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The Oxidative Cost of Acoustic Signals: Examining Steroid Versus Aerobic Activity Hypotheses in a Wild Bird

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Abstract

Vertebrate vocalizations are widespread secondary sexual signals used for mate attraction and territory defence, and variation in signal quality is often condition dependent and impacts reproductive outcomes. Although vocal signal performance is known to reflect various aspects of male quality, few studies have examined the underlying mechanisms mediating its costs and hence its honesty. Using a population of Arctic-breeding snow buntings (*Plectrophenax nivalis*), we compared the '*Oxidation Handicap Hypothesis*', which predicts that testosterone-induced increases in oxidative stress provide a direct mechanistic basis for ensuring the honesty of many secondary sexual signals, to the '*Aerobic Activity Hypothesis*, which predicts that it is the aerobic activity involved with signal production (i.e. vocal performance or defending a large territory) and not testosterone directly that links signal quality and oxidative stress. Males singing at faster rates had higher levels of both reactive oxygen metabolites and non-enzymatic antioxidant capacity in the plasma (i.e. without an increase in overall oxidative stress), enabling certain males to produce high-quality signals while also mitigating the costs of an associated increase in oxidative stress. However, these results were completely independent of plasma testosterone levels, supporting the role of aerobic performance in directly affecting oxidative stress. Although song performance was not linked to reproductive parameters in our data set, our research is the first to test these competing hypotheses in a behavioural trait and results suggest that oxidative stress may be an underlying physiological cost preventing low-quality individuals from producing high-quality signals.

Introduction

Many secondary sexual signals are thought to be condition dependent (Andersson 1994) and as such are assumed to be important components of mate choice by allowing females to assess the direct and indirect benefits males can provide (Garratt & Brooks 2012). Zahavi (1975) was the first to present the handicap principle to explain signal honesty; if signal production or maintenance imposes a cost, and this cost increases with expression, then more elaborate signals should indicate a male's ability to afford larger handicaps. Vocal behaviour (e.g. singing, roaring, chorusing) is a well-known sexual signal and an excellent model for examining the underlying mechanisms driving signal honesty (Vehrencamp 1999). Song rate is one aspect of vocal behaviour that appears important to female vertebrates (e.g. Radesäter et al. 1987; Nolan & Hill 2004; Murphy et al. 2008; Ward et al. 2013). In general, females demonstrate a preference for higher vocal output (e.g. faster rate, more bouts per hour; McComb 1991; Nowicki et al. 2002; Ward et al. 2013) and many studies have revealed interindividual variation in song output (Otter et al. 1997), providing the basis for female choice and subsequent variation in male reproductive success. Although research has suggested that song rate is likely a quality handicap (Vehrencamp 1999), and many studies have now examined the mechanistic costs regulating of song quality (e.g. developmental stress, Buchanan et al. 2003, 2004; stress responsiveness. Macdougall-Shackleton et al. 2009), to date few studies have examined how oxidative mechanisms (i.e. oxidative stress, antioxidant capacity) might relate to or predict variation in song quality or song output (although see Van Hout et al. 2011; Casagrande et al. 2014).

Oxidative status (i.e. the balance between pro-oxidants and antioxidant capacity; Halliwell & Gutteridge 2007) is a condition-related mechanism with a strong potential to explain the honesty of sexual signals. Oxidative stress results when the production of pro-oxidants, or reactive oxygen species, overwhelms an individual's antioxidant capacity and ability to avoid damaging oxidative effects (Sies 1985). It has been suggested that secondary sexual signals may be handicapped by oxidative stress costs (von Schantz et al. 1999; Alonso-Alvarez et al. 2007). Moreover, oxidative stress is thought to be an important factor affecting multiple components of individual fitness (Finkel & Holbrook 2000). Alonso-Alvarez et al. (2007) originally proposed the '*Oxidation Handicap Hypothesis*' arguing that although testosterone influences sexual signalling, it also directly elevates oxidative stress. Metcalfe & Alonso-Alvarez (2010) later added to this hypothesis by suggesting that any signal increasing an individual's aerobic activity level (i.e. increased oxygen demand and metabolic activity) could directly generate higher levels of reactive oxygen species, perhaps independently of testosterone (i.e. '*Aerobic Activity Hypothesis*'). It is entirely possible that testosterone's indirect pathway interacts with the direct energetic and aerobic pathways to induce measureable costs of performing acoustic signals (e.g. Alonso-Alvarez et al. 2007; Mougeot et al. 2009), especially as testosterone's relationship to oxidative stress can be highly complex (Casagrande et al. 2011, 2012a,b). However, although the relationship between testosterone, song output and oxidative stress has been proposed for vertebrate vocalizations (von Schantz et al. 1999; Alonso-Alvarez et al. 2007; Metcalfe & Alonso-Alvarez 2010), they remain to be examined simultaneously.

We examined the interactive relationships between song rate, testosterone, territory size, oxidative stress and reproductive measures in the snow bunting, *Plectrophenax nivalis*, an Arctic-breeding species where males actively sing individually distinctive songs to advertise and defend breeding territories (Baldo et al. 2014) and in which only one successful brood can be produced annually in Arctic regions (Montgomerie & Lyon 2011). Our first objective was to examine the correlative relationships between testosterone, song rate, territory size and oxidative status in males to examine evidence supporting the *Oxidation Handicap* and the *Aerobic Activity Hypotheses*. Briefly, the *Oxidation Handicap Hypothesis* predicts that higher testosterone levels are directly responsible for increased levels of reactive oxygen species, whereas the *Aerobic Activity Hypothesis* predicts that it is the increased level of activity and oxygen consumption as a result of higher song output or defending a larger territory that directly results in increased levels of reactive oxygen species. Although our approach with the current study is a correlative one, it is in fact very difficult to perform manipulative experiments to causally test handicap-signalling hypotheses (Getty 2006) in the case of song production (i.e. to separate the impact of testosterone from that of aerobic activity). This is because increasing song rate or other behaviours associated with territory defence (e.g. via playback experiments) is also expected to increase testosterone levels, while increasing testosterone directly via hormonal manipulations is also expected to increase the aerobic activity of males. As such, within our current study, and with regard to total oxidative status (ratio of reactive oxygen species to antioxidant capacity), we would expect males with higher song rates may be able to afford the oxidative cost of higher song performance via higher antioxidant levels, and we may not detect a relationship with song rate. Our second objective was to investigate the relationships between song performance and reproductive parameters. The timing of breeding is an important predictor of reproductive success in seasonally breeding Arctic birds (Bêty et al. 2004), especially in snow buntings (Guindre-Parker et al. 2013c). As such, we expected to find a negative relationship between song rate and female arrival date, and a positive relationship between song rate and territory quality (measured here as the product of territory size and available nest sites; see methods). Our third objective was to test for relationships between male signalling, female reproductive investment decisions and fitness metrics. We predicted that higher male song rates would be related to earlier laying dates, larger clutch sizes and a greater number of offspring fledged.

Methods

Study System and Sampling

We studied a population of snow buntings at East Bay Island, Nunavut, Canada, from June to August of 2010 (n = 17 pairs) and 2011 (n = 13 pairs). We trapped snow buntings on arrival from spring migration using baited ground traps and banded each bird with an aluminium band and three plastic colour bands to facilitate individual identification. We collected blood samples from the alar veins of all breeding male snow buntings using heparinized microcapillary tubes between early June and July during the late egg-laying and early incubation period, respectively. We centrifuged whole blood (15 000 g for 10 min) within an hour of collection and stored plasma at −20°C in the field and at −80°C in the laboratory until analyses. In 2010, not all blood samples were obtained within 10 min of capture, and it is therefore possible that testosterone levels could have been affected by capture stress (Wingfield et al. 1982; Deviche et al. 2010; but see Hasselquist et al. 1999). All animal work used Canadian Council on Animal Care-approved techniques under Animal Care Committee approval from Environment Canada permits: (NUN-SCI-08-04 and NUN-SCI-11-05) and the University of Windsor (AUPP 09-14), as well as Canadian Wildlife Service banding (10808) and sample collection (0276) permits.

Reproductive Measures

The date of first capture on the island was used as a female's arrival date. Previous geolocation tracking work in this population found that the first date of capture reliably represents actual arrival date (within 12–20 h of arrival, O. P. Love, unpubl. data), especially for females (Macdonald et al. 2012, 2015). Based on geolocation, banding and observational data from 2007 to 2013, we know the majority of males arrive on East Bay Island before females (Macdonald et al. 2012, 2015), supporting the idea that the earliest arriving females have the broadest choice of breeding partners (Guindre-Parker et al. 2013a,b). We located all nests, recorded the laying date of the first egg and then subsequent clutch size. As nestlings leave the nest by day 9 in this species (Hussell 1985), fledging success was calculated as the number of nestlings present on day 7 or 8 of post-natal development. We visited the nests every 2–3 d after hatching to ensure this number accurately reflected fledging success and not premature fledging, or pre-fledging parental abandonment. Breeding territory size (i.e. area) was estimated for all focal males by mapping out all individual breeding territories via detailed behavioural observations (Guindre-Parker et al. 2013c). As a proxy for the number of potential nesting sites within a given territory, we estimated per cent rock cover (via 5% increments) within a 5 m radius of each male's nest (Tinbergen, 1939). Territory 'quality' was defined as the product of territory area and per cent rock cover based upon Guindre-Parker et al. (2013c), representing total potential nest site availability within the territory.

Recording and Analysis of Male Song Performance

We recorded the singing behaviour of 24 different males across the 2 yrs of the study, using a directional microphone (model: Sennheiser ME66/K6) and a solid-state digital recorder (model: Marantz PMD660; recording settings: 44 100 Hz sampling rate, 16 bit accuracy, WAVE format). Three males were recorded in both years, and the remaining males were recorded in only 1 yr. We collected recordings opportunistically throughout the day; however, most recordings were collected in the early morning when male snow buntings are most active vocally (S. Baldo, D. J. Mennill and O. P. Love, unpubl. data; Montgomerie & Lyon 2011). In 2011, we supplemented directional microphone recordings with 24-h recordings obtained using an array of autonomous digital recorders (model: Wildlife Acoustics SM2-GPS; recording settings: 22 050 Hz sampling rate, 16 bit accuracy, WAVE format; Mennill et al. 2012). Individuals were readily identified within the autonomous recordings based on song structure (male song structure is individually distinctive in this species and this population; Baldo et al. 2014) and based on recording location (which corresponded to the territories of males).

Song rates were assessed from recordings obtained over a 2-wk period (in 2010, between June 10 and June 28; in 2011, between June 16 and June 30), between 0230 and 1130 h in 2010, and 0230 and 2330 h in 2011; most recordings were obtained before 1000 h in both years (88% of recordings in 2010 and 90% of recordings in 2011). We calculated song rates for individual males by visualizing our recordings as sound spectrograms in Syrinx-PC sound analysis software (J. Burt, Seattle, WA, USA) and calculating the number of songs divided by the recording length. Estimates were based on song bouts with a minimum of seven songs (mean: 16.1 ± 1.0 songs, range: 7–71 songs) and calculated over multiple song bouts for each individual (mean number of bouts: 5.5 ± 0.3

bouts/individual, range: 4–10 bouts; mean bout length: 4.3 ± 0.3 min, range: 1–27 min), recorded on multiple days (mean: 3.7 ± 0.2 d, range: 3–5 d). We considered a song bout to be finished when the bird flew away from a song post, or a period of silence longer than 60 s began. Unfortunately, two males had song rate recordings of <7 songs, and therefore, these were excluded so as not to bias analyses with less-than-complete data sets. Nonetheless, these two males fell within the mean for song characteristics (where the majority of our data lie) and as such we are confident that leaving these birds out of the analyses did not bias results in any meaningful way.

Physiological Assays

We measured testosterone from field samples using a standard dichloromethane extraction protocol combined with a commercially available enzyme-linked immunoassay (Cayman Chemical Company, USA, Item No. 582701; Pryke et al. 2007), previously validated in this species (see Guindre-Parker et al. 2013a for details). Absorbance was read at 412 nm – where colour intensity is inversely proportional to free testosterone – and concentrations were expressed in ng/mL. Two plates were required, and mean intra- and interassay coefficients of variation were 7.7% and 10.6%, respectively.

We measured pro-oxidants from field samples by assessing plasma reactive oxygen metabolites (ROM) using a previously validated commercially available kit (d-ROMs test, Diacron International, Grosseto, Italy; Guindre-Parker et al. 2013c) modified for 96-well microplates and optimized for snow bunting plasma. Reactive oxygen metabolites are derivatives of reactive oxygen species and are more easily measured than reactive oxygen species because they are more stable. The test primarily measures hydroperoxides, whose concentration is expressed as equivalents of hydrogen peroxide, which provide an early marker of oxidative damage. Plasma samples collected in the field were thawed, and 15 μl of each was diluted with 300 μl of working solution (solution consisted of a chromogenic solution diluted 1:100 in acetate buffer). Each tube was vortexed and incubated at 37°C for 75 min. Tubes were centrifuged at 13 000 g for 30 s and 290 μl of the supernatant pipetted into a new tube. Tubes were then vortexed and 125 μl run in duplicate. Plates were subsequently read at a wavelength of 490 nm. Concentrations were determined using a standard curve (range: 0.28–18 mg H_2O_2/dl) generated using a kit-provided stock solution of 18 mg H_2O_2/dl; colour intensity is proportional to the concentration of reactive oxygen metabolites, and measurements are expressed in mg of H_2O_2/dl. Two plates were required, and intra- and interassay coefficients of variation were 4.9% and 3.0%, respectively.

The total antioxidant capacity (TAC) of plasma samples was measured using a previously validated commercially available kit (OXY-adsorbent test, Diacron International, Grosseto, Italy; Guindre-Parker et al. 2013c) modified for 96-well microplates and optimized for snow buntings. This test measures a sample's non-enzymatic component of plasma antioxidants by measuring the sample's capacity to cope with the oxidizing ability of hypochlorous acid (HClO), a powerful pro-oxidant. Plasma samples were thawed, 2 μl was diluted 1:100 by adding 198 μl of distilled water with vortexing, and then 5 μl of each sample was loaded in duplicate followed by 200 μl of HClO oxidant solution. Plates were shaken for 10 s at 450 RPM and incubated at 37°C for 10 min. Then, 2 μl of chromogenic solution was added to each well, and plates were shaken for 30 s in a plate reader. Concentrations were determined using a standard curve (range: 0.47–5.75 μmol HClO/ml) generated from a kit-provided stock solution of 230 μmol HClO/ml. Plates were read at 490 nm – the chromogenic solution changes colour upon reacting with the remaining HClO, where colour intensity is inversely proportional to the sample's antioxidant capacity (i.e. ability to neutralize the acid). Measurements are expressed in μmol of HOCl/ml neutralized, and two plates were required producing mean intra- and interassay coefficients of variation of 5.1% and 0.9%, respectively. Oxidative status was calculated using ROMs/TAC × 1000 (index of oxidative stress used by Costantini et al. 2006), where higher values equate to higher oxidative stress. When testing this commonly used measure of overall oxidative status in statistical models, results did not differ significantly from models with ROMs as the dependent variable and TAC as an independent covariate.

Statistical Analyses

For the three buntings present in both years of the study, 1 yr of song data was randomly excluded for each male from analyses. Overall, results did not differ using this approach compared to one where the mean variable was used for each bird. We did not detect any interannual differences for our dependent traits (e.g. testosterone, oxidative status measures). We standardized all independent traits of interest (song rate, territory size, territory quality, arrival date, laying date) by correcting an individual's absolute value relative to the mean intra-annual value to account for inherent interannual variation so as not to induce any biases originating from interindividual variation in quality. Sample sizes varied across different analyses because we were not able to obtain all acoustic, hormonal and reproductive measures for all individuals of all pairs. To test our first objective (evidence for the '*Oxidation Handicap*' and '*Aerobic Activity Hypotheses*'), we began with a general linear model (GLM) to confirm the long-held assumption that plasma testosterone should be positively related to song rate (e.g. Romero et al. 1998; Alonso-Alvarez et al. 2007) controlling for male arrival date. We then improved this model by testing oxidative status measures as the dependent variable to examine the relative contribution of plasma testosterone, song rate and territory size. To test our second objective (relationship between song performance and reproductive measures), we used GLMs to examine whether male song rate could be used to predict laying date and territory quality controlling for female arrival date. We included male arrival date as a covariate to control for residual male quality independent of song, as variation in arrival date can indicate intrinsic individual variation (Bêty et al. 2004). Finally, to test our third objective (relationships between male signalling, female reproductive investment decisions and fitness), we used a GLM to examine whether song rate predicted reproductive decisions (laying date; Lepage et al. 2000; Descamps et al. 2011) and an ordinal logistic model to examine whether song rate predicted reproductive output (i.e. clutch size and number of fledglings). All tested variables met the requirements of specific statistical tests, testosterone data were log-transformed due to non-normality, and all analyses were performed in JMP v. 9.0.2. (SAS Institute, Cary, NC).

Table 1: Song rate and plasma testosterone as predictors of breeding reactive oxygen metabolites, antioxidant capacity and oxidative status in male snow buntings (*represents significant relationships at alpha <0.05)

	F-ratio	p-value	Estimate	df	n
Reactive oxygen metabolites					
Whole model	4.13	0.03		12	17
Song rate	5.42	0.04*	0.38 ± 0.16		
Testosterone	0.43	0.52	−0.09 ± 0.14		
Male arrival date	1.59	0.25	−0.03 ± 0.02		
Territory size	0.52	0.49	0.0008 ± 0.0001		
Antioxidant capacity					
Whole model	4.77	0.02		12	17
Song rate	12.9	0.003*	0.37 ± 0.10		
Testosterone	2.99	0.11	−0.15 ± 0.09		
Male arrival date	1.97	0.18	−0.02 ± 0.01		
Territory size	0.02	0.88	0.00014 ± 0.0001		
Oxidative status					
Whole model	0.37	0.82		12	17
Song rate	0.39	0.55	0.79 ± 1.27		
Testosterone	0.41	0.45	0.77 ± 1.27		
Male arrival date	0.002	0.91	0.02 ± 0.21		
Territory size	0.21	0.65	0.00052 ± 0.0001		

Results

Male testosterone levels at arrival on the breeding grounds showed a positive relationship with song rate (whole model: $F_{2,14} = 2.26$, $p = 0.045$, $n = 17$; testosterone: $F_{1,14} = 4.12$, $p = 0.05$; estimate: 0.37 ± 0.19; male arrival date: $F_{1,14} = 2.85$, $p = 0.1$; estimate: 0.06 ± 0.03). However, song rate was positively related to both reactive oxygen metabolites and total antioxidant capacity, independent of testosterone levels and territory size when all variables were included in a global model (Table 1; Fig. 1a, b). Interestingly, neither song rate, testosterone levels nor territory size reflected overall oxidative status (Table 1; Fig. 1c). Song rate was also unrelated to female arrival date, although the earliest arriving females did pair with the earliest arriving males (Table 2). Song rate also failed to predict territory quality, the laying date of the first egg, clutch size or the number of fledglings produced (Table 2). The significance of traits of interest outlined in Tables 1 and 2 was not altered qualitatively whether non-significant terms were backward eliminated from models or not. As such, to provide the reader with full information on the relative contribution of variables, significant or not, we have retained all variables within the models presented in Tables 1 and 2.

Discussion

Male snow buntings that sang at higher rates (i.e. had higher aerobic activity) exhibited higher levels of both reactive oxygen metabolites and total antioxidant capacities, independent of both plasma testosterone and overall territory size. Although recent work has linked an increase in antioxidant capacity and increases in song output (Van Hout et al. 2011; Casagrande et al. 2014), our results are the first to suggest that metabolic aerobic demands during song production can lead to the production of reactive oxygen species. Overall, we found that certain males can perform at a faster rate without suffering the costs of elevated oxidative stress levels through higher or upregulated antioxidant capacities. These results

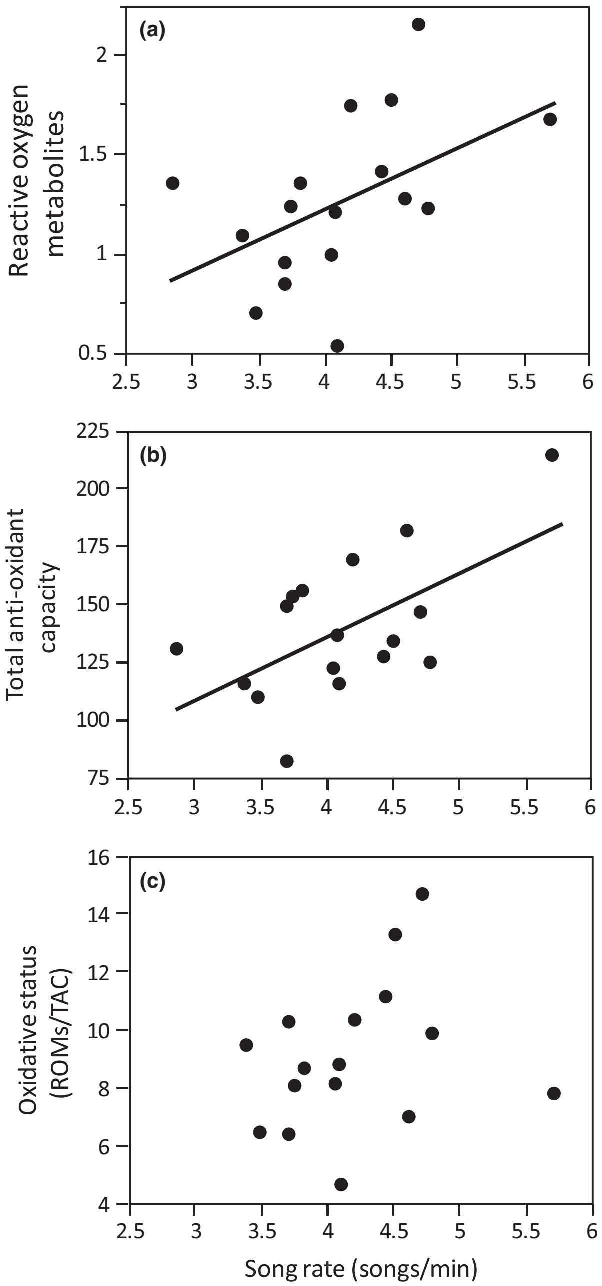

Fig. 1: Male snow buntings that sing at higher rates have both higher reactive oxygen metabolite (ROM; equivalents of H_2O_2 mg/dl) levels (a) and total antioxidant capacities (TAC; μmol of HOCl/ml neutralized) (b), but do not suffer from elevated overall oxidative status (c).

Table 2: Relationships between song rate and breeding parameters in snow buntings (* represents significant relationships at alpha <0.05)

	F-ratio	p-value	Estimate	df	n
Female Arrival Date					
Whole model	7.8	0.004		16	19
Song rate	2.3	0.14	0.7 ± 0.46		
Male arrival date	9.7	0.008*	0.2 ± 0.06		
Territory Quality					
Whole model	1.4	0.27		15	19
Song rate	1.6	0.22	2838.3 ± 2244.2		
Female arrival date	2.7	0.12	−1874.5 ± 1134.7		
Male arrival date	0.002	0.96	18.81 ± 373.7		
Laying Date of First Egg					
Whole model	1.6	0.25		12	16
Song rate	0.001	0.97	−0.033 ± 0.96		
Female arrival date	1.4	0.26	0.61 ± 0.5		
Male arrival date	0.32	0.58	0.10 ± 0.18		
	χ^2	p-value	Estimate	df	n
Clutch Size					
Whole model	25.5	0.03		12	16
Song rate	0.36	0.94			
Female arrival date	0.17	0.98			
Lay date of first egg	54.15	<0.0001*			
Number of Fledglings					
Whole model	25.7	0.04		10	14
Song rate	0.01	0.93			
Clutch size	12.94	0.16			
Lay date of first egg	475.2	<0.0001*			

provide some support for Vehrencamp's (1999) assertion that song rate is likely a quality handicap, as well as recent experimental oxidative stress work in birds (Van Hout et al. 2011; Casagrande et al. 2014), whereby an underlying cost prevents low-quality males from singing at a high rate, maintaining the honesty of this sexually selected signal. To the best of our knowledge, this investigation is the first to test the *Oxidation Handicap Hypothesis* using a behavioural signal; previous studies investigating the relationship between oxidative status and signal quality have focused on visual signals (e.g. plumage and skin; Pike et al. 2007; Galván & Alonso-Alvarez 2008; Alonso-Alvarez et al. 2010). Our study lends support to the idea that sexually selected signals can be reliable indicators of individual quality, the honesty of which is ensured via oxidative costs that are not equally affordable to all individuals. An apparent lack of relationship between song rate and overall oxidative status could be influenced by our relatively small sample size, although it is noteworthy that our sample size did not preclude us from detecting relationships between song rate and both reactive oxygen metabolites and antioxidant capacity.

When examined in isolation as a mechanism, we found the expected positive relationship between testosterone and song rate, supporting previous research on song performance in passerine birds (e.g. Foerster et al. 2002; Ritschard et al. 2011; but see Kunc et al. 2006). As such, we found initial support for testosterone's role in enhancing signal expression and a potential link to oxidative status (i.e. Casagrande et al. 2012a,b), both primary predictions of the original *Oxidation Handicap Hypothesis* (Alonso-Alvarez et al. 2007). However, when examined in combination, song rate was a far stronger predictor of oxidative and antioxidant measures than testosterone and was still a much better predictor than territory size. These results support predictions of the *Aerobic Activity Hypothesis* (Metcalfe & Alonso-Alvarez 2010) suggesting that testosterone need not be a direct driver of the relationship between signal expression and oxidative costs if energetically costly signals (i.e. signals requiring increased oxygen demand such as song rate) generate higher levels of reactive oxygen species. It is certainly possible that individual quality may play an interfering role in our correlative results, as low-quality birds may not be able to increase song rate even when endogenously producing high testosterone levels (i.e. Alonso-Alvarez et al. 2009). As emphasized in the introduction, an experimental manipulative approach would be the ideal manner in which to test handicap hypotheses related to the production of acoustic signals. However, the interrelated nature of song production, testosterone and aerobic activity makes this extremely difficult to examine in free-living birds.

Although many studies have demonstrated a link between song rate and female mate choice (e.g. Mountjoy & Lemon 1996; Buchanan & Catchpole 1997), we found no relationship between male song rate and female arrival date, or territory quality, which we used here as proxies for female mate choice. In snow buntings, Hofstad et al. (2002) reported a negative relationship between song rate and laying date, and Guindre-Parker et al. (2013b) reported that higher quality male plumage signals predicted earlier laying dates. Likewise, Guindre-Parker et al. (2013c) recently reported that male snow buntings with higher quality territories had a greater increase in oxidative costs from arrival to the peak of breeding. However, we found no correlative link between song rate and laying date of the first egg, or reproductive investment (clutch size), contrary to investment predictions regarding oxidative status in this species (e.g. Guindre-Parker et al. 2013c). In laboratory experiments, song quality has been shown to differentially stimulate egg production in common canaries (*Serinus canarius*; Kroodsma 1976). It should be noted that Kroodsma (1976) exposed female canaries to a single stimulus type (low or high quality), while the female snow buntings in this study were able to assess multiple males simultaneously. Finally, interindividual variation in male song rate did not predict the reproductive output (number of fledglings produced) of a male's social partner. High breeding density on East Bay Island may confound this lack of a relationship via unmeasured (but possibly) high levels of extra-pair fertilizations and subsequent offspring. For example, snow bunting plumage signals may be important in selecting a social mate (see Guindre-Parker et al. 2013b), while song quality may play an important role in extra-pair copulation decisions (as in Chiver et al. 2008). If song quality does influence the rates of extra-pair copulations/fertilizations, our current within-pair measure of reproductive success may not be representative of a male's realized reproductive success and future work that incorporates parentage analysis will be needed to investigate these possibilities.

Oxidative stress ecology is a relatively new field of research and many avenues need further investigation (Monaghan et al. 2009; Haussmann et al. 2012). Longitudinal, repeated-measures studies investigating how oxidative stress changes within an individual (e.g. Guindre-Parker et al. 2013c) and in relation to signal expression over time would enhance our understanding of how and why oxidative stress may impact signalling and vice versa (Metcalfe & Alonso-Alvarez 2010). Future studies should continue to explore non-visual signals, particularly those that increase oxygen consumption, especially energetically demanding displays as proposed by Metcalfe & Alonso-Alvarez (2010). An investigation of song amplitude may yield further support for the *Aerobic Activity Hypothesis*, as amplitude is a potentially meaningful signal of individual quality (Ritschard et al. 2010; Brumm and Ritschard 2011) that has been shown to relate to oxygen consumption (Oberweger & Goller 2001). Costantini (2008) urged integrative collaboration between ecology, biochemical and medical disciplines using both *in vivo* and *in vitro* laboratory tests to complement ecological studies to provide a more complete examination of oxidative stress.

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