



Behavior-dependent *cis* regulation reveals genes and pathways associated with bower building in cichlid fishes

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Many behaviors are associated with heritable genetic variation [Kendler and Greenspan (2006) *Am J Psychiatry* 163:1683–1694]. Genetic mapping has revealed genomic regions or, in a few cases, specific genes explaining part of this variation [Bendesky and Bargmann (2011) *Nat Rev Gen* 12:809–820]. However, the genetic basis of behavioral evolution remains unclear. Here we investigate the evolution of an innate extended phenotype, bower building, among cichlid fishes of Lake Malawi. Males build bowers of two types, pits or castles, to attract females for mating. We performed comparative genome-wide analyses of 20 bower-building species and found that these phenotypes have evolved multiple times with thousands of genetic variants strongly associated with this behavior, suggesting a polygenic architecture. Remarkably, F₁ hybrids of a pit-digging and a castle-building species perform sequential construction of first a pit and then a castle bower. Analysis of brain gene expression in these hybrids showed that genes near behavior-associated variants display behavior-dependent allele-specific expression with preferential expression of the pit-digging species allele during pit digging and of the castle-building species allele during castle building. These genes are highly enriched for functions related to neurodevelopment and neural plasticity. Our results suggest that natural behaviors are associated with complex genetic architectures that alter behavior via *cis*-regulatory differences whose effects on gene expression are specific to the behavior itself.

Malawi cichlids | bower building | *cis*-regulatory evolution | genome sequencing

Understanding behavioral evolution requires identifying the genetic and regulatory architectures encoding neural development and function. To characterize the evolution of a complex social behavior, we focused on the remarkable bower-building feats performed by ~200 cichlid fish species in Lake Malawi that live on sandy substrate. Bowers are species-specific sand structures that serve as signals in male–male competition and female mate choice (1). Malawi cichlid species build two basic bower types: (i) pits, which are depressions that resemble nests in the sand, and (ii) castles, which resemble miniature volcanoes (2). Bower building requires highly repetitive activity in which males perform hundreds of scoop–spit bouts with their mouths per hour, interspersing construction with the courtship of females and aggressive encounters with conspecific males (Fig. 1A) (2, 3). To dig pits, males collect sand from the center of the pit and spit it elsewhere, while to build castles, males gather sand from elsewhere and spit it in a targeted location (Fig. 1B and C and Movie S1). Pit and castle bower types are distributed widely across the Malawi cichlid sand-dweller phylogeny, suggesting that parallel evolution and/or hybridization may be responsible (4). Furthermore, bower building is innate. Naive males born in an aquarium, who have experienced neither sand nor other males, perform species-specific bower behavior when housed with sand and gravid females.

Results

Extensive Genetic Differences Exist Between Pit-Digging and Castle-Building Species. To identify genetic variants associated with bower building, we sequenced the genomes of 20 male individuals from 20 sand-dwelling Lake Malawi cichlid species: 11 pit-digging species and nine castle-building species (SI Appendix, Table S1). Species in these two groups construct either pits or castles despite differences within the groups in color pattern, ecology, feeding mode, and other characteristics (2). Sequence reads from each species (mean coverage ~25×) were aligned using the Malawi cichlid reference genome (4) and were mapped to Malawi linkage groups (LGs) (Methods). A maximum-likelihood phylogeny based on variant sites (Fig. 1D) is consistent with repeated evolution of pit-digging and castle-building behavior in our sampled species.

Like other recently evolved species flocks (5), East African cichlids share genetic polymorphisms because of incomplete lineage sorting and hybridization (6–9). Therefore, we used both

Significance

We do not fully understand how behavior evolves. Here we investigate the genomic basis of bower building among Lake Malawi cichlid fishes. Males construct bowers of two major types, pits and castles, to attract females in mating displays. Thousands of genetic variants are strongly associated with divergence in bower behavior. Remarkably, F₁ hybrids of pit-digging and castle-building species perform sequential construction of first pit and then castle bowers. Analysis of brain gene expression in hybrids showed behavior-dependent allele-specific expression with preferential expression of pit-digging alleles during pit digging and castle-building alleles during castle building. Our results suggest that behaviors evolve via complex genetic architectures featuring *cis*-regulatory differences whose effects on gene expression are specific and context dependent.

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The authors declare no conflict of interest.

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Data deposition: All sequencing data in support of the findings of this study have been deposited in the Gene Expression Omnibus (accession no. GSE97490) and in the National Center for Biotechnology Information BioProject databank (accession no. PRJNA380805). Code for the allele-specific expression pipeline is available at <https://github.com/TheFraserLab/ASER>.

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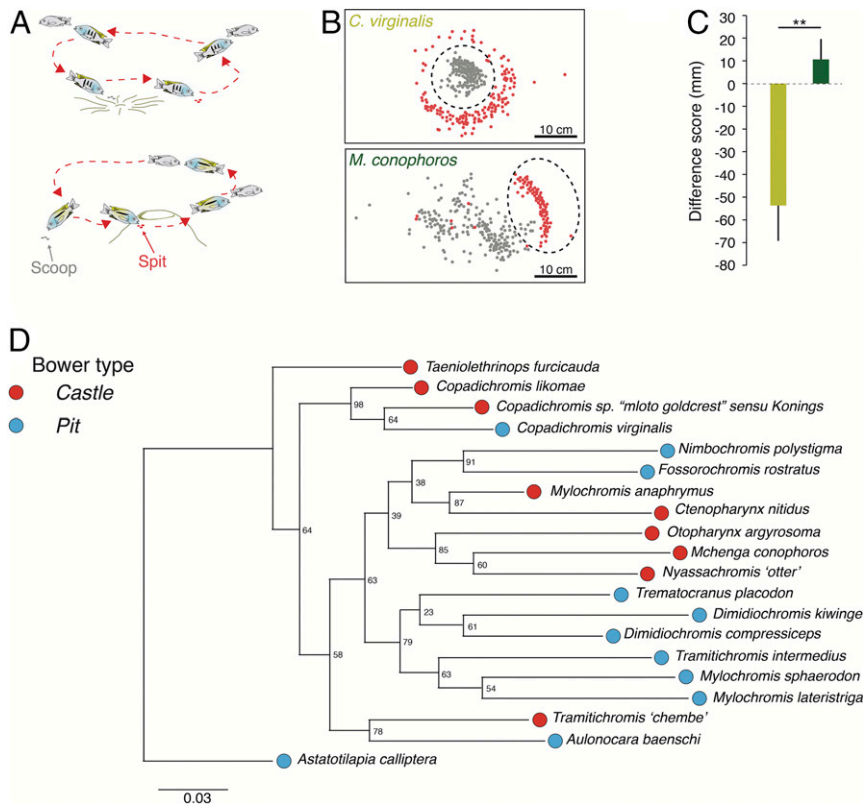


Fig. 1. Bower building. (A) Characteristic behavioral patterns associated with pit digging (Upper) and castle bower building (Lower). (B) Average locations of scoops (gray) and spits (red) during bower-building trials in the pit-digging species *C. virginalis* and the castle-building species *M. conophoros*. Consensus locations of the bowers are indicated by dashed black circles. (C) Results of a Student's *t* test (two-tailed, $P = 0.006$) comparing difference score (in millimeters; mm) between *C. virginalis* (pit digging; yellow) and *M. conophoros* (castle building; green). (** $P < 0.01$.) (D) Maximum-likelihood phylogeny from genome-wide variants of the species sequenced in this study. Numbers at nodes are bootstrap support values.

population-based and phylogeny-based analytical approaches to understand genomic correlates of bower building. We applied the population-based fixation index (F_{ST}) to identify genomic regions differentiating pit-digging vs. castle-building species. We observed ~15.5 million SNPs and ~130,000 insertion/deletions (indels) in the sample set; 1.5% of variable sites were notably divergent between pit-digging vs. castle-building groups ($F_{ST} > 0.2$, compared with 0.08 genome-wide mean). We compared patterns of F_{ST} divergence across the genome (Fig. 2A) with a population-structure-corrected genome-wide association study on bower behavior and found that the two are strongly correlated (SI Appendix, Fig. S1).

We next identified outlier 10-kb regions based on mean F_{ST} of SNPs [10% false-discovery rate (FDR), $F_{ST} > 0.2$] and individual indels (10% FDR, $F_{ST} > 0.1$) (Fig. 2A). Outlier regions were observed on every LG, but peaks on LG2 and LG11 are striking for their size (1.3 Mb and 6 Mb, respectively) and consistency across SNP and indel data. Broad peaks of differentiation on LG2 and LG11 could be caused by structural changes [e.g., inversions (10)] associated with bower behavior or with other traits such as male sex determination (11). However, sex-determination systems are not known for these species, and we could not identify structural variants that could explain the broad peaks of genetic differentiation on LG2 or LG11 (Methods) (12, 13).

Characterizing Variants Associated with Bower Type. We hypothesized that if high- F_{ST} regions across the genome are the product of selection on bower building or associated behavioral traits, then they should be enriched near genes involved in brain function and development. To test this, we examined 1,563 genes located within 25 kb of an outlier 10-kb region (~30% of these genes are located on LGs 2 and 11) for functional enrichment (14). This gene set was significantly enriched for tissue types, pathways, human dis-

orders, and phenotypes associated with brain development and behavior, including axon guidance, synaptic transmission, autism spectrum disorder, and spatial learning (Fig. 2B and Dataset S1). Together, these analyses identify genomic regions and genetic variants associated with bower behavior and demonstrate that genes near these variants are strikingly enriched for putative functions in brain and behavior.

To assess the role of ancestral variation in differentiation of pit-digging vs. castle-building behaviors, we classified SNPs as either “new” if they were only found in sand-dwellers or “ancient” if they were also found in the genomes of non-sand-dwellers, including species from other African rift lakes. For new variants found only in sand-dweller species, we marked alleles as derived if they were not shared with the rock-dweller *Metriaclima zebra* reference genome (SI Appendix, Fig. S2A). Such standing genetic variation has been recruited for rapid adaptation in sticklebacks and other cichlid fish species (8, 15). We used odds ratios (ORs) from a Fisher's exact test comparing SNP-level allele counts to infer whether pit digging and castle building associated preferentially with ancestral or derived alleles (SI Appendix, Fig. S2B). We found that among more genetically diverged SNPs, castle-building species tended to have derived alleles ($P < 0.0001$), while pit-digging species were enriched for ancestral alleles ($P < 0.0001$) (Fig. 2C). Indeed, increasing F_{ST} was related to greater divergence in ORs between derived and ancestral alleles (SI Appendix, Fig. S2C). Furthermore, when comparing the overall distribution of F_{ST} measures, 9% of all SNPs were ancient, but in high- F_{ST} variants ($F_{ST} \geq 0.2$) this proportion was elevated to 20% (χ^2 test, P value $< 2.2e-16$) (SI Appendix, Fig. S3). These data suggest that both standing genetic variation and derived alleles shared by castle-building species contribute to the overall genetic architecture of bower behavior.

higher; building: MC allele 2.75-fold higher) (*SI Appendix, Fig. S8 C and D*; full results are given in *Dataset S2*).

These patterns of differential allelic expression in F_1 animals indicate an unexpected amount of dynamic genomic regulation associated with behavior. Notably we observed that, within the same brain containing alleles from both parental genomes, castle-building *cis*-regulatory elements are specifically activated during the castle-phase, and vice versa for the pit-phase. These results add to a growing body of literature illustrating context-dependent transcriptional response with experience or changes in behavior (27, 28) and further suggest that evolutionary differences between species in relation to brain function and behavior may arise from variation in such context-dependent regulation of gene expression.

Context- and Lineage-Specific Induction Identifies Behavior-Dependent Genes and Pathways. We reasoned that if the context-dependent ASE observed above is biologically relevant, then genes with shared patterns of allelic induction across contexts should be enriched for similar functional roles. To this end we first identified genes with significant diffASE—varying ratios between CV and MC alleles dependent on context—between at least two of the three behavioral contexts (435 genes) (*Methods*). We performed gene-set enrichment analysis using all other genes detected in at least one of the three contexts (9,703 genes) as background. This analysis identified a number of enriched categories spanning various aspects of neural structure and function (*Dataset S3*). For example, genes with diffASE were significantly enriched in a number of neural-specific Reactome pathways such as transmission across chemical synapses (R-HSA-888590; q -value = 8.13×10^{-6}) and GABA synthesis, release, reuptake and degradation (R-HSA-112315; q -value = 5.38×10^{-7}). Enriched gene ontology (GO) biological processes categories were predominately related to synaptic function, neurotransmitter regulation and signaling, and ion transport and binding (Fig. 4C and *Dataset S3*), while cellular component sets included structures and loci important for neuronal function such as clathrin-sculpted vesicles (GO:0060198; q -value = 1.06×10^{-5}) and postsynaptic densities (GO:0014069; q -value = 3.46×10^{-5}).

We further refined these tests by identifying genes that displayed significant differential induction of one or both alleles during building or digging behaviors (building up-regulated genes, $n = 171$; digging up-regulated genes, $n = 174$) (*Methods*). These genes differ from those with diffASE in that the comparison of interest is the expression of individual alleles across rather than within contexts. While diffASE is ascertained by identifying different ratios between alleles, differential induction is more similar to traditional differential expression tests in that it is concerned with the expression

of individual alleles across contexts. Analyzing these genes might then provide insight into the divergence of context-dependent regulation of alleles between CV and MC. To assay the roles of genes with differential induction, we performed gene-set enrichment tests as above. Genes up-regulated during building were enriched for neurotransmitter release (R-HSA-112310; q -value = 4.70×10^{-2}) and ion channel transport (R-HSA-983712; q -value = 1.97×10^{-7}). Similarly, digging-induced genes were associated with ion homeostasis (R-HSA-5578775; q -value = 2.90×10^{-2}) and chemical synaptic transmission (GO:0007268; q -value = 3.20×10^{-2}). These results indicate that genes with differential ASE and induction are coherently enriched for specific neural processes in comparison with the rest of the transcriptome, adding evidence in support of the idea that context-dependent gene regulation may support distinct neural states related to behavior.

We extended this analysis to explore the role of lineage-specific differences in gene regulation via consideration of independent regulation of CV and MC alleles across behavior states. To do so, we applied a sign test to the directionality of CV and MC alleles between the pit- and castle-phases (*Dataset S2*) (24, 29). We compared the allelic counts of individual genes across phases (pit vs. castle), avoiding a bias for the sampled tissue as opposed to a typical GO enrichment test which would compare the complete focal gene list with a background. We found several hierarchically organized gene sets that matched a pattern of significant differential induction of CV and MC alleles between digging and building (*Dataset S3*). The identified gene sets were largely involved in cell signaling and communication (an example is plotted in Fig. 4D). These observations suggest that lineage-specific selection may have played a role in producing differential regulation of neural-signaling genes in CV and MC related to their species-specific bower behavior.

Bower-Associated SNPs and *cis*-Regulatory Variation. Finally, we asked the extent to which genomic divergence among bower-building species is associated with *cis*-regulatory changes inferred from the CV \times MC intercross. If natural selection had acted on regulatory variants associated with pit digging and castle building, it would result in enrichment of highly differentiated SNPs proximal to genes that display ASE. Indeed, we found that ASE genes were significantly enriched near high- F_{ST} SNPs (48 of 621 ASE genes vs. 332 of 1,0221 non-ASE genes; Fisher's exact test, $P = 2.07 \times 10^{-7}$) (Fig. 5A). Furthermore, this enrichment increases when considering those genes displaying diffASE (Fig. 5A). We also detected over 30 pathways that showed significant overlap between ASE and high- F_{ST} gene lists (Bonferroni-corrected $P < 0.05$; hypergeometric test) (Fig. 5B). As in previous analyses of ASE and F_{ST} alone, many of these pathways are involved in neurodevelopment,

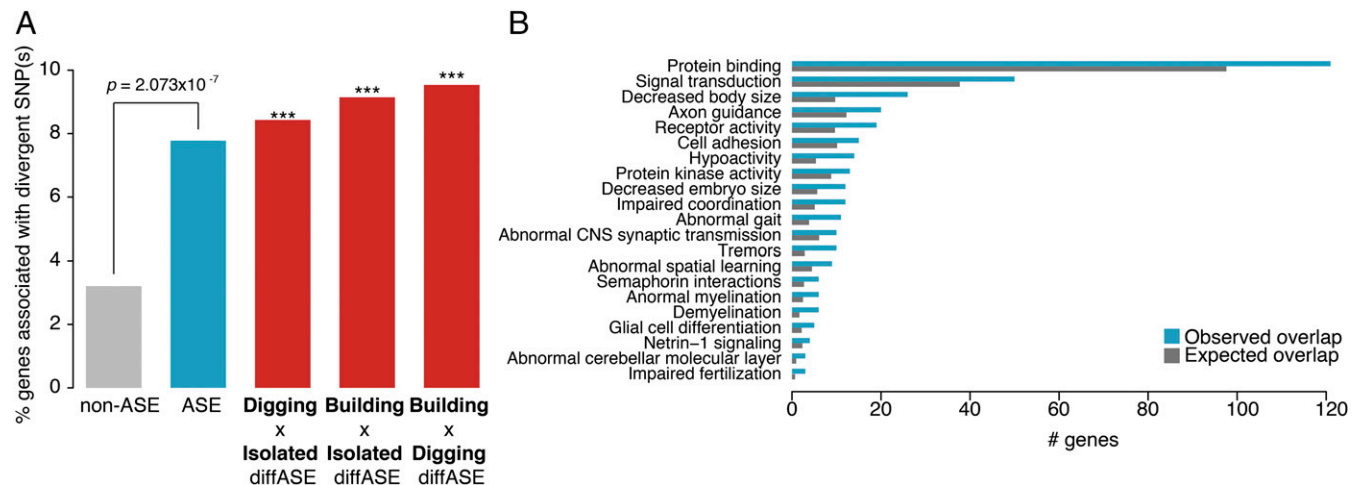


Fig. 5. Intersection of genome-wide SNPs and ASE. (A) Bar plot comparing the number of ASE, non-ASE, and diffASE (across all contexts) genes associated with highly divergent SNPs between pit-digging and castle-building species. $***P < 1 \times 10^{-4}$, Fisher's exact test comparing genes overlapping SNPs and genes not overlapping SNPs. (B) Categories in which the observed amount of overlap (blue) between genes associated with highly divergent SNPs and with ASE is significantly greater than expected (gray).

neuroplasticity, and behavioral regulation, suggesting concordance between patterns of genetic and regulatory divergence among bower-building species.

Discussion

By combining genome sequencing across many closely related species with analysis of ASE in the brains of behaving hybrid animals, we provide a genome-wide view of how a complex behavior has evolved. Our results suggest that the evolution of bower building was associated with polygenic selection on ancient and new genetic variants that regulate genes involved in neural activity and synaptic plasticity in specific behavioral contexts. The observation of context-dependent ASE associated with sequential pit-digging and castle-building behavior in F_1 males suggests how *cis*-regulatory divergence across many genes may combine to produce the evolutionary divergence of a complex behavior.

The elevated F_{ST} values at thousands of variants among the 20 diverse bower-building species examined revealed that bower building is associated with a complex but phylogenetically consistent genetic architecture. The observation that these sites are both ancestral (polymorphisms shared with species outside of Lake Malawi) and derived parallels similar findings from studies of Malawi cichlids (6, 8, 20) and other recently evolved species flocks such as sticklebacks (15) and finches (5). Notably, we found that castle-building species tended to possess derived variants at more genetically diverged sites, suggesting that, at least from a genomic perspective, castle building is the younger, derived behavior. The observation that the species believed to be similar to the common ancestor of Malawi cichlids, *Astatotilapia calliptera*, digs pits and is positioned at the base of the Lake Malawi phylogeny lends credence to this idea (Fig. 1A). The phylogenetic distribution of bower building suggests that repeated instances of selection, be it on standing variation or introgressed alleles, may have acted to differentially fix the genetic architecture associated with castle bowers in a number of sand-dwelling species. That we detect potential genomic signatures of gene flow supports the notion that introgression may have played a role in the propagation of the derived castle-building behavior among sand-dwelling cichlid species. The importance of gene flow across species boundaries has been highlighted before in cichlid fish adaptive evolution (8, 30), but bower building represents a special case of this general phenomenon, as the behavior is sex specific and unlikely to increase male survival. It will be interesting to test specific hypotheses of gene flow between particular bower-building species using more targeted sampling and genetic methods.

Given the large regions of increased genetic divergence identified on LGs 2 and 11, it is intriguing to consider the possibility that there may be “supergene”-like elements underlying bower building similar to those that have been found to be associated with other animal behaviors such as male reproductive morphs in the ruff (31, 32) or insect social organization (33). Our observation that F_1 hybrids of a cross between pit-digging and castle-building species can build both structures and do so in a mutually exclusive, sequential fashion may further support the notion that the varying bower types require genomic regions working in a modular and independent fashion. Such a finding would not be without precedent among the cichlids of Lake Malawi. The orange-blotch (OB) coloration phenotype found among >20 rock-dwelling species is associated with a tightly linked genomic locus resembling a supergene in its size and inheritance and has arisen independently at least three times (34).

Our approach to sequence the genomes of individuals from pit-digging vs. castle-building species has identified numerous genetic variants associated with bower behavior, not unlike the genetic architecture of other complex traits, including human neurological disorders (35). Integrating genome sequencing with RNA-seq from F_1 hybrid brains pairs our strategy with the complexity of the bower trait. Our results fit the general pattern observed in other complex traits: numerous genetic associations with the phenotype (36) and an expectation that many of these variants exert their effects via context-dependent *cis* regulation of gene expression (37, 38). Models like Malawi cichlids may thus occupy a “sweet spot” in complexity, combining a rich genetic, evolutionary,

and phenotypic profile with tractable biology that could yield insights into the origins of behavioral diversity. Of utmost importance will be continued efforts toward correct phenotypic categorization of such complex traits. In this study, we decided to characterize bower building into two qualitative categories and used this definition to perform genome-wide tests across 20 diverse species. However, our species cohort may share other traits correlated with bower type, adding noise to our measures of genetic association, or may represent more than two behavioral strategies (although this scenario seems unlikely, given the behavioral and genetic findings presented here). In general, the use of bower building as a model complex trait will benefit from careful work on the roles of ontogeny, intraspecific variation, and behavioral variability in the regulation of this behavior.

The extensive, context-dependent transcriptomic divergence associated with bower building in F_1 hybrids provides intriguing insights into the regulatory basis of behavioral evolution. For example, it appears that bower building is defined by modularity at multiple levels of biological organization. The transition in CV \times MC F_1 hybrids from pit to castle bowers may be considered as a shift between distinct behavioral modules associated with distinct behavioral patterns reflective of the respective parental species. The finding that these phases are associated with distinct transcriptomic states suggests that the pit and castle alleles function modularly based on behavioral context, best reflected by the significant number of genes displaying discordant ASE across these behavioral modules. This is in opposition to other scenarios in which regulatory divergence might be static across behavioral conditions or minimal in the context of brain function and behavior. Instead, the transcriptomic states associated with the pit- and castle-phases in F_1 hybrids appear to be more similar to those found between tissues in an organism, arising from potentially distinct regulatory or epigenomic cellular environments. In this case, given the genomic and evolutionary signatures identified by the whole-genome analyses, it appears that these differences in regulation are at least partly due to extensive sequence-level variation in a number of functionally related regulatory elements associated with behavior. Notably, this may suggest that the genome harbors regulatory loci specifically involved in the dynamic coordination of behavior and brain function analogous to well-known genetic modulators of morphology and that these loci underlie the evolution of behavioral diversity.

Methods

Bower Behavioral Measurements. Individual adult, reproductive subject males (*C. virginalis*, $n = 4$ and *M. conophoros*, $n = 4$) were each housed with one to three adult, reproductive stimulus females of the same species in $43.2 \times 91.4 \times 40.6$ cm (160-L) glass aquariums maintained on a 12-h:12-h light:dark cycle. A tray (Newbury Black Poly Saucer, SA1412BK; Dynamic Design) 5.1 cm deep and 35.6 cm in diameter was placed in each tank and was filled with sand (Sahara Sand, 00254; CaribSea, Inc.). All animal experiments were approved by the Georgia Tech Institutional Animal Care and Use Committee (protocol no. A11034) and by the Stanford University Animal Care and Use Committee (protocol no. APLAC-28757). For each subject, 90- to 120-min videos were recorded between 3–8 h after lights-on during periods of high bower-building activity using a GoPro camera (Hero4 Silver, CHDHY-401; GoPro) housed in a waterproof compartment (Clear Standard Housing, AHSRH401; GoPro) and placed top-down directly above the sand-filled tray. The behavior of male subjects was scored using The Observer XT 12 software (Noldus) according to the following definitions: “scooping” was defined as opening the mouth and collecting sand, and “spitting” was defined as expulsion of sand from the mouth. For each individual, screenshots were captured at the precise moment of every scoop and spit event, and these screenshots were exported to Paint (Microsoft) to extract spatial coordinates of the mouth for every scoop and spit event.

Bower-building difference scores were calculated to measure the spatial dispersion of scoops compared with the spatial dispersion of spits for each subject male. To calculate the difference score for each subject, we first determined the coordinates for each subject’s average scoop location and average spit location. Using these coordinates, we calculated the absolute distance from the average spit location to each individual spit location and from the average scoop location to each individual scoop location. To generate a quantitative metric of spatial dispersion, these distances were then averaged, yielding two distance scores for each subject, one for scoops, and one for spits. To compare the spatial dispersion of scoops vs. spits, the difference between these distance

scores (scoop distance score minus spit distance score) was calculated for each subject, thus providing an estimate of differences in spatial patterns of scooping and spitting sand for each animal. A two-tailed Student's *t* test was used to compare these difference scores between species.

Genome Sequencing, Alignment, and Variant Identification. We chose diverse representative species of pit-digging and castle-building groups, selected from multiple genera across the phylogenetic tree of sand-dwellers (2). Genomic DNA was extracted from fin clips of 20 individuals collected in Lake Malawi (11 pit diggers and 9 castle builders) (*SI Appendix, Table S1*) using the Qiagen DNeasy kit (catalog no. 69504; Qiagen).

Libraries were constructed following the Illumina TruSeq DNA library-preparation protocol. Paired-end sequencing (2×100) was performed on the Illumina Hi-Seq 2500 system at the Georgia Institute of Technology. Raw sequence reads were quality controlled using the NGS QC Toolkit (39). Quality control was performed as follows: First, raw reads with an average PHRED quality score below 20 were removed. The remaining reads were further trimmed of low-quality bases at the 3' end. Quality-control reads for each of the genomes were aligned to the new *M. zebra* reference genome (4) using bwa-mem (version 0.7.4) and default parameters (40). We used Picard Tools (<https://broadinstitute.github.io/picard/>) to mark PCR duplicates. Sequences were mapped to 87% of the reference genome on average, with mean coverage of $24.31\times$. Variant discovery and filtering were performed using HaplotypeCaller within the Genome Analysis Toolkit (GATK) program according to GATK best-practices recommendations (41–43).

Deletions were identified using modifications to a previously published approach (44). Candidate deletions were first identified using chimeric reads as identified by Burrows–Wheeler Aligner (40) (i.e., reads that included the SA tag) in which each alignment mapped to the same contig and the same strand. These reads were used to infer the breakpoints and insertion sequence of a candidate deletion. Candidate deletions that were also present in the sequencing of *M. zebra* were excluded as likely errors in the reference sequence. Each candidate deletion was then genotyped in each of the pit-digging and castle-building species by collecting all the reads with primary alignments that fell within 10 bp of the candidate deletion and with a mapping-quality score greater than 10. These reads were realigned to both the reference and the candidate deletion sequence using a striped Smith–Waterman alignment from the scikit-bio Python library. Reads were classified as reference, mutant, or undetermined based on their mapping score to the two alleles. Deletions were categorized as homozygous mutant if 80% or more of the reads were categorized as mutant, as homozygous reference if 80% or more of the reads were identified as reference, and as heterozygote if they fell in between. Candidate deletions that were reference homozygous in all species were filtered from the dataset.

Tests of Genetic Divergence and Enrichment. We excluded sites with more than 50% missing genotypes from the whole-genome sequencing data. We calculated F_{ST} per variant and in 10-kb windows using the `-weir-fst-pop` parameter from the VCFtools program (45) with the flags `-fst-window-size 0` for individual sites and `-fst-window-size 10,000` for 10-kb windows. Nucleotide divergence was calculated using VCFtools with the `-window-pi 10,000` flag. Thresholds were estimated using R-package `fdr-tool` (46). F_{ST} values were converted to a normalized scale for visualizing these data on LGs (Fig. 2A) using Fisher's Z-transformation. To compare F_{ST} with a population-structure-controlled genome-wide association analysis, we employed GEMMA v0.96 (47). We first computed a kinship matrix using the filtered sites from which F_{ST} was calculated and then performed the association test on bower type using a linear mixed model (`-lmm` flag) factoring in relatedness via the kinship matrix.

SNP and indel variants were annotated using the SnpEff (4.3i) program (48) and were analyzed for functional enrichment using GeneAnalytics (geneanalytics.genecards.org) (14). Using the binomial distribution, this algorithm tests the null hypothesis that there is no functional overrepresentation. A resulting score is presented for each match in the form of a $-\log_2$ transformed *P* value corrected for multiple comparisons via the FDR method. Scores are arranged into three significance categories: high ($P_{adj} < 0.0001$), medium ($P_{adj} < 0.05$), and low ($P_{adj} > 0.05$). Semantic similarity plots for significantly enriched categories were produced with REVIGO (49).

Identifying Structural Variants. To predict structural variants on a genome-wide basis, we used breakdancer-max (1.1) (12) with default parameters. As read-pair mismatches in size (pairs are farther away than expected) and direction (pairs in the same orientation) can be used to identify inversions, we also used the Integrated Genome Browser (13) to manually validate predicted structural variants from breakdancer-max, particularly those on LGs 2 and 11. Thousands of structural variants were predicted for each species, but none consistently associated with the broad F_{ST} peaks on LGs 2 and 11.

Improved Genome Annotation. In the original National Center for Biotechnology Information (NCBI) release of the latest *M. zebra* reference genome (4), 15,361 of 26,490 predicted protein-coding genes were annotated as hypothetical or without orthologs. To improve this annotation, we identified orthologs for genes in two additional ways: (i) using a phylogenetic method using TreeFam, a curated database of phylogenetic trees of animal genes, and (ii) via reciprocal blast against the human genome and five fish genomes (50). The final annotation merged orthologous genes identified by both methods; 1,900 hypothetical genes remain.

Assigning SNPs and Genome Contigs to Linkage Maps. We used Chromonomer (1.03) (51) to anchor the gap-filled “M_zebra_UMD1” assembly (4) to LGs using two different genetic maps, both generated via traditional F_2 crosses and genotyped with restriction site-associated DNA sequencing (RAD-seq). First, a genetic map from 160 F_2 individuals from an *M. zebra* \times *M. mbenjii* cross resulting in 834 markers in 22 LGs and spanning 1,933 cM (52) was used to anchor the M_zebra_UMD1 assembly. This initial anchored assembly was subsequently reanchored with Chromonomer using a second genetic map. The second genetic map was generated by genotyping 268 F_2 individuals from a *Labeotropheus fuelleborni* \times *Pseudotropheus tropheops* “red cheek” cross, resulting in 946 markers in 24 LGs and spanning 1,453.3 cM (53). BWA mem (version 0.7.12-r1044) (54) was used in both Chromonomer runs to create the input SAM file by aligning respective map-marker sequences to the appropriate assembly or intermediate assembly. A minimum of two markers was required to anchor a contig to a particular LG. The resulting FASTA file of the anchored M_zebra_UMD1 assembly was used for subsequent analysis.

Phylogenetic Analysis. A maximum-likelihood phylogeny was constructed with the variant data using the SNPPhylo pipeline (55). Default parameters were used with an additional flag `-M 0.5`.

Ancestral Allele Reconstruction. Pairwise whole-genome alignments of *Neolamprologus brichardi* (a species belonging to an older radiation from Lake Tanganyika), *A. burtoni* (a riverine species found in East Africa around Lake Tanganyika), and *Pundamilia nyererei* (a species from a recent radiation in Lake Victoria) were each constructed against the latest Lake Malawi cichlid genome, *M. zebra*, using the last alignment algorithm (*A. burtoni* against *M. zebra*, *P. nyererei* against *M. zebra*, *N. brichardi* against *M. zebra*) (56). The generated .maf files from the alignment were converted to sam format using the maf-convert script within the last alignment package. Resulting sam files were converted to bam format via SAMtools (57) and were used to add ancestral allele information into the vcf file obtained from variant discovery.

Detection of Ancient/Derived Allele Enrichment Among Pit-Digging and Castle-Building Species. To assess biases in the presence of ancient (polymorphic within and outside of Lake Malawi) and derived (polymorphic only among sand-dwelling species) alleles among pit-digging and castle-building species, we first intersected our SNP-level F_{ST} measurements with the lists of ancient and derived SNPs as identified through the ancestral allele reconstruction methods outlined above. We then calculated a *P* value and OR on allele counts at each SNP using a Fisher exact test. We assessed both the degree of divergence at each SNP via F_{ST} values and the direction of divergence through the ORs from the Fisher's exact test. For example, at derived SNPs, an OR < 1 indicated that the castle-building species tended to possess the derived allele, while an OR > 1 indicated a bias toward the derived allele for the pit-diggers. For ancient SNPs, an OR < 1 indicated that the castle-building species tended to possess the non-*M. zebra* allele (recall that variants were called in relation to the *M. zebra* reference genome), while an OR > 1 indicated that pit-digging species tended to possess the non-*M. zebra* allele.

To assess systematic differences in the possession of derived or ancient variants between pit-digging and castle-building species, we performed Fisher's exact tests at various F_{ST} thresholds. These tests were applied to both the proportion of SNPs with an OR > 1 and OR < 1 for the derived and ancient lists (as seen in Fig. 2C) in addition to comparing the mean ORs at various *P* value thresholds (as seen in *SI Appendix, Fig. S2C*).

Analyses of Gene Flow and Incomplete Lineage Sorting. Maximum-likelihood phylogenies were produced for 10-kb genomic bins using the python function `phymL_sliding_windows.py` created by Simon Martin (available at https://github.com/simonmartin/genomics_general). This resulted in 1,927 trees that were subsequently visualized using DensiTree v2.2.5 (16).

We used TWISST (17) to analyze the distribution of phylogenetic topologies across genomic windows. To do so the .vcf file containing genotype information for the pit-digging and castle-building species was filtered to include only biallelic sites in which all species possessed genotypes. Indels

were also removed. The function `phyml_sliding_windows.py` was then used to create maximum-likelihood phylogenies from these variants in windows containing 50 informative SNPs. TWISST was then run on these trees, testing for variation in the tree topologies in the following five clades:

Clade 1 (pit diggers): *Trematocranus placodon*, *Dimidiochromis kiwinge*, *Dimidiochromis compressiceps*, *Tramitichromis intermedius*, *Mylochromis sphaerodon*, *Mylochromis lateristriga*

Clade 2 (*C. virginalis*): *C. virginalis*

Clade 3 (Copadichromis castle builders): *Copadichromis* sp. "mlo to gold-crest," *Copadichromis likomae*

Clade 4 (castle builders): *Mylochromis anaphrymus*, *Ctenopharynx nitidus*, *Otopharynx argyrosoma*, *M. conophoros*, *Nyassachromis* sp. "otter"

Clade 5 (*A. calliptera*): *A. calliptera*

Four Population Tests. TreeMix was run on genotype counts using the settings `-k 2000` and `-m 1,2,4,6,8,10` to assess support for admixture events among bower-building species. We extended this analysis by computing the *f*₄ statistic for every possible four-population combination using the `fourpop` function in TreeMix with the setting `-k 500`. *P* values were calculated for every four-population comparison from the reported *z*-scores and adjusted using Bonferroni correction. To use *A. calliptera* as an outgroup in the detection of possible gene flow among sand-dwellers, results for just the combination (A,B; C, *A. calliptera*) were extracted. This led to 14,536 comparisons, of which 3,706 had significant (Bonferroni-corrected *P* < 0.05) *f*₄ statistics (Fig. 3E and Dataset S4). The most significant comparisons for each species pair in which the species were (A,B;C,D) and (A,B;C,D) were then collected.

We used the *fd* statistic to identify patterns of possible introgression across the genome. The *fd* statistic function is similar to *f*₄ in that it compares genotype frequencies between four populations; however, whereas *f*₄ is a genome-wide measure, *fd* can be calculated locally within genomic windows and therefore allows the detection of genomic regions with particularly strong signals of introgression¹⁹. We calculated *fd* using the python function `ABBABABAWindows.py` created by Simon Martin (available at https://github.com/simonmartin/genomics_general) over 10-kb windows containing at least 50 informative SNPs for the four population groups identified as most significant from analyses of the *f*₄ statistic.

RNA-Seq Library Construction. To assess the allele-specific expression, whole brains were obtained from the following animals:

One CV male; digging (sire of all analyzed F₁ hybrids)

Two CV × *M. conophoros* (MC) F₁ hybrids; digging

Two CV × MC F₁ hybrids; building

Two CV × MC F₁ hybrids; isolated

For these experiments individual males were housed with three to five conspecific females and were allowed to develop territories and initiate bower construction. We confirmed that bower behavior was being performed reliably (i.e., was consistent for >24 h), and on the evening before the experiment we separated focal males from females using a transparent divider and flattened the bower. At lights-on the next morning the barrier was removed, and the males' behavior was observed. Males were killed via decapitation 30 min after the initiation of consistent behavior and, to prevent mRNA degradation, brains were dissected into RNAlater (catalog no. AM7020; Thermo Fisher) less than 10 min after the animal was killed. Whole brains were homogenized in TRIzol (catalog no. 12183555; Thermo Fisher) using a pestle. RNA was isolated using a Qiagen RNeasy mini kit (catalog no. 74104; Qiagen). RNA-seq libraries were constructed using Illumina TruSeq kits following the manufacturer's protocols. All libraries were sequenced as multiplexed samples in one lane of an Illumina HiSeq 2000 system. Two biological replicates per context were chosen for analyzing ASE following previously published methods (24) that found a similar sample size was sufficient for reliably detecting allelic biases from RNA-seq data.

RNA-Seq Alignments and SNP Calling. RNA-seq read quality was assessed using FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Illumina adapters were removed using SeqPrep (<https://github.com/jstjohn/SeqPrep>). We obtained the *M. zebra* genome assembly and annotations from NCBI RefSeq (assembly accession: GCF_000238955.2; Assembly name: *M_zebra_UMD1*). RNA-seq reads were aligned to the *M. zebra* genome using STAR 2.4 (58) with the options `-SortedByCoordinate`, `-outSAMattributes MD NH NM`, and `-clip5pNbases 6`.

Read groups were added with `AddOrReplaceReadGroups.jar` in Picard Tools 1.92 (<https://github.com/broadinstitute/picard>). The resulting bam files were sorted using SAMtools (57). Duplicate reads were marked using `MarkDuplicates.jar` in Picard Tools. We then applied GATK 3.3 indel realignment and duplicate removal and performed SNP and indel discovery using `UnifiedGenotyper` following the suggested GATK best practices (41–43).

We filtered the resulting `.vcf` files to identify all heterozygous sites in the F₁ hybrid samples with quality scores >30. We also produced a list of all homozygous sites in the CV parental sample with quality scores >30. To allow proper phasing of heterozygous sites, we filtered the F₁ hybrid list to include only sites that intersected with the CV homozygous SNPs.

Detection and Quantification of ASE. The *M. zebra* reference genome was masked at high-confidence heterozygous sites using the perl script `MaskReferenceFromBED.pl` (<https://github.com/TheFraserLab/ASER>). To control for reference bias, all hybrid samples were then realigned to this masked reference using the same STAR options as above. Duplicates were marked using `MarkDuplicates.jar` in Picard Tools, and the bam files were sorted using Sam Tools. The SNP-level ASE was then calculated with the python script `CountSNPASE.py` (<https://github.com/TheFraserLab/ASER>).

All downstream ASE analyses were conducted using R version 3.2.3 (59). After SNP-level ASE was calculated, we filtered for more than five counts per allele for every gene within each sample. To conduct gene-level analyses, the subset of SNPs that met this expression cutoff were then summed without normalization into gene-level counts using the gene coordinates in the `ref_M_zebra_UMD1_top_level.gff3` annotation (NCBI *M. zebra* annotation release 102). For all genes in each sample, ASE was calculated by taking the log₂ ratio of the gene-level CV allele counts over the gene-level MC allele counts. After alleles were filtered and summed into genes, we investigated the distribution of ASE ratios across all genes for each sample. Distributions consistently skewed toward either allele could be potentially indicative of biases in read alignment or other technical artifacts. Analysis of ASE ratio distributions showed each to be roughly normal and centered around a log₂ ratio of zero, indicating a lack of evidence for strong bias toward either species' allele across samples (SI Appendix, Fig. S9).

We next calculated the significance of ASE per gene. Since allelic counts from RNA-seq data are prone to overdispersion, we identified significant ASE using a beta-binomial test comparing the CV and MC counts at each gene with the R package `MBASED` (60) (1-sample analysis; default parameters; run for 1,000,000 simulations). The resulting *P* values were adjusted for multiple tests using Bonferroni correction. For a gene to be considered significant within a context, we required that both replicates possess Bonferroni All *P* values <0.05 and that the direction of ASE (either CV biased or MC biased) was the same between replicates.

Identifying diffASE. DiffASE and differential allelic induction were identified using the two-sample analysis in `MBASED` (60) (default parameters; run for 1,000,000 simulations) which, as in the one-sample analysis used to assay ASE, employs a beta-binomial model of read counts to control for over dispersion. `MBASED` was used to compare all possible pairings of the three behavioral contexts (digging, building, and isolated). This produced the pairings: digging × building, digging × isolated, and building × isolated. Furthermore, since `MBASED` tests for significance in only one of the two contexts at a time, we also ran all three comparisons in their reciprocal directions (building × digging, isolated × digging, and isolated × building). To identify diffASE, the expression of the CV and MC alleles within each context were compared (i.e., CV allele_{context1} vs. MC allele_{context1} compared with CV allele_{context2} vs. MC allele_{context2}). Significant cases represent scenarios in which the ratios between the alleles diverge between contexts (as represented in Fig. 4A). Differential induction was assayed by testing for significant variation between CV and MC alleles across contexts, using the ratio of each allele to itself for the test of significance (i.e., CV allele_{context1} vs. CV allele_{context2} compared with MC allele_{context1} vs. MC allele_{context2}).

The resulting *P* values for each pairing were combined across replicates using Fisher's method and were adjusted with Bonferroni correction. We then compared these combined and adjusted *P* values across the reciprocal contexts (e.g., digging × isolated vs. isolated × digging) and selected the lowest *P* value for downstream analyses.

Gene Set Enrichment Tests. For the enrichment analyses of diffASE genes and genes with allelic induction in building and digging, the corresponding lists were produced as well as background sets corresponding to all other genes detected in one of the three behavioral contexts. Gene enrichments were calculated with `PANTHER` (61) and filtered for a *q*-value <0.05. The allelic induction lists were produced in a method similar to those for diffASE by using `MBASED`, but instead

of comparing alleles within contexts the analysis was run on alleles across contexts (e.g., comparing CV allele expression in digging vs. building). The resulting *P* values would then reflect the differential induction of each allele across pairs of contexts. For the gene set enrichment tests, only genes that had significant induction (Bonferroni-corrected *P* value <0.05) of at least one allele in either the building or digging contexts alone were selected. Semantic similarity plots for significantly enriched categories were produced with REVIGO (45).

Lineage-specific variation was assayed by comparing the number of genes enriched within gene sets that possessed differential allelic induction of either the CV or MC alleles during building or digging. To do so a .gmt file containing gene sets from the human Kyoto Encyclopedia of Genes and Genomes, GO, Molecular Signatures Database, National Cancer Institute, Institute of Bioinformatics, NetPath, HumanCyc, Reactome, and Panther databases was downloaded from the Bader laboratory website (download.baderlab.org/EM_Genesets/) in April 2018. Genes were then assigned to categories, and a

Fisher's exact test was performed on a 2 × 2 contingency table in which the rows represented digging and building and the columns were the CV and MC alleles. To limit the number of tests performed, we ran the Fisher's exact test on each ontology independently and required each gene set to have 20 genes represented. The resulting *P* values were then adjusted for multiple tests using Bonferroni correction.

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