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Effects of temperature and pCO_2 on lipid use and biological parameters of planulae of *Pocillopora damicornis*

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ABSTRACT

The successful dispersal and recruitment of coral larvae depend on endogenous energy stores that fuel swimming, the search for optimal habitat, and metamorphosis. Ocean acidification and warming threaten to disrupt this critical process within the life cycle of reef-building corals by increasing maintenance costs in the energy budgets of larvae. In this study, lipid utilization and biological parameters of planula larvae of the cauliflower coral *Pocillopora damicornis* under future ocean conditions were examined using manipulative CO_2 experiments. For the first 24 h following their release, planulae were cultured in seawater controlled to mimic a future ocean scenario (1030 μatm pCO_2, 30.7 °C) as well as present-day, ambient ocean conditions (475 μatm pCO_2, 28.1 °C; confirmed by autonomous sensors deployed at our study site). Abundance of wax ester, triacylglycerol, and phospholipids as well as traits of physiological status were measured before and after incubations. High temperature and pCO_2 conditions did not elicit changes in wax ester composition of larvae. Triacylglycerol content increased with temperature but was not sensitive to pCO_2. In general, larvae consumed more total lipid and protein in response to conditions of warming but not high pCO_2, and the day the larvae were released often played a large role in the biological patterns observed. Our results suggest that future ocean warming may influence some organismal properties of coral larvae. High pCO_2 may not have a strong effect on the physiology of this early life history stage.

1. Introduction

Understanding the resilience of early life history stages of marine invertebrates is a research priority in light of co-occurring environmental changes such as ocean acidification (OA) and warming that threaten many coastal marine ecosystems, particularly tropical coral reefs (e.g. Anthony et al., 2011; Pandolfi et al., 2011). Coral larvae are of interest because they rejuvenate degraded reefs and support standing genetic variation (Amar et al., 2007). In this study, the responses of planula larvae of the coral *Pocillopora damicornis* to laboratory-simulated conditions of OA and warming were examined, through shifts in abundance of lipids in planulae. The roles of maternal investment and day of release during planulation in mediating the response of coral planulae to scenarios of future anthropogenic change in coral reef environments were also investigated (Hoegh-Guldberg et al., 2007; IPCC, 2013).

Future acidification and warming of the ocean (IPCC, 2013) may have deleterious effects on various biological processes of young marine invertebrates; for coral larvae specifically, fertilization success, metabolic rate, recruitment, and survival can be suppressed under elevated pCO_2 scenarios (Albright and Langdon, 2011; Albright et al., 2010; Cumbo et al., 2013a,b; Rivest and Hofmann, 2014; Suwa et al., 2010). High pCO_2 can exacerbate the effects of elevated temperatures by narrowing thermal tolerance windows (Hofmann and Todgham, 2010; Lannig et al., 2010; Pörtner, 2008; Walther et al., 2009). A few studies have examined the combined effects of temperature and pCO_2 on the physiology of coral larvae. Larvae of some broadcast-spawning and brooding corals experience metabolic suppression at high pCO_2 (Albright and Langdon, 2011; Nakamura et al., 2011) while other species are more tolerant (Chua et al., 2013). Recent studies on larvae from a Taiwan population of *P. damicornis* have found variable responses of metabolism to elevated temperatures and a lack of response to elevated pCO_2 (Cumbo et al., 2013a,b; Putnam et al., 2013).

The energetic costs of tolerating environmental stress, such as those posed by ocean acidification and warming, can be accommodated through the energy budget, represented by the balance between demand for energy, the amount of endogenous energy, and the ability to utilize external sources of energy. A major biochemical component of planulae, lipids can compose 34–85% of larval biomass (Harii et al.,

Abbreviations: OA, ocean acidification; T, temperature; LTLC, low temperature, low pCO_2; HTLC, high temperature, low pCO_2; LTHC, low temperature, high pCO_2; HTHC, high temperature, high pCO_2; WE, wax ester; TG, triacylglycerol; PL, phospholipid; A_T, total alkalinity.

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2007; Norström and Sandström, 2010; Richmond, 1987). Lipids (e.g. Moran and Manahan, 2004; Nates and McKenney, 2000; Sewell, 2005; Shilling et al., 1996) and protein (Marsh et al., 2001; Vavra and Manahan, 1999) serve as endogenous sources of energy for these larvae during dispersal (Ben-David-Zaslow and Benayahu, 2000; Graham et al., 2013; Harii et al., 2007, 2010; Richmond, 1987). Most coral planulae are rich in maternally derived lipids (e.g. Ward, 1992), whose energy stores largely determine the length of their larval duration and competency period (Alamaru et al., 2009; Arai et al., 1993; Harii et al., 2002, 2007; Richmond, 1987; Wellington and Fitt, 2003).

Two lipid classes serve the majority of energetic demands of coral larvae: triacylglycerols and wax esters. Triacylglycerols can be quickly hydrolyzed for immediate energy needs and are the first lipid class to be consumed during larval development or periods of starvation (Lee et al., 1971, 2006; Moran and Manahan, 2003, 2004; Sewell, 2005). Wax esters have a slower turnover rate, so they serve as long-term energy deposits for larvae. In addition, they play an important role in dispersal of coral larvae, governing buoyancy (Arai et al., 1993; Lee et al., 1971, 2006; Nevenzel, 1970; Villinski et al., 2002) and entraining larvae in surface currents (Wellington and Fitt, 2003; Willis and Oliver, 1990). Other types of lipids have important physiological functions: phospholipids maintain the structure of biological membranes, and other lipids serve as hormones and antioxidants.

OA and elevated temperature may perturb larval energy budgets by challenging both the symbiotic relationship and host physiology. *Symbiodinium*, vertically transmitted from the parent and living in the coral tissue, can translocate ~15% of their fixed carbon to *P. damicornis* planulae (Richmond, 1981), another important source of energy for the host. This species-specific value could be much greater, varying based on factors like *Symbiodinium* clade, environmental parameters, and analytical technique (e.g., Muscatine et al., 1984; Tremblay et al., 2012). Temperature stress can decrease the ratio of photosynthesis to respiration in coral larvae (*Porites astreiodes*, Edmunds et al., 2001). As temperature increases beyond the optimal temperature for photosynthesis, less energy is provided by the *Symbiodinium* to meet the elevated metabolic demands in the host. This decoupling of the symbiosis in brooded larvae could be worsened by the addition of OA, which could lower the thermotolerance ceiling of coral larvae. For example, high pCO_2 can lower the bleaching threshold of corals through a variety of potential mechanisms, including acidosis, changes to carbon-concentrating processes, and effects of high pCO_2 on photorespiration (Anthony et al., 2008). Additionally, OA could increase energetic demands by eliciting cellular stress response, oxidative defense, and ion-balance pathways in the host tissue. Without sufficient metabolite input from *Symbiodinium* under future ocean conditions, coral larvae consume their lipid and protein stores at faster rates (Alamaru et al., 2009; Ben-David-Zaslow and Benayahu, 2000; Harii et al., 2010). In particular, wax ester consumption increases when endosymbiont metabolites are not available (Harii et al., 2010).

Mechanistically, the effects of OA and elevated temperature on the energy metabolism of larvae of brooding corals may be mediated by innate differences in larval physiology based on the day the larvae were released. Developing larvae may experience different microenvironments (dissolved gas, oxygen, light, etc.) that promote the physiological differences observed, as a function of tissue depth as larvae are stacked within coral polyps (Jimenez et al., 2008; Kuhl et al., 1995) as well as the position of the polyp within the colony. Regardless of the mechanism, larvae of *P. damicornis* and other brooding corals can differ by size, *Symbiodinium* density, and photophysiology across the spawning period (Cumbo et al., 2012; Edmunds et al., 2001; Isomura and Nishihira, 2001; Putnam et al., 2010; Rivest and Hofmann, 2014). These innate differences may translate into the between-cohort variation in response to OA and elevated temperature observed to date (Cumbo et al., 2013a; Putnam et al., 2010; Rivest and Hofmann, 2014).

The cauliflower coral, *P. damicornis* (Linnaeus) was selected as the ideal study organism for addressing the physiological consequences of OA and warming on dispersing brooded planulae. This branching scleractinian coral is widely distributed throughout shallow-water habitats of the Indian and Pacific Oceans (Veron, 2000) and broods planula larvae every month according to the lunar cycle (e.g. Fan et al., 2002; Harriott, 1983; Richmond and Jokiel, 1984). The larvae contain endosymbiotic *Symbiodinium* upon release, vertically transmitted from the parent (Harrison and Wallace, 1990). *P. damicornis* larvae are ~70% lipid by dry weight: 60% wax esters, 16.5% triacylglycerol, and 3.2% phospholipid (Harii et al., 2007; Richmond, 1987). The rapid and long-term energy stores, as well as their developmental stage, allow them to settle and metamorphose within hours of release (pers. observation; Isomura and Nishihira, 2001) as well as retain competency in the plankton for more than 100 days (Richmond, 1987).

In this study, the goal was to assess the capacity of present-day coral genotypes to tolerate future conditions of OA and warming by testing a component of their physiological plasticity. To do this, laboratory-based CO_2 manipulation experiments were performed with *P. damicornis* larvae collected in Moorea, French Polynesia and then lipid utilization and biochemical composition of the larvae were used as indices of performance. To help provide context for experimental conditions, autonomous pH and temperature sensors were deployed on the natal reef. During the experiment, the specific aims were: (1) to determine how lipid composition and other biological parameters (here comprising total lipid levels, total protein levels, *Symbiodinium* density, and size) of *P. damicornis* larvae is affected by combinations of pCO_2 and temperature, and (2) to assess whether the effects of OA and warming on these properties differ between cohorts of larvae, based on day of release. Larvae were assessed both upon release and after exposure to experimental conditions in order to evaluate how OA and warming might alter larval performance.

2. Material and methods

2.1. Collection of coral larvae

Eight colonies of adult *P. damicornis* were collected from a fringing reef (17.4803 S, 149.7989 W) on Moorea, French Polynesia, on the new moon on February 21, 2012, from a depth of ~1–3 m. Colonies were maintained in individual aquaria that received indirect natural sunlight and coarsely filtered seawater. Larvae were collected from adult colonies following their lunar pattern of reproduction (Fan et al., 2006) following Rivest and Hofmann (2014). Larval release reached a peak in cohort size around lunar day 7, followed by a decline (see Fig. S1 in the Supplementary material). Due to the small numbers of larvae released from some colonies, all larvae released were used, resulting in unequal genotype ratios in the pool. On each day, larvae from this pool were immediately photographed and preserved to quantify larval quality and lipid metrics (see Sections 2.3 and 2.4) that represented the condition of freshly-released larvae. The remaining larvae in the pool were then randomly assigned to experimental treatments. Data presented here correspond to cohorts of larvae released on lunar day 7 ("Day 7"), lunar day 8 ("Day 8"), and lunar day 9 ("Day 9"). We have chosen to refer to the cohorts by lunar day but acknowledge that there are multiple factors besides day of spawning that may contribute to differences in performance between cohorts.

2.2. Experimental incubations

The daily pool of released larvae was divided among 8 aquaria containing 4 treatment combinations of pCO_2 and temperature. Larvae were incubated in 2 400 mL containers per aquarium at a density of ~0.15–0.25 larvae mL^{-1}. Containers had 100 μm mesh sides and transparent lids, and they were anchored in place within the aquarium to ensure that exposure to photosynthetically active radiation was replicated across aquaria. Larvae were incubated for 24 h under experimental conditions. This short incubation time was chosen due to the rapid

rate of settlement of *P. damicornis* larvae under laboratory conditions. The 24-hour incubation allowed us to maintain individuals in the larval stage until they were sampled for analysis. Due to the time needed to photograph and preserve larvae post-incubation, incubations were staggered by 20 min per aquarium, with the order randomized daily. At the end of each incubation, larvae within aquaria were pooled, and 10 larvae were randomly selected for size measurements. The remaining larvae were aliquoted and frozen at −80 °C for downstream analyses of lipid classes (3 × 25 larvae), total protein content (2 × 5 larvae), and symbiont density (2 × 5 larvae).

For the incubations, two pCO_2 treatments were prescribed. Low-pCO_2 was ~450 μatm CO_2, and High-pCO_2 was ~1000 μatm CO_2. The control treatment approximated the current environmental conditions for the adult coral (verified by environmental data; Section 2.6), while the high treatment represents a level of pCO_2 expected by year 2100 under the RCP8.5 scenario (IPCC, 2013). pCO_2 levels were combined with two experimental temperatures. Low-T and High-T treatments were 28 °C and 31 °C, respectively. The control temperatures approximated the multi-year average temperature for the fringing reefs close to the collection sites for adult *P. damicornis* (Moorea Coral Reef Long-Term Ecological Research site [MCR LTER], unpublished). The elevated temperatures represent an average surface ocean temperature by year 2100 as predicted by global temperature projections (IPCC, 2013). The four treatments created by this experimental set-up are defined as low temperature–low pCO_2 (LTLC), low temperature–high pCO_2 (LTHC), high temperature–low pCO_2 (HTLC), and high temperature–high pCO_2 (HTHC).

Experimental pCO_2 treatments were created using a gas mixing system at the MCR LTER facility with two aquaria for each treatment combination of pCO_2 and temperature (previously described in Edmunds et al., 2012). Aquaria were illuminated on a 13 h:11 h light:dark cycle, including a 4-hour ramping period (Sol LED Module, Aquaillumination, 75 W, Ames, IA, USA). At full intensity, larvae experienced approximately 1800 ± 140 μmol photons m^{-2} s^{-1} (LI-193, Li-Cor Inc., Lincoln, NE, USA) and a Li-Cor LI-1400 meter (Li-Cor Inc., Lincoln, NE, USA).

To verify and monitor the physical parameters of the OA × temperature treatments, pH, temperature, salinity, and total alkalinity of seawater in each aquarium were measured during the incubations. Seawater temperature was measured throughout the experimental exposures (~10 times) using a certified digital thermometer (meter 15-077-8, probe 15-077-7, Thermo Fisher Scientific Inc., Waltham, MA, USA). Seawater salinities were measured using a conductivity meter (YSI 3100, YSI Inc., Yellow Springs, OH, USA). Seawater pH was measured using a spectrophotometric method with an indicator dye, *m*-cresol purple (SOP 6b, Dickson et al., 2007). Total alkalinity (A_T) was measured using automated, closed-cell potentiometric titration (SOP 3b, Dickson et al., 2007) using an automatic titrator (T50 with DG115-SC pH probe, Mettler Toledo, LLC., Toledo, OH, USA). Titrations were performed using a certified acid titrant (~0.1 M HCl, 0.6 M NaCl; A. Dickson Laboratory, Scripps Institute of Oceanography), and A_T was calculated following Dickson et al. (2007). For each day of the experiment, analyzed certified reference materials (A. Dickson Laboratory) were titrated to confirm the precision and accuracy of the process; these reference titrations were accurate within 10 μmol kg^{-1}. pH at 25 °C, A_T, temperature, and salinity were used to calculate the pH and pCO_2 of the treatments using CO2calc (Robbins et al., 2010), with CO_2 constants K1 and K2 from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and pH expressed on the total scale (mol kg SW^{-1}).

2.3. Assessment of biological parameters of larvae

To assess the quality of incubated larvae, size, density of *Symbiodinium*, and total protein were quantified. Larvae were photographed using a dissecting microscope fitted with a camera (objective 5, Western Scientific Company Inc., Valencia, CA, USA; a Q-color 3 camera with QCapture suite software, Olympus American Inc., Center Valley, PA, USA). Larval size was determined via image analysis using ImageJ (Rasband, 1997), calibrated with a stage micrometer. In practice, circumferences of the larvae were traced to calculate larval area, and maximum larval length was measured. Total protein was quantified using the Bradford assay (Bradford, 1976), following Rivest and Hofmann (2014). To determine symbiont density, batches of 5 larvae were homogenized using a pestle. *Symbiodinium* cells in six 10 μL aliquots of homogenate were counted using a hemocytometer.

2.4. Characterization of lipids

Aliquots of 25 larvae were homogenized in 1 × phosphate-buffered saline (PBS) using glass beads (Sigma-Aldrich Corp., St. Louis, MO, USA) and a bead-beater (Biospec Products Inc., Bartlesville, OK, USA). Three 5-minute rounds of homogenization at 30 repetitions per second were spaced with flash-freezing of sample tubes in liquid nitrogen.

Lipid was extracted from the remaining homogenate following Bligh and Dyer (1959), modified by Luo (2008). As an exogenous spike or internal standard, 149 μg ketone (1-hexadecanone, Sigma-Aldrich Corp., St. Louis, MO, USA) was added to the remaining raw homogenate to account for extraction efficiency (Parrish, 1987). Ketone was chosen as the internal standard because it is found in low concentrations in marine tissues (Delmas et al., 1984; Parrish and Ackman, 1985). Briefly, homogenate, chloroform, methanol, and acidic saline were sequentially added to and mixed in glass test tubes for a final volumetric ratio of 0.8:2:2:1, respectively. The chloroform layer was transferred to a new test tube, where it was washed again in a final volumetric ratio of 2:2:1.8, chloroform:methanol:acidic saline. The chloroform layer was removed and evaporated at 37 °C under inert gas (nitrogen). Total lipid was calculated by subtracting the weights of empty, clean vials from weights of vials containing dried lipid. Dried lipid were stored under inert gas at −80 °C until further analysis. All solvents used in the extraction procedure were of HPLC-grade.

Lipid classes of wax ester (WE), triacylglycerol (TG), and phospholipid (PL) were separated and quantified using an Iatroscan MK-5 thin layer chromatography-flame ionization detection (TLC-FID) analyzer (Iatron Laboratories Inc., Tokyo, Japan). Lipid extracts were reconstituted in 100 μL chloroform; 1 μL of this solution was spotted at the origin of a Chromarod® (S-III; Iatron Laboratories Inc., Tokyo, Japan) using a Drummond Digital Microdispenser with Drummond Precision Glass Bores (Drummond Scientific Co., Broomall, PA, USA). The same 10 rods were used to process all samples and standards described here. For each run, three technical replicates per sample as well as a blank were included. To separate the target lipid classes from the total lipid, a two-step solvent system was used, modified from Rodrigues et al. (2008). Each frame of 10 rods was fully developed (25 min) in hexane:ethyl ether:acetic acid (99:1:0.05, v:v:v), then fully developed (25 min) in hexane:ethyl ether:acetic acid (85:15:0.1, v:v:v). The rods were dried at 100 °C after each development. The entire length of the rods was scanned by the Iatroscan under 1200 mL min^{-1} O_2 and 160 mL min^{-1} H_2. Data were collected using LabView software (National Instruments Corp., Austin, TX, USA).

To determine the quantity of lipid classes in each sample, a standard curve generated from mixtures of known lipid standards was run on the frame of Chromarods® at the beginning and end of the sample analysis. The standard curve consisted of: 0.06–1.96 μg $μL^{-1}$ hydrocarbon (5-α-cholestane), 0.06–5.89 μg $μL^{-1}$ wax ester (palmitic acid palmityl ester), 0.12–1.98 μg $μL^{-1}$ ketone (1-hexadecanone), 0.06–3.93 μg $μL^{-1}$ triacylglycerol (tripalmitin), 0.06–3.93 μg $μL^{-1}$ free fatty acid (stearic acid), 0.06–3.93 μg $μL^{-1}$ sterol (stigmastanol), and 0.06–5.89 μg $μL^{-1}$ phospholipid (L-α-phosphatidylcholine). Standard curves of lipid concentration vs. peak area were used to calculate concentrations of lipid classes in resuspended sample extracts. The internal standard was

used to adjust these values to account for extraction efficiency. Amounts of lipid classes were then normalized to total lipid.

2.5. Statistical analyses of biological data

All data were analyzed using R version 3.0.1 (R Core Team 2013). To compare physical conditions between treatments, type III sum of squares was used in which pCO_2 and temperature were fixed factors and aquarium was a random factor. For the initial conditions of larvae, the effect of day of release ("Day") was tested for lipid levels and other biological metrics using a one-way ANOVA. Initial lipid levels were expressed as a percent of total lipid, while other biological metrics were normalized per larva.

To represent changes in response variables over 24 h, post-incubation levels were compared. Lipid class quantities were standardized to total lipid levels to account for the fact that not all larvae are equal, and biological metrics were expressed per larva. Because larval area and length varied significantly by Day (see Section 3, Results), the change in larval size over 24 h was assessed differently. Initial size measurements were subtracted from post-incubation measurements. Therefore, a negative value represents a decrease in larval size during the incubation. For comparative purposes, means and s.e.m. for post-incubation levels of lipid quantities and measures of lipid quality are provided (see Table S1 in the Supplementary material). With pCO_2 ("pCO_2"), temperature ("T"), and day of release ("Day") as fixed factors, effects were estimated using linear mixed-effect models (nlme package in R; Pinheiro and Bates, 2000). As a random effect, we had an intercept for "Aquarium" in all statistical analyses. Model selection was performed incrementally following Burnham and Anderson (2002). At each iteration, the simpler model was chosen if the model AIC value did not increase by 2 or more and if there was not a significant difference in the model log likelihood ratio. Effects of fixed factors were compared using likelihood ratio tests conducted on selected models fit using maximum likelihood (Crawley, 2013; Zuur et al., 2009). When significant differences were detected among treatments, orthogonal contrasts were performed as post-hoc analyses using the multcomp package in R (Hothorn et al., 2008). Tukey's HSD was used for models without significant interactions between terms. Otherwise, general linear hypothesis tests with Bonferroni corrections for multiple comparisons (abbreviated GLHT) were used. The detailed results of the post-hoc analyses are provided (Tables S2B, S3B, S4B, S5B). In all cases, statistical assumptions of normality and homogeneity of variance were met, sometimes following a power-based transformation of the response variable.

2.6. Environmental data collection

At the study site, autonomous sensors were used to characterize the variability of pH and temperature to which our research organisms were likely acclimatized. pH and temperature time series were generated on a fringing reef in Moorea, French Polynesia. pH was recorded continuously from January 20 to March 16, 2012 on the fringing reef approximately 90 m from the collecting location of the adult *P. damicornis* parents. Details of sensor deployment and data analysis can be found in Rivest and Gouhier (2015).

pH and temperature time series contained strong and consistent daily fluctuations (~24-hour period; Fig. 4). During the 56-day deployment, pH variations occurred between extremes of 7.836 and 8.069 (total scale; mean: 7.989). Temperature on the fringing reef oscillated between 27.34 and 30.63 °C (mean: 28.76 °C). pCO_2 averaged 473 μatm. In comparison to our experimental conditions, our Low-pCO_2 (~477 μatm) and Low-T (~28 °C) treatment values approximate the mean environmental conditions during this time period. High-pCO_2 treatment values were not observed in situ during the deployment period (~1035 μatm); High-T treatment values were at the maximum edge of the environmental temperatures observed (~31 °C). Given our assumptions of constant salinity and total alkalinity, our estimates of environmental pCO_2 are conservative by approximately 5–10 μatm (Rivest and Gouhier, 2015).

3. Results

3.1. Condition of P. damicornis larvae immediately after release

Initial condition of larvae did not differ significantly as a function of day of release. Normalized to total lipid, initial levels of WE, TG and PL did not vary significantly by Day (see Table S2 in the Supplementary material). Additionally, initial levels of total lipid, total protein, and symbiont density did not vary significantly by Day (Table S2). Larval area and length did respond significantly to Day (Table S2). Initial larval area was significantly (38.6%) greater on Day 8 than that on Day 7 (Fig. 1; Tukey's HSD; Table S2). Larval length was significantly greater on Day 8 (1308 μm) than those on Days 7 and 9 (924 and 995 μm, respectively; Fig. 1; Tukey's HSD; Table S2).

3.2. Physiological responses of larvae in response to pCO_2 and temperature

During the 24-hour incubations in combinations of pCO_2 and temperature conditions (see Table S6 in the Supplementary material), temperature varied significantly between but not within temperature treatments (see Table S3 in the Supplementary material). Salinity was significantly higher at High-T (Table S3), and total alkalinity did not vary by temperature or pCO_2 treatment (Table S3). pH and pCO_2 of seawater varied significantly by pCO_2 and temperature treatments (Table S3).

In terms of biomolecules that provide long-term energy storage and confer buoyancy, abundance of wax esters (WE) was 47.3 ± 3.2% of the total lipid (mean ± s.e.m.) after 24 h. Post-incubation levels of WE were not significantly affected by pCO_2, Temperature, or Day (see Table S4 in the Supplementary material).

Following 24 h under various conditions of pCO_2 and temperature, the abundance of a biomolecule that confers a rapid source of energy, triacylglycerol (TG), was 9.1 ± 0.5% of total lipid (mean ± s.e.m.). TG content of larvae after 24 h, standardized to total lipid, differed significantly by Temperature and Day, but not by pCO_2 (Table S4). There was a 2.3% average increase in TG from 7.7% of total lipid at Low-T to 10.1% at High-T (Tukey's HSD; Table S4). This difference is particularly noticeable for Day 7 larvae, with higher TG values at HTLC and HTHC than those at LTLC and LTHC (Fig. 2). Day 7 and Day 8 larvae contained significantly more TG than that at Day 9 larvae (9.2% and 11.0% vs. 6.6% of total lipid, respectively, Tukey's HSD; Table S4).

Abundance of phospholipids (PL), an indirect indicator of cell number and important structural molecules, was 11.7 ± 0.9% of total lipid (mean ± s.e.m.) after 24 h. For post-incubation levels expressed per total lipid, PL content of larvae released on different days of the brood varied in response to pCO_2 and temperature (Table S4). For Day 7 larvae, PL content increased by 4.6% at High-pCO_2 under elevated temperature but decreased by 6.5% at High-pCO_2 under low temperature. In contrast, the opposite trends of similar magnitude were present for Day 8 larvae. PL content of Day 9 larvae decreased by 4.9% at High-pCO_2 for both temperatures (Fig. 2)

Total lipid varied between 12.506 μg larva^{-1} (Day 8 HTHC) and 91.531 μg larva^{-1} (Day 9 LTHC; Fig. 3). Post-incubation, total lipid varied significantly by Temperature and Day (see Table S5 in the Supplementary material), with a 15.8% decrease in total lipid from 41.05 μg in larvae incubated at Low-T than to 34.57 μg at High-T (Tukey's HSD; Table S5). Additionally, Day 9 larvae contained significantly more total lipid after 24 h than other cohorts (72%; Tukey's HSD; Table S5). Total lipid increased 73% from ~30 μg larva^{-1} to ~54 μg larva^{-1} on Day 9.

Total protein per larva ranged from 6.353 μg larva^{-1} (Day 8 HTHC) to 14.779 μg larva^{-1} (Day 8 LTHC; Fig. 3) after 24 h; effects of Temperature and Day were significant (Table S5). There was a 13.3% decline in

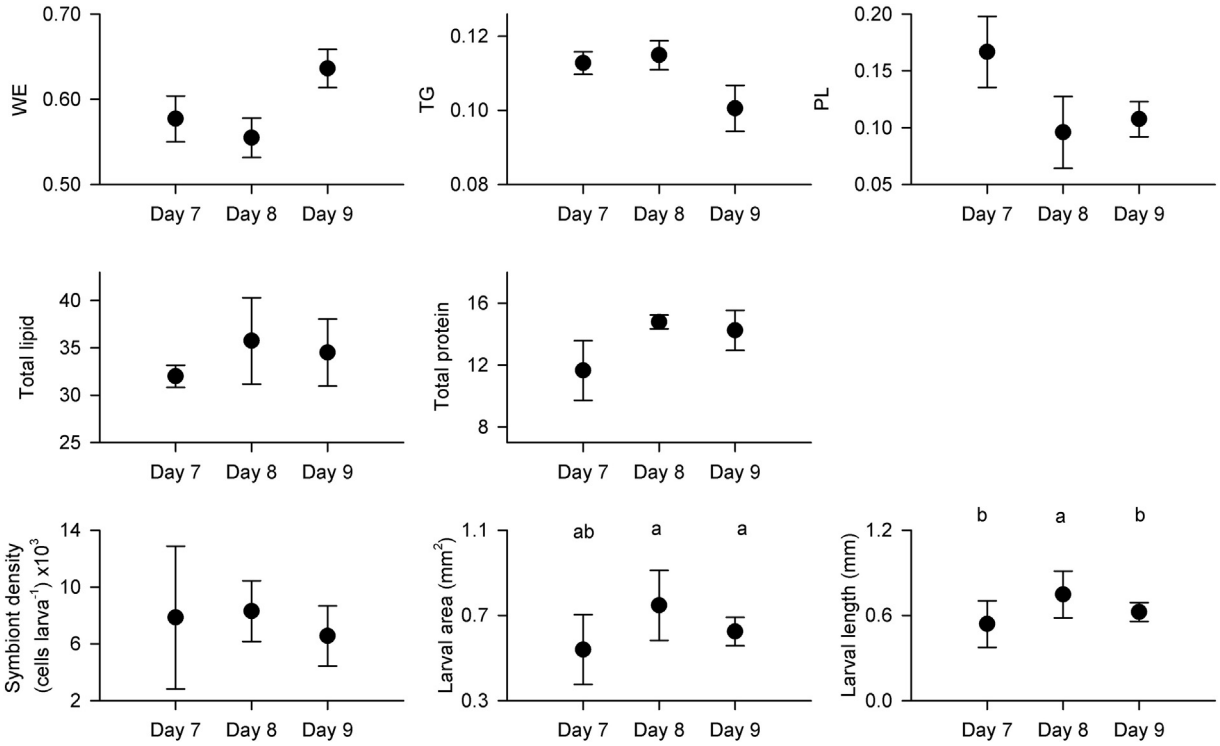

Fig. 1. Traits describing the status of newly-released *P. damicornis* larvae. Mean ± s.e.m. ($n = 3$) quantities of biochemical composition and size of larvae released on Day 7, Day 8, and Day 9. Wax ester (WE), triacylglycerol (TG) and phospholipid (PL) classes are standardized by total lipid. Total lipid and total protein are standardized by larva (μg larva^{-1}).

larval total protein levels from 11.33 μg larva^{-1} to 9.82 μg larva^{-1} at High-T (Tukey's HSD; Table S5). Total protein content of Day 7 larvae (9.51 μg larva^{-1}) was significantly less than that for Day 8 and Day 9 larvae (10.93 and 11.76 μg larva^{-1}, respectively; Tukey's HSD; Table S5).

Post-incubation symbiont abundance ranged from 2322 cells larva^{-1} (Day 9 HTLC) to 10,500 cells larva^{-1} (Day 8 LTLC; Fig. 3). Model effects of pCO_2 × Day and Temperature were significant (Table S5). Only for Day 8 larvae did symbiont abundance change significantly with pCO_2, with lower densities at High-pCO_2 (GLHT; Table S5). Symbiont density decreased significantly (18.4%) with increasing temperatures (GLHT; Table S5). Larval area varied significantly by Day only (Table S5). Area of Day 8 larvae decreased by 0.131 mm^2 while area in larvae from

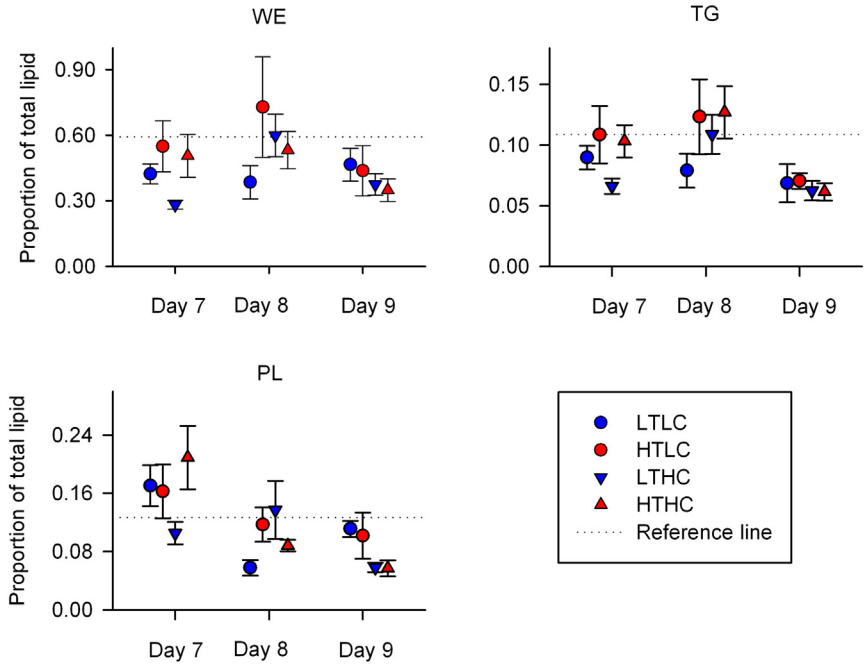

Fig. 2. Lipid composition of *P. damicornis* larvae after 24-hour exposures to combinations of pCO_2 and temperature. Mean ± s.e.m. ($n = 6$) abundance of wax ester (WE), triacylglycerol (TG) and phospholipid (PL) classes for larvae released on Day 7, Day 8, and Day 9. Lipid classes are expressed as a proportion of total lipid. Experimental treatments: Low-T, Low-pCO_2 (LTLC), High-T, Low-pCO_2 (HTLC), Low-T, High-pCO_2 (LTHC) and High-T, High-pCO_2 (HTHC). Dotted reference lines represent the average starting value for each variable (i.e. the value immediately upon release).

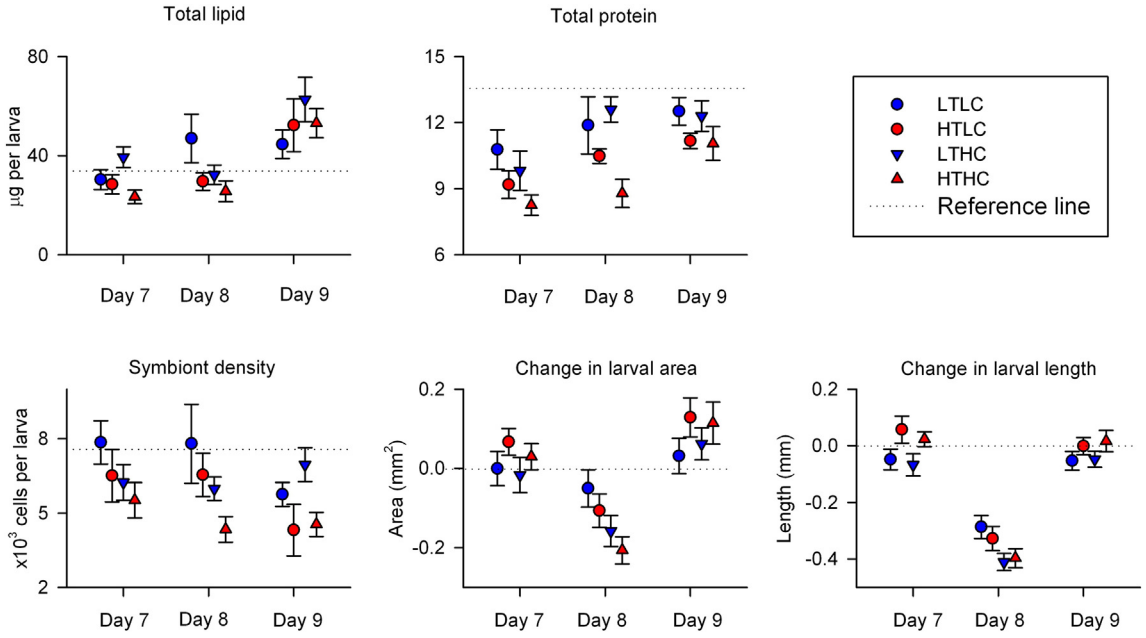

Fig. 3. Larval quality traits for *P. damicornis* larvae after 24-hour exposures to pCO_2 and temperature. Mean ± s.e.m. ($n = 6$) larval composition and size for larvae released on Day 7, Day 8, and Day 9. Quality traits are expressed per larva, unless otherwise noted. Experimental treatments: Low-T, Low-pCO_2 (LTLC), High-T, Low-pCO_2 (HTLC), Low-T, High-pCO_2 (LTHC) and High-T, High-pCO_2 (HTHC). Dotted reference lines represent the average starting value for each variable (i.e. the value immediately upon release).

other cohorts increased (Day 7: +0.019 mm^2; Day 9: +0.084 mm^2; Tukey's HSD; Table S5). Changes in larval length, with respect to the average initial value on each day of release, ranged from −722.14 μm (Day 8 LTHC; 55% reduction of initial size) to +784.81 μm (Day 7 LTHC; 85% increase; Fig. 3); significant effects included pCO_2 and Day (Table S5).

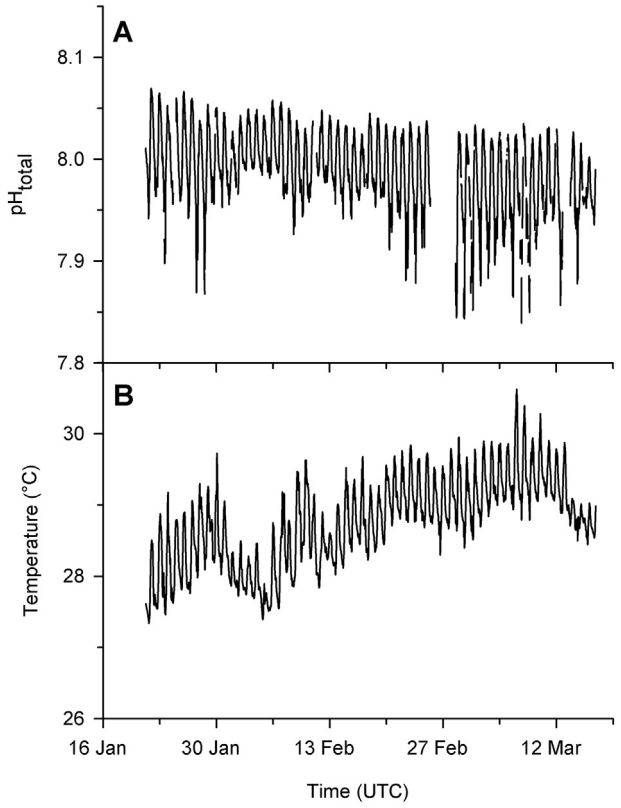

Fig. 4. Time series of pH (A) and temperature (B) at a fringing reef in Moorea, French Polynesia. pH data were collected by a SeaFET sensor and were processed by a one-hour low-pass filter. Temperature data represent averages from duplicate instruments, processed by the one-hour low-pass filter.

Larvae became 34% longer under Low-pCO_2 (+0.153 mm) than under High-pCO_2 (+0.101 mm; Tukey's HSD; Table S5). Length of Day 8 larvae decreased by 0.356 mm during the 24-hour exposure, while larval length in other cohorts decreased by smaller amounts (Day 7: −0.009 mm; Day 9: −0.021 mm; Tukey's HSD; Table S5).

4. Discussion

Our results from the CO_2- and temperature-manipulation experiments demonstrate that, in response to conditions that mimicked warming, *P. damicornis* larvae maintained greater TG levels within their lipid stores, suggesting that future ocean conditions may have consequences for their dispersal potential. Unlike temperature, conditions of OA did not have much effect on the larvae. Additionally, the day the larvae were released often had a strong influence on lipid utilization and biological parameters of the planulae.

4.1. Observations on maternal investment in P. damicornis larvae

Larvae released from *P. damicornis* colonies were similar in biochemical composition regardless of day of release. In addition, symbiont density did not vary between consecutive cohorts. As a result, broods released on each of three days had similar potential to meet energy demands, as ecological units. Larvae may be equally able to meet acute energy demands following release due to similar levels of liquid energy assets (i.e. triacylglycerol, TG). Overall, their energy metabolism may be more influenced by the rate limits of carbon-fixation and translocation by symbionts and by energy conversion from long-term storage molecules (wax ester, WE).

Although phospholipid (PL) levels did not differ between cohorts, larval size did vary. The mechanistic connection between larval size and long dispersal time for brooded larvae, as in those of *P. damicornis*, is that larger size implies greater stores of energy in the form of lipids, protein, and symbionts. In this case, Day 8 larvae were larger and did have slightly higher levels of TG, total lipid, and total protein, though not significantly different in biochemical composition from larvae released on other days. Thus, their size may disguise the limitations of their physiological abilities to produce and use energy, and larval size may not always be a good indicator of dispersal potential.

Our study supports the finding that planulae released close to the population's peak day of release are similarly endowed, while maternal provisioning of *P. damicornis* planulae commonly varies across the entire release period (Cumbo et al., 2012; Putnam et al., 2010). In other brooding species, such as a Caribbean coral, *Porites astreoides*, larvae released later in a reproduction event had higher concentrations of *Symbiodinium* and higher potential for autotrophy (Edmunds et al., 2001). Here, variation in investments between adults and variation in proportions of the larval pool represented by each parent may overshadow subtle changes in maternal provisioning between adjacent release days. Furthermore, in this study, adult corals were not exposed to treatment conditions of pH and temperature. It is possible that exposure to elevated pCO_2 and/or elevated temperature may change maternal investment to larvae as well as any physiological conditioning of larvae during development inside adult coral polyps.

4.2. Responses of larval lipid content and other biological parameters to changes in pCO_2 and temperature

In *P. damicornis* larvae, WE quantified at time of release was provided by the parent. Once these larvae enter the plankton, changes in WE abundance represent a balance of the energy metabolism of the larvae and the carbon fixation activities of its symbionts. Carbon translocated to the coral animal tissue that is not used for processes like respiration and mucus production is stored as lipids (Battey and Patton, 1984; Patton and Burris, 1983; Patton et al., 1977). *P. damicornis* larvae consumed more than 25% of their WE during the first 24 h of their pelagic duration, though this rate was not sensitive to temperature, pCO_2, or day of release and does not reflect any translocated carbon that was consumed as well.

The presence of vertically transmitted symbionts in *P. damicornis* larvae buffer the energetic demands of dispersal (Arai et al., 1993; Harii et al., 2010; Lee et al., 2006); indeed aposymbiotic coral larvae consume about twice as much WE during their first week than do symbiotic larvae (Figueiredo et al., 2012; Harii et al., 2010). Because symbionts provide the means by which larvae can replenish or maintain their maternally-derived lipid reserves, symbiont abundance with respect to changes in WE in larvae may provide important insight into the vulnerability of larvae to future demands on their energy budgets. For example, in adult *Montipora capitata*, recovery of lipid levels following a bleaching event was coincident with increases in gross photosynthesis (Rodrigues and Grottoli, 2007). In this experiment, changes in symbiont abundance and/or rates of carbon fixation and translocation in response to High-T, High-pCO_2 may have resulted in the similar WE levels observed. Indeed, WE abundance did not vary between groups despite significant decreases in symbiont density in response to High-T. While a previous study with *P. damicornis* larvae found no change in photophysiology in response to elevated temperature and pCO_2 after 9 days (Putnam et al., 2013), effects within the first 24 hours may be masked by a general increase in symbiont density over the long exposure duration (e.g. Cumbo et al., 2013b).

TG levels were more sensitive to temperature than pCO_2. Larger pools of post-incubation TG may suggest that under High-T, larvae needed to maintain a larger stock of this energy source to sustain metabolic demands. Because turnover rates of this lipid class are more rapid, it is also possible that equivalent net consumption rates under the two pCO_2 levels used represent different rates of turnover of TG pools. Additionally, for Day 7 and Day 8 larvae particularly, larvae under Low-T may have had lower replenishment rates, such that under conditions of high energy demand by the animal compartment, net TG consumption occurred. At the end of the incubation, Day 9 larvae contained the smallest percentage of TG, leaving them potentially more vulnerable to respond to future energy challenges, including metabolic scope, metamorphosis, and settlement (Gallager et al., 1986; Holland and Walker, 1975; Lee et al., 2006; Moran and Manahan, 2003; Napolitano et al., 1988).

Phospholipids are important for the structural function of coral larvae as well as for formation of cell membranes (Lee et al., 1971, 2006). PL content of *P. damicornis* larvae after 24 h varied depending on cohort and treatment conditions of temperature and pCO_2. In contrast, untreated *Acropora tenuis* planulae did not change in PL content after 30 days in culture (Harii et al., 2007). A decrease in PL composition of total lipid at High-pCO_2 in Day 9 larvae could be explained by metabolic suppression; if larvae lowered their metabolic rates in response to OA, physiological processes involved with structural changes associated with development and preparation for settlement would likely be slowed (e.g. Michaelidis et al., 2005).

For brooded coral larvae, like *P. damicornis* planulae, potential for growth during dispersal comes from the contribution of vertically transmitted *Symbiodinium* to their energy metabolism. Although this contribution can satisfy their metabolic needs, total lipid commonly declines during dispersal (Harii et al., 2002, 2010; Richmond, 1987).

In this study, larval length decreased with pCO_2, in general. Larvae remained long and skinny, even elongating, under Low-pCO_2. However, larvae at High-pCO_2 became shorter and more circular, morphological changes that may indicate preparation for settlement. The shortening of larvae at High-pCO_2 likely represents a behavioral change rather than a developmental response due to ocean acidification. Indeed, levels of WE, TG, total lipid, total protein, and symbiont density did not differ between larvae incubated under the two pCO_2 treatments, suggesting that the morphological changes under High-CO_2 occurred despite both groups having equivalent energy reserves to continue their pelagic larval duration. It is important to note that the morphological change in larvae incubated at High-pCO_2 may not have led to changes in settlement rate or success, as these parameters were not measured in this study. Effects of ocean acidification conditions on induction and rate of settlement in larvae of other coral species are variable (Doropoulos et al., 2012; Nakamura et al., 2011; Webster et al., 2013). In contrast to pCO_2, the size of larvae did not vary significantly in response to temperature, although the biochemical composition of the larvae did change significantly with temperature. While size of larvae has traditionally been associated with dispersal potential (e.g., Marshall and Keough, 2003), in this case, larval size may disguise the physiological ability of larvae to produce and use energy to fuel dispersal.

4.3. The role of day of larval release in energy metabolism

Effects of Day and its interactions with other independent variables commonly explained a significant portion of the variation among groups. Day acted additively with temperature and/or pCO_2 to influence patterns of lipid content and other biological parameters. The differences in performance (or lack thereof) between the three cohorts of larvae could have been influenced by differential mortality in certain treatments and for certain cohorts, different proportions of parents represented in each cohort, slight differences in experimental conditions for each cohort, and/or inherent physiological differences in cohorts based on day of release. Any influence of differences in experimental conditions across days was likely minimal, as differences within treatments were smaller between days than between replicate aquaria within days (Table S6B). While we did not rigorously assess rates of mortality and settlement during the 24-hour incubations, some incidences of death and metamorphosis were observed. These events were aquarium-specific and were not consistent across treatment or across days. The larval pool used for experiments did contain different proportions of parental contributions, so parental influence cannot be parsed from day-specific innate physiology. Consequently, our results represent population-level characteristics of the larval pool. Based on our results, larvae released on particular days may be more resilient or vulnerable to changing environmental conditions based on physiological characteristics associated with their cohort (i.e., related to the combinations of genotypes and phenotypes present). For example, Day 7

larvae contained the least amount of total lipid and total protein, post-incubation. These biological metrics were further diminished by exposure to High-T. Day 9 larvae contained the least amount of TG after 24 h; at Low-T, these larvae had the smallest pool of easily accessible energy reserves, making them more vulnerable to future energy challenges.

Our study along with previous work supports the idea that larvae released from adult colonies at different times can exhibit different sensitivities to elevated temperature and pCO_2 (Cumbo et al., 2013a; Rivest and Hofmann, 2014). Post-incubation symbiont density was sensitive to pCO_2 only for Day 8 larvae, indicating that the sensitivities of the *Symbiodinium*–coral relationship to the experimental treatments may not be equivalent between cohorts. Additionally, the effects of pCO_2 and temperature on larval phospholipid content varied by Day. Through the additive and interactive effects described here, maternal investment can have a large influence on the physiological traits exhibited by *P. damicornis* larvae.

4.4. Variability of environmental pH and temperature

Finally, to place the experimental treatments in an ecologically-relevant context, pH and temperature of the water mass bathing the fringing reef where the study population was located were measured. These measurements approximate the conditions experienced at the reef scale and what the coral larvae may encounter in the water column shortly after release. The mean pCO_2 value recorded (473 μatm) was higher than that recorded in winter at this site in the previous year (374 μatm; Rivest and Hofmann, 2014); lower wind stress in 2012 may have enhanced retention in the lagoon, allowing the biological pCO_2 signal to drive the average pCO_2 levels above the annual global atmospheric mean for 2012 (393 μatm; Conway and Tans, NOAA/ESRL [www.esrl.noaa.gov/gmd/ccgg/trends]). pH variability reported here is similar with that of the SeaFET pH time series data collected from coral reefs in the Northern Line Islands (Price et al., 2012) and is dominated by a diel cycling that has been described previously (Rivest and Hofmann, 2014).

4.5. Conclusions

The results of our study suggest that ocean warming will have greater potential than ocean acidification to affect lipid utilization and biological parameters of *P. damicornis* planulae. In particular, patterns of TG utilization suggest that the metabolic costs of tolerating projected future ocean conditions may be greatest under elevated temperatures. Lower levels of total lipid and total protein may cause planulae to reach the threshold for metamorphosis and settlement more quickly. Furthermore, the physiological responses of *P. damicornis* planulae to OA and warming may have indirect ecological consequences. Despite different physiologies between cohorts of planulae, WE levels, as a percent of total lipid, remained constant, suggesting that the buoyancy of larvae may not be affected by short-term periods of temperature or pCO_2 stress. However, loss of symbionts and lower levels of total lipid and total protein in response to High-T, High-pCO_2 over the first 24 h of dispersal may lead to depletion of energy reserves and decreased buoyancy over longer periods of time. Lower buoyancy may remove larvae more quickly from surface currents and will increase their chance of interaction with benthic substrate, contributing to the likelihood of shorter dispersal distances. Longer exposure times as well as exposures of adults to experimental treatment conditions will add to the results of this study in approximating the response of larval lipid utilization under projected future conditions. However, it is meaningful that under the short exposure times used (24 h), significant changes in certain aspects of larval composition were observed. Consequently, our results generate hypotheses about how OA and warming may affect future population dynamics of a reef-building coral, through the role of lipids as sources of energy and buoyancy for planula dispersal.

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