High temperature inhibits cnidarian-dinoflagellate symbiosis establishment through nitric oxide signaling

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Abstract

Coral reef ecosystems depend on a functional symbiosis between corals and photosynthetic dinoflagellate symbionts (Symbiodiniaceae), which reside inside the coral cells. Symbionts transfer nutrients essential for the corals' survival, and loss of symbionts ('coral bleaching') can result in coral death. Temperature stress is one factor that can induce bleaching and is associated with the molecule nitric oxide (NO). Likewise, symbiont acquisition by aposymbiotic hosts is sensitive to elevated temperatures, but to date the role of NO signaling in symbiosis establishment is unknown. To address this, we use symbiosis establishment assays in aposymbiotic larvae of the anemone model *Exaiptasia pallida* (*Aiptasia*). We show that elevated temperature (32°C) enhances NO production in cultured symbionts but not in aposymbiotic larvae. Additionally, we find that symbiosis establishment is impaired at 32°C, and this same impairment is observed at control temperature (26ºC) in the presence of a specific NO donor (GSNO). Conversely, the specific NO scavenger (cPTIO) restores symbiosis establishment at 32ºC; however, reduction in NO levels at 26°C reduces the efficiency of symbiont acquisition. Our findings indicate that explicit NO levels are crucial for symbiosis establishment, highlighting the complexity of molecular signaling between partners and the adverse implications of temperature stress on coral reefs.

Introduction

The endosymbiotic relationship between photosynthetic dinoflagellates from the family Symbiodiniaceae and marine invertebrates plays a crucial role in coral reefs [1-3], which are ecosystems of immense ecological and economic importance [4, 5]. Particularly, reef-building corals depend on dinoflagellate symbionts because the translocation of photosynthetically fixed

carbon, such as glucose, glycerol, and amino acids, is capable of satisfying up to 95% of the hosts' energy requirements in an otherwise nutrient-poor environment [5, 6]. Accordingly, intracellular symbionts are essential for host nutrition, tissue growth, and biomineralization to create the iconic reef structures, which are home to more than 25% of all marine species [7, 8].

Coral reef decline as a result of 'bleaching' (loss of symbionts) is threatening reefs worldwide. Typically, coral bleaching is triggered by biotic and abiotic stress [9, 10] including diseases, increased seawater temperature, acidification, salinity [9-12], UV radiation [13], and pollution [10]. In fact, coral reef decline by mass bleaching events have become prominent manifestations of the destructive impacts of human activities and climate change on marine environments [14]. Accordingly, various studies have addressed aspects of the molecular mechanisms underlying coral bleaching [e.g. 15-17]. However, environmental change severely impacts coral species' viability in less visible ways posing previously overlooked threats to coral reefs [18]. In this elegant study, it is demonstrated that environmental change impairs sexual reproduction by reducing the synchrony of gamete release leading to reduced fertilization efficiency and thus abundance of new recruits to replenish aging coral communities [18]. Yet another prerequisite for a healthy, rejuvenated coral population, and thus ecosystem function that has received far less attention is the process of symbiosis establishment [19]. In fact most reef-building corals produce progeny which is initially non-symbiotic which has to acquire symbionts anew each generation by phagocytosing free-living microalgae into their endodermal cells [20, 21].

It is known that environmental stress has negative effects on symbiosis establishment in cnidarians. Specifically, it was shown that elevated sea water temperature negatively impacts symbiont uptake efficiency in coral larvae and juvenile polyps [22-24]. Similarly, high light conditions reduce symbiont acquisition, and both stressors may have additive negative consequences for symbiont phagocytosis [24]. Interestingly, symbiotic larvae are more susceptible to heat stress than their aposymbiotic counterparts and it has been hypothesized that oxidative stress originating in the symbiont may cause tissue damage in the host under heat

stress [22, 23]. However, to date no cellular mechanism or molecular pathway of how environmental stress affects symbiosis establishment has been experimentally confirmed.

Nitric oxide (NO) is a ubiquitous lipophilic molecule which plays two distinct roles that can be either harmful or beneficial depending on the circumstances. On one hand, it can be cytotoxic, but on the other hand, it is involved in interspecies signaling and communication. For example, NO is known to play an important role during symbiosis break-down. Specifically, NO levels are elevated in response to thermal stress in the anemone symbiosis model *Exaiptasia pallida* (commonly known as *Aiptasia*) and the dinoflagellate symbionts, a cytotoxic response that could initiate a bleaching cascade [17, 25]. Accordingly, the addition of exogeneous NO leads to bleaching in *Aiptasia,* likely through an apoptotic-like pathway [26]. The enzyme that produces NO, the nitric oxide synthase (NOS), has a number of isoforms that can either be constitutively or inducibly expressed in various organisms, from plant to mammalian cells [27, 28]. The activity of inducible NOS (iNOS) is involved in stress responses and can lead to the production of larger amounts of NO than the constitutive isoforms of NOS in plant cells [29], and it has been hypothesized that the inducible NOS (iNOS) is upregulated in response to the elevated expression of the heat shock protein 70 (Hsp70) in symbionts *in hospite* under heat stress, leading to bleaching of the soft coral *Eunicea fusca* [16]. Moreover, NOS gene expression is downregulated during reinfection of bleached *Ricordea yuma*, a tropical corallimorph [30].

When NO interacts with reactive oxygen species (ROS), it converts into the potent and highly diffusible oxidant peroxynitrite (ONOO–) which is involved in cellular damage [31]. Previous studies have shown that cultured dinoflagellate cells produce NO at much higher concentrations under thermal stress than at normal temperatures, and these higher concentrations can prove harmful to symbiont photophysiology [32]. Moreover, it was hypothesized that NO may elicit a specific innate immune response against their symbiont, reminiscent of an auto-immune condition [17]. However, in plants, NO is known to play a protective role against pathogenic microbes [33]. In diatoms, NO is important for cell-to-cell adhesion and biofilm formation [34]; and in vertebrate

cells, NO is thought to mediate vascular processes [35] as well as function as a neurotransmitter [36]. However, to date the role of NO as a signaling molecule for the communication between symbiont and coral host during symbiosis establishment remains unknown.

Here, we use larvae of the anemone model *Aiptasia* [17, 37, 38] to test the outstanding and ecologically relevant questions of whether, and if so how, NO signaling contributes to symbiosis establishment under control conditions and during temperature stress. *Aiptasia* spawning can be induced under laboratory conditions [39], and like the majority of corals, *Aiptasia* larvae are initially aposymbiotic and must acquire their symbionts horizontally [37, 38, 40]. Symbiont-host pairings in *Aiptasia* larvae are similar to those in corals [37, 41]. Due to the larvae´s small size and transparency they are amenable to high-resolution microscopy and we have developed a fully controlled symbiosis establishment assay to quantitatively assess the efficiency of symbiont uptake [37, 38, 40, 42]. Taking advantage of this quantitative analysis in *Aiptasia* larvae, microscopic observations and chemical perturbation, we find that NO signaling plays multiple, fundamental roles in during establishment of cnidarian-dinoflagellate symbiosis, a process of critical importance for the persistence of coral-reef ecosystems.

Results

Elevated temperature decreases symbiosis establishment efficiency in *Aiptasia* larvae

To assess the effects of temperature on symbiosis establishment, infection assays were performed at four different temperatures in comparison to the control temperature (26 ºC) (Fig. 1). We found no significant difference in the amount of infected larvae in samples subjected to 29 ºC (~21% at 1 day post infection (dpi) and ~37% at 2 dpi) when compared to the control (~22% at 1 dpi and ~33% at 2 dpi) at 1 and 2 dpi (Fig. 1A; 1 dpi p=0.9924 and 2 dpi p=0.5744; Supplementary tables S1 a, b). At 1 dpi, samples at both 30 ºC (~21%) and 31 ºC (9%) also showed no significant difference in amount of infected larvae when compared to the control samples (~34% and ~17% from each experiment, respectively) (Figs. 1B and 1C; 30 ºC p=0.0561

and 31 ºC p=0.1407; Supplementary tables S2 a, b and S3 a, b). However, at 2 dpi there were fewer infected larvae from the samples at 30 ºC (~19%) and 31 ºC (~8%) when compared to the control (~63% and ~31%, respectively) (Figs. 1B and 1C; 30 ºC p<0.0001 and 31 ºC p=0.0003; Supplementary tables S2 a, b and S3 a, b). At 32 ºC, there were basically no infected larvae (~0%) (at 1 and 2 dpi), while the amount of infected larvae in the control samples were consistent with previous results (~18% at 1 dpi and ~32% at 2 dpi) (Fig. 1D; p<0.0001 Supplementary table S4 a, b). This suggests that at a certain threshold elevated temperature negatively impacts symbiosis establishment in *Aiptasia* larvae.

Elevated temperatures increase NO levels and reduce chlorophyll autofluorescence in *B. minutum* cells, while NO levels in *Aiptasia* larvae remain unaltered.

Cultured *B. minutum* cells exposed to elevated temperature (32 ºC), for both 24 and 48 hours, produced significantly higher levels of NO (DAF-FM-DA fluorescence levels of ~5079 A.U. for 24 h and ~6764 A.U. for 48 h) when compared to control samples (26 ºC; DAF-FM-DA fluorescence levels of ~348 A.U. for 24 h and ~1438 A.U. for 48 h) (Figs. 2A-C) (p<0.0001; Supplementary tables S5a, b). Moreover, chlorophyll autofluorescence significantly decreased in samples exposed to high temperature for 24 and 48 hours, when compared to control cells (Figs. 2A, C, and D) (24 h p= 0.0066 and 48 h p<0.0001; Supplementary tables S6 a, b).

Samples exposed for 48 hours to heat (32ºC) had a 33% increase in NO levels in comparison to samples subjected for 24 hours to the same temperature (Fig. 2B) (p=0.0005; Supplementary tables S5 a, b). Although a slight increase in NO was seen in control cells from 24- to 48-hour exposure, chlorophyll autofluorescence remained at the same level in these samples (chlorophyll fluorescence intensity of ~5755 A.U. for 24h and ~5620 A.U. for 48 h) (Fig. 2D). In contrast, for the heat-treated samples, we observed a significant decrease in chlorophyll autofluorescence from 24 hours (~3595 A.U.) to 48 hours (~1175 A.U.) (Fig. 2D) (p=0.0028; Supplementary tables S6 a, b).

There was no significant difference in NO levels in *Aiptasia* larvae when exposed to different temperatures (26 ºC and 32 ºC) or at different time points (24 h or 48 h) (Figs. 2E, F); Moreover, the localization of NO within larvae appeared similar at both temperatures (Fig. 2E) and DAF-DM-DA fluorescence levels stayed between 71 and 126 A.U. (Fig. 2F) ($p>0.05$; Supplementary table S7). Taken together, this suggests that elevated temperature increases NO production in cultured symbionts but not in *Aiptasia* larvae.

Symbiosis establishment is negatively influenced by a specific NO donor

To understand whether elevated levels of NO at control temperature (26 ºC) would influence symbiosis establishment, we used our infection assays using GSNO as a specific NO donor. For this we first performed an infection assay using the same GSNO concentration (1 mM) as used in previous studies [26, 27, 43]. Our results showed that when 1 mM of GSNO was added during infection, the percentage of infected larvae was approximately six times lower than the control samples (~11% for samples treated with GSNO versus ~66% for control samples) (Fig. 3A; $p<0.0001$ Supplementary table S8).

To understand at what level NO begins to affect symbiosis establishment, we performed an infection assay with different concentrations of GSNO (0.05, 0.1 and 0.5 mM). There was no significant difference in the percentage of infected larvae between control (~25%), 0.05 mM GSNO (~28%), and 0.1 mM GSNO (~24%) samples (Fig. 3B, $p>0.5$ Supplementary tables S9 a, b). However, the percentage of larvae containing symbionts was drastically reduced in samples treated with 0.5 mM GSNO (~2% infection) ($p<0.0002$, Supplementary tables S9 a, b). Thus, increasing NO above natural levels impairs symbiont uptake in *Aiptasia* larvae.

NO scavenging increases infection efficiency a under thermal stress, and decreases it at control temperature

To understand the effect of NO depletion on symbiosis establishment, we added a specific NO scavenger (cPTIO) to either the *B. minutum* medium before infection or to the medium during infection (with both *B. minutum* and *Aiptasia* larvae). Interestingly, at the control temperature (26 ºC), the addition of cPTIO and thus reduction of natural NO levels, either before or during the infection, significantly decreases the percentage of infected larvae (~28% and 13%, respectively) in comparison to the control (~35%) (Fig. 4; $p=0.048$ and $p<0.0001$ Supplementary tables S10 a, b). However, depletion of NO during the infection had a much greater negative effect on symbiosis establishment when compared to the effect of treating symbionts alone before infection. Strikingly, we find that at 32 ºC the percentage of larvae containing symbionts was higher with the addition of cPTIO to the *B. minutum* medium before infection (~11%) in comparison to the control (~2%) (Fig. 4; $p=0.0082$ Supplementary tables S10 a, b). However, the proportion of infected larvae when cPTIO was added in the medium during infection (~5%), showed no significant difference when compared to control ($p=0.8006$). This indicates that the elevatedNO levels induced by increased temperature in *B. minutum* negatively affect symbiosis establishment, yet at the same time, certain NO levels are required for efficient symbiont acquisition by *Aiptasia* larvae.

Discussion

Reef-building corals are critically important for coral reef ecosystems dominating shallow, oligotrophic, tropical oceans worldwide. Coral reefs are facing extinction due to an increasing number of mass bleaching events caused by the warming of the oceans due to climate change [44, 45]. However, climate change also has negative consequences for other fundamental aspects of coral biology such as sexual reproduction, symbiosis establishment, calcification and susceptibility to disease [23, 46-49]. Thus, it is imperative to better understand not only the symbiosis disruption, but also the underlying mechanisms and effects of environmental stress in other essential biological processes of corals to protect these critically important ecosystems.

In order to explore the effects of temperature stress on the onset of symbiosis at the cellular level, we dissect the role of NO during symbiosis establishment in naturally, non-symbiotic larvae using the *Aiptasia*-Symbiodiniaceae model. We reveal that (1) high temperature causes an increase in NO production, which may lead to pigment damage indicated by decreased chlorophyll fluorescence in the symbionts; (2) elevated NO levels act as a stressor and result in decreased symbiosis establishment; (3) the scavenging of the stress-induced, elevated NO levels can restore the efficacy of symbiont uptake by larvae; (4) however, reducing natural levels of NO during the acute infection decreases symbiosis establishment efficiency (Fig 5). Thus, NO plays two distinct roles during the establishment of the cnidarian-dinoflagellate symbiosis. On the one hand, NO likely acts as an essential signaling molecule during the initiation of symbiosis, but on the other hand, NO acts as a stress molecule in symbionts under elevated temperatures resulting in the decreased ability to establish symbiosis with their hosts.

NO as a stress molecule in cnidarian-dinoflagellate symbiosis

Temperature stress has been related to disruption in the cnidarian-dinoflagellate symbiosis, ultimately leading to bleaching and coral death [50, 51]. It is postulated that, upon elevated temperature, NO production is increased in various symbiotic animals, such as corals [52-54] and anemones [25, 26, 55]; this also has been reported in Symbiodiniaceae that were freshly isolated from either the coral *Madracis mirabilis* [55] or the anemone *Aiptasia sp.* [32, 56], and in axenic cultures of various lineages of Symbiodiniaceae [16, 32, 43]. More specifically, previous studies suggest that temperature-induced disruption of symbiosis is mediated and caused by *de novo* NO synthesis in both adult *Aiptasia* (as the host) and symbiont [25, 26]. Here we assess the effects of temperature stress on the NO levels in naturally aposymbiotic *Aiptasia* larvae and cultured *B. minutum* symbionts. We confirm that NO levels increase upon temperature stress (32°C) in *B. minutum* cell but, in contrast to previous studies in adult cnidarians, *Aiptasia* larvae do not show any increased NO levels under the same temperature conditions. This suggests that *Aiptasia*

larvae are less susceptible to heat stress than their adult counterparts and that the failure to establish symbiosis at an elevated temperature may be related to an increase in NO production by the *B. minutum* cells. Accordingly, we find that the application of exogenous NO via the specific NO donor GSNO, which is known as an intracellular NO reservoir and a NO carrier throughout the cell that enhances the biological activity of NO [57], resulted in fewer larvae containing symbionts at non-stressful temperature (26ºC).

To date, the molecular mechanism of how elevated NO levels impair symbiont acquisition by *Aiptasia* larvae are unclear. However, during the steady-state of symbiosis it is thought that heat stress causes the upregulation of heat-shock protein 90 (Hsp90), increasing NOS activity and therefore, NO production in the symbionts. Increased production of NO is directly related to increased production of ROS in marine organisms [58, reviewed in 17], both of which ultimately lead to symbiont expulsion (coral bleaching) from host cells [16]. Specifically, previous evidence suggests that high temperature damages the thylakoid membranes of dinoflagellate chloroplasts and causes an increase in reactive oxygen species (ROS) production [59, 60]. In turn, intracellular ROS leads to a decrease of photosystem II (PSII) activity and to oxidative damage to the chloroplast pigments [61]. Accordingly, the decrease in chlorophyll fluorescence seen here in *B. minutum* could be the consequence of the temperature-induced increase in NO levels and we speculate that impaired photosynthetic capacities may reduce the infectability of free-living symbionts.

NO as a signaling molecule in the cnidarian-dinoflagellate symbiosis

Interestingly, NO is not only considered to be an indicator of cellular stress, but it is also an important signaling molecule involved in various processes in marine organisms. For example, NO signaling has been implicated in the interactions between a squid host and its beneficial bacterial partner during the initiation of symbiotic colonization [62], regulating growth in microalgae [63, 64], and it has been shown to have antioxidant properties and photoprotective

effects in microalgae under environmental stresses [65]. Likewise, in response to environmental change, NO can modulate gene expression [66] and the activity of antioxidant enzymes [67], as well as interact with phytohormones [68] and other signal molecules, like calcium and hydrogen peroxide, as a defense mechanism against pathogens [69, 70].

Strikingly, using the specific NO scavenger cPTIO, we show that removal of NO during symbiosis establishment has varying effects depending on the temperature. At elevated temperature (32°C), NO scavenging from *B. minutum* cells before incubation with *Aiptasia* larvae is beneficial for the efficiency of symbiosis establishment; however, this effect is mitigated if NO is only removed during the infection period. In contrast, using the same concentration of cPTIO to remove NO at optimal temperature (26°C) from *B. minutum* cells reduces the efficacy of the initiation of symbiosis in *Aiptasia* larvae. This effect is even more dramatic when NO depletion occurs during the encounter of symbionts and *Aiptasia* larval hosts, indicating that under temperature conditions where symbionts do not produce elevated NO levels, perturbing the natural NO levels has profound negative consequences for establishing this symbiotic partnership. Interestingly, the scavenging of temperature-induced increased NO in adult symbiotic *Aiptasia* has been shown to reduce bleaching [26]. Here, we see a similar effect during the initiation of this ecologically important symbiotic partnership suggesting that elevated NO levels have negative consequences for both, symbiosis establishment and stability. However, in addition to the well-established role of NO as a stress molecule we also provide evidence for the first time that the reduction of NO below natural levels at non-stressful temperature impairs symbiosis establishment in *Aiptasia* larvae, establishing NO as an important signaling molecule during the onset of symbiosis.

We propose that NO is a ubiquitous messenger molecule that plays fundamental roles in cnidarian-dinoflagellate symbiosis. When NO levels are elevated due to temperature stress, symbiont uptake and symbiosis stability are negatively affected. Likewise, when NO levels fall below a certain threshold, important signaling mechanisms between symbiont and host are impaired. Thus, a tight regulation of NO production is key for cnidarian-dinoflagellate symbiosis,

both in health and disease. More broadly, the increase of the seawater temperature due to climate change likely disrupts this globally important symbiosis at various levels with deleterious consequences for coral reef ecosystems.

Materials and Methods

Culture conditions and *Aiptasia* spawning induction

All experiments were performed with clonal axenic cultures of *Breviolum minutum* strain SSB01 [3, 41] maintained in 0.22µm filter-sterilized IMK medium [71] at 26°C and 25 µmol photons m^{-2} s^{-2} light on a 12L:12D cycle, as described previously [41], unless stated otherwise.

Cultivation and spawning of *Aiptasia* from clonal strains CC7 and F003 were performed as previously described previously [39]. *Aiptasia* larvae were kept in filter-sterilized artificial seawater (FASW) in petri dishes at 26 °C on a 12 h light:12 h dark (12L:12D) cycle until further use.

Symbiosis establishment in different temperature regimes

To test if high temperatures would affect the initial uptake of symbionts by the larvae, we performed infection assays. Each experiment had a control temperature (26 ºC) and one of the following treatment temperatures: 29, 30, 31 and 32 ºC. This temperature range was chosen to understand at what point temperature increase starts to have an effect on symbiosis establishment, taking in account thermal bleaching thresholds observed in corals and *Aiptasia* anemones as well as for natural reefs [12, 72]. For each experiment, *Aiptasia* larvae 4 days post-fertilization (dpf) were distributed into 6-well plates at 300 larvae per ml in 5 ml of filtered artificial seawater (FASW) per well. Both *B. minutum* cultures and larvae were acclimated, separately from each other, to the desired temperature for 24 h prior to the infection assay. Then, *B. minutum* cells were added to each well at a final concentration of 10^5 cells/ml. All experiments were carried out in triplicates. Plates were kept in incubators at the desired temperature under white fluorescent light at 20–25 µmol m^{-2} s^{-1} on a 12L:12D cycle. After 24 h and 48 h of exposure

to *B. minutum*, larvae were fixed with 4% formaldehyde, washed 2 times in PBS containing 0.2% Triton X-100 (PBT), one time in PBS (phosphate buffered saline), and mounted in 87% glycerol in PBS for microscopy analysis. Larvae were observed using a Leica SP8 confocal laser scanning inverted microscope using Differential Interference Contrast (DIC) to identify larvae and fluorescence microscopy to identify symbionts through the chlorophyll autofluorescence. For each replicate, ~ 230 larvae were analyzed.

Analysis of temperature-dependent NO production

To investigate the effects of temperature on NO production, *B. minutum* cells and aposymbiotic larvae were subjected to high temperature (32 ºC). For this experiment, *B. minutum* cell suspensions (four replicates of 2 weeks old cultures) were transferred to 24-well plates with round coverslips at the bottom of each well and set to acclimate at regular culture conditions (see above) for 24 h and then, the temperature was either maintained at 26 ºC (control) or increased rapidly to 32 ºC (treatment).

Aiptasia larvae 4 dpf were distributed to 6-well plates (n = 4) at 300 larvae/ml in 5 ml of FASW and acclimated at regular culture conditions for 24 h. Then, they were subjected to 32 ºC (treatment) or kept at 26 ºC (control).

After 24 h and 48 h of exposure, all samples were processed for confocal laser scanning microscopy for fluorometric quantification of NO using a specific fluorescent marker (see below).

Confocal Laser Scanning Microscopy

To measure NO levels in *B. minutum* and *Aiptasia* larvae, the fluorescent NO indicator 4-amino-5-methylamino-20,70-difluorofluorescein diacetate (DAF-FM-DA; Molecular Probes, Oregon USA) was used. In contrast to other NO markers, it is highly sensitive (~ 3 nM detection limit), photostable, and cell permeable. Furthermore, DAF-FM-DA has been successfully used to measure relatively low levels of NO present in diatoms [42], Symbiodiniaceae [22, 39], and in adult *Aiptasia* [31, 32]. DAF-FM-DA stock solution was diluted in DMSO.

For *B. minutum*, live cells were incubated with a final concentration of 15μM of DAF-FM-DA in FASW for 90 minutes, washed three times with FASW, and mounted on slides and coverslips for live imaging.

Similarly, *Aiptasia* larvae were incubated with a final concentration of 15μM of DAF-FM-DA in FASW for 90 minutes, washed three times in FASW and then fixed in 4% formaldehyde for 15 minutes, followed by 2 washes on PBS. Then, they were mounted in 87% glycerol in PBS for imaging.

All imaging and fluorescence analyses were carried out on a Leica SP8 confocal laser scanning microscope with a 63x /1.30 glycerol immersion lens and using Leica LAS X software. Fluorophores were excited at 488 nm for NO-dependent fluorescence (DAF-FM-DA) and 496 nm for chlorophyll fluorescence (on *B. minutum* samples). Acquisition parameters were the same for every sample, as follows: 1) an emission wavelength interval, corresponding to DAF-FM-DA fluorescence, ranging from 500 nm to 550 nm; 2) an emission wavelength interval, corresponding to chlorophyll fluorescence, ranging from 650 nm to 700 nm; 3) an image resolution of 1024 x 1024 pixels; 4) a pinhole setting at 137.2 μm (airy 1); 5) laser powers as 2% for 488 laser and 2% for 496 laser; 6) standardized photomultiplier adjustments (gain and offset); 7) the same step size (0.10 μm for *B. minutum* and 0.5 μm for larvae) and number of steps (30) for each image sequence obtained. For the quantification of NO and chlorophyll, the background was first subtracted from a 3-dimensional stack using Leica LAS X software and cell or larvae size were normalized. Fluorimetric analysis was performed using the same software to obtain the values of pixel intensity per cell or larvae area (mean grey value of pixel sum per pixel count). The number of *B. minutum* cells analyzed from four independent experiments at each time point (24 h or 48 h) was 13. The number of larvae analyzed from four independent experiments at each time point (24 h or 48 h) was 12. Negative control was DMSO only at the same concentration as the DAF-FM-DA.

Perturbation of NO level during symbiosis establishment using a NO donor

To assess whether elevated levels of NO at control temperature (26 ºC) would influence symbiosis establishment, we performed infection assays using the specific NO donor S-nitrosoglutathione (GSNO; Sigma-Aldrich, Missouri USA). First, GSNO was tested at a concentration of 1 mM in FASW, as used in previous studies [26, 32, 72]. For this, 300 larvae/ml at 4 dpf were distributed into 6-well plates with 5ml of FASW and infected with *B. minutum* culture at a density of 10^5 cells/ml. GSNO was added to each well. Infection lasted 24 h at 26 ºC under white fluorescent light at 20–25 µmol m^{-2} s^{-1} on a 12L:12D cycle. After this, we tested three other concentrations of GSNO, to understand at what level NO starts to affect symbiosis establishment: 0.05 mM, 0.1 mM and 0.5 mM. The same infection protocol was used as described above. Experiments were carried out in triplicates. After a 24 h infection, every sample was processed and analyzed as described above.

Perturbation of NO level during symbiosis establishment using a NO scavenger

Infection assays were performed at 32°C and 26°C as a control, with and without the specific NO scavenger: 2 -(4-carboxyphenyl)-4,5-dihydro-4,4, 5,5-tetramethyl-1 H-imidazolyl-1-oxy-3-oxide (cPTIO; Thermo Fisher Scientific, Massachusetts USA). For this, 0.5 mM of cPTIO was added either to *B. minutum* culture 24 h prior to infection (as it was acclimating to the desired temperature) - and then washed with FASW - or to infection medium (with *B. minutum* cells and *Aiptasia* larvae) during infection at each temperature. The treatments were as follows:

1. 26 ºC with no addition of cPTIO
2. 26 ºC + 0.5 mM cPTIO added to *B. minutum* culture
3. 26 ºC + 0.5 mM cPTIO added to infection medium (*B. minutum* + larvae)
4. 32 ºC with no addition of cPTIO
5. 32 ºC + 0.5 mM cPTIO added to *B. minutum* culture
6. 32 ºC + 0.5 mM cPTIO added to infection medium (*B. minutum* + larvae)

Infections were carried out for 48 h. Then, samples were processed as previously described. Triplicates were used for each experiment and ~248 larvae were analyzed per replicate.

Statistical analysis

Data from all experiments were analyzed with One-Way ANOVA followed by Tukey's multiple comparisons test using GraphPad Prism version 8.0.0 for windows (GraphPad Software, California USA).

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Figures

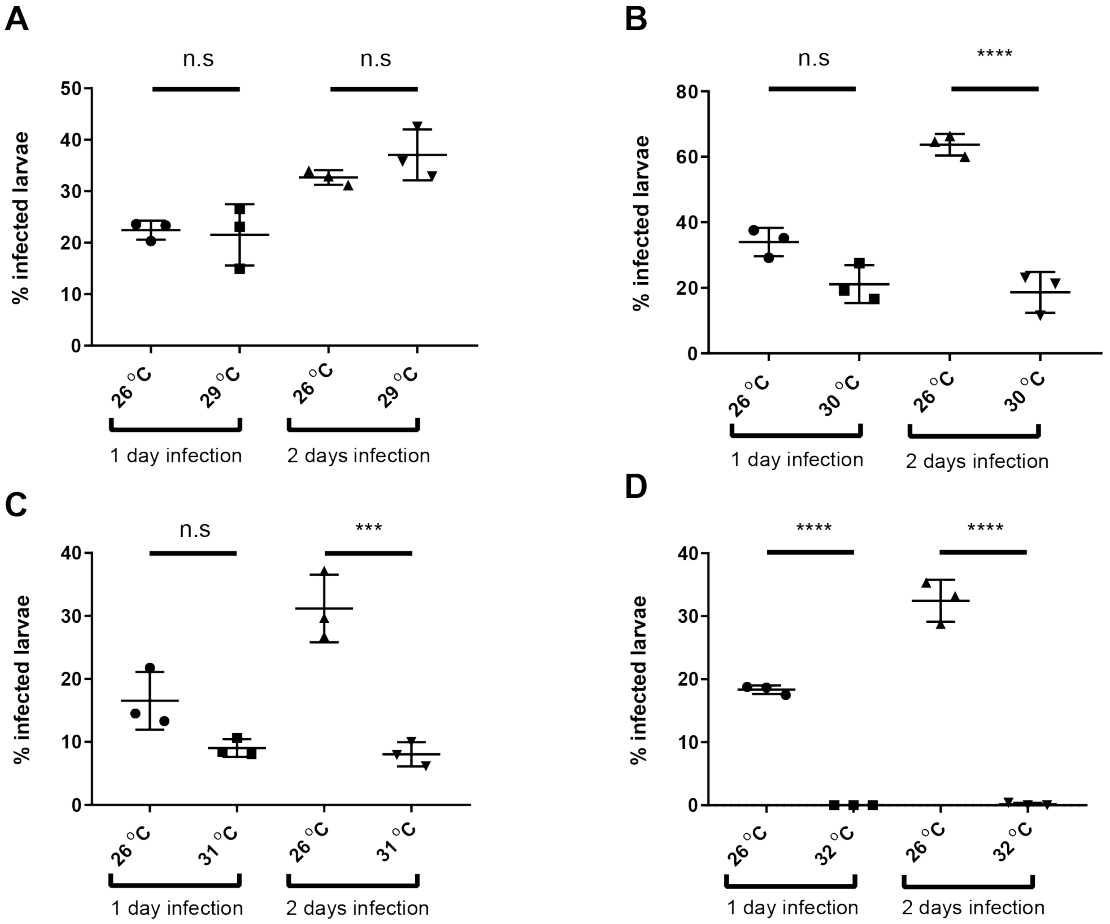

Figure 1. Elevated temperature decreases symbiosis establishment efficiency in *Aiptasia* larvae. Percentage of *Aiptasia* larvae infected by *B. minutum* strain SSB01 at different temperatures after 24 h and 48 h. A – Control temperature (26ºC) versus 29ºC. B – Control temperature versus 30ºC. C – Control temperature versus 31ºC. D – Control temperature versus 32ºC. Asterisks represents significant difference: *** $p<0.01$ and ****$p<0.001$. ANOVA table can be found in supplementary information (S1 - S4). n=3 for each experiment, all error bars are standard deviation.

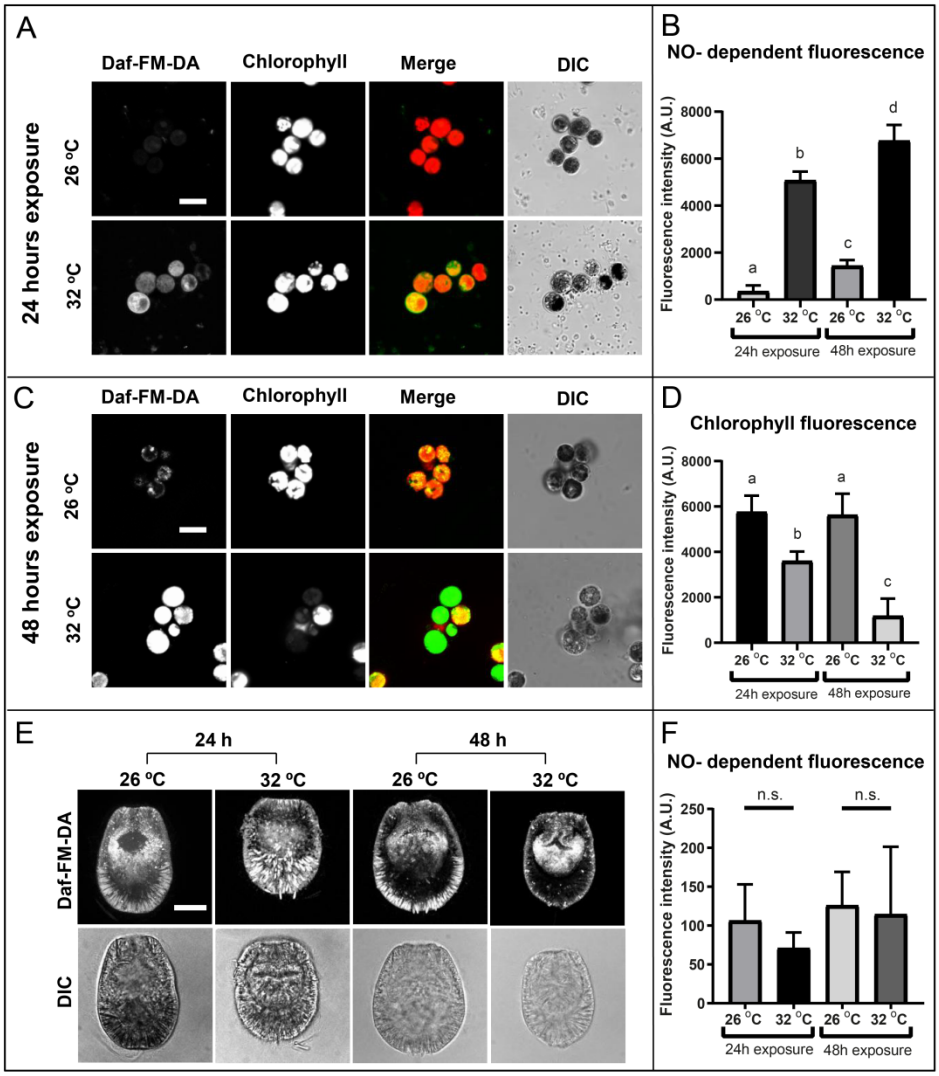

Figure 2. Elevated temperatures increase NO levels and reduce chlorophyll autofluorescence in *B. minutum* cells, while NO levels in *Aiptasia* larvae remain unaltered. A – *B. minutum* cells after 24 h exposure to 26 ºC and 32ºC. Daf-FM-DA corresponds to NO-dependent fluorescence, Chlorophyll autofluorescence and DIC images were also taken (Scale bar represent 10 µm). B – Fluorescence intensity of Daf-FM-DA in *B. minutum* cells subjected to 26 and 32ºC for 24 h and 48 h. C – *B. minutum* cells after 48 h exposure to 26 ºC and 32 ºC. Daf-FM-DA corresponds to NO-dependent fluorescence. Chlorophyll autofluorescence and DIC images were also taken (Scale bar represent 10 µm). D - Fluorescence intensity of chlorophyll in *B. minutum* cells subjected to 26 and 32ºC for 24 h and 48 h. Significant differences are labeled by the different letters above graph bars. ANOVA table can be found in supplementary information (S5 and S6). E - Confocal microscopy and DIC images of *Aiptasia* larvae stained with Daf-FM-DA (labeling NO) after 24 h and 48 h of exposure to 26 ºC and 32 ºC (Scale bar represents 30 µm). F - Fluorescence intensity of Daf-FM-DA in *Aiptasia* larvae subjected to 26 and 32ºC for 24 h and 48 h. No significant differences were observed - ANOVA table can be found in supplementary information (S7). n=13 for *B. minutum* and n=12 for *Aiptasia*, all error bars are standard deviation.

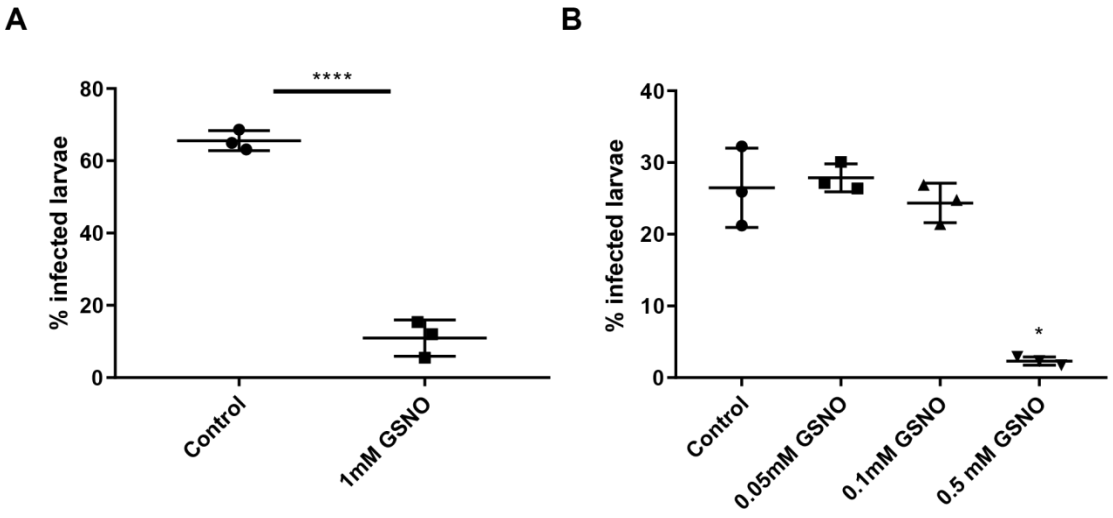

Figure 3. Symbiosis establishment is negatively influenced by a specific NO donor. Percentage of *Aiptasia* larvae infected by *B. minutum* (SSB01) using GSNO at different concentrations as a NO donor after 24 h. A – Pilot infection assay with 1 mM GSNO versus control condition. B – Minimum inhibitory infection assay with different concentrations of GSNO. Asterisks represents significant difference: * $p<0.05$ and ****$p<0.001$ - ANOVA table can be found in supplementary information (S8 and S9). n=3 for each experiment, all error bars are standard deviation.

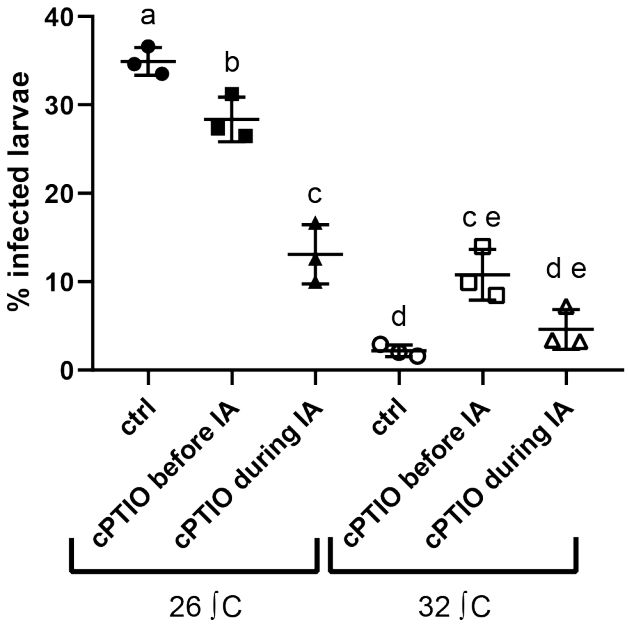

Figure 4. NO scavenging increases infection efficiency a under thermal stress, and decreases it at control temperature. Percentage of *Aiptasia* larvae infected by *B. minutum* (SSB01) after 24 h at different temperatures (26 ºC and 32 ºC) using cPTIO as a NO scavenger. "cPTIO before IA" refers to 0.5 mM of cPTIO added to *B. minutum* culture for 24 h before infection started. "cPTIO during IA" refers to 0.5 mM of cPTIO added to infection medium (with larvae and *B. minutum* cells) right before infection. "Ctrl" refers to control samples, with no addition of cPTIO. Significant differences are labeled by the different letters above graph bars. ANOVA table can be found in supplementary information (S10). n=3 for each experiment, all error bars are standard deviation.

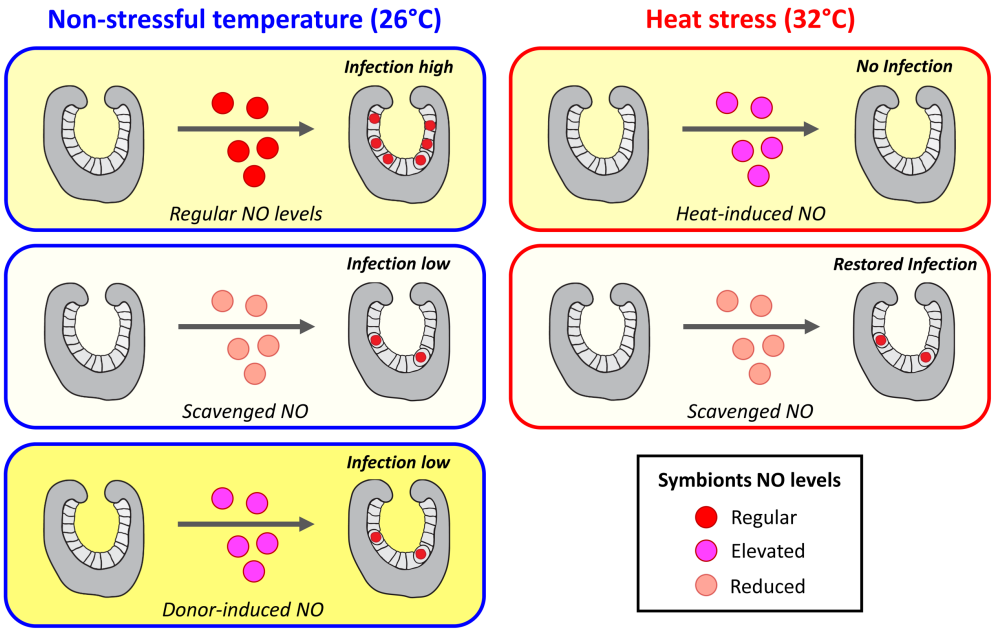

Figure 5. Schematic model representing the influence of NO on symbiosis establishment in *Aiptasia* larvae. Under non-stressful conditions **(**26 ºC – left panels), NO acts as an important signaling molecule for efficient symbiosis establishment (upper panel). Reducing regular NO levels with a specific NO scavenger (cPTIO) decreases infection efficiency (middle panel). Similarly, increasing regular NO levels by adding a specific NO donor (GSNO), reduces symbiont uptake (lower panel). Under heat stress (32 ºC – right panels), symbionts produce an elevated amount of NO which acts as a stress molecule that interferes with symbiosis establishment (upper panel). However, scavenging of excess NO by cPTIO restores infection efficiency in *Aiptasia* larvae (lower panel).

Suplementary Information

Statistical results for each experiment

Table S1 - Infection assay at elevated temperature (29 ºC). Statistical results of One-way ANOVA.

ANOVA table	SS	DF	MS	F (DFn. DFd)	P value
Treatment (between columns)	527.8	3	175.9	F (3. 8) = 10.73	P=0.0035
Residual (within columns)	131.1	8	16.39		
Total	658.9	11			

Table S2 - Infection assay at elevated temperature (29 ºC). Statistical results of Tukey´s multiple comparisons post-hoc test.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
26 ºC (24h) vs. 29 ºC (24h)	0.9	-9.685 to 11.49	No	ns	0.9924
26 ºC (24h) vs. 26 ºC (48h)	-10.24	-20.83 to 0.3419	No	ns	0.0578
26 ºC (24h) vs. 29 ºC (48h)	-14.62	-25.21 to -4.035	Yes	**	0.0095
29 ºC (24h) vs. 26 ºC (48h)	-11.14	-21.73 to -0.5581	Yes	*	0.0395
29 ºC (24h) vs. 29 ºC (48h)	-15.52	-26.11 to -4.935	Yes	**	0.0067
26 ºC (48h) vs. 29 ºC (48h)	-4.377	-14.96 to 6.209	No	ns	0.5744

Table S3 - Infection assay at elevated temperature (30 ºC). Statistical results of One-way ANOVA.

ANOVA table	SS	DF	MS	F (DFn. DFd)	P value
Treatment (between columns)	3847	3	1282	F (3. 8) = 50.49	P<0.0001
Residual (within columns)	203.2	8	25.4		
Total	4050	11			

Table S4 - Infection assay at elevated temperature (30 ºC). Statistical results of Tukey´s multiple comparisons post-hoc test.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
26 ºC (24h) vs. 30 ºC (24h)	12.84	-0.3348 to 26.02	No	ns	0.0561
26 ºC (24h) vs. 26 ºC (48h)	-29.7	-42.88 to -16.52	Yes	***	0.0004
26 ºC (24h) vs. 30 ºC (48h)	15.36	2.182 to 28.54	Yes	*	0.0239
30 ºC (24h) vs. 26 ºC (48h)	-42.54	-55.72 to -29.37	Yes	****	<0.0001
30 ºC (24h) vs. 30 ºC (48h)	2.517	-10.66 to 15.69	No	ns	0.9256
26 ºC (48h) vs. 30 ºC (48h)	45.06	31.88 to 58.24	Yes	****	<0.0001

Table S5 - Infection assay at elevated temperature (31 ºC). Statistical results of One-way ANOVA.

ANOVA table	SS	DF	MS	F (DFn. DFd)	P value
Treatment (between columns)	1029	3	342.9	F (3. 8) = 24.73	P=0.0002
Residual (within columns)	110.9	8	13.86		
Total	1140	11			

Table S6 - Infection assay at elevated temperature (31 ºC). Statistical results of Tukey´s multiple comparisons post-hoc test.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
26 ºC (24h) vs. 31 ºC (24h)	7.5	-2.236 to 17.24	No	ns	0.1407
26 ºC (24h) vs. 26 ºC (48h)	-14.66	-24.39 to -4.921	Yes	**	0.0058
26 ºC (24h) vs. 31 ºC (48h)	8.5	-1.236 to 18.24	No	ns	0.0888
31 ºC (24h) vs. 26 ºC (48h)	-22.16	-31.89 to -12.42	Yes	***	0.0004
31 ºC (24h) vs. 31 ºC (48h)	1	-8.736 to 10.74	No	ns	0.9868
26 ºC (48h) vs. 31 ºC (48h)	23.16	13.42 to 32.89	Yes	***	0.0003

Table S7 - Infection assay at elevated temperature (32 ºC). Statistical results of One-way ANOVA.

ANOVA table	SS	DF	MS	F (DFn. DFd)	P value
Treatment (between columns)	2221	3	740.4	F (3. 8) = 253.2	P<0.0001
Residual (within columns)	23.39	8	2.924		
Total	2244	11			

Table S8 - Infection assay at elevated temperature (32 ºC). Statistical results of Tukey´s multiple comparisons post-hoc test.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
26 ºC (24h) vs. 32 ºC (24h)	18.33	13.86 to 22.80	Yes	****	<0.0001
26 ºC (24h) vs. 26 ºC (48h)	-14.11	-18.58 to -9.636	Yes	****	<0.0001
26 ºC (24h) vs. 32 ºC (48h)	18.19	13.72 to 22.66	Yes	****	<0.0001
32 ºC (24h) vs. 26 ºC (48h)	-32.44	-36.91 to -27.97	Yes	****	<0.0001
32 ºC (24h) vs. 32 ºC (48h)	-0.1433	-4.614 to 4.328	No	ns	0.9996
26 ºC (48h) vs. 32 ºC (48h)	32.3	27.83 to 36.77	Yes	****	<0.0001

Table S9 - Daf-FM-DA fluorescence intensity in *B. minutum* cells subjected to 26 and 32 ºC for 24 and 48h. Statistical results of One-way ANOVA.

ANOVA table	SS	DF	MS	F (DFn. DFd)	P value
Treatment (between columns)	109183532	3	36394511	F (3. 12) = 204.3	P<0.0001
Residual (within columns)	2137492	12	178124		
Total	111321024	15			

Table S10 - Daf-FM-DA fluorescence intensity in *B. minutum* cells subjected to 26 and 32 ºC for 24 and 48h. Statistical results of Tukey´s multiple comparisons post-hoc test.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
26 ºc (24h) vs. 26 ºc (48h)	-1090	-1976 to -204.4	Yes	*	0.0151
26 ºc (24h) vs. 32 ºc (24h)	-4731	-5617 to -3845	Yes	****	<0.0001
26 ºc (24h) vs. 32 ºc (48h)	-6416	-7302 to -5530	Yes	****	<0.0001
26 ºc (48h) vs. 32 ºc (24h)	-3640	-4527 to -2754	Yes	****	<0.0001
26 ºc (48h) vs. 32 ºc (48h)	-5325	-6211 to -4439	Yes	****	<0.0001
32 ºc (24h) vs. 32 ºc (48h)	-1685	-2571 to -798.8	Yes	***	0.0005

Table S11 - Chlorophyll fluorescence intensity in *B. minutum* cells subjected to 26 and 32 ºC for 24 and 48h. Statistical results of One-way ANOVA.

ANOVA table	SS	DF	MS	F (DFn. DFd)	P value
Treatment (between columns)	55377191	3	18459064	F (3. 12) = 33.76	P<0.0001
Residual (within columns)	6560772	12	546731		
Total	61937963	15			

Table S12 - Chlorophyll fluorescence intensity in *B. minutum* cells subjected to 26 and 32 ºC for 24 and 48h. Statistical results of Tukey´s multiple comparisons post-hoc test.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
26 ºc (24h) vs. 26 ºc (48h)	133.9	-1418 to 1686	No	ns	0.9938
26 ºc (24h) vs. 32 ºc (24h)	2160	607.7 to 3712	Yes	**	0.0066
26 ºc (24h) vs. 32 ºc (48h)	4580	3027 to 6132	Yes	****	<0.0001
26 ºc (48h) vs. 32 ºc (24h)	2026	473.7 to 3578	Yes	*	0.0103
26 ºc (48h) vs. 32 ºc (48h)	4446	2893 to 5998	Yes	****	<0.0001
32 ºc (24h) vs. 32 ºc (48h)	2420	867.3 to 3972	Yes	**	0.0028

Table S13 - Daf-FM-DA fluorescence intensity in *Aiptasia* larvae subjected to 26 and 32 ºC for 24 and 48h. Statistical results of Dunn's multiple comparisons after Kruskal-Wallis test results of p=0.0226

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted P Value
26 ºC (24h) vs. 32 ºC (24h)	10.83	No	ns	0.3482
26 ºC (24h) vs. 26 ºC (48h)	-6.667	No	ns	>0.9999
26 ºC (24h) vs. 32 ºC (48h)	0.8333	No	ns	>0.9999
32 ºC (24h) vs. 26 ºC (48h)	-17.50	Yes	*	0.0132
32 ºC (24h) vs. 32 ºC (48h)	-10.00	No	ns	0.4811
26 ºC (48h) vs. 32 ºC (48h)	7.500	No	ns	>0.9999

Table S14 - Infection assay using GSNO 1mM as NO donor. Statistical results of unpaired t-test.

Unpaired t test	
P value	<0.0001
P value summary	****
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t. df	t=16.46. df=4

Table S15 - Infection assay using different concentrations of GSNO as NO donor. Statistical results of One-way ANOVA.

ANOVA table	SS	DF	MS	F (DFn. DFd)	P value
Treatment (between columns)	1306	3	435.3	F (3. 8) = 41.15	P<0.0001
Residual (within columns)	84.62	8	10.58		
Total	1391	11			

Table S16 - Infection assay using different concentrations of GSNO as NO donor. Statistical results of Tukey´s multiple comparisons post-hoc test.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Control vs. 0.05mM GSNO	-1.397	-9.901 to 7.107	No	ns	0.9504
Control vs. 0.1mM GSNO	2.11	-6.394 to 10.61	No	ns	0.8551
Control vs. 0.5 mM GSNO	24.16	15.65 to 32.66	Yes	****	<0.0001
0.05mM GSNO vs. 0.1mM GSNO	3.507	-4.997 to 12.01	No	ns	0.5764
0.05mM GSNO vs. 0.5 mM GSNO	25.55	17.05 to 34.06	Yes	****	<0.0001
0.1mM GSNO vs. 0.5 mM GSNO	22.05	13.54 to 30.55	Yes	***	0.0002

Table S17 - Infection assays subjected to 26 and 32 ºC for 24 and 48h using cPTIO as a NO scavenger. Statistical results of One-way ANOVA.

ANOVA table	SS	DF	MS	F (DFn. DFd)	P value
Treatment (between columns)	2599	5	519.8	F (5. 12) = 91.98	P<0.0001
Residual (within columns)	67.81	12	5.651		
Total	2667	17			

Table S18 - Infection assays subjected to 26 and 32 ºC for 24 and 48h using cPTIO as a NO scavenger. Statistical results of Tukey´s multiple comparisons post-hoc test.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
ctrl (26 ºC) vs. cPTIO before IA (26 ºC)	6.567	0.04723 to 13.09	Yes	*	0.048
ctrl (26 ºC) vs. cPTIO during IA (26 ºC)	21.82	15.30 to 28.34	Yes	****	<0.0001
ctrl (26 ºC) vs. ctrl (32 ºC)	32.75	26.23 to 39.27	Yes	****	<0.0001
ctrl (26 ºC) vs. cPTIO before IA (32 ºC)	24.14	17.62 to 30.66	Yes	****	<0.0001
ctrl (26 ºC) vs. cPTIO during IA (32 ºC)	30.3	23.78 to 36.82	Yes	****	<0.0001
cPTIO before IA (26 ºC) vs. cPTIO during IA (26 ºC)	15.25	8.734 to 21.77	Yes	****	<0.0001
cPTIO before IA (26 ºC) vs. ctrl (32 ºC)	26.18	19.66 to 32.70	Yes	****	<0.0001
cPTIO before IA (26 ºC) vs. cPTIO before IA (32 ºC)	17.57	11.05 to 24.09	Yes	****	<0.0001
cPTIO before IA (26 ºC) vs. cPTIO during IA (32 ºC)	23.74	17.22 to 30.26	Yes	****	<0.0001
cPTIO during IA (26 ºC) vs. ctrl (32 ºC)	10.93	4.407 to 17.45	Yes	**	0.0012
cPTIO during IA (26 ºC) vs. cPTIO before IA (32 ºC)	2.32	-4.199 to 8.839	No	ns	0.831
cPTIO during IA (26 ºC) vs. cPTIO during IA (32 ºC)	8.483	1.964 to 15.00	Yes	**	0.0091
ctrl (32 ºC) vs. cPTIO before IA (32 ºC)	-8.607	-15.13 to -2.087	Yes	**	0.0082
ctrl (32 ºC) vs. cPTIO during IA (32 ºC)	-2.443	-8.963 to 4.076	No	ns	0.8006
cPTIO before IA (32 ºC) vs. cPTIO during IA (32 ºC)	6.163	-0.3561 to 12.68	No	ns	0.0679