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THE EFFECTS OF ARAGONITE SATURATION STATE ON HATCHERY-REARED LARVAE OF THE GREENSHELL MUSSEL *PERNA CANALICULUS*

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ABSTRACT The major cultured mussel species *Perna canaliculus* is now supported by hatchery production, providing the opportunity to explore and optimize environmental parameters to enhance production. Other cultured bivalve larvae have demonstrated performance that is directly correlated to the aragonite saturation state (Ω_{ar}) of their tank water, with low or undersaturated water being detrimental and artificially elevated Ω_{ar} enhancing productivity. Trials were, therefore, designed to specifically explore Ω_{ar} sensitivity in preveliger (0–2 days old, prodissoconch I = "PD1") and veliger (2–21 days old, prodissoconch II = "PD2") stages of *P. canaliculus* separately. For the PD1 experiment, commercial incubation tanks (control Ω_{ar} 1.9) were modified to target Ω_{ar} 0.5 or 0.8 by elevating pCO_2, or 2.9, 4.5, and ~7 by the addition of sodium carbonate. In the control environment, 72.8% ± 2.9% of fertilized eggs formed viable "D" veligers within two days; an increased yield of 82.6% ± 3.8% in Ω_{ar} 4.5 was found to be nonsignificant. In comparison, only 12.7% of the Ω_{ar} ~7 and <1% of the Ω_{ar} 0.5 and 0.8 eggs attained the veliger stage, with the remaining underdeveloped or malformed. By 2 days postfertilization, reactive oxygen species were significantly elevated in the undersaturated treatments, whereas DNA damage, lipid hydroperoxides, and protein carbonyls were significantly higher in the Ω_{ar} 0.5 and ~7 treatments. Antioxidant enzyme levels were significantly lower in these extreme treatments, whereas Ω_{ar} 4.5 larvae showed elevated superoxide dismutase, glutathione reductase, and peroxidase levels. Carry-over effects persisted when veligers were transferred to control conditions, with no net recruitment from undersaturated Ω_{ar}, 29.4% of eggs surviving to pediveliger under control conditions, compared with 33.2% following Ω_{ar} 4.5 exposure or 1.9% from Ω_{ar} ~7. In the PD2 veliger trial, linear shell growth halved in undersaturated water, but was unaffected by elevation of Ω_{ar}. Mortality rate was consistent across all treatments, suggesting relative resilience to different Ω_{ar}. It is recommended that hatcheries trial Ω_{ar} 4–4.5 enrichment in preveliger incubation water to improve yield and minimize oxidative stress. Preveliger stages present a potential survival bottleneck, and focused research exploring sensitivity to near-future ocean acidification is, therefore, needed.

KEY WORDS: *Perna canaliculus*, mussel, larvae, trochophore, veliger, aragonite saturation state, ocean acidification, hatchery, oxidative stress

INTRODUCTION

Increased incursion of high pCO_2, low pH upwelling seawater along the Pacific NW coast of the United States has led to well-documented impacts on hatchery bivalve production (Barton et al. 2012, Barton et al. 2015). A key remedial approach has been to artificially elevate pH and carbonate ion concentrations *via* sodium carbonate dosing (Barton et al. 2015)—a strategy also used in recirculating aquaculture systems to buffer declining pH and alkalinity (Furtado et al. 2011). The dosing strategy has also facilitated research examining the potential benefits of enrichment beyond baseline oceanic levels for cultured larvae. Waldbusser et al. (2015) have subsequently established that the calcium carbonate saturation state (aragonite: Ω_{ar} or calcite: Ω_{ca}) substantially explains performance differences in cultured mussel (*Mytilus galloprovincialis*) and oyster (*Crassostrea gigas*) larvae, rather than individual carbonate system components, including pH and pCO_2. Under hatchery conditions, Barton et al. (2012) correspondingly found that veliger growth reduction was directly correlated with Ω_{ar} experienced during the early larval stages. Culture medium enhancement trials have, therefore, tended to focus on Ω_{ar} manipulations to describe animal sensitivity to carbonate system changes. Reduction of Ω_{ar} below current mean oceanic levels (~2–2.5) is usually achieved by increasing pCO_2, to simulate increasing ocean acidification (Riebesell et al. 2010). These reduced Ω_{ar} environments generally result in reduced net performance (growth, normal development, survival, and recruitment) in larval bivalves (Talmage & Gobler 2009, Barton et al. 2012, Parker et al. 2013, Waldbusser et al. 2015). Conversely, elevated Ω_{ar} environments have supported enhanced performance (Green et al. 2009, Talmage & Gobler 2009, Talmage and Gobler 2010, Waldbusser et al. 2015). For example, Waldbusser et al. (2015) created Ω_{ar} gradients up to ~4 for *M. galloprovincialis* and ~6 for *C. gigas* veligers and observed a progressive decrease in the incidence of abnormality and increase in shell size with Ω_{ar}. Similarly, Talmage and Gobler (2010) described enhanced performance of *Mercenaria mercenaria* and *Argopecten irradians* larvae grown under "pre-industrial" 250 ppm CO_2.

The greenlipped (commercially "Greenshell") mussel *Perna canaliculus* (Gmelin, 1791) supports New Zealand's largest aquaculture

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sector, earning over 250 m USD p.a. in export revenue (Jeffs et al. 2018). The Greenshell mussel industry is now moving toward full domestication, with the construction of a substantial hatchery and commercialization of selective breeding (Camara & Symonds 2014). The mussel *P. canaliculus* naturally inhabits the shallow sublittoral zone (Powell 1979); most wild and farmed populations are, therefore, located within New Zealand coastal areas predicted to experience near-future coastal acidification that exceeds mean ocean acidification rates (Law et al. 2018). Hence, it is of significant value to characterize the response of this keystone species to reduced pH/Ω_{ar} environments, and explore the potential biological and commercial benefits associated with larval development under elevated Ω_{ar} conditions.

During the first few hours of life, a bivalve must undergo fertilization, embryogenesis, and trochophore development and produce its first shell, prodissoconch I. In addition to the obvious vulnerability associated with small size and limited motility, these typically pelagic stages are lecithotrophic and unlikely to possess the ion-regulatory epithelia necessary for acid–base maintenance in suboptimal environments (Parker et al. 2013). The early larva must also maintain a calcium carbonate precipitation rate an order of magnitude higher than in later shelled stages using calcification surfaces that are directly exposed to the surrounding water (Waldbusser et al. 2013). Furthermore, it appears likely that the prodissoconch I shell formation relies on biological induction (Haley et al. 2018), an energetically costly mechanism that imposes a substantial demand on endogenous reserves (Waldbusser et al. 2013). Collectively, these early stages are likely to represent the life stages most sensitive to Ω_{ar} (Barton et al. 2012, Waldbusser et al. 2015). In *Perna canaliculus* aquaculture, these early stages are managed separately, incubating larvae for 2 days postfertilization (pf) in static water containing EDTA (Gale et al. 2016). With the completion of the prodissoconch I shell, the "D"-veligers are then transferred to high-density, flow-through tanks and allowed to feed *ad libitum* on microalgae. Larval culture continues until the advanced pediveliger stage is reached, typically 18–21 days pf (Ragg et al. 2010). The current trials were, therefore, devised to explore the effects of a broad spectrum of Ω_{ar} environments in an effort to understand both environmental vulnerability and commercial opportunities to enhance hatchery production. The preveliger (to the end of prodissoconch I) and veliger (to the end of prodissoconch II) stages were examined in separate experiments, to isolate the effects of the Ω_{ar} treatments on larval performance and net recruitment into the juvenile population.

The effects of a carbonate environment on early life stages may extend beyond the duration of exposure because of either the slow manifestation of measurable effects or a latent effect that carries over to the next life stage. For example, Barton et al. (2012) found that low Ω_{ar} exposure in the 0- to 48-h pf hatchery-reared Pacific oyster *Crassostrea gigas* was correlated with later stage veliger growth and pediveliger yield, but did not apparently influence performance in younger larvae. Gimenez et al. (2018) also describe preveliger incubation Ω_{ar} that influences subsequent presettlement survival in *C. gigas* larvae, but note the effects varied dramatically between cohorts. Similarly, Hettinger et al. (2012) established that the Olympia oyster *Ostrea lurida* larvae exposed to reduced pH seawater demonstrated reduced postsettlement growth, even when juveniles were returned to control pH conditions.

In a fluctuating environment, carry-over effects may be of greater importance to net performance than the acute effects experienced when exposed to challenging conditions (Barton et al. 2012, Hettinger et al. 2012, Gimenez et al. 2018). The present trials, therefore, included an assessment of the subsequent performance when the juveniles were returned to control conditions. In addition, carbonate manipulation (notably simulated ocean acidification) has been associated with oxidative damage (e.g., Tomanek et al. 2011, Matoo et al. 2013). To further elucidate the underpinning mechanisms associated with stress, resilience, or enhanced performance, the appearance of reactive species was monitored in embryos and early veligers, as well as the levels of key antioxidant enzymes and indicators of oxidative damage to organic macromolecules.

METHODS

Experimental Design

Two separate experiments were conducted to specifically isolate the effects of environmental pH/Ω_{ar} manipulation during the first larval shell formation (0–48 h pf = prodissoconch I = "PD1 trial") and the veliger shell development stage (2–21 days pf = prodissoconch II = "PD2 trial"). Both trials examined the acute effects on the larvae and carry-over effects when the juveniles returned to a control environment. The PD1 trial, therefore, included a 3-wk veliger assessment period, whereas the PD2 trial was extended to 10 days postsettlement (Fig. 1).

Seawater was either left unmodified (Ω_{ar} ~2 = control treatments) or adjusted to an approximate Ω_{ar} of ~0.5 and 1.0 by elevating pCO_2, or enriched with sodium carbonate solution to Ω_{ar} ~3, 4, or 7. The latter treatment was chosen as an extreme, likely to result in suboptimal performance because of uncontrolled carbonate deposition (G. Waldbusser, personal communication). Endpoint modification was determined by continuous measurement of free pH (EcoSense EC1030A, YSI, Yellow Springs, OH; calibrated using Merck Hanna pH standards), with target values estimated by CO2calc (v4.0.9; Robbins & Kleypas 2011) using dissociation constants established by Mehrbach et al. (1973) and refitted by Dickson and Millero (1987), using a mean total alkalinity (A_T) of 2,274 μmol kg^{-1} previously measured by Ericson (2010). Carbonate system parameters, including Ω_{ar} and total pH, were subsequently verified by taking 1 L water samples, fixed with 100 μL saturated mercuric chloride, for assessment of total dissolved inorganic carbon (DIC) and A_T (Currie Laboratory, University of Otago). Dissolved inorganic carbon was determined (±1 μmol kg^{-1}) using a coulometer (UIC model 5011) and SOMMA-style CO_2 extraction system (Dickson et al. 2007; SOP2), and A_T was assessed using closed-cell potentiometric titration (±2 μmol kg^{-1}; after Dickson et al. 2007; SOP3). Both assays were calibrated against certified reference material (A. Dickson, Scripps Institution of Oceanography).

Brood Stock and Spawning

Mussel collection and spawning for PD1 took place in November 2014 (austral spring) and in March 2015 (autumn) for PD2. Mature adult *Perna canaliculus* were carefully removed from aquaculture long-lines in Pelorus Sound (South

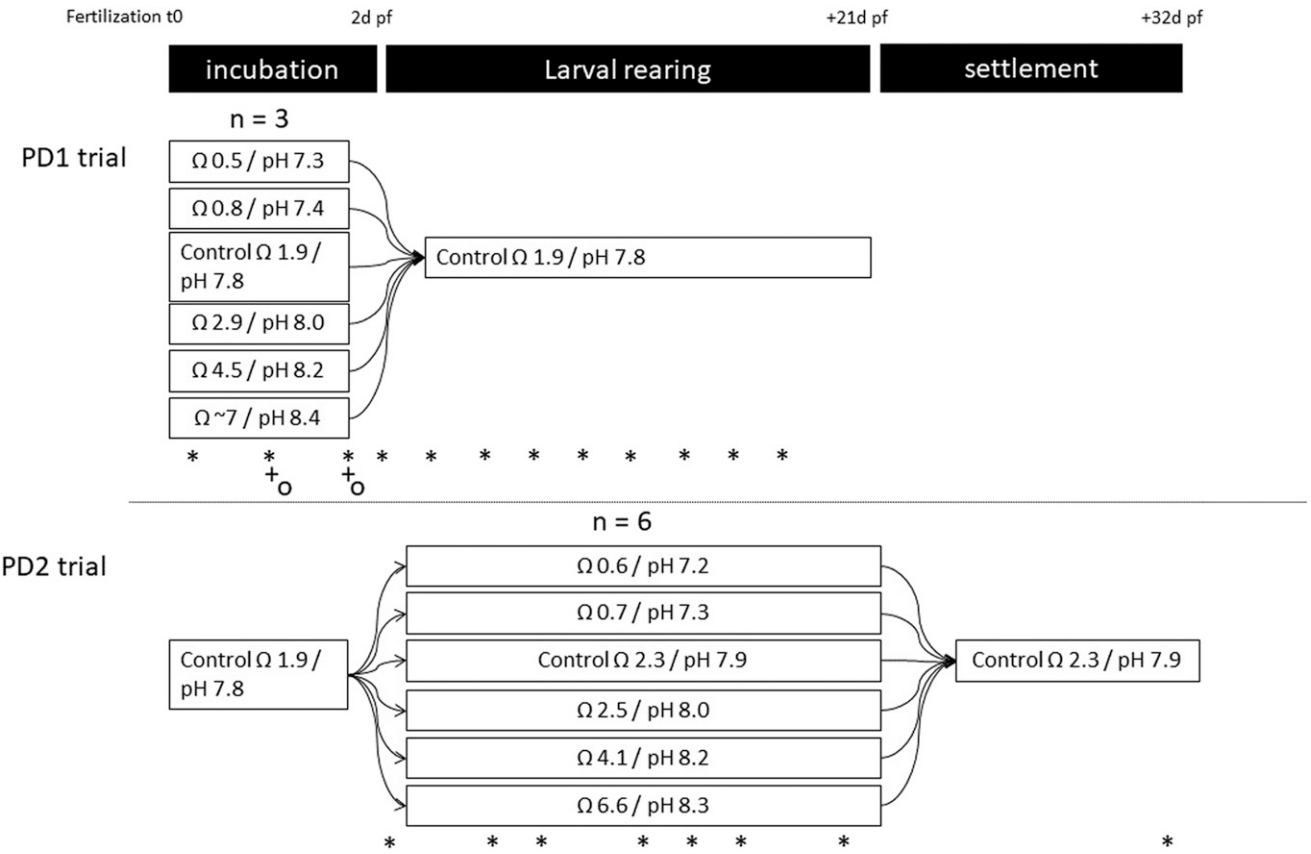

Figure 1. Experimental and sampling design for PD1 and 2 trials. "*" identifies net performance assessment points (population counts, visual assessment, and shell length measurement). "+" identifies sample points for oxidative stress profiling (DNA, lipid, and protein damage markers: 8-OHdG, LP, and protein carbonyls; antioxidant enzymes: SOD, CAT, GR, glutathione peroxidase, and glutathione S-transferase) and dry mass. "o" represents sampling for total ROS. Note expanded time scale (d pf = days postfertilization). Ω and pH refer to mean aragonite saturation state and total pH, respectively.

Island, New Zealand) and transported in humid air (~4 h at 8°C) to the Cawthron Aquaculture Park. Epifauna was removed by mechanical scrubbing, a freshwater rinse followed by 2 min immersion in 0.4% (v/v) sodium hypochlorite, and brief rehydration in seawater. Mussels were then emersed and held in humid air at 16°C for 12 h overnight. Spawning was induced by thermal shock: Mussels were immersed in 1-µm-filtered 34 PSU seawater at 22°C for 2 h and then cycled between 9°C and 22°C at 30 min intervals until spawning was apparent after 2.5–3 h.

Spawning females were rinsed and isolated in 1 L containers with 0.5 L of 8°C seawater, where the negatively buoyant eggs were regularly aspirated and consolidated in a storage vial. Spawning males were wiped dry and "inverted" (siphons directed downward) over a 60-mL collection container in 16°C air, allowing the mussels to eject a concentrated mixture of sperm and residual mantle water. Eggs and sperm from the 30 most fecund individuals of either sex were stored at 4°C for up to 4 h before being pooled and counted. Eggs were added at 1,000 mL^{-1} to 15 L nylon buckets containing 1-µm-filtered, 17°C seawater preincubated with 4 µM EDTA for 24 h (ethylenediamine tetra-acetic acid disodium salt, molecular grade; 5 mM stock prepared in distilled water buffered to pH 8.0), followed by sperm at a ratio of 500 per egg. Fertilization was confirmed by the appearance of polar bodies in greater than 80% of eggs within 30 min. Fertilized eggs were then transferred to 160 L conical nylon tanks containing identical seawater/EDTA (PD2 trial) or a modified Ω_{ar} seawater environment (PD1 trial). Eggs were stocked at 50 mL^{-1} and incubated for 2 days. Air temperature regulation maintained water temperature at 17 ± 1°C, and gentle aeration prevented stratification.

PD1 Trial: Effects of Ω_{ar} on Embryogenesis and Trochophore Development

Manipulation of Water Chemistry

Each Ω_{ar} treatment was established in triplicate 160-L tanks. Reduced Ω_{ar} treatments received CO_2-enriched aeration [using a WMA-5 CO_2 gas analyzer (PP Systems) driving a PLC system admixing CO_2/air via a 3/2-way valve (SMC Pneumatics, type S070)], which equilibrated water to Ω_{ar} ~1; CO_2-saturated seawater was also added at 3 L day^{-1} to create the Ω_{ar} 0.5 treatment. A concentrated sodium carbonate solution was made by dissolving 37 g anhydrous Na_2CO_3 in distilled water at 35°C; 5, 14, and 50 mL of this solution were added slowly to the tank water to create Ω_{ar} 3, 4, and greater than 7, respectively. Small additional volumes were added as required to buffer a gradual pH decline. Temperature and free pH were measured *in situ* in each tank at 2–4 h intervals. Bottle samples were taken from each tank for A_T and DIC analysis immediately before the addition of eggs and following the removal of 2-day-old larvae (Table 1). Total dissolved calcium concentration was also measured in the incubation tank water, 48 h after the addition

TABLE 1.
Carbonate chemistry of 160 L incubation tanks used for the PD1 trial.

Target Ω_{ar}	Free pH	A_T (μmol kg^{-1})	DIC (μmol kg^{-1})	Total pH	Calculated Ω_{ar}	Residual calcium (mg L^{-1})
0.5	7.39 ± 0.006	2,311 ± 4 → 2,311 ± 7	2,359 ± 4 → 2,365 ± 8	7.27 ± 0.01 → 7.27 ± 0.01	0.52 ± 0.01 → 0.50 ± 0.01	430 ± 5.8
1	7.59 ± 0.013	2,298 ± 1 → 2,293 ± 1	2,288 ± 6 → 2,308 ± 3	7.45 ± 0.02 → 7.38 ± 0.01	0.77 ± 0.04 → 0.64 ± 0.01	413 ± 3.3
2 (control)	8.05 ± 0.005	2,287 ± 6 → 2,264 ± 2	2,129 ± 14 → 2,172 ± 5	7.87 ± 0.05 → 7.71 ± 0.01	1.87 ± 0.18 → 1.28 ± 0.03	420 ± 5.8
3	8.19 ± 0.005	2,570 ± 14 → 2,944 ± 35	2,320 ± 12 → 2,745 ± 31	8.03 ± 0.02 → 7.91 ± 0.01	2.86 ± 0.11 → 2.51 ± 0.07	427 ± 3.3
4	8.36 ± 0.003	3,022 ± 3 → 3,816 ± 11	2,633 ± 5 → 3,413 ± 15	8.19 ± 0.01 → 8.13 ± 0.01	4.52 ± 0.05 → 5.00 ± 0.06	417 ± 3.3
7	8.82 ± 0.009	–	–	–	~7	427 ± 3.3
Blank seawater	–	2,305 → 2,307	2039 → 2052	8.10 → 8.08	2.88 → 2.75	410

Fertilized eggs were allowed to develop under these conditions for 48 h to complete embryogenesis and form their first shell. Arrows separate initial from final values, measured after 48 h.

of eggs, by ICP-MS (Hill Laboratories, Hamilton, NZ; protocol: "Saline-ultratrace," APHA 3125 B, 22nd ed. 2012).

Biological Sampling

Visual assessment 18 h pf revealed that most embryos had attained the free-swimming trochophore stage. A 600-mm-diameter plunger was used to gently homogenize the larval population within each tank, and a 30-L sample was removed. Larval counts were established in triplicate volumetric subsamples before dividing the sample into triplicate oxidative stress samples (DNA, lipid, and protein damage markers: 8-hydroxydeoxyguanosine (8-OHdG), lipid hydroperoxides (LP), and protein carbonyls; antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase, and glutathione S-transferase) and a single dry/ash mass sample (Fig. 1, "+" symbols), to provide an indication of net calcification (Ericson 2010). Larvae were concentrated using a 43-μm mesh. The procedure was repeated 48 h pf, when most control treatment larvae had reached the veliger stage. A similar sampling method was also used to obtain samples of ~50,000 individuals 24 and 52 h pf for total reactive oxygen species (ROS) production quantification (Fig. 1, "o" symbols).

Regular visual inspection suggested that larval development had plateaued by 43 h pf; at this point, a formal development assessment was conducted by designating individuals from triplicate 4 mL subsamples as "normal veliger," "pre-veliger," or "abnormal" (deformed shell or unable to retract velum). To avoid the risk of subjective inconsistencies, pre-veliger condition was not further categorized as "normal" or "abnormal." To confirm the development had plateaued, the assessment was repeated 51 and 68 h pf.

It was recognized that some apparently subtle effects of the incubation environment could ultimately have serious longer term implications for the performance of larvae. Carry-over effects on the feeding veliger stages were, therefore, considered by transferring 500,000 individuals from each tank into a corresponding 2.5-L larval rearing tank; these were raised under common conditions for a further 3 wk, as described later for PD2 control treatment. Survival, shell growth, and recruitment to pediveliger were used to compare the latent effects of pre-veliger carbonate treatment.

Biochemical Assessments

Dry mass. Each pre-counted larval sample (approx. 200,000 individuals) was concentrated on a pre-ashed GF/C filter disc (Whatman 25 mm), rinsed with 3.6% ammonium formate (osmotically matched to local seawater), and dried at 60°C for 24 h to establish dry mass (±0.00004 g); filter samples were then ashed at 550°C for 4 h and reweighed to establish inorganic dry mass.

Reactive Oxygen Species

The molecular probe 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA, D399; Invitrogen, Paisley, UK) was used to estimate ROS levels within the larvae. D399 readily permeates cells, where esterases remove acetate groups and activate fluorescence proportional to the concentration of ROS (Anon. 2006). Standard H_2O_2 calibration was, therefore, considered unsuitable, and fluorescence signal was simply standardized by biomass to provide a relative measure of ROS. A

210-µL suspension of larvae was combined with 40 µL of D399/DMSO stock diluted 20× in sterile seawater and incubated in the dark for 1 h before quantification of fluorescence (excitation: 490 nm, emission: 520 nm; PerkinElmer EnSpire plate reader, bottom scan mode).

Oxidative Stress Profile

Larval samples were centrifuged (30 s, 800 rcf) and supernatant seawater removed before snap-freezing. Protein carbonyls and LP were quantified as indicators of the oxidative damage of proteins and lipids, respectively (Fig. 14). Protein carbonyls and LP were extracted and analyzed as described by Gale et al. (2014). Briefly, LP were determined using the ferric thiocyanate method (Mihaljević et al. 1996) adapted for measurement in a microtiter plate reader. Protein carbonyls levels were determined using the 2.4-dinitrophenylhydrazine method (Reznick & Packer 1994) adapted for use with microplates. DNA was extracted using an ISOLATE II Genomic DNA Kit (Bioline, NSW, Australia), following the manufacturer's instructions for standard samples, but with the following modifications. Following the addition of the prelysis buffer, samples were homogenized for 1 min using a Mini-Beadbeater-96 and zirconia/silica beads (Biospec Products, USA). The prelysis was supplemented with 5 mM deferoxamine and 20 mM EDTA, and the lysis buffer with 5 mM deferoxamine. All solutions were deoxygenated by gently bubbling with nitrogen gas for 5 min. The levels of oxidized DNA were estimated by measuring the amount of 8-OHdG present using high-performance liquid chromatography, followed by UV detection of guanine and electrochemical detection (coulometric) of 8-OHdG, as described by Gale et al. (2014).

Total protein was extracted in 100 mM potassium phosphate (pH 7.5) containing 0.1 mM Na_2EDTA, 1% polyvinyl pyrrolidone-40, 1 mM phenylmethylsulfonyl fluoride, and 0.5% TritonX-100 (Sigma-Aldrich). Protein extracts were ultrafiltered (10 kDa molecular weight cutoff), and the semipurified protein was reconstituted in 100 mM potassium phosphate (pH 7.5) as described in Gale et al. (2014) and used for the following assays. Superoxide dismutase activity was measured using the method of Banowetz et al. (2004). Catalase activity was determined using the chemiluminescent method of Janssens et al. (2000). Glutathione peroxidase (GPOX) activity was measured using the spectrophotometric method of Paglia and Valentine (1967). All assays were modified as described in detail in Gale et al. (2014).

PD2: Manipulation of Larval Culture Water 2–21 days pf

Spawning and initial embryogenesis to 2 days pf followed the approach described for the PD1 controls mentioned earlier, using a single 160-L incubation tank. Veliger larvae were then stocked in each of six replicate 2.5-L rearing tanks (500,000 individuals $tank^{-1}$; see Ragg et al. (2010) for design details) which were assigned to one of six Ω_{ar} treatments designed to reproduce the carbonate environments created in PD1. The exception was the extreme "Ω_{ar} 7" treatment, where a slightly reduced target of ~6.5 was used to reduce the risk of spontaneous precipitation. Reduced Ω_{ar} environments were achieved by the continuous dilution of CO_2-saturated seawater in 100 L header tanks, whereas elevated Ω_{ar} required the addition of concentrated sodium carbonate solution (as described earlier). Axenic algal cultures were added to the modified water of each header tank to achieve a mean effluent concentration of 40 cells μL^{-1} [1:2 cell ratio, *Tisochrysis lutea*: *Chaetoceros calcitrans*; Ragg et al. (2010)] and allowed to flow through each rearing tank at 80 mL min^{-1}. Water temperature was maintained at 20°C using air temperature control. Temperature and free pH were measured daily in each tank, and mercuric chloride-fixed water samples were taken on four occasions from each system, as described for PD1, to assess A_T and DIC entering and leaving each larval tank (Table 4).

Biological Sampling

Biological performance assessment and management followed the procedures described by Ragg et al. (2010). In brief, at 2–3 days intervals, the tanks were drained and larvae retained on a screen. Screen mesh size was adjusted to ensure only detritus and dead shells were removed (43–75 µm). Larvae were then resuspended in 1,000 mL of seawater and volumetric subsamples counted to estimate survival. Approximately 200 individuals from each tank were photographed (Olympus C5060 camera mounted on a CK2 inverted microscope, 40× magnification) and shell length determined using image analysis software (ImageJ 1.50i; NIH, USA).

To avoid the compounding effects of diverging population densities and to conform to established best practice for ultradense bivalve larval rearing (Ragg et al. 2010), the resident larval density was standardized to 100 mL^{-1} 9 days pf. Direct estimates of resident population were subsequently corrected for larvae removed for husbandry management or sampling purposes, by assuming the removed animals would experience the same mortality rate as the residual population.

When daily inspection revealed that greater than ~25% of control larvae had attained the settling pediveliger stage (advanced eyespot and active foot), the population was passed through a 178-µm screen to isolate pediveligers (>~215 µm). Pediveligers were counted separately to the residual population and removed from the system. The process was repeated 2 days later and the combined harvest quantified as pediveliger yield (Ragg et al. 2010). In the PD2 trial, a third harvest event was included to ensure all competent individuals had been identified.

On the first day of pediveliger harvesting, a volumetric subsample of 5,000 settling larvae was transferred to a corresponding settlement tank. The settlement system consisted of 8 L cylindrical tanks, each containing 1 m of coconut fiber string to act as settlement substrate (Ragg et al. 2010). The tanks received light aeration, algae, and water flow comparable with the control larval rearing tanks (Ω_{ar} 2.23, pH ~7.9). Settling larvae were allowed 2 wk to form byssus attachment and metamorphose into juveniles (spat). After 13 days, fresh water was added to each tank to induce valve closure, before adding 4% hypochlorite bleach for 10 mins to dissolve the byssus attachment. Spat were then dislodged from the string and tank walls by gentle irrigation and resuspended in a 1,000-mL beaker of seawater for volumetric counting, as described earlier. Net juvenile survival, or "spat yield," was used to compare post-treatment effects following the PD2 veliger treatment trial.

Statistics

Simple one- or two-way analysis of variance was used to detect treatment effects for most response variables,

treating Ω_{ar} as a factor, having transformed data as required to ensure normality and homoscedasticity. *Post hoc* pairwise comparison of means (Tukey's HSD) was used to identify responses that differed significantly from the control treatment ($P \leq 0.05$). Polynomial regression models were fitted to estimate the optimum Ω_{ar} environments required to maximize initial veliger (PD1) and final pediveliger (PD1 and 2) yields. Statistical analyses were performed using R (v.3.3.2) with RStudio (v. 1.0.143) interface (R:Core-Team 2015).

RESULTS

PD1 Trial: Effects of Ω_{ar} on Embryogenesis and Trochophore Development

PD1 Water Chemistry

Carbonate system variables and residual calcium concentrations are summarized in Table 1. Most treatments showed a slight acidosis and corresponding decline in Ω_{ar} during the 48-h incubation (Table 1); this is most apparent in the control and was also observed during the unmodified incubation phase of the PD2 trial. It is suspected that the acidosis was driven by CO_2 from larval and microbial respiration. By contrast, A_T and DIC rise in the Ω_{ar} 4 treatment, driving an increase in calculated Ω_{ar}, may reflect a slower than expected equilibration of added carbonate.

As measured Ω_{ar} deviated from the target levels, all subsequent text will refer to actual starting Ω_{ar} to describe each treatment.

Note that all tanks also contained 4 μM EDTA. Spontaneous precipitation of samples from the Ω_{ar} 7 treatment prevented analysis; Ω_{ar} was, therefore, inferred from potentiometric pH measurements. Values represent mean ± SEM ($n = 3$), reported at the start and end of the 48-h incubation ([Ca] only measured at 48 h). Temperature and salinity remained stable at 16.5 ± 0.2°C and 35.9 ± 0.1 PSU.

PD1 Biological Performance

Treatment had no significant effect on survival 43 h pf ($F_{5,12} = 2.093$, $P = 0.13$; Fig. 2A) but substantially influenced development. Under control conditions (Ω_{ar} 1.9), 72.8% ± 2.9% of eggs developed into apparently normal veliger; this value rose to 82.6% ± 3.8% in the Ω_{ar} 4.5 treatment (Fig. 2A), but the increase was not found to be significant (Tukey's HSD, $P > 0.05$). Development was severely compromised in the extreme treatments, with most larvae displaying physical abnormality in the Ω_{ar} 0.8 and Ω_{ar} ~7 treatments. Larvae reared under Ω_{ar} 0.5 conditions failed to reach the veliger stage (Fig. 2A). Underdeveloped control treatment larvae failed to develop to veliger, even when incubated for a further 15 h ($P > 0.05$; Fig. 2B and C). Further development was, however, apparent in the Ω_{ar} 2.9 treatment, with an initial veliger yield of 70.6% ± 2.8% (Fig. 2A) rising to 77.0% ± 4.2% after 68 h (Fig. 2C); this increase was also found to be nonsignificant ($P > 0.05$). By treating Ω_{ar} as a continuous variable, a quadratic linear model described the (arcsine transformed) 43-h pf veliger yield data very well ($R^2 = 0.90$; Table 2). Peak yield (zero gradient, determined by differential analysis) was predicted to occur at $\Omega_{ar} = 4.03$. Larvae raised under Ω_{ar} ~7 conditions appeared to destabilize and were progressively lost beyond 43 h pf (Fig. 2A–C). Correspondingly, individual dry mass was relatively constant across treatments 18 h pf, but subsequently failed to increase significantly by 48 h pf in the aggressive Ω_{ar} 0.5, 0.8, and ~7 treatments ($P < 0.05$; Fig. 3).

Intracellular ROS concentrations, determined by D399 fluorescence, were statistically similar between treatments and the control (Ω_{ar} 1.9) in samples taken 24 h pf (Fig. 4); only Ω_{ar} 0.8 and Ω_{ar} ~7 levels were significantly different from each other ($F_{5, 12} = 3.685$, $P = 0.03$). By 52 h pf, ROS levels had fallen in control and elevated Ω_{ar} treatments, but remained significantly elevated in low Ω_{ar} treatments ($F_{5, 12} = 21.72$, $P < 0.001$; Fig. 4). DNA and protein damage, quantified as 8-OHdG and protein carbonyl content, respectively, remained stable across treatments and life stage samples 18 and 48 h pf (Fig. 5A and C). The exception was a significant accumulation in the extreme Ω_{ar} 0.5 and ~7 treatments after 48 h. Lipid hydroperoxide levels not only followed a similar pattern, but also notably reduced in the intermediate Ω_{ar} treatments between 18 and 48 h pf (effects of age $F_{1, 24} = 13.21$, $P = 0.001$; Fig. 5B). Defense enzymes showed the reciprocal pattern, with SOD, CAT, GR, glutathione peroxidase, and glutathione S-transferases all being significantly reduced below control treatment levels after 48 h (Fig. 5D–H). Superoxide dismutase, GR, and GPOX showed significant elevation above the control after 48 h in the Ω_{ar} 4.5 treatment (Fig. 5D, F and G).

Despite low or apparently zero veliger yields in extreme treatments (Fig. 2A–C), the large biomass available allowed sufficient veligers to be harvested from all incubation tanks for assessment of carry-over effects. Veligers originating from extreme Ω_{ar} treatments showed rapid attrition (Fig. 6). All individuals previously incubated in Ω_{ar} 0.5 water died within a week. Most larvae previously exposed to Ω_{ar} 0.8 or Ω_{ar} ~7 also

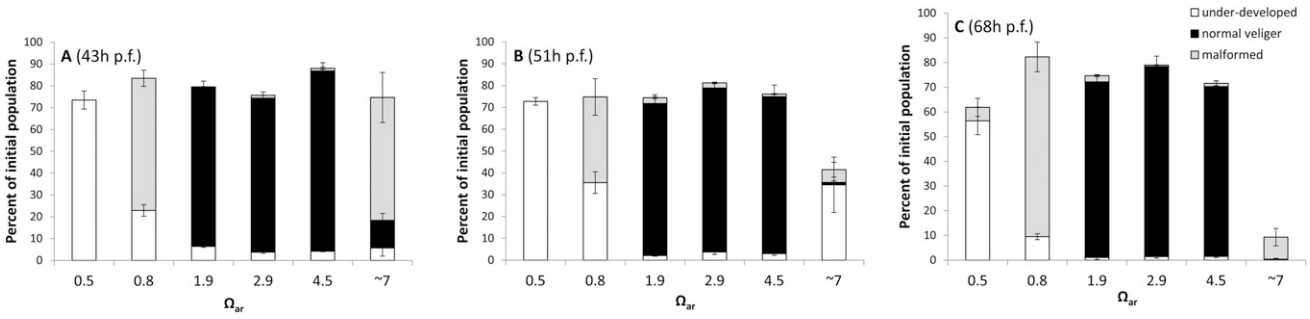

Figure 2. Net survival and proportion of population resembling normal veligers as a percentage of initial number of fertilized eggs stocked in each tank, following (A) 43 h, (B) 51 h, and (C) 68 h of incubation in modified Ω_{ar} seawater.

TABLE 2.

Quadratic regression model fitted to initial veliger yield 43 h pf.

	Regression coefficient	SE	*t*	*P* value
Intercept	–0.422	0.091	–4.63	<0.001
Ω_{ar}	0.817	0.065	12.52	<0.001
Ω_{ar}^2	–0.101	0.009	–11.81	<0.001
Residual	–	0.157 15 df	–	–
Adjusted $R^2 = 0.90$	–	–	–	–

Yield was expressed as a percentage of initial fertilized egg population (arcsine transformed data). Optimum predicted Ω_{ar} (point of zero gradient) = 4.03.

died within 3 days of stocking in veliger rearing tanks, with only 20%–30% of veligers surviving (Fig. 6). Control and moderately elevated treatments (Ω_{ar} 2.9 and 4.5) showed the steady mortality rate typical of commercial *Perna canaliculus* larval rearing (c.f. Ragg et al. 2010), with approximately half of the veligers surviving by 19 days pf (Fig. 6). Similar shell growth rates were observed across all surviving treatments (mean 10.01 ± 0.20 μm day^{-1}), so significant differences in the size of veligers entering the rearing system (3 days pf; $F_{5,11} = 83.08$, $P < 0.001$) were perpetuated to the start of pediveliger harvesting (20 days pf; Fig. 7; $F_{4,9} = 13.4$, $P < 0.001$). When pediveliger yield was expressed as a percentage of population initially added to the veliger tanks (counted 3 days pf; Fig. 8A), the compound effects of reduced growth and survival following Ω_{ar} 0.8 or ~7 treatment were apparent, with a yield of 12%–14% compared with 38%–41% from intermediate treatments. As a result of large within-treatment variability however, differences were nonsignificant ($P > 0.05$). When standardized to original egg population (Fig. 8B), the acute effects experienced during the incubation treatment itself are also accommodated. No net yield occurred in the low Ω_{ar} treatments, and only 1.9% ± 0.6% of eggs yielded pediveligers in the Ω_{ar} ~7 treatment (Fig. 8B). A net control yield of 29.4% ± 5.8% increased to 33.2% ± 3.7% in the Ω_{ar} 4.5 treatment (Fig. 8B), but the increase was not found to be significant ($P > 0.5$). A quadratic regression model fitted the pediveliger yield data well (Fig. 8A, Table 3; $R^2 = 0.69$), predicting maximum yield at $\Omega_{ar} = 3.98$.

PD2 Trial: Effects of Ω_{ar} Veliger Development

Water Chemistry

Although water residence times were relatively short in the continuous flow system, some influence on carbonate chemistry was apparent from the decline in pH_T and Ω_{ar} between "blank" supply seawater and water entering the veliger tanks (Table 4). An increase in pH_T and Ω_{ar} occurred within all veliger tanks, being most apparent in the acidified treatments (Table 4). Treatments have subsequently been identified by the midpoint between in- and out-flowing Ω_{ar} (0.6, 0.7, 2.3 [control], 2.5, 4.1, 6.6).

PD2 Biological Performance

Seawater Ω_{ar} did not appear to significantly influence veliger survival, with all treatments showing steady mortality rates for the first 2 wk of life, plateauing to a final mean survival of 30.6% ± 2.1% of 2 days pf population surviving 20 days pf, when pediveliger harvesting commenced ($F_{5,30} = 0.439$, $P = 0.818$; Fig. 9). Shell growth was not affected by Ω_{ar} elevation (Fig. 10), with mean rates of 5.9–6.6 μm day^{-1}; however, growth fell significantly in the reduced Ω_{ar} treatments, 4.9–5.0 μm day^{-1} ($F_{5,28} = 6.666$, $P < 0.001$). Net pediveliger yield, standardized by 2 days pf population stocked in the tanks, showed substantial within-treatment variability, with only the Ω_{ar} 0.7 yield being significantly lower than the control ($F_{5,30} = 3.243$, $P = 0.02$; Fig. 11). A quadratic regression model failed to adequately describe the yield data ($R^2 = 0.16$), preventing the estimation of an optimum Ω_{ar} level. No statistical differences were detected between either inorganic (ash) or total dry mass of individual pediveligers produced in the various treatments ($F_{5,22} = 1.386$, $P = 0.268$; Fig. 12). It should, however, be noted that some

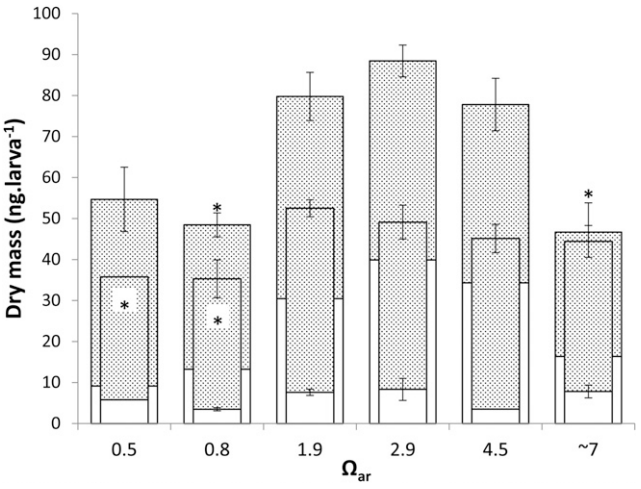

Figure 3. Individual dry mass of larvae sampled at 18 h (narrow bars) and 48 h pf (wide bars). Inorganic (ash) component is represented by the clear area, and organic component by the shaded area. Mean dry mass that differs significantly from the corresponding (18 or 48 h) control is marked * ($P < 0.05$). Bars represent mean ± SEM, $n = 3$.

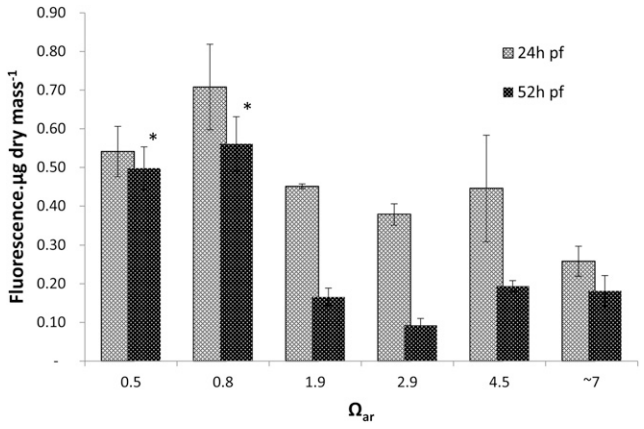

Figure 4. Relative intracellular ROS levels in larvae incubated in a range of Ω_{ar} environments for 24 and 52 h, expressed as fluorescence of the 'D399' molecular probe standardized to larval dry mass. Mean ± SEM, $n = 3$. Signals significantly different to their corresponding control are marked * ($P < 0.05$).

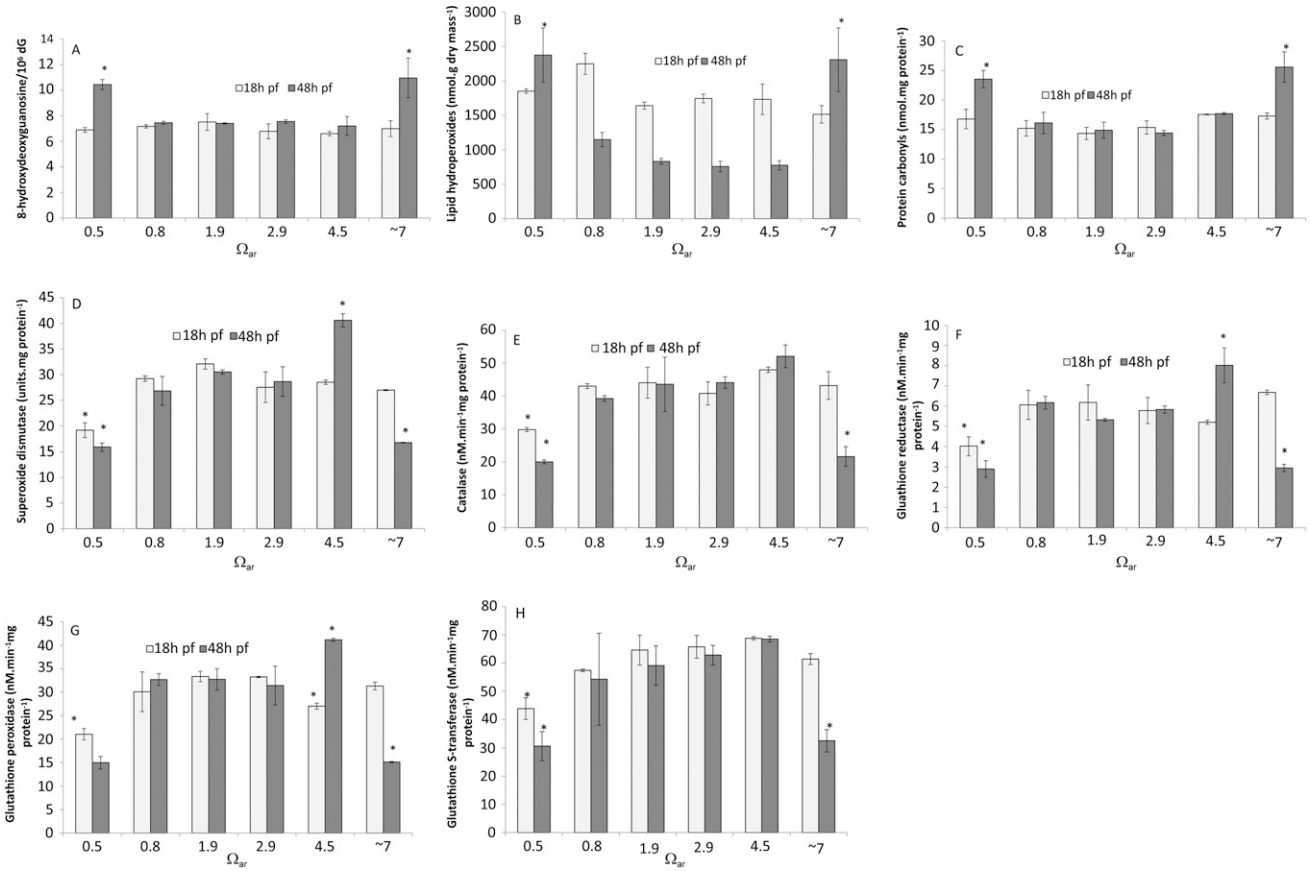

Figure 5. Indicators of oxidative damage and defense in larvae raised for 18 or 48 h in modified Ω_{ar} water. (A) 8-OHdG indicating DNA damage; (B) LP, standardized against larval dry mass; (C) protein carbonyls; (D) SOD; (E) CAT; (F) GR; (G) GPOX; and (H) glutathione S-transferase, standardized against protein mass. Mean ± SEM, $n = 3$. Signals significantly different from their corresponding control are marked * ($P < 0.05$).

tanks failed to produce sufficient larvae for dry matter assessment, resulting in reduced replication and statistical power. Net survival following pediveliger transfer into an unmodified seawater setting system is shown in Figure 13. Juveniles developing from larvae raised in Ω_{ar} 0.7, but not Ω_{ar} 0.6, showed significantly reduced survival ($F_{5,30} = 4.345$, $P = 0.004$; Fig. 13).

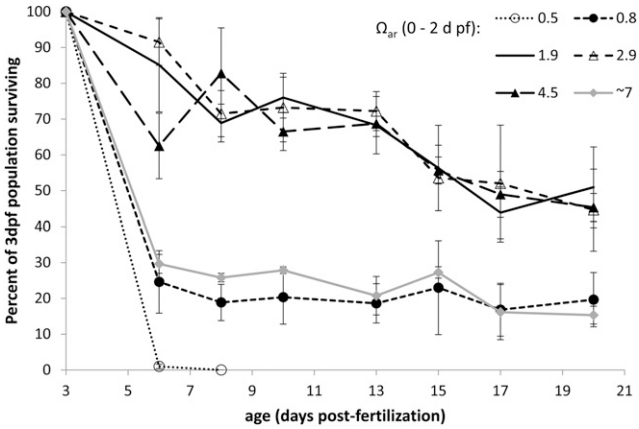

Figure 6. Relative survival of veligers raised in control conditions, following incubation in different Ω_{ar} treatments (mean ± SEM).

DISCUSSION

Water Chemistry Manipulation

Dosing with either CO_2-saturated seawater or concentrated sodium carbonate solution was effective in modulating carbonate chemistry to achieve a range of Ω_{ar} environments. In the large water tanks used for the PD1 incubation, unintended pH decline was also apparent. During an initial 24-h incubation without embryos, pH_T fell from 8.10 to 7.87 and then again to 7.71 following 48 h of incubation with embryos (Table 1). The addition of EDTA may be partially responsible for the initial decrease, but it is suspected that microbial and larval metabolism accounted for most of the subsequent decline. Modified Ω_{ar} treatments also tended to decline, but to a lesser extent than the control seawater; by contrast, Ω_{ar} 4.5 increased to 5.0 during embryo occupancy possibly because of a slight overcorrection during sodium carbonate adjustment. The continuously flowing rearing system used in the PD2 trial was more stable, with only the reduced Ω_{ar} treatments tending to rise significantly within the larval tanks (Table 4) possibly because of progressive equilibration with air bubbling through these tanks.

Effects of Ω_{ar} on Early Larval Stages (0–48 h pf)

Embryogenesis, trochophore development, and initial shell formation were sensitive to the Ω_{ar} environment. Development

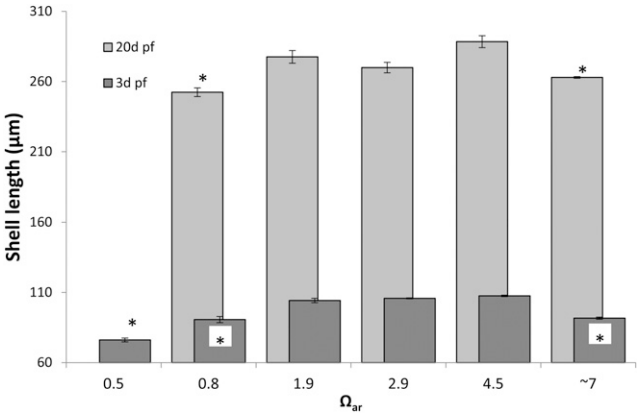

Figure 7. Mean shell length of veligers raised in control conditions, following incubation in different Ω_{ar} treatments. Bars represent mean ± SEM 1 day after stocking in the veliger rearing system (3 days pf) and immediately before pediveliger harvesting, 20 days pf. "*" represent values significantly different from their corresponding control treatment (Tukey's HSD, $P < 0.05$).

was retarded in the undersaturated Ω_{ar} 0.5 and 0.8 treatments; larvae either failed to form a shell or became malformed and apparently nonviable (Fig. 2). Most embryos exposed to the extreme Ω_{ar} ~7 also tended to have malformed shells, showing signs of hypercalcification. These aggressive Ω_{ar} treatments showed little sign of mass gain after 18 h pf (Fig. 3) and began to destabilize by 68 h pf (Fig. 2C). More than 70% of fertilized eggs incubated in the control (Ω_{ar} 1.9) or moderately elevated Ω_{ar} (2.9–4.5) formed normal prodissoconch I shells, becoming apparently viable veligers (Fig. 2). The relative differences between these treatments were fairly subtle; the statistical design ($n = 3$) was, therefore, insufficiently powerful to identify potential significant differences. The mean yield of veligers, however, increased by 13% by 43 h pf if Ω_{ar} was increased to 4.5% and by 8% if veligers were reared in Ω_{ar} 2.9 for 51–68 h (Fig. 2). Larvae incubated in Ω_{ar} 2.9 were also 11% heavier than controls by 48 h pf (Fig. 3). Gray et al. (2017) demonstrated a similar correlation between Ω_{ar} and shell length 2 days pf in *Mytilus californianus* larvae.

Mode of Action

Under control conditions, similar antioxidant enzyme levels (SOD and GPOX) have been described in the eggs (Gale et al. 2014) and 18 h and 43 h pf larvae of *Perna canaliculus* (Fig. 5A and G, present study), suggesting that antioxidant protection is reliant on maternal transfer. For clarity, a simple outline of the mitigation role played by these antioxidants in stress homeostasis is presented in Figure 14. The incubation environment subtly influenced antioxidant enzyme levels of embryos within the first day of life, with significant lowering of SOD, CAT, GR, glutathione peroxidase, and glutathione S-transferase in the most undersaturated Ω_{ar} treatment (18 h pf; Fig. 5D–H). The 18-h pf embryos did not, however, show signs of elevated oxidative damage to DNA, lipids, or proteins, indicated by 8-OHdG, lipid hydroperoxide, and protein carbonyl levels that resembled the control in all treatments (Fig. 5A–C). By the end of the second day (43–52 h pf) however, protective enzyme levels were markedly reduced under extreme Ω_{ar} 0.5 and ~7 conditions (Fig. 5D–H) and indicators of oxidative damage were elevated (Fig. 5A–C), along with intracellular ROS concentration (Fig. 4). Tomanek et al. (2011) suggest that elevated pCO_2 may cause oxidative stress by direct increase of ROS *via* reaction with peroxynitrite, or indirectly by lowering intracellular pH and reducing the efficiency of the electron transport chain and generating ROS. An elevation of Ω_{ar} and pH may, therefore, mitigate these impacts. In the present study, reduced antioxidant enzyme activity appears to precede the elevation of ROS and detectable oxidative damage, suggesting that an impairment of antioxidant synthesis or function may exacerbate the direct elevation of ROS. Interestingly, a significant elevation in antioxidant enzymes GR and GPOX was detected following 43 h incubation in Ω_{ar} 4.5. The mechanisms supporting these increased levels remain to be elucidated, but may relate to energy sparing in the facilitated shell-growing environment. Calcifiers use macromolecules and membrane transporters to enhance shell formation rates beyond passive precipitation rates; however, both mechanisms are energetically costly (Waldbusser et al. 2013). In young *Mytilus californianus* veligers exposed to undersaturated Ω_{ar} conditions, the energetic burden was exacerbated by a delay in the

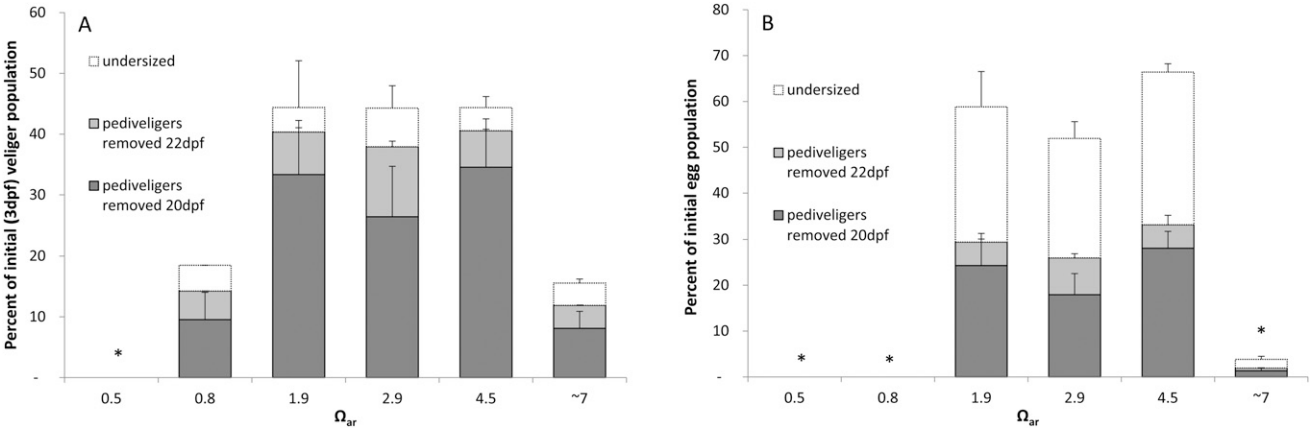

Figure 8. Net survival to pediveliger stage following 2 days of incubation in modified Ω_{ar} water and subsequent rearing under control conditions. Pediveligers are isolated based on size (>~215 µm; Ragg et al. 2010), by removal on a 178-µm mesh 20 and 22 days pf. Values are expressed as (A) percentage of veligers stocked in tanks (3 days pf) and (B) percentage of fertilized eggs initially added to each incubation tank. Mean ± SEM, $n = 3$. Net yields significantly different from their corresponding control are marked * (Tukey's HSD, $P < 0.05$).

TABLE 3.

Quadratic regression model fitted to final pediveliger yield.

	Regression coefficient	SE	t	*P* value
Intercept	–0.0354	0.092	–0.384	0.707
Ω_{ar}	0.410	0.069	5.898	<0.001
Ω_{ar}^2	–0.051	0.009	–5.436	<0.001
Residual	–	0.157 13 df	–	–
Adjusted $R^2 = 0.69$	–	–	–	–

Data were expressed at a percentage of veligers stocked 3 days pf (Fig. 8A; arcsine transformed data). Optimum predicted Ω_{ar} (point of zero gradient) = 3.98.

onset of feeding (Gray et al. 2017); the reverse may, therefore, apply under elevated Ω_{ar}. Adult bivalves (*Crassostrea virginica* and *Mercenaria mercenaria*) also showed signs of oxidative stress following acute exposure to elevated pCO_2 (800 ppm); however, most parameters had returned to baseline by the end of a two-month exposure (Matoo et al. 2013), suggesting resilience may increase in postlarval stages. The bivalve *C. virginica* was, however, shown to upregulate antioxidant proteins, including SOD, when challenged with a more extreme pCO_2 of ~3,500 ppm (Tomanek et al. 2011).

Veligers removed from modified incubation environments and transferred to control seawater rearing conditions failed to compensate for perturbations experienced during the first 2 days of life. Larvae previously exposed to undersaturated (Ω_{ar} 0.5–0.8) or hyper-saturated conditions (Ω_{ar} ~7) displayed elevated mortality; all Ω_{ar} 0.5 larvae died, whereas net survival to pediveliger was substantially reduced in Ω_{ar} 0.8 and ~7 larvae (30%–50% of control levels; Figs. 6 and 8A). Young veligers exposed to these aggressive calcifying conditions were significantly smaller than controls; these differences were still apparent 3 wk later, at the start of metamorphosis (Fig. 7). Gray et al. (2017) developed models suggesting that pediveliger development would be delayed by over 4 days if pre-veliger stages were exposed to undersaturated water. The findings of the present study concur, but final pediveliger yields were reduced rather than delayed, suggesting that additional factors prevent prolongation of the veliger stage. When polynomial models were fitted to yield data, the predicted optimal Ω_{ar} environment for early incubation was estimated to be 4.03 based on initial veliger yield and an almost identical 3.98 based on net pediveliger yield (Tables 2 and 3). Overall, the data suggest that larval status at the start of the veliger prodissoconch II stage has a substantial influence on the net capacity to recruit to the juvenile population.

Effects of Ω_{ar} on Veliger Larvae (2–20 days pf)

Veliger larvae exposed to adjusted Ω_{ar} environments from 2 days pf showed surprising resilience. Survival trajectories appeared unaffected (Fig. 9), but shell length growth was reduced by about 25% in the undersaturated treatments (Fig. 10). Consequently, the net proportion of the initial (2 days pf) population successfully attaining pediveliger size was reduced in the low Ω_{ar} treatments, but only Ω_{ar} 0.7 declines were significant (Fig. 11). Surprisingly, the pediveligers produced in

TABLE 4.

Carbonate chemistry of continuous flow 2.5-L veliger rearing tanks. Values represent mean ± SEM (n = 4), for water flow into and out of each tank.

Target Ω_{ar}	A_T (μmol kg^{-1})	DIC (μmol kg^{-1})	Total pH	Calculated Ω_{ar}
0.5	2,286 ± 32 →2,300 ± 16	2,387 ± 48 →2,294 ± 24	7.09 ± 0.04 →7.40 ± 0.03	0.40 ± 0.02 →0.78 ± 0.04
1	2,293 ± 26 →2,299 ± 15	2,362 ± 23 →2,270 ± 20	7.18 ± 0.01 →7.47 ± 0.02	0.48 ± 0.02 →0.90 ± 0.03
2 (control)	2,283 ± 27 →2,295 ± 14	2090 ± 7 →2083 ± 5	7.90 ± 0.03 →7.95 ± 0.02	2.23 ± 0.19 →2.41 ± 0.09
3	2,389 ± 51 →2,353 ± 1	2,169 ± 15 →2,130 ± 1	7.94 ± 0.06 →7.97 ± 0.01	2.54 ± 0.36 →2.54 ± 0.01
4	2,668 ± 6 →2,664 ± 4	2,313 ± 15 →2,294 ± 5	8.14 ± 0.04 →8.17 ± 0.01	4.07 ± 0.24 →4.19 ± 0.03
7	3,062 ± 60 →3,085 ± 18	2,508 ± 31 →2,499 ± 10	8.33 ± 0.02 →8.37 ± 0.01	6.46 ± 0.35 →6.81 ± 0.09
Blank seawater	2,303 ± 28	2040 ± 3	8.06 ± 0.03	2.89 ± 0.25

Temperature and salinity remained stable at 20.0 ± 0.1°C and 35.5 ± 0.1 PSU.

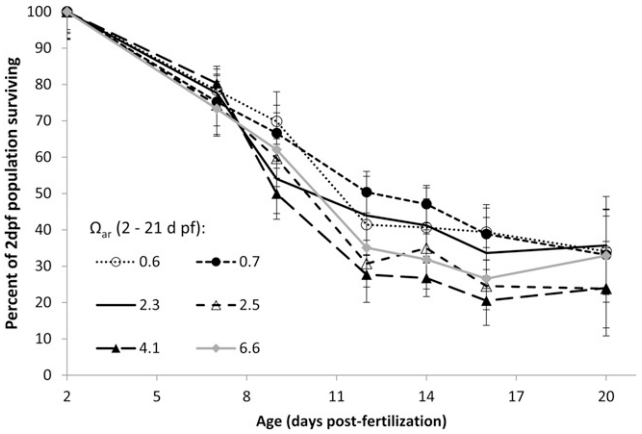

Figure 9. Relative survival of veligers raised in continuous flow tanks (200 mL^{-1}, 2.5 L) receiving Ω_{ar}-modified water. Values are expressed as the mean percentage of initial tank population (2 days pf) ± SEM ($n = 6$) and continue to first harvest of pediveligers, 20 days pf.

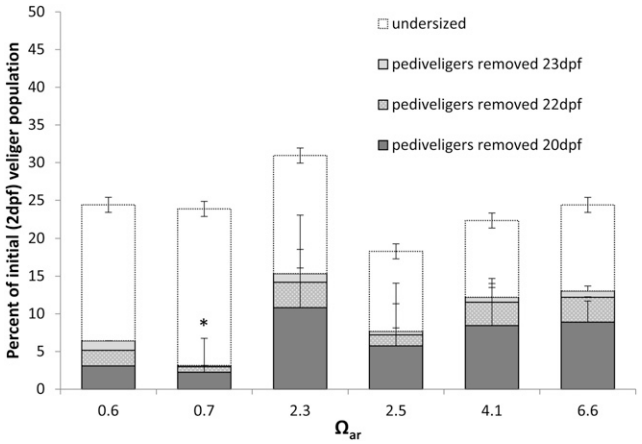

Figure 11. Yield of pediveliger larvae expressed as a percentage of initial tank population 2 days pf; screening was repeated 20, 22, and 23 days pf. Values are expressed as mean ± SEM, $n = 6$. Total yields significantly different from the control are marked * (Tukey's HSD, $P < 0.05$).

undersaturated water appeared to be heavier than those produced in higher Ω_{ar} treatments (Fig. 12). It is suggested that carbonate limitation influenced the deposition of the prodissoconch II shell, slowing longitudinal development, resulting in a deeper shell cavity and a higher mass:shell length ratio.

Unexplained variance may have masked some treatment effects in this trial. Overall batch performance was poorer than in PD1, with 15.3% ± 2.1% of 2 days pf larvae reaching the pediveliger stage, compared with 40.3% ± 10.2% in the PD1 trial, and tank-to-tank variability was frequently higher than between-treatment effects. High between-cohort variability and within-cohort tank effects have been previously observed in *Perna canaliculus* larval trials (Ragg et al. 2010), as well as in other bivalve larvae (e.g., Robert & Gerard 1999). As water quality was carefully controlled during trials, it is suspected that endogenous effects associated with the acquisition of brood stock in a different season (PD1: austral spring, PD2: autumn) may drive the batch differences; however, the reasons for tank-to-tank variability remain elusive.

Carry-over effects were apparent following the removal of pediveligers from the Ω_{ar} 0.7 treatment, with net survival through metamorphosis and juvenile settlement being significantly lower than controls (Fig. 13). Curiously, however, no such legacy effects were apparent following rearing in Ω_{ar} 0.6; again, it is suspected that unexplained variance may mask a treatment effect. Hettinger et al. (2013b) observed reduced juvenile growth as a carry-over effect following rearing of Olympia oyster larvae under low Ω_{ar}, suggesting that the measurement of short-term survival used in the present study may not be the most suitable parameter to quantify. The development of the scallop *Argopecten irradians* and clam *Mercenaria mercenaria* veligers was also significantly influenced by pCO_2, with effects most apparent in the postmetamorphosis juveniles (Talmage & Gobler 2010). The larvae of the slow-growing Olympia oyster *Ostrea lurida* raised under a broad range of Ω_{ar} conditions also showed growth rates that remained relatively independent of Ω_{ar}, but were prone to malformation if grown above Ω_{ar} 6 (Waldbusser et al. 2016).

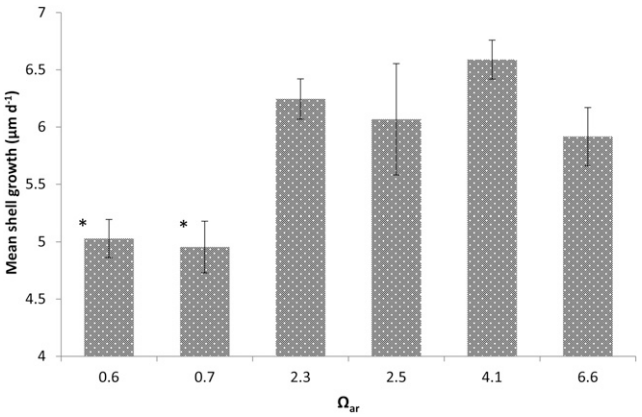

Figure 10. Veliger shell growth between 2 and 14 days pf in a range of Ω_{ar} environments. Bars represent mean daily shell length gain ± SEM. Growth rates significantly different from the control are marked * (Tukey's HSD, $P < 0.05$).

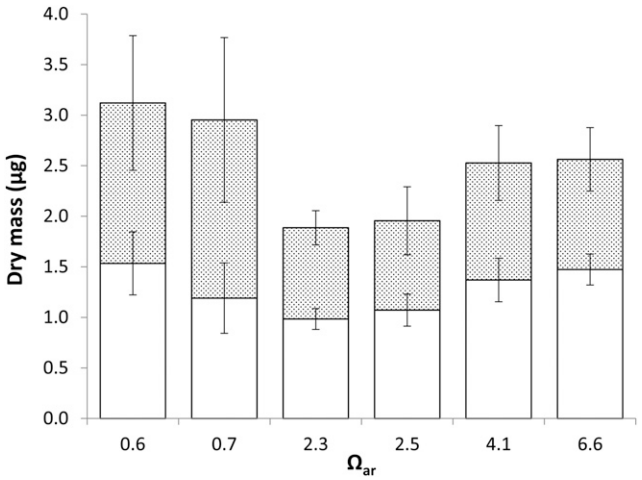

Figure 12. Dry mass of individual pediveliger larvae retained by a 178-µm screen 20 days pf following rearing in different Ω_{ar} environments. Inorganic (ash) component is represented by the clear area, and organic component by the shaded area. Mean ± SEM, $n = 3$–6.

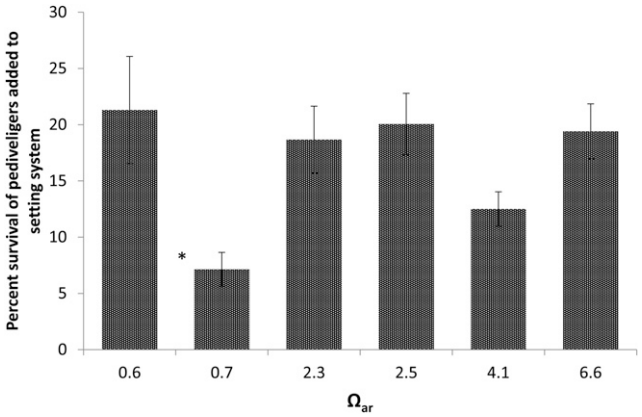

Figure 13. Proportion of 5,000 pediveligers, transferred from modified Ω_{ar} treatments into an unmodified environment, that successfully metamorphosed and survived to 13 days postmetamorphosis (mean ± SEM, $n = 6$).

Ecological Implications

The experimental conditions created were deliberately designed to allow elucidation of relative sensitivity to the pH/carbonate environment, rather than to reproduce ecologically relevant conditions. Some ecological inference can, however, be made from the data presented here. The reduced Ω_{ar} treatments were created by elevating pCO_2 and, therefore, mimic the effects of ocean acidification. The conditions created were extreme (pH_T 7.09–7.47), exceeding likely medium-term projections for the southern oceans (IPCC 2014). The Greenshell mussel *Perna canaliculus*, however, inhabits the shallow sublittoral zone of New Zealand coasts, where local conditions may result in pH and carbonate saturation levels substantially lower than the oceanic mean (Law et al. 2018). Exposure of pre-veliger life stages to pelagic conditions where Ω_{ar} remains below 0.8 could result in a complete recruitment failure (Fig. 8). The parents used in this study, however, were naive, being raised in water that appears to remain above Ω_{ar} of 1.55 (Kim Currie, NZOA-ON, unpublished data); as both selection pressure and conferral of resilience represent possible mechanisms for the production of offspring with increased tolerance to high pCO_2 conditions (e.g., Parker et al. 2012, Munday et al. 2013), the current findings may not fully represent vulnerability in future populations. Veliger stages are less likely to be severely impacted by ocean acidification. In the presence of abundant food, veligers exposed to undersaturated water in the PD2 trial displayed performance that was broadly comparable with the controls. Importantly, development rates were unaffected by the Ω_{ar} environment (Fig. 11). In a natural, food-limited environment, the veliger stage may be protracted and the length of the free-living stage may, in turn, be correlated to reduced recruitment (Alfaro et al. 2010). For example, Gray et al. (2017) observed delayed onset of feeding and lower subsequent ingestion rates in *Mytilus californianus* larvae exposed to undersaturated Ω_{ar}, reducing the scope for growth. Waldbusser et al. (2013) also argue that with a completed prodissoconch shell allowing some degree of isolation from external chemistry, veligers with access to sufficient food to overcome energetic limitations would be far more resilient to varying Ω_{ar} than earlier life stages. Melzner et al. (2011) have demonstrated that abundant food supply can, for example, substantially mitigate nacre dissolution caused by elevated pCO_2. Hettinger et al. (2013a) found that compromised performance of Olympia oyster veligers under elevated pCO_2 was mitigated by the presence of abundant food. An exploration of the interacting effects of ocean acidification and food limitation would, therefore, be useful.

Although the effects of Ω_{ar} elevation are relatively subtle, they support an important hypothesis that progressive acidification from the start of the industrial revolution is already influencing the performance of marine species. For example, in a direct exploration of this hypothesis, Talmage and Gobler (2010) exposed scallop and clam larvae to simulated preindustrial seawater conditions (Ω_{ar} ~3.4) and observed significant increases in the development rate and more robust shell structure. In the present study, net veliger and pediveliger yields conformed to a parabolic, quadratic relationship with Ω_{ar}. Below the apex of

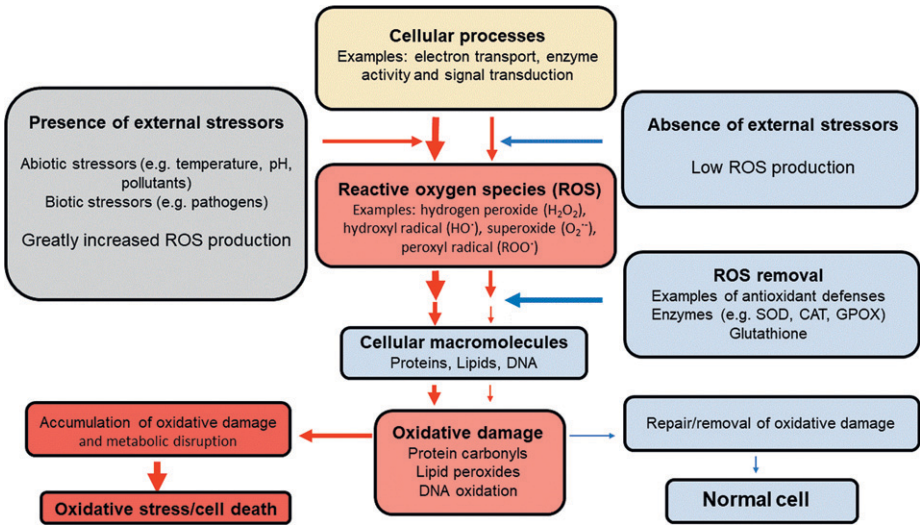

Figure 14. Simple schema of selected processes associated with the development of oxidative stress in shellfish. Red arrows track the development of oxidative stress, whereas blue arrows represent antioxidant defense and return to normality. The antioxidant defense markers targeted in the present study are SOD, CAT, and GPOX.

$\sim\Omega_{ar}$ 4, the response curve was exponential, with the magnitude of response predicted to increase rapidly as Ω_{ar} declines. The implication is that *Perna canaliculus*, and other bivalve larvae, exposed to present-day seawater are already displaying phenotypic responses as part of a continuum, rather than true resistance awaiting a "tipping point" before effects become apparent.

Commercial Application

The importance of Ω_{ar} to commercial bivalve hatcheries operating in the upwelling regions of the Pacific United States has been carefully explored. An economic assessment even proposes a commercial "break-even Ω_{ar}" of 1.7 (Barton et al. 2015). In the present study, elevating Ω_{ar} of the pre-veliger incubation medium to 4.5 (PD1 trial) increased both immediate veliger and subsequent pediveliger yields by an average of 13% (Figs. 2A and 8). The relative economic effects of post-industrialization OA or artificial carbonate enrichment on Greenshell mussel aquaculture would be challenging to calculate. As a broad indication, hatchery production is forecast to supply 30% of *Perna canaliculus* commercial spat by 2020 (Anon. 2015); if the industry size and value remain stable at ~250m USD exports p.a. (Jeffs et al. 2018), a 13% increase in the limited hatchery seed supply would correspond to ~10m USD of increased sales.

Incubation at Ω_{ar} 4.5 also significantly elevated levels of the antioxidant enzymes GR and GPOX (Fig. 5F and G). Quadratic modeling suggested that optimum veliger and pediveliger yields would, in fact, be obtained following incubation in Ω_{ar} ~4 seawater (Tables 2 and 3). It should be noted that the predicted optimum Ω_{ar} of 4 will be influenced by the true saturation state of the hyper-saturated treatment, which was assumed to be 7 for computational purposes, but could not be assessed directly because of precipitation within the sample. Waldbusser et al. (2015) described continuing increases in shell growth of young *Crassostrea gigas* veligers in saturation states as high as Ω_{ar} 6.5, suggesting more focused investigation of high Ω_{ar} environments would be informative. Importantly, Ω_{ar} and pH declined to potentially dangerous levels in the control incubation water in the present study (Ω_{ar} 1.28, pH 7.71). Comparable levels are described in the Pacific NW United States, where sporadic intake of Ω_{ar} undersaturated seawater into commercial bivalve hatcheries can occur (Barton et al. 2012). For both scenarios, carbonate enrichment represents a benign mechanism to buffer these changes. Manipulation of Ω_{ar} in the constantly flowing veliger rearing system is more involved, and the potential benefits of increasing Ω_{ar} are equivocal (PD2 trial). As carbonate enrichment of pre-veliger incubation water is inexpensive and technically straightforward, it is recommended that *Perna canaliculus* tanks be enriched to Ω_{ar} 4 as standard practice. It is also suggested that subsequent veliger culture should be left unmodified, until compelling evidence of potential benefits becomes available. The logistical constraints associated with trials using large-volume commercial tanks resulted in low replication and statistical power in the PD1 trial. It is, therefore, recommended that culture trials continue to contrast performance with unmodified seawater, to develop a robust comparative dataset.

The combined effects of carbonate and EDTA enrichment need further consideration. The presence of EDTA is considered essential in the commercial production of many bivalve veligers (FAO 2004), and recent findings suggest that increasing EDTA concentration above 4 μM will further improve veliger yield (Gale et al. 2016). Metal speciation and bioavailability will vary with both pH and the effects of a chelator, such as EDTA; bioavailability of both beneficial and potentially toxic metals should, therefore, be considered when designing an enriched medium.

Conclusions and Future Work

The pre-veliger larvae of *Perna canaliculus* were found to be sensitive to the aragonite saturation state of their incubation seawater. Embryos and young larvae incubated in Ω_{ar} undersaturated ($\leq$0.8) or hyper-saturated (~7) water showed acute signs of oxidative stress and compromised development. Ongoing performance issues were apparent when the juveniles were returned to normal seawater, resulting in little or no net recruitment. Elevating Ω_{ar} to 4.5 increased antioxidant levels and supported a 13% increase in net recruitment. By contrast, veliger performance differences were dominated by unexplained variability, rather than Ω_{ar}. A valuable enhancement to the approach presented here would be to consider the ramifications of Ω_{ar} manipulation on the other organisms in the hatchery mesocosm, notably the dietary algae and the microbiome associated with the larvae themselves. Repeated commercial trials are also required to improve the robustness of the conclusions presented here and allow a cost–benefit analysis of carbonate enrichment. Young *P. canaliculus* are vulnerable to extreme ocean acidification conditions. Comprehensive trials are now required to characterize the integrated life-cycle responses of this keystone ecological and commercial species to realistic near-future seawater conditions.

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