Immune system activation affects song and territorial defense

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Previous studies have demonstrated that bird song is influenced by infection. We investigated how mounting an immune response by mountain white-crowned sparrows (*Zonotrichia leucophrys oriantha*) affects specific aspects of territorial song and behavior. We used song playback to simulate a territorial intrusion and elicit baseline song and behavioral responses. Individuals were then either injected with a saline control or with a lipopolysaccharide (LPS) solution to induce an acute immune response, and posttreatment territorial song and behaviors in response to playback were recorded 1 day and 7 days later. The immune challenge significantly reduced the number of birds that sang compared with control individuals on Day 1. All birds that sang on Day 1 produced abnormal songs with fewer terminal notes, exhibited elevated alarm behavior, and tended to sing at slower rates. By Day 7, LPS treatment had no effect on the likelihood of singing, and song rates had returned to baseline levels, though songs were still sung with fewer terminal notes. Thus, our study shows that simply mounting an immune response to infection, as opposed to the actual infection per se is sufficient to impact avian song production. This study also suggests that for the most sensitive assessment of immune status, familiarity with an individual's song and behavior is necessary. *Key words:* acute phase response, bird song, immune system response, life-history trade-offs, lipopolysaccharide, territorial behavior, white-crowned sparrow, *Zonotrichia leucophrys.* [*Behav Ecol 21:788–793 (2010)*]

Despite an increasing number of studies assessing the impact of parasites and pathogens on the dynamics of free-living vertebrate populations (for reviews, see Wobeser 1994; Hudson et al. 2002; Combes 2004) and the evolution of vertebrate life history (Hamilton and Zuk 1982; Poulin et al. 2000), many aspects of host–parasite interactions in nature are not well understood (but see Ilmonen et al. 2000; Garamszegi et al. 2004; Owen-Ashley et al. 2006). For example, it is not clear whether costs associated with infection are the result of energy and nutrients being diverted away by the pathogen or the outcome of resources committed to mount an effective immune response. Although infection per se can have dramatic effects on host physiology, recent laboratory investigations have also revealed the surprising cost of actual immune response either in terms of resources committed to responding to infections or because of autoimmune damage to the host's own tissue (McEwen et al. 1997; Norris and Evans 2000; Nilsson et al. 2007).

Because mounting an immune response requires diverting energy and resources away from other functions, one would expect repercussions on multiple aspects of life history including reproduction and investment in soma (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000; Martin 2005; reviewed by Zuk and Stoehr 2002). Bacterial infections are probably the most common challenge that a vertebrate's immune system faces. Lipopolysaccharide (LPS) is a key component of the outer membrane of gram-negative bacteria and is largely responsible for activating the acute phase response

(Rietschel et al. 1994). In house sparrows (*Passer domesticus*), injection with LPS, which mimics a systemic bacterial infection and elicits a generalized sickness response, results in reductions in body mass, depressed parental feeding rates, and decreases in reproductive success (Bonneaud et al. 2003), thus providing support for a trade-off between immune investment and reproductive activity (Ilmonen et al. 2000). LPS was found to affect song-producing structures (Jacot et al. 2005) and song energetics (Fedorka and Mousseau 2007) in male crickets. Worker bees injected with LPS were shown to have increased mortality (Moret and Schmid-Hempel 2000) and reduce the fitness of the colony (Moret and Schmid-Hempel 2004).

Given the demonstrated plasticity in song production (Møller 1988; Hutchinson et al. 1993; Gil et al. 1999), one might expect a trade-off between mounting an immune response and investment in territorial song. Indeed, Gambell's white-crowned sparrows (*Zonotrichia leucophrys gambelii*) injected with LPS experienced elevated corticosterone levels and exhibited short-term hypothermia, depressed feeding, attenuated territorial aggression behavior, and decreased singing during the parental period of the breeding season (Owen-Ashley et al. 2006). We aimed to further understand the basis of behavioral responses to disease by exploring trade-offs between mounting an immune response and territorial song and behavior. We then explore possible repercussions of such trade-offs for inter- and intrasexual selection. Mountain white-crowned sparrows (*Z. l. oriantha*) are an ideal subject because their vocalizations are easy to record, quantify, and analyze (Morton 2002). If energy and resources to generate an immune response are acquired at the expense of territory and defensive behavior, then we would expect that LPS-treated individuals would reduce behaviors that are energetically costly. Because song rate and body reserves are positively related (Thomas 2002), the singing rate of LPS-treated individuals would be expected to decrease.

MATERIALS AND METHODS

Study sites and subjects

The study was conducted in 2 previously established mountain white-crowned sparrow study sites north of the Rocky Mountain Biological Laboratory, Gothic, CO (38°95′N, 106°98′W, 3150 m above sea level). The sites measured approximately 200 by 400 m and were 200–300 m apart. The habitat in both study plots consisted of interspersed patches of alpine meadow and willow (*Salix* spp.) thicket. Experiments were performed during the breeding season (June and July) when male territory defense was high (Morton 2002). Due to the invasive nature of the study, we aimed to use a small sample size of 6 LPS-treated and 6 control individuals per study site, and we made an a priori decision to set our $\alpha = 0.10$ so as not to miss weak but potentially important effects.

All mountain white-crowned sparrows captured on the study sites since 1999 have been banded on first capture with a metal US Fish and Wildlife Service band (Bird Banding Laboratory, Patuxent, MD) and given a unique combination of 3-colored bands for visual identification in the field.

Playback procedure and song recordings

Territories were identified through repeated early season visits on the study plots to determine locations and identities of singing males. Within 2 weeks of identifying territories, we simulated a territory invasion to obtain baseline territorial song and behavior.

Between 0600 and 1200 h, an iHome iH80 directional speaker (SDI Technologies, Rahway, NJ) was placed 1 m off the ground and within 10 m from the perch from where the male was observed singing. One of 4 white-crowned sparrow songs recorded in a previous year from nonresident males and normalized to a peak amplitude of 80 dB measured at 1 m (SPER Scientific 840029 digital sound level meter, accuracy ± 0.7 dB SPL, weighting level A, peak response) was played back from an iPod (Apple Corporation, Cupertino, CA) at a rate of 15 songs every 2 min. The song broadcast was stopped once the resident male was seen by the experimenter, at which time the subject's behavior was recorded for 5 min. All recordings were done using an Audix OM-3xb microphone (Audix Microphones, Wilsonville, OR) onto a Tascam DA-P1 digital audio tape recorder (Tascam, Montebello, CA). Songs were recorded 5–10 m from the focal subject to avoid alarming the individual. Only those males that sang during the baseline playback procedure were included in the study. An individual's singing bout was recorded for a minimum of 2 min to obtain 10 songs with minimum background noise. This playback procedure was repeated for each individual on Day 1 and Day 7 after the injection of either LPS or a saline control.

Song and behavioral measurements

The first 2 min from each singing bout before treatment, and on Days 1 and 7 after treatment, were analyzed using Raven Pro v.1.3 (Cornell Laboratory of Ornithology, Ithaca, NY). We counted the number of terminal notes per song from spectrograms and calculated song rate (songs per minute) from the first 2 min of each recording of a singing bout.

We assessed territory defense behavior by measuring rates of 1) attacking the speaker, 2) flights directly above the speaker, 3) alarm calls ("chink" or "pink" calls—see Morton 2002) produced, and 4) flights greater than 1 m in distance surrounding the playback speaker. Because approaches to speakers have been interpreted as aggressive responses to playback (Slater and Catchpole 1990; Leitao and Riebel 2003), behaviors (1) and (2) were interpreted as direct acts of aggression and behaviors (3) and (4) were interpreted as lower energy alarm behaviors. These behaviors were dictated onto a tape recorder and subsequently analyzed using JWatcher v.1.0 (Blumstein and Daniel 2007).

Trapping and LPS administration

Millet-baited potter traps were used between 0600 and 1200 h to capture recorded individuals. Individuals were captured within 2 weeks of baseline recordings. It should be noted that birds were trapped once in order to administer the treatment injection, at which time morphological measurements were made. On capture, each bird was weighed; cloacal protuberance, left wing, left tarsus, and tail lengths as well as crown and crown stripe were measured. Age was determined from banding history (birds are assumed to be 1 year old on first capture). Birds were alternately administered the treatment or control injection based on capture order. Birds assigned to the immune challenge treatment were subcutaneously injected with approximately 0.2 ml of LPS (resulting in a dose of 0.8 mg/kg tissue) derived from *Escherichia coli* (serotype 055:B5, Sigma product code: L4005) dissolved in saline and emulsified at a 1:1 v/v ratio in Freund's incomplete adjuvant (Sigma Product Code: F5506). This dose is similar to doses used in previous passerine studies (Bonneaud et al. 2003; Owen-Ashley et al. 2006). Injected LPS constitutes an effective target for the immune system, and its injection mimics a disseminated bacterial infection precipitating a brief but generalized sickness syndrome (Adler et al. 2001). Addition of adjuvant to LPS has been shown to extend the acute phase response of white-crowned sparrows in captivity for up to 48 h after treatment (Owen-Ashley et al. 2006). Control individuals received a subcutaneous injection of a saline solution of equal volume (0.2 ml).

Statistical analysis

Age, mass, cloacal protuberance, left wing and tarsus lengths, and crown and stripe widths for each treatment group were compared using Student's *t*-tests, assuming unequal variances, and normality was checked using Shapiro–Wilk tests. We checked for any differential effects playback song on territorial response by performing an analysis of variance on baseline responses against playback song. We used Fisher's exact tests to determine if LPS treatment had a significant effect on whether birds sang during a territorial intrusion and, for each comparison, calculated the contingency coefficient, C, as an index of the effect size.

We then examined how behaviors changed over time after a treatment or control injection by calculating the difference between each individual's behavioral response at each particular time (Day 1 or Day 7) and their baseline response. Due to the small subset of LPS-injected birds that sang on Day 1 (see Results, Table 1), we decided to use Mann–Whitney U tests to compare these difference values. This after–before, control–treatment

Table 1

Number of birds that sang or did not sing during the 3 playback experiments

	Saline		LPS	
	Sang	Did not sing	Sang	Did not sing
Baseline	10	0	11	0
Day 1	10	0	3	8 (1)
Day 7	9	1	8	3 (2)

The numbers in parentheses indicate the number of birds that failed to respond to playback.

comparison is the best way to account for individual variability and isolate the effect of the treatment on subsequent behavior. Due to Type II errors commonly associated with small sample sizes, we also used the standardized difference, Cohen's *d* (calculated with the pooled standard deviation), as a measure of effect size for mean comparisons (Cohen 1988). Cohen suggests that large, moderate, and small magnitudes of difference correspond to *d* values of 0.8, 0.5, and 0.2, respectively.

RESULTS

Treatment groups did not differ in mass, cloacal protuberance, left wing and tarsus lengths, and crown and stripe widths (all data are normally distributed and $P > 0.5$). We obtained quality baseline song recordings from 43 out of the 53 males identified on the study plots. Twenty-one of these 43 males were then successfully trapped; 11 were administered LPS, and 10 were administered saline. Site 1 had 4 control and 7 treatment birds, and Site 2 had 6 control and 4 treatment birds (Table 2). During the Day 1 playback experiment, from those individuals administered LPS, 9 responded to playback but only 3 of those sang. In contrast, all 10 control-treated individuals sang during the Day 1 playback. During the Day 7 playback, 8 out of the 9 LPS-treated individuals that responded to the playback sang, whereas 9 out of the 10 control-treated birds that responded sang. Table 2 summarizes the number of birds that responded to playback and sang, responded to playback but did not sing, and did not respond to playback during baseline, Day 1, and Day 2 experiments. We found no significant effect of playback exemplar on baseline response for all measured behaviors (all *P* values > 0.20).

A summary of behavior responses to playback experiments for LPS- and saline-treated birds is shown in Table 3. We found significant effects of LPS administration when we compared the differences from baseline values across control and treatment (Table 4). No significant effects of LPS treatment were found for analyses that simply compared treatments and controls at the baseline period, Day 1, or Day 7 (Table 4).

When compared with baseline data, on Day 1, LPS-treated birds were significantly less likely to sing ($P = 0.001$, $C = 0.815$) (Table 2), sang fewer terminal notes per song

Table 2

Summary of treatment, capture site, bird mass, and LPS doses

Treatment	Site	Mass (g)	LPS dose (mg)
LPS	1	26.5	0.021
LPS	1	30.5	0.024
LPS	1	28.5	0.023
LPS	1	30.0	0.024
LPS	1	30.5	0.024
LPS	1	28.5	0.023
LPS	1	28.0	0.022
LPS	2	29.0	0.023
LPS	2	31.0	0.025
LPS	2	28.0	0.022
LPS	2	27.0	0.022
Saline	1	27.0	—
Saline	1	30.5	—
Saline	1	28.5	—
Saline	1	30.5	—
Saline	2	28.0	—
Saline	2	26.5	—
Saline	2	26.0	—
Saline	2	30.0	—
Saline	2	28.0	—
Saline	2	29.0	—

Table 3

Results (mean ± SD, *n*) of LPS injection or saline injection on white-crowned sparrows' territorial behavior and song

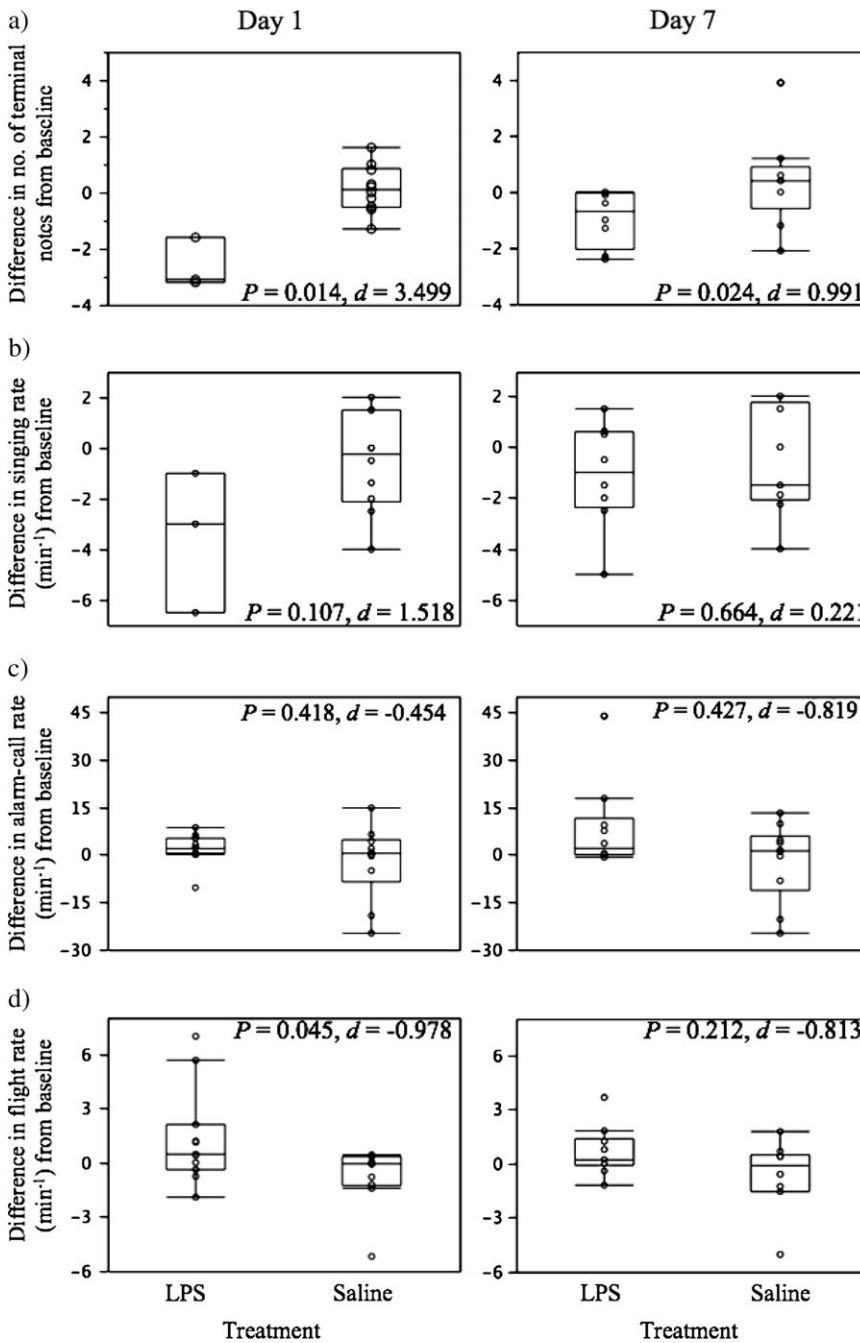|		Saline		LPS	
		Mean ± SD	*n*	Mean ± SD	*n*
Day 1	No. of terminal notes (song^{-1})	0.13 ± 0.85	10	−2.63 ± 0.896	3
	Song (min^{-1})	−0.54 ± 1.944	10	−3.5 ± 2.784	3
	Alarm call (min^{-1})	−2.12 ± 11.77	10	1.691 ± 4.859	11
	Speaker attack (min^{-1})	0 ± 0	10	−0.05 ± 0.106	11
	Flight (min^{-1})	−0.76 ± 1.689	10	1.351 ± 2.693	11
	Flyover (min^{-1})	−0.14 ± 0.33	10	−0.09 ± 0.749	11
Day 7	No. of terminal notes (song^{-1})	0.356 ± 0	9	−0.94 ± 0.063	8
	Song (min^{-1})	−0.67 ± 2.141	9	−1.11 ± 2.097	8
	Alarm call (min^{-1})	−2 ± 12.33	10	8.18 ± 13.85	10
	Speaker attack (min^{-1})	0 ± 0	10	−0.02 ± 0.063	10
	Flight (min^{-1})	−0.64 ± 1.888	10	0.624 ± 1.356	10
	Flyover (min^{-1})	0.002 ± 0.424	10	−0.24 ± 0.558	10

Mean and standard deviation (SD) values are of the difference values with respect to baseline behaviors.

($P = 0.014$, $d = 3.499$), and had increased flight rates ($P = 0.045$, $d = -0.091$) (Table 4, Figure 1). A low *P* and large effect size indicate that LPS-treated birds had a strong tendency to sing at a slower rate ($P = 0.107$, $d = 1.518$). From

Table 4

Results of behavior comparisons using Mann–Whitney *U* tests

		With baselines		Without baselines	
		P	*d*	*P*	*d*
Baseline	No. of terminal notes (song^{-1})	—	—	0.445	0.354
	Song (min^{-1})	—	—	0.318	−0.466
	Alarm call (min^{-1})	—	—	0.159	0.723
	Speaker attack (min^{-1})	—	—	0.178	−0.634
	Flight (min^{-1})	—	—	0.159	0.829*
	Flyover (min^{-1})	—	—	0.614	−0.231
Day 1	No. of terminal notes (song^{-1})	**0.014**	3.499*	0.286	0.928*
	Song (min^{-1})	0.107	1.518*	0.476	1.128*
	Alarm call (min^{-1})	0.418	−0.454	0.572	0.241
	Speaker attack (min^{-1})	0.189	0.634	—	—
	Flight (min^{-1})	**0.045**	−0.978*	0.307	−0.65
	Flyover (min^{-1})	0.769	−0.091	0.189	−0.507
Day 7	No. of terminal notes (song^{-1})	**0.024**	0.991*	0.229	0.592
	Song (min^{-1})	0.664	0.221	0.227	−0.61
	Alarm call (min^{-1})	0.427	−0.819*	0.998	−0.409
	Speaker attack (min^{-1})	0.368	0.471	0.368	−0.471
	Flight (min^{-1})	0.212	−0.813*	0.650	−0.291
	Flyover (min^{-1})	0.136	0.513	0.504	0.553

Significance values that are highlighted in bold indicate significant (<0.10) results; an asterisk denotes a strong trend ($d > 0.8$). Cohen's *d* effect size was calculated using the pooled standard deviation. Significant results were found only when accounting for individual variability by examining responses with respect to baseline behaviors ("with baselines" columns). A second analysis comparing control and treatment birds at a particular time ("without baselines" columns) yielded no statistically significant results.

Figure 1
The effect of LPS or saline injection on white-crowned sparrows' territorial behaviors. Mean changes from baseline levels on Day 1 (left) and Day 7 (right) of (a) number of terminal notes, (b) singing rate, (c) rate of alarm calling, and (d) flight rate. Each open circle is the value for an individual bird, the horizontal line marks the mean, the box ends indicate the 25th and 75th quartiles, and whiskers extend to the most extreme point lying within 1.5 times the interquartile range. P values calculated using Mann–Whitney U tests and Cohen's d are reported in each panel.

a qualitative perspective, all LPS-treated individuals that sang on Day 1 exhibited abnormal songs with respect to baseline spectrograms (Figure 2).

On Day 7, LPS treatment had no effect on whether or not birds sang ($P = 0.587$, $C = 0.220$) (Table 2), and the song rates of LPS-treated individuals recovered to baseline levels ($P = 0.664$, $d = 0.221$) (Table 4, Figure 1). Qualitatively, song spectrograms on Day 7 resembled those of baseline songs. We detected a lingering effect of LPS on the number of terminal notes in songs sung on Day 7 ($P = 0.024$, $d = 0.991$).

DISCUSSION

In contrast to past studies (e.g., Gilman et al. 2007) that revealed the impact of infection on song production, here we

actually show that simply mounting an immune response is sufficient to impact song behaviors. Because treatment groups did not differ in morphological or behavior characteristics prior to injections, we conclude that our results are due to an immunological challenge with LPS injection. The effects of the immunological challenge on territory defense were immediate and profound. As expected, song, a trait selected for by females and used in male–male interactions (Catchpole and Slater 2008), was the behavior most strongly impacted on Day 1 after an immune challenge. By Day 7, most behaviors returned to baseline levels, though lingering costs of mounting an immune response were still detected as a depression in the number of terminal notes and elevated alarm behaviors. The changes in terminal notes, flight rate, and song rate when coupled with the corresponding large effect sizes ($d > 0.8$)
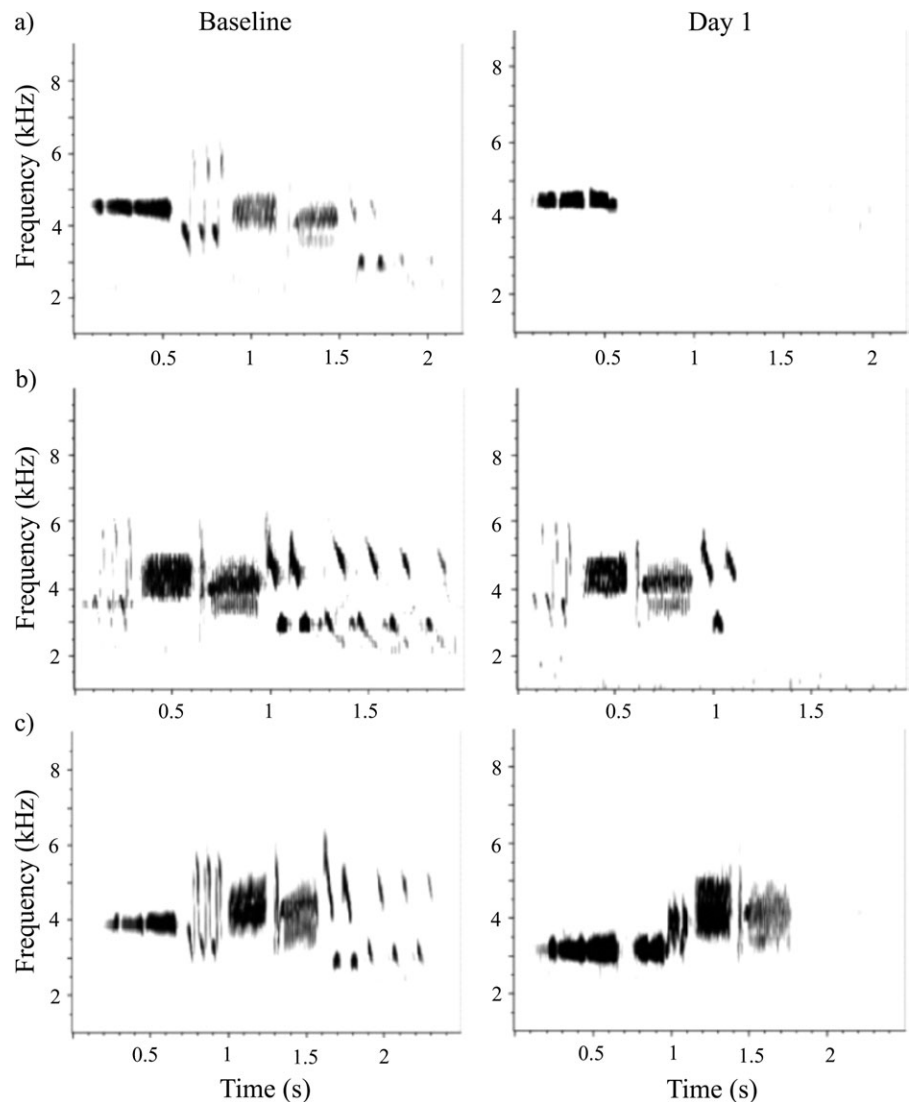
Figure 2
Representative spectrograms of songs from baseline recordings (left) and on Day 1 after an immune challenge (right) of each of the 3 white-crowned sparrow males that sang on Day 1. On Day 1, the focal male (a) only sang the introductory whistle of his song at the same frequency as his baseline whistle of about 4.5 kHz, (b) sang a song similar in structure and frequency to his baseline song but with fewer terminal notes, and (c) sang a song with no terminal notes that differed in structure and frequency from his baseline song.

(Cohen 1988) give us confidence in our results. However, it should be emphasized that due to the dramatic effect of the LPS treatment on the number of birds that sang on Day 1, the sample size of the data sets for number of terminal notes and song rate of LPS-treated birds was reduced to 3, thereby making it difficult to draw broad conclusions.

Surprisingly, 7 of the 11 immune-challenged birds that responded to the intrusion during the playback experiment on Day 1 did not sing but exhibited typical alarm behaviors, suggesting that sickness behavior has specific effects on song. Given any negative repercussions (e.g., decreased mating success) that may follow from communicating an impaired health status, the abstinence from singing that we observed on Day 1 may be a beneficial adaptation acquired by males.

This experimental study demonstrates that terminal notes of mountain white-crowned sparrows are exceptionally susceptible to immune challenge and are thus a potentially valuable indicator of condition. Although song, as a whole, is hypothesized to be a sexually selected trait and therefore condition dependent, few studies have demonstrated that particular portions of song are more affected by infection than others (but see Gilman et al. 2007). The number and quality of terminal notes in a song is associated with female preference (Vallet and Kreutzer 1995; Riebel and Slater 1998) and the

degree of male–male response (Galeotti et al. 1997; Nagle and Couroux 2000; Soha and Whaling 2002; Leitao and Riebel 2003), suggesting that terminal notes may be especially condition dependent. The fact that immune-challenged individuals reduced the number of terminal notes from their baseline on Days 1 and 7 dovetails with the result of Soha and Whaling (2002) in demonstrating that the number of endnotes is indeed an honest and sensitive signal of a singer's quality.

Perhaps, the most interesting result is that we only detected the effects of LPS injection by taking an individual's prior characteristics into account. This suggests that differences within a population are perhaps not as informative as changes in an individual's behavior over time. Thus, "changes" in an individual's singing behavior reflect changes in an individual's health status.

In conclusion, this study demonstrates that mounting an immune response alone, as opposed to infection with an actual pathogen, is enough to affect avian song. Mounting an immune response was found to particularly affect the production of terminal notes. Furthermore, evaluating changes in a male's song will be particularly informative, particularly for neighboring males and resident females that have previous experience with an individual's song.

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