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RESEARCH ARTICLE

# Physiological correlates of symbiont migration during bleaching of two octocoral species

**Sarah E. Netherton*, Daniele M. Scheer, Patrick R. Morrison, Austin P. Parrin and Neil W. Blackstone‡**

## ABSTRACT

Perturbed colonies of *Phenganax parrini* and *Sarcothelia* sp. exhibit migration of symbionts of *Symbiodinium* spp. into the stolons. Densitometry and visual inspection indicated that polyps bleached while stolons did not. When migration was triggered by temperature, light and confinement, colonies of *Sarcothelia* sp. decreased rates of oxygen formation in the light (due to the effects of perturbation on photosynthesis and respiration) and increased rates of oxygen uptake in the dark (due to the effects of perturbation on respiration alone). Colonies of *P. parrini*, by contrast, showed no significant changes in either aspect of oxygen metabolism. When migration was triggered by light and confinement, colonies of *Sarcothelia* sp. showed decreased rates of oxygen formation in the light and increased rates of oxygen uptake in the dark, while colonies of *P. parrini* maintained the former and increased the latter. During symbiont migration into their stolons, colonies of both species showed dramatic increases in reactive oxygen species (ROS), as visualized with a fluorescent probe, with stolons of *Sarcothelia* sp. exhibiting a nearly immediate increase of ROS. Differences in symbiont type may explain the greater sensitivity of colonies of *Sarcothelia* sp. Using fluorescent probes, direct measurements of migrating symbionts in the stolons of *Sarcothelia* sp. showed higher levels of reactive nitrogen species and lower levels of ROS than the surrounding host tissue. As measured by native fluorescence, levels of NAD(P)H in the stolons were unaffected by perturbation. Symbiont migration thus correlates with dramatic physiological changes and may serve as a marker for coral condition.

**KEY WORDS: Oxygen metabolism, *Phenganax parrini*, Reactive oxygen species, Reactive nitrogen species, *Sarcothelia* sp., *Symbiodinium* spp.**

## INTRODUCTION

Octocorals are a major part of cnidarian diversity (Daly et al., 2007). Octocorals of the Holaxonia–Alcyoniina clade of the alcyonaceans (McFadden et al., 2006) typically contain photosynthetic symbionts of *Symbiodinium* spp. As with other *Symbiodinium*-containing cnidarians, these octocorals bleach when perturbed, typically by light or temperature, or both. During bleaching in hexacorals, symbionts may die or exit the colony (Gates et al., 1992). Additionally, in two species of these octocorals, *Phenganax parrini* (Alderslade & McFadden) and *Sarcothelia* sp. (see McFadden et al., 2006), following perturbation involving light, temperature or confinement in small volumes of seawater, symbionts were recently found to migrate from the polyps to the stolons (Parrin et al., 2012). Such migrating symbionts were consistently contained within host cells (Parrin et al., 2010; Parrin et al., 2012). This results in a pattern of bleaching in which the polyps are completely bleached, while the stolons are not (Fig. 1). While bleaching is usually defined as the loss of photosynthetic pigments or *Symbiodinium* spp. symbionts, or both (e.g. Gates et al., 1992; Lesser, 2007; Paxton et al., 2013; Tolleter et al., 2013), it remains unclear whether these observations require modification of this definition. Certainly, the polyps in these colonies are bleached, and the stolons may be difficult to observe on natural substratum.

The Holaxonia–Alcyoniina clade of the octocorals is large and diverse (McFadden and van Ofwegen, 2012). Certainly, it would be premature to generalize from these two species to such a large clade, although *P. parrini* and *Sarcothelia* sp. are only distantly related. Preliminary data from several other species also show evidence of symbiont migration (A.P.P. and N.W.B., unpublished). While clearly more work needs to be done on the phylogenetic extent of symbiont migration, it is worthwhile to also investigate the correlation between symbiont migration and the physiological aspects of the bleaching process.

Indeed, the implications of the process of symbiont migration for the fate of the host cnidarian are unclear. Migration could have positive effects if it represents a sequestration of viable symbionts that can repopulate the polyps once environmental conditions improve. Alternatively, even if migrating symbionts are no longer viable, they could represent a valuable source of nutrition for the host coral. Conversely, if migrating symbionts are no longer capable of photosynthesis and are emitting high levels of, for instance, reactive nitrogen and reactive oxygen species (RNS and ROS, respectively), then migration could be highly detrimental to the host cnidarian (e.g. Dykens et al., 1992).

To gain insight into the implications of symbiont migration for bleaching, we examined physiological correlates of this process. Oxygen metabolism was examined polarographically, and related factors {e.g. nicotinamide adenine dinucleotide [phosphate] (NAD[P]H), RNS and ROS} were investigated in the host tissues and in individual migrating symbionts using fluorescence microscopy. As suggested by previous results (Parrin et al., 2012), symbiont migration and related physiological changes occur at different rates in the two study species, with colonies of *Sarcothelia* sp. exhibiting greater sensitivity. Possibly these differences are related to symbiont type. Nevertheless, considerable similarities in the physiological correlates of symbiont migration are evident as well. In both species, symbiont migration is initiated early in the bleaching process, and a significant number of migrating symbionts appear to remain viable.

## RESULTS

### Densitometric measures of bleaching

Colonies of *Sarcothelia* sp. have broad stolons that neatly frame polyps (Fig. 1A). The difference between polyp and stolon

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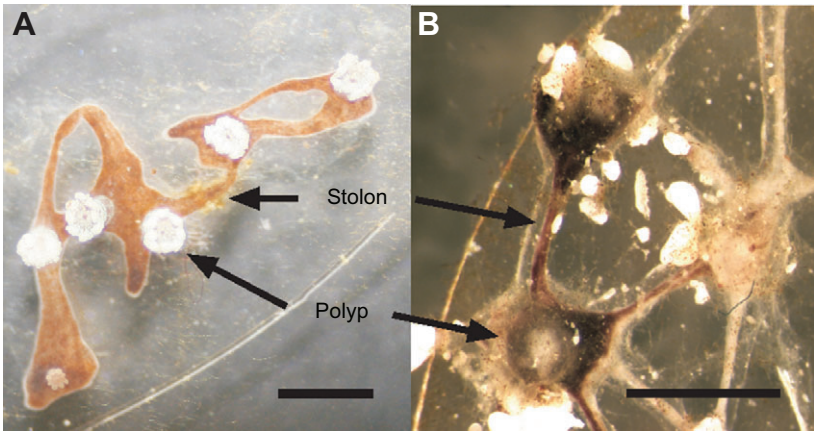


**Fig. 1. Colonies of *Sarcothelia* sp. and *Phenganax parrini* growing on cover glass.** After incubation at 30°C for 12 h, the colony of *Sarcothelia* sp. (A) exhibits bleached polyps and high concentrations of migrating symbionts in the stolons. After incubation at 32°C for 48 h, the colony of *P. parrini* (B) exhibits a similar pattern, but it is more difficult to visualize because the polyps are fully retractable (Alderslade and McFadden, 2011) and symbionts are not evenly distributed in the stolons (white grains of sand are also visible). Scale bars: A, 2 mm; B, 1 mm.

luminance thus nicely captures the effects of symbiont migration. Initially, polyp-minus-stolon values are negative (polyps are darker than stolons), which then shift to positive values following perturbation (Fig. 2A: polyps are lighter than stolons). In contrast, colonies of *P. parrini* have narrow stolons and retractable polyps (Alderslade and McFadden, 2011). Further, migrating symbionts are not evenly distributed in the stolons, particularly after 24 h (Fig. 1B). Polyp-minus-stolon values are thus not particularly informative for this species (Fig. 2B). These values are only significantly different from zero for the 24 h time interval (Fig. 2B). In these experiments, ANOVA showed that within-colony variance is comparable to between-colony variance, thus justifying the paired comparison analysis. The two additional experiments that were carried out showed similar patterns to the data presented in Fig. 2.

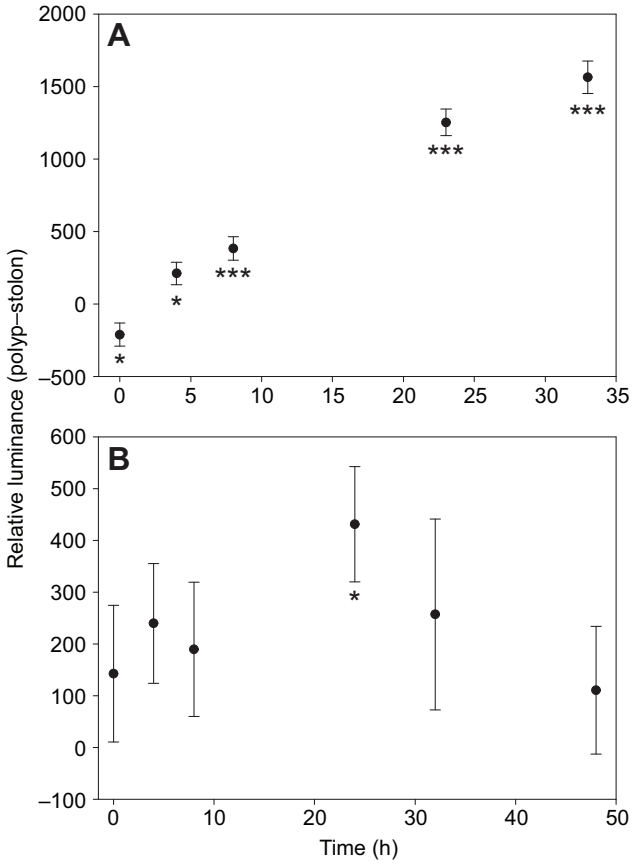


**Fig. 2. Relative luminance in *Sarcothelia* sp. and *P. parrini*.** Mean ± s.e.m. relative luminance due to pigmentation was measured in colonies of *Sarcothelia* sp. (A) and *P. parrini* (B). In A, colonies at 0 h have polyps that are darker than surrounding stolons, hence relative luminance (polyp-minus-stolon values) are significantly less than zero. As colonies are incubated at 30°C, polyps bleach, stolons darken with migrating symbionts, and relative luminance measures become significantly greater than zero. In B, colonies were incubated at 32°C; the pattern is similar but with considerably more variation, particularly after 24 h (see Fig. 1B) (**P*<0.05, ***P*<0.01, ****P*<0.001).

### Measures of oxygen metabolism

For colonies of *P. parrini* that were incubated at 27°C and 55 μE $m^{-2}$ $s^{-1}$ (nominal controls) and 32°C and 140 μE $m^{-2}$ $s^{-1}$ (perturbed), slopes of oxygen concentration versus time were similar in the dark (mean ± s.e.m.: control, −0.037±0.005, $N$=8; perturbed, −0.033±0.004, $N$=10; $t$-test with equal variances, $t$=0.56, d.f.=16, $P$>0.55), but significantly different in the light (control, 0.094±0.014, $N$=8; perturbed, 0.03±0.005, $N$=10; $t$-test with unequal variances, $t$=–4.4, d.f.=8.56, $P$<0.002). The ratio of light to dark slopes (e.g. Kanwisher and Wainwright, 1967) also showed a significant decrease (control, 2.61±0.24, $N$=8; perturbed, 0.99±0.16, $N$=10; $t$-test with equal variances, $t$=5.71, d.f.=16, $P$<<0.001).

Nevertheless, even at 27°C and moderate light levels (55 μE $m^{-2}$ $s^{-1}$), confinement in finger bowls was apparently sufficient to trigger at least some symbiont migration. A repeated-measures design avoided these complications. The oxygen metabolism of each colony was measured directly after it was taken from the aquarium, then measured again after 24 h perturbation involving temperature, light and confinement. Several experiments were carried out in this fashion. In the first experiment (Fig. 3A), colonies of *Sarcothelia* sp. were first measured at 27°C then measured again at 30°C. Dark rates increased and light rates decreased, and both changes were significant (respectively, $t$=–2.97, $P$<0.01; $t$=–3.75, $P$<0.002). In the second experiment (Fig. 3B), colonies of *P. parrini* were first measured at 27°C then measured again at 32°C. While both dark and light rates increased, neither change was statistically significant (respectively, $t$=–1.57, $P$>0.1; $t$=0.91, $P$>0.35). As temperature affects the rate of some processes of photosynthesis and respiration, these experiments were repeated in the absence of thermal perturbation. In the third experiment (Fig. 3C), colonies of *Sarcothelia* sp. were measured at 27°C before and after perturbation involving light and confinement. Dark rates increased and light rates decreased, and both changes were significant (respectively, $t$=–2.34, $P$<0.05; $t$=–11.2, $P$<0.001). In the final experiment (Fig. 3D), colonies of *P. parrini* were measured at 27°C before and after incubation in a finger bowl. Dark rates increased and light rates decreased, but the latter was non-significant (respectively, $t$=–6.31, $P$<0.001; $t$=–2.09, $P$>0.05).

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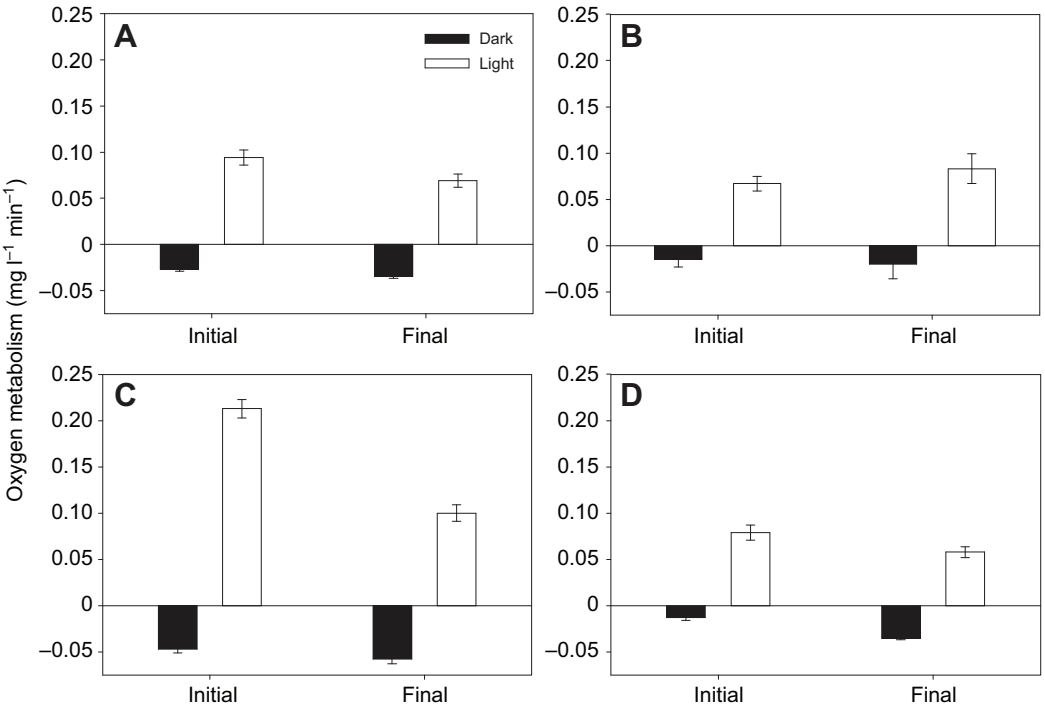


**Fig. 3. Oxygen metabolism in colonies of *Sarcothelia* sp. and *P. parrini*.** *Sarcothelia* sp. are represented in A and C, *P. parrini* in B and D. Slopes of oxygen concentration versus time (mean ± s.e.m.) are shown. All initial measures were taken at 27°C. In A, colonies were incubated at 30°C for 24 h, and final measures were taken at this temperature. In B, colonies were incubated at 32°C for 24 h, and final measures were taken at this temperature. In C and D, colonies were incubated at 27°C for 24 h, and final measures were taken at this temperature.

In the third experiment with *Sarcothelia* sp. (Fig. 3C), the degree of symbiont migration that occurred in the absence of thermal perturbation was assessed with densitometry. In the 13 colonies, 49 polyp-minus-stolon areas were measured before and after perturbation. For the initial measures, polyp-minus-stolon values were not significantly different from 0 (71.6±60.5, paired-comparison *t*-test, $t$=1.18, $P$>0.2). Hence, polyps and stolons were equally pigmented. In contrast, for the final measures, polyp-minus-stolon values were significantly different from 0 (753.5±76.5, $t$=9.85, $P$<0.001). Hence, polyps were brighter (higher luminance, less pigment) than stolons. Even in the absence of temperature changes, incubation in a small dish illuminated at 140 μE $m^{-2}$ $s^{-1}$ was sufficient to trigger measurable differences in pigmentation, confirming visual observations of migrating symbionts.

### Measures of ROS, RNS and NAD(P)H in host stolons

The fluorescent probe 2′,7′-dichlorodihydro-fluorescein diacetate ($H_2$DCFDA) is widely used to assay ROS (e.g. Lesser, 1996; Jantzen et al., 1998; Blackstone, 2001; Franklin et al., 2004; Chen et al., 2012). This probe requires incubation, typically for 1 h in the dark. Measures of ROS for the first experiment showed higher levels for colonies maintained in the aquarium at 27°C and the usual light regime (until 1 h incubation in $H_2$DCFDA) compared with colonies incubated for 13 h at 30°C and 140 μE $m^{-2}$ $s^{-1}$ (plus 1 h incubation in $H_2$DCFDA at 30°C; Fig. 4A; $F$=13.9, d.f.=1,4, $P$<0.02). In the second experiment (a repeat of the first), the same trend was apparent (Fig. 4B), and the effect was again significant ($F$=10.3, d.f.=1,6, $P$<0.02). These results were extremely puzzling because a large body of literature has associated increased ROS with perturbation and bleaching of corals due to light and temperature (e.g. Lesser, 1997; Venn et al., 2008; Weis, 2008). The third experiment provided insight into a possible explanation (Fig. 4C). While there was no overall effect ($F$=1.18, d.f.=1,6, $P$>0.3), the data for the 27°C incubation showed a striking increase in the ROS levels from the first colony measured (colony 1) to the last (colony 4). These results imply that the 1 h incubation in $H_2$DCFDA itself may be sufficient to trigger high levels of ROS, even in the absence of light and temperature perturbation.

Four subsequent experiments focused on detecting any early increase or late decrease in the fluorescence associated with ROS. The first experiment examined three stolons from each of three colonies at 1, 3 and 7 h after the initiation of incubation at 27°C. No trend in ROS levels was apparent (Fig. 5A), e.g. 1 h minus 7 h measures, paired by stolon, showed no significant difference from 0 (171±203, paired comparison *t*-test, $t$=0.84, $P$>0.4). A second experiment examined three stolons from each of three colonies at 8 and 24 h after the initiation of incubation at 27°C (Fig. 5B). A decrease in ROS levels was apparent in many stolons, e.g. 8 h minus 24 h measures, paired by stolon (663±332), but the difference was not significant (paired comparison *t*-test, $t$=2, $P$>0.05). A third experiment examined three stolons from each of six colonies at 1 and 24 h after the initiation of incubation at 30°C (Fig. 5C). The decrease in ROS levels was highly significant, e.g. 1 h minus 24 h measures, paired by stolon (2064±133, paired comparison *t*-test, $t$=15.6, $P$<0.001). The final experiment examined three stolons from each of four colonies at 1 and 30 h after the initiation of incubation at 30°C (Fig. 5D). The decrease in ROS levels again was highly significant, e.g. 1 h minus 30 h measures, paired by stolon (1680±270, paired comparison *t*-test, $t$=6.22, $P$<0.001). In these experiments, ANOVA showed that within-colony variance is comparable to between-colony variance, thus justifying the paired comparison analysis.

To elucidate the relationship between redox state and ROS, native fluorescence of NAD(P)H was measured along with fluorescence from $H_2$DCFDA. This experiment examined three stolons from each of six colonies at 1, 8 and 24 h after the initiation of incubation at 30°C (Fig. 6). The decrease in ROS levels was non-significant for the first interval, e.g. 1 h minus 8 h measures paired by stolon (231±113, paired comparison *t*-test, $t$=2.04, $P$>0.05), but significant for the second interval, e.g. 8 h minus 24 h measures, paired by stolon (603±196, paired comparison *t*-test, $t$=3.07, $P$<0.01). In contrast, NAD(P)H levels showed no consistent trend, e.g. 1 h minus 8 h measures, paired by stolon (−165±117, paired comparison *t*-test,

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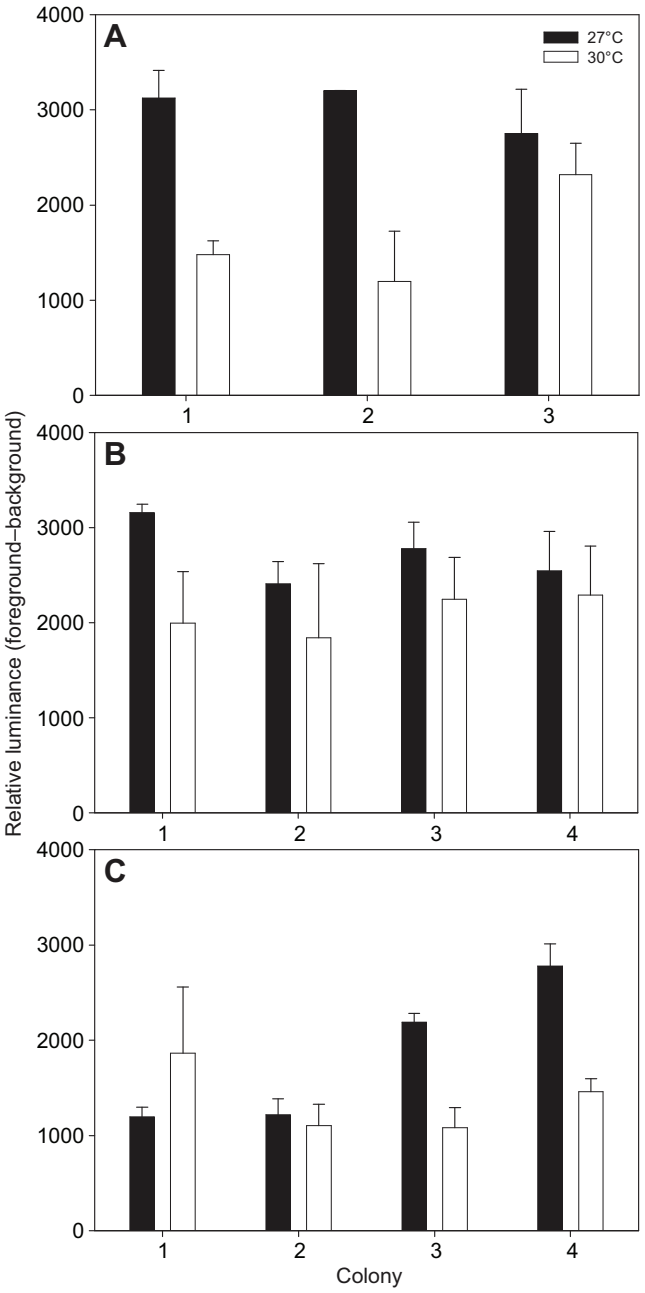


**Fig. 4. ROS in control and perturbed colonies.** Relative luminance (mean ± s.e.m.) of fluorescence due to reactive oxygen species (ROS) was measured in three experiments (A–C) with colonies of *Sarcothelia* sp. Relative luminance was calculated using the stolon as the foreground and an empty cover glass in the same image as the background. By subtracting the background from the foreground, the effect of background luminance is removed. Each experiment followed the same protocol with perturbed colonies incubated at 30°C for 14 h and control colonies incubated at 27°C for 1 h. Surprisingly, the colonies incubated at 27°C exhibit more ROS (A,B). In C, ROS levels in the colonies incubated at 27°C increased rapidly as the colonies were measured (colony 1 was measured first, then colony 2, and so on).

$t$=–1.40, $P$>0.15) and 8 h minus 24 h measures, paired by stolon (311±202, paired comparison $t$-test, $t$=1.54, $P$>0.1).

As NAD(P)H emissions are due to native fluorescence, they can be measured without incubation in a finger bowl containing an exogenous probe, thus more closely assaying an unperturbed colony. This was done in two experiments in which colonies of *Sarcothelia* sp. were incubated at 30°C (Fig. 7). NAD(P)H levels showed no consistent trend. For the first experiment (Fig. 7A), 0 h minus 4 h measures, paired by stolon, showed a near-significant increase (−83±40, paired comparison $t$-test, $t$=–2.06, $P$>0.05). For the second experiment (Fig. 7B), 0 h minus 4 h measures, paired by stolon, showed a non-significant decrease (41±117, paired comparison $t$-test, $t$=0.35, $P$>0.7).

Two experiments with *P. parrini*, which is more resistant to perturbation than *Sarcothelia* sp., better clarified the early stages of bleaching. The first experiment examined three stolons from each of three colonies at 1, 8 and 24 h after the initiation of incubation at 32°C. A clear trend in ROS levels was apparent (Fig. 8A), e.g. 8 h minus 1 h measures, paired by stolon, showed a highly significant increase (1184±197, paired comparison $t$-test, $t$=6.02, $P$<0.001) as did 24 h minus 1 h measures (370±107, paired comparison $t$-test, $t$=3.45, $P$<0.01). A second experiment followed the same protocol, but the increase in ROS occurred during the first measurement interval, and the decrease occurred during the second measurement interval. In Fig. 8B, colonies were measured in the order that they were numbered (i.e. colony 1 first, then colony 2, then 3). Hence, there is no clear overall trend, e.g. 8 h minus 1 h measures, paired by stolon, showed no change (53±197, paired comparison $t$-test, $t$=0.17, $P$>0.8).

The fluorescent probe 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM) is widely used to assay RNS (Perez and Weis, 2006; Cherry Vogt et al., 2011). As with $H_2$DCFDA, this probe requires incubation, typically for 1 h in the dark. One experiment with *Sarcothelia* sp. measured RNS in three stolons from each of five colonies at 1 and 24 h after the initiation of incubation at 30°C (Fig. 9). The difference between the two measurements was not significant (88±70, paired comparison $t$-test, $t$=1.25, $P$>0.2).

### ROS and RNS levels in migrating symbionts

With *Sarcothelia* sp., migrating symbiont-containing cells consistently displayed lower levels of ROS than the surrounding tissue (Fig. 10A). These differences were highly significant (paired-comparison $t$-tests, colony 1: $N$=320, $t$=–36.84, $P$<<0.001; colony 2: $N$=321, $t$=–50.83, $P$<<0.001; colony 3: $N$=348, $t$=–40.73, $P$<<0.001). In contrast, these migrating symbiont-containing cells consistently displayed higher levels of RNS (Fig. 10B). Again, these differences were highly significant (colony 1: $N$=33, $t$=17.41, $P$<<0.001; colony 2: $N$=34, $t$=18.83, $P$<<0.001; colony 3: $N$=53, $t$=13.63, $P$<<0.001). Similar results were obtained in a second experiment with DAF-FM (Fig. 10C; colony 1: $N$=41, $t$=8.23, $P$<<0.001; colony 2: $N$=73, $t$=19.35, $P$<<0.001). Colonies used in these ROS and RNS experiments likely exhibited similar numbers of migrating symbiont-containing cells. Nevertheless, many fewer were detectable with the RNS probe than with the ROS one, suggesting that the differences in luminance were greater in the latter experiment than in the former.

## DISCUSSION

All of the experiments reported were conducted during time periods and conditions when symbionts migrate (Parrin et al., 2012). While symbiont migration has not been widely examined, it is implicit in many discussions of bleaching even in hexacorals (e.g. Gates et al., 1992), i.e. how can symbionts exit the host without entering the gastrovascular fluid and migrating? During perturbation, migrating symbiont-containing cells first appear in the gastrovascular fluid in large numbers and subsequently loosely attach to the tissue of the stolons and cease movement. At 30°C, migration of symbionts in colonies of *Sarcothelia* sp. is largely complete after 12–18 h. At 32°C, migration of symbionts in colonies of *P. parrini* may continue

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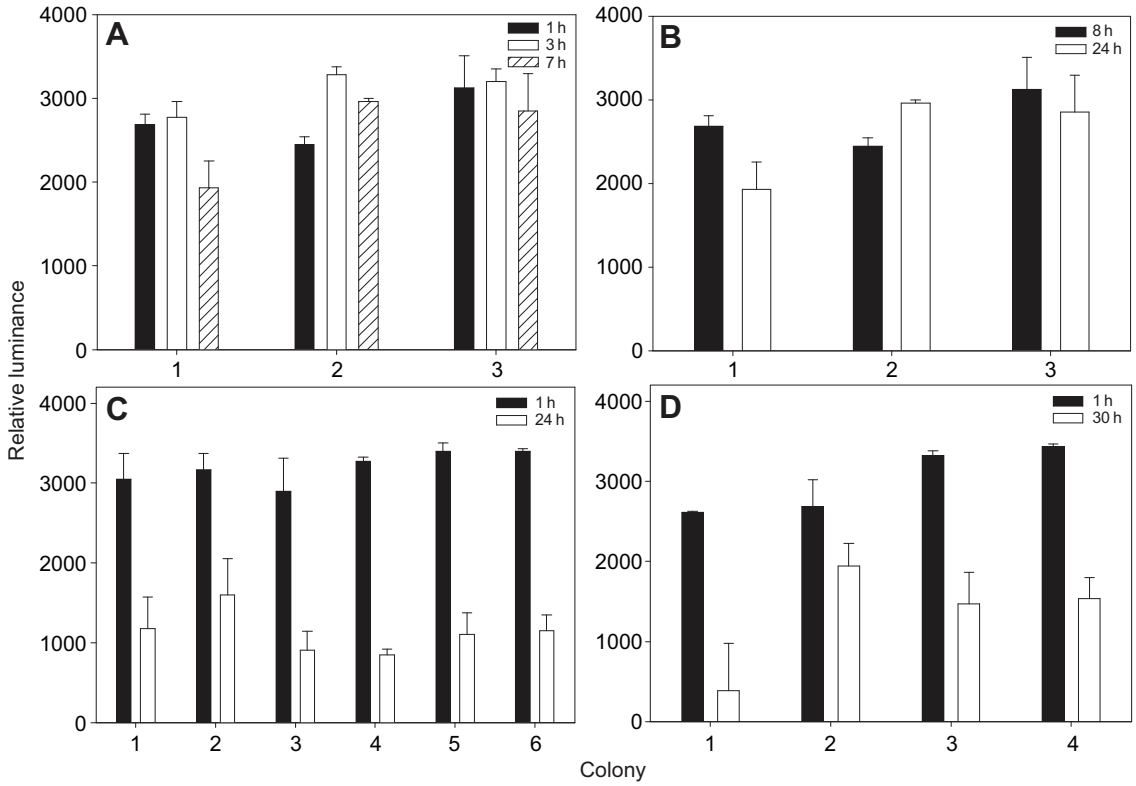


**Fig. 5. Repeated measures of ROS.** Relative luminance (mean ± s.e.m.) of fluorescence due to ROS was measured in four experiments with paired comparisons of colonies of *Sarcothelia* sp. Relative luminance was calculated as in Fig. 4. In A and B, colonies were incubated at 27°C; in C and D, colonies were incubated at 30°C. While no increase in ROS was detected from 1 h to 7 h (A), and no decrease was detected from 8 h to 24 h (B), there was a clear decrease from 1 h to 24 h and 1 h to 30 h at 30°C (C,D).

for more than 24 h. When migration is initiated by perturbation at the same temperature (e.g. 27°C), these time intervals are somewhat increased. Nevertheless, the onset of physiological perturbation is apparently immediate, as shown for instance in the ROS experiments, so even initial measures in many of the experiments described cannot be considered entirely free of perturbation. Merely confining a colony in an oxygen uptake or microscope chamber likely causes some perturbation.

Impairment of photosynthesis is typically associated with bleaching of corals (Jones et al., 1998; Tchernov et al., 2004; Buxton et al., 2012). During symbiont migration, colonies of *Sarcothelia* sp. consistently showed decreased oxygen formation in the light. While oxygen uptake in the dark also increased, the magnitude of the change in the light indicated that photosynthesis was impaired, either because the number of symbionts decreased or because the photosynthetic capabilities of individual symbionts were diminished, or both. Nevertheless, oxygen was still formed at a rapid rate, i.e. photosynthesis was by no means abolished. In colonies of *P. parrini*, the data are more ambiguous. In the first experiment, the nominal controls (incubated at 27°C and 55 μE $m^{-2}$ $s^{-1}$ for 12–18 h) likely exhibited highly perturbed oxygen metabolism, so it is difficult to interpret comparisons with the colonies incubated at 32°C and 140 μE $m^{-2}$ $s^{-1}$. The inherent advantage of the paired comparison experimental design is thus apparent, and all other experiments with both species were conducted in this manner. Such an experimental design may alleviate difficulties found in other circumstances as well (e.g. Ferrier-Pagès et al., 2009). With colonies of *P. parrini*, neither of the paired comparison experiments found a significant difference in oxygen formation in the light. The experiment conducted at 27°C and 140 μE $m^{-2}$ $s^{-1}$ did reveal a substantial increase in oxygen uptake in the dark. Thus, even when symbiont migration was triggered in the absence of thermal perturbation, colonies of both *Sarcothelia* sp. and *P. parrini* showed increased respiration rates. While other studies have found an increase in oxygen uptake in thermally perturbed corals (e.g. Lesser, 1997; Rodolfo-Metalpa et al., 2006; Previati et al., 2010), these findings are confounded by the effects of temperature on metabolism.

In addition to the direct effects of temperature on metabolism, Lesser [see p. 189 of his (Lesser, 1997)] suggests that high rates of respiration in perturbed colonies may be due to ‘an increase in energetic costs associated with repairing damage caused by temperature-induced stress, and the consumption of molecular oxygen during the formation of active oxygen’. While repair costs may still increase when perturbation is triggered at the same temperature, our data suggest that formation of ROS is not likely to be responsible for increased oxygen consumption. For instance, in colonies of *Sarcothelia* sp., merely measuring oxygen metabolism likely perturbs the colony and triggers symbiont migration. Oxygen uptake was nevertheless lower during the first hour of perturbation (i.e. when oxygen metabolism was measured initially) than it was 24 h later. In contrast, ROS formation very likely peaks during that first hour and is much lower 24 h later (see below). ROS formation therefore seems to decrease while oxygen uptake increases. In this context, respiration associated with increased metabolic demand may be a relevant factor. Symbiont migration is associated with decreased velocity of gastrovascular fluid in *P. parrini* (Parrin et al., 2012) and in *Sarcothelia* sp. (K. L. Harmata and N.W.B., unpublished). Possibly, migrating symbionts interfere with the ciliary action that generates the gastrovascular flow in both species (Parrin et al., 2010; Harmata et al., 2013). Consequently, greater metabolic demand from stressed cilium-

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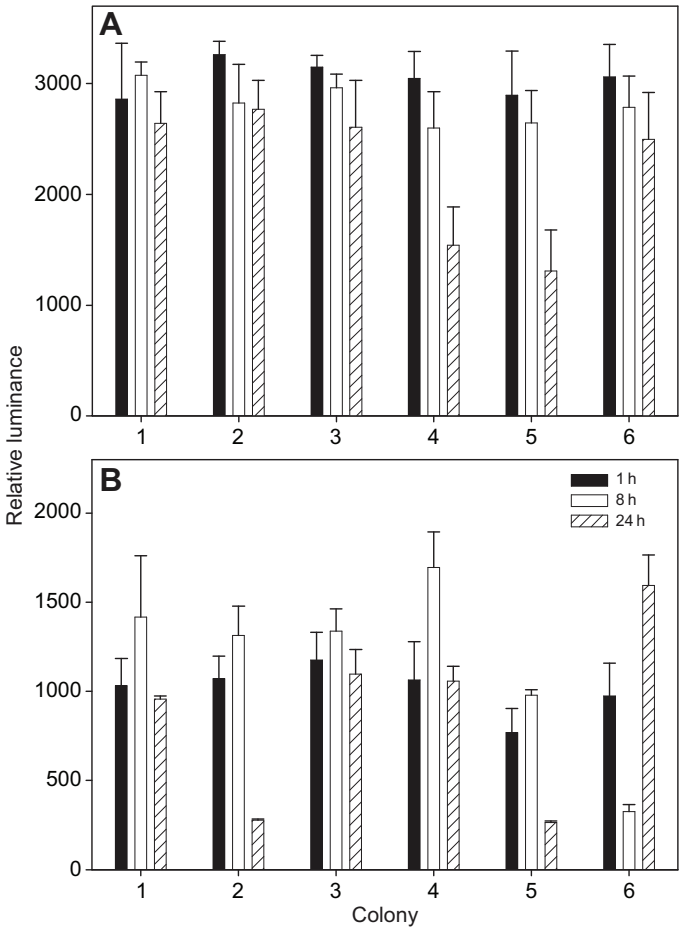


**Fig. 6. ROS and redox state.** Relative luminance (mean ± s.e.m.) of fluorescence due to ROS (A) and NAD(P)H (B) was measured for the same six colonies of *Sarcothelia* sp. incubated at 30°C. Relative luminance was calculated as in Fig. 4. Relative luminance of ROS decreases significantly from 8 h to 24 h but that of NAD(P)H does not.

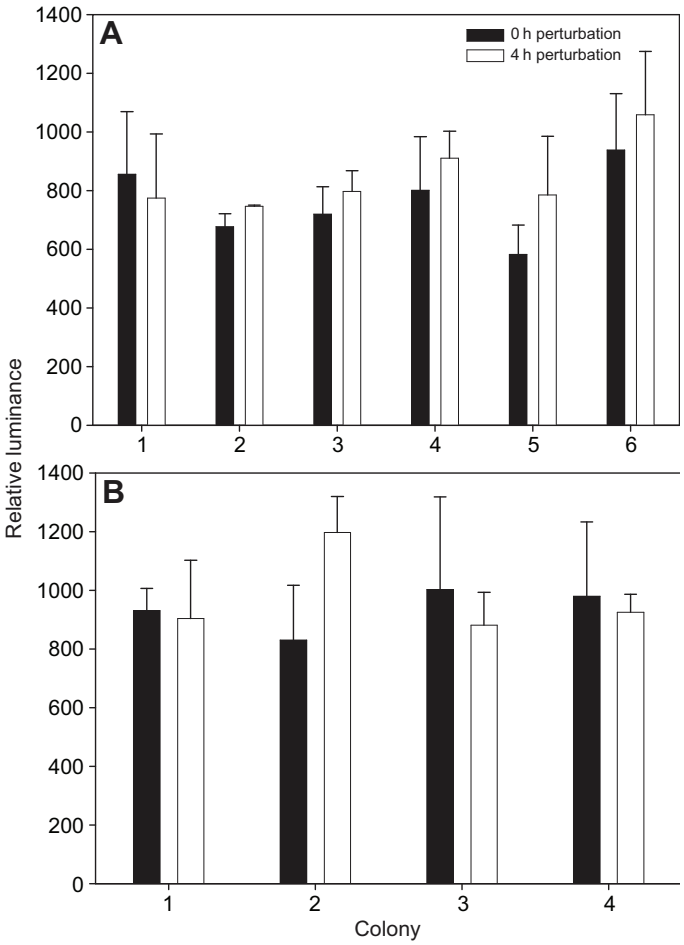


**Fig. 7. Repeated measures of redox state.** Relative luminance (mean ± s.e.m.) of fluorescence due to NAD(P)H was measured in two experiments (A,B) with colonies of *Sarcothelia* sp. Relative luminance was calculated as in Fig. 4. As NAD(P)H does not require incubation in an added probe, it was measured with no incubation (0 h), then re-measured after 4 h of incubation at 30°C. Nevertheless, there is no consistent trend in these measures.

containing cells may underlie the increased respiration rates in colonies with migrating symbionts.

Given the extensive evidence for an association between increased ROS and bleaching (e.g. Lesser, 1997; Venn et al., 2008; Weis, 2008), we were understandably reluctant to accept the results of the first two ROS experiments at face value, i.e. that unperturbed colonies have higher levels of ROS. Indeed, the third experiment provides a plausible explanation for this result: merely by incubating colonies in $H_2$DCFDA, even at 27°C in the dark, a dramatic increase in ROS was triggered. Overall, the data suggest that ROS levels increase to a maximum almost immediately in colonies of *Sarcothelia* sp. As symbiont migration continues to completion, ROS levels then substantially decrease over the next 24 h. These changes do not clearly correlate with the cellular or mitochondrial redox state of the host as measured by NAD(P)H. Further, migrating symbionts exhibit lower levels of ROS than the surrounding host tissue. Thus, the source of the increased ROS is not clear. Possibly, some subset of the symbionts in the polyps (which cannot be visualized *in vivo* because they project out of the focal plane) emit high levels of ROS. These symbionts may subsequently die or exit the colony rather than migrate into the stolons.

Colonies of *P. parrini* contain clade B symbionts and those of *Sarcothelia* sp. contain clade D symbionts (T. L. Goulet, personal communication) [note the contrast to *Sarcothelia edmondsoni*, which contain clade C symbionts (Goulet et al., 2008)]. Evidence suggests that some symbiont types are more sensitive to perturbation (Rowan et al., 1997; Buxton et al., 2012). Certainly, colonies of *Sarcothelia* sp. appeared to be more sensitive to perturbation than those of *P. parrini*. For instance, in the latter, ROS levels do not increase as rapidly even at higher temperatures, and in one of two experiments carried out, the low (initial) ROS levels were successfully measured after 1 h incubation in the fluorescent probe.

The limited investigation of RNS carried out here suggests that these do not increase in the stolon tissue during symbiont migration. However, again in contrast to the results with ROS, some subset of the migrating symbionts exhibits more RNS than the host tissue. As migrating symbionts are contained within host cells in both species (Parrin et al., 2010; Parrin et al., 2012), the production of RNS may still originate with the host (Perez and Weis, 2006).

In both species, symbiont migration initiates early in the bleaching process, and a significant number of migrating symbionts may remain viable. The results suggest that while oxygen formation may decline dramatically or not at all during symbiont migration, it is never abolished. Symbionts thus seem to retain some capacity for photosynthesis even after completing migration into the stolons.

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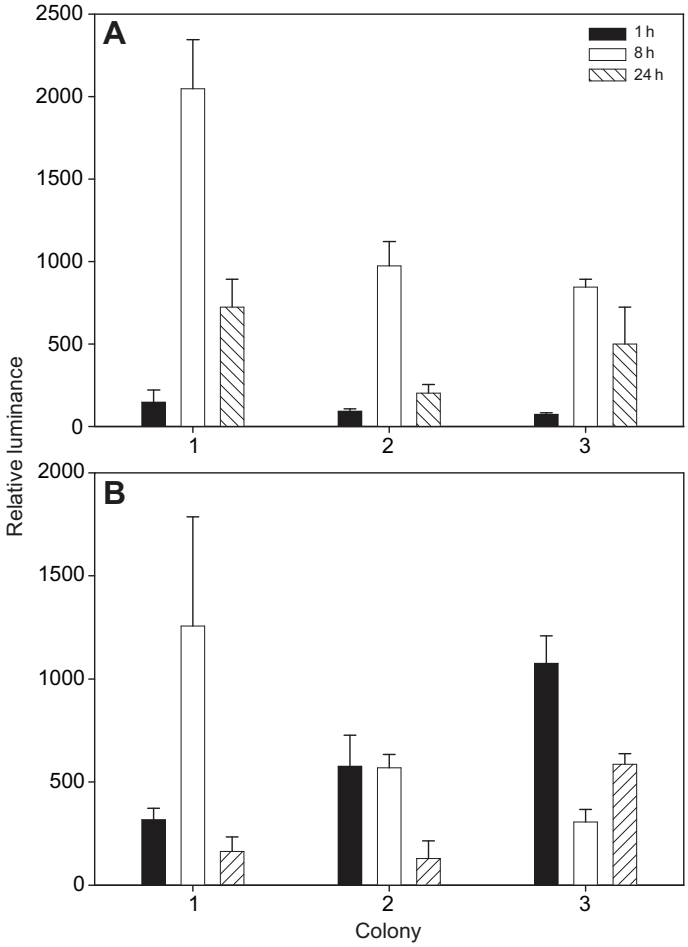


**Fig. 8. Repeated measures of ROS in *P. parrini*.** Relative luminance (mean ± s.e.m.) of fluorescence due to ROS was measured in two experiments (A,B) with colonies of *P. parrini* incubated at 32°C. Relative luminance was calculated as in Fig. 4. In A, relative luminance of ROS increases after 1 h. In B, relative luminance of ROS increased during the time in which the first measurements were taken (filled bars) and decreased during the time in which the second measurements were taken (unfilled bars; at both times, colonies were measured in the order in which they are numbered).

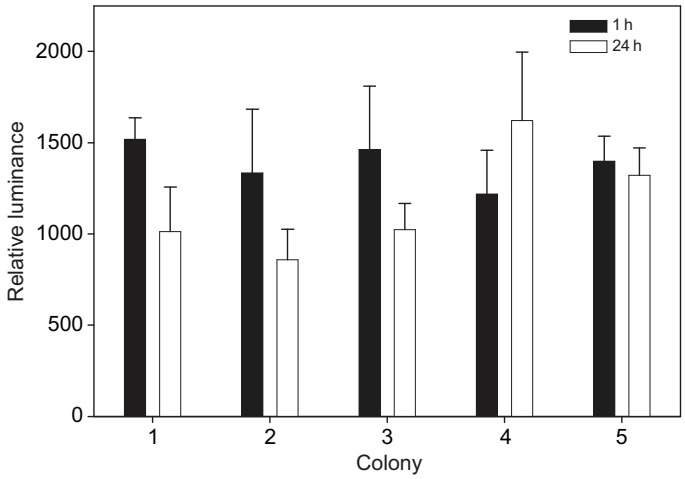


**Fig. 9. Repeated measures of RNS.** Relative luminance (mean ± s.e.m.) of fluorescence due to reactive nitrogen species (RNS) was measured with colonies of *Sarcothelia* sp. incubated at 30°C. Relative luminance was calculated as in Fig. 4. No significant change was detected from 1 h to 24 h.

Migrating symbionts do not seem to be the source of the observed large increase in ROS in the host tissue. In sum, the available data suggest that migrating symbionts are not harmful and may even be beneficial to the host. Further work is needed to clarify the generality of symbiont migration and its relevance to successful outcomes of coral bleaching.

## MATERIALS AND METHODS

### Study species

All experiments were performed on colonies of one clone each of *P. parrini* and *Sarcothelia* sp. While additional clones would better sample within-species diversity (e.g. Shick and Dowse, 1985), presently it is difficult to distinguish within-clone, within-species and between-species diversity. Octocorals exhibit remarkably little variation in conventional genetic markers (McFadden et al., 2011), and much of the biodiversity of small, cryptic species remains undescribed despite recent progress (e.g. Fabricius and Alderslade, 2001). At present, we are unable to generate clones by breeding. Hence, we confined our investigations to one clone of each species. Replicate colonies were produced by explanting material onto 12 and 15 mm diameter cover glass. In each experiment, there was some variability in the numbers of colonies used because of accidental breakage of cover glass and loss of explants prior to the start of the experiment. All colonies were grown under standard conditions (Parrin et al., 2012). Control colonies were maintained in an aquarium at 27°C with illumination of 30 μE $m^{-2}$ $s^{-1}$ (9 h) and 110 μE $m^{-2}$ $s^{-1}$ (3 h). Alternatively, for some experiments nominal controls were contained in 350 ml finger bowls in incubators at 27°C with illumination of 55 μE $m^{-2}$ $s^{-1}$ (12 h). Perturbed colonies were similarly contained with illumination of 140 μE $m^{-2}$ $s^{-1}$ (12 h). Thermal perturbation varied depending on the experiment as noted above and below.

### Densitometric measures of bleaching

To quantify the pattern of bleaching (Fig. 1), colonies were imaged over a time course of 1–2 days. Images were taken using a macro lens attached to a Hamamatsu Orca-100 cooled CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan). Colonies of *Sarcothelia* sp. and *P. parrini* were taken from the aquarium, imaged, then put in a finger bowl and housed in an incubator. Because of the differences between the two species in sensitivity to perturbation (Parrin et al., 2012), the former were treated at 30°C and the latter at 32°C. Additional images were taken throughout the time course. Using Image-Pro Plus 6.3 (Media Cybernetics, Silver Spring, MD, USA), gray-level luminance of the polyp was compared with a similar-sized area of surrounding stolon tissue. For each colony, all mature polyps were analyzed. Relative luminance was calculated by subtracting the stolon luminance from that of the polyp. For each species, paired comparison *t*-tests were used to determine whether the mean relative luminance significantly differed from 0. An assumption of this analysis is that polyps in the same colony bleach independently (or as independently as polyps in different colonies). ANOVA was used to test this assumption. All statistical tests used SAS 9.2 (SAS, Cary, NC, USA). For *Sarcothelia* sp., two experiments were carried out with five and four colonies. For *P. parrini*, two experiments were also performed with eight and four colonies.

### Measures of oxygen metabolism

Oxygen metabolism was measured for individual colonies in the dark and in the light. The first experiment used groups of control and perturbed colonies, while later experiments examined the same individuals, both before and after 24 h of perturbation. As is customary with these experiments, measures in the dark were attributed to respiration, while measures in the light were attributed to the combined effect of photosynthesis and respiration (Blankenship, 2002).

Groups of similar-sized colonies of *P. parrini* subjected to different treatments were compared in the first experiment. The first group ($N$=8), nominally the controls, was placed in finger bowls in an incubator at 27°C with 55 μE $m^{-2}$ $s^{-1}$ illumination for 12–18 h. The perturbed group ($N$=10) was housed in an incubator at 32°C with 140 μE $m^{-2}$ $s^{-1}$ illumination for the same time interval. A drawback of this experimental design was that

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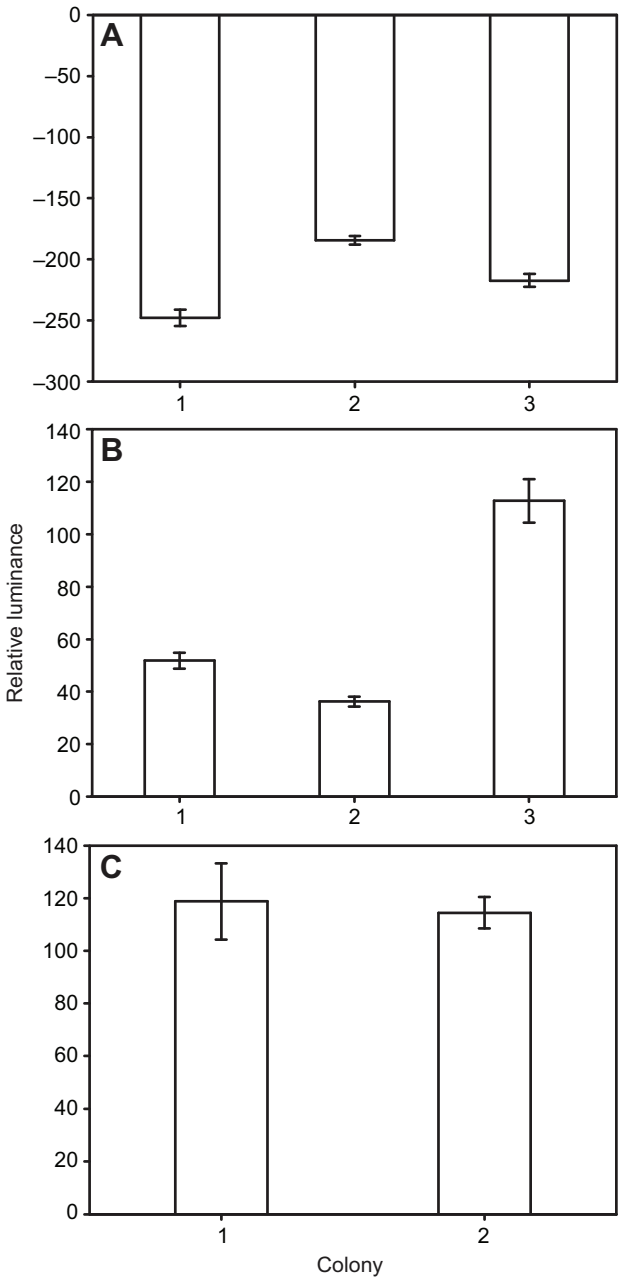


**Fig. 10. ROS and RNS in migrating symbionts.** Relative luminance (mean ± s.e.m.) of fluorescence due to ROS (A) and RNS (B,C) was measured in migrating symbionts. Relative luminance was calculated using the symbiont-containing cell as the foreground and host tissue as the background. By subtracting the background from the foreground, the luminance of the symbiont-containing cell relative to the host tissue can be assessed. Three experiments were performed using colonies of *Sarcothelia* sp. incubated at 30°C for 8 h. Differences between the symbiont-containing cell and the background (host) are shown. Symbiont-containing cells have less ROS than the surrounding tissue (hence negative values in A) and more RNS (hence positive values in B and C).

microscopic examination revealed symbiont migration not only in the perturbed colonies but also in the nominal controls.

Consequently, in a subsequent experiment utilizing colonies of both *P. parrini* (*N*=11) and *Sarcothelia* sp. (*N*=15), a repeated-measures design was used in which each colony served as its own control. Colonies were taken from the aquarium, measured for oxygen metabolism (at 27°C), and then contained in a finger bowl in an incubator (32°C for *P. parrini*, 30°C for *Sarcothelia* sp.) for 24 h. Oxygen metabolism was then re-measured (at 32°C for *P. parrini*, 30°C for *Sarcothelia* sp.). Because some aspects of both photosynthesis and respiration are sensitive to temperature, however, the before and after measures of oxygen metabolism can be confounded by the different temperatures used for measurement. As symbiont migration can be triggered merely by confinement in a finger bowl for 24 h, the experiments were repeated at control temperature (27°C) and the same illumination (140 μE $m^{-2}$ $s^{-1}$) for both species (*P. parrini*, *N*=12 and *Sarcothelia* sp., *N*=13).

In all experiments, measurement of oxygen concentration was performed with a Strathkelvin (North Lanarkshire, UK) 1302 oxygen electrode and 781 meter using standard methods (e.g. Blackstone, 2003). Briefly, a colony on 12 mm diameter cover glass was attached with a small amount of silicone grease to another cover glass cemented to a small magnet. This allowed the water to be stirred in the temperature-controlled Strathkelvin RC300 chamber. A Neslab (Oak Park, IL, USA) RTE-100D recirculating water bath maintained the temperature. Oxygen levels were recorded for 30 min in the dark and 30 min in the light (50 μE $m^{-2}$ $s^{-1}$). Least-squared slopes were calculated for oxygen concentration versus time. Slopes were compared with *t*-tests, either two-sample or paired-comparison tests as appropriate depending on the experimental design. All statistical tests used SAS 9.2.

### Measures of ROS, RNS and NAD(P)H in host stolons

Fluorescent probes (Molecular Probes, Eugene, OR, USA) dissolved in DMSO were used to visualize ROS and RNS (respectively, $H_2$DCFDA and DAF-FM). Both probes must be taken up by living cells and the diacetate groups removed by esterases before they can interact with the target molecule and fluoresce. Similar excitation (blue) and emission (green) wavelengths can be used for the two probes (Cherry Vogt et al., 2011). Negative controls indicate negligible native fluorescence at these wavelengths. In contrast, native fluorescence of NAD(P)H can be visualized by excitation at UV wavelengths and emission at blue wavelengths. An extensive series of experiments with colonies of *Sarcothelia* sp. primarily examined ROS. Some critical follow-up experiments were also done with colonies of *P. parrini*.

In the first experiment, perturbed colonies (*N*=3) were housed in an incubator overnight at 30°C (roughly 14 h total). A small number of colonies were used so that imaging protocols (below) could be completed rapidly for the entire group. At 06:00 h, the colonies were illuminated with compact fluorescent lights (140 μE $m^{-2}$ $s^{-1}$). After several hours, the colonies were incubated in $H_2$DCFDA (10 μmol $l^{-1}$) in the dark for 1 h. Control colonies (*N*=3) were kept in the aquarium until they were incubated in an equivalent amount of $H_2$DCFDA in the dark for 1 h at 27°C. Colonies of *Sarcothelia* sp. are structurally different from hydroid colonies (Harmata et al., 2013). Nevertheless, areas of stolons at the edges of these colonies could be imaged using protocols developed for hydroids (Cherry Vogt et al., 2008; Cherry Vogt et al., 2011). Colonies were contained in a 5 ml microscope chamber in seawater, and three stolons were imaged per colony. Water was frequently changed to maintain temperature (27°C for controls, 30°C for perturbed). Images were taken with Image-Pro Plus, the Hamamatsu Orca, and a Zeiss Axiovert 135 inverted microscope (Carl Zeiss, Jena, Germany). Each image included an area from outside the colony. This area was used to measure background luminance, and relative luminance was obtained by background subtraction. Relative luminance was analyzed as the outcome in a nested ANOVA (stolons within colonies, colonies within treatments) with treatment as a fixed effect.

As described in the results, the surprising findings of the first experiment led to a series of follow-up experiments with colonies of *Sarcothelia* sp. Each experiment was conducted with several control and perturbed colonies. Again, a small number of colonies were used in each experiment to minimize the time differences between imaging the first and last colony. The second and third experiments used the same methodology as the first. The third experiment suggested a possible explanation for the initial surprising result: ROS levels and hence fluorescence first increase, then decrease in colonies in finger bowls at 27°C. To detect such variation, the same stolon areas of the same colonies were imaged over time courses of various lengths with incubation at either 27°C or 30°C. Altogether, four time course experiments were carried out. Colonies were incubated in seawater with 12 h light/12 h dark (140 μE $m^{-2}$ $s^{-1}$). Before measuring fluorescence, colonies were incubated for 1 h in $H_2$DCFDA in the dark. Paired-comparison *t*-tests (paired by stolon) were used to analyze relative luminance. An assumption

of this analysis is that stolons in the same colony develop ROS independently (or as independently as stolons in different colonies). Again, ANOVA was used to test this assumption.

As the high levels of ROS are found in the stolon tissue, they may be the product of host mitochondria (Dunn et al., 2012). The native fluorescence of NAD(P)H indicates mitochondrial redox state and hence can be correlated with ROS production (Chance, 1991). Both ROS and NAD(P)H were measured for three stolons in each of six colonies of *Sarcothelia* sp., 1, 8 and 24 h after perturbation (30°C) was begun. Paired-comparison *t*-tests (paired by stolon) were used to analyze relative luminance. Because NAD(P)H fluorescence does not require incubation in a probe, it can be measured in colonies taken immediately out of the aquarium (i.e. 0 h perturbation). Two experiments were performed that took advantage of this. Fluorescence was measured at 0 and 4 h after perturbation (30°C).

Finally, using similar protocols to those described for *Sarcothelia* sp., colonies of *P. parrini* were assayed for ROS in two experiments (incubation at 32°C). As the latter species is more resistant to perturbation (Parrin et al., 2012), these data clarify aspects of the initial ROS response of both species. Bleaching may also involve RNS, particularly nitric oxide (Perez and Weis, 2006). A single experiment was performed with colonies of *Sarcothelia* sp. incubated in DAF-FM (2 μmol $l^{-1}$). Fluorescence measurements of stolons were taken at 1 and 27 h after perturbation (30°C) was begun.

### Measures of ROS and RNS in migrating symbionts

Colonies of *Sarcothelia* sp. (*N*=3) were housed in a 30°C incubator for 7 h, whereupon they were incubated in $H_2$DCFDA in the dark for an hour. Streaming images (100 frames over 50 s) for two stolon areas of each colony were made using Image Pro Plus, the Hamamatsu Orca and the Zeiss microscope. For each area, all detectable symbiont-containing cells were measured. An area of interest of standard size (135 $\mu m^2$) was placed around a symbiont-containing cell moving in the gastrovascular flow in a given frame of the sequence. The fluorescence of the cell and the background area were measured, and relative luminance was calculated by subtraction. Only the first 30 frames of each film were used for analysis because the probe rapidly bleached. A similar experiment was performed with DAF-FM. A second experiment with DAF-FM was performed with three stolon areas from two colonies.

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#### Competing interests
The authors declare no competing financial interests.

#### Author contributions
All authors participated in the conception, design and execution of the experiments, interpretation of the findings being published, and drafting and revising the article.

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