Ocean acidification impairs the physiology of symbiotic phyllosoma larvae of the lobster *Thenus australiensis* and their ability to detect cues from jellyfish

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Abstract

Ocean acidification (OA) can alter the behaviour and physiology of marine fauna and impair their ability to interact with other species, including those in symbiotic and predatory relationships. Phyllosoma larvae of lobsters are symbionts to many invertebrates and often ride and feed on jellyfish, however OA may threaten interactions between phyllosomas and jellyfish. Here, we tested whether OA predicted for surface mid-shelf waters of Great Barrier Reef, Australia, under Δ pH = - 0.1 (pH ~7.9) and ΔpH = - 0.3 (pH ~7.7) relative to the present pH (~8.0) (P) impaired the survival, moulting, respiration, and metabolite profiles of phyllosoma larvae of the slipper lobster *Thenus australiensis*, and the ability of phyllosomas to detect chemical cues of fresh jellyfish tissue. We discovered that OA was detrimental to survival of phyllosomas with only 20% survival under ΔpH = - 0.3 compared to 49.2 and 45.3 % in the P and ΔpH = - 0.1 treatments, respectively. The numbers of phyllosomas that moulted in the P and ΔpH = - 0.1 treatments were 40% and 34% higher, respectively, than those in the ΔpH = - 0.3 treatment. Respiration rates varied between pH treatments, but were not consistent through time. Respiration rates in the ΔpH = - 0.3 and ΔpH = - 0.1 treatments were initially 40% and 22% higher, respectively, than in the P treatment on Day 2 and then rates varied to become 26% lower (ΔpH = - 0.3) and 17% (ΔpH = - 0.1) higher towards the end of the experiment. Larvae were attracted to jellyfish tissue in treatments P and ΔpH = - 0.1 but avoided jellyfish at ΔpH = - 0.3. Moreover, OA conditions under ΔpH = - 0.1 and ΔpH = - 0.3 levels reduced the relative abundances of 22 of the 34 metabolites detected in phyllosomas via Nuclear Magnetic Resonance (NMR) spectroscopy. Our study demonstrates that the physiology and ability to detect jellyfish tissue by phyllosomas of the lobster *T. australiensis* may be impaired under ΔpH = - 0.3 relative to the present conditions, with potential negative consequences for adult populations of this commercially important species.

Keywords

Behaviour, Metabolomics, pH, Sensory ability, Symbiosis, Zooplankton

Introduction

Different species can interact in a variety of relationships including symbiosis (such as mutualism or parasitism), competition, and as predators and prey (Thrall et al., 2007; Schmitz et al. 2017). An individual may live at the expense of another species (e.g. parasitism and predation) and sometimes both species benefit from the association (e.g. mutualism), such as the interaction between a cleaner wrasse and client fish (Thrall et al., 2007). Positive outcomes from interactions may include dispersal to favourable environments (i.e. phoresy), acquisition of food through phoresy or by directly feeding on the host or prey (Masuda, 2009; Ingram et al., 2017; Schmitz et al. 2017) or protection against predators (Paracer and Ahmadjian, 2000; Masuda, 2009). Inter-species interactions may help support populations of a species and thus benefit biodiversity (Paracer and Ahmadjian, 2000; Schmitz et al. 2017). However, anthropogenic stressors can threaten these interactions (Nagelkerken et al., 2016; Draper and Weissburg, 2019). The exact mechanisms by which stressors impair such interactions is a critical issue (Nagelkerken et al., 2016) since they often contribute towards the regulation of animal populations and can influence the distribution of prey and competitors; thus, disruption may have broad consequences for ecosystems (Draper and Weissburg, 2019).

Increasing atmospheric CO_2 leads to the reduction of pH in oceanic surface waters causing ocean acidification (OA). pH also varies on a diel basis due to organismal respiration and changes in photosynthesis between day and night (Doney et al., 2020). OA can threaten marine fauna and the potential impacts of OA on inter-species interactions need to be examined to predict the fate of marine organisms and ecosystems under increasing atmospheric CO_2 (Nagelkerken and Munday, 2016; Domenici et al., 2019; Doney et al., 2020). Behavioural changes influencing the ability to form inter-species interactions have been described under OA conditions, through disruption of sensory or nervous system function which influences the ability to perceive chemical cues (Bibby et al., 2007; Nagelkerken and Munday, 2016; Draper and Weissburg, 2019). This disruption may result in the failure to locate food, find hosts, or successfully mate (Nagelkerken and Munday, 2016). For example, mud crabs (*Panopeus herbstii*) reduced consumption and handling time of prey oysters (*Crassostrea virginica*) (Dodd et al., 2015) and gastropods exhibited impaired ability to locate food under OA conditions (Domenici et al., 2017). The Ambon damselfish (*Pomacentrus amboinensis*) failed to detect alarm cues of predatory moon wrasses (*Thalassoma lunare*) even under moderately low (but constant) pH of 7.85 in coral reef settings (Chivers et al., 2014). However, behavioural effects of OA are inconsistent since other species such as the humbug damselfish (*Dascyllus aruanus*) survive and do not exhibit impaired behaviour under OA (pH 7.6) (Clark et al. 2020; Munday et al., 2020). Our understanding of behavioural effects of OA are also mainly based on studies of fish and echinoderms (Espinel-Velasco et al., 2018; Munday et al., 2020). We need more research aimed at understanding the potential for OA to negatively impact survival, metabolism, and sensory abilities of individuals, since this can ultimately disrupt important behaviours and inter-species interactions (Watson et al., 2014; Domenici et al. 2019; Draper and Weissburg, 2019).

Lobsters are ecologically and economically important species with planktonic life history stages that are potentially vulnerable to OA (Ross and Behringer, 2019). The phyllosoma larval stage of lobsters feed primarily on marine invertebrate larvae, including small zooplankton and jellyfish (Jones, 2007). They are often observed attached to the aboral surface of jellyfish medusae, where they may ride and feed on the medusae until they metamorphose into the benthic nisto stage (Lavalli et al., 2007; Wakabayashi and Phillips, 2016). Although many lobster species are oceanic, phyllosomas are also abundant in coastal waters and shelf areas with depths of ~ 40 to 50 m (Barnett et al., 1984; Jones, 2007), where coastal acidification is a major environmental concern (Duarte et al. 2013; Wallace et al., 2014). Climate change and OA are predicted to harm the development and sensory systems of some lobster species (Ross and Behringer, 2019). For example, reared in low pH conditions (7.7 vs. 8.1), larvae of *Homarus americanus* exhibited a smaller mean carapace length and spent longer at each larval stage (Keppel et al., 2012). The Caribbean spiny lobster *Panulirus argus* uses chemical cues to find shelter and this ability was impaired when exposed to low pH (7.65) (Ross and Behringer, 2019). Additional evidence suggests that OA may harm the ability of lobsters to interact with other species (Phillips et al., 2017). Therefore, investigations into the effects of OA on sensory ability and interactions between phyllosomas and jellyfish are warranted.

Lobsters exposed to OA must expend energy to maintain biochemical homeostasis to survive, but doing so can be metabolically costly (Pörtner, 2010) and this may affect their ability to interact with jellyfish. For example, juvenile lobsters of *Homarus gammarus* exhibited increased O_2 respiration under high pCO_2 (1,100 μatm; pH 7.7), indicating energetic costs associated with low pH conditions (Small et al., 2020). Metabolic effects are reflected by changes to the biochemical composition of an organism (Bundy et al., 2009). For example, low pH (7.6) upregulated transposable elements in the sea anemone, *Anemonia*

viridis and its *Symbiodinium* sp. endosymbiont, indicating cellular stress and metabolic depression as costs of acclimating under OA (Urbarova et al., 2019). Techniques like untargeted metabolomics enable assessment of organisms' responses to changes in the external environment (Bundy et al., 2009). Metabolomic analyses have been used to characterise changes in response to biological processes such as growth and reproduction and external factors such as changes in pH (Bundy et al., 2009; Mayor et al., 2015). Metabolomics allows assessment of multiple metabolites simultaneously and is not biased to a particular biochemical pathway (Bundy et al., 2009), making it effective in examining metabolic effects of anthropogenic stressors such as OA (Mayor et al., 2015).

Several scenarios of Representative Concentration Pathways (RCP) leading to OA have been described (IPCC, 2014). These pathways range from optimistic (RCP 2.6) to business-as-usual (RCP 8.5), but moderate conditions of the RCP 2.6 are considered the most likely to occur in the future (IPCC, 2014; Hughes et al., 2017; Geraldi et al., 2020). However, the current trajectory of global emissions follows the 'business-as-usual' scenario of RCP 8.5, adding to uncertainty of projecting future climate and OA conditions (van der Zande et al., 2020; Geraldi et al. 2020). Moreover, the optimistic scenario may still cause moderate OA that can result in negative outcomes for key processes in marine invertebrates, including survival, growth (Fabry et al., 2008; Kurihara, 2008) and behaviour (Clements and Hunt, 2015; Nagelkerken and Munday, 2016; Watson et al., 2017). This study examined whether OA influences survival, development, and biochemical composition of phyllosoma larvae, and their ability to detect jellyfish tissue. Specifically, we hypothesised that survival, moulting rates, locomotion, and attraction to jellyfish cues would be reduced in phyllosomas reared in ΔpH = - 0.1 and in ΔpH = - 0.3 relative to present-day pH (P). We also hypothesised that metabolite composition of phyllosomas reared in ΔpH = - 0.1 and in ΔpH = - 0.3would be altered due to metabolic costs of environmental stress.

Materials and Methods

Experimental design

The experiment consisted of three pH treatments including present (pH 7.9-8.1) (P), ΔpH = - 0.1 (= pH 7.8-8.0) and ΔpH = - 0.3 (= pH 7.6-7.8) levels relative to the P. The treatments were based on target atmospheric CO_2 concentrations (in ppm) of 400 (for current pH), 500 (RCP 2.6 scenario with the highest concentration of optimistic greenhouse gas emissions) and 1,000 (the most extreme scenario; RCP 8.5), projected for the year 2100 in Australia (Reisinger et al., 2014). pH levels were deliberately varied on a diel basis to mimic the changes in pCO_2 that occur when photosynthesis ceases at night (Wahl et al., 2016). The magnitude of diel pH variability (0.2 pH range) we used was based on measurements made in an offshore area (Davies Reef) (18˚ 500' S, 147˚ 380' E) in the central Great Barrier Reef with pH range of 7.92 to 8.17 (Albright et al., 2013) and Moreton Bay (27.13˚S, 153.07˚E) (pH 7.90-8.12) (Klein et al., 2017). Diel variation in pH of 0.2 thus corresponds to the diel variability *T. australiensis* phyllosomas experience in their natural habitat (Barnett et al., 1984; Jones, 2007).

Experimental animals

Three-day old stage I phyllosoma larvae of *Thenus australiensis* were obtained from two gravid females from a commercial lobster producer (Australian Bay Lobsters, Inc.). Both phyllosomas and parent lobsters were reared in the dark under constant conditions (mean ± s.e.: pH 8.03 ± 0.01; 35 ± 0.04 PSU salinity; 24 ± 0.01 ˚C temperature; and dissolved O_2 level of 6.98 ± 0.09 mg L^{-1}). Phyllosomas were placed into a plastic bag with air, sealed in an

insulated polystyrene container and transferred from the hatchery to the laboratory within one hour. Phyllosomas were then placed into black mesh baskets (dimension: 18 x 16 x 12 cm.) with mesh size of 3 mm. Each basket was suspended in an acrylic kreisel aquarium filled with 80 L 10-µm filtered and UV-treated seawater. A kreisel is a square aquarium with curved corners, allowing circular water flow. Thirty phyllosomas were placed in each basket and five replicate baskets were randomly allocated to each pH treatment, maintained on a 12:12-h light: dark cycle. Phyllosomas were fed daily with ≃ 30 pieces per basket of ~0.5 cm^2 cubes cut from the bell of *Cassiopea* sp. medusae collected from a local creek (27° 55' 09.0" S, 153° 24' 19.1" E). The basket reduced light exposure that would otherwise aggregate positively phototactic phyllosomas, which could prevent them from feeding on the jellyfish cubes. Each basket was gently bubbled with air to circulate water and facilitate the removal of mucus from the jellyfish food, which can accumulate on the appendages and mouthparts of the larvae and reduce their survival. The phyllosomas were acclimated to laboratory conditions (34.5 PSU salinity, 24.5 ˚C and dissolved O_2 of 7.11 mg L^{-1}) for one day. Water temperature in the kreisels changed at a rate of 0.03 °C/ minute for 30 minutes from the end of acclimation until the start of experimental exposures. To transition larvae to experimental conditions, pH levels were reduced over 12 hours at rates of 0.05 and 0.01 pH units below the present pH (~8.0) per hour for the ΔpH = - 0.1 and ΔpH = - 0.3. treatments, respectively (Table 1) since a 12 -hr gradual transition would reduce shock in introducing lobster larvae to the treatments (Waller et al. 2017). The change of pH levels during transition was achieved by gradually changing the rate of diffusion of gases into the water in each aquarium (see next section: *Manipulation and analyses of water chemistry*). The larvae were maintained at optimal temperature (24 °C) and salinity (35 PSU) corresponding to field conditions (range: 23-24.5 °C and 34.5-35.3 PSU salinity) where phyllosomas survive and grow (Agnalt et al., 2013; Smith et al., 2017) and consistent with conditions in the hatchery. The phyllosomas

were exposed to the treatments for 11 days. Seawater in each kreisel was replaced with 10% of water with the appropriate chemistry every four days.

Manipulation and analyses of water chemistry

pH and pCO_2 levels were maintained and manipulated using a series of gas proportioners that delivered CO_2, N_2, and O_2 gases to the aquaria. The desired gas compositions were mixed from individual gas cylinders using 12 Omega mass flow controllers (FMA-5400 s, 0–20 mL min^{-1} (CO_2), 0–5 L min^{-1} (N_2), 0–2 L min^{-1} (O_2) (*sensu* Bockmon et al., 2013). Gases were passed through reverse osmosis membranes to maximise rates of diffusion into the aquaria. LabVIEW™ software (National Instruments Corp.) was used to regulate the flow of gases through the mass flow controllers and to vary the compositions of gas mixtures on a diel basis by stepping through several time points (0, 4, 8, 12, and 24 hrs) to achieve a 0.2 diel pH difference (Table 1). Gas compositions that produced low pH levels remained constant at night between 18:00 – 06:00 hrs.

Salinity, temperature, dissolved oxygen (DO) and pH were measured in all experimental aquaria at 12:00 and 19:00 every third day. Salinity and temperature were measured using a conductivity–salinity meter (TPS salinity-conductivity meter, MC-84) and thermometer, respectively. DO concentrations were measured using an optic DO sensor (OptiOx, Mettler Toledo Ltd) and pH was measured using a Five Go pH meter equipped with an Inlab Expert Pro Electrode (Mettler Toledo Ltd). Every second day, pH electrodes were calibrated using TRIS/HCl buffers in synthetic seawater to ensure accurate measurements of pH in the seawater carbonate system (Dickson et al., 2007). To accurately measure diel variation, pH measurements were taken hourly (between 07:00 and 19:00 h) from three randomly selected replicates from each of the treatments once per week (Supplementary Fig. 1). Every third day, a 100-mL water sample was collected for analysis of total alkalinity (TA)

from two randomly selected replicates from each treatment (Table 1). Samples of seawater were collected in clean amber glass bottles using a drawing tube and overfilled for 10 s to minimise gas exchange between the sample water and the atmosphere. Samples were filtered through 0.22-µm filters, sealed tightly, and immediately analysed. Samples for TA (80mL) were analysed using a Mettler Toledo titrator, which was calibrated using TRIS/HCl buffers in synthetic seawater and verified with certified TA reference material (provided by A. G. Dickson, batch#162). pCO_2 was calculated using the CO2SYS program with carbonate dissociation constants from Mehrbach et al. (1973) refitted by Dickson and Millero (1987) and KSO_4 according to Dickson (1990). Based on one-way ANOVA, highly significant differences between average levels of the pH ($F = 0.214$, $p = < 0.001$) and pCO_2 treatments ($F = 0.186$, $p = < 0.009$) but not between replicates in each treatment (pH: $F = 1.275$, $p = 0.103$; for pCO_2: $F = 1.682$, $p = 0.092$) were achieved.

Survival and development

All phyllosomas were inspected daily. Dead larvae were counted and removed, and the number of deformed and injured larvae were recorded as an indication of stress or cannibalism. The number of individuals that moulted in each replicate was counted daily. Moulting was determined visually by the presence of exoskeleton cast-off (i.e. exuvia) of the phyllosomas. Survival was measured as the percentage of live individuals remaining in each treatment relative to those at the beginning of the experiment. Moulting was measured as the percentage of live phyllosomas that moulted each day. Five to eight individuals from each replicate were randomly selected each day and observed under a dissecting microscope for 2 to 3 minutes to determine their developmental stage. Developmental stages were assigned based on morphological structures, including sensory setae, antenna, mandible shape,

maxillipeds, presence of pleopod, and pereiopods (Wakabayashi and Phillips, 2016). After examination, larvae were returned to their respective pH treatments. The average number of larvae that reached stage II in each replicate was calculated for the pH 8.0 and 7.9 treatments only, since larvae in pH 7.7 did not survive until the 9th day when most larvae metamorphosed into stage II.

Respiration measurements

Respiration rates of phyllosomas of the same larval stage and similar wet weight (mean ± s.e.: 9 ± 0.13 g) were measured on day 0 and every 1–2 days thereafter. Larvae were weighed by measuring the amount of filtered treatment water displaced by each animal (Spanier et al., 1991). Respiration measurements were made five hours after feeding to ensure measurements were taken in a post-absorptive state. One phyllosoma from each replicate (n=4) were transferred into 0.4 L glass respiration chambers with a FireStingO2 optical oxygen sensor spot attached to their inner wall and filled with 1-µm filtered seawater of the appropriate pH for each treatment and at 24 °C and 35 PSU salinity. Larvae were acclimated for 1 hr and then incubated in chambers for 4 hrs in the dark. Twelve pairs of chambers were used with each pair consisting of a control and a chamber containing a phyllosoma. The controls were used to account for oxygen consumed by microorganisms in the water used for incubations. Chambers were sealed using watch glasses, with care taken to remove air bubbles. Oxygen concentrations were measured using a FireStingO2 optical oxygen sensor at the beginning and end of the incubations. Oxygen levels remained above 80% during the incubations. After incubations, larvae were returned to their respective baskets. Oxygen consumption rates were measured per wet weight of phyllosoma (unit: ng O_2 g^{-1} h^{-1}).

Detection of jellyfish and locomotion assay

The ability of the phyllosomas to detect and respond to the presence of jellyfish tissue was tested after 5 days of exposure to the pH treatments using a choice experiment (adapted from Maibam et al., 2015; Zupo et al., 2015). The test arenas were rectangular plastic containers (17.5 × 12 × 5.5 cm) filled with filtered seawater to a depth of 2 cm. Water in the test arenas was maintained at 24°C. A freshly cut piece of jellyfish tissue and a blank made of silicone (both 1 cm^3), were placed at either end of the arenas. A silicone cube was used to mimic the structure of jellyfish tissue but lacked olfactory cues from jellyfish. The arenas and silicone cubes were soaked in filtered seawater for 15 days before the experiment for conditioning. The test arenas allowed phyllosomas to swim and reduced complex diffusion of odour from the jellyfish attractant because the water was shallow and stagnant. The underside of each arena was marked with a grid of 1 cm^2 squares and was divided into five sections (−2, −1, 0, 1, 2), indicating the position of a phyllosoma relative to the jellyfish tissue, wherein values > 0 indicated attraction towards the end of the arena with the jellyfish cube (Supplementary Fig. 2). The locomotion velocity of phyllosomas was calculated as the number of grid squares crossed (and re-crossed) per second, which was averaged for each replicate.

Phyllosomas are positively phototactic and cannot see red light (Wakabayashi and Phillips, 2016) so choice tests were run in the dark under a red light that was placed above the arena. A GoPro camera was placed on a tripod 34 cm above the arena to record the movements of the phyllosomas. In the choice test (n = 7 trials per treatment), a single phyllosoma (Stage I) was placed in the centre of the arena (Supplementary Fig. 2) under an inverted small glass bowl with treatment water and acclimated for 5 minutes. At the end of the acclimation period, a cube of fresh jellyfish and a silicone blank were placed at opposite ends of the arena and the phyllosoma was released. Trials ran for 10 minutes and each

phyllosoma was only tested once. After testing, they were returned to their aquaria and placed in a separate compartment in the basket. Any preference phyllosomas had for a particular end of the arena was controlled for by switching the location of the jellyfish and silicone cubes between each trial. Half of the phyllosomas used in the behavioural trials were tested in water of their respective pH treatment and the other half were tested in ambient (i.e., pH 8.0) water to determine whether the pH of the water potentially altered the jellyfish odour and whether the ability of phyllosomas to select jellyfish tissue changed under low pH conditions. Videos were analysed using Windows media player with pause, stop and playback options that facilitated recording of the position of the larva in the arena every 10 seconds from the start of the tests.

Metabolomics

One stage I phyllosoma per replicate was harvested on day 5 for analysis of polar metabolites using Nuclear Magnetic Resonance (NMR) spectroscopy. Phyllosomas were blotted dry and transferred to pre-weighed 2.0 mL micro-centrifuge tubes. The tubes were quickly weighed (mg), flash-frozen and samples were stored overnight at −20 °C. The next day, 400 µL ice-cold methanol was added and each sample was ultra-sonicated using a Vibra-Cell VCX-130 probe sonicator (Sonics, USA). This was followed by the addition of 800 µL chloroform and 300 µL ultrapure water, and the samples were vortexed and centrifuged for 10 min (16,000×g at 4°C). After centrifugation, the supernatant methanol fraction, containing hydrophilic metabolites, was transferred to amber glass vials and dried in a GeneVac HT-12 Series 3i centrifugal evaporator (Genevac Technologies, England). Dried samples were reconstituted with deuterium oxide (D_2O) containing 0.05% sodium-3-(tri-methylsilyl)-2,2,3,3-tetradeuteriopropionate (TSP) as an internal standard. The reconstituted samples were transferred to 3 mm NMR tubes using a zero-volume syringe.

NMR spectra were acquired with an 800 MHz Bruker® Avance III HDX spectrometer. The system was equipped with a Triple (TCI) Resonance 5 mm Cryoprobe with Z-gradient and automatic tuning and matching, and a SampleJet automatic sample changer controlled via the software IconNMR™ (Bruker Pty Ltd., Victoria, Australia). Spectra were acquired at 298 K, using D_2O for field locking and TSP (1H δ 0.00) as an internal reference. ^1H spectra were acquired using the zg30 pulse program with 128 scans, 0.8 s relaxation delay, 7.75 μs pulse width and 16 kHz spectral width (^1H δ −3.75–16.28). MestReNova v8.1.4 (Mestrelab Research S.L., Spain) was used for post processing of NMR spectra. ^1H NMR free induction decay (FID) data were Fourier transformed with line broadening of 0.3 Hz and all spectra were manually phase corrected, automatically baseline adjusted (ablative), and referenced and normalised to TSP (^1H δ 0.00). Once processed, individual spectral features were manually integrated and exported to excel for statistical analysis. Chenomx Profiler v8.5 (Chenomx Inc., Edmonton, Canada) was used to identify the metabolites.

Statistical analyses

Generalised linear mixed models (GLMM) were used to analyse survival, moulting and respiration rates of phyllosomas, with pH and days as fixed factors and days also a repeated measure. To determine potential bias due to possible variation between replicates, we included replicates as random factor in the GLMMs. Since replicates did not significantly affect the dependent variables, GLMMs were re-analysed without the random factor. Models were examined using goodness-of-fit statistics (i.e. AIC and BIC) and significant results were further analysed using estimated marginal means. Since data were non-normal, a Mann-Whitney U test was used to compare the average number of larvae in each treatment that reached stage II by the end of the experiment. Comparisons were made only between P and ΔpH = - 0.1 treatments because all larvae in the ΔpH = - 0.3 treatment died by day 9. The

level of selection of phyllosomas towards or against jellyfish and locomotion velocity were analysed using two-way ANOVAs in SPSS. The fixed factors were pH (P, ΔpH = - 0.1 and ΔpH = - 0.3) and test water of the arena (ambient vs reduced pH). Tukey's post-hoc tests were used to analyse differences between the means of significant factors.

Metabolomics data from each replicate were first analysed using MetaboAnalyst 4.0 (Chong et al., 2019). After log normalisation and pareto-scaling, Supervised Partial Least Squares Discriminant Analysis (PLS-DA) was performed to characterise and visualise overall differences in the metabolite profiles of phyllosoma larvae from different treatments. Permutational Multivariate Analysis of Variance (PerMANOVA) in Primer 7 was subsequently used to quantify the differences in metabolite composition among the treatments. All individual metabolites were further analysed using one-way ANOVAs in SPSS and, when significant, Tukey's post-hoc tests were used to determine the differences in their relative abundances between pH treatments.

Results

Less than 60% of phyllosomas survived in each treatment in the experiment (Fig. 1a). pH and day influenced their survival (Table 2). Phyllosomas in the pH ~7.7 under ΔpH = - 0.3 treatment had 20% survival, and survival in P and ΔpH = - 0.1 treatments was 49.2 and 45.3 %, respectively (Fig. 1a). Survival gradually declined each day (Supplementary Fig. 3a). No deformities were observed, and no appendages were lost in any treatment.

The average numbers of phyllosomas that moulted in the P and ΔpH = - 0.1 treatments were 40% and 34% higher respectively than that of treatment under ΔpH = - 0.3 (Table 2; Fig. 1b). A sharp decrease of up to 43 % of larvae that moulted each day occurred

from day 1 to 4 (Supplementary Fig. 3b). The percentage of moulting larvae increased gradually from day 5 to day 8 before it decreased, with the lowest percentage (40%) at the end of the experiment. The percentage of larvae that reached stage II at day 9 did not differ between the P (18% ± 1 of the individuals) and ΔpH = - 0.1 (16% ± 1.8) treatments (U = 9.0, p = 0.419) (Supplementary Fig. 4).

Respiration rates varied between pH treatments, but patterns were not consistent through time (Table 2). Respiration rates in the ΔpH = - 0.3 and ΔpH = - 0.1 treatments were higher than in the P treatment on Day 2 and then rates declined ΔpH = - 0.3 and were lower than the ΔpH = - 0.1 and P treatments on Days 4 and 5 (Fig. 2). Respiration rates in the ΔpH = - 0.1 treatment were higher than those in the pH P treatment from Days 4 to 9 (Fig. 2).

Phyllosomas reared in P and ΔpH = - 0.1 strongly selected the side of the arena with jellyfish tissue but those from ΔpH = - 0.3 avoided the jellyfish tissue and, on average, swam towards the silicone blank, regardless of the test water used in the arena (Table 3; Fig. 3). The rate at which phyllosomas swam (i.e. the number of grid squares crossed by phyllosomas per second in the locomotion assay) did not vary with pH treatment or test water (Table 3; Supplementary Fig. 5).

Clear separation of metabolites extracted from phyllosomas reared in different pH treatments was observed in the sPLS-DA ordination (Fig. 4) and PerMANOVA (p = 0.041). Thirty-four metabolites including a nucleoside, pyridine derivative; carboxylic, amino, keto and sulfinic acids; choline, amine and a sulfone were isolated from phyllosomas and 22 of these were significantly altered by pH treatments (Supplementary Table 1). Most metabolites were significantly decreased in both the ΔpH = - 0.1 and ΔpH = - 0.3 relative to P (Fig. 5). However, four metabolites (homarine, alanine, glutamate, and proline) were reduced only at ΔpH = - 0.1 compared to the other treatments, and a single unidentified metabolite (at 1.41ppm) was increased at ΔpH = - 0.3 compared to the other treatments (Fig. 5).

Discussion

Many jellyfish species contribute to sustaining biodiversity by acting as hosts or prey for a variety of symbiotic (Ohtsuka et al., 2009) or predatory (Hays et al., 2018) marine vertebrates and invertebrates. However, ocean acidification (OA) may threaten interactions between jellyfish and other animals. We found that extreme OA conditions (ΔpH - 0.3 relative to the present) adversely affected the attraction of *Thenus australiensis* phyllosomas to jellyfish tissue, altered their respiration rates and biochemistry (metabolite profiles), and influenced their moulting. Most individual metabolites of phyllosomas were suppressed even in mild pH ~7.9 under ΔpH = - 0.1, thus the suppression of these metabolites support our hypotheses that biological responses would manifest in ΔpH = - 0.1 and ΔpH = - 0.3. Whether the phyllosomas of *T. australiensis* depend on jellyfish as hosts and for food to complete their life cycle is unknown. If this is indeed the case, then impaired jellyfish-phyllosoma interactions may contribute to reduced populations of phyllosomas and, in turn, affect the maintenance of populations of adult lobsters. This would be a detrimental outcome with consequences for a myriad of ecosystem services and could impact the economic benefits these lobsters provide (Jones, 2007). These possible consequences of OA emphasise the need for experiments examining effects of elevated CO_2 on invertebrates, including inter-species interactions like that between lobsters and jellyfish and on behaviours such as host-symbiont or predator-prey interactions (Draper and Weissburg, 2019).

Our results demonstrate that OA may disrupt interactions between an invertebrate and jellyfish. To our knowledge, this is the only study to have examined the effects of OA on behavioural and biochemical endpoints in phyllosomas and to determine effects of OA on the

detection by phyllosomas of jellyfish cues. One previous study investigated the effects of OA on symbiosis between fish and jellyfish, observing similar responses to our own (Nagelkerken et al., 2016). Specifically, fish that commonly associate with jellyfish approached their jellyfish host less frequently and spent less time close to their host under predicted future low pH (7.6) conditions than under present-day pH conditions (8.0) (Nagelkerken et al., 2016). Our study also confirmed that OA impairs the detection of olfactory cues by lobsters and is consistent with Gravinese et al. (2020) who discovered that detection of *Laurencia* spp. macroalgae (a known settlement cue) by the pueruli (postlarva) of Caribbean spiny lobster, *Panulirus argus* was impaired when pueruli were exposed to constantly low pH (7.62). Adverse effects of OA therefore manifest across taxa such as crustaceans and fish and potentially across multiple life stages of lobsters.

Phyllosomas exposed to pH ~7.7 under ΔpH = - 0.3 spent less time interacting with, or in close proximity to, the jellyfish tissue than those reared under ambient pH conditions. This outcome may correspond with impaired chemosensory ability following exposure to low pH treatments and is consistent with studies of other crustaceans. For example, low pH (7.1) impaired olfaction, prey detection and antennule flicking of deep-sea hermit crabs (Kim et al., 2015). Adult Caribbean spiny lobsters, *Panulirus argus*, were also unable to recognize their shelters following exposure to OA (Ross and Behringer, 2019). Similar chemosensory impairments have been observed in vertebrates. After exposure to low pH levels (7.86), adult cardinal fish, *Cheilodipterus quinquelineatus*, failed to recognize their shelters (Devine et al., 2012). In our study, the swimming velocity of phyllosomas was not affected, thus their reduced ability to associate with jellyfish was probably unrelated to their ability to move.

The reduced attraction of phyllosomas to jellyfish tissue under ΔpH = - 0.3 was consistent regardless of the test water used in the arena, suggesting that pH did not affect the chemistry of odours exuding from jellyfish tissues. Chemical cues may degrade quickly in

CO_2-acidified water (Chivers et al., 2014; Roggatz, et al., 2016), making them undetectable by animals that respond to these cues, such as reef fish (Nagelkerken et al. 2016). For example, alarm cues by the predators of Ambon damselfish, *Pomacentrus amboinensis*, took just 5 minutes to degrade under moderately low pH (7.85) (Chivers et al., 2014). The cues became undetectable and elevated the risk of predation on the damselfish that swam in the water with predator cues (Chivers et al., 2014). Here, phyllosomas had reduced selection towards jellyfish even in the ambient test water, suggesting that impaired sensory functions of phyllosomas, not changes to the chemistry of odours, probably reduced their attraction to jellyfish. However, direct chemosensory measurements, such as receptor activation (Domenici et al. 2019) of phyllosomas, and examination of the chemistry of odours would be needed to confirm that their chemosensory functions were disrupted and that degradation of chemical cues (see Draper and Weissburg, 2019) was not a factor influencing the attraction of phyllosomas to jellyfish.

An overall reduction in most of the identified metabolites was observed in phyllosomas reared under OA conditions. Metabolites are key biochemical intermediaries in various physiological processes such as energy metabolism and protein synthesis, which lobsters require to survive and develop (Whiteley, 2011). For example, moulting is critical for growth and development and requires careful regulation of the endocrine system, which itself depends on unimpaired metabolism of amino acids and proteins (Whiteley, 2011; Small et al., 2020). The reduced survival and moulting rates observed under ΔpH = - 0.3 conditions correspond with the overall suppression to energy metabolism of the phyllosomas and both responses are thus likely related, since developmental events are extremely energy intensive and require sufficient metabolite pools (Langenbuch et al., 2006). Similarly, impaired metabolism was observed in juvenile European lobsters, *Homarus gammarus* (pCO_2 = 1,100 and 9,000 μatm; Small et al., 2016) and larvae of the American lobster *H. americanus* under

low pH (1,200 ppm pCO_2) (Keppel *et al*., 2012), however the former did not evaluate effects on moulting. Stressful conditions are well known to impair normal metabolism, including the maintenance of energy reserves in crustaceans like adult lobsters (Whiteley & Taylor, 1990; Whiteley, 2011; Small et al., 2020). Our results suggest an apparent relationship between reduced attraction to cues from jellyfish (as food or host), decreased moulting, and an overall suppression of metabolic status, but it is unclear whether reduced metabolite pools are a cause or consequence of the higher-level effects.

Many marine species increase respiration rates to compensate for increased metabolic demands under low pH conditions (Wood et al., 2008; Portner, 2010). Inability to compensate for the metabolic demand associated with stress may result in mortality (Portner, 2010). This is consistent with the low survival and sharp decline in respiration rates observed in phyllosomas under ΔpH = - 0.3, and the greater survival of those with elevated respiration under the moderate OA scenario (Figure 3). Like those in ΔpH = - 0.1, respiration rates were initially elevated in the ΔpH = - 0.3 treatment at day 2, suggesting phyllosomas reared under extreme OA initially appeared to compensate for the increased metabolic demand associated with the stressful conditions. The subsequent sharp decline in O_2 consumption likely reflects an overall loss of energy reserves due to the inability to compensate metabolically, which ultimately reduced their survival. Impaired energy maintenance under ΔpH = - 0.3 is further reflected in the overall reduced abundance of many metabolites, including important energetic molecules like ketogenic amino acids (e.g. leucine, lysine, phenylalanine, isoleucine, threonine) and the carboxylic acid acetoacetate, which feeds into the tricarboxylic acid (TCA) cycle by replenishing Acetyl-CoA stores (Litwach, 2018; D'Andrea, 2000). The sensitivity of phyllosomas to extreme OA may be linked to less developed physiological systems of early-stage lobsters such as phyllosomas, which are probably less effective in

regulating acid-base changes (Whiteley, 2011; Small et al., 2020). Therefore, comparison of metabolic effects between life stages may be of interest.

Climate change and OA may pose a risk to the future maintenance of lobster populations, since an overall reduction in metabolic content was observed even after exposure of phyllosomas to moderate OA conditions of ΔpH = - 0.1. However, our results suggest that the larvae were better able to compensate against the physiological stress associated with mild acidification, similar to invertebrates such as some corals and mussels that survive low pH conditions through metabolic and acid-base regulation (Wood et al., 2008; Ellis et al., 2014). Indeed, the juvenile American lobster (*H. americanus*), had high survival even in mild OA (600 μatm pCO_2 level) compared to extreme OA (1,200 μatm pCO_2) where survivorship was the lowest (Menu-Courey et al., 2019). The lobster's survival in mild acidification was related to increased aerobic metabolism, examined using electron transport and lactate dehydrogenase metabolism. Therefore, mild OA (ΔpH = - 0.1) can alter metabolism but may not impair overall physiology to the extent that this results in reduced survival, whereas phyllosoma exposed to lower pH (ΔpH = - 0.3) may be unable to compensate and ultimately die.

Throughout the experiment, we tried to minimise stress on phyllosomas. For example, we ensured the incubations used to measure respiration were the minimum duration required to ensure reliable results and phyllosomas were handled gently whenever they were transferred to the microscope or incubation chambers. We acknowledge, however, that handling stress may have influenced overall survival rates of the phyllosomas. Since phyllosomas from all treatments were handled the same, we are confident that our results were not biased. The effects of handling stress are unknown; thus, we recommend obtaining baseline observations of effects of handling stress on lobsters in future studies of lobster eco-physiology. This could be done by comparing responses of stressful handling versus minimal

handling conditions (control) of animals using preferably biochemical methods like lactate and glucose levels in addition to organismal parameters, e.g. survival and respiration, which can determine ecophysiological changes among crustaceans like lobster larvae (Small et al., 2020) under OA stress.

The ecological and physiological effects observed here perhaps indicate thresholds in responses to the OA treatments (e.g., Ventura et al. 2015) such as physiological plasticity under ΔpH = - 0.1 and stress under ΔpH = - 0.3. These response thresholds are evident in the decline in compensating stress in mild pH (ΔpH = - 0.1) conditions, and lethal effects in ΔpH = - 0.3 treatment. Larvae exposed to ΔpH = - 0.1 probably tolerated fluctuating pH levels as an evolutionary adaptation or they exhibited transgenerational acclimation (Torda et al., 2017; Lee et al., 2020) developed from possible exposure of several generations of parent lobsters to slightly reduced but constant pH (~7.98-8.0) in the hatchery. Physiological and transgenerational plasticity of larvae can only be speculated at this stage since examining ecological responses of several generations and between life cycle stages of the lobsters is needed to confirm whether the effects observed here are plastic responses. Regardless of the observed effects, the outcomes here indicate tipping points of ecological responses to present pH ~ 8.06 (range: 8.003-8.113) in mid-shelf waters in the Great Barrier Reef (Fabricius et al. 2020) where the phyllosomas occur. Our study also advanced our understanding on anthropogenic stress on lobster phyllosomas since results were derived from methods that use fluctuating pH levels and conditions which mimic natural and predicted carbonate chemistry (IPCC, 2014).

Conclusions

Our study suggests that the survival, physiology and the ability of *T. australiensis* phyllosomas to detect jellyfish cues may be impaired by extreme (i.e. pH ~7.7) OA conditions under ΔpH = - 0.3 relative to the present pH. Indeed, if extreme OA eventuates, our findings indicate phyllosomas of *Thenus australiensis* may be unable to detect and associate with (potentially as predators or symbionts) jellyfish. If the association between phyllosomas and jellyfish is obligatory, there may be subsequent consequences for later life stages and, ultimately, for populations of adult lobsters. Furthermore, our experiment only considered the effects of changes in ocean pH, but the oceans are also warming. Ocean warming and acidification may interact to generate different outcomes than those observed here and testing multiple climate change stressors is ultimately required to more accurately predict the responses of phyllosomas to future ocean conditions.

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Figures list

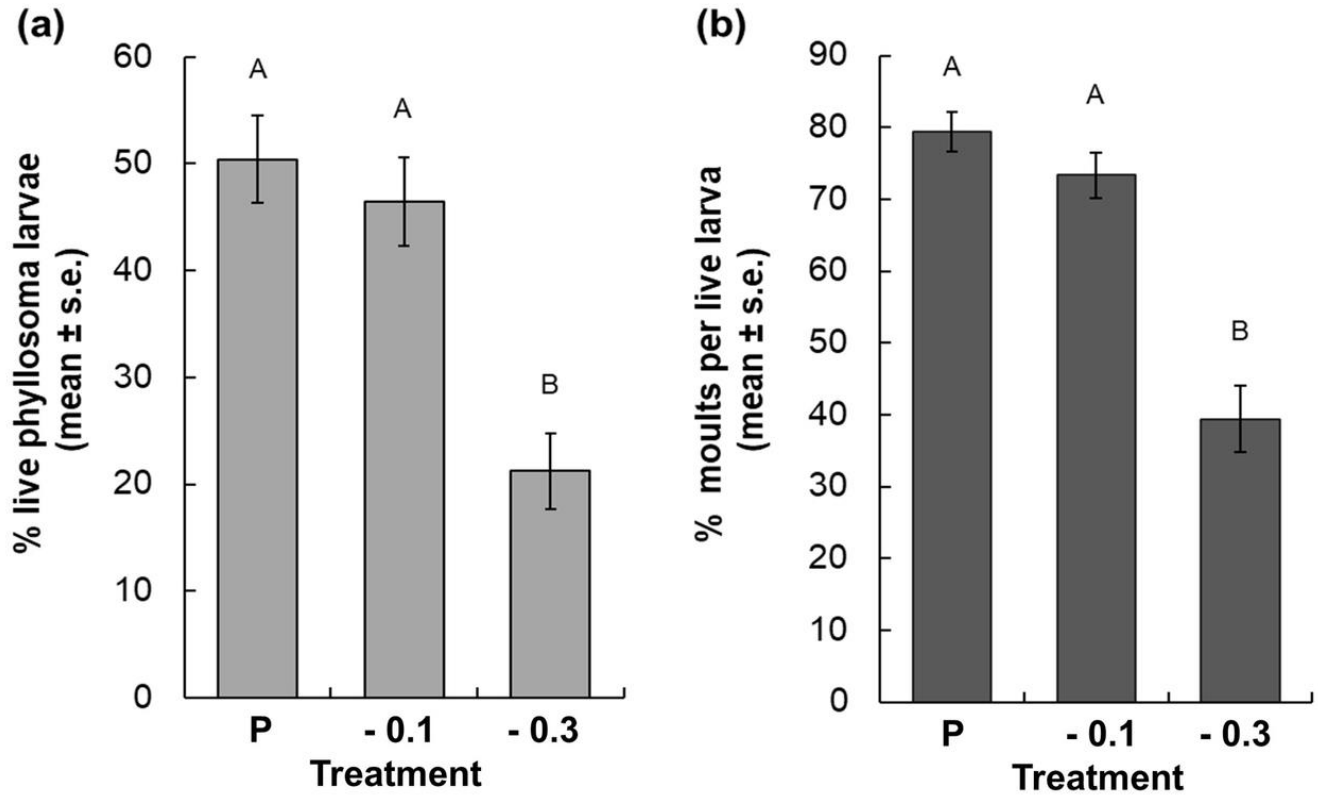

Figure 1. Percentage (mean ± s.e.) survival (a) and moults per live phyllosoma (b) in each treatment: Present pH (P), ΔpH = - 0.1 and ΔpH = - 0.3 relative to P. Letters above data points indicate similarities (e.g. AA) and differences (e.g. AB) between pH treatments based on estimated marginal means.

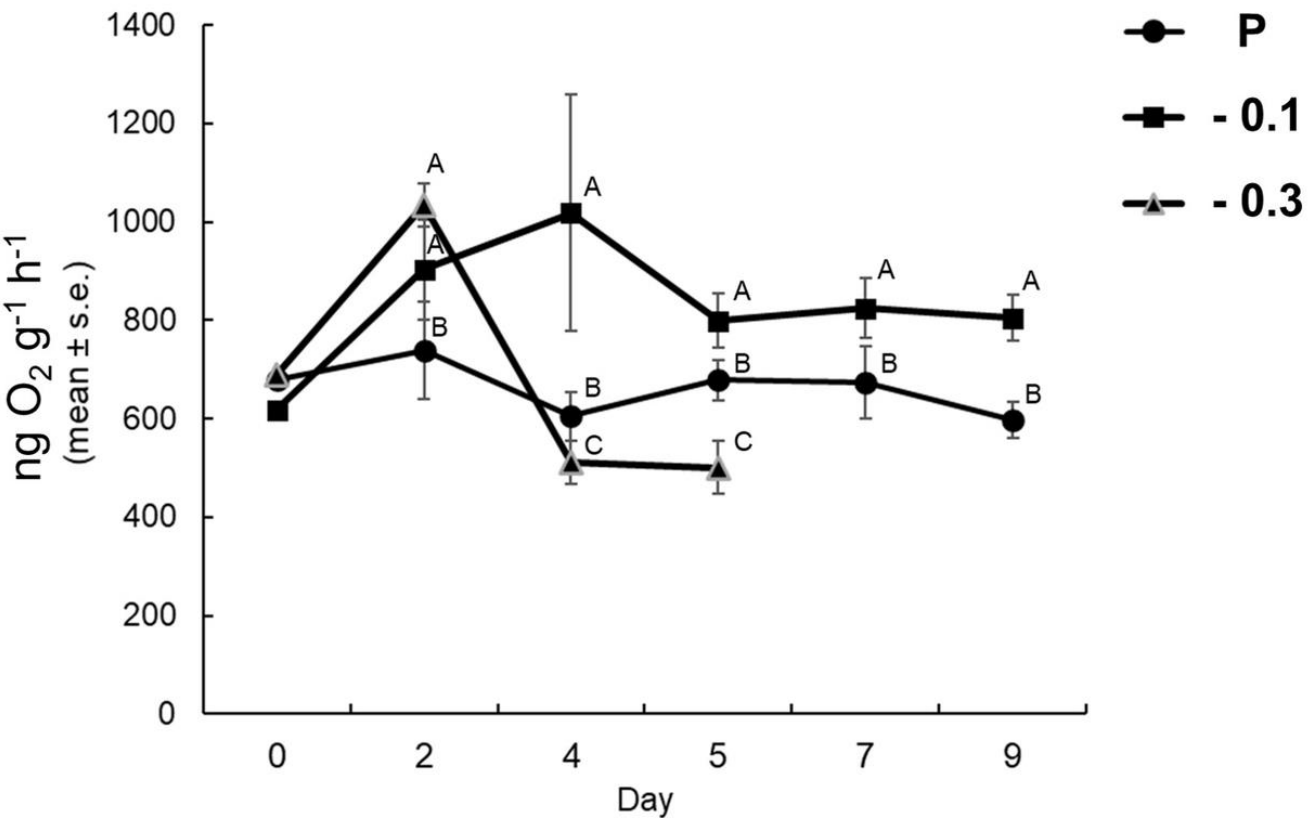

Figure. 2. Respiration rates (mean ± s.e.) of phyllosoma larvae in each treatment (Present pH (P), ΔpH = - 0.1 and ΔpH = - 0.3 relative to P) throughout the experiment. Letters above data points indicate similarities (e.g. AA) and differences (e.g. AB) between pH treatments within each day based on estimated marginal means.

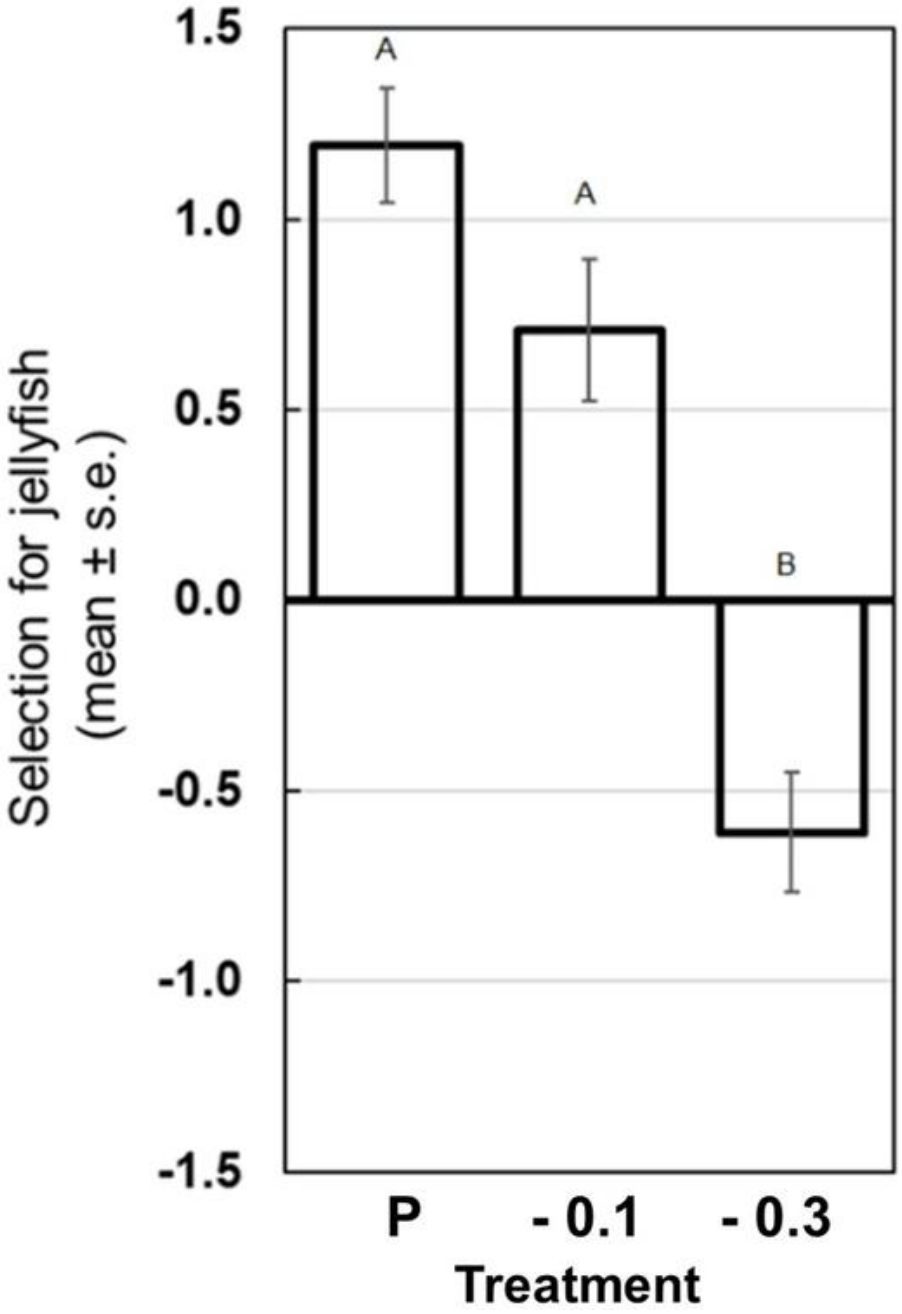

Figure 3. Selection (mean ± s.e.) for jellyfish tissue by phyllosomas reared in different pH treatments: Present pH (P), ΔpH = - 0.1 and ΔpH = - 0.3 relative to P. Values indicate attraction of phyllosomas towards jellyfish cube (> 0) or silicone blank (< 0). Letters above error bars indicate similarities (e.g. AA) and differences (e.g. AB) between pH treatments based on Tukey's HSD results of two-way ANOVA.

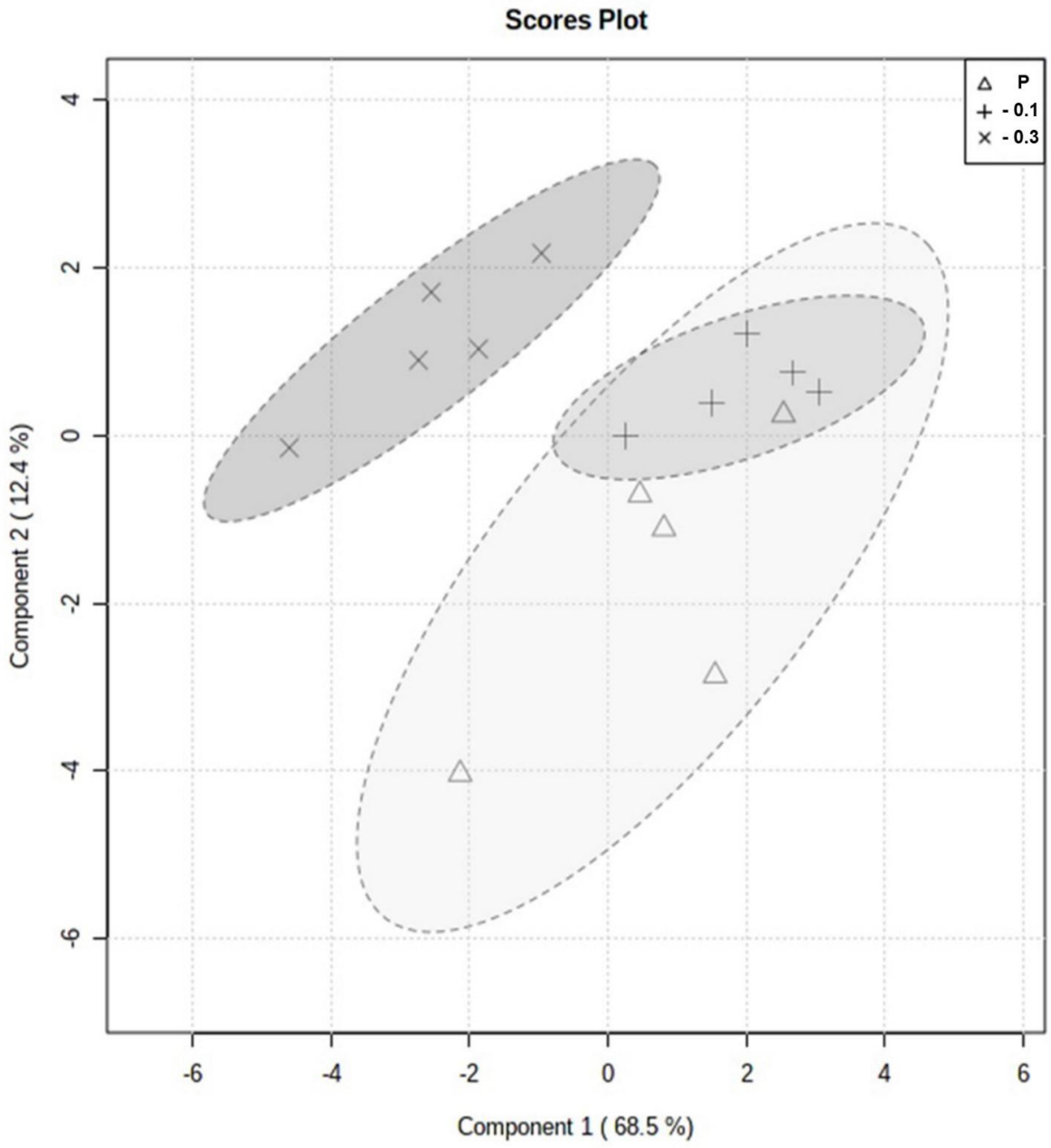

Figure 4. Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) output for the difference of metabolite composition of phyllosoma reared in each pH treatment: Present pH (P), ΔpH = - 0.1 and ΔpH = - 0.3 relative to P. Dashed lines indicate the margins of groups of samples with similar metabolite composition.

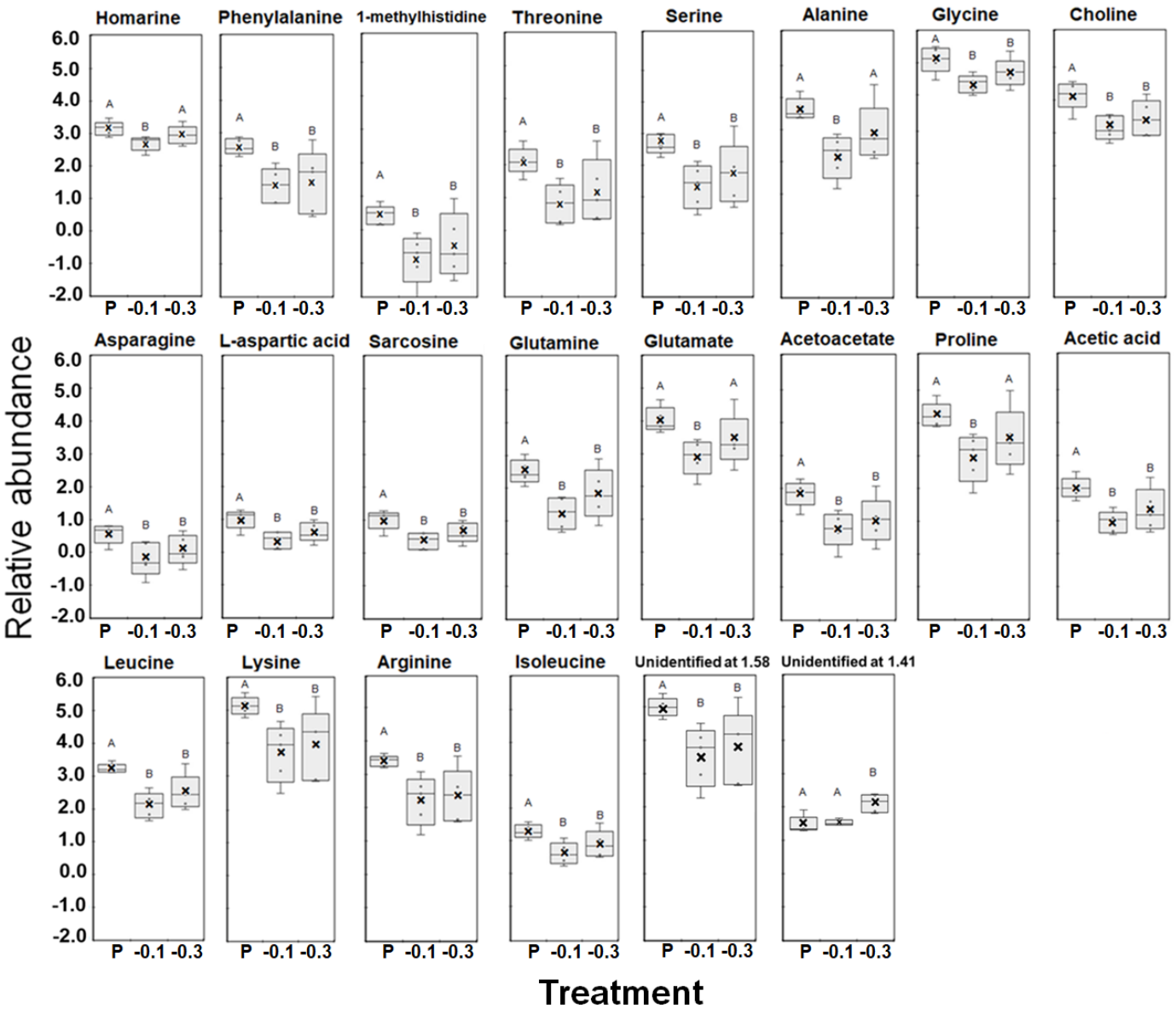

Figure 5. Relative abundance of phyllosoma metabolites that exhibited significant responses to pH treatments: Present pH (P), ΔpH = - 0.1 and ΔpH = - 0.3 relative to P. Boxplots represent the interquartile range, median (horizontal line), min and max (whiskers), and average (×). Letters above whiskers indicate similarities (e.g. AA) and differences (e.g. AB) between the treatments based on Tukey's HSD results of one-way ANOVA.

CRediT author statement

Sheldon Rey Boco: Conceptualization, Methodology, Data curation, Writing- Original draft preparation, Review, Editing. Kylie A. Pitt: Supervision, Conceptualization, Methodology, Data curation, Writing- Original draft preparation, Review, Editing. Steven D. Melvin: Supervision, Methodology, Data curation, Writing- Original draft preparation, Review, Editing

Ocean acidification impairs the physiology of symbiotic phyllosoma larvae of the lobster *Thenus australiensis* and their ability to detect cues from jellyfish

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Declaration of Conflict of Interest:

None.

Tables

Table 1. Average (± s.e.) seawater chemistry measurements and ranges of diel experimental pH conditions in this study. Calculated pCO_2 was determined using total alkalinity, pH, and temperature measurements at 10:00. DO is dissolved O_2.

Treatment	pH	pH range	DO (mg L^{-1})	Salinity (PSU)	Total alkalinity (µmol kg^{-1})	pCO_2 (µatm)
Present pH (P)	8.02 ± 0.03	7.90 – 8.12	6.17 ± 0.12	35.1 ± 0.09	2379 ± 16.8	463.49 ± 5.3
ΔpH = - 0.1	7.91 ± 0.03	7.80 - 7.99	6.23 ± 0.14	34.9 ± 0.06	2333 ± 24.4	591.89 ± 7.4
ΔpH = - 0.3	7.72 ± 0.05	7.61 - 7.74	6.15 ± 0.10	35.2 ± 0.09	2291 ± 28.3	1003.7 ± 12.2

ΔpH is relative to present-day mean pH conditions.

Table 2. Results of three generalized linear mixed model analyses of the number of live phyllosomas (survival), moulting in each day (days 1 – 11) and respiration rates at days 2, 4, 5, 7 and 9 between treatments in the experiment.

	Survival	Moulting	Respiration rate
Source of variation	*p*	*p*	*p*
pH	**<0.01**	**<0.01**	**0.009**
	F = 86.9	F = 13.586	F = 13.59
day	**<0.01**	**<0.01**	**0.001**
	F= 124.72	F = 29.769	F = 29.773
pH × day	0.421	0.098	**0.003**
	F = 1.041	F = 1.562	F = 1.564
Information criterion			
AIC	579.60	45.52	840.20
BIC	607.91	73.20	844.29

Akaike (AIC) and Bayesian information criteria (BIC).

P-values in bold are significant ($p < 0.05$). Numerator degrees of freedom *df* for pH, day and pH × day are 10, 2, and 15, respectively. Denominator *df* for all factors in survival = 104, and in moulting and respiration rates = 100.

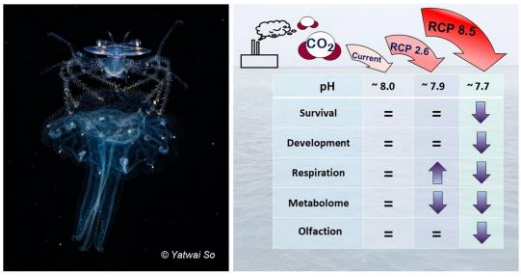
Table 3. Summary of results of two 2-way analyses of variance for the attraction of phyllosoma larvae towards jellyfish prey tissue and locomotion velocity (number of crossed grid square per second) of phyllosomas in the experiment.

Variable		Attraction to jellyfish tissue		Locomotion velocity	
Source of variation	df	F	*p*	F	*p*
pH	2	29.657	< **0.01**	2.003	0.150
Test water	1	0.064	0.801	2.013	0.165
pH × test water	2	0.158	0.854	1.745	0.189

P-value in bold is significant (< 0.05).

Degrees of freedom *df*

Graphical abstract



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Highlights

1. Ocean acidification (OA) under delta (Δ) pH = - 0.3 (pH ~7.7), but not ΔpH = - 0.1 (pH ~ 7.9) relative to the present (~8.0 pH), reduced the survival, respiration and moulting of phyllosomas of *T. australiensis.*

2. OA under pH ~7.7 adversely affected the attraction of *T. australiensis* phyllosomas to jellyfish cues.

3. The majority of individual metabolites of phyllosomas were suppressed even in mild pH ~ 7.9.

4. The interaction between phyllosoma and jellyfish may be impaired under pH ~7.7.