Pre-exposure to simultaneous, but not individual, climate-change stressors limits acclimation capacity of Irukandji jellyfish polyps to predicted climate scenarios

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Abstract

Researchers have investigated the immediate effects of end-of-century climate-change scenarios on many marine species, yet it remains unclear whether we can reliably predict how marine species may respond to future conditions because biota may become either more or less resistant over time. Here we examined the role of pre-exposure to elevated temperature and reduced pH in mitigating the potential negative effects of future ocean conditions on polyps of a dangerous Irukandji jellyfish *Alatina alata*. We pre-exposed polyps to elevated temperature (28 ºC) and reduced pH (7.6), in a full factorial experiment that ran for 14 d. We secondarily exposed original polyps and their daughter polyps to either current (pH 8.0, 25 ºC) or future conditions (pH 7.6, 28 ºC) for a further 34 d to assess potential phenotypically plastic responses and whether asexual offspring could benefit from parental pre-exposure. Polyp fitness was characterised as asexual reproduction, respiration, feeding, and protein concentrations. Pre-exposure to elevated temperature alone partially mitigated the negative effects of future conditions on polyp fitness, while pre-exposure to reduced pH in isolation completely mitigated the negative effects of future conditions on polyp fitness. Pre-exposure to the dual stressors, however, reduced fitness under future conditions relative to those in the control treatment. Under future conditions polyps had higher respiration rates regardless of the conditions they were pre-exposed to, suggesting that metabolic rates will be higher under future conditions. Parent and daughter polyps responded similarly to the various treatments tested, demonstrating that parental pre-exposure did not confer any benefit to asexual offspring under future conditions. Importantly we demonstrate that while pre-exposure to the stressors individually may allow Irukandji polyps to acclimate over short time-scales, the stressors are unlikely to occur in isolation in the long term and thus, warming and acidification in parallel may prevent polyp populations from acclimating to future ocean conditions.

Introduction

Global climate change is predicted to have profound impacts on marine ecosystems. With growing awareness of the vulnerability of marine biota to climate change, many manipulative experiments have exposed biota to predicted end-of-century warming and ocean acidification scenarios to assess their responses to climate change (Munday et al. 2013; Munday 2014). These studies have generally documented negative effects on behaviour (Nagelkerken and Munday 2015; Nagelkerken et al. 2016), physiology (Pörtner 2008), growth, and reproduction for most marine species (Harvey et al. 2013; Kroeker et al. 2013). Evidence is emerging, however, that biota may become more robust to changing ocean conditions over time via genetic adaptation (Munday et al. 2013) and non-genetic processes such as acclimation (Munday 2014; van Oppen et al. 2015; Foo and Byrne 2016).

Genetic adaptation is hypothesised to facilitate the persistence of marine species in the face of climate change (Bell 2013; Logan et al. 2014); however, this process typically occurs over many generations and thus is difficult to test in most metazoans (Bell 2013; Logan et al. 2014). Non-genetic acclimation (see Munday 2014), however, can occur over shorter timescales such as weeks (e.g. Bellantuono et al. 2011) and months (e.g. Form and Riebesell 2012). Most studies that have examined the ability of marine species to acclimate to future ocean conditions pre-expose (or precondition) biota to individual climate-change stressors (e.g. Bellantuono et al. 2011; Miller et al. 2012; Towle et al. 2016). These studies generally demonstrate that pre-exposure to individual climate-change stressors (such as warming or acidification) can induce phenotypically plastic responses that occur within (intra-generational acclimation: Middlebrook et al. 2008; Bellantuono et al. 2011) or across generations (trans-generational acclimation: Donelson et al. 2012; Parker et al. 2015; Thor and Dupont 2015) that help maintain fitness when organisms are re-exposed to the same

climate-change stressor. Recently, however, corals chronically exposed to acidification were found to be less tolerant when exposed to a short-term pulse of thermal stress (i.e. mimicking a summer 'heatwave') than corals pre-exposed to ambient conditions (Towle et al. 2016). Hence, pre-exposure to a single stressor may worsen the response of biota when exposed to different stressors. Pre-exposure of adult corals to the dual stressors of elevated temperature and pCO_2, however, can facilitate trans-generational acclimation, because larvae of pre-conditioned adult corals exhibited greater metabolic acclimation (higher rates of respiration) under future conditions than those that were not pre-conditioned to the dual stressors (Putnam and Gates 2015). Although these results suggest that pre-exposure to warming and acidification in combination may allow biota to acclimate to future conditions, it remains unclear whether warming, acidification, or the stressors in combination, induce physiological changes that may help biota to acclimate to future conditions.

Rapid intra-generational acclimation may be particularly important for marine organisms that rely mostly on asexual reproduction and have long generation times (Sunday et al. 2014; van Oppen et al. 2015). Phenotypically plastic changes that occur in response to changing environmental conditions can occur within a single generation (e.g. Bay and Palumbi 2015) and may be passed to asexually produced clones (van Oppen et al. 2015). Although data are limited in marine species, stress-induced changes have been observed to be inherited across asexual generations of terrestrial plants (Verhoeven et al. 2010). Indeed, the dandelion *Taraxacum officinale* exhibited epigenetic markers after 10–13 weeks of exposure to environmental stress. Genetically identical offspring of pre-exposed *T. officinale* were then maintained under ambient conditions. Although the asexual offspring of *T. officinale* were not exposed to environmental stress, they exhibited the same epigenetic markers as their pre-exposed parents (Verhoeven et al. 2010), demonstrating that phenotypic responses induced by environmental stress can be successfully transmitted to asexual offspring within a single

generation. We must now determine whether asexually produced offspring of marine species may benefit from parental preconditioning under changing ocean conditions.

Of all marine species that may acclimate to changing ocean conditions, venomous Irukandji jellyfish, a group of at least 14 species that cause a suite of debilitating symptoms known as Irukandji syndrome (see Gershwin et al. 2014), are of major concern because of their severe impacts on human health and enterprise (Carrette et al. 2012). Although northern Australia is a 'hotspot' for Irukandji jellyfish, they also occur in many locations worldwide including parts of the continental United States (Grady and Burnett 2003), Hawaii (Yoshimoto and Yanagihara 2002), and the Caribbean (Pommier et al. 2005). Despite their potential to cause severe human health impacts under changing ocean conditions, only one study has examined the immediate effects of warming and acidification on jellyfish polyps (Klein et al. 2014). Although warming enhanced asexual reproduction of the Irukandji jellyfish *Alatina alata*, rates of budding were much slower under reduced pH conditions (pH 7.6) suggesting that polyp populations may not thrive in the future (Klein et al. 2014). The potential role of acclimation in mitigating the apparent negative effects of future conditions on Irukandji polyp populations, however, remains unexplored.

Here we examine the role of pre-exposure to elevated temperature and reduced pH (separately and in combination), in potentially mitigating the effects of future ocean conditions on polyps of *Alatina alata* (Lawley et al., 2016). This study consisted of two parts: (1) the 14-d pre-exposure phase that exposed polyps to either elevated temperature, reduced pH conditions, the stressors in combination, or ambient (control) conditions; and (2) the secondary exposure, which subsequently exposed polyps to either current or future conditions over 34 d. Fitness was characterised as rates of asexual reproduction, respiration, feeding, and protein concentrations of polyps during the secondary exposure. We hypothesised that *A.*

alata polyps pre-exposed to ambient conditions would exhibit negative effects when exposed to future conditions, but that pre-exposure to reduced pH and elevated temperature (separately and simultaneously) would mitigate the negative effects of future conditions. To assess whether pre-exposure of parent polyps would benefit asexual offspring we exposed daughter polyps produced in each pre-exposure treatment to either current or future conditions. We hypothesised that daughter polyps produced by parents pre-exposed to future climate scenarios would exhibit enhanced fitness relative to parent polyps under future conditions. From this experiment, we present novel findings that demonstrate that pre-exposure to simultaneous (but not individual) climate change stressors limits the acclimation capacity of polyps of an Irukandji jellyfish to future climate conditions.

Materials and methods

Species studied

Polyp cultures used in the experiment originated from planula larvae that were collected from adult *A. alata* medusae sampled from spawning aggregations at Osprey Reef, Australia (13.92°S, 146.63°E) during five summer sampling trips between 2000 and 2006 (Carrette et al. 2014). Eight *A. alata* polyps were detached from plastic settling dishes using a stainless steel dissecting probe and transferred into 48 individual glass petri dishes using a glass pipette (i.e. a total of 384 polyps were used). Similarly, additional single polyps were transferred into 24 12.01-mL glass vials. The experiment was done in a controlled-temperature laboratory with the ambient temperature set at 22 ºC so that the temperature of all replicate aquaria could be raised to their respective temperature treatments using aquarium heaters. Polyp populations of Irukandji jellyfish (including those of *A. alata*) have not yet

been located in the field. Polyps of *A. alata*, however, are hypothesised to occur in deeper waters because optimal thermal and salinity conditions match those in waters <120 m at Osprey Reef, Australia (Courtney and Seymour 2013. All polyps were, therefore, acclimated to laboratory conditions for four weeks at 25 ºC (Courtney and Seymour 2013 under a 12:12 light:dark cycle in low light conditions (~97 µmol photons m^{-2} s^{-1}) to mimic conditions at moderate depths and to prevent algal growth. During acclimation and throughout the experiment, polyps were fed newly hatched *Artemia* sp. nauplii every third day (~50 *Artemia* per well, following Courtney and Seymour 2013) and were allowed feed for ca. 2 h.

Pre-exposure phase

The pre-exposure phase consisted of two orthogonal factors: temperature [two levels: ambient (mean ± SE at 1000 hrs = 25 ± 0.01 ºC) and elevated (28± 0.01 ºC)] and pH [two levels: ambient (pH 8.0 ± 0.02) and reduced (pH 7.6 ± 0.01)]. Nominal future pH and temperature conditions (pH 7.6, 28 ºC) were based on RCP8.5 pathway projections for ca. 2100 (IPCC 2014). Many coastal ecosystems exhibit substantial diel fluctuations of pH because pH is reduced at night when photosynthesis ceases and community respiration increases (Hofmann et al. 2011). To accurately mimic natural environmental conditions in coastal ecosystems we therefore exposed polyps in all treatments to diel fluctuations of pH (ambient: ~7.9–8.1, reduced pH: ~7.55–7.75) that were based on 24-h field measurements taken in October 2014 in Moreton Bay, Australia (27.13ºS, 153.07ºE). Six 1-L glass aquaria, each containing two polyp dishes (i.e. 16 polyps in total) and one respiration vial, were randomly allocated to each of the four treatments (n = 24) (Fig. 1). Each replicate aquarium was gently aerated and partially submerged in an individual 5-L water bath. Lids were placed loosely over each aquarium with a header space of ~20 mm to minimise evaporation. Aquarium heaters placed in the water baths maintained the desired temperatures within 0.4 ºC

and aerators circulated water and maintained an even distribution of heat. Approximately 20% of seawater within each replicate aquarium was replaced every second day.

Each day, polyp dishes were removed from all aquaria and polyps were checked for new buds. To ensure that parents had been exposed to their respective pre-exposure treatments for a reasonable time, only daughter polyps produced after 10 d were used in the secondary exposure. It took 4 d to harvest sufficient daughter polyps from all replicate aquaria but the majority of daughter polyps were harvested after 12 d of pre-exposure. Daughter polyps were transferred into separate glass dishes and were randomly assigned to new aquaria that were maintained at the same experimental conditions as their parents to minimise bias. Similarly, daughter polyps produced in the respiration vials after 10 d were transferred into separate glass vials filled with 10-µm filtered seawater for measurements of respiration and also placed in separate aquaria. At the end of the pre-exposure phase there were 48 replicate aquaria, consisting of 24 aquaria containing parent polyps and 24 aquaria containing daughter polyps. Parent and daughter dishes within pre-exposure treatments were pooled and then randomly assigned to their respective replicate aquaria to further minimise bias (Fig. 1). The pre-exposure phase ran for 14 d to allow for sufficient time for polyps to asexually reproduce under the various treatments tested.

Experimental approach of the secondary exposure

The secondary exposure consisted of four orthogonal factors: temperature pre-exposure (two levels: 25ºC and 28ºC), pH pre-exposure (two levels: pH 8.0 and pH 7.6), asexual generation (two levels: parent polyps, daughter polyps) and climate scenario [two levels: current day (pH 8.0, 25 ºC); and future (pH 7.6, 28 ºC)]. Polyps in all treatments were exposed to the same diel fluctuations of pH as those in the pre-exposure phase (current day: ~7.9–8.1, future: ~7.55–7.75). Prior to the secondary exposure, the number of polyps in the dishes and

respiration vials were standardised to four and one, respectively. Three replicate 1-L glass aquaria, each containing two polyp dishes (a total of eight polyps) and one vial, were assigned to each of their respective treatment combinations (total n = 48) (Fig. 1). Within each replicate aquarium, one polyp dish was allocated for asexual reproduction measurements and at the end of the secondary exposure, polyps from both dishes were used for measurements of total protein and prey capture rates. Two polyp dishes were allocated to each replicate aquarium to prevent overcrowding of polyps and for ease of removing excess algal growth. The secondary exposure ran for 34 d to allow sufficient time for polyps to asexually reproduce multiple times (Klein et al. 2014).

Manipulation and analysis of water chemistry

To achieve the desired water chemistry conditions of each treatment, a series of gas proportioners were used to deliver CO_2, N_2, and O_2 gas to seawater. The desired gas compositions were mixed from individual gas cylinders using twelve Omega mass flow controllers [FMA-5400s, 0–20 mL min^{-1} (CO_2), 0–5 L min^{-1} (N_2), 0–2 L min^{-1} (O_2); Bockmon et al. 2013]. Two sets of three mass flow controllers were used to deliver gas mixtures to each pH treatment (pH 8.0 and pH 7.6). The mass flow controllers were operated and functions monitored by a desktop PC running NI LabVIEW software (32-bit version) with communication using a voltage-generating Omega expandable modular data acquisition system (iNET-400) connected with three Omega wiring boxes with screw terminals (iNET-510). After the three gases were mixed in the desired proportion for each treatment the gases were then combined in a stainless-steel manifold before the gas line was split, providing identical gas mixtures to the replicate aquaria. Flow rates to replicate aquaria were manually adjusted using secondary stainless-steel manifolds with control valves. For both pH treatments, two gas compositions were determined to closely mimic diel fluctuations in water chemistry in the natural environment (Electronic supplementary material, ESM, Fig. S1). NI

LabVIEW software was used to linearly transition between the day and night gas mixtures but gas compositions were held constant at night time (1800–0600 hrs) and between 1000 and 1400 hrs.

Every third day, temperature, salinity, pH, and DO were measured at midday (ESM Table S1). Salinity and temperature were measured in each aquarium using a conductivity-salinity meter (TPS salinity-conductivity meter, MC-84) and thermometer, respectively. The dissolved oxygen (DO) concentration in each aquarium was recorded using an optic DO sensor (Mettler Toledo OptiOPx, Mettler Toledo Ltd) and exceeded 85% O_2 saturation in all replicates throughout the experiment. The pH of each aquarium was measured using a FiveGo pH meter (Mettler Toledo Ltd) equipped with an Inlab Expert Pro Electrode (Mettler Toledo Ltd). Every 2–3 d, 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 patterns of pH during the experiment, pH measurements were taken hourly (between 0600 and 1800 hrs) from one randomly selected replicate from each of the treatments once per week (ESM Fig. S1). Levels of pCO_2 were calculated based on measured levels of total alkalinity (TA), pH, temperature, and salinity using the program CO2SYS (Lewis et al. 1998) (ESM Table S1). Every third day, a 100-mL water sample was collected for analysis of TA from one randomly selected replicate from each of the treatments. Samples of seawater (100 mL) 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. All samples were filtered through 0.22-µm filters, spiked with 20 µL of mercuric chloride, sealed tightly, and stored at 3 ºC to prevent biological activity until samples were analysed. All TA samples were analysed using a Mettler Toledo T50 automatic titrator, which was also calibrated using TRIS/HCl buffers in synthetic

seawater. TA measurements on 50-mL samples of certified reference material (provided by A. G. Dickson, batch #138) were used to verify TA values.

Data collection

Four response variables were measured: asexual reproduction, respiration, protein concentration, and prey capture rate. Each day during the pre-exposure phase, polyps were removed from aquaria and viewed under a dissecting microscope and the number of polyps and their developmental stage (i.e. polyp, budding or partial fission or juvenile medusae) was recorded. During the secondary exposure, polyps were counted every third day and at the end of the experiment, polyp dishes in each replicate aquarium were removed to count and collect polyps for estimations of total protein and rates of prey capture.

Respiration measurements

The respiration rates of polyps in all replicate aquaria were measured during the secondary exposure at Days 1, 4, 14, 24, and 34 using a Unisense PA200 picoammeter equipped with a 20–30-µm tip O_2 microsensor (OX-25 Unisense, Denmark). One control (blank) vial was also placed in each replicate aquarium to account for the respiration of bacterial communities and other micro-organisms that had formed during the experiment. All vials were sealed with double-wadded caps designed for multi-sampling and were partially submerged in temperature-controlled water baths that were heated to the same temperature as their respective thermal treatments. The seawater was 0.22-µm filtered and of the same water chemistry as the respective treatments. Water within each vial was gently mixed during the incubation and O_2 saturation was never <70%. O_2 concentrations were determined using a linear equation calculated from a two-point calibration curve (pA at 0% and 100% O_2 saturation). For all replicate vials, O_2 consumed by the corresponding blank vials were subtracted from polyp vials to obtain respiration rates of polyps (units: ng O_2 $polyp^{-1}$ h^{-1}).

Protein estimations

Total protein estimations were measured to investigate potential up- (or down-) regulation of protein synthesis as a means of assessing potential trade-off mechanisms by which polyps acclimated. Ten polyps from each replicate aquarium were transferred into pre-labelled cryogenic centrifuge tubes that contained a cocktail of protease inhibitors (P8340, Sigma-Aldrich) to inhibit protein degradation, diluted to a volume ratio of 1:100 (inhibitor: TRIS buffer). The QuantiPro bicinchoninic acid (BCA) kit (QPBCA, Sigma-Aldrich) was used to measure protein concentrations and bovine serum albumin (BSA, 1.0 mg mL^{-1} in 0.15M NaCl with 0.05% sodium azide, Sigma-Aldrich) was used as the protein standard. For standard curve determinations, BSA standards (0.5– 30 µg mL^{-1}) were prepared in TRIS and NaCl (artificial seawater) buffers. All samples (in TRIS buffer) that formed a precipitate were centrifuged after colour development and the absorbance of the supernatant measured. The absorbance of all samples at 562 nm was measured against a blank using a UV spectrophotometer (UV-1800, Shimadzu). We compared the standard curves of TRIS and NaCl buffer standards and confirmed that the formation of the precipitate in TRIS buffer samples did not affect colour development, and thus did not affect protein estimations of polyp samples.

Measurements of prey capture rates

At the end of the secondary exposure, four polyps from each replicate aquarium were transferred into individual wells of a 96-well plate. *Artemia* sp. nauplii were used as prey to examine the prey capture rate of polyps. Each well contained 10-µm filtered seawater. Polyps were allowed ~1 h to acclimate to well conditions. During the acclimation period, seawater was partially replenished every 10 min to maintain normoxic conditions. Approximately 15 *Artemia* sp. nauplii were added to each well, and the time and exact number of nauplii added

to each well was recorded. To ensure that DO concentrations within individual wells remained normoxic during the feeding experiments, polyps were allowed to feed for 20 min before the number of remaining prey was recorded in each well. Prey capture rates of polyps from each replicate aquarium were summed across the four polyp wells for statistical analyses (i.e. the unit of replication was the average percentage of prey captured across the four polyp wells for each aquarium).

Statistical analyses

The dependent variables—number of polyps, protein concentration (ng $polyp^{-1}$), and prey capture rates (% prey captured) at Day 34— were analysed using linear mixed models (LMMs) in SPSS. The four fixed factors were temperature pre-exposure, pH pre-exposure, asexual generation, and climate scenario. The dependent variable respiration (ng O_2 $polyp^{-1}$ h^{-1}) in all treatments was analysed using repeated measures LMMs. The five fixed factors were temperature pre-exposure, pH pre-exposure, asexual generation, climate scenario, and time, which was the repeated measure. A range of models were investigated to assess the model of best fit by comparing various goodness-of-fit statistics (e.g. -2 Restricted Log Likelihood, Akaike's Information Criteria (AIC) and Bayesian Information Criterion (BIC)). To test for potential aquarium bias, we included a random factor aquarium (or block) into all LMMs analyses. Preliminary analyses for all dependent variables, however, revealed no significant effects of aquarium, so the factor was removed and the analyses re-run. Data were checked for normality and homoscedasticity using standardised residual and Q-Q plots and if required, data were either ln or ln(x+1) transformed. If significant differences were found, estimated marginal means (post-hoc comparisons of least-squares means) were used to determine which means differed using the EMMEANS sub-command to obtain estimated marginal means for the significant, highest-order terms. Multiple comparisons were done for all relevant fixed factors.

Results

Survival and asexual reproduction

All polyps of *A. alata* survived experimentation and the number of polyps increased in all treatments during the secondary exposure. There was no difference between parent and daughter polyps for any of the response variables tested (Tables 1, 2). At the end of the secondary exposure, numbers of polyps varied substantially among treatments, resulting in a significant pH × temp × climate scenario interaction (Table 1; Fig. 2). Specifically, polyps pre-exposed to ambient conditions produced 56% fewer polyps when exposed to future conditions than polyps that remained in ambient conditions. Polyps pre-exposed only to elevated temperature produced 29% fewer polyps when exposed to future conditions relative to those in the control treatment, but 52% more than the treatment that had been pre-exposed to ambient conditions and transferred to future conditions (Fig. 2). Polyps pre-exposed to reduced pH alone produced a similar number of polyps when exposed to future conditions to those in the control treatment but substantially more polyps than the treatment that had been pre-exposed to ambient conditions and transferred to future conditions. Polyps pre-exposed to the stressors simultaneously, however, produced approximately 43% fewer polyps than those in the control treatment, regardless of whether they had been transferred to ambient or future conditions (Fig. 2). Thus, pre-exposure to elevated temperature alone appeared to partially mitigate the negative effects of future conditions on polyp fitness, while pre-exposure to reduced pH alone completely mitigated the negative effects of future conditions on polyp fitness. Pre-exposure to the stressors in combination, however, did not appear to sustain polyp fitness under future conditions.

Respiration rates

329 The temporal variation in respiration rates differed among treatments, resulting in a
330 significant pH × climate scenario × day interaction (Table 2; Fig. 3a, b). Overall, respiration
331 rates were higher under future conditions compared to ambient conditions, regardless of
332 whether polyps had been pre-exposed to low or ambient pH (Fig. 3a, b). The only exception
333 was for Day 1 of the secondary exposure where respiration rates did not differ between
334 ambient and future conditions for polyps pre-exposed to ambient conditions (Fig. 3a). Polyps
335 pre-exposed to ambient pH had respiration rates that were ~63% higher under future
336 conditions than those in ambient conditions (Fig. 3a). Similarly, after Day 1 of the secondary
337 exposure, respiration rates of polyps pre-exposed to reduced pH were ~52% higher when
338 exposed to future conditions than those under ambient conditions (Fig. 3b).

339 **Protein concentrations**

340 At the end of the secondary exposure, protein concentrations varied substantially among
341 treatments, resulting in the pH × temp × climate scenario interaction (Table 1; Fig. 4). At the
342 end of the secondary exposure, polyps pre-exposed to ambient conditions had 55% lower
343 protein concentrations when exposed to future conditions than polyps that remained in
344 ambient conditions. Conversely, polyps pre-exposed to high temperature and reduced pH
345 individually and in combination had similar protein concentrations regardless of whether they
346 were exposed to ambient or future conditions (Fig. 4). Overall, pre-exposure to high
347 temperature and reduced pH individually appeared to mitigate the negative effects of future
348 conditions on protein concentrations because the protein content of these polyps was greater
349 than for the polyps that had been pre-exposed to ambient conditions and transferred to future
350 conditions. Polyps pre-exposed to the dual stressors, however, had 48% lower protein
351 concentrations when exposed to future conditions than those in the control treatment. The
352 response of these polyps was no different to the polyps raised in ambient conditions and
353 subsequently transferred to future conditions (Fig. 4).

Rates of prey capture

We examined the ability of polyps to capture prey at the end of the secondary exposure to evaluate energy intake and thus, their ability to support basal maintenance costs. Specifically, prey capture rates varied among pre-exposure treatments, but patterns were not consistent among climate change scenario treatments (Table 1; Fig. 5). Polyps pre-exposed to ambient conditions exhibited similar rates of prey capture when exposed to future conditions to those that remained in ambient conditions (Fig. 5). Similarly, polyps pre-exposed to high temperature and reduced pH individually exhibited similar rates of pre-capture regardless of whether they were subsequently exposed to ambient or future conditions. Overall, polyps pre-exposed to high temperature and reduced pH individually had (on average) 32% lower prey capture rates than those in the control treatment, regardless of the conditions they were subsequently exposed to. These polyps, however, produced ~65% more polyps than the treatment that had been pre-exposed to the dual stressors and remained in future conditions. Prey capture rates of polyps pre-exposed to reduced pH and high temperature in combination were 75% lower than those that were subsequently exposed to ambient conditions (Fig. 5). Polyps that were pre-exposed to high temperature and reduced pH in combination and remained in future conditions were exposed to the dual stressors for a longer period of time overall than other treatments and exhibited lower prey capture rates than those in all other treatments (Fig. 5).

Discussion

Pre-exposure to individual climate-change stressors can induce phenotypically plastic responses that allow marine biota to cope under future conditions (e.g. Bellantuono et al. 2011; Miller et al. 2012; Towle et al. 2016). Our data are consistent with these observations,

specifically that pre-exposure to reduced pH appeared to completely sustain fitness under future conditions, whereas pre-exposure to elevated temperature appeared to only partly mitigate the negative effects of future conditions. Ocean warming and acidification, however, are occurring concurrently on a global scale (IPCC 2014) and thus, biota are likely to be exposed to these stressors simultaneously. Most importantly, we observed that polyps pre-exposed to elevated temperature and reduced pH in combination did not sustain fitness under future climate-change conditions. Our observations suggest that Irukandji polyp populations, such as those of *A. alata*, are likely to persist but asexually reproduce at a slower rate in response to the dual stressors. Studies that pre-expose biota to individual climate-change stressors may be limited in their ability to provide a realistic understanding of how biota are likely to acclimate to warming and acidification in parallel. These observations highlight the importance of investigating how pre-exposure to multiple stressors may confer effects that differ from those when biota are pre-exposed to the stressors individually.

Marine organisms may acclimate to future conditions by altering the relative amount of energy allocated to metabolic processes to maintain fitness (Sunday et al. 2014). Although studies of the effects of warming and acidification on physiological trade-offs in marine organisms are limited, observations of biota exposed to acidification demonstrate that marine organisms may acclimate to high CO_2 conditions by re-allocating energy to different functions (e.g. Reipschläger and Pörtner 1996; Pörtner and Bock 2000; Michaelidis et al. 2005). Indeed, exposure to reduced pH conditions (pH 7.7–6.8) over 40 d increased rates of calcification and respiration in the brittle star *Amphiura filiformis* (Wood et al. 2008). These compensatory mechanisms, however, coincided with the partial resorption of arm muscles of *A. filiformis* and suggest that while the upregulation of metabolism and calcification may potentially mitigate the effects of acidification conditions on *A. filiformis*, these mechanisms may come at a substantial cost because of elevated energy demands under elevated pCO_2

403 conditions (Wood et al. 2008). In our study, respiration rates of polyps were higher under
404 future conditions regardless of the conditions to which polyps were pre-exposed.
405 Observations of asexual reproduction and protein concentrations, however, demonstrate that
406 only pre-exposure to the stressors individually mitigated the negative effects of future
407 conditions. Pre-exposure to the stressors individually probably induced physiological changes
408 that allowed polyps to cope under future conditions, but polyps pre-exposed to the dual
409 stressors may have re-allocated energy away from reproduction and protein synthesis to
410 support basal maintenance costs under future conditions. Although we did not investigate
411 specific compensatory mechanisms, these results further support the observations that marine
412 organisms may increase metabolic rates to compensate for the additional energy expenses of
413 basal maintenance under changing ocean conditions (Calow 1991; Pörtner et al. 2006;
414 Sokolova 2013).

415 The ability of marine species to acquire and assimilate food may ultimately determine
416 their ability to support basal maintenance costs and survive under changing ocean conditions
417 (Sokolova 2013). Indeed, reduced rates of feeding and assimilation may decrease the fitness
418 of biota under future climate-change conditions because there is less energy available for
419 growth and reproduction after energy is allocated to maintaining basal maintenance. For
420 example, consumption rates of the sea urchin *Lytechinus variegatus* increased when
421 temperature was elevated to 29 ºC but decreased when exposed to 31 ºC (Lemoine and
422 Burkepile 2012). Respiration rates of *L. variegatus*, however, increased exponentially with
423 increasing temperature (20–31 ºC). Reduced consumption rates and elevated rates of
424 metabolism under more extreme temperature conditions (31 ºC) reduced the ingestion
425 efficiency of *L. variegatus* by ~50% and was hypothesised to reduce overall fitness (Lemoine
426 and Burkepile 2012). In the current study, polyps pre-exposed to high temperature and
427 reduced pH in combination had the lowest food consumption rates when exposed to future

conditions, which suggests that basal maintenance costs of polyps will probably be higher in response to the dual stressors and ultimately reduce rates of asexual reproduction, similar to the results of asexual reproduction and protein concentrations. We therefore advocate that for experiments to accurately predict the response of marine organisms over longer timeframes, they should measure multiple responses to assess potential trade-offs that may lead to overall reductions in fitness.

Parent and daughter polyps responded similarly to the various treatments tested. These observations do not support the hypothesis that pre-exposure of parent polyps to elevated temperature and reduced pH would confer benefits to daughter polyps under future conditions. We cannot determine whether non-genetic changes were passed on to genetically identical offspring because this study did not identify potential non-genetic mechanisms (such as the transmission of specific proteins and hormones) or epi-genetic factors (e.g. DNA methylation) because they typically require advanced whole transcriptomic approaches (e.g. Moya et al. 2012; Pespeni et al. 2013). To better determine whether genetically identical offspring inherit phenotypically plastic responses from parents that are pre-exposed to climate-change stressors, we must now identify potential non-genetic mechanisms and epi-genetic markers that may allow asexual offspring to benefit from parental pre-exposure.

Our finding that pre-exposure to the combined effects of reduced pH and elevated temperature did not mitigate the effects of future conditions on *A. alata* polyps contrasts with those of the only other study to examine whether preconditioning to warming and acidification in combination could mitigate the negative effects of future conditions (Putnam and Gates 2015). Preconditioning of the symbiotic and calcifying coral, *Pocillopora damicornis*, to constant levels of elevated temperature and reduced pH over 1.5 months resulted in greater metabolic acclimation of their larvae under future conditions than those of colonies that were preconditioned to ambient conditions (Putnam and Gates 2015). These

453 results suggest that trans-generational acclimation may be important in the persistence of
454 marine species under the effects of the dual stressors. Results of our study, however, suggest
455 that although polyps of *A. alata* pre-exposed to the dual stressors survived, they may have a
456 limited ability to acclimate to the combined effects of warming and acidification within a
457 single generation. *Pocillopora damicornis* may have acclimated to the combined effects of
458 warming and acidification because symbiotic dinoflagellates (such as *Symbiodinium*) can
459 exert significant control over the internal pH of host tissues (Laurent et al. 2013, 2014;
460 Gibbin et al. 2014; Klein et al. 2017) and thus, symbionts were probably important in
461 mitigating the negative effects of acidification on *P. damicornis*. Consequently, differences in
462 results between Putnam and Gates (2015) and this study could be attributed to the absence of
463 endosymbionts in polyps of *A. alata*. These observations highlight how symbiotic and non-
464 symbiotic cnidarians may acclimate differently to warming and acidification in combination.

465 The extent to which we can accurately predict how marine species, such as *A. alata*,
466 may respond to changing ocean conditions depends partly on whether biota can acclimate or
467 adapt over longer time scales than those tested in manipulative experiments. The duration of
468 pre-exposure in studies that have examined the ability of marine species to acclimate to future
469 ocean conditions can vary from hours (e.g. Middlebrook et al. 2008) or days (e.g.
470 Bellantuono et al. 2011), to months (e.g. Form and Riebesell 2012). Indeed, recent evidence
471 suggests that the duration of pre-exposure (or preconditioning) can either limit or enhance the
472 ability of marine species to acclimate (Foo and Byrne 2016). For example, adult sea urchins,
473 *Psammechinus miliaris*, pre-exposed to reduced pH for 70 d produced smaller larvae than
474 those produced by parents pre-exposed to reduced pH over 28 and 42 d (Suckling et al.
475 2014). Conversely, larvae of adult sea urchins, *Strongylocentrotus droebachiensis*,
476 acclimated to reduced pH for 16 months had greater survival rates than those of adults
477 acclimated to reduced pH for only 4 months (Dupont et al. 2013). In the current study, polyps

of *A. alata* were pre-exposed to warming and acidification (separately and in combination) over 14 d to allow for sufficient time for polyps to asexually reproduce under the various treatments tested. Polyps appeared to acclimate to warming and acidification in isolation, but their acclimation capacity was limited when the stressors co-occurred. It is possible that a longer pre-exposure phase that exposed multiple asexual generations of *A. alata* polyps to the dual stressors may have yielded a different response. Combined, these studies highlight the need to consider the duration of reproduction and development of individual marine species when assessing appropriate acclimation periods because the rates of such processes may differ considerably among marine species.

Marine biota are often subject to fluctuations in environmental conditions due to changes associated with tidal and diel cycles (Hofmann et al. 2011), nutrient input (Frieder et al. 2014) and seasonal extremes (Pennington and Chavez 2000). In some cases, diel fluctuations in pH and temperature exceed future climate change projections (e.g. pH: Hofmann et al. 2011; temperature: Oliver and Palumbi 2011) and may act to precondition or pre-expose biota to elevated temperature and reduced pH conditions; thus, biota may become more robust to future ocean conditions (Byrne and Przeslawski 2013). To persist under future climate-change conditions, however, phenotypically plastic responses induced by environmental fluctuations must persist when environmental conditions return to ambient. In our study, polyps pre-exposed to elevated temperature and reduced pH individually (but not simultaneously) had similar protein concentrations, rates of asexual reproduction and prey-capture regardless of whether they subsequently transferred to future or ambient conditions. Although data are limited, our observations are generally consistent with studies that demonstrate that phenotypically plastic responses persist when biota are subsequently returned to ambient (or control) conditions (e.g. Middlebrook et al. 2008; Hettinger et al. 2012; Putnam and Gates 2015). For example, exposing larvae of the Olympia oyster (*Ostrea*

lurida) to acidification reduced juvenile shell sizes and these changes persisted for >1.5 months after juveniles were subsequently transferred to ambient conditions (Hettinger et al. 2012). These observations thus demonstrated persistent carry-over effects from the larval phase. Taken together these observations further highlight the need to examine the duration and magnitude of acclimation processes and that phenotypically plastic responses that occur during environmental fluctuations may ultimately determine the response of biota to future climate-change conditions.

Northern Australia is a 'hot spot' for dangerous cubozoan jellyfish but envenomations occur throughout the tropics worldwide (Gershwin et al. 2010). Our results suggest that, if other cubozoan jellyfish respond similarly, polyp populations are likely to persist but reproduce asexually at a slower rate in response to the dual stressors. To more accurately predict how Irukandji jellyfish, as a group, are likely to respond to future conditions, we must now determine whether results of this study are consistent with other species and other life-history stages. To better determine whether asexual offspring of other non-calcifying cnidarians may benefit from parental pre-exposure in the face of climate change, we must also identify potential non-genetic mechanisms and epi-genetic factors that may be passed to asexual offspring. Importantly, we highlight the need to investigate how pre-exposure to individual stressors may impart effects that differ from those of pre-exposure to multiple stressors and demonstrate that biota may acclimate to future climate conditions over short time scales. Only through the combination of manipulative experiments, such as this one, and monitoring studies that measure long-term changes in abundance and distribution of marine species (e.g. Chivers et al. 2017), is it possible to accurately assess how biota respond to changing ocean conditions.

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Figures

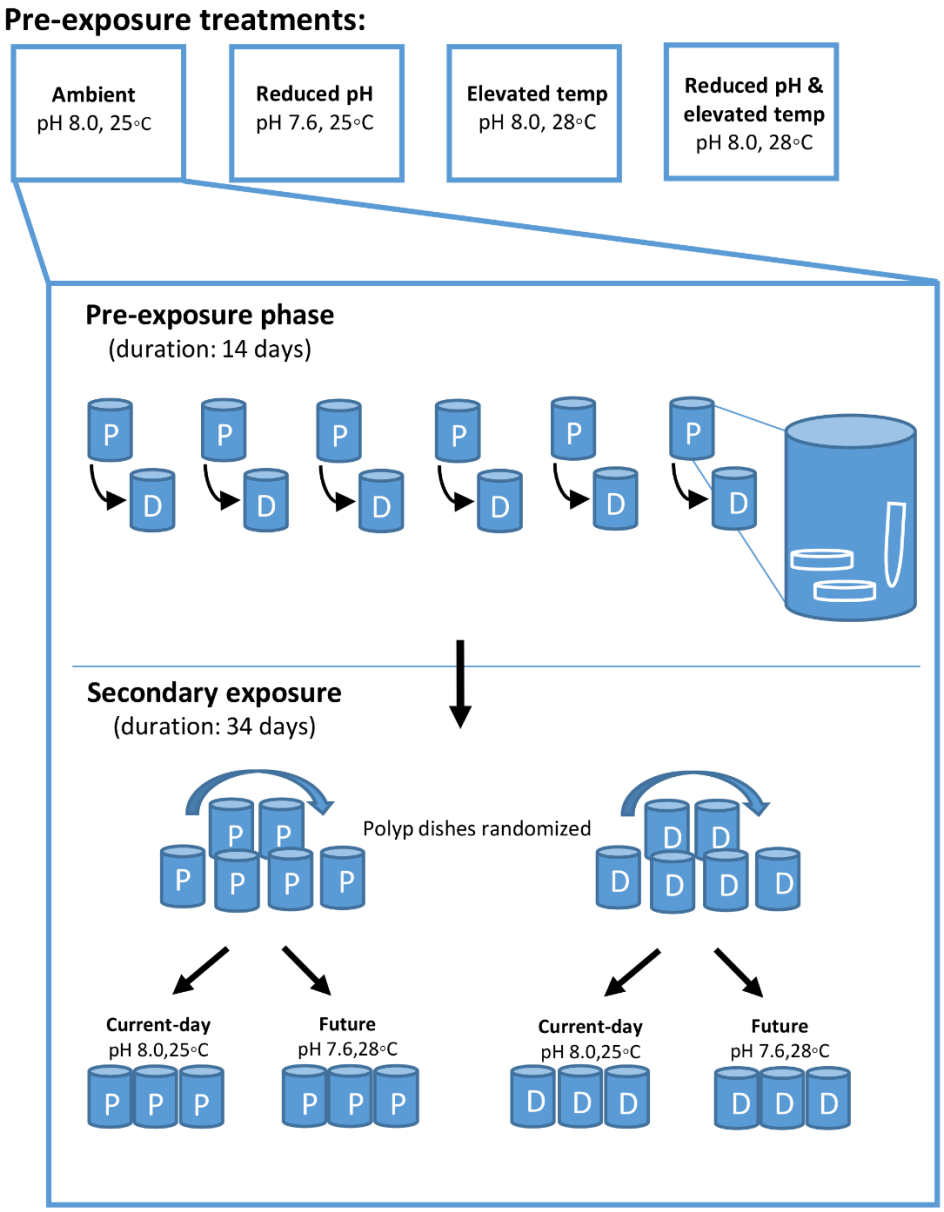
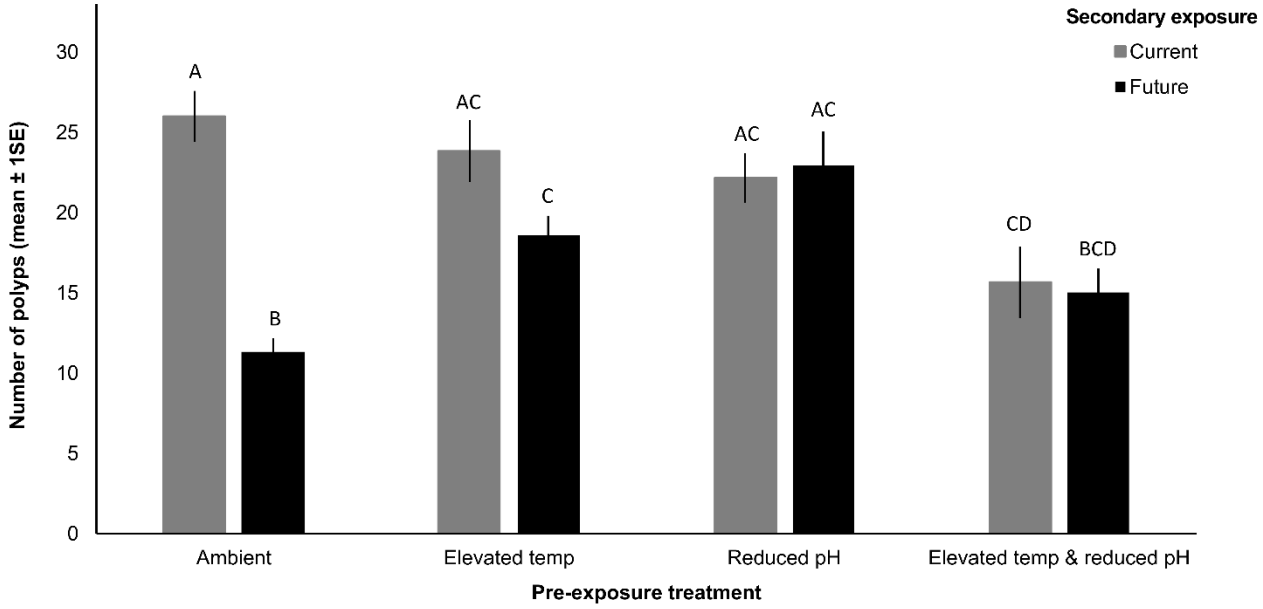
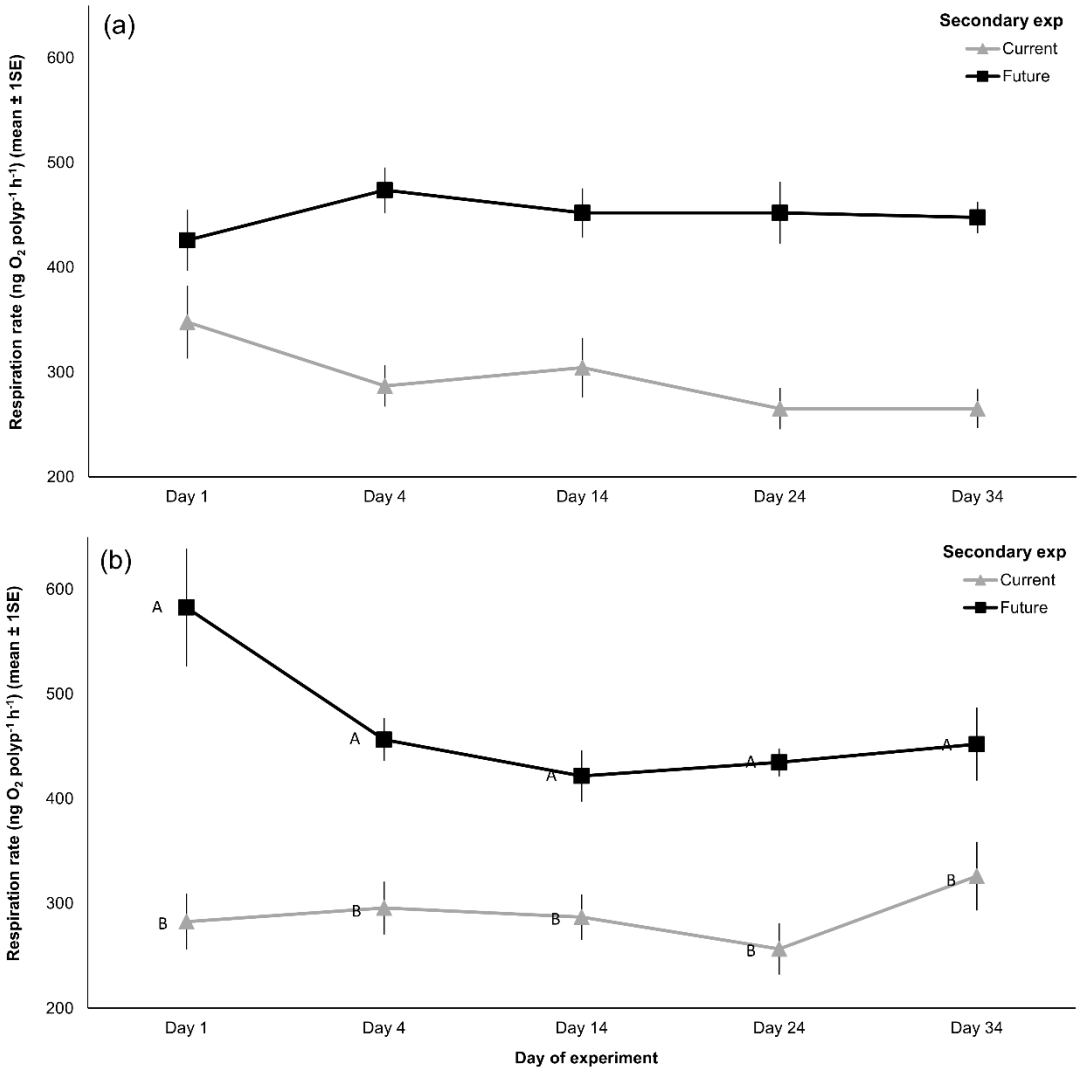


Fig. 1 Schematic diagram of experimental design that consisted of two parts: a 14-d pre-exposure phase that exposed polyps to either elevated temperature, reduced pH conditions, the stressors in combination, or ambient (control) conditions; and a secondary exposure, which subsequently exposed parent (*P*) and daughter (*D*) polyps to either current or future conditions over 34 d. Each 1-L glass aquarium contained two polyp dishes and one respiration vial. Note: for each pre-exposure treatment, polyp dishes for parent and daughter polyps were pooled across replicate aquaria and randomly assigned to secondary exposure treatments to reduce bias

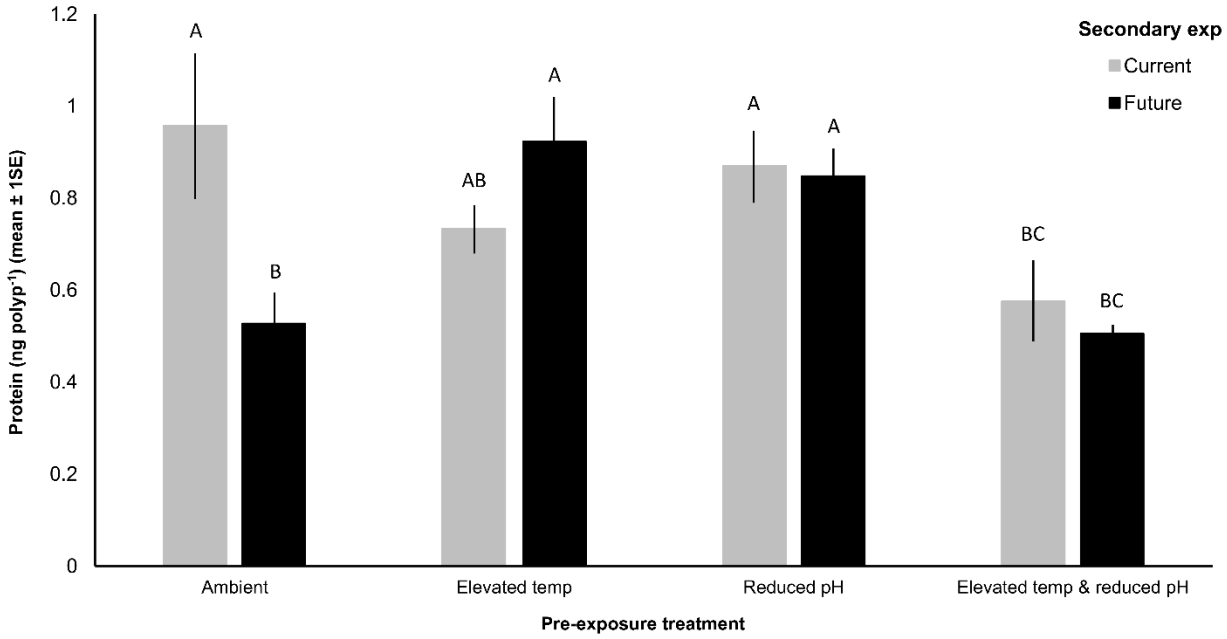


697 **Fig. 2** Number of polyps (mean ± SE) recorded at Day 34 of the secondary exposure (n = 48).
698 *Letters above data points* indicate similarities (e.g. AA) and differences (e.g. AB) among all
699 treatments, as determined by estimated marginal means

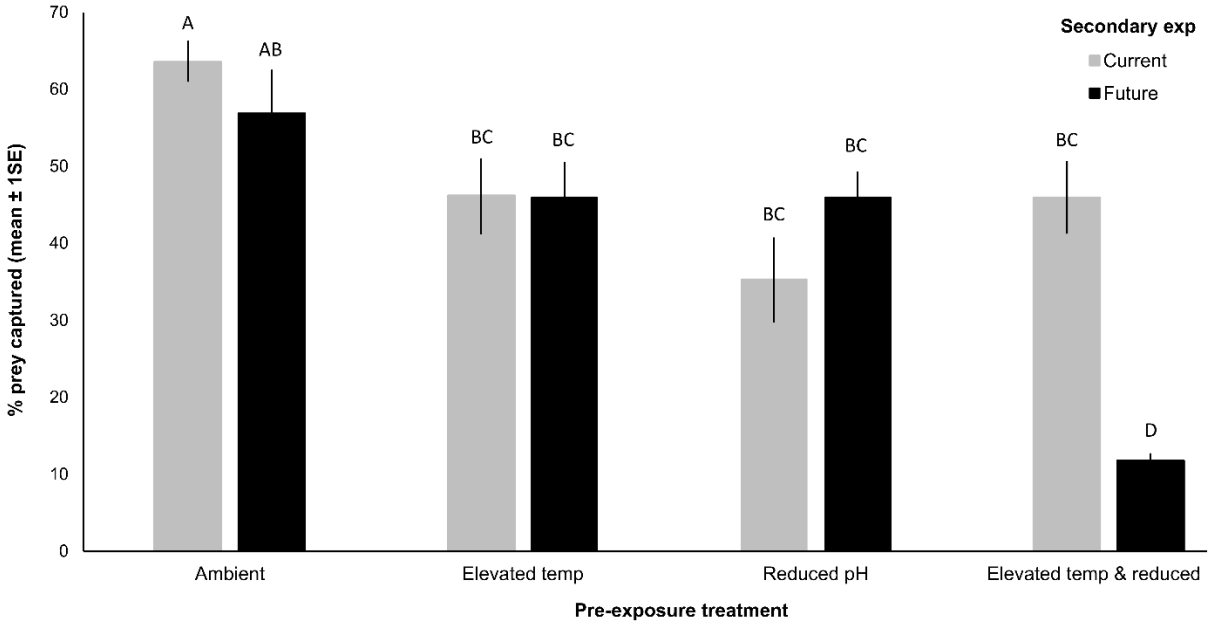


700

701 **Fig. 3** Respiration rates of polyps (mean ± SE) pre-exposed to ambient pH (**a**) and reduced
702 pH (**b**) conditions recorded at Days 1, 4, 14, 24 and 34 of the secondary exposure (Days 1–
703 34, n = 48). *Letters above data points* indicate similarities (e.g. AA) and differences (e.g. AB)
704 among treatments, as determined by estimated marginal means



705 **Fig. 4** Protein concentrations (mean ± SE) of polyps sampled at Day 34 of the secondary
706 exposure (n = 48). *Letters above data points* indicate similarities (e.g. AA) and differences
707 (e.g. AB) among all treatments, as determined by estimated marginal means



708 **Fig. 5** Percentage prey captured (mean ± SE) recorded at Day 34 of the secondary exposure
709 (n = 48). *Letters above data points* indicate similarities (e.g. AA) and differences (e.g. AB)
710 among all treatments, as determined by estimated marginal means