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The role of oxidative stress as an underlying mechanism in life-history trade-offs

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Abstract

A key aspect underpinning life-history theory is the existence of trade-offs. Trade-offs occur because resources are limited, meaning that individuals cannot invest in all traits simultaneously, leading to costs for traits such as growth and reproduction. Such costs may be the reason for the sub-maximal growth rates that are often observed in nature, though the fitness consequences of these costs would depend on the effects on lifetime reproductive success. Recently, much attention has been given to the physiological mechanism that might underlie these life-history trade-offs, with oxidative stress (OS) playing a key role. OS is characterised by a build-up of oxidative damage to tissues (e.g. protein, lipids and DNA) from attack by reactive species (RS). RS, the majority of which are by-products of metabolism, are usually neutralised by antioxidants, however OS occurs when there is an imbalance between the two. There are two main theories linking OS with growth and reproduction. The first is that traits like growth and reproduction, being metabolically demanding, lead to an increase in RS production. The second involves the diversion of resources away from self-maintenance processes (e.g. the redox system) when individuals are faced with enhanced growth or reproductive expenditure.

Previous research investigating trade-offs involving growth or reproduction and self-maintenance has been equivocal. One reason for this could be that associations among redox biomarkers can vary greatly so that the biomarker selected for analysis can influence the conclusion reached about an individual's oxidative status. Therefore the first aim of my thesis was to explore the strength and pattern of integration of five biomarkers of OS (three antioxidants, one damage and one general oxidation measure) in wild blue tit (*Cyanistes caeruleus*) adults and nestlings (Chapter 2). In doing so, I established that all five biomarkers should be included in future analyses, thus using this collection of biomarkers I explored my next aims; whether enhanced growth (Chapters 3 and 4) or reproductive effort (Chapter 5) can lead to increased OS levels, if these traits are traded off against self-maintenance. I accomplished these aims using both a meta-analytic and experimental approach, the latter involving manipulation of brood size in wild blue tits in order to experimentally alter growth rate of nestlings and provisioning rate (a proxy for reproductive expenditure) of adults. I also investigated the potential for redox integration to be used as an index of body condition (Chapter 2), allowing predictions about future fitness consequences of changes to oxidative state to be made.

A growth – self-maintenance trade off was supported by my meta-analytic results (Chapter 4) which found OS to be a constraint on growth. However, when faced with experimentally enhanced growth, animals were typically not able to adjust this trade-off so that oxidative damage resulted. This might support the idea that energetically expensive

growth causes resources to be diverted away from the redox system; however, antioxidants did not show an overall reduction in response to growth in the meta-analysis suggesting that oxidative costs of growth may result from increased RS production due to the greater metabolism needed for enhanced growth. My experimental data (Chapter 3) showed a similar pattern, with raised protein damage levels (protein carbonyls; PCs) in the fastest growing blue tit chicks in a brood, compared with their slower growing sibs. These within-brood differences in OS levels likely resulted from within-brood hierarchies and might have masked any between-brood differences, which were not observed here.

Despite evidence for a growth – self-maintenance trade off, my experimental results on blue tits found no support for the hypothesis that self-maintenance is also traded off against reproduction, another energetically demanding trait. There was no link between experimentally altered reproductive expenditure and OS, nor was there a direct correlation between reproductive effort and OS (Chapter 5). However, there are various factors that likely influence whether oxidative costs are observed, including environmental conditions and whether such costs are transient. This emphasises the need for longitudinal studies following the same individuals over multiple years and across a wide range of habitats that differ in quality. This would allow investigation into how key life events interact; it might be that raised OS levels from rapid early growth have the potential to constrain reproduction or that high parental OS levels constrain offspring growth. Any oxidative costs resulting from these life-history trade-offs have the potential to impact on future fitness. Redox integration of certain biomarkers might prove to be a useful tool in making predictions about fitness, as I found in Chapter 2, as well as establishing how the redox system responds, as a whole, to changes to growth and reproduction. Finally, if the tissues measured can tolerate a given level of OS, then the level of oxidative damage might be irrelevant and not impact on future fitness at all.

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Author's Declaration

I declare that, except where explicit reference is made in the Acknowledgements to the contribution of others, this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or at any other institution.

Shona M. Smith

October 2016

Abbreviations

CAT	Catalase
D_M	Mahalanobis distance
FRAP	Ferric Reducing Ability of Plasma (measure of TAC)
GC-MS	Gas Chromatography-Mass Spectrometry
GLM	General Linear Model
GPX	Glutathione Peroxidase
HPLC	High Performance Liquid Chromatography
KRL	Kit Radicaux Libres test (measure of TAC)
LC-MS	Liquid Chromatography-Mass Spectrometry
LRT	Likelihood Ratio Test
MDA	Malondialdehyde
ORAC	Oxygen Radical Absorbance Capacity (measure of TAC)
OS	Oxidative Stress
OXY	OXY- Adsorbent Assay (measure of TAC)
PCA	Principal Components Analysis
PC1	First Principal Component
PC2	Second Principal Component
PCs	Protein Carbonyls
RBCs	Red Blood Cells
ROMs	Reactive Oxygen Metabolites
RS	Reactive Species
SOD	Superoxide Dismutase
TAC	Total (Non-Enzymatic) Antioxidant Capacity
TBARS	Thiobarbituric Acid Reactive Substances
TEAC	Trolox Equivalent Antioxidant Capacity (measure of TAC)
UPGMA	Unweighted Pair-Group Method with Arithmetic Average (cluster analysis)

Chapter 1

General Introduction

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Abstract

Trade-offs play an essential role in the evolution of life-history strategies. Such trade-offs are often a result of limited resources being allocated to one trait at the expense of another, but might also arise if the performance of one trait has direct negative consequences for another. Resultant costs emphasise the important role trade-offs play in life-history theory. Long-term costs, for instance effects on future reproduction or survival, allow the fitness consequences of these trade-offs to be examined and there is growing interest into the underlying physiological mechanism of this. One such mechanism, OS, is receiving increasing attention as a key player in this area. Therefore, this general introduction to my thesis will begin by discussing life-history trade-offs and the costs of growth and reproduction that support the idea of such trade-offs, with reference to the potential role of OS as an underlying mechanism, before going on to provide an overview of redox homeostasis. In this overview, details will be given on what leads to OS, what physiological defences the body has against it and how it can be measured in the laboratory. Next my thesis aims will be given, followed by a summary of my study species, the blue tit, explaining why it is a suitable species for this kind of research, before ending with an outline of my thesis structure.

1.1 Life-history trade-offs and the costs of growth and reproduction

Evolutionary theory predicts that organisms should strive to maximise fitness. In order to do so, individuals must alter life-history components so that they can achieve an optimal life-history strategy and contribute genetic material to future generations (Fisher 1930). An underlying concept of this idea is that animals are unable to maximise all life-history components at once because resources tend to be limited, thus resulting in trade-offs between them. Such trade-offs might be based on resource allocation (e.g. of time, energy, nutrients, etc.) and form the basis of the disposable soma theory of ageing. This theory stipulates that limited resources allocated to one trait (e.g. growth or current reproduction) become unavailable for another (e.g. somatic maintenance). Reduced investment leads to deterioration of somatic maintenance, and reduced survival and longevity (Kirkwood & Holliday, 1979). Thus trade-offs can transpire when the performance of one trait negatively impacts another; for instance whereby rapid growth or enhanced reproductive effort lead to a reduction in lifespan (Zera & Harshman 2001). The existence of life-history trade-offs is supported by the fact that a multitude of costs can be observed in relation to certain traits.

Costs of accelerated growth may be the reasoning behind the sub-maximal growth rates that are observed across numerous species (Blanckenhorn 2000), even though attaining a large size quickly is beneficial in terms of improving lifetime reproductive success. Larger individuals experience a lower risk of predation (Arendt 1997), earlier time to sexual maturity and so reproduction, better competitive ability and so improved access to resources and mates, and potentially higher fecundity (Metcalfe & Monaghan 2003; Dmitriew 2011). Yet various costs of enhanced growth are apparent. Animals that grow quickly might be more vulnerable to starvation, especially if resource availability declines, because enhanced growth is an energetically expensive trait that renders resources unavailable for other important functions, for instance physiological development (Blanckenhorn 2000). At the same time, large individuals can store disproportionately greater energy reserves than small individuals which might decrease starvation risk (Ludsin & DeVries 1997). Another cost of growth is that structures that are grown quickly may lack quality and this could have detrimental consequences for fitness (Metcalfe & Monaghan 2003); for example, skeletal deformities from fast growth, as has been demonstrated in greater sandhill cranes (*Grus canadensis tabida*) (Serafin 1982), might limit escape from a predator. Increased predator exposure may also result from a greater time spent foraging to meet the associated increased energy demands with enhanced growth (Metcalfe & Monaghan 2003; Dmitriew 2011). Finally, this increased foraging effort could increase the risk of injury from competition, if resource availability is low (Dmitriew 2011).

Costs are not only associated with growth, but have been observed with reproduction too. In fact, if there were no costs to reproduction, there would be a “Darwinian demon” that matures instantly and produces an infinite number of offspring (Reznick, Nunney & Tessier 2000). Like growth, reproduction is a metabolically demanding process requiring additional energy and nutrients. Thus a greater foraging effort during this time might expose individuals to an increased predation risk (Zera & Harshman 2001). In fact this increased energetic demand is a cost in itself with breeding animals greatly modifying their internal anatomy, for example, increasing the size of the alimentary tract as well as making modifications at the structural and cellular level, to cope with the increased food intake during reproduction (Speakman 2008). However, it has been suggested that some animals might not be able to increase food intake sufficiently to keep up with an increased reproductive demand, as is seen in lactating mice that are physiologically limited by heat dissipation (Król & Speakman 2003). In this case, larger litter sizes would result in less milk per pup, lower weaning size and potentially lower offspring survival (Speakman 2008). Costs to current reproduction have also been implicated by a meta-analysis that demonstrated no link between female reproductive effort and survival – the authors

speculated that females may be transferring the costs to offspring, in terms of reduced weight or recruitment (Santos & Nakagawa 2012). In support of this, though parental provisioning was increased in enlarged great tit (*Parus major*) broods, offspring mass and survival were reduced (Wegmann, Voegeli & Richner 2015a). Finally, an increased reproductive effort may negatively impact on immune defence (Knowles, Nakagawa & Sheldon 2009) and parasitic resistance (Knowles *et al.* 2009; Christe *et al.* 2012).

If lifetime reproductive success and survival remain unaffected, there may be no fitness consequences of accelerated growth and increased reproductive effort (Metcalfe and Monaghan 2003), despite these apparent costs. On the contrary, both enhanced growth and reproductive effort have been found to negatively impact on future reproduction (Hanssen *et al.* 2005; Lee, Monaghan & Metcalfe 2012), survival (Nur 1984a; Santos & Nakagawa 2012) and lifespan (Jennings *et al.* 1999; Lee, Monaghan & Metcalfe 2013a). Yet it may be difficult to tease apart long-term consequences of growth and those of reproduction, since in species with flexible ages at maturity faster growing animals would be expected to reach reproductive age sooner. Thus any negative effects from fast growth might actually result from a higher number of reproductive events in individuals that started reproducing earlier compared with those that grew more slowly (Metcalfe & Monaghan 2003). Furthermore, long term negative effects of enhanced growth and reproductive effort are not always observed (Carlson, Hendry & Letcher 2004; Parejo & Danchin 2006; Dmitriew & Rowe 2007). Of course any effects may only become evident under demanding conditions, for instance, during a poor quality year (Sinervo & DeNardo 1996) or a particularly demanding phase of reproduction, for example, provisioning of chicks in birds or lactation in mammals (Speakman 2008). There might also be sex differences; a meta-analysis found increased parental effort to reduce survival of male, but not female, birds (Santos & Nakagawa 2012). The authors speculated that females may already be working close to their maximum provisioning level with not much capacity to increase this; whilst males are more flexible and increase effort if they perceive enhanced reproductive success, even if this is at the expense of survival (Santos & Nakagawa 2012).

Despite consequences of accelerated growth and enhanced reproductive effort possibly only occurring under specific conditions, it is evident that there are costs associated with these life-history traits, supporting the role of trade-offs in shaping life-histories. At the same time, it might be interesting to explore the physiological mechanism that could underlie such trade-offs. OS, which can be defined as the rate of increase of oxidative damage to biomolecules, has been implicated in playing such a role here (von Schantz *et al.* 1999; Monaghan, Metcalfe & Torres 2009; Dowling & Simmons 2009), either indirectly

through a reduction in antioxidant resources which are traded-off against growth or reproduction (Alonso-Álvarez *et al.* 2004; Monaghan *et al.* 2009), or via increased oxidative damage as a direct consequence of the increased metabolic rate needed for enhanced growth or reproduction (Nilsson 2002; Mangel & Munch 2005).

In order to understand how increased metabolic rate might lead to greater oxidative damage, it is important to know firstly how RS, which attack biomolecules causing oxidative damage, are generated. In the final phase of cellular respiration, electrons are passed down a chain of progressively more electronegative acceptors, with oxygen being the final electron acceptor. This provides energy for protons to be pumped across the membrane creating an electrochemical proton gradient that causes protons to flow back across the membrane – this releases energy to create ATP. Some electrons do not pass along all acceptors and leak directly to oxygen, donating an electron and causing the superoxide radical to be formed (Speakman & Selman, 2011). If not neutralised, further reactions take place with superoxide, and additional RS are generated. However, currently there is debate about whether increased metabolic rate actually raises RS production. Anything that slows the flow of electrons leads to a build-up of reduced electron acceptors, which are more likely to donate an electron to oxygen (Barja, 2007). Therefore an increase in oxygen consumption from greater metabolic rate actually reduces RS production per unit of oxygen consumed, because it eliminates oxygen from the surrounding tissue and speeds up the electron flow (Barja, 2007). Lowering the membrane potential will also increase the flow of electrons; this occurs if ADP is non-limiting or by mitochondrial uncoupling (Speakman *et al.* 2004).

Mitochondrial uncoupling proteins carry protons back across the mitochondrial membrane, avoiding the pathway that generates ATP and thus lowering the membrane potential (Brand 2000; Stier *et al*. 2014a). Studies have demonstrated that greater mitochondrial uncoupling lowers RS production per unit of oxygen consumed (Salin *et al.* 2012) and thus leads to lower oxidative damage (Salin *et al.* 2012; Stier *et al*. 2014a). This is supported by a study of brown trout that found a negative relationship between mass-independent standard metabolic rate and RS levels (Salin *et al*. 2015); though the authors pointed out that increased scavenging from antioxidants could also lower RS. Although previous work implies greater mitochondrial uncoupling with increased energy expenditure (Speakman *et al*. 2004), evidence suggests uncoupling is reduced during energetically demanding reproductive phases, like lactation (Speakman 2008). As uncoupling reduces mitochondrial efficiency (Salin *et al.* 2012), it seems logical that this would be reduced during such an intensely energy demanding time (Speakman 2008). This would result in higher RS production for a given level of metabolic rate (Speakman 2008) and may

explain why research of breeding individuals has found a link between daily energy expenditure and oxidative damage levels (Fletcher *et al*. 2013), while that of non-breeding individuals has not (Selman *et al.* 2008). The considerable yet transient increase in energy expenditure that occurs with reproduction, and also during growth, might overwhelm antioxidant defences (Selman *et al.* 2008), exasperating effects of RS.

Regardless of the mechanism, it is unclear whether consequences from increased oxidative damage from greater growth rate or reproductive effort would extend to the long term. Raised damage levels might be transient, or animals might be able to mitigate any effects (Speakman *et al.* 2015). The implication of mitigation of oxidative damage can be observed in naked mole rats (*Heterocephalus glaber*) that produce RS at a similar rate to mice, but have unremarkable antioxidant levels and relatively high oxidative damage levels (Edrey *et al*. 2011). However, they are extremely long-lived when compared with similar sized mice, and one explanation might be their ability to mitigate oxidative damage (Edrey *et al*. 2011). It has been suggested that certain evolutionary lineages that have been selected for long lives through ecological factors (e.g. low age-independent mortality), might also have evolved these mechanisms alongside increased longevity (Selman *et al.* 2012). Furthermore, because most species lack such mechanisms, the consequences of OS may not be of great importance to overall fitness (Selman *et al.* 2012).

In contrast to this, there are studies that have linked high levels of oxidative damage with reduced survival or longevity (e.g. Reichert *et al*. 2014; Vitikainen *et al*. 2016; Zhang *et al*. 2016). Additionally, a review of rodent studies revealed that the mechanism behind the extended lifespan that is observed with dietary restriction (DR) might well involve OS (Walsh, Shi & Van Remmen, 2014). The authors suggest that the observed reduction in oxidative damage with DR could be a result of changes to protein turnover, as well as removal and repair of damaged molecules, rather than changes to antioxidant enzyme activity or RS production. This emphasises the complexity of the mechanism by which OS could affect fitness. The increased levels of the endogenous antioxidant glutathione (GSH) with DR that was observed in Walsh *et al.*'s study could imply that it is a shift in the oxidative balance causing altered gene transcription and protein function, that inevitably leads to ageing (Walsh *et al*. 2014). Therefore, it is still relevant to investigate how OS might be involved in life-history trade-offs.

1.2 Redox homeostasis

The strict balance in the cells and tissues of organisms between RS and antioxidants is what encompasses redox homeostasis – if this is upset and antioxidants can no longer overcome the RS that is produced, oxidative damage can occur. This may result from an increase in RS that is not matched by an increase in antioxidants; or a reduction in antioxidant defences (Sies 1997). OS is never likely to be absolutely zero since RS are being continually produced (Costantini & Verhulst 2009; Pamplona & Costantini 2011). These RS can consist of free radicals – atoms/compounds with one or more unpaired electron (e.g. superoxide, hydroxyl, nitric oxide), which exist for just nanoseconds – or non-radical oxidants (e.g. hydrogen peroxide, hypochlorous acid, singlet oxygen) that can persist for longer thus can travel to other parts of the body and propagate oxidation (Yu 1994; Pamplona & Costantini 2011).

The majority of RS are endogenously produced by-products of metabolism (Yu 1994; Dröge 2002). Chronic stress can also result in greater OS, although the extent to which will depend on duration of exposure, tissue, sex and age (Costantini, Fanfani & Dell'omo 2008; Costantini, Marasco & Møller 2011a). Not all endogenous RS have negative effects however, about 10% are involved in important cell functions – for instance, cell signalling, transformation and immune defence (Yu 1994; Dröge 2002). The other 90% can go on to cause damage (Monaghan *et al.* 2009). It is important to note that exogenous sources of RS also exist (e.g. UV, pollutants, ozone) (Monaghan *et al.* 2009).

Unstable RS accept electrons from other atoms, which become secondary free radicals. These go on to accept electrons from other molecules, which become tertiary free radicals and so on – an oxidative cascade that results in damage to biomolecules (Yu 1994; Sies 1997; Dröge 2002). This can lead to damage of DNA (base modifications, telomere attack), proteins (formation changes that impair function) and lipids (alteration to membrane structure and function) (Yu 1994; Sies 1997). Molecules may differ in their susceptibility to RS attack. In lipid membranes, polyunsaturated fatty acids (PUFA) are most susceptible to attack (Hulbert *et al.* 2007); in DNA guanine is the most susceptible base (Mateos & Bravo 2007); while in proteins, methionine is particularly susceptible (Stadtman, Moskovitz & Levine 2003). It is essential that the nature of damage is specified in studies since increased damage to one area will not necessarily mean there is increased damage to another (Costantini 2010a).

The first line of defence against OS involves minimising the initial release of RS. This takes place at the main site of RS production: the mitochondrion. The mitochondrial

membrane composition and density will affect RS production, as will uncoupling of oxygen consumption and ATP production which, as described earlier, can result in greater energy production with fewer RS (Brand 2000; Hulbert *et al.* 2007). However, as discussed, the role of uncoupling in protecting tissues from RS is still debated and evidence from oysters (Buttemer, Abele & Costantini 2010) and birds (Costantini 2010a) is not strong. If RS levels do increase, then antioxidants can be up-regulated. These can be defined as "any mechanism, structure and/or substance that prevents, delays, removes or protects against oxidative non-enzymatic chemical modification (damage) to a target molecule" (Pamplona & Costantini 2011).

Antioxidants can be grouped into enzymatic molecules, that are endogenously produced, and non-enzymatic antioxidants, which can be endogenous or exogenous. The main enzymes involved in RS neutralisation are superoxide dismutase (SOD) that catalyses the breakdown of the superoxide anion to hydrogen peroxide; and catalase (CAT) and glutathione peroxidase (GPX) that convert the resulting hydrogen peroxide to water (Yu 1994). CAT and GPX complement each other: while CAT works quickly but with low affinity for hydrogen peroxide (useful during peaks of production), GPX work slowly but with high affinity (useful for removing continuously produced hydrogen peroxide) (Pamplona & Costantini 2011). GPX also plays an important role in catalysing the breakdown of hydroperoxides, forming glutathione disulphide, water and alcohol (Yu 1994; Dickinson & Forman 2002).

Non-enzymatic antioxidants tend to be of low molecular weight so can access additional parts of the body (Pamplona & Costantini 2011). An example of an endogenous non-enzymatic antioxidant are thiols, which are protein or non-protein molecules that contain a cysteine residue; glutathione and thioredoxin are the most commonly synthesised thiols in animals (Dickinson & Forman 2002). Though their pathways are linked, cell concentrations of glutathione are far greater than those of thioredoxin and they operate independently (Bindoli, Fukuto & Forman 2008). Glutathione is a cofactor of GPX described above, while thioredoxin neutralise hydrogen peroxide via peroxiredoxin enzymes (Bindoli *et al.* 2008). Another endogenous non-enzymatic antioxidant is ascorbic acid (vitamin C). Although it is synthesised endogenously in most vertebrates, primates, guinea pigs, fruit bats and some birds must obtain vitamin C from their diet (Pamplona & Costantini 2011).

The two main groups of exogenous (i.e. dietary) non-enzymatic antioxidants are tocopherols (vitamin E) and carotenoids (e.g. α/β carotene, lutein, lycopene, zeaxanthine and cryptoxanthine; (Surai & Speake 1998)). The former inhibit the lipid peroxidation

chain reaction by reducing lipid peroxyls to hydroperoxides (Yu 1994; Pamplona & Costantini 2011), while carotenoids protect lipids from peroxidation as well (Yu 1994). The two antioxidants are integrated, with vitamin E being recycled by carotenoids (Costantini 2008), so that these antioxidants might have synergistic effects (Orledge *et al.* 2012). In fact, there are potentially complex interactions between various antioxidants (Yu 1994; Costantini 2008, 2010a; Costantini *et al.* 2011a; Costantini, Monaghan & Metcalfe 2011b; Orledge *et al.* 2012), for instance thiols and GPX have been found to be highly integrated in the blood (Costantini *et al.* 2011b). These interactions might be complicated by the fact that certain antioxidants have other important functions, for example, carotenoids are also involved in enhancing immune function and sexual signals (Perez-Rodriguez *et al.* 2008; del Cerro *et al.* 2010).

If the antioxidant machinery fails to prevent OS, damaged molecules must be removed or repaired (Sies 1997; Monaghan *et al.* 2009), for instance, by DNA repair enzymes or phospholipases (membrane repair) (Yu 1994). Modifications to amino acids are quite often irreversible, thus the resulting protein must be catabolised; although a few can be repaired by methionine sulfoxide reductase (Msr) (Stadtman *et al.* 2003). Additionally, healthy cells must rid themselves of damaged proteins to prevent aggregation (Pamplona & Costantini 2011). In cell membranes, peroxidised lipids are removed and resulting reactive carbonyl species are scavenged (Pamplona & Costantini 2011).

The vast array of antioxidant molecules that might respond to greater RS production and the complex interactions that can occur between specific antioxidants, as well as the large number of damage compounds that can be produced if RS is not neutralised, make it difficult when selecting the best method for analysing OS (Halliwell & Gutteridge 2007). In addition it can be difficult to interpret antioxidant levels: a high antioxidant capacity could indicate either a response to increased RS concentration, or alternatively naturally higher antioxidant levels in certain individuals, despite no change to RS production (i.e. the animal is not troubled by OS) (Costantini & Verhulst 2009). While the idea of symmorphosis suggests that antioxidants should be produced in line with their functional need (Weibel *et al*. 1991) – i.e. on demand in response to raised RS production, rather than in excess – variation in baseline antioxidant levels may still occur, for instance, as a result of differential antioxidant gene expression or dietary differences between individuals (Costantini & Verhulst 2009). Either way, it does not necessarily mean damage is occurring, thus it is important to measure more than one type of marker of OS. Potentially RS might be measured, but due to the high instability of these compounds it likely proves more realistic to measure both the level of antioxidant protection and the damage that occurs (Monaghan *et al.* 2009).

There are numerous methods for measuring oxidative damage. Firstly malondialdehyde (MDA), an end product of lipid peroxidation, can be measured using the thiobarbituric acid reactive substances (TBARS) assay (Hulbert *et al.* 2007) or high performance liquid chromatography (HPLC) (Pike *et al.* 2007). However, absorption of MDA via the GI tract may reduce reliability of these tests (Mateos & Bravo 2007) and thiobarbituric acid reacts with substances other than MDA potentially making TBARS unreliable (Mateos & Bravo 2007). Alternatively, isoprostanes (more stable lipid peroxidation products) can be measured using gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS) (Mateos & Bravo 2007). GC-MS and LC-MS can also be used to measure oxidised derivatives of guanine in DNA, as can HPLC and electrophoresis (Mateos & Bravo 2007). Carbonyl groups (C=O) that are indicative of protein damage (which is often irreversible) can be measured using ELISA assays, electrophoresis and western blotting (Mateos & Bravo 2007) or via derivatisation of carbonyl groups to 2,4-dinitrophenylhydrazone (Levine *et al.* 1990). More general markers of oxidation, reactive oxygen metabolites (ROMs, intermediate compounds primarily including hydroperoxides), come from lipid, protein and nucleic acid oxidation that can be measured using the d-ROMs test (Costantini *et al.* 2006; Costantini & Dell'Omo 2006a; b).

Recently there has been some debate around the dROMs assay and it's capacity for measuring hydroperoxides, since certain components (e.g. ceruloplasmin-CP) might interfere with the values of the assay (Kilk *et al.* 2014). However, previous research has found no correlation between CP and d-ROMs readings (Colombini *et al.* 2016; Costantini 2016a). Additionally, adding CP to human serum samples did not cause a significant rise in d-ROMs values (Colombini *et al.* 2016); likewise testing with a CP inhibitor did not lead to a significant reduction in absorbance (Costantini 2016a) – this suggests CP does not interfere with the d-ROMs test to a huge extent. Given that the assay has been previously demonstrated to detect hydroperoxides for a large range of values (Costantini 2016a), and that it has been linked to a number of traits, including growth and reproduction (Table 1:1), it is likely that it is a relevant measure of OS. This is emphasised by the fact that it has repeatedly been shown to correlate with other OS measures, for example, GPX and thiols (Costantini *et al*. 2011b), as well as total antioxidant capacity (van de Crommenacker *et al.* 2011a).

While it might prove interesting to measure the activity of repair enzymes or overall repair capacity, this is rarely attempted (Monaghan *et al*. 2009). Instead the antioxidant system itself can be measured, however, it is difficult to interpret levels of individual antioxidants due to the complex interplay between various antioxidants. Instead, total antioxidant

capacity (TAC) can be measured which assesses how all the antioxidants in a given matrix (e.g. plasma) might respond to a given oxidative challenge (Cohen, Klasing & Ricklefs 2007; Costantini 2010b). One issue with measuring TAC is that a large proportion of the measured capacity may be due to uric acid so this must be corrected for in analyses, however the correlation between TAC and uric acid depends on the method used (Costantini 2010b). Another point to bear in mind is that uric acid may increase in blood as an active response to OS, or as a passive response to protein catabolism (Costantini 2010b).

There are numerous assays for measuring TAC; generally they measure the ability of a blood sample to quench a free radical (Costantini *et al.* 2006; Costantini & Dell'Omo 2006a; b). Examples include oxygen radical absorbance capacity (ORAC), trolox equivalent antioxidant capacity (TEAC), ferric reducing ability of plasma (FRAP) and the OXY-Adsorbent test (Costantini 2010b). The FRAP test measures uric acid, while the outcome of the OXY test is not influenced by levels of uric acid so this would not need to be corrected for (Costantini 2010b). OXY uses an endogenously produced pro-oxidant (HOCl) but does not always correlate with specific antioxidants, while FRAP uses an indirect measure (Fe) and is repeatable (Costantini 2010b). Most likely, the best method is to combine more than one assay to attain a 'more rounded picture' (Costantini 2010b).

The Kit Radicaux Libres (KRL) test is another method for assessing antioxidant capacity. This measures the time to haemolyse 50% of red blood cells (RBCs) – more time indicates greater antioxidant status, which is sometimes equated to OS, although oxidative damage must also be measured here to confirm this (e.g. Alonso-Álvarez *et al.* 2004; Losdat *et al.* 2010). One problem with this test and the other TAC assays is that each measures the response to different free radicals, which is likely to cause discrepancies among assays. Additionally, non-blood tissues utilise enzymatic antioxidants and it is unclear how these correlate with circulating antioxidants (Monaghan *et al.* 2009). Therefore other antioxidants should be measured in conjunction with TAC, including both enzymatic (e.g. GPX and SOD, which can be measured using the Ransel and Ransod assays, respectively (Abiaka *et al.* 2002)) and non-enzymatic (e.g. uric acid and thiols (Cohen *et al.* 2007; Costantini *et al.* 2011b)) molecules. A summary of biomarkers is given in Table 1:1, with reference to their relevance in biology and ecology.

1.3 Thesis aims

Life-history trade-offs are clearly important in shaping organisms to produce optimal strategies that maximise fitness. As a consequence of such trade-offs, costs of both

growth and reproduction are apparent, although consequences for fitness (i.e. long-term costs) might depend on conditions like year, reproductive phase and sex. Regardless of these costs, research is becoming more focussed on the underlying physiological mechanism that may underlie such trade-offs – OS has been implicated to play a role here. As discussed, selecting biomarkers for OS measurement can prove tricky, due to the huge range of damage compounds and antioxidants that exist as well as the interactions that occur between them. This thesis uses a suite of markers of OS to explore the potential mechanisms underlying trade-offs between growth or reproductive effort and self-maintenance. It uses both a meta-analytic and experimental approach, with the experiments involving manipulations of both growth rate and reproductive effort in wild blue tits, chosen because of their high rates of growth and reproductive effort combined with experimental tractability.

1.4 My study species

The blue tit is a small passerine that is widespread throughout Britain and easily distinguishable by its blue plumage (Perrins 1979). Blue tits tend to inhabit broad-leaved deciduous woodland, for example oak woodland, where they feed primarily from the leaves during the breeding season when caterpillars are most abundant (Perrins 1979). Caterpillar abundance increases to a peak before rapidly declining over the breeding season so may only be available for a very short period (as little as two weeks) making it crucial that birds can match the time at which their chicks are most demanding and so require most food (at approximately 11 days in blue tits; (Blondel *et al.* 1991)) to this short-lived caterpillar peak. Since it takes time for females to form, lay and hatch eggs (likely about 24 or more days), the birds must be preparing to breed about four weeks before caterpillars will become abundant (Perrins 1979). In order to shift their breeding season to match this peak, they must rely on certain environmental cues that fluctuate in parallel with the caterpillars. The most obvious factor that is thought to contribute to the timing of breeding is day length, although the great variation in day length from year to year suggests it is likely to be more complicated than this, involving a “complex series of different factors”, including temperature, habitat and whether the female can get enough food early on to initiate breeding (Perrins 1979). In any case this strong reliance upon the caterpillar peak, and the need to time reproduction to coincide with this, makes breeding relatively synchronous among breeding pairs, which is of advantage to the researcher who can obtain lots of data in a relatively short space of time.

At the beginning of the breeding season, which tends to start in March in Britain, males and females explore potential nest sites together. Typically they will choose a small hole

in a tree that is considerable distance above the ground, but are quite willing to use man-made nestboxes if available (Perrins 1979). It is this willingness to breed in nestboxes that makes the blue tit an ideal study species – in study sites where numerous nestboxes have been placed within oak deciduous woodland, occupancy can be as much as 75% by pairs of blue tits leading to potentially large samples. Furthermore, nestboxes can be easily accessed, taken down to ground level so that chicks can be measured and blood sampled, and adults can be easily trapped on the nest. Nests, which are composed of moss and lined with feathers or sometimes fur, are entirely built by the female (Perrins 1979). Once the nest is complete, egg-laying will commence. Since females tend to lay one egg a day first thing in the morning (Perrins 1979), it is easy to estimate a clutch completion and expected hatching date; another advantage for the researcher.

Clutch size in blue tits ranges from 7 to 13 eggs, although as large a range as 4 to 21 eggs has previously been reported in Wytham Wood in Oxfordshire in 1974 (Perrins 1979). This makes blue tit clutches the largest of all the tits (and likely of any altricial species) and might suggest significant costs to both chicks and adults, in terms of growth and reproduction. Moreover, with up to 70% of adults not surviving to breed the following year, blue tits are a short-lived species, tending to live on average only about one and a half to two and a half years (Perrins 1979). Short-lived species would be expected to sacrifice self-maintenance (e.g. OS defence and repair) for current reproduction (Parejo & Danchin 2006), making the blue tit a good candidate for investigating the oxidative costs of reproduction (Bergeron *et al.* 2011). Only the female incubates the eggs, having a brood patch, which becomes well developed and highly vascularised near the end of egg-laying and allows sex determination of adult birds in the hand (Perrins 1979). Incubation will not usually begin until the clutch is completed, however if it is particularly late in the season, incubation may start before clutch completion, which leads to asynchronous hatching of chicks (Perrins 1979).

Large clutch, and so brood, sizes of blue tits result in frequent feeding visits to the nest by both parents. Males will feed chicks, as well as his partner, early on while she is still brooding, with females tending to feed more later in the nestling period as the male “slows down” (Perrins 1979). Either parent will bring a single food item (usually a caterpillar) per visit so that each visit will result in the feeding of only one chick (Perrins 1979). Since brooding stops at approximately day 9, non-feeding visits are likely to be rare by this time (Nur 1984b), making it relatively easy to measure parental provisioning rate in the latter part of the nestling period by counting the number of nest visits. Fledging success tends to be high in blue tits and once the first nestling has fledged parents will encourage the others to do so also, however survival after fledging is low, with only about one fledgling

per nest living long enough to breed (Perrins 1979). One final but crucial point that makes this species advantageous to the researcher is its resilience to disturbance, once the sensitive incubation period has passed. Chicks can be removed from the nest and biometrics taken on a regular basis, as well as adults caught on the nest, with little risk of abandonment. Furthermore, the species has not evolved the ability to recognise its own young (Perrins 1979), meaning that brood manipulations can be carried out successfully.

1.5 Thesis structure

In Chapter 2 ("Integration of blood redox state in wild blue tits (*Cyanistes caeruleus*)") I explore and describe patterns of integration among five biomarkers of OS, including a damage marker, a general oxidation measure and three antioxidants, in chicks and breeding adult birds. Here, I determine the usefulness of redox integration as a tool for investigating OS, as well as a proxy for condition, potentially allowing predictions of short-term future fitness consequences to be made. To determine the role OS might play in life-history trade-offs, Chapter 3 ("Oxidative costs of growth only become apparent under stressful conditions") investigates whether OS is a cost of enhanced growth in blue tit nestlings subjected to experimental indirect manipulations of growth rate. Chapter 4 ("Meta-analysis indicates that oxidative stress is both a constraint on and a cost of growth") employs meta-analytic techniques to examine the OS-growth relationship further, determining if OS is a cost of, or alternatively a constraint on, growth. In Chapter 5 ("Experimental increase of reproductive expenditure does not affect blood oxidative status in wild blue tits (*Cyanistes caeruleus*)") I explore the hypothesis that enhanced reproductive effort in adult blue tits leads to OS. Finally, the findings of the thesis are brought together and evaluated in the last (Discussion) chapter, which focusses on the evidence for OS being an underlying physiological mechanism in life-history trade-offs.

Table 1:1 Summary of various damage (A), oxidation (A) and antioxidant (B) markers of oxidative stress. Examples are also given of various life-history traits that biomarkers might be involved in, though results tend to vary depending on, for example, tissue and sex. Note that this list of biomarkers is not exhaustive.

Biomarker	**Description**	**Biological Relevance**
A. DAMAGE AND OXIDATION MEASURES		
protein carbonyls (PCs)	Protein oxidation product	If not removed, damaged proteins can aggregate in the cell, causing damage to other proteins and parts of the cell (e.g. the cell membrane) (Pamplona & Costantini 2011). PCs have been demonstrated to be altered in response to certain traits, for example, reproduction (Blount *et al*. 2015) and migration (Jenni-Eiermann *et al*. 2014).
isoprostanes	Lipid peroxidation product	Generated from RS attack of PUFA acids in membrane phospholipids, and so isoprostanes can potentially affect cell membrane function. They have been previously used as lipid peroxidation biomarkers in human disease (Mateos & Bravo 2007).
malondialdehyde (MDA)	Lipid peroxidation product	Though the TBARS assay method may react with other compounds, HPLC could be a more reliable method of measuring MDA (Mateos & Bravo 2007). Previous work has found MDA levels to be related to some traits, for instance growth (Larcombe *et al*. 2010), reproductive effort (Bergeron *et al.* 2011) and fitness (Alonso-Álvarez *et al.* 2010).
oxidised derivatives of guanine	DNA base peroxidation product	Damage to DNA can lead to changes in gene expression, which can affect protein function (Mateos & Bravo 2007). DNA damage has been related to growth (Mustafa *et al*. 2011), reproduction (Olsson *et al.* 2012) and survival (Freeman-Gallant *et al.* 2011).
reactive oxygen metabolites (ROMs)	Intermediate product from lipid, protein and DNA peroxidation	If not removed, ROMs can attack biomolecules causing oxidative damage (Halliwell & Gutteridge 2007). ROMs have been related to a number of traits, including dominance (van de Crommenacker *et al.* 2011b), immunity (Costantini & Dell'Omo 2006b), diet (Costantini 2010c), growth (Gieger *et al.* 2011) and reproduction (Costantini *et al.* 2006).

B. ANTIOXIDANT MEASURES

total antioxidant capacity (TAC)	Ability of biological matrix (often blood) to quench a given free radical	Measures the non-enzymatic antioxidants, though results will depend on the assay. Various measures of TAC have been related to traits such as age (Alonso-Álvarez *et al.* 2010), growth (Geiger *et al.* 2011) and reproduction (van de Crommenacker *et al.* 2011b).
Kit Radicaux Libres (KRL) test	Measures the time taken to haemolyse 50% RBCs; more time equates to greater antioxidant status	Oxidative stress resistance measured by this assay has previously been demonstrated to be affected by variations in reproductive effort (Alonso-Álvarez *et al.* 2004; Losdat *et al*. 2010; Christe *et al.* 2012).
Thiols	Protein and non-protein antioxidant molecules containing an -SH group	Glutathione (GSH) and thioredoxin (Trx) are the two most commonly synthesised thiols in animals (Dickinson & Forman 2002). GSH is involved in the neutralisation of hydrogen peroxide and hydroperoxides via GPX (Yu, 1994). Trx neutralises hydrogen peroxide via peroxiredoxins enzymes (Bindoli *et al*. 2008). Studies have linked thiols with traits like growth (Leggatt *et al*. 2007) and reproduction (Garratt *et al.* 2011).
superoxide dismutase (SOD)	Antioxidant enzyme	Catalyses the breakdown of the superoxide radical to form hydrogen peroxide and singlet oxygen (Yu 1994).
glutathione peroxidase (GPX)	Antioxidant enzyme	Catalyses the breakdown of hydrogen peroxide, forming water; and of hydroperoxides, forming alcohol (Yu 1994).
catalase (CAT)	Antioxidant enzyme	Catalyses the breakdown of hydrogen peroxide, forming water (Yu 1994); works more quickly than GPX so useful during peaks of hydrogen peroxide production (Pamplona & Costantini 2011).
		Antioxidant enzymes can be altered in response to, for example, growth (DeBlock & Stoks 2008; Yengkokpam *et al.* 2013) and reproduction (Wiersma *et al.,* 2004; Yang *et al.* 2013).

Chapter 2

Integration of blood redox state in wild blue tits (*Cyanistes caeruleus*)

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Abstract

Redox homeostasis involves a complex interplay between potentially tissue-damaging RS and antioxidant defences. If the RS are not neutralised by the antioxidant defences then OS can result. By measuring redox biomarkers (i.e. antioxidants or markers of oxidative damage) we can obtain some insight into an individual's level of OS. However, associations among these biomarkers have been found to vary greatly, which can lead to different conclusions about redox status depending on what biomarker has been measured. One solution is to consider the redox system as a whole and explore the level of integration that occurs among biomarkers of OS – this has rarely been done. In this study, I investigated integration of five biomarkers of OS in blue tit adults and nestlings. Though OXY and ROMs were strongly and positively correlated in both adults and nestlings, the overall level of integration was not found to be high, suggesting that for this study population a more traditional univariate approach, investigating each biomarker individually, might be more appropriate. None of the five biomarkers should be excluded from future analyses as they appear to be providing independent information on the redox system. Different sets of biomarkers or those in different populations could be more strongly integrated and so better suited to integration analysis, allowing us to further disentangle the complicated interactions among redox biomarkers. Finally, my results suggested that integration of four out of the five redox biomarkers investigated may be useful as an index of body condition in nestling, but not adult, blue tits, potentially allowing short-term future fitness consequences to be predicted.

2.1 Introduction

Redox homeostasis involves a balance between RS and antioxidants. RS are highly unstable molecules, produced endogenously as by-products of metabolism and the immune system (Yu 1994; Dröge 2002), that can damage biomolecules (Yu 1994; Dröge 2002); the build-up of damage is termed OS. When RS levels increase within the body, then antioxidants that protect the body from the damage induced by RS can be mobilised or up-regulated to neutralise them. Antioxidants can be categorised into enzymatic and non-enzymatic (Yu 1994). Enzymatic antioxidants include SOD, CAT and GPX. Non-enzymatic antioxidants include endogenously produced (thiols – e.g. thioredoxin and glutathione, uric acid and ascorbic acid – i.e. vitamin C, though some species cannot synthesise this endogenously, e.g. apes and bats) and dietary (tocopherols – vitamin E, and carotenoids) antioxidants (Yu 1994). Finally, if the antioxidant machinery fails to prevent OS, damaged molecules can be removed or repaired by lipolytic and proteolytic enzymes (Yu 1994). In certain cases where oxidative damage accumulates leading to high levels within cells, the removal of whole cells prevents further spread of damage (Sies 1997).

In redox homeostasis, the level of integration among biomarkers of enzymatic and non-enzymatic defence against and damage from oxidation is indicative of how an individual is responding to an oxidative threat (Costantini, Monaghan & Metcalfe 2013). The strength of this integration depends on the degree of interdependency among biomarkers. For instance if antioxidants A and B are strongly integrated and A becomes impaired, this could reduce the efficacy of B and constrain the whole system (Costantini *et al.* 2011b). If failure of one antioxidant could compromise the whole system, then the balance that must be maintained between different components of the antioxidant machinery has the potential to constrain the overall response (Costantini 2010a). One 'fail-safe' mechanism to this that organisms can adopt is the involvement of unrelated components (Costantini 2010a), such as independent non-enzymatic and enzymatic modules, as is seen in sub-adult captive zebra finches (*Taeniopygia guttata*; (Costantini *et al.* 2011b)). Thus it might be important to consider the redox system, not as a single unit, but as a number of different components with covariation between them. This has rarely been done.

The idea that the redox system is integrated is not a new one (Yu 1994) and some of the known interactions are presented in Figure 2:1. For example, the synergistic interactions between vitamins C and E have been recognised for decades (Yu 1994) and it is now accepted that oxidised vitamin E is recycled back to its reduced form by carotenoids (Biard, Surai & Møller 2006; Orledge *et al.* 2012), which in turn are recycled by vitamin C

(Palozza 1998) (Figure 2:1). However, interactions among redox biomarkers are highly variable, for instance, baseline levels of vitamin E were not found to be correlated with carotenoid levels across species in a study of 99 bird species (Cohen & McGraw 2009). Variations in the strength and direction of correlations among other redox biomarkers have been found too, as demonstrated by a meta-analysis whereby differing associations were found among different OS assays (Dotan, Lichtenberg & Pinchuk 2004). Specific examples emphasise these variations; both positive and negative relationships have been demonstrated between GPX and thiols (positive: Costantini *et al*. 2011b; negative: Costantini *et al.* 2013) and between the non-enzymatic antioxidant capacity of the plasma and ROMs (positive: van de Crommenacker *et al*. 2011a; Costantini *et al*. 2011b; negative: Costantini, Carello & Fanfani 2010a). The variable nature of OS biomarker interactions among individuals and species makes it very difficult to generalise the redox system from one or two measures and reveals the need for more studies that investigate the interrelationships of various biomarkers to obtain a better understanding of OS.

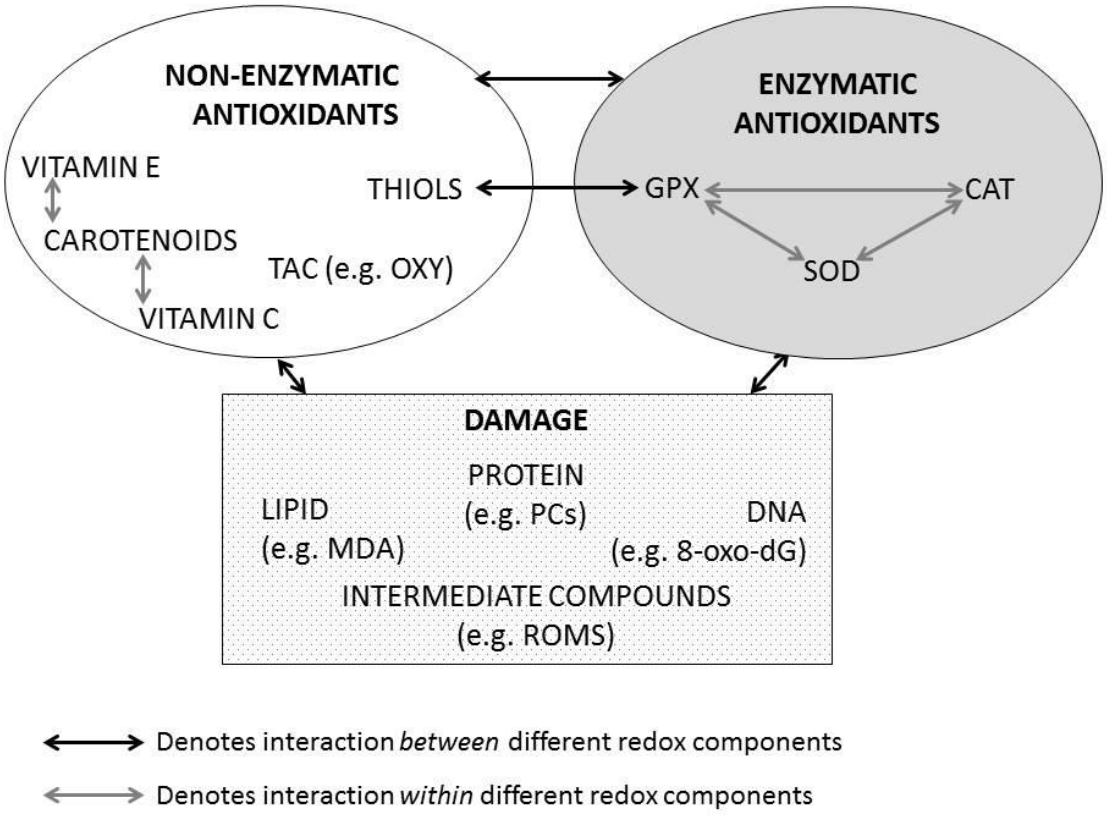

Figure 2:1 Known interactions among different components of the redox system. Only a few examples within each group have been given and just the main interactions that are discussed in the text are shown for simplicity. Interactions among damage biomarkers are not shown.

The complexity of the redox system integration is not surprising, given that these biomarkers can have marked biochemical and ecological differences (Costantini 2010c). From a biochemical perspective, specificity varies between antioxidants and this will affect their antioxidant potential as well as their physiological strategy for neutralising RS (Cohen & McGraw 2009; Costantini *et al.* 2011b). Additionally, the wide range of RS that exist has given rise to numerous pathways of neutralisation, leading to an array of oxidation products (Dotan *et al.* 2004). Another source of variation comes from the fact that antioxidants obtained through the diet might be more affected by environmental fluctuations (Costantini 2010c) and it could be that if one antioxidant becomes temporarily unavailable, another may compensate (Cohen & McGraw 2009). Furthermore, integration will depend on the physiological need of the organism, as well as on certain pathophysiological conditions and activity level (Dotan *et al.* 2004). This is further complicated by the fact that certain antioxidants, for example vitamin E, can also have pro-oxidant effects at high levels (Cohen *et al.* 2007).

Hence it is clear that correlations between different OS biomarkers are likely to vary considerably, both within individuals over time and between individuals and species, making the redox system difficult to define in "universal terms" with few univariate measures (Dotan *et al.* 2004; Cohen & McGraw 2009). One approach is therefore to measure the overall integration of the redox system using a variety of different biomarkers from different antioxidant and damage groups (for examples of biomarkers, see Figure 2:1). Investigating redox integration might prove to be a useful method for overcoming the vast differences that exist amongst biomarkers of OS, allowing an overall picture of an individual's redox state to be observed. If only specific damage biomarkers were increased in response to an oxidative threat or if only certain antioxidants responded to raised damage levels, then these changes could easily be missed by considering a small number of biomarkers, if we happened to look at the 'wrong' biomarkers. By considering the redox system as a whole, a better indication of the redox response to such an oxidative threat could be obtained.

Furthermore, measures of this overall redox integration could be related to other factors, such as body condition, often measured using body mass corrected for body size. Body condition has been related to probability of survival in passerines (Tinbergen & Boerlijst 1990; Magrath 1991) therefore redox integration might provide insight into long term fitness prospects for adults and chicks. For the latter there may be consequences if chicks are fledged in a poor body condition. This could become important when considering how well parents match the time at which their chicks are most demanding (i.e. requiring most food) with the time of peak food abundance. For parents that don't

match peak chick demand with peak food availability well, chicks will inevitably receive less food, resulting in reduced body condition of chicks by the time of fledging and potentially lower reproductive success (e.g. Reed, Jenouvrier & Visser 2013). If such parents are working harder to provide food for chicks, then they also may suffer reduced body condition. If redox integration could serve as an index of body condition, it might then be expected to also be related to this match between the peaks of chick demand and food availability.

Since associations among redox biomarkers vary substantially (Dotan *et al*. 2004; Cohen & McGraw 2009), it can be difficult to select an appropriate array for integration analysis. One must take into consideration the fact that antioxidant responses and specificity are not universal (Cohen & McGraw 2009) which means there are numerous damage products that could result (Dotan *et al.* 2004). Additionally, a high antioxidant level might reflect a response to greater RS production or higher antioxidant levels in some individuals unrelated to RS production, as a result of variations in diet or antioxidant gene expression (Costantini & Verhulst 2009) – neither provide conclusive evidence of oxidative damage, and so OS. Thus it is important to include a broad range of biomarkers from different components of the redox system (Figure 2:1), including antioxidant groups (e.g. enzymatic antioxidants, non-enzymatic antioxidants) in addition to markers of damage, to obtain a more complete picture of redox integration.

Thus when selecting which biomarkers to include in integration analysis, it is important that different groups be included. Markers of damage and oxidation include protein carbonyls, that are generated within the protein structure by reaction of proteins with radicals and lipid peroxidation products (Halliwell & Gutteridge 2007), and ROMs, which are intermediate compounds from lipid, protein and nucleic acid oxidation, primarily including hydroperoxides (Halliwell & Gutteridge 2007). Formation of protein carbonyls is often irreversible and so is a well-established indicator of oxidative damage to proteins (Dalle-Donne *et al.* 2003), whilst ROMs provide a broader picture of the level of oxidation. In terms of antioxidants, the enzyme GPX that catalyses the breakdown of hydrogen peroxide and hydroperoxides into water and alcohol, respectively (Yu 1994), might be considered alongside thiols, the most abundant of which, glutathione, is a cofactor of GPX (Dickinson & Forman 2002). To get more general information on the antioxidant system, the overall antioxidant barrier, for example, of the plasma, could be measured. One method of doing so is the OXY-adsorbent test that determines the non-enzymatic antioxidants that can react with HOCl, including protein thiols, ascorbate, vitamin E and carotenoids (Costantini 2010b).

Previous work (Costantini *et al.* 2011b; Dong *et al.* 2013) has demonstrated that principal component analyses (PCAs) and cluster analyses can be useful when investigating the strength and patterns of integration among redox biomarkers. These analyses can reveal valuable information about how biomarkers are associated with each other, giving some indication as to which antioxidants may be interacting and how such antioxidants might covary with oxidative damage (Costantini *et al.* 2011b). The eigenvalues from the PCA can also be used to obtain an overall value for the level of integration, which can be compared between groups (Pavlicev, Cheverud & Wagner 2009; Haber 2011).

Another approach to exploring redox integration involves estimating physiological dysregulation of individual redox integration using the Mahalanobis distance (D_M) (Mahalanobis 1936; De Maesschalck, Jouan-Rimbaud & Massart 2000). This can be accomplished by scoring the individual covariance among redox biomarkers according to their distance from that of a reference population or the population average, which is assumed to represent the average or 'normal' state (Cohen *et al.* 2013; Milot *et al.* 2014). Deviation from such a population 'norm' might be considered to be disadvantageous (Milot *et al.* 2014); this can be further investigated by comparing D_M with other physiological biomarkers or markers of body condition.

Therefore, the main aim of the present study was to investigate how five different biomarkers of the redox system are integrated in wild blue tit adults and nestlings and to describe patterns of integration that appear amongst the biomarkers. This allowed me to determine whether redox integration could be considered a useful method for investigating OS for future analyses and which biomarkers should be included. In addition, I aimed to determine if chick and adult redox integration and Mahalanobis distance could be useful as indices of body condition, providing information that might not be detected from individual biomarkers and allowing us to make predictions of future fitness consequences, at least in the short term.

2.2 Materials and methods

2.2.1 Field observations 2012 & 2013

Field work was carried out in the sessile oak *Quercus petraea* woodland surrounding the university field station, SCENE, at Loch Lomond, west Scotland (56°07'44''N, 4°36'46"W) (see Ramsay & Houston 1997 for details). Before egg-laying from late March onwards nestboxes were visited weekly to determine the birds' breeding status. Female blue tits lay one egg per day (Perrins 1979), so once eggs were present, they were counted so that the day the clutch was initiated could be back calculated. Then on subsequent visits the number of eggs laid since the previous visit was used to establish the date of clutch completion, again assuming one egg was laid per day. This could then be used to estimate an expected earliest hatching date, since the shortest incubation period of a blue tit is ten days from clutch completion. Once the clutch had been completed the nest was not visited until the expected hatching date to reduce disturbance during incubation which might lead to abandonment. From the expected hatching date, nestboxes were visited daily until the first egg was found to have hatched.

2.2.1.1 Chicks

In the 2012 breeding season I focused on chicks. When nestlings were 13 days old (day 0=day of hatching of the first chick), chicks were transferred to a holding bag from which six chicks per nest could be blindly selected for collection of a blood sample; all chicks were ringed. The brachial vein of these six chicks was punctured using a 25 G needle, then a heparinised capillary tube was used to collect up to 100 µl blood (~10% of the total blood volume). The blood sample was then kept in a cool box until returning to the SCENE laboratory within three to four hours; note that sampling time was not found to significantly affect any of the OS measures for chicks (from mixed models with each biomarker in turn as the response variable, time of sampling as a fixed effect and nestbox identity as a random effect – GPX: $t=0.65$, $p=0.52$; thiols: $t=0.66$, $p=0.51$; OXY: $t=1.03$, $p=0.30$; PCs: $t=0.43$, $p=0.66$; ROMs: $t=0.95$, $p=0.34$). Samples were centrifuged at 10,000 rpm for ten minutes and plasma and RBCs were pipetted into 5 µl aliquots as soon as possible – a maximum of four hours since collection. The aliquots were then frozen at -20°C before being transferred to Glasgow in dry ice and stored at -80°C within two weeks of field collection. All bleeding was conducted under licence from the UK Home Office.

2.2.1.2 Adults

In the 2013 breeding season my focus was on adult blue tits. Between days 8 and 11 of nestling rearing (day 0=day of hatching of the first chick), adults were caught on the nest using door traps (upon entering the nestbox, the bird triggered a mechanism which covered the hole). The birds were individually marked and their maximum wing chord (to 0.5 mm), tarsus length (using callipers, to 0.1 mm) and mass (to 0.1 g) were recorded. Additionally, birds were sexed by the presence (females) or absence (males) of a brood patch and aged by the presence of dull blue primary coverts in younger breeders born the previous year (these are brighter blue in birds greater than one year old (Svensson 1992)). Blood samples were taken from the brachial vein, centrifuged and stored using the same procedure as for chicks – again sampling time had no effect (GPX: t=0.12, p=0.90; thiols: t=1.41, p=0.16; OXY: t=0.09, p=0.93; PCs: t=0.82, p=0.41; ROMs: t=1.77, p=0.08). After taking measurements the trap was reset to attempt to catch the mate. The total time of nest disturbance from first setting the trap to removing it was never more than 90 minutes and all trapping took place between 08:00 h and 18:30 h.

As described in the Introduction, a greater 'mismatch' between peak chick demand and peak food availability could result in reduced chick and adult body condition and/or lower reproductive success (Reed *et al.* 2013). Therefore the time of peak caterpillar (blue tit chicks' main food source during the breeding season) abundance over the 2012 and 2013 breeding seasons was measured by collecting, drying and weighing caterpillar frass (Zandt 1994). Frass collectors were placed underneath the tree canopy (predominantly mature sessile oaks) in six transects of five collectors each throughout the study site. Each frass collector consisted of an 86 x 110 cm cotton sheet, which was emptied twice a week throughout the breeding season. To estimate how well birds had matched chick demand to caterpillar availability, I subtracted the day of peak caterpillar abundance from the day on which chicks were 11 days old, since this is when peak food demand occurs in blue tit broods (Blondel *et al.* 1991). I used the absolute values in analyses so that the greater the value, the greater the mismatch in timing between peak food demand and peak caterpillar abundance. Henceforward this variable will be termed 'mismatch'.

2.2.2 Laboratory analyses

2.2.2.1 Summary of Protocols

The following biomarkers were measured in RBCs: GPX – an antioxidant enzyme, total thiols – important antioxidant molecules, and protein carbonyls – indicative of protein damage. In plasma, I measured OXY, and ROMs – a marker of oxidation.

2.2.2.2 Analysis of GPX

The Ransel assay (RANDOX Laboratories, Crumlin, UK) was applied to determine GPX concentration in RBCs. Reagents were prepared according to the instructions provided with the assay kit. The Ransel Quality Control (RANDOX Laboratories, Crumlin, UK) was used to estimate random variation among assays and this was diluted 1:40 with diluting agent provided with the assay kit. RBC samples (approximately 5 µl aliquots in duplicate) were prepared by adding 45 µl (1:10 dilution; chicks) or 195 µl (1:40 dilution; adults) diluting agent, vortexing and centrifuging. The resulting supernatant was used in the assay, which is described in detail in Costantini *et al.* (2011b).

The original RBC supernatant (after adding 45 µl (chicks) or 195 µl (adults) diluting agent) was used to measure the protein concentration of the sample (according to Quick Start™ Bradford assay; Bio-Rad Laboratories, Inc. California) so that the ratio of GPX to proteins could be calculated (GPX concentration in U/ml divided by protein concentration in mg/ml); the final GPX concentration was in U per mg proteins. This accounted for the difficulty in pipetting exactly 5 µl RBCs for this assay, as a result of their high viscosity. The intra-assay coefficient of variation (mean±SE) was 5.38±0.29% (chicks) and 4.08±0.41% (adults). The inter-assay coefficient of variation was 2.31±0.50% (chicks) and 2.85% (adults; only over one day so no SE).

2.2.2.3 Analysis of total (protein and non-protein) thiol groups

Total thiol groups in RBCs were measured using the –SHp test (Diacron International, Grosseto, Italy). Firstly, RBC samples (approximately 5 µl aliquots in duplicate) were prepared by adding 100 µl distilled water, vortexing and centrifuging. The resulting supernatant was diluted 1:10 (chicks) or 1:30 (adults) with distilled water. Zebra finch plasma was used as an internal control and this was diluted 1:8 with distilled water. The remaining protocol was completed as described in Costantini *et al.* (2011b).

The original RBC supernatant (after adding 100 µl distilled water) was used to measure the protein concentration of the sample (according to Quick Start™ Bradford assay; Bio-Rad Laboratories, Inc. California) so that the ratio of thiols to proteins could be calculated (thiol concentration in µmol/ml divided by protein concentration in mg/ml); the final thiol concentration was in µmol thiols per mg proteins. This is important as the exact volume of RBCs used in the assay was unknown due to the viscous nature of the RBCs. The intra-assay coefficient of variation (mean±SE) was 5.40±1.13% (chicks) and 6.23±2.50% (adults). The inter-assay coefficient of variation was 5.85±1.35% (chicks) and 10.82±5.23% (adults).

2.2.2.4 Analysis of non-enzymatic antioxidant capacity (OXY)

The OXY-adsorbent test (Diacron International, Grosseto, Italy) was used to measure the non-enzymatic antioxidant capacity of the plasma. Duplicates of the calibrator, samples and zebra finch plasma acting as an internal control were diluted 1:100 and 2 µl of each was used in the assay (for detailed description see Costantini & Dell'Omo 2006a). The intra-assay coefficient of variation (mean±SE) was 5.15±0.37% (chicks) and 4.17±0.32% (adults). The inter-assay coefficient of variation was 3.33±0.63% (chicks) and 5.01±1.23% (adults).

2.2.2.5 Analysis of protein carbonyls

To quantify protein carbonyls in RBCs, the method from Levine *et al.* was used (Levine *et al.* 1990; see also Cao & Cutler 1995; Jenni-Eiermann *et al.* 2014). Duplicate samples were first diluted with distilled water in order to have a concentration of 1 mg/ml protein (as given by Quick Start™ Bradford assay; Bio-Rad Laboratories, Inc. California) and the protocol was carried out as described in Jenni-Eiermann *et al.* (2014). A small number of chick samples (5.43%) were below the detection limit, thus values were substituted with half the detection limit, as suggested by Sinha, Lambert & Trumbull (2006). This means that if, for example, I had values of 1, 2, 3, 4 and 5, I used 0.5 (i.e. half the lowest value, which in this case is 1) for the undetected samples. The intra-assay coefficient of variation (mean±SE) was 6.79±0.48% (chicks) and 7.90±0.74% (adults). The inter-assay coefficient of variation was 5.42±1.28% (chicks) and 4.67±1.46% (adults).

2.2.2.6 Analysis of ROMs

To determine ROMs in the plasma, the d-ROMs test (Diacron International, Grosseto, Italy) was applied. In duplicate, 8 µl (chicks) or 4 µl (adults; zebra finch plasma as a quality control) aliquots of samples were pipetted into 0.5 ml Eppendorf tubes, and 2 µl calibrator (diluted 1:1) was added to each (chicks only). Additionally, 2 µl for each of the points of a standard curve and 2 µl blank (distilled water) were pipetted into 0.5 ml Eppendorf tubes. The remaining protocol is as described in Costantini & Dell'Omo (2006a), except that absorbance was read at 505 nm (Thermo Scientific Multiskan Spectrum; ThermoFisher, Vantaa, Finland). Reading at 505nm minimises any effect of plasma colour, which is important here as haemolysis of some of the samples led to slightly pink or red plasma.

The equation of the calibration curve was used to calculate the ROMs value (mM H_2O_2 equivalents) of the samples. The calibrator value was then subtracted from this (chicks

only) and the volume was correct for. The intra-assay coefficient of variation (mean±SE) was 6.28±0.69% (chicks) and 7.92±0.07% (adults). The inter-assay coefficient of variation was 7.03±1.65% (chicks) and 4.43±0.45% (adults).

2.2.3 Statistical methods

For adults and chicks, PCAs and cluster analyses were performed in PAST version 2.17 (Hammer, Harper & Ryan 2001) on correlation matrices of all five redox biomarkers (GPX, total thiols, OXY, protein carbonyls, ROMs). For this analysis I used residuals from mixed models (lme4 package in R; Bates *et al.* 2014) with nestbox identity as a random factor (to account for the non-independence of the two members of a pair in adults and of the six members of a brood in chicks) and (in the case of the adult OXY and ROMs analyses) plasma colour as a fixed factor (categorised into 'yellow', 'pink' or 'red') to account for some plasma samples being slightly pink or red due to haemolysis – this release of proteins from RBCs can lead to high OXY values but lower ROMs values if iron and GPX are also released. Transformed data were used for some of the biomarkers (adult protein carbonyls, thiols, OXY and chick OXY were log-transformed; chick protein carbonyls were square root-transformed) to improve residual normality and heterogeneity.

From the PCAs, biplots were generated and the loadings (expressed as correlation coefficients) of each OS variable on each principal component were extracted, as well as the eigenvalues (λ). To estimate how strongly integrated the biomarkers were, an integration index was obtained by calculating the relative standard deviation (s.d.) of the eigenvalues (Pavlicev *et al.* 2009; Haber 2011), where n is the number of variables, in this case biomarkers:

$$relative\ s.d.(\lambda) = \frac{\sqrt{\mathrm{Var}(\lambda)}}{\sqrt{\mathrm{n}-1}}$$

Dendrograms were generated from the cluster analyses (unweighted pair-group method with arithmetic average; UPGMA) and the cophenetic correlation coefficient was extracted – this indicates how well the dendrogram reflected the original data structure; the closer the value is to 1, the better the fit. The term 'association' was used in the Results and Discussion text to refer to any grouping or clustering identified by the PCA and/or cluster analyses, whether significant or not. The term 'correlation' was only used when a specific association between two biomarkers had been tested for separately using Pearson's product-moment correlation and was statistically significant.

To investigate the integration of redox state further, the residuals used in the PCA and cluster analyses were standardised to have a mean of zero and unit variance and used to calculate the Mahalanobis distance (Mahalanobis 1936; De Maesschalck *et al.* 2000) for each individual. D_M can be calculated from the following equation (De Maesschalck *et al.* 2000; Milot *et al.* 2014):

$$D_M(x) = \sqrt{(x - \mu)^T S^{-1} (x - \mu)}$$

where x=multivariate observation; μ=vector of reference population means for each variable; T=indicates that the vector should be transposed; S^{-1}=inverse variance-covariance matrix of the reference population, which was calculated using the MMULT function in Excel. Multivariate normality was confirmed using Shapiro tests in R (R Core Team 2013). Within-brood repeatability of chick D_M, and the first (PC1) and second (PC2) principal components from the PCA, was calculated according to Lessells & Boag (1987) with standard errors according to Becker (1992).

To determine how redox integration might be related to body condition, general linear models (GLMs) were carried out in R (R Core Team 2013). In order to obtain a value for body condition in chicks, residuals were extracted from mixed effects models (lme4 package in R; Bates *et al.* 2014) with mass as the response variable, tarsus length as a fixed effect and nestbox identity as a random effect (to account for the non-independence of members of the same brood). This value for body condition was then included in linear models as a response variable, however in adults the mass-tarsus and mass-wing relationships were not significant so mass was used as the measure of body condition and the response variable. This was deemed suitable since there was no significant difference in mass between adult males and females (males = 11.1±0.3g; females = 11.1±0.2g; t=0.10; p=0.92). The three measures of redox integration (D_M and PC1 and PC2 from the PCA) were added as fixed effects. The relationships between each measure of redox integration and the mismatch between peak chick demand and peak food availability were not found to be significant when tested for separately in linear models with mismatch as the response variable and the three integration measures as fixed effects for chicks (D_M: t=0.55, p=0.59; PC1: t=0.15, p=0.88; PC2: t=0.15, p=0.88) or adults (D_M: t=0.28, p=0.79; PC1: t=1.73, p=0.10; PC2: t=0.02, p=0.99); therefore it was deemed appropriate to include all three integration measures as explanatory variables in one model. Furthermore, variance inflation factors were calculated to check for collinearity between the three integration measures; since GVIF<2 in all cases this was not considered to be a problem. Additional fixed effects were brood size at day 7 (chicks),

number of chicks after manipulation (adults), age (adults; split into younger and older breeders) and mismatch between peak chick demand and peak food (caterpillar) availability (chicks and adults; see 2.2.1.2 for more detailed description). Simplification of the models was carried out in a backward stepwise manner by the removal of non-significant terms and residuals were checked for normality and heteroscedasticity.

2.2.4 Robustness of the analysis

There was large variation in blood volumes acquired from both chicks and adults, resulting in different numbers of plasma and red blood cell aliquots from each individual, with not all samples being large enough to measure all five biomarkers. Complete sets of all five biomarkers were measured in 39 chicks from 17 nests in 2012 and 19 adults (13 females, 6 males) attending 17 nests in 2013. Despite the small sample size, these nests spanned a good proportion of the breeding season (Figure S1, Appendix 1). The analyses did not consider sex because chick samples had insufficient quantities of RBCs to be sexed molecularly and there were only a small number of adult males.

To test the robustness of the integration analyses, the data were re-analysed excluding those biomarkers that most reduced sample size (so as to increase the sample size included in each analysis). For chicks the removal of the ROMs assay from the integration analysis increased the sample size to 67 chicks from 21 nests, while the alternative option of removal of the thiols assay increased it to 55 chicks from 23 nests. In both cases the results were qualitatively similar but inevitably led to the loss of significant correlations involving the excluded variables (OXY-ROMs and GPX-thiols, respectively). Therefore, all five biomarkers were retained in the analyses. For adults, removing biomarkers did not greatly increase the sample size because the samples with high volumes of plasma (used in OXY and ROMs analysis) were not necessarily the same ones that had high quantities of RBCs (used in GPX, thiol and protein carbonyl analyses).

2.3 Results

2.3.1 Patterns of integration among five redox biomarkers in the chick and adult populations

Descriptive statistics for the five redox biomarkers are reported in Table 2:1. The PCA (Figure 2:2) and cluster analyses (Figure 2:3) showed patterns of associations among redox biomarkers in wild blue tits and provided corroborating evidence for some integration between redox biomarkers. Note that the PCA and cluster analyses were exploratory techniques allowing me to investigate patterns in redox integration and so I have attempted to describe such patterns, even when individual correlations were not significant, to give an overall impression of the redox system; hence I only used the term 'correlation' when the relationship was statistically significant, and 'association' when it was not (see Section 2.2.3 "Statistical methods" for a full definition). For chicks and adults, there was a strong and significant positive correlation between OXY (plasmatic non-enzymatic antioxidants) and ROMs (intermediate oxidation metabolites in the plasma) (Table 2:2). For chicks, plasma OXY and ROM levels were also moderately positively associated with the antioxidant enzyme GPX (Table 2:2 A), whereas in adults GPX was positively associated with damage to proteins (protein carbonyls) (Table 2:2 B).

Both chick and adult thiols were negatively associated with plasma OXY, ROMs and GPX levels, although only the association with GPX in chicks was a significant correlation (Table 2:2 A). Lastly, the cluster analysis suggested that there was a positive association between the damage compound protein carbonyls and total thiols in chicks only (Figure 2:3 A), but this was weak and non-significant (Table 2:2 A). The integration index, measuring the overall strength of redox integration, was similar between chicks (0.28) and adults (0.29) (Figure 2:4).

Table 2:1 Means±1 standard error (SE) and the range of data (in brackets) of the five redox biomarkers for (A) chicks (n=39 chicks from 17 nests) and (B) adults (n=19 adults from 17 nests). RBC measures were GPX, thiols and protein carbonyls. Plasma measures were OXY and ROMs.

	(A) Chick	**(B) Adult**
GPX (U/mg protein)	0.003±0.0002	0.12±0.01
	(0.002 - 0.006)	(0.07 - 0.19)
THIOLS (µmol/mg protein)	0.10±0.003	0.85±0.10
	(0.05 - 0.14)	(0.39 - 2.05)
OXY (mM HOCl neutralised)	154.08±6.02	182.83±7.28
	(87.05 - 259.51)	(134.20 - 262.46)
PCs (nmol/mg protein)	1.58±0.29	4.27±0.42
	(0.03 - 8.52)	(1.30 - 8.08)
ROMs (mM H_2O_2 equivalents)	0.23±0.02	0.12±0.02
	(0.03 - 0.46)	(0.02 - 0.34)

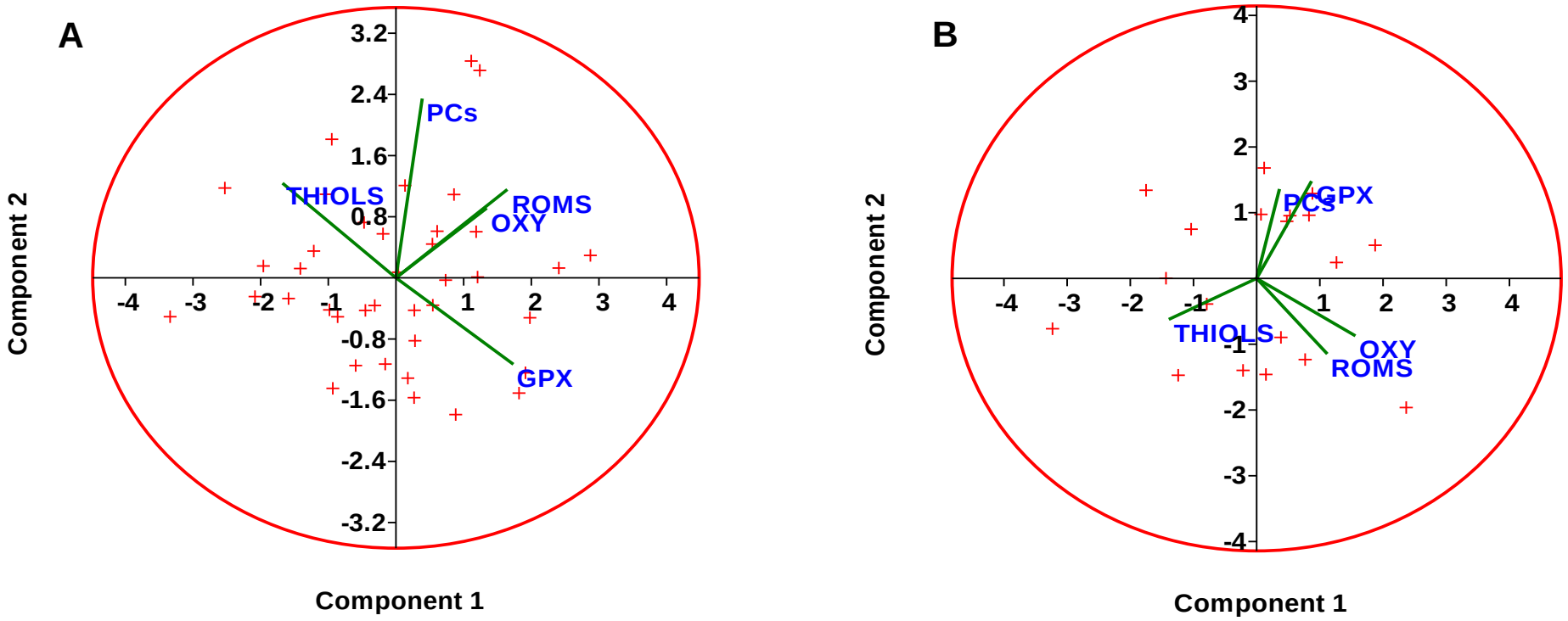

Figure 2:2 Biplots from the PCA displaying loadings of the five redox biomarkers onto the first two principal components (PC1-Component 1, PC2-Component 2) for chicks (A; n=39 chicks from 17 nests) and adults (B; n=19 adults from 17 nests); individuals are indicated by red crosses. The loadings (expressed as correlation coefficients) of the five OS biomarkers onto PC1 and PC2 have also been plotted with a line projected through the data point for each. For chicks and adults, the closeness of the lines for OXY and ROMs indicates their similar loadings onto both principal components and a strong positive association. For chicks, GPX also loaded positively onto PC1 suggesting a positive association with OXY and ROMs, whilst in adults GPX is associated with protein carbonyls, as demonstrated by the closeness of the lines for these variables. Finally, the negative loadings of thiols onto PC1 imply a negative association of thiols with GPX, OXY and ROMs in both chicks and adults. PC1 explained 37.40% and 35.88% of the total variance and had eigenvalues of 1.87 and 1.79 for chicks and adults, respectively. For PC2 the values were 23.29% and 26.52%, and 1.16 and 1.33.

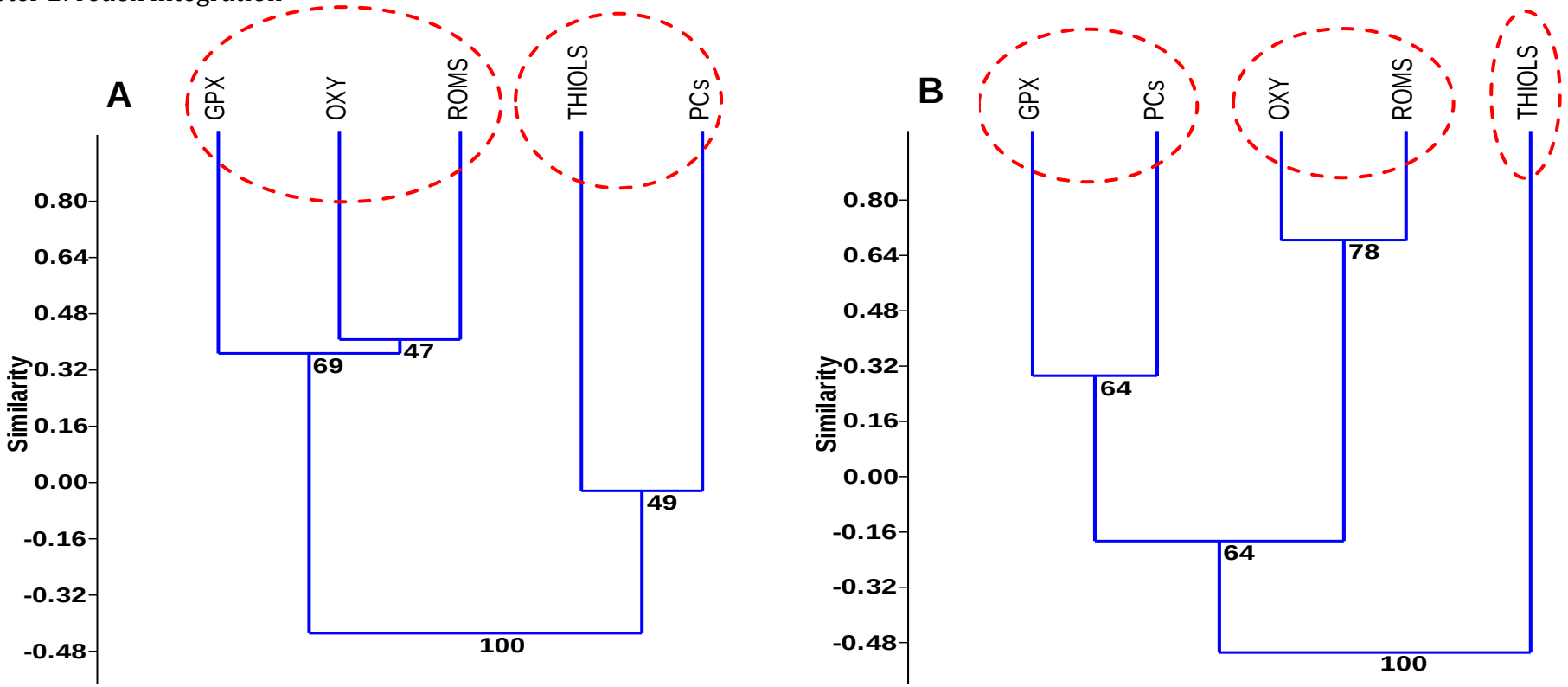

Figure 2:3 Dendrograms generated by cluster analyses for chicks (A; n=39 chicks from 17 nests) and adults (B; n=19 adults from 17 nests). At the root of each cluster the bootstrapping value (from 1000 replicates) is given; this is the percentage of replicates in which the cluster is still supported. The left hand scale indicates the level of similarity between groups, ranging from -1 (no association) to +1 (perfect association). The cophenetic correlations of 0.83 (A) and 0.91 (B) imply the dendrograms reflected the similarity structure in the original data very well. For chicks (A) two clusters have been identified: the first contains OXY, ROMs and GPX (a moderate association is indicated by the reasonable level of similarity); the second contains protein carbonyls and thiols (a weak association is indicated by the lower level of similarity). The adults differ in that OXY and ROMs are in their own cluster with a high level of similarity, implying a strong association. Additionally, there is a second cluster containing protein carbonyls and GPX, with thiols being the least associated with the other biomarkers.

Table 2:2 Correlations (Pearson's r values) among redox biomarkers for (A) chicks (n=39 chicks from 17 nests) and (B) adults (n=19 adults from 17 nests). For description of these biomarkers see Table 2:1. Correlations were tested for significance in the control birds of this study using the residuals used in the PCA and cluster analyses, which corrected for pair in adults, brood in chicks and plasma colour for adult OXY and ROMs (see section 2.2.3 for full description), in Pearson's product-moment correlation tests in R (R Core Team 2013). Significance denoted by * for $p<0.05$ and ** for $p<0.01$.

(A) Chick	GPX	THIOLS	OXY	PCs	ROMs
GPX					
THIOLS	-0.49**				
OXY	0.24	-0.11			
PCs	-0.03	0.04	0.07		
ROMs	0.23	-0.29	0.34*	0.22	

(B) Adult	GPX	THIOLS	OXY	PCs	ROMs
GPX					
THIOLS	-0.33				
OXY	0.16	-0.42			
PCs	0.23	-0.14	-0.15		
ROMs	-0.09	-0.10	0.51*	0.10	

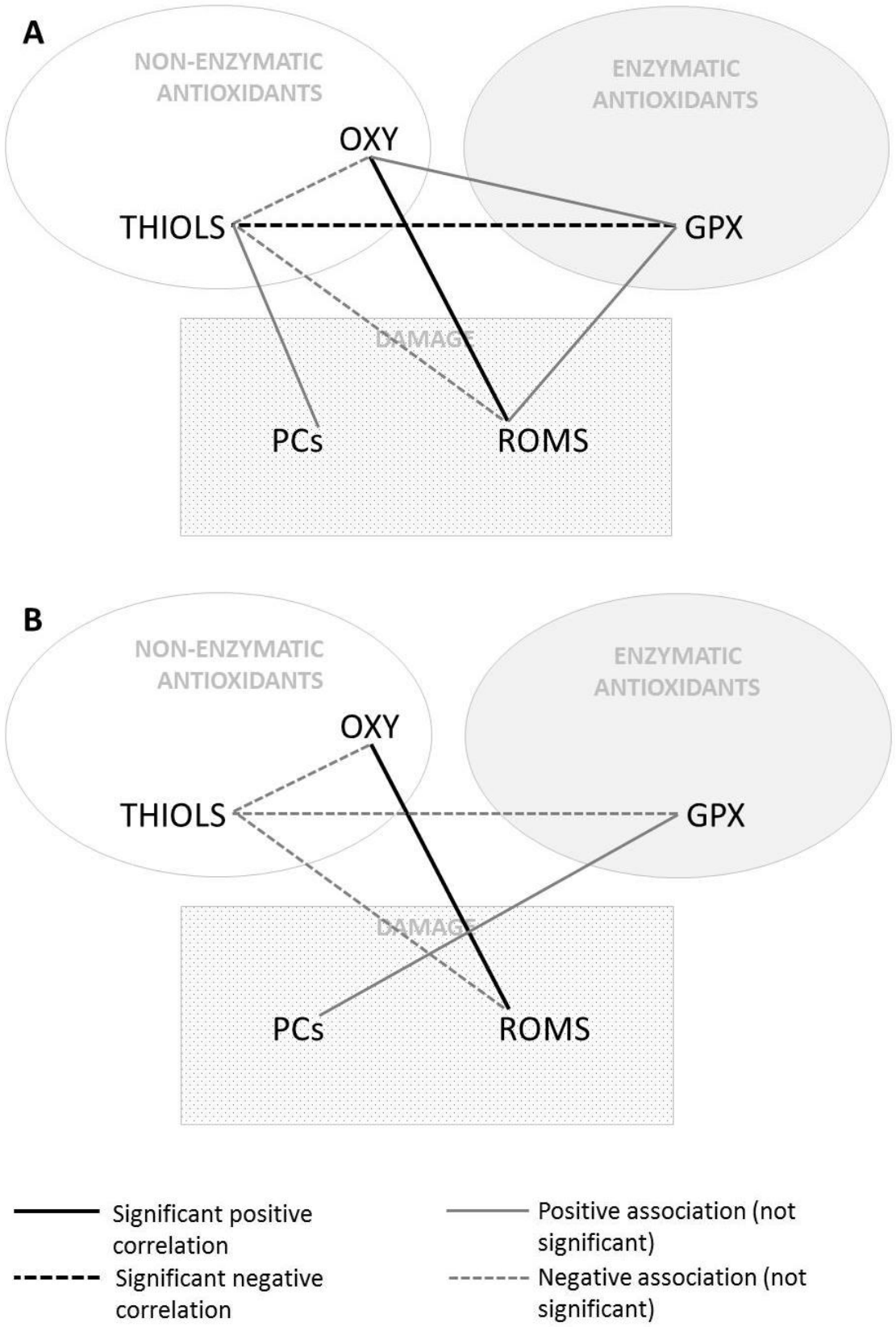

Figure 2:4 Network model demonstrating associations identified by the PCA and cluster analyses among the five redox biomarkers for chicks (A; n=39 chicks from 17 nests) and adults (B; n=19 adults from 17 nests). The integration index, as measured by the standard deviation of eigenvalues, was 0.28 for chicks and 0.29 for adults.

2.3.2 Redox integration and Mahalanobis distance as indices of body condition

For chicks, D_M tended to be repeatable within broods (r=0.32±0.20, $F_{16,22}$=2.07) although this was not statistically significant (p=0.06). D_M was significantly positively correlated with protein carbonyls (Table 2:3 A). Given that a higher value for D_M means that the individual has a redox integration profile that is further away from the population 'norm', this suggests individuals that had less 'normal' profiles also had greater protein damage. However, from Figure 2:5 it is evident that there were four values pulling this trend and this suggests that only very high protein carbonyl levels (greater than 1.85 standard deviations above the mean; the next lowest value was 0.73 standard deviations above the mean) led to abnormal redox profiles. In adults, the significant negative correlation between D_M and protein carbonyls (Table 2:3 B) implies less 'normal' individuals had *lower* levels of protein damage. D_M was not significantly correlated with any of the other redox biomarkers in either chicks or adults (Table 2:3), nor did chick or adult D_M significantly correlate with body condition (Table 2:4).

There was no evidence of within-brood repeatability for chick PC1 (r=-0.40, $F_{16,22}$=0.35, p=0.98) or PC2 (r=-0.41, $F_{16,22}$=0.34, p=0.98). Significant correlations between PC1 and four of the redox biomarkers indicate that PC1 varied negatively with thiols (r=-0.71, p<0.001 and r=-0.73, p<0.001 in chicks and adults, respectively) and positively with GPX (r=0.73, p<0.001 and r=0.46, p=0.049), OXY (r=0.57, p<0.001 and r=0.82, p<0.001) and ROMs (r=0.70, p<0.001 and r=0.59, p=0.008). PC1 was significantly positively correlated with body condition in chicks (Figure 2:6), but not adults; whilst PC2 did not correlate with body condition in chicks or adults (Table 2:4). Finally, body condition was not significantly related to brood size at day 7 in chicks, nor in adults was it related to their age class or the number of chicks after manipulation (Table 2:4). Finally, there was no significant relationship between body condition and the mismatch between the caterpillar peak and timing of peak chick demand in chicks or adults (Table 2.4).

Table 2:3 Correlations (Pearson's r values) of D_M with the redox biomarkers for (A) chicks (n=39 chicks from 17 nests) and (B) adults (n=19 adults from 17 nests). Correlations were tested for significance using Pearson's product-moment correlation tests in R (R Core Team 2013). For biomarker descriptions and significance codes, see Table 2:1 and Table 2:2, respectively.

	(A) Chick	**(B) Adult**
GPX	-0.07	-0.44
THIOLS	0.04	0.34
OXY	-0.05	-0.02
PCs	0.32*	-0.49*
ROMs	0.13	0.08

Table 2:4 Results from GLMs investigating the relationship between body condition and measures of redox integration; D_M and the first two principal components from the PCA. For (A) chicks (n=39 chicks from 17 nests), brood size at day 7 was also included in the model. For (B) adults (n=19 adults from 17 nests), age and number of chicks after manipulation were included. The mismatch between the caterpillar peak and timing of peak chick demand (see text for details) was included for both chicks and adults. Parameter estimates are only given when variables were included in the final model.

(A) Chick	parameter estimate	SE	df	t	p
D_M			35	-1.15	0.26
PC1	0.11	0.05	37	2.06	**0.046**
PC2			36	1.35	0.19
brood size (d7)			33	-0.23	0.82
mismatch			34	-0.67	0.51

(B) Adult	parameter estimate	SE	df	t	p
D_M			12	0.15	0.89
PC1	-0.08	0.11	17	0.79	0.44
PC2			14	0.36	0.73
age			16	0.76	0.46
no. chicks after manipulation			15	0.34	0.74
mismatch			13	0.32	0.75

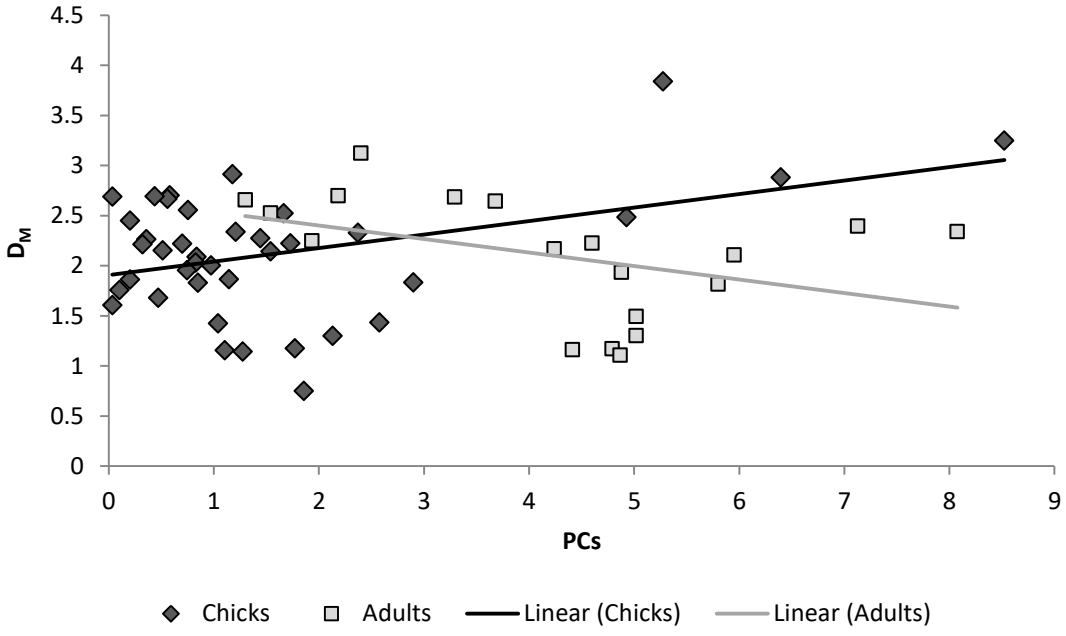

Figure 2:5 Relationship between protein carbonyls and D_M for chicks and adults.

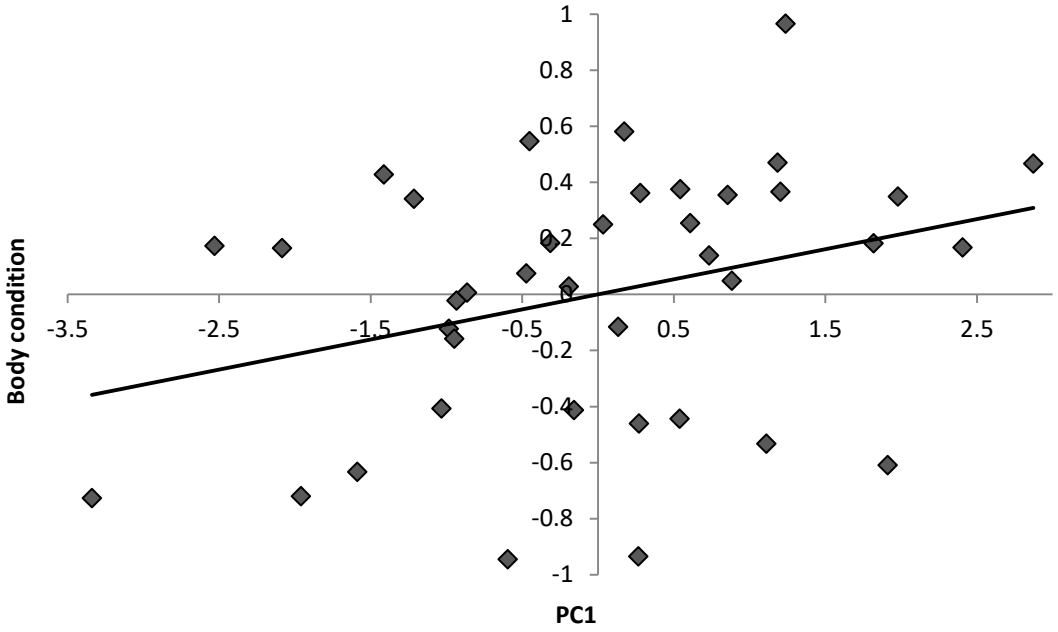

Figure 2:6 Relationship between the first principal component from the PCA on redox biomarkers and body condition in chicks. See Table 2:4 A for statistical analysis.

2.4 Discussion

The overall strength of integration was found to be similar between chicks (0.28) and adults (0.29) and is comparable with unmanipulated zebra finches (0.28; Costantini *et al*. 2013). While I found some consistent associations among OS biomarkers in chicks and adults, most of the biomarkers were not strongly integrated given the low correlation coefficients and lack of significance for the associations. This is contrary to the situation in adult zebra finches where GPX, thiols and ROM levels were found to be highly integrated (Costantini *et al.* 2011b), thus emphasising the between-species differences that can occur when considering patterns of redox integration. Though the sample size was larger in Costantini *et al.*'s (2011b) study (n=78; compared with 39 chicks and 19 adults in the present work), the cophenetic correlation that they measured (0.95) was comparable with mine (0.83 for chicks and 0.91 for adults). Since lower cophenetic correlations (e.g. <0.7) imply the clusters identified by the analysis are less robust (Rohlf 1970), both the present study and Costantini *et al.*'s work can be considered to be relatively robust.

The fact that the integration among most of the biomarkers was not found to be strong implies that, for my study population at least, perhaps a traditional univariate approach is more appropriate. This should still include all five biomarkers since the lack of strong integration implies each biomarker revealed independent information on the redox system. Yet the choice of biomarkers will also have an effect on the strength of integration between biomarkers; it has been shown that within-group correlations of antioxidants are stronger than those between groups (Cohen & McGraw 2009). Future research on this population including similar OS measures (i.e. within the same antioxidant or damage groups) might well be suited to integration analysis. There may also be differences between populations of blue tits; more challenging environments such as those with high pollutant levels may affect oxidative status (Isaksson 2010) leading to differences in redox integration. However, the effect of stressors on integration is hard to predict; while one might expect an increase in covariation of biomarkers as a redox response is mounted, previous work has found redox integration to be reduced under stress, perhaps due to an increase in modularity (Costantini *et al.* 2013). As was shown in adult zebra finches, integration among different biomarker groups could be high when considering other species. Furthermore, sex differences in the pattern and strength of redox integration might be apparent as a result of differing reproductive behaviours and investments of male and female blue tits (Perrins 1979), as well as hormonal differences during reproduction – evidence suggests male and female sex hormones have differential effects

on RS production (Isaksson *et al.* 2011b). Unfortunately the current study did not have the statistical power to consider sex differences, but it might be fruitful for future studies.

Chick body condition was found to be unrelated to the degree of mistiming of breeding (i.e. the mismatching of peak food availability with peak chick demand). This might be because the chicks were studied in a good quality year whereby food availability and sibling competition put less pressure on chicks. Indeed, caterpillar frass (indicating caterpillar abundance) collected during 2012 (when chicks were studied) was significantly greater compared with the following year (2012: 320±20 mg frass per m^2 per day; 2013: 99±7 mg frass per m^2 per day; $t=11.07$, $df=582.02$, $p<0.0001$; data were logged to improve normality). However, there was no difference between years in how well breeding was timed with peak prey abundance (mismatch in 2012: 5.6±0.45 days; 2013: 5.1±0.50 days; $t=0.62$, $df=138.28$, $p=0.53$), nor was there an overall difference in temperature between years when date was controlled for (mixed model with date as a random effect: $t=0.69$; $p=0.49$); nonetheless there was a heatwave that occurred between 23rd and 28th May 2012, during which mean daily temperatures ranged from 20-25^{o}C, compared with 2013 when the highest mean daily temperature was 20.8^{o}C. This period of raised temperature may have led to the observed greater caterpillar abundance in 2012 and provided increased foraging opportunities for parents. However, if there were fewer foraging opportunities for adults in 2013 and parents that missed the caterpillar peak had to work harder than those that did not, this did not affect their body condition, which was unrelated to mismatch.

Of course it cannot be disregarded that my measure of body condition for chicks, mass corrected for body size, was not suitable. Green (2001) suggests that certain key assumptions are likely to be violated when this method is employed, for example, that the relationship between mass and tarsus is linear, though for the chicks in the present study the mass-tarsus relationship was linear and significant. In addition, mass corrected for body size has been suggested to be a reliable method when (as here) the assumption of homoscedasticity is met (Jakob *et al.* 1996). However, in adults, mass alone was used as the measure of body condition because the mass-tarsus and mass-wing relationships were not significant and this may have been an unsuitable measure. Additionally, physiological, rather than morphological, estimates of body condition might be more relevant when considering physiological aspects of the redox system.

2.4.1 Patterns of integration among five redox biomarkers in the chick and adult populations

Patterns of redox integration in chick and adult blue tits showed similar trends, though it is difficult to compare directly between them as they were measured in different years. Firstly, a significant positive correlation between OXY and ROMs was present in both chicks and adults. A positive relationship between these variables has previously been reported in other species, including wild adult Seychelles warblers (*Acrocephalus sechellensis*) (van de Crommenacker *et al.* 2011a) and captive adult zebra finches (Costantini *et al.* 2011b), however for the latter this was weak and not significant. For the zebra finches in Costantini *et al.*'s study (2011b), ROMs were highly integrated with the antioxidant enzyme GPX and thiols, and the authors reasoned that the weak positive connection between OXY and ROMs could be an indirect result of GPX being significantly correlated with both biomarkers. In the present study, chick GPX showed a tendency to be positively associated with both OXY and ROMs so perhaps there is some upregulation of this antioxidant enzyme in response to raised ROM levels. Yet neither connection was particularly strong nor significant, and in adults there was little association between ROMs and GPX, so possibly in blue tits plasma OXY is more responsive to raised ROM levels than the antioxidant enzyme GPX. Of course this might arise from the fact that both OXY and ROMs were measured in plasma while GPX was measured in RBCs, since it has been suggested that there is not a direct interaction between RBC GPX and plasma ROMs (Costantini *et al.* 2011b). Nonetheless Costantini *et al.* (2011b) found both RBC and plasma GPX to correlate positively with plasma ROMs and they suggest a high integration between RBC and plasma redox status.

Unlike in zebra finches (Costantini *et al.* 2011b), a negative association was found between GPX and thiols for chicks and adults in the present study, although only the former was statistically significant. Since the thiol glutathione is a cofactor of GPX, when activity of GPX increases, it might reduce its availability leading to this negative relationship (Brown-Borg & Rakoczy 2003). This may suggest that the GPX response is stronger in chicks than it is in adults – the r value was higher in chicks (-0.49), compared with adults (-0.33). However the adult data could still lack statistical power to find a significant relationship. Additionally, GPX was positively associated with raised protein damage (protein carbonyls) in adults, however as GPX does not directly interact with protein carbonyls it is difficult to explain this; possibly they both increase independently when there is an oxidative insult. At the same time, both chicks and adults are likely to have other antioxidant molecules and enzymes that may influence these relationships – both GPX and the enzyme CAT are involved in hydrogen peroxide breakdown and they

complement each other. Moreover, the removal of hydrogen peroxide will depend upon its production in the first place from the superoxide ion, where the antioxidant enzyme SOD is involved (Yu 1994).

2.4.2 Redox integration and Mahalanobis distance as indices of body condition

The Mahalanobis distance measures distance from the population 'norm', with low values being near the population norm and higher values indicating greater distance from the norm, which has been suggested to be disadvantageous to the individual (Milot *et al.* 2014). In other words, extremes can be sub-optimal (Cohen *et al.* 2013); for example, low antioxidant levels mean lower protection against OS but if too high, they can act as pro-oxidants (Cohen *et al.* 2007). For blue tit chicks in the present study, only very high protein carbonyl levels were associated with less 'normal' oxidative profiles (i.e. those with greater D_M), suggesting that deviations from the population 'norm' are only disadvantageous at extreme levels. However, the significant negative correlation between adult D_M and protein carbonyls implies that lower levels of protein damage are associated with being further away from the population norm. Since the adults in the present study were all breeding when blood samples were taken, perhaps it is more normal for them to have raised levels of protein damage during this demanding period. Conversely, this could be the result of redox integration differing between different life stages or years. Finally, there were no significant correlations between D_M and the other biomarkers implying that D_M is providing information that is independent of them.

The idea that a high value for D_M is disadvantageous in some way is also challenged by the fact that neither adult nor chick D_M was significantly correlated with body condition. On the contrary, previous work has found D_M to be negatively related to body condition in red knots (*Calidris canutus*) (Milot *et al.* 2014). That study used a larger range of physiological biomarkers to calculate D_M, mainly from the immune system, which may be more sensitive to fluctuations from the 'norm' than the redox system. This could arise from the fact that deviations from a healthy functioning immune system would likely have more immediate fitness effects, while effects of a sub-optimal oxidative profile might not be seen until later in life. Milot *et al.* (2014) used maximal thermogenic capacity and a score of inflammation to measure body condition, compared with mass (adults) or a combination of mass and structural size (chicks) in the present study; perhaps a physiological measure of body condition might yield similar results to their study.

From the PCA, PC1 was significantly positively correlated with body condition in chicks. Since PC1 in chicks was positively associated with OXY and ROMs, a better body

condition may be associated with a non-enzymatic antioxidant plasma capacity that is more responsive to general oxidation levels (ROMs). Likewise, a negative relationship between GPX and thiols was suggested by PC1, potentially implying an increased GPX activity in chicks in a better body condition. This is supported by the significant positive correlations of PC1 with GPX, OXY and ROMs; and its significant negative relationship with thiols. Thus, the integration of these four biomarkers (GPX, OXY, ROMs and thiols) might reveal information about the body condition of blue tit nestlings and so future fitness consequences, since body condition at fledging can be related to future survival probability (Tinbergen & Boerlijst 1990; Magrath 1991); however, the same was not true for PC2 in chicks, or PC1 and PC2 in adults, none of which correlated significantly with body condition. Once more, it must be emphasised that my body condition measure is possibly not the most optimal here yet if it is, this has some implications. First, integration of protein damage with the other biomarkers is possibly not a good indicator of body condition in blue tit chicks, since it was the only biomarker not to correlate with PC1, though this may result from protein carbonyls not being strongly integrated with the other biomarkers. Second, redox integration might not be useful as an index of body condition in adults – perhaps because consequences of sub-optimal redox integration are not reflected immediately in adults due to an increased tolerance of redox imbalance. It is also important to note that there may be no long-term consequences of a sub-optimal redox profile. Evidence form certain long-lived species with high OS levels, such as the naked mole rat (Edrey *et al*. 2011), suggests that it is the ability to mitigate OS, rather than the level *per se*, that is important for longevity (Selman *et al*. 2012). As most species do not have the capacity to cope with OS in this way, it may not impact on overall fitness (Selman *et al*. 2012).

2.4.3 Conclusion

Due to the variable nature of the redox system, with correlations among biomarkers fluctuating both within individuals over time and between individuals and species, (Dotan *et al.* 2004; Cohen & McGraw 2009) analyses of only one or two redox biomarkers no longer seem appropriate. The present study has investigated two approaches to analysing multiple biomarkers at once, thus avoiding the loss of information that may occur otherwise. Certainly all five biomarkers measured here appear to provide independent information and so none can be excluded from future analyses of this species, although the low levels of integration observed suggest a more traditional univariate approach might serve better. However, future research including different biomarkers, populations or species, or considering sex differences, may well be suited to integration analysis and this would allow us to tease apart complicated relationships,

giving some insight into how antioxidants might respond to each other and to oxidative damage.

One potential drawback with this type of analysis is that the correlations among biomarkers of OS are constantly changing over time (Sepp *et al.* 2014). Ideally, one should take repeated measurements from the same individual to assess how the redox profile changes over different life-history events, for instance reproduction or senescence; as well as over different years and habitats to account for environmental fluctuations. Secondly, each biomarker is not 100% specific to a given molecule and there are structural variations of certain antioxidants, for instance, plasma and RBC GPX (Halliwell & Gutteridge 2007). This means that results are likely to include some degree of noise so it might be useful to extend the range of biomarkers and tissues included, to obtain a fuller picture of the redox system; of course this is not always feasible in ecological studies. Additionally, these should be accompanied by complementary univariate analyses to investigate any trends further. Lastly, when repeating the analysis on different populations and species, caution must be taken since the associations among biomarkers are likely to vary between species.

Body condition and survival can be related in passerines and so if redox integration could be used as an index of body condition, it might allow predictions of short term fitness consequences to be made. It has been demonstrated here that integration of four redox biomarkers (GPX, OXY, ROMs and thiols) by PCA correlated with body condition and so could be useful in predicting future fitness outcomes, at least in blue tit nestlings. Yet none of the adult measures of redox integration correlated with body condition; perhaps because sub-optimal redox profiles do not become apparent in adults immediately or my chosen measure of body condition was not suitable here. Alternatively, sub-optimal redox profiles may have no long-term fitness consequences. Future studies could compare various indices of body condition, including those from both morphological and physiological parameters, to investigate which might be best related to redox integration.

Chapter 3

Oxidative costs of growth only become apparent under stressful conditions

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Abstract

Sub-maximal growth rates are prevalent in nature and might suggest accelerated growth comes at a cost. This cost could result from a diversion of resources away from other important processes, like self-maintenance, and it has been suggested that OS might be involved. OS is tissue oxidative damage caused by a build-up of RS that are no longer neutralised by the antioxidants that normally overcome them. Enhanced growth is thought to lead to oxidative damage either by resulting in an increase in metabolic rate and so RS production, or indirectly by diverting resources away from antioxidant protection. Previous work exploring the relationship between growth and OS has found differing results, depending on, for instance, the biomarkers selected. Therefore, the present study experimentally altered blue tit chick growth rates by manipulating brood size and then measured the levels of five biomarkers of OS. My results showed that while between-brood growth differences led to changes in the antioxidant enzyme GPX, there was no corresponding change to oxidative damage levels, suggesting no oxidative costs of growth. However, when considering within-brood growth differences, oxidative protein damage was greater in chicks that had grown faster than their sibs. Furthermore, this pattern was only apparent in the experimental broods, so perhaps oxidative costs of growth only occur in the presence of an additional stressor that utilises antioxidants making them unavailable for any growth-induced rise in RS levels.

3.1 Introduction

Trade-offs form the basis of life-history theory and result from resources being limited, so that individuals cannot invest in all traits at once (Kirkwood & Holliday, 1979). One such trade-off occurs when increased investment in growth negatively impacts on other requirements of the offspring, for instance self-maintenance, and may explain why sub-maximal growth rates are observed, despite the greater vulnerability (e.g. to predators) of individuals during growth (Arendt 1997). Investigation into the mechanism that underlies these physiological and life-history trade-offs is a growing aspect of ecological research and it has been suggested that OS could be involved (von Schantz *et al.* 1999; Isaksson, Sheldon & Uller 2011a; Stier *et al.* 2014b).

OS is defined as a build-up of oxidative damage that results from the production of RS, the majority of which are by-products of metabolism, that cannot be neutralised by the antioxidants that usually overcome them (Yu 1994). This may result from an increase in RS that is not matched by an increase in antioxidants; or a reduction in antioxidant defences (Sies 1997). It has been suggested that enhanced growth can lead to an increase in RS production through a higher metabolic rate (Rosa *et al.* 2008), although the extent to which will depend on the partial oxygen pressure in the surrounding tissue (Barja 2007) and the degree of uncoupling in mitochondria (Stier *et al.* 2014a; Salin *et al.* 2015). A second hypothesis linking enhanced growth to OS is that there is a resource allocation trade-off between growth and antioxidant defences, so that any increase in the former would cause a reduction in OS resistance and so an increase in OS even if RS production is unchanged (Alonso-Álvarez *et al*. 2007). However, the two hypotheses are not likely to be mutually exclusive and may interact to exacerbate effects. For instance, if enhanced growth led to greater RS production as well as a diversion of resources away from antioxidant defences, the RS might be less likely to be neutralised and oxidative damage more likely to occur.

In Chapter 2, I demonstrated that five different biomarkers of OS, including endogenous and exogenous antioxidants, as well as markers of damage and oxidation, provided independent information on the redox system. This is hardly surprising given the variability of the antioxidant response and large range of potential damage markers (Halliwell & Gutteridge 2007), and might explain the inconsistencies in studies that have investigated growth and OS (e.g. Guerra *et al.* 2012; Aziza, Awadin & Orma 2013; Dong *et al*. 2013; Xu & Pan 2014). Thus in order to investigate fully how growth might lead to OS, studies should include multiple measures of both damage and antioxidant molecules.

Furthermore, determination of causality of the OS-growth relationship must involve experimental manipulation of growth rates between groups which can then be compared in terms of OS, since correlational studies can rarely demonstrate trade-offs (van Noordwijk & de Jong 1986). One method of experimentally altering growth rates in young animals is brood/litter manipulations. By altering brood/litter size, growth rate can be changed (e.g. Alonso-Álvarez *et al.* 2007) and the resulting OS biomarkers compared. Compensatory growth might also be induced. This type of growth occurs when an individual's growth rate is accelerated in response to an increase in resources, following a period of nutritional deficit (or alternatively following a growth depression by unusually cold temperatures in ectotherms); compensatory growth can also occur if animals are relatively small for the time of year (e.g. having been born particularly late in the season) (Metcalfe & Monaghan 2001; Dmitriew 2011). There is increasing evidence for the negative impacts of compensatory growth, such as reduced reproductive performance (Morgan & Metcalfe 2001; Lee *et al.* 2012), lifespan (Jennings *et al.* 1999; Lee *et al*. 2013a) and changes to redox homeostasis (De Block & Stoks 2008; Roark *et al.* 2009; Zheng *et al.* 2012).

Relating growth to OS is complicated by factors that might have consequences for growth trajectories. For instance, in birds there may be within-brood hierarchies that can occur as a result of hatching asynchrony (e.g. Magrath 1990), which occurs when eggs in a brood do not hatch close together (typically over more than 24 hours) so that age and size differences occur. Differential maternal investment in eggs (e.g. Saino *et al*. 2011a) and the onset of incubation before clutch completion so that earlier-laid eggs hatch first (e.g. Perrins 1991) can both cause hatching asynchrony. First-hatched nestlings have the potential to initiate fledging, with those that hatched later following closely behind (Nilsson & Svensson 1993). This puts pressure on the late-hatched nestlings to alter growth patterns so they can attain a similar size by fledging; great tit late-hatched chicks were found to reach the same tarsus length as their early-hatched nest mates by the time of fledging, suggesting that they had a greater growth rate (Kilgas *et al*. 2010). Thus different growth trajectories between chicks within a brood, as well as between synchronous and asynchronous broods, can be expected. If there is a hierarchy of growth rates within a brood, then this might mask any differences between broods, therefore within-brood growth differences should be accounted for in analysis of the OS-growth relationship.

Environmental factors that can influence OS also have the potential to impact on the OS-growth relationship. A cross-fostering experiment of wild kestrel nestlings (*Falco tinnunculus*) demonstrated that while 23.5% of the variance in ROMs could be explained by nest of origin, indicating a genetic component of oxidative damage, 52.8% of the

variance in the serum non-enzymatic antioxidant barrier was explained by nest of rearing (Costantini & Dell'Omo 2006a). This implies a strong environmental influence on antioxidant status; this is not surprising given that a number of antioxidants are obtained through the diet (Catoni, Peters & Schaefer 2008), which will depend on environmental quality. Environmental variation might also arise from other factors that can influence nestling antioxidant status, for example, parental foraging skills or brood size (Alonso-Álvarez *et al*. 2006).

An additional effect of the environment on oxidative status can arise at habitat edges (i.e. edge effects). Edge effects can occur as a result of changes to the microclimate that might be associated with a habitat edge, for instance, increased sun exposure at a woodland's edge making temperatures warmer during the day (Murcia 1995), as well as alterations to air/soil moisture and light intensity (Murcia 1995). These changes can lead to changes in species interactions, for example, greater light exposure at a woodland edge may lead to increased plant growth. This in turn could attract herbivorous insects, which attract nesting birds, and so this leads to more predators or brood parasites at such habitat edges (Murcia 1995). Thus environmental quality might be altered as one moves away from a habitat edge (Hinsley, Rothery & Bellamy 1999). Moreover, for species that must match the time of peak food abundance to that when their offspring are most demanding, a mismatch in timing relative to peak prey availability could reduce the period over which food is plentiful and so create costs for offspring. For instance, OS may arise if chicks obtain fewer antioxidants from their diet or as a result of resources being diverted away from antioxidant defence.

The main aim of the present study was to test the hypothesis that enhanced growth leads to OS by comparing OS between groups that have been experimentally manipulated to have different growth trajectories. Experimental alteration of growth trajectories allows determination of causality of the effects of growth on OS; this was achieved using a brood manipulation regime in wild blue tit nestlings. I expected a rise in OS levels, in terms of greater oxidative damage and/or changes to antioxidant levels, in broods that had been experimentally manipulated to grow more quickly. From Chapter 2, it was clear that a wide array of both antioxidant and oxidative damage biomarkers should be included in future analyses since all five biomarkers investigated provided independent information on the redox system. Therefore, all five biomarkers were measured here so that a broad picture of an individual's oxidative status could be obtained, which could then be related to growth. This study included data on within-brood growth differences and hatching synchronicity that can cause variation in growth, allowing a more in-depth investigation

into the OS-growth relationship, since within-brood hierarchies may mask any between-brood growth differences.

3.2 Materials and methods

3.2.1 Field observations and brood manipulation

Field work took place in 2012 in the sessile oak woodland along the eastern shore of Loch Lomond at the university field station, SCENE (see Chapter 2 for details). Weekly nestbox checks were carried out from late March onwards in order to obtain an expected hatching date for each brood, after which nestboxes were visited daily until the first eggs had hatched. The first chick measurements were taken on day 1 (day 0=day of hatching of the first chick) unless there were still unhatched eggs in the nest, in which case they were taken on day 2 (after the last egg had hatched) or 3 (even if there were still unhatched eggs in the nest) and then repeated every three days until day 13 (day 14/15 for the latter). Those nests in which all eggs had hatched by day 1 were defined as synchronous broods, whilst those where first measurements were not taken until day 2 or 3 were defined as asynchronous broods. It is important to note that those first measured on day 3 might have had eggs that never hatched and hence eggs could have hatched synchronously, however this was only the case for three nests and the eggs that did hatch in those nests hatched asynchronously. Chicks were given individual marks on their heads and legs using permanent non-toxic marker pens (Sharpie, Lichfield, UK), weighed to 0.1 g using a Pesola spring balance (10 g capacity) and measured for tarsus length to the nearest 0.5 mm.

Since the study focused on the effect of growth rate on OS, I used a brood manipulation regime to alter growth rates (Figure S2, Appendix 2). Approximately half of the nests in the experiment (26 out of 60) were allocated at random to the experimental treatment; broods in this treatment were enlarged on day 1 (or day 2/3 for asynchronous broods); the remaining nests were assigned to the control treatment. Two newly-hatched chicks taken from a pool of ‘donor’ nests in a neighbouring part of the same woodland not included in the experiment were added to each of the nests in the experimental treatment; there was never more than a one-day age difference between donor and experimental chicks. After six days the two extra chicks were removed and returned to their original nest. At least three chicks were left in the donor nests to ensure the parents would not abandon those nests in the meantime. I expected this treatment to reduce the growth rate of all chicks in the experimental treatment during the period in which experimental broods had two extra chicks, as increasing brood size has been found to reduce nestling growth in this species (Nur 1984c). Once the extra chicks were removed, so also removing the constraints on growth, I predicted that the experimental chicks would exhibit compensatory growth, since similar compensatory growth has been observed in other species (Alonso-Álvarez *et al*.

2007). All chicks from both experimental and control nests were measured on the same days (as described above) so that the level of disturbance was similar. Six chicks per nest were randomly selected to be blood sampled by puncture of the brachial vein (under UK Home Office licence) on the last day of measurements (day 13, 14 or 15 as above) (see Chapter 2 for further details), so that OS biomarkers could be measured. Chicks were all sampled between 08:00 h and 18:30 h and the time of sampling did not have a significant effect on the OS measures.

3.2.2 Environmental factors potentially influencing chick OS

During the breeding season, blue tits are heavily reliant on caterpillars (Lepidopteran larvae) as the main food source given to their chicks (Perrins 1979). Thus the timing of their breeding is crucial, with broods that miss the period of peak caterpillar abundance potentially suffering from reduced body condition and reproductive success (van Noordwijk, McCleery & Perrins 1995). Therefore seasonal change in caterpillar abundance was estimated using caterpillar frass production (Zandt 1994) along six irregularly spaced transects, each consisting of five collectors approximately 50 m apart, situated throughout the study site. The frass collectors were 86 x 110 cm cotton sheets that were placed under the same oak tree branches each year around the time of budburst to collect frass falling from the leaves above for the duration of the breeding season. Frass was collected twice a week (dry weather permitting), dried, sieved to remove non-frass items like seeds, and weighed. Assessment of the seasonal change in the amount of frass allowed determination of the period of peak caterpillar abundance, defined as when frass production peaked; this was estimated as the 26th May (Figure 3:1). To quantify how well parents had matched this peak caterpillar abundance to the period of peak chick demand, I calculated the absolute difference between the day of the caterpillar peak and the day on which chicks were 11 days old (when peak food demand occurs in blue tit broods; Blondel *et al*. 1991) – this variable will henceforth be termed ‘mismatch’. The greater the value for this variable, the greater the mismatch in timing between chick peak food demand and prey peak abundance.

Previous work has found blue tit breeding performance to be reduced in smaller woodland areas with greater edges (Hinsley *et al*. 1999) and so the same topographical feature could have implications for chick growth and OS levels. The nestboxes were located in an area of continuous woodland, but since this woodland ran along the shore of Loch Lomond, this might act as a habitat edge and so there may be differences in environmental quality between those nestboxes that were situated closer to the loch shore and those that were further away. Thus to determine the position of each nestbox relative

to the loch shore, the distance between each nestbox and the closest point on the shoreline was estimated to the nearest tenth of a metre from a map on which all nestboxes had been plotted according to their GPS coordinates.

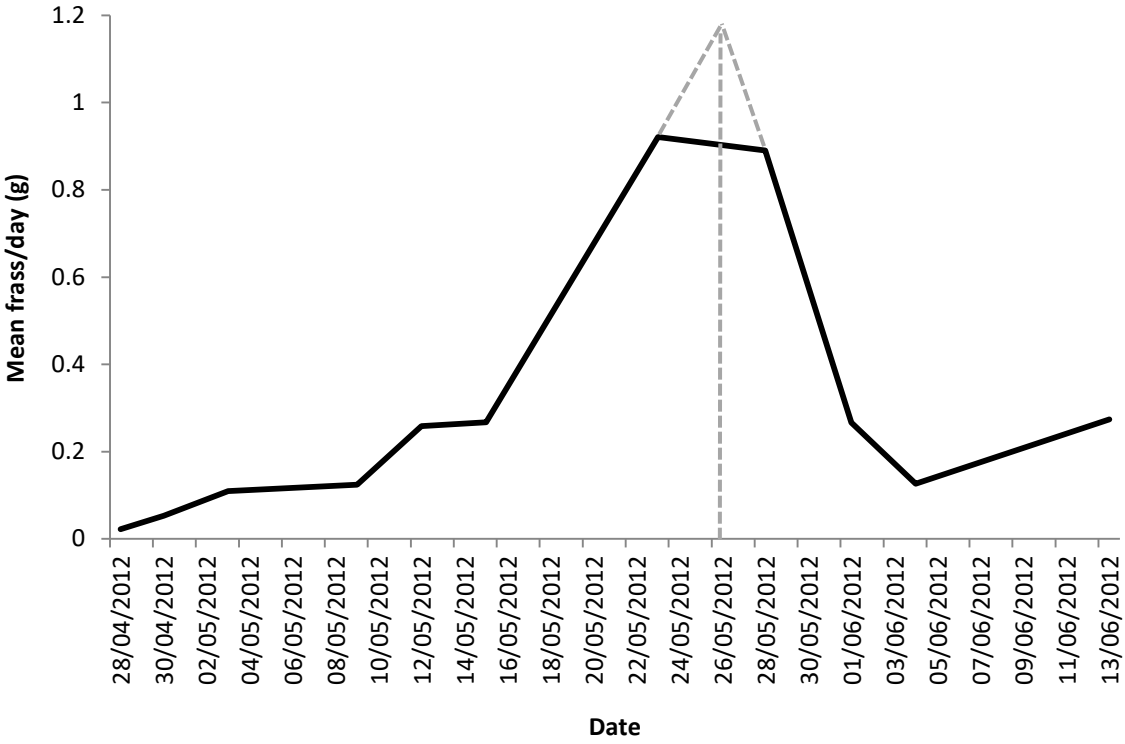


Figure 3:1 Mean frass production over the 2012 breeding season. The peak lay between the 23rd and 28th May; by extrapolating the lines on the graph (see grey dashed lines), the most likely estimate for the peak was the 26th May.

Additionally, territorial differences might affect growth and OS levels through dietary differences. For instance, exogenous antioxidants, as well as the precursor molecules for endogenous antioxidants, are both dependent on dietary intake (Isaksson *et al.* 2011a). When these nutrients are limiting in the environment, antioxidant levels might be insufficient to neutralise RS and OS could result. This has the potential to exacerbate potential OS from increased growth rates. The study site was predominantly comprised of oak woodland, which can be subdivided into two areas depending on the underlying rock and undergrowth type. To the west, Ross Wood (Figure S3, Appendix 2) is dominated by blueberry (*Vaccinium myrtillus*) undergrowth, while Sallochy Wood (Figure S3, Appendix 2) to the South has bluebells (*Hyacinthoides non-scripta*) as its dominant undergrowth. This difference in vegetation could indicate a difference in soil type, which in turn might affect leaf quality and caterpillar abundance. This might have influenced growth and potentially OS levels however this was not found to be the case here and so this habitat difference was not considered further.

3.2.3 Laboratory analyses

When considering which redox biomarkers to analyse, it is important to remember that there are a vast array of damage products that might result from OS (Dotan *et al.* 2004). There is also a large number of potential antioxidant molecules, whose responses and specificities vary (Cohen & McGraw 2009). Therefore, it is essential to include biomarkers of both damage and antioxidant defences (preferably from different groups, e.g. enzymes and non-enzymatic antioxidants) when analysing OS. This is further emphasised by the fact that levels of antioxidants can both be reduced (if they are used up) or increased (if upregulated) in response to increased RS production (Costantini & Verhulst 2009) so it can be difficult to judge whether OS is occurring from measurements of antioxidants alone.

Thus I chose to analyse a wide range of biomarkers from different damage and antioxidant groups which all gave independent information on OS (Chapter 2). The damage biomarker measured was PCs in RBCs (Levine method, Levine *et al*. 1990) produced by oxidative damage to protein molecules, and the general oxidation marker was ROMs (d-ROMs test, Diacron International) in plasma. If enhanced growth led to OS, I expected an increased level of both these biomarkers in the experimental broods that had been manipulated to show compensatory growth, compared with controls. The non-enzymatic antioxidant capacity (OXY-adsorbent test, Diacron International) of the plasma was also measured, in addition to RBC thiol antioxidant molecules (–SHp test, Diacron International). Lastly, the antioxidant enzyme GPX (Ransel assay, RANDOX Laboratories) was measured in RBCs. The protocols for all of these assays are described in detail in Chapter 2. For the three antioxidant measures it is difficult to predict an expected direction, as described in the previous paragraph, so their response must be considered alongside that of the damage biomarkers.

3.2.4 Statistical analysis

When considering if the treatment had successfully altered growth rates, I was interested in mass/tarsus growth during the two experimental periods. Firstly, the six-day period where experimental broods had extra chicks; henceforth this will be termed the early growth period, during which I expected growth to be reduced in the experimental group. Secondly, the three-day linear growth period directly after the extra chicks had been removed from the experimental broods; from now on this will be referred to as the late growth period, during which increased growth was expected in the experimental group, since they were predicted to exhibit compensatory growth.

GLMs were carried out in R (R Core Team 2013) to compare laying date (of the first egg), hatching date (of the first chick), mismatch, clutch size, initial brood size, unhatched eggs (as a measure of hatching success) and percentage of chicks fledged between control and experimental broods, with each variable in turn as the response variable and treatment as an explanatory variable. In a separate analysis, these variables were also compared between synchronous and asynchronous broods.

Mixed effects models (R Core Team 2013; lme4 package, Bates *et al*. 2014) were used to analyse chick growth trajectories, with mass or tarsus in turn as the response variable, age (categorised into the five measurement periods; A=days 1/2/3, B=days 4/5/6, C=days 7/8/9, D=days 10/11/12, E=days 13/14/15; day within each period differed depending on the first day of biometric measurements), treatment (experimental or control) and synchronicity (synchronous or asynchronous broods) as fixed effects and chick identity nested within nestbox identity as random effects. To investigate whether the effect of treatment on growth trajectories was influenced by whether the chicks had hatched synchronously or asynchronously, the three-way interaction between age, treatment and synchronicity was also included. A likelihood ratio test (LRT) was used to determine if this three-way interaction was significant overall, if so, this justified testing the effects of treatment on growth trajectories separately for the synchronous and asynchronous broods, using similar mixed models including the treatment*age interaction to determine if the experimental treatment had successfully altered chick growth trajectories. The final models were checked for residual normality, a lack of heterogeneity and potential influential data points (identified by a Cook's distance of greater than n/4; Bollen & Jackman 1985). There were two potential outliers for the asynchronous broods and so the models were re-run excluding these data; on no occasion did this qualitatively alter the results and so presented results always include all data points.

Since within-brood growth differences might mask any overall between-brood differences, these should be accounted for in analyses of the OS-growth relationship. To do so, chicks within a brood were rated according to their mass and tarsus growth during the late growth period and categorised into three groups: high growth (defined as the two fastest growing chicks in a brood), low growth (the two slowest growing chicks in a brood) and mid growth (all the remaining chicks); these relative measures of growth rate were termed relative growth^{M} and relative growth^{T} for mass and tarsus growth ratings during the late growth period, respectively.

To investigate whether differences in growth trajectories affected OS, further mixed effects models were applied with each OS biomarker in turn as the response variable (note that

multivariate analysis was not used since the low level of integration among biomarkers observed in Chapter 2 implied a univariate approach would serve better for my study population). To improve residual normality and reduce heterogeneity, some of the variables were transformed: PCs were cubic root-transformed and thiols and OXY were log-transformed. The fixed effects were relative growth^{M} (high, mid, low), treatment (control or experimental), distance from the loch shore, mismatch and synchronicity (synchronous, asynchronous). Nestbox identity was included as a random effect. Since the effect of treatment on growth trajectories was found to differ between synchronous and asynchronous broods, I included the treatment*synchronicity interaction in the OS models. This would allow the determination of differences in OS where there had been differences in growth. As well as treatment*synchronicity, I also investigated individually the interactions of treatment with relative growth^{M}, mismatch and distance from the loch shore, and between mismatch and synchronicity/relative growth^{M}. The models were repeated replacing relative growth^{M} with relative growth^{T} to see if within-brood tarsus growth differences might affect OS but this was never significant. For GPX, the relationship with mismatch was non-linear, so the model included a quadratic term for mismatch. GPX was found to decrease with mismatch up to a mismatch of approximately six days and increase with mismatch if the mismatch was greater than this. To obtain a specific estimate for this break point between the two relationships, a broken-stick regression with GPX as the response and mismatch the explanatory variable was run using the segmented package in R (Vito & Muggeo 2003, 2008). Then the two relationships (i.e. only including values either less or greater than this break point) were tested separately in mixed models, with GPX as the response variable and treatment, synchronicity, mismatch and the treatment*synchronicity interaction that had been significant in the original model as fixed effects and nestbox identity as a random effect.

Backward stepwise deletion of non-significant terms, starting with interactions (only significant interactions are reported) simplified the models, using an LRT to compare the fit of the full and reduced model at each stage. Model checks were carried out as before, with one influential data point being detected for the GPX model but having no influence on the results when removed. To confirm the robustness of the statistical models I compared the significant terms between full and final models – in all cases where a variable was significant in the final model, it had also been significant in the full model.

3.3 Results

3.3.1 Descriptive statistics

Descriptive statistics for the two treatment groups are given in Table 3:1. There was no significant difference in laying date, hatching date, mismatch, clutch size, initial brood size, number of unhatched eggs or percentage of chicks fledged between control and experimental nests (Table 3:1 A). Parents of asynchronous broods started and finished laying eggs significantly later than those of synchronous broods (Table 3:1 B), but there was no significant difference in clutch size or date of hatching of the first egg (Table 3:1 B); this implies that parents of asynchronous broods started incubation before clutch completion, so causing their chicks to hatch asynchronously. Since synchronous broods were defined as having all eggs hatched by day 1, the number of unhatched eggs was zero and so significantly less than that for asynchronous broods. Furthermore, asynchronous broods were no more likely to miss the caterpillar peak, as mismatch was not significantly different between the two groups (Table 3:1 B). Initial brood size and percentage of chicks fledged also did not differ significantly between synchronous and asynchronous broods (Table 3:1 B). In addition, the proportion of synchronous (n=14 control and 6 experimental nests) and asynchronous (n=20 control and 20 experimental nests) broods was comparable between control and experimental groups (from GLM with binomial errors; $z=1.46$, $p=0.15$).

Table 3:1 Descriptive statistics (mean±SE) and GLM results comparing control (n=34 nests) and experimental (n=26 nests) broods (A), as well as synchronous (n=20 nests) with asynchronous (n=40 nests) broods (B). Laying and hatching dates are relative to 1st April; so that 1st April=1, 1st May=31, and so on, allowing dates to be compared statistically.

A	**Laying date**	**Hatching date**	**Mismatch (days)**	**Clutch size**	**Initial brood size**	**No. unhatched eggs**	**Percentage fledged**
Control	28.6±0.8	50.4±0.6	5.7±0.6	8.5±0.3	7.8±0.3	0.7±0.2	82.5±5.5
Experimental	28.8±1.0	50.4±0.6	5.5±0.7	8.5±0.4	7.8±0.4	0.5±0.2	92.1±3.3
t	0.17	0.02	0.18	0.06	0.05	0.63	1.41
p	0.86	0.99	0.86	0.95	0.96	0.53	0.17

B	**Laying date**	**Hatching date**	**Mismatch (days)**	**Clutch size**	**Initial brood size**	**No. unhatched eggs**	**Percentage fledged**
Synchronous	26.7±0.8	49.4±0.7	4.8±0.6	8.7±0.4	8.3±0.4	0.0±0.0	91.8±4.3
Asynchronous	29.8±0.8	51.0±0.5	6.0±0.6	8.4±0.3	7.5±0.3	0.9±0.2	84.2±4.7
t	2.54	1.78	1.30	0.54	1.45	3.85	1.07
p	**0.01**	0.08	0.20	0.59	0.15	**0.0003**	0.29

3.3.2 Testing treatment assumptions

The three-way interaction between treatment, age and synchronicity had a significant effect on both mass and tarsus growth (mass: χ^2=15.22, df=4, p=0.004; tarsus: χ^2=66.00, df=4, p<0.0001; n=60 nests, 447 chicks, 2139 measurements for mass and tarsus each) which means that the effect of treatment on growth trajectories was influenced by whether or not the broods had hatched asynchronously. This justified the separation of synchronous and asynchronous broods for further investigation of how growth trajectories might have been affected by the experimental treatment. The interaction between treatment and age was statistically significant for both mass and tarsus growth in synchronous (mass: χ^2=21.61, df=4, p=0.0002; tarsus: χ^2=48.81, df=4, p<0.0001; n=20 nests, 161 chicks, 777 measurements for mass and tarsus each) and asynchronous (mass: χ^2=12.56, df=4, p=0.01; tarsus: χ^2=18.40, df=4, p=0.001; n=40 nests, 286 chicks, 1362 measurements of both mass and tarsus) broods. However, the nature of the effect of treatment on growth trajectories was not the same for synchronous and asynchronous broods. For synchronous broods (Figure 3:2 A&C), experimental chicks grew faster between 1 and 7 days old (i.e. the early growth period), and then more slowly between 7 and 10 days old (i.e. the late growth period), after the extra chicks were removed from experimental nests. The asynchronous broods showed the reverse pattern (Figure 3:2 B&D): the experimental chicks' mass and tarsus grew more slowly than the control group during the early growth period, after which experimental chick tarsus, but not mass, growth increased so that tarsus growth was faster than control chicks during the late growth period.

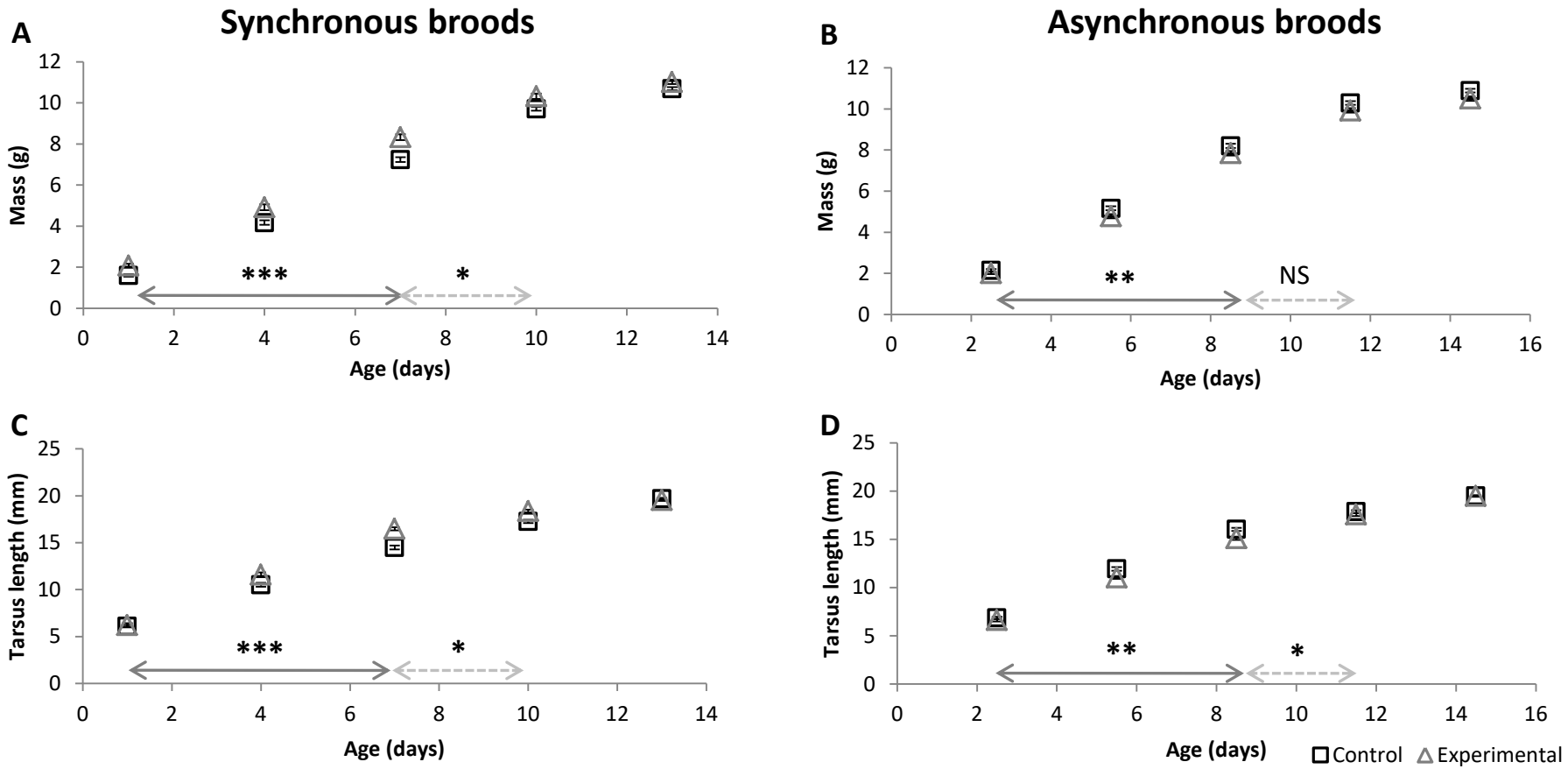


Figure 3:2 Comparison of mass (A & B) and tarsus (C & D) growth over the nestling period between control and experimental (had two extra chicks during the early growth period, which were removed at the start of the late growth period) groups for synchronous (A & C) and asynchronous (B & D) broods. ⟷ denotes the early growth period and ⇢⇠ denotes the late growth period. * denotes a significant treatment-by-age interaction when the early and late growth periods were tested separately, with * $p<0.05$, ** $p<0.01$ and *** $p<0.001$; NS is not significant. If significant, this indicates that the growth slopes were significantly different between control and experimental broods for that period. Means±SE are given; due to the large sample size the error bars are very small and can be viewed inside the squares (control) and triangles (experimental).

3.3.3 Effect of growth rate on OS

When considering whether known growth differences alter redox state, I included the treatment-by-synchronicity interaction in the OS models since growth trajectories had been found to be different between control and experimental groups and the effect differed between synchronous and asynchronous broods. The treatment-by-synchronicity interaction was only significant for GPX, implying it was affected by the experimental manipulation of growth trajectories (Table 3:2 A). GPX was significantly higher in the experimental group than the controls, but only within synchronous broods (Figure 3:3 A), which was the group where experimental chicks showed accelerated growth during the early growth period when they had two extra chicks; whilst control chicks had greater growth rates during the late growth period. The treatment-by-synchronicity interaction was not significant for any of the other biomarkers (Figure 3:3 B-E), implying that the observed differences in growth between control and experimental groups of synchronous and asynchronous broods did not alter these components of the redox system.

Differences in growth trajectories within a brood (relative growth^{M}) were found to affect PC levels, although this depended on treatment (Table 3:2 D): PCs tended to be higher in experimental compared with control broods, but only in the chicks growing fastest within each brood (Figure 3:4), though this difference was not statistically significant. Additionally, these fast-growing chicks in experimental broods had significantly higher PC levels than their slower growing sibs (Figure 3:4). In terms of whether the treatment alone affected OS levels, only thiols differed significantly, with the experimental group having higher thiol levels than controls (although this effect was borderline; Table 3:2 B and Figure 3:3 B).

Synchronicity had a significant effect on both damage biomarkers (Table 3:2 D&E); PCs (Figure 3:3 D) and ROMs (Figure 3:3 E) were significantly higher in asynchronous than synchronous broods. In terms of environmental effects, only PCs were affected by distance of the nest from the loch shore (Table 3:2 D), increasing as one moved further away from the loch shore (Figure 3:5). GPX and thiols were related to mismatch (Table 3:2 A&B), although the relationships varied. GPX showed a non-linear relationship and declined slightly but significantly with mismatch up to a mismatch of approximately six days (exact estimate given from the broken-stick regression was 5.87±0.56 days) and increased significantly with mismatch when this was greater than six days (Figure 3:6 A), while thiols declined slightly but significantly with increasing mismatch (Figure 3:6 B).

Table 3:2 Results from mixed models investigating whether differences in growth (including within-brood effects) altered redox state. Parameter estimates are only included for those variables that were present in the final model and t/p values of non-significant terms were taken from the last model that included each variable, during model simplification. LRTs were used to test the significance of interactions; only significant interactions are shown. The relationship between GPX and mismatch was non-linear so a quadratic term is included.

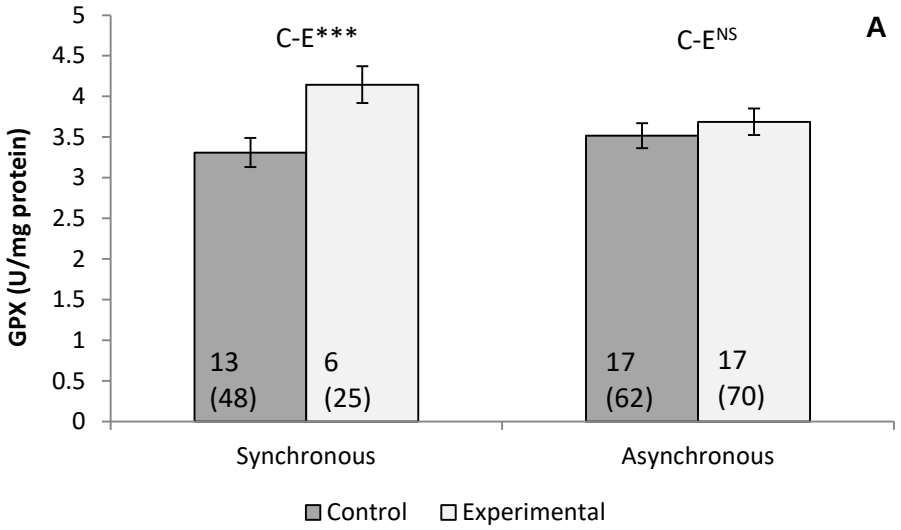
A. GPX, n=53 nests (205 chicks)	**Estimate**	**SE**	**t**	**p**
Relative growth^{M} (low)			0.17	0.86
Relative growth^{M} (mid)			1.55	0.12
Treatment	0.05	0.27	0.19	0.85
Distance from loch shore (m)			0.48	0.63
Mismatch (days)	-0.43	0.13	3.33	**0.001**
Mismatch squared	0.04	0.01	3.87	**0.0001**
Synchronicity	-0.32	0.30	1.07	0.29
Treatment*Synchronicity (LRT)	**χ^2**	**df**	**p**	
	4.95	1	**0.03**	

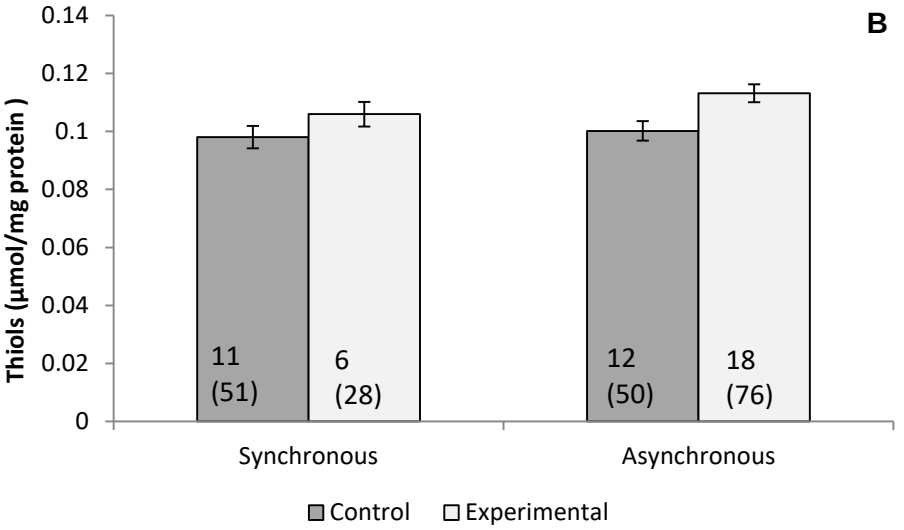
B. Thiols, n=47 nests (205 chicks)	**Estimate**	**SE**	**t**	**p**
Relative growth^{M} (low)			0.72	0.47
Relative growth^{M} (mid)			1.06	0.29
Treatment	0.11	0.06	1.92	**0.05**
Distance from loch shore (m)			0.79	0.43
Mismatch (days)	-0.02	0.01	2.16	**0.03**
Synchronicity			0.95	0.34

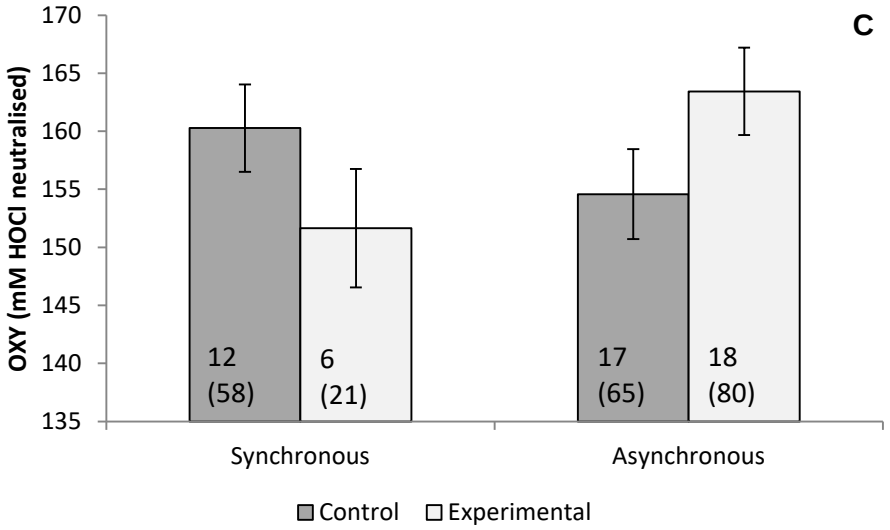
C. OXY, n=53 nests (224 chicks)	**Estimate**	**SE**	**t**	**p**
Relative growth^{M} (low)	-0.04	0.03	1.52	0.13
Relative growth^{M} (mid)	-0.01	0.03	0.50	0.62
Treatment			1.17	0.24
Distance from loch shore (m)			1.20	0.23
Mismatch (days)			0.09	0.93
Synchronicity			0.30	0.76

D. PCs, n=55 nests (318 chicks)	**Estimate**	**SE**	**t**	**p**
Relative growth^{M} (low)	0.09	0.07	1.33	0.18
Relative growth^{M} (mid)	0.16	0.07	2.23	**0.03**
Treatment	0.21	0.09	2.52	**0.01**
Distance from loch shore (m)	0.001	0.0006	2.14	**0.03**
Mismatch (days)			1.35	0.18
Synchronicity	-0.13	0.06	2.13	**0.03**
Treatment* Relative growth^{M} (LRT)	**χ^2**	**df**	**p**	
	10.19	2	**0.006**	

E. ROMs, n=42 nests (111 chicks)	**Estimate**	**SE**	**t**	**p**
Relative growth^{M} (low)			0.42	0.67
Relative growth^{M} (mid)			0.22	0.83
Treatment			0.61	0.54
Distance from loch shore (m)			1.24	0.21
Mismatch (days)			0.54	0.59
Synchronicity	-0.06	0.03	2.31	**0.02**


A
C-E***
C-E^NS
GPX (U/mg protein)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
13
(48)
6
(25)
17
(62)
17
(70)
Synchronous
Asynchronous
Control
Experimental


B
Thiols (µmol/mg protein)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
11
(51)
6
(28)
12
(50)
18
(76)
Synchronous
Asynchronous
Control
Experimental


C
OXY (mM HOCl neutralised)
170
165
160
155
150
145
140
135
12
(58)
6
(21)
17
(65)
18
(80)
Synchronous
Asynchronous
Control
Experimental

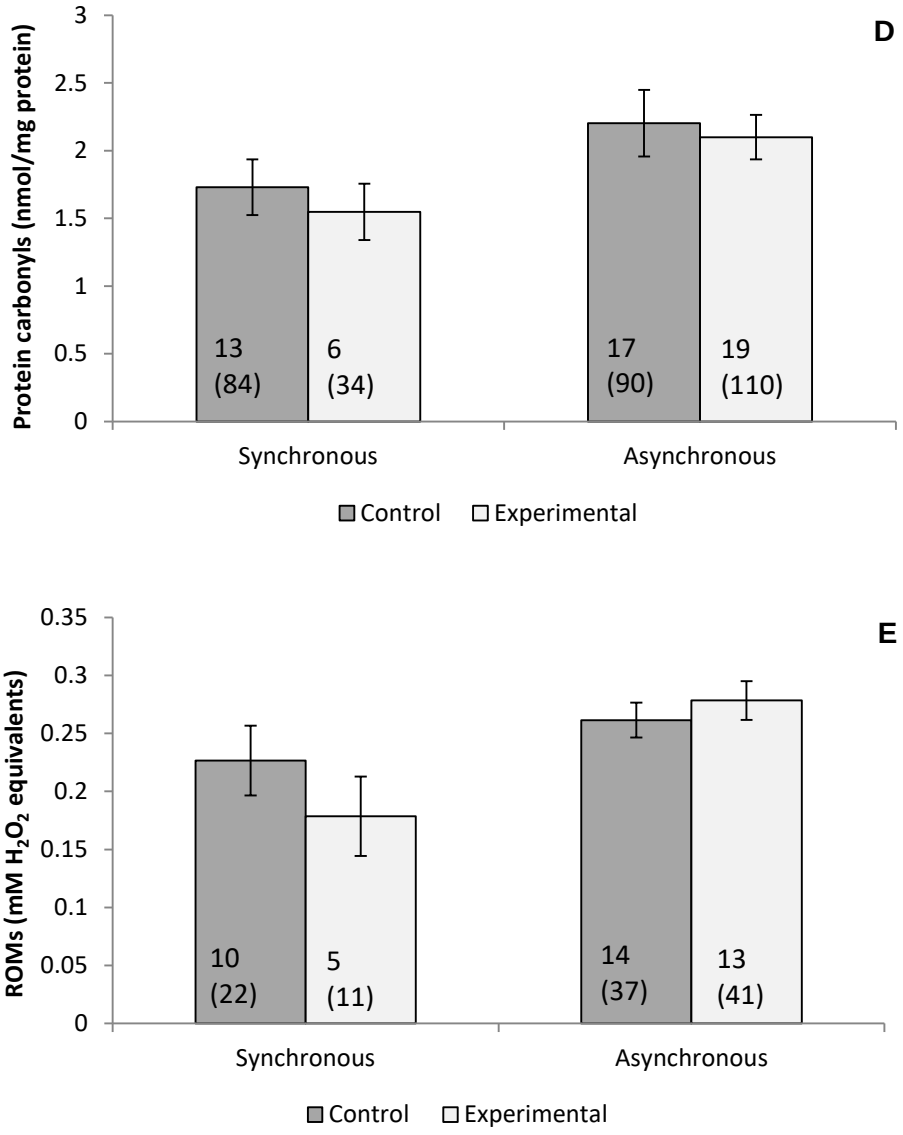


Figure 3:3 Treatment effects on each OS biomarker (GPX (A), thiols (B), OXY (C), PCs (D) and ROMs (E)) within synchronous and asynchronous broods. The treatment*synchronicity interaction was only significant for GPX, where *** indicates a significant difference between control and experimental broods (C-E) at $p<0.001$; ^{NS} is not significant (taken from mixed models with GPX as the response, nestbox identity as a random effect and treatment (for synchronous and asynchronous broods separately) and mismatch (since this had been significant in the original model) as fixed effects). Sample sizes on the bars are number of broods (number of chicks). Means ± SE are given.

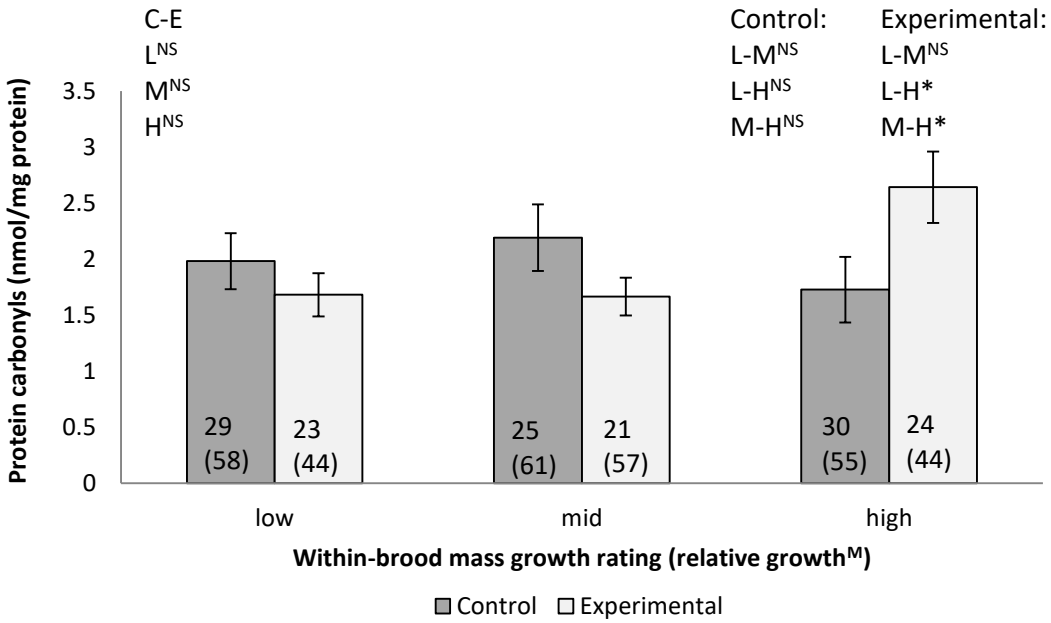


Figure 3:4 Mean PC levels (± SE) within each category of relative growth^{M} for control and experimental broods. Sample size is number of broods (number of chicks). * Indicates significant difference between low and mid (L-M), low and high (L-H) or mid and high (M-H) growth ratings, or between control and experimental broods (C-E), where * is $p<0.05$; ^{NS} is not significant. Results are taken from individual mixed models with PCs as the response variable, nestbox identity as a random effect and treatment (for each growth rating separately) or relative growth^{M} (for control and experimental broods separately) as fixed effects. Distance from the loch shore and synchronicity were also included as they had been significant in the original model.

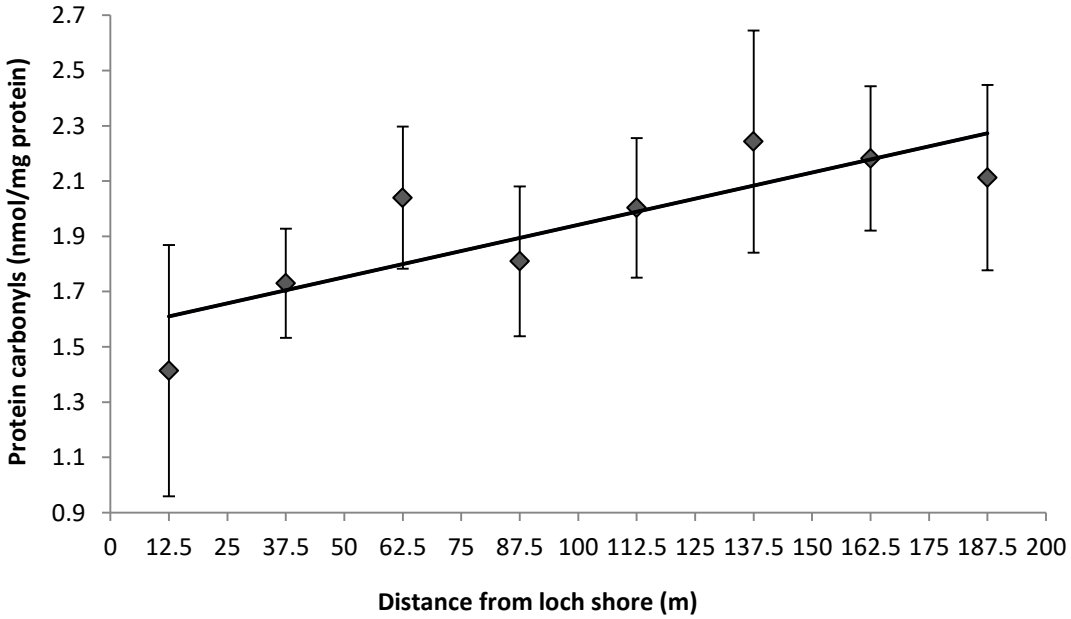


Figure 3:5 Relationship between PCs and distance of the nestbox from the loch shore. For graphical presentation, distance has been grouped into 25 m categories and the mean±1 SE have been plotted for each category. n=55 nests; 318 chicks.

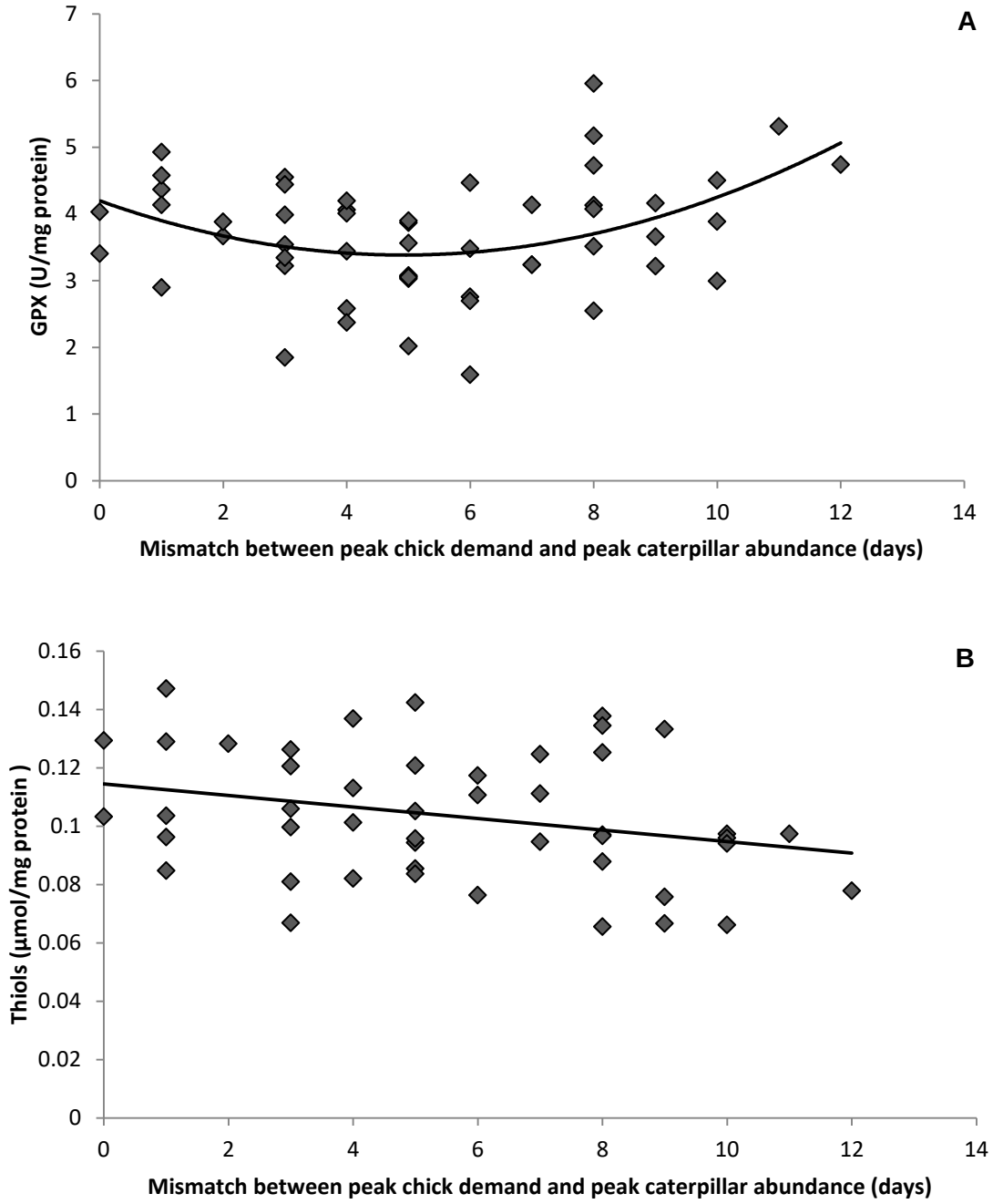


Figure 3:6 Relationship of GPX (A) and thiols (B) with the mismatch of peak chick demand with caterpillar peak abundance. For GPX (A), a broken-stick regression gave the break point between the negative and positive relationships as 5.87±0.56 days. Both the negative relationship from 0 to 5.87 days (parameter estimate=-0.23±0.08, t=2.82, p=0.005) and the positive relationship between 5.87 and 12 days (parameter estimate=0.36±0.12, t=2.97, p=0.003) were found to be statistically significant. Data points are brood means; A: n=47 nests; 205 chicks; B: n=53 nests; 205 chicks.

3.4 Discussion

I have demonstrated that experimentally altered growth trajectories can affect oxidative status. Greater GPX levels were found in the experimental group that had accelerated growth during the early part of the experiment when they had been enlarged, though this group had slower growth than controls once extra chicks had been removed. It is unclear, therefore, if the enhanced early growth in the experimental group led to greater GPX levels or whether lower GPX levels in controls have resulted from their faster growth during the latter part of the experiment. Either way it implies GPX responded to changes in growth trajectories but this was not accompanied by greater oxidative damage levels so these growth changes have not led to OS. However, there were costs of accelerated growth when within-brood growth differences were taken into account but these did not become apparent unless the enhanced growth was combined with another stressor, namely enlarged brood size. The fastest growing chicks in a brood thus had greater protein damage levels than their slower growing sibs, but only in the experimental group.

The experimental manipulation of brood size successfully altered the growth trajectories of blue tit nestlings, but the effect differed according to the hatching synchronicity of the nest. In synchronous broods, the experimental chicks showed the opposite pattern to that expected, growing more quickly than controls during the early growth period when there were extra chicks, but slowing down once the extra chicks had been removed. Potentially, the increase in brood size caused an increase in begging intensity in the experimental broods; as has been demonstrated in great tits (Neuenschwander *et al*. 2003). In blue tits, parents are highly responsive to the begging cues of their chicks (Grieco 2001) so the parents of synchronous broods in the present experiment may have over-compensated for the greater begging intensity of their enlarged broods, allowing chicks to grow more quickly. Alternatively, thermoregulatory costs might be reduced in enlarged broods due to insulation provided by a larger number of nest mates (Neuenschwander *et al*. 2003) allowing the experimental broods to grow faster. When the extra chicks were removed there may have been a reduced provisioning rate (due to satiated chicks and a reduction in brood size) and/or an increase in thermoregulatory costs, resulting in reduced growth rate so there was no difference in mass or tarsus between experimental groups by the time of fledging.

However, this was not the pattern observed in asynchronous broods. In fact, they showed the predicted pattern, with a reduction in growth when extra chicks were present, followed by an increase in tarsus (although not mass) growth once the brood was returned to its original size. This implies that the parents of asynchronous broods in the experimental

group did not fully compensate for the larger brood size by increasing provisioning rate, so each nestling received less food than controls. The lack of compensation in these parents is intriguing. Female blue tits cannot store in advance all the nutrients that they will require for egg laying, so must be able to find abundant food at this time; if food is not sufficiently abundant here the onset of egg laying could be delayed (Perrins 1970), leading to asynchronous broods. This might be more likely if females have a reduced foraging ability (e.g. due to reduced quality or experience (Perrins 1970)), in which case they also may not be able to compensate for extra chicks by increasing provisioning. The foraging ability of these females might be further reduced by the overlap of laying and incubation that must have occurred in asynchronous broods as these females started incubation before clutch completion. Once the extra chicks (and hence the constraints on growth) were removed from asynchronous broods, each chick would receive more food, which might have allowed experimental chicks to increase growth rate in an effort to catch up with controls, but only in terms of tarsus growth. Perhaps chicks prioritise tarsus growth to obtain a competitive advantage over nest mates with smaller tarsi, as (skeletally) bigger chicks might receive more food (Price & Ydenberg 1995).

Since I was interested in whether growth trajectories affected an individual's oxidative status, I looked for differences in the OS variables that corresponded to differences in growth rate. Growth differed between control and experimental groups but the effect of treatment depended on hatching synchronicity, so I investigated the treatment-by-synchronicity interaction in the OS models. This was significant for GPX, which was greater in the experimental group of synchronous broods, which had accelerated growth during the early part of the experiment when they had two extra chicks but reduced growth once the brood was returned to its original size. It is difficult to determine if GPX had been upregulated in response to increased RS production as a result of faster early growth in the experimental group, or whether lower GPX levels occurred in the control group because their growth was faster in the second part of the experiment. In the case of the latter, GPX levels might have been reduced by mopping up the RS that had been produced in response to the increased growth, or alternatively, enhanced growth may have caused resources to be diverted away from antioxidant defence.

It is important to consider that the greater GPX levels in the experimental group of synchronous broods could be unrelated to growth. Firstly, upregulated GPX levels might have been a response to the increased competition and begging intensity resulting from the enlarged brood size, which has previously been linked to OS (Costantini *et al.* 2006, 2010a), although this interpretation is not supported by the fact that asynchronous broods did not have increased GPX in the experimental group. Alternatively, if parents of

synchronous broods had over-compensated their provisioning when they had extra chicks, then this might have increased the supply of nutrients from the diet, allowing GPX to be upregulated, as has been demonstrated in broiler chickens (*Gallus gallus domesticus*) that were supplemented with vitamin C and E (Cinar *et al*. 2014). In this case we would expect dietary antioxidants to be greater in this group, but there is no evidence for this since the interaction between treatment and synchronicity was not significant for OXY (which includes the dietary antioxidant component of the antioxidant defences).

Furthermore, the raised GPX levels in experimental chicks of synchronous broods might be expected to be associated with changes to thiol levels, because glutathione is a cofactor of GPX (Dickinson & Forman 2002). However, predicting the direction of the effect on thiols is difficult, since glutathione has been shown to have different dynamics to other biomarkers of OS (i.e. ROMs and OXY) in great tits (Isaksson 2013). GPX activity might reduce thiol availability, leading to a negative relationship between the two molecules (Brown-Borg & Rakoczy 2003), which I demonstrated in chapter 2, but previous work has found a positive relationship here (Costantini *et al.* 2011b) In any case, the treatment-by-synchronicity interaction was not significant for thiols, suggesting growth differences have not affected this parameter.

Despite alteration to GPX levels in response to changing growth trajectories, there was no effect on either the measure of damage or general oxidation (PCs or ROMs, respectively) as the treatment-by-synchronicity interaction was not significant. It is possible that any increased RS production from enhanced early growth in the experimental group of synchronous broods was neutralised by raised GPX levels or other antioxidants not measured here, so that OS did not occur. Alternatively, in the case of controls where GPX levels were lowered (possibly in response to faster growth later on), the lack of raised damage levels suggests that GPX might have been utilised in overcoming increased RS levels (thus avoiding OS) rather than being reduced as antioxidant resources are diverted away due to increased growth. Finally, it must be noted that the sample size for growth was rather large (n=161 chicks from 20 synchronous nests; n=286 chicks from 40 asynchronous nests) so interactions were significant, despite small effects (Cohen's w was less than 0.3 for all the comparisons of growth slopes between control and experimental chicks during the late growth period). Therefore, the effects on growth rate may not have been biologically relevant; hence oxidative damage was not affected. The weak effects on growth I observed are unexpected, given that previous research has found blue tit chicks from experimentally enlarged broods to be smaller by fledging (Nur 1984c; Kunz & Ekman, 2000), suggesting reduced growth rate. Addition of extra chicks (i.e. three instead of two) is debatable because there is no guarantee parents would cope

– for instance, the smallest natural brood size in my study was four, so this would almost double it. As the effect on compensatory growth was not strong, instead of increasing the number of chicks, future work could leave the extra chicks for the duration of the nestling period to potentially get a stronger effect on overall growth.

The apparent lack of a treatment effect of growth rate on oxidative damage might suggest that enhanced growth does not divert resources from antioxidant defence or cause an increase in metabolism that is sufficient to raise RS levels to cause OS, but so far we have only considered between- and not within-brood growth differences. In fact, growth differences could occur as a result of within-brood hierarchies (Magrath 1990) so it is important to consider these differences in relation to OS. Indeed, the damage molecule PCs were significantly related to within-brood mass growth rating, but this depended on treatment. Within the experimental group, PC levels were significantly higher in the fastest-growing chicks, compared with sibs that had grown more slowly. This implies that there were costs of enhanced growth for the experimental chicks, in terms of OS. The faster-growing control chicks, compared to experimental chicks, did not show this pattern, suggesting that the oxidative costs of enhanced growth only become apparent when another stressor, in this case an enlarged brood during the first half of the nestling period, is present. Previous research has linked greater brood size to OS (Costantini *et al*. 2006, 2010a) and it might be that the stress of an increased brood size (e.g. from greater competition) utilises antioxidant defences, which are then unavailable when RS production rises with enhanced growth – thus damage is increased. It is interesting that I did not find this effect with within-brood tarsus growth, suggesting skeletal growth may be less detrimental in terms of OS.

If OS only becomes apparent under stressful conditions, then it may be that in addition to there being oxidative costs of growth only in the experimental broods that were enlarged for the first part of the experiment, we might only see oxidative costs of other variables in this group too. For instance, there may be raised OS levels in the experimental group only in those broods that were more likely to miss the caterpillar peak (i.e. those with a higher value for mismatch) or for those that were closer to the loch shore. However, there were no significant interactions between treatment and either mismatch or distance from the loch shore for any of the OS measures (as tested in the OS mixed models described in Section 3.2.4; $p>0.05$ for all the treatment-by-mismatch and treatment-by-distance from the loch shore interactions). At the same time, it might be possible that any within-brood effects are intensified by within-territory differences in caterpillar abundance, with stronger effects whereby parents missed the caterpillar peak relative to local trees. Though total frass production was found to vary substantially between different areas of the study site

in 2012, the time of caterpillar peak abundance was less variable, with the majority occurring over two sampling days (MacLeod 2012). Additionally, even if there was strong individual variation in tree phenology, over the breeding season blue tits defend territories of, on average, approximately 40m in radius (Dhondt *et al*. 1982), so it likely that they forage over a large range.

Mistiming breeding in relation to peak caterpillar abundance also did not increase oxidative damage levels in chicks. However, GPX and thiols were both related to this mismatch, but they showed different patterns despite both being antioxidants. The significant increase in GPX in chicks in broods that missed the caterpillar peak by more than approximately six days might be attributed to an increase in GPX activity, suggesting a redox response is initiated below a certain threshold of caterpillar availability. There are a number of reasons why lower caterpillar numbers might result in an initiation of a redox response; for instance, reduced food availability making dietary antioxidants less available to counteract RS. The declining thiol levels with increasing mismatch provide some support for this interpretation, since thiols are used up as a cofactor as GPX levels increase.

For the second environmental variable, PCs were found to be positively related to distance from the loch shore, though this implies that habitats further away from the loch shore were of lower quality. The lack of spatial heterogeneity detected in ambient temperature during the breeding season (mixed model with mean daily temperature as the response variable, distance from the loch shore as a fixed effect and date as a random effect to account for the change in temperature across the season; $t=0.45$, $p=0.65$) and lack of significant difference in caterpillar abundance with distance from the loch shore (Macleod 2012) in the current study make it difficult to determine the cause of this difference in habitat quality. Possibly the antioxidant content of caterpillars at the loch shore was higher, allowing a greater antioxidant intake and thus lower PC levels. Caterpillar antioxidant content was not measured in this study, but has previously been found to vary spatially (Isaksson & Andersson 2007).

Another variable that could affect the levels of oxidative damage is hatching synchronicity, as both the measure of damage and general oxidation (PCs and ROMs, respectively) were significantly higher in asynchronous than synchronous broods, regardless of treatment. Again this could be due to lower foraging ability of parents of asynchronous broods, leading to them bringing back fewer or lower quality prey items with lower antioxidant content resulting in greater damage levels in their chicks. As previously discussed, a lower foraging ability might stem from reduced experience or quality of

parents of asynchronous broods (Perrins 1970). As endogenous parts of the redox system have been shown to exhibit some degree of heritability (Kahar *et al*. 2016; Lopez-Arrabe *et al*. 2016), it is possible that these lower quality parents had compromised antioxidant defences, which were then reflected in chicks leading to greater oxidative damage levels. Unfortunately, I could not test this because I did not measure the OS of parents in the same year as chicks. It is also important to point out that synchronous and asynchronous broods differed in the age at which blood samples were taken from chicks, with synchronous broods being sampled a day (or two if compared with asynchronous broods first measured at day 3) earlier. However, this age difference is not considered to be large enough to cause detectable natural variation in OS.

In conclusion, due to the existence of within-brood hierarchies in growth rates, attempting to make a connection between growth and OS can prove problematic if between-brood effects are masked. Though I did find a response of the antioxidant enzyme GPX to changes in growth trajectories, this was difficult to interpret and was not accompanied by changes to other antioxidants nor raised oxidative damage. These results might lead to the conclusion that there are no oxidative costs of growth, yet I found oxidative protein damage to be greater in those chicks that had grown faster than their sibs. Furthermore, the fact that this pattern was only observed in the experimental group suggests that these costs might only become apparent when enhanced growth is combined with another stressor that uses up antioxidants and renders them unavailable to combat growth-induced RS production. The reason for the lack of effect growth had on OS in previous work may arise from ideal laboratory conditions where there are no additional pressures on an individual's antioxidant resources. However, in general, studies that have attempted to link growth with OS have produced variable results; for this reason, I suggest a meta-analytic review to summarise previous findings and investigate whether a consistent relationship between OS and growth exists.

Chapter 4

Meta-analysis indicates that oxidative stress is both a constraint on and a cost of growth

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Abstract

OS as a proximate mechanism for life-history trade-offs is widespread in the literature. One such resource allocation trade-off involves growth rate and theory suggests that OS might act as both a constraint on and a cost of growth, yet studies investigating this have produced conflicting results. Here I use meta-analysis to investigate whether increased OS levels impact on growth (OS as a constraint on growth) and if greater growth rates can increase OS (OS as a cost of growth). The role of OS as a constraint on growth was supported by the meta-analysis. Greater OS, in terms of either increased damage or reduced levels of antioxidants, was associated with reduced growth although the effect depended on the experimental manipulation used. My results also support an oxidative cost of growth, at least in terms of increased oxidative damage, though faster growth was not associated with a change in antioxidant levels. These findings that OS can act as a constraint on growth support theoretical links between OS and animal life-histories and provide evidence for a growth – self-maintenance trade-off. Furthermore, the apparent oxidative costs of growth imply individuals cannot alter this trade-off when faced with enhanced growth. I offer a starting platform for future research and recommend the use of oxidative damage biomarkers in non-lethal tissue to investigate the growth-OS relationship further.

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4.1 Introduction

Animals do not appear to grow at the maximum rate (Blanckenhorn 2000) which is peculiar given the potential benefits of reaching an increased size quickly, including reduced predation risk, earlier time to sexual maturity and so increased lifetime reproductive success (Dmitriew 2011). This implies there must be constraints on rapid or accelerated growth through a resource allocation trade-off where energetically expensive growth causes resources to be diverted away from other processes like physiological development (Metcalfe & Monaghan 2003). If individuals are not able to completely adjust that trade-off it may result in costs of rapid growth in terms of reduced self-maintenance.

Growth is an energetically demanding process that diverts resources away from self-maintenance processes. One of these processes that can be negatively affected in faster growing individuals is the level of antioxidant protection (Alonso-Álvarez *et al.* 2007). An alternative mechanism by which growth might relate to self-maintenance processes is that the increased cellular activity needed for enhanced growth leads to increased production of RS as a by-product of metabolism (Mangel & Munch 2005; Dmitriew 2011). Indeed there are several studies linking increased growth rate with increased metabolic rate (Criscuolo *et al.* 2008; Careau *et al.* 2013; Stier *et al*. 2014b) and daily energy expenditure (Careau *et al.* 2013). Increased metabolic rate can increase RS production (Mangel & Munch 2005; Dmitriew 2011; but see Barja 2007; Fletcher *et al.* 2013; Stier *et al*. 2014a; Salin *et al.* 2015 for a debate) – when this occurs antioxidants will be mobilised or upregulated in order to neutralise the RS (Yu 1994). If there is an imbalance between the levels of RS and antioxidant protection this can result in oxidative damage to tissues (Yu 1994) and a state of OS. It has been suggested that OS might play a key role as a constraint on, and cost of, growth (von Schantz *et al.* 1999; Monaghan *et al.* 2009; Costantini *et al*. 2010b; Costantini 2014).

Yet inconsistent patterns have been reported for the relationship of growth with OS when considering within-species patterns in both correlational and experimental studies investigating the cost of growth. Faster growing individuals have been linked to both raised (Leggatt *et al.* 2007; Salomons 2009) and reduced (Kilgas *et al.* 2010; Almroth *et al.* 2012) antioxidant levels, while other studies have found no effect (Rosa *et al.* 2008; Larcombe *et al.* 2010; Geiger *et al.* 2011). Moreover enhanced growth rates have been associated with increased oxidative damage within species that cover a number of different taxa (Nussey *et al.* 2009; Almroth *et al.* 2012; Stier *et al.* 2014b) but not always (Rosa *et al.* 2008). This confounds our understanding of the growth-OS relationship.

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The apparent discrepancies between studies investigating the growth-OS relationship could be the result of different studies measuring different components of the redox system. Antioxidants have varying specificity for the vast array of RS that exist (Halliwell & Gutteridge 2007) so different antioxidants will be mobilised or upregulated depending on the RS that have been produced. Moreover utilisation of such antioxidants could result in a decrease, rather than increase, in antioxidant levels. However, either of these responses may be sufficient to neutralise the increased RS levels. If RS are not neutralised, there are numerous oxidation products that can result (Dotan *et al.* 2004) but many studies do not measure more than one damage biomarker. The variability in the redox response emphasises the importance of including a number of different biomarkers of both oxidative damage and antioxidant defence when assessing whether the changes in RS and antioxidant levels impact on the organism.

In correlational studies in which phenotypic flexibility in growth has been associated with OS it is unclear whether the relationship is causal. Experimental alteration of growth rates and comparison of OS between manipulated and unmanipulated individuals within the same species could clarify causality. For instance reducing brood size (Salomons 2009) and increasing lipid/protein composition of the diet (Costantini 2010d) have both led to increased growth rates and higher oxidative damage. The induction of compensatory growth (Metcalfe & Monaghan 2001; Dmitriew 2011) is sometimes linked to increased oxidative damage (Tarry-Adkins *et al.* 2008; Hall *et al.* 2010) but not always (Savary-Auzeloux *et al.* 2008; Noguera *et al.* 2011a).

Additionally, the growth-OS relationship might depend on the developmental stage of the organism; for instance, at certain points in development, an individual could be more vulnerable to OS. This is particularly notable at birth/hatching due to changes in the partial oxygen pressure and metabolic rate (Surai 2002; Davis & Auten 2010). Since the enzymatic antioxidant system takes time to become fully mature, there may be a greater reliance upon non-enzymatic antioxidants at earlier stages of development and mothers may compensate by increasing deposition of antioxidants into prenatal stages (Surai 2002).

The aim of this study was therefore to use meta-analytic techniques to review evidence for the relationship between growth rate and OS within species, whilst taking into account some of the confounding factors, such as what biomarkers were measured, in what tissue and at what developmental stage. A diverse range of eight taxonomic classes were considered. Two hypotheses were tested; the first was whether OS constrains growth as I

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might expect if a resource allocation trade-off is present between the two systems. In this case increased OS levels (i.e. greater damage and/or reduced antioxidant levels) would lead to a reduction in growth rate. To test this, the first meta-analysis (hereafter termed the constraint meta-analysis, constraint-MA) included studies where growth could be compared between groups that had been experimentally manipulated to differ in OS levels. This allowed us to investigate whether lower antioxidant levels might lead to a greater investment in antioxidant protection at the expense of growth, as well as if greater oxidative damage to tissues could limit growth. If a trade-off between growth and self-maintenance does exist, then one might expect there also to be costs of growth, if individuals cannot adjust the trade off in the face of accelerated growth. Therefore, the second hypothesis to be tested was that OS is a cost of growth, where I would expect that increased growth rates would lead to greater OS levels (i.e. greater damage and/or reduced antioxidant levels). Therefore the second meta-analysis (henceforth referred to as the cost meta-analysis, cost-MA) included studies that had experimentally manipulated growth rate between individuals, which could then be compared in terms of OS to determine if these within-species growth differences impacted on OS. By including a vast array of biomarkers of OS over numerous species covering eight taxonomic classes, my meta-analyses provide an in-depth investigation of the complex interplay between growth and OS.

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4.2 Materials and methods

4.2.1 Data collection

I focused on studies that manipulated either aspects of OS or growth within species. A systematic literature search was carried out in Web of Knowledge using combinations of the keywords "growth", "growth rate" and "compensatory growth" with "oxidative stress", "antioxidant" and "oxidative damage". In addition a more detailed search with the same keywords but also including "supplementation", "oxidised lipid" and "oxidised fat" was carried out to obtain studies that had manipulated OS through dietary changes, for instance antioxidant supplementation or inclusion of oxidised fats in the diet. Singular rather than plural words were used where appropriate and the following truncated words were used: supplement* (thus incorporating supplement, supplemented and supplementation) and oxid* (to include oxidised and oxidative). The last search was conducted on 7th August 2014 and citations of key papers were also searched. This resulted in the screening of approximately 2410 papers and relevant studies were included (Appendix 3). If possible for each included study multiple effect sizes were extracted if separate measures of various antioxidant levels and damage biomarkers were provided. For testing the first hypothesis, whether OS constrains growth (constraint-MA), there were 184 effect sizes in growth between groups where OS was manipulated from 61 studies. To investigate whether OS is a cost of growth (cost-MA) there were 120 effect sizes in OS between groups where growth was manipulated from 28 studies. Further details of the selection procedure for inclusion of studies into the meta-analyses, standardisation of growth measurements across studies and tests that revealed the absence of any publication bias are provided in Appendices 3 and 4 (Figure S4).

4.2.2 Effect size calculation

The *compute.es* package (Del Re 2013) in R (R Core Team 2013) was used to calculate the standardised effect size Hedges' *g* from test statistics (e.g. t values or F ratios) and sample sizes that were reported in papers; this package applies appropriate formulae described in Cooper *et al*. (2009). To calculate effect sizes, the standardised mean difference (Hedges' *d*) was first calculated – this accounts for the use of different units (i.e. different redox biomarkers) between studies by dividing the raw difference by the within-group standard deviation. For Hedges' *d* the type I and II error rates can increase if the number of studies is very low (<15) but the precision of the estimate increases with increasing number of studies (unlike other effect size measures; e.g. log response ratio)

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(Lajeunesse & Forbes 2003). Thus given the large sample size of the current meta-analyses, Hedges' *d* was deemed an appropriate effect size estimate.

With small within-study sample sizes Hedges' *d* can be over-estimated, so to correct for this it was converted to Hedges' *g* by multiplying by a correction factor calculated from the degrees of freedom (Cooper *et al.* 2009; Del Re 2013). Where appropriate test statistics were not reported, means, standard errors and sample sizes were extracted from tables or figures using ImageJ (Abràmoff, Magalhães & Ram 2004), which could then be entered into *compute.es*. If mixed models had been carried out in the original study and there was access to the model output, *r* was calculated from equation 24 in Nakagawa & Cuthill (2007) and then converted to Hedges' *g* as described above. Where appropriate, the number of families contributing to the dataset was used as the total sample size rather than the number of offspring, to account for non-independence of siblings sharing the same rearing environment.

4.2.3 Moderators included and categorisation

Since the relationship between growth rate and OS can be influenced by various factors, several explanatory variables (termed moderators in meta-analysis) were considered to be included in the analyses. The nature of the experimental manipulation might be influential, so is an essential moderator. For constraint-MA, three types of experimental manipulation were considered (Table 4:1 A). For supplementation with both antioxidants and natural compounds, I expected an improvement in the antioxidant status of supplemented individuals. Therefore unsupplemented individuals would suffer higher levels of OS and this would lead to a reduction in growth. On exposure to stressors (i.e. environmental challenges that increased OS), exposed individuals were expected to reduce their growth. For cost-MA, I included three different types of experimental manipulation and four correlational studies (Table 4:1 B). Regardless of treatment, I expected a greater level of OS (so increased damage and/or reduced antioxidants) in the faster growing groups.

Secondly, the growth-OS relationship is likely to depend on which biomarker is considered, because the antioxidants responding to, as well as the damage molecules produced from, OS can vary greatly. Therefore biomarker type was included and categorised into (1) damage biomarkers that included markers of protein (e.g. PCs), DNA (e.g. 8-oxo-dG) and lipid (e.g. MDA) damage; (2) non-enzymatic antioxidants (e.g. thiols, carotenoids and measures of total antioxidant capacity); and (3) antioxidant enzymes (e.g.

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CAT, GPX and SOD). A list of all the specific biomarkers is given in Tables S1 and S2 of Appendix 5.

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Table 4:1 Summary of the experimental manipulations for constraint-MA (A) and cost-MA (B). Note that some studies provided data for more than one experimental manipulation. None included comparisons between younger and older individuals with different growth rates, initially small late-hatched and larger early-hatched individuals and between individuals living at different elevations.

A. constraint-MA	**Details**	**Sample size: studies (effect sizes)**
Supplementation with antioxidants	E.g. carotenoids, vitamins, synthetic compounds that led to a reduction in OS (i.e. decreased damage and/or greater antioxidant levels), compared with the unsupplemented group.	24 (63)
Supplementation with natural compounds	Compounds with potential antioxidant properties (e.g. prebiotics, probiotics, herbs, plant extracts) that led to a reduction in OS (i.e. decreased damage and/or greater antioxidant levels), compared with the unsupplemented group.	23 (63)
Exposure to stressors	Environmental stressors that induce OS (i.e. increased damage and/or reduced antioxidants), e.g. inclusion of oxidised lipids in the diet, exposure to hypoxia, high stocking density, heat stress, toxins.	21 (58)
B. cost-MA		
None	Correlational studies in which the growth difference between groups was natural and statistically significant.	4 (18)
Compensatory growth	Food restriction followed by a period of *ad libitum* food, leading to compensatory growth in the experimental group.	7 (34)
Brood manipulation	Altering the number of chicks or hatching synchrony within a brood in avian studies. This led to increased growth in reduced broods compared with controls. Enlarged broods had decreased growth rates compared with controls, as did chicks that hatched asynchronously compared with synchronously. For one study, compensatory growth occurred later in life after an initial growth decrease of enlarged broods.	4 (8)
Dietary changes	Changes to protein and lipid composition of the diet. This included diets of differing quality with greater growth rates in high quality diet groups, as well as comparisons of different types of dietary proteins (e.g. fish meal, maggot meal or soy bean meal) and lipids (e.g. cod liver oil or vegetable oil) that had different effects on growth.	13 (60)

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The developmental stage of an organism is likely to have consequences for the growth-OS relationship because at certain developmental stages animals may become more susceptible to OS. The dataset spanned eight taxonomic classes – Actinopterygii, Amphibia, Aves, Gastropoda, Holothuroidea, Malacostraca, Mammalia and Reptilia. Therefore developmental stage was standardised by categorisation into: i) early juveniles (larvae of fish/insects/Malacostraca, tadpoles, nestling birds, mammals not yet weaned); ii) older juveniles (juvenile fish, fledged birds not yet of reproductive age, weaned mammals not yet of reproductive age, post-larval Malacostraca); iii) adults (i.e. of reproductive age). In constraint-MA there was a large sample imbalance between groups, with only two studies being from adults. Therefore the analysis was repeated excluding the two studies on adults so that older juveniles and early juveniles could be compared – no significant difference was found so developmental stage was not included as a moderator in constraint-MA.

Another moderator that was considered was sampling method, which was categorised into non-lethal (e.g. of blood, urine) and lethal (e.g. of liver, muscle), in order to determine whether the same effects could be obtained with non-lethal sampling. The sampling method will also affect the tissue type available for analysis, which has been suggested to lead to variations in the growth-OS relationship (Brown-Borg & Rakoczy 2003; Leggatt *et al.* 2007). For a full list of studies included in both meta-analyses and a breakdown of the categorisation of specific biomarkers and tissues see Tables S1 and S2 of Appendix 5.

4.2.4 Meta-analytic technique

Meta-analytic multilevel mixed-effects models were implemented using the rma.mv function in the *metafor* package (Viechtbauer 2010) in R (R Core Team 2013). The extracted Hedges' *g* values were the response variables in my statistical models. For constraint MA, the Hedges' *g* values denoted whether groups of the same species that had been found to differ in OS levels also had significantly different growth rates (growth is the response variable). Since here I was interested in whether OS constrains growth, a positive Hedges' *g* value meant that the group with the highest level of OS (i.e. greater damage and/or reduced antioxidants since OS is associated with *higher* levels of damage but *lower* levels of antioxidants) also had the *lowest* growth rate. For cost-MA, the Hedges' *g* values indicated if there was a significant difference in OS between two groups of the same species that had been found to differ phenotypically in growth rate (OS is the response variable), with a positive value signifying that the group with greater growth rates

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also had greater OS levels (defined as increased oxidative damage and/or decreased antioxidants).

Estimates were weighted according to the inverse sampling variance to account for different sample sizes across studies. This multi-level approach allowed the inclusion of study identity (to accounting for the non-independence of effect sizes from the same study) and taxonomic class (to partly control for phylogeny, which is difficult to do since the dataset was rather unevenly distributed across eight taxonomic classes) as random effects. Experimental manipulation, biomarker type, developmental stage (cost-MA only) and sampling method were included as moderators. The model output included the Q_E-test for residual heterogeneity, indicating whether the unexplained variance is greater than expected by chance – if it was then there is some variance that is not accounted for by the moderators. An omnibus test of model coefficients indicated whether there were significant differences among moderator levels; the test statistic for this is $Q_{M.}$ Models were simplified in a stepwise manner by the removal of non-significant terms, using an LRT to compare the fit of the full and reduced model at each stage. Post-model fitting checks were carried out, firstly by plotting the (restricted) log-likelihood against each variance component in the model, to ensure the function peaked at the parameter estimates. A flat surface around the parameter estimate would suggest the model was over-parameterised (Viechtbauer 2014a). Additionally model residuals were checked and met the requirements for normality and a lack of heterogeneity. Finally, since an outlier in cost-MA was detected where the Hedge's *g* was more than double the value of the next highest point (and so might have been influencing the results), models were repeated excluding it. Since it made no qualitative difference to the outcome, all presented results include the outlier.

When moderators are included in meta-analysis, it is difficult to get an overall average effect size because this will be influenced by the distribution of studies amongst moderators (Viechtbauer 2007). Therefore, the models that contained moderators were used to calculate predictions for particular 'sets' of moderators rather than an overall effect size (e.g. for each treatment type in constraint-MA and each biomarker type in cost-MA).

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4.3 Results

For investigation into whether the manipulations successfully altered OS (constraint-MA) and growth (cost-MA), plus a discussion of whether the manipulations produced differences between groups that were similar in magnitude to the differences that occurred under unmanipulated conditions, see Appendices 3, 4 (Figure S5), 5 (Table S3), 6 and 7.

There was an association between raised OS levels (i.e. greater damage, such as to proteins, lipids or DNA, and/or reduced antioxidants) and reduced growth in the constraint-MA implying OS was constraining growth. This effect was significantly different among different experimental manipulations (Q_M=36.64, df=2, p<0.0001; Table 4:2 A for pairwise comparisons), but not between different biomarkers (Q_M=3.65, df=2, p=0.16) or sampling methods (Q_M=0.41, df=1, p=0.52). Stressor exposure produced the largest effect and this was positive and significant (Figure 4:1 A), suggesting exposure to environmental challenges that raised OS levels reduced growth. Supplementation with natural compound (e.g. plant extracts, probiotics, herbs that have potential antioxidant properties; not specific antioxidants) also produced a significant positive effect but the effect for antioxidant supplementation was not significant (Figure 4:1 A). Therefore supplementing with natural compounds, but not specific antioxidants, also supports the hypothesis that increased OS constrains growth; increased OS (increased damage and/or lower antioxidant levels) in the unsupplemented group was associated with reduced growth.

For the analyses of the effect of differences in growth on OS (effect sizes in the cost-MA), a positive effect signified that increased growth was associated with increased OS (i.e. increased damage and/or reduced antioxidants). This effect was not significantly different between different experimental manipulations (Q_M=0.38, df=3, p=0.95), developmental stages (Q_M=0.35, df=2, p=0.84) or sampling methods (Q_M=2.44, df=1, p=0.12). However, there was a significant difference among different biomarkers of OS (Q_M=49.46, df=2, p<0.0001; Table 4:2 B for pairwise comparisons), with markers of oxidative damage producing significantly larger effects than those of enzymatic or non-enzymatic antioxidants (Figure 4:1 B). Furthermore, only oxidative damage produced a significant positive effect (Figure 4:1 B).

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Table 4:2 Pairwise comparisons between effect sizes for constraint-MA (A) and cost-MA (B). A: comparison of the effect sizes (slower growth in groups with experimentally increased oxidative damage and/or reduced antioxidant defences) between the different experimental manipulations; natural and antioxidant supplementation and stressor exposure. There was no significant difference among biomarkers for constraint-MA; only experimental manipulation was left in the final model. B: comparison of effect sizes (greater oxidative damage and/or lower antioxidant defences in the experimental group with faster growth) among the different OS biomarkers; enzymatic and non-enzymatic antioxidants and oxidative damage. There was no significant difference among manipulations for cost-MA; only biomarker type was left in the final model. Significance did not change following adjustment of p values using the sequential Bonferroni method (Rice 1989). The variance explained by the random factors was 0.07 and 0.51 (taxonomic class) and 1.25 and 5.92 (study), for constraint-MA and cost-MA, respectively.

A. Pairwise comparisons (constraint-MA)	**z value**	**p value**	**adjusted p value**
Natural-Antioxidant	2.60	0.009	**0.01**
Natural-Stressor	2.80	0.005	**0.01**
Antioxidant-Stressor	6.03	<0.0001	**0.0003**

B. Pairwise comparisons (cost-MA)	**z value**	**p value**	**adjusted p value**
Enzymatic-Non-enzymatic	0.94	0.35	0.35
Enzymatic-Damage	6.17	<0.0001	**0.0002**
Non-enzymatic-Damage	6.45	<0.0001	**0.0002**

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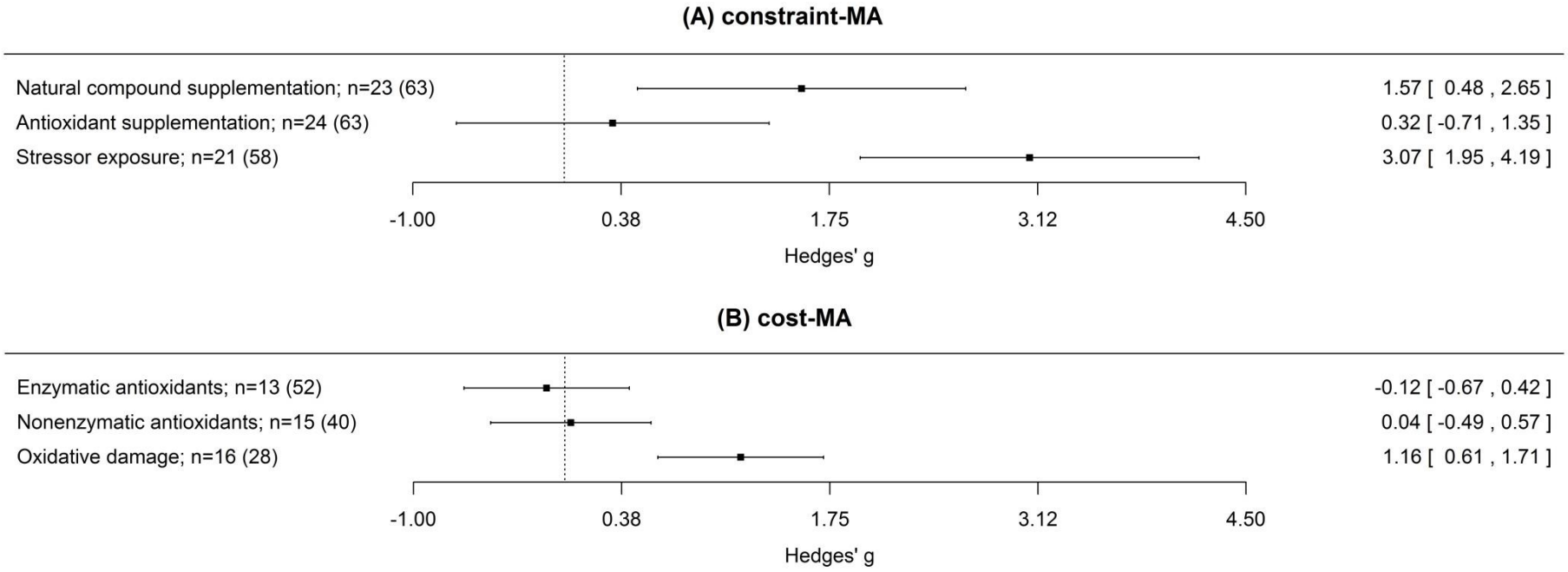

Figure 4:1 Predicted effect sizes (mean and 95% confidence interval, CI, at right) for (A) constraint-MA (slower growth in groups with experimentally increased oxidative damage and/or reduced antioxidant defences) and (B) cost-MA (greater oxidative damage and/or lower antioxidant defences in the experimental group with faster growth). A: a positive effect size indicates increased OS is associated with reduced growth. Separate effect sizes are given for each experimental manipulation, since the effect size differed significantly between these. B: a positive effect size indicates increased growth is associated with increased OS. Since the effect size differed significantly between biomarkers of OS, separate effect sizes are given for each biomarker. When the CI does not include zero, the effect size is significant. 'n' is number of studies (number of effect sizes).

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The Q_E-test revealed significant levels of residual heterogeneity (constraint-MA: Q_{E181}=1108.40, p<0.0001; cost-MA: Q_{E117}=553.86, p<0.0001) implying that the variance not accounted for by the moderators was significantly greater than expected and thus it is likely that there are additional moderators not considered here that might be responsible for the residual variation.

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4.4 Discussion

In using meta-analysis to review available data on the relationship between growth rate and OS within species, I found that experimentally altering OS levels so that OS was increased (increased damage and/or reduced antioxidants) through exposure to environmental stressors or being unsupplemented (compared with groups that were supplemented with natural compounds but not antioxidants) was associated with a reduction in growth rate, suggesting OS constrains growth. This effect was significantly influenced by the type of experimental manipulation but not biomarker type or sampling method. Furthermore animals with phenotypically greater growth rates had higher levels of oxidative damage, though no difference in antioxidant levels. This indicates that there are costs in terms of oxidative damage for individuals that cannot adjust the trade-off between growth and self-maintenance when fast growth occurs. This effect did not differ significantly between the types of experimental manipulation leading to the growth difference, the developmental stages at which OS was measured or the sampling methods.

The constraint-MA provides evidence for OS as a constraint on growth as implied by the positive effect I found; increased OS (greater damage and/or reduced antioxidants) was associated with reduced growth. Both exposure to environmental stressors (e.g. inclusion of oxidised lipids in the diet, exposure to hypoxia, high stocking density) and supplementation with natural compounds (e.g. plant extracts, probiotics, herbs that have potential antioxidant properties; not specific antioxidants) produced significant positive effects. In groups exposed to environmental challenges (i.e. what I term stressors), a reduction in antioxidants might have occurred as they are utilised in neutralising the RS that have been produced in response to the stressor. Since antioxidants might improve the physiological status of cells, when they are reduced in response to these environmental challenges, as well as being lower in the unsupplemented (compared with supplemented) group, oxidative damage might increase cell deterioration and death (Costantini 2008) which could limit growth. Additionally, lower antioxidant levels might necessitate the need for a greater level of investment in antioxidant protection which could divert resources from growth.

This positive effect that provides evidence for OS as a constraint on growth was significant for natural compound but not antioxidant supplementation and this could be due to the nature of these compounds. The antioxidants that were supplemented were of a specific type, while the natural compounds, which included plant extracts and herbs, had

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the potential to include a combination of numerous antioxidant molecules. Due to the synergistic effect certain antioxidants can have (Yu 1994) this might have enabled the neutralisation of multiple RS molecules as well as the recycling of antioxidants that have been utilised. Therefore this would allow reduced investment in antioxidant resources, compared with the unsupplemented group that might need to divert resources from growth. This also suggests that supplementing one type of antioxidant does not provide enough of an impact on the antioxidant status to affect growth.

All in all, the constraint-MA implies there is a resource allocation trade-off between growth and self-maintenance – when greater resources are invested into maintaining oxidative status, this has a negative impact on growth, which is reduced. Moreover my results suggest that individuals are unable to adjust this trade-off when experimentally forced to grow faster and so are faced with OS, in terms of increased oxidative damage levels, as a cost of growth (cost-MA). This corroborates previous work that has linked increased growth with increased damage (Nussey *et al.* 2009; Almroth *et al.* 2012; Stier *et al*. 2014b); where other studies have not found such a link, this might be a result of using transgenic animals that have been genetically programmed to grow faster (Rosa *et al.* 2008) and so may have other physiological differences making OS less notable.

The fact that the cost-MA demonstrated that increased growth had no impact on either enzymatic or non-enzymatic antioxidants – the similar number of studies between the three biomarker groups (Figure 4:1 B) indicates this is not a power issue – might not be surprising given the variable results of previous studies, which have found increased growth to be associated with greater (Leggatt *et al.* 2007; Tobler & Sandell 2009) or lower (Kilgas *et al.* 2010; Almroth *et al.* 2012; Yengkokpam *et al.* 2013) antioxidant levels, as well as no change in antioxidants (Rosa *et al.* 2008; Larcombe *et al.* 2010; Geiger *et al.* 2011). Perhaps the variability of the antioxidant response to OS explains the lack of effect here; if increased growth caused greater RS production, levels of enzymatic and/or non-enzymatic antioxidants may be utilised in neutralising RS and so decline or become upregulated in response to the RS therefore increase (Costantini & Verhulst 2009). The lack of clear trend in antioxidants demonstrates the complexity of the growth-OS relationship, implying that the oxidative cost of growth might not be the direct result of a diversion of resources from antioxidant protection to growth since in this case I would have expected an overall reduction in antioxidants (i.e. a positive effect). Despite debate around whether increased metabolic rate (e.g. from enhanced growth) leads to greater RS levels (Barja 2007; Fletcher *et al.* 2013; Stier *et al*. 2014a; Salin *et al.* 2015), my results suggest that this might be a more likely mechanism for the oxidative cost of growth.

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One potential drawback to the data for cost-MA is that it might be difficult to manipulate growth without altering other traits as well. For instance, the initial food restriction to reduce growth in the 'compensatory growth' group may have reduced antioxidant status simply due to there being fewer antioxidants in the diet, which might have led to OS unrelated to growth. However, food was subsequently provided *ad libitum* in these groups so they had the opportunity to make up any antioxidant deficit. Likewise, enlarged broods in the 'brood manipulation' group might have suffered both reduced growth and increased OS due to sibling competition but my results do not indicate this. In fact, the different experimental manipulations included in this meta-analysis do have the potential to affect traits unrelated to growth, however, the lack of significant difference between these manipulations in how they affected growth implies their main effect was on growth and not on other traits.

Another limitation of cost-MA is that I cannot distinguish between increased oxidative damage due to compensation and damage that might have occurred during the initial food restriction in the 'compensatory growth' group because OS was only measured once in each individual. Since the studies here measured OS after the period of compensatory growth, later than the initial reduction in growth rate, I could speculate the damage is due to compensation, but cannot be sure. When considering constraint-MA, the data might be limited by the fact that some of the growth differences observed might be unrelated to OS. For instance, exposure to environmental stressors could inhibit growth independently of the oxidative status of the cell, via alternative biochemical or physiological pathways (e.g. inhibition of cell signalling) (Inagaki *et al.* 2008). However, if this were the case, the association between increased OS and reduced growth that was observed in my study might not have been so apparent.

There are a number of other factors that might have affected the variation in effect sizes for both constraint-MA and cost-MA. Firstly, when investigating the effects of developmental stage in cost-MA, I found no significant difference between adults, older juveniles and early juveniles. However, one issue with including adults in my analysis is that they would come from indeterminate growing species, making it difficult to distinguish between taxonomic class and developmental stage. Furthermore, the uneven distribution of data across eight taxonomic classes that were included in these meta-analyses makes it difficult to include phylogeny. This might mask any within-class differences, although taxonomic class was included as a random effect to partly account for this and the variance due to taxonomic class was low.

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Secondly, sampling method is important for ecological studies with a preference for non-lethal methods and this is likely to affect the tissue used in analysis (e.g. blood in non-lethal versus organs in lethal sampling), which in turn may affect the growth-OS relationship (Brown-Borg & Rakoczy 2003; Leggatt *et al.* 2007). In my meta-analyses, most studies using non-lethal sampling were conducted in the wild and thus any pattern may be confounded by the collinearity of sampling method with whether the study was conducted in the laboratory or wild (for a more detailed discussion see Appendix 7). However, I found no significant difference between lethal and non-lethal sampling for either meta-analysis.

Lastly, the significant levels of residual heterogeneity in both meta-analyses suggest there might be other factors affecting the variation in effect sizes, for example sex (excluded since the majority of studies were of mixed/unknown sex) or tissue type (excluded due to the vast array of tissues sampled). While important, considerable residual heterogeneity is common in ecological meta-analyses (Costantini & Møller 2009; Isaksson 2010; Hector & Nakagawa 2012) and could be due to random variation.

In summary, despite having a heterogeneous sample, spanning a large variety of tissues and biomarkers of OS, my results demonstrated two clear patterns. Firstly, I found OS appears to constrain growth, since greater OS (i.e. increased damage and/or reduced antioxidants) was associated with reduced growth. This could imply that lower antioxidant levels lead to resources being diverted directly from growth in a bid to enhance antioxidant protection or that a reduction in antioxidant protection leads to a deterioration of the physiological status of the cell causing oxidative damage and eventually cell necrosis and death. This would necessitate greater investment in repairing or replacing damaged cells and tissues, thus reducing that which could be put into building new cells and tissues, leading to a reduction in growth. However, the lack of significant effect when specific antioxidants were supplemented, compared with natural compounds that likely contain a mix of various antioxidant molecules, suggests that in order to have an effect on growth a combination of antioxidants must be supplemented.

Secondly, my work supports the idea of an oxidative cost of growth, at least in terms of oxidative damage to proteins, lipids and DNA, and implies individuals might not be able to adjust the growth – self-maintenance trade-off when forced to grow quickly. Conversely, antioxidants do not appear to have been affected by growth demonstrating the complexity of this trade-off and I hypothesise that the oxidative cost of growth might result from an increase in metabolic rate and so RS production. This emphasises that oxidative damage

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might be more relevant than antioxidants for future studies investigating the oxidative costs of growth. Overall, my results provide evidence that OS might act as both a constraint on and a cost of growth. For researchers who want to investigate this further I recommend focussing on markers of oxidative damage rather than antioxidants and suggest that non-lethal sampling is appropriate, especially in ecological studies.

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Chapter 5

Experimentally altering reproductive expenditure does not affect blood oxidative status in wild blue tits (*Cyanistes caeruleus*)

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Abstract

Life-history theory predicts that reproductive expenditure will be traded off against various traits, such as future reproduction and survival. A growing body of research has explored potential underlying mechanisms of such trade-offs and it has been suggested that OS might play a key role. OS occurs when RS, primarily metabolic by-products, can no longer be neutralised by the antioxidants that usually overcome them and so can cause oxidative tissue damage. Raised reproductive expenditure is thought to lead to oxidative damage either by diverting antioxidant resources away from the redox system, or by directly raising RS production via increased metabolism. However, studies investigating how OS might be related to reproduction have proved to be equivocal, with different conclusions depending on factor such as species, tissues and biomarkers used. Therefore, the current study used a brood manipulation to experimentally alter reproductive expenditure in adult blue tits. The results showed that an experimental increase in the size of brood that parents were feeding did not lead to elevated parental OS levels. Furthermore, there was no direct correlation between reproductive effort (as measured by parental provisioning frequency) and OS. Nevertheless, a true effect might exist and be missed if it is limited to specific biomarkers and tissues, is only apparent in poorer environmental conditions or is either transient or takes time to become apparent. Thus future analyses should include longitudinal sampling of individuals within and between years, ideally over a range of environmental qualities and using a broad range of biomarkers and tissues, to reveal any relationship between reproduction and OS.

5.1 Introduction

A greater investment into current reproduction could compromise resources put into other bodily functions, potentially leading to negative consequences for other aspects of current reproduction (e.g. reduced offspring weight with increasing brood size (Nur 1984c)), or future reproduction and fitness (Stearns 1992), as well as survival (see Santos & Nakagawa 2012 for a meta-analytic review). Evidence for these reproductive costs supports the idea that reproductive expenditure is traded off against various life-history traits and a growing area of research involves investigation into the potential physiological mechanisms that underlie such trade-offs. One such mechanism is OS, which results from an imbalance between RS, produced mainly as by-products of metabolism, and the antioxidants that overcome them (Yu 1994).

The hypothesised OS-reproduction relationship may be generated by a greater reproductive expenditure that utilises limited antioxidant resources, leading to a reduced defence against OS and thus a decline in somatic maintenance (Costantini 2008). This is based on the assumption that antioxidant defences are either energetically costly (Speakman & Garratt 2014) or are derived from biomolecules that are in short supply (e.g. limiting dietary micronutrients). Another route by which increased reproduction might lead to greater OS is through the increase in metabolism associated with a greater reproductive expenditure (Nilsson 2002; Speakman 2008; Bergeron *et al*. 2011), which may result from increased energy intake and/or digestion efficiency to cope with the greater demands (Nilsson 2002). This makes the assumption that a greater metabolic rate will result in a direct increase in RS production, though the extent to which will depend on the degree of uncoupling of the mitochondria by uncoupling proteins (Brand 2000; Stier *et al*. 2014a). Reproduction may also influence OS due to changes in hormones (Isaksson *et al.* 2011b)) or body composition.

Studies exploring the relationship between experimentally altered reproductive expenditure and either antioxidant defence level (e.g. Wiersma *et al*. 2004; Garratt *et al*. 2013) or oxidative damage (e.g. Garratt *et al.* 2013; Wegmann *et al.* 2015a) are currently conflicted. Discrepancies amongst studies might be an artefact of the vast array of responding antioxidant molecules and resulting damage products that can be altered as a result of raised RS levels (Halliwell & Gutteridge 2007). Therefore, different biomarkers are likely to provide independent information on the redox system, as demonstrated in Chapter 2, highlighting the importance of combining several OS biomarkers of both antioxidants and damage in the analyses. Finally, experimental manipulations of reproductive expenditure may be necessary (Metcalfe & Monaghan 2013) since any

trade-offs could be masked by some individuals being naturally better able to acquire resources (e.g. antioxidants) and so invest in both a higher reproductive output and self-maintenance (van Noordwijk & de Jong 1986). One method of experimentally manipulating reproductive expenditure is to alter brood or litter size. This is supported by a meta-analysis of 11 bird species that found a positive relationship between parental expenditure during chick rearing and experimental manipulation (of brood or clutch size), suggesting that these manipulations are successful in altering parental expenditure (supporting information of Santos & Nakagawa 2012).

Another factor that might affect the OS-reproduction relationship is a species' life-history. Long-lived species would be expected to compromise reproduction for self-maintenance and survival. Thus, we may expect greater defences with increased reproductive expenditure in such species but no change in oxidative damage (Beaulieu *et al*. 2011, 2015; Stier *et al*. 2012). Conversely, short-lived species would be expected to sacrifice self-maintenance (and so OS defence and repair) for current reproduction (Parejo & Danchin 2006), making them a better candidate for revealing oxidative costs of reproduction (Bergeron *et al*. 2011); yet by doing so, species might suffer consequences for future survival or fecundity (Costantini *et al*. 2014a).

Environmental conditions can also have profound effects for the OS-reproduction relationship. Sacrificing self-maintenance for reproduction might be more likely to have consequences under certain environmental conditions that may limit recovery, such as poor food availability (Cram, Blount & Young 2014). A poor quality habitat with low food availability could reduce nutrient intake (e.g. of antioxidants) as well as force individuals to increase metabolically intensive foraging effort, both potentially leading to OS (Costantini, Casasole & Eens 2014b). For species that must match the time during which their offspring are most demanding with that of peak food availability, a poor quality environment could result in a shortened time over which food is available and thus increase the likelihood that this will not coincide with peak offspring demands. Mistimed breeding such as this creates a poorer quality environment, whereby parents need to increase foraging effort to meet offspring demands and this raised effort could increase OS levels.

The main aim of the current study was to test the hypothesis that experimentally altered reproductive expenditure leads to OS. I experimentally enlarged brood size in wild blue tits in order to modify parental provisioning rates (a proxy for reproductive expenditure); I expected provisioning to be increased in parents with enlarged broods, compared with controls. To relate reproductive expenditure to OS, I measured a range of antioxidants

and oxidative damage biomarkers that were designed to give a broad summary of oxidative status that could be related to variation in reproductive expenditure. My study provided a detailed investigation of the OS-reproduction relationship by including data on initial brood sizes in analyses, considered important because there may be an upper limit to reproductive expenditure which is reached more quickly at larger natural brood sizes.

5.2 Materials and methods

5.2.1 Field observations

Field work was carried out in March to June 2013 (for further details of the field site, including references, see Chapter 2). Weekly nestbox checks from late March allowed the identification of species, onset of laying, clutch size and an expected hatching date. Hatching was confirmed by daily visits after the expected hatching date until the first egg hatched (day 0). Nests were not visited again until day 2 and so whether hatching was synchronous or asynchronous could not be measured in this study. I investigated whether OS was a cost of reproduction by manipulating brood size; 40 nests were chosen at random to be enlarged on day 2 (or day 3 if not all eggs had hatched by day 2) by the addition of two chicks from a pool of donor nests not included in the study (Figure S6, Appendix 8). Enlarging by two chicks was deemed appropriate so that brood size was kept close to natural limits – if additional chicks were added this would have led to unrealistic brood sizes and the potential abandonment of chicks if parents failed to cope. The average initial brood size was comparable between control (7.7±0.4 chicks) and experimental (8.2±0.3 chicks) broods (see Results and Table 5:1 A for details). This treatment was expected to increase provisioning rate of parents in the experimental group, since feeding rate has been found to be positively related to brood size in this species (Nur 1984b).

For 32 out of the 40 enlarged nests, there was no difference in chick age between donor and experimental recipient nests; for the remaining eight nests, there was a maximum of a one-day age difference. 42 control nests were left at the natural brood size but were also visited and chicks counted on day 2/3 to create a comparable level of disturbance. This total of 82 nests was deemed an appropriate sample size as 62 nests were estimated sufficient to see a significant effect of reproduction on OS – from a power analysis (GPower, Version 3.1) with power=80% and the effect size from Blount *et al*. (2015; g=0.34; 95% CI: 0.04 – 0.64) for the correlation between reproductive expenditure and oxidative damage in blood markers of OS.

Between days 8 and 11, adults were caught (from 28 out of 42 control nests and 35 out of 40 experimental nests) in the nestbox using door traps, which have a mechanism that covers the hole when triggered by the adult bird entering the nestbox. The birds were colour ringed, with one of the colour rings carrying a Passive Integrated Transponder (PIT) tag (Appendix 9). PIT tags consisted of a small rod (12 mm in length; 2 mm in diameter) containing a silicon chip and weighing 0.1 g (Macleod, Gosler & Cresswell

2005). The maximum wing chord (wing ruler to 0.5 mm), tarsus length (callipers to 0.1 mm) and mass (digital balance to 0.1 g) of the bird were also recorded. Additionally, sexing was carried out by identification of a brood patch in females, while the primary coverts were used to classify whether birds were born in the previous year (dull blue) or were older (brighter blue) (Svensson 1992). A blood sample was taken (under UK Home Office licence) so that OS biomarkers could be measured (see Chapter 2 for further details); for three individuals from two nests, the bird escaped before a sample could be taken, therefore individuals from a total of 28 control and 33 experimental nests were bled. After taking measurements and releasing the adult, the trap was reset in an attempt to catch its mate; the trap was removed if the mate was not trapped within 90 minutes of first setting the trap. All trapping took place between 08:00 h and 18:30 h; sampling time was not found to have a significant effect on any of the OS measures. On day 13, chicks were ringed, weighed using a digital balance (to 0.1 g) and tarsus length was measured using callipers (to 0.1 mm).

To determine if provisioning rate was greater (indicating greater reproductive expenditure) in parents of the experimental nests, nest visit rate was monitored using the PIT tags over two days between days 11 and 14 in a subsample of 32 nests (14 control; 18 experimental; 40 marked adults in total for which provisioning rate could be measured). This subsample was deemed to be representative of the whole dataset (see Results for details). Nest visit rate is a good proxy for provisioning rate since non-feeding visits to the nestbox are likely to be rare by this point (Nur 1984b) because brooding stops at approximately day 9 (Perrins 1979) and, in great tits, 97.4 - 99.8% of total visits throughout chick rearing were feeding visits (Eguchi 1980). Nest visit rate was quantified using those nests in which one or more parents had been fitted with a PIT tag. When close to an electromagnetic field emitted by a PIT tag reading device placed at the nestbox entrance (Francis Scientific Instruments, Cambridge, UK), the tag reflected a signal allowing identification of the bird (Macleod *et al*. 2005). This electromagnetic field was emitted three times per second to detect if a bird was present; if triggered, bird identity, as well as date and time of the event, were recorded on a data logger. The nest visit rate (i.e. number of visits per hour, henceforth referred to as provisioning rate) was calculated for each tagged bird during two days over two four-hour time periods each day: 05:00-09:00 h and 09:00-13:00 h; repeatability (Lessells & Boag 1987) was high and significant between the four four-hour measurements ($r=0.81$, $F_{17,54}=18.55$, $p<0.0001$). Provisioning rate after 13:00 h was not considered because the PIT tag reading devices were typically deployed or moved in the afternoon and the birds were given an overnight acclimatisation time before measurements were begun. The exclusion of afternoon provisioning rates was not considered to be a problem since previous work found no

diurnal pattern in great tit provisioning, except that it tended to be slightly higher at the start of the day (Eguchi 1980), which my study has included.

5.2.2 Environmental factors potentially influencing provisioning rate

Caterpillars (Lepidopteran larvae) are the main food source parents provide for chicks during blue tit provisioning (Perrins 1979), making the timing of breeding in relation to caterpillar peak abundance an important factor for determining chick condition and future reproductive success (Naef-Daenzer & Keller 1999; Reed *et al.* 2013). Thus, I estimated the change in caterpillar abundance over the season by collecting, drying and weighing caterpillar frass (Zandt 1994) (see Chapter 2 for more details) twice a week. Although this would also include sawfly larvae frass which cannot be distinguished from caterpillar frass, a previous study found the proportion of sawflies to be low and not to interfere with caterpillar abundance estimates (Zandt 1994). Estimating caterpillar abundance allowed the approximate day of peak caterpillar abundance to be established as 10th June (Figure 5:1). I then calculated the absolute difference between this day and the day of peak food demand of chicks (day 11 in blue tits (Blondel *et al*. 1991)) to determine how well parents had matched their chick-rearing with the caterpillar peak – this variable is termed mismatch.

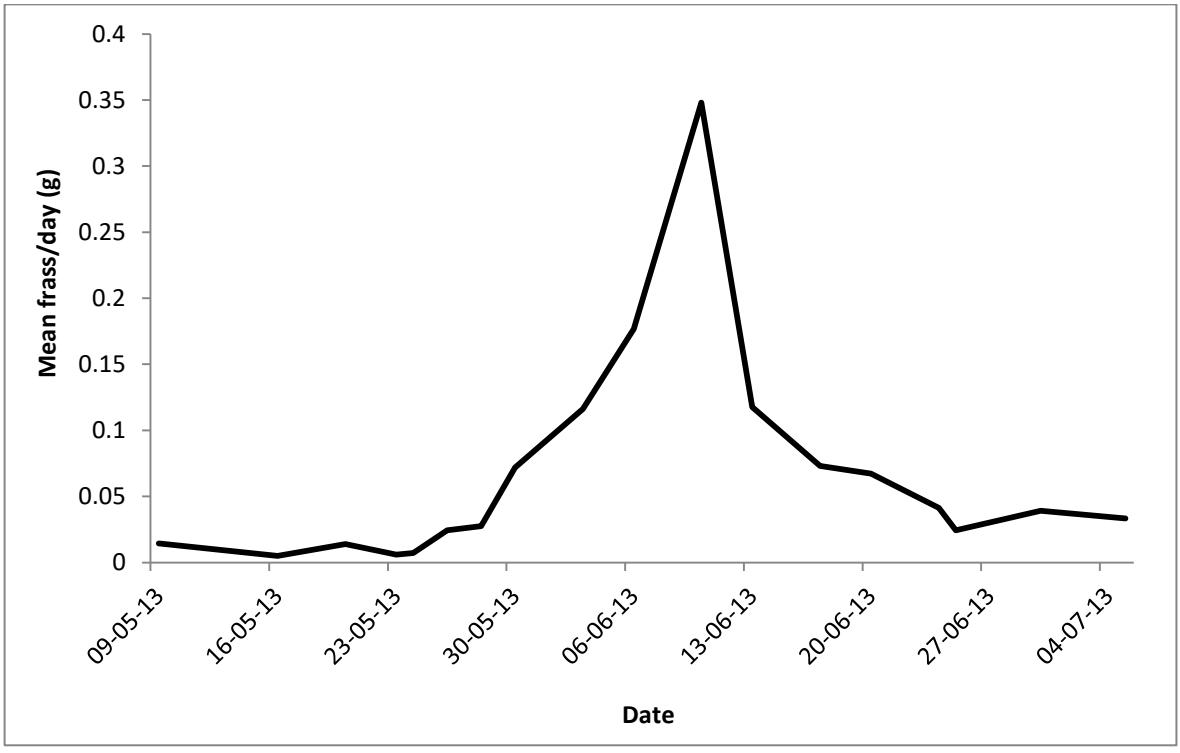


Figure 5:1 Mean caterpillar frass production over the 2013 breeding season. The peak in caterpillar abundance was approximately the 10th June.

5.2.3 Laboratory analyses

Due to the large number of damage products that can result from OS (Dotan *et al.* 2004), as well as the variety of antioxidant defences that can be upregulated or utilised in response to it (Cohen & McGraw 2009), it is beneficial to measure a wide range of biomarkers of both antioxidants (enzymatic and non-enzymatic) and damage when evaluating its extent. In the current study, I measured one marker of oxidative damage: PCs in RBCs (n=61 nests, 72 adults; Levine method, Levine *et al*. 1990), and one marker of general oxidation: ROMs in plasma (mainly organic hydroperoxides, n=37 nests, 43 adults; d-ROMs test, Diacron International). Additionally, three antioxidant markers were measured: the non-enzymatic antioxidant capacity of the plasma (n=60 nests, 70 adults; OXY-adsorbent test, Diacron International), RBC thiol antioxidant molecules (n=46 nests, 51 adults; –SHp test, Diacron International) and RBC levels of the antioxidant enzyme GPX (n=40 nests, 48 adults; Ransel assay, RANDOX Laboratories). The discrepancy in sample sizes between assays is due to the large variation in blood volumes acquired, resulting in different numbers of plasma and red blood cell aliquots from each individual, with not all samples large enough to measure all five biomarkers. All assays are described in full detail in Chapter 2.

5.2.4 Statistical analysis

For the subsample of 32 nests (40 adults), to determine whether parental provisioning rate was greater in the experimental group, mixed effects models (R Core Team 2013; lme4 package, Bates *et al*. 2014) were applied, using a Poisson distribution. The response variable was the total number of visits over 16 hours (across the four time periods; 05:00-09:00 h and 09:00-13:00 h over the two days) for each bird. However, not all birds were measured in all four periods and therefore an additional piece of coding was added to the model to account for the actual hours measured; this is preferential to an average visit rate which might be influenced by birds that had only been measured during one of the time periods. Nestbox identity was included as a random effect to account for the eight nests in which both the males and females were sampled. Due to the limited sample size in which provisioning rate had been measured, preliminary univariate analyses were run to investigate individual effects of variables in order to minimise the number of variables that went into the full model. Adult age (split into younger birds born in the previous year and older birds born in earlier years), adult sex, tarsus and wing length, mean chick mass and tarsus length for each brood at day 13 and mismatch (of peak chick demand with caterpillar peak abundance), as well as their interactions with treatment, were all non-significant so were not considered further. Hence the full model was run with treatment (experimental or control), initial brood size, adult mass and an interaction term for

treatment*initial brood size. This interaction was included because the effect of the experimental treatment might be restricted at larger brood sizes if there is a limit to the number of provisioning trips a parent can make. In this case, the difference in provisioning rate between parents of control and enlarged broods could depend on the size of the brood before manipulation. Since the interaction was significant, brood size was split into below and above average for investigative purposes and pairwise comparisons were made between control and experimental birds within each group. To account for overdispersion, which is common in Poisson models, an observation level random effect was also added (Harrison 2014).

To investigate whether experimentally-induced known differences in reproductive expenditure (as revealed by the parental provisioning model above) affected OS, mixed effects models (R Core Team 2013; lme4 package, Bates *et al*. 2014) with a Gaussian distribution were implemented, with each OS biomarker being used as the response variable in a separate model (see N in Table 5:2 for sample sizes). Some plasma samples were pink or red due to haemolysis – the red samples resulted in significantly higher readings for both OXY and ROMs when compared with yellow and pink samples; note that the remaining biomarkers were measured in RBCs and so were not affected. This may be due to the release of molecules, such as antioxidants and pro-oxidants, or metal ions that can trigger oxidative processes from lysed RBCs (Costantini *et al*. 2007). Therefore, OXY and ROMs values were corrected for this by extracting residuals from mixed models with plasma colour (yellow, pink or red) as a fixed effect and nestbox identity as a random effect and these residuals were then used as the response variable; a similar approach has previously been applied to account for haemolysed samples in OXY and ROMs analysis (De Coster *et al.* 2012). To minimise the number of variables going into the full model, preliminary univariate analyses were used again to explore individual variables. The variables that were excluded from the full model due to being non-significant in these preliminary univariate analyses were adult mass, tarsus and wing length, mean chick mass and tarsus length for each brood at day 13 and mismatch, as well as their interactions with treatment. The interaction between adult sex and treatment was also excluded since males and females did not differ in their response to the treatment. Therefore, the fixed effects included in the full model were treatment (experimental or control), initial brood size, adult sex and age. Since the effect of treatment on provisioning rate was found to depend on initial brood size, the treatment-by-initial brood size interaction was included in the OS models. This would allow differences in OS to be determined where there had been differences in parental provisioning (i.e. reproductive expenditure) – I expected an improvement in redox state (i.e. reduced oxidative damage, though the direction of antioxidants is harder to predict) where I had

experimentally-induced a reduction in provisioning rate (note that the effect on provisioning rate was the opposite from what I predicted). Nestbox identity was added as a random effect.

Some of the variables were transformed to improve residual normality or reduce heterogeneity (GPX and thiols were log-transformed). There was a slight deviation from normality for ROMs which transforming did not improve. Since parametric tests should be robust against mild normality violations when the kurtosis (peakedness of the data distribution) lies between -1 and 2 (Chiarotti 2004) (for ROMs the kurtosis=-0.75), this was not deemed to be a problem. When outliers were identified (by a Cook's distance greater than n/4; Bollen & Jackman 1985), these data points were more than two standard deviations above or below group means (e.g. for males or females, or control or experimental broods) and considered to be aberrant, therefore they were excluded from analysis. One such outlier was identified for GPX, three for OXY and two for PCs.

To investigate the direct relationship between provisioning rate and OS, mixed models (R Core Team 2013; lme4 package, Bates *et al*. 2014) were carried out on a subset of data for which data on provisioning rate and OS biomarkers were available (see N2 in Table 5:2 for sample sizes). Each OS biomarker was taken in turn as the response variable (with corrected values being used for OXY and ROMs as above) and nestbox identity was added as a random effect. The fixed effects were treatment, provisioning rate (mean nest visits per hour) and the interaction between the two. Sex and age were also included where they had been significant in the original models presented above. For all biomarkers, there was a mild deviation from normality in the model residuals, which remained after transformation. As the kurtosis for each was within the limits suggested by Chiarotti (2004) (GPX=-0.76, thiols=-0.46, OXY=-0.5, PCs=-0.52, ROMs=-0.8), this was considered to be acceptable.

For thiols, there were only three nests for which I had provisioning rate measurements of both parents, so the model could not distinguish between residual and nest variance. This means residual variance was estimated to be almost zero (which is unlikely in reality), because it was explained by nest variance. To overcome this, the thiols model was run as a simple GLM with only one parent per nest (by randomly excluding one of the parents from the three nests in which there were data for two) and the results were qualitatively the same.

To investigate the effects of sex, treatment and OS on survival, mixed effects Cox models (R Core Team 2013; coxme package, Therneau, 2015) were applied. Survival was

estimated using the proportion of birds that were recaptured in the following breeding season (2014), or during the winters of 2013 and 2014. The response variable was the date of recapture, with a censoring variable (1=recaptured; 0=not recaptured) added to specify any individuals that had not been recaptured and so were considered dead. For those individuals that were not recaptured, the last recapture date was used to emphasise that they had still not been recaptured (indicated by the coding '0' in the censoring variable) by the end of the recapture period. Since there were three individuals that escaped before being bled and therefore could not be included in the OS analysis, the first model was run with sex, treatment and initial brood size as fixed effects. As already discussed, there was a discrepancy in sample size between the OS biomarkers so the model was repeated with each one in turn as a fixed effect. Nestbox identity was included as a random effect.

Where appropriate, all models were simplified by stepwise backward deletion of non-significant terms, starting with interactions, using an LRT at each step to compare the fit of the full and reduced model and then residual normality and a lack of heterogeneity were confirmed. Significance was assumed when $p<0.05$ and standard errors are given. Finally, for all models, terms that were significant in the final model were also found to be significant in the full model, thus confirming the robustness of the statistical tests.

5.3 Results

Descriptive statistics for basic breeding parameters, comparing control and experimental groups for the whole dataset, are given in Table 5:1 A. The two groups were comparable since there were no significant differences in their laying date, clutch completion date, hatching date, mismatch, clutch size, number of unhatched eggs or initial brood size. As expected, experimental broods had significantly more chicks than controls after manipulation (Table 5:1 A). In addition, the sample sizes for the number of sampled males (n=13C and 11E) and females (n=21C and 27E), as well as the number of younger (n=16C and 20E) and older (n=18C and 17E) birds (for one bird the age had not been noted), were comparable between control and experimental groups (from GLMs with binomial errors; $z=0.83$, $p=0.41$ for sex and $z=0.59$, $p=0.56$ for age). The subsamples for which provisioning rate was measured were deemed to be representative of the whole dataset because all breeding parameters except brood size after manipulation, which was significantly greater in the experimental group, did not differ significantly between control and experimental broods (Table 5:1 B). Table 5:2 gives the sample sizes for each OS measure and the size of the subsamples for which provisioning rate had been measured for each OS measure.

The difference in provisioning rate between control and experimental nests depended on the initial brood size prior to the manipulation (significant interaction between treatment and initial brood size; Table 5:3). To investigate further, the data were split into below and above average brood size and mixed models were used to determine whether there was any significant difference between provisioning of control and experimental broods (Figure 5:2 for details). Although experimental parents of initially below average broods appear to have a greater provisioning rate than controls (Figure 5:2), this difference was not statistically significant ($z=1.44$; $p=0.15$), suggesting that for these smaller broods parents raising experimental broods did not significantly increase their provisioning rate in response to having extra chicks. Furthermore, when broods were initially above average size, parents of experimental broods had significantly lower provisioning rate than control birds ($z=3.20$; $p=0.001$). There was also a significant negative relationship between provisioning rate (measured between days 11 and 14) and adult body mass (measured between days 8 and 11) (Table 5:3; Figure 5:3).

Table 5:1 Comparison of control (n=28 nests) and experimental (n=35 nests) groups from which adults were caught on the nest (A), and between control (n=14 nests) and experimental (n=18 nests) groups for which provisioning data were available (B), using GLMs (means±SE are given). In order to allow statistical comparison of dates, values for laying, clutch completion and hatching dates are given relative to 1st April; 1st April=1, 1st May=31, etc.

A	Laying date	Clutch completion date	Hatching date	Mismatch (days)	Clutch size	No. unhatched eggs	Initial brood size	Brood size after manipulation
Control	44.5±1.4	52.1±1.2	66.3±0.9	6.4±0.9	8.6±0.4	0.9±0.2	7.7±0.4	7.7±0.4
Experimental	42.1±0.9	49.9±0.8	65.0±0.7	5.2±0.7	8.9±0.3	0.6±0.1	8.2±0.3	10.2±0.3
t	1.51	1.59	1.16	1.06	0.44	1.22	0.98	4.89
p	0.14	0.12	0.25	0.30	0.66	0.23	0.33	**<0.0001**

B	Laying date	Clutch completion date	Hatching date	Mismatch (days)	Clutch size	No. unhatched eggs	Initial brood size	Brood size after manipulation
Control	43.3±2.0	51.5±1.7	65.9±1.4	6.1±1.3	9.2±0.5	0.9±0.3	8.3±0.5	8.3±0.5
Experimental	42.6±1.3	50.7±1.1	65.5±0.9	5.5±0.9	9.1±0.4	0.5±0.2	8.6±0.5	10.6±0.5
t	0.26	0.38	0.28	0.37	0.23	1.33	0.45	3.26
p	0.79	0.71	0.78	0.71	0.82	0.20	0.66	**0.003**

Table 5:2 Summary of sample sizes for the five biomarkers of OS. The full samples are given in the column headed N, while N2 only includes those birds for which provisioning rate was measured. For both N and N2 the sample sizes are expressed as the number of nests (number of adults). N.B. these sample sizes do not include outliers that were excluded from analyses (see 5.2.4 Statistical analysis).

OS Biomarker	N	N2
GPX	38 (46)	20 (26)
Thiols	46 (51)	25 (28)
OXY	57 (67)	30 (37)
PCs	60 (70)	30 (37)
ROMs	37 (43)	18 (22)

Table 5:3 Results from the mixed model investigating whether provisioning rate (nest visits per hour) was affected by the experimental manipulation of brood size. The Poisson model gives estimates on a log scale so exponentiated values are given. These values are ratios so for mass, 0.8 is a 20% reduction in provisioning rate with a 1 g increase in mass; for initial brood size, 1.07 is a 7% increase in provisioning rate for a one chick increase in initial brood size. Provisioning rate was higher in experimentally increased broods, although the interaction with initial brood size makes this difficult to interpret alone (see Figure 5:2). The confidence intervals are significant when they do not overlap with 1.00. For details of variables included in preliminary analyses see section 5.2.4 Statistical analysis in the Methods.

Parameter	Exp(estimate)	Exp(lower CI)	Exp(upper CI)	z	p
Treatment	2.34	1.04	5.24	2.12	**0.03**
Initial brood size	1.07	1.00	1.15	2.06	**0.04**
Parental mass	0.80	0.68	0.94	2.80	**0.01**
Treatment*Initial brood size	0.90	0.82	0.98	2.47	**0.01**

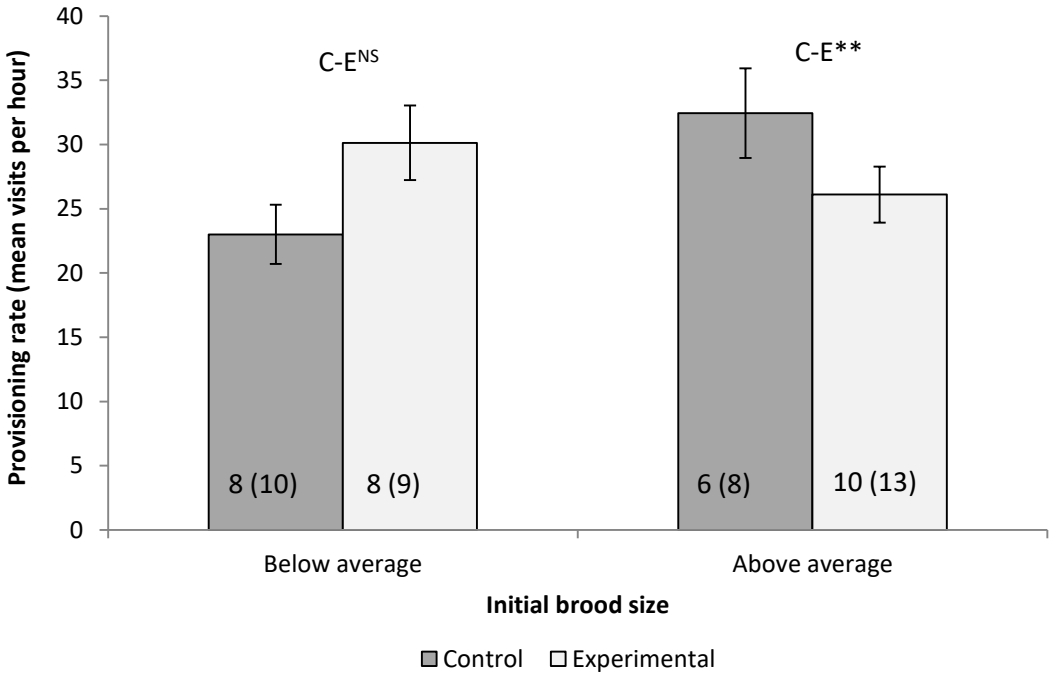


Figure 5:2 Provisioning rate by parents feeding control and experimental broods, depending on initial brood size. Overall there was a significant interaction between treatment and initial brood size; for investigative purposes, the data has been split into below and above average brood size. ** denotes a significant difference at $p<0.01$ between control and experimental (C-E) nests when below and above average brood sizes were subsequently tested separately in mixed models with a Poisson distribution, with treatment and parental mass as fixed effects and nestbox identity as a random effect; NS is not statistically significant. Results are mean±SE. Sample sizes are given as number of nests (number of adults).

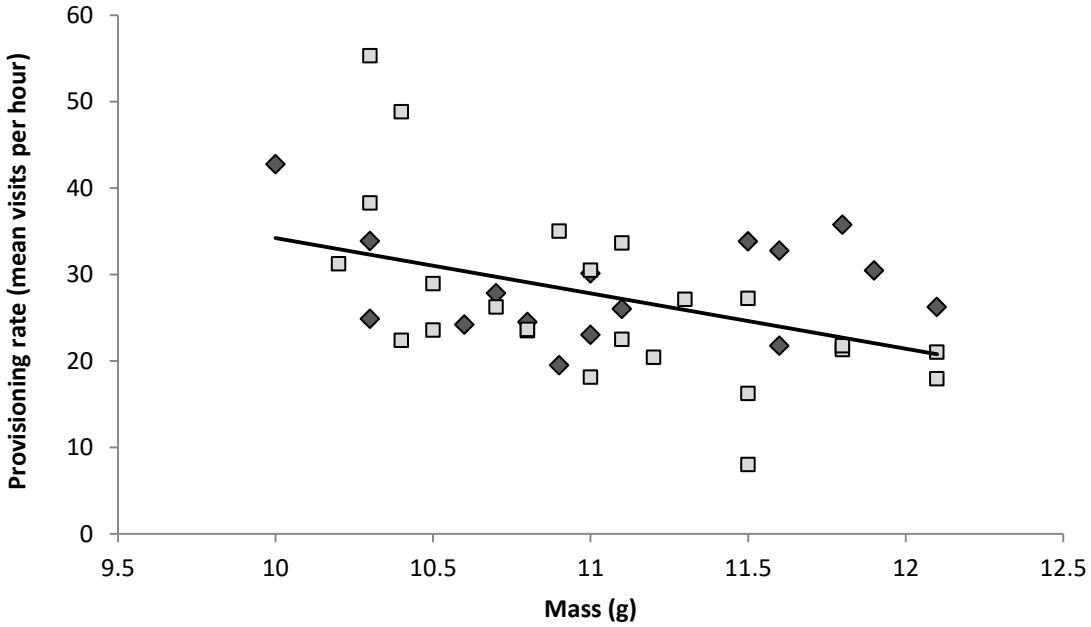


Figure 5:3 Relationship between parental mass and provisioning rate (n=32 nests, 40 adults). This plot is to illustrate the effect shown in the full analysis (Table 5:3) that controls for non-independence in cases where data were obtained from both the male and female feeding the same nest. Males (dark grey diamonds) and females (light grey squares) are shown separately for illustrative purposes, though the linear trend line goes through all data.

As provisioning rate had been found to be different between control and experimental groups but this effect depended on initial brood size, I included the treatment-by-initial brood size interaction in the OS models. This allowed the investigation of whether the experimentally-induced reduction in reproductive expenditure (i.e. provisioning rate) in the parents of enlarged broods, which had initially been above average in size, improved redox state. Neither treatment nor the treatment-by-initial brood size interaction was significant for any of the OS biomarkers (Table 5:4 A-E), suggesting treatment did not alter redox state. Additionally, analysing a sub-sample of birds with known provisioning rate showed that none of the biomarkers were significantly related to a parent's provisioning rate (Table 5:5 A-E). Regardless of treatment, GPX was positively and significantly related to initial brood size (Table 5:4 A; Figure 5:4).

The five OS biomarkers are shown broken down by adult sex in Figure 5:5, although from preliminary analyses males and females did not differ in their response to the treatment (treatment-by-sex interaction was not significant for provisioning rate; $z=1.23$; $p=0.22$). GPX, OXY and PC levels differed significantly between males and females (Table 5:4 A, C & D), with females having lower levels of GPX and non-enzymatic antioxidants (Figure 5:5 A&C) and higher PC levels (Figure 5:5 D) than males. Thiols and ROMs also tended to be higher in females (Figure 5:5 B&E) but this was not statistically significant (Table 5:4 B&E). Lastly, GPX was found to be significantly higher in older, compared with younger, birds (Table 5:4 A; Figure 5:6).

There was no significant difference in survival between males and females ($z=1.60$; $p=0.11$; seven females from 47 nests and eight males from 29 nests were recaptured) or between birds from control and those from experimental nests ($z=1.45$; $p=0.15$; four control birds from 28 nests and ten experimental birds from 35 nests were recaptured). Initial brood size did not significantly affect survival ($z=0.61$; $p=0.54$). Additionally, survival was not significantly related to any of the OS biomarkers: GPX ($z=0.59$; $p=0.56$), thiols ($z=0.15$; $p=0.88$), OXY ($z=0.49$; $p=0.63$), PCs ($z=0.07$; $p=0.95$) or ROMs ($z=0.92$; $p=0.36$).

Table 5:4 Results from mixed models investigating the effect of treatment on OS. Parameter estimates are only included for those variables that were present in the final model; t and p values of non-significant terms were taken from the last model that included each variable during model simplification.

A. GPX, n=38 nests (46 adults)	**Estimate**	**SE**	**t**	**p**
Treatment			1.38	0.17
Initial brood size	0.05	0.02	2.79	**0.01**
Sex	0.23	0.07	3.12	**0.002**
Age	0.20	0.07	2.81	**0.005**
Treatment*Initial brood size			1.00	0.32

B. Thiols, n=46 nests (51 adults)	**Estimate**	**SE**	**t**	**p**
Treatment			0.44	0.66
Initial brood size			0.66	0.51
Sex	0.17	0.13	1.28	0.20
Age			0.58	0.56
Treatment*Initial brood size			1.59	0.11

C. OXY, n=57 nests (67 adults)	**Estimate**	**SE**	**t**	**p**
Treatment			0.35	0.72
Initial brood size			0.67	0.50
Sex	0.08	0.04	2.14	**0.03**
Age			0.50	0.62
Treatment*Initial brood size			0.33	0.74

D. PCs, n=60 nests (70 adults)	**Estimate**	**SE**	**t**	**p**
Treatment			0.25	0.81
Initial brood size			0.27	0.79
Sex	-0.95	0.42	2.28	**0.02**
Age			1.18	0.24
Treatment*Initial brood size			1.31	0.19

E. ROMs, n=37 nests (43 adults)	**Estimate**	**SE**	**t**	**p**
Treatment			0.67	0.50
Initial brood size			0.36	0.72
Sex	-0.01	0.01	1.59	0.11
Age			0.96	0.34
Treatment*Initial brood size			0.17	0.87

Table 5:5 Results of mixed model (GLM for thiols) analyses investigating whether OS was influenced by provisioning rate (based on a subset of parents for which provisioning data were available). Parent age and sex were only included if they were significant in the original models above (see Table 5:4). Parameter estimates are given for variables that were present in the final model. For non-significant terms, t and p values were taken from the last model that included them, during model simplification.

A. GPX, n=20 nests (26 adults)	**Estimate**	**SE**	**t**	**p**
Treatment			1.25	0.21
Provisioning rate			0.10	0.92
Sex	0.29	0.09	3.37	**0.001**
Age	0.32	0.08	3.74	**0.0002**
Treatment*Provisioning rate			0.22	0.83

B. Thiols, n=25 nests (28 adults)	**Estimate**	**SE**	**t**	**p**
Treatment			0.27	0.79
Provisioning rate	0.01	0.01	0.63	0.54
Treatment*Provisioning rate			0.49	0.63

C. OXY, n=30 nests (37 adults)	**Estimate**	**SE**	**t**	**p**
Treatment			0.13	0.89
Provisioning rate			0.36	0.72
Sex	0.06	0.05	1.15	0.25
Treatment*Provisioning rate			0.51	0.61

D. PCs, n=30 nests (37 adults)	**Estimate**	**SE**	**t**	**p**
Treatment			0.86	0.39
Provisioning rate			0.68	0.50
Sex	0.55	0.49	1.12	0.26
Treatment*Provisioning rate			1.74	0.08

E. ROMs, n=18 nests (22 adults)	**Estimate**	**SE**	**t**	**p**
Treatment			0.69	0.49
Provisioning rate	-0.001	0.001	1.53	0.13
Treatment*Provisioning rate			0.94	0.35

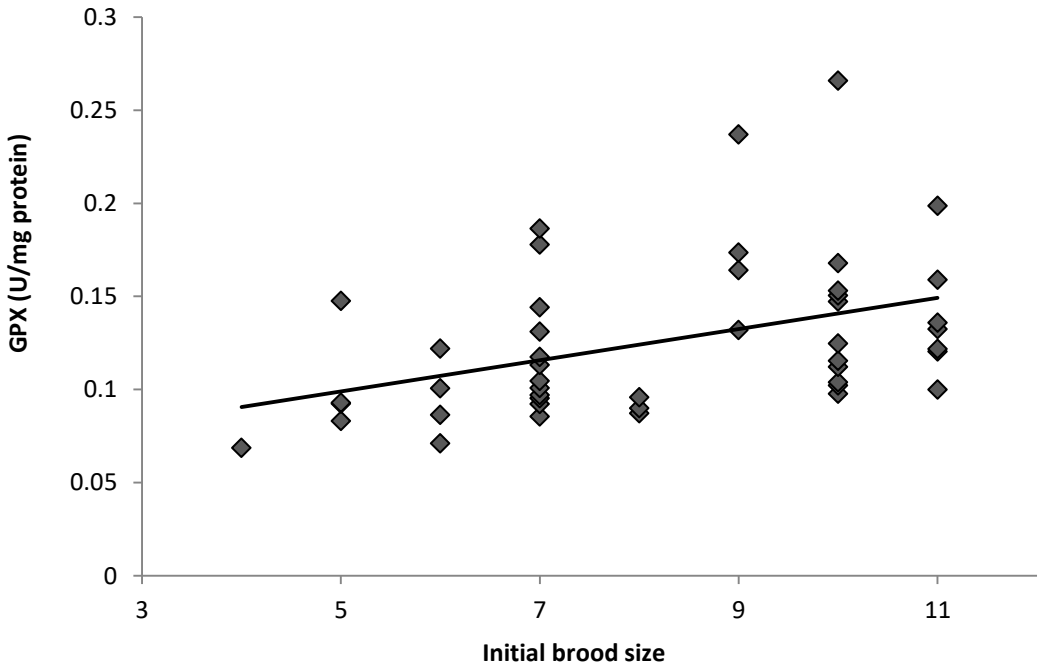
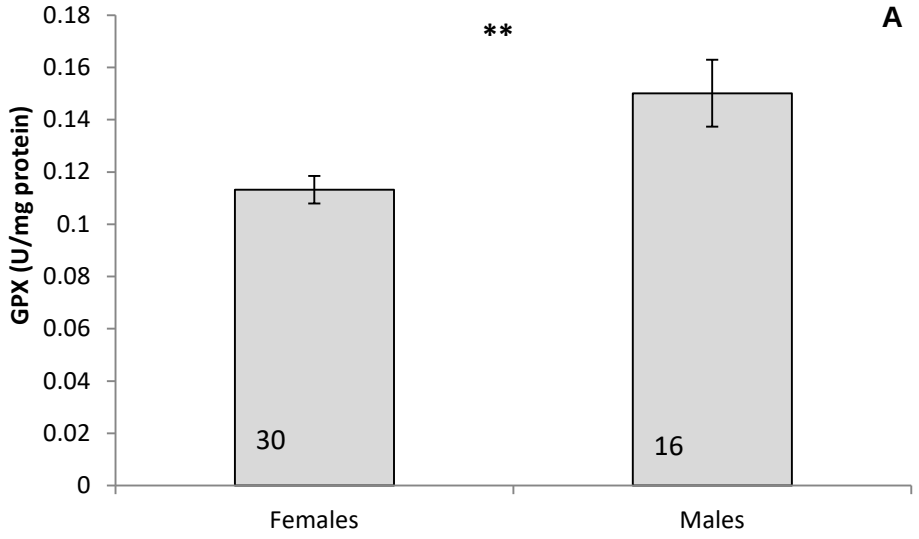
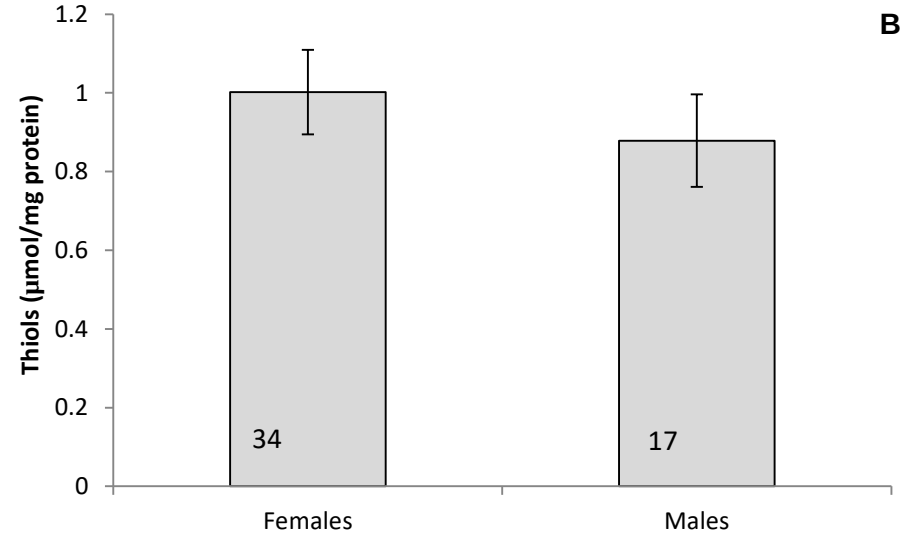
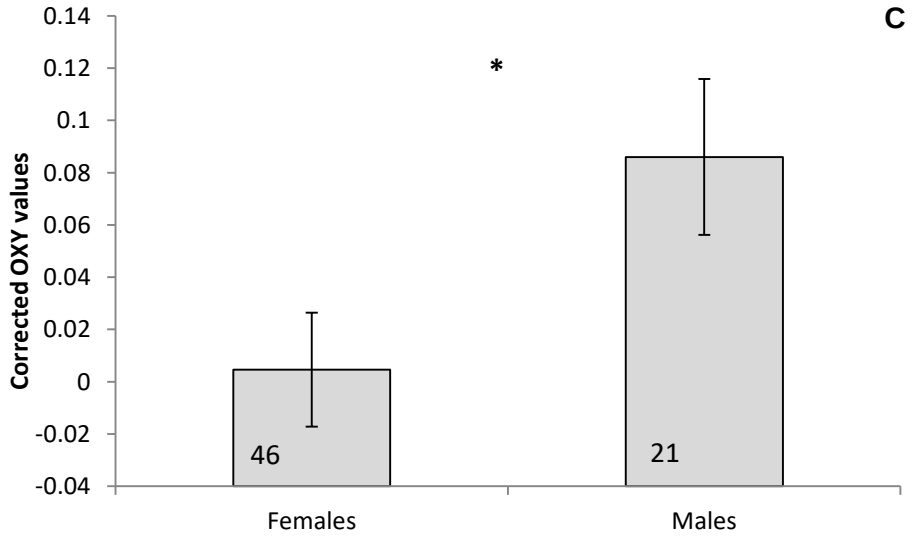


Figure 5:4 The relationship between GPX and initial brood size considering control and experimental nests together. n=38 nests (46 adults).


A
**
GPX (U/mg protein)
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
30
16
Females
Males


B
Thiols (µmol/mg protein)
1.2
1
0.8
0.6
0.4
0.2
0
34
17
Females
Males


C
*
Corrected OXY values
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
-0.02
-0.04
46
21
Females
Males

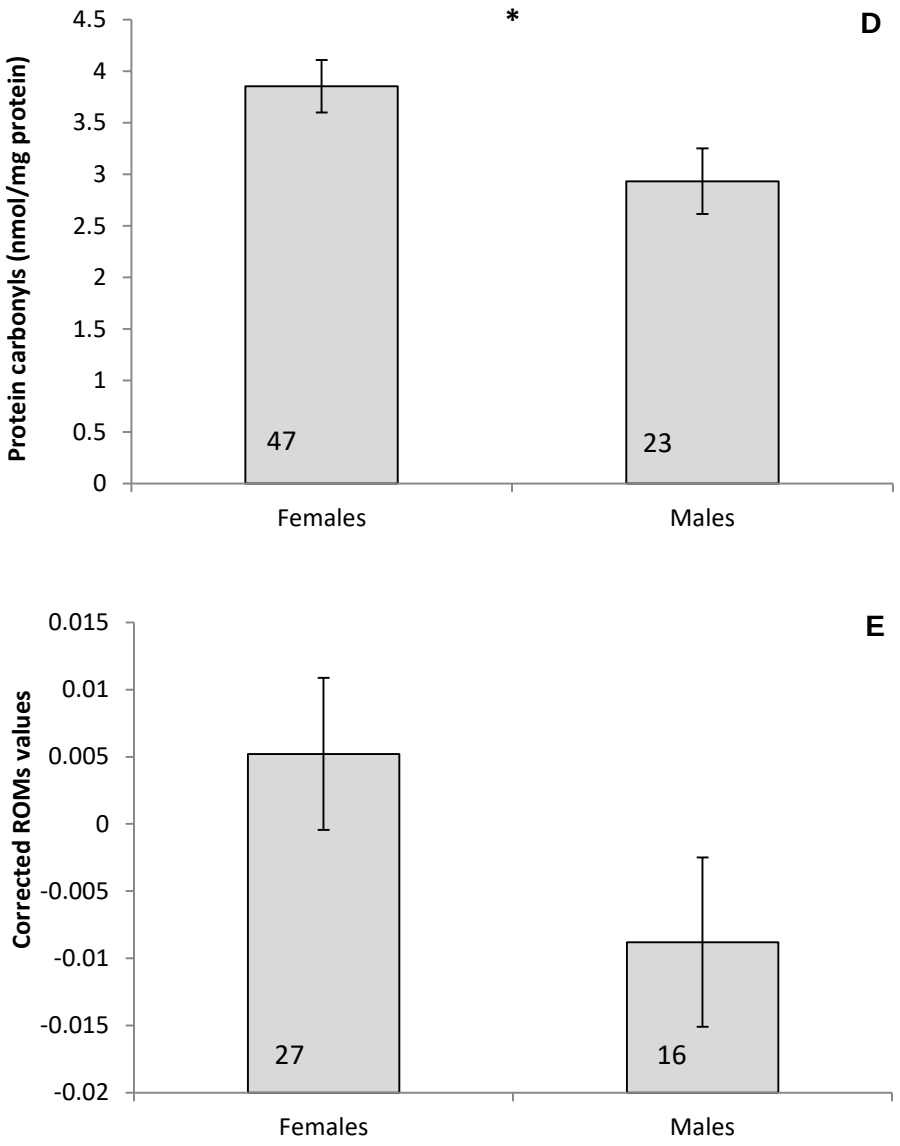


Figure 5:5 Comparison of the five OS biomarkers, GPX (A), thiols (B), OXY (C), PCs (D) and ROMs (E) between male and female parents. Sex was significant for GPX (** denotes $p<0.01$), OXY and PCs (* denotes $p<0.05$). Sample size is noted on the bars; means ± standard errors are given. OXY and ROMs samples have been corrected for plasma colour.

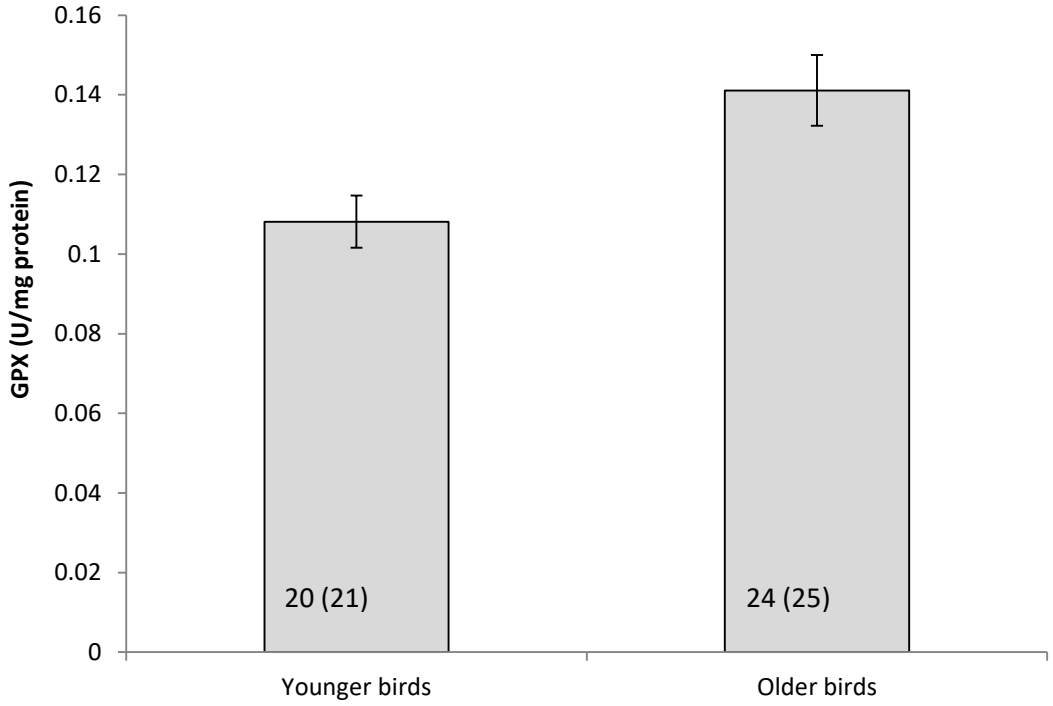


Figure 5:6 Comparison of GPX levels between younger (born in the previous year) and older (born earlier) parents. The sample size on the bars gives the number of nests (number of adults). The error bars denote the standard error of the mean.

5.4 Discussion

None of the five OS biomarkers measured here responded to experimentally-induced changes to provisioning rate; however, since the current study triggered a reduction, rather than increase, in reproductive expenditure, it is difficult to conclude that enhanced reproductive expenditure does not lead to greater OS levels. Furthermore, contrary to previous work, I did not find any correlational link between OS and reproduction. The apparent lack of association between reproductive expenditure and OS is unexpected and a number of contributing factors are now discussed, including the choice of biomarkers and tissues used for analysis, as well as the possibility of good environmental conditions masking any effect.

For those parents that had an initially below average brood size, the experimental manipulation did not lead to a significant difference in provisioning rate between control and experimental birds. Given the results of a meta-analysis of 11 bird species that found a positive relationship between parental expenditure and experimental manipulation (i.e. of brood or clutch size) (supporting information of Santos & Nakagawa 2012), the lack of increase in provisioning rate in the experimental group that had an enlarged brood size is unexpected. It has been suggested that parents might be limited by a physiological ceiling that prevents them from adjusting to manipulations of brood or litter size that are carried out after birth (Speakman & Garratt 2014), for instance, the need to dissipate heat in rodents might limit the capacity to increase food processing and milk production when litter size is increased (Król & Speakman 2003). However, this may be species-specific and does not appear to be the case for the 11 bird species in Santos & Nakagawa's meta-analysis. Another explanation for the lack of increase in provisioning in these birds could be that the quality of the territory was not sufficient to support the increased foraging required to feed more chicks than the initial brood size. The relatively low fledging success in this year (66.7±6.7% chicks fledged) compared with the previous year (93.5±2.7% chicks fledged; GLM with binomial errors: $z=7.39$; $df=75$; $p<0.0001$) indicates this could be the case. Finally, when considering Figure 5:2, the provisioning rate of experimental birds of initially below average brood sizes was higher than the controls; the lack of statistical significance might be due to a lack of statistical power. From a power analysis (GPower, Version 3.1), I would need a total sample size of 25 nests (30 chicks) in the below average group to get a significant difference in provisioning rate between control and experimental broods at the effect size I observed ($\rho=0.5$).

A lack of statistical power might explain why blue tits in the current study do not appear to have significantly increased provisioning levels when feeding enlarged broods but it does

not explain why provisioning would actually be lower in the enlarged than control broods, as it is for those experimental parents that had initially above average brood sizes. This is the opposite of what I predicted, even if we consider that there may have been an upper limit to the number of provisioning trips a parent can make. One explanation is that this could have been a result of the lower thermoregulatory costs associated with having a larger number of nest mates, which provide more insulation (Neuenschwander *et al*. 2003). This would mean a reduced food demand of chicks and hence lower provisioning rate of parents. Alternatively, experimental parents may have been sacrificing current reproduction for self-maintenance under the demanding conditions of this experiment (i.e. raising two additional chicks above their natural brood size when this was already above average), as has been demonstrated in great tits (Wegmann *et al*. 2015a) and *Bicyclus anynana* butterflies (Beaulieu *et al*. 2015).

If parents of initially above average-sized broods were sacrificing current reproduction for self-maintenance when faced with demanding conditions, then it might be expected that OS levels would reflect this. In this case, antioxidant levels would be predicted to be higher in the experimental than control parents with no change in damage levels as they invest more into prevention, repair and replacement of damaged cells; whilst no difference in OS levels between control and experimental parents of initially below average-sized broods that did not differ significantly in provisioning rate would be expected. However, the treatment-by-initial brood size interaction was not significant for any of the five OS biomarkers: GPX, thiols, OXY, PCs or ROMs. It is possible that there was a transient increase in OS levels, after an initial increase in provisioning rate, in those experimental birds of initially above average-sized broods which was no longer observable by the time of sampling. This brief increase in OS could have led parents to compensate by reducing provisioning rate so that it was lower than that of controls by the time of measurement. So possibly provisioning rate and OS were measured too late and it would therefore be interesting to investigate the dynamics of this behavioural response to the treatment over the whole chick-rearing period.

Furthermore, the known experimentally-induced reduction in the parental provisioning rate of parents of initially above average-sized broods did not appear to have improved redox state. This suggests that an experimentally altered reproductive expenditure had no effect on OS, though it must be noted that the lack of effect might be due to a lack of statistical power – samples for OXY (n=60 nests) and PCs (n=61 nests) were the only biomarkers that approached the sample size of 62 nests suggested by my power analysis, while GPX (n=40 nests), thiols (n=46 nests) and ROMs (n=38 nests) were all below this. In any case, there was no clear evidence of any experimental link between reproductive investment

and OS: while the positive relationship between GPX and brood size in the current study might support the idea that greater brood sizes are more demanding, as it suggests an upregulation of GPX that may have been in response to raised RS levels, it should be noted that none of the damage biomarkers showed any such relationship with brood size.

Other recent experimental studies investigating the OS-reproduction relationship also found reproduction to be unrelated to parental oxidative damage (Costantini *et al*. 2014a; b; Wegmann *et al*. 2015a), which raises the question as to whether enhanced reproductive expenditure leads to OS. To the contrary, a recent meta-analysis investigating the relationship between reproductive expenditure and OS found a positive association between reproductive expenditure and oxidative damage (Blount *et al*. 2015) but when correlational studies were excluded from that analysis, the relationship lost significance. The authors suggested that the lack of effect in experimental studies might be due to individuals' reproductive expenditure being restrained by the physiological ceiling that I have described earlier, although it has previously been demonstrated that individuals can be forced to invest more in reproduction (supporting information of Santos & Nakagawa 2012). It has been suggested that manipulations of reproductive expenditure need to be large and quantifiable in order to relate these to OS (Speakman & Garratt 2014), so the differences in provisioning levels between control and experimental birds in my study may have been insufficient to affect OS levels.

It must be noted that the current study induced a reduction, rather than increase, in reproductive expenditure in the experimental parents that had initially above average-sized broods. It has previously been demonstrated that experimentally reduced reproductive effort was not found to affect parental survival (meta-analysis by Santos & Nakagawa 2012) and the authors suggested the extra resources were allocated to aspects other than survival (e.g. in France, blue tits with reduced reproductive effort had a higher chance of producing a second clutch; Parejo & Danchin 2006). Hence birds with an experimentally reduced reproductive effort will not necessarily improve oxidative status, and this might indicate that there needs to be a forced increase in reproductive expenditure to affect OS. Furthermore, birds with reduced provisioning rate are unlikely to be sitting on the nest and so will still face costs of flight, for instance, when foraging or evading predators. These stressors could make it challenging for them to improve oxidative status.

Although there was no relationship between experimentally altered reproductive expenditure and OS, I investigated the direct relationships between parental provisioning rate and each of the five OS biomarkers, but none of these were found to be significant.

This contrasts Blount *et al*.'s meta-analysis (2015) whereby the association between reproductive expenditure and oxidative damage was only significant if correlational studies were included. In comparison with Blount *et al*.'s finding of an effect size of 0.34 (Hedges' g) for the correlation between reproductive expenditure and oxidative damage in blood markers of OS, the effect size for the damage biomarker in my study was 0.23 (PCs), and, for the marker of general oxidation, was 0.65 (ROMs). This suggests that for ROMs, the lack of a significant relationship with reproductive expenditure could be due to a lack of statistical power; for PCs and the antioxidant markers it is difficult to make this assumption.

The lack of significant relationship between reproductive expenditure and OS in my study could be a result of the difference in metabolism between different levels of reproductive expenditure (e.g. provisioning levels for different brood sizes) being much smaller than between different reproductive and non-reproductive phases (e.g. lactating versus non-breeding mice) (Speakman & Garratt 2014). It is possible that a comparison between breeding and non-breeding individuals is more appropriate (Speakman & Garratt 2014). In the current study, it was not feasible to include non-breeding individuals since the breeding status of an adult bird captured away from the nest could not be determined for certain. Moreover, it has been argued that using non-breeding individuals as controls to test the oxidative costs of reproduction might be inappropriate; differences in aspects such as diet and hormone levels, which can affect oxidative status independently of reproduction, might render breeding and non-breeding individuals incomparable (Costantini 2016b). In addition, in the absence of a method of experimentally manipulating breeding status, naturally non-breeding individuals (even if they could be identified and caught) could be of poorer genetic or phenotypic quality than breeders (Costantini 2016b), thus making it difficult to interpret any differences in redox state. Furthermore, interpretation of redox state will also differ between changes to reproductive status (i.e. breeders versus non-breeders), where the cost comes from the decision of whether or not to reproduce, and changes to reproductive expenditure (i.e. changes to brood or litter size), where the cost is from deviations from an optimal investment (Stier *et al*. 2012).

Despite no detectable relationship between reproductive effort and OS in this study, other costs of reproduction were apparent, as revealed by the negative relationship between adult body mass and parental provisioning rate. This might suggest parents that worked harder (i.e. had a greater reproductive expenditure) consequently lost body mass; as body condition can be related to survival in passerines (Tinbergen & Boerlijst 1990; Magrath 1991), this may imply long-term consequences of raised reproductive expenditure. This is

supported by a meta-analysis of 19 bird species that demonstrated a reduction in survival with increased parental expenditure (Santos & Nakagawa 2012). Alternatively, the birds could have been strategically shedding mass since the energetic cost of making provisioning flights (and their ability to evade predators) will be dependent on their body mass and hence wing loading (Kullberg, Metcalfe & Houston 2002). At the same time, it must be noted that blue and great tit provisioning rates can be negatively related to prey size, implying that fewer visits may not necessarily mean the chicks are getting less food (Nour *et al*. 1998). Lastly, it cannot be ruled out that larger individuals invest less in provisioning because they incur greater metabolic costs (Kleiber 1947).

Whether or not we see an association between reproduction and OS could depend on the choice of biomarkers. The direction of an antioxidant response might be inconsistent, depending on whether it is upregulated in response to raised RS levels or utilised in combating the RS (Costantini & Verhulst 2009). Additionally, the response of biomarkers to raised reproductive expenditure might be unpredictable, simply because there are so many potential antioxidant molecules that might respond to increased levels of RS production, as well as resulting damage products (Halliwell & Gutteridge 2007). This could explain why previous studies have found only some damage biomarkers to be affected by reproduction (e.g. Ołdakowski *et al*. 2012). Furthermore, certain tissues or organs of the body might be more sensitive to the effects of OS and it has been demonstrated that the OS-reproduction relationship is likely to depend on the tissue chosen for analysis (Garratt *et al*. 2012; Xu *et al*. 2013; Yang *et al*. 2013). However, though investigating the effects of growth, not reproduction, on OS, my meta-analyses in Chapter 4 found no difference between non-lethal (e.g. of blood, urine) or lethal (e.g. of liver, muscle) sampling, suggesting blood-based biomarkers might be just as sensitive as other tissues.

Environmental factors are also likely to affect the OS-reproduction relationship. Under optimal conditions, manipulations of reproductive expenditure may have no effect – parents can cope with the raised expenditure with minimum additional cost (Costantini *et al*. 2014b). This is emphasised by the fact that the oxidative costs of reproduction have been found to be reduced upon supplementation with either antioxidants directly (Bertrand *et al*. 2006) or with food that led to an increase in antioxidant protection (Fletcher *et al*. 2013). Negative effects of reproduction on OS might therefore only be seen in a low quality environment – emphasised by a study of female pigeons (*Columba livia*) in which the increase in non-enzymatic antioxidant capacity over the chick rearing phase was reversed when they received a decreased quality diet (Costantini 2010e). Thus, I might have expected a treatment effect on OS in my study for birds that had a greater value for

mismatch so that there was a larger gap between the timing of chicks being most demanding and that of peak food availability. However, I found neither an effect of mismatch nor any treatment-by-mismatch interaction. It is possible that the present study was conducted in a favourable year, allowing changes in reproductive expenditure with no consequences for OS.

One method of assessing environmental quality would be to compare this timing of breeding in relation to peak food demand between years, as this can have consequences for chick condition and potentially future reproductive success (Naef-Daenzer & Keller 1999; Reed *et al*. 2013). I did not find a significant difference in mismatch between this study year and the preceding one (mismatch in previous year, 2012: 5.6±0.45 days; this year, 2013: 5.1±0.50 days; t=0.62, df=138.28, p=0.53). Additionally, my values for mismatch were comparable with previous work on blue tits (Blondel *et al*. 1991) and great tits (Reed *et al*. 2013), which might suggest 2013 was not an especially 'good' year, although the latter study revealed an increase in mismatch over recent years attributable to climate change (Reed *et al*. 2013). Furthermore, as previously mentioned, the fledging success in 2013 was significantly lower when compared with that of 2012, suggesting a poorer year in 2013. This lower fledging success may have been the result of a reduced overall caterpillar abundance in 2013 (2013: 99±7 mg frass per m^2 per day; 2012: 320±20 mg frass per m^2 per day; t=11.07, df=582.02, p<0.0001; data were logged to improve normality) leading to reduced foraging opportunities for parents. This makes it seem unlikely that the lack of effect of reproduction on oxidative status in my study is a result of 2013 being a good quality year.

Even if a greater reproductive expenditure does lead to an increase in oxidative damage, effects may be transient and have no effect on future reproduction or survival (Metcalfe & Monaghan 2013). Transient effects of reproduction on OS have been demonstrated in female breeding rats (*Rattus norvegicus*) that had lower levels of damage at six months than non-breeders but no difference by 24 months (da Silva *et al*. 2013). Effects may be even more short-lived than this; in male great tits, an enlarged brood size led to a reduced OS resistance compared with controls five days after the start of the manipulation, but effects were no longer evident by day 13 (Losdat *et al*. 2011). Short-lived effects on OS could be caused by compensatory antioxidant mechanisms being activated after a short lag period (Losdat *et al*. 2011) or compensatory behavioural responses such as reduced reproductive expenditure after an initial increase. Alternatively, removal of damaged cells (e.g. via apoptosis) might increase during reproduction, thus resulting in effects on OS that are transient, as has been demonstrated in Caterina scallops (*Argopecten ventricosus*) (Guerra *et al*. 2012). These short-term effects may be missed if the OS

measurements are taken too late, for instance if all RS or damage has been neutralised or removed by the time measurements are taken (Stier *et al*. 2012). Conversely, effects on damage levels could take longer to become apparent and may not occur unless antioxidant levels drop below a critical threshold (Costantini *et al*. 2014a).

Irrespective of reproductive expenditure, sex differences were evident in some of the biomarkers. Protein damage (PCs) was significantly higher in breeding females than males and levels of the general oxidation marker, ROMs, also tended to be higher in females. Higher PC levels in females compared with males have previously been demonstrated in captive canaries (*Serinus canaria*) that were rearing reduced or enlarged broods (Costantini *et al*. 2014b). Since male and female blue tits differ in their reproductive behaviour and investment, with males defending the territory while females do all the nestbuilding, egg production, incubation and brooding of chicks (Perrins 1979), the greater damage levels in females might be a result of their greater overall level of reproductive investment, when compared with males. If this were true, then it might be expected that female blue tits would have a lower survival, as this has been found to be negatively impacted by increased reproductive expenditure (Santos & Nakagawa 2012). Indeed, previous work found female, but not male, blue tit survival to decrease with an increase in brood size (Nur 1984a), however, there was no significant sex difference in survival in my study nor was there any relationship between survival and initial brood size. Sex differences in antioxidant levels were also present, with GPX and OXY being significantly lower in females. This corroborates findings from great tits, in which non-enzymatic antioxidant capacity (OXY) was lower in females than males (Wegmann, Voegeli & Richner 2015b), and may have resulted from the potential greater reproductive investment of females, compared with males, causing antioxidant resources to be diverted away from antioxidant protection.

GPX was the only biomarker related to age, being significantly higher in birds that were born prior to the previous year, compared with birds that were one year old. A higher GPX level in older birds has previously been demonstrated in migrating robins (*Erithacus rubecula*) (Jenni-Eiermann *et al*. 2014); the authors suggested a number of reasons why this might be so. Firstly, the difference with age could arise from antioxidant defences maturing so that older birds have antioxidants that are more responsive to damage compounds (Surai 2002). Secondly, previous experience of a mildly demanding reproductive event in older birds might lead to activation of oxidative defence systems (i.e. hormesis; (Costantini 2014)), which does not occur in one-year-old birds because this is their first breeding attempt. However, it cannot be guaranteed that all older birds would have previously had the opportunity to breed. Thirdly, higher GPX in older birds could

result from selective disappearance of younger birds with poorer antioxidant defences (i.e. lower GPX levels).

In conclusion, my findings suggest that experimentally altered reproductive demands do not necessarily affect OS, nor is there a direct correlation between reproductive expenditure and OS. However, my experimental manipulation of brood size produced the opposite pattern to that expected, in that it reduced parental provisioning rate in parents of initially above average-sized broods. This makes it difficult to deduce that enhanced reproductive effort does not cause OS. Furthermore, a recent meta-analysis of homeothermic species (Blount *et al*. 2015) suggests a reproduction-OS relationship might well exist, depending on factors such as the biomarkers and tissues chosen for analysis, the time of sampling, as well as the environmental conditions. Future investigation of the oxidative costs of reproduction should involve longitudinal studies with repeated sampling of individuals within the same breeding period, as well as over many years and environments, to assess how this relationship might change over space and time.

Chapter 6

General Discussion

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In summary, my thesis has aimed to investigate OS and its mechanistic role in life-history trade-offs. From Chapter 2 it became clear that all five of the OS biomarkers considered here (GPX, thiols, OXY, PCs and ROMs) provided independent information on the redox system and therefore should be included in the subsequent analyses. Thus, using these biomarkers, I considered how OS and life-history might be linked. My meta-analyses in Chapter 4 suggested OS constrains growth and thus provided evidence for a growth – self-maintenance trade-off. Moreover, when forced to grow quickly, individuals could not adjust this trade-off and were faced with greater oxidative damage levels as a cost of growth. However, in my study species, these costs only became apparent when considering within-brood growth differences in the presence of an additional stressor (Chapter 3).

In my study it was not possible to evaluate whether any increased oxidative damage had long-term consequences and so in future analyses it might be interesting to determine whether there are fitness consequences to this OS. Since integration of four of the redox biomarkers considered here was correlated with body condition, which may relate to survival (Chapter 2), the covariance of these biomarkers could allow predictions about future fitness to be made. If a growth – self-maintenance trade-off exists, then we might also find self-maintenance to be traded off against other life-history traits, for instance, reproduction, leading to an oxidative cost of reproduction. Indeed, from Chapter 2, my results suggested a lower level of protein damage in breeding adults was associated with being further away from the population norm for covariance among redox biomarkers and I speculated that increased levels of oxidative damage may be normal during this demanding life-history phase. Yet in Chapter 5 I did not find any correlational or experimental link between reproductive expenditure and OS.

Measurements of the level of redox system integration provide an exciting new perspective in investigating how the redox response might be involved in life-history trade-offs. I found that the five OS biomarkers were not strongly integrated in breeding blue tits, contrary to a study of zebra finches that found GPX, thiols and ROM levels to be highly integrated (Costantini *et al.* 2011b). However, another study that included seven OS biomarkers (five antioxidant enzymes, glutathione and total antioxidant capacity) found differences both among species (gibel carp, *Carassius auratus gibelio* versus darkbarbel catfish, *Pelteobagrus vachelli*) and tissues in the level of integration among biomarkers (Dong *et al.* 2013). Overall the strength and direction of correlations among redox biomarkers has been found to be highly variable (Dotan *et al.* 2004), so perhaps the discrepancies are not surprising. My own and others' results have revealed the complexity of the redox system and show that this type of integration analysis benefits

from the inclusion of a large number of biomarkers of OS in different tissues (and ideally species), in addition to complementary univariate analyses to further investigate any trends. Furthermore, it has previously been demonstrated that within-group correlations of antioxidant levels are stronger than between group correlations (Cohen & McGraw 2009), so it might be beneficial to cluster groups of antioxidant biomarkers with similar properties together (e.g. antioxidant enzymes). Unfortunately I only had three antioxidant measures that covered separate parts of the redox system so I could not do this but it could be one aspect that warrants further investigation.

As the integration analyses had indicated, I used all five biomarkers to investigate how OS might be involved in life-history trade-offs. Experimental studies in which growth rate and reproductive expenditure have been manipulated may offer the best chance of identifying any oxidative costs of such trade-offs (Metcalfe & Monaghan 2013). This is because in purely correlational studies good quality individuals might be able to grow fast, enhance reproductive expenditure and not incur oxidative costs – possibly as a result of a better foraging ability and capacity to acquire antioxidants (Metcalfe & Monaghan 2013). At the same time, a recent meta-analysis found a significant positive effect between reproductive expenditure and oxidative damage, but only when correlational studies were included in the analysis (Blount *et al.* 2015); this suggests that correlational studies may be capable of revealing life-history trade-offs. Moreover, in my meta-analysis there was an oxidative cost of growth regardless of whether the study was correlational or experimental. It should, however, be noted that the two meta-analyses test different trade-offs and so are perhaps non-comparable. Thus while Blount *et al*. (2015) speculated that females engaged in parental care (which formed the majority of their data) cannot adjust physiologically to manipulations that are carried out after birth, as they are already working close to their maximum capacity, my meta-analysis focussed on growth rates, which are usually presumed to be submaximal and so unlikely to be limited by a physiological ceiling in the same way. While correlational studies clearly should not be discounted, experimental studies can offer a more robust system for investigating life-history trade-offs, hence in my empirical work I used brood manipulations in blue tits to investigate evidence for trade-offs between growth or reproduction and self-maintenance.

The results of the constraint meta-analysis (constraint-MA) in Chapter 4 supported the idea of a resource allocation trade-off between growth and self-maintenance, whereby growth would cause resources to be diverted away from self-maintenance processes like the repair and replacement of damaged cells. In general, individuals were not found to be able to adjust this trade-off when faced with enhanced growth (cost-MA), leading to oxidative costs in terms of raised damage levels. In addition, I suggested that oxidative

costs may result from increased RS production from an increased metabolism that occurs with enhanced growth. These patterns were also found in the empirical study of growth in blue tits (Chapter 3), in which being the fastest growing chick in a brood was associated with higher levels of protein damage (PCs) compared with slower growing sibs. Though I found no oxidative cost of growth when considering between-brood differences, the large brood size of blue tits (up to 13 chicks in my study) and within-brood hierarchies in growth rates may have meant that within-brood differences in oxidative status (i.e. PC levels) masked any between-brood differences. Due to the nature of the meta-analysis that considered OS differences between groups that had been experimentally manipulated to grow at different rates (cost-MA, Chapter 4), it was not possible to consider within-brood changes. A potential and exciting avenue for future research could be to review evidence for an oxidative response to within-brood growth differences in species whereby within-brood hierarchies occur. Since the within-brood effects in my study only became apparent in the presence of an additional stressor to enhanced growth rate (i.e. adding two extra chicks to the brood for the first half of the nestling period), it might also be interesting to determine how different environmental stressors affect this relationship.

Although my meta-analysis revealed that growth potentially carried a cost of increased oxidative damage, antioxidant levels remained unaffected by changes to growth rate. This contradicts the results of my empirical study of blue tits, in which levels of the antioxidant enzyme GPX varied in response to between-brood differences in growth rate (Chapter 3). In Chapter 4, I attributed the lack of change in overall antioxidant levels with changing growth rate to the variability in the antioxidant response to increased RS production, since antioxidant levels could potentially increase if upregulated or decrease if utilised in combating RS (Costantini & Verhulst 2009). We might obtain a clearer picture of the overall antioxidant response to growth if we considered the strength of integration amongst redox biomarkers and how this changes in response to alterations to growth rate. I found no difference in the integration of OS biomarkers between control and experimental nests (from mixed models with either D_M ($t=1.15$, $p=0.25$), PC1 ($t<0.0001$, $p=0.99$) or PC2 ($t<0.0001$, $p=0.99$) as the response variable, treatment as a fixed effect and nestbox identity as a random effect), however the effect of treatment on growth was found to depend on the synchronicity of hatching within the brood. Unfortunately I could not include the treatment-by-synchronicity interaction as there were only five asynchronous experimental chicks for which all five biomarkers had been measured, but this does not preclude future work from investigating how redox integration might relate to growth.

Even though altering growth rates did not affect antioxidant levels, the constraint-MA revealed that OS constrained growth in the unsupplemented group compared with those supplemented with natural compounds but not specific antioxidants. I speculated in Chapter 4 that the synergistic nature of antioxidant molecules might have allowed RS neutralisation and antioxidant recycling only when numerous antioxidants are supplemented together. Investigating how redox integration changes in response to supplementation may reveal the specific pathways that lead to improvements in antioxidant status when supplemented. Furthermore, it might be interesting to consider whether any changes to the integration of biomarkers resulting from either supplementation or exposure to environmental stressors could constrain various life-history traits, like growth and reproduction.

If self-maintenance is traded off against growth, then it might be expected to trade off against other life-history traits as well. Reproduction is another energetically demanding event that has the potential to cause OS, either directly by increasing RS production through raised metabolism, or by diverting resources away from self-maintenance. As mentioned in the summary, results from Chapter 2 (the negative association between Mahalanobis distance and PCs) suggested that oxidative damage might be a normal occurrence in reproducing animals. However, in Chapter 5 I did not find redox state to be altered where reproductive expenditure was experimentally reduced, nor was there any correlational evidence for a link between reproductive expenditure and OS. The reasoning behind the lack of a link between reproductive expenditure and OS is discussed in detail in Chapter 5 and further emphasises the need for longitudinal studies that include multiple biomarkers and tissues as there is a potential for transient effects to be missed.

In fact, repeated within-individual measures from longitudinal studies are especially important because cross-sectional studies might give the impression that oxidative damage is lower in breeders (versus non-breeders) and older (versus younger) individuals, if those with higher damage levels are less likely to reproduce or more likely to die earlier. Obtaining longitudinal data from the field can prove to be difficult, with issues surrounding recapturing the same individual repeatedly, especially where mortality risk is high. Whilst laboratory studies solve the problem of resampling, they might not be reflective of natural conditions and so impractical for testing life-history trade-offs. For instance, *ad libitum* food given so that resources are not limited could negate the need to trade-off different processes. Constant temperature and photoperiod in the laboratory also reduce the potential impact of environmental stressors that occurs under natural conditions, for instance thermoregulatory demands (Speakman *et al.* 2015).

Repeated sampling in the field, while possible, is further limited in species that have a high risk of abandoning their breeding attempt when disturbed or those whereby sampling (e.g. of blood) is restricted. Yet, even a small number of within-individual longitudinal samples targeted at various key life-history events (e.g. over the period of growth, or from repeated reproductive attempts) would prove to be useful in determining interactions between OS across an individual's life. Obtaining longitudinal data would reinforce the need for non-lethal sampling (e.g. of blood, feathers), which I was restricted to in my study. This could prove to be problematic if effects on OS depended on the tissue chosen for analysis, as has been demonstrated for both the OS-growth relationship (Brown-Borg & Rakoczy 2003; Leggatt *et al.* 2007) and the OS-reproduction relationship (Garratt *et al.* 2012; Xu *et al.* 2013; Yang *et al.* 2013). Furthermore, certain tissues and organs may be prioritised and protected, if, for example, their functions are essential for reproductive success (e.g. digestive organs that optimise nutrient absorption and waste processing) and offspring quality (e.g. ovarian follicles) (Blount *et al.* 2015). In such tissues, the effects on oxidative damage might be masked, however, the fact that I found an oxidative cost of growth in blue tit chicks suggests blood is not protected in such a way. Additionally results from my meta-analyses did not differ between non-lethal (e.g. of blood, urine) and lethal (e.g. of liver, muscle) sampling, implying that blood-based biomarkers are at least as sensitive as other tissues. Furthermore, the significance of an oxidative cost to tissues obtained via non-lethal methods would depend on whether these costs entail consequences for organismal fitness (Speakman *et al.* 2015).

When considering fitness consequences it is likely that the two trade-offs of growth and reproduction with self-maintenance are not unrelated. Individuals that experienced raised oxidative damage levels as a result of experimentally enhanced growth rates might suffer in terms of reproductive output – previous work has found litter size at birth to be negatively affected by oxidative damage levels before reproduction in laboratory mice (Stier *et al.* 2012). While pre-reproductive OS levels influenced reproductive decisions (e.g. clutch size) in canaries, this did not affect reproductive success (Costantini *et al.* 2015). Additionally, effects on reproduction may not be seen if the oxidative costs of growth are transient and any damage has been repaired or removed by the time of the following breeding season. Likewise, if adults with high oxidative damage levels reproduced successfully, this might impact on offspring OS levels, which in turn could constrain their growth.

Moreover, raised oxidative damage levels as a result of fast growth or high reproductive expenditure could have consequences later in life. Indeed a trade-off between early life events, such as growth and reproduction, and late life performance, such as the rate of

ageing and longevity, has previously been demonstrated in a number of studies (Lee *et al*. 2013a; Reichert *et al.* 2014; Sudyka *et al.* 2014; Badás *et al.* 2015) including one review of 26 studies of free-ranging vertebrates (Lemaître *et al.* 2015). Yet the role of OS here is unclear, with discussion as to whether increased oxidative damage can cause ageing or negatively impact on lifespan (for a review, see Speakman & Selman 2011; Selman *et al.* 2012). At the same time, previous work has linked deteriorated oxidative status with reduced survival (Saino *et al.* 2011b; Noguera, Kim & Velando 2011b; Reichert *et al.* 2014). It is evident that antioxidants are essential for the 'normal' functioning of the organism: when certain antioxidants (e.g. vitamins C and/or E) dropped below a critical threshold, becoming deficient, survival was found to be reduced (Lee & Dabrowski 2003; Zhou *et al*. 2013). Additionally supplementation of various dietary antioxidants (e.g. vitamins C and/or E) has been shown to increase activity of endogenous antioxidant enzymes (e.g. GPX, SOD) (Ebeid *et al*. 2013; Zhou *et al.* 2013; Cinar *et al.* 2014). Antioxidant supplementation reduced the amount of telomere decay (indicative of ageing) between breeding seasons in blue tits (Badás *et al.* 2015), yet various studies have shown antioxidant supplementation not to affect survival (e.g. de Ayala *et al.* 2006; Gao *et al*. 2013a; Gao *et al*. 2014a). Therefore, further investigation is needed here to elucidate future fitness consequences of oxidative costs.

One method of assessing the fitness consequences of OS could be to explore redox integration and how this relates to a measure of fitness (e.g. body condition that can be related to future survival in passerines (Tinbergen & Boerlijst 1990)), as I did in Chapter 2. I found that integration of four out of five redox biomarkers (GPX, OXY, ROMs and thiols) correlated with body condition in nestling, but not adult, blue tits. Therefore the degree of integration of these biomarkers and how this changes in response to growth rate could allow fitness consequences of the oxidative costs of growth to be estimated. The lack of correlation between redox integration and condition in adult blue tits could be the result of the tissue chosen for analysis; perhaps if tissues that are more sensitive to the negative effects of OS were considered (e.g. those important for producing the germline DNA), we might see impacts on condition and future fitness. However, these tissues are likely to be the ones that are protected from OS (Blount *et al.* 2015), as already discussed – this emphasises the complexity of the OS response and the need to investigate many different tissues to tease apart which are protected. Of course adults may simply have a greater tolerance for redox imbalance than do young animals and so the consequences to changes in redox integration do not become evident immediately in adults; in this case another physiological measure of body condition (e.g. maximal thermogenic capacity or an inflammation score as used by Milot *et al.* 2014) might have served better.

The conclusions drawn about these life-history trade-offs are likely to depend on a particular species' life-history strategy. For instance, in rodents the capacity to process more food and produce more milk when litter size is increased is limited by the need to dissipate heat (Król & Speakman 2003), meaning they cannot raise reproductive expenditure, so it might appear that OS does not increase with reproduction. On the other hand, many birds will increase reproductive expenditure, in terms of provisioning of chicks, when faced with an enlarged brood size (Santos & Nakagawa 2012), though in my study parental response to increased brood size depended on the initial brood size. Short-lived species with a small window for growth, such as the blue tit, might be expected to sacrifice self-maintenance for growth and current reproduction (Parejo & Danchin 2006). This could mean that oxidative costs are more apparent, compared with longer-lived indeterminate growing species that grow for a larger proportion of their life (e.g. mammals and fish) and may be able to delay growth and reproduction when OS arises, thus prioritising self-maintenance. However the choice of the blue tit as my study species had the disadvantage that the blood samples obtained were not always large enough to analyse all five OS biomarkers, limiting my sample size. Furthermore, time constraints meant I had to focus on either chicks or adults so they were measured over two separate years – this made it difficult to directly compare between them.

Environmental quality usually differs between years and this is likely to have a considerable impact on the extent to which we can observe resource allocation trade-offs. It may be expected that we only see the costs of growth and reproduction during suboptimal conditions that render individuals' repair and defence mechanisms less able to cope (Speakman *et al.* 2015) and during which individuals are unable to invest in all processes simultaneously. Therefore comparing habitats of differing quality (either through experimental manipulation or environments that naturally differ in quality) or between-year effects might help to tease apart the circumstances under which we can observe such trade-offs. While my study attempted this by analysing distance from the loch shore, the treatment effect on OS did not depend on this variable for chicks (Chapter 3) or adults (Chapter 5; distance from the loch shore was excluded due to non-significance in preliminary tests) and the treatment effects on growth and reproduction were complicated by other factors. Perhaps if environmental quality is not suboptimal then additional stressors must be present to reveal any effects; it would be interesting to determine whether the presence of such stressors might reveal oxidative costs of reproduction. For instance, it could be that parents are more likely to show a cost of greater oxidative damage levels for the increased foraging effort required to feed additional offspring if they have increased maintenance costs (e.g. endotherms during a cold spell). Furthermore, environmental stressors could impact on the overall redox

response as well as the interactions among biomarkers – in zebra finches short-term high flight activity resulted in a decline in redox integration (Costantini *et al.* 2013). What is needed here is further exploration into the effects of such environmental stressors on redox integration amongst a larger variety of species and environments.

In conclusion, my thesis has highlighted the complex and variable nature of the redox system and its response to changes in animal life-histories, as well as a number of potential areas for future research. Firstly, measuring redox integration of numerous biomarkers and tissues can be used as a tool for investigating the overall antioxidant response regardless of its direction, potentially overcoming the issue of its variability and uncovering the individual interactions behind the synergistic nature of various antioxidant molecules. This could help to clarify how antioxidants respond to changes to growth and reproduction, in addition to any oxidative damage that might be present. Secondly, while my results suggest OS may both constrain and be a cost of growth, there was no correlational or experimental link between OS and reproduction; yet oxidative costs are likely to depend greatly on environmental conditions, possibly only becoming apparent if these are suboptimal. This emphasises the need to make comparisons across a range of different habitats and conditions, as well as over different years that are likely to vary in quality. While experimental manipulations remain an important method for assessing such costs, my results also imply correlational ones should not be discounted as there was no difference between correlational and experimental studies in trends shown in the cost-MA. Additionally, comparisons across numerous taxonomic groups with varying life-history strategies would prove to be interesting; for instance, those species that might be more likely to sacrifice self-maintenance due to a short growth window compared with indeterminate growing species, as well as comparisons of different reproductive strategies, with some species being limited by a physiological ceiling. Furthermore, there is likely to be a complex interplay between different key events in an individual's life; high OS levels from fast growth could constrain reproduction later in life and likewise high parental OS could constrain offspring growth. Within-individual sampling from longitudinal studies could help to tease apart how various life-history events interact, as well as overcoming the highly variable nature of biomarker interactions over time. Any change to the oxidative status has the potential to have long-term consequences and inevitably impact on future fitness, though further study is needed here before firm conclusions can be drawn about how OS might impact on ageing and survival. Finally, if important tissues are prioritised and protected from oxidative damage, while others can tolerate a given level of damage, then there may be no long-term detrimental effects of any oxidative costs.

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Appendices

Appendix 1: supplementary figure for Chapter 2

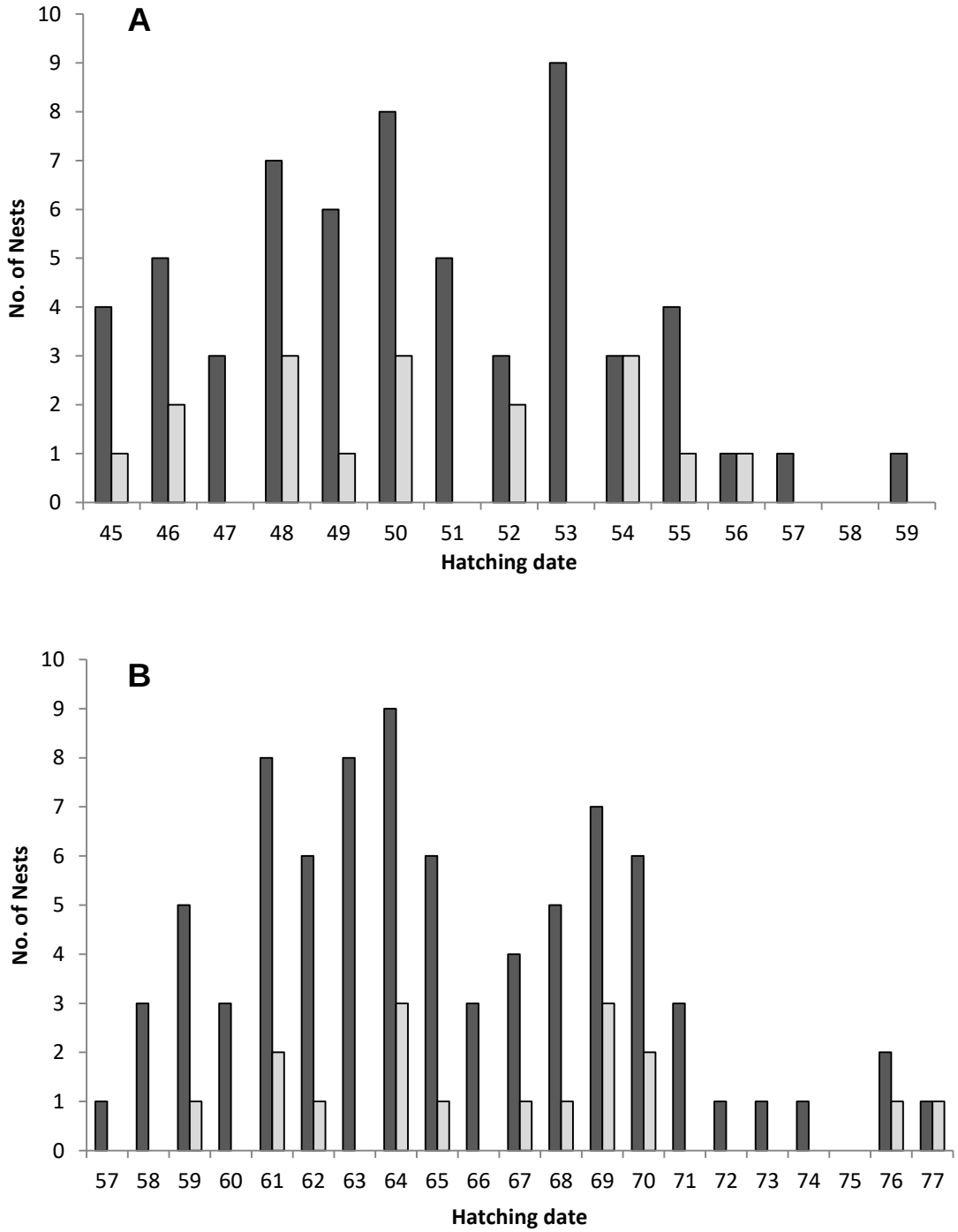


Figure S1 Frequency histograms demonstrating the number of nests at each hatching date for 2012 when chicks were sampled (A) and 2013 when adults were sampled (B); for the whole population (dark grey bars, n=60 for 2012, n=82 for 2013) and only those nests included in Chapter 2 (light grey bars, n=17 for 2012, n=17 for 2013). Note that hatching date is given as 'April days' so that 1 = 1st April, thus 45 = 15th May and 57 = 27th May; 2013 was a later and more prolonged breeding season than 2012.

Appendix 2: supplementary figures for Chapter 3

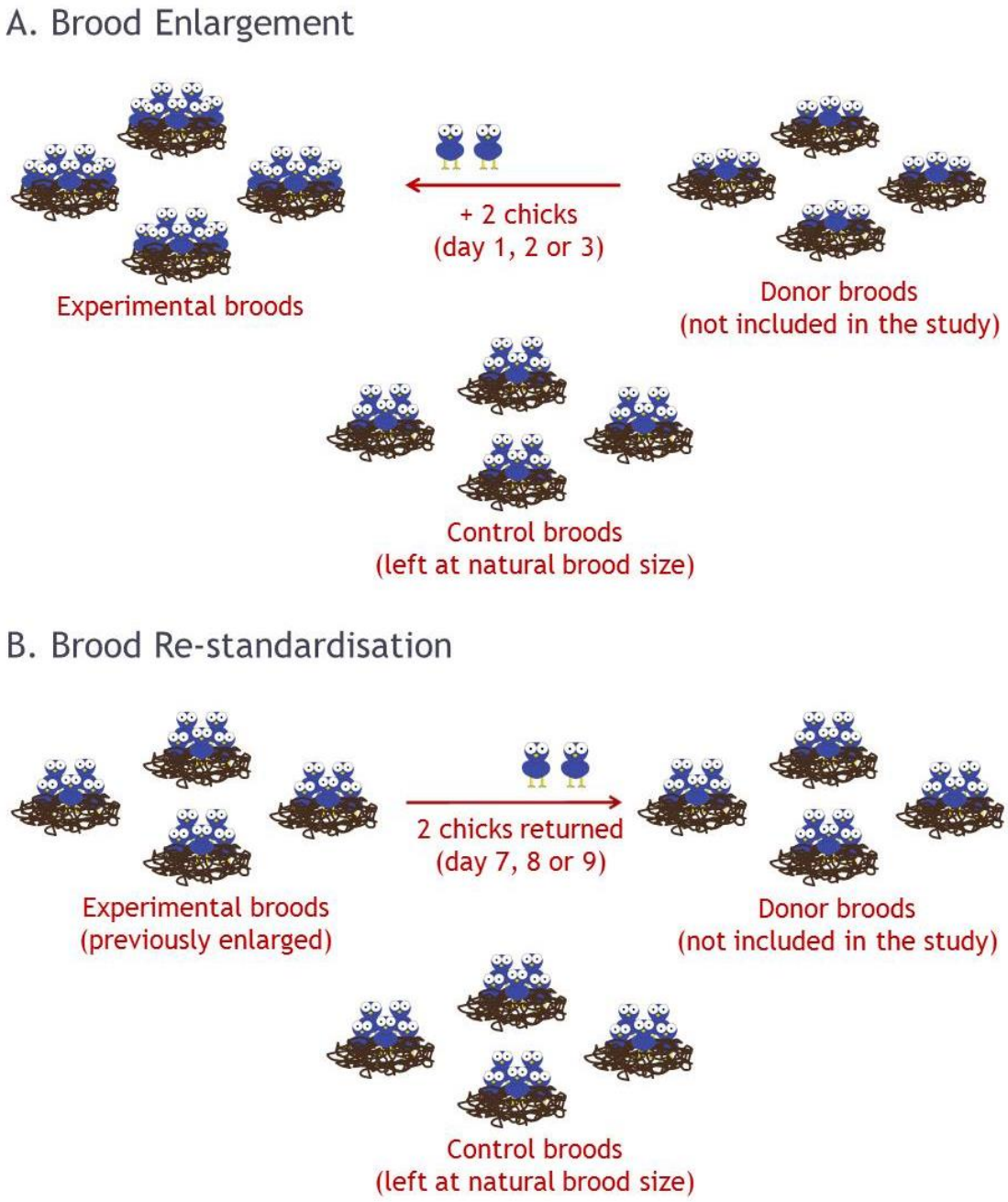


Figure S2 Brood manipulation regime for 2012. Brood sizes shown have been standardised for presentation purposes. Day 0 = day of hatching of the first chick. For full details please see the text.

Figure S3 Map of the study site demonstrating the two main habitat types. Ross Wood has blueberries, whilst Sallochy Wood has bluebells, as dominant underlying undergrowth. The boundary between these habitats is given by the red line

Appendix 3: supplementary methods for Chapter 4

Data acquisition from authors

There were 30 papers where an English copy of the text could not be accessed, in which case authors were contacted; 19 out of 30 authors responded, through which eight more papers were obtained, the remaining 11 were not available in English. To calculate effect sizes for each paper in meta-analysis, either appropriate test statistics (e.g. t values or F ratios) or means, standard errors and sample sizes must be available. In 21 papers these values were not provided so the authors were contacted for additional information with a reminder being sent after one month, of which ten responded and provided the missing information.

Inclusion/exclusion criteria

Inclusion and exclusion criteria were applied to select papers to include in analyses. For constraint-MA, papers were selected if they contained a comparison of growth rates between two groups that differed significantly in OS. In some cases for constraint-MA, studies did not measure OS until after the growth period was measured (e.g. if animals were sacrificed for tissue samples) so it might be questionable that this would have affected growth. However, since many redox biomarkers show intra-individual consistency, even taking into account experimental manipulations (Costantini *et al.* 2007), any differences in OS between control and experimental groups observed after the growth period would have been relatively the same before the growth period. Therefore the final OS measure was taken as a validation that OS had been significantly altered in the treatment groups (note that OS was not the response variable in the main analysis where I was interested in growth for constraint-MA). Furthermore, the time of OS measurement (i.e. before or after the growth period) was included as a factor in the statistical analysis for constraint-MA and was not found to have a significant effect. For cost-MA, papers were selected if they contained a comparison of OS levels between two groups that differed significantly in growth rate.

The following studies were excluded from both meta-analyses: those that measured RS, since they do not provide direct evidence of OS (RS might be mopped up before OS arises) (Costantini *et al.* 2011a); those that measured gene expression rather than activity of antioxidant molecules because I was interested in the biochemistry and not genetics of the relationship; or studies where necessary information for calculating effect size was

unavailable. Where there was more than one experimental group, the two most extreme groups in terms of OS (constraint-MA) or growth rate (cost-MA) were compared.

Standardisation of growth data

The method by which growth rate was defined differed between studies. For most studies, growth rate was defined as the change in mass (i.e. final mass – initial mass) over time (days of experimental period). Some studies used specific growth rate (SGR), which was defined as:

$$SGR = \frac{\ln(final\ mass) - \ln(initial\ mass)}{number\ of\ days\ elapsed}$$

At times studies did not measure growth rate directly but assumed different growth rates where two groups were measured to have the same initial mass but ended with significantly different masses. The majority of studies used mass growth, except three studies in the constraint-MA that used developmental rate and one in the cost-MA that used structural size. These variations in growth measures were of no concern for either meta-analysis because standardised effect sizes for the growth difference between groups were extracted from each study where growth rates were given, thus overcoming any differences in units between studies.

Publication bias

Publication bias was assessed by examining funnel plots of effect size against the log of sample size for each dataset (Møller & Jennions 2001). The plot should be in the shape of a 'funnel' with larger variance in effect sizes at small sample sizes and a decreasing variance with increasing sample size. If only significant findings were published, one might expect there to be a 'gap' in the lower left of the graph, where for small samples effect sizes must be relatively large to be statistically significant. The funnel plots in the present study indicate there was no publication bias (Figure S4, Appendix 4). This is confirmed by the fact that sample size was not significant when added as a predictor in the models (constraint-MA: Q_M=0.45, df=1, p=0.50; cost-MA: Q_M=1.69, df=1, p=0.19); this method of assessing funnel plot asymmetry is recommended by Viechtbauer (2014b) since it takes into account moderators (unlike the fail-safe N and rank correlation).

The effect of manipulation type on OS (constraint-MA) and growth (cost-MA)

To investigate the relationship between growth and OS for each MA, I first had to determine whether the treatments successfully altered OS (constraint-MA) and growth (cost-MA). Note this is not a test of the main hypotheses, where I was interested in growth (constraint-MA) and OS (cost-MA) as my response variables. Hedges' *g* values were extracted for the treatment effect on OS (constraint-MA, extracted from all studies) and on growth (cost-MA, data were unavailable for one study). Since the Hedges' *g* values indicated the effectiveness of the experimental manipulation, a positive value denoted a difference in OS (constraint-MA) or growth rate (cost-MA) in the treatment group, compared with controls. The Hedges' *g* values were the response variable in similar mixed models to that described in Chapter 4, with experimental manipulation as a moderator. Biomarker and sampling method were also included as moderators for constraint-MA. Lastly, model simplification was carried out as before.

Appendix 4: supplementary figures for Chapter 4

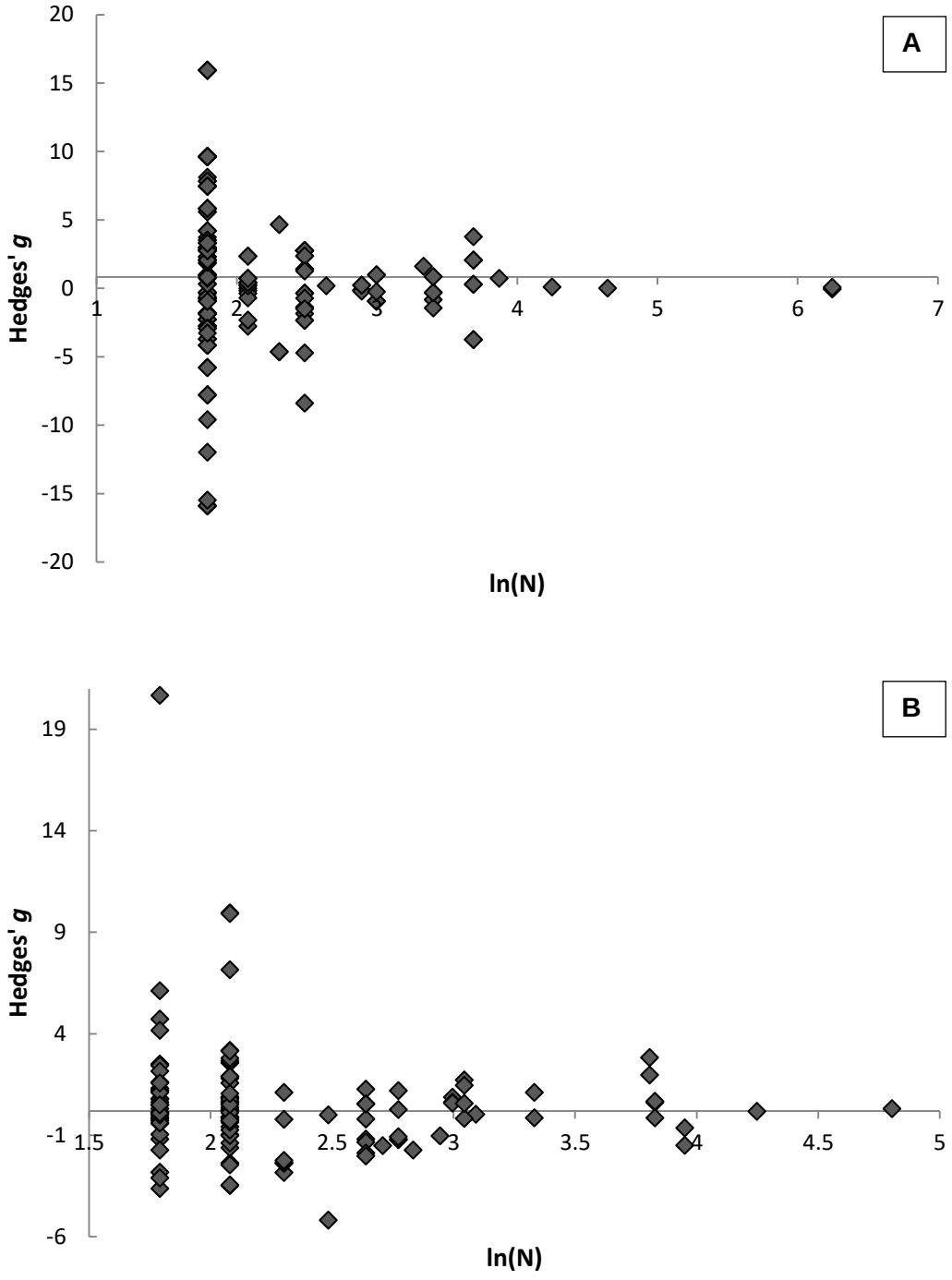


Figure S4 Funnel plots for constraint-MA (A) and cost-MA (B) showing the change in effect size (Hedges' g) with the natural log of sample size (N). The X-axes have been centred on the average effect sizes.

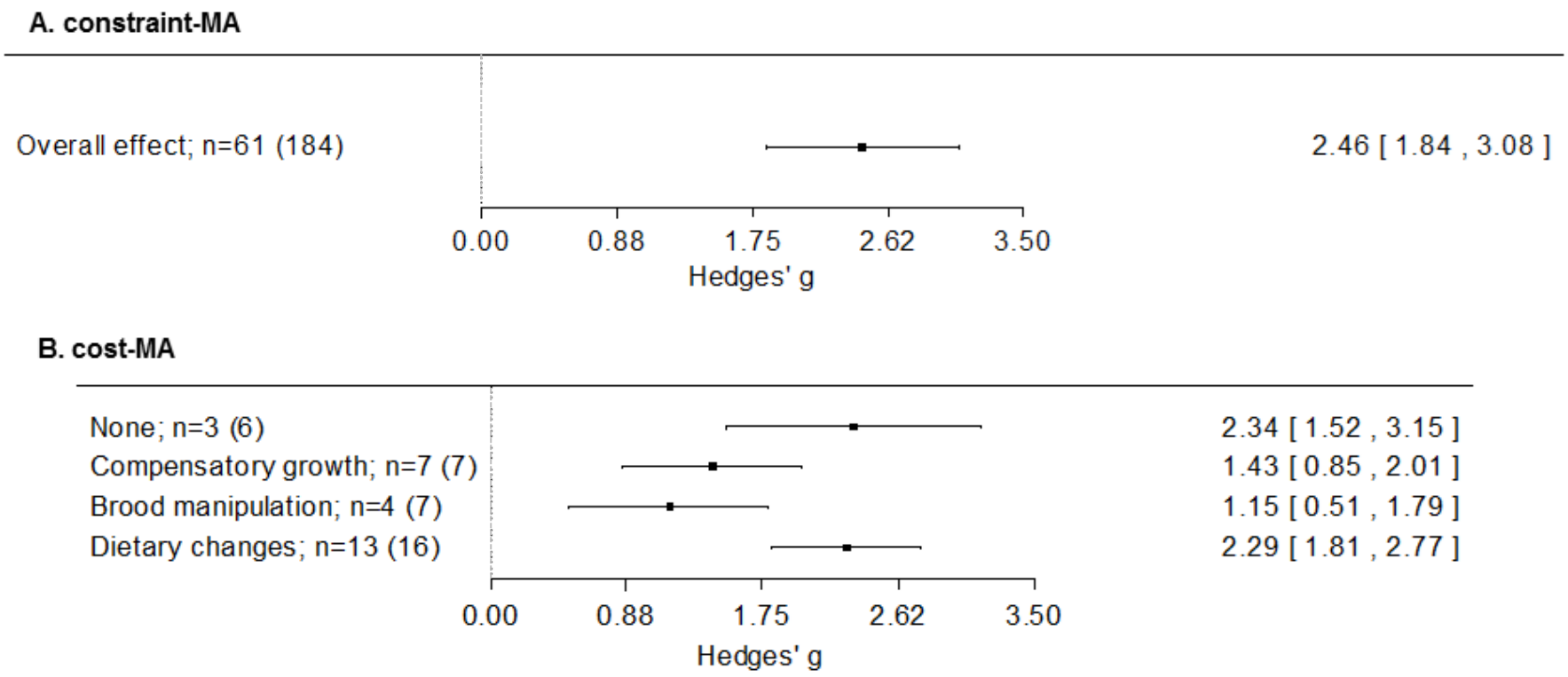


Figure S5 Forest plots for constraint-MA (A) and cost-MA (B). These do not test the main hypotheses but provide evidence that treatments were successful in manipulating OS (A) and growth (B). Since the effect on growth in cost-MA (B) depended on the type of manipulation, the plot has been separated accordingly (there was no difference between experimental manipulations for constraint-MA, therefore an overall effect size is given). Mean and 95% confidence interval, CI, are given on the right hand side. When the CI does not include zero, the effect size is significant. Sample sizes given are number of studies (number of effect sizes). 'None' indicates observational studies that found natural and significant growth differences between two groups, for example from different populations or habitats. For full details of growth manipulations, see Table 4:1 in Chapter 4.

Appendix 5: supplementary tables for Chapter 4

Table S1 Summary details for studies included in constraint-MA investigating the effects of OS on growth rate.

Species	Taxonomic Class	Experimental Manipulation	Biological Matrix	Sampling Method	Biomarker	Biomarker Category	N	Hedges' g	Authors
Acipenser baerii	Actinopterygii	stressor exposure	whole body	lethal	isoprostanes	damage	40	3.75	(Fontagné *et al.* 2006)
Acipenser baerii	Actinopterygii	stressor exposure	whole body	lethal	TBARS	damage	40	3.75	(Fontagné *et al.* 2006)
Acipenser baerii	Actinopterygii	stressor exposure	whole body	lethal	Se-GPX	enzyme	40	3.75	(Fontagné *et al.* 2006)
Alectoris chukar	Aves	natural compound supplementation	blood	non-lethal	DNA damage (Comet)	damage	6	0.63	(Yurtseven *et al.* 2008)
Anguilla japonica	Actinopterygii	natural compound supplementation	liver	lethal	CAT	enzyme	6	5.58	(Lee *et al*. 2013b)
Anguilla japonica	Actinopterygii	natural compound supplementation	liver	lethal	SOD	enzyme	6	5.58	(Lee *et al*. 2013b)
Apostichopus japonicus	Holothuroidea	stressor exposure	whole body	lethal	TBARS	damage	6	5.78	(Gao *et al*. 2013a)
Apostichopus japonicus	Holothuroidea	antioxidant supplementation	whole body	lethal	TBARS	damage	6	0.8	(Gao *et al*. 2013a)
Apostichopus japonicus	Holothuroidea	antioxidant supplementation	whole body	lethal	vitamin E	non-enzymatic	6	0.8	(Gao *et al*. 2013a)
Apostichopus japonicus	Holothuroidea	stressor exposure	whole body	lethal	vitamin E	non-enzymatic	6	5.78	(Gao *et al*. 2013a)
Bos taurus	Mammalia	natural compound supplementation	serum	non-lethal	MDA	damage	8	0.17	(Wang *et al.* 2011)

Bos taurus	Mammalia	natural compound supplementation	serum	non-lethal	SOD	enzyme	8	0.17	(Wang *et al.* 2011)
Carassius auratus gibelio	Actinopterygii	natural compound supplementation	liver	lethal	MDA	damage	30	0.32	(Yi *et al.* 2012)
Carassius auratus gibelio	Actinopterygii	natural compound supplementation	serum	non-lethal	MDA	damage	30	0.32	(Yi *et al.* 2012)
Cavia porcellus	Mammalia	antioxidant supplementation	liver	lethal	vitamin C	non-enzymatic	12	1.25	(Kato, Kawai & Yoshida 1981)
Coturnix japonica	Aves	stressor exposure	liver	lethal	MDA	damage	20	0.96	(Onderci *et al.* 2005)
Coturnix japonica	Aves	stressor exposure	muscle	lethal	MDA	damage	20	0.96	(Onderci *et al.* 2005)
Coturnix japonica	Aves	stressor exposure	serum	non-lethal	MDA	damage	20	0.96	(Onderci *et al.* 2005)
Coturnix japonica	Aves	stressor exposure	serum	non-lethal	vitamin C	non-enzymatic	20	0.96	(Onderci *et al.* 2005)
Coturnix japonica	Aves	stressor exposure	serum	non-lethal	vitamin E	non-enzymatic	20	0.96	(Onderci *et al.* 2005)
Cyprinus carpio	Actinopterygii	stressor exposure	liver	lethal	GPX	enzyme	12	1.35	(Mustafa *et al.* 2011)
Gallus gallus	Aves	antioxidant supplementation	plasma	non-lethal	vitamin A	non-enzymatic	18	-0.22	(Cinar *et al.* 2014)
Gallus gallus	Aves	antioxidant supplementation	blood	non-lethal	GPX	enzyme	18	0.22	(Cinar *et al.* 2014)
Gallus gallus	Aves	antioxidant supplementation	plasma	non-lethal	vitamin E	non-enzymatic	18	0.22	(Cinar *et al.* 2014)
Gallus gallus	Aves	natural compound	liver	lethal	TBARS	damage	516	0.06	(Díaz-Cruz *et al.* 2003)

		supplementation							
Gallus gallus	Aves	natural compound supplementation	liver	lethal	tGSH	non-enzymatic	516	0.06	(Díaz-Cruz *et al.* 2003)
Gallus gallus	Aves	natural compound supplementation	muscle	lethal	MDA	damage	14	-0.17	(Ijiri *et al.* 2013)
Gallus gallus	Aves	natural compound supplementation	plasma	non-lethal	TBARS	damage	8	0.03	(Khempaka, Pudpila & Molee 2013)
Gallus gallus	Aves	antioxidant supplementation	plasma	non-lethal	TBARS	damage	20	0.27	(Lu *et al.* 2014)
Gallus gallus	Aves	stressor exposure	heart	lethal	GSH:GSSG	non-enzymatic	12	2.75	(Simitzis *et al.* 2012)
Gallus gallus	Aves	stressor exposure	heart	lethal	tGSH	non-enzymatic	12	2.75	(Simitzis *et al.* 2012)
Gallus gallus	Aves	stressor exposure	liver	lethal	GSH:GSSG	non-enzymatic	12	2.75	(Simitzis *et al.* 2012)
Gallus gallus	Aves	stressor exposure	liver	lethal	tGSH	non-enzymatic	12	2.75	(Simitzis *et al.* 2012)
Gallus gallus	Aves	stressor exposure	spleen	lethal	GSH:GSSG	non-enzymatic	12	2.75	(Simitzis *et al.* 2012)
Gallus gallus	Aves	stressor exposure	testes	lethal	GSH:GSSG	non-enzymatic	12	2.75	(Simitzis *et al.* 2012)
Gallus gallus	Aves	stressor exposure	testes	lethal	tGSH	non-enzymatic	12	2.75	(Simitzis *et al.* 2012)
Gallus gallus	Aves	stressor exposure	liver	lethal	MDA	damage	12	1.85	(Taniguchi, Ohtsuka & Hayashi 1999)
Gallus gallus	Aves	antioxidant supplementation	liver	lethal	MDA	damage	12	1.51	(Taniguchi *et al.* 1999)
Gallus gallus	Aves	stressor exposure	plasma	non-lethal	MDA	damage	8	2.78	(Thiamhirunsopit *et al.* 2014)

Gallus gallus	Aves	natural compound supplementation	plasma	non-lethal	MDA	damage	8	-0.26	(Thiamhirunsopit *et al.* 2014)
Gallus gallus	Aves	natural compound supplementation	serum	non-lethal	MDA	damage	8	0.4	(Zhang *et al*. 2013a)
Gallus gallus	Aves	natural compound supplementation	serum	non-lethal	GPX	enzyme	8	0.4	(Zhang *et al*. 2013a)
Gallus gallus	Aves	natural compound supplementation	serum	non-lethal	SOD	enzyme	8	0.4	(Zhang *et al*. 2013a)
Gallus gallus	Aves	natural compound supplementation	serum	non-lethal	MDA	damage	8	0.72	(Zhang *et al.* 2009)
Gallus gallus	Aves	natural compound supplementation	serum	non-lethal	GPX	enzyme	8	0.72	(Zhang *et al.* 2009)
Gallus gallus	Aves	natural compound supplementation	serum	non-lethal	SOD	enzyme	8	0.72	(Zhang *et al.* 2009)
Gallus gallus	Aves	natural compound supplementation	liver	lethal	MDA	damage	6	0.97	(Zheng *et al.* 2013)
Gallus gallus	Aves	natural compound supplementation	liver	lethal	GPX	enzyme	6	0.97	(Zheng *et al.* 2013)
Gallus gallus	Aves	natural compound supplementation	liver	lethal	SOD	enzyme	6	0.97	(Zheng *et al.* 2013)
Gallus gallus	Aves	natural compound supplementation	liver	lethal	T-AOC	non-enzymatic	6	0.97	(Zheng *et al.* 2013)

Gallus gallus	Aves	natural compound supplementation	serum	non-lethal	GPX	enzyme	6	0.97	(Zheng *et al.* 2013)
Haliotis discus hannai	Gastropoda	natural compound supplementation	hepato-pancreas	lethal	GPX	enzyme	6	2.75	(Zhang *et al.* 2010)
Haliotis discus hannai	Gastropoda	natural compound supplementation	hepato-pancreas	lethal	GSH	non-enzymatic	6	2.75	(Zhang *et al.* 2010)
Haliotis discus hannai	Gastropoda	natural compound supplementation	hepato-pancreas	lethal	SOD	enzyme	6	2.75	(Zhang *et al.* 2010)
Haliotis discus hannai	Gastropoda	natural compound supplementation	hepato-pancreas	lethal	T-AOC	non-enzymatic	6	2.75	(Zhang *et al.* 2010)
Hirundo rustica	Aves	antioxidant supplementation	plasma	non-lethal	vitamin E	non-enzymatic	104	-0.0001	(de Ayala, Martinelli & Saino 2006)
Hyphessobrycon callistus	Actinopterygii	antioxidant supplementation	serum	non-lethal	ALT	damage	6	0.76	(Wang, Chien & Pan 2006)
Hyphessobrycon callistus	Actinopterygii	antioxidant supplementation	serum	non-lethal	AST	damage	6	0.76	(Wang *et al.* 2006)
Hyphessobrycon callistus	Actinopterygii	antioxidant supplementation	serum	non-lethal	GPX	enzyme	6	-0.76	(Wang *et al.* 2006)
Hyphessobrycon callistus	Actinopterygii	antioxidant supplementation	serum	non-lethal	SOD	enzyme	6	-0.76	(Wang *et al.* 2006)
Hypophthalmichthys nobilis	Actinopterygii	stressor exposure	liver	lethal	MDA	damage	40	-0.29	(Sun *et al.* 2013)
Hypophthalmichthys nobilis	Actinopterygii	stressor exposure	liver	lethal	SOD	enzyme	40	0.29	(Sun *et al.* 2013)
Ictalurus punctatus	Actinopterygii	natural compound	plasma	non-lethal	CAT	enzyme	6	3.49	(Zheng *et al.* 2009)

		supplementation							
Ictalurus punctatus	Actinopterygii	natural compound supplementation	plasma	non-lethal	SOD	enzyme	6	3.49	(Zheng *et al.* 2009)
Larus michahellis	Aves	antioxidant supplementation	RBCs	non-lethal	DNA damage	damage	70	-0.09	(Noguera *et al.* 2011a)
Leporinus elongatus	Actinopterygii	stressor exposure	blood	non-lethal	tGSH	non-enzymatic	12	-1.41	(Filho *et al.* 2005)
Leporinus elongatus	Actinopterygii	stressor exposure	liver	lethal	tGSH	non-enzymatic	12	-1.41	(Filho *et al.* 2005)
Leporinus elongatus	Actinopterygii	stressor exposure	blood	non-lethal	GST	enzyme	12	1.41	(Filho *et al.* 2005)
Litopenaeus vannamei	Malacostraca	natural compound supplementation	liver	lethal	MDA	damage	6	5.82	(Shen *et al.* 2010)
Litopenaeus vannamei	Malacostraca	natural compound supplementation	liver	lethal	GPX	enzyme	6	5.82	(Shen *et al.* 2010)
Litopenaeus vannamei	Malacostraca	natural compound supplementation	liver	lethal	T-AOC	non-enzymatic	6	5.82	(Shen *et al.* 2010)
Litopenaeus vannamei	Malacostraca	natural compound supplementation	haemolymph	non-lethal	GPX	enzyme	6	3.3	(Yang *et al.* 2010)
Litopenaeus vannamei	Malacostraca	antioxidant supplementation	haemolymph	non-lethal	CAT	enzyme	10	-4.65	(Zhang *et al*. 2013b)
Litopenaeus vannamei	Malacostraca	antioxidant supplementation	haemolymph	non-lethal	SOD	enzyme	10	-4.65	(Zhang *et al*. 2013b)
Litopenaeus vannamei	Malacostraca	antioxidant supplementation	haemolymph	non-lethal	TAS	non-enzymatic	10	4.65	(Zhang *et al*. 2013b)
Macrobrachium rosenbergii	Malacostraca	natural compound supplementation	hepato-pancreas	lethal	vitamin C	non-enzymatic	6	7.46	(Radhakrishnan *et al.* 2013)

Macrobrachium rosenbergii	Malacostraca	natural compound supplementation	hepato-pancreas	lethal	vitamin E	non-enzymatic	6	7.46	(Radhakrishnan *et al.* 2013)
Macrobrachium rosenbergii	Malacostraca	natural compound supplementation	muscle	lethal	vitamin C	non-enzymatic	6	7.46	(Radhakrishnan *et al.* 2013)
Macrobrachium rosenbergii	Malacostraca	natural compound supplementation	muscle	lethal	vitamin E	non-enzymatic	6	7.46	(Radhakrishnan *et al.* 2013)
Marsupenaeus japonicus	Malacostraca	antioxidant supplementation	flesh	lethal	carotenoids	non-enzymatic	6	0.71	(Chien & Shiau 2005)
Micropterus salmoides	Actinopterygii	stressor exposure	liver	lethal	CAT	enzyme	6	-1.82	(Chen *et al.* 2013)
Micropterus salmoides	Actinopterygii	stressor exposure	liver	lethal	MDA	damage	6	1.82	(Chen *et al.* 2013)
Micropterus salmoides	Actinopterygii	stressor exposure	muscle	lethal	MDA	damage	6	1.82	(Chen *et al.* 2013)
Micropterus salmoides	Actinopterygii	stressor exposure	serum	non-lethal	MDA	damage	6	1.82	(Chen *et al.* 2013)
Micropterus salmoides	Actinopterygii	stressor exposure	liver	lethal	GSH	non-enzymatic	6	1.82	(Chen *et al.* 2013)
Micropterus salmoides	Actinopterygii	stressor exposure	liver	lethal	vitamin E	non-enzymatic	6	-2.29	(Chen *et al.* 2012)
Micropterus salmoides	Actinopterygii	stressor exposure	muscle	lethal	vitamin E	non-enzymatic	6	-2.29	(Chen *et al.* 2012)
Micropterus salmoides	Actinopterygii	stressor exposure	serum	non-lethal	vitamin E	non-enzymatic	6	-2.29	(Chen *et al.* 2012)
Micropterus salmoides	Actinopterygii	stressor exposure	muscle	lethal	MDA	damage	6	-2.29	(Chen *et al.* 2012)
Micropterus salmoides	Actinopterygii	stressor exposure	serum	non-lethal	MDA	damage	6	-2.29	(Chen *et al.* 2012)
Oncorhynchus kisutch	Actinopterygii	antioxidant supplementation	liver	lethal	vitamin E	non-enzymatic	6	-0.3	(Huang *et al.* 2004)

Oncorhynchus kisutch	Actinopterygii	antioxidant supplementation	muscle	lethal	vitamin E	non-enzymatic	6	-0.3	(Huang *et al.* 2004)
Oncorhynchus kisutch	Actinopterygii	antioxidant supplementation	liver	lethal	MDA	damage	6	-0.3	(Huang *et al.* 2004)
Oncorhynchus kisutch	Actinopterygii	antioxidant supplementation	muscle	lethal	MDA	damage	6	-0.3	(Huang *et al.* 2004)
Oncorhynchus mykiss	Actinopterygii	stressor exposure	whole body	lethal	lipid-soluble fluorescent products	damage	6	0.4	(Fontagné *et al.* 2008)
Oncorhynchus mykiss	Actinopterygii	antioxidant supplementation	liver	lethal	GR	enzyme	12	-2.35	(Hisar *et al.* 2012)
Oncorhynchus mykiss	Actinopterygii	antioxidant supplementation	liver	lethal	MDA	damage	12	2.35	(Hisar *et al.* 2012)
Oncorhynchus mykiss	Actinopterygii	antioxidant supplementation	liver	lethal	GST	enzyme	12	2.35	(Hisar *et al.* 2012)
Oncorhynchus mykiss	Actinopterygii	antioxidant supplementation	liver	lethal	SOD	enzyme	12	2.35	(Hisar *et al.* 2012)
Oncorhynchus mykiss	Actinopterygii	stressor exposure	kidney	lethal	MDA	damage	6	15.91	(Keleştemur & Seven 2013)
Oncorhynchus mykiss	Actinopterygii	stressor exposure	liver	lethal	MDA	damage	6	15.91	(Keleştemur & Seven 2013)
Oncorhynchus mykiss	Actinopterygii	stressor exposure	muscle	lethal	MDA	damage	6	15.91	(Keleştemur & Seven 2013)
Oncorhynchus mykiss	Actinopterygii	natural compound supplementation	kidney	lethal	MDA	damage	6	4.18	(Keleştemur & Seven 2013)
Oncorhynchus mykiss	Actinopterygii	natural compound supplementation	liver	lethal	MDA	damage	6	4.18	(Keleştemur & Seven 2013)
Oncorhynchus mykiss	Actinopterygii	natural compound supplementation	muscle	lethal	MDA	damage	6	4.18	(Keleştemur & Seven 2013)
Oncorhynchus mykiss	Actinopterygii	antioxidant supplementation	kidney	lethal	MDA	damage	6	2.76	(Keleştemur & Seven 2013)

Oncorhynchus mykiss	Actinopterygii	antioxidant supplementation	liver	lethal	MDA	damage	6	2.76	(Keleştemur & Seven 2013)
Oncorhynchus mykiss	Actinopterygii	antioxidant supplementation	muscle	lethal	MDA	damage	6	2.76	(Keleştemur & Seven 2013)
Oncorhynchus mykiss	Actinopterygii	antioxidant supplementation	kidney	lethal	vitamin E	non-enzymatic	6	2.76	(Keleştemur & Seven 2013)
Oncorhynchus mykiss	Actinopterygii	antioxidant supplementation	liver	lethal	vitamin E	non-enzymatic	6	2.76	(Keleştemur & Seven 2013)
Oncorhynchus mykiss	Actinopterygii	antioxidant supplementation	muscle	lethal	vitamin E	non-enzymatic	6	2.76	(Keleştemur & Seven 2013)
Oncorhynchus mykiss	Actinopterygii	natural compound supplementation	kidney	lethal	vitamin C	non-enzymatic	6	4.18	(Keleştemur & Seven 2013)
Oncorhynchus mykiss	Actinopterygii	natural compound supplementation	liver	lethal	vitamin C	non-enzymatic	6	4.18	(Keleştemur & Seven 2013)
Oncorhynchus mykiss	Actinopterygii	natural compound supplementation	muscle	lethal	vitamin C	non-enzymatic	6	4.18	(Keleştemur & Seven 2013)
Oncorhynchus mykiss	Actinopterygii	stressor exposure	kidney	lethal	vitamin A	non-enzymatic	6	15.91	(Keleştemur & Seven 2013)
Oncorhynchus mykiss	Actinopterygii	stressor exposure	kidney	lethal	vitamin C	non-enzymatic	6	15.91	(Keleştemur & Seven 2013)
Oncorhynchus mykiss	Actinopterygii	stressor exposure	liver	lethal	vitamin A	non-enzymatic	6	15.91	(Keleştemur & Seven 2013)
Oncorhynchus mykiss	Actinopterygii	stressor exposure	liver	lethal	vitamin C	non-enzymatic	6	15.91	(Keleştemur & Seven 2013)
Oncorhynchus mykiss	Actinopterygii	stressor exposure	muscle	lethal	vitamin A	non-enzymatic	6	15.91	(Keleştemur & Seven 2013)
Oncorhynchus mykiss	Actinopterygii	stressor exposure	muscle	lethal	vitamin C	non-enzymatic	6	15.91	(Keleştemur & Seven 2013)
Oreochromis niloticus	Actinopterygii	natural compound supplementation	liver	lethal	MDA	damage	12	8.4	(Özlüer-Hunt *et al.* 2011)

Oreochromis niloticus X O. aureus	Actinopterygii	antioxidant supplementation	liver	lethal	MDA	damage	6	3.72	(Huang & Huang 2004)
Oreochromis niloticus X O. aureus	Actinopterygii	antioxidant supplementation	muscle	lethal	MDA	damage	6	3.72	(Huang & Huang 2004)
Oreochromis niloticus X O. aureus	Actinopterygii	antioxidant supplementation	liver	lethal	tGSH	non-enzymatic	6	3.72	(Huang & Huang 2004)
Oryctolagus cuniculus	Mammalia	natural compound supplementation	plasma	non-lethal	MDA	damage	30	1.44	(Liu *et al.* 2010)
Pagrus major	Actinopterygii	stressor exposure	liver	lethal	vitamin E	non-enzymatic	6	-0.0001	(Gao *et al*. 2013b)
Pagrus major	Actinopterygii	stressor exposure	liver	lethal	TBARS	damage	6	-0.0001	(Gao *et al*. 2013b)
Pagrus major	Actinopterygii	stressor exposure	liver	lethal	vitamin E	non-enzymatic	6	0.94	(Gao *et al.* 2012)
Pagrus major	Actinopterygii	stressor exposure	muscle	lethal	vitamin E	non-enzymatic	6	0.94	(Gao *et al.* 2012)
Paralichthys olivaceus	Actinopterygii	stressor exposure	liver	lethal	TBARS	damage	6	0.94	(Gao *et al*. 2014a)
Paralichthys olivaceus	Actinopterygii	stressor exposure	liver	lethal	vitamin C	non-enzymatic	6	0.94	(Gao *et al*. 2014a)
Paralichthys olivaceus	Actinopterygii	stressor exposure	liver	lethal	vitamin E	non-enzymatic	6	0.94	(Gao *et al*. 2014a)
Paralichthys olivaceus	Actinopterygii	antioxidant supplementation	gill	lethal	vitamin C	non-enzymatic	6	2.05	(Wang, Kim & Bai 2002)
Paralichthys olivaceus	Actinopterygii	antioxidant supplementation	kidney	lethal	vitamin C	non-enzymatic	6	2.05	(Wang *et al.* 2002)
Paralichthys olivaceus	Actinopterygii	antioxidant supplementation	liver	lethal	vitamin C	non-enzymatic	6	2.05	(Wang *et al.* 2002)
Paralichthys olivaceus	Actinopterygii	antioxidant supplementation	muscle	lethal	vitamin C	non-enzymatic	6	2.05	(Wang *et al.* 2002)
Parus major	Aves	antioxidant supplementation	RBCs	non-lethal	KRL	non-enzymatic	48	0.71	(Marri & Richner 2014)

Pelobates cultripes	Amphibia	stressor exposure	whole body	lethal	CAT	enzyme	40	2.06	(Gomez-Mestre, Kulkarni & Buchholz 2013)
Pelobates cultripes	Amphibia	stressor exposure	whole body	lethal	SOD	enzyme	40	2.06	(Gomez-Mestre *et al.* 2013)
Pelodiscus sinensis	Reptilia	antioxidant supplementation	liver	lethal	MDA	damage	30	0.84	(Huang & Lin 2004)
Pelodiscus sinensis	Reptilia	antioxidant supplementation	liver	lethal	vitamin E	non-enzymatic	30	0.84	(Huang & Lin 2004)
Pelodiscus sinensis	Reptilia	antioxidant supplementation	muscle	lethal	vitamin E	non-enzymatic	30	0.84	(Huang & Lin 2004)
Penaeus monodon	Malacostraca	antioxidant supplementation	digestive gland	lethal	MDA	damage	6	7.81	(Niu *et al.* 2013)
Penaeus monodon	Malacostraca	antioxidant supplementation	digestive gland	lethal	PCs	damage	6	7.81	(Niu *et al.* 2013)
Penaeus monodon	Malacostraca	antioxidant supplementation	digestive gland	lethal	SOD	enzyme	6	-7.81	(Niu *et al.* 2013)
Penaeus monodon	Malacostraca	antioxidant supplementation	digestive gland	lethal	GPX	enzyme	6	7.81	(Niu *et al.* 2013)
Penaeus monodon	Malacostraca	antioxidant supplementation	digestive gland	lethal	TAS	non-enzymatic	6	7.81	(Niu *et al.* 2013)
Penaeus monodon	Malacostraca	antioxidant supplementation	haemolymph	non-lethal	ALT	damage	6	1.88	(Niu *et al.* 2012)
Penaeus monodon	Malacostraca	antioxidant supplementation	haemolymph	non-lethal	AST	damage	6	1.88	(Niu *et al.* 2012)
Penaeus monodon	Malacostraca	antioxidant supplementation	haemolymph	non-lethal	SOD	enzyme	6	-1.88	(Niu *et al.* 2012)
Penaeus monodon	Malacostraca	antioxidant supplementation	haemolymph	non-lethal	TAS	non-enzymatic	6	1.88	(Niu *et al.* 2012)
Perca flavescens	Actinopterygii	antioxidant supplementation	plasma	non-lethal	TBARS	damage	6	2.82	(Lee & Dabrowski 2003)
Perca flavescens	Actinopterygii	antioxidant supplementation	liver	lethal	vitamin C	non-enzymatic	6	2.82	(Lee & Dabrowski 2003)
Perca flavescens	Actinopterygii	antioxidant	liver	lethal	vitamin E	non-	6	2.82	(Lee &

		supplementation				enzymatic			Dabrowski 2003)
Platichthys stellatus	Actinopterygii	natural compound supplementation	liver	lethal	MDA	damage	6	2.95	(Wang *et al.* 2014)
Platichthys stellatus	Actinopterygii	natural compound supplementation	serum	non-lethal	MDA	damage	6	2.95	(Wang *et al.* 2014)
Platichthys stellatus	Actinopterygii	natural compound supplementation	liver	lethal	SOD	enzyme	6	2.95	(Wang *et al.* 2014)
Platichthys stellatus	Actinopterygii	natural compound supplementation	liver	lethal	T-AOC	non-enzymatic	6	2.95	(Wang *et al.* 2014)
Platichthys stellatus	Actinopterygii	natural compound supplementation	serum	non-lethal	CAT	enzyme	6	2.95	(Wang *et al.* 2014)
Platichthys stellatus	Actinopterygii	natural compound supplementation	serum	non-lethal	SOD	enzyme	6	2.95	(Wang *et al.* 2014)
Rachycentron canadum	Actinopterygii	antioxidant supplementation	liver	lethal	TBARS	damage	6	3.29	(Zhou *et al.* 2013)
Rachycentron canadum	Actinopterygii	antioxidant supplementation	liver	lethal	vitamin E	non-enzymatic	6	3.29	(Zhou *et al.* 2013)
Rachycentron canadum	Actinopterygii	antioxidant supplementation	plasma	non-lethal	SOD	enzyme	6	3.29	(Zhou *et al.* 2013)
Rana temporaria	Amphibia	stressor exposure	whole body	lethal	MDA	damage	28	-1.59	(Salin *et al.* 2012)
Rattus norvegicus (Sprague-Dawley)	Mammalia	stressor exposure	liver	lethal	MDA	damage	12	4.74	(Ohtsuka *et al.* 1998)
Rattus norvegicus (Sprague-Dawley)	Mammalia	antioxidant supplementation	liver	lethal	GST	enzyme	12	-0.37	(Ohtsuka *et al.* 1998)
Rattus norvegicus	Mammalia	antioxidant	liver	lethal	vitamin E	non-	12	-0.37	(Ohtsuka *et al.*

(Sprague-Dawley)		supplementation				enzymatic			1998)
Salmo coruhensis	Actinopterygii	antioxidant supplementation	liver	lethal	CAT	enzyme	18	-0.16	(Can *et al.* 2012)
Salmo coruhensis	Actinopterygii	antioxidant supplementation	liver	lethal	MDA	damage	18	0.16	(Can *et al.* 2012)
Spondyliosoma cantharus	Actinopterygii	natural compound supplementation	plasma	non-lethal	MDA	damage	6	9.61	(Ma *et al.* 2008)
Spondyliosoma cantharus	Actinopterygii	natural compound supplementation	liver	lethal	CAT	enzyme	6	9.61	(Ma *et al.* 2008)
Spondyliosoma cantharus	Actinopterygii	natural compound supplementation	liver	lethal	GPX	enzyme	6	9.61	(Ma *et al.* 2008)
Spondyliosoma cantharus	Actinopterygii	natural compound supplementation	liver	lethal	SOD	enzyme	6	9.61	(Ma *et al.* 2008)
Spondyliosoma cantharus	Actinopterygii	natural compound supplementation	muscle	lethal	GST	enzyme	6	9.61	(Ma *et al.* 2008)
Spondyliosoma cantharus	Actinopterygii	natural compound supplementation	muscle	lethal	SOD	enzyme	6	9.61	(Ma *et al.* 2008)
Spondyliosoma cantharus	Actinopterygii	natural compound supplementation	plasma	non-lethal	GPX	enzyme	6	9.61	(Ma *et al.* 2008)
Spondyliosoma cantharus	Actinopterygii	natural compound supplementation	plasma	non-lethal	GST	enzyme	6	9.61	(Ma *et al.* 2008)
Spondyliosoma cantharus	Actinopterygii	antioxidant supplementation	liver	lethal	MDA	damage	6	15.49	(Peng *et al.* 2009)
Spondyliosoma cantharus	Actinopterygii	stressor exposure	liver	lethal	MDA	damage	6	11.98	(Peng *et al.* 2009)

Sus scrofa domesticus	Mammalia	natural compound supplementation	serum	non-lethal	MDA	damage	8	2.34	(Wang *et al*. 2012a)
Sus scrofa domesticus	Mammalia	natural compound supplementation	serum	non-lethal	GPX	enzyme	8	2.34	(Wang *et al*. 2012a)
Sus scrofa domesticus	Mammalia	natural compound supplementation	serum	non-lethal	SOD	enzyme	8	2.34	(Wang *et al*. 2012a)
Sus scrofa domesticus	Mammalia	stressor exposure	serum	non-lethal	MDA	damage	12	0.74	(Wang, Chi & Kim 2012b)
Trachinotus marginatus	Actinopterygii	natural compound supplementation	muscle	lethal	TBARS	damage	6	-8.1	(Kütter *et al.* 2012)

Table S2 Summary details for studies included in cost-MA investigating the effects of growth rate on OS.

Species	Taxonomic Class	Experimental Manipulation	Biological Matrix	Sampling Method	Biomarker	Biomarker Category	N	Hedges' g	Reference
Aptenodytes patagonicus	Aves	none	plasma	non-lethal	ROMs	damage	28	1.1	(Geiger *et al.* 2011)
Aptenodytes patagonicus	Aves	none	plasma	non-lethal	OXY	non-enzymatic	28	0.14	(Geiger *et al.* 2011)
Argopecten ventricosus	Bivalvia	none	gills	lethal	TBARS	damage	8	1.57	(Guerra *et al.* 2012)
Argopecten ventricosus	Bivalvia	none	gills	lethal	PCs	damage	8	3.17	(Guerra *et al.* 2012)
Argopecten ventricosus	Bivalvia	none	mantle	lethal	TBARS	damage	8	1.89	(Guerra *et al.* 2012)
Argopecten ventricosus	Bivalvia	none	mantle	lethal	PCs	damage	8	0.51	(Guerra *et al.* 2012)
Argopecten ventricosus	Bivalvia	none	muscle	lethal	TBARS	damage	8	7.15	(Guerra *et al.* 2012)
Argopecten ventricosus	Bivalvia	none	muscle	lethal	PCs	damage	8	1.89	(Guerra *et al.* 2012)
Argopecten ventricosus	Bivalvia	none	gills	lethal	CAT	enzyme	8	-9.95	(Guerra *et al.* 2012)
Argopecten ventricosus	Bivalvia	none	gills	lethal	SOD	enzyme	8	-2.66	(Guerra *et al.* 2012)
Argopecten ventricosus	Bivalvia	none	mantle	lethal	CAT	enzyme	8	-9.92	(Guerra *et al.* 2012)
Argopecten ventricosus	Bivalvia	none	mantle	lethal	SOD	enzyme	8	-2.79	(Guerra *et al.* 2012)
Argopecten ventricosus	Bivalvia	none	muscle	lethal	CAT	enzyme	8	-3.14	(Guerra *et al.* 2012)
Argopecten ventricosus	Bivalvia	none	muscle	lethal	SOD	enzyme	8	-1.05	(Guerra *et al.* 2012)
Carassius auratus gibelio	Actinopterygii	dietary changes	intestine	lethal	SOD	enzyme	6	-0.05	(Dong *et al.* 2013)

Carassius auratus gibelio	Actinopterygii	dietary changes	intestine	lethal	CAT	enzyme	6	-0.46	(Dong *et al.* 2013)
Carassius auratus gibelio	Actinopterygii	dietary changes	intestine	lethal	GPX	enzyme	6	1.73	(Dong *et al.* 2013)
Carassius auratus gibelio	Actinopterygii	dietary changes	intestine	lethal	GR	enzyme	6	0.12	(Dong *et al.* 2013)
Carassius auratus gibelio	Actinopterygii	dietary changes	liver	lethal	SOD	enzyme	6	-0.77	(Dong *et al.* 2013)
Carassius auratus gibelio	Actinopterygii	dietary changes	liver	lethal	CAT	enzyme	6	2.82	(Dong *et al.* 2013)
Carassius auratus gibelio	Actinopterygii	dietary changes	liver	lethal	GPX	enzyme	6	0.18	(Dong *et al.* 2013)
Carassius auratus gibelio	Actinopterygii	dietary changes	liver	lethal	GR	enzyme	6	0.96	(Dong *et al.* 2013)
Carassius auratus gibelio	Actinopterygii	dietary changes	intestine	lethal	T-AOC	non-enzymatic	6	0.28	(Dong *et al.* 2013)
Carassius auratus gibelio	Actinopterygii	dietary changes	intestine	lethal	GSH	non-enzymatic	6	-0.63	(Dong *et al.* 2013)
Carassius auratus gibelio	Actinopterygii	dietary changes	liver	lethal	T-AOC	non-enzymatic	6	-1.22	(Dong *et al.* 2013)
Carassius auratus gibelio	Actinopterygii	dietary changes	liver	lethal	GSH	non-enzymatic	6	-0.13	(Dong *et al.* 2013)
Chelonia mydas	Reptilia	compensatory growth	liver	lethal	GPX	enzyme	17	1.74	(Roark *et al.* 2009)
Chelonia mydas	Reptilia	compensatory growth	liver	lethal	BIOXYTECH	non-enzymatic	15	1.51	(Roark *et al.* 2009)
Columba livia	Aves	dietary changes	serum	non-lethal	ROMs	damage	21	1.72	(Costantini 2010d)
Columba livia	Aves	dietary changes	serum	non-lethal	OXY	non-enzymatic	21	-1.46	(Costantini 2010d)
Columba livia	Aves	dietary changes	serum	non-lethal	Thiols	non-enzymatic	21	0.17	(Costantini 2010d)
Corvus monedula	Aves	brood manipulation	plasma	non-lethal	ROMs	damage	46	0.67	(Salomons 2009)

Corvus monedula	Aves	brood manipulation	plasma	non-lethal	ROMs	damage	46	-0.14	(Salomons 2009)
Corvus monedula	Aves	brood manipulation	plasma	non-lethal	OXY	non-enzymatic	46	-0.61	(Salomons 2009)
Cuculus canorus	Aves	none	plasma	non-lethal	ROMs	damage	20	0.58	(Hargitai *et al.* 2012)
Cuculus canorus	Aves	none	plasma	non-lethal	FRAP	non-enzymatic	21	-0.57	(Hargitai *et al.* 2012)
Gallus gallus	Aves	dietary changes	heart	lethal	MDA	damage	122	0.3	(Nain *et al.* 2008)
Labeo rohita	Actinopterygii	dietary changes	liver	lethal	SOD	enzyme	8	2.46	(Yengkokpam *et al.* 2013)
Labeo rohita	Actinopterygii	dietary changes	liver	lethal	CAT	enzyme	8	3.45	(Yengkokpam *et al.* 2013)
Larus michahellis	Aves	compensatory growth	plasma	non-lethal	trolox	non-enzymatic	70	-0.17	(Noguera *et al.* 2011a)
Lestes viridis	Insecta	compensatory growth	whole body	lethal	SOD	enzyme	45	-1.96	(De Block & Stoks 2008)
Lestes viridis	Insecta	compensatory growth	whole body	lethal	CAT	enzyme	45	-2.82	(De Block & Stoks 2008)
Litopenaeus vannamei	Malacostraca	dietary changes	haemolymph	non-lethal	SOD	enzyme	8	-0.85	(Xu & Pan 2014)
Litopenaeus vannamei	Malacostraca	dietary changes	hepato-pancreas	lethal	SOD	enzyme	8	0.36	(Xu & Pan 2014)
Litopenaeus vannamei	Malacostraca	dietary changes	haemolymph	non-lethal	T-AOC	non-enzymatic	8	-1.8	(Xu & Pan 2014)
Litopenaeus vannamei	Malacostraca	dietary changes	haemolymph	non-lethal	GSH:GSSG	non-enzymatic	8	-0.77	(Xu & Pan 2014)
Litopenaeus vannamei	Malacostraca	dietary changes	hepato-pancreas	lethal	T-AOC	non-enzymatic	8	-2.56	(Xu & Pan 2014)
Litopenaeus vannamei	Malacostraca	dietary changes	hepato-pancreas	lethal	GSH:GSSG	non-enzymatic	8	0.28	(Xu & Pan 2014)
Macrobrachium nipponense	Malacostraca	compensatory growth	haemolymph	non-lethal	ROIs	damage	6	0.8	(Li *et al.* 2009)

Macrobrachium nipponense	Malacostraca	compensatory growth	muscle	lethal	SOD	enzyme	6	0.42	(Li *et al.* 2009)
Macrobrachium nipponense	Malacostraca	compensatory growth	muscle	lethal	CAT	enzyme	6	1.2	(Li *et al.* 2009)
Misgurnus anguillicaudatus	Actinopterygii	dietary changes	whole body	lethal	CAT	enzyme	6	3.63	(Gao *et al*. 2014b)
Misgurnus anguillicaudatus	Actinopterygii	dietary changes	whole body	lethal	SOD	enzyme	6	-6.12	(Gao *et al*. 2014b)
Misgurnus anguillicaudatus	Actinopterygii	dietary changes	whole body	lethal	GPX	enzyme	6	3.1	(Gao *et al*. 2014b)
Oreochromis niloticus	Actinopterygii	dietary changes	liver	lethal	MDA	damage	12	-5.19	(Aziza, Awadin & Orma 2013)
Oreochromis niloticus	Actinopterygii	dietary changes	muscle	lethal	MDA	damage	12	0	(Aziza *et al.* 2013)
Oreochromis niloticus	Actinopterygii	dietary changes	liver	lethal	SOD	enzyme	10	2.84	(Saïdi *et al.* 2010)
Oreochromis niloticus	Actinopterygii	dietary changes	liver	lethal	CAT	enzyme	10	2.39	(Saïdi *et al.* 2010)
Oreochromis niloticus	Actinopterygii	dietary changes	liver	lethal	GST	enzyme	10	2.35	(Saïdi *et al.* 2010)
Oreochromis niloticus	Actinopterygii	dietary changes	liver	lethal	GPX	enzyme	10	2.25	(Saïdi *et al.* 2010)
Ovis aries (Ile de France X INRA 401)	Mammalia	compensatory growth	muscle	lethal	PCs	damage	8	0.22	(Savary-Auzeloux *et al.* 2008)
Ovis aries (Ile de France X INRA 401)	Mammalia	compensatory growth	muscle	lethal	PCs	damage	8	0.26	(Savary-Auzeloux *et al.* 2008)
Ovis aries (Ile de France X INRA 401)	Mammalia	compensatory growth	muscle	lethal	PCs	damage	8	-0.26	(Savary-Auzeloux *et al.* 2008)
Ovis aries (Ile de France X INRA 401)	Mammalia	compensatory growth	muscle	lethal	GPX	enzyme	8	-0.66	(Savary-Auzeloux *et al.* 2008)

Ovis aries (Ile de France X INRA 401)	Mammalia	compensatory growth	muscle	lethal	SOD	enzyme	8	0.74	(Savary-Auzeloux *et al.* 2008)
Ovis aries (Ile de France X INRA 401)	Mammalia	compensatory growth	muscle	lethal	GPX	enzyme	8	-0.84	(Savary-Auzeloux *et al.* 2008)
Ovis aries (Ile de France X INRA 401)	Mammalia	compensatory growth	muscle	lethal	SOD	enzyme	8	-0.11	(Savary-Auzeloux *et al.* 2008)
Ovis aries (Ile de France X INRA 401)	Mammalia	compensatory growth	muscle	lethal	GPX	enzyme	8	-0.61	(Savary-Auzeloux *et al.* 2008)
Ovis aries (Ile de France X INRA 401)	Mammalia	compensatory growth	muscle	lethal	SOD	enzyme	8	-0.89	(Savary-Auzeloux *et al.* 2008)
Ovis aries (Ile de France X INRA 401)	Mammalia	compensatory growth	muscle	lethal	TAS	non-enzymatic	8	3.48	(Savary-Auzeloux *et al.* 2008)
Ovis aries (Ile de France X INRA 401)	Mammalia	compensatory growth	muscle	lethal	tGSH	non-enzymatic	8	0.98	(Savary-Auzeloux *et al.* 2008)
Ovis aries (Ile de France X INRA 401)	Mammalia	compensatory growth	muscle	lethal	carnosine	non-enzymatic	8	0.15	(Savary-Auzeloux *et al.* 2008)
Ovis aries (Ile de France X INRA 401)	Mammalia	compensatory growth	muscle	lethal	anserine	non-enzymatic	8	0.13	(Savary-Auzeloux *et al.* 2008)
Ovis aries (Ile de France X INRA 401)	Mammalia	compensatory growth	muscle	lethal	TAS	non-enzymatic	8	1.62	(Savary-Auzeloux *et al.* 2008)
Ovis aries (Ile de France X INRA 401)	Mammalia	compensatory growth	muscle	lethal	tGSH	non-enzymatic	8	0.1	(Savary-Auzeloux *et al.* 2008)

Ovis aries (Ile de France X INRA 401)	Mammalia	compensatory growth	muscle	lethal	carnosine	non-enzymatic	8	0.59	(Savary-Auzeloux *et al.* 2008)
Ovis aries (Ile de France X INRA 401)	Mammalia	compensatory growth	muscle	lethal	anserine	non-enzymatic	8	1.02	(Savary-Auzeloux *et al.* 2008)
Ovis aries (Ile de France X INRA 401)	Mammalia	compensatory growth	muscle	lethal	TAS	non-enzymatic	8	2.38	(Savary-Auzeloux *et al.* 2008)
Ovis aries (Ile de France X INRA 401)	Mammalia	compensatory growth	muscle	lethal	tGSH	non-enzymatic	8	1.39	(Savary-Auzeloux *et al.* 2008)
Ovis aries (Ile de France X INRA 401)	Mammalia	compensatory growth	muscle	lethal	carnosine	non-enzymatic	8	-0.51	(Savary-Auzeloux *et al.* 2008)
Ovis aries (Ile de France X INRA 401)	Mammalia	compensatory growth	muscle	lethal	anserine	non-enzymatic	8	-0.36	(Savary-Auzeloux *et al.* 2008)
Pagrus major	Actinopterygii	dietary changes	plasma	non-lethal	ROMs	damage	6	20.66	(Kader *et al.* 2010)
Pagrus major	Actinopterygii	dietary changes	plasma	non-lethal	BAP	non-enzymatic	6	-0.09	(Kader *et al.* 2010)
Parus major	Aves	brood manipulation	plasma	non-lethal	BIOXYTECH	non-enzymatic	19	1.04	(Kilgas *et al.* 2010)
Pelteobagrus vachelli	Actinopterygii	dietary changes	intestine	lethal	SOD	enzyme	6	-1.62	(Dong *et al.* 2013)
Pelteobagrus vachelli	Actinopterygii	dietary changes	intestine	lethal	CAT	enzyme	6	0.39	(Dong *et al.* 2013)
Pelteobagrus vachelli	Actinopterygii	dietary changes	intestine	lethal	GPX	enzyme	6	-0.3	(Dong *et al.* 2013)
Pelteobagrus vachelli	Actinopterygii	dietary changes	intestine	lethal	GR	enzyme	6	-1.1	(Dong *et al.* 2013)
Pelteobagrus vachelli	Actinopterygii	dietary changes	liver	lethal	SOD	enzyme	6	-1.31	(Dong *et al.* 2013)

Pelteobagrus vachelli	Actinopterygii	dietary changes	liver	lethal	CAT	enzyme	6	-0.49	(Dong *et al.* 2013)
Pelteobagrus vachelli	Actinopterygii	dietary changes	liver	lethal	GPX	enzyme	6	-2.51	(Dong *et al.* 2013)
Pelteobagrus vachelli	Actinopterygii	dietary changes	liver	lethal	GR	enzyme	6	-1.2	(Dong *et al.* 2013)
Pelteobagrus vachelli	Actinopterygii	dietary changes	intestine	lethal	T-AOC	non-enzymatic	6	-0.12	(Dong *et al.* 2013)
Pelteobagrus vachelli	Actinopterygii	dietary changes	intestine	lethal	GSH	non-enzymatic	6	0	(Dong *et al.* 2013)
Pelteobagrus vachelli	Actinopterygii	dietary changes	liver	lethal	T-AOC	non-enzymatic	6	-2.43	(Dong *et al.* 2013)
Pelteobagrus vachelli	Actinopterygii	dietary changes	liver	lethal	GSH	non-enzymatic	6	-4.72	(Dong *et al.* 2013)
Periparus ater	Aves	none	RBCs	non-lethal	8-OHDG	damage	14	1.27	(Stier *et al*. 2014b)
Periparus ater	Aves	none	plasma	non-lethal	OXY	non-enzymatic	14	-0.54	(Stier *et al*. 2014b)
Rattus norvegicus (Fischer 344)	Mammalia	dietary changes	blood	non-lethal	5-OHmdU	damage	20	0.87	(Djuric *et al.* 2009)
Rattus norvegicus (Fischer 344)	Mammalia	dietary changes	mammary gland	lethal	5-OHmdU	damage	20	0.62	(Djuric *et al.* 2009)
Rattus norvegicus (Sprague-Dawley)	Mammalia	compensatory growth	muscle	lethal	SOD	enzyme	16	1.22	(Zheng *et al.* 2012)
Rattus norvegicus (Sprague-Dawley)	Mammalia	compensatory growth	muscle	lethal	CAT	enzyme	16	1.2	(Zheng *et al.* 2012)
Rattus norvegicus (Sprague-Dawley)	Mammalia	compensatory growth	muscle	lethal	GPX	enzyme	16	1.08	(Zheng *et al.* 2012)
Rattus norvegicus (Wistar)	Mammalia	dietary changes	brain	lethal	PCs	damage	10	-0.22	(Langley-Evans & Sculley 2006)
Rattus norvegicus (Wistar)	Mammalia	dietary changes	liver	lethal	PCs	damage	10	1.1	(Langley-Evans & Sculley 2006)
Rattus norvegicus (Wistar)	Mammalia	compensatory growth	urine	non-lethal	8-oxo-dG	damage	22	0.03	(Tarry-Adkins *et al.* 2008)

Rattus norvegicus (Wistar)	Mammalia	compensatory growth	urine	non-lethal	8-oxo-dG	damage	14	0.55	(Tarry-Adkins *et al.* 2008)
Sparus aurata	Actinopterygii	dietary changes	liver	lethal	GR	enzyme	14	1.88	(Sitjà-Bobadilla *et al.* 2005)
Sparus aurata	Actinopterygii	dietary changes	muscle	lethal	GR	enzyme	14	2.02	(Sitjà-Bobadilla *et al.* 2005)
Sparus aurata	Actinopterygii	dietary changes	blood	non-lethal	GSH:GSSG	non-enzymatic	14	1.19	(Sitjà-Bobadilla *et al.* 2005)
Sparus aurata	Actinopterygii	dietary changes	liver	lethal	GSH:GSSG	non-enzymatic	14	1.34	(Sitjà-Bobadilla *et al.* 2005)
Sparus aurata	Actinopterygii	dietary changes	muscle	lethal	GSH:GSSG	non-enzymatic	14	0.2	(Sitjà-Bobadilla *et al.* 2005)
Synechogobius hasta	Actinopterygii	dietary changes	liver	lethal	MDA	damage	6	1.57	(Luo *et al.* 2012)
Synechogobius hasta	Actinopterygii	dietary changes	liver	lethal	GPX	enzyme	6	-0.49	(Luo *et al.* 2012)
Synechogobius hasta	Actinopterygii	dietary changes	liver	lethal	SOD	enzyme	6	-4.16	(Luo *et al.* 2012)
Synechogobius hasta	Actinopterygii	dietary changes	liver	lethal	CAT	enzyme	6	-2.15	(Luo *et al.* 2012)
Taeniopygia guttata	Aves	brood manipulation	plasma	non-lethal	carotenoids	non-enzymatic	52	1.5	(Alonso-Álvarez *et al.* 2007)
Taeniopygia guttata	Aves	brood manipulation	RBCs	non-lethal	KRL	non-enzymatic	52	0.64	(Alonso-Álvarez *et al.* 2007)
Taeniopygia guttata	Aves	brood manipulation	blood	non-lethal	8-oxo-dG	damage	16	0.26	Sophie Reichert, unpublished
Taeniopygia guttata	Aves	brood manipulation	plasma	non-lethal	OXY	non-enzymatic	16	-1.2	Sophie Reichert, unpublished

Table S3 Pairwise comparisons of the treatment effect on growth for the different experimental approaches (cost-MA). Adjusted p values have been calculated using the sequential Bonferroni correction method. The variance explained by the random factors was 0.33 (study) and 0 (taxonomic class).

Pairwise comparisons	**z value**	**p value**	**adjusted p value**
none-compensatory growth	1.78	0.08	0.45
none-brood manipulation	2.24	0.03	0.15
none-dietary changes	0.10	0.92	1.00
compensatory growth-brood manipulation	0.63	0.53	1.00
compensatory growth-dietary changes	2.25	0.02	0.15
brood manipulation-dietary changes	2.79	0.01	**0.03**

Appendix 6: supplementary results for Chapter 4

The effect of manipulation type on OS (constraint-MA) and growth (cost-MA)

All treatments significantly altered OS (constraint-MA, Figure S5 A, Appendix 4) and growth (cost-MA, Figure S5 B, Appendix 4). Among the categories manipulating OS (constraint-MA), all experimental manipulations had a similar sized effect on OS (QM=0.73, df=2, p=0.69; see Figure S5 A, Appendix 4, for the overall treatment effect) and there were no significant differences irrespective of what biomarkers (i.e. damage, enzymatic antioxidants, non-enzymatic antioxidants; QM=2.75, df=2, p=0.25) or sampling methods (i.e. lethal versus non-lethal; QM=0.35, df=1, p=0.55) were measured. For cost-MA, the effect of treatment on growth depended on the type of experimental manipulation (QM=11.20, df=3, p=0.01), with dietary changes producing a significantly larger effect than did brood manipulations (Table S3, Appendix 5; Figure S5 B, Appendix 4).

Appendix 7: supplementary discussion for Chapter 4

Did the experimental manipulations produce differences that were similar in magnitude to unmanipulated levels?

All three categories of growth manipulation in cost-MA (compensatory growth, brood manipulation and dietary changes) produced growth differences that were similar in magnitude to those occurring in the unmanipulated group (manipulation category 'None', see Table 4:1 in the main paper for full description), since the effects of treatment on growth did not differ significantly between manipulated and unmanipulated groups (Table S3, Appendix 5). However, given that there were no observational studies in which differences in OS between unmanipulated groups had been compared, it is difficult to tell whether the treatments altering OS produced comparable differences with unmanipulated groups (constraint-MA); as was determined for the treatment effects on growth in cost-MA. This could imply that the experimental manipulations led to unrealistic differences in OS. However strong effects of OS on growth were reported for studies in which species were exposed to stressors that would be encountered naturally (e.g. pond desiccation; Hedges' g=2.06±1.13 (Gomez-Mestre *et al.* 2013)) or that are potentially encountered in farming situations (e.g. hypoxia in fish; Hedges' g=-1.41±1.35 (Filho *et al.* 2005); high stocking density in broiler chickens; Hedges' g=2.75±1.73 (Simitzis *et al.* 2012)). Therefore the results are still considered relevant for assessing how OS can affect growth rate and might prove to be a useful consideration for animal welfare issues.

The issue with sampling method

The high collinearity (cost-MA) of sampling method with whether the study was conducted in the laboratory or wild (excluded from analysis) makes it difficult to determine the underlying cause of any differences between lethal and non-lethal sampling. The lack of environmental challenges that reduce fitness in the laboratory might mean effects are only evident in field studies of wild animals. Since field studies tended to use non-lethal sampling, a greater effect here could be wrongly interpreted as a greater effect with non-lethal sampling. Yet I found no significant difference between the two sampling methods for either constraint-MA or cost-MA, which might suggest the tissues sampled by non-lethal methods are sufficient for investigating the growth-OS relationship. The constraint-MA included only three studies conducted in the wild so it is difficult to determine if this collinearity would have been an issue and when the analysis was repeated excluding these, similar results were obtained – there was no significant difference between lethal and non-lethal sampling.

Appendix 8: supplementary figure for chapter 5

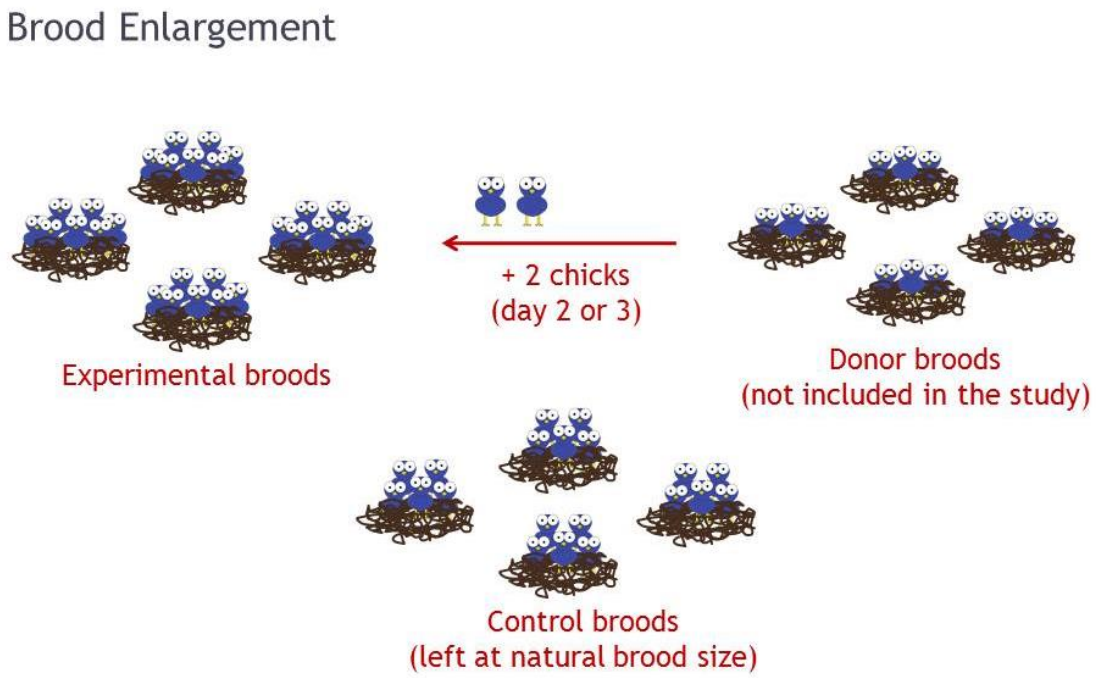


Figure S6 Experimental manipulation of brood size in 2013. For presentation, standardised brood sizes have been shown. Day 0 = day of hatching of the first chick. For full details please see the text.

Appendix 9: PIT tag details

written by Nosrat Mirzai, Head of BioElectronics Unit, CMVLS, University of Glasgow, 2013

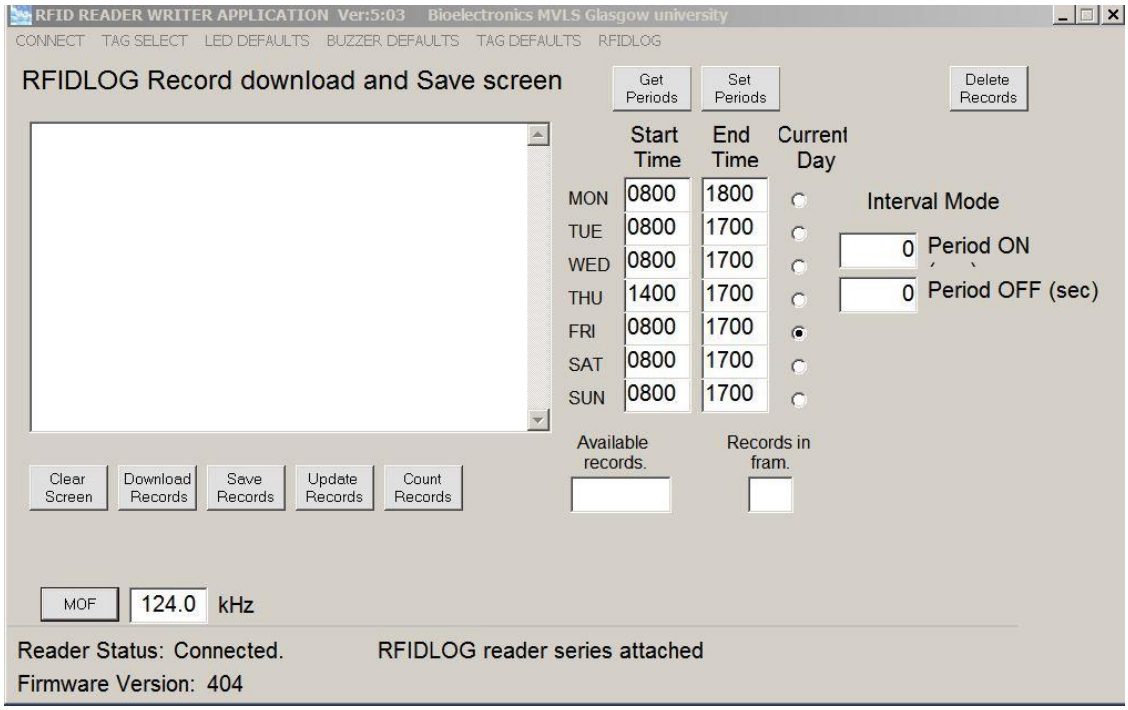
Data logger – With external antenna and SD card

This logger is a module capable of reading tags using FDX-B and HDX.
It features a time and date circuit and SD card interface to allow scanned codes to be recorded.
This logger continually scans for tags (transponders).
When one is detected a record entry is stored in the log file containing the time and date of detection, tag designation and identity code.

Records are stored inside a SD memory card which can be removed from the unit and replaced as needed.
SD cards have a limited number of write cycles so in order to increase the life span, records are not written immediately when they are scanned.
First accumulate 16 records which are stored in FRAM (Ferro magnetic memory) and then 16 records are written in one go.
For this reason the SD card is updated on power up and on card insertion.
Example: If you scan 82 tags and then pull out the SD card you'll find 80 records, while 2 are still being stored in FRAM.
The saved file is in "DAT" format and can be manipulated as required, i.e. in Excel application

Time/Date:

To set time and date for new SD card, RFID needs to be connected to PC and running the RFID software provided.
Connect the RFID to PC via serial cable to Comport,
Run RFID reader software. Select **"connect"** Select communication port.
Software:



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http://www.gla.ac.uk/colleges/mvls/informationforstaff/facilities/bioelectronicsunit/

By using the standard SD card reader connected to PC, data can be down loaded by clicking on, first **Update records** and use SD card adaptor to connect to PC.

To see all the records use **Download records** you can save the file by **save records**. The saved file is in "TXT" format and can be manipulated as required, i.e. in Excel application.
To delete records click on **Delete records** then **update records**

When you use the PC RFID reader application, click on "**Connect**" select appropriate COM port, the application detects that the logger is connected and jumps to the **RFIDLOG screen**. This logger has no options for setting different tags as it is designed only for animal tags (or in this case EM4100 tags).
Get Period and Set Period (start time and end time) and **Current day** have to be set, after setting, click on **Set Periods** and then **Get Periods** *now real time and date are stored in SD card.*

To find out about the frequency of antenna, click on **MOF** and wait for few seconds.
Finally click on **Update records**.
Disconnect the system from PC, system is ready for operation.

RFID Transponders

RFID transponders (Tags) are devices carrying digital information that can be read from a distance by a
RFID transceiver (Reader). In order to be able to read the information stored on the RFID tags the reader
must know how the information is stored and the protocol for extracting it. One of the more common data formats for RFID transponders is the EM4100 protocol, named so because the microchip at the heart of the Tag is based on the controller chip made by the company EM Microelectronic.

Reading an EM4100 RFID Transponder

EM4100 compatible RFID transponders carry 64 bits of Read Only memory. This means that information can be read from the Tag but no data can be changed, or new data written to the card once the card has been programmed with the initial data. The format of the data is as shown here.

When the Tag enters the electromagnetic field transmitted by the RFID reader it draws power from the field and will commence transmitting its data as shown above.

More information can be found at the under noted website

http://download.mikroe.com/documents/accessories/rfid/125khz/rfid-card-125khz-em4100-datasheet.pdf