identifies a rate of warming of 0.10°C decade⁻¹ in coastal waters of southern New Zealand (Shears & Bowen 2017). The observed rate of coastal warming in conjunction with climate models forecasts average aerial temperature increases of 0.7–3.0°C by 2090 (Ministry for the Environment 2018). Along with the increasing probability of rising temperatures, the duration and intensity of MHWs and associated low oxygen events are pre-

dicted to continue to increase (Hobday et al. 2016, Frölicher et al. 2018). For example, New Zealand experienced sequential heatwaves in the austral summers (Dec–Feb; hereafter ‘DJF’) of 2017–2018 and 2018–2019 (Salinger et al. 2019). The coupled atmospheric–marine heatwave of 2017–2018 resulted in +3.7°C SST anomalies compared to the 1981–2010 averages (Salinger et al. 2019). The observed increase in heatwave events and subsequent increase in mean water temperatures can induce MMEs as organisms experience the synergistic effects of thermal stress, hypoxia and multiple associated stressors.

Consequently, the present research focussed on understanding the implications of midday emersion following chronic oxygen and thermal stress on *Austrovenus*, a scenario that has been observed to cause mortality events in New Zealand and globally among intertidal bivalves. Therefore, the specific aims of the current research were to investigate the exposure thresholds and tolerances of adult *Austrovenus* exposed to multiple stressor events. These scenarios included chronic low oxygen, elevated water temperatures along with increased intensity of heat and desiccation exposure during a simulated summertime low tidal series. Two key questions were addressed: (1) How do siphon activity and survivorship in *Austrovenus* respond to the combined effects of hypoxia and elevated water temperature? (2) How does *Austrovenus* respond to midday low tide thermal challenges given preconditioning to low oxygen and elevated water temperature? Accordingly, we assessed (1) siphon activity, (2) respiration rates and (3) survival of *Austrovenus* subjected to multiple stressors and subsequent heat/desiccation exposure challenges. We predicted that lower DO and warmer waters would cause stress to *Austrovenus* and negatively impact survival. Results obtained in the present study are important for understanding how cumulative environmental stressors affect a critical endemic New Zealand estuarine bivalve.

2. MATERIALS AND METHODS

2.1. *Austrovenus* collections

Austrovenus were collected within the mid and low intertidal zones at Doctors Point, Waitati Inlet, Otago, New Zealand (45.7341°S, 170.5954°E), between March and September 2019 on 5 separate occasions and transported to Portobello Marine Laboratory, located on the Otago Peninsula. Individuals were maintained in flow-through 200 l tanks without sedi-

ment, containing 10 µm sand-filtered fully aerated seawater, for 7 to 14 d at a salinity fluctuating between 34.0 and 34.2. During this time water temperature was increased gradually from ambient early autumn to early spring seawater temperatures of 7–16°C to 22°C or 25°C, respective of the experiment, using aquarium heaters (Eheim Thermocontrol 300 W) at an average rate of 1°C per day. Once at 22°C and 25°C, animals remained in the acclimation tanks until commencement of the experiment. Biofilm and epibionts living on the *Austrovenus* were removed by scrubbing the shell to reduce biological activity that could confound experimental treatments. Active supplementary feeding was withheld for the entirety of the acclimation and experimental treatments, with the only food source being the natural Otago Harbour phytoplankton communities present in the sand-filtered seawater (G. McCarthy pers. comm.). Standardisation of food availability helped to ensure metabolic rates were as close to their basal rates as possible, isolating treatment effects in respiratory response (e.g. Aguirre-Velarde et al. 2016).

2.2. Experimental setup

Experiments were conducted in modified 5 l sealed tanks with a mean (\pm SE) seawater tank flowrate of 4.18 l h⁻¹ (0.02). Using a 200 l seawater reservoir and an IKS AquaStar Oxygen Module and control panel, seawater was deoxygenated with N₂ to 10 \pm 3% (mean \pm SE) or 20 \pm 3% saturation (hypoxic). Normoxic water (\geq 100% saturation) was continuously aerated in a separate 200 l reservoir. Deoxygenated water temperatures in each tank were maintained at 22 or 25°C for respective experiments using 2 aquarium heaters (Eheim Thermocontrol 300 W), a fine-tuned thermostat and a water pump (Hailea HX6530).

A flow-through system from the reservoirs to experimental tanks was developed to mitigate the effects of excretion, sulfide accumulation, bacterial production and dramatic shifts in tank pH (de Zwaan et al. 2002, Coffin et al. 2021). Daily water temperature and DO saturation measurements (YSI ProODO Optical Dissolved Oxygen meter; resolution 0.1%) were taken from the top 3 cm of the tank. Furthermore, daily morning visual counts were made of protruding *Austrovenus* siphons, as a proxy for stress and activity. Dead individuals were removed daily with total shell lengths measured. After daily measurements, steps to mitigate waste product accumulation were taken; existing water was discarded, and each experimental tank was wiped down to remove surface biofouling.

2.3. Effects of DO and temperature

An initial comparison of how increasing water temperature from 22°C to 25°C affected response variables of *Austrovenus* was conducted at 10, 20 and 100% DO using the experimental design described in Section 2.2. Three treatments each with 6 replicates were systematically assigned to either 22°C or 25°C and an oxygen level, with each replicate containing 20 *Austrovenus*. Experiments were run for 35 and 25 d for the 22°C and 25°C water temperatures, respectively.

2.4. Low tide emersion

A separate set of experiments aimed to mimic exposure to summertime aerial conditions and the thermal capacity of sediment. Experiments were carried out in 25°C water immersion in either hypoxic (20% DO) or normoxic (100% DO) oxygen conditions and emersion at either 25°C or 33°C for 36 and 16 d, respectively. Four treatments were assigned systematically, each with 3 replicates and each containing 15 (33°C) or 17 (25°C) *Austrovenus*. Individuals in emersion treatments were removed from the tanks and placed on a 3 cm layer of sediment under heat lamps (4 Philips Flood 240 V 120 W) for 5 h (Fig. 1) before being re-immersed in their respective tanks. Three temperature loggers (Onset, HOBO Pendant) were used to monitor emersion temperature.

Low tide exposure at 33°C was selected due to summer air temperatures within Waitati Inlet, Otago, regularly surpassing 30°C (Southern Clams Ltd. unpubl. data). Furthermore, expected aerial warming of up to 3°C by 2090 due to climate change (Ministry for the Environment 2018) strongly implies that 33°C is realistic of future conditions *Austrovenus* may experience within the intertidal zone in Otago. Water temperatures during the DJF 2017–2018 MHW within Waitati Inlet, reached ~25°C, with prominent temperature peaks occurring at low tide and with mean 24 h water temperatures in February 2018 also reaching 20°C (Southern Clams Ltd. unpubl. data). Furthermore, SST at Portobello during the same DJF heatwave event reached a maximum mean intensity of 5.7°C above the climatology (Salinger et al. 2019). Consequently, heatwaves such as those experienced in 2017–2018 and 2018–2019 (Salinger et al. 2019) have the potential to cause extensive warming of waters beyond 22°C and 25°C for extended periods of time.

The current study focused on respiration during the recovery and reoxygenation (normoxia) phase,

simulating a flood tide carrying oxygenated water onto intertidal habitats harbouring *Austrovenus*. Respiration rates were assessed in 7 (0.24 l) sealed acrylic respiratory chambers. Individuals used for the respiration measurements were randomly chosen and labelled prior to the experiment to avoid selection bias. If a specified individual died prior to the test, the next live individual in numerical order was used. Six chambers contained a singular *Austrovenus* while the seventh was a control chamber, which only contained seawater to account for bacterial respiration and temperature flux. A magnetic flea ensured continuous mixing of the water during the incubation, while a mesh divider ensured the individual was not disturbed by the magnetic flea. Incubations were 6 to 6.5 h in duration. The incubation duration was selected based on natural semi-diurnal tidal submersion experienced by intertidal species in New Zealand (O'Connell-Milne et al. 2020). Oxygen concentration was recorded at 30 min intervals using a non-invasive fibre optic oxygen sensor and sensor spots (PreSens OXY-1 SMA meter and PreSens Oxygen-Sensitive Spots) and PreSens Measurement Studio 2 software.

Per treatment, respiration rates were measured for 6–17 individuals at regular intervals during the experiment following the acclimation period. All respiration rates were measured at least 3 wk after bivalves were moved into the laboratory setting. All soft tissue was dissected from each *Austrovenus*, placed into individual pre-weighed crucibles, and dried at 60°C for a minimum of 4 d to obtain the tissue dry weight. Ash-free dry weight (gAFDW) was determined as the weight change from the dry weight based on the loss-on-ignition approach following exposure to 450°C for 4 h.

Measurement of respiration was restricted to actively feeding or gaping bivalves to test differ-



Fig. 1. Experimental emersion of cockles *Austrovenus stutchburyi* to midday low tide conditions

ences between treatments: due to the experimental design of the present study respiration occurring within closed bivalves was unable to be measured. As a consequence, siphon activity was included as a response variable in the statistical design. Respiration rate ($\text{mg O}_2 \text{ l}^{-1}$) was calculated using Eq. (1) whereby ΔD is the change in DO ($\text{mg O}_2 \text{ l}^{-1}$) between the start and end DO readings for the period an individual was respiring, V is the volume of water in each chamber (0.24 l) and ΔT is the duration of time (h) between when an individual began and finished respiring to the closest oxygen measurement. The time used was based on observations of *Austrovenus* valves opening and closing and how these coincided with inflexion points of the relationship with DO (Fig. 2).

$$\text{Oxygen consumption rate} = \left(\frac{\Delta D}{\Delta T} \right) V \quad (1)$$

Individual respiration rate ($\text{mg O}_2 \text{ gAFDW}^{-1} \text{ h}^{-1}$) was calculated using Eq. (2). OCR_{Aust} and OCR_{Cont} are the oxygen consumption rates ($\text{mg O}_2 \text{ h}^{-1}$) in the *Austrovenus* and control chambers respectively.

$$\text{Respiration} = \frac{\text{OCR}_{\text{Aust}} - \text{OCR}_{\text{Cont}}}{\text{gAFDW}} \quad (2)$$

2.5. Theoretical oxygen saturation and Portobello TDMO anomaly

The theoretical daily maximum dissolved oxygen concentration (TMDO, mg l^{-1}) was calculated from temperature measurements using the following 2 formulae (Eqs. 3 & 4) derived from Sverdrup et al. (1942) for chlorinity (Cl , ‰) and DO ($\text{mg O}_2 \text{ l}^{-1}$), respec-

tively, whereby t represents temperature ($^{\circ}\text{C}$) and other values are constants. Chlorinity was included in the calculation due to the reduced solubility of oxygen in solution when chloride and other dissolved electrolytes are present, known as the salting-out effect (Lang & Zander 1986).

$$\text{Cl} = \frac{(\text{Salinity} - 0.03)}{1.805} \quad (3)$$

$$\text{ml O}_2 \text{ l}^{-1} = 10.291 - 0.2809t + 0.006009t^2 - 0.0000632t^3 - \text{Cl}(0.1161 - 0.003922t + 0.0000631t^2) \quad (4)$$

Further conversion from ml l^{-1} calculated in Eq. (4) to mg l^{-1} was performed using the ratio of molar volume at standard temperature and pressure (STP) to the molar weight of oxygen:

$$\text{mg l}^{-1} = \frac{\text{ml l}^{-1}}{0.69976} \quad (5)$$

These theoretical oxygen measurements are based on saturation at a given temperature and do not account for biological productive and consumptive processes (Rabalais et al. 2014) or non-biological factors including winds, tides, solar radiation and precipitation (Sawabini et al. 2015, Nelson et al. 2017) which all influence oxygen concentrations.

Austral summer periods (DJF) were extracted from the 65 yr sea surface temperature (SST; 1953–2018) time series from Portobello Marine Laboratory. Salinity was not available for the dataset, and subsequently was estimated to be 33.5 based on vertical CTD profiles taken throughout the Otago Harbour. Eqs. (3)–(5) were applied to manual SST measurements taken daily at 09:00 h to determine the TMDO. A baseline was derived from the average TMDO across the 65 yr, while a 31 d moving average was performed on TMDO measurements to derive a corresponding daily TMDO anomaly.

To highlight the implications of thermal extremes on TMDO anomalies, a ‘cold’ period and a ‘warm’ period were compared within the SST and TMDO time series. Shears & Bowen (2017) identified the early 1960s as a decade with persistent cold anomalies, with 1963–1964 identified to have the most numerous and extreme cold DJF temperatures for coastal Otago. The corresponding TMDO anomalies were compared with the TMDO anomalies for 2017–2018 when Otago and New Zealand experienced a coupled atmospheric–marine heatwave that broke the previous 1974–1975 record for hottest DJF mean temperature (Salinger et al. 2019). TMDO anomalies were rounded to the nearest 0.05 mg l^{-1} and a Student’s t -test was performed

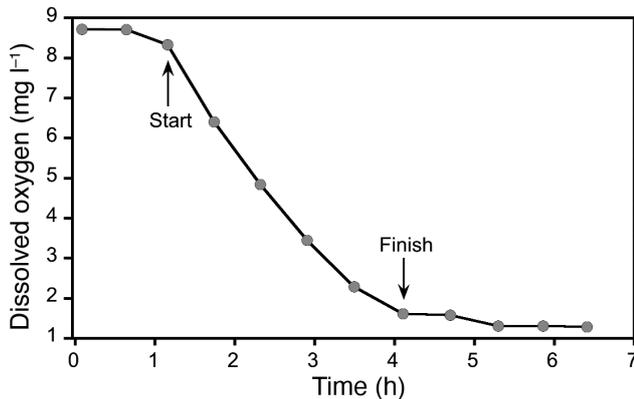


Fig. 2. Inflexion points representing the start and end times of cockle *Austrovenus stutchburyi* respiration during experimental manipulations

on frequency of days with negative anomalies between time periods.

2.6. Statistical analysis

The statistical models for temperature-DO and emersion were applied to the data for siphon activity, respiration rate and survivorship to determine the relationship between stressors and the responses of *Austrovenus*. As individuals died throughout the experiment, the weighting of each surviving individuals siphon observation increased due to a reduced experimental sample size. Repeated daily siphon counts lacked temporal independence. Consequently, siphon activity was reported as the mean of all observations across the first half of each experiment when the majority of individuals were still alive and subjected to the stressor. A linear section of the relationship between dissolved oxygen and time was selected to represent respiration rate (e.g. Fig. 2). Respiration values less than $0.2 \text{ mg O}_2 \text{ gAFDW}^{-1} \text{ h}^{-1}$ and/or r^2 values less than 0.85 were withheld from the analysis due to respiration being intermittent. A cut-off r^2 value of 0.85 was selected after visual examination of oxygen curves similar to Fig. 2 that indicated individuals were either not respiring or had irregular depletion patterns associated with it, prior to a linear regression between the start and finish of the respiration being applied to determine the r^2 value. Statistical differences in siphon activity and respiration rates were performed using a 2-way ANOVA with factors Oxygen (fixed, 10%, 20% and 100% or hypoxic vs. normoxic) and either Temperature (fixed, 22°C vs. 25°C) and/or Emersion (fixed, 25°C vs 33°C).

Cumulative survival was analysed with 2 different general linear models. Model 1 analysed factors Oxygen (fixed, 10%, 20% and 100% or hypoxic vs. normoxic), Day (continuous) and Temperature (fixed, 22°C vs. 25°C). Cumulative survival of *Austrovenus* exposed to simulated low tide emersion was analysed with a mixed model with factors Oxygen (fixed, hypoxic vs. normoxic), Day (continuous) and Emersion (fixed, 25°C vs 33°C). All statistical analyses were performed in JMP 11.0 Pro with post-hoc Tukey's HSD, with alpha applied at 5% confidence.

3. RESULTS

3.1. Effects of DO and temperature

Oxygen saturation ($F_{2,30} = 83.51$, $p < 0.001$), temperature ($F_{1,30} = 22.17$, $p < 0.001$) and the whole statisti-

cal model ($F_{2,30} = 10.66$, $p < 0.001$) influenced the average siphon activity of *Austrovenus*. At 22°C, siphon activity increased with reducing oxygen saturation, while at 25°C, activity was greatest at 20% saturation (Fig. 3a). Increasing temperature reduced siphon activity at 10% DO, while 20 and 100% DO treatments showed no difference between water temperature treatments ($p > 0.05$).

Survivorship decreased with reducing oxygen saturation ($F_{2,220} = 58.60$, $p < 0.0001$) and increasing temperature ($F_{1,220} = 5.12$, $p = 0.0243$). The cumulative survival decreased through time (day: $F_{1,220} = 66.95$, $p < 0.0001$). The interaction between low oxygen and warmer waters was significant ($F_{2,220} = 15.30$, $p < 0.0001$), with oxygen decreased to 10–20% DO while concurrently increasing temperature to 25°C. Cumulative survival was also significantly lower at 25°C 10% DO than at 22°C 10% DO (Fig. 3B).

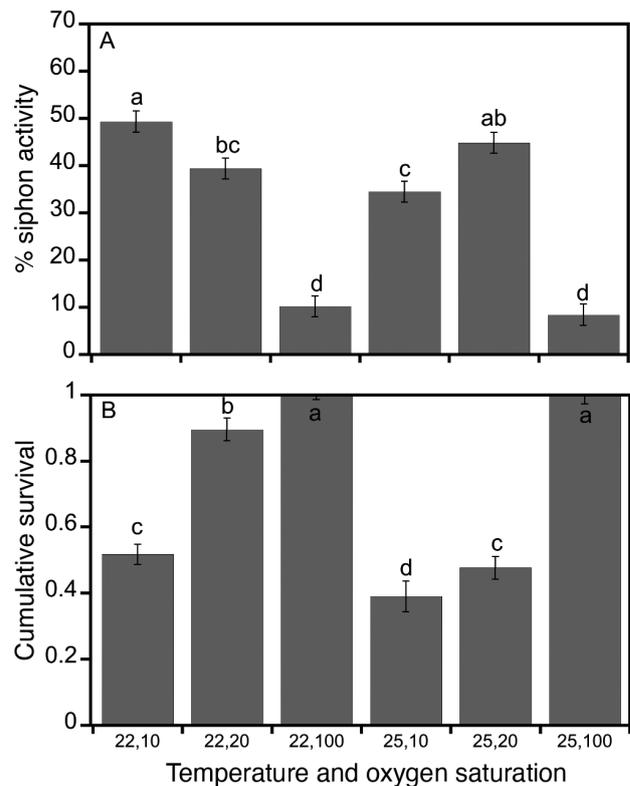


Fig. 3. (A) Mean \pm SE siphon activity of cockles *Austrovenus stutchburyi* exposed to 10, 20 and 100% dissolved oxygen saturations at 22°C or 25°C water temperatures ($n = 6$ replicates per treatment). (B) Mean \pm SE least-squares cumulative survival of *A. stutchburyi* immersed in either 10, 20 or 100% dissolved oxygen at 22°C or 25°C seawater. Treatments not connected by the same letter were significantly different ($p < 0.05$) based on a Tukey's HSD

3.2. Emersion challenge

3.2.1. Siphons

Along with oxygen ($F_{1,8} = 108.37$, $p < 0.0001$), oxygen \times emersion ($F_{1,8} = 10.24$, $p = 0.012$) was a predictor of siphon activity when *Austrovenus* were held at 25°C water temperatures (Fig. 4A). A difference between siphon activity comparing emersion temperature was not resolved ($p = 0.11$). Siphon activity in hypoxic treatments was suppressed in 33°C compared to 25°C emersion, whereby siphon activity occurred 45.9 and 65.7% of the time, respectively.

3.2.2. Respiration

Effect tests identified that oxygen ($F_{1,28} = 5.65$, $p = 0.02$) but not emersion ($p = 0.08$), nor their interaction ($p = 0.14$), influenced respiration rates. A Tukey's

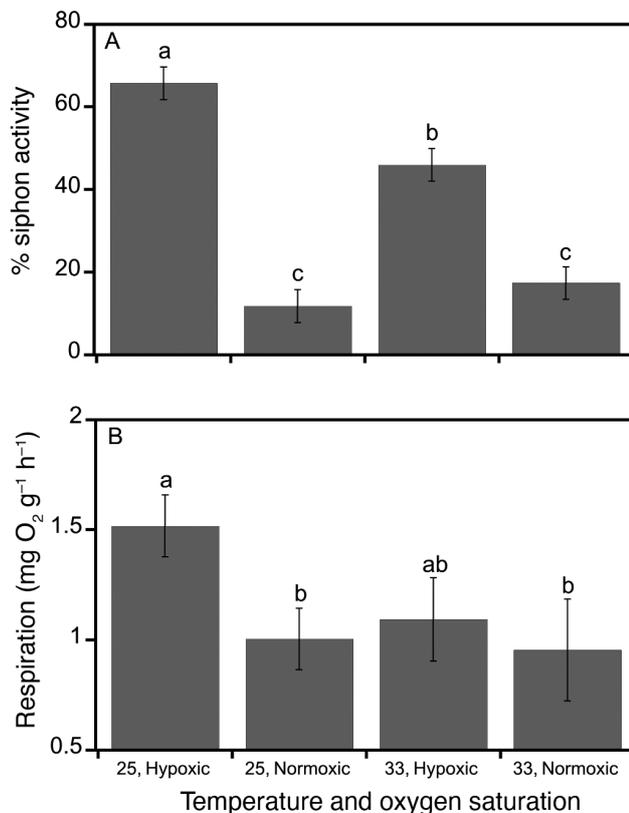


Fig. 4. Mean \pm SE (A) siphon activity (%) ($n = 3$ replicates per treatment) and (B) respiration rate of cockles *Austrovenus stutchburyi* under multiple stressor conditions in either hypoxia or normoxia. $n =$ (x-axis, left to right) 11, 11, 6, 4 cockles. Treatments not connected by the same letter were significantly different ($p < 0.05$) based on a Tukey's HSD

HSD test identified a statistical difference ($p = 0.02$) between 25°C hypoxic and oxygenated treatments with higher respiration in the hypoxic treatment, but not when compared with hypoxic 33°C emersion (Fig. 4B).

3.2.3. Survivorship

Hypoxia was a significant driver of the survival of *Austrovenus* when comparisons of the first 9 d of low tide treatment were conducted ($F_{1,103} = 6.02$, $p = 0.015$). *Austrovenus* that were subjected to a larger 33°C emersion had reduced survival compared to 25°C counterparts ($F_{1,103} = 71.91$, $p < 0.0001$) (Fig. 5A), with Day also being significant ($F_{1,103} = 47.70$, $p < 0.0001$). Regardless of oxygen concentration, emersion stress at 33°C resulted in substantial mortality to eventuate across 9 d. Differences between 25°C hypoxic and normoxic treatments and the interaction between factors ($p = 0.35$) could not be resolved across the initial 9 d (Fig. 5B). Instead, exclusively looking at the full duration of the 25°C challenge (29 d) where day also was a significant predictor of mortality ($F_{1,54} = 18.31$, $p < 0.0001$), the cumulative survival was significant with hypoxic treatments suffering greater mortality ($F_{1,54} = 47.96$, $p < 0.0001$) reaching its median lethal exposure time (LT₅₀) on Day 11, while only a single individual within the normoxic treatment died on Day 27 (Fig. 6).

3.3. Portobello TMDO extreme summer anomaly

The distribution of DO anomalies for 2 extreme summers were normally distributed but offset in opposing directions in relation to the 65 yr baseline (Fig. 7). During DJF 2017–2018, 88.9% of the days were below the average TMDO compared to 13.3% of days in DJF 1963–1964 ($p < 0.0001$).

4. DISCUSSION

The data and results of the current study demonstrated the sublethal and lethal effects of combined hypoxic and thermal stress on siphon activity, respiration and survival of the critical estuarine bivalve *Austrovenus stutchburyi*. A lethal emersion thermal threshold was surpassed at 33°C for treatments exposed to both hypoxic and normoxic treatments, suggesting that extreme thermal events such as heatwaves, which are increasing in frequency in the

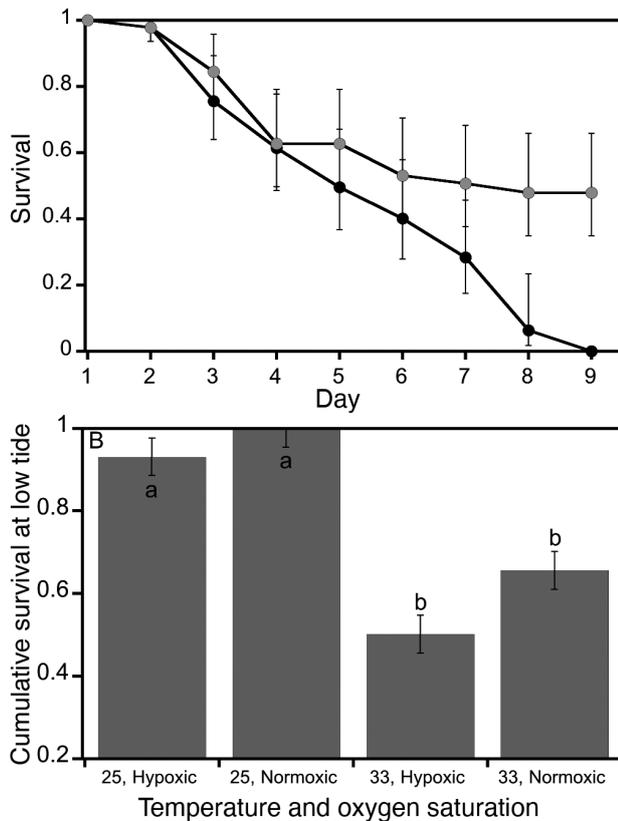


Fig. 5. Survival of cockles *Austrovenus stutchburyi* during exposure to a 9 d repeated stressor of low tide emersion across treatments ($n = 3$ replicates per treatment). (A) Mean survival probability with a 95% CI ($n = 3$ replicates per treatment). Grey and black circles represent normoxic and hypoxic treatments respectively under 33°C emersion. (B) Mean \pm SE cumulative survivorship of individuals immersed in 25°C hypoxic or normoxic water and subsequently exposed to 25°C or 33°C low tide conditions. Treatments not connected by the same letter were significantly different ($p < 0.05$) based on a Tukey's HSD

study system, could have substantial detrimental effects on *Austrovenus* populations and their vital ecosystem functions and services. Further, the ability of bivalves to survive heat and desiccation of midday low tide events was much reduced when they were exposed to even minor heat and hypoxia stress when immersed. These results have important implications for our understanding the combined effects of warming and reductions in oxygen saturation, evident from long-term temperature records, as well as oxygen depletion from eutrophication, on *Austrovenus* specifically, and bivalve populations in coastal seas more generally.

In highly dynamic systems such as estuaries that experience fluctuating environmental conditions over small temporal and spatial scales, intensification of multiple extremes in physical conditions can strongly

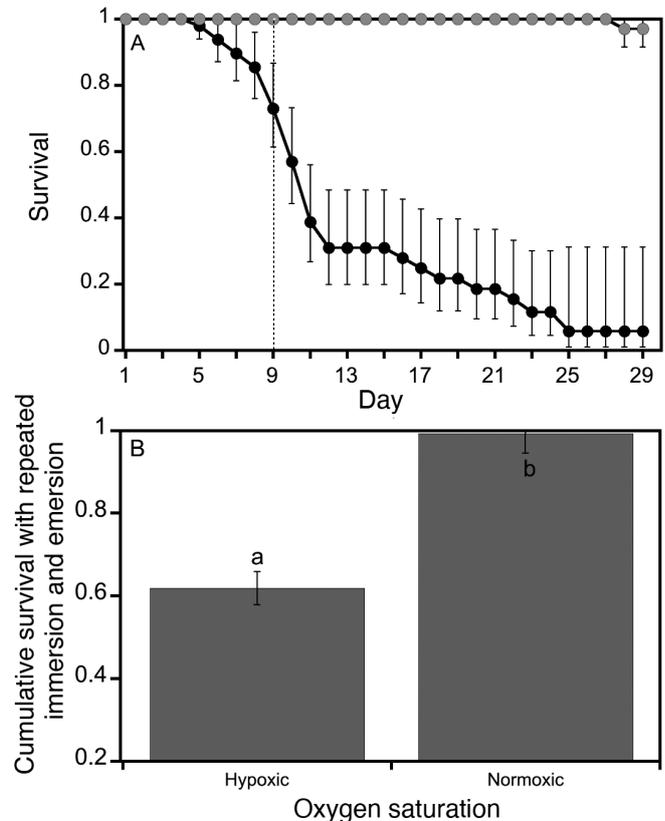


Fig. 6. Survivorship of cockles *Austrovenus stutchburyi* to repeated 25°C immersion and subsequent 25°C low tide emersion. (A) Survival probability across the duration of the experiment with a 95% CI ($n = 3$ replicates per treatment). Grey and black circles represent normoxic and hypoxic treatments respectively. Dashed vertical line represents Day 9 when cumulative survival was calculated (see Fig. 5). (B) Mean \pm SE cumulative survivorship across the full 29 d experiment. Treatments not connected by the same letter were significantly different ($p < 0.05$) based on a Tukey's HSD

influence survival of key ecosystem engineers (Gaines & Denny 1993, Rutger & Wing 2006, Schliehman et al. 2022). Intertidal bivalves in particular are subject to multiple environmental 'extremes' including long duration exposures to low salinity events (Rutger & Wing 2006, Wing & McLeod 2007, McLeod & Wing 2008), low salinity combined with heat stress during MHWs (Domínguez et al. 2021) and exposure to high nutrient concentrations and hypoxia (Salmond & Wing 2022). Though bivalves are resilient to strong fluctuations in physical conditions, multiple extremes can result in tipping points for the population if their effects combine in an additive or synergistic fashion (McLeod & Wing 2008, Salmond & Wing 2022).

Our analysis of the temporal trends in temperature and oxygen saturation in the Portobello Marine Labo-

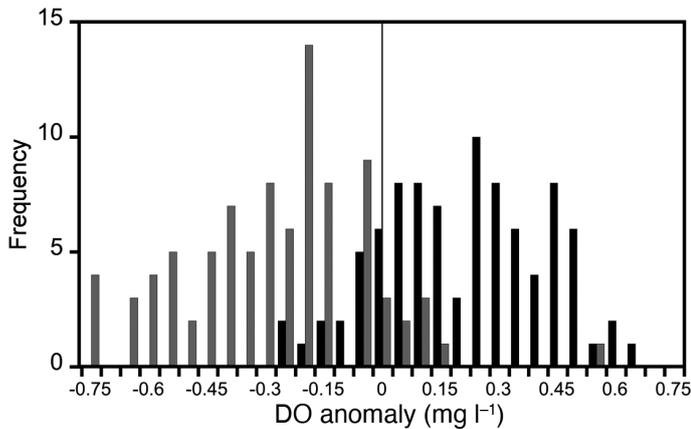


Fig. 7. Frequency distribution of daily theoretical dissolved oxygen (DO) anomaly between 1 December and 28 February as deviations away from the daily mean DO of the 65 yr Portobello Marine Laboratory SST time-series. 1963–1964 represents a cold summer (black bars) while 2017–2018 represents a coupled atmospheric–marine heatwave (grey bars). Positive and negative values represent an increase and decrease in measured oxygen saturation, respectively

ratory time series provide support for the idea that there are long-term shifts in the baseline temperature and the dissolved oxygen saturation point, as well as increased probability of extreme events. These environmental data provided important empirical context for the experiments on responses of *Austrovenus* to combined stressors. As coastal SSTs around south-eastern New Zealand warm, with associated declines in oxygen solubility and MHWs becoming more frequent (Salinger et al. 2019), the risk of intertidal organisms reaching their upper limit of thermal tolerance is expected to rise (Madeira et al. 2012). Accordingly, an increased occurrence of bivalve MMEs is expected, altering species distributions, ranges and community structure, as has been observed globally. For example, increased frequency of MHWs over a 10 yr period is believed to be the mechanism behind a population decline of a subtropical bivalve which has had detrimental effects on customary and commercial harvests (Carneiro et al. 2020). Similarly, the occurrence of midday low tides and increasing aerial temperatures is the dominant predictor for sublethal and lethal responses of mussels and limpets on North American intertidal rocky shores (e.g. Helmuth et al. 2006). For example, in June 2019, when individual mussels (*Mytilus californianus*) in Bodega Bay, California, USA, were exposed to 37°C at low tide following a record-breaking heatwave, it caused the largest MME in 15 yr (Cagle 2019).

MMEs of wild populations of intertidal and shallow subtidal mussels in New Zealand have also been

observed following similar patterns in heat stress to those that *Austrovenus* were exposed to in the current study. For example, 3 d of aerial temperatures averaging 31°C (max. 36.9°C, highest weekly average 23°C in 2001–2004) in Christchurch during January 2005 saw 35.4% of the local population of *Perna canaliculus* perish (Petes et al. 2007). Similarly, low spring tides and hot dry aerial temperatures were speculated to have triggered a *P. canaliculus* MME in north-western New Zealand in February 2020, killing half a million individuals (Graham-McLay 2020). Recurrent moderate heat stress during both of these events rather than a single extreme heat event is a probable mechanism for the mass die-offs as Seuront et al. (2019) demonstrated experimentally, with reduced survivorship of the blue mussel *M. edulis*.

Similarly, lowered resilience of *Austrovenus* to infection as a result of thermal stress due to exposure to high aerial temperatures coinciding with afternoon spring low tides is hypothesised to have contributed to the mortality and stress observed within *Austrovenus* populations in Whangateau Harbour, New Zealand, in mid-February 2009. The mortality event disproportionately affected adults (≥ 30 mm) with 80–84% mortality compared with 60% for the total *Austrovenus* population (Tricklebank et al. 2021). Furthermore *Austrovenus* collected during the 2009 and subsequent smaller 2014 mortality events identified many individuals undergoing gonadal resorption/degeneration (Tricklebank et al. 2021). Consequently, species that spawn in late spring and/or summer may have increased susceptibility to heat stress as energy is diverted into reproduction rather than somatic growth or immune response.

Both global and local observations of population level responses of bivalves to coupled thermal and hypoxic stresses highlight how relatively small changes in the thermal baseline can influence behaviour and metabolism, and trigger tipping-points in population survival. For example, our experiments on *Austrovenus* demonstrate that increasing water temperature from the current summer maximum of 22°C in Otago Harbour to a projected maximum of 25°C under low oxygen conditions of 10% DO resulted in decreased siphon activity, indicating the combined effects of hypoxic and thermal stress and consistent with hypoxia-induced aerobic stress. Under warm high tide conditions at 25°C, reducing DO from 100 to 10% resulted in high mortality, while changes in DO to 10% at 22°C had a similar effect on survival as a reduction to 20% DO at 25°C. Low tide emersion at 33°C initiated earlier onset of mortality than 25°C

emersion, and importantly chronic preconditioning to hypoxia when immersed at high tide initiated much earlier onset of mortality when *Austrovenus* was exposed to 33°C at low tide. Comparably, *Austrovenus* preconditioned in the same water conditions but subjected to 25°C emersion had significantly lower mortality rates across the first 9 d of the experiment, thus indicating that a low tide thermal tolerance was surpassed between 25°C and 33°C. Importantly, individuals preconditioned to chronic hypoxic stress had greater associated cumulative mortality than those exposed to normoxic treatments in 25°C emersion.

The observed patterns demonstrate how the addition of hypoxic stress can lower thermal tolerances which for many intertidal marine organisms can quickly be exceeded in a warming coastal zone (Madeira et al. 2012). For example, European cockles have upper thermal limits similar to the 33°C emersion used in the current study. Specifically, upper thermal limits of 29°C to 31°C exist for *Cardium edule*, and *C. tuberculatum* and 33°C to 35°C for *C. glaucum* (Ansell et al. 1981). In addition, the aragonite composition of *Austrovenus* shells and those of other economically important bivalves give the shells greater thermal conductivity, causing further heat transfer and stress to the bivalve. The observed combined responses to lowered DO and warming was indicative of a synergistic effect of stressors on survival and narrowing of the thermal niche. These results are consistent with the reported general physiological responses of bivalves to hypoxia and thermal stress.

Below a critical oxygen concentration (PO_2), an organism loses the ability to oxyregulate and shifts to oxyconformity (Pörtner 2010). Intertidal species often live close to their upper thermal tolerance, so small shifts in the thermal baseline can result in large influences on survival (Hofmann & Todgham 2010). Heat stress increases an organism's PO_2 and causes the thermal tolerance to narrow, influencing growth, survival and the accumulation of intermediary metabolic products (Wu 2002). The change was visible in the patterns of survival for *Austrovenus*, whereby moving from 22°C, 10% DO to 25°C, 20% DO conditions increased the rate of mortality and reduced the LT_{50} from 14 to 10 d. The results reflect the oxygen- and capacity-limited thermal tolerance (OCLTT) concept by Pörtner (2010). The OCLTT describes the relationship between aerobic scope, oxygen supply and organismal fitness, whereby at a threshold there is a narrowing of the thermal niche, resulting from an imbalance in supply and demand of DO (Pörtner 2010). For example, Artigaud et al. (2014) demon-

strated a similar result whereby the PO_2 of the king scallop *Pecten maximus* increased from 18.3% DO at 10°C to 36.1% at 25°C. Likewise, concurrent exposure to hypoxia and hypercapnia at 22.5°C water compared to 25°C warming alone reduced the LT_{50} of *P. maximus* (Götze et al. 2020), also indicating a narrowing of thermal niche and synergistic response to multiple stressors.

Reoccurring exposure to 25°C emersion and unfavourable immersion conditions (hypoxic 25°C water) reduced the physiological resilience of *Austrovenus*, with cumulative mortality increasing drastically between Days 9 and 12. The result highlights important nonlinear biological responses to the combined exposure to stressors. The resolution of an upper thermal threshold, over which mass mortality may occur, indicates a tipping point into an alternate state if critical species in a system such as filter feeding bivalves were temporarily or permanently lost from an estuarine system. During MMEs, proliferation of bacteria and pathogens as well as increases in ammonia concentrations can further stress and damage the soft tissues of surviving animals which can result in a positive feedback on mortality rates (de Zwaan et al. 2002). In these circumstances within enclosed bays and inlets the residence times of water, clearance rates of suspension feeders and uptake rates of nitrogenous wastes by phytoplankton (Jones et al. 2017, O'Connell-Milne et al. 2020) play significant roles in how bivalve populations respond to combined heat and hypoxic stressors (Diaz & Rosenberg 1995).

An organism's response to reoxygenation following low DO is also important for understanding the mechanisms of recovery and thresholds for bivalves' tolerance to hypoxia. Tolerance can vary depending on proximity to their upper thermal limit and environmental temperature (e.g. Madeira et al. 2012) and the physiological hysteresis and recovery of a system. Within the current study, *Austrovenus* were likely to shift into a state of anaerobiosis both when submerged in hypoxic water (10 and 20% DO) and when emersed on the sediment. Bivalves have 2 general responses to emersion from the water. They can either seal their 2 valves shut and enter an anaerobic state and accumulate nitrogenous waste or they can remain aerobic and periodically gape, risking desiccation and predation. Gaping at the sediment surface is a phenomenon observed during mortality events of clams and cockles alike when individuals were moribund (Tricklebank et al. 2021).

In the current study, when emersed, individuals were observed gaping more commonly under 33°C

conditions than 25°C. The lack of sufficient recovery time between emersion and immersion under hypoxia and warming within the current study suggests that exposure to multiple stressors in quick succession resulted in increased mortality, while recovery in normoxic water from 25°C emersion proved to be sufficient for survival. Emersion during the current study was conducted on 30 mm deep sediment, allowing for partial burial of the *Austrovenus*. Under normal environmental conditions, *Austrovenus* would often be more fully buried in sediments which are often water-saturated and therefore partially buffered from extreme aerial temperatures (O'Connell-Milne et al. 2020). The experimental design used for emersion in the current study simulated partially and surfaced individuals, a phenomena that is commonly observed following the thinning of the oxic sediment layer, or infection by parasites (O'Connell-Milne et al. 2016). Further caveats to the current study include the possibility that mortality rates were underestimated relative to those in natural conditions, where proliferation of pathogens and ammonia may accentuate a MME (de Zwaan et al. 2002).

The results of the current study provide important evidence for how multi-stressor conditions and increases in frequency of extreme temperature and hypoxic events influence the survival of the critical New Zealand bivalve *Austrovenus stutchburyi*. Temperatures used in the present study align with future extreme conditions or chronic MHW events for New Zealand. Extreme heatwave events such as the one observed in 2017–2018 (Salinger et al. 2019) demonstrate that DO becomes reduced and that shallow bodies of water such as estuaries are particularly susceptible to extreme temperature fluctuations. The lack of recovery time and repeated exposure to 33°C emersion followed by re-immersion in warm 25°C waters resulted in a synergistic effect of multiple stressors on survivorship of *Austrovenus*. The results underscore the importance of understanding how *Austrovenus* and other key species respond to multiple stressor environments and extreme events and how preconditioning under stressful conditions lessens the probability of surviving a thermal challenge during low tide. Observations of long-term changes in SST, associated reductions in oxygen solubility and increasing frequency of marine and terrestrial heatwaves indicate that *Austrovenus* populations will become increasingly vulnerable to environmental change. As the frequency and duration of MHWs and associated low oxygen events increase, understanding and mitigating biological responses of *Austrovenus* will be of critical impor-

tance to maintaining healthy coastal ecosystems in New Zealand.

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