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The causes and consequences of inter-individual variation in corticosterone in the blue tit, *Cyanistes caeruleus*

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If you knew what you were doing it wouldn’t be called research.

– **Albert Einstein**

General Abstract

Corticosterone (CORT), the main glucocorticoid in birds, plays a fundamental role in maintaining homeostasis and energy-balance, and is therefore tightly linked to an individual's energetic state and the prevalent environmental conditions. CORT also has pleiotropic effects, ranging from reproductive function, the regulation of behaviour, morphology and immune function. Thus, inter-individual variation in CORT can potentially underpin a range of life-history traits, and through its pleiotropic effects act as a physiological mediator of reproductive decisions, causing birds to direct resources towards reproduction or self-maintenance dependent upon energetic condition and/or environmental quality. In turn, the role of CORT as a mediator of life-history traits has lead to the suggestion that inter-individual variation in CORT may be associated with individual differences in fitness. Despite this, the causes and consequences of large inter-individual variation in baseline CORT, specifically during reproduction, remain relatively unknown. The main aim of this thesis was to address these knowledge gaps by monitoring a nest-box population of blue tits, *Cyanistes caeruleus*, breeding on the east banks of Loch Lomond, UK over three years (2008-2010), and measuring baseline CORT concentrations in both adult and nestling birds at a standard stage of breeding in each year. Although environmental quality is often linked to variation in baseline CORT in breeding birds, this has rarely been investigated at the individual level. **Chapter 2** focuses on the relationship between foraging conditions measured at the territory-scale and baseline CORT in adult and nestlings in 2008-2010. Synchrony with the peak in caterpillar abundance was the only factor to influence nestling CORT, and only in 2008. However, I found that synchrony between breeding and the peak in caterpillar abundance, weather variables and the density of oak trees influenced baseline CORT in adult birds. Importantly, the relationships between adult baseline CORT and these foraging conditions were only evident in some years; when conditions were most demanding. In addition, the effects of the foraging conditions measured upon adult baseline CORT appear to be synergetic and/or additive in nature. As inclement environmental conditions are often associated with elevated baseline CORT and reduced fitness in birds, it has been suggested that elevated baseline CORT should also be associated with reduced fitness (the 'CORT-Fitness' Hypothesis). However, this may not be the case, as modulation of CORT in the face of environmental challenges can adaptively influence physiology and behaviour to improve breeding performance and/or survival. In **Chapter 3**, I tested these assumptions and my results indicate that the foraging conditions linked to maternal baseline CORT differ to those associated with a proxy of fitness i.e. reproductive success. Specifically, maternal baseline CORT appears to be linked with factors that affect energetic demand, i.e. movement between trees, rather than reproductive success, i.e. total number of prey provided to offspring. In addition, in 2009 only, maternal baseline CORT was positively correlated with fledging number. In **Chapter 4,** I investigate whether there is a link between maternal baseline CORT and brood sex ratio adjustment over three years. I discovered that maternal baseline CORT was not correlated with brood sex ratio in any year. Maternal body condition, however, was linked to brood sex ratio adjustment in one year. Furthermore, experimental manipulation of maternal CORT during egg laying did not result in brood sex ratio adjustment or affect maternal condition, hatching success or chick development. **Chapter 5** investigates the role of maternal baseline CORT in reproductive trade-offs. I reduced the costs of egg laying through supplemental feeding and compared maternal baseline CORT, brood care and maternal return rates between manipulated and control mothers. Reducing costs negated the physiological stress associated with provisioning effort in manipulated mothers and improved their return rates the following year compared with controls. Therefore, maternal CORT may mediate reproductive trade-offs in this species. As baseline CORT is often linked with energetic status and environmental conditions, and there is some evidence that CORT affects feather growth, I hypothesised that it may be linked to the expression of UV colouration in the crown feathers of female birds (**Chapter 6**). The results show that baseline CORT was indeed negatively correlated with UV colouration, and that UV colouration was positively correlated with reproductive success consistently over the three years, thus suggesting this trait signals maternal quality. Finally, **Chapter 7** summarizes the main findings and considers how my results add to our knowledge base and discusses pertinent avenues of future research. This thesis presents compelling evidence that inter-individual variation in baseline CORT is significant, as the results show that it is associated with foraging conditions, reproductive success and may also influence reproductive trade-offs and UV plumage colouration. However, the results do not support a role for baseline CORT in brood sex ratio adjustments in blue tits. The results also reveal the complexity of the relationships between inter-individual variation in baseline CORT, environmental conditions and reproductive success. Specifically, both foraging conditions and proxies of fitness, i.e. reproductive success were linked to baseline CORT differently between years, most likely due to the contrasting conditions experienced in those years. Therefore, although inter-individual variation in CORT is linked to life-history traits in breeding birds, relating this variation to individual fitness is challenging. Furthermore, there remains a lack of knowledge concerning the repeatability of baseline CORT concentrations in blue tits. Ultimately, my thesis suggests that in order to achieve a full understanding of how inter-individual variation in baseline CORT is linked to fitness, single year or short-term studies are inadequate; instead, researchers must relate individual differences to long-term measures of fitness.

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Figures and Tables

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Candidate's Declaration

I declare that the work recorded in this thesis is entirely my own and is of my own composition. No part of this thesis has been submitted for another degree.

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Chapter 1: General Introduction

1.1 Primer

Maintaining physiological function in the face of a fluctuating, and at times harsh environment is integral to survival (Sapolsky 2002). While navigating environmental challenges endotherms must maintain their internal environment within precise parameters for the body to properly function (Randall, Burggren & French 2001), and this physiological equilibrium is defined as homeostasis (Wingfield & Romero 2001; Sapolsky 2002; McEwen & Wingfield 2003). When an animal encounters a challenge (often defined as a stressor) that disrupts this balance, this initiates a cascade of physiological processes that influence bodily function and behaviour to help maintain homeostasis. Claude Bernard (1813–1878), a French physiologist, was the first to define the term milieu intérieur, now known as homeostasis. Later stress physiology, the study of how animals cope with and are affected by stress, emerged as a discipline through the work of Walter Cannon (1871-1945) and Hans Selye (1907-1982) who respectively coined the term homeostasis, and identified the role of a specific set of hormones integral to the body's response to stress, the glucocorticoids (GCs). When animals encounter a stressor the body responds by releasing GCs into the blood stream through the activation of the Hypothalamic-Pituitary-Adrenal (HPA) axis. A stressor can range from minor fluctuations in temperature and blood glucose level (McEwen & Wingfield 2003), to the more severe circumstances animals face, such as storms (Breuner & Hahn 2003) or conspecific aggressive encounters (Landys *et al*. 2007; Landys *et al*. 2010). Therefore, GCs play a fundamental role in physiological functions and are indispensible for an individual's survival (Randall, Burggren & French 2001).

As GCs are constantly fluctuating in response to both external and internal stressors they are variable in nature (McEwen & Wingfield 2003). However, under standard conditions, there is evidence of marked between individual variation, which in turn, has been linked to behaviour (Silverin 1998; Cockrem 2007; Landys *et al*. 2007; Angelier 2008), reproductive success (Müller *et al*. 2007; Bonier *et al*. 2009b; Bonier, Moore & Robertson 2011; Breuner 2011), morphology (Douglas *et al*. 2008; Roulin *et al*. 2008) and other physiological factors such as immune function (Harvey *et al*. 1984; Saino *et al*. 2002). Furthermore, in both wild animals and those kept under controlled conditions there is evidence that GCs are repeatible within individuals (Jones, Satterlee & Ryder 1994; Cockrem & Silverin 2002a; Romero & Reed 2008) and the extent to which an animal can change in response to environmental cues, i.e. phenotypic plasticity, can also be regulated by GCs (Moore & Hopkins 2009). Therefore, evolutionary changes in GCs may be an important mechanism by which a variety of traits can evolve, or be maintained within populations via selection on heritable individual variation or through adaptive phenotypic plasticity (Dufty *et al*. 2002; Williams 2008; Zera *et al*. 2007). Although GCs have been studied in free-ranging animals for over 30 years (Wingfield & Farner 1976a), it is still common to compare mean concentrations between groups of individuals and populations, rather than attempt to explain the significant inter-individual variation evident within populations (Williams 2008). However, as variation between individuals is fundamental for the evolution and maintinace of traits through natural selection (Darwin 1959), it is important to understand the causes and consequences of inter-individual variation in GCs.

In this thesis I will investigate the causes and consequences of inter-individual variation in corticosterone (CORT) the main GC in birds, in the blue tit, *Cyanistes caeruleus*. The blue tit is a common model species for avian breeding ecology, therefore, I will explicitly consider inter-individual variation in baseline CORT in relation to the quality of the breeding habitat, reproductive success, offspring phenotype, reproductive trade-offs and UV plumage colouration that is involved in mate choice. I will begin this introduction by first outlining the biology and function of CORT in birds, specifically in relation to energy-balance and coping with environmental perturbations. Then I will go on to describe how CORT is measured and the magnitude and significance of inter-individual variation in baseline CORT. Finally, I will describe the role of CORT in avian reproduction, particularly how CORT can influence reproductive success, offspring phenotype and condition-dependent traits, such as plumage colouration.

1.2 Glucocorticoids: biology, function and inter-individual variation

1.2.1 Biology & function

The steroid hormone corticosterone (CORT) is the main glucocorticoid in birds. Although often characterized as a ‘stress’ hormone, its primary role is the maintenance of homeostasis through the regulation of the body’s essential processes, including water/salt balance (Landys, Ramenofsky & Wingfield 2006), energy usage (Harvey *et al*. 1984; Sapolsky, Romero & Munck 2000; McEwen & Wingfield 2003) and immune function (Munck, Guyre & Holbrook 1984b; Butler, Leppert & Dufty 2010). As CORT plays a central role in a range of physiological processes, CORT is constantly present at baseline concentrations within the blood, although these concentrations can vary throughout the day and seasonally (see figure 1.1, Wingfield & Romero 2001). CORT concentrations can also increase rapidly after an acute stressor, such as severe weather or exposure to a predator, and in this case CORT is realised in association with catecholamines, which include the fight or flight hormone adrenaline (Sapolsky 2002). In passerine birds, concentrations can increase between 4- and 10-fold within minutes (Breuner, Patterson & Hahn 2008), usually significantly exceeding baseline levels after three minutes and reaching their peak within 10-30 minutes (see figure 1.1, Romero 2004). This rapid increase in circulating CORT is often termed the ‘stress-response’, and the CORT concentrations experienced are regarded as stress-induced rather than baseline concentrations. The stress-response allows animals to mount an adaptive behavioural and physiological response to an acute stressor (Breuner, Patterson & Hahn 2008).

The release of CORT into the blood stream is the end product of the stimulation of the Hypothalamic-Pituitary-Adrenal (HPA) axis (see figure 1.2). The HPA is stimulated by neural input regarding external stimuli such as, weather conditions and internal stimuli, such as low blood glucose (Sapolsky 2002). The higher brain centres perceive these stimuli and convey this information to the hypothalamus, which initiates the release of corticotropin-releasing hormone (CRH) from the paraventricular nucleus (PVN) (Sapolsky 2002). In turn this stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH), which signals the adrenal cortex to release CORT into the blood stream (Sapolsky 2002). Once in the circulation glucocorticoids exert their effects through intra-cellular receptors, which are ubiquitously present in the cells of the body (Romero 2004). The process is influenced by feedback loops, which ease activation of, or further stimulate the HPA axis dependent upon whether the stressor has been rectified (see figure 1.2, Sapolsky 2002). Glucocorticoids can also influence gene expression via the hormone receptor complex, as they enhance transcription by acting on glucocorticoid response elements (GREs), which are upstream from specific genes (Jantzen *et al.* 1987). The ubiquity of cellular receptors throughout the cells of the body and the influence of CORT upon gene expression, causes the hormone to have pleiotropic effects ranging from immune function, morphology, reproductive function and behaviour (Romero 2004). Therefore inter-Individual variation in baseline CORT could ultimately have important repercussions for a wide range of life-history traits (Romero 2004; Williams 2008) and also be involved in trade-offs between them (Moore & Hopkins 2009). Overall, inter-Individual variation in baseline CORT could mediate fundamental life-history traits and deserves further attention (Williams 2008).

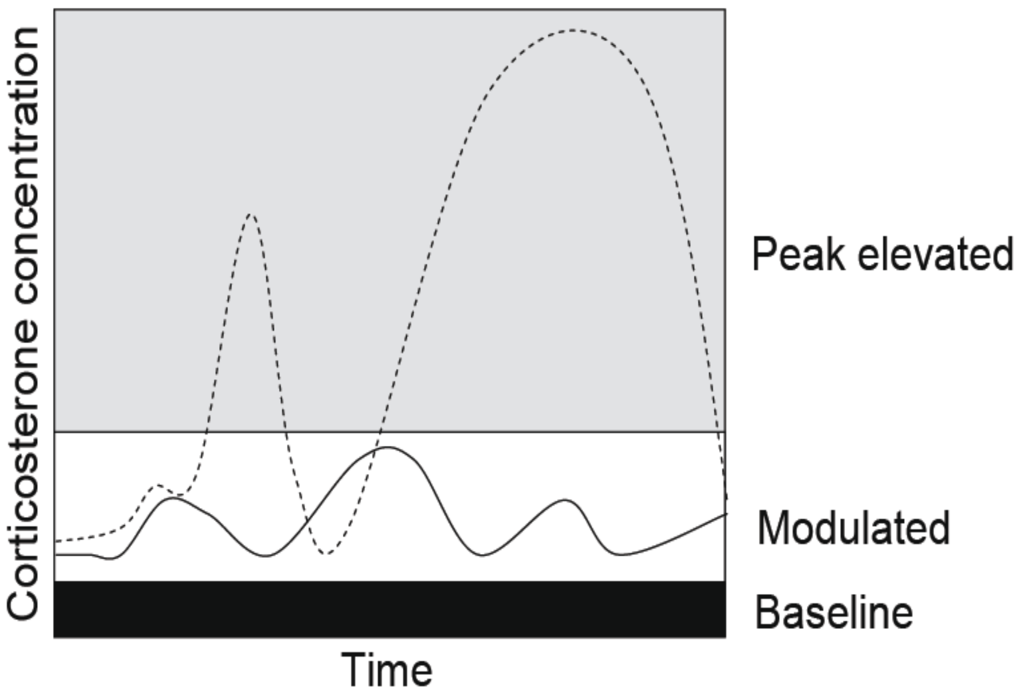


Figure 1.1. Schematic of the range of baseline CORT concentrations required for basic function, (black shaded zone). Baseline concentrations are also modulated in response to daily, seasonal and life-history demands within a range specific to the individual and species (solid line in non-shaded zone). In response to a severe acute stressor, CORT concentrations can rapidly increase to concentrations considerably higher than baseline for a temporary period (gray shaded zone and dotted line), which is also specific to the individual or species. Diagram taken from Busch & Hayward (2009).

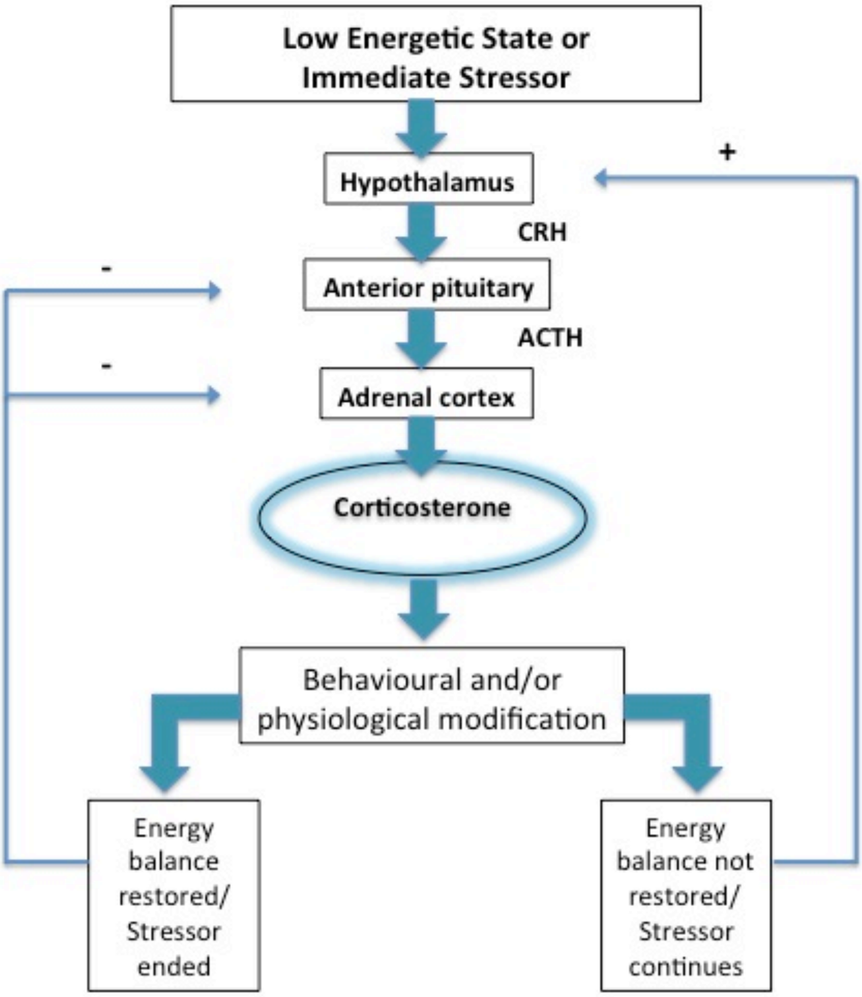


Figure 1.2. The Hypothalamic-Pituitary-Adrenal (HPA) axis, and its activation associated with energetic state and an immediate stressor. Lines connecting the endpoint of the flow chart with earlier stages illustrate both negative (-) and positive (+) feedback loops, which reduce activation of, or further stimulate the HPA axis dependent upon whether the stressor has been rectified. CRH signifies corticotrophin-releasing hormone that is released from the hypothalamus and causes the anterior pituitary to release ACTH - adrenocorticotropic hormone, which, in turn causes the adrenal cortex to release corticosterone. Diagram was modified from Randall, Burggren & French (2001).

1.2.2 Biology & function: Energy-balance

The main metabolic role of CORT is the regulation of circulating glucose levels (Sapolsky, Romero & Munck 2000), with both acute and chronic elevation of CORT concentrations causing the mobilization and storage of glucose in a context-dependent manner (Sapolsky 2002). Firstly, minor fluctuations in food availability or other environmental conditions can lead to a slight elevation in baseline CORT concentrations, which in turn can increase blood glucose via stimulation of foraging behaviour and appetite (Sapolsky 2002). Secondly, if CORT elevation occurs for several hours due to food deprivation, individuals can accrue a negative energy balance and under these circumstances elevated baseline CORT can stimulate multiple energetic pathways that both generate and store glucose for future use (Wingfield & Romero 2001; Sapolsky 2002). These include the stimulation of glycogenolysis and gluconeogenesis by glucagons and catecholamines, the storage of glycogen in the liver and the inhibition of peripheral glucose transport and utilization (Sapolsky 2002). Thirdly, when baseline CORT is chronically elevated due a longer-term lack of resources, elevated CORT can mobilize lipids through lipolysis and amino acids through the catabolism of protein stores, both of which are converted into glucose for use by the tissues (Sapolsky 2002).

Baseline CORT shows both diurnal and seasonal fluctuations, allowing animals to manage their energy requirements and maintain homeostasis in fluctuating environments (Wingfield & Romero 2001). For example, in birds baseline CORT is usually elevated at the end of the inactive period, typically first thing in the morning (Breuner, Wingfield & Romero 1999; Romero & Remage-Healey 2000), which is thought to influence behaviour and resource acquisition, ultimately inducing glucose mobilization at the beginning of the day when blood glucose may be low. Seasonal increases in baseline CORT during energetically expensive life-history stages, such as migration and reproduction are also thought to aid energy mobilization (Romero, Ramenofsky & Wingfield 1997a), for example, they have been shown to increase feeding rate and the storage of fat during the pre-migration phase (Holberton 1999) and have been associated with high nestling provisioning rates in birds (Doody *et al.* 2008).

Stress-induced rather than baseline CORT concentrations can also manage energy requirements, for example, during a short-term reduction in the quality of the environment due to severe weather conditions, the acute elevations of stress-induced CORT can lead to an escalation of foraging behaviours (Wingfield & Silverin 1986; Astheimer, Buttemer & Wingfield 1992; Breuner & Hahn 2003; Pravosudov 2003) or short-distance migration allowing individuals to reach areas where food may still be available (Breuner & Hahn 2003).

Therefore, although stress-induced CORT plays an important role in maintaining energy balance during discrete stressors, the daily management of baseline CORT in relation to energetic state is vital to maintain sufficient plasma glucose for the tissues (Sapolsky 2002). The role of baseline CORT in daily management of energy-balance, and its constant presence in the blood stream is the main rationale for choosing baseline as opposed to stress-induced CORT concentrations as the focus of this thesis.

1.2.3 Biology & function: Environmental perturbations

In birds, CORT concentrations are modulated in response to a number of factors within the environment, which in turn allows them to cope with these perturbations (Sapolsky 2002). These include the social environment (Kotrschal, Hirschenhauser & Moestl 1998; Hirschenhauser *et al.* 2000), the presence of predators (Cockrem & Silverin 2002b; Clinchy *et al.* 2004), food availability (Kitaysky, Piatt & Wingfield 2007) and habitat quality (Marra & Holberton 1998; Müller *et al.* 2007). For example, both the defense of territories (Landys *et al.* 2007; Landys *et al.* 2010; Lundberg & Alatalo, 1992; Silverin, 1998), and mates (Hirschenhauser *et al.* 2000; Kotrschal *et al.* 1998) against conspecifics has been associated with elevated CORT concentrations, in addition to testosterone. Furthermore, great tits, *Parus major* exhibit elevated plasma CORT concentrations in the presence of a stuffed predator, a Tengham's owl, *Aegolius funereus* (Cockrem & Silverin, 2002a) and song sparrows, *Melospiza melodica* also elevate plasma CORT concentrations when there are predators present (Clinchy *et al.*, 2004). The elevation of CORT in response to conspecific aggression or the presence of predators stimulates aggressive and/or avoidance behaviours (Wingfield & Romero 2001), as well as making glucose available for these energetically demanding activities (Sapolsky 2002). There is also evidence that reduced food availability is associated with elevated CORT concentrations in a wide range of bird species; e.g. Adelie penguins, *Pygoscelis adeliae* (Vleck *et al.*, 2000), black-legged kittiwakes, *Rissa tridactyla* (Kitaysky *et al.* 1999; Buck *et al.* 2007), white storks, *Ciconia ciconia* (Corbel & Groscolas, 2008) and white-crowned sparrows, *Zonotrichia leucophrys* (Breuner & Hahn, 2003). In this case, the elevation of baseline CORT may be linked to the increased foraging effort associated with reduced food abundance, as elevated baseline CORT concentrations have also been associated with increased locomotor activity (Astheimer, Buttemer & Wingfield 1992; Breuner 2000), foraging duration (Angelier 2008), and food intake rate (Astheimer, Buttemer & Wingfield 1992; Lõhmus, Sundström & Moore 2006).

As the focus of this thesis is inter-individual variation in baseline CORT during breeding in a woodland passerine, I will concentrate on describing some of the factors within the breeding habitat that could, and have been linked to baseline CORT in birds. Ultimately, during reproduction the quality and abundance of food is extremely important, as parents must vastly increase foraging effort to provide themselves and their developing offspring with sufficient nutrition. A number of species time their reproduction to coincide with maximal abundance of their prey (Perrins 1991; Kitaysky, Wingfield & Piatt 1999), however individuals often differ in the level of synchrony between breeding and prey abundance (Perrins 1991; Naef-Daenzer & Keller 1999; Naef-Daenzer, Naef-Daenzer & Nager 2000; Visser, Holleman & Gienapp 2005). In the common murre, *Uria aalge*, for example, in years where breeding does not coincide with the peak abundance of their fish prey, population level baseline CORT was higher than in matched years, furthermore, elevated baseline CORT was associated with higher foraging effort only in mismatch years (Doody *et al.* 2008). In woodland birds, like the blue tit, timing reproduction to coincide with the peak in caterpillar abundance, their predominate prey, has important consequences for reproductive success (Van Noordwijk, McCleery & Perrins 1995; Naef-Daenzer & Keller 1999; Visser, Holleman & Gienapp 2005). However, the influence of asynchrony between breeding woodland birds and the peak in caterpillar abundance upon baseline CORT has not previously been investigated.

In the woodland habitat, the density of trees caterpillars feed upon can also influence the number of invertebrates available to provisioning birds, and the work required to obtain them (Hinsley *et al.* 2008; Wilkin, King & Sheldon 2009). In the closely related great tit, *Parus major* provisioning effort is strongly influenced by the distribution and density of oak foliage surrounding the nest (Hinsley *et al.* 2008). In turn there is evidence that broad scale habitat differences are related to variation in baseline CORT between populations of blue tits (Müller *et al.* 2007), but the specific influence of tree density at the territory scale upon CORT has not been examined. Furthermore, weather variables including temperature and rainfall have been linked to baseline CORT in birds (Romero, Reed & Wingfield 2000), and may also influence baseline CORT in breeding birds as they can impede provisioning behaviours (Bolger, Patten & Bostock 2005; Geiser, Arlettaz & Schaub 2008). Although, multiple factors within the breeding habitat may be linked to both reproduction and baseline CORT concentrations, they have not been previously addressed within a single study. Additionally, as the above examples illustrate there is a lack of information regarding the influence of the breeding habitat upon baseline CORT concentrations at the individual level (Bonier *et al.* 2006; Kitaysky, Piatt & Wingfield 2007; Müller *et al.* 2007), which is necessary to explain inter-individual variation.

1.2.4 Biology & function: Measurement

CORT is most commonly measured from blood plasma using Radioimmunoassay or Enzyme Immunoassay, but CORT exists in the blood stream in two states: 1) the free molecule which has the potential to bind to intra-cellular receptors and 2) bound to corticosterone-binding proteins (CBPs) and therefore not active. When CORT is measured from blood plasma, however, it is both the free and bound CORT that is quantified (Silverin 1986; Breuner & Orchinik 2002). Therefore, measuring CORT from blood plasma is a crude method of estimating the amount of CORT available to the tissues. Furthermore, both hormone receptor density or affinity can influence an animal’s response to circulating CORT, but these factors are rarely measured (Ball & Balthazart 2008). In spite of this, selection studies that choose individuals based upon their circulating hormone concentrations provide evidence for the functional significant of these crude hormonal measures. For example, in Japanese quail, *Coturnix coturnix japonica*, selection for low or high stress-induced CORT, also leads to a change in behavioural phenotype, specifically the display of fear-related behaviour in the high-selection lines (Jones, Satterlee & Ryder 1994). In addition, both natural variation and manipulation of hormone titres have been shown to be correlated with behaviour (Breuner 2000; Carlson *et al*. 2006; Angelier 2008) and other physiological measures such as immune response (Martin *et al*. 2005; Bourgeon & Raclot 2006). Taken together these results suggest that this crude method of estimating or manipulting CORT concentrations is valid. Moreover, quantifying factors, such as receptor density, would require destructive methods, thus reducing the type of questions that could be addressed. For example, in my thesis in order to relate baseline CORT to reproductive success and behaviours it would not have been appropriate to sacrifice individuals after blood sampling. Therefore, I have measured baseline CORT from plasma, the most common method for quantifying concentrations in birds (Breuner & Orchinik 2002).

1.2.5 Inter-individual variation

CORT concentrations are fundamentally flexible, fluctuating to maintain homeostasis. In addition, individuals show significant plasticity across seasons (Romero 2004; Romero, Cyr & Romero 2006), life-history stages (Wingfield & Sapolsky 2003) and throughout their lifetime (Heidinger, Nisbet & Ketterson 2006b; Heidinger, Nisbet & Ketterson 2008). However, under standard conditions studies have found significant variation between individuals in CORT concentrations that can range from 5- to 25-fold (reviewed in Williams 2008). In fact, in most species where it has been investigated large inter-individual variation in CORT concentrations exist (Williams 2008). This inter-individual variation has often been overlooked because it has been considered to be susceptible to measurement error or unrepeatable within individuals (Ball & Balthazart 2008). However, there is growing evidence from bird species that hormone titres are repeatable within individuals (Cockrem & Silverin 2002a; Love, Bird & Shutt 2003; Romero & Reed 2008). Romero & Reed (2008) found baseline CORT levels to be relatively consistent between repeated bleeds in captive house sparrows, *Passer domesticus*, over a six week period and further studies on individual variation in CORT titres from wild birds provide evidence of consistent individual responses (Cockrem & Silverin 2002a; Love, Bird & Shutt 2003; but see Cockrem *et al*. 2009). There is also evidence that inter-individual variation is genetically determined, as individuals can be selectively bred to show either high or low circulating levels of CORT (Satterlee & Johnson 1988; Evans *et al*. 2006). Furthermore, variation between individuals in baseline CORT is related to other physiological indices (Berger *et al*. 2005; Cyr *et al*. 2007; Butler, Leppert & Dufty 2010), behaviour (Breuner 2000; Kitaysky, Wingfield & Piatt 2001; Angelier 2008) and morphology (Douglas *et al*. 2008; Roulin *et al*. 2008).

In avian species, inter-individual variation in CORT concentrations have been the subject of research since the 1970s (Wingfield & Farner 1976a), and since then a great deal of information has been gained in relation to their function (Sapolsky, Romero & Munck 2000; Sapolsky 2002) and mediation of life-history traits (Douglas *et al*. 2008; Angelier, Holberton & Marra 2009; Bokony *et al*. 2009; Angelier *et al*. 2010). Yet, comparing the mean difference in baseline CORT concentrations between groups of individuals is still commonplace (Williams 2008). This approach, however, does not capitalize on the significant inter-individual variation within populations, and prevents the analysis of this variation for evolutionary questions, as selection acts at the level of the individual (Darwin 1959). For example, is inter-individual variation in baseline CORT concentrations linked to fitness and what maintains the high level of inter-individual variation within populations?

Ultimately, there is evidence that significant inter-individual variation in baseline CORT concentrations exists within populations and may be consistent within individuals. In this thesis I measured baseline CORT at a standardised point in blue tits during breeding (day 5-6 after hatching) over three years, and related them to environmental conditions at the individual level and key life-history traits. Unfortunately, due to the duration of the research and methodological constraints it was not possible to address the consistency of baseline CORT concentrations within individuals.

1.3 The role of glucocorticoids in avian reproduction: an evolutionary perspective

CORT plays a significant role in avian reproduction, both directly by affected reproductive function (McEwen & Wingfield 2003) and indirectly by modifying behaviour (Silverin & Wingfield 1982; Silverin 1998; Kitaysky, Wingfield & Piatt 2001; Angelier 2008), reproductive investment (Bonier *et al.* 2009b; Bonier, Moore & Robertson 2011) and condition-dependent traits involved in mate choice (Husak & Moore 2008). The relationship between baseline CORT and reproductive success is complex, as there is evidence that elevated CORT concentrations are associated with both reduced investment in reproduction (Cyr & Romero 2007; Ellenberg *et al.* 2007; Angelier *et al.* 2010) and high reproductive success (for review see Bonier *et al.* 2009a). Additionally, there is evidence that maternal baseline CORT can influence offspring phenotype, including sex (Love *et al.* 2005; Pike & Petrie 2005a; Bonier, Martin & Wingfield 2007), mass (Hayward & Wingfield 2004; Love *et al.* 2005) and even the stress-response itself (Love & Williams 2008a). It is also becoming evident that CORT has the potential to influence mate choice (Husak and Moore 2008), as it mediates condition-dependent traits that serve as honest signals of mate quality, such as song (Spencer *et al.* 2003) and feather colouration (Roulin *et al.* 2008). This complexity and the range of factors within avian reproduction that CORT can influence are explored in this thesis, and in the following sections I will outline how CORT is thought to influence and/or be associated with reproductive success, offspring phenotype and condition-dependent traits.

1.3.1 CORT and reproductive success

There is evidence from biomedical research that chronic elevation of glucocorticoids can inhibit reproductive function, for example in females glucocorticoids suppress reproductive function by reducing the production of gonadotropin-releasing hormone (GnRH) (Harvey *et al.* 1984; McEwen & Wingfield 2003; Charmandari, Tsigos & Chrousos 2005) which, in turn reduces the release of luteinizing hormone (LH) from the pituitary (Sapolsky, Romero & Munck 2000). In addition, elevated glucocorticoids can affect the gonads directly, inhibiting the secretion of reproductive androgens (Charmandari, Tsigos

& Chrousos 2005). In birds, there is evidence that elevated CORT concentrations are associated with nest abandonment, reduction in reproductive behaviours and reduced reproductive success (Cyr & Romero 2007; Ellenberg *et al.* 2007; Angelier *et al.* 2010). However, there is also evidence that elevated baseline CORT is associated with high reproductive success and investment (for review see Bonier *et al.* 2009a). These findings suggest that as with energy-balance, the relationship between baseline CORT and reproductive success may be context-dependent, with elevated CORT associated with reduced or increased investment in reproduction dependent upon individual condition and/or the prevalent environment (Wingfield & Sapolsky 2003). This may be because during mild fluctuations in weather conditions or food availability, elevated baseline CORT can enhance foraging and thus energy mobilisation to maintain reproductive effort (Wingfield & Sapolsky 2003). Whereas, when conditions become very poor, CORT elevation may more appropriately direct investment from reproduction to survival (Wingfield & Sapolsky 2003). Under these contrasting conditions both of these functions would be adaptive and enhance lifetime reproductive success (LRS) (Bonier *et al.* 2009a). Yet this causes the relationship between reproductive success and baseline CORT to be difficult to predict, as it may vary between breeding stages, individuals or years (Bonier *et al.* 2009a).

There is a common assumption within the ecology and conservation biology literature that elevated baseline CORT concentrations are linked to poor conditions and low fitness (Bonier *et al.* 2009a; Breuner 2011). In response to this and the growing use of CORT as a measure of the relative condition or health of individuals and populations, the validity of this hypothesis was questioned in a recent literature review by Bonier *et al.* 2009 (see figure 1.3). The concept was termed the “CORT-fitness” hypothesis (see figure 1.3), and the review provided evidence that variation in baseline CORT was in fact positively (Love *et al.* 2004; Müller *et al.* 2007; Bonier *et al.* 2009b), negatively (Buck, Oreilly & Kildaw 2007; Williams *et al.* 2008; Angelier *et al.* 2010), and non-significantly (Eeva *et al.* 2005; Ellenberg *et al.* 2007; Müller *et al.* 2007) related to estimates of fitness, including reproductive success and survival. In addition, these relationships varied within populations (Müller *et al.* 2007; Bonier *et al.* 2009b), between years (Lanctot 2003; Chastel *et al.* 2005) and even within individuals at different life history-stages (Bonier *et al.* 2009b; Ouyang *et al.* 2011). This is consistent with the idea the the realtionship between baseline CORT and reproductive success is context-dependent.

The threshold over which it is adaptive for baseline CORT elevation to be associated with nest abandonment rather than an increase in reproductive investment can vary between individuals, breeding stages or between species (Bonier *et al.* 2009a; Wingfield & Sapolsky 2003). For example, individuals that begin reproduction with lower energy reserves may abandon a reproductive event earlier in the face of inclement conditions and elevated CORT, than those with greater energy reserves (see figure 1.4b, Wingfield & Sapolsky 2003). In contrast, older individuals with limited potential breeding opportunities may continue breeding despite elevated CORT and poor environmental conditions (Heidinger, Nisbet & Ketterson 2006a; Heidinger, Nisbet & Ketterson 2008). Similarly, in species with strictly seasonal breeding, individuals may continue their breeding attempt in spite of harsh conditions and elevated CORT (Wingfield & Sapolsky 2003). Inter-species variation in life-history may also be important, with longer-lived species directing resources towards survival in years where conditions are not suitable for reproduction, to improve LRS (Bokony *et al.* 2009; Heidinger, Nisbet & Ketterson 2008). In addition, the breeding stage at which hormone concentrations are measured can influence the relationship between CORT and reproductive success (see figure 1.4a), with a positive relationship between CORT and breeding success in later stages of reproduction when investment and the value of the offspring are higher (Bonier et al. 2009b). This may be because at the start of a reproductive event when investment has been minimal, elevated CORT may signal that circumstances are not conducive to breeding, therefore individuals may invest in self-maintenance over reproduction. However, at the later stages of reproduction when individuals have already invested heavily in reproduction, elevated baseline CORT may facilitate reproduction by increasing brood care.

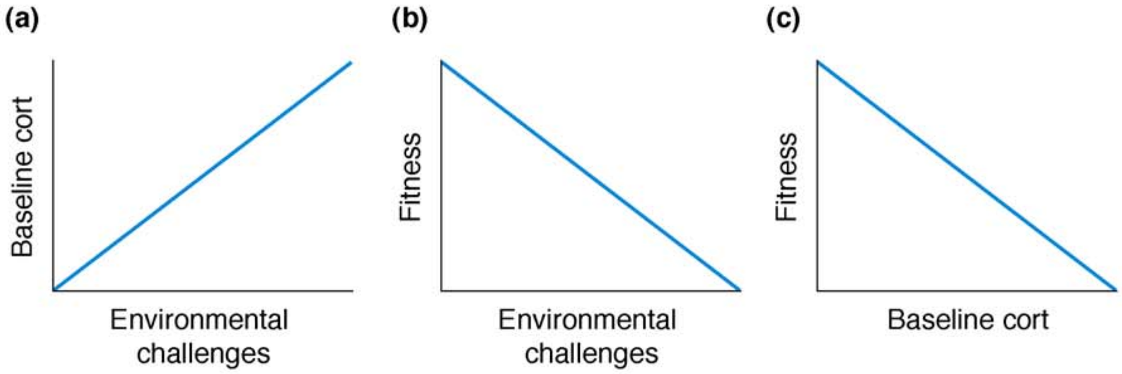


Figure 1.3. The theoretical foundation of the "Cort–fitness" Hypothesis. (a) Baseline CORT concentrations are predicted to increase with environmental challenges. (b) Increasing environmental challenges are associated with decreasing fitness because resources must be reallocated toward survival at the expense of reproduction and/or self-maintenance. (c) In combination, these two tenets lead to the prediction that baseline CORT is negatively correlated with fitness. Taken form Bonier *et al.* (2009a).

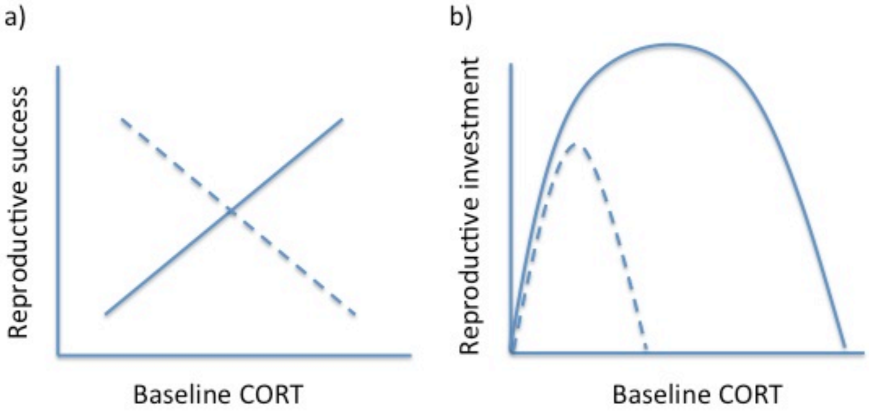


Figure 1.4. a) The relationship between baseline CORT and reproductive success between different breeding stages, dashed line indicates early reproduction i.e. egg laying and solid line indicates late reproduction, i.e. brood rearing. b) The alternate associations between elevation in baseline CORT and reproductive investment dependent upon individual condition, dashed line indicates the relationship for an individual in poor condition and the solid line shows the relationship for an individual in superior condition. Modified from concepts in Bonier *et al.* (2009b).

As yet the majority of studies that have investigated the link between baseline CORT and reproductive success in breeding birds have done so within a single year (see review of studies in Bonier *et al.* 2009a). As there is evidence that the relationship between baseline CORT and reproduction is context-dependent, this relationship could differ between years (Bonier *et al.* 2009a). However, this has rarely been investigated at the individual level (Buck, O'Reilly & Kildaw 2007; Williams *et al.* 2008). Furthermore, the cost of reproduction is a central concept in evolutionary biology, where increased investment in current reproduction is predicted to decrease future fecundity and survival (Lack 1947; Williams 1966). There is evidence that experimental enlargement of brood size in tree swallows, *Tachycineta bicolor*, caused mothers to have higher baseline CORT, than mothers with reduced broods (Bonier, Moore & Robertson 2011). But, elevated baseline CORT associated with high investment in current reproduction could negatively influence future survival and reproduction (Love *et al.* 2004; Love *et al.* 2005; Bonier, Moore & Robertson 2011). For example, elevated CORT during reproduction could reduce survival through negative effects upon immune function (MacDougall-Shackleton *et al.* 2009; Goutte *et al.* 2010). Alternatively, elevating CORT during reproduction can re-direct behaviours from reproduction to survival when conditions are poor (Bonier *et al.* 2009a). In this case, elevating baseline CORT may reduce the costs of the current reproductive event, having positive implications for future reproduction. To address these questions studies that employ multiple years are required to investigate how the modulation of baseline CORT within individuals in response to current reproductive investment influences future reproduction and survival.

Overall, further research is required to understand how inter-individual variation in baseline CORT is linked to reproductive success. Specifically, how consistent is the relationship between baseline CORT and reproductive success during the same breeding stage across multiple years that vary in harshness. Furthermore, the role of CORT in reproductive trade-offs required investigation, for example how is baseline CORT associated with differential investment within a reproductive event, and does this influence future reproductive success and survival?

1.3.2 Offspring phenotype

Reproductive success is not only influenced by the number of offspring reared, but also offspring phenotype (Love & Williams 2008b; Monaghan 2008). Maternal CORT has the potential to influence offspring phenotype, not only during development (Love *et al.* 2005; Love & Williams 2008a) but also into adulthood (Seckl 2004). Experiments have shown that maternal CORT concentrations during laying correlate with CORT concentrations within the yolk (Hayward & Wingfield 2004). In turn, elevation of maternal CORT during egg production has been associated with reduced growth, immune function and body size of offspring (Hayward & Wingfield 2004; Saino *et al.* 2005; Eriksen *et al.* 2006). In addition, evidence from the biomedical literature suggests that prenatal “stress” is linked to long-term health disorders (for review see Viltart & Vanbesien-Mailliot, 2007). However, these seemingly negative consequences of elevated maternal CORT may be adaptive, enhancing offspring phenotype under certain conditions. For example, Gluckman & Hanson (2004) proposed that maternal stress is a developmental cue to offspring, programming their future phenotype to suit the harsh environmental conditions they are brought into. In addition, maternal stress could match offspring need to maternal ability to provide, with increased exposure to maternal CORT causing reduced growth rate in chicks associated with reduced parental workloads (Breuner 2008; Love & Williams 2008b). In turn this would provide benefits for mothers by reducing the costs of current reproduction, thus improving future survival and reproductive success (Breuner 2008; Love & Williams 2008b). Reduced growth rates may also be advantageous for chicks when food availability is low, reducing the probability that parents will not be able to rear nestlings to fledging (Hayward and Wingfield 2004).

The facultative adjustment of offspring sex can also have fitness benefits for parents if sexes differ in their survival and reproductive potential. In many systems the sexes can differ in their survivorship and breeding potential dependent upon the prevalent conditions. For example, daughters are often more likely to reproduce than sons, regardless of their quality (Kruuk *et al.* 1999b; Widdig *et al.* 2004). Whereas high-quality sons can leave many more offspring than daughters and low-quality sons may fail to reproduce at all (Hewison & Gaillard 1999). Therefore, in circumstances that enable a female to produce high-quality offspring she should produce more sons, conversely a female constrained to produce low-quality offspring should produce more daughters (Trivers & Willard 1973). However, the opposite pattern may be observed in species where the sex roles are reversed (West 2009).

Although this hypothesis was originally applied to polygamous species, it can equally apply to any species where maternal condition or environmental conditions differently influence the quality of offspring and their reproductive success dependent upon their sex (West 2002). In agreement with theory, there have been a number of studies documenting the ability of birds to manipulate the sex ratio of their offspring in response to maternal condition and/or environmental factors (reviewed in West 2009). In birds, the female is the heterogametic sex (producing Z- and W-bearing ova) and this has led to the suggestion that mechanisms of sex-ratio adjustment could potentially be under maternal control (Oddie 1998). Experimental and empirical studies have demonstrated that brood sex-ratio is associated with numerous factors, including food abundance (Austad & Sunquist 1986), mate quality (Svensson & Nilsson 1996; Sheldon *et al.* 1999a; Pike & Petrie 2005c), habitat quality (Komdeur *et al.* 1997; Komdeur, Magrath & Krackow 2002b; Desfor, Boomsma & Sunde 2007), and maternal condition (Nager *et al.* 1999; Parker 2002; Pike & Petrie 2005a). However, it should be noted that there are a number of studies that have not found the predicted adjustments (reviewed in West 2009).

As CORT has been linked with the factors associated with brood sex ratio adjustment (Food availability: (Schoech, Bowman & Reynolds 2004), Habitat quality: (Müller *et al.* 2007), Mate attractiveness (Pike & Petrie 2005c; Pryke *et al.* 2011) and maternal condition (Love *et al.* 2005; Pike & Petrie 2005c), the hormone as been investigated as a possible mechanism. As circulating CORT concentrations can link the external conditions with the internal environment, maternal CORT could reliably indicate conditions that might favour brood sex ratio adjustment (Pike & Petrie 2003; Love *et al.* 2005). For example, elevated baseline CORT has been associated with poor body condition (Schoech, Mumme & Wingfield 1997; Kitaysky, Wingfield & Piatt 1999; Love *et al.* 2005; Pike & Petrie 2005a); therefore it would be expected to be associated with investment in the sex whose survival and reproductive success is least effected by poor developmental conditions. In agreement with this hypothesis correlative and experimental studies have found a link between CORT and female biased brood sex ratios, in species were males are the larger sex and therefore may be more sensitive to poor natal conditions (Love *et al.* 2005; Pike & Petrie 2005a; Bonier, Martin & Wingfield 2007).

The mechanism by which CORT could influence the sex of offspring is currently unknown (Pike & Petrie 2003). However, CORT has been suggested to influence brood sex ratio at both the pre-laying stage, by directly influencing offspring sex by causing segregation distortion of the sex chromosomes during meiosis (Rutkowska & Badyaev 2008) or selective reabsorption of ova dependent upon sex (Pike & Petrie 2003). Alternatively, yolk CORT concentrations can influence hatching success (Saino *et al.* 2005), nestling growth (Hayward & Wingfield 2004) and survival (Love *et al.* 2005; Cyr & Romero 2007), thus could affect brood sex ratio post-laying, through early embryo death or sex-specific nestling mortality. Indeed, studies provide both correlative and experimental evidence of a relationship between maternal baseline CORT and brood sex ratio at laying (primary sex ratio: Pike & Petrie 2005a; Bonier, Martin & Wingfield 2007; Gam, Mendonça & Navara 2011) and at fledging (secondary sex ratio: Love *et al.* 2005). Therefore, although there is growing evidence that maternal CORT is linked to brood sex ratio, the mechanism through which this is achieved remains unclear. In addition, further work is required to establish whether CORT is consistently associated with brood sex ratio adjustment in birds.

1.3.3 Mate choice & condition-dependent traits

Inter-individual variation in baseline CORT may be associated with, or directly influence the expression of condition-dependent traits that act as honest signals of quality to potential mates (Spencer *et al.* 2003; Buchanan *et al.* 2004; Leary, Garcia & Knapp 2006; Roulin *et al.* 2008). As baseline CORT is intrinsically linked to an individual's energetic state, it is often negatively correlated with measures of condition, such as mass, body condition index and fat scores (Holberton et al 1996; Kitaysky et al. 1999a, 1999b; Romero and Wikelski 2001; Love et al. 2005). In turn, individuals in superior condition can often invest more in costly signals, such as plumage colouration and song (Husak & Moore 2008), therefore, this could lead to a correlation between baseline CORT and these condition-dependent traits. However, there is also evidence that CORT can directly influence the expression of condition-dependent traits involved in mate choice. For example, the quality of male song in the zebra finch, *Taeniopygia guttata*, determines the attractiveness of males to potential mates, and is negatively affected by both dietary stress and experimental elevation of CORT concentrations during development (Spencer *et al.* 2003). CORT elevation directly affects song quality by reducing the size of the high vocal center in the brain, which is responsible for song complexity (Buchanan *et al.* 2004). Another study has demonstrated the link between CORT and plumage colouration (Roulin *et al.* 2008). In barn owls, *Tyto alba*, the size and colour of melanic plumage spots are sexually selected, as both sexes show a preference for this plumage colouration (Roulin *et al.* 2008). Furthermore, an experimental increase in circulating CORT in barn owl nestlings was associated with reduced expression of the melanic plumage spots (Roulin *et al.* 2008). The authors suggest this was due to the inhibiting effect of CORT upon the secretion of melanocortins and tyrosinase, and thus melanogenesis in plumage (Roulin *et al.* 2008).

As the name implies, blue tits have bright blue plumage, however, their feathers are also highly UV reflectant (Hunt *et al.* 1998). The sexes are slightly dimorphic, with females on average having lower intensity and chroma of the UV-blue crown feathers (Hunt *et al.* 1999; Appendix II), but some females can be as bright as males. Mate choice studies have shown that both males and females choose mates dependent upon their crown colouration (Hunt *et al.* 1999) and there is evidence of assortative mating based on UV crown reflectance (Andersson, Örnborg & Andersson 1998). The expression of structural feather colouration, such as the UV reflectance of blue tit feathers, is influenced by both food availability (McGraw *et al.* 2002; Siefferman & Hill 2005b) and feather growth rate (Griggio *et al.* 2009). Elevated CORT can negatively influence the microstructure of the feathers during growth, which is responsible for the strength of their UV reflectance (DesRochers *et al.* 2009), perhaps explaining why CORT is often down regulated during moult (Romero, Strochlic & Wingfield 2005). Therefore, CORT could directly or through its link with energetic state be associated with UV plumage colouration. However, as yet this has not been investigated in female birds.

1.4 The study species

The blue tit, *Cyanistes caeruleus* is a small (10-13 g) hole-nesting bird belonging to the family Paridae. The species is widely distributed across Europe and commonly inhabits broadleaf woodland, but is also abundant in urban parks and gardens. The blue tit was employed as a model for my PhD as it readily breeds in nest boxes, allowing easy access to both adults and offspring during reproduction. Also, the UK blue tit population has remained relatively stable over the last few decades, unlike other woodland passerine species which have experienced significant declines (Hewson et al. 2007).

Blue tits are a socially monogamous species, but previous studies have found a relatively high occurrence of extra-pair paternity, with populations ranging from 29% (Gullberg, Tegelström & Gelter 1992) to 68% (Charmantier et al. 2004) of broods containing extra-pair young. In Scotland, the species breeds between March and June, with pairs defending nest sites from late March and egg laying beginning in April or May. Blue tits often have large broods, and over the three years of this study the average clutch was 9.9±2.1, with a clutch of 15 recorded in one year. The female alone incubates eggs (see figure 1.5) and her mate will bring her food items during this time (Perrins 1979). Both parents, however, provision the offspring once they hatch and chicks remain in the nest for 18-20 days until fledging (Perrins 1979). Like other Parid species, they are entirely insectivorous during the breeding season, with moth larvae like the Winter Moth, *Operophtera brumata* constituting the majority of their diet (Perrins 1991). However, they also provision their nestlings with other invertebrates such as spiders (Arnold *et al.* 2007). Their large brood size causes nestling provisioning to be particularly demanding and parents can provision at a rate of one visit per minute throughout the day (Perrins 1991). By mid June all nestlings have usually fledged, and as with the UK as a whole (Perrins 1979), I did not find evidence of second broods at my study site.

The blue tit is one of the most intensively studied passerines in behavioural ecology and evolutionary biology, covering topics ranging from quantitative and population genetics (Taberlet, Meyer & Bouvetv 1992; Merilä & Wiggins 1995; Johnsen et al. 2003), sexual selection (Andersson, Örnborg & Andersson 1998;

Delhey et al. 2003; Charmantier et al. 2004; Limbourg et al. 2004; Dreiss 2005; Doutrelant et al. 2008), foraging ecology (Ramsay & Houston 1997; Lambrechts et al. 2004; Stauss, Burkhardt & Tomiuk 2005), climate change (Sanz 2002; Visser *et al.* 2003; Visser, Both & Lambrechts 2004), and personality (Arnold *et al.* 2007; Herborn *et al.* 2010). Comparatively few studies have employed the species to address questions relating to hormonal titres (but see Müller *et al.* 2006; Peters *et al.* 2006; Müller *et al.* 2007; Kempenaers, Peters & Foerster 2008; Kurvers *et al.* 2008; Robert, Ras & Peters 2009). One reason for this may be their small size, which restricts the ease with which blood can be drawn and also the absolute amount of blood that can be taken over a short period of time. In addition, in the UK, Home Office regulations suggest the restriction of blood sampling to 1% of body mass per 30 days (mass ~10 g = 100 µl). Therefore, it is difficult to blood sample individual birds on multiple occasions within the breeding season or while held in captivity for a short period. This poses a problem when wishing to investigate the repeatability of hormone measures within individuals. In addition, when validating hormone manipulations it is not possible to blood sample the same individual before and after a manipulation.

Specifically, the causes and consequences of variation in CORT have rarely been investigated in the blue tit (Müller *et al.* 2006; Peters *et al.* 2006; Landys *et al.* 2007; Müller *et al.* 2007). Previous studies have addressed the influence of broad scale habitat quality upon CORT concentrations in breeding adults (Müller *et al.* 2007) and the role of human disturbance upon elevating CORT during measurement (Müller et al. 2006). In addition, CORT concentrations have also been related to the expression of feather colouration in male blue tits (Peters et al. 2006) and male social encounters (Landys *et al.* 2007). Overall we know very little about how external factors influence inter-individual variation in baseline CORT and in turn how this is related to reproductive success.

Figure 1.5. A female blue tit photographed on her nest at the study site while incubating in 2009, photograph by Dr T. Lislevand.

1.5 The field site

The nest boxes used in this study were situated within mixed deciduous woodland on the east banks of Loch Lomond surrounding the University of Glasgow field station, the Scottish Centre for Ecology and the Natural Environment, UK (figure 1.6, SCENE, 56.13 ° N, 4.13° W). The woodland consists of a mixture of deciduous trees, with oak species, sessile, *Quercus petraea* and English, *Quercus robur* the most dominant, but also birch *Betula* spp., ash, *Fraxinus excelsior*, hazel, *Corylus avellana* and sycamore, *Acer pseudoplatanus* interspersed throughout the site. The woodland lies within the Loch Lomond & Trossachs National Park and is designated as a Site of Special Scientific Interest (SSSI) and a Special Area for Conservation (SAC). The woodland is considered ancient, as the site has had continuous woodland cover for at least 250 years. This is rare within the national park and the UK as a whole, as ancient woodland covers only 2.5% of the total park area and 2% of the land cover of the UK (The Woodland Trust 2011).

The nest boxes are situated in two sites within continuous woodland, with one site immediately surrounding SCENE, Ross Woods (~120 ha, see figure 1.7a) and the second < 3 km to the south Cashel Woods (~60 ha, see figure 1.7b). The woodland sites are functionally connected, as birds move between the sites. On a few occasions nestlings that were ringed at one of the sites were recorded breeding in the other as adults. There were approximately 430 woodcrete nest boxes (Schwegler) hung from mature trees approximately 2.5 m from the ground. The occupancy differed between years with 40% in 2008, 33% in 2009 and 27% in 2010. However, the percentage of nests to successfully fledge young varied only slightly between years with 72% in 2008, 71% in 2009 and 79% in 2010. During the course of my PhD a number of the nest boxes were no longer used as part of the study, because of pine marten, *Martes martes*, predation. The crosshatched area in figure 1.7b is where nestlings were predated. In 2009 all broods (35) within nest boxes were depredated, therefore in 2010 the nest boxes were no longer in use. This is the first time pine marten predation has been recorded at the site and was thought to be due to the placement of the nest boxes directly upon trees in this area rather than from perpendicular brackets as in the rest of the site (see Appendix III).

Figure 1.6. The location of the Scottish Centre for Ecology and the Natural Environment (SCENE) on the east banks of Loch Lomond in Scotland, UK. The map has been taken from the SCENE website (www.gla.ac.uk/researchinstitutes/bahcm/researchcentres/scene/whereisscene).

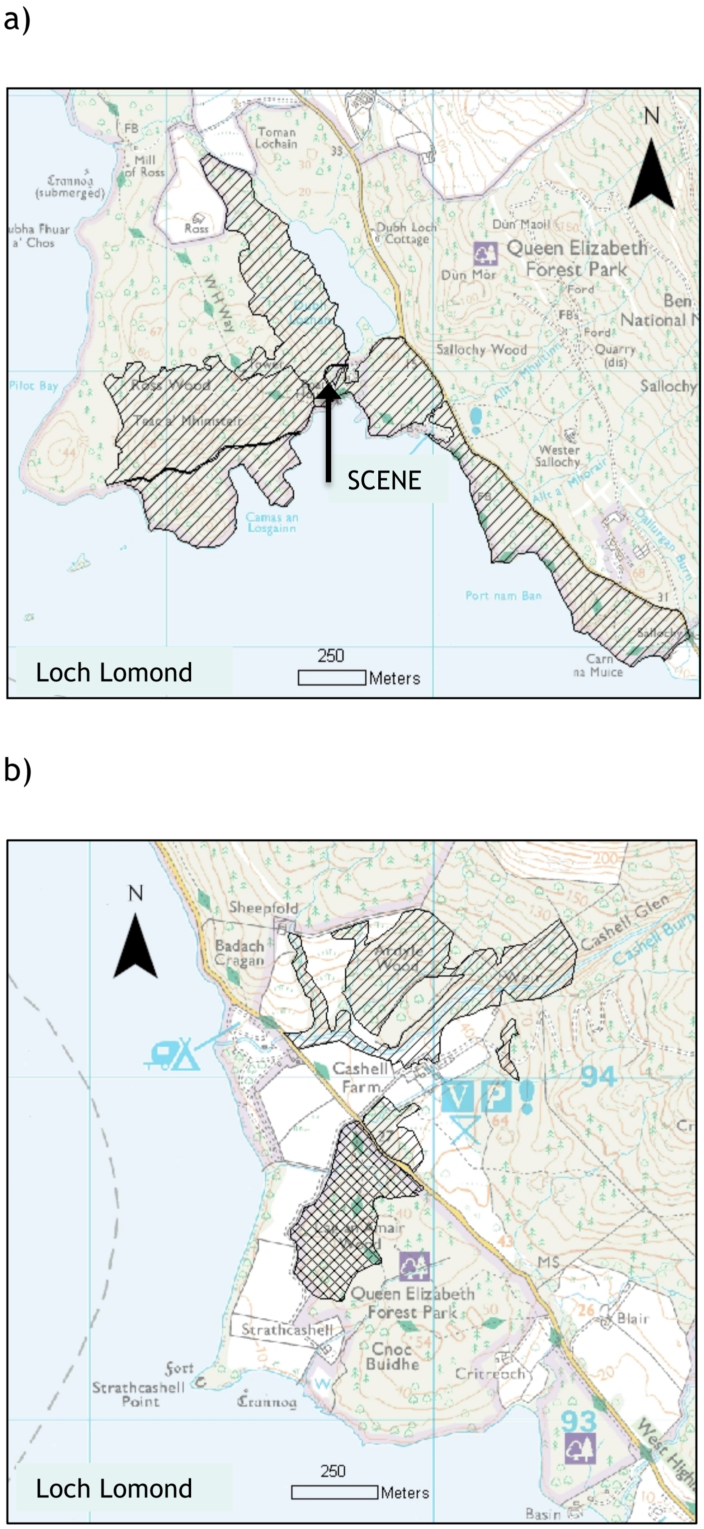


Figure 1.7. The location of a) Ross Woods, the woodland immediately surrounding the Scottish Centre for Ecology and the Natural Environment (SCENE) and b) Cashel Wood, the woodland situated by Cashel farm about 3 km south of SCENE. The woodland areas denoted by the diagonal lines contain nest boxes used in this study and the area denoted by crosshatched lines contained nest boxes that ceased to be used due to predation. Both sites are situated on the east banks of Loch Lomond, UK. Digitised Ordinance Survey maps (1:10,000) courtesy of EDINA were used.

1.6 Thesis content

This thesis examines both the causes and consequences of inter-individual variation in baseline CORT in breeding blue tits. **Chapter 2** aims to assess how the foraging conditions during provisioning, measured at the territory-scale, influence baseline CORT in adults and nestlings over three years. **Chapter 3** considers whether similar foraging conditions are correlated with both maternal baseline CORT and reproductive success over three years, and ultimately whether maternal baseline CORT is correlated with reproductive success consistently over three years. **Chapter 4** investigates whether maternal body condition, maternal baseline CORT and experimental elevation of CORT is associated with brood sex ratio adjustment in blue tits. I also address whether CORT is involved in reproductive trade-offs in **Chapter 5**, and in **Chapter 6** I examine whether inter-individual variation in maternal baseline CORT is linked to UV plumage colouration in female blue tits.

Chapter 2: The influence of foraging conditions upon stress hormones in adult and nestling blue tits, *Cyanistes caeruleus*

The aim of Chapter 2 was to investigate how foraging conditions measured during provisioning, such as weather, oak density and synchrony between breeding and the peak in their caterpillar prey, influence baseline CORT in breeding adults and their dependent offspring across three years (2008-2010). My chapter aims were;

- Quantify the inter-year variation in both foraging conditions and baseline CORT in adult and nestling birds.
- Establish whether synchrony between breeding and the peak in their caterpillar prey, oak density, rainfall and temperature explained variation in parental and nestling baseline CORT.

Chapter 3: Are baseline glucocorticoids associated with fitness? Linking environmental conditions, corticosterone and reproductive success in the blue tit, *Cyanistes caeruleus*

Chapter 3 considers whether the foraging conditions outlined in Chapter 2, that are correlated with maternal baseline CORT are also correlated with reproductive success, and ultimately whether maternal baseline CORT is correlated with reproductive success consistently across three years (2008-2010). My chapter aims were;

- Quantify the inter-year variation in both foraging conditions and maternal baseline CORT.
- Test whether similar foraging conditions are associated with reproductive success and/or maternal baseline CORT.
- Examine whether there is evidence that maternal baseline CORT is linked to reproductive success.

Chapter 4: Maternal condition but not corticosterone is linked to brood sex ratio adjustment in a passerine bird

This chapter investigates whether maternal body condition, maternal baseline CORT and experimentally elevated CORT are linked to brood sex ratio adjustment in the blue tit. My chapter aims were;

- Investigate whether maternal body condition and/or maternal baseline CORT are correlated with brood sex ratio and nestling condition over three years (2008-2010).
- Examine whether maternal body condition and maternal baseline CORT are correlated.
- Investigate whether experimental elevation of maternal baseline CORT was associated with brood sex ratio adjustment and nestling condition.

Chapter 5: Experimental manipulation of the early costs of reproduction influence maternal brood care and return rates

In this chapter I investigated whether maternal baseline CORT is involved in reproductive trade-offs. To do so, I reduced the costs of egg laying through supplemental feeding and compared maternal baseline CORT, brood care and maternal survival between manipulated mothers and controls. My chapter aims were;

- Investigate whether the treatment influenced reproductive success, nestling condition or maternal return rates.
- Investigate whether maternal brood care differed between treatment groups.
- Examine whether maternal body condition and maternal baseline CORT differed between treatment groups.

Chapter 6: UV crown colouration in female blue tits, *Cyanistes caeruleus*, predicts baseline corticosterone and reproductive success

In Chapter 6 I investigate whether UV crown colouration, is linked to indices of condition and reproductive success in female birds over three years (2008-2010). My chapter aims were;

- Investigate whether maternal UV crown colouration differs in relation to year and age.
- Examine whether indices of condition, i.e. baseline CORT, body condition and haematocrit are linked to UV crown colouration.
- Investigate whether UV crown colouration is associated with measures of reproductive success.

Chapter 2: The influence of foraging conditions upon stress hormones in adult and nestling blue tits, *Cyanistes caeruleus*

2.1 Abstract

For insectivorous woodland birds, the synchrony between breeding and peak caterpillar abundance, tree density, rainfall and temperature can all influence the energetic demands of foraging parents and their nest-bound offspring. Elevated baseline corticosterone (CORT) is associated with increased foraging and food intake, and may allow birds to meet the needs of reproduction under harsh conditions. However, elevating baseline CORT in response to harsh environments may be costly in the long-term, as it has also been associated with reduced survival and immune responses in birds. This study aimed to measure key aspects of the foraging conditions experienced at the level of the individual nest, and to assess their influence upon baseline CORT in adult and nestling blue tits, *Cyanistes caeruleus* over three years (2008-2010). There was significant inter-annual variation in baseline CORT, rainfall, temperature and synchrony between breeding and the caterpillar peak. At the population level, both adults and nestlings had elevated baseline CORT concentrations, in the year characterised by most asynchrony (2008). However in 2009, the year in which birds experienced the most rainfall, population level baseline CORT was elevated in adults but not nestlings, despite birds being most synchronous in this year. In 2008 only, baseline CORT was negatively correlated with synchrony with the caterpillar peak in adults and nestlings. Oak density was not related to nestling CORT, but in 2008 and 2010 when birds were more asynchronous than 2008; it was negatively correlated with adult baseline CORT. Weather variables were also only associated with adult CORT; rainfall was a significant predictor of adult baseline CORT in 2009, the year characterised by the most rainfall and temperature was negatively correlated with adult baseline CORT, in 2008 and 2009, the years characterised by the most asynchrony and rainfall respectively. This study suggests that contrasting aspects of the foraging conditions affect baseline CORT concentrations in adult and nestling birds. Importantly, these effects are context-dependent and relative to the severity of the conditions within each year.

2.2 Introduction

In birds the availability and abundance of prey during brood rearing has important consequences for reproductive success and the energy required to rear offspring (Tinbergen & Dietz 1994; Naef-Daenzer & Keller 1999; Naef-Daenzer, Naef-Daenzer & Nager 2000; Tremblay *et al.* 2005). To minimise the costs of foraging, breeding birds often time reproduction to co-inside with maximal abundance of prey (Perrins 1991; Naef-Daenzer & Keller 1999), however, temporal and spatial availability of food items can cause parents to experience differential costs of foraging (Naef-Daenzer, Naef-Daenzer & Nager 2000). Birds possess physiological mechanisms that allow them to maintain homeostasis and reproduction in spite of inclement conditions (Wingfield & Sapolsky 2003). Baseline corticosterone (CORT) is the main glucocorticoid in birds, and is elevated in response to environmental challenges to adjust physiology and behaviour appropriately for the prevalent conditions (Wingfield & Romero 2001). The release of CORT in response to environmental perturbations can be beneficial for breeding birds, as elevated baseline CORT promotes gluconeogenesis, which can mobilise fat reserves for energetically demanding breeding behaviours (Wingfield & Romero 2001). Indeed elevated baseline CORT has been correlated with the increased energetic demand of raising offspring (Bonier *et al.* 2009b). Furthermore, CORT elevation can adaptively redirect behaviour from breeding to survival when conditions become unsuitable (Wingfield & Sapolsky 2003). However, if inclement conditions persist stressors can become chronic and in this case elevated CORT has been associated with reduced survival, immune response, juvenile recruitment and reproductive success (see Blas *et al.* 2007; Cyr & Romero 2007; Goutte *et al.* 2010; Harvey *et al.* 1984; Martin 2009; Wingfield & Sapolsky 2003).

Elevated baseline CORT concentrations have been associated with increased locomotor activity (Astheimer *et al.* 1992; Breuner & Wingfield 2000), foraging duration (Kitaysky et al. 2001), and food intake rate (Astheimer, Buttemer & Wingfield 1992; Lõhmus, Sundström & Moore 2006). Therefore, the hormone has been hypothesized to play an integral role in mediating foraging behaviours. For example, in the Adélie penguin, *Pygoscelis adeliae* individuals with elevated pre-foraging CORT concentrations spent proportionally more time foraging closer to the colony than out at sea compared with those with low concentrations (Angelier 2008). In addition, in years when common murres, *Uria aalge*, mistime breeding with the peak abundance of their fish prey, population level baseline CORT is higher than in years breeding coincides with peak fish abundance. Furthermore, elevated baseline CORT was associated with higher foraging effort only in mismatch years (Doody *et al.* 2008a). This suggests that not only is baseline CORT associated with low food abundance and foraging behaviour, but that the relationship between foraging and baseline CORT is dependent upon the prevalent conditions, with harsher conditions linking foraging effort with physiological stress.

The circulating CORT concentrations experienced by dependent offspring are also influenced by parental foraging conditions. Both food quality and provisioning effort have been found to influence nestling CORT concentrations (Kitaysky *et al.* 2001; Corbel & Groscolas 2008). Elevation of baseline CORT may be adaptive for altricial young, as it can influence begging rate or alternatively reduce activity in the nest to conserve energy when food abundance is low (Kitaysky 2003). Furthermore, it is important to investigate the conditions that may result in elevated baseline CORT in developing offspring, because CORT concentrations experienced during development can have long lasting repercussions, affecting future survival, behaviour and the stress-response (Monaghan 2008; Spencer *et al.* 2010).

Despite mounting evidence that foraging conditions are linked to CORT concentrations in both adult and nestling birds, few studies have focused on woodland birds (but see Pravosudov *et al.* 2001; Suorsa *et al.* 2003). In addition, few studies have employed multiple variables and years to investigate the influence of foraging conditions upon baseline CORT (but see Doody *et al.* 2008). However, this approach may be insightful as in reality individuals are influenced by multiple factors, which may affect stress hormones in an additive, synergistic or antagonistic manner. In addition, foraging conditions often vary between years (Perrins 1991; Visser, Holleman & Gienapp 2005), therefore studying multiple years may reveal the relative importance of different environmental factors. There is also a lack of information regarding the physiological effects of poor foraging conditions at the individual or territory scale (but see Suorsa *et al.* 2003). The majority of studies compare stress hormones between birds breeding in contrasting habitats or populations (Bonier *et al.* 2006; Kitaysky, Piatt & Wingfield 2007; Müller *et al.* 2007), but within habitats there is often both spatial and temporal variation in environmental quality (Wilkin, King & Sheldon 2009).

I aimed to address these knowledge gaps by investigating the influence of foraging conditions upon baseline CORT concentrations in adult and nestling blue tits, *Cyanistes caeruleus* over three years (2008-2010). Blue tits predominately feed Lepidopteran larvae to their young, which are found at the highest densities on the foliage of oak trees, *Quercus* spp. (Perrins 1991). Caterpillar availability varies across the breeding season, with peak abundance persisting for only a short period (Perrins 1991). Thus birds that breed asynchronously with the caterpillar peak can suffer negative consequences for reproductive success, and may experience greater workloads and provision smaller quantities of food to offspring than synchronous birds (Tinbergen & Dietz 1994; Van Noordwijk, McCleery & Perrins 1995; Naef-Daenzer & Keller 1999; Naef-Daenzer, Naef-Daenzer & Nager 2000; Visser & Holleman 2001). The density of oak trees within territories can also influence the abundance of caterpillars available to provisioning adults. For example, in the closely related great tit, *Parus major* provisioning effort is strongly influenced by the distribution and density of oak foliage surrounding the nest (Hinsley *et al.* 2008). When there is only a short window of plentiful food for parents to provision their young, the prevalent weather conditions such as persistent rainfall could significantly reduce foraging ability (Geiser, Arlettaz & Schaub 2008). In addition, variation in temperature can influence both thermo-regulation costs of adults and nestlings, but also caterpillar activity (Tinbergen & Dietz 1994) that may influence their detectability for foraging birds.

Evidently both the abundance of prey items and the effort required to obtain them is influenced by multiple factors. In this study I quantified the main factors that have been previously shown to affect foraging efficiency in breeding woodland birds at the individual level; synchrony between breeding and the caterpillar peak (Naef-Daenzer & Keller 1999; Naef-Daenzer, Naef-Daenzer & Nager 2000; Tremblay *et al.* 2003; Tremblay *et al.* 2005), territory-scale oak density (Wilkin, King & Sheldon 2009) and weather conditions (Bolger, Patten & Bostock 2005; Geiser, Arlettaz & Schaub 2008). The aims of this study were three-fold; firstly, to quantify inter-year variation in both foraging conditions and baseline CORT in adult and nestling birds. Secondly, within years I wished to establish which of the foraging conditions measured explained variation in parental baseline CORT. Thirdly, identify which of the foraging conditions measured affected nestling baseline CORT.

2.3 Methods & Materials

2.3.1 Field site & nest monitoring

Blue tits breeding in nest boxes in oak-dominated woodland around Loch Lomond, Scotland (56^{o} 13′ N, 4^{o} 13′ W) were studied for three years from April to June 2008-2010. Nest boxes (*n* = 357, % occupancy 2008 = 40%, 2009 = 33% and 2010 = 27%) were monitored regularly from the onset of nest building to establish laying date and clutch size. When eggs were found to be warm and no new eggs had been layed on two consecutive visits, incubating mothers were left undisturbed for 10 days. Nests were then visited every day to establish hatching date, when >50% eggs had hatched this was considered day 1.

2.3.2 Adult blood sampling

In order to measure baseline CORT for breeding adults, birds were captured on the nest by blocking the entrance hole during provisioning on day 5-7 after chicks hatched. Adults were captured between 8:00-20:00. The majority of birds entered the nest box without any sign that they were disturbed by our presence. However, when researchers had to wait any length of time for birds to enter the nest box, the duration was noted. A small blood sample was obtained (about 80–100 µl) with the aid of a standard heparinised capillary tube after puncture of the brachial vein with a 25 gauge needle. Blood samples were immediately stored on ice and separated through centrifugation within 2h of collection. The plasma portion of the sample was removed and stored at -20^{o}C until assay.

All samples were collected within three minutes of the initial blockage of the nest box entrance. CORT samples were considered to be baseline because time spent at the nest before capture, time between sampling and initial disturbance of the nest and time of day were not related to maternal CORT when controlling for year (GLMM: time of day; $t_{109} = 1.55$, $P = 0.12$, sampling time, 2.16±0.05 mins; $t_{109} = 0.90$, $P = 0.37$ and time at nest box, 2.40±0.40 mins; $t_{109} = 1.21$, $P = 0.23$). Birds were sexed based on presence or absence of a brood patch and aged based on plumage characteristics (Svensson, 1994).

2.3.3 Nestling blood sampling

Nestlings were blood sampled (as described above) on day 14 after hatching. The nest box was taken down from the tree and all nestlings were removed and placed on heat pads in a cloth bag. The time between initial disturbance of the nest and blood sampling was recorded for each chick. For this reason a maximum of two nestlings per brood were blood sampled within 3 minutes of initial disturbance of the nest, but on the majority of occasions only one nestling was sampled. CORT samples taken within three minutes were considered to be baseline because time between sampling and initial disturbance of the nest and time of day were not related to nestling CORT when controlling for year and sex (GLMM: time of day; $t_{45} = 1.54$, $P = 0.13$, sampling time; $t_{45} = 1.66$, $P = 0.20$). To identify the sex of nestlings a small portion of whole blood was put in 100% ethanol for genetic sexing. Nests were not disturbed for longer than 30 minutes.

2.3.4 Oak density

To assess habitat quality within territories, the distance to the closest oak tree was measured as caterpillars are found at highest densities on oak foliage (Wint 1983; Keller & van Noordwijk 1994; Foss & Rieske 2003). This is a time efficient method of assessing oak density and potentially foraging effort within the breeding territory for woodland birds (Wilkin, King & Sheldon 2009). A measuring tape was used to record the distance to the nearest oak tree relative to the tree the nest box was placed upon. For a sub-sample of nest boxes at the field site, I measured the number of oak trees within a 25 m radius and found density to be negatively correlated with the distance to the nearest oak tree (Pearson's Correlation; $r = -0.94$, $n = 10$, $P < 0.01$, see also Wilkin, King & Sheldon 2009). Therefore, in analyses distance to the nearest oak tree from the focal nest box was employed as a proxy measure of the oak density immediately surrounding the nest box.

2.3.5 Caterpillar abundance

To assess relative caterpillar abundance throughout the breeding period, frass fall (caterpillar droppings) was collected from April to June each year, a method that has been widely used in previous studies (Fischbacher, Naef-Daenzer & Naef-Daenzer 1998; Tremblay *et al.* 2003; Visser, Holleman & Gienapp 2005; Blondel *et al.* 2006). Caterpillars are at their highest densities in oak foliage therefore frass fall was measured from 20 mature oak trees (circumference: 1.2-1.7 m). To take account of any variation across the field site, trees selected for frass collection were distributed throughout the woodland. Two nets were placed underneath each frass tree, 1 m from the trunk on the east and west side (figure 2.2). Each frass net was 50x50 cm therefore $1m^2$ of area beneath an oak tree was sampled for frass fall. Frass nets were emptied every 3 days unless rain prevented collection, in which case they were emptied once they were dry or on the next day frass was collected. Samples were then stored at -20°C prior to analysis.

In order to establish frass fall all samples were weighed individually. Firstly, larger debris such as leaves and bark were separated from frass using a medium sieve (mesh size 1.4mm). To obtain dry mass, the remaining samples were placed in a convection oven and dried for 48 hours at 60°C. A trial using 20 frass samples dried for 24, 36, 48, and 60 hours showed that there was no significant change in the mass of the samples after 48 hours in the oven (Paired t-Test; 48 hr vs. 60 hr, $n = 20$, $t = 1.45$, $P = 0.16$). Samples were then stored in a desiccator to avoid absorption of moisture as samples cooled. Samples were sorted further with a smaller sieve (mesh size 0.67mm) to separate frass from other small pieces of debris. Frass pellets from each sample were then weighed to the nearest mg. When heavy rain had dissolved the frass pellets and reduced them to a powder, this material was weighed and included in the total weight of the frass.

To assess the synchrony between blue tit breeding and the peak in caterpillar abundance the number of days between date of maximum frass abundance (mean calculated from all trees) and the date when nestlings were 10 days old was calculated for each nest. This date was chosen because at 10 days old nestlings are growing at their fastest rate (Perrins 1991). In addition, to compare between years, dates were converted to Julian with 0 = 1st April. Figure 2.1 depicts the seasonal abundance of caterpillar frass for each year of the study. Synchrony between breeding and the peak in caterpillar abundance was used rather than absolute abundance because weather conditions can influence frass fall and collection (Fischbacher, Naef-Daenzer & Naef-Daenzer 1998). For example, frass fall was lower in 2009 than the other two years, which was the year with the most rainfall, however, the seasonal change in frass fall is still evident (see figure 2.1).

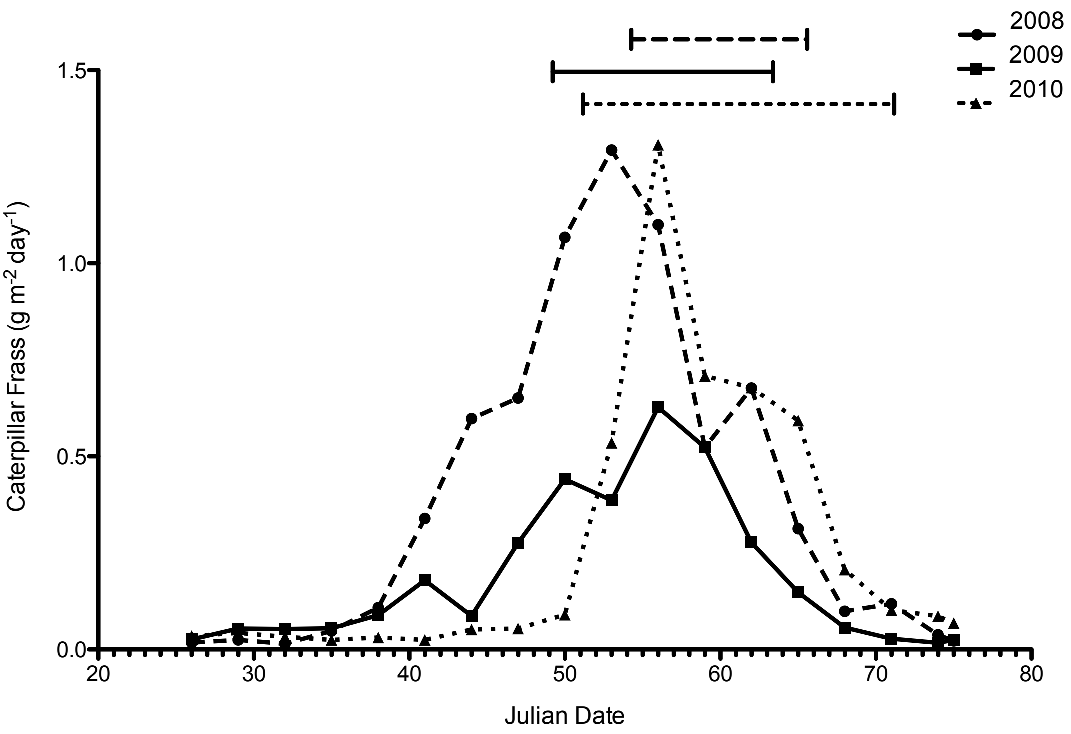


Figure 2.1. Caterpillar abundance measured by frass fall collection (g m^{-2} day^{-1}) in April to June 2008-2010 (Julian date, 1st April = 0). Horizontal lines indicate the period when blue tit nestlings were 10 days old in each year. Breeding blue tits were significantly more synchronous with the peak in caterpillar abundance in 2009 than the other two years and birds in 2010 were significantly more synchronous than 2008.

Figure 2.2. Example of frass nets used to estimate caterpillar abundance. Two were placed under each oak tree 1m from the trunk on the east and west side (20 trees in total were sampled). Frass nets were 50x50 cm therefore 1m^2 of area beneath each tree was sampled.

2.3.6 Weather variables

Weather data were collected at a meteorological station in Gartocharn (56^{o} 2′ N, 4^{o} 31′ W), less than 10 miles from the field site. The total rainfall (mm) and maximum temperature (^{o}C) were collected every 24 hrs (between 09:00-09:00) throughout the breeding season (onset of laying until all nestlings had fledged 27th April- 15th June) for all years of the study. To assess the impact of prolonged weather conditions upon adult and nestling baseline CORT a mean of the rainfall and maximum temperatures experienced 72 hr proceeding blood sampling was calculated for each individual and used in the analysis.

2.3.7 Hormone assays

Circulating corticosterone concentrations were measured using a double antibody radioimmunoassay (Wingfield, Vleck & Moore 1992). Samples were extracted from 5-20µl aliquots of plasma in diethyl ether and anti-corticosterone antiserum primary antibody (Esoterix B183), secondary antibody (Sigma goat anti-rabbit) and [3H]-corticosterone label (GE Healthcare, UK) were used. The extraction efficiency was 85–100%. Recoveries were measured for each sample independently and adjustments to the final assayed concentrations were made. CORT was measured in 6 assays for which the detection limit was 0.03 ng/ml (calculated as 2 SD from B0) and the averaged intra-and inter-assay variation was 9±6% and 10±6% respectively.

2.3.8 Molecular sex identification

DNA was extracted from blood samples using both a salt extraction based upon the methods used in Nicholls *et al.* (2000) or Qiagen DNeasy kits. Primers P2/P8 were employed to identify sex of nestlings (Griffiths *et al.* 1998a). PCR amplification was carried out in a total volume of 10 μl. The final reaction conditions were as follows: 0.8μM of each primer, 200 μM of each dNTP, target DNA, 0.35 units GoTaq polymerase (Promega), 2μM (5x) GoTaq Flexi Buffer (Promega) and 2μM of 25mM MgCl2. Thermal cycling was carried out in a Biometra UnoII: 94°C/2 min, 30 cycles of (49°C/40 s, 72°C/40 s and 94°C/30 s

49°C/1 min, 72°/5 min. PCR products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide.

2.3.9 Statistical Analysis

To investigate how synchrony with the peak in caterpillar abundance, oak density, rainfall and temperature differed between years Kruskal-Wallis tests were employed. This non-parametric test was used because data were not normally distributed and were resilient to transformation. In addition, a Generalized Linear Model with a binomial error structure was employed to assess the difference in the number of rain days between years (raining = 1 or not = 0).

All CORT data were square root transformed because of non-normality. General Linear Mixed Models (GLMMs) were employed to assess the effect of year and sex upon adult and nestling baseline CORT, with CORT concentrations as the dependent variable in all models. This is because some adult birds were sampled in more than one year ($n = 5$) therefore to avoid pseudo-replication ring number was used as a random factor within models. Mated pairs were not blood sampled for CORT concentrations therefore only one individual from each pair was included in analysis. For nestling models, GLMMs were used because more than one nestling was sampled from some broods ($n = 6$) within years therefore brood ID was used as a random factor.

General Linear Models (GLMs) were employed to assess the effect of environmental conditions upon adult baseline CORT within years, with CORT concentrations as the dependent variable. GLMMs were employed to assess the affect of environmental conditions upon nestling baseline CORT, with brood ID used as a random factor; CORT concentration was also the dependent variable in nestling models. As maximum temperature and rainfall were significantly correlated (Spearman’s Rho: $r = -0.38$, $n = 150$, $P < 0.001$), models were run twice, once with rainfall and once with temperature. Due to the lack of degrees of freedom in nestling models, interactions could not be investigated.

Models were optimised using backward elimination of non-significant terms when this improved the AIC (Akaike Information Criteria; Burnham & Anderson 2002). Model validations were applied and the underlying statistical assumptions of normality and homogeneity of variance were verified. All statistical analyses were conducted using R version 2.8.0.

2.4 Results

2.4.1 Inter-annual variation in foraging conditions

The environment that breeding birds experienced differed between years in respect to the synchrony between breeding and the peak in caterpillar abundance and weather conditions. In 2009 breeding birds were more synchronous with the peak in caterpillar abundance than the other two years, and in 2010 breeding birds were more synchronous than in 2008 (figure 2.3a, $H_{2, 325} = 155.73$, $P < 0.001$). In 2009, median asynchrony with the caterpillar food peak was 2 days, but it was 8 and 5 days in 2008 and 2010, respectively. Oak density, measured by distance to the nearest oak tree from each occupied nest box, did not differ between years (figure 2.3b, $H_{2, 244} = 2.16$, $P = 0.34$). During the breeding season in 2009 there was significantly more rainfall and rain days than in the other two years of the study (Rainfall: $H_{2, 148} = 7.10$, $P = 0.03$, Rain days: $t_{148} = 2.16$, $P = 0.03$, see figure 2.4a). For example, it rained on 62% of days in 2009 compared with 40% and 50% of days in 2008 and 2010 respectively. In addition, in 2008 maximum temperatures were warmer than the other two years by about 2^{o}C (see Figure 2.4b, $H_{2, 148} = 6.29$, $P = 0.04$).

2.4.2 Inter-annual variation in CORT concentrations

Adult baseline CORT was found to differ significantly between years. In 2010, adult baseline CORT was significantly lower than the other two years of the study (see figure 2.5a, $t_{111} = 4.51$, $P < 0.001$). In addition, male baseline CORT was significantly lower than female baseline CORT in all years of the study (see figure 2.5a, Sex; $t_{111} = 2.43$, $P = 0.02$, Sex x Year; $t_{111} = 0.11$, $P = 0.91$).

Nestling baseline CORT also differed significantly between years. Similar to adult CORT, nestling baseline CORT was significantly lower in 2010, but also in 2009 when compared with 2008 (see figure 2.5b, 2009; $t_{47} = 2.36$, $P = 0.02$ and 2010; $t_{47} = 4.81$, $P < 0.001$). There was also a trend that nestling baseline CORT was lower in male nestlings when compared with female nestlings (see figure 2.5b, Sex; $t_{47} = 2.04$, $P = 0.10$, Sex x Year; $t_{45} = 0.05$, $P = 0.96$).

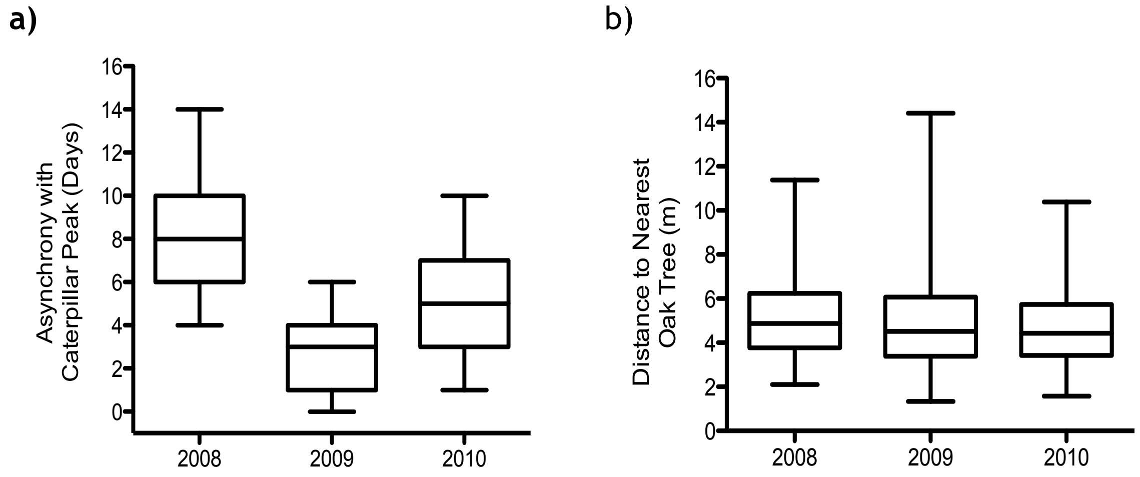


Figure 2.3. Box-and-whisker plots (showing median and interquartile range) of inter-annual variation in a) asynchrony between 10 day old blue tit nestlings and the peak in caterpillar abundance measured by frass fall, 0 days = completely synchronous, *n* = 327, and b) distance to the nearest oak tree from each occupied nest box, *n* = 346.

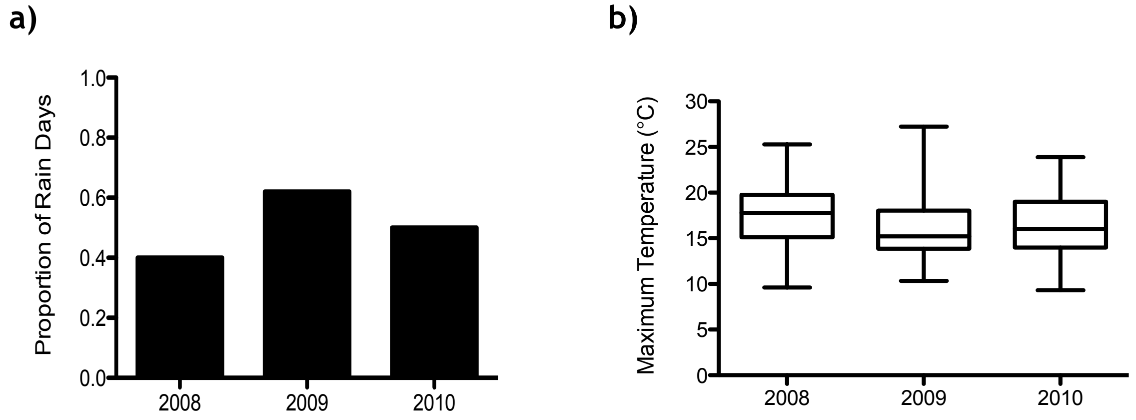


Figure 2.4. An inter-annual comparison of a) the proportion of rain days and b) box-and-whisker plots (showing median and interquartile range) of maximum daily temperature (°C). Weather variables were measured during the blue tit breeding season, onset of laying 27th April until all chicks had fledged 15th June (2008-2010).

2.4.3 Influence of foraging conditions on CORT concentrations within years

2.4.3.1 Adults

The years were analysed separately as CORT concentrations and the foraging conditions measured showed significant inter-annual variation. The environmental factors that explained the variance in adult baseline CORT differed between years. In 2008, adult baseline CORT was negatively correlated with synchrony with the caterpillar food peak. In addition, adult birds that were breeding in oak-dense territories and that were more synchronous with the caterpillar food peak had lower baseline CORT concentrations (see table 2.1 & figure 2.6a). The mean temperature experienced 72hr prior to blood sampling was also related to baseline CORT concentrations, with lower temperatures associated with higher baseline CORT (see table 2.1). Rainfall was not significantly related to baseline CORT concentrations in 2008 (see table 2.1). All interactions with sex were non-significant (see table 2.1).

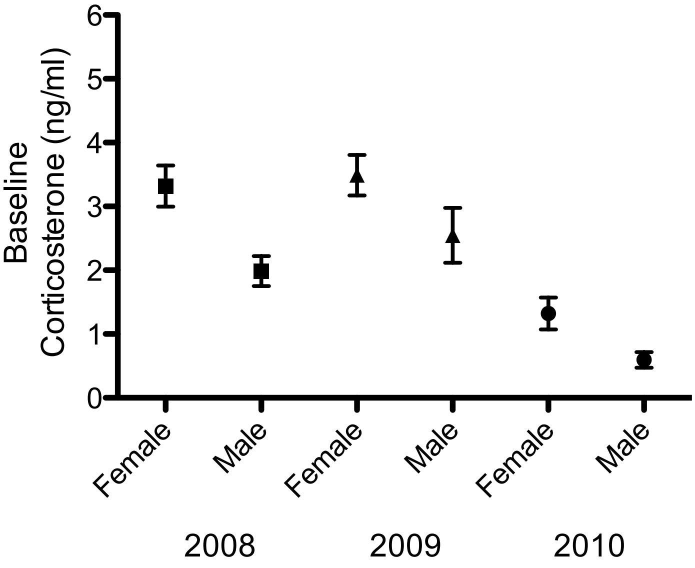
In 2009, adult baseline CORT was not related to oak density or synchrony with the caterpillar food peak (see table 2.1 & figure 2.6b). However, rainfall was related to adult baseline CORT in 2009; individuals that experienced more rainfall in the proceeding 72 hr had higher baseline CORT than those who experienced drier conditions (see table 2.1). There was a trend that temperature was negatively related to baseline CORT concentrations in 2009 (see table 1). All interactions with sex were non-significant (see table 2.1).

In 2010, the only environmental factor that significantly influenced adult baseline CORT was oak density. Similar to 2008, adults breeding in a territory with higher oak density had lower baseline CORT (see table 2.1 & figure 2.6c). Synchrony with the caterpillar food peak and weather variables were not significantly related to baseline CORT in 2010 (see table 2.1). All interactions with sex were non-significant (see table 2.1).

2.4.3.2 Nestlings

When the years were analysed separately the environmental factors that explained the variance in nestling baseline CORT also differed between years. In 2008, synchrony with the caterpillar food peak was significantly related to nestling baseline CORT (see table 2.2 & figure 2.7a). Nestlings from broods that were more synchronous with the caterpillar food peak had lower baseline CORT than more asynchronous broods. In 2008, oak density and weather variables were not related to nestling baseline CORT (see table 2.2). In 2009 and 2010 none of the environmental factors considered were associated with nestling baseline CORT (see table 2.2).

a)



b)

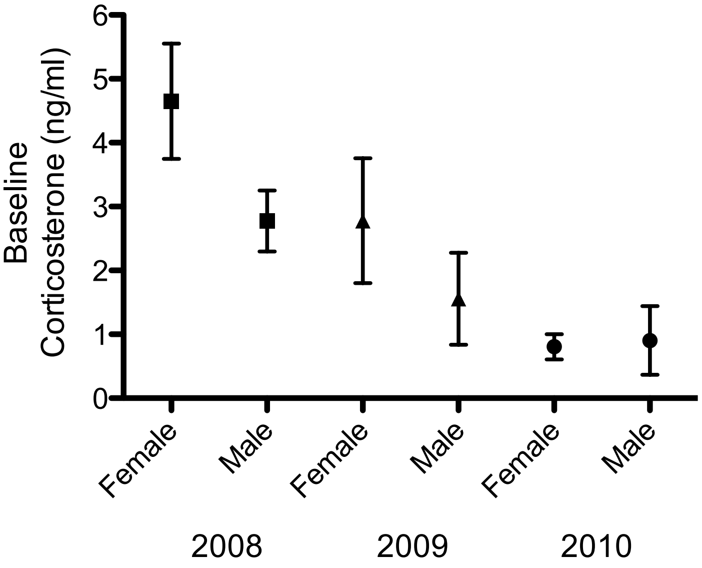


Figure 2.5. a) Baseline CORT in breeding adult blue tits differed between the sexes and years (*n* = 2008: M: 14 F: 34, 2009: M: 11, F: 33 and 2010: M: 6, F: 15). Females consistently had significantly higher baseline CORT than males. Baseline CORT was also significantly lower in 2010 than the other two years of the study. b) Baseline CORT in nestling blue tits differed between years (*n* = 2008: M: 15, F: 8, 2009: M: 8, F: 5 and 2010: M: 3, F: 11). Nestling baseline CORT was significantly lower in 2010 and 2009 when compared to 2008 and there was a trend that male nestlings had lower baseline CORT than females. Graph shows mean±SE.

Table 2.1. The results of General Linear Models assessing the determinants of adult baseline CORT in blue tits during the breeding season for three years (2008; n = 48, 2009; *n* = 44, 2010; *n* = 21). Factors in bold were significant, denotes borderline significance at *P*<0.06, *denotes significance at *P*<0.05 and ** denotes significance at *P*<0.01. Models were optimized using backward elimination of non-significant terms if this improved the AIC. Due to collinearity of the weather variables, rainfall and temperature were analysed in separate models. The model outputs for rainfall and rainfall x sex alone were from models that included rainfall; all other values are from models that included temperature. Terms that were removed from final models are denoted by rm.

Dependent/ Independent factor	**2008**					**2009**					**2010**				
Adult Baseline CORT	**SE**	***t***	***d.f.***	***P***		**SE**	***t***	***d.f.***	***P***		**SE**	***t***	***d.f.***	***P***	
Constant	1.086	3.512	44	0.001		1.667	3.285	41	0.002		0.176	3.415	18	0.003	
Sex	**0.141**	**-2.977**	**44**	**0.005****		**0.174**	**-2.124**	**41**	**0.040***		**0.132**	**-2.023**	**18**	**0.059**	
Oak Density	**0.027**	**2.922**	**44**	**0.006****		0.025	1.422	41	0.162		**0.035**	**2.596**	**18**	**0.019***	
Synchrony	**0.035**	**2.241**	**44**	**0.030***		0.049	0.491	40	0.626	rm	0.032	-0.357	16	0.726	rm
Temperature	**0.072**	**-2.461**	**44**	**0.018***		**0.108**	**-2.367**	**40**	**0.023***		0.025	-1.293	16	0.215	rm
Rainfall	0.085	1.115	44	0.271	rm	**0.040**	**2.172**	**40**	**0.036***		0.107	0.671	16	0.512	rm
Oak Density x Sex	0.062	-0.532	41	0.598	rm	0.069	1.043	37	0.304	rm	0.445	-1.127	13	0.282	rm
Synchrony x Sex	0.088	0.295	41	0.399	rm	0.128	-0.082	37	0.935	rm	0.160	-1.318	13	0.212	rm
Temperature x Sex	0.295	0.666	41	0.509	rm	0.407	-0.165	37	0.869	rm	0.208	1.063	13	0.349	rm
Rainfall x Sex	0.185	-0.305	41	0.762	rm	0.255	0.371	37	0.713	rm	0.349	-0.974	13	0.775	rm

Table 2.2. The results of General Linear Mixed Models (2008 & 2010) and General Linear Models (2009) assessing the determinants of nestling baseline CORT in blue tits from a three year study (2008; n = 23, 2009; *n* = 13, 2010; *n* = 14). General Linear Mixed Models were used in 2008 and 2010 because there were multiple nestlings sampled form the same brood (random factor = brood ID). General Linear Models were used in 2009 because only one nestling from each brood was included in the analysis. Factors in bold are significant, *denotes significance at *P*<0.05. Due to collinearity of the weather variables, rainfall and temperature were analysed in separate models. The model outputs for rainfall and rainfall x sex alone were from models that included rainfall; all other values are from models that included temperature. Terms that were removed from final models are denoted by rm.

Dependent/ Independent factor		**2008**						**2009**						**2010**	
Nestling Baseline CORT	**SE**	***t***	***d.f.***	***P***		**SE**	***t***	***d.f.***	***P***		**SE**	***t***	***d.f.***	***P***
Constant	1.060	2.466	21	0.024		1.482	3.677	11	0.010		1.773	2.070	10	0.072
Sex	-0.554	-2.281	21	0.150		-0.907	-2.085	11	0.082		0.017	0.076	10	0.951
Oak Density	0.002	0.039	19	0.970	rm	0.033	1.387	11	0.215		-0.007	-0.811	10	0.441
Synchrony	**0.124**	**2.670**	**21**	**0.015***		0.201	0.907	9	0.416	rm	0.005	0.099	10	0.923
Temperature	0.057	0.452	19	0.678	rm	0.087	0.656	9	0.548	rm	-0.047	-1.093	10	0.306
Rain	-0.100	-1.081	19	0.295	rm	-3.635	-1.053	9	0.352	rm	0.074	1.152	10	0.283

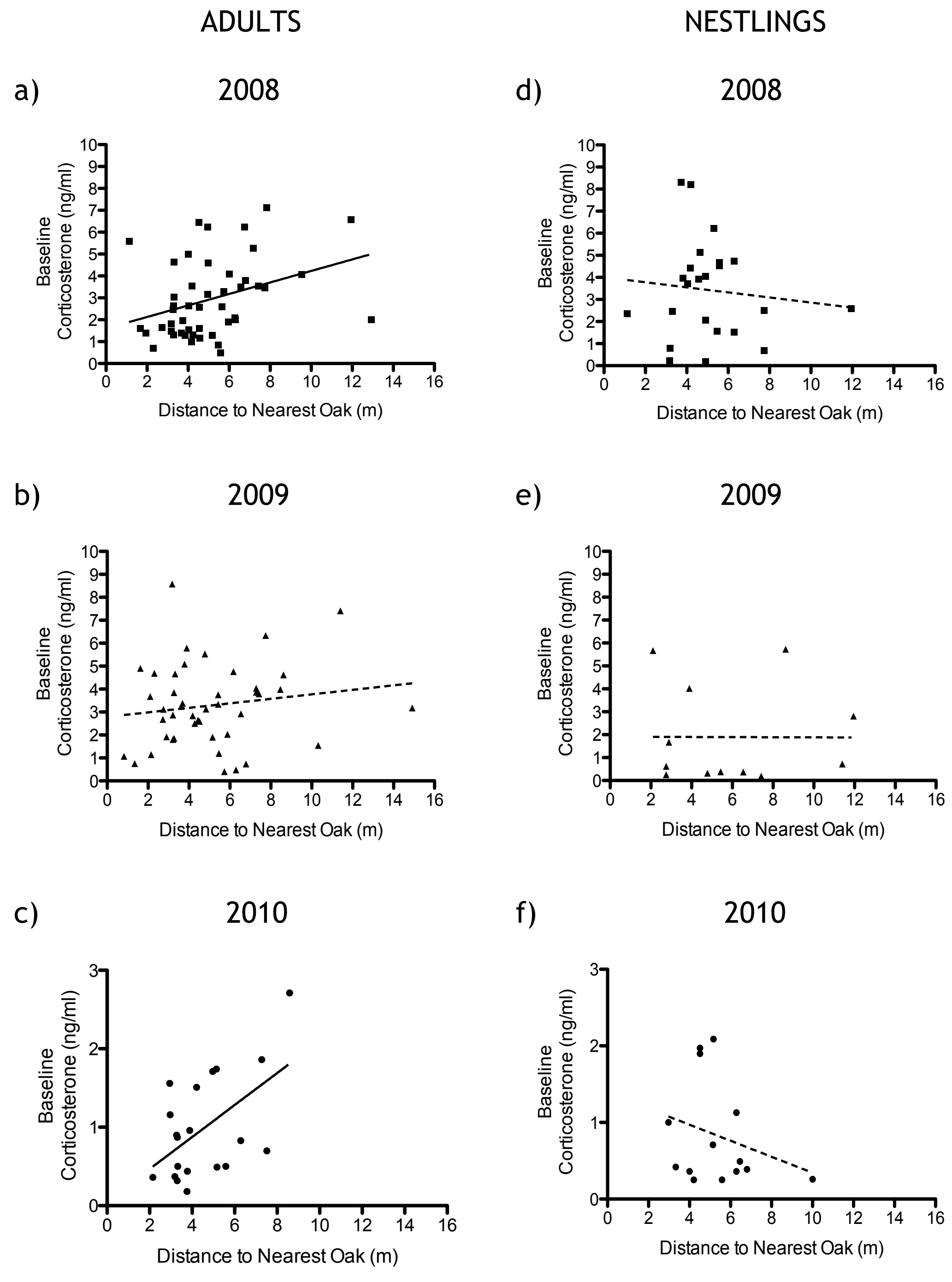


Figure 2.6. Relationship between adult a) 2008, (*n* = 48), b) 2009, (*n* = 44) and c) 2010, (*n* = 21) and nestling c) 2008 (n = 23), d) 2009, (*n* = 13) and e) 2010, (*n* = 14) baseline CORT (ng/ml) and density of oak trees immediately surrounding the nest box in blue tits over three years. Solid lines indicate significant correlations and dashed lines indicate non-significant correlations. Note the different scales for baseline CORT between years.

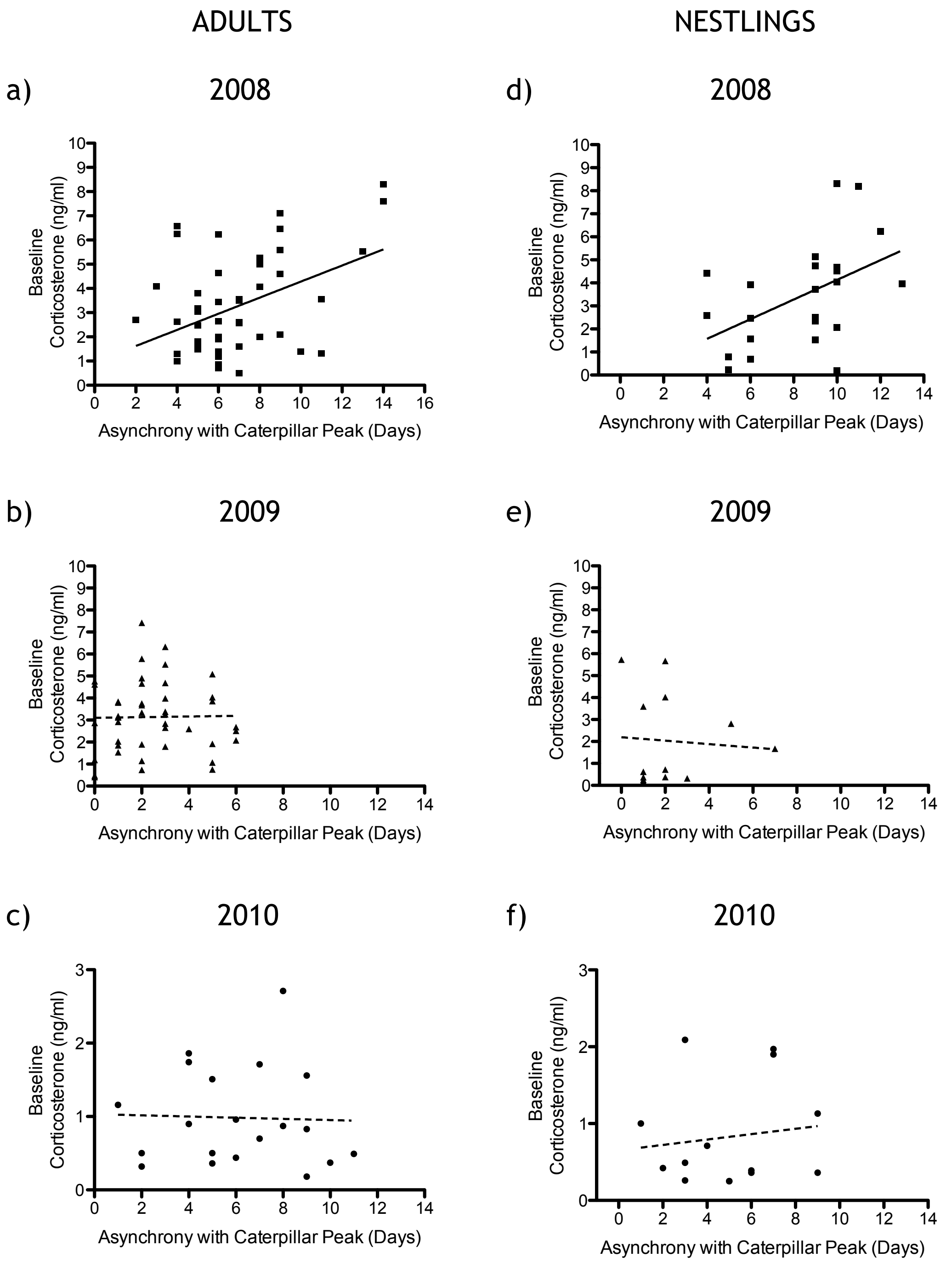


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Figure 2.7. Relationship between adult a) 2008, (*n* = 48), b) 2009, (*n* = 44) and c) 2010, (*n* = 21) and nestling c) 2008 (n = 23), d) 2009, (*n* = 13) and e) 2010, (*n* = 14) baseline CORT (ng/ml) and synchrony with the caterpillar food peak over three years in blue tits. Solid lines indicate significant correlations and dashed lines indicate non-significant correlations. Asynchrony was measured by the number of days between the date nestlings were 10 days old and the peak date of caterpillar abundance, 0 = complete synchrony. Note the different scales for baseline CORT between years.

2.5 Discussion

My results suggest that variation in adult and nestling baseline CORT is associated with the prevailing foraging conditions experienced by breeding birds. At the population level baseline CORT in adult and nestling birds did not show the same pattern across years. In 2008, when breeding birds were most asynchronous with the caterpillar peak, baseline CORT concentrations were elevated in both adults and nestlings (figure 2.5a & b). However, in 2009 when birds experienced the wettest conditions, adults but not nestlings showed elevated baseline CORT (figure 2.5a & b). Synchrony between breeding and the peak in caterpillar abundance was not an important determinant of baseline CORT for either nestling or adult birds, except in 2008 the most asynchronous year of the study. Oak density was negatively correlated with adult baseline CORT in 2008 and 2010, the two years birds were most asynchronous, but was not related to nestling CORT in any year. Weather variables were also related to baseline CORT in adults only, with rainfall positively correlated with baseline CORT in 2009, the year characterised by the most rainfall. Also in 2008 and 2009, when birds were most asynchronous and experienced the most rainfall respectively, temperature was negatively correlated with adult baseline CORT. These results suggest that some aspects of the foraging conditions measured have an influence upon adult rather than nestling baseline CORT. Furthermore, foraging conditions are associated with baseline CORT concentrations only when conditions are more demanding, suggesting that there is a threshold level over which asynchrony, oak density, temperature and rainfall or a combination of these factors elicit a physiological response in breeding blue tits.

Asynchrony between breeding birds and the peak abundance of their caterpillar prey can increase the energetic demands of provisioning parents and their growing nestlings, through reduced foraging efficiency and provisioning (Tremblay *et al.* 2003; Tremblay *et al.* 2005; Visser, Holleman & Gienapp 2005). Therefore it is likely to increase physiological stress in adult birds through increased work rate and possibly through increased food deprivation in nestling birds. The results from 2008 would indeed support this hypothesis because a significant negative relationship was found between synchrony between breeding and peak caterpillar abundance and baseline CORT in both adult and nestling birds. The absence of this relationship in the other two years indicates that birds respond physiologically to asynchrony dependent upon its severity. A similar effect has been found in seabirds, where foraging effort in the common murre was positively correlated with baseline CORT only in a year of low prey abundance (Doody *et al.* 2008).

Recent studies have documented the consequences of physiological stress during development in birds, presenting evidence that dietary restrictions are associated with elevated CORT and begging rate in nestlings (Kitaysky *et al.* 2001; Spencer *et al.* 2003; Williams, Kitaysky & Buck 2008). Elevated baseline CORT could be beneficial for nestlings receiving fewer food items, by increasing begging activity to stimulate parental provisioning, or alternatively through reducing activity to conserve energy (Kitaysky 2003). However, physiological stress during the nestling phase can also have negative long-term consequences for behaviour and cognition after fledging (Kitaysky *et al.* 2001; Spencer *et al.* 2003). Therefore, high levels of CORT during the nestling phase associated with asynchrony with the caterpillar peak could have long lasting effects upon cognition and survival for nestling blue tits.

There is evidence that climate change has the potential to disrupt synchrony between insectivorous woodland birds and their prey, as they respond at divergent rates to increasing temperatures (Visser, Holleman & Gienapp 2005; Both *et al.* 2009). As my results indicate that asynchrony between breeding and the caterpillar peak is associated with elevated baseline CORT, climate change could increase physiological stress in breeding birds over the coming years. Although modulation of CORT concentrations can allow animals to alter their physiology and behaviour in an adaptive manner, chronic stress can be associated with reduced survival (Goutte *et al.* 2010), juvenile recruitment (Kitaysky *et al.* 2006) and reproductive success (Bonier *et al.* 2009b). Indeed, elevated physiological stress in seabird chicks due to mismatched breeding with their fish prey has been suggested as a mechanism of recent seabird declines (Kitaysky *et al.* 2006). Therefore even if asynchrony between breeding and the caterpillar peak does not influence reproductive success, it may increase circulating baseline CORT concentrations in adult and nestling birds, causing them to pay higher physiological costs. Overall, measuring baseline CORT in woodland birds may be a valid line of research when trying to understand the full effects of asynchrony with the caterpillar peak.

Importantly my results also suggest that both temperature and rainfall are associated with baseline CORT in adult blue tits. As the climate may change in respect to these variables over the coming years (Houghton *et al*. 2001), this could influence baseline CORT in breeding birds in conjunction with asynchrony between breeding and the caterpillar peak, in an additive, synergetic or antagonistic manner. For example, if breeding birds grow more asynchronous with the peak abundance of their caterpillar prey, and springs also become wetter (Osborn & Hulme 2002; Leech & Crick 2007), this may have more severe consequences for the physiological stress and reproductive success of breeding birds than asynchrony alone. However, if growing asynchrony is associated with warmer temperatures, the costs may be attenuated, as birds may require less energy for thermoregulation (Stevenson & Bryant 2000), caterpillars may increase the feeding rates (Tinbergen & Dietz 1994), grow larger at a faster rate, thus becoming easier to detect, and leaf growth may be enhanced (Myneni *et al*. 1997) extending the period caterpillars are available (Leech & Crick 2007). Overall the consequences of climate change for woodland birds, and thus the effects on baseline CORT are difficult to predict (Leech & Crick 2007).

Blue tits forage predominately within the vicinity of their nest, usually no further than 50 m from their nest (Stauss, Burkhardt & Tomiuk 2005). Therefore the density of oak trees surrounding the nest can have a significant affect upon foraging distances and hence adult workload (Tremblay *et al*. 2005; Hinsley *et al*. 2008). Specifically, oak density could impose greater physiological stress upon parents when caterpillar abundance is low, by increasing the foraging time required to collect sufficient prey items. My results support this hypothesis as oak density was negatively correlated with adult baseline CORT, only in the two most asynchronous years of the study, 2008 and 2010, suggesting these effects are additive. The absence of an effect upon nestling baseline CORT indicates that adult birds were able to alleviate the influence of oak density upon nestlings, possibly by increasing their work rate to provide a similar amount of food items to nestlings in oak-poor territories.

In 2009 birds bred most synchronously with the caterpillar peak, but in this year adult birds had significantly higher baseline CORT than 2010, a less synchronous year. Analysis of the other environmental variables indicated that rainfall was significantly higher in this year and was positively related to adult baseline CORT in 2009 alone. This may have been the cause of elevated adult baseline CORT in this year as previous studies have reported that rainfall can significantly affect foraging and reproductive success in breeding birds, through increased energetic costs of foraging and reduced ease of detecting prey (Bolger, Patten & Bostock 2005; Geiser, Arlettaz & Schaub 2008). In the present study rainfall did not influence nestling CORT in any year of the study, which again suggests that parents suffered the energetic costs associated with these inclement conditions and buffered the nestlings from these impacts. In addition, lower temperatures were also associated with higher baseline CORT in adult but not nestling birds. Blue tits are cavity-nesting birds and therefore nestlings are considerably shielded from inclement conditions. Furthermore, there is evidence from other species that parents can buffer the effects of inclement conditions upon their offspring through changes in provisioning behaviour (Kitaysky, Wingfield & Piatt 2001; Tremblay *et al.* 2005). Therefore, the differential exposure in 2009 between nestlings and adult birds to rainfall, and the potential for parental compensation regarding food intake may explain why at the population level, nestling and adult baseline CORT did not follow a similar pattern in this year.

This study presents evidence for sex differences in baseline CORT in adult but not nestling birds. In all years of the study, adult males had significantly lower baseline CORT than females. Previous studies present mixed evidence for affect of sex upon baseline CORT concentrations during breeding (Romero, Ramenofsky & Wingfield 1997b; Marra & Holberton 1998; Lormée *et al.* 2003; Bokony *et al.* 2009; Angelier *et al.* 2010). On the whole, the sex that invests more into offspring care, is expected to have an attenuated stress-response to prevent nest desertion (Bokony *et al.* 2009), but this refers to an acute stress-response as opposed to fluctuations in baseline levels. The opposite trend may be evident in baseline concentrations due to the differential workload experienced by the sexes. In blue tits, females build the nest, lay large clutches and incubate them alone, the male will provide food for his mate during incubation and then the pair will provision their brood together (Perrins 1979). Therefore, female birds would have been expected to have greater energetic demands during breeding and may have required elevated baseline CORT concentrations to maintain reproductive behaviours (Wingfiled & Sapolsky 2003). In addition, baseline CORT was measured between day 5-7 after hatching, during which time female birds continue to show brooding behaviour (Pers. Obs.) and may not be able to feed as frequently as males.

A limitation of this study was that it was not possible to sample pairs for baseline CORT analysis. Therefore I cannot state that the affect of sex upon baseline CORT is evident within breeding pairs. Male nestlings also had marginally lower baseline CORT than female nestlings, particularly in 2009 (P = 0.082), but as the sample size for nestlings was small, I therefore cannot interpret the data further. However previous studies have also failed to find evidence for sex differences in hormone concentrations at the nestling stage (Schwabl 1999; Love, Bird & Shutt 2003).

This study presents evidence for synergistic impacts of foraging conditions upon baseline CORT, e.g. lower temperatures were only associated with elevated baseline CORT in the years characterized by the greatest asynchrony or rainfall. This finding is not unprecedented; there is evidence that similar patterns exist for other physiological measures of stress. For example in humans, lipid peroxidation, the oxidative degradation of lipids by free radicals, and plasma antioxidant levels that inhibit this oxidation, are often uncorrelated in healthy individuals. Whereas, correlations are found in individuals suffering from disease or undergoing demanding exercise (for review see Dotan, Lichtenberg & Pinchuk 2004).

2.5.1 Conclusions

This study presents evidence that foraging conditions are associated with basal CORT concentrations in breeding woodland birds. Importantly this study highlights the complexity of the role of foraging conditions upon baseline CORT concentrations. Intuitively, harsher conditions may place higher energetic demands upon breeding birds, but understanding the threshold levels over which individuals experience physiological stress in response to these factors provides greater insight into how birds respond to their environment. The results also indicate that baseline CORT concentrations in nestlings are unaffected by some aspects of the foraging environment that influence adult birds, suggesting that the nest environment or parental behaviours act to buffer their influence upon offspring. This study cannot, however, assign causation to the patterns observed and experimental manipulation of foraging conditions, baseline CORT and foraging effort would greatly advance our understanding.

Chapter 3: Are glucocorticoids associated with fitness? Linking foraging conditions, corticosterone and reproductive success in the blue tit, *Cyanistes caeruleus*

3.1 Abstract

Poor environmental conditions are often associated with reduced reproductive success and elevated baseline CORT in birds. This has lead to the assumption that elevated baseline CORT should be associated with reduced fitness, which was recently coined the “CORT-fitness” Hypothesis. However, this may not follow, as modulation of CORT in the face of environmental challenges can adaptively influence physiology and behaviour to improve breeding performance and/or survival. In this study I tested the “CORT-fitness” Hypothesis using free-living blue tits, *Cyanistes caeruleus* over three years (2008-2010). To gauge environmental quality I quantified key aspects of the foraging conditions, namely, synchrony between breeding and the peak in caterpillar abundance, territory-scale oak density and weather variables. I investigated whether similar harsh foraging conditions during brood rearing were associated with elevated maternal baseline CORT and reduced reproductive success. In turn, I considered whether maternal baseline CORT was related to indices of reproductive success consistently across years. In 2008 and 2010, the years characterised by the greatest asynchrony between breeding and the peak in caterpillar abundance, broods that were more synchronous fledged more offspring, a higher proportion of the clutch (2010: $P = 0.087$) and had heavier chicks than less synchronous broods. Whereas, there was only a trend in 2008 that synchrony with the caterpillar peak was negatively correlated with maternal baseline CORT. Oak density was not linked to indices of reproductive success, but was positively correlated with maternal baseline CORT in 2008 and 2010. In 2009, however, when birds experienced the wettest conditions, heavy rainfall and low temperatures were associated with low nestling mass and elevated maternal baseline CORT. Maternal baseline CORT was positively correlated with the number of chicks fledged, and negatively correlated with nestling mass in 2009 only. These results indicate that the foraging conditions associated with reproductive success are not consistently those linked to maternal baseline CORT. Furthermore, correlations between maternal CORT and proxies of fitness may be dependent upon the prevalent conditions. Ultimately, single year studies may not be sufficient to provide evidence that inter-individual variation in baseline CORT is linked to fitness.

3.2 Introduction

Poor environmental conditions are often associated with reduced reproductive success (Keller & Van Noordwijk 1994; Naef-Daenzer & Keller 1999; Garant *et al.* 2007; DeGabriel *et al.* 2009; Grimardias *et al.* 2010) and elevated glucocorticoids (GCs) (Marra & Holberton 1998; Kitaysky *et al.* 2001; Homan, Reed & Romero 2003; Cash & Holberton 2005; Kitaysky, Piatt & Wingfield 2007) in a variety of vertebrates. This has lead to the assumption that elevated GCs may be evident in individuals with reduced fitness (Bonier *et al.* 2009a), however, this has rarely been validated (but see Angelier *et al.* 2010; Bonier *et al.* 2009b; Cook *et al.* 2011) and is currently the subject of debate (Bonier *et al.* 2009a; Bonier *et al.* 2010; Dingemanse, Edelaar & Kempenaers 2010). Baseline concentrations of corticosterone (CORT), the main GC in birds, are primarily involved in maintaining daily homeostatic energetic balance (Harvey *et al.* 1984; Remage-Healey & Romero 2001a; Charmandari, Tsigos & Chrousos 2005). Therefore, CORT is intrinsically linked with an individual's energetic state, which in turn is often dependent upon the prevalent environmental conditions (Holberton, Parrish & Wingfield 1996; Kitaysky *et al.* 2001; Love *et al.* 2005; Kitaysky *et al.* 2006). Although CORT is commonly referred to as a "stress" hormone, the modulation of CORT concentrations enable animals to cope with and maximize fitness in the face of fluctuating and unpredictable environmental conditions (Sapolsky & Wingfield 2003; Wingfield 2005). Yet, the link between environmental conditions and circulating CORT, has lead to the hormone being increasingly used as a tool in ecology and conservation science for monitoring the health of species and populations of concern (reviewed in Busch & Hayward 2009). The assumption that harsh environmental conditions are linked to elevated baseline CORT and therefore elevated CORT is associated with reduced fitness has recently been coined as the "CORT-Fitness Hypothesis" in a review article by Bonier *et al.* (2009a). However, a review of the empirical evidence within this article and subsequent studies (Bonier *et al.* 2009b; Angelier *et al.* 2010; Dingemanse, Edelaar & Kempenaers 2010; Ouyang *et al.* 2011) suggest that there is mixed support for the hypothesis.

Due to the role of CORT in maintaining daily energy balance and its pleiotropic affects upon physiology and behaviour the hormone has been suggested to have an integral role in life-history trade-offs, and play an adaptive role in maximising reproductive output under a range of conditions (Sapolsky & Wingfield 2003). Indeed a positive relationship between baseline CORT and indices of fitness might be predicted when environmental conditions are poor (Bonier *et al.* 2009a: Sapolsky & Wingfield 2003), as baseline CORT mobilises energy stores enabling individuals to maintain reproduction in the face of inclement conditions (Sapolsky & Wingfield 2003). Furthermore, individuals with large broods might be expected to elevate baseline CORT to meet the energetic demands associated with a greater number of offspring (Bonier *et al.* 2009b; Bonier, Moore & Robertson 2011). For example, experimentally enlarging brood size in the tree swallow, *Tachycineta bicolor*, caused an increase in maternal baseline CORT relative to mothers with reduced broods (Bonier, Moore & Robertson 2011). It could also be hypothesised that such a relationship would be more apparent in short-lived species with high fecundity, because it may be prudent to invest heavily in reproduction when the likelihood of future reproduction is minimal (Wingfield & Sapolsky 2003: Bokony *et al. 2009*), and this was supported in a recent meta-analysis that showed baseline CORT is positively correlated with the value of current reproduction relative to lifetime reproductive output in birds (Bokony *et al.* 2009).

Breeding stage can also affect the relationship between baseline CORT and proxies of fitness (Bonier *et al.* 2009b; Ouyang *et al.* 2011). Studies report a negative relationship between CORT during egg-laying and reproductive success, but a positive relationship between CORT during brood rearing and reproductive success in both populations and individuals (Bonier *et al.* 2009b; Ouyang *et al.* 2011). The contrasting relationship between CORT and fitness under these circumstances may be adaptive (Bonier *et al.* 2009b), as elevated CORT at an early stage may indicate poor individual condition and therefore a high probability of reproductive failure, whereas at later stages may facilitate nestling provisioning (Bonier *et al.* 2009b). Overall, the growing knowledge of the role of CORT in avian reproduction, indicates that an assumption of a negative relationship between parental CORT and reproductive success is simplistic (Love *et al.* 2005; Breuner 2008; Love & Williams 2008b; Williams 2008; Bonier *et al.* 2009a).

Importantly, when interpreting a CORT-fitness relationship, the links between CORT, environmental conditions and fitness must first be validated. This is because under certain circumstances the variation in or severity of the environmental conditions experienced during reproduction may not have consequences for baseline CORT or indeed fitness, therefore undermining the main assumptions of the “CORT-Fitness Hypothesis”. In this case, contrasting conditions between years or habitats, may cause the relationship between CORT and fitness to differ. For example, in an island population of blue tits, *Cyanistes caerulus*, where provisioning was more challenging than the mainland, baseline CORT was positively correlated with brood size, whereas this relationship was not evident in a mainland population (Müller *et al.* 2007).

The aim of this study was to investigate whether inclement foraging conditions, specifically those experienced during brood rearing were associated with reduced fitness and/or elevated baseline CORT consistently over three years (2008-2010). In turn, I considered whether maternal baseline CORT is consistently linked to indices of fitness, specifically reproductive success over multiple years. A free-living population of blue tits was studied as there is evidence that both foraging conditions (Chapter 2) and reproductive success (Müller *et al.* 2007) are associated with baseline CORT concentrations in this species. To quantify foraging conditions during brood rearing, synchrony between breeding and the peak in caterpillar abundance and territory-scale oak density, which influence both reproductive success and foraging efficiency (Naef-Daenzer & Keller 1999; Tremblay *et al.* 2005) was measured. To assess fitness, fledging success, proportion of the clutch to fledge and mean nestling mass on day 14 after hatching was recorded. To control for effects of breeding stage, maternal baseline CORT was measured at the same point during brood rearing across all years.

3.3 Methods & Materials

3.3.1 Field site & reproductive success

Blue tits breeding in nest boxes in oak-dominated woodland around Loch Lomond, Scotland (56° 13′ N, 4° 13′ W) were studied for three years from April to

June 2008-2010. Nest boxes (n = 357, % occupancy 2008 = 40%, 2009 = 33% and 2010 = 27%) were monitored regularly from the onset of nest building to establish laying date, clutch size, hatching date and number fledged. In addition, nest boxes were visited after fledging to check for unfledged offspring and establish fledging success. Proportion fledged was calculated as the proportion of the clutch that fledged. To measure nestling condition, nestlings were weighed to the nearest 0.01g with a digital balance on day 14 after hatching. In addition, on day 14 chicks were fitted with a uniquely numbered aluminium ring (British Trust for Ornithology).

3.3.2 Maternal baseline CORT

In order to measure maternal baseline CORT, birds were captured on the nest during brood provisioning on day 5-7 after hatching. All adults were captured between 08:00-20:00. The majority of birds entered the nest box without any sign that they were disturbed by human presence. However, when researchers had to wait any length of time for birds to enter the nest box, the duration was noted. A small blood sample was obtained (about 80–100 µl) with the aid of a standard heparinised capillary tube after puncture of the brachial vein with a 25 gauge needle. Blood samples were immediately stored on ice and separated through centrifugation within 2h of collection. The plasma portion of the sample was removed and stored at -20^{o}C until assay.

All blood samples were collected within three minutes of the initial blockage of the nest box entrance. CORT samples were considered to be baseline because time spent by researchers at the nest before capture, time between sampling and initial disturbance of the nest and time of day were not related to plasma CORT (GLM: time at nest; t_{84} = 1.56, P = 0.12, sampling time; t_{84} = 0.67, P = 0.51 and time of day; t_{84} = 1.52, P = 0.13). Birds were sexed based on presence or absence of a brood patch (Svensson 1992).

3.3.3 Foraging conditions

The foraging conditions experienced by breeding birds were assessed by 1) the synchrony between breeding and the peak in caterpillar abundance, 2) the density of oak trees immediately surrounding the nest and 3) the weather conditions.

1) Caterpillar abundance was calculated by the collection of frass (caterpillar droppings) from April to June each year. This method has been widely used in previous studies (Fischbacher, Naef-Daenzer & Naef-Daenzer 1998; Tremblay *et al.* 2003; Visser, Holleman & Gienapp 2005; Blondel *et al.* 2006). Caterpillars are at their highest densities in oak foliage therefore frass fall was collected from 20 mature oak trees and assessed by measuring the dry weight (see Chapter 2 for full methods). To calculate the synchrony between breeding blue tits and the peak in caterpillar abundance the number of days between date of maximum frass abundance (mean calculated from all trees) and the date when nestlings were 10 days old was calculated for each nest. At 10 days of age, nestlings are growing at their fastest rate (Perrins 1991), so their nutritional requirements are at their highest. To allow comparisons between years, dates were converted to Julian with 0 = 1st April.

2) The density of oak trees immediately surrounding the nest can influence the availability of caterpillars for provisioning adults and therefore the energy required to gather them (Hinsley *et al.* 2008). Within territories, a measuring tape was used to record the distance to the nearest oak tree relative to the tree the nest box was placed upon. Distance to the closest oak tree from the focal nest is correlated with oak density immediately surrounding the nest (see Chapter 2; Wilkin, King & Sheldon 2009), and was therefore used in the analysis as a proxy for oak density.

3) As both rainfall and temperature have been linked to maternal baseline CORT in blue tits (see Chapter 2), both variables were measured across years. Weather data were collected at a meteorological station in Gartocharn (56° 2′ N, 4° 31′ W), less than 10 miles from the field site. The total rainfall (mm) and maximum temperature (°C) were collected every 24 hrs (between 09:00-09:00) throughout the breeding season (onset of laying until all nestlings had fledged 27th April- 15th June) for all years. To assess the impact of prolonged weather conditions upon maternal baseline CORT a mean of the rainfall and maximum temperatures experienced 72 hr proceeding blood sampling was calculated for each individual and used in the analysis. Also, to investigate the impact of prolonged weather

conditions upon reproductive success, a mean of the rainfall and maximum temperatures experienced between day 9-11 after hatching (72 hr) was calculated for each nest and used in the analysis. The period was chosen as nestlings are growing at their fastest rate during this time (Perrins 1991), and may be most sensitive to inclement weather conditions.

3.3.4 Hormone assays

Circulating CORT concentrations were measured using a double antibody radioimmunoassay (Wingfield, Vleck & Moore 1992). Samples were extracted from 5-20µl aliquots of plasma in diethyl ether and anti-corticosterone antiserum primary antibody (Esoterix B183), secondary antibody (Sigma goat anti-rabbit) and [3H]-corticosterone label (GE Healthcare, UK) were used. The extraction efficiency was 85–100%. Recoveries were measured for each sample independently and adjustments to the final assayed concentrations were made. CORT was measured in 3 assays for which the detection limit was 0.03 ng/ml (calculated as 2 SD from B0) and the averaged intra-and inter-assay variation was 9±2% and 10±5% respectively.

3.3.5 Statistical analysis

To investigate how synchrony with the peak in caterpillar abundance, oak density, rainfall and temperature differed between years Kruskal-Wallis tests were employed. This non-parametric test was used because data were not normally distributed and were resilient to transformation. In addition, a Generalized Linear Model with a binomial error structure was employed to compare the number of rain days between years (raining = 1 or not = 0).

To assess how measures of reproductive success differed between years and how they were related to foraging conditions and maternal baseline CORT Generalized Linear Models were employed. All CORT data were square root transformed because of non-normality. Reproductive success data did not conform to the underlying assumptions of normality therefore a range of error structures were used. For the number of chicks fledged and the proportion of chicks that fledged, quasipoisson and binomial error structures were employed respectively. General Linear Models (GLMs) were employed to assess the influence of foraging conditions and maternal baseline CORT upon mean nestling mass on day 14. In all models, measures of reproductive success were dependent variables and maternal baseline CORT or the foraging conditions were explanatory variables. To assess the influence of the foraging conditions upon maternal baseline CORT GLMs were also employed with maternal CORT as the dependent variable. As maximum temperature and rainfall were significantly correlated (Spearman's Rho: $r = -0.376$, $n = 150$, $P < 0.001$), all models including these terms were run twice, once with rainfall and once with temperature. Sample sizes differ, as the number of nests where measures of reproductive success were recorded was larger than the number of nests where mothers were measured for baseline CORT.

Models were optimised using backward elimination of non-significant terms. For most models, terms were eliminated when this improved the AIC (Akaike Information Criteria; Burnham & Anderson 2002). Models assessing the determinants of number fledged, however, were compared using ANOVA, as AIC cannot be calculated when quasipoisson error structures are used. In the ANOVA comparisons, terms were dropped from the model if *P*-values were found to be non-significant (>0.05). Models were validated where appropriate to verify that underlying statistical assumptions were not violated; normality was assessed by plotting theoretical quantiles versus standardised residuals (quantile-quantile plots), homogeneity of variance was evaluated by plotting residuals versus fitted values, non-linearity was evaluated by plotting residuals versus explanatory variables, and influential data points were identified using Cook's distance (Quinn & Keough 2002). There were three data points in baseline CORT models identified as outliers and removed. All statistical analyses were conducted using R version 2.8.0.

3.4 Results

3.4.1 Inter-annual variation in foraging conditions

The foraging conditions that breeding birds experienced differed between years with respect to both the synchrony between breeding and the peak in caterpillar abundance and weather conditions. In 2009 breeding birds were more synchronous with the peak in caterpillar abundance than the other two years, and in 2010 breeding birds were more synchronous than in 2008 (table 3.1, $H_{2,\ 325} = 155.73$, $P < 0.001$). During the breeding season in 2009 there were significantly more rain days than in the other two years of the study (table 3.1, $t_{148} = 2.16$, $P = 0.03$). In addition, in 2008 maximum temperatures were warmer than the other two years (table 3.1, $H_{2,\ 148} = 6.29$ $P = 0.04$). However, oak density did not differ between years (table 3.1, $H_{2,\ 244} = 2.161$, $P = 0.34$).

Table 3.1. Inter-annual variation in synchrony between breeding birds and the caterpillar peak, territiory-scale oak density, rainfall, maximum temperature, maternal baseline CORT (*n* = 2008: 36, 2009: 36 and 2010: 18), number of chicks fledged (*n* = 2008: 129, 2009: 71 and 2010: 46) and mean nestling mass on day 14 (*n* = 2008: 85, 2009: 38 and 2010: 40) for free-living blue tits (2008-2010). Kruskal-Wallis tests were employed to assess Inter-annual variation in synchrony between breeding birds and the caterpillar peak, territiory-scale oak density, rainfall and maximum temperature. GLMs were employed to assess Inter-annual variation in maternal baseline CORT, number of chicks fledged and mean nestling mass on day 14. Mean±SE are shown, values in bold differ significantly from the other years, * denotes significance at *P*<0.05 ** denotes significance at *P*<0.001.

	2008	**2009**	**2010**
Synchrony with caterpillar peak (days)	8.28±0.25	**2.68±0.24****	**5.28±0.40**** **Compared with 2008 only**
Territory-scale oak density (m)	5.41±0.26	6.15±0.88	5.74±1.36
Rainfall (% rain days)	40	**62***	50
Temperature (°C)	**17.39±0.39***	16.16±0.42	16.42±0.47
Maternal baseline CORT (ng/ml)	3.57±0.42	3.63±0.38	**1.73±0.22****
Number fledged	7.06±0.35	**6.78±0.51****	8.02±0.54
Mean nestling mass on day 14	11.0±0.10	11.3±0.09	11.4±0.12

3.4.2 Foraging conditions and reproductive success

Reproductive success varied significantly between years, with 2009 characterised by lower reproductive success than the other two years (table 3.1, Number fledged; $z = 5.05$, $P < 0.001$, Proportion fledged; $z = 4.52$, $P < 0.001$). However, mean nestling mass on day 14 after hatching did not differ between years (table 3.1, $t_{163} = 0.36$, $P = 0.72$).

Synchrony with the caterpillar peak explained significant variation in the number of offspring fledged, proportion of the clutch to fledge and mean nestling mass on day 14 in a year-specific manner (table 3.2). In 2008 and to a slightly lesser extent 2010, broods that were more synchronous with the caterpillar peak fledged a greater number of offspring (figure 3.1a, 2008: $z = 4.28$, $P < 0.001$, 2009, $z = 0.05$, $P = 0.962$, 2010: $z = 2.81$, $P = 0.007$), proportion of the clutch (2008: $z = 3.89$, $P < 0.001$, 2009: $z = 0.04$, $P = 0.965$, 2010: $z = 1.71$, $P = 0.087$), and had heavier nestlings on day 14 (figure 3.1b, 2008: $t_{84} = 2.57$, $P = 0.012$, 2009: $t_{37} = 0.35$, $P = 0.732$, 2010: $t_{39} = 3.38$, $P = 0.002$), however, these relationships were not evident in 2009.

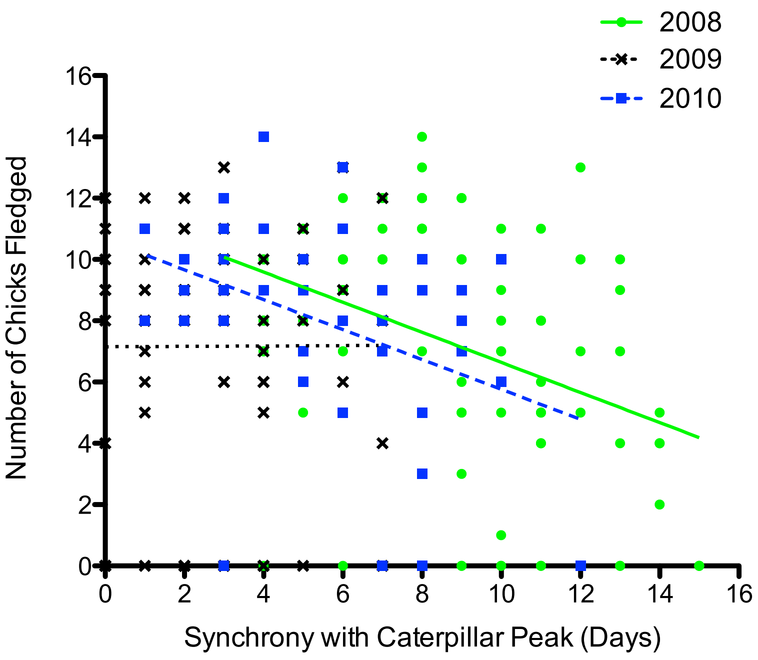
Oak density was not correlated with number fledged, proportion fledged or mean nestling mass in any year (Number fledged: 2008: $z = 0.29$, $P = 0.775$, 2009: $z = 0.52$, $P = 0.601$, 2010: $z = 0.16$, $P = 0.875$, Proportion fledged: 2008: $z = 0.27$, $P = 0.787$, 2009: $z = 0.76$, $P = 0.445$, 2010: $z = 0.28$, $P = 0.782$ and Nestling mass: 2008: $t_{84} = 0.43$, $P = 0.671$, 2009: $t_{37} = 0.33$, $P = 0.743$, 2010: $t_{39} = 0.64$, $P = 0.523$).

In all years temperature and rainfall were unrelated to number fledged (Temperature: 2008: $z = 0.73$, $P = 0.47$, 2009: $z = 1.53$, $P = 0.13$, 2010: $z = 0.64$, $P = 0.52$, Rainfall: 2008: $z = 1.46$, $P = 0.15$, 2009: $z = 1.29$, $P = 0.20$, 2010: $z = 1.05$, $P = 0.301$) and proportion of the clutch fledged (Temperature: 2008: $z = 0.98$, $P = 0.34$, 2009: $z = 1.51$ $P = 0.13$, 2010: $z = 0.80$, $P = 0.42$, Rainfall: 2008: $z = 0.51$, $P = 0.61$, 2009: $z = 0.64$, $P = 0.52$, 2010: $z = 1.20$, $P = 0.23$). In 2009 only, mean nestling mass was significantly negatively correlated with rainfall (2008: $t_{84} = 0.50$, $P = 0.62$, 2009: $t_{37} = 2.35$, $P = 0.02$, 2010: $t_{39} = 0.28$, $P = 0.78$), and in the same year there was a trend that nestling mass was positively correlated with temperature (2008: $t_{84} = 0.22$, $P = 0.82$, 2009: $t_{37} = 1.92$, $P = 0.06$, 2010: $t_{39} = 0.09$, $P = 0.93$).

Table 3.2. The table presents the significant correlates of maternal baseline CORT, number fledged, proportion of the clutch fledged and mean nestling mass on day 14 ($P < 0.05$ unless otherwise stated in brackets). **-ve** = negative correlation and **+ve** = positive correlation. General Linear Models was used to analyse the factors which explian variation in baseline CORT and mean nestling mass on day 14. Generalized Linear Models with quasipoisson and binomal error structures were used to analyse which factors explained variation in number fledged and proportion of the clutch fledged respectively.

	Explanatory variables		
	2008	**2009**	**2010**
Maternal baseline CORT	Synchrony **-ve** (0.052) Temperature **-ve** Oak density **-ve**	Temperature **-ve** Rainfall **+ve**	Oak density **-ve**
Number of chicks fledged	Synchrony **+ve**	Maternal CORT **+ve**	Synchrony **+ve**
Proportion of clutch fledged	Synchrony **+ve**		Synchrony **+ve** **(0.087)**
Mean nestling mass on day 14	Synchrony **+ve**	Temperature **+ve** **(0.063)** Rainfall **-ve** Maternal CORT **-ve**	Synchrony **+ve** Maternal CORT **-ve** **(0.080)**

a)



b)

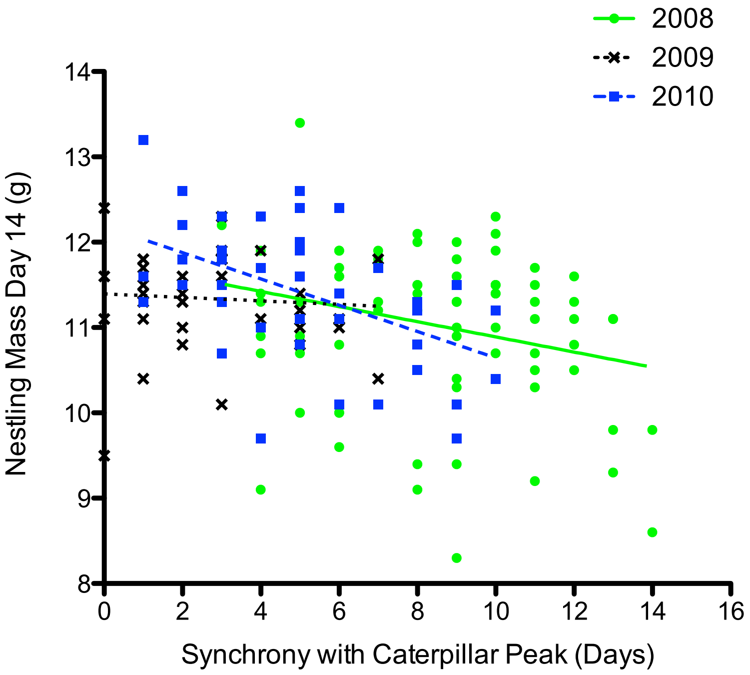


Figure 3.1. The year-specific relationships between synchrony with the caterpillar peak and a) number of blue tit chicks fledged (2008: *n* = 129, 2009: *n* = 71, 2010: *n* = 46) and b) mean nestling mass on day 14 after hatching (2008: *n* = 85, 2009: *n* = 38, 2010: *n* = 40). For both number fledged and nestling mass there was a significant negative correlation with synchrony in 2008 and 2010, but this was not evident in 2009.

3.4.3 Foraging conditions and maternal baseline CORT

Maternal baseline CORT differed significantly between years, being lower in 2010 than the other two years (table 3.1, $t_{90} = 4.08$, $P < 0.001$). Synchrony with the caterpillar peak explained variation in maternal baseline CORT in a year-specific manner. In 2008 only, there was a trend that mothers that were more synchronous with the caterpillar peak had lower baseline CORT than mothers that bred asynchronously (table 3.2, 2008: $t_{30} = 2.03$, $P = 0.05$, 2009: $t_{29} = 0.35$, $P = 0.73$ and 2010: $t_{10} = 0.21$, $P = 0.84$).

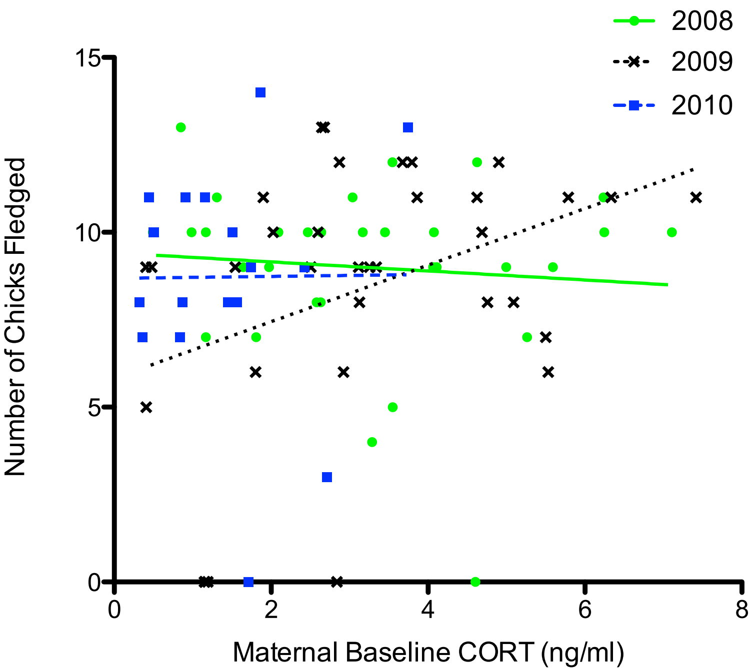
Mothers breeding in oak dense territories had significantly lower baseline CORT in 2008 and 2010 (table 3.2, 2008: $t_{30} = 2.33$, $P = 0.03$ and 2010: $t_{12} = 2.71$, $P = 0.02$) than mothers in oak sparse territories, but this was not evident in 2009 ($t_{29} = 0.16$, $P = 0.87$).

Both temperature and rainfall were related to maternal baseline CORT in a year-specific manner (table 3.2). Temperature was negatively correlated with maternal CORT in 2008 and 2009 (2008: $t_{30} = 2.35$, $P = 0.03$ and 2009: $t_{31} = 2.65$, $P = 0.013$) but not 2010 ($t_{10} = 0.28$, $P = 0.781$). Rainfall was positively correlated with maternal baseline CORT in 2009 only (2008: $t_{30} = 1.01$, $P = 0.32$, 2009: $t_{31} = 2.43$, $P = 0.02$ and 2010: $t_{10} = 0.42$, $P = 0.68$).

3.4.4 Maternal baseline CORT and reproductive success

Maternal baseline CORT explained significant variation in the number of chicks fledged in 2009 only (table 3.2 & figure 3.2a, 2008: $z = 0.80$, $P = 0.43$, 2009: $z = 2.94$, $P = 0.003$ and 2010: $z = 0.11$, $P = 0.91$). There was no relationship evident between proportion of the clutch fledged and maternal baseline CORT in any year (2008: $z = 0.72$, $P = 0.47$, 2009: $z = 1.22$, $P = 0.22$ and 2010: $z = 0.51$, $P = 0.61$). Maternal baseline CORT was negatively correlated with mean nestling mass on day 14 in 2009 and there was a similar trend in 2010 (table 3.2 & figure 3.2b, 2009: $t_{22} = 2.68$, $P = 0.01$ and 2010: $t_{15} = 1.88$, $P = 0.08$), but this was not evident in 2008 (figure 3.2b, $t_{29} = 1.42$, $P = 0.17$).

a)



b)

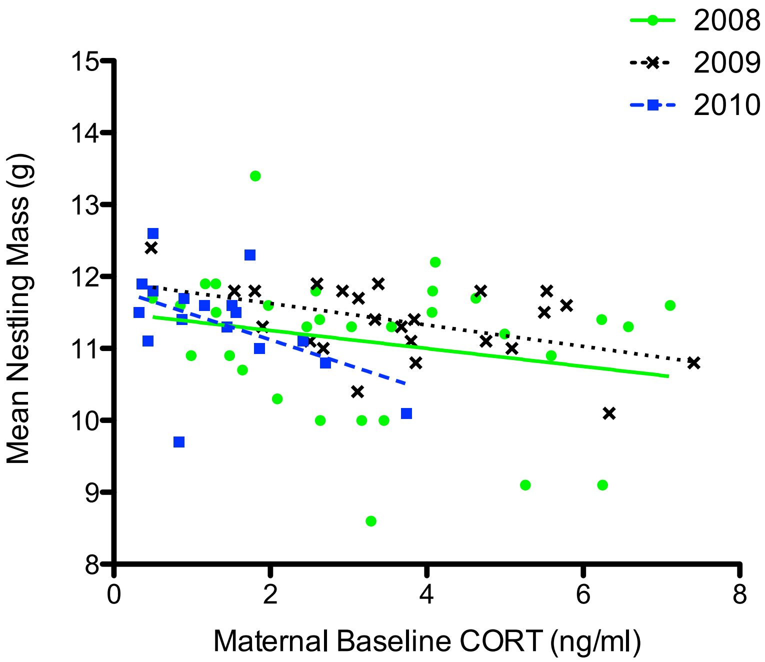


Figure 3.2. The relationship between maternal baseline CORT and a) the number of chicks fledged (2008: *n* = 36, 2009: *n* = 36, 2010: *n* = 18) and b) mean nestling mass on day 14 after hatching (2008: *n* = 30, 2009: *n* = 23, 2010: *n* = 16) in blue tits over three years (2008-2010). Maternal baseline CORT was significantly correlated with number fledged and nestling mass in 2009 only.

3.5 Discussion

The results of this study indicate that in female blue tits, the foraging conditions associated with reduced reproductive success are not consistently those linked to elevated maternal baseline CORT. For example, in 2008 and 2010, the years characterised by the greatest asynchrony between breeding and the caterpillar peak, broods that were more synchronous with the caterpillar peak fledged more offspring, a greater proportion of the clutch (marginally non-significant in 2010) and had heavier nestlings than less synchronous broods. Whereas, there was a marginally non-significant trend that synchrony with the caterpillar peak was negatively correlated with maternal baseline CORT in 2008 only. In addition, the density of oak trees within territories was not linked to indices of reproductive success, however, in 2008 and 2010 mothers breeding in oak dense territories had significantly lower baseline CORT than mothers in oak sparse territories. However, in 2009, the year characterised by the wettest conditions, nestling mass was negatively correlated with rainfall and there was a trend it was also positively correlated with temperature. In the same year, heavy rainfall and low temperatures were also associated with elevated maternal baseline CORT. The results also indicate that contrary to the CORT-Fitness hypothesis, maternal baseline CORT was positively correlated with the number of chicks fledged in 2009.

Parents may be able to buffer the effects of asynchrony with the caterpillar peak to their offspring by increasing foraging effort (Kitaysky, Wingfield & Piatt 2001; Tremblay *et al*. 2005). Therefore, up to a point, the amount of food nestlings receive may not be affected by asynchrony with the caterpillar peak, whereas, parental workload and thus baseline CORT may be elevated. This may explain why territory-scale oak density was associated with maternal baseline CORT, rather than measures of reproductive success. The number of oak trees in a territory can influence parental foraging, with fewer trees causing parents to forage further to provide a similar number of food items (Hinsley *et al*. 2008). Overall, these results suggest that maternal baseline CORT may be linked with factors that affect energetic demand rather than reproductive success. In which case, one might not expect a negative correlation between baseline CORT and indices of fitness. However, in 2009 heavy rainfall and low temperatures were associated with both low nestling mass and elevated maternal baseline CORT. An inter-year comparison indicates that rainfall was significantly highest in this year, and may have been responsible for the reduced population level reproductive success in this year (see table 3.1). Rainfall can significantly affect reproductive success and physiological stress in breeding birds by significantly reducing the ability of parents to forage and increasing the costs associated with thermo-regulation (Bolger, Patten & Bostock 2005; Geiser, Arlettaz & Schaub 2008). Importantly this result suggests there are factors that concurrently cause an elevation of maternal CORT, a reduction in nestling quality and potentially reproductive success.

Interestingly, although elevated maternal CORT was linked to harsh environmental conditions, maternal CORT was positively associated with number fledged in 2009. Functionally, elevated baseline CORT may be beneficial for breeding birds during brood rearing as baseline CORT has been associated with increased foraging duration (Kitaysky, Wingfield & Piatt 2001), foraging efficiency (Pravosudov 2003), and can promote gluconeogenesis which mobilises fat reserves for breeding behaviours (Wingfield & Romero 2001). Indeed, maternal baseline CORT has been positively correlated with brood size in a number of bird species (Kotrschal, Hirschenhauser & Moestl 1998; Silverin 1998; Love *et al*. 2004; Müller *et al*. 2007; Love & Williams 2008b), including the house sparrow, *Passer domesticus*, (Ouyang *et al*. 2011) and the tree swallow, *Tachycineta bicolor,* (Bonier *et al*. 2009b). Furthermore, experimentally increasing brood size in tree swallows (Bonier, Moore & Robertson 2011) has been associated with an elevation in baseline CORT. Importantly, in my study the relationship between maternal baseline CORT and reproductive success was only evident in one year, and this year may have been most challenging for breeding birds as population reproductive success was significantly lower in this year (see table 3.1). Overall this result indicates that the relationship between reproductive success and baseline CORT may be dependent upon the prevalent conditions, specifically, baseline CORT may only be associated with brood size when the environment is particularly challenging.

This study adds to the growing evidence that the link between baseline CORT and proxies of fitness are context-dependent. For example, the relationship between baseline CORT and fledging number differed between blue tits breeding within island and mainland populations; with a positive relationship between brood size and baseline CORT in the mainland but not the island population (Müller *et al.* 2007). Furthermore, the sex of parental birds can influence the relationship between CORT and fitness in black-browed albatrosses, *Thalassarche melanophrys* (Angelier *et al.* 2010). Accumulative reproductive success over five years was negatively related to baseline CORT in males but not in females in this species, which the authors suggest could be explained by differential breeding investment, with males investing more in chick provisioning than females (Angelier *et al.* 2010). In addition, experimental elevation of baseline CORT in female side-blotched lizards *Uta stansburiana*, differentially affected individuals dependent upon their life-history strategy, with K-strategists investing in survival rather than reproduction and r-strategists investing in reproduction at the cost of higher mortality (Lancaster *et al.* 2008). The context-dependence of these relationships highlights the importance of validating the relationship between baseline CORT and fitness before employing CORT as a proxy of population health.

Greater investment in current reproduction associated with elevated baseline CORT could reduce future survival and reproductive success of mothers (Love & Williams 2008b). For example, elevated CORT during reproduction could influence survival through effects on immune function (MacDougall-Shackleton *et al.* 2009; Goutte *et al.* 2010) and thus future reproductive success. Furthermore, when elevated baseline CORT was associated with increased fledging number, it was also linked to reduced nestling condition (see table 3.2). As nestling mass is often positively correlated with survival (Magrath 1991; Ringsby, Sæther & Solberg 1998; Smith & Bruun 1998), this suggests that although mothers fledged a greater number of offspring they may have been of reduced quality, ultimately making it difficult to establish whether mothers with elevated baseline CORT had greater reproductive success in 2009 than with mothers with lower baseline CORT. However, reduced offspring condition in response to maternal baseline CORT may not indicate a decline in fitness for offspring, as this phenotype may be adaptive under poor environmental conditions (Monaghan 2008). However, there is limited empirical evidence for this hypothesis, coined the thrifty phenotype (Bateson *et al.* 2004).

This study, therefore, highlights the importance of measuring multiple measures of fitness, and provides evidence that relating inter-individual variation in baseline CORT to reproductive success in a single year may not be indicative of individual fitness. Ideally studies should address whether CORT is linked to lifetime reproductive success or other longer-term estimates of fitness. Furthermore, CORT may also influence offspring phenotype rather than number, undermining the predicted association between elevated CORT and reduced fitness, particularly when number fledged is employed as a proxy of fitness. Maternal CORT can influence both the sex of offspring (Love *et al*. 2005; Bonier, Martin & Wingfield 2007) and/or their condition (Love *et al*. 2005; Love & Williams 2008b), which in turn can maximise reproductive output and maternal survival (Breuner 2008; Love & Williams 2008b). Therefore, offspring phenotype and individual fitness may differ between breeding adults dependent upon baseline CORT concentrations, but absolute number of offspring may not.

To date, the relationship between food abundance and stress hormones during breeding has been best described in seabirds (Kitaysky, Wingfield & Piatt 2001; Kitaysky *et al*. 2006; Benowitz-Fredericks, Shultz & Kitaysky 2008). A number of studies provide evidence that population level CORT concentrations are associated with years of low food abundance and reduced reproductive success in seabirds species, specifically the black-legged kittiwake, *Rissa tridactyla* (Kitaysky et al. 2001; Kitaysky, Wingfield & Piatt 2001; Kitaysky et al. 2006; Kitaysky, Piatt & Wingfield 2007; Benowitz-Fredericks, Shultz & Kitaysky 2008; Goutte et al. 2010). If the data in my study were analysed at the population level, the conclusions would have been different. 2008 and 2009 had significantly higher mean maternal baseline CORT than 2010, and these two years also had lower mean reproductive success compared to 2010 (see table 3.1, number fledged significantly lower in 2009 only). This result would support the association between elevated baseline CORT and low reproductive success. Therefore, my results highlight not only the complexity of linking baseline CORT to measures of fitness, but also the importance of using inter-individual variation in addition to population means to fully understand the links between baseline CORT, environmental conditions and reproductive success.

Unfortunately in this study it was not possible to get repeated measures from individuals within or across multiple years. There is evidence that individual baseline CORT concentrations are highly repeatable between years (Angelier *et al.* 2010). However for animals in variable environments the influence of the prevalent conditions upon CORT may make it difficult to assess the repeatability of baseline CORT concentrations. Indeed, there is evidence that baseline CORT is not always repeatable in vertebrates (Cockrem *et al.* 2009), which suggests that it may not be straight forward to relate baseline CORT with fitness, particularly from one sample (Bonier *et al.* 2009a). For future studies, it would be insightful to establish whether individuals differ in their baseline CORT concentrations relative to the environmental conditions and reproductive success at different stages of reproduction and years in future studies.

3.5.1 Conclusions

This study is consistent with previous findings that suggest harsh environmental conditions are associated with reduced reproductive success and elevated maternal baseline CORT (Kitaysky, Piatt & Wingfield 2007; Bonier *et al.* 2009a). However, the foraging conditions that explained variation in baseline CORT and reproductive success differed. Therefore, although harsh conditions may be linked to low reproductive success and elevated maternal baseline CORT, indices of fitness and baseline CORT may not be correlated in a predictable manner. In fact, maternal baseline CORT was positively correlated with number fledged in one year of the study. This relationship was only evident in one year, which suggests that the relationship between maternal CORT and proxies of fitness may be dependent upon the prevalent conditions. Importantly, future studies should employ inter-individual variation in baseline CORT in addition to population means to fully appreciate the links between baseline CORT, environmental conditions and reproductive success. Ultimately, single year studies are unlikely to elucidate whether inter-individual variation in baseline CORT is linked to fitness.

Chapter 4: Maternal condition but not corticosterone is linked to brood sex ratio adjustment in a passerine bird

4.1 Abstract

Sex allocation theory predicts that mothers should adjust brood sex ratio in relation to their condition to maximise fitness. In birds, evidence suggests that mothers can adjust the sex of offspring in relation to their condition. Moreover, maternal concentrations of the steroid hormone corticosterone (CORT) may play a role in this adjustment, possibly through their relationship with energy-balance. However, further research is needed to validate the link between maternal condition, maternal CORT and sex ratio adjustment in wild birds. In this study I measured maternal baseline CORT and body condition in free-living blue tits, *Cyanistes caeruleus* over three years and related these factors to brood sex ratio, nestling mass and growth. In addition, a non-invasive technique was employed to experimentally elevate maternal CORT during egg laying, and its effects upon brood sex ratio and indices of nestling condition were measured. Unlike previous studies maternal condition was not linked to maternal CORT in any year. Furthermore, maternal CORT was not correlated with brood sex ratio or nestling growth, but was negatively correlated with nestling mass in all years. In one year of the study, mothers in good body condition produced male biased broods and there was a trend that maternal condition was positively correlated with both indices of nestling condition in all years. Experimental elevation of maternal CORT did not influence brood sex ratio or indices of nestling condition. This study provides some evidence that maternal condition is linked to brood sex ratio manipulation in blue tits, but baseline CORT may not be involved in this adjustment. This may be because maternal baseline CORT was not linked to maternal condition, and thus may not be indicative of circumstances that might favour sex ratio adjustment. Fundamentally, it is unclear whether sex ratio manipulation would be beneficial for blue tits, and long-term studies that measure lifetime reproductive success are required to establish this.

4.2 Introduction

Natal conditions can affect the survival and reproductive potential of offspring in a sex-specific manner (Trivers & Willard 1973; Jones, Nakagawa & Sheldon 2009). Therefore mothers breeding in favourable conditions may gain fitness benefits from investing in the sex that will benefit most from those conditions, whereas mothers breeding in poor conditions should invest in the sex least affected (Trivers & Willard 1973; Charnov 1982). Maternal quality has been proposed to influence the direction of this investment, specifically for polygynous ungulate mammals that show high variance in male as opposed to female reproductive success (Trivers & Willard 1974; Sheldon & West 2004). In these species, producing sons when conditions would ensure that they are of superior quality could result in greater fitness benefits for mothers than the production of daughters. However, the theory can also be extended to other systems. The best-known example comes from a cooperatively breeding bird, the Seychelles warbler, *Acrocephalus sechellensis* in which one sex is highly philopatric and assists parents to raise offspring (Komdeur et al. 2002; Ewen et al. 2003). In this species mothers improve their fitness by investing in the philopatric sex when conditions are good, and alternatively producing the dispersing sex when conditions are poor. Furthermore, in size-dimorphic species where the energetic demands during postnatal growth differ between the sexes (Lessells, Mateman & Visser 1996; Korpimäki *et al.* 2000; Radford & Blakey 2000), maternal condition and/or the quality of the natal environment could also influence the fitness benefits of differential investment in the sex of offspring (Hardy 2002).

In agreement with sex allocation theory there are both experimental and correlative studies that demonstrate that brood sex ratio adjustment is associated with maternal condition in avian species (Ketterson *et al.* 1996; Nager *et al.* 1999b; Pike & Petrie 2005a; Stauss *et al.* 2005). However, the replication of results has proved difficult, with outcomes differing between years and studies (Radford & Blakey 2000; Ewen, Cassey & Moller 2004). Moreover, predicting the direction of a sex ratio bias has been problematic, with evidence of no bias or the opposing bias from that expected in empirical studies (see West

2009 for review). Overall, the variety of avian life histories, extended parental care and the array of factors that could influence the benefits of sex ratio manipulation cause the prediction of sex ratio adjustment in birds to be complex (Komdeur & Pen 2002). In this case, identifying a mechanism of sex ratio adjustment, would offer insight into the potential costs of manipulation and may improve predictions of when sex ratio adjustment is expected to occur (Pike & Petrie 2003).

In birds females are the heterogametic sex (producing Z- and W-bearing ova), therefore it has been suggested that primary sex ratio adjustment (occurring prior to laying) could be under maternal control (Oddie 1998). Baseline corticosterone (CORT) has been proposed to play a role in brood sex ratio adjustment, as the hormone is often associated with factors implicated in sex ratio adjustment, such as, food availability: (Schoech, Bowman & Reynolds 2004), mate attractiveness (Pike & Petrie 2005c; Pryke *et al.* 2011) and maternal body condition (Love *et al.* 2005; Pike & Petrie 2005c). CORT concentrations are elevated in response to internal and external challenges, to maintain homeostasis and energy-balance by modulating behaviour and physiological processes (Wingfield 2005). Due to this, elevated baseline CORT has been associated with poor body condition (Schoech, Mumme & Wingfield 1997; Kitaysky, Wingfield & Piatt 1999; Love *et al.* 2005; Pike & Petrie 2005a) and inclement environmental conditions in birds (Marra & Holberton 1998; Kitaysky *et al.* 2001; Buck, O'Reilly & Kildaw 2007; Kitaysky, Piatt & Wingfield 2007), therefore it would be expected to be associated with investment in the sex whose survival and reproductive success is least effected by poor developmental conditions (Pike & Petrie 2003; Love *et al.* 2005). In agreement with this hypothesis correlative and experimental studies have found a link between CORT and female biased brood sex ratios, in species where males are the larger sex and therefore may be more sensitive to poor natal conditions than females (Love *et al.* 2005; Pike & Petrie 2005a; Bonier, Martin & Wingfield 2007).

The mechanism by which CORT could influence the sex of offspring is currently unknown, but could potentially act at the pre- and/or post-laying stage. There is evidence that the concentration of CORT deposited in the yolk of developing oocytes, reflects the circulating CORT concentrations of laying mothers (Hayward & Wingfield 2004). The germinal disc, where sex determination occurs, is located in the layer of yolk deposited in the oocyte during the last day of vitellogenesis, with one side against the yolk layer and the other against the vitelline membrane. Therefore, this could create a hormonal gradient, and it has been suggested that this could influence segregation of the sex chromosomes during meiosis and therefore offspring sex (Rutkowska & Badyaev 2008). In addition, after sex determination yolk CORT concentrations could induce selective reabsorption of ova dependent upon sex (Pike & Petrie 2003). Alternatively, yolk CORT concentrations can influence hatching success (Saino *et al.* 2005) and nestling growth (Hayward & Wingfield 2004) and survival (Love *et al.* 2005; Cyr & Romero 2007), thus could affect brood sex ratio through early embryo death or sex-specific nestling mortality. Studies provide evidence for a correlative and causal relationship between maternal baseline CORT and brood sex ratio both at laying (primary sex ratio, Bonier, Martin & Wingfield 2007; Pike & Petrie 2005a; Gam, Mendonça & Navara 2011) and at fledging (secondary sex ratio, Love *et al.* 2005). Importantly, CORT may not act directly but rather influence other aspects of physiology. For example, high levels of glucose have been associated with differential development of male blastocysts in mammals (Cameron 2004), and circulating CORT concentrations are involved in the regulation and availability of glucose in the bloodstream (Remage-Healey & Romero 2001b). When examining the role of CORT in brood sex ratio adjustment, one must consider whether it is associated with the sex ratio at laying and/or fledging. By doing so, researchers can investigate how CORT may influence the sex of offspring and also the potential costs of this adjustment.

There have been a number of studies that present evidence of brood sex ratio adjustment in blue tits, *Cyanistes caeruleus* (Sheldon *et al.* 1999b; Griffith *et al.* 2003; Dreiss 2005; Korsten 2006; Delhey *et al.* 2007). Specifically, studies have examined whether females mated to attractive males, may increase their fitness by investing in sons rather than daughters, as sons may inherit their fathers attractiveness (Sheldon *et al.* 1999b). Although there is correlative and causal evidence of a link between paternal attractiveness and male-biased brood sex ratio in blue tits, the results have proved difficult to replicate and have varied between years (Griffith *et al.* 2003; Korsten 2006). Maternal baseline CORT could be the mechanism through which females could adjust brood sex ratio in response to male attractiveness. As maternal baseline CORT in bird species has been shown to be higher in females with unattractive mates (Pike & Petrie 2005b; Griffith, Pryke & Buttemer 2011), which in turn has been associated with brood sex ratio adjustment (Pryke *et al.* 2011). There is also evidence for assortative mating in blue tits (Hunt *et al.* 1999), thus females paired with high quality attractive partners may also be of superior quality. However, to date the relationship between maternal condition, maternal baseline CORT and brood sex ratio has not been considered in blue tits.

In this study, a free-living population of blue tits were monitored for three years to assess the relationship between maternal baseline CORT, maternal body condition and brood sex ratio. To identify the potential mechanisms of sex ratio adjustment, both primary and secondary sex ratio was established. In addition, laying gaps were recorded to identify evidence of potential reabsorption of ova, and un-hatched eggs where development was not evident were recorded. Nestling mass and growth were also measured to investigate whether maternal baseline CORT and condition had sex-specific effects on nestlings, therefore indicating the potential benefits of brood sex ratio adjustment. In the final year of the study a field-based experiment was conducted to investigate whether transient elevation of maternal CORT during egg laying influenced brood sex ratio. Sex determining meiotic division occurs 2-4 hrs before ovulation (Olsen & Fraps 1950, data from chicken), with oviposition occurring approximately 24 hrs later (Romanoff 1960; Sturkie 2000). As blue tits lay in the early hours of the morning (Perrins 1996), sex determination is expected to occur during the night, approximately 28 hrs before oviposition. Therefore, mothers were fed exogenous CORT in the evening to coincide with sex determination to establish whether maternal CORT influenced the sex ratio at laying.

The main aims of this study were; 1) to ascertain whether endogenous maternal baseline CORT and maternal body condition were correlated with primary or secondary brood sex ratio, 2) establish if maternal condition and/or baseline CORT concentrations were related to nestling mass and growth, 3) investigate whether maternal baseline CORT and body condition are linked in this species and 4) employ a non-invasive technique to elevate maternal CORT during egg laying and investigate its influence upon brood sex ratio and offspring quality.

4.3 Methods & Materials

4.3.1 Empirical study

Blue tits breeding in nest boxes in oak-dominated woodland around Loch Lomond, Scotland (56° 13′ N, 4° 13′ W) were studied for three years from April to June 2008-2010. Nest boxes were monitored regularly from the onset of nest building to establish laying date. Nests were then checked every second day and eggs were counted to establish clutch size; as blue tits lay one egg per day (Perrins 1979), this allowed laying gaps to be identified. When eggs were found to be warm and no new eggs had been laid on 2 consecutive visits, incubation was deemed to have started and mothers were left undisturbed for 10 days. Nests were then visited every day to establish hatching date, when >50% of eggs had hatched this was considered day 1. All un-hatched eggs and dead nestlings were collected for molecular sexing (see below).

To measure maternal baseline CORT, birds were caught throughout the day during provisioning (between 08:00-20:00), on day 5-7 after hatching. Mothers were captured on the nest by blocking the entrance hole, and a small blood sample was obtained (about 80–100 ul) after puncture of the brachial vein with a 25-gauge needle and with the aid of a standard heparinised capillary tube. All samples were collected within 3 minutes of initial blockage of the nest box entrance. Blood samples were immediately stored on ice and separated through centrifugation within 2h of collection. The plasma portion of the sample was removed and stored at -20°C until assay. Occasionally researchers had to wait for a short period near the nest for birds to enter the nest box, when this occurred the duration of the time at the nest was noted. CORT samples were considered to be baseline because time spent at the nest before capture, time between sampling and blockage of the nest box entrance and time of day were not related to maternal CORT (GLM: time at nest; $t_{84} = 1.56$, $P = 0.12$, sampling time; $t_{84} = 0.67$, $P = 0.51$ and time of day; $t_{84} = 1.52$, $P = 0.13$). Circulating corticosterone concentrations were measured using a double antibody radioimmunoassay (Wingfield, Vleck, Moore 1992, for full details see Chapter 2). CORT was measured in three assays for which the detection limit was 0.03 ng/ml (calculated as 2 SD from B0) and the intra-and inter-assay variation was 9±2% and 10±5%, respectively.

To establish body condition, mothers were weighed to the nearest 0.05g with a Pesola spring balance and wing length was measured. Maternal condition was established by mass (g)/ wing length (mm). This metric was chosen rather than using residuals from a linear regression because mass and wing length were not significantly correlated in any year (All three years; $t_{82} = 1.06$, $P = 0.29$; Green 2001). Wing length as opposed to tarsus length was employed because wing length has been shown to be a significantly more repeatable measure and therefore a better predictor of body size in passerines (Gosler *et al.* 1998). Parental birds were sexed based on presence/absence of a brood patch, aged based on feather characteristics (Svensson 1992) and all birds captured were fitted with a uniquely numbered aluminium ring (British Trust for Ornithology).

Baseline CORT was measured during provisioning rather than egg-laying for three reasons, i) in previous studies that have found a link between maternal baseline CORT and brood sex ratio, mothers have been blood sampled post egg-laying (see Pike & Petrie, 2005a; Bonier, Martin & Wingfield, 2007), ii) baseline CORT concentrations did not differ significantly between the breeding stages in this population (figure 4.1, sub-sample 2009, Paired *t*-Test: $t = 1.80$, $n = 14$, $P = 0.10$). It should be noted that birds were blood sampled during egg-laying or provisioning due to UK Home Office regulations that restrict blood sampling to 1% of body mass per 30 days (mass ~10 g = 100 µl). iii) It was extremely difficult to obtain blood from mothers during egg-laying; blood samples were only obtained from <30% of mothers caught on their nest. In addition, in 2009 when a sub-sample of birds were blood sampled during egg-laying all broods were depredated and consequently it was not possible to establish brood sex ratio (see Appendix III).

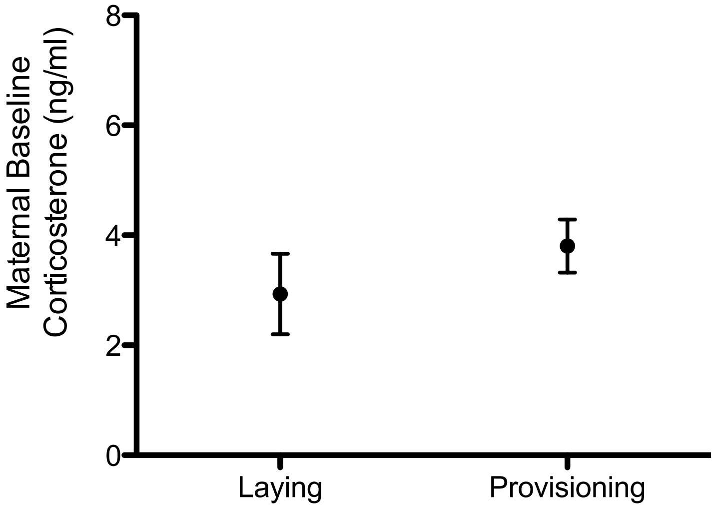


Figure 4.1. Maternal baseline plasma CORT concentrations did not differ between breeding stages in blue tits. 'Laying', includes mothers sampled on day 7 after initiation of egg laying (*n* = 7), and 'Provisioning', includes mothers sampled on day 5-7 after hatching (*n* = 7). Mothers were paired based on lay date and clutch size. Graph shows means±SE.

Figure 4.2. Picture shows an example of a plastic eyebath that was secured onto the inside of nest boxes to provide a tray for mealworms during the experimental study.

4.3.2 Experimental study

To deliver CORT non-invasively to breeding blue tits in 2010, CORT solution was injected into mealworms that were placed in nest boxes during egg-laying. Crystalline CORT (Sigma) was dissolved directly into peanut oil (Sigma, St. Louis, MO) through sonification. To ensure the CORT was dissolved evenly in the peanut oil, the solution was sonicated before each use. Mealworms (approx. 20 mm length and 0.1g) were injected with 20µl of peanut oil containing one of the following concentrations of CORT: (1) control, no CORT or (2) CORT, 0.3 mg/ml. Hence, mothers received 0 or 6µg CORT per mealworm. I injected solution into mealworms with a 25-µl Hamilton syringe using a 26-gauge ½-inch needle. Prior to injection, mealworms were kept at -20°C to reduce movement during injection. The needle was inserted ventrally, into the anterior abdomen, between two segments. If fluid leaked from the mealworm after injection, it was not used. I validated this methodology under lab conditions and found that blue tits fed 6µg CORT injected mealworms had significantly higher baseline CORT 10 minutes after ingestion than blue tits fed Control mealworms (see Appendix I, CORT: n = 4, 39.9±4.0 ng/ml, Control: n = 4, 3.1±0.95 ng/ml). However, CORT concentrations did not differ significantly between groups after 30 minutes (see Appendix I). The circulating CORT concentrations achieved after spiked mealworm consumption were within 1 SE of the mean concentrations (n = 5, 28.2±11.2 ng/ml) found in blue tits 25 minutes after subjection to a standard stressor (see Appendix I). Therefore, these results suggest CORT concentrations were elevated within the natural range for this species.

In 2010 prior to the onset of nest building, green plastic eyebaths, (figure 4.2, 3 x 1.5 x 2 cm) were secured on the inside of all nest boxes to later provide a tray for mealworms. Nest boxes were monitored weekly to establish the onset of nest building. When nests were found to be half to fully built, they were checked daily for eggs. When the first egg was laid, the nest was randomly assigned by the toss of a coin to the CORT or Control group. Beginning that day, a CORT spiked (6 µg) or Control mealworm was placed into the plastic tray every evening between 17:30 and 19:30, until no more eggs had been laid on 2 consecutive visits. This time was chosen because female blue tits have been found to roost as early as 19:00 during egg laying in Scotland (Pendlebury & Bryant 2005).

Mothers received their first mealworm to coincide with the sex determination of their third egg. The mean clutch size was 10.9±1.5, consequently on average >80% of eggs laid were manipulated.

To check that the treatment targeted female rather than male blue tits, a hide was erected close to a sub-sample of nest boxes during egg-laying (*n* = CORT: 4, Control: 3). The nests were then monitored after the mealworm was placed in the nest until sunset and then checked the following morning before 06:00. This was to record activity at the nest during this time and to establish if the mealworm had been consumed during the night. The mealworm was consumed by 06:00 for each nest box checked. On only one occasion a bird was recorded to enter the nest on more than one occasion before roosting. For all other nests only one bird was recorded to enter the nest and not leave. As female rather than male breeding blue tits roost in the nest box during laying (Pendlebury & Bryant 2005), when only one bird was seen entering and not leaving the nest before sunset, it was assumed to be the female. Therefore our observations suggest that mealworms were consumed between 19:00-06:00, and that female breeders and not their mates consumed mealworms. The progress of all manipulated nests were followed as stated in the empirical study section, and the chicks weighed and sexed as below.

4.3.3 Nestling condition

All nestlings were weighed to the nearest 0.01g with a digital balance on day 4, 6, 8, 10 and 14 after hatching. On day 4, nestlings were individually marked by colouring patches of down using non-toxic pens. On day 10, chicks were re-marked because feather growth can obscure the coloured down, this was achieved by individually blunting chicks' toenails in unique combinations. Nestling growth rate was calculated individually as mass gain day^{-1} from day 4-14 after hatching. For mothers that had been captured to measure baseline CORT and body condition, 66 broods and 608 nestlings (Broods: 2008; 26, 2009; 23 and 2010; 17) were measured for mass on day 14, however there were 59 broods and 540 nestlings measured for nestling growth over all years (Broods: 2008; 24, 2009; 20 and 2010; 15). As some mothers were measured for body condition but not baseline CORT sample sizes differ in the analysis. For mothers that were included in the experimental study (both control and treatment groups), 59 broods and 505 nestlings were measured for mass and growth rate. On day 14 chicks were fitted with a uniquely numbered aluminium ring (British Trust for Ornithology).

4.3.4 Molecular sex identification

At the age of 14 days, nestlings were blood sampled to provide DNA for molecular sex identification. All nestlings that were collected dead before this time and un-hatched eggs where development was evident were also sexed. Therefore, where possible both the primary sex ratio, i.e. the sex ratio of all eggs laid, and the secondary sex ratio, i.e. the sex ratio of all nestlings that fledged was calculated for each nest. However, for some of the un-hatched eggs collected there was no evidence of development, therefore they could not be sexed and are hereon referred to as 'unsexed eggs'. In this case, the nests were still included in primary sex ratio analysis if all the remaining offspring were sexed.

For some nests it was not possible to calculate primary sex ratio as dead nestlings or unviable eggs were lost prior to sexing, usually because the mother removed them from the nest. In this case only secondary sex ratio was calculated. Nests were not included in the analysis if any eggs were accidentally broken or molecular sexing was not successful for an individual egg or chick.

A salt extraction based upon the methods used in Nicholls *et al.* (2000) or Qiagen DNeasy kits were employed for DNA extraction. Primers were P2/P8 (Griffiths *et al.* 1998b). PCR amplification was carried out in a total volume of 10 μl. The final reaction conditions were as follows: 0.8μM of each primer, 200 μM of each dNTP, target DNA, 0.35 units GoTaq polymerase (Promega), 2μM (5x) GoTaq Flexi Buffer (Promega) and 2μM of 25mM MgCl2. Thermal cycling was carried out in a Biometra UnoII: 94°C/2 min, 30 cycles of (49°C/40 s, 72°C/40 s and 94°C/30 s) 49°C/1 min, 72°/5 min. PCR products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide.

4.3.5 Ethical note

In order to minimise disruption to parents and nestlings a number of precautions were followed. All adult birds were captured and blood sampled within 15 minutes of initial disturbance at the nest, on the majority of occasions birds were disturbed for less than 10 minutes. Furthermore, nestlings were held on heat pads to prevent them from getting chilled and never disturbed for longer than 30 minutes. During the experimental study, nest visits were carried out as quickly as possible to minimise disturbance, and because maternal CORT was elevated within the natural range recorded for this species, the physiological stress mothers were subjected to was comparable to concentrations commonly experienced by the birds.

4.3.6 Statistical analysis

Brood sex ratios were calculated by: Males / (Males + Females). Generalized Linear Models with binomial errors and no explanatory terms were used to test if brood sex ratios differed from a binomial distribution (Wilson & Hardy 2002; Korsten *et al*. 2006). In addition, to investigate whether year, maternal condition, maternal baseline CORT, lay date, and maternal age (explanatory factors) explained variation in brood sex ratio (dependent factor), Generalized Linear Models with a binomial error structure and a logit link function, weighted by brood size were used (Wilson & Hardy 2002). Overdispersion was not deemed a problem as the residual mean deviance (residual deviance / residual d.*f*.) was always less than 1.5 (Wilson & Hardy 2002). All CORT data were square root transformed because of non-normality.

Some nests contained unsexed eggs (where development was not detected) and these were included in the primary sex ratio analysis. To investigate whether this influenced the outcome of analysis, a sub-set of nests where all offspring produced had been sexed were analysed separately, hereon referred to as PrimaryC (for complete primary sex ratio). In addition, to investigate whether a sex-bias of unsexed eggs obscured evidence of a relationship between maternal CORT and/or body condition and primary sex ratio, all unsexed eggs were considered male and primary sex ratio was re-analysed. This is because in blue tits, males tend to be larger than females and may be more sensitive to poor laying conditions that result in early embryo death.

GLMs with a Poisson error structure were employed to establish whether laying gaps and the number of unsexed eggs were related to year, experimental treatment, maternal baseline CORT or maternal condition. GLMs were also used to establish whether maternal condition, lay date and age (explanatory factors) explained variance in maternal baseline CORT (dependent factor).

General Linear Mixed Models (GLMMs) were employed to assess the determinants of nestling mass on day 14 after hatching and nestling growth rate. Two models were run, and nestling mass on day 14 after hatching or nestling growth rate were the dependent factors, and year, sex, maternal baseline CORT and maternal condition were the explanatory factors. As multiple nestlings from each brood were included in the analysis, brood identity was always fitted as a random effect to avoid pseudo-replication.

For the experimental study in 2010, GLMs were used to establish whether maternal condition and lay date differed between control, CORT and un-manipulated broods. GLMs with a Poisson error structure were used to compare clutch size and number fledged between treatment groups. Similar to the empirical study, Generalized Linear Models with a binomial error structure and a logit link function, weighted by brood size were used to investigate whether the treatment group affected brood sex ratio (Wilson & Hardy 2002). Also GLMMs were used to establish whether nestling mass on day 14 after hatching and nestling growth rate were affected by the treatment.

Models were optimised using backward elimination of non-significant terms when this improved the AIC (Akaike Information Criteria; Burnham & Anderson 2002). Model validations were applied where appropriate and the underlying statistical assumptions of normality and homogeneity of variance were verified. All statistical analyses were conducted using R version 2.8.0, and the nlme library was used for GLMMs.

4.4 Results

4.4.1 Population brood sex ratio

Overall 93.1% of eggs laid were sexed (n = 1360) from 145 un-manipulated broods. Neither the population primary nor the secondary brood sex ratio departed from a binomial distribution (see figure 4.3, Primary: z = 1.33, n = 145, P = 0.18, PrimaryC: z = 0.73, n = 83, P = 0.46, Secondary: z = 0.73, n = 142, P = 0.46). Furthermore, population brood sex ratios did not differ between years (Primary: z = 0.49, P = 0.63, PrimaryC: z = 1.14, P = 0.25, Secondary: z = 1.01, P = 0.31).

When both un-hatched eggs and nestlings that died before fledging were included, there was no indication of sex-biased offspring mortality (Yates' corrected: χ^2 = 0.82, P = 0.37). Un-hatched eggs and dead nestlings were combined, as samples sizes were very small when analysed independently. Also the number of laying gaps exhibited by mothers did not differ between years (z = 0.69, P = 0.49). However, the number of unsexed eggs (where development was not detected) was significantly lower in 2010 compared with the other two years, with 50%, 32% and 15% of broods containing unsexed eggs in 2008-2010 respectively (z = 2.86, P = 0.004).

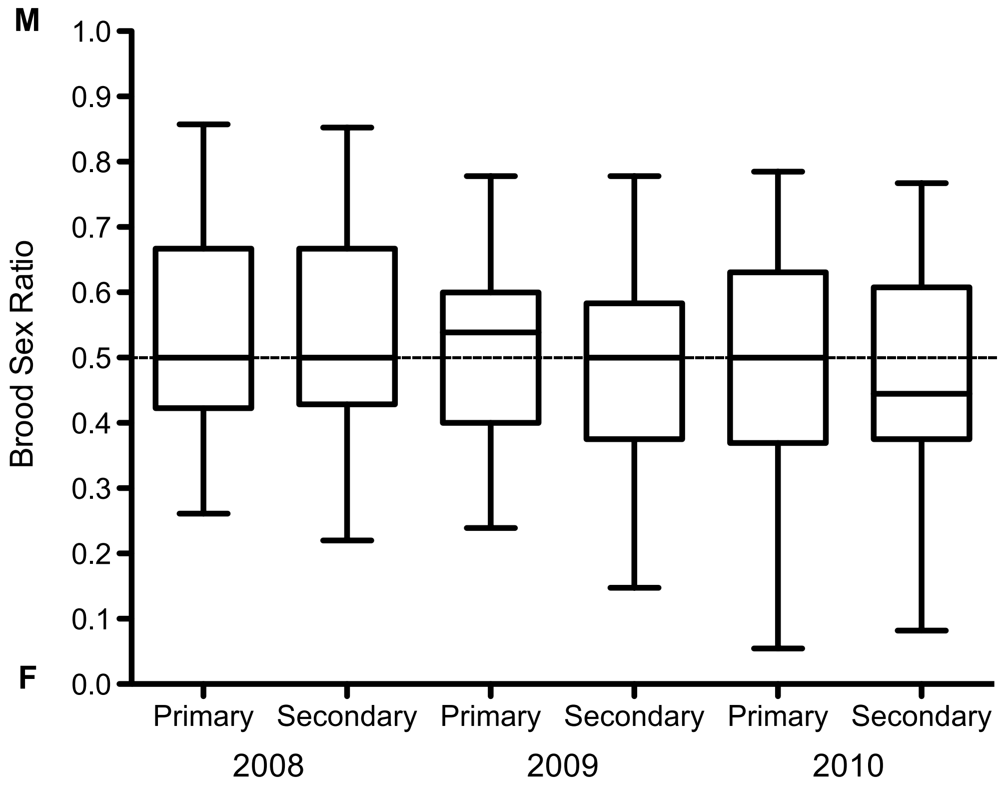


Figure 4.3. The primary (at laying) and secondary (at fledging) brood sex ratio in blue tits in three years (Primary: *n* = 2008: 89, 2009: 31, and 2010: 25, Secondary: *n* = 2008: 87, 2009: 27, and 2010: 28). Graph shows box-and-whisker plots with median and interquartile range. M indicates a male biased brood sex ratio and F indicates a female biased brood sex ratio. The dashed line denotes a 50:50 brood sex ratio.

4.4.2 Maternal baseline CORT, body condition and brood sex ratio

Maternal baseline CORT did not predict variance in primary or secondary brood sex ratio in any year of the study (Primary: CORT; z = 0.44, P = 0.66, CORT x Year; z = 0.63, P = 0.53, PrimaryC: CORT: z = 0.46, P = 0.65, CORT x Year: z = 0.18, P = 0.85, Secondary: CORT; z = 0.85, P = 0.39, CORT x Year; z = 1.14, P = 0.25). In addition, when all unsexed eggs were included in the analysis and considered male, maternal baseline CORT was unrelated to the primary brood sex ratio (CORT; z = 0.68, P = 0.49, CORT x Year; z = 0.48, P = 0.63).

Maternal body condition was significantly correlated with both primary and secondary brood sex ratio in a year-specific manner (see figure 4.4, Primary: Body Condition x Year; z = 2.85, P = 0.004, PrimaryC: Body Condition x Year; z = 1.90, P = 0.06, Secondary: Body Condition x Year; z = 2.66, P = 0.007). In 2010 only, mothers that were in good condition had more male biased broods (see figure 4.4). Furthermore, this result was evident when unsexed eggs were included in the analysis and considered male (Body Condition x Year; z = 2.60, P = 0.009).

Maternal age and lay date were not related to brood sex ratio in any year (Primary: Age; z = 0.09, P = 0.93, Age x Year; z = 0.71, P = 0.47, Lay Date; z = 0.04, P = 0.97, Lay Date x Year; z = 1.31, P = 0.19, PrimaryC: Age; z = 0.28, P = 0.78, Age x Year; z = 0.17, P = 0.86, Lay Date; z = 0.28, P = 0.77, Lay Date x Year; z = 0.85, P = 0.40, Secondary: Age; z = 0.61, P = 0.54, Age x Year; z = 0.53, P = 0.59, Lay Date; z = 0.23, P = 0.81, Lay Date x Year; z = 1.52, P = 0.13).

In addition, maternal body condition and baseline CORT were unrelated to the number of laying gaps mothers exhibited (Body Condition; z = 1.08, P = 0.28, Body Condition x Year; z = -0.30, P = 0.77 and CORT; z = -0.62, P = 0.53, CORT x Year; z = 0.04, P = 0.97). Also, maternal body condition and baseline CORT were not associated with the number of unsexed eggs (Body Condition; z = 1.38, P = 0.17, Body Condition x Year; z = -0.04, P = 0.97 and CORT; z = 0.49, P = 0.63, CORT x Year; z = -0.22, P = 0.82).

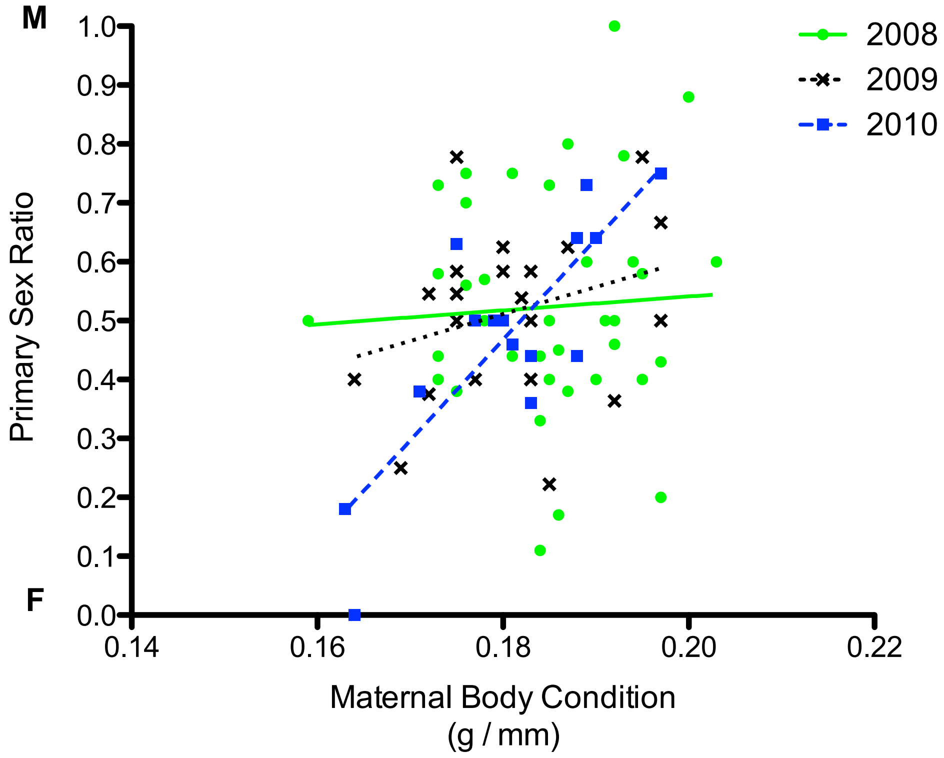


Figure 4.4. The relationship between maternal body condition (mass (g)/wing length (cm)) and the primary brood sex ratio (sex ratio at laying) in blue tits over three years (2008-2010). In 2010 only, maternal body condition was positively correlated with primary sex ratio (*n* = 2008: 39, 2009: 22, and 2010: 15). Mothers in superior body condition had more male biased broods in 2010 only. M indicates a male biased brood sex ratio and F indicates a female biased brood sex ratio.

4.4.3 Are maternal baseline CORT and maternal body condition correlated?

Maternal baseline CORT differed significantly between years. In 2010 values for maternal CORT were significantly lower than the other two years (see figure 4.5a, $t_{86} = 4.41$, $P < 0.001$). However, maternal body condition was significantly lower in 2009 compared with the other years (see figure 4.5b, $t_{110} = 2.18$, $P = 0.03$). Maternal body condition did not explain variation in maternal baseline CORT in any year of the study (see figure 4.5c, Maternal Condition; $t_{86} = 1.35$, $P = 0.18$, Maternal Condition x Year; $t_{82} = 0.29$, $P = 0.77$). In addition, maternal age and lay date did not explain variation in maternal baseline CORT in any year (Age; $t_{85} = 0.49$, $P = 0.62$, Age x Year; $t_{80} = 0.29$, $P = 0.77$, Lay Date; $t_{84} = 0.05$, $P = 0.96$ and Lay Date x Year; $t_{78} = 0.83$, $P = 0.41$).

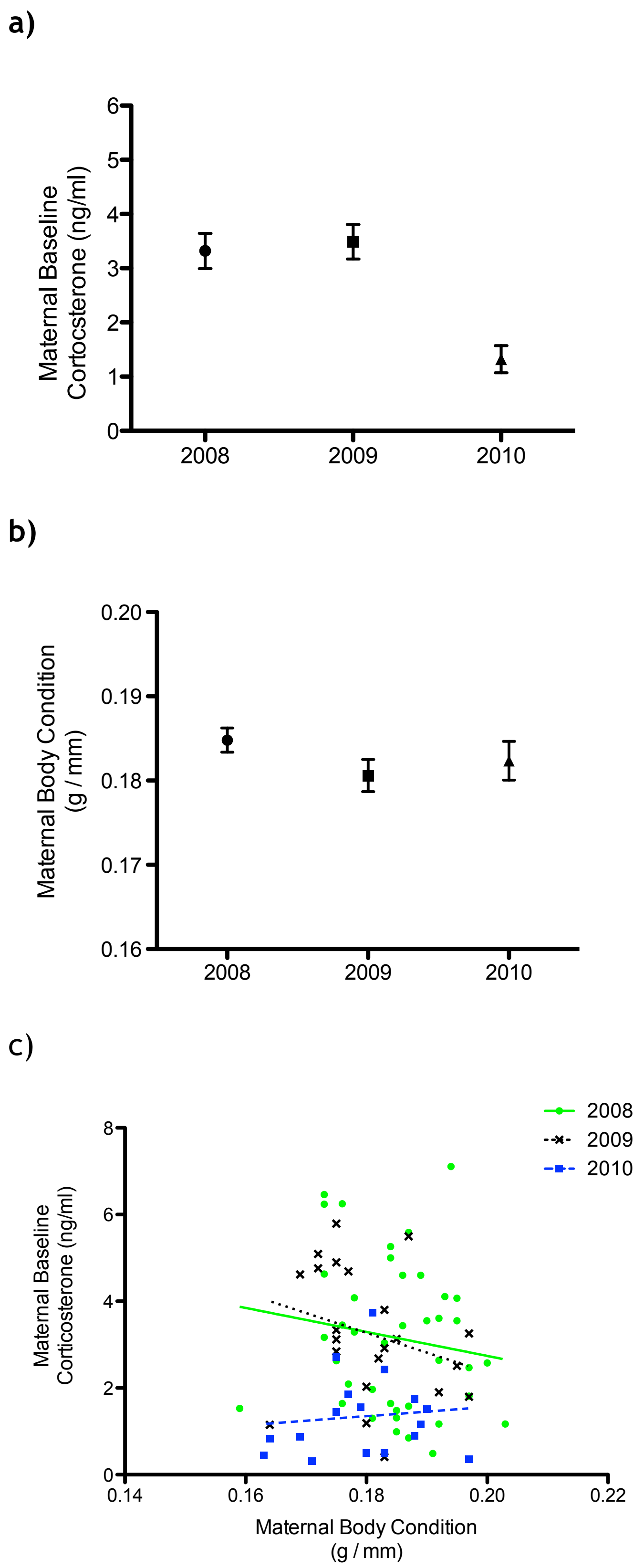


Figure 4.5. The variation in maternal baseline CORT (ng/ml) and maternal body condition (mass (g) / wing length (mm)) over three years in blue tits (2008-2010). a) Maternal baseline CORT, (*n* = 2008: 34, 2009: 34, and 2010: 15), was significantly lower in 2010 compared with the other years studied; b) Maternal body condition, (*n* = 2008: 40, 2009: 22, and 2010: 17), was significantly lower in 2009 compared with the other years studied. Bars show mean±SE. c) Maternal baseline CORT was not correlated with maternal body condition in any year.

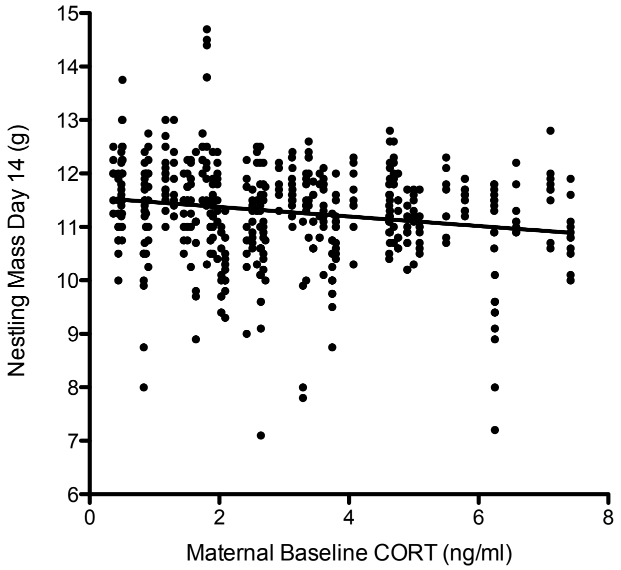
4.4.4 Are maternal baseline CORT and body condition linked to nestling condition?

Nestling mass on day 14 and growth rate did not significantly differ between years (Mass: $t_{63} = 0.33$, $P = 0.74$ and Growth: $t_{56} = 0.08$, $P = 0.93$). Male nestlings were consistently heavier on day 14 and grew at a faster rate than their female siblings in all years (Mass: Sex; $t_{541} = 11.55$, $P < 0.001$, Sex x Year; $t_{539} = 0.04$, $P = 0.97$ and Growth: Sex; $t_{480} = 5.40$, $P < 0.001$, Sex x Year; $t_{478} = 0.41$, $P = 0.68$).

Maternal baseline CORT was negatively correlated with nestling mass on day 14 in all years, but not nestling growth (Mass: figure 4.6a, CORT; $t_{64} = 2.05$, $P = 0.04$, CORT x Year; $t_{57} = 0.27$, $P = 0.78$ and Growth: CORT; $t_{57} = 0.64$, $P = 0.52$, CORT x Year; $t_{53} = 0.55$, $P = 0.58$). However, maternal baseline CORT did not explain variation in nestling mass or growth in a sex-specific manner (Mass: Sex x CORT; $t_{498} = 1.47$, $P = 0.14$, Growth: Sex x CORT; $t_{479} = 0.68$, $P = 0.49$).

In all years there was a trend that mothers in superior body condition had heavier and faster growing offspring than mothers in poor condition (Mass: figure 4.6b, Maternal Condition; $t_{64} = 2.50$, $P = 0.06$, Body Condition x Year; $t_{60} = 0.87$, $P = 0.39$ and Growth: Body Condition; $t_{57} = 1.96$, $P = 0.05$, Body Condition x Year; $t_{53} = 0.84$, $P = 0.40$). Maternal body condition did not influence nestling mass or growth in a sex-specific manner (Mass: Body Condition x Sex; $t_{540} = 1.20$, $P = 0.23$ and Growth: Body Condition x Sex: $t_{479} = 0.85$, $P = 0.40$).

a)



b)

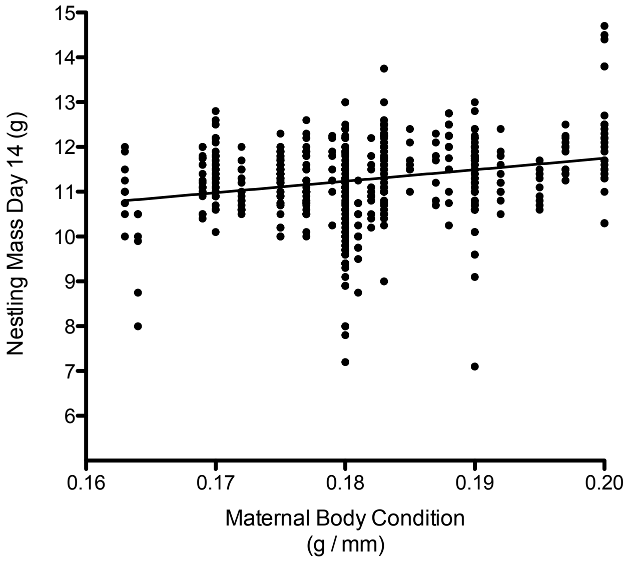


Figure 4.6. The relationship between a) maternal baseline CORT (ng/ml) (*n* = nestlings: 503, broods: 66) and b) maternal body condition (mass (g) / wing length (cm)) (*n* = nestlings: 545, broods: 66) and nestling mass on day 14 after hatching over three years (2008-2010) in blue tits.

4.4.5 Experimental study

In 2010 there were 57 nests manipulated and monitored (n = Control 29, CORT 28). In addition, 31 un-manipulated control nests were monitored. Treatment did not affect nest failure, with 5 nests in the control group failing prior to clutch completion, 4 in the CORT group and 6 in the un-manipulated controls. Therefore, there were 25 Control, 24 CORT and 25 un-manipulated nests successfully monitored until fledging.

Lay date ($t_{70} = 0.34$, $P = 0.74$), clutch size (see figure 4.7, $z = 0.22$, $P = 0.83$), number fledged ($z = 0.32$, $P = 0.74$, Control; 9.8±1.8, CORT; 9.5±2.1, Un-manipulated; 9.0±3.0) and maternal body condition (see figure 4.7, $t_{50} = 0.30$, $P = 0.78$) did not differ significantly between treatment groups. The number of laying gaps and unsexed eggs also did not differ between the treatment groups (Laying Gaps: $z = 0.41$, $P = 0.24$, Unsexed Eggs: $z = 1.62$, $P = 0.11$).

Primary and secondary brood sex ratios did not differ between treatment groups (see figure 4.8, Primary: $z = 0.47$, $P = 0.64$, PrimaryC: $z = 0.60$, $P = 0.55$, Secondary: $z = 0.05$, $P = 0.96$). In addition, nestling mass on day 14 and growth rate did not differ between groups (Mass: $t_{57} = 0.89$, $P = 0.38$ and Growth: $t_{57} = 0.59$, $P = 0.62$, Broods: 59, Nestlings: 505). Furthermore, nestling mass and growth were not affected by treatment in a sex-specific manner (Mass: Sex x Treatment; $t_{500} = 0.002$, $P < 0.99$ and Growth: Sex x Treatment; $t_{500} = 0.68$, $P = 0.50$). Similar to un-manipulated nests, male nestlings from manipulated broods grew at a significantly faster rate than their female siblings ($t_{503} = 5.12$, $P < 0.001$) and were heavier on day 14 after hatching ($t_{503} = 8.47$, $P < 0.001$).

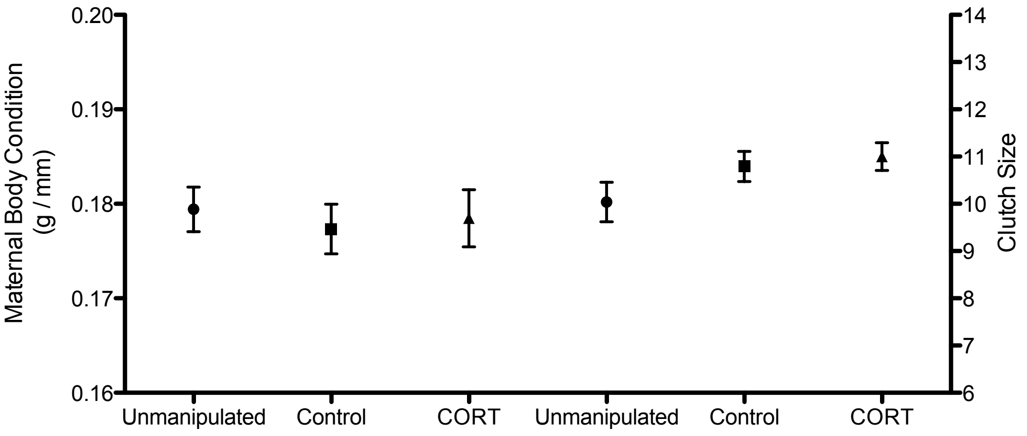


Figure 4.7. Maternal body condition (mass (g)/ Wing Length (g)) and clutch size did not differ between mothers fed CORT spiked mealworms during egg laying (*n* = 24), Control mealworms (*n* = 24) or un-manipulated mothers (*n* = 25). Bars show mean±SE.

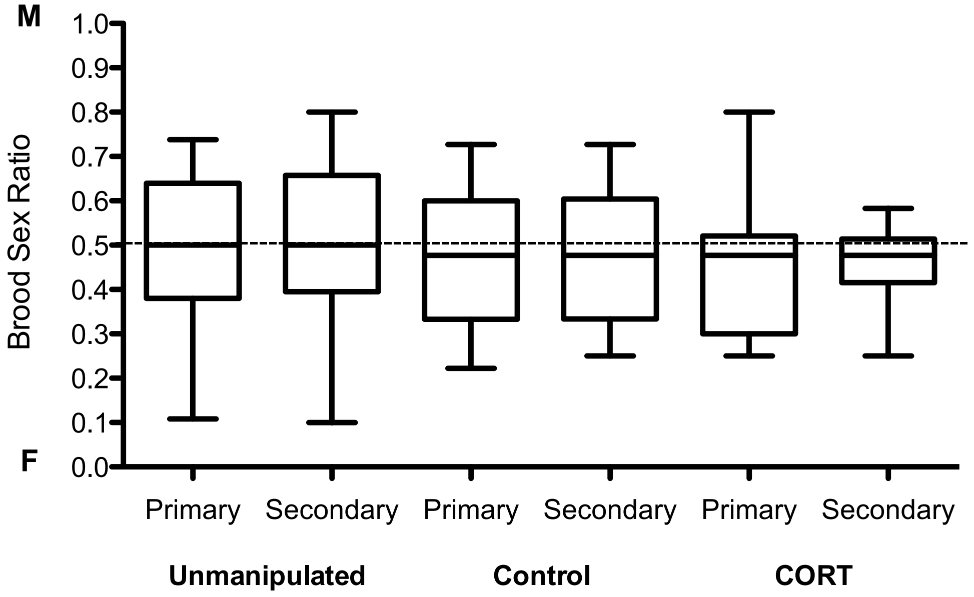


Figure 4.8. Primary (sex ratio at laying) and secondary (sex ratio at fledging) brood sex ratio did not differ between mothers fed CORT spiked mealworms during egg laying (*n* = 24), Control mealworms (*n* = 24) or un-manipulated mothers (*n* = 25). Graph shows box-and-whisker plots with median and interquartile range. M indicates a male biased brood sex ratio and F indicates a female biased brood sex ratio. Dashed line denotes a 50:50 brood sex ratio.

4.5 Discussion

This study suggests that maternal baseline CORT measured during brood rearing is not related to the sex ratio of offspring at laying or fledging in blue tits. Furthermore, exogenous elevation of maternal CORT during egg laying did not influence offspring sex, nestling mass or nestling growth. Interestingly, maternal baseline CORT was not correlated with maternal body condition in any year. However, mothers in good condition produced male biased primary and secondary brood sex ratios in one year of the study. Both maternal body condition and baseline CORT were associated with indices of nestling quality. In all years, maternal baseline CORT was negatively correlated with nestling mass on day 14, but not with nestling growth rate. In addition, there was a trend that maternal body condition was positively correlated with nestling mass and growth in all years. Although male nestlings were heavier and grew at a faster rate than female nestlings, maternal body condition and baseline CORT did not affect sons and daughters differently.

The majority of studies that have found a link between maternal CORT and brood sex ratio have also found maternal CORT to be associated with factors that could influence the adaptive significance of manipulating offspring sex, i.e. maternal condition: (Love *et al.* 2005; Pike & Petrie 2005a) and mate attractiveness (Pike & Petrie 2005c; Pryke *et al.* 2011). Therefore, maternal baseline CORT may be linked with brood sex ratio only when it is also associated with factors that would favour brood sex ratio adjustment. In the present study however, maternal condition was linked to brood sex ratio in one year, but was not correlated with maternal CORT in any year. Furthermore, a previous study that linked maternal CORT to sex ratio adjustment, also found sex-specific effects of elevated maternal CORT upon nestling mass (Love *et al.* 2005). Whereas, in my study maternal baseline CORT was negatively correlated with nestling mass in all years, but the effect was not sex-specific. Therefore in blue tits maternal baseline CORT may not be indicative of circumstances that might favour sex ratio adjustment, and thus may not be expected to affect brood sex ratio.

These results provide evidence that CORT may not be linked to sex ratio adjustment consistently across bird species. Previous studies that present evidence of a link between maternal CORT and brood sex ratio differ in the timing of sex ratio adjustment, and therefore the potential mechanisms employed. For example, there is evidence of a pre-laying mechanism in peafowl, *Pavo cristatus*, Japanese quail, *Coturnix coturnix japonica* and white-crowned sparrows, *Zonotrichia leucophrys* (Pike & Petrie 2005a; Pike & Petrie 2006; Bonier, Martin & Wingfield 2007), as maternal baseline CORT was found to be correlated and causally linked to the primary sex ratio in these species. However, Love *et al.* (2005) provided evidence from the European starling, *Sturnus vulgaris*, that exogenous elevation of maternal CORT during egg laying was associated with secondary brood sex ratio adjustment through male nestling mortality. The lack of a relationship between maternal CORT and brood sex ratio in my study and the contrasting findings of previous studies highlight the need for additional studies to establish the generality of hormonal mechanisms in sex ratio manipulation and the timing of these adjustments. Specifically, studies that explicitly investigate whether maternal CORT can directly influence the sex of eggs laid through ova reabsorption dependent upon sex or segregation distortion would be insightful.

Importantly the limitations of the methods employed in this study may have obscured the link between maternal CORT and brood sex ratio. Firstly, similar to previous studies (Pike & Petrie 2005a; Bonier, Martin & Wingfield 2007), baseline CORT was measured post laying. However, it would have been preferable to measure CORT during or prior to egg laying, as this is when primary sex ratio adjustment would occur. Furthermore, although CORT concentrations did not differ between breeding stages in this population, and there is evidence to suggest maternal baseline CORT concentrations are consistent between breeding stages within individuals (Wingfield & Farner 1976b; Wingfield & Farner 1978), this could not be ascertained in this study. Therefore, the CORT concentrations measured during brood rearing may not have reflected those experienced by mothers during egg laying. However, it is important to note the caveats associated with measuring CORT during the egg laying stage. For example, blood sampling itself is stressful for breeding birds, and could differentially affect mothers in poorer condition, who may be more sensitive to the disturbance. In turn the disturbance could differentially cause poor quality mothers to manipulate the sex of offspring, or alter brood care that could result in sex-specific embryo/nestling mortality.

There were also shortcomings associated with the method employed to manipulate maternal CORT during egg laying. The CORT elevation elicited by consuming mealworms was transitory; therefore if mothers did not consume mealworms close to the time of sex determination it may have been unlikely to affect offspring sex. In addition, as mothers began the treatment once they had laid their first egg, only ~80% of each clutch was manipulated. As the study was field based I could not monitor hatching, therefore it was not possible to establish hatching order or identify which nestlings hatched from un-manipulated eggs. Thus it would be insightful to repeat the manipulation under lab conditions, which would enable mealworm consumption and hatching to be monitored more closely to assure confidence in the results. The CORT manipulation also did not influence nestling mass or growth, although endogenous baseline CORT was linked with nestling mass in all years. This may have been because of the duration maternal CORT was elevated. In birds yolk is deposited over a period of ~10 days before oviposition (Sturkie 2000), therefore the treatment was unlikely to have influenced the concentration of CORT in the yolk. In this case unlike previous studies that have employed implants to elevate maternal CORT for longer periods (Love *et al.* 2005; Pike & Petrie 2006; Bonier, Martin & Wingfield 2007), it was unlikely to have influenced nestling condition through yolk mediated maternal effects. Therefore, it is important to note that the manipulation used in this study probably only investigated whether CORT affected offspring sex at the pre- rather than the post-laying stage.

Maternal condition was related to the primary brood sex ratio in one year of the study. The literature provides evidence of a both a correlative and causal link between maternal condition and brood sex ratio from a wide range of avian species (Kilner 1998; Nager *et al.* 1999b; Clout, Elliott & Robertson 2002; Pike & Petrie 2005a), however, there are also studies that have found no such relationship (Koenig & Dickinson 1996; Radford & Blakey 2000; Leech *et al.* 2001; Whittingham, Dunn & Nooker 2005). In addition, studies that have measured maternal condition and offspring sex ratio over multiple years in birds are rare, and where contrasting patterns between years have been found convincing biological explanations are lacking (Korpimäki *et al.* 2000; Radford & Blakey 2000). There is evidence to suggest that the link between maternal condition and offspring sex ratio is influenced by the prevalent conditions. In red deer, *Cervus elaphus* the tendency of dominant females to produce more male offspring disappeared as population density increased (Kruuk *et al.* 1999a), which has been suggested to have been caused by increased mortality of male foetuses as conditions became less favourable (Hardy 2002). In the year maternal condition was linked to brood sex ratio in the present study, there were significantly fewer unsexed eggs (where no development was found) laid compared with the two other years, thus if the unsexed eggs in the previous two years were male this may have obscured the effect of maternal condition upon sex ratio in these years. However, the number of unsexed eggs was not linked to maternal condition in any year and when unsexed eggs were considered male and re-analysed, maternal condition remained non-significantly correlated with brood sex ratio in two out of the three years. Therefore it is unlikely that early embryo death or sex-biased fertilization of ova obscured sex ratio adjustment in these years.

Variation in the breeding conditions between years could influence the fitness benefits of sex ratio adjustment in relation to maternal condition. In the great tit, *Parus major*, a closely related species, natal conditions influence lifetime reproductive success (LRS) more strongly in male compared with female birds (Wilkin & Sheldon 2009). Therefore, mothers in superior condition may derive fitness benefits from investing in sons only when breeding conditions are also good. This is because their male offspring will not only be competing for mates against males hatched the same year as them, but also males hatched in superior quality years. This may have been the reason for a year-specific relationship in the present study, as the year that brood sex ratio was linked to maternal condition was characterised by high food availability and favourable weather conditions compared with the other years (see Chapter 2). However, it is important to note that as maternal condition was correlated with sex ratio in only one year it is possible the relationship evident in this study was caused by random variation or chance (Ewen, Cassey & Moller 2004). Therefore, manipulative studies are required to provide convincing evidence of a link between maternal condition and brood sex ratio adjustment in this species.

This study provides evidence of a weak positive correlation between maternal condition and indices of nestling condition, but these effects were not sex specific. Therefore, although male nestlings grew at a faster rate and were heavier than females, maternal condition did not influence growth or mass more strongly in sons compared with daughters. In spite of this, mothers may have improved their fitness by investing in sons when they were in good condition, as improved nestling mass and growth during the nestling phase may have beneficial long-term effects for male but not female birds. For example in the great tit, improved nestling mass close to fledging was linked to greater reproductive success in male but not female birds (Tilgar *et al.* 2010). Unfortunately, in my study it was not possible to investigate the effects of maternal condition upon the future reproductive success of offspring, as very few nestlings were re-captured in subsequent years.

Fundamentally, there is a lack of knowledge concerning the benefits of sex ratio adjustment in blue tits. Although the sexes are size dimorphic the difference is relatively small (~5%), and the level of extra-pair paternity often differs between years and populations (Gullberg, Tegelström & Gelter 1992; Charmantier *et al.* 2004), thus variance in reproductive success between the sexes is not as extreme as in polygynous species. Therefore, for the blue tit the potential benefits of investing in males when conditions are favourable may never be as extreme as for species with contrasting mating systems, but long-term studies that address the fitness benefits of brood sex ratio adjustment for parents and offspring are required to establish this.

4.5.1 Conclusions

This study does not provide evidence for a link between maternal baseline CORT and brood sex ratio adjustment in blue tits. However, limitations of the methods employed, specifically the measurement and manipulation of maternal CORT may have obscured this relationship. The results do provide evidence that maternal condition was linked to brood sex ratio adjustment, but this was only evident in one year of the study. In addition, it was not possible to establish whether there were fitness benefits associated with sex ratio adjustment relative to maternal condition in this year. Fundamentally, it is not clear whether there would be fitness benefits to blue tit mothers of investing differentially in sons or daughters. Further studies that manipulate maternal condition and investigate effects upon the LRS of offspring would be valuable. In addition, improved methods for the manipulation of hormone concentrations are required for future research. Overall, this study serves to highlight the complexity of sex ratio adjustment in birds and the difficulties associated with identifying sex biasing mechanisms.

Chapter 5: Experimental manipulation of the early costs of reproduction influence maternal brood care and return rates

5.1 Abstract

The evolution of life-history traits is influenced by trade-offs in the division of finite resources among stages within and between breeding attempts. In birds, reducing the costs associated with egg laying may lead to increased investment in the later stages of reproduction and/or influence future survival. To investigate this in the blue tit, *Cyanistes caeruleus*, I supplemented mothers with a modest amount of food during egg laying. I subsequently measured indices of reproductive success, maternal care and condition. Supplemented mothers laid on average one more egg than controls, therefore, I removed an egg from food supplemented nests to standardize clutch size between treatment groups. The treatment did not influence either baseline corticosterone (CORT), a measure of physiological stress or maternal body condition. The results also indicate that food supplementation of mothers during laying did not influence hatching or fledging success. However, experimental mothers had heavier nestlings on day 4 and 10 after hatching compared with controls. In addition, supplemented mothers incubated their clutch for a longer period and there was a trend that they spent longer brooding nestlings than controls. While maternal provisioning rates were not influenced by the treatment, the positive correlation between maternal baseline CORT and provisioning rate in the control group was not evident in manipulated mothers. Finally, food supplemented mothers were more likely to return to breed the following year than controls. Thus, reducing costs during laying improved offspring quality, and negated the physiological stress associated with provisioning effort. Furthermore, reducing the costs of current reproduction lead to improved maternal survival. Overall, this study provides evidence that reducing the costs of early reproduction influences the trade-offs in the allocation of resources within and between reproductive events.

5.2 Introduction

Lack (1947) hypothesised that clutch size should reflect the number of offspring that can be successfully reared to independence. Empirical evidence, however, suggests birds tend to lay and incubate fewer eggs than they can rear (Vander Werf 1992; Monaghan & Nager 1997; Nager 2006). This has been explained, for iteroparus species, by the concept of reproductive trade-offs which exist between investment in current reproduction versus future survival and reproduction (Lindén & Møller 1989). Specifically, as animals have finite resources, increasing investment in current reproduction may negatively influence their future survival and breeding success (Williams 1966). In birds, a number of studies have explored trade-offs between current and future reproductive investment through brood size manipulations, as this stage was considered the most energetically demanding (reviewed in Sterns 1992; Monaghan & Nager 1997). These studies experimentally increased brood size, and illustrated that the increased costs of chick rearing had negative consequences for offspring quality, parental survival and future breeding success (Daan, Deerenberg & Dijkstra 1996; Ilmonen *et al*. 2003). However, similar experiments also presented non-significant results (Lessells 1986; Orell *et al*. 1996). These mixed results caused the earlier stages of reproduction, i.e. egg production and incubation, to also be included in studies examining reproductive trade-offs, and they have now been shown to be as energetically demanding as the brood rearing stage (Nager 2006).

Studies that have experimentally increased investment in egg production and/or incubation effort have shown that increasing the costs of early reproduction can have negative consequences for nestling quality and the survival and reproductive success of parents (for review see Nager 2006). In the lesser black-backed gull, *Larus fuscus*, experimentally causing females to lay four rather than their usual clutch of three eggs, caused them to fledge significantly fewer fledglings than controls (Monaghan, Nager & Houston 1998). Furthermore, increasing egg production reduced the local return rates for mothers in this species (Nager, Monaghan & Houston 2001). These results suggest that increased investment during egg production caused a decline in investment during the brood rearing stage, providing evidence that trade-offs exist within, in addition to between breeding attempts.

In the most part, previous studies that have investigated reproductive trade-offs, have experimentally increased the costs of reproduction at one or more stages during breeding (Daan, Deerenberg & Dijkstra 1996; Orell *et al*. 1996; Monaghan & Nager 1997; Monaghan, Nager & Houston 1998; Nager, Monaghan & Houston 2001; Ilmonen *et al*. 2003; Dobbs, Styrsky & Thompson 2006). However, manipulative studies that reduce the costs of reproduction may also influence trade-offs within or between reproductive events (Verhulst & Hut 1996; Verhulst & Tinbergen 1997; Wernham & Bryant 1998; Reid, Monaghan & Ruxton 2000). Reducing the costs associated with egg production through supplemental feeding for example, may allow birds to lay larger clutches without suffering negative consequences associated with the increased incubation and provisioning effort required (Robb *et al*. 2008). Indeed, there is evidence to suggest that supplementary feeding prior to and during egg laying can result in increased clutch size, nestling condition and/or adult survival (Hörnfeldt & Eklund 1990; Ramsay & Houston 1997; Robb *et al*. 2008; but see Harrison *et al*. 2010). However, supplementary feeding during the early stages of reproduction alone, may mismatch nestling demand with parental ability to provide food, as mothers may not longer be able to provide for a larger brood (Nager, Rueegger & Van Noordwijk 1997). In this case the benefits of the treatment to the mother and her offspring may be negated.

To isolate the influence of supplementary feeding during egg laying, from the confounding effects of clutch and brood size, egg number can be controlled through egg removal. This approach would allow researchers to isolate the effects of supplemental feeding upon the later stages of reproduction and future reproductive success and survival. Under these circumstances, birds that experience reduced costs of egg production may invest more in the later stages of reproduction. Specifically, reduced costs of egg production could allow mothers to invest in incubation and brood rearing. There is evidence that supplemental feeding during egg laying reduces incubation duration, potentially reducing the costs of this stage upon mothers (Nilsson & Smith 1988; Sanz 1996; Harrison *et al*. 2010). However, few studies have investigated the effects of supplemental feeding during egg production upon brood care (but see Helfenstein *et al*. 2008; Hinde, Buchanan & Kilner 2009), with the majority of studies manipulating food supply during the nestling phase (Cook & Hamer 1997; Wernham & Bryant 1998; Hoodless *et al*. 1999; González *et al*. 2006). However, in the great tit (*Parus major*) mothers that were supplemented with dietary carotenoids prior to and during egg laying provisioned at a higher level than control birds (Helfenstein *et al*. 2008). This suggests supplementing birds during egg laying can influence their investment in the later stages of reproduction.

The steroid hormone corticosterone (CORT) is involved in the maintenance of daily homeostatic energetic balance (Wingfield 2005), and is often elevated in response to poor body condition (Love *et al*. 2005; Romero 2004) and inclement environmental conditions (Chapter 2; Marra *et al*. 1998; Kitaysky, Piatt & Wingfield 2007). Inter-individual variation in baseline CORT is also associated with reproductive investment, such as provisioning behaviours (Carlson *et al*. 2006; Doody *et al*. 2008; Nguyen *et al*. 2008). Thus, CORT may mediate reproductive trade-offs, for example high concentrations may indicate conditions where individuals should re-direct behaviour from reproduction to survival (Bonier *et al*. 2009a). Alternatively, experimentally improving conditions through food supplementation may lessen the physiological stress associated with a reproductive event, and be associated with a reduction in circulating CORT. In addition, measuring baseline CORT in addition to body condition may provide a more holistic approach to quantifying how demanding mothers perceive their environment to be. Particularly because there is mixed evidence that reducing the costs of egg production through supplemental feeding is linked to improved body condition for mothers (Wernham & Bryant 1998; Hoodless *et al*. 1999; Karell *et al*. 2008).

In my study the costs of egg production in free-living blue tits were reduced by supplementing mothers with food (one mealworm day^{-1}) during egg laying to investigate the effects upon measures of reproductive success, maternal care and maternal return rates. As clutch size can increase in response to increased food intake during laying (Hörnfeldt & Eklund 1990; Nilsson 1991; Soler & Soler 1996; Robb *et al*. 2008), and can therefore confound effects of the treatment, an egg was removed from manipulated clutches prior to incubation. The main aims of the study were to investigate whether supplementing mothers with food during laying 1) influenced hatching success, fledging success and nestling mass, 2) altered maternal baseline CORT and/or body condition, 3) increased investment in maternal care, and lastly 4) affected maternal return rates in the following breeding season.

5.3 Methods & Materials

5.3.1 Breeding parameters

Blue tits breeding in nest-boxes in oak-dominated woodland around Loch Lomond, Scotland (56.13 ° N, 4.13° W) were studied from April to June 2009-2010. Nest-boxes were monitored regularly to establish laying date and clutch size. Incubation period was defined as the number of days between clutch completion date and hatching date. A mean incubation period of 12–13 days has been recorded in blue tits (Perrins 1979) therefore, daily checks began 10 days after clutch completion to establish hatching date. When >50% of eggs had hatched this was considered day 1. Nestlings were weighed to the nearest 0.01g with a digital balance, on day 4 and day 10 after hatching to establish mean nestling mass. Nests were checked for nestlings that did not fledge, which allowed fledging number to be calculated.

5.3.2 Food supplementation and egg removal

In order to alleviate the costs of egg production, mothers were food supplemented during egg laying. When the first egg was laid, the nest was randomly assigned to the manipulated or control treatment. Manipulated nests received a mealworm, *Tenebrio molitor* (2.5 kcal/g, for nutritional information see Finke 2002) every evening between 17:30 and 19:30 throughout the laying period, until no more eggs had been laid on two consecutive visits. The time mealworms were placed in the nest was chosen because female blue tits roost as early as 19:00 during egg laying in Scotland (Pendlebury & Bryant 2005), and therefore only mothers were expected to consume the mealworm. Thus the supplement was relatively modest and targeted towards the mother. Furthermore, only mothers that consumed each mealworm provided were included in the experiment. Control nests were also visited but a food supplement was not provided. To isolate the effects of the food supplementation upon the later stages of reproduction and maternal return rates from the potentially confounding effects of clutch size, egg removal was used to standardise clutch size, this was achieved by removing an egg before incubation had begun.

To validate that the treatment targeted female rather than male blue tits, a sub-sample of nests (n = 7) were monitored from a distance after the mealworm was placed in the nest until sunset and then checked the following morning before 06:00 to establish if the mealworm had been consumed during the night. The mealworm was consumed by 06:00 for each nest and on only one occasion an individual was seen entering the nest more than once after mealworm placement, for the remaining nests only one bird was recorded entering and not leaving the nest thereafter. Therefore, our observations suggest that female breeders and not their mates consumed the mealworms.

5.3.3 Maternal condition

To measure baseline CORT and body condition, mothers were captured in the nest-box during brood provisioning when chicks were 5 days old, by blocking the entrance hole. A small blood sample was obtained (about 80–100 ul) after puncture of the brachial vein with a 25 gauge needle and with the aid of a standard heparinised capillary tube. Blood samples were immediately stored on ice and separated through centrifugation within 2h of collection. The plasma portion of the sample was removed and stored at -20°C until assay. All blood samples were collected within 3 minutes of initial blockage of the nest box entrance. Breeding females were blood sampled throughout the day, from 8:00 to 18:00. Both the time taken to blood sample birds and time of day samples were collected was not found to influence maternal CORT (Time of Day: t_{25} = 1.99, P = 0.17 and Sampling Time: t_{25} = 1.02, P = 0.26). Therefore, the values for maternal CORT are considered to be baseline.

Parental birds were sexed using presence or absence of the brood patch and weighted to the nearest 0.05g with a Pesola spring balance and skeletal measures recorded. Maternal condition was established by dividing mass (g) by wing length (cm). A small blob of Tip-pex was applied to one parent per nest on the crown and rump to identify sex from video recordings of provisioning behaviour (see below). To investigate the effects of the treatment on maternal return rate, all birds caught were fitted with a uniquely numbered aluminium ring (British Trust for Ornithology).

5.3.4 Brood care

To assess the provisioning behaviour of parental birds, a small infrared video camera was placed in the nest-box opposite the entrance hole so the adults could be observed entering. The camera was placed in the nest-box between 16:00 and 19:00 on day 6 after hatching and the provisioning adults were recorded between 06:00 and 10:00 the following morning, when the chicks were aged 7 days. The identity of the provisioning adult was determined by the presence or absence of a small blob of Tip-Pex on the crown and rump. The recordings allowed the measurement of provisioning rate of both parents and for the analysis the provisioning rate brood^{-1} hour^{-1} was used. In addition, the total time spent in the nest by the mother was used in analysis, hereafter called brooding duration. Only visits in which the parent had food in its beak were classed as provisioning visits.

5.3.5 Hormone analysis

CORT concentrations were measured after extraction of 5-20 µl aliquots of plasma in diethyl ether, using a double antibody radioimmunoassay (Wingfield, Vleck & Moore 1992). Primary antibody Esoterix B183, secondary antibody Sigma goat anti-rabbit and [3H]-corticosterone label (GE Healthcare, UK). The extraction efficiency was 85–100%. Recoveries were measured for each sample independently and adjustments to the final assayed concentration were made. Samples were run within one assay with a detection limit (calculated as 2 SD from B0) of 0.01 ng/ml and an intra-assay coefficient of variation of 10.1%.

5.3.6 Statistical analysis

General Linear Models (GLMs) with a Poisson error structure were used to establish whether clutch size differed between treatment groups prior to and after egg removal. In addition, due to evidence of over-dispersion, GLMs with a quasipoisson error structure were used to investigate whether lay date differed between treatment groups.

I also used GLMs to investigate which factors explained variance in nestling mass on day 4 and day 10, maternal body condition, maternal baseline CORT, incubation length, brooding duration, provisioning rate and maternal return rate. Mean nestling mass on day 4 or day 10 were the dependent variables and treatment, brood size and lay date were the explanatory variables. Maternal body condition or baseline CORT, were the dependent variables and treatment, brood size and lay date were the explanatory variables. All CORT data were square root transformed because of non-normality. Incubation length, brooding duration or provisioning rate, were the dependent variables and treatment, brood size and lay date were the explanatory variables. GLMs with a binomial error structure were used to investigate whether treatment, lay date or brood size explained maternal returns rates the following breeding season (0 or 1).

Models were optimised using backward elimination of non-significant terms when this improved the AIC (Akaike Information Criteria; Burnham & Anderson 2002). However the model assessing the determinants of lay date were compared after backward stepwise elimination of non-significant terms, using ANOVA. If *P*-values were found to be non-significant (>0.05) terms remained dropped from the model. This was because AIC cannot be calculated when quasipoisson error structures are used. Where appropriate, model validations were applied and the underlying statistical assumptions of normality and homogeneity of variance were verified. All statistical analyses were conducted using R version 2.8.0.

5.4 Results

5.4.1 Reproductive success

Nests assigned to the control (n = 19) or manipulated group (n = 8) did not differ significantly in their lay date (t_{25} = 0.18, P = 0.86), original clutch size or clutch size after egg removal (Clutch Size before: z = 1.00, P = 0.32 and Clutch Size after: z = 0.28, P = 0.78). However, the clutch size of manipulated mothers prior to egg removal was larger, on average, by one egg (Control: 10.4±2.2, Manipulated before: 11.7±1.0 and Manipulated after: 10.7±1.0). Brood size on day 7 after hatching, when provisioning was recorded, did not differ between treatment groups (z = 0.73, P = 0.47). Also hatching and fledging success did not differ between groups (Hatching Success: z = 0.32, P = 0.74, see figure 5.1a, and Fledging Success: z = 0.29, P = 0.77).

Mean nestling mass on day 4 was significantly higher for broods of manipulated mothers (see figure 5.1b, t_{23} = 2.64, P = 0.01). In addition, brood size was negatively correlated with mean nestling mass on day 4 (Brood Size: t_{23} = 3.34, P = 0.003, Brood Size x Treatment: t_{21} = 1.12, P = 0.27) and there was a trend that lay date was negatively correlated with mean nestling mass on day 4 (Lay Date: t_{23} = 1.76, P = 0.09, Lay Date x Treatment: t_{22} = 1.64, P = 0.11). Mean nestling mass on day 10 was also significantly higher for broods of manipulated mothers (see figure 5.1c, t_{23} = 2.70, P = 0.01) and brood size was negatively correlated with mean nestling mass on day 10 (Brood Size: t_{23} = 3.15, P = 0.004, Brood Size x Treatment: t_{21} = 0.11, P = 0.91). However, lay date was not related to mean nestling mass on day 10 (Lay Date: t_{23} = 0.15, P = 0.88, Lay Date x Treatment: t_{22} = 1.65, P = 0.12).

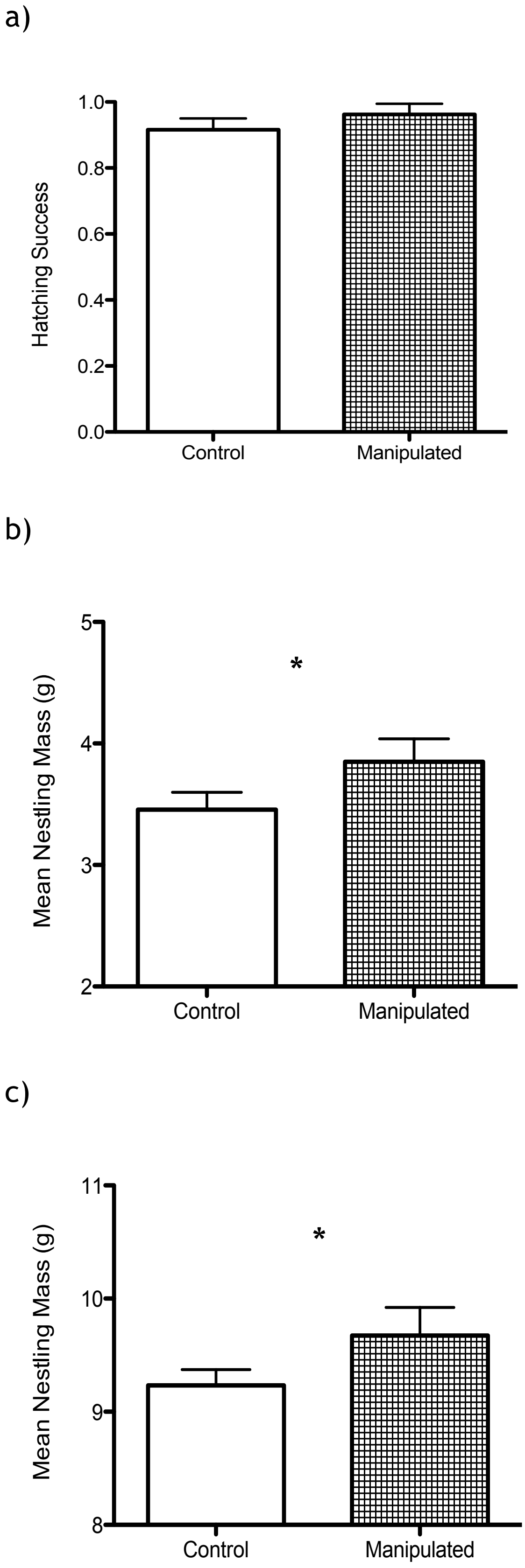


Figure 5.1. Comparison of a) hatching success (proportion of clutch that hatched), b) mean nestling mass (g) on day 4 after hatching and c) mean nestling mass (g) on day 10 after hatching between nests that received food supplementation during egg laying and controls. *n* = Control: 19, Manipulated: 8. Graphs show mean±SE. * denotes $P < 0.05$

5.4.2 Maternal baseline CORT

Maternal baseline CORT, measured when chicks were 5 days old, was not affected by the treatment (Manipulated: 2.87±1.40 ng/ml and Control: 3.23±1.87 ng/ml, $t_{23} = 0.52$, $P = 0.61$), nor was it correlated with lay date (Lay Date: $t_{24} = 0.76$, $P = 0.45$, Lay Date x Treatment: $t_{21} = 1.39$, $P = 0.18$). However, irrespective of treatment maternal baseline CORT was positively correlated with brood size (Brood Size: $t_{25} = 2.75$, $P = 0.01$, Brood Size x Treatment: $t_{21} = 0.01$, $P = 0.99$).

Maternal body condition was also not affected by the treatment (Manipulated: 1.18±0.01 and Control: 1.18±0.01, $t_{24} = 1.63$, $P = 0.12$). However, maternal body condition was positively correlated with lay date (Lay Date: $t_{24} = 3.64$, $P = 0.001$, Lay Date x Treatment: $t_{21} = 1.09$, $P = 0.29$), but was not correlated with brood size (Brood Size: $t_{24} = 0.21$, $P = 0.84$, Brood Size x Treatment: $t_{21} = 0.51$, $P = 0.62$).

5.4.3 Maternal care

The eggs of supplemented mothers had a longer incubation period relative to controls (see figure 5.2a, $z = 2.18$, $P = 0.03$). Neither clutch size nor lay date influenced incubation duration (Clutch Size: $z = 0.18$, $P = 0.86$ and Lay Date: $z = 0.66$, $P = 0.51$).

In the model investigating variation in maternal provisioning rate, the interaction term Maternal Baseline CORT x Treatment was significant ($t_{20} = 2.42$, $P = 0.02$), indicating that the relationship linking baseline CORT and provisioning rate differed between treatment groups (see figure 5.3a). *Post hoc* analysis revealed that there was a significant positive correlation between maternal baseline CORT and provisioning rate in the control group only (see figure 5.3a, Manipulated: $r = -0.46$, $n = 8$, $P = 0.25$, Control: $r = 0.54$, $n = 19$, $P = 0.02$). In addition, the interaction term Brood Size x Treatment was borderline significant ($t_{20} = 2.03$, $P = 0.06$). *Post hoc* analysis revealed that there was a significant positive correlation between brood size and provisioning rate in both groups, but there was a steeper correlation between brood size and provisioning rate in the manipulated group (see figure 5.3b, Manipulated: $r = 0.62$, $n = 8$, $P < 0.001$, Control: $r = 0.64$, $n = 19$, $P = 0.003$). Lay date was not related to provisioning rate (Lay Date: $t_{20} = 0.58$, $P = 0.56$, Lay Date x Treatment: $t_{19} = 0.76$, $P = 0.46$.

On the morning of the day 7, there was a trend that supplemented mothers spent longer brooding, and less time off the nest during the 4 hour recording period than control mothers (figure 5.2b, $t_{24} = 1.96$, $P = 0.06$). In addition, brood size was negatively correlated with maternal brooding duration (Brood Size: $t_{24} = 2.21$, $P = 0.04$, Brood Size x Treatment: $t_{21} = 0.14$, $P = 0.89$). Lay date was not related to maternal brooding duration (Lay Date: $t_{23} = 0.77$, $P = 0.45$, Lay Date x Treatment: $t_{22} = 0.52$, $P = 0.61$).

5.4.4 Maternal local return rate

Mothers that received the supplementary food treatment were more likely to return to breed the following year than controls (see figure 5.2c, $z = 2.54$, $P = 0.01$). Neither brood size ($z = 1.06$, $P = 0.29$) nor lay date ($z = 1.21$, $P = 0.23$) influenced return rates the following year.

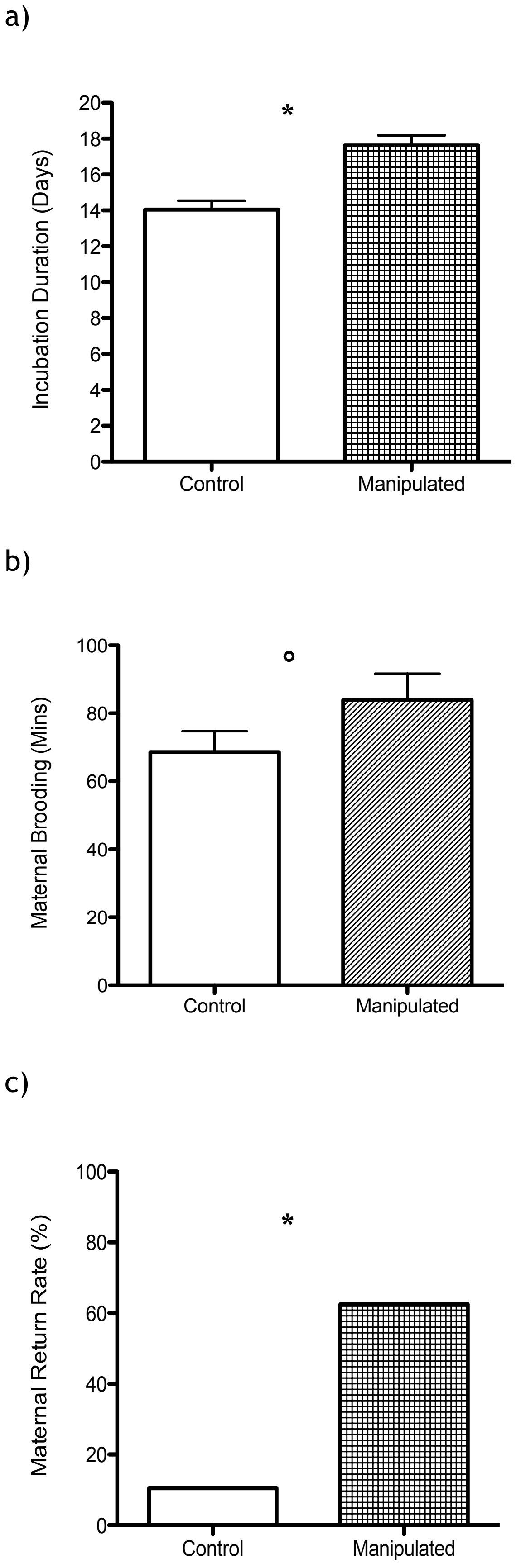
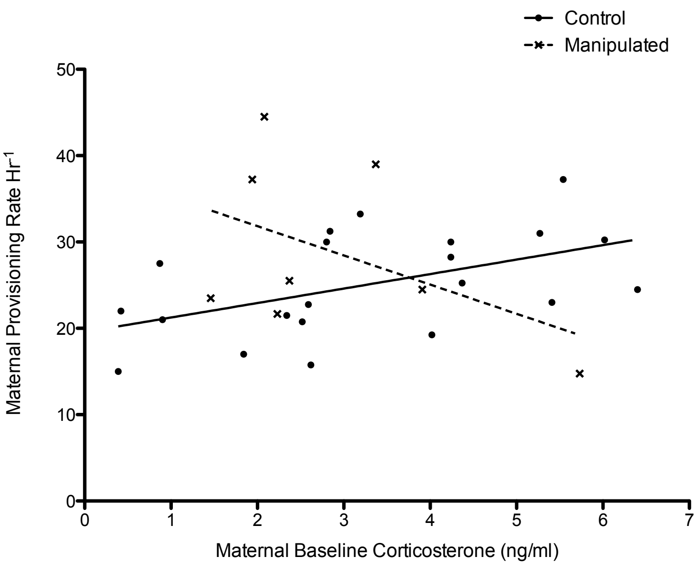


Figure 5.2. Comparison of a) the duration of incubation in days, b) the duration of maternal brooding (minutes within 4 hours of recording) and b) the % of control and manipulated blue tit mothers that returned to breed the following year. *n* = Control: 19, Manipulated: 8. ° denotes $P = 0.06$ and * denotes $P < 0.05$. Graphs show mean±SE.

a)



b)

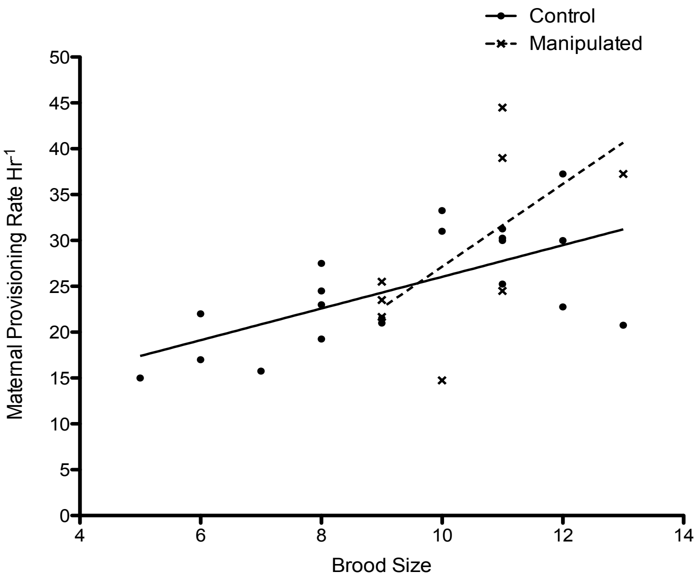


Figure 5.3. The relationship between a) maternal baseline CORT and maternal provisioning rate hour^{-1} and b) brood size and maternal provisioning rate hour^{-1} for control and manipulated blue tits. Manipulated broods received a food supplementation and egg removal treatment prior to incubation. *n* = Control: 19, Manipulated: 8.

5.5 Discussion

This study presents evidence that supplementary feeding during egg laying influenced nestling mass, maternal behaviour and maternal return rates. Primarily, mean nestling mass on day 4 and day 10 after hatching was significantly higher for the broods of supplemented mothers. Furthermore mothers that received the manipulation incubated their clutch for a longer period, and there was a trend that they spent longer brooding nestlings compared with controls. While provisioning rate did not differ between groups, the rate of increase in provisioning rate with brood size was higher for manipulated mothers. There was also a positive correlation between provisioning rate and baseline CORT for controls only, indicating the treatment negated the relationship between maternal baseline CORT and provisioning rate. Maternal body condition and baseline CORT concentrations, however, were unaffected by the treatment. Finally, mothers that received supplemental feeding during egg laying were more likely to return to breed the following year.

The study indicates that supplementing mothers with food during laying did not significantly influence the size of the clutch laid. However, clutch size was on average larger by one egg in the manipulated group (Control: 10.4±2.2, Manipulated before egg removal: 11.7±1.0 and Manipulated after egg removal: 10.7±1.0) and power analysis indicated that the sample size was not adequate to identify this minor increase in size. The removal of one egg from manipulated nests, therefore, resulted in a more similar mean clutch size for both groups. Therefore this modest egg removal should, while not statistically affecting clutch size, have ensured that the effects of the treatment were not confounded by an increase in clutch size between groups.

This study suggests that although hatching and fledging success were unaffected by the treatment, nestlings from manipulated mothers were heavier compared with controls. The results imply that maternal provisioning rates were not responsible for the difference in nestling mass between the treatments; furthermore the provisioning rate of male partners did not differ between groups ($t_{23} = 1.46$, $P = 0.156$). Importantly this does not mean provisioning behaviour was not the cause, as the size and quality of prey may have differed between treatments, which in turn could have influenced nestling quality (Arnold *et al.* 2007). Additional aspects of brood care may have been responsible for the difference in nestling mass between the treatment groups. There was a trend that manipulated mothers spent longer brooding nestlings, which can reduce the thermal costs to nestlings allowing them to invest more energy into growth (Visser 1998). However, the maternal care data were collected over 4 hours on one day so may not have fully represented the variation between the groups.

In addition, food supplementation during egg-laying can stimulate mothers to differentially invest nutrients (Ramsay & Houston 1997) or differing hormone concentrations into their eggs (Verboven *et al.* 2003), both of which can influence nestling mass (Mousseau & Fox 1998). As the difference in mass was evident as early as day 4 after hatching, this indicates that egg quality may have influenced nestling mass. Unfortunately egg mass was not measured, which could have provided at least crude information regarding egg quality. Also a cross-fostering approach was not employed thus it was not possible to disentangle the effects of laying conditions and egg quality from post laying maternal care (Nager 2006).

Incubation is an energetically demanding reproductive stage (Reid, Monaghan & Ruxton 2000; Deeming 2002) and previous studies have found that supplemental feeding prior to and during incubation reduces incubation length (Nilsson & Smith 1988; Sanz 1996; Harrison *et al.* 2010). This is thought to be caused by a reduction in time mothers spend foraging during incubation, so eggs are warmed for longer, speeding up development. This may be advantageous as it reduces the risks of predation and time-dependent egg mortality (Lack 1968; Bosque & Bosque 1995). In contrast mothers that received the manipulation in the present study incubated their clutch for approximately 4 days longer than controls. Previous studies have found that food supplementing mothers during egg laying increases egg size (for review see Christians 2002), and as there is evidence that larger eggs require longer incubation periods (Deeming 2002), this may explain this result. Food availability has also been shown to influence incubation length, with low food availability associated with a longer incubation period through a decrease in nest attentiveness as mothers spend longer foraging (Rauter & Reyer 1997; Deeming 2002). In the present study, mothers could have perceived the discontinuation of food at the onset of incubation as a reduction in food availability. Therefore this may have been responsible for the increase in incubation length. However, as total incubation length rather than the time spent on the eggs was recorded, it is not possible to establish whether the extended incubation shown by manipulated mothers was due to more time spent incubating or more foraging breaks.

The influence of supplementary feeding of female birds prior to and during egg laying has had mixed effects upon measures of maternal condition (Draycott *et al.* 1998; Verboven *et al.* 2003; Schoech *et al.* 2007; Robertson 2009). There is evidence that food quality and availability prior to chick hatching influences maternal baseline CORT (Schoech *et al.* 2007; Robertson 2009). Whereby increased food availability or quantity was associated with reduced baseline CORT. Maternal body condition and baseline CORT concentrations, however, were not affected by the treatment in this study, but the food supplementation was modest compared with previous studies (Schoech *et al.* 2007; Karell *et al.* 2008; Robertson 2009). Rather this study suggests that supplementary feeding during laying negated the relationship between provisioning rate and baseline CORT concentrations. Among controls, mothers with higher baseline CORT on day 5, provisioned at a higher rate on day 7. This indicates that the relationship is state-dependent as mothers that experienced reduced costs during early reproduction did not show an increase in baseline CORT with higher provisioning rates. D'Alba *et al.* (2011) found a similar result in the common eider (*Somateria mollissima*), where the influence of nest shelter upon maternal CORT was investigated. CORT concentrations did not differ between groups dependent upon the level of nest shelter, instead, baseline CORT was negatively correlated with hatching success in birds that nested at exposed sites only. This relationship was not evident in birds that nested in naturally sheltered sites. In addition, females nesting in naturally sheltered sites had greater reproductive success than those at exposed sites. This indicates that in eiders, female quality may influence the relationship between CORT and measures of reproductive success. In my study control mothers did not benefit from lowered costs of early reproduction, therefore higher provisioning rates may have required elevated CORT to mobilise extra energy reserves (Sapolsky, Romero & Munck 2000).

Importantly although mean brood size did not differ between treatment groups, there is evidence that there was greater range of brood sizes for control mothers compared with supplemented mothers (see figure 5.3b). This may have been due to the manipulation itself, or due to the smaller sample size of manipulated broods. Ultimately the difference between the groups could be responsible for the lack of relationship between maternal baseline CORT and provisioning rate in supplemented mothers. However, the range of provisioning rates did not differ considerably between groups (see figure 5.3a). Overall, although logistically difficult particularly when supplementing birds and measuring hormone titers, it would have been preferable to have equal sample sizes between groups.

Surprisingly the treatment employed in this study also influenced maternal local return rate the following breeding season, with food supplemented mothers more likely to return to breed than controls. There is evidence that increasing the costs of reproduction within one year reduces maternal return rates and future breeding performance (Wernham & Bryant 1998; Reid, Monaghan & Ruxton 2000; Nager, Monaghan & Houston 2001; Brommer, Karell & Pietiäinen 2004). However, studies have also failed to find these effects (Verhulst & Tinbergen 1997; Dobbs, Styrsky & Thompson 2006). In the year the experiment was conducted, breeding success was significantly poorer compared with the preceding and following year (see Chapter 3). Therefore the modest reduction in the costs of early reproduction through supplemental feeding may have had a stronger influence upon maternal survival and therefore return rates in this year, compared with more benign years.

5.5.1 Conclusions

This study indicates that reducing the costs of egg production through maternal feeding affects maternal investment in the later stages of reproduction and maternal return rates the following year. This study also presents evidence that it is not physiological stress *per se* that was affected by reducing early costs, but rather the relationship between baseline CORT and provisioning rates. Fundamentally, this study suggests reducing the costs of early reproduction influences trade-offs in the allocation of resources within and between reproductive events.

Chapter 6: UV crown colouration in female blue tits, *Cyanistes caeruleus*, predicts baseline corticosterone and reproductive success

6.1 Abstract

A growing number of avian studies provide evidence that female colouration is condition-dependent and can influence male mate choice. In blue tits, both sexes exhibit bright UV-blue crown feathers, which have previously been shown to be attractive to the opposite sex. In males there is evidence that the UV reflectance from the crown feathers is an honest signal of quality in this species, but it is unclear whether this is the case for females. In this study, I measured maternal UV crown colouration from sampled feathers during brood rearing over three years (2008-2010). In addition, I measured indices of maternal condition i.e. body condition, baseline corticosterone (CORT) and haematocrit, and indices of reproductive success, i.e. lay date, clutch size and number of chicks fledged. Female UV crown colouration varied significantly between years, but was not linked to maternal age. In all years maternal baseline CORT was negatively correlated with UV crown colouration. However, maternal UV signal was also negatively related to body condition in one out of three years (2010) and was unrelated to haematocrit. In addition, maternal UV crown colouration was related to indices of reproductive success. UV crown colouration was negatively correlated with lay date in 2008 and marginally in 2010 but not in 2009. Furthermore, in all years maternal UV crown colouration was positively correlated with number of chicks fledged, but was not correlated with clutch size in any year. This study adds to evidence that female plumage colouration is related to measures of individual quality. Interestingly, this study presents the first evidence that maternal baseline CORT is associated with UV plumage colouration in free-living birds. Indeed it is intriguing that maternal CORT measured during chick rearing would be linked to the colouration of plumage produced months before. However, these findings are correlative and further research is required to elucidate a mechanistic link between stress hormones, breeding success and UV colouration in female blue tits.

6.2 Introduction

Birds are the most colourful of the terrestrial vertebrates, (Amundsen & Pärn 2006) with plumage colouration ranging from the cryptically coloured Nightjar, *Caprimulgus europaeus*, to the extravagant greater bird-of-paradise, *Paradisaea apoda*. This vast array of colouration is broadly caused by two mechanisms; the pigment molecules laid down within the feather, such as melanins, psittacofulvins or carotenoids, and the microstructure of the feather itself (Hill & McGraw 2006). Additionally, in some cases both the structure and the pigment content influence feather colour (Shawkey & Hill 2005). Unsurprisingly, birds possess colour vision and can perceive a larger portion of the light spectrum than humans, specifically wavelengths within the ultraviolet (UV) range, as they possess four rather than three retinal cones. The inability of humans to perceive the same range of wavelengths as birds has necessitated the use of objective methodologies, such as spectrophotometers to quantify feather colouration. The most common components of feather colour measured are firstly, intensity (or brightness) an achromatic component of colour, secondly, hue (spectral location) and finally, chroma (or saturation), both of which describe the chromatic component (Endler 1990). The sensitivity of retinal cones from a number of avian species has become available (Hart & Voroboyev, 2005), and has allowed researchers to estimate the neural excitation of each cone type induced by a specific wavelength for a given species (Heindl & Winkler, 2003; Uy & Endler, 2004; Hadfield & Owens, 2006; Stoddard & Prum, 2008). This provides researchers with an ability to assess the perception of feather colouration by the species of interest.

The function of extravagant plumage colouration has been the subject of much investigation over the last hundred years (reviewed in Hill & McGraw 2006). Darwin's (1871) theory of sexual selection was the first hypothesis to explain the occurrence of these elaborate but seemingly functionless traits. However, the theory predominately addressed the colourful plumage of male birds, which on the whole tend to be showier than females (Hill & McGraw 2006). As feather colouration shares a common genetic basis in both sexes, female colouration was thought to be a by-product of selection acting on male ornaments (Darwin 1871; Lande 1980). Therefore, historically male birds have been the focus of studies, although in many species females exhibit brightly coloured feathers equal to or more elaborate than males (Amundsen & Pärn 2006). In addition, as in male birds, there is evidence that female plumage colouration is condition-dependent (Velando, Lessells & Márquez 2001; Massaro, Davis & Darby 2003; Amundsen & Pärn 2006) and linked to competitive ability (Midamegbe *et al.* 2011). In this case plumage colouration may signal female quality and be used by males during mate choice, potentially providing them with fitness benefits if females honestly signal reproductive potential or genetic quality (Burley 1977; Pizzari et al. 2003; Simmons & Kvarnemo 2006). Indeed, mate choice studies provide evidence that male birds often prefer more ornamented females (Amundsen, Forsgren & Hansen 1997; Amundsen & Forsgren 2001; Pizzari et al. 2003; Griggio et al. 2005; Baldauf et al. 2010). Overall, female colouration may act as an honest indicator of quality, and therefore may be linked to measures of condition and reproductive success.

Feather colouration has been linked to an individual's diet (Hill, Inouye & Montgomerie 2002; McGraw *et al.* 2002; Siefferman & Hill 2005b; Sorensen *et al.* 2010), nutritional status (Hill & Montgomerie 1994) and environmental conditions (Vergara *et al.* 2009). Both the content of and the amount of food consumed can influence plumage colouration, for example, the amount of carotenoids consumed within the diet can influence the expression of carotenoid based plumage (Hill, Inouye & Montgomerie 2002). Furthermore, in Eastern bluebirds, *Sialia sialis*, females given ad libitum access to food exhibited more ornamented structural coloration than females on food-restricted diets (Siefferman & Hill 2005b) and in male brown-headed cowbirds, *Molothrus ater* nutritional stress has been shown to reduce the expression of structural based plumage colour (McGraw *et al.* 2002; but see Peters *et al.* 2011). As such parameters that are associated with diet, nutrition and environmental conditions, such as body condition (Naef-Daenzer & Keller 1999; Lovvorn *et al.* 2003; Brown & Sherry 2006) and baseline corticosterone (CORT) (Kitaysky *et al.* 2001; Kitaysky *et al.* 2006; Müller *et al.* 2007) may be linked to plumage colouration. A number of studies have explicitly investigated whether female colouration is linked to body condition, but evidence is mixed. Some studies that have related body condition to feather colour have found positive correlations (Piersma & Jukema 1993; Velando, Lessells & Márquez 2001; Massaro, Davis & Darby 2003; Siefferman &

Hill 2005b), whereas others have found no such correlations (Hill 1993; Tella, Forfero & Donázar 1997; Peters *et al.* 2011).

Studies that focus on avian colouration have rarely employed hormone titres as indicators of condition, or related hormone concentrations to the expression of feather colouration (Husak & Moore 2008, but see Peters *et al.* 2006; Roberts, Ras & Peters 2009). Yet, CORT the primary glucocorticoid in birds, is intrinsically linked to energetic status through its role in the glucose regulation (Sapolsky, Romero & Munck 2000) and has been linked to environmental conditions (Wingfield, Moore & Farner 1983; Romero, Reed & Wingfield 2000; Kitaysky, Piatt & Wingfield 2007; Müller *et al.* 2007), diet (Kitaysky *et al.* 2001; Kitaysky *et al.* 2006) and body condition in birds (Love *et al.* 2005; Pike & Petrie 2005a). In this case, CORT may provide a holistic reflection of individual condition, as it reveals an individual's response to various factors that constitute their environment (Sapolsky, Romero & Munck 2000). Additionally, baseline CORT concentrations have been shown to be repeatable within individuals (Breuner & Orchinik 2002; Cockrem & Silverin 2002a; Doody *et al.* 2008a; Romero & Reed 2008; Cockrem *et al.* 2009; Bonier, Moore & Robertson 2011), thus may provide a long-term measure of individual quality. There is also growing evidence that circulating CORT concentrations influence both the colour (Roulin *et al.* 2008) and growth (Romero, Strochlic & Wingfield 2005) of feathers. As feather growth has been shown to influence the expression of UV plumage colouration (Griggio *et al.* 2009), circulating CORT concentrations could have a direct influence upon the expression of this trait.

If plumage colouration signals individual quality, more colourful individuals may have greater reproductive success than less colourful individuals (Siefferman & Hill 2003; Jawor *et al.* 2004). However, studies that have investigated the link between maternal colouration and reproductive success have provided mixed results. For example, while the intensity of carotenoid-based colouration is positively correlated with clutch size in blue tits, *Cyanistes caeruleus*, this effect was only seen in females that were forced to lay a second clutch (Doutrelant *et al.* 2008). Also, in eastern bluebirds, structural colouration predicted maternal provisioning rates and nestling condition, but not fledging number (Siefferman & Hill 2005). Whereas, in bluethroats, *Luscinia s. svecica*, a mate removal experiment did not provide evidence that parental care or reproductive success was linked to female colouration (Smiseth & Amundsen 2000). Evidently further studies are required to establish the generality of the link between female colouration and measures of reproductive success.

Blue tits are a slightly sexually dimorphic species, with females on average having lower intensity and chroma of the UV-blue crown feathers (Hunt *et al.* 1999; Appendix II), but some females can be as bright as males. Several studies suggest that male colouration has an important signalling function in the context of sexual selection in this species (Sheldon *et al.* 1999b; Griffith *et al.* 2003; Johnsen *et al.* 2003; Alonso-Alvarez, Doutrelant & Sorci 2004; Delhey *et al.* 2006). In addition, mate choice studies have shown that males also choose females dependent upon their crown colouration (Hunt *et al.* 1999) and there is evidence of assortative mating based on UV crown reflectance in free-living birds (Andersson, Örnborg & Andersson 1998). As such, blue tits provide an ideal system for investigating the occurrence of extravagant female plumage colouration, as the UV-blue crown colouration may act as an honest signal of female quality and be used in mate choice.

In this study I examined whether female UV-blue crown colouration measured during chick rearing was linked to individual quality in free-living blue tits over three years. Firstly, I investigated the variability of female UV colouration in relation to year and age. Secondly, variation in maternal UV signal was related to measures of condition, i.e. body condition, baseline CORT and haematocrit. Finally, I considered whether maternal UV signal predicted measures of reproductive success, specifically; lay date, clutch size and number of chicks fledged.

6.3 Methods & Materials

6.3.1 Field site & reproductive success

Blue tits breeding in nest boxes in oak-dominated woodland around Loch Lomond, Scotland (56.13° N, 4.13° W) were studied for three years from April to June 2008-2010. Nest boxes were monitored regularly from the onset of nest building to establish laying date and clutch size. Nests were then visited every second day to establish hatching date and brood size; when >50% eggs had hatched this was considered day 1. Two weeks after nestlings were 14 days old, nests were checked for unfledged young to establish the number that successfully fledged. To compare between years, lay dates were converted to Julian with 0 = 1st April.

6.3.2 Feather colouration

During provisioning on day 5 after hatching, parental birds were captured on the nest. Birds were captured at this stage to reduce the chance of nest desertion. On average 6±2 feathers were plucked from a standard point on the crown for each bird (see figure 6.1a) and stored in paper envelopes. At a later date in the lab, UV crown reflectance was assessed from feathers, which has been shown to be repeatable and comparable with measurements of UV reflectance taken directly from the bird (see Appendix II; Quesada & Sena 2006). Feather samples were placed upon black velvet (100% cotton) to minimise background reflectance. Feathers were then stacked on top of one another to resemble how they would lay on the bird's crown (see figure 6.1b) and three colour measurements were recorded sequentially, with the probe being lifted and replaced on the feather sample between each scan.

Spectral data were recorded in the lab with an Ocean Optics S2000 spectrometer (range 250–880 nm; Dunedin, Florida) using a micron fibre-optic probe at a 45° angle to the feather surface. Ambient light was excluded with a cylindrical plastic sheath affixed to the probe tip, and the sheath was placed against a feather specimen with the probe held a fixed distance of 6 mm from the feather surface. The reading area was a ~11 mm^2 diameter of light, illuminated with both a deuterium bulb (UV light source) and a tungsten halogen bulb (visible light source). We generated reflectance data relative to a white standard (Labsphere, Inc.). The spectra acquisition software package OOIBase was employed to record spectra.

Using the SPEC package (Hadfield et al. 2006) for R (http://www.R-Project.org), spectral data was reduced into four quantal cone catches that quantify the amount of light captured by each of the avian single cones, specific to the spectral sensitivity of the blue tit visual system (Hart 2001). Irradiance spectra and ocular media transmittances were not specified. The four cone types are UVS (very short – UV), SWS (short – blue), MWS (medium – green) and LWS (long – red) and refer to the range of wavelengths to which they are most sensitive (Hart, 2001). As our analysis focused on the UV-blue crown feathers we utilised the results from the UVS and SWS cone types only. To calculate the chromatic signal, hereafter 'UV signal', for each individual the UVS and SWS cone catches were standardised by dividing them by their sum to eliminate variation in intensity (achromatic component), which is highly sensitive to the method of measurement. We calculated the UV signal so that higher values indicate a signal with a stronger UV component (formula from Osorio, Miklosi & Gonda 1999; Larcombe *et al*. 2010);

$$\text{UV signal} = (\text{UVS} - \text{SWS}) / (\text{UVS} + \text{SWS})$$

This method was employed rather than other commonly used descriptors of reflectance spectra, such as UV chroma and intensity, because they were found to differ significantly when generated directly from birds as opposed to feather samples thereof (see Appendix II). Feather number was not found to influence UV signal (see Appendix II) and was therefore not included in the analysis. Within individuals, values for UV signal were highly repeatable (ANOVAS; 2008: $r = 0.81$, $n = 31$, $P < 0.001$, 2009: $r = 0.86$, $n = 49$, $P < 0.001$, 2010: $r = 0.80$, $n = 23$, $P < 0.001$, Lessells & Boag 1987).

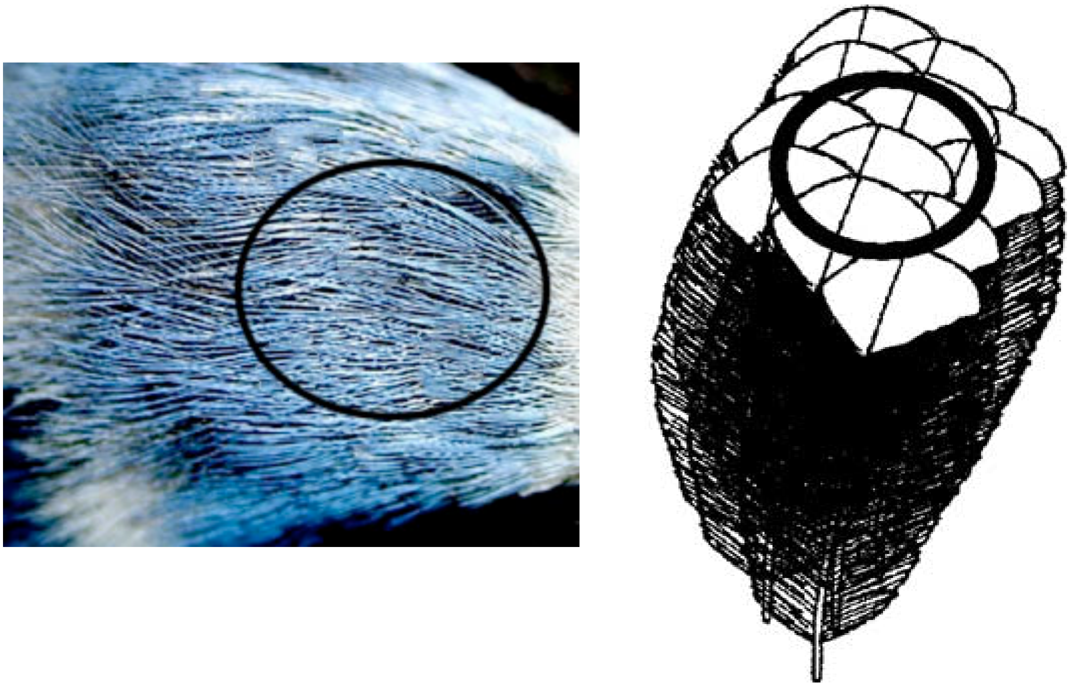


Figure 6.1. Schematic showing a) the area of the blue tit crown where feathers were plucked for colour assessment, modified from Delhey (2005) and b) how collected feathers were arranged for measurement by a spectrometer, from Quesada & Sena (2006). Respectively, the circles illustrate the area where feathers (a) and measurements (b) were taken.

6.3.3 Maternal baseline CORT and body condition

To measure baseline CORT in breeding blue tits, mothers were blood sampled during provisioning when they were captured for feather collection. A small blood sample (about 80–100 µl) was obtained after puncture of the brachial vein with a 25-gauge needle and collected with a standard heparinised capillary tube. All samples were collected within 3 minutes of initial blockage of the nest box entrance. Blood samples were immediately stored on ice and the red blood cells and plasma were separated through centrifugation within 2h of collection. For a sub-set of birds, haematocrit, another measure of condition, was calculated by assessing the proportion of the total blood that was constituted by red blood cells. Then the plasma portion of the sample was removed and stored at -20ºC until assay.

The majority of birds entered the nest box without any sign that they were disturbed by our presence. However, when individuals made alarm calls or hesitated before entering the nest box, the duration of disturbance was noted. CORT concentrations were not influenced by time of day, time taken to sample or disturbance duration at the nest ($t_{80} = 0.78$, $P = 0.44$, $t_{80} = 0.73$, $P = 0.47$, $t_{80} = 0.28$, $P = 0.78$ respectively). Therefore they were considered to be baseline samples.

Mothers were also weighed to the nearest 0.05g with a Pesola spring balance and wing length was measured. Maternal body condition was established by mass (g)/ wing length (mm). This metric was chosen rather than using residuals from a linear regression because mass and wing length were not significantly correlated ($t_{102} = 1.62$, $P = 0.11$). Wing length as opposed to tarsus length was employed because wing length has been shown to be a significantly more repeatable measure and therefore a better predictor of body size in passerines (Gosler *et al.* 1998). Females were aged as juveniles (<1 year) or >2 years based on plumage (Svensson 1992). Unfortunately, some birds in each year were not measured for baseline CORT and/or body condition therefore sample sizes vary between analyses.

6.3.4 Hormones assays

CORT concentrations were measured after extraction of 5-20μl aliquots of plasma in diethyl ether, by radioimmunoassay (Wingfield et al. 1992) using anti-corticosterone antiserum primary (Esoterix B183), secondary antibody (Sigma goat anti-rabbit) and [3H]-corticosterone label (GE Healthcare, UK). The extraction efficiency was 85–100%. Recoveries were measured for each sample independently and adjustments to the final assayed concentration were made. The assay detection limit (calculated as 2 SD from B0) was 0.03 ng/ml. Samples were run in three assays, intra-assay variation was 9±2% and inter-assay variation was 10±5%.

6.3.5 Statistical analysis

There were some birds captured in more than one year (n = 3), but individual birds were only included once in the analysis when they were first captured to avoid pseudo-replication.

Firstly, I analysed variation of female colouration in relation to year and age using general linear models (GLMs). Secondly, I used GLMs to investigate which factors explained variance in maternal body condition, baseline CORT and haematocrit. Body condition was the dependent variable and year, UV signal, baseline CORT, lay date, age and brood size were independent variables. When baseline CORT was the dependent variable, year, UV signal, body condition, lay date and age were independent variables. All baseline CORT data were square root transformed because of non-normality. With haematocrit as the dependent variable, year, UV signal, body condition, baseline CORT and lay date were independent variables. Age was not included in this analysis as only 2 age class 6 birds had a measure for haematocrit. Haematocrit was not an independent variable in the other two models as this metric was recorded for only a sub-sample of females (n = 66).

I also used GLMs to investigate whether maternal UV signal predicted measures of reproductive success. Lay date was the dependent variable and year, UV signal, body condition and age were the explanatory variables. Due to over-dispersion in the model a quasipoisson error structure was employed. GLMs with clutch size and number fledged as the dependent variables were used to investigate whether year, UV signal, body condition, lay date and age explained variation in these measures of reproductive success. As these reproductive measures were counts, a Poisson error structure was employed. Two-way interactions between the explanatory variables and year were fitted in all models.

Models were optimised using backward elimination of non-significant terms. For most models, I eliminated terms when this improved the AIC (Akaike Information Criteria; Burnham & Anderson 2002). Models assessing the determinants of lay date, however, were compared using ANOVA, as AIC cannot be calculated when quasipoisson error structures are used. In the ANOVA comparisons, terms remained in the model if *P*-values were found to be non-significant (>0.05). Models were validated where appropriate to verify that underlying statistical assumptions were not violated; normality was assessed by plotting theoretical quantiles versus standardised residuals (quantile-quantile plots), homogeneity of variance was evaluated by plotting residuals versus fitted values, non-linearity was evaluated by plotting residuals versus explanatory variables, and influential data points were identified using Cook's distance (Quinn & Keough 2002). There were three data points in baseline CORT models identified as outliers and removed. All statistical analyses were conducted using R version 2.8.0.

6.4 Results

6.4.1 Year & age

There were 31, 49 and 23 females measured for UV signal in 2008, 2009 and 2010, respectively. Inter-individual variation in UV signal was evident from spectrograms, specifically in the UV portion of the spectrum (figure 6.2). In addition, female UV signal differed significantly between years, with values for UV signal higher in 2009 compared with the other two years of the study (table 6.1, $t_{100} = 2.57$, $P = 0.01$). Maternal age did not explain variation in UV signal in any year (Age: $t_{99} = 1.31$, $P = 0.19$, Year x Age: $t_{97} = 0.68$, $P = 0.50$).

Table 6.1. Inter-annual variation in maternal UV signal, maternal baseline CORT and number of chicks fledged for free-living blue tits (2008-2010). Values in bold indicate years where vaules significantly differed (*P* < 0.01) from the other years shown. Values are expressed as mean±SE.

	2008	**2009**	**2010**
Maternal UV signal (UVS – SWS) / (UVS + SWS)	0.07±0.01	**0.09±0.004**	0.07±0.01
Maternal baseline CORT ng/ml	3.37±0.38	3.48±0.29	**1.37±0.21**
Number of chicks fledged	8.43±0.60	**7.53±0.59**	9.00±0.56

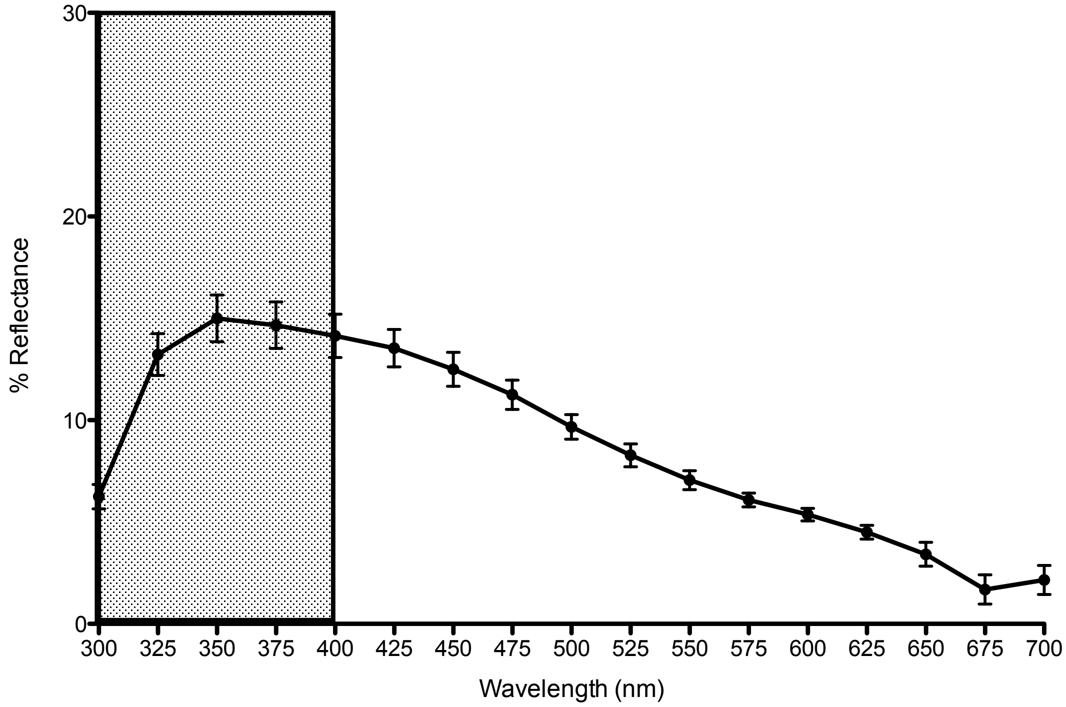


Figure 6.2. The mean crown reflectance curve of female blue tits from 2008-2010 (*n* =103). Standard errors around the means are depicted at 25-nm intervals. The shaded area indicates the UV range of the light spectrum.

6.4.2 Female condition

6.4.2.1 Body condition

In the model investigating the determinants of body condition, the term ‘Year x UV signal’ was significant indicating that the relationship between UV signal and body condition was year specific (t_{96} = 2.43, P = 0.02). Analysis of the years individually revealed that UV signal was negatively correlated with body condition in 2010 only (2008: t_{26} = 0.59, P = 0.56, 2009: t_{44} = 0.57, P = 0.57 and 2010: t_{19} = 2.43, P = 0.02). Therefore females in good body condition had lower UV signal than females in poor condition in 2010. Body condition was not related to lay date, age, baseline CORT or brood size in any year (see table 6.2).

6.4.2.2 Baseline CORT

Baseline CORT was negatively correlated with UV signal in all years (see table 6.2 & figure 6.3a). However, lay date, body condition and age were not related to baseline CORT in any year (see table 6.2). In addition, baseline CORT was significantly lower in 2010 compared with the other two years (see table 6.1 & 6.2).

6.4.2.3 Haematocrit

Haematocrit was not related to UV signal in any year (see table 6.2). Haematocrit was negatively correlated with baseline CORT in all years (see table 6.2). Lay date and body condition were not related to haematocrit (see table 6.2).

Table 6.2. The results of General Linear Models assessing the determinants of maternal body condition, baseline CORT and haematocrit in blue tits over three years (2008-2010). Factors in bold were significant, *denotes significance at *P* < 0.05 and ** denotes significance at *P* < 0.01. Models were optimised using backward elimination of non-significant terms when this improved the AIC. Terms that were removed from final models are denoted by rm. If terms were dropped the full model results are shown, if not, the final model results are shown.

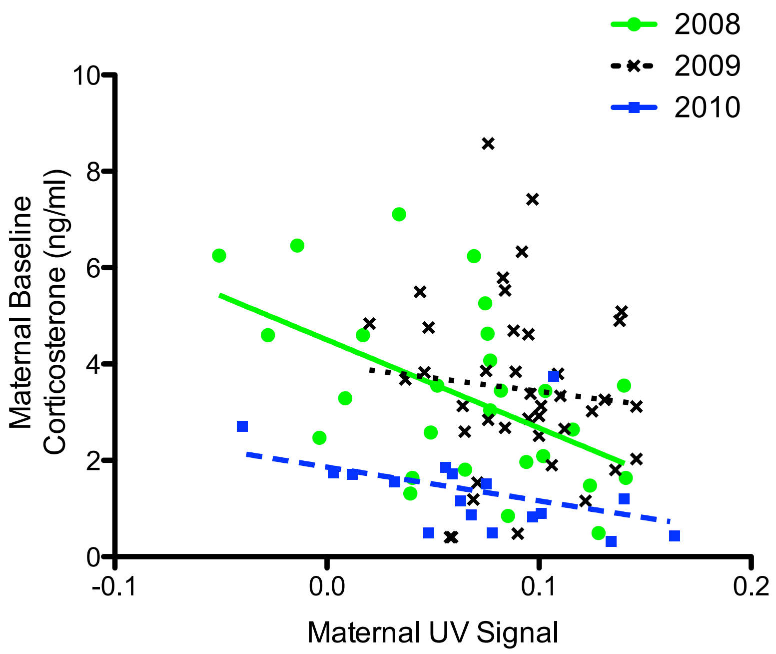
Dependent/
Independent factor

Body condition/	**SE**	***t***	***d.f.***	***P***	
Year	**0.005**	-1.805	96	0.074	
UV signal	**0.028**	0.630	96	0.530	
Baseline CORT	0.004	-1.375	59	0.174	rm
Lay date	0.001	0.218	59	0.828	rm
Age	0.003	-1.009	59	0.317	rm
Brood size	0.001	0.405	59	0.687	rm
UV signal x Year	**0.048**	**-2.431**	**96**	**0.017***	
Baseline CORT x Year	0.005	0.429	59	0.669	rm
Lay date x Year	0.001	0.506	59	0.614	rm
Age x Year	0.005	0.664	59	0.509	rm
Brood size x Year	0.001	-1.156	59	0.252	rm

Baseline CORT/	**SE**	***t***	***d.f.***	***P***	
Year	**0.146**	**-4.236**	**80**	**0.001****	
UV signal	**1.224**	**-2.869**	**80**	**0.005****	
Body condition	11.586	-1.268	68	0.209	rm
Lay date	0.028	0.380	68	0.705	rm
Age	0.194	-1.093	68	0.278	rm
UV signal x Year	3.402	1.741	68	0.086	rm
Body condition x Year	15.351	0.075	68	0.941	rm
Lay date x Year	0.039	-0.690	68	0.493	rm
Age x Year	0.259	0.450	68	0.654	rm

Haematocrit/	**SE**	***t***	***d.f.***	***P***	
Year	25.476	-0.813	43	0.421	rm
UV signal	16.030	-0.767	43	0.447	rm
Body condition	97.624	-0.753	43	0.466	rm
Baseline CORT	**0.669**	**-2.411**	**57**	**0.019***	
Lay date	0.249	-0.474	43	0.638	rm
UV signal x Year	23.944	-0.561	43	0.578	rm
Body condition x Year	129.22	0.333	43	0.741	rm
Baseline CORT x Year	7.111	-0.884	43	0.697	rm
Lay date x Year	0.340	1.736	43	0.089	rm

a)



b)

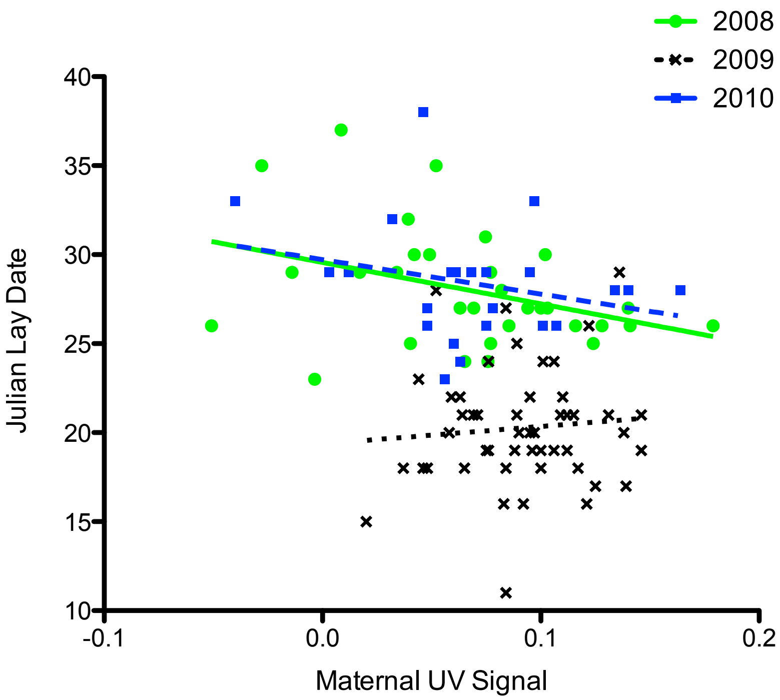


Figure 6.3. Maternal UV signal measured from crown feathers was a) negatively correlated with maternal baseline CORT measured during chick rearing in three years (*n* = 2008: 27, 2009: 40 and 2010: 17) and b) negatively correlated with Julian lay date in 2008, marginally in 2010 but not 2009 (April 1^{st} = 0, *n* = 2008: 31, 2009: 49 and 2010: 23).

6.4.3 Reproductive success

6.4.3.1 Lay date

In the model investigating the determinants of lay date, the term ‘Year x UV signal’ was borderline significant indicating that the relationship between UV signal and lay date was year specific (see table 6.3). Analysis of the years individually revealed that UV signal was negatively correlated with lay date in 2008 and marginally in 2010 but not 2009 (see figure 6.3b, 2008: $t_{27} = 2.06$, $P = 0.05$, 2009: $t_{44} = 0.78$, $P = 0.44$ and 2010: $t_{19} = 2.04$, $P = 0.06$). In addition, the term ‘Year x Age’ was borderline significant indicating that the relationship between age and lay date was also year specific (see table 6.3). Further analysis revealed that older mothers laid earlier than younger birds in 2009 only (2008: $t_{27} = 0.13$, $P = 0.90$, 2009: $t_{44} = 2.74$, $P = 0.009$ and 2010: $t_{19} = 0.16$, $P = 0.87$). Body condition was not related to lay date in any year of the study (see table 6.3).

6.4.3.2 Clutch size

UV signal did not explain variation in clutch size in any year (see table 6.3). In addition, body condition and age were not related to clutch size (see table 6.3). Clutch size was, however, negatively correlated with lay date, with earlier laying mothers producing larger clutches in all years (see table 6.3). Also clutch sizes were significantly smaller in 2009 compared with the other years of the study (see table 6.3).

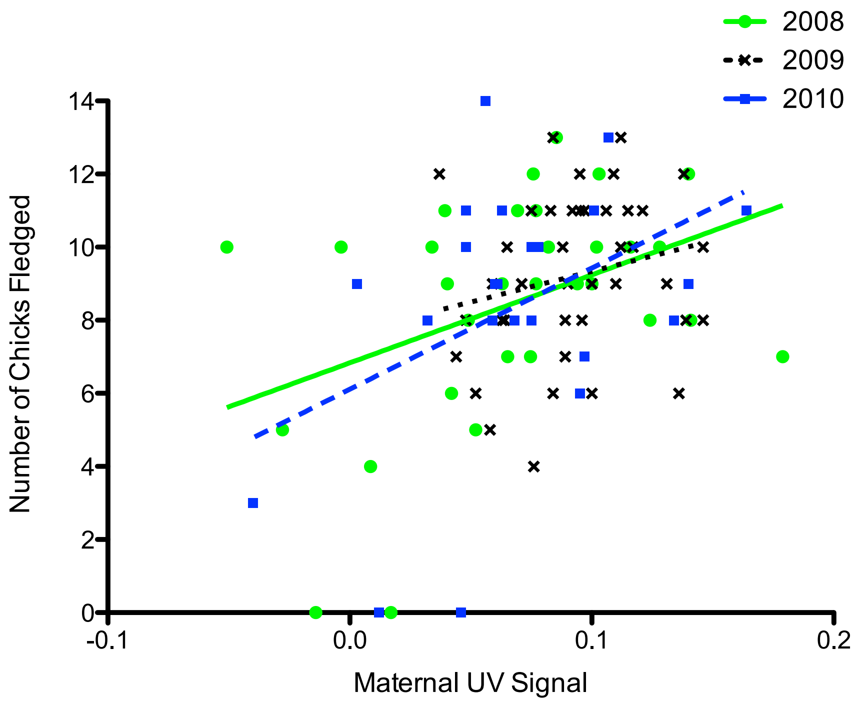
6.4.3.3 Number of chicks fledged

UV signal was positively correlated with number of chicks fledged in all three years of the study (see table 6.3 & figure 6.4a). In addition, lay date was negatively correlated with number fledged in all years (see table 6.3 & figure 4b). However, body condition and age did not explain variation in the number of chicks fledged (see table 6.3). Similar to clutch size the number of chicks fledged was significantly lower in 2009 than the other years of the study (see table 6.1 & 6.3).

Table 6.3. The results of Generalized Linear Models assessing the determinants of lay date, clutch size and number of chicks fledged in blue tits over three years (2008-2010). Factors in bold were significant, °denotes borderline significance at *P*<0.06, *denotes significance at *P*<0.05 and ** denotes significance at *P*<0.01. Models were optimised using backward elimination of non-significant terms when this improved the AIC. Models assessing the determinants of lay date, however, were compared using ANOVA. Terms that were removed from final models are denoted by rm. If terms were dropped the full model results are shown, if not, the final model results are shown.

Dependent/ Independent factor					
Lay date/	**SE**	***t***	***d.f.***	***P***	
Year	0.083	4.650	92	<0.001	
UV signal	0.430	1.990	92	0.049	
Body condition	1.649	0.704	90	0.483	rm
Age	0.045	0.095	92	0.924	
UV signal x Year	**0.867**	**1.924**	**92**	**0.057°**	
Body condition x Year	3.699	1.504	90	0.136	rm
Age x Year	**0.063**	**1.918**	**92**	**0.058°**	
Clutch Size/	**SE**	***z***	***d.f.***	***P***	
Year	**0.105**	**2.734**	**98**	**0.006****	
UV signal	1.163	0.201	86	0.841	rm
Body condition	7.212	0.063	86	0.950	rm
Lay date	**0.009**	**2.461**	**44**	**<0.001****	
Age	0.117	0.412	86	0.680	rm
UV signal x Year	**2.014**	0.895	86	0.371	rm
Body condition x Year	9.347	0.483	86	0.629	rm
Lay date x Year	0.025	0.018	86	0.986	rm
Age x Year	0.158	0.691	86	0.489	rm
Number of chicks fledged/	**SE**	***z***	***d.f.***	***P***	
Year	**0.118**	**3.254**	**89**	**0.001****	
UV signal	**0.884**	**2.405**	**89**	**0.016***	
Body condition	8.016	0.139	79	0.890	rm
Lay date	**0.016**	**4.485**	**89**	**<0.001****	
Age	0.131	1.200	79	0.230	rm
UV signal x Year	2.379	0.051	79	0.959	rm
Body condition x Year	10.610	0.515	79	0.606	rm
Lay date x Year	0.029	0.452	79	0.651	rm
Age x Year	0.179	0.991	79	0.322	rm

a)



b)

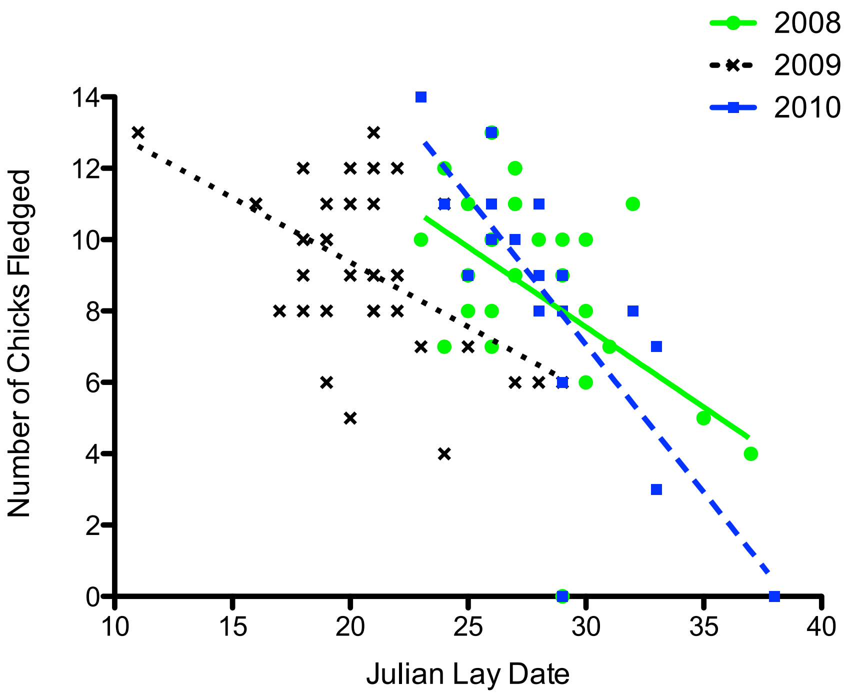


Figure 6.4. Variation in the number of chicks fledged per nest was related to a) maternal UV signal measured from crown feathers (*n* = 2008: 31, 2009: 40 and 2010: 23) and b) Julian lay date (April 1st = 0, *n* = 2008: 31, 2009: 40 and 2010: 23).

6.5 Discussion

This study shows that UV crown colouration in female blue tits varies significantly between years, but is not linked to an individual's age. In addition, in all years mothers with higher values of UV signal had lower baseline CORT than mothers with lower UV signal. However, maternal UV signal was negatively related to body condition in 2010 and unrelated to haematocrit. Maternal UV signal was also correlated with indices of reproductive success across years. Specifically, maternal UV signal was correlated with lay date in a year specific manner. Mothers with a higher UV signal laid significantly earlier compared with females with lower UV signal in 2008, but this effect was marginal in 2010 (P = 0.056), and non-significant in 2009. Lay date was also a significant predictor of clutch size and fledging number in this population. Finally, maternal UV signal was positively correlated with number of chicks fledged in all years, but not clutch size.

To my knowledge this study presents the first evidence that maternal baseline CORT is negatively correlated with UV crown colouration in free-living birds. Birds respond to environmental challenges, such as food shortage, by elevating baseline CORT concentrations, which in turn modulates both physiology and behaviour (Wingfield & Romero 2001). Therefore, elevated baseline CORT has been associated with reduced resources (Astheimer, Buttemer & Wingfield 1992; Kitaysky, Piatt & Wingfield 2007) and/or body condition in birds (Love et al., 2003; Pike & Petrie, 2005a), and in this study was associated with low haematocrit values. This suggests females that exhibit higher UV signal on their crown feathers experienced lower physiological stress during the chick rearing stage. Importantly, although baseline CORT was measured during chick rearing, it was associated with plumage colouration that was produced months before, therefore, UV signal in female blue tits may be indicative of an individual's baseline CORT over the long-term and vice versa.

Mechanistically, the link between maternal UV signal and baseline CORT could be causal. Females that exhibit low baseline CORT during breeding may also maintain low physiological stress in the face of other demanding life-history stages, such as moult. During moult when feathers are being replaced, CORT concentrations are down regulated (Romero 2002), which has been shown to aid feather growth and quality (Romero, Strochlic & Wingfield, 2005). DesRochers *et al.* (2009) provide evidence that both endogenous and exogenous CORT can influence the microstructure of feathers. As the UV reflectance of feathers is caused by their microstructure, specifically the size and arrangement of nanostructural elements within the medullary layer of feather barbs (Prum *et al.* 1999; Prum, Andersson & Torres 2003), circulating CORT concentrations could directly influence UV plumage colouration. Therefore, if females with elevated CORT during breeding also exhibit elevated concentrations during moult, UV signal and baseline CORT could be causally linked. Indeed, a recent study suggests that in blue tits nutritional deprivation during moult does not influence UV colouration, and the authors suggest physiological stress as an alternative (Peters *et al.* 2011). However, as yet researchers have not measured baseline CORT and feather colouration over multiple life-history stages or manipulated baseline CORT during moult, which would be necessary to establish causality.

Female UV signal at the population level was significantly higher in 2009 than the other two years of the study. As feathers are grown each year in the autumn, this suggests that the trait was affected by contrasting conditions between the years, and therefore may be condition-dependent. Previous studies also provide evidence for inter-annual variation in feather colouration within populations (Masello, Lubjuhn & Quillfeldt 2008; Vergara *et al.* 2009). For example, in female Eurasian kestrels, *Falco tinnunculus,* the expression of melanin based colouration co-varied positively with the environmental conditions in the previous year (Vergara *et al.* 2009). In my study, crown feathers measured for UV signal in 2009 were produced during moult in the autumn of 2008. The breeding season in 2008 was characterised by relatively high reproductive success and good weather conditions (see Chapter 3), which suggests that the favourable conditions experienced in that year may have lead to the stronger UV crown colouration within the population in 2009. However, to provide convincing evidence of the link between environmental conditions and inter-annual variation in plumage, it would be essential to monitor the population for a greater number of years and manipulate conditions within years.

Interestingly, maternal age was not linked to UV signal in any year. Age has often explained inter-individual variation in plumage colouration (for review see Amundsen & Pärn 2006). This has been suggested to occur because older individuals tend to be more dominant, and therefore may have additional access to resources, alternatively only high quality individuals may survive to older age (Brown 1975). However, in short-lived species such as the blue tit, where the average life span is three years (British Trust for Ornithology, 2011) these effects may not be apparent. In addition, in this study the relationship between age and UV signal may have been obscured because only two age classes were used. This is because plumage characteristics, do not enable the observer to exactly age birds older than one year (Svensson 1992). In order to address the relationship between UV signal and age, a population of known age individuals would be required.

Surprisingly in one year of the study, mothers with higher values of UV signal had poorer body condition during chick rearing than mothers with lower UV signal. In all years maternal UV signal was correlated with number fledged, therefore females with higher values of UV signal may have invested more energy in reproduction than those with lower UV signal, thus accounting for the negative relationship with body condition. However, this relationship was unexpected as females with high UV signal had low baseline CORT concentrations, which would suggest they had superior energetic status. Yet, body condition and baseline CORT were not correlated in this population (see Chapter 4; General Discussion) suggesting they may be indicative of differing aspects of condition during chick rearing. For example, body mass, a component of body condition, is often lowest and least variable during chick rearing (Macleod *et al.* 2005), hence may not be the best measure of condition during this stage.

Lay date is often a strong predictor of reproductive success in blue tits (Perrins 1979), and in this study lay date was positively correlated with clutch size and number fledged in all years. As individual condition and food quality can influence structural feather colouration (Siefferman & Hill 2005b; Masello, Lubjuhn & Quillfeldt 2008), females that can defend resources, forage effectively and maintain good body condition during moult could produce feathers with a relatively strong UV signal (Keyser & Hill 1999). Furthermore, these females may be more able to maintain their condition over winter, and thus begin breeding earlier (Nager, Rueegger & Van Noordwijk 1997; Ramsay & Houston 1997). This result suggests that UV signal may reflect maternal quality in blue tits, however, the relationship between maternal UV signal and lay date was weak and requires further investigation to establish the strength of this association.

Maternal UV signal was positively correlated with the number of chicks fledged rather than clutch size in all years. This result suggests that mothers with a stronger UV signal did not differ in the number of eggs laid, but rather they were able to rear a greater number of chicks to fledging. This may have been because maternal UV signal reflects the quality of the eggs laid or the level of investment in incubation. Egg quality and incubation behaviour have important consequences for the fledging success of nestlings (Reid, Monaghan & Nager 2002; Nager 2006), yet little is known about the relationship between maternal plumage colouration and these early stages of reproduction. In a recent study the intensity of UV crown colouration in female blue tits was found to be positively correlated with egg size (Szigeti *et al*. 2007). Therefore, differential egg quality dependent upon maternal colouration could lead to a relationship between this UV signal and fledging success. Also, the relationship between maternal UV signal and fledging number could have been caused by maternal provisioning behaviour. Previous studies provide correlative evidence that provisioning behaviour is linked to maternal colouration (Linville, Breitwisch & Schilling 1998; Siefferman & Hill 2005b), but a similar number of studies do not support this relationship (Rhode, Johnsen & Lifjeld 1999; Smiseth & Amundsen 2000; Hill 2002). Of course these factors are not mutually exclusive and further investigation into the relationship between maternal UV signal and egg quality, incubation and provisioning behaviour is required to explain the link between maternal UV signal and fledging number in this population.

Maternal UV signal may not simply reflect female reproductive capacity but also the quality of care provided by her partner. There is evidence that assortative mating occurs in blue tits with regard to UV crown colouration (Andersson, Örnborg & Andersson 1998; Hunt *et al*. 1999), thus maternal UV signal may have reflected the UV signal of her partner. There is evidence that males that exhibit brighter UV colouration provision nestlings more frequently (Keyser & Hill 2000; Siefferman & Hill 2003) and provide a higher number of food items to incubating mothers (Siefferman & Hill 2005a) than less colourful males. In blue tits, male UV colouration has also been positively associated with female provisioning rate (Limbourg *et al.* 2004) and the deposition of androgens within the eggs (Kingma *et al.* 2009). Therefore, male UV signal could influence both nestling quality directly through paternal behaviour or indirectly through its effects upon maternal behaviour and egg deposits. Unfortunately, in this study very few male birds were captured, consequently, I cannot exclude the possibility that the relationship between maternal colouration and fledging success is not an indirect effect of paternal quality. However, for a sub-sample of breeding pairs, both male and female UV signal was measured and these data do not present strong evidence for assortative mating in this population (GLM: $r = -0.55$, $n = 14$, $P = 0.98$). Importantly, even if information regarding male partners were available, it would be difficult to separate the effects of male and female investment upon reproductive success in species where both sexes provide care, particularly as brood care is often correlated between parents (Hinde 2006).

6.5.1 Conclusions

This study presents intriguing results, that female plumage colouration is associated with baseline CORT and signals reproductive potential. However, the presence of confounding factors, such as male quality, causes the link between maternal UV signal and fledging number to require further investigation before definite conclusions can be drawn. The fact a single measure of baseline CORT measured during chick rearing can be indicative of plumage colouration produced months before, suggests baseline CORT may provide long-term information regarding individual condition.

Chapter 7: General Discussion

> ***"The more you know, the more you realise you know nothing".***
> **- Socrates**

The measurement of glucocorticoids, specifically CORT in free-living birds has been conducted for over 30 years (Wingfield & Farner 1976a), and a great deal of information has been gained in relation to their function and mediation of life-history traits (Williams 2008; Wingfield, Williams & Visser 2008). However, the majority of studies continue to compare mean baseline CORT concentrations between groups and populations, and the causes and consequences of inter-individual variation in baseline CORT remain relatively unknown (Williams 2008). The physiology/life-history nexus suggests that in order to understand variation in life-history traits and the constraints imposed upon them, an understanding of their underlying physiological mechanisms is essential (Ricklefs & Wikelski 2002; Hau 2007; Hau *et al.* 2010). Furthermore, to do so it is necessary to relate baseline CORT to key-life history traits at the individual level (Ricklefs & Wikelski 2002; Hau 2007; Hau *et al.* 2010). In this thesis I have employed this approach and investigated how variation in environmental conditions measured at the territory-scale explains variation in baseline CORT concentrations between individuals. Furthermore, I investigated whether inter-individual variation in baseline CORT was linked to measures of reproductive success, reproductive trade-offs, offspring phenotype and UV plumage colouration. Importantly, I related baseline CORT to these key life-history traits across multiple years that differed in the quality of the prevailing conditions. The results suggest that the relationships between baseline CORT, environmental conditions and reproductive success are context-dependent, as they varied between years dependent upon the conditions experienced. Within the endocrinology literature the context-dependence of relationships between CORT and other physiological measures are considered fundamental for the understanding of the actions of CORT (Sapolsky 2002). For example, a short term challenge and a transitory increase in circulating CORT concentrations enhances the immune system, whereas prolonged elevation of CORT in response to a chronic challenge suppresses it (Munck, Guyre & Holbrook 1984a). Furthermore, as CORT elevation can negatively influence the immune system, birds living where parasite threats are high may maintain low concentrations of CORT to conserve their immune defenses. Indeed exogenous elevation of CORT in a population of house sparrows, *Passer domesticus*, in the tropics (Panama) where parasite threats are high did not affect immune function, whereas in a population in North-America (New Jersey) where parasite threats are lower, immune function was suppressed. These issues are rarely investigated or accounted for in studies that address the link between CORT and reproductive success. However, as my results show the relationship between baseline CORT and key life-history traits may also differ dependent upon the prevalent conditions. Overall, this thesis provides evidence that inter-individual variation in baseline CORT between breeding birds is significant, and it is important to consider the prevalent conditions when trying to explain this variation.

7.1 Review of findings

7.1.1 Environmental conditions and baseline CORT at the individual level

There is a large body of evidence that suggests inclement environmental conditions are associated with elevated baseline CORT in birds (Wingfield, Smith & Farner 1982; Romero, Reed & Wingfield 2000; Suorsa *et al*. 2003; Cash & Holberton 2005; Kitaysky *et al*. 2006; Buck, Oreilly & Kildaw 2007; Kitaysky, Piatt & Wingfield 2007; Benowitz-Fredericks, Shultz & Kitaysky 2008; Busch & Hayward 2009). This is thought to be due to the role of baseline CORT in metabolism and energy-balance, with elevated baseline CORT stimulating behaviour and/or gluconeogenesis to resolve a deficit in energy resources (Wingfield & Romero 2001; Sapolsky 2002). However, there is a striking lack of knowledge concerning the influence of environmental factors upon baseline CORT at the individual level (but see Suorsa *et al*. 2003). This is of fundamental importance, not only when attempting to identify factors within the environment that influence stress hormones, but also when trying to ascertain whether baseline CORT mechanistically links poor environmental conditions with the costs of reproduction and reproductive success. In Chapter 2 I addressed these knowledge gaps by investigating whether foraging conditions at the territory-scale were correlated with adult and nestling baseline CORT over three years (2008-2010). I measured synchrony between breeding and the peak in caterpillar abundance, the main prey of provisioning blue tits (Perrins 1991), oak density, which affects the availability of caterpillars (Hinsley *et al*. 2008; Wilkin, King & Sheldon 2009) and weather variables that can influence foraging efficiency (Romero, Reed & Wingfield 2000; Geiser, Arlettaz & Schaub 2008).

The results showed that synchrony between breeding and the peak in caterpillar abundance negatively correlated with baseline CORT for both nestling and adult birds, but only in 2008, when birds were most asynchronous with the caterpillar peak. Whereas, oak density was negatively correlated with adult baseline CORT in the two years birds were most asynchronous (2008 & 2010), but was not related to nestling CORT in any year. Weather variables were also related to baseline CORT in adults only, with rainfall positively correlated with baseline CORT only in the year characterised by the most rainfall (2009), and low temperatures were negatively correlated with elevated baseline CORT in the two years of the study characterised by either the most severe asynchrony or rainfall. These results suggest that foraging conditions are associated with baseline CORT concentrations when they are more demanding. For example, synchrony between breeding and the peak in caterpillar abundance was only linked to baseline CORT in the years birds were most asynchronous, suggesting that there is a threshold level over which asynchrony elicits a physiological response in breeding birds. Furthermore, the effects of the foraging conditions measured appear to be additive and/or synergistic, e.g. low temperatures were only linked to adult baseline CORT in two years characterised by either the greatest asynchrony or the heaviest rainfall. A study on nestling treecreepers, *Certhia familiaris*, provides a similar result, as low density and quality of prey was linked to elevated CORT in young, in small forest patches only, but not in older, larger forest patches (Suorsa *et al*. 2003). These results advocate measuring multiple surrogates of environmental conditions over multiple years, as this allows researchers to establish whether there are potentially additive or synergistic effects of environmental factors, threshold levels over which stress hormones are elevated, or consistent effects of specific environmental factors associated with CORT concentrations. In addition, by first establishing the correlative links between foraging conditions and baseline CORT, experimental approaches required to elucidate the causal effects will be better informed, providing information on how best to manipulate the environment.

In addition, my results suggest that the foraging conditions that influence adult and nestling baseline CORT differ. This is an interesting finding and perhaps not unexpected, as blue tits are cavity nesting and therefore chicks are somewhat shielded from the prevalent conditions (Perrins 1979). Importantly this suggests that in cavity nesting birds it is not necessarily possible to draw conclusions about the effect of the breeding habitat upon nestling baseline CORT from the effects upon adult CORT and vice versa. Also, this result suggests that parental behaviour as well as environmental conditions may influence baseline CORT concentrations in nestlings. For example, although oak density was linked to adult baseline CORT in the two years in which birds were most asynchronous, it was not linked to nestling CORT. This suggests that parents in more oak sparse territories worked harder in these years to provide nestling with similar amounts of food. Therefore, in addition to the nest environment, parental behaviour may buffer chicks from inclement conditions (Kitaysky, Wingfield & Piatt 2001; Tremblay *et al.* 2005). Of course there may also be a threshold over which parental behaviour can no longer buffer the nestling from the prevalent conditions, for example, in 2008 when synchrony between breeding and the caterpillar peak was linked to nestling CORT. In addition, in the Florida scrub-jay, *Aphelocoma coerulescens*, the longer parents spent away from the nest was associated with a significant increase in nestling CORT concentrations. It would be interesting to investigate whether provisioning behaviour is linked to nestling baseline CORT in blue tits, and whether this relationship differs between years dependent upon the environmental conditions.

7.1.2 Inter-individual variation in baseline CORT and reproductive success

In agreement with the literature (Marra & Holberton 1998; Kitaysky *et al.* 2001; Homan, Reed & Romero 2003; Cash & Holberton 2005; Kitaysky, Piatt & Wingfield 2007), the results of Chapter 2 indicate that inclement conditions experienced during breeding are associated with elevated baseline CORT in breeding birds. As poor environmental conditions are also associated with low reproductive success (Keller & Van Noordwijk 1994; Naef-Daenzer & Keller 1999; Garant *et al.* 2007; DeGabriel *et al.* 2009; Grimardias *et al.* 2010), it has been suggested that elevated CORT in breeding adults may be linked to reduced reproductive success (Bonier *et al.* 2009a; Busch & Hayward 2009). This may be adaptive, as birds experiencing poor conditions and elevated CORT may invest in self-maintenance over reproduction and through doing so improve their lifetime reproductive success (LRS). In Chapter 3 I investigated whether there was evidence of a link between low reproductive success and elevated baseline CORT over multiple years. I also examined whether this was because the foraging conditions (as measured in Chapter 2) that were associated with elevated maternal baseline CORT were similarly linked with low reproductive success.

The results showed that in the most part the foraging conditions that were associated with elevated baseline CORT differ to those associated with low reproductive success. In 2008 and 2010, the years characterised by the greatest asynchrony between breeding and the caterpillar peak, broods that were more synchronous fledged more offspring, a higher proportion of the clutch (marginally non-significant in 2010) and had heavier chicks than less synchronous broods. However, oak density was not linked to reproductive success. Synchrony with the caterpillar peak was also associated with low maternal baseline CORT, but in 2008 only, the most asynchronous year. Whereas, in 2008 and 2010, mothers breeding in oak dense territories had significantly lower baseline CORT than mothers in oak sparse territories. In 2009 the wettest year of the study, inclement weather conditions (high rainfall and low temperatures) were associated with elevated maternal baseline CORT and reduced mean nestling mass. These results indicate, that in female blue tits, the foraging conditions associated with reduced reproductive success are not consistently those linked to elevated maternal baseline CORT. Specifically, maternal baseline CORT appears to be linked with factors that affect energetic demand, i.e. movement between trees, rather than reproductive success, i.e. total number of prey provided to offspring. However, inclement weather conditions appear to link maternal baseline CORT and nestling mass, as nesting mass was negatively correlated with maternal baseline CORT in 2009.

Interestingly, in 2009 when reproductive success was lower than the other years, suggesting it was the harshest year for breeding birds, maternal baseline CORT was positively correlated with the number of chicks fledged. Brood rearing is a demanding breeding stage (Perrins 1991; Tremblay *et al*. 2005), and the positive correlation between fledging number and maternal baseline CORT may be due to the increased energetic demand associated with the need to provide for a larger brood, particularly when conditions are challenging (Saino *et al*. 2002; Bonier, Moore & Robertson 2011). Indeed, in 2009 provisioning rate per brood was positively correlated with maternal baseline CORT (Chapter 5). The result is not unprecedented, as previous studies report a negative relationship between CORT during egg-laying and reproductive success, but a positive relationship between CORT during brood rearing and reproductive success in both populations and individuals (Bonier *et al*. 2009b; Ouyang *et al*. 2011). This has been suggested to be adaptive as individuals that begin breeding in good condition with lower CORT, may be able to invest more in egg laying than birds in poorer condition with higher baseline CORT concentrations. In this case, this may lead to the negative relationship between CORT and reproductive success during egg-laying. However, during brood rearing when individuals have already invested heavily in the reproductive event, good quality individuals that have reared larger broods may be able to increase CORT concentrations to meet the needs of brood rearing, causing baseline CORT to positively correlate with reproductive success during this stage. As maternal baseline CORT was positively correlated with number fledged but not the proportion of the clutch to fledge, this suggests that elevated baseline CORT in mothers fledging more offspring was not through increased work to fledge a higher proportion of the clutch, but rather mothers that laid larger clutches may have been able to elevate baseline CORT to meet the needs of their offspring. Indeed, maternal condition can modify the effects of experimental CORT elevation. In Black-legged kittiwakes, *Rissa tridactyla*, experimental CORT elevation causes breeding adults in superior condition to invest in brood care, whereas adults in poor condition invested in self-maintenance over brood care.

The results of Chapter 3 also suggest that the relationship between CORT and reproductive success may vary between years dependent upon the prevalent conditions. For example, elevated baseline CORT may only be associated with brood size in years where conditions are most challenging, and the energetic demands of the parents are higher. In an island population of blue tits, maternal baseline CORT was positively correlated with the number of nestlings, but this relationship was not evident in a population on the mainland (Müller *et al.* 2007). The authors suggest this was because the energetic demands of provisioning were greater in the island population compared with mainland (Müller *et al.* 2007). This result may explain why studies based on a single year, whether experimental or correlative have not consistently found correlations between maternal baseline CORT and reproductive success (Saino *et al.* 2002; Eeva *et al.* 2005; Ellenberg *et al.* 2007).

Importantly if these data were only analysed at the population level, the conclusions would have been different. 2008 and 2009 had significantly higher mean maternal baseline CORT than 2010, and these two years also had lower mean reproductive success compared to 2010 (see figure 3.1, number fledged significantly lower in 2009 only). This result would support the association between elevated baseline CORT and low reproductive success. Therefore, my results highlight not only the complexity of linking baseline CORT to measures of fitness, but also the importance of using inter-individual variation in addition to population means. In conservation led studies, CORT concentrations are increasingly used as a proxy of population or individual health, with elevated CORT assumed to be associated with reduced survival or reproductive success (Busch & Hayward 2009). However, my results emphasize the importance of validating the relationship between baseline CORT and measures of fitness before employing measures of CORT in this way.

7.1.3 Inter-individual variation in baseline CORT and brood sex ratio

Chapter 3 indicates that maternal baseline CORT is not consistently linked to number of chicks fledged over years. However, maternal CORT could adaptively influence other aspects of offspring phenotype, to the benefit of offspring and mothers (Love *et al.* 2005; Breuner 2008). Sex allocation theory predicts that mothers should adjust brood sex ratio in relation to their condition and/or the prevalent conditions to maximise fitness (West, Reece & Sheldon 2002). As maternal CORT links the quality of the environment with internal physiology (Wingfield & Romero 2001), circulating concentrations may signal conditions that influence the quality of offspring. Indeed in birds there is evidence that mothers adjust the sex of offspring in relation to their condition and/or environment (Sheldon *et al.* 1999b; Komdeur, Magrath & Krackow 2002a; Pike & Petrie 2005a; Stauss *et al.* 2005; Pryke *et al.* 2011) and that maternal concentrations of CORT play a role in this adjustment (Love *et al.* 2005; Pike & Petrie 2005a; Bonier, Martin & Wingfield 2007). Therefore, in Chapter 4 I investigated whether maternal baseline CORT was linked to brood sex ratio at laying (primary) and/or fledging (secondary), and if experimental elevation of maternal CORT during laying influenced brood sex ratio.

The results suggest that maternal baseline CORT measured during brood rearing was not related to the sex ratio of offspring at laying or fledging, but was negatively correlated with nestling mass on day 14. Exogenous elevation of maternal CORT during egg laying did not influence offspring sex, nestling mass or nestling growth. However, maternal condition was correlated with primary sex ratio, with mothers in good condition producing male biased broods in one year of the study, and in all years, there was a trend that maternal condition was positively correlated with nestling mass and growth. Although male nestlings were consistently heavier and grew at a faster rate than female nestlings, maternal condition and baseline CORT were not related to indices of nestling quality in a sex-specific manner.

My results are in contrast to previous findings, as all reported studies that have investigated the link between maternal baseline CORT and brood sex ratio have found significant correlations (Love *et al.* 2005; Pike & Petrie 2005a; Pike & Petrie 2006; Bonier, Martin & Wingfield 2007; Pryke *et al.* 2011). Unlike previous studies (Love *et al.* 2005; Pike & Petrie 2005a) maternal baseline CORT was not linked to maternal condition in any year, therefore may not be indicative of circumstances that favour sex ratio adjustment, and thus may not be expected to affect brood sex ratio. Furthermore, in the blue tit, mothers may not benefit from sex ratio adjustment. Although the sexes are size dimorphic the difference was relatively small (~5%), and it is unclear whether males, as the larger sex would be more sensitive to poor developmental conditions and thus have lower LRS when conditions are poor. Also the level of extra-pair paternity often differs between years and populations in blue tits (Gullberg, Tegelström & Gelter 1992;

Charmantier *et al.* 2004), thus variance in reproductive success between the sexes may not be as extreme as in other species, perhaps reducing the benefits of investing in sons when the conditions ensure they are of good quality. As with the majority of studies that have addressed sex ratio adjustment in birds, the studies which have related maternal CORT to sex ratio manipulation have yet to find evidence for the selective advantage of this adjustment (Pike & Petrie 2003), and perhaps this should be a focus of future research. Specifically, for my research it would have been interesting to know whether sexes differed in their survival and reproductive success dependent upon maternal condition and/or the environmental conditions. However, there is no reason to believe brood sex ratio should be evident across all bird species (West & Sheldon 2002).

I employed mealworms injected with CORT to experimentally elevate maternal CORT during egg-laying and test the effect on brood sex ratio and nestling condition. Unfortunately, there were a few drawbacks associated with this method. For example, I was not able to guarantee when and if females consumed the mealworms. As the cameras I used for nest recording could only be placed at the back of the nest box, it would have been difficult to see females eating mealworms from feeder trays placed on the side. If they had been placed on the lid looking down, I may have been able to document females eating the mealworms on the nest. The technique did successfully cause a temporary elevation of CORT in captive blue tits (Appendix I). Therefore, under lab conditions where females can be closely monitored it would be interesting to employ this method, perhaps in a species where CORT has already been shown to be linked to brood sex ratio. This would provide insight into whether transitory elevation of CORT during sex determination, as well as the continuous elevation used in previous studies could influence brood sex ratio. As continuous elevation of CORT may also influence maternal condition (Love *et al*. 2005; Pike & Petrie 2005a), this approach could separate the effects of maternal condition and CORT upon brood sex ratio adjustment.

7.1.4 Inter-individual variation in baseline CORT and reproductive trade-offs

The cost of reproduction is a central concept in evolutionary biology, where increased investment in current reproduction is predicted to decrease future fecundity and survival (Lack 1947; Williams 1966). The physiological mechanisms that mediate these life-history trade offs are less well known (Zera & Harshman 2001), but CORT may play a significant role in life-history trade-offs and reproductive decisions based around energetic state and/or the prevalent environmental conditions. As discussed above, in a context-dependent manner, elevated baseline CORT can be linked to increased investment in reproduction or a diversion of investment from reproduction to self-maintenance (Wingfield & Sapolsky 2003). In addition, mechanistically, elevated baseline CORT during breeding due to increased investment, could negatively influence reproduction in future years as elevated CORT has been associated with a reduced survival (MacDougall-Shackleton *et al.* 2009; Goutte *et al.* 2010) and decreased disease resistance (Harvey *et al.* 1984; Saino *et al.* 2002).

To investigate the role of CORT in reproductive trade-offs I reduced the costs of egg laying through supplemental feeding and compared maternal baseline CORT, brood care and maternal return rates between manipulated and control mothers. The results showed that supplementary feeding during egg laying improved nestling mass, influenced incubation and brooding behaviour and increased maternal return rates relative to controls. Therefore this suggests reducing the costs of early reproduction influenced investment in the later stages of the reproduction and increased maternal survival. Maternal baseline CORT concentrations, however, were unaffected by the treatment. While provisioning rate did not differ between groups, there was a positive correlation between provisioning rate and baseline CORT for controls only, suggesting that the treatment negated the relationship between maternal baseline CORT and provisioning rate. In this case, supplemented mothers with higher provisioning effort would not have suffered any costs associated with increased CORT, which in turn, may have been beneficial for their survival.

A recent study suggests elevating investment in current reproduction through increasing brood size is associated with elevated baseline CORT (Bonier, Moore & Robertson 2011). However, the authors did not investigate whether this had consequences for survival or future reproductive success. Alternatively, elevated CORT during reproduction may be beneficial for survival and thus reproductive success, by matching offspring need to maternal ability to provide (Breuner 2008). Mothers with high baseline CORT during egg laying may be of lower quality, and less able to invest in reproduction than mothers with lower baseline CORT. In turn, elevated maternal CORT during egg laying has been shown to reduce hatching success and nestling growth (Hayward & Wingfield 2004; Love & Williams 2008b). In which case, mothers with high baseline CORT during egg laying may produce less demanding broods than mothers with low baseline CORT, and potentially not suffer costs for their future reproduction. To test this, Love & Williams (2008b) elevated maternal CORT during laying to produce offspring that may be less demanding. In addition, they clipped the wings of half the CORT elevated and control mothers to reduce their provisioning ability. This created four treatment groups; CORT-Clipped, Control-Clipped, CORT-NonClipped and Control-NonClipped. The results showed that in the un-matched group (Control-Clipped), mothers had lower return rates in the following year than all the other groups. The authors suggest this was because in the CORT-Clipped group broods were smaller due to nestling mortality and nestlings grew at a slower rate, therefore, raising these broods was less demanding. This suggests that CORT may match nestling demand with maternal ability to provide, stabilizing the costs of reproduction between mothers regardless of their condition. My results and these previous findings suggest the role of CORT in reproductive trade-offs requires further investigation and should be an exciting line of research over the coming years.

7.1.5 Baseline CORT and feather colouration

Stress hormones can be key mediators of condition-dependent traits that serve as honest signals of quality (Husak & Moore 2008; Moore & Hopkins 2009). These include song quality (Spencer *et al*. 2003; Buchanan *et al*. 2004), ornament size (Douglas *et al*. 2008) and feather colouration (Roulin *et al*. 2008). However, this line of research is in its infancy and especially lacking are examples of the role of CORT in mediating feather colouration (Roulin *et al*. 2008). Roulin *et al*.

(2008) provide evidence that manipulating CORT concentrations can influence the expression of melanin based feather colouration, in barn owls, *Tyto alba*, a species showing heritable variation in the degree of phaeomelanism. The authors show that experimental elevation of CORT in nestlings during feather growth caused them to produce feathers with less phaeomelanic colouration than controls. Mechanistically, there is reason to believe CORT concentrations could directly influence melanin based colouration as elevated glucocorticoids have inhibiting effects upon melanogenesis (Slominski *et al*. 2004). In structural feather colouration such as the UV colouration in blue tits, the rate and consistency of feather growth has been shown to influence the expression of UV colouration (Shawkey *et al*. 2003) and CORT has been shown to influence feather growth (Romero, Strochlic & Wingfield 2005; DesRochers *et al*. 2009). Therefore, variation in baseline CORT between individuals has the potential to influence UV colouration, and in chapter 6 I investigated whether UV crown colouration in female blue tits was correlated with baseline CORT. The results showed that UV crown colouration in female blue tits varied significantly between years. In addition, in all years mothers with higher values of UV signal had lower baseline CORT than mothers with lower UV signal. As far as I know this is the first evidence of a link between inter-individual variation baseline CORT and UV feather colouration in birds. Importantly, CORT was measured during breeding, months after the autumn moult when feathers were produced, raising interesting questions about how and why this correlation might exist. If baseline CORT concentrations are consistent within individuals, females that exhibit high baseline CORT during reproduction may also do so during moult. In this case inter-individual variation in CORT may be mechanistically linked to UV feather colouration. However, through the link between CORT and energy-balance, the correlation between the hormone and UV colouration may simply be because it is linked to other aspects of individual quality, such as ability to forage for and defend food resources, as diet and nutritional status have also been linked to structural colouration in birds (McGraw *et al*. 2002; Siefferman & Hill 2005; but see Peters *et al*. 2011). Either way this result suggests that a single measure of baseline CORT can reflect feather colouration laid down months before, providing new insight into how inter-individual variation in CORT concentrations can be associated with longer-term indicators of quality. However, experimental studies are required to establish a causal link between UV plumage colouration and circulating CORT concentrations.

7.2 Baseline CORT: a measure of condition?

There is evidence that baseline CORT is often correlated with measures of body condition in birds (Marra & Holberton 1998; Lormée *et al*. 2003; Chastel *et al*. 2005; Love *et al*. 2005; Pike & Petrie 2005a; Williams *et al*. 2008). As CORT regulates metabolism and energy-balance, it is understandable that a measure of condition based upon mass or fat score may be linked to CORT. However, my findings do not support a link between maternal body condition (Wing (mm)/ Mass (g)) and baseline CORT measured over three years. Importantly, my study was conducted in brood rearing blue tits, and a literature review would support my finding that the correlation between maternal body condition and baseline CORT may not be evident in brood rearing birds (see table 7.1, review includes the first papers presenting data regarding the relationship between baseline CORT and body condition from a Google Scholar search (Search terms = corticosterone AND body condition AND bird – date searched September 2011). For example, in the black-legged kittiwake, a negative correlation between body condition and baseline CORT that was evident during the earlier stages of reproduction in one study (Kitaysky, Wingfield & Piatt 1999), was not evident during the later stages of reproduction in another study (Chastel *et al*. 2005). In addition, there is evidence that body condition modulates the influence of CORT upon activity budgets in brood rearing birds (Angelier *et al*. 2007). To examine how experimental elevation of CORT affected the activity budget of male black-legged kittiwakes, researchers used miniaturized activity loggers to record behaviour (Angelier *et al*. 2007). The results showed that the effect of the CORT treatment upon time-spent flying/foraging was dependent upon body condition, as CORT-implanted males in good condition spent more time flying/foraging than controls, whereas poor condition males did not (Angelier *et al*. 2007). Therefore, elevated CORT for birds in superior body condition may cause an increase in the time spent foraging and investment in brood provisioning, whereas poor body condition birds may invest in self-maintenance rather than provisioning. Overall birds that begin provisioning in superior condition may lose more mass than those in poorer condition, due to a difference in their activity budgets (Angelier *et al.* 2007). Indeed in the black-legged kittiwake, birds that were in better condition at the start of recording lost more mass than birds in poorer condition (Angelier *et al.* 2007). This may explain why in the later stages of reproduction body condition is not consistently correlated with baseline CORT.

Although my results do not provide evidence for a correlation between maternal body condition and baseline CORT during brood rearing, this does not mean it may not be evident at alternative breeding stages or seasons in this species. Importantly, baseline CORT can still be regarded as a measure of condition, without necessarily being correlated with body condition. In fact, baseline CORT may be a more holistic measure of condition, as it reveals an individual's response to various factors that simultaneously constitute their "condition", i.e. external factors such as, habitat quality (Marra & Holberton 1998; Müller *et al.* 2007) and food abundance (Kitaysky *et al.* 2006; Kitaysky, Piatt & Wingfield 2007) and internal factors such as immune function (Saino *et al.* 2002) and energy-balance (Romero 2004).

Table 7.1. A summary of studies relating body condition to baseline CORT in adult birds. Direction of correlation denoted by; **+ve** indicates a positive correlation, **-ve** a negative correlation and **ns** a non-significant correlation. Measurement employed column shows the method used to establish body condition, PCs = principal component score.

Breeding stage / Season	**Species** (Sample size)	**Direction of Correlation**	**Measurement employed**	**Source**
Egg-laying	European starling, *Sturnus vulgaris* (*n* = 35)	**- ve**	Residuals from a regression of body mass against first PCs calculated from exposed culmen, tarsus and wing length	Love *et al.* (2005)
Incubation and early brood rearing	Black-legged kittiwakes, *Rissa tridactyla* (*n* = 46)	**-ve**	Ratio of body mass and (bill length + tarsus length)	Kitaysky, Wingfield & Piatt (1999)
Incubation and early brood rearing	Peafowl, *Pavo cristatus* (*n* = 11)	**-ve**	Residuals from a regression of body mass against tarsus length	Pike & Petrie (2005a)
Brood rearing	Tufted puffin, *Fratercula cirrhata* (*n* = 94)	**ns**	Residuals from a regression of body mass against first PCs calculated from bill, tarsus and wing length	Williams et al. (2008)
Brood rearing	Black-legged kittiwakes, *Rissa tridactyla* (*n* = 24)	**ns**	Residuals from a regression of body mass against skull length	Chastel et al.(2005)
Brood rearing	Blue tit, *Cyanistes caeruleus* (*n* = 79)	**ns**	Ratio of body mass to wing length	Chapter 4
All breeding stages	Red-footed boobie, *Sula sula* (*n* = 103)	**ns**	Residuals from a regression of body mass against body size (Females: tarsus length, Males: wing length)	Lormée et al.(2003)
Non-breeding season (winter)	American redstart, *Setophaga ruticilla* (*n* = 84)	**-ve**	Residuals from a regression of body mass against first PCs calculated from tarsus and wing length	Marra & Holberton (1998)

7.3 Inter-individual variation in baseline CORT

I measured baseline CORT at a standardised point during brood rearing (day 5-7 after hatching) in blue tits over three years, and there was significant evidence of inter-individual variation in this hormone titer. Furthermore, although mean concentrations differed significantly between the years (see figure 4.5a, GLM: $t_{86} = 4.41$, $P < 0.001$), the variation between individuals was similar across the three years (mean over three years in table 7.2, 2008: 0.5-14.0 ng/ml, 28-fold, 2009: 0.4-12.3 ng/ml, 30-fold, 2010: 0.3-9.6 ng/ml, 30-fold). Baseline CORT concentrations are inherently variable, showing marked diurnal, age-dependent and seasonal variation (Wingfield & Romero 2001). Therefore, standardization is difficult, especially in field studies, but by attempting to do so, it is clear large inter-individual variation exists and can explain variation in environmental conditions (Chapter 2), reproductive success (Chapter 3 & 4), behaviour (Chapter 5) and expression of UV feather colouration (Chapter 6).

A recent review of the data regarding inter-individual variation in baseline CORT confirms that there is large variation in baseline CORT, varying 6- to 25-fold among individuals for a given physiological state in both free-living and captive birds (see table 7.2). For example, in the European starling there is evidence of a 25-fold difference between individuals measured during breeding (see table 7.2, Love *et al.* 2004). In fact, inter-individual variation in hormone titres may be greater than for other physiological traits, such as heat shock proteins, basal metabolic rate and carotenoids (Williams 2008). There is also evidence that the duration and magnitude of elevated CORT concentrations (stress-induced) in response to a standardised stressor is also variable between individuals (Appendix I; Cockrem & Silverin 2002), and has lead to the suggestion that in addition to variation in baseline CORT, variation in the stress-response between individuals may be linked to fitness (Breuner, Patterson & Hahn 2008). For example, a rapid increase in CORT and a fast induction of negative feedback, causing CORT to return to baseline concentrations quickly after the stressor, is generally considered a ‘good’ response (Breuner, Patterson & Hahn 2008). This is because an immediate increase can facilitate a quick behavioural and physiological response, while fast clearance of elevated CORT concentrations would alleviate any negative effects of sustained CORT (Breuner, Patterson &

Hahn 2008). However, whether inter-individual variation in the stress-response is linked to indices of fitness is still unclear (Breuner, Patterson & Hahn 2008).

Table 7.2. Examples of the magnitude of inter-individual variation for plasma baseline CORT, modified from Williams (2008). **NB** some values are taken from scatter plots.

Range	**Difference**	**Standardised conditions or state and species**	**Captivity (C)/ free-living (F)**	**Reference**
0.6–10.4 ng/ml	15-fold	Non-breeding great tit	C	Cockrem & Silverin (2002a)
0.9-17.5 ng/ml	19.4-fold	Pre-breeding, house sparrows	F	Ouyang *et al.* (2011)
1.8-46.5 pg/ml	25-fold	Breeding, European starling	F	Love *et al.* (2004)
10-60 ng/ml	6-fold	Incubation, eider	F	Bourgeon *et al.* (2006)
0.9-7.7 ng/ml	8.5-fold	Brood rearing, black- browed albatrosses	F	Angelier *et al.* (2010)
1.9-9.7 ng/ml	5.1-fold	Brood rearing, blue tit - Island population (Corsica)	F	Müller *et al.* (2007)
1.9-14.9 ng/ml	7.8-fold	Brood rearing, blue tit – Mainland population (France)	F	Müller *et al.* (2007)
0.3-14.0 ng/ml	29-fold	Brood rearing, blue tit	F	See section 7.3

The large inter-individual variation in baseline CORT for brood rearing blue tits does pose the question; have I successfully measured baseline CORT concentrations? When designing the blood sampling procedure I was aware that one must obtain blood samples as quickly as possible after the initial disturbance of the focal bird. Specifically, evidence suggests that blood samples must be obtained within 3 mins of initial disturbance, and ideally after 2 mins. Romero & Reed (2005) explicitly investigated whether collecting blood samples within 3 mins reflects baseline concentrations for six species in 14 data sets. Their study showed that for five species and seven of the data sets, there was no significant increase in CORT within 3 min of capture. In six of the 14 data sets, CORT increased significantly after 2 min, and in one data set, the increase started at 1.5 min. Therefore, samples collected within 2–3 min are likely to represent baseline concentrations. As I was only able to capture birds within 3 mins, I wanted to test that time between initial disturbance and blood sampling was not correlated with baseline CORT. Indeed my results showed that baseline CORT concentrations were not correlated with the time between initial disturbance at the nest box and blood sampling in any year. This held true even if only birds blood sampled between 2-3 mins were included in analysis (GLM: $t_{56} = 1.35$, $P = 0.18$). However, to ensure CORT concentrations are baseline it may also be important to assess any disturbance researchers cause birds before they have actually been caught and handled. In another study where plasma CORT was measured in blue tits, the authors investigated how the duration of time spent at the nest prior to capture influenced CORT concentrations in parental birds (Müller *et al*. 2006). The study showed that the presence of humans near the nest did not elicit an CORT response, whether birds showed a behavioural reaction to their presence or not (Müller *et al*. 2006). When I captured birds for blood sampling I often had to wait for provisioning adults to return to their nest after foraging, close by the nest usually hidden by undergrowth. As it is not always possible to establish whether the parents were aware that I was waiting near the nest, as they may not necessarily make alarm calls, I recorded the duration of time I waited at the nest in addition to the time it took to blood sample birds after disturbance at the nest box. My results showed that CORT concentrations were not correlated with time spent at nest before capture. This further suggests that I measured baseline CORT concentrations in this thesis.

There is also evidence that baseline CORT concentrations can be affected by the time of day blood samples are taken (Wingfield & Romero 2001; Romero 2004; Romero & Reed 2008). I was unable to blood sample birds at a standard time during the day, in fact, I sampled birds between 08:00-20:00. However, I tested the influence of time of day upon baseline CORT concentrations and did not find any evidence that it affected the concentrations recorded in any year (GLM: $t_{109} = 1.55$, $P = 0.12$). This may be because during May in Scotland sunrise is at about 05:00 and sunset at 23:00. In this case I was not blood sampling birds close to the start or end of the light period (Breuner, Wingfield & Romero 1999; Romero & Remage-Healey 2000). In addition, I tested for a linear relationship between baseline CORT and time of day, however, CORT could potentially vary with time of day non-linearly. Yet, visual inspection of the data suggests that no such relationship exists between baseline CORT and time of day across years (see figure 7.1).

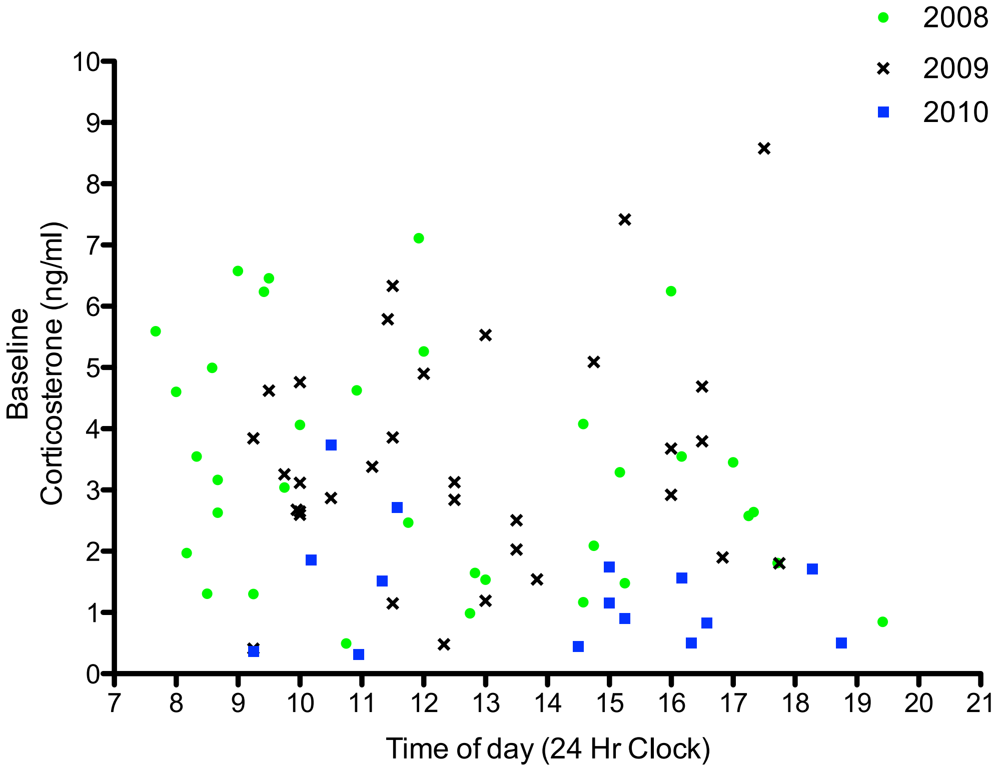


Figure 7.1. The relationship between baseline CORT concentrations and time of day blood samples were taken for provisioning blue tits measured over three years (2008-2010).

7.4 Inter-individual variation and fitness

Unfortunately, it continues to be rare for authors to present inter-individual variation (but see Williams 2008). Williams (2008) presents a review of the proportion of articles that present the range of inter-individual variation, with only 8% of 57 research articles in General and Comparative Endocrinology (2006), and 14% of 40 research articles in Physiological Biochemistry and Zoology (2005) presented inter-individual data. Therefore, my study may be one of the first studies to document the consistency of inter-individual variation in baseline CORT concentrations during a standardised breeding stage over multiple years. Overall, more studies are required to establish the ubiquity of the large inter-individual variation in baseline CORT. Furthermore, if individuals consistently show high or low absolute values of baseline CORT and/or similar values relative to conspecifics, this may influence how individuals behave and/or cope within a range of circumstances or life-history stages, which could ultimately influence their fitness (Bonier *et al.* 2009a; Dingemanse, Edelaar & Kempenaers 2010).

The persistence of large inter-individual variation in baseline CORT is intriguing; specifically how and why would this level of variation be maintained within populations? If baseline CORT concentrations are consistent within individuals, inter-individual variation may be maintained through differential selection across environments (Dingemanse *et al.* 2004). For example, inter-individual variation in baseline CORT has been proposed to underpin consistent differences in personality between individuals (Carere *et al.* 2003; Cockrem 2007). Personality, is defined as behavioural responses that are consistent within individuals over time and/or contexts (Sih, Bell & Johnson 2004). Both personality (Dingemanse *et al.* 2004) and baseline CORT concentrations (Angelier *et al.* 2009; Angelier, Holberton & Marra 2009) can cause individuals to respond differently to challenges in their environment. Dingemanse *et al.* (2004) present evidence that selection on a personality trait with high heritability fluctuates across years within a free-living bird population. Annual adult survival and the number of offspring surviving to breeding were related to personality, but the effects were reversed between years. The difference between years in selection pressures coincided with changes in environmental conditions that affected competition between birds. Therefore, fluctuations within the environment may lead to fluctuations in the competitive ability between birds, and this may maintain inter-individual variation in personalities within populations. If inter-individual variation in baseline CORT is linked to personality traits this mechanism could also maintain this variation within populations. Thus individual variation in CORT might represent fundamental differences in how individuals cope with environmental challenges (Bonier *et al*. 2009a; Dingemanse, Edelaar & Kempenaers 2010), and there might not be a single optimal response (Dingemanse, Edelaar & Kempenaers 2010).

Plasticity in baseline CORT concentrations between individuals may also be under selection, as the ability to respond flexibly to environmental challenges may be advantageous (Dingemanse *et al*. 2010). When investigating whether inter-individual variation in baseline CORT is related to fitness it is important to establish whether individuals vary in their CORT response to a challenge, i.e. environmental or physiological challenges. For example, individuals may respond to environmental challenges similarly, with individuals increasing CORT in response to a challenge, and if these individuals differ in the average CORT concentrations they may consistently showing high or low concentrations under a range of conditions (see figure 7.2a). Alternatively, the slope of the relationship between CORT and a challenge can also differ between individuals, i.e. their plasticity (see figure 7.2b). In this case, under contrasting circumstances individuals may or may not show differences in CORT concentrations (see figure 7.2b). These relationships are termed reaction norms, and, future work would benefit from attempting to measure individual reaction norms when relating variation in baseline CORT to measures of fitness (Dingemanse, Edelaar & Kempenaers 2010).

Throughout this thesis I have investigated how variation between individuals in baseline CORT can be explained by environmental conditions, and how the hormone is linked to key life-history traits such as reproductive investment, offspring phenotype and plumage colouration. I have made the assumption that as baseline CORT is linked to metabolism and energy-balance (Wingfield & Romero 2001), it is a holistic measure of current condition, and therefore is linked to these traits. To address whether inter-individual variation in baseline CORT is linked to fitness, it is essential to assess repeatability within individuals and individual reaction norms. Unfortunately it was not possible to assess repeatability within individuals during this research or establish individual reaction norms, as few birds were captured on multiple occasions across years (recaptures 2009: 4, 2010: 5). Furthermore UK Home Office restrictions prevented the repeated blood sampling of individuals within a breeding season. Indeed the number of studies which have been able to achieve this goal in regard to other traits is limited (Clutton-Brock & Sheldon 2010), and therefore this information may be a long time coming. In addition, there may be both ethical and methodological drawbacks of blood sampling individuals multiple times over a breeding stage or their lifetime. For example, collecting blood in itself is a stressor, and some individuals, particularly those of poor quality, may be differentially affected by the procedure. If this is the case blood sampling may differentially influence the CORT concentrations, behaviour or life-history of individuals dependent upon their quality. Furthermore, multiple blood sampling may influence CORT concentrations, as long-term, repeated stressors can attenuate or increase CORT concentrations (Cyr & Romero 2007), although this may be particularly relevant for stress-induced rather than baseline concentrations.

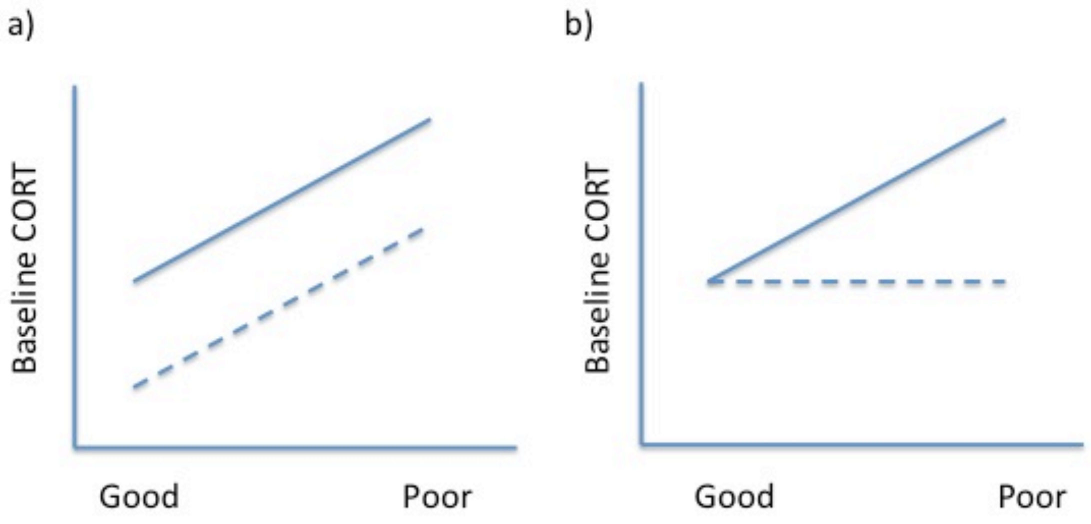


Figure 7.2. This diagram shows hypothetical relationships between baseline CORT concentrations and environmental conditions (Good/ Poor) in two individuals (solid and dashed line). a) Represents two individuals that differ in baseline CORT concentrations and respond similarly to contrasting conditions, whereas b) represents two individuals that differ in their response to contrasting conditions and therefore differ in baseline CORT only under certain circumstances. Modified from Dingemanse, Edelaar & Kempenaers (2010).

7.5 Method of measurement

In the majority of studies that have investigated variation in baseline CORT concentrations (Breuner & Orchinik 2002) and throughout this thesis, I have measured circulating CORT concentration from blood plasma. However, the cellular and organismal responses to baseline CORT can be influenced and modified by a number of other physiological factors (Breuner & Orchinik 2002; Ball & Balthazart 2008). For example, plasma corticosteroid binding globulins (CBGs) and hormone receptor density and/or affinity can regulate the availability of CORT to the tissues, and the delivery of hormones to specific sites within the body (Ball & Balthazart 2008). When CORT is measured from blood plasma it is total CORT that is quantified, this includes both free CORT, which is able to bind to hormone receptors, and bound CORT, which is attached to CBGs and thus cannot bind to receptors. In the house sparrow, total CORT concentrations vary seasonally, but CBG concentrations change with them, so that free CORT concentrations do not in fact differ across the seasons (Breuner & Orchinik 2002). Whereas, in white-crowned sparrows, CBG concentrations vary when total CORT does not, this results in significant changes in circulating free CORT (Breuner & Orchinik 2002), which would be overlooked if CBG concentrations were not measured. Therefore, if individuals differ in their CBG concentrations relative to their total CORT, concentrations of free CORT may differ to those of total CORT. In this case, the range of inter-individual variation and the rank order of individual's CORT concentrations within populations may change when CBGs are accounted for. Unfortunately time constraints and also limitations on how much plasma could be taken form individual birds prevented CBG analysis in this thesis. However, considering CBG concentrations may be essential when trying to understand inter-individual variation in baseline CORT and how it mediates reproduction and behaviour.

7.6 Final thoughts

Addressing both ultimate and proximate explanations for differences between individuals is by no means novel (Tinbergen 1963), but combining these approaches remains challenging (Williams 2008; Wingfield, Williams & Visser 2008; Moore & Hopkins 2009). In this study I have used an interdisciplinary approach to further understand the large variation between individuals in baseline CORT. A number of the findings I have reported are in agreement with previous results; the link between elevated baseline CORT and inclement environmental conditions (Kitaysky, Wingfield & Piatt 1999; Suorsa *et al*. 2003; Kitaysky, Piatt & Wingfield 2007; Müller *et al*. 2007; Busch & Hayward 2009), the complexity of linking CORT to reproductive success (Bonier *et al*. 2009a; Angelier *et al*. 2010; Dingemanse, Edelaar & Kempenaers 2010). I also present evidence that variation in maternal baseline CORT may be involved in reproductive trade-offs (Moore & Hopkins 2009; Bonier, Moore & Robertson 2011) and mediate UV plumage colouration (Roberts et al. 2007a; Roulin et al. 2008; Wada et al. 2008). My results for the blue tit, however, differ from the majority of literature for other birds that maternal baseline CORT is linked to brood sex ratio adjustment in birds (Love *et al*. 2005; Pike & Petrie 2005a; Pike & Petrie 2006; Bonier, Martin & Wingfield 2007; Pryke *et al*. 2011). Overall, the results provide evidence that inter-individual variation in baseline CORT explains variation in key life-history traits and may be involved in reproductive trade-offs through their pleiotropic effects. Together these results highlight the importance of employing inter-individual variation in baseline CORT when investigated the influence of baseline CORT upon key life-history traits as opposed to population means. Furthermore, studying populations over multiple years may be particularly insightful, as the relationship between CORT and life-history traits may differ between years and contexts. Further work is required to fully understand the role of baseline CORT in avian reproduction and there remain major gaps in knowledge regarding the repeatability of CORT measures within individuals, the importance of other physiological factors such as CBGs and how variation is maintained within populations.

Figure 7.3. Sunset on the east banks of Loch Lomond, UK.

7.7 References

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Appendix I: Biologically relevant elevation of corticosterone using a non-invasive method in the blue tit, *Cyanistes caeruleus*

I.1 Abstract

The temporary elevation of corticosterone (CORT) in response to a stressor is commonly observed and considered to be adaptive in birds, but experimental manipulations are required to test this. However, designing a method of experimental CORT elevation that is comparative to a naturally occurring stress-response is challenging. Conventional methods of CORT elevation such as silastic implants, involve handling animals directly, which in itself causes a stress-response. These methods also tend to elevate CORT for prolonged periods, thus are not suitable for investigating the effects of transient CORT elevation. Alternatively, non-invasive methods have been employed to elevate CORT for short periods. Feeding mealworms (*Tenebrio molitor*) injected with exogenous CORT to birds has been found to successfully elevate CORT for a biologically relevant period and within naturally occurring concentrations. In this study I tested the effects of this technique upon circulating CORT concentrations and activity levels in a common model species for behavioural ecology studies, the blue tit (*Cyanistes caeruleus*). In addition, I used a standard restraint procedure to establish the natural range of CORT concentrations elicited in response to a stressor. Consumption of CORT spiked mealworms resulted in significant plasma CORT elevation after 10 minutes relative to birds fed control mealworms, but had declined to control levels 30 minutes after consumption. Furthermore, the CORT concentrations achieved were within the natural stress-induced concentrations found after a standard restraint procedure for this species. Although the method elevated plasma CORT there were no effects upon activity levels after mealworm consumption between control and CORT fed birds. To conclude, this technique may be an effective means of mimicking the stress-response in the blue tit, as it causes short-term CORT elevation within the natural range.

I.2 Introduction

There are an increasing number of experiments that aim to understand the causal role of glucocorticoids in regulating avian behaviour (Silverin 1986; Astheimer, Buttemer & Wingfield 1992; Breuner, Greenberg & Wingfield 1998; Lõhmus, Sundström & Moore 2006). Corticosterone (CORT), the main glucocorticoid in birds is the principal mediator of allostasis (Sterling & Eyer 1988), with the hormone being released by the Hypothalamic-Pituitary-Adrenal (HPA) axis under stressful conditions to maintain homeostasis (McEwen & Wingfield 2003). CORT is always present in the bloodstream at baseline concentrations that show both diurnal and seasonal fluctuations to maintain energy-balance (Sapolsky, Romero & Munck 2000). However, in response to a "life-threatening" stressor, such as a storm or exposure to a predator, circulating CORT concentrations can increase from 4- to 10-fold higher than baseline levels within minutes, usually significantly exceeding baseline levels after three minutes and reaching their peak within 10-30 minutes (Breuner, Patterson & Hahn 2008). This rapid and transient increase in circulating CORT concentrations in response to a stressor is defined as a stress-response, and the concentrations reached under these circumstances are known as "stress-induced" levels. Baseline and stress-induced concentrations of CORT interact with different intra-cellular receptors, and therefore can have contrasting effects upon physiology and behaviour (Romero 2004). In this case, when addressing the causal role of CORT upon behaviour, it is important to address elevation in baseline and stress-induced levels independently, as this approach will provide further insight into the mechanisms that maintain behavioural variation in avian species.

However, the majority of studies have manipulated circulating CORT using subcutaneous implants, which raise circulating levels of CORT for a prolonged period, often for days or weeks (Silverin 1986; Wingfield & Silverin 1986; Buttemer, Astheimer & Wingfield 1991; Astheimer, Buttemer & Wingfield 1992; Kitaysky 2003; Criscuolo *et al.* 2005; Pike & Petrie 2006). This may be an effective method of elevating baseline CORT, but these methods do not replicate a stress-response (Breuner, Greenberg & Wingfield 1998). To date there are relatively few studies that have investigated the effects of transient

CORT elevation upon behaviour, but the results from these studies suggest that short-term CORT elevation has immediate effects upon behaviour in birds (Breuner, Greenberg & Wingfield 1998; Lõhmus, Sundström & Moore 2006).

Alternative methods that can be used to elevate CORT concentration for short periods include injections (Madison, Jurkevich & Kuenzel 2008) or the application of CORT-soaked bandages (Knapp & Moore 1997). However, similar to implants both of these techniques can stimulate an immediate and temporary increase in circulating CORT concentration through handling. Therefore non-invasive methods that increase circulating CORT in avian species have been devised. Breuner *et al.* (1998) injected CORT into mealworms (*Tenebrio molitor*) and fed them to captive White-Crowned Sparrows (*Zonotrichia leucophrys gambelii*). This method was shown to elicit a rapid and temporary increase in CORT, mimicking the circulating CORT concentrations that occur naturally in response to a perturbation without the need for handling or surgery.

In this study I validate a method of non-invasive CORT delivery for the blue tit, (*Cyanistes caeruleus*), a common model species in behaviour ecology. The main aims of this study were; 1) to investigate the effects of CORT spiked mealworm consumption upon circulating CORT concentrations 10 and 30 minutes after consumption, 2) establish if CORT elevation had an effect upon activity levels, and (3) investigate if CORT elevation mimics natural surges of CORT caused by subjection to a standard restraint procedure.

I.3 Methods & Materials

I.3.1 Mealworm Injection

To deliver CORT non-invasively, CORT solution was injected into mealworms. Crystalline CORT (Sigma) was dissolved directly into peanut oil, 0.3 mg/ml (Sigma, St. Louis, MO) through sonification. To ensure the CORT was dissolved evenly in the peanut oil, the solution was sonicated before each use. Mealworms were injected with 20µl of either, (i) peanut oil, which served as a control, or (ii) peanut oil with dissolved CORT. Hence, consumption of a mealworm caused birds to receive 0 or 6µg of exogenous CORT. The mealworms used were approximately 20 mm in length and 0.1g in weight; their size was important as they had to be small enough for the blue tits to eat completely, but large enough to hold 20 µl of injected solution. CORT was administered by injection using a 25-µl Hamilton syringe with a 26-gauge 1/2-inch needle. Prior to injection, mealworms were placed at -20°C to reduce movement during injection. The needle was inserted ventrally, into the anterior abdomen, between two segments. If fluid leaked from the mealworm after injection, it was not used.

I.3.2 Validation of non-invasive technique

Blue tits were captured in mist nets in March 2009, in woodland on the east banks of Loch Lomond, UK (56° 13′ N, 4° 13′ W). Birds were then transported and realised into cages at the University of Glasgow Field Station, SCENE (n = 24, 12 birds collected on 2 days). Birds were housed in single cages (1m x 0.5m x 0.5m) in 2 rooms (2.4m x 1.7m x 2.2m) with air, temperature and light control. The birds were kept under long-day photoperiod (12L:12D) which was comparable to the light regime they would have been experiencing in the wild and at 20 °C during the experiment. Birds were maintained in captivity for three days and given insectivorous mix, mealworms, waxworms and water, which were available ad libitum at all times. Birds were held in captivity for 24 hours prior to feeding experiments to allow them to acclimatise to their new environment.

Experiments were conducted between 09:00 and 16:00 on the second day of captivity. In order to investigate if CORT can be non-invasively increased, pairs of birds were food deprived for half an hour and then individuals were presented with either a control or CORT spiked mealworm. Birds were paired to control for diurnal effects and disturbance, which may influence baseline CORT (Romero 2004). The birds were monitored from behind a screen and their consumption of the mealworm was noted. To investigate if birds fed CORT-injected mealworms had elevated CORT compared with controls, 7 minutes after the mealworm had been consumed, birds were caught and blood sampled. Samples were collected within 3 minutes of capture to minimise effects of capture on circulating CORT concentrations (Wingfield, Smith & Farner 1982; Romero & Reed 2005). The timing of blood sample collection was informed by previous work employing this technique that has shown that concentrations peak at ~7 minutes after consumption (Breuner, Greenberg & Wingfield 1998). This procedure was repeated on a second group of birds, but on this occasion they were blood sampled 30 minutes after consumption to determine the duration of CORT elevation.

I.3.3 Behavioural observations

To investigate if activity levels were related to CORT elevation, the number of movements made by birds during trials was monitored. A movement was counted when birds changed position in the cage or on a perch. For example, movements of the bird while stationary, such as wing stretches or preening were noted but not counted. Prior to placing the mealworm into the cage the number of movements made by the focal bird were counted for 7 minutes. When the mealworm had been consumed the bird's movements were again monitored for 7 minutes until they were captured for blood sampling. For those birds blood sampled 30 minutes after mealworm consumption, their movements were monitored for 27 minutes after the mealworm was eaten. Observers were blind to the status of the mealworms.

I.3.4 Natural range of baseline and stress-induced CORT

In order to establish the CORT concentrations experienced by blue tits during a stress-response, birds were caught and blood sampled at a range of time points after capture and handling. Blue tits were captured in mist nets on two days in March 2010, on the same site as birds caught for mealworm validation. Mist nets were monitored continuously and birds were extracted within a minute of flying into the net. Once extracted from the net, birds were immediately placed in bags until blood sampling. Individual birds were blood sampled at only one time point due to UK home office regulations that restrict blood sampling to 1% of body mass per 30 days (blue tit body mass ~10 g = 100 µl). In addition, to measure baseline CORT, birds were caught in the nest-box in May 2010. All birds were blood sampled during brood provisioning when chicks were 6 days old and within 3 minutes of disturbance at the nest box.

I.3.5 Hormone analysis

All blood samples were obtained after puncture of the brachial vein with a 25-gauge needle and with the aid of a standard heparinised capillary tube (about 80–100 ul). Blood samples were immediately stored on ice and separated through centrifugation within 2h of collection. The plasma portion of the sample was removed and stored at -20ºC until assay.

Circulating corticosterone concentrations were measured using a double antibody radioimmunoassay (Wingfield, Vleck & Moore 1992). Samples were extracted from 5-20µl aliquots of plasma in diethyl ether and anti-corticosterone antiserum primary antibody (Esoterix B183), secondary antibody (Sigma goat anti-rabbit) and [3H]-corticosterone label (GE Healthcare, UK) were used. The extraction efficiency was 85–100%. Recoveries were measured for each sample independently and the final assayed concentrations were adjusted accordingly. CORT was measured in three assays for which the detection limit was 0.03 ng/ml (calculated as 2 SD from B0) and the averaged intra-and inter-assay variation was 9±2% and 10±5% respectively.

I.3.6 Statistical analysis

To compare CORT concentrations and activity levels between birds fed CORT and control mealworms paired *t*-Tests were employed. CORT data were square root transformed because of non-normality and analyses were conducted in R 2.8.0.

I.4 Results

I.4.1 Mealworm validation

On average birds consumed the entire mealworm within 2 minutes, and in the majority of cases the mealworm was completely consumed; only birds that consumed the entire mealworm were considered to have been successfully manipulated. 8 pairs of birds (16 individuals) from the 24 taken into captivity were successfully manipulated and blood sampled. 4 pairs were sampled 10 minutes after consumption and 4 pairs were sampled 30 minutes after consumption. 8 birds could not be used; only one bird was rejected, as it did not consume the entire mealworm. The other 7 birds were not included as they were not successfully blood sampled.

Circulating CORT concentrations observed 10 minutes after mealworm consumption were significantly elevated (x13) in birds that received a CORT injected mealworm (see figure I.1, t = 5.54, n = 8, P = 0.001, Control: 3.1±1.9 ng/ml, CORT: 39.9±7.96 ng/ml). CORT concentrations did not significantly differ between birds fed CORT or control mealworms when blood sampled 30 minutes after consumption (t = 0.96, n = 8, P = 0.41). However, birds fed a CORT injected mealworm, were on average twice as high as those fed the control mealworm (see figure I.1, Control: 4.7±0.3 ng/ml, CORT: 8.6±4.3 ng/ml).

I.4.2 CORT and behaviour

Prior to mealworm consumption birds in the control or CORT group did not differ in their activity levels (t = 0.32, n = 24, P = 0.76). In addition, activity levels did not differ between groups when monitored for 7 or 27 minutes after consumption (7 mins: t = 0.25, n = 22, P = 0.80, 27 mins: t = 0.27, n = 10, P = 0.79).

I.4.3 Natural range of CORT concentrations

Birds were sampled at 3 (n = 17), 10 (n = 6), 15 (n = 7), 20 (n = 5), 25 (n = 5), 30 (n = 3) and 35 (n = 2) minutes after extraction from the mist net. The CORT concentrations found in blue tits that had been subjected to a standard restraint procedure varied considerably within and between time points. Birds sampled 25 minutes after initial capture in a mist nest were found to have the highest mean CORT concentrations, 28.2±11.7ng/ml with a maximum of 74.6ng/ml (see figure I.2). Therefore CORT concentrations elicited by feeding birds CORT injected mealworms are on average within 1 SE of circulating levels found after a bird experienced a standard restrain procedure (see figure I.3). Furthermore manipulated CORT levels are within the maximum concentration of CORT found in birds after a standard restraint procedure.

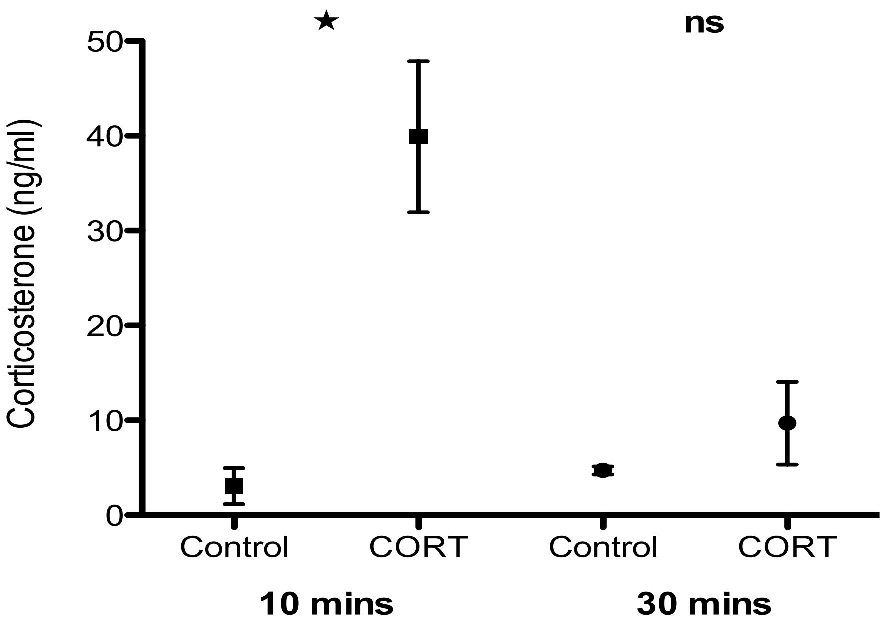

Figure I.1. Circulating CORT concentrations were significantly higher in captive blue tits fed CORT spiked rather than control mealworms 10 minutes after consumption (*n* = 8). However, this effect was no longer evident 30 minutes after consumption (*n* = 8). Graph depicts mean±SE

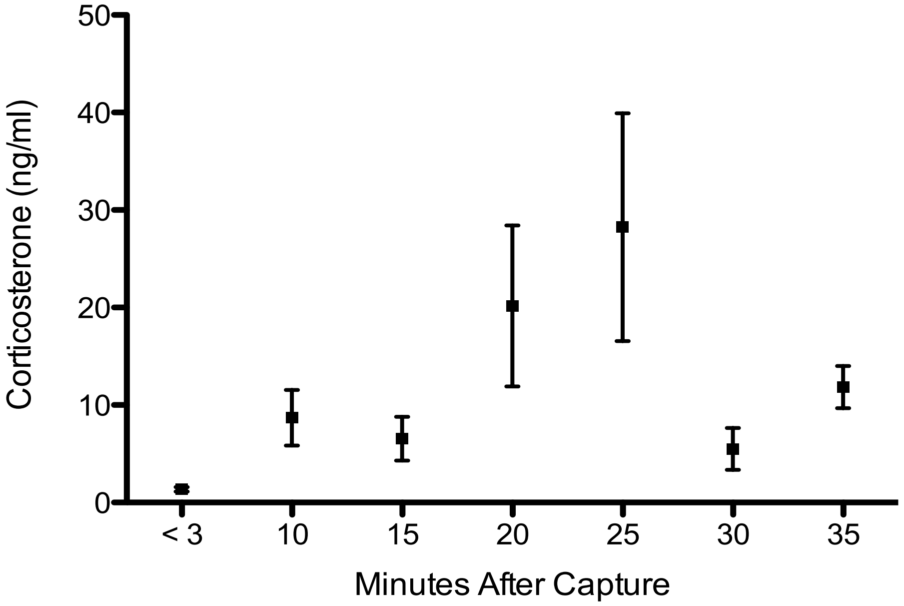

Figure I.2. Circulating CORT concentrations measured in free-living blue tits at a range of time points after subjection to standard stress procedure. CORT concentrations were highest 25 minutes after a stressor. Baseline <3 mins: *n* = 17, 10 mins: *n* = 6, 15 mins: *n* = 7, 20 mins: *n* = 5, 25 mins: *n* = 5, 30 mins: *n* = 3 and 35 mins: *n* = 2. Graph depicts mean±SE

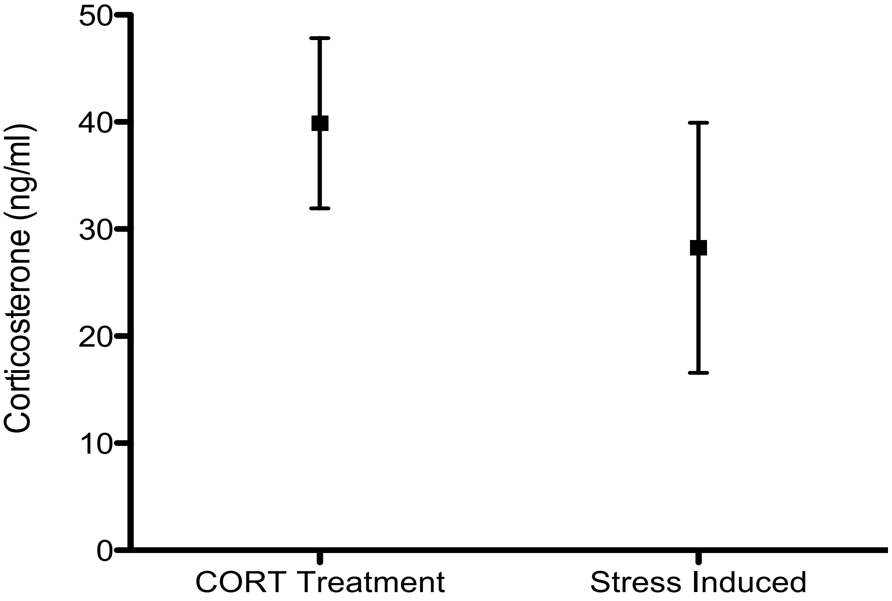

Figure I.3. Blue tits fed CORT spiked mealworms in captivity had CORT concentrations within 1 SE of the concentrations found in free-living birds 25 minutes after a standard restraint procedure (CORT Treatment: *n* = 4 and Stress induced: *n* = 5). Graph depicts mean±SE.

I.5 Discussion

This study has validated a method for the non-invasive delivery of CORT in the blue tit. Only one out of 24 birds taken into captivity did not consume the entire mealworm. In addition, the dosage used elicited a CORT response at ~10 minutes after consumption that was comparable with the CORT concentrations induced by a standard restraint procedure. Consumption of a CORT spiked mealworm, however, did not result in elevated CORT concentrations 30 minutes after consumption, indicating that the effect upon hormone concentrations was transient. CORT elevation was not associated with increased activity levels up to blood sampling, 7 or 27 minutes after mealworm consumption.

These results are similar to those presented by Breuner *et al.* (1998), where CORT-injected mealworms were used to achieve non-invasive CORT elevation in the White-Crowned Sparrow. Similar to the current study, peak CORT concentrations were reached at ~7 minutes after consumption and declined after

30 minutes to an hour. However, in Bruener *et al.* (1998) two dosages were tested, 4μl and 20μl, eliciting a peak response from baseline levels of x2 and x30, respectively. The 20μl dosage was effective because it resulted in CORT concentrations comparative to maximal concentrations found during a stress response. When the average mass of a White-Crowned Sparrow is taken into account (25g), this is equivalent to 0.76 μl CORT per g. In my study a dosage of 6μl was employed, which resulted in an x13 increase in circulating CORT. This is equivalent to 0.57 μl CORT per g (blue tit mass 10.5g). Therefore, the difference in the dosage required and the response elicited indicates that it is important to validate this methodology for each species.

Importantly CORT concentrations were only measured 10 and 30 mins after mealworm consumption. This was because previous work employing this technique has shown that concentrations peak at ~7 minutes after consumption (Breuner, Greenberg & Wingfield 1998). However, as the dosage and response elicited differed between this study and Breuner *et al.* (1998), I cannot be completely confident that the concentrations measured after 10 mins were the maximum elicited after mealworm consumption.

The CORT manipulation did not have an affect upon activity levels, with individuals fed CORT or control mealworms showing similar levels of activity. This result is in contrast to previous studies that have found non-invasive CORT elevation is associated with a rapid increase in activity within minutes of hormone administration. Both Breuner *et al.* (1998) and Lõhmus *et al.* (2006) have shown that non-invasive CORT administration was associated with an increase in perch hopping and feeding behaviour, respectively. However, the individuals used in this study varied considerably in their activity levels (Movements: 306±290, range = 0-978), therefore the small sample size employed may not have had the power to show a moderate increase in activity between the groups.

In conclusion, I have demonstrated that CORT-injected mealworms are an effective and non-invasive means of rapidly increasing blood CORT concentrations in the blue tit. To date there are very few studies that have manipulated CORT levels within their natural range and for a biologically meaningful time period, therefore this technique may be beneficial for future studies.

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Appendix II: In-situ versus ex-situ: Are feather samples a reliable method of quantifying UV crown colouration in the blue tit?

II.1 Abstract

Avian plumage colouration has been the focus of numerous studies engaged in determining its role in sexual selection, social interactions and status signalling. In the past two decades objective methodologies, such as spectrometers have become commonplace, allowing plumage colouration to be assessed from the perspective of the avian visual system, specifically colour in the UV range. Measurement of plumage colouration with a spectrometer may be taken directly on the bird or, alternatively by collecting feathers and measuring them in the laboratory. However, little is known about the validity of comparing measures obtained from feathers rather than the whole bird. In this study I compared intensity, UV chroma and UV signal measured directly from the crown of the blue tit *Cyanistes caeruleus* (in-situ) or from a collection of feathers from the same individual (ex-situ). In addition, I investigated the influence of the methods used to generate colour measures upon demographic analysis. The results show that values generated from each method were significantly different for intensity, UV chroma and UV signal. Furthermore, for UV chroma and intensity there was no correlation between the values obtained via in-situ and ex-situ methods. However, values for UV signal generated from the different methods was significantly correlated. Methodology also influenced the relationship between colouration and demographic data. Values for UV chroma and UV signal were significantly higher for males compared with females when either method was used. But the same pattern was only evident for intensity when values were generated from ex-situ methods. My results indicate some colour descriptors may not be comparable when generated from different techniques. Importantly this study provides evidence that UV signal was reliable regardless of whether the whole bird or just feathers were analysed.

II.2 Introduction

Avian plumage colouration has been a key paradigm for testing theories of sexual selection (Darwin 1871; Andersson 1994). Therefore, the reliability of measuring colouration, and repeatability between studies in the methodologies employed, has profound implications for our understanding of the evolution of avian plumage colouration. In recent years the most common method of measuring plumage colouration has been spectrometers (reviewed in Hill & McGraw 2006). Birds have a fourth retinal cone that allows them to see ultraviolet (UV) colouration, whereas humans are 'blind' to these wavelengths (Hart 2001). Spectrometers provide an objective and reliable tool to determine plumage colouration in this range of the spectrum (300-400 nm), although it can also be used in the visible range (400-700 nm). Measurements of plumage colouration with the spectrometer may be taken directly on the bird, but with wild populations this requires the devices to be carried into the field. This is an important drawback because of the fragility of equipment and the logistics of carrying heavy equipment over rough terrain. An alternative has been to collect feathers and to measure them in the laboratory. Although there are a growing number of studies using feather samples for colour assessment (Szigeti *et al.* 2007; Doutrelant *et al.* 2008; Remy *et al.* 2010; Midamegbe *et al.* 2011) there is little known about the repeatability of using one or the other procedure (but see Quesada & Sena 2006).

The blue tit, *Cyanistes caeruleus*, is a common biological model in plumage colouration studies (Andersson, Örnborg & Andersson 1998; Hunt *et al.* 1999; Sheldon *et al.* 1999; Delhey *et al.* 2003; Delhey & Kempenaers 2006; Hadfield *et al.* 2006). Blue tits have vivid blue plumage, which also strongly reflects light in the UV spectrum (Hunt *et al.* 1999). Several studies suggest that plumage colouration in this species has an important signalling function in the context of sexual selection (Sheldon et al. 1999; Griffith et al. 2003; Johnsen et al. 2003; Alonso-Alvarez, Doutrelant & Sorci 2004; Delhey et al. 2006). Furthermore, there are a growing number of studies that have investigated plumage colouration in this species using sampled feathers (see table II.1). However, the reliability of these methods to generate values similar to those generated directly from the bird has not been tested. This may be most important for smaller feathers that are harder to measure accurately, like the crown feathers of blue tits.

In this study, we obtained colour measurements from the crown feathers of breeding blue tits both directly from the animal (in-situ) and from collected feathers from the same individual which I later analysed in the laboratory (ex-situ). Three descriptors of plumage colouration that have been used previously in studies of blue tit colouration were employed (Andersson, Örnborg & Andersson 1998; Sheldon *et al.* 1999; Griffith *et al.* 2003; Delhey 2005; Hadfield *et al.* 2006; Szigeti *et al.* 2007; Doutrelant *et al.* 2008; Remy *et al.* 2010; Midamegbe *et al.* 2011). Firstly, intensity was quantified, which is the average light reflectance of the feathers across the light spectrum ($R_{320\text{-}700}$). Secondly, UV chroma, which is the strength of UV reflectance relative to reflectance within the rest of the light spectrum ($R_{320\text{-}400}$/ $R_{400\text{-}700}$) (Hadfield *et al.* 2006). Thirdly, UV signal was calculated, which is a measure of the reflectance captured by the blue tit retinal cones (Hadfield *et al.* 2006). Specifically, the two retinal cones that are most sensitive to the UV and blue portion of the spectrum.

The aims of this study were: 1) to determine the repeatability of measuring plumage colouration using both in-situ and ex-situ methods, 2) to determine whether the three colour descriptors differ when generated from in-situ as opposed to ex-situ methods and 3) investigate whether the method employed influences the outcome of analyses with demographic variables.

Table II.1. A summary of previous studies that have employed sampled feathers to measure UV colouration to address patterns of colour variation in adult blue tits.

Covariates analysed	Number of feathers	*n*	Colour parameters	Reference
Lay date, clutch size, fledging success, offspring recruitment and adult survival	6	80	Intensity, UV chroma, hue	C. Doutrelant *et al.* (2007)
Egg quality - yolk carotenoid concentration	5-8	14	Intensity, UV contrast, UV chroma	B. Szigeti *et al.* (2007)
Male aggressiveness and social interactions with conspecifics	6	48	Intensity, UV chroma	A. Rémy *et al.* (2010)
Female aggressiveness and social interactions with conspecifics	6	28	UV chroma, hue	A. Midamegbe *et al.* (2011)

II.3 Methods & Materials

A population of blue tits breeding in nest boxes situated in oak-dominated woodland around Loch Lomond, Scotland (56.13° N, 4.13° W) were studied from April to June 2008. On day 5 after chicks had hatched, parental birds were captured on the nest. A total of 25 adults were measured for UV crown colouration, 7 males and 18 females. No paired birds were captured and sampled; therefore no pairs were included in the analysis. Parental birds were sexed based on presence/absence of a brood patch and aged based on plumage characteristics (Svensson 1992). In addition, all birds captured were fitted with a uniquely numbered aluminium ring (British Trust for Ornithology).

Two colour measurements were taken on a standard patch on the crown of each individual. Subsequently, 6±2 feathers were plucked from the same region of the crown for all birds (see figure II.1a). Spectral data were recorded in the field and the lab with an Ocean Optics S2000 spectrometer (range 250–880 nm; Dunedin, Florida) using a micron fibre-optic probe at a 45° angle to the feather surface. Ambient light was excluded with a cylindrical plastic sheath affixed to the probe tip, and the sheath was placed against an individual bird and a feather specimen with the probe held a fixed distance of 6 mm from the feather surface. The reading area was a ~ 11 mm^2 diameter of light illuminated with both a deuterium bulb (UV light source) and a tungsten halogen bulb (visible light source). We generated reflectance data relative to a white standard (Labsphere, Inc.). The spectra acquisition software package OOIBase was employed to record spectra.

To assess UV crown colouration from sampled feathers, feathers were stacked on top of one another to resemble how they would lay on the bird's crown (see figure II.1b). Feather samples were placed upon black velvet (100% cotton) and 3 colour measurements were recorded sequentially, with the probe being lifted and replaced on the feather sample between each scan. Recorders were blind to the sex and age of the birds.

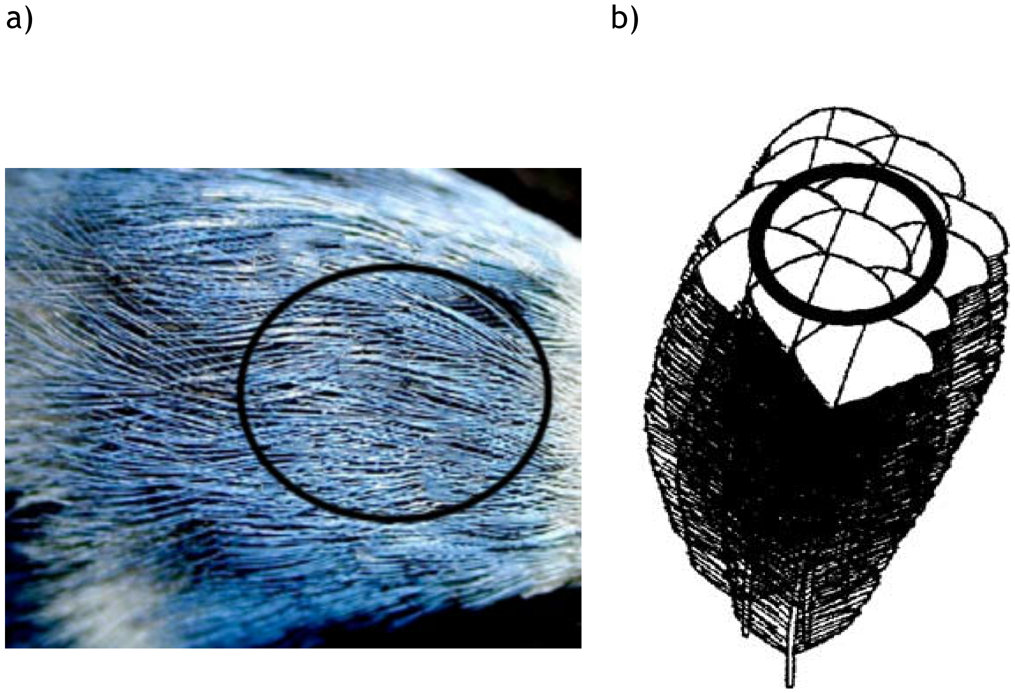

Figure II.1. Schematic showing a) the area on the blue tit crown where feathers were plucked for colour assessment and b) how collected crown feathers were arranged for measurement by a spectrometer. The circle illustrates the area where feathers were lucked and where measurements were taken. Diagram a) has been modified from Delhey (2005) and b) has been taken from Quesada & Sena (2006).

Feather colour was quantified using two standard descriptors of reflectance spectra, UV chroma and intensity, which have been used previously for studies on blue tit colouration. UV chroma (Sheldon *et al*. 1999; Griffith *et al*. 2003) was calculated as the sum of the blue-UV reflectance, 320-400nm divided by the intensity of reflected light summed for wavelengths between 400-700nm ($R_{320-400}$/ $R_{400-700}$). Intensity was estimated by the sum of reflectance from 320 nm to 700 nm divided by the total number of wavelengths measured.

In addition, using the SPEC package (Hadfield *et al*. 2006) for R (http://www.R-Project.org) UV signal was calculated. Spectral data was reduced into four quantal cone catches that quantify the amount of light captured by each of the avian single cones, specific to the spectral sensitivity of the blue tit visual system (Hart 2001). Irradiance spectra and ocular media transmittances were not specified. The four cone types are UVS (very short – UV), SWS (short – blue), MWS (medium – green) and LWS (long – red) and refer to the range of wavelengths to which they are most sensitive (Hart, 2001). As our analysis focused on the blue crown feathers we utilised the results from the UVS and SWS cone types (see figure II.2). To calculate the UV chromatic signal for each individual the UVS and SWS cone catches were standardised by dividing them by their sum to eliminate variation in intensity (achromatic component), which is highly sensitive to the method of measurement (see Hill & McGraw 2006). We calculated the chromatic signal so that higher values indicate a signal with a stronger UV component (Osorio, Miklosi & Gonda 1999; Larcombe *et al*. 2010);

UV signal = (UVS – SWS) / (UVS + SWS)

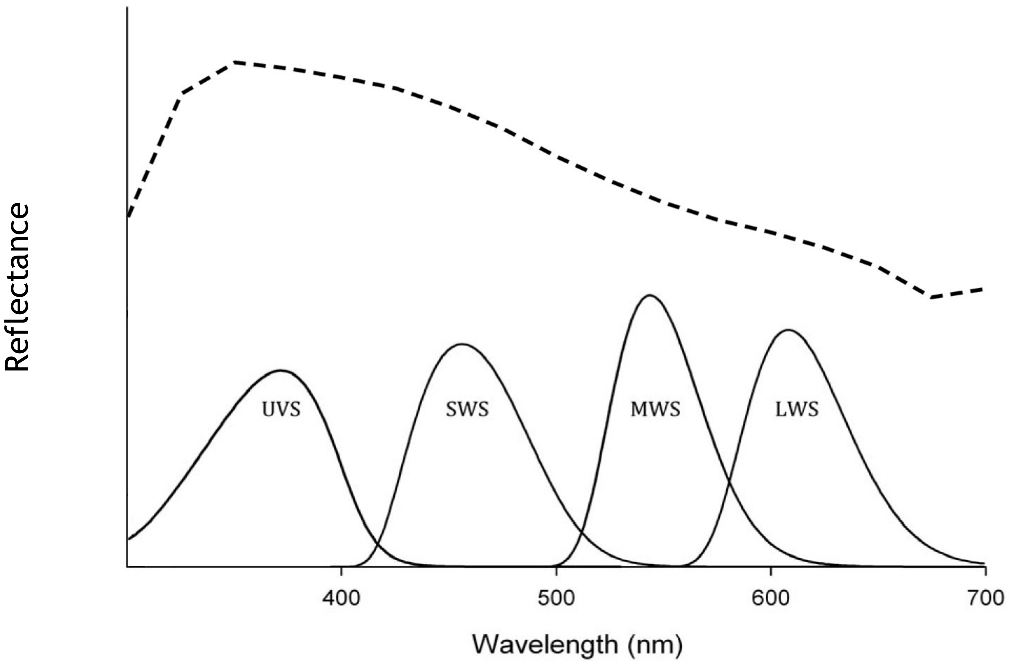

Figure II.2. The spectral sensitivity of the four single-cones (solid lines), with the mean reflectance spectrum generated from crown feathers (dashed line) plotted above. Passerine cone sensitivities are taken from the data provided in Endler & Mielke (2005). Diagram modified from Evans & Sheldon (2008).

II.3.1 Statistical analysis

Single-factor ANOVAs were employed to assess repeatability of feather colouration within birds and the equation, $r = S^2A / (S^2 + S^2A)$ was employed to generate the r-value (See Lessells and Boag, 1987). Paired *t*-Tests were employed to compare in-situ and ex-situ generated values for intensity, UV chroma and UV signal. In addition, Pearson correlations were employed to assess the relationships between the values for each colour descriptor generated from the different methods.

General Linear Models (GLMs) were employed to assess colour variation with respect to age and sex. Colour measures generated from either in-situ or ex-situ methods were dependent factors, with sex and age as explanatory variables.

The underlying statistical assumptions of the tests used were not violated; the data used and the residuals generated from the GLMs were normally distributed and there was no evidence of heterogeneity of the variance. All statistical analyses were conducted using R version 2.8.0.

II.4 Results

II.4.1 Repeatability and feather number

Within individuals, measurements of UV chroma, intensity, and UV signal were highly repeatable with values of $P < 0.001$ for both in-situ and ex-situ methods (ex-situ: UV chroma, $r = 0.75$, intensity, $r = 0.67$, UV Signal, $r = 0.81$; in-situ: UV chroma, $r = 0.77$, intensity, $r = 0.56$, UV Signal, $r = 0.75$; see Lessells & Boag, 1987).

Feather number was positively correlated with values for intensity ($t_{2,23} = 2.799$, $P = 0.011$), but was not correlated with UV chroma ($t_{2,23} = -0.233$, $P = 0.818$) or UV signal ($t_{2,23} = 0.155$, $P = 0.879$). Therefore, as the number of feathers used increased, the values for intensity also increased, but this was not evident for UV chroma or UV signal.

II.4.2 In-situ vs. ex-situ

For each bird, UV chroma and intensity differed significantly depending on whether they were generated from in-situ or ex-situ methods (see figure II.3, UV chroma, $t = 4.95$, $n = 25$, $P < 0.001$, intensity, $t = -10.04$, $n = 25$, $P < 0.001$). Values for UV chroma generated from ex-situ methods were significantly higher than those generated from in-situ methods. However, the opposite trend was evident for intensity, with values from in-situ methods being significantly higher than ex-situ methods. Furthermore, UV chroma and intensity generated from the different methodologies were not correlated (UV chroma, $r = 0.25$, $n = 25$, $P = 0.25$, Intensity, $r = 0.24$, $n = 25$, $P = 0.26$).

In addition, when values for UV signal were compared between sampling methods, there was a significant difference between in-situ and ex-situ methods, with those generated from ex-situ methods being higher ($t = 5.33$, $n = 25$, $P < 0.001$). However, values for UV signal generated from the different methodologies were significantly correlated (see figure II.4, $r = 0.51$, $n = 25$, $P = 0.01$).

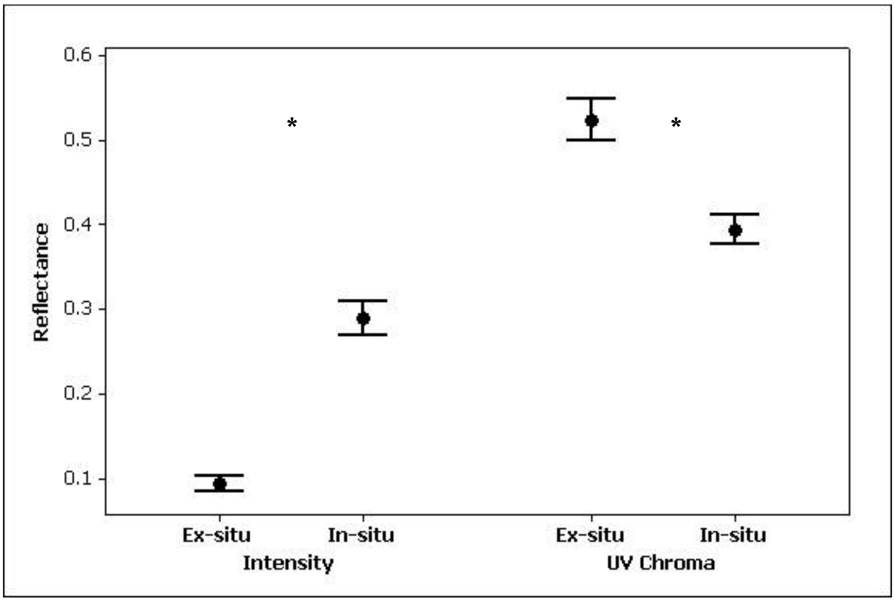

Figure II.3. Comparison of intensity and UV chroma from blue tit crown feathers generated from in-situ (directly from bird) and ex-situ (sample of feathers from same individual) methods. Values for both UV chroma and intensity differed significantly (*) dependant upon the method employed. Figure shows mean±SE.

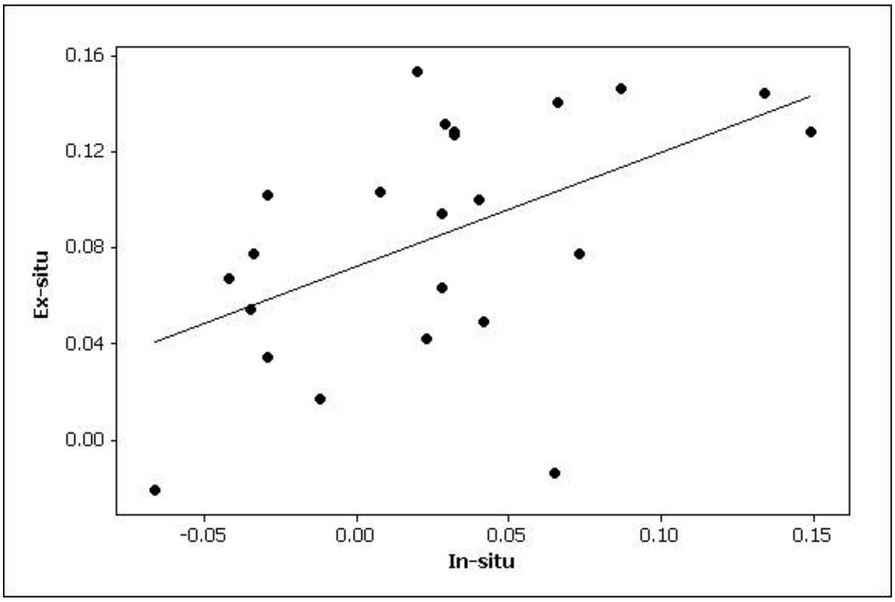

Figure II.4. UV signal (UVS–SWS/UVS+SWS) from blue tit crown feathers generated from in-situ (directly from bird) and ex-situ (sample of feathers from same individual) methods were significantly correlated.

II.4.3 Demographic analysis

To establish whether the methodology used to generate the colour measures influenced the outcome of analysis, I assessed colour variation with respect to age and sex. UV Chroma and UV signal generated from in-situ methods differed significantly with sex but not age (UV Chroma: Sex, $t_{23} = 3.32$, $P = 0.003$, Age, $t_{22} = 1.01$, $P = 0.33$, UV signal: Sex, $t_{23} = 3.01$, $P = 0.007$, Age, $t_{22} = 0.67$, $P = 0.51$). Male birds were found to have significantly higher values for both UV Chroma and UV signal. When ex-situ colour measures were used the same result was found (UV Chroma: Sex, $t_{23} = 2.10$, $P = 0.05$, Age, $t_{22} = 0.80$, $P = 0.43$, UV signal: Sex, $t_{23} = 3.91$, $P < 0.001$, Age, $t_{22} = 0.83$, $P = 0.42$).

When intensity was analysed, methodology influenced the outcome of analysis. Males were found to have significantly higher intensity than females when ex-situ methods were used (see figure II.5, Sex, $t_{23} = 2.53$, $P = 0.02$) but not when in-situ methods were used (see figure II.5, Sex, $t_{23} = 0.37$, $P = 0.71$). Intensity did not differ between age classes when either method was used (in-situ: $t_{22} = 0.12$, $P = 0.91$ and ex-situ: $t_{22} = -0.76$, $P = 0.46$).

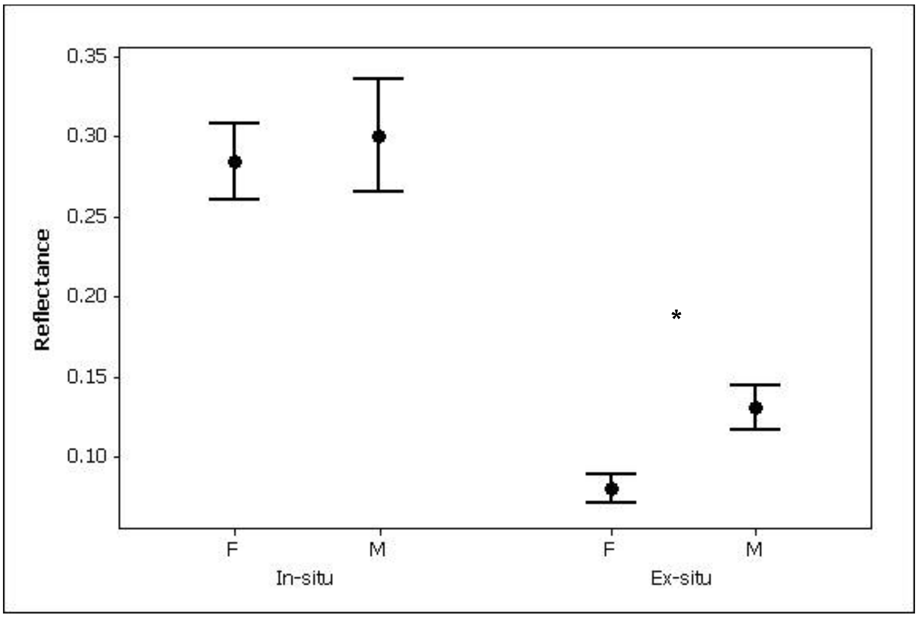

Figure II.5. Values for intensity ($R_{320\text{-}700}$) from blue tit crown feathers were significantly (*) higher for males (M) when compared with females (F) when generated from ex-situ methods (sample of feathers). This was not evident when in-situ methods (directly from bird) were used. Figure shows mean±SE.

II.5 Discussion

This study provides evidence that the methods employed to measure structural crown colouration in the blue tit affect the values obtained. UV chroma and intensity differed significantly and were not correlated when obtained from in-situ or ex-situ methods. In addition, feather number was positively correlated with intensity, but not UV chroma or UV signal. Although values for UV signal also differed between methods, they were significantly correlated. Methodology also affected the outcome of demographic analysis with respect to intensity. Males had significantly higher UV chroma and UV signal than females irrespective of the methods used, and age was not related to any colour metric for both methods. Whereas, sex explained significant variation in intensity when ex-situ methods were used but not when in-situ methods were used. Yet, for the colour descriptors employed, values were highly repeatable within individuals irrespective of the method used to generate them. This suggests that it was not a lack of repeatability for either of the methods that caused the colour metrics to differ.

This study indicates that values for intensity, UV chroma and UV signal are significantly repeatable within individuals when generated from the bird directly or from sampled feathers thereof. However, *r* values for intensity were lower than the other colour metrics, which indicates that this metric is more sensitive to measurement error (see also Hill & McGraw 2006). Furthermore, feather number was found to influence intensity but not UV chroma or UV signal. As intensity is a measure of the mean reflectance across wavelengths, it is understandable that increasing the number of feathers may increase the total light reflectance. Indeed, a previous study that validated the use of feather samples for colour measurements in the great tit, *Parus major* found a similar result, but this effect disappeared when more than 10 feathers were used (Quesada & Sena 2006).

The values for intensity, UV chroma and UV signal varied significantly dependent upon the method used to generate them, but not consistently. For example, when values from the two differing methods were compared for UV chroma and UV signal, ex-situ methods were significantly higher than in-situ methods.

Whereas, for intensity values generated from in-situ methods were significantly higher. Values for intensity between methods may differ because sampled feathers cannot resemble the density of feathers on the bird, therefore intensity would usually be lower for ex-situ methods. However, it may not be ethical to collect a large number of feathers from breeding individuals. Also as birds have been shown to differ in their reproductive investment dependant upon a partner's colouration (Limbourg *et al*. 2004), doing so could bias the outcome of the study.

Importantly, values for intensity and UV chroma were not correlated. In this case, values for these metrics differed in their rank order dependent upon which methods were used. This is particularly alarming because this indicates that the method used could have an affect upon the outcome of analysis and therefore any interpretation of intraspecific colour variation. When colour metrics generated from the two methodologies were analysed in respect to sex and age, the results were consistent for UV chroma but not intensity. For UV chroma male blue tits had significantly higher UV colouration when compared to females regardless of the method used, which is consistent with previous studies (Andersson, Örnborg & Andersson 1998; Hunt *et al*. 1998; Delhey 2005). But when intensity was analysed with respect to age and sex, the method employed affected the result. When ex-situ generated values were used, males were found to have higher values for intensity compared with females, but this was not the case when in-situ generated values were used. This suggests, that intensity may not be reliable colour metric when using ex-situ methods, however, it has been used in previous studies (see table II.1).

Values for UV signal were significantly correlated, and when UV signal was analysed in respect to sex and age the results were consistent. This suggests that values for this metric are more robust to the method used. This is probably because UV signal was calculated as a ratio, which would eliminate variation in intensity. While these results indicate that this colour descriptor is less sensitive to the method used, to date this metric has not been employed in studies that have used feather samples to assess plumage colouration (but see chapter 6).

To summarise, this study highlights the importance of validating the methods used to measure structural crown colouration in blue tits, because they can have an influence upon the values of the colour metrics generated and the outcome of analysis. Using colour descriptors that employ avian cone sensitivities may be a more reliable method of describing UV colouration when using ex-situ methods. Overall, by validating the methods used to assess plumage colouration, researchers can maximise the insight gained for our understanding of avian colouration.

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Appendix III: Pine martens, *Martes martes* as predators of nestling blue tits, *Cyanistes caeruleus*

For ~20 years nest boxes around the Scottish Centre for Ecology and the Natural Environment (SCENE) and Cashel Farm on the east banks of Loch Lomond have been employed to study the breeding behaviour of blue tits, *Cyanistes caeruleus*. The nest boxes have also been used albeit infrequently by Pied Flycatchers, *Ficedula hypoleuca* and Redstarts, *Phoenicurus phoenicurus* both amber listed species. The woodcrete nest boxes (Schwegler) are suspended from mature oak trees by a 35cm metal bracket perpendicular to the trunk. In previous years birds breeding in the nest boxes have rarely encountered predation. On only one occasion a greater spotted woodpecker, *Dendrocopos major*, a common predator of nestling passerines, successfully consumed blue tit nestlings (Pers. Obs.).

For the past two years I have been monitoring the breeding blue tits for my PhD thesis, and in order to expand the field site additional boxes were added. However, unlike the older boxes they were hung directly on the tree from a nail rather than a bracket (see figure III.1). The second breeding season after these boxes were put in place, three out of ~100 broods (shortly after hatching) were found dead, although there was no apparent injury to the nestlings themselves. The nestlings were also covered in blood which was not their own. Therefore, I concluded that the breeding adult had been predated while brooding and the nestlings had died of starvation. Later in the nestling stage, always after nestlings were 10 days old, blue tit broods began disappearing prior to fledging. The characteristics of each predation event were the same and as before, predation only occurred in nest boxes hung directly upon trees. The nest was always removed from the box and found directly beneath on the ground. In some cases there were also a few nestling feathers (identified because they were still in pin) found on the nest box or below. I was able to infer that the predator was mammalian from these remains as feathers had been bitten through rather than plucked as seen after avian predation (Hudson, Newborn & Robertson 1997).

Figure III.1. a) The recently established nest boxes hung from nails placed directly onto trees and b) the older nest boxes hung from a metal bracket.

At the Cashel Farm site where the majority of the new boxes had been placed, every occupied nest box (35 broods) was predated in the same manner, with not one nestling surviving to fledge. On one occasion when going to weigh nestlings as part of my research I interrupted a pine marten, *Martes martes* sitting on top of one of the nest boxes pulling out nestlings through the entrance hole. When I was about 20m from the nest the individual saw me and ran into the woodland. When I arrived the majority of the nest had been pulled out of the box presumably bringing the nestlings with it. The pine marten had consumed the majority of the brood, one was found partially consumed on the ground below and only two of the ten nestlings remained in the nest box with little remaining nest material. When returning to the nest box a few days later the last two nestlings had disappeared.

My experience serves to highlight how effective a predator pine martens can be for hole nesting passerines. In addition, the fact that the majority of the broods were predated 10 days after hatching when nestlings are almost adult sized indicates that something about this stage of development attracts pine martens. There are a couple of reasons why this may be the case; fledglings are very vocal at this time and may draw attention to their presence, or simply that pine martens cannot reach nestlings until they are larger and therefore closer to the entrance hole. It is important to note that nests placed on brackets between and within 20m of predated boxes did not experience predation. Therefore this simple precaution may help to avoid predation events. The predated nests were predominately at one site but they also extended over 3km. There is evidence that elsewhere in Europe pine martens can move 8.5km per day during the summer months (Zalewski, Jedrzejewski & Jedrzejewska 2004), therefore it is possible that these predation events were carried out by the same individual or more than one individual from a neighbouring territory.

Pine martens have been documented to prey upon birds in Scotland (Lockie 1964; Balharry 1993; Gurnell *et al.* 1994; Halliwell 1997; Putman 2000; Paterson & Skipper 2008) with particular reference being made to passerine predation in some cases (e.g. Putman, 2000; Halliwell, 1997). There is also evidence that bird predation by pine martens is seasonal having a higher occurrence in the diet during winter months (e.g. Putman, 2000; Gurnell *et al.*, 1994). However, bird predation does occur at other times of the year with at least one study showing an increase in bird predation during spring and summer (Balharry 1993). My observations indicate that pine martens are potentially a voracious predator of breeding passerines. However, as the predation event documented here was related to man-made nest structures only, this level of predation rate may not be similar for naturally nesting birds.

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