Prior heat stress increases pathogen susceptibility in the model cnidarian *Exaptasia diaphana*

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Abstract

Anthropogenic climate change has significantly altered terrestrial and marine ecosystems globally, often in the form of climate-related events such as thermal anomalies and disease outbreaks. Although the isolated effects of these stressors have been well documented, a growing body of literature suggests that stressors often interact, resulting in complex effects on ecosystems. Recent declines in coral cover from a variety of climate-related stressors make reef ecosystems well-suited to investigate sequential associations between climate impacts, particularly heat stress and disease. Here we used the model cnidarian *Exaiptasia diaphana* to investigate mechanisms linking heat stress to increased disease susceptibility. We examined anemone pathogen susceptibility and physiology (symbiosis, immunity, and energetics) following recovery from heat stress. We observed significantly increased pathogen susceptibility in anemones exposed to heat stress, likely linked to observed suppression of multiple immunological parameters. Furthermore, our data suggest that changes in resource allocation might drive observed reductions in immunity following thermal stress. These findings are an important first step towards understanding temporal associations between two of the most significant climate-associated stressors, heat and disease.

Keywords

cnidarian, heat stress, symbiosis, disease, multiple stressors, immunity

Introduction

Anthropogenic pressure has led to environmental and ecological changes in terrestrial and aquatic ecosystems across the world, often in the form of climate-related events. (Hansen & Stone, 2015; Rosenzweig et al., 2008). Marine ecosystems, in particular, have been significantly affected by multiple climate-induced stressors, such as ocean warming and disease outbreaks (Doney et al., 2011; Hoegh-Guldberg & Bruno, 2010; Scavia et al., 2002). Both of these stressors directly affect organismal physiology through alteration of metabolic function, and indirectly affect a variety of ecological processes, such as oxygen availability, nutrient cycling, and ocean circulation (Doney et al., 2011; Smith et al., 2023). As such, ocean warming, specifically marine heatwaves, are a leading cause of mass mortality in marine organisms (Genin et al., 2020; Rivetti et al., 2014; Smale, 2020; Wild et al., 2019). Similarly, increases in the frequency and severity of disease outbreaks pose significant threats to marine ecosystems. Marine taxa are affected by a large diversity of pathogens ranging from viruses and bacteria to fungi and metazoans (Bateman et al., 2020), many of which have triggered significant mortality events in a variety of marine ecosystems (Hamilton et al., 2021; Patterson, 1996; Petton et al., 2015; Sanderson & Alexander, 2020; Short et al., 1987).

Although the isolated effects of ocean warming, disease outbreaks, and other climate-associated stressors have been well documented within marine ecosystems (Doney et al., 2011; Hoegh-Guldberg & Bruno, 2010), a growing body of literature suggests that these stressors often coincide. Depending on the circumstances and stressors in question, interactions may range from cumulative to synergistic, or even antagonistic in nature (Adams, 2005; Crain et al., 2008). Associations between heat stress and disease outbreaks are particularly prevalent, and have often contributed to extensive marine mortality (Harvell et al., 2002). Consequently, studies have attempted to improve understanding of associations between heat stress and disease in marine ecosystems, but most have focused only on simultaneous synergy between the two stressors (Ban et al., 2014; Harvell et al.; Olsen & Duarte, 2015). However, frequent increases in disease outbreaks in the weeks to months following marine heatwaves suggest that these two stressors may more commonly accumulate in a sequential, rather than simultaneous, manner, (Case et al., 2011; Godwin et al., 2020; Marcogliese, 2008; Vezzulli et al., 2013). Despite these observations, the mechanisms driving sequential temporal patterns between heat stress and disease are poorly understood and necessitate further investigation.

Coral reef ecosystems present a unique opportunity to investigate sequential associations between heat stress and disease. These ecosystems are commonly faced with a variety of climate stressors (Smith & Buddemeier, 1992), which have resulted in unprecedented declines in coral cover over the past several decades (Bruno & Selig, 2007; Gardner et al., 2003). Increasing sea surface temperature (SST), in particular, has exacerbated the frequency and severity of coral decline. Two major drivers of these declines are heat-induced bleaching—loss of endosymbiotic algae (Brown, 1997)—and disease (Harvell et al., 2007). Although isolated heat-induced bleaching (Johnston et al., 2019; McClanahan et al., 2007; Winter et al., 1998) and disease are common (Richardson et al., 1998; Subhan et al., 2020), these events have also been frequently observed in close spatial and temporal associations (Brandt & McManus, 2009; Burke et al., 2023; Harvell et al., 2001; Howells et al., 2020). In many cases, coral disease follows bleaching (Croquer & Weil, 2009), with extensive disease-related mortality often reported in the weeks to months after heat stress (Jones et al., 2021; Miller et al., 2009; Miller et al., 2006). Despite these observations, nearly all experimental studies have focused on the synergistic effects of simultaneous heat stress and disease, specifically documenting increases in disease susceptibility when these stressors occur at the same time (Palmer, 2018; Zaragoza et al., 2014). Consequently, many questions remain regarding the mechanisms linking sequential associations between heat stress and disease outbreaks. The increasing prevalence of heat-induced bleaching events and disease outbreaks highlight the need for an improved understanding of how biotic and abiotic stressors interact through time within the context of changing coral reef environments.

While tropical scleractinian corals are exceptionally important from an ecological standpoint, their precarious species statuses and inherent experimental limitations complicate the laboratory investigation of complex biological phenomenon in these species. Consequently, there has been a rise in the development of closely related model species with traits better suited to experimental manipulation. Of these cnidarian models, *Exaiptasia diaphana*, commonly known as Aiptasia, is a species of anemone from the same class as scleractinian corals (Hexacorallia). Aiptasia is widely regarded for its ecological abundance and generation of genetically identical clonal lines, allowing for control of genetic variation in experimentation (Baumgarten et al., 2015). Furthermore, like scleractinian corals, *E. diaphana* hosts endosymbiotic algae of the family Symbiodiniaceae (LaJeunesse et al., 2018). We leveraged the *E. diaphana* model system to investigate the mechanisms linking heat stress and disease susceptibility in cnidarians.

Specifically, we examined host immune and energetic responses to pathogen exposure following heat-stress in two genetically distinct clonal lines of *E. diaphana*. Here we document a significant association between heat stress and increased pathogen susceptibility and highlight the potential physiological mechanisms of this association. The results described here are an important first step towards understanding temporal links between two of the most significant climate-associated stressors faced by cnidarians.

Methods

Experimental Design

Anemones were obtained from laboratory-maintained clonal lines of *E. diaphana* (VWB9 and H2; V. Weis). Anemones from each clonal line were randomly assigned to one of four possible treatment combinations: control or heat stress and placebo or live bacteria. Individuals were transferred accordingly into sterile 6-well culture plates filled with 10 mL of filter-sterile artificial sea water (FASW). Control plates were maintained in a temperature-controlled Percival incubator at 27°C for the duration of the experiment. Plates assigned to heat treatment were randomly divided between two separate temperature-controlled incubators designated for heat stress. Prior to the start of the experiment, all individuals were acclimated to well plates for three days at 27°C in their respective incubators. Throughout the entire experiment, anemones in all three incubators were maintained under the same light conditions (12hr:12hr light: dark cycles with approximately 40-60 $\mu mol \cdot m^{-2}$ PAR) and received bi-weekly feeding and water changes until the point of infection, Plate locations were randomized within and between incubators at the time of water changes, as appropriate.

Experimental Heat Stress & Immune Challenge

Heat stress consisted of a seven-day ramping period where temperatures were raised 1°C per day until they reached 34°C. Temperatures were then maintained at 34°C for five days based on data from pilot studies **(Supplemental Table 1).** During the experiment, bleaching was confirmed by visual assessment of anemone color and was found to be consistent with the expected timeline. Following heat stress, anemones were immediately returned to 27°C and allowed to recover for two weeks. At the conclusion of this recovery period, surviving anemones from both control and heat treatments were inoculated with either a pathogen or a placebo

(FASW) treatment according to *a priori* assignments. Pathogen exposure was conducted using the known coral pathogen *V. coralliilyticus* BAA 450 (Ben-Haim et al., 2003) at a predetermined LD50 dosage of 1 x 10^8 CFU·mL^{-1} (Zaragoza et al., 2014). Final bacterial concentrations were confirmed with serial dilutions and plating. Following exposure, a subset of anemones was monitored for survivorship (**Supplemental Table 2)**: mortality data was collected every two hours for the first 36 hours, and then daily for the remainder of the experiment (96 hours post-infection) at which point 89% of exposed anemones had died. Individuals were considered dead when they exhibited more than 75% tissue degradation or had 75% tissue degradation for more than 24 hours.

Sample Collection and Processing

Twelve hours after inoculation, a second subset of randomly selected anemones was sampled for the assessment of symbiont density, immune activity, and energetic reserves (**Supplemental Table 2**). Anemones were anesthetized in 10 mL of $MgCl_2$ (0.37 M) prior to sampling. First, we excised a single tentacle for estimates of symbiont density. Subsequent tentacle processing was modified from existing protocols (Tortorelli et al., 2020). Following tentacle excision, the remaining anemone was halved using a sterile razor blade; one half was flash frozen for the immune and energetic assays described herein. Frozen tissue samples were homogenized with 3 mm glass beads in 1.5 mL of 100 mM Tris + 0.05 M DTT buffer (pH 7.8) for 1 minute at 5 m·s^{-1} (Tim Bateman, *pers. comm.*) using a bead mill (Fisherbrand). For each sample, a 200 µL aliquot of homogenate was transferred into a separate microcentrifuge tube and stored at -20°C for melanin assays. The remaining homogenate for each sample was then transferred into new 2 mL microcentrifuge tubes and centrifuged at 2250xg at 4°C for five minutes. Resultant sample supernatants were aliquoted accordingly for each set of assays (protein, lipid, carbohydrates, ash-free dry weight) and stored at -80°C (protein fraction) or -20°C (all other fractions) prior to downstream analyses.

Symbiont Density Estimation

Mounted tentacles were imaged using brightfield and fluorescence microscopy on the Cytation1 Cell Imaging Multimode Reader (Biotek). Approximate tentacle area was determined via brightfield microscopy. Symbiont autofluorescence was measured using a chlorophyll a fluorescent filter (Agilent; EX 445/45 nm, EM 685/40 nm). Brightfield and fluorescent images of

each tentacle were combined to standardize fluorescence by tentacle area. The average pixel intensity of the chlorophyll a fluorescence within each standardized tentacle area was used as a proxy for symbiont density.

Immune Assays

Cnidarian immunity was measured using a suite of standardized immune assays originally developed for scleractinian corals (Mydlarz & Palmer, 2011; Pinzón C et al., 2014) and adapted for use in *E. diaphana* (**Supplemental File 1**). We measured responses related to three major innate immune pathways: phenoloxidase, antibacterial, and antioxidant pathways. The phenoloxidase pathway is an enzymatic cascade resulting in the synthesis of melanin – an effector molecule involved in wound healing and pathogen encapsulation (Mydlarz et al., 2016). Here we quantify the phenoloxidase cascade using both total phenoloxidase activity (enzymatic activity of prophenoloxidase + phenoloxidase) and melanin concentration. An additional essential component of coral immunity is the production of antimicrobial peptides and metabolites that can neutralize a wide variety of pathogenic bacteria (Mydlarz et al., 2016; Palmer & Traylor-Knowles, 2018). Antibacterial activity here was quantified by measuring bacterial growth rates of *Vibrio coralliilyticus* in the presence of protein extracts generated from host tissue. Finally, we also measured activity of the important antioxidant catalase, using established methods (Pinzón et al., 2014). Cnidarians produce antioxidant enzymes as a mechanism to protect against reactive oxygen species generated by both hosts and pathogens during infection and associated immune responses (Parisi et al., 2020)

All immune assays were run in triplicate on separate 96-well plates and measured using the Cytation1 Cell Imaging Multimode Reader and Gen5 Software (Biotek). 100mM Tris + 0.05 mM DTT buffer was used as a negative control for all assays. All enzymatic immune assays were standardized by protein concentration using Red660 colorimetric assays, while melanin concentration was standardized by desiccated tissue weight.

Energetic Assays

Total lipids and total carbohydrates assays were used to determine approximate host energetic budget. Lipids and carbohydrates are commonly used proxies for energetic budgets in multiple cnidarian species (Lesser, 2013). Lipid and carbohydrate concentrations were measured and standardized by ash-free dry weight following modifications to previously established

protocols (Cheng et al., 2011; Fitt et al., 2000; Masuko et al., 2005). Total lipid and total carbohydrate assays were run in triplicate and duplicate, respectively, and measured in separate 96-well plates using the Cytation1 Cell Imaging Multimode Reader and Gen5 Software (Biotek). Tris + DTT buffer was used as a negative control for both assays.

Statistical Analyses

All statistical analyses were performed using R Version 4.3.0 (2023-04-21; R Core Team 2023). Prior to hypothesis testing and modeling sample outliers were identified using the Rosner test from the EnvStats package (v2.7.0; Millard, SP., 2013) and removed. Shapiro-Wilke and Bartlett tests were used to confirm the assumptions of normal distribution and equal variances, respectively. Several assays failed to meet assumptions necessary for parametric testing, even following transformation, necessitating the use of non-parametric analyses.

<u>Mortality</u>

Survivorship data from our first subset of anemones were used to assess the effects of pathogen exposure, heat stress and genotype (clonal line) on mortality. First, the effects of pathogen exposure on mortality were assessed using a Cox proportional hazard's model (Survival ~ immune challenge; n = 188; survival package v3.5.5; Therneau, T, 2023). We then used a secondary Cox model to investigate the effects of heat stress and genotype on mortality within the subset of pathogen-exposed anemones only (Survival ~ treatment + clonal line; n = 90).

<u>Univariate hypothesis testing</u>

A second subset of anemones reserved for assessment of symbiont density, energetic reserves, and immune activity, was used to investigate hypotheses related to mechanisms of increased mortality. To start, we tested for differences in symbiont density (mean chlorophyll fluorescence intensity) or energetic reserves (total lipids and total carbohydrates) between treatment groups (control vs. heat) and clonal lines. The impacts of heat stress or clonal line on symbiont density, lipids, and carbohydrates were assessed independently with separate Welch's two-sample t-tests (symbiont density and lipids) or non-parametric Wilcoxon Rank Sum tests (carbohydrates).

Redundancy analysis

To investigate the impact of our independent variables (heat stress, pathogen exposure, and clonal line) and covariates (symbiont density, total lipids, and total carbohydrates) on host immune activity, we ran a redundancy analysis (RDA) using the vegan package (v2.6.4; Oksanen, J. et al., 2022). Before starting with this multivariate approach, we tested for and confirmed no collinearity among our variables. All continuous variables were centered and scaled before separation into predictor (i.e., symbiont density, lipids, carbohydrates) and response (i.e., catalase, total phenoloxidase, and antibacterial activity, and melanin concentration) data frames. An RDA was used to identify which variables best predicted variation in immunity across our samples. We started by running an RDA with all possible predictor variables (i.e. clonal line, temperature treatment, pathogen exposure, lipids, and carbohydrates). From there, we used a forward selection to identify which predictor variables explained significant variation in our dataset. After identifying significant predictor variables, we ran a final RDA using the best-fit model determined by forward selection (~ Treatment + Clonal Line + Immune Challenge + Lipids).

Linear modeling

Finally, we used linear modeling to assess variation in individual immune parameters with respect to the statistically relevant factors determined via RDA. For normally distributed immune parameters (catalase and antibacterial activity) we used general linear models (GLMs). Global models with all possible additive and interactive variables were analyzed for catalase and antibacterial activity independently. All models were dredged and ranked via AICc using the MuMIn package (v1.47.5; Barton, K., 2023), where applicable. Models with delta AICc values less than or equal to 2 were averaged to determine the best-fit models for each immune parameter. For non-normally distributed parameters, we used a similar approach with modifications to account for unequal variances and zero-inflated data. To account for unequal variances within total phenoloxidase data we used the nlme package (v3.1.162; Pinheiro, J. et al., 2023) to run generalized least squares (GLS) modeling with the same model dredging and averaging approach as described above. Finally, for melanin, we adopted a two-tiered strategy that was motivated by the high number of zero measurements (see results). First, we used a probit model (i.e. a generalized linear model with binomial

error structure) to predict the presence of melanin among samples using heat treatment, clonal line, immune challenge and lipid concentration as predictors. We then removed samples in which melanin was not detected and used multiple regression to predict melanin concentrations employing the same predictors.

Results

Mortality

Anemone mortality was significantly greater in anemones exposed to pathogens (Cox proportional hazards model; hazard ratio = 103; 95% CI [25 – 421]; $p < 0.001$; **Supplemental Fig. 1**). Furthermore, within pathogen exposed anemones, mortality rate was significantly greater in the heat stressed anemones relative to controls (hazard ratio = 2.15; 95% CI [1.34 – 3.46]; $p = 0.002$) and among H2 anemones relative to VWB9 (hazard ratio = 0.26; 95% CI [0.16-0.42]; $p < 0.001$; **Fig. 1**).

Symbiont Density & Energetic Reserves

Symbiont density, estimated by mean chlorophyll a fluorescence pixel intensity, was marginally, but not significantly, higher in heat stressed anemones compared to controls (Welch's two sample t-test; t = -1.93, df = 68.7, $p = 0.0579$. 95% CI [-3740 – 63.3]; **Fig. 2a**). However, symbiont density did significantly vary across clonal lines (Welch's two sample t test; t = 2.04, df = 49.4, $p = 0.0472$; 95% CI [26.5 – 4100]); H2 had significantly greater symbiont density than VWB9 anemones **(Fig. 2b).** For energetic reserves, total carbohydrates varied between treatment groups. Carbohydrates were significantly lower in heat treated anemones compared to control anemones (Wilcoxon Rank Sum test; W = 1460, $p < 0.01$; **Fig. 2c**) and in the VWB9 clonal line compared to the H2 clonal line (Wilcoxon Rank Sum test; W = 733, p = 0.0116; **Fig. 2d**). Total lipids did not significantly differ between either temperature treatment (Welch's two sample t-test; t = -0.945, df = 77.3, $p = 0.348$, 95% CI [0.0774 – 0.0846], **Fig 2e**) or clonal lines (Welch's two sample t-test; t = -0.436, df = 84.0, $p = 0.664$, 95% CI [-0.0184 – 0.0117], **Fig 2f**).

Immune Responses

The RDA explained significant variation along the first three axes of the ordination. These axes explained 14.32% (RDA1), 7.84% (RDA2), and 7.57% (RDA3) for a cumulative proportion

explained of 29.74% **(Fig. 3)**. Treatment (ANOVA, $p = 0.001$), clonal line (ANOVA, $p = 0.001$), pathogen exposure (ANOVA, $p = 0.003$), and lipid concentration (ANOVA, $p = 0.032$) all explained significant variation in immune responses along these axes. Temperature treatment and lipid concentration explained variation mostly along the first RDA axis, while clonal line and pathogen exposure explained variation along both the first and second RDA axes. When considering immune responses, the variables that drove the variation along the first axis (RDA1, 14.32%) were catalase activity and melanin concentration while PPO activity drove the variation on the second axis (RDA2, 7.84%).

Linear modeling of the effects of each of our significant predictors on individual immune metrics revealed metric specific effects (**Table 1**). Both catalase activity and melanin concentration were significantly lower in heat treated anemones relative to control temperatures (Catalase GLM, $p < 0.001$; Melanin GLM, $p < 0.001$; **Fig. 4).** Catalase activity was also significantly different between clonal lines (GLM, $p < 0.001$); VWB9 anemones had significantly greater catalase activity than H2 (**Supplemental Fig. 2**). Both antibacterial activity and melanin concentration were significantly correlated with lipid concentration (**Fig 5**). Notably, antibacterial activity was significantly different as a result of the interaction between heat treatment and lipid concentration (GLM, $p < 0.01$; **Fig. 5a**). Heat stressed anemones had a significant negative association between lipids and antibacterial activity (LM; $R^2_{adj} = 0.103$, $F(1, 25) = 4.66$, $p = 0.0396$). However, control anemones maintained a positive, but insignificant, association between lipids and antibacterial activity (LM; $R^2_{adj} = 0.0557$, $F(1, 24) = 3.196$, $p = 0.0865$). While heat treatment predicted the presence or absence of melanin (GLM, $p < 0.001$), melanin concentration, when present, was significantly positively associated with lipid concentration independent of other predictors (0s removed GLM, $p < 0.001$; **Fig. 5b**). Total phenoloxidase was the only metric to vary as a result of pathogen exposure; activity was reduced in anemones following pathogen exposure (GLS, $p < 0.01$; **Supplemental Fig. 3**).

Discussion

As anthropogenic climate change worsens, marine ecosystems are faced with a multitude of stressors that may form complex associations with each other (Doney et al., 2011; Hoegh-Guldberg & Bruno, 2010). However, empirical study of interactive stressors, particularly those occurring asynchronously, has been limited, leaving many questions regarding the nature and

mechanisms of associations amongst stressors (Pirotta et al., 2022). Cnidarians, which face a diversity of climate related stressors(Ban et al., 2014; Maor-Landaw & Levy, 2016) , provide a relevant model to investigate the effects of multiple stressors on marine species. Here we conducted one of the first experimental studies investigating the consequences of heat stress on cnidarian pathogen susceptibility. Our results support a long held theory (Muller et al., 2018) that previous heat stress increases disease susceptibility and provide important new insight regarding the mechanisms of these patterns. Specifically, we highlight changes in host cnidarian immunity following heat stress which are likely driven by shifting resource allocation which may contribute to observed patterns of differential mortality.

Mortality differs across temperature treatments and clonal lines

Susceptibility to pathogen exposure was significantly higher among those anemones exposed to heat stress. This result is in agreement with documented increases in disease and disease-related mortality following coral bleaching in the wild (Harvell et al., 2001; Matsuda et al., 2020; Miller et al., 2009; Precht et al., 2016; Sakai et al., 2019; Wright et al., 2017). Notably, while previous laboratory experiments have documented impacts of simultaneous heat stress and pathogen exposure (Muller et al., 2018; Zaragoza et al., 2014) ours is the first to document extended consequences of heat stress on pathogen susceptibility, more accurately representing observed environmental patterns in nature. Future studies combining our approach with emergent experimental disease techniques (Huntley et al., 2022; Traylor-Knowles et al., 2021) will allow for more broadscale applications to complex cnidarian and marine disease systems.

We also observed significant genotypic differences in mortality. Anemones from the VWB9 clonal line were less susceptible to pathogen-induced mortality than H2. Both clonal lines originate from similar geographic regions and host the same species of algal endosymbiont (*Brevolium minutum*) (Thornhill et al., 2013), suggesting that fine scale genetic variation likely contributes to these differences. While intraspecific variation in cnidarian disease resistance has been linked to genetic variation in the wild (Miller et al., 2019; Vollmer & Kline, 2008), there are a lack of studies investigating intraspecific variation in laboratory settings (Wright et al., 2017) . Similarly, few experimental studies investigating disease dynamics in Aiptasia incorporate genetic variation (Krediet et al., 2014; Zaragoza et al., 2014)**.** Our findings suggest that inclusion of genetic variation in cnidarian studies, including those involving *E. diaphana*, may provide increased insight regarding the roles of genetic variability in contributing to

resistance or susceptibility to stress.

Mechanisms of differential mortality: roles of symbiont density and energetic reserves

Beyond documenting differences in mortality as a result of environmental and genetic factors, we employed an integrative approach to investigate the underlying mechanisms driving these differences. First, we tested for differences in symbiont density and energetic reserves between heat stressed and control anemones. Both symbiont densities and energetic reserves may be affected during heat stress events (Rodrigues & Grottoli, 2007). Furthermore, changes in symbiont densities or energetic reserves may impact host immunity (Changsut et al., 2022; Valadez-Ingersoll et al., 2023), and consequently pathogen susceptibility (Palmer et al., 2010). We failed to detect any differences in symbiont density after two weeks of recovery, likely due to *E. diaphana*'s capacity for exceptionally rapid recovery from dysbiosis (Tortorelli et al., 2020). Thus, the two weeks prior to pathogen exposure in which anemones were allowed to recover from heat stress was likely ample time for symbiont densities to partially recover in our heat stress group. In contrast, we did observe differences in carbohydrate concentration as a result of heat stress: anemones from heat stress treatments had significantly lower carbohydrate concentration than anemones from control treatments. Thus, while symbiont density recovers quickly, carbohydrate concentration appears to recover more slowly, potentially contributing to increased pathogen susceptibility. More fine-scale approaches leveraging metabolomic or carbon-flux modeling may be useful in determining the complex effects of heat stress on host energetic budget, and subsequent effects on pathogen susceptibility.

Mechanisms of differential mortality: roles of host immunity

Next, we focused on the potential roles of shifts in immunological activity which might contribute to observed patterns of mortality. When considering immunity holistically using multivariate statistics, we saw significant effects of temperature, clonal line, immune challenge, and lipid concentration on immune response. Here we discuss the impacts of temperature and lipid concentration on immunity as these factors had the most prevalent impacts on holistic immunity and individual immune parameters. Examination of the effects of heat stress on individual immune parameters revealed strong suppression of both catalase activity and melanin concentration. While numerous studies have evidenced the short term effects of heat stress (days

– weeks) on cnidarian host immunity (Krueger et al., 2015; Palmer, 2018; Caroline V. Palmer et al., 2011; Wall et al., 2018) the long-term effects of heat stress (months – years) on immune activity are not well documented (Mydlarz et al., 2009; Wall et al., 2021). Generally, heat stress is believed to induce activation of a variety of immune parameters, including antioxidant (e.g., catalase) and phenoloxidase enzymes (Krueger et al., 2015; Palmer, 2018). However, here we document pronounced suppression of immunity (catalase, antibacterial activity, melanin) after two weeks of recovery from heat stress, suggesting that immune activation is dampened during recovery. Our approach provides new insight suggesting suppression manifests quickly after heat stress and highlights the need for frequent, repeated sampling during bleaching and recovery to better understand the prolonged effects of increasing SSTs on cnidarian immunity.

Investigating the role of symbiotic immunosuppression

While immune suppression following thermal stress may be the result a number of mechanisms, one of the most commonly proposed mechanisms involves dynamic changes in symbiosis. Suppression of host immunity by symbionts has been hypothesized as an important mechanism required for the successful establishment and maintenance of symbiotic relationships (Mansfield & Gilmore, 2019; Merselis et al., 2018). Previous laboratory studies have confirmed negative associations between symbiosis and immunity across several cnidarians (Detournay et al., 2012; Fuess et al., 2020a; Fuess, et al., 2020b; Jacobovitz et al., 2021; Mansfield et al., 2017; Merselis et al., 2018). Nonetheless, few studies have examined the dynamics of immune-symbiosis interplay during the breakdown and re-establishment of symbiosis. It is possible that the rapid repopulation of endosymbionts following heat-induced dysbiosis exacerbates symbiotic immunosuppression, potentially explaining, in part, naturally observed patterns of disease after thermal bleaching events. While symbiont density neither significantly differed between temperature treatments, nor significantly predicted holistic immunity, it is possible that indirect or time lagged effects of changing symbiont densities may be influencing observed patterns of differential mortality. Specifically, rapid and excessive symbiont proliferation (evidenced by marginally higher symbiont densities in heat treated anemones) may have had significant prolonged effects on host immunity. Future studies incorporating time-series approaches to sampling may be used to test this hypothesis.

Investigating the role of energetic immunosuppression and the importance of resource

allocation

A second potential hypothesis to explain observed associations between heat stress and immunity is that changes in resource allocation as a result of resource limitations during dysbiosis may result in immune suppression. According to resource allocation theory, organisms must distribute a finite amount of resources amongst diverse biological processes—growth, reproduction, metabolism, immunity, etc. (Ng'oma et al., 2017). This may involve trade-offs where resources are shifted between competing needs in order to maximize fitness (Garland et al., 2022; Zera & Harshman, 2001). Consequently, reduction in the total amount of resources may alter the proportions in which resources are distributed, potentially resulting in the suppression of one or more biological processes. As such, immune suppression is one of the most commonly documented energetic trade-offs associated with resource limitation (Norris & Evans, 2000; Rantala & Roff, 2005; Rauw, 2012; Sadd & Schmid-Hempel, 2009; van der Most et al., 2011). In cnidarians, the loss of symbionts during a heat induced bleaching event may severely limit the number of energetic resources available to the host. These limitations likely exacerbate resource tradeoffs and result in changes to resource allocation, including allocation to host immune responses. Alternatively, immune-energetic trade-offs may also occur in the absence of resource limitation in response to biotic or abiotic pressures: organisms may dynamically shift resources between processes in response to pressing needs caused by environmental stress or other changes in biological condition (McNamara & Buchanan, 2005).

In congruence with this hypothesis, the relationship between antibacterial activity and lipids differed as a result of temperature treatment potentially due to differences in resource allocation. We observed a negative association between lipids and antibacterial activity only following heat stress. Increased investment of lipids towards antibacterial activity under control conditions is likely indicative of immunological "front-loading"; increased constitutive activity of potent preventative immune metrics which confer increased pathogen resistance (Mydlarz et al., 2016). However, under heat stress, lipids may be directed away from preventative mechanisms (i.e. pathogen defense) in favor of more pressing stress response pathways. The most frequently measured response to heat stress is production of antioxidant enzymes which mitigate oxidative damage to host tissues (Krueger et al., 2015; Yakovleva et al., 2005). While we did not detect significant associations between antioxidant enzymes and lipids, it is possible that resources were allocated to other, unmeasured stress-related mechanisms such as fluorescent pigments,

mycosporine-like amino acids, or heat-shock proteins (Baird et al., 2009). In contrast, melanin concentration was significantly positively associated with lipid concentration regardless of temperature treatment, likely due to its multi-faceted roles in cnidarian host fitness (C. V. Palmer et al., 2011; Palmer & Traylor-Knowles, 2018).

Other factors contributing to variation in immune activity

Finally, both genotype and pathogen exposure were also significant predictors of immune activity. Specifically, catalase activity was significantly different between clonal lines: VWB9 anemones had higher catalase activity than H2 anemones. Given that antioxidant activity is an essential component of coral immunity (Parisi et al., 2020), these differences may be related to differential clonal line mortality. Additionally, total phenoloxidase activity was lower in anemones exposed to pathogens. Rapid changes in immune activity, and in particular the phenoloxidase cascade, have been observed following pathogen exposure in cnidarians (Caroline V. Palmer et al., 2011). Notably, the phenoloxidase cascade results in the production of melanin which has been linked to pathogen defense and wound healing (Mydlarz et al., 2010). Thus, observed declines in phenoloxidase could indicate increased investment in melanin synthesis in anemones exposed to pathogenic challenge. While we did not observe significant differences in melanin concentrations between placebo and infected anemones, it is possible that sampling timing (12 hours post infection) coincided with intermediate stages of the signaling cascade.

Conclusion & Future Directions

Multiple stressors are a common consequence of anthropogenic climate change and can have pervasive effects on a variety of marine ecosystems (Adams, 2005; Crain et al., 2008). As such, an improved understanding of organismal responses to climate-induced stressors is imperative to the conservation of these ecosystems. Here we detail one of the first studies to investigate the mechanistic links between prior heat stress and increased disease susceptibility in a marine species. Our study establishes the ability to reproduce heat stress and disease associations under laboratory conditions using a tractable model system, allowing for more nuanced and robust investigations of ecologically relevant phenomena. Using this experimental approach, we were able to demonstrate prolonged effects of prior heat stress on host immunity, which may contribute to observed pathogen-induced mortality. Furthermore, our results implicate changes in resource allocation as a potential mechanism of heat-induced immunosuppression. Further

studies incorporating time series approaches will be necessary to fully disentangle the observed patterns and associated mechanisms and assess their broad applicability to marine systems. Specifically, future work should aim to sample across multiple timepoints from the onset of bleaching through acute and long-term recovery to determine the effects of dynamic changes in symbiont density and energetic reserves on host immunity and pathogen susceptibility. Additional comparative experimentation using scleractinian corals and non-cnidarian species will also help determine the applicability of our results to other systems. In summary, our results provide an important starting point for understanding the mechanistic links between heat stress and disease that will aid in the conservation of marine species, including cnidarians, in the face of a changing climate. Most importantly, they generate testable hypotheses that will be invaluable in better understanding the effects of multiple stressors on diverse species and ecosystem functions.

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Conflict of Interest

The authors declare they have no competing interests.

Author Contributions

SCD was responsible for experimental design, sample collection, data analysis and interpretation, and drafting/revising the manuscript. LEF developed the study concept, contributed to experimental design and data analysis, conducted critical revision of the

manuscript and provided funding for the study. EMB supported statistical analyses, graphical generation, and manuscript revision. PYA assisted with data collection.

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Tables and Figures

Table 1: Best-fit linear models for each immune parameter when including temperature treatment, clonal line, immune challenge, and lipids as predictors. † indicate models that did not undergo model dredging and averaging. Asterisks (*) represent significant p-value (α = 0.05).

Catalase					
Predictors	**Estimates**	**SE**	**SEadj**	**z**	**p value**
(Intercept)	336	29.3	29.7	11.3	**< 0.001 *****
Treatment	-126	31.8	32.2	3.90	**< 0.001 *****
Clonal Line	62.8	29.0	29.4	2.13	**0.0329 ***
Immune Challenge	43.6	37.8	38.1	1.14	0.253
Treatment: Immune	-14.9	36.5	36.7	0.404	0.686
Clonal: Immune	7.33	26.8	27.0	0.271	0.786
Antibacterial Activity					
Predictors	**Estimates**	**SE**	**SEadj**	**z**	**p value**
(Intercept)	1.86	0.112	0.114	16.3	**< 0.001 *****
Treatment	0.327	0.157	0.160	2.04	**0.0411 ***
Immune Challenge	0.00205	0.103	0.104	0.020	0.984
Lipids	2.32	1.21	1.23	1.88	0.0605
Treatment: Immune	-0.0487	0.0884	0.0891	0.547	0.584
Treatment: Lipids	-4.66	1.63	1.65	2.82	**< 0.01***
Immune: Lipids	-0.369	1.03	1.04	0.355	0.723
Total Phenoloxidase					
Predictors	**Estimates**	**SE**	**SEadj**	**z**	**p value**
(Intercept)	0.0233	0.00136	0.00138	16.9	**< 0.001 *****
Treatment	-3.04E-02	1.10E-02	1.12E-02	2.71	**< 0.01 ****
Clonal Line	-0.00156	0.00272	0.00275	0.569	0.569
Immune Challenge	0.00478	0.00176	0.00178	2.68	**< 0.01 ****
Treatment: Clonal	-0.00356	0.00413	0.00415	0.857	0.391
Treatment: Immune	0.000337	0.00137	0.00138	0.244	0.807
Clonal: Immune	-0.00133	0.00268	0.00270	.494	0.621
Melanin†					
Predictors	**Estimates**	**SE**	**t**	**p value**	
(Intercept)	2.31	1.36	1.70	0.089	
Treatment	-2.45	0.675	-3.62	**< 0.001*****	
Clonal Line	1.12	0.635	1.77	0.077	
Immune Challenge	0.494	0.624	0.791	0.429	
Lipids	-11.4	12.5	-0.916	0.360	
0s removed Melanin†					
Predictors	**Estimates**	**SE**	**t**	**p value**	
(Intercept)	-1.49E-04	1.01E-04	-1.47	0.149	
Treatment	1.62E-05	5.19E-05	0.311	0.757	
Clonal Line	-1.24E-06	4.96E-05	-0.025	0.980	
Immune Challenge	5.35E-05	4.83E-05	1.11	0.275	
Lipids	4.02E-03	9.34E-04	4.31	**<0.001 *****	

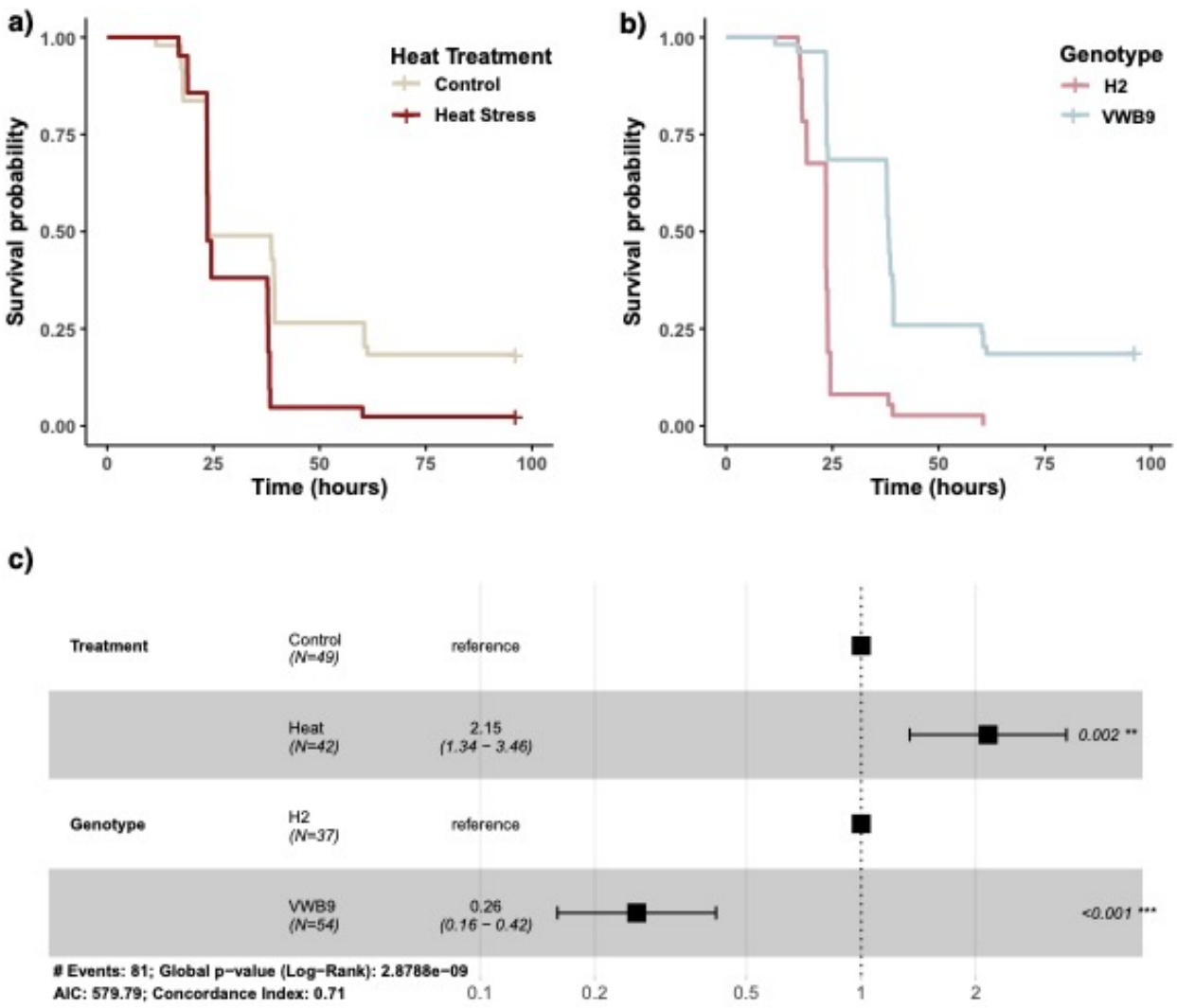

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Figure 1: (a-b) Kaplan-Meier survivorship curves for pathogen exposed anemones split based on comparisons across **(a)** temperature treatment and **(b)** genotype (clonal line). **(c)** Cox proportional hazards models of pathogen exposed anemones compared across temperature treatment and genotype. Vertical dotted line at 1 indicates the reference to which the second group is compared. The distance from 1 indicates the probability of mortality (i.e. heat stress is about twice as likely to die from immune challenge as heat). Both treatment ($p < 0.01$) and genotype ($p < 0.001$) had significant impacts on survival. Hazards ratios are displayed for both predictors with 95% confidence intervals.

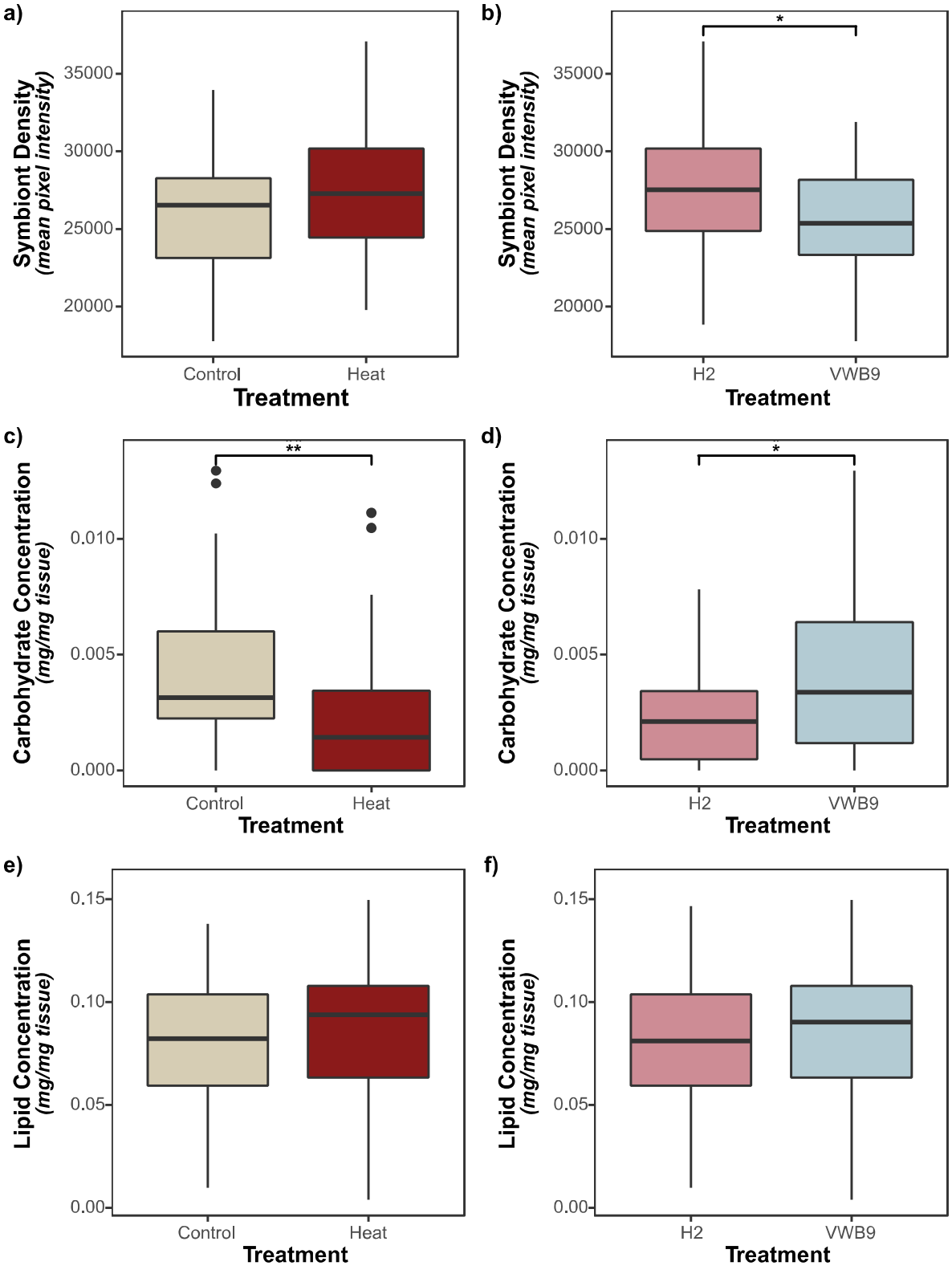

Figure 2: Box plots displaying symbiont density (**a-b**), carbohydrate concentration (**c-d**), and lipid concentration (**e-f**0 across temperature treatments and clonal lines. **(a)** Symbiont density was non-significantly different between temperature treatments (Welch's two sample t-test; p = 0.058); **b)** However, symbiont density was significantly higher in H2 anemones compared to VWB9 (Welch's two sample t-test; p = 0.046). Carbohydrate concentrations, on the other hand, were **(c)** significantly lower in heat-stressed anemones compared to control (Wilcoxon Rank Sum; $p < 0.01$) and **(d)** higher in the VWB9 clonal line compared to H2 (Wilcoxon Rank Sum; $p < 0.01$). Lipid concentrations did not differ across either **(e)** temperature treatments (Welch's two sample t-test; p = 0.348) or **(f)** clonal lines (Welch's two sample t-test; p = 0.664).

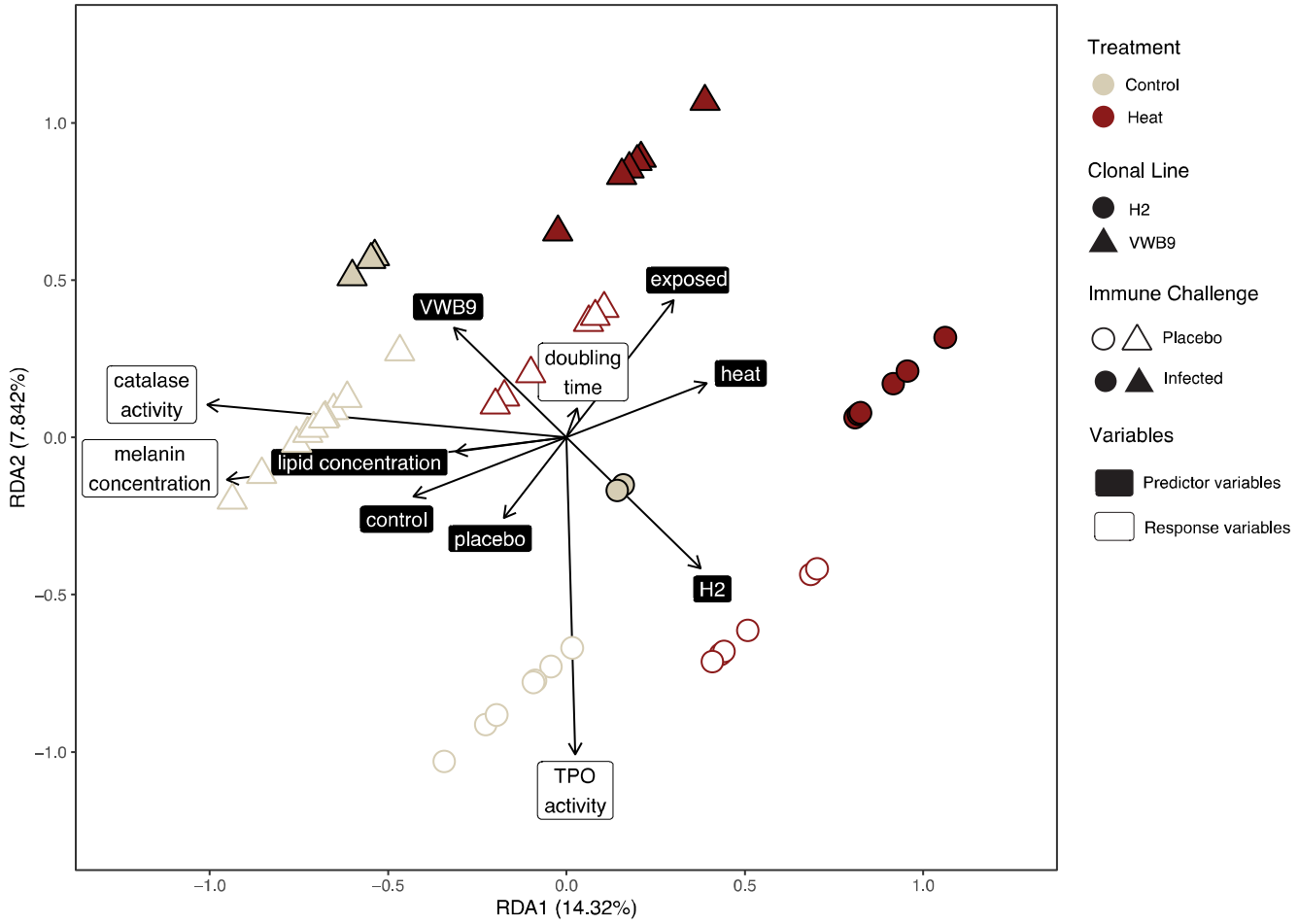

Figure 3. RDA plot showing significant predictors for explaining variation in holistic immunity. Data points represent individual samples. Ordination was constructed from response variables which in this case were immune parameters. Color indicates temperature treatment, shape indicates clonal line, and open shape/filled shape indicates immune challenge. Black boxes represent predictor variables and white boxes represent response variables. Arrows represent strength and directionality of each variable in multi-variate space.

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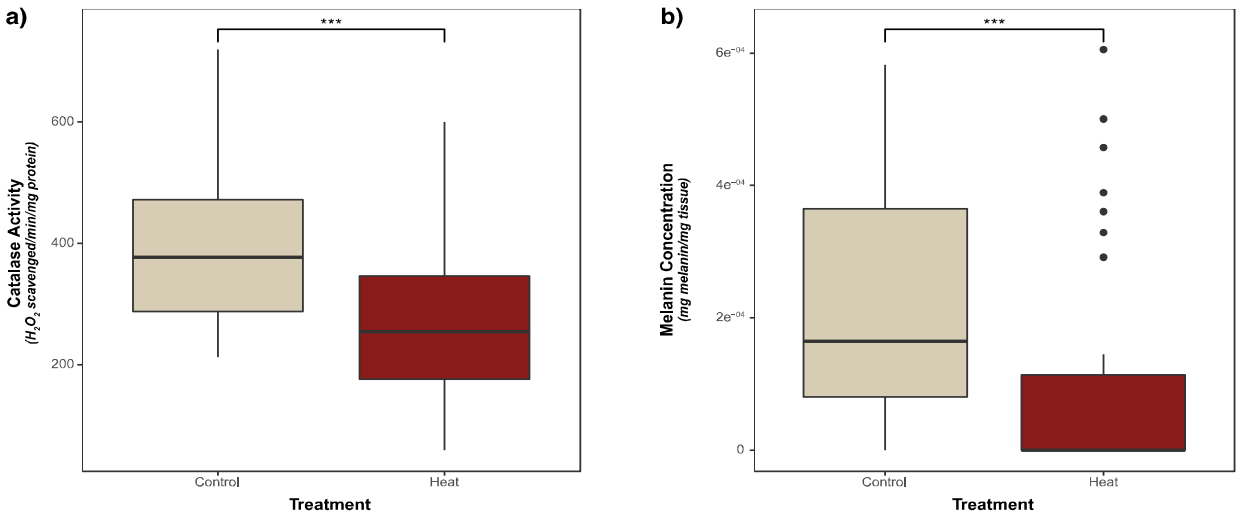


Figure 4: Differences in individual immune parameters as a result of temperature treatment. Prior heat treatment significantly affected **(a)** catalase activity (GLM, $p < 0.001$) and **(b)** melanin concentration (GLM, $p < 0.001$; 0s removed GLM, $p < 0.001$).

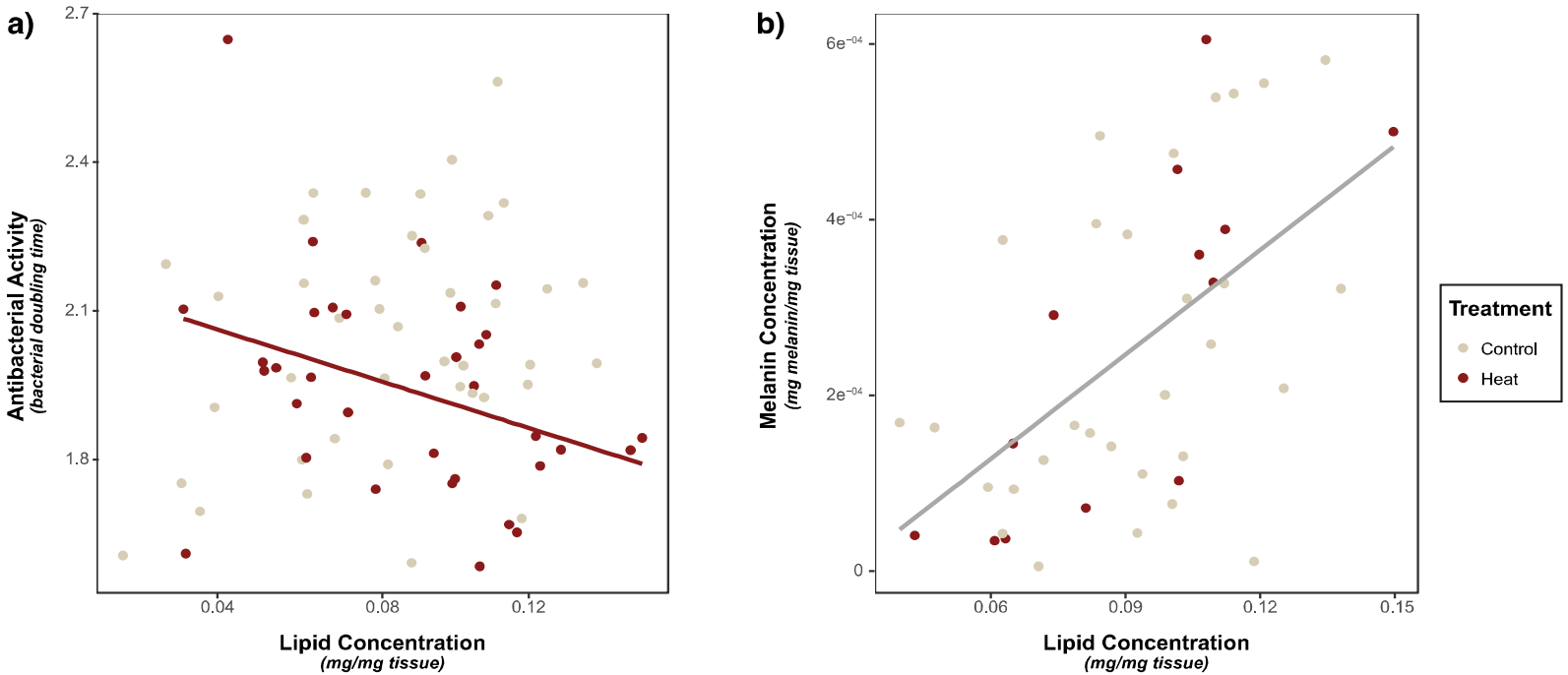
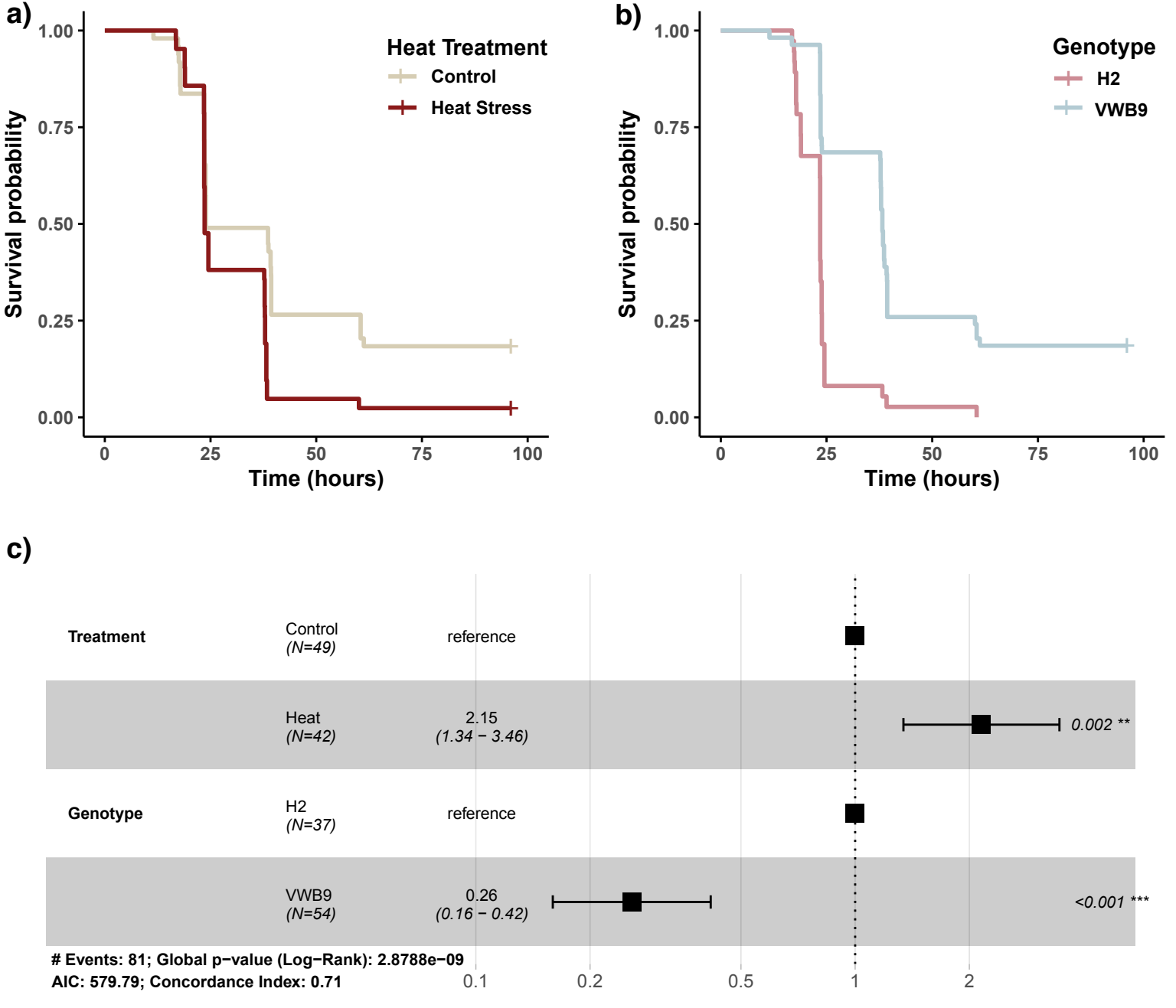
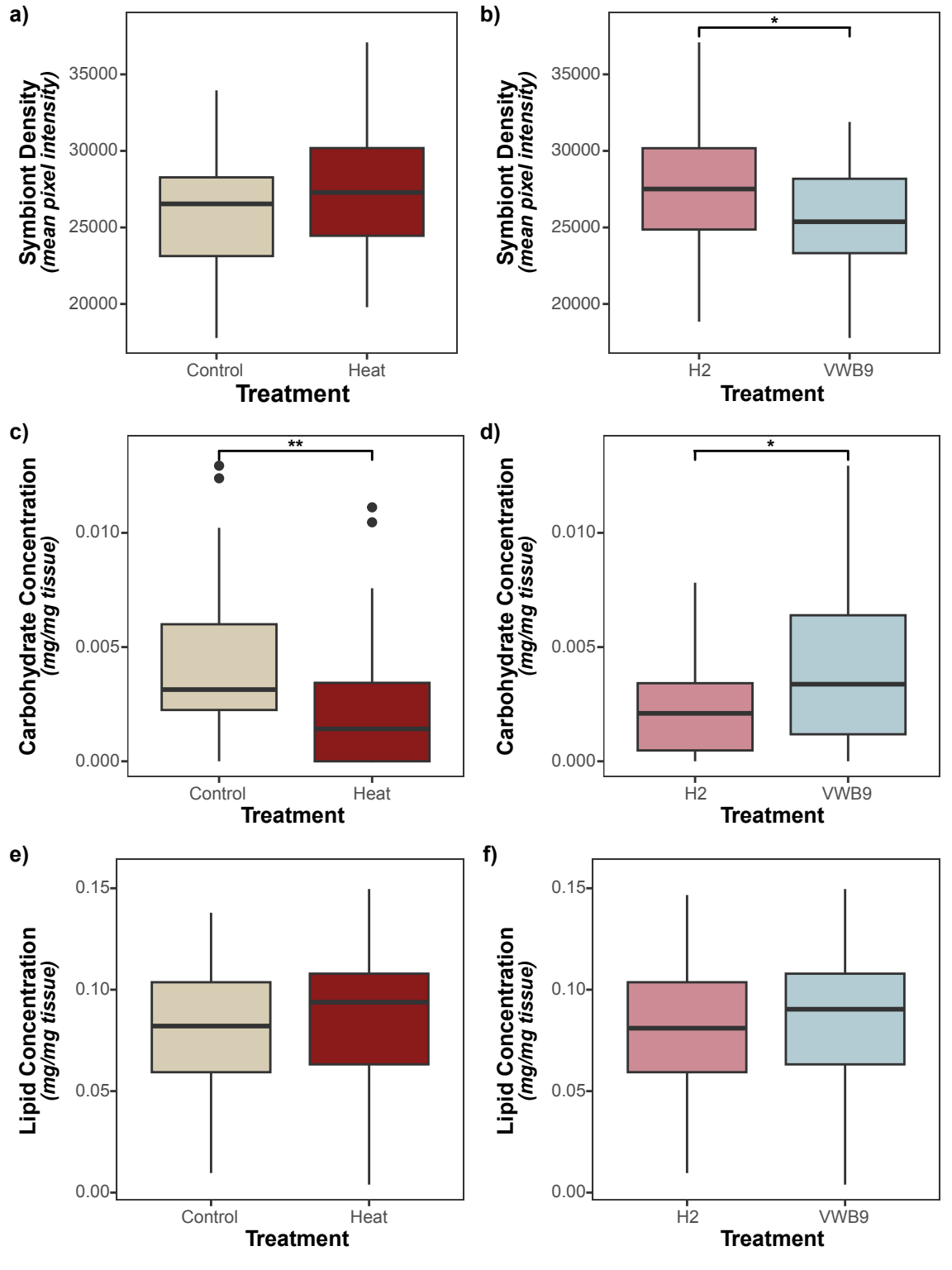
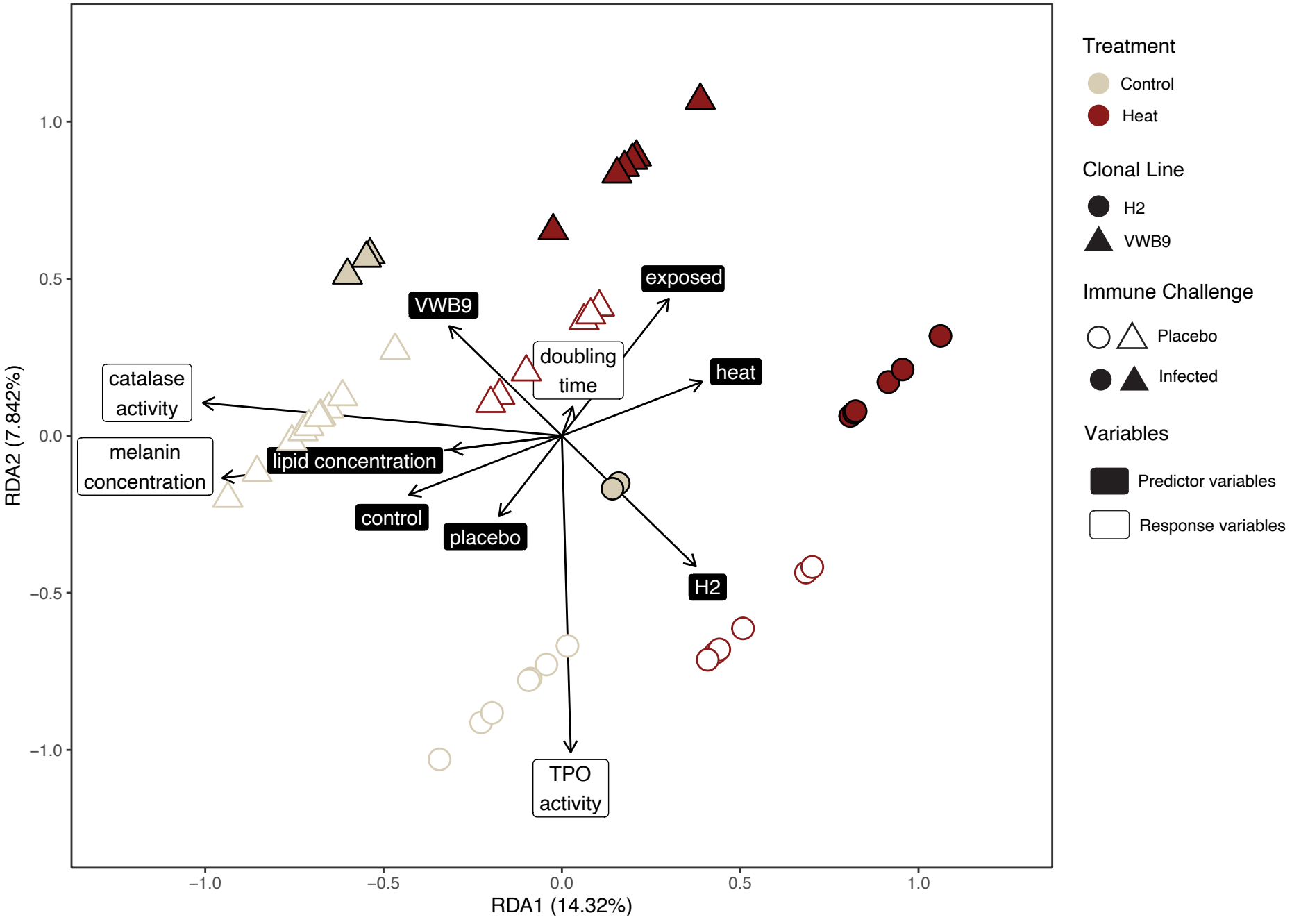
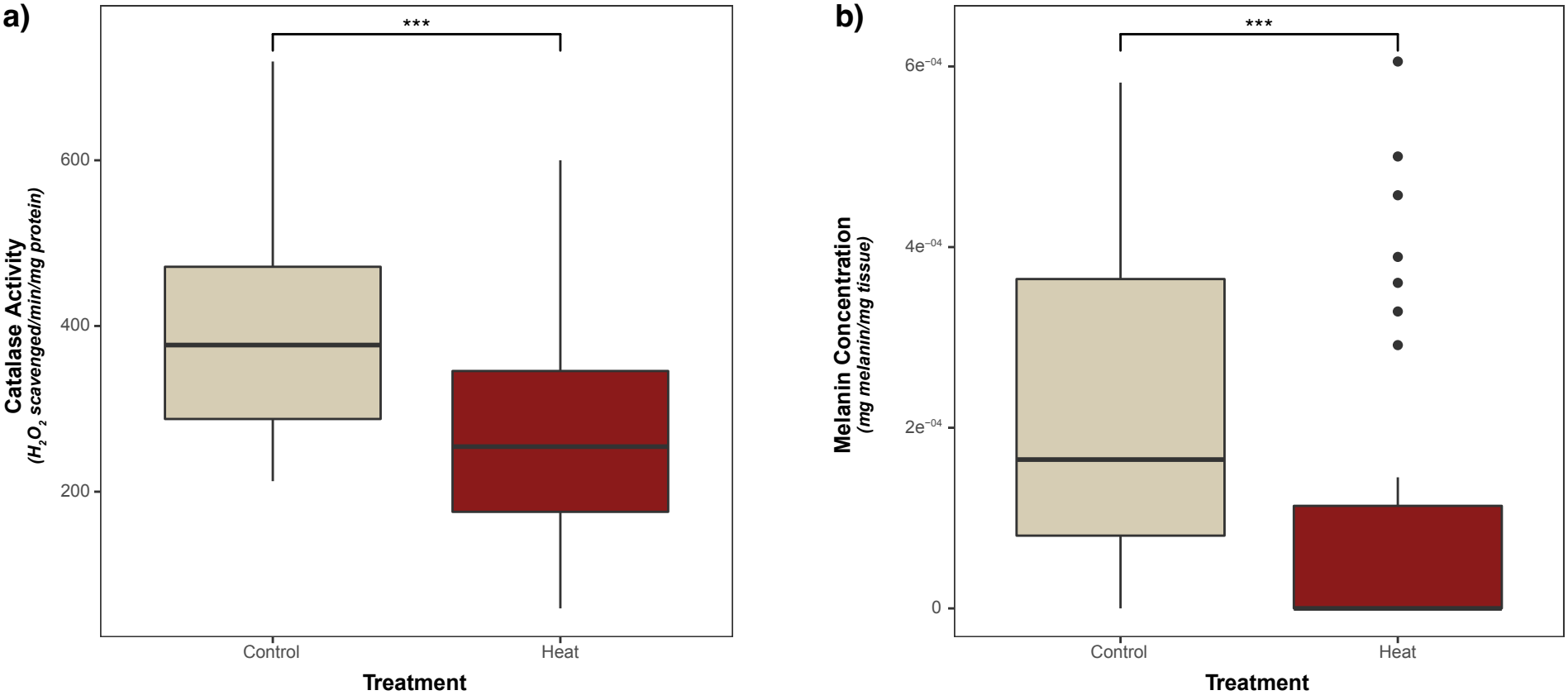


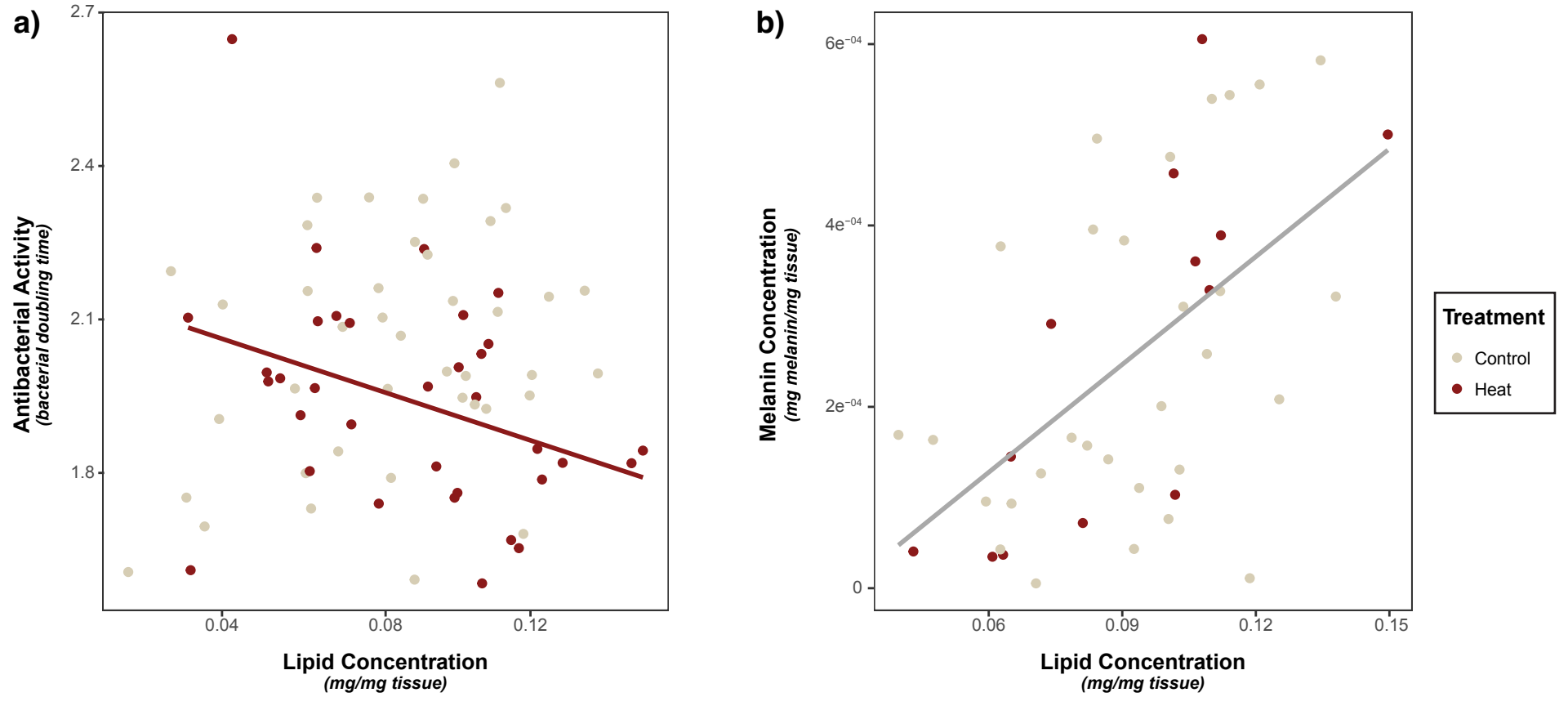
Figure 5: Best-fit linear models of the association between lipids and **(a)** antibacterial activity and **(b)** melanin concentration. **(a)** Heat treatment significantly affected associations between standardized lipid concentration and antibacterial activity (GLM, $p < 0.01$). **b)** However, melanin concentration was consistently associated with lipid concentration regardless of treatment group (GLM, $p < 0.001$; 0s removed GLM, $p < 0.001$). Points represent individual samples color coded by treatment group. Red trendline represents linear model of temperature- dependent association. Grey trendlines represent linear models of associations independent of temperature.


a)
Survival probability
1.00
0.75
0.50
0.25
0.00
0
25
50
75
100
Time (hours)
Heat Treatment
Control
Heat Stress
b)
Survival probability
1.00
0.75
0.50
0.25
0.00
0
25
50
75
100
Time (hours)
Genotype
H2
VWB9
c)
Treatment
Control
(N=49)
reference
Heat
(N=42)
2.15
(1.34 − 3.46)
0.002 **
Genotype
H2
(N=37)
reference
VWB9
(N=54)
0.26
(0.16 − 0.42)
<0.001 ***
Events: 81; Global p−value (Log−Rank): 2.8788e−09
AIC: 579.79; Concordance Index: 0.71
0.1
0.2
0.5
1
2


a)
Symbiont Density
(mean pixel intensity)
35000
30000
25000
20000
Control
Heat
Treatment
b)
*
Symbiont Density
(mean pixel intensity)
35000
30000
25000
20000
H2
VWB9
Treatment
c)
**
Carbohydrate Concentration
(mg/mg tissue)
0.010
0.005
0.000
Control
Heat
Treatment
d)
*
Carbohydrate Concentration
(mg/mg tissue)
0.010
0.005
0.000
H2
VWB9
Treatment
e)
Lipid Concentration
(mg/mg tissue)
0.15
0.10
0.05
0.00
Control
Heat
Treatment
f)
Lipid Concentration
(mg/mg tissue)
0.15
0.10
0.05
0.00
H2
VWB9
Treatment


Treatment
Control
Heat
Clonal Line
H2
VWB9
Immune Challenge
Placebo
Infected
Variables
Predictor variables
Response variables
exposed
VWB9
doubling time
heat
catalase activity
melanin concentration
lipid concentration
control
placebo
H2
TPO activity
1.0
0.5
0.0
−0.5
−1.0
RDA2 (7.842%)
RDA1 (14.32%)


a)
***
Catalase Activity
(H2O2 scavenged/min/mg protein)
600
400
200
Control
Heat
Treatment
b)
***
Melanin Concentration
(mg melanin/mg tissue)
6e−04
4e−04
2e−04
0
Control
Heat
Treatment


a)
Antibacterial Activity
(bacterial doubling time)
2.7
2.4
2.1
1.8
0.04
0.08
0.12
Lipid Concentration
(mg/mg tissue)
b)
Melanin Concentration
(mg melanin/mg tissue)
6e−04
4e−04
2e−04
0
0.06
0.09
0.12
0.15
Lipid Concentration
(mg/mg tissue)
Treatment
Control
Heat