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Cite this article: Winkler NS, Pandolfi JM, Sampayo EM. 2015 *Symbiodinium* identity alters the temperature-dependent settlement behaviour of *Acropora millepora* coral larvae before the onset of symbiosis. *Proc. R. Soc. B* **282**: 20142260.
http://dx.doi.org/10.1098/rspb.2014.2260

Received: 16 September 2014
Accepted: 9 December 2014

Subject Areas:
ecology

Keywords:
Symbiodinium, thermal tolerance, species interactions, coral recruitment, habitat selection

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Electronic supplementary material is available at http://dx.doi.org/10.1098/rspb.2014.2260 or via http://rspb.royalsocietypublishing.org.

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Symbiodinium identity alters the temperature-dependent settlement behaviour of *Acropora millepora* coral larvae before the onset of symbiosis

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The global distribution of marine species, many of which disperse during the larval stages, is influenced by ocean temperature regimes. Here, we test how temperature and the coral symbionts (*Symbiodinium*) affect survival, symbiont uptake, settlement success and habitat choice of *Acropora millepora* larvae. Experiments were conducted at Heron Island (Australia), where larvae were exposed to 22.5, 24.5, 26.5 and 28.5°C. Within each temperature treatment, larvae were offered symbionts with distinct characteristics: (i) homologous *Symbiodinium* type C3, (ii) regionally homologous thermo-tolerant type D1, and (iii) heterologous thermo-tolerant type C15, as well as controls of (iv) un-filtered and (v) filtered seawater. Results show that lower instead of higher temperatures adversely affected recruitment by reducing larval survival and settlement. Low temperatures also reduced recruit habitat choice and initial symbiont densities, both of which impact on post-settlement survival. At lower temperatures, larvae increasingly settle away from preferred vertical surfaces and not on crustose coralline algae (CCA). Surprisingly, substrate preference to CCA was modified by the presence of specific symbiont genotypes that were present *ex-hospite* (outside the coral larvae). When different symbionts were mixed, the outcomes were non-additive, indicating that symbiont interactions modify the response. We propose that the observed influence of *ex-hospite* symbionts on settlement behaviour may have evolved through ecological facilitation and the study highlights the importance of biological processes during coral settlement.

1. Introduction

Coral reefs are productive ecosystems that provide habitats to over a million marine species and offer a range of ecosystem services. Reefs are now confronting a crisis due to a range of local and global anthropogenic stressors. Climate change has been highlighted as an important threat to the persistence of coral reef ecosystems as increasing ocean temperatures have decimated coral populations globally [1,2].

As sessile organisms, corals are regulated by their supply-side ecology [3]. Population recovery after disturbances relies on the influx of propagules and their subsequent successful recruitment [4]. Recruitment further underlies genetic diversity and connectivity within and between adult populations [5,6]. For many reef-building corals (Scleractinia) that reproduce during an annual spawning event, dispersal depends on pelagic larval competency which can last from several days to weeks [5]. After this, larvae actively search the benthos for a suitable hard substrate, attach and metamorphose from a planula larvae to a single polyp recruit. Rising temperatures adversely affect these early life stages by disrupting embryogenesis, decreasing larval survivorship and competency, or promoting premature metamorphosis [7,8]. To exacerbate this, the coral

reproductive season coincides with the season most likely to experience thermal stress and many tropical coral species are already near upper thermal limits [9]. Understanding how thermal increases affect coral reproduction, dispersal and settlement is critical given the importance of successful recruitment in population recovery and maintenance.

Initial larval habitat selection can directly impact on post-settlement survival [10,11]. Any reductions in propagule abundances as well as recruitment represent a bottleneck for coral populations and can lead to changes in community structure [12]. Corals do not settle randomly [5], and larvae exhibit active searching behaviour of benthic substrates (microhabitat), with multiple sensory cues involved in habitat selection [13]. Light and sediment conditions underlie the choice for settlement orientation [14,15] while specific substrate selection has been linked to crustose coralline algae (CCA) and associated microbial communities [16,17]. Despite the lifelong consequences linked to substrate discrimination [13], the potential effects of temperature on this process are poorly understood [8].

An important yet largely unstudied aspect that may affect recruitment is the mutualistic association of corals with single-celled dinoflagellates (*Symbiodinium*). In adult corals, specific symbionts confer advantages under certain environmental conditions [18,19]. Studies have explored how different *Symbiodinium* influence juvenile growth rates and thermo-tolerance [20,21], but this work has been done post-settlement. Currently, no data exist on the contribution of the symbionts as a settlement cue. Here, we test whether temperature and *ex-hospite* (outside the host) symbionts affect larval survival, settlement success and habitat choice (orientation and substrate preference). The common Great Barrier Reef (GBR) coral *Acropora millepora* was used because adults associate with thermally tolerant or sensitive symbionts [19,22] and it produces aposymbiotic larvae. *Acropora millepora* larvae were exposed to four temperatures in combination with seven *Symbiodinium* treatments. The results show temperature-dependent larval survival, recruitment and habitat selection under the influence of distinct *Symbiodinium* genotypes.

2. Material and methods

(a) Study site and larval collection

In 2012, a 'split spawning' event occurred and instead of spawning in a single month, some colonies delay spawning for a month. Three *A. millepora* colonies containing mature gametes were collected prior to the late October and November full moons from Heron Island reef (23°26.7′ S, 151°54.7′ E) (figure 1*a*). Colonies were housed in flow-through outdoor aquaria and monitored from 18.00 until midnight. During the consecutive spawning months, *A. millepora* colonies released egg/sperm bundles between 21.30 and 22.30, 11 and 6 days after the full moon (10 November 2012 and 5 December 2012, respectively). Gametes were collected immediately and cross-fertilized in 0.45 µm filtered seawater (FSW). After the initial division stages (approx. 2 h), the eggs were gently rinsed to remove sperm and transferred to fresh FSW. Fertilized eggs were kept indoors at 25°C, and FSW changes were done one to two times daily. After 3–4 days, eggs developed into swimming planula larvae and were transferred to experimental containers.

(b) Temperature treatments and experimental design

Ecologically relevant temperature treatments were selected using long-term mean temperatures at four sites spread across *A. millepora's* distribution range (figure 1*a*; distributional limits at the sGBR, but rare occurrence at Flinders reef [23]). Monthly mean values over a 10-year period were acquired from the MODIS aqua satellite (http://disc.sci.gsfc.nasa.gov/giovanni) (figure 1*b*). The first treatment of 22.5°C represents the mean beyond the distributional limits of *A. millepora* (Solitary Islands) when spawning occurs around November at Heron Island. The second treatment, 24.5°C, represents the temperature at Heron Island during spawning. The third treatment, 26.5°C, represents the summer mean (based on the three warmest months, December–February) at Heron Island and is also the temperature during spawning at Lizard Island. Finally, the 28.5°C treatment represents the summer mean near Lizard Island but, for Heron Island, exceeds the seasonal mean by about 4°C and the bleaching threshold by 1.7°C (figure 1*b*) [24,25].

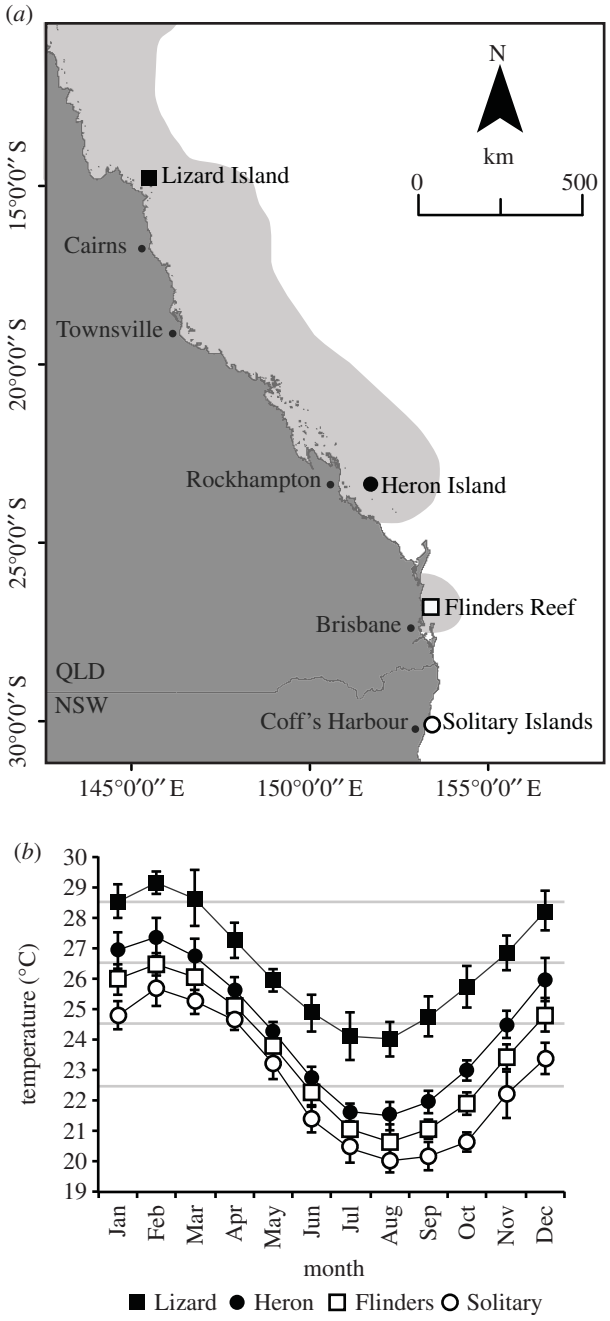

Figure 1. (*a*) Distribution of *A. millepora* (grey areas) along the eastern Australian coastline from Queensland (QLD) into New South Wales (NSW). (*b*) Long-term (10 years) monthly average temperatures (°C ± s.e., $n = 10$ per site for each month) at the four sites that represent locations where *A. millepora* occurs in high abundance (Lizard, Heron Island) to areas where it is rare (Flinders Reef) or absent (Solitary Islands).

Four outdoor tubs with flowing seawater were electronically controlled with heater–chiller units to $\pm 0.5^{\circ}$C of each treatment. In each tub, we placed sets of 50 ml Falcon tubes for the larval survival experiment and 500 ml containers with a single limestone tile ($5 \times 5 \times 1$ cm) for the settlement experiments. Prior to use, all tiles were left in the field for six to seven weeks (23°25.8′ S; 151°55.8′ E) for local conditioning (to accumulate benthic substrate that induces settlement). Considering irradiance affects settlement [10,15], all tiles were placed in the same orientation and received natural light intensities of around 95 µmol quanta $m^2\,s^{-1}$ (equivalent to Heron Island reef conditions at 7–10 m depth; E. M. Sampayo, unpublished data).

(c) Symbiont treatments and isolation of symbiont cells for infection

Larvae were offered *Symbiodinium* treatments (triplicate) that consisted of several single symbiont genotypes, a mix of two specific symbionts, seawater or no symbionts at all. The seawater treatment (SW) used local unfiltered seawater, while recruits in the remaining treatments were kept in FSW (refreshed one to two times daily) to prevent acquisition of 'wild' symbionts as opposed to 'treatment' symbionts. 'Single' symbiont treatments consisted of three distinct *Symbiodinium* types independently, i.e. type 'C3' (homologous = found in local conspecifics), 'D1' (regionally homologous, thermo-tolerant) or 'C15' (heterologous, thermo-tolerant) [19,22]. 'Mixed' treatments were a 50 : 50 cell-density mix resulting in three treatments, 'C3 × D1', 'C3 × C15' and 'D1 × C15'. In addition, larvae were kept in FSW (no symbionts) to test effects of symbiont presence or absence.

Symbionts were freshly isolated from fragments of *A. millepora* (C3), *Isopora palifera* (D1) and *Porites lobata/lutea* (C15). *Symbiodinium* cells were isolated by airbrushing coral fragments in FSW, homogenizing the tissue slurry and centrifugation (3300*g*, 5 min; Eppendorf 5810R). The supernatant (host tissue, soluble proteins) was discarded and the remaining symbiont pellet re-suspended in FSW. This process was repeated until the supernatant became clear. The final symbiont pellet was re-suspended in FSW and cell densities calculated (haemocytometer, eight replicates) to standardize the concentration of symbiont cells across treatments to 1500 cells ml^{-1}. As the SW treatment contained unfiltered seawater, the concentration of cells was unknown. A 50% water change was done 24 h after addition of the symbionts; after 48 h complete water changes were done daily.

(d) Measurements

A 100 five-day-old larvae were added to the 50 ml tubes placed inside the temperature treatments. To measure survival and potential symbiont uptake during the planktonic stage, these larvae were not provided with any settlement surface. Tubes were inoculated with each of the symbiont treatments. After 6 days, the number of swimming larvae was recorded and eight larvae were randomly sampled to assess symbiont acquisition. To maintain symbiont chlorophyll auto-fluorescence, larval samples were fixed in 4% paraformaldehyde prepared in phosphate buffered saline (1× PBS), and placed in the fridge for 2 days, after which the fixative was discarded and replaced with 1× PBS. Larvae were visually inspected for the presence of symbiont cells using fluorescence microscopy (Olympus BX41; Texas Red U-MWIY filter).

To assess the effect of temperature on settlement success in the absence of symbionts, 100 seven-day-old larvae (first spawning) were added to each settlement container and allowed 3 days to settle. After this, the number of recruits was recorded (10 days post-spawning). *Acropora millepora* is known to produce aposymbiotic larvae but this was confirmed by visually inspecting squashed larvae under light and fluorescence microscopy. The effect of temperature on settlement success in the presence of symbionts was tested with larvae from the second spawning. A total of 200 four-day-old larvae were added to each settlement container and immediately offered each of the *Symbiodinium* treatments. The number of recruits was recorded 6 days post-infection (10 days post-spawning) and five recruits were randomly sampled to assess if symbiont cells were acquired (as above).

Habitat choice was recorded by scoring each recruit as settled on either 'top', 'vertical' or 'back' tiles surfaces. Top surfaces experienced maximum light exposure and back surfaces received no direct light. Recruits were also scored as being settled on top of a CCA or not. Although specific CCA may differentially induce settlement, identification to the species level was not possible due to limited taxonomically informative structures in young CCA (size < 1 cm). Considering all tiles were conditioned in a similar environment and randomly distributed across treatments, it is unlikely that substrate selection scored here in two categories, 'on CCA' and 'not on CCA', resulted from different CCA species.

(e) Data analysis

All data were tested for normality and homogeneity of variances (spread versus residual plots, Q–Q plots, Levene's test; Statistica v. 11.0, StatSoft, USA) under several transformations. The log-transformation achieved normality for all factors but homogeneity of variances was not met for the factor temperature. Therefore, all analyses were done using permutation-based generalized linear models (PERMANOVA) because these are less sensitive to deviations from homoscedasticity as p-values are calculated from permutations (Primer-e v. 6 [26,27]).

Larval survival during the swimming stage (% of larvae alive) and settlement success (% of larvae settled) were analysed using a univariate two-way PERMANOVA with the fixed factors 'temperature' (four levels: 22.5, 24.5, 26.5 and 28.5°C) and 'symbiont presence/absence' (two levels: present or absent). Additionally, settlement under the various specific symbiont treatments was analysed using a univariate three-way PERMANOVA with fixed factors 'tile orientation' (three levels: top, vertical and back surface), 'temperature' (four levels) and '*Symbiodinium* type' (seven levels: SW, three single and three mixed treatments) or 'infection category' (three levels: SW, single or mixed). 'Single' category represents all individual treatments grouped (C3, D1 and C15), whereas 'Mixed' category represents all mixed treatments grouped (C3 × D1, C3 × C15 and D1 × C15). All PERMANOVA analyses used Euclidean distance for resemblance matrices and were run with 9999 permutations, a Type III error sum of squares (SS) and unrestricted permutation of residuals. The model was optimized by removing factors with $p > 0.25$ (see [28]) unless a single-level insignificant factor occurred in a significant higher order interaction. *A posteriori* pairwise tests were done within significant main effects.

Given that the substrate preference data consisted of only two choices for a recruit (either 'settled on CCA = 1' or 'not settled on CCA = 0'), the most appropriate statistic is a binomial logistic regression analysis [29]. It assumes a binomial distribution and is based on probability calculations from odds ratios rather than deviations from a continuous numerical mean. The probability is calculated from the total number of larvae settled in a binary substrate preference category, 'on CCA' or 'not on CCA'. The analysis was done with the 'stats' and 'popbio' packages in R (R Development Core [30,31]), using general linear models with a binomial data family and Wald χ^2 tests for the main significant effects [29,31]. Substrate preference was tested against the predictor variables 'temperature' (continuous), 'tile orientation' (categorical), '*Symbiodinium* type' (categorical) or 'infection category' (categorical). To test for spatial correlation between larvae from a single container, we comparatively analysed the data with and without the inclusion of 'tile' as a random factor. No differences existed in the main effects, and

subsequent analyses were done with the simpler model (excluding 'tile'). A predicted probability curve (with a 95% CI) was plotted for significant categorical predictors (orientation, symbiont treatment or infection category) against the continuous predictor (temperature). The probability (P) for each temperature treatment was calculated from the number of recruits (n) settled on CCA (success, k) as $P = k/n$. The probability, P, is given with a precision, which may be expressed in terms of a probability-based standard error s.e.(P) or confidence interval CI(P) as $\sqrt{(Pq/n)}$ (P = probability of success, settlement on CCA; $q = (1 - P)$ probability of failure, not on CCA). Finally, post hoc pairwise comparisons were done to test for significant differences within predictor variables.

3. Results

(a) Larval survival and *Symbiodinium* uptake

Symbiont availability, type or infection category did not affect larval survival. Although marginally insignificant ($p = 0.065$, $F = 2.528$; electronic supplementary material, table S1a), larval survival appeared slightly reduced at the lowest temperature at 14% compared with 21–30% at higher temperature treatments (figure 2*a*). Post hoc tests indicated significantly less larval survival at 22.5 compared with 26.5°C ($p = 0.009$; electronic supplementary material, table S2a).

After 6 days, none of the swimming larvae contained symbionts; symbionts were only observed in tissues of recruits 6 days post-settlement. The initial cell densities were unrelated to symbiont treatment but did differ significantly with temperature ($p < 0.001$, $F = 25.548$; electronic supplementary material, table S1b). Recruits exposed to 22.5°C acquired significantly fewer *Symbiodinium* cells compared with recruits exposed to all other temperatures (figure 2*b*; $p < 0.05$; electronic supplementary material, table S2b).

(b) Habitat choice: orientation

Settlement success (% of larvae settled) was not influenced by symbiont presence/absence or the specific symbiont treatment (type or infection category) but a significant interactive effect was present between tile orientation and temperature ($p = 0.012$, $F = 2.806$; electronic supplementary material, table S1c). Recruits preferentially settled on 'vertical' surfaces across temperature treatments (figure 2*c*; $p \leq 0.05$; electronic supplementary material, table S2c). At 22.5°C, differences in settlement success among surfaces were small (less than 17%) compared with higher temperatures where up to 46% more settlement occurred on vertical as opposed to back surfaces (figure 2*c*). On vertical surfaces, settlement was significantly reduced at low temperatures (42% at 22.5°C versus a maximum of 62% at 26.5°C; figure 2*c*). This pattern was absent in the other orientations. On back tile surfaces, already low settlement (32%) at 22.5°C further decreased with increasing temperatures to an overall minimum of 10% at 28.5°C. Settlement on top surfaces did not show a distinct up- or downward trend and varied between 20 and 33% across temperatures.

(c) Habitat choice: substrate preference (crustose coralline algae selectivity)

The binomial logistic regression showed that recruits settle preferentially on CCA with increasing temperature across all symbiont treatments (figure 3, top, dark grey bars). The probability curves are based on the number of larvae settled on either one of the two possible substrate choices (figure 3; number of recruits in the 'on CCA' category increases the probability of settlement on CCA (P)). While there was a significant three-way interaction (temperature × symbiont type × orientation, $p < 0.0001$; electronic supplementary material, table S3), all orientations within a symbiont followed the above-mentioned trend of increased CCA selectivity with temperature. However, there were two interesting exceptions. In the SW treatment, an inverse trend occurred only in the 'top' orientation, with recruits

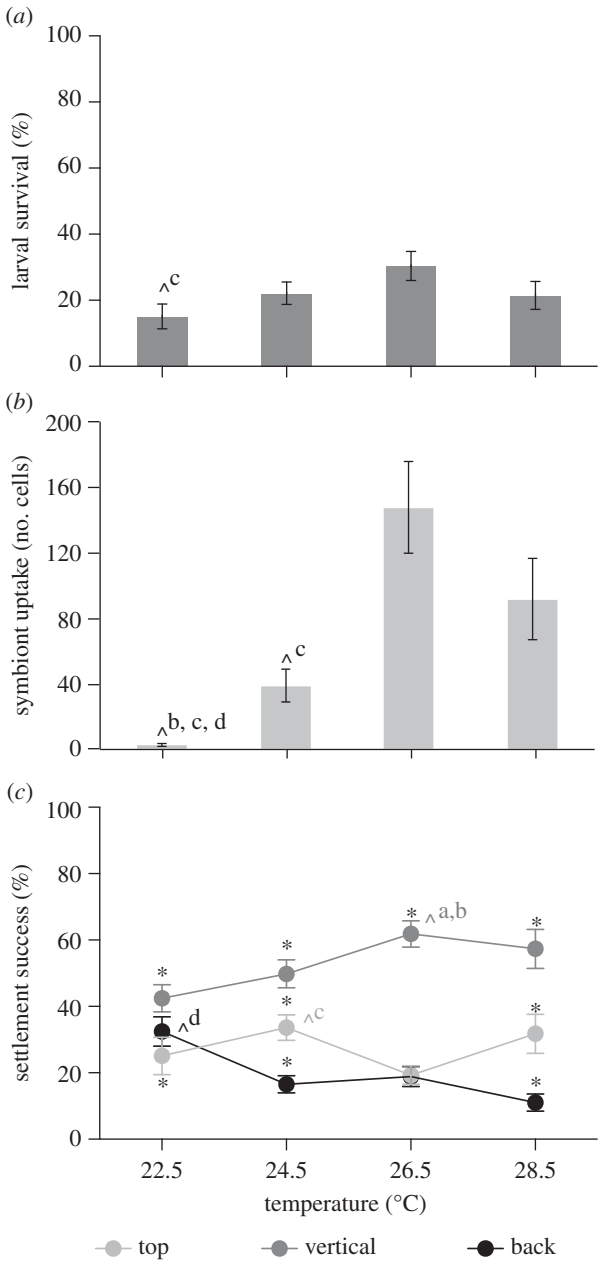

Figure 2. *Acropora millepora* (*a*) larval survival as percentage of larvae alive (±s.e., $n = 21$ per temperature), (*b*) symbiont cell density in 6-day-old recruits (±s.e., $n = 21$ per temperature) and (*c*) settlement success as the percentage of recruits settled on tile surfaces in one of three orientations: top, vertical and back (±s.e., $n = 21$ per temperature for each orientation). Significant differences ($p < 0.05$) between temperatures within a surface orientation are indicated with '∧' with temperatures as 'a' = 22.5°C, 'b' = 24.5°C, 'c' = 26.5°C and 'd' = 28.5°C. Significant differences between surfaces within the same temperature treatment are shown by '*' (for detailed *p*-values, see electronic supplementary material, table S2).

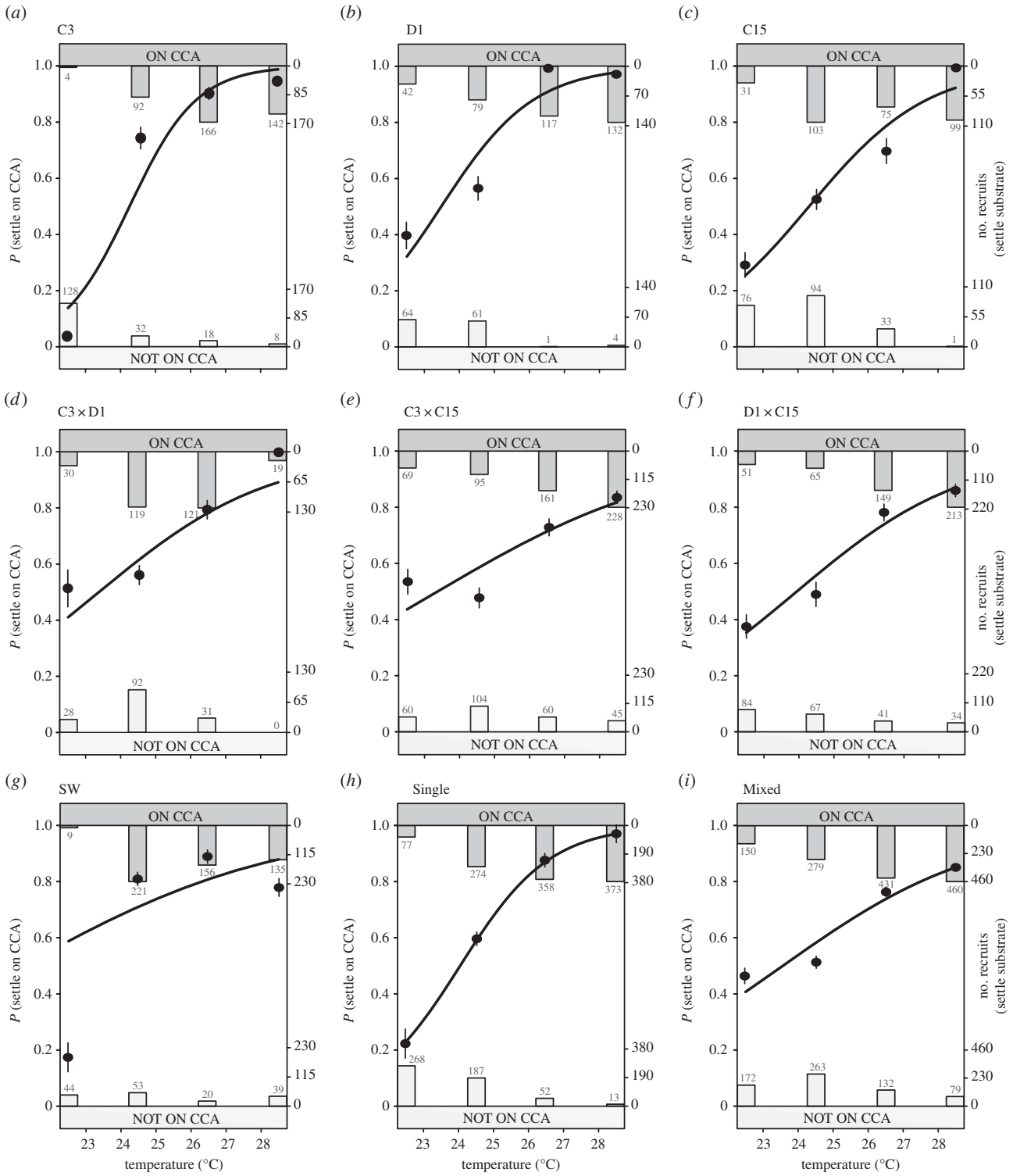

Figure 3. Logistic regression curves, probability at each temperature and raw data of recruit substrate preference, shown for (*a–c*) individual symbiont treatments, (*d–f*) mixed symbiont treatments, (*g*) SW and (*h*, *i*) grouped variable 'infection category' (single = all individual treatments grouped; mixed = all mixed treatments grouped). The curve (line) represents the predicted probability (left *y*-axis) of a larva settling on CCA (1) or not on CCA (0) in relation to temperature (*x*-axis). The probability at each temperature (black dots, ± CI) is calculated from the total number of recruits (right *y*-axis; grey bars with total values) in either one of two options of the response variable 'settlement substrate' (axis values split with top = on CCA, bottom = not on CCA) for each temperature treatment.

settling away from CCA as temperature increased (electronic supplementary material, figure S1g; $p < 0.05$; electronic supplementary material, table S4). For larvae exposed to symbiont C15 at low temperatures (less than 25°C), settlement on back surfaces leads to a pronounced reduction of CCA selectivity (electronic supplementary material, figure S1c; $p < 0.05$; electronic supplementary material, table S4).

As most orientations followed a similar trend, we focus here on the response related to distinct symbiont treatments (temperature × symbiont type, $p < 0.0001$; electronic supplementary material, table S3). Post hoc pairwise comparisons showed that larval CCA preference was different in larvae exposed to *Symbiodinium* C3 or C15 compared with SW, but those with D1 did not differ from SW (figure 4*a*; $p < 0.05$;

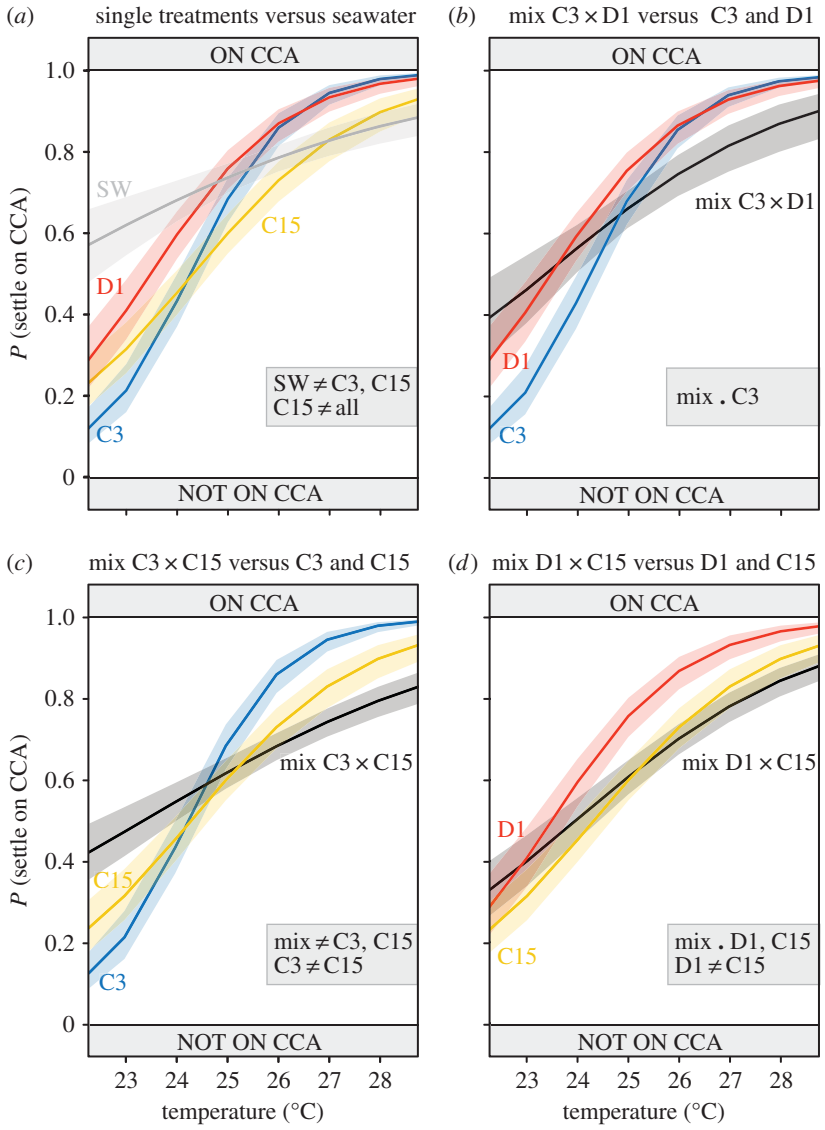

Figure 4. Logistic regression analyses showing significant pairwise differences in substrate choice ('on CCA', 'not on CCA') between (*a*) individual symbiont treatments (C3, D1 and C15) and seawater (SW) and (*b–d*) mixed symbiont treatments and their respective individual components. The curve (line plus confidence interval shaded) represents the probability (*y*-axis) of a larva settling on CCA (1) or not on CCA (0) in relation to temperature (*x*-axis). Significance differences between symbiont treatments (grey box) are shown as '$\neq$' for $p < 0.05$ and '.' for $p < 0.1$ (for specific *p*-values, see electronic supplementary material, table S5).

electronic supplementary material, table S5). Additionally, CCA preference differed when larvae were offered C15 compared with locally homologous type C3 and heterologous type D1 (figure 4*a*, $p < 0.05$; electronic supplementary material, table S5). Across all individual symbiont treatments, low temperatures reduced the probability of a larva settling on CCA to less than 50%. Larvae offered D1 had higher than 50% probability of settling on CCA between 24 and 25°C, while for larvae with C3 or C15 this temperature was 1°C lower. Below 24°C, larvae provided with C3 had the lowest CCA preference but at temperatures above 26°C CCA preference became identical to larvae supplied with the 'thermo-tolerant' D1 symbiont. Larvae settling in the presence of 'heterologous thermo-tolerant' C15 had the lowest CCA preference at higher temperatures (figure 4*a*).

When comparing each mix of symbionts to its respective individual components, the response was non-additive. Pairwise comparisons showed that most mixes were significantly different from their individual components. Interestingly, the mix of homologous symbionts C3 × D1 differed only marginally from C3 and not from D1 (figure 4*b*; $p < 0.1$; electronic supplementary material, table S5). C3 × C15 differed from both C3 and C15 (figure 4*c*; $p < 0.001$; electronic supplementary material, table S5), and finally D1 × C15 differed from C15 and marginally from D1 (figure 4*c*; $p < 0.01$ and <0.1, respectively; electronic supplementary material, table S5). Across mixed treatments, only C3 × C15 differed from SW ($p < 0.01$; electronic supplementary material, table S5), while mixes C3 × D1 and D1 × C15 were similar to SW.

Finally, habitat preference between infection categories showed a significant interaction with temperature ($p < 0.0001$; electronic supplementary material, table S3). Overall, the SW (figure 3*g*) and Mixed (figure 3*i*) were similar and both differed from the Single category (figure 3*h*) ($p < 0.1$ and 0.001, respectively; electronic supplementary material, table S5). Settlement on CCA reached 90–100% probability for single treatments, whereas in mixed and SW treatments the maximum probability was 80–90% at higher

temperatures. However, at lower temperatures, CCA preference was two to three times lower in single treatments compared with those containing multiple symbionts (figure 3*g–i*).

4. Discussion

In contrast to previous studies, we find negative effects on recruitment success and settlement habitat orientation at lower as opposed to higher than normal temperatures. We further present the novel finding that the presence of specific symbionts has the capacity to modulate temperature-dependent habitat preference (selection of CCA for settlement).

(a) Temperature-dependent larval survival and settlement success

Studies have shown that the larval pre-competence period is adversely affected by high temperatures in several broadcast spawning species, including *A. millepora* [32], and this is expected to reduce successful recruitment [3,5]. Here, we found that lower instead of higher temperatures reduced larval survival (15%) and settlement (up to 20%) (figure 2*a*,*c*). This discrepancy with previous work might arise from the fact that our highest experimental temperature was 28.5°C, which is low compared with other studies (30–32°C) [32,33]. However, under the worst Intergovernmental Panel on Climate Change (IPCC) scenario projections (scenario A1FI; warming of +4°C), the maximum temperature at our experimental location, Heron Island, is not expected to exceed 30°C by the end of the century [34]. The highest temperature treatment represents an increase of approximately 3°C above the long-term seasonal average during the reproductive season and 1.7°C above the summer average for the southern GBR [24,25]. The findings therefore have ecological relevance when estimating the response to projected local seawater temperature changes. Based on this, settlement rates for *A. millepora* in the southern GBR are not likely to suffer negative effects, with current projections of a 1–2°C increase above the long-term summer mean for the next several decades. Nevertheless, fertilization success or larval development may be substantially influenced within the experimental thermal range and this represents a perspective for follow-up studies.

The reduction of recruitment at lower temperatures corresponds with the distributional range of *A. millepora* which is not commonly found south of the GBR. During the annual spawning at Heron Island, sea surface temperatures are between 24.5 and 25.5°C, while reef environments along the southern Queensland and New South Wales coast experience temperatures between 22 and 23°C (figure 1). Our experiments indicate that southward recruitment will be limited by local temperatures as both larval survival and settlement success are decreased at 22.5°C (figure 2*a*,*c*). Thus, larval dispersal distance is dependent not only on biophysical factors (ocean currents) but also on temperature-dependent biological responses.

(b) Habitat selection

For corals, habitat choice is critical for post-settlement survival [10,11], with subsequent lifelong consequences on community composition, species abundances and genetic structure [13,15]. The selection of a suitable settlement habitat relies on salinity, light, depth, water motion, surface orientation and sedimentation [13,14,35]. Similar to previous findings, larvae of *A. millepora* settled preferentially on vertical surfaces within their natural thermal distribution range (greater than 24.5°C) (e.g. [11,15,35,36]). Vertical surfaces represent an intermediate state between maintenance of algal growth and destructive grazing [36]. Larval behaviour towards a light-dependent settlement response might also be linked to the intra-cellular symbionts that rely on the optimization of light for photosynthesis. Settlement in suboptimal light environments likely reduces photo-assimilation and might lead to reduced metabolite translocation to the host. This may have negative consequences on post-settlement survival or growth. Preferential settlement towards intermediate-exposed, vertical surfaces is likely to have evolved as a behavioural mechanism to maximize survival by escaping high grazing pressure [11,36] while at the same time optimizing light environment.

Some corals (i.e. *Stylophora pistillata*, *Favia fragum* or *Porites porites*) settle haphazardly, while others (various agaricid or acroporid species) have a distinct substrate preference [37,38]. In general, coral larvae settle near or on CCA, which serve as a recognition cue for appropriate habitat and act as a strong inducer of settlement and metamorphosis [11,13,16]. Here, *A. millepora* larvae showed a temperature-dependent selectivity towards settlement on CCA. Preferential settlement to CCA was significantly reduced at low temperatures, while at high temperatures it became almost absolute (always selecting CCA as a substrate for settlement; figures 3 and 4). Similar behaviour has been reported for *S. pistillata* larvae [8], which did not experience any negative effects of increased temperatures. Contrary to our findings, reduced settlement on CCA with increasing temperatures has been reported as well [39]. It is interesting to consider that biofilm composition associated with CCA may change significantly with prolonged minor temperature increases and reduce their capacity to induce settlement [17,39]. As tiles were not pre-conditioned under the various temperature treatments, it is possible that changes in the CCA themselves or associated biofilms [39] negatively influence settlement even if temperatures remain within predicted future limits. Here, substrate selection became suboptimal at lower temperatures, with larvae increasingly settling away from CCA (figure 4). This, combined with decreases in larval survival and settlement on preferred vertical surfaces (figure 2), reduces recruitment success of *A. millepora* beyond the southern limits of the GBR.

(c) Modulation of substrate preference by the coral symbionts

The specific symbionts offered in our experiments were present *ex-hospite* (outside the host tissues), yet their presence during recruitment significantly influenced temperature-dependent selectivity towards CCA. Studies have shown that specific symbionts affect survival, growth or competitive abilities in juvenile corals (e.g. [20,40,41]) but their role in larval substrate selection has never been examined. Here, the onset of symbiosis in *A. millepora* occurred after settlement (but see [42]) and, given the aposymbiotic nature of *A. millepora* larvae, it was surprising that symbiont identity modulated the selection of CCA as a settlement substrate.

The selected symbionts had varying levels of host-compatibility and thermo-tolerance. *Symbiodinium* type C3 is the commonly found symbiont of adult *A. millepora*

populations around Heron Island (homologous) [22] and is sensitive to temperature increases. Thermally tolerant type C15, generally found in *Porites* or *Montipora* spp., never associates with *A. millepora* and is considered non-compatible (heterologous) [22]. Finally, thermally tolerant *Symbiodinium* type D1 is found in *A. millepora* at certain GBR locations (regionally homologous) (e.g. [19,22]). This symbiont has been linked to unfavourable trade-offs such as reductions in growth rate or nutrient translocation (e.g. [40]) and likely represents a more opportunistic symbiont [43]. The temperature-dependent CCA selectivity was significantly different between larvae offered the homologous C3 symbiont during settlement versus those provided with heterologous symbiont C15, but did not differ from regionally homologous type D1. CCA selectivity also varied over the temperature range. At temperatures over 26°C, CCA selection in the presence of D1 was similar to that of larvae provided with C3. However, at lower temperatures, *Symbiodinium* D1 had the highest selectivity of CCA, which is interesting considering its thermo-tolerant characteristics (figure 4*a*). Despite its thermo-tolerant characteristics in its usual host, *Porites* spp., larvae settling in the presence of C15 showed the lowest CCA selectivity above 24°C (figure 4*a*). Our findings show that substrate seeking behaviour of coral larvae not only involves appropriate light conditions and CCA availability but also differs depending on the presence of specific symbionts.

(d) The link between crustose coralline algae and coral symbionts

Interestingly, corals with vertical symbiont transmission (symbionts passed on from parent to offspring) settle haphazardly compared with corals with a horizontal symbiont transmission (acquire symbionts from the environment) [11,13,16]. For example, symbiotic larvae of *S. pistillata*, *Pocillopora damicornis*, *P. porites*, *Stylarea punctata* and *F. fragum* readily settle in the absence of CCA [37,38]. By contrast, aposymbiotic larvae of *Goniastrea retiformis* and various acroporids exhibit a strong CCA preference [5]. Although some exceptions exist, this link between symbiont transmission strategy and substrate selectivity, together with our findings that symbionts modulate CCA selectivity, indicates that *ex-hospite Symbiodinium* acts as a contributing biological factor to settlement in coral larvae that must acquire their symbionts after settlement.

Under normal conditions, corals continuously expel symbionts to regulate cell densities. Where and how these symbionts survive outside the host still remains largely unclear (e.g. [44]). As the symbionts receive nitrogen and phosphate as metabolic waste products from their host [45], the nutrient levels surrounding *Symbiodinium* cells *in-hospite* are likely significantly higher compared with *ex-hospite* nutrient-poor waters. Very few symbiont types can be kept in culture and most are never found in free-living stages. This inability to survive *ex-hospite* is likely due to nutrient limitation or dependence on specific host-derived compounds.

We propose that after symbionts are expelled from their host, nutrient limitation causes them to actively aggregate onto surfaces containing dissolved organic compounds. Findings from several studies align with this suggestion. First, nutrient enrichments in *Symbiodinium* cultures show no direct effects of nitrogen toxicity, common to other marine phytoplankters, and confirming that *Symbiodinium* thrive under higher nutrient levels [46]. Secondly, free-living (although not *per se* symbiotic) *Symbiodinium* have been found in sand or on macro-algae [44,47], both of which are a likely source of dissolved organic and inorganic compounds. Surprisingly, the presence of *Symbiodinium* on or near CCA surfaces has never been examined. While highly speculative, we propose that *Symbiodinium* aggregate on CCA as they may represent a source of metabolic waste products similar to macro-algae. Specific symbiont genotypes may differentially aggregate on CCA surfaces depending on their energy requirements or metabolite quantity/quality released from the CCA. Our finding that CCA selectivity is reduced at lower temperatures aligns with a link between CCA and the aggregation of symbionts on their surfaces. Low temperatures may decrease CCA metabolism, thereby reducing metabolites that attract symbionts and likely decrease the gregarious response towards CCA surfaces.

Studies have shown that *Symbiodinium* occurs in ordinary seawater at low densities [44] and coral larvae with a horizontal transmission strategy can initially acquire multiple symbionts (e.g. [41,48]). Here, we found that the presence of multiple symbionts (SW and mixed treatments) leads to a significantly different habitat preference compared with the presence of individual symbionts. As both single and mixed treatments were offered a standardized number of *Symbiodinium* cells, we conclude that co-occurrence of *Symbiodinium* genotypes leads to a stronger selection of CCA as a settlement substrate. Different combinations of symbiont genotypes had non-additive effects, indicating that interactions between symbionts change larval habitat selection and that the response is dependent on the specific composition of symbionts. These findings bear similarities to, for example, co-infecting parasites which cause non-additive interactive effects due to internal competition or ecological facilitation among species [49]. The observed differential larval settlement to CCA depending on the symbiont identity suggests either that specific differences between symbionts modulate the settlement cue from the CCA or that the symbionts themselves act as a co-inducer for settlement.

5. Conclusion

We show that the distributional limits of coral populations can be significantly altered by temperature-dependent biological responses. The restriction of *A. millepora* to the southern GBR is linked to a temperature-dependent change in larval survival and settlement success combined with a disruption of habitat selectivity at lower temperatures. The most striking finding is that the coral symbionts alter settlement behaviour despite the fact that they are present *ex-hospite*. These findings provide the first evidence of the involvement of *Symbiodinium* in the settlement process and are expected to shift our perspective on how biotic factors regulate coral settlement. The mechanisms underlying these biological drivers of coral settlement present exciting avenues for further research. Changes in biological settlement cues under the influence of changing thermal regimes and potential links to suboptimal habitat selectivity will have negative lifelong consequences for corals as sessile organisms.

Acknowledgements. We thank Steve Dalton, George Roff, Tessa Turnbull, Giovanni Bernal and Aaron Chai for assistance during fieldwork.

Funding statement. The study was supported by the Australian Research Council (ARC) Centre of Excellence for Coral Reef Studies.

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