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NOTE

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Normalizing physiological data for scleractinian corals

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Introduction

Increasing body size exposes the structural, functional, and geometric constraints of phenotype design (Schmidt-Nielsen 1984) which result in scaling effects that have a pervasive and profound influence on organismic physiology (Gould 1966; Schmidt-Nielsen 1984). In experimental research, the confounding effects of size (i.e., scaling) can be minimized by comparing organisms of similar size, or by comparing organisms of different size after normalizing the results to a metric that reflects size. Size normalization assumes a proportional relationship (i.e., isometry) between size and the trait of interest (Packard and Boardman 1988), yet the assumption of isometry is rarely demonstrated and most traits scale disproportionately with size (i.e., allometry). Isometry characterizes only a small number of taxa, and includes the colonial modular organisms like bryozoans (Hughes and Hughes 1986) and, theoretically, the scleractinian corals (Jackson 1979; Sebens 1987).

Corals present a number of problems for normalization of physiological data. Their tissue biomass (i.e., size) is difficult to quantify due to its gelatinous and contractile nature, and the tissues are closely intertwined with a morphologically complex skeleton. Although tissue area can serve as a proxy for biomass in isometric organisms, in corals the actual tissue area varies with polyp expansion, and this, in turn, is plastic and highly responsive to environmental conditions. Faced with these problems, early coral physiologists normalized their results by colony (e.g., rate/colony; Yonge et al. 1932), or by weight of the entire colony (e.g., rate/g coral; Kawaguti 1937), so that colonies of equivalent shape, weight, and density could be compared. In rare cases, individual polyps have been used for normalizing (e.g., rate/polyp; Marshall 1996), but the diversity of polyp and corallite dimensions (Porter 1976), interspecific variation in polyp density (Clayton 1985), and plastic corallite morphology (Bruno and Edmunds 1997) restricts useful comparisons to species with conserved corallite dimensions and densities. Presently, the two most common size-normalizing parameters for scleractinian corals are the surface area of the skeleton covered by living tissue, and tissue biomass. Here we revisit the question of which normalizing parameter is most suitable for studies of coral physiology, and suggest that biomass is often more appropriate than surface area for this purpose.

The surface area of the skeleton has been used to standardize physiological data for >60 years (e.g., Motoda 1940; Kanwisher and Wainright 1967), but it gained widespread acceptance with the publication of the simple "aluminum foil" method (Marsh 1970). Although alternative techniques such as dye-dipping (Hoegh-Guldberg 1988) provide greater resolution for morphologically complex skeletons, none of the available methods quantifies the actual area of coral tissue, which arguably is more biologically relevant than skeletal area in normalizing the flux of important solutes (Patterson 1992). Coral tissue conforms closely to the surface of the skeleton, and sometimes penetrates the outer layer of the skeleton to a depth of several millimeters (e.g., *Porites*), but there is no clear relationship between skeletal area and actual tissue area. The actual tissue area is determined by the instantaneous area of polyps and tentacles, and therefore varies hugely depending on the degree of contraction. Normalizing to

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biomass avoids having to equate skeleton area to actual tissue area, but the common practice of treating biomass and area-normalized data as equivalent (Edmunds and Davies 1986) is misleading if biomass varies.

A substantial hurdle to quantifying coral biomass is the separation of living tissue from dead skeleton. One approach is to dissolve the skeleton in acid and quantify the organic content of the slurry, for example as total nitrogen content (Goreau and Goreau 1959). If the tissue is fixed prior to decalcification, biomass can be determined by drying and weighing the tissue tunic remaining (Davies 1980). Regardless of the analytical technique, the unpleasant task of decalcification deterred the use of biomass for normalizing until alternative techniques were developed. Such techniques involve using a jet of high-pressure water from a dental hygiene tool (a WaterPik®; Johannes and Wiebe 1970), or a jet of compressed air and water from an artist's air brush, to blast the tissue off the skeleton (e.g., Szmant et al. 1990). The WaterPik® provides a quantitative tool for the removal of coral tissue in a slurry suitable for proximal analysis including, for example, the quantification of biomass as protein content. The effectiveness of the WaterPik® in removing coral tissue varies depending on the perforate nature of the skeleton of the coral species. However, WaterPik® is the method of choice for the majority of researchers, and in our hands we have obtained >92% efficiency using this tool to remove zooxanthellae from the perforate coral *Porites porites* (Edmunds 1994). This suggests that the majority of the coral tissue is removed, assuming that the zooxanthellae provide an effective proxy for coral tissue, a condition that is likely to be correct where the zooxanthellae are distributed uniformly throughout the tissue.

The suite of analytical techniques employed by coral physiologists has advanced significantly in the last decade. For instance, while simple photosynthesis to respiration ratios provided insight into coral physiology in the 1960s and 1970s (Beyers 1966; Wells et al. 1973), contemporary studies employ advanced techniques such as the analysis of protein expression (Black et al. 1995), metabolite concentration (Lesser et al. 1994), short-term biochemical dynamics (Brown et al. 1999a), and the intracellular microenvironment (Kühl et al. 1995). Given the precision and dynamic nature of these measures, it is essential to normalize to a parameter that accurately reflects the size of the animal on a temporally relevant scale. In this context, we examine the suitability of the surface area of the skeleton and soluble protein as biologically relevant metrics for normalizing physiological data in scleractinian corals.

To support our discussion, we draw upon data originally collected for different purposes. First, we present data from a manipulative experiment with *Montastraea franksi* that demonstrates differing results for area-normalized versus protein-normalized data. This experiment was completed in Jamaica during January 1994, with the goal of comparing bleaching susceptibility among coral clones. Second, we use results from a mensurative experiment with *M. franksi* to demonstrate a disproportionate relationship between zooxanthella content normalized to area and protein, and variable protein content among adjacent conspecifics. This experiment was completed in the Florida Keys during August 1997 and July 1998, with the initial goal of quantifying the characteristics of coral tissue during a natural bleaching event.

Methods

The manipulative experiment compared bleaching between clones of *M. franksi* transplanted from deep to shallow water. Clone-mates were prepared from two plates of *M. franksi* that were collected from 21-m depth on the fore reef at Discovery Bay. Each plate was broken into clone-mates (i.e., ramets of each genet) with a mean area of 12.0 ± 0.6 cm^2 ($\pm$SE, $n = 32$), and these were glued to tiles and returned to the collection depth. After 5 days of recovery, some ramets were kept at 21-m depth as controls ($n = 7$/genet), and some were moved to 3-m depth (transplants; $n = 8$–10/genet) to induce bleaching. The protein content of the two clones was measured at the start of the experiment, and after 7 days the controls and transplants were collected and their area, protein, and zooxanthellae content were measured. Area was assessed with aluminum foil (Marsh 1970); zooxanthellae were quantified using a hemocytometer to count the cells in the tissue slurry removed with a WaterPik® (Johannes and Wiebe 1970) and concentrated by centrifugation. Soluble protein in the tissue slurry (i.e., of animal and algal origin) was measured using the Coomassie Brilliant Blue assay (Bradford 1976; Edmunds et al. 2001). Zooxanthella densities were normalized to the area and protein content of each ramet, and compared between clones and treatments with a mixed model, two-way ANOVA (clone = random factor, treatment = fixed factor). The residuals from the ANOVA were used to test the assumptions of normality and homoscedasticity, and we assumed that the ramets were statistically independent replicates of each genet because they were physiologically autonomous, and had recovered from the initial fragmentation. Protein content was compared between clones with a t-test.

The mensurative experiment quantified the effects of bleaching on *M. franksi* that was part of the widespread event coincident with the severe El Niño of 1997/1998. The study population was at 15-m depth on Conch Reef, and was sampled at times coincident with a previously planned research trip (1997), and a time point approx. 1 year later (1998), respectively. Because the research trip was not specifically scheduled to quantify the effects of bleaching, we have no time-course data to determine when these corals first were affected by thermal stress, or how long they had been pale when we collected our first samples. In 1997, 75 colonies were selected haphazardly to assess bleaching severity, and the majority (93%) was bleached. By July 1998, 23 of the same colonies were normally colored (i.e., khaki brown/green) and these are used in the present analysis to equate area and protein normalization in relatively "healthy" corals. Since these corals were sampled only 11 months after a severe bleaching event, and at a time when seawater temperatures again were unusually high (NOAA 2001), it is possible that recovery was incomplete, and that another bleaching event already was underway. Several Caribbean corals appear normally colored even when their zooxanthella populations are substantially reduced (Fitt et al. 2000), and in the present study the zooxanthella densities in *M. franksi* that were khaki brown/green in 1998 may have been depressed compared to normal densities. A fragment of skeleton and tissue was removed from the 23 colonies and processed immediately for area, zooxanthella densities, and protein content using the methods described above. Zooxanthella densities were normalized to area and protein, and tested for proportional variation using Model II regression and a t-test of the null hypothesis that b (slope) corresponds to a doubling in zooxanthellae per milligram of protein for a doubling in the

zooxanthellae per square centimeter. A histogram was used to examine the variability in protein content of the sampled colonies.

Results and discussion

The results of the manipulative experiment differed qualitatively in magnitude and direction depending on whether zooxanthellae were normalized to area or protein (Fig. 1). Zooxanthella densities were higher in controls for clone 1 versus clone 2 when normalized to protein, but not when normalized to area. Transplantation reduced zooxanthella densities in clone 1 but not in clone 2, regardless of the normalizing method. When normalized to area, there was a significant interaction of clone and treatment ($F = 5.763$, $df = 1, 28$, $P = 0.023$) and no significant difference between clones ($F = 4.168$, $df = 1, 28$, $P = 0.051$). The interaction of clone and treatment was highly significant when zooxanthella densities were normalized to protein ($F = 7.971$, $df = 1, 28$, $P = 0.009$), *and* there was a significant difference between clones ($F = 4.993$, $df = 1, 28$, $P = 0.038$). Thus, the normalizing method modified the interpretation of the experiment by strengthening the interaction between clone and treatment, and accentuating the difference between clones. These effects were probably a result of between-clone differences in protein content which, for the control corals (i.e., unstressed) was 0.22 ± 0.03 mg/cm^2 for clone 1 and 0.33 ± 0.06 mg/cm^2 for clone 2 (both mean $\pm$SE, $n = 7$ and 8, respectively). Protein content differed 50% between clones, but this difference was not statistically significant ($t = 1.695$, $df = 13$, $P = 0.114$).

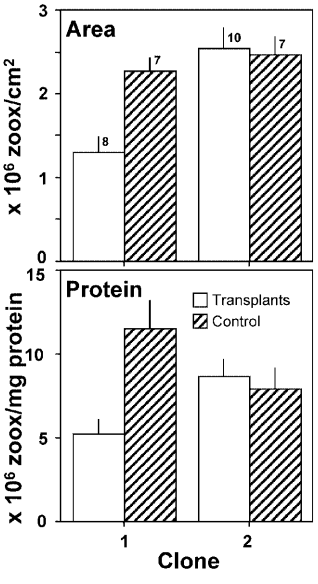

Fig. 1. Zooxanthellae content of two clones (1 and 2) of *Montastraea franksi*, clone mates of which either were kept at 21-m depth for 7 days (controls), or were transplanted from 21-m depth to 3-m depth (transplants) for 7 days. *Above* Results normalized to surface area; *below* the same results normalized to protein content. Mean $\pm$SE is shown, with sample sizes above each *bar*. Refer to text for further details

The mensurative experiment demonstrated that zooxanthellae per square centimeter was correlated significantly with zooxanthellae per milligram of protein ($r = 0.633$, $n = 23$, $P = 0.001$; Fig. 2), but the slope of the relationship ($b = 0.259$) differed significantly from the slope necessary for proportional variation ($b = 0.356$ and passing through the centroid, $t = 2.205$, $df = 21$, $P = 0.039$). Thus, there was disproportional variation in zooxanthella densities normalized to area and protein. For instance, a doubling in zooxanthellae per milligram of protein (from 1×10^6 to 2×10^6) corresponded only to a 60% increase in zooxanthellae per square centimeter (from 0.43×10^5 to 0.69×10^5) (Fig. 2a). The histogram showing the protein content for the same corals (Fig. 2b) reveals positive skewing and a four-fold difference between the extremes for colonies on the same reef. Variation in protein biomass can account for at least some of the discrepancies between the two methods of normalizing zooxanthellae, because the corals with the smallest biomass (filled squares in Fig. 2a) tend to depress the slope of the line relating zooxanthellae per square centimeter and zooxanthellae per milligram of protein. However, some of the variance in zooxanthella densities is independent of protein biomass, as shown by the similar ranges of zooxanthella densities (regardless of normalizing method) for the first two protein biomass size classes (< 0.4 mg/cm^2) and for the data set as a whole (Fig. 2b). Variation of zooxanthella densities within a range of protein biomass values could reflect differences in host cell size caused by, for example, varying lipid and carbohydrate content, and different numbers of zooxanthella within each cell (Muscatine et al. 1998). The discrepancy between area- and biomass-normalized results might also have been accentuated by protein losses associated with bleaching (Porter et al. 1989) that occurred in 1997, or were beginning in 1998.

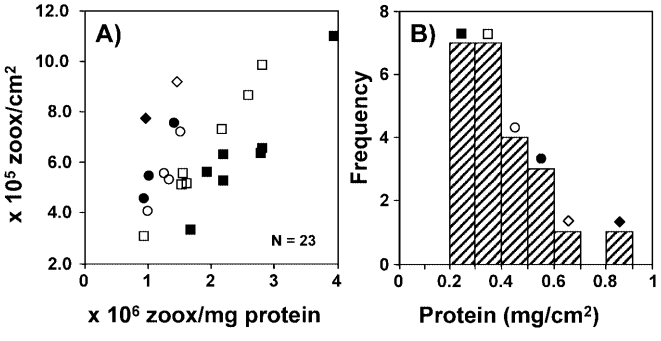

Fig. 2. **a** Zooxanthellae content of 23 colonies of *Montastraea franksi* at 15-m depth on Conch Reef in July 1998. Results are normalized to area and to protein, with the *six symbols* representing class intervals for protein content (shown in **b**). Overall (i.e., for pooled class intervals), there is a significant but disproportional relationship between zooxanthellae per milligram of protein and zooxanthellae per square centimeter (refer to text for further details). **b** Protein content of the 23 colonies of *M. franksi* that were analyzed for zooxanthellae content (**a**). The zooxanthellae content of the colonies belonging to each protein class interval are shown in **a** using the *symbols* shown above each *bar* of the histogram

Regardless of the causes, the present data show that colony areas are *not* equivalent in terms of protein content, and that biomass can serve as a confounding covariate in comparisons based on area-normalized data. In the case of zooxanthella densities, normalizing to colony area obscures potentially important variation caused by differences in protein biomass as well as other factors, such as the packing of zooxanthella within host cells.

The value of area for normalizing coral physiology lies in the potential for valid comparisons independent of the confounding effects of size. Area is easy to measure and varies little during most experiments (i.e., changes in area due to growth are small), but its use in normalizing relies on two assumptions. First, it assumes that biomass does not vary over time, so that results from different periods can be compared directly. Although coral biomass is known to vary with water depth (Davies 1980; Lasker 1981), the common practice of comparing corals from similar depths at different sites using area-normalized data relies on biomass per area being similar at all sites. Second, area-normalization assumes that the colonial, modular design of most scleractinians provides freedom from allometric constraints (Jackson 1979). Thus, unlike unitary organisms (Schmidt-Nielsen 1974), the tissue biomass per unit area should be constant across a wide range of colony sizes. Several studies show that both of these assumptions are incorrect. For example, coral biomass and tissue thickness (a proxy for biomass) vary two-fold between winter and summer (Brown et al. 1999b; Fitt et al. 2000), and mean tissue thickness for *Porites* varies 2.3-fold among locations on the Great Barrier Reef (Barnes and Lough 1992). In addition, the assumption of isometric scaling for colonial scleractinians has been questioned based on theoretical analyses of mass transfer characteristics (Patterson 1992) and resource capture (Kim and Lasker 1998), as well as empirical studies of respiration and photosynthesis (Kim and Lasker 1998; Vollmer and Edmunds 2000). In 1985, W.S. Clayton reported 35% difference in polyp density (polyps/cm^2) between colonies of *Pocillopora damicornis* growing at the same depth, and drew attention to the need to understand this variation in order to advance the field of coral energetics (Clayton 1985). In more than 15 years, little attention has been paid to variation in biomass per area of skeleton (but see Brown et al. 1999b; Fitt et al. 2000), and coral physiologists still normalize their data to area or biomass without addressing the assumptions implicit in their choice of normalizing parameter. Although we have used zooxanthella densities to draw attention to the potential advantages of biomass normalization in studies of coral physiology, the same volumetric arguments also should apply to the measurement of most solutes within coral tissue.

Together with published examples of variable coral biomass (cited above), the data presented here illustrate the consequences of normalizing physiological data to colony area, and suggest that similar effects may be widespread within the Scleractinia. Neither area nor biomass is "right" or "wrong" for normalizing, but there are inherent advantages (and drawbacks) to each method. In most cases, we believe that normalizing to biomass will be most effective in accounting for coral size in comparative studies, because area does not fully incorporate the intricacies of coral design (i.e., tissue and skeleton morphology) or its spatio-temporal variability (Clayton 1985). Protein provides one tractable measure of biomass (e.g., Zamer et al. 1989), although care must be taken to ensure its complete extraction from the coral skeleton and accurate quantification. Because the efficiency of removing tissue with a WaterPik® might vary inversely with tissue thickness or its penetration into a perforate skeleton, further studies are necessary to compare biomass estimates obtained with a WaterPik® with those from alternative methods, such as crushing and extracting pieces of coral skeleton (Lesser et al. 1994). *Montastraea* does not have a perforate skeleton, and therefore a WaterPik® probably is quite efficient at removing tissue, although we did not quantify extraction efficiency with this technique. Area still may, however, be better than biomass for normalizing processes that are, or have the potential to be, mass-transfer limited. For example, in corals with thick tissues, oxygen transfer through their surface area could limit aerobic respiration (Patterson 1992). In such cases, the effects of size on respiration would be better adjusted for by normalizing to area, preferably the actual area of the tissue. Similar reasoning also could apply to normalizing zooxanthella densities in corals with thick tissue, but with the zooxanthellae localized in the outer layer of the tissue, or to normalizing metabolites and other solutes that tend to concentrate near the outer surface of the tissue. Because surface area normalization remains the best approach for certain parameters, it will be important to develop and refine techniques – such as three-dimensional photography – to quantify the area of expanded coral tissue.

In summary, this note draws attention to the consequences of normalizing physiological data to area and biomass for scleractinian corals, and demonstrates the importance of matching the normalizing parameter to the research hypothesis and dependent variable. While there is no "correct" method, we believe that normalizing to biomass offers the greater potential to better understand, and experimentally detect, subtle variation in coral biology that could have important consequences.

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