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Research Article

Experimentally Induced Bleaching in the Sea Anemone *Exaiptasia* Supports Glucose as a Main Metabolite Associated with Its Symbiosis

Víctor Hugo Molina,[1] Raúl Eduardo Castillo-Medina,[2] and Patricia Elena Thomé[3]

[1]*Instituto Tecnológico de Morelia, Av. Tecnológico 15000, 58120 Morelia, MICH, Mexico*
[2]*Programa de Posgrado en Ciencias del Mar y Limnología, Mexico City, Mexico*
[3]*Unidad Académica de Sistemas Arrecifales Puerto Morelos, Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México, Apartado Postal 13, 77500 Cancún, QROO, Mexico*

Correspondence should be addressed to Patricia Elena Thomé; thome@cmarl.unam.mx

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Our current understanding of carbon exchange between partners in the *Symbiodinium*-cnidarian symbioses is still limited, even though studies employing carbon isotopes have made us aware of the metabolic complexity of this exchange. We examined glycerol and glucose metabolism to better understand how photosynthates are exchanged between host and symbiont. The levels of these metabolites were compared between symbiotic and bleached *Exaiptasia pallida* anemones, assaying enzymes directly involved in their metabolism. We measured a significant decrease of glucose levels in bleached animals but a significant increase in glycerol and G3P pools, suggesting that bleached animals degrade lipids to compensate for the loss of symbionts and seem to rely on symbiotic glucose. The lower glycerol 3-phosphate dehydrogenase but higher glucose 6-phosphate dehydrogenase specific activities measured in bleached animals agree with a metabolic deficit mainly due to the loss of glucose from the ruptured symbiosis. These results corroborate previous observations on carbon translocation from symbiont to host in the sea anemone *Exaiptasia*, where glucose was proposed as a main translocated metabolite. To better understand photosynthate translocation and its regulation, additional research with other symbiotic cnidarians is needed, in particular, those with calcium carbonate skeletons.

1. Introduction

Tropical symbiotic cnidarians are characterized by the metabolic exchange of organic carbon and nutrients with their symbiotic dinoflagellate partners, allowing them to prosper in oligotrophic waters [1, 2]. The chemical identity of the organic carbon that is translocated from symbiont to host seems to be different depending on the organism. Muscatine and Trench [3, 4] identified glycerol as a metabolite potentially translocated to animal hosts, including a coral, a giant clam, an anemone, and a zoanthid, although this earlier work was conducted with freshly isolated symbionts. Many studies employing labeled ^{14}C-bicarbonate have since demonstrated that after its uptake and fixation by the photosynthetic activity of symbionts, the two labeled, free carbohydrates identified in symbiotic animal tissues were mainly glycerol and glucose [5–13]. Overall, several contributions have suggested that the carbon translocated from the dinoflagellate partner may exist as pools of molecules with high turnover rates [5, 10, 11, 14] which make it difficult to identify with certainty.

Considering the current threat to reef ecosystems worldwide, carbon translocation in the symbiosis of reef corals represents an essential research topic. Defining the chemical identity of transferred metabolites in the symbiosis is essential to understand the mechanisms that stimulate their formation as well as their transfer. Excess carbon fixed by the symbionts cannot be used to their own advantage (i.e., increase in cell numbers), mainly due to the limitation of nitrogen and phosphorous of the symbiotic animals [15–19], a result of the nutrient-poor waters where they live. This excess

carbon is thus translocated to the animal host, being used mostly for respiration, calcification, and mucus production [20–22]. Considering the photophysiology of the symbiotic dinoflagellates, an opposing explanation of mechanisms for glucose and glycerol release/translocation by the symbionts can be presently inferred: (1) glucose production by the symbiont may be subject to feedback inhibition by photosynthesis [23] and would not allow glucose to represent an important overflow mechanism. (2) On the other hand, the release of glycerol from symbionts may represent a more important overflow mechanism, since glycerol has been suggested to have a photoprotective function as a sink for excess carbon and reducing power [24, 25].

In the model sea anemone *Exaiptasia pallida*, Burriesci and collaborators [13] specified that glucose is the major translocated photosynthate in this symbiosis. However, the mechanisms that potentially regulate the translocation of glucose exposed above suggest the need for further experimental evidence. With this purpose, we examined glycerol and glucose metabolism in this symbiotic animal and its response to the loss of symbionts.

2. Materials and Methods

2.1. Organisms and Experimental Conditions. We worked with the symbiotic tropical sea anemone, recently renamed *Exaiptasia (Aiptasia) pallida* [26]. Free growing anemones were collected from the aquaria water system and identified according to Grajales and Rodríguez [26]. Anemones with a 0.5 cm pedal disc were maintained for 3 months in 4 L aquaria with a bubbling air supply. Seawater temperature was maintained at room temperature (27°C ± 1°C); animals were fed twice weekly with freshly hatched *Artemia* sp. nauplii, following a full replacement of fresh seawater. Light was provided with fluorescent lamps delivering 250 (high light) or 50 μmol photons m^{-2} s^{-1} (low light) in a 12 hrs light/dark cycle. Bleached anemones were obtained by exposing the animals twice to 4°C for 4 h in the dark, in consecutive days [27, 28], with daily water replacements. After this treatment, anemones were returned to aquaria in the dark for one week before being collected and processed, being fed as above.

2.2. Animal Homogenates and Quantification of Metabolites. Collected animals were briefly washed in PBS buffer, blotted to filter paper (3 mm) and weighted. Crude protein extracts were prepared by homogenizing animal tissues (2-3 anemones for enough material) with a Glass-Potter homogenizer in one volume (relative to tissue mass) of extraction buffer [50 mM KH_2PO_4; 0.01 mM EDTA pH 7.8] with a protease inhibitor cocktail (complete, Sigma, USA) on ice, until complete breakdown (less than 2 minutes). Each sample was processed separately. The homogenates were centrifuged at 4°C for 15 min at 10,000 ×g; 0.1 mL aliquots were taken for Bradford protein determinations, measured with a commercial dye assay (Sigma, USA) according to the manufacturer, employing BSA as the standard. Pelleted algal cells were fixed with Lugol for determination of symbiont density. For total glycerol and total glucose determinations, three aliquots (200 μL) from each homogenate were boiled for 10 min and centrifuged 15 min, and the supernatants frozen at −70°C until processed. We used the Free Glycerol Reagent and the GO Glucose Assay Kit (both from Sigma, USA) according to the protocols of the manufacturer. Readings were obtained with a SmartSpec spectrophotometer (BioRad) and compared to the respective standards. Values were normalized to wet mass.

Glycerol 3-phospate (G3P) and glucose 6-phosphate (G6P) were quantified according to protocols described in Bergmeyer and Graβl [29]. Briefly, 1 g of animal wet mass was ground in liquid nitrogen with a mortar and pestle. The resulting powder was transferred to 15 mL falcon tubes, containing 5 mL of 0.6 M perchloric acid, and centrifuged for 10 min at 3,000 ×g. The supernatant transferred to a clean tube, and the pellet was reextracted with 1 mL of 0.6 M perchloric acid diluted 1 : 2 with nanopure water and centrifuged as before. Supernatants were pooled, the pH adjusted to 3.5 with 5 M K_2CO_3 (indicator paper), and water added to a final volume of 8 mL. Extracts were allowed to sit on ice for 15 min and the supernatant was transferred to a clean tube. The reaction assay for G3P consisted of hydrazine (189 mM)/glycine (470 mM)/EDTA (2.7 mM) buffer pH 9.5, 2.3 mM NAD, and 100 μL of sample [29]. Absorbance readings for NAD (339 nm) were taken until constant (A_1); then 20 μL of a 1 kU L^{-1} solution of GPDH was added, taking second readings after 5 min (A_2). The reaction assay for G6P consisted of 200 mM TEA buffer pH 7.6, 20 μM NADP, 5 mM MgCl2, and 100 μL of sample [29]. Readings at 339 nm (A_1) were taken at 3 min intervals until stable. Then, 5 μL of a 35 kU L^{-1} suspension of G6PDH was added; the reaction allowed to proceed taking a second set of readings (A_2) after 10 min. The difference in absorbance was used for calculations of metabolite concentrations normalized to wet mass and adjusted for dilution factors introduced during the extraction and neutralization of samples [29].

2.3. Symbiont Density. Sedimented cells obtained from the preparation of protein and metabolite extracts were fixed with 20% Lugol solution (Sigma). Symbiont density normalized to protein mass was determined by directly counting four aliquots in a NewBauer hemocytometer chamber. Dilutions were done when needed, adjusting the cell numbers accordingly.

2.4. Enzymatic Assays. Glycerol 3-phosphate dehydrogenase (GPDH) was assayed following a modified procedure [24]. Animal homogenates were prepared for metabolite determination, but in a TrED buffer (10 mM TEA, 1 mM EDTA, 1 mM DTT, and pH 7.5) with a protease inhibitor cocktail (Complete, Sigma, USA). The crude extracts were clarified by centrifugation at 10,000 ×g for 15 min at 4°C. The supernatant was used for protein determination and enzymatic assays, in which case PEG (3,500 MW) was added to a final concentration of 15% for enzyme stabilization. Some samples were concentrated before analysis by centrifugation with Millipore centrifugal filters (30 kDa MW cutoff) at 4°C. The specific activity was measured in 2 mL assays containing

Table 1: Symbiont density in anemones before and after bleaching. Values are the total number of cells per mg of protein mass, isolated from 3 individuals for each biological replicate. For each sample, four technical replicates were counted.

Anemones	With symbionts	Bleached
(1)	5.31×10^6	4.37×10^5
(2)	5.80×10^6	3.14×10^5
(3)	3.71×10^6	4.34×10^5
Mean	4.94×10^6	3.95×10^5
SD	1.09×10^6	0.70×10^5

160 μM NADH and 1 mM dihydroxyacetone phosphate. For each sample, triplicate assays were initiated by adding 25 or 50 μL of animal extract and followed for 5 min. The rate of decrease in absorbance of NADH (339 nm) measured in a BioSpectrometer (Eppendorf, USA) against air was used to calculate the specific activity reported as units (μmol of substrate converted min^{-1}) g^{-1} of protein mass.

For the determination of G6P dehydrogenase (G6PDH) enzyme activity, we followed the protocol described in Bergmeyer and Graβl [30]. Briefly, the reaction assay contained 50 mM Tris buffer pH 7.5, 380 μM NADP, 6.3 mM $MgCl_2$, 3.3 mM G6P, and 5 mM maleimide. The reaction was started by adding 20 μL of clarified animal extract, reading at 339 nm against nanopure water. After the reaction was linear, measurements were taken at 1 min intervals for 5 min. Enzyme specific activity is reported as units per gram of protein mass, one unit being the increase in absorbance that corresponds to the conversion of 1 μmole of substrate per minute.

2.5. Western Blotting. For the detection of glycerol kinase (GK) and glucokinase (GCK), similar concentrations of total protein were resolved in an SDS-PAGE system at 12% acrylamide concentration. The resolved proteins were transferred to nitrocellulose membranes and incubated with commercial specific antibodies raised against human antigens in mouse (GK (H-132), SC-366975 and GCK (H-88), SC-7908; Santa Cruz Biotechnology, USA). We checked for the compatibility of the antibody epitope in the amino acid sequences for both enzymes of several cnidarians. Both antibodies were used at 1 : 500, detected with an anti-mouse secondary antibody coupled to alkaline phosphatase (Sigma). The bound antibodies were developed with a blue substrate kit (BCIP/NBT, Sigma, USA).

2.6. Statistical Analysis. All experiments had three biological independent replicates, with at least two technical replicates. Data are presented as means ± standard deviations. For the comparison of metabolites, results were compared with ANOVA analysis followed by a post hoc Tukey HSD test set at $p = 0.01$ significance. Specific activity of enzymes was compared between symbiotic and bleached animals by Student's t-test.

3. Results

We obtained on average, a 92% reduction of symbionts for bleached anemones. Results are presented in Table 1. Total glycerol levels were detected in symbiotic anemones under both light regimes (high and low) at similar concentrations, while G3P levels were significantly higher for symbiotic anemones under low light (Figure 1). By contrast, bleached anemones showed a significantly higher concentration of total glycerol but its phosphorylated form was similar to anemones under low light (Figure 1). The concentration of total glucose was 10 times higher than that for glycerol in symbiotic anemones under both light regimes and significantly reduced in bleached animals (Figure 2). G6P levels were very low in all conditions (Figure 2).

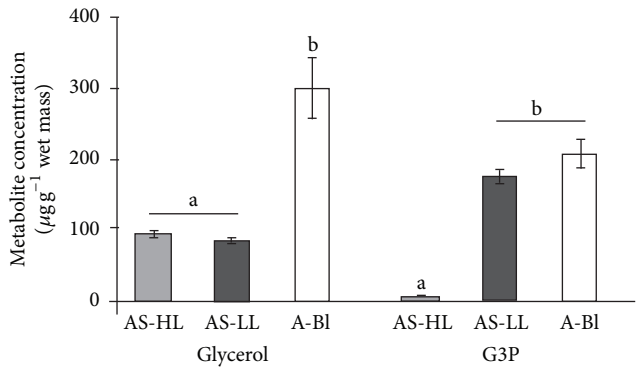

Figure 1: Glycerol levels measured in symbiotic and bleached anemones. Animals in the symbiotic state were maintained at two light conditions (HL = 250 μmol photons cm^{-2} s^{-1}; LL = 50 μmol photons cm^{-2} s^{-1}). Bleached animals were maintained in the dark. Columns represent the average of three independent determinations ± SD (bars). Values are in $\mu\text{g g}^{-1}$ of wet mass. Small letters group treatments that were similar for each metabolite (Tukey HSD, $p < 0.01$). AS = symbiotic anemones; A-Bl = bleached anemones.

The specific activity of GPDH and G6PDH was measured in extracts from symbiotic animals kept under low light and from bleached animals. The results showed a significant decrease of GPDH enzyme activity in bleached animals as compared to animals in the symbiotic state (Table 2). On the other hand, results showed an increase of about 30% the specific activity for G6PDH enzyme in bleached animals (Table 2).

Results for the detection of glucokinase, which specifically phosphorylates glucose, were not conclusive. Glycerol kinase was not detected in animals, symbiotic or bleached, but only in *Symbiodinium* extracts, detecting a polypeptide with an apparent molecular weight of 55 kDa (data not shown).

Table 2: Glycerol 3-phosphate dehydrogenase (GPDH) and glucose 6-phosphate dehydrogenase (G6PD) specific activity in anemones in the symbiotic state compared to bleached anemones. Values are the means of three independent determinations ± SD. Specific activity in units (μmol substrate converted min^{-1}) per gram of protein mass. For each sample, three assays were performed (technical replicates) and averaged.

Enzyme	Enzyme specific activity		t-Student
	Symbiotic	Bleached	p
GPDH	49.78 ± 6.62	0.20 ± 0.08	12.95 0.0001
G6PDH	0.65 ± 0.28	0.85 ± 0.10	3.11 0.036

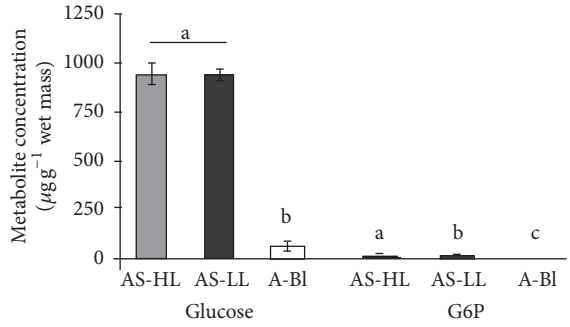

Figure 2: Glucose levels measured in symbiotic and bleached anemones. Animals in the symbiotic state were maintained at two light conditions (HL = 250 μmol photons cm^{-2} s^{-1}; LL = 50 μmol photons cm^{-2} s^{-1}). Bleached animals were maintained in the dark. Columns represent the average of three independent determinations ± SD (bars). Values are in μg g^{-1} of wet mass. Small letters group treatments that were similar for each metabolite (Tukey HSD, $p < 0.01$). AS = symbiotic anemones; A-Bl = bleached anemones.

4. Discussion

4.1. Shifts in Metabolites and Enzyme Activities after Bleaching. Even though evidence for glycerol and glucose as being translocated metabolites in the cnidarian-*Symbiodinium* symbioses may be yet insufficient, our results showed that glucose levels were close to 50 times higher in anemones with symbionts as opposed to bleached anemones. By contrast, glycerol levels increased about three times in bleached anemones suggesting a catabolism of lipids, as has been documented to occur due to a deficit in nutrition [31, 32]. The values we measured for phosphorylated glycerol (G3P) agree with this interpretation, since this metabolite does not accumulate when lipids are being synthesized. It is interesting, however, that, under low light, G3P levels were higher and more similar to bleached animals than to symbiotic animals under high light, which suggests that at low light anemones are obtaining less carbon from their symbionts. This possibility did not reflect on glucose levels, perhaps due to secondary synthesis. Correspondingly, the specific activity of the enzyme GPDH in bleached anemones was significantly lower than that of anemones in the symbiotic state. It seems likely that lipid degradation is compensating the massive decrease of glucose in bleached anemones. Garrett et al. [33] performed lipid profiles of *E. pallida* both symbiotic and aposymbiotic. Among their findings, they identified that nonpolar lipids, like tri- (TAGs) and diacylglycerols (DAGs), were more abundant in anemones in the symbiotic state. As for our experiments, lipid degradation may explain the increased levels of glycerol in aposymbiotic anemones. Further, the specific activity of GPD in bleached anemones was too low, strongly indicating that almost no lipid synthesis was taking place in this condition. GPDH catalyzes the reversible conversion of G3P to dihydroxyacetone phosphate (DHAP), serving as a major link between glycolysis, gluconeogenesis, and lipid metabolism. Low levels of activity for GPDH would direct the flux of carbon to glycolysis, through lipid catabolism. This assessment concurs with the results we obtained for the phosphorylated forms and the levels of glucose and glycerol in bleached anemones.

As opposed to anemones with symbionts, bleached anemones showed a significant increase in the specific activity for G6PDH, suggesting a compensation effort by the bleached animals to alleviate a deficit of fatty acids due to the catabolism of lipids. G6PDH is the first step in the pentose phosphate pathway that regenerates NADPH for anabolic metabolism, as well as pentoses like ribose-5 phosphate, a precursor in the synthesis of nucleotides. When increased, the level of enzymes involved in oxidative pentose phosphate pathway (e.g., G6PDH and 6-P gluconate dehydrogenase), rather than leading to the accumulation of carbon skeletons for cellular growth, regenerates NADPH, essential for the reduction in fatty acid biosynthesis [34].

4.2. The Effects of Light and Feeding. Our results showed similar levels of glycerol and glucose in the symbiotic state under two light levels, which may be indicative that the light levels we used were not qualitatively too different. Also, we maintained the animals with moderate feeding, but we clearly distinguished the effects of the significant reduction of symbionts (bleaching) in the metabolites we measured. Tremblay et al. [35] showed that, in well-fed coral *Stylophora pistillata*, irradiance levels determine the amount of translocated carbon, observing higher translocation under high light, as well as higher fixation by the symbiont, resulting in an increase of host biomass, while, at low light, they measured less translocation but higher host and symbiont biomass. For unfed colonies, the irradiance levels did not show differences in translocation or utilization of carbon. Since we did not measure differences in the pools of glycerol and glucose between high and low light treatments on anemones with

symbionts, we can conclude that the anemones were not well-fed. Finally, if glycerol and/or glucose were translocated from symbiont to host, we would expect to detect glucokinase and/or glycerol kinase in symbiotic animals. Since our results were negative, we hypothesize either that (1) the levels of these enzymes may be low but sufficient enough to phosphorylate these metabolites, after their putative uptake by animal cells, or (2) that better suited antibodies might be able to detect these enzymes clearly. We did not assay hexokinase since this is not a specific enzyme for glucose, but this enzyme could be more important in phosphorylating potentially translocated glucose.

4.3. Symbiont-Dependent Metabolism in Exaiptasia. Our results strongly indicate that the metabolism of symbiotic *E. pallida* depends on a fresh supply of glucose, in agreement with published observations that detected ^{13}C-labeled glucose in animal tissues exposed for 2 min to ^{13}C-bicarbonate [13]. In contrast, thermally induced bleaching seems to have a minor effect on glucose metabolism for this anemone, as has been shown by Hillyer et al. [36].

Fatty acids and lipids represent major stores of carbon and energy in host animals; the transfer of organic carbon between partners affects these stores in the symbiosis [33, 37, 38]. Autotrophically derived carbon in *Exaiptasia* sp. is preferably stored in lipids that may be quickly consumed by the host [39]. Hillyer and collaborators [40] measured losses of multiple PUFAs following a thermal stress in these anemones. When faced with a reduction of symbionts as occurs during bleaching, animal hosts shift their metabolism towards the catabolism of lipid stores to provide the necessary energy [41–43]. We were able to differentiate shifts in metabolites with our experimental setup through (1) the reduction of glucose pool; (2) an increase in glycerol and G3P pools, (3) a diminished specific activity for G3PDH, related to the synthesis of glycerol, and (4) an increase in the specific activity of the G6PDH enzyme.

The possibility of glycerol being translocated and used for the synthesis of glucose in the symbiosis cannot be ruled out by our experimental approach. In such instance, glycerol would need to be phosphorylated (by glycerol kinase, which we could not detect in symbiotic or bleached anemones) and oxidized back to DHAP, to be condensed into 1,6 bisphosphate (by the reversible action of aldolase), needing 2 molecules of ATP and being therefore an energy-consuming pathway. Four key enzymes regulate gluconeogenesis in humans: pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose 1,6 bisphosphatase, and glucose 6-phosphatase. The first two are involved when carbohydrates are produced from amino acids; the latter two are involved in the final steps of gluconeogenesis. It could seem likely to expect glucose to be produced by secondary synthesis during bleaching or starvation (from either lipid or protein catabolism), but the energy-consuming alternate pathway for its synthesis in symbiotic animals would be difficult to support. Further, Burriesci and collaborators [13] were unable to detect changes in labeled glucose after evaluating light incubated samples of anemones in the presence of the gluconeogenesis inhibitor 1-thioglycerol.

Finally, the two key enzymes in the glyoxylate cycle have been identified in the anemone's transcriptome, but only isocitrate lyase showed overexpression in symbiotic animals as opposed to aposymbiotic ones [44]. Through this pathway, four-carbon succinate is produced from acetyl CoA that can enter gluconeogenesis and is therefore an alternate path for the synthesis of glucose from glycerol. However, here again, glycerol would have to be phosphorylated, oxidized to DHAP, and fed into glycolysis to produce acetyl CoA. This alternative for the synthesis of glucose may be active in symbiotic animals under particular circumstances.

5. Conclusions

The effect of the loss of symbionts in the anemones was clearly observed as a significant reduction on the levels of glucose. The consistency of our results with the literature [13, 36, 45] strongly supports the hypothesis that glucose is a major chemical form in the translocation of reduced carbon in the symbiosis of *E. pallida*. The possibility of glucose being synthesized from glycerol seems unlikely, being an energy-consuming path. However, we still need to explain why would glucose be released from symbionts, given its regulatory effect on photosynthesis [46], not expecting glucose to be a carbon overflow mechanism. Conversely, high nitrogen conditions may override the feedback effect of glucose on photosynthesis, but such conditions are still controversial in the *Symbiodinium*-cnidarian symbioses under nutrient-poor waters [16, 47–49].

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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