

Research



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Anemone bleaching increases the metabolic demands of symbiont anemonefish

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Increased ocean temperatures are causing mass bleaching of anemones and corals in the tropics worldwide. While such heat-induced loss of algal symbionts (zooxanthellae) directly affects anemones and corals physiologically, this damage may also cascade on to other animal symbionts. Metabolic rate is an integrative physiological trait shown to relate to various aspects of organismal performance, behaviour and locomotor capacity, and also shows plasticity during exposure to acute and chronic stressors. As climate warming is expected to affect the physiology, behaviour and life history of animals, including ectotherms such as fish, we measured if residing in bleached versus unbleached sea anemones (*Heteractis magnifica*) affected the standard (i.e. baseline) metabolic rate and behaviour (activity) of juvenile orange-fin anemonefish (*Amphiprion chrysopterus*). Metabolic rate was estimated from rates of oxygen uptake (\dot{M}_{O_2}), and the standard metabolic rate ($\dot{M}_{O_{2,min}}$) of anemonefish from bleached anemones was significantly higher by 8.2% compared with that of fish residing in unbleached anemones, possibly due to increased stress levels. Activity levels did not differ between fish from bleached and unbleached anemones. As $\dot{M}_{O_{2,min}}$ reflects the minimum cost of living, the increased metabolic demands may contribute to the negative impacts of bleaching on important anemonefish life history and fitness traits observed previously (e.g. reduced spawning frequency and lower fecundity).

1. Background

Mass bleaching of corals and sea anemones is occurring at unprecedented rates throughout the tropics as a consequence of climate warming and increased sea surface temperatures [1,2]. The frequency of severe bleaching events worldwide has increased from once every 27 years in the early 1980s to currently once every 5.9 years [3]. Furthermore, since 2000, a third of all bleaching events have occurred every 1–3 years [3]. Bleaching occurs following an environmental stress (elevated water temperature or solar radiation), causing the density of algal symbionts (zooxanthellae, *Symbiodinium* spp.) harboured within the coral or anemone tissue to decline, resulting in a loss of host coloration and a white appearance [4]. The ecological effects of bleaching are becoming increasingly clear, with negative impacts on, among others, fish communities associated with coral reefs [5,6].

Anemonefish (genus *Amphiprion*) form a symbiotic relationship with sea anemones and spend most of their lives in close association with their host anemone [7]. The anemone's stinging cells offer the fish protection against predators, especially in the juvenile stage where the fish are most vulnerable. The anemonefish also attach their eggs close to the base of the anemone

where they are well protected [7]. The fish thus rely on their host for both defence and reproduction. Studies have shown negative effects of anemone bleaching on anemonefish, including reduced reproductive success and decreased abundance [8–10]. The underlying causes for these negative effects are not clear, but changes in the fish's physiology may be involved. Indeed, recent work has found increased levels of stress hormones in anemonefish associated with bleached anemones [10]. Such physiological responses to bleaching may also be reflected in other fundamental traits such as whole-animal metabolic rate, which on other occasions have been found to be negatively affected by increased temperatures (e.g. reduced scope to increase metabolic rate above baseline requirements [11]) and which is believed to be a key pathway through which climate change impacts ectothermic animals [12,13].

Using wild-caught orange-fin anemonefish (*Amphiprion chrysopterus*), we investigated if heat-induced bleaching of their host, the magnificent sea anemone (*Heteractis magnifica*), directly affected the anemonefish's standard metabolic rate, which is a measure of the baseline (resting) energetic requirements of an ectothermic animal and represents the cost of living. For diurnally active animals, such as the anemonefish, standard metabolic rate is usually reached at night when the fish are resting. We therefore also evaluated if the anemonefish from bleached and unbleached anemones differed in their day-time metabolic rates, which represents the elevation in metabolic rate caused by increased activity in response to daylight. In addition to being an indicator of stress, increases in metabolic rate among anemonefish could have effects on energy allocation and a range of behaviours, particularly those associated with foraging and risk-taking, and affect the fish's fitness and ability to cope with changing environmental conditions. We therefore also measured if activity levels during the day differed between anemonefish from bleached and unbleached hosts.

2. Methods

Ten juvenile orange-fin anemonefish were collected from unbleached magnificent sea anemones in and around Opunohu Bay, Moorea, French Polynesia, in November 2016 using hand nets while snorkelling or scuba diving. The fish were transported by boat to the CRIOBE research station located at inner Opunohu Bay and subsequently placed in individual aquaria (25 × 29 × 52 cm; approx. 30 l of water) containing either bleached ($n = 5$ fish) or unbleached ($n = 5$ fish) *H. magnifica* (mean ± s.e. anemone surface area: bleached = 388.3 ± 52 cm², unbleached = 383.4 ± 84 cm²). This was done over 2 days, with fish being placed on bleached anemones on the first day and unbleached anemones on the second day, to ensure that the fish experienced the same amount of time in each treatment before experimental measurements. To increase the sample size, another six fish were collected in November 2017 and placed in bleached ($n = 3$ fish) or unbleached ($n = 3$ fish) anemones as for the fish from 2016. The fish were initially maintained on their bleached or unbleached anemones for 2 weeks in filtered, protein-skimmed and UV-treated seawater in a closed re-circulation system (mean ± s.e. temperature in aquaria: bleached = 28.54 ± 0.11 °C, unbleached = 28.55 ± 0.11 °C), during which time fish (and anemones) were fed granulated food twice daily and also supplemented with live artemia. After 2 weeks in either bleached or unbleached anemone treatments, metabolic rate was recorded overnight followed by activity in the morning (only for fish from

2016), as detailed below, after which all fish were swapped between aquaria and anemones, such that fish from bleached anemones were transferred to unbleached anemones and vice versa. The fish were then allowed another 2 weeks with their bleached or unbleached hosts before metabolic rate and activity were measured again in the same fish. This paired design produced a total sample size of 16 fish, of which all 16 were measured for metabolic rate while 10 were also measured for activity, all in both treatments (bleached versus unbleached anemones).

The *H. magnifica* were collected from the wild 2 weeks prior to the introduction of fish, and half were bleached by subjecting the anemones to 31°C for 5–6 days, at which point the anemones were still alive but had lost their symbiont algae and appeared white. Successful bleaching was clearly visible but also confirmed by measuring photosynthetic activity of the anemones (details and data in electronic supplementary material).

After each of the 2-week treatment periods, the fish from either bleached or unbleached anemones were transferred to a respirometry set-up (along with anemones from their respective treatments) for measurements of the fish's oxygen uptake rates (\dot{M}_{O_2}), which was used as a proxy for their metabolic rates. This was done over 2 consecutive days such that fish were measured after 14 days in both of the anemone treatments. Prior to transferring fish to the respirometry set-up, food had been withheld for 20 h to ensure the fish were post-absorptive for the metabolic rate measurements. The respirometry set-up was shielded from surrounding disturbances and comprised a 40 l (water volume) tank receiving flow-through normoxic seawater at 28.58 ± 0.12 °C (mean ± s.e.), eight glass respirometry chambers (35 or 110 ml volume, depending on fish size) in which the \dot{M}_{O_2} of the fish could be measured by oxygen meters and probes (FireStingO2; PyroScience GmbH, Germany), a peristaltic pump with gas-tight tubing which recirculated water through the chambers and past the oxygen probes, and a set of flush pumps which intermittently flushed fresh and fully aerated seawater through the respirometry chambers for 4 or 5 min in every 9 to 12 min intermittent-closed respirometry cycle (flush and close durations were adjusted based on chamber volumes and fish sizes; see [14] for details on respirometry). The respirometry chambers were supported by two plastic pipes in between which anemones from the respective treatment were placed such that their tentacles touched the bottom and sides of the respirometry chambers (figure 1). This allowed the fish to be surrounded by and see (but not touch) the anemones, and also receive olfactory cues from the anemones in the flush water. Fish were introduced to respirometry chambers a few minutes before the first automated \dot{M}_{O_2} recordings were started in the afternoon and remained there for at least 14 h (average duration was 16.8 h) until the following morning, which produced between 77 and 99 \dot{M}_{O_2} recordings per fish (figure 2).

After completion of the overnight \dot{M}_{O_2} measurements, the 10 fish measured in 2016 were filmed for 27 min in both treatments, starting at approximately 11:00 (while the fish were still inside their respirometry chambers), by a camera mounted directly above the respirometry set-up. Fish activity was calculated from these 20 videos by digitally splitting the respirometry chambers into four sections of equal length and counting the number of times each fish crossed the divides between these areas. The fish were then removed from the respirometry chambers and their body masses recorded to the nearest 0.001 g, which meant that growth could be calculated over the final 2 weeks of the experiment for eight fish from each treatment. The exact food intake was not monitored, but all fish were manually fed approximately equal amounts. All fish and anemones survived and appeared healthy (except for the deliberate loss of zooxanthellae) throughout the experiment, and also after when they were returned to the lagoon.

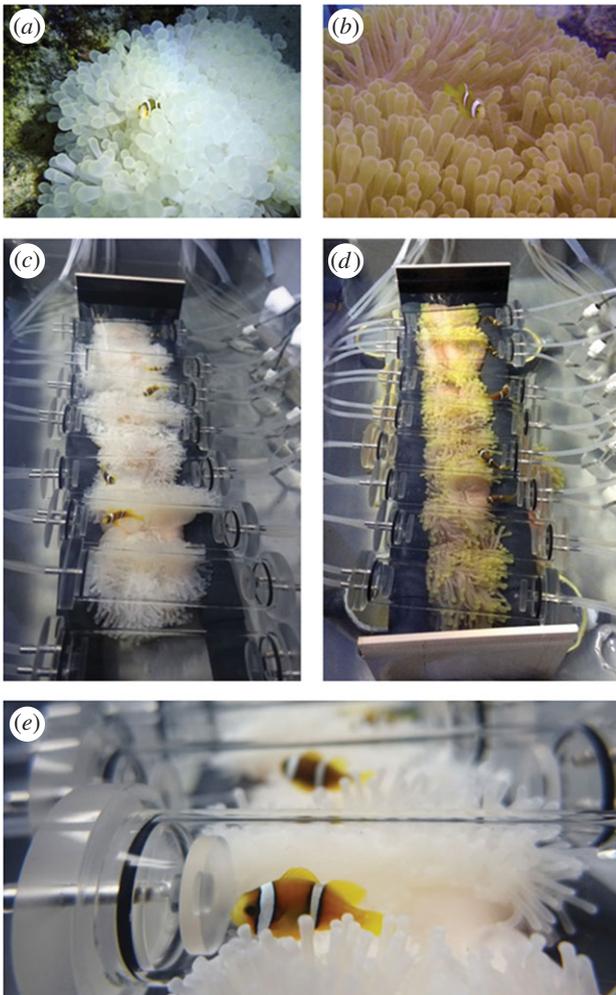


Figure 1. Photographs of juvenile orange-fin anemonefish (*Amphiprion chrysopterus*) in a (a) bleached or (b) unbleached anemone in the wild, and in the respirometry set-up used to measure the metabolic rates of the anemonefish in either (c,e) bleached or (d) unbleached anemones.

Fish \dot{M}_{O_2} was calculated by multiplying the slopes (over 3–5 min) for the decline in oxygen inside the respirometry chambers during the closed phases of the respirometry cycles by the volume of the respirometry chamber after subtracting fish volume and background bacterial respiration (calculated from a respirometry chamber without fish). The standard metabolic rate ($\dot{M}_{O_{2,min}}$) of each individual fish was determined as the 0.25 quantile of the \dot{M}_{O_2} data obtained over the entire respirometry trial [15]. One fish had to be excluded from the \dot{M}_{O_2} data analyses because a detached piece of anemone had got sucked into the respirometry chamber at some point during the overnight measurements (fish 4 in figure 2), making the \dot{M}_{O_2} recordings unreliable. As $\dot{M}_{O_{2,min}}$ primarily occurred during the night when the fish were resting, we also calculated day-time \dot{M}_{O_2} as the mean of each fish's \dot{M}_{O_2} values from 7:00 (coinciding with an increase in \dot{M}_{O_2} as room lights came on) and until the respirometry trials were stopped later in the morning (figure 2).

As any differences in metabolic rates between fish would affect their oxygen demands while residing in their anemone hosts, we also measured the environmental oxygen availability among the tentacles of bleached and unbleached anemones in the field at both day (approx. 11:30; $n = 5$ bleached, $n = 10$ unbleached) and night (approx. 04:50; $n = 11$ bleached, $n = 18$ unbleached). Measurements were taken across two days in January 2018 using an oxygen probe (ProPlus multiparameter instrument; YSI Inc., Yellow Springs, USA) inserted between the anemone tentacles.

All statistical analyses were performed in R [16] as follows. Differences in \dot{M}_{O_2} and activity between treatment groups were evaluated from linear mixed effect (LME) models (lme4 package)

with either \log_{10} -transformed whole-animal \dot{M}_{O_2} or \log_{10} -transformed activity as the response variable, trial (i.e. first round of measurements versus second round of measurements 2 weeks later), treatment, and \log_{10} -transformed body mass as the predictor variables (fixed effects), and fish ID and year of experiments (only for \dot{M}_{O_2}) as random effects. Differences in growth were evaluated from an LME with \log_{10} -transformed body mass gain as the response variable, \log_{10} -transformed initial body mass and treatment as fixed effects, and year as a random effect. Differences in oxygen availability among the anemone tentacles were evaluated from a linear model (LM) with oxygen as the response variable, and time (day versus night), treatment and site as predictor variables, including an interaction between time and treatment. Assumptions of homoscedasticity and normality of residuals were confirmed by visual inspection of residual-fit plots. Statistical significance (p -values) in LME models was evaluated from the lmerTest package, which uses Satterthwaite approximations of degrees of freedom.

Although overall mean (\pm s.e.) body mass was similar between treatment groups (bleached = 0.821 ± 0.183 g, unbleached = 0.836 ± 0.181 g), individual fish differed in mass, which (as expected) had a strong effect on both $\dot{M}_{O_{2,min}}$ (LME, effect of body mass: $t = 17.581$, d.f. = 12.965, $p < 0.0001$) and day-time \dot{M}_{O_2} (LME, effect of body mass: $t = 12.844$, d.f. = 13.508, $p < 0.0001$). Each of the 77–99 \dot{M}_{O_2} recordings for each fish was therefore adjusted to the overall mean fish body mass of 0.829 g (for graphical presentation in figure 2) using the calculation $\dot{M}_{O_{2,adjusted}} = \dot{M}_{O_{2,measured}} \cdot (M_{measured}/M_{mean})^{(1-b)}$, where M is body mass and b is the mean of the mass-scaling exponents for $\dot{M}_{O_{2,min}}$ (0.694) and day-time \dot{M}_{O_2} (0.696), which was 0.695. $\dot{M}_{O_{2,min}}$ and day-time \dot{M}_{O_2} are presented as model-derived estimates in both the text and in figure 3. Body mass had no effect on activity ($p = 0.781$) and these results are presented as raw (measured) values. Oxygen levels among the anemone tentacles are also presented as measured values.

3. Results

Estimated standard metabolic rate ($\dot{M}_{O_{2,min}}$) of the anemonefish was elevated in 12 out of the 15 fish in bleached anemones (figures 2 and 3), which led to $\dot{M}_{O_{2,min}}$ being significantly higher by 8.2% when living in bleached (0.335 ± 0.006 mg O_2 h^{-1} , mean \pm s.e.) as compared with unbleached (0.310 ± 0.007 mg O_2 h^{-1}) anemones (LME, effect of treatment: $t = 2.986$, d.f. = 12.932, $p = 0.011$; figure 3). Day-time \dot{M}_{O_2} did not differ between fish from bleached (0.495 ± 0.019 mg O_2 h^{-1}) and unbleached (0.480 ± 0.018 mg O_2 h^{-1}) treatments (LME, effect of treatment: $t = -0.982$, d.f. = 12.822, $p = 0.344$). Fish activity also did not differ between bleached (549 ± 43 line crossings, mean \pm s.e.) and unbleached (599 ± 94 line crossings) treatments (LME, effect of treatment: $t = -0.090$, d.f. = 16.000, $p = 0.929$). The effect of trial (i.e. whether the fish were with bleached or unbleached anemones first) was not significant for $\dot{M}_{O_{2,min}}$ ($p = 0.860$), day-time \dot{M}_{O_2} ($p = 0.359$) or activity ($p = 0.072$).

Fish residing on bleached anemones gained 0.088 ± 0.018 g in body mass, while fish from unbleached anemones gained 0.148 ± 0.020 g (means \pm s.e.) over the 2 weeks. However, the seemingly lower growth of fish from bleached anemones did not reach significance (LME, effect of treatment: $t = 1.770$, d.f. = 13.000, $p = 0.100$).

Oxygen availability among the tentacles of bleached and unbleached anemones decreased from 105.5 ± 2.0 and $108.9 \pm 1.9\%$ air saturation during the day to 74.5 ± 0.5 and $72.5 \pm 1.0\%$ air saturation at night, respectively. The

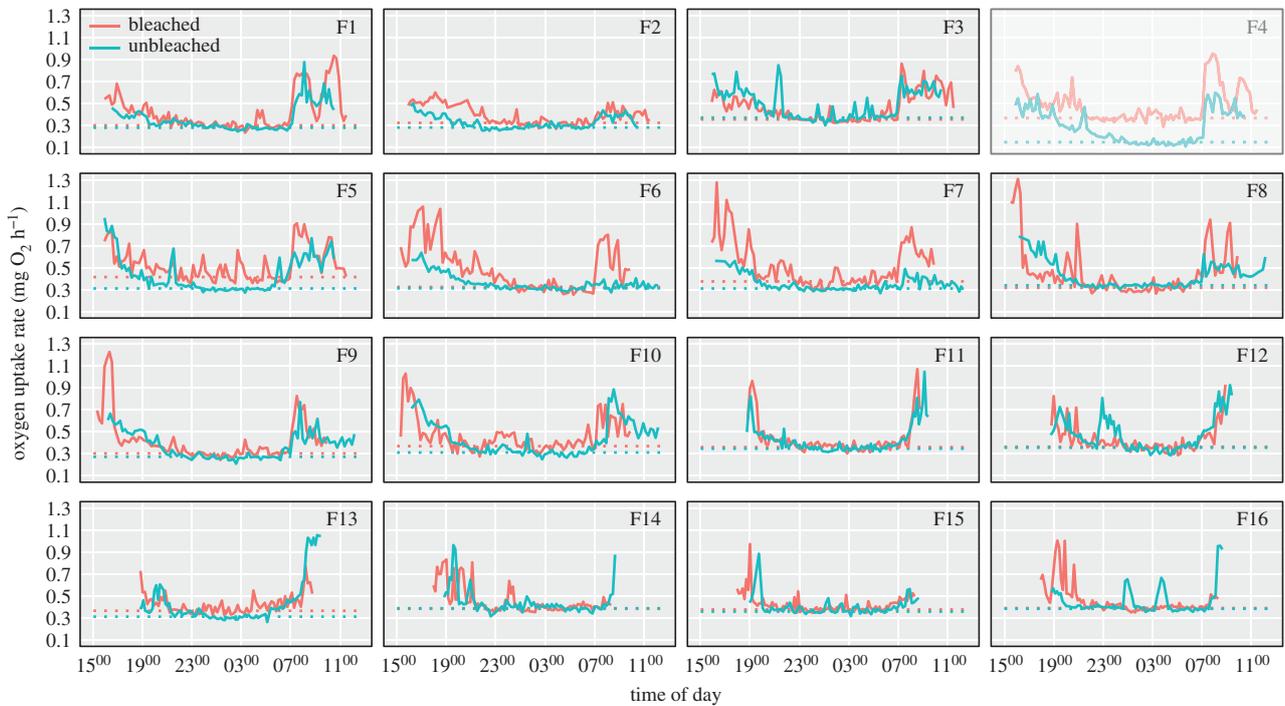


Figure 2. Body-mass-adjusted oxygen uptake rate (\dot{M}_{O_2}) traces for the overnight respirometry trials for the 16 anemonefish from bleached or unbleached anemones. Traces are based on individual \dot{M}_{O_2} recordings (data points omitted for clarity) collected every 9–12 min depending on fish and respirometry chamber size. Dotted horizontal lines represent $\dot{M}_{O_{2,min}}$. Fish 4 (F4) was excluded from further data analyses (i.e. not included in the linear mixed effects models) because a detached piece of anemone had got sucked into the respirometry chamber at some point during the unbleached trial. Data for fish 1–10 are from 2016, while data for fish 11–16 are from 2017.

difference between day and night was the same for bleached and unbleached anemones (LM, time \times treatment interaction: $t = 1.438$, d.f. = 38, $p = 0.159$) but, overall, night-time oxygen levels were significantly lower than day-time levels (LM, effect of time: $t = -14.193$, d.f. = 38, $p < 0.0001$).

4. Discussion

Anemonefish living with bleached host anemones had significantly higher $\dot{M}_{O_{2,min}}$ compared with fish from unbleached anemones, which is probably reflective of an autonomic stress response in fish exposed to bleached anemones [10]. This suggests that fish associated with bleached anemones are at an energetic disadvantage and may experience decreased growth rates as observed previously for fish with elevated $\dot{M}_{O_{2,min}}$ [17]. In the present study, fish exposed to unbleached anemones had growth rates that were 68% higher than when they were with bleached anemones, although this difference was not significant ($p = 0.100$). Activity levels did not differ between anemonefish from bleached and unbleached anemones, and this finding is corroborated by day-time \dot{M}_{O_2} which also did not differ between treatments. In the wild, however, anemonefish with a higher $\dot{M}_{O_{2,min}}$ may be able to compensate for this elevated baseline energy demand with increased foraging activity, but this would also expose them to a greater risk of predation. Increased predation risk may be further exacerbated by the shrinking of anemones when bleached [9] due to reduced shelter area. Overall, the results here suggest that residing in bleached anemones may exacerbate the classical trade-off between foraging and growth on the one hand and predation risk on the other in juvenile anemonefish. The elevated $\dot{M}_{O_{2,min}}$ may also manifest as trade-offs with

other life-history and fitness traits. Indeed, high basal or standard metabolic rate (i.e. elevated $\dot{M}_{O_{2,min}}$) has been linked to reduced reproductive success and reduced survival in a range of animal species, including fish [18–20]. This could explain the decreased fecundity and spawning frequency observed elsewhere for anemonefish associated with bleached anemones [9,10].

The higher oxygen demands of fish living with bleached anemones could also make the anemonefish more susceptible to aquatic hypoxia, if environmental oxygen levels dropped enough to affect the fish's $\dot{M}_{O_{2,min}}$. Night-time oxygen levels within the branches of coral colonies can decrease below 20% air-saturation on tropical reefs [21], but our measurements of oxygen levels among the anemone tentacles suggest that night-time reductions are modest. Despite this, the intermittent occurrence of hypoxic 'dead zones' on coral reefs has recently been reported to be widespread [22], suggesting that coral reef fish do experience reduced oxygen availability on a regular basis.

Sensing of bleached versus unbleached anemones could occur through visual, tactile or olfactory cues in the wild or in the fish's holding aquaria, while the anemonefish were able to use either visual or olfactory cues while in the respirometry chambers. Previous studies have shown that olfactory cues are pivotal for anemonefish recognition of their anemone host, and that anemonefish can distinguish between bleached and unbleached anemones through olfaction alone [23–25]. Despite this, anemonefish appear to settle on bleached anemones as much as on unbleached anemones [9]. This could be due to limited anemone (i.e. habitat) availability or simply because bleached anemones are encountered by larval anemonefish at the same rate as unbleached anemones during a bleaching event. It is also possible that, despite there being a fitness disadvantage to choosing a

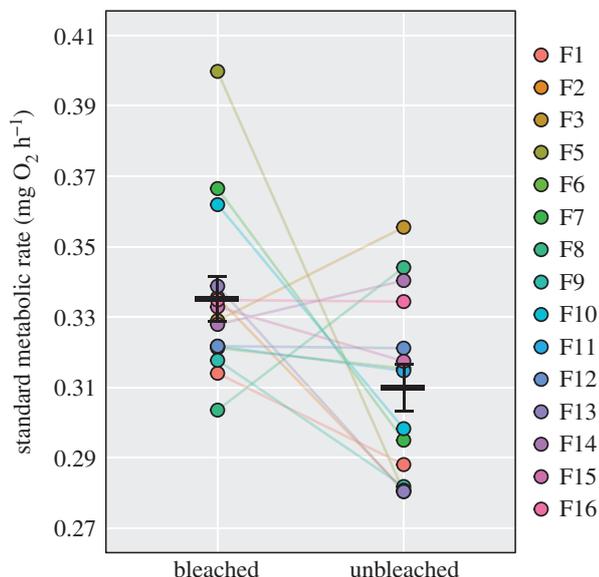


Figure 3. Body-mass-adjusted standard metabolic rates ($\dot{M}_{O_{2,\min}}$) of the 15 anemonefish (F4 is excluded) after the two 2-week periods of living with either bleached or unbleached anemones. Mean $\dot{M}_{O_{2,\min}}$ for each treatment is presented as wide horizontal black bars with black error bars (± 1 s.e.), while $\dot{M}_{O_{2,\min}}$ values for each fish are presented as coloured circles with lines connecting the two data points from each fish.

bleached anemone, bleaching episodes are not frequent enough (currently) for selection to act on habitat choice; spending more time in search for a healthy host would increase predation risk, so settling quickly may outweigh the negative consequences of choosing a bleached host.

The anemonefish in our experiment were exposed to bleached anemones for only 2 weeks, while anemones remained bleached for 2–5 months during the 2016 bleaching event in Moorea [10]. It is possible that anemonefish exposed to bleached anemones over a longer period will acclimate in terms of their metabolic and stress responses. Another species of coral reef fish has been reported to show some capacity for developmental and trans-generational acclimation of metabolic rate to elevated temperatures [26,27], so acclimation to host bleaching may be possible but remains to be tested.

Our study evaluated the metabolic effect of host anemone bleaching at a temperature (28.6°C) equivalent to the current summer average sea surface temperature at the study location [10]. Future temperature increases will not only increase the chance of bleaching but also exert a direct effect on fish metabolic rate and hormonal stress levels [28]. As metabolic rate typically increases exponentially with temperature, it is likely that the combined stressors of bleaching and elevated temperature may increase the metabolic demands of future anemonefish far more than the 8.2% increase observed here

for $\dot{M}_{O_{2,\min}}$. Increasing carbon dioxide levels (i.e. ocean acidification) may add an additional stressor and elevate metabolic rate of anemonefish even further [29], although this may be relieved, to some extent, by trans-generational acclimation [29]. The combined effects of ocean acidification and elevated temperature can also cause disruptions to foraging behaviour and food consumption in juvenile anemonefish [30]. In addition, solar radiation may be a better discriminator of bleaching for inshore reefs [31] and, in a similar manner to temperature (and ocean acidification), may exert a direct effect on fish physiology, although this requires further studies.

Finally, our results show, for the first time, a causal link between host bleaching and a fish's increased energetic demand, adding to the accumulating evidence showing negative consequences of bleaching events, and of climate warming in general. In coral reef ecosystems, there are a considerable number of fish—as well as invertebrates—that strictly depend on hosts amenable to bleaching, such as corals and anemones [10]. The effects of bleaching on metabolic rate shown here for anemonefish may extend to a considerable number of other organisms and alter the trade-offs in such symbiotic associations. If predictions of future temperature regimes hold, mass bleaching is expected to occur every 1–3 years [1,3], which is within the lifespan of most coral reef fish. Selection on individuals that are able to acclimate should therefore increase, but whether or not this allows coral reef organisms to persist is unknown.

Ethics. Approval was granted from the institutional animal ethics committee at CRIOBE (permit no. 006725).

Data accessibility. Data are available in the electronic supplementary material.

Authors' contributions. S.C.M. and R.B. conceived the study and provided research facilities. T.N., S.C.M., R.B. and S.S.K. designed the experiments, and T.N. and S.S.K. provided experimental equipment. T.N., A.C. and D.C. conducted the experiments, and T.N., R.B. and S.C.M. analysed the data. T.N. drafted the manuscript and all authors revised it.

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