

## CORAL REEFS

# The role of floridoside in osmoadaptation of coral-associated algal endosymbionts to high-salinity conditions

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The endosymbiosis between *Symbiodinium* dinoflagellates and stony corals provides the foundation of coral reef ecosystems. The survival of these ecosystems is under threat at a global scale, and better knowledge is needed to conceive strategies for mitigating future reef loss. Environmental disturbance imposing temperature, salinity, and nutrient stress can lead to the loss of the *Symbiodinium* partner, causing so-called coral bleaching. Some of the most thermotolerant coral-*Symbiodinium* associations occur in the Persian/Arabian Gulf and the Red Sea, which also represent the most saline coral habitats. We studied whether *Symbiodinium* alter their metabolite content in response to high-salinity environments. We found that *Symbiodinium* cells exposed to high salinity produced high levels of the osmolyte 2-O-glycerol- $\alpha$ -D-galactopyranoside (floridoside), both in vitro and in their coral host animals, thereby increasing their capacity and, putatively, the capacity of the holobiont to cope with the effects of osmotic stress in extreme environments. Given that floridoside has been previously shown to also act as an antioxidant, this osmolyte may serve a dual function: first, to serve as a compatible organic osmolyte accumulated by *Symbiodinium* in response to elevated salinities and, second, to counter reactive oxygen species produced as a consequence of potential salinity and heat stress.

## INTRODUCTION

Coral reefs are biodiversity hot spots of immense biological and economical value (1). The relationship between *Symbiodinium*—an autotrophic endosymbiotic dinoflagellate—and scleractinian corals form the basis of coral reef ecosystems (2). The coral host provides inorganic nutrients and carbon dioxide to the dinoflagellate in exchange for energy in the form of photosynthetically produced carbohydrates (3, 4). This symbiotic relationship is highly sensitive to environmental disturbance. For instance, increases in temperature, salinity, nutrients, and/or high solar irradiance can impair photosynthetic efficiency and enhance the formation of harmful reactive oxygen species (ROS) that must be detoxified by the organism via antioxidants and ROS scavengers (5–8). Otherwise, environmental stress can ultimately lead to coral bleaching, the visual whitening of corals due to loss of their endosymbionts (5, 8). As a consequence, increasing exposure to environmental stress, in particular to rising seawater temperatures, is threatening the existence of coral reefs at a global scale (9, 10).

In comparison to the detrimental effects of elevated seawater temperatures, much less is known about the effects of increased salinities on corals and their endosymbionts. Yet, reef corals from the Red Sea and the Persian/Arabian Gulf (PAG) are commonly found at salinities of up to 41 [Practical Salinity Scale 1978 (PSS-78)] in the Red Sea and up to 50 in the PAG, at summer temperatures exceeding 32° and 35°C in parts of the Red Sea and PAG, respectively (11–14). Both salinity and temperature in these regions are the highest globally to support reef growth (15). Although the osmotic response of *Symbiodinium* at the molecular level is virtually unknown (16–18), studies on free-living algae suggest that

production and accumulation of compatible organic osmolytes (COOs), referred to as osmoadaptation (19), is the most widespread mechanism for adjusting intracellular osmotic pressure in response to elevated salinity; accumulation of inorganic ions and cell volume changes may occur as well. However, because the latter two processes are stressful and can disturb cellular function, they are not considered to represent viable long-term solutions for osmoadaptation (20–23). In contrast, the accumulation of COOs adjusts the osmotic pressure and protects proteins from increased ion concentrations (22).

To test whether *Symbiodinium* is capable of synthesizing COOs, we subjected *Symbiodinium* strains from different clades and origins to conditions of high salinity, both in vitro and in their coral hosts (hereafter referred to as in hospite), and screened for the presence of COOs using gas chromatography–mass spectrometry (GC-MS). We hypothesized that *Symbiodinium* would increase cellular concentrations of COOs in response to elevated salinities and that the response would be similar in vitro and in hospite. Among the COOs employed by *Symbiodinium*, we identified the carbohydrates floridoside, inositol, and mannitol in vitro and in hospite. These compounds can act as both osmolytes and antioxidants, thereby having the potential capacity to convey osmoadaptation to increased salinities and the ability to counter ROS produced as a consequence of salinity or other forms of stress, including heat stress (6, 8, 24–30).

## RESULTS

### High levels of floridoside in *Symbiodinium* exposed to high salinities

We screened *Symbiodinium* cultures exposed to different salinities (that is, 25 for low salinity, 38 for ambient salinity, and 55 for high salinity) for the presence of carbohydrate COOs. A markedly high abundance of a compound at 31.5-min retention time in the GC-MS trace was detected in all four tested *Symbiodinium* cultures under high-salinity conditions (that is, *Symbiodinium microadriaticum* type A1,

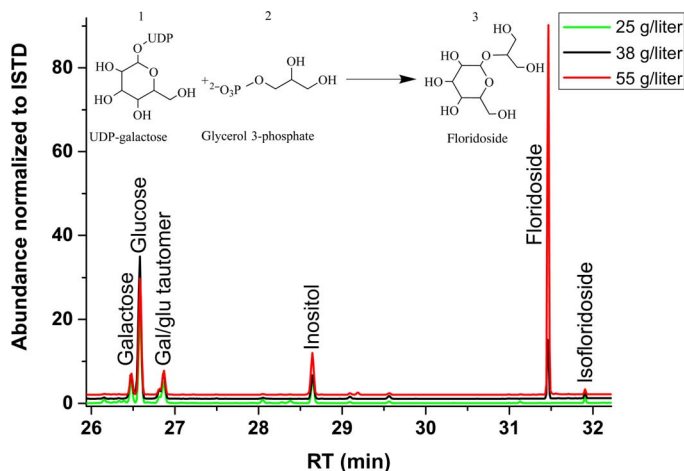
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**Fig. 1. GC analysis of carbohydrate osmolyte diversity in *S. psymgophilum* at three salinity levels.** Green, low-salinity level of 25; black, ambient-salinity level of 38; red, high-salinity level of 55. The trace from 26- to 32-min retention time (RT) shows a floridoside peak at 31.5-min RT. The abundances are normalized to an internal standard (ISTD). The chemical reaction depicts synthesis of floridoside (3) as a product of uridine diphosphate (UDP)-galactose (1) and glycerol 3-phosphate (2). Notably, nearly identical traces were obtained for the other three *Symbiodinium* strains. Gal/glu, galactose/glucose.

*Symbiodinium* sp. type A1, *Symbiodinium minutum* type B1, and *Symbiodinium psymgophilum* type B2) (Fig. 1 and Table 1). This compound was identified as the derivative of floridoside [2-*O*-glycerol- $\alpha$ -D-galactopyranoside-(hexa-trimethylsilane)] by a search against the National Institute of Standards and Technology Mass Spectrometry (NIST MS) library with a reverse match factor of 971 of 1000 (table S1). Floridoside levels of *Symbiodinium* strains exposed to high salinity ranged from  $50.3 \pm 4.3$  nmol (*S. minutum*) to  $489.5 \pm 31.9$  nmol (*S. psymgophilum*) and were consistently represented among the most abundant carbohydrates quantified in this analysis (Table 1). In contrast, floridoside was nondetectable under low-salinity conditions for all *Symbiodinium* strains (Fig. 2A and Table 1). At an ambient salinity level of 38, floridoside was only detected in *S. psymgophilum* at a level of  $51.0 \pm 8.0$  nmol (Fig. 2A and Table 1). This strain also accounted for the highest measured amounts of floridoside under a high-salinity level of 55 ( $489.5 \pm 31.9$  nmol). In comparison, inositol and mannitol were consistently present at low salinities and showed reduced levels at higher salinities for some strains (Table 1).

We identified several other metabolites in the same GC-MS trace and could quantify a total of five additional carbohydrates (that is, glycerol, glucose, galactose, ribose, and fructose) and four amino acids (that is, glycine, alanine, valine, and proline) that serve as putative osmolytes for each of our samples. Metabolite levels were significantly different

**Table 1. COO levels at three salinities of four *Symbiodinium* strains.** Carbohydrates (floridoside, inositol, mannitol, glycerol, glucose, galactose, ribose, and fructose) and amino acids (glycine, alanine, valine, and proline) were quantified by GC-MS. COO levels are provided in nanomoles and normalized to the GC-MS internal standard hydroxy benzylic acid (HBA) and  $1 \times 10^5$  cells  $\text{ml}^{-1}$  [that is, concentration (Conc.) is in nanomoles per  $10^5$  cells or in nanomoles per milliliter culture]. *S. microadriaticum*, type A1; *S. minutum*, type B1; *S. psymgophilum*, type B2.

Metabolite	Salinity	<i>S. microadriaticum</i>		<i>Symbiodinium</i> sp. type A1		<i>S. minutum</i>		<i>S. psymgophilum</i>	
		Conc.	SE	Conc.	SE	Conc.	SE	Conc.	SE
Floridoside	25	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	38	0.0	0.0	0.0	0.0	0.0	0.0	51.0	8.0
	55	144.2	15.0	273.8	28.5	50.3	4.3	489.5	31.9
Inositol	25	0.7	0.1	1.2	0.2	3.7	0.5	19.6	2.5
	38	2.2	0.1	0.4	0.1	1.2	0.1	51.5	8.1
	55	14.3	1.5	2.6	0.3	35.2	3.0	111.1	7.2
Mannitol	25	3.1	0.3	3.0	0.4	3.0	0.4	0.1	0.0
	38	3.7	0.2	2.5	0.2	7.6	0.5	1.8	0.3
	55	2.8	0.3	2.0	0.2	4.6	0.4	1.6	0.1
Glycerol	25	59.5	6.2	92.9	11.7	25.7	3.4	29.1	3.6
	38	130.2	8.3	198.8	15.2	68.2	4.3	37.4	5.9
	55	107.0	11.1	37.2	3.9	6.9	0.6	10.9	0.7
Glucose	25	85.3	8.9	181.0	22.7	27.3	3.6	50.8	6.4
	38	111.9	7.2	69.9	5.4	32.7	2.0	193.9	30.5
	55	152.8	15.9	232.8	24.2	25.0	2.1	429.4	27.9

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Metabolite	Salinity	<i>S. microadriaticum</i>		<i>Symbiodinium</i> sp. type A1		<i>S. minutum</i>		<i>S. psygmophilum</i>	
		Conc.	SE	Conc.	SE	Conc.	SE	Conc.	SE
Galactose	25	89.5	9.3	106.3	13.4	21.9	2.9	12.3	1.6
	38	94.4	6.1	63.6	4.8	26.0	1.6	41.3	6.5
	55	127.2	13.2	166.2	17.3	27.2	1.4	74.3	4.8
Ribose	25	4.6	0.5	8.8	1.1	2.8	0.3	2.9	0.3
	38	8.3	0.5	0.2	0.0	1.0	0.1	5.9	0.9
	55	8.3	0.9	17.9	1.8	1.4	0.1	8.1	0.5
Fructose	25	1.6	0.2	1.7	0.2	8.3	1.1	1.1	0.1
	38	2.6	0.2	1.2	0.1	0.2	0.0	2.9	0.5
	55	2.4	0.2	7.8	0.8	1.3	0.1	3.7	0.2
Glycine	25	18.6	2.0	6.2	0.8	4.1	0.5	0.0	0.0
	38	31.5	2.0	1.3	0.1	5.1	0.5	13.3	2.1
	55	37.9	3.9	11.9	1.2	63.0	5.4	86.4	5.6
Alanine	25	21.3	2.2	20.8	2.6	26.1	3.5	30.0	3.8
	38	154.9	9.9	27.0	2.1	169.7	10.6	14.8	2.3
	55	53.5	5.6	108.3	11.3	30.0	2.5	108.2	7.0
Valine	25	19.1	2.0	14.2	1.8	2.6	0.3	27.7	3.5
	38	1.0	0.1	6.0	0.5	2.0	0.1	8.3	1.3
	55	7.7	0.8	9.8	1.0	3.6	0.3	10.7	0.7
Proline	25	6.5	0.7	3.7	0.5	19.1	2.5	0.0	0.0
	38	12.2	0.8	5.3	0.4	8.5	0.5	5.4	0.9
	55	9.8	1.0	3.4	0.3	3.5	0.3	19.1	1.3

between different salinities (except proline) and *Symbiodinium* strains, as well as combinations thereof (all  $P_{ANOVA} < 0.01$ ) (Table 2). This indicates that these metabolites are differentially regulated under changing salinities and in different *Symbiodinium* strains. Only the production of floridoside showed a substantial increase at high salinities, whereas the levels of all other metabolites (including mannitol and inositol) showed inconsistent patterns (Table 1).

Because floridoside can be derived from glycerol and glucose/galactose (31), we investigated changes in the abundance of these molecules in detail (Fig. 3). In all *Symbiodinium* strains, a decrease in glycerol coincided with the accumulation of floridoside when comparing low- to high-salinity conditions (Fig. 3 and Table 1). Notably, glucose and galactose were enriched under high-salinity conditions in *S. microadriaticum*, *Symbiodinium* sp. type A1, and *S. psygmophilum*; it was only in *S. minutum* that these sugars remained at the same level or showed a slight decrease between low- and high-salinity conditions (Fig. 3 and Table 1).

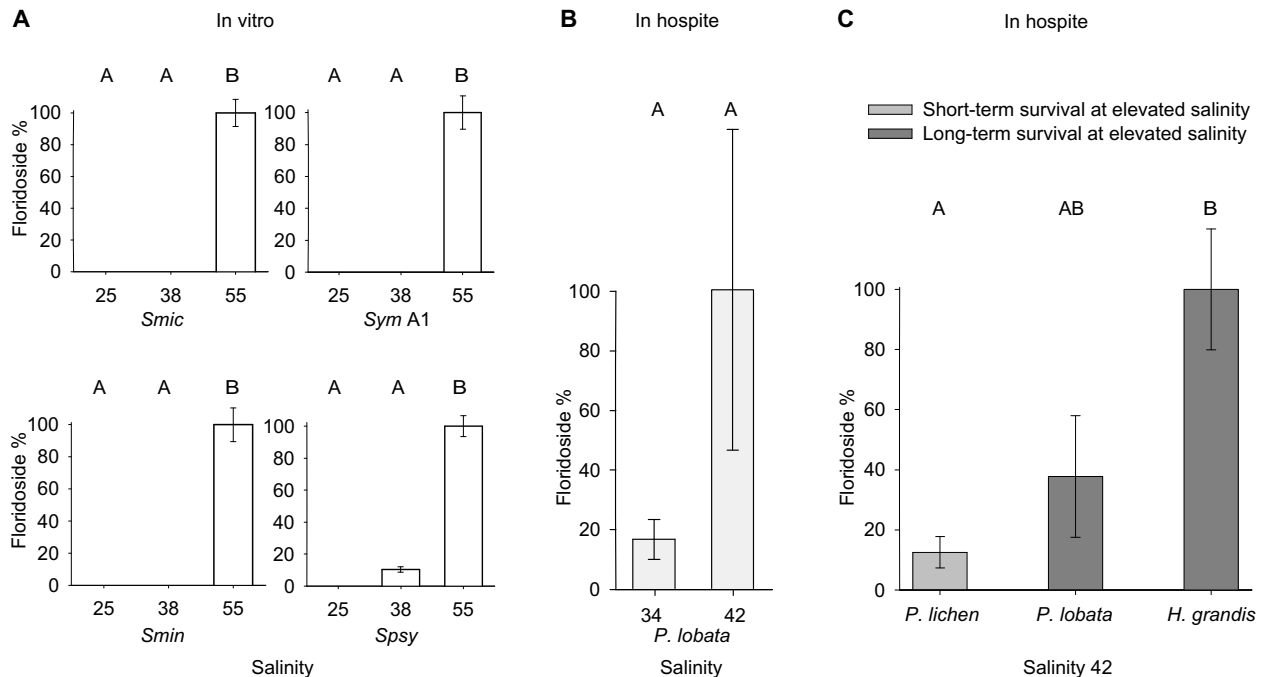
### High levels of floridoside in *Symbiodinium* from coral holobionts exposed to high salinities

To assess the importance of floridoside in coral holobionts, we exposed the coral *Porites lobata* associated with *Symbiodinium thermophilum* originating from the southern PAG (11) to different salinities and measured

floridoside levels. All samples exposed to high salinity displayed a substantial increase of floridoside (~6-fold increase) (Fig. 2B and table S2).

We then compared floridoside levels in *Symbiodinium* from coral holobionts that show different capacities to survive at high salinities. We found that corals that were actively growing and surviving at a salinity of 42 for >24 months (that is, *P. lobata* and *H. grandis*) had higher floridoside levels than *Porites lichen*, which is only capable of surviving for a short period of time at this salinity (Fig. 2C and table S2) (11).

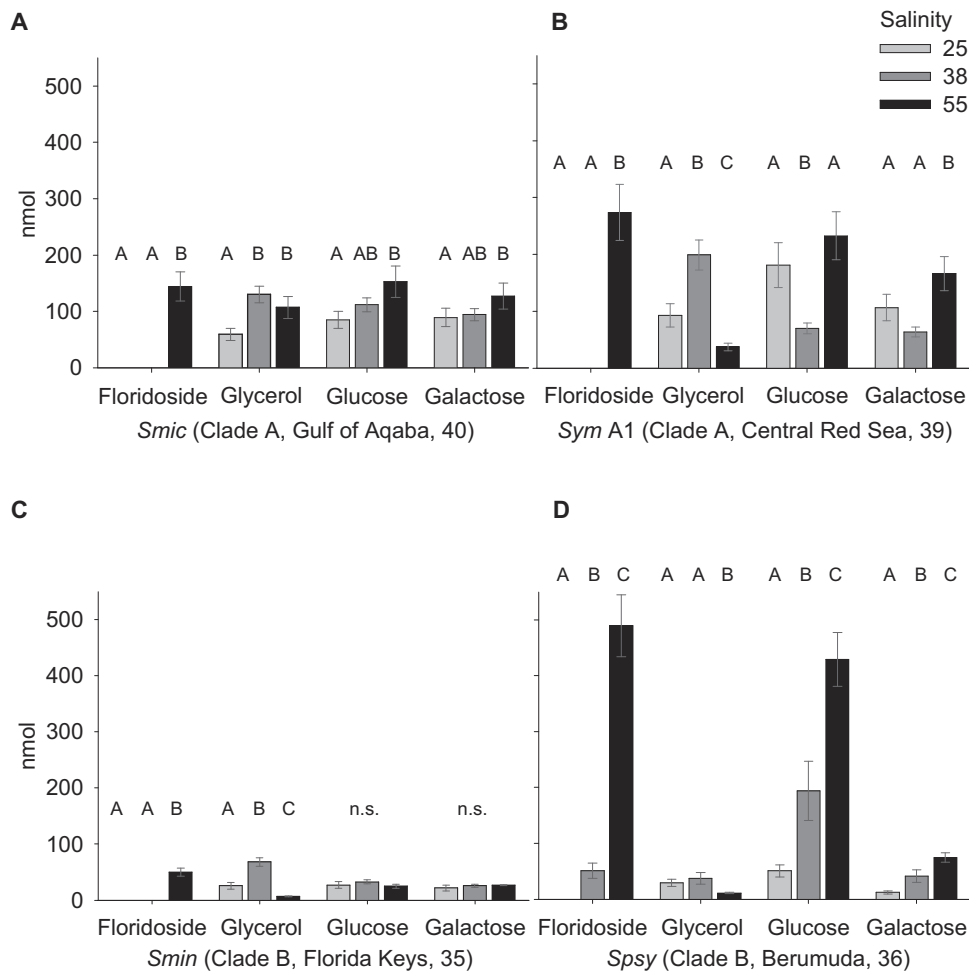
We also checked for homologs of the putative enzyme that converts glycerol 3-phosphate to floridoside in the available genomes of the corals *Acropora digitifera* and *Stylophora pistillata* to assess whether coral hosts are, in principle, able to synthesize floridoside. Following the study of Pade *et al.* (31), we used the gene sequence that encodes the enzymatically active floridoside phosphate synthase/phosphatase from the red alga *Galdieria sulphuraria* (Gasu\_26940) to search for homologs in coral. We found no homologs for the corals *A. digitifera* and *S. pistillata*. Conversely, we found putative full-length homologs of this gene in all available *Symbiodinium* genomes, that is, *S. microadriaticum* (Smic14738, Smic32192, and Smic6078), *S. minutum* (symbB.v1.2.003359, symbB.v1.2.013114, and symbB.v1.2.013196), and *Symbiodinium kawagutii* (Skav203497). Hence, the coral genomes investigated do not harbor the floridoside phosphate synthase/phosphatase enzyme required to produce floridoside.



**Fig. 2. Floridoside levels of *Symbiodinium* in vitro and in hospite at different salinities.** Floridoside levels represent measured amounts (in nanomoles) per  $10^5$  cells  $ml^{-1}$  for cultured *Symbiodinium* and per dry weight (in milligrams) of isolated *Symbiodinium* for coral samples. Data obtained for each experiment were normalized to the highest value (set to 100%). (A) Floridoside levels in four cultured *Symbiodinium* strains in vitro at low (25), ambient (38), and high salinities (55). *Smic*, *S. microadriaticum* (type A1); *Sym A1*, *Symbiodinium* sp. type A1; *Smin*, *S. minutum* (type B1); *Spsy*, *S. psygmophilum* (type B2). (B) Floridoside levels for *S. thermophilum* isolated from *P. lobata* cultured at salinities of 34 and 42. (C) Floridoside levels of *Symbiodinium* from corals with a different long-term survival capacity at high salinities after incubation at a salinity of 42. Floridoside levels were determined for *Symbiodinium* sp. type C96 (*P. lichen*), *S. thermophilum* (*P. lobata*), and *Symbiodinium* sp. type C40 (*Hydnophora grandis*). Error bars denote SE. Letters indicate Tukey's HSD (honestly significant different) post hoc test differences based on pairwise comparisons of analysis of variance (ANOVA) results [groups with different letters are significantly different at  $P < 0.01$  for (A) and  $P < 0.05$  for (C)]. Osmolyte levels represent measured amounts (in nanomoles) per  $10^5$  cells  $ml^{-1}$ .

**Table 2. Statistical evaluation of metabolite changes of carbohydrates and amino acids at three salinities across four *Symbiodinium* strains.** *Symbiodinium* strain and salinity level are fixed factors, and strain\*salinity serves as the interaction effect. Two-way ANOVA was used. Significance levels at  $P < 0.01$  are in boldface.

Measured metabolite	<i>Symbiodinium</i> strain		Salinity		Strain*salinity	
	F	P	F	P	F	P
Floridoside	84.9329	<0.0001	408.1242	<0.0001	61.0533	<0.0001
Inositol	199.4686	<0.0001	116.6508	<0.0001	37.0999	<0.0001
Mannitol	86.3291	<0.0001	30.0316	<0.0001	14.8469	<0.0001
Glycerol	97.7188	<0.0001	92.3146	<0.0001	22.3281	<0.0001
Glucose	74.5158	<0.0001	66.2231	<0.0001	32.1908	<0.0001
Galactose	77.2113	<0.0001	31.7549	<0.0001	7.7703	0.0001
Ribose	45.7089	<0.0001	47.2761	<0.0001	33.2876	<0.0001
Fructose	6.0851	0.0031	23.1370	<0.0001	52.0966	<0.0001
Glycine	55.2650	<0.0001	282.0940	<0.0001	56.3923	<0.0001
Alanine	15.1440	<0.0001	123.7890	<0.0001	97.8385	<0.0001
Valine	42.2583	<0.0001	71.2012	<0.0001	12.3394	<0.0001
Proline	23.6038	<0.0001	2.8518	0.0774	57.3734	<0.0001



**Fig. 3. Osmolyte levels of floridoside and intermediates (glycerol, glucose, and galactose) at three salinities across four *Symbiodinium* strains.** Glucose and galactose can be metabolized to glycerol (via the Calvin cycle) and cover UDP-galactose and glycerol 3-phosphate demands for floridoside synthesis. Bar graphs show floridoside, glycerol, glucose, and galactose levels for *S. microadriaticum* (type A1) (A), *Symbiodinium* sp. type A1 (B), *S. minutum* (type B1) (C), and *S. psygmophilum* (type B2) (D) cultures at low salinity (25; light gray), ambient salinity (38; gray), and high salinity (55; black) after 4 hours at  $108 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Clade designation, origin of strain, and respective average salinity are provided following strain abbreviations. Error bars denote SE. Letters indicate Tukey's HSD post hoc differences based on pairwise comparisons of ANOVA results (groups with different letters are significantly different at  $P < 0.05$ ). Tukey's post hoc tests were not performed if ANOVAs yielded a nonsignificant  $F$  ratio, designated as n.s. (not significant). Osmolyte levels represent measured amounts (in nanomoles) per  $10^5$  cells  $\text{ml}^{-1}$ .

## DISCUSSION

Here, we determined levels of the carbohydrates floridoside, inositol, and mannitol in response to high salinity in *Symbiodinium* in vitro and in hospite to assess the capacity of these COOs to fulfill a function in osmoadaptation to high salinities. Notably, other osmolytes that were not measured in our study, for example, taurines, betaines, and dimethylsulfoniopropionate (32–34), might also contribute to the osmoadaptation of *Symbiodinium*. Consequently, our data should not be considered a complete assessment of all osmolytes in *Symbiodinium*. However, we identified the osmolyte floridoside consistently and in increased amounts in *Symbiodinium* at high salinities. This shows that cultured *Symbiodinium* cells produce floridoside in response to salinity stress. We also found elevated floridoside levels in *Symbiodinium* of corals capable of long-term survival under high-salinity conditions (*P. lobata* and *H. grandis*). Hence, our work has uncovered a putative key COO that allows *Symbiodinium* to osmoadapt to extreme salinities in vitro and in hospite. The fact that our results show a consistent accumulation of floridoside across a range of *Symbiodinium* strains and

experimental conditions provides strong support for the notion that increased floridoside levels constitute part of the osmoadaptive response to high salinities. Our findings also provide insight for our understanding of the role of osmoadaptation in the coral-*Symbiodinium* endosymbiosis, with implications for the coral stress response, as further discussed below.

### Floridoside as a key osmolyte in *Symbiodinium*

Synthesis of the osmolyte floridoside has been identified as a conserved pathway in evolutionary distinct organisms, such as red algae, green algae, and Cryptophyceae (24, 31, 35). Floridoside is produced by UDP galactosyltransferases via condensation of glycerol 3-phosphate and UDP-galactose (31). UDP-galactose demands can be supplied via starch mobilization, resulting in increased glucose/galactose pools, as described for the green algae *Dunaliella* sp. under conditions of high salinity (36). A similar mechanism might explain the increased levels of glucose (in cultures of *S. microadriaticum* and *Symbiodinium* sp. type A1) and galactose (in all cultured strains) that we measured in *Symbiodinium* at high salinity (Fig. 3 and Table 1). The consistent

increase of glucose and galactose in concert with up-regulation of floridoside suggests that these compounds fulfill a conserved osmotic adjustment function within the genus *Symbiodinium* (Fig. 3 and Table 1). Notably, the different *Symbiodinium* strains exhibited a differential response in regard to absolute floridoside production levels, but these differences did not follow a geographic (considering salinity levels at origin) or phylogenetic pattern (Fig. 3). Besides UDP-galactose, the second component required for floridoside synthesis, glycerol 3-phosphate, is likely supplied from photosynthesis or via the Calvin cycle (37). Glycerol 3-phosphate can be produced from glycerol, which is considered to be one of the main COOs in marine algae (38), although it has been shown to be released under osmotic pressure in *Symbiodinium* (16, 18, 39). Given that our analysis on available *Symbiodinium* genomes confirmed the presence of homologs for the enzyme that produces floridoside, it will be interesting to check for the presence and identity of the enzyme(s) required for floridoside synthesis in ecologically relevant *Symbiodinium* (for example, *S. thermophilum*) and to determine whether gene expression or duplication can be aligned with strain- or species-specific differences (40).

### Osmoadaptation in the coral-*Symbiodinium* endosymbiosis, with implications for the coral stress response

Our results demonstrate that exposure to high salinities leads to higher endosymbiont floridoside levels in vitro and in hospite. This may point to the fact that elevated floridoside levels increase not only the capacity of *Symbiodinium*, but also that of the holobiont, to cope with the effect of high osmotic pressure in extreme environments. Furthermore, our results suggest that osmolarity changes within the coral tissue are noticed by endosymbiotic *Symbiodinium*; thus, both the coral and *Symbiodinium* respond to salinity changes, presumably by adjusting the inner osmolarity to the higher outside salinity. However, it remains to be determined whether *Symbiodinium* adjust their inner osmolarity the same way in hospite as in vitro (32). Since potentially any metabolite contributes to the osmolarity in hospite, the endosymbiotic environment in coral cells might differ from the in vitro seawater environment (38).

Beyond its function as an osmolyte, floridoside has been shown to act as an antioxidant with ROS-scavenging properties (25, 26). Hence, floridoside has the capability to convey osmoadaptation as well as to counter ROS produced in response to salinity or other forms of stress (6, 8, 24, 26–28). In particular, increased ROS is detrimental to photosystem II in photosynthetic organisms (5, 41–43). Therefore, the production of antioxidants at high salinities is potentially important for *Symbiodinium*, and floridoside represents an osmolyte that fulfills a ROS-scavenging function at the same time (26). Increasing levels of floridoside and oxidative stress in response to increased salinities were shown in the red algae *Gracilaria sordida* (44) and *Gracilaria corticata* (45), respectively. Future work should determine the exact role that floridoside plays in response to conditions of high salinity, as either an antioxidant, a COO, or both, for example, by comparing floridoside and ROS levels at ambient and increased salinities.

The notion that ROS-producing mechanisms for photosynthetic organisms are similar (if not identical) under salinity and heat stress (42) also has interesting implications for our understanding of the response to heat stress in *Symbiodinium* and, by extension, for the coral hosts. Similar to salt stress, heat stress results in malfunction of the photosynthetic machinery of *Symbiodinium* and in the production of ROS that may damage the algal cells and, in the case of the coral-algal endosymbiosis, may trigger bleaching (46). We find that *Symbiodinium* exposed to high salinity in vitro and in hospite accumulate high amounts

of floridoside. Hence, elevated floridoside levels in high-salinity environments may increase the ability to tolerate heat stress in *Symbiodinium* and, by extension, their coral hosts through scavenging of increased ROS levels. Consequently, the thermal resilience of coral holobionts may potentially increase under conditions of high salinity because of the accumulation and inherent antioxidative capabilities of floridoside. Experimental data connecting increased floridoside levels to decreased ROS and bleaching levels at increased salinities are in demand to support this potential link.

## MATERIALS AND METHODS

### *Symbiodinium* cultures

*S. microadriaticum* CCMP2467 (type A1; originally isolated from *S. pistillata*, Red Sea, Gulf of Aqaba) (40), *Symbiodinium* sp. type A1 (originally isolated from *Astreopora* sp., Central Red Sea) (47), *S. minutum* Mf1.05b (type B1; isolated from *Orbicella faveolata*, Florida Keys, United States) (48, 49), and *S. psymphilum* Mf10.14b.02 (type B2; isolated from *Oculina diffusa*, Bermuda, UK) (50, 51) were cultured in f/2 medium without silicium under a photon flux of  $108 \mu\text{mol m}^{-2} \text{s}^{-1}$  at 26°C (52). The f/2 medium was prepared from sterile filtered Red Sea water [with a salinity of 38 and complemented with  $\text{NaNO}_3$ ,  $\text{NaH}_2\text{PO}_4$ , vitamins, and trace metals (53)]. For each strain, we used replicate culture flasks and prepared three salt-adjusted f/2 media [salinities of 25, 38, and 55; following the study of van der Merwe *et al.* (54)] either by adding appropriate amounts of NaCl or by diluting the media with double-distilled  $\text{H}_2\text{O}$  ( $\text{ddH}_2\text{O}$ ). Triplicates of 5 ml of *Symbiodinium* cultures at exponential growth ( $10^5$  to  $10^6$  cells  $\text{ml}^{-1}$ ) were transferred to 35 ml of salt-adjusted f/2 media for each salinity and incubated for 4 hours under culturing conditions. *Symbiodinium* cells were subsequently harvested by centrifugation (4500g, 10 min, 4°C). Cells were counted by fluorescence-activated cell sorting (FACS). To do this, 1 ml of each *Symbiodinium* culture was collected and fixed with formalin. After washing, samples were resuspended in 1 ml of  $\text{ddH}_2\text{O}$  and labeled with SYBR Gold (Thermo Fisher Scientific), of which 150- $\mu\text{l}$  aliquots were supplied for FACS (50- $\mu\text{l}$  counting volume). FACS measurements were conducted in triplicate on a cell analyzer (LSRFortessa, BD Biosciences). Stained DNA or RNA was excited via a 488-nm blue laser and emission-detected for total nucleotide detection (Alexa Fluor 488 filters, Life Technologies). Detection of valid signals was a combined measure of forward and side scattering and of both fluorescence signals (that is, SYBR Gold and chlorophyll autofluorescence). FACS data were analyzed by FlowJo 10 flow cytometry analysis software (FlowJo LLC).

### Coral cultures

Corals were kept in long-term culture (>24 months) in different compartments of the experimental coral mesocosm facility at the University of Southampton at salinity levels mimicking those of their habitats of origin [salinity of 42 for *P. lobata* from the PAG (11) and salinity of ~36.5 for *P. lichen* and *H. grandis* from the Indo-Pacific (11, 55, 56)]. *P. lobata* was additionally cultured under a reduced salinity condition of 34 for >24 months. Corals were kept at a temperature of 26°C with a 10-hour/14-hour light/dark cycle under a photon flux of  $150 \mu\text{mol m}^{-2} \text{s}^{-1}$  (11). Light and temperature levels suitable for long-term culture of the corals were established during previous work (55, 56). These three species were studied owing to their different capacity for survival at elevated salinities: *P. lichen* associated with *Symbiodinium* sp. type C96 exhibits short-term survival (11),

whereas *P. lobata* with *S. thermophilum* (11, 14) and *H. grandis* with *Symbiodinium* sp. type C40 both show long-term survival.

Survival capacity was determined before the experiment by incubating 10 replicate colonies for >24 months at a salinity of 42. In contrast to other Indo-Pacific species (including *P. lichen*) (11), *P. lobata* and *H. grandis* have not suffered any mortality and have been actively growing during this time (56). In the present experiments, replicate colonies of *P. lichen* and *H. grandis* previously cultured at lower salinities (34 and 36.5) were gradually adjusted to a high salinity of 42 over 2 days before being moved to the high-salinity compartment for 12 days before sampling. Replicate colonies were produced by earlier fragmentation.

Using an airbrush, coral tissue was blasted off the skeleton with ice-cold, sterile-filtered, freshly prepared artificial seawater with the same salinity as the culture-rearing water. Three coral colonies were used per species to extract zooxanthellae. *Symbiodinium* cells were precipitated from the homogenate by centrifugation at 2500g for 5 min at 4°C. The cells were washed twice (to remove the host tissue fraction and to prevent residual salt to infer with the downstream GC-MS analyses), first in precooled seawater and then in ddH<sub>2</sub>O, each step followed by centrifugation and resuspension. Exposure to ddH<sub>2</sub>O was limited to ~30 s to minimize potential effects of the hypo-osmotic environment. It should also be noted that because of the slow diffusion of water through the cell wall/membrane, the *Symbiodinium* cells experienced only a minimal influx of water and were protected from bursting by their rigid cell walls. Furthermore, any water that had entered the cells was removed in the process of freeze-drying and hence could not interfere with osmolyte downstream analysis. After the final centrifugation step, all liquid was removed, and the cell pellet was lyophilized for 14 hours. Although this procedure might still retain traces of host animal tissue/cells and osmolyte profiles may contain a host component, we consider the potential carry-over minor, in particular because corals do not seem to harbor the gene to produce floridoside.

### Metabolite extraction and recovery

Cell pellets from *Symbiodinium* cultures and *Symbiodinium* extracted from coral tissues were resuspended and washed with 30 ml of sterile seawater on ice, pelleted, and washed for ~30 s, with further 5 ml of ddH<sub>2</sub>O to remove residual salt. After a further centrifugation step, pellets were resuspended in 5 ml of ddH<sub>2</sub>O, and cells were disrupted by tip ultrasonication for 4 min at 3-s pulsing and 6-s pause. Cell debris was removed by centrifugation at 20,000g for 20 min at 4°C. Proteins, DNA, or RNA was removed by ethanol precipitation by adding nine parts of -20°C ethanol to one part of supernatant. The precipitate was pelleted and removed by centrifugation, whereas the supernatant was frozen in liquid nitrogen and lyophilized. Dry samples were dissolved in 240 µl of ddH<sub>2</sub>O, spiked with 10 µl of internal standard [HBA (1 µg/µl) in ddH<sub>2</sub>O], transferred into GC vials, and dried under vacuum. For derivatization, 50 µl of MOX reagent (2% methoxamine HCl in pyridine) was added to each sample, and the solution was heated to 75°C for 1 hour. Afterward, 100 µl of MSTFA solution [*N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide, 1% trimethylchlorosilane; Thermo Scientific] was added, and samples were heated for 1 hour at 75°C. Each sample vial was centrifuged at 2000g for 10 min, and 100 µl of the supernatant was transferred to glass inserts placed inside GC vials.

### GC-MS analysis, quantification, and analysis

Derivatized carbohydrates, amino acids, and further intracellular compounds were characterized and quantified by GC-MS. For separa-

tion, an HP-5ms column (Agilent Technologies) and a temperature profile starting at 70°C were chosen. Temperature was increased in increments of 6°C min<sup>-1</sup> up to 230°C, followed by increments of 60°C min<sup>-1</sup> to a maximum of 280°C where it was held for 4 min. Metabolites were quantified by standard curves produced with pure glucose (99.5%; Sigma) and glycerol (≥99.5%, ACS Reagent-grade; Sigma), with 60, 30, 10, 1, and 0.1 µg of both compounds. The calibration standards were spiked with 1 µg of HBA, derivatized for GC-MS, and analyzed as described above. All samples were prepared and measured in triplicate. GC-MS data were processed (that is, background subtraction, peak picking, and integration; OpenChrom v. 0.901, Lablicate UG) and MS ionization spectra-identified (NIST MS Software 2.0, Agilent Technologies). Statistical testing was conducted on normalized quantities of metabolites (in nanomoles) using ANOVAs and Tukey's HSD post hoc tests to assess differences between pairwise comparisons. In the case of cultured *Symbiodinium*, we normalized to 10<sup>5</sup> cells ml<sup>-1</sup>. For corals, *Symbiodinium* extracts were normalized over dry weights in milligrams.

### Floridoside homologs in coral and *Symbiodinium* genomes

We searched for homologs of the putative enzyme that converts glycerol 3-phosphate to floridoside (31) in the available coral and *Symbiodinium* genomes via BLASTp on reefgenomics.org (57) using an *e* value cutoff of <10<sup>-5</sup>. Briefly, the amino acid sequence for the gene (*Gasu\_26940*) coding for floridoside phosphate synthase/phosphatase from the red alga *G. sulphuraria* was queried against the genomes of *A. digitifera* (58) and *S. pistillata* (59), as well as against the genomes of *S. microadriaticum* (40), *S. minutum* (49), and *S. kawagutii* (60).

### SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/3/8/e1602047/DC1>

table S1. Metabolites in GC-MS traces and identification information for NIST MS 2.0 Library Search.

table S2. Overview of floridoside amounts from *Symbiodinium* of coral holobionts at different salinities.

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agreement no. 311179 to J.W.). **Author contributions:** M.A.O., T.R., and C.R.V. designed and conceived the experiments. M.A.O., T.R., C.R.V., C.D., and J.W. generated, analyzed, and interpreted data. C.R.V., C.D., and J.W. contributed cultures, reagents, and materials. C.R.V. wrote the manuscript, with contributions from T.R., C.D., J.W., and M.A.O. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors. Contact the corresponding author for animal specimens.

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## The role of floridoside in osmoadaptation of coral-associated algal endosymbionts to high-salinity conditions

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