

## The oxidative costs of territory quality and offspring provisioning

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territory quality.

### Abstract

The costs of reproduction are an important constraint that shapes the evolution of life histories, yet our understanding of the proximate mechanism(s) leading to such life-history trade-offs is not well understood. Oxidative stress is a strong candidate measure thought to mediate the costs of reproduction, yet empirical evidence supporting that increased reproductive investment leads to oxidative stress is equivocal. We investigated whether territory quality and offspring provisioning increase oxidative stress in male snow buntings (*Plectrophenax nivalis*) using a repeated sampling design. We show that arrival oxidative stress is not a constraint on territory quality or the number of offspring fledged. Nevertheless, owners of higher-quality territories experienced an oxidative cost, with this cost increasing more rapidly in younger males. Males that provisioned offspring at a high rate also experienced increased oxidative stress. Together, these findings support the potential role of oxidative stress in mediating life-history trade-offs. Future work should consider that reproductive workload is not limited to offspring care, and other activities – including territory defence – may contribute significantly to the costs of reproduction.

### Introduction

Life-history theory predicts that reproduction is costly (Williams, 1966), and evolutionary biologists have long been interested in the evolutionary role of reproductive costs in shaping variation in life histories. As a result, there is ample evidence that ultimate costs of reproduction are important drivers of life-history variation across a variety of taxa (birds: Erikstad *et al.*, 1998; plants: Miller *et al.*, 2008; mammals: Hamel *et al.*, 2010; insects: Tsuji *et al.*, 2012). Recently, the attention of evolutionary biologists has shifted towards improving our understanding of the proximate physiological costs that constrain and shape the ultimate costs of reproduction, as these remain far less understood (Hausmann & Marchetto, 2010; Selman *et al.*, 2012; Metcalfe &

Monaghan, 2013). Identifying physiological costs of reproduction that have the potential to be ubiquitous across all species is challenging, yet crucial to further our understanding of life-history evolution (Harshman & Zera, 2007). Oxidative stress – the ratio of harmful reactive oxygen species to neutralizing antioxidants (Costantini, 2008) – is a strong candidate for mediating these costs (Selman *et al.*, 2012; Metcalfe & Monaghan, 2013) as increased metabolic demands during reproduction should cause the production of reactive oxygen species that can damage lipids, DNA and proteins (Monaghan *et al.*, 2009). In turn, the oxidative stress theory of ageing suggests that this accumulation in oxidative damage over time leads to senescence (Harman, 1956). Together, these ideas form a framework linking current reproductive investment with survival and future reproductive success.

Empirical work, although limited, has shown that reproduction can be associated with increased oxidative stress in both captive (Alonso-Alvarez *et al.*, 2004; Wiersma *et al.*, 2004) and free-living systems (Bize *et al.*, 2008; Losdat *et al.*, 2011; Christe *et al.*, 2012). However, many studies do not find the predicted positive

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relationship between reproductive investment and reactive oxygen species production (Wheelwright *et al.*, 1991; Beaulieu *et al.*, 2011; Van de Crommenacker *et al.*, 2012). These discrepancies could in part be explained by differing methodologies (discussed in Selman *et al.*, 2012), but may also result from focusing primarily on costs during parental care. For most species, successful reproduction requires more than offspring provisioning. Although researchers have begun exploring how physiology can mediate carry-over effects from one life-history stage to another (Mark & Rubenstein, 2013), little attention has been paid to how various reproductive behaviours may contribute to the costs of reproduction within a single life-history stage. One behaviour crucial for reproduction, territory defence can be intense, time-consuming, can increase the risk of injury and is energetically costly (Ros *et al.*, 2006; Finerty *et al.*, 2009). Furthermore, variation in territory quality can have important implications for annual reproductive success (e.g. Ens *et al.*, 1992), and males that are unable to defend a territory often do not secure a mate or have reduced reproductive success (Kempnaers *et al.*, 2001). Few studies have considered how prebreeding costs figure prominently as a driver of the evolution of life histories (see Legagneux *et al.*, 2012), and none have examined whether territory quality has an oxidative cost.

We addressed whether territory quality and offspring provisioning are constrained by, or have a cost of, oxidative stress in males of a free-living population of Arctic-breeding snow buntings (*Plectrophenax nivalis*). We consider constraints to occur when individual condition prior to a life-history stage limits the success of individuals, whereas costs occur when a particular life-history stage leads to reduced condition. The two can be distinguished by sampling condition at various times (i.e. before the life-history stage or the change in condition from the beginning to the end of the life-history stage).

Male snow buntings have an unusually prolonged prenesting period, presumably due to intense competition for territories (Meltofte, 1983): territory owners react aggressively to intruders, attacking them repeatedly (Romero *et al.*, 1998). Nesting sites are limiting, and males must begin defending territories weeks before the arrival of females. High-quality territories provide access to multiple rocky crevices from which females choose one nesting site (Tinbergen, 1939). Intense male territory defence ends after hatching as males and females must obligately provision offspring together to achieve maximal reproductive success, and all birds feed communally around ponds (Montgomerie & Lyon, 2011). Snow buntings are an excellent model for disentangling the relationship between condition and territory quality given that their territories are not traditional resource-based ones where birds defend an area for access to food. Although we might predict prebreeding condition to affect the quality of a territory

that a male can acquire, we would not expect defending a higher-quality territory to have enhancing effects on condition (which could otherwise mask the condition cost of holding a high-quality territory). Similarly, because territory defence ends at the onset of provisioning, this system is ideal to examine the relationships between oxidative stress and territory quality or offspring provisioning, independently from one another.

## Materials and methods

### Field data collection

We studied breeding snow buntings from late May to August of 2010 ( $N = 17$  pairs) and 2011 ( $N = 13$  pairs) at East Bay Island, Nunavut (Canada; 64°02'N, 81°47'W). We mapped territories by observing male–male territorial interactions (Tinbergen, 1939), defining territory boundaries as the site where two males give up a territorial fight and mutually retreat. We digitized territory maps and manually traced the boundaries using a tablet to measure territory area in ImageJ (v1.45; National Institute of Health, Bethesda, MD, USA). Two observers visually estimated the per cent rock cover (by 5% increments) within a 5-m radius of the nest as a proxy of the number of potential nesting sites within the territory (scores were averaged; Tinbergen, 1939). We defined territory quality as the product of territory area and per cent rock cover, making it a measure of suitable nesting site habitat throughout the territory.

We trapped males following arrival in late May with seed-baited ground traps (potter and walk-in traps), which we checked *ca.* every 30 min. Previous research showed that capture and restraint of birds do not alter oxidative stress levels over this time frame, so our trapping method is unlikely to affect oxidative stress levels (Costantini *et al.*, 2007; Costantini & Lipp, 2010). Data from a geolocation-based migratory study in this population (Macdonald *et al.*, 2012) confirmed that the date of first capture represents a male's approximate arrival date to the breeding grounds. We applied a numbered metal band and unique colour band combination to all individuals and aged birds as second year (first breeding year) or after second year (minimum second breeding year; Smith, 1992). We collected an arrival blood sample for oxidative stress analyses; briefly, we punctured the brachial vein with a 26-G needle and collected blood in heparinized capillary tubes. Whole blood was centrifuged for 10 min at 13 300 *g* within an hour of collection, after which plasma was frozen at  $-20^{\circ}\text{C}$ . We retrapped males during their mate's egg-laying period to collect an incubation blood sample, and in 2011, we also collected a post-breeding blood sample during late nestling provisioning (nestling age: 8–15 days).

We tracked breeding pairs by locating nests and visiting them every 2–3 days to determine the day the first

egg was laid (i.e. laying date). At 8 days of age – the day before fledging – we counted the number of nestlings present in the nest as our estimate of the number of fledglings. We trust that this is an accurate estimate of fledging success as there is no nest predation at our island study site and nestling deaths occur due to starvation (discussed in Guindre-Parker *et al.*, 2013). Although these deaths may be related to genetic or health problems, we feel that it is more likely that they represent brood reduction from starvation, as suggested previously for snow buntings (Hussell, 1985).

During the nestling provisioning stage in 2011 only, we assessed male feeding rates by observing each nest for an hour per day on two consecutive, nonrainy days. We restricted observations between 08:00 and 12:00 and when offspring were between 2 and 5 days of age, corresponding to the linear phase of chick growth (Montgomerie & Lyon, 2011), although male feeding rates remain approximately constant as nestlings age (Falconer *et al.*, 2007). Direct observations were conducted from a blind with a spotting scope when time permitted, but the majority of nests were filmed with a weatherproof HD digital video camcorder (Sharper Image WPVC-50) placed 2–6 m from the nest entrance. For both methods, we differentiated between males and females by their individual colour bands and/or plumage, and we noted whether parents came with or without food. We defined male feeding rate as the rate of food delivery visits per hour (as in Falconer *et al.*, 2007).

### Physiological assays

We measured reactive oxygen metabolites (measure of early oxidative damage) using a commonly used commercial kit (dROM; Diacron International, Grosseto, Province of Grosseto, Italy). We assayed for reactive oxygen metabolites according to existing protocols (Costantini *et al.*, 2008) with slight modifications. In this assay, the acidic buffer causes the release of iron in the plasma when it reacts with reactive oxygen metabolites in the sample. This reaction causes the oxidation of an alkylamine solution causing a colour change – the intensity of the colour is indicative of reactive oxygen metabolite concentrations in the original sample. We diluted 15  $\mu\text{L}$  of plasma with 300  $\mu\text{L}$  of a 1 : 100 mixture of the alkylamine solution and acetate buffer (optimized for snow buntings). Similar to a previous study (Costantini *et al.*, 2011b), we found a precipitate (i.e. lipids) at the bottom of the wells, so we ran the assay in small tubes after a 75-min incubation at 37 °C, centrifuged tubes at 10 000 rpm for 30 s and pipetted the upper 280  $\mu\text{L}$  into a second tube. After a second centrifuging step, we added 130  $\mu\text{L}$  to duplicate wells of a flat-bottomed 96-well plate after vortexing the sample a second time. Absorbance was read at a wavelength of 490 nm, and concentrations of reactive

oxygen metabolites are given in  $\text{mg H}_2\text{O}_2 \text{ dL}^{-1}$ . The mean intra-assay coefficient of variation was 8.5%.

We assayed samples for antioxidants using a commonly used commercial kit (OXY; Diacron International), which measures the general capacity of plasma antioxidants to neutralize HOCl (a type of oxidant). The absorbance is inversely proportional to the amount of HOCl absorbed by the plasma sample or the concentration of antioxidants. Similar to published protocols (Costantini *et al.*, 2008, 2011a,b), we diluted samples to 1 : 100 (optimized for snow buntings) with deionized water and added 5  $\mu\text{L}$  of diluted samples in triplicate to wells of a flat-bottomed 96-well plate with 200  $\mu\text{L}$  of the HOCl solution before shaking the plate at 450 rpm for 10 s and incubating the plate at 37 °C for 10 min. We then added 2  $\mu\text{L}$  of the colour-changing chromogen solution (N,N-diethylparaphenyldiamine) and read the plate at 490 nm after shaking for 30 s. Concentrations of antioxidants are in  $\mu\text{mol of HOCl mL}^{-1}$ , and the mean intra-assay coefficient of variation was 3.4%.

We calculated oxidative stress as the ratio of reactive oxygen metabolites to antioxidants (as in Costantini *et al.*, 2007) to generate a single variable that captured interindividual variation in oxidative balance. We computed a ratio rather than a subtracted oxidative index (see Vassalle, 2008) because it is unclear how many units of antioxidants are required to neutralize one reactive oxygen metabolite (see Van de Crommenacker, 2011).

### Statistical analyses

All raw numerical variables were standardized within year (mean = 0, SD = 1) prior to analyses. We originally included year as a covariate in all models, but removed it as it was not a statistically significant term ( $P > 0.05$ ); similarly, standardizing by year means that our results from 2010 to 2011 were comparable. Three males returned from 2010 to 2011; randomly excluding one sample per male did not alter the results, thus they were included in the analyses.

To determine whether oxidative stress constrains breeding, we assessed whether oxidative stress prior to territory acquisition (at arrival) or prior to nestling provisioning (at incubation) was a significant predictor of either territory quality or feeding rate. Conversely, to examine whether oxidative stress is a cost of territory quality or feeding rate, we considered the change in oxidative stress from the beginning until the end of the time period where males defended territories or fed nestlings, respectively. These constraints and cost models are similar (including many of the same variables), but the key difference lies in when the oxidative stress samples were measured.

To assess whether oxidative stress acts as a constraint on territory quality or paternal feeding rates, we used general linear models (GLMs) with territory quality or

the number of fledglings as the dependent variable and oxidative stress prior to each stage as the independent variable (arrival oxidative stress or incubation oxidative stress, respectively). We included timing (arrival or laying dates, respectively) and male age as covariates.

To test whether oxidative stress is a cost of territory quality, we used a GLM with incubation oxidative stress as the dependent variable and territory quality as the independent variable, including a territory quality by male age interaction (arrival oxidative stress, male age and arrival date were covariates). To assess whether rearing nestlings has an oxidative cost, we used a GLM with post-breeding oxidative stress as our dependent variable; the number of offspring and feeding rate were the independent variables (controlling for incubation oxidative stress and laying date, which were included as covariates). We could not include male age as a covariate for this analysis as post-breeding oxidative stress was only measured in 2011, when there were too few young males in this year alone.

We used a GLM to assess whether territory quality influenced reproductive success with the number of nestlings as the dependent variable and territory quality as the independent variable. Male age and laying date were included as covariates, as well as a territory quality by male age interaction. The residuals of all GLMs were normally distributed (Shapiro–Wilk test,  $P > 0.05$ ). Analyses were performed in JMP (V9; SAS Institute Inc., Cary, NC).

Some of the above-mentioned analyses are based on small sample sizes ( $N < 15$ ), so we calculated the statistical power of these analyses to show whether the sample sizes were suitable to avoid committing type II errors. We calculated post hoc power in JMP using an alpha level of 0.05, as well as the effect size and the sample size from our analyses. The power of detecting a male age by territory quality interaction and an effect of feeding rate on oxidative stress were 0.8 and 0.7, respectively. Conventionally, power of 0.8 is targeted for behavioural studies (Cohen, 1988), although using an arbitrary power threshold is often inappropriate (Di Stefano, 2003). This power analysis suggests that although our sample sizes are small, the effect sizes are large enough that our analyses are likely to successfully detect oxidative costs of reproduction.

## Results

We found that timing (both for arrival and for breeding) was a key constraint on territory quality and reproductive output, respectively (Table 1; Fig. 1). Laying date ( $t = -2.81$ ,  $N = 21$ ,  $P = 0.01^*$ ), but not incubation oxidative stress ( $t = 1.11$ ,  $N = 21$ ,  $P = 0.28$ ), predicted the number of offspring fledged. Similarly, male arrival date ( $t = -3.13$ ,  $N = 26$ ,  $P = 0.005^*$ ), but not arrival oxidative stress ( $t = 0.63$ ,  $N = 26$ ,  $P = 0.53$ ), predicted the quality of a territory that a male acquired,

so that males that arrived the earliest acquired the higher-quality territories (Fig. 1).

Males that defended higher-quality territories had a greater increase in oxidative stress from arrival to incubation; the slope of this relationship was steeper for younger males than for older males (territory quality by age interaction:  $t = -1.74$ ,  $N = 25$ ,  $P = 0.008^*$ ; Table 2). The within-individual change in oxidative stress over the course of offspring rearing was unrelated to the number of nestlings a male cared for ( $t = -1.49$ ,  $N = 7$ ,  $P = 0.38$ ; Table 2), although males that fed offspring at a greater rate increased in oxidative stress to a greater extent ( $t = 13.13$ ,  $N = 7$ ,  $P = 0.04^*$ ; Fig. 2).

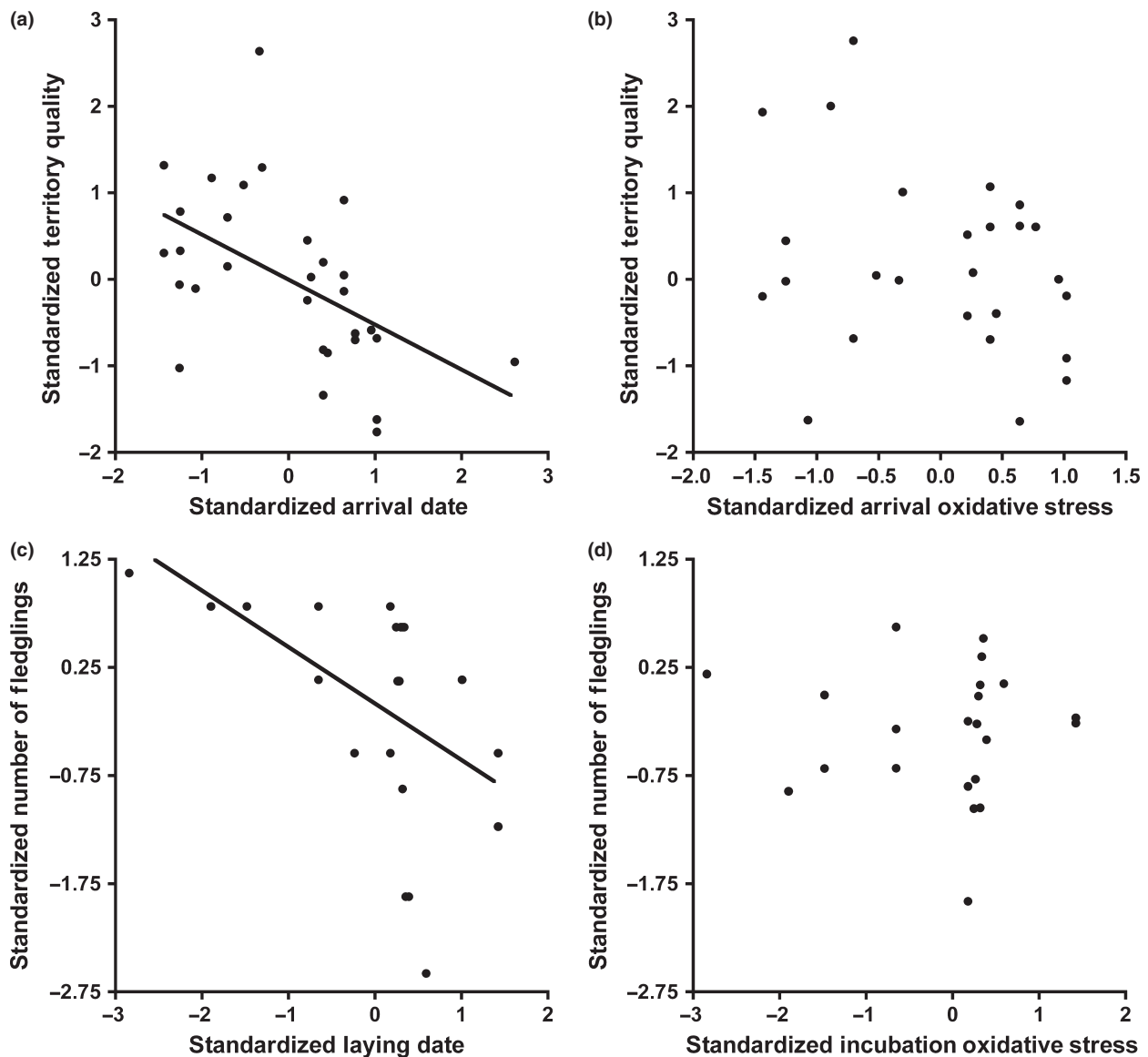
The number of offspring fledged was significantly related to territory quality ( $t = 2.69$ ,  $N = 23$ ,  $P = 0.015^*$ ), and there was a significant territory quality by male age interaction ( $t = -2.11$ ,  $N = 23$ ,  $P = 0.049^*$ ) where the positive effect of increased territory quality on reproductive success was more pronounced in young males (Table 3; Fig. 3).

## Discussion

Our results support the idea that oxidative stress can play a role in mediating key life-history trade-offs. This is the first evidence to suggest that territory quality is associated with an oxidative cost. This could be due to the increased metabolic demands of patrolling territories, performing territorial displays or defending territories from intruders (Ros *et al.*, 2006; Finerty *et al.*, 2009). Owners of larger or higher-quality territories have a greater area to patrol and are more likely to suffer intrusions (Eberhard & Ewald, 1994), this is expected to increase their metabolic rate, and subsequently led to an increased production of reactive oxygen species and oxidative stress (Fletcher *et al.*, 2013). Territory quality and territorial intrusions may also relate to hormones that could alter the oxidative balance: chronic increase in both testosterone (increased

**Table 1** Male arrival oxidative stress does not act as a constraint on (a) reproductive success ( $R^2 = 0.26$ ,  $N = 21$ ) or (b) on territory quality ( $R^2 = 0.42$ ,  $N = 26$ ). Instead, the timing of laying and arrival, respectively, seem to drive reproduction and territoriality. \* $P < 0.05$ .

Predictor	Estimate	<i>t</i> ratio	<i>P</i> -value
(a) Dependent: number of fledglings			
Intercept	-0.502	-1.66	0.11
Age	0.568	1.74	0.10
Laying date	-0.514	-2.81	0.01*
Incubation oxidative stress	0.235	1.11	0.28
(b) Dependent: territory quality			
Intercept	-0.123	-0.55	0.59
Age	0.267	1.18	0.25
Arrival date	-0.653	-3.13	0.005*
Arrival oxidative stress	0.102	0.63	0.53



**Fig. 1** The timing of both male arrival to the breeding grounds and laying constrained territory quality (a) and reproductive success (c), respectively. Conversely, oxidative stress was not a significant constraint on territory quality (b) or reproductive success (d).

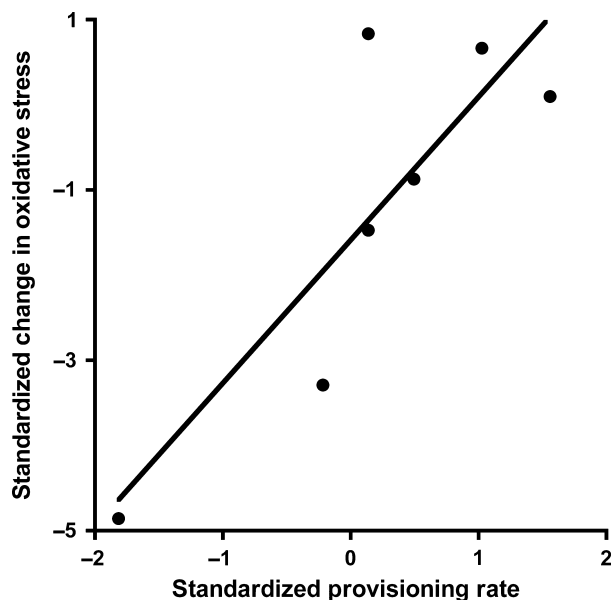
during aggressive interactions) and glucocorticoids (increased in response to social conflict) is proposed to produce reactive oxygen metabolites (discussed in Alonso-Alvarez *et al.*, 2007; Costantini *et al.*, 2011a,b), although empirical evidence is limited. Although our study cannot elucidate the causal link between territory quality and oxidative stress, our finding that oxidative stress does not constrain territory quality, yet increases over the period of territoriality, suggests that there is an oxidative cost of owning a higher-quality territory.

Although many studies have related breeding effort to oxidative damage (Alonso-Alvarez *et al.*, 2004; Wieersma *et al.*, 2004; Heiss & Schoech, 2012), this study is

the first to relate increased behavioural workload (feeding rate) with an increase in oxidative stress across reproduction. The increased energetic demands of provisioning young likely increase oxidative stress during this time (Fletcher *et al.*, 2013). Whereas other studies have suggested that oxidative stress can constrain reproduction (Metcalf & Alonso-Alvarez, 2010; Stier *et al.*, 2012), our study does not lend support to this idea – arrival oxidative stress was unrelated to territory quality, and incubation oxidative stress was unrelated to offspring provisioning rates. It is likely that oxidative stress acts either as a cost or as a constraint (but not both simultaneously), such that male snow buntings

**Table 2** Increases in oxidative stress occur during (a) offspring provisioning ( $R^2 = 0.98$ ,  $N = 6$ ) and (b) territory quality ( $R^2 = 0.46$ ,  $N = 25$ ). \* $P < 0.05$ .

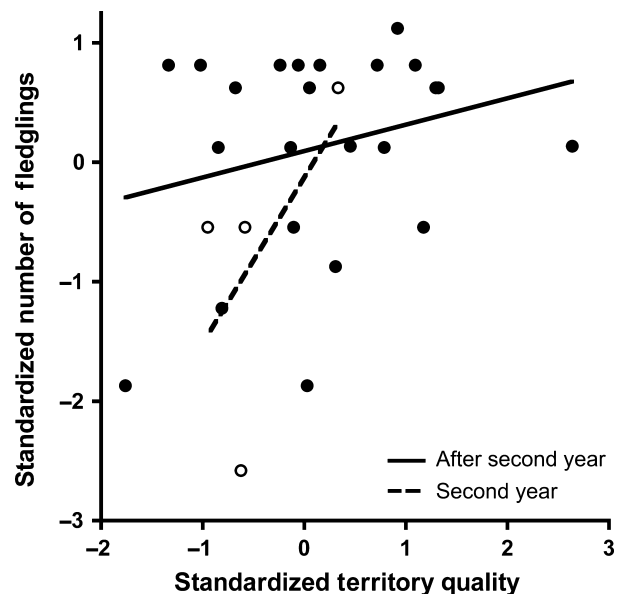
Predictor	Estimate	<i>t</i> ratio	<i>P</i> -value
(a) Dependent: post-breeding oxidative stress			
Intercept	-1.57	-13.15	0.04*
Incubation oxidative stress	1.73	6.12	0.10
Laying date	-0.80	-6.28	0.10
Number of fledglings	-0.26	-1.49	0.38
Feeding rate	2.13	13.13	0.04*
(b) Dependent: incubation oxidative stress			
Intercept	1.03	3.25	0.004*
Arrival oxidative stress	0.10	0.56	0.58
Arrival date	0.52	1.84	0.08
Age	-1.53	-4.00	0.0008*
Territory quality	1.99	3.07	0.006*
Territory quality by age	-1.74	-2.94	0.008*



**Fig. 2** Males that provision offspring to a greater degree experience increase in oxidative stress from incubation to post-breeding.

**Table 3** Male territory quality has a significant effect on the number of fledglings produced ( $R^2 = 0.54$ ,  $N = 23$ ). \* $P < 0.05$ .

Predictor	Estimate	<i>t</i> ratio	<i>P</i> -value
Dependent: number of fledglings			
Intercept	-0.050	-0.18	0.86
Age	-0.328	-0.88	0.39
Laying date	-0.444	-2.70	0.015*
Territory quality	1.371	2.69	0.015*
Territory quality*age	-1.080	-2.11	0.049*



**Fig. 3** The number of offspring fledged successfully increased with territory quality, although this effect is more pronounced in younger males (open circles, dashed line) than older males (closed circles, solid line).

invest in reproduction regardless of their prebreeding physiology, but experience an oxidative cost of reproduction as a result. Alternatively, the timing of our sampling may not have captured constraining effects. For example, oxidative stress may drive which individuals do and do not breed (Van de Crommenacker *et al.*, 2011) or could affect the timing or speed of migration. Exploring variation in oxidative stress from one life-history stage to another, or one breeding season to another, will be key to fully assess whether oxidative stress has constraining effects on reproduction.

We showed that a greater proximate cost of territory quality occurs for young males. This increased cost of territory quality may result from a lack of experience in efficiently defending a territory (Pärt, 2001) or from young males being subjected to more territorial intrusions because they are less aggressive (Arcese, 1987). If females preferentially mate with experienced males (Weatherhead, 1984), being able to defend a high-quality territory may benefit younger males by allowing them to secure a mate. Alternatively, younger males may have to spend more time performing courtship displays to attract a mate, something that our study could not quantify. Our results suggest that young males defending higher-quality territories increase their annual reproductive success relative to other second-year birds; thus, young males may be willing to endure a greater oxidative cost to do so. It remains unclear what specific factors mediate variation in second-year male territory quality.

Territory quality, estimated through the amount of suitable nesting site habitat, significantly predicted the

number of fledglings produced by a pair. Although the link between territory quality and reproductive success is indirect in snow buntings, nest microclimate is particularly important for this species (Montgomerie & Lyon, 2011) and is a potential mechanism linking nest site availability and fledging success. Snow bunting nests cool rapidly compared with other Arctic-breeding passerines (Lyon & Montgomerie, 1987), and this may vary with nest cavity characteristics. The availability of nest cavities whose orientation, depth and size limit thermal stress is expected to be limiting (discussed in Lyon & Montgomerie, 1987), and the most successful pairs may identify a superior nest cavity, given a greater availability of nesting site choices within their territory.

Previously, much attention has been devoted to parental care alone as a costly component of reproduction, despite the fact that reproduction is associated with many different energetically costly activities, which may include migration, territory defence, courtship displays, mate attraction, mate searching, mate guarding, incubation feeding and overlapping moulting with breeding. Future studies of the costs of reproduction should consider these activities as potentially important drivers of life-history evolution. Furthermore, it may be critical to explore the interaction between multiple costly activities and the potential long-term carry-over effects associated with high post-breeding oxidative stress. It is also important to explore how such carry-over effects may ultimately influence lifespan and individual fitness (i.e. lifetime reproductive success). We hope that future work will explore the oxidative costs of breeding through experimental manipulations (i.e. changing territory quality and breeding workload); this will improve our understanding of the causality between these variables.

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