Science Magazine Article Guidelines

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* Materials and Methods should be included in supplementary materials and must provide sufficient detail to allow replication of the study. This should be followed by additional data and figures needed to support the paper’s conclusions.
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**Article title:** Rapid Evolutionary Response to a Virulent, Introduced Parasite in Darwin’s Finches

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Parasites exert some of the strongest selective forces on host taxa, yet the early stages of host-parasite coevolution are poorly understood. Human-mediated redistribution of parasites has introduced novel parasites to naïve hosts, providing a unique opportunity to characterize the biological processes that evolve in hosts during the initial stage of host-parasite coevolution, the timeline over which selection on hosts is detectible, and the extent to which evolutionary patterns are parallel across species. Such insights are essential for understanding and managing the increasing introduced parasitic threats to biodiversity worldwide.

Darwin’s finches are a classic example of rapid evolution, with well-studied beak traits that evolve on short time scales through character displacement resulting from fluctuating climate conditions and competition for food (*1*, *2*). This clade consists of 18 species in the tanager family (Thraupidae), 17 species on the Galápagos Islands and one on Cocos Island, that have diversified over 1-2 million years (*3*). But a virulent and introduced parasite has reshaped the selective landscape for Darwin’s finches over the last 30 years. In 1998, larvae of the vampire fly (*Philornis downsi*) were first observed in the nests of breeding birds in the Galápagos. Several endemic passerine species on the Galápagos are now nearing extinction including two species of Darwin’s finch, the Mangrove finch (*Camarhynchus heliobates*) and the medium tree finch (*C. pauper*, *4*). High rates of parasitism affect all Darwin’s finch species, resulting in a reduced fledging success rate of ~50%. The larvae of the vampire fly feed on the blood of nestling and occasionally adult nesting passerine birds, and they cause anemia, reduced body condition, life-long facial and nare damage, and often death (*5*). It is unknown if naïve hosts in the Galápagos can adapt to a novel and highly virulent ectoparasite or if the high costs observed today will persist and ultimately drive some populations and species to extinction (*6*). Despite similarly high rates of parasitism across Darwin’s finches, only one species, the vegetarian finch (*Platyspiza crassirostris*), experiences minimal negative effects of parasitism (*5*, *7*).

We sequenced 24 birds from three species of Darwin’s finch collected in 2019, 21 years after the first observation of vampire fly larvae in the Galápagos in 1998 (*5*). We compared these post-invasion samples to previously sequenced pre-invasion samples collected in the 1980s and 1990s (*8*). Birds from both sampling time points were collected on Santa Cruz Island and were sequenced to the same depth of coverage (~10x) using 2x125 base-pair paired-end reads. All were aligned to the STF\_HiC small tree finch genome (1.28 GB; Genbank accession number: GCF\_901933205.1, *6*). Disentangling the signals of selection from those of drift over such a short time frame (~3.5-7 generations) is challenging, so we used a conservative approach that identified outlier genomic regions with evidence of both divergence over time and shifts in the site frequency spectrum associated with selective sweeps. We classified outliers as the top 0.1% of the sum of the scaled and centered FST and the inverse of ∆Tajima’s D, using 50kb windows with a 12.5kb step in ANGSD. We used a less conservative cutoff of 1% to test for parallel section on the same genes across all three species. We tested for overrepresented Gene Ontology (GO) terms associated with genes within the outlier windows from our additive model of *Z-*FST and the inverse of *Z-*∆Tajima’s D for each species.

**Low genome-wide divergence over 20 years of selection**

All three species showed low levels of divergence between the two time points (Genome-wide FST = 0.029-0.038, maximum windowed FST = 0.299-0.369). Historic population size inferences in MSMC could change over time in the presence of novel patterns of introgression or gene flow (*9*); however, we did not observe differences between models of pre- and post-invasion samples in any species. [*Alternatively, briefly describe observed differences here* – *depends on Logan’s final results*].

**Signals of selection emerged from two species**

Two of the three taxa, the vegetarian finch and the medium ground finch, demonstrated significantly overrepresented GO terms. All overrepresented terms in the vegetarian finch were associated with blood vessel development or immune function (Table 2). The overrepresented GO terms in the vegetarian finch are likely candidates for a response to parasitism by the vampire fly, and we further explored the function of and mutations within each gene. We investigated the SNPs with the highest FST within and around each gene for evidence of coding changes or mutations in a CpG site, which would indicate a change in DNA methylation and epigenetic gene regulation.

The overrepresented categories for the medium ground finch were associated with regulation of various metabolic processes. Seven genes drove the signal for these overrepresented GO terms, and four of these genes have associations with blood physiology or immune functions. *CHST4* influences lymphocyte adhesion onto endothelial cells, *CELF1* affects heme oxygenase-1 (*10*), *TERF2IP* regulates of innate and adaptive immune function, inflammation (*11*), and *PRTFDC1* suppresses immune related pathways (*12*). The remaining three – *ADAT1*, *FARS2*, *MCM9* – are involved in gene expression and DNA-related processes (*13*–*15*).

While no GO terms were significant in the small tree finch, regions of the genome did show local peaks in our composite statistic. This suggests selection on some genes, although perhaps without a strong enough signal of selection across multiple genes involved in a single biological process. Our findings do not rule out ongoing selection on relevant physiological processes that could be better detected over a longer time period.

**Angiogenesis and immune functions are under selection in the vegetarian finch**

Four genes drove the angiogenesis signal in the vegetarian finch: *ANGPT1*, *HPSE*, *ITGA2B*, and *PODXL*. Both *ANGPT1* and *ITGA2B* contain noncoding blocks of single nucleotide polymorphisms (SNPs) with high FST, which occur between exon 1 and 2 in *ANGPT1* and in the region immediately following *ITGA2B*. *ANGPT1* encodes the protein angiopoietin-1, which reduces vascular leakage and inflammation at wound sites (*16*). ITGA2B encodes the subunit α2b of the αIIbβ3 integrin protein which is found in the cell membrane of platelets and is essential for clotting (*17*, *18*). No SNPs were found in coding regions, nor were any SNPs in a CpG site, but the high FST region near *ITGA2B* is within 1kb and could affect a promoter region.

**Selection signals linked to mutations in translated regions in HPSE and PODXL**

*HPSE* contained one genotype within the transcribed region with a relatively high FST (0.327), specifically within the 3`UTR. RNA-binding proteins (RBPs) that bind to the 3`UTR regulate gene expression, and we used RBPmap (*19*) to predict RBP binding sites that may differ between the ancestral and derived allele in this region. We found one binding motif associated with both the derived and ancestral genotype (wgcaugm), and two associated only with the derived genotype (cgucca and rygcgcb). In all three circumstances, the derived genotype matched the motif and the ancestral did not. The derived allele at this site increased from a frequency of 0.5 pre-invasion to 0.875 post-invasion, suggesting selection for an increased ability to regulate *HPSE* using miRNA. HPSE encodes the enzyme heparanase, which regulates clotting, inflamation, and the innate immune response, among other functions (*20*, *21*).

We found five mutations within the coding sequence of *PODXL*, four of which were synonymous but one of which (P295A) was a missense mutation. We used SIFT to predict if this mutation resulted in a nonfunctional protein and found that it is expected to be tolerated (tolerance score = 0.35, median sequence conservation 4.32, *13*), but it may have impacts on protein folding and structure. The frequency of this mutation decreased over time from 1.0 to 0.333, indicating selection against the derived protein. *PODXL* encodes the protein Podocalyxin Like-1 which is found in hematopoetic progenetior cells and plays a role in the production of new blood cells (*23*).

**Intronic CpG mutations underlie parallel signals of selection on genes related to inflammation and angiogenesis**

All pairwise comparisons of the top 1% of genes found evidence of significant overlap. We identified 32 genes in both the vegetarian and medium ground finch (OR = 3.32 [2.20 – 4.86], q < 0.001), 30 in both the vegetarian and small tree finch (OR = 2.18 [1.44- 3.21], q < 0.001), and 14 in both the medium ground finch and the small tree finch (odds ratio = 1.83 [1.04 - 3.01], q = 0.030). GO term analysis of genes identified in two or more species found no significantly overrepresented terms. However, the three genes identified in all species, *BMPER*, *NUDT4*, and *TENM3*, function in biological processes relevant to a response to parasitism by the vampire fly. *BMPER* encodes the protein Bone Morphogenetic Protein-binding Endothelial Regulator, which facilitates endothelial cell sprouting, an essential step in angiogenesis (*24*). It also regulates dysregulated systemic inflammation at injury sites (*25*). *TENM3* encodes the protein Teneurin Transmembrane Protein 3 which influences the developing craniofacial mesenchyme and dermis (*26*, *27*). *NUDT4* encodes the protein Nudix Hydrolase 4 and has been identified as a key gene involved in sepsis (*28*), and is correlated with the abundance of various immune cells (*29*), and is also upregulated during erythropoiesis (*30*). We identified CpG sites with relatively high FST values within the intronic regions of each of the three genes in each of the three species (Supplementary Table XXX). All CpG sites in *NUDT4* decreased in frequency following invasion of the vampire fly, while CpG sites in *BMPER* and *TENM3* decreased for the small tree finch, increased for the vegetarian finch, and both increased and decreased for the medium ground finch (Figure 2). In the small tree finch, we also identified a CpG site within the 5’-UTR of *NUDT4* that decreased from a frequency of 0.4 to 0 in the post-invasion samples. These results demonstrate evidence of selection for and against various regulatory sites, with species specific trends.

We document evidence of selection in response to a novel parasite in the vegetarian finch, the only taxa in the clade that does not experience high costs associated with parasitism by the vampire fly. Our results show that this tolerance evolved through selection on standing variation in regulatory regions of angiogenesis and clotting genes. Across all three species studied, we also document evidence of selection on three genes involved in blood clotting, craniofacial and dermis development, and immune function especially as it relates to sepsis. These findings suggest that fitness reductions are driven by blood loss, tissue damage, and a costly immune response that depletes the limited resources of developing nestlings.

**Table 1:** Counts of individuals sequenced and population genetic statistics that compare pre- and post-invasion sequences.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Common name** | **Species** | **No.pre-invasion** | **No. post-invasion** | **FST** | **∆Tajima** |
| Vegetarian finch | *Platyspiza crassirostris* | 5 | 9 | 0.038 | 0.338 |
| M. ground finch | *Geospiza fortis* | 10 | 8 | 0.031 | -0.176 |
| S. tree finch | *Camarhynchus parvulus* | 12 | 7 | 0.029 | -0.173 |

**Table 2:** Significantly overrepresented GO terms and FDR corrected q-values from candidate gene lists of each species, and from the list of candidate genes identified in two or more species (pairwise overlap).

|  |  |  |  |
| --- | --- | --- | --- |
| **Species** | **PANTHER GO-Slim Biological Process** | **GO Term** | **q-value** |
| Vegetarian finch | regulation of cell adhesion mediated by integrin | GO:0033628 | 0.043 |
|  | angiogenesis | GO:0001525 | 0.014 |
|  | blood vessel morphogenesis | GO:0048514 | 0.011 |
|  | blood vessel development | GO:0001568 | 0.008 |
|  | vasculature development | GO:0001944 | 0.007 |
|  | tube morphogenesis | GO:0035239 | 0.005 |
|  | tube development | GO:0035295 | 0.006 |
| Medium ground finch | tRNA metabolic process | GO:0007519 | <0.001 |
|  | nucleic acid metabolic process | GO:0060538 | <0.001 |
|  | nucleobase-containing compound metabolic process | GO:0007517 | <0.001 |
|  | heterocycle metabolic process | GO:0060537 | <0.001 |
|  | cellular aromatic compound metabolic process | GO:0010720 | <0.001 |
| Small tree finch | None | None | NA |
| Pairwise overlap | None | None | NA |

**Table 3**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Motif** | **Ancestral** | **Z-score** | **P-value** | **Derived** | **Z-score** | **P-value** |
| wgcaug**m** | ...g..**g** | 2.855 | 0.0022 | ...g..**c** | 3.329 | 0.0004 |
| cguc**c**a | ...g**g**. | NA | NA | ...g**c**. | 2.241 | 0.0125 |
| ryg**c**g**cb** | ...u.ga | NA | NA | ...u.**c**a | 1.784 | 0.0372 |

**Figure 1:** Manhattan plots of composite statistic for each species. The genes associated with a significant GO term identified for a particular species are mapped to the plot and presented in the color theme of the respective species. The three genes in black were identified as candidate genes in all three species.

A diagram of a number of colorful lines

AI-generated content may be incorrect.

**Figure 2: A**. Counts of candidate genes identified in the top 1% of windows of each species using the composite statistic. **B**. Change in allele frequency of CpG sites identified within the three genes identified within all three focal taxa, the three genes in the center of the venn diagram in *A*. Allele frequencies are encoded to show the frequency of the allele that contains the CpG pattern at a particular site rather than the alternative allele.

A diagram of different types of trees

AI-generated content may be incorrect.

**Methods**

**DNA Sequencing**

We accessed published whole genome sequence data (~9x coverage) from samples taken in the 1980s and 1990s from three species of Darwin’s finch: the medium ground finch (*Geospiza fortis,* N=10), vegetarian finch (*Platyspiza crassirostris,* N=5), and the small tree finch (*Camarhynchus parvulus*, N=12). These samples constitute our “pre invasion” treatment. We collected blood samples from these same species in 2019, (N=8, N=9, and N=7, respectively), extracted the DNA using Qiagen DNeasy kits [confirm extraction with Sabrina], and sequenced the whole genomes on Illumina … [get sequencing info from Sabrina] to an average depth of ~9x coverage. These samples constitute our “post invasion” treatment, and were taken 21 years or 3.5-7 finch generations after the fly was first recorded in an avian nest in 1998 (CITE).

**DNA Processing**

To assess and address potential batch effects between the genomic datasets in our pre and post treatments, we carefully followed guidelines for data processing to ameliorate batch effects outlined in Lou et al. (CITE). We also prioritized analyses that leverage genotype likelihoods for all of our analyses. For analyses of specific mutations in candidate genes, we report allele frequencies using genotype calls for ease of interpretability of results.

We trimmed adapters from the raw sequence fasta files using Trimmomatic. We then trimmed polyg tails with a minimum poly-g length of 10 in the program fastp. Following this, we trimmed for length and quality using Trimmomatic with the settings LEADING:20 TRAILING:20 SLIDINGWINDOW:4:20 MINLEN:90. We assessed sequence data using fastqc at each step.

We then aligned files using bowtie2 to the chromosome level *Camarhynchus parvulus* STF-HiC reference genome (1.28 GB;Genbank accession number: GCF\_901933205.1), converted the output to bam files, and sorted each bam file in samtools. We clipped overlapping read pairs using the function clipOverlap in bamUtil and we realigned around indels using gatk (3.7-0). Finally, we added read groups using the AddOrReplaceReadGroups function in picard. We then curated a list of SNPs for use in downstream analyses in angsd using the default SNP p-value cutoff of 1e-6, minimum quality and mapping quality scores of 30, and minimum minor-allele frequency of 0.05.

**DNA Analysis**

We assessed population structure of our samples using a principal component analysis implemented in PCAngsd, which confirmed species clusters and showed no bias by treatment. For all windowed analyses, we eliminated windows with either an average depth of coverage or average mapability below or above 2 standard deviations from the mean. We tested for selection between our two time points by analyzing FST and change in Tajima’s D (∆Tajima) across the genome using angsd. For each, we performed overlapping windowed analyses using 50,000 base pair windows with a step size of 12,500 base pairs. For FST, we directly compared pre- and post-invasion treatments within each species. We computed the difference in ∆Tajima by subtracting the value estimated in each window from the pre-invasion treatment from the value estimated in the post-invasion treatment. We computed average depth of coverage and mapability (cite snpable) for each window and then pruned out windows with depth greater or less than two standard deviations from the mean, or with mapability less than two standard deviations below the mean. These windows were pruned prior to the computation of outlier windows.

We first identified outlier windows under selection using a composite statistic of FST and Tajima’s D. A soft sweep is expected to drive relatively high FST values and reductions in Tajima’s D over time. For each species, we computed the Pearson’s correlation coefficient and found no evidence of a strong correlation between FST and ∆Tajima. We scaled and centered both statistics, took the inverse of ∆Tajima, and took the sum. This produced our composite statistic in which the highest values exhibit characteristics of an ongoing selective sweep. We conservatively took the top 0.1% of windows as candidate windows for selection, and we took the top 1% of windows for analysis of parallel evolution across taxa.

We identified the biological processes under selection by testing for overrepresented gene ontology (GO) terms in PANTHER. From the lists of the top 0.1% outlier windows from each analysis, we curated a list of genes that overlapped with part of any window using bedtools2. We curated background gene lists for each analysis that only included the genes within the reference genome’s GFF that overlapped with any window kept in each analysis after depth and mapability filtering, i.e. the genes that had the potential to be identified as an outlier. In PANTHER, we used *Gallus gallus* as our reference organism, performed the statistical overrepresentation test using the PANTHER GO-Slim Biological Process annotation dataset, and a Fisher’s Exact Test with no correction for multiple testing. We downloaded the full list of all GO terms and performed a Benjamini-Hochberg procedure to control for False Discovery Rate (FDR), keeping only terms with q<0.05. We visualized significant GO terms using the R package rrvgo, which produces a hierarchical clustering analysis of semantic similarities between G terms. These plots groups GO terms by similar meaning and visualizes them in a scatterplot similar to a PCA, with arbitrary and therefore unlabeled axes.

To test for evidence of parallel evolution across species, we curated a list of genes that overlapped with part of any window using bedtools2 from the less stringent lists of the top 1% outlier windows from each analysis. We performed a Fisher’s Exact Test pairwise between species on counts of genes and used a Benjamini-Hochberg procedure to control for False Discovery Rate (FDR) with a q-value threshold of <0.05. We exported lists of genes that were identified by more than one taxa and tested for overrepresented GO terms following the methods described above.

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