Exploratory behavior is associated with microhabitat and evolutionary radiation 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 a total of 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 demonstrate generally conserved behavioral response patterns to novel stimuli in Lake Malawi cichlids, spanning multiple assays and paralleling other teleost and rodent lineages. Next, we demonstrate that more specific dimensions of exploratory behavior vary strongly among Lake Malawi cichlids, and that a large proportion of this variation is explained by species differences. We further show that species differences in open field behavior are associated with microhabitat and with major evolutionary radiations between mbuna and benthic/utaka lineages in Lake Malawi. Lastly, we track individuals across multiple behavioral assays and show that patterns of behavioral covariation across contexts are characteristic of modular complex traits. Taken together, our results tie ecology and evolution to natural behavioral variation, and highlight Lake Malawi cichlids as a powerful system for understanding the biological basis of exploratory behaviors.

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

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 et al., 2004; Ferrari, McCormick, Meekan et al., 2015; Lapiedra, Schoener, Leal et al., 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, Wright, Kazem et al., 2007). For example, a comparative study across 61 species of parrots showed that microhabitat predicts 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 data support the idea that habitat divergence is associated with variation in exploratory behaviors. 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 avian species (Garland & Adolph, 1994; Réale, Reader, Sol et al., 2007). Furthermore, different behavioral assays and testing parameters have been used across these studies, making it difficult to identify common factors 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 in new vertebrate systems are needed.

Lake Malawi cichlid fishes are well-suited for comparative investigations of phenotypic variation, and have attracted the attention of evolutionary biologists for more than a century (R. C. Albertson, Markert, Danley et al., 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, diversifying through multiple major evolutionary radiations into an estimated 500-1000 species that vary in morphology, coloration, diet, habitat preference, and behavior (Brawand, Wagner, Li et al., 2014; Kocher, 2004; Malinsky, Svardal, Tyers et al., 2018). 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, although many species can be grouped into two canonical ecotypes, rock-dwelling and sand-dwelling (Kocher, 2004), a large number of species occupy the intermediate habitat, or the interface between rocky and sandy substrate. Thus, the Lake Malawi species assemblage is an excellent system for studying relationships between evolution, ecology, and phenotypic variation.

Comparative studies in Lake Malawi cichlids have already generated insights into the evolution of complex traits. Species differences in morphology, color patterning, sex determination, and bower building behavior have been mapped to specific genomic loci (R. Craig Albertson, Streelman, & Kocher, 2003; Bloomquist, Parnell, Phillips et al., 2015; Conith, Hu, Conith et al., 2018; Kratochwil, Liang, Gerwin et al., 2018; Roberts, Ser, & Kocher, 2009; Ser, Roberts, & Kocher, 2010; R. A. York, Patil, Abdilleh et al., 2018). Ecological factors have also been linked to phenotypic variation, including species differences in jaw morphology and behaviors such as aggression and bower-building (R. Craig Albertson, 2008; Danley, 2011; Ryan A. York, Patil, Hulsey et al., 2015). Several studies have also demonstrated modular patterns of phenotypic variation in Malawi cichlids (R. Craig Albertson, Powder, Hu et al., 2014; Parsons, Cooper, & Albertson, 2011). Briefly, evolutionary modularity and integration refer to patterns of covariance within a set of traits across divergent populations and/or species (e.g. patterns of covariance among the lengths of different oral jaw bones across species), and they are thought to be related to trait evolvability (Raff & Raff, 2000; Wagner, Pavlicev, & Cheverud, 2007). Integration refers to

more uniform patterns of covariation, while modularity refers to non-uniform patterns of covariation and is generally considered to reflect enhanced trait evolvability; however, integration does not necessarily suggest a constraint on evolvability, and patterns of covariation by themselves are not sufficient for proving lesser or greater evolutionary potential (Armbruster, Pélabon, Bolstad et al., 2014).

Although the Lake Malawi species assemblage is an excellent system for comparative investigation, few comparative behavioral studies have been conducted in this system. We aim to address this gap by investigating exploratory behavior using four classic behavioral assays (Stewart, Cachat, Wong et al., 2011; Stewart, Gaikwad, Kyzar et al., 2012) across a total of 23 species, which collectively span three Lake Malawi microhabitats: rock, sand, and rock/sand intermediate. We test the following hypotheses: (i) Malawi cichlids exhibit responses to novel stimuli that are similar to other teleosts and other vertebrates; (ii) natural evolution has resulted in a high degree of phenotypic variance in exploratory behaviors among Lake Malawi cichlids; (iii) variation in exploratory behaviors is explained by divergence between species; (iv) species differences in exploratory behaviors are associated with microhabitat and major evolutionary radiations in Malawi cichlids; and (v) like other complex traits in this species assemblage, exploratory behaviors are modular.

2. Methods

2.1 Subjects

Subjects were maintained at two institutions, Georgia Institute of Technology (INSTITUTION 1) in Atlanta, GA and North Carolina State University (INSTITUTION 2) in Raleigh, NC. Both institutions house laboratory cichlid lines derived from wild-caught animals collected in Lake Malawi. Similar housing and husbandry conditions were maintained at both institutions: (i) age- and size-matched individuals were socially housed in mixed-sex tanks at similar densities (ranging between 0.67-1.33 cm of fish/liter) and co-cultured as necessary to reduce aggression; (ii) room temperature ranged from 26.5-28.0ºC and humidity was maintained at

155 approximately 40%; (iii) tank water temperature ranged between 27-28ºC, pH between 7.8-
156 8.2, and conductivity between 230-260 uS; and (iv) 12:12 hour light:dark cycles were
157 maintained with transitional dim light periods.
158
159 INSTITUTION 1 animals were maintained in the Engineered Biosystems Building cichlid
160 aquaculture facilities at INSTITUTION 1 in accordance with the Institutional Animal Care and
161 Use Committee (IACUC) guidelines (protocol numbers A100028 and A100029). Subjects
162 were housed on a 12:12-hour light:dark cycle with full lights on between 8am-6pm Eastern
163 Standard Time (EST) and dim lights on for 60 minutes between the light-dark transition
164 (7am-8am and 6pm-7pm EST). All subjects were housed in 190-liter or 95-liter glass tanks
165 measuring 92 cm (long) x 46 cm (wide) x 42 cm (high) or 46 cm (long) x 46 cm (wide) x 42
166 cm (high), respectively, and fed daily (Spirulina Flake; Aquatic Ecosystems). Male and
167 female subadults (age 90-180 days) were analyzed in the novel tank test and light-dark test
168 (described below), and male and female reproductive adults (>180 days) were tested in the
169 open field test (described below).
170
171 INSTITUTION 2 animals were maintained in the INSTITUTION 2 Roberts Lab cichlid
172 aquaculture facility in Raleigh, NC. Subjects were housed on a 12:12-hour light:dark cycle
173 with dim lights on for 15 minutes during the light-dark transition periods, and were fed daily
174 (Best Flake 70% Vegetable/30% Brine mix; Worldwide Aquatics). All experiments were
175 conducted under the approval of the Institutional Animal Care and Use Committee (IACUC)
176 guidelines (protocol number 14-138-O). For all thirteen INSTITUTION 2 species tested in the
177 open field test, subjects were housed in 189-liter or 473-liter tanks measuring 92 cm (long) x
178 47 cm (wide) x 48 cm (high) or 184 cm (long) x 47 cm (wide) x 60 cm (high), respectively,
179 and were tested as male and female reproductive adults (>180 days). For all five
180 INSTITUTION 2 species tested in the novel object test, subjects were socially housed in
181 mixed-species groups in a single 473-liter aquarium and were tested as male and female
182 reproductive adults (>180 days).
183
184 2.2 Animal welfare

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186 At both institutions, the utmost care was taken to minimize stress from handling and housing,
187 both in general husbandry and during behavioral experiments. Non-experimental fish were
188 communally housed to provide social interaction, and monitored carefully to ensure that
189 animals had access to territories and were not the target of aggression. Monitoring allowed
190 for intermittent reorganization of co-housed fish if needed. During behavioral experiments,
191 fish were gently netted out of their home tanks by an experienced handler and carefully
192 moved to reduce stress as much as possible. Transfer containers were covered by nets to
193 reduce stress, as well. For the subset of tests at INSTITUTION 2 that required isolation in
194 aquaria to allow the focal fish to establish ownership of territory, visual contact was
195 maintained with surrounding tanks providing opportunity for social interaction. To prevent
196 influence of neighbor species on behavior, a blocked design was used such that each
197 species had an equal number of times with neighbors of every other species—a step
198 necessary to reduce stress from social isolation.

199

200 2.3 Behavioral assays

201

202 A total of 525 subjects spanning 23 Lake Malawi cichlid species were tested in one or more
203 assays that are well-established and designed to measure exploratory behaviors in teleosts.
204 Pilot data indicated strong effects of species but no effects of sex on exploratory behavior
205 across multiple assays. Based on these data, subjects for the present study were sampled
206 randomly from mixed sex tanks but were not euthanized and dissected to determine gonadal
207 sex, with the exception that visually identified dominant males were sampled at a proportion
208 consistent with the composition of the home tank, and maternal mouthbrooding females
209 were not sampled. All assays were performed between 10:00 and 16:00 Eastern Standard
210 Time EST. Each assay is described in detail by institution (INSTITUTION 1 and
211 INSTITUTION 2), species, sample size, and experimental design in the following sections.

212

213 *Assays by test site*

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The novel tank and light-dark tests were conducted at INSTITUTION 1 only. 110 subjects from eight species were tested in the novel tank test; 67 of these subjects were also tested in the light-dark test, and four additional subjects were tested exclusively in the light-dark test (see Supplementary Tables 1, 2, and 5 for sample sizes by species). The novel object test was conducted at INSTITUTION 2 only, and 70 subjects from five species were tested. Motivated by convergent patterns found independently at both institutions, the open field test was then conducted across a larger species and subject pool spanning both INSTITUTION 1 and INSTITUTION 2. For the open field test, 341 subjects from 19 species were tested: 227 subjects from 13 species at INSTITUTION 2, and 113 subjects from seven species at INSTITUTION 1, with one species (*Labeotropheus fuelleborni)* tested at both institutions (See Supplementary Table 3 for sample sizes by species).

To assess phenotypic integration versus modularity of exploratory behaviors, correlated behaviors across novel contexts were measured by applying Modularity Modular Clustering analysis (MMC; described below) to three independent datasets in which subjects were tracked across multiple assays. The first dataset included 67 subjects from eight Malawi cichlid species that were tested in both the novel tank test and light-dark test at INSTITUTION 1 (Supplementary Table 4). The second dataset included 70 subjects from five Malawi cichlid species that were tested in the novel object test, open field test, and resident intruder test at INSTITUTION 2 (Supplementary Table 4). As a control, a third dataset was re-analyzed from a previously published study in selectively bred high- and low-exploratory strains of wild-derived zebrafish. In this study, 99 subjects from three selection lines were tested across a battery of behavioral assays (Wong, Perrin, Oxendine et al., 2012).

Novel tank test

The novel tank test is a classic assay designed to measure exploration of a tall and narrow transparent tank, with primary focus on exploration of the upper half (Fig. 1A-B). Individual subadult subjects (90-180 days; 1.75-2.5 cm standard length, SL) spanning eight species

were collected between 11:00-15:00 Eastern Standard Time from their home tank, transferred to a 300 mL holding beaker, and habituated for 30 minutes prior to behavioral testing. Water for both habituation beakers and test tanks was collected from a circulating aquaculture 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 time spent in the top half, entries/exits to and from the top half, latency to enter top half, and average distance from the bottom and corners, and total distance traveled.

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). As in rodents, this assay is designed to investigate place preferences between a dark versus illuminated environment, and exploration of the illuminated environment. 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 30 minutes prior to testing. All water was collected from the same circulating aquaculture 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 the 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, at which point two inserts were 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 time spent in the light versus dark halves, as well as latency to enter, number of entries, total time spent, and total distance traveled in the light half.

Novel object test

The novel object test has been employed across a wide range of vertebrate species and is designed to test behavioral responses (e.g. patterns of approach and retreat) toward an unfamiliar object (Fig 1E-F). Subjects were introduced to a 38-liter (50 cm x 28 cm x 33 cm) aquarium containing a single terracotta flowerpot territory and acclimated for three days. To assess activity and motivation during the acclimation period, latency to feed was measured at each meal. All subjects ate within 60 seconds of feeding by the final day of acclimation. Following the acclimation period, a camera was placed overhead, and water and air flow was stopped five minutes prior to the beginning of the test to enable clear video recording and to allow time for subjects to habituate to the change. 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. 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; distance from the object; and 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 teleosts is 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. For teleosts, vertical motion is restricted by shallow water depth, and the test is thus designed to measure behavioral responses to a large and open shallow water environment (Fig 1G-H). For the present study, 19 species were analyzed in the open field test at two test sites (INSTITUTION 1 and INSTITUTION 2). MMC (described below) also included re-analysis of a separate open field (and resident intruder) dataset collected as part of a

different study (Moore & Roberts, *in preparation*) from five species under different parameters (described below) at INSTITUTION 2 (see Supplementary Table 4).

All subjects 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. At both institutions, larger subjects exceeding 4.5 cm standard length (SL) were introduced to a 49.6 cm-wide square arena filled to a depth of 15 cm, while smaller subjects ranging from 2.5-4.5 cm SL were introduced to a 25.5 cm-wide square arena filled to a depth of 10 cm.

For all open field trials, tank water was replaced between every subject. 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.

2.4 Designations of microhabitat, evolutionary radiation, and genus

Previous genomic analyses suggest that Lake Malawi cichlids have diversified through multiple major evolutionary radiations of (i) pelagic species, (ii) shallow/deep benthic and “utaka” species, and (iii) “mbuna” species (Malinsky, Svardal, Tyers et al., 2018). The species sampled in the present study represented the latter two radiations (shallow/deep benthic and utaka, B/U; and mbuna). These radiations are well-characterized, and designations for evolutionary radiation as well as genus were made according to Konings (Konings, 2007). Microhabitat designations (rock, sand, or intermediate) for each species

were made according to Ribbink et al. and Konings (Konings, 2007; Ribbink, Marsh, Marsh et al., 1983).

2.5 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 biases between zones in the novel tank and light-dark tests across species, a linear regression model with time spent as the outcome variable, and zone (e.g. top vs. bottom) and species as categorical predictor variables, was fit to the data.

Time spent in zone ~ zone + species

Because the open field test was performed at two test sites using two arena sizes, these factors were added to the model as categorical variables, and time spent in central versus peripheral regions were analyzed:

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

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

Species differences in exploratory behavior

When appropriate, one-way ANOVA was used to test for species differences in behaviors. Effect size (Eta-squared) was calculated by dividing the individual effects' sum of squares by the total sum of squares. 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.

Effects of microhabitat and radiation on exploratory behavior

Associations between microhabitat and behavior were assessed through linear mixed effects models using the "lme4" package in R. Each behavior of interest was designated as the outcome variable, microhabitat and evolutionary radiation (mbuna vs. B/U) as fixed effects, species nested within genus as a random effect, and both arena size and lab as random effects. In this model microhabitat and evolutionary radiation directly competed to explain variatiance in exploratory behavior, controlling for variance explained by other phylogenetic factors and batch-like effects such as arena size and test site. This model was used to test six open field behavioral metrics, including time spent in the corners, entries into the corners, time spent in the center, entries into the center, total distance traveled, and change in speed over time. The model was organized as follows, (with bold italicized terms representing fixed effects, and non-bold italicized terms representing random effects, with nested terms in parentheses):

OF behavior ~ *microhabitat* + *radiation* + *(genus/species) + test site + arena size*

Because the mbuna radiation tends to inhabit rock microhabitats, and the B/U radiation tends to inhabit sand microhabitats, the fixed effects (radiation and microhabitat) in the above model were correlated, potentially masking additional relationships between microhabitat, evolutionary radiation, and exploratory behaviors. To further disentangle the relationships between the intermediate microhabitat, evolutionary radiation, and exploratory behavior, we applied a second model in which the original microhabitat term (rock, sand, or intermediate) was simplified into an intermediate (versus non-intermediate) term. This model thus tested how divergence into the intermediate microhabitat was associated with exploratory behaviors. To account for the possibility that divergence into the intermediate habitat is differentially related to exploratory behaviors in the mbuna versus B/U radiations, we also included an intermediate*radiation interaction term:

OF behavior ~ *intermediate + radiation + intermediate*radiation +* *(genus/species) + test site + arena size*

To test whether mbuna rock-dwellers and B/U sand-dwellers exhibited differences in novel tank behavior, a simpler model was used (all species came from a unique genus, and all subjects were tested in identical tanks at the same test site). Notably, because all INSTITUTION 1 mbuna species tested inhabited rock habitats, and all INSTITUTION 1 B/U species tested inhabited sand habitats, “radiation” and “microhabitat” could be interchanged in the model with identical results:

NT behavior ~ *radiation +* *species*

For all linear mixed effects models, estimates for fixed effects were calculated by maximum likelihood estimation using the ‘lme4’ package in R, and significance for fixed effects was calculated using Satterthwaite approximation through the ‘lmerTest’ package and the anova function in R. Estimates of pairwise differences between levels for each fixed effect were calculated using estimated marginal means (least squared means), and the significance of these differences were determined using Satterthwaite approximation corrected for multiple comparison families with Tukey’s adjustment, using the ‘emmeans’ and ‘multcomp’ packages in R

426

427 To analyze movement in the open field test over time, the numbers of slow or stopped
428 instances were summed over each minute, and one minute bins were used as the input for a
429 repeated measures MANOVA. Time points within individuals were analyzed at one level,
430 differences between microhabitat were analyzed at an additional level, microhabitat*time
431 was included as an interaction term, and additional terms were included to control for test
432 site and arena size. The overall change in velocity (average velocity in minute 1 – average
433 velocity in minute 5) throughout the assay was analyzed with an ANOVA by microhabitat (a
434 positive value indicates that the subject swam faster at the start of the assay, and a negative
435 value indicates the subject swam faster at the end of the assay).

436

437 *Effects of Test Site*

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439 To assess the extent to which test site may have influenced open field behavior and/or
440 downstream analyses, we analyzed its effect on behavior for the only species that was
441 housed and tested at both sites, *Labeotropheus fuelleborni* (INSTITUTION 1, n=16;
442 INSTITUTION 2, n=7). Controlling for arena size, linear regression showed that test site was
443 not significantly associated or trending with any of the six analyzed open field behaviors:
444 corner time (t=1.33, p=0.20), corner entries/exits (t=0.15, p=0.88), center time (t=0.56,
445 p=0.58), center entries/exits (t=0.86, p=0.39), distance traveled (t=0.54, p=0.60), and speed
446 change (t=1.56, p=0.14). We also conducted all open field analyses with and without test site
447 included in the above linear mixed effects models, and found that the vast majority (11/14) of
448 significant or trending relationships were also significant or trending when test site was
449 excluded. The few results that changed from statistically significant to p>0.10, as well as all
450 results that were significant or trending in both models, are indicated in Tables 1 and 2.

451

452 *Behavioral modularity test*

453

454 To examine behavioral correlations within and across assays, we performed Modulated
455 Modularity Clustering (MMC) analysis (Stone & Ayroles, 2009). This test identifies clusters of

456 covariance in multivariate data. Although this method was developed to analyze gene
457 expression data, it is effective for any large, multivariate datasets where many phenotypes
458 have been measured across a large sample of subjects. To demonstrate as a proof-of-
459 principle that MMC analysis can reveal behavioral correlations across these assays, we re-
460 analyzed a previously published zebrafish dataset in which individuals from selectively bred
461 high- and low-exploratory strains were tracked across multiple assays and behavioral
462 correlations across assays were identified (Wong, Perrin, Oxendine et al., 2012). We then
463 separately performed MMC on two independent Lake Malawi cichlid datasets: an
464 INSTITUTION 1 dataset in which individuals were tracked across the novel tank and light-
465 dark tests (Supplementary Table 4), and an INSTITUTION 2 dataset to analyze behavioral
466 modules across the open field, novel object, and resident-intruder tests (Supplementary
467 Table 4). In all MMC analyses, each individual behavioral metric within each assay (such as
468 speed, position, time spent in a specific zone, etc.) was included in the analysis. Since these
469 assays are of different measurement types, Spearman rank-order correlation was used in
470 place of Pearson's correlation.
471
472 **3. Results**
473
474 3.1 Malawi cichlids exhibit consistent place biases across assays
475
476 The three novel environment assays used in this study have been used widely in teleosts,
477 particularly in zebrafish, and variations of these tests are well-established in rodents. We first
478 investigated how Lake Malawi cichlids respond to these novel environments by measuring
479 their place biases between different zones (e.g. light half versus dark half). In general, Lake
480 Malawi cichlids exhibited strong place biases for specific zones in all three novel
481 environment assays, spending more time in the bottom half of the novel tank test, the dark
482 half of the light-dark test, and the periphery of the open field test. The direction of the place
483 biases were the same in all species tested, and were consistent with other teleosts and
484 rodents. More detailed results are organized by assay below:
485

Malawi cichlids prefer the bottom region in the novel tank test

Linear regression 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 bias between *Mchenga conophoros*, a B/U sand-dwelling species, and all other species tested, with *Mchenga conophoros* spending significantly more time in the top half (Supplementary Figure 1A). More detailed results by species are shown 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 2. Notably, one B/U 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 (Supplementary Figure 1B). Additional results are presented by species in Supplementary Table 2.

Malawi cichlids prefer peripheral regions in the open field test

Malawi cichlids spent more time in the peripheral regions 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. Two-tailed paired t-tests revealed these differences to be significant in every species tested ($p<0.05$ for all species tested, Supplementary Table 3). Additional results are presented by species in Supplementary Table 3. Notably, the B/U intermediate species *Aulonocara baenschi* and the mbuna rock-dweller *Metriaclima mbenjii* spent significantly less time in corner regions compared to multiple other species (Supplementary Figure 1C).

3.2 Malawi cichlids exhibit a high degree of phenotypic variance in exploratory behaviors

We next investigated the degree of phenotypic variance in exploratory behaviors that has resulted from natural evolution in Lake Malawi. For a frame of reference, we compared phenotypic variance in exploratory behaviors among Lake Malawi cichlids and among three strains of zebrafish: two wild-derived strains that have been selectively bred for divergent exploratory behaviors and a common domesticated wild-type strain (AB). Notably, genetic divergence between common strains of zebrafish is greater than between Malawi cichlid species (Loh, Katz, Mims et al., 2008). For this analysis, we compared phenotypic variance in novel tank behaviors, because the test parameters used in the present study were the same as those used in the zebrafish study. For time spent in the top half, Malawi cichlids collectively exhibited greater phenotypic variance compared to the high- and low-exploratory zebrafish strains (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; F-test, $p=0.006$). This pattern 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 half (variance for zebrafish = 15.56 vs. variance for cichlids = 15.59; F-test, $p=0.996$). Phenotypic variance in the novel tank test is represented in Figure 1A-B.

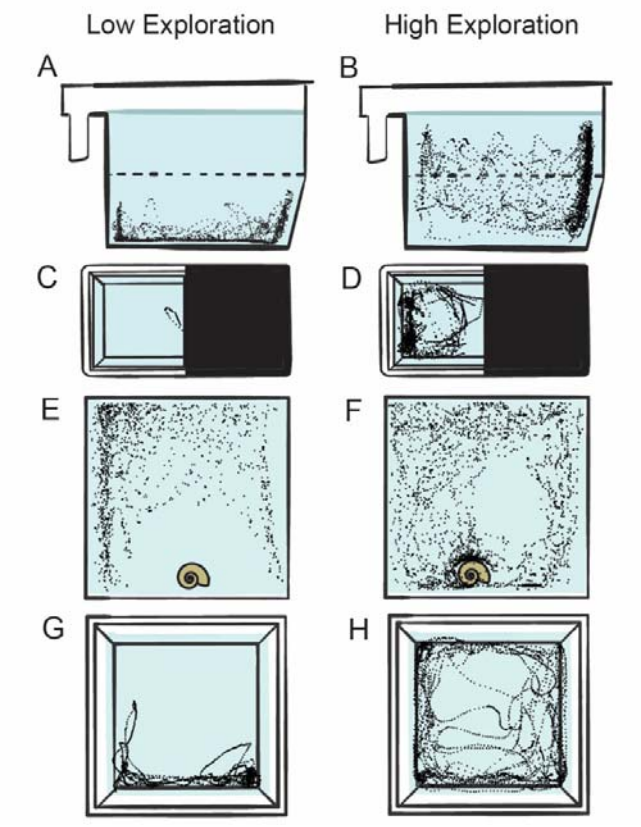


Figure 1. Lake Malawi cichlids exhibit high phenotypic variation in exploratory behaviors. Behavioral variation illustrated by representative traces from the four behavioral assays used in this study, the novel tank test (A-B), light-dark test (C-D), novel object test (E-F), and open field test (G-H). Individual points illustrate the position of the subject in the arena at a single timepoint. A high degree of phenotypic variance was observed across assays, ranging from stereotypically low exploratory phenotypes (A,C,E,G) to high exploratory phenotypes (B,D,F,H). For each assay, the schematic reflects the camera angle from which video recordings were collected for each trial.

3.3 Malawi cichlids exhibit strong species differences in exploratory behaviors

We next investigated the degree to which phenotypic variance in exploratory behaviors (e.g. see Figure 1) is explained by divergence along species lines. Across all four behavioral assays, nearly every dimension of exploratory behavior measured differed strongly among species. More detailed results are organized by assay below:

Novel tank test

In the novel tank test (Fig. 1A-B), several standard metrics of exploratory behavior were analyzed: total time spent in the top half, latency to enter the top half, total number of entries into the top half, and total distance traveled. 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 time spent in the top half ($F_{7,102}$=8.64; p=2.74x10^{-8}; Eta-squared=0.37, Fig. 2A), 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 distance traveled ($F_{7,102}$=8.30; p=5.38x10^{-8}; Eta-squared=0.36), 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). Pairwise differences between species are shown in Supplementary Figures 1A and 2A-D. Notably, the B/U sand-dweller *Mchenga conophoros* differed strongly from multiple other species in every dimension of behavior analyzed in this test, in every case exhibiting “more exploratory” phenotypes.

Light-dark test

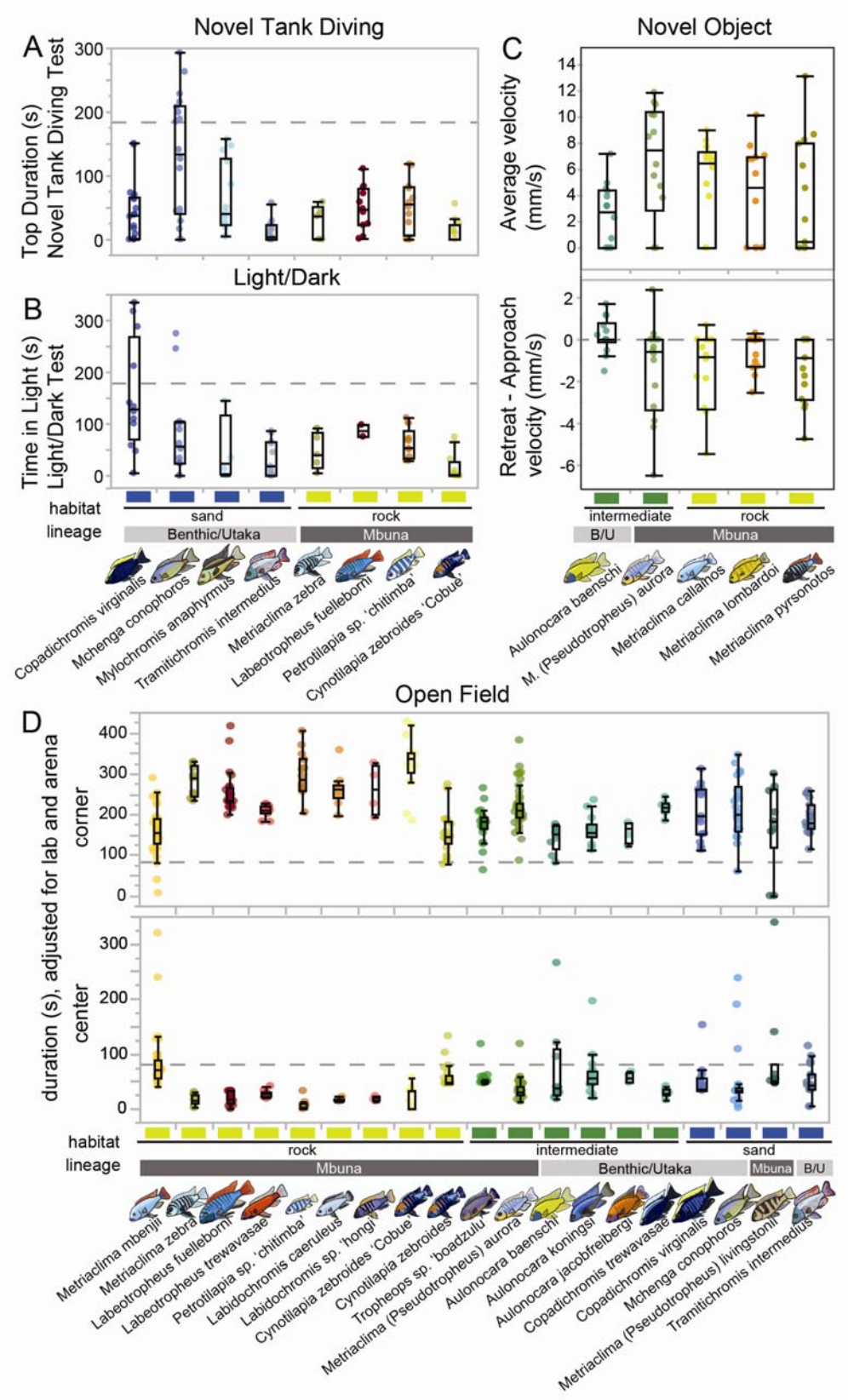
For the light-dark test (Fig 1C-D), total time spent in the light half (Fig 2B), latency to enter the light half, total number of entries into the light half, and total distance traveled in the light half were analyzed. One-way ANOVAs revealed a significant effect of species on total time spent in the light half ($F_{7,63}$=4.95; p=1.67x10^{-4}; Eta-squared=0.35, Fig 2B), 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 distance traveled in the light half ($F_{7,63}$=2.87; p=0.012; Eta-squared=0.24). Pairwise differences between species are shown in Supplementary Figures 1B and 2E-G. Notably, the mbuna rock-dweller *Cynotilapia zebroides ‘Cobue’* exhibited the longest latencies to enter the light half of any species, differing significantly from several other species (Supplementary Figure 2F).

Novel object test

In the novel object test (Fig. 1E-F) 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). Species also differed in swimming velocity throughout 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 2C, top panel) all differed strongly by species. Notably, the *Metriaclima* spp. were faster when retreating from the shell than when approaching it, whereas *Auloncara baenschi* approached and retreated with the same speed (Wilcoxon/Kruskal-Wallis test, χ^2=20.42, p=0.0004, Fig 2C, bottom panel). Pairwise species differences are shown in Supplementary Figure 2H-J.

Open field test

In the open field test (Fig. 1G-H), time spent in corner regions, corner entries/exits, time spent in the center, center entries/exits, total distance traveled, and speed change over time 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 strong species differences in time spent in the corner regions ($F_{18,319}$=8.928; p<2.00x10^{-16}; Eta-squared=0.33, Fig. 2D top panel), corner entries/exits ($F_{18,319}$=8.901, p<2x10^{-16}, Eta-squared=0.33), time spent in the center region ($F_{18,319}$=4.77; p=2.00x10^{-9}; Eta-squared=0.21, Fig. 2D bottom panel), center entries/exits ($F_{18,319}$=8.57; p<2x10^{-16}; Eta-squared=0.33), total distance traveled ($F_{18,319}$=6.03; p=1.34x10^{-12}; Eta-squared=0.25), and speed change over time ($F_{18,319}$=9.20; p<2.00x10^{-16}; Eta-squared=0.34). There were many pairwise differences between species in open field behavior, as shown in Supplementary Figure 1C and 3A-E.



620

621 **Figure 2. Exploratory behaviors differ strongly between species in Lake Malawi**
622 **cichlids.** Exploratory behaviors differed strongly among species in all four assays. This
623 figure illustrates representative dimensions of behavior in each assay to highlight these
624 differences: time spent in the top half of the novel tank test (A), time spent in the light half of

the light-dark test (B), average velocity and approach/retreat velocity in the novel object test (C), and time spent in the corner and center regions in the open field test (D). Species differences were observed for every behavioral measure. For all panels, microhabitat (rock=yellow; intermediate=green; sand=blue) and evolutionary radiation (Mbuna=dark gray; shallow/benthic and utaka=light gray) are color coded and labeled. Dotted lines in all panels indicate null expected values.

3.4 Microhabitat predicts species differences in exploratory behaviors

We next investigated whether variation in exploratory behavior was associated with microhabitat. In order to test this, we subjected a larger set of species (n=19) representing three Lake Malawi microhabitats (rock, sand, and intermediate) to the open field test. Controlling for variation explained by phylogenetic factors (evolutionary radiation, genus, and species), we found significant associations between microhabitat and exploratory behavior in multiple open field behaviors. These results are organized into three lines of analysis below (two linear mixed effect regression models, and one MANOVA model; see “Effects of microhabitat on behavioral responses to novel stimuli” under “Methods” above for full statistical models).

Linear mixed effects regression revealed significant relationships between microhabitat (rock, sand, or intermediate) and open field behavior. Controlling for variation explained by phylogenetic factors, microhabitat was significantly associated with the number corner entries/exits ($F=5.61$, $p=0.014$, Fig. 3A). This effect was driven by intermediate species entering and exiting the corners more than sand-dwellers (39.4 ± 11.84 more entries, $t=3.329$, Tukey’s HSD $p=0.0096$), and a trend toward rock-dwelling species entering and exiting the corners more than sand-dwellers (36.2 ± 15.11 more entries, $t=2.40$, Tukey’s HSD $p=0.069$). This effect was consistent in direction and statistically significant (Tukey’s $p<0.05$) regardless of whether test site was included in the model. Microhabitat was also associated with entries/exits to and from the center region ($F=12.66$, $p=5.72\times10^{-6}$, Fig. 3C). When controlling for evolutionary radiation, the rock microhabitat was associated with more

center entries/exits than sand (6.5 ± 2.14 more entries, t=3.04, Tukey's HSD p=0.0074) and intermediate (4.4 ± 0.94 more entries, t=4.70, Tukey's HSD $p=1.15x10^{-5}$). Notably, the relationship between microhabitat and center entries/exits was not statistically significant or trending when test site was removed from the model (Tukey's HSD p>0.10 for both effects). A trend was also observed between microhabitat and total distance traveled (F=4.42, p=0.053, Fig. 3E), with intermediate species swimming farther during the test compared to sand-dwellers (1015 ± 358 cm further, t=2.84, Tukey's HSD p=0.0571), and this effect was consistent in direction and statistically significant when test site was removed from the model (Tukey's HSD p=0.036). In this model, microhabitat was not significantly associated with time spent in corner regions (F=0.41, p=0.673, Fig. 3B) or time spent in the center region (F=0.70, p=0.512, Fig. 3D), or change in speed over time (F=0.240, p=0.79, Fig. 3F).

To further investigate the relationships between microhabitat and behavior, we tested a second model in which each microhabitat was designated as either intermediate (rock/sand interface) or non-intermediate (rock or sand). This model allowed effects of microhabitat to be more fully dissociated from effects of evolutionary radiation. The model also included an interaction term to test whether the intermediate microhabitat was differentially associated with behavior between evolutionary radiations. Consistent with findings from above, this model revealed a strong association between the intermediate microhabitat and entries/exits to and from the corner regions (F=27.08, p=0.0011, Fig. 3A), and this relationship differed between evolutionary radiations (F=6.7945, p=0.041): although intermediate species made more entries/exits to and from the corner regions than non-intermediates in both lineages, the difference was much greater within the B/U radiation (estimated difference of 50.2 ± 10.36 more entries by intermediates vs. non-intermediates, t=2.61, p=0.00056) compared to the mbuna radiation (estimated difference of 17.1 ± 7.57 more entries by intermediates vs. non-intermediates, t=2.26, p=0.10). The model also supported the association between intermediate microhabitat and distance traveled (F=9.17, p=0.018, Fig. 3E), with intermediate species traveling farther than non-intermediates (estimated difference of 729 ± 241 cm farther, t=3.028, p=0.018). Lastly, the model revealed that the intermediate microhabitat was differentially related to swimming speeds in the mbuna versus B/U

radiations (F=5.70, p=0.030): controlling for microhabitat, mbuna intermediate species slowed down more than their non-intermediate counterparts during the test (32.1 ± 12.46 mm/s greater decrease in swimming speed, t=2.572, p=0.027), and this pattern was reversed but not statistically significant in B/U species (12.9 ± 15.52 mm/s greater *increase* in swimming speed, t=0.34, p=0.41). All of the above effects were statistically significant (Tukey's p<0.05) when test site was excluded from the model, with the exception of the interaction between radiation, microhabitat, and change in speed (Tukey's p>0.10). The full linear regression results for open field behavior, including estimates for pairwise differences between microhabitats, are presented in Tables 1 and 2.

Microhabitat was also associated with additional patterns of movement over time in the open field test (repeated measures MANOVA, full model $F_{(4,336)}$=11.81, p<0.0001). Both frequency of freezing ($F_{(2,336)}$=15.64 p<0.0001) and the pattern of freezing over time (Wilks' Lambda value 0.866, approx. $F_{(8,666)}$=6.23, p<0.0001) were associated with microhabitat. Intermediate species initially froze more frequently and exhibited a decrease in slowed swimming as the assay progressed, whereas sand species initially froze less but tended to freeze more as the assay progressed (Fig. 3G).

3.6 Open field behaviors differ between mbuna and benthic/utaka radiations

The same linear mixed effects regression models described above were used to test for relationships between evolutionary radiation and behavior. Controlling for variance explained by microhabitat, these models revealed that mbuna vs. B/U radiations differed in time spent in corner regions (F=7.065, p=0.018, Table 2, Fig. 3H), time spent in the center region (F=5.32, p=0.047, Table 2), and entries/exits to and from the center region (Model 1, F=13.25, p=0.0029, Table 1; Model 2, F=5.55, p=0.043, Table 2). In comparison to B/U species, mbuna species spent more time in the corner regions (45.6 ± 17.2 seconds, t=2.658, p=0.0175), less time in the center region (24.0 ± 10.4 seconds, t=2.306, p=0.0472), and made fewer entries/exits to and from the center region (4.5 ± 1.9 fewer entries/exits, t=2.356, p=0.0428). The direction of all three of these effects was the same at both test

sites, and all three of these effects were consistent in direction and statistically significant or trending towards significance when test site was excluded from the model (Tukey's $p<0.10$ for all). A trend toward differences in speed change over time was also observed between radiations ($F=3.62$, $p=0.086$), with mbuna species slowing more as the assay progressed compared to B/U species (19.1 ± 10.1 mm/s greater decrease in swimming speed, $t=1.902$, $p=0.0863$). This effect was consistent in direction at both test sites and was statistically significant and consistent in direction when test site was excluded from the model ($p=0.032$). Notably, for all (6/6) open field behaviors analyzed, significant or trending relationships with microhabitat and/or evolutionary radiation were found regardless of whether test site was included in the model.

Novel tank test

Because of the strong differences between mbuna versus B/U radiations in open field behavior, we also reanalyzed novel tank data, in which four mbuna rock-dwelling species and four B/U sand-dwelling species were tested. Consistent with differences in corner behavior in the open field test, a linear mixed effects regression showed that mbuna rock-dwellers remained significantly closer to outer corner regions compared to B/U sand-dwellers in the novel tank test (0.56 ± 0.23 cm closer, $t=2.43$; $p=0.038$, Fig. 3l), but did not differ in the other analyzed dimensions of behavior.

OF behavior ~ *microhabitat* + *radiation* + *(genus/species) + test site + arena size*						
Behavior	**Fixed effect**	**Comparison**	**Estimate**	**S.E.**	**t**	**Tukey's p**
Time in corners (s)	Microhabitat	Rock-Sand	41.5	32.4	1.281	0.4237
		Rock-Inter	26.6	20.7	1.282	0.4249
		Sand-Inter	-14.9	24.6	-0.61	0.8189
	Radiation	Mbuna-Benthic/Utaka	23.9	24.4	0.979	0.3404
Corner entries and exits	**Microhabitat ***	Rock-Sand	36.2	15.1	2.398	0.0689 .
		Rock-Inter	-3.2	9.3	-0.344	0.9371
		Sand-Inter	**-39.4**	**11.8**	**-3.329**	**0.0096 ****
	Radiation	Mbuna-Benthic/Utaka	-16.4	13	-1.265	0.2283
Time in center (s)	Microhabitat	Rock-Sand	-18.2	18.8	-0.966	0.6070
		Rock-Inter	-13.4	11.9	-1.125	0.5138
		Sand-Inter	4.8	14.4	0.332	0.9412
	Radiation	Mbuna-Benthic/Utaka	-3.6	14.2	-0.251	0.8046
Center entries and exits	**Microhabitat ****	**Rock-Sand**	**6.5**	**2.1**	**3.041**	**0.0074 ***
		Rock-Inter	**4.4**	**0.9**	**4.701**	**1.2×10^{-5} ******
		Sand-Inter	-2.1	2.1	-1.008	0.5724
	Radiation **	**Mbuna-Benthic/Utaka**	**-9.6**	**2.6**	**-3.640**	**0.0029 ****
Distance traveled (cm)	Microhabitat .	Rock-Sand	728	452	1.611	0.2731
		Rock-Inter	-288	290	-0.991	0.5959
		Sand-Inter	-1015	358	-2.839	0.0572 .
	Radiation	Mbuna-Benthic/Utaka	-308	338	-0.909	0.3771
Speed change (mm/s)	Microhabitat	Rock-Sand	-28.9	20.1	-1.441	0.3419
		Rock-Inter	-18.3	13.0	-1.406	0.3604
		Sand-Inter	10.6	16.9	0.630	0.8054
	Radiation	Mbuna-Benthic/Utaka	27.7	15.2	1.819	0.1005

Table 1. Effects of microhabitat and evolutionary radiation on open field behavior. Summary of linear mixed effect regression output for associations between microhabitat, evolutionary radiation (mbuna versus B/U), and exploratory behaviors in the open field test. The full regression model is shown at the top of the table and was fit to open field behavioral data, with bold italicized terms representing fixed effects, and non-bold italicized terms representing random effects, with nested terms in parentheses. For each behavior, the standard output from linear regression in R is summarized, organized by fixed effect and then by pairwise comparisons between each level of each fixed effect. The output includes the estimated difference between levels of each fixed effect, as well as the standard error, t-statistic, and Tukey's HSD p-value for each difference. Behaviors, fixed effects, and levels of

(Tukey's HSD p<0.05) in the model. Behaviors, fixed effects, and levels of fixed effects that are underlined were found to be statistically significant or trending in this model and also in a second model excluding test site from the model (Tukey's HSD p<0.10 in both models). Asterisks indicate levels of significance (* for p<0.05; ** for p<0.005; *** for p<0.0005; **** for p<0.00005; and "." for p<0.10).

OF behavior ~ *intermediate + radiation + intermediate*radiation +* *(genus/species) + test site + arena size*						
Behavior	**Fixed effect**	**Comparison**	**Estimate**	**S.E.**	**t**	**Tukey's p**
Time in corners (s)	Inter	Inter - Non-inter	-1.1	17.2	-0.066	0.9481
	Radiation *	**Mbuna - Benthic/Utaka**	**45.6**	**17.2**	**2.658**	**0.0175 ***
	Inter*Radiation	Mbuna (Inter - Non-inter)	-16.9	23.7	-0.711	0.8912
		Benthic/Utaka (Inter - Non-inter)	14.6	25.0	0.583	0.9358
Corner entries and exits	**Inter ****	**Inter - (Non-inter)**	**33.7**	**6.5**	**5.196**	**0.0011 ****
	Radiation	Mbuna - Benthic/Utaka	4.69	10.4	0.452	0.6650
	Inter*Radiation *	Mbuna (Inter - Non-inter)	17.1	7.6	2.264	0.2775
		Benthic/Utaka (Inter - Non-inter)	**50.2**	**10.4**	**4.848**	**0.0027 ****
Time in center (s)	Inter	Inter - (Non-inter)	-16.4	9.7	-1.685	0.1138
	Radiation *	**Mbuna - Benthic/Utaka**	**-24.0**	**10.4**	**-2.306**	**0.0472 ***
	Inter*Radiation	Mbuna (Inter - Non-inter)	-16.1	11.0	-1.473	0.5404
		Benthic/Utaka (Inter - Non-inter)	-16.7	15.2	-1.100	0.6941
Center entries and exits	Inter	Inter - (Non-inter)	2.0	1.7	1.190	0.2531
	Radiation *	**Mbuna - Benthic/Utaka**	**-4.52**	**1.9**	**-2.356**	**0.0428 ***
	Inter*Radiation	Mbuna (Inter - Non-inter)	0.0	2.06	0.014	1.00
		Benthic/Utaka (Inter - Non-inter)	4.0	2.6	1.545	0.4366
Distance traveled (cm)	**Inter ***	**Inter - (Non-inter)**	**729**	**241**	**3.028**	**0.0183 ***
	Radiation	Mbuna - Benthic/Utaka	127	230	0.552	0.5901
	Inter*Radiation	Mbuna (Inter - Non-inter)	433	315	1.376	0.5368
		Benthic/Utaka (Inter - Non-inter)	1024	351	2.914	0.0753 .
Speed change (mm/s)	Inter	Inter - (Non-inter)	9.56	10.4	0.915	0.3718
	Radiation .	Mbuna - Benthic/Utaka	19.1	10.1	1.902	0.0863 .
	Inter*Radiation *	Mbuna (Inter - Non-inter)	32.06	12.5	2.573	0.1060
		Benthic/Utaka (Inter - Non-inter)	-12.94	15.5	-0.834	0.8377

Table 2. Effects of intermediate microhabitat and evolutionary radiation on open field behavior. Linear mixed effect regression output testing associations between intermediate (versus non-intermediate) microhabitat, evolutionary radation (mbuna versus B/U), the interaction between microhabitat and radiation, and exploratory behaviors in the open field (OF) test. For each behavior, the standard output from linear regression in R is summarized

as explained above for Table 1. Behaviors, fixed effects, and levels of fixed effects in bold indicate associations that were found to be statistically significant (Tukey's HSD $p<0.05$) in the model. Behaviors, fixed effects, and levels of fixed effects that are underlined were found to be statistically significant or trending in this model and also in a second model excluding test site (Tukey's HSD $p<0.10$ in both models). Asterisks indicate levels of significance (* for $p<0.05$; ** for $p<0.005$; "." For $p<0.10$).

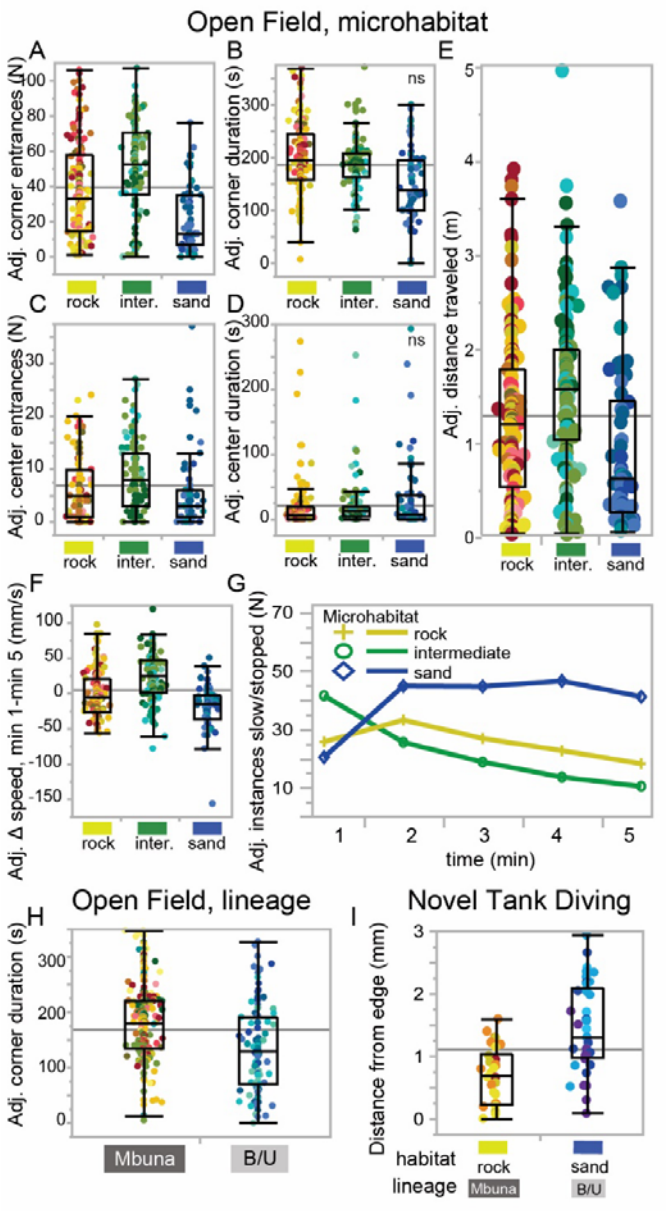


Figure 3. Exploratory behavior is associated with microhabitat and evolutionary radiation. Variation in open field and novel tank behaviors associated with microhabitat and evolutionary radiation. Open field behaviors are adjusted for lab and arena size based on estimates from linear regression. Controlling for phylogenetic factors, corner entrances/exits

779 (p=0.014). Center entries/exits (C), but not time in the center (D), are also associated with
780 microhabitat (p<0.0001). The association between microhabitat and distance traveled (E) is
781 trending towards significance (p=0.053). Microhabitat is not associated with change in speed
782 over the course of the open field test (F); however, microhabitat is significantly associated
783 with instances of stopping and slowed swimming (G) throughout the open field test
784 (p<0.0001). Exploratory behaviors are also associated with evolutionary radiation (mbuna
785 versus shallow/deep benthic and utaka, B/U). Controlling for variance explained by
786 intermediate versus non-intermediate microhabitat, Mbuna species spent significantly more
787 time in corners (H), less time in the center (not shown), and made more entries/exits into the
788 center compared to B/U species (p<0.05 for all). Mbuna rock-dwellers also remained
789 significantly closer to the corners in the novel tank test compared to B/U sand-dwellers (I;
790 p=0.038).
791
792 3.6 Exploratory behaviors are not strongly correlated across contexts in Lake Malawi cichlids
793
794 We next investigated evidence for phenotypic integration versus phenotypic modularity of
795 exploratory behaviors in Lake Malawi cichlids. To do this, we analyzed correlations of
796 exploratory behaviors across novel contexts using MMC, which identifies clusters of
797 covariation in large multivariate datasets. We reasoned that if exploratory behaviors are
798 phenotypically integrated, we would expect to observe strong correlations in exploratory
799 behaviors across novel contexts. In contrast, if exploratory behaviors are modular, we would
800 expect to observe weak or no correlations in exploratory behaviors across contexts. As a
801 ground truth and control, we first demonstrated that MMC could reveal clusters of correlated
802 behaviors across contexts by re-analyzing a previously published dataset from selectively
803 bred high- and low-exploratory strains of wild-derived zebrafish. In this study, subjects were
804 phenotyped across a battery of assays (including the novel tank, light-dark, and open field
805 tests among others) and were found to exhibit correlated behaviors across assays (Wong *et*
806 *al*, 2012). As expected, MMC revealed extensive across-assay clustering in this dataset, with
807 five of the eight (62.5%) clusters spanning multiple assays (including clustering across novel
808 tank and light-dark assays). We then applied MMC to two independent Lake Malawi cichlid

datasets, one in which subjects were phenotyped across two behavioral assays (novel tank test and light-dark test) at INSTITUTION 1, and a second in which subjects were phenotyped across three behavioral assays (open field test, novel object test, and resident intruder test) at INSTITUTION 2. For both Malawi cichlid datasets, behavioral clusters grouped exclusively within assay rather than across assays—zero of ten (0%) modules from the INSTITUTION 2 data set and zero of three (0%) modules from the INSTITUTION 1 data spanned multiple assays.

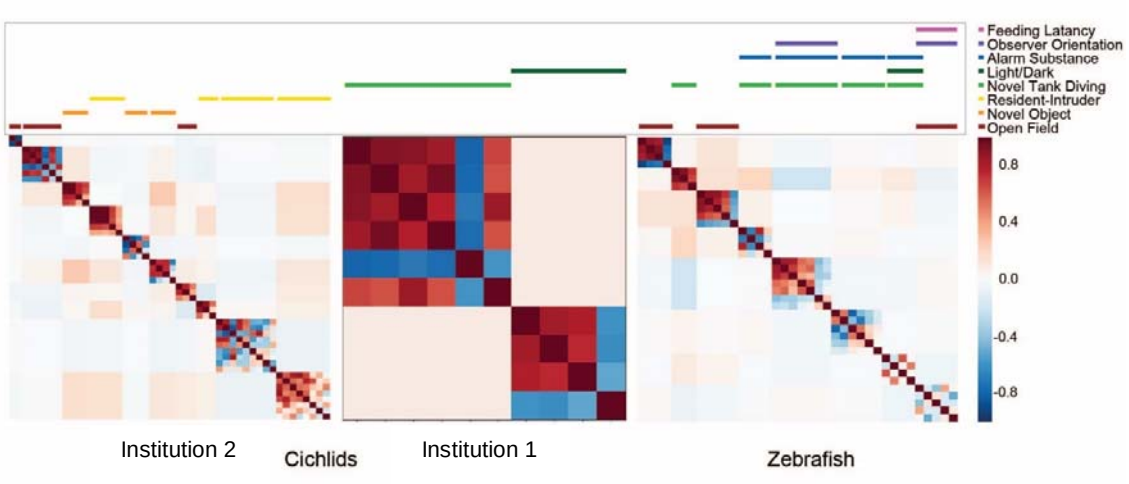


Figure 3. Behavioral modularity analysis across assays in Lake Malawi cichlids and high- and low-anxiety strains of zebrafish. MMC analysis of correlated behaviors across contexts shows extensive clustering within assays in cichlids (INSTITUTION 2 and INSTITUTION 1). In contrast, high- and low-exploratory strains of zebrafish show extensive clustering across assays, indicating strong correlations in behaviors across contexts. 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 pairwise correlations between behavioral measurements across all individuals, with dark red indicating a strong positive correlation and dark blue indicating a strong negative correlation. The color-coded line(s) above each heatmap indicate behavioral assay(s) represented in each module.

4 Discussion

We phenotyped a wide array of Lake Malawi cichlid species in three classic novel environment assays for the first time. Collectively, Lake Malawi cichlids showed strong behavioral patterns that mirrored those of other teleost lineages in all three assays (novel tank test, light-dark test, open field test), 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, de Brito, de Moraes et al., 2007; Stewart, Cachat, Wong et al., 2010; Stewart, Gaikwad, Kyzar et al., 2012; Yoshida, Nagamine, & Uematsu, 2005). The directions of bias in the light-dark and open field tests also match biases displayed by terrestrial vertebrates in similarly designed assays: for example, 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 et al., 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.

Although the direction of these biases was consistent in all species tested, some species exhibited significantly weaker or stronger biases compared to others. For example, the B/U sand-dweller *Mchenga conophoros* spent significantly more time in the top half of the novel tank test compared to every other species tested; and the B/U sand-dweller *Copadichromis virginalis* spent significantly more time in the light half of the light-dark test compared to several other species. Future studies are needed to understand the ecological and/or biological factors contributing to these differences. The ability to hybridize Lake Malawi cichlids across species boundaries is a promising strategy for identifying natural genetic variants contributing to these behavioral differences.

We also investigated the degree of phenotypic diversity in exploratory behaviors in Lake Malawi cichlids. To place our analyses in a frame of reference, we measured phenotypic variance in novel tank behavior among Lake Malawi cichlids and among three laboratory strains of zebrafish that were tested with the same parameters in a previous study: two wild-derived strains that were selectively bred for extreme and opposite exploratory behaviors, and a common wild-type laboratory strain (AB). It is worth noting that previous studies have

demonstrated that the average genetic divergence between Lake Malawi cichlid species is less than between common laboratory strains of zebrafish (Loh, Katz, Mims et al., 2008). We found that Lake Malawi cichlids collectively exhibited significantly greater variance in multiple dimensions of exploratory behavior compared to the zebrafish strains, including time spent in the top half and entries into the top half. These results suggest that natural evolution in Lake Malawi cichlids has resulted in extreme phenotypic diversity in exploratory behaviors, similar to other complex traits such as morphology and color patterning.

We tested the extent to which this phenotypic diversity is explained by species differences. Strong species differences were observed for nearly every dimension of exploratory behavior analyzed across all assays. Taken together, these results show that the extreme diversity in exploratory behaviors in Lake Malawi cichlids is explained in part by patterns of strong divergence along species lines. This is consistent with findings in other vertebrate lineages, in which 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 et al., 2013; Claudia Mettke-Hofmann, Winkler, & Leisler, 2002). Considering the low genetic divergence and ability to hybridize between species, these results further demonstrate that Lake Malawi cichlids are a powerful complementary system to traditional laboratory models for understanding the genetic basis of naturally evolved species differences in exploratory behaviors.

To investigate the ecological basis of species differences in exploratory behavior, we phenotyped 19 species spanning three Lake Malawi microhabitats (rock, sand, and intermediate) in the open field test, and analyzed the relationship between microhabitat and behavior. Controlling for variation explained by phylogenetic factors, microhabitat was associated with multiple dimensions of open field behavior, including entries/exits to and from the corners, entries/exits to and from the center, and total distance traveled. Notably, intermediate species traveled significantly farther and made significantly more entries/exits to and from the corners compared to non-intermediate species, suggesting that intermediate species exhibit distinct exploratory behavioral phenotypes compared to rock- and sand-

dwelling species. Interestingly, the relationship between intermediate habitat and behavior also differed between the mbuna and B/U radiations for multiple dimensions of open field behavior, including corner entries/exits and speed change over time. These results support the idea that unique behavioral specializations are associated with divergence into the intermediate habitat between the mbuna and B/U radiations.

We also investigated whether two major Lake Malawi cichlid radiations (mbuna versus B/U) are associated with species differences in exploratory behaviors. Controlling for variation explained by microhabitat, multiple dimensions of exploratory behavior differed significantly between the mbuna and B/U radiations, including time spent in the corners, time spent in the center, and center entries/exits. In all three cases, the mbuna species exhibited less exploratory phenotypes compared to B/U species. Consistent with this pattern, mbuna rock-dwellers also remained significantly closer to the corner regions in the novel tank test compared to B/U sand-dwellers. Taken together, these results provide evidence for behavioral divergence between two major cichlid radiations in Lake Malawi. One potential explanation for these data is that behavioral preferences for edges or corners helps mediate behavioral preferences for the narrow crevasses and caves characteristic of rocky habitats; inversely, a reduced aversion toward open environments may facilitate preferences for and/or invasion of new and potentially more exposed habitats. Future experiments are needed to understand how differences in exploratory behaviors are linked to variation in neural structure and function. Notably, mbuna versus B/U lineages exhibit fixed genetic differences as well as neurogenetic and neuroanatomical specializations (e.g. volume of the cerebellum and telencephalon) (Huber, van Staaden, Kaufman et al., 1997; Sylvester, Rich, Loh et al., 2010), highlighting potential substrates for behavioral divergence.

Comparative studies in Lake Malawi cichlids have previously demonstrated modular patterns of covariation for several complex traits that are thought to have played a central role in cichlid diversification, including oral jaw morphology and color patterning (R. Craig Albertson, Powder, Hu et al., 2014; Parsons, Cooper, & Albertson, 2011). Briefly, evolutionary modularity and integration refer to distinct 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 more modular and are generally considered to be more evolvable, although see Armbruster et al. (Armbruster, Pélabon, Bolstad et al., 2014). Similarly, we reasoned that, because behaviors in response to a given context are measurable traits, behavioral correlations across contexts can provide evidence for behavioral integration versus behavioral modularity.

Following this logic, we tracked individual subjects across assays to investigate whether patterns of covariation in Lake Malawi cichlid exploratory behaviors are modular or integrated. To do this, we applied MMC, a statistical approach designed to identify clusters of covariation in large multivariate datasets. We first applied MMC to a previously published dataset in which laboratory strains of zebrafish were found to exhibit correlated, or syndromic, behaviors across contexts (Baker, Goodman, Santo et al., 2018; Wong, Perrin, Oxendine et al., 2012). This analysis revealed extensive clustering across assays, indicating that behaviors were correlated across contexts. We then applied MMC to two independent Malawi cichlid datasets, in which subjects were phenotyped in different assays at two separate institutions. In both datasets, behaviors clustered exclusively within assay. Taken together, these results support the hypothesis that, like other complex traits, Lake Malawi cichlids exhibit modular patterns of behavioral variation. Future studies are needed to investigate whether exploratory behaviors are more evolvable in this species assemblage, and whether they have played a causal role in cichlid diversification.

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 natural environments. 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 social context. These questions were beyond the scope of this study and are promising areas for future research.

Despite these limitations, these experiments constitute a large comparative investigation of exploratory behavioral variation in a previously untested vertebrate system. We phenotype a total of 23 new species in a variety of classic behavioral assays and show conserved behavioral responses that mirror other teleosts and rodents. We demonstrate high phenotypic variance in exploratory behaviors that segregates along species lines. We further link exploratory behavioral variation to microhabitat and to the major mbuna and benthic/utaka evolutionary radiations. Lastly, we provide evidence for behavioral modularity in Lake Malawi cichlids. Taken together, these findings provide new insights into the ecology and evolution of exploratory behaviors, and demonstrate Lake Malawi cichlids as a powerful complement to traditional models for investigating the ecological, genetic, and neural factors underlying natural behavioral diversity.

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Species	Microhabitat	Rad	Inst	N	Bottom time (seconds)	Top time (seconds)	S.E.	$P_{top\ vs.\ bottom}$
Metriaclima zebra	Rock	Mbu	1	5	332.46	27.54	13.15	2.05×10^{-4} ***
Labeothropheus fuelleborni	Rock	Mbu	1	12	310.40	49.60	10.11	3.50×10^{-8} ***
Petrotilapia sp. 'chitimba'	Rock	Mbu	1	12	306.82	53.18	12.66	4.71×10^{-7} ***
Cynotilapia zebroides 'Cobue'	Rock	Mbu	1	13	348.23	11.77	4.99	1.32×10^{-12} ***
Copadichromis virginalis	Sand	B/U	1	18	316.60	43.40	11.29	5.75×10^{-10} ***
Mchenga conophoros	Sand	B/U	1	18	229.88	130.12	23.35	0.0421 *
Mylochromis anaphyrmus	Sand	B/U	1	14	295.95	64.05	15.30	2.70×10^{-6} ***
Tramitichromis intermedius	Sand	B/U	1	18	346.99	13.01	4.46	5.47×10^{-18} ***

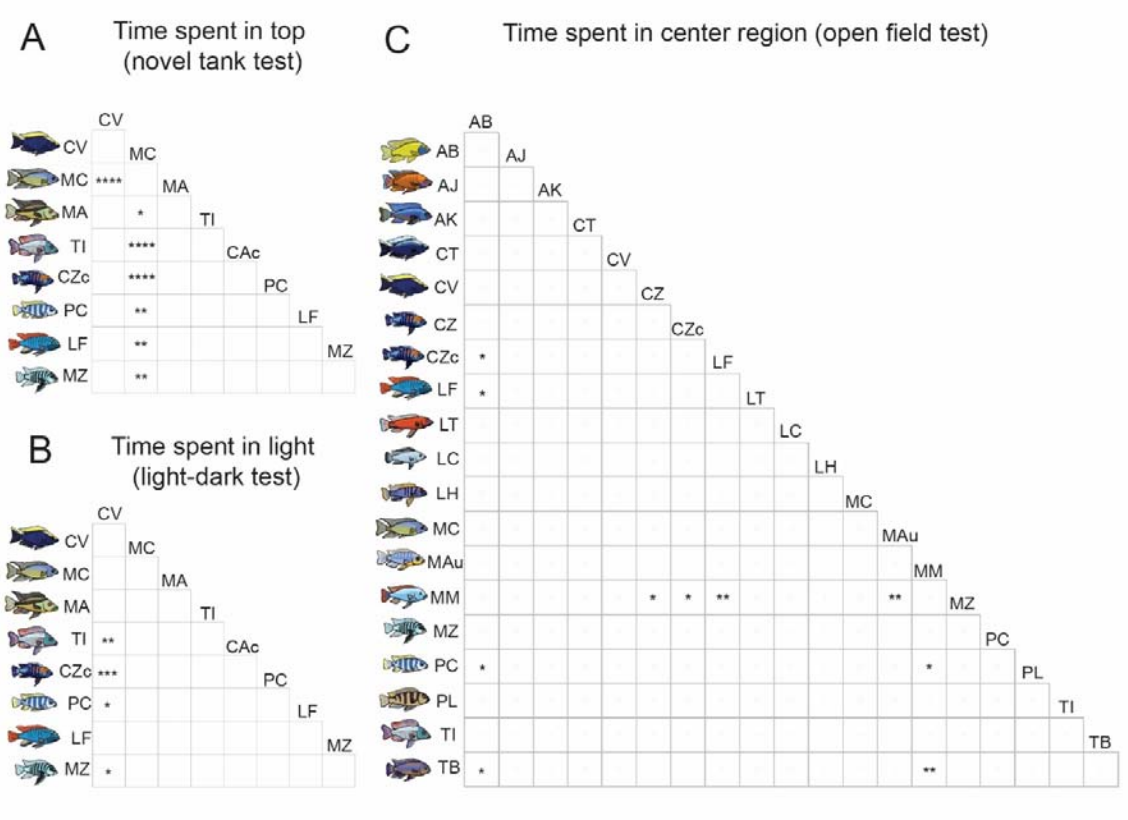
Supplementary Table 1. Novel tank place bias between bottom and top regions by species. Each row corresponds to the species labeled in the left column. The following are presented for each species: microhabitat designation, evolutionary radiation (mbuna, Mbu; shallow/deep benthic and Utaka, B/U), test site (Inst.; INSTITUTION 1 vs. INSTITUTION 2), sample size, 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.

Species	Microhabitat	Rad	Inst	N	Dark time (seconds)	Light time (seconds)	S.E.	$P_{light\ vs.\ dark}$
Metriaclima zebra	Rock	Mbu	1	5	312.67	47.33	17.67	0.0011 **
Labeothropheus fuelleborni	Rock	Mbu	1	2	272.91	87.09	16.19	0.078
Petrotilapia sp. 'chitimba'	Rock	Mbu	1	13	297.82	62.18	8.34	4.87×10^{-9} ***
Cynotilapia zebroides 'Cobue'	Rock	Mbu	1	12	344.07	15.93	8.15	3.12×10^{-10} ***
Copadichromis virginalis	Sand	B/U	1	12	203.77	156.23	32.73	0.46
Mchenga conophoros	Sand	B/U	1	12	275.42	84.58	27.02	0.0036 **
Mylochromis anaphyrmus	Sand	B/U	1	4	311.83	48.17	38.02	0.028 *
Tramitichromis intermedius	Sand	B/U	1	11	325.74	34.26	11.07	7.74×10^{-8} ***

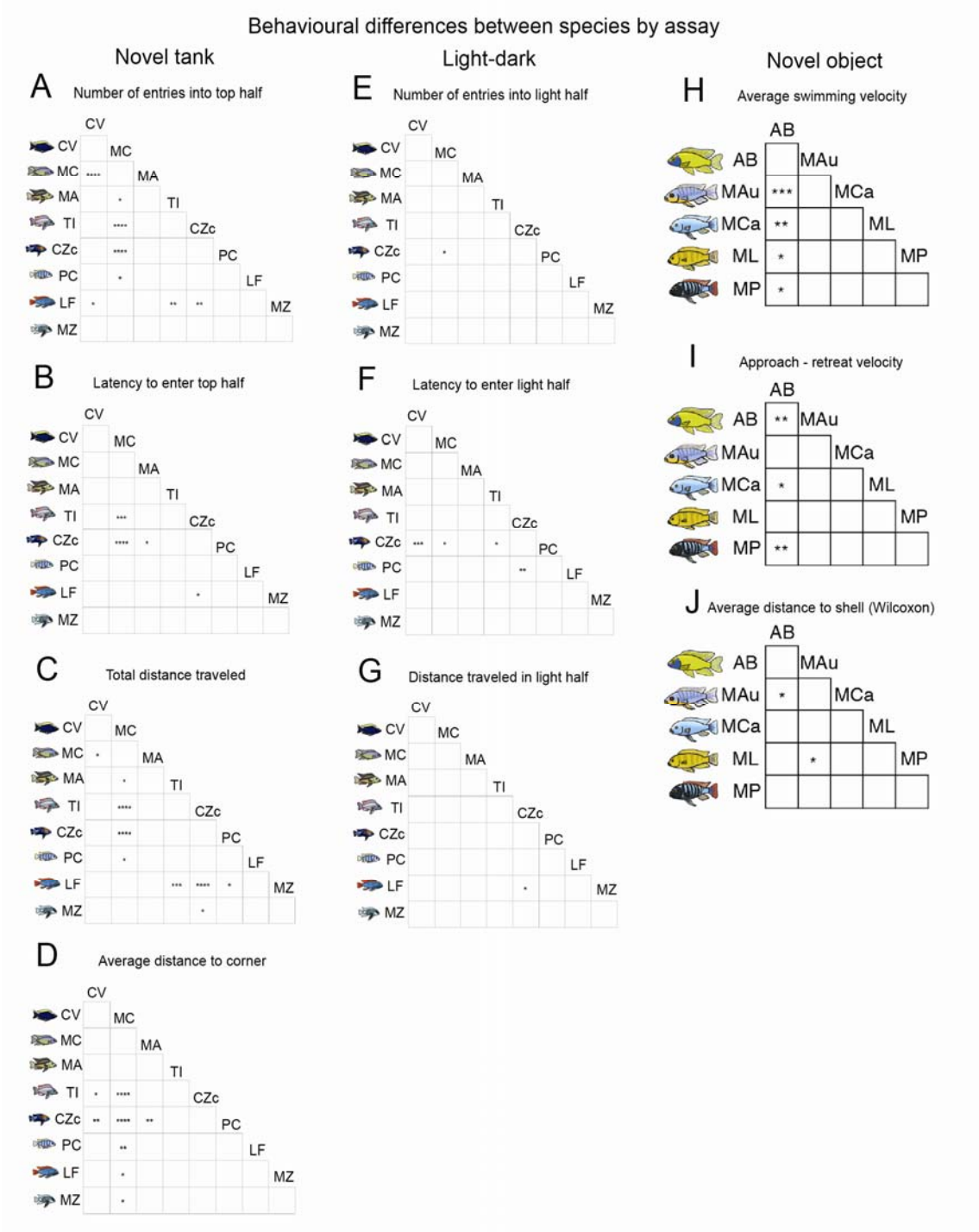
Supplementary Table 2. Place bias between light and dark halves of the light-dark test by species. Each row corresponds to the species labeled in the left column. The following are presented for each species: sample size, microhabitat designation, evolutionary radiation (mbuna, Mbu; shallow/deep benthic and Utaka, B/U), 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.

Species	Microhab	Rad	Inst	N	MD	LG	Center time (seconds)	Periphery time (seconds)	S.E.	$P_{center\ vs.\ periphery}$
Metriaclima mbenjii	Rock	Mbu	2	39	9	30	62.06	257.94	10.13	$7.89x10^{-12}$ ***
Metriaclima zebra	Rock	Mbu	1	9	9	0	17.32	302.68	3.31	$5.83x10^{-11}$ ***
Labeotropheus fuelleborni	Rock	Mbu	1,2	23	15	8	9.27	310.73	2.13	$1.10x10^{-27}$ ***
Labeotropheus trewavasae	Rock	Mbu	2	11	11	0	28.09	291.91	2.01	$1.04x10^{-14}$ ***
Petrotilapia sp. 'chitimba'	Rock	Mbu	1	14	12	2	2.56	317.44	1.09	$1.91x10^{-22}$ ***
Labidochromis caeruleus	Rock	Mbu	2	10	10	0	17.17	302.83	0.80	$1.75x10^{-17}$ ***
Labidochromis sp. 'hongi'	Rock	Mbu	2	4	4	0	19.37	300.63	2.50	$8.08x10^{-6}$ ***
Cynotilapia zebroides	Rock	Mbu	2	21	0	21	26.22	293.78	4.94	$1.92x10^{-17}$ ***
Cynotilapia zebroides 'Cobue'	Rock	Mbu	1	18	9	9	4.90	315.10	3.30	$1.20x10^{-19}$ ***
Tropheops sp. 'Boadzulu'	Intermediate	Mbu	2	29	0	29	20.29	299.71	2.48	$1.37x10^{-30}$ ***
Metriaclima (Pseudotropheus) aurora	Intermediate	Mbu	2	55	48	7	29.94	290.06	2.20	$5.78x10^{-51}$ ***
Aulonocara baenschi	Intermediate	B/U	2	9	9	0	73.77	246.23	28.32	0.0121 *
Aulonocara koningsi	Intermediate	B/U	2	18	10	8	47.91	272.09	10.50	$3.87x10^{-9}$ ***
Aulonocara jacobfreibergi	Intermediate	B/U	2	4	0	4	36.52	283.48	13.98	0.00201 **
Copadichromis trewavasae	Intermediate	B/U	2	11	11	0	29.75	290.25	2.23	$3.24x10^{-14}$ ***
Copadichromis virginalis	Sand	B/U	1	15	0	15	16.99	303.01	8.23	$4.52x10^{-11}$ ***
Mchenga conophoros	Sand	B/U	1	22	10	12	30.84	289.16	14.11	$5.99x10^{-9}$ ***
Tramitichromis intermedius	Sand	B/U	1	19	9	10	35.07	284.93	8.03	$4.39x10^{-12}$ ***
Metriaclima (Pseudotropheus) livingstonii	Sand	Mbu	2	10	0	10	56.48	263.52	30.74	0.00622 ***

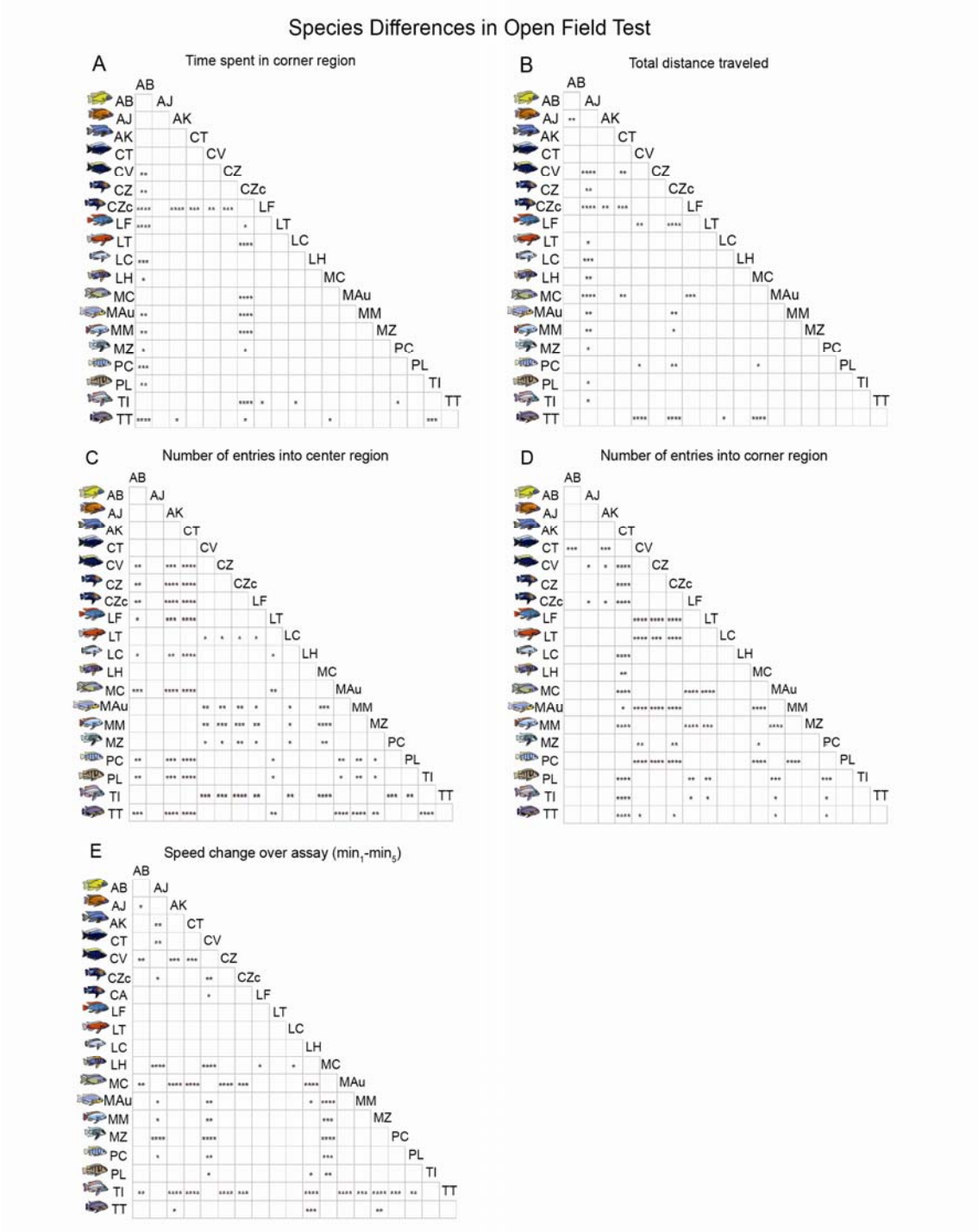
Supplementary Table 3. Place bias between central and peripheral regions of the open field test by species. Each row corresponds to the species labeled in the left column. The following are presented for each species: sample size, microhabitat designation, estimate for mean time in center, estimate for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



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 'Cobue'*), LF (*Labeotropheus fuelleborni*), LT (*Labeotropheus trewavasae*), LC (*Labidochromis caeruleus*), LH (*Labidochromis* sp. 'hongi'), MC (*Mchenga conophoros*), MA (*Mylochromis anaphyrmus*), Mau (*Metriaclima aurora*), MM (*Metriaclima mbenjii*), MZ (*Metriaclima zebra*), PC (*Petrotilapia sp. 'chitimba'*), PL (*Pseudotropheus livingstonii*), TI (*Tramitichromis intermedius*), TB (*Tropheops sp. 'Boadzulu'*).



Supplementary Figure 2. Pairwise species differences in behavior across assays, including the novel tank (A-D), light-dark (E-G), and novel object (H-J) tests. Asterisks indicate levels of significance for post-hoc Tukey's HSD tests of the pairwise differences between species (* $p<0.05$, ** $p<0.005$, *** $p<0.0005$, ****$p<5x10^{-5}$).



Supplementary Figure 3. Pairwise species differences in open field behaviors (A-E). 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	Institution	N	Age (days)	Novel tank	Light-dark	Resident intruder	Novel object	Open field
Copadichromis virginalis	1	11	90-180	X	X			
Cynotilapia zebroides 'Cobue'	1	11	90-180	X	X			
Labeotropheus fuelleborni	1	2	90-180	X	X			
Mchenga conophoros	1	12	90-180	X	X			
Metriaclima zebra	1	5	90-180	X	X			
Mylochromis anaphyrmus	1	4	90-180	X	X			
Petrotilapia sp. 'chitimba'	1	12	90-180	X	X			
Tramitichromis intermedius	1	10	90-180	X	X			
Aulonocara baenschi	*2*	14	>180			X	X	X
Metriaclima (Pseudotropheus) aurora	*2*	14	>180			X	X	X
Metriaclima callainos	*2*	14	>180			X	X	X
Metriaclima lombardoi	*2*	14	>180			X	X	X
Metriaclima pyrsonotos	*2*	14	>180			X	X	X

Supplementary Table 4. Samples analyzed for Modularity Modular Clustering analysis, organized by species, institution, sample size, age, and assay. For each species, each "X" indicates the assays in which all subjects sampled were tested. Two independent datasets were analyzed, the first dataset is represented by the first eight species listed, and the second dataset is represented by the last five species listed.

Species	Microhab	Rad	Inst	NT	LD	NO	OF	RI	Total
Metriaclima mbenjii	Rock	Mbu	2	0	0	0	39	0	39
Metriaclima zebra	Rock	Mbu	1	5	(5)	0	9	0	14
Labeotropheus fuelleborni	Rock	Mbu	1	12	(2)	0	16	0	28
(Labeotropheus fuelleborni)	Rock	Mbu	2	0	0	0	7	0	7
Labeotropheus trewavasae	Rock	Mbu	2	0	0	0	11	0	11
Petrotilapia sp. 'chitimba'	Rock	Mbu	1	(12)	13	0	14	0	27
Labidochromis caeruleus	Rock	Mbu	2	0	0	0	10	0	10
Labidochromis sp. 'hongi'	Rock	Mbu	2	0	0	0	4	0	4
Cynotilapia zebroides	Rock	Mbu	2	0	0	0	21	0	21
Cynotilapia zebroides 'Cobue'	Rock	Mbu	1	13	(11) + 1	0	18	0	32
Tropheops sp. 'Boadzulu'	Inter	Mbu	2	0	0	0	29	0	29
Metriaclima (Pseudotropheus) aurora	Inter	Mbu	2	0	0	14	55+*(14*)*	*(14*)*	69
Aulonocara baenschi	Inter	B/U	2	0	0	14	9+*(14*)*	*(14*)*	23
Aulonocara koningsi	Inter	B/U	2	0	0	0	18	0	18
Aulonocara jacobfreibergi	Inter	B/U	2	0	0	0	4	0	4
Copadichromis trewavasae	Inter	B/U	2	0	0	0	11	0	11
Copadichromis virginalis	Sand	B/U	1	18	(11) + 1	0	15	0	34
Mchenga conophoros	Sand	B/U	1	18	(12)	0	22	0	40
Mylochromis anaphyrmus	Sand	B/U	1	14	(4)	0	0	0	14
Tramitichromis intermedius	Sand	B/U	1	18	(10) + 1	0	19	0	38
Metriaclima (Pseudotropheus) livingstonii	Sand	Mbu	2	0	0	0	10	0	10
Metriaclima callainos	Rock	Mbu	2	0	0	14	*(14*)*	*(14*)*	14
Metriaclima lombardoi	Rock	Mbu	2	0	0	14	*(14*)*	*(14*)*	14
Metriaclima pyrsonotos	Rock	Mbu	2	0	0	14	*(14*)*	*(14*)*	14
TOTALS				110	(67) + 4	70	341+*(70*)*	*(70*)*	525

Supplementary Table 5. Total subject counts by species, microhabitat (Rock, Sand, Intermediate=Inter), evolutionary radiation (Mbuna=Mbu, shallow/deep benthic and utaka=B/U), institution (INSTITUTION 1 vs. INSTITUTION 2), and assay (novel tank=NT, light-dark=LD, novel object=NO, open field=OF, resident intruder=RI). Subjects that were tracked in multiple assays are indicated by parentheses, and are only counted once. Numbers that are italicized and marked by asterisks within parentheses indicate individuals that were subjected the novel object test as well as a different open field test and a resident intruder test that are part of another study (Moore & Roberts, *in preparation*); because these subjects were tracked across multiple assays, their behavior across all three assays was analyzed in MMC. One species, *Labeotropheus fuelleborni,* was housed at both institutions, and is represented in two separate rows to show information about subjects at each institution (indicated as a duplicate species by parentheses).