Shared transcriptional responses to con- and heterospecific behavioral antagonists in a wild songbird

Matthew I. M. Louder[1,2]*, Michael Lafayette[3], Amber A. Louder[1], Floria M. K. Uy[4], Christopher N. Balakrishnan[5], Ken Yasukawa[3], and Mark E. Hauber[1,]

[1]Department of Evolution, Ecology, and Behavior, School of Integrative Biology, University of Illinois at Urbana-Champaign, USA

[2]International Research Center for Neurointelligence, University of Tokyo

[3]Department of Biology, Beloit College, Beloit, WI, USA

[4]Department of Neurobiology and Behavior, Cornell University, Ithaca, NY, USA

[5]Department of Biology, East Carolina University, Greenville, NC, USA

*Correspondence: mckimlouder@gmail.com

Abstract: The recognition of and differential responses to salient stimuli are among the main drivers of behavioral plasticity, yet, how animals evolve and modulate functional responses to novel classes of antagonistic stimuli remain poorly understood. We studied free-living male red-winged blackbirds (*Agelaius phoeniceus*) to test whether gene expression responses in blood are distinct or shared between patterns of aggressive behavioral responses directed at simulated conspecific versus heterospecific intruders. In this species, males defend territories against conspecific males and respond aggressively to female brown-headed cowbirds (*Molothrus ater*), a brood parasite that commonly lays eggs in blackbird nests. Both conspecific songs and parasitic calls elicited aggressive responses from focal subjects and caused a downregulation in genes associated with immune system response, relative to control calls of a second, harmless heterospecific species. In turn, only the conspecific song treatment elicited an increase in singing behavior and an upregulation of genes associated with metabolic processes relative to the two heterospecific calls. Our results suggest that aspects of antagonistic responses to both conspecifics and brood parasites can be based on similar physiological responses, suggestive of shared molecular and behavioral pathways involved in the recognition and reaction to both evolutionarily old and new enemies.

Key words: auditory recognition, biomarker, blood, host-parasite interactions, RNA sequencing, territory defense,

Introduction

Faced with a suite of relevant and irrelevant stimuli, animals must rapidly perceive, process, and decide whether and how behaviorally to respond to diverse cues [1]. Upon the recognition of salient stimuli, for example, a cascade of neurophysiological and motor responses can be engaged to facilitate and modulate a reaction [2]. Yet, how recognition systems adapt and enable responses to evolutionary novel stimuli remain poorly understood [3]. In particular, the timely and accurate recognition of heterospecific antagonists, such as parasites, predators, or competitors, typically have substantial fitness advantages [4]. Can evolutionarily established behavioral and physiological responses to conspecific competitors be co-opted in parallel to adaptively respond to heterospecific antagonists?

Hosts of avian brood parasites pay the costs of raising unrelated young, often with the additional expense of losing some or all of their own offspring [5]. Many hosts have evolved to combat brood parasitism by attacking adult parasites, abandoning parasitized nests, and/or rejecting parasitic offspring [6]. Anti-parasitic defenses can take categorically different responses from competitive (e.g. territorial defense with song and overt aggression against same-sex conspecific intruders) or anti-predatory behaviors, and can even be evoked by a partial suite of (visual or auditory only) sensory cues [7,8].

What constitutes the physiological basis of anti-parasite responses remains largely unknown in avian host-parasite systems [9]. For example, recent work in wild-caught juvenile male red-winged blackbirds (*Agelaius phoeniceus*; hereafter: redwings) found no differences in immediate early gene (IEG) expression levels within the auditory forebrain in response to the calls of adult female brood parasites (brown-headed cowbirds *Molothrus ater*; hereafter: cowbirds)

60 versus a harmless control species (mourning dove *Zenaida macroura*; hereafter: doves), whereas
61 responses were stronger to conspecific adult female calls [10]. In the wild, however, juvenile redwings
62 do not hold territories and may not (yet) have been exposed to parasitism by cowbirds. In contrast,
63 breeding adult male redwings are well known for territory defense and anti-brood parasite
64 aggression [11] (Fig. 1). The species-typical "conk-a-ree" song is a long-range "keep-out" signal [12,13];
65 and is the most common conspecific territory defense display of this species [14]. Male redwings also
66 respond strongly to parasitic female cowbirds by approaching and attacking the parasites [15], but
67 they respond little to harmless sympatric species. Therefore, we set out to test whether antagonistic
68 responses to conspecifics and parasites involve distinct or shared behavioral and physiological
69 responses in freely-behaving adult male redwings on their breeding territories.

70 Linking evolutionarily relevant and ecologically salient stimuli with their proximate
71 recognition system responses has been difficult in wild animals. This is because, beyond the well-
72 known complexities of field work, lethal collection is required to sample neural tissues during or
73 following the undertaking of a recognition task. Furthermore, such terminal collection prohibits
74 repeated contrasts or ontogenetic comparisons within the same subjects. Alternatively, non-lethal
75 neuroimaging-based techniques, including functional Magnetic Resonance Imaging or micro
76 Positron Emission Tomography, require that subjects become captive during or soon after the
77 recognition task [16,17].

78 Transcriptomic analyses have become a critical tool to analyze functionally relevant cell
79 types and tissues both in model species and, increasingly in non-model species collected from the
80 wild [18,19]. Moreover, non-terminal collection, such as gene expression within peripheral, whole
81 blood of birds, may reveal functional parallels in the recognition of salient stimuli. For example, a
82 con- vs. heterospecific acoustic playback paradigm in captive zebra finches (*Taeniopygia guttata*)

found that gene expression patterns correlated for a subset of genes between the auditory forebrain and in peripheral blood [20].

Here we presented playback stimuli of unfamiliar conspecific, parasitic heterospecific, and control heterospecific vocalizations to free-living adult male redwings on their breeding territories, recorded their behavioral responses, then caught them to collect blood samples, and assessed peripheral gene expression patterns. Given the well-known behavioral repertoire of territorial male redwings [11], we expected them to respond to playback of conspecific song by increasing their own singing and approaching and remaining in proximity to the playback speaker when compared to playback of a harmless heterospecific song. We expected them to approach and maintain proximity to playback of parasite calls, but not to increase singing, in comparison to the harmless heterospecific. We also predicted distinct and parallel gene activation and behavioral patterns in response to conspecific vs. parasitic stimuli relative to controls.

Fig. 1. Male red-winged blackbird responding to model presentation of a stuffed female brown-headed cowbird. Photo credit: K. Yasukawa.

Results

Behavioral response to playbacks

We performed playbacks to 34 territorial male red-wings. Whether or not a subject was caught within 30 min. had no impact on its response behaviors statistically (all $z > -1.9$, $p \geq 0.05$), nor were there differences in behavioral responses between the 1st and 3rd playback trial segment (see Methods), therefore behavioral data from all subjects and territories in response to the playback paradigm were analyzed statistically ($n_{\text{redwing}} = 11$, $n_{\text{cowbird}} = 12$, $n_{\text{dove}} = 12$). However, transcriptomic data were only available for subjects caught within 30 min of its respective playback set's delivery (see below for sample sizes). Generalized linear mixed models, with playback type as the independent predictor, revealed that male redwings responses were significantly variable during active playback periods: number of songs $z = -2.82$, $p = 0.005$, number of approaches $z = -2.50$, $p = 0.01$, and nearest distance to the playback speaker ($z = -2.10$, $p = 0.04$), but not in the time spent near the playback speaker ($z = -0.90$, $p = 0.17$) (Fig. 2). Tukey-corrected post-hoc analyses revealed that subjects sang significantly more in response to Redwing than Cowbird ($p = 0.02$) or Dove ($p = 0.02$) playbacks and did not differ significantly in songs in response to Cowbird and Dove playbacks (p = 0.99). Territorial male redwings approached more frequently to Redwing versus Dove ($p = 0.005$) and Cowbird versus Dove playbacks ($p = 0.03$), but did not differ between Redwing versus Cowbird playback (p = 0.79). Territorial male redwings approached more closely to Cowbird than Dove playbacks ($p = 0.003$), but did not differ in response to Redwing vs. Dove ($p = 0.10$), nor to Redwing vs. Cowbird playbacks ($p = 0.38$).

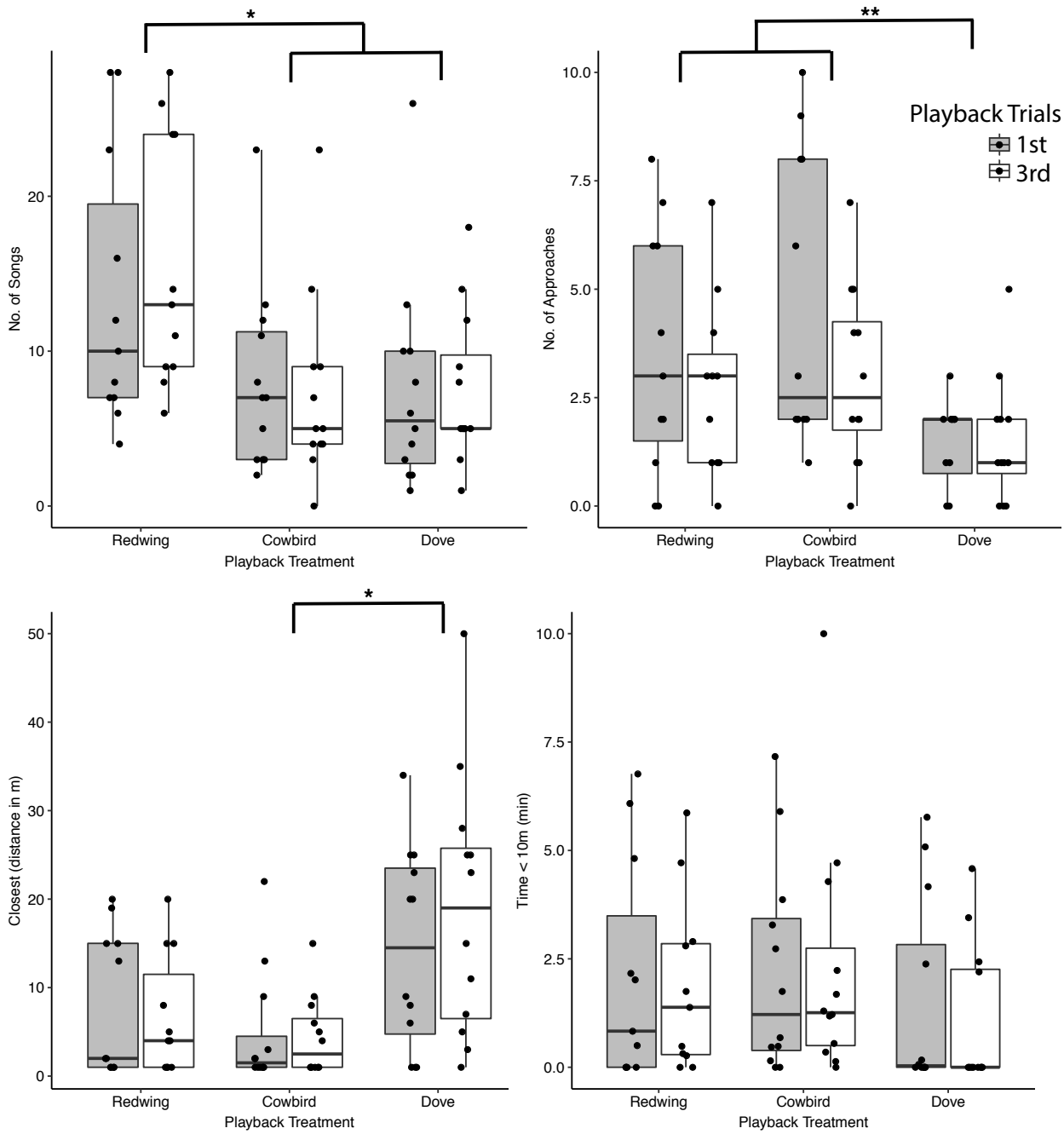


Fig. 2. Boxplots depicting the behavioral responses to playback type for male red-winged blackbirds in the 1st (grey boxes) and 3rd (white boxes) trials. Stars denote *: $p < 0.05$ and **: $p < 0.01$ between indicated groups.

126

127 *Gene expression*

128 We were able to extract enough RNA from the blood of 21 males upon experimental presentations
129 of playback treatments ($n_{redwing}$ = 6, $n_{cowbird}$ = 8, n_{dove} = 7). For these samples, we sequenced an
130 average of 15.9 million reads per sample (range = 11.7 – 19.1 million reads).

131 We first tested for differential gene expression for 20 candidate biomarker genes, previously
132 identified from peripheral (whole blood) in Louder et al. [20]. This analysis yielded no significant
133 differentially expressed genes in response to conspecific songs versus dove coo playback
134 treatments (all Bonferroni corrected *p*-values > 0.50).

135 After filtering for lowly expressed genes, we then incorporated the read counts of 7202
136 genes for co-expression analysis (WGCNA). Two modules were significantly correlated with
137 playback treatments (Figs. 3 and 4), the "dark-turquoise" module included upregulated genes in
138 response to Redwing song treatment (r = 0.52, p = 0.01) and the "yellow" module included genes
139 downregulation in response to both Redwing songs and Cowbird chatter relative to Dove coo (r =
140 0.64, p = 0.002).

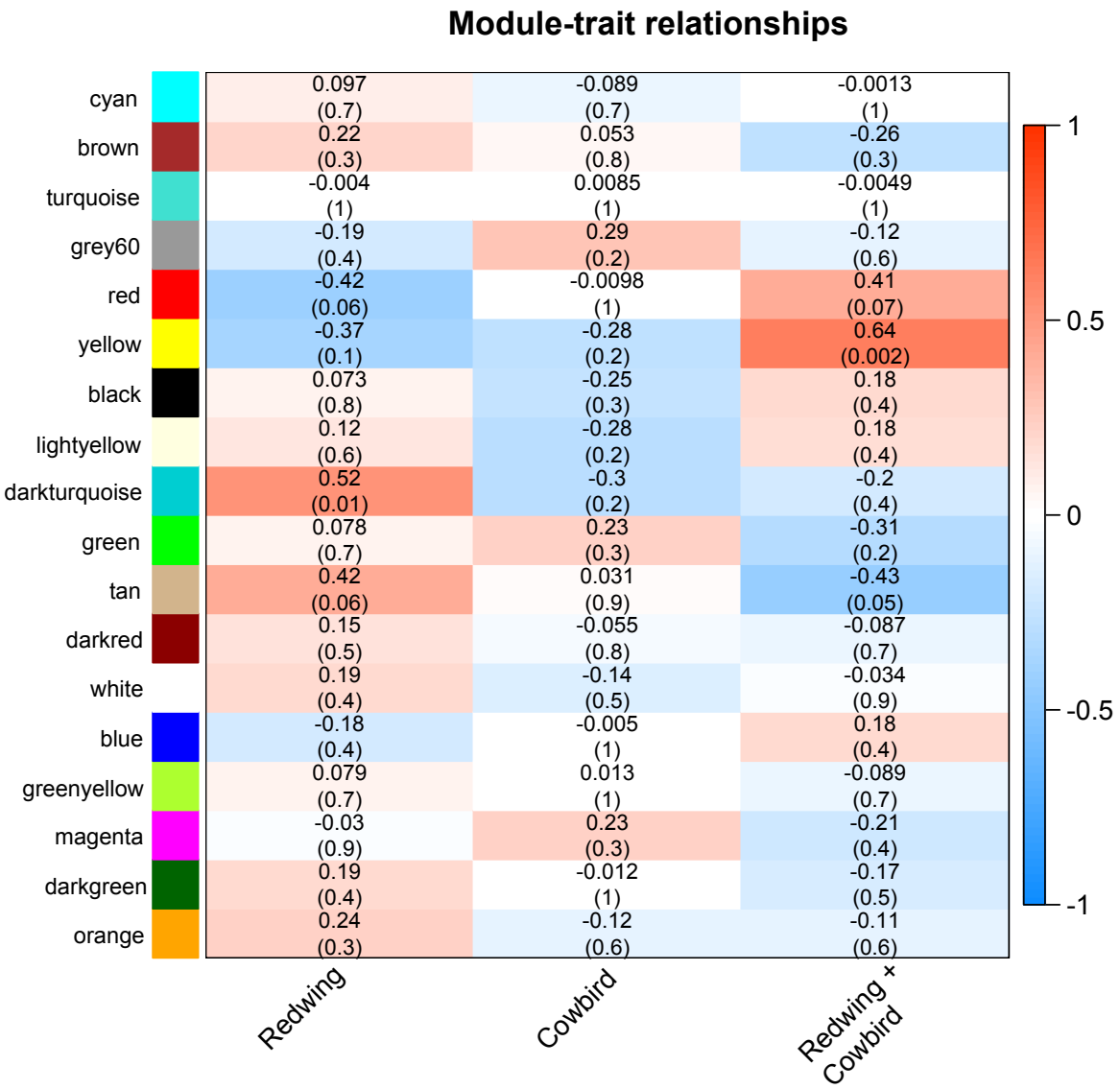


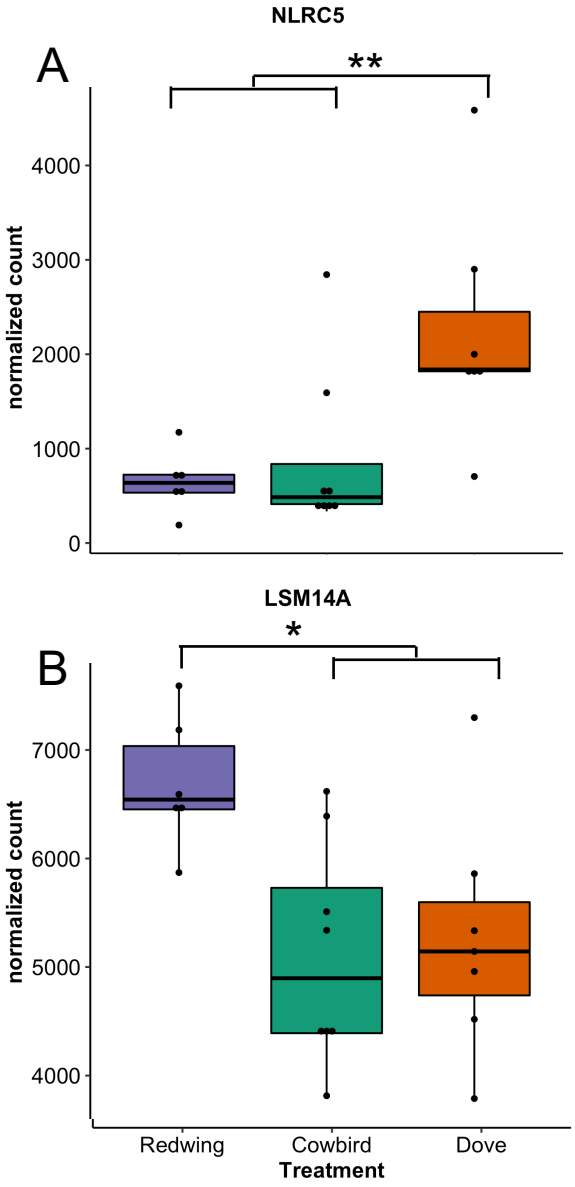
Fig. 3. Statistical associations between expression profiles of each WGCNA reconstructed modules and the playback groups. Presented are correlation coefficients and associated *p*-values (within brackets).

Using a rank-ordered approach, we identified an enrichment for gene ontology (GO) terms for each significant module. For the "dark-turquoise" module, correlated with a response to Redwing song playback, we found significant GO terms associated with the metabolism, regulation of gene expression, and protein ubiquitination (Table 1). For the "yellow" module, correlated with

150 a response to both Redwing song and Cowbird playback, we find significant GO terms associated

151 with immune system response, such as defense responses to virus and cytokine-mediated pathways

152 (Table 1).



153

154 **Fig. 4. The top gene's expression profiles from each of the (A) the "yellow" module, which**
155 **includes genes significantly downregulated in song and chatter treatments relative to dove**
156 **coo and (B) the "dark-turquoise" module, which includes genes associated with an**
157 **upregulation in response to the red-winged blackbird song treatment. Stars denote *: $p < 0.05$**
158 **and **: $p < 0.01$ between indicated groups.**

Discussion

Our expectations that subjects would sing in response to conspecific playback and approach, spend and perch close to redwing and cowbird playback were confirmed. We are therefore confident that we produced meaningful behavioral differences in aggressive responses of our subject male red-winged blackbirds. By studying free-living male territorial red-winged blackbirds, we detected patterns of co-expressed genes matching patterns of variation in aggressive behavioral responses to different classes of playbacks. In one behavioral response metric (approach distance) and in a co-expressed gene module, responses to conspecific songs and brood parasitic heterospecific calls were similar and both were significantly different from responses to harmless heterospecific calls. In turn, in a second behavioral response (number of songs) and another co-expressed gene module, responses to conspecific songs were significantly different from responses to both brood parasitic heterospecific and harmless heterospecific calls. Integrating the discriminability generated by these two patterns of responses provides for unique encoding for each of the three different playback types in both the behavioral and gene expression domains, separating conspecific songs from brood parasitic calls from harmless heterospecific calls.

Our results demonstrate that antagonistic responses to both conspecifics and brood parasites can involve similar physiological responses. The gene module correlated with both conspecific songs and cowbird chatters, relative to dove coos, was enriched for gene ontology immune system terms (Table 1), including defense responses to viruses and regulation of type I interferon production. For example, the top gene from the module, NLRC5 (NOD-like receptor family CARD domain containing 5 gene; Fig. 4), regulates adaptive immune responses against pathogens,

182 including the activation of MHC class I genes [21]. Unsurprisingly, these genes from the module were
183 downregulated relative to the control playbacks, perhaps as a trade-off between aggressive
184 responses and immune function seen elsewhere in avian and other vertebrate lineages[22]. Similarly,
185 luteinizing hormone decreases in male red-wing blackbirds in response to simulated territorial
186 intrusion [23]. However, the source of the mRNA expression in peripheral blood, such as
187 erythrocytes, leukocytes or exosomes [24], as well as the physiological function of these genes in
188 either immunosuppressive or enhancement of immune response to acute stress remains unclear.

189 Overall, the behavioral and gene expression data indicate that similar physiological
190 pathways may be involved in the cognitive recognition and motor responses to distinct antagonistic
191 threats, in this case conspecific and heterospecific intruders. Many host species of brood parasites
192 have evolved aggressive anti-parasitic behaviors [6], yet the cognitive and physiological mechanisms
193 involved in this behavioral evolution remain unclear [9]. Our study suggests that some of proximate
194 responses to conspecific intruders were co-opted in the evolution of anti-parasitic aggression
195 towards female cowbirds.

196 The gene expression responses found here in adult, territorial redwing males are different
197 from the IEG patterns detected from juvenile redwing males, which showed that the only
198 conspecific calls generated differential responses in the auditory forebrain relative to cowbird and
199 dove calls [10]. In turn, our behavioral data from adult red-winged blackbirds confirm previous
200 findings in this and other avian hosts of brood parasitic species, which demonstrated behavioral
201 responses to heterospecific parasitic models and auditory stimuli [15,25–27].

202 We did not find differential gene expression in a candidate set of potential biomarker genes,
203 previously identified from peripheral whole blood in a conspecific vs. harmless heterospecific
204 (dove) acoustic playback paradigm in captive female zebra finches [20]. Given that our experimental

205 treatments in the present study also included conspecific (redwing songs) vs. irrelevant
206 heterospecific (dove) comparisons, we predicted that we would detect some of these same potential
207 biomarker genes to be differentially expressed in free-living redwings. However, the use of
208 different study species (zebra finches vs. red-winged blackbirds) or the different sexes of our
209 subjects (female finches vs. male blackbirds) likely contributed to our inability to identify
210 consistent gene expression differences within whole blood for conspecific recognition in songbirds.
211 In conclusion, our study demonstrates a parallel in behavioral and gene expression
212 responses to simulated antagonistic threats. In particular, we find support for physiological
213 responses involved in conspecific territorial aggression co-opted in the evolved recognition of
214 heterospecific brood parasites. In addition, our study further demonstrates the utility for peripheral
215 gene expression to study avian recognition and behavioral responses to social stimuli.
216
217
218 **Methods**
219 *Study Species and Study Area*:
220 We studied red-winged blackbirds at Newark Road Prairie in Rock County, Wisconsin, USA (42°
221 32' N, 89° 08' W) during the breeding season of 2018. Newark Road Prairie is a 13-ha wet-mesic
222 remnant prairie and sedge meadow habitat that supports about 35 male redwing territories [28]. All
223 males were captured in Potter traps baited with non-viable sunflower seeds and were banded with
224 United States Geological Survey numbered aluminum bands and unique color combinations of
225 plastic wraparound bands for individual identification (USGS permit # 20438 to KY); most females
226 at this study site were not banded.

227 We used observations of territorial behaviors to place additional seed-baited traps within
228 breeding territories of potential subjects and allowed them to use the traps without being captured
229 for 1–3 weeks prior to playbacks. Previous work at this site on redwings' responses to model
230 cowbirds vs. harmless heterospecifics, coupled with their respective call playbacks, showed
231 strongly graded aggressive responses to the former relative to the latter [15].
232
233 *Playbacks:*
234 We presented one of three playback types at each territory: (1) male conspecific songs ("Redwing";
235 highly salient), (2) female cowbird chatter calls ("Cowbird"; salient heterospecific vocalization of
236 a brood parasite of redwing nests), or (3) dove coo ("Dove"; non-salient vocalizations of a harmless
237 sympatric heterospecific) for broadcasts. Given that male red-winged blackbirds exhibit greater
238 behavioral responses to female vs. male cowbird models [15], the chatter call, a specific call of female
239 cowbirds was chosen to acoustically simulate brood parasite intrusion. To address pseudo-
240 replication [29], one out of five available exemplars were assigned at random for each playback type.
241 Audacity v 2.2.0 was used to filter playback stimuli above 2000 Hz and below 500 Hz, and
242 normalize mean amplitude of all stimuli. Acoustic stimuli were matched in peak amplitude and
243 duration.
244 Playback types and exemplar files were randomly assigned to territorial male redwings
245 using a stratified balanced design to keep sample sizes per type similar. For each playback type we
246 randomly chose one of the exemplars to broadcast from an iPhone 5 or 6 (Apple Inc., Cupertino,
247 California, USA) connected to an Ecoxgear ECOXBT speaker (Grace Digital Audio, Peterborough,
248 Ontario, Canada) via a 30-m auxiliary cable. Playbacks were broadcasted at 80–85 dB SPL at 1 m

249 from the source (as measured by a sound pressure meter: Pyle PSPL01, Pyle Audio Inc., Brooklyn,
250 New York, USA), which approximated natural amplitude (KY personal observations).

251 We used a 30 min. paradigm to induce (differential) gene expression (e.g., [20]), but to avoid
252 habituation in the field, each playback consisted of three 10-min segments for a total period of 30
253 min. The first and third segments were active sound broadcast periods and the second segment was
254 a 10-min silent period. Each active period consisted of 10 1-min sub-segments in which an
255 exemplar played at 0, 10, 20, 30, and 40 s, followed by 20 s of silence. As soon as the 30-min
256 playback was completed, we removed the playback equipment and baited and set the trap; we aimed
257 to capture each subject within 30 min of the end of the playback, banded all previously unmarked
258 subjects as described above, took a blood sample of approximately 100 µL (see below), and
259 released the subject. We recorded the time to capture for all samples obtained within the 30-min
260 maximum.

261 Prior to playback, we placed two markers each 10 m from the speaker to facilitate measuring
262 proximity time to the speaker within 10 m. During each 10-min segment we recorded (1) number
263 of songs (songs), (2) number of flights towards the speaker (approaches), (3) time spent within 10
264 m of the speaker (time in proximity), and (4) the distance (m) of the closest approach (closest
265 approach). These behavioral variables are well known to indicate a male redwing's aggressiveness
266 [30]. We analyzed behavioral data collected during the 1st and 3rd 10-min periods, which preceded
267 the capture timepoint by up to 30 min and, thus, is representative of the time sampled for the gene-
268 expression patterns, too. Number of songs and number of approaches were the totals for periods 1
269 and 3 (active playback). Time (min) within 10 m was the total time for the active playback periods.
270 Closest approach was the shortest distance between the subject and the speaker during the two
271 active playback periods.

272 We used generalized linear mixed models with a negative binomial response and individual
273 male identity as a random effect with glmmTMB in R (version 3.5.1) to analyze the responses of
274 male redwings to the three playbacks. Each model included playback treatment, trail period, and
275 whether the male was captured as explanatory variables. A significant result was further examined
276 with a Tukey post-hoc analysis to identify significantly different pairs of treatment. The alpha level
277 was set at the $p < 0.05$.
278
279 *RNA extraction and sequencing*:
280 A blood sample from each subject was collected from the brachial vein using a 1.27 cm 27 g needle
281 and heparinized capillary tube (Fisher Scientific, Pittsburgh, Pennsylvania, USA). Blood was
282 placed in 500 µl of RNAlater and then stored in a −80°C freezer for processing. RNA was extracted
283 with RiboPure blood kits (Life Technologies, Carlsbad, California, USA) and treated with DNAse
284 for purification. We assessed the quality of purified RNA on a Bioanalyzer (Agilent, Wilmington,
285 Delaware, USA) (RIN > 7.0).
286 All library preparations and sequencing were performed at the University of Illinois at
287 Urbana-Champaign Roy J. Carver Biotechnology Center, Urbana, IL, USA. A library for each
288 sample was prepared with an Illumina TruSeq Stranded RNA sample prep kit. All libraries were
289 pooled, quantitated by qPCR, and sequenced on one lane of an Illumina HiSeq 4000 with a HiSeq
290 4000 sequencing kit version 1, producing single-end 100 bp reads. Fastq files were demultiplexed
291 with bcl2fastq v 2.17.1.14 (Illumina).
292
293 *Preparation of reference genome:*
294 Lacking an annotated reference genome for the red-winged blackbird, we created a proxy reference

295 following the pseudo-it pipeline (https://github.com/bricesarver/pseudo-it). Briefly, we extracted
296 DNA from liver and muscle tissue of a male red-winged blackbird (cataloged at the Museum of
297 Southwestern Biology MSB:Bird:40979) and performed paired-end (200 bp) whole-genome
298 sequencing on one lane of HiSeq (2500) at the Duke Genome Center. After removing the Illumina
299 adapters with Trim Galore! v 0.3.7
300 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), which incorporates Cutadapt v
301 1.7.1 [31], we aligned the DNA reads to the closest related species publicly available, the white-
302 throated sparrow (*Zonotrichia alibicolis*) genome (assembly Zonotrichia_albicollis-1.0.1), with
303 BWA-mem [32]. Using GATK [33] we then identified and inserted red-winged blackbird SNPs into the
304 white-throated sparrow reference genome. To improve the proxy reference genome, we performed
305 an additional iteration of the pseudo-it pipeline.
306
307 *Gene expression*:
308 We removed Illumina adapters from RNA reads with Trim Galore! v 0.3.7. We then aligned the
309 reads to the proxy reference genome with Hisat2 [34] and quantified read abundance with HTSeq-
310 count [35]. With DeSeq2 [36], we then included the playback treatments and time to capture (minutes)
311 to analyze the gene expression patterns of the 20 top genes identified from the parallel expression
312 patterns of peripheral (whole blood) RNA-sequencing study of Louder et al. [20].
313 Next, we sought to identify networks of genes specifically responsive to Redwing song,
314 Cowbird chatter or both Redwing and Cowbird relative to Dove coo. We performed weighted gene
315 co-expression network analysis (WGCNA), which is used for finding clusters (modules) of highly
316 correlated genes and determine the relationship of modules to treatments. We used the WGCNA
317 package in R (Langfelder & Horvath 2008) to identify modules of co-expressed genes in our

318 dataset. To remove genes with low read abundance, we filtered for genes with < 1 count per million
319 in at least 10 samples. We then normalized for read-depth and extracted variance stabilizing
320 transformed (vst) read counts from DEseq2 into WGCNA. To build the co-expression matrix, we
321 chose a soft thresholding power (β) value of 12, at which at which we observed a plateau in Mean
322 Connectivity, thus representing a scale-free topology [37]. We generated a signed network with
323 minimum module size of 30 genes and merged highly correlated modules (dissimilarity threshold
324 = 0.25). We then correlated the eigengene, which is the first principal component of a module, of
325 these merged modules with playback treatments (Redwing, Cowbird, Dove). Modules with $p \leq$
326 0.05 were considered significantly correlated with a given trait.

327 Finally, we tested for functional enrichment of gene ontology (GO) categories with GOrilla
328 [38]. For each module, genes were ranked based on their module membership score determined in the
329 WGCNA analysis. We preferred this rank-order based approach (as opposed to strict module
330 assignment) as it reflects the correlation among modules, and because some genes could be
331 assigned to multiple modules [39]. GOrilla performs ranked-order analyses with human gene IDs, so
332 we identified orthologous genes from the annotated red-winged blackbird proxy-genome.
333 Statistical significance of GO categories was determined with *p*-values corrected for multiple
334 hypothesis testing (*FDR* < 0.05). We used REVIGO to remove redundant and overlapping GO
335 categories, with an allowed semantic similarity measure of 0.5 [40].

336

337 **Data Accessability**

338 The data that support the findings of this study are being submitted to NCBI, and will be available
339 prior to publication.

340

341 **Author contributions**

342 M.I.M.L., K.Y., F.M.K.U. and M.E.H. designed the project; M.L., K.Y. collected data and
343 samples; A.A.L. collected laboratory data, M.I.M.L., C.B. and M.E.H. analyzed the data; M.I.L,

and M.E.H. wrote the first draft, and all authors provided critical feedback, reviewed, and edited the manuscript. All authors approve the manuscript.

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Additional Information

Competing Interests: The authors declare no competing interests.

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450 **Table 1. Significant GO terms associated with "dark-turquoise" module, specific to**
451 **conspecific song playback and "yellow" module, shared responses for conspecific song and**
452 **cowbird chatter playback. FDR represents the *p*-value adjusted for false discovery rate.**

Significant GO terms associated with "yellow" module

term_ID	description	FDR
GO:0051607	defense response to virus	3.16E-05
GO:0060759	regulation of response to cytokine stimulus	0.006
GO:0032480	negative regulation of type I interferon production	0.01
GO:2000042	negative regulation of double-strand break repair via homologous recombination	0.04
GO:0009607	response to biotic stimulus	0.04

GO terms associated with "dark-turquoise" module

term_ID	description	FDR
GO:0019219	regulation of nucleobase-containing compound metabolic process	3.90E-05
GO:0044265	cellular macromolecule catabolic process	3.94E-05
GO:0016567	protein ubiquitination	5.74E-05
GO:0070647	protein modification by small protein conjugation or removal	6.24E-05
GO:0044267	cellular protein metabolic process	0.001
GO:0043412	macromolecule modification	0.003
GO:0017148	negative regulation of translation	0.006
GO:0032482	Rab protein signal transduction	0.03

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