The Deadly Trio: Do warming, acidification & deoxygenation destabilize the anemone-algae symbiosis?

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Keywords: hypoxia, hypercapnia, thermal tolerance, CTmax, Cnidaria, photosymbiosis

Abstract

Anthropogenic climate change is primarily driven by carbon dioxide release, which causes a domino effect of warming, acidification, and hypoxia in aquatic habitats. Using a fully-crossed experimental design, we investigated how exposure to this "deadly trio" of environmental stressors affects the sea anemone, *Exaiptasia diaphana* and its endosymbiotic dinoflagellates. To mimic conditions found on tropical reefs, we cycled hypoxic treatments between high oxygen saturation during the day (100 %) and hypoxia (40 %) at night. We increased ocean warming and acidification treatments to 30 °C and 7.7 pH in accordance with the "worst case" predictions in the IPCC 2023 report. After exposure for two weeks, we measured each anemone's metabolic rate, thermal tolerance, and heat tolerance plasticity. We also assessed algal density and photosynthetic efficiency, both of which remained consistent across all treatments, indicating negligible stress levels and a healthy symbiosis. Interestingly, metabolic depression was observed in anemones exposed to the deadly trio, which is likely an energy conservation strategy due to its co-occurrence with increased thermal tolerance. Furthermore, we found an interaction between ocean acidification and nightly hypoxia as combined exposure improved heat tolerance plasticity, potentially because increased carbon dioxide enhanced photosynthetic activity. We therefore conclude that *E. diaphana* will survive short-term exposure to the "worst case" levels of deadly trio stressors. To verify this interpretation, we determined their acute stress tolerance, finding that they can withstand termporary exposure to more than 34 °C seawater, a minimum pH of 6.01 and anoxia for more than 4 days. These conditions greatly exceed worst-case predictions, confirming that this species should survive acute exposure to the deadly trio, potentially due to their symbiosis. These findings highlight the importance of considering all three stressors in climate change ecophysiological studies.

1. Introduction

Ocean warming, acidification and deoxygenation are the main climate change related stressors affecting marine ecosystems and have thus been given the somber moniker, the "deadly trio" due to their interconnectivity (Calvin et al., 2023; Götze et al., 2020). Much of the carbon dioxide (CO_2) emitted by anthropogenic activities is absorbed by the oceans, decreasing the pH in a process termed hypercapnia (Gattuso et al., 2015). Simultaneously, increased atmospheric CO_2 levels warm the planet via the greenhouse effect (Calvin et al., 2023). Warming leads to an expansion of, and increase in the number of marine hypoxic areas because O_2 solubility decreases (Matear & Hirst, 2003; Woods et al., 2022) and O_2 consumption increases at higher temperatures (Allan et al., 2006; Glazier, 2015; Killen et al., 2010), even though O_2 diffusibility increases (Verberk et al., 2011). Increased respiration and O_2 uptake also lead to increased CO_2 output, meaning hypoxia and hypercapnia often co-occur (Woods et al., 2022). So far, there have been efforts to research the effects of the stressors individually (e.g. Cereja, 2020; Figuerola et al., 2021; Guinotte & Fabry, 2008; Spicer, 2014; Vinagre et al., 2019) or in pairs (e.g. Bennett et al., 2017; Gobler & Baumann, 2016; Klein et al., 2017; Steckbauer et al., 2015; Tripp-Valdez et al., 2019), but investigations into all three stressors are extremely limited (Götze et al., 2020; Lucey et al., 2020; Pimentel et al., 2023), likely because they require large numbers of samples and complicated experimental designs/equipment (e.g. Cornwall & Hurd, 2016). Due to these hurdles, understanding interactions between these stressors has remained elusive, despite their interdependency, but filling this gap in knowledge is crucial if we are to understand how biological communities will change due to anthropogenic pressure.

The deadly trio stressors have been shown to affect the performance and fitness of marine organisms in numerous ways, when investigated individually and in pairs. Warmer water leads to exponential increases in the rates of biological processes, which in turn increases an ectotherm's need for cellular energy. Organismal functions such as locomotion, growth and reproduction are primarily fueled by aerobic respiration, which depends on O_2 availability and an organism's ability to deliver that oxygen to its tissues (Duncan et al., 2023; Woods et al., 2022). Therefore, warming oceans cause anincreased demand for O_2, which can lead to a mismatch between the amount of O_2 an ectotherm needs to acquire to sustain aerobic activity and the amount it can obtain from the surrounding seawater (Pörtner, 2001; Seebacher et al., 2014). An insufficient supply of O_2 to maintain metabolic functions could determine thermal limits (Cereja, 2020; Pörtner et al., 2017) and numerous investigations support this premise (summarized in Pörtner et al., 2017), however its general applicability is also contested (e.g. Jutfelt et al., 2018; Verberk et al., 2016) and factors beyond oxygen dynamics also likely play a role in thermal tolerance (Laetz & Verberk, 2024).

Hypercapnia also affects metabolism and oxygen delivery since high internal CO_2 levels can impair the capacity of oxygen transport proteins and their affinity for oxygen-binding (Coates et al., 2022), causing changes in circulation and internal acid-base regulation (Lehtonen & Burnett, 2016). Hypercapnia and hypoxia have been shown to synergistically and negatively affect survival, growth, activity, and metabolism (Gobler & Baumann, 2016; Gu et al., 2019; Sui et al., 2016), amongst other key organismal functions, although not all species are affected equally (summarized in Woods et al., 2022). Hypercapnia and warming also have been linked to reduced growth and lower survival in marine organisms like corals, demonstrating either additive or synergistic effects (Carbonne et al., 2022; Prada et al., 2017) although they may also interact antagonistically in other marine ectotherms (Lefevre, 2016).

Stress tolerance is not a fixed property of a species, and variation in environmental conditions can lead to changes in tolerances through acclimation processes (Stewart et al., 2023). Heat tolerance is frequently measured by determining an organism's Critical Thermal maximum (CTmax): a physiological threshold, commonly defined as the loss of muscle control causing an inability to flee the stressor (Cowels & Bogert, 1944) or the point at which an organism enters a heat coma (Lutterschmidt & Hutchison, 1997). Prolonged exposure to the CTmax temperature is often lethal. Exposure to elevated (but non-lethal) thermal regimes, can cause thermal acclimation processes such as heat hardening, an adaptive response that confers increased heat tolerance (Georgoulis et al., 2021; Stewart et al., 2023). One way to measure acclimation capacity as a proxy for heat tolerance plasticity is via Acclimation Response Ratios (ARRs, Claussen, 1977), which are defined as the rise in CTmax for each degree of acclimation (Hutchison, 1961; Claussen, 1977; Morley et al., 2019). Acclimation capacity can be modulated or mitigated by other factors, such as hypoxia, because O_2 limitation is expected to cause a decrease in thermal tolerance and consequently an inability to acclimate to elevated thermal regimes (Healy & Schulte, 2012).

To investigate how acute exposure to the deadly trio of environmental stressors affected organismal performance, we examined a control treatment (current average levels of each stressor), specimens exposed to individual deadly trio stressors, pairs of stressors and the combination of all three (deadly trio) resulting in eight treatments total, yielding a fully factorial design. Temperature and pH levels were chosen based on the "worst case" predictions presented in the report published by the International Panel on Climate Change (IPCC) in 2023 (Calvin et al., 2023). While temperature and pH in tropical environments are relatively stable on a short-term basis (days to weeks), O_2 levels fluctuate greatly within 24 hour periods (Bianchi et al., 2013; Johansson et al., 2006). This is mainly due to photosynthetic activity which releases O_2 during the day but ceases at night. Since aerobic organisms constantly consume O_2 but photosynthesis only generates O_2 during the day, many shallow coastal areas experience nightly hypoxia (Gobler & Baumann, 2016; Woods et al., 2022). Therefore, in this study, we

chose to allow O_2 saturation to fluctuate according to daily cycles, mimicking the natural drops that tropical reef habitats experience (Deleja et al., 2022; Nelson & Altieri, 2019), although we made these hypoxic swings more intense for the treatments where we investigated hypoxia (Fig. 2). We therefore refer to this as nightly hypoxia. This design allowed us to disentangle the individual effects of each stressor and evaluate if they function synergistically, neutrally or antagonistically.

Marine ectotherms that form photosymbioses (i.e. animals that have developed a symbiosis with single-celled algae), provide an ideal lens with which to empirically study the physiological effects of exposure to the deadly trio for a few reasons. Their endosymbiotic algae perform photosynthesis, releasing O_2 that can be used to maintain aerobic respiration and potentially prevent declining thermal tolerance due to O_2 limitation). Furthermore, the increased amounts of CO_2 and its associated inorganic carbon ions in seawater due to hypercapnia may improve carbon acquisition by the algae, thus improving photosynthetic output (Suggett et al., 2012), meaning exposure to the deadly trio could benefit the algae. Because the endosymbiotic algae usually provide the host animal with O_2 and energy in the form of photosynthates, exposure to hypercapnia could also benefit the animal host. Furthermore, numerous photosymbioses are key reef building species (stony coral, giant clams, some sponges) that comprise the foundation of coral reef ecosystems, on which millions of species and humans depend (Costanza et al., 1997; West & Salm, 2003). These ecosystems are often considered to highly vulnerable to climate change, so understanding how the deadly trio affect organismal performance in photosymbiotic animals is of critical biological importance.

We conducted these investigations in the photosymbiotic model species *Exaiptasia diaphana* (Rapp, 1829), (Cnidaria: Anthozoa), a (sub)tropical sea anemone species commonly used as a model system for other cnidarian-zooxanthellae symbioses, such as reef-building corals (Dungan et al., 2020; Roberty et al., 2024). Photosymbiotic cnidarian species (corals, anemones, many jellyfish, etc.) such as *E. diaphana* host dinoflagellate algae (family Symbiodiniaceae) within their gastrodermal tissue, called zooxanthellae once incorporated in an animal host (Meyer & Weis, 2012), allowing us to measure a number of algal and animal performance indicators.

To examine algal performance, we examined the maximum quantum yield of photosystem II (F_VF_M), using Pulse-Amplitude Modulated (PAM) fluorometry. This non-invasive technique gives an indication of how efficient photosystem II is functioning, and has been widely used to detect algal stress responses when lower efficiency values are detected (Schreiber, 2004). Algal cell density is frequently used as an essential indicator of the health and stability of the symbiotic relationship between cnidarians and their zooxanthellae (Davy et al., 2012), with decreases in algal density (bleaching) corresponding to signs of cellular stress and symbiosis breakdown (Weis, 2008). We also measured algal cell reproduction using the mitotic index, which is defined as the percentage of zooxanthellae within a sample in a paired

stage of cytokinesis (Wilkerson et al., 1988). Changes in the mitotic index would indicate changes in algal reproduction due to stress exposure (Suharsono & Brown, 1992). Lastly, we investigated algal ultrastructure to visually assess if deadly trio exposure altered algal morphology, since exposure to high temperatures (30 °C - 32 °C) has been linked to organelle distortion and the appearance of apoptotic bodies (e.g. Strychar et al., 2004). Under severe stress exemplified by long-term exposure to high temperatures, the cell membranes become crumpled, and the cell wall begins to break, and internal organelles start to degrade (Zhang et al., 2018).

To examine anemone performance, we examined a variety of physiological and behavioral metrics. Closed-circuit respirometry experiments allowed us to determine if photosynthetic O_2 production was enough to meet or exceed the aerobic demands of the anemone. Differences in an anemone's O_2 scavenging behavior when subjected to the deadly trio of stressors could indicate a shifting metabolic strategy in animals. The anemones were also subjected to an acute hyperthermal stress test to determine their CTmax. Differences in CTmax could indicate if certain deadly trio stressors or combinations thereof compromise or enhance thermal tolerance. Finally, ARRs were also calculated to examine if these animals are able to increase their thermal tolerance via plasticity.

2. Methodology

2.1 Specimen husbandry

For all treatments, artificial seawater was prepared by dissolving Instant Ocean salts (Spectrum Brands, USA) in distilled water to achieve a salinity of 35 ± 1 PSU. Seawater was bubbled with pressurized air that was integrated into the climate-controlled room (set to maintain 25 °C) in which these experiments took place. Specimens were donated by private aquarists who acquired them accidentally alongside purchases of other tropical specimens. They were exposed to 50-60 μmol m^{-2} s^{-1} full-spectrum lighting for 12 hours a day (6500K LEDs, Beijing Yuji International Co. Ltd., China). They were fed ad libitum three times a week with freshly hatched *Artemia sp.* shrimp.

2.2 Experimental setup

Each experimental tank contained 51 liters of artificial seawater. The temperature, pH and salinity were monitored by an Apex controller (Neptune Systems, USA). Each specimen was randomly assigned to one of eight treatment groups: control; the deadly trio stressors individually: hyperthermia - HT, hypercapnia - HC, nightly hypoxia - NH; and in all possible combinations (Fig. 1). Two individuals were randomly assigned to each experimental tank for the following experiments.

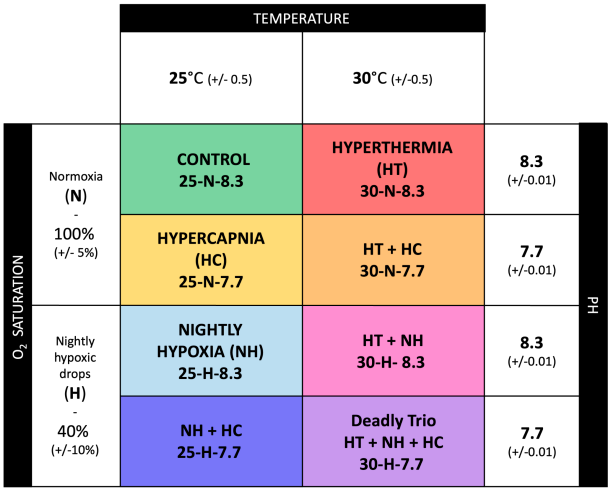

Figure 1. Schematic of experimental treatments

The experimental period lasted two weeks including the four days it took to acclimate the specimens (Supplementary Fig. 1). Aquarium water heaters were used for thermal control of all the high temperature (HT) treatment tanks (300W, Superfish - Aquadistri, Global). The temperature was increased by 1 °C per day until it reached 30 °C. The water pH was lowered from an average of 8.3 to 7.7 by 0.2 points decrease per day via CO_2 gas; which was bubbled in the water. We programmed the Apex aquarium controller to maintain and monitor our target temperatures and pH levels using real time temperature and pH readings from Apex temperature and lab grade pH probes that were calibrated once a week to ensure stable readings (Neptune Systems, USA). Nightly oxygen saturation drops were achieved with the use of Sodium Sulfite (SSI), a non-toxic salt that scavenges oxygen from water to form sodium sulfate, inducing hypoxia (Pinzaru & Dehelean, 2024;). SSI was added manually once a day at 17:00. We gradually increased the concentration over three days starting from 0.075 g/L to the final concentration of 0.125 g/L, which effectively induced a drop in O_2 level down to 40 ± 0.49 % O_2 saturation (Fig. 2). This was timed to occur when the lights turned off. Following the wave of nightly hypoxia, the oxygen saturation was allowed to increase back up to control conditions 100 % (Fig 2).

2.3 Carbon chemistry

The pH was measured daily for each treatment tank using a HI98190 pH/ORP meter (Hanna instruments, USA). Calibration was carried out once a week with National Institute of Standards and Technology (NIST) standard solutions (Hanna instruments, USA). The average pH_{NIST} values for each treatment were then converted to the "total hydrogen ion concentration scale" (pH_T) following Badocco et al. (2021). A multiparameter photometer (HI83399 - Hanna instruments, USA) was used to measure alkalinity using their seawater alkalinity reagent (HI755-26). Total alkalinity and pH_T were used to calculate the other carbon chemistry parameters via the R package "seacarb" (Gattuso et al., 2024) (Table

1), using R (v. 4.1.2; R Development Core Team, 2021) and RStudio (v. 2022.07.2+576; RStudio Team, 2022). The package "tidyverse" (Wickham et al., 2019) was used for data handling.

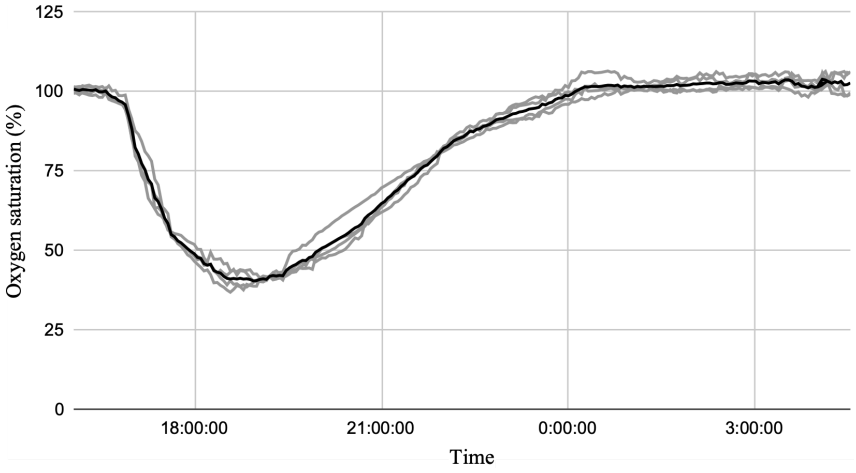

Figure 2. Oxygen depletion during the nightly hypoxic treatments. Following the addition of sodium sulfite, oxygen saturation dropped to ~40 % saturation. Moderate aeration was provided to reoxygenate the seawater, mix it, and ensure gradients did not form.

Table 1. Average carbon chemistry conditions for each treatment. Abbreviations: HC - hypercapnia (pH 7.7), HT - hyperthermia (30°C), Ω_{arag} - aragonite saturation state, pCO2 - the partial pressure of CO_2 in µatm, pH_T - pH according to the total hydrogen ion concentration scale, NH - nightly hypoxia (40 %), TA - the total alkalinity in µmol kg^{-1}, Temp - the temperature in °C for each treatment.

	Control	HC	HT	NH	HC + HT	HC + NH	HT + NH	Deadly trio
Temp	25 °C	25 °C	30 °C	25 °C	30 °C	25 °C	30 °C	30 °C
pH_T	8.320	7.690	8.351	8.338	7.746	7.726	8.331	7.713
pCO_2	128.050	813.523	118.933	113.650	738.266	728.371	162.177	766.166
TA	1733.314	1800.053	1887.366	1640.528	1914.006	1771.160	1622.082	1817.084
Ω_{arag}	4.153	1.449	5.442	4.006	2.083	1.531	3.796	1.850

2.4 Photosynthetic efficiency, algal cell density, and mitotic index

The following methodologies were carried out on one of the specimens in each aquarium. The maximum quantum yield of photosystem II (PSII) was measured using a Junior PAM pulse amplitude modulated fluorometer (Walz GmBH, Germany). Prior to measurement, anemones underwent a 15-minute dark acclimation period before the maximum quantum efficiency of photosystem II (F_v/F_m) was recorded for all specimens.

Subsequently one tentacle was vivisected from each individual in order to capture fluorescence images displaying the presence of algae. The samples were then mounted on microscope slides. Fluorescence images were taken with a Nikon Eclipse E800 epi-fluorescent microscope (Nikon USA, USA) using custom filter cubes with the following peak excitation, dichroic and long-pass emission: 425 nm, 515 nm, 610 nm for chlorophyll fluorescence (algal imaging) (Chroma, USA). Images were analyzed using Fiji (Schindelin et al. 2012) and the plugin 3D-AMP (Laetz et al., 2017), which counted the total area covered by chlorophyll and the total endoderm (algae-containing tissue) in each sample, to provide an estimation of the total area covered that was filled with algae (%). The mitotic index was also calculated using images of chlorophyll fluorescence. First, the number of algae present in each sample was estimated by dividing the total area covered by chlorophyll by the average size of an algal cell (also measured with 3D-AMP). Then, the number of algae undergoing cytokinesis was counted visually and the mitotic index was calculated as the percentage of dividing algae out of the total number of algae in each picture (as described in Wilkerson et al., 1988).

Lastly, tentacles of *E. diaphana* from the control treatment and the "deadly trio" treatment were fixed in 2.5 % glutaraldehyde (Cat. no. G5882, Merck, Germany). Post-fixation staining was performed with OsO_4 followed by an overnight staining with uranyl acetate. Samples were dehydrated with a graded series of ethanol washes and embedded by slowly replacing the ethanol with epon resin. Samples were mounted on copper grids, which were stained with uranyl acetate (1 %) and lead citrate for 3 minutes each. Imaging was performed on a CM12 electron microscope (Phillips, the Netherlands). Algal ultrastructure was examined visually for signs of cellular abnormalities or damage.

2.5 Metabolic rate

The second specimen from each tank was used in the following experiments. Following two weeks of exposure to a given treatment, the metabolic rate of each anemone was measured via closed-circuit respirometry. We placed each anemone in a glass chamber (volume of 2 or 22.2 mL) containing an oxygen microsensor (sensor type PSt3, PreSens, GmBH, Germany). They were allowed to recover from the stress of handling inside the chambers with the lids open for 30 minutes before measurements. We considered full extension of the tentacles and attachment to the surface of the chamber as signs of relaxation, preliminary testing demonstrated that 30 minutes was enough time to accommodate this. After 30 minutes, we sealed the chamber with 100 ± 3 % saturated seawater and the specimen inside. We then used a Fibox Trace LCD 4 (PreSens GmBH, Germany) to measure the oxygen saturation, temperature, and atmospheric pressure during the experiment. We measured the oxygen saturation every 10 minutes for 3 hours, during which specimens were exposed to full-spectrum lighting at an intensity of 50-60 μmol m^{-2} s^{-1} (Superfish SLIM LED 45). Prior to experimentation, the oxygen meter was calibrated

using nitrogen gas (0 % oxygen) and freshly aerated sea water (100 % saturation). To calculate their metabolic rates, specimens were weighted, following the CTmax experiment (detailed below).

To calculate the amount of O_2 in the chamber when the experiment began, we determined the amount of water actually in each chamber by subtracting the volume of the specimen (derived from the specimen's mass and a density proxy (1.13 g/L) measured using larger anemones (Laetz & Verberk, 2024) from the total chamber volume. We then converted oxygen saturation values to concentration values (mgO_2/L) using an online calculator (https://water.usgs.gov/water-resources/software/DOTABLES/). We could then determine how much O_2 (mg) was consumed (or produced) during experimentation. This was divided by the experiment duration producing the mg O_2 consumed/produced per hour (mgO_2h^{-1}). Since metabolic rate scales allometrically with body mass, we also derived a scaling exponent for this species (detailed in Laetz et al., 2024; Laetz & Verberk, 2024). Our subsequent metabolic rates are therefore presented in $mgO_2\ g^{-0.61}\ h^{-1}$. This allows us to examine changes in metabolic rates while excluding variation caused by body mass since it has already been corrected for via the scaling exponent. The experiment was carried out at the respective treatment temperature of each treatment group.

2.6 Critical thermal limits and acclimation response ratios

Following the metabolic rate measurements, each specimen was placed in a glass container with freshly aerated seawater from their treatment tanks. These containers were placed within a temperature controlled water bath. Anemones were allowed to recover from potential stress of handling for approximately 30 minutes, before we began gradually increasing the water temperature at a rate of 1 °C every 10 minutes. We observed each anemone until it displayed two behaviors consistent with heat stress, defined here as a sudden retraction of the anemone's tentacles and detachment of its pedal disk from the substrate. Unlike other studies examining heat stress behavior in anemones (Laetz & Verberk, 2024; Qin et al., 2024), the specimens in this study did not display these behaviors sequentially, so both behaviors were considered necessary before the CTmax temperature was recorded.

As a proxy for heat tolerance plasticity, ARRs were calculated using the average CTmax of individuals exposed to 25 °C treatments and the average CTmax of individuals exposed to 30 °C treatments. This was calculated for all four combinations of pH and O2, according to Equation 1).

$$\frac{CTmax\ 30\ °C - CTmax\ 25\ °C}{30\ °C - 25\ °C}$$

Equation 1: Calculating Acclimation Response Ratios, a proxy for thermal plasticity. Perfect acclimation (ARR = 1) would mean that for every 1 °C of acclimation the specimen gains 1 °C of thermal tolerance (CTmax) (Claussen, 1977).

2.7 Statistical analyses

Data analysis was performed using R (v. 4.1.2; R Development Core Team, 2021) and RStudio (v. 2022.07.2+576; RStudio Team, 2022). The package "tidyverse" (Wickham et al., 2019)was used for data handling. The packages "lme4" and "lmerTest" were used for model building (Bates et al., 2015; Kuznetsova et al., 2013). To analyze the effect of the treatments on F_V/F_M, algal density, mitotic index, metabolic rate and critical thermal limits, outliers were first identified and subsequently removed based on the interquartile range. We then used the function "glmulti" (Calcagno, 2020) to perform a multi-selection analysis of linear models (LMs) including "temperature", "pH" and "oxygen" as predictor variables. We selected the best model for each as the one with the lowest Akaike Information Criterion (AICc). All pairwise comparisons within the models were then examined by computing estimated marginal means via the package "emmeans" (Lenth, 2024). All plots were produced using the package "ggplot2" (Wickham et al., 2019). The package "multcomp" was used to compute letters included in the boxplots, used to visualize significant differences (Hothorn et al., 2002).

2.8 Oxygenic and pH stress limits

To examine the oxygen tolerance limits, all specimens were placed into 3 L jars and allowed to acclimate for 48 h (n = 6) under control conditions (Temp = 25 °C, pH = 8.3, O_2 saturation ~100 %). After acclimation, we reduced the O_2 saturation in each tank by 5 % every ten minutes by adding a 12.5 g/L sodium sulfite solution) until it stabilized at 3 ± 2 %. During this process, each anemone was monitored for behavioral signs of stress, e.g. tentacle retraction, acontia discharge or pedal disk detachment. Since none of the anemones displayed any of these signs of stress while we were reducing the O_2 saturation, we maintained anoxia conditions until they did.

To determine the pH tolerance limits of the model organisms, anemones (n = 6) were kept in 51 L tanks filled with aerated seawater. We decreased the pH by 0.1 every 10 min by bubbling in CO_2 and the Apex controller was used to monitor the pH (Neptune Systems, USA). The experiment was ended when anemones displayed one of the stress behaviors described above. We verified temperature and oxygen saturation during both experiments using the Fibox system detailed in section 2.5 above (Fibox Trace LCD 4, PreSens GmBH, Germany).

3. Results

3.1 Photosynthetic efficiency, algal cell density and mitotic index

Neither photosynthetic efficiency nor algal density was affected by our treatments and null models provided the best-fit for our data, i.e. none of the predictors we included (temperature, pH and O_2) held significant explanatory power (Supplementary Table 1 and 2, respectively; Fig. 3A-B). Photosynthetic efficiency averaged 556 ± 69.82 (mean ± s.d) across all treatments, while algal cell density ranged broadly between individuals, averaging 71.11 ± 23.36 %. Mitotic index was not affected by pH and O_2, however it was significantly affected by temperature ($p = 0.032$, Supplementary Table 3). Post hoc test revealed a significant decrease in mitotic index between 25 °C and 30 °C (EMM, $t42=2.218$, $p=0.0320$; Fig. 3D).

Algal ultrastructure was analyzed for the experimental deadly trio treatment (30-H-7.7) and for the control (25-N-8.2). In both samples, the tentacles housed round and intact algae, containing well defined organelles with distinct boundaries (Fig. 4A-B). All of the algae that were observed contained a distinct chloroplast, with clear thylakoid membranes located within its lobes. Starch and lipid bodies were numerous and clearly visible, particularly in the deadly trio samples, which also contained larger, darker accumulation bodies (Fig. 4B).

3.2 Metabolic rate

Temperature, oxygen and pH conditions were included in the best model, and metabolic rate was significantly affected by the interaction between temperature and oxygen (see Supplementary Table 4). Anemones exposed to deadly trio conditions (30-H-7.7) were found to have a significantly lower metabolic rate in comparison to ones exposed to 25 °C+hypoxia+hypercapnia (25-H-7.7, $t_{43}=3.196$ $p=0.0486$, Fig. 5A). Anemones exposed to all other treatments displayed intermediate metabolic rate to the ones exposed to the deadly trio and 25 °C+hypoxia+hypercapnia at control conditions ($p > 0.05$) (Fig. 5A). Furthermore, 100 % of anemones belonging to the deadly trio treatment (30-H-7.7) displayed net oxygen production (i.e. they oxygenated the surrounding seawater and displayed a negative MO_2 value). Contrarily 100 % of the organisms belonging to the control temperature and nightly hypoxia treatment, at both 8.3 and 7.7 pH, took up oxygen from their environments and therefore displayed positive MO_2 values (Fig. 5A).

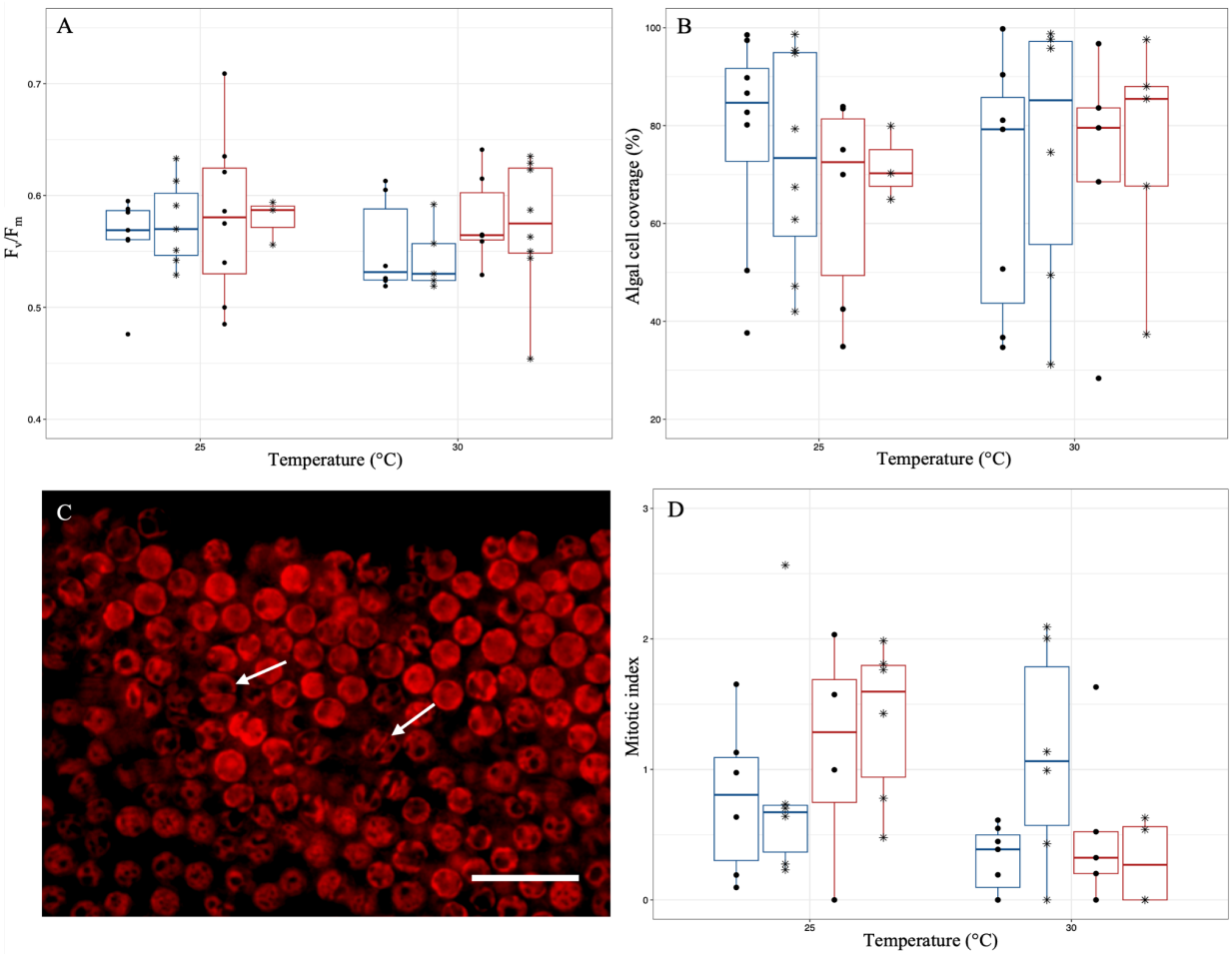

Figure 3. Photosynthetic efficiency, algal cell density and mitotic index were mostly unaffected by exposure to single and multi-stressor treatments. All eight treatments are depicted in all of the plots, with normoxia in blue and nightly hypoxia in red, while pH 8.3 is shown with dots and pH 7.7 is indicated by asterisks. Temperatures are listed on the x-axis. A) the maximum photochemical quantum yield (F_V/F_M) and B) the percentage coverage of algae in the endoderm tissue of the anemones measured after two-weeks exposure. C) Chlorophyll fluorescence (red) allowed us to count algal cell densities and rates of algal reproduction in the anemone's endoderm. Dividing algae are indicated by arrows. Scale bar is 32 µm. D) The mitotic index (percentage of algal cells undergoing cytokinesis compared to those that are not,) decreased after exposure to 30 °C in comparison to 25 °Cdid not change in any treatment.

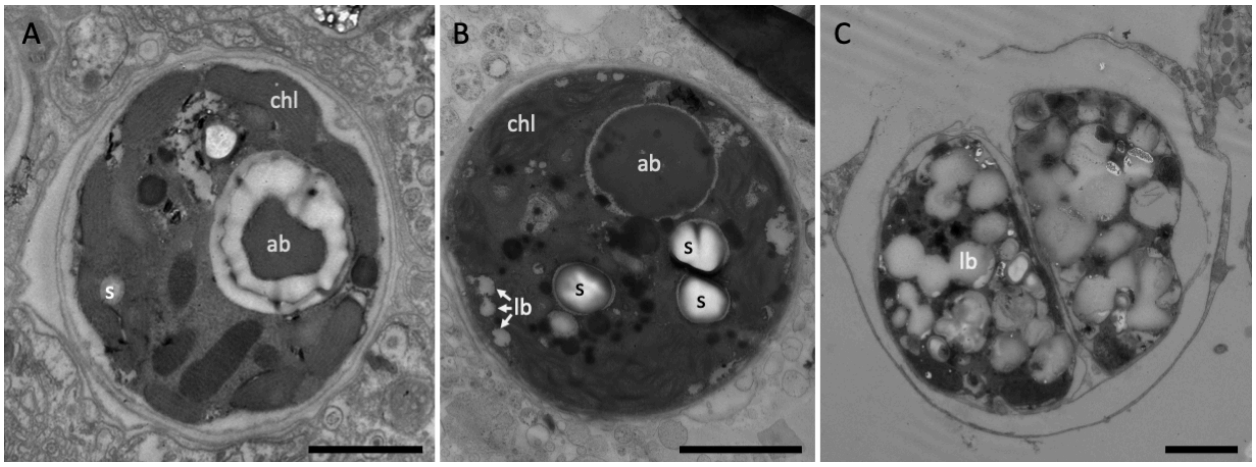

Figure 4. Micrographs of zooxanthellae. A) An algal symbiont in a tentacle from an anemone in the control treatment, B) one from the "deadly trio" treatment, and C) an algal cell undergoing cytokinesis in a tentacle from the deadly trio treatment. Abbreviations used in micrographs: accumulation body (ab), lobes of the single large chloroplast (chl), lipid bodies (lb), and starch granules (s), All scale bars are 2.5μm.

3.3 Critical Thermal maxima and Acclimation Response Ratios

Temperature, oxygen and pH conditions were found to have significant, additive effects on the anemones' CTmax (see Supplementary Table 5). In all cases, the CTmax of anemones exposed to 30 °C treatments were significantly higher than anemones exposed to 25 °C treatments ($p < 0.0001$) within each pH and oxygen condition (e.g. at pH 8.3+normoxia, CT limits were significantly different between specimens acclimated to 25 °C and 30 °C). Within the 25 °C treatments, anemones exposed to nightly hypoxia+control pH (25-H-8.3) have a significantly lower CTmax than specimens exposed to normoxia+hypercapnia (25-N-7.7) (t_{45}=-3.316, p=0.0352, designated by the letters "a" and "c" in Fig. 5B) and the other two treatments (25-N-8.3 and 25-H-7.7) were found to have an intermediate, but not significantly different, CTmax ($p < 0.05$, designated with the letters "a" and "b" in Fig. 5B). The same pattern was found within the 30 °C treatments (t_{45}=-3.316, $p = 0.0352$; $p < 0.05$). (Fig. 5B).

The highest increase in thermal plasticity occurred in nightly hypoxia+hypercapnia conditions, as the ARRs for these stressors demonstrated the steepest slope, while the lowest plasticity was observed in nightly hypoxia+control pH conditions (H-8.3; Table 2; Fig. 6). Under normoxic conditions however, the ARR was higher at pH 8.3 than at pH 7.7 (Table 2, Fig. 6).

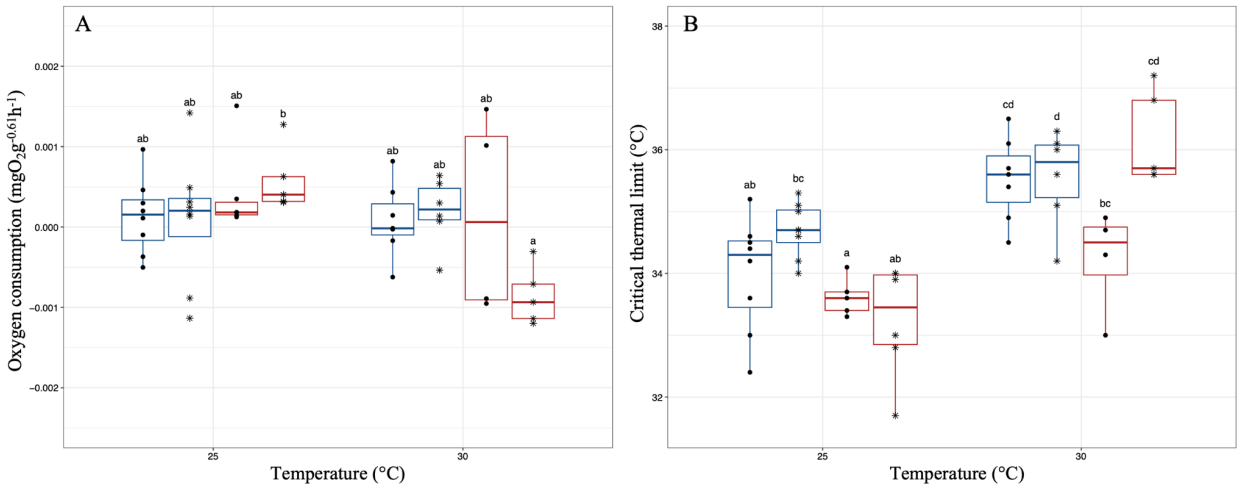

Figure 5. Metabolic rates and critical thermal limits in *Exaiptasia diaphana.* A) Oxygen consumption (in $mgO_2g^{-0.61}h^{-1}$) and B) CTmax (°C) after two-week exposure to all combinations of temperature (x-axis), oxygen saturation (normoxia is depicted with blue boxes and hypoxia is in red), and pH (8.3 is depicted with dots while pH 7.7 is delineated by asterisks).

Table 2. Acclimation response ratios for *E. diaphana*, calculated using Equation 1 and the mean CTmax of specimens exposed to 25 °C and 30 °C, under both oxygen and pH conditions.

O_2	pH	CTmax 25 °C	CTmax 30 °C	ARR
Normoxia	8.3	34.0	35.5	0.308
Normoxia	7.7	34.7	35.6	0.170
Nightly hypoxia	8.3	33.6	34.2	0.121
Nightly hypoxia	7.7	33.2	36.2	0.589

3.5 Oxygenic and pH stress limits

Exaiptasia diaphana was able to maintain attachment to the substrate until the pH decreased to 6.00 ± 0.11 (mean ± standard deviation), which occurred after 170 ± 10.95 minutes. After 4.00 ± 0.63 days of exposure to anoxic conditions, specimens retracted their tentacles and after 5.83 ± 1.47 days, the anemones were no longer able to remain attached to the substrate.

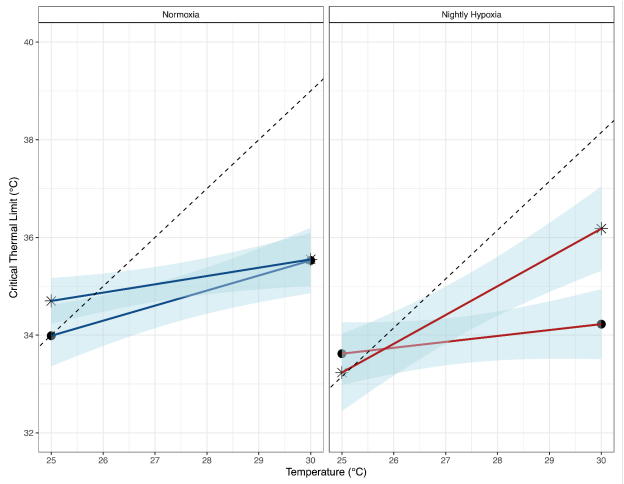

Figure 6. Mean CTmax plotted against the acclimation temperature of *E. diaphana*, when exposed to normoxia (blue) and nightly hypoxia (red); at pH 8.3 (dots) and pH 7.7 (asterisks). A steeper slope indicates a higher heat tolerance plasticity. The dashed lines have a slope of 1, indicating perfect acclimation.

4. Discussion

4.1 Photosynthetic efficiency, algal cell density and mitotic index

Exposure to the deadly trio of environmental stressors did not impact photosynthetic efficiency after two weeks of exposure to any of our treatments. These results both align with and contrast previous studies on *Acropora spp.* corals exposed to individual stressors. For example, corals exposed to nightly hypoxia demonstrated compromised photosynthetic capacity, but an overall tolerance of the symbiosis to weather nightly hypoxic stress (Deleja et al., 2022), as long as hypoxia is limited to short timescales and oxygen concentrations remain above certain thresholds (Haas et al., 2014). Similarly, the photosynthetic efficiency of algae in *Acropora spp.* decreases following exposure to higher temperatures, especially if the coral contains heat-sensitive algae (e.g. Oliver & Palumbi, 2011). Under elevated pH levels, changes in F_V/F_M have been reported to increase (e.g. Crawley et al., 2010) or decrease (e.g Iguchi et al., 2012), depending on the coral species studied. In context, the results from this study and these previous works indicate that a number of environmental and biological factors are involved in maintaining high rates of photosynthetic efficiency, including the deadly trio stressors, algal symbiont clade, cnidarian host species, amongst others.

Algal cell density was similarly unaffected by our treatments, but individual variation within each treatment did vary considerably, ranging from > 40 to < 90 % symbiont coverage in the endoderm. The fact that symbiont density remained stable across all treatments suggests that the symbiosis remained intact, even when anemones were exposed to multiple stressors, i.e. there was no evident sign of bleaching. Previous reports also detailed that bleaching does not occur in specimens exposed to 32 °C, (Doering et al., 2023; Herrera et al., 2021), however specimens exposed to more extreme thermal regimes,

e.g., 34 °C (Dungan et al., 2022) did show signs of bleaching. In comparison, certain clades of algae within corals have been shown to be more sensitive to hyperthermal stress, bleaching at temperatures of 28.7 °C (e.g. Sully et al., 2019). This indicates that there is large interspecific variation, amongst both cnidarian hosts and algal species (detailed in Abrego et al., 2008; Mizerek et al., 2018)).

Mitotic index was the only algal health related response affected by the experimental exposure, however, it was only affected by increased temperature and not by pH or O_2. Surprisingly, the rate of algal cell division decreased at 30 °C, an unexpected result considering the rates of cellular processes often increase at higher temperatures due to thermodynamics. Unlike in *Anthopleura elegantissima*, hypercapnia did not significantly increase the rate of algal cell division (Towanda & Thuesen, 2012). Our results also contrast previous reports that examined mitotic indices in symbiotic algae inhabiting the anemone, *Anemonia viridis*, and the coral, *Galaxea fascicularis*, when they were subjected to increased temperatures of 10 °C and 6 °C, respectively (Bhagooli & Hidaka, 2002; Suharsono & Brown, 1992). The decrease in mitotic index we observed in this study leads us to conclude that warmer temperatures had a negative impact on algal reproduction under our laboratory conditions or in *E. diaphana*, however the contrast with other photosymbiotic systems warrants further study.

Ultrastructure images of zooxanthellae exposed to the deadly trio showed intact morphologies, almost indistinguishable from the control anemones (Fig. 4A-B). Lipid bodies and starch grains inside these zooxanthellae indicate that these algae are still photosynthetically productive (Luo et al., 2009), and capable of mitosis (Fig. 4C), even after two weeks of exposure to the deadly trio of stressors. The lack of visible cell degradation and/or necrosis indicates that exposure to the deadly trio of stressors did not visibly alter algal morphology, as has been observed in other studies examining stress in zooxanthellae (e.g. Pasaribu et al., 2016; Rosset et al., 2017). Since ultrastructure degradation has reported from thermally sensitive strains (Tchernov et al., 2004), our findings suggest that the algae examined in this study are thermally (and pH/hypoxia) tolerant, although the increased sizes of the accumulation bodies that we observed in the deadly trio treated specimens could signal that degradation was beginning and had not completely set in (Rosset et al., 2015; Trench, 1974).

4.2 Critical oxygen saturation and pH limits

Tentacle retraction and pedal disk detachment are well-documented stress behaviors in anemones, yet *E. diaphana* displayed extraordinary resilience to both decreasing pH levels and oxygen deprivation before these behaviors were observed. It was able to withstand a pH more than 2.0 lower (pH 6.00 ± 0.11) than current ocean conditions (pH ~8.1) before displaying behavioral signs of stress. Little is known about the exact pH tolerances in other sea anemone species, however numerous studies have examined them under predicted acidification conditions, often finding that anemones may thrive in acidic waters,

especially if they are photosymbiotic (Suggett et al., 2012). Since the IPCC “worst case predictions indicate the average ocean pH could drop to 7.6 by the year 2100 (Calvin et al., 2023), we predict that *E. diaphana* will likely survive ocean acidification.

Even more surprisingly *E. diaphana* was able to withstand 4 days of exposure to anoxic conditions before displaying a stress behavior. We hypothesize this could be due to its symbiotic relationship with algae and consequential ability to produce oxygen (Laetz et al., 2024; Laetz & Verberk, 2024), although extreme resilience to anoxia has been previously recorded in anemones, with *Calliactis parasitica*, showing mortality after 52h exposure (Riedel et al., 2012). From this we can conclude that our treatment conditions did not expose *E. diaphana* to conditions near or exceeding its oxygen saturation and pH limits. This allowed us to investigate potential additive effects of the stressors, since we know that hypoxia and hypercapnia exposure alone at the levels we used were not extreme enough to elicit severe behavioral stress responses.

4.3 Metabolic Rate

The metabolic rate of the anemones was affected by temperature, oxygen conditions, and pH. Under normoxia+control pH (8.3) conditions, an increase in temperature did not affect metabolic rate (Fig. 5A). This contrasts previous reporting of increased oxygen uptake in ectotherms subjected to increased temperatures, which is often attributed to higher metabolic demands (Allan et al., 2006; Glazier, 2015; Killen et al., 2010). However, when the experimental regime included a cycle of nightly hypoxia+hypercapnia, we observed a significant decrease in metabolic rate between anemones exposed to 25 °C and 30 °C (Fig. 5A). In comparison to control conditions, specimens exposed to nightly hypoxia+hypercapnia tended to display an increased metabolic rate at 25 °C, with 100 % of these specimens consuming oxygen from the seawater (i.e. they had a positive MO_2; Fig. 5A). This exclusive oxygen uptake occurs only for anemones of one other treatment: control temperature+nightly hypoxia+control pH, although the metabolic rate is statistically not different from the control.

At 30 °C we observe an opposite, decreasing trend in metabolic rates after exposure to nightly hypoxia+hypercapnia, with 100 % of the organisms having a negative MO_2 value, i.e. oxygen produced via photosynthesis diffused into the surrounding seawater (Fig. 5A). Due to the lack of significant changes in symbiont density (Fig. 3B), the oxygen production of these anemones is most likely explained by a decrease in respiration rate of the host organism rather than an increase in oxygen production. This is likely metabolic depression - an extended period in which an animal reduces its metabolic rate to conserve energy (Withers & Cooper, 2010). Previous studies show evidence of reduced respiration rate in two species of anemone, *Anemonia alicemartinae* and *Phymactis papillosa*, after exposure to the

combination of hypercapnia and hypoxia, which acted in an additive manner (Steckbauer et al., 2015). Interestingly, we here observed a similar response in our deadly trio samples, but not in specimens subjected to hypercapnia+hypoxia at control temperatures.

4.4 Critical Thermal Limits and Acclimation Response Ratio

Temperature, O_2 conditions and pH all affected the anemones' CTmax. Under all possible combinations of pH and O_2 conditions, the anemones exposed to 30 °C displayed a significantly higher CTmax in comparison to the ones exposed to 25 °C, indicating that acclimating to warmer conditions conferred an advantage when faced with acute heat stress (Fig. 5B). This implies that neither nightly hypoxia or hypercapnia disrupted or counteracted the process of thermal acclimation to 30 °C.

Hypercapnia may have led to increased thermal tolerance in comparison to control pH. In particular, under the combination of nightly hypoxia+hyperthermia, the difference in the average CTmax between 7.7 (deadly trio) and 8.3 pH is the highest (1.955 °C; Fig. 5B). Considering that exposure to hyperthermia+nightly hypoxia+control pH led to the lowest average CTmax within the 30 °C treatments, we conclude that the combination of hyperthermia and nightly hypoxia seems detrimental to the thermal tolerance of the anemones, however hypercapnia offsets this negative effect.

The amplification of hypoxia and hypercapnia is especially clear when observing the ARRs of the four combinations of pH and O_2 conditions, calculated using the average CTmax at 25 °C and 30 °C (Table 2). Whilst the ARRs of anemones exposed to either pH level under normoxic conditions barely deviated (0.138 difference), under nightly hypoxic conditions, hypercapnia leads to a much higher level of thermal tolerance plasticity in comparison to control pH conditions (0.419 difference) (Table 2, Fig. 6). From this we conclude that exposure to nightly hypoxia+hypercapnia leads to the highest level of heat tolerance plasticity. Since nightly hypoxia is not normally considered in studies examining heat tolerance plasticity, yet it is a normal process to which marine organisms are adapted (Deleja et al., 2022; Gobler & Baumann, 2016; Nelson & Altieri, 2019; Woods et al., 2022), our results indicate that ARRs presented for other marine organisms are likely underestimated, which highlights the importance of examining all three stressors in combination.

Hypercapnia has been shown to benefit various photosymbiotic cnidarians in multiple studies, and although the exact mechanisms have yet to be described, most hypotheses center around the increased availability of carbon for photosynthesis, which in turn supports the host cnidarian energetically (e.g. (Towanda & Thuesen, 2012). While this is possible in our specimens, we cannot conclude that this is why hypercapnia had positive effects on thermal tolerance because we did not observe higher symbiont density in any of our low pH treatments. Since the average algal percent coverages in our treatments spanned 58.32 - 77.9 %, the lack of an effect from pH is probably not caused by the endoderm being at a

maximum density of algae already (other treatments had percent coverages up to 99.77 %). We can also exclude that the positive effect of hypercapnia on CTmax is caused by a higher oxygen production of the organism, because pH did not affect metabolic rates. Therefore, alternate explanations are needed to explore the reasons why hypercapnia exposure significantly increased thermal tolerance plasticity and nearly increased CTmax.

In this study, exposure to hypercapnia counteracted the effects of hypoxia, which has been previously observed in *E. diaphana* (Gibbin & Davy, 2014), other anemones (Jarrold et al., 2013; Suggett et al., 2012) and other photosymbiotic cnidarian species such as the upside-down jellyfish, *Cassiopea andromeda* (Klein et al., 2017). This mitigation could be due to elevated carbon levels promoting photosynthesis and reducing photorespiration under hypercapnia (Crawley et al., 2010), increasing net O_2 production in the cnidarian's tissues, which may help alleviate the O_2 limitation caused by hypoxia (Laetz et al., 2024; Malcolm & Brown, 1977). Increased photosynthesis could also mitigate any harmful effects from increased acidosis in the host's tissues due to higher rates of CO_2 diffusion from the seawater (Klein et al., 2017), however confirming these hypotheses will require detailed studies with aposymbiotic specimens.

4.5 Metabolic depression as an energy conservation strategy

Metabolic depression is frequently observed as a stress response to cold or heat shock (Guppy & Withers, 1999), and/or a strategy for conserving energy (Marshall & McQuaid, 2011) when faced with stressful environments (Liao et al., 2021). Our results show an association between increased CTmax and metabolic depression: the anemones that were exposed to deadly trio conditions likely entered metabolic depression, yet they also displayed the overall highest average CTmax (Fig. 5). Continuing this pattern, all of the anemones with the lowest average CTmax consumed O_2 (two treatments: 25 °C+nightly hypoxia+hypercapnia, and 25 °C+nightly hypoxia+8.3 pH). We therefore hypothesize that depressing metabolic rates under deadly trio conditions is a strategy *E. diaphana* utilizes to conserve energy, which confers higher thermal tolerance. This physiological adaptation has been demonstrated in numerous marine invertebrates allowing them to withstand extreme environments (Liao et al., 2021; Marshall & McQuaid, 2011), but it is less understood and possibly less common in cnidarians, having been reported in a coral species *Pocillopora damicornis* (Jiang et al., 2023), but not in *Cassiopea andromeda* (Aljbour et al., 2017). Further research is necessary to investigate the long-term effects of this strategy as there are also suggestions that it might be maladaptive, particularly from a multi-generational standpoint (Jiang et al., 2023).

Observing a decreased metabolic rate and increased thermal tolerance supports the hypothesis linking oxygen acquisition and delivery with thermal tolerance (Pörtner, 2001; Pörtner et al., 2017).

Examining the individual effects of hyperthermia and hypoxia as well as their combination revealed that hyperthermia alone (control pH+normoxia) did not lead to an increase in metabolic rate or a decrease in thermal tolerance in comparison to control conditions (Fig. 5), however hyperthermia+hypoxia (control pH), led to a decreasing trend in CTmax, suggesting that oxygen availability is likely limiting thermal tolerance (Fig. 5). Furthermore, increased metabolic rate in response to decreased oxygen availability by itself (25 °C+nightly hypoxia+control pH) led to decreased thermal tolerance, in comparison to the control (25 °C+normoxia+control pH; Fig. 5). This clearly shows that exposure to hyperthermia alone does not impair thermal tolerance, but exposure to nightly hypoxia (at control pH) for both 25 and 30 °C acclimated specimens leads to the lowest CTmax averages amongst other treatments at these respective temperatures.

4.6 Conclusions

Neither *E. diaphana* nor their incorporated dinoflagellates experienced significant negative effects in response to treatments simulating IPCC worst case scenario climate change projected conditions. While algal reproduction may have slowed somewhat at high temperatures, it was not enough to measurably decrease algal cell density in anemone tissues and algal reproduction was still observed under all conditions and photosynthetic efficiency remained high. Surprisingly, the anemones displayed resilience to the stressors, exhibiting minimal signs of physiological stress and demonstrating the ability to plastically adjust to new conditions. Furthermore, they displayed thermal limits far higher than the IPCC 2100 predicted warming conditions of 30 °C, ranging from 33.23 °C ± 0.918 to 36.18 °C ± 0.762. This study therefore identifies symbiotic anemones as potential "winners" under future predicted climate change conditions. Uncovering the pH and deoxygenation limits in *E. diaphana* further support this conclusion, as specimens tolerated a far lower pH (6.0 ± 0.11) than the worst case predictions (7.6 pH). Predicting hypoxia is difficult due to daily, seasonal and regional fluctuations, but more than 4 days of anoxia tolerance is remarkable for any aerobic organism.

Notably, exposure to the deadly trio led to metabolic depression and increased thermal tolerance. Hypercapnia and nightly hypoxia had a synergistic effect on *E. diaphana* heat tolerance plasticity, which could be due to algal activity, but this will require more study. Perhaps most importantly, this study shows clear evidence that examining all three deadly trio stressors, in combination and individually, was critical to understand how these factors interact to influence physiological responses in *E. diaphana.* Including nightly hypoxia allowed us to observe temperature driven changes in metabolic rates that are not present in normoxic conditions. Secondly, the heat tolerance plasticity varied according to pH conditions, but only under nightly hypoxia conditions. This study demonstrates that the deadly trio stressors are not only interconnected in the environment, but also in the ways they affect biological processes and that their

effects vary in different combinations. We therefore conclude that it is necessary to examine all three in order to fully understand how marine ecosystems will respond to the deadly trio.

Lastly, although *E. diaphana* is an efficient model to investigate questions on the processes underlying photosymbiosis in cnidarians (Roberty et al., 2024), our research confirms previous assertions that it is not a good model for research in the context of climate change (Dungan et al., 2020). Its high stress tolerance makes it a poor model for other cnidarian species, namely corals, that instead display higher sensitivity to hyperthermia (e.g. Schoepf et al., 2015; Sully et al., 2019), hypoxia (e.g. Alderdice et al., 2022; Pontes et al., 2023) and hypercapnia (e.g. Godefroid et al., 2022; Kavousi et al., 2016). We therefore suggest taking the extraordinary stress tolerance of *E. diaphana* into consideration in future studies using this anemone as a model species and caution against comparisons to other cnidarians.

Author contributions

BAP: Conceptualization; data curation; formal analysis; methodology; visualization; writing - original draft; writing- review and editing; **IS:** Data curation; methodology; visualization; writing - review and editing; **LBP:** Data curation; formal analysis; methodology; writing - review and editing; **ZQ:** Data curation; methodology; writing - review and editing; **EMJL:** Conceptualization; data curation; formal analysis; methodology; visualization; resources; writing - original draft; writing - review and editing.

Acknowledgments

We would like to thank Nadine Speelpenning and the rest of the RUG aquarium facility staff, Gerard Overkamp, Joke Bakker for data management, as well as Hinke Tjoelker and Paul Steerenberg for administrative support (all RUG). We are also grateful to members of the Dutch public who donated anemones. This work was supported by a Dutch Research Council (NWO) award to EMJL (VI.Veni.202.218), and a Chinese Scholarship Council award to ZQ (202208520004).

Conflict of interests statement

All authors declare no conflict of interest.

Data availability statement

The data and codes used to support this study will be made available upon reasonable request.

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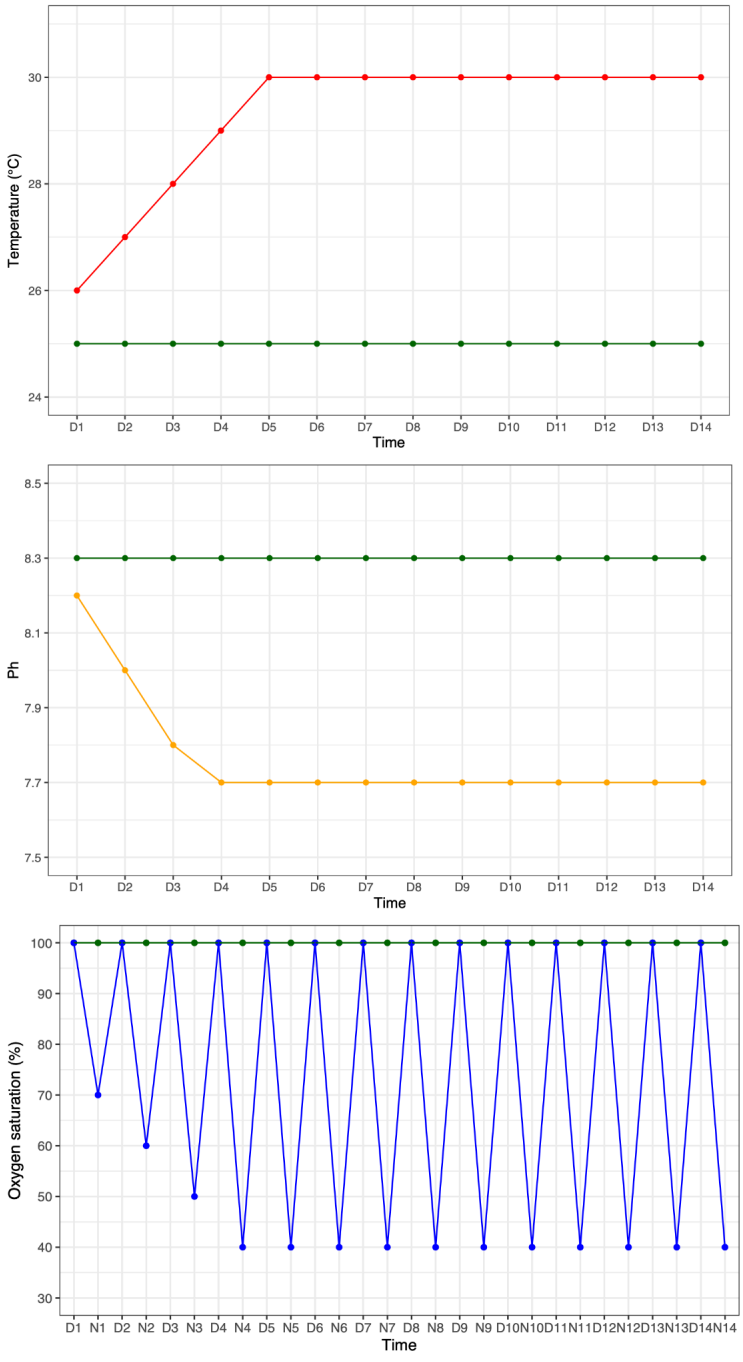
Supplementary Materials

Supplementary Figure 1. Parameters of temperature, pH and oxygen saturation throughout the experimental exposure to the stressors or to control conditions (D = Day - 5:00 to 17:00; N = Night - 17:00 to 5:00). Green indicates control conditions; red, yellow and blue respectively indicate hyperthermia, hypercapnia and hypoxia. Exposure to increased temperatures, hypercapnia and hypoxia were gradually introduced so as not to shock the organisms (Grottoli et al., 2018).



Supplementary Table 1. Summary table of the model used in evaluating the effects of temperature, oxygen conditions and pH on photosynthetic efficiency.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.579000	0.028811	20.097	<2e-16
Temperature30	-0.005875	0.033784	-0.174	0.863
Ph8.3	0.002375	0.033784	0.070	0.944
O2normoxia	5.6903	6.9712	0.816	0.419
temperature30:pH8.3	0.003333	0.043216	0.077	0.939
temperature30:O2normoxia	-0.025296	0.044667	-0.566	0.574
pH8.3:O2normoxia	-0.015946	0.043045	-0.370	0.713
temperature30:pH8.3:O2normoxia	0.019838	0.059095	0.336	0.739

Supplementary Table 2. Summary table of the model used in evaluating the effects of temperature, oxygen conditions and pH on algal density.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	58.323	12.264	4.755	2.45e-05
Temperature30	16.879	16.454	1.026	0.311
Ph8.3	6.639	15.833	0.419	0.677
O2normoxia	14.871	15.021	0.990	0.328
temperature30:pH8.3	-10.487	22.167	-0.473	0.639
temperature30:O2normoxia	-15.511	21.124	-0.734	0.467
pH8.3:O2normoxia	-1.925	20.028	-0.096	0.924
temperature30:pH8.3:O2normoxia	-1.276	28.775	-0.044	0.965

Supplementary Table 3. Summary table of the best-found model used in evaluating the effects of temperature on mitotic index.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.0305	0.1447	7.122	9.72e-09
Temperature30	-0.4538	0.2046	-2.218	**0.032**

Supplementary Table 4. Summary table of the best-found model used in evaluating the effects of temperature and oxygen conditions on metabolic rate (MO_2).

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	5.175e-04	2.333e-04	2.219	0.03184
Temperature30	-1.078e-03	3.372e-04	-3.196	0.00261
Ph8.3	-4.562e-05	2.447e-04	-0.186	0.85295
O2normoxia	-3.817e-04	2.488e-04	-1.534	0.13231
Temperature30:Ph8.3	3.934e-04	3.656e-04	1.076	0.28792
Temperature30:O2normoxia	8.864e-04	3.718e-04	2.384	0.02161

Supplementary Table 5. Summary table of the best-found model used in evaluating the effects of temperature, oxygen conditions and pH on critical thermal limit (CTmax).

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	33.8261	0.2404	140.702	< 2e-16
Temperature30	1.4932	0.2401	6.218	1.48e-07
Ph8.3	-0.5177	0.2395	-2.162	0.0360
O2normoxia	0.6517	0.2435	2.676	0.0103