INTERACTIONS AMONG CORAL REEF HABITAT AND THE BEHAVIOR AND STRESS PHYSIOLOGY OF BICOLOR DAMSELFISH (*STEGASTES PARTITUS*)

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS v

DEDICATION vii

LIST OF TABLES viii

LIST OF FIGURE ix

CHAPTER 1: SPATIAL PATTERNS OF INTRASPECIFIC BEHAVIORAL VARIATION IN THE DEMERSAL FISH *STEGASTES PARTITUS* ASSOCIATE WITH PHYSICAL AND SOCIAL ENVIRONMENTAL VARIATION ON A CORAL REEF 1

SUMMARY 2

INTRODUCTION 4

MATERIALS AND METHODS 8

Study site 8

Fish behavior assessments 10

Assessment of social and physical habitats 11

Statistical analyses 12

RESULTS 18

Relationships among physical and social habitat conditions 18

Variation in bicolor damselfish behavior 23

Relationships between intraspecific variation in behavior and habitat 27

Path analysis of relationships between behaviors and environmental variation 29

DISCUSSION 35

Individual variation in relationships between behaviors in bicolor damselfish 37

Interacting influences of physical and social habitat conditions on damselfish demography and behavior 39
Spatial variation in damselfish behavior associates with physical reef conditions 44
Summary 46
REFERENCES 48
CHAPTER 2: EVIDENCE FOR HABITAT-ASSOCIATED INTRASPECIFIC VARIATION IN THE STRESS PHYSIOLOGY OF A CORAL REEF FISH, *STEGASTES PARTITUS* 57
ABSTRACT 58
INTRODUCTION 60
MATERIALS AND METHODS 64
Identification of stress-associated cDNAs from bicolor damselfish 64
Comparison of stress reactivity in bicolor damselfish from different reef habitats 68
Statistical analyses 75
RESULTS 76
Bicolor damselfish behaviors in rubble vs. reef habitats 76
Comparison of stress-associated mRNA responses in fish from rubble vs. reef habitats 78
DISCUSSION 83
Stress hormones and the environment 83
Responses of neural mRNAs to acute stress 87
Conclusions 93

REFERENCES 94

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DEDICATION

I dedicate this manuscript to my parents, Bryan and Gloria, who taught me how to be me. You encouraged me to follow my dreams . . . and you would not let anything stand in my way. I love you.

LIST OF TABLES

CHAPTER 1: Page

1. Summary of model fit parameters of all possible path analysis models for large and small bicolor damselfish 17

2. Factor loadings for the Principal Components Analysis performed on the five measured physical habitat variables 19

3. Relationships between bicolor damselfish behaviors and social environmental conditions.....28

CHAPTER 2:

1. Nucleotide sequences for degenerate primers used for isolation of partial cDNAs 66

2. Nucleotide sequences for primers used in quantitative real time RT-PCR 73

LIST OF FIGURES

CHAPTER 1: Page

1. Map of Curaçao, the Netherlands Antilles, in the southern Caribbean Sea, with the three sampling sites indicated 9

2. Principal Components Analysis (PCA) for the five habitat variables. Original output is in the upper right corner....20

3. Relationships of bicolor damselfish densities and physical habitat PC1 for a) total bicolor damselfish density, b) small fish only, and c) large bicolor damselfish only 22

4. Relationship between coral reef fish diversity and a) habitat PC1 and b) habitat PC2 24

5. Relationships of bicolor damselfish densities and physical habitat PC2 for a) total bicolor damselfish density, b) small fish only, and c) large bicolor damselfish only 25

6. Behavioral variation between large (>4 cm, TL) and small (<4cm, TL) bicolor damselfish for a) aggression, b) shelter use, and c) courtship dips 26

7. Relationships between behavioral frequencies and physical habitat PC1 for large bicolor damselfish a) aggression, b) shelter use 30

8. Path analysis model for large bicolor damselfish 32

9. Path analysis model for small bicolor damselfish....34

CHAPTER 2:

1. Map showing the location of the Playa Kalki fringing reef sampling site on the leeward side of Curaçao, the Netherlands Antilles in the southern Caribbean Sea..69

2. Behavioral variation in adult bicolor damselfish (> 4 cm, TL) from rubble and reef habitats....77

3. Real time quantitative RT-PCR comparison of CRH and CRH-BP mRNA levels in the brain of bicolor damselfish from two different habitats and stress levels 79

4. Real time quantitative RT-PCR comparison of CRH-R1 and CRH-R2 mRNA levels in the brain of bicolor damselfish from two different habitats and stress conditions....81

5. Real time quantitative RT-PCR comparison of urotensin 1 mRNA levels in the brain of bicolor damselfish 82

CHAPTER 1

SPATIAL PATTERNS OF INTRASPECIFIC BEHAVIORAL VARIATION IN THE DEMERSAL FISH *STEGASTES PARTITUS* ASSOCIATE WITH PHYSICAL AND SOCIAL ENVIRONMENTAL VARIATION ON A CORAL REEF

This chapter has been prepared in the style of the Journal of Animal Ecology

SUMMARY

1. Many animals exhibit behavioral variation across environmental gradients, but often it is unclear how abiotic and biotic environmental conditions interact to shape these geographic patterns of behavioral variation.
2. This study identifies spatial patterns of intraspecific behavioral variation in the bicolor damselfish (*Stegastes partitus*) across a range of coral reef habitats and examines how this behavioral variation associates with environmental conditions. Specifically, we characterized the behavior of bicolor damselfish across the fringing coral reefs of Curaçao, the Netherlands Antilles to determine how behavioral variation related to physical (e.g., hole number, hole size, rugosity, coral cover %, depth) and social (e.g., conspecific density, species diversity) conditions.
3. Principal Components Analysis (PCA) reduced the physical habitat variables to two independent components. Principal Component 1 (PC1) included the habitat variables of depth, coral cover (%), rugosity, and average hole size (cm^2), and increased along the reef slope as the habitat transitioned from rubble to live coral. The number of holes loaded most strongly onto Principal Component 2 (PC2), which did not show a predictable pattern across the transition from rubble to reef slope.
4. Increased values of PC1 were associated with reduced densities of bicolor damselfish – but increased fish species diversity – as the habitat transitioned from rubble to reef.
5. Aggression, shelter use and courtship differed between large (> 4 cm, TL) or small (< 4 cm, TL) bicolor damselfish, with large fish showing higher levels of aggression, using shelter more frequently, and courting at higher rates. Spatial variation in the behavior of

large (>4 cm, TL) bicolor damselfish was associated with PC1, with fish behaving more aggressively and using shelters more often in shallow, rubble habitats on the coral reef.

6. Interrelationships between environmental and behavioral variables were examined using path analysis, which identified robust associations between physical habitat characteristics and fish behavior. This finding points to the importance of physical habitat variation for shaping spatial patterns of behavioral variation in some coral reef fishes, and suggests that this tight coupling between behavior and physical habitat may lead to altered behaviors in areas affected by coral reef habitat degradation.

INTRODUCTION

Environmental variation holds a central role in determining the distribution and diversity of species. For any given taxon, the number and type of environmental variables that govern its distribution can range widely, but commonly include both abiotic parameters (e.g., temperature, salinity, wave energy, substratum shelter) and biotic variables such as food and social factors (e.g, predation risk, competition). Spatial heterogeneity in environmental conditions often equates to variation in habitat quality which ultimately leads to variation in the habitat use and distribution of species. Less commonly considered, however, is the role that this environmental variation plays in generating spatial variation in behavior within a species. Whether along environmental gradients or among populations, individuals that experience differing environmental conditions can vary in behavior as they cope with disparate abiotic and biotic challenges in their local habitats (Foster 1999).

Similar to other phenotypic traits, behavior can be strongly influenced by variation in physical and social environmental conditions. Such intraspecific behavioral variation can occur either through evolutionary divergence (Magurran et al. 1995) or via plastic changes in behavioral development and expression (Carroll & Corneli 1999; Ghalambor, Angeloni & Carroll 2010). With behavior responsive to such a multitude of environmental variables, it is difficult to determine how many facets of environmental variation interact to generate spatial variation in behavior in the wild (Foster & Endler 1999). While controlled laboratory studies show that specific variables ranging from temperature to water flow can be linked directly to changes in behavior (Vehanen et al. 2000; Lema 2006), geographic variation in behavior in the wild is likely shaped by the interacting influences of several environmental variables. In such

cases, relationships between behavioral variation and specific environmental variables may be informative for identifying the conditions responsible for shaping spatial variation in behavior in the wild. Field studies of geographic variation in behavior in terrestrial species have previously documented associations between behavioral variation and environmental conditions over large spatial scales (kilometers) (e.g., Boinski, 1999; Thompson 1990; Reichert 1974). Mechanisms generating patterns of behavioral variation over smaller spatial scales, however, have received considerably less attention, especially for marine taxa, even though a high degree of spatial heterogeneity that can generate variation in behaviors relevant to survival and reproduction is frequently observed in marine habitats.

Coral reef ecosystems are heterogeneous environments commonly having variation in both physical and social conditions over spatial scales on the order of meters. This variation makes coral reef ecosystems ideal for exploring how habitat heterogeneity contributes to variation in the distribution and behavior of fishes. Previous studies have found that the overall diversity and relative density of coral reef fishes is related to the structural complexity of the reef habitat (Holbrook, Forrester & Schmitt 2000; Holbrook, Brooks & Schmitt 2002; Roberts & Ormond 1987; Öhman & Rajasuriya 1998; Luckhurst & Luckhurst 1978; Chabanet et al. 1997; Nanami et al. 2005). Such relationships between fish diversity and reef habitat structure appear to be mediated by the interacting influences of predation pressure and competition for suitable shelter and feeding sites (Johnson 2007; Schmitt & Holbrook 2000). More recently, several studies have indicated that variation in fish growth and demography can be linked to coral reef habitat structure (Feary, McCromick & Jones 2009; Paddack, Sponaugle, & Cowen 2009; Afonso, Morato, & Santos 2008; Kingsford & Hughes 2005). Accordingly, spatial variation in fish demography appears to be associated with geographic variation in behavior. In the three-

spot damselfish (*Stegastes planifrons*), for instance, agonistic interactions between juvenile fish were more frequent on small lagoonal patch reefs where damselfish density was greater, compared to continuous back reef habitat (Levin et al. 2000). Taken together, findings from this and other studies suggest that physical and social environmental variation can interact to generate spatial variation in reef fish behavior, although the relative contributions of these environmental parameters or whether one is more influential than the other is debatable.

Among coral reef fishes, demersal species are well suited as models for investigating habitat effects on intraspecific behavioral variation. The bicolor damselfish (*Stegastes partitus*) is a small (<10 cm total length (TL)), demersal fish species common to coral reef environments in the Caribbean Sea. After settling from a planktonic larval life stage, bicolor damselfish exhibit high site fidelity, defending a small territory from settlement to adulthood (Booth & Hixon 1999). The distribution of bicolor damselfish is strongly influenced by the structural complexity and live coral cover of the local reef habitat (Booth & Beretta 2002; Holbrook, Brooks & Schmitt. 2002), with the greatest densities of these fish located in reef areas with high current flow (Nemeth 1997). Bicolor damselfish exhibit high site fidelity, but the type of reef microhabitats occupied by these fish is quite variable. Nemeth (1998) documented bicolor damselfish in habitats ranging from *Montrastea*-dominated reef to *Acropora* patches and even coral rubble. Within these habitats, however, bicolor damselfish have been shown to exhibit differences in juvenile growth and survival. Juveniles from the fore reef have greater growth rates than those from the back reef, and juveniles from boulder coral habitats have higher mortality than those from rubble habitats (Nemeth 1998, 2005; Figueira et al. 2008). Behavioral interactions among bicolor damselfish are readily observed, and may be important in determining settlement distributions, especially in preferred, high quality habitats. When

examining aggressive interactions between adult and juvenile damselfish, Harrington (1993) noted increased levels of aggression by adult bicolor damselfish that were dependent upon the size and species identity of the juvenile recruits. However, after repeated exposure to juveniles, Harrington (1995) also confirmed that habituation of aggression toward juveniles occurs in the bicolor damselfish. Social conditions as well as physical habitat characteristics (i.e. the structural complexity of the reef) affect the distribution of bicolor damselfish and their behavior; therefore, the bicolor damselfish provides a tractable model for examining the interrelationships between environmental variation – both physical structure and social conditions – and intraspecific behavioral variation.

This study examines the interacting influences of physical and social environmental variation on intraspecific variation in the behavior of bicolor damselfish on the fringing coral reefs of Curaçao, the Netherlands Antilles. Specifically, we quantified variation in the physical (e.g., hole number, rugosity, coral cover) and social (e.g., conspecific density, species diversity) conditions across the transition zone from coral rubble to the reef slope, and examined how patterns of behavior in the bicolor damselfish varied across a spatial gradient in physical and social conditions. This approach identified broad spatial patterns of intraspecific behavioral variation that associated with variation in key physical and social variables of the coral reef habitat. Moreover, examination of the interrelationships between these environmental and behavioral variables identified variation in physical habitat conditions – rather than social conditions – as having the strongest relationships with spatial variation in behavior in this species, suggesting that changes in the physical conditions of coral reefs may impact patterns of behavioral variation for the fishes that rely on these habitats.

MATERIALS AND METHODS

Study Site

Bicolor damselfish were studied on the fringing reefs of Curaçao, the Netherlands Antilles, in the southern Caribbean Sea from 16 to 31 May 2009. Three coral reefs along the southern, leeward side of the island were used as sampling sites: Playa Kalki, Playa Jeremi, and Daaibooibaai (Fig. 1). At each site, reef habitats varied in structure with dead coral rubble located inshore and live coral cover located near the reef crest and reef slope. The majority of live coral at these sites consisted of mixed *Montastrea* species, which is typical of leeward reefs in Curaçao and other islands of the southern Caribbean Sea (Bruckner & Bruckner 2003).

To assess how the behavior of bicolor damselfish varied relative to the physical and social characteristics of the reef habitat, we used SCUBA to establish ten transect lines at each of the three sampling sites. At each site, a starting point was haphazardly selected in the rubble habitat and a single 30 m transect was placed parallel to the reef contour. The nine subsequent transects were then placed at 5 m distances offshore of this initial transect, so that all transects were parallel. The goal was to sample the distribution of habitats through the coral rubble, the transitional zone, and live coral habitats at each of the three sampling sites. Eight 1 m^2 quadrats were sampled along each transect, resulting in a total of n = 80 quadrats per site, or N = 240 sampled quadrats for the three sites combined. Quadrat locations along the transect line were randomized, as determined by a random number generator that assigned the quadrat's distance along the transect (with a minimum distance of 2 m between quadrats) as well as the placement of the quadrat on either the left or right side of the transect line. Within each quadrat, we characterized the behavior of a single bicolor damselfish and quantified several parameters that defined the immediate physical and social environment.

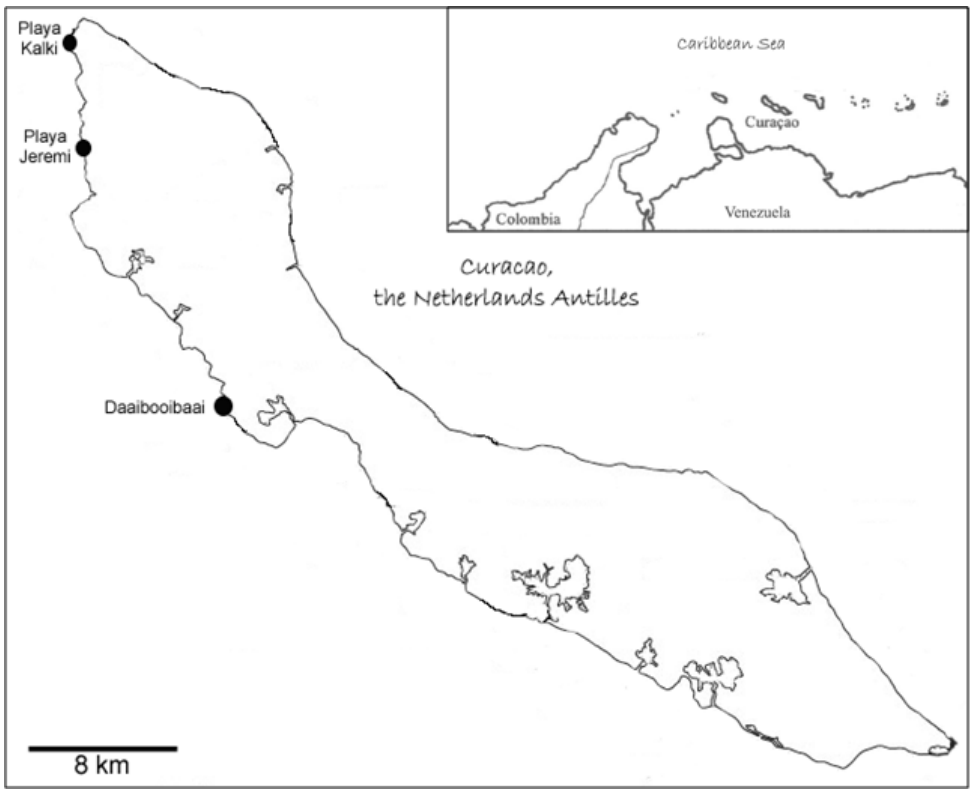


Figure 1. Map of Curacao, the Netherlands Antilles, in the southern Caribbean Sea, with the three sampling sites indicated. The depth ranges for the three sampling sites are as follows: Playa Kalki: 2.7 m – 12.8 m; Playa Jeremi: 6.1 m – 12.2 m; Daaibaaibooi: 3.6 m – 13.4 m.

Fish Behavior Assessments

We observed the behavior of bicolor damselfish during 6 min focal observations on individual fish. All behavior observations were conducted between 1000 and 1800 hr. During each observation period, the frequency of aggressive chases and nips, shelter use, and courtship behaviors (courtship dips) was recorded. These behaviors were categorized according to descriptions provided by Myrberg (1972). For aggressive behavioral exchanges, we also recorded which fish (focal or nonfocal) initiated the social interaction, and noted whether this interaction occurred between two bicolor damselfish or between the focal fish and some other species. The behavior of a single bicolor damselfish was recorded for each quadrat location. In order to avoid disturbing the behavior of damselfish, focal behavioral observations were conducted at each quadrat site from a distance, prior to laying either the transect line or quadrat. Divers used the measuring lines on the transect tape to identify where the quadrat would be placed, and observed an individual damselfish from 2 – 4 m away, a distance sufficient to avoid disturbing the behavior of the fish. At each quadrat location, the behavior of either one 'small' (<4 cm, TL) or one 'large' (>4 cm, TL) bicolor damselfish was observed. The demarcation between size classes was based on published body sizes at which bicolor damselfish have been shown to become sexually mature (Aguilar et al. 2008). Selection of either a large or small damselfish was predetermined for each quadrat in accordance with whether the quadrat was to be on the left or right side of the transect line. Individual fish fitting the predetermined size category were selected haphazardly within the quadrat area by the observer, using either the markings on the transect tape or pre-drawn markings on the quadrat to check each fish's size. If a fish of the predetermined size class was not present, the largest bicolor damselfish in the quadrat area was observed if a large fish was supposed to be observed, or the smallest of the fish

in the given quadrat was observed if a small fish was supposed to be observed. If no bicolor damselfish were present, the designated quadrat area was moved to the opposite side of the transect line. If there were still no bicolor damselfish present at this alternative quadrat location, observers continued further down the transect line, adjusting the predetermined quadrat positions based on the distance traveled, until the next bicolor damselfish was encountered along the transect.

Assessment of Social and Physical Habitats

The social environmental conditions were measured after each fish behavioral observation. A 1 m^2 quadrat was placed at the predetermined random position along the transect line, and observers waited 6 min without disturbing the quadrat or immediate surroundings. Preliminary experiments that estimated fish numbers in the habitat area before and after quadrat placement found that 6 min was sufficient time for fish to resume normal activities including returning to the area if the fish fled or retreated into shelter during the quadrat's placement. Following this 6 min period, divers recorded the number (calculated as # fish m^{-2}) and species designation of all fish within the quadrat area. Small and large bicolor damselfish and all other fish species were counted as instantaneously as possible.

The physical characteristics within each quadrat were determined by measuring the size and number of holes or crevices, rugosity, and percent (%) substrate cover. Holes, defined as any crevice deeper than the width, so that a bicolor damselfish could reasonably enter the hole for safety, were measured along two lines attached to the quadrat parallel to the direction of the transect. These two strings were placed at distances of 25 cm and 75 cm from the quadrat's edge overlapping the transect line. Any holes located directly underneath these two strings were

counted and measured. Hole size was quantified as an area (cm^2) by measuring the length of string spanning the hole opening and the width at the hole's widest point (Nemeth 1998) perpendicular to the string.

Rugosity, used as a measure of coral reef structural complexity, was measured with the 'chain and tape' method (Risk 1972; Luckhurst & Luckhurst 1978) (individual link length = 1.45 cm) at the 50 cm mark of the quadrat, running perpendicular to the transect line. All quadrats were photographed (Canon Powershot 990 IS camera, Canon USA Inc., Lake Success, NY, USA), and the percent composition was determined for each quadrat using Coral Point Count with Excel Extensions 3.6 (CPCe 3.6) (Kohler & Gill 2006). A grid of 81 uniformly distributed points was placed within each 1 m^2 quadrat, and the substrate cover beneath each point was identified and used to generate the overall percent live coral cover for each quadrat.

Statistical Analyses

Preliminary data analyses were conducted to examine homogeneity of coefficients of variation (Zar 1996) among the three sampling sites. An extended χ^2 (Feltz & Miller 1996) was performed to test whether the samples from the different sites had the same relative variability in the physical habitat variables and behaviors. Coefficients of variation were statistically similar among the three sites for three habitat variables and all behaviors (habitat variables: $0.583 \leq p \leq 0.935$; behaviors: $0.332 \leq p \leq 0.943$). Because the purpose of this study was to examine patterns of behavioral variation in bicolor damselfish as physical and social conditions of the habitat varied, we pooled the data from the three sampling sites because the coefficients of variation were similar among all three sites.

Principal Components Analysis (PCA) was performed using the number of holes, average size of holes (cm^2), rugosity, live coral cover (%), and depth to determine associations among these physical habitat variables and establish patterns of physical habitat variation within the sampling areas. Because physical habitat variables were measured on different scales, data for each variable were normalized by subtracting the mean and dividing by the standard deviation (McGarigal et al. 2000). Normalization equalizes the variance of all variables so that each variable has equal importance in determining the principal components. This procedure transforms the variables into dimensionless and comparable units so that relationships among variables will not result simply because of a difference in measurement scales. PCA condensed the measured habitat variables into a smaller set of derived components by combining those variables with similar or highly correlated information. The number of principal components (PCs) retained was based on the eigenvalues; only those PCs with an eigenvalue greater than one (> 1.0) were retained. The extracted PCs were then used as independent variables in subsequent analyses regarding the relationship between the density of bicolor damselfish and the physical habitat.

The relationship between the physical habitat and social conditions was examined in two ways: 1) relationships between the density of bicolor damselfish and the physical habitat PCs were assessed using quantile regression and 2) the relationship between fish diversity and physical habitat was examined using least squares regression. Preliminary analysis of relationships between damselfish density and physical habitat revealed a wedge-shaped pattern when the number of bicolor damselfish in a quadrat was plotted against either physical habitat PC (PC1 or PC2, obtained above). These wedge-shaped abundance patterns have been previously encountered in stock assessment studies (Terrell et al. 1996), and indicate unequal

variance for the response variable (density of bicolor damselfish) along the range of the independent variable (either habitat PC). This unequal variance makes analysis by an ordinary least squares regression technique inappropriate, given that such methods estimate a measure of central tendency. Quantile regression, on the other hand, is suitable for assessing the upper and lower boundaries of a distribution, which may be different than the relation of the response variable's central tendency to the independent variable (Terrell et al. 1996). Quantile regression is similar to ordinary least squares regression, but is more robust to outliers because the model minimizes the least absolute values of the residuals (as opposed to the square of the residuals in ordinary least squares regression). In this study, multiple quantiles (10^{th}, 50^{th}, and 90^{th}) were calculated, and the slopes were analyzed for statistically significant differences from zero (Stata, StataCorp, TX, USA). Quantile regression analysis thus revealed whether an upper threshold existed in terms of the maximum number of bicolor damselfish present for any given value of a physical habitat PC.

Relationships between physical habitat PCs and social conditions were also assessed by calculating Shannon-Weiner diversity indices (H′) for each of the 240 quadrats using PAST software (Hammer, Harper & Ryan 2001), and then using linear regression to examine the relationship between H′ and the habitat PCs. H' accounts for the total number of species present and the number of individuals representing each species, so the index also provides information on evenness. An adjusted H' was also calculated after excluding bicolor damselfish from the data set of species within each quadrat in order to allow for statistically independent assessment of how bicolor damselfish density related to overall fish diversity.

Bicolor damselfish behavior was analyzed first by comparing the frequencies of aggression (with aggressive chases and nips performed by the focal fish and received by the

focal fish analyzed separately), shelter use, and courtship displays between large (>4 cm, TL) and small (< 4 cm, TL) fish categories using t-tests. Because large and small bicolor damselfish differed in their behavior, subsequent analyses using behavior were separated by fish size.

Spearman rank correlations were used to examine whether there were associations between the bicolor damselfish behaviors and physical (e.g., principal components) and social (e.g., H′ and bicolor damselfish density) environmental condition parameters (Zar 1996). Given that statistically significant associations were found between the physical habitat PCs and social conditions, as well as between behavior and several of the physical and social conditions, we also performed a path analysis to elucidate the structure of the dependence among variables. Path analysis was performed using AMOS™ 5.0 (Arbuckle 2003) with SPSS 16.0 (SPSS Inc., Chicago, IL, USA) to investigate which variables affected behavior directly or indirectly, and which pathways or relationships were strongest. This analysis is similar to multiple regression, and allows the investigator to test *a priori* defined direct and indirect relationships; however, predictor variables can serve as both independent and dependent variables.

Given that the behavior of small and large bicolor damselfish differed – and that relationships of these fishes' behaviors to environmental parameters also differed – a path analysis was performed separately for small and large fish, using maximum likelihood estimation. Multiple models were built for each damselfish size class using original variables from the physical habitat PCA, social conditions, and behaviors, and then each model was tested for goodness of fit. Because the use of strongly correlated variables within a single path model can generate biased results, a single variable (depth) was chosen (see below) to represent habitat PC1, which originally contained four correlated habitat variables. All path analysis models used

the number of holes to represent habitat PC2, and the final dependent variables in the models were the behavior variables.

For all models constructed, general goodness-of-fit measures were calculated: χ^2 was the difference between the observed covariance from the expected, CFI (comparative fit index) provided an indication of the lack of fit accounted for by going from the null model to our defined model and should be close to 1, and RMSEA (root mean square error of approximation) allowed for comparison of non-nested models and should be less than 0.05 (<0.05). For each model determined to be of general good fit, a bootstrap approach with 1000 bootstrap samples with replacement was used to choose the single best-fitting model for each fish size (Linhart & Zucchini 1986). The model with the smallest average discrepancy between the implied moments obtained from the bootstrap samples and those of the overall sample (ML discrepancy) was determined to be the overall best-fit model (Table 1).

After testing four different models with each one containing a different variable to represent habitat PC 1, the model using depth was selected as the best overall fit, given that this model maximized the variance explained in each dependent variable and had the lowest ML discrepancy ($\chi^2 = 8.831$; df = 8; $p = 0.357$ for small bicolor damselfish and $\chi^2 = 11.338$; df = 10; $p = 0.332$ for large bicolor damselfish) (Table 1). Regardless of which variable was chosen to represent habitat PC1, however, the direction of PC1's influence on other parameters in the model was similar.

The final models for small and large bicolor damselfish contained an adjusted fish diversity index (H´) that did not consider bicolor damselfish in the fish species data set. This adjusted diversity measure was used to ensure independence from another variable in the model:

Table 1. Summary of model fit parameters for Path Analysis models of large and small bicolor damselfish. The single best-fitting model was selected from the five possibilities from each size class. X^2 = chi-square, CFI = comparative fit index, RMSEA = root mean square error of approximation, ML = ML discrepancy.

Habitat PC1 Variable Included	X^2	df	p	CFI	RMSEA	ML (mean ± s.e.)
Small bicolor damselfish						
average hole size	5.385	7	0.613	1.000	0.000	38.242 ± 0.617
% coral cover	11.517	8	0.174	0.944	0.061	41.630 ± 0.566
rugosity	6.695	8	0.570	1.000	0.000	38.668 ± 0.621
depth	8.831	8	0.357	0.992	0.030	35.087 ± 0.475
depth + adjusted diversity	4.624	7	0.706	1.000	0.000	31.343 ± 0.479
Large bicolor damselfish						
average hole size	13.652	11	0.253	0.978	0.045	58.910 ± 1.004
% coral cover	17.377	12	0.136	0.959	0.061	57.405 ± 0.901
rugosity	12.333	11	0.339	0.989	0.032	52.741 ± 0.899
depth	11.338	10	0.332	0.992	0.033	51.236 ± 0.881
depth + adjusted diversity	12.791	13	0.464	1.000	0.000	48.984 ± 1.218

df= number of unspecified parameters

the density of bicolor damselfish. Using the same bootstrap approach as discussed above, the model with the adjusted diversity measure was determined to be a better fit than the original depth model ($\chi^2 = 4.624$; df = 7; $p = 0.706$ for small bicolor damselfish and $\chi^2 = 12.791$; df = 13; $p = 0.464$ for large bicolor damselfish) (Table 1).

RESULTS

Relationships among Physical and Social Habitat Conditions

Principal components analysis (PCA) reduced the five measured physical habitat variables (hole number, average hole size (cm^2), rugosity, % live coral cover, and depth) down to two independent PC axes, which together accounted for 66.34% of the variation observed in physical habitat conditions. The physical habitat PC1 (eigenvalue = 2.296) explained 45.92% of the variation in physical habitat, while habitat PC2 explained an additional 20.14%. Rugosity, average hole size (cm^2), % coral cover, and depth all clustered along the PC1 axis – with each of these variables having positive loadings on PC1 (Table 2, Fig. 2). The positive loadings of each variable indicate that these environmental parameters varied together positively, so that as depth increased, increases were also seen in rugosity, % coral cover and average hole size. Habitat PC2 (eigenvalue = 1.021), in contrast, was only represented by a significant loading with the number of holes, which also loaded positively (Table 2, Fig. 2). The bifurcation of the number of holes and average hole size into separate PCs indicates that these two physical habitat parameters varied independently across the range of habitats sampled.

Social environmental conditions showed significant relationships with variation in the physical coral reef habitat. When analyzed as all bicolor damselfish together, small fish alone, or

Table 2. Factor loadings for the Principal Components Analysis performed on the five measured physical habitat variables. Bold loadings indicate the axis of strongest loading for each variable.

Variable	PC1	PC2
Rugosity	**0.5155**	0.0279
Number of holes	-0.0614	**0.9728**
Average hole size (cm2)	**0.4963**	-0.1514
% coral cover	**0.4939**	0.1234
Depth	**0.4902**	0.1215

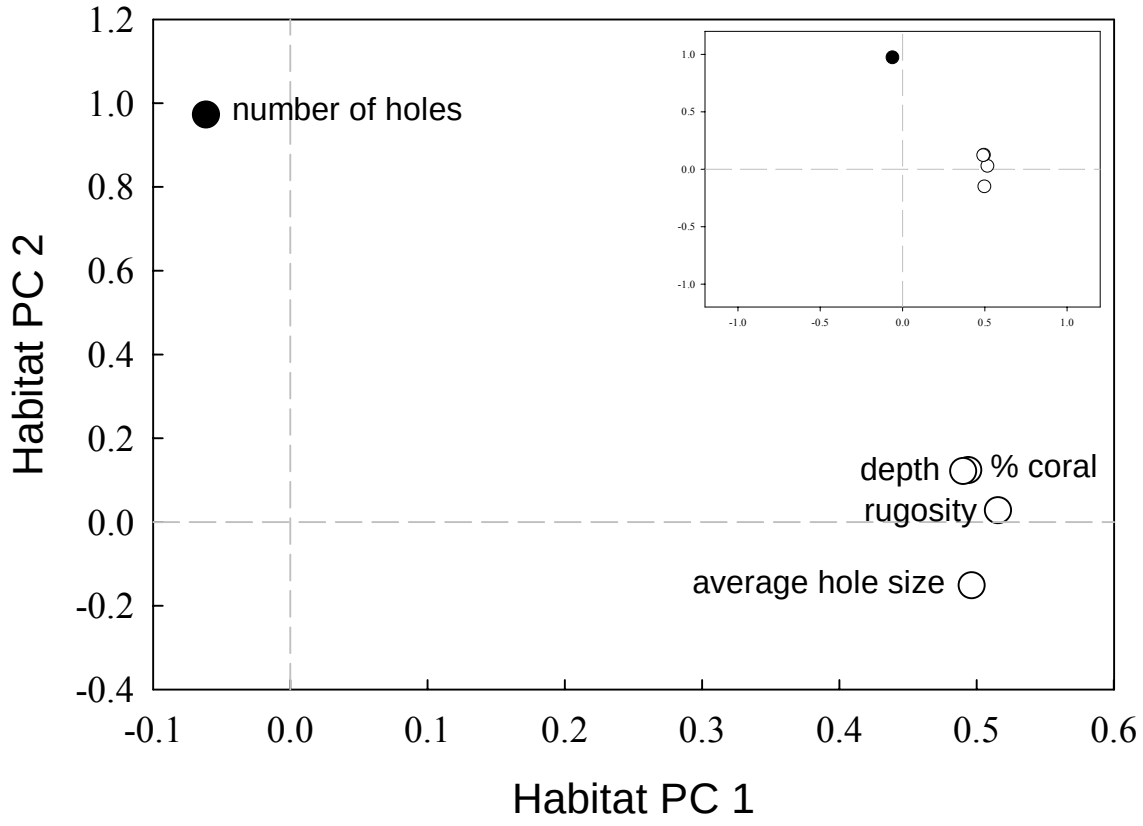


Figure 2. Principal Components Analysis (PCA) for the five habitat variables. Original output is in the upper right corner. PCA reduced the five variables into two independent PCs.

large damselfish alone, the density of bicolor damselfish declined with increasing values of habitat PC1 (Fig. 3). The density of bicolor damselfish plotted against either habitat PC1 or PC2 showed a wedge-shaped distribution indicating that the central tendency of the relationship may not be the best indicator of the overall pattern; rather, the bounds of the distribution better represent the relationship (Scharf , Juanes & Sutherland 1998; Cade & Noon 2003). Quantile regression was therefore used to test for relationships between bicolor damselfish density and physical habitat characteristics.

When the total density of bicolor damselfish was analyzed against PC1, a significantly negative slope was found at the 90^{th} quantile ($t= -2.597$; $df = 239$; $p = 0.010$) and the 50^{th} quantile or median ($t = -3.614$; $df = 239$; $p = 0.010$), but not at the lower bound (10^{th} quantile), which had a slope of zero (Fig. 3a). The relationship between PC1 and the density of small damselfish showed a similar pattern with increasing values of PC1, with significantly negative slopes at the 90^{th} ($t = -2.780$; $df = 239$; $p = 0.006$) and 50^{th} quantiles ($t = -5.015$; $df = 239$; $p = 0.0001$), but a slope of zero at the 10^{th} quantile (Fig. 3b). The density of large damselfish, however, showed a different pattern relative to changes in PC1. Slopes of the relationships between large damselfish density and PC1 were not significant at the 10^{th} ($t = 0.000$; $df = 239$; $p = 1.000$), 50^{th} ($t = 0.000$; $df = 239$; $p = 1.000$), or 90^{th} quantiles ($t = -1.910$; $df = 239$; $p = 0.057$) (Fig. 3c), indicating that the density of large bicolor damselfish did not vary significantly with changes in the PC1 dimension of physical habitat structure. Rather, the change in overall bicolor damselfish density with PC1 appeared to be caused by decreases in the abundance of small bicolor damselfish as PC1 increased in the range of habitats examined. Concomitantly, the diversity of fish species (H′) increased with an increase in physical habitat PC1 ($r^2 = 0.19$; $p <$

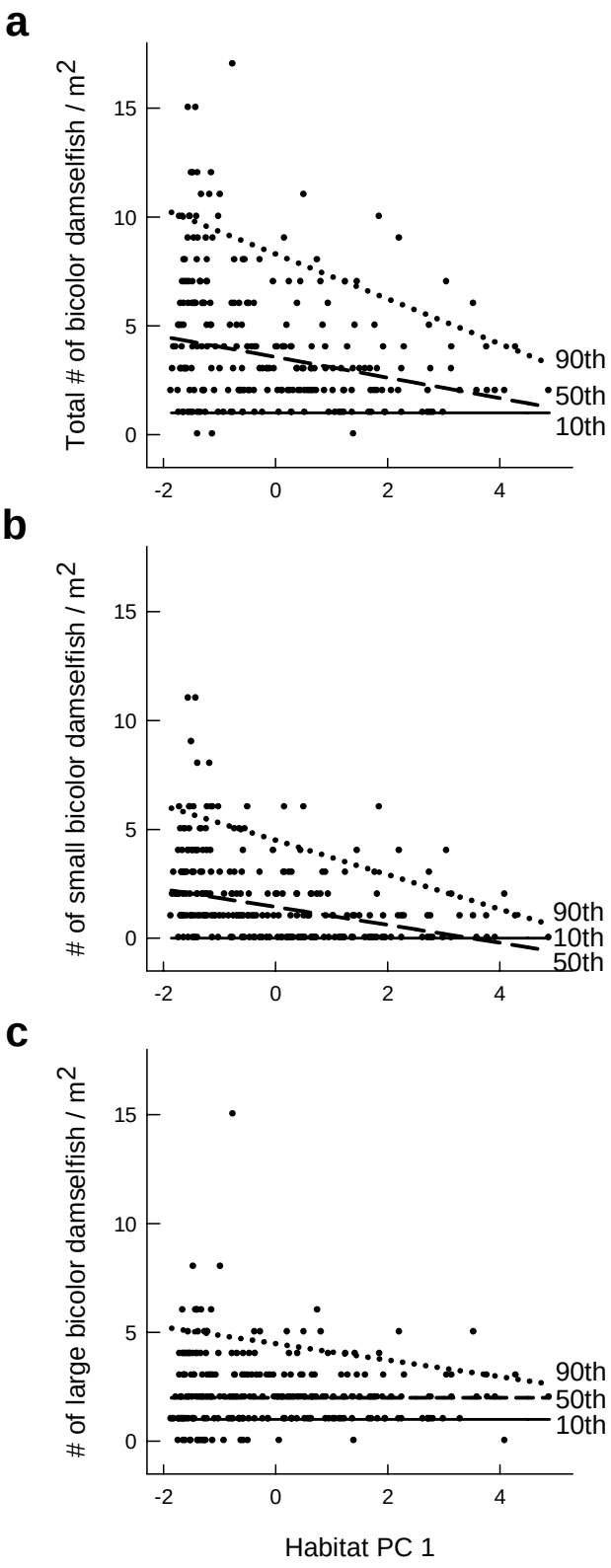


Figure 3. Relationships of bicolor damselfish densities and physical habitat PC1 for a) total bicolor damselfish density, b) small fish only, and c) large bicolor damselfish only.

0.0001) (Fig. 4a). This relationship still holds if the H' values of zero (quadrats that only contained bicolor damselfish) are removed ($r^2 = 0.21$; $p < 0.0001$).

The density of bicolor damselfish also showed associations with habitat PC2, although density varied in a pattern opposite to that observed with PC1 (Fig. 5). The total density of bicolor damselfish in relation to PC2 showed a significantly positive slope at the 90th quantile ($t = 4.131$; $df = 239$; $p = 0.0001$) and 50th quantile ($t = 2.315$; $df = 239$; $p = 0.021$), but not at the 10th quantile (Fig. 5a). Similarly, the density of small damselfish increased with PC2 when examined at the 90th quantile ($t = 3.391$; $df = 239$; $p = 0.001$) and 50th quantile ($t = 2.888$; $df = 239$; $p = 0.004$), but again not at the 10th quantile (Fig. 5b). Large bicolor damselfish showed increasing densities with greater values of habitat PC2 at the 90th quantile ($t = 3.902$; $df = 239$; $p = 0.0001$), but no significant relationships at the 50th ($t = 0.000$; $df = 239$; $p = 1.000$) or 10th quantiles (Fig. 5c). Unlike with PC1, there was no significant relationship between fish species diversity (H′) and habitat PC2, the number of holes ($r^2 = 0.0003$; $p = 0.802$) (Fig. 4b). This relationship also holds when the H' values of zero are excluded from the analysis ($r^2 = 0.0011$; $p = 0.632$).

Variation in Bicolor Damselfish Behavior

Large and small bicolor damselfish differed significantly in the frequency of all three behaviors observed: aggression, shelter use, and courtship displays (Fig. 6). Large bicolor damselfish exhibited higher rates of 'by focal' aggression ($t = -5.685$; $df = 238$; $p < 0.0001$), averaging nearly 4 times the number of chases directed at opponents by small fish. However, the large bicolor damselfish also received less aggression ('at focal') than small fish ($t = 4.413$; $df = 238$; $p < 0.0001$). Large bicolor damselfish were involved in more aggressive interactions

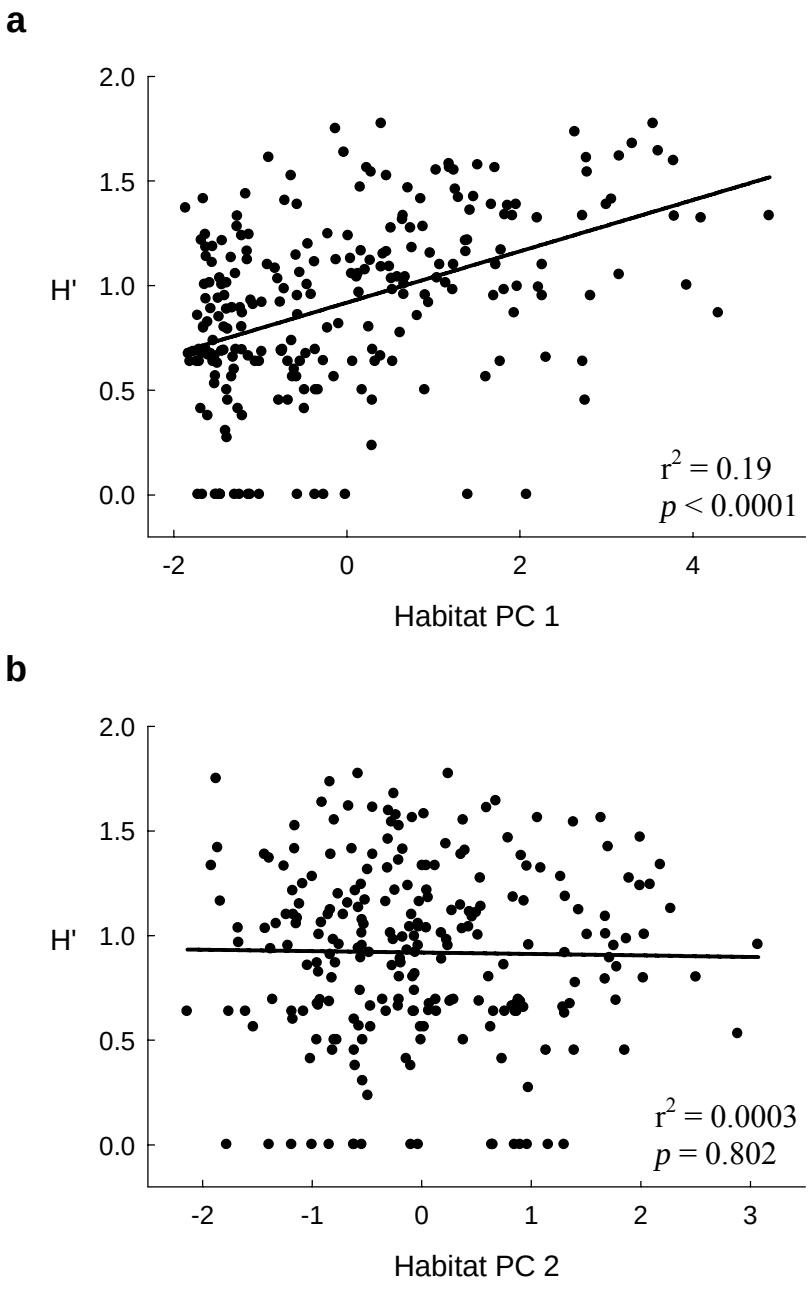


Figure 4. Relationship between coral reef fish diversity and a) habitat PC1 and b) habitat PC2. H' in this figure includes bicolor damselfish because H' is being related to a variable that is not associated with bicolor damselfish.

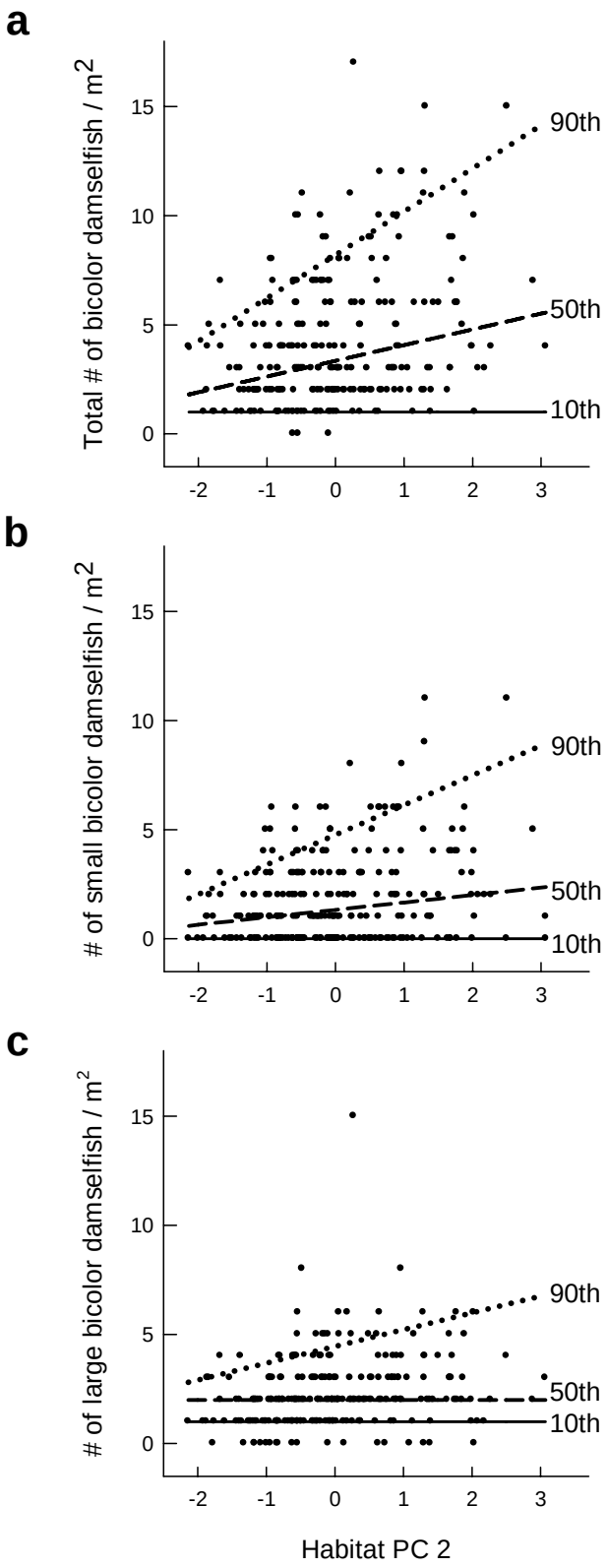


Figure 5. Relationships of bicolor damselfish densities and physical habitat PC2 for a) total bicolor damselfish density, b) small fish only, and c) large bicolor damselfish only.

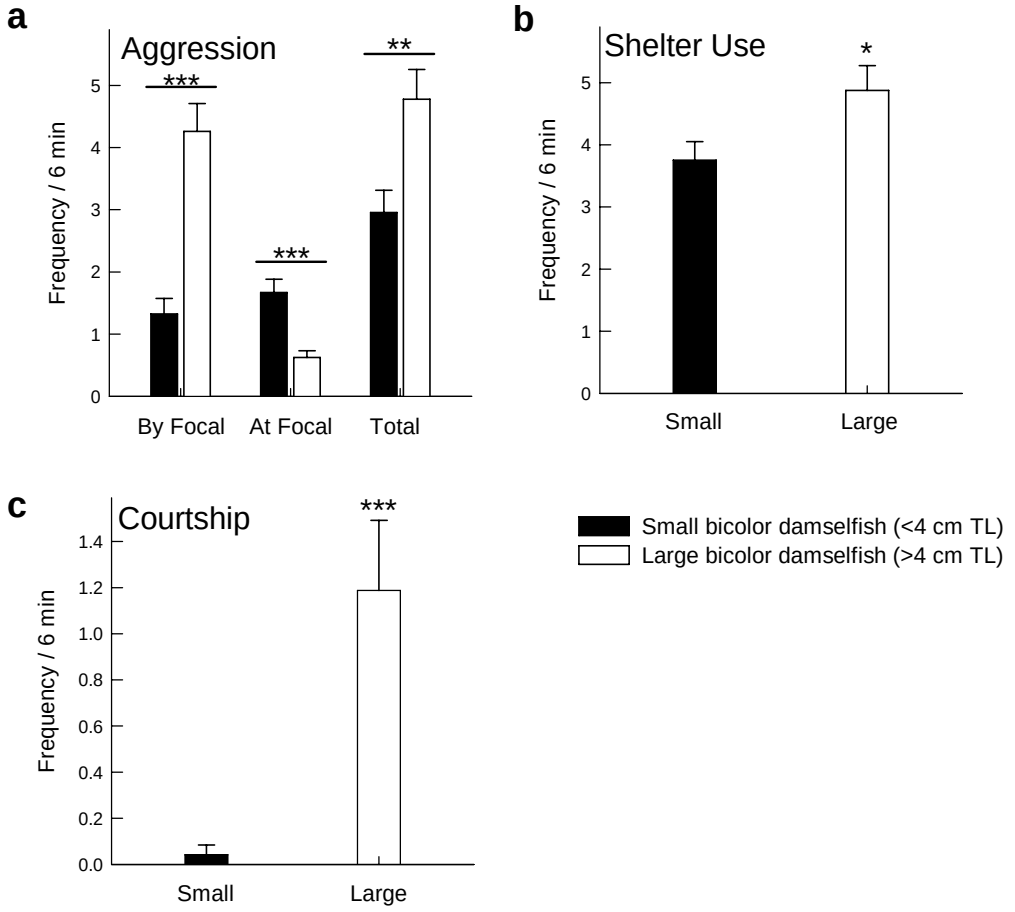


Figure 6. Behavioral variation between large (>4 cm, TL) and small (<4cm, TL) bicolor damselfish for a) aggression, b) shelter use, and c) courtship dips. Asterisks indicate a statistically significant difference detected by t-tests between large and small bicolor damselfish (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

overall ('by focal' and 'at focal' aggression frequencies combined) compared to small damselfish ($t = -3.050$; $df = 238$; $p = 0.0025$). Large bicolor damselfish also entered shelters more often than small fish ($t = -2.246$; $df = 238$; $p = 0.0256$), and showed significantly elevated frequencies of courtship ($t = -3.679$; $df = 239$; $p = 0.0003$); only one of the 118 small bicolor damselfish was observed to court.

Relationships between Intraspecific Variation in Behavior and Habitat

In addition to behavioral differences between the large and small size classes of bicolor damselfish, considerable variation in behavior was observed among fish within each size class. Pairwise relationships between this behavioral variation within a size class and the physical characteristics of the habitat (habitat PCs 1 and 2) – as well as the social conditions of the habitat – were examined using Spearman's rank correlations. For large bicolor damselfish, 'by focal' and 'total aggression' were significantly correlated ($\rho = 0.931$; $p < 0.0001$); we will only discuss 'by focal' aggression for large fish since it is statistically similar to 'total aggression'. For small bicolor damselfish, 'at focal' and 'total aggression' were highly significantly correlated ($\rho = 0.850$; $p < 0.0001$), so likewise, we will discuss 'at focal' aggression for the small bicolor damselfish as opposed to 'total aggression'. Large bicolor damselfish also showed statistically significant positive correlations between 'by focal' aggression and both shelter use and courtship displays, even though no similar relationships were seen among the behaviors of small bicolor damselfish (Table 3).

Significant relationships were also found between intraspecific variation in bicolor damselfish behavior and variation in physical habitat conditions. The frequencies of aggression, shelter use and courtship by large bicolor damselfish each showed significant negative

Table 3. Relationships between bicolor damselfish behaviors and social environmental conditions for large (>4 cm, TL) and small (<4cm, TL) bicolor damselfish.

Size	Correlated Variables		Spearman's ρ	p-value
Large:	by focal aggression	shelter use	0.5382	< 0.0001
		courtship dips	0.4562	< 0.0001
		total BC density	0.3181	0.0004
		small BC density	0.3439	0.0001
		large BC density	0.1528	0.0928
	total fish diversity	by focal aggression	-0.3073	0.0006
		shelter use	-0.3210	0.0003
		courtship dips	-0.1485	0.1026
Small:	at focal aggression	shelter use	0.2173	0.0181
		courtship dips	0.0114	0.9026
		total BC density	-0.0959	0.3013
		small BC density	0.1173	0.2057
		large BC density	-0.0114	0.9025
	total fish diversity	at focal aggression	-0.2649	0.0038
		shelter use	-0.2085	0.0235
		courtship dips	-0.0950	0.3059
All Fish:	total fish diversity	total BC density	-0.1243	0.0051
		total fish density	0.3348	< 0.0001

BC=bicolor damselfish

relationships with increasing values of habitat PC1 (Fig. 7). Similarly, aggression and shelter use by small bicolor damselfish were also negatively associated with PC1 (Fig. 7). Habitat PC2, in contrast, was not found to be correlated with behavioral variation among large bicolor damselfish; however, all measures of aggression by small bicolor damselfish (e.g., 'at focal', 'by focal' and total) were found to be negatively correlated with habitat PC2, indicating that the intraspecific variation in the behavior of small bicolor damselfish was associated with the number of holes present in the local habitat area.

Variation in the behavior of bicolor damselfish was also found to be related to variation in the social conditions of the local habitat. Fish diversity was negatively correlated with aggression and shelter use for large and small bicolor damselfish, and also with courtship dips for large fish (Table 3). For large bicolor damselfish, by focal aggression was positively correlated with small and total bicolor damselfish densities, but not large bicolor damselfish density. For small bicolor damselfish, 'at focal' aggression was not significantly correlated with the density of bicolor damselfish (Table 3).

Path Analyses of Relationships between Behavioral and Environmental Variation

Path analyses were used to examine the relative influences of physical and social habitat conditions on intraspecific variation in bicolor damselfish behavior. Separate path analysis models were generated for the large and small categories of damselfish, given that fish from these categories differed significantly in behavior. In each model, relationships between habitat variation (PC1 and PC2), variation in social conditions (bicolor damselfish density and H') and bicolor damselfish behavior were analyzed. Path diagrams and accompanying standardized path

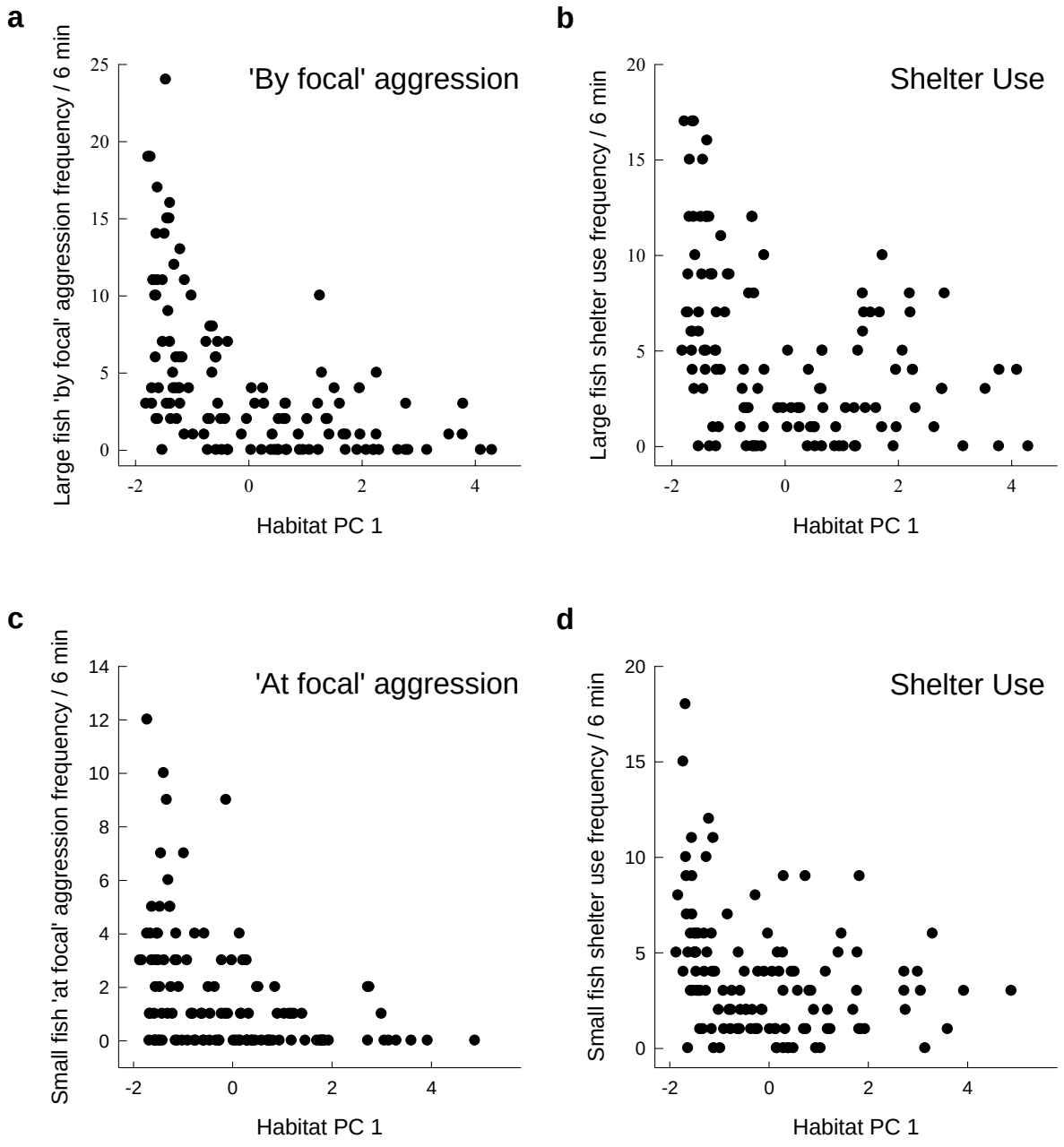


Figure 7. Relationships between behavioral frequencies and physical habitat PC1 for large bicolor damselfish a) 'by focal' aggression, b) shelter use, and for small bicolor damselfish c) 'at focal' aggression, and d) shelter use.

coefficients for the two best fit models are shown in Figures 8 and 9.

The path model for large bicolor damselfish behavior explained 34% of the variation seen in 'by focal' aggression, 34% of the variation in shelter use, and 13% of the variation in courtship behaviors (Fig. 8). Direct effects outweighed indirect effects in all cases except for the association between the proxy for PC1 (depth) and courtship displays because there was no direct relationship between depth and courtship. In this case, the relationship between the physical conditions and the courtship behavior was mediated by either the total bicolor damselfish density or 'by focal' aggression. The strongest relationship seen in the large damselfish model occurred between depth and 'by focal' aggression, where the negative path coefficient indicated a decrease in aggression with increasing habitat depth, or habitat PC1. Strong relationships were also present between depth and shelter use, where again the negative coefficient indicates a negative association between variation in these variables. Total bicolor damselfish density showed a negative relationship with depth but a positive relationship with the number of holes (indicative of habitat PC 2) in the habitat. These results indicate that bicolor damselfish density decreased with depth, but increased with the number of holes in the benthos. A strong relationship was also found between 'by focal' aggression and shelter use, where the positive coefficient indicated that large damselfish that were more aggressive also tended to use shelter more frequently. Courtship displays were more weakly positively associated with both the total density of bicolor damselfish and the frequency of 'by focal' aggression. Overall, this path analysis model suggests that variation in aggression and shelter use among large bicolor damselfish are most strongly associated with the proxy for physical habitat PC1 (depth), as opposed to either measure of social environmental variation. This is further supported by the lack of relationships between the diversity of fish species and any of the behaviors.

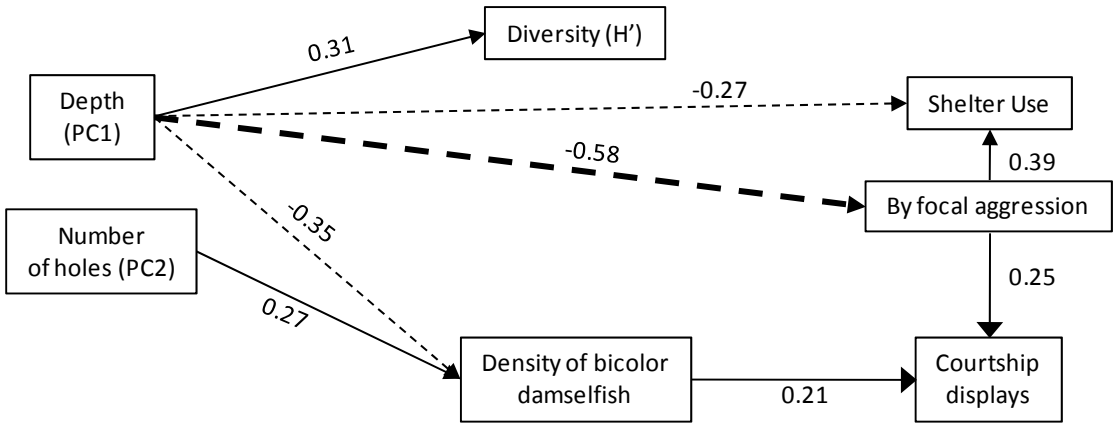


Figure 8. Path analysis model for large bicolor damselfish. Depth is used as a proxy for habitat PC1 and the number of holes represents habitat PC2. Path coefficients are the standardized path coefficients, and the thickness of arrows is proportional to the strength. Dashed arrows indicate negative associations among the variables. All arrows are statistically significant in the model; non-significant arrows have been removed.

In the path analysis model for small bicolor damselfish behavior, all direct effects outweighed indirect effects. The best fit model for small damselfish behavior effectively explained 23% and 27% of the variation in small bicolor damselfish shelter use and 'at focal' aggression, respectively (Fig. 9). Similar to the model with large damselfish behavior, the physical habitat variables were directly associated with behavioral and social environmental variation, but neither of the social environmental variables had significant direct effects on behavior. The strongest relationships in the path model for small damselfish behavior were again seen between depth and the two behavioral variables: shelter use and 'at focal' aggression. Negative path coefficients between depth and these two behaviors indicate that as depth increased the frequency of 'at focal' aggression and shelter use by small bicolor damselfish decreased. Similar to the model of large damselfish behavior described above (Fig. 8), the number of holes (PC2) was found to be positively associated with the total density of bicolor damselfish. A weak negative relationship, however, was found between the number of holes and 'at focal' aggression by small damselfish, even though no similar relationship was seen with large damselfish. The two social variables, fish diversity and bicolor damselfish density, were positively associated with each other, while the two behavior variables of aggression and shelter use were negatively associated. Similar to the model for the behavior of large bicolor damselfish, the best fit model for small bicolor damselfish suggests physical habitat characteristics, and not social environmental characteristics, have the strongest role in predicting variation in the behavior of small bicolor damselfish.

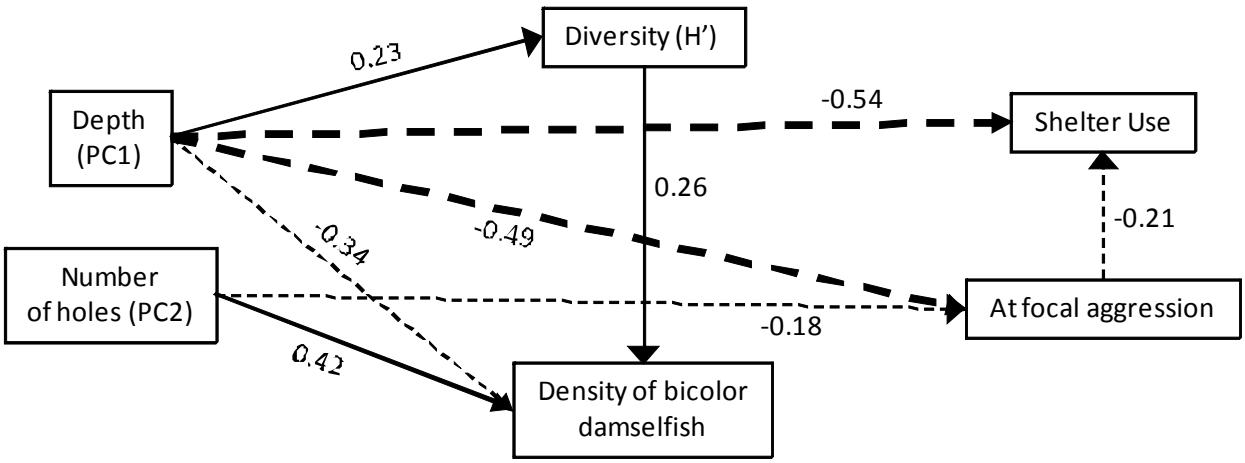


Figure 9. Path analysis model for small bicolor damselfish. Depth is used as a proxy for habitat PC1 and the number of holes represents habitat PC2. Path coefficients are the standardized path coefficients, and the thickness of arrows is proportional to the strength. Dashed arrows indicate negative associations among the variables. All arrows are statistically significant in the model; non-significant arrows have been removed.

DISCUSSION

Geographic variation in habitat conditions is known to influence the distribution and abundance of species. However, it is less well recognized that the same habitat variation can also promote intraspecific diversity in behaviors of consequence to fitness (Foster, 1999). Understanding the environmental origins of such behavioral variation is critical to understanding the evolutionary ecology of the species, as it may either reflect or ultimately result in evolved adaptations to local selective pressures. In the present study, we provide evidence that the demersal bicolor damselfish exhibits behavioral variation over small spatial distances (meters) across a coral reef. The variation in behavior was observed in patterns strongly associated with variation in the physical and social environmental conditions of the coral reef. Path analyses revealed the strongest relationships between variation in the behaviors of large (> 4 cm) bicolor damselfish and habitat PC1 (a composite variable of physical habitat conditions consisting of the size of holes in the benthos, rugosity, % coral cover and depth) so that as PC1 values increased as transects moved further offshore toward the reef slope, the frequencies of aggression, shelter use and courtship by large damselfish decreased. Spatial variation in aggression and shelter use by small bicolor damselfish also declined with increasing PC1 values. Taken together, these results indicate that aggressive interactions were less frequent in the deeper reef slope habitats (higher PC1 values) and provide support for the hypothesis that variation in bicolor damselfish behavior is associated with spatial variation in the physical structure of coral reef habitat. Furthermore, the spatial variation in bicolor damselfish behavior was observed over distances of only ~35 m as the reef transitioned from shallower areas of *Acropora cervicornis* rubble to the *Montastrea sp.*-dominated fringing reef slope.

The overall density of bicolor damselfish also varied with the PC1 variable of physical habitat conditions, with a significantly greater density of bicolor damselfish in the shallow coral rubble (low PC1 values) than the deeper reef slope where there were more live corals (higher PC1 values). The spatial variation in overall damselfish density appears to result from a change in the abundance of small bicolor damselfish (< 4 cm, TL), and not large damselfish (> 4 cm, TL), as only the density of small bicolor damselfish declined with higher PC1 values. Quantile regression analysis revealed that the relationship between small bicolor damselfish density and the habitat PC1 axis showed an upper bound, as indicated by a significant slope at the 90^{th} quantile, but not at the 50^{th} (median) or 10^{th} quantiles. Because the 10^{th} quantile (lower bound) slopes were consistently found to be zero, and the 50^{th} and 90^{th} quantiles were not, the upper bound (90^{th} quantile) was influencing the median slope, indicating that the value of PC1 for a given quadrat best predicted the *maximum* number of bicolor damselfish, not the average number, within that local habitat. The quantile regression results, therefore, suggest that habitat PC1 acts as a limiting factor for the density of bicolor damselfish and that other factors not accounted for in the quantile regression models must be interacting with habitat PC1 to determine the actual number of damselfish in a given quadrat.

Although the predominant spatial relationships between bicolor damselfish behavior and reef habitat structure involved PC1, we also found significant relationships between damselfish behavior and PC2, the physical habitat dimension representing the number of holes in the local habitat. Unlike PC1, which varied with depth and therefore position along the reef slope, the spatial distribution of PC2 values among quadrats was seemingly random across the reef as a whole: the number of holes in a quadrat did not show any statistically significant association with the depth, rugosity, % coral cover, or average size of holes (habitat PC1) in that quadrat.

Nevertheless, the frequency of aggressive behaviors by small (< 4 cm) bicolor damselfish, but not large (> 4 cm) bicolor damselfish, varied negatively with PC2.

The total damselfish density also increased with increasing PC2 values, indicating more bicolor damselfish were present in local habitats containing more holes. Bicolor damselfish rely on shelter for protection from predators, as well as for nesting sites for reproduction, and significant quantile regression associations between maximum bicolor damselfish density and the number of holes in the benthos indicate that the number of holes acts as an upper bound, limiting the density of bicolor damselfish occupying a particular local habitat. Finding relationships between the density of small, but not large, bicolor damselfish and PC2 implies that small and large bicolor damselfish may be using available coral reef habitats differently, likely through differences in behavior or relative fitness (e.g., survivorship) in structurally distinct reef habitats (see also Nemeth 2003, 2005).

Individual variation in relationships between behaviors in bicolor damselfish

Supporting the idea that large and small bicolor damselfish may be using available coral habitats differently, frequencies of behaviors were found to differ between large and small bicolor damselfish, with large fish behaving more aggressively, using substrate shelters more frequently and courting more often. Contrastingly, small bicolor damselfish received significantly more aggression than large fish, which were found to generally be the initiators of intraspecific agonistic interactions (Harrington 1993, 1995). Harrington's (1993, 1995) observations are consistent with our observation of large damselfish showing high rates of overt aggression and small damselfish receiving a majority of the aggression.

In large damselfish, high rates of aggression may be related to reproductive activity because male damselfish defend territories with nesting holes in the substratum where eggs are laid by females (Knapp & Warner 1991). In the present study, large fish showed more courtship displays and more frequent use of available substrate shelters, which may be indicative of reproductively active males defending nesting holes containing eggs. Bicolor damselfish become sexually mature around 3.5 cm total length (Aguilar et al. 2008), and since large and small bicolor damselfish were distinguished by being either larger or smaller than 4 cm, inherent differences in behavior were expected between the two size classes of fish. Our finding of only a single fish less than 4 cm, TL displaying any courtship dips suggests that only fish from the large category may have been reproductively active. Moreover, our behavioral observations occurred in May, which is one of the peak spawning months for bicolor damselfish (Myrberg 1972).

Perhaps of greater consequence for understanding how large and small bicolor damselfish might be using habitats differently, we also found that suites of correlated behaviors differed between the two size classes. Frequencies of 'by focal' aggression, courtship and shelter use were all positively correlated in large bicolor damselfish, while small bicolor damselfish only showed a significant positive relationship between 'at focal' aggression and shelter use. Size-related variation in the type of aggression associated with substrate shelter use suggests that large and small bicolor damselfish may be using substrate holes for different functions. Because overtly aggressive, high courting males from the large fish category also used substratum shelters more frequently, large males were likely defending eggs within substrate nesting holes. Large male bicolor damselfish need to constantly maintain the nest and protect it from intruders and nest predators (Myrberg 1972), which would explain positive relationships between offensive 'by focal' aggression, courtship, and shelter use in large male bicolor damselfish (Knapp &

Kovach 1991). Similarly, Myrberg (1972) found male bicolor damselfish to visit eggs in shelters 1 – 2 times per minute during peak spawning months, which is slightly higher than our observation of large bicolor damselfish entering shelters an average of 0.8 times per minute (see Fig. 6b).

For small bicolor damselfish, the positive correlation between 'at focal' aggression and shelter use may instead result from agonistic encounters with larger bicolor damselfish or potential predators. Adult bicolor damselfish routinely attack juveniles that enter their territories, seemingly because the small fish are becoming more competitive for the same shelters as they grow larger (Harrington 1993). Small bicolor damselfish that stray too far from shelter are likely subject to frequent aggression from larger males or potential predation from other fishes. The positive relationship between shelter use and 'at focal' aggression, therefore, is best interpreted as small bicolor damselfish using substrate shelters more for individual protection as opposed to nesting sites. It is also important to note, however, that a higher rate of shelter use (as measured by the number of times that focal fish entered substrate shelters per time) should not to be confused with the time spent within shelters. The average time spent within a substrate shelter was not recorded in the present study, but the time may be similar across size classes or even greater in small fish, especially since small fish appear to be using shelters largely to avoid predators.

Interacting influences of physical and social habitat conditions on damselfish demography and behavior

Our observations indicate that bicolor damselfish exhibit spatial patterns of behavioral variation associated with the physical conditions of the local coral reef habitat. While similar

intraspecific variation in behavior has been documented for several coral reef fishes at much larger geographic scales (e.g., kilometers) (Afonso, Morato & Santos 2008), the presence of such repeatable patterns of spatial variation at relatively small scales (<35 m) suggests that environmental variation on a coral reef may generate intraspecific behavioral variation in reef-associated species at previously unacknowledged spatial scales. Species occupying broad geographic ranges frequently experience a range of environmental variation, which can lead to phenotypic diversification through developmental and, ultimately, evolutionary changes (Foster 1999). However, finding habitat-associated phenotypic diversity in the bicolor damselfish occurring at smaller spatial scales suggests that habitat structural heterogeneity may contribute underappreciated influences on phenotypic variation among individuals of the same species.

Unraveling the relative influences of specific environmental parameters on intraspecific behavioral variation can be difficult, especially considering how several environmental factors probably interact when shaping behavior in either a developmental or evolutionary context (West et al., 2003; Stamps, 2003). Nevertheless, previous laboratory and field studies do provide some insight into the spatial patterns of behavioral variation among bicolor damselfish observed here. Relationships between behavior and physical habitat structure have been examined in several fishes under controlled laboratory conditions. Female, sexually mature zebrafish (*Danio rerio*), for instance, show reduced aggression when housed in structurally complex aquaria compared to bare tanks (Carfagnini et al. 2009). Similar results were observed for laboratory Australian freshwater crayfish, in which the frequency of aggressive interactions and overall time spent interacting with conspecifics was reduced among individuals maintained in a complex habitat (Baird, Patullo & Macmillan 2006). Reductions in aggressive interactions in complex habitats have been suggested to result from several causes including visual impairment (in lobsters:

Atema et al. 1979), a reduction in territory size because vision is limited by the structural complexity (Eason & Stamps 1992), and the interference with transmitted stimuli used to detect conspecifics (e.g., Atema et al. 1979). More recent studies in fishes provide evidence proposing the brain itself may be affected by the structural complexity of the habitat an individual experiences (Lema et al., 2005; Kihslinger, Lema & Nevitt 2006; Gonda, Herczeg & Merila 2009), suggesting a link between the behavioral impacts of habitat complexity and fundamental changes in the development of neural pathways. Habitat complexity should be recognized as a factor capable of altering behavior in several ways; however, the functional consequences of behavioral changes often remain to be determined. For instance, Rilov et al. (2007) suggest the structural complexity may actually be detrimental to the fitness of territorial bicolor damselfish because the fish may not be able to appropriately assess predation risk in structurally complex habitats where visual distances can be limited.

Determining how habitat complexity directly influences patterns of behavior can be difficult because habitat complexity rarely varies independently. Rather, social environmental conditions commonly vary with habitat structure, resulting in the covariation of multiple environmental parameters. Several studies have found increased fish diversity to be associated with increased habitat complexity for coral reefs and other marine habitats (e.g., Luckhurst & Luckhurst, 1978; Roberts & Ormond 1987; Ohman & Rajasuriya, 1998; Holbrook, Forrester & Schmitt 2000; Holbrook, Brooks & Schmitt 2002; Lingo & Szedlmayer 2006; Piko & Szedlmayer 2007). Previous studies have also documented a connection between habitat characteristics and fish assemblages, with depth (a component of habitat PC1 in the current study) explaining the majority of variation in fish composition in coral reef habitats (González-Sansón et al. 2009). In general, associations between habitat conditions and fish density and

diversity appear to result from differences in resource availability, shelter sites for predation protection, and species variation in habitat use (e.g., Itzkowitz 1977; Ormond, Roberts & Jan 1996). For example, small bicolor damselfish may be more numerous in rubble areas because of food resource availability or differential predation pressures. If bicolor damselfish forage a mixed diet of benthic algae and zooplankton, as has been described by Booth & Hixon (1999), then differences in algae and zooplankton availability between rubble and reef areas may provide some explanation for the density differences observed in bicolor damselfish between rubble and reef habitats in the current study. Ontogenetic shifts in dietary resource use by bicolor damselfish – juveniles feeding more on benthic algae and adults feeding more on zooplankton - may also lead to greater abundances of juveniles in areas with greater benthic algae cover, similar to what is found on coral rubble. Furthermore, variation in predation risk whether via spatial variation in predator types and abundance, differences in shelter site characteristics, or differences in risky behaviors of damselfishes themselves may also contribute to variation in spatial distribution and demography. Nemeth (1998) observed bicolor damselfish swimming farther away from shelter to collect drifting zooplankton when they inhabited *Montastrea* coral heads as opposed to *Porites porites* rubble habitats. Increased foraging distances from substrate shelter would likely increase predation pressure (Nemeth 1998) (especially for small bicolor damselfish), and predation may be selecting against small bicolor damselfish in the reef slope areas of high structural complexity and more live coral cover. Relative predation risk may also be affected by the number and size of shelter holes, as a shelter of similar size to the body of a prey item is important for increasing survivorship from predation (Hixon & Beets 1989, 1993). Further support for interacting influences of predation pressure and habitat structure on damselfish distribution patterns is evidenced by juvenile (small) bicolor damselfish having

higher rates of survival in rubble habitats as compared to structurally complex coral habitats (Nemeth 1997).

Habitat selection influences the establishment of fish distribution patterns, especially as juvenile fishes settle from planktonic larval stages (Montgomery, Tolimieri & Haine 2001). Although differences in juvenile survival among physically dissimilar habitats may alter the distribution and relative abundance of damselfish post-settlement via both density-dependent and density-independent competition and mortality (Booth 2002; see also Nemeth 1998), habitat complexity itself can alter the relative contributions of these processes to survival (Johnson 2007). Competition, often expressed behaviorally as aggressive interactions, is important in establishing spatial variation in fish density among habitats, as adult fish will aggressively displace juveniles from preferred habitats to lower quality habitats where food availability may be lower or predation risk greater (Bay, Jones & McCormick 2001; Figueira et al., 2008). Because adults will aggressively displace juveniles, the frequency of aggressive behaviors often correlates with fish density (e.g., Osório et al., 2006), although the direction of the relationship varies depending on whether the interactions are intraspecific or interspecific. For example, the frequency of intraspecific aggressive interactions was found to be greater in habitats with higher conspecific densities for black triggerfish (*Melichthys niger*) (Kavanagh and Olney, 2006), as well as several Caribbean parrotfish species (Mumby and Wabnitz, 2002). Conversely, however, interspecific agonistic interactions were more numerous at lower densities of parrotfish (Mumby and Wabnitz, 2002). This is consistent with our findings in which bicolor damselfish aggression increased with greater conspecific densities, but was unrelated to fish species diversity.

Furthermore, fish density is commonly considered a major factor in the frequency and intensity of reproductive behaviors, and we observed more courtship displays in rubble habitats

with higher bicolor damselfish densities. Contrastingly, in a different territorial reef fish (*Chromis dispilus*), more time was spent on courtship displays in areas of low population density (Barnett & Pankhurst 1996). When accounting for physical and social conditions simultaneously, Semmens, Brunmaugh & Drew (2005) found no difference in aggression rates of blue tang (*Acanthurus coeruleus*) between flat, low relief carbonate rock and the high relief reef crest, even though the density of blue tangs was more than four times greater on the reef crest than the pavement. The results from Semmens, Brunmaugh & Drew (2005) indicate that behavioral variation in the blue tang may be more associated with physical habitat characteristics as opposed to conspecific density as was observed by Barnett & Pankhurst (1996). The general inconsistencies among studies, however, emphasizes that patterns of intraspecific behavioral variation are collectively influenced by the interaction of several physical and/or social environmental parameters, but the relative contributions of each are not readily distinguished.

Spatial variation in damselfish behavior associates with physical reef conditions

Although the results of the current and previous studies indicate several physical and social environmental parameters are interacting to shape spatial patterns of fish behavior, we nevertheless detected a strong influence of physical habitat on the expression of intraspecific behavioral variation in the bicolor damselfish. The strongest statistical correlations detected between behavioral variation and environmental conditions were observed between 'by focal' aggression by large damselfish and habitat PC1 in the independent correlation analyses and the path analyses. In contrast, for small damselfish habitat PC1 showed the strongest relationship with variation in shelter use. Path analysis also revealed a significant positive relationship between social environment (conspecific density) and behavior (courtship frequency) in large

bicolor damselfish, but no such relationship for small fish. At present, the mechanisms underlying the establishment of the habitat-behavior association patterns remain unknown. Potential mechanisms may include plastic developmental responses of behavior to local environmental conditions (e.g. West, King & White 2003; Stamps 2003), distinct patterns of habitat selection by developmentally or genetically distinct juvenile fishes during settlement (Nemeth 2005), post-settlement selection against particular behavioral phenotypes via predation or competitive exclusion (Figueira et al., 2008), or some combination of these and other factors. Nevertheless, our findings suggest intraspecific behavioral variation among bicolor damselfish is tightly coupled with physical habitat conditions, but the patterns of habitat-behavior relationships vary with fish size.

While our work has established spatial patterns of behavioral variation associated with habitat conditions in bicolor damselfish, the functional significance of the intraspecific behavioral variation is unclear. Previous studies of bicolor damselfish and other demersal coral reef fishes found individuals relegated to less preferred habitats may suffer from lower reproductive outputs, lower survival, or reduced growth rates (Munday 2001; Caley and Munday 2003; Nemeth 2003). Whether similar fitness-related differences occur in bicolor damselfish in habitats with differing values of PC1, however, is not clear. Furthermore, the majority of *A. cervicornis* coral rubble found in habitats with low PC1 values in the present study was created as a result of the extensive loss of *A. cervicornis* and other branching coral species from white band disease outbreaks during the 1980's (Bries et al., 2004; Wapnick et al., 2004 and citations within). The mass losses from disease – combined with impacts of coral bleaching, coastal development, and pollution – have significantly reduced live coral cover in shallow regions of the fringing reefs of Curaçao and nearby islands since the 1970's (Bak & Nieuwland 1995; Bak,

Nieuwland & Meesters 2005). The spatial variation observed here in bicolor damselfish behavior along a gradient of physical habitat variation, therefore, may not have been present 30 years ago, but rather may be a recent result of changes in the coral composition of Curaçao's fringing reefs since the 1970-1980's.

Summary

Species occupying broad geographic ranges frequently experience a range of environmental variation that can lead to phenotypic diversification through developmental and, ultimately, evolutionary changes. In this study, we document spatial patterns of variation in bicolor damselfish behavior associated with variation in physical (e.g., substrate hole number, rugosity, % substrate coral cover) and social (e.g., conspecific density, fish diversity) conditions of the local habitat. Intraspecific behavioral variation in bicolor damselfish was most strongly related to variation in the physical habitat structure, and the patterns of association between physical habitat and behavioral variation differed between large (> 4 cm, TL) and small (< 4 cm, TL) bicolor damselfish. Spatial patterns of intraspecific behavioral variation in bicolor damselfish are likely the result of complex interactions between physical and social conditions and life history stage. Our findings, combined with other recent studies identifying links between variation in fish demography and coral reef habitat structure (Paddack, Sponaugle & Cowen 2009; Afonso, Morato & Santos 2008; Kingsford and Hughes, 2005), indicate that spatial heterogeneity in coral reef habitat structure may have more substantial effects on intraspecific variation in reef fish behavior than previously recognized. Therefore, habitat structure (and alteration) may have a strong influence on critical aspects of coral reef fish ecology, including behaviors associated with reproduction and survival. Considering that coral reefs are continuing

to undergo major structural changes resulting from the combined impacts of coastal development, coral bleaching, pollution and disease (e.g. Knowlton 2001), future studies examining how the behavior and demography of reef-obligate species responds to variation in the physical and social habitat may provide insights into the response of reef species to changes occurring to the world's coral reefs.

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CHAPTER 2

EVIDENCE FOR HABITAT-ASSOCIATED INTRASPECIFIC VARIATION IN THE STRESS PHYSIOLOGY OF A CORAL REEF FISH, *STEGASTES PARTITUS*

This chapter has been prepared in the style of the journal Marine Ecology Progress Series

ABSTRACT

Relationships between geographic variation in behavior and spatial variation in environmental conditions have been observed in many species, but only rarely have the physiological bases for such behavioral variation been explored. Given the importance of hormones in regulating animals' responses to environmental conditions, endocrine signaling is likely to play a role in mediating habitat-associated variation in behavior. In a companion study, bicolor damselfish (*Stegastes partitus*) were found to display distinct patterns of behavior associated with spatial variation in the physical complexity of their coral reef environment. In this study, we examined stress-associated hormonal correlates of spatial variation in the behavior of bicolor damselfish inhabiting two areas of a fringing coral reef that differ in habitat structure: areas dominated by dead coral rubble (< 2% live coral cover), and areas near the reef slope with > 25% live coral cover. Bicolor damselfish in these two habitat types differed in behavior, with fish from rubble habitats showing more frequent aggression, shelter use, and courtship. Fish sampled from these two habitats at either < 2.5 min after collection ('baseline') or 20 min after collection ('stressed') showed differences in neural levels of mRNAs encoding the neuropeptides corticotrophin-releasing hormone (CRH) and urotensin 1, CRH binding protein (CRH-BP), and CRH receptors 1 (CRH-R1) and 2 (CRH-R2), to acute capture stress. In both male and female damselfish, CRH mRNA levels in the brain were altered by acute stress, although the direction of this change varied between females from rubble and reef habitats. Habitat origin also influenced CRH-binding protein (CRH-BP) mRNA levels in the brains of both sexes, with females again showing habitat-specific patterns of CRH-BP transcript changes in response to stress. Neural CRH-R2 mRNA levels were greater in males inhabiting rubble areas, but increased in males from both habitats following acute stress. Transcript abundance for urotensin

1 also varied among females both with habitat origin and stress condition, but was not affected by either factor in males. Taken together, these results demonstrate sex-specific variation in transcriptional responses of bicolor damselfish to acute stress, and provide the first evidence that environmental conditions of the local coral reef habitat can influence a fish's physiological response to stress.

INTRODUCTION

Spatial and temporal variation in the environment can have profound effects on animals as they cope behaviorally and physiologically with differing environmental conditions. Such changes often include not only variation in physical conditions like temperature, water flow, shelter availability, and structural complexity of the habitat, but also associated variation in social conditions (e.g., conspecific density, predator density). In coral reef ecosystems, for instance, habitat characteristics can vary considerably across different areas of the reef, and accordingly have been found to relate to changes in patterns of density and diversity of fishes that live there (i.e. Luckhurst and Luckhurst 1978; Roberts & Ormond 1987; Holbrook et al 2000; Paddack et al. 2009). More recently, spatial variation in coral reef habitat conditions has been linked to intraspecific variation in the demography (Kingsford and Hughes 2005; Afonso et al. 2008; Paddack et al. 2009) and behavior (Mumby and Wabnitz 2002; Osório et al. 2006; Kavanagh and Olney 2006; see Schrandt Chapter 1) of reef fish. Intraspecific demographic variation appears to result from changes in the behavioral and life history strategies of fishes under the varying physical and social pressures of the local habitats. Whether behavioral and demographic variation represents a developmentally plastic response to local environmental conditions, or differential selective pressures on different phenotypes in dissimilar environments, is not clear. Nonetheless, some of the variation may stem from immediate responses of behavior to current conditions, or may reflect developmental responses of growth, fecundity, and other life history traits (e.g. West-Eberhard 1989).

Regardless of the developmental and evolutionary context of intraspecific variation, the phenotypic changes seen among fish inhabiting dissimilar regions of a coral reef may be

mediated in part by changes in endocrine signaling. Hormones have a fundamental role in linking changes in environmental conditions with physiologic responses, ultimately resulting in phenotypic shifts (e.g., behavioral, life history). Variation in phenotypic expression within a species may be regulated by multiple endocrine pathways, although changes in the stress physiology of animals have been implicated in environmentally-associated differences in behavior in several vertebrate species (e.g., Boinski 1999). Stress in vertebrates has been defined many different ways, but ultimately deals with an individual's attempt to re-establish homeostasis when a stress signal is perceived (Schreck et al 2001). The glucocorticoid (e.g., cortisol) and catecholaminergic (e.g., epinephrine, norepinephrine) responses to short-term (or acute) stress are generally considered to be adaptive and function to help the animal reestablish physiological homeostasis or respond behaviorally to the environmental stressor (Ramsay et al. 2006). Stress, however, becomes chronic with continuous or repeated activation of glucocorticoid secretion over longer time scales. In such cases, the physiological stress response can become maladaptive and detrimental to growth, reproduction, and immune function (reviewed by Wendelaar Bonga 1997).

In fish, the glucocorticoid stress response occurs via activiation of the hypothalamo-pituitary-interrenal (HPI) axis, which is analogous to the mammalian hypothalamo-pituitary-adrenal (HPA) stress axis, and is a vertebrate adaptation for coping with a dynamic environment (Wendelaar Bonga 1997; Mommsen et al. 1999). Activation of the HPI axis commences when a stress signal is received by the hypothalamus, which then produces and secretes corticotropin releasing hormone (CRH). CRH peptide, which is normally bound to a carrier protein termed CRH-binding protein (CRH-BP) (Potter et al. 1991), then acts upon the anterior pituitary gland to stimulate the release of other hormones including adrenocorticotropic hormone (ACTH) (Metz

et al. 2004). ACTH stimulates the interrenal cells of the head kidney to synthesize and release the glucocorticoid steroid hormone cortisol into blood circulation (Flik et al. 2006), which has several key physiological functions including the mobilization of energy reserves, reallocation of energy away from growth and reproduction, temporary inhibition of immune function, and even changes in behavior that help animals cope with the stressor (Wendelaar Bonga 1997; Mommsen et al. 1999). Levels of both cortisol and ACTH in blood plasma therefore have been widely used as an indicator of stress condition.

Changes up-axis in the endocrine signals regulating these hormones, however, can also be indicative of stress condition since it is these hormones that ultimately activate cortisol secretion (Denver 2009). The release of ACTH from the pituitary gland occurs via CRH activation of CRH receptor-1 (CRH-R1) intracellular signaling pathways (Huising et al. 2004). Accordingly, the abundance of mRNAs encoding CRH and CRH-R1 in the hypothalamus and pituitary gland has been observed to change following exposure to an environmental stressor (Bernier et al. 2008; Chen and Fernald, 2008). A second CRH receptor, CRH receptor-2 (CRH-R2), has also been found in the piscine brain (Chen and Fernald 2008), and appears to function by interacting with the broader family of CRH-like neuropeptides including mammalian urocortin 1 and its teleost homolog urotensin 1 (Uroten1) (Bale and Vale, 2004; Denver, 2009). While binding affinities of the teleost CRH-R2 receptor have not been examined, mammalian CRH-R2 has a high affinity for urocortin 1 (Wei et al 1998; Hsu and Hsueh 2001; Bale and Vale, 2004). However, in fish, Uroten1 has been demonstrated to activate CRH-R1 with an affinity similar to CRH (Arai et al. 2001; Huising et al. 2004), suggesting that Uroten1 likely plays a key role along with CRH in regulating the HPI fish stress response (Bernier et al. 2008).

While the response of the HPI axis to stress has been examined in several model fish species (reviewed by Wendelaar Bonga 1997), little is known about how stress signaling pathways might relate to intraspecific behavioral variation among individuals or populations in the wild. In other vertebrates, changes in stress reactivity – or the response of glucocorticoids and other hormones involved in HPA axis signaling – have been shown to differ among populations occupying ecologically dissimilar habitats (e.g., Boinski 1999). Also, differences in stress physiology have been found among populations of birds occupying natural and anthropogenically-impacted environments (Lucas et al. 2006; Müllner et al. 2004; Romero and Wilkelski 2002; Wasser et al. 1997), again indicating a relationship between local habitat conditions and stress physiology. Similar relationships have yet to be explored in fish even though fish in some habitats, such as coral reefs, can display considerable intraspecific variation in behavioral and life history traits.

Here we examine whether bicolor damselfish (*Stegastes partitus*), a demersal coral reef fish found in the Caribbean Sea, from different coral habitats have differing responses of the HPI axis to acute stress. In a previous study with bicolor damselfish, Schrandt and coworkers (see Chapter 1) identified distinct patterns of association between intraspecific behavioral variation in this species and variation in the coral reef environment. More specifically, large (> 4, cm TL) bicolor damselfish inhabiting shallow coral rubble areas exhibited higher rates of aggression, shelter use and courtship than bicolor damselfish in areas with higher coral cover nearer the reef slope (see Chapter 1). Although this previous work revealed distinct relationships between physical conditions of the coral reef and behavioral variation in bicolor damselfish, the physiological bases for this behavioral variation have not been examined.

In this study, we explore whether the intraspecific behavioral variation seen among bicolor damselfish in different coral reef habitats may be related to variation in stress reactivity. Specifically, we assessed whether differences in the response of the HPI axis to acute stress were present in large (> 4 cm total length (TL)) bicolor damselfish occupying two discrete types of coral reef habitat: 1) dead coral 'rubble' characterized by a low percentage of live coral cover on the substrate, and 2) live coral 'reef' with a comparatively high percent coral cover. Observations of the behavior of bicolor damselfish from each habitat type were first performed to confirm the behavioral differences previously observed (see Schrandt Chapter 1) between fish from the two habitats. We then collected bicolor damselfish from each habitat type and quantified changes in several stress-associated mRNAs (e.g., CRH, CRH-BP, CRH-R1, CRH-R2, and Uroten1) in the brain following acute capture stress. The integrated experimental design allowed us to assess associations among variations in the physical habitat of the coral reef, behavior of adult bicolor damselfish, and the response of the bicolor damselfish HPI axis to acute stress.

MATERIALS AND METHODS

Identification of stress-associated cDNAs from bicolor damselfish

Isolation and sequencing of partial cDNA sequences

Using scuba, divers collected two adult male bicolor damselfish (standard lengths: 45.30 mm and 65.05 mm) by hand net (SlicDive Inc., Gilbert, SC, USA) on 14 November 2006, from the fringing coral reefs of Curaçao, the Netherlands Antilles, in the southern Caribbean Sea. Each fish was euthanized in tricaine methanesulfonate (MS-222) (Argent Chemical, Redmond,

WA, USA), and the whole brain was immediately dissected and placed in RNAlater (Ambion, Inc., Austin, TX, USA) at 4°C for 24 hrs before being stored at -20°C. Total RNA was extracted from the brains of the fish using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) with bromochloropropane as the phase separation reagent, and then quantified by spectrophotometry (NanoDrop 1000, NanoDrop Technologies, Wilmington, DE, USA). RNA quality was confirmed by electrophoresis of the RNA on a 0.8% agarose gel.

Total RNA was reverse transcribed in a 20 µL reaction by first incubating 2 µg of total RNA template with 0.5 µL random hexamer (10 µM), 1 µL dNTPs (10 mM), and 8.43 µL water at 65˚C for 5 min. The mixture was placed on ice for 1 min and 4 µL of 5X First Strand Buffer was added, along with 2 µL of 0.1M DTT, 1 µL of RNase inhibitor, and 1 µL of Superscript III reverse transcriptase (SuperScript III Reverse Transcription kit, Invitrogen, Carlsbad, CA, USA.). The mix was then incubated under a thermal profile of 25˚C for 10 min, 42˚C for 50 min, and 70˚C for 5 min, before being stored at -20˚C.

PCR was performed using degenerate primers designed to consensus regions of cDNA sequences from other teleost fishes (Table 1). Degenerate primer PCR was performed in 50 µL reactions containing 36.6 µL water, 5.0 µL 10X buffer, 3.0 µL of 25 mM $MgCL_2$, 0.4 µL GoTaq DNA polymerase (5 u/µL), 1.0 µL of 10 mM dNTPs, 1.0 µL each of forward and reverse primers (50 µM), and 2.0 µL of cDNA template. The following thermal profile was used: 95˚C for 2 min, 35 cycles of 95˚C for 30 s, 51˚C for 30 s, 72˚C for 1 min, and then 72˚C for 5 min. When examination on a 1.2% agarose gel revealed a band of predicted size, the cDNA was purified (QIAquick PCR purification Kit, Qiagen Inc., Valencia, CA) and sequenced on an ABI PRISM 3100 Genetic Analyzer using Big Dye Terminator Cycle Sequencing Kit v 3.1. The resulting sequences were then aligned using Sequencher v. 4.8 (GeneCodes, Ann Arbor, MI) and

Table 1. Nucleotide sequences of degenerate primers used for isolation of partial cDNAs.

Transcript	Species Used for Consensus Regions	GenBank Accession Number	Primers Developed	Primer Sequence (5' - 3')
CRH	*Cyprinus carpio*	AJ317955	CRHfor1	CTCAATTT(A/T)(C/T)TCG(G/T)(C/T)ACCAC
	Danio rerio	BC085458	CRHfor2	GTG(A/G)(C/T)TCTGCT(A/C)GTTGCCTT
	Oreochromis mossambicus	AJ011835	CRHrev1	AGCAG(A/G)TG(A/G)AAGGTCAG(A/G)TC(C/T)AGGGA
			CRHrev2	GATGTT(C/T)CCAACTTT(C/G)CCCT
CRH-R1	*Ameiurus nebulosus*	AF229359	CRHRdegFor1	GTCCGHTACAACACCACCAATAA
	Carassius auratus	AY688837	CRHRdegFor2	AAGAGCAAGCTGCA(C/T)TACCACAT
	Cyprinus carpio	AJ576244	CRHRdegRev1	TGAAAGGACTG(G/T)AGGAAAGA(A/G)TT(A/G)AA(A/G)TA
	Epinephelus coioides	AY820281	CRHRdegRev2	TTCCTGTACTG(A/G)AT(C/G)GTCTCTGA(C/G/T)GTG
			CRHRdegRev3	ATCAG(A/C/T)AG(A/G)AC(A/C/G)AGGATCATGGG
CRH-R2	*Ameiurus nebulosus*	AF229360	CRH-R2degFor1	GAGCC(G/T)TGGTG(C/T)CG(C/T)CT(C/T)ATAAC
	Danio rerio	XM_681362	CRH-R2degFor2	GGTGAC(C/G)AATTTTTTCTGGAT
	Oncorhynchus keta	AJ277158	CRH-R2degFor3	ATGAC(A/C/T)TA(C/T)TC(C/T)AC(A/C)GACAAG
			CRH-R2degRev1	GGTGA(G/T)GTGGGRA(G/T)GGACAT
			CRH-R2degRev2	AACAGCATGTA(G/T)GTGAT(C/G/T)CC
			CRH-R2degRev4	CCAAACCAGCA(C/T)TGTTC(A/G)TTTTC
CRH-BP	*Cyprinus carpio* (CRH-BP1)	AJ490880	CRHBPfor2	CAG(A/G)GGAGG(A/G)GA(C/T)TTCAT(A/C)AAGGT
	Cyprinus carpio (CRH-BP2)	AJ490881	CRHBPfor3	TTTGATGG(C/G)TGGGTGATGAAGGG
	Oncorhynchus mykiss	NM_001124631 &	CRHBPfor4	AAAC(C/T)CATCAA(C/T)CC(G/T)TTCCCCTG
		AY363677	CRHBPrev2	CACCAT(C/T)CT(C/G)A(C/T)CAC(A/C)GTGTTATC
			CRHBPrev3	CAGTTCCTGTGCTGCTG(G/T)GG
Uroten1	*Carassius auratus*	AF129115	Uroten1-degF1	ATGAAGCC(C/G/T)GTC(C/T)C(A/C/T)TTG(A/C/G)TCCTGCTC
	Cyprinus carpio	M11671	Uroten1-degF2	TTG(A/C/G)TCCTGCTC(A/C/T)T(A/C/T)(A/G/T)C(C/T)TC(A/C/T)GTC
	Danio rerio	NM_001030180	Uroten1-degF3	TCCTGCTC(A/C/T)T(A/C/T)(A/G/T)C(C/T)(A/T)C(A/C/T)GT(C/T)(C/T)T(A/C)CT
	Oncorhynchus mykiss	AJ005264	Uroten1-degR2	CGCCAT(G/T)T(C/G)GATCAT(A/G)TT(C/T)CT(C/G)AG
	Platichthys flesus	AJ571694	Uroten1-degR3	GTGGAA(A/G)GT(C/G)AGGTCGATGGA
EF-1α	*Carassius auratus*	AB056104	EF1αfor1	GGGAAAGGAAAA(A/G)A(C/T)CCACAT
	Oryzias latipes	NM_001104662	EF1αfor2	CACAT(C/T)AACATCGTGGT(C/T)ATTGGC
	Pagrus major	AY190693	EF1αrev1	C(C/T)TTGAC(A/G)GACACGTTCTT(G/C)A
	Seriola quinqueradiata	AB032900	EF1αrev2	ACGTTGTCACCAGG(A/C/G)(A/G)(C/T)(A/G)GC
β-actin	*Carassius auratus*	AB039726	BAfor1	ATCATGTT(C/T)GAGACCTTCAACACCC
	Cirrhinus molitorella	DQ007446	BArev1	TACTCCTGCTTGCT(A/G)ATCCACAT
	Danio rerio	AF057040	BArev2	GCAATGCC(A/G)GGGTACATGGT
	Spinibarbus denticulatus	DQ656598		
	Rivulus marmoratus	AF168615		
18S	*Cyprinidon variegatus*	EF535030	18Sfor1	CCTGCGGCTTAATTTGACCCAACA
			18Srev1	GACATCTAAGGGCATCACAAGACCT
			18Srev2	TTGCTCAATCTCGTGTGGCTCAAC

their identities confirmed by BLASTX searching against known teleost sequences provided in GenBank.

For corticotropin-releasing hormone (CRH), nested primers were designed to *Cyprinus carpio* (GenBank accession no. AJ317955), *Danio rerio* (BC085458), and *Oreochromis mossambicus* (AJ011835). The outer and inner nested primers amplified a 396-bp partial sequence of bicolor damselfish CRH provided at GenBank accession no. HM047108. Degenerate primers for CRH receptor 1 (CRH-R1) were designed to consensus regions of CRH-R1 cDNAs from *Ameiurus nebulosus*, (AF229359), *Carassius auratus* (AY688837), *Cyprinus carpio* (AJ576244), and *Epinephelus coioides* (AJ820281), and for CRH receptor 2 (CRH-R2) to cDNAs from *Ameiurus nebulosus* (AF229360), *Danio rerio* (XM_681362), and *Oncorhynchus keta* (AJ277158). Theses primers amplified a 575-bp partial sequence of CRH-R1 (GenBank accession no. HM047110) and a 407-bp sequence of CRH-R2 (GenBank accession no. HM047111) from bicolor damselfish. For CRH binding protein (CRH-BP), nested degenerate primers were designed to CRH-BP cDNAs from *Cyprinus carpio* CRH-BP1 (AJ490880), *Cyprinus carpio* CRH-BP2 (AJ490881), and *Oncorhynchus mykiss* (NM_001104662 and AY363677), which amplified a 503-bp partial sequence of bicolor damselfish CRH-BP (GenBank accession no. HM047112). Lastly, a 344-bp partial cDNA sequence of urotensin 1 from bicolor damselfish (GenBank accession no. HM047113) was amplified using degenerate primers designed to the urotensin 1 cDNAs of *Carassius auratus* (AF129115), *Cyprinus carpio* (M11671), *Danio rerio* (NM_001030180), *Oncorhynchus mykiss* (AJ005264), and *Platichthys flesus* (AJ571694).

A 754-bp, partial cDNA sequence for elongation factor-1α (EF-1α) was also isolated and sequenced from bicolor damselfish (GenBank accession no. HM047114) using degenerate

primers designed to consensus regions of cDNAs for EF-1α from *Carassius auratus* (AB056104), *Oryzias latipes* (NM_001104662), *Pagrus major* (AY190693), and *Seriola quinqueradiata* (AB032900). Partial cDNAs encoding 18S ribosomal gene and β-actin were also isolated and sequenced from bicolor damselfish as alternative control genes. Degenerate primers for 18S were designed to consensus regions of the 18S gene from *Cyprinidon variegatus* (EF535030), and resulted in a 272-bp partial cDNA sequence (GenBank accession no. FJ707475). A 691-bp partial cDNA sequence of β-actin for bicolor damselfish (GenBank accession no. HMO47109) was amplified using degenerate primers designed to consensus regions from *Carassius auratus* (AB039726), *Cirrhinus molitorella* (DQ007446), *Danio rerio* (AF057040), *Spinibarbus denticulatus* (DQ656598), and *Rivulus marmoratus* (AF168615).

Comparison of stress reactivity in bicolor damselfish from different reef habitats

Habitat and behavioral assessments

From 2 to 7 June 2009, adult bicolor damselfish were collected from two distinct coral reef habitats: coral rubble and live *Montastrea sp.* dominated reef. All fish were collected at Playa Kalki, located at the western end of the southern leeward coast (12˚22'31.63" N, 69˚09'29.62"W) of Curaçao, the Netherlands Antilles, in the southern Caribbean Sea (Fig. 1). Offshore of Playa Kalki is a fringing reef that transitions from dead coral rubble (largely *Acropora cervicornis* remnants) in the shallows to live corals on the reef slope (Bruckner and Bruckner 2003).

Bicolor damselfish were studied in two areas characterized as belonging to distinct coral reef habitat types: coral rubble ('rubble'), and *Montastrea*-dominated reef ('reef') habitats. These two habitat types were identified previously when Schrandt and coworkers (see Chapter 1)

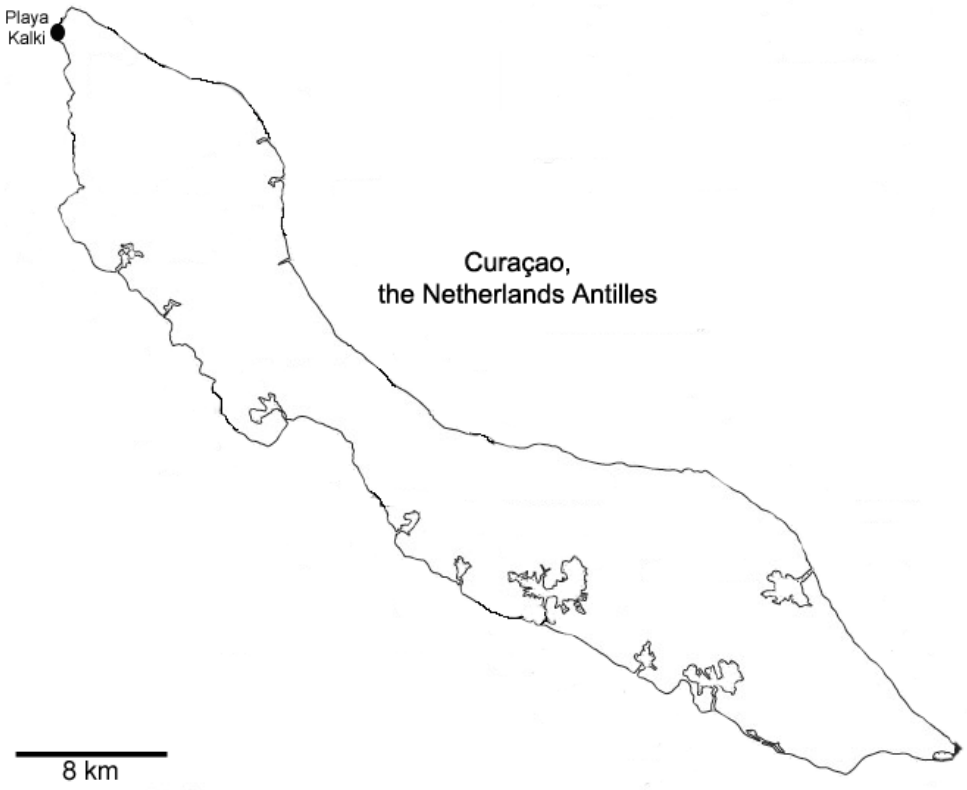


Figure 1. Map showing the location of the Playa Kalki fringing reef sampling site on the leeward side of Curaçao, the Netherlands Antilles in the southern Caribbean Sea.

documented that the behavior of bicolor damselfish exhibited spatial patterns of variation associated with coral reef habitat conditions at the Playa Kalki site. Transects (20 m in length and 3 m apart) were established parallel to the shoreline, with two transects located in the rubble and two within the live coral habitat. Previous work revealed that rubble and reef habitats could be distinguished by the % live coral cover on the benthos (see Schrandt Chapter 1); therefore, to confirm that the transects were located in the appropriate habitat types, photographs were taken at 1 m intervals along each of the four transects using a Canon Powershot 990 IS camera (Canon USA Inc., Lake Success, NY, USA). Photographs were later analyzed for percent coral cover using Coral Point Count 3.6 (Kohler and Gill 2006). As in our previous characterization of habitat at this site (see Schrandt Chapter 1), rubble transects were found to contain a mean benthos cover of < 2% live coral (range among individual quadrats along the transects: 0.0 to 9.9% live coral cover), while the reef transects had a mean percent live coral cover of > 25% (range: 4.9 to 58.0% live coral cover).

The behavior of bicolor damselfish was characterized by focal observations of individual fish within the two habitat types. At 1 m intervals along each of the 4 transects, a single bicolor damselfish (>4 cm, TL) was haphazardly selected and its behavior recorded for 6 min by an observer on scuba. All behavioral observations were conducted between 1050 and 1600 hr. Observers remained at least 1 m away from the focal fish at all times so as to not disturb the fish's behavior. The frequency of aggressive chases (focal fish either chasing or being chased by another fish), shelter use (focal fish entering a shelter), and courtship behaviors (discrete swimming dips performed by males) was recorded (see also behavior descriptions by Schrandt et al. (see Chapter 1) and Myrberg 1972). A total of n = 38 and n = 40 bicolor damselfish were observed from the rubble and reef habitats, respectively. The frequencies of behaviors were

averaged among fish observed within each habitat to obtain behavioral profiles of bicolor damselfish for each habitat type.

Damselfish Collection

Adult bicolor damselfish (>4 cm, TL) were collected (between 1200 and 1630 hr) by scuba divers using hand nets from the areas between each set of transects within the rubble and reef habitats. These collections provided a sample of fish from each habitat type. For each fish collected, the time at which the fish first entered the net during capture was noted, and written on waterproof paper (DuraCopy Waterproof paper, Rite in the Rain, J.L. Darling Corporation, Tacoma, WA, USA) by the divers. While still underwater, divers quickly transferred the captured fish from the hand net to a plastic bag, which was then immediately passed to a free diver who swam the fish and paper (with the time written) to an anchored kayak floating at the surface. Once at the surface, collected bicolor damselfish were euthanized (MS-222) at one of two time points: 1) within 2.5 min of capture for a 'baseline' assessment of stress indices, or 2) at 20 min after capture for a 'stressed' assessment. Fish held until the 20 min 'stressed' sampling time were maintained in the bag under ambient water temperatures until the time of euthanization.

For each fish, blood was collected from the caudal artery and placed on ice before being centrifuged at room temperature for plasma collection. Plasma was stored at -80˚C for later measurement of cortisol levels. In addition, the brain was dissected and stored in RNAlater for subsequent extraction and quantification of stress associated mRNA levels. A total of n = 52 and n = 53 fish were collected from the rubble and reef habitats, respectively, with n = 4 – 22 fish per sex and sampling time for each habitat. Gonads were also dissected from each fish and fixed for

24 hr in 4% paraformaldehyde before being stored in 70% ethanol at 4˚C for later histological confirmation of gonadal sex. Both gonads were collected from each fish because visual assessment at the time of dissection suggested that some fish possessed two gametogenically distinct gonads.

Quantitative Real-Time RT-PCR of Stress-Responsive Transcripts

Total RNA was extracted from the whole brains of bicolor damselfish using TRI-Reagent (Molecular Research Center, Cincinnati, OH, USA) with bromochloropropane as the phase separation reagent. The resulting RNA was DNase I treated (DNA-free Treatment kit, Ambion, Austin, TX, USA) and quantified by spectrophotometry (Nanodrop 2000, ThermoScientific, Wilmington, DE, USA). The DNase I treated total RNA was then reverse transcribed in 5 µl reactions containing 1.0 µl 5x First Strand Buffer, 0.25 µl dNTPs (10 mM), 0.5 µl random primers (10 µM), 0.5 µl DTT (0.1 M ; Invitrogen), 0.125 µl RNaseOut inhibitor, 0.25 µl Superscript III reverse transcriptase (Invitrogen), and 2.375 µl (105.26 ng/µl) of total RNA template. All RT reactions were run in 96 well plates under a thermal profile of 25˚C for 10 min, 50˚C for 50 min, and 85˚C for 5 min (MyCycler thermal cycler, Bio-Rad).

Primers for SYBR green quantitative real-time PCR assays were designed (Primer Quest, Integrated DNA Technologies, and Primer Express 2.0, Applied Biosystems, Inc.) to the partial cDNAs for CRH , CRH-R1, CRH-R2, CRH-BP, and urotensin 1 identified previously from bicolor damselfish (see above). Primers were also designed for EF-1α from bicolor damselfish for comparison as a control gene. All primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA) and are provided in Table 2.

Table 2. Nucleotide sequences for primers used in quantitative real time RT-PCR.

Transcript	Primer	Sequence (5' - 3')	Amplicon Size (bp)	PCR efficiency (avg. %)
CRH	Forward	GCGGCTTGGAGAGGAGTATTTCAT	121	94.7%
	Reverse	CAGCTGGAGTTGTAACGCTCTGTT		
CRH-R1	Forward	ATGTTCGGAGAGGGCTGCTA	101	94.8%
	Reverse	GGTATACACCAGCCGATGCA		
CRH-R2	Forward	AGCTGAGAAAGTGGGTCTTCCTCT	273	91.4%
	Reverse	TCGCTTTCACGGCTTTCCTGTACT		
CRH-BP	Forward	CATGGTCTTCTTCCGCATCCA	101	94.9%
	Reverse	CTGGTGACTGGGAGATGACATTACA		
Uroten1	Forward	TGAGCGACAACATCCTGAGGTT	103	98.4%
	Reverse	GTCCTCACCGCCTCATCGT		
EF-1α	Forward	ACAAGTGCGGAGGAATCGACAAGA	366	92.6%
	Reverse	CAACAATGAGCTGCTTCACACCGA		

Quantitative real-time PCR reactions were conducted in 25 µl reactions. Each reaction contained 6.5 µl nuclease-free water (Sigma, St. Louis, MO, USA), 12.5 µl iQ SYBR green Supermix (Bio-Rad, Hercules, CA, USA), 1.0 µl each of forward and reverse primers (3.75 µM – 10 µM), and 4.0 µl of reverse-transcribed cDNA template. The PCR thermal profile for each reaction was 50°C for 2 min, 95°C for 10 min, 42 cycles of 95°C for 15 s and 59°C for 1 min, and all assays were run on a Bio-Rad iCycler with a MyiQ™ Single Color PCR Detection System (Bio-Rad, Hercules, CA, USA). Melt curve analysis was performed to assess amplification of a single product and the absence of primer-dimers. Specificity of these SYBR green primer sets for the desired stress-related genes was confirmed by sequencing selected PCR products. For each gene of interest, a serially diluted standard curve was made from a pool of RNA from samples representing all habitats, times, and sexes. All standards were assayed in triplicate. DNA contamination was assessed for each gene measured by analyzing a total RNA sample that was not reverse-transcribed, and each qPCR run included two samples without cDNA template to further control for contamination. EF-1α was quantified as the normalizing gene. The mean EF-1α transcript abundances were similar between both habitats ($t = -1.835$; $df = 94$; $p = 0.0694$) and baseline and stressed sampling times ($t = 1.209$; $df = 94$; $p = 0.2295$). For each gene, correlation coefficients (r^2) for the standard curve ranged from 0.982 – 0.992. PCR efficiencies for each gene were calculated using the equation: efficiency = $10^{(-1/slope)} - 1$, and are provided in Table 2. For each gene, relative mRNA levels were subsequently calculated using the standard curve and normalized to EF-1α mRNA expression. Finally, expression of each gene of interest was expressed as a relative level by dividing the resulting values by the baseline stress value of male fish collected from the rubble habitat.

Histological confirmation of gonadal sex

After 24 hr fixation in 4% paraformaldehyde and storage in 70% ethanol at 4˚C, gonadal tissues were dehydrated in a graded ethanol series. Ethanol was removed from the tissues with toluene. Tissues were then paraffin infiltrated overnight at 60°C before being embedded in paraffin. Gonads were sectioned (10 μm) by rotary microtome, and three sections spaced at distances 100 - 400 μm apart were obtained from each gonad and mounted onto albumin-coated glass slides. Slides were then stained with hematoxylin and eosin, and the resulting tissues were photographed with a digital camera (SPOT RT KE, Diagnostic Instruments, Inc., Sterling Heights, MI, USA) attached to an Olympus BX-60 light microscope operating in brightfield mode. The sex of each bicolor damselfish was confirmed by visual identification of spermatogenic or oogenic cells within each gonad (Leino et al. 2005).

Statistical Analyses

Non-parametric Mann-Whitney U tests were used to examine differences in the frequencies of behaviors of fish from the rubble and reef habitat areas (SPSS 16.0, SPSS Inc., Chicago, IL, USA) because data failed to conform to the assumptions of parametric statistics and could not be transformed successfully. Spearman's correlations were also performed to determine whether particular behaviors were correlated in rubble and reef habitats (JMP 7.1 software, SAS, Cary, NC, USA). All data are shown as mean ± SEM.

Relative mRNA values were log transformed to yield normal distributions and analyzed separately for male and female bicolor damselfish using two-way ANOVA models with habitat origin ('rubble' or 'reef') and stress condition ('baseline' or 'stressed') as factors (JMP 7.1 software, SAS, Cary, NC, USA). When main factor effects or interaction effects were found to

be significant, pairwise comparisons between transcript abundance values for the two levels within that factor were calculated using Fisher's protected LSD tests.

RESULTS

Bicolor damselfish behaviors in rubble vs. reef habitats

The frequencies of aggressive interactions, shelter use, and courtship behaviors differed between bicolor damselfish in rubble and reef habitats (Fig. 2). Bicolor damselfish in the rubble habitat initiated aggressive interactions ('by focal' aggression) nearly four times more often than fish from reef habitats ($U = 294$; $z = -4.738$; $p < 0.001$) (Fig. 2a). Bicolor damselfish from the rubble habitat also showed more frequent aggression overall (total aggression, or the sum of 'by' and 'at focal' aggression) than fish from the reef habitat ($U = 219$; $z = -5.450$; $p < 0.001$) with nearly three times more total aggression in the rubble; however, there was no significant difference in 'at focal' aggression frequencies for fish in the rubble and reef habitats ($U = 675$; $z = -0.940$; $p = 0.347$) (Fig. 2a). Shelter use also differed for fish in the rubble and reef habitats, with rubble fish entering shelters more often than fish from the reef ($U = 547$; $z = -2.138$; $p = 0.032$) (Fig. 2b). Lastly, the frequency of courtship dips was more than three times greater in male bicolor damselfish in the rubble than in fish in the reef ($U = 542.5$; $z = -2.484$; $p = 0.013$) (Fig. 2c).

We also observed several significant correlations among the behaviors performed by an individual focal damselfish, although these correlations were not always the same for fish in the rubble and reef habitats. The frequency of 'by focal' aggression showed a significant correlation with total aggression for fish in both the rubble ($\rho = 0.8947$; $p < 0.0001$) and reef habitats ($\rho =$

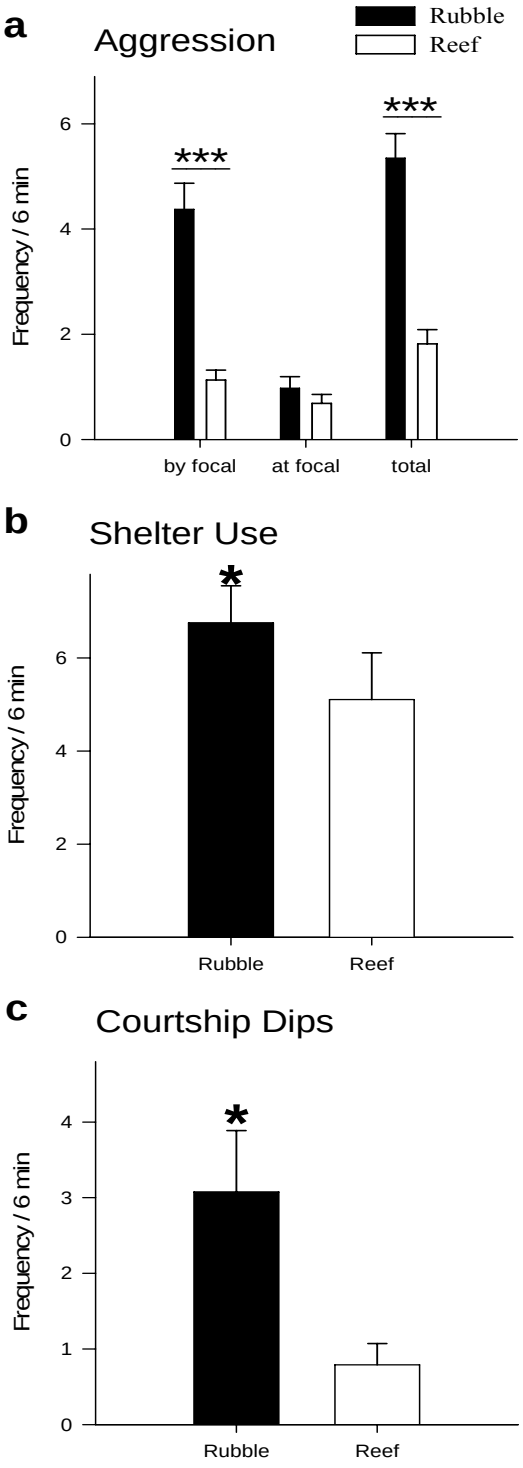


Figure 2. Behavioral variation in adult bicolor damselfish (> 4 cm, TL) from rubble and reef habitats. Mann-Whitney U tests revealed more 'by focal' and 'total' aggression in rubble habitats (a), as well as more shelter use (b) and courtship dips (c) in rubble habitats when compared to reef habitats. Asterisks indicate statistically significant differences (* $p < 0.05$, *** $p < 0.001$).

0.7971; $p < 0.0001$), while the frequency of 'at focal' aggression was only correlated with total aggression in fish occupying the reef habitat $\rho = 0.6368$; $p < 0.0001$) and not the rubble ($\rho = -0.0663$; $p = 0.6960$). Aggression (any measure) was not correlated with shelter use, but was correlated with courtship in the rubble. In the rubble habitat, courtship dips and 'by focal' aggression were positively correlated ($\rho = 0.4682$; $p = 0.0023$), along with courtship dips and total aggression ($\rho = 0.5243$; $p = 0.0005$). These associations, however, were not seen in bicolor damselfish in the reef habitat ($\rho = 0.2500$; $p = 0.1301$ for 'by focal' and $\rho = 0.1850$; $p = 0.2660$ for total aggression vs. courtship). Shelter use and courtship dips were also positively correlated in the reef habitat ($\rho = 0.6506$; $p < 0.0001$) but not in the rubble habitat ($\rho = 0.1243$; $p = 0.4449$).

Stress-associated mRNA responses in fish from rubble vs. reef habitats

In male bicolor damselfish, acute capture stress significantly affected neural mRNA levels of CRH, with stressed fish having higher relative CRH expression ($F_{(1,34)} = 5.165$; $p = 0.0271$) (Fig. 3a). This acute stress effect on CRH mRNA levels was similar for males collected from the rubble and reef habitats ($F_{(1,34)} = 2.336$; $p = 0.1323$). In female bicolor damselfish, acute stress also affected the relative expression of CRH, but the direction of change was different for females from the two habitats (habitat*time interaction $F_{(1,34)} = 3.989$; $p = 0.0538$) (Fig. 3b). In female fish from rubble habitats, the relative abundance of CRH transcript decreased with acute stress, while females from the reef habitat showed the opposite response: an increase in relative CRH transcript abundance following acute stress.

For CRH-BP, higher relative mRNA levels were observed in male fish from the reef habitat compared to the rubble ($F_{(1,54)} = 4.955$; $p = 0.0302$) (Fig. 3c). Relative CRH-BP mRNA levels were similar between baseline and stressed fish, and there was no interaction between

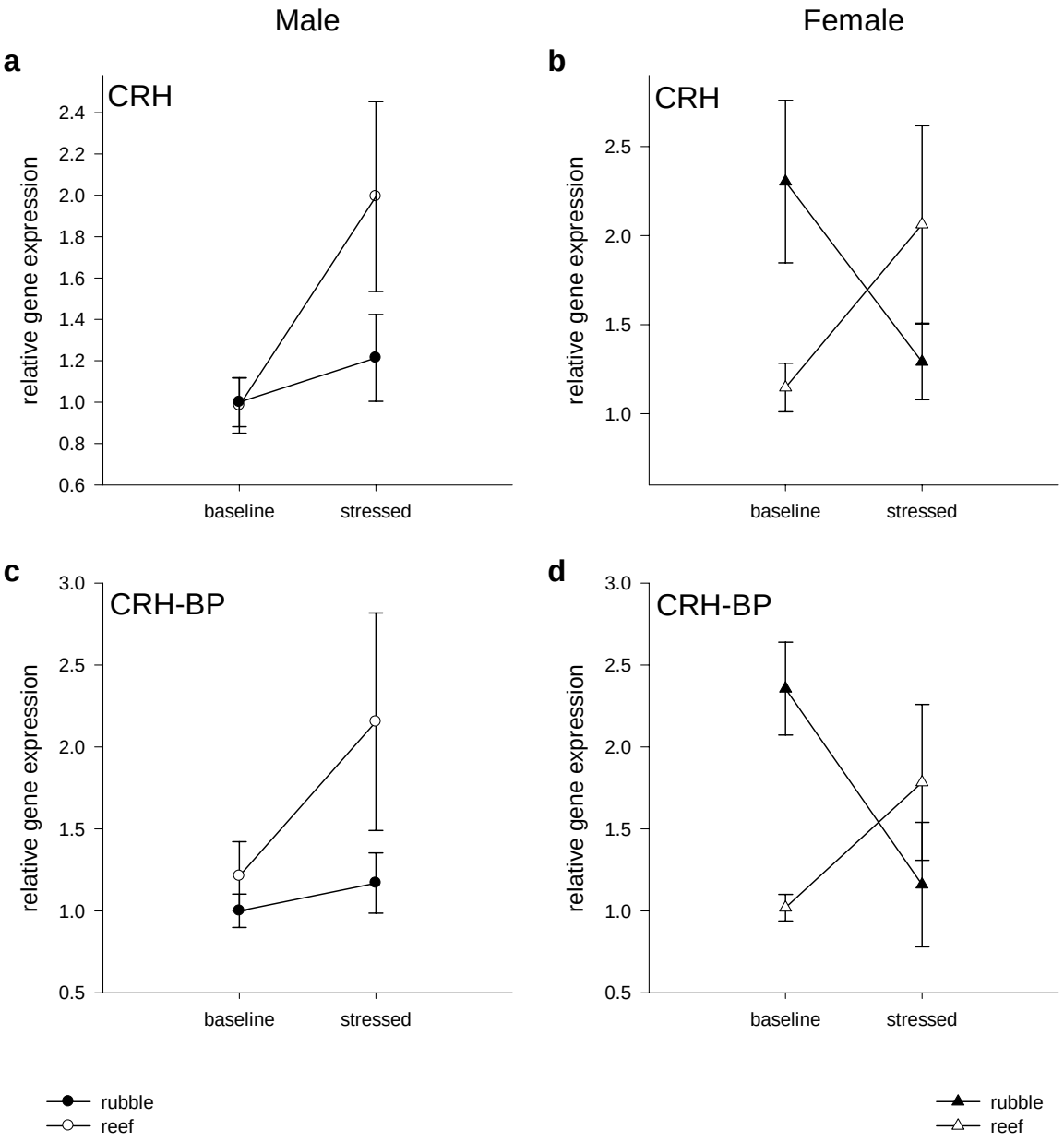


Figure 3. Real time quantitative RT-PCR comparison of CRH and CRH-BP mRNA levels (± SEM) in the brain of bicolor damselfish from two different habitats and stress levels ('baseline' representing < 2.5 min after capture or 'stressed' representing 20 min after capture). Two-way ANOVA models with habitat origin and stress condition as factors were used to compare relative mRNA levels within male and female fish separately. Significant pairwise differences were also found for selected factors identified previously as significant in the ANOVA models. In females, transcript abundances of CRH and CRH-BP were higher at baseline in the rubble habitat than in the reef habitat ($t = 2.781$; $df = 20$; $p = 0.0115$ for CRH; and $t = 4.430$; $df = 20$; $p = 0.0003$ for CRH-BP). CRH mRNA levels showed a near significant increase from baseline to stressed condition in male bicolor damselfish collected from the reef habitat ($t = 2.088$; $df = 18$; $p = 0.0513$).

habitat and time. For females, CRH-BP mRNAs showed a pattern of change similar to those of CRH mRNAs following acute stress. While there was no difference in CRH-BP transcript expression between habitats or times for females, there was a significant habitat*time interaction ($F_{(1,34)} = 9.188$; $p = 0.0046$) (Fig. 3d). Similar to CRH, relative mRNA abundances decreased following acute stress in females from the rubble habitat, but increased in females from the reef habitat.

Relative transcript levels of CRH-R1 were not affected by either habitat or stress condition in either males or females (Fig. 4a,b). CRH-R2 transcript levels in males, however, were greater in males from rubble habitats than in males from reef habitats ($F_{(1,54)} = 5.749$; $p = 0.0200$). Additionally, acute capture stress increased CRH-R2 transcript abundance in the brain of stressed males ($F_{(1,54)} = 6.165$; $p = 0.0162$) (Fig. 4c). Conversely, CRH-R2 mRNA levels in females were not affected by either habitat origin or stress condition (Fig. 4d).

Transcript abundance of urotensin 1 did not vary with either habitat origin or stress condition in male damselfish (Fig. 5a), but was affected by acute stress in females, although the direction of that effect varied depending on which habitat the fish occupied (habitat*time interaction $F_{(1,34)} = 11.550$; $p = 0.0017$) (Fig. 5b). In females from rubble habitats, relative urotensin 1 mRNA levels decreased 20 min after the stress of capture, but increased after stress in females collected from the reef habitat.

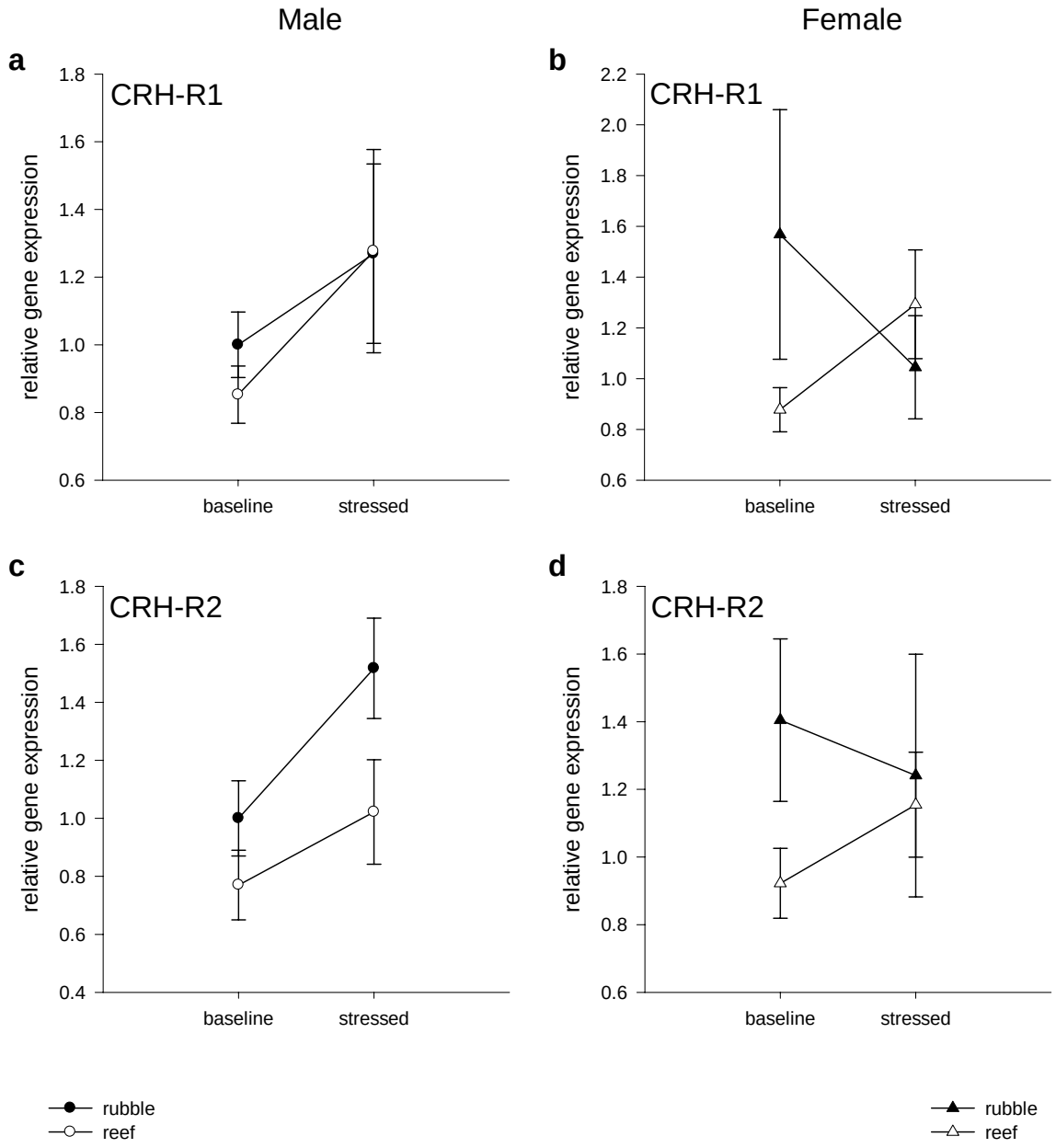


Figure 4. Real time quantitative RT-PCR comparison of CRH-R1 and CRH-R2 mRNA levels (± SEM) in the brain of bicolor damselfish from two different habitats and stress conditions. Two-way ANOVA models using habitat origin (rubble or reef) and stress condition (baseline or stressed) were used to compare relative mRNA levels within male and female fish separately. CRH-R1 mRNA levels did not vary with either habitat origin or stress condition in either sex. In contrast, CRH-R2 mRNA levels were greater in males from rubble habitat, and increased in males from both habitats 20 min after acute capture stress. Pairwise comparisons indicated significant increases in CRH-R2 mRNA levels following acute stress in males from rubble habitats ($t = 2.682$; $df = 36$; $p = 0.0110$) and from reef habitats ($t = 2.235$; $df = 26$; $p = 0.0342$).

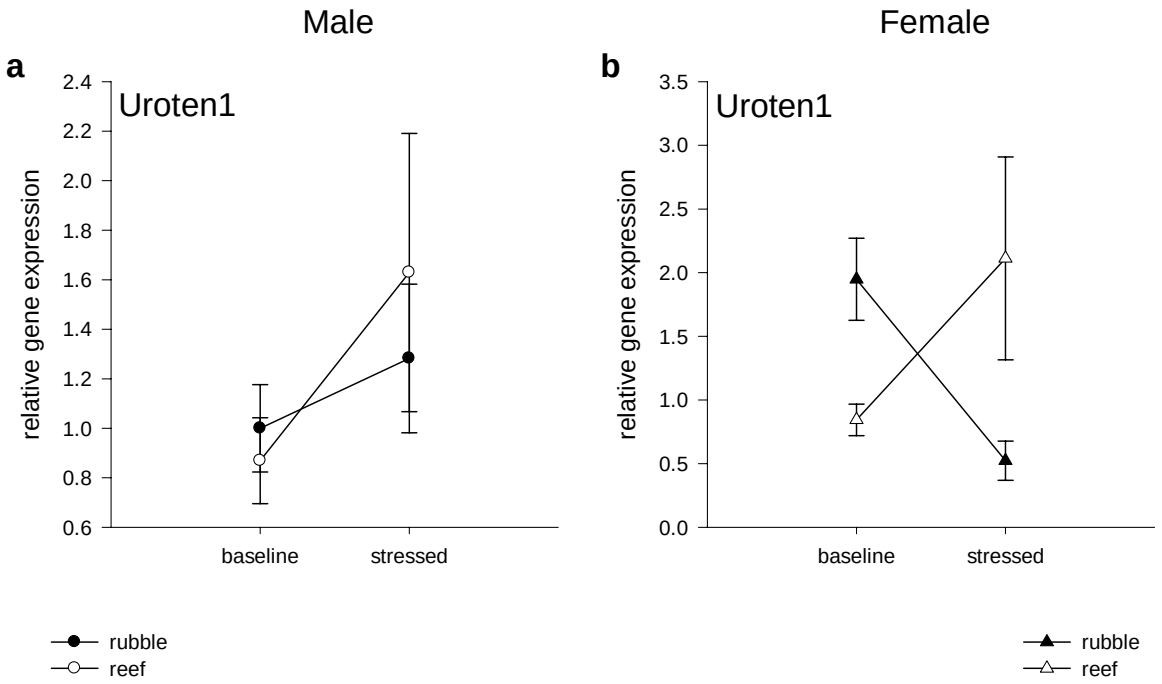


Figure 5. Real time quantitative RT-PCR comparison of urotensin 1 mRNA levels (± SEM) in the brain of bicolor damselfish. Two-way ANOVA models were used to test for effects of habitat origin (rubble or reef) and stress condition (baseline or stressed) on relative mRNA levels within male and female fish. Urotensin 1 transcript abundance in females showed significant changes following acute capture stress. Pairwise comparisons following a significant ANOVA model revealed that urotensin 1 mRNA levels decreased following acute capture stress in females from rubble habitats ($t = -4.378$; $df = 6$; $p = 0.0047$). Furthermore, baseline urotensin 1 transcript abundance differed between females collected from rubble and reef habitats ($t = 2.822$; $df = 20$; $p = 0.0105$).

DISCUSSION

Animals experiencing different environmental conditions in the wild will often show differences in behavior, but the physiological bases for this behavioral variation are rarely known. This study revealed that wild bicolor damselfish from two coral reef habitats differed in how mRNAs in the brain encoding CRH, CRH-BP, CRH-R2 and Uroten1 responded to acute capture stress. Although the responses of these transcripts to acute capture stress sometimes varied between male and female damselfish, the finding that bicolor damselfish from the rubble and reef habitats differed in how brain mRNA levels respond to acute stress indicates that local environmental conditions can lead to intraspecific variation in stress physiology. As was found previously by Schrandt et al. (see Chapter 1), the behavior patterns of bicolor damselfish were also different between rubble and reef habitats. These results provide evidence that variation in local habitat conditions can influence how coral reef fish respond behaviorally and physiologically to environmental stressors.

Stress hormones and the environment

Because the endocrine system mediates interactions between the environment, physiology and behavior in patterns that ultimately affect an individual's fitness (Ricklefs and Wikelski 2002, Wingfield et al. 2008), studying endocrine mechanisms in an ecological context can provide insights into how individuals respond to alterations in their environment. In fishes, it is well established that ecological conditions can affect endocrine processes other than the stress response. For example, social conditions such as conspecific density and the frequency of territorial intrusions have been shown to alter hypothalamic-pituitary-gonadal (HPG) axis

signaling with implications for reproductive condition (Pankhurst and Barnett 1993; Pankhurst et al. 1997; reviewed by Pankhurst and Van Der Kraak 1997). Similarly, neuroendocrine pathways, such as the vasotocin system, that mediate osmotic balance and regulate sociosexual behaviors have been shown to respond to both the physical and social conditions that an individual experiences (Godwin et al. 2000; Lema 2006).

In the current study, we found differences in the response of stress-associated gene transcripts in the brain of bicolor damselfish from two discrete areas of a fringing coral reef that differ in physical habitat structure: shallow rubble and *Montastrea*-dominated reef habitats, indicating the physiological stress response varies among fish from different habitats. The differential responsiveness of brain gene transcript levels may indicate that bicolor damselfish have to cope with different stressors in the two habitats. Baseline levels of neural CRH, CRH-BP, and Uroten1 mRNAs were greater in female bicolor damselfish from rubble habitats compared to those from reef habitats. This elevation in baseline CRH mRNA levels may indicate that the HPI axis is "hyper-responsive" (McCormick et al. 1995) in female fish from rubble habitats. Exposure to stressful stimuli during early development can cause a hyper-responsiveness of HPA/HPI activity, which can lead to elevated expression of fearful behaviors and anxiety (Meaney 2001; Ellis et al. 2006), or more intense or prolonged responses of CRH to acute stressors (Meaney 2001).

Causes of these habitat-associated differences in stress reactivity are not clear, but likely are related to differences in the physical and social conditions experienced by fish in their habitats. Bicolor damselfish were previously found to be more abundant in coral rubble areas on the same fringing reef sampled in this study, and bicolor damselfish from rubble areas were also found to be involved in more agonistic interactions (see Schrandt Chapter 1). Confirming the

findings of Schrandt and coworkers, bicolor damselfish in the rubble habitat in this study showed higher rates of aggressive interactions than fish in the reef habitat. Habitat-associated variation in the frequency of agonistic interactions may contribute to differences in stress reactivity between bicolor damselfish living in rubble and reef habitats. It is well established that agonistic social interactions activate physiological stress responses (Summers 2002), as high fish densities could lead to more aggressive interactions and therefore, more stress. Reproductively active female damselfish (*Pomacentrus amboinensis*) exposed to high densities of heterospecific individuals experience more frequent agonisitic interactions, leading to higher ovarian cortisol levels in these females (McCormick 2009). In the current study, we found that bicolor damselfish from rubble areas experience more frequent aggressive interactions, which may explain why male fish from the rubble did not show significant neural transcript responses to stress, as well as why female fish from rubble habitats showed a decrease in transcript levels with stress. Chronic social stress can lead to blunting of the stress response, or an inability to elicit the typical physiological stress response when exposed to an acute stressor (reviewed by Busch and Hayward 2009).

Physical conditions of a habitat have also been suggested to lead to changes in stress physiology. Recently, several studies have documented habitat effects on the physiological stress response of tetrapods, usually as assessed by plasma levels of the glucocorticoids corticosterone or cortisol. For instance, physical habitat degradation has been shown to induce higher corticosterone in male midday gerbils (*Meriones meridianus* Pall.), even though population density was not related to corticosterone levels (Kuznetsov et al. 2004). Likewise, in American redstarts (*Serophaga ruticilla*), corticosterone levels 30 min after acute capture stress varied among individuals in patterns reflecting both a bird's physiological condition and the

quality of habitat experienced during the non-breeding season (Marra and Holberton 1998). Spatial variation in the distribution of food resources – and the associated competition that arises from this variation – has been implicated as a cause of variation in baseline cortisol levels and the magnitude of cortisol response among individuals from two different populations of squirrel monkey (Boinski 1999). Furthermore, there is evidence that habitat fragmentation can impact cortisol levels in black howler monkeys (*Alouatta pigra*), with monkeys from fragmented habitats having higher fecal levels of cortisol metabolites (Martinez-Mota et al 2007). In several studies, increased levels of stress indicators have been found to be associated with sub-optimal or degraded habitats (reviewed in Busch and Hayward, 2009). Variation in local habitat quality resulting from environmental variation, therefore, may generate these physiological differences in how fish are responding to stress, and may have differential consequences for the relative fitness of individuals among various habitats. It is important to note, however, the physical and social conditions that characterize high quality habitat can differ between the sexes, as males and females often use habitats differently. Such sex differences might explain why females in rubble habitats showed higher baseline levels of CRH, CRH-BP and Uroten1 mRNA levels. The rubble habitat may have been suboptimal for females either due to the physical or social conditions of the habitat, but still optimal for males.

Functionally, the importance of these habitat- and sex-associated differences in physiological stress reactivity is not clear. Several stress-related neuropeptides including CRH have been demonstrated to regulate ecologically-significant behaviors by acting centrally as a neurotransmitter within the brain (Lowry and Moore, 2006), and any habitat-associated differences in the production and release of these neuropeptides in the brain may contribute to the behavioral differences observed between fish in the rubble and reef habitats. Moreover, there

is recent evidence from coral reef fishes that the exposure of reproductively-active females to stress may have significant effects on offspring fitness. Female damselfish (*Pomacentrus amboinensis*) on the Great Barrier Reef, Australia, that were involved in more frequent aggressive interactions had higher circulating levels of cortisol, as well as significant reductions in the size of hatchling larvae spawned by these females (McCormick 2006, 2009). Experimentally increasing cortisol levels in the eggs of this damselfish species result in increased egg mortality and delayed hatching (Gagliano and McCormick 2009), indicating a direct effect of oocyte glucocorticoid levels on offspring development. Although agonistic interactions are a naturally occurring stressor in coral reef habitats, any synthetic or anthropogenic stressor that affects the physiological stress status of reproductively active adult damselfish could result in the same multi-generational effects on fitness.

Responses of neural mRNAs to acute stress

Here, we found that gene transcripts encoding several proteins (CRH, CRH-BP, CRH-R2, and Uroten1) involved in the HPI axis showed significantly different responses in their abundance between pre- and post-stressed damselfish. The specific responses following acute stress varied depending on the transcript, and in some cases, depending on the habitat from which fish were collected, indicating that experience with different habitat conditions may influence gene transcription associated with the HPI axis stress response. Explanations for why certain gene transcripts were affected by acute capture stress, or habitat origin, are not entirely clear, but are likely related to the specific functions of each protein in the physiological stress response.

The principal function of CRH from the hypothalamus in teleostean fishes is the regulation of the stress response (Huising et al. 2004; Flik et al. 2006), and previous studies have shown increases in mRNAs encoding CRH after acute stress (Plotsky 1991; Alexander et al. 1996). Neural levels of CRH mRNAs increased in response to the capture/confinement stressor in male bicolor damselfish, as has been observed previously in other teleost fishes (Huising et al. 2004; Bernier et al. 2008; see also Yao and Denver 2007). While the response of CRH mRNA levels to acute stress was similar between males from the rubble and reef habitats, female bicolor damselfish showed different CRH mRNA responses depending on habitat origin. In reef habitats, acute capture stress increased brain mRNA levels of CRH in males. Contrastingly, female bicolor damselfish from the rubble showed reductions in CRH mRNA levels following capture/confinement stress. This decrease in levels of CRH mRNAs in females from rubble habitats suggests they may have exhausted the response of the HPI axis by being chronically stressed in this habitat. Previous work in teleosts has observed higher basal cortisol levels in chronic stress scenarios and a failure to increase cortisol levels after subjection to an acute stressor while under chronic stress (reviewed by Mommsen 1999). Although these fish are still responding to stressors, they fail to elicit the typical signs of stress as measured by cortisol. Because the mRNAs we measured encode key proteins regulating the HPI axis and production of cortisol, either a lack of response or decreased magnitude of response of these mRNAs to acute stress may indicate that the HPI axis has an altered response to stress stemming from sex- and habitat-dependent chronic stresses that fish experienced prior to subjection to our capture/confinement stress. Analysis of the cortisol levels of these fish in concert with the stress-associated mRNAs may provide insights for the regulation of the mechanisms that ultimately produce cortisol.

In this study, changes in the abundance of transcripts encoding CRH and its binding protein (CRH-BP) were similar following acute capture stress, although the pattern of response was different for males and females (see Fig. 3). Similar responses of CRH and CRH-BP mRNA levels within each sex may reflect the role that CRH-BP plays in the bioactive circulation of CRH. The mammalian form of CRH-BP has binding sites for both CRH and Uroten1 (Huising et al. 2008), and CRH-BP is thought to regulate the biological activity of both CRH and Uroten1 by modulating the concentrations of these hormones that are bioavailable to bind CRH receptors (Potter et al. 1991; Seasholtz et al. 2002). CRH and CRH-BP mRNAs and proteins have been shown to both be expressed in the parvocellular and magnocellular regions of the preoptic area of common carp (*Cyprinus carpio*), and mRNAs encoding CRH, Uroten1, and CRH-BP colocalize to the hypothalamus of adult zebrafish (*Danio rerio*) (Alderman and Bernier 2007). These patterns of protein and mRNA colocalization suggest a role for CRH-BP as a key regulator of CRH and Uroten1 bioavailability within – and release from – the hypothalamus (Huising et al. 2004). In this study, both male and female bicolor damselfish showed stress-induced changes in CRH-BP mRNA levels following patterns similar to those of CRH and Uroten1 mRNAs, consistent with a role for CRH-BP in regulating bioavailability of these peptide hormones. In rainbow trout (*Oncorhynchus mykiss*), hypoxic stress has been shown to increase CRH-BP mRNA levels in the telencephalon and hypothalamus (Alderman et al. 2008). Similarly, agonistic social interactions resulting in social subordination have been shown to increase CRH-BP mRNA levels in the telencephalon of this same species, while interactions leading to a dominant social status decrease CRH-BP mRNA levels in the hypothalamus (Alderman et al. 2008). Given these findings in rainbow trout, the differential response in CRH-BP transcript abundance seen between male and female bicolor damselfish may be related to

differences in the type and frequency of agonistic social interactions experienced by the sexes. Moreover, the opposing patterns in females from rubble and reef habitats in how their brain CRH-BP mRNA levels respond to acute stress provide evidence that habitat conditions influence the stress responses of females.

The differential responses of the CRH receptors to acute stress in this study may stem from the presumed opposing roles of these receptors in the stress response. Signals from CRH are transduced across cell membranes via activation of CRH-R1 and CRH-R2 (see review by Grammatopoulos and Chrousos 2002; Flik et al. 2006), but these two receptors appear to have fundamentally different roles (Dautzeberg et al. 2001). CRH-R1 has been thought to regulate HPA/HPI responses to stress (Timpl et al 1998). Contrastingly, CRH-R2 appears to be involved in the fine tuning of the stress response in mammals, including longer term changes in behaviors including stress-coping and emotional behaviors (Dautzenberg et al. 2001). CRH-R2 has been proposed to mediate an anxiolytic response, whereas the activation of CRH-R1 by CRH is thought to invoke anxiety in mice (Kishimoto et al. 2000). Variations in mRNA expression of these two receptors in different brain regions, as determined by laboratory studies of animals, suggests that the systems may be separate yet interrelated, and that the expression of CRH-R1 and CRH-R2 may be simultaneously regulated in the same or opposite direction (Skelton et al. 2000).

Supporting the hypothesized different roles of the two receptors, the abundance of transcripts encoding CRH-R1 was not affected by 20 minute confinement stress in either sex of bicolor damselfish collected from the rubble and reef coral habitats. In a laboratory study of female prairie voles subjected to acute restraint stress, CRH-R1 mRNA levels in the hypothalamus and hipppocamus were unaffected, but CRH-R1 mRNA levels in the pituitary

gland increased (Pournajafi-Nazarloo et al 2009). The lack of response by CRH-R1 in our study may be partly due to CRH-R1 having a lower affinity for CRH than CRH-BP (Potter et al. 1991, Cortright et al. 1995). Changes in the levels of CRH-R1 may not always manifest when there are changes in levels of CRH because CRH-BP may be more tightly coupled with the fluctuations in CRH. However, other studies have seen biphasic changes in CRH-R mRNA levels: levels decreased in the pituitary of Sprague Dawley rats 2 hr after onset of a stressor, but then recovered or increased by 4 hr (Rabadan-Diehl et al 1996). This biphasic response between 2 and 4 hr does not appear to be universal. In common carp (*Cyprinus carpio*), Huising et al. (2004) found downregulation of CRH-R1 in the pituitary pars distalis after response to a 24 hr restraint stress, while Mazon et al. (2006) observed significant downregulation in the gills and skin following both infection and restraint stressors. In this study, it is possible that the acute stressor was not experienced long enough to induce changes in CHR-R1 mRNA levels since we held the fish for 20 min as opposed to 2 hr. On the other hand, the exposure to the stressor may have been sufficient, but associated stress hormone changes (CRH, arginine vasopressin/vasotocin) may have inhibited the increase of CRH-R1 transcripts. Laboratory studies on rats found that CRH-R mRNA levels in the pituitary decreased (within 2 hr) after injection of CRH (Rabadan-Diehl et al. 1996; Ochedalski et al. 1998).

CRH-R2 mRNA levels were affected by acute stress and habitat origin in this study, but only for male bicolor damselfish. Our results were opposite those seen in prairie voles where CRH-R2 mRNA levels in the hypothalamus decreased after 4 weeks of daily exposure to an acute stressor (Pournajafi-Nazarloo et al. 2009). CRH-R2 may also be affected by circulating levels of steroid hormones. In rats, administering high levels of corticosterone can induce CRH-R2 mRNA expression (Makino et al. 1998). Conflicting responses of the CRH receptors

between acute and chronic stressors have been previously documented, and Pournajafi-Nazarloo and coworkers (2009) suggest that the response of these receptors to stress is dependent on the stimulating intensity and the stress type; however, the mechanisms driving the regulation are not clear.

The neural mRNA expression of UrotenI in female bicolor damselfish was also affected by acute stress, but the direction of the response depended on the habitat. Females from rubble habitats subjected to the acute stressor had decreased levels of Uroten1 whereas those from reef habitats had elevated mRNA levels of Uroten1 after 20 min confinement stress. This response is similar to that seen for CRH and CRH-BP in female bicolor damselfish. Increases in the abundance of mRNAs encoding both Uroten1 and CRH in the brain have been observed previously in other teleost fishes following acute stressors, although the magnitude of the change appears to depend on the type of stressor (Bernier et al. 2008). Uroten1 and CRH are closely related (Pittman and Hollenerg 2009) and may explain why their neural mRNA responses to acute stress appear similar. Originally the role of Uroten1 was proposed to be only important with exposure to osmotic stressors after a study that transferred fish from freshwater to seawater revealed that Uroten1 release from the urophysis was inhibited (reviewed by Wendellaar-Bonga 1997). Since then, however, Uroten1 has been demonstrated to have roles in regulating food intake in fish (Bernier and Peter 2001), and it has been demonstrated that Uroten1 and CRH co-occur in the caudal neurosecretory system of flounder (*Platichthys flesus*), suggesting shared roles in regulating stress-induced changes in interrenal cortisol secretion (Lu et al. 2004). This indicates a greater response of Uroten1 to potentially many different stressors.

Conclusions

Results presented here provide evidence that bicolor damselfish from two physically distinct coral reef habitats – 'rubble' habitat characterized by abundant *A. cervicornis* coral rubble and comparatively little live coral cover (< 2%), and 'reef' habitat dominated by *Montastrea* corals and a higher % of the benthos occupied by live corals (> 25%) – show differences in how several key stress-associated gene transcripts in the brain respond to acute stress. Given that bicolor damselfish in the rubble and reef habitats differ significantly in behavior (see Schrandt Chapter 1), our findings of differences in stress reactivity between fish in these habitats provides a physiological link between the differing physical and social conditions of these two habitats, and the intraspecific variation in damselfish behavior. This variation in physiological stress responses is particularly notable because it occurs over very small spatial scales (~35 m distance) on the coral reef, and also appears to vary between the sexes. Taken as a whole, our findings suggest that local environmental variation may generate physiological differences in how fish respond to stressors. Given this finding, we propose that future studies of the behavior and stress physiology of animals in an ecologically-relevant context could provide novel insights into how animals respond to changes in their habitat's physical and social conditions.

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