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**Corals through the light : phylogenetics, functional diversity and adaptive strategies of coral-symbiont associations over a large depth range**

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*Citation for published version (APA):*

Rodrigues Frade, P. (2009). Corals through the light : phylogenetics, functional diversity and adaptive strategies of coral-symbiont associations over a large depth range.

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# CHAPTER 5

## **Abundant betaines in reef-building corals: Phenotypic plasticity and evidence for photosystem-protective roles**

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Submitted to *Proceedings of the Royal Society B - Biological Sciences*



## ABSTRACT

Betaines, although well known as compatible solutes that exert protein- and membrane-stabilizing effects, have received minimal attention in reef-building corals. Here we employ metabolite profiling, based on isotope dilution LC/MS with six deuterated internal standards, to show that glycine betaine (GlyB), proline betaine (ProB), and at least five other betaines (plus the related metabolite dimethylsulfoniopropionate) occur in physiologically significant concentrations, totaling 20 to  $\geq$  150 mM, in all of 10 species of reef-building corals collected at Curaçao. Moreover, betaine concentrations vary in field-collected corals in patterns that match *a priori* predictions for molecules that defend photosynthesis against negative effects of high irradiance: in *Madracis mirabilis* – which occupies unshaded locations – GlyB and ProB are 44-78% more abundant at 5 than 20 m depth, and in *Madracis pharensis* – which occupies unshaded and shaded locations - the two betaines are 32-44% more abundant in unshaded colonies. Such patterns demonstrate that coral betaines are phenotypically plastic and that they likely participate in acclimatisation and play photosystem-protective roles. Betaines may well defend against bleaching: they are documented in vascular plants and free-living algae to protect photosystem II against stresses of high temperature and high irradiance – a protective effect that in corals would be targeted at the putative chain of bleaching causation.

## INTRODUCTION

Betaines are amino (or imino) acids fully methylated at the N position, resulting in a permanent positive charge on nitrogen. They are widely recognised as metabolites active in the regulation of cellular physiology, serving often as compatible solutes that stabilize proteins and membranes (Rhodes and Hanson 1993; McNeil *et al.* 1999). Betaines, however, have been studied minimally in corals. Of all the many betaines, only one, glycine betaine (GlyB), has been mentioned in scleractinian (stony) corals, and it has been reported just three times (Moore and Huxley 1976; Suenaga 2004; Andrell *et al.* 2006), including only one instance (Andrell *et al.* 2006) in which it was identified definitively and quantified (in a non-reef-building species). We were drawn to the study of betaines in reef-building corals by hints in our prior research that these corals in fact possess multiple betaines in abundance.

Our motivation to develop definitive knowledge of coral betaines has been stimulated in particular by the roles that betaines are already known to play in modulating and defending photosynthesis in vascular plants and free-living algae. In these groups, betaines stabilize photosystem II in the photosynthetic apparatus (Papageorgiou and Murata 1995; Schiller and Dau 2000), protecting photosystem II against stresses of high temperature and high irradiance (Yang *et al.* 1996; Allakhverdiev *et al.* 2003; Klimov *et al.* 2003). These effects are sufficiently established that multiple crops (or plants used as models in crop research) are genetically engineered to increase betaine expression to enhance stress resistance (McNeil *et al.* 1999; Sakamoto and Murata 2002; Prasad and Saradhi 2004; Shirasawa *et al.* 2006; Yang *et al.* 2007; Chen and Murata 2002).

Urgent attention deserves to be focused on metabolites with the potential to protect photosystem II in corals against stresses of high temperature and high irradiance, because corals are presently threatened by those stresses. Reef-building corals are animal-algal symbioses in which photosynthesis carried out by endocytic dinoflagellate symbionts (zooxanthellae) is essential. Corals are in decline (Hoegh-Guldberg *et al.* 2007; Carpenter *et al.* 2008), and one of the most important causes of this decline is bleaching, a reduction or loss of the symbiont populations and/or their photopigments (Warner *et al.* 1999; Fitt *et al.* 2001; Lesser and Farrell 2004). Bleaching most commonly results from stresses of elevated water temperature and high light intensity acting synergistically; moreover, when corals bleach because of such stresses, the primary insult is often to photosystem II, or to pathways of electron flow downstream from photosystem II (Warner *et al.* 1999; Fitt *et al.* 2001; Jones and Hoegh-Guldberg 2001; Hill *et al.* 2004; Lesser and Farrell 2004). Global warming is anticipated to drive an upward trend in bleaching because many species of corals experience temperature stress when sea temperature is elevated by only a few degrees Celsius above traditional norms (Hill *et al.* 2004; Hoegh-Guldberg *et al.* 2007; Carpenter *et al.* 2008). The fact that betaines are established protective agents – defending photosystem II in plants and free-living algae against heat and irradiance stresses – has strongly motivated our research on corals.

Our thinking has been particularly influenced by observations such as the following: In vascular plants, GlyB protects the D1 protein in photosystem II against heat- and irradiance-induced inactivation (Allakhverdiev *et al.* 2003). Simultaneously, one of the most probing studies of coral bleaching identifies the D1 protein in reef-building corals as the primary focus of damage during bleaching caused by synergistic heat and light stresses (Warner *et al.* 1999). Such observations persuade us that knowledge of coral betaines is likely to be imperative for

a full understanding of bleaching – and might even be useful for combating bleaching.

In addition to possible bleaching-protective roles, betaines are likely also to modulate photoinhibition in corals, just as they do in crop plants. Healthy reef-building corals commonly experience photoinhibition when exposed to the high light intensities of midday in shallow, clear, tropical waters (Gorbunov *et al.* 2001; Jones and Hoegh-Guldberg 2001; Winters *et al.* 2003). Photosystem II is the principal locus of this photoinhibition (Gorbunov *et al.* 2001; Jones and Hoegh-Guldberg 2001). This fact highlights the pertinence of agents such as betaines that are known (from studies of plants and free-living algae) to stabilize photosystem II.

Betaines may also be important to reef-building corals in several ways in addition to the defense of photosynthesis that has particularly stimulated our interest. The most firmly established role of betaines in animals, for example, is as intracellular osmotic agents (Anthoni *et al.* 1991; Yancey 2005) – suggesting that betaines serve as osmolytes in corals (Andrell *et al.* 2006).

We had two primary goals for this research. One goal was to determine with definitive methods the concentrations of multiple, soluble betaines in a diverse set of reef-building corals collected from natural habitats. This work, carried out by stable isotope dilution LC/MS, entailed the synthesis of multiple deuterated standards inasmuch as most betaines are unavailable commercially in any form. Our second goal was to articulate and test bold, *a priori* hypotheses regarding patterns of variation of betaine concentrations in wild corals – hypotheses predicated on the postulate that betaines defend photosynthesis against negative effects of high irradiance.

We here report that multiple betaines are present in abundance in all of 10 species of reef-building corals studied. Moreover, we report strong evidence that corals substantially modulate tissue concentrations of betaines in response to environmental conditions – pointing to betaines as important, heretofore unrecognised agents of acclimatisation and stress response. Finally, based on tests of two out of three *a priori* hypotheses, we report that, in field populations of corals, colonies exposed to relatively high irradiance accumulate betaines to higher concentrations than conspecific colonies exposed to lower irradiance – pointing to the use of betaines by corals as photoprotective agents.

## MATERIALS AND METHODS

Field collections of 10 species of reef-building corals were carried out in March 2007 at Curaçao, Netherlands Antilles. Curaçao was chosen because highly detailed studies have been completed there on the light relations of corals of the genus *Madracis* (Vermeij and Bak 2002), permitting well informed *a priori* hypotheses regarding spatial and temporal variation in *Madracis* betaine concentrations. Coral specimens, taken from exposed and upward-facing colony locations unless otherwise stated, were collected by scuba near Buoy One, 1 km from the laboratories of CARMABI Foundation. Possession of specimens and transfer to Michigan State University (MSU) were sanctioned by CITES permit issued by CARMABI.

Each specimen of *Madracis mirabilis*, which grows in multiple fingerlike extensions, consisted of the tips (outer 1-2 cm) of several extensions from a colony. Specimens of other species were pieces of superficial skeleton, covered by living tissue, removed from colonies by chisel. Each specimen was from a different colony, was placed immediately in an individual plastic bag with local seawater, and promptly protected from light within a black plastic bag until transferred to a light-tight box in the field. Later, each specimen was

cleaned of any extraneous organisms, cut to fit a 15-mL Teflon vial (Nalgene), and frozen by liquid nitrogen or dry ice. The total number of specimens was limited to 110 by constraints on cryogenic transport. Specimens were kept and traveled to MSU in dry ice, then (4-8 days after collection) transferred to a -80°C freezer.

For analysis, tissue was removed from coral skeleton by blasting with ice-cold distilled water using a WaterPik®. The area blasted was measured by digital image processing (SCION IMAGE) using direct images or images of foil previously fitted to coral contours. Immediately after blasting, a blastate subsample was acidified to 3% formic acid, mixed with methanol (2 parts blastate: 1 part methanol), internal standards added, sonicated (10 min), and centrifuged (3000 x g, 5 min). Supernatant (shown to contain 99% of blastate betaines) was evaporated dry, redissolved in pH 3.85 citrate buffer, and analysed by means of a Supelco Discovery-HS F5 HPLC column coupled to a Waters LCT Premier LC/MS using electrospray ionization in positive mode. In most cases, extraction and LC/MS were carried out twice.

Five betaines – GlyB, alanine betaine (AlaB), b-alanine betaine (bAlaB), proline betaine (ProB), and hydroxyproline betaine (HProB) – were quantified against matching deuterated internal standards ( $d_9$ -GlyB,  $d_9$ -AlaB,  $d_9$ -bAlaB,  $d_6$ -ProB, and  $d_6$ -HProB) synthesised from amino acids and  $CD_3I$  (Chen and Benoiton 1976). Tissue dimethylsulfonylpropionate (DMSP) was also quantified using a deuterated standard:  $d_6$ -DMSP synthesised from  $CD_3I$  and 3-mercaptopropanoic acid. The purities and concentrations of all internal standards were established using  $^1H$ -NMR and LC/MS. Taurine betaine (TauB) and trigonelline (Trig) – which we had not anticipated to be in the tissues – were quantified against  $d_9$ -GlyB.

In the coral literature, metabolite concentrations, whereas commonly normalised to specimen surface area, are sometimes normalised to protein or photopigment. To permit comparison, protein and photopigment were measured in subsamples of each blastate. Protein was quantified in triplicate by Bradford assay (Bio-Rad) using standards of bovine serum albumin (Pierce). To analyse photopigment, a light-protected blastate subsample was mixed with nine times its volume of acetone. The mix was acidified to 3 mM HCl to convert all chlorophyll to pheophytin (to remove effects of variable native degradation of chlorophyll to pheophytin), and pheophytin *a* was quantified with a Turner TD-700 fluorometer and standards of acidified spinach chlorophyll *a* (Sigma-Aldrich).

Statistical calculations were carried out in SPSS 15.0 for WINDOWS. Normality and homoskedasticity were assessed by probit plots and Levene's test. In the minority of cases that displayed non-normality or heteroskedasticity, log transformation often corrected the problem. Otherwise nonparametric Mann-Whitney or Kuskal-Wallis tests were used. Null hypotheses were rejected if the likelihood of the results obtained was  $< 0.05$  under the null hypothesis.

## RESULTS

Multiple betaines and DMSP were present in all specimens of the 10 coral species studied (Tables 5.1-5.4). In the great majority of specimens, betaines were the most abundant metabolites detected within the range of molecular masses examined, 50-1500 u. Some specimens also contained similar concentrations of a metabolite putatively assigned as a methylated guanine based on MS/MS and high resolution MS data. Concentrations of glycerol and other polyols, determined by separate GC/MS analyses, were lower than betaine concentrations by 10- to

100-fold.

For study of patterns of variation in betaine concentrations in field populations of corals, *Madracis mirabilis* was the focal species because its ecological distribution permits straightforward prediction of spatial variation. *Madracis mirabilis* is common at Curaçao at a wide range of depths, where it grows almost always in fully exposed (unshaded) locations (Vermeij and Bak 2002), so that (on a given reef) the depth of a colony is an excellent proxy for the irradiance the colony experiences. Our *a priori* hypothesis was that betaine concentrations vary inversely with depth. Our design was to collect equal numbers ( $n = 8$ ) of specimens from 5, 10, and 20 m. We tested our hypothesis by two-way ANOVA, the two factors being the specimen depth and the time of specimen collection: morning (09:30-11:00 local time) or afternoon (15:30-17:00). Time effects and time-depth interactions (assessed by two-tail test) were not observed. As we hypothesised, however, the concentrations of several betaines – GlyB, AlaB, ProB, and HProB – varied inversely with depth ( $P < 0.05$ , one-tail test) (Figure 5.1, Table 5.1). Concentrations of protein and pheophytin did not vary significantly with time or depth. When betaine levels were normalised to protein or pheophytin, results paralleled those described.

Our second *a priori* hypothesis was focused on *Madracis pharensis*, which – in contrast to *M. mirabilis* – occurs in both exposed and highly shaded locations at most depths (Vermeij and Bak 2002). We hypothesised that betaine concentrations are elevated in exposed colonies, and as a test, we analysed approximately equal numbers ( $n = 9-10$ ) of specimens of *M. pharensis* from two sharply different light regimes – locations as exposed to full illumination and as shaded as we could find – at one depth, 10 m. Based on two-way ANOVA, there were no effects of collection time (morning or afternoon) and no time-exposure interactions. As hypothesised, however, concentrations of several betaines averaged significantly higher ( $P < 0.05$ , one-tail test) in exposed than shaded colonies (Table 5.2).

Our third *a priori* hypothesis was intended to provide a strong test of time-of-day effects. We hypothesised that specimens of *M. mirabilis* and *M. pharensis* collected at dawn have lower betaine concentrations than ones obtained later in the day. To test the prediction, we collected

**Table 5.1** Concentrations (in  $100 \times \mu\text{mol cm}^{-2}$ ; average  $\pm$  SE) in *Madracis mirabilis* collected from exposed (unshaded) locations. Values for specific depths are listed only for compounds that showed statistically significant differences in concentration as a function of depth. AlaB = alanine betaine,  $\beta$ AlaB =  $\beta$ -alanine betaine, DMSP = dimethylsulfoniopropionate, GlyB = glycine betaine, HProB = hydroxyproline betaine, ProB = proline betaine, TauB = taurine betaine, Trig = trigonelline.  $n = 24, 8, 8$  and  $8$  for the four columns. Pheophytin and protein are in units of  $100 \times \text{mg cm}^{-2}$ .

Compound	All colonies sampled	Colonies at 5 m	Colonies at 10 m	Colonies at 20 m
DMSP	6.01 $\pm$ 0.23			
GlyB	39.8 $\pm$ 2.6	52.6 $\pm$ 3.2	37.2 $\pm$ 1.9	29.5 $\pm$ 3.4
AlaB	3.55 $\pm$ 0.18	4.05 $\pm$ 0.32	3.64 $\pm$ 0.27	2.96 $\pm$ 0.24
$\beta$ AlaB	2.23 $\pm$ 0.45			
ProB	1430 $\pm$ 71	1640 $\pm$ 112	1520 $\pm$ 94	1140 $\pm$ 92
HProB	2.73 $\pm$ 0.31	3.32 $\pm$ 0.46	3.16 $\pm$ 0.65	1.71 $\pm$ 0.28
TauB	23.7 $\pm$ 2.7			
Trig	4.58 $\pm$ 0.39	5.90 $\pm$ 0.51	4.04 $\pm$ 0.34	3.79 $\pm$ 0.88
Pheophytin	0.489 $\pm$ 0.041			
Protein	7.56 $\pm$ 0.70			

**Table 5.2** Concentrations (in  $100 \times \mu\text{mol cm}^{-2}$ ; average  $\pm$  SE) in *Madracis pharensis* at 10 m. Values for exposed and shaded colonies are listed only for compounds that showed statistically significant differences in concentration as a function of exposure.  $n = 19, 10$  and  $9$  for the three columns. Pheophytin and protein are in units of  $100 \times \text{mg cm}^{-2}$ . See Table 5.1 for abbreviations.

Compound	All colonies sampled	Exposed colonies	Shaded colonies
DMSP	7.05 $\pm$ 0.82		
GlyB	38.7 $\pm$ 3.2	43.7 $\pm$ 5.0	33.2 $\pm$ 3.2
AlaB	2.99 $\pm$ 0.25	3.36 $\pm$ 0.32	2.58 $\pm$ 0.37
$\beta$ AlaB	5.96 $\pm$ 1.9		
ProB	1020 $\pm$ 87	1190 $\pm$ 127	829 $\pm$ 84
HProB	4.56 $\pm$ 2.5		
TauB	16.4 $\pm$ 2.1		
Trig	2.70 $\pm$ 0.33		
Pheophytin	2.13 $\pm$ 0.24		
Protein	28.5 $\pm$ 2.5	33.5 $\pm$ 3.5	23.0 $\pm$ 2.7

**Table 5.3** Concentrations (in  $100 \times \mu\text{mol cm}^{-2}$ ; average  $\pm$  SE) in *Madracis senaria* collected from exposed locations. Values for colonies collected in morning (09:30-11:00) and afternoon (15:30-17:00) are listed only for compounds that showed statistically significant differences in concentration between the times.  $n = 25, 7$  and  $18$  for the three columns. Pheophytin and protein are in units of  $100 \times \text{mg cm}^{-2}$ . See Table 5.1 for abbreviations.

Compound	All colonies sampled	Morning-collected colonies	Afternoon-collected colonies
DMSP	8.84 $\pm$ 0.67		
GlyB	55.1 $\pm$ 4.1	35.9 $\pm$ 6.1	62.6 $\pm$ 4.0
AlaB	2.27 $\pm$ 0.20	1.63 $\pm$ 0.26	2.51 $\pm$ 0.23
$\beta$ AlaB	0.601 $\pm$ 0.21		
ProB	27.2 $\pm$ 3.4		
HProB	0.861 $\pm$ 0.083	0.546 $\pm$ 0.072	0.983 $\pm$ 0.099
TauB	24.0 $\pm$ 2.4	14.4 $\pm$ 1.3	27.8 $\pm$ 2.9
Trig	11.2 $\pm$ 0.94	8.50 $\pm$ 0.82	12.3 $\pm$ 1.2
Pheophytin	1.36 $\pm$ 0.12		
Protein	14.5 $\pm$ 1.1		

specimens ( $n = 8$  for each species) from exposed locations at 10 m at dawn (07:00-08:00) and compared them with exposed colonies collected at 10 m during morning and afternoon. No significant differences were evident (one-tail tests).

*Madracis senaria* (unlike *M. mirabilis* or *M. pharensis*) exhibits a dramatic tendency to shift its use of exposed and shaded locations with depth in a way that opposes depth effects on irradiance experienced. When Vermeij and Bak (2002) ranked the locations of *M. senaria* colonies at Curaçao from 1 (fully exposed) to 5 (highly shaded), the colonies at 5 m occurred only at ranks 4-5, whereas colonies at 10 and 20 m occurred, respectively, at ranks 2-5 and 1-5. Without making *a priori* predictions, we collected the most exposed colonies available at three depths (5, 10, and 20 m) at two times (morning and afternoon). No significant depth effects or time-depth interactions were observed (two-tail tests). However, GlyB, AlaB, and HProB were more concentrated ( $P < 0.05$ , two-tail test) in afternoon- than morning-collected specimens (Table 5.3): a result compatible with our hypothesis that betaines are elevated with



**Table 5.4** Concentrations (in 100 × μmol cm<sup>-2</sup>; average ± SE) in seven scleractinian coral species collected from exposed locations. Sample size (*n*) and depths of collection are listed with each species. *n* = 2 for pheophytin in *Siderastrea*. Pheophytin and protein are in units of 100 × mg cm<sup>-2</sup>. See Table 5.1 for abbreviations.

Compound	<i>Agaricia agaricites</i> <i>n</i> = 3 5, 10, 20 m	<i>Agaricia humilis</i> <i>n</i> = 3 All 5 m	<i>Agaricia lamarcki</i> <i>n</i> = 3 All 20 m	<i>Meandrina meandrites</i> <i>n</i> = 1 20 m	<i>Montastraea faveolata</i> <i>n</i> = 3 5, 10, 20 m	<i>Porites astreoides</i> <i>n</i> = 3 5, 10, 20 m	<i>Siderastrea siderea</i> <i>n</i> = 3 5, 10, 20 m
DMSP	9.47 6.1–13	2.92 2.8–3.2	10.2 4.1–20	45.9	6.66 4.9–9.4	11.3 6.2–17	3.97 2.2–6.8
GlyB	185 139–227	259 231–275	181 106–277	1320	196 127–245	7.08 5.1–8.9	188 139–263
AlaB	2.87 2.3–3.8	5.55 4.0–6.4	2.26 1.2–3.5	30.5	3.41 2.2–5.5	58.9 11–147	1.97 1.3–3.1
βAlaB	0.445 0.12–0.99	0.248 0.13–0.37	0.0957 0.03–0.14	12.3	1.34 0.20–3.5	0.288 0.22–0.36	0.111 0.046–0.18
ProB	25.5 13–40	58.6 29–112	19.3 9.4–28	189	31.6 12–47	1820 848–2670	17.3 14–23
HProB	0.405 0.28–0.56	0.902 0.75–1.0	0.313 0.17–0.54	5.73	0.444 0.40–0.50	103 57–165	9.03 0.44–26
TauB	0.226 0.18–0.30	4.63 3.7–5.9	0.409 0.19–0.71	4.53	1.19 0.050–1.9	44.3 19–72	0.541 0.023–1.0
Trig	2.06 1.6–2.4	2.15 1.9–2.4	1.36 0.89–2.1	26.6	3.90 2.2–6.2	2.85 1.7–4.7	1.26 1.1–1.6
Pheophytin	1.82 1.4–2.2	1.72 0.89–2.5	2.09 1.8–2.4	4.73	3.10 2.9–3.5	2.71 1.5–4.2	0.865 0.55–1.2
Protein	20.7 17–23	40.0 27–61	24.3 16–31	150	35.2 27–45	24.7 11–34	43.7 22–77

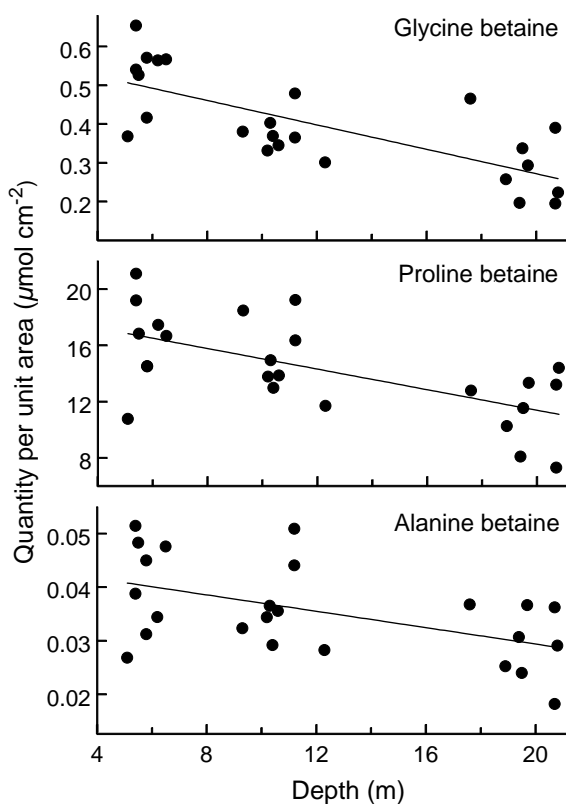
increased light exposure.

Dominant algal symbionts were genetically typed (ITS2-rDNA) in all specimens of *Madracis*. Symbionts consistently belonged to *Symbiodinium* clade B (B7 and B13 in *M. mirabilis*; nearly always B7 in *M. pharensis* and *M. senaria*).

## DISCUSSION

Multiple betaines are demonstrated (Tables 5.1–5.4) to be abundant in all 10 species of reef-building corals studied. Betaines, in fact, are the most abundant metabolites detected within the range of molecular masses examined and are far more concentrated than polyols. In regards quantitative chemistry, our objective was a definitive study. Accordingly, we employed stable isotope dilution LC/MS (without chemical derivatization) using deuterated internal standards for most compounds of interest. Moreover, we systematically confirmed all chemical identifications by measures of multiple criteria: chromatographic retention time, exact mass, signatures of naturally occurring stable isotopes, and molecular fragmentation products.

For expressing concentrations of coral metabolites, we have followed common practice. Coral tissue thickness and tissue volume are imperfectly known. Concentrations therefore are commonly expressed per cm<sup>2</sup> of coral surface area sampled (Tables 5.1–5.4). We have



**Figure 5.1** Betaine concentration is inversely related to water depth in *Madracis mirabilis*. Each symbol represents a single coral colony. All colonies grew in fully exposed, unshaded locations. Lines, fitted by linear regression, are for description only; statistical analysis was by ANOVA because colonies were collected at discrete nominal depths. Where Y is betaine concentration ( $\mu\text{mol cm}^{-2}$ ) and D is depth (m),  $Y = 0.587 - 0.0157D$  for glycine betaine;  $Y = 18.7 - 0.366D$  for proline betaine; and  $Y = 0.0447 - 0.000764D$  for alanine betaine.

also estimated molar concentrations, however, by assuming a model tissue thickness of 1 mm (probably an overestimate for *Madracis* species but an underestimate for some other species). With this approach, we estimate total betaine concentrations of 20 to  $^3$  150 mmol per liter of tissue volume. Operative betaine concentrations are probably substantially higher because of subcellular betaine compartmentalization. Using our conservative estimates, tissue molar concentrations in corals are high enough for betaines to play roles as compatible solutes capable of stabilizing protein and membrane functions (Prasad and Saradhi 2004; Shirasawa *et al.* 2006; Yang *et al.* 2007; Chen and Murata 2008).

Betaines have been minimally recognised in reef-building corals heretofore. GlyB is the only betaine mentioned in previous reports, and the only roles ascribed to it have been that it is an osmolyte (Andrell *et al.* 2006) or a potential taste factor affecting *Acanthaster* predation (Moore and Huxley 1976; Suenaga 2004). The present report represents the discovery in reef-building corals of all the other betaines reported. DMSP, a tertiary sulfonium metabolite often addressed in studies of quaternary ammonium compounds (e.g. betaines) because of its related chemical structure, has previously been known in corals from Hill *et al.* (1995) and subsequent reports.

Our motivation to study coral betaines (as stressed in the Introduction) has been stimulated

by our interest in key, but heretofore unrecognised, roles that betaines are likely to play in coral tissue function. Betaines probably represent a newly discovered defense system against environmental stresses, adding to the list of known defense systems in corals (Gorbunov *et al.* 2001). Based on studies of both vascular plants (Papageorgiou and Murata 1995; Yang *et al.* 1996; Schiller and Dau 2000; Allakhverdiev *et al.* 2003) and free-living algae (Papageorgiou and Murata 1995; Schiller and Dau 2000; Klimov *et al.* 2003; Allakhverdiev *et al.* 2007; Hema *et al.* 2007), betaines protect photosystem II against photoinhibition and other light and temperature stresses – so much so that the genetic manipulation of crop plants to increase betaine levels is a prominent tactic in crop bioengineering (*see* Introduction). It would seem likely in principle that betaines exert similar protective effects in reef-building corals, where such effects could be especially important because the initial insult in coral bleaching is often to photosystem II (Warner *et al.* 1999; Jones and Hoegh-Guldberg 2001; Hill *et al.* 2004; Lesser and Farrell 2004).

Direct support for betaine protective roles in corals is provided by our tests of *a priori* hypotheses focused on patterns of spatial and temporal variation in coral field populations. Recognizing that corals are subject to photoinhibition under high irradiance (Gorbunov *et al.* 2001; Jones and Hoegh-Guldberg 2001; Winters *et al.* 2003) and that betaines are implicated in protecting photosynthesis against the stresses of high irradiance (Allakhverdiev *et al.* 2003; Prasad and Saradhi 2004; Chen and Murata 2002), we predicted positive relations between betaine concentrations in coral colonies and the irradiance the colonies experience.

We found in *Madracis mirabilis* – a species that generally lives in fully exposed locations at Curaçao (Vermeij and Bak 2002) – that shallow-water colonies receiving relatively high irradiance have 37-94% higher concentrations of GlyB, AlaB, ProB, and HProB than deep-water colonies (Figure 5.1, Table 5.1). In *M. pharensis* – a species that occupies both exposed and highly shaded locations at any single depth (Vermeij and Bak 2002) – several betaines are 30-44% more concentrated in exposed than shaded colonies studied at 10 m (Table 5.2). In *M. senaria*, some betaines are 54-80% more concentrated in the afternoon than the morning (Table 5.3). These patterns are expected if betaines protect photosynthesis against negative effects of high irradiance in reef-building corals, as they do in vascular plants.

Further support for a positive relation between betaine concentrations and irradiance in reef-building corals comes from our study of non-*Madracis* species (Table 5.4). The data on those species provide descriptive information only, because sample sizes were too small for statistical testing. Nonetheless, in the *Porites* and *Siderastrea* – sampled from depths of 5, 10, and 20 m – concentrations of GlyB, AlaB, ProB, and HProB were dramatically higher at 5 m than at the other depths, paralleling the pattern in *M. mirabilis*. For example, in *Siderastrea* the concentration of GlyB at 5 m was 2.6  $\mu\text{mol cm}^{-2}$ , compared to an overall average of 1.9  $\mu\text{mol cm}^{-2}$  at all three depths and to concentrations of 1.4-1.6  $\mu\text{mol cm}^{-2}$  at 10-20 m. Betaine concentrations were also higher in the three specimens of *Agaricia humilis* – a species that occurs principally in shallow waters and was collected exclusively at 5 m – than in the three specimens of the congener *A. lamarcki* – which occurs only in deep waters and was collected at 20 m. The range of concentrations of many betaines in *A. humilis* did not overlap the range in *A. lamarcki*. These results in *Agaricia* again parallel the pattern in *M. mirabilis*.

The protein- and membrane-stabilizing effects of betaines – widely recognised – are attributed for the most part to influences of betaines on the structure of water (McNeil *et al.* 1999; Bennion and Daggett 2004; Street *et al.* 2006). In contrast to disrupting solutes such as urea

and inorganic ions, which tend differentially to displace water of hydration from surfaces of proteins, betaines evade participation in such displacement and leave hydration layers intact. Existing theories of betaine action stress universal properties of betaines. Accordingly, different chemical species of betaines are often viewed as being relatively interchangeable and additive (Rhodes and Hanson 1993; Yancey 2005).

Despite the argument for interchangeability, Anthoni *et al.* (1991) predicted that biologists ultimately will recognize that natural selection has favored various betaines in various contexts because of distinctive properties. Concordant with this proposition, some corals have dramatically different betaine signatures than others (Tables 5.1-5.4). *Madracis senaria* and the *Agaricia* species, for example, have high GlyB concentrations in relation to their ProB concentrations, whereas *M. mirabilis*, *M. pharensis*, and *Porites astreoides* are high in ProB relative to GlyB.

The metabolic source of betaines in corals (and location of expression of genes involved) is a key question for future research. GlyB is synthesised by a wide variety of animals and algae (Blunden and Gordon 1986; Anthoni *et al.* 1991; McNeil *et al.* 1999; Yancey 2005), suggesting that the source of GlyB in corals could be animal, algal, or both. The same seems likely for at least some other betaines. DMSP in corals is almost surely algal in origin because no animal is known to synthesize DMSP. We hasten to add that, in an animal-algal symbiosis, the source of a metabolite is not necessarily well correlated with the subcellular compartments (animal or algal) where the metabolite is accumulated.

A puzzling aspect of our results is that time-of-day effects on betaine concentrations, although absent in *Madracis mirabilis* and *M. pharensis*, are present in *M. senaria* (Table 5.3). With the preliminary knowledge of *Madracis* comparative physiology that exists (Frade *et al.* 2008), it would be speculative to postulate why *M. senaria* is particularly poised to respond to diurnal light rhythms. The response in *M. senaria*, nonetheless, seems commensurate with the species' comparatively low levels of photoprotective xanthophyll pigments (Frade *et al.* 2008) and its avoidance of high irradiance (Vermeij and Bak 2002).

The discovery of multiple betaines in abundance in reef-building corals calls for focused consideration of the full range of roles that betaines play. We have already emphasised the well known protein- and membrane-stabilizing roles of betaines, with specific applications to photosystem II. Now we itemize other roles, the first three of which are not mutually exclusive to the stabilizing roles already stressed:

(1) Our results on *Madracis* (Tables 5.1-5.3, Figure 5.1) demonstrate that coral betaine concentrations are phenotypically plastic and responsive to environmental conditions – pointing to betaines as heretofore unknown agents of coral acclimatisation. Coral defense systems exhibit acclimatisation (Edmunds and Gates 2008; Middlebrook *et al.* 2008). Betaine dynamics should be considered as potential mechanisms.

(2) Betaines are potentially agents by which the expression of animal genes in the coral symbiosis affects function of the algal symbionts. Betaine levels in specific subcellular compartments depend on the concentrations and regulation of enzymes and other proteins involved in betaine biosynthesis, transport, and degradation in both animal and alga. Within this complex set of interacting processes, one possibility is for betaines synthesised in the animal tissue to be exported and accumulated in the symbionts. This sort of process could be one of the specific mechanisms by which symbiont function is modulated by the animal

tissue, perhaps especially in cases of photosystem II modulation (Brown *et al.* 2002b; Abrego *et al.* 2008).

(3) Betaines could also be agents by which clades or types of symbionts differ in the susceptibility of photosystem performance to environmental stressors (Rowan 2004; Jones *et al.* 2008). Within the set of interacting processes noted in (2), symbiont types might differ in betaine biosynthesis, transport, or degradation in ways that result in divergent intracellular betaine concentrations.

(4) Betaines are well recognised as signals that mediate fish attraction to prey (Kasumyan and Døving 2003). Thus fish (among other applications) might maintain awareness of predation on corals by sensing betaines released through damage to coral tissues, much as documented for DMSP at Curaçao (DeBose *et al.* 2008).

(5) Betaines and DMSP are increasingly recognised as key substrates of microbial metabolism in marine ecosystems (DeLong and Karl 2005).

(6) The most firmly established function of betaines in animals is as osmotic agents (Anthoni *et al.* 1991; Yancey 2005) – suggesting osmolyte function in corals (Andrell *et al.* 2006).

Betaines long been recognised to play crucial roles in cellular metabolism. For the understanding of reef-building corals, it is striking to discover that betaines are among the most abundant metabolites and clearly are modulated in response to environmental conditions. Both concentrations and plasticity point to betaines as metabolites of significance.

## ACKNOWLEDGEMENTS

We thank R.P.M. Bak, J.W.H. Dacey, D.A. Gage, S.D. Hill, and E.H. Gladfelter for insightful advice and encouragement. M.T. Cook measured coral surface areas. J.M. Hanna assisted with GC/MS analyses of glycerol and other polyols. We feel privileged to have used the resources of the Caribbean Research and Management of Biodiversity (CARMABI) Foundation in Curaçao, and we thank A.O. Debrot, the Director, and B. Leysner for invaluable assistance during our work there. This research was funded by the Department of Zoology and the Department of Biochemistry and Molecular Biology, Michigan State University.

### Author contributions

R.W.H., A.D.J. and P.R.F. designed the study; R.W.H., P.R.F. and J.P.G. collected and processed the samples in Curaçao; C.L. synthesized and established the purity of standards; C.L., A.D.J. and R.W.H. carried out the tissue assays; P.R.F. genetically typed the *Madracis* specimens; R.W.H. collated the data, performed the statistical analyses, and wrote the first draft of the manuscript. All authors reviewed the manuscript prior to submission.