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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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Research article

Abstract

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

Introduction

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

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

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

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

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

Materials and Methods

Maintenance of coral colonies

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

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

Treatments

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

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

Analyses of trace and major element concentrations

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

Analyses of copper and zinc isotopic compositions

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

HNO₃ (0.5 mol L⁻¹). The purification step helps removing elements (e.g. Ni, Ti, Al or Ba), which may interfere with copper and zinc during the instrumental measurement (Chen et al., 2016; Sossi, Halverson, Nebel, & Eggins, 2014). Indeed, it has been shown that small amounts of Ni and Ti residues, for example, can produce isobaric interferences and shift the measured $\delta^{66}\text{Zn}$ values by more than 0.07 ‰ if the Ni/Zn and Ti/Zn ratios are higher than 0.001 and 0.01, respectively (Chen et al., 2016).

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

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

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

were measured on a Nu Plasma (Nu 500) MC-ICP-MS in wet plasma conditions. Instrumental mass fractionation was corrected with an exponential law using an elemental-doping method as recommended by Maréchal, Télouk, & Albarède, (1999). $\delta^{66}\text{Zn}$ and $\delta^{65}\text{Cu}$ were then calculated by standard bracketing using Zn JMC 3-0749L (Johnson Matthey Royston, UK) and Cu SRM 976 (NIST, Gaithersburg, MD, USA) as reference standards. Note that these standards were repeatedly measured between each sample to correct for the instrumental drift throughout the analytical sequence; a method called standard bracketing. The accuracy of the isotopic compositions was assessed based on the analyses of an in-house (sheep plasma; OEP) and international (bovine liver, 1577c, Sigma Aldrich, France) standard solution at the

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

Statistics

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

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

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

Results

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

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

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

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

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

Discussion

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

1- Effect of the dietary regime at 25°C

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

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

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

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

The $\delta^{65}\text{Cu}$ and $\delta^{66}\text{Zn}$ values were significantly different between fed and unfed hosts and symbionts (Fig. 2), suggesting, for the first time, that corals fractionate Cu and Zn isotopes depending on their dietary regime. The higher $\delta^{65}\text{Cu}$ values in fed colonies is linked to the consumption of Cu in the form of particulate organic matter, while unfed colonies only rely on the uptake of dissolved inorganic Cu by the symbionts. The $\delta^{65}\text{Cu}$ signature in fed corals indeed follows the rule that organic matter is enriched in the heavier Cu isotope compared to dissolved inorganic Cu (Petit et al., 2013). The lower $\delta^{66}\text{Zn}$ signature of fed compared to unfed colonies is also in agreement with the observation that carnivores are slightly ^{66}Zn -depleted relative to herbivores (Jaouen, Szpak, & Richards, 2016). Overall, the significant effect of feeding on the $\delta^{66}\text{Zn}$ and $\delta^{65}\text{Cu}$ signatures demonstrates that metal isotopes represent useful proxies to trace nutrient flows within a symbiotic association, although they have poorly been studied in marine food webs in general (Jaouen, Szpak, & Richards, 2016).

2- Effect of thermal stress

After three weeks of thermal stress, which induced bleaching in unfed corals (Fig. 2), significant changes occurred in metal concentrations and Cu-Zn isotopic compositions of symbionts and host tissue under both fed and unfed conditions. We observed a clear increase in the $\delta^{65}\text{Cu}$ and $\delta^{66}\text{Zn}$ values of both hosts and symbionts (Fig. 3A). Such isotopic variations can be related to an increase in reactive oxygen species (ROS) generated by thermal stress in coral tissue (Cunning & Baker, 2013). As light isotopes create weaker links with molecules compared to heavy isotopes (Schauble, 2004), these bonds are more easily disrupted by ROS

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

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

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

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

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

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

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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

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

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

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

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

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

Figure 1

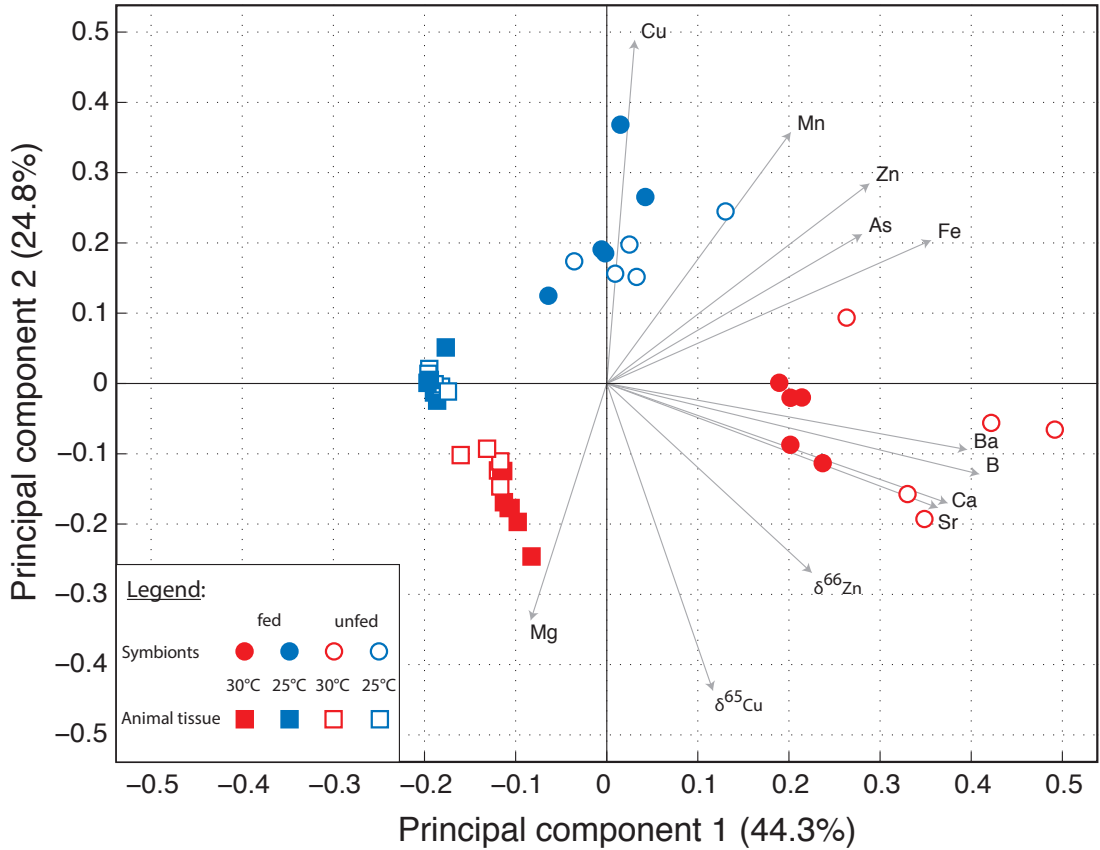


Figure 2

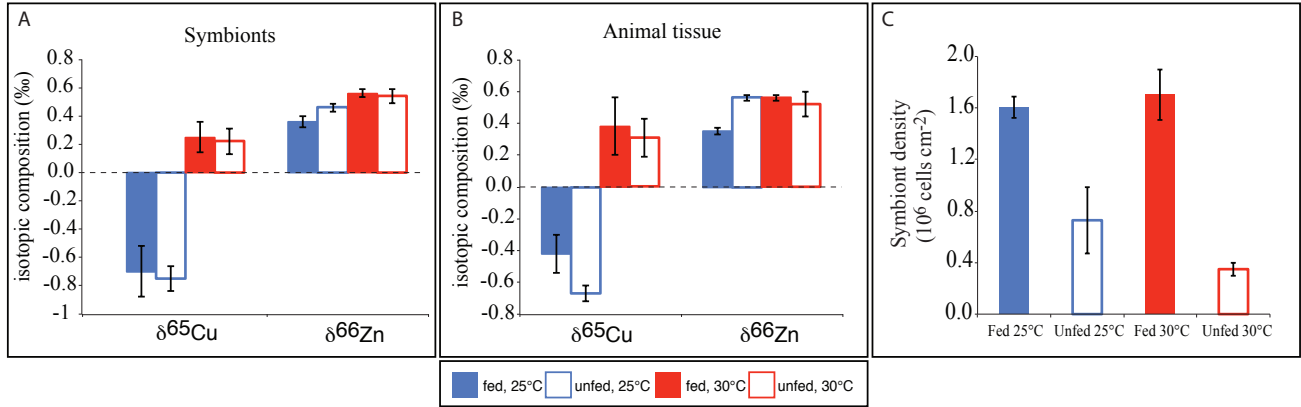
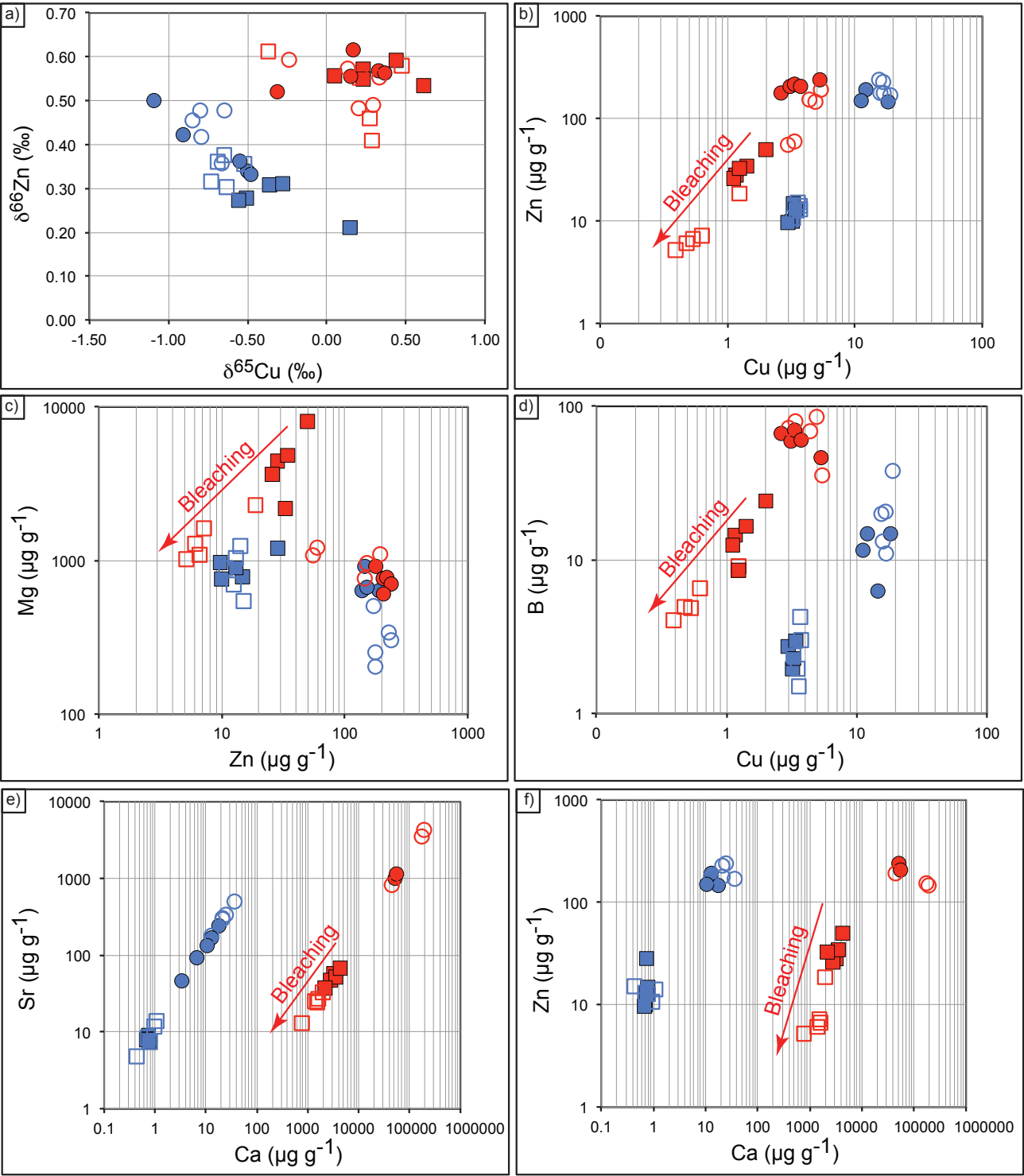


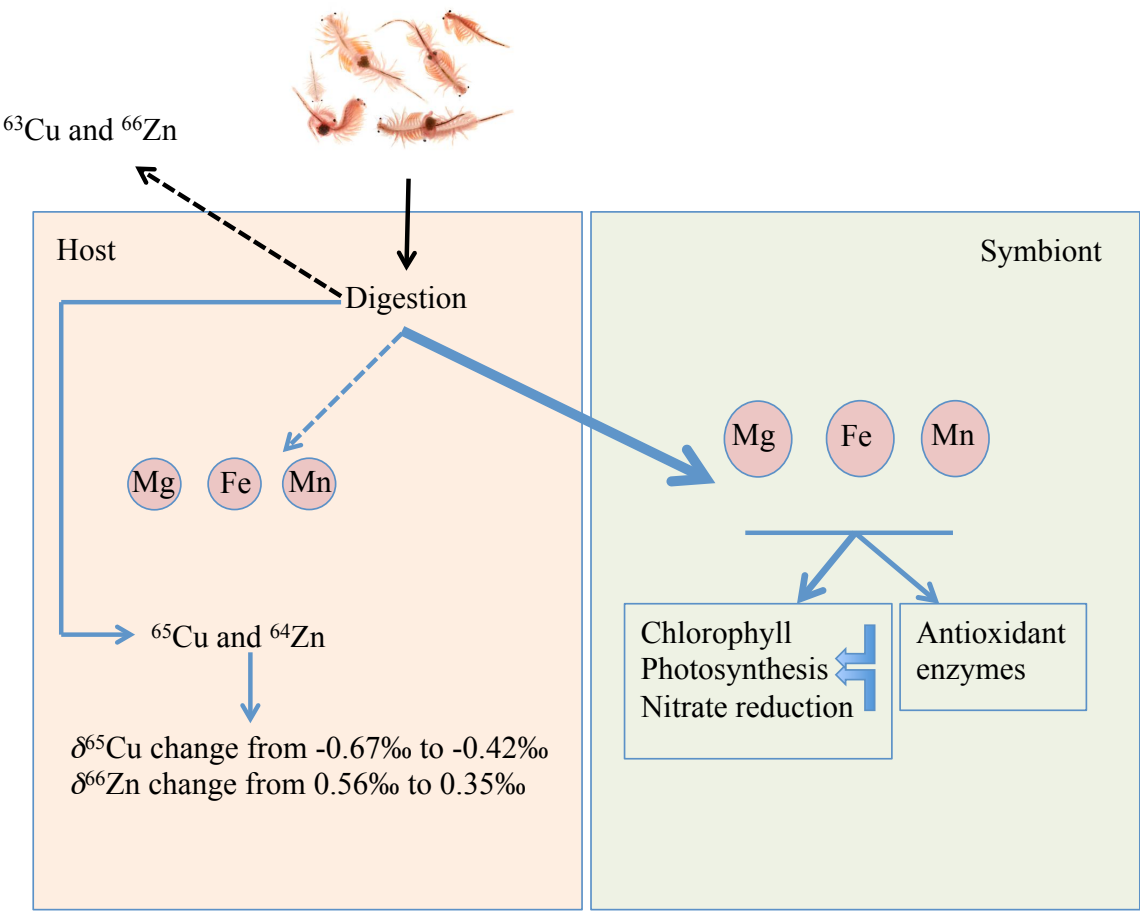
Figure 3



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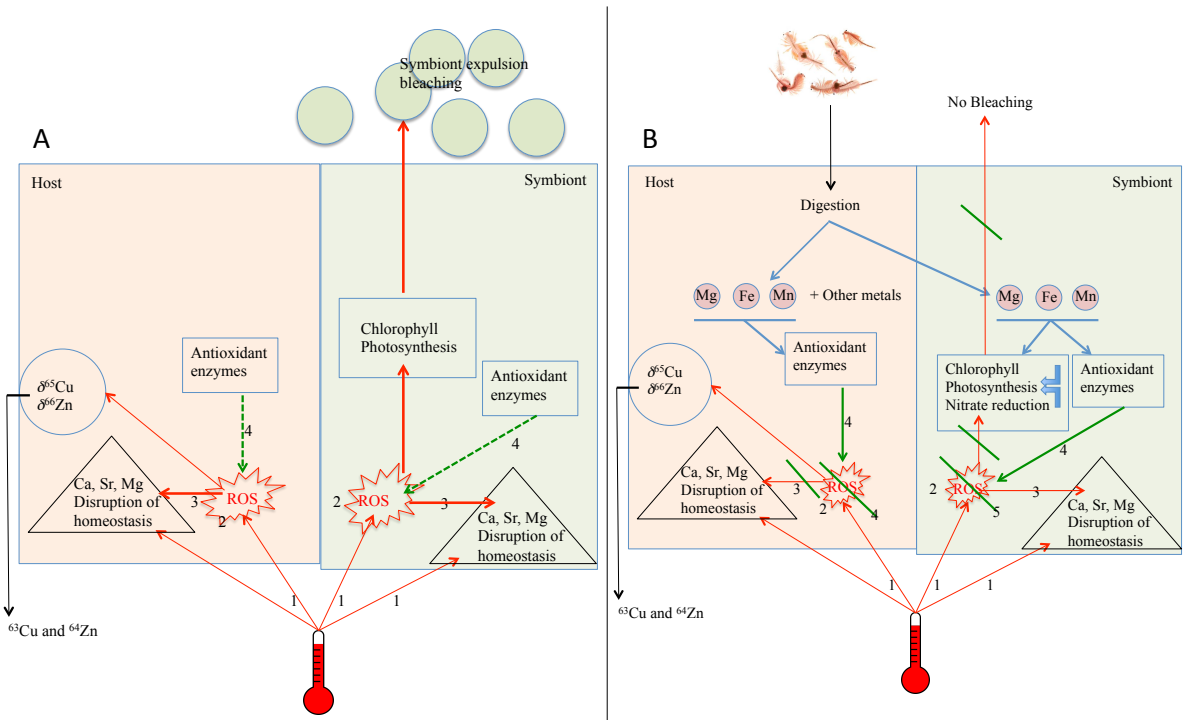
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Figure 4



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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								
		Mn	Fe	Mg	Zn	Sr	As	Ba	Ca	B	Cu	δ ⁶⁵ Cu (‰)	δ ⁶⁶ Zn (‰)
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		