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METHODS & TECHNIQUES

Menthol-induced bleaching rapidly and effectively provides experimental aposymbiotic sea anemones (*Aiptasia* sp.) for symbiosis investigations

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ABSTRACT

Experimental manipulation of the symbiosis between cnidarians and photosynthetic dinoflagellates (*Symbiodinium* spp.) is crucial to advancing the understanding of the cellular mechanisms involved in host–symbiont interactions, and overall coral reef ecology. The anemone *Aiptasia* sp. is a model for cnidarian–dinoflagellate symbiosis, and notably it can be rendered aposymbiotic (i.e. dinoflagellate-free) and re-infected with a range of *Symbiodinium* types. Various methods exist for generating aposymbiotic hosts; however, they can be hugely time consuming and not wholly effective. Here, we optimise a method using menthol for production of aposymbiotic *Aiptasia*. The menthol treatment produced aposymbiotic hosts within just 4 weeks (97–100% symbiont loss), and the condition was maintained long after treatment when anemones were held under a standard light:dark cycle. The ability of *Aiptasia* to form a stable symbiosis appeared to be unaffected by menthol exposure, as demonstrated by successful re-establishment of the symbiosis when anemones were experimentally re-infected. Furthermore, there was no significant impact on photosynthetic or respiratory performance of re-infected anemones.

KEY WORDS: Cnidarian–dinoflagellate symbiosis, *Symbiodinium*, Coral reefs

INTRODUCTION

The symbiosis between corals and dinoflagellate algae of the genus *Symbiodinium* promotes the success of coral reefs in nutrient-poor tropical seas. However, there are still large gaps in our knowledge about how this symbiosis is established and maintained (Davy et al., 2012; Weis and Allemand, 2009; Weis et al., 2008). Moreover, we do not fully understand how and why this symbiosis breaks down under stress (e.g. bleaching) (Weis, 2008). These knowledge gaps hinder our capacity to understand the function of coral reefs, and to predict how coral reefs might respond to our changing environment.

To elucidate the cellular basis of the coral–dinoflagellate relationship, the symbiotic sea anemone *Aiptasia* sp. has been widely adopted as a model system because of its robust nature and the ability to produce large populations easily in the laboratory (Weis et al., 2008). Furthermore, *Aiptasia* sp. can be cleared of symbionts, maintained in an aposymbiotic state for extended periods, and successfully re-infected with a variety of *Symbiodinium* types (Belda-Baillie et al., 2002; Schoenberg and Trench, 1980; Starzak et al., 2014). The ease of experimental manipulation, combined with the rapidly increasing resolution of genomic, proteomic and metabolic databases for *Aiptasia* sp., makes this symbiotic anthozoan an ideal candidate for biochemical and genetic experiments that will further our understanding of the cellular processes underlying the cnidarian–dinoflagellate symbiosis (Lehnert et al., 2012; Peng et al., 2010; Sunagawa et al., 2009; Weis et al., 2008).

Currently, the most commonly used method to render anemones (including *Aiptasia* sp.) aposymbiotic combines temperature stress (heat or cold shock) followed by dark treatment and/or chemical inhibition of photosynthesis with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (e.g. Belda-Baillie et al., 2002; Lehnert et al., 2014; Xiang et al., 2013). This is a slow, laborious process that often requires months of preparation (Starzak et al., 2014; Xiang et al., 2013), and may not result in the full eradication of *in hospite* endosymbionts (Belda-Baillie et al., 2002; Schoenberg and Trench, 1980; Wang and Douglas, 1998).

Recently, Wang et al. (2012) described a method of menthol-induced bleaching in the corals *Isopora palifera* and *Stylophora pistillata*. Menthol is known to cause local anaesthetic effects in neuronal and skeletal muscles via blocking voltage-operated sodium channels (Haeseler et al., 2002). This cellular response has led to its use as a marine anaesthetic (Alexander, 1964; Lauretta et al., 2014). Menthol is known to act on a variety of different membrane receptors, including transient receptor potential (TRP) M8, which results in an increase in intracellular Ca^{2+} concentrations and causes a cold sensation in vertebrates (Hans et al., 2012; McKemy et al., 2002; Okazawa et al., 2000; Peier et al., 2002). As proposed by Wang et al. (2012), the mechanism of menthol-induced bleaching might be attributable to Ca^{2+}-stimulated exocytosis (Pang and Südhof, 2010). Preliminary experiments conducted by Wang et al. (2012) also suggest that menthol inhibition of *Symbiodinium* photosystem II activity may play a role in the expulsion of the algal cells or the digestion of the *Symbiodinium* cells by the host. Although the exact mechanism by which menthol induces symbiont expulsion is not yet clear, the findings of Wang and co-authors provide a platform for developing the use of menthol to generate aposymbiotic *Aiptasia* sp.

We therefore tested the applicability of this approach to *Aiptasia* sp., in terms of its effectiveness and impact on the capacity for symbiosis re-establishment. In particular, we determined whether menthol can rapidly and effectively produce aposymbiotic anemones, and whether these anemones can be experimentally re-infected with symbiotic dinoflagellates at cell densities similar to

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those of untreated and healthy symbiotic anemones. Ultimately, we describe a method to assist the many researchers around the world who use the *Aiptasia* model system to further our understanding of the coral–dinoflagellate symbiosis.

MATERIALS AND METHODS

Experimental organisms

Symbiotic individuals of *Aiptasia* sp. (n=300) were harvested from a long-term laboratory stock maintained in 1 µm filtered seawater (FSW) at 25°C, with light provided by AQUA-GLO T8 fluorescent bulbs at ∼95 µmol photons m^{-2} s^{-1} (12 h:12 h light:dark cycle). Anemones were maintained in 1 µm FSW aerated with a Hailea® aquarium air pump at 2 l min^{-1} and at 25°C using the WEIPRO® Temperature Controller MX-1019. These lighting and aquarium conditions were maintained throughout the 19-week experiment. The anemones were evenly divided among the treatments (menthol, cold shock and untreated) and allowed to settle for 72 h before beginning a 4-week treatment period.

The *Symbiodinium* culture (ID: FLAp2) was grown at 25°C and an irradiance of 100 µmol photons m^{-2} s^{-1} on a 12 h:12 h light:dark cycle. *Symbiodinium* cells were sub-cultured from a laboratory stock and grown in silica-free f/2 medium (AusAqua Pty, SA, Australia) for 6 weeks before use in the infection study. To genotype the *Symbiodinium* cells, DNA was extracted as described by Hill et al. (2014) from an algal pellet derived from a 1 ml aliquot of the *Symbiodinium* suspension. Samples were immediately used for PCR, using the thermal cycling regime and reaction mixture of Hill et al. (2014), and the outer primers ITSintfor2 and ITS2Rev2. PCR products were cleaned with ExoSAP-IT (USB Corporation, OH, USA) and sequenced by the Macrogen Sequencing Service (Macrogen, Seoul, South Korea). Sequences were aligned with Geneious v. 7.0 (Biomatters, Auckland, NZ) and a BLAST search was carried out against *Symbiodinium* ITS2 sequences in GenBank. Using this approach, the *Symbiodinium* genotype was confirmed as B1.

Menthol versus cold-shock treatment

Menthol (20% w/v in ethanol; Sigma-Aldrich, Auckland, NZ) was added to 1 µm FSW at a final concentration of 0.19 mmol l^{-1}. This concentration resulted in successful bleaching without causing mortality. A higher concentration of 0.38 mmol l^{-1} as suggested by Wang et al. (2012) was trialled prior to this experiment, but caused mortality of over 50% of the anemones (J.L.M., unpublished data). The anemones were incubated in the menthol/FSW solution for 8 h, after which the menthol/FSW was removed and the anemones were incubated in 1 µm FSW for 16 h. DCMU (100 mmol l^{-1} dissolved in EtOH, Sigma-Aldrich) was added to a final concentration of 5 µmol l^{-1} to prevent nuisance algal blooms and to limit the re-establishment of any residual symbiont cells by inhibiting photosynthesis. This 24-h cycle was repeated for four consecutive days. The 8-h menthol/FSW incubation occurred during the 12-h light period. The 4-day treatment was repeated after a 3-day break, during which anemones were maintained under the lighting and temperature conditions stated above, with biweekly feeding with *Artemia* sp. nauplii.

For cold shock, the water was replaced with 4°C 1 µm FSW, and placed in a refrigerator at 4°C for 4 h. Afterwards, the water was replaced with 25°C 1 µm FSW containing a final concentration of 50 µmol l^{-1} DCMU (100 mmol l^{-1} dissolved in EtOH, Sigma-Aldrich) and held under the lighting and aquarium conditions

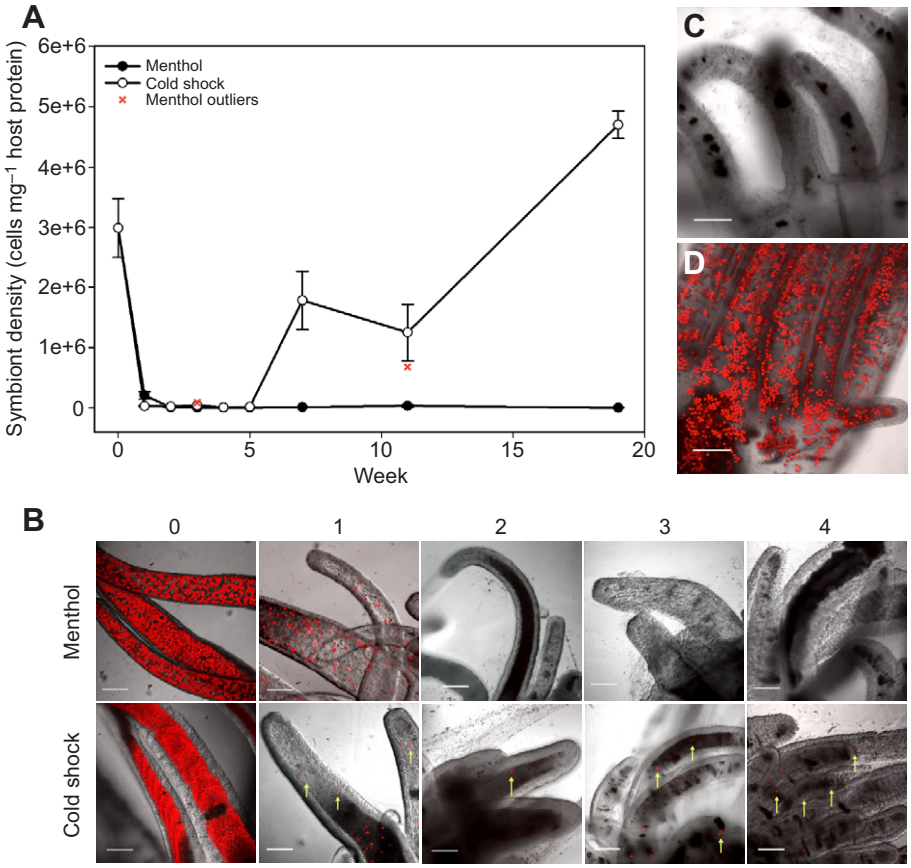

Fig. 1. Effects of menthol and cold shock on algal cell density in *Aiptasia* sp. (A) Density of *Symbiodinium* cells in menthol-treated (closed circles) and cold-shocked (open circles) anemones over a 19-week period. The anemones were treated for the first 4 weeks, then left to recover in regular filtered seawater for the remaining 15 weeks. Outliers (defined as exceeding 1.5× interquartile range) were detected in the menthol-treated anemones at weeks 4 and 11, and are indicated by a red cross. Values are mean±s.e.m.; n=5. (B) Confocal microscopy images of anemones during menthol treatment and cold shock (weeks 1–4). Yellow arrows indicate *Symbiodinium* cells in host tissues. (C,D) Confocal fluorescence images of menthol-treated and cold-shocked anemones, respectively, 15 weeks post-treatment (i.e. week 19). Scale bars are 100 µm.

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described above. The anemones were incubated under these conditions for 3 days, and the treatment was then repeated. The anemones were unfed for the duration of the cold shock, as per the protocol described by Steen and Muscatine (1987). Anemones were cold shocked a total of eight times (twice per week) during the treatment period.

After the 4-week treatment period, all anemones were maintained in 1 µm FSW at 25°C under the irradiance regime described above, with biweekly feeding, for an additional 15 weeks. Their symbiotic status was assessed via confocal fluorescence microscopy and cell density estimates (described below) at weeks 1–5, 7, 9, 11 and 19.

The untreated group of anemones was kept under the same thermal and light regimes as the menthol-treated and cold-shocked anemones, with biweekly feeding throughout the entire 19-week experiment. They were assessed for symbiont density at the same time points as the experimental anemones, to obtain an average symbiont density of untreated, healthy anemones.

Re-infection of aposymbiotic anemones

The capacity to re-infect menthol-treated anemones with algal symbionts was tested; this was not possible with cold-shocked anemones because they were not fully aposymbiotic. Anemones were infected with cultured homologous *Symbiodinium* type B1, i.e. symbionts originally isolated from *Aiptasia* sp. A drop of concentrated *Symbiodinium* suspension (3×10^6 cells ml^{-1}) was pipetted onto the oral disc of each anemone, followed by a drop of *Artemia* sp. nauplii to invoke a feeding response and encourage *Symbiodinium* uptake (Davy et al., 1997). This process was repeated 4 h later, and the water was replaced with 1 µm FSW 8 h after the second infection.

Confocal microscopy

Whole anemones from each treatment were examined at the same time points as cell density assessments by confocal microscopy (Olympus Provis AX70, at 100× magnification) to detect the chlorophyll autofluorescence of any dinoflagellates. Anemones were selected at random from each treatment, and placed in a 'relaxation solution' [50% FSW, 50% 0.37 mol l^{-1} magnesium chloride ($MgCl_2$)] for 15 min before being moved to a FluoroDish™ glass-bottom confocal dish (World Precision Instruments, FL, USA). The autofluorescence emission of the *Symbiodinium* cells was excited using a 559 nm laser and captured at 647±10 nm. This was performed to check the rates of bleaching and re-infection, as well as to detect any background *Symbiodinium* populations in the post-treatment anemones.

Symbiont density and protein content

Symbiodinium populations were quantified with a haemocytometer and light microscope (100× magnification; n=8 replicate counts per sample) using whole-anemone homogenate (n=5). Cell density was normalised to soluble protein content, measured via the Bradford assay (Bradford, 1976). Cell density was measured at weeks 1–5, 7, 9, 11 and 19.

O_2 flux

Fifteen weeks after reinfection, maximum photosynthetic and dark respiratory O_2 fluxes were measured to compare re-infected menthol-treated anemones against untreated anemones (n=4). A FIBOX 3 fibre optic oxygen transmitter and oxygen probe (PreSens, Germany) were used as described by Starzak et al. (2014). The respiration rate (ml O_2 h^{-1}) was measured for 30 min in the dark, after which the rate of net photosynthesis was measured for 30 min at an irradiance of ~100 µmol photons m^{-2} s^{-1}. Net photosynthesis and respiration were corrected for background levels using a FSW-only control, and gross photosynthesis was calculated by the addition of net photosynthesis to dark respiration (Muscatine et al., 1981). The average ratio of gross photosynthesis to respiration (P:R) for 24 h was calculated for each anemone, by assuming a 12 h:12 h light:dark cycle and that respiration was constant in the light and dark, and the average ratio for each treatment was calculated.

Statistical tests

The non-parametric Mann–Whitney U-test was used to test for differences between treatments with respect to symbiont density (SPSS statistical software, v. 20, IBM Corporation). Separate analyses were run for the bleaching and post-bleaching periods.

A one-way ANOVA was performed on the photosynthetic rate, respiration rate and log-transformed P:R values to determine whether significant differences were present between the re-infected menthol-treated and untreated symbiotic anemones at the end of the 19-week experimental period.

RESULTS AND DISCUSSION

Menthol treatment induced 97–100% symbiont loss within 14–28 days, with 60% of anemones losing all symbionts. In comparison, cold shock never induced 100% loss within the same

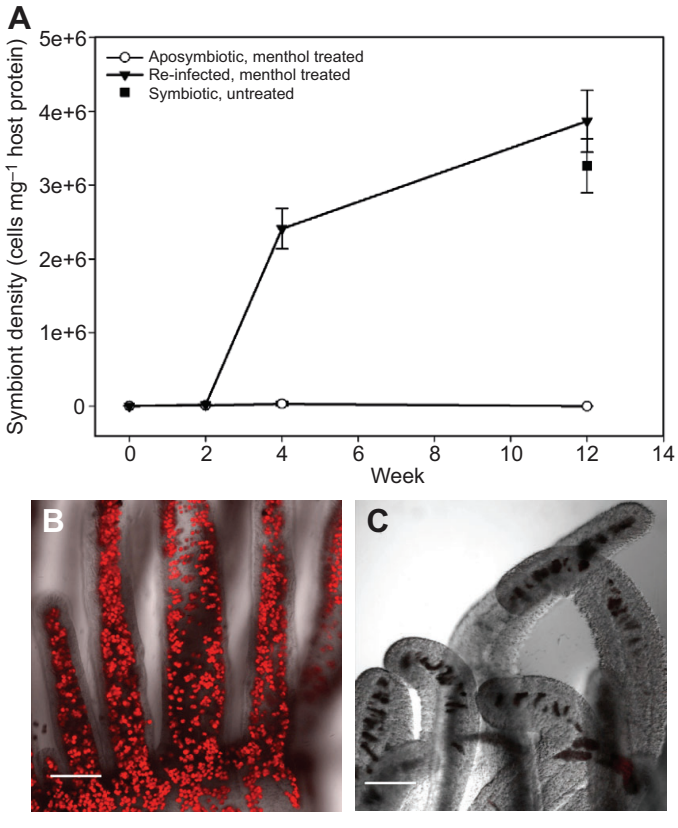

Fig. 2. Repopulation of menthol-treated *Aiptasia* sp. with algal symbionts. (A) Symbiont cell density over a 12-week period after anemones were experimentally re-infected with the homologous *Symbiodinium* type B1 (closed triangles). Also shown are symbiont densities in symbiotic, untreated anemones (closed square) and menthol-treated anemones that were not experimentally re-infected (open circles). Values are means±s.e.m.; n=5. (B,C) Confocal images showing, respectively, the re-established symbiont population 12 weeks after reinfection versus tissues in a non-infected aposymbiotic anemone. Scale bars are 100 µm.

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time frame (Fig. 1A,B), and any residual symbionts in the menthol-treated anemones did not re-establish a full symbiosis once returned to normal seawater conditions over the course of our experiment (Fig. 1A,C).

Although statistically, the patterns of symbiont loss during weeks 1–4 were not different between the menthol and cold-shock treatments (Mann–Whitney U=113, d.f.=4, P=0.423, n=5; Fig. 1A), only the menthol-treated anemones successfully maintained aposymbiotic status in the longer term. In both treatments, symbiont density remained near zero during the first week of recovery (weeks 4–5; Fig. 1A); however, subsequently there was a significant difference between the cell densities of menthol- and cold-shock-treated anemones (Mann–Whitney U=35, d.f.=4, P<0.0005; n=5), with the menthol-treated anemones remaining mostly symbiont-free (0–0.05% of pre-bleaching density) and the cold-shocked anemones becoming fully re-populated (weeks 5–9; Fig. 1A). Confocal microscopy confirmed these measurements (Fig. 1C). Menthol treatment therefore has considerable potential for the rapid and effective generation of aposymbiotic *Aiptasia* sp.

One key use for aposymbiotic *Aiptasia* sp. is for experimental work that involves re-infection with symbiotic algae of different types and from different host species. It was therefore important to test the infectivity of the menthol-treated anemones, and establish whether there were any significant long-term impacts of menthol treatment versus no treatment on physiological performance (photosynthesis and respiration) of the re-infected anemones. When infected with *Symbiodinium* cells (ITS2 type B1), symbiont density increased slowly during the initial 2 weeks, followed by a rapid increase during weeks 2–12 (Fig. 2A). Confocal microscopy confirmed that menthol-treated anemones were fully repopulated after 12 weeks (Fig. 2B). The infection pattern and maximum cell density were similar to those of other re-infection studies using heat- and cold-shock bleached *Aiptasia* and type B1 *Symbiodinium*, where a steep increase in cell density was observed in the first 4 weeks, followed by stabilisation (Belda-Baillie et al., 2002; Schoenberg and Trench, 1980; Starzak et al., 2014). Additionally, the photosynthetic rate (P=0.432; n=4), respiration rate (P=0.347; n=4) and ratio of photosynthesis to respiration (P=0.368; n=4) in these anemones were similar to those of untreated symbiotic anemones. This indicates that their capacity to re-form a functional symbiosis was not compromised, further highlighting the value of this method.

As expected, given its anaesthetic properties (Alexander, 1964; Lauretta et al., 2014), menthol induced tentacle relaxation and unresponsiveness in anemones for the duration of the treatment. Once the seawater was replaced with regular FSW, the anemones were able to regain full movement and feeding capabilities within ~15 min. This is in stark contrast to the residual effects of cold shock on anemones, where long-lasting tentacle retraction and occasional mortality were observed.

In summary, we provide a rapid and effective method for the generation of aposymbiotic *Aiptasia* sp. that will facilitate studies of symbiosis establishment and maintenance, host–symbiont recognition, and symbiosis function with this model system. In particular, menthol treatment has the potential to rapidly create large stocks of aposymbiotic anemones without compromising their infectivity. Ultimately, this method will help to accelerate the rate of discovery as we try to better understand the function of coral reefs, and their response and adaptive capacity in the face of climate change.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

J.L.M., A.E.S., C.A.O., A.R.G., V.M.W. and S.K.D. conceived and designed the experiments. J.L.M. and A.E.S. carried out the experiments and subsequent data analysis. J.L.M., A.E.S., C.A.O., A.R.G., V.M.W. and S.K.D. wrote the manuscript.

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