Causes and consequences of oxidative stress in a cooperatively breeding bird

Submitted by

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to the University of Exeter as a thesis for the degree of

Doctor of Philosophy in Biological Sciences

July 2013

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SUMMARY

Oxidative stress has recently been highlighted as a potential physiological mechanism underpinning life-history trade-offs in animals. While the role of oxidative stress in mediating such trade-offs is receiving increasing attention, its importance in wild populations remains poorly understood. In this thesis, I use a wild population of cooperatively breeding white-browed sparrow weavers *Plocepasser mahali* to investigate the role that oxidative stress plays in mediating the costs of reproduction and immune defence. Cooperative animal societies offer a unique opportunity to investigate the costs of reproduction, because dominants frequently monopolise breeding opportunities (exhibiting higher reproductive effort than subordinates), and subordinate cooperative contributions frequently lighten reproductive workloads. My findings reveal, first, that dominants' reproductive monopolies do not arise because they exhibit superior oxidative balance, as no such rank-related differences in oxidative state exist *prior* to breeding (Chapter 2). However, the higher reproductive effort of dominant females may underpin their differential declines in antioxidant protection *after* the breeding season (Chapter 2). Second, experimental manipulation of reproductive effort reveals marked oxidative damage and body mass costs incurred during reproduction. However, these costs are entirely mitigated in large social groups, suggesting that the cooperative contributions of helpers may offset the costs of reproduction for all group members (Chapter 3). While this represents rare evidence of an oxidative stress cost of reproduction in the wild, longitudinal data suggests that these costs do not endure after the breeding season (Chapter 4), highlighting that circulating markers of oxidative balance are unlikely to mediate long-term costs of reproduction. Finally, an immune activation experiment reveals that, while mounting an immune response causes no net change in oxidative balance, the scale of the response can be adjusted according to baseline antioxidant protection in an oxidative-condition-dependent manner (Chapter 5). Together my results provide support for the role of oxidative stress in shaping life histories in the wild. Furthermore, evidence of rank-related disparities in oxidative balance and the avoidance of reproductive costs in large social groups may have important implications for our understanding of both the evolution of cooperative breeding and the patterns of health and ageing in societies.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES

AUTHOR'S DECLARATION

All chapters in this thesis were written by Dominic L. Cram, with comments and editing from Dr Andrew J. Young and Dr Jonathan D. Blount. Dr Jennifer E. York also provided comments for each chapter. Guidance was provided throughout by Dr Andrew J. Young and Dr Jonathan. D. Blount. The wild population of white-browed sparrow weavers (*Plocepasser mahali*) used in this thesis is part of a long-term study led by Dr Andrew J. Young at the University of Exeter (Cornwall Campus) with support from Dr Jennifer E. York.

All laboratory analysis was carried out by Dominic L. Cram, with the support of Christopher Mitchell and Dr Jonathan D. Blount.

CHAPTER 1

General introduction
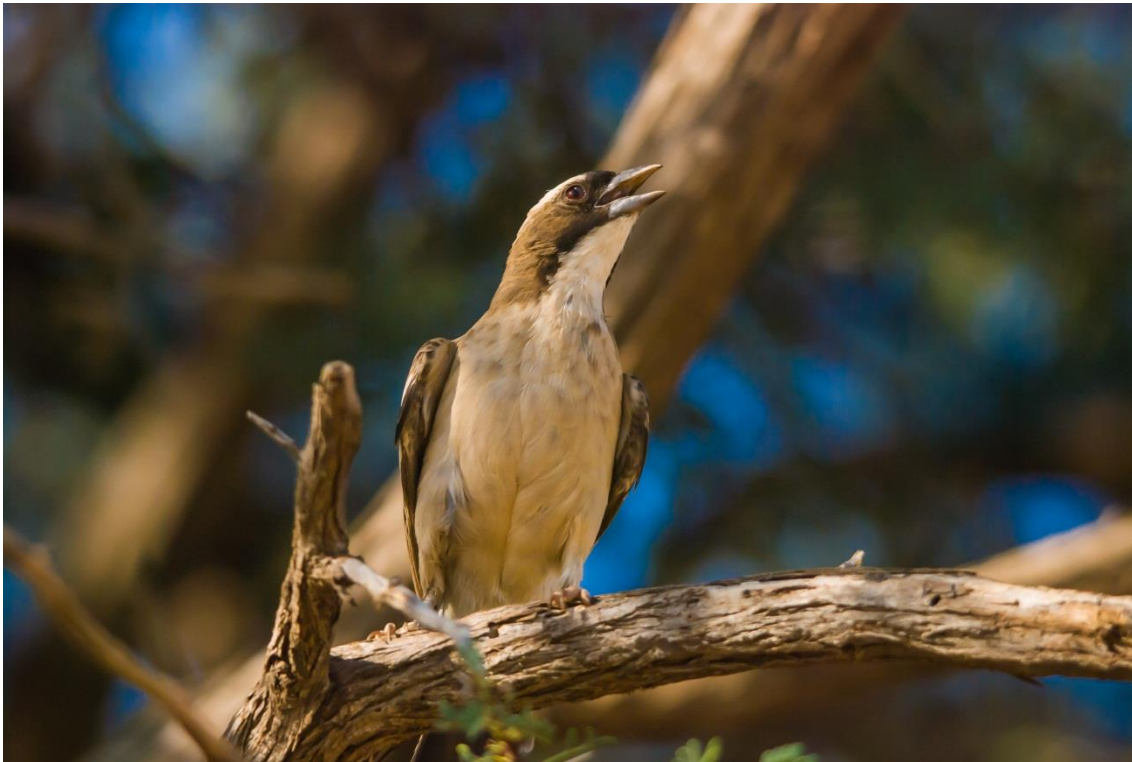
1.1 OVERVIEW

Despite increasing interest, the role of oxidative stress in shaping life-histories remains poorly understood, particularly in wild contexts. In this chapter, I discuss the importance of life-history trade-offs and the emerging role that oxidative stress may play in mediating them. I then consider three key factors that may influence oxidative balance, yet have received little attention in studies of wild populations. Finally, I highlight a number of methodological considerations when studying oxidative stress in the wild, and outline the aims of this thesis.

1.2 LIFE HISTORY TRADE-OFFS

Life-history theory provides a fundamental framework within which to investigate key questions in evolutionary biology (Williams, 1966). Central to this theory was the discovery that life-history traits are frequently negatively associated with each other, leading to the view that investment in one trait is traded-off against investment in others. Selection is therefore expected to favour patterns of investment across traits that maximise fitness, subject to the constraints imposed by any such trade-offs among traits (Stearns, 1989). While these trade-offs have been instrumental in developing our understanding of evolutionary biology, their proximate basis remains unclear (Rose and Bradley, 1998; Zera and Harshman, 2001; Monaghan *et al.*, 2009). Without an understanding of the physiological mechanisms that underpin life-history trade-offs, we cannot understand why they occur or how they are regulated.

Early studies proposed that a finite supply of energy was the core limiting factor driving trade-offs, and that energetic investment in one trait was necessarily at the

expense of others (Williams, 1966; Stearns, 1989). However, more recent work has suggested that non-energetic resources may play a key role (Zera and Harshman, 2001). In particular, oxidative stress has received increasing attention as a physiological mechanism which may have far-reaching implications for our understanding of life-history trade-offs (Costantini, 2008; Dowling and Simmons, 2009; Monaghan *et al.*, 2009; Metcalfe and Alonso-Alvarez, 2010; Selman *et al.*, 2012; Metcalfe and Monaghan, 2013).

1.3 OXIDATIVE STRESS

Oxidative stress occurs when reactive oxygen species (ROS) cause damage to cellular components (Finkel and Holbrook, 2000; Halliwell and Gutteridge, 2007). While low levels of ROS are produced by a number of specialised enzymes (e.g. NADPH oxidases) to serve valuable functions in cell-signalling and immune defence (Klebanoff and Clark, 1978; Finkel, 2003), ROS are primarily produced as a by-product of oxidative phosphorylation in the mitochondria (Chance *et al.*, 1979; Balaban *et al.*, 2005). ROS are highly reactive and unstable, and rapidly trigger chain reactions resulting in damage to DNA, proteins and lipids (Ames *et al.*, 1991; Dröge, 2002; Hulbert *et al.*, 2007).

Protection against oxidative stress is critical in maintaining homeostasis, and a complex antioxidant system has therefore evolved to prevent, delay or repair oxidative damage (Surai, 2002). Intracellular antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase form the first line of defence by converting the highly reactive superoxide anion to hydrogen peroxide (itself a ROS), and ultimately water (Godin and Garnett, 1992). However, these enzymes do not offer complete protection, and the antioxidant system therefore

also comprises a complex group of compounds that further neutralize ROS. These chain-breaking antioxidants are primarily found in the circulatory system (Cohen *et al.*, 2007), and are either produced endogenously or obtained from the diet (Felton and Summers, 1995; Krinsky and Yeum, 2003; Halliwell and Gutteridge, 2007; Catoni *et al.*, 2008). Dietary antioxidants include some vitamins (e.g. vitamin E) and carotenoids (e.g. lutein), while endogenously produced antioxidants include non-enzymatic proteins (e.g. glutathione), other vitamins (e.g. vitamin C) and uric acid (reviewed in Monaghan *et al.*, 2009).

Under normal circumstances, the balance between ROS production and antioxidant protection ("oxidative balance") is maintained such that oxidative damage is minimised. However, when the production of ROS is elevated, or antioxidant defences are reduced, ROS can overwhelm antioxidant protection and cause oxidative stress. Prolonged exposure to oxidative stress can cause significant disruption to normal cell function, and has been implicated in the pathogenesis of a number of diseases and accelerated rates of ageing (Harman, 1956; Ames *et al.*, 1991; Ames *et al.*, 1993; Beckman and Ames, 1998; Selman *et al.*, 2012).

1.4   ECOLOGICAL FACTORS THAT MAY INFLUENCE OXIDATIVE BALANCE

1.4.1. Physical activity and reproduction

The majority of ROS are produced by mitochondria during ATP synthesis, and it was therefore expected that increased demand for ATP during hard physical exercise would lead to a concomitant increase in ROS generation (Beckman and Ames, 1998). Indeed, early evidence suggested that exercise can result in elevated

ROS production in mammalian muscle tissue (Davies *et al.*, 1982; Quiroga and Barja, 1992). However, the relationship is not straightforward, and mitochondrial ROS production is not always directly proportional to oxygen consumption (Barja, 2007). While acute exposure to intensive physical exercise can result in elevated ROS generation, moderate exercise can have a negligible or even positive effect on oxidative balance (Leeuwenburgh and Heinecke, 2001; Selman *et al.*, 2002; Navarro *et al.*, 2004). Furthermore, individuals habituated to high levels of exercise can avoid oxidative stress, perhaps via the expression of mitochondrial uncoupling proteins (Ji, 1999). Uncoupling proteins bypass normal oxidative phosphorylation pathways and thus reduce ROS production (Criscuolo *et al.*, 2005). Despite this complexity in the mitochondrial production of ROS (Murphy, 2009), hard physical work can lead to oxidative stress, particularly in unaccustomed individuals or when antioxidant protection is weak (Reddy *et al.*, 1998; Leeuwenburgh *et al.*, 1999; Costantini *et al.*, 2008).

Reproduction may influence patterns of energy expenditure, temporarily raising metabolic rate and therefore potentially increasing the generation of ROS (Deerenberg *et al.*, 1995; Moreno *et al.*, 1995; Verhulst and Tinbergen, 1997; Nilsson, 2002). While the link between metabolic rate and ROS generation is not straightforward (see above), a body of evidence does now suggest that reproduction can indeed elevate exposure to oxidative stress (Behrman *et al.*, 2001; Toescu *et al.*, 2002; Murdoch and Martinchick, 2004; Myatt and Cui, 2004; Murdoch *et al.*, 2005; Bergeron *et al.*, 2011; Fletcher *et al.*, 2012; Heiss and Schoech, 2012; Stier *et al.*, 2012).

Antioxidants are also directly required for a number of processes during reproduction, including the development of sexual signals (von Schantz *et al.*, 1999), the production of eggs and sperm (Blount *et al.*, 2000; Blount *et al.*, 2001; Bertrand *et al.*, 2006a; Helfenstein *et al.*, 2010) and the expression of parental care (Alonso-Alvarez *et al.*, 2004b; Wiersma *et al.*, 2004; Pike *et al.*, 2007). Any resulting diversion of antioxidants away from their function in self-maintenance could therefore act (in isolation or in concert with any increase in ROS production) to mediate the fundamental trade-off between reproduction and survival *if* it precipitates oxidative stress (Alonso-Alvarez *et al.*, 2008; Catoni *et al.*, 2008; Costantini, 2008; Monaghan *et al.*, 2009; Metcalfe and Alonso-Alvarez, 2010).

While reproduction frequently does lead to a reduction in circulating markers of antioxidant protection (Alonso-Alvarez *et al.*, 2004b; Wiersma *et al.*, 2004; Losdat *et al.*, 2011; Christe *et al.*, 2012), a number of studies do not find the predicted increase in oxidative damage during reproduction (reviewed in Metcalfe and Monaghan, 2013). This may be due to the fact that the majority of studies rely on correlations between natural reproductive effort and oxidative balance, and are carried out almost exclusively in a captive environment (Metcalfe and Alonso-Alvarez, 2010; Metcalfe and Monaghan, 2013). Experimental studies investigating the 'true' oxidative stress consequences of investment in reproduction in wild populations are therefore much-needed.

1.4.2. Group-living and cooperative behaviour

If investment in reproduction does lead to oxidative stress, natural selection should favour strategies that help mitigate such costs for an individual and its kin.

One such strategy may be cooperative breeding. In cooperatively breeding species, subordinate individuals delay dispersal from their natal territory and help raise the offspring of their family group (Brown, 1987; Emlen, 1991; Emlen, 1997; Solomon and French, 1997; Clutton-Brock, 2002; Koenig and Dickinson, 2004). In some species, helpers' contributions towards offspring care enhance the total amount of care received by young, thereby increasing offspring survival (Brown *et al.*, 1982; Emlen and Wrege, 1991; Clutton-Brock *et al.*, 2001). However, in other species, dominant individuals receiving help reduce their own investment, either partially or fully compensating for the additive effects of helper contributions (Crick, 1992; Hatchwell, 1999; Heinsohn, 2004). These 'load-lightened' dominants can gain survival and fecundity benefits as a result of their decreased breeding effort (Khan and Walters, 2002; Russell *et al.*, 2003), yet the mechanism that underpins these fitness benefits remains unclear.

If reproduction does negatively impact oxidative stress, reductions in offspring care by load-lightened dominants may in turn alleviate the oxidative burden placed on them during breeding, reducing their exposure to oxidative stress and delaying senescence (Selman *et al.*, 2012). This raises the largely unexplored possibility that the long-term benefits enjoyed by dominants in large social groups arise following 'oxidative load-lightening,' whereby dominants in larger groups reduce investment in offspring care thus minimizing the oxidative stress it can entail.

Social status may play a key role in determining oxidative balance in animal societies, because dominant individuals typically breed more frequently than subordinates (Keller and Reeve, 1994; Magrath *et al.*, 2004; Hodge, 2009), and may

therefore have different patterns of energy expenditure and ROS production. As such, dominants may be disproportionately exposed to oxidative stress during reproductive episodes. Indeed, a recent study of cooperatively breeding Seychelles warblers *Acrocephalus sechellensis* reported evidence that dominant males alone suffered elevated oxidative damage during costly periods of mate-guarding, suggesting that dominant-specific traits can indeed result in oxidative stress (van de Crommenacker *et al.*, 2011b). However, evidence that dominants show extended lifespans in some cooperative vertebrates (Arnold and Owens, 1998; Carey, 2001; Dammann and Burda, 2006) suggests that somehow, despite their differential exposure to the costs of reproduction, dominants in some societies may enjoy reduced levels of oxidative damage relative to their subordinates. This might arise, for example, because dominants tend to be phenotypically superior to subordinates (Creel, 2001; Heg *et al.*, 2004; Young and Bennett, 2010). This enhanced competitive ability may grant dominants differential access to the resources necessary for effective antioxidant defences, thus allowing them to cope with the elevated oxidative stress burdens dominance may entail. Indeed, prior to breeding, dominant female Seychelles warbler 'breeders' had lower blood concentrations of oxidative damage than 'non-helper' subordinates that do no invest in reproduction (van de Crommenacker *et al.*, 2011b), suggesting that dominant individuals may enjoy superior oxidative balance in some social species.

Tenure as dominant may therefore conceivably impact oxidative stress either positively or negatively, and this may vary seasonally depending on breeding stage. Such rank-related disparities in oxidative balance could have important implications for the health and senescence trajectories associated with differing social status in animal societies (Balaban *et al.*, 2005; Dammann and Burda, 2006).

Moreover, cooperatively breeding societies offer unique opportunities to study the impact of reproduction on oxidative balance, because variable levels reproductive effort occur within closely-related individuals sharing the same territory. Furthermore, reproductive effort can also vary between small groups and those benefiting from abundant cooperative contributions from subordinates.

1.4.3. Immune defence and infection status

The activation of the immune system following infection by pathogens and parasites may also impact oxidative balance. A number of studies have reported impaired fecundity and survival following experimental immune activation (reviewed in Zuk and Stoehr, 2002; Hasselquist and Nilsson, 2012), yet the mechanism mediating the costs of mounting an immune response remains unclear. Oxidative stress is a particularly promising candidate, because the generation of ROS may be elevated during an immune response for two reasons. First, immune responses typically raise metabolic rate, which may elevate the production of ROS (Demas *et al.*, 1997; Ots *et al.*, 2001; Martin *et al.*, 2003). Second, a number of immune cells produce ROS directly to target pathogens during "respiratory burst" (Holmes *et al.*, 1967; Babior, 1984; Dahlgren and Karlsson, 1999). While such defence can successfully destroy foreign cells, the destructive activity of ROS is non-specific and may therefore also damage host tissues and cause oxidative stress. Although experimental immune activation can promote oxidative stress, the evidence is somewhat mixed (reviewed in Costantini and Møller, 2009), and much-needed studies in the wild are scarce (Hasselquist and Nilsson, 2012).

1.5 OXIDATIVE STRESS IN THE WILD

Until relatively recently, studies of oxidative stress were primarily restricted to medical physiology (McGraw *et al.*, 2010). However, in the last 15 years, the evolutionary and ecological implications of oxidative stress have received increasing attention (Costantini, 2008; McGraw *et al.*, 2010), due in large part to oxidative stress being highlighted as a potential mediator of life-history trade-offs (Dowling and Simmons, 2009; Monaghan *et al.*, 2009; Metcalfe and Alonso-Alvarez, 2010). Indeed, recent evidence suggests that oxidative stress may play an important role in shaping investment in reproduction, immune defence and the development of sexual signals (von Schantz *et al.*, 1999; Costantini and Møller, 2009; Metcalfe and Monaghan, 2013). However, the majority of early ecological studies of oxidative balance were conducted in captivity, and studies in wild contexts remain scarce (Metcalfe and Alonso-Alvarez, 2010).

Recent evidence suggests that measures of oxidative balance taken in captivity do not accurately reflect those in the wild (Sepp *et al.*, 2010; Casagrande *et al.*, 2011), calling into question the generality of the findings of studies in captivity. Furthermore, captive conditions typically feature *ad lib* high-quality food, favourable thermal conditions and an absence of predation and competition. These relatively favourable conditions are likely to both increase the resource pool available for investment in life-history traits (e.g. by providing unlimited food), and reduce the need to invest in certain traits (e.g. reduced investment in vigilance in the absence of predators). As such, the need for captive study animals to make the precise resource allocation trade-offs under investigation may be greatly diminished or distorted, compared to their wild counterparts (Garratt *et al.*, 2012).

While captive studies are valuable, it is critical that researchers also rise to the challenge of conducting such studies under realistic environmental conditions, to test the generality of findings from studies in captivity (Metcalfe and Alonso-Alvarez, 2010; Selman *et al.*, 2012).

Although research examining oxidative balance in wild populations is much-needed (Metcalfe and Alonso-Alvarez, 2010), such studies involve new challenges not encountered in captivity. The oxidative balance of wild animals is likely to be influenced by a variety of intrinsic and extrinsic factors, including early life conditions (Blount *et al.*, 2003; Alonso-Alvarez *et al.*, 2007), diet (McGraw *et al.*, 2005; Selman *et al.*, 2006; Catoni *et al.*, 2008; Cohen *et al.*, 2009) and territory quality (van de Crommenacker *et al.*, 2011a). In captivity, these factors can often be standardised, minimising the amount of noise they introduce. By contrast, in wild populations such factors are expected to result in considerable inter-individual variation in oxidative balance, which may conceal key relationships or even generate misleading confounds. While such variation can never be fully controlled for, studies of oxidative balance in the wild must attempt to account for likely sources of variation (Hõrak and Cohen, 2010).

In this thesis, I seek to minimise the effects of inter-individual variation in oxidative balance wherever possible, by: (i) controlling for potential sources of variation (e.g. age, sex, dominance status etc.); (ii) investigating within-individual changes in metrics of oxidative balance across different contexts; and (iii) employing experimental approaches wherever tractable. I also use random factors in mixed models to statistically control for variation among social groups in metrics of oxidative balance, which is likely to absorb variation arising, for

example, from differences in territory quality or genotype (van de Crommenacker *et al.*, 2011a).

1.6   MEASURING OXIDATIVE DAMAGE AND ANTIOXIDANT PROTECTION

As outlined above, oxidative balance is a complex, multi-faceted physiological state, and a comprehensive assessment of oxidative balance is therefore challenging to achieve. Direct measurement of ROS production is intrinsically difficult due to their high reactivity and short half-lives (but see Olsson *et al.*, 2008). Furthermore, elevated ROS generation itself may not necessarily indicate oxidative stress, if there is sufficient antioxidant protection to neutralize any threat that this may pose (Monaghan *et al.*, 2009). Levels of oxidative damage products are therefore more straightforward to both measure and interpret. As such, molecular markers for oxidative damage to lipids, proteins and DNA have been developed and are now used extensively in studies of oxidative balance (reviewed in Monaghan *et al.*, 2009). In particular, their use in ecological studies is highly encouraged (Hõrak and Cohen, 2010), as many ecological studies of oxidative balance have measured only antioxidant components and therefore provide an incomplete assessment of whether oxidative stress is actually occurring (e.g. Alonso-Alvarez *et al.*, 2004b; Wiersma *et al.*, 2004; Losdat *et al.*, 2011; Christe *et al.*, 2012).

A number of techniques exist to measure individual antioxidants, both enzymatic (e.g. superoxide dismutase activity) and non-enzymatic (e.g. vitamin E concentrations). While such data can be informative, the antioxidant system is highly complex, and synergistic and antagonistic interactions between individual

22

antioxidants make it challenging to draw inference regarding overall antioxidant capacity from information about the levels of multiple individual antioxidants. For example, vitamin E has been highlighted as a key circulating antioxidant (Costantini, 2008), yet, without sufficient levels of other antioxidants, it can itself act as a powerful pro-oxidant and cause extensive damage to lipids (Bowry *et al.*, 1992). A more straightforward estimate of antioxidant protection is therefore provided by assays that estimate overall antioxidant defence (Cohen *et al.*, 2007; Somogyi *et al.*, 2007). Such techniques measure the ability of a sample to quench a free radical attack *in vitro*, thus providing a functional measure of "total antioxidant capacity" (TAC). Despite the generality of these metrics of TAC, they only provide information about circulatory antioxidants, as most intracellular antioxidant activity is enzymatic and thus not measured by the TAC assay. The use of multiple markers of antioxidant protection is therefore encouraged (Monaghan *et al.*, 2009; Hõrak and Cohen, 2010).

In this thesis, I employ a suite of molecular markers to assess oxidative balance. As a metric of oxidative damage, I measured circulating plasma concentrations of malondialdehyde (MDA), a lipid peroxidation product. Lipids are major targets of ROS, and their oxidative damage can lead to the breakdown of cell membranes and disruption of cell function (Halliwell and Chirico, 1993; Hulbert, 2005). Furthermore, lipid peroxidation products can themselves act as highly reactive intermediates in a chain reaction that propagates oxidative damage to DNA and proteins (Hulbert *et al.*, 2007). Elevated plasma MDA concentrations can therefore indicate increased oxidative stress suffered by an animal.

I also measured both enzymatic and non-enzymatic antioxidant protection. As a metric of intracellular enzymatic antioxidant protection, I assessed the activity of superoxide dismutase within erythrocytes. Superoxide dismutase plays a vitally important role in the first line of defence against ROS (Godin and Garnett, 1992; Parkes *et al.*, 1998). As a measure of non-enzymatic antioxidant protection I measured plasma TAC. It has recently been highlighted that up to 90% of the variation in antioxidant activity measured by TAC can be due to the 'incidental' antioxidant effects of the uric acid (Cohen *et al.*, 2007). Uric acid's *in vitro* antioxidant activity may be incidental to its primary role as a waste product. Furthermore, increased uric acid levels can indicate stress (Cohen *et al.*, 2008), and uric acid production can itself generate ROS (Dröge, 2002). As a result, plasma TAC values comprise the activities of important antioxidants, as well as that of uric acid, which may at best add noise or at worst confound analyses (Cohen *et al.*, 2007; Cohen *et al.*, 2008; Cohen and McGraw, 2009). In order to factor out the impact of uric acid on plasma TAC, I therefore measured plasma uric acid concentrations and calculated residuals as recommend by Cohen *et al.* (2007).

1.7   THESIS AIMS

This thesis aims to clarify the role that oxidative balance may play in mediating life-history trade-offs and the implications of group living for patterns of oxidative stress, using a wild population of the cooperatively breeding white-browed sparrow weaver *Plocepasser mahali* as a model system. Specifically, I first examine how social rank and group size impact oxidative balance in a wild cooperative breeder (Chapter 2). I then investigate whether the costs of reproduction may be mediated by impacts on oxidative balance in both the short-term (Chapter 3) and

long-term (Chapter 4), and the degree to which cooperation can offset these costs.

Finally, I experimentally investigate whether the documented costs of immune

defence may be mediated by oxidative stress arising during an immune response

(Chapter 5).

1.8   STUDY SYSTEM

White-browed sparrow weavers *Plocepasser mahali* are a cooperatively breeding

bird with a range spanning the arid and semi-arid regions of sub-Saharan Africa

(Sinclair and Ryan, 2010). Groups defend year-round territories, and are composed

of a dominant male and female and up to 10 subordinate helpers of both sexes, that

may be of natal or immigrant origin (Collias and Collias, 1978; Lewis, 1981; Lewis,

1982; Wingfield and Lewis, 1993). Breeding takes place during the wet season,

typically from October to March. Reproductive opportunities are monopolised by

dominants (Harrison *et al.*, 2013). The dominant female is the sole egg producer

and incubator as well as the primary provisioner of nestlings (Lewis, 1981). The

dominant male monopolises within-group paternity (Harrison *et al.*, 2013; 12-18%

of young are sired by extra-group males). Dominant males also sing dawn song

throughout the breeding season (Voigt *et al.*, 2006); while subordinate males do

occasionally sing, they are invariably out-sung by their dominants (York, 2012).

Sparrow weavers exhibit well-developed cooperation, with group members

contributing to the care of young, sentinelling, territory defence and the weaving of

roost chambers (Collias and Collias, 1978; Lewis, 1981).

A number of traits make sparrow weavers an ideal study system with which to ask

questions about the physiological mechanisms that underpin life-history trade-

offs, both in terms of their unusual tractability and their breeding biology. Groups

remain in their territories year round, and birds can reliably be captured individually from their roost chambers at night, facilitating the collection of both repeated longitudinal and time-sensitive physiological samples. Breeding nests are conspicuous and easily accessible, permitting close observation of breeding stage and allowing individual contributions to nestling care to be recorded with video cameras. Furthermore, sparrow weavers exhibit high reproductive skew, yielding clear rank-related contrasts in reproductive effort which could potentially lead to associated asymmetries in oxidative balance. Subordinates of both sexes help at the nest, but the dominant female provisions at the highest rate. While contributions to nestling care by all group members are typically reduced in larger groups (Lewis, 1981, Young *et al.,* unpublished data), the oxidative balance consequences of this remain to be explored.

1.9   THESIS STRUCTURE

In **chapter 2**, I investigate whether rank-related differences in oxidative balance and body mass exist before, or emerge after, the lengthy breeding season. Prior to breeding, superior oxidative balance may allow dominants to monopolise reproduction. Equally, dominants invest more heavily in reproduction, territory defence and sentinelling duties during the breeding season, and therefore may suffer differential declines in oxidative balance over the course of the breeding season.

In **chapter 3**, I experimentally investigate whether reproduction negatively impacts oxidative balance and body mass in a wild cooperative breeder, using a clutch-removal manipulation and within-individual sampling. Furthermore, I

assess the extent to which cooperative contributions of helpers can mitigate the costs of reproduction in larger social groups.

Chapter 4 investigates whether the immediate costs of reproduction (revealed in Chapter 3) endure or accumulate, thus resulting in long-term impacts on oxidative balance after the breeding season is complete. Oxidative stress may play an important role in mediating carryover effects between seasons (Harrison *et al.,* 2011), yet longitudinal data on individuals' oxidative balance and reproductive effort is limited.

In **chapter 5**, I experimentally investigate the oxidative stress costs of mounting an immune response in a wild, free-roaming bird. Furthermore, I examine whether oxidative balance mediates condition-dependent adjustment of immune defence, by investigating whether baseline oxidative balance predicts the subsequent size of response following immune challenge.

In **chapter 6** I summarise the findings of this thesis and discuss the wider implications of my results for our understanding of the role that oxidative stress plays in mediating life-history trade-offs and its distribution in society and suggest future research directions.

CHAPTER 2

Rank-related variation in oxidative balance reflects reproductive skew but does not cause it in a wild cooperative breeder

2.1 ABSTRACT

While oxidative stress has been proposed as an important mediator of life-history trade-offs, the factors that affect the distribution of oxidative stress in animal societies remain virtually unexplored. Where dominant and subordinate individuals differ in their reproductive success, variation in oxidative balance could also be marked, with implications for patterns of survival, health and senescence. Here we investigate rank-related differences in oxidative state in wild cooperatively breeding white-browed sparrow weavers (*Plocepasser mahali*) before and after a lengthy breeding season. Immediately before the breeding season, neither sex showed rank-related variation in circulating markers of oxidative damage or enzymatic and non-enzymatic antioxidant protection, suggesting that the reproductive monopolies of dominants do not arise from rank-related differences in oxidative state. Over the course of the breeding season, however, females (who provision young at higher rates than males) exhibited a rise in oxidative damage and dominant females (the primary provisioners and only birds to lay and incubate eggs) suffered differential declines in plasma antioxidant capacity. While males also showed reduced plasma antioxidant capacity after the breeding season, this decline was rank-independent and not associated with elevated oxidative damage. Together, our findings suggest that variation in oxidative balance in societies may arise in large part from the division of labour among individuals, with the hardest working dominance and sex classes experiencing differential deficits in oxidative state after reproductive episodes, raising the possibility of hitherto unexplored downstream effects on patterns of health and senescence.

2.2 INTRODUCTION

Oxidative stress occurs when the generation of reactive oxygen species (ROS), over-powers the body's antioxidant protection system, resulting in damage to biomolecules (Finkel and Holbrook, 2000; Dröge, 2002; Halliwell and Gutteridge, 2007). It has been proposed that oxidative stress might therefore be an important mechanism underpinning life-history trade-offs and senescence (Beckman and Ames, 1998; Dowling and Simmons, 2009; Monaghan *et al.*, 2009; Metcalfe and Alonso-Alvarez, 2010; Selman *et al.*, 2012). Indeed, mounting evidence does suggest that oxidative stress could mediate the costs of reproduction (Alonso-Alvarez *et al.*, 2004b; Wiersma *et al.*, 2004; Losdat *et al.*, 2011; Christe *et al.*, 2012; Fletcher *et al.*, 2012; Stier *et al.*, 2012) and shape rates of ageing (Devevey *et al.*, 2010; Saino *et al.*, 2011; Selman *et al.*, 2012). A historical focus of research on oxidative stress in laboratory populations, in which tractability might conceivably trade-off against ecological validity (Garratt *et al.*, 2012), is now being met with increased research on wild populations (Bergeron *et al.*, 2011; Losdat *et al.*, 2011; Christe *et al.*, 2012). However, such work has focussed almost exclusively on solitary or pair-breeding species, leaving the distribution of oxidative stress in social species largely unexplored. This is perhaps surprising, as the marked divisions of labour seen in many animal societies, including our own, have the potential to either drive or reflect individual differences in oxidative state, with implications for the patterns of survival, health and senescence in society.

In many animal societies, for example, dominant individuals breed at markedly higher rates than their subordinates (Keller and Reeve, 1994; Magrath *et al.*, 2004; Hodge, 2009; Koenig *et al.*, 2009), raising the possibility that variation among

individuals in oxidative balance in such 'high skew' species could be particularly marked. While the endocrine effects of social dominance and their implications for reproductive skew in animal societies have received considerable attention (Creel, 2001; Young *et al.*, 2006; Young, 2009), whether rank-related differences in oxidative state also underpin (or indeed arise from) rank-related differences in reproduction remains unknown.

Dominant individuals could differ from subordinates in their oxidative balance prior to reproductive episodes, as they may be phenotypically superior (Heg *et al.*, 2004; Russell *et al.*, 2004; Dengler-Crish and Catania, 2007; Young and Bennett, 2010) and thereby have stronger antioxidant defences, either endogenously or via differential access to key dietary resources (Bart and Earnst, 1999; Candolin and Voigt, 2001). Such superior antioxidant defences might thereby permit differential investment in reproduction (Bertrand *et al.*, 2006a; Pike *et al.*, 2007; Heiss and Schoech, 2012), while disruptive effects of oxidative stress on reproduction (Kaur *et al.*, 2006; Stier *et al.*, 2012) might conceivably underpin reproductive suppression among subordinates. Indeed in the pre-nesting phase, dominant female Seychelles warbler *Acrocephalus sechellensis* 'breeders' had lower blood concentrations of reactive oxygen metabolites (a proxy for oxidative damage), compared with 'non-helper' subordinates that do not invest in reproduction (van de Crommenacker *et al.*, 2011b), suggesting that subordinate individuals may indeed be more susceptible to oxidative stress in some social species.

Although dominant individuals may be phenotypically superior, tenure as a dominant is likely to entail physiological challenges not faced by reproductively suppressed subordinates (Creel, 2001). Dominant individuals breed more

frequently (Keller and Reeve, 1994; Magrath *et al.*, 2004), which can incur costs while defending a territory and mate (Vehrencamp *et al.*, 1989; Castro *et al.*, 2006; Hasselquist and Bensch, 2008), and producing and caring for young (Nilsson, 2002; Hanssen *et al.*, 2005; Vézina and Williams, 2005). Increased physical activity and elevated metabolic rate during these behaviours can promote the production of ROS (Reddy *et al.*, 1998; Leeuwenburgh *et al.*, 1999; Powers and Jackson, 2008; Radak *et al.*, 2008), leaving dominant individuals differentially exposed to oxidative stress during breeding periods. As such, rank-related differences in oxidative balance might also emerge after breeding, with dominants suffering differential deficits relative to their state prior to such periods. Indeed, a recent study of cooperatively breeding Seychelles warblers found evidence that male breeders suffered increased oxidative damage when guarding their nest and mate, relative to the pre-nesting phase (van de Crommenacker *et al.*, 2011b). This result was not evident in helper males, highlighting that dominance-specific traits can promote oxidative stress. If dominant individuals do suffer differential ROS challenges during breeding periods, selection might also be expected to favour the pre-emptive elevation of antioxidant defences among dominants before such periods begin (Beaulieu *et al.*, 2011). Evidence that dominant individuals in some animal societies actually live for longer than their non-reproductive subordinates (Arnold and Owens, 1998; Carey, 2001; Dammann and Burda, 2006) suggests that dominants do somehow mitigate the oxidative impact of their differential investment in reproduction.

Aside from dominance status, the extent to which group size influences oxidative state in social species has also received little attention. Animals can accrue a wide array of benefits from living in larger groups, including decreased predation risk

(Krause and Godin, 1995; Ebensperger and Wallem, 2002), increased foraging time (Caraco, 1979; Clutton-Brock *et al.*, 1999; Lian *et al.*, 2007) and lightened workloads during reproduction (Crick, 1992; Hatchwell, 1999; Heinsohn, 2004). Such benefits of group-living might also improve oxidative balance (by impacting ROS production and/or the intake of resources that support antioxidant defences), which could thereby mediate, at least in part, the fitness advantages that can arise from living in larger groups (e.g. enhanced fecundity: Brown et al 1982; Mumme 1992; Russell et al 2003 and survival: Clutton-Brock et al 1999).

Here we investigate rank-related differences in oxidative balance and body mass in wild populations of the cooperatively-breeding white browed sparrow weaver (*Plocepasser mahali*), before and after a lengthy breeding season. White-browed sparrow weavers are an ideal model system for this investigation, because they live in year-round territorial groups of 2-12 birds in which within-group reproduction is completely monopolised by the dominant male and female (Harrison *et al.*, 2013). While 12-18% of young are sired by extra-group males, the vast majority of extra-group sires are also dominant males (Harrison *et al.*, 2013). The dominant female is the sole egg producer and incubator as well as the primary provisioner of nestlings, but is assisted with provisioning by all group members (Lewis, 1982; Harrison *et al.*, 2013). The dominant male sings dawn song throughout the breeding season (Voigt *et al.*, 2006; York, 2012); while subordinate males do sometimes sing, they are invariably outsung by their dominants (York, 2012). Dominants of both sexes also invest more heavily than subordinates in territorial defence (York & Young unpublished data). We therefore investigate whether these marked rank-related differences in reproductive success and associated investment during the breeding season (i) reflect rank-related

differences in oxidative state and/or body mass that exist at the start of the breeding season and/or (ii) give rise to differential rank-related declines in oxidative state or body condition over the course of the breeding season. We also investigate whether sparrow weavers of either sex enjoy improved oxidative states when living in larger groups, in which *per capita* investment in both provisioning young and sentinel behaviour are known to decline, affording individuals more time to forage (Young *et al.*, unpublished data).

Oxidative balance is a complex, multi-faceted physiological state that can only be characterised through multiple markers of antioxidant protection and oxidative damage (Hõrak and Cohen, 2010). We therefore characterise oxidative state through a suite of metrics, including circulating levels of a lipid oxidative damage product (malondialdehyde, MDA), levels of a key intra-cellular antioxidant enzyme (superoxide dismutase, SOD) and circulating non-enzymatic antioxidant activity (total antioxidant capacity, TAC). Furthermore, oxidative state can show considerable inter-individual variation, as it can be influenced by a variety of factors, including early life conditions (Blount *et al.*, 2003; Alonso-Alvarez *et al.*, 2007), diet (de Ayala *et al.*, 2006; Selman *et al.*, 2006) and territory quality (van de Crommenacker *et al.*, 2011a). We therefore employ a paired-sampling approach, and all individuals were captured both before and after the breeding season.

2.3 METHODS

2.3.1. Study species and population

Data collection was conducted in the context of a long-term study, monitoring a population of 40 cooperative groups of white-browed sparrow weavers in an area of approximately 1.5 km² in Tswalu Kalahari Reserve, South Africa (27°16'S, 22°25'E). All birds were fitted with a single metal ring and three colour rings for identification (under SAFRING license 1444). Males and females were distinguished by beak colour: males have dark brown beaks while females have paler horn-coloured beaks (Leitner *et al.*, 2009). Dominant birds were identified following weekly behavioural observations and criteria detailed elsewhere (Harrison *et al.*, 2013). Briefly, dominant males consistently sang dawn song during the breeding season, and were behaviourally dominant to all other males. Dominant females were the only females to lay and incubate eggs in each group. Group size was assigned by assessing the number of birds roosting in the group's tree(s) and consistently seen in close contact with other group members during routine behavioural observations.

Blood sampling was conducted during two phases. Our population breeds during the wet season, which typically lasts from October to March. Blood sampling took place in September 2011 and April 2012, corresponding to immediately before ('pre phase') and after the breeding season ('post phase'), respectively. Confirmation of each group's non-breeding status was confirmed by nest searches conducted every other day. The data included 44 females (20 dominants and 24 subordinates) and 49 males (21 dominants and 28 subordinates, four of these

subordinates became dominant during the season) from 32 social groups. Sample sizes for the three markers of oxidative balance may vary according to the availability of the required samples. All birds were captured during both the pre- and post-season phases, and were at least five months old.

2.3.2. Capture and blood-sampling

All capture, blood sampling and measurements were conducted by one person (DC) under license (SAFRING license 1444). Birds were captured individually at night, by flushing them from their individual roost chambers into a custom capture bag. A blood sample (approximately 160 µl) was immediately collected from the brachial vein with a 26g needle. The lag between bird capture and completion of blood sampling was minimized, but recorded to examine potential effects of capture stress on metrics of oxidative balance. Body mass was then recorded to the nearest 0.01 g (Durascale 100 , MyWeigh, UK) and tarsus length measured to the nearest 0.1 mm using callipers. After processing, birds were returned to their roosts to pass the remainder of the night. Although it has received little attention, it is possible that the oxidative balance of diurnal animals changes as they rest overnight. Sparrow weavers go to roost shortly after sunset, so the period of time between sunset and sample collection was calculated for each bird. This allowed an investigation of whether oxidative balance metrics recover, or deteriorate, as the night progresses.

2.3.3. Blood processing and oxidative balance metric determinations

After collection, blood was immediately separated by centrifugation ($12 \times g$ for 3 minutes, Haematospin 1400; Hawksley Medical and Laboratory Equipment, UK). Erythrocytes drawn from the cellular phase of the separated whole blood were

lysed in four times their volume of ice-cold distilled water. This solution was mixed, placed on ice for 5 minutes, and centrifuged for 3 minutes ($12 \times g$). The supernatant (erythrocyte lysate) was then drawn off. Plasma from the separated whole blood (for the determination of MDA, TAC and uric acid levels) and lysed erythrocytes (for the determination of SOD activities) were stored on ice until they could be transferred to liquid nitrogen (mean ± S.D. time lag from processing to storage on liquid nitrogen = 131 ± 60 minutes). Samples were later transported from the field site to our laboratory in the UK on dry ice (approximately 48 hours), where they were stored at -80°C until analysis.

Oxidative damage to lipids

Lipids are a major target of ROS during oxidative stress, and concentrations of lipid peroxidation products therefore offer a measure of *in vivo* oxidative damage (Halliwell and Chirico, 1993). Plasma concentrations of malondialdehyde (MDA; a product of lipid peroxidation) were determined by high performance liquid chromatography (HPLC), following Agarwal & Chase (2002) with some modifications. This is a widely used and precise method of measuring MDA concentrations in biological samples (Monaghan *et al.*, 2009). A 10 μl aliquot of plasma was vortex mixed with 10 μl of butylated hydroxytoluene (0.05% w/v in 95% ethanol), 80 μl of phosphoric acid (0.44M) and 20 μl of 2-thiobarbituric acid (TBA) solution (42 mM) in screw-cap centrifuge tubes. These tubes were heated at 100 °C for exactly 1 hour in a dry bath incubator and then cooled on ice (5 minutes). After removal from the ice, 100 μl of n-butanol was added and the mixture was then vortex mixed for 20 seconds. After centrifugation for 3 minutes (4°C, $13 \times g$), 40 μl of the upper phase (containing the MDA-TBA adduct) was

drawn off and injected into an HPLC system (Dionex Corporation, USA) fitted with a Hewlett-Packard Hypersil 5μ ODS 100 x 4.6 mm column and a 5μ ODS guard column, maintained at 37°C. The mobile phase was methanol-buffer (40:60 v/v) running isocratically over 3.5 min at a flow rate of 1 ml/min. The buffer was an anhydrous solution of potassium monobasic phosphate (50 mM) at pH 6.8 (adjusted using 5M potassium hydroxide solution). Fluorescence detection was performed at 515 nm (excitation) and 553 nm (emission) (RF2000; Dionex Corporation, USA). A standard curve was generated in a parallel assay, using serial dilutions of 5 μM 1,1,3,3-tetraethoxypropane (which hydrolyses to produce MDA) in 40% ethanol. A subset of plasma samples run in duplicate were highly repeatable ($F_{66,67}$ = 15.92, r = 0.88, p < 0.001, see Lessells and Boag 1987).

Superoxide dismutase antioxidant protection

Superoxide dismutase (SOD) is a key intracellular antioxidant enzyme, which forms part of the first line of defence against oxidative stress (Godin and Garnett, 1992; Parkes *et al.*, 1998). Erythrocyte SOD activity was determined using a colorimetric assay involving the activation of a chromogen by the superoxide ion. SOD activity inhibits chromogen activation by converting the superoxide ion to hydrogen peroxide, and can therefore be read using a spectrophotometer (Spectramax M2; Molecular Devices, USA). Erythrocyte lysate samples prepared in the field (see above) were analysed using an assay kit (Cayman Chemicals, USA) following kit instructions. SOD activities were calibrated using a serial dilution of bovine SOD run on each plate. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical; enzyme activities are reported as units/ml. A subset of samples were analysed in duplicate on separate

plates; SOD activities were highly repeatable ($F_{37,38}$ = 6.07, r = 0.72, p < 0.001, see Lessells and Boag 1987).

Total Antioxidant Capacity

Non-enzymatic antioxidants represent a key defence against the harmful effects of ROS (Cohen *et al.*, 2007). This complex antioxidant protection comprises many endogenous and dietary components, and it is therefore not feasible to measure the concentrations of all potential antioxidants separately. Furthermore, synergistic and antagonistic relationships between antioxidants make it difficult to interpret concentrations of individual antioxidants in isolation (e.g. Bowry *et al.*, 1992). These problems can be avoided by measuring the ability of a sample to quench free radicals, thus providing a functional measure of total antioxidant capacity (TAC).

Plasma TAC was determined using a colorimetric assay kit (Cayman Chemicals, USA) in which the activation of a chromogen by hydrogen peroxide is inhibited by antioxidants in the sample. Antioxidant activity can therefore be read using a spectrophotometer (Spectramax M2; Molecular Devices, USA). This is a commonly employed approach for estimating an integrated parameter of antioxidant capacity in biological samples (reviewed in Halliwell and Gutteridge, 2007). Plasma samples were analysed following kit instructions (Cayman Chemicals, USA). Absorbance values were calibrated using a serial dilution of Trolox (a water-soluble vitamin E analogue) run on each plate. Plasma TAC values are expressed as trolox-equivalent antioxidant concentrations. A subset of plasma samples were run

in duplicate on two plates; TAC values were highly repeatable between plates ($F_{41,42}$ = 8.20, r = 0.78, p < 0.001, see Lessells and Boag 1987).

It has recently been highlighted that up to 90% of the variation in antioxidant activity that makes up TAC can be due to the 'incidental' antioxidant effects of uric acid (Cohen *et al.*, 2007). Uric acid is a waste product of protein metabolism in birds, and its *in vitro* antioxidant activity may be incidental to this primary role. The *in vivo* importance of uric acid as an antioxidant remains unclear. Furthermore, increased uric acid levels can indicate stress (Cohen *et al.*, 2008), and uric acid production can itself generate ROS (Dröge, 2002). As a result, plasma TAC values comprise the activities of important antioxidants, as well as that of uric acid, which may at best add noise or at worst confound key analyses (Cohen *et al.*, 2007; Cohen *et al.*, 2008; Cohen and McGraw, 2009).

In order to factor out the impact of circulating uric acid on plasma TAC, we therefore calculated the residuals of the relationship between uric acid and TAC (hereafter termed 'residual TAC') as recommend in Cohen *et al.* (2007). A linear mixed model was first used to confirm the relationship between uric acid concentration (set as a predictor) and TAC (as the response). Bird identity was the random factor (as two measures of each blood marker were taken from each bird). Plasma uric acid concentration significantly predicted plasma TAC values (χ^2_1 = 37.89, p < 0.001, n = 102 samples). The residuals were therefore extracted from a separate linear model (with TAC as the response and uric acid as the only predictor), to yield a measure of plasma antioxidant capacity excluding that arising from plasma uric acid levels.

Uric acid

Plasma concentrations of uric acid were determined using a fluorescence assay kit (Cayman Chemical, USA). In this assay, uricase oxidizes uric acid, releasing hydrogen peroxide. The resulting hydrogen peroxide activates a highly fluorescent chromogen. Uric acid concentrations can therefore be read using a spectrophotometer (Spectramax M2; Molecular Devices, USA). Plasma samples were analysed following kit instructions (Cayman Chemical, USA). Plasma uric acid levels were calibrated using a serial dilution of pure uric acid on each plate. A subset of plasma samples were run in duplicate on separate plates; uric acid concentrations were highly repeatable between plates ($F_{39,40} = 8.35$, $r = 0.79$, $p < 0.001$, see Lessells and Boag 1987).

Body Mass

We calculated the Scaled Mass Index (SMI) (Peig and Green, 2009) to compare body mass among dominant and subordinate individuals of both sexes. The SMI avoids the problems associated with residual-based measures of "body condition," which have recently been highlighted (Labocha and Hayes, 2012), instead scaling each individual's body mass to the value expected if all birds were all of identical skeletal size, by using the inherent power relationship between mass and size modelled from the data (Peig and Green, 2009). We used body mass and tarsus length measures from 186 capture records (before and after the breeding season) of 93 birds. We scaled the masses of all birds to the mean tarsus length (24.6 mm), using a Secondary Major Axis slope of 2.64 (Peig and Green, 2009). This Scaled Mass Index is hereafter referred to as "body mass."

2.3.4. Statistical Analyses

Statistical analyses were carried out using R (R Development Core Team, 2013), using a step-wise model simplification approach (Crawley, 2007). Initially all fixed terms of interest were fitted, followed by the stepwise removal of terms whose removal from the model resulted in a non-significant change in deviance (using a likelihood-ratio test for model comparison), until the minimal adequate model (MAM) was obtained, in which only significant terms remained. Dropped terms were then added back in to the MAM to confirm their non-significance and were retained in the MAM when found to be significant in this context. The homoscedasticity and normality of residuals were inspected visually and where necessary response terms were transformed to satisfy these criteria. The significance of all terms was tested either by removing the terms from the MAM (if the term was in the MAM) or adding the terms back in to the MAM and then removing them (if the term was not included in the MAM).

The focus of the current study is intra-sexual rank-related differences in oxidative balance and body mass. Furthermore, dominance is likely to entail different challenges in males and females in this species (see introduction). For this reason, males and females were analysed in separate models. For each response, a model was fitted comprising season phase and dominance rank, as well as their interaction, as the major predictor of interest. Group size (the number of birds in the social group), the number of hours after sunset that the bird was caught and bled, and the time lag between bird capture and blood sampling ('capture-to-bleed' lag in seconds) were also fitted as fixed effect predictors (capture-to-bleed lag was not included in models of body mass). Bird ID was nested within social group as a random factor, to account for the sampling of multiple individuals

within a group and the repeated sampling of individuals at different season phases (no bird was sampled more than once within a given season phase).

Initially, the effect of age on markers of oxidative balance and body mass was investigated using a subset of known-age individuals captured in either the pre- or post-season phase (84 males and 80 females from a total of 29 groups). The above models were used, with the addition of age in days as a fixed effect. Age in days did not significantly predict levels of any of the response terms (MDA, SOD, residual TAC or body mass), in either males or females (all $p > 0.18$). The datasets were therefore expanded to include individuals of unknown age, and models were fitted without the age term; it is these models that are presented in the results section.

2.4 RESULTS

2.4.1. Female rank-related oxidative balance

Do dominant and subordinate females differ in oxidative damage?

Plasma levels of the lipid peroxidation product MDA did not differ significantly between dominant and subordinate females (χ^2_1 = 1.34, p = 0.25, n = 88 captures of 44 females), and this relationship was not dependent on season phase (Figure 2.1a, dominance × season phase interaction: χ^2_1 = 0.31, p = 0.58). However, MDA levels were significantly higher in the post-season phase (χ^2_1 = 8.40, p = 0.004). We found no evidence that female plasma MDA levels were affected by social group size, capture-to-bleed lag or time since sunset (all χ^2_1 < 0.38, p > 0.54).

Do dominant and subordinate females differ in enzymatic antioxidant protection?

We found no evidence that female erythrocyte SOD activities differed significantly with dominance rank, either as a single term (χ^2_1 = 0.06, p = 0.80, n = 54 captures of 27 females) or in the interaction with breeding season phase (Figure 2.1b, χ^2_1 = 1.09, p = 0.30). Erythrocyte SOD activity was not significantly predicted by season phase (χ^2_1 = 1.62, p = 0.20), or by group size, capture-to-bleed lag or the time since sunset (all χ^2_1 < 2.41, p > 0.12).

Do dominant and subordinate females differ in non-enzymatic antioxidant protection?

The interaction between dominance rank and season phase revealed significant differences in residual TAC in females (Figure 2.1c, χ^2_1 = 6.39, p = 0.011, n = 74

captures of 37 females). Dominant females showed significantly decreased residual TAC after the breeding season, while subordinate females exhibited no clear decline. This effect was confirmed using paired t-tests to compare a dominant female's residual TAC to the mean residual TAC of her own female subordinates, in the six social groups for which data was available for females of both ranks in both phases. In the pre-season phase, a dominant female's residual TAC did not significantly differ from the mean residual TAC of her own female subordinates (t_5 = 0.15, p = 0.89). However, this within-group rank-related difference in female plasma residual TAC emerged in the post season phase (t_5 = 3.09, p = 0.015). Residual TAC in females was not significantly predicted by group size, capture-to-bleed or time since sunset (all $\chi^2_1 < 1.94$, p > 0.16).

Do dominant and subordinate females differ in body mass?

There was a significant interaction between dominance rank and season phase in the model of female body mass (Figure 2.1d, dominance x season phase interaction: $\chi^2_1 = 9.54$, p = 0.002, n = 88 captures of 44 females). Dominant females were heavier than subordinates before the breeding season, but subordinate body mass increased over the course of the season, such that no rank-related difference in female body mass was evident in the post-season phase. This effect was further confirmed by comparing a dominant female's change in body mass over the course of the season with the mean change of her own female subordinates, in the nine social groups for which data was available for females of both ranks in both phases (paired t-test: $t_8 = 5.65$, p < 0.001; body mass change: dominants: 0.11 g ± 0.65, subordinates: 1.70 g ± 0.51, mean ± S.E.). Female body mass was not significantly affected by time since sunset or group size (both $\chi^2_1 < 0.77$, p > 0.38).
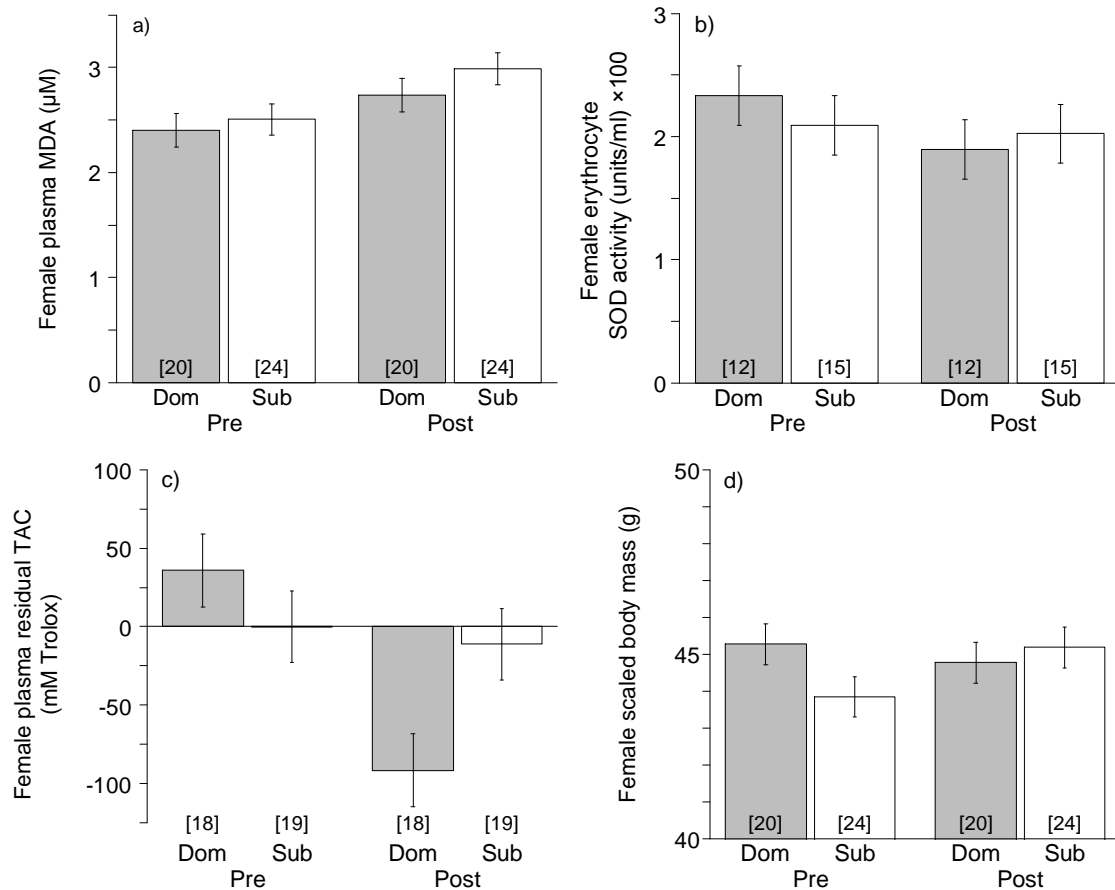
Figure 2.1 – Female rank-related differences in oxidative state and body mass, before (Pre) and after (Post) the breeding season, in dominants (grey bars) and subordinates (white bars): **(a)** oxidative damage (MDA), **(b)** intracellular enzymatic antioxidant protection (SOD), **(c)** non-enzymatic antioxidant protection (non-uric acid residual TAC), **(d)** body mass. Bars show model predicted mean ± SE from the interaction of season phase and dominance rank, while controlling for other significant predictors. Numbers in parentheses are sample sizes (numbers of females sampled).

2.4.2. Male rank-related oxidative balance

Do dominant and subordinate males differ in oxidative damage?

Dominance rank did not significantly predict male plasma concentrations of the lipid peroxidation product MDA (χ^2_1 = 0.01, p = 0.94, n = 96 captures of 48 males), and this was not dependent on season phase (Figure 2.2a, dominance × season phase interaction: χ^2_1 = 0.46, p = 0.50; season phase: χ^2_1 = 0.51, p = 0.47). Furthermore, plasma MDA concentration was not significantly predicted by group size, capture-to-bleed lag or time after sunset (all χ^2_1 < 0.58, p > 0.48) in males.

Do dominant and subordinate males differ in enzymatic antioxidant protection?

Similarly, the activity of a key intracellular antioxidant enzyme (SOD) was not predicted by dominance rank (χ^2_1 = 0.01, p = 0.93, n = 62 captures of 31 males), or season phase (either as a single term: χ^2_1 = 0.08, p = 0.78; or in the interaction with dominance rank: Figure 2.2b, χ^2_1 = 0.34, p = 0.56). Group size, capture-to-bleed lag and time after sunset were also non-significant predictors of male SOD activity (all χ^2_1 < 0.37, p > 0.54).

Do dominant and subordinate males differ in non-enzymatic antioxidant protection?

Male dominance rank did not significantly predict plasma residual TAC, either as a single term (χ^2_1 = 0.11, p = 0.74, n = 88 captures of 44 males), or in the interaction with breeding season phase (Figure 2.2c χ^2_1 = 2.89, p = 0.089). However, after controlling for other important predictors (see below), residual TAC did differ significantly between pre- and post-breeding season phases (χ^2_1 = 4.96, p = 0.026):

males had stronger non-enzymatic antioxidant protection before the breeding season than they did after it. There was also a significant negative effect of group size on residual TAC (χ^2_1 = 6.92, p = 0.009), a significant positive effect of capture-to-bleed lag (χ^2_1 = 5.11, p = 0.024) and a significant negative effect of time since sunset (χ^2_1 = 15.93, p < 0.001).

Do dominant and subordinate males differ in body mass?

There was no evidence that dominant and subordinate males differed significantly in body mass (χ^2_1 = 2.59, p = 0.11, n = 98 captures of 49 males), while controlling for the significant negative effect of time since sunset (χ^2_1 = 13.55, p < 0.01). There was also no significant effect of the season phase × dominance interaction (Figure 2.2d, χ^2_1 = 3.44, p = 0.064), although there was a trend reflecting the result found in females: dominant males did not gain body mass during the season, while subordinates did. Season phase as a single term was also of borderline significance in predicting body mass (χ^2_1 = 3.70, p = 0.054), with a tendency towards higher body mass in the post-season. There was no significant effect of group size on male body mass (χ^2_1 = 0.18, p = 0.67).
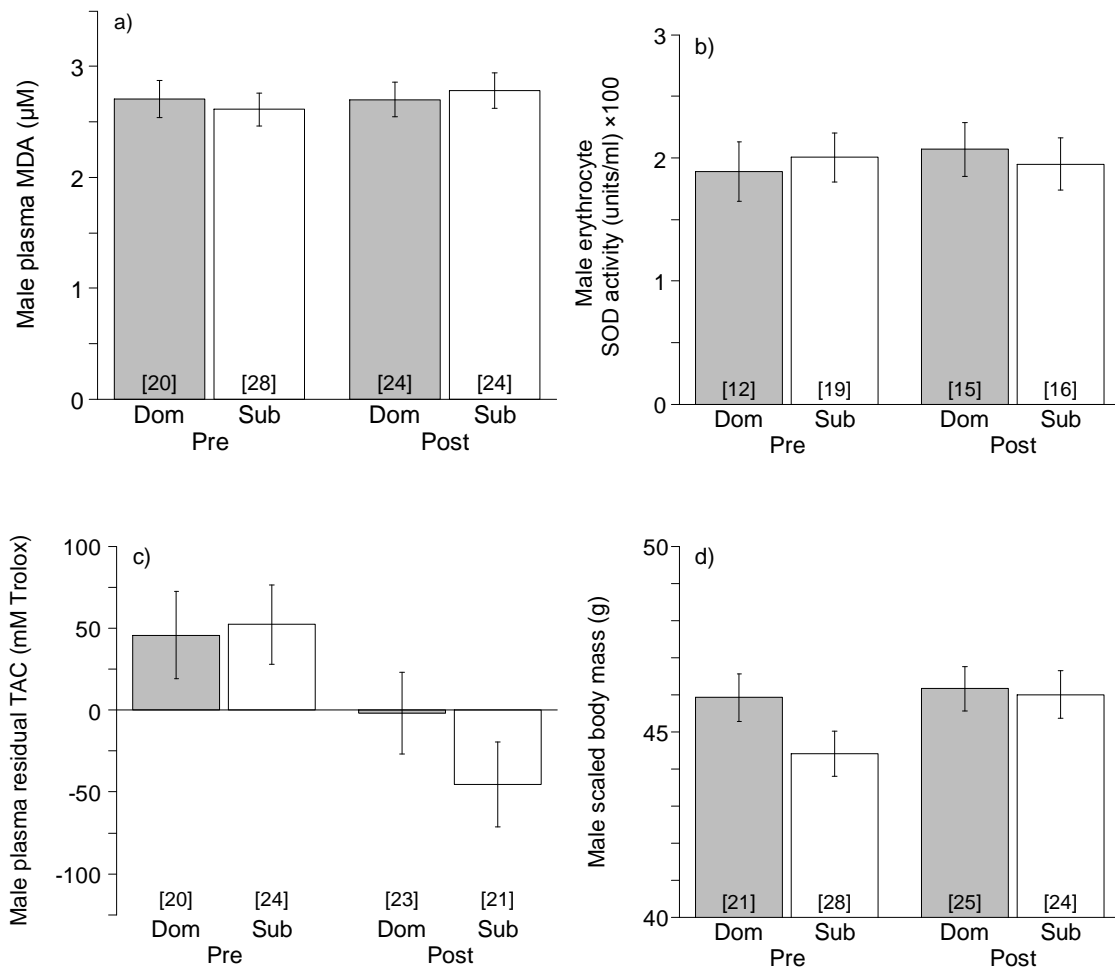
Figure 2.2. – Male rank-related differences in oxidative balance and body mass, before (Pre) and after (Post) the breeding season, in dominants (grey bars) and subordinates (white bars): **(a)** oxidative damage (MDA), **(b)** intracellular enzymatic antioxidant protection (SOD), **(c)** non-enzymatic antioxidant protection (non-uric acid residual TAC), **(d)** body mass. Bars show model predicted mean ± SE from the interaction of season phase and dominance rank, while controlling for any (other) significant predictors. Numbers in parentheses are sample sizes (number of males sampled).

2.5 DISCUSSION

This study provides rare evidence of rank-related differences in oxidative balance in a wild social vertebrate. That such differences only became apparent after the breeding season, strongly suggests that they reflect rather than cause rank-related differences in reproduction. Neither dominant males nor females entered the breeding season with stronger oxidative balance than their same-sex subordinates. However, both sexes showed declines in non-enzymatic antioxidant protection over the course of the breeding season. In males, this decline was mild, independent of rank and not accompanied by a rise in oxidative damage. By contrast, in females, the decrease in antioxidant defences was marked but restricted to dominant females, while all females showed rank-independent increases in oxidative damage. Consistent with dominant females suffering differential impacts on state over the course of the breeding season, dominant females also entered the breeding season in stronger body condition than their subordinates, but this contrast was no longer apparent at the end of the breeding season. While group size had no impact on oxidative state among females, males living in larger groups showed reduced plasma residual TAC.

In the pre-season, neither dominant males nor females differed from their same-sex subordinates in oxidative balance. This lack of rank-related oxidative balance disparity before the breeding season is perhaps surprising, as dominant birds might have been expected to elevate antioxidant levels in preparation for the breeding season (Beaulieu *et al.*, 2011), when investment in territory defence, egg production, incubation and nestling care may differentially challenge their oxidative balance relative to that of their subordinates. Indeed, dominant females

were heavier than subordinates before the breeding season began, suggesting that they may pre-emptively acquire energetic reserves, but do not appear to do so with regard to antioxidant protection. Strong antioxidant protection and lower pre-breeding levels of oxidative damage have previously been shown to predict greater reproductive effort in some species (van de Crommenacker *et al.*, 2011b; Heiss and Schoech, 2012; Stier *et al.*, 2012). It is possible that, in our study species, dominants are simply unable to pre-emptively elevate their antioxidant protection because they do not enjoy differential access to antioxidant-rich dietary resources, or such resources are scarce following the lengthy dry non-breeding season (April – October). It is also conceivable, however, that dominants actually pre-emptively store additional antioxidants in tissues, whose levels may not be reflected in the circulating markers measured here (Veskoukis *et al.*, 2009; Garratt *et al.*, 2012). Alternatively, subordinates may also be selected to invest in increased antioxidant protection, in the event that they are able to secure a dominance position during the breeding season. Indeed, in our dataset, four males attained dominance between the pre- and post-season sampling periods. Reproduction may be particularly costly for inexperienced breeders (Sanz-Aguilar *et al.*, 2008) and cooperative breeders founding new groups without helpers (Heinsohn, 2004), so newly dominant individuals might differentially benefit from having raised their levels of antioxidant protection.

At the end of the breeding season, we again found no significant differences between the oxidative states of dominant and subordinate males, although males of both ranks exhibited declines in their antioxidant protection. Throughout the breeding season, dominant males produce a costly dawn song more regularly, and for longer durations, than male subordinates (York, 2012). Dominant males also

contribute more to sentinelling duties than their same-sex subordinates and invest equally in nestling care (Lewis 1981; Young *et al.*, unpublished data). As a result, dominant males could be expected to suffer an oxidative stress cost following the breeding season. However, any cost of dawn song performance may in fact be relatively transient, and decreased dawn song output late in the breeding season (York 2012) could allow dominant males to recover from any accumulated deficits in oxidative state during the final stages of the breeding season, before our post-season sampling took place. Alternatively, subordinate males may be suffering an equal oxidative stress burden over the course of the breeding season, arising from costs associated with prospecting for dispersal opportunities (e.g. Young and Monfort, 2009), as this species (unusually for cooperatively breeding birds) shows male-biased dispersal.

After the breeding season, females showed evidence of increased oxidative damage to lipids. While this apparent increase in oxidative stress among females was independent of dominance rank, we found evidence of marked rank-related differences in the effect of the breeding season on the antioxidant defences and body mass of females. Following the breeding season, subordinate females showed no reduction in antioxidant protection and gained body mass, while dominant females exhibited reduced antioxidant protection and did not gain body mass. These findings are unlikely to be due to between-individual differences in genotype or territory quality, because it remains even when dominant females are compared with their own subordinate females, with whom they share close relatedness and territories, but who invest much less in reproduction (Lewis, 1981; Harrison *et al.*, 2013). This post-breeding rank-related difference in oxidative balance therefore suggests a cost of reproduction paid primarily by

dominant females. Dominant females in white-browed sparrow weaver societies are the only birds to lay and incubate eggs, and invest most in nestling care (Lewis, 1981; Harrison *et al.*, 2013). As such, their differential decline in antioxidant defences and failure to gain body mass during the breeding season is consistent with evidence that reproduction can incur an energetic cost (Cox *et al.*, 2010; Lane *et al.*, 2010; Bergeron *et al.*, 2011) and promote oxidative stress (Bergeron *et al.*, 2011; Fletcher *et al.*, 2012; Stier *et al.*, 2012). It should be noted that dominant and subordinate females did not differ in circulating levels of lipid oxidative damage (MDA), and that lower circulating levels of antioxidant protection do not necessarily signify oxidative stress *per se* (Costantini and Møller, 2009). However, the impaired antioxidant defences of dominant females do raise the possibility that they are suffering oxidative damage either to biomolecules whose damage products were not measured here (e.g. proteins and DNA), or to tissues whose damage levels may be uncorrelated with those reflected by circulating markers (Veskoukis *et al.*, 2009; Garratt *et al.*, 2012). The reduced antioxidant defences of dominant females at the end of the breeding season may also be expected to entail downstream costs as sparrow weavers are likely to suffer poor food availability during the dry non-breeding season.

Interestingly, we found no evidence supporting a benefit of living in larger social groups. In fact, male non-enzymatic antioxidant protection was lower in larger groups, perhaps reflecting elevated intra-group competition. In larger white-browed sparrow weaver groups, shared workloads typically decrease individual investments in a number of activities, including nestling care, territory defence and sentinelling duty (Lewis 1981, Young *et al.,* unpublished data). For this reason, it was expected that individuals in larger groups might enjoy lower levels of

oxidative damage, higher levels of antioxidant protection and/or greater body mass. However, social group size may not accurately reflect the amount of cooperation occurring in a group, as there is great variation between helpers in cooperative contributions in this species. Detailed reproductive effort data, for example, will allow a better investigation into whether the oxidative impact of reproduction is lower when individual contributions to nestling care are reduced.

The findings of our study provide rare evidence of physiological burdens which fall heaviest on dominant individuals in animal societies. Specifically we find that, in sparrow weaver societies, dominant individuals can suffer differentially large declines in antioxidant defences and fail to gain body mass over the course of a breeding season, and that this can occur in a sex-specific manner. Importantly, our results also suggest that reproductive skew does not arise from rank-related disparities in oxidative state that exist before breeding begins. Our findings may have important implications for our understanding of the effect of dominance tenure on health and senescence trajectories in societies, and suggest that the increased longevity of dominants in some cooperatively breeding vertebrates (Arnold and Owens, 1998; Carey, 2001; Dammann and Burda, 2006) might actually be in spite of, and not due to, their antioxidant protection.

CHAPTER 3

Oxidative stress and the costs of reproduction: experimental evidence from a wild cooperative breeder

3.1 ABSTRACT

Life-history theory predicts that reproduction should entail a cost, and research on cooperatively breeding societies suggests that cooperation can mitigate these costs to some degree. However, the molecular mechanisms that underpin the costs of reproduction remain poorly understood. Recently, it has been suggested that oxidative stress may mediate these costs, with increased investment in reproduction resulting in elevated production of damaging reactive oxygen species, leading to impaired future reproductive success and senescence. However, experimental evidence of the role of oxidative stress in mediating the cost of reproduction in the wild remains scarce. Here, we use a clutch-removal manipulation to investigate the oxidative balance and body mass costs of reproduction in a wild cooperatively breeding bird. Our experimental findings reveal that the effect of treatment was dependent on social group size: relative to groups whose clutch was removed, reproduction was costly in small groups both in terms of reduced body mass and elevated oxidative damage, while in larger groups such costs were less pronounced. Furthermore, within breeding groups, higher natural offspring provisioning rates *per se* appeared to carry a cost in terms of reduced body mass and impaired antioxidant protection. Our results provide rare evidence that reproduction can negatively impact both body mass and oxidative balance in the wild, and that these costs can be mitigated in cooperative societies by the presence of additional helpers. These findings have implications for our understanding of the roles that macro- and micro-nutrients play in mediating both life-history trade-offs and the benefits of group-living and cooperation in animal societies.

3.2 INTRODUCTION

Life-history theory predicts that reproduction should entail a cost, and that investment in reproduction is therefore subject to trade-offs with other traits (Williams, 1966; Stearns, 1989). Indeed, there is extensive evidence that investment in reproduction can have a detrimental effect on future reproduction and survival (Clutton-Brock *et al.*, 1983; Nur, 1988; Landwer, 1994; Nager *et al.*, 2001; Nussey *et al.*, 2006; Cox *et al.*, 2010; Bowers *et al.*, 2012). Central to our understanding of these trade-offs is the identification of the physiological mechanisms that underpin them (Rose and Bradley, 1998; Zera and Harshman, 2001). Traditionally, the costs of reproduction have been considered in terms of the allocation of macronutrients (e.g. energy stores) to breeding, at the expense of self-maintenance (Stearns, 1989). As such, many studies have investigated the energetic costs of reproduction, frequently finding that breeding can entail marked reductions in body mass (Dijkstra *et al.*, 1990; Merila and Wiggins, 1997; Alonso-Alvarez *et al.*, 2004a; Bertrand *et al.*, 2006a; Cox *et al.*, 2010; Mitchell *et al.*, 2012). However, such macronutrient deficits may be relatively straightforward to recover or may be avoided entirely by increasing food intake (Nilsson, 2002). It is therefore questionable whether macronutrients alone mediate the documented long-term costs of reproduction, such as impaired future reproduction and curtailed survival (Nilsson, 2002).

More recently, oxidative stress has been highlighted as a potential mediator of important life-history trade-offs (Dowling and Simmons, 2009; Monaghan *et al.*, 2009). Oxidative stress occurs when the generation of reactive oxygen species (ROS) causes damage to proteins, lipids and DNA (Finkel and Holbrook, 2000;

Finkel, 2003; Halliwell and Gutteridge, 2007) ROS are primarily produced as a by-product of aerobic respiration, and increased energy turnover during reproduction may therefore elevate pro-oxidant production (Deerenberg *et al.*, 1995; Moreno *et al.*, 1995; Behrman *et al.*, 2001; Salmon *et al.*, 2001; Casanueva and Viteri, 2003; Murdoch and Martinchick, 2004; but see Barja et al 2007). Under normal circumstances, the damaging effects of ROS are limited by the body's complex antioxidant system (Ames *et al.*, 1993; Yu, 1994; Surai, 2002; Halliwell and Gutteridge, 2007). However, demand for antioxidants may be particularly high during reproduction, when antioxidant investment in sexual signals (von Schantz *et al.*, 1999), egg development (Blount *et al.*, 2000; Bertrand *et al.*, 2006a), sperm production (Blount *et al.*, 2001; Helfenstein *et al.*, 2010) and parental care (Alonso-Alvarez *et al.*, 2004a; Wiersma *et al.*, 2004; Pike *et al.*, 2007)may divert key antioxidants away from self-maintenance. As such, investment in reproduction may be traded-off against other life-history traits by the allocation of micronutrient antioxidants (Alonso-Alvarez *et al.*, 2008; Catoni *et al.*, 2008), and the combined effects of elevated pro-oxidant production and re-allocation of antioxidants during reproduction may promote oxidative stress (Costantini, 2008; Metcalfe and Alonso-Alvarez, 2010). Furthermore, oxidative stress can lead to increased rates of senescence, impaired future reproductive success and curtailed survival (Harman, 1956; Bize *et al.*, 2008; Monaghan *et al.*, 2008; Ricklefs, 2008; Selman *et al.*, 2012), making it a key candidate for the mediation of the costs of reproduction (Costantini, 2008; Metcalfe and Alonso-Alvarez, 2010; Metcalfe and Monaghan, 2013).

Results from correlative studies suggest that investment in reproduction can negatively impact oxidative balance, either by elevating circulating markers of

oxidative damage (van de Crommenacker *et al.*, 2011b; Fletcher *et al.*, 2012; Heiss and Schoech, 2012; Olsson *et al.*, 2012) or reducing circulating levels of antioxidant protection (Alonso-Alvarez *et al.*, 2004b; Wiersma *et al.*, 2004). However, other correlative studies found weak or no evidence of an oxidative stress cost of reproduction (Beaulieu *et al.*, 2011; Isaksson *et al.*, 2011; Markó *et al.*, 2011; Ołdakowski *et al.*, 2012). Notably, none of these studies manipulated reproductive effort, and individuals were therefore allowed to manage their own investment in breeding. Under such circumstances, an individual may be expected allocate resources optimally, by investing in reproduction up to the level at which further effort will result in oxidative damage (Metcalfe and Monaghan, 2013). This threshold level may vary between individuals, but all individuals can avoid incurring costs during reproduction by remaining beneath their threshold. Experimental manipulation of reproductive effort can push individuals beyond these thresholds, and thus offers a greater insight into the true costs of breeding (Metcalfe and Monaghan, 2013). While there is experimental evidence from studies in captivity that increased care for young can reduce antioxidant protection (Alonso-Alvarez *et al.*, 2004b; Wiersma *et al.*, 2004) and increase oxidative damage (Stier *et al.*, 2012), most studies report no negative effect on oxidative balance following experimentally elevated investment in reproduction (Garratt *et al.*, 2011; Ołdakowski *et al.*, 2012; Garratt *et al.*, 2013).

Experimental studies of the impact of reproduction on oxidative balance have been conducted almost exclusively in captivity to date. Such studies typically feature *ad lib* high-quality food located close to breeding areas, favourable thermal conditions and an absence of predation and competition. These relatively favourable conditions may greatly diminish the need for study animals to make the precise

trade-offs being investigated (Metcalfe and Alonso-Alvarez, 2010; Fletcher *et al.*, 2012). Advancing our understanding of the impacts of reproduction on oxidative balance therefore demands experimental studies on wild animals (Metcalfe and Alonso-Alvarez, 2010; Selman *et al.*, 2012; Metcalfe and Monaghan, 2013). To our knowledge, only two studies in the wild have investigated the impact of experimentally manipulated reproductive effort on oxidative balance: Losdat *et al.* (2011) and Christe *et al.* (2012) both reported reduced red blood cell antioxidant protection following experimental brood enlargement. While these studies provide valuable evidence that reproduction may negatively impact oxidative balance in the wild, the effects of reproductive investment on oxidative damage were not investigated. Variation in antioxidant levels can be difficult to interpret without measures of oxidative damage, as a reduction in antioxidant protection does not necessarily indicate oxidative stress (Costantini and Verhulst, 2009; Monaghan *et al.*, 2009; Selman *et al.*, 2012). Our understanding of the oxidative costs of reproduction in the wild therefore remains limited.

If investment in reproduction does entail physiological costs, evolution is expected to favour strategies that mitigate such costs. One such strategy may be cooperative breeding (Solomon and French, 1997; Koenig and Dickinson, 2004), whereby breeders' reproductive efforts are frequently reduced when 'helpers' aid in the rearing of the breeders' offspring (Brown, 1987; Crick, 1992; Hatchwell, 1999; Heinsohn, 2004). In such species, breeders receiving help often enjoy increased fecundity (Brown *et al.*, 1982; Mumme, 1992; Komdeur, 1994; Russell *et al.*, 2003) and survival (Reyer, 1984; Khan and Walters, 2002). However, the physiological mechanisms that underpin the long-term benefits of cooperation remain unclear. If care for young entails an oxidative stress cost, lowered workloads in cooperative

societies may lead to concomitant reduced risks of oxidative stress, with larger groups enjoying 'oxidative load-lightening.' Remarkably, the oxidative stress costs of reproduction and the benefits of cooperation in group-living species remain largely unexplored.

Here, we use a clutch removal experiment to investigate the impact of reproduction on oxidative balance and body mass in a wild population of cooperatively breeding white-browed sparrow weavers *Plocepasser mahali*. White-browed sparrow-weavers live in year-round territorial groups of 2-12 birds throughout the semi-arid regions of sub-Saharan Africa (Collias and Collias, 1978; Lewis, 1981). Groups comprise a single dominant pair that completely monopolise within-group reproduction (Harrison *et al.*, 2013; 12-18% of young are sired by extra-group males) and up to 10 subordinate males and females in approximately equal sex ratio (Lewis, 1981; Harrison *et al.*, 2013). The species shows well-developed cooperation, with most group members contributing to the care of young, sentinelling, territory defence and weaving (Collias and Collias, 1978; Lewis, 1982). With regard to reproduction, clutches of 1-4 eggs (mode: 2) are laid and incubated solely by the dominant female (Lewis, 1981; Harrison *et al.*, 2013), while most group members contribute to the cooperative provisioning of nestlings and fledglings (Lewis, 1981; Lewis, 1982). The presence of helpers significantly lightens the provisioning rates of breeders (Lewis, 1981; Lewis, 1982; Young *et al.*, unpublished data) and is associated with enhanced reproductive success (Lewis, 1981). Whether reproduction entails short-term costs in terms of impacts on either body mass or oxidative balance has yet to be investigated in this species.

Oxidative balance is a complex, multi-faceted physiological state that can only be characterised through multiple markers of antioxidant protection and oxidative damage (Hõrak and Cohen, 2010). We therefore investigate a suite of metrics of oxidative balance, including circulating levels of a lipid oxidative damage product (malondialdehyde, MDA), the levels of intra-cellular enzymatic antioxidant protection in the form of superoxide dismutase (SOD) and circulating non-enzymatic antioxidant activity (total antioxidant capacity, TAC).

Specifically, we use a clutch removal experiment to investigate the costs associated with reproduction, by contrasting the plights of individuals in breeding groups whose clutches of eggs were experimentally removed at clutch completion ('*clutch-removal*' treatment) with those of individuals in breeding groups that were allowed to hatch and rear their clutches ('*control*' treatment). Individuals in both treatments were caught (for the determination of their body mass and oxidative balance) both at clutch completion and again one month later, when the *control* groups were provisioning their broods at their highest rates, and the *clutch-removal* groups were not breeding. First, we test whether reproduction entails a short-term cost in terms of differential body mass reductions and/or deficits in oxidative balance in the *control* treatment relative to the *clutch-removal* treatment, and whether such costs may be mitigated in larger social groups. Second, we focus our attention on the *control* breeding groups, to investigate whether higher rates of offspring provisioning *per se* are associated with body mass reductions or deficits in oxidative balance at peak provisioning.

3.3 METHODS

3.3.1. Study population

Data collection was conducted in the context of a long-term study, monitoring a population of white-browed sparrow weavers in an area of approximately 1.5 km² in Tswalu Kalahari Reserve, South Africa (27°16'S, 22°25'E). All birds were fitted with a single metal ring and three colour rings for identification. Males and females were distinguished by beak colour: males have dark brown beaks while females have paler horn-coloured beaks (Leitner *et al.*, 2009). Dominant birds were identified following weekly behavioural observations and criteria detailed elsewhere (Harrison *et al.*, 2013). Briefly, dominant males consistently sang dawn song during the breeding season, and were behaviourally dominant to all other males. Dominant females were the only females to lay and incubate eggs in each group. Group size was assigned by assessing the number of birds consistently seen foraging and travelling in close contact with other group members during routine behavioural observations during the day.

All capture, blood sampling and measurements were conducted by one person (DC) under license (SAFRING license 1444). Birds were captured individually at night, by flushing them from their roost into a custom capture bag. Time from sunset to capture was 4.1 ± 0.8 hours (mean ± S.D.). A blood sample (approximately 160 µl) was immediately collected from the brachial vein with a 26g needle. The lag between bird capture and completion of blood sampling was minimized (213 ± 14 seconds, mean ± S.E.). Body mass was recorded to the nearest 0.01 g (Durascale 100, MyWeigh, UK). After processing, birds were returned to their roosts to pass the remainder of the night.

3.3.2. Clutch-removal experiment

Nest searches were conducted every one to two days from November 2011 to April 2012. In our species, the dominant female lays one egg each morning on consecutive days. When eggs were discovered, the date of clutch completion could therefore be determined by re-visiting the nest every afternoon until the same number of eggs was encountered on two consecutive visits ("*clutch completion*"). On the evening of the *clutch completion* day, all birds except the dominant female were captured and blood sampled. Capture of the incubating dominant female from the nest may have caused clutch abandonment; dominant females were therefore excluded from this study. Groups were then assigned to one of two treatments: clutches of eggs were either collected from the nest (*clutch-removal* treatment; n = 9 groups), or handled and returned allowing them to be incubated and reared as normal (*control* treatment; n = 11 groups). The subsequent breeding activity of all groups was then monitored, to confirm hatching dates for *control* groups, and to confirm the continued non-breeding status in the *clutch-removal* groups. Any control groups whose clutches failed to survive to peak provisioning were excluded from the analysis. All birds were then captured again between 25 and 30 days after *clutch completion*, when the nestlings in *control* groups were 10-12 days of age and therefore being provisioned at peak rates (Young *et al.,* unpublished data), while *clutch-removal* groups were not breeding. At this point, all birds were weighed and blood sampled again, to investigate '*final*' oxidative balance and body mass (see Figure 3.1).

'Clutch Completion' state (Day 1)	Treatment	*'Final'* state (Day 25 – 30)

Body mass and oxidative balance measures

Clutch-removal → Non-breeding
Body mass and oxidative balance measures

Control → Peak provisioning
Body mass and oxidative balance measures
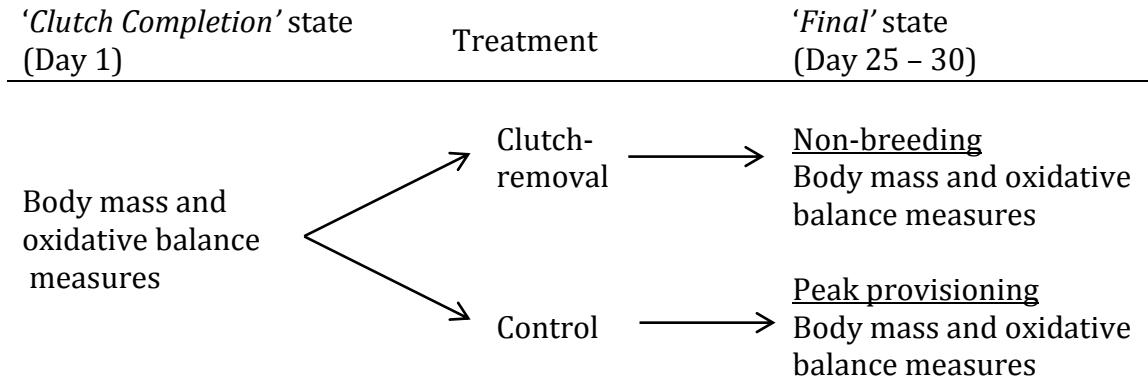
Figure 3.1 – Schematic representation of experimental treatments and data collection

3.3.3. Provisioning observations

When the nestlings in the control groups were aged 9-12 days, provisioning data were collected prior to the '*final*' blood sampling of adults. In the late incubation phase or early nestling phase, all breeding individuals except the dominant female were captured and given unique dye marks on their white vent feathers. This allowed the identification of individual provisioning birds using video cameras placed on tripods beneath the entrance to the nest. A tripod was placed at the nest at least two days before filming commenced, to allow the birds to habituate to its presence. On at least two mornings when nestlings were aged 9-12 days, approximately three hours of video data (186 ± 16 minutes, mean ± S.D.) collected, beginning at 0652 am ± 16 minutes (mean ± S.D.). Individual feeding rates were then calculated by dividing the total number of visits to the nest by the total duration of video data collected at that nest.

3.3.4. Determination of oxidative balance in blood

Lipids are a major target of ROS during oxidative stress, and concentrations of the lipid peroxidation products offer a measure of *in vivo* oxidative damage (Halliwell and Chirico, 1993). We therefore measured plasma concentrations of malondialdehyde (MDA). We also measured superoxide dismutase (SOD) activity in erythrocytes. SOD is a key intracellular antioxidant enzyme, which forms part of the first line of defence against oxidative stress (Godin and Garnett, 1992; Parkes *et al.*, 1998). Finally, we measured the ability of a plasma sample to quench free radicals *in vitro*, thus providing a functional measure of total antioxidant capacity (TAC). Details of all sample collection, processing, transport and laboratory analysis can be found in the Methods section of Chapter 2.

It has recently been highlighted that up to 90% of the variation in antioxidant activity measured in this reaction can be due to the 'incidental' antioxidant effects of uric acid (Cohen *et al.*, 2007). Uric acid is a waste product of protein metabolism in birds, and its *in vitro* antioxidant activity may be incidental to this primary role. The *in vivo* importance of uric acid as an antioxidant remains unclear. Furthermore, increased uric acid levels can indicate stress (Cohen *et al.*, 2008), and uric acid production can itself generate ROS (Dröge, 2002). As a result, plasma TAC values comprise the activities of important antioxidants, as well as that of uric acid, which may at best add noise or at worst confound key analyses (Cohen *et al.*, 2007; Cohen *et al.*, 2008; Cohen and McGraw, 2009).

In order to factor out the impact of circulating uric acid on plasma TAC, we therefore calculated the residuals of the relationship between uric acid and TAC (hereafter termed 'residual TAC') as recommend in Cohen *et al.* (2007). A linear

mixed model was first used to confirm the relationship between uric acid concentration (set as a predictor) and TAC (as the response). Bird identity was the random factor (as two measures of each blood marker were taken from each bird). Plasma uric acid concentration significantly predicted plasma TAC values ($\chi^2_1 = 74.64$, p < 0.001, n = 89 samples). The residuals were therefore extracted from a separate linear model (with TAC as the response and uric acid as the only predictor), to yield a measure of plasma antioxidant capacity excluding that arising from plasma uric acid levels.

3.3.5. Statistical Analyses

Statistical analyses were carried out using R (R Development Core Team, 2013), using a step-wise model simplification approach (Crawley, 2007). Initially all fixed terms of interest were fitted, followed by the stepwise removal of terms whose removal from the model resulted in a non-significant change in deviance (using a likelihood-ratio test for model comparison), until the minimal adequate model (MAM) was obtained, in which only significant terms remained. Dropped terms were then added back in to the MAM to confirm their non-significance and were retained in the MAM when found to be significant in this context. The homoscedasticity and normality of residuals were inspected visually and where necessary response terms were transformed to satisfy these criteria. The significance of all terms was tested either by removing the terms from the MAM (if the term was in the MAM) or adding the terms back in to the MAM and then removing them (if the term was not included in the MAM).

First, the effect of treatment on *final* measures of oxidative balance and body mass was assessed. The *final* measure of a given metric was set as the response, and the level of that same metric at *clutch completion* was fitted as a predictor. This approach is statistically more powerful than modelling the effect of treatment on the change in a given metric, and can also account for the effects of chance biases in the treatment groups in the initial levels of a given metric (Crawley, 2007). Treatment, group size and their two-way interaction were also fitted as predictors. Class was included as a three-level factorial predictor (dominant male, subordinate male, subordinate female). Dominant females were excluded from all analyses as those in the control treatment could not be sampled at *clutch completion*, given the risk of clutch abandonment. Social group was fitted as the single random effect (while each individual had both *clutch completion* and *final* measures in the analysis, the former was a predictor and the latter a response in each case, and so the response contained no repeated measures of individuals).

Second, the effect of natural variation in individual provisioning rates on *final* measures of oxidative balance and body mass was assessed (necessarily using data solely from the control groups). Linear mixed effects models were fitted with an individual's provisioning rate (nest visits/hour), class and brood size included as predictors with social group fitted as the single random effect. Initially, we investigated whether the levels of a given oxidative balance metric or body mass at *clutch completion* predicted the *final* (peak provisioning) levels of that same metric, using the subset of birds captured at both stages. However, the levels of each oxidative balance metric at *clutch completion* did not significantly predict the subsequent *final* levels of that metric for the same bird (MDA (n = 22 birds), SOD (n = 21 birds) and residual TAC (n = 14 birds): all $\chi^2_1 < 1.94$, all p > 0.16), nor did

they do so in the full data set for both treatments (see results). For the analyses investigating the effect of provisioning rate on the *final* measures of each of the oxidative balance metrics, the datasets were therefore expanded to include those individuals sampled only at peak provisioning (to enhance the power of our analyses: MDA: n = 58 birds, SOD: n = 34 birds, residual TAC: n = 39 birds), and the levels of that metric at *clutch completion* were no longer fitted as a predictor. By contrast, as body mass at *clutch completion* was a strong positive predictor of body mass at peak provisioning (χ^2_1 = 51.41, n = 24, p < 0.01), the dataset for this analysis remained restricted to those birds captured at both *clutch completion* and peak provisioning.

3.4 RESULTS

3.4.1. Does reproduction affect body mass and oxidative balance?

Body Mass

An individual's *final* body mass (at peak provisioning in *control* groups and when peak provisioning would have been in *clutch-removal* groups) was strongly positively predicted by its body mass at *clutch completion* (χ^2_1 = 59.47, p < 0.001, n = 34 birds), and dominant and subordinate males were significantly heavier than subordinate females (χ^2_2 = 10.42, p = 0.005). Controlling for these effects, there was also a significant interaction between treatment and group size (Figure 3.2a, χ^2_1 = 12.50, p < 0.001). The effect of treatment was strongest in smaller groups, where birds whose clutch had been experimentally removed showed higher *final* body masses than those that were left to hatch and provision their nestlings. By contrast, in larger groups there was no effect of treatment.

Plasma MDA Concentration

As for the body mass findings above, *final* MDA concentrations were significantly predicted by the interaction between treatment and group size (Figure 3.2b, χ^2_1 = 5.79, p = 0.016, n = 32 birds). The data set contained a single outlying high MDA value (indicated with an arrow in Figure 3.2b), but this point was not driving the interaction; its exclusion only enhanced the interaction's significance (χ^2_1 = 9.69, p = 0.002). In smaller groups, control birds (who were provisioning nestlings) had higher plasma MDA concentrations than *clutch-removal* birds (who were not). In larger groups, the effect of treatment was less clear; there was a tendency towards higher MDA levels in the *clutch-removal* treatment, though just two MDA measures

were available for *clutch-removal* birds in groups of more than five individuals. An individual's *final* plasma MDA concentration was not significantly predicted either by its plasma MDA concentration at *clutch completion* or its class (both $\chi^2 < 1.63$, p > 0.44).

Erythrocyte Superoxide Dismutase

Treatment did not significantly predict *final* SOD enzyme activity, either as a single term (Figure 3.2c, $\chi^2_1 = 1.86$, p = 0.17, n = 31 birds) or via an interaction with group size ($\chi^2_1 = 0.19$, n = 31, p = 0.66). SOD activity at *clutch completion* was a marginally non-significant positive predictor of *final* SOD activity ($\chi^2_1 = 3.07$, p = 0.08), but its retention or exclusion from the *final* model had no qualitative impact on the significance of treatment. Neither group size nor class significantly affected *final* SOD activities (both $\chi^2 < 2.12$ p > 0.35).

Plasma Residual Total Antioxidant Capacity

Treatment did not significantly predict *final* plasma residual TAC, either as a single term (Figure 3.2d, $\chi^2_1 = 0.08$, p = 0.78, n = 22 birds,) or via an interaction with group size ($\chi^2_1 = 0.05$, p = 0.82). Class significantly predicted *final* residual TAC ($\chi^2_2 = 8.43$, p = 0.015); subordinate females had lower residual TAC than both classes of male (dominant male: -60.00 ± 35.45, subordinate male: 29.40 ± 66.43, subordinate female: -216.93 ± 46.85, means ± S.E.). Residual TAC at *clutch completion* was a marginally non-significant predictor of *final* residual TAC ($\chi^2_1 = 3.12$, p = 0.078), with a weak trend towards consistency between the two time-points. Blood sampling lag and group size have previously been reported to impact

residual TAC in this species (Chapter 2), but neither affected *final* residual TAC in

this dataset (both $\chi^2_1 < 0.31$, p > 0.58).
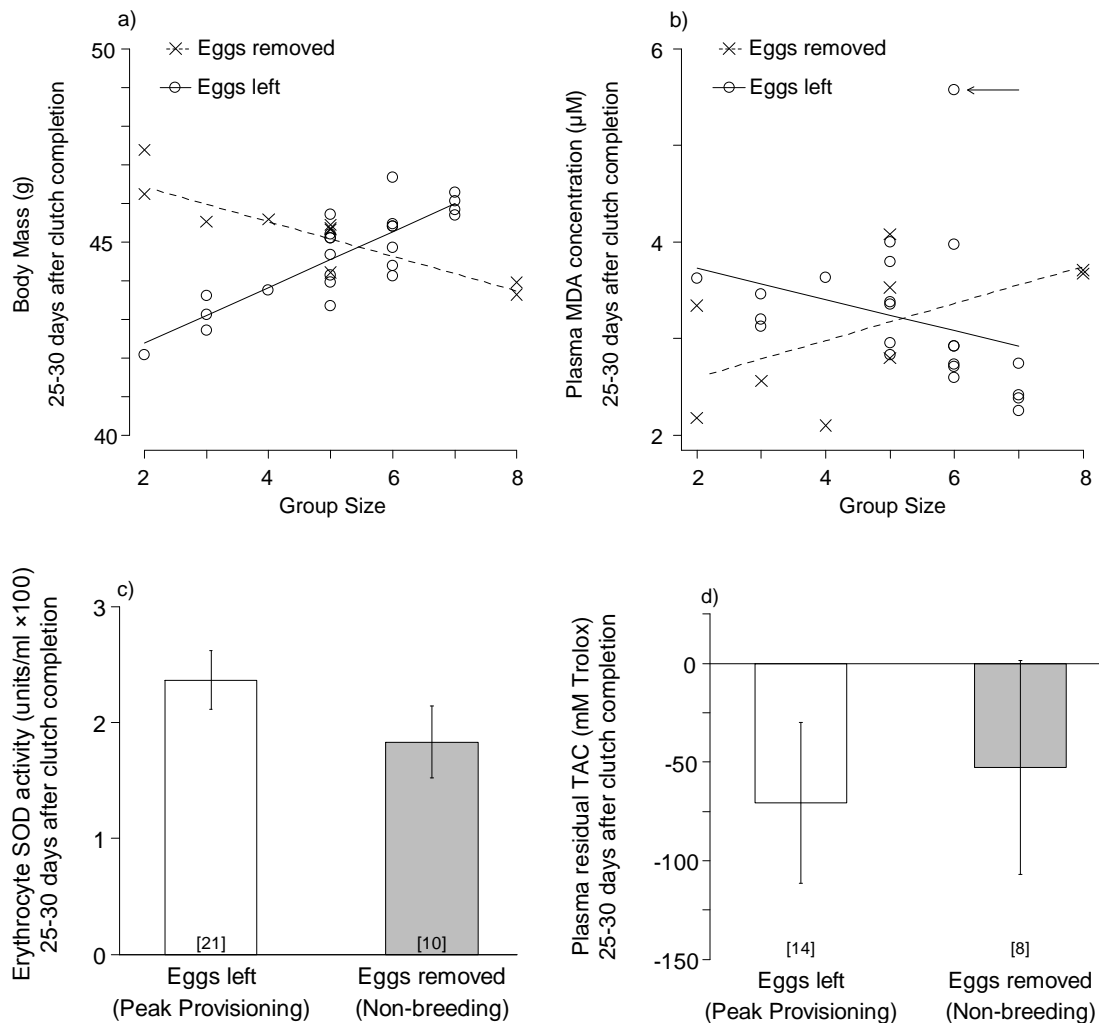
Figure 3.2. – The effect of treatment on **(a)** body mass (a significant interaction between treatment and group size), **(b)** plasma MDA concentrations (a significant interactions between treatment and group size), **(c)** erythrocyte SOD activity (no significant effect of treatment), **(d)** plasma residual TAC (no significant effect of treatment). In (a) and (b), lines represent the model predictions from the minimal adequate model and the points represent the model residuals. In (b) the outlying high MDA value indicated by an arrow is not driving the interaction; its removal enhances the significance of the interaction (see results). In (c) and (d), bars represent model predictions from the treatment predictor in the minimal adequate model. Error bars represent standard errors from these predictions and numbers in parentheses are sample sizes.

3.4.2. Within control birds, do birds provisioning at a higher rate suffer greater deficits in body mass and oxidative balance?

Body Mass

Body mass at *clutch completion* was a strong positive predictor of body mass at peak provisioning (χ^2_1 = 51.41, p < 0.001, n = 24 birds), and dominant and subordinate males were significantly heavier than subordinate females (χ^2_2 = 12.91, p < 0.001). Controlling for these effects, an individual's body mass at peak provisioning was significantly predicted by its provisioning rate in the preceding days (Figure 3.3a, χ^2_1 = 5.50, n = 24, p = 0.02). Birds provisioning at higher rates subsequently had lower body masses.

Oxidative balance

Controlling for the significant effect of class (χ^2_2 = 7.78, p = 0.02, n = 39 birds, means ± S.E.: dominant male 14.79 ± 49.15, subordinate male 159.65 ± 47.76, subordinate female 36.50 ± 62.05), an individual's plasma residual TAC at peak provisioning was significantly predicted by its provisioning rate in the preceding days (Figure 3.3b, χ^2_1 = 7.69, p = 0.006). Birds provisioning at higher rates subsequently had lower residual TAC measures. Plasma residual TAC at peak provisioning was not significantly predicted by an individual's group size or the brood size that it was tending (all χ^2_1 < 0.06, p > 0.81).

An individual's plasma MDA concentration at peak provisioning was not significantly predicted by its provisioning rate in the preceding days, brood size, class or group size (all χ^2_1 < 0.70, all p > 0.40, n = 58 birds).   An individual's erythrocyte SOD activity at peak provisioning was not significantly predicted by its

provisioning rate in the preceding days, group size or class (all χ^2_1 < 2.58, all p > 0.28, n = 34 birds, SOD activity was square-root transformed for normality of residuals). Brood size marginally significantly predicted *final* SOD activity (χ^2_1 = 3.99, p = 0.046): SOD activities were higher in groups with two nestlings, compared with those with only one nestling.
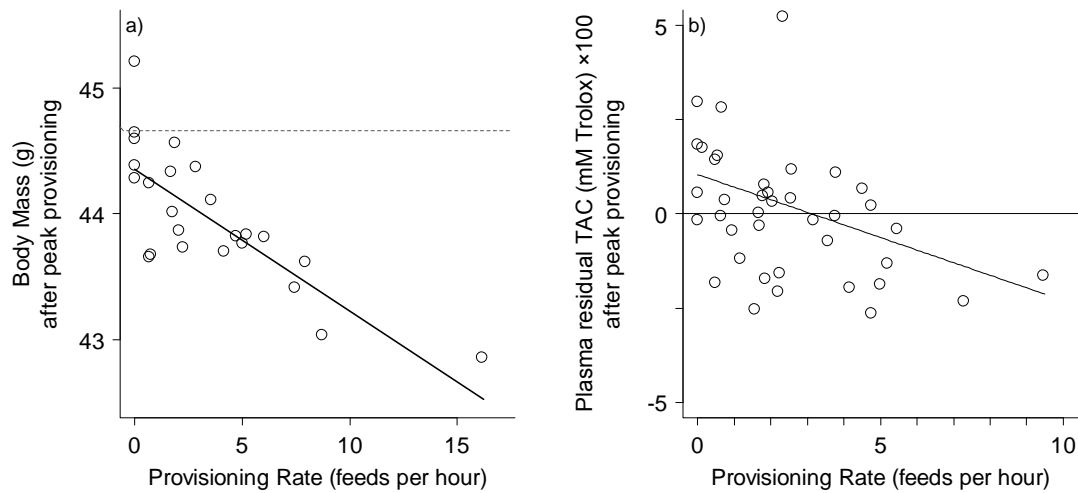
Figure 3.3 – (a) The effect of natural variation in individuals' provisioning rates on their body mass at peak provisioning. Birds provisioning at a higher rate subsequently had a significantly lower body mass at peak provisioning, while controlling for the effects of body mass at *clutch completion* and class. The solid line represents the model predictions from a model containing *clutch completion* mass and provisioning rate as predictors. The predictions are for an individual of mean *clutch completion* body mass (44.66 g – also indicated by the dotted line) and the points represent residuals from this model. **(b)** The effect of natural variation in individuals' provisioning rates on their plasma residual TAC at peak provisioning. Birds provisioning at a higher rate subsequently had lower plasma residual TAC. The diagonal solid line represents the model predictions from the minimum adequate model (in which provisioning rate was the only predictor), while the points represent residuals from this model.

3.5 DISCUSSION

Our results provide rare experimental evidence of a cost of reproduction in a wild cooperative breeder, both in terms of reduced body mass and increased exposure to oxidative stress. Compared to birds in groups whose eggs were removed, birds who subsequently reared their young suffered a decline in body mass, but this was only evident in smaller groups. Similarly, levels of a circulating marker of oxidative damage to lipids (MDA) were elevated in birds left to rear young relative to those from which eggs were removed, but this was evident only in small groups. Furthermore, our findings suggest that investment in nestling care *per se* may contribute to the above costs, as, within groups that were allowed to rear their young, individuals that provisioned at higher rates lost the most body mass and suffered more impaired antioxidant protection. Together, these results provide new evidence that reproduction bears a two-fold cost in the wild, and suggest that such costs may be mitigated for individuals in cooperatively breeding societies through the cooperative lightening of individual workloads.

In smaller groups, breeding birds suffered lower body mass and higher plasma oxidative damage, compared with groups whose eggs had been experimentally removed. Evidence suggests that investment in reproduction can increase energetic turnover (Deerenberg *et al.*, 1995; Moreno *et al.*, 1995), and breeding frequently leads to depleted fat reserves and declines in body mass (Dijkstra *et al.*, 1990; Merila and Wiggins, 1997; Alonso-Alvarez *et al.*, 2004b; Cox *et al.*, 2010; Crocker *et al.*, 2012; Mitchell *et al.*, 2012). Increased energy turnover during intensive exercise can lead to increased production of ROS (Davies *et al.*, 1982; Leffler, 1993; Reddy *et al.*, 1998; Ji, 1999; Costantini *et al.*, 2008; Powers and

Jackson, 2008); but see Selman *et al.*, 2002; Barja, 2007), which may lead to oxidative damage and increased exposure to oxidative stress. Although most captive experiments yield weak or no support for this theory (Garratt *et al.*, 2011; Ołdakowski *et al.*, 2012; Garratt *et al.*, 2013), wild experiments suggest reproduction can impair antioxidant protection (Losdat *et al.*, 2011; Christe *et al.*, 2012), and, in our species, result in elevated oxidative damage and impaired antioxidant defences.

We find evidence for a body mass and oxidative damage cost of reproduction only in small social groups. The effect of group size suggests that the costs of reproduction can be partially or fully mitigated in larger groups, representing evidence of a benefit of group-living. By contrast, Chapter 2 revealed that, during nonbreeding periods before and after the breeding season, group size did not affect oxidative damage, enzymatic antioxidant protection or body mass, and *negatively* affected non-enzymatic antioxidant protection in males. Taken together, these results suggest that group-living can have beneficial consequences for oxidative balance and body condition, but only during periods of intensive reproduction. Furthermore, our results suggest that the cost of reproduction in small groups may result, at least in part, from contributions to nestling care *per se*. Individuals of this species in small groups contribute more to provisioning nestlings, compared with larger groups (Lewis 1981, Young *et al*., unpublished data), and we find evidence that birds working hardest to provision nestlings pay correspondingly large costs, both in terms of lost body mass and impaired antioxidant protection. While provisioning rate did not directly affect oxidative damage, decreased antioxidant resistance resulting from intensive provisioning for nestlings may exacerbate the impact of other burdens that fall heaviest on smaller

groups. For example, individuals in smaller groups typically spend more time vigilant, and therefore have less time to forage (Lima, 1995; Wright *et al.*, 2001), which may result in greater loss of body mass and dietary antioxidants regardless of provisioning rate. Furthermore, such effects are likely to be particularly pronounced in smaller groups during the incubation phase, when the temporary absence of the incubating dominant female further reduces functional group size. Costs incurred during the incubation phase are rarely considered in cooperatively breeding species. While the current study did not manipulate provisioning rate (in contrast to brood-size manipulation experiments: Alonso-Alvarez *et al.*, 2004b; Wiersma *et al.*, 2004; Losdat *et al.*, 2011; Christe *et al.*, 2012), clutch-removal experiments may more comprehensively reveal the true costs of reproduction, comprising costs arising during both the incubation and provisioning phases.

In this species, dominant females are the only birds to lay and incubate eggs, and are the primary providers of nestling care (Lewis, 1981; Harrison *et al.*, 2013). Dominant females therefore directly invest more in breeding compared with other classes, and might therefore be expected to incur correspondingly large costs of reproduction. Indeed, Chapter 2 revealed that over the course of the breeding season, dominant females suffered differential declines in antioxidant protection and failed to gain body mass, in contrast with their female subordinates. Although dominant females could not be included in the current study (as their capture may have risked nest desertion), our results nonetheless provide a plausible explanation for the disproportionate decline in condition of dominant females over the course of the breeding season. In particular, the current study's finding that elevated rates of nestling provisioning impact both body mass and antioxidant protection suggest that dominant females (who provide the majority of nestling

care; Lewis 1982) may pay the largest cost, and that this could lead to the long-term body condition and antioxidant protection deficits they suffer (Chapter 2).

Experimental evidence from wild animals is critical in order to advance our understanding of the oxidative costs of reproduction (Metcalfe and Alonso-Alvarez, 2010; Selman *et al.*, 2012; Metcalfe and Monaghan, 2013). Our results provide rare support for the theory that investment in reproduction, and specifically care for nestlings, entails a cost in terms of reduced body mass and elevated exposure to oxidative stress. However, our findings also suggest that cooperative breeding can entirely offset these costs in large social groups. Together, these results have implications for our understanding of the physiological costs of reproduction, which can arise at both the macro- and micro-nutrient levels, and the evolution of cooperative breeding in animal societies, which may have evolved to minimize these costs.

CHAPTER 4

Oxidative stress and the long-term costs of reproduction in a wild cooperative breeder

4.1 ABSTRACT

Despite the importance of the fundamental life-history trade-offs between reproduction and self-maintenance, our understanding of the physiological mechanisms that underpin them remains poorly developed. Oxidative stress has recently been proposed as a proximate cost of reproduction and a mediator of the trade-off between current and residual reproduction. While interest in the impact of reproduction on oxidative balance is increasing, the majority of studies focus on short-term effects of breeding effort. Whether these short-term costs are enduring, and may therefore mediate the marked survival costs of reproduction, remains unclear. Here, we use a wild population of cooperatively breeding white-browed sparrow weavers (*Plocepasser mahali*) to investigate whether natural variation in reproductive effort impacts circulating markers of oxidative balance and body mass after the breeding season is complete. We find no evidence that reproductive effort, measured either as total fledglings produced or individual provisioning rate, impacts oxidative balance or body mass after the breeding season. One explanation for such a lack of apparent cost is that individuals adjust their investment in reproduction according to their ability to cope with its associated challenges. However, our findings do not support such condition-dependent regulation of reproductive effort, as a female's likelihood of initiating reproduction early in the breeding season was independent of her oxidative balance or body condition before the season began. Our findings reveal that, while short-term costs of reproduction can be evident in circulating markers of oxidative stress, long-term costs are not, suggesting that variation in these markers reflects only recent oxidative state.

4.2 INTRODUCTION

The trade-off between reproduction and self-maintenance is a core assumption of life history theory (Williams, 1966; Stearns, 1989), yet the physiological mechanisms that underpin such trade-offs remain poorly understood (Rose and Bradley, 1998; Zera and Harshman, 2001; Ricklefs and Wikelski, 2002). Recently, oxidative stress has been highlighted as a potential mediator of the costs of reproduction (Costantini, 2008; Monaghan *et al.*, 2009; Metcalfe and Alonso-Alvarez, 2010; Metcalfe and Monaghan, 2013). Oxidative stress occurs when the generation of reactive oxygen species (ROS) overwhelms the body's protective antioxidant system, resulting in damage to lipids, proteins and DNA (Finkel, 2003; Halliwell and Gutteridge, 2007). During reproduction, ROS generation may be elevated and demand for antioxidants may be particularly high (Blount *et al.*, 2000; Blount *et al.*, 2001; Alonso-Alvarez *et al.*, 2004b; Wiersma *et al.*, 2004; Christe *et al.*, 2012), which may lead to oxidative stress. While studies investigating the impact of reproductive effort on oxidative stress are accumulating (reviewed in Metcalfe and Monaghan, 2013), the vast majority of this research focuses on short-term effects, typically only sampling individuals during peak reproductive effort. If oxidative stress does indeed mediate the marked long-term costs of reproduction (Nur, 1988; Landwer, 1994), reproduction should lead to an enduring impact on oxidative balance, which cannot be easily recovered. Our understanding of the long-term oxidative stress costs of reproduction remains poorly developed.

Correlative evidence that reproductive effort leads to oxidative stress is somewhat equivocal (reviewed in Metcalfe and Monaghan, 2013). However, experimental evidence suggests that elevated investment in breeding can lead to impaired

antioxidant protection (Alonso-Alvarez *et al.*, 2004b; Wiersma *et al.*, 2004; Losdat *et al.*, 2011; Christe *et al.*, 2012) and elevated oxidative damage (Chapter 3; but see Garratt *et al.*, 2011; Ołdakowski *et al.*, 2012; Garratt *et al.*, 2013). While these studies reveal the impact of breeding on oxidative balance during or immediately after reproduction, in order to understand whether changes in oxidative balance mediate longer-term trade-offs between current reproduction and future reproduction and survival, we must investigate whether such deficits persist over time and accumulate with continued investment in reproduction. Evidence of the long-term effects of reproduction on oxidative balance remains rare and inconsistent (Chapter 2; Cohen *et al.*, 2012; Heiss and Schoech, 2012).

Two effects have the potential to hamper attempts to investigate the long-term impacts of reproduction on oxidative balance. First, considerable inter-individual variation may result in high levels of noise. Oxidative balance can be influenced by a wide variety of factors, including early life conditions (Blount *et al.*, 2003; Alonso-Alvarez *et al.*, 2007), diet (Selman *et al.*, 2006; Orledge *et al.*, 2012), territory quality (van de Crommenacker *et al.*, 2011a) and immune status (Costantini and Møller, 2009). Second, correlative studies of the effect of reproduction on oxidative balance may be difficult to interpret because individuals may adjust their reproductive effort in order to avoid oxidative stress (Metcalfe and Monaghan, 2013). Indeed, there is growing evidence that reproductive effort can be regulated according to oxidative balance prior to breeding, and that individuals with poor antioxidant protection or elevated oxidative damage subsequently invest correspondingly little in reproduction (Heiss and Schoech, 2012; Stier *et al.*, 2012). Such condition-dependent mechanisms may be particularly important in the production of eggs, which requires antioxidant investment (Blount *et al.*, 2000),

and might therefore be delayed or reduced in individuals with low antioxidant reserves (Harrison *et al.*, 2011), in order to avoid depletion of antioxidants and oxidative stress (Heiss *et al.*, 2011).

Both of these potential problems can be addressed by employing a repeated-measures method, sampling individuals both before they begin reproduction and again during or after it is completed. Such longitudinal sampling allows an investigation of the effects of reproductive effort on oxidative balance during or after reproduction, whilst controlling for any inter-individual variation in oxidative balance that existed *before* breeding began. Furthermore, where experimental manipulation of reproductive effort is not feasible, sampling individuals prior to breeding also permits an investigation of whether pre-breeding oxidative balance predicts reproductive effort (Heiss *et al.*, 2011; Heiss and Schoech, 2012; Stier *et al.*, 2012). Repeated-measures designs therefore offer greater insight into the effect of reproductive effort on markers of oxidative stress.

Here, we use a wild population of white-browed sparrow weavers *Plocepasser mahali* to investigate whether natural variation among individuals in reproductive investment during the breeding season is reflected in enduring differences in oxidative balance and body mass at the end of the breeding season. We take a longitudinal within-individual sampling approach, investigating the effects of reproductive effort on an individual's post breeding season state while controlling for their state prior to the breeding season. Furthermore, we investigate whether mothers mount oxidative condition-dependent reproductive responses (adjusting their probability of clutch initiation according to their pre-season oxidative

balance and body condition), which might conceivably mask the long-term oxidative costs of reproduction.

White-browed sparrow-weavers live in year-round territorial groups, comprising a single dominant pair that completely monopolise within-group reproduction (Harrison *et al.*, 2013; 12-18% of young are sired by extra-group males) and up to 10 subordinate males and females in approximately equal sex ratio (Collias and Collias, 1978; Lewis, 1981; Lewis, 1982). The species shows well-developed cooperation, with most group members contributing to the care of young, sentinelling, territory defence and weaving (Collias and Collias, 1978; Lewis, 1982). Clutches of 1-4 eggs (mode: 2) are laid and incubated solely by the dominant female (Lewis, 1982; Harrison *et al.*, 2013), while most group members contribute to the cooperative provisioning of nestlings and fledglings (Lewis 1981). Up to six clutches may be laid during the relatively long breeding season (typically lasting 6-7 months from the first rains in October), raising the possibility that the costs of reproduction may accumulate with sustained breeding effort. Indeed, evidence suggests that investment in reproduction can entail a short-term cost in this species, in terms of decreased body mass, impaired antioxidant protection and elevated oxidative damage (Chapter 3). However, whether these deficits endure beyond the reproductive period and may thereby exert downstream impacts on future reproduction and survival is unknown. Dominant females do, however, suffer differential declines in antioxidant defences over the course of the breeding season (Chapter 2), which raises the possibility that such deficits do indeed reflect long-term oxidative costs arising from their reproductive effort during the season (which is higher than that of all other group members; Lewis 1982). Specifically, we investigate i) whether natural variation among

groups in the number of fledglings reared (a proxy for total reproductive effort) negatively impacts the post-season oxidative balance and body mass of group members, (ii) whether natural variation in nestling care (rates of provisioning at the nest) negatively affect post-season oxidative balance and body mass, and iii) whether dominant females with stronger pre-season oxidative state and/or body condition are more likely to initiate reproduction early in the season.

4.3   METHODS

4.3.1. Study species and population

Data collection was conducted in the context of a long-term study, monitoring a population of 40 cooperatively breeding groups of white-browed sparrow weavers in an area of approximately 1.5 km² in Tswalu Kalahari Reserve, South Africa (27°16'S, 22°25'E). All birds were fitted with a single metal ring and three colour rings for identification (under SAFRING license 1444). Males and females were distinguished by beak colour: males have dark brown beaks while females have paler horn-coloured beaks (Leitner *et al.*, 2009). Dominant birds were identified following weekly behavioural observations and criteria detailed elsewhere (Harrison *et al.*, 2013). Briefly, dominant males consistently sang dawn song during the breeding season, and were behaviourally dominant to all other males. Dominant females were the only females to lay and incubate eggs in each group. Group size was assigned by assessing the number of birds roosting in the group's tree(s) and consistently seen in close contact with other group members during routine behavioural observations conducted every 1-2 weeks.

Birds were captured and blood sampled immediately before and after the breeding season that ran from October 2011 to April 2012 (Figure 4.1a). All captures, blood sampling and measurements were conducted by one person (DC) under license (SAFRING license 1444). Birds were captured individually at night, by flushing them from their roost into a custom capture bag. A blood sample (approximately 160 µl) was immediately collected from the brachial vein with a 26g needle. The lag between bird capture and completion of blood sampling was minimized (182 ± 55 sec, mean ± S.D.). Body mass was then recorded to the nearest 0.01 g (Durascale

100, MyWeigh, UK) and tarsus length measured to the nearest 0.1 mm using callipers. After processing, birds were returned to their roosts to pass the remainder of the night.

4.3.2. Monitoring of reproductive effort

Nest searches were conducted every one to two days. Where the date of egg-laying could not be determined to the day from nest search data, it was calculated retrospectively from hatching dates using the mean incubation period of 17 days from clutch initiation. In order to investigate whether maternal pre-season state determined whether or not she initiated a clutch in the early breeding season, dominant females were divided into those that laid eggs before the 35th day of the breeding season (day 0 was defined as the day the first egg was laid in our population) and those that did not (comprising both later-laying females and those that did not lay all season; Figure 4.1b).This date was selected as it divided the females according to two naturally occurring peaks in the date of first clutch initiation (at approximately 25 and 85 days, figure 4.1b). Results were not qualitatively different if clutches were instead divided at 60 days. Fledging success was monitored by targeted observations and captures 30 days after hatching (fledging typically takes place between day 20 and 25).

The contributions of all group members to nestling provisioning were observed when nestlings were 9-13 days old ("peak provisioning") in a subset of groups (51 individuals in 16 groups). Several days prior to peak provisioning, all birds except the dominant female (who was incubating eggs or nestlings in the nest) were captured and given unique dye marks on their white vent feathers. These unique

marks allowed individual identification using a video camera attached to a tripod under the nest. A tripod was placed under the nest 48 hours before data collection to habituate birds to its presence. Video data was collected for approximately three hours on at least two mornings (186 ± 16 min, mean ± S.D), for a minimum total of six hours of provisioning data (8.4 ± 1.2 hours, mean ± S.D.). Visits to the nest by each bird were extracted from the videos and summarised to yield individual feeding rates per hour across all monitored breeding attempts (n = 51 individuals from 16 groups).
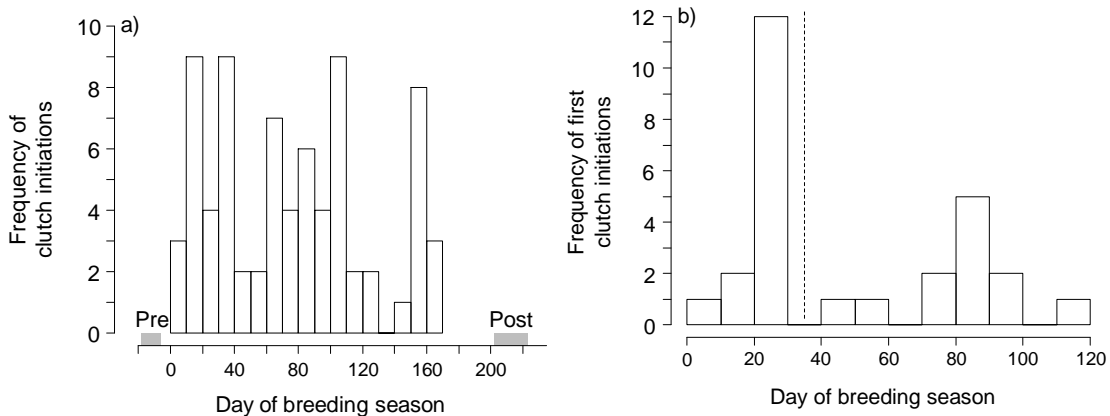
Figure 4.1 – (a) The distribution of clutch initiations from all dominant females throughout the breeding season. The grey bars indicate the pre-season (days -18 to -6) and post-season (days 202 to 223) sampling periods. **(b)** The distribution of *first* clutch initiations across the breeding season, for females blood-sampled in the pre-season. The dotted line indicates day 35, the date used to divide females into those that initiated early in the season, and those that did not.

4.3.3. Determination of oxidative balance metrics

Lipids are a major target of ROS during oxidative stress, and concentrations of the lipid peroxidation products offer a measure of *in vivo* oxidative damage (Halliwell and Chirico, 1993). We therefore measured plasma concentrations of malondialdehyde (MDA). We also measured superoxide dismutase (SOD) activity in erythrocytes. SOD is a key intracellular antioxidant enzyme, which forms part of the first line of defence against oxidative stress (Godin and Garnett, 1992; Parkes *et al.*, 1998). Finally, we measured the ability of a plasma sample to quench free radicals *in vitro*, thus providing a functional measure of total antioxidant capacity (TAC). Full details of sample collection, processing, transport and laboratory analysis can be found in the Methods section of Chapter 2.

It has recently been highlighted that up to 90% of the variation in antioxidant activity measured in this reaction can be due to the 'incidental' antioxidant effects of uric acid (Cohen *et al.*, 2007). Uric acid is a waste product of protein metabolism in birds, and its *in vitro* antioxidant activity may be incidental to this primary role. The *in vivo* importance of uric acid as an antioxidant remains unclear. Furthermore, increased uric acid levels can indicate stress (Cohen *et al.*, 2008), and uric acid production can itself generate ROS (Dröge, 2002). As a result, plasma TAC values comprise the activities of important antioxidants, as well as that of uric acid, which may at best add noise or at worst confound key analyses (Cohen *et al.*, 2007; Cohen *et al.*, 2008; Cohen and McGraw, 2009).

In order to factor out the impact of circulating uric acid on plasma TAC, we therefore calculated the residuals of the relationship between uric acid and TAC

(hereafter termed "residual TAC") as recommend in Cohen *et al.* (2007). A linear mixed model was first used to confirm the relationship between uric acid concentration (set as a predictor) and TAC (as the response). Bird identity was the random factor (as two measures of each blood marker were taken from each bird). Plasma uric acid concentration significantly predicted plasma TAC values (χ^2_1 = 74.31, p < 0.001, n = 118 samples). The residuals were therefore extracted from a separate linear model (with TAC as the response and uric acid as the only predictor), to yield a measure of plasma antioxidant capacity excluding that arising from plasma uric acid levels.

4.3.4. Body mass and body condition

We controlled for pre-season body mass in statistical models investigating whether an individual's contributions to nestling care or the number of fledglings reared over the course of the season impacted body mass at the end of the season. As such, these within-individual analyses did not necessitate the use of size-corrected mass. However, when investigating whether dominant female condition before the breeding season predicted whether they initiate a clutch early in the season, we calculated the Scaled Mass Index (SMI) (Peig and Green, 2009). The SMI allows a between-individual comparison of size-corrected body mass, yet avoids the problems associated with residual-based measures of body condition (Labocha and Hayes, 2012) by instead scaling each individual's body mass to the value expected if all birds were of identical skeletal size. This scaling is based on the power relationship between mass and tarsus length modelled from the data (Peig and Green, 2009). For this analysis we therefore scaled the pre-season masses of

the 30 dominant females to their mean tarsus length (24.5 mm), using a secondary major axis slope of 3.36. This SMI value is hereafter referred to as 'body condition.'

4.3.5. Statistical Analyses

Statistical analyses were carried out using R (R Development Core Team, 2013), using a step-wise model simplification approach (Crawley, 2007). Initially all fixed terms of interest were fitted, followed by the stepwise removal of terms whose removal from the model resulted in a non-significant change in deviance (using a likelihood-ratio test for model comparison), until the minimal adequate model (MAM) was obtained, in which only significant terms remained. Dropped terms were then added back in to the MAM to confirm their non-significance and were retained in the MAM when found to be significant in this context. The homoscedasticity and normality of residuals were inspected visually and where necessary response terms were transformed to satisfy these criteria. The significance of all terms was tested either by removing the terms from the MAM (if the term was in the MAM) or adding the terms back in to the MAM and then removing them (if the term was not included in the MAM).

1) Does the number of fledglings that an individual rears impact its post-season oxidative balance and body mass?

Linear mixed effects models were used to investigate the effect of the number of fledglings that a group reared during the season on an individual's post-season body mass and oxidative balance, controlling for their pre-season levels of the same metric in each case. The number of fledglings produced was selected as a proxy for total reproductive effort. This measure correlated strongly with the

number of nestlings reared to both day 9 and day 17, and results did not qualitatively differ when these proxies of effort were used.

A linear mixed effects model was fitted with the post-season measure as the response and the pre-season measure as a covariate predictor. This approach is statistically more powerful than using the change in a given oxidative balance measure and can also account for the effects of chance biases in the treatment groups in the initial levels of the oxidative balance metric (Crawley, 2007). The individual's sex, dominance rank and the number of fledglings reared during the breeding season (n = 12, 31 & 16 birds rearing zero, one & two fledglings respectively) were included as factorial predictors. Two-way interactions of the number of fledglings reared with sex and dominance allowed an investigation of whether the costs of raising fledglings varied depending on a bird's dominance status and sex. Social group was fitted as the single random term to account for measures from multiple birds within the same group (59 birds in 17 groups).

2) Does an individual's provisioning rate impact its post-season oxidative balance and body condition?

While the above models investigate the impact of reproductive effort at the group level (number of fledglings produced), it is possible that this effort is divided unequally amongst members of the group, and that the long-term costs of reproduction fall most heavily on those that invest the most. To account for potential unequal contributions to reproduction, we investigated the impact of individual rates of nestling provisioning on post-season oxidative balance and body mass, using linear mixed effects models. Linear mixed effects models were fitted with the post-season measure as the response and the individual

provisioning rate (feeds per hour averaged across all broods for which data was available). as a covariate predictor. As sex, dominance and group size are known to co-vary with provisioning rate in this species (females typically provision more than males, the dominant female provisions more than other classes and individuals in larger groups provision at lower rates; Lewis, 1982; Young *et al.,* unpublished data), sex, dominance and group size were omitted from the model to avoid these terms absorbing variation in the response term that in fact arises from correlated differences in provisioning rate. Social group was fitted as the single random term.

3) Does a dominant female's pre-season state predict whether she breeds early in the breeding season?

To investigate whether pre-season oxidative balance and body condition affect subsequent investment in reproduction, we focused on dominant females, as they are the only birds to lay eggs in this population (Harrison *et al.*, 2013). If individuals adjust their reproductive effort in a condition-dependent way to avoid incurring oxidative stress (Metcalfe and Monaghan, 2013), we might expect that females in poor condition in the pre-season would delay egg-laying until they are in sufficiently good condition to breed. A binomial general linear model was used to investigate whether a dominant female's body condition and oxidative balance in the pre-season determined her likelihood to initiate breeding early or late in the season. Data from 23 dominant females were included, for whom measures of body condition and all oxidative balance metrics were available from the pre-season period. The response term was a binary term, specifying whether a given dominant female laid her first egg before or after the 35th day of the breeding season. The predictor terms were the dominant female's oxidative balance metrics

(plasma MDA concentration, erythrocyte SOD activity, plasma residual TAC), body

condition and group size at the time of pre-season sampling.

4.4   RESULTS

4.4.1. Does the number of fledglings that an individual rears impact its post-season oxidative balance and body mass?

Body Mass

The number of fledglings that an individual reared during the breeding season did not significantly predict its body mass after the breeding season, either as a single term (χ^2_2 = 0.33, p = 0.85, n = 59 birds, Figure 4.2a) or in two-way interactions with either dominance rank or sex (both χ^2_2 < 2.46, p > 0.29), while controlling for the significant positive effect of its pre-season mass (χ^2_1 = 69.43, p < 0.001). Males were significantly heavier than females in the post-season (χ^2_1 = 11.52, p < 0.001) and dominants were heavier than subordinates (χ^2_1 = 12.07, p < 0.001), while controlling for inter-individual variation in pre-season mass. There was no significant effect of group size on post-season body mass (χ^2_1 = 0.88, p = 0.35).

Plasma MDA concentration

The number of fledglings that an individual reared during the breeding season did not significantly predict its plasma MDA concentrations after the breeding season, either as a single term (χ^2_2 = 1.62, p = 0.44, n = 58 birds, Figure 4.2b) or in two-way interactions with either dominance rank or sex (both χ^2_2 > 1.23, p > 0.44). Post season plasma MDA levels were significantly higher in subordinates than dominants (χ^2_1 = 5.88, p = 0.015), but pre-season plasma MDA levels, sex and group size did not significantly predict post-season plasma MDA levels (all χ^2_1 < 2.48, all p > 0.12).

Plasma residual TAC

The number of fledglings that an individual reared during the breeding season did not significantly predict its plasma residual TAC at the end of the breeding season, either as a single term (χ^2_2 = 0.10, p = 0.95, n = 51 birds, Figure 4.2c) or in two-way interactions either with dominance rank or sex (both χ^2_2 < 4.27, p > 0.12). The interaction between sex and dominance rank significantly predicted post-season residual TAC (χ^2_1 = 6.93, p = 0.009); dominant females showed the lowest residual TAC in the post-season (dominants: female = -98.5 ± 33.3 and male = -20.7 ± 30.6; subordinates: female = 23.8 ± 33.8 and male = -60.4 ± 31.2). Group size and pre-season residual TAC did not significantly predict post-season residual TAC (both χ^2_1 < 1.55, p > 0.21).

Erythrocyte SOD activity

The number of fledglings that an individual reared during the breeding season did not significantly predict its SOD activity at the end of the breeding season (Figure 4.2d, χ^2_2 = 0.79, p = 0.67, n = 34 birds). Two-way interactions between the number of fledglings reared and sex or dominance status could not be tested due to our limited sample size. SOD activities after the breeding season were not significantly predicted by dominance rank, sex, pre-season SOD activity or group size (all χ^2_1 < 1.04, p > 0.31).
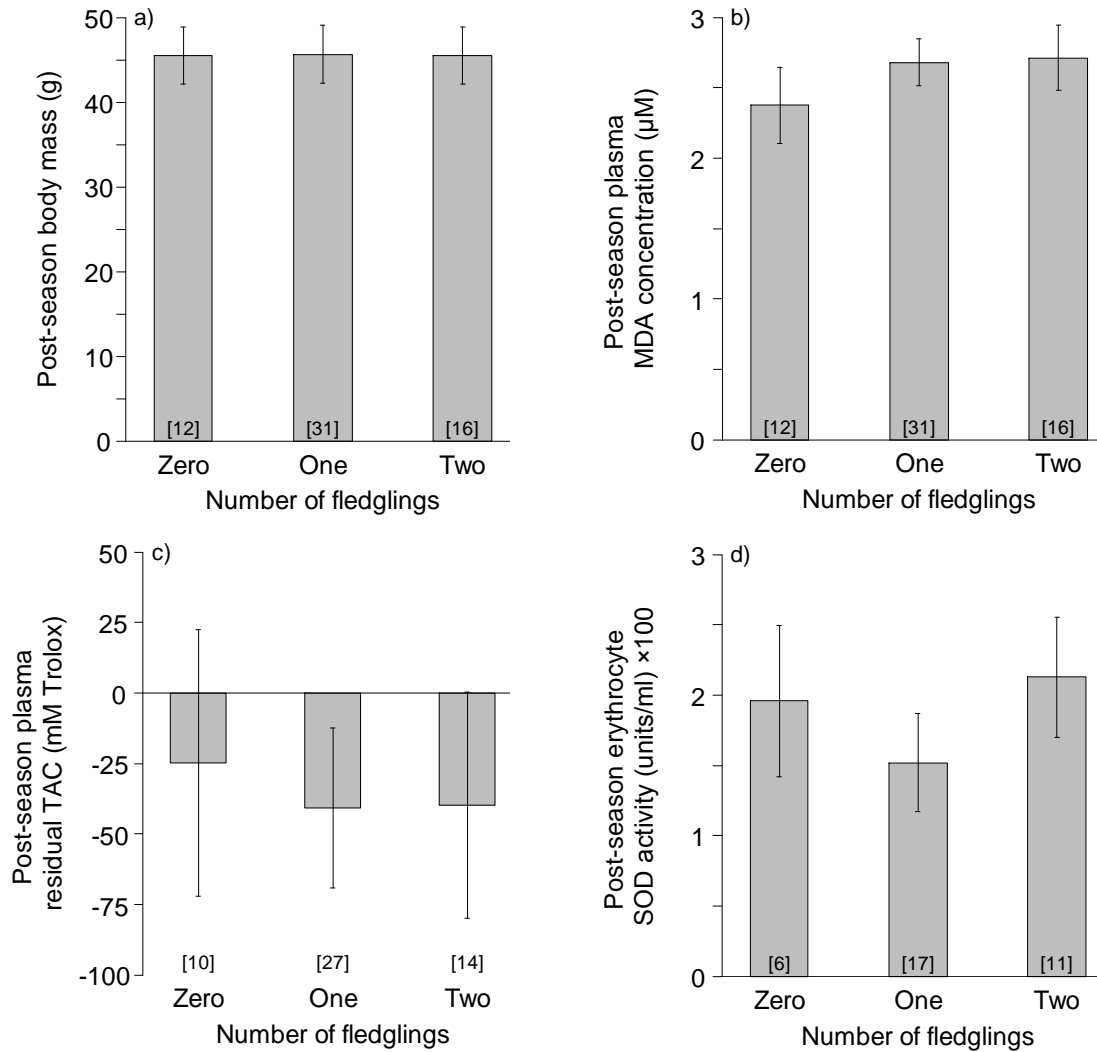
Figure 4.2. – The effect of raising zero, one or two fledglings on oxidative balance and body mass at the end of the breeding season. **(a)** body mass, **(b)** plasma MDA, **(c)** plasma residual TAC, **(d)** erythrocyte SOD activity. Bars represent the predicted means ± SE from the minimal adequate models while controlling for the effects of other significant predictors.

4.4.2. Does an individual's provisioning rate impact its post-season oxidative balance and body mass?

An individual's provisioning rate during the breeding season was a marginally non-significant negative predictor of its post-season body mass (χ^2_1 = 3.44, p = 0.063, n = 51 birds). There was no evidence that an individual's provisioning rate during the breeding season predicted either its erythrocyte SOD activity (n = 32 birds), its plasma MDA concentration (n = 51 birds) or its plasma residual TAC (n = 38 birds) at the end of the breeding season (all χ^2_1 < 0.90, p > 0.34).

4.4.3. Does a dominant female's pre-season state predict whether she breeds early in the breeding season?

Whether a dominant female initiated a clutch in the first 35 days of the breeding season was not significantly predicted by her pre-season plasma MDA, erythrocyte SOD activity, plasma residual TAC, body condition or social group size (all p > 0.46, n = 23 dominant females).

4.5 DISCUSSION

This study investigated whether reproductive effort yielded long-term impacts on oxidative balance and body condition in a wild, cooperatively breeding bird. Our findings reveal no evidence that oxidative balance and body mass at the end of the breeding season were impacted by the number of fledglings that an individual reared during the course of the season. While this finding could be due in part to the fact that not all individuals contribute strongly to rearing offspring, further analyses revealed that individuals that provisioned offspring at higher rates (which has been shown to be associated with differential short-term reductions in plasma residual TAC and body mass; Chapter 3) showed no evidence of differential deficits in post-season oxidative balance, though there was a tendency towards such an effect for post-season body mass. One possible explanation for this apparent lack of a long-term impact of reproductive effort on body mass and oxidative balance is that dominant females might only have initiated reproduction when they (and perhaps by extension their fellow group members) were in sufficiently good condition to raise offspring. However, we found no evidence that dominant females in better state at the start of the breeding season (either with regard to oxidative balance or body condition) were more likely to initiate reproduction during the early part of the breeding season.

We found no evidence that an individual's post-season oxidative balance and body mass were affected by the number of fledglings that it reared during the breeding season (a proxy for reproductive effort). The number of fledglings reared is a group-level proxy for reproductive effort expended in provisioning young, and so it is possible that any long-term cost of such effort is suffered only by those

individuals that provision at the highest rates. However, post-season oxidative balance and body mass were also not significantly influenced by individual provisioning rates. Our findings suggest that the previously-reported short-term impacts of reproductive effort on oxidative balance and body mass in this species (measured at peak provisioning; Chapter 3) are transient and can be recovered before the end of the breeding season. Such transience in the impact of reproductive efforts on circulating markers of oxidative state has previously been reported by Losdat *et al.* (2011), who found that male great tits *Parus major* feeding enlarged broods suffered impaired antioxidant protection after five days, but had recouped this deficit after 13 days.

Although fleeting, the impact of reproduction on circulating markers of oxidative balance may nonetheless have important repercussions for health and survival. The recovery of antioxidant protection/oxidative damage during and after reproduction reported here and elsewhere (Losdat *et al.*, 2011; Cohen *et al.*, 2012) may arise by the up-regulation of endogenous antioxidants (Beaulieu *et al.*, 2011) and allocation of additional resources to antioxidant protection, which is likely to entail a potentially costly reduction in investment in other traits. Alternatively, reduced investment in reproduction shortly after an imbalance in oxidative balance arises may directly allow the recovery of homeostasis, improving future condition at a cost to current reproductive success (Losdat *et al.*, 2011). Long-term costs of reproduction may also exist but not be evident in the circulatory markers of oxidative balance reported here. While we found no effects of reproductive effort on oxidative damage to lipids, ROS can also cause damage to proteins and DNA (Finkel, 2003; Halliwell and Gutteridge, 2007). In particular, extensive oxidative damage to DNA could occur even during brief exposure to oxidative

stress, resulting in cell death, the generation of tumours and reduced survival (Beckman and Ames, 1998; Chandra *et al.*, 2000; Kryston *et al.*, 2011). Oxidative damage to telomeres may be particularly damaging, as it may be repaired less effectively than similar damage to other regions of the genome (Petersen *et al.*, 1998; von Zglinicki, 2002), and can lead to curtailed survival (Heidinger *et al.*, 2012). Finally, the circulatory markers of oxidative balance measured here may also not reflect potentially important oxidative stress occurring in other tissues (Sohal *et al.*, 1995; Gaál *et al.*, 1996; Selman *et al.*, 2012). Markers of oxidative damage in blood may be more straightforward to recover and therefore reflect only recent oxidative state (Nussey *et al.*, 2009), or may not indicate more serious oxidative stress in, for example, liver, skeletal muscle, brain and heart (Veskoukis *et al.*, 2009; Garratt *et al.*, 2012). Transient increases in circulating markers of oxidative damage products (such as lipid peroxidation products e.g. MDA) may therefore constitute ephemeral signals that permanent damage is occurring elsewhere, in tissues or molecular markers not investigated in our study (Selman *et al.*, 2012).

Dominant females in this population are the only females to produce and incubate eggs (Harrison *et al.*, 2013), and provision offspring at higher rates than any other group members (Lewis, 1982). We have previously reported that individuals that provision at higher rates subsequently show impaired antioxidant defences (Chapter 3), and that dominant females suffer a differential fall in circulating antioxidant protection over the course of the breeding season (Chapter 2). It was therefore expected that dominant females that reared more fledglings would show more marked declines in antioxidant defences and elevated oxidative damage. While limited sample size meant our analyses could not focus directly on dominant

females, we found no evidence of long-term costs of high provisioning rates, which suggests that dominant females (by far the highest provisioners; Young *et al.,* unpublished data) do indeed suffer no long-term costs of reproduction in terms of circulating markers of oxidative balance. As such, investment in post-hatching provisioning *per se* may not be responsible for dominant females' differential decline in circulating TAC residuals over the course of the breeding season. This effect could arise if the primary oxidative cost of reproduction for dominant females is in fact incurred during egg production (Blount *et al.*, 2000) and incubation (Monaghan and Nager, 1997; Hanssen *et al.*, 2005; de Heij *et al.*, 2006). Almost all dominant females in our study incubated at least one clutch (27/31), and almost all clutches in the breeding season were two eggs (69/79), so we lacked the natural variation required to specifically investigate whether dominant females' post-season state was impacted by variation in investment in egg production and/or incubation. The decline in antioxidant protection of dominant females over the course of the breeding season might also reflect their differential investment in activities other than reproduction *per se*, such as defence of territory or their dominant position.

One way by which dominant females may avoid incurring excessive costs whilst breeding is to adjust their investment according to their physiological ability to cope with reproduction. For example, female Florida scrub jays *Aphelocoma coerulescens* with low circulating vitamin E levels subsequently laid eggs later (Heiss *et al.*, 2011). However, we found no evidence that antioxidant protection or oxidative damage influenced dominant females' likelihood of laying in the first 35 days of the breeding season. Dominant females in better body condition or in larger social groups were also no more likely to initiate breeding early in the

season, suggesting that the decision to reproduce may be based on other criteria, such as impacts of territory quality that are not mediated through effects on the metrics measured (e.g. antioxidants stored in tissues).

In conclusion, we find no evidence that natural variation in reproductive effort (assessed either as the number of offspring fledged or individual provisioning rates), affected post-season oxidative balance or body mass, suggesting that the short-term oxidative and body mass deficits previously reported in this species (Chapter 3) can be recovered relatively quickly. While our findings do not necessarily undermine the potential importance of such short-term impacts on oxidative state, they suggest that mechanisms other than impacts on the circulating metrics of oxidative state measured here may underpin the long-term costs of reproduction, such as lasting oxidative damage to proteins and DNA, or in key tissues whose oxidative states may not correlate strongly with that of circulating blood.

CHAPTER 5

Oxidative stress costs of mounting an immune response in a wild bird: an experimental study

5.1 ABSTRACT

The immune system provides vital protection against pathogen infection, but this protection comes at a cost. Despite widespread evidence of reduced fecundity and survival following immune activation, our understanding of the molecular mechanisms that underpin the costs of mounting an immune response remains poorly developed. Recently, oxidative stress has been highlighted as a potential mediator of such costs, as the activation of an immune response could enhance the generation of reactive oxygen species (ROS), yet this possibility has rarely been tested in wild populations. Here, we investigate the effects of a phytohaemagglutinin (PHA) immune challenge on oxidative balance in a wild adult bird, the white-browed sparrow weaver, *Plocepasser mahali*. Our findings suggest that PHA-induced immune activation does not elicit oxidative stress in the wild. Compared with controls, PHA-injected birds showed no difference in circulating markers of oxidative damage to lipids and enzymatic and non-enzymatic antioxidant protection 24 hours after challenge. Consistent with this finding, the scale of an individual's swelling response to PHA injection did not predict their associated change in oxidative state. Finally, we found that the pre-injection activity of a key antioxidant enzyme (superoxide dismutase, SOD) predicted the scale of the swelling response to PHA challenge: birds with a stronger initial SOD activity produced smaller swellings. Our findings suggest that wild birds can mount clear immune responses without suffering oxidative stress, and raise the possibility that they mitigate such costs by adjusting the strength of their immune response in relation to their oxidative state.

5.2 INTRODUCTION

Complex immune systems have evolved to minimize the detrimental effects of pathogen infection on host fitness. Activating these protective systems can however be costly, as evidenced by the selective triggering of immune responses only when needed and the clear fitness costs that can arise from immune challenge experiments (reviewed in Zuk and Stoehr, 2002; Hasselquist and Nilsson, 2012). Investment in active immune responses is known to trade-off against the resource demands of other functions (Sheldon and Verhulst, 1996), including growth (Fair *et al.*, 1999), reproductive effort (Knowles *et al.*, 2009) and the expression of sexual signals (Faivre *et al.*, 2003). As a result of these trade-offs, mounting a wide variety of immune responses can ultimately lead to reduced fecundity (Uller *et al.*, 2006) and curtailed survival (Moret and Schmid-Hempel, 2000; Hanssen *et al.*, 2004). Despite the importance of such trade-offs, our understanding of the molecular mechanisms that mediate the costs of mounting an immune response remains poorly developed (Hasselquist and Nilsson, 2012).

Traditionally, the costs of diverse immune responses have been thought to arise from energetic trade-offs, as costly immune components may divert energy away from other functions (Stearns, 1989; Sheldon and Verhulst, 1996). Indeed, a recent meta-analysis found evidence of significant energetic costs resulting from mounting immune responses following experimental immune activation (Hasselquist and Nilsson, 2012). However, on average these energetic costs were relatively small (5-15% elevated energy consumption), and might therefore be readily recovered in the short-term (e.g. Verhulst *et al.*, 2005). Thus, the

mechanisms underpinning the apparent long-term costs of immune activation (Hanssen *et al.*, 2004; Uller *et al.*, 2006) remain largely unknown.

Recently, it has been suggested that the long-term costs of an immune response may be mediated by the damaging effects of reactive oxygen species (ROS), which may be more difficult to alleviate than a small energy deficit (Costantini and Møller, 2009; Hasselquist and Nilsson, 2012). ROS are primarily produced as a by-product of aerobic respiration, and cause damage to DNA, lipids and proteins (Finkel, 2003; Halliwell and Gutteridge, 2007). The harmful effects of ROS are usually minimized by the body's complex antioxidant system, but when the generation of ROS overwhelms antioxidant protection, a state of oxidative stress results (Ames *et al.*, 1993). Oxidative stress can lead to long-term impairments including decreased reproductive success and senescence (Beckman and Ames, 1998; Bize *et al.*, 2008; Monaghan *et al.*, 2008; Ricklefs, 2008; Selman *et al.*, 2012), and has been highlighted as a potential mediator of life-history trade-offs (Dowling and Simmons, 2009; Monaghan *et al.*, 2009; Metcalfe and Alonso-Alvarez, 2010). Immune responses in particular could enhance ROS production via two key pathways. First, mounting an immune response can increase metabolic rate (Demas *et al.*, 1997; Ots *et al.*, 2001; Martin *et al.*, 2003), leading to higher oxygen consumption and, potentially, greater ROS production (but see Barja, 2007). Second, macrophages and heterophils can produce ROS directly in response to foreign antigens, so as to kill pathogens during 'respiratory burst' (Holmes *et al.*, 1967; Babior, 1984; Dahlgren and Karlsson, 1999). As the destructive action of ROS is non-specific, excessive ROS production during respiratory burst can result in damage to host tissues (Weitzman and Stossel, 1981; Gordon and Weitzman, 1988; Anderson and Theron, 1990; Güngör *et al.*, 2010). Were such elevated ROS

production to overpower the body's antioxidant protection system it could induce generalised oxidative stress and thereby elicit the documented long-term costs of immune activation.

A number of studies have now investigated the oxidative stress effects of immune activation using experimental immune challenges. Some studies have demonstrated increased oxidative damage and/or reduced antioxidant protection in response to a novel antigen challenge (bacterial lipopolysaccharide (LPS): (Bertrand *et al.*, 2006b; Torres and Velando, 2007); phytohaemagglutinin (PHA, Costantini and Dell'Omo, 2006; Hõrak *et al.*, 2007); sheep red blood cells (sRBC, Casagrande *et al.*, 2012); Tetravac vaccine: (Stier *et al.*, 2009)), while others found no effects of such challenges (LPS: (Alonso-Alvarez *et al.*, 2004a; Cohen *et al.*, 2007); PHA: (Perez-Rodriguez *et al.*, 2008); sRBC: (Hõrak *et al.*, 2006)). Indeed, a meta-analysis found that experimental immune activation typically caused oxidative stress, though there was considerable heterogeneity among studies (Costantini and Møller, 2009). However, these studies have been conducted almost exclusively in captivity; whether wild birds in natural populations respond in similar ways therefore remains virtually unexplored (but see Costantini *et al.,* 2006 for a study of wild Eurasian kestrel *Falco tinnunculus* nestlings).

It has recently been highlighted that oxidative stress measures taken in captivity may not reflect natural oxidative states in the wild (Sepp *et al.*, 2010; Casagrande *et al.*, 2011). Limited exercise, unlimited access to food and water, favourable diet compositions and unpredictable external stressors may all impact the scale and associated costs of an immune response. Furthermore, captive studies exclude potentially important challenges only faced by wild animals in natural

environments, potentially diminishing the pressure to make life-history trade-offs in the relatively favourable captive conditions. For example, the need to counter predation risk and defend territories may amplify costs in wild populations that might otherwise appear minor in captivity. Alternatively, greater scope to mount compensatory adjustments to behaviour and foraging regimes may leave wild birds better able to mitigate the costs of mounting an immune response. In order to fully understand the oxidative costs of mounting an immune response, it is therefore critical that future studies challenge wild, free-ranging birds (Hasselquist and Nilsson, 2012).

Furthermore, if mounting an immune response does indeed elicit oxidative stress, we might predict selection to favour modulation of the strength of immune responses according to an individual's oxidative state. Thus, individuals with low concentrations of circulating antioxidants might be predicted to mount correspondingly weaker immune responses so as to mitigate the extent of oxidative damage that results (dependent on the extent to which such benefits outweighed any concomitant cost of reduced impact on the pathogen). Such regulation of immune response according to baseline oxidative balance further highlights the importance of natural baseline oxidative balance, and the need for studies in the wild.

In this study, we use a PHA immune challenge experiment to test whether immune activation causes oxidative stress in a wild bird, the white-browed sparrow weaver *Plocepasser mahali*. White-browed sparrow weavers are cooperatively breeding birds that live in territorial groups of 2-12 individuals (Collias and Collias, 1978; Lewis, 1982). They are an unusually tractable model for immune challenge studies

in the wild as they can be readily caught from their individual roost chambers after dusk, greatly facilitating the repeat captures at precise intervals that are typically required for such studies. By sampling the birds' oxidative state at the time of challenge and again on recapture exactly 24 hours later, we address three key aims. First, we investigate whether birds subjected to a PHA challenge differ in oxidative state after 24 hours from control birds injected with saline buffer. Second, we investigate whether the strength of an individual's swelling response to PHA challenge predicts the magnitude of their change in oxidative state. Wing-web swelling is a frequently measured physical response to PHA injection (Smits *et al.*, 1999), and there is evidence that it scales with levels of infiltration of a number of immune cell types (Martin *et al.*, 2006). Whether wing-web swelling following PHA-injection indicates elevated oxidative stress remains unknown. Finally, we investigate whether an individual's oxidative state immediately prior to PHA challenge predicts the strength of their swelling response to challenge (which might be predicted if individuals mitigate the oxidative costs of immune responses by modulating their response according to their oxidative state).

PHA injection is a widely used challenge in ecological studies of immunity and is known to induce a complex immunological cascade, including the initiation of an inflammatory response, the proliferation of lymphocytes and the recruitment and activation of heterophils and macrophages involved in respiratory burst (Kennedy and Nager, 2006; Martin *et al.*, 2006; Vinkler *et al.,* 2010). Oxidative balance is a complex, multi-faceted physiological state that can only be characterised through multiple markers of antioxidant protection and oxidative damage (Hõrak and Cohen, 2010). We therefore investigate a suite of metrics of oxidative balance, including circulating levels of a lipid oxidative damage product (malondialdehyde,

MDA), the levels of intra-cellular enzymatic antioxidant protection in the form of superoxide dismutase (SOD), circulating non-enzymatic antioxidant activity (total antioxidant capacity, TAC) and a circulating antioxidant whose *in vivo* antioxidant function is poorly-understood (uric acid) leading to calls to be cautious of its contribution to TAC (Cohen *et al.*, 2007).

5.3 METHODS

5.3.1. Study population and field methods

The experiment was conducted in the context of a long-term study, monitoring a population of 40 cooperative groups of white-browed sparrow weavers in an area of approximately 1.5 km² in Tswalu Kalahari Reserve, South Africa (27°16'S, 22°25'E). Data collection took place between November 2011 and February 2012 during the breeding season, but when group members were not provisioning offspring. Breeding stage was confirmed by nest searches every one to two days.

All experimental birds were subordinate group members aged at least seven months, and were fitted with a single metal ring and three colour rings for identification (under SAFRING license 1444). Males and females were distinguished by beak colour: males have dark brown beaks while females have paler horn-coloured beaks (Leitner *et al.*, 2009). Dominant birds were excluded to avoid disrupting propensity to breed (e.g. Ilmonen *et al.*, 2000). Dominance was assigned following weekly behavioural observations and criteria detailed elsewhere (Harrison *et al.*, 2013). Briefly, dominant males were behaviourally dominant to all other males and consistently sang dawn song during the breeding season. Dominant females were the only females to lay eggs in each group.

5.3.2. Experimental design

Birds were alternately assigned to one of two treatment groups: PHA-challenged (n = 27 birds) or control-injected (n = 25 birds). During the evening of Day 1, birds were captured and blood sampled for the determination of their baseline levels of oxidative damage and antioxidant protection. Morphometric measures body mass,

tarsus length and wing-web thickness were then taken (see below). Individuals were then injected with either PHA solution or phosphate buffered saline (PBS) and returned to their roost chambers to pass the remainder of the night and freely range the following day. On the subsequent evening (Day 2), these same birds were recaptured 24 hours after their injection (24.02 ± 0.8 hours, mean ± S.D.) and blood samples and morphometric measures were repeated as on night one, to reveal states after treatment.

5.3.3. Captures, blood-sampling and immune challenge

All capture, blood sampling and measurements were conducted by one person (DC) under license (SAFRING license 1444). Birds were captured individually at night, by flushing them from their individual roost chambers into a custom capture bag. A blood sample (approximately 160 μl) was immediately collected by nicking the brachial vein with a 26g needle (mean ± S.D. time lag from capture to bleed completion = 188 ± 78 seconds). All blood samples were collected from the left wing and all injections were administered to the right wing (as described below), to avoid possible localized effects of blood sampling on any swelling response to injection. Following blood sampling, body mass was recorded to the nearest 0.01 g (Durascale 100; MyWeigh, USA) and the thickness of the wing-web (patagium) of the right wing was measured three times using a pressure sensitive calliper (Model 700-118; Mitutoyo, Japan). These patagium thickness measures were highly repeatable (Lessells and Boag, 1987) both pre-injection ($F_{51,104} = 35.643$, r = 0.920, p < 0.001) and post-injection ($F_{51,104} = 242.78$, r = 0.988, p < 0.001), and can reflect an individual's pro-inflammatory potential and the infiltration of key immune cells to the injection site (Martin *et al.*, 2006, Vinkler and Albrecht, 2010). The injection

was administered as follows: the patagium was sterilized with ethanol and injected subcutaneously with either a solution of 0.02 mg PHA (L8754; Sigma, UK) in 0.04 ml autoclaved PBS (P4244; Sigma, UK), or a control solution of 0.04 ml PBS (following Spottiswoode, 2008). The 'swelling response' to injection was calculated as the difference between the means of the patagium thickness measures pre- and post-injection.

5.3.4. Blood processing and oxidative balance metric determinations

Lipids are a major target of ROS during oxidative stress, and concentrations of the lipid peroxidation products offer a measure of *in vivo* oxidative damage (Halliwell and Chirico, 1993). We therefore measured plasma concentrations of malondialdehyde (MDA). We also measured superoxide dismutase (SOD) activity in erythrocytes. SOD is a key intracellular antioxidant enzyme, which forms part of the first line of defence against oxidative stress (Godin and Garnett, 1992; Parkes *et al.*, 1998). Finally, we measured the ability of a plasma sample to quench free radicals *in vitro*, thus providing a functional measure of total antioxidant capacity (TAC). Full details of sample collection, processing, transport and laboratory analysis can be found in the Methods section of Chapter 2.

It has recently been highlighted that up to 90% of the variation in antioxidant activity that makes up TAC can be due to the 'incidental' antioxidant effects of uric acid (Cohen *et al.*, 2007). Uric acid is a waste product of protein metabolism in birds, and its *in vitro* antioxidant activity may be incidental to this primary role. The *in vivo* importance of uric acid as an antioxidant remains unclear. Furthermore, increased uric acid levels can indicate stress (Cohen *et al.*, 2008), and

uric acid production can itself generate ROS (Dröge, 2002). As a result, plasma TAC values comprise the activities of important antioxidants, as well as that of uric acid, which may at best add noise or at worst confound key analyses (Cohen *et al.*, 2007; Cohen *et al.*, 2008; Cohen and McGraw, 2009).

In order to factor out the impact of circulating uric acid on plasma TAC, we therefore calculated the residuals of the relationship between uric acid and TAC (hereafter termed 'residual TAC') as recommend in Cohen *et al.* (2007). A linear mixed model was first used to confirm the relationship between uric acid concentration (set as a predictor) and TAC (as the response). Bird identity was the random factor (as two measures of each blood marker were taken from each bird). Plasma uric acid concentration significantly predicted plasma TAC values ($\chi^2_1 =$ 37.89, p < 0.001, n = 102 samples). The residuals were therefore extracted from a separate linear model (with TAC as the response and uric acid as the only predictor), to yield a measure of plasma antioxidant capacity excluding that arising from plasma uric acid levels. Partitioning TAC in this way allowed the assessment of treatment effects on both uric acid and non-uric-acid derived plasma antioxidant capacity.

5.3.5. Statistical analysis

Statistical analyses were carried out using R (R Development Core Team, 2013), using a step-wise model simplification approach (Crawley, 2007). Initially all fixed terms of interest were fitted, followed by the stepwise removal of terms whose removal from the model resulted in a non-significant change in deviance (using a likelihood-ratio test for model comparison), until the minimal adequate model

(MAM) was obtained, in which only significant terms remained. Dropped terms were then added back in to the MAM to confirm their non-significance and were retained in the MAM when found to be significant in this context. The homoscedasticity and normality of residuals were inspected visually and where necessary response terms were transformed to satisfy these criteria. The significance of all terms was tested either by removing the terms from the MAM (if the term was in the MAM) or adding the terms back in to the MAM and then removing them (if the term was not included in the MAM). Sparrow weaver social group was fitted as a random term in all models, to control for sampling of multiple individuals within social groups (n = 52 individuals were sampled across 20 social groups). The effect of treatment on wing-web thickness was tested using a linear mixed model. The change in wing-web thickness was fitted as the response term, and treatment, sex and their interaction were fitted as predictors.

1) Does PHA challenge affect an individual's oxidative state?

Four linear mixed models were used to assess the effect of treatment (PHA challenge vs PBS injection) on an individual's post-challenge levels of each oxidative balance metric (MDA concentration, SOD activity, TAC residuals and uric acid concentration). The post-challenge level of a given oxidative balance measure was fitted as the response term and the pre-challenge level of the same measure was fitted as a covariate predictor alongside treatment. This approach is more statistically more powerful than modelling the effect of treatment on the change in a given oxidative balance measure and can also account for the effects of chance biases in the treatment groups in the pre-challenge levels of the oxidative balance metric (Crawley, 2007). Bird sex was also included as a factorial predictor, as well

as all two-way interactions among terms. Post-treatment plasma concentrations of MDA and uric acid were log-transformed, and post-treatment SOD activity was square-root-transformed.

2) Does an individual's swelling response to PHA challenge predict the magnitude of their associated change in oxidative state?

A similar set of four mixed models were used to examine whether the magnitude of the swelling response to PHA challenge predicted an individual's post-challenge oxidative balance metrics (control birds were therefore excluded from this analysis). The pre-challenge level of the focal oxidative balance measure was fitted as a covariate predictor, along with the size of the swelling response, sex, and all two-way interactions among terms. Post-treatment plasma concentrations of MDA and uric acid were log-transformed, and post-treatment SOD activity was square-root-transformed.

3) Does an individual's pre-challenge oxidative state predict the strength of their swelling response to PHA challenge?

Finally, a single linear mixed model was used to test whether pre-challenge oxidative balance metrics predicted the magnitude of the bird's swelling response to PHA challenge (control birds were therefore excluded from this analysis). The size of the swelling response was the response term and the pre-challenge levels of all oxidative balance measures were fitted as covariate predictors. Sex was fitted as a factor. Swelling response was square-root transformed for normality, and SOD activity was log-transformed for normality of residuals.

5.4 RESULTS

5.4.1. Does PHA challenge affect an individual's oxidative state?

As expected, birds injected with PHA show significantly larger wing-web swellings after 24 hours than those injected with PBS (mean change in wing-web thickness ± S.E.: PHA = 0.42 ± 0.03 mm, PBS = 0.07 ± 0.01 mm, GLMM: χ^2_1 = 73.49, p < 0.001, n = 52 birds). There was no significant effect of sex on the extent of the swelling response either as a single factor or in an interaction between sex and treatment (both χ^2_1 < 0.67, p > 0.41). Moreover, as outlined below, we found no significant effect of treatment (PHA challenge versus PBS injection) on plasma MDA concentrations, erythrocyte SOD activity or plasma residual TAC.

There was no significant effect of treatment on post-treatment plasma MDA concentrations (Figure 5.1a, χ^2_1 = 2.07, p = 0.15, n = 52 birds), nor were there significant effects of sex or pre-treatment MDA levels (both χ^2_1 < 1.39, p > 0.24). Similarly, there was no significant effect of treatment on post-treatment erythrocyte SOD activity (Figure 5.1b, χ^2_1 = 0.39, p = 0.53, n = 49 birds) while controlling for a significant interaction between sex and pre-treatment SOD activity (χ^2_1 = 7.70, p = 0.006). This interaction arose because pre- and post-treatment SOD activities were similar in females (model estimate ± S.E.: 1.02 ± 0.07), but post-treatment SOD was typically lower than pre-treatment SOD in males (model estimate ± S.E.: 0.83 ± 0.05). There was also no significant effect of treatment on plasma residual TAC (Figure 5.1c, χ^2_1 = 0.03, p = 0.87, n = 50 birds), nor were there significant effects of pre-treatment residual TAC (χ^2_1 = 1.31, p = 0.25) or sex (χ^2_1 = 2.25, p = 0.13). There was, however, an effect of treatment on post-treatment plasma uric acid concentrations, as evidenced by a significant

interaction between treatment and pre-treatment uric acid concentration (Figure 5.1d, χ^2_1 = 5.93, p = 0.01, n = 50 birds), while controlling for a significant interaction between sex and pre-treatment uric acid concentration (χ^2_1 = 7.26, p = 0.007). Treatment with PHA (relative to PBS) had a negative effect on post-treatment uric acid levels in birds with low pre-treatment uric acid levels, but no clear effect on post-treatment uric acid levels in those with high pre-treatment uric acid levels (Figure 5.1d).
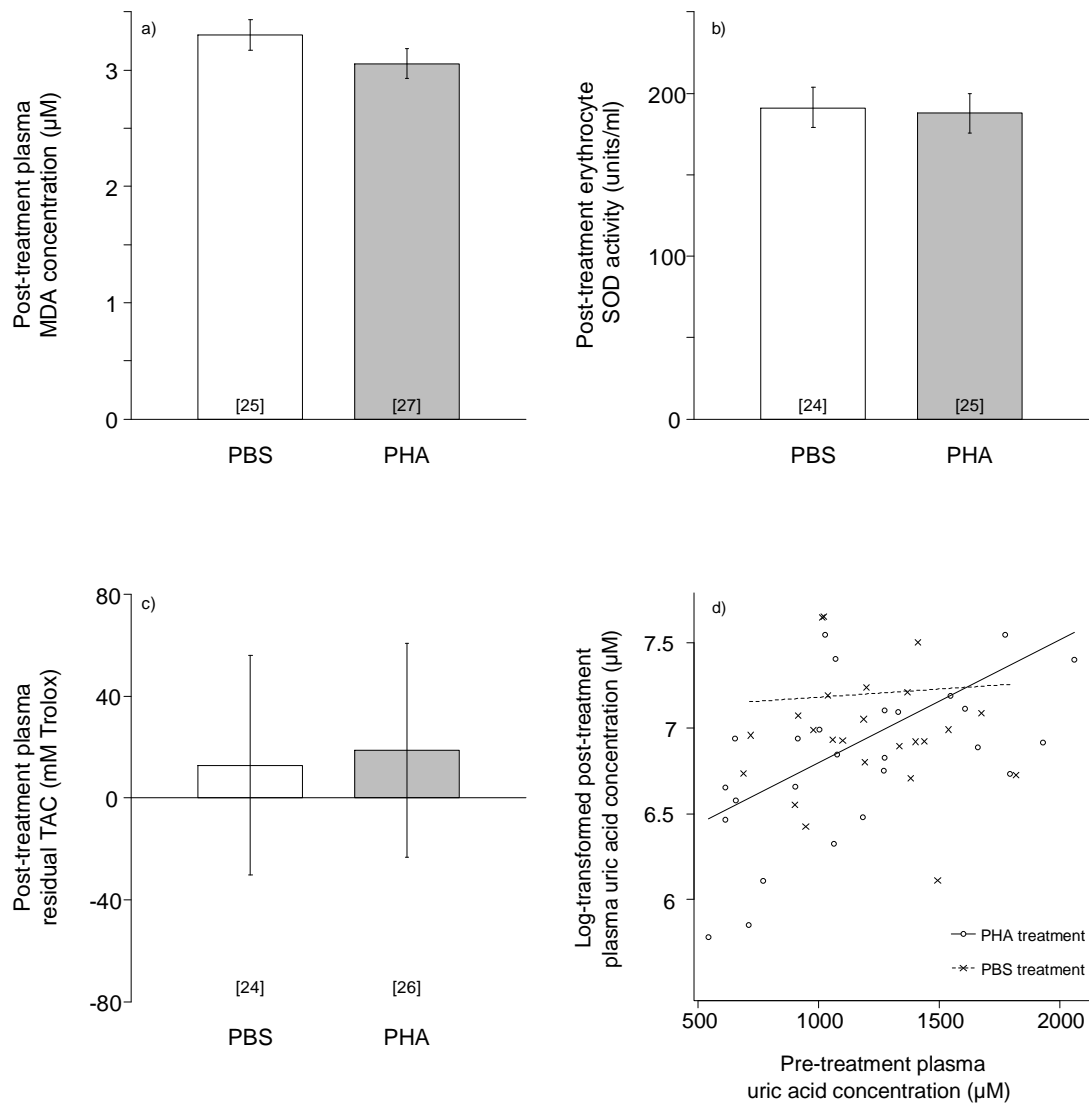
Figure 5.1– The effect of a PHA challenge on markers of oxidative balance: **(a)** plasma concentrations of the lipid peroxidation product malondialdehyde (MDA); **(b)** activity the antioxidant enzyme superoxide dismutase in erythrocytes (SOD); **(c)** plasma total antioxidant capacity excluding the effects of uric acid (residual TAC) and **(d)** plasma uric acid concentrations. In (a), (b) and (c), bars show model predictions ± S.E from GLMMs with treatment as the only predictor. Numbers in parentheses are sample sizes. The significant treatment × pre-treatment uric acid interaction is show in (d). Open circles are PHA birds, crosses are PBS control birds. Lines represent model predictions for PHA (solid line) and PBS-injected birds (dashed line) from a GLMM containing the interaction of treatment and pre-treatment uric acid levels.

5.4.2. Does an individual's swelling response to PHA challenge predict the magnitude of their associated change in oxidative state?

Among PHA challenged birds, the scale of an individual's swelling response to PHA challenge did not significantly predict any aspect of its post-challenge oxidative state. The scale of the swelling response to PHA challenge did not significantly predict post-treatment plasma MDA concentration (χ^2_1 = 1.05, p = 0.31, n = 27 birds), while controlling for a significant positive effect of pre-treatment MDA concentration (χ^2_1 = 5.20, p = 0.023). Similarly, the extent of the swelling response to PHA challenge did not significantly predict post-treatment erythrocyte SOD activity (χ^2_1 = 0.33, p = 0.57, n = 25 birds) while controlling for a significant interaction between pre-treatment SOD activity and sex (χ^2_1 = 5.40, p = 0.020). Post-treatment residual TAC was also not significantly predicted by the size of the swelling response following PHA challenge (χ^2_1 = 0.14, p = 0.71, n = 26 birds). Finally, post-treatment uric acid concentration was not significantly predicted by the strength of the swelling response to PHA challenge (χ^2_1 = 0.65, p = 0.41, n = 26 birds) while controlling for a significant interaction between pre-treatment uric acid concentration and sex (χ^2_1 = 6.33, p = 0.012).

5.4.3. Does an individual's pre-challenge oxidative state predict the strength of their swelling response to PHA challenge?

Pre-treatment erythrocyte SOD activity significantly predicted the size of the subsequent swelling response to PHA challenge (Figure 5.2, χ^2_1 = 5.94, p = 0.015, n = 26 birds); those birds with higher pre-treatment SOD activities showed smaller swelling responses to PHA challenge. The extent of the swelling response to PHA challenge was not predicted by pre-treatment plasma concentrations of MDA (χ^2_1 = 0.28, p = 0.58), uric acid (χ^2_1 = 0.76, p = 0.38) or residual TAC (χ^2_1 = 0.13, p = 0.72).

Figure 5.2 – Birds with higher pre-treatment erythrocyte SOD activities mounted smaller swelling responses to PHA challenge (n = 26 birds). The solid line depicts model predictions from the log pre-treatment SOD activity in the minimal adequate model; the points show raw data.

5.5 DISCUSSION

To our knowledge this is the first experimental study to investigate the effects of a PHA immune challenge on oxidative balance in a wild, free-ranging adult bird. The results suggest that PHA-induced immune activation does not cause changes in key circulating metrics of oxidative balance, including oxidative damage to lipids and measures of both enzymatic and non-enzymatic antioxidant protection. Immune activation did affect levels of plasma uric acid, though not in a straightforward way; PHA-challenge (relative to PBS) had a negative effect on post-treatment uric acid levels in birds with low pre-treatment uric acid levels, but no clear effect on post-treatment uric acid levels in those with high pre-treatment uric acid levels. Within PHA challenged birds, the size of the swelling response at the injection site (a proxy for the strength of the inflammatory response; Vinkler and Albrecht, 2010) did not predict associated changes in any of the oxidative balance metrics. Finally, pre-treatment erythrocyte SOD activity predicted the strength of the swelling response following PHA challenge: birds with higher initial SOD activity subsequently produced a smaller swelling after PHA challenge. Pre-treatment plasma concentrations of MDA, uric acid and residual TAC did not affect the size of the PHA-induced swelling response. Together, these findings suggest that immune activation by PHA challenge may have a minimal net effect on circulating markers of oxidative balance in the wild, but that the scale of the swelling response to such a challenge may be regulated by baseline oxidative state.

A subcutaneous PHA challenge of the type administered here has been shown to trigger the infiltration and proliferation of a number of immune cell types (Martin *et al.*, 2006), provoke an inflammatory response (Kennedy and Nager, 2006;

Vinkler and Albrecht, 2010) and elevate metabolic rate (Martin *et al.*, 2003), and it has been suggested that these responses may elicit oxidative stress (Costantini and Dell'Omo, 2006). While studies on captive birds have suggested that this may indeed be the case (Costantini and Dell'Omo, 2006; Hõrak *et al.*, 2007), our findings reveal no clear impact of PHA challenge on circulating markers of oxidative damage or antioxidant protection, suggesting that costs reflected in these currencies may be minimal. Consistent with this finding, our results also indicate that individuals that produced larger swelling responses in response to PHA challenge (which may indicate greater infiltration of immune cells at the injection site (Martin *et al.*, 2006), and a larger inflammatory response following injection (Vinkler and Albrecht, 2010) did not experience larger changes in oxidative balance. While we examined a suite of oxidative stress markers, PHA immune activation might impact other markers not investigated here (e.g. damage to DNA or proteins), or cause oxidative damage primarily sustained in tissues. Alternatively, net impact of PHA-challenge on oxidative balance may be minimized in the wild by compensatory behavioural and physiological adjusts that reduce other sources of ROS. Indeed, evidence from our study population suggests that dominant males, for example, markedly reduce their dawn song output when administered an identical PHA challenge (York, 2012). Immune activation is also known to compromise a number of other behaviours, including the provisioning of young (Råberg *et al.*, 2000) and overall activity (Bonneaud *et al.*, 2003). Strategic reductions in these behaviours may therefore alleviate the impact of an immune response on oxidative balance, allowing animals to actively sacrifice investment in certain behaviours in order to minimise the net oxidative costs incurred during immune activation.

PHA-challenge did negatively affect levels of uric acid, but only in those birds that had low initial circulating uric acid concentrations. Uric acid's contribution to plasma measures of antioxidant activity has only recently been highlighted (Cohen *et al.*, 2007; Cohen and McGraw, 2009; Costantini, 2011), and little is known about its importance and regulation as an antioxidant *in vivo*. Uric acid is the primary form of nitrogen excretion in birds, so reduced uric acid levels may simply result from decreased foraging and activity, which are symptomatic of typical 'sickness behaviours' during an immune response (Bonneaud *et al.*, 2003; Adelman and Martin, 2009). Alternatively, reduced uric acid may indicate oxidative stress, if ROS convert uric acid to its oxidation product allantoin (Simoyi *et al.*, 2003; Yardim-Akaydin *et al.*, 2004; Kand'ár and Žáková, 2008). Further work is required to investigate the ratio of uric acid and allantoin, which is likely to provide greater insight into the antioxidant properties of uric acid (Tsahar *et al.*, 2006).

We found that, following PHA challenge, the magnitude of swelling response was predicted by the activity of a key antioxidant enzyme (SOD). Previous work has shown that the magnitude of PHA-induced swelling is not affected by supplementation with dietary antioxidants including vitamin E and carotenoids (de Ayala *et al.*, 2006; Hõrak *et al.*, 2007), suggesting that PHA response may be modulated by endogenous (e.g. enzymatic), rather than dietary, antioxidants. The synthesis of such antioxidant enzymes is likely to be costly, and investment in SOD is therefore likely to be greatest in individuals with elevated ROS generation, thereby requiring more powerful antioxidant protection. As such, higher levels of pre-treatment SOD activity may indicate birds suffering from oxidative stress. If this is the case, our findings support the view that individuals may modulate the strength of their immune responses to avoid oxidative stress. Standing oxidative

state may therefore be a critical determinant of future oxidative balance fluctuations, whether in captivity or in the wild. Recent evidence suggests that oxidative balance metrics from captive birds can be distinct from those of wild birds (Sepp *et al.*, 2010; Casagrande *et al.*, 2011), and captive birds may therefore respond to challenges such as immune activation in a way which does not reflect the strategies of their wild counterparts. Our finding that baseline antioxidant protection predicts swelling response to PHA-challenge highlights the importance of natural baseline oxidative balance and the need for immune challenge experiments in the wild (Hasselquist and Nilsson, 2012).

In conclusion, our findings indicate that PHA-induced immune activation does not have a net effect on circulating metrics of oxidative balance in a wild, social bird. We suggest two likely mechanisms that might act in isolation or concert to account for this result. First, our findings provide novel evidence suggesting that individuals may regulate the strength of their immune responses according to their oxidative state at the time of challenge; a mechanism that may mitigate the oxidative costs of mounting an immune response. Second, individuals may also make compensatory behavioural or physiological adjustments during an immune response, mitigating the net impact of the immune response on oxidative balance by reducing ROS production through other activities. As such, our findings highlight the need to examine the effects of immune activation on oxidative balance in the wild, where natural baseline oxidative balance and compensatory mechanisms are likely to reveal the true costs of mounting an immune response.

CHAPTER 6

GENERAL DISCUSSION

6.1 OVERVIEW

Oxidative stress has repeatedly been highlighted as a potential mechanism mediating life-history trade-offs (Costantini, 2008; Dowling and Simmons, 2009; Monaghan *et al.*, 2009; Metcalfe and Alonso-Alvarez, 2010). Despite increasing interest in oxidative balance from behavioural ecologists, its role in mediating the costs of key life history traits, such as reproduction and immune defence, remains unclear. Recent reviews have also highlighted that much-needed experimental studies in wild contexts are particularly scarce (Metcalfe and Alonso-Alvarez, 2010; Selman *et al.*, 2012; Metcalfe and Monaghan, 2013). Furthermore, the factors that affect the distribution of oxidative stress in animal societies, where high reproductive skew, social status and cooperation may impact oxidative balance, remain largely unexplored. In this thesis I aimed to address these shortfalls in our understanding by using experimental and observational approaches to investigate the causes and consequences of oxidative stress in a wild population of the cooperatively breeding white-browed sparrow weaver *Plocepasser mahali*. In this Chapter, I will briefly discuss my findings and their wider implications, and suggest potential future research directions.

6.2 OXIDATIVE STRESS AND REPRODUCTION

Experimental studies are crucial in advancing our understanding of the link between reproduction and oxidative stress (Metcalfe and Monaghan, 2013). My experimental and correlative results revealed that reproductive effort can result in declines in antioxidant protection and elevated oxidative damage (Chapter 3). However, Chapter 4 suggested that such effects on circulating makers of oxidative balance do not endure, and contributions to nestling care do not promote oxidative

stress after the breeding season is complete. These results add to growing evidence that any impacts reproductive effort may have on oxidative balance do not persist long enough to directly mediate the observed long-term costs of investment in reproduction (Losdat *et al.*, 2011; Cohen *et al.*, 2012).

The transient nature of the impact of reproduction on circulating markers of oxidative balance may be particularly surprising for dominant females, given their high investment in breeding and disproportionate loss of antioxidant protection post-breeding season (Chapter 2). As dominant females are the sole egg-layers and incubators in this species, costs associated with these activities (Monaghan and Nager, 1997; Nager *et al.*, 2001; Visser and Lessells, 2001) would fall solely on this class, and this may be reflected in their oxidative balance. In particular, egg production can require significant investment, both in energetic terms and in the form of key antioxidants (Blount, 2004; Vézina and Williams, 2005; Bertrand *et al.*, 2006a), which may intensify antioxidant trade-offs for dominant females (Catoni *et al.*, 2008). Furthermore, incubation is frequently overlooked as a potentially costly breeding stage, and is often (wrongly) assumed to be less energetically demanding than periods of intensive care for young (Williams, 1996; Monaghan and Nager, 1997). Clutch size manipulations suggest that incubation can incur energetic costs, which in some cases can even result in marked decreases in survival (Williams, 1996; Thomson *et al.*, 1998; de Heij *et al.*, 2006). Incubation is also likely to impact normal foraging regimes. Reduced opportunities to forage may lead to declines in dietary antioxidants (Cohen *et al.*, 2009; van de Crommenacker *et al.*, 2011a), potentially leaving mothers with weak antioxidant defences immediately before intensive provisioning of young. In short, the combined effects of antioxidant deposition during egg production, reduced intake of dietary antioxidants during

incubation, and increased oxidative damage during nestling care may therefore result in the poor antioxidant protection exhibited by dominant females after breeding.

It was not possible to investigate the costs of egg production and incubation in this thesis using the data available. Almost all clutches contained two eggs, leaving insufficient variation to investigate whether producing large clutches entailed greater antioxidant costs. Furthermore, the females that produced the highest number of clutches over the course of the season were typically those whose eggs never hatched (therefore prompting them to re-clutch), thus confounding greater investment in egg production with lower investment in care for nestlings and fledglings.

A key goal in understanding the oxidative stress costs of reproduction should now be to experimentally disentangle the impacts on oxidative balance of reproductive effort during different breeding stages, from securing a mate, to egg production, incubation, and care for nestlings and fledglings (van de Crommenacker *et al.*, 2011b). Investment of antioxidants in developing eggs may have particularly important fitness consequences, as it may negatively impact a mother's oxidative balance while differentially improving that of her developing young (Surai *et al.*, 1998; Surai and Speake, 1998; Blount *et al.*, 2000). The maternal strategies that determine the levels of antioxidant deposition in eggs in wild contexts are as-yet poorly understood (Saino *et al.*, 2002; Remeš *et al.*, 2007; Safran *et al.*, 2008; Plummer *et al.*, 2013). Forthcoming analysis of levels of oxidative damage and antioxidant protection in sparrow weaver egg yolk, together with matched maternal blood samples at clutch completion, will likely shed light on the link

between maternal oxidative balance and that of her eggs. Such a link may have particularly important implications for the development of eggs produced later in the season, as they may suffer from the reduced antioxidant reserves dominant females experience as the season advances (Chapter 2).

Perhaps surprisingly, despite finding that reproduction can cause oxidative stress, I found no evidence that reproductive effort is reduced in individuals with poor pre-breeding oxidative balance. Recent evidence from other species suggests that investment in reproduction may be regulated according to oxidative balance (Heiss *et al.*, 2011; van de Crommenacker *et al.*, 2011b; Stier *et al.*, 2012), suggesting that individuals may adjust their reproductive effort according to their ability to cope with the costs it might entail (Metcalfe and Monaghan, 2013). By contrast, Chapter 2 revealed that dominant and subordinate individuals do not differ in their oxidative balance before the breeding season, despite the fact that dominants subsequently invest differentially more in reproduction. Moreover, a dominant female's pre-season oxidative balance did not predict her likelihood of laying early in the breeding season (Chapter 4). Nonetheless, despite limited support for the role of oxidative balance in shaping reproductive effort in my study species, there was evidence for such a role in immune defence.

6.3   OXIDATIVE STRESS AND IMMUNITY

In Chapter 5, I used an immune challenge to experimentally investigate the oxidative stress costs associated with mounting an immune response. My results revealed that, despite mounting a clear immune response, experimental birds exhibited no net change in their oxidative balance compared to control birds. However, individuals with elevated enzymatic antioxidant protection (SOD

activity) before the challenge subsequently mounted smaller immune responses, suggesting that individuals may adjust the strength of their immune responses in an oxidative-condition-dependent manner, potentially to mitigate the costs that might otherwise arise. That immune responses are scaled according to baseline oxidative balance suggests that the impacts of other factors (e.g. reproduction: Deerenberg *et al.*, 1997; Nordling *et al.*, 1998) on immune responses could also be mediated via their effects on oxidative balance.

6.4   SYNTHESIS – TRADE-OFFS BETWEEN LIFE-HISTORY TRAITS

While early ecological studies of oxidative stress (including the current study) have focussed on single life-history traits in isolation, future work should investigate the combined effects of multiple traits on oxidative balance. In particular, recent evidence suggests that the oxidative stress costs of reproduction and infection may be particularly severe when both are experienced simultaneously. For example, malarial infection in Seychelles warblers *Acrocephalus sechellensis* caused marked increases in reactive oxygen metabolites, but only during nestling care (van de Crommenacker *et al.*, 2012). Similarly, experimentally enlarged broods led to both reduced antioxidant protection and increased malarial parasitaemia in male Great tits *Parus major* (Christe *et al.*, 2012).

Experimental immune challenges during already-demanding periods of nestling care may shed further light on the impacts of both on oxidative balance, and the strategies used to minimise oxidative stress. If investment in reproduction exacerbates the oxidative burden of pathogen infection (and vice versa), selection

may favour strategic reduction of one trait, in order to protect investment in the other and maintain homeostasis. For example, experimentally activated immune responses may be suppressed during nestling care (Nordling *et al.*, 1998; Moreno *et al.*, 1999), in order to maintain investment in breeding and avoid oxidative stress. Alternatively, other species may sacrifice rates of offspring care in order to fight infection (Forbes, 1993; Ilmonen *et al.*, 2000) whilst avoiding oxidative stress, thus safeguarding future survival and fecundity. Such evidence will be critical in advancing our understanding of the true role played by oxidative stress in shaping life-histories in the wild.

6.5   CONSISTENCY AND THE VALUE OF CIRCULATING MARKERS OF OXIDATIVE BALANCE

Given my focus on repeated within-individual sampling over time, the studies in this thesis shed new light on the consistency of circulating markers of oxidative balance over time. Although the precise repeatability of measures of oxidative balance is likely to depend on the markers, species and contexts involved, it is nonetheless informative to investigate the degree to which these measures fluctuate over time. Specifically, high inconsistency in circulating markers of oxidative balance could undermine the importance of these currencies in mediating the long-term costs of investment in, for example, reproduction and defence against infection. Understanding within-individual variation in these markers over extended periods of time is therefore critical in assessing the extent of their role in shaping life-histories.

Chapter 5 revealed that SOD activity and levels of uric acid can be consistent over a 24 hour period under some circumstances, while concentrations of peroxidised

lipids (MDA) and non-enzymatic antioxidant protection (residual TAC) were not. Chapter 3 found weaker trends in consistency over a period of one month: only SOD and residual TAC were marginally consistent over this timescale. Chapter 4 found no evidence of consistency in any of the focal markers over a period of six months. While a number of studies have reported similar consistencies in measures of oxidative balance over the course of weeks (even over a year: Saino *et al.*, 2011), others have found no evidence of consistency after less than a week (Costantini *et al.*, 2007; Galván and Alonso-Alvarez, 2009; Norte *et al.*, 2009; Sepp *et al.*, 2010; Losdat *et al.*, 2011; Beamonte-Barrientos and Verhulst, 2013). It is increasingly believed that circulating markers may reflect only recent oxidative stress, because of the relatively high turn-over of plasma and serum and the short half lives of many markers of oxidative balance (Halliwell and Chirico, 1993; Nussey *et al.*, 2009).

Body mass can show similar fluctuations, with the losses experienced during investment in key life-history traits often being recovered shortly afterwards (Nilsson, 2002; Verhulst *et al.*, 2005). This has led to questions over the role of macro-nutrient deficits in mediating survival costs of investment in reproduction and immunity (Verhulst *et al.*, 2005), with some authors even highlighting that body mass losses may be adaptive (Witter and Cuthill, 1993). Given the variable stability of circulating markers of oxidative stress, the mechanism by which these markers may signal potential long-lasting impairments must also be questioned. However, unlike deficits in body mass, even fleeting increases in oxidative damage may cause irreversible DNA damage, potentially leading to the generation of tumours, senescence and reduced survival (Beckman and Ames, 1998; Chandra *et al.*, 2000; von Zglinicki, 2002; Kryston *et al.*, 2011). Although transient, elevated

circulating markers of oxidative damage may therefore indicate permanent damage occurring to other cellular components and/or in other tissues.

Such transience in circulating markers of oxidative state may diminish the need for longitudinal studies controlling for baseline levels of oxidative balance (with the exception of studies of ageing - Selman *et al.*, 2012). It has been suggested that oxidative damage to tissues may reveal individuals suffering from more chronic oxidative stress (Nussey *et al.*, 2009). However, empirical evidence of this remains scarce. Although evidence is mounting that tissue and blood oxidative balances may differ (Sohal *et al.*, 1995; Veskoukis *et al.*, 2009; Garratt *et al.*, 2011; Garratt *et al.*, 2012; Garratt *et al.*, 2013), the implications of these differences are not well understood. Moreover, the use of plasma and serum samples in ecological studies is likely to continue, because of the logistical and ethical considerations of collecting tissue samples from wild animals. As such, future studies should seek to clarify what circulating markers can tell us about oxidative balance in a range of tissues, and under what circumstances tissue samples could provide important variation that would not be evident in circulating markers alone.

6.6 CONCLUDING REMARKS

Oxidative stress represents an exciting new candidate for the physiological mediation of a number of life-history trade-offs. In this thesis, I have investigated the role played by oxidative balance in mediating the costs of reproduction and immune defence. I have also examined the extent to which dominance rank and group size modulate oxidative stress in cooperative animal societies. My findings offer valuable new insights into the oxidative stress costs of reproduction and immune defence in wild populations, and the oxidative benefits of cooperative

breeding, which may have evolved in part to mitigate these costs. Furthermore, my results highlight potentially widespread differences between the roles of oxidative stress in wild and captive contexts. While costs of investment in life-history traits may be evident in captive studies, such effects may be either relaxed (e.g. due to compensatory mechanisms) or more intense (e.g. due to harsher environmental conditions) in the wild. Progress in clarifying the extent to which oxidative stress shapes life histories will rely on the widespread use of experimental approaches in wild contexts, employing a suite of physiological markers. Studies investigating concurrent investment in multiple life-history traits are likely to provide novel insights into the way oxidative stress mediates life-history trade-offs in the wild.

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