Molecular parallelism in signaling function across different sexually selected ornaments in a warbler

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Extravagant ornaments are thought to signal male quality to females choosing mates, but the evidence linking ornament size to male quality is controversial, particularly in cases in which females prefer different ornaments in different populations. Here, we use whole-genome sequencing and transcriptomics to determine the genetic basis of ornament size in two populations of a widespread warbler, the common yellowthroat (*Geothlypis trichas*). Within a single subspecies, females in a Wisconsin population prefer males with larger black masks as mates, while females in a New York population prefer males with larger yellow bibs. Despite being produced by different pigments in different patches on the body, the size of the ornament preferred by females in each population was linked to numerous genes that function in many of the same core aspects of male quality (e.g., immunity and oxidative balance). These relationships confirm recent hypotheses linking the signaling function of ornaments to male quality. Furthermore, the parallelism in signaling function provides the flexibility for different types of ornaments to be used as signals of similar aspects of male quality. This could facilitate switches in female preference for different ornaments, a potentially important step in the early stages of divergence among populations.

sexual selection | ornament | parallelism | bird

Ornaments are often thought to signal male qualities, such as health and vigor, that may increase the fitness of females choosing mates. However, the evidence for a connection between ornaments and the quality of males is mixed, and it remains unclear if females in different populations prefer males with ornaments that signal similar aspects of quality (1). Several hypotheses have been proposed at the genetic level to link the production of sexually selected ornaments to honest information about male quality. These hypotheses (2–4) define male quality in relation to genes controlling core cellular processes, including cellular respiration and oxidative balance, growth, and immunity. Thus, we expect genes related to these processes to be associated with the production of larger or more elaborate sexually selected ornaments, although their influence is likely to be indirect, if these traits are controlled by many genes.

The preferences of females for particular male ornaments can vary across populations and time (5, 6), providing an important driver for divergence and reproductive isolation among populations (1, 7). In theory, female preferences for an ornament can shift to a new ornament, if the newly preferred ornament is an equally informative signal of male quality (8). For example, birds often possess multiple colors in their plumage, including black and brown produced by melanins, and yellow and red produced by carotenoids. Potentially either type of pigment can signal male quality (9), but how each pigment type might relate to core cellular processes underlying male quality, and if they are the same processes, remains to be established (3).

Here we determine if two different male plumage ornaments provide signals of male quality in terms of core cellular functions, and if those functions are similar in two populations that differ in the ornament preferred by females. Males of the common yellowthroat (*Geothlypis trichas*), a widespread North American warbler, possess two sexually selected ornaments, a black facial mask and a yellow breast ("bib"). Females in a Wisconsin (WI) population prefer males with larger black masks as mates, while females in a New York (NY) population prefer males with larger yellow bibs (10). Birds in these two populations belong to the same subspecies (*G. t. trichas*), have low genetic divergence (mean F_{ST} = 0.013) (11), and have similar mean bib sizes, although the mask is 5% larger and 6% more variable in size in WI than in NY (10). Despite being produced by different pigments (eumelanin in mask and lutein in bib) (12), both ornaments signal similar aspects of male quality in their respective populations. For example, survival, resistance to oxidative stress, antibody production, and allelic diversity at the major histocompatibility complex (MHC) are all positively correlated with mask size among WI males (13, 14) and with bib size or color among NY males (11, 13, 15, 16). Here we focus on the size of ornaments, because size is the primary target of mate choice in aviary experiments (10) and it is correlated with both social and extrapair mating success

Significance

Ornaments are thought to signal the genetic quality of males to females choosing mates, but the qualities they signal and why they sometimes vary between populations are poorly understood. Here, we show, within a single warbler species, that two plumage ornaments signal similar aspects of male quality (e.g., immunity and oxidative balance) at the level of genetic functions, even though the ornament preferred by females differs between populations, involves different body parts, and is produced by different pigments (melanins and carotenoids). This parallelism at the functional level provides the flexibility for different types of ornaments to be used as signals of similar aspects of male quality, allowing for switches in female preferences between ornaments and potentially facilitating divergence and speciation.

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(15, 17). Although the bib in NY and mask in WI are correlated with several of the same physiological processes, it is not known if the same genes are involved in producing these different ornaments in different populations (i.e., if there is parallelism at the molecular level). We used both whole-genome sequencing and transcriptomics to determine if the same core genetic functions are associated with the preferred male trait in their respective populations, as predicted by theoretical models for divergence in mating preferences (8, 18).

Results

Ornament Size Is Highly Polygenic. We found that ornament size was related to many genes that were widely distributed throughout the genome (Fig. 1 and *SI Appendix*, Table S1). Using a whole-genome analysis comparing pooled samples of DNA (pool-seq) of males with large and small ornaments (top and bottom 25th percentiles; $n = 35$ to 47 males per pool; *SI Appendix*, Figs. S1 and S2), we found up to 256 genes associated with significant F_{ST} outlier peaks. We defined outlier peaks as those that contained at least two single-nucleotide polymorphisms (SNPs) with a genome-wide significance level of $P < 5 \times 10^{-8}$ (19) (Table 1 and Dataset S2). We also examined the genetic architecture of ornament size variation using SNPs in messenger RNA (mRNA) transcripts obtained from developing feathers. These transcripts were from individual males ($n = 25$ males in NY, 23 males in WI; *SI Appendix*, Fig. S2 and Dataset S4), in contrast to the pooled DNA samples. Based on these transcripts, 72 to 76% of the variation in ornament size was explained by all of the SNPs, while 44 to 47% of the variation was due to large-effect SNPs, implying a substantial proportion of the variation in ornament size was controlled by many genes of small effect [based on a Bayesian sparse linear mixed model (20); *SI Appendix*, Table S2]. We also found that the proportion of variance in the ornaments explained by SNPs increased with chromosome size (*SI Appendix*, Fig. S3 and Table S2), as expected if a trait is polygenic (21).

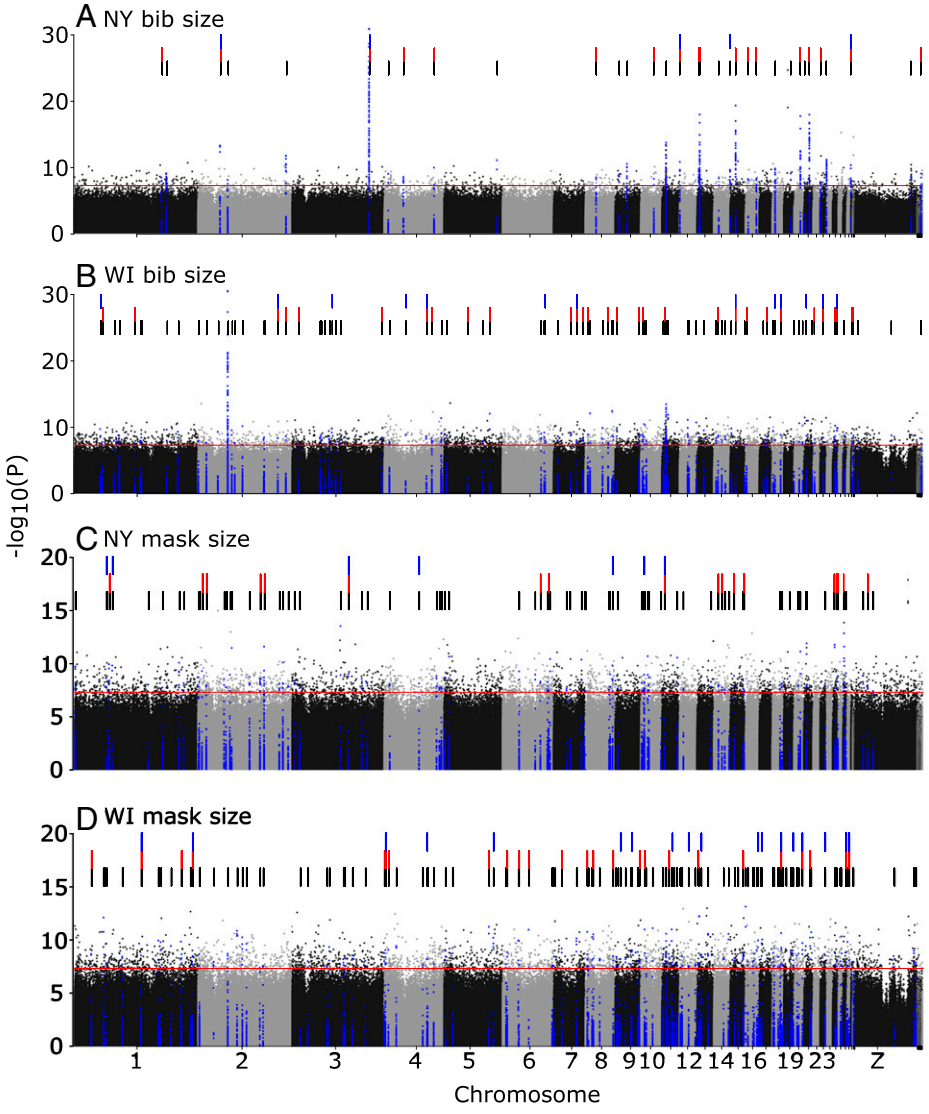

Fig. 1. Manhattan plots of genomic divergence between large and small ornament size pools. Fisher's exact test $-\log_{10}(P)$ values are shown for F_{ST} comparisons of bib (*A* and *B*) and mask (*C* and *D*) size in NY (*A* and *C*) and WI (*B* and *D*). Sample sizes were 35 to 47 males per pool. Red lines indicate a genome-wide significance of $-\log_{10}(P > 5 \times 10^{-8})$. Blue points and black vertical lines indicate the position of outlier peaks (identified by GenWin) with at least two SNPs. Note that all SNPs under the divergence peaks are colored blue for clarity, including SNPs below the significance threshold. Colored vertical lines above the Manhattan plots indicate the position of outlier peaks with immunity- (red) or oxidative balance– (blue) related functions (defined by the GO terms listed in Table 1). The largest peak in *A* has been truncated for space [$-\log_{10}(P) = 34.7$].

Table 1. Number of genes related to immunity, oxidative balance, and growth (GO terms) in outlier peaks

Category/GO term	No. of reference genes in GO term	Feather ornaments			
		Preferred		Nonpreferred	
		NY bib	WI mask	WI bib	NY mask
No. of outlier peaks		80	213	204	182
No. of genes in outlier peaks		111	257	200	161
Immune function					
Immune system process (GO:0002376)	3,171	25	32	40	20
Immune response (GO:0006955)	2,271	17	22	27	9
Innate immune response (GO:0045087)	926	7	8	10	2
Adaptive immune response (GO:0002250)	445	4	3	6	4
Antigen processing and presentation (GO:0019882)	234	6	5	4	2
Oxidative balance					
DNA repair (GO:0006281)	581	3	11	7	5
Response to oxidative stress (GO:0006979)	458	2	9	6	2
Cellular response to oxidative stress (GO:0034599)	307	2	4	4	2
Oxidative phosphorylation (GO:0006119)	147	0	1	3	0
Growth (IGF)					
IGF receptor signaling pathway (GO:0048009)	38	0	0	2	1

The numbers of genes within 25 kb of F_{ST} outlier peaks are from the pool-seq analysis of individuals with large and small ornaments (Fig. 1). The total number of genes in each GO term in humans is given for reference.

Ornament Size Is Related to Genes Signaling Male Quality. As predicted if ornaments signal core cellular functions, individual genes in the pool-seq peaks were related to immunity, oxidative phosphorylation, and response to oxidative stress (Fig. 1, Table 1, and Dataset S2), and they were found in 13 to 31% of outlier peaks (WI mask: 17.8%, 38/213; NY bib: 31.3%, 25/80; WI bib: 18.6%, 38/204; NY mask: 13.7%, 25/182). Additionally, protein-interaction networks constructed from genes in the outlier peaks were significantly enriched for the Gene Ontology (GO) terms "innate immune response" in NY bib (64 gene hits, 30.6 expected, false discovery rate [FDR]–adjusted $P = 1.3 \times 10^{-7}$) and "DNA repair and response to oxidative stress" in WI mask (19 gene hits, 5.95 expected, FDR-adjusted $P = 2.3 \times 10^{-4}$ and 10 gene hits, 3.09 expected, FDR-adjusted $P = 8.3 \times 10^{-3}$, respectively; Dataset S3). Genes regulating growth (e.g., insulin-like growth factor [IGF]) have previously been associated with the size of ornaments (22), but we found only a small number of these genes in outlier peaks, and they were only outliers in size comparisons of nonpreferred ornaments (NY mask, WI bib; Table 1). In addition to our specific hypothesis functions, we found that genes that differed between large and small ornaments were related to a diversity of other functions potentially affecting ornament quality, such as regulation of cell proliferation, growth, and development (*SI Appendix*, Table S3). Thus, there were individual genes related to immunity and oxidative balance associated with ornament size in all four comparisons, and these quality-related functions may be important in the protein interactions of female-preferred ornaments in particular.

Genes Related to Ornament Size Differ between Populations but Signal Similar Core Functions. There was little overlap of genes related to ornament size between populations or ornaments in the pool-seq analyses (Table 2). Even within the same ornament, the overlap of genes related to size was only 1 to 1.5% between populations (Table 2). Between populations, the overlap of genes from different ornaments ranged from 0.8 to 1.9% (Table 2). Nevertheless, randomization tests show that the overlap in genes, while low, was higher than expected (i.e., >95% quantile of 100 simulations) between NY bib and WI mask, the ornaments preferred by females, as well as between the bib and mask in WI (Table 2). Pairwise overlap of genes in the remaining comparisons was not different from random. Despite limited overlap between ornaments and populations at the level of individual genes, there was greater overlap at the level of function (36 to 44% overlap in GO categories for each gene list; Table 2). Greater overlap at the functional level is expected simply because these GO categories are broader and, in fact, we did not find more overlap of GO terms than expected by chance (Table 2). Nonetheless, genes underlying ornament size variation had considerably more overlap in their functional roles, and in all four comparisons there were GO functions related to immunity and oxidative balance (Table 1), indicating their potential importance to signaling male quality.

Molecular Parallelism of Ornaments Preferred by Females. If there is parallelism between ornaments in different populations in terms of the genes underlying their size, then we would predict similar patterns of gene expression in each population, despite

Table 2. Overlap among ornaments in individual genes and enriched GO categories from the pool-seq analyses

Overlap in	Female-preferred	Nonpreferred	Same ornament,different populations		Different ornaments,same population	
	NY bib vs.WI mask	NY mask vs.WI bib	NY bib vs.WI bib	NY mask vs.WI mask	NY bib vs.NY mask	WI bib vs.WI mask
Individual genes	1.9% (7/359)*	0.8%(3/356)	1.0% (3/306)	1.5% (6/410)	0.0% (0/270)	2.0% (9/446)**
Gene GO terms	38.3% (1,695/4,421)	39.6% (1,815/4,577)	37.2% (1,590/4,270)	40.6% (1,920/4,728)	36.2% (1,386/3,834)	44.5% (2,243/5,045)
Enriched networks	36.0% (63/175)	32.8% (58/177)	33.3% (59/177)	20.9% (41/196)	14.5% (30/207)	24.2% (46/190)

Percentages are based on the total number of unique genes, gene GO term membership, or significantly enriched interaction network GO:BP terms in the two ornaments in each comparison (total counts are in parentheses). Randomization tests indicated greater than expected overlap in comparisons marked with asterisks (i.e., >95% of 100 random trials). *95th percentile: 1.66% overlap; $P = 0.02$; **95th percentile: 1.82%; $P = 0.02$.

occurring in different ornaments. To test for parallel patterns of gene expression, we first filtered potential candidate genes based on a nominally significant slope (either positive or negative at $P < 0.05$) between ornament size and gene expression (normalized transcript counts; Dataset S6). Here, we assumed that genes underlying revealing ornaments will be significantly expressed in both populations (Fig. 2; see *SI Appendix*, Fig. S4 for an example with the MHC gene BLB2), so a gene had to be significantly related to an ornament in both populations to be included in this analysis (but see below). As a consequence, sample sizes differed between comparisons (Fig. 2; $n = 43$ to 98 per test). We did not apply an FDR correction at this stage, because we were simply filtering candidate genes prior to our test of parallelism. The test of parallelism was based on a χ^2 test of the number of similar (both slopes positive or both negative) or dissimilar (positive slope in one ornament or population and a negative slope in another) slopes in each comparison of ornaments and populations (Fig. 2 *A*–*D*). As expected if there were parallelism for preferred ornaments, we found that there were more genes with similar, rather than dissimilar, slopes in a comparison of preferred ornaments (bib in NY, mask in WI; Fig. 2*D*). There was no similar size-related pattern of gene expression in any of the other comparisons (Fig. 2 *A*–*C*). The genes with similar slopes in preferred ornaments had multiple functions and interactions, including immune system function (e.g., MHC class II, BLB2), oxidative phosphorylation (ND5), and DNA repair (TERF1), but in this case no genes were related specifically to growth (IGF) (Fig. 2*E*).

We also conducted a similar analysis using genes that were significantly related to ornament size (mask or bib) in one population but not the other ($n = 3{,}668$), because it is possible that some genes may lose their relationship (or fail to gain one) with ornaments in one population. This analysis also revealed a significant positive relationship between the slopes for NY bib and WI mask ($F_{1,\ 3666} = 201.3$, $P < 0.0001$), indicating similar effects of these genes on ornaments (*SI Appendix*, Fig. S5). In contrast, we found nonsignificant relationships for WI bib slopes vs. NY mask and NY bib slopes (*SI Appendix*, Fig. S5), similar to Fig. 2, but a slightly positive slope for WI vs. NY mask slopes (standardized slope = 0.05, $F_{1,\ 3666} = 9.76$, $P = 0.002$). Thus, there was some similar directionality for the genes underlying mask size in each population, but it was not as strong as for the preferred ornaments (WI mask vs. NY bib; standardized slope = 0.22 in *SI Appendix*, Fig. S5). These results are interesting because the mask is sexually selected by male–male competition in both NY and WI [e.g., males with larger masks are dominant over other males in aviary experiments (11)] and, thus, we might expect mask size to also show some underlying relationship with male quality, although it is less consistent here than in the preferred-ornament comparison.

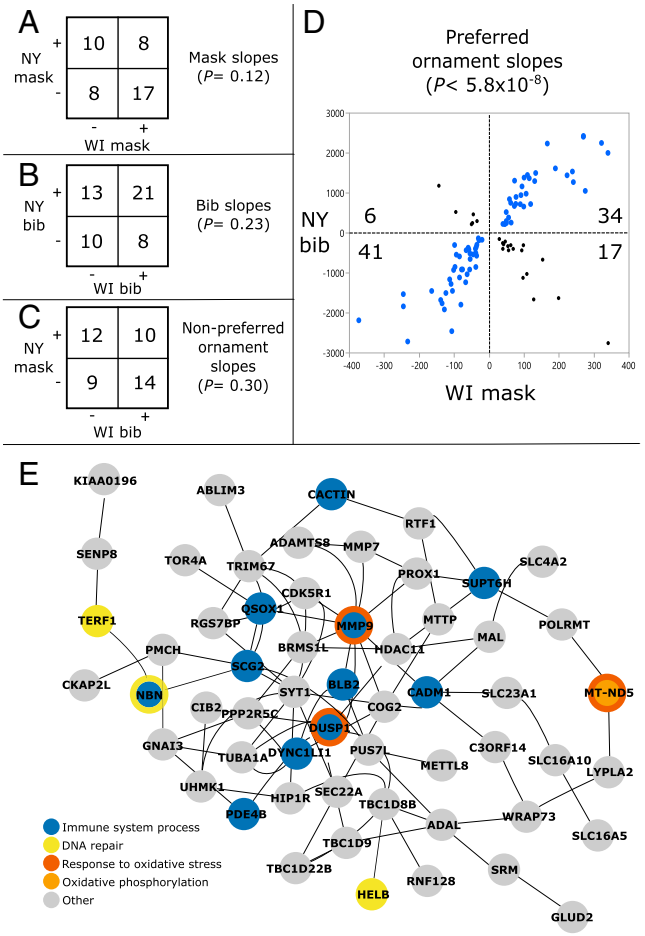

Fig. 2. Tests of parallelism of genes related to the size of sexually selected ornaments. Numbers in boxes indicate the number of ornament size and gene expression slopes that were similar (both positive or both negative) or dissimilar (different directions) for each combination of ornaments in NY and WI ($n = 224$ genes overall). The size-expression slopes between the same ornaments in different populations (*A* and *B*) and in nonpreferred ornaments (*C*) did not show any pattern of similarity. However, the slopes for preferred ornaments (*D*; NY bib and WI mask) were more often similar (both positive or both negative) than expected ($\chi^2 = 29.4$, $P = 5.84 \times 10^{-8}$). The gene network (*E*) shows the STRING interaction network for 63 annotated genes from the 75 congruent slopes (blue points in *D*). Select functional GO:BP categories are indicated in color.

Discussion

We demonstrate that the size of male ornaments preferred by females is linked to multiple genes that involve core cellular processes, confirming several hypotheses for the honest signaling of male quality (2, 4, 23). Furthermore, we show that the preferred ornaments in different populations signal some of the same core functions, despite being produced by different pigments. These patterns occur in both the whole-genome (pool-seq) and feather expression RNA-sequencing (RNA-seq) results, indicating that quality-related genes are expressed in the developing ornament and are also potentially heritable. Our previous studies demonstrated that the preferred ornament in each population (mask in WI, bib in NY) was correlated with multiple measures of male quality at the physiological level, but these new results provide evidence that the size of preferred ornaments is related to expression of numerous genes involved in quality-related functions, particularly immunity and oxidative balance. In contrast, we found limited evidence that genes associated with growth, particularly IGF (22), were related to ornament size. Our results also reveal that some of the same core genes are related to the size of both carotenoid- (bib) and melanin- (mask) based ornaments, confirming that both types of pigments can be used as signals of male quality. Overall, these results suggest that changes in female preference for an honest signaling trait can occur within a species if the new signal already has links to male quality at the genetic level.

Our previous studies indicated that the mask and bib of common yellowthroats were related to immunity, including MHC class II variation and immunoglobin G concentration (10, 24). Here we found MHC class II gene expression was positively correlated with the size of the preferred ornament in each yellowthroat population (*SI Appendix*, Fig. S4), supporting the hypothesis that females select the ornament that is the best indicator of resistance to pathogens in each population (4). However, we also found dozens of other immune genes linked to the size of each ornament through expression patterns or SNP frequencies (Table 1 and Dataset S2). This suggests that

ornaments are reflective of many aspects of the immune system and may not indicate resistance to specific pathogens as originally envisioned by Hamilton and Zuk (4). Instead, ornaments may signal a more general ability to resist infection or disease that also affects the ability to produce ornaments (25).

Resistance to oxidative stress provides one mechanism by which ornament expression could be linked to immunity (26, 27). Here, we found ornament size was correlated with SNPs and gene expression in oxidative phosphorylation genes (e.g., ND5), response to oxidative stress (e.g., DUSP1), and DNA repair (e.g., NBN), suggesting that more ornamented individuals are better able to mitigate oxidative stress through efficiency in oxidative phosphorylation, reduction in reactive oxygen species, or mitigation of oxidative damage to DNA (16) (Fig. 2 and Dataset S2). von Schantz et al. (26) predicted that individuals with lower variation at the MHC will recognize fewer pathogens, leading to prolonged immune responses, greater oxidative stress, and, ultimately, reduced expression of ornaments. Oxidative stress can interfere with melanin synthesis (28) and carotenoid deposition (29, 30) in ornaments. Therefore, higher-quality plumage ornaments can signal both genetic resistance to oxidative stress and better overall quality of the immune system. Alternatively, ornament production could be tied directly to oxidative phosphorylation, as occurs when carotenoid pigments are converted from yellow to red in a ketolation reaction in liver mitochondria (31). However, this mechanism is unlikely to explain the yellow carotenoid-based bib in common yellowthroats because the carotenoid in the blood (lutein) is deposited into the feather with little modification (12).

Across multiple analyses, we found a considerable number of genes related to ornament size similar to other continuous traits in birds, such as beak size (32, 33). The polygenic control of ornaments is consistent with the genic capture model of sexual selection (34), which suggests that ornaments remain useful indicators of male quality, despite strong selection from female choice, because many genes affect fitness and this allows ornaments to maintain genetic variance. Our results are also consistent with an omnigenic model in which quantitative variation in a complex trait is associated with many interconnected pathways outside the "core" genes that directly produce the trait (i.e., pigmentation genes in this study) (35, 36). Under this type of genetic architecture, genes involved in immunity and oxidative balance have an indirect role in producing ornaments and are not necessarily predicted to be a majority of the candidate genes (i.e., they may not be enriched). These results stand in contrast to several previous studies in birds (37, 38) and fish (39) that have found only a few pigmentation genes control the production of discrete patches of color that distinguish closely related species. This difference in genetic architecture might suggest that more genes are involved in the production of ornaments used for mate choice, which often exhibit continuous variation, than in the production of discrete traits, which are often involved in species recognition (37, 40). However, it remains to be determined whether this is simply a difference between continuous and discrete traits, or a more fundamental difference between ornaments that covary with condition (and thus undergo genic capture) and other types of signaling traits.

Despite the large number of genes associated with ornament size, there was very little overlap between ornaments in the individual genes associated with size (Table 2). This might be surprising if we assume that trait size is controlled by the same genes in different populations. However, the genic capture model proposes that traits under sexual selection will be controlled by many genes of small effect (34), which can potentially allow independently evolving populations to converge on a similar quality-indicating ornament using different sets of genes (41).

Our results revealed molecular parallelism in the signaling function of ornaments at two levels. First, we found that different ornaments, while using largely different sets of genes, show overlap in function, including functions linked to male quality as predicted by genic capture models (Tables 1 and 2). Second, we show limited, but significant, parallelism in gene expression in female-preferred ornaments, including genes with these same quality-related functions (Fig. 2). The parallelism between the yellow bib in NY and black mask in WI might be unexpected, given that melanins and carotenoids are produced by independent biochemical processes; however, prior studies indicate that both pigments have independent pleiotropic links to cellular processes like immunity and oxidative stress (42), which could produce parallelism at a functional level. Thus, parallelism in functions relating to male quality may give these two different pigments the flexibility to both be used as honest signals of similar aspects of male quality. This flexibility in species with multiple ornaments could facilitate the divergence of choice and signals across populations, and ultimately has the potential to lead to the initial stages of speciation.

Materials and Methods

Sample Collection and Measurements. We sampled common yellowthroats from 2005 to 2019 in Saratoga Springs, NY, and Saukville and Eagle, WI (see *SI Appendix* for details). All animal care use was approved by the Institutional Animal Care and Use committees at Skidmore College (protocol 163) and the University of Wisconsin–Milwaukee (protocols 11–12 #33, 15–16 #42, 17–18 #31). We obtained morphometric measurements, blood samples for pooled DNA analysis (n = 35 to 47 males per pool), and feather samples for mRNA analysis (n = 23 in WI, 25 in NY) from males caught with mist nets on their breeding territories. Feather samples were obtained in August and September from males that were simultaneously molting mask, bib, and back feathers (molt occurs prior to migration; see *SI Appendix* for details). We focused on sampling feather tips because melanin is produced in the feather tip (43), and lutein, which produces the yellow bib, is incorporated into the feather relatively unmodified, unlike some other carotenoids (12, 31). It is possible that ornament size is also influenced by gene expression in other tissues, but that was beyond the scope of our study.

Ornament Measurements. Ornament (mask and bib) size was estimated from photographs (*SI Appendix*, Fig. S1) of captured males by tracing the outlines of the bib and mask (both sizes summed) in ImageJ (https://imagej.nih.gov/ij/) (*SI Appendix*). Repeatabilities of these measurements are >0.90 for different pictures and different persons performing the measurements (15, 44).

DNA Extraction and Pooled Sequencing. We constructed a total of eight genomic DNA pools for WI and NY yellowthroats. Within each population, we designed four pools of individual males. Two large and small mask size pools were composed of individuals in the top and bottom 25% of mask size in the population but which did not differ in mean bib size (*SI Appendix*, Fig. S1). Two large and small bib size pools were composed of individuals in the top and bottom 25% of bib size in the population but which did not differ in mean mask size. Individuals were only used in a single pool. We extracted DNA from blood, quantified the concentration using NanoDrop and Qubit fluorometers, and then combined equal amounts of DNA from each individual to create pools (*SI Appendix*). The pooled samples were sequenced to 30× coverage on an Illumina HiSeq by Novogene.

RNA Extraction and Sequencing. RNA was extracted from feathers after they were first homogenized using a TissueLyser (*SI Appendix*). RNA samples were checked for quality and concentration on an Agilent BioAnalyzer (RNA Integrity Number > 8.4) and Qubit fluorometer. Complementary DNA libraries were prepared at the University of Wisconsin Biotech Center or at Novogene with the TruSeq v3 Stranded mRNA Sample Preparation Kit (Illumina) and then sequenced on an Illumina HiSeq 2000.

Reference Genome and Annotation. A chromosome-level reference genome of the common yellowthroat (GCA_009764595.1) was constructed by the G10K-Vertebrate Genomes Project (PRJNA589703) with 52× coverage (*SI Appendix*). We annotated the reference genome using the MAKER pipeline (v2.31.10) (45) (*SI Appendix*). This produced a final total of 18,740 gene annotations, of which we assigned identities to 17,695 genes using BLAST (Dataset S1).

Pool-Seq Filtering and Analysis. After sequencing the DNA pools, we removed Illumina adapter sequences and trimmed low-quality and short reads (*SI Appendix*). These steps resulted in the retention of an average 96% of read

pairs per pool. We aligned paired and trimmed reads to the common yellowthroat reference genome using bwa mem with default settings (v0.7.17; http://bio-bwa.sourceforge.net/bwa.shtml) (46). Next, we dropped reads with a quality score below 20 and converted the SAM files to BAM format using SAMtools (v1.7) (47). These steps resulted in aligned pools with an average genomic read depth of 40.6×. We used PoPoolation2 (v1.201) (48) to compute F_{ST} and Fisher's exact test values for individual SNPs between ornament pools (*SI Appendix*). SNPs were considered significant if they exceeded a genome-wide threshold of $-\log(P < 5 \times 10^{-8})$ from the Fisher's exact test. We used spline-based windows that contained at least two significant SNPs to define peaks of divergence between pools (*SI Appendix*). Candidate genes for analysis were those genes in or within 25 kb of a peak (Dataset S2).

RNA-Seq Filtering and Analysis. For each RNA-seq sample, we used HISAT2 (v2.1.0) (49) to map reads to the common yellowthroat genome. Prior to analyses, we performed adapter removal and quality filtering as described above. This resulted in an average decrease of 9.6% of reads per sample. We used the BAM files from the HISAT2 alignment and the GTF file from MAKER to construct a gene count file [using the featureCounts command in the R package Rsubread (50); *SI Appendix*]. We further removed any genes that had fewer than 10 counts total, resulting in a final gene count file with 17,835 putative genes (Dataset S5).

Analysis of Parallelism. For the analysis of molecular parallelism in different populations and ornaments, we compared the slopes of the relationship between ornament size (mask or bib) and gene expression in the same feather type between ornaments and populations. We estimated gene expression using the normalized counts (log10-transformed) produced by DESeq2 (51). Slopes of the relationship between ornament size and gene expression were estimated using generalized linear models with ornament size as the response, gene expression in the ornamental feather (mask or bib [log-transformed]) as the main predictor and sequencing batch, and expression in the back feathers (log-transformed) of the same individual as covariates to control for batch effects and individual variation in expression in nonornamental (back) feathers. We then filtered potentially relevant genes by retaining those with positive or negative slopes (nominal $P < 0.05$) in both NY and WI for a particular ornament comparison, as shown in *SI Appendix*, Fig. S4. The number of these slopes in both populations was then tabulated and compared to test for parallelism with χ^2 tests as shown in Fig. 2.

Interaction Network Analysis. We constructed protein–protein interaction networks for candidate gene lists produced by the pool-seq analyses using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) interactome database with humans for the reference species and default settings (900 confidence score cutoff and requiring experimental evidence) in NetworkAnalyst 3.0 (52). We used a protein interaction network (built using STRING), in contrast to pathway models like the Kyoto Encyclopedia of Genes and Genomes, because these networks incorporate interactions that are often not included in a specific pathway. These interactions are particularly relevant in our study because different types of quality-related genes may be interacting in a protein network that are not necessarily included in a pathway model. We used the minimum network tool in NetworkAnalyst, which adds the minimum number of additional genes necessary to connect the input genes into a single interaction network. Functional enrichment of GO:BP (biological process) terms (from http://geneontology.org) in each resulting network was tested using the human reference and hypergeometric tests as implemented in NetworkAnalyst.

Number of Genes Associated with Ornament Size. We scanned the transcriptome for SNPs associated with ornament size using a Bayesian sparse linear mixed model in the GEMMA package (20). These SNPs were obtained from transcripts in developing feathers and were discovered following the GATK (v4.2.1.0) (53) RNA-seq short-variant discovery pipeline (*SI Appendix*). For each ornament, we ran 10 independent runs of the model, each with 5 million burn-in runs followed by 20 million iterations. The proportion of variation explained and the number of large-effect SNPs were estimated by the median of the hyperparameters from all runs (*SI Appendix*, Table S2).

Data Availability. Raw sequencing reads from pooled whole-genome and RNA-seq analyses have been deposited in the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) (PRJNA734331). All other study data are included in the article and/or supporting information.

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1. E. S. Scordato, L. B. Symes, T. C. Mendelson, R. J. Safran, The role of ecology in speciation by sexual selection: A systematic empirical review. *J. Hered.* **105** (suppl. 1), 782–794 (2014).
2. D. J. Emlen, I. A. Warren, A. Johns, I. Dworkin, L. C. Lavine, A mechanism of extreme growth and reliable signaling in sexually selected ornaments and weapons. *Science* **337**, 860–864 (2012).
3. G. E. Hill, Condition-dependent traits as signals of the functionality of vital cellular processes. *Ecol. Lett.* **14**, 625–634 (2011).
4. W. D. Hamilton, M. Zuk, Heritable true fitness and bright birds: A role for parasites? *Science* **218**, 384–387 (1982).
5. A. S. Chaine, B. E. Lyon, Adaptive plasticity in female mate choice dampens sexual selection on male ornaments in the lark bunting. *Science* **319**, 459–462 (2008).
6. A. M. Siepielski *et al.*, The spatial patterns of directional phenotypic selection. *Ecol. Lett.* **16**, 1382–1392 (2013).
7. S. P. Turbek *et al.*, Rapid speciation via the evolution of pre-mating isolation in the Iberá seedeater. *Science* **371**, eabc0256 (2021).
8. Y. Iwasa, A. Pomiankowski, The evolution of mate preferences for multiple sexual ornaments. *Evolution* **48**, 853–867 (1994).
9. S. C. Griffith, T. H. Parker, V. A. Olson, Melanin- versus carotenoid-based sexual signals: Is the difference really so black and red? *Anim. Behav.* **71**, 749–763 (2006).
10. P. O. Dunn, L. A. Whittingham, C. R. Freeman-Gallant, J. DeCoste, Geographic variation in the function of ornaments in the common yellowthroat *Geothlypis trichas*. *J. Avian Biol.* **39**, 66–72 (2008).
11. L. A. Whittingham, P. O. Dunn, C. R. Freeman-Gallant, C. C. Taff, J. A. Johnson, Major histocompatibility complex variation and blood parasites in resident and migratory populations of the common yellowthroat. *J. Evol. Biol.* **31**, 1544–1557 (2018).
12. K. J. McGraw, M. D. Beebee, G. E. Hill, R. S. Parker, Lutein-based plumage coloration in songbirds is a consequence of selective pigment incorporation into feathers. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **135**, 689–696 (2003).
13. P. O. Dunn, J. C. Garvin, L. A. Whittingham, R. Freeman-Gallant, D. Hasselquist, Carotenoid and melanin-based ornaments signal similar aspects of male quality in two populations of the common yellowthroat. *Funct. Ecol.* **24**, 149–158 (2010).
14. A. E. Henschen, L. A. Whittingham, P. O. Dunn, Oxidative stress is related to both melanin- and carotenoid-based ornaments in the common yellowthroat. *Funct. Ecol.* **30**, 749–758 (2015).
15. C. R. Freeman-Gallant *et al.*, Sexual selection, multiple male ornaments, and age- and condition-dependent signaling in the common yellowthroat. *Evolution* **64**, 1007–1017 (2010).
16. C. R. Freeman-Gallant *et al.*, Oxidative damage to DNA related to survivorship and carotenoid-based sexual ornamentation in the common yellowthroat. *Biol. Lett.* **7**, 429–432 (2011).
17. K. J. Thusius, K. A. Peterson, P. O. Dunn, L. A. Whittingham, Male mask size is correlated with mating success in the common yellowthroat. *Anim. Behav.* **62**, 435–446 (2001).
18. D. Schluter, T. Price, Honesty, perception and population divergence in sexually selected traits. *Proc. Biol. Sci.* **253**, 117–122 (1993).
19. J. Fadista, A. K. Manning, J. C. Florez, L. Groop, The (in)famous GWAS *P*-value threshold revisited and updated for low-frequency variants. *Eur. J. Hum. Genet.* **24**, 1202–1205 (2016).
20. X. Zhou, P. Carbonetto, M. Stephens, Polygenic modeling with Bayesian sparse linear mixed models. *PLoS Genet.* **9**, e1003264 (2013).
21. J. Yang *et al.*, Genome partitioning of genetic variation for complex traits using common SNPs. *Nat. Genet.* **43**, 519–525 (2011).
22. I. A. Warren, H. Gotoh, I. M. Dworkin, D. J. Emlen, L. C. Lavine, A general mechanism for conditional expression of exaggerated sexually-selected traits. *BioEssays* **35**, 889–899 (2013).
23. G. E. Hill, J. D. Johnson, The mitonuclear compatibility hypothesis of sexual selection. *Proc. Biol. Sci.* **280**, 20131314 (2013).
24. L. A. Whittingham, C. R. Freeman-Gallant, C. C. Taff, P. O. Dunn, Different ornaments signal male health and MHC variation in two populations of a warbler. *Mol. Ecol.* **24**, 1584–1595 (2015).
25. D. F. Westneat, T. R. Birkhead, Alternative hypotheses linking the immune system and mate choice for good genes. *Proc. Biol. Sci.* **265**, 1065–1073 (1998).
26. T. von Schantz, S. Bensch, M. Grahn, D. Hasselquist, H. Wittzell, Good genes, oxidative stress and condition-dependent sexual signals. *Proc. Biol. Sci.* **266**, 1–12 (1999).
27. R. E. Koch, C. C. Josefson, G. E. Hill, Mitochondrial function, ornamentation, and immunocompetence. *Biol. Rev. Camb. Philos. Soc.* **92**, 1459–1474 (2017).

28. I. Galván, C. Alonso-Alvarez, The expression of melanin-based plumage is separately modulated by exogenous oxidative stress and a melanocortin. *Proc. Biol. Sci.* **276**, 3089–3097 (2009).
29. C. Alonso-Alvarez, I. Galván, Free radical exposure creates paler carotenoid-based ornaments: A possible interaction in the expression of black and red traits. *PLoS One* **6**, e19403 (2011).
30. M. J. Simons, A. A. Cohen, S. Verhulst, What does carotenoid-dependent coloration tell? Plasma carotenoid level signals immunocompetence and oxidative stress state in birds—A meta-analysis. *PLoS One* **7**, e43088 (2012).
31. G. E. Hill *et al.*, Plumage redness signals mitochondrial function in the house finch. *Proc. Biol. Sci.* **286**, 20191354 (2019).
32. M. Bosse *et al.*, Recent natural selection causes adaptive evolution of an avian polygenic trait. *Science* **358**, 365–368 (2017).
33. L. P. Lawson, K. Petren, The adaptive genomic landscape of beak morphology in Darwin's finches. *Mol. Ecol.* **26**, 4978–4989 (2017).
34. L. Rowe, D. Houle, The lek paradox and the capture of genetic variance by condition dependent traits. *Proc. Biol. Sci.* **263**, 1415–1421 (1996).
35. E. A. Boyle, Y. I. Li, J. K. Pritchard, An expanded view of complex traits: From polygenic to omnigenic. *Cell* **169**, 1177–1186 (2017).
36. G. Sella, N. H. Barton, Thinking about the evolution of complex traits in the era of genome-wide association studies. *Annu. Rev. Genomics Hum. Genet.* **20**, 461–493 (2019).
37. R. Price-Waldman, M. C. Stoddard, Avian coloration genetics: Recent advances and emerging questions. *J. Hered.* **112**, 395–416 (2021).
38. D. P. Toews *et al.*, Plumage genes and little else distinguish the genomes of hybridizing warblers. *Curr. Biol.* **26**, 2313–2318 (2016).
39. K. Hench, M. Vargas, M. P. Höppner, W. O. McMillan, O. Puebla, Inter-chromosomal coupling between vision and pigmentation genes during genomic divergence. *Nat. Ecol. Evol.* **3**, 657–667 (2019).
40. D. P. L. Toews, N. R. Hofmeister, S. A. Taylor, The evolution and genetics of carotenoid processing in animals. *Trends Genet.* **33**, 171–182 (2017).
41. N. Barghi, J. Hermisson, C. Schlötterer, Polygenic adaptation: A unifying framework to understand positive selection. *Nat. Rev. Genet.* **21**, 769–781 (2020).
42. A.-L. Ducrest, L. Keller, A. Roulin, Pleiotropy in the melanocortin system, coloration and behavioural syndromes. *Trends Ecol. Evol.* **23**, 502–510 (2008).
43. S. J. Lin *et al.*, Topology of feather melanocyte progenitor niche allows complex pigment patterns to emerge. *Science* **340**, 1442–1445 (2013).
44. J. C. Garvin, P. O. Dunn, L. A. Whittingham, D. A. Steeber, D. Hasselquist, Do male ornaments signal immunity in the common yellowthroat? *Behav. Ecol.* **19**, 54–60 (2008).
45. B. L. Cantarel *et al.*, MAKER: An easy-to-use annotation pipeline designed for emerging model organism genomes. *Genome Res.* **18**, 188–196 (2008).
46. H. Li, R. Durbin, Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* **26**, 589–595 (2010).
47. H. Li *et al.*, 1000 Genome Project Data Processing Subgroup, The sequence alignment/map format and SAMtools. *Bioinformatics* **25**, 2078–2079 (2009).
48. R. Kofler, R. V. Pandey, C. Schlötterer, PoPoolation2: Identifying differentiation between populations using sequencing of pooled DNA samples (pool-seq). *Bioinformatics* **27**, 3435–3436 (2011).
49. D. Kim, J. M. Paggi, C. Park, C. Bennett, S. L. Salzberg, Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat. Biotechnol.* **37**, 907–915 (2019).
50. Y. Liao, G. K. Smyth, W. Shi, The R package Rsubread is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads. *Nucleic Acids Res.* **47**, e47 (2019).
51. M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).
52. J. Xia, M. J. Benner, R. E. W. Hancock, NetworkAnalyst—Integrative approaches for protein-protein interaction network analysis and visual exploration. *Nucleic Acids Res.* **42**, W167–W174 (2014).
53. G. A. Van der Auwera, B. D. O'Connor, *Genomics in the Cloud: Using Docker, GATK, and WDL in Terra* (O'Reilly Media, Sebastopol, CA, 2020).