THE EFFECTS OF NUTRIENT STATUS AND THERMAL STRESS IN THE SYMBIOTIC SEA ANEMONE *EXAIPTASIA PALLIDA*

by

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ABSTRACT

Corals reefs are rapidly disappearing under the damage of exposure to high temperature caused by climate change, which results in bleaching (a loss of algal symbionts). Corals that feed heterotrophically on zooplankton display less stress under increased temperature and recover faster from bleaching events; however it is not understood how coral preacclimation to available nutrient levels (and in turn nutrient availability to symbionts) impacts thermal tolerance. The symbiotic sea anemone *Exaiptasia pallida* was exposed to two different feeding regimes: fed *Artemia salina* nauplii once weekly, or starved (unfed) for 31 days. In addition, another set of animals were withheld food, but were treated with low-levels of nitrate and phosphate (5 μM and 0.5 μM respectively). Anemones were subsequently heated to 32 ºC for an additional 10-14 days and continued nutrient treatment. During nutrient exposure, fed anemones maintained maximum quantum yield of photosystem II (F_v/F_m) and wet weight, while both unfed groups lost wet weight. Furthermore, starved anemones consumed more *Artemia* after 31 days of nutrient treatment. High temperature led to signs of bleaching stress, observed as a decline in symbiont density and a drop in F_v/F_m; however the percentage of carbon translocated to the host increased in starved anemones. Together, these results indicate that heterotrophic feeding gives symbiotic anemones a potential advantage in combating bleaching stress. However, the documented advantage of heterotrophic feeding to corals was increased symbiont density and photosynthetic efficiency of photosystem II, but in this study the observed advantage to sea anemones was maintenance of animal size and respiration rate.

Understanding the ecological implications of how sessile marine invertebrates respond to bleaching will be key in understanding the ecological impacts of global warming on coral reefs.

Chapter 1

INTRODUCTION

1.1 Overview of Cnidarian Symbiosis

Coral reefs are one of the most biodiverse environments on Earth, and provide food and resources to over 450 million people (Hoegh-Guldberg 2011), as well as providing economic revenue for local fishermen and commercial fisheries, eco-tourism, cosmetics, jewelry, and pharmaceuticals. Coral reefs generated an estimated $125 trillion/yr revenue for the year 2011 (Costanza et al. 2014; Hughes et al. 2017). Reefs provide a multitude of ecosystem services to coastal waters such as nurseries for juvenile fish, cleaning stations for invertebrates and vertebrates to interact, and coastal protection against wave/storm damage (Moberg and Folke 1999). Despite all the benefits coral reefs provide, their health is rapidly declining due to climate change (Downs et al. 2005; Hoegh-Guldberg 2011; Tsounis and Edmunds 2017). Global warming has a detrimental effect on an already delicate cnidarian-algal symbiosis (Muscatine and Porter 1977). Cnidarians, including reef-building stony corals (order Scleractinia) and sea anemones (class Anthozoa), represent a rich symbiosis with endosymbiotic dinoflagellates from the family Symbiodiniaceae. This family contains both symbiotic and free-living algae in seven formally described genera: *Symbiodinium, Breviolum, Cladocopium, Durusdinium, Effrenium, Fugacium,* and *Gerakladium*, as well as at least three other lineages that await further characterization (LaJeunesse et al. 2018). These symbionts obtain their energy via photosynthesis (autotrophy), providing the cnidarian host primarily with photosynthate products in the

form of glucose or glycerol (Davy et al. 2012). This symbiosis is critical to coral reef success in an oligotrophic ocean due to efficient nutrient recycling between partners, and the translocated organic carbon from the symbiont which accounts for 75%-100% of the host's daily metabolic carbon requirements (Grottoli et al. 2006). In the dynamic marine environment, there are multitudes of abiotic and biotic stressors to the holobiont (i.e., the coral and algal symbiont) including changing temperature, light intensity, wave action and flow, availability of nutrients, disease, competition and predation (Hughes and Connell 1999).

1.2 Symbiodiniaceae Biodiversity

There is an incredible range of genetic diversity within the family Symbiodiniaceae. Until recently, all of these organisms were placed in the single genus, *Symbiodinium*, with nine clades (designated by the letters A–I) that were based on multiple genetic markers, including the internal transcribed spacer-2 region (ITS2) (LaJeunesse 2002; Pochon et al. 2006; Pochon and Gates 2010; LaJeunesse et al. 2014; Suggett et al. 2017; Thornhill et al. 2017). LaJeunesse et al. (2018) recently reclassified the genus into seven genera corresponding to the aforementioned clade-level designations. Importantly, each genus contains multiple species, many of which have yet to be formally characterized and represent a range of physiological performance. LaJeunesse et al. (2012) revealed that physiological diversity did not match up with genetic diversity within clades, so cladal generalization does not hold across different species of symbionts. This distinction is key when comparing multiple strains even within the same species (LaJeunesse et al. 2012). Current research in dinoflagellate-cnidarian symbioses is focused at the genus or species level, while previous research focused at the clade level of organization. The dominant algal

symbiont varies across the globe, for example, clade B in the Caribbean and clade C in the Indo-Pacific (Thornhill et al. 2013). Some species of algae are specialists, forming a symbiosis only with a specific host, while some are generalists, forming a symbiosis with a variety of hosts (LaJeunesse et al. 2003). Some corals host multiple types of symbionts at the same time, while others only have one type of host-specific symbiont (LaJeunesse et al. 2010; Hambleton et al. 2014; Matthews et al. 2017). Bleaching is the loss of algal symbionts or photosynthetic pigments due to stress (Jokiel and Coles 1977). When a symbiosis is disrupted (i.e. bleaching), some corals gain new symbionts from their surrounding environment in a process called symbiont switching (Baker 2003). Symbionts are expelled due to stress, and then new symbionts recolonize the host from the surrounding water column. Some corals experience a change in algal dominance within their tissues, called symbiont shuffling (Baker 2003), where there are multiple different algae already present within the host tissue but there is a change in dominance. Experiencing a change in symbiont population also means a change in species-specific functionality (LaJeunesse et al. 2012). Different algae function differently under environmental stressors, and that functional diversity impacts the symbiotic relationship accordingly. For example, some species are more thermally tolerant than others and maintain photosynthetic output even at high temperatures (Hawkins et al. 2016). Functional diversity is crucial to ecological success in a changing environment.

Studies have examined the effects of bleaching between populations of the sea anemone *Exaiptasia pallida* colonized with the natal (homologous) symbiont *Breviolum minutum* and the non-native (heterologous) symbiont, *Durusdinium trenchii*, which has high thermal tolerance and repopulates some Caribbean corals

after bleaching events (Grottoli et al. 2014; Cunning et al. 2015; Pettay et al. 2015; Silverstein et al. 2015). *D. trenchii* exhibits greater antioxidant defense, which could contribute to enhanced holobiont thermotolerance (Matthews et al. 2017); however, *D. trenchii* did not translocate as much carbon to the anemone and displayed decreased productivity, and growth compared to *B. minutum*. In fact, anemones harboring *D. trenchii* shifted toward carbohydrate and lipid catabolism and mobilization as well as increased host immune response, thereby suggesting the anemone could not meet daily metabolic requirements when harboring *D. trenchii* (Matthews et al. 2017). Even though *D. trenchii* exhibits tolerance to high temperature, it does not provide as much energy to the host as other species do. Symbiont switching may be an appropriate tactic for short-term survival, but due to the low energetic output to the host compared to other species does not present a long-term solution to bleaching.

Symbiont specificity is primarily limited by cellular mechanisms between the host and the symbiont that we do not entirely understand (Rowan 1991; Baumgarten et al. 2015). Other determinants for successful symbioses include nutrient exchange, such as carbon, between partners and response to intracellular oxidative stress once the symbiont is established (Matthews et al. 2017). A successful relationship isn't just the capacity to form a symbiosis, but also whether or not the symbiont benefits the host in some way. If the alga isn't translocating enough carbon to the host, producing too many harmful reactive oxygen species (discussed in further detail below), or if the algal population becomes too dense, the host expels its symbionts. Keeping in mind Symbiodinaceae diversity, new research is moving away from the older clade-level generalizations and toward investigating physiological and ecological variation between species. Recent work has demonstrated high intra-cladal (and now

intraspecific) variability in thermal and light tolerance, indicating that greater taxonomic resolution is needed for more accurate comparisons across symbionts (Tchernov et al. 2004; Robison and Warner 2006; LaJeunesse et al. 2014).

1.3 Bleaching

Bleaching is the forceful expulsion of algal symbionts or loss of photosynthetic pigmentation due to thermal stress (Jokiel and Coles 1977). Chronic high temperatures over 1-2 ºC above the typical average summer time maximum are the primary cause of widespread global bleaching. Coral bleaching is increasing in frequency and severity across the globe (Glynn 1993; Brown 1997; Manzello 2015), and is predicted to occur annually as soon as the year 2020 in Florida (Manzello 2015). The host loses a vital energy source necessary for survival if it does not acquire new symbionts or if remaining symbionts do not recover (Houlbrèque and Ferrier-Pagès 2009). If the coral is not recolonized by symbionts, it will eventually die (Glynn 1993; Brown 1997; Houlbrèque and Ferrier-Pagès 2009).

During normal photosynthetic processes, oxygen occasionally forms singlet oxygen, hydrogen peroxide, superoxides, or hydroxyl radicals; these are highly reactive oxygen species (ROS) which contain high chemical energy and are known to be a cause of bleaching (Lesser 1997). Environmental stressors cause an increase in the production of ROS inside the chloroplasts and mitochondria, which further damage the host by chemically reacting with and damaging photosynthetic machinery, cellular machinery, or even DNA (Lesser 1997, 2011; Tchernov et al. 2004; Falkowski and Raven 2007). This damaging behavior of ROS makes them dangerous for both the alga and the animal host, and excessive ROS production promotes bleaching (Lesser 1997; Dunn et al. 2012; Goyen et al. 2017).

Previous work (Schreiber et al. 1986; van Kooten and Snel 1990; Maxwell and Johnson 1999) established active chlorophyll fluorescence as a standard method to measure photochemical efficiency in plants. When photosystem II (PSII) is illuminated, some energy is used for photosynthesis while some energy is lost as fluorescence or heat dissipation. If the light is strong enough, the fluorescence will increase from a low constant level (F_o) to some maximum level (F_m). The difference between F_o and F_m is the variable fluorescence (F_v). Photochemical efficiency is measured as a ratio of chlorophyll fluorescence (F_v/F_m). It describes the ability of the photochemical apparatus to convert light energy into chemical energy through photosynthesis, or the photochemical efficiency. This ratio has been established as a metric for photochemical efficiency of the photosynthetic apparatus in plants and has been applied to marine algae energy *in hospite* (Gorbunov et al. 2001; Warner et al. 2002; Falkowski and Raven 2007).

Symbionts acclimate to different light levels through photoacclimation or photoprotection (Iglesias-Prieto; Behrenfeld et al. 1998). Photoacclimation is the long-term acclimation to irradiance and usually manifests by a five to ten-fold increase in chlorophyll per cell or per unit surface area as irradiance decreases (Rodrigues et al. 2008). Photoprotection, on the other hand, refers to either photochemical quenching or non-photochemical quenching of the fluorescence signal (Hill et al. 2012). Photoinhibition is either a reversible or an irreversible process (dependent upon the extent of inhibition) in which photosynthesis is slowed when an alga is exposed to excess light (Hoegh-Guldberg and Jones 1999). It manifests as either a reduction in the number of light harvesting complexes and PSII reaction centers or by an increase in the maximum time it takes for protein turnover (Hoegh-Guldberg and Jones 1999).

Under high light, PSII begins to breakdown (in a process called photoinhibition) due to an excess of excitation energy in the chloroplast that cannot either be used for photosynthesis or that PSII dissipates it as heat (by a process known as non-photochemical quenching; Hill et al. 2005). Photoacclimation to different light levels occurs naturally on a seasonal and daily basis *in situ*, but in conjunction with increased heat in summer causes thermal damage (Rodrigues et al. 2008). High temperature leads to a net loss in PSII proteins, specifically the D1 protein on PSII and thus a loss of photochemical capacity, which outpaces the PSII reaction center repair pathways and results in net damage (Warner et al. 1999).

A combination of high light and high temperature are especially damaging to the holobiont (Warner et al. 1999). High temperature damages multiple parts of algal cells, including thylakoid membrane stability (Tchernov et al. 2004; although countered by Díaz-Almeyda et al. 2011) and photosystem II (PSII) (Warner et al. 1999; Warner and Suggett 2016). Algal symbionts acclimate to high temperature similarly to how they acclimate to high light by decreasing population density and decreasing chlorophyll *a* content (Maxwell and Johnson 1999; Warner et al. 2006). Active chlorophyll *a* fluorescence is used to measure symbiont stress within the host, however, it is possible that the animal could become stressed before we observe any significant changes in algal photochemistry (Parkhill et al. 2001; Wiedenmann et al. 2013). Researchers should not rely on F_v/F_m as the sole indicator for algal health within the symbiosis. Although far from perfect, active chlorophyll *a* fluorescence is the most widely used indicator of algal stress to date (Suggett et al. 2010).

Thermal tolerance varies across different species of algae. Warner et al. (1999) observed that a thermally tolerant alga had a faster PSII turnover rate when compared

to a thermally sensitive alga, and thus hypothesized that differences in photorepair rates account for the differences in coral bleaching susceptibility. Ragni et al. (2010) studied net versus gross photoinhibition in two different strains of *Symbiodinium* (ITS2-type A1 and A1.1) with different thermal tolerances to elucidate the tradeoffs between photoprotection and photorepair, finding that high light increases the rate of the D1 (psbA) protein degradation. The thermally tolerant alga used high photorepair rates to minimize net photoinhibition, while the thermally sensitive alga showed a greater drop in F_v/F_m (net photoinhibition) when exposed to high light. However, gross photoinhibition was similar in both strains, demonstrating that both phylotype and acclimation state play a role in susceptibility to light stress, as well as a link between light and temperature stress. Differences in thermal tolerance are caused by species-specific functional diversity and depends upon PSII turnover rates.

1.4 *Exaiptasia* as a Model System

The sea anemone *Exaiptasia pallida* (formerly *Aiptasia,* see Grajales and Rodríguez 2014) is a widely adopted model system for investigating cnidarian-dinoflagellate symbioses and hence for scleractinian corals as well (Muller-Parker 1984; Baumgarten et al. 2015; Rädecker et al. 2018). *E. pallida* naturally contains *Symbiodinium* A4 in the Florida Keys, but harbors *Breviolum minutum* in most other locations around the world (Lajeunesse et al. 2012; Thornhill et al. 2013; Grajales et al. 2016); however, anemones from Key Largo, FL contain at least two different strains of *Symbiodinium* A4, and it is thought that one algal strain is dominant at a time (Hawkins et al. 2016). *E. pallida* reproduces asexually which maintains populations in the lab, and has previously described genetic data (Baumgarten et al. 2015; Matthews et al. 2017). It is easily rendered aposymbiotic (without symbionts)

enabling further infection with a range of different symbionts, including symbioses not found in nature (Matthews et al. 2017). Several studies have manipulated *E. pallida* symbioses in order to investigate the physiological result of different animal-algal partner combinations (Goulet et al. 2005; LaJeunesse et al. 2010; Leal et al. 2015; Hawkins et al. 2016; Matthews et al. 2017).

Chapter 2

PHYSIOLOGICAL RESPONSES OF THE SYMBIOTIC SEA ANEMONE *EXAIPTASIA PALLIDA* TO NUTRIENT STATUS AND THERMAL STRESS

2.1 Abstract

Corals reefs are rapidly disappearing under the damage of exposure to high temperature caused by climate change, which results in bleaching (a loss of algal symbionts). Corals that feed heterotrophically on zooplankton display less stress under increased temperature and recover faster from bleaching events; however it is not understood how coral preacclimation to available nutrient levels (and in turn nutrient availability to symbionts) impacts thermal tolerance. The symbiotic sea anemone *Exaiptasia pallida* was exposed to two different feeding regimes: fed *Artemia salina* nauplii once weekly, or starved (unfed) for 31 days. In addition, another set of animals were withheld food, but were treated with low-levels of nitrate and phosphate (5 μM and 0.5 μM respectively). Anemones were subsequently heated to 32 ºC for an additional 10-14 days and continued nutrient treatment. During nutrient exposure, fed anemones maintained maximum quantum yield of photosystem II (F_v/F_m) and wet weight, while both unfed groups lost wet weight. Furthermore, starved anemones consumed more *Artemia* after 31 days of nutrient treatment. High temperature led to signs of bleaching stress, observed as a decline in symbiont density and a drop in F_v/F_m; however the percentage of carbon translocated to the host increased in starved anemones. Together, these results indicate that heterotrophic feeding gives symbiotic anemones a potential advantage in combating bleaching stress. However, the documented advantage of heterotrophic feeding to corals was increased symbiont density and photosynthetic efficiency of photosystem II, but in this study the observed advantage to sea anemones was maintenance of animal size and respiration rate.

Understanding the ecological implications of how sessile marine invertebrates respond to bleaching will be key in understanding the ecological impacts of global warming on coral reefs.

2.2 Introduction

2.2.1 Nutrient Status within Symbiosis

Reef building corals obtain a portion of their energy via heterotrophic feeding, which in turn provide nutrients to their endosymbiotic algae. Macronutrients such as carbon, nitrogen, and phosphorous are key requirements for the synthesis of cellular machinery, but coral reefs thrive in the tropics where light and temperature are high, and nutrients are low. The holobiont is typically nitrogen limited in the oligotrophic ocean (T. A. V. Rees 1991; Rädecker et al. 2017). If either the host or the symbionts experience nutrient limitation, cellular repair mechanisms, synthesis, or function is impaired (Rees 1991). Due to the low availability of nutrients in the environment, the cnidarian host withholds nutrients from the algal symbionts and exerts control of the algal population (Falkowski et al. 1993). Falkowski et al. (1993) and Ezzat et al. (2016) demonstrated that an excess of nutrients (ambient: 0.5 μM N and 0.1 μM P, treatment: +2 μM N and +0.5 μM P) leads to a bloom in the symbiont population within the host. The holobiont is adapted to the specific nutrient ratios available in its environment (Ezzat et al. 2016); symbionts inside the host (*in hospite*) grow in an unbalanced fashion and are typically nutrient limited, but still use low amounts of nutrients for their low growth rates (>0.1 day^{-1} while typical cell specific growth in culture may be as high as 0.3–0.45 day^{-1}) (Brading et al. 2011). When one nutrient is added into the system (i.e., nitrogen), the ratio of nitrogen to phosphorous (N:P) shifts

out of balance. Imbalance in N:P disrupts cellular pathways such as the formation of protein complexes, cellular repair, or synthesis of cellular machinery necessary for cellular reproduction (Wiedenmann et al. 2013; Kamalanathan et al. 2016; Wooldridge 2016). Symbiont populations are forced to incorporate other molecules (i.e., sulfur) into compounds when phosphorous levels are low (Ezzat et al. 2016). Algal cells can increase phosphatase activity, resulting in phosphorous starvation (Ezzat et al. 2016). Nutrient starvation results in a decline in the population. Without a thriving symbiont population and the energy received from it, the host's capacity to combat environmental stressors (such as temperature or high light) is reduced (Wiedenmann et al. 2013).

When cells become nitrogen limited, previously it was thought that F_v/F_m decreases as key proteins are lost that transfer excitation energy from the light harvesting chlorophyll proteins to the reaction centers (Falkowski et al. 1992, Kolber et al. 1988). However, more recently studies have demonstrated that nutrient limitation does not impact photochemical efficiency (F_v/F_m) (Parkhill et al. 2001; Wiedenmann et al. 2013). Therefore, researchers cannot rely on F_v/F_m alone to determine nutrient status. On the other hand, increased nutrient input benefits the holobiont if symbiont populations are struggling under nutrient limitation and temperature stress combined (Ezzat et al. 2016). The N:P ratio should be considered when discussing nutrient status (Wiedenmann et al. 2013) but has often been overlooked in previous studies.

2.2.2 Heterotrophic Feeding on Zooplankton

Fed corals are more resilient to bleaching, and recover faster from bleaching stress (Muller-Parker 1985; Grottoli et al. 2006; Rodrigues and Grottoli 2007; Palardy et al. 2008; Ezzat et al. 2016). Heterotrophy accounts for 15%–35% of a healthy

coral's daily metabolic needs (Houlbrèque and Ferrier-Pagès 2009). When corals are bleached and lack input of photosynthetic carbon from symbionts, consuming zooplankton can even provide up to 100% of a coral's daily metabolic needs (Grottoli et al. 2006; Houlbrèque and Ferrier-Pagès 2009). Consuming zooplankton provides a source of organic carbon that promotes increased tissue growth, calcification, photosynthetic rates, and symbiont, chlorophyll, protein, and lipid contents in scleractinian corals when comparing fed versus starved treatments (Muller-Parker 1985; Rodrigues and Grottoli 2007; Houlbrèque and Ferrier-Pagès 2009).

In *Exaiptasia pallida*, symbionts translocate far less carbon (relative to net photosynthesis) than in corals (Hoadley et al. 2016), so sea anemones tend to increase heterotrophic feeding rates as compared to corals. Photosynthetically derived carbon is translocated to the host and quickly respired in cnidarians such as *E. pallida* (Rädecker et al. 2017), but the host cannot get other important macronutrients from its symbionts alone. Nitrogen and phosphorous are only obtained through heterotrophic feeding on zooplankton, dissolved organic matter, and other particulate organic matter (Houlbrèque and Ferrier-Pagès 2009). Under thermal stress, cellular damage occurs and cellular repair mechanisms increase. Nitrogen is a necessary component for synthesizing new proteins, which cannot be obtained through photosynthetic carbon translocation from symbionts. When corals undergo thermal stress, heterotrophic feeding increases (Grottoli et al. 2006; Houlbrèque and Ferrier-Pagès 2009), logically due to a requirement for more nitrogen to synthesize proteins in order to replace proteins damaged by thermal stress and maintain energy reserves, but this conclusion has not been tested to date. The ability to switch between auto- and heterotrophy in response to changing environmental conditions are a key component of surviving and

dominating a coral reef under future climate change. However, not all corals have demonstrated this ability and the exact physiological mechanism of the benefit of heterotrophic feeding is not currently known.

2.2.3 Research Goals

The goal of this study was to describe the effects of nutrient status and the effects of preacclimation to nutrient levels combined with chronic thermal stress on the physiological response of *Exaiptasia pallida.* Comparing different nutrient treatments highlighted the benefit of heterotrophic feeding in the holobiont which has been demonstrated by previous studies (Muller-Parker 1985; Grottoli et al. 2006; Rodrigues and Grottoli 2007; Palardy et al. 2008; Ezzat et al. 2016). Animal and algal physiology were compared across three different nutrient treatments (fed, unfed, and inorganic nutrient addition) and discussed in the context of environmental thermal stress. Based on previous short-term experiments using *E. pallida,* we anticipated the heterotrophic feeding group would be more resilient to thermal stress; specifically, we anticipated maintenance of wet weight and respiration rate in the fed anemones, as well as maintenance of photosynthetic carbon fixation in the alga (Clayton and Lasker 1984). We expected the inorganic addition anemones would respond similarly to the starved anemones due to the lack of zooplankton, and also predicted thermal stress would lead to symbiont photosystem damage and possible patterns of bleaching (decreased F_v/F_m and lower symbiont density).

Climate change is progressing at an unprecedented rate, as seen by rapidly increasing global temperatures, sea level rise, and ocean acidification. Heterotrophy alleviates the detrimental effects of high temperature stress (Grottoli et al. 2006; Rodrigues and Grottoli 2007; Palardy et al. 2008; Wiedenmann et al. 2013; Ezzat et al.

2016), but the mechanism is not yet clear. Research in the field has revealed the importance of the rate of photorepair in relation to bleaching recovery (Behrenfeld et al. 1998; Warner et al. 1999; Grottoli et al. 2006; Davy et al. 2012), which heterotrophic feeding is thought to support. Cnidarian heterotrophic plasticity is vital for surviving such effects as elevated water temperature and coral bleaching. It is still unknown what the energetic difference between fed versus starved corals is, or by what exact mechanism bleaching recovery occurs with feeding. The goal of this study was to describe the effects of nutrient status and the effects of chronic thermal stress on the physiological response of *Exaiptasia pallida*.

2.3 Materials and Methods

2.3.1 Animal Collection and Husbandry

Exaiptasia pallida sea anemones were collected from Key Largo, Florida in July 2016. After transport back to Delaware, animals were held in glass bowls filled with UV sterilized artificial seawater (Instant Ocean, salinity 34 psu) at room temperature (27 ºC) under a bank of cool white LED lights (Cree XP-G2) set to 100 µmol photons m^{-2} s^{-1} (scalar irradiance recorded by a small quantum sensor; model US-SQS1L, Heinz Walz Gmb, Germany and LI-250A, LiCor Biosciences) set to a 12:12h light-dark cycle. Anemones were fed freshly hatched juvenile *Artemia salina* brine shrimp 1 time $week^{-1}$, and seawater was replaced in each bowl approximately 18 hours after feeding.

E. pallida from Key Largo, FL typically harbors Symbiondinium A4, which previous studies have noted as thermally tolerant (Hawkins and Warner 2017). A second alga, *Breviolum minutum* (ITS2 type B1, reference number 83, originally

isolated from *E. pallida*) was selected based on its thermal sensitivity in culture (Mansour et al. 2018). This strain of *B. minutum* (donated by T.C. LaJeunesse, The Pennsylvania State University) was maintained in log-phase growth in sterile f/2 culture media (Guillard and Ryther 1962; Guillard 1975) at 28pall C and 80 µmol photons m^{-2} s^{-1} on a 12:12 light:dark cycle in an incubator (Percival scientific). In order to create Florida anemones with *B. minutum*, their original symbionts were removed via menthol exposure (Matthews et al. 2016) and were reinfected with *B. minutum*. Anemones were rendered aposymbiotic by treating previously collected animals to a series of menthol exposures similar to that described by Matthews et al. (2016). In brief, *E. pallida* were exposed to a 0.1% w/v menthol solution in 1.0% ethanol (working concentrations) mixed in ASW for 7 hours day^{-1}, 5 days $week^{-1}$, for several months (August 2016 – January 2017). During the menthol treatment, anemones were kept in the dark in artificial seawater at a constant temperature of 26 ºC and fed twice weekly with freshly hatched *Artemia* nauplii. Complete symbiont loss was confirmed using fluorescence microscopy (EVOS system, ThermoFisher) at 40 x magnification with a CY5 filter to visualize cells by algal chlorophyll a fluorescence.

Aposymbiotic animals were held in constant darkness for approximately one year after menthol treatment and prior to re-infection with new symbionts in August 2018. All aposymbiotic animals were fed 2 times $week^{-1}$, followed by full seawater replacement. Anemones were held at room temperature and under the same LED lighting as described above. Re-infection were performed by pipetting 2 mL of algal culture (containing ~300,000 cells mL^{-1}) into each 1 L bowl containing ~70 anemones. This was repeated 7 days later and anemones were simultaneously fed brine shrimp

during both infections. The algal population grew within its host for a minimum of three months prior to further use.

2.3.2 Experimental Design

The complete experimental design is shown in Figure 1. The experiment consisted of two parts designed to test nutrient effects on animal and algal physiology, and to test the effect of chronic thermal stress combined with the effect of nutrient status. First, the anemones were exposed to 1 of 3 different nutrient treatment over the course of 28-31 days (hereafter called Part-1). Measurements were collected over a period of 4 days, so this sampling was referred to as 28-31 days nutrient treatment. Four days were necessary to collect all the data for one sampling period. Animals were separated into a fed group (n = 48) and fed 1 time $week^{-1}$ with freshly hatched *Aremia salina* nauplii), while another group was starved (unfed, n = 48). Lastly, another set of animals (n = 48) were withheld food, but were treated with a low-level exposure to nitrate and phosphate (final concentration of 5 µM nitrate, 0.5 µM phosphate, 10:1 N:P). Sodium nitrate and sodium phosphate were added to the anemone container 2 times $week^{-1}$ and mixed with a stir bar for 3 minutes. Water samples were collected before inorganic nutrient addition, then after 6 and 24 hours to quantify nutrient draw-down in a 300 mL bowl of 4 anemones (water samples to be analyzed at a later date). The anemones were moved into clean 1.5 L glass bowls in artificial seawater (Instant Ocean, 33 psu, changed twice weekly) and placed in incubators (Percival Scientific) set to 27 ºC with fluorescent light set to 120 µmol photons $m^{-2}s^{-1}$ (cool white, Phillips fluorescent) on a 12:12 hour light:dark cycle. For each treatment group, 4 anemones were placed into each of 12 glass bowls (300 mL). One entire bowl of anemones was used for each assay (respiration and biomass, ^{14}C and biomass, or feeding rate) at each

of three sampling periods (day 0, 28-31 days of nutrient treatment, and 10-14 days of high temperature treatment). As entire 3 bowls were used at each sampling period, we were unable to test for a bowl effect.

At the start of the experiment and before anemones were separated into treatment groups, a subsample of anemones were measured for wet weight, symbiont density, chlorophyll *a*, and F_v/F_m (n = 4). After 28-31 days of nutrient treatment, individuals from each nutrient group were measured for wet weight, symbiont density, chlorophyll *a*, F_v/F_m, carbon fixation, carbon translocation, heterotrophic feeding rate, and respiration rate (further details below, n = 4 for each assay). Following Part-1, all remaining anemones were separated into control and high temperature groups (n = 32 for each nutrient treatment). The high temperature group was exposed to 32 ºC for 10-14 days while continuing their respective nutrient treatments. Part-2 began with 5 days of thermal ramping (1 ºC/day until reaching 32 ºC, followed by a sampling period (10-14 days at 32 ºC). The maximum temperature used in this study was selected based on bleaching thresholds observed globally, but usually under lower light levels in Key Largo (Dr. M. E. Warner, personal communication). After 10 days of chronic high temperature exposure, measurements were collected for wet weight, symbiont density, chlorophyll *a*, C:N, F_v/F_m, carbon fixation, carbon translocation, heterotrophic feeding rate, and respiration rate (n = 8 for each assay: n = 4 for control 27 ºC and thermal treatment 32 ºC respectively). Measurements were collected over a period of 4 days, so this sampling was referred to as 10-14 days chronic thermal treatment. On the first day, heterotrophic feeding rate was measured, the second day respiration rate was measured, the third day PSII protein turnover was measured (data not shown), and the

fourth day ^{14}C translocation was measured. The figures below reflect this in the day of the experiment (days 48 - 51).

F_v/F_m and the number of juveniles produced by asexual pedal laceration were measured (1 time week^{-1}) over the course of the entire experiment (Part 1 and 2, non-destructive techniques) to quantify photochemical efficiency of PSII and to quantify reproductive output. All bowls of anemones were examined each week for asexually produced juveniles with visible tentacles at the base of the adult pedal disk (i.e. the foot). Juveniles were counted by treatment group and then transferred to a separate bowl and held within the same respective incubator as their parents.

2.3.3 Processing for Animal and Algal Biomass

The processing protocol used for non-radioactive anemones is shown in Figure 3. The wet weight of each anemone was recorded before they were frozen at -80 ºC. Animals were thawed on ice and 0.5 mL cold lysis buffer (50 mM potassium phosphate, 0.1 M EDTA, pH 7.8) was added to each 2 mL screw cap vial with two stainless steel beads (3.2 mm diameter). Anemones were homogenized in a bead beater (MP Bio Bead Beater Pro Fast Prep 24) for 1 min at 5 ms^{-1}. Following homogenization, a subsample was removed for symbiont enumeration and fixed with glutaraldehyde (0.4% final concentration), and another subsample was removed for chlorophyll *a*. The remaining homogenate was centrifuged at 4,000 x g for 10 min (5415R Eppendorf) to pellet the symbiont cells separately from the animal host fraction, then a subsample was removed for protein quantification from the host fraction.

Algal cells were counted manually via an improved Neubauer hemocytometer on a light microscope (100 x). Chlorophyll a content was quantified by extracting the

pigment from the algal pellet with cold 100% methanol. Pigment absorbance was read on a FLUOstar Omega microplate reader (BMG LABTECH, Ortenburg, Germany) at 632 nm, 665 nm, and 750 nm (turbidity correction) wavelengths (Ritchie 2006; Warren 2008).

Carbon to nitrogen ratios for both host and symbiont fractions were measured on a CosTech Elemental Analyzer. Anemones from both the 27 ºC and 32 ºC treatment groups (n = 4) were weighed on a balance, homogenized following the above protocol, and homogenate was loaded into tin cups (10 x 12 mm, CE Elantech). Samples were dried in a drying oven at 60 ºC for one week. Standards were Phenylalanine and EDTA weighing 0.5-2 mg.

2.3.4 Photosynthesis and Carbon Translocation

F_v/F_m was measured over the course of the experiment on a Pulse Amplitude Modulation (PAM) fluorometer (measurement light = 6, saturation intensity = 12, saturation pulse = 0.8, gain = 10, damping = 2) on anemones dark acclimated for 20 min. The complete ^{14}C carbon fixation/translocation protocol is shown in Figure 2. Prior to measuring carbon uptake, anemones from each nutrient treatment/temperature were quickly weighed and then placed into glass vials containing artificial seawater. Carbon fixation was measured as gross ^{14}C uptake and normalized to algal cell number and DIC present in the seawater. Freshly collected seawater samples from anemone bowls were analyzed on an acid-sparging analyzer (AS-C5 DIC Analyzer, Apollo Sci Tech with a LI-COR LI-850 CO2 Analyzer, Newark, DE, USA) and compared to a seawater standard (Carbon Dioxide Information Analysis Center, Scripps, UCSD, USA). ^{14}C ($NaH^{14}CO_3$) label was added (0.15 µCi in 1 mL) to 2 mL artificial seawater containing a single anemone for each nutrient group/thermal treatment (n = 4).

Anemones (n = 3) were placed in a temperature controlled photosynthetron set to either 27 ºC or 32 ºC. Light was provided by a bank of LEDs (cool white, Cree XP-G2) set to 120 µmol photons m^{-2} s^{-1} for 60 minutes, or placed in the dark as a control for animal ^{14}C uptake alone (n = 1). Anemones were removed and then blot-dried on paper towels, placed in 2 mL screw cap vials, and immediately stored at -80 ºC until further processing.

Anemones were processed for biomass by thawing frozen samples on ice and suspending anemones in 0.5 mL lysis buffer (50 mM potassium phosphate, 0.1 M EDTA, pH 7.8) and were then homogenized with two stainless steel beads (3.2 mm diameter) in a bead beater (Fast Prep 24, MP Bio) for 1 min at 5 ms^{-1}. The homogenate was centrifuged at 8,161 x g for 5 min to pellet the algal cells, and a subsample for protein quantification was removed from the separated animal fraction. The algal pellet was washed in 500 µL lysis buffer with vortexing, and was centrifuged a second time at 8,161 x g for 5 min, and the resulting supernatant was collected for the second host fraction. The algal pellet was resuspended in 800 µL 0.2 µm filtered seawater and vortexed well. Following this final resuspension, a subsample was removed for symbiont enumeration and fixed with glutaraldehyde (0.4% final concentration), and a second subsample was removed for chlorophyll *a*.

2.3.5 Heterotrophic Feeding

Heterotrophic feeding rate was measured for each nutrient/thermal treatment group. Prior to feeding measurements, anemones were quickly weighed on a balance. Feeding chambers consisted of 50 mL glass beakers filled with fresh artificial seawater and held a small PVC platform covered in mesh (100 µm) that sat over a stir bar set to 200 rpm. Individual anemones were placed on the mesh platform and left

undisturbed in the dark for one hour to allow for attachment and tentacle relaxation. *Artemia* nauplii stock solution (approximately 555 *Artemia* mL^{-1}) was added to each feeding chamber, anemones were allowed to feed for 2 hours in the dark at room temperature, and remaining *Artemia* were counted. Anemones were removed, then feeding chambers were filtered onto 100 µm mesh, rinsed 3 times, then the sample was fixed with 70% ethanol solution and counted manually via dissection scope within 48 hours. Control *Artemia* samples (no anemone present) were quantified by manual counting via dissection scope. Feeding rate was calculated as the number of *Artemia* consumed per hour, normalized to anemone wet weight.

2.3.6 Anemone Respiration

Oxygen consumption was measured with a four channel contactless fiber-optic oxygen system (FireSting O_2, PyroScience). Anemones were placed into individually sealed 20 mL glass chambers fitted with an oxygen sensor strip and then held in a constant temperature water bath (27 ºC or 32 ºC respectively) connected to a recirculating heater/chiller. Prior to use, each sensor strip was calibrated to 0 and 100% saturation. In each vial, anemones sat on a 100 µm mesh platform, above a constantly stirring magnetic stir bar set to 200 rpm. Anemones were left undisturbed for one hour in unsealed vials to allow for attachment and tentacle relaxation, and respiration was recorded in the dark for approximately 40 minutes. Oxygen levels were not permitted to drop below 5 mg O_2 L^{-1} during any trial in order to avoid animal mortality. Following measurements in sealed chambers, anemones were removed, gently blotted on a paper towel and immediately weighed prior to homogenization for further biomass analyses described above. Background oxygen consumption was recorded in empty vials previously containing anemones for 30 minutes and was

negligible relative to the animal respiration vials. Respiration rates (g O_2 hr^{-1} mg wet $weight^{-1}$) were calculated by fitting the raw data to a linear function in R and then normalized to anemone wet weight.

2.3.7 Statistical Analysis

All statistical analyses were conducted in the open source software R with 'stats', 'FSA', 'Rmisc', and 'multcompView' packages (R Core Team, 2017). Each variable was tested for assumptions of normality of distribution and homogeneity of variance using the Shapiro-Wilk and Levene's test, respectively. If the data passed both assumptions, either a one-way ("Nutrient Group" as the fixed factor) or a two-way ("Nutrient Group" and "Temperature" as fixed factors) analysis of variance (ANOVA) was conducted to test the main effects of nutrient treatment and temperature and any potential interactive effects of the two. When significance of a main effect was found ($\alpha = 0.05$), a Tukey *post hoc* test was conducted to detect the significance between the different nutrient treatments or temperatures. When an interaction was discovered between effects, pairwise comparisons of the simple main effects (nutrient effect and temperature effect) were conducted with an adjusted $P < 0.017$ considered significant to account for the number of different nutrient treatments ($n = 3$). All statistical outputs are included in Tables 1 and 2.

2.4 Results

2.4.1 Part-1: Nutrient Treatment

2.4.1.1 Animal Biomass

All anemones started at the same wet weight on day 0 ($P = 0.718$ across groups, Figure 4). Wet weight significantly decreased in the starved group by almost half of its initial weight compared to the day 0 control ($P = 0.024$), while the fed group gained weight ($P = 0.031$). However, wet weight did not vary significantly in the inorganic group over the 31-day treatment period ($P = 0.882$). Individual animal wet weight was used to normalize symbiont density, respiration rate, and feeding rate below.

2.4.1.2 Algal Symbionts

Animals in all three nutrient groups had similar symbiont densities at the start of the experiment ($P = 0.346$ across groups, Figure 5). Both the fed ($P = 0.041$) and starved groups ($P = 0.002$) significantly decreased symbiont densities after 31 days of nutrient treatment. While symbiont density in the inorganic group did not significantly change, it also showed a decreasing trend after 31 days of nutrient treatment.

There was no difference in the starting cellular chlorophyll *a* content on day 0 ($P = 0.084$ across groups, Figure 6). After 31 days of treatment, chlorophyll *a* was approximately twice as high in the starved ($P < 0.001$) and inorganic groups ($P < 0.001$) when compared to their starting concentrations, but chlorophyll *a* cell^{-1} did not change in the fed group ($P = 0.084$). The maximum quantum yield of PSII (F_v/F_m) was similar across all groups at the start of the experiment ($P = 0.152$ across groups, Figure 7). Likewise, F_v/F_m did not change between day 0 and day 31 in the fed group

($P = 0.597$) or starved group ($P = 0.070$) but dropped significantly in the inorganic group ($P < 0.001$). With regard to photosynthetic carbon fixation, 31 days of nutrient treatment had no distinguishable effect on photosynthesis in any treatment group when normalized to either algal cell ($P = 0.139$) or chlorophyll *a* content ($P = 0.519$, Figure 8 Panes A and B).

2.4.1.3 Carbon Translocation, Holobiont Respiration, and Heterotrophy

For each nutrient treatment, the percentage of photosynthetically fixed carbon translocated to the host did not change ($P = 0.707$, Figure 9). However, feeding rate varied across the nutrient groups (Figure 10). The inorganic and fed groups were statistically similar ($P = 0.078$), as well as the starved and inorganic groups ($P = 0.079$), however, the fed and starved groups were significantly different from each other, with the starved group consuming almost twice as many *Artemia* as the fed group ($P = 0.002$). Respiration rate remained the same and did not significantly vary ($P = 0.747$) within nutrient treatment groups after 31 total days (Figure 11).

2.4.2 Part-2: Chronic Thermal Treatment

2.4.2.1 Animal Biomass

At day 51 of the experiment, we observed a continued effect of the three different nutrient treatment on *E. pallida* wet weight ($P < 0.01$, Figure 12). The inorganic and starved groups were similar in weight ($P = 0.990$), however, the starved and inorganic groups were significantly smaller than the fed group and roughly half the wet weight of the fed group ($P < 0.001$ and $P = 0.010$ respectively). There was no discernable effect of temperature on anemone wet weight. Likewise, there was no interaction between nutrient group and temperature for wet weight ($P = 0.249$).

2.4.2.2 Algal Symbionts

Symbiont density decreased with high temperature ($P = 0.011$) as well as across nutrient treatments ($P = 0.018$, Figure 13). The starved group decreased its symbiont density by about half of the fed group ($P = 0.020$), with a similar but non-significant trend in the inorganic group ($P = 0.065$). No interaction for nutrient group and temperature was found for symbiont density ($P = 0.968$). In contrast, there was an increase in cellular chlorophyll *a* in the unfed nutrient treatments ($P < 0.01$), but chlorophyll *a* was not affected by temperature ($P = 0.124$), nor was there a significant interaction of nutrient group and temperature ($P = 0.529$, Figure 14). Cellular chlorophyll *a* in the starved anemones was almost twice that of the fed group ($P = 0.020$), with the inorganic group appearing moderate between the other two groups ($P = 0.835$). Fewer algal cells had higher chlorophyll *a* content ($cell^{-1}$) in the starved nutrient treatment. Nutrient condition and temperature had no significant effect in the animal fraction C:N ($P = 0.878$ and $P = 0.640$ respectively, Figure 15). For algal C:N, while there was no significant effect of high temperature ($P = 0.345$) there was a significant effect of nutrient group ($P = 0.002$). Specifically, algal C:N in the inorganic and starved groups were significantly higher compared to the fed group ($P = 0.002$ and $P = 0.041$ respectively).

There was a significant effect of both nutrients and temperature on F_v/F_m ($P = 0.002$ and $P = 0.030$, Figure 16). Again, both inorganic and starved groups were lower than the fed group, but no different from one another ($P = 0.005$, $P = 0.011$, and $P = 0.973$ respectively). No significant interaction was detected in the F_v/F_m between nutrient group and temperature ($P = 0.347$). When cellular photosynthesis was compared, there was a significant interaction for carbon fixation normalized to algal cell between nutrient group and temperature ($P = 0.009$, Figure 17 Pane A).

Specifically, the interaction of photosynthesis (carbon fixation) algal cell^{-1} was driven by the simple main effect of nutrient treatment. There was a decrease in carbon fixation in the fed group, but an increase in carbon fixation in the starved group with temperature ($P = 0.020$ and $P < 0.001$). Meanwhile, carbon fixation normalized to chlorophyll *a* decreased across nutrient groups ($P = 0.001$), and with high temperature ($P < 0.001$, Figure 17 Pane B). The inorganic and starved groups fixed less than half the carbon that the fed group did ($P < 0.001$ and $P < 0.001$), but the same as one another ($P = 0.863$). There was no significant interaction between the nutrient groups and temperature for carbon fixation normalized to chlorophyll *a* ($P = 0.253$).

2.4.2.3 Carbon Translocation, Holobiont Respiration, Heterotrophy, and Fitness

An interaction between nutrient group and temperature was detected in the percent of fixed carbon translocated to the host ($P = 0.022$, Figure 18). Specifically, the interaction was driven by the simple main effect of nutrient group. There was an increase in translocation in the starved group and a decrease in the fed group with high temperature ($P = 0.025$) and between the fed and starved groups ($P = 0.013$), but there was no simple main effect of nutrients when comparing the starved and inorganic groups ($P = 0.512$) or any simple main effect of temperature ($P = 0.770$). Carbon translocation only increased in the starved group with high temperature. Respiration rate also exhibited a significant nutrient group and temperature interaction ($P = 0.013$, Figure 19). The interaction had a simple main effect of nutrient group, which occurred between both the fed and starved groups ($P = 0.019$), and a simple main effect of temperature ($P = 0.007$). However, the trend was an increase in respiration with high temperature as expected, but was about 10 times higher than the control rate which

was a much larger magnitude change than previously observed (Hawkins and Warner 2017).

Due to sample loss and extremely high variance across samples, I was unable to statistically examine the effect of high temperature and nutrient treatment on the feeding rate for this part of the project. While the asexual reproduction data are only considered on a qualitative basis here, some interesting patterns emerged from this experiment (Figure 20). Cumulative juvenile bud production increased over the duration of the experiment, but did not appear to differ between groups. Once the temperature was raised, asexual budding continued at a similar rate to the control temperature animals. Adult anemones were removed from each treatment group at the same time during the experiment. Reproduction normalized to number of adults fluctuated throughout the experiment, but generally declined in the starved group. Budding increased rapidly with temperature, within the first week of temperature ramping up to 32 ºC, by double in the starved group. It is also possible that reproduction is limited by the absolute size of the animal - that anemones will not invest energy into reproduction until they achieve a certain body size. This idea has not yet been empirically tested to date, but could explain the decline in starved group reproduction in conjunction with a decline in anemone size (wet weight).

2.5 Discussion

2.5.1 Part-1: Nutrient Treatment

2.5.1.1 Animal Response to Nutrient Treatment

The goal of this study was to describe the effects of nutrient status on the physiological response of *Exaiptasia pallida*. Manipulation of three different nutrient

treatments over 31 days, designed to test the benefits of heterotrophic feeding or inorganic nutrient addition compared to starvation in symbiotic cnidarians, resulted in increased fitness in the fed *E. pallida* by proxy of wet weight. Fed animals were able to maintain their size as expected from previous studies (Muller-Parker 1985). Animal protein is widely used to normalize data to animal size, but in this study wet weight was used due to low protein content in the homogenate samples. A wet weight-protein regression will be examined at a later date to provide an ease of comparison between these two normalization techniques.

Across all three nutrient treatments, there was no change in respiration despite changes in the symbiont number, indicating that the host did not increase its metabolic rate even when losing symbionts. However, the decline in symbiont density should not significantly affect the respiration rate and symbiont density is not typically accounted for in respiration calculations (Hawkins and Warner 2017). Perhaps the combination of symbiont density and the loss of host tissue in the unfed anemones led to no net change in respiration. Overall, anemone wet weight declined in the unfed groups, indicating a benefit of heterotrophic feeding on animal size.

Starved animals had notably higher prey capture than continuously fed animals had after 31 days of starvation. This further demonstrates that starved anemones are poised to capture prey and the reliance this anemone has for energy input provided by zooplankton. Starved anemones possibly diverted greater energetic resources into synthesizing more nematocysts used to capture prey. However, it is possible that normalizing feeding rate to body size resulted in the appearance of higher feeding rates in starved anemones. Thorington et al. (2010) found that anemones fed to repletion were not inhibited in prey capture or prey ingestion, as well as partially

inhibited in prey killing when compared to starved anemones. A recent study by Hoepner et al. (2019) examined the quantity and quality of nematocyst venom in the sea anemone *Entacmaea quadricolor* with bleaching and found that venom was maintained in these anemones, indicating the resilience of these animals when confronted with climate change and environmental stressors. Additionally, studies predict zooplankton decline in climate change conditions in locations around the world, including the tropical Atlantic, the Bering Sea, and the North Pacific (Piontkovski and Castellani 2009; Eisner et al. 2014; Woodworth-Jefcoats et al. 2017), which would limit the animal's ability to take advantage of the benefits of feeding observed in this study. Cnidarians exhibit a prey preference for small size 200 – 400 μm (Palardy et al. 2006) and is representative of feeding in the field, which was the reason for selecting *Artemia salina* nauplii in this study. Some types of prey (e.g. copepods) avoid capture in anemone tentacles via escape or avoidance behavior, and despite their abundance in the field are not commonly consumed by corals (Sebens et al. 1996). Together, this study and others suggest a beneficial impact of heterotrophic feeding on *E. pallida* by providing another source of organic carbon for the animal.

2.5.1.2 Algal Response to Nutrient Treatment

The net decline in algal symbionts in the unfed groups may have been a result of those algae diverting more energy into maintaining photosynthesis and increased chlorophyll *a* instead of investing energy into population growth. While F_v/F_m was significantly lower in these treatments compared to the fed treatment, the biological implications of such small declines in photochemistry should be interpreted with caution as these algae were still able to maintain photosynthetic carbon fixation and carbon translocation to the host. This dynamic also demonstrates the tight nutrient

recycling in cnidarian symbioses (Piniak and Lipschultz 2004; Ferrier-Pagès et al. 2016; Matthews et al. 2017). For example, the animals respire and produce CO_2, which the algae use for photosynthesis and translocate organic carbon back to the animal. The alga receives more macronutrients in a concentrated area as compared to algae grown in monoculture or free-living in the ocean. Based on previous longer (5 week) experiments on *Aiptasia pulchella*, we anticipated that the starved group would exhibit higher symbiont density (Muller-Parker 1985), but lower F_v/F_m compared to fed anemones. We observed the opposite effect on symbiont density in this study, - all nutrient treatments lost algal cells. Similarly, Muller-Parker (1985) found higher chlorophyll *a* (cell^{-1}) in starved *Aiptasia pulchella* sea anemones, which was also observed in this study.

Cell size, chloroplast arrangement, pigment content (including accessory pigments), and pigment packaging into the thylakoids all play a role in the total amount of light that can be absorbed by the photosynthetic pigments in marine algae (Glover et al. 1987). Spectral quality of light can affect photosynthetic efficiency as well. In this study, cool white lights were used and only chlorophyll *a* pigment was quantified. Chlorophyll *a* absorbs light in both the red and blue wavelengths in the visible light spectrum. Cool white fluorescent lights emit a continuous spectrum of visible light (Glover et al. 1987), which is representative of ambient light in the field and is the typical light source utilized when growing marine phytoplankton in the lab. The other metrics of cell size, chloroplast arrangement, and other pigments were not measured in this study.

When algae live within the tissues of a cnidarian, host tissue affects the light that the algae receive. Wet weight declined the most in the starved group, which

constrains symbionts to fewer gastrodermal cells in the animal. Taken in the context of a smaller area to inhabit, would mean the algal cells are packed more densely (despite loss in symbiont density) with increased symbiont self-shading. With more self-shading, each algal cell is exposed to a lower irradiance, which explains the increased chlorophyll *a* observed (Warner and Madden 2007). Decrease in symbiont density could release the remaining symbionts from carbon limitation *in hospite*.

Despite the reduction in cell densities, algal carbon fixation did not significantly change across nutrient groups but were still relatively high compared to values observed in Hawkins and Warner (2017). However, the starved group showed an increasing trend of photosynthesis (algal cell^{-1}). Starved anemones may have been investing more energetic resources (e.g. organic carbon) into supporting the photochemistry of their symbionts, since these unfed anemones were completely autotrophic. Another possible resource anemones may utilize to support their symbionts is increased carbonic anhydrase expression and activity, which enhances inorganic carbon delivery to the algae for photosynthesis (Weis et al. 1989). Total carbon translocation was maintained across all three nutrient groups, however, all groups experienced a decline in algal cells. This observation agrees with previous studies on *E. pallida* (Clayton and Lasker 1984; Davy and Cook 2001), and corals (Tremblay et al. 2014) that also found percent carbon translocation did not change with starvation. However, it is important to consider that the percentage of carbon translocated does not mean symbionts translocated the same absolute amount of carbon. There could be a difference in the rate of carbon translocation across nutrient treatment groups, but it was not statistically examined at this time for this study. When compared to anemones, coral symbionts translocate a higher percentage of

photosynthetic carbon to their hosts (Houlbrèque and Ferrier-Pagès 2009; Hoadley et al. 2016), similar to observations in this study. Overall, *E. pallida* responded to starvation by decreasing animal size while the symbionts still maintained photosynthesis and carbon translocation.

While partners in the symbiosis recycle nutrients, nitrogen may be more abundant than carbon (Davy and Cook 2001; Ezzat et al. 2016). Earlier work with *E. pallida* by Davy and Cook (2001) observed an increase in net photosynthesis and photosynthate translocation after 20-30 days of starvation, and symbiont density declined in these starved anemones as well. The increase in net photosynthesis was thought to be the result of greater carbon availability per algal cell (Davy and Cook 2001; Wooldridge 2009; Hoadley et al. 2016; Rädecker et al. 2017), demonstrating that carbon limitation is relieved when symbiont density decreases. Early work with free-living phytoplankton assumed that carbon limitation did not play a large role, where subsequent studies have observed increases in productivity associated with elevated DIC, suggesting that carbon limitation may indeed play an important role in productivity rates within the symbiosis (Goreau 1977; Muscatine et al. 1989; Weis 1993).

Muller-Parker (1988) found that algae took up more inorganic nutrients when they were isolated from starved anemones, and continued to take up more nutrients the longer the host was starved. Interestingly, the algal inorganic group responded similarly to the starved group throughout this experiment. This effect is likely driven by anemone starvation as seen in the starved group, but inorganic nutrient addition had little effect. The inorganic nutrients used in this current study were relatively low and may not have been high enough to detect a significant effect. In the Florida Keys

where *E. pallida* were collected, ammonium may reach concentrations of 5–10 µM (Dr. M. E. Warner, personal communication; Falkowski et al. 1993). Another consideration is the form of nitrogen addition, as nitrate is more energetically costly to incorporate into cells than ammonia (Eppley et al. 1969; Wilkerson and Muscatine 1984). Overall, inorganic nutrient addition had no discernable effect on the alga. Overall, *E. pallida* responded to starvation by decreasing animal size while the symbionts still maintained photosynthesis and carbon translocation.

2.5.2 Part-2: Chronic Thermal Treatment

2.5.2.1 Animal Response to High Temperature

The goal of this study was to describe the effects of elevated temperature on a thermally sensitive alga preacclimated to three different nutrient scenarios. Chronic elevated temperature (10-14 days), designed to mimic high temperatures observed in the field, resulted in further decline in wet weight, increased respiration, and increased asexual reproduction.

Not surprisingly, unfed wet weight started at about half of the fed group weight (not an *a priori* assumption going into Part-2; inorganic vs. fed: $P > 0.001$, starved vs. fed: $P > 0.001$). However, increased temperature did not continue to drive any decline in weight. In agreement with the Grottoli et al. (2006) study on corals, animal weight was maintained with high temperature in fed anemones. Respiration increased with temperature across nutrient groups in agreement with previous studies on increased temperature in *E. pallida* (Hawkins and Warner 2017). The large jump in respiration in the starved group at 32 ºC was supported by increased carbon translocation from the

symbionts - starved anemones appear to be respiring the extra carbon they are receiving from their algae.

Q_{10} is a measure of the proportional increase in a biological chemical reaction (e.g. respiration or photosynthesis) for every 10 ºC increase in the measured temperature, typically around 2 in plants (Atkin et al. 2005). This proportional value could indicate that the increased respiration and photosynthesis observed in Part-2 of this study were simply due to a faster rate of the basic chemical reactions, not due to any sort of host control. The Q_{10} values for respiration and photosynthesis in this study will be examined at a later date.

While reproduction data was qualitative in this study, it is interesting that the fed group appeared to maintain asexual budding better than the starved and inorganic groups. There was a declining trend of reproduction in the starved group over the whole experiment, which may have continued to decline if the experiment were conducted longer. A general decrease in reproduction with high temperature across the three nutrient groups was possibly driven by reallocation of resources for greater cellular maintenance and repair. Starved anemone carbon translocation could have contributed the energy needed to increase reproduction, but was unlikely due to increased respiration and thus maintenance of metabolism.

2.5.2.2 Algal Response to High Temperature

Algal symbiont population decreased and photosynthetic efficiency of PSII was lost in the unfed groups, but symbionts in unfed anemones were still able to maintain photosynthetic carbon fixation and carbon translocation to the host. Previous studies have noted that high temperature decreases photochemical efficiency in Symbiodiniaceae (Goulet et al. 2005; Ragni et al. 2010; Karim et al. 2015; Davies et

al. 2018). However, the decline in relative F_v/F_m in this study was not as large compared to acute heating described in Hawkins and Warner (2017). Symbiont density declined with high temperature exposure across all groups, but decreased most in the fed group. The unfed groups started with about half of the algal density of the fed group, and hence did not drop by as much with high temperature. As discussed above, a decline in symbiont density may be driven by a decrease in host biomass. High temperature leads to increased production of ROS in both chloroplasts and mitochondria, and unfavorable conditions for the algae within their anemone hosts as discussed above. It is possible that heterotrophically fed anemones are providing some sort of ROS protection to their symbionts, and thus there is less risk for fed anemones to lose their symbionts. Hawkins and Warner (2017) found that thermally pre-conditioning *E. pallida* protected mitochondrial function and symbiosis integrity. Recent work also hypothesized that the anemone could be assimilating various forms of nitrogen in the seawater and sharing that nitrogen with its algae (Roberts et al. 2001; Yellowlees et al. 2008).

Cook et al. (1988) found that algal symbiont C:N increased from 7.5 to 16 when *Aiptasia pallida* were starved for 20-30 days in the laboratory, but was slightly higher in field-collected hosts (9.4); however C:N in this study remained near 5 in all groups after 50 days of nutrient treatment and in both high temperature and control groups. Algal C:N was higher in the unfed groups, indicating their lower N content due to lack of feeding (Goldman et al. 1979). However, the C:N did not start at a high value, indicating that symbionts were not truly nitrogen limited to begin with. Photosynthetic carbon products can be considered “junk food”, deficient in nitrogen (Falkowski et al. 1984), which further implicates the importance of C:N of the alga

because C:N shows the ratio of carbon and nitrogen present. If the algae are nitrogen limited, their translocated photosynthetic products are also low in nitrogen. The C:N is used as an indication of the quality of translocated products from the alga to the host, with a lower C:N indicating a better quality (due to a higher nitrogen content). Nitrogen is an essential macronutrient used to synthesize cellular proteins as discussed above.

F_v/F_m declined with high temperature in all groups, with the greatest drop observed in the fed group where the symbiont density also decreased the most. Although statistically significant, the decline in F_v/F_m from approximately 0.63 to 0.57 is not a biologically relevant decrease with regards to photoinactivation. Other studies have shown much greater relative decline (almost half the initial value) in F_v/F_m (Hawkins et al. 2016). This minimal decline in F_v/F_m combined with a decrease in symbiont population may indicate the initial signs of bleaching stress. Nevertheless, this result was unexpected when considering previous studies with high temperature fed vs. starved corals (Grottoli et al. 2006), but the high temperature study by Grottoli et al. (2006) was conducted for 5-6 weeks as compared to only 2 weeks in this study and was also performed with corals. It is possible that the animals were not exposed to increased temperature treatment long enough to discern much effect of high temperature on photochemistry. Hawkins et al. (2016) reported that increasing temperature even by 1.5 ºC (from 32 ºC to 33.5 ºC) led to a significant decline in F_v/F_m, which demonstrates that anemones can easily be pushed over the edge to bleaching. However, relying on PSII photochemistry alone as a determinant of algal stress can lead to misinterpreted results. For example, functional absorption cross-section (σ_{PSII}), maximum rate of photosynthesis (P_{max}), rate approaching P_{max} (α), non-

photochemical quenching (NPQ), and compensatory electron flow between the light harvesting complexes of PSII could all be examined in conjunction with F_v/F_m to provide a more complete indication of photochemistry (Behrenfeld et al. 1998), but will not be discussed in detail here.

Photosynthetic carbon fixation normalized to algal cell or chlorophyll *a* must be interpreted carefully due to a decrease in algal cells and in chlorophyll *a*. Cell density was decreasing, but the data indicate the starved group truly did fix more carbon with increased temperature. Elevated temperature associated with global warming is known to decrease photosynthesis, damage proteins, increase ROS production, and increase metabolic rate (Goulet et al. 2005). Increases in carbon translocation were previously observed for fed corals (Grottoli et al. 2006), but the opposite was found in this study when fed anemones were exposed to high temperature. Starved algae increased carbon translocation with high temperature in conjunction with increased carbon fixation. This point could influence what has come be known as the selfish alga hypothesis, wherein an alga may withhold photosynthetic carbon from its host when under stress (Stat and Gates 2011; Starzak et al. 2014). Some algae may be considered opportunists, especially if a coral host has lost its homologous symbionts due to bleaching, and 'new' algae translocate less carbon to the host. While not an exact analog, results here run counter to this hypothesis in the starved anemones where carbon fixation increased, but the percentage of carbon translocation also increased. Overall these results indicate that despite a decline in symbiont population, the alga can still function with chronic high temperature stress.

2.5.3 Limitations and Future Directions

As discussed previously, it is possible this study wasn't conducted long enough to see significant changes in holobiont physiology. It should also be noted that the *Exaiptasia pallida* model system is widely used to study the cnidarian symbiosis, but it is not identical to the symbiosis of scleractinian corals. Stony corals have additional factors such as calcification to consider in carbon budgets, unlike in the anemone model system. This could be one reason why *E. pallida*'s symbionts translocated less carbon than is typically observed in corals (Tremblay et al. 2014). Another consideration when comparing this study to the literature is the identity of the algal symbiont and the cnidarian host. *E. pallida* forms a symbiosis with *B. minutum* in its natural habitat, but not all corals harbor *B. minutum*. Thornhill et al. (2013) found two genetically distinct populations of *E. pallida* from samples collected around the world, one genetic population is located in FL and the other genetic population is spread across oceans of the world. The FL population of *E. pallida* participates in diverse symbioses not seen in other locations (where they were only found harboring *B. minutum*). The identities of the host and the symbiont could have a major effect on the holobiont response to heterotrophic feeding or to high temperature (Sampayo et al. 2008; Grottoli et al. 2014). The cnidarian symbiosis is especially diverse and dynamic in *E. pallida*, which can lead to an ecological advantage for certain host-symbiont combinations. For example, some species of Symbiodiniaceae are more thermally tolerant than others. The strain of *B. minutum* used was previously shown to be thermally sensitive and common in the field where these animals were collected, which is why it was chosen for this study as discussed above. Anemones harboring *B. minutum* may see a greater impact of climate change in the near future as compared to anemones harboring a more thermally tolerant species.

2.5.4 Broader Impacts and Conclusion

I would suggest further field studies be conducted with zooplankton concentrations collected from FL in the native habitat, as well as further study with higher replication. The observed response of *E. pallida* to heterotrophic feeding with thermal stress was not the same response observed in corals under similar conditions. In corals, symbiont density, photosynthetic efficiency of PSII, and carbon translocation rates were maintained with feeding and high temperature. In anemones in this study, wet weight was maintained with feeding and high temperature, but I did not note the same type of advantage with heterotrophic feeding as seen in the coral literature.

Despite similarities in the starting *E. pallida* population, changes were observed between different nutrient and thermal treatment groups, indicating a response to the respective treatments. As discussed previously, the change in animal size undoubtedly influenced changes in algal physiology in turn, and may indicate some host control over the algal symbionts. The response of sea anemones and corals to nutrient availability and elevated temperatures will become increasingly important under climate change conditions to understand the future of coral reefs. Similarly, the ability to differentiate between host and symbiont physiological responses and how those separate responses affect the holobiont symbiosis is another important area of symbiosis research that should be investigated further.

TABLES

Table 1. One-way ANOVAs for the effects of nutrient treatment for (Part-1). Significant *P*-values are depicted in bold.

Variable	**Fig. No.**	**Effect Tested**	**Degrees of Freedom**	**F-value**	**p-value**
Wet Weight	6	Nutrients	3	4.60	**> 0.001**
Symbiont Density per Wet Weight	7	Nutrients	3	0.135	**0.001**
Chlorophyll per Algal Cell	8	Nutrients	3	22.6	**> 0.001**
Carbon Fixed per Algal Cell	10	Nutrients	2	2.79	0.139
Carbon Fixed per Chlorophyll	10	Nutrients	2	0.734	0.519
Fv/Fm	9	Nutrients	3	1.11	**> 0.001**
Feeding rate	12	Nutrients	2	12.5	**0.003**
Respiration	13	Nutrients	2	0.302	0.747
Percent Carbon Translocation	11	Nutrients	2	0.367	0.707

Table 2. Two-way ANOVAs for the effects of nutrient treatment, temperature, and the interaction of effects (Part-2). Significant *P*-values are depicted in bold.

Variable	**Figure No.**	**Effect Tested**	**Degrees of Freedom**	**F-value**	**p-value**
Wet weight	14	Nutrients	2	46.89	**> 0.001**
		Temp	1	0.927	0.339
		Interaction	1	1.42	0.249
Symbiont Density	15	Nutrients	2	5.03	**0.018**
		Temp	1	7.95	**0.011**
		Interaction	1	0.032	0.968
Chlorophyll	16	Nutrients	2	14.8	**> 0.001**
		Temp	1	2.46	0.124
		Interaction	1	0.647	0.529
C:N (animal)	17	Nutrients	2	0.132	0.878
		Temp	1	0.231	0.640
		Interaction	1	0.003	0.997
C:N (alga)	17	Nutrients	2	10.8	**0.003**
		Temp	1	0.974	0.345
		Interaction	1	0.929	0.424
Fv/Fm	18	Nutrients	2	6.41	**0.002**
		Temp	1	4.86	**0.030**
		Interaction	1	1.07	0.347
Carbon fixed per algal cell	19	Nutrients	2	2.84	0.105
		Temp	1	0.005	0.946
		Interaction	1	7.74	**0.009**
Carbon fixed per chlorophyll	19	Nutrients	2	15.4	**0.001**
		Temp	1	29.8	**< 0.001**
		Interaction	1	1.61	0.253
Percent Carbon translocation	20	Nutrients	2	9.71	**0.003**
		Temp	1	0.240	0.632
		Interaction	1	5.33	**0.022**
Respiration rate	22	Nutrients	2	8.12	**0.003**

		Temp	1	24.5	**< 0.001**
		Interaction	1	5.59	**0.013**

FIGURES

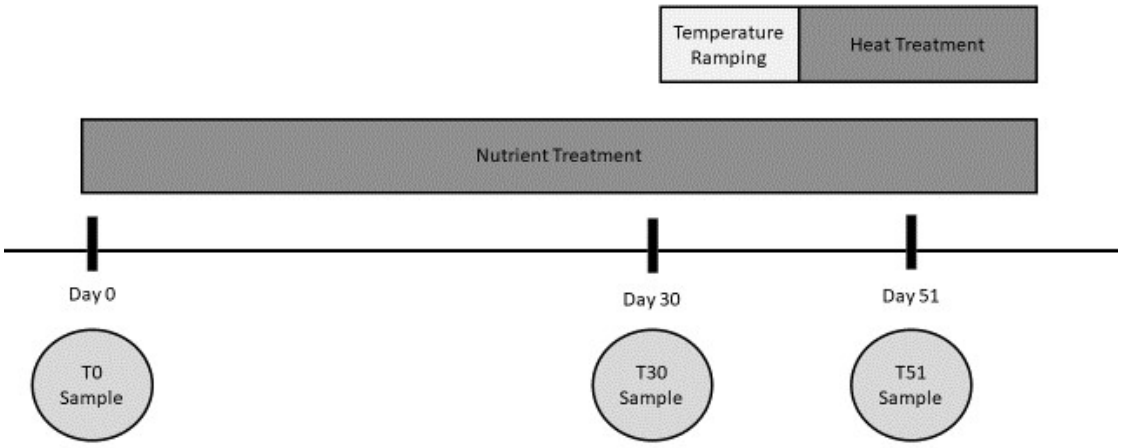


Figure 1. Experimental time line. Sampling periods are depicted in circles going from left to right under the timeline, while treatments are depicted in rectangles above the timeline.

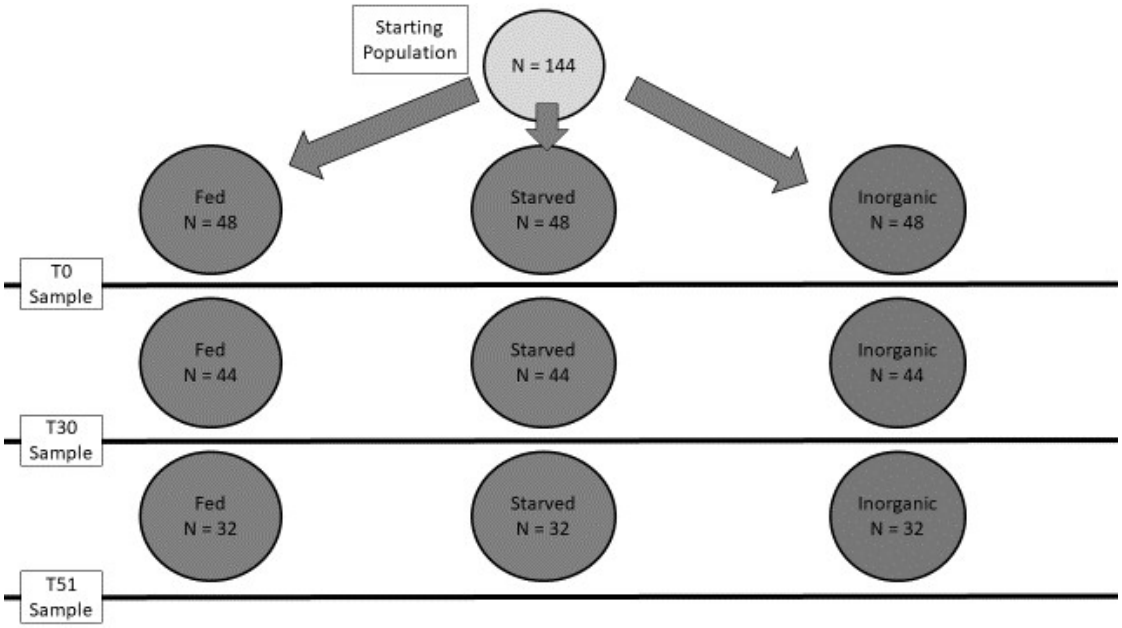


Figure 2. Anemone numbers throughout the experiment. A starting population of 144 *E. pallida* anemones were separated into three different nutrient treatment groups. n = 4 anemones were removed at T0 sampling, n = 12 were removed for the T30 sampling, and n = 32 were removed for the T51 sampling for each nutrient treatment group.

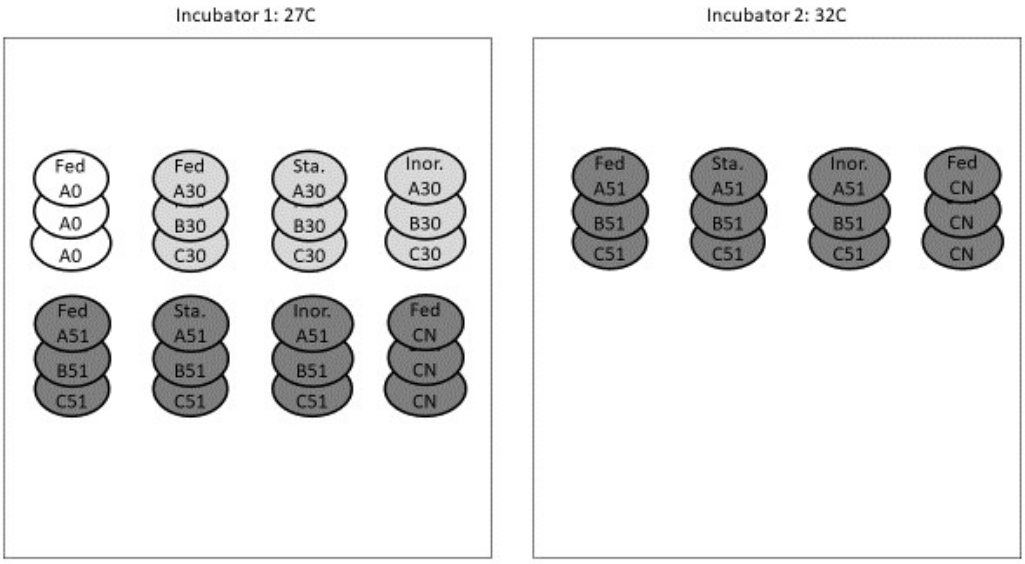


Figure 3. Bowls (circles) containing each n = 4 individual *E. pallida* anemones for each sampling period. Bowls were stacked three high inside the incubators. White "0" bowls were used for the T0 sampling, light gray "30" bowls were used for the T30 sampling, and dark gray "51" bowls were used for the T51 sampling. "CN" bowls were only used at the day 51 sampling. Each bowl was rotated daily to remove any spatial effects within the incubator. Figure not drawn to scale.

*

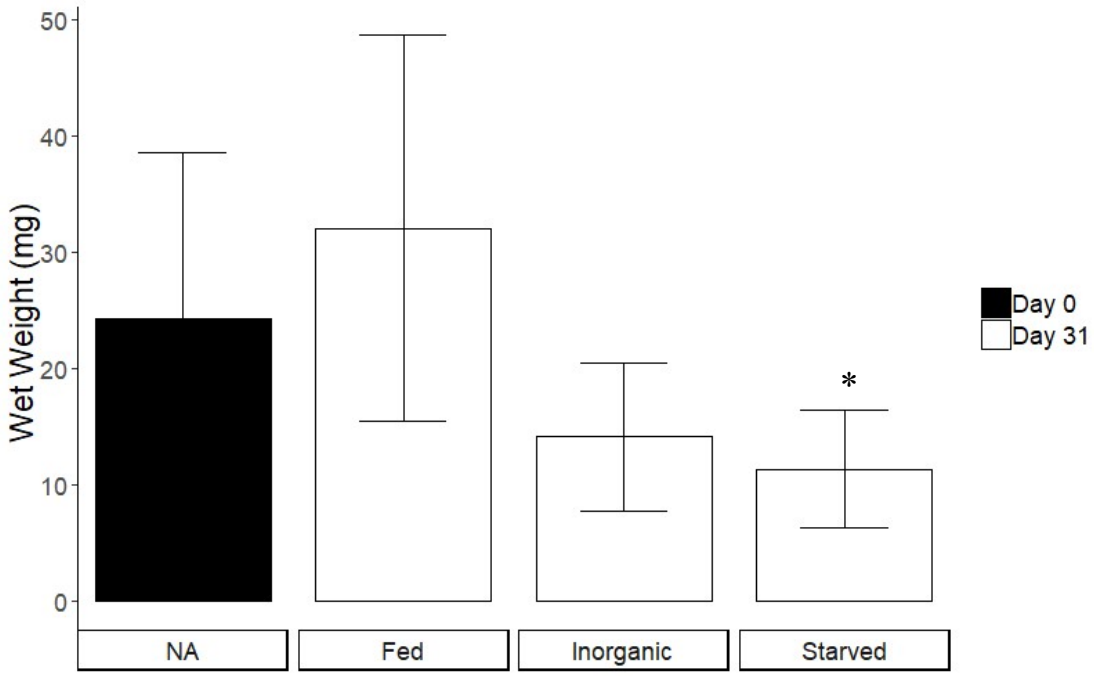


Figure 4. *E. pallida* mean wet weight after 31 days under three different nutrient conditions. Error bars are ±1 SD (n = 4, day 0 and n = 12, day 31). Asterisks designate a significant difference of a day 31 nutrient group compared to the day 0 control.

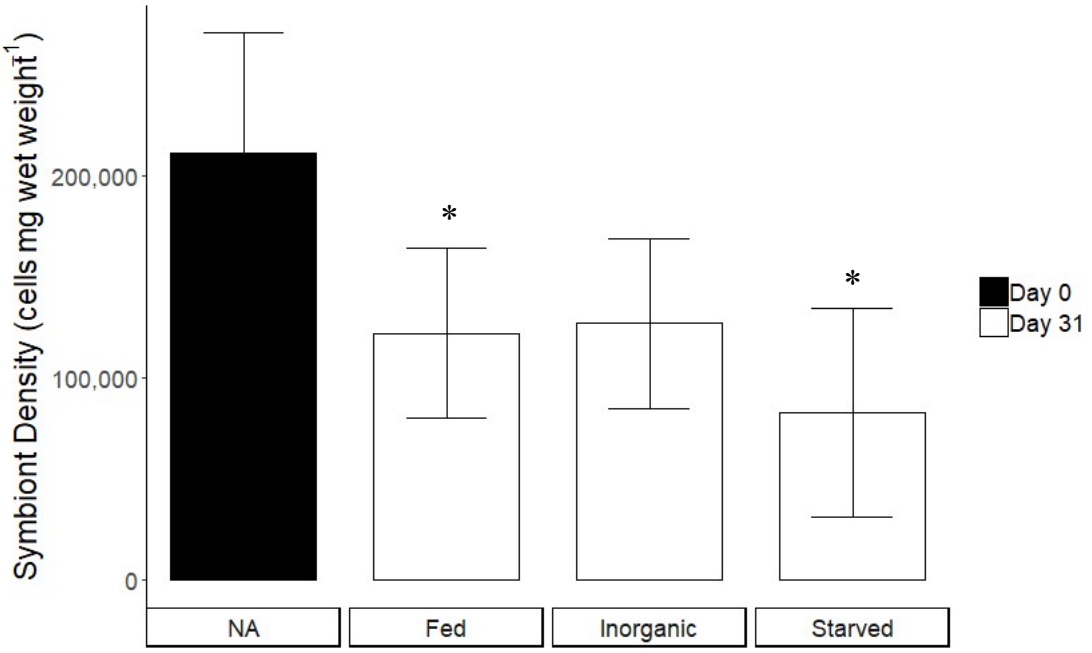


Figure 5. Mean symbiont density in *E. pallida* after 31 days under three different nutrient conditions. Error bars are ±1 SD (n = 4 at each point). Asterisks designate a significant difference of a day 31 nutrient group compared to the day 0 control.

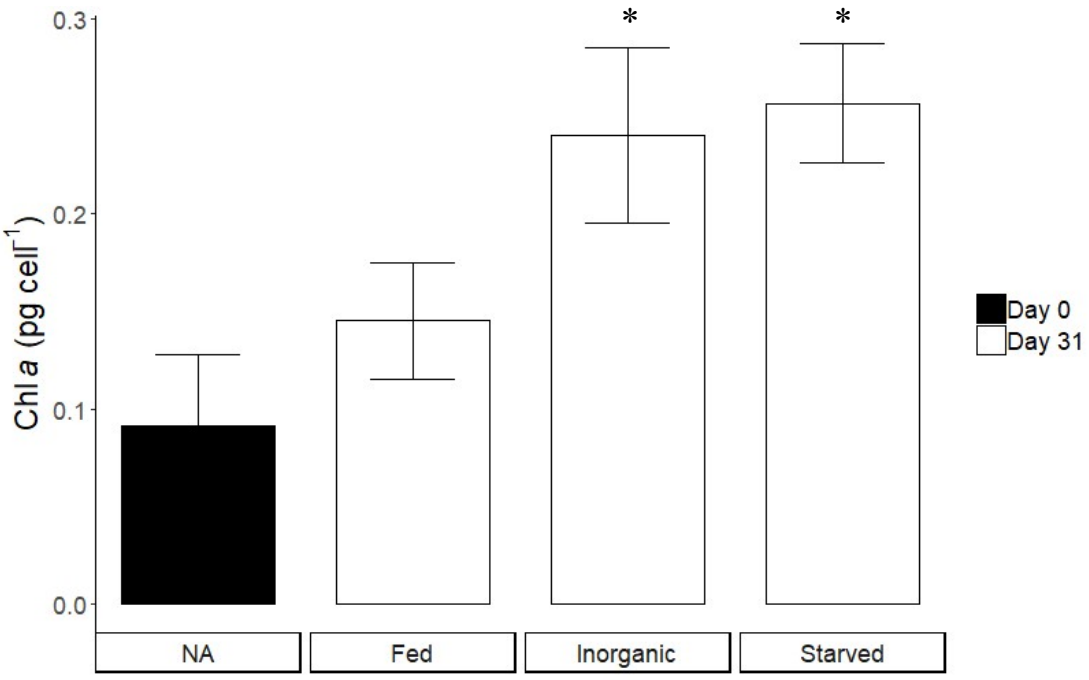


Figure 6. Symbiont chlorophyll *a* content in *E. pallida* after 31 days under three different nutrient conditions. Error bars are ±1 SD (n = 3–4). Asterisks designate a significant difference within a nutrient group.

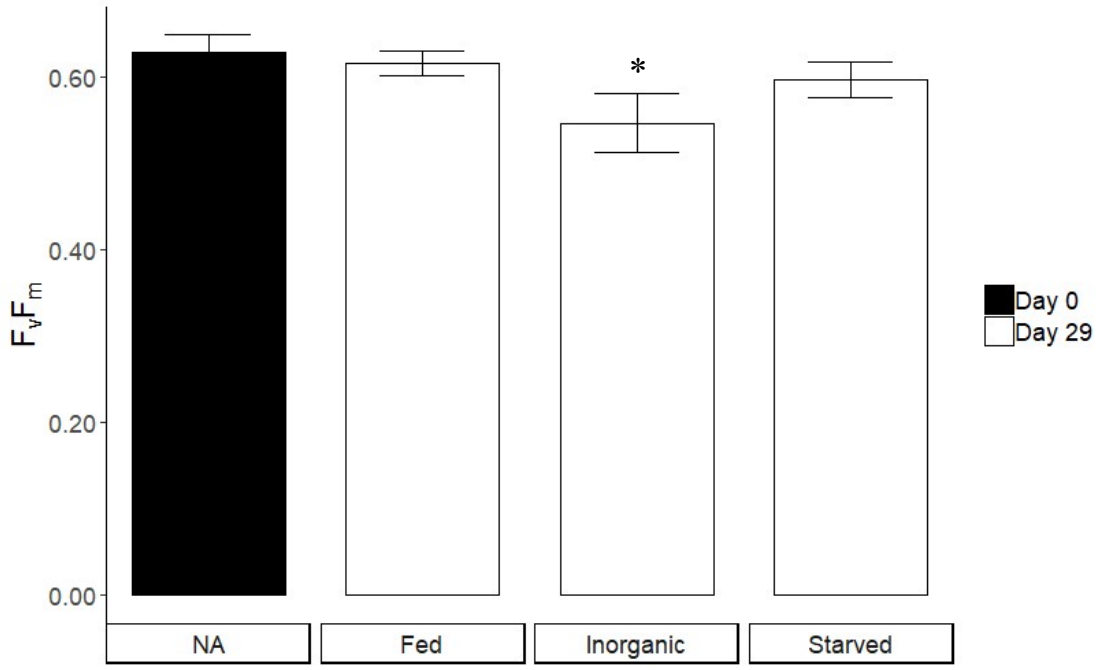


Figure 7. Maximum quantum yield of PSII for *E. pallida* after 29 days under three different nutrient conditions. Error bars are ±1 SD (n = 4). Asterisk designates a significant difference within a nutrient group.

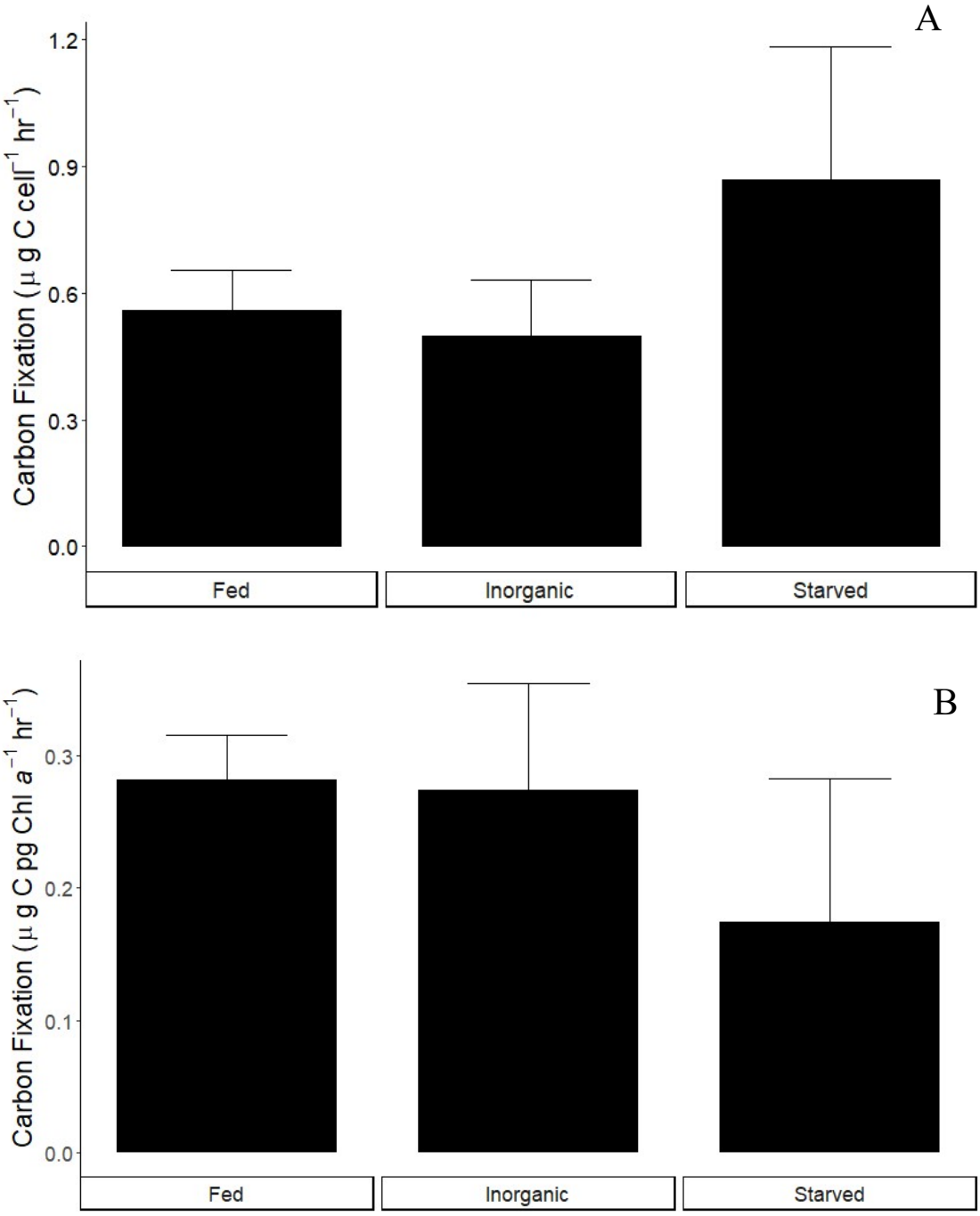


Figure 8. Maximal photosynthetic rate normalized to algal cell (A) or chlorophyll a content (B) in *E. pallida* after 30 days under three different nutrient conditions. Error bars are ±1 SD (n = 3).

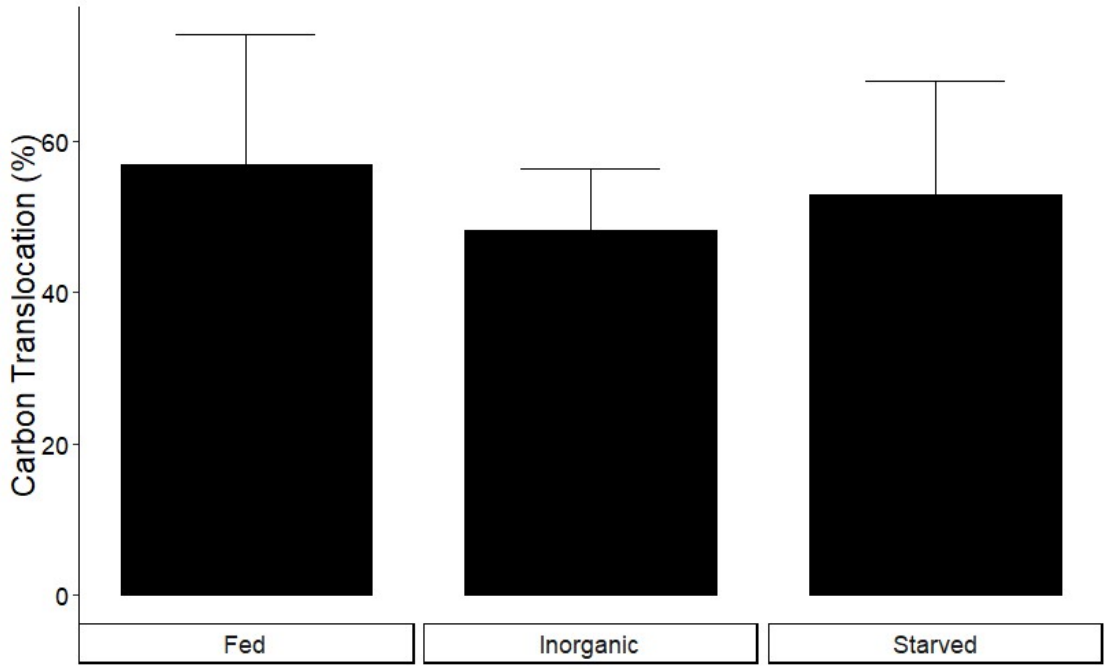


Figure 9. Percentage of photosynthetically fixed carbon translocated to *E. pallida* after 30 days under three different nutrient conditions. Error bars are ±1 SD (n = 3).

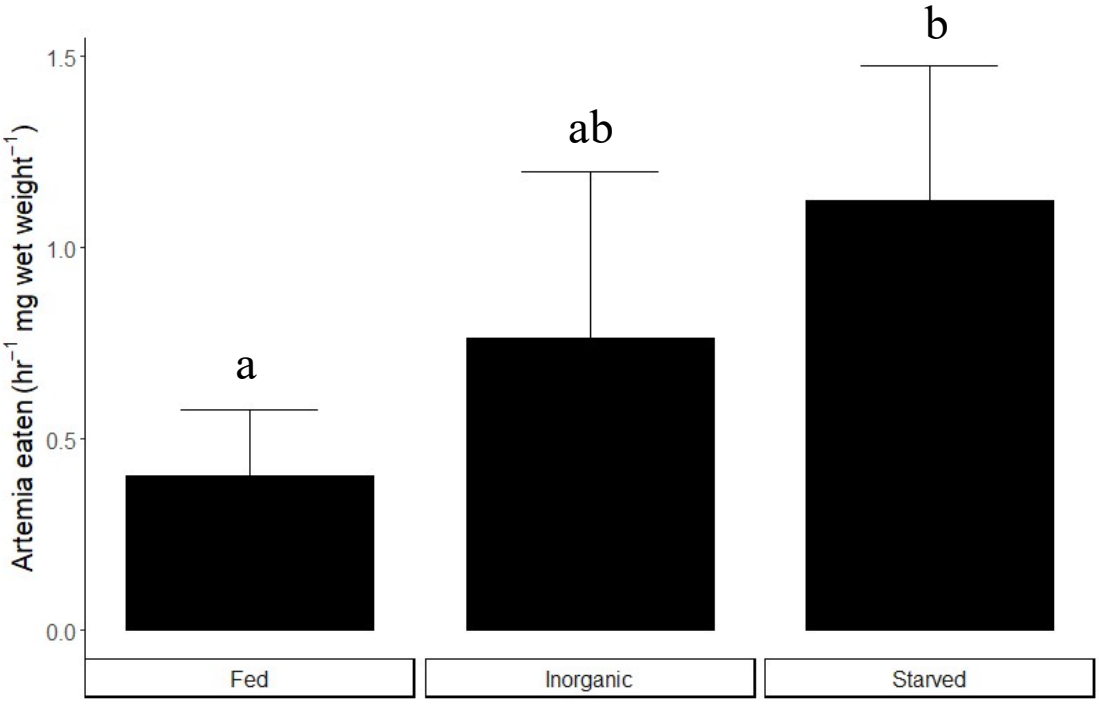


Figure 10. *E. pallida* feeding rate after 28 days under three different nutrient conditions. Error bars are ±1 SD (n = 4). Letters designates a significant difference between nutrient groups.

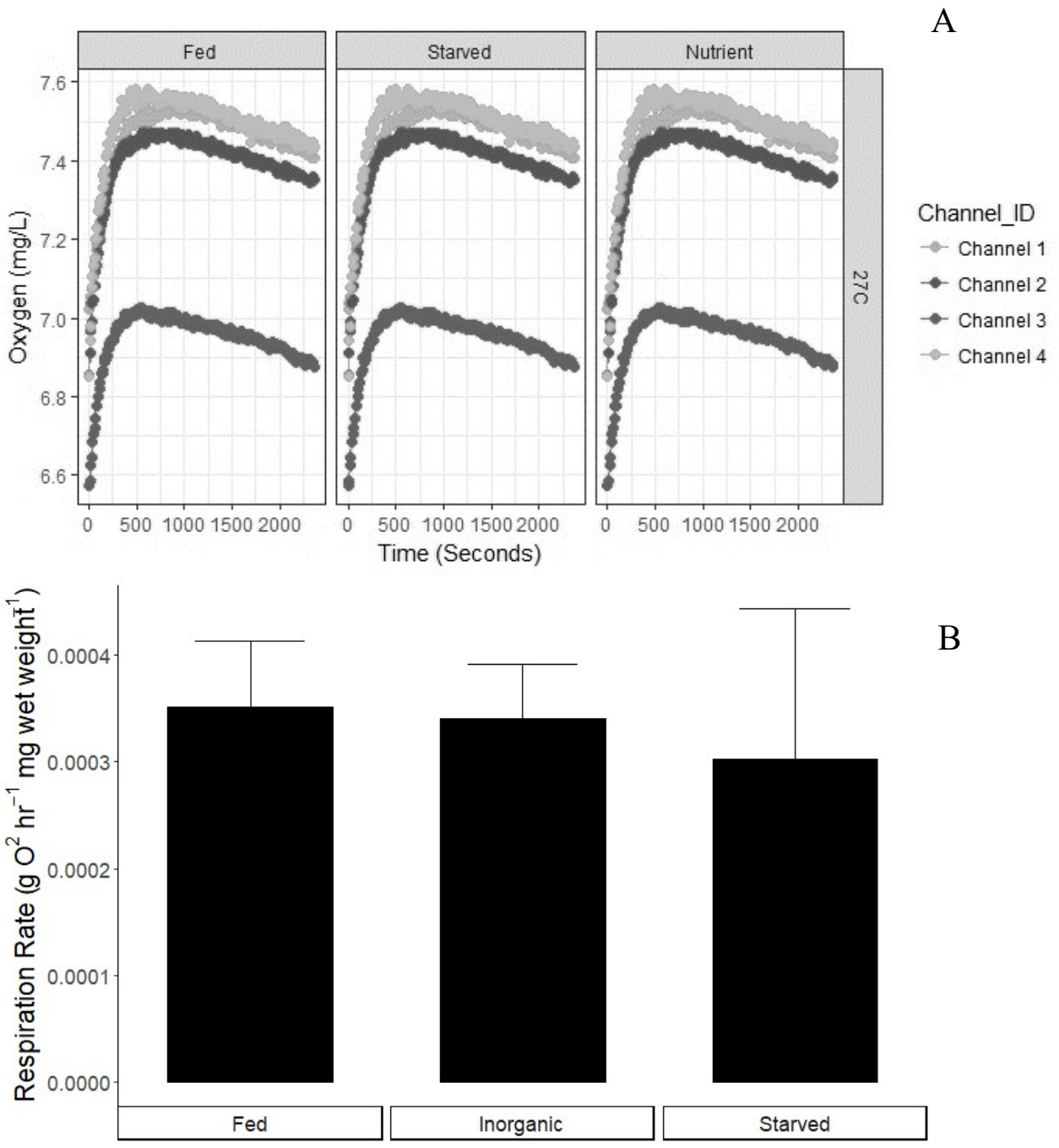


Figure 11. Oxygen consumption (A) or mean respiration rate (B) of *E. pallida* after 29 days under three different nutrient conditions. Error bars are ±1 SD (n = 4).

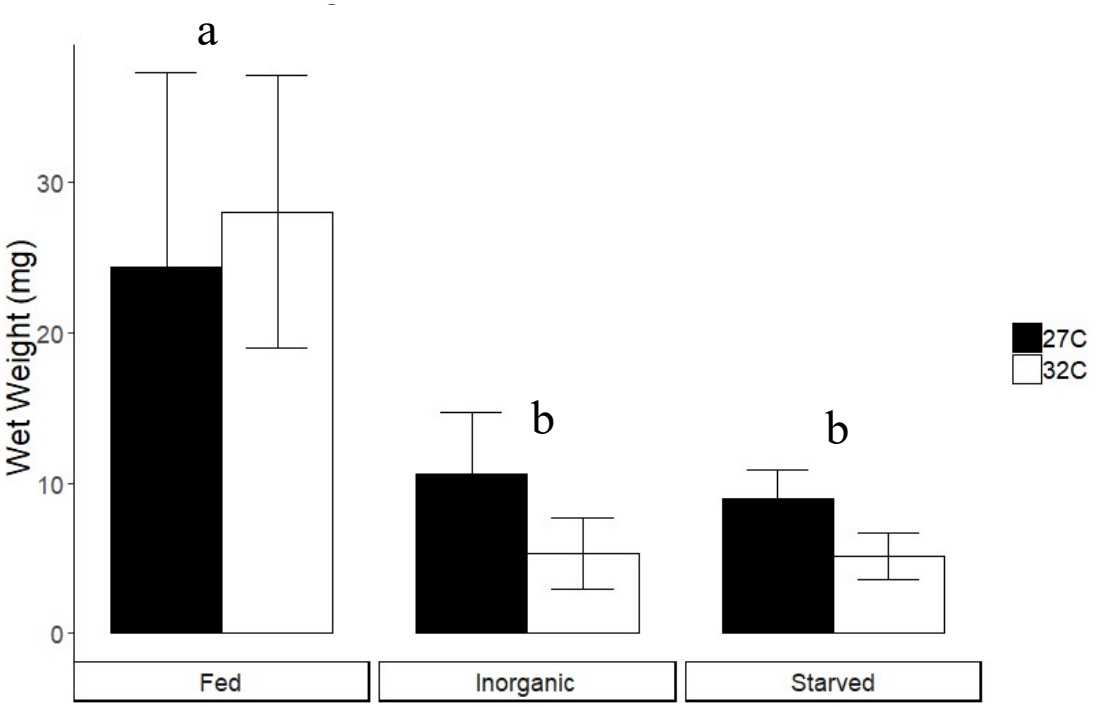


Figure 12. *E. pallida* wet weight after 51 days under three different nutrient conditions, followed by 14 days at 27 or 32 ºC. Error bars are ±1 SD (n = 12). Letters designate a significant difference between nutrient groups.

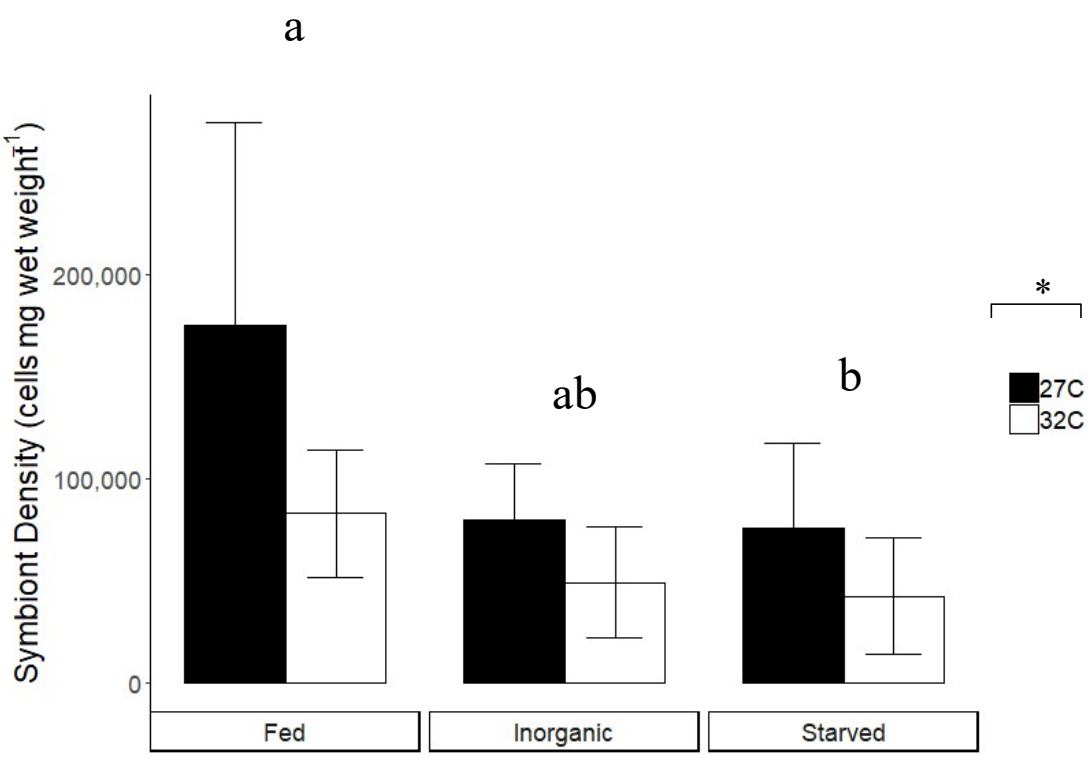


Figure 13. Symbiont density in *E. pallida* after 51 days under three different nutrient conditions, followed by 14 days at 27 or 32 ºC. Error bars are ±1 SD (n = 4). Asterisk designates a significant difference between temperatures and letters designate a significant difference between nutrient treatments.

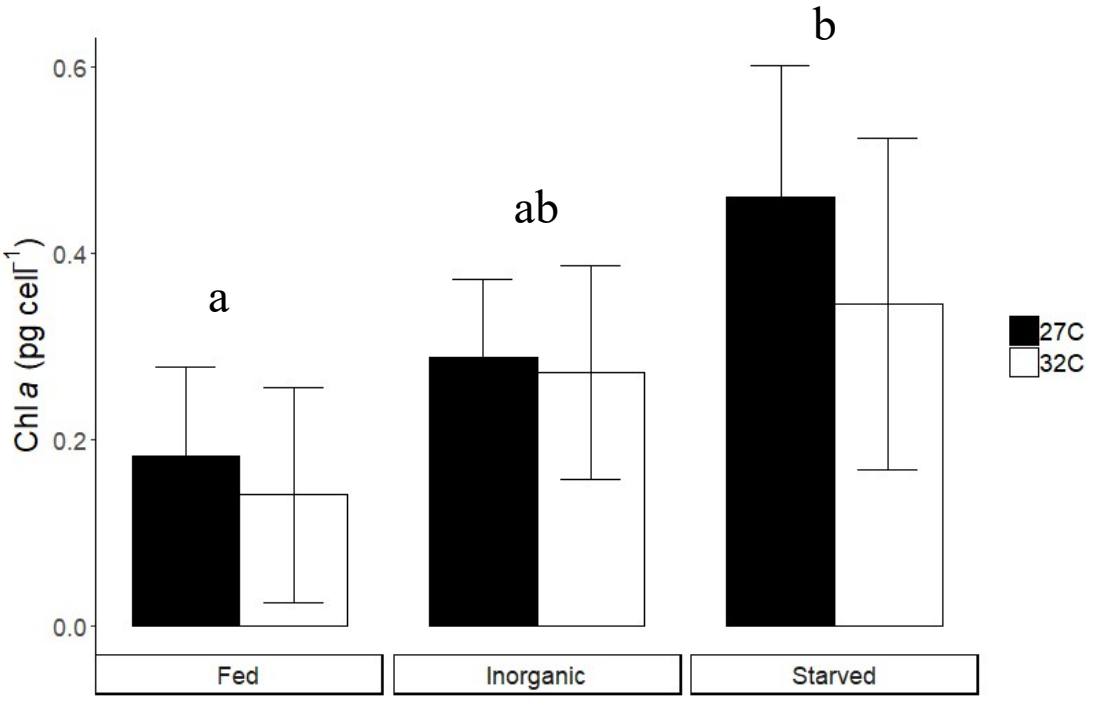


Figure 14. Symbiont chlorophyll *a* content in *E. pallida* after 51 days under three different nutrient conditions, followed by 14 days at 27 or 32 ºC. Error bars are ±1 SD (n = 8). Letters designate a significant difference between nutrient groups.

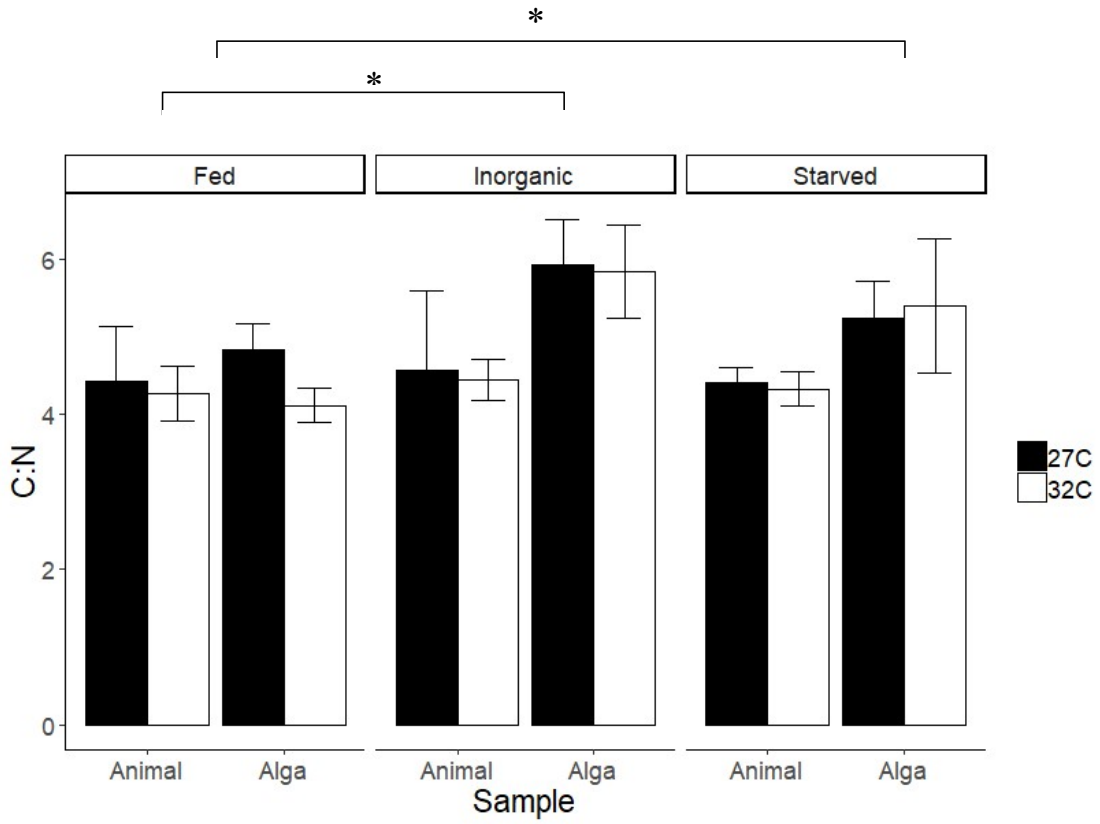


Figure 15. *E. pallida* animal and symbiont carbon to nitrogen ratios after 51 days under three different nutrient conditions, followed by 14 days at 27 (closed symbols) or 32 ºC (open symbols). Error bars are ±1 SD (n = 3). Asterisk designates a significant difference between nutrient groups in the algal fraction.

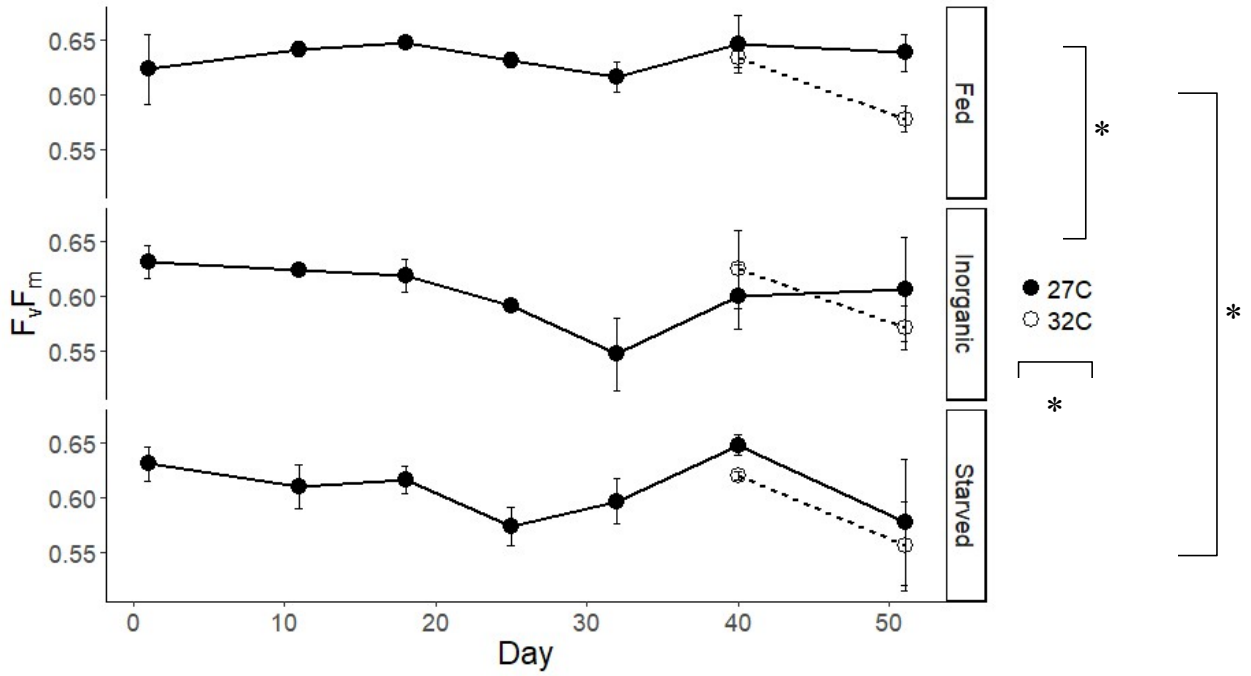
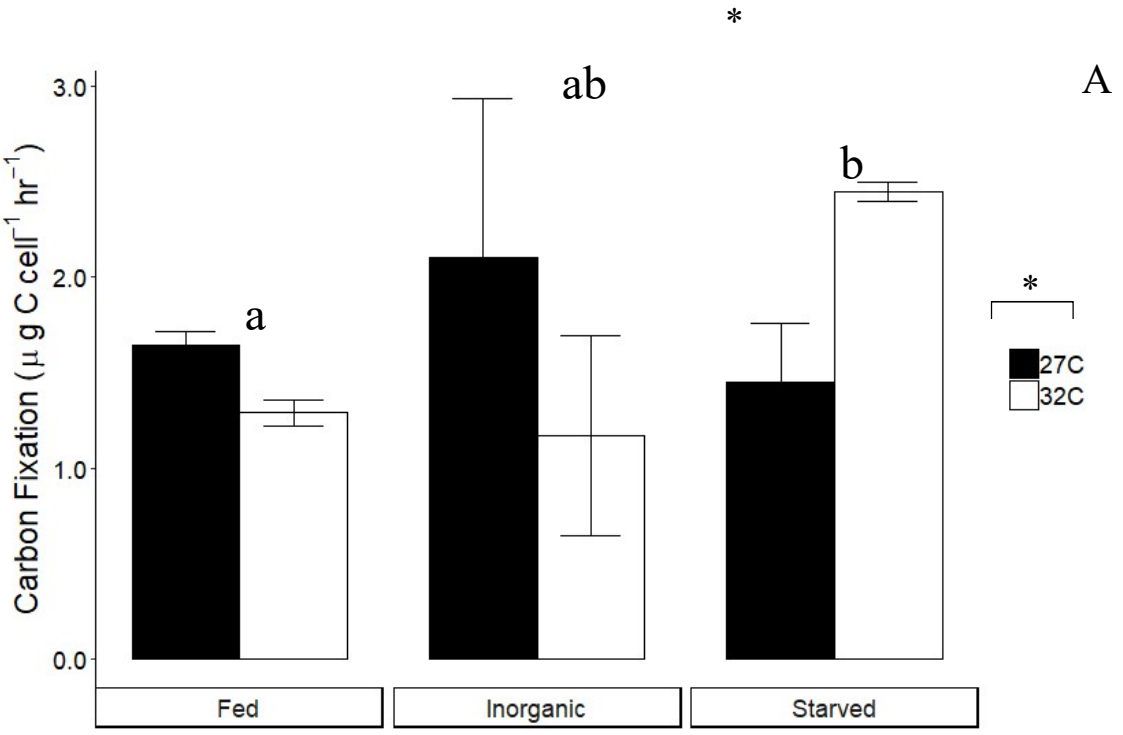


Figure 16. Maximum quantum yield of PSII (F_v/F_m) in *E. pallida* after 51 total days under three different nutrient conditions, followed by 14 days at 27 (closed symbols) or 32 ºC (open symbols). Error bars are ±1 SD (n = 4). Asterisk designates a significant difference between nutrient groups and between temperatures.


*
A
ab
b
a
*
27C
32C
Carbon Fixation (µ g C cell^{-1} hr^{-1})
3.0
2.0
1.0
0.0
Fed
Inorganic
Starved

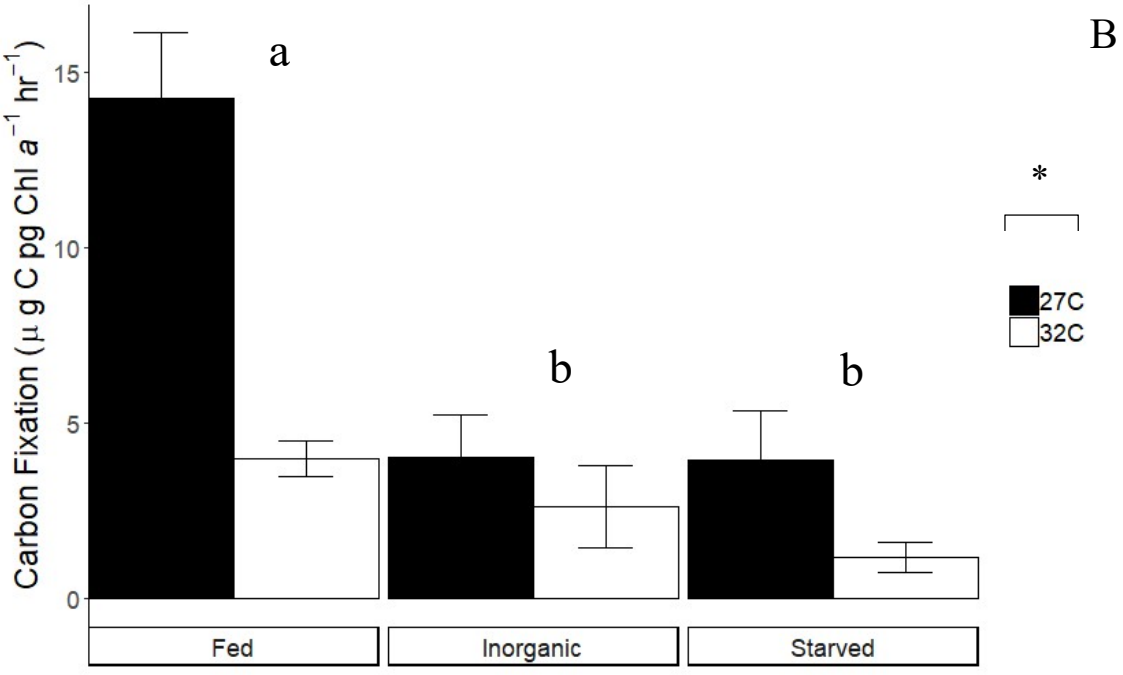

B
a
b
b
*
27C
32C
Carbon Fixation (µ g C pg Chl a^{-1} hr^{-1})
15
10
5
0
Fed
Inorganic
Starved

Figure 17. Maximal photosynthetic rate normalized to algal cell (A) or chlorophyll *a* content (B) in *E. pallida* after 49 days under three different nutrient conditions, followed by 12 days at 27 or 32 ºC. Error bars are ±1 SD (n = 3). Asterisk designates a significant difference between temperatures. Letters designate a significant simple effect between nutrient groups (A) or a significant main effect of nutrient treatment (B).

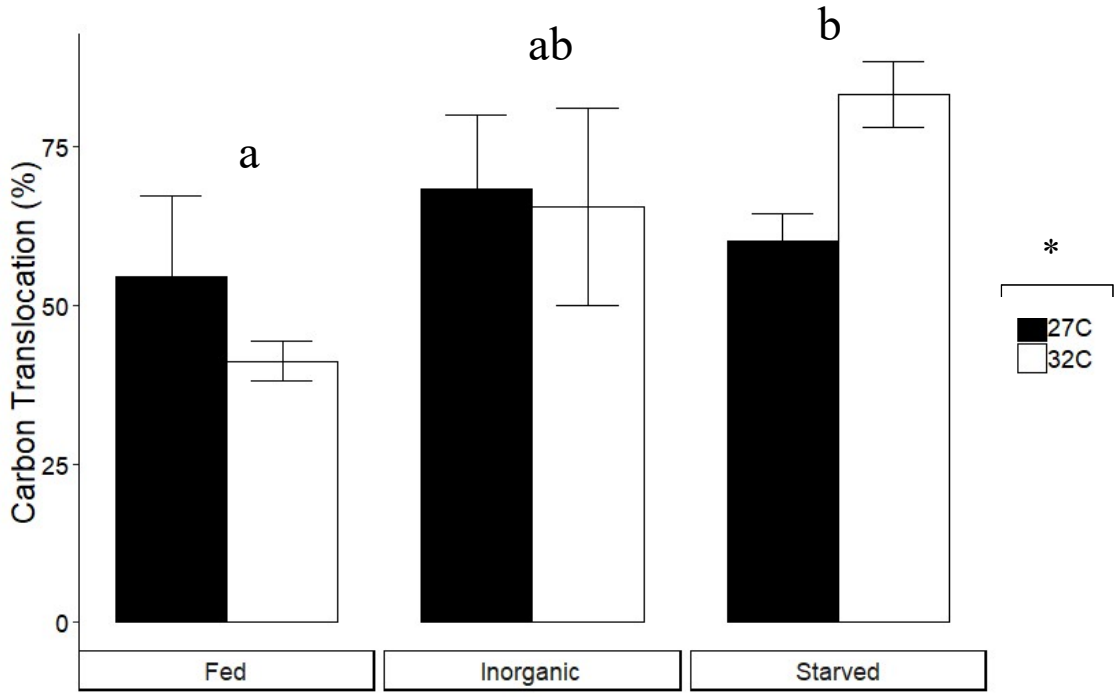


Figure 18. Percentage of photosynthetically fixed carbon translocated to *E. pallida* after 49 days under three different nutrient conditions, followed by 12 days at 27 or 32 ºC. Error bars are ±1 SD (n = 3). Asterisk designates a significant difference between temperatures. Letters designate a significant simple effect between nutrient groups.

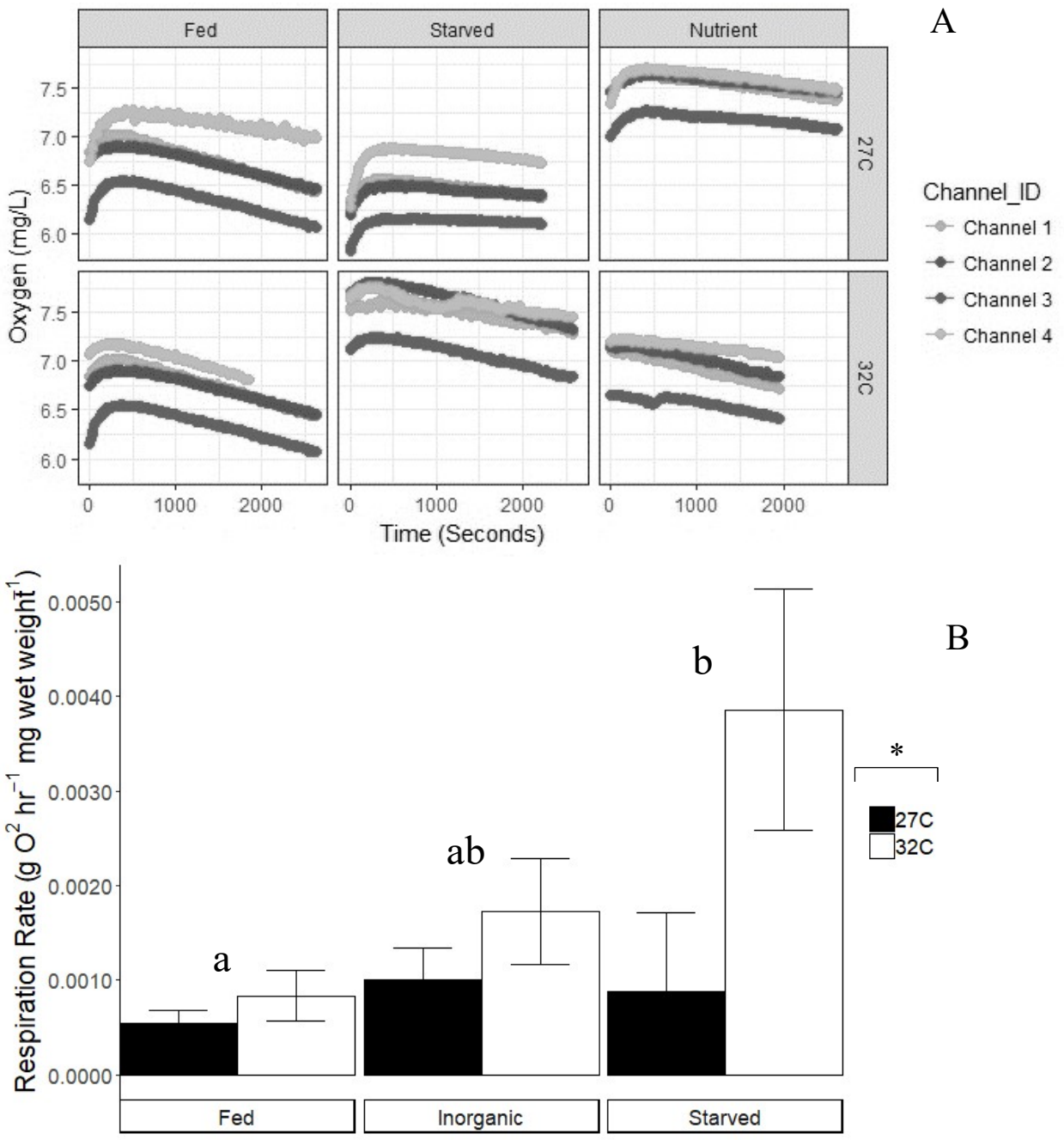
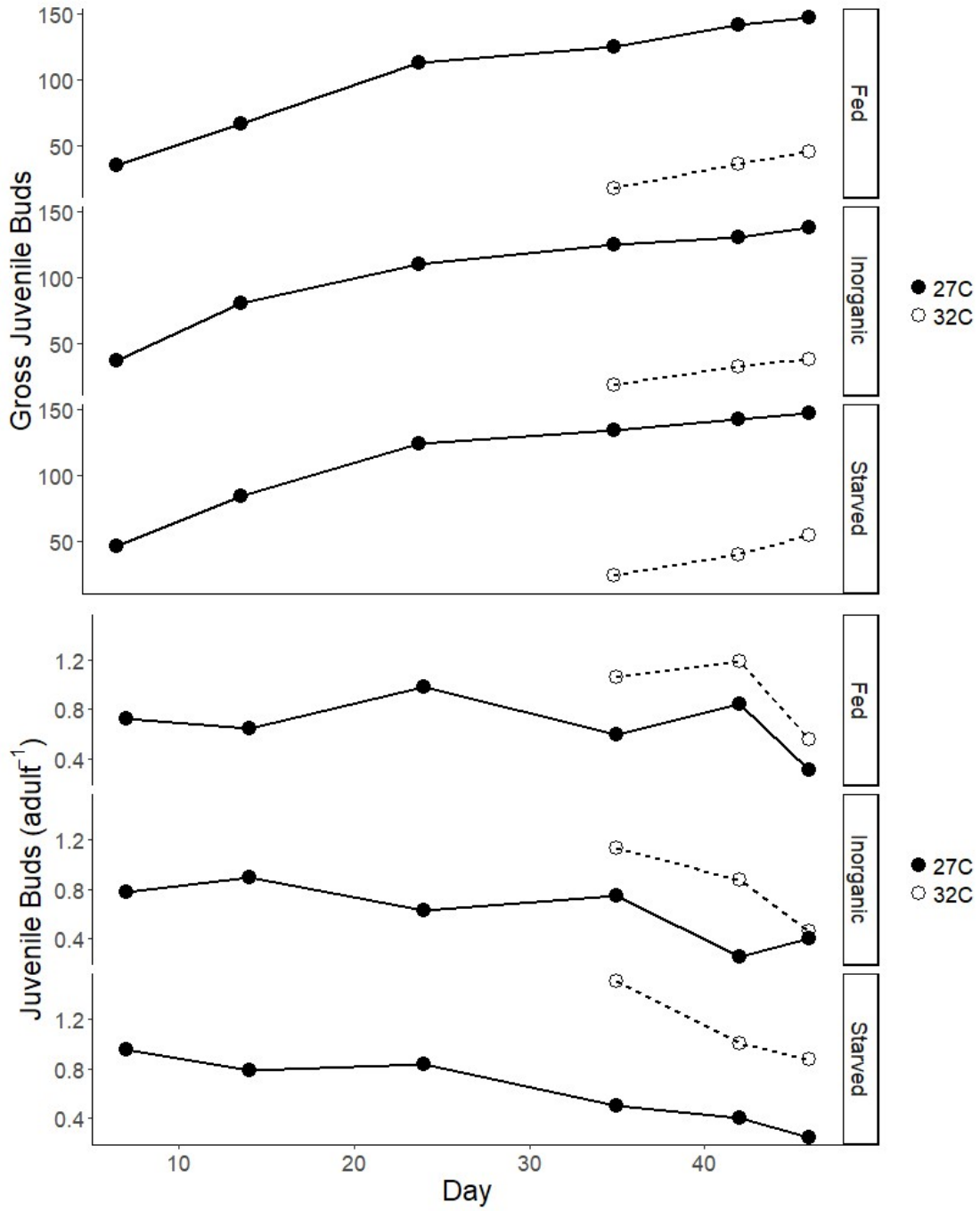


Figure 19. Oxygen consumption (A) or mean respiration rate (B) of *E. pallida* after 48 days under three different nutrient conditions, followed by 10 days at 27 or 32 ºC. Error bars are ±1 SD (n = 4). Asterisk designates a significant difference between temperatures. Letters designate a significant simple effect between nutrient groups.



20. Asexual reproduction of *E. pallida* gross number of juvenile buds (A) or number of juvenile buds (adult^{-1}) (B) after 51 total days under three different nutrient conditions, followed by 14 days at 27 (closed symbols) or 32 ºC (open symbols).

These data were unable to be statistically analyzed, but provide valuable qualitative information.

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