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

Effects of NO in coral symbiosis establishment

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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.

Keywords: *Aiptasia*, *Symbiodiniaceae*, endosymbiosis, nitric oxide signaling

Introduction:

The endosymbiotic relationship between photosynthetic dinoflagellates from the family Symbiodiniaceae and marine invertebrates plays a crucial role in coral reefs [1, 2, 3, 4,

5], which are ecosystems of immense ecological and economic importance [3, 4, 6]. 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 [7, 8]. 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 [9, 10].

Coral reef decline as a result of bleaching is threatening reefs worldwide. Typically, coral bleaching is triggered by biotic and abiotic stress [11, 12, 13, 14] including diseases, increased seawater temperature, acidification, salinity [13, 14, 15, 16, 17], UV radiation [18, 19], and pollution [14]. Failure to re-acquire symbionts within a relatively short time frame leads to coral death [20]. Thus, it is imperative to better understand the underlying mechanisms of cnidarian-dinoflagellate symbiosis in health and disease to protect these ecosystems.

The family of Symbiodiniaceae comprises several genera including *Symbiodinium* (formerly Clade A), *Breviolum* (formerly Clade B), *Cladocopium* (formely Clade C), *Durusdinium* (formerly Clade D), and *Gerakladium* (formerly Clade G), many of which are known for engaging in symbiotic relationships with cnidarians, such as corals and sea anemones [21, 22, 23, 5]. Interestingly, most reef-building corals acquire symbionts anew each generation by phagocytosing free-living microalgae into their endodermal cells [24, 25]. Thus, the successful acquisition of symbionts by aposymbiotic larvae is a fundamentally important process during coral development and for environmental adaptation by genetic variation [26]. Moreover, horizontal transmission of symbionts may also allow the host to adjust symbiont composition throughout development or in

63 response to the environment [26]. Thus, ex-*hospite* populations of Symbiodiniaceae are
64 essential for the horizontal transmission mechanisms [27].

65 Currently, our understanding of the initiation, maintenance and break-down of the host-
66 symbiont relationship at a molecular level is not well understood. This is mostly due to
67 that fact that most corals produce larvae only once annually [26], severely limiting
68 access to larvae for controlled laboratory experiments. To overcome this, the sea
69 anemone *Exaiptasia pallida* (commonly known as *Aiptasia*) has been established as a
70 model to investigate fundamental aspects of coral symbiosis [28, 29, 30]. Like the
71 majority of corals, *Aiptasia* also produces aposymbiotic larvae that must acquire their
72 symbionts horizontally [29, 30, 31]. Symbiont-host pairings are similar to those in
73 corals [32, 30] and powerful resources have been developed for this model including
74 defined and cultured symbiont strains and clonal *Aiptasia* lines [32, 33, 34], a protocol
75 for regular spawning induction in *Aiptasia* [34], genome sequences for host and
76 symbiont [35, 36, 37], transcriptomes of aposymbiotic and symbiotic life stages [38, 39,
77 30], symbiosis establishment assays [29, 31], as well as microinjection as the basis for
78 functional approaches [40]. Importantly, *Aiptasia* also recapitulates bleaching in
79 response to environmental stress like corals [41, 42, 43, 44].

80 Nitric oxide (NO), a ubiquitous lipophilic molecule, is thought to play an important role
81 during symbiosis break-down. Specifically, NO levels are elevated in response to
82 thermal stress in both the host and the symbiont, acting as a direct signaling molecule
83 between the symbiotic partners as a cytotoxic response that could initiate a bleaching
84 cascade [45, 28]. Moreover, the addition of exogeneous NO leads to bleaching in
85 *Aiptasia,* likely through an apoptotic-like pathway [46]. The enzyme that produces NO,
86 the nitric oxide synthase (NOS), has a number of isoforms that can either be
87 constitutively or inducibly expressed in various organisms, from plant to mammalian

88 cells [47, 48]. Specifically, the activity of inducible NOS (iNOS) is involved in stress
89 responses and can lead to the production of larger amounts of NO than the constitutive
90 isoforms of NOS in plant cells [50, 51], and it has been hypothesized that the inducible
91 NOS (iNOS) is upregulated in response to the elevated expression of the heat shock
92 protein 70 (Hsp70) in symbionts *in hospite* under heat stress, leading to bleaching of the
93 soft coral *Eunicea fusca* [49].

94 NO has two distinct functions that can be either harmful or beneficial depending on the
95 circumstances. On one hand, it can be cytotoxic, but on the other hand, it is involved in
96 important signaling pathways in organisms spanning the tree of life. For example, in
97 plants, NO is known to play a protective role against pathogenic microbes [52, 53]. In
98 diatoms, NO is important for cell-to-cell adhesion and biofilm formation [54]; and in
99 vertebrate cells, NO is thought to mediate vascular processes [55] as well as function as
100 a neurotransmitter [56]. However, when NO interacts with reactive oxygen species
101 (ROS), it converts into the potent and highly diffusible oxidant peroxynitrite, ONOO–
102 involved in cellular damage [57]. Moreover, studies have shown that cultured
103 dinoflagellate cells produce NO at much higher concentrations under thermal stress than
104 at normal temperatures, and these higher concentrations can prove harmful to symbiont
105 photophysiology [58]. As for *Aiptasia,* it was hypothesized that NO may elicit a specific
106 innate immune response against their symbiont, reminiscent of an auto-immune
107 condition [28].

108 Although NO has been implicated in cnidarian-dinoflagellate symbiosis break-down, it
109 is unclear whether NO plays a role during the initial uptake of dinoflagellates by
110 aposymbiotic larvae. Here, we use the *Aiptasia* model to test whether elevated
111 temperature influences symbiont uptake efficiency and how temperature- and chemical-
112 induced perturbations of NO levels influence symbiosis establishment in *Aiptasia*

113 larvae. Symbiont acquisition from the environment by aposymbiotic coral progeny is a
114 vital step during coral development. Thus, it is key to better understand the effects of
115 heat stress on symbiosis establishment at the cellular and molecular levels, with the
116 ultimate goal of helping to predict the long-term effects of elevated seawater
117 temperature due to global climate change.

118

119 Material and Methods:

120 Culture conditions and *Aiptasia* spawning induction

121 All experiments were performed with clonal axenic cultures of *Breviolum minutum*
122 strain SSB01 [32, 5] maintained in 0.22µm filter-sterilized IMK medium [59] at 26°C
123 and 25 µmol photons m^{-2} s^{-2} light on a 12L:12D cycle, as described previously [32],
124 unless stated otherwise.

125 Cultivation and spawning of *Aiptasia* from clonal strains CC7 and F003 were performed
126 as previously described previously [34]. *Aiptasia* larvae were kept in filter-sterilized
127 artificial seawater (FASW) in petri dishes at 26 °C on a 12 h light:12 h dark (12L:12D)
128 cycle until further use.

129 Symbiosis establishment in different temperature regimes

130 To test if high temperatures would affect the initial uptake of symbionts by the larvae,
131 we performed infection assays. Each experiment had a control temperature (26 ºC) and
132 one of the following treatment temperatures: 29, 30, 31 and 32 ºC. This temperature
133 range was chosen to understand at what point temperature increase starts to have an
134 effect on symbiosis establishment, taking in account thermal bleaching thresholds

observed in corals and *Aiptasia* anemones as well as for natural reefs [15, 60]. 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).

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

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

163 Confocal Laser Scanning Microscopy

164 To measure NO levels in *B. minutum* and *Aiptasia* larvae, the fluorescent NO indicator
165 4-amino-5-methylamino-20,70-difluorofluorescein diacetate (DAF-FM-DA; Molecular
166 Probes, Oregon USA) was used. In contrast to other NO markers, it is highly sensitive
167 (~ 3 nM detection limit), photostable, and cell permeable. Furthermore, DAF-FM-DA
168 has been successfully used to measure relatively low levels of NO present in diatoms
169 [54], Symbiodiniaceae [49, 58], and in adult *Aiptasia* [45, 46]. DAF-FM-DA stock
170 solution was diluted in DMSO.

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

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

178 All imaging and fluorescence analyses were carried out on a Leica SP8 confocal laser
179 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 [46, 58, 60]. For this, 300

204 larvae/ml at 4 dpf were distributed into 6-well plates with 5ml of FASW and infected
205 with *B. minutum* culture at a density of 10^5 cells/ml. GSNO was added to each well.
206 Infection lasted 24 h at 26 ºC under white fluorescent light at 20–25 µmol m^{-2} s^{-1} on a
207 12L:12D cycle. After this, we tested three other concentrations of GSNO, to understand
208 at what level NO starts to affect symbiosis establishment: 0.05 mM, 0.1 mM and 0.5
209 mM. The same infection protocol was used as described above. Experiments were
210 carried out in triplicates. After a 24 h infection, every sample was processed and
211 analyzed as described above.

212 Perturbation of NO level during symbiosis establishment using a NO 213 scavenger

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

221 1. 26 ºC with no addition of cPTIO
222 2. 26 ºC + 0.5 mM cPTIO added to *B. minutum* culture
223 3. 26 ºC + 0.5 mM cPTIO added to infection medium (*B. minutum* + larvae)
224 4. 32 ºC with no addition of cPTIO
225 5. 32 ºC + 0.5 mM cPTIO added to *B. minutum* culture

226 6. 32 ºC + 0.5 mM cPTIO added to infection medium (*B. minutum* + larvae)

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

230 Statistical analysis

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

234

235 Results:

236 Elevated temperature decreases symbiosis establishment efficiency in *Aiptasia*
237 larvae

238 To assess the effects of temperature on symbiosis establishment, infection assays were
239 performed at four different temperatures in comparison to the control temperature (26
240 ºC) (Fig. 1). We found no significant difference in the amount of infected larvae in
241 samples subjected to 29 ºC (~21% at 1 day post infection (dpi) and ~37% at 2 dpi) when
242 compared to the control (~22% at 1 dpi and ~33% at 2 dpi) at 1 and 2 dpi (Fig. 1A; 1
243 dpi p=0.9924 and 2 dpi p=0.5744; Supplementary tables S1 a, b). At 1 dpi, samples at
244 both 30 ºC (~21%) and 31 ºC (9%) also showed no significant difference in amount of
245 infected larvae when compared to the control samples (~34% and ~17% from each
246 experiment, respectively) (Figs. 1B and 1C; 30 ºC p=0.0561 and 31 ºC p=0.1407;
247 Supplementary tables S2 a, b and S3 a, b). However, at 2 dpi there were fewer infected

248 larvae from the samples at 30 ºC (~19%) and 31 ºC (~8%) when compared to the
249 control (~63% and ~31%, respectively) (Figs. 1B and 1C; 30 ºC $p<0.0001$ and 31 ºC
250 $p=0.0003$; Supplementary tables S2 a, b and S3 a, b). At 32 ºC, there were basically no
251 infected larvae (~0%) (at 1 and 2 dpi), while the amount of infected larvae in the control
252 samples were consistent with previous results (~18% at 1 dpi and ~32% at 2 dpi) (Fig.
253 1D; $p<0.0001$ Supplementary table S4 a, b). This suggest that at a certain threshold
254 elevated temperature negatively impacts symbiosis establishment in *Aiptasia* larvae.

255

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

258 Cultured *B. minutum* cells exposed to elevated temperature (32 ºC), for both 24 and 48
259 hours, produced significantly higher levels of NO (DAF-FM-DA fluorescence levels of
260 ~5079 A.U. for 24 h and ~6764 A.U. for 48 h) when compared to control samples (26
261 ºC; DAF-FM-DA fluorescence levels of ~348 A.U. for 24 h and ~1438 A.U. for 48 h)
262 (Figs. 2A-C) ($p<0.0001$; Supplementary tables S5a, b). Moreover, chlorophyll
263 autofluorescence significantly decreased in samples exposed to high temperature for 24
264 and 48 hours, when compared to control cells (Figs. 2A, C, and D) (24 h $p= 0.0066$ and
265 48 h $p<0.0001$; Supplementary tables S6 a, b).

266 Samples exposed for 48 hours to heat (32ºC) had a 33% increase in NO levels in
267 comparison to samples subjected for 24 hours to the same temperature (Fig. 2B)
268 ($p=0.0005$; Supplementary tables S5 a, b). Although a slight increase in NO was seen in
269 control cells from 24- to 48-hour exposure, chlorophyll autofluorescence remained at
270 the same level in these samples (chlorophyll fluorescence intensity of ~5755 A.U. for
271 24h and ~5620 A.U. for 48 h) (Fig. 2D). In contrast, for the heat-treated samples, we

272 observed a significant decrease in chlorophyll autofluorescence from 24 hours (~3595
273 A.U.) to 48 hours (~1175 A.U.) (Fig. 2D) (p=0.0028; Supplementary tables S6 a, b).

274 There was no significant difference in NO levels in *Aiptasia* larvae when exposed to
275 different temperatures (26 ºC and 32 ºC) or at different time points (24 h or 48 h) (Figs.
276 2E, F); Moreover, the localization of NO within larvae appeared similar at both
277 temperatures (Fig. 2E) and DAF-DM-DA fluorescence levels stayed between 71 and
278 126 A.U. (Fig. 2F) (p>0.05; Supplementary table S7). Taken together, this suggests that
279 elevated temperature increases NO production in cultured symbionts but not in *Aiptasia*
280 larvae.

281

282 Symbiosis establishment is negatively influenced by a specific NO 283 donor

284 To understand whether elevated levels of NO at control temperature (26 ºC) would
285 influence symbiosis establishment, we used our infection assays using GSNO as a
286 specific NO donor. For this we first performed an infection assay using the same GSNO
287 concentration (1 mM) as used in previous studies [46, 58, 61]. Our results showed that
288 when 1 mM of GSNO was added during infection, the percentage of infected larvae was
289 approximately six times lower than the control samples (~11% for samples treated with
290 GSNO versus ~66% for control samples) (Fig. 3A; p<0.0001 Supplementary table S8).

291 To understand at what level NO begins to affect symbiosis establishment, we performed
292 an infection assay with different concentrations of GSNO (0.05, 0.1 and 0.5 mM). There
293 was no significant difference in the percentage of infected larvae between control
294 (~25%), 0.05 mM GSNO (~28%), and 0.1 mM GSNO (~24%) samples (Fig. 3B, p>0.5

295 Supplementary tables S9 a, b). However, the percentage of larvae containing symbionts
296 was drastically reduced in samples treated with 0.5 mM GSNO (~2% infection)
297 ($p<0.0002$, Supplementary tables S9 a, b). Thus, increasing NO above natural levels
298 impairs symbiont uptake in *Aiptasia* larvae.

299

300 NO scavenging increases infection efficiency a under thermal stress, 301 and decreases it at control temperature

302 To understand the effect of NO depletion on symbiosis establishment, we added a
303 specific NO scavenger (cPTIO) to either the *B. minutum* medium before infection or to
304 the medium during infection (with both *B. minutum* and *Aiptasia* larvae). Interestingly,
305 at the control temperature (26 ºC), the addition of cPTIO and thus reduction of natural
306 NO levels, either before or during the infection, significantly decreases the percentage
307 of infected larvae (~28% and 13%, respectively) in comparison to the control (~35%)
308 (Fig. 4; $p=0.048$ and $p<0.0001$ Supplementary tables S10 a, b). However, depletion of
309 NO during the infection had a much greater negative effect on symbiosis establishment
310 when compared to the effect of treating symbionts alone before infection. Strikingly, we
311 find that at 32 ºC the percentage of larvae containing symbionts was higher with the
312 addition of cPTIO to the *B. minutum* medium before infection (~11%) in comparison to
313 the control (~2%) (Fig. 4; $p=0.0082$ Supplementary tables S10 a, b). However, the
314 proportion of infected larvae when cPTIO was added in the medium during infection
315 (~5%), showed no significant difference when compared to control ($p=0.8006$). This
316 indicates that the elevatedNO levels induced by increased temperature in *B. minutum*
317 negatively affect symbiosis establishment, yet at the same time, certain NO levels are
318 required for efficient symbiont acquisition by *Aiptasia* larvae.

319

320 # Discussion:

321 Temperature stress is known to disrupt the cnidarian-dinoflagellate symbiosis,
322 ultimately leading to bleaching and coral death [62, 63, 64]. Upon elevated temperature,
323 NO production is increased in various symbiotic animals, such as corals [65, 66, 67] and
324 anemones [45, 46, 68]; this also has been reported in Symbiodiniaceae that were freshly
325 isolated from either the coral *Madracis mirabilis* [68] or the anemone *Aiptasia sp.* [58,
326 69], and in axenic cultures of various lineages of Symbiodiniaceae [49, 58, 61]. More
327 specifically, previous studies suggest that temperature-induced disruption of symbiosis
328 is mediated and caused by *de novo* NO synthesis in both adult *Aiptasia* (as the host) and
329 symbiont [45, 46]. Thus, temperature-induced increase in NO levels is considered a
330 major factor for inducing coral bleaching.

331 In addition to symbiosis stability, symbiosis establishment in coral larvae has been
332 found to be impaired by temperature stress [70]. However, the role of NO during the
333 initiation of this fragile partnership between cnidarian hosts and their symbionts has not
334 been assessed to date. Here we show that elevated temperature negatively affects
335 symbiosis establishment in *Aiptasia* larvae. Moreover, NO levels increase upon
336 temperature stress (32°C) in *B. minutum* but not in *Aiptasia* larvae under similar
337 temperature conditions, suggesting that the failure to establish symbiosis at an elevated
338 temperature may be related to an increase in NO production by the *B. minutum* cells.
339 Accordingly, we find that the application of exogenous NO via the specific NO donor
340 GSNO, which is known as an intracellular NO reservoir and a NO carrier throughout
341 the cell that enhances the biological activity of NO [71], resulted in fewer larvae
342 containing symbionts at non-stressful temperature (26ºC). This again supports the idea

343 that high temperature induced NO production in symbionts has negative consequences
344 on the establishment of cnidarian-dinoflagellate symbiosis.

345 Interestingly, NO is not only considered to be an indicator of cellular stress, but it is
346 also an important signaling molecule involved in various processes in marine
347 organisms. For example, NO signaling has been implicated in the interactions between a
348 squid host and its beneficial bacterial partner during the initiation of symbiotic
349 colonization [72], regulating growth in microalgae [73, 74, 75], and it has been shown
350 to have antioxidant properties and photoprotective effects in microalgae under
351 environmental stresses [76, 77]. Likewise, in response to environmental change, NO can
352 modulate gene expression [78] and the activity of antioxidant enzymes [79], as well as
353 interact with phytohormones [80] and other signal molecules, like calcium and
354 hydrogen peroxide, as a defense mechanism against pathogens [81, 82].

355 Strikingly, using the specific NO scavenger cPTIO, we show that removal of NO during
356 symbiosis establishment has varying effects depending on the temperature. At elevated
357 temperature (32°C), NO scavenging from *B. minutum* cells before incubation with
358 *Aiptasia* larvae is beneficial for the efficiency of symbiosis establishment; however, this
359 effect is mitigated if NO is only removed during the infection period. In contrast, using
360 the same concentration of cPTIO to remove NO at optimal temperature (26°C) from *B.*
361 *minutum* cells reduces the efficacy of the initiation of symbiosis in *Aiptasia* larvae. This
362 effect is even more dramatic when NO depletion occurs during the encounter of
363 symbionts and *Aiptasia* larval hosts, indicating that under temperature conditions where
364 symbionts do not produce elevated NO levels, perturbing the natural NO levels has
365 profound negative consequences for establishing this symbiotic partnership. The
366 scavenging of temperature-induced increased NO in adult symbiotic *Aiptasia* has been
367 shown to reduce bleaching [46]. Here, we see a similar effect during the initiation of

368 this ecologically important symbiotic partnership. Moreover, we also provide evidence
369 for the first time that the reduction of NO below natural levels at non-stressful
370 temperature can also be prejudicial for symbiosis establishment in *Aiptasia* larvae.

371 Taken together, we show that NO plays two distinct roles during the establishment of
372 the cnidarian-dinoflagellate symbiosis. On the one hand, NO likely acts as an essential
373 signaling molecule during the initiation of symbiosis, but on the other hand, NO acts as
374 a stress molecule in symbionts under elevated temperatures resulting in the decreased
375 ability to establish symbiosis with their hosts.

376 To date, the molecular mechanism of how elevated NO levels impair symbiont
377 acquisition by *Aiptasia* larvae are unclear. However, during the steady-state of
378 symbiosis it is thought that heat stress causes the upregulation of heat-shock protein 90
379 (Hsp90), increasing NOS activity and therefore, NO production in the symbionts.
380 Increased production of NO is directly related to increased production of ROS in marine
381 organisms [83, 84, reviewed in 28], both of which ultimately lead to symbiont expulsion
382 (coral bleaching) from host cells [49]. Specifically, previous evidence suggests that high
383 temperature damages the thylakoid membranes of dinoflagellate chloroplasts and causes
384 an increase in reactive oxygen species (ROS) production [85, 86]. In turn, intracellular
385 ROS leads to a decrease of photosystem II (PSII) activity and to oxidative damage to
386 the chloroplast pigments [87]. Thus, we speculate that the decrease in chlorophyll
387 fluorescence seen here in *B. minutum* could be the consequence of the temperature-
388 induced increase in NO levels.

389 In summary, we propose that several related processes affect symbiosis establishment in
390 *Aiptasia* larvae under thermal stress (Fig. 5): (1) High temperature causes an increase in
391 NO production, which may lead to pigment damage indicated by decreased chlorophyll

fluorescence in the symbionts; (2) elevated NO levels act as stress molecules 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. More broadly, 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.

Author contribution:

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Formal analysis: Lilian J. Hill.

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Competing Interests

The authors have declared that no competing interests exist.

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662

663 ## Figure legends:

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

671

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

689

690 **Figure 3 – Symbiosis establishment is negatively influenced by a specific NO**
691 **donor.** Percentage of *Aiptasia* larvae infected by *B. minutum* (SSB01) using GSNO at
692 different concentrations as a NO donor after 24 h. A – Pilot infection assay with 1 mM
693 GSNO versus control condition. B – Minimum inhibitory infection assay with different
694 concentrations of GSNO. Asterisks represents significant difference: * $p<0.05$ and

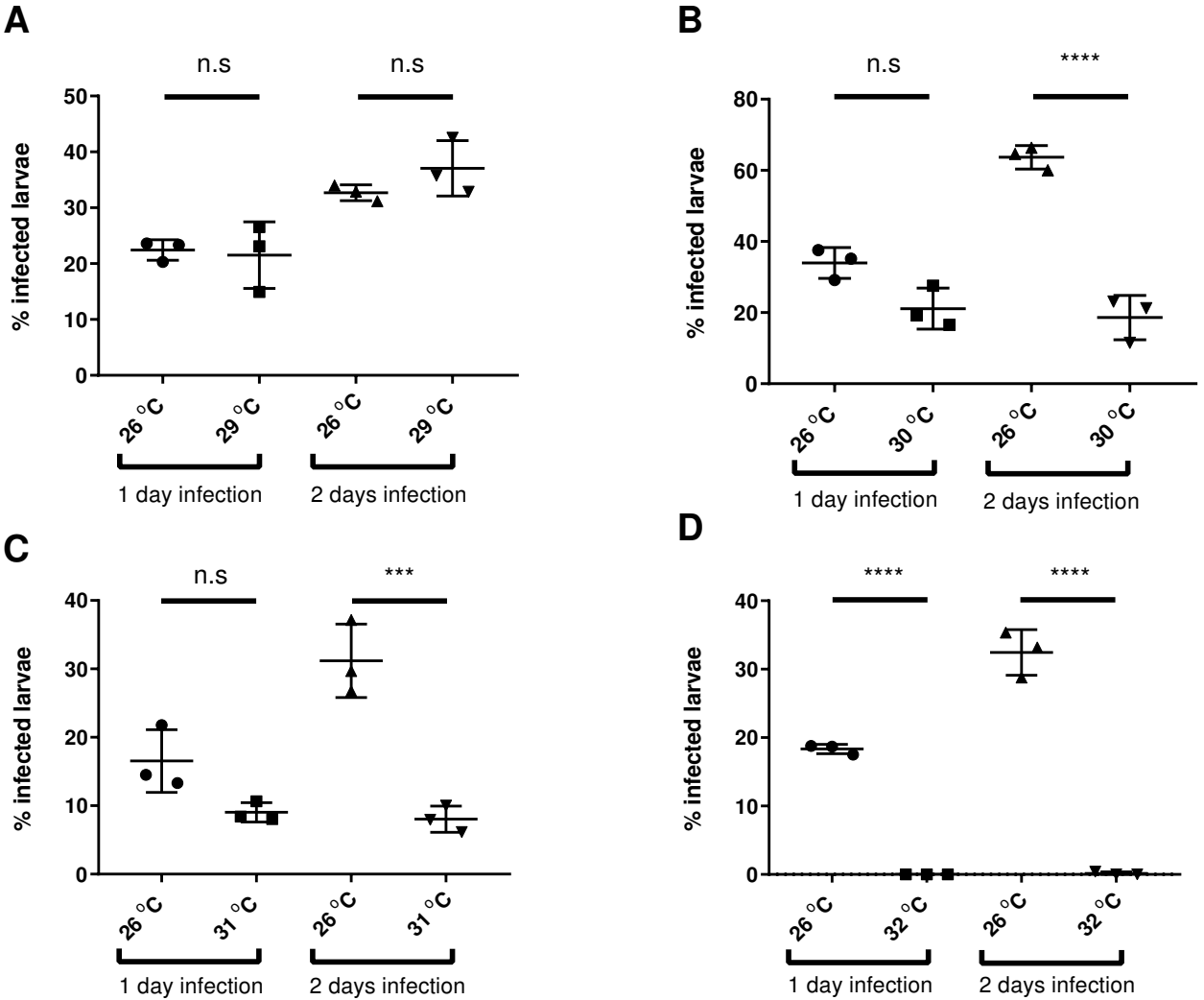
695 ****p<0.001 - ANOVA table can be found in supplementary information (S8 and S9).
696 n=3 for each experiment, all error bars are standard deviation.

697

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

708

709 **Figure 5 – Schematic model representing the influence of NO on symbiosis**
710 **establishment in *Aiptasia* larvae.** Under non-stressful conditions **(**26 ºC – left panels),
711 NO acts as an important signaling molecule for efficient symbiosis establishment (upper
712 panel). Reducing regular NO levels with a specific NO scavenger (cPTIO) decreases
713 infection efficiency (middle panel). Similarly, increasing regular NO levels by adding a
714 specific NO donor (GSNO), reduces symbiont uptake (lower panel). Under heat stress
715 (32 ºC – right panels), symbionts produce an elevated amount of NO which acts as a
716 stress molecule that interferes with symbiosis establishment (upper panel). However,
717 scavenging of excess NO by cPTIO restores infection efficiency in *Aiptasia* larvae
718 (lower panel).


A
n.s
n.s
% infected larvae
50
40
30
20
10
0
26 °C
29 °C
26 °C
29 °C
1 day infection
2 days infection
B
n.s
****
% infected larvae
80
60
40
20
0
26 °C
30 °C
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30 °C
1 day infection
2 days infection
C
n.s
***
% infected larvae
40
30
20
10
0
26 °C
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1 day infection
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% infected larvae
40
30
20
10
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26 °C
32 °C
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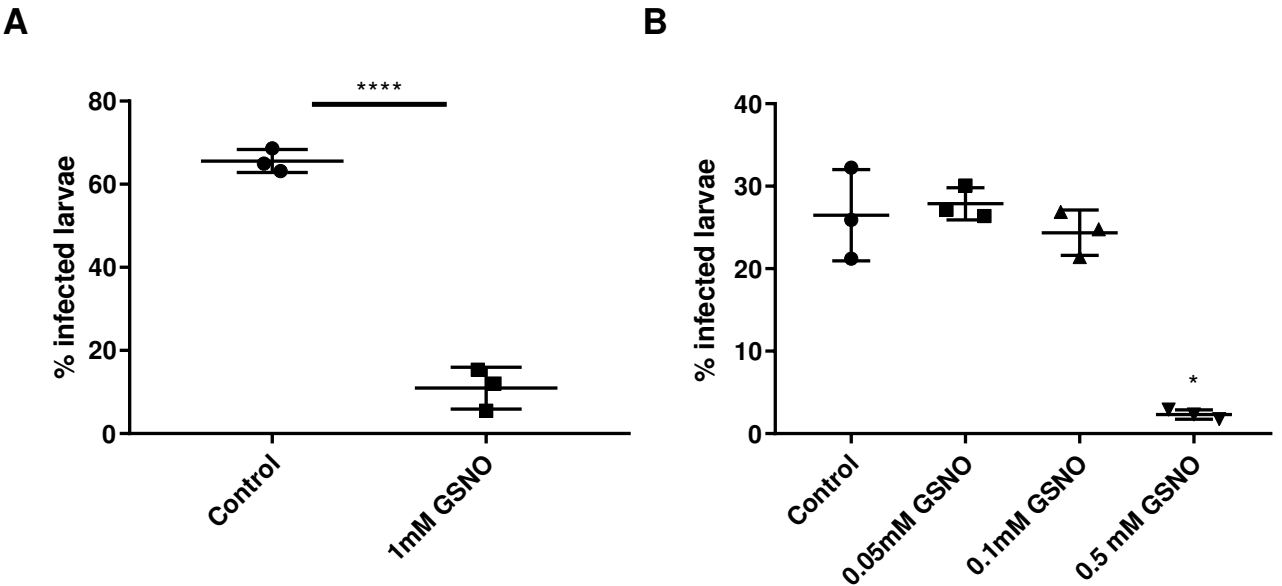
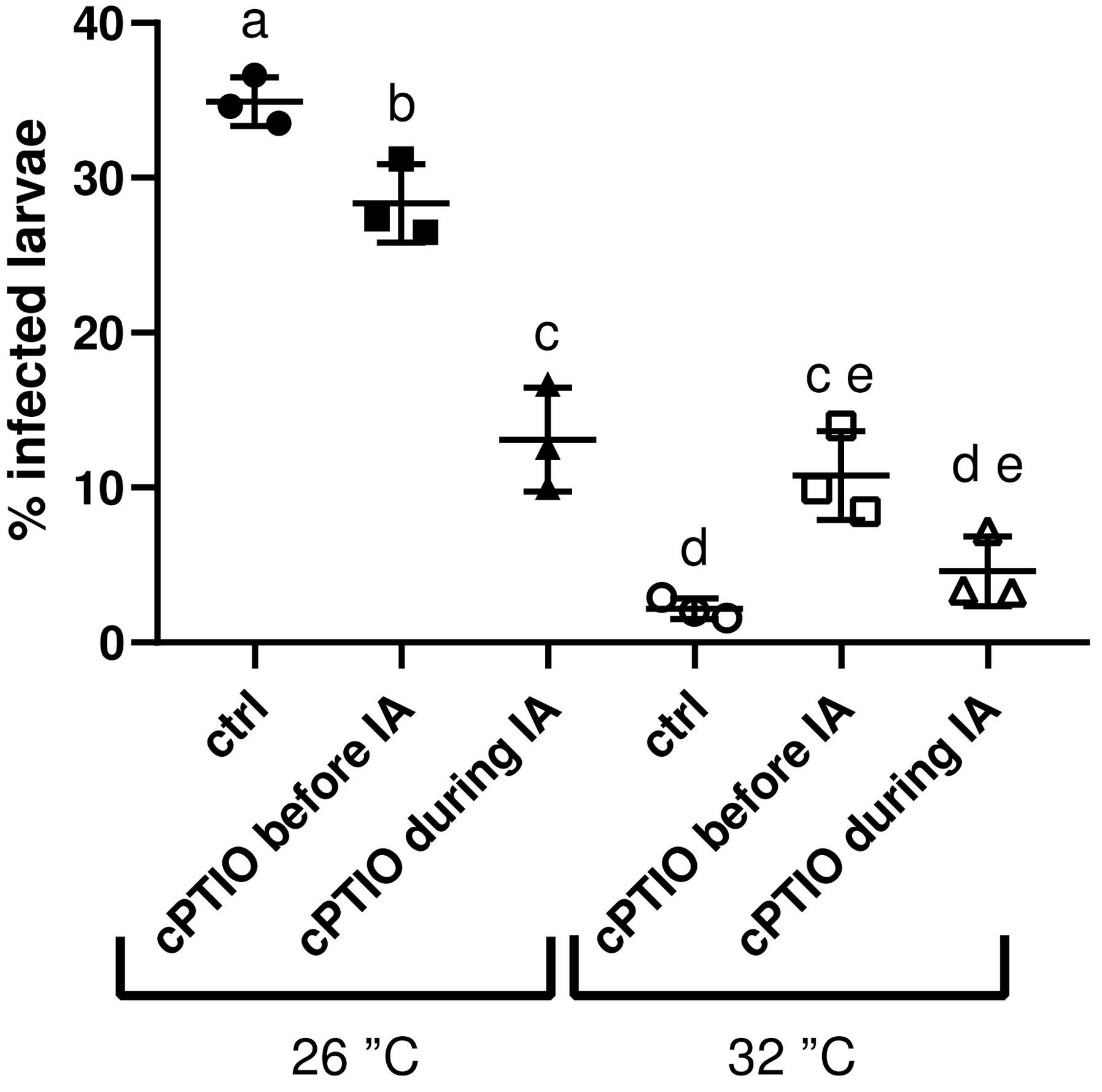
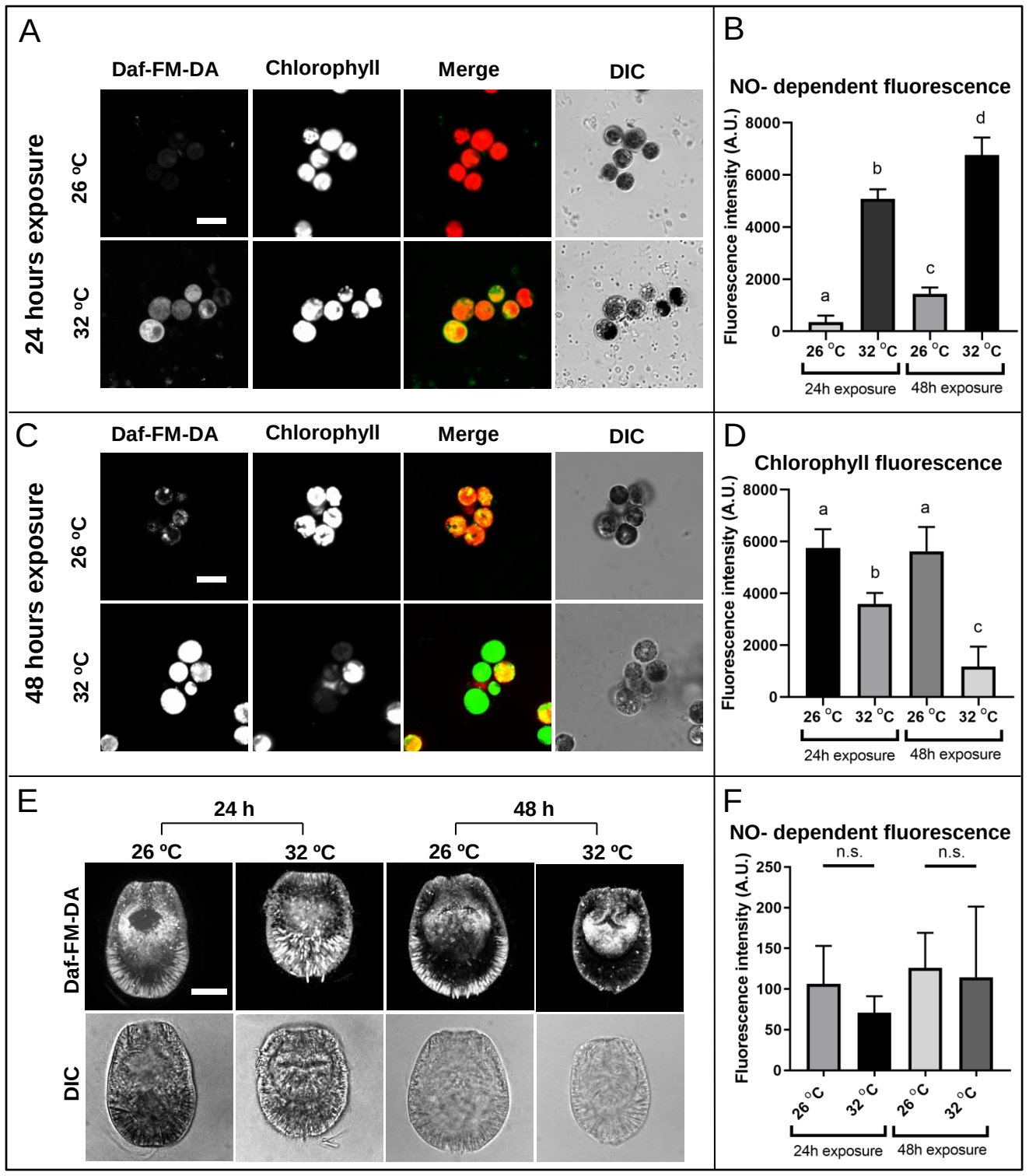
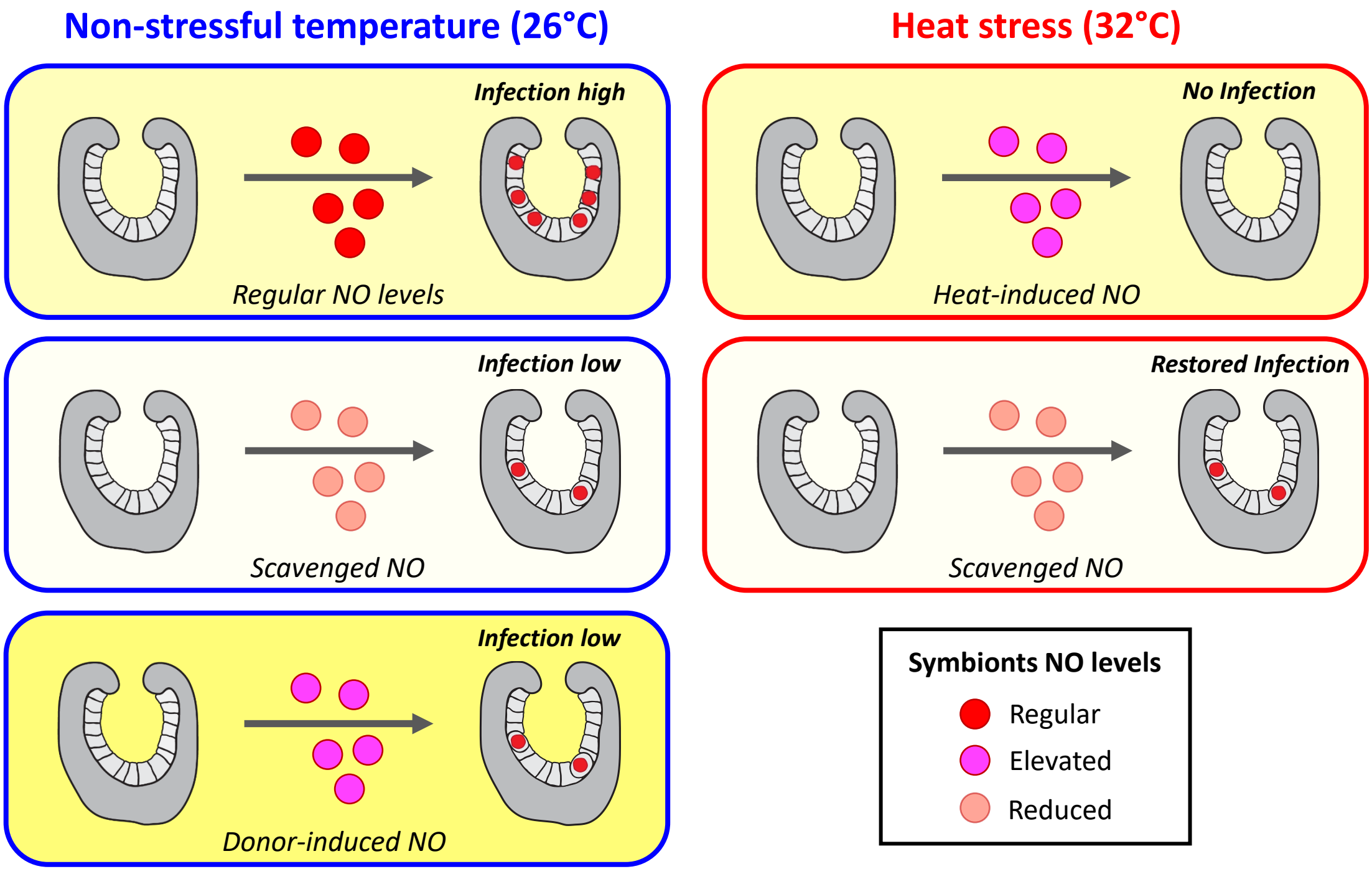
32 °C
1 day infection
2 days infection


A
****
80
60
40
20
0
% infected larvae
Control
1mM GSNO
B
40
30
20
10
0
% infected larvae
*
Control
0.05mM GSNO
0.1mM GSNO
0.5 mM GSNO


% infected larvae
40
30
20
10
0
a
b
c
d
c e
d e
ctrl
cPTIO before IA
cPTIO during IA
ctrl
cPTIO before IA
cPTIO during IA
26 ”C
32 ”C


A
Daf-FM-DA
Chlorophyll
Merge
DIC
24 hours exposure
26 ºC
32 ºC
B
NO- dependent fluorescence
Fluorescence intensity (A.U.)
8000
6000
4000
2000
0
a
b
c
d
26 °C
32 °C
26 °C
32 °C
24h exposure
48h exposure
C
Daf-FM-DA
Chlorophyll
Merge
DIC
48 hours exposure
26 ºC
32 ºC
D
Chlorophyll fluorescence
Fluorescence intensity (A.U.)
8000
6000
4000
2000
0
a
b
a
c
26 °C
32 °C
26 °C
32 °C
24h exposure
48h exposure
E
24 h
48 h
26 ºC
32 ºC
26 ºC
32 ºC
Daf-FM-DA
DIC
F
NO- dependent fluorescence
n.s.
n.s.
Fluorescence intensity (A.U.)
250
200
150
100
50
0
26 °C
32 °C
26 °C
32 °C
24h exposure
48h exposure


Non-stressful temperature (26°C)
Heat stress (32°C)
Infection high
Regular NO levels
No Infection
Heat-induced NO
Infection low
Scavenged NO
Restored Infection
Scavenged NO
Infection low
Donor-induced NO
Symbionts NO levels
Regular
Elevated
Reduced

Supplementary information

Statistical results for each experiment

Table S1 a. 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 S1 b. 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 ^{o}C (24h) vs. 29 ^{o}C (24h)	0.9	-9.685 to 11.49	No	ns	0.9924
26 ^{o}C (24h) vs. 26 ^{o}C (48h)	-10.24	-20.83 to 0.3419	No	ns	0.0578
26 ^{o}C (24h) vs. 29 ^{o}C (48h)	-14.62	-25.21 to -4.035	Yes	**	0.0095
29 ^{o}C (24h) vs. 26 ^{o}C (48h)	-11.14	-21.73 to -0.5581	Yes	*	0.0395
29 ^{o}C (24h) vs. 29 ^{o}C (48h)	-15.52	-26.11 to -4.935	Yes	**	0.0067
26 ^{o}C (48h) vs. 29 ^{o}C (48h)	-4.377	-14.96 to 6.209	No	ns	0.5744

Table S2 a. 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 S2 b. 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 ^{o}C (24h) vs. 30 ^{o}C (24h)	12.84	-0.3348 to 26.02	No	ns	0.0561
26 ^{o}C (24h) vs. 26 ^{o}C (48h)	-29.7	-42.88 to -16.52	Yes	***	0.0004
26 ^{o}C (24h) vs. 30 ^{o}C (48h)	15.36	2.182 to 28.54	Yes	*	0.0239

30 ^{o}C (24h) vs. 26 ^{o}C (48h)	-42.54	-55.72 to -29.37	Yes	****	<0.0001
30 ^{o}C (24h) vs. 30 ^{o}C (48h)	2.517	-10.66 to 15.69	No	ns	0.9256
26 ^{o}C (48h) vs. 30 ^{o}C (48h)	45.06	31.88 to 58.24	Yes	****	<0.0001

Table S3 a. 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 S3 b. 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 ^{o}C (24h) vs. 31 ^{o}C (24h)	7.5	-2.236 to 17.24	No	ns	0.1407
26 ^{o}C (24h) vs. 26 ^{o}C (48h)	-14.66	-24.39 to -4.921	Yes	**	0.0058
26 ^{o}C (24h) vs. 31 ^{o}C (48h)	8.5	-1.236 to 18.24	No	ns	0.0888
31 ^{o}C (24h) vs. 26 ^{o}C (48h)	-22.16	-31.89 to -12.42	Yes	***	0.0004
31 ^{o}C (24h) vs. 31 ^{o}C (48h)	1	-8.736 to 10.74	No	ns	0.9868
26 ^{o}C (48h) vs. 31 ^{o}C (48h)	23.16	13.42 to 32.89	Yes	***	0.0003

Table S4 a. 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 S4 b. 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 ^{o}C (24h) vs. 32 ^{o}C (24h)	18.33	13.86 to 22.80	Yes	****	<0.0001
26 ^{o}C (24h) vs. 26 ^{o}C (48h)	-14.11	-18.58 to -9.636	Yes	****	<0.0001
26 ^{o}C (24h) vs. 32 ^{o}C (48h)	18.19	13.72 to 22.66	Yes	****	<0.0001
32 ^{o}C (24h) vs. 26 ^{o}C (48h)	-32.44	-36.91 to -27.97	Yes	****	<0.0001

32 ^{o}C (24h) vs. 32 ^{o}C (48h)	-0.1433	-4.614 to 4.328	No	ns	0.9996
26 ^{o}C (48h) vs. 32 ^{o}C (48h)	32.3	27.83 to 36.77	Yes	****	<0.0001

Table S5 a. 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 S5 b. 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 S6 a. 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 S6 b. 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 S7. 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 S8. 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 S9a. 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 S9b. 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 S10a. 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 S10b. 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