Light enhanced calcification in *Stylophora pistillata*: effects of glucose, glycerol and oxygen

Zooxanthellate corals have long been known to calcify faster in the light than in the dark, however the mechanism underlying this process has been uncertain. Here we tested the effects of oxygen under controlled pCO2 conditions and fixed carbon sources on calcification in zooxanthellate and bleached microcolonies of the branching coral Stylophora pistillata. In zooxanthellate microcolonies, oxygen increased dark calcification rates to levels comparable to those measured in the light. However in bleached microcolonies oxygen alone did not enhance calcification, but when combined with a fixed carbon source (glucose or glycerol), calcification increased. Respiration rates increased in response to oxygen with greater increases when oxygen is combined with fixed carbon. ATP content was largely unaffected by treatments, with the exception of glycerol which decreased ATP levels.

1   Light enhanced calcification in *Stylophora pistillata*: effects of glucose, glycerol and oxygen

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16   **Abstract**

17   Zooxanthellate corals have long been known to calcify faster in the light than in the dark,

18   however the mechanism underlying this process has been uncertain. Here we tested the effects of

19   oxygen under controlled pCO_2 conditions and fixed carbon sources on calcification in

20   zooxanthellate and bleached microcolonies of the branching coral *Stylophora pistillata*. In

21   zooxanthellate microcolonies, oxygen increased dark calcification rates to levels comparable to

22   those measured in the light. However in bleached microcolonies oxygen alone did not enhance

23   calcification, but when combined with a fixed carbon source (glucose or glycerol), calcification

24   increased. Respiration rates increased in response to oxygen with greater increases when oxygen

25   is combined with fixed carbon. ATP content was largely unaffected by treatments, with the

26   exception of glycerol which decreased ATP levels.

27   **Introduction**

28   Rising ocean temperatures and declining pH have received considerable attention in recent years

29   (e.g. Hoegh-Guldberg et al., 2007; McCulloch et al., 2012). Among the major concerns are the

30   impacts on coral reefs, particularly on corals themselves. As temperatures rise, bleaching events

31   (the loss of symbiotic zooxanthellae) become more frequent and severe, reducing the ability of

32   the coral to cope with other stressors, leading to the loss of coral reefs (e.g. McWilliams et al.,

33   2005). Rising CO_2 levels further compound the problem by reducing aragonite saturation state

34   and potentially calcification (e.g. Langdon and Atkinson 2005; Holcomb et al., 2010).

35   Central to our understanding of the response of corals to changing environmental conditions is an

36   understanding of the role the symbiotic zooxanthellae play in coral calcification. It has long been

37   established that calcification rates increase in zooxanthellate corals during periods in which

38   photosynthesis is occurring (Yonge, 1931), a process known as light-enhanced calcification (e.g.

39   Vandermeulen et al., 1972; Gattuso et al., 1999). Comparisons of facultatively symbiotic corals

40   with and without zooxanthellae similarly show increased calcification in the presence of

41   symbionts (Jacques et al., 1983). Yet, comparisons of zooxanthellate corals to azooxanthellate

42   corals show that some azooxanthellate corals can grow just as rapidly (Marshall, 1996;

43   Mortensen and Rapp, 1998), bleached corals can continue calcifying for some time at rates

44   similar to those of unbleached corals (Rodrigues and Grottoli, 2006), in corals such as acroporids,

45   the fastest growing regions are largely azooxanthellate (Pearse and Muscatine 1971), and light

46 does not necessarily stimulate calcification in zooxanthellate corals (e.g. Rinkevich and Loya,

47 1984). Thus, it is not clear that symbionts per se enhance calcification.

48 There are several mechanisms which could be responsible for the increase in calcification

49 generally associated with symbiosis (for reviews see: Gattuso et al., 1999; Allemand et al., 2011;

50 Tambutté et al., 2011). Calcification could increase due to the drawdown in CO_2 as a direct result

51 of photosynthesis, leading to increased pH, shifting the dominate CO_2 species from bicarbonate

52 to carbonate and increasing the saturation state (Kawaguti and Sakumoto, 1948). Other likely

53 mechanisms by which calcification could be increased include the use of fixed carbon from

54 photosynthesis as fuel to increase ion pumping and elevate saturation state (Goreau, 1959), or the

55 use of fixed carbon for organic matrix synthesis, which may facilitate aragonite precipitation

56 (Young et al., 1971). Oxygen produced by the zooxanthellae may relieve internal hypoxia and

57 thus enhance calcification (Rinkevich and Loya, 1984; Rands et al., 1992, Nakamura et al.,

58 2013). Or symbionts may remove compounds which inhibit calcification or metabolism (e.g.

59 Goreau, 1961; Vandermuelen and Muscatine, 1974).

60 Though numerous studies have examined the production of fixed carbon by zooxanthellae and

61 suggested that various forms of fixed carbon (glycerol (Muscatine, 1967), glucose (Burriesci et

62 al., 2012), among other compounds (e.g. Whitehead and Douglas, 2003)) are translocated to the

63 coral host, the role of fixed carbon specifically in calcification has received little attention.

64 Various carbon compounds are known to be taken up by corals. Stephens (1960, 1962), and

65 Lewis and Smith (1971) demonstrated that corals have the ability to take up fixed carbon

66 compounds supplied exogenously. Stephens (1962) suggested that a glucose concentration of

67 ~80 µM is sufficient for glucose uptake to balance respiration in *Fungia*. Oku et al. (2003a,b)

68 claimed corals have the ability to incorporate a range of exogenous sugars into their tissues, and

69 that supplying sugars exogenously can improve the survival of bleached corals. Thus, exogenous

70 fixed carbon may substitute for that provided by zooxanthellae.

71 The effect of fixed carbon supplementation on calcification has been examined in five studies.

72 Taylor (1977) showed that fixed carbon compounds supplied exogenously are transported to the

73 regions of most rapid growth. Vandermeulen and Muscatine (1974) and Chalker (1975) exposed

74 corals in the light and in the dark to glucose, glycerol and alanine. Calcification rates were either

75 unchanged or declined as a result of exposure to fixed carbon sources at concentrations from 10

76 µM – 10mM. A similar result was found by Al-Horani et al. (2007) for both glucose and feeding

77   with *Artemia*. In contrast, Colombo-Pallotta et al. (2010) found that in bleached corals, glycerol

78   could stimulate calcification, though not in zooxanthellate corals. Such results suggest that fixed

79   carbon by itself is not generally limiting the calcification process.

80   In addition to fixed carbon, zooxanthellae also provide oxygen to the coral host. Several studies

81   have documented the daily cycle in dissolved oxygen levels adjacent to the coral tissue, with

82   hyperoxia persisting for much of the day, and hypoxia persisting for much of the night (e.g.

83   Rands et al., 1992; Shashar et al., 1993; Marshall and Clode, 2003; Agostini et al., 2009).

84   Respiration and calcification rates are linked to this cycle in oxygen saturation, with lower rates

85   at reduced oxygen levels (Rinkevich and Loya, 1984; Shick, 1990; Al-Horani et al., 2007) and

86   increased calcification rates under elevated oxygen (Colombo-Pallotta et al., 2010; Wijgerde et

87   al., 2012, Nakamura et al., 2013). In both the studies of Vandermeulen and Muscatine (1974) and

88   Chalker (1975), corals were incubated in unstirred, un-aerated containers, thus any potential

89   benefit from the supplied carbon sources may have been masked by oxygen limitation. Under

90   normal conditions, photosynthate is supplied and oxygen levels are elevated by photosynthesis,

91   thus it may be the combination of oxygen and fixed carbon that allow the coral to respire and

92   calcify faster.

93   A lack of, or insufficient, flow may be an important factor limiting the potential benefits of fixed

94   carbon in early experiments. Corals, like many benthic organisms, depend upon water movement

95   for their supply of nutrients, food, and waste removal. Water flow plays a major role in

96   determining the diffusive boundary layers surrounding corals, affecting transport rates of O_2, CO_2,

97   nutrients, etc. likely affecting all aspects of coral physiology (Dennison and Barnes 1988;

98   Atkinson and Bilger 1992; Kuhl et al., 1995; Nakamura and van Woesik 2001). Respiration in

99   particular may be limited by the diffusion of oxygen under stagnant or low-flow conditions

100 (Shick 1990; Kuhl et al., 1995), which would in-turn limit energy availability to other processes,

101 such as calcification. Thus adequate flow is likely critical if any benefit is to be seen to

102 supplementation with fixed carbon.

103 Given that respiration and calcification rates respond to elevated oxygen levels, dark calcification

104 may indeed be energy limited due to insufficient oxygen to maintain oxidative phosphorylation.

105 Fixed carbon availability may also limit calcification if sufficient oxygen is available. Thus,

106 elevated oxygen levels in combination with fixed carbon supplementation may be sufficient to

107 increase dark calcification rates to levels comparable to light calcification rates. The primary

108 energy carrier used to convert energy derived from respiration into ion pumping and other energy

109 requiring activities is adenosine triphosphate (ATP), thus energy limitation may be reflected in

110 cellular ATP content. Here combinations of different fixed carbon sources and concentrations

111 and elevated oxygen levels were tested on zooxanthellate and bleached microcolonies of the

112 branching coral *Stylophora pistillata* to determine whether oxygen limitation or a combination of

113 oxygen and fixed carbon limitation can explain light enhanced calcification. In addition to

114 calcification, respiration and ATP were measured to provide insight into the potential underlying

115 processes.

116

117 **Materials & Methods**

118 Experimental organisms

119 Colonies of the branching coral *Stylophora pistillata* were maintained in the laboratories of the

120 Centre Scientifique de Monaco in flow-through aquaria receiving Mediterranean seawater (S=38)

121 heated to 25 °C. Experimental microcolonies were prepared as described by Al-Moghrabi et al

122 (1993) and Ferrier-Pagès et al (2003), composed of ~2 cm x ~1 cm terminal portions of branches

123 on nylon monofilament line. Microcolonies were used after at least three weeks of recovery,

124 allowing tissue to fully cover the cut skeleton. Lighting was provided by JBL Marin Day T5

125 bulbs providing an irradiance of ~195 μmol photons $m^{-2} s^{-1}$ at the position of the microcolonies

126 with a 12 h light/dark cycle. To prepare bleached microcolonies, microcolonies were maintained

127 in the dark for a minimum of six weeks prior to use, at which point microcolonies appeared pure

128 white. Microcolonies were fed twice a week with newly hatched *Artemia sp.* A subset of

129 microcolonies were weighed periodically to estimate growth rates (buoyant weight technique,

130 Jokiel et al. 1978; Davies 1989).

131

132 Experimental incubations

133   Exposures to treatment conditions, calcification, and respiration rate measurements were carried

134   out in 250 ml Erlenmeyer flasks (made of borosilicate glass). Flasks were sealed using

135   transparent acrylic stoppers. A small hole in the center of the acrylic stopper allowed a

136   microcolony to be suspended in the center of the flask; a rubber stopper was used to seal the hole

137   once the microcolony had been placed level with the 200 ml mark (Supplemental Figure S1).

138   Preliminary experiments showed oxygen influx into nitrogen sparged seawater and oxygen efflux

139   from oxygen sparged seawater were negligible with this sealing system. Flasks were placed on a

140   multiposition magnetic stir plate and stirred at 145 rpm with teflon coated stir bars. Different stir

141   bar sizes (7.8 x 39.2 mm, 5.9 x 15 mm, and 4.5 x 11.95 mm – values are average measured

142   diameter and length, the manufacturer's stated size was generally slightly larger) were tested

143   initially (Fig. 1), different stirring speeds were tested as well (data not shown, see supplemental

144   material). Flasks were held within a constant temperature bath set at 25°C. Incubations were

145   carried out either in the dark or under ~195 μmol photons m^{-2}s^{-1} provided by JBL Marin Day T5

146   bulbs. Microcolonies were starved for at least two days prior to being used in experiments to

147   reduce nutrient efflux. All dark incubations for zooxanthellate colonies were started in the

148   morning such that microcolonies had been pre-conditioned to dark conditions for 12 hr prior to

149   the start of experiments. Light incubations were carried out during normally lit hours. Corals

150   were maintained under treatment conditions for at least 20 min prior to starting the experiment to

151   allow the coral to acclimate to treatment conditions. All seawater used for incubations was

152   passed through two 0.45 μm membrane filters prior to use. The seawater mass present in each

153   incubation was estimated based on the mass of seawater held by each incubation chamber

154   without a coral and the displacement volume of the coral skeleton calculated from skeletal dry

155   weight and assuming a density of 2.9 g/cm^3. For each experimental run, in addition to treatment

156   incubations, seawater only and treatment seawater incubations were carried out to check for

157   background changes and corals not exposed to treatment conditions were included as well to

158   check for day-to-day variations in growth/metabolic rates. Following incubations, water from a

159   subset of flasks which had previously contained corals was pooled and used for an additional

160   incubation (without coral) to assess the potential for microbial growth in the treatment seawater

161   to impact the measured values. Dark incubations of corals generally resulted in a ~14% decline

162   in oxygen and a ~4% decline in alkalinity by the end of the incubation. In some instances (for

163   bleached microcolonies), the change in alkalinity was below the resolution of the measurement

164   method, calcification data for such incubations were discarded.

165

166   Water chemistry

167   Oxygen

168   Experimental oxygen and CO_2 levels were set by bubbling seawater held at 25 °C with

169   humidified air or mixed gas, the composition of the mixed gas was set using mass flow

170   controllers to mix ambient air, oxygen, and CO_2 to achieve ~2x ambient pO_2 at ambient pCO_2.

171   CO_2 levels were verified using a Licor 6262 CO_2 analyzer. Oxygen (% saturation) was measured

172   using a fiber optic oxygen measurement system (PreSens) with Pst3 sensors mounted on the

173   inside of each flask. Sensors were calibrated with air (100%) and N_2 (0%) sparged seawater, a

174   linear response was assumed. Atmospheric pressure was recorded at the time each experiment

175   was performed. Oxygen concentrations were calculated as follows. The oxygen concentration

176   for air saturated (100%) seawater at the experimental salinity and pressure was calculated using

177   data from Benson and Krause (1984) as fit by equation 8 of Garcia and Gordon (1992), the first

178   A_3 term was ignored. Concentrations calculated in this manner are for 1 atm total pressure,

179   values were corrected for the measured atmospheric pressure and used to convert percent

180   saturation measurements to concentrations.

181

182 Carbonate chemistry

183 Alkalinity samples were collected at the start and end of each experiment in pre-cleaned 20 ml

184 scintillation vials and stored at 4 °C till measured. Semi-automated titrations with 0.03 N HCl

185 (containing 41g/L NaCl) were carried out on ~4 g replicated samples using a Metrohm Titrando

186 808 dosimat. Certified reference material (supplied by the laboratory of Andrew Dickson,

187 Scripps Institute of Oceanography) and an internal seawater standard were run each time samples

188 were measured, alkalinity was calculated via regression per Holcomb et al. (2012).

189 Samples for pH measurement were collected in 20 ml syringes, capped, and kept in a 25 °C water

190 bath until measured. An initial sample was injected into a 10 cm fiber optic flow cell for

191 collecting the reference spectrum, then ~1 µl of a 1.47 mM meta Cresol Purple (Acros

192 199250050 lot: A0264321) solution was added per ml seawater in the syringe using a micrometer

193 syringe (Gilmont) to inject and mix the mCP. The solution was then injected into the flow cell

194 and the pH measured, calculations followed DOE (1994).

195 Alkalinity, ammonia, and pH were then used to estimate calcification assuming calcification

196 alters alkalinity by 2 moles per mole $CaCO_3$ produced, ammonia was assumed to increase

197 alkalinity by an amount equal to its concentration. Carbonate chemistry parameters were

198 calculated using CO2Sys (van Heuven et al., 2009), additional species contributing to alkalinity

199 were included as appropriate.

200

201 Fixed carbon

202 All incubations with fixed carbon substrates included antibiotics (35 IU penicillin, 0.035 mg

203 streptomycin per ml) and antibiotics were included in the associated control incubations. The

204 effects of this antibiotic concentration on calcification and respiration were tested as well.

205 Glucose or glycerol was added as a concentrated stock solution. Target concentrations were ~1

206 % (120 mM) glycerol, 1 mM glycerol, 1 mM glucose, and 0.02 mM glucose. Concentrations

207 were chosen based on the works of Whitehead and Douglas (2003) and Colombo-Pallotta et al.

208 (2010). Concentrations of each respiratory substrate were verified at the start and end of each

209 incubation using one of the following kits, protocols were modified slightly to improve results in

210 seawater matrices: d-glucose kit (Megazyme) for glucose, glycerol GK kit (Megazyme) for

211 glycerol. Measured concentrations were: 110 - 130 mM and 1 - 1.4 mM for glycerol, 0.9-1 mM

212 and 0.019 - 0.021 mM for glucose. Concentrations of all fixed carbon compounds changed by

213 less than 10% during the incubations, except for the 0.02 mM glucose treatment for which

214 concentration declined by ~40% by the end of the incubation, which is consistent with estimates

215 of the uptake of fixed carbon substrates (Stephens 1960; Whitehead and Douglas 2003).

216

217 Nutrients

218 Ammonia and phosphate concentrations were measured at the start and end of a subset of

219 incubations using a Proxima autoanalyzer (Alliance Instruments) following the manufactures

220 recommended protocols. No nutrient release was detected for zooxanthellate microcolonies, only

221 for bleached microcolonies were nutrient releases measureable and thus alkalinity data corrected

222 for changes in nutrient concentrations.

223

224 ATP

225 ATP was extracted with sulfuric acid following a protocol modified from Fang et al. (1987;

226 1989). Microcolonies were placed in 0.6 N sulfuric acid (kept on water/ice prior to use) and

227 placed immediately in a sonicator (Branson 200 Ultrasonic Cleaner) filled with water/ice.

228 Samples were sonicated for 15 min, tubes with microcolonies re-weighed, and a sample of the

229 extract transferred to a 1.5 ml polypropylene tube (Eppendorf) and stored at -80 ºC until

230 measured. See supplementary materials for a comparison of this method with other techniques.

231

232   ATP measurements were made using a luciferin/luciferase based ATP detection kit (Roche ATP

233   Bioluminescence Assay Kit HS II) with light emission measured using either a Lumat 9507 or

234   9508 luminometer (Berthold), and concentrations calculated based on standard additions. A

235   detailed protocol is included in the supplemental materials.

236

237   Normalizations:

238   Protein was extracted from the tissue following ATP extraction by adding NaOH to produce a ~1

239   N NaOH solution which was held at 90 ºC for ~20 min. Protein was measured using a Pierce

240   BCA protein assay kit; protein concentrations were calculated using standard addition (using

241   BSA as the standard).

242   Surface area was determined via the wax method of Stimson and Kinzie (1991). Normalization

243   data are summarized in the supplementary materials (Table S2).

244

245   Statistics

246   To calculate total protein and ATP per coral it was assumed that the internal water volume of the

247   microcolony equilibrated with the surrounding solution, thus the extract volume consisted of the

248   volume of the extraction solution added plus the volume of seawater added with the microcolony

249   (approximated by the change in weight of the tube upon addition of the coral minus the dry

250   weight of the coral).

251   ATP extracted from corals was normalized to surface area, protein, and skeletal dry weight and

252   compared using a one-way ANOVA. Measurements of calcification and respiration under

253   treatment conditions were normalized to measurements on the same coral under ambient (dark)

254   conditions, to account for day to day variations in rates, all rates were normalized to untreated

255   controls measured at the same time (per Holcomb et al., 2012), data were subsequently compared

256   using ANOVA. When the overall ANOVA model showed a significant treatment effect,

257   treatments significantly different from each other were detected using Dunnett's multiple

258   comparison to compare treatments to controls. Residuals were plotted against predicted values

259   and on normal probability plots to assess violations of ANOVA assumptions. The SAS software

260   package was used for all statistical tests.

261

262   **Results**

263   Effects of stirring on physiological parameters

264   Several stirring rates and stir bar sizes were tested to establish appropriate flow rates for the

265   experimental incubations (Fig. 1). Significant differences were detected in dark respiration rates

266   among stirring treatments ($F_{2,13}=18.68$, $p=0.0002$), with smaller stir bars being associated with

267   lower respiration rates. No significant differences in calcification were detected, though there

268   was an apparent trend toward higher dark calcification rates with larger stir bar sizes.   A stirring

269   speed of 145 rpm with a 39.2 x 7.8 mm (manufacturers stated size: 40 x 8 mm) stir bar was

270   chosen for the experiments since it allowed maximal respiration and calcification rates while

271   maintaining normal polyp expansion (visually assessed, not quantified).

272

273   Effect of treatments on calcification

274   Dark calcification was significantly affected by treatment conditions ($F_{7,125} = 10.69$, $p<0.0001$ for

275   zooxanthellate and $F_{6,38} = 3.99$, $p=0.0034$ for bleached microcolonies, Fig. 2A, C). Exposure to

276   light or oxygen significantly ($p<0.05$) increased calcification of zooxanthellate microcolonies

277   relative to dark controls while 1% glycerol with oxygen led to a significant decline in dark

278   calcification (Fig. 2A). Treatment with glucose and oxygen or lower concentrations of glycerol

279   with oxygen was not associated with a significant change in dark calcification for zooxanthellate

280   microcolonies. In contrast, for bleached microcolonies light or oxygen alone had no effect but

281   treatment with oxygen combined with fixed carbon increased dark calcification, with a significant

282   increase (p=0.0035) for the 1% glycerol plus oxygen treatment (Fig. 2C).

283

284   Effects of treatments on respiration

285   Respiration rates were significantly affected by treatment conditions ($F_{6,65} = 26.96$, p<0.0001 for

286   zooxanthellate and $F_{6,43} = 23.48$, p<0.0001 for bleached microcolonies, Fig. 2B, D). All

287   treatments (except antibiotics) were associated with higher oxygen consumption in the dark

288   (p<0.05) for zooxanthellate microcolonies with the largest increases for corals treated with

289   oxygen and fixed carbon (Fig. 2B) (incubation in the light led to oxygen production, data were

290   not compared with dark incubations). Similarly in bleached microcolonies treatment with fixed

291   carbon combined with oxygen had the largest effect (p<0.001) on respiration (Fig. 2D) (light had

292   no effect on respiration in bleached colonies).

293

294   Effect of treatments on ATP

295   Treatments significantly affected ATP levels in zooxanthellate microcolonies regardless of the

296   normalization procedure ($F_{6,32}=3.18$, p = 0.0146 for normalization to protein, $F_{6,32} = 6.41$,

297   p=0.0002 for dry weight, $F_{6,32} = 5.71$, p=0.0004 for surface area), with reduced ATP levels being

298   associated with glycerol (Fig. 3). For bleached microcolonies similar trends were observed,

299   however only for normalization to surface area was the 1% glycerol with oxygen treatment

300   significantly lower than control ($F_{5,26} = 3.84$, p=0.0097, pairwise 1% glycerol to control p=0.011).

301

302   **Discussion**

303   Stirring

304   Flow limitation may in-part explain the wide range of estimates for 'light enhanced calcification'

305   (Gattuso 1999; Al-Horani et al., 2007). In the initial experiments with different sized stir bars

306   (Fig. 1) light calcification and oxygen production remained nearly constant, while smaller stir

307   bars (and consequent reduced flow) were associated with declines in dark respiration and

308   calcification. If such trends extend to still lower flow regimes, as may be suggested by the larger

309   declines in dark calcification observed by Dennison and Barnes (1988) under unstirred

310   conditions, large differences in light v dark calcification could be generated by incubating corals

311   in static or poorly stirred containers. As suggested by experiments with elevated oxygen (Fig. 2),

312   and experiments under aerated versus un-aerated conditions (Rinkevich and Loya 1984), oxygen

313   limitation may be the primary factor responsible for reduced calcification in the dark. The effects

314   of oxygen limitation are likely to be amplified under low flow conditions due to the increased

315   thickness of the diffusive boundary layer (e.g. Kuhl et al., 1995), as has been established for

316   respiration (e.g. Shick 1990).

317

318   Calcification

319   For corals with intact symbiosis (zooxanthellate microcolonies), oxygen appears to be the

320   primary factor limiting dark calcification (Fig. 2A). A similar rate enhancement was seen with

321   both light and oxygen in zooxanthellate microcolonies, suggesting oxygen production by the

322   symbionts is the primary mechanism for light enhanced calcification. Similar observations have

323   been made previously (Colombo-Pallotta et al., 2010; Wijgerde et al., 2012), however earlier

324   studies have not monitored pCO_2 when adjusting pO_2, thus the possibility of changes in saturation

325   state being responsible for the reported increase in calcification could not be ruled out. Here

326   pCO_2 has been controlled during oxygen manipulation to ensure that the observed effect is a

327   result of oxygen elevation and not concomitant degassing of CO_2. In contrast to Wijgerde et al

328   (2012), we found no indication that oxygen tensions of ~200% (~400 μmol/kg sw) negatively

329   affected coral growth. Indeed it seems surprising that such a modest increase in external pO_2

330 would be associated with substantial declines in calcification given that higher pO_2 is present at

331 the surface of the coral tissue when photosynthesis is occurring (Shashar et al., 1993), and that

332 such conditions are usually associated with enhanced calcification e.g. light enhanced

333 calcification.

334 Oxygen alone is not sufficient to increase calcification, as seen in bleached microcolonies (Fig.

335 2C). A respiratory substrate must also be present to allow the coral to benefit from increased

336 oxygen tension. The precise nature of the respiratory substrate appears to be of less importance

337 than its concentration as suggested by an increase in calcification for both glucose and glycerol

338 which appears to be enhanced at high substrate concentrations. However, high concentrations of

339 fixed carbon in the seawater are not necessarily beneficial to calcification as glycerol

340 concentrations which enhanced calcification in bleached microcolonies reduced dark calcification

341 in zooxanthellate microcolonies (Fig 2; Colombo-Pallotta et al., 2010).

342 The similarity of the results of the current study and those of Colombo-Pallotta et al. (2010)

343 suggest that oxygen limitation in the dark for zooxanthellate corals is a common phenomenon,

344 affecting both branching (*S. pistillata*, current study), and massive corals (*Montastraea faveolata*,

345 Colombo-Pallotta et al., 2010). However, although oxygen may generally be limiting

346 calcification at night in zooxanthellate corals, there is likely a gradual depletion in respiratory

347 substrates over the course of the night and eventually respiratory substrates will become limiting

348 even for zooxanthellate corals. This may help to explain why declines in calcification with

349 prolonged dark incubations have been observed in some studies (Al-Horani et al., 2007) but not

350 others (Moya et al., 2006), and be partly responsible for the lack of an enhancement of

351 calcification in earlier studies of fixed carbon supplementation (Vandermeulen and Muscatine

352 1974; Chalker 1975). For corals in the natural environment, there is likely to be a high degree of

353 variability amongst corals as to whether fixed carbon or oxygen is limiting in the dark, depending

354 upon such factors as flow, light history, photosynthetic rates, etc.

355

356   Respiration

357   Respiration was similarly affected by oxygen and fixed carbon for both zooxanthellate and

358   bleached microcolonies (Fig 2B, D), with increased rates at elevated pO_2 and further increases

359   with added fixed carbon. Despite increases in respiration, calcification did not necessarily

360   increase, with different patterns being observed in bleached versus zooxanthellate microcolonies

361   (Fig. 2). Since treatments had different effects on calcification but similar effects on respiration

362   for zooxanthellate v bleached microcolonies, there could be fundamentally different processes

363   occurring e.g. additional fixed carbon stressing the carbon replete zooxanthellate microcolonies,

364   yet benefiting the fixed carbon limited bleached colonies, or use of additional fixed carbon (and

365   energy) in biosynthetic pathways for zooxanthellate microcolonies and respiration for bleached

366   colonies. However it is also possible that the additional fixed carbon is having similar effects for

367   both zooxanthellate and bleached corals. Since the oral tissue layers (which do not carry out

368   calcification) are most exposed to the surrounding environment, they are likely to be more

369   affected by changes in the composition of the external seawater. So oxygen and fixed carbon in

370   the seawater are likely to have a greater effect on the oral tissue layers than the aboral tissue

371   layers, thus the changes in respiration may reflect primarily the oral tissue layers. For

372   zooxanthellate microcolonies with their high respiration rates (Table S2) and greater tissue

373   thicknesses (assumed from a higher protein content per unit surface area in zooxanthellate

374   microcolonies relative to bleached (Table S2), which may be associated with a greater diffusion

375   distance through the tissue), increases in respiration in the oral tissue layers in response to fixed

376   carbon may reduce oxygen availability to the calicoblasts and thus reduce calcification

377   (alternatively the increased CO_2 produced by respiration could generate similar patterns). In

378   bleached microcolonies, respiration rates are lower and the tissue thickness (protein content) is

379   reduced, thus the increase in respiration by oral tissue layers may be insufficient to restrict the

380 oxygen supply to the calicoblasts, thus allowing the calicoblasts to benefit from the supply of

381 fixed carbon. Zooxanthellae are likely to be more effective than external substrates in enhancing

382 calcification due to the production of oxygen and fixed carbon within the coral tissue, and thus

383 closer to the calicoblasts. Though even photosynthesis may not be sufficient to completely

384 eliminate hypoxia in parts of the calicodermis, as suggested by low pO_2 values measured within

385 some corals (Agostini et al., 2011), so hypoxia may still affect calcification even under light

386 conditions.

387 Estimates of gross photosynthesis which have been made by combining net oxygen release in the

388 light and oxygen consumption in the dark have long been known to be underestimates (Edmunds

389 and Davies 1988; Shick 1990). Our results suggest that light respiration is stimulated by both

390 oxygen and fixed carbon produced by photosynthesis, and, based on our observed stimulation,

391 respiration rates are likely to be at least 20% higher in the light than in the dark. Even greater

392 enhancements have been reported for short-term respiration measurements made immediately

393 after light incubations (Edmunds and Davies 1988; Colombo-Pallotta et al., 2010). However,

394 much of this enhancement of respiration may be due to respiration by the zooxanthellae, with a

395 relatively small enhancement in the animal host (Agostini et al., 2013).

396

397 ATP

398 Despite different fixed carbon sources having similar effects on respiration and calcification,

399 glucose and glycerol had markedly different effects on ATP (Fig. 3). Glycerol was associated

400 with reduced ATP levels while little change was observed for other treatment conditions.

401 Although it is not clear why such a difference is observed, it could be associated with high

402 concentrations of glycerol stressing the corals as reduced ATP levels have been associated with

403 stress events (Fang et al., 1991). Or it may reflect different pathways being used for glycerol

404 versus glucose (e.g. Whitehead and Douglas 2003), and the demands of those respective

405 pathways on cellular ATP. Light v dark conditions appear to make relatively little difference in

406 ATP levels – here we measured slightly lower values in the light, while previously slightly higher

407 levels have been measured in the light (Al-Horani et al., 2003), in neither case were the

408 differences statistically significant, suggesting that corals generally regulate metabolic activities

409 to maintain stable ATP levels, though glycerol may perturb this balance (Fig. 3). ATP levels have

410 been suggested to be potentially useful indicators of coral health (Fang et al., 1991), however our

411 results suggest that such an interpretation is far from clear – calcification, which is also used as

412 an indicator of coral health, showed little relationship to ATP levels. However, ATP appears to be

413 a potential indicator of coral biomass. ATP normalized to protein was similar for zooxanthellate

414 and bleached microcolonies, but when normalized to either skeletal mass or surface area, ATP

415 was lower in bleached microcolonies, suggesting that ATP is closely linked to biomass as has

416 been observed in deep-sea corals (Hamoutene et al., 2008), and since tissue biomass is a potential

417 indicator of coral health, ATP may be similarly useful.

418 Due to the nature of the measurements, we cannot, however, rule out a role for ATP levels in

419 controlling calcification rates. Our measurements estimate total ATP and not the ATP content of

420 the calicoblastic cells, nor the turn-over of ATP within the calicoblasts. Thus though the ATP

421 content of the full tissue layer does not track calcification, we cannot assess whether ATP levels

422 within different cell layers respond differently to treatments and what the ATP content of the

423 calicoblasts may be.

424

425 **Environmental context**

426 Although aquarium conditions are far removed from natural environmental conditions in

427 many respects – with corals often experiencing constant temperatures, flow regimes, and light

428 intensities under aquarium conditions, many of the parameters measured agree well with

429 measurements made on corals taken from natural environments. ATP levels for a variety of

430 corals collected from the wild were reported to range from $8.2 - 52.6$ μg/g (Fang et al., 1991),

431 our measurements (~$9.3 - 46.8$ μg/g) fall well within this range, however Fang et al. (1991) did

432 not report values for *S. pistillata*. Our respiration rate of 12.5 μmol O_2/d/cm^2 (for zooxanthellate

433 control corals) falls within the range measured in *S. pistillata* in the field: $0.5 - 16.6$ μmol

434 O_2/d/cm^2 (Mass et al., 2007). Calcification rates (23.7 μmol $CaCO_3$/d/cm^2 for zooxanthellate

435 control corals), were however higher than recently reported in the field for *S. pistillata* $-0.24 - 9.6$

436 μmol $CaCO_3$/d/cm^2 (Mass et al., 2007). However, in other species, higher calcification rates have

437 been reported during the light: 24 μmol $CaCO_3$/d/cm^2 (Schneider et al., 2009), 88 μmol

438 $CaCO_3$/d/mg protein (Dennison and Barnes 1988), and in the dark: 44 μmol $CaCO_3$/d/mg protein

439 (Dennison and Barnes 1988) versus our 10.2 μmol $CaCO_3$/d/mg protein. Despite the differences

440 between aquarium and natural conditions, for the range of physiological parameters measured,

441 values fall within the range encountered in the natural environment. Thus, results from the

442 current laboratory experiment likely apply to the natural environment, though with the diversity

443 of species, growth forms, and environmental conditions found in nature, it is difficult to assess

444 how common oxygen or fixed carbon limitation may be in corals growing under natural

445 conditions.

446

447 **Conclusions**

448 Oxygen limitation plays a role in explaining reduced calcification rates during dark periods for

449 scleractinian corals. Factors such as flow rate and tissue structure likely contribute to the degree

450 of oxygen limitation and thus influence 'light enhanced calcification'. Fixed carbon can also be

451 limiting, as was the case for bleached microcolonies. However changes in total ATP in the tissue

452 did not reflect changes in calcification, but rather changed in response to the form of fixed

453   carbon. This suggests that any regulation of calcification by energy availability occurs via a

454   pathway which is not responding directly to the total ATP level in the tissue.

455

456

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References

Agostini S, Suzuki Y, Higuchi T, Casareto BE, Yoshinaga K, Nakano Y, Fujimura H. 2011.
Biological and chemical characteristics of the coral gastric cavity. *Coral Reefs* 31:147-156.

Agostini S, Fujimura H, Fujita K, Suzuki Y, Nakano Y. 2013. Respiratory electron transport
system activity in symbiotic corals and its link to calcification. *Aquatic Biology* 18:125-139.

Al-Horani FA, Al-Moghrabi SM, de Beer D. 2003. The mechanism of calcification and its
relation to photosynthesis and respiration in the scleractinian coral Galaxea fascicularis.
Marine Biology 142: 419-426.

Al-Horani FA, Tambutté É, Allemand D. 2007. Dark calcification and the daily rhythm of
calcification in the scleractinian coral, Galaxea fascicularis. *Coral Reefs* 26:531-538.

Al-Moghrabi S, Allemand D, Jaubert J. 1993. Valine uptake by the scleractinian coral Galaxea
fascicularis: characterization and effect of light and nutritional status. *Journal Comparative
Physioliogy B* 163: 355-362.

Allemand D, Ferrier-Pagès C, Furla P, Houlbrèque F, Puverel S, Reynaud S, Tambutté É,
Tambutté S, Zoccola D. 2011. Biomineralization in reef-building corals : from molecular
mechanisms to environnemental control. *CR Acad Sc Paris Palévol* 3:453-467.

Benson BB, Krause JD. 1984. The concentration and isotopic fractionation of oxygen dissolved
in freshwater and seawater in equilibrium with the atmosphere. *Limnology Oceanography*
29:620-632.

Burriesci MS, Raab TK, Pringle JR. 2012. Evidence that glucose is the major transferred
metabolite in dinoflagellate–cnidarian symbiosis. *Journal of Experimental Biology* 215:3467-
3477.

Chalker BE. 1975. Calcification, metabolism, and growth by the staghorn coral, Acropora
cervicornis (Lamarck). Thesis; University of Miami.

Chida J, Yamane K, Takei T, Kido H. 2012. An efficient extraction method for quantitation of
adenosine triphosphate in mammalian tissues and cells. *Anal Chim Acta* 727:8-12.

Cohen AL, Holcomb M. 2009. Why corals care about ocean acidification. *Oceanography* 22:
118-127.

Colombo-Pallotta MF, Rodríguez-Román A, Iglesias-Prieto R. 2010. Calcification in bleached
and unbleached Montastraea faveolata: evaluating the role of oxygen and glycerol. *Coral
Reefs* 29:899-907.

Crossland CJ, Barnes DJ. 1974. The role of metabolic nitrogen in coral calcification. *Marine
Biology* 28: 325-332.

Davies PS. 1989. Short-term growth measurements of corals using an accurate buoyant weighing
technique. *Marine Biology* 101:389-395.

498 Dennison WC, Barnes DJ. 1988. Effect of water motion on coral photosynthesis and
499   calcification. *Journal Experimental Marine Biology Ecology* 115:67-77.
500 DOE. 1994. Handbook of methods for the analysis of the various parameters of the carbon
501   dioxide system in sea water version 2
502 Edmunds PJ. 2011. Zooplanktivory ameliorates the effects of ocean acidification on the reef coral
503   Porites spp. *Limnology Oceanography* 56: 2402-2410.
504 Edmunds PJ, Davies S. 1988. Post-illumination stimulation of respiration rate in the coral *Porites*
505   *porites*. *Coral Reefs* 7:7-9.
506 Falter JL, Atkinson MJ, Coimbra CFM. 2005. Effects of surface roughness and oscillatory flow
507   on the dissolution of plaster forms: Evidence for nutrient mass transfer to coral reef
508   communities. *Limnology Oceanography* 50: 246-254.
509 Fang L-S, Chen Y-W, Chen C-S. 1991. Feasibility of using ATP as an index for environmental
510   stress on hermatypic coral. *Marine Ecology Progress Series* 70: 257-262.
511 Fang L-S, Chen Y-WJ, Chen C-S. 1989. Why does the white tip of stony coral grow so fast
512   without zooxanthellae? *Marine Biology* 103: 359-363.
513 Fang L-S, Chen Y-WJ, Soong KY. 1987. Methodology and measurement of ATP in coral.
514   *Bulletin Marine Science* 41: 605-610.
515 Ferrier-Pages C, Witting J, Tambutte E, Sebens KP. 2003. Effect of natural zooplankton feeding
516   on the tissue and skeletal growth of the scleractinian coral Stylophora pistillata. *Coral Reefs*
517   22: 229-240.
518 Garcia HE, Gordon LI. 1992. Oxygen solubility in seawater: Better fitting equations. *Limnology*
519   *Oceanography* 37:1307-1312.
520 Gattuso JP, Allemand D, Frankignoulle M. 1999. Photosynthesis and calcification at cellular,
521   organismal and community levels in coral reefs: a review of interactions and control by
522   carbonate chemistry. *American Zoologist* 39: 160-183.
523 Gladfelter EH. 1982. Skeletal development in Acropora cervicornis: I. patterns of calcium
524   carbonate accretion in the axial corallite. *Coral Reefs* 1:45-51.
525 Goreau TF. 1959. The physiology of skeleton formation in corals I. a method for measuring the
526   rate of calcium deposition by corals under different conditions. *Biological Bulletin* 116:59-75.
527 Goreau TF. 1961. On the relation of calcification to primary productivity in reef building
528   organisms. In Lenhoff, HM, Loomis, WH ed. *The biology of hydra and some other*
529   *coelenterates*. 269-285.
530 Hamoutene D, Burt K, Samuelson S, Wareham V, Miller-Banoub J. 2008. Adenosine triphosphate
531   (ATP) and protein data in some species of deep-sea corals in Newfoundland and Labrador
532   Region (Northwest Atlantic Ocean). *Canadian Technical Report Fisheries Aquatic Science*
533   2801: 1-18.
534 Hand SC, Hardewig I. 1996. Downregulation of cellular metabolism during environmental stress:
535   mechanisms and implications. *Annual Review Physiology* 58: 539-563.
536 Hoegh-Guldberg O, Mumby PJ, Hooten AJ, Steneck RS, Greenfield P, Gomez E, Harvell CD,
537   Sale PF, Edwards AJ, Caldeira K, Knowlton N, Eakin CM, Iglesias-Prieto R, Muthiga N,
538   Bradbury RH, Dubi A, Hatziolos ME. 2007. Coral reefs under rapid climate change and
539   ocean acidification. *Science* 318:1737-1742
540 Holcomb M, Cloud JG, Woolsey J, Ingermann RL. 2004. Oxygen consumption in unfertilized
541   salmonid eggs: an indicator of egg quality? *Comparative Biochemistry Physiology A:*
542   *Comparative Physiology* 138: 349-354.
543 Holcomb M, McCorkle DC, Cohen AL. 2010. Long-term effects of nutrient and CO2 enrichment
544   on the temperate coral Astrangia poculata (Ellis and Solander, 1786). *Journal Experimental*
545   *Marine Biology Ecology* 386: 27-33.

546 Holcomb M, Cohen AL, McCorkle DC. .2012. An investigation of the calcification response of
547   the scleractinian coral *Astrangia poculata* to elevated pCO2 and the effects of nutrients,
548   zooxanthellae and gender. *Biogeosciences* 9:29-39

549 Jacques TG, Marshall N, Pilson MEQ. 1983. Experimental ecology of the temperate scleractinian
550   coral Astrangia danae II. Effect of temperature, light intensity and symbiosis with
551   zooxanthellae on metabolic rate and calcification. *Marine Biology* 76: 135-148.

552 Jokiel PL, Maragos JE, Franzisket L. 1978. Coral growth: buoyant weight technique. In: Stoddart
553   DR, Johannes RE. eds), Coral Reefs: Research Methods. UNESCO monographs on
554   oceanographic methodology, Paris: 529–542.

555 Kardish N, Ronen R, Bubrick P, Garty J. 1987. The influence of air pollution on the concentration
556   of ATP and on chlorophyll degradation in the lichen, Ramalina duriaei (de not.) Bagl. *New
557   Phytologist* 106: 697-706.

558 Kawaguti S, Sakumoto D. 1948. The effect of light on the calcium deposition of corals. *Bull
559   Oceanograpaical Inst Taiwan* 4:65-70.

560 Kuhl M, Cohen Y, Dalsgaard T, Jorgensen BB, Revsbech NP. 1995. Microenvironment and
561   photosynthesis of zooxanthellae in scleractinian corals studied with microsensors for O2, pH
562   and light. *Marine Ecology Progress Series* 117: 159-172.

563 Langdon C, Atkinson MJ. 2005. Effect of elevated pCO2 on photosynthesis and calcification of
564   corals and interactions with seasonal change in temperature/irradiance and nutrient
565   enrichment. *J Geophys Res* 110:C09S07

566 Lannig G, Eilers S, Portner HO, Sokolova IM, Bock C. 2010. Impact of ocean acidification on
567   energy metabolism of oyster, Crassostrea gigas--changes in metabolic pathways and thermal
568   response. *Marine Drugs* 8: 2318-2339.

569 Larsson C-M, Olsson T. 1979. Firefly assay of adenine nucleotides from algae: Comparison of
570   extraction methods. *Plant Cell Physiology* 20:145-155.

571 Lewis DH, Smith DC. 1971. The autotrophic nutrition of symbiotic marine coelenterates with
572   special reference to hermatypic corals. 1. Movement of photosynthetic products between
573   symbionts. *Proceedings of the Royal Society London (B)* 178:11 1-129.

574 Marshall AT, 1996. Calcification in hermatypic and ahermatypic corals. *Science* 271: 637-639.

575 Marshall AT, Clode PL. 2003. Light-regulated Ca2+ uptake and O2 secretion at the surface of a
576   scleractinian coral Galaxea fascicularis. *Comparative Biochemistry and Physiology* 136A:
577   417-426.

578 Mass T, Einbinder S, Brokovich E, Shashar N, Vago R, Erez J, Dubinsky Z. 2007.
579   Photoacclimation of Stylophora pistillata to light extremes: metabolism and calcification.
580   *Marine Ecology Progress Series* 334: 93-102.

581 McCulloch M, Falter J, Trotter J, Montagna P. 2012. Coral resilience to ocean acidification and
582   global warming through pH up-regulation. *Nature Climate Change* 2:623-627

583 McWilliams, J.P., Cote, I.M., Gill, J.A., Sutherland, W.J., Watkinson, A.R., 2005. Accelerating
584   impacts of temperature-induced coral bleaching in the caribbean. *Ecology* 86: 2055-2060.

585 Mortensen PB, Rapp HT. 1998. Oxygen and carbon isotope ratios related to growth line patterns
586   in skeletons of Lophelia pertusa (L) (Anthozoa, scleractinia): implications for determination
587   of linear extension rates. *Sarsia* 83: 433-446.

588 Moya A, Tambutté S, Tambutté E, Zoccola D, Caminiti N, Allemand D. 2006. Study of
589   calcification during a daily cycle of the coral Stylophora pistillata: implications for 'light-
590   enhanced calcification'. *Journal Experimental Biology* 209:3413-3419

591 Muscatine L. 1967. Glycerol excretion by symbiotic algae from corals and tridacna and its
592   control by the host. *Science* 156:516-519

593 Nakamura T, Nadaoka K, Watanabe A. 2013. A coral polyp model of photosynthesis, respiration
594   and calcification incorporating a transcellular ion transport mechanism. *Coral Reefs* 32:779-
595   794.
596 Oku H, Yamashiro H, Onaga K. 2003. Lipid biosynthesis from [14C]-glucose in the coral
597   Montipora digitata. *Fisheries Science* 69: 625-631.
598 Oku H, Yamashiro H. 2003. Effect of glucose addition on the coral Montipora digitata. Sangusho
599   ni kansuru Chosa Kenkyu Hokokusho Heisei 14 Nendo 40-45.
600 Pearse VB, Muscatine L. 1971. Role of symbiotic algae (Zooxanthellae) in coral calcification.
601   *Biological Bulletin* 141: 350-363.
602 Ries JB. 2011. A physicochemical framework for interpreting the biological calcification
603   response to CO2-induced ocean acidification. *Geochimica Cosmochimica Acta* 75: 4053-
604   4064.
605 Rands ML, Douglas AE, Loughman BC, Ratcliffe RG. 1992. Avoidance of hypoxia in a cnidarian
606   symbiosis by algal photosynthetic oxygen. *Biological Bulletin* 182: 159-162.
607 Rinkevich B, Loya Y. 1984. Does light enhance calcification in hermatypic corals? *Marine*
608   *Biology* 80: 1-6.
609 Rodrigues LJ, Grottoli AG. 2006. Calcification rate and the stable carbon, oxygen, and nitrogen
610   isotopes in the skeleton, host tissue, and zooxanthellae of bleached and recovering Hawaiian
611   corals. *Geochimica Cosmochimica Acta* 70: 2781-2789.
612 Schneider K, Levy O, Dubinsky Z, Erez J. 2009. In situ diel cycles of photosynthesis and
613   calcification in hermatypic corals. Limnol. Oceanogr. 54: 1995-2002.
614 Shashar N, Cohen Y, Loya Y. 1993. Extreme diel fluctuations of oxygen in diffusive boundary
615   layers surrounding stony corals. *Biological Bulletin* 185: 455-461.
616 Shick JM, 1990. Diffusion limitation and hyperoxic enhancement of oxygen consumption in
617   zooxanthellate sea anemones, zoanthids, and corals. *Biological Bulletin* 179: 148-158.
618 Smith JE, Shaw M, Edwards RA, Obura D, Pantos O, Sala E, Sandin SA, Smriga S, Hatay M,
619   Rohwer FL. 2006. Indirect effects of algae on coral: algae-mediated, microbe-induced coral
620   mortality. *Ecology Letters* 9: 835-845.
621 Stephens GC. 1960. Uptake of glucose from solution by the solitary coral, Fungia. *Science* 131:
622   1532.
623 Stephens GC. 1962. Uptake of organic material by aquatic invertebrates. I. uptake of glucose by
624   the solitary coral, Fungia scutaria. *Biological Bulletin* 123: 648-659.
625 Stimson J, Kinzie RA III. 1991. The temporal pattern and rate of release of zooxanthellae from
626   the reef coral Pocillopora damicornis (Linnaeus) under nitrogen-enrichment and control
627   conditions. *Journal Experimental Marine Biology Ecology* 153: 63-7
628 Tambutté S, Holcomb M, Ferrier-Pagès C, Reynaud S, Tambutté É, Zoccola D, Allemand D.
629   2011. Coral biomineralization: From the gene to the environment. *Journal Experimental*
630   *Marine Biology Ecology* 408: 58-78
631 Taylor DL. 1977. Intra-colonial transport of organic compounds and calcium in some atlantic reef
632   corals. *Proceedings of the 3rd International Coral Reef Symposium*, 431-435.
633 van Heuven S, Pierrot D, Lewis E, Wallace DWR. 2009. MATLAB Program Developed for CO_2
634   System Calculations. ORNL/CDIAC-105b. Carbon Dioxide Information Analysis Center,
635   Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, Tennessee
636 Vandermeulen JH, Davis ND, Muscatine L. 1972. The effect of inhibitors of photosynthesis on
637   zooxanthellae in corals and other marine invertebrates. *Marine Biology* 16:185-191
638 Vandermeulen JH, Muscatine L. 1974. Influence of symbiotic algae on calcification in reef
639   corals: critique and progress report. In Vernberg WB, ed. *Symbiosis in the Sea* 1-19.

640   Whitehead LF, Douglas AE. 2003. Metabolite comparisons and the identity of nutrients
641   translocated from symbiotic algae to an animal host. *Journal of Experimental Biology* 206:
642   3149-3157.
643   Wijgerde T, Jurriaans S, Hoofd M, Verreth JAJ, Osinga R. 2012. Oxygen and Heterotrophy Affect
644   Calcification of the Scleractinian Coral *Galaxea fascicularis*. *PLoS ONE* 7(12): e52702.
645   doi:10.1371/journal.pone.0052702
646   Yonge CM. 1931. The significance of the relationship between corals and zooxanthellae. *Nature*
647   128:309-311
648   Young SD, O'Connor JD, Muscatine L. 1971. Organic material from scleractinian coral skeletons
649   - II. Incorporation of 14C into protein, chitin and lipid. *Comparative Biochemistry and*
650   *Physiology* 40B: 945-958.
651
652

Figure 1

Effect of stir bar size

Figure 1. Effect of stir bar size on relative calcification rates (A) and oxygen consumption (B) by zooxanthellate microcolonies maintained in the light or in the dark, the 40 mm stir bar was used as the control value for calculating relative rates. N=4 or greater for all treatments, symbols are mean, error bars are standard deviation.
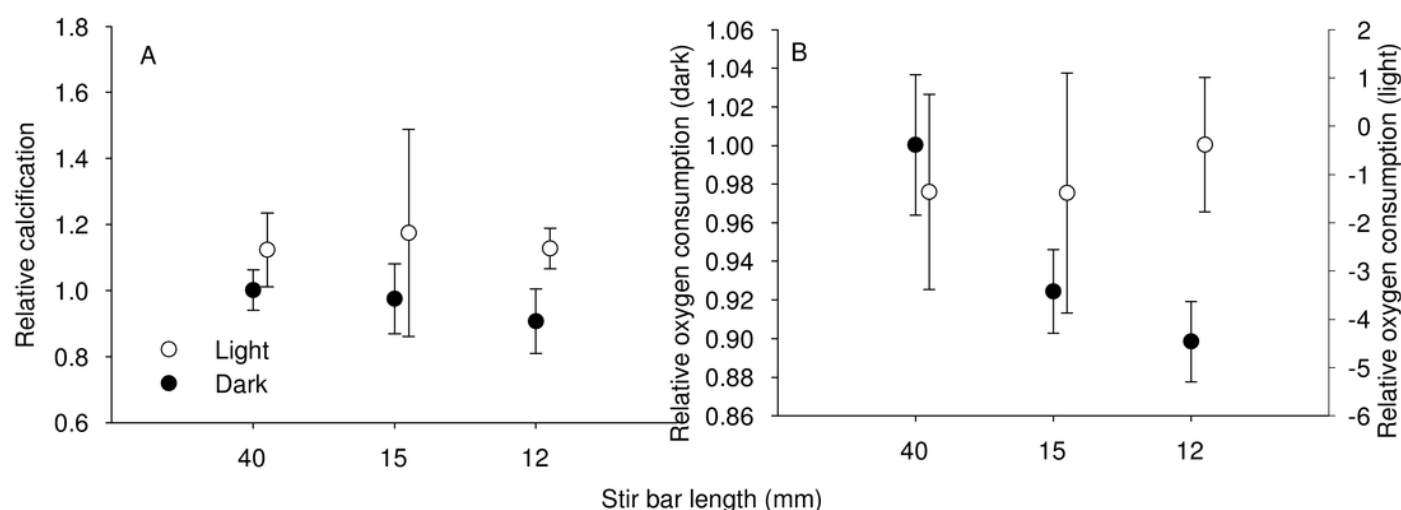
Figure 2

Relative calcification and respiration rates

Figure 2. Relative calcification (A, C) and respiration (B, D) rates for zooxanthellate (A, B) and bleached (C, D) microcolonies maintained under each treatment condition. Treatments were: cont = control, ab = antibiotics, O2 = ~2x atmospheric pO2, glu20um = ~20 uM glucose with oxygen, glu1mm = ~1 mM glucose with oxygen, gly1mm = ~1 mM glycerol with oxygen, gly120mM = 120 mM glycerol with oxygen, light = light. With the exception of the light treatment, all incubations were conducted in the dark. N=4 or greater for all treatments, symbols are mean, error bars are standard deviation, * = treatment significantly different from control at p = 0.05.
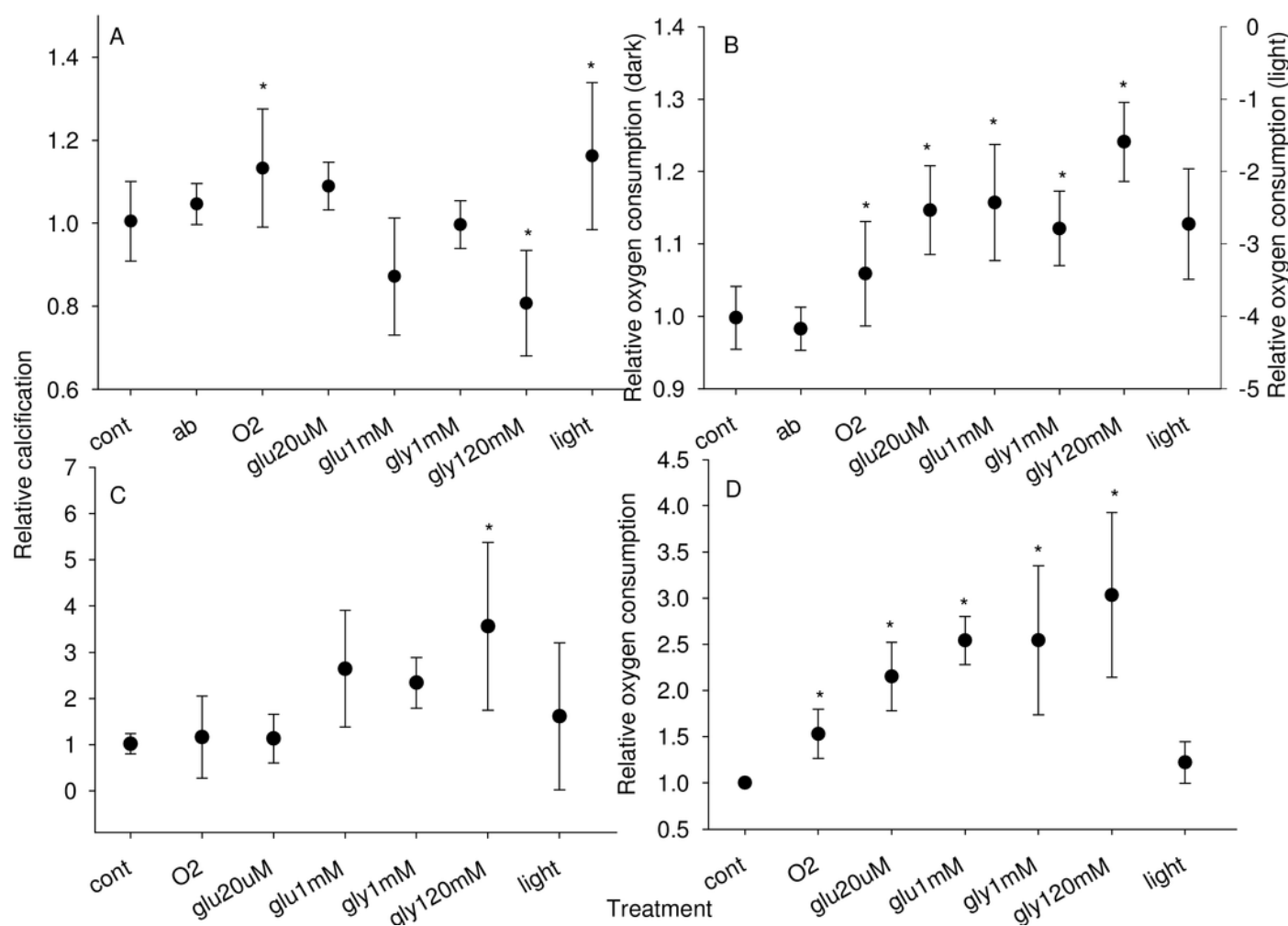
Figure 3

ATP measurements

Figure 3. ATP contents (ug) normalized to: mg protein (up triangle, plotted against left axis), cm2 surface area (filled circles, plotted against left axis), and g skeletal dry weight (squares, plotted against the right axis) for zooxanthellate (A) and bleached (B) microcolonies exposed to each treatment (per Fig. 2). N=2 or greater for all treatments, symbols are mean, error bars are standard deviation, * = treatment significantly different from control at p = 0.05.