Plasticity of a coral reef fish, *Abudefduf saxatilis*, to thermal stress and habitat degradation

by

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Abstract

Sea surface temperatures are rising at unprecedented rates due to anthropogenic activities. Warm conditions have the potential to push marine species beyond their physiological limits, which can lead to challenges on the individual and community level. Major changes are being observed in tropical and subtropical species, where many species are already living close to their thermal maxima. For example, reef-forming corals are particularly sensitive to warming, and increases in temperature can lead to bleaching and ultimately degradation of the complex three-dimensional habitats they form. Thus, in the case of organisms associated with coral reefs, oceanic warming will lead to detrimental effects that could be compounded by irreversible changes in reef structure. Studies have shown that, for marine ectotherms, thermal stress leads to increased aerobic demands, which in turn causes physiological, molecular, and behavioral changes. Despite recent advances evaluating the effect of temperature in ectotherms, questions remain on the behavioral responses to habitat complexity and warming, and how these traits also influence the molecular processes of the main sensory organ, the brain. Here, I evaluated how thermal stress and habitat loss are acting independently and synergistically as stressors in a damselfish of the Tropical Western Atlantic, *Abudefduf saxatilis*. Commonly known as the sergeant major, this species is omnivorous, territorial, and abundant in coral reefs, seagrass beds, and rocky habitats throughout the Atlantic. In chapter 1, I acutely exposed juvenile *A. saxatilis* to warm temperatures and simplified environments to evaluate molecular changes in the brain and the oxidative stress of liver and muscle. The results indicate that warming resulted in increased oxidative damage in the liver and changes in molecular pathways including neurotransmission, immune function, and DNA and tissue repair, while habitat loss did not have a significant effect on its own. In chapter 2, I exposed juveniles to habitat loss throughout 3 months of development

and found that individuals in low complexity habitat became more active, bold, and aggressive than those in a high complexity habitat. The results found here are parallel to observations in the field, highlighting the relevance of controlled aquarium studies to address how marine fishes respond to abiotic stressors. This thesis addresses key gaps in the literature on how fishes are responding to coral reef degradation and thermal stress and enhances our understanding on the plastic responses that enable generalist species to persist in a changing world.

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With the guidance of all those mentioned above and several others that I'm sure have been unintentionally omitted, I am proud to present my master's thesis!

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List of Abbreviations

ROS – Reactive oxygen species
RAS – Recirculating aquarium system
C-HC – Control temperatures and high complexity habitat
C-LC – Control temperatures and low complexity habitat
HW-HC – Heatwave temperatures and high complexity habitat
HW-LC – Heatwave temperatures and low complexity habitat
HC – High complexity
LC – Low complexity
IACUC – Institutional Animal Care and Use Committee
LRT – Likelihood ratio test
DEG – Differentially expressed gene
PCA – Principal component analysis
GO – Gene ontology
MWU – Mann-Whitney U test
WGCNA – Weighted gene co-expression network analysis
ELISA – Enzyme-linked immunosorbent assay
glmm – generalized linear mixed-effects model

Background

Global climate change is considered one of the main threats for the persistence of species and entire ecosystems. These changes are primarily driven by the unprecedented emission of greenhouse gasses, which have been increasing steadily since the Industrial Revolution and are promoted by burning fossil fuels, industrial processes, changes in land-use and deforestation, fabrication of building materials, etc. (Lamb et al. 2021). Increases of greenhouse gas emissions have resulted in average global warming of surface temperatures by 1.1°C since 1950 (IPCC 2019). However, these changes are not equally distributed around the planet, as 90% of the radiative energy is absorbed by the top 100 meters of sea surface waters (Levitus et al. 2012). Taking this into consideration, marine environments could be particularly affected by climate change, and estimates suggest warming of 1.5-4°C depending on the greenhouse emission scenarios (IPCC 2019). This is a worrisome trend, given that warming temperatures can trigger extreme weather events, extreme seasonality, heatwaves, and biodiversity loss (Hobday et al. 2015).

Of particular concern under these warming scenarios are ectothermic marine species, as their metabolic rate and multiple physiological processes are regulated by their environmental temperature. Phenotypic plasticity, or the expression of different phenotypes by the same genotype under different environmental conditions (DeWitt et al. 1998), may provide a buffer to rapid environmental changes to facilitate adaptive responses (Donelson et al. 2019). The most immediate response to increasing temperatures is behavioral plasticity, which may allow individuals to alter activity levels, microhabitat preferences, feeding behavior, and inter- and intra-species interactions to regain homeostasis (Snell-Rood 2013). Morphological plasticity such as changes in growth, reproductive rates, and variation in color patterns may also benefit

homeostasis maintenance (McGaughran et al. 2021). Lastly, molecular plasticity such as gene expression may increase thermotolerance by upregulating genes associated with heat shock response, electron-transport chain activity, and oxidative stress response (Bernal et al. 2020). Studies also indicate the response to warming is dependent on activating critical neuroendocrine pathways in the brain to orchestrate an appropriate response that uses stress hormones to communicate to the rest of the body (Alfonso et al. 2021). Thus, the survival of marine species occupying tropical reef systems is dependent on their ability to acclimate and/or adapt to meet energetic demands as these changes progress (Fox et al. 2019).

Tropical reef-building corals, for example, are highly susceptible to temperature changes. These ecosystem engineers of the sea create complex three-dimensional structures that support one of the most biodiverse and productive ecosystems on the planet (Blackall et al. 2015). Coral reefs support over 25% of marine life, directly housing over 2.4 million species who depend on the reef habitat for food and refuge (Spalding et al. 2001). Humans depend on coral reefs for many resources including food, cultural significance, economy, and coastal protection. During a heatwave, reef habitats are especially sensitive to a phenomenon called coral bleaching, or the release of algal symbionts by corals under thermal stress (Douglas 2003). In a bleached state, coral starvation and subsequent death is imminent if symbionts are not recovered within 8 weeks (Glynn 2009). Immediately after death, rapid deterioration of calcium carbonate coral skeletons ensues, while turfing algae covers the barren substrate left behind (Baker et al. 2008). Within months, the vibrant, three-dimensional, and diverse coral reef ecosystem evolves into a barren, rocky habitat. For groups of specialists that are reliant on corals for food or shelter (such as coral reef fishes) populations decline or have been extirpated following coral death (Pratchett et al. 2008). Generalist species occupying corals also face population declines following a bleaching

event, however, they have been shown to recover over time (Robinson et al. 2019; Garpe et al. 2006). Still questions remain on the generality of these patterns, and the physiological, behavioral, and molecular changes experienced by fishes during these habitat changes. Studies evaluating the phenotypic plasticity of generalist species to rapidly changing community dynamics, habitat availability, and diets must be prioritized to predict consequences of coral bleaching on entire reef communities.

Ocean warming represents two synergistic stressors for tropical reef fish: warming waters that can affect the physiology of a species and the challenges associated with habitat simplification. Here, I focus on evaluating the molecular, physiological, and behavioral responses of an abundant, generalist species to ecologically relevant stressors of temperature and habitat loss. While some studies have addressed these questions separately in marine fishes, questions remain on the generality of these patterns, the synergistic effects of warming and habitat simplification, and the molecular effects on the brain of tropical species. Overall, the two data chapters presented in this thesis address knowledge gaps that will advance our understanding on the synergistic effects of habitat shifts and warming temperatures on the phenotypic plasticity of a very robust damselfish.

Chapter 1

Warming and habitat loss influence brain gene expression and oxidative damage in a coral reef fish

Introduction

Anthropogenic activities are driving rapid changes in sea surface temperatures worldwide. These changes can be particularly detrimental for marine species, where a large portion of organisms conform to the conditions of their surrounding environment (i.e. ectotherms). These changes can be seen as slow increases in average ocean temperature through time, as well as an increase in the intensity and frequency of acute heatwaves (Jacox 2019; Holbrock et al. 2019). Given their commercial, cultural, and ecological importance worldwide, it is imperative to study how marine organisms will respond to these changes in coming decades.

Ocean warming is leading to the transformation of key marine ecosystems, including coral reefs (Guan et al. 2020). Over the past decades, intense heat waves lasting several days to weeks have resulted in severe episodes of coral bleaching (e.g. Hughes et al. 2018, Eakin et al. 2019; Loya et al. 2001). In many cases coral reef communities have changed irreversibly, shifting from complex three-dimensional structures, to barren/rocky landscapes (Hughes et al. 2018; Hughes et al. 2017). These changes can inflict stress on organisms that depend on reefs for habitat, such as coral reef fishes. Thus, to accurately predict how marine fishes are responding to warming oceans, it is necessary to evaluate the effects of warming with the addition of habitat simplification.

Most marine fishes are ectotherms, and their rate of metabolic processes is regulated by environmental temperatures. Resting metabolic rates increase with warming, which translates to increases in oxygen consumption and foraging to sustain increased metabolic demands (Pörtner

2021; Clarke and Johnston 2002; Clark et al. 2013). An increase in metabolic rates places constraints on optimal life history and behavior of fishes (Holt and Jorgensen 2015), immune capacity (Avtalion et al. 1973), and increases production of natural by-products in the cell, including reactive oxygen species (ROS) that promote oxidative stress to DNA, proteins, and lipids (Pörtner and Farrell 2008; Pörtner 2010; Pörtner and Peck 2010). If warm water conditions persist and the aerobic demands are not met, the physiological changes experienced by fish can lead to changes in metabolism, reproduction, growth, development, and physical condition (Alfonso et al. 2021).

Phenotypic plasticity in the form of physiological, molecular, and behavioral changes has been identified as a key mechanism to compensate for the effects of warming. In this context, previous studies have shown that fishes exposed to elevated temperatures upregulate molecular pathways associated with oxygen uptake, oxidative stress, mitochondrial function, electron transport chain, and cellular stress responses (Eaton et al., 2022; Madeira et al., 2013; Beemelmanns et al., 2021; Madaro et al. 2018; Neubauer and Andersen 2019; Somero 2020; Bernal et al. 2022). However, most of this information corresponds to molecular measurements in tissues such as muscle and liver, which are well known to track changes in the aerobic metabolism of marine ectotherms (Eaton et al., 2022; Seebacher et al. 2010). Questions remain on the effects warming can have on other organs of central importance such as fish brains, which initiate sensorimotor, stress, and behavioral responses to temperature fluctuations (van den Burg et al. 2005). One of the few studies evaluating molecular effects of warming in the brain of a tropical damselfish found functional differences in oxygen transport, neurotransmission, immunity, and ion channel regulation/formation (Bernal et al. 2022). Still, questions remain on the molecular responses of the brain to changes in temperature and habitat complexity.

We aim to address these knowledge gaps in the sergeant major, *Abudefduf saxatilis*. This is an abundant damselfish that inhabits coral reefs, rocky habitats, and seagrass beds throughout the Western Atlantic from Rhode Island to Uruguay (Froese and Pauly 2011). This species plays a key role in reef community structures as they have a broad omnivorous diet, are very territorial and aggressive, and adults show great site fidelity. They swim in small schools of 30 individuals or more when traveling through the water column or near reef edges and serve as common prey for larger fish in marine communities, including species of commercial importance such as snappers and groupers (Bester 2021). A relatively long larval dispersal stage (17-20 days; Wellington and Victor, 1989) allows high genetic connectivity between populations throughout the species range (Piñeros et al. 2017). Given the abundance in numbers and tolerance to a wide range of environmental conditions, *A. saxatilis* represents a well-suited model to understand the responses of coral reef fishes to global environmental changes.

In this study, we exposed *A. saxatilis* to different treatments of habitat complexity and temperature to evaluate the effects on gene expression of the brain and oxidative stress of the liver and muscle. This project addresses three key knowledge gaps: 1) what is the effect of warming on brain gene expression and oxidative stress of sergeant majors?; 2) what is the effect of simplified habitat on brain gene expression and oxidative stress?; 3) does oxidative stress as an effect of warming and habitat loss differ between liver and muscle tissues?; 4) are there synergistic effects between warming and habitat loss that should be considered in future climate change projections?. We hypothesize that both temperature and loss of habitat complexity will have a significant effect on the differential expression of sergeant major brains, and significant increase in oxidative stress of the liver and muscle. The results of this study highlight the

relevance of evaluating rapid environmental changes on the sensory organs of marine fishes, as several key processes could be affected with projected changes in coming decades.

Methods

Fish collections

We collected juvenile *A. saxatilis* (average mass: 10.6 ± 2.8 g) in September of 2021 using clove oil, hand nets, and cast nets in <1m of water depth along the jetties of St. Andrews State Park, Florida (30.122785, -85.732821; Florida Park Service permit #10042121). Live fish were transported in aerated buckets to aquarium facilities at Auburn University (Auburn, AL, USA). Fish acclimated to aquaria maintained at 27°C and a salinity of 28 ppt for four-weeks to maintain the seasonal average temperatures at the collection site (National Data Buoy Center). Throughout the acclimation period and experimental exposure, fish were fed tropical fish flakes (Ocean Nutrition Formula One Flakes) twice daily with a 12:12 hour day:night photoperiod using LED light strips. The fish were housed in six independent recirculating aquarium systems (RAS), each one with 8 tanks of 30 gallons each. Due to the aggressive ecology of the species, we housed individuals in separate, 30-gallon chambers of the RAS (40 fish total for the experiment).

Experimental exposure

We implemented a fully factorial experimental design to assess the interactive effects of temperature and habitat complexity (Figure 1). For this, the temperature treatments chosen were Control (27°C) and Heatwave (31°C), considering the average temperatures for the month of October (2015-2020) and the upper 5% of temperatures recorded at the collection site during the same period, respectively (National Buoy Data Center; Hobday et al. 2018; Oliver et al. 2018). These conditions were replicated across three independent RAS, to avoid issues with pseudoreplication. For the heatwave treatments, temperatures were increased 2°C per day over two days to avoid thermal shock. After heatwave temperatures were reached, habitat complexity

treatments of low complexity or high complexity were assigned to half of the individuals on each of the RASs. Low complexity tanks contained no habitat structure, and high complexity tanks contained two 12-inch artificial aquarium plants and two PVC pipes to provide shelters. This combination of treatments results in four experimental treatment groups: control temperatures / high complexity (C-HC; n= 10), control temperatures / low complexity (C-LC; n=10), heatwave / high complexity (HW-HC; n=10), heatwave / low complexity (HW-LC; n=10). Individuals were acutely exposed to these conditions for a period of 10 days.

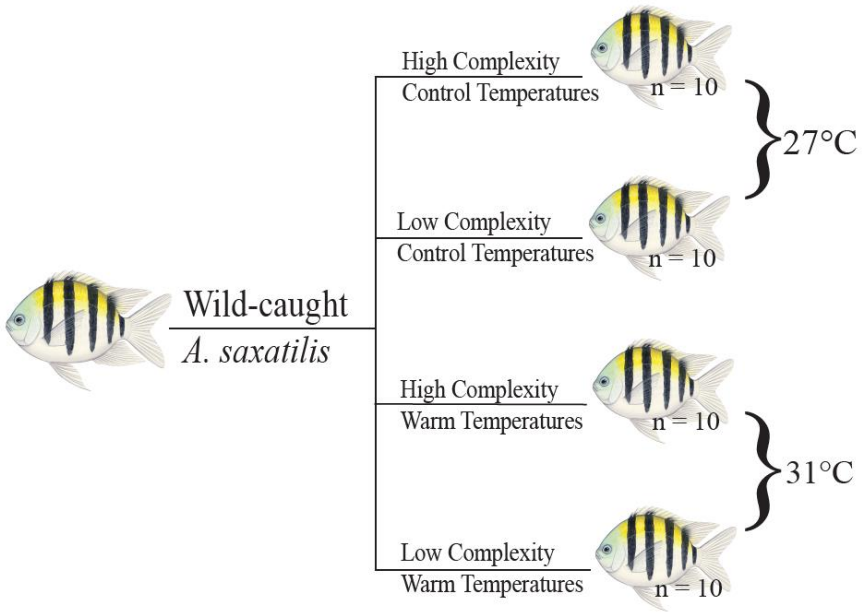

Figure 1. Experimental design to assess effects of temperature and habitat complexity. Image of *Abudefduf saxatilis* illustrated by Julie Johnson (Life Science Studios, LLC).

At the end of the 10-day exposure, fish were euthanized by cervical transection using sharp dissection scissors following the Institutional Animal Care and Use Committee (IACUC) of Auburn University (protocol #2020-3708). We measured the total length, standard length, and weight of each fish. Whole brains were removed and stored in 500 µL DNA/RNA shield (Zymo)

at -80°C. Full brains were used to ensure our results were not skewed by the differences in gene expression among different brain regions. Livers and muscle filets were flash frozen in liquid cdnitrogen and stored at -80°C for oxidative stress analysis.

Transcriptome RNA extractions, sequencing, assembly, and annotation

Gill, brain, muscle, fin, liver, and eye of one non-experimental individual of *A. saxatilis* were removed from the fish and stored in DNA/RNA shield. The tissues were individually extracted using the ZYMO Quick-RNA Miniprep Plus kit according to the manufacturer's protocol. The tissues were combined into a single extraction, with the following proportions: gill = 6.5%; muscle = 19.4%; brain = 15.1%; fin = 17.4%; liver = 25.3%; eye = 16.2%. RNA extractions were sent to Novogene for library preparation with Illumina TruSeq Kit, and sequencing was done with an Illumina NextSeq.

Raw reads were trimmed using trimmomatic v. 0.39 (Bolger et al., 2014) with the following parameters: -phred33 ILLUMINACLIP:TruSeq3-SE:2:30:10 LEADING:3 TRAILING:3 MINLEN:25. The transcriptome was subsequently assembled using Trinity v. 2.11.0 (Grabherr et al., 2011) and completeness of the assembled transcriptome was determined using BUSCO v. 4 (Manni et al., 2021). We then used CD-HIT v. 4.8.1 (W. Li & Godzik, 2006) to reduce the redundancy on the assembled transcriptome by cluster sequences based on similarity, using a clustering threshold of 95% identity.

The assembled transcriptome was annotated using the program BLAST+ ver 2.11.0 (Camacho et al. 2009) to search for sequence similarity against closely related unspliced gene sets on Ensembl (Cunningham et al. 2022), using a minimum e-value of 1e-10. We filtered the BLAST results to exclude any matches with a query coverage <40%, or a percent identity <60%.

We used custom bash scripts to identify the best match for each transcript by the highest bit-score. SeqKit ver 0.10.1 was used to isolate transcripts with low quality matches from the reference transcriptome. We repeated this process iteratively for transcripts that were not matched in BLAST results in the following order of Ensembl gene sets: *Amphiprion percula* (orange clownfish), *Acanthochromis polyacanthu*s (spiny chromis), *Danio rerio* (zebrafish), *Stegastes partitus* (bicolor damselfish), and *Amphiprion ocellaris* (clown anemonefish). Any remaining unannotated contigs were compared to the UniProt Swiss-Prot protein database (Apweiler et al. 2004), using the same parameters described above other than a looser query coverage of <48%. We additionally removed any matches to non-chordates to improve annotation accuracy. The full pipeline for annotation of the transcriptome can be found at: https://github.com/allyswank/Abudefduf-Brains/Annotation.

Experimental RNA extractions, sequencing, and differential gene expression

RNA was extracted from full brain samples using the Zymo Quick-RNA Miniprep kit according to the manufacturer's protocol with minor modifications described here. Approximately 50mg of sample was homogenized in 250µL DNA/RNA shield. Samples were incubated in 15µL Proteinase K for four hours in a 32°C water bath or until cell lysates were fully digested. We treated each sample with DNase to remove DNA contamination, and the final elution was done with 100 µL of elution buffer. Quality of each extraction was assessed visually on a 1% agarose gel and with a Nanodrop 2000 spectrophotometer. For samples with Nanodrop A260/A230 ratios below 1.60 were purified using the Zymo RNA Clean & Concentrator-25 kit and an additional DNase step following the manufacturer's protocol. For brain samples with more than 50mg of tissue, two separate RNA extractions were done to avoid clogging the kit columns. These

samples with two extractions were consolidated into one sample using the clean and concentrator kit as described above. The final concentration of RNA was measured with a Qubit 4 fluorometer. Quantity of purified RNA was standardized to 100 ng/µL, and total RNA samples were shipped to the University of Texas at Austin Genomic Sequencing and Analysis Facility for library preparation and Tag-Seq sequencing using the Illumina NovaSeq6000 (Meyer et al. 2011). This library protocol results in 100-bp single-end Illumina reads that were mapped to the *A. saxatilis* transcriptome.

Sequence quality was assessed with FastQC v0.11.9. We used the program cutadapt v1.13 to trim Illumina adapters (command parameters: cutadapt - -a AAAAAAAA -a AGATCGG -q 15 -m 25). Quality of trimmed reads were again assessed with FastQC before mapping the reads to the *de novo A. saxatilis* reference transcriptome with Bowtie2 v2.3.0. We compiled Tag-Seq counts of aligned reads to each transcript into a single table for downstream analyses. Custom scripts and a comprehensive review of the read-sequencing pipeline are available at: https://github.com/z0on/tag-based_RNAseq.

The analyses of differential gene expression were conducted with DESeq2 ver 1.34.0 (Love et al. 2014) package in RStudio ver 4.1.3 (RStudio Team, 2019). We used variance stabilized data to evaluate for outliers based on gene counts. A likelihood ratio test (LRT) was conducted, controlling for the placement in the aquarium towers (top or bottom row), to evaluate the differential expression between all four treatment groups. Differentially expressed genes (DEGs) were identified as those with an adjusted p-value < 0.05. We transformed the gene count matrix using the variance stabilizing transformation function. To visualize DEGs across treatments, we generated a principal component analysis (PCA) of significant variance transformed gene counts from all samples. We used a Wald test for pairwise comparisons to

identify DEGs between the individual treatment groups: C-HC vs. C-LC, HW-HC vs. HW-LC, C-HC vs. HW-HC, C-LC vs. HW-LC, C-LC vs. HW-HC, and C-HC vs. HW-LC.

A Mann-Whitney U test was used to identify significantly enriched Gene Ontology (GO) categories for the DEGs from each pairwise comparison (GO-MWU; Wright et al. 2015). For this, the Log2Fold change estimates resulting from the pairwise comparisons described above were used to estimate GO category enrichment. The GO-MWU is a test of ranks, and estimates enrichment of GO categories based on the distribution at the top (upregulated) or bottom (downregulated) of a list of all genes, making the analysis independent from the estimates of differential expression described above (Wright et al. 2015). Significant GO categories were identified as those with a false discovery rate of < 0.1. All custom scripts were adapted from https://github.com/z0on/GO_MWU and are publicly available at: https://github.com/allyswank/Abudefduf-Brains.

Weighted Gene Co-expression Network Analysis

A weighted gene co-expression network analysis (WGCNA) was used to identify clusters of highly correlated genes within the experimental conditions (Langerfelder and Horvath 2008). To ensure results were not skewed by genes with low expression, we filtered the RNA-seq dataset to eliminate genes with less than 50 reads across samples from further analysis. Filtered gene counts were normalized and transformed using the variance stabilizing transformation function in DESeq2 (Love et al. 2014). The power parameter was set to 10 based on the scale-free topology criterion. A signed topological overlap matrix was used to build gene modules. We ran a linear model on each module to identify the most significantly differential expressed modules. Scripts for WGCNA analysis are available at: https://github.com/allyswank/Abudefduf-Brains.

Co-expressed genes from each module were analyzed with a Fisher's test to identify significant GO categories within each significant module. GO analyses were conducted using the GO-MWU program adapted from https://github.com/z0on/GO_MWU, and scripts are available at: https://github.com/z0on/GO_MWU).

Oxidative stress analysis

To analyze the effects of complexity and temperature on oxidative stress, we used a protein carbonyl enzyme-linked immunosorbent assay (ELISA) kit to quantify the protein oxidation levels of liver and muscle tissues separately (Cell Biolabs, Inc., San Diego, CA). For each sample, 15mg of tissue was homogenized in 500mL of 10X PBS buffer. To avoid overestimation of carbonyl content by nucleic acid content, samples were treated with a 0.5% concentration of PEI followed by centrifugation at 4°C for 10 minutes at 6000rpm. The supernatant was collected after centrifugation, and a Bradford assay was used to assess protein concentration (Thermo Scientific). Samples were diluted to 10 ± 2 µg/mL in 10X PBS before implementing the ELISA assay according to the manufacturer's protocol (OxiSelect Protein Carbonyl ELISA Kit, Cell Biolabs, VWR). Both samples and standards were assayed in duplicate. The website MyAssays.com (Cook 2006) was used to analyze the spectrophotometer output using a four-parameter logistic curve to estimate unknown carbonyl concentrations. Statistical analysis was done in RStudio ver 4.1.3 (RStudio Team, 2019). A linear model was conducted to confirm no significant correlation of raw protein concentration per assayed sample and carbonyl quantification. To account for size variation between fish, we used weight as a covariate in the linear mixed model:

lme(CarbonylConcentration~Weight+Temperature+Complexity+Temperature:Complexity, data = elisa, random=~1|Tower). A qqplot confirmed that model residuals were normally distributed and that the data set contained no outliers.

Results

Transcriptome assembly and annotation

The transcriptome of *A. saxatilis* had a total of 264,002,416 assembled bases, an average contig length of 1,268.73bp, a median contig length of 462bp, and a contig N50 of 3,023bp. The Trinity assembly had 208,084 total trinity transcripts and 144,719 trinity 'genes', with a percent GC of 45.51%. The assessment of completeness with BUSCO v.4 against the Actinopterygii odb10 database (total of 3640 genes) resulted in 3203 complete genes (88%; made up of 1642 single copy and 1561 duplicated genes), 114 fragmented genes (3%), and 323 missing genes (9%) in the Trinity assembly. CD-HIT identified 178,505 unique clusters out of the total 208,084 transcripts. These unique clusters were used for annotation of the transcriptome and as reference for the analyses of differential expression. The efficacy of annotation was 41%, as annotation with the different genomes from ENSMBL resulted in 72,332 annotated genes out of 178,505.

Tag-seq processing and Differential Gene Expression

After trimming adapters and removing PCR duplicates, there was an average of 3,045,823 (+/- 359,436) Tag-Seq reads per sample. These trimmed and filtered reads were mapped to the *A. saxatilis* reference transcriptome with an average mapping rate of 91.7%.

One individual from the C-HC and one from the HW-HC treatment groups had a much higher number of reads compared to other samples. To avoid any biases in DGE analyses caused by variance associated with sequencing, these two samples were removed from the DESeq2 analyses.

The LRT between all four treatment groups revealed a total of 285 significant DEGs with temperature being the dominant effect (171 upregulated and 114 downregulated; **Figure 2**).

When isolating the effects of temperature and removing the effect of habitat complexity, the LRT revealed 124 upregulated and 131 significantly downregulated genes. To evaluate the effects of habitat complexity, temperature was excluded as a factor from the analysis, and this test resulted in no significant DEGs.

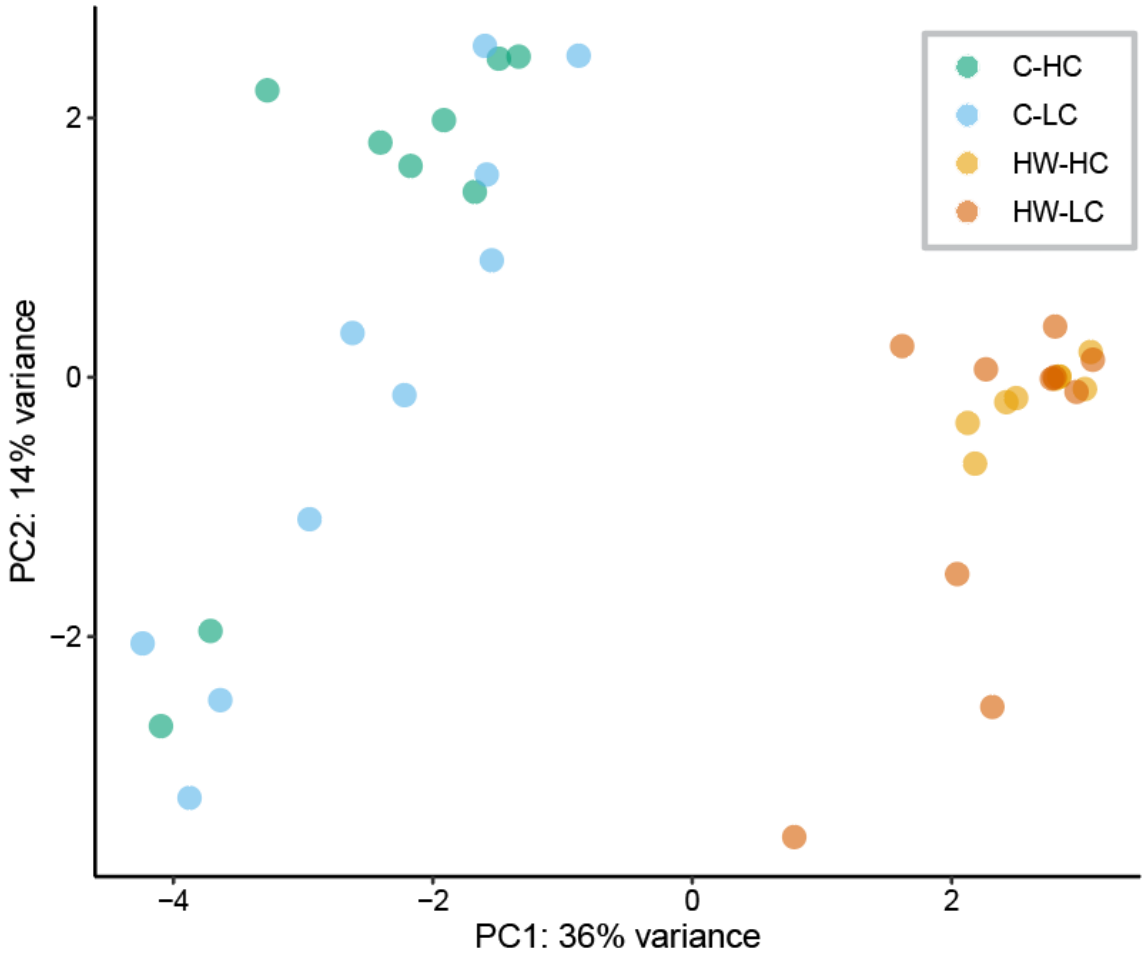

Figure 2. Principal component analysis (PCA) of all four treatment groups with one outlier removed from both C-HC and HW-HC based on gene read counts: control temperatures / high complexity (C-HC; n= 9), control temperatures / low complexity (C-LC; n=10), heatwave / high complexity (HW-HC; n=9), heatwave / low complexity (HW-LC; n=10).

The number of significant DEGs for each pairwise comparison are reported in **Table 1**. The largest number of DEGs were found between C-LC and HW-LC (n = 122). Functions that were upregulated in HW temperatures for this comparison include heat shock proteins (*HSPA2*; Wang et al. 2015), immunity and cytokine pathways (*ivns1abpa*; Hotter et al. 2020), neuron synaptogenesis (*sypb*; Du et al. 2018), and downregulated functions included DNA damage response (*dazap2*; Liebl et al. 2021) and central nervous system regeneration and repair (*zgc:77056*; Maheras et al. 2018). These results suggest that acute thermal stress has a significant effect on the molecular processes of the brain.

Table 1. Number of DGE and GO categories of each significant pairwise comparison.

Comparison	**Direction**	**DEGs**	**Significant GO Terms**			**General Functions**
			BP	**MF**	**CC**	
Control Temperatures vs. Heatwave Temperatures	Up	171	0	0	0	
	Down	114	0	0	0	
Control Temperatures / High Complexity vs. Heatwave Temperatures / High Complexity	Up	32	0	0	0	cytokine activity, calcium:cation antiporter activity, active ion transmembrane transporter activity, obsolete ATP hydrolysis activity
	Down	24	0	4	0	
Control Temperatures / Low Complexity vs. Heatwave Temperatures / Low Complexity	Up	58	0	0	0	ion transmembrane transporter activity, voltage-gated channel activity, ATPase activity, G protein-coupled receptor kinase activity
	Down	64	1	7	0	
Control Temperatures / Low Complexity vs. Heatwave Temperatures / High Complexity	Up	49	1	2	0	electron-transferring-flavoprotein dehydrogenase activity, oxidoreductase activity acting on the CH-NH group of donors
	Down	53	3	15	0	cytoskeleton protein binding, ATPase-coupled cation transmembrane transporter activity, P-type transmembrane transporter activity
Control Temperatures / High Complexity vs. Heatwave Temperatures / Low Complexity	Up	42	0	0	0	
	Down	54	0	0	0	

As with the LRT, habitat complexity did not result in any DEGs (Table 1). However, the number of DEGs in the comparisons between control and heatwave in low habitat complexity were almost double (C-LC vs. HW-LC = 122) than the number of DEGs in the treatments with high habitat complexity (C-HC vs. HW-HC = 56 DEGs; Table 1). This result indicates a synergistic effect of habitat complexity on thermal stress response, even when habitat complexity alone did not result in differential expression.

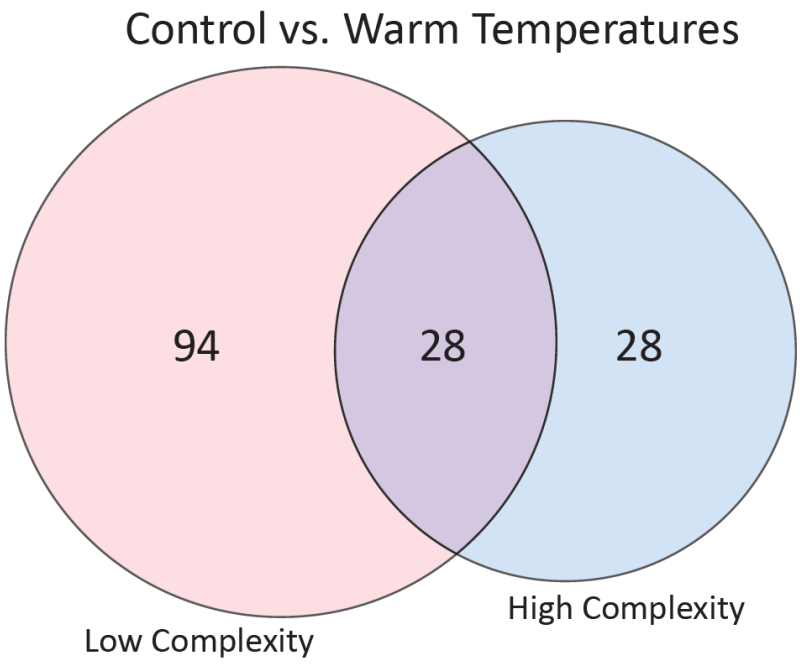

Figure 3. Venn diagram showing numbers of shared differentially expressed genes from acute thermal stress in the high complexity and low complexity comparisons.

To understand potential underlying effects of habitat complexity on thermal stress response, we identified DEGs found exclusively in the high complexity groups and those found exclusively in the low complexity comparisons (**Figure 3**). There were 28 DEGs exclusive to the high complexity thermal comparison (C-HC vs. HW-HC), but these lacked annotation in the reference transcriptome. Meanwhile, there were the 94 DEGs exclusive to the low complexity

comparison (C-LC vs. HW-LC). Upregulated genes in the HW treatment were involved in eye lens development (*dnajb1b*; Khan et al. 2016), heat stress (*hsp90aa1.2*; Rebl et al. 2013), and circadian clock (*cry3a*; Sacksteder and Kimmey 2022), while downregulated genes were involved in neurogenesis and neuronal cell differentiation (*ctdsp2*; Dill et al. 2012), growth suppression (*RERG*; Mou et al. 2022), inflammation and tissue reconstruction response (*hmgb1a*; Bianchi et al. 2009), and neurotransmission (*cplx2*; Nichterwitz et al. 2020). The gene that was most downregulated in both low and high complexity treatment groups when comparing control and heatwave temperatures was *smtla*, which is a hormone regulator involved in many physiological functions of fishes including sexual maturation, plasma ion and pH regulation, lipid metabolism, and body color-regulation (Kakizawa 2021).

When comparing C-LC vs. HW-HC, 49 upregulated and 53 downregulated genes were identified (102 total DEGs). Meanwhile, C-HC vs. HW-LC comparisons revealed 42 up- and 54 downregulated genes (96 total DEGs). There were 33 DEGs shared between these two comparisons which are likely key regulators in plasticity to heatwaves (**Figure 4**). Functions of these genes included neuron communication (*sptssb*; Bryosis et al. 2022), neuron synaptogenesis (*sypb*; Du et al. 2018), DNA damage response (*dazap2*; Liebl et al. 2021), central nervous system regeneration and repair (*zgc:77056*; Maheras et al. 2018), and inflammation and tissue reconstruction response (*hmgb1a*; Bianchi et al. 2009).

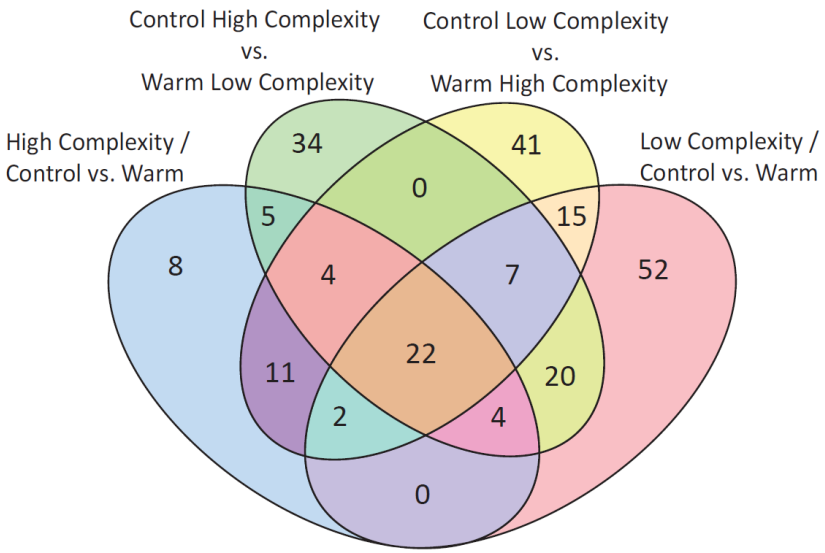

Figure 4. Venn diagram showing the number of genes that are shared and unique for the different pairwise comparisons.

Specific functional categories that were significantly enriched due to the effect of temperature were identified using the GO-MWU analysis. In high complexity treatments, the heatwave temperatures expressed downregulation of the functional GO categories "active ion transmembrane transporter activity", "calcium:sodium antiporter activity", "obsolete ATP hydrolysis activity", and "cytokine activity." In low complexity treatments, the heatwave temperature treatment showed downregulation for the functional GO categories "ion transmembrane transporter activity", "potassium ion transmembrane transporter activity", "cation transmembrane transporter activity", "voltage-gated cation channel activity", "voltage-gated channel activity", "transporter activity", and "G protein-coupled receptor kinase activity." C-LC vs. HW-HC contained the highest number of GO categories (n = 21), including "pore complex assembly", "ion transport", "oxidoreductase activity, acting on the CH-NH group of donors", "cytoskeletal protein binding", "obsolete ATP hydrolysis activity", "ATP-dependent

activity", "large conductance calcium-activated potassium channel activity", "P-type calcium transporter activity".

Weighted Gene Correlation Network Analysis (WGCNA)

Our WGCNA analysis revealed 21 modules of co-expressed genes. Several of these modules were associated with effects of temperature and contained genes that overlapped with shared DEGs reported in the pairwise comparisons. **Figure 5** shows the top statistically significant module which contains genes associated with synaptic function (*serbp1a*; Baudin et al. 2021), peroxisomal membrane formation (*pex19*; Snyder et al. 2000), and growth suppression (*RERG*; Mou et al. 2022). The Fisher's test did not indicate any significant functional GO categories within any set of WGNCA modules.

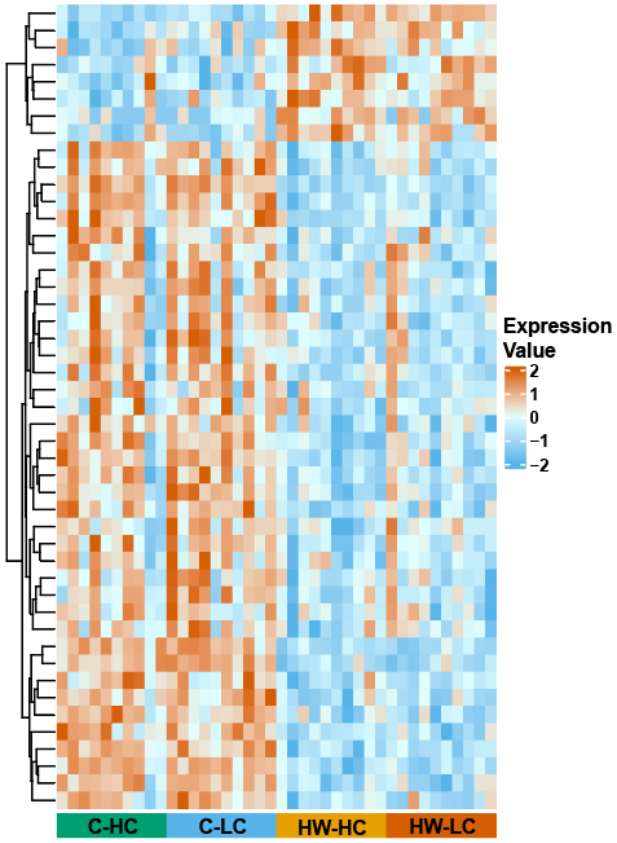

Figure 5. WGCNA gene network showing the differential expression related to temperature. The tree on the y-axis represents the relatedness genes being expressed in the module.

Oxidative stress

The general linear model revealed that there was no interaction between temperature and complexity (p = 0.071), so we analyzed the effects of these variables separately. Acute thermal stress resulted in a trend of greater protein carbonyl concentrations in the liver (32%; p = 0.007; **Figure 6**), while habitat complexity had no visible effect (p = 0.15). Muscle protein had carbonyl concentrations below detectable limits by the ELISA assay kit when tested at the same protein concentration as the liver tissue. However, the C-LC treatment group contained 8 samples with detectable concentrations, while no other treatment group had detectable oxidative stress.

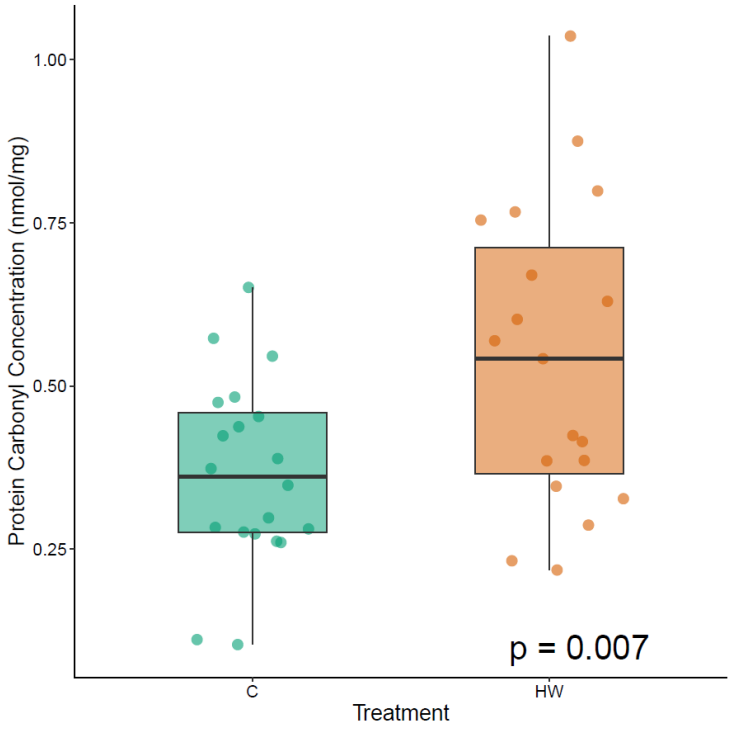

Figure 6. Protein carbonyl concentrations from liver in Control (27°C) vs. Heatwave (31°C) samples.

Discussion

Abudefduf saxatilis is a widespread and ecologically relevant damselfish in coastal and offshore ecosystems of the Tropical Western Atlantic. Despite the importance of this species, there is a current knowledge gap in its response to environmental stress, and more broadly, how fish brains are affected by warming and habitat complexity. To bridge this gap, we analyzed gene expression responses in the brain and oxidative stress responses in the liver and muscle. Overall, the largest effects for brain gene expression were observed with acute warming. Meanwhile, reduction of habitat complexity did not lead to differential gene expression but could increase stress through synergistic effects with warming. Levels of oxidative stress in the liver were significantly different with acute warming, but not with reduction of habitat. Muscle tissue contained oxidative damage below detectable limits by the ELISA assay with the exception of samples exposed to habitat reduction. The results of this study are relevant to understand how changes of habitat complexity and temperature will affect marine communities, while informing specific phenotypic and molecular responses of damselfishes to stressors predicted in coming decades.

Effects of temperature on fish brains:

Analyses of gene expression revealed functional differences between temperature treatments in the brain of *A. saxatilis*. Specifically, we found that heatwave temperatures resulted in downregulation of functional categories associated with ion transport, including potassium ion transport, calcium:sodium antiporter activity, and voltage-gated channel activity. Ion transportation in the brain is critical for the generation, recovery, and maintenance of neuropathic responses to external stimuli. Thus, when a stimulus occurs, voltage-gated sodium and calcium

channels open and allow positively charged ions to rush into cells (i.e. depolarization) to create a nerve impulse that is transferred to the synaptic terminal and the release of neurotransmitters is initiated (Raz and Perouansky 2013). To repolarize the cells, potassium channels open to end action potentials and return to resting membrane potential (Raz and Perouansky 2013). The downregulation of potassium ion transport channels seen in this study could substantially delay or inhibit neurons from returning to resting potential, a mechanism commonly associated with neuron overexcitability and locomotor impairment of zebrafish (Issa et al. 2011; Chatterjee et al. 2021). In a study evaluating their role in mice brains, calcium:sodium antiporters were also shown to engage in spatial learning and memory consolidation (Molinaro et al. 2013), an interesting result which could be associated with exposure to low complexity environments. These findings are indicative of synaptic plasticity of *A. saxatilis* under warming conditions, with potential interplay of stress induced by habitat complexity changes.

In addition, cellular communications (i.e., G protein-coupled receptor kinase, cytokine activity, and energy production) were also downregulated. Cytokine activity is associated with immune response, brain development, motor activity, and behavioral responses such as motivation, anxiety, arousal, and alarm (Miller et al. 2013). Further research on the role of cytokine function in the central nervous system of fishes should be prioritized, as decreased expression of cytokine activity has only been evaluated in other tissues including the spleen and liver. In studies on largemouth bass (*Micropterus salmoides*), heat stress decreased gene expression of cytokine activity in the spleen and was positively correlated with risk of infection and persistence of pathogenic bacteria when compared to fish in control temperatures (Yang et al. 2022). Additionally, we found consistent downregulation of genes associated with DNA damage repair and regeneration of central nervous system tissue and neurons in heatwave groups

(*ctdsp2*, *hmgb1a*, *dazap2*, and *zgc*:*77056*). Taken together, these results could indicate that metabolic resources within the neural-immune network are inhibited, reducing innate immune response to infectious pathogens, behavioral cues, and brain development in juvenile fishes under thermal stress.

Interestingly, we found that downregulation of cytokine activity is paired with the downregulation of G protein-coupled receptor kinases, which has been linked to oxidative stress in human brains (Lombardi et al. 2002). These kinases play a key role in the responsiveness of G protein-coupled receptors that are responsible for signaling responses to neurotransmitters, neuropeptides, and other molecules in the brain (Lombardi et al. 2004). Downregulation of these receptor kinases supersensitizes the receptor sites, which could indicate a plastic response with a lower concentration of neurotransmitters and other signaling molecules. Based on these findings, we hypothesize that marine fishes could be eliciting a compensatory trade-off where neurotransmission activity decreases, while the receptor sites to initiate signal transduction to other organs may be hypersensitive to a lower concentration gradient of these molecules.

Pairwise comparisons of individuals exposed to acute warming showed downregulation of *smtla*. This gene codes for a growth hormone specific to teleost fishes and is involved with endocrine regulation (Kakizawa 2021). While this gene has been previously associated with sexual maturation and energy homeostasis of juvenile fishes (Herkenhoff et al. 2020; de Celis et al. 2003), it may also regulate hormones responsible for behavior such as locomotion, body color regulation, feeding, cognitive functions, and aggression (Fukamachi et al. 2004). Thus, it is possible that downregulation of this relevant gene could affect regulation of important molecular processes and behavior during marine heatwaves.

Effects of habitat complexity on fish brains:

Habitat structure can minimize effects of natural disturbances such as heatwaves on reef fish assemblages (Emslie et al. 2014). In this study, the results of the habitat complexity were not as clear as the effects of temperature on gene expression of the brain. However, even when there were no significant differences between the habitat complexity treatments, it is possible that habitat complexity is compounding the detrimental effects of warming.

Omnivorous and herbivorous fishes have been shown to thrive in rocky environments of low coral cover and complexity due to the abundant dietary resources of algal overgrowth following coral death (Pratchett et al. 2018). In a study evaluating habitat choice of black-axil chromis (*Chromis atripectoralis*), Nay et al. (2020) showed that damselfish strongly preferred complex habitats, even when the complex habitat had higher temperatures. However, the molecular implications of these stressors have not been evaluated, which leaves long-term implications of habitat choice unknown. Our results indicate there were nearly double the amount of DEGs in low complexity treatments than in high complexity treatment groups when evaluating thermal stress. Specifically, we found upregulation of *dnajb1b*, which is associated with eye lens development pathways (Kahn et al. 2016), in heatwave samples exposed to habitat loss. This could indicate plasticity in visual acuity in low complexity treatments, where previous studies in cichlids have suggested that fish living in high complexity environments have enhanced spatial memory and visual acuity (Dobberfuhl et al. 2005). There were also several GO categories associated with ion transmembrane transport in the low complexity groups, which could be associated with decreased synaptic function at elevated temperature, as previously reported for damselfishes (Bernal et al. 2022).

There were some potential caveats that could have also influenced the results of gene expression in our study. First, the artificial habitat used in this experiment may not reflect the density of natural three-dimensional structures *A. saxatilis* encounters in nature. With this in mind, future experiments with habitat complexity in captivity should have longer acclimation periods to the specific habitats, as well as increased density of the artificial habitats. Another factor that could have limited our results of gene expression is sequencing whole brains, rather than isolating regions of the brain associated with visual acuity and spatial learning (Shumway 2008; White and Brown 2015). For example, the evolution of cichlid fishes to environments with varying levels of complexity has been suggested to only influence the size of five of seven regions of brain morphometrics (Pollen et al. 2007). In addition, the optic tectum and hypothalamus brain size increase in low complexity environments, while high complexity increases the size of telencephalon of intertidal gobies (White and Brown 2015). Future studies could also include the evaluation of gene expression in different brain regions of known significance to spatial awareness. It is also possible that a molecular response to habitat complexity loss was masked by an overwhelming response to acute thermal stress, as this has been described as the dominant factor in other multi-stressor comparisons (Laubenstein et al. 2020; Allan et al. 2017; Miller et al. 2015).

Effects of temperature and habitat complexity on oxidative stress:

Oxidative stress occurs when there is a disturbance in the ratio of reactive oxygen species (ROS) to the antioxidants that regulate these free radicals (Harman 1956). As ROS increases and inflicts damage to biotic compounds, proteins are often vulnerable to oxidative damage including protein oxidation, DNA damage, and enzyme inactivation which can affect cellular integrity.

ROS lead to the formation of carbonyl groups to protein side chains, leading to the formation of a peptide which blocks the N-terminal amino acid. Because they are well conserved and easily detected, protein carbonyl formations provide insight to oxidative stress levels across tissues and taxa (Birnie-Gauvin et al. 2017). Increasing temperatures are known to promote oxidative damage, as an increase in metabolic demands increases the production of metabolic by-products including ROS (Madeira et al. 2013; Birnie-Gauvin et al. 2017).

The liver plays a central role in an organism's metabolic activity by regulating nutrient, carbohydrate, protein, and fat metabolism (Rui 2014). Our results indicate that increased temperature increases oxidative damage to proteins of *A. saxatilis* liver, which can lead to dysfunction of the hepatic system (Cichoż-Lach and Michalak 2014). This can weaken the liver's ability to detoxify blood after pollutant exposure (Mahboob 2013), reduce immune response (Sun et al. 2020), and reduce important biological processes of the liver (Rui 2014; Birnie-Gauvin et al. 2017). Gene expression results of multiple damselfish species' livers from previous studies have indicated strong molecular responses to oxidative stress (Bernal et al. 2020), potentially mitigating oxidative damage by free radicals. Coupled with findings of increased oxidative damage in this study, we conclude that acute exposure to high temperatures may trigger physiological strategies for metabolic adjustments that allow fish to adapt to a rapidly changing environment.

Protein carbonyls were undetectable in muscle samples of the same protein concentrations. Tissue-specific responses are dependent on their physiological function and their antioxidant capacity. For example, liver, muscle, and gill all showed significant differences in oxidative stress biomarkers in *Gobius paganellus* exposed to thermal increases, but muscle consistently presented the lowest concentrations of biomarkers (Vinagre et al. 2014). This result

is consistent with our finding of undetectable concentrations of protein carbonyls in muscle tissue and strong signals of oxidative damage in the liver with thermal stress. However, we did detect traceable amounts of carbonyls in the muscle of control temperature samples exposed to habitat loss. Decreases in habitat complexity can be correlated with increased motor activity of damselfishes (da Silva-Pinto et al. 2020), which increases metabolic and energy demands of muscle tissue in fish (Martinez et al. 2003; Drumond and Black 1960). Although these results cannot be conclusive without direct comparison to our other treatment groups, these results suggest that oxidative stress levels may be higher in muscle of marine fishes in low complexity environments.

Chapter 2

Developmental exposure to degraded habitat induces plasticity in boldness and aggression of a coral reef fish

Introduction

Coral reefs are some of the most complex and diverse ecosystems in the world (Jackson 1991). The three-dimensional complexity of reefs provides several services including: habitat and refugia for thousands of marine species; protection of coasts against hurricanes, cyclones, and tsunamis; protection from anthropogenic nutrient run-off; regulation of water flow, light, and other abiotic factors (Graham and Nash 2013; Moberg and Folke 1999). This essential ecosystem, however, is currently being threatened by ocean warming, as a direct result of anthropogenic climate change (Crabbe 2008). Extended periods of warming can lead to coral bleaching, and if conditions are not restored to average temperatures, there will be massive coral die-offs, which can lead to irreversible damage to these hyper-diverse systems (Hughes et al. 2018). Once corals die, wave action, currents and erosion caused by marine organisms will eventually lead to a simplification of the habitat, transforming the ecosystem and directly impacting the services provided (Eakin et al. 2019). Evaluating how organisms associated with coral reefs are impacted by the transition from complex three-dimensional structures to barren sites is essential to predict the consequences of ocean warming.

One of the main consequences after a massive coral die-off is a change in reef fish assemblages (Pratchett et al. 2018; Krajewski and Floeter 2011). In turn, a decline of coral reef fish populations can limit the services they provide to the reef such as: algal grazing, predatory influence on the dynamics of benthic invertebrate communities, and nutrient cycling (Jones 1988; Garcia-Herrera et al. 2017; Li et al. 2018; Van Doan et al. 2019; Gupstra et al. 2021;

Gupstra et al. 2023). The effects can also be different based on the diet of specific species: fishes that are obligate coral-feeders have consistently declined in abundance following bleaching events, but long-term patterns in herbivorous and omnivorous fishes are less clear (Komyakova et al. 2013; Pratchett et al. 2018). These groups benefit from increased algal productivity following coral mortality, but the benefits may be offset by their dependence on reef structure for recruitment of juvenile fishes, refuge from predation, and foraging (Johnson 2007; Chivers et al. 2016). Overall, the transformation of the ecosystem can lead to broad changes in biodiversity and community dynamics, where effects on reef fishes are dependent on their diet and ability to acclimate/adapt to a changing habitat (Komyakova et al. 2013).

At the individual level, long-term survival and fitness on a degraded reef is dependent on several factors, including behavioral plasticity. For example, low complexity environments have been linked to increased predator-prey interactions and high mortality rates in juvenile recruits post-settlement (Almany 2004; Scharf et al. 2006). Further, previous studies in healthy reef systems of the Great Barrier Reef show that increased habitat structure allows fish to avoid risk-taking behaviors, whereas fish in low complexity environments show increases in foraging, aggression, boldness, and overall activity levels (McCormick and Lonnstedt 2013; Pratchett et al. 2018). A meta-analysis on the effects of habitat simplification in terrestrial and marine organisms suggests that territorial species in low complexity environments have increased capacity for detecting competitors and predators, decreasing the cost of defense, and leading to larger territory sizes per individual (Eason and Stamps 1992; Church et al. 2022). However, questions remain on the generality of behavioral shifts during habitat transitions for marine fishes, and the time scale at which these changes can operate.

The sergeant major, *Abudefduf saxatilis*, is a schooling damselfish that inhabits coral reefs, rocky habitats, and seagrass beds throughout the Western Atlantic, from Rhode Island to Uruguay (Froese and Pauly 2011). This species plays a key role in benthic community structures as they have a broad omnivorous diet of algae and small invertebrates, are highly territorial, show great site fidelity, and exhibit demersal spawning where males guard nests and provide care for egg clutches (Foster 1987; Fishelson 1970). In this species, the larval dispersal stage (17-20 days; Wellington and Victor, 1989) allows high genetic connectivity between populations throughout the species' range (Piñeros et al. 2017). They have also shown great plasticity in morphology based on geography and diet (Piñeros et al. 2015). They are abundant throughout the Western Atlantic and successfully occupy a wide range of habitats, from pristine coral reef ecosystems to degraded habitats, allowing us to explore potential changes in behavior in response to changes in habitat complexity. Here, we aim to evaluate how developmental exposure (~3 months) to a barren habitat will affect the aggression and boldness of juvenile *A. saxatilis*. We hypothesize that low complexity fish will display higher levels of aggression and increased boldness when compared to the high complexity groups. This experimental approach allows us to understand how tropical marine species will respond to the transition of coral reef ecosystems from complex to simplified environments in the coming decades.

Methods

Fish collection and experimental exposures:

Experiments were conducted on 48 juvenile *A. saxatilis* collected in Marathon, Florida in October of 2022 (Dynasty Marine Associates Inc., Marathon, FL). Fish were treated with copper at 0.15 ppm and nitrofurazone as an antimicrobial before being shipped overnight to Auburn University. Upon arrival, fish were acclimated to aquaria with a temperature of 24°C and a salinity of 28 ppt for 2 weeks to represent the seasonal averages (October-January) of their collection location (temperature data based on National Buoy Data Center). Throughout the acclimation period and experimental exposure, fish were fed tropical fish flakes (Ocean Nutrition Formula One Flakes) twice daily with a 12:12 hour day:night photoperiod using LED light strips. The fish were housed individually in 30-gallon chambers of a recirculating aquarium system (RAS). There were six total independent RAS towers with two 120-gallon tanks per system divided in 4 subsections of 30 gallons each, which allowed us to house 8 individuals per system.

In each RAS, half of the samples were assigned to low complexity (LC; n=24, 4 per RAS) and half were assigned to high complexity (HC; n=24, 4 per RAS) enclosures. LC tanks were completely barren (n = 24), while HC tanks (n = 23) contained a 4-inch PVC pipe, two terracotta pots (6-inch and 4-inch diameters) glued to a 12-inch ceramic tile for additional shelter, one 12-inch artificial aquarium plant, one 18-inch artificial aquarium plant, and a black paper covering the lower front of the tank to limit visual stimulus by humans at the time of feeding or cleaning (Figure 1). Differences in sample sizes were due to one fish death in HC during the exposure period. All fish were held in these conditions at control temperatures (24°C) for 11 weeks before behavioral assays.

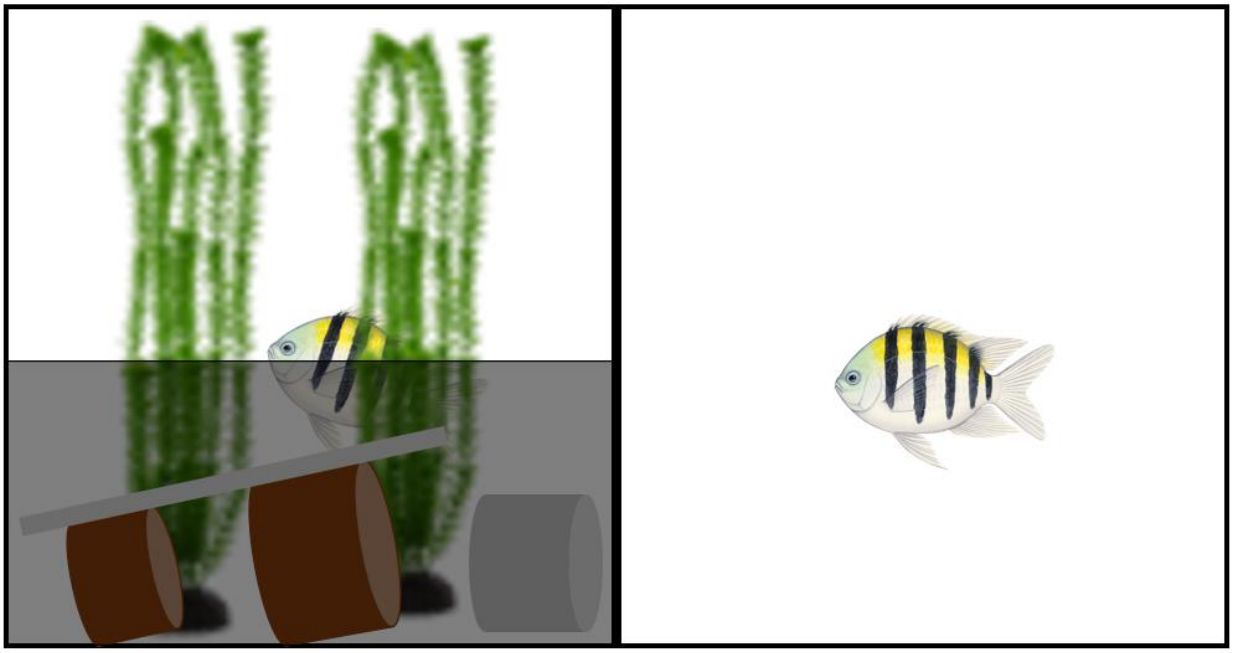

Figure 1. Habitat exposure design: high complexity (HC; n = 23) and low complexity (LC; n = 24).

Behavioral assay conditions:

Behavioral assays were conducted within a 3-hour period (between 9:00 and 12:00 CST), to avoid any diurnal behavioral differences in measurements. Only 8 fish were tested per day during the 3-hour window: 2 individuals exposed to HC and 2 individuals exposed to LC samples from one RAS system, and the same combination from another RAS tower. This allowed evaluating two RAS towers (i.e., 8 individuals each, 16 individuals total) over a period of two days.

Fish were moved to assay tanks at 17:00 the evening prior to testing to allow the fish to acclimate and establish territory in the arena for ~14 hours prior to testing. A plastic mesh lid was placed on the tank to prevent animals from escaping overnight. Test tanks had a swimming

area of (20x15x15cm) with adequate oxygenation of water with a pump, LED lighting under the same photoperiod as their home tanks, and water temperatures maintained with a heater (24°C). An acrylic divider was placed in the tank to prevent fish from utilizing the heater and pump as shelter during the assay. The tanks were placed on top of a grid that was divided into 4, 5 cm zones. The sides of the tank were covered by black corrugated plastic sheets to prevent the fish from recognizing their reflection while acclimating. A digital video camera (Canon G7x) was situated with a tripod ~80 cm above the testing arena to record two trials simultaneously.

After behavioral trials were completed, fish were moved back to their home tanks for 7 days, and then euthanized by transection of the spinal cord with dissection scissors. All fish were weighed and measured for standard and total length, and then whole brains were dissected and flash frozen in liquid nitrogen.

Pretrial Period

Prior to the initiation of behavioral stimuli, the mesh tank lid was removed and a 5-minute recording was made to analyze baseline activity levels. This pretrial period ensured that behaviors recorded during the aggression and boldness trials were not spontaneously performed in unstimulated contexts.

Aggression assay

Aggression in fishes is necessary to acquire and defend territory, monopolize resources, and successfully reproduce (Conrad et al. 2011). This behavior can be highly plastic in variable environmental conditions (Bhat et al. 2015). To assess aggression as a response to habitat complexity, we conducted a mirror aggression test (Figure 2). This assay has been reliably used

to quantify aggression across several fish genera (Baran and Streelman 2020; Oliveira et al. 2022; Silva-Pinto et al. 2020; Eriksson et al. 2010). For this a mirror was placed at one end of the tank, and the corrugated plastic sheet was removed to allow visual access to the fish's reflection. A 15-minute recording was collected, but to minimize the effect of disturbances when moving the mirror and corrugated plastic sheet only behaviors in the last 10 minutes of recordings were scored (i.e., 5-minute acclimation period after the barrier was removed). Variables collected included the number of bites the fish made against the mirror, the number of lateral displays, the number of frontal displays (defined as frontal contact with the mirror while flicking the tail with dorsal fins erect), amount of time the fish was active away from the mirror, the amount of time the fish spent inactive in the 5 cm zone in front of the mirror, and the time spent active within 5 cm of the mirror. An individual was scored as within a zone based on the location of their eyes.

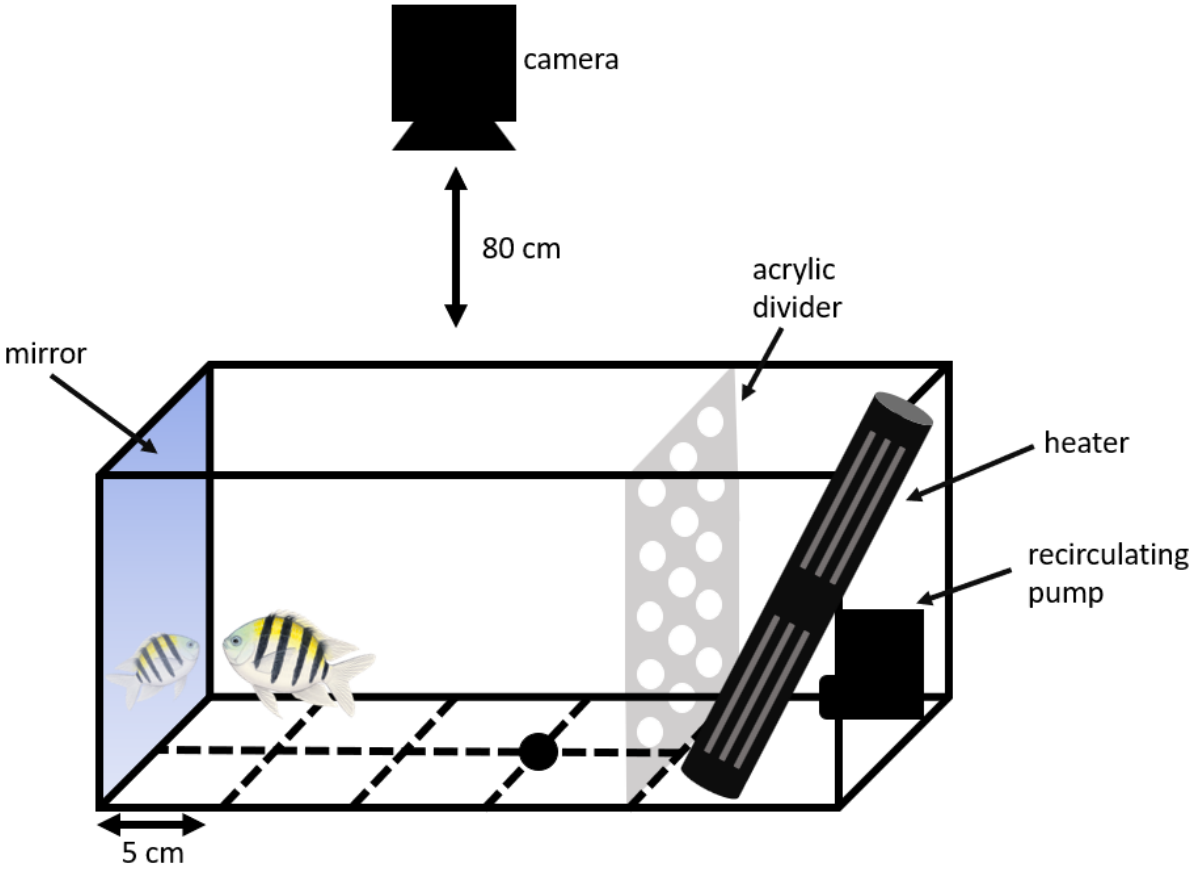

Figure 2. Mirror assay to assess aggression in *A. saxatilis*. Zone One is represented by the 5 cm zone in front of the mirror. An acrylic divider was used to ensure fish were unable to hide behind the heater and recirculating pump during trials.

Boldness assay

Boldness is a large determinant for survival as variation in this trait influences everyday ecological challenges in fishes including competition for resources, foraging under predation pressure, and habitat selection (Church et al. 2022; Magnhagen and Borcherding 2008). To assess *A. saxatilis* boldness with habitat complexity loss, we conducted a novel object test (Figure 3). This assay has been used to quantify boldness across several fish species (Bensky and Bell 2022; Frost et al. 2006; Hamilton et al. 2021; Swank et al. 2021). Immediately following the aggression assay, the corrugated plastic sheet was replaced to block the mirror used in the aggression assay. The assay tank was divided into two 10 cm zones, one containing a novel object placed in the center of the zone ('novel object zone') and the other zone undisturbed ('outside of novel object zone'). An acrylic model of a starfish (5.5x2.5cm, Petco) with pink and green colors was used as a novel object, placed 5 cm from the end opposite of the covered mirror. A 15-minute recording began immediately after the placement of the novel object. Only behaviors in the last 10 minutes were scored to allow the fish a 5-minute acclimation period after the novel object was placed. Variables collected included the time spent active and inactive in each zone.

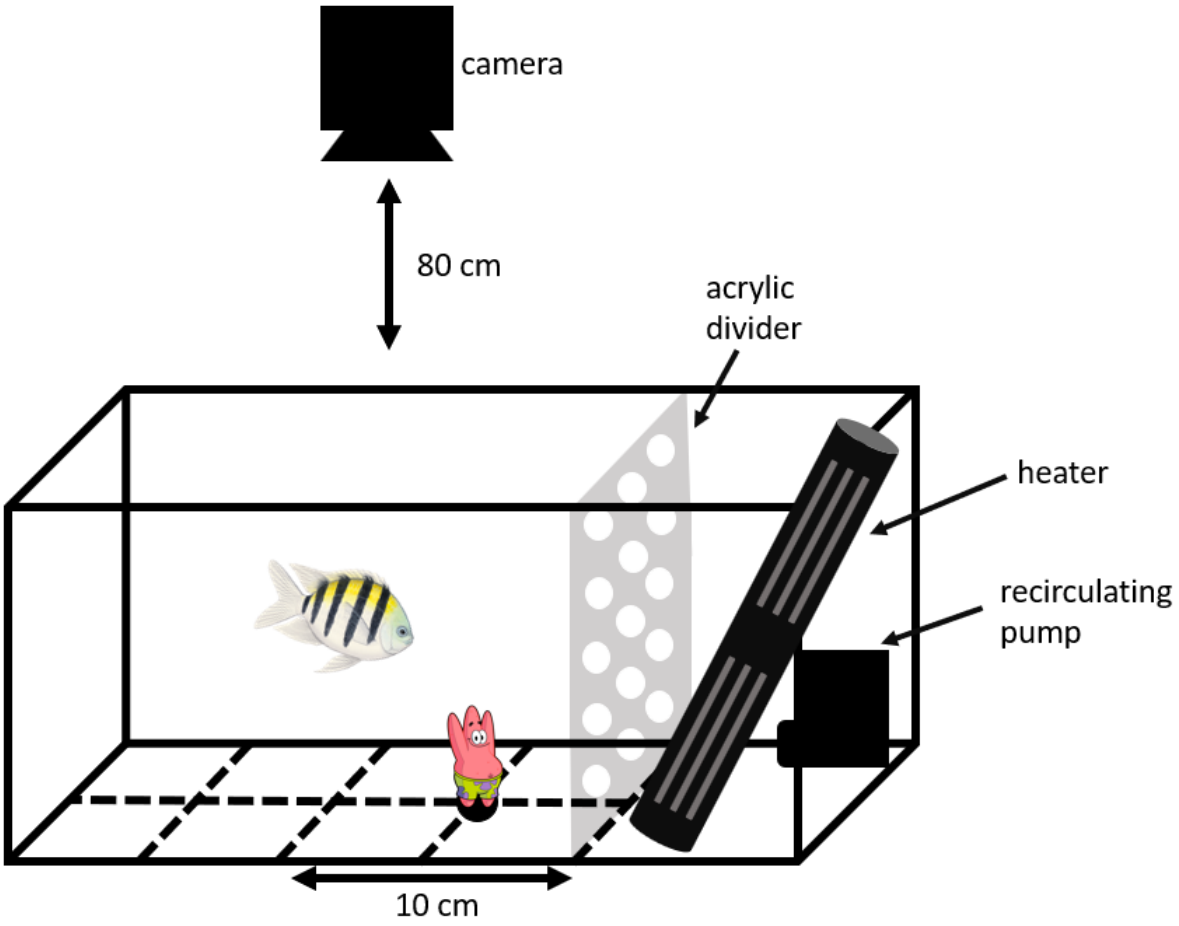

Figure 3. Novel object assay to assess boldness in *A. saxatilis*. The novel object zone is represented by the 10 cm zone containing the novel object. An acrylic divider was used to ensure fish were unable to hide behind the heater and recirculating pump during trials.

Statistical analyses

Raw data failed normality assumptions using a Shapiro-Wilk's normality test (Shapiro and Wilk 1965). All variables were tested using a generalized linear mixed-effects model (*glmm*) with a poisson distribution. The *glmm* is robust to non-normal distributions of data and zero-inflated data (Stroup 2015). For the model, habitat complexity and weight were set as nested fixed effects, and RAS and Observer as random effects [Eg. glmer(Bite ~ Habitat + Weight +

(1|RAS/Observer), family = "poisson", data = data)]. To control for effects of multiple comparisons, a Bonferroni correction was used. Prior to running statistics on variables of interest, we used a *glmm* to evaluate differences in behavioral responses across the 3-hour daily testing period and found no significant effect with time of day. A qqplot was used to assess normality of residuals for each model by ensuring residuals followed a linear pattern on the plot. All statistical tests and figures were completed using RStudio version 2022.12.0+353 (RStudio Team 2019).

Results

Pretrial Period

Prior to stimulus introduction, activity within the novel object zone was not significant between treatment groups ($p = 0.43$), but LC fish spent more time inactive ($p < 0.001$). Outside of the novel object zone, LC fish were more active ($p < 0.001$), and HC fish spent more time inactive ($p < 0.001$). LC fish spent more time active overall prior to receiving any stimulus ($p < 0.001$). No aggressive displays were recorded in any treatment groups during this time.

Aggression Assay

The aggression assays showed that LC fish had significantly more bites ($p < 0.001$; Figure 4E), performed more lateral displays ($p < 0.001$; Figure 4I), and more frontal displays ($p < 0.001$; Figure 4G) than HC fish. However, the number of attacks to the mirror was not significantly different between the two treatments ($p = 0.174$). The amount of time spent active within 5 cm of the mirror was not different between treatment groups ($p = 0.08$), but the amount of time spent inactive within 5 cm of the mirror was higher in HC groups ($p < 0.001$; Figure 4C). Meanwhile, the amount of time spent active outside of the 5cm zone was higher in LC fish ($p < 0.001$; Figure 4A). There was also a trend by size, where larger fish spent more time active in front of the mirror ($p < 0.001$) and more time active overall ($p < 0.001$; Figure 4B), while smaller fish spent more time inactive within 5 cm of the mirror than larger fish ($p < 0.001$; Figure 4D). Heavier fish also performed more bites ($p < 0.001$; Figure 4F), less lateral displays ($p = 0.01$; Figure 4J), and more frontal displays ($p < 0.001$; Figure 4H). Importantly, there was no significant effect of testing time in aggression (within the 3-hour margin of evaluations; $p > 0.05$). Presence or absence of aggressive behavior throughout the entire assay was not different

between treatment groups ($p = 0.7$) or fish weight ($p = 0.99$). This indicates that the magnitude of aggression differences between treatment groups is an effect of habitat complexity exposure, not zero-inflation driven by individual differences.

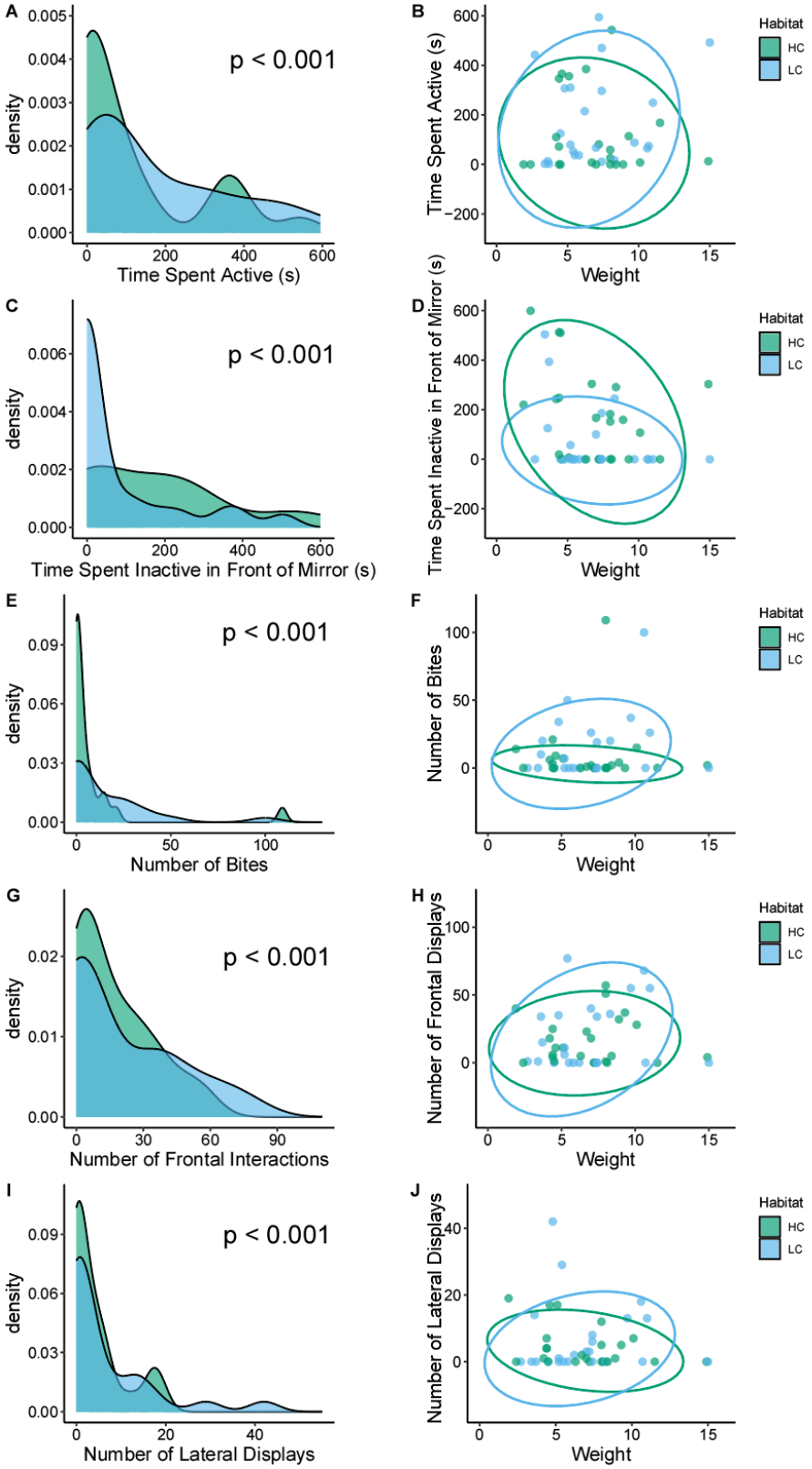
A
p < 0.001
density
Time Spent Active (s)
B
Time Spent Active (s)
Weight
Habitat
HC
LC
C
p < 0.001
density
Time Spent Inactive in Front of Mirror (s)
D
Time Spent Inactive in Front of Mirror (s)
Weight
Habitat
HC
LC
E
p < 0.001
density
Number of Bites
F
Number of Bites
Weight
Habitat
HC
LC
G
p < 0.001
density
Number of Frontal Interactions
H
Number of Frontal Displays
Weight
Habitat
HC
LC
I
p < 0.001
density
Number of Lateral Displays
J
Number of Lateral Displays
Weight
Habitat
HC
LC

Figure 4. Density distribution plots for time spent active (A,C) and number of aggressive displays (E,G,I). Scatterplots for the time spent active (B,D) and number of aggressive displays (F,H,J) distributed by weight on the x-axis.

Boldness Assay

LC fish spent more time investigating the novel object than the HC group ($p < 0.001$; Figure 5A). LC fish also spent more time active outside of the novel object zone ($p < 0.001$; Figure 5E). The amount of time spent inactive in the novel object zone was not significantly different between groups ($p = 0.25$; Figure 5C), but HC fish spent more time inactive outside of the novel object zone ($p < 0.001$; Figure 5G). There were no bites, lateral displays, or attacks towards the novel object recorded in either treatment group. Smaller fish spent more time interacting with the novel object ($p < 0.001$; Figure 5B and 5D). Outside of the novel object's zone, larger fish were more active ($p < 0.001$; Figure 5F), but smaller fish spent more time inactive ($p < 0.001$; Figure 5H). These trends are opposite of those found in the baseline activity of fish prior to stimulus introduction, demonstrating that our assay efficiently elicited bold behaviors.

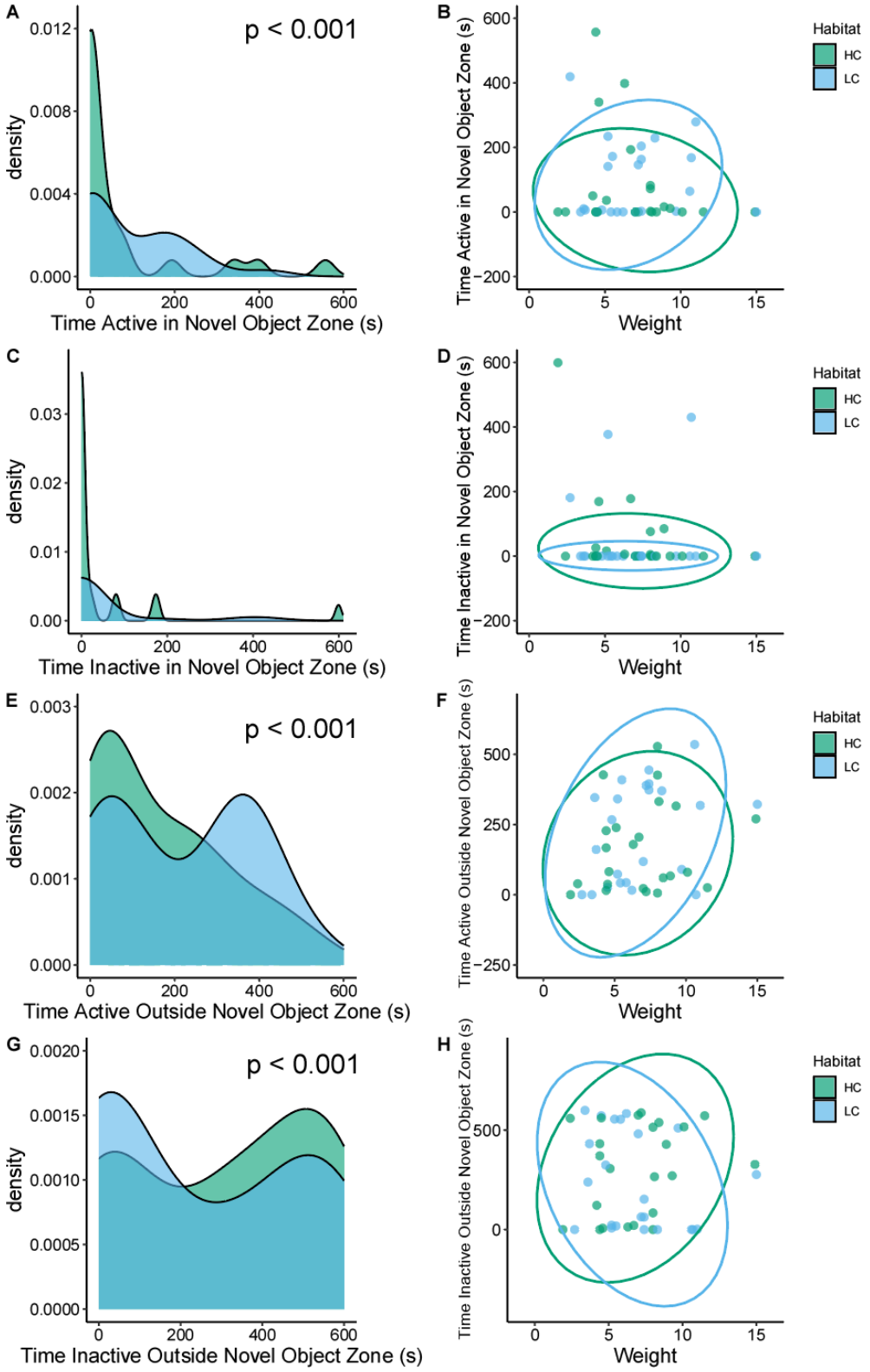
A
p < 0.001
density
Time Active in Novel Object Zone (s)
B
Time Active in Novel Object Zone (s)
Weight
Habitat
HC
LC
C
Time Inactive in Novel Object Zone (s)
D
Time Inactive in Novel Object Zone (s)
E
Time Active Outside Novel Object Zone (s)
F
Time Active Outside Novel Object Zone (s)
G
Time Inactive Outside Novel Object Zone (s)
H
Time Inactive Outside Novel Object Zone (s)

Figure 5. Density distribution plots for time spent active (A,E) and time spent inactive (C,G) in each zone. Scatterplots for the time spent active (B,F) and time spent inactive (D,H) in each zone distributed by weight on the x-axis.

Discussion

Ocean warming is accelerating mass coral die-offs worldwide, decreasing habitat availability and species richness on reef ecosystems (Graham and Nash 2013). Early exposure to differing habitats will influence behavioral plasticity of marine fish communities in the future (Magurran and Higham 1988). Here, we show that a wide scope of behavioral plasticity exists in juvenile *A. saxatilis* where developmental exposure to decreased habitat complexity results in increased aggression and increased boldness, for individuals from the same population of origin. The capacity of this territorial species to modify their behavior in an environment with heightened competition for resources may provide insight to their ability to acclimate to a wide variety of habitats across the Western Atlantic. Responses of *A. saxatilis* to habitat modifications can help biologists predict responses of other fishes with similar functional roles (i.e., territorial, omnivorous, demersal egg layers) and at a similar developmental stage (i.e. juvenile) in reef communities.

Our results show that developmental exposure to low habitat complexity increases aggression in *A. saxatilis* across several metrics, including those with direct contact (biting and frontal displays) and in a non-contact display of aggression (lateral displays). Loss of habitat complexity leads to an increase in the frequency of interactions between competitors and increases competition for resources (Boström-Einarsson et al. 2013), and organisms often use aggression to compete for territories, food, and refuge from predators (Pratchet et al. 2018; Carr et al. 2002). The plasticity of *A. saxatilis* aggression in degraded habitats may allow them to obtain better territories with access to more resources while pushing less aggressive individuals to less suitable habitat (i.e., reef's edge; McCormick 2009).

Interestingly, larger fish displayed more contact aggression while smaller fish displayed more aggression via lateral displays. This finding is consistent with studies in freshwater and marine systems that have shown larger, more aggressive fish have higher settlement and survival rates in less complex systems (Church and Grant 2018; McCormick et al. 2016). Meanwhile, studies in the Great Barrier Reef have shown that adaptation to degraded environments over multiple generations results in less propensity of smaller fish to attack larger conspecifics (McCormick 2009; Webster and Hixon 2000). While the aforementioned studies have shown long-term behavioral shifts of juveniles settling on degraded reefs, it is possible that the plasticity shown in the present study could lead to advantageous genetic changes and behavioral shifts in future generations (Fox et al. 2019).

In terms of boldness, it is hypothesized that fishes with long-distance dispersal and the capacity to occupy novel habitats show increased boldness (Fraser et al. 2001). Coral reef fishes that lack a bold behavioral phenotype are more closely associated with their territory and have lower mortality rates than risk-taking individuals (McCormick et al. 2016). Therefore, bolder individuals increase the risk of mortality (Blake et al. 2018), but at the same time, increase opportunity for food acquisition and increased territory size (Church et al. 2022; Wilson et al. 1993). Thus, plasticity in bold behaviors and appropriate decision-making under rapid environmental changes is expected to enhance colonization success by coral reef fishes in less suitable habitats (Rehage et al. 2005; Blake et al. 2018). Here we show that habitat complexity loss increases boldness in *A. saxatilis*, which could be a critical contribution to the persistence of this species in a wide range of habitats.

Interestingly, we found that smaller fish were bolder than larger fish, where larger fish tended to inspect the novel object from further away. This finding is consistent with patterns in

previous studies with *Brachyraphis episcopi* (Brown and Braithwaite 2004), suggesting that smaller fish have lower fat reserves and faster metabolic rates which compels them to investigate novel objects as a food source (i.e., metabolic hypothesis; Brown and Braithwaite 2004; Krause et al. 1998). However, other studies have suggested that sex is a greater influence than size in individual boldness (Ingley et al. 2014), and sex was not a factor controlled for in this study due to sexual immaturity of our samples.

These results support the well-established, positive relationship of habitat exploration and individual boldness (Sih and Del Guidice 2012) and the hypothesis that increased habitat complexity decreases risk-taking behaviors (Church et al. 2022; McCormick et al. 2016). *Abudefduf saxatilis* is a generalist species with great phenotypic plasticity that allows them to inhabit a broad range of habitats, both geographically and in terms of habitat type (Piñeros and Gutiérrez-Rodríguez 2017). For many specialist species, such as corallivores, transitions in habitat complexity could lead to local extirpation of populations, which could translate to extinctions in species with restricted geographic ranges. While our study highlights the behavioral plasticity of damselfish juveniles from the same population, additional research should evaluate the population-level effects of habitat complexity loss on long-term changes in development, growth rates, body conditioning, genetics, diet, and fecundity over several generations. In addition, it is key to disentangle the influence of plasticity from that of adaptation, as this could help understand short- vs long-term responses of reef fish communities in global change scenarios.

Conclusions

The studies presented here have revealed that exposure to habitat loss and thermal stress can have a direct influence on brain function, oxidative stress, and behavior in a coral reef fish. Plasticity of juvenile fishes in response to environmental changes may be linked to adaptive response for survival during key life stages between egg fertilization, pelagic stages, and settlement. Thus, the ability of juvenile fishes to rapidly acclimate provides a fitness advantage when settling on habitats of different quality, after the larval dispersal stage. Settlement success can influence population dynamics and connectivity, especially in species such as *A. saxatilis* that show high site fidelity post-settlement.

In chapter 1, I showed that acute exposure to ecologically relevant heatwave temperatures influenced gene expression of the brain and oxidative damage in the liver, where fishes from a low complexity habitat showed a stronger thermal stress response than those in a high complexity habitat. As the main sensory organ, it is surprising to encounter how many gaps in knowledge we have for the molecular responses of the brain to environmental stress. The experiments in this chapter indicated that elevated temperature leads to downregulation of genes associated with DNA damage repair, regeneration of central nervous system tissue, and growth hormone regulation. Further, downregulation of functional categories that suggest impairment to immune function and neurotransmission were observed. Heatwave exposed individuals also show a trend of oxidative damage in the liver. While this study has provided a broad view of synergistic effects of habitat loss and thermal stress, future studies could focus on evaluating gene expression in specific regions of the brain, as there could be additional differences among regions related with spatial awareness, learning, and behavior. Overall, these results highlight the energetic trade-offs, physiological consequences, and molecular mechanisms that damselfish

undergo when under thermal stress and can be informative on how damselfishes may respond to predicted global change scenarios in coming decades.

In chapter 2, an aquarium experiment showed that developmental exposure to habitat loss increases boldness, aggression, and activity in *A. saxatilis*. The experiment also indicated that larger fish were more aggressive, while smaller fish were bolder in a novel object test. The plasticity of juvenile behavioral phenotypes undergoing rapid habitat shifts could play a role in long-term adaptations of future generations exposed to transformed habitats. Future studies evaluating effects of habitat degradation on coral reef fishes are necessary to understand differences in behavior between developmental and transgenerational exposure, changes in growth rates, body conditioning, and ultimately effects on fecundity as habitats shift. In addition, questions remain on how changes in complexity differ in fishes across functional roles in the community (i.e., generalists/specialists, territorial/non-territorial, diet type, reproductive life histories, etc.), as there could be relevant patterns among different ecological guilds.

Taken together, the experiments conducted during my thesis address gaps in our understanding of how coral reef fishes are responding to both increases in global oceanic temperatures and habitat loss as a consequence of coral reef degradation. Many of the results presented here coincide with observations on coral reef fishes in the field, highlighting the relevance of controlled aquarium experiments for addressing these questions. Based on these results, we expect that as temperatures increase and habitats become simplified, coral reef fishes will experience challenges in metabolic and immune function and oxidative stress, while increasing their boldness and aggression in the environment. These plastic responses could lead to advantageous genetic changes and behavioral shifts in future generations that allow damselfish populations to persist in a changing planet.

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