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Sarah Baldo

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SONG AS AN HONEST INDICATOR OF OXIDATIVE DAMAGE AND ANTI-OXIDANT
CAPACITY: EXPLORING THE RELATIONSHIPS BETWEEN SIGNAL QUALITY, OXIDATIVE
STATUS, AND REPRODUCTION IN THE SNOW BUNTING (*PLECTROPHENAX NIVALIS*)

by

Sarah Baldo

A Thesis
Submitted to the Faculty of Graduate Studies
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in Partial Fulfillment of the Requirements for
the Degree of Master of Science
University of Windsor

Windsor, Ontario, Canada

2012

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Declaration of Co-Authorship

I hereby declare that this thesis incorporates material that is result of joint research, as follows: This thesis examines data collected in collaboration with Sarah Guindre-Parker under the supervision of Daniel Mennill and Oliver Love. Chapters 1 and 3 were written by me, though Daniel Mennill and Oliver Love improved both chapters with valuable feedback. Chapter 2 was co-authored by me, Sarah Guindre-Parker, Grant Gilchrist, Daniel Mennill, and Oliver Love. Sarah Guindre-Parker aided in data collection and provided helpful suggestions on statistical analyses and writing. Grant Gilchrist provided extensive logistical support and helped with data collection in the field. Daniel Mennill and Oliver Love were heavily involved with data analysis and interpretation, as well as with writing, and also provided substantial logistical support.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis.

I certify that, with the above qualification, this thesis, and the research to which it refers, is the product of my own work.

I certify that the above material describes work completed during my registration as a graduate student at the University of Windsor.

I declare that, to the best of my knowledge, my thesis does not infringe upon anyone's copyright nor violate any proprietary rights and that any ideas, techniques, quotations, or any other material from the work of other people included in my thesis, published or otherwise, are fully acknowledged in accordance with the standard referencing practices.

I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee and the Graduate Studies office, and that this thesis has not been submitted for a higher degree to any other University or Institution.

Abstract

Sexual selection has led to the evolution of elaborate signals which enhance mate attraction and reproductive success. Often there is marked variation in signal quality between individuals yet little is known about the physiological mechanisms underlying this variation. Bird song is a signal used to attract mates and repel rivals and can vary between individuals in both content and performance. Oxidative stress is a potential mechanism that may affect song content and performance, explaining some of the inter-individual variation in signal quality. I investigated the relationship between song, oxidative status, and reproduction in the Snow Bunting (*Plectrophenax nivalis*) and found support for the Oxidation Handicap Hypothesis. Birds that sang at a higher rate had higher levels of reactive oxygen metabolites, but also had higher anti-oxidant capacities. Therefore, the ability to avoid oxidative stress is reliably indicated via song performance and reactive oxygen species may be the handicap ensuring signal honesty.

"No shore seems too desolate, no rock ledge too bare, for the snow-bunting. Everywhere through-out the North, wherever man has been in summer, the snow-bunting has greeted him. Even the explorers who have crossed the ice-cap of Greenland have reported hearing his song, or seeing him, as they sledged along their lonely, dreary way. The snow-bunting, and the poppy, and the [Inuit], are all alike in their fearlessness, their cheeriness, and their love of the North."

-Walter Elmer Ekblaw, 1919

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Chapter 1

General Introduction

Chapter 1 – General Introduction

Darwin (1871) proposed the theory of sexual selection to explain the evolution of elaborate ornaments and displays. Individuals, usually males, often possess exaggerated ornaments or perform complex displays that may decrease their probability of survival, but are beneficial because they serve as signals to rivals and potential mates, and can thereby increase reproductive success (Andersson and Iwasa 1996). However, despite the potential benefits, there still exists a large degree of inter-individual variation in the quality of elaborate ornaments and displays, and an important area of behavioural ecology aims to understand the causes and consequences of this variation.

Zahavi (1975) proposed the handicap principle as a means of explaining inter-individual variation in the quality of secondary sexual signals. The handicap principle states that ornaments and displays carry an underlying production or maintenance cost, so that low quality individuals are unable to produce high quality signals because of an inability to afford these costs (Zahavi 1975). Many studies have supported the idea that some sexual signals are influenced by the handicap principle (e.g. Pryke et al. 2001; Berg et al. 2005). Alternatively, signal honesty can exist for reasons outside of the traditional explanation of signal costs (Hill 2011). The honesty of an 'index signal', for example, is maintained by a physical or physiological constraint that cannot be altered, such as body size. These constraints render index signals honest advertisements of individual quality without invoking a direct cost (i.e. without creating a handicap; Smith and Harper 1995; Vehrencamp 1999; Hill 2011). Many studies have demonstrated that some sexual signals

are physically or physiologically constrained to provide honest information, and as such, serve as index signals (e.g. Zimmitti 1999; Scheuber et al. 2003).

Acoustic Communication and Bird Song

Acoustic communication is a widespread form of signalling used by many animals. Birds rely heavily on vocalizations to facilitate various functions, including individual recognition (e.g. parent recognition: *Aptenodytes patagonicus*; Jouventin et al. 1999), and to convey rank (e.g. submissive calls: *Copsychus saularis*; Kumar and Bhatt 2001), as well as to inform other individuals about environmental factors, such as predation threat (e.g. alarm calls: *Sericornis frontalis*; Leavesley and Magrath 2005) and food sources (e.g. food calls: *Corvus corax*; Bugnyar et al. 2001). Bird vocalizations are divided into two categories: calls and songs (Catchpole and Slater 2008). Calls are typically shorter and simpler vocalizations used to fulfill a wide variety of functions, while songs are generally longer and more complex vocalizations used primarily for territory defence and mate attraction (Tinbergen 1939; Smith 1959; Marler 1970; Catchpole and Slater 2008).

The Order Passeriformes, or songbirds, consists of oscine passerines and suboscine passerines (Gill 2007). In general, oscine passerines learn their song, while suboscine passerines are born with innate song (Catchpole and Slater 2008). The 'auditory template model' suggests that oscines are born with a crude template of species-specific song which becomes more refined as young birds memorize and begin producing songs to match the initial template (Marler and Tamura 1964; Konishi 1965;

Marler 1970). As song develops, it becomes louder and less variable; a young bird first produces subsong, then plastic song, and lastly full adult song, in an ontogenetic pattern that mirrors the development of human speech (Marler and Tamura 1964; Konishi 1965; Marler 1970; Nottebohm 1970). Most oscines are thought to learn song within their first year of life, after which point song content (see list of key terms in glossary contained in Chapter 1 Appendix) is rather invariable (Catchpole and Slater 2008; but see Payne 1985; McGregor and Krebs 1989; Gil et al. 2001; Beecher and Brenowitz 2005). Bird song provides an excellent study system for investigating how natural and sexual selection shape animal communication as it is comprised of multiple traits shaped by a variety of factors at different life history stages (Gil and Gahr 2002). Factors as diverse as habitat structure, weather, developmental homeostasis, tutor availability, female preferences, conspecific interactions, and hormone levels all influence the songs produced by adult birds (Beecher and Brenowitz 2005; Catchpole and Slater 2008). In the temperate zone, song is produced primarily by males, stimulated, at least in part, by testosterone, and is tightly associated with breeding behaviour (Foerster et al. 2002; Catchpole and Slater 2008; Fusani 2008).

Recent technological advances have made studying song increasingly feasible as recording, playback, and analysis equipment become less expensive and more widely available. Additionally, powerful sound analysis software facilitates increasingly accurate and quantitative measurement of song (Mennill 2011). Song quality can be measured in two ways: on the basis of content or performance (see glossary; Ritschard et al. 2011). Song content deals with song structure (see glossary) and complexity (see glossary). For

example, song content measurements may include song length, note duration, repertoire size, and song versatility. Song performance deals with the quantity and amplitude of song. For example, song performance measurements may include song rate or total output. Because multiple constraints shape song traits, different traits comprising song or associated with singing behaviour may convey different messages to receivers (Gil and Gahr 2002; Catchpole and Slater 2008). Taken together, the measurement of both song content and performance can potentially provide an integrated measure of an individual's "quality" as advertised via song. Song content represents, at least in part, a bird's past developmental condition, whereas song performance may better represent an individual's current condition (Ritschard et al. 2011). As such, by studying both content and performance, we may gain insight into the past and current pressures shaping song quality, making song a very informative signal for research (Gil and Gahr 2002).

Bird Song, Intra-sexual Competition, and Inter-sexual Choice

The structure and complexity of secondary sexual signals and the way in which they are used can have pronounced effects on conspecific interactions and ultimately fitness. Song is no exception, as both content and performance can affect the outcome of intra-sexual competition and inter-sexual choice. In terms of male-male competition, birds use song to repel rivals from their territories (e.g. *Plectrophenax nivalis*; Tinbergen 1939; *Ammodramus savannarum*; Smith 1959; *Mionectes oleagineus*; Westcott 1992; *Melospiza melodia*; Nowicki et al. 1998; de Kort et al. 2009). While singing behaviour

during contests can greatly influence intra-sexual encounters (e.g. song type and repertoire matching: *Melospiza melodia*; Burt et al. 2001; overlapping and frequency matching: *Poecile atricapillus*; Mennill and Ratcliffe 2004), song content and performance are also important factors which may deter competitors. For example, song content appears influential in intra-sexual encounters, as speakers broadcasting larger repertoires in speaker-replacement experiments successfully defend territories longer than those broadcasting smaller repertoires (e.g. *Parus major*; Krebs et al. 1978), and playback of longer songs elicits stronger responses from resident males than shorter songs (*Sylvia communis*; Balsby and Dabelsteen 2001). Song performance also appears related to male-male competition, as song bout length has been found to correlate with male status (dominance rank: *Poecile atricapillus*; Otter et al. 1997), and song rate with the quality of defended resources (nest site quality: *Sylvia atricapilla*; Hoi-Leitner et al. 1995).

In terms of inter-sexual choice, many studies have demonstrated female preference for certain song attributes. For example, male songbirds that sing larger repertoires often pair earlier (e.g. *Sturnus vulgaris*; Mountjoy and Lemon 1996; *Acrocephalus schoenobaenus*; Buchanan and Catchpole 1997), have higher annual and lifetime reproductive success (e.g. *Melospiza melodia*; Hiebert et al. 1989), and sire more extra-pair offspring (e.g. *Acrocephalus arundinaceus*; Hasselquist et al. 1996). Song versatility (number of different syllables in a song or a sample of songs/total number of syllables in a song or a same sample of songs, respectively) and song length are other song content measures of interest to females, with preference for more versatile over

less versatile songs (e.g. *Phylloscopus trochilus*; Järvi 1983; *Ficedula hypoleuca*; Lampe and Espmark 2003), and for long over short songs (e.g. *Taeniopygia guttata*; Neubauer 1999; *Ficedula hypoleuca*; Lampe and Espmark 2003; *Carpodacus mexicanus*; Nolan and Hill 2004; but see *Phylloscopus trochilus*; Järvi 1983). In addition to song content, song performance also influences female choice and, consequently, male reproductive success. Song rate, for example, is a well-studied performance measure which appears to play an important role in mate choice. Male songbirds that sing at a faster rate often pair earlier (e.g. *Phylloscopus trochilus*; Radesäter et al. 1987), pair to earlier breeding females (e.g. *Plectrophenax nivalis*; Hofstad et al. 2002; *Tyrannus tyrannus*; Murphy et al. 2008), receive more copulation solicitations (e.g. *Carpodacus mexicanus*; Nolan and Hill 2004), and pair to females that lay larger clutches (e.g. *Tyrannus tyrannus*; Murphy et al. 2008).

Oxidative Stress and Bird Song

Reactive oxygen species (see 'reactive oxygen metabolites' in glossary), or pro-oxidants, are unstable molecules that can detrimentally affect cells, and consequently organism health, by damaging various biomolecules, such as proteins, lipids, and DNA (Nordberg and Arnér 2001; Monaghan et al. 2009). Anti-oxidants are molecules that neutralize reactive oxygen species preventing oxidative damage (Silverthorn 2007). An individual's oxidative status (see glossary) depends on the interplay between the two, and oxidative stress can result from "a disturbance in the pro-oxidant/anti-oxidant systems in favour of the former" (Sies 1985). In mammals, oxidative stress is thought to contribute to the

development of a number of ailments, such as muscular dystrophy, liver cirrhosis, hemolytic anemia, cataracts, and cancer, and is also thought to be a cause of cellular and whole organism senescence (Sies 1985, 1991; Finkel and Holbrook 2000). Although a heavily studied topic in medical and biochemical research, oxidative stress has not been well-studied in ecological and evolutionary studies on non-human animals despite its potential role in driving life history trade-offs and shaping organism phenotype (Costantini 2008; Monaghan et al. 2009; Metcalfe and Alonso-Alvarez 2010).

Reactive oxygen species are by-products of normal cellular metabolism, as well as products of detoxification pathways and immune systems, produced to destroy toxins and pathogens (Finkel and Holbrook 2000; Costantini and Dell’Omo 2006). Von Schantz et al. (1999) suggested that underlying genetic differences, which relate to the tolerance and resistance of the detoxification and immune systems, may affect the expression of secondary sexual signals, as less tolerant individuals will generate more reactive oxygen species promoting oxidative stress, and many signals are sensitive to oxidative stress. Although von Schantz et al. (1999) did not name this concept, I refer to this idea as the Oxidation Sensitivity Hypothesis throughout my thesis.

The Oxidation Handicap Hypothesis was later formulated by Alonso-Alvarez et al. (2007) proposing another way in which signal quality may be related to oxidative stress; individuals with lower resistance to reactive oxygen species display lower quality secondary sexual traits because of their inability to afford the oxidative stress inducing costs associated with signal production or maintenance caused by elevated levels of the

steroid hormone testosterone (Alonso-Alvarez et al. 2007). Metcalfe and Alonso-Alvarez (2010) later added that signals which are unrelated to testosterone, but increase the activity level of an individual, could also cause an increase in reactive oxygen species as a result of increased oxygen consumption and energy expenditure, rendering these signals most affordable to high quality individuals.

The Oxidation Sensitivity Hypothesis suggests that low quality individuals may be prevented from producing high quality signals because of a physiological inability to “cheat” (i.e. cannot prevent signal quality from reliably revealing oxidative stress), while the Oxidation Handicap Hypothesis suggests that low quality individuals cannot afford to produce high quality signals (i.e. do not have sufficient anti-oxidant capacity (see glossary) to bear the cost of higher song production). Several components of song are predicted to function as honest signals providing information regarding an individual’s oxidative status in at least two ways based on the aforementioned hypotheses:

(1) The *Oxidation Sensitivity Hypothesis*. Von Schantz et al. (1999) suggested that song repertoire size may honestly reflect an individual’s level of oxidative stress. The brain is particularly vulnerable to oxidative stress because it (i) consumes a large amount of oxygen, (ii) contains enzymes which produce reactive oxygen species, and (iii) contains high levels of polyunsaturated fatty acids which are especially vulnerable to oxidative damage (von Schantz et al. 1999). Because neurogenesis is associated with song development and maintenance throughout a bird’s life (e.g. Nordeen and Nordeen 1990; Scharff et al. 2000) and is negatively affected by reactive oxygen species (Saito et

al. 1997), a large repertoire may be indicative of low oxidative stress. Song features other than repertoire size are also associated with neurogenesis (e.g. Scharff et al. 2000) and, therefore, I predict that other song content measures of potential importance to females (e.g. song length, note duration, and song versatility) may also be sensitive to oxidative stress and consequently serve as honest signals of oxidative status. The Oxidation Sensitivity Hypothesis (von Schantz et al. 1999) is in line with the idea that a signal does not need to be costly per se, to serve as an honest signal (Vehrencamp 1999; Hill 2011).

(2) The *Oxidation Handicap Hypothesis*. Alonso-Alvarez et al. (2007) promoted the idea that high levels of testosterone, which enhance the expression of many secondary sexual characteristics and behaviours, may induce oxidative stress by inhibiting anti-oxidant defences or increasing reactive oxygen species production. Therefore, testosterone-enhanced song traits may be indicative of an individual's ability to combat the actions of reactive oxygen species production. More recently, it has been suggested that traits which are unrelated to testosterone, but increase the activity level of an individual, could also serve as an honest signal of an individual's ability to manage the cost of increased reactive oxygen species, because these traits may generate higher levels of reactive oxygen species as a result of increased metabolic activity (Metcalf and Alonso-Alvarez 2010). Song rate has been shown to be related to testosterone concentration (e.g. *Agelaius phoeniceus*; Johnsen 1998; *Vireo solitarius*; Van Roo 2004; *Parus caeruleus*; Foerster et al. 2002; but see Kunc et al. 2006) and oxygen consumption is higher during singing compared to oxygen consumption prior to singing (Oberweger

and Goller 2001). As such, I predict song rate may reflect an individual's ability to avoid oxidative stress, as song rate has often been shown to be promoted by testosterone and also relates to the activity level of an individual. The Oxidation Handicap Hypothesis (Alonso-Alvarez et al. 2010) is in line with Zahavi's (1975) suggestion that signal production or maintenance is associated with a direct cost which is more affordable to high quality individuals.

Both males and females may benefit from being able to use inter-individual variation in song traits to distinguish between conspecific males of differing quality. For example, rival males would benefit from an ability to distinguish between males with differing fighting ability, which could be communicated via inter-individual variation in song quality. Oxidative stress affects individual condition (Costantini et al. 2007) and potentially song, and as such may link song quality and fighting ability. Females also stand to benefit from using variation in song to distinguish between males of differing quality. Females may be able to compare males using song and obtain indirect benefits (e.g. good genes) if song quality is indeed sensitive to oxidative stress which in turn is associated with genetic differences in detoxification and immune system resistance (von Schantz et al. 1999). Using song, females may also be able to discriminate between males that would provide differing levels of direct benefits. If song rate is subject to the handicap of increased reactive oxygen species, females may gain social partners better able to invest in reproduction by choosing males singing at higher rates as they may have higher anti-oxidant capacities (Nolan and Hill 2004; Alonso-Alvarez et al. 2007). Thus, a signal providing information about oxidative status could prove beneficial to

both rivals and potential mates as it could provide valuable information relating to male condition or potential benefits.

Study Species

Snow Buntings (*Plectrophenax nivalis*) are circumpolar Arctic-breeding songbirds. Males are slightly larger than females (males: mean mass=40 g, mean wing chord=110 mm; females: mean mass=37 g, mean wing chord=104 mm; Montgomerie and Lyon 2011) and arrive on the breeding grounds several weeks earlier than females (Tinbergen 1939; Meltofte 1983; Montgomerie and Lyon 2011). Snow Buntings are socially monogamous, but genetically promiscuous (Tinbergen 1939; Espmark and Moksnes, unpublished data cited in Hofstad et al. 2002). Females lay a single clutch per season (Tinbergen 1939; Sutton and Parmalee 1954; but see Nethersole-Thompson 1966) and are solely responsible for nest-building and incubation (Tinbergen 1939; Sutton and Parmalee 1954). Males are responsible for mate feeding during incubation and biparental care, both of which are required to maximize reproductive success (Tinbergen 1939; Hussell 1972; Lyon and Montgomerie 1985).

My study was conducted on East Bay (Mitivik) Island (64°02'N, 81°47'W), Nunavut, Canada. East Bay Island is largely covered by loose granite rock which provides ideal nesting habitat for Snow Buntings and is thought to contribute to the high breeding density on the island (~65 pairs/km², 2008-2011; Love, unpubl. data). The detailed reproductive ecology of this population has been studied via the individual marking of breeding adults using unique colour-band combinations since 2008. Snow

Buntings arrive at East Bay Island from wintering grounds in late-May to early-June. Females lay a single clutch of approximately 6 eggs (5.9 ± 0.8 eggs, $n=51$ nests, 2008-2011; Love, unpubl. data) in mid-June (mean Julian lay date: 169.6 ± 0.5 , $n=37$ nests, 2008-2011; Love, unpubl. data) and on average, 4 nestlings fledge from each nest (number of fledglings/nest: 4.0 ± 1.6 , $n=55$ nests, 2008-2011; Love, unpubl. data).

Song in Snow Buntings

As with the majority of temperate passerines, only male Snow Buntings sing. Males produce the majority of their songs from several favoured song perches, often atop rocks or boulders (Tinbergen 1939; Nethersole-Thompson 1966). Each male, with rare exception, produces a single song type (Espmark 1995, 1999). Although syllable sharing does occur, most males' songs are easily distinguished from one another as "an enormous amount of individual variation in song" exists (Tinbergen 1939; Borror 1961; Drury 1961; Nethersole-Thompson 1966; Espmark 1995; Fig. 1.1), although occasionally neighbours' songs can be nearly identical (Tinbergen 1939). Tinbergen (1939) noted the occurrence of restricted, local dialects in Greenland, as did Montgomerie and Lyon (2011) in the Canadian Arctic, although Espmark (1995, 1999) did not find evidence of dialects in Norway.

Snow Buntings use song for both intra-sexual purposes (territory defence) and inter-sexual purposes (mate attraction; Tinbergen 1939; Nethersole-Thompson 1966). Although males generally sing from perches, they also produce songs during song-flights in response to male intruders, supporting the idea that song has a territorial function

(Tinbergen 1939). If intruders do not retreat, chase and attack ensues (Tinbergen 1939). Additionally, males begin singing before females arrive on the breeding grounds, and intruders that sing elicit stronger responses than silent males (Tinbergen 1939). Moreover, after territory establishment, newcomers cause changes in the singing positions of territory owners, which begin singing closer to the contested boundaries than before (Tinbergen 1939). Ekblaw (1919) also writes of “males engage[ing] in competitive antiphonal concert”, which likely refers to territorial counter-singing interactions.

Snow Bunting song also serves a mate attraction function. Males that have attracted females generally sing less, but increase singing intensity when visually separated from their mates (Tinbergen 1939). Additionally, Tinbergen (1939) noted that males resumed singing during the laying period of their social mates, perhaps attempting to attract extra-pair partners. However, Espmark (1995) wrote that male Snow Buntings in Spitsbergen, Norway, maintain their singing activity after pairing and suggested that this may be the case in areas of high breeding intensity, where extra-pair mating is more prevalent, and mate guarding more important.

Hofstad et al. (2002) explored the relationship between song and reproduction and found several results supporting the idea that song is important to females as a means to assess individual male quality. First, higher song rate (songs/minute) predicted earlier breeding in males, suggesting that females prefer males that can sing at higher rates. Secondly, song rate was found to be positively correlated to male feeding rate of

8-9 day old nestlings, illustrating a potential benefit to females for discriminating between males using song (Hofstad et al. 2002). Lastly, a near significant relationship was reported between song length and the number of fledglings produced, indicating a potential link between some aspect of male quality and fitness (Hofstad et al. 2002). Although other measures of song content were also explored, they were not found to correlate with parental effort or reproductive output. However, the authors suggest that findings may vary depending on how complexity is measured, and that their lack of findings could be an artefact of a small sample size or a short study period (Hofstad et al. 2002).

Although the study conducted by Hofstad et al. (2002) is interesting, there are a number of improvements which could be made. Firstly, birds were not banded and as such individual identification relied solely on song content. Although male Snow Buntings sing individually distinctive songs, songs are occasionally nearly identical between neighbours (Tinbergen 1939). Thus, individual marking of birds (via distinctive colour-banding) would significantly strengthen identification ability and certainty. Secondly, many males received a song rate score of zero songs/minute if they did not sing during recording sessions. While these song rates may certainly reflect performance during the specific recording sessions in this study, these rates are not representative of the rate at which birds sing during actual song bouts. As such, a measure of zero songs per minute could be a significantly biased underestimate of a given male's true song performance potential. Lastly, Hofstad et al. (2002) did not examine possible mechanisms underlying their observed relationships. As such, we know little about the

evolutionary pressures shaping song content and performance in this species. Thus, the relationship between Snow Bunting song and reproduction warrants further investigation, with particular attention paid to the proximate reason(s) for the presence of inter-individual variation in song.

Research Questions

My research explores a potential proximate mechanism underlying inter-individual variation in song content and performance in Snow Buntings and investigates how this variation relates to fitness-related measures. Specifically, I examine whether Snow Buntings can be discriminated between using song content. I then investigate two hypotheses concerning oxidative stress and signal expression to understand the underlying mechanism driving individual variation in song content and performance: (1) Following from the Oxidation Sensitivity Hypothesis, I predict that oxidative stress negatively affects song content measures, potentially as a result of impaired neurogenesis, (2) Following from the Oxidation Handicap Hypothesis, I predict that the cost of enhanced reactive oxygen species production or reduced anti-oxidant capacity, either through testosterone or increased activity, ensures the honesty of song performance. Finally, my research investigates the relationship between Snow Bunting song and reproductive measures attempting to elucidate a link between signal quality and fitness. To the best of my knowledge, my thesis is the first to use any behavioural signal to investigate either the Oxidation Sensitivity Hypothesis or the Oxidation Handicap Hypothesis and adds to an emerging body of literature examining the

mechanisms behind complex signals, as well as to oxidative stress ecological research in general.

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Chapter 1 Appendix – Glossary of Terms

The following terms are defined specifically for use in this thesis:

Physiology Terms:

Reactive oxygen metabolites (ROM): An early marker of oxidative damage caused by reactive oxygen species which damage biomolecules such as proteins, lipids, and DNA, if not neutralized by anti-oxidants. In this thesis, I measured reactive oxygen metabolites, primarily hydroperoxides, in plasma.

Total anti-oxidant capacity (TAC): A measure of an individual's enzymatic and non-enzymatic anti-oxidant capacity to combat reactive oxygen species. In this thesis, I assessed total anti-oxidant capacity as the plasma's ability to absorb hypochlorous acid, a powerful pro-oxidant.

Oxidative status: The balance between oxidative damage and anti-oxidant capacity, assessed in this thesis as the ratio between reactive oxygen metabolites and total anti-oxidant capacity.

Song Terms

Song content: A measure of the make-up of song, assessed in this thesis as song structure and complexity (see below).

Song structure: A component of song content related to the temporal and frequency components of song. In this thesis, I assessed song structure by measuring song length and note duration.

Song complexity: A component of song content related to the number and kinds of sounds produced by an animal within songs. In this thesis, I assessed song complexity by measuring syllable repertoire size and song versatility.

Song performance: A measure of a bird's singing behaviour in terms of output; assessed in this thesis using song rate.

Fitness-related Terms

Arrival date: The day a bird arrived at the study site from migration; approximated in this thesis using banding date. Geolocator data has confirmed that banding date is an appropriate estimate of arrival date.

Clutch size: The number of eggs in a nest upon clutch completion.

Fledglings: Nestlings that successfully leave the nest; calculated on day 7 or 8 of post-natal development, as birds are sufficiently active by day 9 to leave the nest.

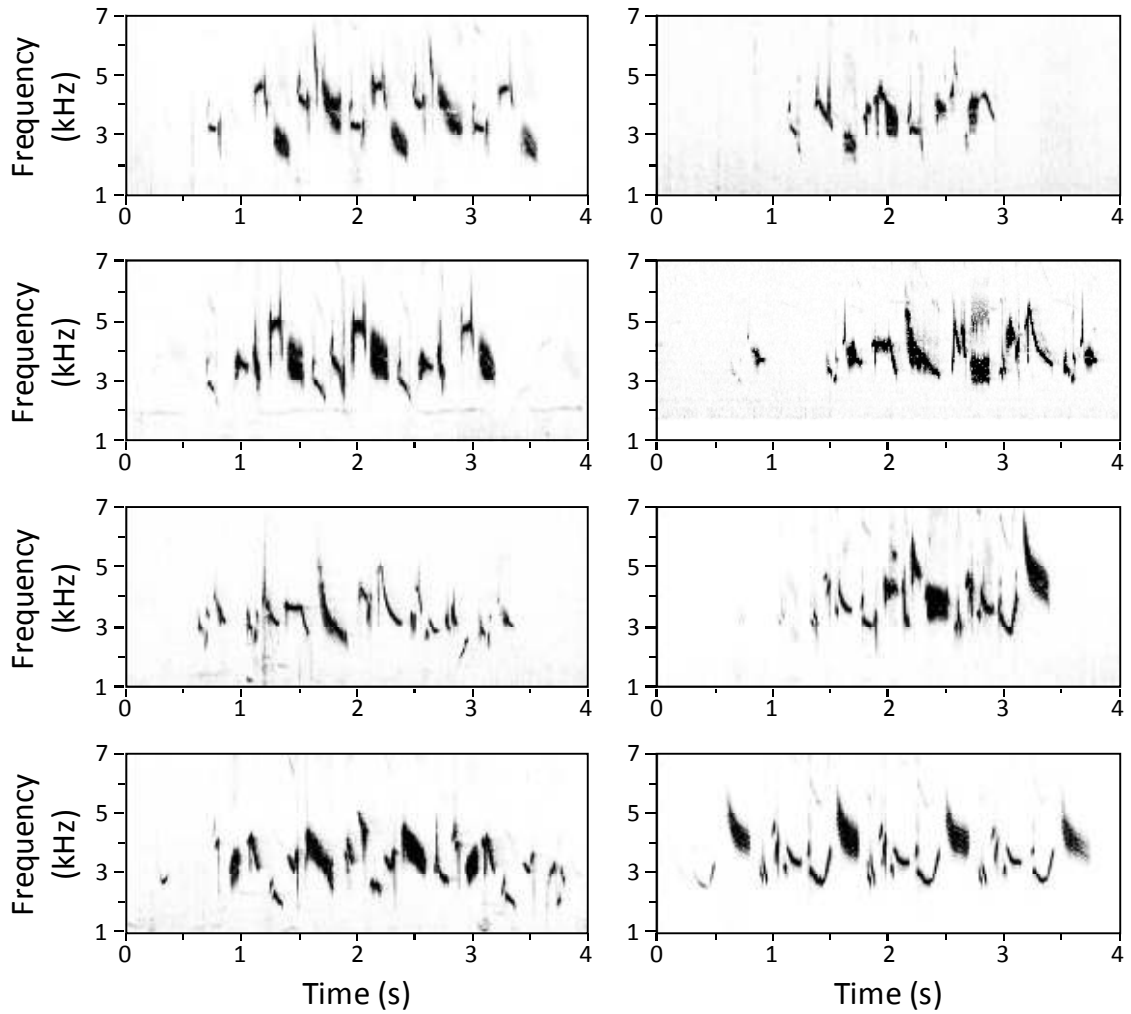


Fig. 1.1 Each male Snow Bunting produces a single song type which is individually distinctive. Shown here are sound spectrograms of eight males' songs recorded on East Bay Island, Nunavut.

Chapter 2

Song performance as an honest indicator of oxidative damage and anti-oxidant capacity: support for the Oxidation Handicap Hypothesis

This chapter is the outcome of joint research and is co-authored with Sarah Guindre-Parker, Grant Gilchrist, Daniel Mennill, and Oliver Love. Sarah Guindre-Parker aided in data collection and analysis. Grant Gilchrist helped with fieldwork and provided extensive logistical support. Daniel Mennill and Oliver Love contributed substantially to data analysis and interpretation, and with writing, and provided financial support.

Chapter 2 – Song performance as an honest indicator of oxidative damage and anti-oxidant capacity: support for the Oxidation Handicap Hypothesis

Chapter Summary

Elaborate secondary sexual signals are used by males across a wide variety of species for mate attraction and the quality of these signals is often condition-dependent. Variation in signal quality can have a substantial effect on male reproductive success, as females can use this variation to assess male quality and make reproductive choices. Bird song is a mate attraction signal, and both song content and performance have been shown to accurately reflect various aspects of male quality, potentially providing valuable information to prospective mates. Fairly recently, oxidative stress has been proposed as a mechanistic basis for ensuring the honesty of many secondary sexual signals, including bird song. We investigated two hypotheses which relate oxidative stress to signal quality: the Oxidation Sensitivity Hypothesis and the Oxidation Handicap Hypothesis. Our results do not support the Oxidation Sensitivity Hypothesis, as song content was unrelated to oxidative stress. However, song performance reflected oxidative damage and total anti-oxidant capacity, supporting the Oxidation Handicap Hypothesis; males that sang at a faster rate had higher levels of reactive oxygen metabolites as well as higher anti-oxidant capacities, allowing them to avoid increased levels of oxidative stress. Song content and song performance did not relate to reproductive measures, although future studies incorporating extra-pair paternity and offspring recruitment would yield more comprehensive fitness estimates. This research investigates oxidative status as a potential mechanism shaping bird song, and suggests that oxidative stress

may be an underlying physiological cost preventing low quality individuals from producing high quality signals.

Introduction

The theory of sexual selection explains the adaptive advantage of producing seemingly maladaptive, conspicuous signals; individuals producing the most elaborate ornaments and displays often achieve the highest reproductive success (Darwin 1871; Andersson 1994). Many secondary sexual signals are thought to be effective at attracting mates or repelling rivals because they are condition-dependent (Andersson 1994; Garratt and Brooks 2012), condition being defined as a physiological state “that relates to health, vigour, acquired resources and ultimately reproductive value” (Garratt and Brooks 2012). As such, condition-dependent signals can be important components of mate choice allowing females to accurately assess the direct and indirect benefits individual males may provide (Darwin 1871; Andersson 1994; Garratt and Brooks 2012).

Zahavi (1975) proposed the handicap principle to explain how the honesty of secondary sexual signals might be ensured. If signal production or maintenance imposes a cost on the signaller, and that cost increases with signal expression, then individuals with more elaborate signals are demonstrating their ability to afford larger handicaps (Zahavi 1975). Alternatively, a signal may still be honest and condition-dependent even if it is not costly per se; ‘index signals’ are honest signals because signal production is physically or physiologically constrained, independent of any cost of signal production (Smith and Harper 1995; Vehrencamp 1999; Hill 2011). Recently, there has been a growing emphasis placed on integrating function and mechanism, as researchers recognize how integrating ultimate and proximate questions enhances our

understanding of behavioural ecology (Ricklefs and Wikelski 2002; McNamara and Houston 2009; MacDougall-Shackleton 2011).

Bird song is a well-known sexual signal (Tinbergen 1939; Smith 1959; Marler 1970a; Catchpole and Slater 2008) and is composed of a large number of traits that make it an ideal model for examining the underlying mechanisms driving the honesty of sexual signals. Much observational and experimental evidence confirms that bird song plays a critical role in mate attraction, and certain song features play a particularly important role, such as song length (Neubauer 1999; Lampe and Espmark 2003; Nolan and Hill 2004), repertoire size (Hiebert et al. 1989; Reid et al. 2004; Hasselquist et al. 1996), and song versatility (Järvi 1983; Poesel et al. 2001; Lampe and Espmark 2003). In addition to song content, song performance, most often measured as song rate (Berg et al. 2005), also appears important for mate attraction (Radesäter et al. 1987; Nolan and Hill 2004; Murphy et al. 2008). In general, many female songbirds demonstrate a preference for increased complexity and increased output (Nowicki et al. 2002) and many studies have revealed inter-individual variation in song content and song performance (e.g. Espmark 1995; Otter et al. 1997), providing the basis for female choice and subsequent variation in male reproductive success.

The Order Passeriformes, or songbirds, can be broken down into two subdivisions: the oscine passerines and the suboscine passerines (Gill 2007). One main differentiation between oscine passerines and suboscine passerines is that oscines must hear conspecific tutors to learn normal adult song, whereas suboscines can develop

normal adult song without tutoring (Gill 2007). Most oscine passerines are thought to learn songs early in life (Catchpole and Slater 2008; Beecher and Brenowitz 2005) and various factors affect the quality of song development including social, environmental, and physiological factors (e.g. tutor availability and interaction: Baptista and Morton 1981; nutritional stress: Nowicki et al. 2002; testosterone concentration: Korsia and Bottjer 1991). Like content, song performance is also affected by various factors, again including social, environmental, and physiological factors (e.g. neighbour aggressiveness: Hyman and Hughes 2006; temperature: Reid 1987; testosterone concentration: Ritschard et al. 2011). Ritschard et al. (2011) suggested that measures of song content may better represent an individual's past condition because song content is largely established early in life, whereas measures of song performance may better represent an individual's current condition, as it is more flexible.

A condition-related mechanism with a strong potential to explain the honesty of song as a sexual signal, and hence the basis for individual variation in signal quality, is an individual's oxidative status (i.e. the balance between reactive oxygen species and anti-oxidant capacity; von Schantz et al. 1999; Alonso-Alvarez et al. 2007; Metcalfe and Alonso-Alvarez 2010; Garratt and Brooks 2012). Pro-oxidants are by-products of normal cellular metabolism and are also produced by the immune and detoxification systems to destroy pathogens and toxins (Finkel and Holbrook 2000; Costantini and Dell'Omo 2006). Oxidative stress results when the production of pro-oxidants, or reactive oxygen species, overwhelms an individual's anti-oxidant capacity and ability to avoid negative oxidative effects (Sies 1985, 1991; Monaghan et al. 2009). Oxidative stress has been

linked to a number of diseases in humans, in addition to cellular and whole organism senescence (Sies 1985, 1991; Finkel and Holbrook 2000). Although a longstanding focus of biomedical research, oxidative stress has only recently become a focus in ecological studies, with a relatively new field of oxidative stress ecology emerging over the past several decades (Metcalfe and Alonso-Alvarez 2010; Beaulieu et al. 2010; Hausmann et al. 2012). It has been argued that secondary sexual signals may be sensitive to, or 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 and Holbrook 2000).

Von Schantz et al. (1999) predicted that differences between individuals in the tolerance and resistance of their immune and detoxification systems are responsible for variation in signal quality between individuals, and that oxidative stress is a mechanism constraining signal quality. For example, a less tolerant immune system generates more reactive oxygen species, leading to higher oxidative stress, which detrimentally affects signal quality. Here we formalize this concept as the Oxidation Sensitivity Hypothesis. Alonso-Alvarez et al. (2007) later proposed the Oxidation Handicap Hypothesis, arguing that testosterone is associated with sexual signaling and also actively promotes oxidative stress as a by-product by generating increased reactive oxygen species or suppressing anti-oxidant capacity. Metcalfe and Alonso-Alvarez (2010) recently broadened this hypothesis by adding that signals that increase an individual's activity level (i.e. increased oxygen demand and metabolic activity) should also generate higher levels of reactive oxygen species, independent of testosterone. Both the Oxidation

Sensitivity Hypothesis and the Oxidation Handicap Hypothesis suggest that oxidative stress may be responsible for inter-individual variation in signal quality. Each suggests a different mechanistic pathway by which oxidative stress and signal quality are related, yet the two hypotheses are not mutually exclusive. Studies to date linking oxidative stress and secondary sexual signals have focused on visual signals, particularly carotenoid-dependent signals (Metcalf and Alonso-Alvarez 2010), and more recently melanin-based signals (Garratt and Brooks 2012). Although the authors that proposed these hypotheses have suggested that there may be a relationship between an individual's oxidative status and song (von Schantz et al. 1999; Alonso-Alvarez et al. 2007; Metcalf and Alonso-Alvarez 2010), this relationship has not yet been investigated.

Here we explore the relationship between song quality (in terms of both song content and song performance), individual oxidative status, and fitness-related measures in the Snow Bunting (*Plectrophenax nivalis*). Snow Buntings are oscine passerines which use song for both mate attraction and territory defence (Tinbergen 1939; Nethersole-Thompson 1966). We first confirm the long-held qualitative assertion that male Snow Buntings sing individually distinctive songs (Tinbergen 1939; Borror 1961; Drury 1961; Nethersole-Thompson 1966; Espmark 1995). Following this, our primary objective was to explore the Oxidation Sensitivity and Oxidation Handicap Hypotheses by investigating the relationships between song and oxidative status. We focus on song features predicted to be attractive to females, including both features related to song output and song complexity. Our song content measures were song

length, note duration, syllable repertoire size, and song versatility, and our song performance measure was song rate. According to the Oxidation Sensitivity Hypothesis, the quality of song content may be negatively affected by oxidative stress via the negative effects oxidative stress can have on neurogenesis, and therefore reactive oxygen species and oxidative stress should be higher in males with lower quality songs (i.e. shorter songs, shorter notes, smaller syllable repertoire, less versatility; Table 2.1). Following from the predictions of the Oxidation Handicap Hypothesis, we predict that song performance will be positively correlated with levels of reactive oxygen species (Table 2.1), a result of either elevated testosterone levels or the increased level of activity and oxygen consumption caused by higher song output. Importantly, males with higher song performance should be able to afford the cost of higher song rates via higher anti-oxidant levels.

Our second objective was to investigate the relationship between song and fitness-related measures using measures of song content and song performance shown to relate to female choice and reproductive success in other species. As such, we predicted a negative relationship between song quality measures and female arrival date, a proxy for pairing date. Pairing date is an important measure that can have subsequent effects on reproductive output because earlier breeding birds often achieve higher reproductive success (e.g. Lepage et al. 2000; Descamps et al. 2011). Additionally, we predicted a positive relationship between song quality measures and reproductive output, because females should preferentially chose to mate with males that will maximize their reproductive success. This research represents the first investigation of

oxidative stress as a proximate mechanism for honest song production, and attempts to couple function and mechanism by exploring the relationship between signal quality, oxidative status, and reproduction.

Methods

Study Site and Species

We studied Snow Buntings on East Bay (Mitivik) Island in Nunavut, Canada, from June to August of 2010 and 2011. This small island (800 m × 400 m) is located in the low Canadian Arctic (64°02'N, 81°47'W) and is covered largely by loose granite rock, providing ideal nesting habitat for Snow Buntings. Snow Buntings are socially monogamous, although occasionally polygyny does occur (Tinbergen 1939; Hofstad et al. 2002). Males do not assist with either nest-building or incubation (Tinbergen 1939; Sutton and Parmalee 1954), but do partake in incubation feeding and offspring provisioning, both of which are necessary to maximize a pair's reproductive success (Tinbergen 1939; Hussell 1972; Lyon and Montgomerie 1985). Male Snow Buntings use song for mate attraction and territory defence (Tinbergen 1939; Nethersole-Thompson 1966). Each male typically sings a single song type (Espmark 1995, 1999), and while syllable sharing does occur between individuals, many researchers have anecdotally noted the individuality of each male's song (Tinbergen 1939; Borror 1961; Drury 1961; Nethersole-Thompson 1966; Espmark 1995).

Trapping, Banding, and Blood Sampling

We trapped Snow Buntings on arrival from spring migration using baited ground traps (Potter and walk-in) and banded each bird with an aluminum Canadian Wildlife Service band and three colour bands to facilitate individual identification. We took standard morphometric measurements (mass, wing chord, tarsus length) and determined sex and age using plumage characteristics (Montgomerie and Lyon 2011). We collected blood samples from the brachial veins of all breeding male Snow Buntings using heparinised microcapillary tubes between June 9 and June 24 in 2010, and between June 26 and July 14 in 2011, during the egg-laying or incubation period. We centrifuged whole blood (10,000 RPM for 10 minutes) within an hour of collection and stored plasma at -20°C in the field and at -80°C in the laboratory until analyses. All animal work used Canadian Council on Animal Care-approved techniques under Animal Care Committee approval from Environment Canada (Canadian Wildlife Service permits: NUN-SCI-08-04 and NUN-SCI-11-05) and the University of Windsor (permit: 09-14).

Reproductive Measures

We used female arrival date (first trapping date) as a proxy for pairing date, implying female mate choice under the assumption that the earliest-arriving females have the choice of the highest quality males to pair with and that breeding earlier is advantageous (e.g. Lepage et al. 2000; Descamps et al. 2011). Geolocation studies in 2010-12 indicate that the first trapping date is a highly accurate measure of female arrival (i.e. within hours; Macdonald and Love, unpubl. data). We located all Snow

Bunting nests in 2010 and 2011, and noted clutch size and the number of nestlings reaching day 7 or 8 of post-natal development, which we used as an estimate of the number of fledglings as nestlings are sufficiently active to leave the nest by day 9 (Hussell 1985). There were 26 nests where we counted the number of fledglings directly. For 1 nest, we visited the nest too late and the nestlings had already fledged. For this nest we estimated the number of fledglings based on the average loss between hatchlings and fledglings calculated from nests where both values were known (average loss between number of hatchlings and number of fledglings within a nest was one nestling; mean: 1.1 ± 0.2 nestlings, $n=26$ nests from 2010 and 2011).

Song Recording

In June and July 2010 and 2011, 24 different males were recorded using a directional microphone (Sennheiser K6/ME66) and a solid-state digital recorder (model: Marantz PMD 660; recording settings: 44,100 Hz sampling rate, 16 bits, WAVE format). Three males were recorded in both years. Focal recording took place opportunistically, but the majority of focal recording was conducted in the morning. Male identification (i.e. colour band combination) was noted whenever bands were visible. In 2011, we supplemented directional-microphone recordings with 24-hour recordings obtained at nine recording locations spread across the island using autonomous digital recorders (model: SM2-GPS; recording settings: 22,050 Hz sampling rate, 16 bits, WAVE format).

Analysis of Song Content

All recordings were filtered to remove background noise outside of the range of Snow Bunting song (high-pass frequency: 867 Hz; low-pass frequency: 13,939 Hz). Recordings were normalized to -3 dB in Audition (Adobe, San Jose, CA, USA) prior to analysis. We performed detailed measurements of song structure (i.e. length and frequency measures) in Avisoft SAS Lab Pro (R. Sprecht, Berlin, Germany) using the 'automated parameter measurement' tool which minimizes human measurement error and subjectivity (temporal resolution: 5.8 ms; frequency resolution: 86 Hz). Syllable counts and typing were determined by visual inspection; syllables are elements or several elements that fall out into clear groupings, and syllables that looked different from one another were considered to be different types. The following song features were measured: (1) *song length*, calculated as the time elapsed from the start to the end of a song (sec); (2) *note duration*, calculated as the sum of all note lengths (sec); (3) *total number of syllables*, calculated as the total number of syllables within a song; (4) *number of different syllables*, calculated as the number of unique syllable types within a song; (5) *minimum song frequency*, calculated as the minimum frequency of a song; (6) *maximum song frequency*, calculated as the maximum frequency of a song; (7) *average minimum syllable frequency*, calculated as the average minimum syllable frequency within a song; (8) *average maximum syllable frequency*, calculated as the average maximum syllable frequency within a song; (9) *syllable repertoire*, calculated as the total number of unique syllables produced across a sample of 10 songs; and (10) *song*

versatility, calculated as the number of unique syllables in a sample of 10 songs divided by the total number of syllables in the same sample of 10 songs.

Most content measures were determined from a sample of 10 songs for each male (following from Espmark et al. 1995, 1999; Hofstad et al. 2002). To measure syllable repertoire size and song versatility, songs did not need to be of extremely high quality because the 'automated parameter measurement' tool was not used; 21 males had at least 10 songs of sufficient quality to assess song complexity. However, because the 'automated measurement tool' was used to measure length and frequency measures, in those cases songs had to be of very high quality (i.e. high signal-to-noise ratio). In a few cases (n=2), we recorded fewer than 10 songs of high enough quality to be measured using the 'automated measurement tool'. Consequently, on average, 9.7 ± 0.2 songs per individual were analysed to measure structural traits (i.e. song length and note duration: range=6-10 songs/individual).

Analysis of Song Performance

Song rate was calculated as the number of songs divided by recording length (min). Estimates of song rate were based on song bouts with a minimum of 7 songs (mean: 16.1 ± 1.0 songs, range: 7-71 songs), and calculated over multiple 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 days, range: 3-5 days). A song bout was considered finished when a period of silence longer than 60 seconds began or the focal male flew away. In 2010, all song rates were

calculated from focal recordings; in 2011, song rates were calculated from both focal and autonomous recordings (individual identity in autonomous recordings was readily assessed using both song content and recording location). Song rates were assessed from recordings obtained over an approximate two-week period (in 2010, between June 10 and June 28; in 2011, between June 16 and June 30), and between 02:30 and 11:30 in 2010, and 02:30 and 23:30 in 2011, although most recordings were obtained before 10:00 in both years (88% of recordings in 2010 and 90% of recordings in 2011). We used Syrinx-PC (J. Burt, Seattle, WA, USA) to assess song rate.

Testosterone Assay

Dichloromethane extractions were performed to extract testosterone from plasma samples using a modified protocol from Wingfield and Farner (1975; mean extraction efficiency: $71.4 \pm 27.9\%$, $n=16$). We measured plasma testosterone in triplicate using a previously validated commercially-available enzyme-linked immunoassay (Cayman Chemicals, USA, Item No. 582701; Pryke et al. 2007) optimized in-house for Snow Buntings. Briefly, 50 μ l of plasma was added to each sample well, combined with testosterone AChE tracer (50 μ l), except for the Total Activity and Blank wells, and testosterone EIA antiserum (50 μ l) was then added. Plates were incubated for two hours at 25°C and then emptied and rinsed five times with Wash Buffer. Ellman's Reagent (200 μ l) was added to all wells and tracer (5 μ l) was added to Total Activity wells. Plates were incubated for one hour at 25°C and read at a wavelength of 412 nm. Mean intra- and inter-assay coefficients of variation were 7.7% and 10.6%, respectively.

Reactive Oxygen Metabolite and Total Anti-oxidant Capacity Assays

Our measure of pro-oxidants was reactive oxygen metabolites (ROM) which we measured in plasma samples using a previously validated d-ROM kit (Diacron, Grosseto, Italy; Costantini et al. 2006) modified for 96-well microplates and optimized for avian plasma. Reactive oxygen metabolites are derivatives of reactive oxygen species which are more easily measured than reactive oxygen species because they are more stable. The test primarily measures the concentration of hydroperoxides, which are the resulting metabolites of lipid peroxidation and provide an early marker of oxidative damage. Plasma samples 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 minutes. Tubes were centrifuged at 10,000 RPM for 30 seconds and 290 μL of the supernatant pipetted into a new tube. Tubes were then vortexed and 125 μL was pipetted into the plate in duplicate. Plates were subsequently read at a wavelength of 490 nm. Concentrations were determined using a standard curve (range: 0.28125–18 mg $\text{H}_2\text{O}_2/\text{dL}$) generated using a stock solution of 18 mg $\text{H}_2\text{O}_2/\text{dL}$; colour intensity is proportional to the concentration of reactive oxygen metabolites and measurements are expressed in mg of $\text{H}_2\text{O}_2/\text{dL}$. Mean intra- and inter-assay coefficients of variation were 4.9% and 3.0%, respectively.

The total anti-oxidant capacity (TAC) of plasma samples was measured using a previously validated OXY-adsorbent kit (Diacron, Grosseto, Italy; Costantini et al. 2006)

modified for 96-well microplates and optimized for avian plasma. The OXY-adsorbent test measures a sample's ability to "absorb" and quench 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 seconds at 450 RPM and incubated at 37°C for 10 minutes. Before reading at 490 nm, 2 μL of chromogenic solution was added to each well and plates were shaken for 30 seconds by a plate reader. Concentrations were determined using a standard curve (range: 0.4702–5.7500 $\mu\text{mol HClO/mL}$) generated from a stock solution of 230 $\mu\text{mol HClO/mL}$. The chromogenic solution changes colour upon reacting with the remaining HClO. Therefore, colour intensity is inversely proportional to the plasma's anti-oxidant capacity (i.e. ability to neutralize the acid). Measurements were multiplied by 100 to account for dilution and are expressed in $\mu\text{mol of HOCl/mL}$ neutralized. Mean intra- and inter-assay coefficients of variation were 5.1% and 0.9%, respectively.

Oxidative status was calculated using ROMs/TAC \times 1000 (an index of oxidative stress used by Costantini et al. 2006), where higher values equate to higher oxidative stress.

Statistical Analyses

We recorded 24 different male Snow Buntings in 2010 and 2011. Three males were recorded in both years so one year of data was excluded for each male (for two birds the recording quality was markedly better in one year so data from years with poorer

quality were excluded; a random number generator determined which year of data to exclude for the third bird). Sample sizes for our analyses vary, as indicated below, due to the fact that we were not able to obtain all acoustic, hormonal, and fitness measures for all males.

To examine whether Snow Bunting songs can be discriminated on an individual basis, we conducted a canonical discriminant function analysis to test whether songs could be correctly assigned to the males which produced them using the following eight content measures: song length, note duration, total number of syllables, number of different syllables, minimum song frequency, maximum song frequency, average minimum syllable frequency, and average maximum syllable frequency. The analysis was based on 10 songs from each of 19 males recorded in 2010 and 2011; only 19 males were used because we excluded males with fewer than 10 high quality songs recorded. We ran the discriminant analysis using a cross-validation approach. We randomly selected 75% of the data and performed the discriminant analysis on these data. We then tested the accuracy of the discriminant analysis on the remaining 25% of the data.

To investigate the Oxidation Sensitivity Hypothesis and the Oxidation Handicap Hypothesis, we first ran a principal component analysis, with varimax rotation, to summarize the four song content variables which we predicted females may be attentive to as they relate to output and complexity. Details of the principal component analysis, including the loadings of the four song content variables on the two principal component factors that had eigenvalues > 1.0 , are given in Table 2.2. Factor 1

summarized song length and note duration and explained 53% of the variance; higher Factor 1 scores correspond to longer songs with greater note duration, and we labelled this factor *song structure*. Factor 2 summarized syllable repertoire and song versatility and explained an additional 44% of the variance; higher Factor 2 scores correspond to larger syllable repertoires with higher song versatility. We labelled this second principal component *song complexity*. Song rate was not included in this principal component analysis because we were interested in analyzing song content (i.e. the above principal component factors) and song performance (i.e. song rate) separately. In the end, we had three song measures: two principal component factors that summarized variation in song structure and complexity providing a measurement of song content, and song rate providing a measurement of song performance.

We used standard least squares models to test for relationships between oxidative status measures and song structure (Factor 1). We included year as a fixed effect in these analyses because the variables that loaded onto Factor 1 showed significant differences between years. We used linear regressions to explore the relationships between oxidative status measures and song complexity (Factor 2), as the variables that loaded onto Factor 2 did not differ between years. We used standard least squares models to investigate the relationships between song rate and oxidative status measures, with testosterone level included as a covariate, given it has been shown to relate to both song rate and oxidative status measures.

We used standard least squares models to test for relationships between song measures and relative female arrival date (i.e. female arrival date relative to mean intra-annual arrival dates), our proxy for pairing date. We included relative male arrival date (i.e. male arrival date relative to mean intra-annual arrival dates) in all models including relative female arrival date to control for residual male quality independent of song. We used ordinal logistic models to explore the relationships between song measures and reproductive output (i.e. clutch size and number of fledglings). We controlled for year in all ordinal logistic models including Factor 1, as the variables that went into creating Factor 1 differed significantly between years. We included relative hatch date (i.e. hatch date relative to mean intra-annual hatch dates) in all models with number of fledglings because laying/hatching date can influence fledging success (Lepage et al. 2000; Descamps et al. 2011). All statistical analyses were performed in JMP v. 9.0.2. (SAS Institute, Cary, NC).

Results

Individual Distinctiveness of Songs

The discriminant analysis confirmed that male Snow Buntings sing individually-distinctive songs (Fig. 2.1), with cross-validation assigning 91.5% of songs to the correct male, significantly higher than the 5.3% expected by chance for 19 males (binomial test, $p < 0.0001$). Variables that contributed most strongly to the first canonical axis were 'average minimum syllable frequency' and 'average maximum syllable frequency', and this axis explained 32% of the variation between males' songs; variables that

contributed most strongly to the second canonical axis were 'number of different syllables' and 'average maximum syllable frequency', and this axis explained 27% of the variation between males' songs.

The Oxidation Sensitivity Hypothesis

The Oxidation Sensitivity Hypothesis states that secondary sexual signals are sensitive to oxidative stress and thus individuals experiencing oxidative stress should display lower quality signals. This led to our prediction that song structure (i.e. song length and note duration) and song complexity (i.e. syllable repertoire and song versatility) would be negatively related to reactive oxygen metabolites and oxidative stress (Table 2.1).

However, in our analysis of Snow Bunting song content we found no significant relationship between reactive oxygen metabolites and song structure (Factor 1; whole model: $F_{2,15}=1.8$, $p=0.20$, $n=18$; year: $F_{1,1}=3.2$, $p=0.09$; ROM: $F_{1,1}=0.7$, $p=0.42$; Fig. 2.2), total anti-oxidant capacity and song structure (Factor 1; whole model: $F_{2,16}=0.7$, $p=0.51$, $n=19$; year: $F_{1,1}=1.4$, $p=0.25$; TAC: $F_{1,1}=0.3$, $p=0.61$; Fig. 2.2), or oxidative stress and song structure (Factor 1; whole model: $F_{2,14}=2.0$, $p=0.17$, $n=17$; year: $F_{1,1}=3.6$, $p=0.08$; oxidative stress: $F_{1,1}=1.9$, $p=0.19$; Fig. 2.2). We also found no relationship between reactive oxygen metabolites and song complexity (Factor 2; ROM: $R^2=0.07$, $p=0.28$, $n=18$; Fig. 2.2), total anti-oxidant capacity and song complexity (Factor 2; TAC: $R^2=0.002$, $p=0.84$, $n=19$; Fig. 2.2), or oxidative stress and song complexity (Factor 2; oxidative stress: $R^2=0.12$, $p=0.17$, $n=17$; Fig. 2.2). These analyses, therefore, provide no support for the Oxidation Sensitivity Hypothesis.

The Oxidation Handicap Hypothesis

The Oxidation Handicap Hypothesis states that secondary sexual signal expression may generate increased levels of reactive oxygen metabolites creating a handicap, and that only individuals able to afford this cost (via higher anti-oxidant capacities) will be able to produce and maintain high quality signals. This led to our prediction that song performance (i.e. song rate) would be positively related to both reactive oxygen metabolites and total anti-oxidant capacity (Table 2.1). Additionally, we predicted that oxidative stress levels would be unrelated or negatively related to song rate (i.e. males that sang at a high rate would be able to afford the costs of increased reactive oxygen metabolites without having higher oxidative stress; Table 2.1). We found a significant positive relationship between song performance and reactive oxygen metabolites (whole model: $F_{2,14}=2.4$, $p=0.13$, $n=17$; testosterone: $F_{1,1}=0.02$, $p=0.65$; song rate: $F_{1,1}=4.7$, $p=0.049$; Fig. 2.3), and between song performance and total anti-oxidant capacity (whole model: $F_{3,13}=4.4$, $p=0.02$, $n=17$; testosterone: $F_{1,1}=0.007$, $p=0.93$; song rate: $F_{1,1}=9.6$, $p=0.008$; Fig. 2.3). There was no significant relationship between song rate and oxidative stress (whole model: $F_{2,13}=0.5$, $p=0.64$, $n=16$; testosterone: $F_{1,1}=0.007$, $p=0.93$; song rate: $F_{1,1}=0.9$, $p=0.36$; Fig. 2.3). We, therefore, found support for the Oxidation Handicap Hypothesis.

Song and Fitness-related Measures

We found no relationship between song structure (Factor 1) and relative female arrival date (whole model: $F_{3,17}=2.37$, $p=0.11$, $n=20$; year: $F_{1,1}=0.01$, $p=0.92$; relative male

arrival date: $F_{1,1}=6.5$, $p=0.02$; Factor 1: $F_{1,1}=0.002$, $p=0.97$; Fig. 2.4), clutch size (whole model: $\chi^2=2.2$, $p=0.33$, $n=17$; year: $\chi^2=0.4$, $p=0.52$; Factor 1: $\chi^2=1.1$, $p=0.29$; Fig. 2.4), or number of fledglings (whole model: $\chi^2=13.3$, $p=0.004$, $n=13$; year: $\chi^2=0.9$, $p=0.34$; relative hatch date: $\chi^2=12.0$, $p=0.0005$; Factor 1: $\chi^2=1.9$, $p=0.17$; Fig. 2.4). We also found no relationship between song complexity (Factor 2) and relative female arrival date (whole model: $F_{2,18}=4.3$, $p=0.03$, $n=21$; relative male arrival date: $F_{1,1}=7.7$, $p=0.01$; Factor 2: $F_{1,1}=0.7$, $p=0.40$), clutch size ($\chi^2=0.2$, $p=0.63$, $n=17$; Fig. 2.4), or number of fledglings (whole model: $\chi^2=10.7$, $p=0.005$, $n=13$; relative hatch date: $\chi^2=8.7$, $p=0.003$; Factor 2: $\chi^2=1.6$, $p=0.21$; Fig. 2.4). Finally, we found no relationship between song rate and relative female arrival date (whole model: $F_{3,15}=8.2$, $p=0.004$, $n=19$; relative male arrival date: $F_{1,1}=9.7$, $p=0.007$; song rate: $F_{1,1}=2.8$, $p=0.11$; Fig. 2.4), clutch size ($\chi^2=1.3e-5$, $p=1.0$, $n=15$; Fig. 2.4), or number of fledglings (whole model: $\chi^2=5.5$, $p=0.06$, $n=11$; relative hatch date: $\chi^2=4.9$, $p=0.03$; song rate: $\chi^2=0.01$, $p=0.93$; Fig. 2.4).

Discussion

Male Snow Buntings sing individually distinctive songs and males performing at higher rates exhibit higher levels of reactive oxygen metabolites and higher total anti-oxidant capacities, but not oxidative stress, than males performing at lower rates. These results suggest that males that sing at higher rates may be bearing the cost of increased reactive oxygen species, but that they have higher or up-regulated anti-oxidant capacities which allow them to afford singing at a faster rate without suffering the costs of elevated levels of oxidative stress. This is in line with Vehrencamp's (1999) assertion

that song rate is likely a quality handicap, whereby some cost prevents low quality males from singing at a high rate. To the best of our knowledge, our investigation is the first to provide support for the Oxidation Handicap Hypothesis using a behavioural secondary sexual signal; previous studies investigating the relationship between oxidative status and signal quality have focused on morphological signals (e.g. Pike et al. 2007; Galván and Alonso-Alvarez 2008; Alonso-Alvarez et al. 2010). Furthermore, this study lends support to the idea that sexually-selected signals can be reliable indicators of individual quality, the honesty of which is ensured via physiological costs that are not equally affordable to all individuals.

Although testosterone and song performance have been found to be associated in some passerine birds (e.g. Johnsen 1998; Van Roo 2004; Foerster et al. 2002; Ritschard et al. 2011; but see Kunc et al. 2006), we found no effect of testosterone on song rate when we included it in our Standard Least Squares Models. As such, we found no evidence for the notion that testosterone causes an increase in reactive oxygen metabolites or enhances signal expression, a prediction of the original Oxidation Handicap Hypothesis (Alonso-Alvarez et al. 2007). There are a number of possible explanations for this. First, testosterone may be unrelated to song production in Snow Buntings. We find this possibility unlikely because Snow Bunting song output mirrors gonadal growth (P. Johnsen 1953 in Nethersole-Thompson 1966) and testosterone implants have been shown to enhance singing in male Snow Buntings during periods of low testosterone (Romero et al. 1998). However, testosterone may be important for

stimulating song production, but once a threshold is reached, the exact concentration of testosterone may be unrelated to variation in song production (Hews and Moore 1997).

Alternatively, because blood samples collected in 2010 were not all obtained within ten minutes of capture, testosterone levels could have been affected by capture stress (Wingfield et al. 1982; Deviche et al. 2010; but see Hasselquist et al. 1999). All samples in 2011 used for testosterone analyses were obtained in less than 10 minutes, and in this restricted subset the effect of testosterone on reactive oxygen metabolites approached significance, suggesting that capture stress may be a confounding effect. Another methodological issue may be that a male's testosterone concentration was calculated from a single sample, which may not be an adequate representation of an individual's testosterone level, because testosterone levels change with time of day (Foerster et al. 2002) and the behavioural state of an individual (Wingfield et al. 1990). The relationship between testosterone and behaviour is complex and many researchers have found contradictory patterns on what factors affect testosterone levels and also how testosterone subsequently affects behaviour (Kempnaers et al. 2008), warranting further investigation. Regardless, Metcalfe and Alonso-Alvarez (2010) noted that testosterone need not be a driver of the relationship between signal expression and oxidative costs if energetically-costly signals (i.e. those which require increased oxygen demand, such as song production; Oberweger and Goller 2001) generate higher levels of reactive oxygen species.

We found no support for the Oxidative Sensitivity Hypothesis, as song content measures were unrelated to reactive oxygen metabolites and level of oxidative stress. However, song structure or complexity may still be sensitive to oxidative stress. Most oscine passerines learn song during their first year of life (e.g. *Taeniopygia guttata*; Immelmann 1969; *Zonotrichia leucophrys*; Marler 1970b; *Fringilla coelebs*; Nottebohm 1970; Catchpole and Slater 2008; but see Beecher and Brenowitz 2005). Unfortunately, no information on song learning in Snow Bunting exists. However, if Snow Bunting song is learned early in life, as we expect based on the widespread pattern in other temperate oscine Passeriformes, then song content could be related to the oxidative stress levels experienced during song development but not the oxidative stress levels experienced as an adult. Longitudinal studies are necessary to determine when Snow Bunting song development occurs and whether oxidative status measures are repeatable across an individual's life.

Although many studies have demonstrated a link between song rate and pairing date (e.g. Mountjoy and Lemon 1996; Buchanan and Catchpole 1997), we found no relationship between Snow Bunting song rate and female arrival date, which we used as a proxy for pairing date. Perhaps song rate does not relate to pairing date, although Hofstad et al. (2002) found that song rate was negatively related to laying date. Alternatively, female arrival date may be an inappropriate proxy for pairing date. We also found no significant relationship between any song measure and clutch size. In laboratory experiments, song quality has been shown to differentially stimulate egg production in Common Canaries (*Serinus canarius*; Kroodsma 1976). However,

Kroodsma (1976) exposed females to a single stimulus type (low or high quality), while in a natural environment, females are able to hear many males simultaneously. We also found no relationship between any song measure and number of fledglings. High breeding density, like that found on East Bay Island, likely provides ample opportunity to obtain extra-pair fertilizations. Consequently, our measure of apparent reproductive success may not be representative of a male's actual reproductive success. Future work should assess extra-pair paternity to determine a male's true, genetic reproductive success. Also, measuring offspring recruitment the following breeding season may provide a more accurate fitness estimate than number of fledglings. However, the content measures we explored in our study may be unimportant to females, and could potentially be signals directed at rivals rather than mates.

Oxidative stress ecology is a relatively new field of research and as such there are many avenues in need of further investigation (Monaghan et al. 2009; Beaulieu et al. 2010; Hausmann et al. 2012). For one, longitudinal studies investigating how oxidative stress changes within an individual and in relation to signal expression over time would enhance our understanding of the possible effects oxidative stress may have on signalling and vice versa (Metcalf and Alonso-Alvarez 2010). Future studies should continue to explore signals other than visual signals, particularly those which may increase an individual's oxygen consumption, such as energetically demanding displays (Metcalf and Alonso-Alvarez 2010). An investigation of song amplitude may yield further support for the Oxidation Handicap Hypothesis, as it is a meaningful signal (Ritschard et al. 2010; Brumm and Ritschard 2011) which has been shown to relate to

oxygen consumption (Oberweger and Goller 2001). Costantini (2008) urges collaboration between ecology, biochemical, and medical disciplines, suggesting that in vivo and in vitro laboratory tests would complement ecological studies, providing a more holistic understanding of oxidative stress. As our knowledge of reactive oxygen species, anti-oxidants, and oxidative stress continues to evolve, and our ability to measure each improves, we are sure to better understand the signalling implications individual oxidative status may have.

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Table 2.1 Predictions following from the Oxidation Sensitivity Hypothesis and the Oxidation Handicap Hypothesis between song measures and reactive oxygen metabolites (ROM), total anti-oxidant capacity (TAC), and oxidative stress. – and + indicate directionality of predicted relationships; NR=no relationship; grey shading indicates no predicted relationship.

Hypothesis	Song Measure	ROM		TAC		Oxidative Stress	
		Expected	Observed?	Expected	Observed?	Expected	Observed?
Oxidation Sensitivity Hypothesis	Song structure (length and note duration)	-	✘			-	✘
	Song complexity (syllable repertoire size and song versatility)	-	✘			-	✘
Oxidation Handicap Hypothesis	Song performance (song rate)	+	✓	+	✓	NR or -	✓

Table 2.2 Factor loadings for four song content variables after varimax rotation. Bolded values indicate variables that loaded strongly onto principal component factors. Factor 1 (song structure) explained 53% of the variation and Factor 2 (song complexity) explained an additional 44% of the variation.

Song Content Variable	Factor 1	Factor 2
Song length	0.97	-0.03
Note duration	0.96	-0.05
Syllable repertoire size	0.14	0.96
Song versatility	-0.24	0.94
Eigenvalue	2.12	1.75
Percentage of variation explained	52.95	43.83
Cumulative variation explained	52.95	96.79

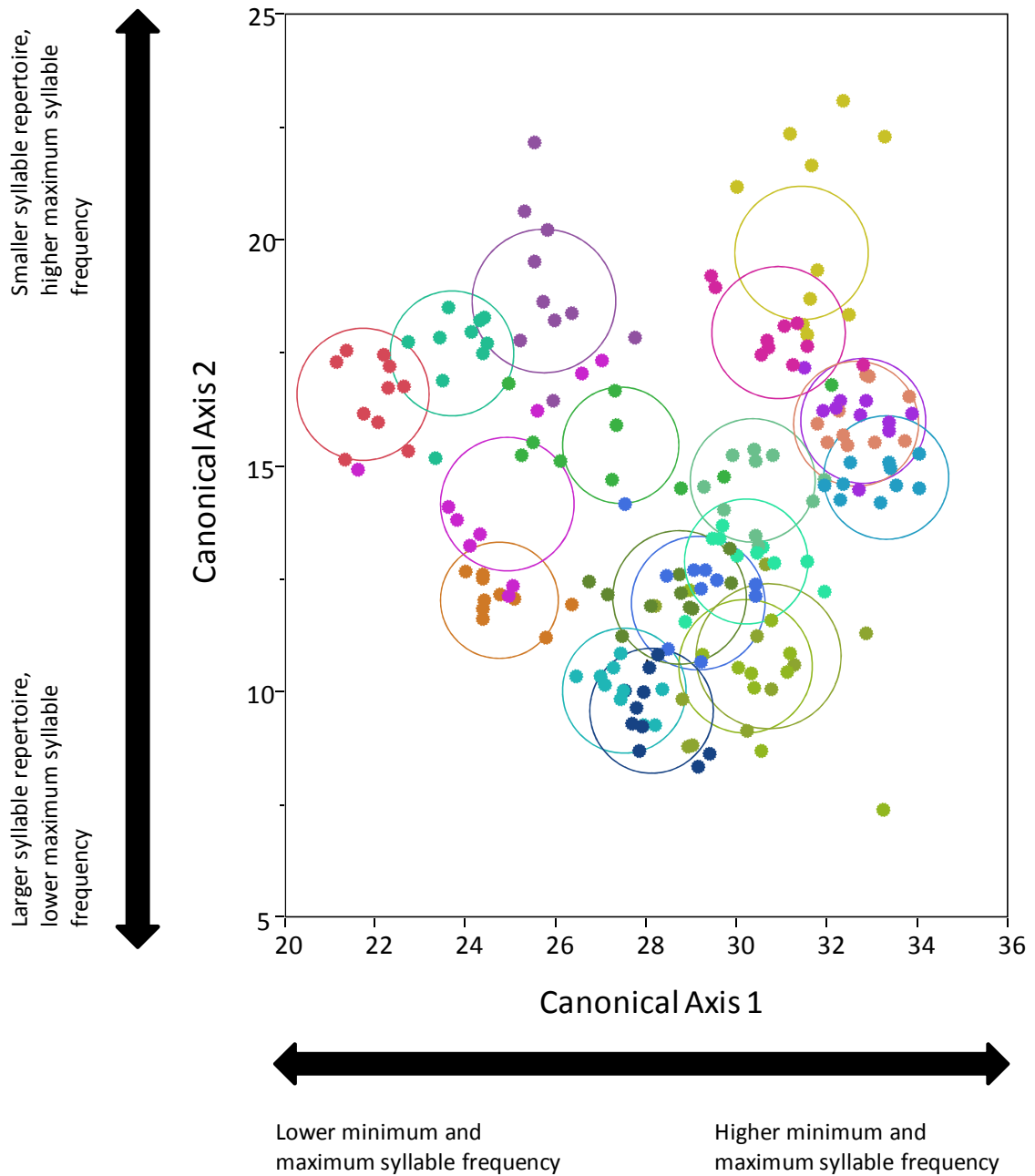


Fig. 2.1 Male Snow Buntings' songs can be accurately discriminated between using song content. Shown here are the first and second canonical axes from a discriminant analysis based on songs recorded from 19 different Snow Buntings. Circles represent the 95% confidence region containing the true mean for each male. The analysis classified 91.5% of songs correctly; binomial test, $p < 0.0001$.

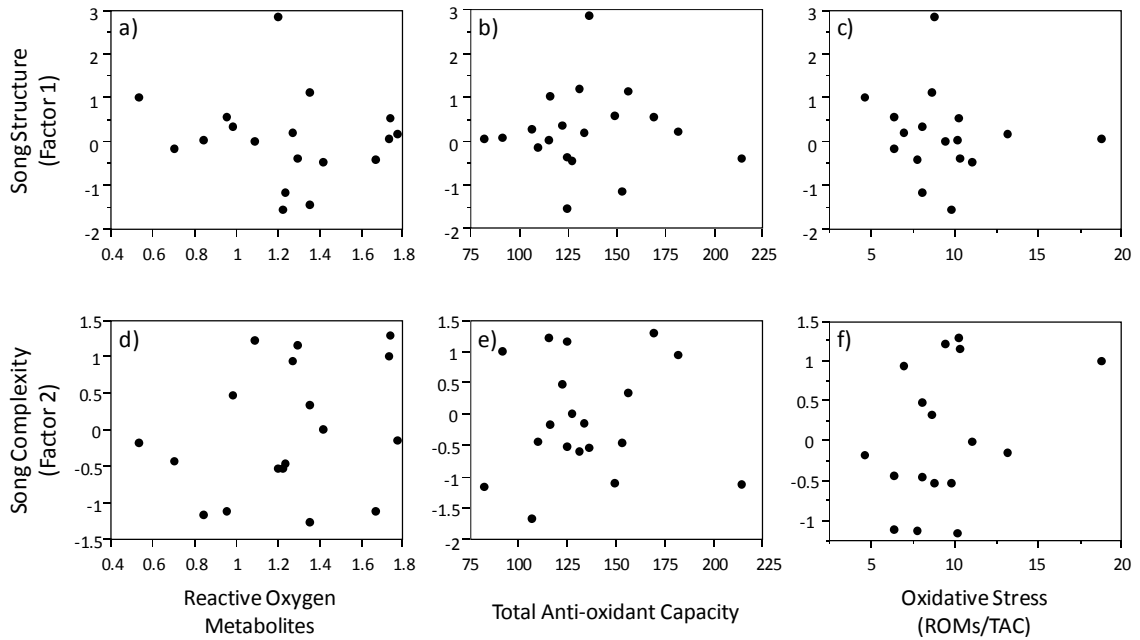


Figure 2.2 Reactive oxygen metabolites (ROM; mg H₂O₂/dL), total anti-oxidant capacity (TAC; μmol of HOCl/mL neutralized), and oxidative stress were unrelated to a-c) song structure or d-f) song complexity, providing no support for the Oxidation Sensitivity Hypothesis. See Results for statistical details.

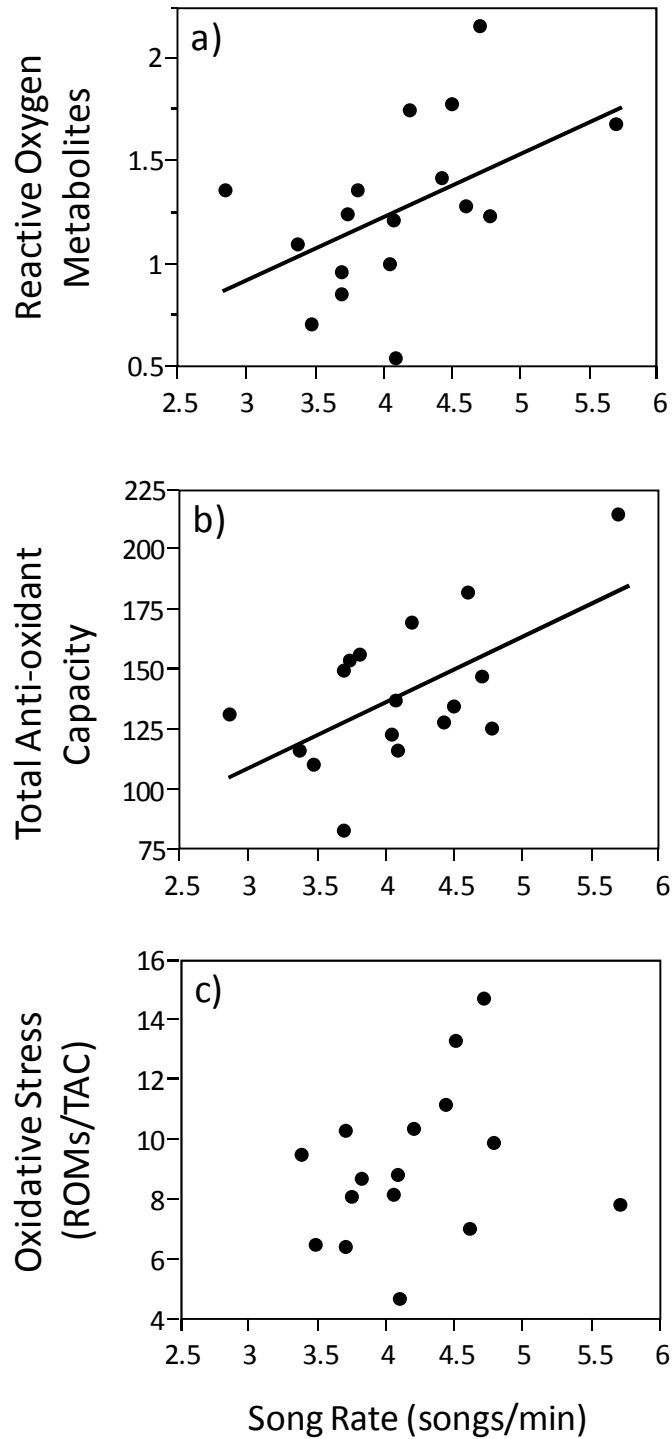


Figure 2.3 Song rate (songs/min) was positively related to a) reactive oxygen metabolites (ROM; mg H₂O₂/dL) and b) total anti-oxidant capacity (TAC; μmol of HOCl/mL neutralized), but unrelated to c) oxidative stress, supporting the predictions of the Oxidation Handicap Hypothesis. See Results for statistical details.

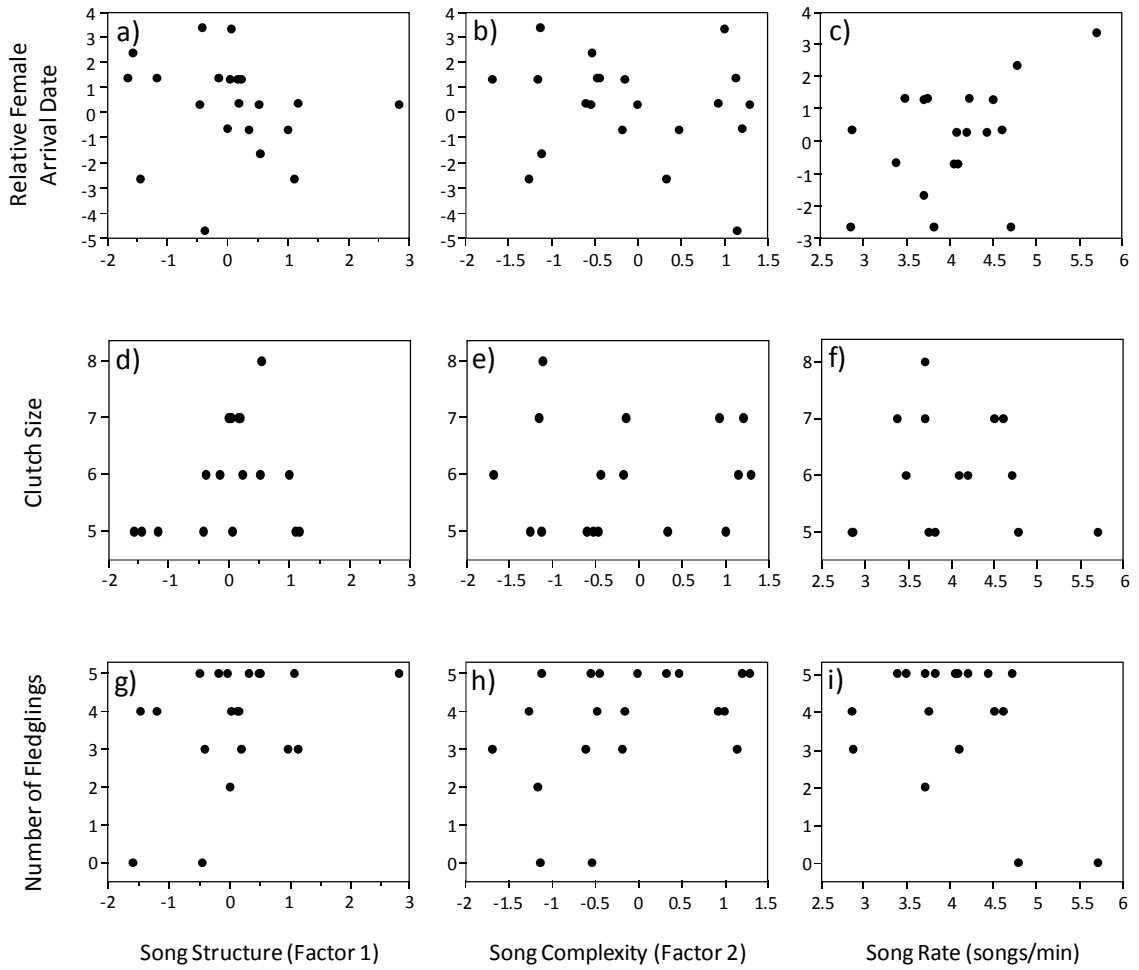


Figure 2.4 Song structure (Factor 1), song complexity (Factor 2), and song rate (songs/min) were all unrelated to a-c) relative female arrival date, d-f) clutch size, and g-i) number of fledglings. See Results for statistical details.

Chapter 3 – Summary and Future Directions

Oxidative stress has long been important in biomedical research, known to affect individual health (Harman 1956; Sies 1985). In recent decades, increasing attention has been paid to the potential ecological and evolutionary implications of oxidative stress in non-human animals (Costantini 2008). The Oxidation Sensitivity Hypothesis and the Oxidation Handicap Hypothesis both predict relationships between oxidative status and the quality of secondary sexual signals (von Schantz et al. 1999; Alonso-Alvarez et al. 2010). The ideas comprising the Oxidation Sensitivity Hypothesis were proposed by von Schantz et al. (1999) and suggest that secondary sexual signals are sensitive to oxidative stress which detrimentally affects signal quality. The Oxidation Handicap Hypothesis was later proposed by Alonso-Alvarez et al. (2010), and broadened by Metcalfe and Alonso-Alvarez (2010), and suggests that secondary sexual signals promote oxidative stress by generating increased reactive oxygen species or suppressing anti-oxidant capacity, and as such, only high quality individuals can afford to produce and maintain high quality signals.

My thesis provides the first test of either the Oxidation Sensitivity Hypothesis or the Oxidation Handicap Hypothesis that focuses on song as the sexual trait. Song content (i.e. song structure and complexity) was unrelated to any measure of oxidative status providing no support for the Oxidation Sensitivity Hypothesis. However, song performance (i.e. song rate) was positively correlated to both reactive oxygen metabolites and total anti-oxidant capacity, yet unrelated to overall oxidative stress,

providing support for the Oxidation Handicap Hypothesis. These results suggest that high quality individuals may be advertising their ability to combat the negative consequences of increased reactive oxygen metabolites (i.e. the handicap) via higher or up-regulated anti-oxidant capacities, allowing them to avoid higher levels of oxidative stress.

Although my findings are based on correlative data, and as such cannot confirm causation, they add to a growing body of literature demonstrating a relationship between secondary sexual signals and individual oxidative status (Markó et al. 2011; Moreno et al. 2011; Garratt and Brooks 2012). Correlative data obtained from observational field studies, as described in this thesis, are necessary for demonstrating that relationships between animal signals and oxidative status are biologically relevant and hold true under natural conditions (Searcy and Yasukawa 1996). Additionally, longitudinal studies which measure signal quality and oxidative status of the same individuals across years would yield valuable insight into the relationship between the two.

In the future, manipulative studies that alter either signal quality or oxidative status could provide experimental support that complements my observational research. Altering song quality will be particularly challenging; manipulating song content in many species would require raising birds from nestlings in order to alter song development, while altering song performance often involves some type of supplemental administration (e.g. food: Reid 1987; hormone: Van Roo 2004) which

could have confounding effects on oxidative status. Alternatively, interactive playback experiments simulating territorial intrusions could be conducted to alter song performance (e.g. Wunderle 1978; but see Romero et al. 1998). However, the frequency and duration of simulated intrusions needed to cause a sufficient change in song performance to alter oxidative status measures is unknown. Manipulating some component of individual oxidative status and observing the effects on song quality may be easier, although not free from complications. Increasing exogenous anti-oxidant intake can negatively affect endogenous anti-oxidant production and pharmacological dosages of anti-oxidants can lead to enhanced oxidative damage (Herbert 1994). Exposing birds to detrimental environmental conditions (e.g. ultraviolet light, ionizing radiation, environmental toxins; Finkel and Holbrook 2000) may increase reactive oxygen species production, but would likely cause other damaging effects which could independently affect signal quality and would be of questionable ethical integrity. Some signals, such as morphological traits, can be relatively easily manipulated (e.g. tail length manipulation: Andersson 1982), and may provide a more feasible starting point for investigating the relationship between oxidative status and signal quality.

One area for continued research is the exploration of that factors which cause inter-individual differences in oxidative damage and anti-oxidant capacity. Research, largely conducted over the past decade, has helped to elucidate some of the many factors affecting individual oxidative status, which include both genetic and environmental factors. For example, selective breeding has created lines of mice (*Mus musculus*) with different anti-oxidant capacities (Costantini et al. 2008) supporting the

idea that there is a genetic component to oxidative status. Neonatal nutrition, laying order, and rearing conditions all affect the anti-oxidant capacities of birds (Blount et al. 2003; Rubolini et al. 2006), while brood size affects levels of reactive oxygen metabolites and oxidative stress, the effect being attributed to the intensity of sibling rivalry (Costantini et al. 2006). The factors affecting individual oxidative status are many and can be complicated by additional variables such as species, sex, and age (Costantini et al. 2006; Rubolini et al. 2006; Costantini et al. 2007; Markó et al. 2011). I was unable to conduct a compelling analysis of the relationship between age and oxidative status due to the low incidence of second-year males and the inability to differentiate between males two years and older based on plumage (Montgomerie and Lyon 2011). Future studies should investigate the potential effect of age using a species where a more definitive ageing method exists or where long-term data can be used to determine the age of individuals.

The relationship between reactive oxygen species and anti-oxidants, and the cost and benefit of each, also warrants further investigation. Although reactive oxygen species are traditionally understood to have negative health consequences, a more recent understanding shows that low to moderate levels of reactive oxygen species are beneficial and necessary for normal cell signalling, effective immune and defence systems, muscle force, and exercise-related endurance (Finkel and Holbrook 2000; Brigelius-Flohé 2009). Additionally, the relationship between physical activity and oxidative stress is not as straightforward as once thought. While exercise and physical activity generate reactive oxygen species, low to moderate increases in pro-oxidants can

cause an up-regulation in anti-oxidant capacity, priming an individual for future pro-oxidant exposure (Brigelius-Flohé 2009). This phenomenon, termed hormesis, is analogous to how a vaccination primes an individual for a future antigen attack and subsequently leaves the individual more prepared to deal with immune challenges (Finkel and Holbrook 2000; Brigelius-Flohé 2009).

Although methods for measuring oxidative status are continuously improving, there are still several methodological issues which require further attention. Though widely used, there have been questions concerning what the d-ROMs test (Diacron, Grosseto, Italy) is actually detecting. Harma et al. (2006) deny its validity claiming that it measures ceruloplasmin, a copper-carrying protein. However, although Kostikas et al. (2006) admit that the test may measure ceruloplasmin in addition to reactive oxygen metabolites, they claim the difference in absorbance as a result of ceruloplasmin is small. Additionally, the OXY-Adsorbent test (Diacron, Grosseto, Italy) may underestimate lipid-soluble anti-oxidants as it only measures the anti-oxidant capacity of serum or plasma (Bartosz 2010; but see Cohen et al. 2007). Recently, Costantini and Dell'Omo (2006) have suggested measuring pro-oxidant and anti-oxidant levels in various tissues to obtain a more comprehensive measure of oxidative status. Even more recent, is the suggestion that measuring the activity of specific anti-oxidants may be more valuable than measuring total anti-oxidant capacity (Garratt and Brooks 2012; but see Constantini and Verhulst 2009). Lastly, measuring both oxidative damage and anti-oxidant capacity is critical in order to determine oxidative status. Measuring only one component yields incomplete and potentially misleading results, and although this point

is widely recognized, it is still often overlooked (Costantini and Verhulst 2009; Monaghan et al. 2009).

Oxidative damage, anti-oxidant capacity, and oxidative stress have been linked to signal quality (Garratt and Brooks 2012), individual condition (Costantini et al. 2007), ageing (Finkel and Holbrook 2000), reproduction (Wiersma et al. 2004), and even personality (Costantini et al. 2008), and thus are important physiological factors which can have fitness implications (Metcalf and Alonso-Alvarez 2010). Oxidative stress ecology is a promising new field that will likely continue to pervade many streams of ecology and, although great progress has been made over the past several decades, there are still many exciting avenues for future research (Costantini 2008b; Metcalfe and Alonso-Alvarez 2010). My research investigated oxidative stress as a mechanism ensuring signal honesty and was the first to do so using song. My findings revealed that reactive oxygen metabolites may be a proximate cost associated with signal quality, and that higher quality individuals may be better able to afford this cost through higher anti-oxidant capacities.

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