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Title: Microhabitat predicts species differences in exploratory behavior in Lake Malawi cichlids

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Abstract

Encountering and adaptively responding to unfamiliar or novel stimuli is a fundamental challenge facing animals and is linked to fitness. Behavioral responses to novel stimuli, or exploratory behavior, can differ strongly between closely related species; however, the ecological and evolutionary factors underlying these differences are not well understood, in part because most comparative investigations have focused on only two species. In this study, we investigate exploratory behavior across 23 species in a previously untested vertebrate system, Lake Malawi cichlid fishes, which comprises hundreds of phenotypically diverse species that have diverged in the past one million years. We investigate behavioral variation across species, across microhabitats, and across environmental contexts. We find strong species differences in behavior that are associated with microhabitat, demonstrate that intermediate microhabitats are associated with higher levels of exploratory behavior, show that patterns of behavioral covariation across contexts are characteristic of modular complex traits, and contrast Malawi cichlid data with behavioral data from selectively bred high- and low-exploratory zebrafish. Taken together, our results tie ecology to species differences in behavior, and highlight Lake Malawi cichlids as a powerful system for understanding the evolution, ecology, and biology of natural behavioral variation.

Keywords: teleosts, neophobia, neophilia, anxiety-like behavior, bold shy axis, stress response, habitat preference, behavioral syndromes, behavioral modularity, behavioral integration

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Highlights

- Malawi cichlids exhibit high phenotypic variance in exploratory behaviors
- Species differences in exploratory behavior are explained by microhabitat
- Rock-dwelling species exhibit strong edge preferences across assays
- Intermediate habitats are associated with “high exploratory” open field behavior
- Patterns of behavioral covariance across contexts are modular in Malawi cichlids

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1. Introduction

Deciding how to respond to unfamiliar or novel stimuli is a fundamental aspect of animal life that has important implications for fitness. For example, how individuals respond to novel conspecifics, heterospecifics, physical environments, food resources, or objects can directly impact survival (N. J. Dingemanse, Both, Drent, & Tinbergen, 2004; Ferrari, McCormick, Meekan, & Chivers, 2015; Lapiedra, Schoener, Leal, Losos, & Kolbe, 2018; Smith & Blumstein, 2008). Behavioral responses to novel stimuli can vary strongly between individuals, populations, and closely-related species; however, the factors underlying this behavioral variation are not well resolved.

At the interspecies level, large scale comparative studies are a promising strategy for identifying evolutionary and ecological factors contributing to variation in behavioral responses to novel stimuli (Niels J. Dingemanse et al., 2007). For example, a comparative study across 61 species of parrots showed that species differences in microhabitat predict species differences in behavioral responses to novel objects: species inhabiting intermediate habitats between the forest and the savannah more readily approached novel objects compared to species inhabiting more uniform savannah habitats (R. Greenberg, 2003; Greenberg & Mettke-hofmann, 2001; Claudia Mettke-Hofmann, Winkler, & Leisler, 2002). These and other findings support hypotheses that ecological plasticity or habitat variability is associated with higher levels of exploratory behavior. However, it is unclear how well this model generalizes across species and vertebrate lineages, in part because many comparative studies of behavioral responses to novel stimuli have compared just two species and have been conducted in birds (Garland & Adolph, 1994; Réale, Reader, Sol, McDougall, & Dingemanse, 2007). Furthermore, different behavioral assays and testing parameters have been used across studies, making it difficult to identify organizing principles that explain species differences in behavior. To better elucidate relationships between ecological factors, such as microhabitat, and species differences in exploratory behavior, larger comparative studies employing multiple assays and in new vertebrate systems are needed.

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Lake Malawi cichlid fishes are well-suited for comparative investigations of phenotypic variation (R. C. Albertson, Markert, Danley, & Kocher, 1999; Johnson & Young, 2018; Rupp & Hulsey, 2014; Ryan A. York & Fernald, 2017). These fishes have recently (within the past one million years) undergone explosive speciation, radiating into an estimated 500-1000 species that vary in morphology, coloration, diet, habitat preference, and behavior (Brawand et al., 2014; Kocher, 2004). Additionally, within Lake Malawi, ecological conditions vary across small spatial scales, resulting in diverse species occupying different microhabitats while living in close geographic proximity. For example, while many species can be grouped into two canonical ecotypes, rock-dwelling and sand-dwelling (Kocher, 2004), a large number of species inhabit intermediate habitats, including the interface between rocky and sandy substrate, and shallow sediment-rich bays near rocks and reed stands. Thus, the Lake Malawi species assemblage is well positioned to test relationships between microhabitat and species differences in behavior.

Comparative studies in Lake Malawi cichlids have already begun generating insights into the evolution of complex traits (Streelman & Danley, 2003; Sylvester et al., 2010; R. A. York et al., 2018). For example, ecological factors have been associated with species differences in aggression and bower-building behavior (Danley, 2011; Ryan A. York et al., 2015). Other studies have investigated the evolution of non-behavioral traits such as oral jaw morphology and color patterning, and have demonstrated modular patterns of phenotypic variation (R. Craig Albertson et al., 2014; Parsons, Cooper, & Albertson, 2011). Briefly, evolutionary modularity and integration refer to patterns of covariation among sets of traits (e.g. dimensions of different oral jaw bones), and they are thought to be related to trait evolvability (Raff & Raff, 2000; Wagner, Pavlicev, & Cheverud, 2007). Phenotypic integration refers to more uniform patterns of covariation, while modularity refers to non-uniform patterns of covariation and may reflect increased trait evolvability, although these relationships are complex (Armbruster, Pélabon, Bolstad, & Hansen, 2014).

Although Lake Malawi cichlids are primely positioned to link ecological and evolutionary factors to species differences in behavior, only a small number of comparative behavioral

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investigations have been conducted in this species group. We aim to address this gap by investigating species differences in exploratory behavior using four established behavioral assays (Stewart et al., 2011; Stewart et al., 2012) across a total of 23 species, which collectively span five Lake Malawi microhabitats: rock, sand, rocky intermediate, sandy intermediate, and shallow silt. We test the following hypotheses: (i) species differ in behavioral responses to novel stimuli; (ii) intermediate microhabitats are associated with higher levels of exploratory behavior; and (iii) patterns of behavioral variation across Lake Malawi cichlids are modular. We also contrast behavioral variance and covariance among Lake Malawi cichlids to previously published behavioral data in high and low exploratory strains of zebrafish to highlight patterns of behavioral diversity in this species assemblage.

2. Methods

2.1 Subjects

Subjects were maintained at two institutions, Georgia Institute of Technology (GT) in Atlanta, GA and North Carolina State University (NCSU) in Raleigh, NC. The open field test was conducted with animals from both facilities; the novel tank test and light-dark test were conducted with GT animals only; and the novel object test was conducted with NCSU animals only. Both institutions house laboratory cichlid lines derived from wild-caught animals collected in Lake Malawi. Behavioral data from two separate studies were re-analyzed for Modularity Modular Clustering analysis (described below): one in which 70 subjects from five species were tested in the novel object test, open field test, and resident intruder test at NCSU; and a second previously published behavioral study in zebrafish (Ryan Y. Wong et al., 2012).

GT animals were maintained in the Engineered Biosystems Building cichlid aquaculture facilities at GT in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines. Age- and size-matched male and female individuals were socially housed on a 12:12-hour light:dark cycle with full lights on between 8am-6pm Eastern Standard Time (EST) and dim lights on for 60 minutes between the light-dark transition (7am-8am and 6pm-7pm

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EST). All fish were housed at densities of approximately 0.67 cm of fish/liter in 190-liter or 95-liter glass tanks measuring 92 cm (wide) x 46 cm (deep) x 42 cm (high) or 46 cm (wide) x 46 cm (deep) x 42 cm (high), respectively. Male and female subadults (age 90-180 days) were analyzed in the novel tank test and light-dark test (described below), and male and female reproductive adults (>180 days) were tested in the open field test (described below).

NCSU animals were maintained in the NCSU Roberts Lab aquaculture facility in Raleigh, NC, under a 12:12-hour light:dark cycle with dim lights on for 15 minutes during the light-dark transition periods. All experiments were conducted under the approval of the Institutional Animal Care and Use Committee (IACUC) guidelines (protocol number 14-138-O). For the five NCSU species included in the novel object and behavioral module analysis, age matched male and female individuals from at least two families per species were raised in mixed-species groups in a 473-liter (184 cm x 47 cm x 60 cm) aquarium until onset of reproductive age, when behavioral assays began. For all thirteen NCSU species tested for open field behavior, fish were housed in size-matched general husbandry tanks and co-cultured as necessary to reduce aggression.

2.2 Behavioral assays

A total of 520 subjects spanning 23 Lake Malawi cichlid species were tested in one or more exploratory behavioral assay(s), described in detail by institution (GT and NCSU) below. 110 subjects from eight species were tested at GT in the novel tank test, and 77 of these subjects were also tested in the light-dark test (see Supplementary Tables 1 and 2 for sample sizes by species). 340 subjects from a total of 19 species were tested in the open field test: 227 subjects from 13 species were tested at NCSU and 113 subjects from seven species were tested at GT, with one species (*Labeotropheus fuelleborni)* tested at both institutions (See Supplementary Table 3 for sample sizes by species). Pilot data indicated strong effects of species but no effects of sex on exploratory behavior across multiple assays. Based on these data, subjects for the present study were sampled randomly from mixed sex tanks but were not euthanized and dissected to determine gonadal sex, with the exception that visually

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identified dominant males were sampled at a proportion consistent with the composition of the home tank, and maternal mouthbrooding females were not sampled. All assays were performed between 10:00 and 16:00 Eastern Standard Time EST.

Novel tank test

The novel tank test is designed to measure exploratory behavior in a narrow, tall tank with transparent sides (Fig 1A,B). Individual subadult subjects (90-180 days; 4-6.5 cm length) spanning eight species were each collected between 11:00-15:00 Eastern Standard Time from their home tank, transferred to a 300 mL beaker of water, and habituated for 30 minutes prior to behavioral testing. Water for both habituation beakers and test tanks was collected from a circulating system supplying all home tanks, ensuring that water was consistent across the home tank, transfer, habituation, and testing environments. Following habituation, subjects were introduced to a plastic 1.8-L novel tank (Aquaneering; 29.7 cm long x 7.5 cm wide 15.2 cm high) and were side-view video recorded for 6 minutes using a GoPro Hero4 camera. Species composition was counterbalanced across trials to control for potential effects of testing round. EthoVision (Noldus) software was used to analyze distance traveled, latency to enter top half, total duration spent in top half, and average distance from the corners.

Light-dark test

In the light-dark test, subjects can freely move between an opaque black chamber and a backlit semi-opaque white chamber (Fig 1C,D). This test is thus designed to investigate exploratory behavior between environments that vary in light intensity. Individual subadult subjects (90-180 days; 4-6.5 cm length) from all eight tested species were transferred to a 300 mL beaker of water and habituated for 60 minutes prior to testing. All water was collected from the same circulating system (described above). Following habituation, subjects were first introduced to a 6.5 cm x 7.5 cm habituation chamber (half white, half black) within a larger custom built acrylic “light-dark” tank (half white, half black; 24 cm long x 6.5 cm wide x 16.5 cm high). Individual subjects habituated for 5 minutes in the central habituation chamber, after

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which both inserts were immediately and simultaneously removed, allowing subjects to swim freely throughout the entirety of the light-dark tank. Species were counterbalanced across trials. All subjects were top-down video recorded for 6 minutes using a GoPro Hero4 camera. EthoVision (Noldus) software was used to analyze distance traveled in the light half (total distance traveled in the dark half could not be calculated because automated tracking from RGB video is not possible when the subject is in this region of the tank), latency to enter the light half, number of entries into the light half, and total duration in the light half.

Novel object test

The novel object test is designed to test an animal's behavioral response (i.e. approach, avoidance, and exploration) toward an unfamiliar object. Subjects were introduced to a 38-liter (50 cm x 28 cm x 33 cm) aquarium with a single flowerpot territory for three days of acclimation. To assess activity and motivation during the acclimation period, latency to feed was measured at each meal. All subjects ate within 1 minute of feeding by the final day of acclimation. Once acclimated, a camera was placed overhead and water and air flow was turned off for five minutes before commencement of the novel object test. A snail shell from Lake Malawi was then introduced into the home aquarium and behavior was recorded for 30 minutes with a digital video camera (Fig 1G,H). The position of the most rostral aspect of the head was scored with Manual Tracking plug-in (Cordelieres 2005) for ImageJ (Schneider et al. 2012) in 0.2 second intervals (5 frames per second). Aquarium positioning prevented the entire arena from being filmed, so position analysis was restricted to the front-most 25.4 cm x 26 cm of the tank for all subjects. For the novel object test, total time spent stationary, approaching, and retreating from the object; as well as approach velocity, retreat velocity, average velocity, and change in velocity over the course of the assay were analyzed.

Open field test

The open field test for fish is generally similar in design to the open field test used in mice and other rodents, in which subjects are allowed to move freely throughout a large open arena. In

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fishes, the open field test is thus used to investigate behavioral responses to an unfamiliar large and open shallow water environment (Fig 1E,F). 19 species were analyzed at two sites and using two arena sizes. Fish were gently netted from their home tank and placed in the center of a white, opaque container filled with aquaculture system water at shallow depths to restrict vertical movement as much as possible. Tank water was replaced between every individual. For the five NCSU species measured for multiple assays, a rectangular 76 cm x 46 cm arena was filled with 6 cm of water; for all GT fish and all additional NCSU fish, the size of the arena was scaled to the size of the fish. In these experiments, the large 49.6 cm square arena was filled to a depth of 15 cm of water for fish > 4.5 cm SL and the medium 25.5 cm square arena was filled to a depth of 10 cm of water for fish 2.5-4.5 cm SL. Video recordings were taken for 5.5 minutes from an overhead position. The first 10 seconds of the video files were trimmed (Quicktime Player 7) to remove footage of fish placement, and processed at 10 frames per second (fps) using C-trax (0.5.4, (Branson et al. 2009)) to generate XY coordinates of fish position in arena. Custom scripts were used to generate position and speed in the arena (R v3.3.1). For place analysis, the arena was divided into a grid of 16 squares, with the outer ring of squares forming the “peripheral” regions, the central four squares forming the “center” region, and the four corner squares forming the “corner” regions.

2.3 Statistics

All statistics analyses were performed in R (R v3.3.1 and R v3.4) unless otherwise specified.

Place bias in novel environment assays

To measure general place bias for specific arena zones in the novel tank and light-dark tests across species, a linear regression model with time spent in zone as the outcome variable, and zone and species as categorical predictor variables, was fit to the data.

Time spent in zone ~ zone + species

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Because the open field test was performed at two test sites using two arena sizes, these were added to the model as categorical variables to analyze place bias for central versus peripheral regions in the open field test:

Time spent in zone ~ zone + species + test site + arena size

Within species, paired t-tests were used to test the significance of differences in time spent between zones.

Effect of species on behavioral responses to novel stimuli

When appropriate, one-way ANOVA was used to test for species differences in behaviors for the assays where only a subset of all species was tested. For some of the measurements taken, there were unequal variances between species. Because unequal variance between groups violates the assumptions of one-way ANOVA, non-parametric tests were used in these cases, including the one-way ANOVA equivalent Wilcoxon/Kruskal-Wallis test and the Wilcoxon Product-Limit survival fit for latency measures. To be considered to have unequal variances, at least one of O'Brien, Brown-Forsythe, or Levene's tests of unequal variance had to be significant at the p=0.05 level. Pairwise contrasts were performed with Tukey-Kramer honest significant difference test (HSD) for measurements with equal variance between groups, and Wilcoxon multiple comparisons was conducted for those requiring non-parametric analysis. To examine behavioral responses to a novel object over time, we used a MANOVA repeated measures, where time points within individuals were analyzed at one level, and differences between species were analyzed as an additional level, with a species*time interaction term. Since Mauchly's Test of Sphericity indicated violations to the sphericity assumption (criterion=0.346; Chi^2=67.95; df=14, $p=4.53x10^{-9}$) we used the Huynd-Feldt correction to adjust for unequal covariances between groups.

Effect of microhabitat on behavioral responses to novel stimuli

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The association between microhabitat and behavior was assessed through multiple linear regression using the "lm" package in R. The behavior of interest was the outcome variable and microhabitat, lineage (Mbuna vs. non-Mbuna), genus, species, arena size, and test site were predictor variables. This allowed behavioral variation explained by microhabitat to be measured while simultaneously controlling for variance explained by phylogenetic relatedness and batch-like effects. The model was organized as follows:

Open field behavior ~ microhabitat + lineage + genus + species + test site + arena size

This model was used to test several core open field behavioral metrics, including distance traveled, time spent in the corners, time spent in the center region, and total number of entries into the center region. To assess the relationship between intermediate habitats and species differences in behavior, a categorical predictor variable representing intermediate vs. non-intermediate was added to the model:

Open field behavior ~ intermediate + microhabitat + lineage + genus + species + test site + arena size

To assess the relationship between microhabitat and time spent in the corner regions in the novel tank test, a similar linear model was used except (i) genus was excluded as all species in this test were members of a unique genus, and (ii) test site and arena size were excluded as all animals were tested at the same site using identical tanks. Thus, this model was organized as follows:

Novel tank behavior ~ microhabitat + lineage + species

To examine changes in open field movement over time, we used a MANOVA repeated measures, where time points within individuals were analyzed at one level, and differences between microhabitat were analyzed as an additional level, with a microhabitat*time interaction term, controlling for lab and arena size. Change in velocity (minute 1 velocity – minute 5 velocity) was analyzed with an ANOVA by microhabitat, where a positive value

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indicates the subject swam faster at the start of the assay, and a negative value indicates the subject swam slower at the start of the assay.

Behavioral modularity test

To examine behavioral correlations within and across assays, we performed Modulated Modularity Clustering (MMC) analysis (Stone & Ayroles, 2009). This test identifies clusters of covariance in multivariate data. Although this method was developed to analyze gene expression data, it is effective for any large, multivariate datasets where many phenotypes have been measured across a large sample of subjects. We separately performed MMC on two independent Lake Malawi cichlid datasets: a GT data set to analyze behavioral modules between the novel tank test and light-dark test, and a NCSU dataset to analyze behavioral modules between the open field, novel object, and resident-intruder behavioral tests. We also applied this test to a previously published zebrafish behavioral dataset where individuals were measured across multiple assays and correlations of behaviors between assays were identified (Ryan Y. Wong et al., 2012). Each individual behavioral metric within each assay (such as speed, position, time spent in a specific zone, etc.) was included in the analysis. Since these assays are of different measurement types, Spearman rank-order correlation was used in place of Pearson's correlation.

3. Results

3.1 Malawi cichlids exhibit strong place biases across assays

In general, Lake Malawi cichlids exhibited strong place biases for specific arena zones across the three novel environment assays, spending more time in the bottom half of the novel tank test, the light half of the light-dark test, and the corner regions of the open field test. The direction of the place biases were the same in all species tested. More detailed results are organized by assay below:

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Malawi cichlids prefer corner regions in the open field test

Malawi cichlids spent more time in the outer region of the open field test compared to the center region. Linear regression controlling for species, test site, and arena size showed a strong place bias between the central versus peripheral regions (n=340; t=89.24; p<0.0001); spending an average of 298.9±2.2 seconds in the periphery compared to 21.1±2.2 seconds in the center. Both *Aulonocara baenschi* and *Metriaclima mbenji* spent significantly less time in corner regions compared to multiple other species (Supplementary Figure 1C). Additional results are presented by species in Supplementary Table 3.

Malawi cichlids prefer the bottom region in the novel tank test

A linear model controlling for species revealed that Malawi cichlids generally expressed a strong place preference for the bottom half in the novel tank test (n=110; t=20.982; p<0.0001), spending an average of 307.5±6.1 seconds in the bottom half compared to 52.5±6.1 seconds in the top half. The direction of the preference was consistent across all species tested, and two-tailed paired t-tests showed that this preference was significant within each species (p<0.05 for all species tested, Supplementary Table 1). Notably, *post-hoc* Tukey's HSD tests showed significant differences in the strength of the preference between *Mchenga conophoros*, a sand-dwelling species, and all other species tested (Supplementary Figure 1A). Detailed results are presented by species in Supplementary Table 1.

Malawi cichlids prefer the dark region in the light-dark test

Malawi cichlids exhibited a strong place bias in the light-dark test (n=77; t=16.07; p<0.0001), spending more time in the dark half (an average of 283.2±8.9 seconds in the dark half versus 76.8±8.9 seconds in the light half). Detailed results are presented by species in Supplementary Table 1. Notably, one sand-dwelling species, *Copadichromis virginalis*, did not exhibit a significant place bias between the light and dark zones (n=12; two-tailed paired t-test, p=0.46; Supplementary Table 2), and this differed significantly from several other species

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(Supplementary Figure 1B). Additional results are presented by species in Supplementary Table 2.

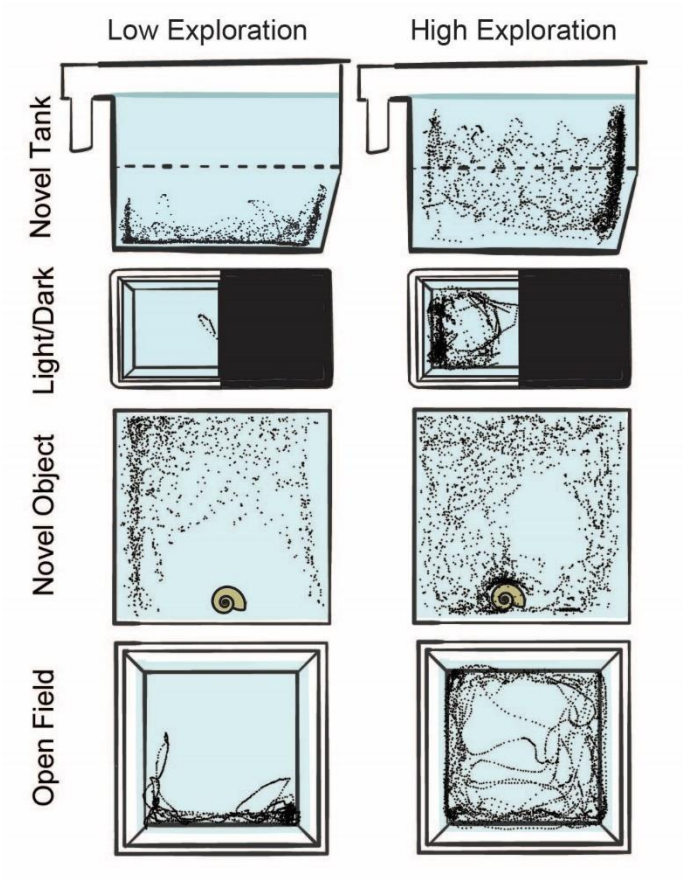

Figure 1. Variation in exploratory behavior across assays in Lake Malawi cichlids. Representative traces of the four assays used in this study, with individual points illustrating the position of the fish in the arena at a single moment. Individual fish can show little exploration (left column), or high levels of exploration (right column). Schematics reflect the angles at which video cameras were positioned for recording behavior for each assay.

3.2 Malawi cichlids exhibit high phenotypic variance in exploratory behavior

Because the novel tank test parameters conducted in Lake Malawi cichlids were the same as those conducted in zebrafish, we measured phenotypic variance across Lake Malawi cichlid species and compared it to phenotypic variance across strains of zebrafish that have been selected for high and low exploratory behavior. For duration in the top, a primary measure of exploratory behavior in this assay, Malawi cichlids exhibited higher behavioral variance compared to zebrafish (n=110 Malawi cichlid individuals from eight species, n=99 zebrafish from three selection lines; variance for cichlids = 134.6 versus variance for zebrafish = 72.7;

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F-test, p=0.006;). This was also true for latency to enter the top (variance for cichlids = 19,941 versus variance for zebrafish = 10,653; F-test, p=0.004), but not for frequency of entries into the top (p=0.996, variance for zebrafish = 15.56 vs. variance for cichlids = 15.59).

3.3 Malawi cichlids exhibit strong species differences in exploratory behavior

We next tested whether Lake Malawi cichlid species differed in more detailed dimensions of behavior within each assay. These results are organized by assay below:

Open field test

In the open field test, total distance traveled, total number of entries into the center region, total time spent in the center region, and total time spent in the corners were analyzed. Because this assay was conducted using two different square arena sizes at two different test locations, the data was analyzed using a one-way ANOVA including an error term with arena size nested within test site. These analyses revealed a significant effect of species on total distance traveled ($F_{18,318}$=6.01; p=1.53x10^{-12}; Eta-squared=0.25), total number of entries into the center region ($F_{18,318}$=8.50; p<2x10^{-16}; Eta-squared=0.32), total time spent in the center region ($F_{18,318}$=4.75; p=2.28x10^{-9}; Eta-squared=0.21), and total time spent in the corners ($F_{18,318}$=8.83; p<2x10^{-16}; Eta-squared=0.33) Fig 2A.

Novel tank test

In the novel tank test, several aspects of exploratory behavior were analyzed: total distance traveled, latency to enter the top half, total number of entries into the top half, and total time spent in the top half. In addition to these metrics, we also analyzed the average distance from the tank bottom, and the average distance from the tank corners. One-way ANOVAs revealed strong effects of species on total distance traveled ($F_{7,102}$=8.30; p=5.38x10^{-8}; Eta-squared=0.36), latency to enter the top half ($F_{7,102}$=5.44; p=2.50x10^{-5}; Eta-squared=0.27), total number of entries into the top half ($F_{7,102}$=8.56; p=3.21x10^{-8}; Eta-squared=0.37), total time

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spent in the top half ($F_{7,102}$=8.64; p=2.74x10^{-8}; Eta-squared=0.37, Fig 2B), average distance from the tank bottom ($F_{7,102}$=12.48; p=1.86x10^{-11}; Eta-squared=0.46), and average distance from the tank corners ($F_{7,102}$=8.21; p=6.49x10^{-8}; Eta-squared=0.36).

Light-dark test

For the light-dark test, latency to enter the light half, total number of entries into the light half, total time spent in the light half, and total distance traveled in the light half were analyzed. One-way ANOVAs revealed a significant effect of species on total distance traveled in the light half ($F_{7,63}$=2.87; p=0.012; Eta-squared=0.24), latency to enter the light half ($F_{7,63}$=4.42; p=4.75x10^{-4}; Eta-squared=0.33), total number of entries into the light half ($F_{7,63}$=2.54; p=0.023; Eta-squared=0.22), and total time spent in the light half ($F_{7,63}$=4.95; p=1.67x10^{-4}; Eta-squared=0.35, Fig 2C).

Novel object test

In the novel object test there were strong species differences in time spent approaching the object (Wilcoxon/Kruskal-Wallis: χ^2=14.04, df=4, p=0.0072), swimming away from the object, (Wilcoxon/Kruskal-Wallis: χ^2=15.06, df=4, p=0.0046), and remaining stationary (Wilcoxon/Kruskal-Wallis: χ^2=10.92, df=4, p=0.0275). Time spent approaching and retreating were strongly correlated with each other (Pearson's r = 0.976), but stationary, or 'freezing,' responses were only partially correlated with approach patterns (Pearson's r, approach = 0.662; retreat = 0.648). Differences were also detected in swimming velocity during the test; approach velocity (ANOVA Adj. R^2= 0.227712, $F_{(4, 70)}$ = 6.1599, p=0.0003), retreat velocity (Wilcoxon/Kruskal-Wallis test, χ^2=27.49, p<0.0001), and overall average velocity (Wilcoxon/Kruskal-Wallis test, χ^2=22.54, p=0.0002, Fig 2D, top panel) were all different by species. Additionally, the *Metriaclima* spp. were faster when retreating from the shell than when approaching it, whereas *Auloncara baenschi* approached and retreated with the same speed (Fig 2D, bottom panel).

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3.4 Microhabitat is associated with species differences in exploratory behavior

Open field test

To investigate the relationship between microhabitat and behavior, open field behavior was analyzed in 19 species representing five Lake Malawi microhabitats (rock, sand, sandy intermediate, rocky intermediate, and shallow silt intermediate). Several behavioral metrics were analyzed including total distance traveled, total number of entries into the center region, total time spent in the center region, and total time spent in the corner regions. There was a significant relationship between microhabitat and total time spent in the center region ($t=2.887$; $p=0.00415$), as well as total time spent in the corners ($t=-3.056$; $p=0.00243$); but not number of entries into the center region ($t=-0.915$; $p=0.3608$) or total distance traveled ($t=0.773$; $p=0.44043$). The strongest behavioral differences were observed for time spent in the corner regions, where rock-dwelling species spent significantly more time compared to species inhabiting rocky intermediate ($t=-2.070$; $p=0.039292$), sandy ($t=-3.056$; $p=0.002430$), sandy intermediate ($t=-3.106$; $p=0.002064$); and shallow silt habitats ($t=-3.326$; $p=0.000985$). Additional differences between microhabitats are represented in Supplementary Figure 2, and the full results of the linear modeling for open field behavior, including estimates for time spent in each zone by microhabitat, are presented in Table 2.

There were also differences in pattern of movement over time associated with microhabitat when controlling for arena size and lab (repeated measures MANOVA, full model $F_{(6,334)}=5.86$, $p<0.0001$). Microhabitat was associated with both frequency of freezing ($F_{(4,334)}=4.94$, $p=0.0007$) and the pattern of freezing over time (Wilks' Lambda value 0.793, approx. $F_{(16,1011.9)}=4.99$, $p<0.0001$). Both rocky and sandy interface species initially froze more frequently and exhibited a decrease in slowed swimming as the assay progressed, whereas open sand species initially froze less but increased freezing behavior as the assay progressed (Fig 2E). These patterns were also reflected by differences in swimming velocity over the course of the assay (Fig 2F, ANOVA significance groups by letter, $p<0.05$).

Novel tank test

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The strong differences in open field behavior—specifically in preference for corner regions—between rock-dwelling species and species inhabiting other microhabitats motivated us to test whether behavioral differences might persist in other types of novel environments, such as the novel tank test. Although behavior relative to corner regions is not a traditionally analyzed behavioral metric in the novel tank test, we reasoned that the most open area of the tank (center-top region) was the most distant from corner regions and, in contrast, that the outermost edges of the tank (from the side view video) were in immediate proximity to the corners. We therefore measured the average distance to the outer edge/corners of the tank for each subject. Consistent with findings from the open field test, rock-dwelling species exhibited a strong preference for the corners, remaining significantly closer to the corners compared to sand-dwelling species ($t=2.082$; $p=0.03984$), but not compared to shallow silt species ($t=0.024$; $p=0.98050$).

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Behavior ~ microhabitat + lineage + *genus* + species + *test site* + *arena size*						
Dataset	**Behavior**	**Microhabitat**	**Y**	**Standard Error**	**t-statistic**	**p**
Open field (19 species)	**Time spent in corners**	(Intercept)	239.014	31.875	7.498	6.45×10^{-13} ***
		Rocky intermediate	176.111	30.394	-2.070	0.039292 *
		Sand	129.838	35.721	-3.056	0.002430 **
		Sandy intermediate	59.651	57.740	-3.106	0.002064 **
		Silt	108.750	39.171	-3.326	9.85×10^{-4} ***
	Time spent in center	(Intercept)	-10.409	22.269	-0.467	0.64053
		Rocky intermediate	6.393	21.234	0.791	0.42937
		Sand	61.644	24.956	2.887	0.00415 **
		Sandy intermediate	86.634	40.339	2.406	0.01671 *
		Silt	65.242	27.366	-2.351	0.00603 *
	Total entries into center	(Intercept)	5.4230	2.9792	1.820	0.06966
		Rocky intermediate	3.2069	2.8407	-0.780	0.43591
		Sand	2.4176	3.3387	-0.915	0.36081
		Sandy intermediate	7.1277	5.3967	0.316	0.75229
		Silt	10.5824	3.6611	1.409	0.15974
	Total distance traveled	(Intercept)	1112.44	436.96	2.546	0.011370 *
		Rocky intermediate	1779.07	416.64	1.600	0.110587
		Sand	1490.68	489.67	0.7772	0.440432
		Sandy intermediate	2423.06	791.52	1.656	0.098738
		Silt	2090.37	536.97	1.821	0.069511
Novel tank (8 species)	**Average distance from corners**	(Intercept)	0.778672	0.266971	2.917	0.00435 **
		Sand	1.406998	0.301781	2.082	0.03984 *
		Silt	0.786065	0.301781	0.024	0.98050

Table 1. Effects of microhabitat on open field behavior and distance to the corners in the novel tank test. This table is a summary of linear regression output for models fitting behavioral data to microhabitats, phylogenetic factors, and batch-like factors. The table presents the slope coefficient estimate (Y), standard error, t-statistic, and p-value for the intercept and different microhabitats (note that p-values for microhabitats represent significance relative to rock-dwellers). The full model is presented at the top and was fit to open field behavioral data. For novel tank diving data, italic terms were removed from the model as each species represented a unique genus, and test site and tank dimensions were identical for all subjects. Rock-dwelling species remained closer to corner regions compared to sand-dwelling species in both the open field and novel tank tests. Asterisks indicate levels of significance (* for $p<0.05$; ** for $p<0.005$; *** for $p<0.0005$).

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3.5 Intermediate microhabitats are associated with high exploratory phenotypes in the open field test

We next tested whether species inhabiting intermediate habitats express increased exploratory behavior compared to members of the canonical rock and sand Lake Malawi ecotypes. Indeed, a linear model controlling for variance explained by microhabitat, arena size, test site, and phylogenetic factors revealed that species inhabiting intermediate habitats spent significantly more time in the center region ($t=2.764$, $p=0.0060$) and significantly less time in the corner regions ($t=-3.326$, $p=0.0010$) compared to rock- and sand-dwelling species, but did not differ in the total number of entries into the center ($t=1.409$, $p=0.1597$) or total distance traveled ($t=1.821$, $p=0.0695$).

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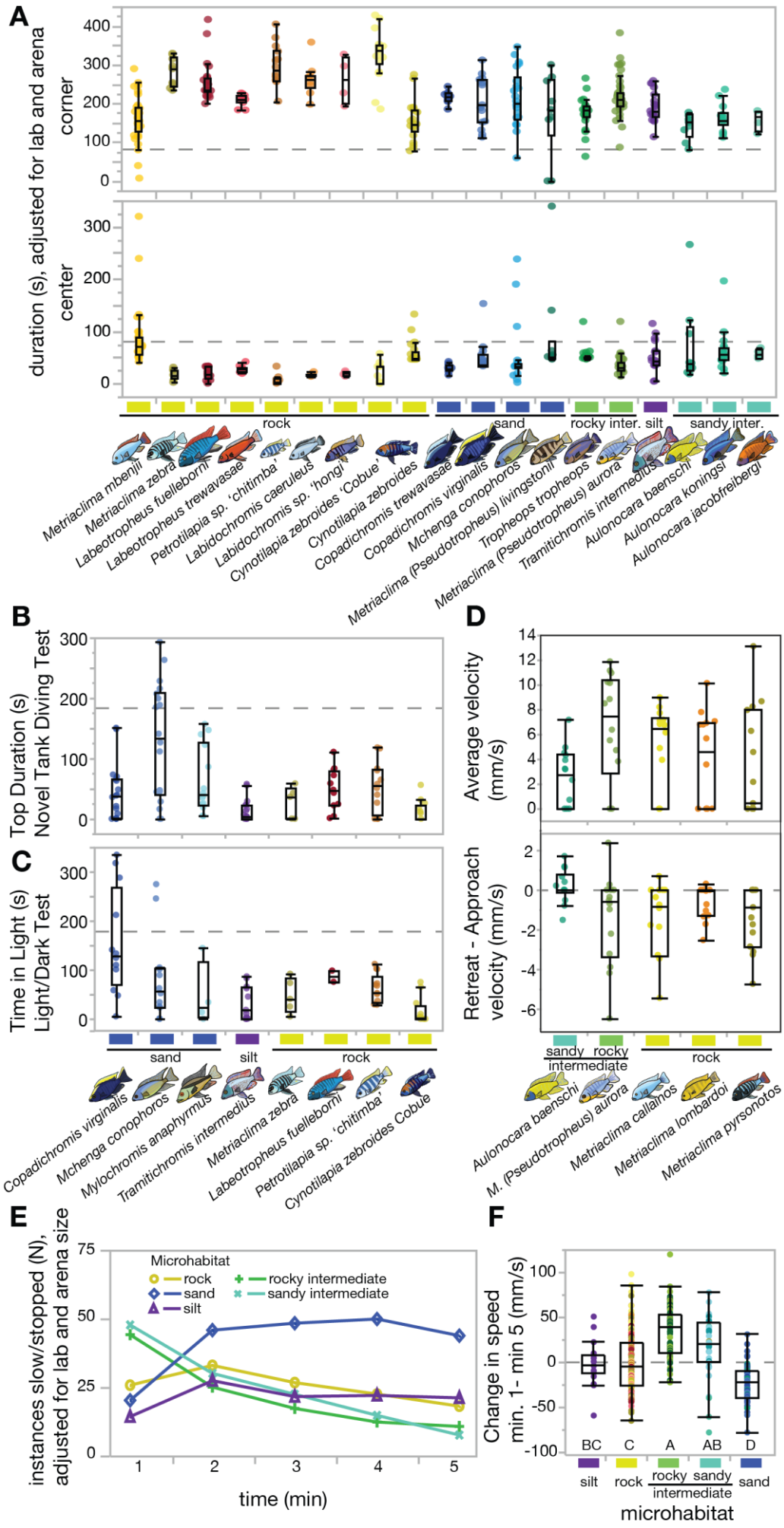

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Figure 2. Behavioral responses to novel stimuli across species and microhabitats. Behavioral responses to novel stimuli differed by species and microhabitat across all four assays. This Fig illustrates representative dimensions of behavior in each assay to highlight these differences: time spent in the center and corner regions in the open field test (A); time spent in the top half in the novel tank test (B); time spent in the light half in the light-dark test (C); average velocity and differences in approach and retreat velocities in the novel object test (D); instances of slow/stopped movement in the open field test over time (E); and change in swimming velocity over the course of the open field test (F). Species differences were observed for every behavioral measure. Microhabitat differences were observed for time spent in center and corners in the open field test (A); distance to the edge in the novel tank test (B); instances of slowed or stopped movement over time in the open field test (E); and change in velocity over time in the open field test (F). For Panels A-D, individual species tested are illustrated and labeled below. For all panels, microhabitat is color coded and labeled. Dotted lines in all panels indicate null expected values for time spent in each zone based on zone area.

3.6 Malawi cichlids exhibit modular patterns of behavioral covariation across contexts

We next investigated whether behavioral phenotypes covaried across different novel contexts in Malawi cichlids. To assess the relative evolutionary integration vs. modularity of exploratory behaviors, we correlated individual subjects' behaviors across multiple assays in two independent experiments and identified clusters of strongest covariation using MMC. For comparison, we applied the same analysis to a previously published zebrafish data set in which high and low exploratory strains exhibited syndromic patterns of behavior (Wong *et al*, 2012). As expected, modularity analysis revealed extensive clustering between assays in zebrafish: five of the eight (62.5%) modules encompassed multiple behavioral assays, indicating that behavioral phenotypes in zebrafish correlated strongly across novel contexts. In stark contrast, for both Malawi cichlid data sets, behavioral modules grouped exclusively within assay rather than between assay—zero of ten (0%) modules from the NCSU data set and zero of three (0%) modules from the GT data spanned multiple assays.

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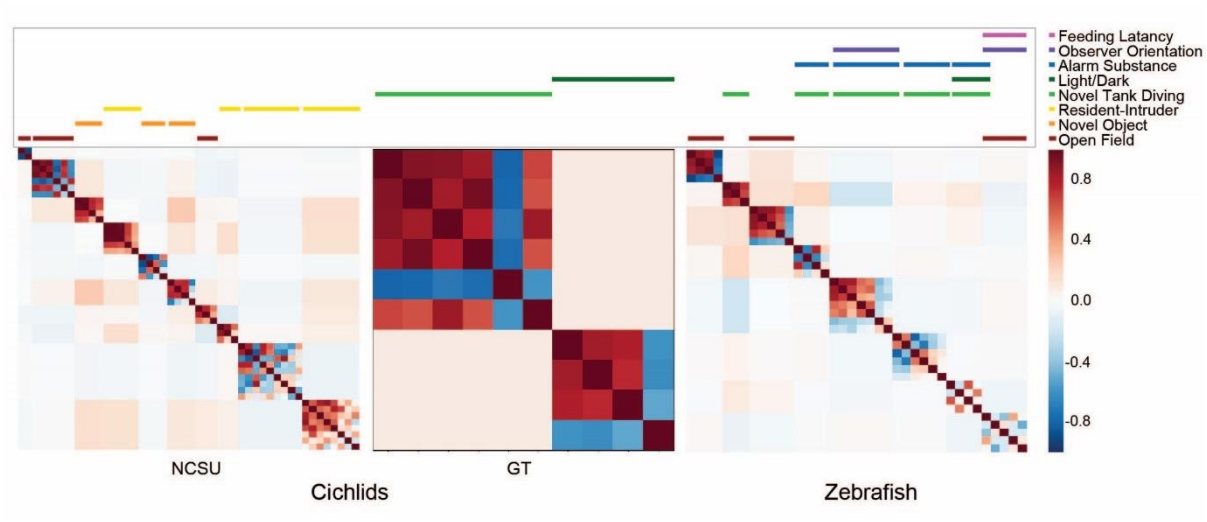

Figure 3. Behavioral modularity analysis across novel environment assays in Lake Malawi cichlids and high- and low-anxiety strains of zebrafish. Clustering by assay in cichlids (NCSU and GT), but across assays in zebrafish. Each entry into the matrix is a single behavioral measurement (such as seconds in the corner [open field], or latency to enter the top of the arena [novel tank]. The modules show the correlations between the measurements across all individuals, with dark red indicating a strong positive correlation and dark blue indicating a strong negative correlation. The lines at the top indicated which assays each module represents.

4 Discussion

Across all three novel environment behavioral assays (novel tank test, light-dark test, open field test), Lake Malawi cichlids showed place biases that mirrored those of other teleosts, spending less time in the top half in the novel tank test, the light half in the light-dark test, and the center region in the open field test (Maximino et al., 2007; Stewart et al., 2010; Stewart et al., 2012; Yoshida, Nagamine, & Uematsu, 2005). The patterns in the light-dark and open field tests also reflect behavioral patterns observed in response to similar novel environments in terrestrial vertebrates such as mice and rats: mice and rats spend less time in the light zone in the light-dark test and the center region in the open field test (Bailey & Crawley, 2009; Ramos, Berton, Mormède, & Chaouloff, 1997). Taken together, these results support conserved behavioral and/or stress responses to specific types of novel stimuli that are shared between Lake Malawi cichlids and other teleosts, and more broadly across vertebrates.

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More specific dimensions of behavior differed strongly between species in all four assays (e.g. strength of place biases between zones, number of zone entries, latencies to enter arena zones, and total distance traveled among others). Because Lake Malawi cichlids are thought to have diverged <1 mya, these results suggest that behavioral responses to novel stimuli have rapidly evolved in this species group. This is consistent with previous work showing that behavioral responses to novel stimuli have rapidly diverged between closely-related species of birds and mammals (Cowan, 1977; R. S. Greenberg, 2003; C. Mettke-Hofmann, Winkler, Hamel, & Greenberg, 2013; Claudia Mettke-Hofmann et al., 2002). We further investigated this behavioral variation by comparing Lake Malawi cichlid behavioral variation in the novel tank test with behavioral variation in wild-derived strains of zebrafish that have been selectively bred for high and low exploratory behavior. We found that, compared to high and low exploratory zebrafish, Lake Malawi cichlids exhibited a higher degree of phenotypic variance in multiple dimensions of behavior, including the amount of time spent in the top and the number of entries into the top. These data further support the high degree of behavioral variation in Lake Malawi cichlids.

We next demonstrated a strong association between microhabitat and exploratory behavior in Lake Malawi cichlids. Rock-dwelling species spent significantly more time in the corner regions and less time in the center region compared to species inhabiting open sand, rocky intermediate, sandy intermediate, and shallow silt habitats. Rock-dwelling species also remained closer to the tank corners in the novel tank test compared to sand-dwelling species, suggesting that this preference persists when exploration is restricted in different spatial dimensions. One potential explanation for these data is that a behavioral preference for edges or corners, and/or an aversion toward open and exposed sandy environments, helps mediate behavioral preferences for the narrow crevasses and caves characteristic of rocky habitats; inversely, higher exploratory behavior may facilitate preference for or invasion of new and potentially more exposed ecological niches. In Lake Malawi cichlids, microhabitat has also been associated with species differences in neuroanatomy (e.g. volume of the cerebellum and telencephalon) and aggression (Danley, 2011; Huber, van Staaden, Kaufman, & Liem, 1997;

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Sylvester et al., 2010). Taken together, these data suggest that microhabitat may play a central role in cichlid brain and behavioral evolution.

Exploratory behaviors may have different tradeoffs in more uniform versus variable environments (Greenberg & Mettke-hofmann, 2001). Consistent with this idea, a comparative study in parrots found that species inhabiting more “intermediate” habitats between the forest and savannah more readily approach novel objects compared to species inhabiting more uniform savannah habitats (Claudia Mettke-Hofmann et al., 2002). We tested whether intermediate habitats are associated with higher levels of exploratory behavior in Lake Malawi cichlids compared to the canonical rock-dwelling and sand-dwelling ecotypes. Across 19 species, we found that species inhabiting intermediate habitats are more exploratory in the open field test compared to the rock-dwellers and sand-dwellers, spending more time in the center region and less time in the corner regions of the open field arena. The direction of the association between habitat and behavior is thus consistent between parrots and cichlids. Although habitat variability or complexity is difficult to measure, these data are consistent with the hypothesis that intermediate habitat zones are associated with increased exploratory behavior in both birds and teleosts. Interestingly, *Tramitichromis intermedius*, which inhabits intermediate shallow silt habitats near read stands, readily explored the center region in the shallow open field test but exhibited a strong corner preference in the novel tank test, highlighting that exploratory behavior can vary strongly depending on the behavioral assay and the spatial features of the environment.

Evolutionary integration and modularity refer to patterns of covariation among sets of traits across taxa. For example, if the dimensions of different oral jaw bones are correlated in the same way across species, then they are considered to be evolutionarily integrated. In contrast, if they are uncorrelated or are correlated non-uniformly across taxa, they are considered to be more modular and may be more evolvable, although see Armbruster et al. (Armbruster et al., 2014). Comparative studies in Lake Malawi cichlids have demonstrated modular patterns of covariation for several complex traits that are thought to have played a central role in cichlid

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diversification, including oral jaw morphology and color patterning (R. Craig Albertson et al., 2014; Parsons et al., 2011).

We investigated patterns of covariation in Lake Malawi cichlid behavior, in which our traits of interest were behavioral outputs in response to different sets of novel stimuli. Correlated behaviors across contexts, or behavioral syndromes, have been demonstrated in many species, including teleost fishes (Andrew Sih, Alison M Bell, J Chadwick Johnson, & Robert E Ziemba, 2004; Sih, Bell, & Johnson, 2004; Sih & Bell, 2008). We reasoned that if behavioral phenotypes in different novel contexts are strongly correlated, or syndromic, this would constitute evidence for phenotypic integration. In contrast, weakly or uncorrelated behavioral phenotypes across contexts would support modular patterns of behavioral variation. We found that exploratory behavior was weakly correlated across assays in Lake Malawi cichlids in two separate experiments, consistent with modular patterns of behavioral variation. As a reference, we applied the same analysis to a previously published dataset from high and low exploratory strains of zebrafish that exhibit syndromic behavior (Baker, Goodman, Santo, & Wong, 2018; Ryan Y Wong et al., 2012). As expected, and in contrast to Lake Malawi cichlids, this analysis revealed patterns of strong behavioral covariation across assays. Taken together, these results are consistent with the notion that, like other complex traits, Lake Malawi cichlids exhibit modular patterns of behavioral variation, and raise the possibility that exploratory behavior is highly evolvable in this species assemblage.

There are several limitations to these experiments. First, these assays do not reflect environmental conditions in Lake Malawi, and therefore it is unclear how behavioral phenotypes in these experiments map onto behavior in the wild. Additionally, although the number of species investigated was larger than most comparative behavioral investigations, larger samples of species and individuals may uncover additional links between more specific dimensions of ecology and behavioral variation. For example, factors such as diet, resource distribution, population density, turbidity, depth, and/or predation risk may explain species differences in behavioral responses to novel stimuli. Additional factors may also influence behavioral responses to novel stimuli across species, such as developmental stage, sex, or

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social context. These questions were beyond the scope of this study and are promising areas for future research. Lastly, the sample sizes here were not sufficiently large to investigate syndromic behavior within individual Lake Malawi cichlid species; thus, interspecies differences in behavioral syndromes may be revealed by future comparative investigations with larger samples.

Despite these limitations, these experiments constitute a large comparative investigation of exploratory behavioral variation in a previously untested and species-rich vertebrate system. We show strong species differences in exploratory behavior, demonstrate links to microhabitat, and show that intermediate microhabitats predict higher levels of exploratory behavior. Our data also supports modular patterns of exploratory behavioral variation across Lake Malawi cichlids. Taken together, these findings demonstrate that Lake Malawi cichlids are positioned as a powerful complement to traditional model systems for investigating the ecological, neural, and genetic factors underlying behavioral evolution.

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Species	n	Microhabitat	Mean time spent in bottom (seconds)	Mean time spent in top (seconds)	Standard Error	$P_{top\ vs.\ bottom}$
Copadichromis virginalis	18	Sand	316.60	43.40	11.29	5.75×10^{-10} ***
Mchenga conophoros	18	Sand	229.88	130.12	23.35	0.0421 *
Mylochromis anaphyrmus	14	Sand	295.95	64.05	15.30	2.70×10^{-6} ***
Tramitichromis intermedius	18	Silt	346.99	13.01	4.46	5.47×10^{-18} ***
Metriaclima zebra	5	Rock	332.46	27.54	13.15	2.05×10^{-4} ***
Labeothropheus fuelleborni	12	Rock	310.40	49.60	10.11	3.50×10^{-8} ***
Petrotilapia sp. 'chitimba'	12	Rock	306.82	53.18	12.66	4.71×10^{-7} ***
Cynotilapia zebroides cobue	13	Rock	348.23	11.77	4.99	1.32×10^{-12} ***

Supplementary Table 1. Place bias between bottom and top regions of the novel tank test by species. Each row corresponds to the species labeled in the left column. The following are presented for each species: sample size, microhabitat designation, mean time in bottom zone, mean time in top zone, standard error for time spent in both zones, and two-tailed paired t-test p-values for the difference in time spent between the two zones.

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Species	n	Microhabitat	Mean time spent in dark (seconds)	Mean time spent in light (seconds)	Standard Error	$P_{light\ vs.\ dark}$
Copadichromis virginalis	12	Sand	203.77	156.23	32.73	0.46
Mchenga conophoros	12	Sand	275.42	84.58	27.02	0.0036 **
Mylochromis anaphyrmus	4	Sand	311.83	48.17	38.02	0.028 *
Tramitichromis intermedius	11	Silt	325.74	34.26	11.07	7.74×10^{-8} ***
Metriaclima zebra	5	Rock	312.67	47.33	17.67	0.0011 **
Labeothropheus fuelleborni	2	Rock	272.91	87.09	16.19	0.078
Petrotilapia sp. 'chitimba'	13	Rock	297.82	62.18	8.34	4.87×10^{-9} ***
Cynotilapia zebroides Cobue	12	Rock	344.07	15.93	8.15	3.12×10^{-10} ***

Supplementary Table 2. Place bias between light and dark halves of the light-dark test by species. Each row corresponds to the species labeled in the left column. The following are presented for each species: sample size, microhabitat designation, mean time in dark zone, mean time in light zone, standard error for time spent in both zones, and two-tailed paired p-value for the difference in time spent between the two zones.

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Species	n	Microhabitat	Mean time center (seconds)	Mean time in periphery (seconds)	Standard Error	$P_{center\ vs.\ periphery}$
Aulonocara baenschi	9	Sandy intermediate	73.77	246.23	28.32	0.0121 *
Aulonocara koningsi	18	Sandy intermediate	47.91	272.09	10.50	3.87×10^{-9} ***
Aulonocara jacobfreibergi	4	Sandy intermediate	36.52	283.48	13.98	0.00201 **
Copadichromis trewavasae	11	Sand	29.75	290.25	2.23	3.24×10^{-14} ***
Copadichromis virginalis	15	Sand	16.99	303.01	8.23	4.52×10^{-11} ***
Mchenga conophoros	22	Sand	30.84	289.16	14.11	5.99×10^{-9} ***
Tramitichromis intermedius	19	Silt	35.07	284.93	8.03	4.39×10^{-12} ***
Tropheops tropheops	29	Rocky intermediate	20.29	299.71	2.48	1.37×10^{-30} ***
Metriaclima (Pseudotropheus) livingstonii	10	Rocky intermediate	56.48	263.52	30.74	0.00622 ***
Metriaclima (Pseudotropheus) aurora	55	Rocky intermediate	29.94	290.06	2.20	5.78×10^{-51} ***
Metriaclima mbenjii	38	Rock	62.06	257.94	10.13	7.89×10^{-12} ***
Metriaclima zebra	9	Rock	17.32	302.68	3.31	5.83×10^{-11} ***
Labeotropheus fuelleborni	23	Rock	9.27	310.73	2.13	1.10×10^{-27} ***
Labeotropheus trewavasae	11	Rock	28.09	291.91	2.01	1.04×10^{-14} ***
Petrotilapia sp. 'chitimba'	14	Rock	2.56	317.44	1.09	1.91×10^{-22} ***
Labidochromis caeruleus	10	Rock	17.17	302.83	0.80	1.75×10^{-17} ***
Labidochromis sp. 'hongi'	4	Rock	19.37	300.63	2.50	8.08×10^{-6} ***
Cynotilapia zebroides	21	Rock	26.22	293.78	4.94	1.92×10^{-17} ***
Cynotilapia zebroides Cobue	18	Rock	4.90	315.10	3.30	1.20×10^{-19} ***

Supplementary Table 3. Place bias between central and peripheral regions of the open field test by species. Each row corresponds to the species labeled in the left column. The following are presented for each species: sample size, microhabitat designation, mean time in central regions, mean time in peripheral regions, standard error for time spent in both regions, and two-tailed paired p-value for the difference in time spent in central versus peripheral regions.

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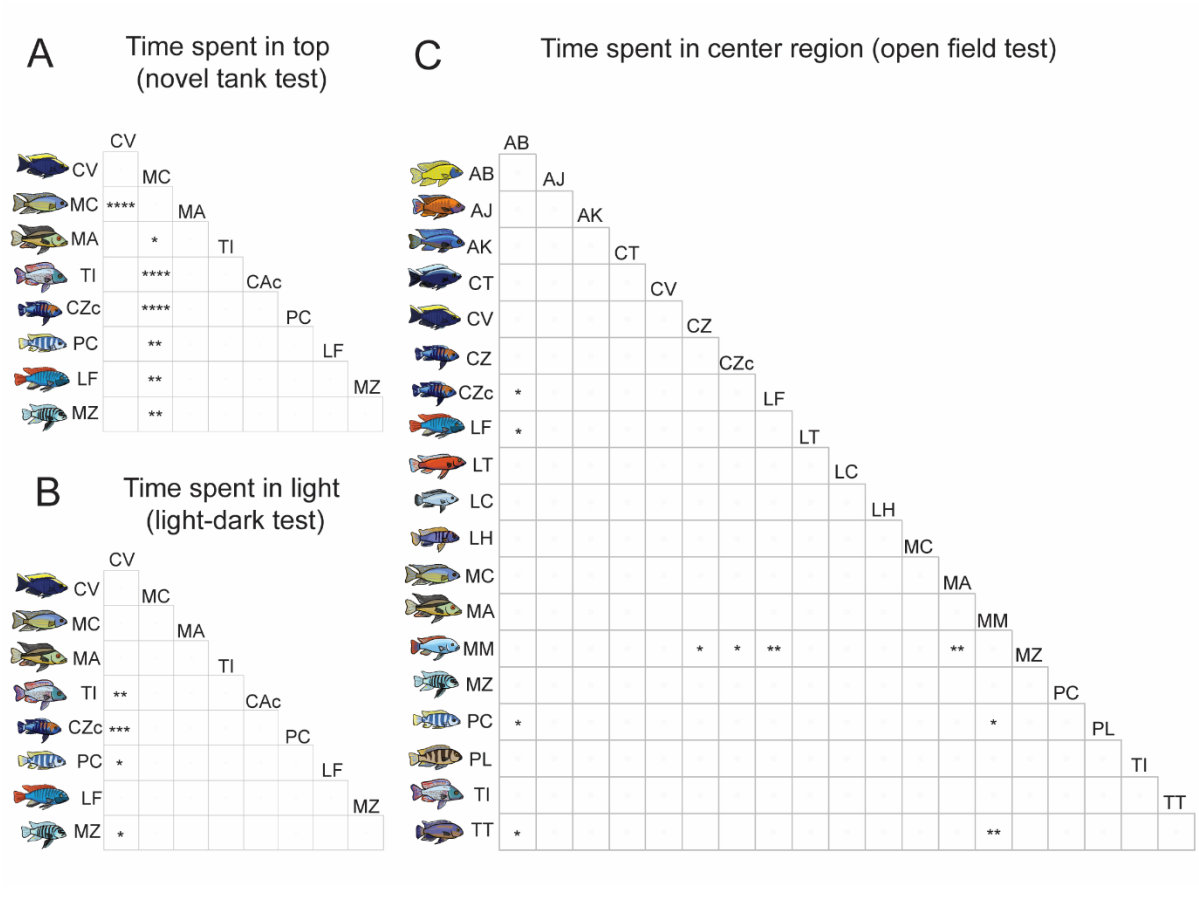

Supplementary Figure 1. Pairwise species differences in strength of zone preferences across assays. Species differences were present in the amount of time spent in the top half of the novel tank test (A), light half in the light-dark test (B), and center region in the open field test (C). Asterisks indicate levels of significance for post-hoc Tukey's HSD tests of the difference between species (* $p<0.05$, ** $p<0.005$, *** $p<0.0005$, ****$p<5x10^{-5}$). Species abbreviations are as follows: AB (*Aulonocara baenschii*), AJ (*Aulonocara jacobfreibergi*), AK (*Aulonocara koningsi*), CT (*Copadichromis trewavasae*), CV (*Copadichromis virginalis*), CZ (*Cynotilapia zebroides*), CZc (*Cynotilapia zebroides sp. 'afra cobue')*, LF (*Labeotropheus fuelleborni*), LT (*Labeotropheus trewavasae*), LC (*Labidochromis caeruleus*), LH (*Labidochromis* sp. 'hongi'), MC (*Mchenga conophoros*), MA (*Mylochromis anaphyrmus*), MM (*Metriaclima mbenjii*), MZ (*Metriaclima zebra*), PC (*Petrotilapia sp. 'chitimba'*), PL (*Pseudotropheus livingstonii*), TI (*Tramitichromis intermedius*), TT (*Tropheops tropheops*).

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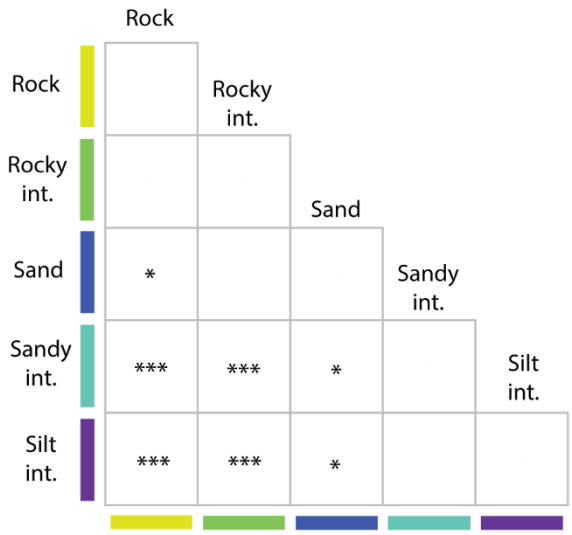

Supplementary Figure 2. Differences in open field behavior by microhabitat. Time spent in corner regions in the open field test differed significantly by microhabitat. The significance of each pairwise combination of different microhabitats (coded by color) is illustrated, with asterisks indicating the level of significance determined by post-hoc Tukey's HSD tests (* $p<0.05$, ** $p<0.005$, *** $p<0.0005$, ****$p<5x10^{-5}$).