



Title	Heat-stress Memory is Responsible for Acquired Thermotolerance in <i>Bangia fuscopurpurea</i>
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Citation	Journal of phycology, 55(5), 971-975 https://doi.org/10.1111/jpy.12895
Issue Date	2019-10
Doc URL	http://hdl.handle.net/2115/79385
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1 Manuscript ID: JPY-19-058-L

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3 HEAT-STRESS MEMORY IS RESPONSIBLE FOR ACQUIRED
4 THERMOTOLERANCE IN *BANGIA FUSCOPURPUREA*¹

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14 Running title: Heat-stress memory in *Bangia*

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16 ¹Received 27 March 2019, Accepted 18 June 2019.

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25 **The environmental stresses that sessile organisms experience usually fluctuate**
26 **dramatically and are often recurrent. Terrestrial plants can acquire memory of**
27 **exposure to sub-lethal heat stress to acquire thermotolerance and survive**
28 **subsequent lethal high-temperature stress; however, little is known concerning**
29 **whether seaweeds acquire thermotolerance via heat-stress memory. We have**
30 **demonstrated that the red seaweed *Bangia fuscopurpurea* can indeed acquire**
31 **memory of sub-lethal high-temperature stress, resulting in the acquisition of**
32 **thermotolerance that protects against subsequent lethal high-temperature stress.**
33 **Moreover, the maintenance of heat-stress memory was associated with a slight**
34 **increase in the saturation level of membrane fatty acids. This suggests that the**
35 **modification of membrane fluidity via changes in membrane fatty acid**
36 **composition is involved in the establishment and maintenance of heat-stress**
37 **memory in *B. fuscopurpurea*. These findings provide insights into the physiological**
38 **survival and growth strategies of sessile red seaweeds to cope with recurrent**
39 **changes in environmental conditions.**

40

41 ***Key index words: Bangia fuscopurpurea, Fatty acid, Heat stress, Rhodophyta,***
42 **Stress memory, Thermotolerance**

43

44 Temperature fluctuation represents one of the most prominent environmental stresses
45 for sessile plants (Cramer et al. 2011, Kumar et al. 2012, Pandey et al. 2017). Increases
46 in ambient temperature can cause heat stress and affect the survival, growth,
47 development and reproduction of plants (Barnabás et al. 2008, Zinn et al. 2010;
48 Hartfield and Prueger 2015). In the natural environment, heat stress is often chronic or

49 recurring. Although the molecular mechanisms that regulate the response to a single
50 acute heat stress have been analyzed in detail, the response to recurring stress is only
51 beginning to be elucidated. In plants, priming by exposure to a sub-lethal heat stress
52 promotes the acquisition of thermotolerance, which allows plants to survive a
53 subsequent and temporally distinct second potentially lethal heat stress (Bruce et al.,
54 2007, Bäurle 2016, Sanyal et al., 2018). These findings indicate that plants can maintain
55 the primed state to respond strongly to a recurring stress, a phenomenon that is termed
56 heat-stress memory (Bruce et al. 2007, Bäurle 2016, Crisp et al. 2016, Sanyal et al.
57 2018).

58 Bangiales is an industrially and economically important marine resource and a
59 large-scale mariculture system has been established for *Pyropia yezoensis* in Japan and
60 *P. haitanensis* and *Bangia fuscopurpurea* in China (Mumford and Miura 1988, Wang et
61 al. 2008, Blouin et al. 2011). Because an increase in temperature affects the growth and
62 productivity of maricultured seaweeds, much effort has been invested to establish and
63 analyze heat stress-tolerant strains of Bangiales (Yan et al. 2010, Ding et al. 2016, Lee
64 et al 2017, Wang et al 2018a). In addition, transcriptome analyses have generated
65 information concerning heat-inducible and -repressible genes (Choi et al. 2013, Im et al.
66 2015, Sun et al. 2015, Cao et al. 2017, Wang et al. 2018a, 2018b), which has indicated
67 that Bangiales can respond to heat stress. However, knowledge concerning the
68 regulatory mechanisms underlying the heat-stress response, tolerance and memory in
69 Bangiales and other seaweeds is lacking.

70 The effects of heat stress on survival of the red seaweed *B. fuscopurpurea* have
71 been investigated using strains harvested from various locations (Sommerfeild and
72 Nichols 1973, Notoya and Iijima 2003, Wang et al. 2008, Mikami and Kishimoto 2018).

73 Sub-lethal heat stress promotes the asexual life cycle, which releases large numbers of
74 asexual spores to increase the number of clones (Notoya and Iijima 2003, Wang et al.
75 2008, Mikami and Kishimoto 2018). Because these findings suggest that *B.*
76 *fuscopurpurea* responds to heat stress like other Bangiales, we addressed whether *B.*
77 *fuscopurpurea* can acquire heat-stress tolerance via acquiring heat-stress memory.

78 We first examined whether *B. fuscopurpurea* can acquire heat-stress tolerance
79 (see Appendix S1 for details of experimental procedure). After growth at 15°C, *B.*
80 *fuscopurpurea* was primed at the sub-lethal high temperature 28°C for 1 week and was
81 further incubated at 30, 32 or 34°C for 1 week (Fig. 1A). As reported previously
82 (Mikami and Kishimoto 2018), the direct transfer of unprimed control samples from
83 15°C to 32 or 34°C for 1 week strongly reduced their viability (Figs. 1B, S1). By
84 contrast, *B. fuscopurpurea* samples that were primed at 28°C for 1 week could survive
85 exposure to lethal conditions for 1 week (Fig. 1B). In this case, priming for 3 days was
86 enough to acquire the heat-stress tolerance (Fig. S2). These findings indicate that the
87 priming of *B. fuscopurpurea* by sub-lethal high temperature promotes the acquisition of
88 heat-stress tolerance, enabling survival of subsequent exposure to lethal high
89 temperature as a short-term response.

90 To assess whether this priming in *B. fuscopurpurea* leads to heat-stress
91 memory, we designed experiments that included the insertion of a recovery phase by
92 returning the samples to non-stress 15°C conditions between the priming phase and
93 triggering phase as a secondary heat-stress exposure to a lethal temperature (Fig. 1C).
94 Importantly, the viability of samples exposed to a 1-day recovery period was equal to
95 that of primed samples without a recovery phase (Fig 1D). Thus, we concluded that *B.*
96 *fuscopurpurea* can acquire heat-stress memory and maintain it after being returned to

97 non-stress conditions. However, increasing the duration of the recovery phase by 1 to 5
98 days gradually decreased viability and 6- and 7-day recovery periods abolished heat-
99 stress memory (Fig. 1D), indicating that increasing the length of the recovery phase
100 reduces heat-stress memory. Thus, we conclude that heat-stress memory is detectable
101 for only 5 days after transfer to normal growth conditions.

102 There is a close relationship between membrane rigidification via a reduction
103 in fluidity and heat-stress tolerance in Poikilothermic organisms, where membrane fatty
104 acid composition influences the fluidity of membranes (Carratù et al. 1996, Horváth et
105 al. 1998, Murakami et al. 2000, Sangwan et al. 2002, Königshofer et al. 2008, Leach
106 and Cowen 2014). We therefore addressed whether the acquisition of the heat-stress
107 memory in *B. fuscopurpurea* is related to changes in the composition of membrane fatty
108 acids (Appendix S1). Firstly, we examined whether the membrane fatty acid
109 composition was modulated by heat stress. When *B. fuscopurpurea* was cultured at
110 15°C followed by 32°C for a week, the relative amounts of saturated fatty acids and
111 monoenes increased compared to those in the 15°C controls, whereas the relative
112 amounts of polyenes decreased (compare the white and black bars in Fig. 2A–C). Thus,
113 heat stress induces an increased saturation level of membrane fatty acids in *B.*
114 *fuscopurpurea*, which likely results in increased membrane rigidification. Secondly, we
115 investigated the effects of priming by exposure to sub-lethal high temperatures on the
116 composition of membrane fatty acids. Cells from samples induced to acquire heat-stress
117 memory exhibited intermediate levels of fatty acid saturation and unsaturation
118 compared to those in the control and non-primed 32°C-shifted samples (Fig. 2A–C;
119 gray bars at 0 days). Thus, the modification of fatty acid composition by priming
120 appears to be important for the acquisition and maintenance of heat-stress tolerance.

121 Lastly, we investigated the relationship between the loss of heat-stress memory (Fig.
122 1D) and changes in fatty acid composition. The relative levels of monounsaturated fatty
123 acids (monoenes) and polyunsaturated fatty acids (polyenes) in cells induced to acquire
124 heat-stress memory gradually became more similar to those observed in non-primed
125 controls (black bars), depending on the length of the recovery phase (Fig. 2B and C).
126 Thus, the loss of heat-stress memory, which resulted in more dead cells at 32°C was
127 closely associated with changes in membrane fatty acid composition. These findings
128 indicate that priming of *B. fuscopurpurea* can lead to the acquisition of heat-stress
129 memory and recovery treatments to its loss, and that both states are associated with a
130 specific membrane fatty acid composition. We therefore conclude that the maintenance
131 of heat-stress memory requires a specific membrane physical state and an appropriate
132 level of membrane fluidity.

133 Our data demonstrate that *B. fuscopurpurea* can acquire heat-stress memory as
134 a heat-stress tolerance strategy (Figs. 1 and 2), confirming that this phenomenon is
135 widely conserved among seaweeds and terrestrial plants. This represents an important
136 strategy that enables plants to adapt and survive under recurring or chronic heat stress in
137 natural environments. Although heat-stress memory involves the maintenance of a heat-
138 stress tolerance-specific composition of membrane fatty acids (Fig. 2), the regulatory
139 mechanisms that alter membrane fatty acid composition by priming are largely
140 unknown. The establishment and maintenance of heat-stress memory, which plays a
141 central role in the maintenance of thermotolerance in response to recurring heat stress in
142 terrestrial plants, is regulated epigenetically (Crisp et al. 2016, Lämke and Bäurle 2017,
143 Friedrich et al. 2019). For example, a relationship exists between the maintenance of
144 heat-stress memory and the accumulation of histone methylation markers H3K4me3

145 and H3K4me2 within chromatin at the promoters of genes that are highly expressed for
146 several days during heat-stress memory (Kusch et al. 2014, Lämke et al. 2016; Liu et al.
147 2018). Thus, heat-stress memory is maintained by structural chromatin changes caused
148 by H3 methylation during the memory phase. Because little is known concerning
149 epigenetic regulation in response to abiotic stress in any Bangiales species, it is
150 necessary to investigate whether genes associated with memory and their epigenetic
151 control potentially regulate membrane fatty acid composition and maintain heat-stress
152 memory in *B. fuscopurpurea*.

153

154 This work was supported in part by the KAKENHI grant (15H04539) from the Ministry
155 of Education, Culture, Sports, Science and Technology, Japan.

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287 **Supporting Information**

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289 **Fig. S1.** Priming-induced thermotolerance in *Bangia fuscopurpurea*. Viability of *B.*
290 *fuscopurpurea* under lethal high temperatures (32 and 34°C) following prior priming at
291 28°C were examined. For primed experiments (right half), samples grown at 15°C were
292 primed at 28°C for a week, then triggering was performed at 30, 32 or 34°C for a further
293 week. For non-primed control experiments (left half), samples grown at 15°C were
294 directly transferred to 30, 32 or 34°C and incubated for a further week. Mean values \pm
295 SD per 0.1 g sample fresh weight were calculated from three independent experiments.
296 Letters denote statistically significant differences ($p < 0.05$) as determined by one-way
297 ANOVA as described in Mikami et al. (2018).

298

299 **Fig. S2.** Time course analysis for acquisition of heat-stress tolerance during the priming
300 phase in *Bangia fuscopurpurea*. Changes in viability associated with the duration of the
301 priming phase were examined. Algal samples grown at 15°C were separately primed at
302 28°C for one to seven days, then triggering was performed for all of samples at 32°C for
303 a further week. Mean values \pm SD per 0.1 g sample fresh weight were calculated from
304 three independent experiments. Letters denote statistically significant differences ($p <$
305 0.05) as determined by one-way ANOVA as described in Mikami et al. (2018).

306

307 **Appendix S1.** Details of experimental procedure.

308

309

310

311 **Legends to Figures**

312

313 Fig. 1. Thermotolerance is dependent on heat-stress memory in *Bangia fuscipurpurea*.

314 (A) Schematic representation of the experimental design to assess the ability of *B.*

315 *fuscopurpurea* to acquire thermotolerance. After growth at 15°C and priming at 28°C

316 for a week, triggering was performed at 30, 32 or 34°C for a further week. The upper

317 panel indicates the control treatment without priming. (B) Priming-induced

318 thermotolerance; *B. fuscopurpurea* survived lethal high temperatures (32 or 34°C)

319 following prior priming under sub-lethal conditions. (C) Schematic representation of the

320 experimental design to assess the ability of *B. fuscopurpurea* to acquire heat-stress

321 memory. Varying durations of the recovery phase at 15°C were inserted between

322 priming and triggering phases. (D) Changes in viability associated with the duration of

323 the recovery phase. Mean values \pm SD per 0.1 g sample fresh weight were calculated

324 from three independent experiments. Letters denote statistically significant differences

325 ($p < 0.05$) as determined by one-way ANOVA as described in Mikami et al. (2018).

326

327 Fig. 2. Changes in membrane fatty acid composition are associated with the acquisition

328 and loss of heat-stress memory in *B. fuscopurpurea*. Changes in the relative amounts of

329 saturated fatty acids (Saturates, A), monounsaturated fatty acids (Monoenes, B) and

330 polyunsaturated fatty acids (Polyenes, C) were analyzed in control samples (white bars)

331 or those triggered either with a lethal high temperature (32°C) without priming (black

332 bars) or with priming and recovery treatments (gray bars). Mean values \pm SD per 0.1 g

333 sample fresh weight were calculated from three independent experiments. Letters

334 denote statistically significant differences ($p < 0.05$) as determined by one-way ANOVA
335 as described in Mikami et al. (2018).

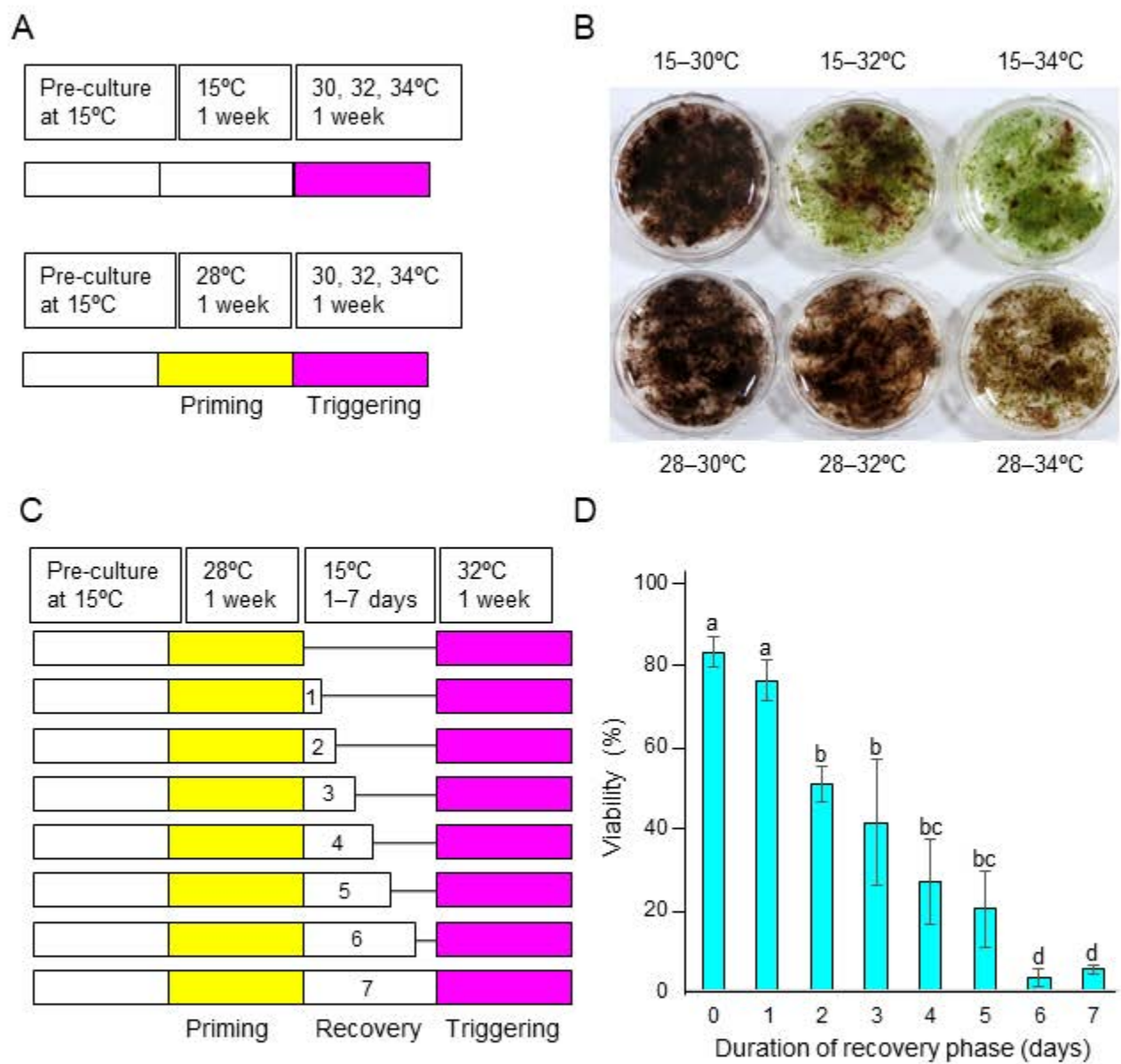


Fig. 1

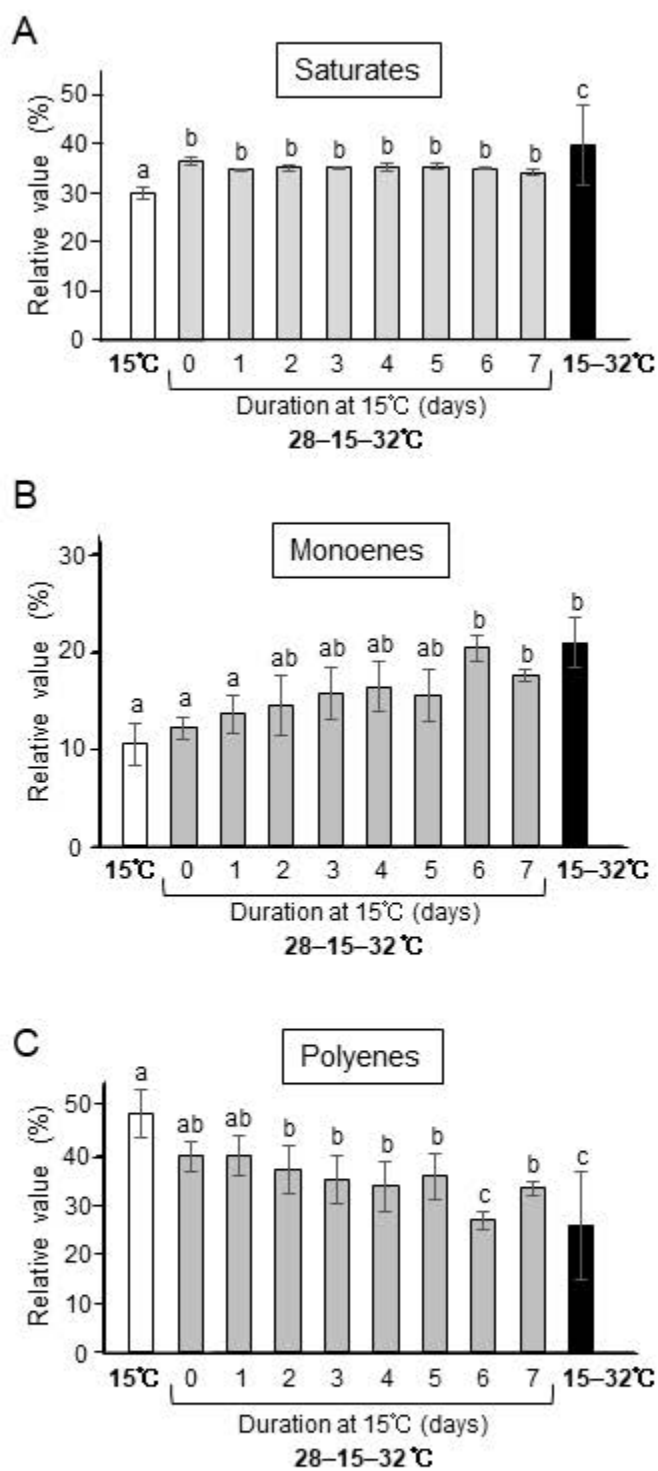


Fig. 2

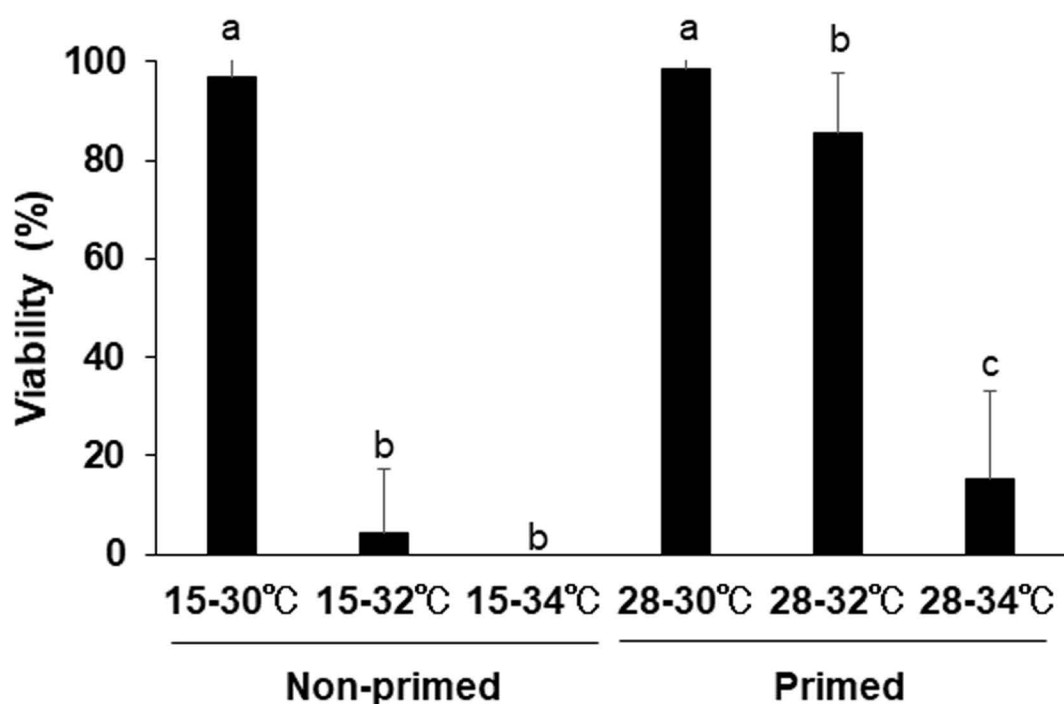


Fig. S1 Priming-induced thermotolerance in *Bangia fuscopurpurea*.

Viability of *B. fuscopurpurea* under lethal high temperatures (32 and 34°C) following prior priming at 28°C were examined. For primed experiments (right half), samples grown at 15°C were primed at 28°C for a week, then triggering was performed at 30, 32 or 34°C for a further week. For non-primed control experiments (left half), samples grown at 15°C were directly transferred to 30, 32 or 34°C and incubated for a further week. Mean values \pm SD per 0.1 g sample fresh weight were calculated from three independent experiments. Letters denote statistically significant differences ($p < 0.05$) as determined by one-way ANOVA as described in Mikami et al. (2018).

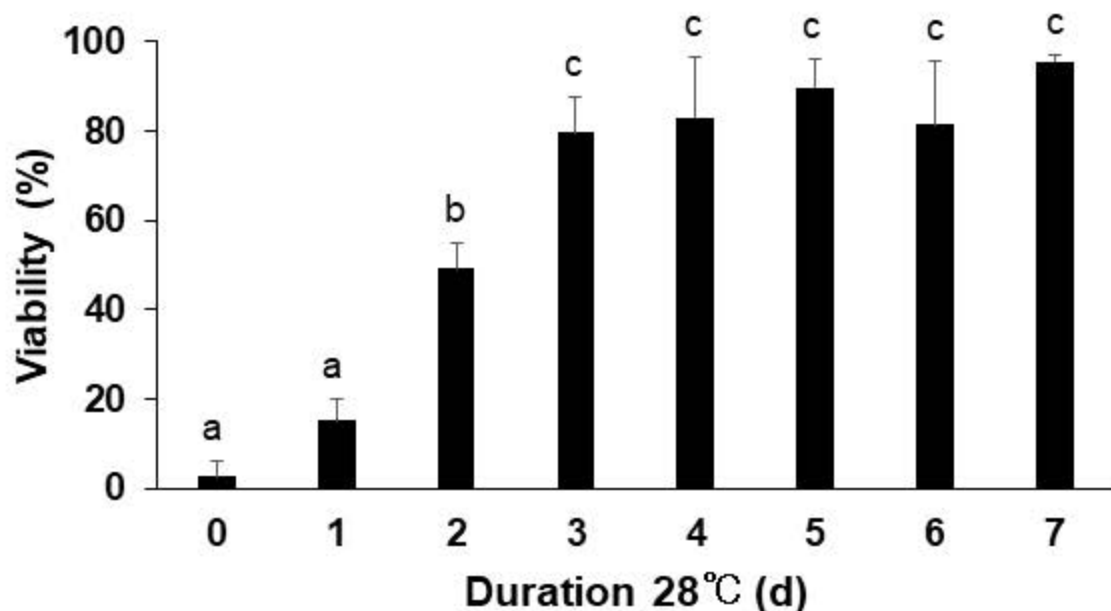


Fig. S2 Time course analysis for acquisition of heat-stress tolerance during the priming phase in *Bangia fuscopurpurea*.

Changes in viability associated with the duration of the priming phase were examined. Algal samples grown at 15°C were separately primed at 28°C for one to seven days, then triggering was performed for all of samples at 32°C for a further week. Mean values \pm SD per 0.1 g sample fresh weight were calculated from three independent experiments. Letters denote statistically significant differences ($p < 0.05$) as determined by one-way ANOVA as described in Mikami et al. (2018).

Appendix S1

Detailed Materials and Methods

Algal material and heat-stress treatment

Gametophytic thalli of the marine red seaweed *Bangia fuscopurpurea* were collected at Esashi, Hokkaido, Japan on 14 May 2010, and a clean single thallus of unknown sex was maintained in the laboratory via the asexual propagation producing clones as an experimental line. To prepare control and starting samples, each 0.1 g (fresh weight) of *B. fuscopurpurea* thalli was cultured in dishes (Azunoru dish $\phi 90 \times 20$ mm height, As One) containing 50 mL esterized artificial seawater at 15°C under $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ irradiation (see Mikami and Kishimoto 2018, for details). Priming of *B. fuscopurpurea* thalli was performed by cultivating 15°C-grown samples at 28°C as a sub-lethal high temperature for 1 week. To analyze thermotolerance acquisition, primed samples were exposed to 32 or 34°C as lethal triggering conditions for 1 week. To assess the acquisition of heat-stress memory, recovery phases consisting of cultivation at 15°C for one to seven days were inserted between phases of priming at 28°C and triggering at 32 or 34°C. To determine the duration required for establishment of heat-stress tolerance, samples were separately primed at 28°C for one to seven days and then all samples were transferred to 32°C and incubated for seven days. Every treatment was repeated three times. The viability of all samples was tested, and the membrane fatty acid composition was analyzed as follows.

Viability test

As described previously (Mikami and Kishimoto, 2018), 0.1 g (fresh weight) of *B.*

fuscopurpurea thalli exposed to priming, recovery and triggering conditions as described above, were visualized daily by staining with ESL medium containing 0.01% erythrosine (Wako Pure Chemical Industries, Japan). After staining for 5 min at room temperature, thalli were gently rinsed with ESL medium to remove excess erythrosine and were mounted on a slide with ESL medium. Thalli were observed and photographed using an Olympus IX73 light microscope equipped with an Olympus DP22 camera. Cells stained by the dye were defined as dead cells. Viability was calculated from the number of living and dead cells obtained using micrographs. Analysis of every treatment was repeated three times and statistical analysis was performed as described in Mikami et al. (2018).

Extraction of lipids and preparation of fatty acid methyl esters

Fresh samples of *Bangia fuscopurpurea* were immersed in boiling water for 3 min to deactivate lipid hydrolytic enzymes and were then freeze-dried and homogenized using a grinder. Lipids were extracted via the Bligh-Dyer method (Bligh and Dyer, 1959) with some modifications. In brief, 0.1 g powdered algal sample was homogenized in a mixture of 2 mL MeOH and 1 mL CHCl₃, 1 mL CHCl₃ was added and the sample was vigorously mixed. After filtration, 2 mL CHCl₃ was added to the algal residue, which was then homogenized and filtered. All the obtained solvent fractions were combined, 1 mL water was added, and the sample was mixed. The organic solvent layer was recovered by centrifugation and evaporated to dryness under reduced pressure. The resultant lipids were resuspended to a known concentration with CHCl₃/MeOH (2:1, v/v) and stored at -30°C until use.

Lipids were converted to fatty acid methyl esters by heating at 90°C for 1 h in a

solution of 1–2% H₂SO₄/MeOH (Christie and Han, 2010) and were then purified in a Pasteur pipette filled with silica gel using hexane-Et₂O (95:5, v/v) as the mobile phase. The purified fatty acid methyl esters were confirmed on a silica gel 60 F₂₅₄ aluminum TLC sheet (Merck, Darmstadt, Germany) using hexane-Et₂O (90:10, v/v) as the developing solvent (Christie and Han, 2010), and were then dissolved in 5 mg/mL hexane for gas chromatography.

GC analysis of fatty acid methyl esters

Fatty acid methyl esters were analyzed using a Shimadzu GC-14A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with an Omegawax 320 column (30 m × 0.32 mm i.d., Supelco, PA, USA). Helium was used as the carrier gas at a constant flow-rate of 2.2 mL/min. The split ratio was 1:25. The column temperature was maintained at 200°C isothermally. The injector and flame-ionization detector (FID) temperatures were set at 230°C and 240°C, respectively. Peaks were monitored on a Chromatopac C-R6A and identified by comparison with the retention data of some authentic standards (GLC-462, Nu-Chek Prep., Elysian, MN), and those of known fatty acids from marine algae (Takagi et al. 1985). Analysis of samples for every treatment was repeated three times and statistical analysis was performed as described in Mikami et al. (2018).

References

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