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Respiratory electron transport system activity in symbiotic corals and its link to calcification

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ABSTRACT: Scleractinian corals host photosynthetic endosymbionts, making direct measurement of the host respiration rate via incubation methods based on O_2 consumption impossible. We tested the use of the respiratory electron transport system activity (ETSA) for measuring host potential respiration. The applied method, modified from a previous study, is based on the reduction of (4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) to formazan. After the development of a protocol suitable for corals, the method was tested on 5 different species. Metabolism, including photosynthesis, dark respiration and light and dark calcification, was measured through incubation. Host and zooxanthellae fractions were separated and their ETSAs, protein contents and zooxanthellae densities were measured. Mean ETSA/dark respiration (ETSA/R) ratios for host corals ranged from 1.7 ± 0.2 to 3.5 ± 0.6, while ratios for zooxanthellae ranged from 3.7 to 7.8. The high ratios observed for zooxanthellae indicate that their respiration may be 5 times higher under light conditions than in the dark. Considering the obtained ratios, host respiration in light could increase at most by a factor of 3.5 compared with dark respiration rates. Ratios close to 1 were found for some specimens, which suggests that higher respiration rates under light compared with dark conditions are not possible. Therefore, increased respiration in light cannot explain the observed enhancement of calcification under light conditions. ETSA was correlated with zooxanthellae density, suggesting adaptation of the levels of host ETS enzymes to the amount of translocated photosynthetates under optimal conditions. Estimated dark host respiration was correlated with photosynthesis, which suggests that it is determined mainly by the amount of energy available but also the amount of electron transport system enzymes. This constrains the amount of ATP available for calcification. Hence, we propose a mechanism by which respiration limits the calcification rate.

KEY WORDS: Electron transport system · ETS · Respiration · Calcification · Symbiotic corals

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INTRODUCTION

Respiration measurements are usually based on O_2 consumption, but such measurements have important limitations. In the case of laboratory experiments, stresses due to the incubation conditions required for measurement may affect the respiration rates of the studied organisms. To overcome the limitations of these methods, indirect biochemical techniques have been developed. However, to the best of our knowledge, such methods have never been used in symbiotic organisms. Corals are symbiotic organisms that

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host photosymbionts known as zooxanthellae, which is the name given to a wide array of different algae of the genus *Symbiodinium*.

The biochemical methods proposed for conducting respiratory measurements include the quantification of respiratory electron transport system activity (ETSA) (Packard 1971), which was later modified by Owens & King (1975). This method measures the activities of the enzymes that constitute the electron transport system (ETS) at saturated substrate concentrations. Therefore, ETSA represents the potential respiratory rate and has been used to estimate oxygen consumption in various environments and community assemblages, including sediment meiofauna (Olanczukneyman & Vosjan 1977, Broberg 1985, Relexans 1996a,b), plankton (Kenner & Ahmed 1975a, Owens & King 1975, Packard et al. 1975, Bamstedt 1980, Hernández-Leó & Gómez 1996) and marine invertebrates (Cammen et al. 1990, St-Amand et al. 1999, Martínez et al. 2010). The method proposed by Packard in 1971 and modified for application to corals in the present study is based on the reduction of an artificial substrate, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT), by the coenzyme Q cytochrome-*b* complex. The mitochondrial and microsomal ETSs are saturated with substrates, and the reduction of INT to formazan is followed by spectrophotometry. The transfer of electrons between the coenzyme Q cytochrome-*b* complex and complex III or INT is known to be the rate-limiting step in the electron transport chain (Packard 1971). Therefore, ETSA represents the maximum potential respiration, which is assumed to be correlated with the maximum potential ATP production. Finally, ETSA measurements present the advantage of removing problems due to the stressful conditions during the confinement required for measurement with traditional methods, which can confound the obtained respiration rates. To apply the ETSA method to the coral holobiont system, a suitable protocol must be developed that will allow the measurement of the ETSAs of the different components, i.e. the host and the zooxanthellae.

Corals and other calcifying organisms that harbor photosymbionts or are themselves photosynthetic show strong coupling between calcification and photosynthesis at the organismal and community levels. Increased calcification is commonly known to occur under light in photosynthetic organisms; this phenomenon is referred to as light-enhanced calcification. The calcification rates in corals are on average 3 times higher in the light than in the dark (Gattuso & Allemand 1999, Goreau 1959, Barnes & Chalker 1990), and have been reported to be 6 to 10 times higher in the light for the coral *Stylophora pistillata* (Houlbreque et al. 2004). Several hypotheses (see Gattuso & Allemand 1999 for a review) have been proposed to explain this enhancement, but the interactions between calcification and photosynthesis remain unresolved and are a matter of continuous controversy (McConnaughey et al. 2000, Gattuso et al. 2000), with some arguing that repression of calcification occurs during night (Marshall 1996). The most recent hypothesis is based on the titration of protons (H^+) produced by calcification with hydroxyl ions (OH^-) produced during the photosynthetic process (Furla et al. 1998, 2000, Moya et al. 2006). Some of the recurrent hypotheses that have been put forth to explain this phenomenon are as follows: removal of metabolites as phosphate compounds that may inhibit calcification (Simkiss 1964); removal of CO_2, shifting the chemical equilibrium of calcification towards the formation of calcium carbonates (Goreau 1959); and provision of photosynthetates used for the construction of the organic matrix or as an energy source that is then used for calcification (Barnes & Taylor 1973, Barnes & Chalker 1990, McConnaughey et al. 2000). As ATP produced by the zooxanthellae cannot be used directly by the coral host, translocated photosynthetates need to be converted to ATP by the host itself through aerobic respiration. Therefore, the light enhancement of calcification due to the translocation of photosynthetates during the day should involve higher respiration rates than during the night.

An increase of respiratory rates under light that could be linked to the enhancement of calcification was observed by Al-Horani et al. (2003) using microsensors. Employing the same technique, higher respiration rates have been found for photosynthetic organisms (Jørgensen et al. 1985), including coral holobionts (Kühl et al. 1995). However, this method requires microsensors with specific characteristics (response time <1 s) that are difficult to acquire. It may also be biased, as the respiration of the holobiont as a whole is measured, even if only the host respiration is of interest. By measuring the ETSAs of the host and zooxanthellae separately, we can assess their potential respiratory rates. This potential can be compared with the actual respiration in the dark, making it possible to determine whether coral hosts exhibit the ETS that is required to increase their respiration rate in the light and if it can therefore explain the phenomenon of light-enhanced calcification. Moreover, higher respiration rates in the light have strong implications for coral physiology, rang-

ing from carbon budgets to calcification. Thus, knowing how much of the organic carbon produced by zooxanthellae is actually consumed by the host through respiration is essential to the understanding of coral carbon and energy budgets. Therefore, it is important to develop new techniques to assess the respiration of the holobiont system in the light and in the dark.

The ETSA technique has been widely used to estimate the respiration of plankton *in situ*, but a ratio or algorithm is required for conversion to *in vivo* respiration. ETSA/respiration (ETSA/R) ratios can be determined for isolated zooxanthellae. It is possible to estimate the amount of respiration to be attributed to zooxanthellae by measuring the zooxanthellae ETSA of a coral holobiont and the associated ETSA/R ratios. Therefore, coral host respiration can also be calculated, which will allow investigation of the relationship between host respiration and calcification.

A protocol suitable for the measurement of the ETSA of the coral holobiont and its different components, i.e. the host and the zooxanthellae, was developed. Using this protocol, we investigated whether the coral host shows higher respiration rates under light that may explain the enhancement of calcification in the light. To test this hypothesis, we compare the ETSA/R ratios of different species of coral hosts. Finally, the link between photosynthesis, ETSA, respiration and calcification is discussed.

MATERIALS AND METHODS

Study species

Colonies of *Galaxea fascicularis*, *Porites cylindrica*, *Pocillopora damicornis*, *Montipora digitata* and massive *Porites* sp. were collected from a coastal region off Okinawa Island, Japan, with permission from the Okinawa Prefecture government (no. 23-7). The colonies were fractionated to obtain small fragments, and were maintained for several months in an outdoor aquarium with running seawater at the Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, Okinawa, Japan. A month before measurements were performed, the coral colonies were transferred to an indoor aquarium with a 12 h: 12 h light:dark cycle. The water in the aquarium (15 l) was replaced every week with fresh seawater, replacing half of the water every 3 to 4 d. The aquarium only contained the specimens (n = 5 to 7) of one species at a time.

Host–zooxanthellae separation, homogenization and enzyme extraction for ETSA assays

To measure ETSA in coral hosts and zooxanthellae, the 2 fractions need to be separated and homogenized to extract the ETS. Both steps need to be conducted while preserving enzyme activity. Finally, the assay conditions have to be adapted to the enzyme properties of the coral host and zooxanthellae. To develop a suitable protocol for corals, colonies of *Galaxea fascicularis* were used.

Separation of the host and zooxanthellae fractions

Coral tissues were removed using an airjet filled with ice-cold 50 mmol l^{-1} phosphate buffer containing 10 g l^{-1} NaCl. The resulting slurry was first slightly homogenized by applying 5 strokes in a Teflon homogenizer. Then, it was centrifuged at 414 × *g* for 20 min to separate the host and zooxanthellae fractions. The pellet containing the zooxanthellae fraction was re-suspended in saline phosphate buffer, and the centrifugation step was repeated 3 times, after which no activity was detected in the supernatant.

Extraction and preparation of the ETS for assays

Cell-free extracts can be obtained through mechanical action, such as bead beating or grinding, or via sonication to break up cells and mitochondria to release enzymes. Both methods were tested. Different durations of sonication were tested (1 to 20 min) with the power of the sonicator (Smurt NR-50 M, Microtec) set at 25% in one fraction each from the host and zooxanthellae. The effects of the presence of the commonly used chemicals polyvinylpyrrolidone K30 (PVP) at 1.5 mg ml^{-1} (Wako Pure Chemical Industries), $MgSO_4$ at 75 µmol l^{-1} (Wako Pure chemical Industries), Triton-X at 0.20% (Triton-X100, Sigma Aldrich) and EDTA 2Na at 10 mmol l^{-1} (Kanto Chemical) were also investigated in both the host and zooxanthellae fractions. The treatment containing PVP at 1.5 mg ml^{-1}, $MgSO_4$ at 75 µmol l^{-1} and Triton X at 0.20%, which is the composition recommended by Packard et al. (1975), was used as the control treatment, and its ETSA was fixed at 100%. The activities obtained under other treatments, omitting PVP, $MgSO_4$ or Triton X or adding EDTA to the buffer used in the control treatment, were expressed as the percentage of activity com-

pared with the control treatment. ANOVA followed by a Dunnett's test was used to assess the differences between the different homogenization buffers. The R software package (R Development Core Team 2011) was used to perform these tests.

After homogenization, the homogenates were cleared via centrifugation at 10 000 × *g* for 5 min. While this step is not required for host fractions, it reduced the turbidity blank of the zooxanthellae-containing fractions, without affecting their activities. All steps were conducted on ice or in a cooling centrifuge at 3°C.

Optimization of the ETSA assay

Substrate concentrations

To measure the maximum enzymatic rate (V_{max}), substrates should be at saturation concentrations. The saturation concentrations reported in the literature range from 0.835 to 1 mmol l^{-1} for NADH, from 0.15 to 0.25 mmol l^{-1} for NADPH and from 0 to 133 mmol l^{-1} for succinate. As these concentrations were adjusted mainly for plankton, investigations of the concentration kinetics of the host and zooxanthellae fractions were performed. All ETSA measurements were carried out within 2 h of homogenization. The optimum concentrations and saturation concentrations of the substrates were investigated in 3 different colonies of the model species *Galaxea fascicularis*. Under the assumption that the reduced form of NADH is the main substrate for mitochondrial ETS, while the reduced form of NADPH is the main substrate for microsomal ETS, we studied their kinetics by adding different concentrations of these substances to the assays. In addition, succinate, an initial electron donor in the mitochondrial ETS, was added to saturated NADH and NADPH concentrations. The tested concentrations were 0–3 mmol l^{-1} NADH (Wako Pure Chemicals), 0–0.3 mmol l^{-1} NADPH (Wako Pure Chemicals) and 0–300 mmol l^{-1} succinate (disodium succinate, anhydrous, Wako Pure Chemicals). The concentration of INT (Wako Pure Chemicals) was 0.4 mg ml^{-1}, as indicated in Packard (1971). Higher and lower INT concentrations were also tested, but the chemical blank increased too greatly at higher concentrations, without a detectable increase in activity. The reactions were stopped using a solution of 50% formalin after 20 min. The absorbance was read immediately at 490 nm in a UV-Vis spectrophotometer (U-2001, Hitachi). The obtained substrate kinetics were fitted against a non-linear model based on the Mickaelis-Menten equation.

Length of incubation and linearity of the reaction

To determine the optimal incubation length, the absorbances in 3 samples were followed continuously. The linearity in function of the activities of the samples was tested in 3 extracts by increasing the volume of extract used in the assay. The tested volume ranged from 0 to 600 µl. For volumes less than 300 µl, the extract was diluted to a volume of 300 µl using saline phosphate buffer. For volumes greater than 300 µl, the volume of buffer used for dilution of the substrates was reduced to maintain the same final assay volume (1.6 ml). The reaction was incubated for 20 min and stopped with 50% formalin (1 ml).

Temperature and pH effects

The effects of pH and temperature were tested in 3 different colonies, one from each of *Galaxea fascicularis*, *Montipora digitata* and *Porites cylindrica*. Incubations were conducted at room temperature (ca. 25°C) and in a temperature-controlled water bath to study the variation in activity with temperature. All solutions were prepared in phosphate buffer (with the exception of INT, which was prepared in MilliQ water) at 50 mmol l^{-1} and adjusted to pH 8.5 or a pH ranging from 7.0 to 9.0 to study the effects of pH. The pH of the phosphate buffer was adjusted using NaOH or HCl. The effect of temperature on the reaction kinetics was investigated in a temperature range from 10°C to 40°C. The variation in activity with temperature was fitted against the Arrhenius law using Qtiplot software (http://soft.proindependent.com/qtiplot.html). The energy of the activation constant (E_a) for the reactions was determined by nonlinear fitting.

Calculation of the ETS activities

The volumes of the substrate solution (containing NADH, NADPH and/or succinate) and cell-free extract used in the protocol were 1 ml and 300 µl, respectively. The reaction was started with 300 µl of INT and stopped with 1 ml of 50% formalin. The increase in absorbance was linear over 20 min at the temperature tested (25°C), and all subsequent samples were therefore incubated for 20 min. The absorbance of each sample was corrected using a turbidity blank (300 µl cell-free extract and 2.3 ml phosphate buffer) and a chemical blank, where the cell-free extract was replaced by a solution of phos-

phate buffer and homogenization buffer, as indicated in Packard (1971). Changes in absorbance were converted into ETSA expressed in mg O_2 h^{-1} mg^{-1} protein using the following formula:

$$\mathrm{ETSA} = \frac{A}{\varepsilon_{490}} \times \frac{V_a}{t} \times \frac{1}{2} \times M \times \frac{D}{V_s C_{prot}} \quad (1)$$

where A is the corrected absorbance at 490 nm; ε_{490} is the molar extinction coefficient, which is 15.9 l $mmol^{-1}$ cm^{-1} in 0.16% Triton X (Kenner & Ahmed 1975a); t is the incubation time of the assay in hours (here t = 1/3 h); V_a is the final volume of the assay (V_a = 2.6 ml); M is the molecular mass of O_2 (M = 32); D is the dilution applied to the sample; V_s is the volume of sample used in the assay (V_s = 0.3 ml); and C_{prot} is the concentration in protein of the homogenate in mg ml^{-1}. A fraction of 1/2 is required to convert the reduction of INT into O_2, as 2 moles of INT is electrochemically equivalent to 1 mole of O_2. For our assay, the formula becomes:

$$\mathrm{ETSA} = 7.85 \times A \times \frac{D}{V_s C_{prot}} \quad (2)$$

Application of the ETSA assay and metabolic measurements

Respiration rates and ETSAs of isolated zooxanthellae

Colonies of *Galaxea fascicularis*, *Porites cylindrica*, *Pocillopora damicornis*, *Montipora digitata* and massive *Porites* sp. were used in this experiment. The respiration rates and ETSAs were measured in isolated zooxanthellae from 2 colonies of each species (n = 10). For respiration measurements, zooxanthellae were isolated from a coral tissue slurry obtained from half a colony using an airjet with filtered seawater. After 3 consecutive washes via repeated centrifugation/re-suspension in filtered seawater, approximately 5 ml of the obtained zooxanthellae suspension was placed in a stirred, airtight micro-chamber fixed on a luminescence-based dissolved oxygen (RDO) probe (Orion 4 stars, Thermo Scientific). The suspension was incubated for a minimum of 30 min, or until dissolved oxygen reached ~9 mg l^{-1}, under white LED light at 50 µmol photons s^{-1} m^{-2}. The suspension was then placed in the dark. Respiration was calculated from the consumption of dissolved oxygen during the first 20 min in the dark. Zooxanthellae abundances were determined by counting individual cells in a hemocytometer. Respiration rates were expressed in mg O_2 h^{-1} $zoox^{-1}$.

To measure the ETSA per zooxanthellae, tissues from the remaining halves of the colonies were removed using an airjet with saline phosphate buffer. The ETSA and zooxanthellae abundances in this slurry were determined using the optimized methods. The ETSAs were expressed in mg O_2 h^{-1} $zoox^{-1}$. These activities and the respiration rates measured in the micro-chamber were used to calculate the ETSA/R ratio for the zooxanthellae. The average of all samples (n = 10) was used in later analyses.

Measurement of the metabolic rates of whole colonies

Colonies of *Galaxea fascicularis*, *Porites cylindrica*, *Pocillopora damicornis*, *Montipora digitata* and massive *Porites* sp. were incubated in freshly collected seawater at Sesoko Station, Okinawa, Japan, at room temperature (ca. 25°C) for 3 h in light (metal halides, 150 µmol photons s^{-1} m^{-2}, measured with a LiCor quantum sensor) and 2 h in the dark in individual vessels. The metabolic rates in terms of photosynthesis, dark respiration and calcification were measured for each colony. The dissolved oxygen and pH were measured at the beginning and end of each incubation using an Orion 4-Star pH-DO sensor equipped with an RDO probe and a pH electrode (8156 BNUWP, Thermo Scientific) calibrated to a seawater scale. Sub-samples of the incubation water were sampled at the beginning and end of each incubation period and filtered through a 0.45 µm membrane filter to measure total alkalinity. Total alkalinity was determined via titration with HCl at 0.1 mol l^{-1} with a Metrohm titrator (785 DMP titrino). Calcification rates were calculated using the alkalinity anomaly method (Gattuso et al. 1996), and photosynthesis and respiration were calculated based on the variation in dissolved oxygen during the light and dark incubations, respectively. The photosynthesis rates presented here are therefore net photosynthesis rates. The protein contents and zooxanthellae densities were determined via the Bradford method and by counting cells in the tissue slurry using a hemocytometer (Neubauer modified), respectively. The ETSAs of the total and the zooxanthellae fractions were measured using the optimized protocol.

Calculations and expression of the results

Coral metabolic rates, including those associated with photosynthesis, calcification, total respiration (dark) and zooxanthellae, were expressed relative to

milligrams of host protein. ETSAs were expressed in mg O_2 h^{-1} mg^{-1} protein, whether for the host or zooxanthellae, as shown in Eq. (1). The host ETSA was calculated as the difference between the holobiont ETSA (before zooxanthellae separation) and the zooxanthellae ETSA, as the recovery of the host ETSA after separation via centrifugation was low. The zooxanthellae ETSA of the whole colony was calculated. Then, the zooxanthellae ETSA was converted to zooxanthellae respiration, the total O_2 consumption of the zooxanthellae, using the average of the ETSA/R ratios for all species (2 specimens per species) obtained in the experiment with isolated zooxanthellae. Host respiration was calculated from the difference between the zooxanthellae respiration and the O_2 consumption in the dark for the whole colony measured during the incubation of the corals. The correlations between the different metabolic rates and parameters were determined via the least-squares methods.

RESULTS

ETS extraction: homogenization technique and buffer

Several methodological tests were conducted to optimize the homogenization procedure. Bead beating was compared with sonication, but the obtained ETSA recovery was only 20%, and this method was soon discarded. Different sonication durations were tested for both the host and zooxanthellae fractions. The power of the sonicator was adjusted to 25% when the volume of homogenate was 5 ml because under a higher power foam formed, which may reduce the measured activity (Packard et al. 1975). Maximum zooxanthellae activity was obtained after 2 min of sonication and decreased with time (Fig. 1A). Host activity reached a maximum after 5 min. As the difference in the zooxanthellae activity after 2 and 5 min was small, a sonication length of 5 min was chosen for both the zooxanthellae and host fractions.

The tests using different extraction buffers revealed that both the host and zooxanthellae ETSAs were significantly decreased when PVP was omitted from the homogenization buffer ($p = 0.0254$ and 0.0387, respectively, $n = 3$, Dunnett's test, omission of PVP vs. control; Fig. 1B). The addition of EDTA at a final concentration of 10 mmol l^{-1} did not affect the zooxanthellae ETSA ($p > 0.05$, $n = 3$, Dunnett's test, addition of EDTA vs. control), but host activity was significantly increased ($p = 0.0142$, $n = 3$, Dunnett's test, addition of EDTA vs. control). The other treatments (omission of $MgSO_4$ and omission of Triton X) did not affect the activities of either component when compared with the control solution ($p > 0.05$, $n = 3$, Dunnett test).

Optimal assay conditions

Substrate concentrations, incubation length and linearity of the assay

The substrate kinetics of both the host and zooxanthellae ETSs follow Michaelis-type kinetics with regard to NADH, showing a linear increase of the velocity as a function of the NADH concentration for the lowest concentrations and reaching a plateau at concentrations higher than 1 mmol l^{-1} (Fig. 2). Hence, the kinetics were fitted using the Michaelis-

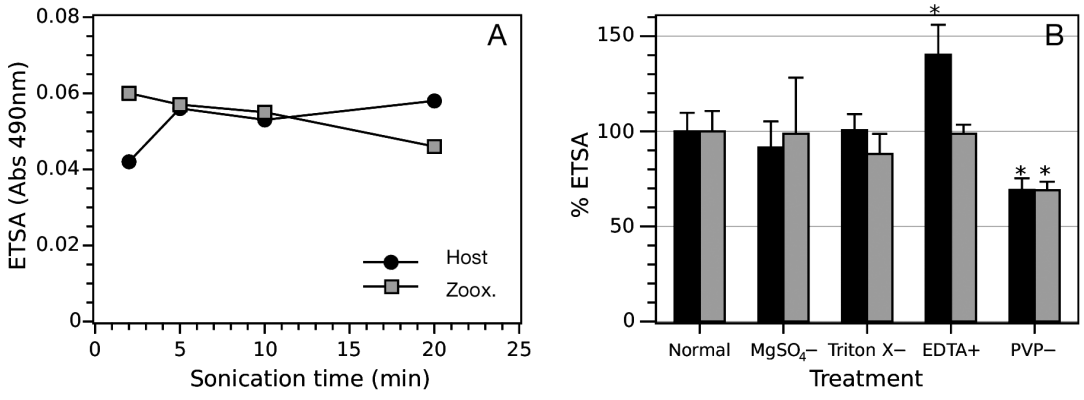

Fig. 1. (A) Variations in ETSA as a function of sonication time for the host and zooxanthellae fractions. (B) Effect of the addition of different components to the homogenization buffer on the host (black bars) and the zooxanthellae (grey bars) fractions. Normal indicates the control treatment, in which $MgSO_4$, PVP, and Triton X are present. $MgSO_4$–, Triton X–, and PVP– indicate the treatments where each of the compounds was omitted, and EDTA+ represents the treatment where 10 mmol l^{-1} EDTA was added to the normal buffer. Error bars indicate SD ($n = 3$). Asterisk indicates a significant difference compared to the normal treatment (Dunnett's test, $n = 3$, $p < 0.05$)

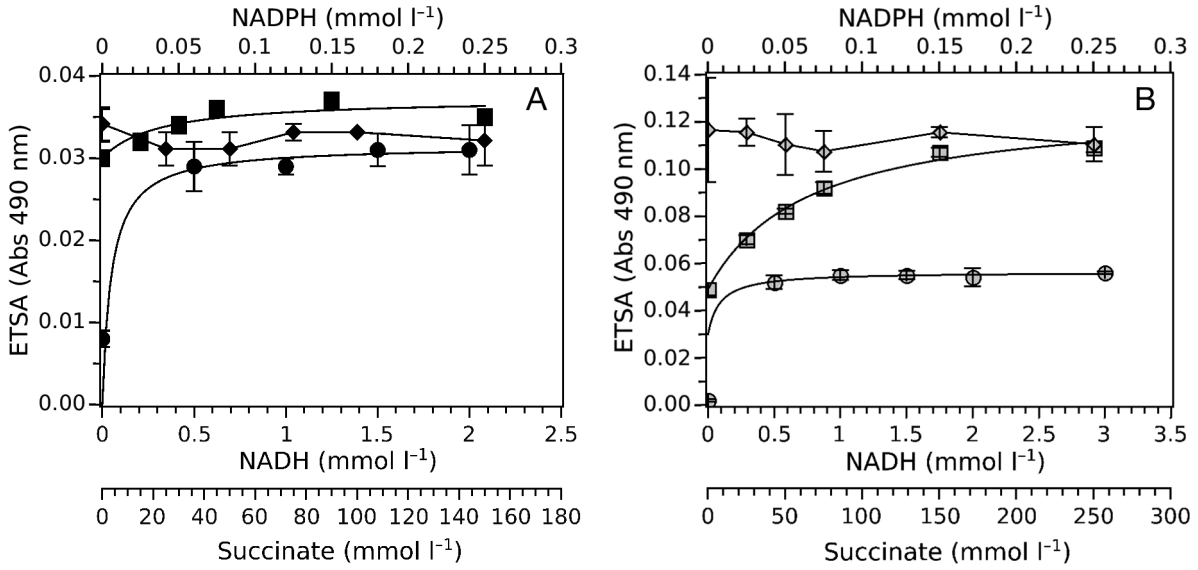

Fig. 2. Substrate kinetics of the ETS of (A) the coral host and (B) zooxanthellae. NADH (circles) and NADPH (squares) curves were fitted using the Michaelis-Menten equation. Succinate symbols (diamonds) are connected with straight lines. Error bars indicate SD (n = 3). NADPH was added to a saturated NADH concentration (2 mmol l^{-1}) and succinate to saturated NADH and NADPH concentrations (2 mmol l^{-1} and 0.15 mmol l^{-1}, respectively)

Menten equation, and the characteristics of the enzyme, apparent Michaelis constant (K_m) and V_{max} were calculated. The apparent K_m for NADH in the host fraction was 0.049 mmol l^{-1}, and the minimum saturated concentration was estimated to be 1.5 mmol l^{-1}. The zooxanthellae fraction exhibited a lower apparent K_m of 0.038 mmol l^{-1}, and a 1 mmol l^{-1} concentration could be considered saturated. The addition of 0.15 mmol l^{-1} NADPH to a saturated concentration of NADH (1.5 mmol l^{-1}) more than doubled the zooxanthellae activity, increasing it by 118% compared with using NADH alone. The addition of 0.15 mmol l^{-1} NADPH increased the host ETSA by only 20%. The addition of succinate did not increase the ETSA in either fraction, and the ETSA was slightly reduced in the host fraction at the highest succinate concentrations. According to these results, the final substrate mixture used contained NADH at 1.5 mmol l^{-1} and NADPH at 0.15 mmol l^{-1}.

At these concentrations, the reactions were linear for 20 min. The measured activity was proportional to the amount of homogenate (r^2 = 0.992, $p < 0.01$ and r^2 = 0.999, $p < 0.01$ for the host and zooxanthellae, respectively; Fig. 3).

Temperature and pH

The ETSA at 25°C was fixed as 100% for all specimens, and other activities were expressed as the percentage of the activity at 25°C. The activity showed an exponential increase with temperature until 40°C, which was the maximum temperature tested. The variation in activity with temperature fits the Arrhenius law well, with r^2 = 0.9508 for the host fraction and r^2 = 0.9149 for the zooxanthellae (Fig. 4A). The host ETS presented an E_a of 47.2 kJ mol^{-1}, and the zooxanthellae constant was 30.3 kJ mol^{-1}. The optimal pH was found to be approximately 8.5 for both the host and the zooxanthellae fractions, and ETSA was therefore fixed as 100% at pH 8.5. The activity of the ETS increased at pH levels between 7.5 and 8.5 for both the host and zooxanthellae fractions. It

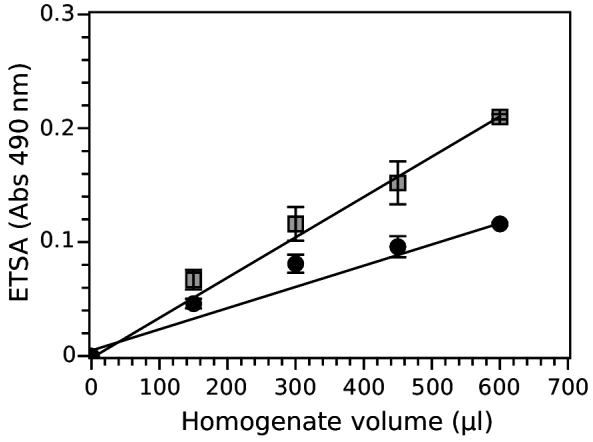

Fig. 3. Increase in ETSA of the xooxanthellae (grey squares) and host (black circles) fractions as a function of the volume of homogenate used. Straight lines represent the best fit by the least square methods for volumes ranging between 0 and 600 µl. Error bars indicate SD (n = 3)

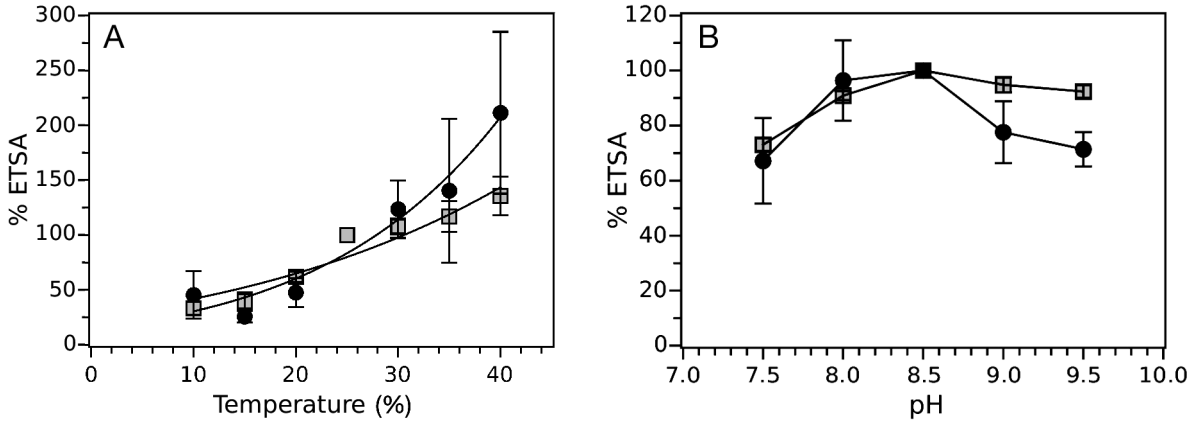

Fig. 4. Variations in ETSA as a function of (A) temperature and (B) pH for zooxanthellae (grey squares) and host (black circles) fractions. Error bars indicate SD (n = 3). Temperature curves were fitted using the Arrhenius law, and pH values are connected with straight lines

decreased at the higher pH levels tested, up to 9.5, especially for the host fraction (Fig. 4B).

Application to coral colonies

Respiration and ETSA of isolated zooxanthellae

The respiration rates of freshly isolated zooxanthellae were only measurable in filtered seawater. Zooxanthellae isolated in phosphate buffer did not show any detectable O_2 consumption. Therefore, ETSAs and respiration were measured in 2 fragments from each of the examined coral colonies. The respiration measurements showed an almost linear decrease in oxygen during the initial 20 min after darkening (Fig. 5). The slope of the decrease was used as the respiration rate. The respiration rates ranged between 3.3 and 9.4 ng O_2 h^{-1} 1000 $zoox^{-1}$ among all coral species (n = 10). The ETSA per zooxanthellae ranged from 25.8 to 35.5 ng O_2 h^{-1} 1000 $zoox^{-1}$ (n = 10). The resulting ETSA/R ratio was 5.2 ± 1.3 (n=10) on average and ranged between 3.7 and 7.8, with the maximum ratio obtained for zooxanthellae isolated from *Galaxea fascicularis* and the minimum for zooxanthellae isolated from *Pocillopora damicornis* (Table 1).

ETSA and metabolism of coral holobionts

Because the water temperatures during the respiration measurements and ETSA assays were the same (25°C), no temperature correction was needed. The zooxanthellae exhibited a significantly higher ETSA per mg of protein than the hosts, with values ranging from 5.41 ± 0.66 to 12.37 ± 2.40 mg O_2 h^{-1} mg^{-1} zoox protein, compared with 0.88 ± 0.18 to 4.68 ± 0.67 mg O_2 h^{-1} mg^{-1} host protein for the host. For calculation of zooxanthellae respiration, we used an ETSA/R of 5.2, the average for all species examined in the isolated zooxanthellae experiment. Zooxanthellae respiration accounted for less than 40% of the total respiration, with the exception of *Pocillopora damicornis*, for which zooxanthellae respiration represented 54% of the total respiration. Host respiration, or the O_2 consumption accounted for by the host, ranged from 0.26 ± 0.06 (*P. damicornis*) to 1.39 ± 0.23 mg O_2 h^{-1} mg^{-1} host protein (*Galaxea fascicularis*) (Table 2). The host species *G. fascicularis*,

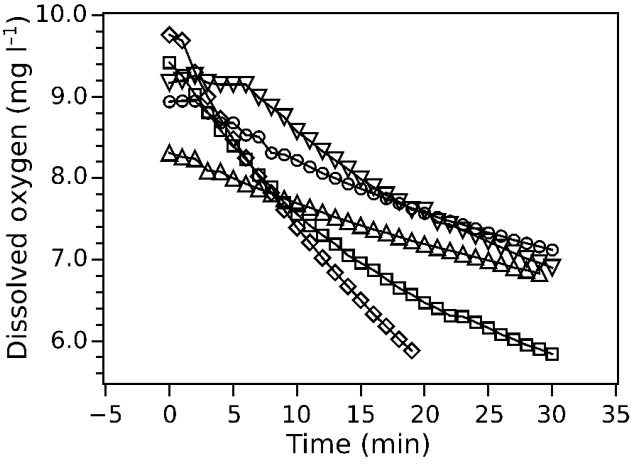

Fig. 5. Representative variations in the dissolved oxygen concentration recorded for zooxanthellae suspensions incubated in the dark in an air-tight micro-chamber; 0 min indicates the initiation of the dark incubation. Zooxanthellae were isolated from *Montipora digitata* (squares; 1.6 × 10^6 cells ml^{-1}), *Pocillopora damicornis* (diamonds; 1.3 × 10^6 cells ml^{-1}), *Porites cylindrica* (inverted triangles; 0.5 × 10^6 cells ml^{-1}), *Galaxea fascicularis* (circles; 1.2 × 10^6 cells ml^{-1}) and massive *Porites* sp. (triangles; 0.8 × 10^6 cells ml^{-1})

Table 1. Respiration rate (R; ng O_2 h^{-1} 1000 $zoox^{-1}$) and ETSA of zooxanthellae isolated from 5 different coral species; 2 specimens per species

Species	R	ETSA	ETSA/R
Galaxea fascicularis	7.66	39.3	5.13
	3.32	25.9	7.80
Porites cylindrica	5.67	35.5	6.26
	3.99	23.0	5.77
Pocillopora damicornis	9.38	34.6	3.69
	6.23	29.4	4.72
Montipora digitata	6.39	36.9	5.77
	4.83	28.2	5.83
Massive *Porites* sp.	6.35	33.9	5.34
	6.80	26.1	3.83
Mean ± SD	6.06 ± 1.70	31.3 ± 4.9	5.2 ± 1.3

Porites cylindrica and *P. damicornis* showed ETSA/R ratios of 3.53 ± 0.57, 2.56 ± 0.60 and 3.43 ± 0.53, respectively, whereas *Montipora digitata* and massive *Porites* sp. exhibited ETSA/R ratios closer to 1, at 1.67 ± 0.33 and 1.38 ± 0.20, respectively (Fig. 6).

The average metabolic rates for each species are shown in Table 3. Light calcification ranged from 6.89 ± 0.66 (*Pocillopora damicornis*) to 34.98 ± 8.54 µmol $CaCO_3$ h^{-1} mg^{-1} protein (massive *Porites* sp.) and dark calcification from 3.70 ± 0.53 (*P. damicornis*) to 21.35 ± 6.05 µmol $CaCO_3$ h^{-1} mg^{-1} protein (massive *Porites* sp.) for all species. Photosynthesis rates ranged from 0.24 ± 0.07 (*P. damicornis*) to 1.07 ± 0.24 mg O_2 h^{-1} mg^{-1} protein (*Galaxea fascicularis*), while respiration rates ranged from 0.57 ± 0.08 (*P. damicornis*) to 1.39 ± 0.23 mg O_2 h^{-1} mg^{-1} protein (*G. fascicularis*). The zooxanthellae density, expressed per mg of host protein, varied among species, with the highest density of 15.99 ± 1.57 zoox mg^{-1} protein found for *G. fascicularis* and the lowest of 4.04 ± 0.63 zoox mg^{-1} protein observed for *P. damicornis*.

Comparing host respiration rates with other metabolic rates, strong correlations were found between respiration and photosynthesis ($r^2 = 0.8454$, $p < 0.01$) and respiration and light calcification ($r^2 = 0.7399$, $p < 0.01$), and weaker correlations were detected between respiration and zooxanthellae density ($r^2 = 0.4916$, $p < 0.01$) and respiration and dark calcification ($r^2 = 0.4947$, $p < 0.01$; Fig. 7). Regarding the host ETSA and its correlation with other metabolic rates, the strongest correlation was found between ETSA and zooxanthellae ($r^2 = 0.7320$, $p < 0.01$). A weak correlation between photosynthesis and the host ETSA was also found ($r^2 = 0.4687$, $p < 0.01$). However, calcification (in the light or dark) was not correlated with the host ETSA ($r^2 = 0.2098$, $p < 0.05$ and $r^2 = 0.0666$, $p = 0.21$, respectively; Fig. 8).

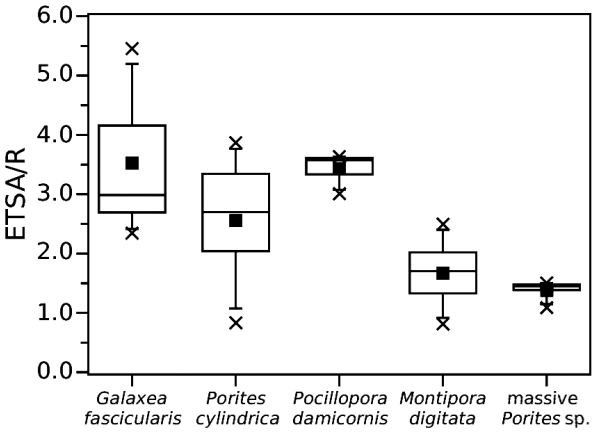

Fig. 6. Box plots of the ETSA/R ratios for the different host species tested. Black squares indicate the average values (n = 5); whiskers represent the 5–95% CIs, × the minimum and maximum, and the boxes the 25–75% CIs

DISCUSSION

ETSA methodology

After a series of tests, it was possible to identify the optimal procedure for performing ETSA measurements in symbiotic corals. Separation of the host and zooxanthellae fractions was achieved via homogenization using a hand-manipulated Teflon homogenizer and centrifugation. Using this method, the zooxanthellae were released from the host cells. Live mitochondria are usually isolated from animal tissue using a rotor-manipulated homogenizer (Pallotti & Lenaz 2001). Therefore, we assumed that host mitochondria were not disrupted in this first homogenization step.

Table 2. Mean ± SE ETSA (mg O_2 h^{-1} mg^{-1} protein) and calculated respiration rates (R) (mg O_2 h^{-1} mg^{-1} protein) of the host and the zooxanthellae for each coral species (5 replicates per species)

Species	Host ETSA	Host R	Zoox. ETSA	Contribution of zoox. to total R (%)
Galaxea fascicularis	4.68 ± 0.67	1.39 ± 0.23	5.41 ± 0.66	28 ± 3
Porites cylindrica	1.63 ± 0.26	0.76 ± 0.18	6.77 ± 1.38	38 ± 6
Pocillopora damicornis	0.88 ± 0.18	0.26 ± 0.06	12.37 ± 2.40	54 ± 7
Montipora digitata	1.11 ± 0.23	0.77 ± 0.21	9.75 ± 1.79	28 ± 5
Massive *Porites* sp.	1.78 ± 0.40	1.28 ± 0.26	8.48 ± 1.42	20 ± 3

Table 3. Mean ± SE metabolic rates of the coral colonies during incubation

Species	Calcification (μmol $CaCO_3$ h^{-1} mg^1 protein) Light	Dark	Photosynthesis (μmol O_2 h^{-1} mg^{-1} protein)	Respiration (μmol O_2 h^{-1} mg^{-1} protein)	Zooxanthellae density (10^7 cell mg^{-1} protein)
Galaxea fascicularis	29.27 ± 2.99	8.63 ± 2.69	1.07 ± 0.24	1.39 ± 0.23	15.99 ± 1.57
Porites cylindrica	18.09 ± 6.28	7.66 ± 1.32	0.59 ± 0.16	1.18 ± 0.21	6.93 ± 1.29
Pocillopora damicornis	6.89 ± 0.66	3.70 ± 0.53	0.24 ± 0.07	0.57 ± 0.08	4.04 ± 0.63
Montipora digitata	19.29 ± 5.12	6.52 ± 1.71	0.50 ± 0.16	1.07 ± 0.30	5.60 ± 1.30
Massive *Porites* sp.	34.98 ± 8.54	21.35 ± 6.05	0.93 ± 0.22	1.60 ± 0.30	10.61 ± 2.34

Sonication produced better results in terms of ETSA recovery than mechanical homogenization, similar to previous results (Broberg 1985). The homogenization buffer used in this study differs from that described in the original methods mainly due to the addition of 10 mmol l^{-1} EDTA. The specific effect of EDTA on host activity is not yet understood, though it may be caused by a greater amount of divalent cations (e.g. Ca^{2+}, metals) in the host fraction (than in the zooxanthellae fraction) interfering with the assay. Divalent cations are known co-factors of phospholipase, which may damage the isolated ETS (Pallotti & Lenaz 2001). Therefore, the final homogenization buffer chosen consisted of 1.5 mg ml^{-1} PVP, 75 mmol l^{-1} $MgSO_4$, 10 mmol l^{-1} EDTA and 10 g l^{-1} NaCl in phosphate buffer at 50 mmol l^{-1}, adjusted to pH 8.5.

For this study, the substrate concentrations required to reach saturation of the ETS differed slightly from previously reported concentrations. The addition of NADPH strongly increased the ETSA of the zooxanthellae. This result can be interpreted in different ways. First, the zooxanthellae presented a higher microsomal ETSA, which utilizes NADPH, than did the hosts, thus indicating that the microsomal ETS is a major site of energy conversion and oxygen consumption in the zooxanthellae. High microsomal ETSAs have also been shown for the unicellular green alga *Dunaliellae tertiolecta* (Kenner & Ahmed 1975a). Second, the zooxanthellae fraction exhibited high transhydrogenase activity, which transfers electrons from NADPH and NADH. However, because NADH was already at saturation, this interpretation can be re-

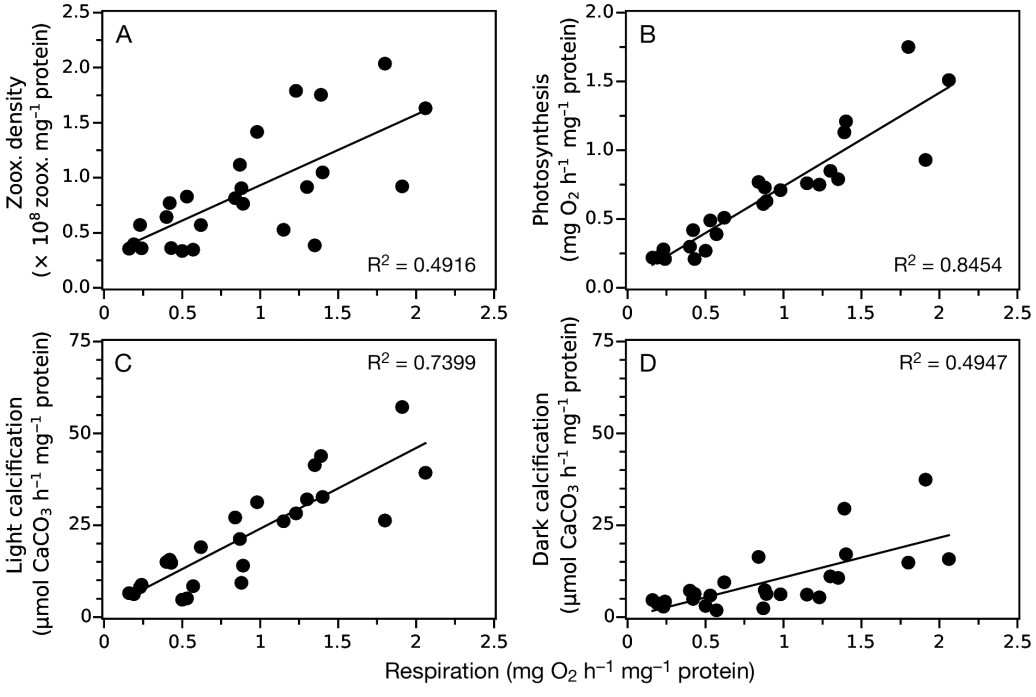

Fig. 7. Correlation of host respiration with (A) zooxanthellae density, (B) photosynthesis, (C) light calcification and (D) dark calcification. Each point represents the values for one specimen of either *Galaxea fascicularis*, *Porites cylindrica*, *Pocillopora damicornis*, *Montipora digitata* or the massive *Porites* sp. Straight lines indicate the linear regression by the least square method. The R^2 for each regression is indicated on the respective plot

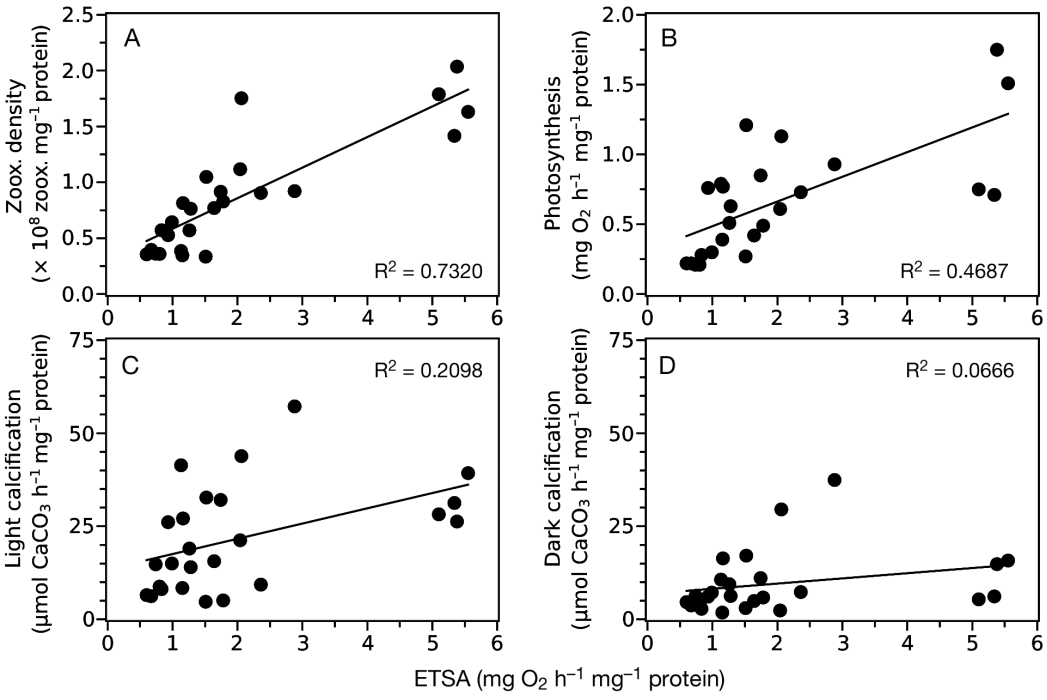

Fig. 8. Correlation of ETSA with (A) zooxanthellae density, (B) photosynthesis, (C) light calcification and (D) dark calcification. Each point represents the values for one specimen of either *Galaxea fascicularis*, *Porites cylindrica*, *Pocillopora damicornis*, *Montipora digitata* or the massive *Porites* sp. Straight lines represent the linear regression by the least square method. The R^2 for each regression is indicated on the respective plot

jected. Third, the zooxanthellae mitochondrial ETS can also use NADPH as an electron donor (Savenkoff et al. 1995a). The addition of succinate did not increase the measured ETSA in either fraction, and even had a small inhibitory effect. A similar impact has been observed on the ETSA of *Daphnia* (Owens & King 1975) and zooplankton (Simičič & Brancelj 2004), even though a positive effect was shown in the original protocol (Packard 1971). Therefore, the selected substrate mixture was NADH at 1.5 mmol l^{-1} and NADPH at 0.15 mmol l^{-1}. The activity was proportional to the volume of homogenates used in the assay up to 600 µl with a 20 min incubation. For samples with very high activity, the reaction may be become substrate limited, and a lower sample volume than the chosen volume of 300 µl or shorter incubation time should therefore be considered in such a case.

An optimum pH of 8.5 was identified. The same optimum pH has been found for other organisms (Kenner & Ahmed 1975a, Christensen & Packard 1979), which can be explained by the similar pH dynamics observed in the mitochondria across a variety of organisms and their general alkaline character (Takahashi et al. 2001, Balut et al. 2008, Orij et al. 2009). Although the buffering capacity of phosphate buffer is weak at pH 8.5, the measurements of Owens & King (1975) and our own measurements showed very little variation in pH within the assay mixture, with pH differences of <0.1 being recorded.

Across the range of temperatures inhabited by scleractinian corals, from 20 to 35°C, activity increased with temperature. Simičič & Brancelj (2004) showed maximum ETSA of different *Daphnia* hybrids at temperatures corresponding to their naturally occurring temperature range. The dependence of the ETSA on temperature may explain the observed increase in respiration with temperature for some species (Edmunds 2004, Colombo-Pallotta et al. 2010). However, these studies only showed the effects of temperature on ETSA during the assay, and caution should be used when interpreting results from temperature stress experiments, where corals are incubated for long periods at elevated temperatures (>30°C), and damage to the mitochondrial ETS may occur (Moller 2001, Taylor et al. 2004).

The obtained E_a values were 47.2 and 30.3 kJ mol^{-1} for the host and zooxanthellae, respectively, which are lower than those previously reported for zooplankton of 65.9 kJ mol^{-1} (Packard et al. 1975) and 57.6 kJ mol^{-1} (Simičič & Brancelj 2004). The temperature chosen for our assay was 25°C. At this temperature, we were able to obtain a strong signal, and the reaction remained linear for 20 min. Higher or lower temperatures may be used, but the linearity of the

reaction should be checked and the incubation time modified accordingly. The E_a presented here can be employed to correct the ETSA using the Arrhenius law (Packard et al. 1975). This can be particularly useful for estimating ETSA in the field when the temperature at which the assay is performed differs from the *in situ* temperature.

Respiration and ETSA of isolated zooxanthellae

The ETSA/R ratios were 5.2 on average for the zooxanthellae. This ratio falls within the ETSA/R range of 5.6 to 9.4 reported for 10 species of phytoplankton (Kenner & Ahmed 1975b), and is higher than those reported for zooplankton, which range between 1.0 and 2.0 (Hernández-Leó & Gómez 1996). The high ETSA/R ratio of phytoplankton suggests that photosynthetic organisms do not exploit their full respiratory potential in the dark, due to substrate limitation (Peñuelas et al. 1988). Higher respiration rates for phytoplankton and corals have been shown previously using light/dark shift methods with microsensors (Jørgensen et al. 1985, Kühl et al. 1995, Al-Horani et al. 2003). Our results support the idea that measuring the respiration rates of the zooxanthellae based on O_2 consumption in the dark may have led to underestimation of the daytime respiration rates of zooxanthellae and, consequently, of the coral holobiont.

ETSA/R ratio and host respiration

An ETSA/R ratio of 1 is the theoretical minimum value (Packard & Gómez 2008). Considerable intraspecific and interspecific variation in the ETSA/R ratio has been observed for coral hosts. The higher ratios reported here may be due to lower-than-optimal respiration rates. Indeed, coral respiration depends on numerous factors, and sub-optimal conditions during measurement, such as confinement stress, limited availability of food, low light conditions and therefore low photosynthesis rates, or conditions to which corals are not acclimated, may decrease measured respiration rates. In such cases, the measured respiration rates will be lower than the potential rates represented by the ETSA, as the limiting factor for respiration is not the number of ETS chains present, but the availability of the substrate (NADH). Therefore, we suggest that the high ratios observed in some specimens of the different species tested were due to respiration in the dark being substrate limited in our experiment. The ratios measured under optimal conditions may not differ significantly among species, and as respiration is not limited, coral hosts may show an ETSA/R ratio closer to 1, as was the case for some of our specimens, especially in the massive *Porites* sp. and *Montipora digitata*.

The ETSA/R ratios reported here show that the respiration rates of hosts in the dark could theoretically increase in light by a factor ranging from 1.38 to 3.53, with an average of 2.5. A ratio close to 1, as was found for some specimens, indicates that the coral hosts were reaching their maximum respiration rates in the dark. Therefore, an increase in respiration, which would increase the amount of energy available under light conditions, would not be possible.

Due to the small amount of zooxanthellae compared with host tissue and/or their high ETSA/R ratios, zooxanthellae respiration represents less than half of the total respiration in the dark. If zooxanthellae increase their respiration rates by a factor of 5.2 in light, as shown by their ETSA/R ratios, the amount of respiration contributed by the zooxanthellae may become higher than the amount attributed to the host. Taken together, if the zooxanthellae increase their respiration by a factor of 5.2 in the light and the host by factor of 2.5, the total holobiont respiration would increase by a factor of 3.4. This maximum possible increase in the respiration of the holobiont predicted by ETSA/R is lower than that reported from microsensor studies, which have shown an increase of respiration by a factor of 7 to 12 for whole corals (Kühl et al. 1995, Al-Horani et al. 2003). The higher respiration in light recorded using microsensors may be due to the extreme location specificity of such measurements, which measure variation in oxygen only in the most external tissues (oral layer), where zooxanthellae are present in high numbers compared with deeper tissue. However, the reported increase of 7 to 12 times is higher than the ETSA/R ratio of the zooxanthellae. Further investigation of the increased respiration observed under light in the coral holobiont is still required.

ETSA was correlated with the zooxanthellae density. This result suggests an adaptation of the potential respiration rate to the density of zooxanthellae. The translocation of carbon from the zooxanthellae represents up to 90% of the total carbon requirement of corals (Edmunds & Davies 1986, Leletkin 2000). Thus, organic matter produced by the zooxanthellae serves as the main substrate source for respiration. Therefore, the zooxanthellae density constrains the amount of substrate available for host respiration. Consequently, we can hypothesize that the amount of ETS in corals is adapted to the amount of substrate

available and therefore to the zooxanthellae density. The ETSAs measured for micro-picoplankton in the open ocean have been found to be correlated with the amount of organic carbon available for respiration (Kenner & Ahmed 1975b, Savenkoff et al. 1995b, Lefevre et al. 1996, Arístegui et al. 2005) and deep-ocean ETSAs can be equated with new production in surface waters (Packard et al. 1988). Similarly, in the case of symbiotic corals, the host ETSA may be equivalent to the rate of carbon translocation from the zooxanthellae to the host.

ETSA, respiration and calcification

In the present study, host respiration was correlated with calcification both in the light and in the dark. This is not unexpected because host respiration is the source of ATP, which is used, in part, for calcification (Allemand et al. 2004). Energy in the form of ATP is required for up-regulation of the pH at the calcification site, which is essential for the formation of aragonite. Direct measurements with microsensors have indicated pH values at the calcification site as high as 9.3 (Al-Horani et al. 2003), which was recently confirmed using boron isotopes in the skeleton as a proxy (McCulloch et al. 2012) and live imaging of the subcallicoblast pH (Venn et al. 2009, 2011).

In our experiment, calcification was enhanced in light. As discussed earlier, an increase in host respiration under light may not be possible, and the mechanism involving the titration of H^+, pumped into the coelenteron by OH^- produced from photosynthesis (Furla et al. 2000), may therefore be the main factor enhancing calcification under light. ETSA was not correlated with calcification, and the ETSA/R ratios were not correlated with the ratio between light and dark calcification. As the high ETSA/R ratio obtained suggests that host respiration, and therefore energy production, was substrate limited, the amount of ETS enzymes available was not the limiting factor. Under optimal conditions, where host respiration increases up to the potential respiration, calcification and ETSA may be correlated.

The typical relationship observed between photosynthesis and calcification may be indirect. The photosynthetic rate limits the host respiration rate through the supply of the required substrate. Consequently, the production of ATP is limited, which in turn, limits the Ca-ATPase activity and the pH up-regulation required for calcification. An alternative substrate source for respiration may be the organic matter ingested and digested by the coral, i.e. heterotrophy. The calcification rates of fed corals are higher than those of starved corals, showing the importance of heterotrophy for coral growth (Ferrier-Pagès et al. 2003, Houlbrèque et al. 2003, Houlbrèque & Ferrier-Pagès 2009). Dark calcification has been reported to be especially simulated by feeding compared with light calcification (Houlbrèque et al. 2004). Heterotrophy provides the substrate required for respiration, especially in the dark, increasing the amount of ATP that can be produced by the ETS and used for calcification. It also provides the substrate essential for the construction of the organic matrix.

Different parameters, such as light, food availability and temperature, may influence the availability of the substrate for host respiration and/or the rate at which O_2 and therefore ATP is produced, limiting

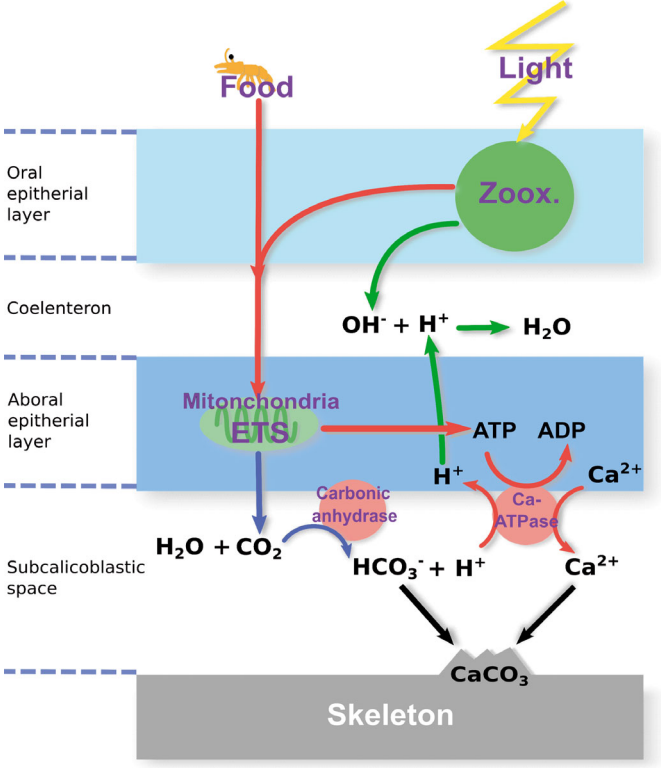

Fig. 9. Calcification model based on Allemand et al. (2004). Substrate limitation of calcification is shown by the red arrows: the calcification rate is limited by the availability of the substrate for respiration, and the substrate may be acquired via heterotrophy or translocation from the zooxanthellae. Thus, the respiration rate determines the amount of energy produced by the mitochondrial ETS and, in turn, the amount of ATP available. This limits the activity of Ca-ATPase, which regulates the pH in the subcalicoblastic space. Light enhancement of calcification (green arrows) is due to the titration of protons in the coelenteron by OH^- produced from photosynthesis. The blue arrows represent the CO_2 supplied for calcification by respiration. CO_2 is then converted to HCO_3^- by carbonic anhydrase. Black arrows represent the precipitation of calcium carbonates

calcification. If host respiration is not limited by substrate availability, it will be limited by the ETSA, which will determine the energy available for calcification. Thus, we propose a 'respiration-limited calcification model' based on the model described in Allemand et al. (2004) and the trans-calcification model of McConnaughey (1997). In our model, illustrated in Fig. 9, respiration occupies a central region, as it determines calcification rates, and it furthermore produces CO_2, which is then converted into carbonate by carbonic anhydrase and used for calcification (Moya et al. 2008). In this model, the enhancement of calcification by light is only due to the increase in OH^- in the coelenteron, as an increase in host respiration under light may not be possible due to the low ETSA/R ratio exhibited by coral hosts.

The proposed method for the measurement of ETSA in symbiotic corals provides a new and useful tool for the study of coral physiology. The obtained ETSA/R values showed that the zooxanthellae may increase their respiration in light by a factor of 5.2, while that of the coral host may increase by a factor of 2.5. Some host specimens showed an ETSA/R ratio of 1, suggesting that the higher ETSA/R values observed for other specimens may be due to respiration being limited by a factor other than the ETS at the time of measurement. Further research will be needed to determine whether the hosts can show increased respiration in light, but our results suggest that an increase in host respiration in light may not explain the enhancement of calcification in light. We demonstrated that host respiration is correlated with calcification. Taken together, our results suggest that host respiration, limited by substrate availability or the ETSA, determines calcification rates.

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