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***Coral bleaching is linked to the capacity of the animal host
to supply essential metals to the symbionts***

Running head: coral bleaching and metal availability

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zinc isotope

Research article

25 **Abstract**

26 Massive coral bleaching events result in extensive coral loss throughout the world. These
27 events are mainly caused by seawater warming, but are exacerbated by the subsequent
28 decrease in nutrient availability in surface waters. It has therefore been shown that nitrogen,
29 phosphorus, or iron limitation contribute to the underlying conditions by which thermal stress
30 induces coral bleaching. Generally, information on the trophic ecology of metals in corals,
31 and on how they modulate the coral response to thermal stress is lacking. Here, we
32 demonstrate for the first time that heterotrophic feeding (*i.e.* the capture of zooplankton prey
33 by the coral host) and thermal stress induce significant changes in the trace metal
34 concentrations and isotopic signatures of the scleractinian coral *Stylophora pistillata*. The
35 results obtained first reveal that coral symbionts are the major sink for the heterotrophically
36 acquired metals and accumulate manganese, magnesium and iron from the food. These metals
37 are involved in photosynthesis and anti-oxidant protection. In addition, we show that fed
38 corals can maintain high metal concentrations in the host tissue during thermal stress and do
39 not bleach, while unfed corals experience a significant decrease in copper, zinc, boron,
40 calcium and magnesium in the host tissue and bleach. In addition, the significant increase in
41 $\delta^{65}\text{Cu}$ and $\delta^{66}\text{Zn}$ signature of symbionts and host tissue at high temperature suggests that these
42 isotopic compositions are good proxy for stress in corals. Overall, present findings highlight a
43 new way in which coral heterotrophy and metal availability contribute to coral resistance to
44 global warming and bleaching.

45

46 **Introduction**

47 Mutualistic nutritional symbioses between animals and microorganisms are
48 widespread in aquatic and terrestrial ecosystems, because they convey ecological advantages
49 to the partners by allowing them to exploit a large panel of food sources (Douglas 1998; Saffo,
50 1992; Venn, Loram, & Douglas, 2008). For example, cyanobacteria in symbiosis with lichens,
51 or bacteria associated to insects, supply their host with essential amino acids (Douglas, 1998;
52 Usher, Bergman, & Raven, 2007). Prominent and widely recognised nutritional symbioses in
53 the marine environment are those formed between dinoflagellate algae and sea anemones,
54 jellyfish, sponges, clams, or corals (Muscatine & Porter, 1977; Norton, Shepherd, Long, &
55 Fitt, 1992). In particular, the association between corals and dinoflagellates of the genus
56 *Symbiodinium* is at the basis of tropical reef ecosystems, which are as biodiverse as the rain
57 forest, gathering more than 25% of all known marine species (Moberg & Folke 1999).

58 Dinoflagellates in symbiosis with corals transform inorganic into organic nutrients via
59 photosynthesis and translocate most of their photosynthates to the animal host for its own
60 nutritional needs (Muscatine, Falkowski, Porter, & Dubinsky, 1984; Tremblay, Grover,
61 Maguer, Legendre, & Ferrier-Pagès, 2012). In turn, the host provides shelter to the
62 dinoflagellates, but also nutrients from its metabolic waste products and/or its heterotrophic
63 nutrition, i.e. the capture of zooplankton prey (Tremblay, Maguer, Grover, & Ferrier-Pagès,
64 2015; Yellowlees, Rees, & Leggat, 2008). Since photosynthates cover almost 100% of the
65 energetic needs of the symbiotic association in optimal living conditions, most studies until
66 now have focused on better understanding the many services provided by dinoflagellates to
67 corals under varying environmental conditions (reviewed in Davy, Allemand, & Weis, 2012;
68 Tremblay, Grover, Maguer, Hoogenboom, & Ferrier-Pagès, 2014). The contrary, i.e., the
69 amount and nature of heterotrophic nutrients translocated from the host to the symbionts have
70 been very poorly studied (Hughes, Grottoli, Pease, & Matsui, 2010; Piniak, Lipschultz, &

71 McClelland, 2003; Tremblay, Maguer, Grover, & Ferrier-Pagès, 2015). Heterotrophy can
72 however maintain the growth and metabolism of both symbionts and hosts whenever
73 conditions are not favourable to autotrophy, such as in low-light environments (Anthony,
74 2000), or during seawater warming, which leads to oxidative stress, symbiont expulsion
75 (bleaching) and nutritional starvation (Ferrier-Pagès, Rottier, Beraud, & Levy, 2010; Grottoli,
76 Rodrigues, & Palardy, 2006). The processes by which heterotrophy sustains coral metabolism
77 during bleaching are not all known, except that particle capture is a major source of
78 macronutrients (carbon, nitrogen and phosphorus), which enter into the composition of
79 energetic reserves (Grottoli, Rodrigues, & Palardy, 2006; Hughes & Grottoli, 2013). In
80 addition, heterotrophy promotes the re-establishment of photosynthate translocation after heat
81 stress, suggesting that the symbionts are one of the main beneficiaries of this nutrient source
82 (Tremblay, Gori, Maguer, Hoogenboom, & Ferrier-Pagès, 2016). Recently, genomic analyses
83 also showed, in corals fed with zooplankton, an up-regulation of genes involved in the anti-
84 oxidant response (Levy et al., 2016). Such response may be mediated through the
85 heterotrophic supply of micronutrients (trace metals), which are constituents of anti-oxidant
86 enzymes (Richier, Furla, Plantivaux, Merle, & Allemand, 2005), or which enter into the
87 composition of photosynthetic pigments of the coral symbionts and are essential during
88 bleaching (Shick et al., 2011).

89 Although the acquisition and allocation of autotrophic and heterotrophic
90 macronutrients in corals start to be well studied via the use of stable isotope ratios such as
91 $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ (Hughes, Grottoli, Pease, & Matsui, 2010; Tremblay, Maguer, Grover, &
92 Ferrier-Pagès, 2015), the trophic ecology of trace metals remains almost unknown, both under
93 normal growth conditions and during thermal stress. To date, the wide majority of studies
94 have focused on metal concentrations in coral skeletons to follow pollution events (Barnes,
95 Taylor, & Lough, 1995; Bastidas & Garcia, 1999; David, 2003; Fallon, White, & McCulloch,

96 2002). Those performed at the tissue level showed that algal symbionts actively take up and
97 accumulate trace metals dissolved in seawater (Ferrier-Pagès et al., 2005; Harland & Nganro,
98 1990; Reichelt-Brushett & McOrist, 2003), suggesting that coral bleaching may result in
99 metal limitation through symbiont loss. However, the changes in metal concentrations or
100 isotopic signature of symbionts and host tissue with feeding and environmental stress are still
101 unknown. As shown in other organisms such as mice and humans, isotopic signature of
102 metals can be significantly affected by pathological conditions (e.g. Balter et al., 2015) as
103 well as by dietary conditions (e.g. Jaouen, Pons, & Balter, 2013; Jaouen, Szpak, & Richards,
104 2016, Costas-Rodríguez, Van Heghe, & Vanhaecke, 2014). This suggests that a change from
105 autotrophic to heterotrophic feeding caused by bleaching conditions could also be marked by
106 isotopic deregulations. In this study, we have investigated the trophic ecology of trace metals
107 in the scleractinian coral *Stylophora pistillata* as well as the changes in the isotopic signature
108 of copper and zinc under normal and thermal stress conditions. The first aim was to assess
109 whether heterotrophy brings essential metals to the symbiotic association, and contributes, by
110 this process, to increase the resistance of corals to thermal-stress induced bleaching. The
111 second aim was to evaluate whether heterotrophy, and/or thermal stress, can be traced through
112 a change in the isotopic signature of copper and zinc. Investigating those aspects allows for a
113 better understanding of the processes leading to coral bleaching. The results will show
114 whether bleaching is exacerbated by the lack of trace metals in coral tissue and whether
115 zooplankton capture can supply these metals to corals.

116

117 **Materials and Methods**

118 Maintenance of coral colonies

119 Five colonies of the scleractinian coral *Stylophora pistillata* (Esper 1797), originating
120 from the Red Sea (Aqaba, Jordan), were used to generate 40 large nubbins of ca. 10 cm long

121 (8 nubbins from each of the five colonies). Nubbins were then equally divided into 4
122 treatments and two 20 L aquaria per treatment (8 aquaria in total). During the first five weeks,
123 4 aquaria were fed during 2h and three times a week with 4000 *artemia salina* nauplii while
124 the other 4 aquaria were kept unfed. All aquaria were maintained in an open flow system,
125 with a water renewal rate of 10 L h⁻¹ and at a constant temperature of 25°C ± 0.5°C. Light was
126 provided by hydrargyrum quartz iodide (HQI) lights at a photosynthetic active radiation level
127 (PAR) of 200 μmol m⁻²s⁻¹ (measured using a spherical quantum sensor; LiCor LI-193,
128 Lincoln, NE, USA), with a 12h:12h dark:light cycle. After these first 5 weeks, and for each
129 feeding conditions, two aquaria were kept at 25°C while temperature was slowly raised (1°C
130 every two days) in the two other aquaria to reach 30°C. Temperature and trophic conditions
131 were maintained for three additional weeks prior measurements of trace metal concentrations
132 and symbiont density were performed as described below.

133

134 Treatments

135 Five nubbins in each condition were sampled for the determination of the symbiont
136 density. Coral tissue was extracted from the skeleton using an air pick and homogenized with
137 a Potter tissue grinder. The symbiont density was quantified with three sub-samples of 100 μL
138 using a Z1 Coulter Particle Counter (Beckman Coulter). The five remaining nubbins in each
139 condition were sampled, rinsed using artificial seawater (ASW) made with ultrapure sodium
140 chloride (NaCl) (Trace Select for trace analysis ≥ 99.999%, Sigma Aldrich). They were then
141 individually placed in 50 mL beakers (precombusted at 480°C for 4 h in a ThermolyneH
142 62700 oven and rinsed with ASW) containing 5 mL of ASW. For each sample, tissue was
143 completely removed from the skeleton with an air pick and homogenized with a Potter tissue
144 grinder (treated as beakers). The homogenate was divided into a host and a symbiont fraction.
145 For the host fraction, the homogenate was centrifuged (Biofuge 17RS Heraeus) at 3000 g for

146 10 min to pellet most of the symbionts. The supernatant was re-centrifuged twice to eliminate
147 the remaining symbionts, then flash frozen with liquid nitrogen, and freeze-dried using a Heto
148 (model CT 60) drier. For the symbiont fraction, the pellet was washed several times in ASW,
149 flashed frozen and freeze-dried.

150

151 Analyses of trace and major element concentrations

152 All chemical analyses were carried out in clean laminar flow hoods using double-
153 distilled acids to avoid any exogenous contaminations. Freeze-dried coral and symbiont
154 samples were dissolved in a concentrated HNO₃-H₂O₂ mixture, in Savillex beakers, at 120°C
155 for at least 72h. Major and trace element concentrations were first measured in a small aliquot
156 on an ICP-AES (iCAP 6000 Radial) and a quadrupole ICP-MS Thermo iCap-Q respectively
157 at the Ecole Normale Supérieure (ENS) of Lyon following the method described in Garçon et
158 al. (2017). Oxide interference and analytical drift were corrected using indium (In) and
159 scandium (Sc) addition as internal standards for trace and major elements respectively (Sigma
160 Aldrich, France). In-house (sheep plasma, OEP) and international standards (bovine liver,
161 1577c, Sigma Aldrich) as well as complete duplicate and re-run analyses were measured to
162 ensure the validity and assess the precision of our results. Metal concentrations are all
163 reported in µg g⁻¹ (ppm) dry weight.

164

165 Analyses of copper and zinc isotopic compositions

166 Copper and zinc isotopic compositions were then measured following the procedure
167 described by Maréchal, Télouk, & Albarède, (1999). Briefly, before each isotopic
168 measurement, samples were purified by ion-exchange chromatography using quartz columns
169 filled with 1.8 mL of Bio-Rad AGMP-1 (100-200 mesh) anion-exchange resin. Copper was
170 first eluted with 20 mL of HCl (7 mol L⁻¹) + 0.001% H₂O₂ followed by zinc with 10 mL of

171 HNO₃ (0.5 mol L⁻¹). The purification step helps removing elements (e.g. Ni, Ti, Al or Ba),
172 which may interfere with copper and zinc during the instrumental measurement (Chen et al.,
173 2016; Sossi, Halverson, Nebel, & Eggins, 2014). Indeed, it has been shown that small
174 amounts of Ni and Ti residues, for example, can produce isobaric interferences and shift the
175 measured δ⁶⁶Zn values by more than 0.07 ‰ if the Ni/Zn and Ti/Zn ratios are higher than
176 0.001 and 0.01, respectively (Chen et al., 2016).

177 On the day of the analyses, an aliquot of the Zn and Cu purified solutions, previously
178 evaporated to dryness, are dissolved in a Cu (Cu SRM 976, National Institute of Standards
179 and Technology, Gaithersburg, MD, USA) or Zn-doped solution (Zn JMC 3-0749L, Johnson
180 Matthey Royston, UK) respectively to reach sample concentration of about 300 µg L⁻¹ that is
181 similar to the concentration of the standard solution that was run between each sample. Both
182 copper and zinc isotopic compositions, expressed as

$$183 \quad \delta^{65}\text{Cu}_{\text{sample}} (\text{in } \text{‰}) = \left[\frac{\left(\frac{{}^{65}\text{Cu}}{{}^{63}\text{Cu}} \right)_{\text{sample}}}{\left(\frac{{}^{65}\text{Cu}}{{}^{63}\text{Cu}} \right)_{\text{standard}}} - 1 \right] * 1000$$

$$184 \quad \delta^{66}\text{Zn}_{\text{sample}} (\text{in } \text{‰}) = \left[\frac{\left(\frac{{}^{66}\text{Zn}}{{}^{64}\text{Zn}} \right)_{\text{sample}}}{\left(\frac{{}^{66}\text{Zn}}{{}^{64}\text{Zn}} \right)_{\text{standard}}} - 1 \right] * 1000$$

185 were measured on a Nu Plasma (Nu 500) MC-ICP-MS in wet plasma conditions. Instrumental
186 mass fractionation was corrected with an exponential law using an elemental-doping method
187 as recommended by Maréchal, Télouk, & Albarède, (1999). δ⁶⁶Zn and δ⁶⁵Cu were then
188 calculated by standard bracketing using Zn JMC 3-0749L (Johnson Matthey Royston, UK)
189 and Cu SRM 976 (NIST, Gaithersburg, MD, USA) as reference standards. Note that these
190 standards were repeatedly measured between each sample to correct for the instrumental drift
191 throughout the analytical sequence; a method called standard bracketing. The accuracy of the
192 isotopic compositions was assessed based on the analyses of an in-house (sheep plasma; OEP)
193 and international (bovine liver, 1577c, Sigma Aldrich, France) standard solution at the

194 beginning and during the analytical sequence. The average $\delta^{66}\text{Zn}$ measured for the in-house
195 standard solution was $+0.75\text{ ‰} \pm 0.07$ and $-1.19\text{ ‰} \pm 0.07$ for $\delta^{65}\text{Cu}$ which is in good
196 agreement with our previous reference in-house values ($\delta^{65}\text{Cu} = -1.15\text{ ‰} \pm 0.20$ (standard
197 deviation (sd), $n = 35$) and $\delta^{66}\text{Zn} = +0.73\text{ ‰} \pm 0.09$ (sd, $n = 20$). Our results for the
198 international standard (1577c) are also in good agreements with previous results: $\delta^{66}\text{Zn}_{\text{this study}}$
199 $= -0.20\text{ ‰} \pm 0.06$ (sd, $n = 10$) compared to $\delta^{66}\text{Zn}_{1577c} = -0.16\text{ ‰} \pm 0.15$ (sd, $n = 45$) and
200 $\delta^{65}\text{Cu}_{\text{this study}} = +0.30\text{ ‰} \pm 0.13$ (sd, $n = 10$) compared to $\delta^{65}\text{Cu}_{1577c} = +0.37\text{ ‰} \pm 0.14$ (sd, $n =$
201 43). Based on re-run samples and complete duplicate analyses, we estimate the precision of
202 our measurements at ± 0.1 (sd) for both $\delta^{65}\text{Cu}$ and $\delta^{66}\text{Zn}$. The long-term precision based on
203 the repeated measurements of standard Zn JMC 3-0749L and Cu SRM 976 alone is similar (\pm
204 0.07 ‰ (sd, $n = 480$)).

205

206 Statistics

207 A correlation-based principal component analysis (PCA) was used to quantify the effect of
208 thermal stress and heterotrophic feeding on the chemical composition of symbionts and host
209 tissue of colonies of *Sylophora pistillata*. The method consists in identifying new variables
210 called principal components (PCs), which are linear combination of the original variables and
211 along which data variation is maximal. For each principal component (PC), linear regressions,
212 using standard least-squares techniques, were thus used to estimate the relationship between
213 parameters. In this study, the variables include the chemical concentrations of the trace
214 elements measured in host tissue and symbionts (manganese, iron, magnesium, zinc,
215 strontium, arsenic, barium, calcium, boron and copper), as well as copper ($\delta^{65}\text{Cu}$) and zinc
216 ($\delta^{66}\text{Zn}$) isotopic compositions. All data were log transformed and normalized, and samples
217 with incomplete data were excluded. PCA was implemented in MATLAB™.

218 Statistical analyses: all parameters were expressed as mean \pm standard deviation (SD) of five

219 measurements. Statistical analyses were performed using Systat 13 software (Systat Software,
220 Chicago, IL, USA). Data were checked for normality using the Kolmogorov-Smirnov test
221 with Lilliefors correction and for homoscedasticity using Levene test. Data were transformed
222 with a natural logarithm transformation when required (i.e. Fe, Sr, Ca and B in symbionts; Fe,
223 Mg, Zn, Ba, Ca and B in coral host). Factorial ANOVA with two factors (feeding an
224 temperature) were performed and when there were significant differences between treatments,
225 analyses were followed by a posteriori testing (Tukey's test). Differences between factors
226 were considered significant for p-values < 0.05.

227

228 **Results**

229 The PCA yielded two principal components (PC1 and PC2) that explain ca. 70% of
230 the total variance (Fig. 1). In this figure, grey arrows graphically represent the loading factors
231 (i.e. weight of each variable on PC1 and PC2, and Table S1). The first component (PC1),
232 which explains 44% of the total variance, is defined by positive correlations with several trace
233 elements such as iron (Fe), barium (Ba) or boron (B). This principal component demonstrates
234 that symbionts contained higher metal concentrations ($\mu\text{g g}^{-1}$ dry weight) than the animal
235 tissue, suggesting that metals were not distributed equally in these two compartments.
236 Principal component 2 (PC2) explains 25% of the total variance and is defined by relatively
237 high weights from Cu, but negative correlation with magnesium (Mg), $\delta^{65}\text{Cu}$ and $\delta^{66}\text{Zn}$. This
238 principal component separates both symbionts and animal tissues maintained at 25°C and
239 30°C, showing that thermal stress was associated with variation in both their metal content
240 and isotopic composition. As demonstrated by the PCA and the results summarised in Table 1,
241 for both temperatures investigated (25°C and 30°C), and in all feeding conditions, symbionts
242 presented higher metal concentrations ($\mu\text{g g}^{-1}$ dry weight) than the animal tissue, except for
243 Mg, whose concentration tended to be slightly higher in the host tissue (Fig.1).

244 The two factors ANOVA (Table 2) shows significant effect of both feeding and
245 temperature, alone or in combination, on the micronutrient concentrations of symbionts and
246 animal tissue. At the normal growth temperature (25°C), symbionts of fed colonies presented
247 higher concentrations of Mg (post-hoc, p-value= 0.002), Fe (p-value= 0.003) and Mn, (p-
248 value <0.001), but lower concentrations of strontium (Sr), and arsenic (As) (p-value= 0.01
249 and 0.004 respectively) than symbionts of unfed colonies (Table S2). Inversely, there was no
250 significant difference in trace metal concentrations between fed and unfed coral tissue at 25°C
251 (Table S3, post-hoc, p value >0.1). Host tissue of fed corals had however a higher $\delta^{65}\text{Cu}$
252 signature ($-0.42\text{‰} \pm 0.12$, ANOVA, p value = 0.0455) than the host tissue of unfed corals ($-$
253 $0.67\text{‰} \pm 0.05$ respectively) (Fig. 2).

254 Thermal stress (30°C) induced a significant bleaching in unfed colonies (t-test, p-value
255 = 0.0016), with a 50% reduction in symbiont density (Fig. 2). On the contrary, it had no effect
256 on the symbiont density of fed colonies, which also presented two times more symbionts (Fig.
257 2). Thermal stress significantly changed the metal concentrations (Tables 1, 2 and Tables S2,
258 S3) of the symbiotic association. There was a significant increase of Sr, Ca, Zn and B
259 concentrations in both symbionts and host of fed and unfed colonies (Tables 1 and 2). High
260 temperature also significantly increased Mg concentrations in fed host tissue and unfed
261 symbionts, Ba in fed and unfed symbionts, as well as Mn in the host tissue of fed corals
262 (Tables 2, S2, S3). On the contrary, the Cu content of fed and unfed colonies was
263 significantly decreased (Table 2). Finally, thermal stress significantly increased the $\delta^{65}\text{Cu}$ and
264 $\delta^{66}\text{Zn}$ values of both host and symbionts, independently of the feeding status (Fig. 2).

265 The binary plot of Fig. 3a shows that there was an overall increase in both the $\delta^{65}\text{Cu}$
266 and $\delta^{66}\text{Zn}$ values with thermal stress in corals and symbionts. The other binary plots
267 performed with metal concentrations (Fig. 3b to 3f) exhibit two thermal stress patterns on fed
268 and unfed coral colonies: data corresponding to fed and unfed host or fed and unfed

269 symbionts are tightly clustered at 25°C, suggesting that at this temperature there is no effect
270 of the dietary regime. The same pattern is observed for all symbionts at 30°C, suggesting that
271 they obtain the same amount of metals from their respective fed and unfed hosts. On the
272 contrary, metal concentrations in the host show a distillation process at 30°C, associated to a
273 metal depletion in unfed hosts compared to fed ones (red arrows bleaching, Fig. 3b to f). The
274 amplitude by which hosts are depleted increases as the metal concentrations decrease.

275

276 **Discussion**

277 Understanding the nutritional requirements of scleractinian corals is critical to assess
278 their health status and predict how reefs will change in response to rising seawater
279 temperatures (Connolly, Lopez-Yglesias, & Anthony, 2012; Ferrier-Pagès, Rottier, Beraud, &
280 Levy, 2010). The main finding of this study is that heterotrophic feeding brings essential
281 metals to corals, which are mainly accumulated in the symbionts, and help avoid thermal
282 stress damages and subsequent bleaching. During thermal stress, symbionts retain metals at
283 the expense of the coral host, thus being more parasites than symbionts. We also demonstrate
284 a significant disruption in the Ca, Mg, Sr and B homeostasis in coral tissue during thermal
285 stress, as well as a significant increase in the $\delta^{65}\text{Cu}$ and $\delta^{66}\text{Zn}$ values of coral host and
286 symbionts. These isotopic compositions thus constitute good proxies for thermal stress in
287 corals.

288

289 **1- Effect of the dietary regime at 25°C**

290 Symbionts were the main sink for micronutrients within the symbiotic association
291 (Fig. 1), although these nutrients were provided to corals as particulate organic food, first
292 digested by the animal. This observation is contrary to the usual view that symbionts are the
293 main food suppliers of the symbiotic association, and provides one of the rare evidence of

294 inverse nutrient translocation, from the animal host to the symbionts (Piniak, Lipschultz, &
295 McClelland, 2003; Tremblay, Maguer, Grover, & Ferrier-Pagès 2015). Although the above
296 studies showed that symbionts profited from heterotrophic macronutrients (carbon, nitrogen)
297 entering the symbiotic association, the transfer was restricted to less than 20% of the total
298 gain, against 100% in this study.

299 Plankton feeding significantly increased, in symbiont cells, the concentrations of three
300 main micronutrients, namely Mn, Fe and Mg (Table 1). Among other functions, Mn, Fe and
301 Mg are all involved in the composition of anti-oxidant enzymes (Krueger et al., 2015). These
302 enzymes, such as catalases, peroxidases and Fe/Mn or Mn-superoxide dismutases (SOD), are
303 present in *Symbiodinium* (Lin et al., 2015; Zhang, Zhuang, Gill, & Lin, 2013), and are used to
304 scavenge reactive oxygen species (ROS) continuously produced through photosynthesis and
305 over-produced during thermal stress (Weis, 2008). Mn and Fe also play a major role in
306 photosynthesis, nitrate reduction, as well as in chlorophyll and amino acid synthesis (Twining
307 & Baines, 2013). This involvement of Fe and Mn in photosynthetic processes is one of the
308 explanations why symbiont density, chlorophyll content, and photosynthetic rates are
309 significantly enhanced in heterotrophically-fed coral colonies compared to unfed ones, both in
310 normal growth and stress conditions (this study and reviewed in Houlbrèque & Ferrier-Pagès,
311 2009). On the contrary, a lack of Fe during thermal stress increases the bleaching
312 susceptibility of corals (Shick et al., 2011). Overall, these results highlight the importance of
313 Fe and other key micronutrient availability for coral photosynthesis and health. Whereas
314 concentrations of Fe, Mn, Mg were enhanced in fed symbionts under normal growth
315 conditions, concentrations of Ca and Sr, which are main components of coral skeletons, were
316 significantly decreased (Table 1). Such changes may be linked to a higher translocation of
317 these elements to the skeleton, since fed corals present higher calcification rates than unfed
318 corals (Houlbrèque & Ferrier-Pagès, 2009). The schematic diagram of Fig. 4 summarizes the

319 findings observed in this study and the potential role of metals in symbionts, under normal
320 growth conditions of *S. pistillata*.

321

322 The $\delta^{55}\text{Cu}$ and $\delta^{66}\text{Zn}$ values were significantly different between fed and unfed hosts
323 and symbionts (Fig. 2), suggesting, for the first time, that corals fractionate Cu and Zn
324 isotopes depending on their dietary regime. The higher $\delta^{55}\text{Cu}$ values in fed colonies is linked
325 to the consumption of Cu in the form of particulate organic matter, while unfed colonies only
326 rely on the uptake of dissolved inorganic Cu by the symbionts. The $\delta^{55}\text{Cu}$ signature in fed
327 corals indeed follows the rule that organic matter is enriched in the heavier Cu isotope
328 compared to dissolved inorganic Cu (Petit et al., 2013). The lower $\delta^{66}\text{Zn}$ signature of fed
329 compared to unfed colonies is also in agreement with the observation that carnivores are
330 slightly ^{66}Zn -depleted relative to herbivores (Jaouen, Szpak, & Richards, 2016). Overall, the
331 significant effect of feeding on the $\delta^{66}\text{Zn}$ and $\delta^{55}\text{Cu}$ signatures demonstrates that metal
332 isotopes represent useful proxies to trace nutrient flows within a symbiotic association,
333 although they have poorly been studied in marine food webs in general (Jaouen, Szpak, &
334 Richards, 2016).

335

336 **2- Effect of thermal stress**

337 After three weeks of thermal stress, which induced bleaching in unfed corals (Fig. 2),
338 significant changes occurred in metal concentrations and Cu-Zn isotopic compositions of
339 symbionts and host tissue under both fed and unfed conditions. We observed a clear increase
340 in the $\delta^{55}\text{Cu}$ and $\delta^{66}\text{Zn}$ values of both hosts and symbionts (Fig. 3A). Such isotopic variations
341 can be related to an increase in reactive oxygen species (ROS) generated by thermal stress in
342 coral tissue (Cunning & Baker, 2013). As light isotopes create weaker links with molecules
343 compared to heavy isotopes (Schauble, 2004), these bonds are more easily disrupted by ROS

344 (Demidov, 2007; Shepinov & Pestov, 2010). Light isotopes will thus preferentially be
345 released in the medium. Although this assumption still needs to be verified, we can already
346 suggest that $\delta^{65}\text{Cu}$ and $\delta^{66}\text{Zn}$ signatures represent good proxies for thermal stress in corals. In
347 addition, the correlation which existed between $\delta^{65}\text{Cu}$ and $\delta^{66}\text{Zn}$ at 25°C was lost at 30°C
348 (Fig. 3), suggesting that animals and symbionts were not in equilibrium any more at 30°C.

349 For all corals, thermal stress also induced a very large increase of the Mg, Sr, Ca and
350 B concentrations compared to normal growth conditions, in particular in symbionts of unfed
351 colonies. Although such changes have never been investigated in corals, an increase in
352 intracellular Ca concentration has been monitored in insects (Teets, Yi, Lee, & Denlinger,
353 2013), chicken lymphocytes (Han et al., 2010) or terrestrial plants (Zhu, 2001) during thermal
354 stress, while an increase in Mg was observed in the shrimp *Crangon crangon* (Sartoris &
355 Pörtner, 1997). Genomic studies, which have investigated the molecular basis of cnidarian
356 bleaching, also suggested that Ca homeostasis was disrupted in heat-stressed corals (DeSalvo
357 et al., 2008; Levy et al., 2016; Rodriguez-Lanetty, Harii, & Hoegh-Guldberg, 2009). Finally,
358 Dishon et al., (2015) demonstrated a decline of $\delta^{11}\text{B}$ as a result of coral bleaching, likely due,
359 according to our results, to the observed large increase in B concentration, which should have
360 changed the intracellular pH. All these metals play important cellular roles. Ca is an
361 intracellular messenger, while Mg has a protective role against oxidative damage (Freedman
362 et al., 1992; Sartoris & Pörtner, 1997), which suggests that the significant Mg increase in
363 coral cells followed the temperature-induced ROS production (Weis, 2008). Finally, B is
364 needed, among others, for membrane structural integrity and carbohydrate metabolism
365 (reviewed in Pilbeam & Kirkby, 1983), two important processes in corals.

366 Despite the large internal changes induced by thermal stress, mixotrophic corals (fed
367 with artemia) did not bleach while autotrophic corals did. The lack of bleaching, as well as the
368 maintenance of high rates of photosynthesis and calcification in mixotrophic corals have been

369 observed several times in previous studies (Borell & Bishof, 2008; Connolly, Lopez-Yglesias,
370 & Anthony, 2012; Ferrier-Pagès, Rottier, Beraud, & Levy, 2010). The binary plots of metal
371 concentrations in fed and unfed coral colonies (Fig. 3B to 3F) highlight a new mechanism by
372 which heterotrophy sustains coral metabolism during thermal stress, e.g. via the supply of
373 essential metals to the host and symbionts. These plots show similar metal concentrations in
374 symbionts of both fed and unfed colonies during thermal stress. In other words, symbionts
375 sequestered the same amount of metals irrespective of the feeding status of their host. As
376 metals are mostly translocated from the host to the symbionts, this led to two different metal
377 conditions in host tissue depending on the feeding status of the colonies. In fed corals,
378 heterotrophy supplied enough metals to the host to sustain symbiont's needs and no bleaching
379 is observed. In unfed corals, symbiont repletion lead to a significant depletion of metals in
380 host tissue (Fig. 3B to F), and a cost for the symbiotic association, which underwent
381 bleaching and nutrient starvation. The amplitude by which hosts were depleted increased as
382 the metal concentrations decreased. These results thus demonstrate the significant effect of
383 feeding in providing metals to the animals during thermal stress. The significant bleaching in
384 unfed colonies further suggests that, contrary to fed corals, metal concentrations in unfed host
385 tissue were not sufficient to sustain high densities of symbionts. Finally, they also suggest that
386 thermal stress has promoted symbiont parasitism in unfed corals. A similar selfish behaviour
387 of symbionts during thermal stress has very recently been observed for macronutrients (Baker
388 et al. 2018). Figure 5 summarizes the findings and hypotheses on the role of metals in coral
389 bleaching.

390 In summary, our results indicate that the $\delta^{65}\text{Cu}$ and $\delta^{66}\text{Zn}$ values represent good
391 proxies for stress in corals. They also show that heterotrophy and thermal stress induce
392 significant changes in trace metal concentrations and isotopic signatures of the scleractinian
393 coral *Stylophora pistillata*. The results obtained first reveal that symbionts are the major sink

394 for trace metals in the symbiotic association, and accumulate Mn, Mg and Fe from the
395 heterotrophic feeding of the host. They also demonstrate that bleaching is exacerbated by the
396 shortage of trace metals in coral tissue (especially Cu, Zn, B, Mg, Ca) and that heterotrophic
397 feeding can supply these essential metals to corals. Such results thus highlight a new way by
398 which heterotrophy contributes to coral resistance to bleaching, and have major implications
399 for the resilience of coral reefs under threat of global change.

400
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576 Figure and Table captions:

577 Figure 1: Principal Component Analysis (PCA) taking into account all data (micronutrient
578 concentrations in symbionts and host tissue, as well as the isotopic signature of zinc and
579 copper in each compartments, n = 5 samples for each condition) at 25°C (normal growth
580 temperature) and 30°C (stress)

581

582 Figure 2: Isotopic signature of copper (d65Cu) and zinc (d66Zn) in (a) symbionts and (b) host
583 tissue of colonies maintained fed and unfed at 25°C or 30°C; (c) Symbiont density (106
584 cell/cm²) (data are mean and standard deviation of 5 samples per experimental conditions)

585

586 Figure 3: Relationship between the d65Cu and d66Zn values, or between the concentration
587 (lg/g) in Cu and Zn, Mg and Zn, B and Cu, Ba and Ca, Zn and Ca (n = 5 samples for each
588 experimental condition). Open circles and squares represent unfed symbionts and host tissue

589 at 25°C (blue) or 30°C (red) respectively. Filled circles and squares represent fed symbionts
590 and host tissue at 25°C (blue) or 30°C (red) respectively. The arrow indicates the direction of
591 the distillation process during bleaching

592

593 Figure 4: Heterotrophic supply of micronutrients within the coraldinoflagellate association.
594 Zooplankton predation increases the amount of Mg, Mn and Fe in symbionts. These metals
595 are involved in photosynthesis processes, in nitrate reduction and in the structure of
596 antioxidant enzymes. Prey digestion preferentially releases light copper isotope (i.e. ^{63}Cu)
597 and inversely heavy zinc isotope (^{66}Zn), increasing the $\delta^{65}\text{Cu}$ signature and decreasing the
598 $\delta^{66}\text{Zn}$ signature of coral tissue. Blue arrows: positive effect

599

600 Figure 5: Schematic diagram summarizing the effect of thermal stress on micronutrient
601 concentrations in unfed (a) and fed (b) corals. Thermal stress (1) increases ROS (reactive
602 oxygen species) in coral tissue (2), and induces the disruption of Ca, Sr, Mg and B
603 homeostasis (3). In unfed corals (a), micronutrient concentrations and antioxidant enzymes
604 are not produced in sufficient amount to significantly reduce ROS (4). Photosynthesis is
605 impaired and symbionts are expelled (bleaching). In fed corals (b), feeding increases the
606 amount of micronutrients in host tissue and symbionts. Antioxidant enzymes are produced in
607 sufficient amount to significantly reduce ROS (4). Photosynthesis is not impaired and
608 symbionts are not expelled (no bleaching). Red arrows: negative effect, green arrows:
609 positive effect

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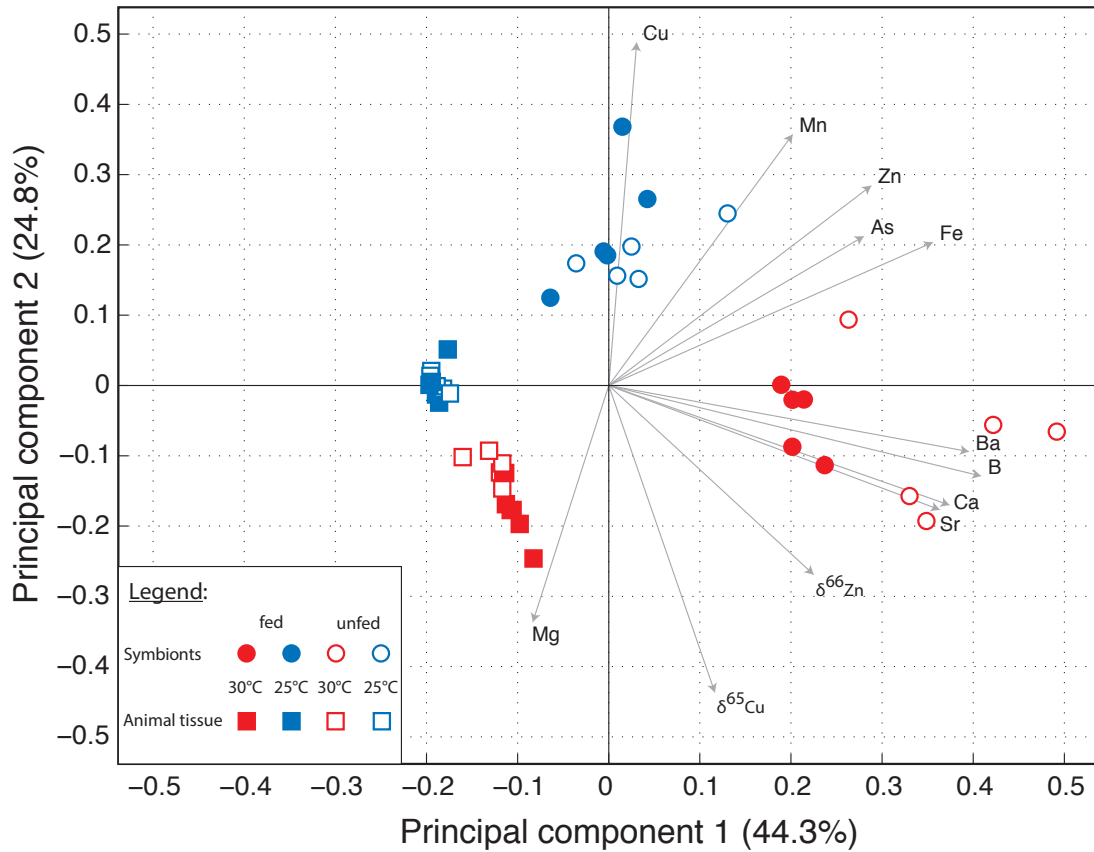
611 Table 1: Micronutrient concentrations (lg/g dry weight) in symbionts and host tissue of fed
612 and unfed *S. pistillata* maintained under normal (25°C) and thermal stress (30°C) conditions

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614 Table 2: Results of the two factors ANOVA testing the effects of temperature and feeding on
 615 the nutrient concentrations and isotopic signatures

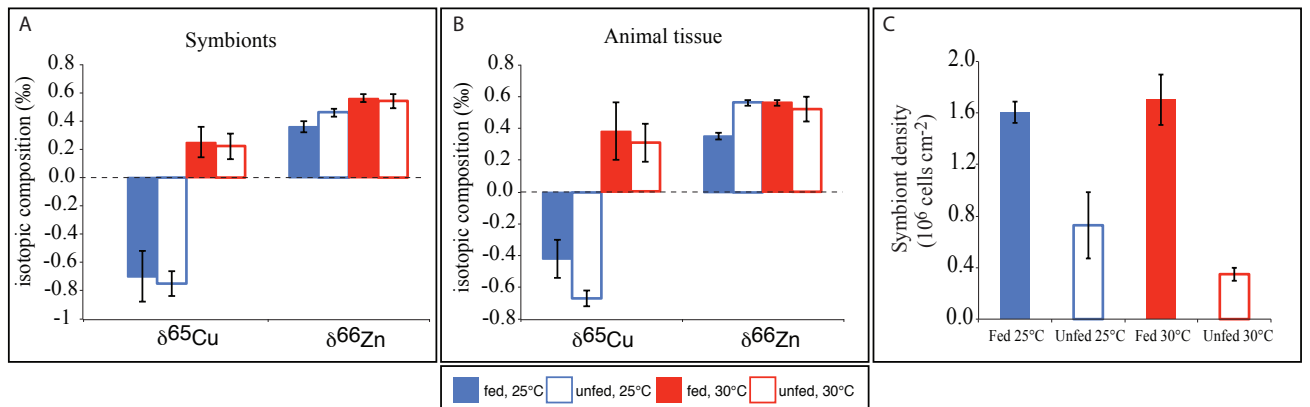
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Figure 1



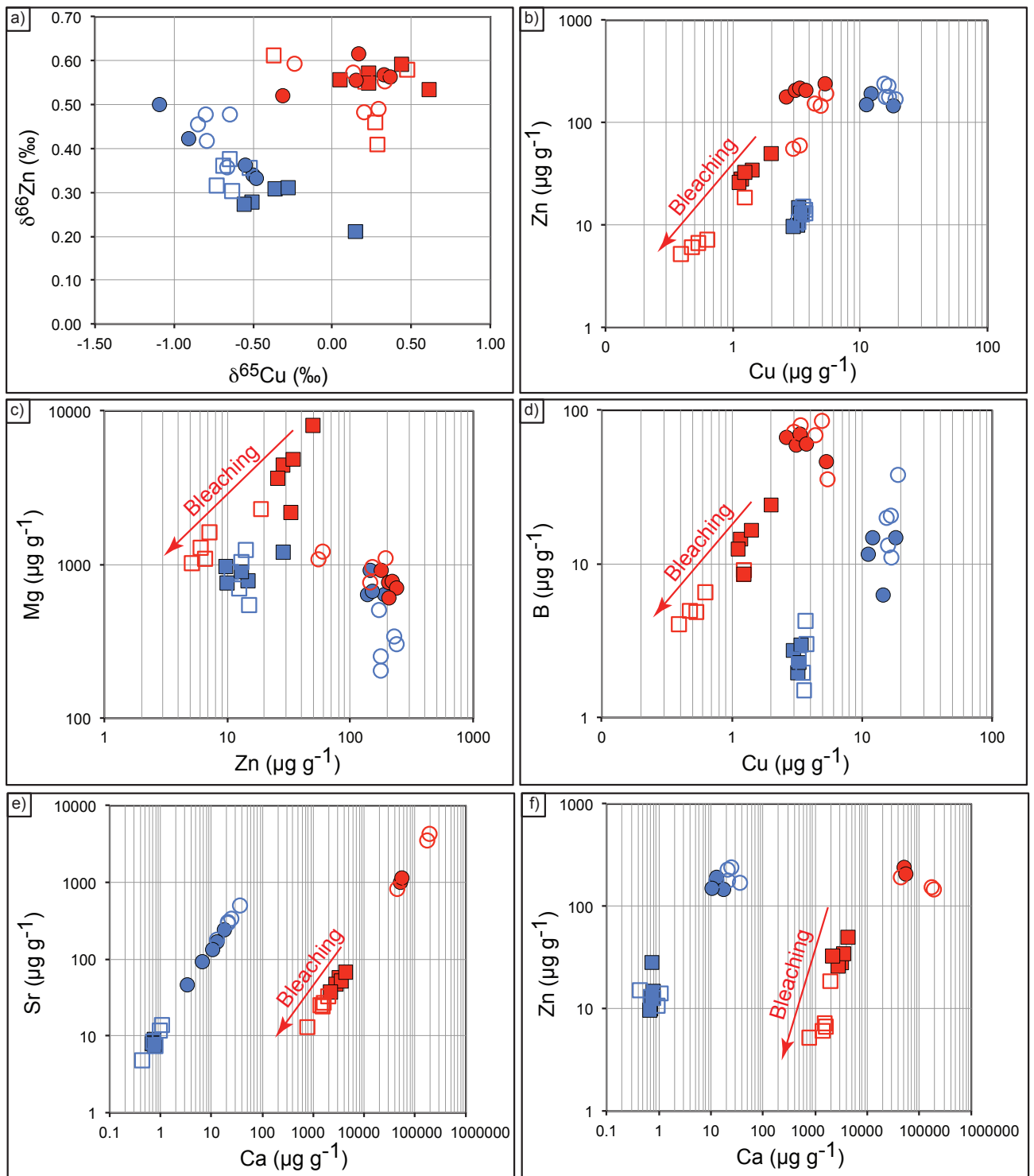
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Figure 2



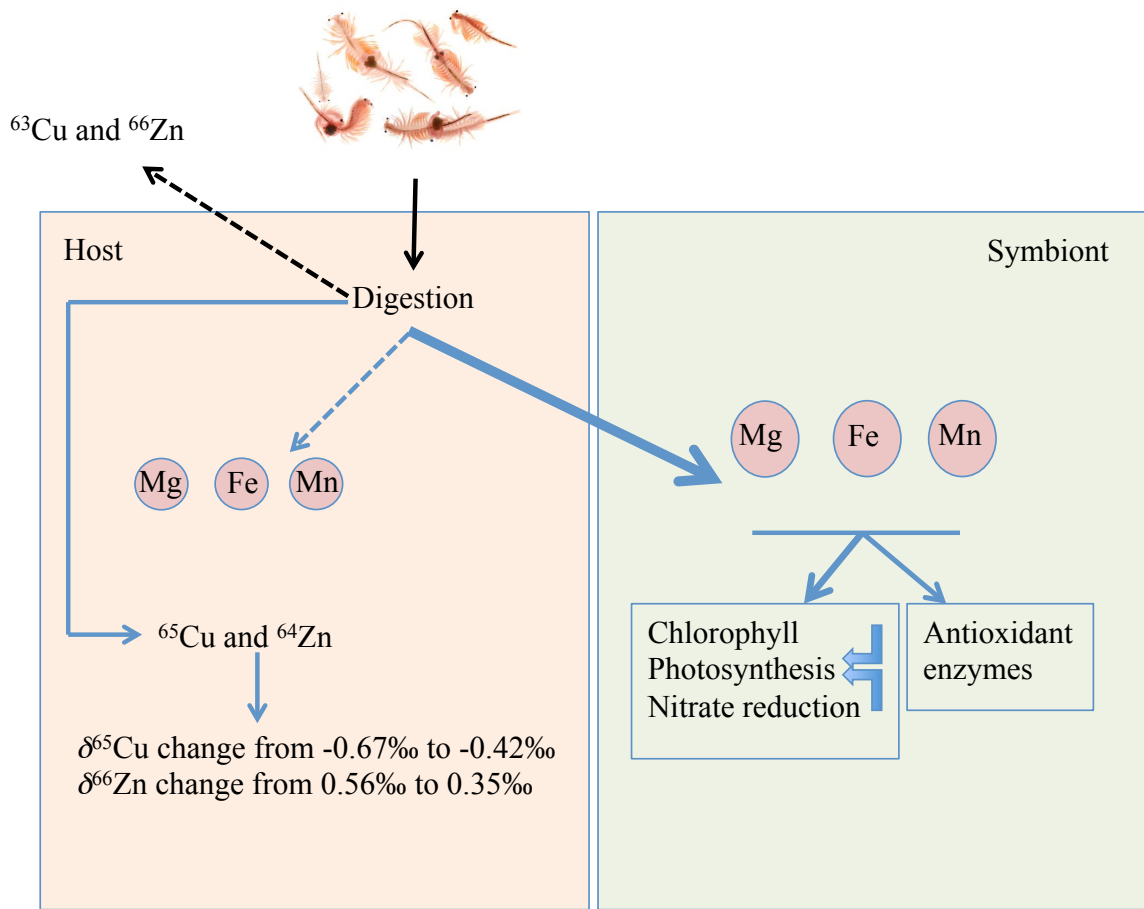
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Figure 3



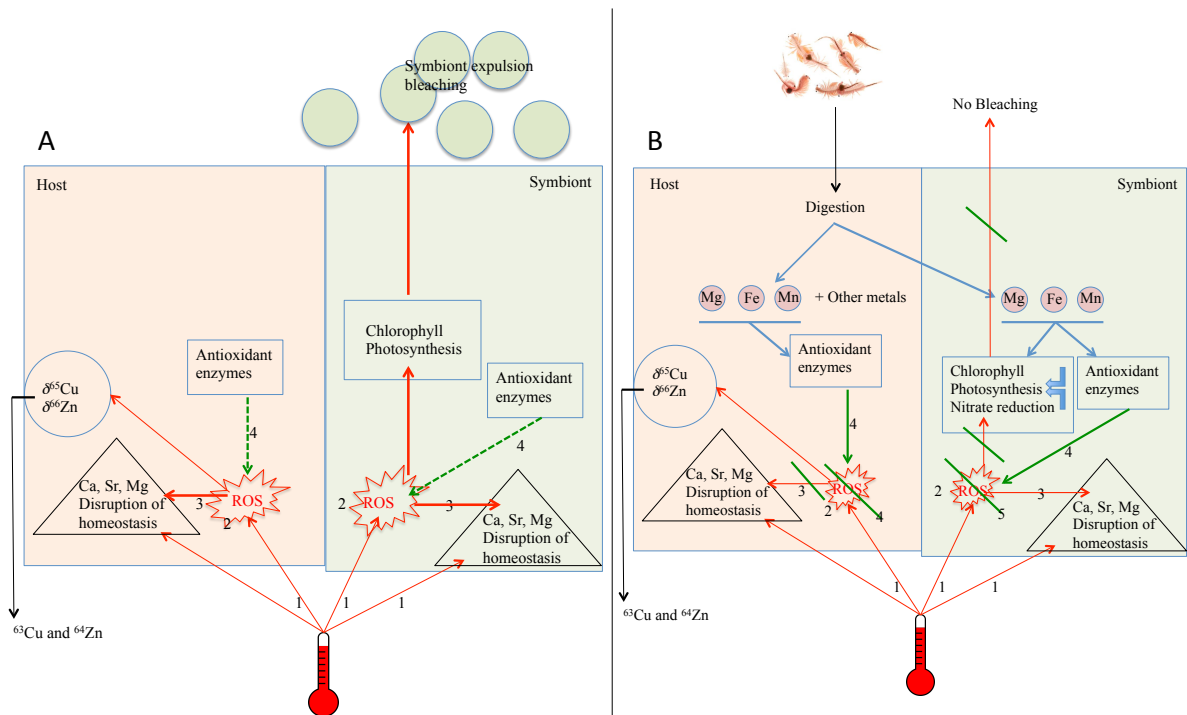
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Figure 4



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Figure 5



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Table 1

		Higher concentrations in fed symbionts at 25°C			Lower concentrations in fed symbionts at 25°C			Ba	Ca	B	Cu	δ ⁶⁵ Cu (‰)	δ ⁶⁶ Zn (‰)
		Mn	Fe	Mg	Zn	Sr	As						
Symbionts 25°C	Fed	6.12*	81.73	659.15	156.61*	160.35*	4.48	0.59*	12.02*	12.86*	14.02*	-0.70*	0.36*
	SD	1.22	13.89	168.28	23.70	64.89	0.86	0.17	4.72	2.27	3.13	0.18	0.04
Symbionts 25°C	Unfed	0.22*	45.83*	275.15*	198.14*	358.77*	10.77	1.22*	23.39*	16.24*	16.76*	-0.75*	0.46
	SD	0.25	4.89	58.82	31.84	94.19	4.07	0.61	8.55	4.79	1.38	0.09	0.03
Symbionts 30°C	Fed	1.19*	83.61	810.45	209.40*	1992*	5.99	2.98*	96242*	60.01*	3.56*	0.25*	0.56*
	SD	0.33	35.15	132.33	20.13	840.41	1.17	1.18	73835	8.28	0.93	0.11	0.03
Symbionts 30°C	Unfed	2.32*	100.84*	864.49*	111.33*	3984*	6.48	4.89*	116904*	59.53*	3.57*	0.22*	0.54
	SD	1.52	57.38	360.29	72.34	1975	3.91	2.16	65108	25.66	1.40	0.09	0.05
Host 25°C	Fed	0.07*	4.22*	924.45*	11.87*	8.02*	1.83*	0.04	0.73*	3.02*	6.59	-0.42*	0.35*
	SD	0.07	0.74	178.86	2.46	0.67	0.28	0.01	0.05	1.25	7.52	0.12	0.02
Host 25°C	Unfed	0.04	3.63	881.99	13.14*	9.24*	1.74*	0.09	0.80*	2.62*	3.55*	-0.67*	0.56
	SD	0.08	0.44	273.63	1.68	3.59	0.27	0.10	0.25	1.07	0.19	0.05	0.02
Host 30°C	Fed	0.35*	11.11*	5670*	31.24*	46.62*	3.32*	0.19	3581*	16.63*	1.36	0.38*	0.56*
	SD	0.10	2.44	2002	3.37	8.49	1.36	0.18	583.86	4.53	0.30	0.18	0.02
Host 30°C	Unfed	0.10	4.45	1360	6.28*	27.06*	3.21*	0.04	1393*	5.84*	0.63*	0.31*	0.52
	SD	0.03	2.06	461.09	0.74	3.51	1.29	0.01	446.35	1.79	0.31	0.12	0.08

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Table 1: Metal concentrations (µg g⁻¹ dry weight) in symbionts and host tissue of fed and unfed *S. pistillata* maintained under normal (25°C) and thermal stress (30°C) conditions. Data are mean and standard deviation (SD) of 5 samples per condition; * represents significant differences in metal concentrations between the two temperature conditions for both symbionts and host under fed or unfed conditions.

Table 2

	Symbionts			Tissue		
	df	<i>p</i>	f	df	<i>p</i>	f
Manganese (Mn)						
Feeding	1	0.0010	17.27	1	0.0010	16.04
Temperature	1	0.0273	6.07	1	0.0002	22.89
Feeding × Temperature	1	< 0.001	61.84	1	0.0075	9.36
Error	14					
Iron (Fe)						
Feeding	1	0.0467	4.65	1	0.0004	20
Temperature	1	0.0004	19.61	1	0.0003	21.75
Feeding × Temperature	1	< 0.0001	65.32	1	0.0044	10.95
Error	16			16		
Magnesium (Mg)						
Feeding	1	0.3671	0.86	1	0.0014	14.84
Temperature	1	< 0.0001	48	1	< 0.0001	43.72
Feeding × Temperature	1	< 0.0001	28.43	1	0.0039	11.38
Error	16			16		
Zinc (Zn)						
Feeding	1	0.0419	4.89	1	< 0.0001	45.67
Temperature	1	0.2934	1.18	1	0.4327	0.64
Feeding × Temperature	1	< 0.0001	30.4	1	< 0.0001	38.65
Error	16			16		
Strontium (Sr)						
Feeding	1	0.0105	8.39	1	0.0005	18.89
Temperature	1	< 0.0001	90.03	1	< 0.0001	119.91
Feeding × Temperature	1	0.3487	0.93	1	0.0002	22.85
Error	16			16		
Arsenic (As)						
Feeding	1	0.0048	10.7	1	0.5212	0.43
Temperature	1	0.6682	0.17	1	0.0008	18.83
Feeding × Temperature	1	0.1279	2.58	1	0.6728	0.19
Error	16			16		
Barium (Ba)						
Feeding	1	0.0021	13.49	1	0.5284	0.18
Temperature	1	< 0.0001	52.96	1	0.2393	0.34
Feeding × Temperature	1	0.0438	4.79	1	0.0357	5.66
Error	16			16		
Calcium (Ca)						
Feeding	1	0.0223	7.02	1	0.0075	8.75
Temperature	1	< 0.0001	744.93	1	< 0.0001	650.5
Feeding × Temperature	1	0.82	0.05	1	0.0035	12.11
Error	11			16		

Boron (B)						
Feeding	1	0.0052	10.43	1	0.0042	11.15
Temperature	1	< 0.0001	132.15	1	< 0.0001	56.67
Feeding × Temperature	1	0.0294	5.71	1	0.0275	5.89
Error	16			16		
Copper (Cu)						
Feeding	1	0.0336	5.4	1	0.2796	1.25
Temperature	1	< 0.0001	222.68	1	0.0286	5.78
Feeding × Temperature	1	0.141	2.4	1	0.5027	0.47
Error	16			16		
$\delta^{65}\text{Cu}$						
Feeding	1	0.8399	0.04	1	0.0455	4.7
Temperature	1	< 0.0001	72.23	1	< 0.0001	44.26
Feeding × Temperature	1	0.8398	0.04	1	0.3745	0.83
Error	16			16		
$\delta^{66}\text{Zn}$						
Feeding	1	0.6455	0.22	1	0.5186	0.44
Temperature	1	< 0.0001	33.11	1	< 0.0001	103.31
Feeding × Temperature	1	0.1159	2.76	1	0.0331	5.44
Error	16			16		

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