Body surface temperature as an indicator of physiological state in wild birds

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Thesis abstract

Understanding physiological processes is key to answering the questions of why organisms behave in the way they do, and how they interact with each other, and their environment. Despite technological innovations in recent decades, assessment of physiological state in free-living animals still generally requires subjects to be trapped and handled, so tissues or blood can be sampled, or so measurement devices can be attached or implanted. Such methods limit research to species and individuals that can be caught, potentially restricting the generalisability of findings, and introducing bias. Additionally, natural behaviours are interrupted, and subsequent physiology, behaviour or performance may be altered as a result of the stress of capture, the burden of attached apparatus, or the effects of surgery. Consequently, alternative techniques such as inferring physiological state from traits that do not require invasive sampling would be a valuable development. Body temperature is a particularly promising candidate trait, linked with an array of physiological functions, and having previously been used as a proxy for metabolic activity, stress state and immune challenge. With the advent of low cost, highly portable thermal imaging cameras, physiological ecologists are now presented with unprecedented opportunities to measure body surface temperature non-invasively, and at high frequencies from free-living animals. In this thesis, I investigated relationships between body surface temperatures, measured using thermal imaging from free-living blue tits or captive zebra finches, with physiological measures or situations relevant to the assessment of physiological state. I developed reliable thermal imaging techniques to take non-invasive measurements of body surface temperatures in a variety of contexts, allowing characterisation of physiological responses in real time. My studies of captive birds revealed that activity levels influence body surface temperatures measured from free moving animals, and so should be accounted for in experimental designs. I also successfully acquired body surface temperatures from overwintering blue tits visiting food-baited traps, and from breeding blue tits entering and leaving their nest. Using this data, I showed that body surface temperature exhibits a characteristic response to acute stress, which differs with stressor type. While the mechanisms require explanation, much potentially useful information

appears to be stored within body surface temperature dynamics during acute stress. Additionally, I established links between body surface temperature and longer term physiological processes in free-living blue tits. I observed near identical correlations between body surface temperature and body condition across differing seasons and life history stages. Also, I found evidence suggesting both that repeated acute stressors (predation risk and human disturbance) had a chronic effect on body condition breeding blue tits, and that surface temperature in those birds was linked to body condition. If confirmed, these results would be particularly interesting in a conservation physiology context, as it may prove possible to detect a signal of persistent physiological effect(s) relating to human disturbance, non-invasively. Furthermore, my discovery of a further correlation between baseline plasma glucocorticoids and body surface temperature in overwintering birds implies links with the hypothalamic-pituitary-adrenal axis. All of these results combined suggest that body surface temperatures measured using thermal imaging are highly likely to prove useful in determining aspects of physiological state non-invasively from free-living animals. While further investigation and validations are necessary, this work has laid the foundations for an exciting new methodology that could help solve many questions that remain unanswerable using current techniques.

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Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work, and has not been submitted for any other degree at the University of Glasgow, or any other institution. The work was funded by the Biotechnology and Biological Sciences Research Council UK doctoral training programme, and conducted by the author under the supervision of Ruedi Nager, Dominic McCafferty, and Dorothy McKeegan.

Chapter 2 was published on 11/06/2015 in the Journal of Visualized Experiments as '*Thermal imaging to study stress non-invasively in unrestrained birds*' (doi:10.3791/53184). Although the methods described, and the results presented were the result of my own work, the introduction and discussion sections were predominantly written by Ruedi Nager and Katherine Herborn. Also, the hormonal analyses in Chapters 4 and 5 were carried out by Susanne Jenni-Eiermann and Juanita Olano Marin at the Swiss Ornithological Institute. Additionally, the behavioural and breeding success data presented in Chapter 6 were collected by Nicole Tipple and Ian Woodman as part of their respective University of Glasgow Masters and Undergraduate projects.

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1. General Introduction

Understanding physiological processes is key to answering the questions of why organisms behave in the way that they do, and how they interact with each other, and their environment (Karasov & Martínez del Rio 2007). Despite the technological innovations of recent decades, assessment of physiological state in free-living animals still generally requires subjects to be trapped and handled, so that tissues or blood can be sampled, or so measurement devices can be attached or implanted. Such methods necessarily limit research to those species and individuals that can be caught, potentially restricting the generalisability of findings, and introducing bias (Weatherhead & Greenwood 1981; Boon et al. 2008; Garamszegi et al. 2009; Carter et al. 2012; Stuber et al. 2013). Additionally, natural behaviours are interrupted, and subsequent physiology, behaviour or performance may be altered as a result of capture and handling (Le Maho et al. 1992; Davis 2005; Templeton et al. 2005; Romero & Reed 2005; van Oers & Carere 2007; Lynn et al. 2010b), the burden of attached apparatus (Casper 2009; McMahon et al. 2011), or the effects of surgery (Hawkins 2004). Combined, these factors severely curtail the potential for investigators to examine physiological dynamics in most wild species, and during many commonplace activities. As a consequence, alternative techniques that avoid these constraints would be a valuable development. One potential strategy is to assess traits which do not require invasive sampling, inferring physiological state via established links between the trait and underlying processes, rather than relying on direct measurement. Perhaps the most promising candidate trait, offering the most diverse opportunities for this purpose, is body temperature.

Body temperature is linked with an array of physiological functions. It has previously been used as a proxy for metabolic activity (McCafferty et al. 1998; Ovadia et al. 2002; Ward et al. 2004; Ward & Slater 2005; Evangelista et al. 2010), stress state (Carere & van Oers 2004; Bouwknecht et al. 2007; Kohlhause et al. 2011) and immune challenge (Adelman et al. 2010a; Møller 2010; Marais et al. 2011a). Historically, measurement of body temperature typically required physical contact between a temperature sensitive probe and the target body region. As such, assessment of body temperature in wild animals was almost as logistically challenging and invasive as the collection of blood or tissue samples. However, with

the recent advent of low cost, highly portable thermal imaging cameras, physiological ecologists are now presented with unprecedented opportunities to measure body surface temperature of free-living animals non-invasively, and at very high frequencies. Nevertheless, taking advantage of this situation will require rigorous validation of precisely which physiological processes contribute to variation in body surface temperature in any given context. As such, the broad aim of the work contained in this thesis is to explore relationships between body surface temperatures measured non-invasively using thermal imaging, and situations or aspects of physiology that have relevance to the assessment of physiological state in free-living individuals.

Body temperature; maintenance and influencing factors

Although their boundaries are somewhat blurred, there are two main approaches to the maintenance of body temperature in the animal kingdom – ectothermy and endothermy (Davenport 2012). Ectotherms generally lack internal thermogenesis or physiological mechanisms for heat retention (although either may be present, but make negligible contributions to body temperature). In contrast, endotherms (the focus of this work) possess internal sources of heat, and are able use both their anatomy and physiology to control heat loss, allowing maintenance of a high, relatively constant core temperature (McNab 2002). In endotherms, the temperature of a given body region, however defined, is primarily dependent on the metabolic activity within it, on the amount and temperature of blood flow through it, and on the temperature gradients to surrounding tissues or the environment (Morrison & Blessing 2011). Body temperature as a whole is controlled by the autonomic nervous system through dedicated neural pathways, adjusting thermoregulatory effectors to maintain an equilibrium with the physiological parameters that influence them (Werner 1980). In addition to basal heat production (by combustion of cellular fuels, driven by ATP consumption at the myofilaments (Hohtola 2004)), specific effectors involved in thermogenesis include skeletal muscle, the heart, and mammalian brown adipose tissue, whereas cutaneous circulation and various species-specific mechanisms of evaporative heat loss (sweating, panting or saliva spreading) are used to expel heat to the environment (Morrison & Nakamura 2011). Effector mechanisms used to combat cold include behavioural changes to reduce heat loss, cutaneous vasoconstriction (conserving heat in the body core), piloerection (of hair in mammals, or feather fluffing in birds), non-shivering thermogenesis in brown adipose tissue, and shivering thermogenesis in skeletal muscle. Conversely, thermoregulatory behaviour to induce heat loss, cutaneous vasodilation (conducting heat from the body core to the surface) and evaporative cooling are all used to defend against overheating (Morrison & Nakamura 2011). Maintenance of body temperature requires the rate of heat gain to equal that of heat loss (Bicudo et al. 2010). Usually, the environment is cooler than body temperature, meaning that endotherms typically lose heat from their bodies, via radiation, convection, conduction or evaporation

(McCafferty et al. 2011). Much of the heat needed to maintain body temperature against this gradient is generated by metabolic heat production (Bicudo et al. 2010). Exactly how much heat is lost, and the relative importance of the four pathways, is dependent on features of the organism, such as its surface area and posture, and on physical environmental conditions, including air temperature, wind speed and humidity (Mcnab 1980).

While body temperature in endotherms is held relatively constant, it is influenced by a range of endogenous and exogenous factors. Oscillations in body temperature occur over a number of timescales. Stable longer term (infradian) oscillations are rare, but have been observed in relation to oestrous in a variety of mammal species (Hurnik et al. 1985; Refinetti & Menaker 1992). Short period (ultradian) oscillations, have also been described in a number of mammal and bird species (Møller & Bojsen 1974; Bitman et al. 1984; Heldmaier et al. 1989; Almirall et al. 2001; Benstaali et al. 2001; Petrovski et al. 2010; Tattersall et al. 2016). However, circadian rhythmicity of body temperature appears commonplace among mammals and birds (Refinetti 2010). Circadian oscillations of body temperature are sustained by systems of endogenous clocks, comprising 'master clocks', such as the suprachiasmatic nuclei in the hypothalamus (Klein & Moore 1991), and peripheral 'pacemakers', coupled to synchronizing external environmental cues ('*zeitgebers'*) (Herzog 2007). Entrainment cues include the light-dark cycle (Johnson et al. 2003), air temperature cycles (Rensing & Ruoff 2002), and food availability (Mendoza 2007). As exercise can elevate body temperature (Zerba & Walsberg 1992; Golombek et al. 1993; Piccione et al. 2004), locomotor activity may also have an enhancing or disrupting effect on circadian rhythms of body temperature, especially as oscillations in locomotor activity often exhibit a similar time-course to that of body temperature (Refinetti 2010). Body temperature and its rhythmicity may also be influenced by two immune system processes – inflammation and fever. Inflammation is an adaptive response to adverse stimuli such as pathogenic infection or tissue injury (Medzhitov 2008), and involves a host of processes, such as vasodilation, which increases blood flow (and hence transport of heat from the body core) to the affected area (Blatteis 2003). If inflammation or infection becomes systemic or chronic, endogenous

pyrogens (cytokines) are released into the bloodstream, inducing the preoptic-anterior hypothalamus to raise the body's thermoregulatory set point to a febrile temperature (Roth et al. 2009). Elevated body temperature during fever acts to enhance immune function in a variety of ways, including increased neutrophil/ monocyte motility and pathogen clearance, and antibody production (Harden et al. 2015). Finally, body temperature may also be modulated by the postprandial metabolic response (also known as specific dynamic action, SDA); the increase in metabolic activity that occurs subsequent to ingestion of a meal (McCue 2006). Whilst much research has been conducted assessing the effect of body temperature on features of SDA (Secor 2009), relatively little attention seems to have been given to how the associated increase in metabolic activity affects body temperature. Increased metabolic activity would generally be expected to increase body temperature. However, such increases are likely to be offset by the cooling effect of the meal itself, if (as would generally be the case in endotherms) meal temperature is below body temperature (Wilson & Culik 1991).

Physiological processes related to body surface temperature

Links between body surface temperature and physiology have been established in four main physiological areas – metabolic activity, thermoregulation, stress and immune response. While the main focus of this thesis will be the relationships between physiological stress and body surface temperature, it is not possible to assess such relationships without considering the three other influencing processes. Given the historic difficulties in measuring body temperature and other physiological processes in free-living individuals, almost all research in all four areas has been carried out under controlled laboratory conditions.

Metabolic Activity

Because body temperature is generally higher than ambient temperature, a substantial fraction of metabolic energy is lost to the environment as heat. Such metabolic heat loss has been estimated for a variety of species using heat transfer modelling from body surface temperatures (reviewed in McCafferty et al. 2011). It is possible to convert the rate of heat transfer to the environment into metabolic rate because the majority of energy released during the metabolisation of food is converted to heat, with only a small proportion being used for chemical or mechanical work (Ward & Slater 2005). If an individual is to maintain a constant body temperature during sustained activity, heat production must equal heat loss (Bicudo et al. 2010). Therefore, sustained changes in heat loss indicate changes to metabolic rate (Ward & Slater 2005). Validations of this relationship have been carried out, with varying degrees of success, both against previous measurements of metabolic rate (Williams 1990; Phillips & Sanborn 1994; McCafferty et al. 1998), and against respirometry and/or doubly labelled water measurements from the same individuals (Boily et al. 2000; Ward et al. 2004; Evangelista et al. 2010). Williams (1990), McCafferty et al. (1998), and Ward et al. (2004) (also Evangelista et al. (2010), but only at lower temperatures) reported relatively similar estimates of metabolic expenditure between methods (≤14% difference). Variability in agreement between methods is likely to relate to the limited sample sizes evident in all of these

studies. Also, in the case of Evangelista et al. (2010), the breakdown of the relationship between respirometry and the estimates provided by heat transfer modelling at higher temperatures was thought to relate to an increase in the importance of heat transfer modes that were not accounted for in the models.

The introduction of thermal imaging to measure body surface temperatures allowed researchers to estimate heat transfer non-invasively during activity. McCafferty et al. (1998), Ward et al. (2004) and Evangelista et al. (2010) all used thermal imaging to establish metabolic heat loss in birds during flight (in barn owl (*Tyto alba*), European starling (*Sturnus vulgaris*) and Anna's hummingbirds (*Calypte anna*), respectively). Flight in the barn owl was associated with an increase metabolic rate approximately 13x above BMR (McCafferty et al. 1998), while metabolic activity was found to increase linearly with flight speed in starlings (Ward et al. 2004). Mechanical output in Anna's hummingbirds hovering at a nectar feeder averaged 0.11±0.01 W (Evangelista et al. 2010). Additionally, Ward & Slater (2005) estimated the metabolic cost of song in the canary (*Serinus canaria*) to be 14±5% higher than during standing. The ability to assess metabolic rate non-invasively in the laboratory suggests that equivalent measurements would also be possible in free-living individuals, presuming that the increased variation in confounding variables can be accounted for. As such, variation in body surface temperature may not only reveal the comparative metabolic costs of various activities in wild populations, but also longer term changes in energy use over the course of development, and/or different life history stages (McCafferty 2013).

Thermoregulation

In addition to variation in overall metabolic rate, the physiological regulation of body temperature in endotherms is achieved through variation in peripheral blood flow, evaporative cooling and shivering/non-shivering thermogenesis. Vasodilation and vasoconstriction in peripheral tissues that have contact with the environment allows controlled variation of heat loss to the environment, and thus maintenance of a relatively constant core body temperature. Consequently, the temperature of

vascularised areas on the periphery may be used to assess the relative heat stress an individual is under (Tattersall & Cadena 2010). If an animal is heat stressed, then body surface temperature at vascularised sites should approach that of core body temperature, whereas body surface temperatures approaching ambient temperatures would indicate cold stress (Tattersall & Cadena 2010). Richly vascularised areas of body surface with variable (or high) heat exchange, known as 'thermal windows' (Tattersall 2016), have been observed in a wide variety of species. One of the earliest studies to describe this phenomenon reported vasodilation and vasoconstriction in the horns of goats subjected to heat and cold stress, respectively (Taylor 1966). Similar thermal windows have since been observed on bird bills (Tattersall et al. 2009), feet (Hillman et al. 1982; Wilson et al. 1998), combs, wattles and naked facial skin (Buchholz 1996; Negro et al. 2006), elephant and rabbit ears (Hill et al. 1980; Phillips & Heath 1992), the flippers of newborn seals (Blix et al. 1979), the trunk of adult seals (Mauck et al. 2003), and the dorsal fin of the bottlenose dolphin (Williams et al. 1999). Peripheral tissues used as thermal windows are often associated with higher densities of arteriovenous anastomoses (Gemmell & Hales 1977; Midtgård 1984a; b). Anastomoses act as shunts, effectively opening or closing blood supply from the body core to the periphery, and in so doing, opening or closing a window of heat exchange with the environment (Tattersall 2016). The capacity for heat loss from thermal windows seems particularly variable, even between similar sites in closely related species. For example, the capacity for heat loss from the ears of African bush elephants (*Loxodonta africana*) was found to be 10 times greater than that of Indian elephants (*Elephas maximus*), despite an only three-fold difference in ear surface area (Williams 1990). Impressively, the toco toucan (*Ramphastos toco*) appears capable of transferring up to five times the resting metabolic heat production to the environment via its bill (Tattersall et al. 2009). In addition to thermal windows, blood flow to peripheral body regions may be controlled to maintain tactile sensory capabilities. Both the mystacial vibrissal follicals of harbour seals (*Phoco vitulina*), and the follical crypts on the rostrum of the Guiana dolphin (*Sotalia fluviatilis guianensis*) were found to be maintained at higher temperatures than surrounding areas due to selective provision of warm blood (Mauck et al. 2000). As mechanoreceptor sensitivity has been found to decrease with

ambient temperature (Verrillo & Bolanowski 1986), Mauck et al. (2000) hypothesised that increased blood flow around these sense organs was specifically intended to preserve high tactile sensitivity.

The latent heat of vaporisation of water is used by many endotherms to increase heat transfer to the environment (Robertshaw 2006). Evaporative cooling mechanisms include sweating, panting, and saliva spreading, although the red kangaroo (*Megaleia rufa*) is thought to be the only species to utilise all three means (Dawson et al. 1974). Sweating, which occurs only in mammals, increases evaporative cooling from the skin surface through secretion of fluid from either apocrine or eccrine glands in the dermis (Folk & Semken 1991). Sweating rate has been reported to relate to both body surface temperature, and the rate of change of body surface temperature in humans (Nadel et al. 1971). However, the current consensus is that sweating is controlled by brain temperature, and modulated by mean skin temperature (Shibasaki & Crandall 2010). Panting is defined as an increase in respiratory frequency, with a proportionate decrease in tidal volume (Robertshaw 2006). Optimisation of heat transfer during panting depends on three main factors; increased ventilation (through increased breathing rate), flow of exhaled air across as much of the surface of the upper respiratory tract as possible, and increased blood flow to evaporative surfaces (Bech & Johansen 1980). In mammals, the nasal mucosa (and the tongue, in dogs (Krönert et al. 1984)), exhibit prominent systems of arteriovenous anastomoses, facilitating control of blood flow control to these evaporative surfaces in much the same way as has been described for external thermal windows (Widdicombe 1997). However, there are no arteriovenous anastomoses in the tracheobronchial tree (Widdecombe 1993). Instead, control of blood flow to the airway mucosa is reliant solely on vasoconstriction or vasodilation. During panting, internal evaporative surfaces such as the pharynx and respiratory tract typically exhibit temperatures lower than core body temperatures (Menuam & Richards 1975).

Shivering occurs in all endotherms as a response to cold exposure, and generates heat through an increase in contractile activity (and increased metabolic activity) in skeletal muscle (Rowland et al. 2015). Shivering is modulated by the respiratory cycle, but differs between birds and mammals in that shivering intensity is facilitated by inspiration in mammals (Kleinebeckel & Klussmann 1990), and by expiration in birds (Hohtola & Johansen 1987; Tøien 1993). This differential control, and differences in body shape mean that while shivering in mammals usually involves visible tremors, such tremors are rarely seen in birds (Hohtola 2004). During particularly intense shivering, muscle oxygen consumption can account for up to 90% of whole body oxygen uptake (Zurlo et al. 1990), and therefore 90% of heat production. In humans, the shivering threshold is linearly related to mean skin temperature, with skin temperature accounting for ~20% of the control of shivering (Cheng et al. 1995). While all endotherms employ shivering to generate heat (Hohtola 2004), non-shivering thermogenesis is somewhat less universal. The use of brown adipose tissue to generate heat (probably the best studied mechanism of non-shivering thermogenesis), is unique to mammals (Cannon & Nedergaard 2004). Brown adipose tissue generates heat by uncoupling mitochondrial respiration from ATP production (through the action of the UCP1 protein) to increase energy expenditure (van Marken Lichtenbelt et al. 2009). Depending on the size of deposit, and its location, activation of brown adipose tissue may affect body surface temperatures. For example, skin surface temperature above activated interscapular brown adipose tissue in mice (*Mus musculus*) increased similarly to temperatures measured from the brown adipose tissue (Hodges et al. 2008). Regional blood flow may also increase simultaneously with brown adipose tissue activation (Tattersall 2016), possibly further affecting surface temperatures. Although brown adipose tissue is absent in birds, there is evidence for the existence of non-shivering thermogenesis in avian skeletal muscle (reviewed in Rowland et al. 2015). While the precise mechanisms remain unclear, avian non-shivering thermogenesis is also hypothesised to involve mitochondrial uncoupling (Stier et al. 2014). As avian non-shivering thermogenesis has been typically studied in terms of metabolic rate and molecular processes (Barré et al. 1985; Duchamp & Barre 1993; Bicudo et al. 2001, 2002; Walter & Seebacher 2009), it's relationship with body surface temperature is

poorly characterised. However, sufficient heat production in skeletal muscle would most likely result in both local and whole body increases in body surface temperature.

Stress state

While endotherm body temperature oscillates with rhythms tuned to predictable environmental changes (Refinetti 2010), alterations to body temperature are also made in response to unpredictable events that threaten homeostasis (i.e. stressors). Stress-induced hyperthermia (SIH, also referred to as emotional, or psychogenic fever) is characterised by a consistent increase in core body temperature resulting from activation of the autonomic nervous system in response to a stressor (Vinkers et al. 2009b). The response has been described in a wide variety of endotherm species, including mice (Groenink et al. 1994; Van der Heyden et al. 1997; Oka et al. 2001; Keeney et al. 2001; Veening et al. 2004; Pardon et al. 2004; van Bogaert et al. 2006; Vinkers, et al. 2009; Vianna & Carrive 2012), rats (*Rattus norvegicus*) (Briese & Cabanac 1991; Terlouw et al. 1996; Bhatnagar et al. 2006; Barnum et al. 2007; Vinkers et al. 2010; Hayashida et al. 2010), humans (Marazziti et al. 1992; Briese 1995), ground squirrels (*Spermophilus beecheyi*) (Muchlinski et al. 1998), rabbits (*Oryctolagus cuniculus*) (Yang et al. 1987), chipmunks (*Tamias striatus*) (Careau et al. 2012), pigs (*Sus scrofa domesticus*) (Judge et al. 1973; Parrott et al. 1995), sheep (*Ovis aries*) (Lowe et al. 2005; Pedernera-Romano et al. 2010; Sanger et al. 2011) horses (*Equus caballus*) (Yarnell et al. 2013), and several bird species(Nomoto 1996; Cabanac & Aizawa 2000; Cabanac & Guillemette 2001; Carere & van Oers 2004; Gray et al. 2008; Miller et al. 2010; Bittencourt et al. 2015). SIH has been detected in response to both acute and chronic stressors, including environmental, physical and psychological stimuli (reviewed in Bouwknecht et al. 2007). SIH appears closely associated with hypothalamic-pituitary-adrenal (HPA) axis, and sympathetic-adrenal-medullary (SAM) system activation (Groenink et al. 1994). However whether the HPA axis actually mediates SIH remains an open question (Carrasco & Van de Kar 2003). In mice, the timing of the body temperature response to acute stress is similar to that of the corticosterone response (Groenink et al. 1994; Spooren

& Schoeffter 2002; Veening et al. 2004). Analogous relationships have also been reported in sheep (Lowe et al. 2005), and domestic horses (Yarnell et al. 2013), although the results are somewhat inconsistent, with further studies failing to find associations (Engert et al. 2014; Soroko et al. 2016). Additionally, there is evidence, from lab rodents, chickens, and horses that both the degree of SIH response, and its duration are related to the intensity of a given stressor (Van der Heyden et al. 1997; Herborn et al. 2015; Fenner et al. 2016).

Although sometimes described as a fever, stress-related body temperature changes are predominantly mediated by different mechanisms to those involved in infection-induced fever (Vinkers et al. 2010). Stress-induced increases in core body temperature are achieved in part via sympathetically mediated cutaneous vasoconstriction (Blessing 2003; Busnardo et al. 2010). Blood flow is also redirected from the periphery to the core (Briese & Cabanac 1991), presumably in order to minimise blood loss from vulnerable areas in the event of injury, and to prepare for a 'fight-or-flight' reaction by diverting blood to areas with the greatest metabolic demands (Blessing 2003). As with 'thermal windows', redirection of blood flow occurs via sympathetically innervated arteriovenous anastomoses, permitting blood to bypass the surface by being shunted between arteries and veins on precapillary blood vessels (Rimm-Kaufman & Kagan 1996). The process can be detected either by direct measurement of blood flow, or via associated reductions in body surface temperature, and has been described in a number of mammal species. For example, rabbits exposed to an unexpected sound reduced blood flow to their pinnae (Blessing 2003), reduced ear surface temperature by almost 3 ºC (Ludwig *et al.* 2007). However, more extensive measurement of dairy calves revealed a more complex response after painful disbudding, where a rapid, but short-term decline in eye temperature was followed by a prolonged elevation above baseline levels (Stewart et al. 2008). This suggests that body surface temperature changes are eventually reversed, either as a result of vasodilation, or hyperthermia within the core increasing heat flow to the surface where it can be dissipated to the environment. Further stress mediated changes in cutaneous temperatures have been demonstrated, although again, relationships between stimuli and response are potentially complex. The paws

and tails of rats underwent considerable surface temperature reductions during re-exposure to a conditioned fear stimulus (Vianna & Carrive 2005), and nasal temperature has been found to decrease when rhesus macaques (*Macaca mulatta*) were subjected to a variety of threatening stimuli (Nakayama et al. 2005; Kuraoka & Nakamura 2011). In contrast, dogs subjected to veterinary examination showed an increase in lachrymal caruncle temperature (Travain et al. 2015), although in a more recent study, ear pinnae temperature in dogs (*Canis lupus familiaris*) fell in response to social isolation (Riemer et al. 2016). Similarly the forehead surface temperature of human infants also fell when they were separated from their mothers (Mizukami et al. 1987, 1990). However, positive stimuli have also elicited similar responses from the facial skin of laughing infants (Nakanishi & Imai-Matsumura 2008), highlighting that decreases in body surface temperature may only reflect emotional arousal, and not stress in some cases.

Research into stress-induced body surface temperature changes in birds remains relatively limited, and restricted to acute stress responses. The response in chickens (*Gallus gallus domesticus*) has probably been the most comprehensively characterised, although temporal resolution is typically low. Cabanac & Aizawa (2000) measured surface temperature from the comb and inter-digital membrane of the foot using an infrared thermometer, in cockerels subjected to handling at 3 min intervals. Whilst the cloacal temperature of all birds (measured using a thermocouple) responded hyperthermically, only two out of the three subjects exhibited initial drops in surface temperature. However, in a similar pattern to that seen in disbudded calves (Stewart et al. 2008), body surface temperature values from all individuals, except the comb temperature of one bird, was higher at the end of the experiment than at first handling. More recently, analogous comb and whole head surface temperature responses (measured at 1 min intervals via thermal imaging) were recorded from hens subjected to handling followed by mild restraint (Edgar, et al. 2013). Additionally, Herborn et al. (2015) subsequently reported that differing intensities of physical restraint induced distinct patterns of comb and wattle temperature changes.

A variety of factors not directly associated with a given stressor may potentially modulate stress-induced body temperature responses. Although the general pattern of the stress-induced core temperature response appears relatively similar in most species studied thus far, it is expected that there will be differences, not only between species, but also between sexes and individuals (Vinkers et al. 2008). Individual variability may also be affected by past experiences, and social context. For example, chronic social defeat (a severe stressor) over 7 days induced increased hyperthermic response amplitude in response to a novel restraint in rats (Bhatnagar et al. 2006). Also, maternal vocalisations reduced the magnitude of peripheral temperature stress responses in domestic chicks (Edgar et al. 2015). Additionally, repeated daily testing with more moderate stressors has also been found to reduce hyperthermic amplitude, indicating some potential for habituation (Thompson et al. 2003; Barnum et al. 2007). Ambient conditions are also likely to influence the possible magnitude of temperature dynamics. For example, the effects of emotional arousal on comb surface temperature reported in Moe et al. (2012) were only apparent when initial comb temperature exceeded 30ºC. While the sample size was small (n = 5), this result highlights the likelihood of lower ambient temperature limits, beneath which thermoregulatory vasoconstriction will occur to conserve heat. Such thermoregulatory vasoconstriction could potentially restrict, or even prevent stress/arousal-related vasoconstriction, meaning that stress-induced hyperthermia could be restricted within safe biological limits for body temperature. Indeed, the only endotherm species not to exhibit a pronounced stress-induced hyperthermia is the guinea pig (*Cavia porcellus*), with this having been attributed to its comparatively high basal body temperature (Dymond & Fewell 1998). Restricted capacity for change in body temperature means that both the circadian rhythm of body temperature, and activity levels are also likely to affect the stress-induced hyperthermia response. Further potential modifiers include fever state, dehydration and food deprivation. Whilst fever states may reduce the magnitude of hyperthermic responses due to ceiling effects (Vinkers et al. 2008), dehydration limits avian capacity to use evaporative cooling to thermoregulate, leading to increased hyperthermia at high ambient temperatures (Whittow 2000). Food

deprivation, by contrast, has been found to increase the depth of nocturnal hypothermia (e.g. Graf et al. 1989).

Immune Response

Inflammatory and febrile responses are partially mediated by changes in peripheral circulation, and are thus closely linked with body surface temperatures. Inflammation always involves peripheral vasodilation, and so is typified by increased heat (a 'cardinal sign', in medical terminology), and therefore increased surface temperature at the affected area (Blatteis 2003). In cows (*Bos taurus)*, udder surface temperatures have been shown to increase linearly with mastitis infection severity (Colak et al. 2008). Similarly, in humans, skin surface temperature at the kneecap correlates with the severity of knee osteoarthritis (Denoble et al. 2010). The relationship between inflammation and body surface temperature is sufficiently characteristic that measurements of body surface temperature are frequently used in diagnosis of inflammation in medical (Collins & Ring 1972; Ring & Ammer 2012), pharmacological (Tanaka et al. 1987; Giraudel et al. 2005; Sanchez et al. 2008) and veterinary contexts (Hurnik et al. 1984; Schwartzkopf-Genswein & Stookey 1997; Colak et al. 2008; Alsaaod & Büscher 2012). The use of such diagnostic techniques is rare in free-living species. Nonetheless, measurement of skin surface temperatures was shown to be more accurate than routine skin sampling when diagnosing sarcoptic mange in wild Spanish ibex (*Capra pyrenaica*) (Arenas et al. 2002). Additionally, surface temperatures around gray seal pup (*Halichoerus grypus*) flipper tag sites were found to increase after tag application, but return to pre-tagging values before they went to sea, (Paterson et al. 2011). This was taken to indicate that inflammation relating to application of the tags had subsided by this point in time.

The increased body temperature associated with fever is initially accomplished partly through reduced heat loss (Blessing 2004). The interaction of cytokines with the brain results in activation of neurons in the basal forebrain and hypothalamus (Elmquist et al. 1997; Zhang et al. 2000), leading to vasoconstriction (Ohashi & Saigusa 1997), reducing surface temperatures in peripheral tissues. For example, Rabbits injected with *E. coli* exhibited an immediate decrease in ear pinnae temperature, followed by an increase to

within 3ºC of hypothalamic temperature at 120 min post injection (Vybíral et al. 1987). As the rabbits were handled during administration of the endotoxin, this drop could also have been related to handling stress-induced hyperthermia, however intravenous injection of lipopolysaccharide (LPS) into catheterized mice also induced a similar decrease in tail surface temperature without handling (Rudaya et al. 2005). Elevated body surface temperatures may also occur in inflamed tissues during fever. Elevated skin surface temperatures are increasingly used for fever diagnosis in travelling passengers (Ring & Ammer 2012). Also, infection of calves with bovine diarrhoea virus induced facial surface temperature increases of up to 4ºC, several days before clinical scores indicated illness (Schaefer et al. 2004). In one of the only studies investigating infection-related surface temperatures in free-living species, song sparrows (*Melospiza melodia*) from populations living at different latitudes were found to have differing magnitudes of long-term skin temperature increase in response to LPS injection (Adelman et al. 2010a). Rather than being attributable entirely to contrasting environmental conditions, these differences are likely to have had a genetic component, as a subsequent study which brought birds from each population into captivity at the same location, also found similar population level differences in skin temperature response to LPS challenge (Adelman et al. 2010b). Further work in wild-caught captive great tits (*Parus major*) found that LPS injection induced long term increases in skin temperature with consistent magnitude despite different ambient temperatures (Nord et al. 2013). This suggests that at least in these birds, that the energetic cost of the febrile response could be met regardless of heat loss to the environment. However, in more challenging environmental situations, suppression of the fever response in free-living species may occur during winter to reduce metabolic expenditure (Owen-Ashley & Wingfield 2006). This hypothesis is supported by evidence that LPS induced fever in Pekin ducks is attenuated with decreased ambient temperature (Marais et al. 2011b)

Thermal imaging as a tool for measuring surface temperature from free-living animals

Thermal imaging cameras detect the near-infrared (~8-12μm) electromagnetic radiation emitted from all surfaces with a temperature above absolute zero (i.e. 0 K, or −273.15°C) (Speakman & Ward 1998). If the surface's ability to emit and absorb radiation (emissivity, ε) is known, the temperature of the surface (T ºK) can be calculated from the amount of infrared radiation emitted (R Wm^{-2}) using the Stefan-Boltzmann equation:

$$R = \varepsilon \sigma T^4$$

where σ is the Stefan-Boltzmann constant (5.67 x 10^{-8} W m^{-2} K^{-4}), and ε is specifically the ratio of the radiant energy emitted by a surface to that which would be emitted by a perfect blackbody of the same area, at the same temperature (Monteith & Unsworth 2013). A surface with an emissivity of zero does not absorb or emit energy, instead reflecting all energy falling on it, whereas a surface with an emissivity of one (a perfect blackbody) reflects no energy, absorbing all energy falling on it (Heppner 1970). The emissivity of most animal integuments is similar to that of a blackbody, with values in the approximate range of 0.95-0.98 (Hammel 1956a; Best & Fowler 1981; Monteith & Unsworth 2013).

Initially the preserve of the military, thermal imaging technologies developed during the second world war became available for civilian use in the 1950s (McCafferty et al. 2011). Early thermal imaging devices predominantly used photon detectors, where infrared radiation is absorbed within the detecting material through interaction with electrons, generating an electrical signal due to changes in energy distribution (Rogalski 2012). However, such detectors are highly sensitive to the thermal generation of charge carriers, and so require cryogenic cooling, making devices which employ them costly (Rogalski 2002). The practicality, economy and availability of thermal imaging technology was revolutionised in the 1990s by the

public release of previously classified technological advances in uncooled thermal detectors made by the United States military (Rogalski 2012). The exploitation of these advances by manufacturers means that today, thermal imaging cameras suitable for use in scientific research can cost as little as a professional digital SLR. In modern uncooled thermal imaging cameras, the detection of infrared radiation is carried out by focal plane arrays of silicon or vanadium microbolometers, each of which provides an individual picture element (pixel) (Rogalski 2002). The surface of the detector material is heated by infrared radiation striking it, which changes its electrical resistance, proportionally to the amount of radiation absorbed (Wood 1993). This change in resistance is then used to calculate the temperature of the surface from which the radiation was emitted. To make the thermal image, the data from each pixel is combined into a monochrome rendering, visually summarising the combined energy emitted, transmitted and reflected by the subject and its environment. These monochrome images are typically displayed using a false-colour spectrum to aid identification of areas differing in temperature (Tattersall 2016) (Figure 1-1). Many contemporary thermal imaging cameras are also capable of combining multiple images into thermographic videos with frame-rates typically ranging from 5-60 Hz, permitting rapid repeated sampling.

As infrared radiation obeys the simplest laws of optics (Rogalski 2012), many of the concepts associated with visible light photography are also relevant to infrared thermography. Lenses are used to focus infrared radiation onto the sensor array in much the same way as with visible light cameras. However, as silicate glass and the majority of plastics do not transmit infrared radiation well, other compounds including germanium crystals and chalcogenide glass are typically used for thermal imaging camera lenses (Zhang et al. 2003). Relationships between focal length, the distance between the front of the lens and the subject, field of view, and depth of field (the range of distances from the lens within which objects appear in sharp focus) are all analogous to those of visible light cameras. Nonetheless there are a number of considerations specific to infrared thermography which must be taken into account. While focus is often only an aesthetic consideration in photography, a lack of focus in thermography results in low spatial resolution, effectively averaging

Figure 1-1 Thermal image of woodland passerines visiting a feeding station, illustrating the use of a false colour spectrum to aid identification of areas differing in surface temperature. Comparison of temperatures between areas using a false colour spectrum is only possible between objects of similar emissivity (e.g. biological tissue). *Image: Paul Jerem.*

temperatures across pixels, and so introducing error (Tattersall 2016). If the subject is a warm area on a cool background, such averaging will result in underestimation of the subject temperature as details are 'blended' together (and vice-versa for cool subjects on warm backgrounds). Spatial resolution also decreases with distance from the camera to the subject. Instantaneous field of view values, typically provided by camera manufacturers, allow calculation of the minimum measurement spot size detectable by a given camera, which can then be used to calculate appropriate camera-to-subject distances for capture of sufficient levels of detail (Tattersall 2016). One further consideration concerns the measurement drift inherent in cameras that use microbolometer arrays. Each microbolometer in the array is subject to random changes in sensitivity which require regular correction, usually when non-uniformity exceeds a threshold of 0.04% (Ring & Ammer 2012). Thermal imaging cameras can generally be set either to manually or automatically correct for non-uniformity.

However, depending on the length of time since the previous correction, such recalibrations can have a substantial impact on the value of temperatures measured, and temporarily halt recording (potentially losing a number of frames if recording high frequency thermographic video). As a consequence, it is often necessary to make a trade-off between recording duration and accumulating error when wishing to capture unbroken sequences of video data.

Ambient conditions, and the surface characteristics of animal subjects must also be considered when using thermal imaging in the field. Both direct solar radiation and reflected infrared radiation can affect measured values of surface temperatures (McCafferty et al. 2011). However, the effect of solar radiation can be avoided by restricting thermography to overcast periods, where sunlight is diffused, or actively shielding the subject. Error due to reflected radiation is likely to be minimal (< 5%) given the high emissivity (and therefore low reflectance) typically exhibited by biological tissues (Tattersall 2016). Additionally, as infrared radiation is absorbed by water, it is impossible to record thermal images through atmospheric water vapour such as thick fog. Water and dirt on the surface of an animal will also effect emissivity, potentially introducing measurement error if such effects are not accounted for (McCafferty 2007). Furthermore, both dirt and integument colour affect the absorptive properties of the body surface, and therefore the temperatures measured from affected regions. Dark surfaces have much greater solar absorptivity than pale surfaces, meaning that dark integument is likely to be considerably warmer in strong sunlight (McCafferty 2007). Finally, it should also be noted that the fine structure of both mammal fur and avian plumage mean that radiative exchange occurs within the pelage, rather than at its surface. For this reason, the radiative temperatures measured from pelage by thermal imaging are usually higher than the temperature at the actual pelage surface (McCafferty et al. 2011). This issue can be avoided by taking measurements only from exposed areas of skin or other thermal windows such as the eyes.

In addition to the data provided by the camera's sensor, precise calculation of absolute radiative surface temperatures also requires air temperature, reflected

temperature, relative humidity, measurement distance and surface emissivity to be measured. Given the potential for accumulated marginal errors involved in such measurements, a simpler and more accurate alternative is to place an object of known temperature (and similar emissivity to the subject) in the field of view (Jerem et al. 2015). This allows a straightforward calibration of the subject's temperature using the difference between the actual temperature of the object, and the value provided by the thermal imaging camera, without the need for measuring additional variables. Once calibrated, numerical temperature values can then be extracted from regions of interest using proprietary software, and exported to suitable analytical platforms.

While taking into account all of these varied factors may make thermography appear substantially more demanding than photography, the reality is that both techniques are remarkably similar. In fact, as long as appropriate procedures are established, it can be helpful to think of thermography as essentially being photography, only using a different wavelength of electromagnetic radiation. This philosophy is particularly advantageous when it comes to capturing data from wild animals, as there is a long established industry already dedicated to the capture of images and footage of wild animals, from which techniques can be usefully co-opted. Moreover, the implication is that if a species has already been filmed or photographed, then it follows that it is in all likelihood possible to collect thermal imaging data from it too. As such thermal imaging offers a multitude of opportunities to investigate the physiology of previously unstudied free-living species.

Subject species and study site

Two study species were used in the research presented in this thesis. The majority of the work (Chapters 2-4 and 6) was carried out on free-living Eurasian blue tits (*Cyanistes caeruleus*, Figure 1-2), whilst the single aviary study (Chapter 5) used captive bred zebra finches (*Taeniopygia guttata*).

Figure 1-2 The Eurasian blue tit (*Cyanistes caeruleus*). *Image: Paul Jerem*.

The Eurasian blue tit is a small cavity nesting passerine belonging to the Paridae family. As the name implies, the species is widely distributed, inhabiting predominantly temperate regions from Western Europe to the Middle East. In the UK, the blue tit is commonly found in lowland deciduous woodland, but is also a familiar sight in urban parks and gardens. There are a number of reasons why blue tits are generally an attractive study species for research, and particularly suitable for this study. Probably the most compelling argument for using blue tits is their population size. In Britain, there are thought to be approximately 3.4 million breeding pairs each year (British Trust for Ornithology 2016), making it one of the

most common, and therefore available bird species in the country. Currently, the resident breeding population size in the UK is stable, with the species listed by the International Union for Conservation of Nature as being of least conservation concern, across its range (Butchart et al. 2016). Blue tits also readily take advantage of winter feeding stations and artificial nest boxes. Indeed, their general curiosity and exploratory nature means that traps and experimental set ups using food as bait are usually rapidly investigated, accepted, and repeatedly visited by the same individuals. This situation allows easy access, both for thermal imaging and capture (either to mark individuals, or obtain physical samples) during the majority of the year, and across multiple life-history stages. Blue tits are also one of the most intensively studied species of wild birds, so a substantial body of research continues to accumulate, which is particularly helpful when attempting to interpret results. Topics previously investigated include behaviour (Hegner 1985; Lind et al. 2002; Gorissen et al. 2006; Steinmeyer et al. 2013), breeding biology (Nadav 1984; Clamens & Isenmann 1989; Svensson & Nilsson 1995; Adriaensen et al. 1998; Blondel et al. 1998; Ramsay & Houston 1998; Naef-Daenzer & Keller 1999; Stauss et al. 2005; Tremblay et al. 2005; Nour et al. 2008; García-Navas & Sanz 2010, 2011, 2012; Mainwaring et al. 2012; Mutzel et al. 2013; Henderson et al. 2013; Mainwaring & Hartley 2016), stress physiology (Müller et al. 2006, 2007; Lobato et al. 2008; Landys et al. 2013; Sudyka et al. 2014), disease ecology (Lucas & Heeb 2005; Wood et al. 2007; Cosgrove et al. 2008; Ferrer et al. 2012) and thermal biology (Nord et al. 2009; Nord & Nilsson 2011; Andreasson et al. 2016).

In Scotland, blue tits breed between March and June, with pairs defending territories from late March. Egg laying of a single brood usually takes place during April or May. Owing to particularly high mortality rates (Snow 1956), broods are often large, with average clutch sizes of 9.06 ± 2.14 eggs (British Trust for Ornithology 2016). Only the female incubates her eggs, with her mate bringing food during incubation (Perrins 1979). Once the eggs hatch, both parents provision the nestlings (predominantly with moth larvae (Perrins 1991), which remain in the nest for 18-20 days until fledging (Perrins 1979). Given the large brood size, provisioning rates of individual parents can exceed 60 visits per hour (Chapter 6, Figure 6-6). The blue tit

population studied for this thesis breeds in nest boxes (Schwegler 1B, 32mm entrance hole, Schwegler, Schorndorf, Germany) installed in the mixed deciduous woodland surrounding the Scottish Centre for Ecology and the Natural Environment (SCENE), on the eastern shore of Loch Lomond (56.13ºN, 4.13ºW) (Figure 1-3). As the birds in this population generally overwinter in mixed species flocks at the same site, a programme of baited mist-netting and colour-ringing during winter aids identification of individuals both during the winter months, and the breeding season. In recent years, ringed birds have also been fitted with leg mounted RFID tags (125 kHz 2.3mm EM4102 Bird Tag, IB Technology Glenfield, Leicestershire), enabling automatic identification at suitably equipped feeders and nest boxes.

Figure 1-3 Map of the study site for the blue tit fieldwork, and its location relative to Loch Lomond, and within Scotland. The dashed line indicates the area within which nest boxes have been installed. The shadowed yellow box gives the location of the Scottish Centre for Ecology and the Natural Environment (SCENE).

The zebra finch (Figure 1-4), Central Australia's most common Estrildid finch is an exceptionally important model species in many fields of research (Zann 1996), including sperm competition (Birkhead 2010), maternal effects (Griffith et al. 2010), the functional control of song learning (Hauber et al. 2010), and cognition (Healy et al. 2010). The zebra finch was the first passerine, and only the second bird ever to have its entire genome sequenced (after the chicken) (Warren et al. 2010) illustrating its value to the scientific community. The species was first studied in captivity in the 1950s by Desmond Morris (Morris 1954), who along with Klaus Immelmann was influential in establishing the zebra finch as the avian model of choice across an array of disciplines (Zann 1996). To directly quote Morris, the principle reasons why the species is so popular among researchers are that it is:

'...ideally suitable for laboratory observations. It will nest and rear young in small indoor aviaries... There are no seasonal difficulties, as it breeds all through the year. The species is exclusively a seed-eater and the nestlings require no special diet in captivity.'

Also, as zebra finches are readily available for purchase, required sample sizes are easily obtained. The species was chosen for the aviary study reported in Chapter 5, both for convenience (as facilities to house the birds were already available within the University of Glasgow), and as the species is relatively close, in taxonomic terms, to the blue tit - the main subject of this thesis. Therefore, relationships observed between body surface temperatures and physiological measures in free-living blue tits may reasonably be expected to exist in captive zebra finches also.

Figure 1-4 Male (left), and female (right) captive zebra finches (*Taeniopygia guttata*). *Image: Max Planck Institute for Ornithology*

Thesis content

This thesis investigates relationships between body surface temperatures, measured using thermal imaging from free-living blue tits or captive zebra finches, and physiological measures or situations relevant to the assessment of physiological state. Chapter 2 presents a method developed to reliably measure surface temperatures from blue tits during the acute stressor of confinement within a trap. Chapter 3 compares surface temperature responses between blue tits experiencing differing acute stressors. Chapter 4 investigates correlations between body surface temperature and body condition/baseline plasma glucocorticoid concentrations observed in overwintering blue tits. In Chapter 5 plasma glucocorticoid and body surface temperature responses of zebra finches to a putative chronic stressor are experimentally assessed, while Chapter 6 contrasts effects of human disturbance and predation risk on behaviour, body surface temperature, and breeding success in breeding blue tits. Finally, in Chapter 7, key findings, limitations/caveats, and potential future directions are discussed, with broad conclusions drawn regarding the content of the thesis.

Specific aims

Chapter 2:
Thermal imaging to study stress non-invasively in unrestrained birds

The aims of Chapter 2 were to:

a) Establish a thermal imaging method to reliably measure body surface temperatures at high frequencies from free-living blue tits during the acute stressor of trapping and confinement.

b) Demonstrate the technique by collecting representative data using the method described.

Chapter 3:
Surface temperature responses to acute stress differ with stressor type in a wild bird

The aims of Chapter 3 were to:

a) Extend the method described in Chapter 2, by adding handling to the established protocol, thus providing a contrasting acute stressor.

b) Establish whether surface temperature responses differ with stressor type by comparing body surface temperature responses between the method described in Chapter 2 (trapping and release without handling), and the extended trapping and handling protocol.

Chapter 4:
Body condition and glucocorticoids relate to variation in body temperature in a wild bird

The aims of Chapter 4 were to:

a) Compare body surface temperatures with body condition overwintering blue tits.

b) Compare baseline plasma total and free corticosterone levels with baseline body surface temperature in the same individuals.

Chapter 5:
An experimental test of the relationship between baseline plasma glucocorticoids and surface temperatures measured from free-moving birds

The aims of Chapter 5 were to:

a) Compare baseline plasma total and free corticosterone levels, and baseline body surface temperatures in captive zebra finches exposed to a reduced enrichment environment intended to induce chronic stress.

b) To establish whether the relationships between body surface temperature and free corticosterone / body condition observed in free-living blue tits (Chapter 4) were also present in captive zebra finches.

Chapter 6:
Human disturbance vs Predation risk; Do fitness consequences differ?

The aims of Chapter 6 were to:

a) Experimentally assess the effects of human disturbance and predation risk on breeding blue tits, in terms of the birds' behavioural responses, body surface temperatures, and breeding success.

b) Compare body surface temperatures with body condition in the subset of birds for which condition data was available.

Chapter 7:

General Discussion

The aims of Chapter 7 were to:

a) Establish the key findings from those presented in the thesis

b) Acknowledge general limitations of the work.

c) Discuss potential future directions that would build on the presented data.

d) Draw broad conclusions from the thesis as a whole.

2. Thermal imaging to study stress non-invasively in unrestrained birds

Abstract

Stress, a central concept in biology, describes a suite of emergency responses to challenges. Among other responses, stress leads to a change in blood flow that results in a net influx of blood to key organs and an increase in core temperature. This stress-induced hyperthermia is used to assess stress. However, measuring core temperature is invasive. As blood flow is redirected to the core, the periphery of the body can cool. This paper describes a protocol where peripheral body temperature is measured non-invasively in wild blue tits (*Cyanistes caeruleus*) using infrared thermography. In the field we created a set-up bringing the birds to an ideal position in front of the camera by using a baited box. The camera takes a short thermal video recording of the undisturbed bird before applying a mild stressor (closing the box and therefore capturing the bird), and the bird's response to being trapped is recorded. The bare skin of the eye-region is the warmest area in the image. This allows an automated extraction of the maximum eye-region temperature from each image frame, followed by further steps of manual data filtering removing the most common sources of errors (motion blur, blinking). This protocol provides a time series of eye-region temperature with a fine temporal resolution that allows us to study the dynamics of the stress response non-invasively. Further work needs to demonstrate the usefulness of the method to assess stress, for instance to investigate whether eye-region temperature response is proportional to the strength of the stressor. If this can be confirmed, it will provide a valuable alternative method of stress assessment in animals and will be useful to a wide range of researchers from ecologists, conservation biologists, physiologists to animal welfare researchers.

Introduction

Stress is a central concept in biology, describing the emergency response of an organism in response to an environmental challenge attempting to restore homeostasis (McEwen & Wingfield 2003; Romero et al. 2009). Under stress, blood glucose, fatty acids and amino acids levels, heart rate, respiratory rate and metabolic rate all increase, and blood is diverted from the periphery to the core organs (McEwen & Wingfield 2003). This generic pattern of physiological changes primes the animal to be able to respond quickly and adaptively to an array of social and physiological challenges. Whilst recognising and understanding stress is at the heart of both pure and applied animal research, assessing stress in unrestrained animals remains a challenge.

A widely used physiological marker of stress is an increase in the levels of circulating glucocorticoid hormones, such as cortisol and corticosterone (McEwen & Wingfield 2003; Romero et al. 2009). A great strength of this approach is that their concentrations increase in proportion to stressor intensity, allowing stress to be quantified. However, glucocorticoids are not 'stress' hormones *per se*, but mobilisers of energy stores (McEwen & Wingfield 2003). As such glucocorticoid levels change also with energy requirements, time of day, age and reproductive status (Wingfield 2008; Rensel & Schoech 2011), as well as in response to apparently positive situations, such as mating opportunity (Buwalda et al. 2012). Glucocorticoid levels, therefore, must be interpreted cautiously and within context. Measuring the glucocorticoid response to acute stress has also some limitations. An acute stressor triggers a dynamic response, initially increasing glucocorticoid levels and subsequently returning to a baseline level (McEwen & Wingfield 2003; Romero et al. 2009). Glucocorticoid samples are typically obtained invasively by blood sampling, which is a stressor in itself, and therefore can affect the measured glucocorticoid levels (Romero & Reed 2005). Moreover, glucocorticoid levels can only be measured at one or very few time points, which may not capture variation in peak levels and timing or response duration. This limits our ability to examine change through time of the dynamic stress response within individuals. Non-invasive

methods of hormone sampling, such as from faeces (Harper & Austad 2000), hair or feathers (Bortolotti et al. 2008), measure average glucocorticoid levels over a longer time scale, of days or months, although useful to study chronic stressors, are not applicable to the study of acute stress. As even the best established methods offer only a limited perspective on variation in acute stress amongst individuals there is a need for an alternative methodology in physiological stress measurements.

As the stress response involves a number of physiological effects there are numerous candidates that could indicate stress. Among others, sympathetically-mediated vasoconstriction channels blood from the periphery to the body core. This concentration of blood, and hence heat, along with various forms of stress-induced thermogenesis, warms the core (Oka et al. 2001). As such, core warming, termed stress-induced hyperthermia (SIH) has also been used as a marker of acute stress in pharmaceutical research (Bouwknecht et al. 2007). SIH typically raises core body temperature by 0.5-1.5°C within 10-15 min of an acute stressor (Bouwknecht et al. 2007). It is a relatively well documented phenomenon that the commonly applied experimental stressor of catching and handling an animal can raise core body temperature in a range of mammal and bird species (Moe & Bakken 1997; Cabanac & Aizawa 2000; Cabanac & Guillemette 2001; Carere & van Oers 2004; Meyer et al. 2008; Gray et al. 2008; Busnardo et al. 2010). Importantly, SIH correlates with other established indicators of stress, such as heart rate (Kramer et al. 2004), glucocorticoid levels (Lowe et al. 2005) and behaviour (Ahola et al. 2000; Harri et al. 2003). And like glucocorticoid levels, core temperature has been linearly related to stress level in some species (van Bogaert et al. 2006). However, as with blood sampling, the measurement of core temperature is invasive in itself, requiring the insertion or implantation of a probe (Bouwknecht et al. 2007). Baseline core temperature gradually increases each time when the stress-inducing handling or probe insertion must be repeated (Van der Heyden et al. 1997). Recent developments in temperature recording equipment that allow remote data access may provide a solution, at least for large animals (Signer et al. 2010).

Within the same mechanism that generates SIH, though, lies yet another potential marker of stress. The peripheral vasoconstriction that moves blood (and with it heat) to the core simultaneously cools the skin (Busnardo et al. 2010). Unlike core temperature, skin temperature can be measured completely non-invasively, by infrared thermography (IRT). IRT converts the infrared radiation emitted from the surface of an object into temperature (McCafferty 2013). Provided access to a suitable exposed area of skin, and the animal can be kept in the field of view of the camera, IRT cameras may be used to collect continuous skin temperature measurements remotely. Such data would allow the complete temperature response to acute stress to be filmed and compared between individuals. It has already been shown in chickens that surface temperature responds to acute stress (Edgar et al. 2013). However, the novelty of this study is that it measures surface body temperature of wild animals in a finer temporal resolution than previous studies, and also shows that the expected skin temperature drop can be detected in the field where temperature, humidity and weather are variable. The aim of this paper is to describe the necessary methodology to measure skin temperature of an unconstrained animal using thermal imaging. We use capture to induce a mild acute stress in free-living blue tits (*Cyanistes caeruleus*) and describe how to obtain suitable imagery, analyse the images and process the data to produce temperature response curves against time that demonstrate a significant cooling of the birds' skin temperature in response to the mild stressor. This can provide a valuable alternative method to assess stress in wild and captive animals, useful to a wide range of researchers from ecologists, conservation biologists and physiologists to animal welfare researchers.

The study was carried out in the period between 17th December 2013, and 4th January 2014 in a deciduous oak (*Quercus* spp.) forest at the Scottish Centre for Ecology and the Natural Environment, University of Glasgow at the eastern shore of Loch Lomond, west central Scotland (56.13ºN, 4.13ºW). The protocol involves three main steps: (1) Setting up suitable conditions under which to capture thermal images from a free-ranging animal, (2) applying an acute stressor while taking a thermal video, and (3) extracting and processing data from the thermal images that then can be used to characterise the animal's response to the acute stressor. In our case we attracted a

free-living passerine, the blue tit to a feeding box (filming set-up) where conditions for thermal image capture were optimised, and then applied a capture stress by closing the feeding box remotely when the bird was inside. The description of thermal image capture and data extraction is specific to the equipment we used, and may vary between thermal imaging systems. Data processing is described using open-source software.

Protocol

Ethics statement

The work involved a routine trapping method of free-living small passerines approved by the British Trust for Ornithology and the capture protocol eliciting a mild stress in the bird was carried out under UK Home Office licences.

1. Set-up for filming

1. Create a filming set-up where birds are encouraged to position themselves in front of the camera (Figure 2-1). The bird enters the set-up through a hole in one end wall and has access to food close to the opposite end wall.
2. In order to habituate the birds to the set-up, provide a suitable food (e.g. peanut kernels) at the feeder for several weeks prior to recording. Apart from provision of the food, leave the set-up undisturbed. During this period, place a dummy tripod in front of the feeder to allow the birds to also habituate to the camera.
3. On the day of recording, reduce the availability of peanut kernels in the set-up by removing all remaining food from the feeder. Replace it with food contained in a transparent container with only a small hole at the centre, on the top, through which birds can access the food. Position this food container in the centre of the cage at the end opposite to the entrance hole.
4. Place a small square of black insulation tape (similar in emissivity to that of natural integument (Fluke Corporation 2009)) in one of the corners of the mesh of the box so it appears in all images. Attach the black insulation tape to a thermocouple connected with a temperature logger that records the temperature of the black insulation tape with a resolution of 0.1 °C every 1 s.

Figure 2-1 The filming arena. The set-up in the field was designed to attract the birds to the field of view of the camera where a mild acute stressor could be applied, using a feeding box. The set-up consisted of a 25cm x 14 cm x 16.5 cm box built from plywood, with a front panel of galvanised wire mesh (aperture size 1 x 2.5 cm). The mesh allows infrared radiation to pass through, making it possible to film birds whilst inside the set-up. Wire mesh was chosen, as glass and most plastics do not transmit infrared radiation. A hole of 60 mm diameter was cut in the left end wall to allow birds entering the set-up to gain access to the food placed at the right end of the box. A temperature logger probe sandwiched between two squares of black insulation tape (marked by black arrow) is attached to the top right corner of the front panel to record a reference temperature. The mild acute stressor is applied to the bird inside by closing the box. Pulling on a fishing line attached to a rotating door at the entrance hole allowed the experimenter to close the box remotely when a bird is inside. The thermal imaging camera set in front of the trap records the whole sequence of events.

5. Place the thermal imaging camera 50 cm from the centre of the box trap so that all of the box fits within the camera's field of view, and birds feeding from the clear container will be positioned in the camera's zone of focus. Connect the camera to a laptop, set the camera to record images at an average of 7.5 frames per second (with a time stamp), and send images to the laptop's hard disk to be saved there.

6. Attach a fishing line to the rotating door of the set-up and roll it out to a position where the experimenter is hidden from view of the birds, but the set-up can still be observed - about 20 m from the set-up.

2. Filming the bird's response to a mild acute stress

1. Once a bird enters the feeding box, leave the bird undisturbed in the box for approximately 5 s.
2. Pull the fishing line after the bird has spent ca. 5 s in the box to close the feeding box. Take care that the bird is still at the far end of the box (away from the closing door) to minimise the risk of injury.
3. Immediately approach the feeding box and stand motionless behind the camera for approximately 3 min. Then retrieve the bird from the box and let it go.

3. Extraction of eye-region temperature from thermal images

1. Extract the maximum temperature from each frame.

   Note: The maximum temperature was virtually always recorded from within the region around the eye, bounded by the exposed skin of the periopthalmic ring (Figure 2-2), and is hereafter referred to as eye-region temperature.

2. In the Thermal Imaging Analysis Software right click on the image, 'add' a new plot for the image maximum. Then, right click on the plot to export the data (eye-region temperature and the time each frame was taken) to a CSV file.
3. Delete from the CSV file all lines from frames where the eye-region of the bird was not visible.
4. From the plot of maximum temperature against time in R (R Team 2014), identify manually points where temperature spikes > +0.2 ºC between two successive readings (when the nictating membrane was pulled over the eye - blinking), and points with low values outside the range of body temperatures for birds (Prinzinger et al. 1991) (i.e. when the eye-region was visible but blurred through motion for multiple frames). Then remove the relevant lines from the CSV data file produced during step 3.1.

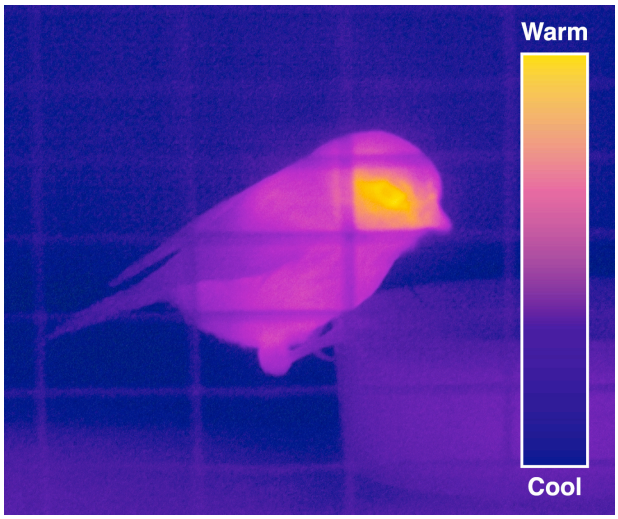

Figure 2-2 Thermal Image of a Blue Tit. The majority of a passerine's body is well insulated by feathers (or to a lesser extent by leg scales), but the skin around the eye is exposed and radiates most heat under normal circumstances, and is surrounded by cooler integument or background. This is shown in this thermal image by the yellow colour (highest temperature) around the eye, the orange, red, purple and blue colours signify cooler and cooler temperatures.

5. Measure ambient temperature.
    1. Download the data (time and probe temperature) from the temperature logger onto a computer and export into a spreadsheet.
    2. To extract the ambient temperature from the thermal image, draw a square over the black insulation tape that covered the temperature logger probe in the Thermal Image Analysis Software, then right click on the square and select 'add new plot' for the square's average temperature.
    3. Then, right click on the plot, and export the data (time and IRT temperature) to a CSV file. Merge the two resulting temperature time series for temperature logger and thermal image, matching for time, into one spreadsheet.
6. Correct eye-region temperature against ambient temperature. Export the spreadsheet created in step 3.4 into the CSV file, matching for time. To each retained eye-region temperature add the difference between the temperature logger and the thermal imaging-derived temperature values (logger temp – IRT temp) measured at the same time.

7. Carry out automated filtering to remove less accurate low eye-region temperature values using the peak search algorithm (see Appendix 1 a. for Supplementary R Code File) to automatically extract the highest (and therefore most accurate) points in the data.

   Note: The peak search algorithm re-organises the temperature data into a vector with a user-defined width (span), recommended span = 3, extracting the central value in rows where the numbers either side are lower, i.e. the peaks.

8. Use linear interpolation to close the gaps left by sequences when no peak was extracted, and when the eye-region wasn't visible. Use the R function *na.approx* (R package: zoo v1.7-11 (Zeileis & Grothendieck 2005)) to give a single temperature value per second for each individual.
9. In the CSV data file, add a value of 0.2 to each eye-region temperature to correct for the effect of taking the images through a mesh.

   Note: Tests showed that when a black body, heated to the approximate body temperature of a bird in its active phase (~41 ºC) (Prinzinger et al. 1991), was imaged through a mesh window with the same mesh size as used in the filming set-up. Temperatures recorded by the thermal imaging camera in areas not obscured by the wires were 0.2ºC lower than values obtained without the intervening mesh (mean difference±SD = -0.2±0.09 ºC, n = 30, temperature range of black body = 41.6-42.5 ºC).

   Note: This is the correction factor specific to the conditions in this study. It is likely to vary with instrument make, and type and width of the mesh between the animal and the camera. Therefore the correction needs to be established for the specific study conditions in each situation.

10. From the maximum eye-region temperatures recorded before the box was closed, select the highest value, which will constitute the baseline eye-region temperature

of the undisturbed bird. Add this baseline eye-region temperature as a new column to the CSV file. Then subtract the baseline value from each retained maximum eye-region temperature value, generating a new column in the CSV data file.

Note: This new value now expresses the bird's response to mild acute stress as deviation from its undisturbed baseline temperature.

11. Plot the deviations in maximum eye-region temperature from the baseline eye-region temperature of all individuals after the trap was closed using the R function *ggplot* (R package: ggplot2 v1.0.0 (Wickham 2009)). Generate bootstrapped 95% confidence intervals using the option *mean_cl_boot* (R package Hmisc v3.17-4 (Harrell 2014)). For R code see Appendix 1.

Representative results

Results from 9 free-living blue tits are presented, illustrating the information that can be obtained from thermal imaging, and demonstrating that the predicted signal of stress in the bird's eye-region temperature can be detected in free-ranging animals. Each bird was filmed for an average of 5.1±0.9 s (n = 9) before the box' door was closed. This allowed the calculation of the undisturbed bird's eye-region temperature (baseline temperature) to which all subsequent measures can be referred to. In tests conducted on 20 s thermal video clips of undisturbed blue tits arriving and feeding within a trap (n = 9 birds - different from those subjected the application of a mild stressor), correlations of $r>0.7$ were found to exist between the maximum eye-region temperature recorded during the first 5 s, and the maximum recorded in the first 10, 15 and 20 s (Figure 2-3). This was interpreted as indicating that the maximum value recorded in the initial few seconds after trap entry was representative of the longer-term level. Therefore, the mean eye-region temperature measured during the (approximately) 5 s immediately preceding box closure were used as the baseline value. The blue tits in this sample had a mean baseline eye-region temperature of 38.4±0.5 ˚C (mean±SE), and a range of 35.8- 39.9 ˚C (n = 9). Once the bird had been left undisturbed for 5 s (to allow the calculation of the baseline eye-region temperature), the box door was closed by the experimenter. All birds noticed the closure of the box as evidenced by their attempted escape flights.

Upon closure of the trap, the eye-region temperature dropped rapidly and reached a minimum eye-region temperature ~1.3 ˚C below the baseline temperature after ~10 s (Figure 2-4). Figure 2-4 shows the composite curve based on the maximum eye-region temperature of all nine individuals, averaged for every second. As the timing and the magnitude of the eye-region temperature drop varies between individuals the composite curve masks the true extent of the temperature response. The mean temperature drop calculated for each individual separately is 2.0±0.2 ˚C (mean±SE) and the lowest point is reached after 9.4±2.8 s. From then on, eye-region temperature gradually returned towards baseline eye-region temperature, but did not reach the baseline by the end of the trial.

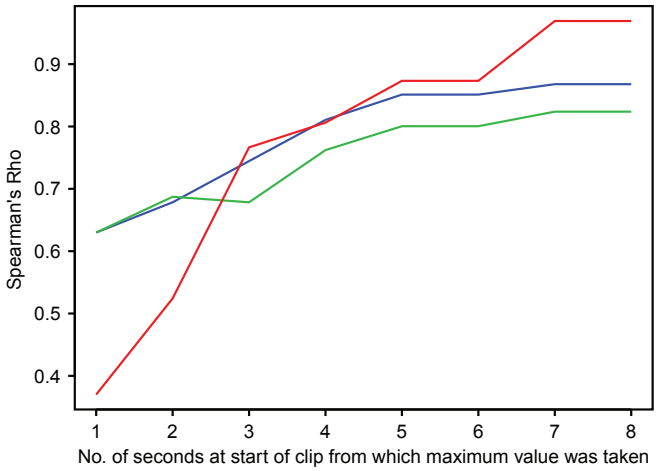

Figure 2-3 Repeatabilities of baseline temperature measured over different periods of time. Correlation coefficients between the maximum temperature recorded in the first 1-8 s of a clip and the 'mean of the maxima' within 0.5º C of the highest value recorded for the first 10, 15 and 20 s of the clip (represented by red, blue and green lines, respectively). Maximum temperatures recorded in the first ≥3 s did not differ significantly from the 'mean of the maxima' within 0.5º C of the highest value recorded for the first 10, 15 and 20 s of the clip (see Appendix 1 b.). All clips were 20 s in duration (n = 14). During initial analysis using a larger dataset, a number of the 'means of the maxima' were calculated from just 1 value, and gave the same value for rho across all comparisons. These clips were removed from the analysis, leaving only those where means were calculated from more than three measurements.

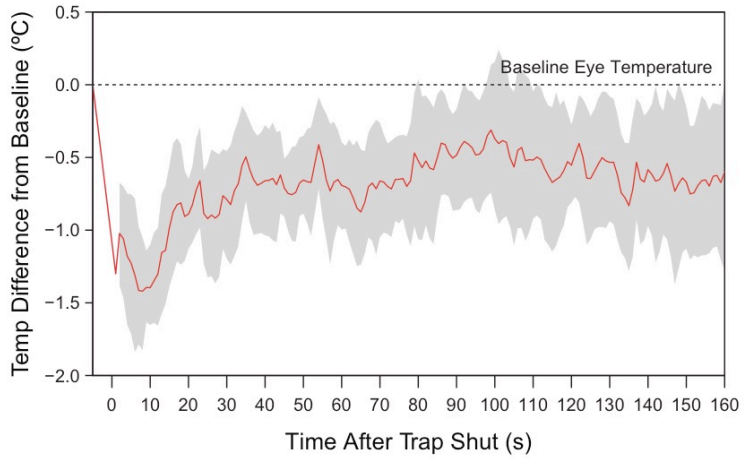

Figure 2-4 Response of the eye-region temperature to trapping. There is a clear and pronounced signal of the trapping event reflected in the change of eye-region temperature over time. To compare across individuals that vary in their baseline temperature, residual eye-region temperature (the difference between the individual's current temperature and baseline temperature), is plotted on the vertical axis. This is plotted against time, with the closure of the trap set as zero. The bird was removed from the trap after 3 min. The red line shows the mean residual temperature and the shaded area represents the 95% confidence interval.

Discussion

The aim of this paper is to describe the necessary methodology to measure changes in skin temperature of a free-living animal in response to an acute stressor. This study demonstrated that rapid changes in skin temperature associated with an acute stress response in wild birds can be captured non-invasively using IRT. This procedure involved five important stages:

1. Design of field set-up using a highly portable thermal imaging system
2. Measurement of baseline temperature
3. Application of a mild stressor involving capture of the bird in the set-up
4. *Post hoc* automated sampling of eye-region temperature
5. Calibration of extracted temperature data

The methodology described here was applied to the capture of wild blue tits. Representative baseline eye-region temperatures could be captured in as little as 5 seconds. The estimated time required to record one bird in the field was about one hour, with a further hour required to process the 3 min thermal video sequence.

Collecting good data on an animal's skin temperature requires high quality thermal images. Thermal imaging cameras only differ fundamentally from visible light cameras in terms of the wavelength of the electromagnetic radiation that they detect, and thus many of the concepts that apply in photography (e.g. field of view, depth of field) also apply in thermography. As commonly practiced by wildlife photographers, any location that an animal visits predictably, whether natural (e.g. nest sites) or artificial (e.g. feeders), could be used for collecting thermal images. Attracting animals to the camera, rather than attempting to follow them though their habitat, has the advantage of allowing us to set up the camera in advance, and collect high-quality image with minimal disturbance as soon as the animal comes into view. However, with this approach, it is vital to examine or account for the effects of the

specific context of measurement on stress or skin temperature. For example, here, birds were measured in a feeding context, and food was used to actively lure blue tits into the set-up. Research on chickens suggests that acute positive experiences (such as the anticipation and consumption of a food reward in an associative learning trial), can also lower eye temperature (Moe et al. 2012). As such, 'baseline temperatures' of individuals entering this particular set up may be influenced by the association of this act with a food reward. This possibility warrants further investigation, though if present, it would be expected to enhance the drop from baseline levels already observed. Additionally, thermogenesis occurs with the digestion of food, though the specific effects of food consumed during baseline measurement are not expected to significantly elevate temperature within the 3 min trapping period (Secor 2009). This limitation considered, measuring all animals within the same context is also a great strength of this approach. The representative results showed that there was a large variation in eye-region temperature between individuals before the acute stressor was applied (baseline eye-region temperature). The variation in baseline eye temperature (coefficient of variation = 3.9%) could be partly due to measurement error, or a true reflection of between-individual differences. When the IRT camera recorded the temperature of the black insulation tape over 5 s (when the tape's temperature did not change), the mean coefficient of variation was 0.26% (range 0.08-0.59%). Although measurement error is likely to be larger in a moving bird, this nonetheless indicates that most of the variation in baseline eye-region temperature is indeed between individuals. The notion of relatively small measurement error on moving birds is further supported by the high repeatability of eye-region temperature over 20 s during the baseline measurements (Figure 2-3). By controlling for context, we can ensure that all birds were engaging in the same activity. We can also minimise environmental contributions at the time of measurement to between-individual variation in skin temperature, for example, by ensuring that the locality is protected from confounding incident radiation that would, for thermal imaging, lead to an overestimation of the animal's surface temperature. This can be done by working in the shade, or by strategically placed shades, including most transparent plastics that block infra-red wavelength. The protocol presented here shows a simple set-up that could be easily applied to the situation of wintering blue tits. The blue tit

was filmed while in a feeding box with a solid roof which avoided exposure to direct sunlight. This set-up might also be applied to many other passerines that regularly come to artificial feeders at certain times of the year. As blue tits are comparatively small animals, they need to be filmed 'close-up' to capture a good level of detail. As with photographic cameras, getting close to the subject – both in terms of actual distance, and magnification – dramatically reduces depth of field. To have the birds as large as possible in the field of view required filming them from the camera's minimum focus distance (MFD). In this case, the MFD was 500 mm, and filming at this distance reduced depth of field to 40 mm. Therefore, all features of the filming set-up need to be designed to encourage the animals to display their head within the relatively narrow zone of sharp focus of the particular camera used.

In response to a well-established acute mild stressor to an animal we expected rapid changes in the pattern of blood flow, from the periphery to the core via sympathetically mediated vasoconstriction that reduces skin temperature (Busnardo et al. 2010). The periorbital region was chosen, as this provides the only region of the body that is uninsulated. It is also associated with a rich intermingled network of small blood vessels, the rete ophthalmicum that can affect the heat loss from the eye (Midtgård 1983). The fast frame rate of the thermal videoing technique was able to show a drop in eye-region temperature of approximately 2 ˚C in 10 s, followed by a subsequent rise in temperature to within 0.5 ˚C of baseline by 160 s after the bird was trapped. Although peripheral cooling in response to mild stress has been recorded previously at around 1 minute intervals (Busnardo et al. 2010; Edgar et al. 2011, 2013), the technique here showed that maximum drop in temperature may be extremely rapid. With lower time resolution the magnitude of this effect may be missed. This may be important when comparing differences in the stress response of different individuals. The eye region temperature of the bird did not return to baseline level after the rapid 'fight or flight' response ,while the bird remained within the box, indicating that the bird remained physiologically stressed. The birds were released from the experimental set-up after an arbitrarily chosen cut-off point of ca. 3 minutes to minimise distress. However, in future, trials may need to be extended to record the complete temperature response (i.e. until the animal's eye-region

temperature has returned to baseline level), if logistically possible. This new protocol, involving a series of images collected before and after the stressor was applied, now allows detailed study of the dynamic of the response to acute stress, and comparisons of multiple time-points between individual animals. Repeating this trial within individuals over different seasons or environmental conditions may allow the effects of environment on baseline or post-acute stress skin temperature to be disentangled, as a possible avenue into chronic stress assessment as well.

The skin temperature of a bird is dependent not only on metabolic heat production and blood flow but also on heat exchange from solar radiation, wind speed and wetting (McCafferty 2013). This precludes wet and windy conditions from accurate thermographic recordings. The eye-region temperature recorded by a thermal imaging camera can at times either be underestimated (negative error) or overestimated (positive error). Substantial positive error would require energy input, but this was avoided by the bird in the feeding box being shielded from the sun. One other source of positive error included when the bird blinked. Occasionally the bird briefly pulls its nictating membrane over the eye-region. As it is stored internally at a temperature closer to that of the body core, blinking gives an anomalously high temperature reading when it covers the eyeball. This is, however, easily detected as it leaves a very marked signature and the affected frame can be removed. The main reason for a negative error in our records was motion blur. Any movement too swift to be captured sharply by the frame rate of the camera confounds the data captured from the small warm eye-region of the image with that of the surrounding cool area (motion blur), resulting in an underestimation of eye-region temperature. Thus after removing positive errors this makes the maximum temperature measured from the eye-region the most accurate measurement. Subsequent automated filtering using the peaks function also removes less likely lower values of maximum eye-region temperatures.

In addition, as infrared radiation is absorbed by water vapour, surface temperature recorded will be influenced by the relative humidity of the environment. This can be accounted for by entering air temperature, relative humidity and distance to object

into the analytical Thermal Image Analysis Software. However, a more accurate and efficient approach (as undertaken here) is to include a reference body of known temperature and emissivity within the field of view. In our case this was a square of black insulation tape with an emissivity of 0.97, which is approximately equivalent to that of natural integument (Hammel 1956b). This allows calibrating the eye-region temperature by using the difference between the actual and thermal imaging-derived temperature values measured for the insulation tape. The surface temperature of the blackbody can then be used to calibrate images continuously throughout the measurement period.

Thanks to the technological advances in the development in thermal cameras, they can now be small, lightweight, and able to collect many frames per second over extended periods of time. Although thermal imaging is a widely used technique in avian research (McCafferty 2013), the size and expense of thermal imaging systems have historically restricted its use in the wild. In this study, the system was highly portable, cost approx. £6,000, and provided high resolution thermal video capable of non-invasively capturing accurate temperature data from free-living animals without the need to handle them. Recording at multiple frames per second allows measurements of high resolution time series of peripheral temperature and thus provides the possibility to explore the dynamics of the acute stress response. This is difficult to achieve with the conventional glucocorticoid sampling, constrained by the number of samples that can be taken within a period of time. Here we have extracted a time series of eye-region temperature with an interval of 1 second, which was sufficient to demonstrate the eye-region temperature response to an acute stressor (capture), but higher temporal resolution would also be possible. The accumulation of a large number of images, however, requires some level of automation in the extraction of information from the images. The protocol describes a simple semi-automated process to extract the maximum temperature from each image. We were able to do this as the region of interest, the eye-region, was always the warmest spot in the image. Analysis could be more problematic if regions of interest are more complex and may require custom designed pattern recognition software (e.g. Khaliq et al. 2014). In this study some manual filtering was required

for situations where the eye-region temperature was over- or under-estimated but these were easily detected. Further automation would, of course, be desirable. The measurements of stress-induced peripheral cooling by thermal imaging provides a valuable addition to other physiological measures of stress and the non-invasive aspect of this technique is highly advantageous for further studies involving captive and wild animals.

Capturing the complete stress response, infrared thermography clearly has great potential as a tool for stress assessment. To become a non-invasive alternative to established hormonal and core temperature assays though, it will be necessary to cross-validate and determine if skin temperature shows the same proportionality with stressor intensity, i.e. the extent of the skin temperature response can reflect the strength of the stressor. Future research should also address whether skin temperature captures chronic stress. Whilst SIH in response to an acute stressor is expected to be transient, recurrent exposure to physical or psychological acute stressors can generate chronic elevation in core body temperature (Kant et al. 1991; Endo & Shiraki 2000). Whether ongoing vasoconstriction contributes to this core elevation has not been tested explicitly. However, correlative studies on humans suffering from chronic stress-related illness do show reduced finger temperatures (Lin et al. 2011). A final attribute to explore is valence: the ability to distinguish positive from negative events. Hormonal assays cannot distinguish valence (Buwalda et al. 2012), with glucocorticoid levels appearing to reflect level of excitation rather than stress specifically. Research on comb temperature in domestic chickens exposed to aversive and positive stimuli suggest skin temperature may similarly reflect general arousal (van Bogaert et al. 2006; Moe et al. 2012). However, in humans, different emotional states elicit regionally specific changes in skin temperature. For example startle responses involve periorbital warming and cheek cooling (Pavlidis et al. 2002), whereas laughing is associated with an overall decreased skin temperature laughing (Nakanishi & Imai-Matsumura 2008). Comparisons between different regions may yet reveal emotional state. Using the recommendations laid out in this paper, it will be possible to address all of these questions, and validate skin temperature as a non-invasive marker of stress.

3. Surface temperature responses to acute stress differ with stressor type in a wild bird

Abstract

Knowledge regarding the initiation, regulation and modulation of responses to acute stress in free living animals is scant, primarily due to challenges in measuring physiological processes in the field. Two main physiological systems are triggered during the vertebrate 'fight-or-flight' response – the hypothalamic-pituitary-adrenal (HPA) axis, and the sympathetic-adrenal-medullary (SAM) system. The rapidity of the SAM response, combined with the ephemeral nature of catecholamines released, means it has largely been ignored in studies of stress in natural environments. However, SAM system activation also results in stress-induced hyperthermia (SIH), which can be detected non-invasively from surface temperature (T_s) by thermal imaging. Nonetheless, SIH responses in free-living populations remain poorly characterised. Therefore, to gain a more detailed understanding of T_s responses to acute stress in the natural environment, we measured T_s of wild blue tits (*Cyanistes caeruleus*) throughout trapping and handling using thermal imaging. We also compared T_s responses with previously published data where blue tits were trapped, but were then released without handling. We observed a characteristic pattern of T_s change during trapping and handling, which differed from T_s responses to trapping without handling. We show for the first time that T_s responses to acute stress in a free-living animal species are reproducible, and exhibit pronounced differences with stressor type. We conclude that the abundance of information contained within these T_s responses represents an unprecedented opportunity to investigate 'fight or flight' responses non-invasively in free-living populations.

Introduction

Stress represents a challenge to homeostasis (McEwen & Wingfield 2003), and is commonly categorised as either acute (short term), or chronic (long term). Acute stress is the type most frequently experienced in the natural environment, as the majority of stressors act only temporarily. 'Fight-or-flight' reactions to acute stressors such as predator attack or agonistic social interactions are naturally occurring emergency responses beneficial for both survival and fitness. However, knowledge regarding the initiation, regulation and modulation of such responses in free living animals is scant, primarily due to challenges in measuring physiological processes in the field (Romero & Wingfield 2015).

Two main physiological systems are triggered during the vertebrate 'fight-or-flight' response – the hypothalamic-pituitary-adrenal (HPA) axis, and the sympathetic-adrenal-medullary (SAM) system (Sapolsky 2002). Activation of the HPA axis leads to the release of glucocorticoids, which are relatively stable, and so can be readily assayed (Abraham 1975). Consequently, glucocorticoid levels have become the most commonly investigated indicator of stress in natural populations (Romero & Wingfield 2015), despite the difficulties involved in obtaining samples from free-living animals. In contrast, the rapidity of the SAM response, combined with the ephemeral nature of catecholamines released (Sapolsky 2002), make collecting samples prohibitively difficult. Given this, the SAM response has largely been ignored in studies of stress in natural environments. However, a number of physiological changes occur during catecholamine release that may be more practical to assess in field situations than glucocorticoid levels. SAM system activation also results in elevated respiration and heart rates, and shunting of blood to the organs and muscles with the greatest metabolic need (Sapolsky 2002). Investigation of respiration rates in free-living animals is rare, and has been limited to large species due to technological constraints (Samson et al. 2011; Laubscher et al. 2015). Although some progress has been made towards assessing heart rate responses in wild caught animals via telemetry (Cochran & Wikelski 2005; Cyr et al. 2009; Steiger et al. 2009; Bisson et al. 2011), the techniques are invasive, and therefore

may impact on natural behaviours. A third alternative measure of SAM system activation is presented in the associated changes of blood flow. Sympathetically mediated cutaneous vasoconstriction reduces the amount of blood travelling from the warmer core to the cooler exterior (Busnardo et al. 2010), reducing heat exchange with the environment. As a result, core temperature increases, whilst surface temperature (T_s) decreases, in the phenomenon known as stress-induced hyperthermia (SIH). Importantly, such T_s dynamics can be detected non-invasively from uninsulated skin by thermal imaging (Edgar et al. 2013; Herborn et al. 2015; Jerem et al. 2015; Lecorps et al. 2016). In addition to circumventing the need for the invasive procedures associated with respiratory rate, heart rate telemetry and core body temperature, thermal imaging also allows immediate, continuous and high frequency measurement. Such fine resolution data collection may allow characterisation of the integrated SAM response to acute stress, including both initiation and return to baseline, and therefore ability to cope. Additionally, the ability to simultaneously record behaviour may shed light on underlying physiological processes driving stress-induced behavioural change. However, thus far most investigations of stress-related T_s dynamics have been carried out on domesticated animals in controlled environments, and SIH responses in free-living populations remain poorly characterised.

To gain a more detailed understanding of surface temperature responses to acute stress events in the natural environment, we measured T_s of wild blue tits (*Cyanistes caeruleus*) throughout trapping and handling using thermal imaging. We predicted that T_s would exhibit a characteristic pattern of change during acute stress across all individuals. As body temperature can be affected by air temperature (Aschoff 1979) and humidity (Lin et al. 2005), we predicted that both environmental variables would also relate to patterns of body temperature change associated with acute stress. The SIH response has previously been shown to vary with stressor intensity in domestic chickens (Herborn et al. 2015), where stressor application led to an immediate drop in T_s in response to both mild, and more stressful stimuli. However, the drop was larger after application of the more intense stressor, and was followed by an increase in T_s above baseline that did not occur in response to the mild stressor. It is not

known whether T_s responses vary between stressors in natural populations. Therefore, we compared T_s responses with published data where blue tits (a different group from the same population) were subjected to an identical trapping protocol, but were then released without handling, after a period of confinement with the experimenter in full view of the bird (Jerem et al. 2015). We predicted that T_s responses in blue tits would reflect those found in chickens, and differ from the time-point where the two test-types diverged. An identical response prior to the divergence would demonstrate that the phenomenon is reproducible, whilst differences post divergence would indicate that the T_s response to acute stress contains potentially useful information relating to the nature of the stressor.

Methods

Field Site & Trapping Method

Fieldwork took place from December 2013 to March 2014 in the Atlantic oak forest surrounding the Scottish Centre for Ecology and the Natural Environment (56.13ºN, 4.13ºW). Four walk-in box traps (120mm deep x 140mm high x 250mm wide, with a 60mm diameter entrance hole at one end, and a 10mm x 25mm aperture galvanised wire mesh window on the longest side (Jerem et al. 2015)) were installed 0.8±0.2 km (mean±SEM) apart. Traps were continuously baited with granulated peanuts for ≥ 1 month prior to trapping in order to habituate the birds to the experimental set-up. On six separate trapping days a total of 40 blue tits (*Cyanistes caeruleus,* Linnaeus, 1758) were trapped and took part in one of two test-types. 31 birds underwent 'trapping and handling', whilst 9 birds experienced 'trapping only' during a previous study (Jerem et al. 2015). The sample size in this study was chosen to ensure adequate power to detect an effect size of 0.65 calculated from pilot work carried out on captive zebra finches (R. Nager, unpublished data) with a power of 80% at p=0.05. As soon as the trap containing a bird was closed, the experimenter immediately ran to the front of the closed trap. For the 'trapping and handling' procedure, birds in the trap were caught by hand 21.6±0.8 s after trap closure. The bird was then removed from the trap and held (in the hand) for a further ~2 min 30 s before being measured, ringed and released. Only unringed individuals were selected to take part in the 'trapping and handling' tests to avoid pseudoreplication. For the 'trapping only' procedure, the experimenter stood in front of the trap without handling the bird for a similar amount of time after which the trap door would be opened, and the bird released. Only already ringed individuals were selected to take part in the 'trapping only' tests (excepting one unringed individual), to ensure each individual was only tested once. Total trapping/handling time was limited to ~3 min to avoid detrimental effects of prolonged handling, whilst providing adequate data for the most likely period of interest suggested by earlier work (Jerem et al. 2015). The study was carried out under Home Office authority.

Data Collection

Eye region surface temperatures (T_{eye}) were collected from when the bird first entered the trap, until it was released, using the thermal imaging and data extraction/processing techniques described in Jerem et al. (2015). To summarise, an A65 thermal imaging camera (FLIR Systems, Wilsonville, Oregon, US) was used to record thermal video at ~7.5 frames per second). To film the birds throughout testing, the camera was positioned such that individuals were always within the field of view, both within the trap, and during handling. During 'trapping and handling' tests, the bird was removed from the trap and placed in the centre of field of view once the trap (mounted on hinges) had been swung out of the field of view. To ensure that the eye was visible during handling, birds were then held as shown in Figure 3-1.

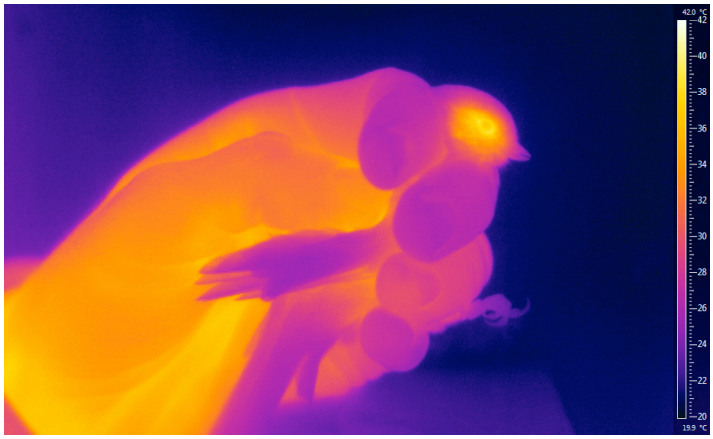

Figure 3-1 The position in which birds were held after capture during 'trapping and handling' tests to ensure that the eye region was always visible to the thermal imaging camera.

Eye region temperature T_{eye} was extracted from the thermal video as the maximum temperature in each frame, which almost always occurred within the periopthalmic ring. In the rare cases where the maximum temperature occurred in other locations (for example where a bird's activity caused the feathers to open over an area of muscle), these frames were removed from the analysis. Maximum temperature was used, since T_{eye} is most likely only underestimated because of motion blur when measuring eye temperature from moving subjects such as birds. When thermal

imaging small passerines, where the majority of the body is insulated by feathers, the exposed eye region is surrounded by cooler integument. Motion blur (which occurs when activity is too rapid to be captured by the camera's frame rate) causes data from the small warm eye area of the image to be confounded with that of larger neighbouring cooler areas, resulting in an underestimation of T_{eye}. In contrast, overestimation of T_{eye} requires energy input. This was avoided by shielding the trap interior from the sun. So, the maximum temperature measured from the eye region was always likely to be the most accurate measurement recorded.

Filtering of raw data was carried out to further minimise underestimation using a 'peak search' algorithm to extract the highest values in the data. The function re-organises data into a vector with user-defined width, the 'span'. Central values in rows where the numbers either side are lower (peaks), are then extracted, giving a temperature curve with most underestimation removed. A span of 15 was used in all filtering across both test-types; in tests performed on the raw data, this value removed the most negative error, whilst retaining the most peaks. Time was standardised by setting trap closure as $t = 0$, whilst temperature data was standardised by subtracting baseline T_{eye} from the test T_{eye} data. The combination of filtering of the raw data (Jerem et al. 2015), and periods where the bird's eye was not visible to the camera resulted in incomplete time series (mean inter-T_{eye} measurement gap (all tests) = 2.87±0.17 s). Therefore, to give one temperature value per second per individual, the standardised data was interpolated using *na.approx* (R package: zoo 1.7-11, Zeileis & Grothendieck, 2005). Basic nonparametric bootstrapped 95% confidence intervals were also added to each plot using *mean.cl.boot* (R Package: Hmisc 3.14-6, Harrell, 2014).

An individual's surface temperature when undisturbed (baseline T_{eye}) was defined as the maximum temperature recorded during the first few seconds (4.2±0.2 s) after an individual entered the trap, but prior to trap closure (Jerem et al. 2015). Maximum T_{eye} during the initial seconds after trap entry has been shown to be representative of maximum T_{eye} over a longer period of time (Jerem et al., 2015), and also correlates with longer term measures of individuals' state, such as body condition and plasma

free corticosterone concentrations (Chapters 4 and 6). Consequently, T_{eye} measured in this way can be considered a suitable baseline measure.

As body temperature can be affected by air temperature (Aschoff 1979) and humidity (Lin et al. 2005), both were measured during all tests. Air temperature and relative humidity were recorded at 30 min intervals by a weather station (Minimet, Skye Instruments, Llandrindod Wells, Powys, UK) situated centrally (approximately) between trapping sites.

Statistical Analysis

Two approaches were combined to create a comprehensive description of the T_{eye} responses. To explore broad differences between test-types, whole responses were compared. Then, to determine precisely where T_{eye} responses differed, a more detailed comparison was carried out, comparing specific curve features (Figure 3-2) between test-types, and within individuals.

All analyses were completed using R v3.1.2 (R Core Team, 2014). The mean T_{eye} response of all individuals over time was plotted for each test-type using *ggplot* (R package: ggplot2 1.0.0, Wickham, 2009). As T_{eye} responses over time during both test-types were non-linear, a generalized additive model was used to determine whether response curves differed as a whole (R function: *gamm*, R package: 'mgcv' 1.8-3, Wood, 2011). T_{eye} was specified as the response variable, test-type, air temperature and humidity as fixed effects, time as a thin plate regression spline smooth, and time by test-type as a tensor product interaction. The interaction was included to test whether responses differed beyond the time point at which the protocols diverged. Bird ID was included as a random effect, to allow for repeated measures from each individual.

Whilst averaging by time provides a good indication of overall response shape, such calculations also mask a considerable proportion of the variation between

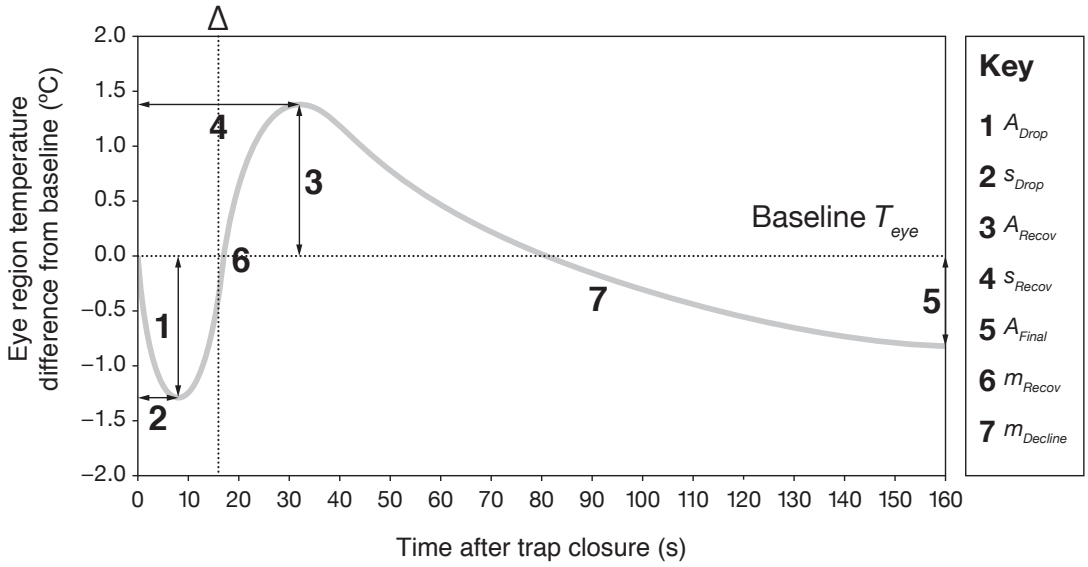

Figure 3-2 Representative eye region temperature (T_{eye}) response to 'trapping and handling', identifying separate features analysed. Δ marks the mean time point at which the experimenter's hand entered the trap in the 'trapping and handling' tests.

individuals. Therefore, a combination of univariate and multivariate linear models (R function: *lm*), and a linear mixed effect model fitted by REML (R function: *lme*, R package: nlme 3.1-118, Pinheiro et al., 2014) were used to assess differences in the scale of specific features of the T_{eye} response curve (Figure 3-2) between test-types. For the multivariate linear models, significance of specific explanatory variables (critical two-tailed $p = 0.05$) was determined using backwards-stepwise (from most to least complex), pairwise model selection (R function: *drop1*). Model selection for the linear mixed effect model followed Zuur et al. (2009).

Features were chosen to be included in the analyses based on their potential connection with stress induced hyperthermia (SIH). The amplitude of the immediate drop from baseline (A_{Drop}), and its timing (s_{Drop}) were considered likely to result from the reduction in warm blood passing from the body core to its surface during SIH (see introduction). Equally, the maximum amplitude of the subsequent recovery (A_{Recov}), its slope (m_{Recov}) and its timing (s_{Recov}) were hypothesised to relate to dissipation of heat generated in the body's core following the initial SIH response, with the post recovery decline slope ($m_{Decline}$) reflecting the progress of this process. Finally, the amplitude from baseline at 160 s (A_{Final}) marks the progress of recovery

towards baseline state by that point in time. While test length was approximately 3 min, the shortest tests were only 160 s in length and therefore measuring A_{Final} at 160 s permitted all tests to be included in the analysis.

A_{Drop} and s_{Drop} occurred before the point at which the two test protocols diverged (marked on Figure 3-2 as Δ), hence it was predicted that neither would differ between test-types. Conversely, as A_{Final} and $m_{Decline}$ were measured after Δ, whilst m_{Recov} was measured over the period during which Δ occurred, all three of these variables were predicted to vary with test-type. Any variation in A_{Final}, m_{Recov}, and $m_{Decline}$ between test-types was expected to result from differing thermal conditions (i.e. being insulated by the experimenter's hand during handling), and/or differences in the nature of the stressor, and/or recovery processes. To compare A_{Drop}, s_{Drop}, and A_{Final} (Figure 3-2) between test-types, each curve feature was specified as the response variable in separate multivariate models with test-type, air temperature and humidity as explanatory variables. To compare m_{Recov} between test-types, a mixed effect model was used, with bird ID included as a random effect to account for repeated measures from individuals. T_{eye} was the response variable, and air temperature, humidity, and interactions between test-type/air temperature/humidity and time/time^2 were explanatory variables. The quadratic term was introduced to account for the recovery's apparent curvature (Figure 3-2). Only T_{eye} data collected between A_{Drop} and A_{Recov} (the part of the curve where the recovery took place) was included in this analysis for the 'trapping and handling' tests. For the 'trapping only' tests, which did not exhibit such a clearly demarcated recovery, this period was defined as that between A_{Drop}, and the mean time of A_{Recov} calculated from the 'trapping & handling' tests.

As there are physiological limits on how fast SIH related changes in blood distribution can occur, we predicted that the speed of the response would be related to its magnitude. For example, smaller responses might be more rapid (and vice versa), both in terms of initiation, and recovery to baseline state. To investigate this possibility, relationships between A_{Drop}, and s_{Drop} or A_{Final} within individuals (pooled

from both test-types) were assessed using univariate models with A_{Drop} as the response variable, and s_{Drop} or A_{Final} as explanatory variables. To account for possible metabolic effects on T_{eye} related to the process of capture within the 'trapping and handling' tests, the relationship between recovery maximum timing s_{Recov} and timing of capture from stressor onset was assessed using a mutlivariate model. In this analysis, s_{Recov} was the response variable, and timing of capture from stressor onset, air temperature and humidity were the explanatory variables. Finally, a multivariate model was used to investigate whether $m_{Decline}$ was a result of passive cooling due to heat loss to the environment, possibly also influenced by the scale and/or speed of the initial response (see above). As the decline slope was considered exponential, $m_{Decline}$ was calculated as k in $y(t) = a*e^{kt}$, where y was T_{eye}, t was 160 s (the latest point at which data was available from all tests –see above), and a was T_{eye} at A_{Recov}. $m_{Decline}$ was specified as the response variable, and air temperature and humidity, A_{Drop}, s_{Drop}, A_{Recov} and s_{Recov} as explanatory variables.

Assumptions for all model types were tested for and met with. Influential outliers were evaluated within the linear models using Cook's Distances. Cook's Distances calculated for all models were <0.5, which was interpreted as meaning no datapoint exercised undue influence.

Results

Characteristic changes in T_{eye} over time were observed during both 'trapping and handling' (n=31), and 'trapping only' (n=9) tests (Figure 3-3). The overall response differed between test-types, but not with air temperature or humidity (GAMM, Test-type by time interaction: $F_{3.96,3.96} = 107.45$, p<0.001; air temperature: $t = 1.08$, p<0.29, df=35; humidity: $t = 1.21$, p<0.23, df=35). During both 'trapping only' and 'trapping and handling' tests, T_{eye} dropped rapidly after trap closure to a similar extent (see Table 3-1 for summary statistics relating to specific curve features from both test-types). The amplitude of this initial drop in T_{eye} below baseline (A_{Drop}) decreased with increased air temperature and humidity (Table 3-2 a.). However s_{Drop} (the timing of A_{Drop}), was unrelated to air temperature or humidity (Table 3-2 b.). During 'trapping and handling', T_{eye} increased after the initial drop, reaching a maximum above baseline. This was followed by a gradual decline to below baseline at the end of the test (Figure 3-3). In contrast, during 'trapping only' the initial drop was followed by a more limited recovery (likelihood-ratio test of test-type x time interaction, accounting for non-linear effect of time: $\chi^2 = 250.6$, p < 0.0001, df = 2), with T_{eye} remaining below baseline for the remainder of the test. The slope of the recovery after the initial drop (m_{Recov}) exhibited a non-linear relationship with air temperature, but was not associated with humidity (Table 3-3). Bird ID also explained T_{eye}, indicating consistent differences between individuals (Table 3-3). A_{Final} did not differ between test-types, or with air temperature or humidity (Table 3-2 c.). T_{eye} at both A_{Drop} and A_{Final} was significantly below baseline (Fig. 3, paired Wilcoxon signed-rank test, A_{Drop} ; v = 780, p = 0.0001, A_{Final}; v = 536.5, p = 0.016), and A_{Drop}, was not related to s_{Drop} (GLM: $F_{1,37} = 1.32$, p = 0.26) in either test-type. During 'trapping and handling', neither A_{Recov} (Table 3-4 a.), or s_{Recov} (Table 3-4 b.) were related to the time taken to capture from stressor onset, air temperature, or humidity. Finally, $m_{Decline}$ (only calculated for 'trapping and handling') was not related to air temperature or humidity (Table 3-4 c.). Neither was it related to A_{Drop}, s_{Drop}, or A_{Recov}. However increased $m_{Decline}$ was positively associated with later s_{Recov}.

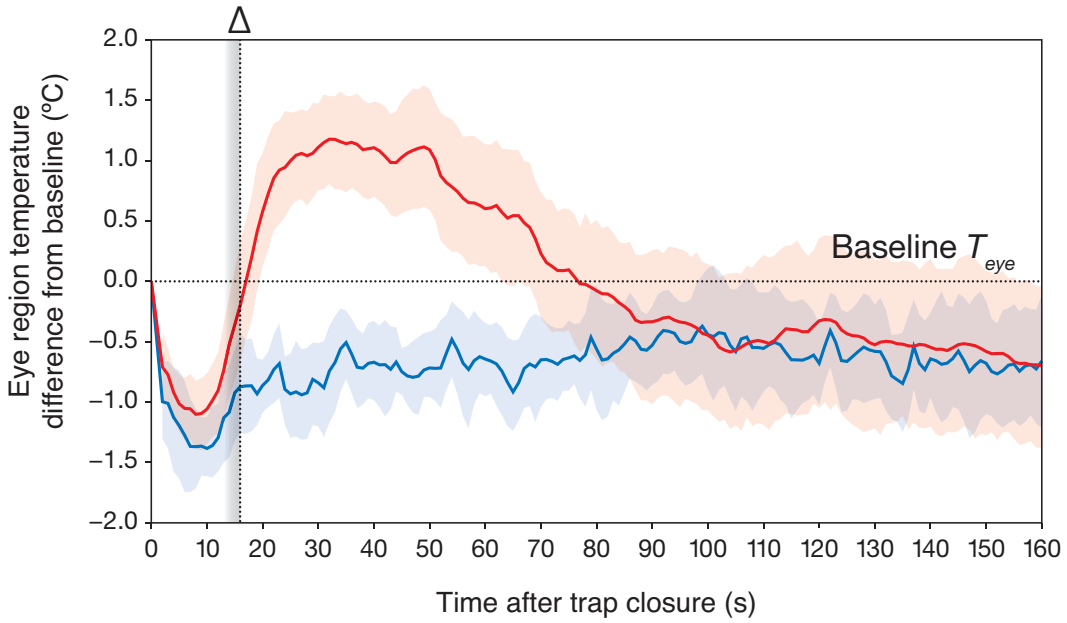

Figure 3-3 Mean effects of 'trapping and handling' (red line, n=31), and 'trapping only' (blue line, n=9) on blue tit eye region temperature (T_{eye}). Shaded areas indicate 95% confidence intervals. Δ marks the mean time point at which the experimenter's hand entered the trap during 'trapping and handling' (this did not occur during 'trapping only' tests). Δ is also the approximate point at which the two test-type protocols deviated, although there would likely be some prior divergence during the experimenter's final approach to the trap, illustrated by the grey gradation. 'Trapping only' data reproduced from (Jerem et al. 2015).

Curve Feature				Trapping Only (n=9)		Trapping & Handling (n=9)	
Key	Abbreviation	Description	Unit	Mean	SEM	Mean	SEM
1	A_{Drop}	Initial drop amplitude from baseline	ºC	-1.98	0.21	-1.60	0.15
2	s_{Drop}	Timing of initial drop minimum	s	11.42	2.22	10.30	0.83
3	A_{Recov}	Recovery maximum amplitude from baseline	ºC	-	-	1.90	0.22
4	s_{Recov}	Timing of recovery maximum	s	-	-	41.40	2.72
5	A_{Final}	Amplitude from baseline at 160 s	ºC	-0.90	0.23	-0.70	0.33
6	m_{Recov}	Recovery slope	-	0.05	0.01	0.14	0.02
7	$m_{Decline}$	Post recovery decline slope	-	-	-	-0.001	0.00004

Table 3-1 Descriptive statistics for curve features identified in Figure 3-2. Key numbers refer to those used in the figure.

a.

Response Variable: A_{Drop}

Fixed Effects	Estimate	t-value	p-value
Test-type	-0.15±0.51	0.56	0.58
Air Temperature	0.19±0.10	3.95	0.0003
Humidity	0.03±0.02	3.21	0.003

b.

Response Variable: s_{Drop}

Fixed Effects	Estimate	t-value	p-value
Test-type	-3.20±3.57	1.75	0.09
Air Temperature	-0.08±0.64	0.25	0.80
Humidity	0.03±0.12	0.40	0.69

c.

Response Variable: A_{Final}

Fixed Effects	Estimate	t-value	p-value
Test-type	-0.12±1.35	0.17	0.86
Air Temperature	-0.04±0.25	0.29	0.77
Humidity	0.02±0.05	0.64	0.52

Table 3-2 GLM parameter estimates (± 95% confidence intervals), t-statistics and p-values for fixed effects predicted to relate to a) A_{Drop}, b) s_{Drop} and c) A_{Final} (see Figure 3-2 and Table 3-1 for definitions).

Response Variable: T_{eye}

Fixed Effects	Estimate	t-value	p-value
Test-type	-0.43±0.56	1.50	0.14
Air Temperature	0.15±0.09	3.25	0.002
Humidity	0.02±0.013	3.61	0.0008
Time	0.17±0.008	40.64	<0.0001
$Time^2$	-0.002±0.0002	21.87	<0.0001
Test-type (Trapping Only) x Time	-0.09±0.03	6.60	<0.0001
Test-type (Trapping Only) x $Time^2$	0.0009±0.0008	1.98	0.047
Air Temperature x Time	0.0016±0.0022	1.41	0.16
Air Temperature x $Time^2$	-0.00004±0.00001	1.66	<0.0001
Humidity x Time	0.0002±0.0003	1.02	0.31
Humidity x $Time^2$	-0.000001±0.000002	1.47	0.14
Random Effect	**Variance**	**SD**	**p-value**
Bird ID	0.45	0.67	<0.0001

Table 3-3 Parameter estimates (±95% confidence intervals), t-values and p-values for fixed effects included in a linear mixed model relating maximum eye region temperature (T_{eye}), measured between A_{Drop} and A_{Recov} (see Figure 3-2 and Table 3-1 for definitions) to test-type, controlling for the effects of air temperature, humidity and time from A_{Drop}. Also, variance, standard deviation and p-values for the random effect included in the model.

a.

Response Variable: A_{Recov}

Fixed Effects	Estimate	t-value	p-value
Time taken to capture	0.02±0.12	0.28	0.78
Air Temperature	0.15±0.19	1.49	0.15
Humidity	0.03±0.04	1.66	0.11

b.

Response Variable: s_{Recov}

Fixed Effects	Estimate	t-value	p-value
Time taken to capture	-8.22±11.34	1.42	0.17
Air Temperature	1.51±18.24	0.16	0.87
Humidity	-0.86±3.38	0.50	0.62

c.

Response Variable: $m_{Decline}$

Fixed Effects	Estimate	t-value	p-value
Air Temperature	-10.86±37.95	0.56	0.58
Humidity	-1.75±5.02	0.68	0.50
A_{Drop}	34.90±167.89	0.41	0.69
s_{Drop}	-1.21±21.97	0.11	0.92
A_{Recov}	44.58±74.53	1.17	0.25
s_{Recov}	6.92±5.33	2.54	0.02

Table 3-4 GLM parameter estimates (± 95% confidence intervals), t-statistics and p-values for fixed effects predicted to relate to a) A_{Recov}, b) s_{Recov} and c) $m_{Decline}$ during 'trapping & handling' (see Figure 3-2 and Table 3-1 for definitions). Parameter estimates in b. and c. have been multiplied by 10^4, and 10^6 respectively to avoid rounding errors.

Discussion

We observed a characteristic pattern of eye region temperature (T_{eye}) change in free-living blue tits during trapping and handling. The T_{eye} response to trapping and handling differed from previously reported T_{eye} dynamics found in response to an identical trapping protocol, minus the handling. The responses differed only from the time point at which the two protocols diverged.

The identical initial T_{eye} responses produced by both test-types demonstrates that the rapid initial drop and recovery reported in Jerem, et al. (2015) is a reproducible phenomenon. Additionally, only the amplitude of the initial drop (A_{Drop}) and the slope of the subsequent recovery (m_{Recov}) were related to environmental conditions, suggesting a predominant role for physiological processes in determining the overall shape of observed T_{eye} responses. Subsequent to the shared initial phase, the pattern of T_{eye} change observed in response to trapping and handling exhibited a pronounced increase above baseline that was not found in birds subjected to trapping without handling. Both the increase above baseline, and the higher rate of increase (m_{Recov}) during trapping and handling are likely to be related to a number of non-mutually exclusive factors. Firstly, it is possible that T_{eye} increased as a result of exercise-related heat production and heat loss (Taylor et al. 2014) associated with the struggle to escape the experimenter's hand before and/or after capture. However, exercise related increases in T_{eye} seem unlikely, as neither the recovery maximum T_{eye} (A_{Recov}) or its timing (s_{Recov}) were related to the length of time between trap closure and capture (a proxy for the amount of energy exerted while attempting to evade capture). Moreover, the escape attempts made by 'trapping only' birds were similarly vigorous, and continued throughout their confinement. As handled birds were calm once captured (21.6±0.8 s after trap closure), this suggests a greater overall level of exertion when flying around the trap whether caught by hand or not. Yet, T_{eye} in these birds remained below baseline throughout. Secondly, handling may have resulted in direct heat transfer from the experimenter's hand to the bird, or a reduction in heat loss to the environment relating to the warmer microclimate created by the enclosing hand. While both are possible, if the held bird was being warmed or

insulated by the experimenter's hand it might be expected that T_{eye} at 160s would be higher in the 'trapping and handing' than T_{eye} at the same point in the 'trapping only' tests. This was not the case, with no difference in A_{Final} found between test-types. Similar increases in surface temperature (T_s) have been observed in domestic chickens in relation to handling (Edgar et al. 2013; Herborn et al. 2015). Admittedly, T_s dynamics took place on longer timescales than reported here, however this is likely to be a result of different volume to surface area ratios associated with larger body sizes. Specifically, comb and T_{eye} responses both shared a rapid initial drop followed by an increase above baseline (Herborn et al. 2015), as seen in the birds in this study. As the post-drop increase in T_s reported in Herborn et al. 2015 was measured subsequent to release from handling, this suggests that stress-related physiological processes (independent of any thermal effects of being handled) were responsible. As such, similar processes may also have contributed to the pattern of T_{eye} change observed in this study. Indeed, while the similarly negative A_{Final} observed at the end of both test-types may be coincidental, the implication that SIH was still occurring at that point remains. Additionally, the lack of a relationship between cooling slope ($m_{Decline}$**)** and ambient conditions, suggests that T_{eye} dynamics during the post-increase cooling phase were not due only to passive cooling (i.e. through heat loss to the environment); $m_{Decline}$ would be expected to be steeper at cooler air temperatures if the reduction in T_{eye} was a predominantly passive process (Ricklefs 1987). The lack of such an association could relate to the microclimate within the experimenter's hand, which would be expected to be warmer than air temperature. However, later A_{Recov} was associated with steeper $m_{Decline}$, suggesting that $m_{Decline}$ was linked to physiological processes taking place during the stress response. Variation in SIH responses with differing stressors has previously been attributed to stressor intensity (van Bogaert et al. 2006; Herborn et al. 2015). It is likely that humans are viewed as a physical threat by a trapped bird. Thus, the two differing test-types could be said to represent different intensities of physical threat, possibly analogous to a predation attempt (Frid & Dill 2002), with handling expected to be interpreted by the bird as more severe than close proximity alone. As such, the differences in the shape of the response curve between test-types reported here may also relate to stressor intensity. However confirmation would require comparison of

the T_{eye} response with other empirically derived measures of stressor intensity, such as plasma glucocorticoid levels (Hennessy & Levine 1978; Kant et al. 1983).

The amplitude of T_s changes in response to handling reported here are within the range of those observed in other bird species (Miller et al. 2010; Edgar et al. 2013; Herborn et al. 2015). Direct comparisons of response shape with these studies are not possible, as methods, regions of interest, and test durations differed. Also, measurement frequencies were substantially lower than those achieved here. Nonetheless, it is interesting to note that a similar rapid initial drop in T_{eye} was observed from a variety of alternative measurement sites in both chicken studies (Edgar et al. 2013; Herborn et al. 2015). The time-course of comb temperature changes is approximately seven times slower than our observations of T_{eye}. highlighting the need for species specific studies. Nonetheless, it remains striking that both chicken comb and blue tit T_{eye} responses to the milder stressor exhibited a rapid initial drop below baseline, and remained between ~0.5-0.75ºC below baseline for the remainder of the tests. Equally, the comb and T_{eye} responses to the more severe stressor both exhibited a rapid initial drop followed by an increase above baseline. It is also interesting to note that the less severe handling stressor ('cradling') used by Herborn et al. (2015) was likely to be more insulating. Cradling involved contact with both the experimenter's hands, arms and torso, whereas the more severe 'side-pinning', only involved contact with the experimenter's hands. Additionally, the potential for heat dissipation in a chicken released from such holds is likely to be greater than that of a blue tit held within the hand. The existence of similarities between studies, independent of thermal circumstances supports the argument that the T_{eye} dynamics reported here are related predominantly to stress physiology.

Our results suggest that in principle, it may be possible to assess acute stress non-invasively from wild animals by thermal imaging of SIH. However, a number of further validations would be necessary before the method could be established as a practicable means of stress assessment. Firstly, as A_{Drop} decreased with increased air temperature and humidity, the range of environmental conditions within which a

response can be detected needs to be established. Secondly, if proportionality can be confirmed, responses to stressors of a substantial range of intensities should then be compared within individuals, both to define the physiological limits demarcating the possible extent of T_s changes (Vinkers et al. 2009b), and to determine response linearity. Corticosterone responses to graded intensities of acute stressor have been shown to be linear (Hennessy & Levine 1978; Kant et al. 1983). Also, SIH shares a number of features with the hypothalamic-pituitary-adrenal (HPA) axis response to acute stress (Groenink et al. 1994; Veening et al. 2004). Therefore, it might be expected that variation in SIH with stressor intensity is also linear. Finally, comparison of surface temperature responses with both core body temperatures, and hormonal/behavioural measures of stress would be useful in allowing separation of stress-related variation in surface temperature from other sources.

In conclusion, we have shown for the first time that T_s responses to acute stress in a free-living animal species are reproducible, and exhibit pronounced differences with stressor type. Further investigations will be required to explain the causes and processes underlying such differences. However, it is clear that an abundance of potentially useful information relating to the nature of a stressor is contained within the T_s response. As such, we believe the use of thermal imaging in this context represents an unprecedented opportunity to investigate of 'fight or flight' responses in free-living populations, not least in circumventing a number of the limitations associated with current invasive methods.

4. Body condition and glucocorticoids relate to variation in body temperature in a wild bird

Abstract

Substantial within- and between-individual variation in body temperature exists in endotherms, but the sources of this variation are poorly understood, especially in wild animals. Variation in body temperature may reflect how individuals cope with their environment via activation of stress-induced-hyperthermia or metabolic effects related to depletion of energy reserves. Using thermal imaging, we examined the relationship between body surface temperature and physiological state in a wild passerine. We show that individuals in poorer body condition had lower surface temperatures in non-breeding and breeding seasons, and that body surface temperature was related to baseline glucocorticoid levels in non-breeding birds. The results suggest remotely measured body surface temperature could be used to track ecologically relevant annual variation in physiological state of free-living organisms.

Introduction

Endotherms maintain a high and relatively constant body temperature (T_b). T_b is a key parameter for understanding thermoregulation, physiology, behaviour and responses to environmental change (McCafferty et al. 2015). There is substantial within- and between-individual variation in T_b in free-living animals (McCafferty et al. 2015), but little is known about factors that cause this variation (Adelman et al. 2010a; Nord et al. 2015; Nilsson et al. 2016), partly due to difficulties in measuring T_b in the wild (McCafferty et al. 2015).

Variation in T_b can reflect how individuals cope with their environment. Individuals challenged by poor environmental conditions may show altered hypothalamic-pituitary-adrenal (HPA) axis activity, and glucocorticoid secretion (Romero & Wingfield 2015). Activation of the HPA axis can also trigger the sympathetic-adrenal-medullary (SAM) axis, causing changes in heart rate, respiration and blood flow (Romero & Wingfield 2015). The diversion of blood elevates core T_b, resulting in stress-induced-hyperthermia (SIH) (Bouwknecht et al. 2007), a widespread phenomenon amongst endotherms. Redirection of blood from the periphery, also lowers body surface temperature (Busnardo et al. 2010). SIH appears closely associated with HPA axis activation for short-lived (< 1 h) responses to single and repeated acute stressors, and longer-term (≥ 24 h) responses to social stress (Keeney et al. 2001; Bouwknecht et al. 2007). Alternatively, T_b can reflect metabolic effects with lowered T_b linked to energy reserve depletion (Reinertsen 1986), due to decreased heat production from metabolism in individuals experiencing poor feeding conditions (MacLeod et al. 1993). Hence, variation in T_b is predicted to be associated with levels of glucocorticoids, energy reserves or both.

New methods of measuring body temperature remotely and non-invasively using thermal imaging (Jerem et al. 2015) provide an opportunity to determine variation in body temperature, which reflects the physiological state of animals. In this study, we examine the relationship between body surface temperature measured

from the eye region (T_{eye}) and physiological state in wild blue tits *(Cyanistes caeruleus)*. Specifically, we test the hypotheses that lower T_{eye} relates to higher glucocorticoid concentrations and poorer body condition.

Methods

Data Collection

Data were collected from overwintering or breeding blue tits occupying oak woodland at Loch Lomond (56.13ºN, 4.13ºW). Birds were recorded on thermal videos either entering walk-in box traps (winter), or their nest box (breeding season), (see below). In winter, we trapped 31 blue tits. Once a single bird entered the trap it was allowed to feed undisturbed while thermal videos were recorded. The trap was then closed, the bird retrieved and a blood sample taken to measure baseline total and free corticosterone (see Laboratory Analyses). During the breeding season thermal videos were recorded of 14 birds while visiting their nest on day 13 after hatching.

During the winter of 2013/14, walk-in box traps (for details of the trap see (Jerem et al. 2015)) were installed at four locations (mean±SE inter-trap distance 0.8±0.2 km) across the study site. To habituate visiting birds and avoid biases, the traps were continuously baited with granulated peanuts for >1 month before sampling. The distribution of individual condition index from birds sampled during this study was not significantly different to the distribution of individual condition index from mist-netted birds caught at the same site, during the same time of year (first two weeks in March) on three previous years (2011, 2012, 2013) (Kolmogorov-Smirnov test, $D=0.224$, $p=0.225$, Figure 4-1). This indicates no detectable effect of sampling bias in terms of body condition relating to the use of baited traps in this study, and notably no bias towards birds in particularly poor condition. The 31 blue tits caught in the winter were trapped during 10-13 March 2014 between 08:18 and 16:52 (mean sampling time 12:50±32 min). The end of winter was chosen as the period in winter when the greatest differences in individual state would be expected, and so where relationships between body temperature and state would be most detectable. The relatively short period was chosen to minimise (and control for) environmental variation, as much as possible. Daytime sampling was chosen to minimise the effect of circadian changes in body temperature. Single birds entering the trap were filmed using a thermal imaging camera, while feeding

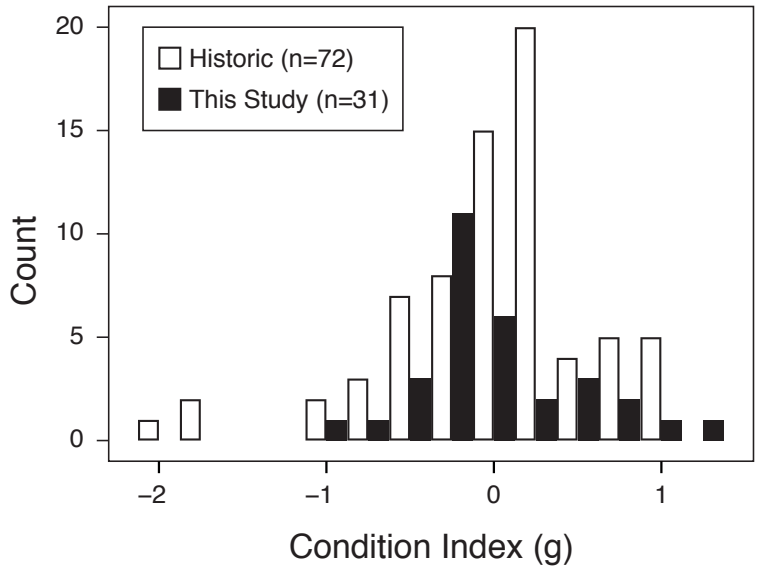

Figure 4-1 Condition index distributions for the birds sampled in this study, and mist-netted birds caught at the same site, during the same time of year in three previous years (2011, 2012, 2013). A condition index of zero represents mean condition, with positive and negative values indicating weight above or below average for given wing length.

undisturbed, for an average of 4.2±0.16 s before the trap was closed by the experimenter (using a fishing line, from a concealed position). The bird was then retrieved by hand 17.4±0.75 s after trap closure, and blood was sampled from the jugular by venipuncture within 112.3±3.2 s of trap closure, and immediately placed on ice. Blood samples were subsequently separated into plasma and red blood cells by centrifugation (10 min at 2000 rpm), and the plasma used to measure corticosterone (CORT). Before the birds were released we sexed the birds from plumage characteristics, and measured body mass (to the nearest 0.1 g), and maximal wing chord (to the nearest 0.5 mm). Individual condition index was calculated as the residuals of mass regressed on wing chord[3].

During the breeding season, breeding parents were filmed while entering or exiting their nest box using a thermal imaging camera mounted in front of the nest box. Breeding birds used Schwegler 1B nest boxes (32mm entrance hole, Schwegler, Schorndorf, Germany) installed across the site. To habituate the birds to the presence of the camera, a dummy camera was installed at each nest ≥ 7 days prior to filming,

and left in place. The dummy camera was replaced with the actual camera immediately prior to filming on day 13 after hatching (day of hatching=day 0; filming took place during 5-16 June 2015, between 10:09 and 20:31, mean sampling time 13:05±8 min). Daytime sampling was again chosen to minimise the effect of circadian changes in body temperature. Once the camera was installed, thermal video was recorded for 45 minutes. To avoid acute stress related changes in body temperature (resulting from disturbance associated with installation), only body temperature measures from video recorded after the initial 15 minutes were included in the analyses. To distinguish between the two individuals that attended each nest at least one of the attending parents (14 individuals breeding in 12 randomly selected nests) was caught on day 8-9 after hatching, between 01-11 June 2015 and fitted a RFID tag (125 kHz, 2.3mm, EM4102 Bird Tag, IB Technology Glenfield, Leicestershire) mounted on a leg ring. Parents were then identified on the thermal videos using a combination of the records of the RFID tag logger mounted in the nest box entrance (Nature Counters, Maidstone, Kent; IB Technology Glenfield, Leicestershire; Francis Scientific Instruments, Ltd., Huntingdon, Cambridgeshire; University of Glasgow Bioelectronics Dept., Glasgow), and distinguishing features (e.g. leg rings) visible in the thermal images. During RFID fitting, body mass and wing chord were measured, sex identified, and body condition index calculated using the same techniques as described for the overwintering birds. In both winter and the breeding season, there was no significant correlation between condition index and body size (Spearman's Rho; Winter=0.06, p=0.74; Breeding Season=0.06, p=0.84). Consequently, our condition index represents body mass, controlling for body size.

During all thermal imaging, the camera (FLIR A65, FLIR Systems, Wilsonville, Oregon) was mounted (at a distance of 50 cm) so individuals were recorded passing through the camera's field of view and within the camera's zone of focus, either within the trap (Jerem et al. 2015), or as they entered and left the nest box. Maximum eye region temperatures (T_{eye}) were extracted from the thermal video following the principles described in (Jerem et al. 2015), where the highest temperature measured from the eye region is assumed to be the most accurate. To avoid underestimation from motion blur, T_{eye} was taken to be the maximum value; overestimation was

avoided by shielding the trap interior from sunlight in winter (Jerem et al. 2015). Baseline T_{eye} was defined as the maximum temperature measured from the eye region of an individual while feeding undisturbed within the trap (winter), or during entry/exit from the nest box, or during periods where the bird was in the camera's field of view, if it did not enter or leave the nest box (breeding season). The type of event from which T_{eye} was measured during the breeding season appeared to influence the value of T_{eye} and so was noted and included in the analysis. Visits to the nest box were defined as 'in' when an individual entered the nest box, 'out' when an individual left the nest box, and 'no entry/exit' when an individual entered the field of view of the camera, but did not enter or exit.

Accurate absolute temperatures can be extracted from thermal images by the inclusion of an object of known temperature and emissivity within the field of view, against which the temperature measured from the bird's surface by the thermal imaging camera can be calibrated. To achieve this, a thermistor probe coated in black insulation tape (Tesa UK, Milton Keynes, Buckinghamshire) was installed onto the front of the trap (winter) or the nest box perch (breeding season), and connected to a Tinytag Talk 2 Temperature Logger (Gemini Data Loggers UK, Chichester, West Sussex). The logger was set to record at 1 s intervals, and the resulting temperature data used to calibrate the individual frames from which T_{eye} was extracted.

CORT shows circadian rhythms, responds to environmental conditions, and can differ between sexes (Romero & Wingfield 2015). Also, T_b fluctuates with a circadian rhythm modulated by air temperature (T_a) (Aschoff 1979), and humidity (Lin et al. 2005), and may differ between sexes (Bonier et al. 2007). Therefore, time of day, T_a, humidity, and sex were considered as potential confounding explanatory variables. T_a was recorded from the trap mounted temperature logger described above. During winter data collection, relative humidity was recorded every 30 min (Minimet, Skye Instruments, Wales) at the centre of the study site. During the breeding season, weather station malfunction meant that humidity data was instead obtained from the MIDAS MET Office weather station at Bishopton (approximately 25 km from the study site; 55.91ºN, -4.53ºW); for a short period during the breeding

season where we had data from both weather stations, the correlation between humidity data was high (GLM: $F_{1,15}$=35.68 p<0.0001). Sex was determined by crown colouration. T_b may also be influenced by solar radiation (Lustick et al. 1970). While birds in the trap were shielded from the sun, the breeding birds were not completely in shade. Therefore, the presence/absence of direct solar radiation falling on the nest box during thermal imaging was recorded as a categorical measure during the breeding season.

Laboratory Analyses

Total CORT levels were assessed using a commercial ELISA kit (Enzo Life Sciences, Switzerland), following the manufacturer's instructions. One reading was below the detection limit (1 ng/ml), so was set to 0.999 ng/ml. CBG affinity and capacity were assessed, and free CORT levels estimated following (Breuner et al. 2003). For individual birds, CBG was estimated using 20 nM [^{3}H] corticosterone. Maximum site binding capacity (*Bmax)*, and the dissociation constant (*Kd)* from the saturation analysis were calculated using iterative, least-squares curve-fitting (GraphPad Prism, GraphPad Software, US) to fit untransformed data to a single site binding hyperbola (y = *Bmax**x/(*Kd*+x)). *Kd* was 2.58 nM, whilst *Bmax* was 321.9±13.27 nM (Figure 4-2, Figure 4-3). The intra- and inter-assay coefficients of variation were 11.25% and 3.85%, respectively.

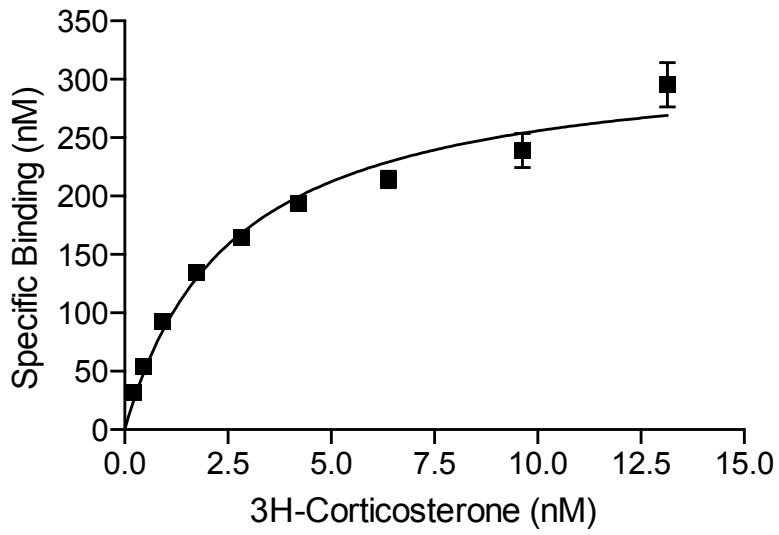

Figure 4-2 Specific binding of [3H] corticosterone to blue tit plasma in relation to increasing concentrations of radiolabeled corticosterone. Solid squares represent means ± standard error.

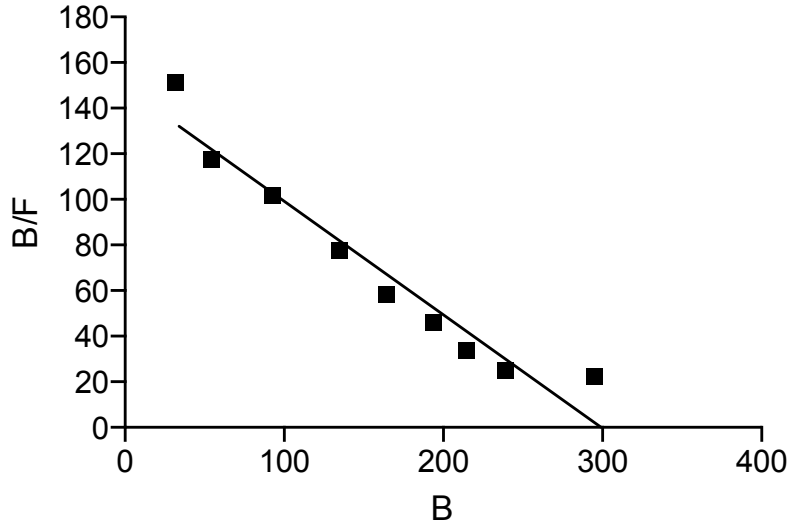

Figure 4-3 Scatchard-Rosenthal re-plot of Figure 4-2, where F = free, and B = bound [3H] corticosterone fraction.

Statistical Analyses

All statistical analyses were performed using R v3.1.2 (R Core Team 2016). Relationships between T_{eye} (response variable), and condition index, free CORT and total CORT during winter were analysed in separate general linear models (GLMs - *lm* command). As repeated measures of T_{eye} were taken from breeding birds, the relationship between T_{eye} (response variable), and condition index during the breeding season was analysed analysed using a general linear mixed model (GLMM - *lmer* (package:lme4 1.1-12, (Bates et al. 2014)) with Bird ID specified as a random effect. From the full models, significance of explanatory variables (critical two-tailed $p<0.05$) was determined via backwards-stepwise model selection using *drop1*. P-values for fixed/random effects in the GLMM were calculated using *lmerTest/rand* (package: lmerTest 1.0, (Kuznetzova et al. 2016)). Effect size *r* was calculated for parameters in the final models according to (Nakagawa & Cuthill 2007), while R^2 was calculated for the GLMM following (Nakagawa & Schielzeth 2013). Variance inflation factors (*vif*, 'car' package v2.0-22, (Fox & Weisberg 2011)) calculated post-hoc for explanatory variables included in the GLMs suggested no issue with collinearity. All model assumptions were met.

We considered the following potentially confounding explanatory variables when analysing variation in T_{eye}: T_a, humidity, sex (as CORT can differ between sexes), solar radiation, time of day (as CORT and T_b show circadian rhythms) and whether they exited or entered the nest (event type). Potentially confounding explanatory variables were included in full models only if they were significantly associated with T_{eye} in preliminary univariate tests (critical p=0.1). In these preliminary analyses, T_{eye} differed between event types, so event type was included in the breeding season analysis. However, T_{eye} did not differ between sexes ($F_{1,29}$=2.57, p=0.12) or presence/absence of direct sunlight (t=0.81, p=0.43), therefore these variables were not included in full models. All other potential confounding explanatory variables were related to T_{eye} in the univariate tests (p<0.017). As time of day was correlated with T_a and humidity, we could not distinguish between diurnal changes in T_{eye} or effects of T_a. Consequently, only T_a was included in the models.

Results

T_{eye} ranged from 26.5-31.2 ºC (28.7±0.23 ºC, mean±SE, n=31) in the overwintering birds, and from 25.2-33.6 ºC (29.62±0.0.9 ºC, n=372 observations from 14 individuals) in breeding birds. T_{eye} increased with T_a in both seasons and decreased with humidity, but only in the models considering CORT (Table 4-1, Table 4-2). T_{eye} also increased with body condition in both seasons (Figure 4-4). Additionally, when breeding birds entered the nest box, T_{eye} was lower than when they left, or did not enter or leave (Table 4-1). In winter, free CORT concentrations varied from 0.007-0.381 ng/ml (0.11±0.02 ng/ml, n=31), whilst total CORT concentrations varied between 0.999-18.61 ng/ml (8.29±0.65 ng/ml, n=31). Of the total CORT concentration, 1.41±0.18% was available as free CORT. Sampling latency was unrelated to free or total CORT (free CORT R^2=0.00006, p=0.97, total CORT R^2=0.03, p=0.34). Variation in T_{eye} in winter was associated with free CORT concentration (Table 4-2a, Figure 4-5), but not with total CORT concentration (Table 4-2b).

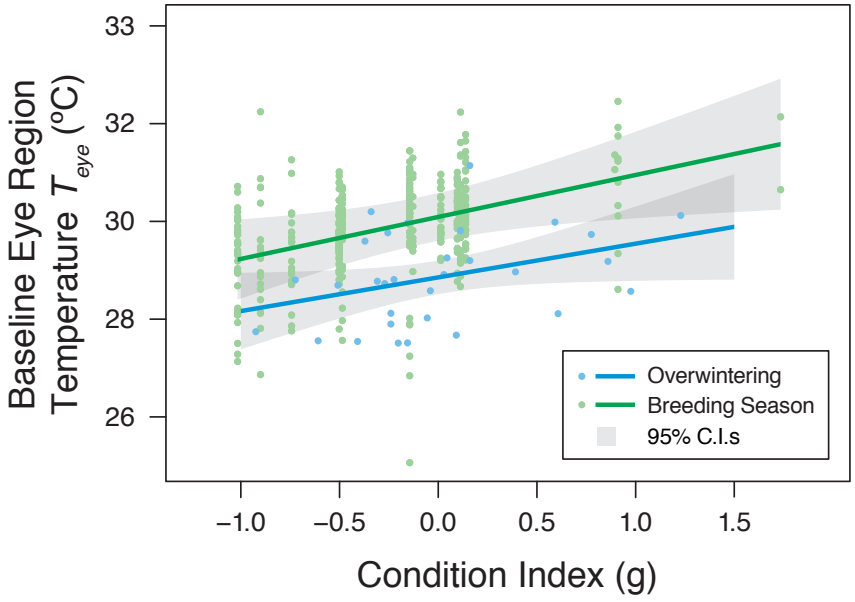

Figure 4-4 Model predictions of relationships between baseline eye region temperature and body condition index. Zero represents mean condition, with positive and negative values indicating weight above or below average for given wing length.

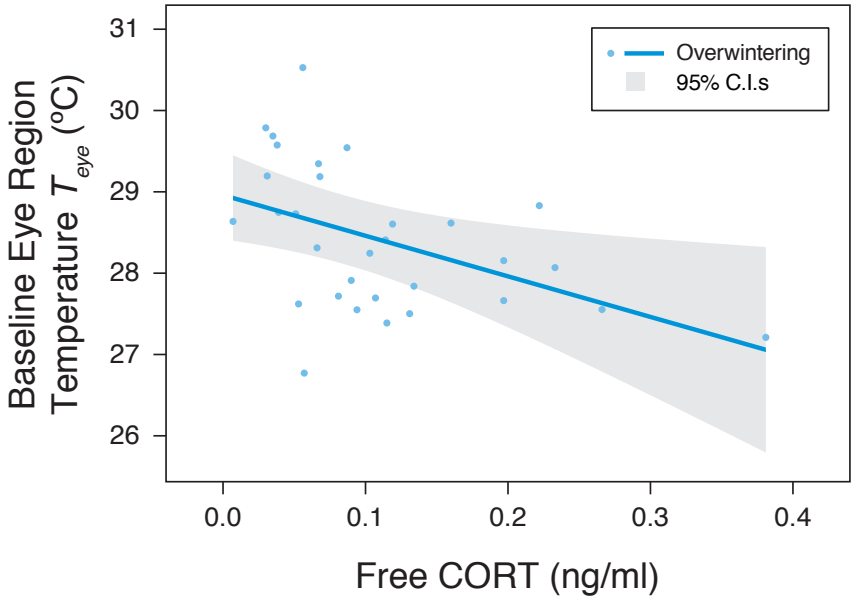

Figure 4-5 Model prediction of relationship between baseline eye region temperature and free CORT.

Response Variable: Baseline Eye Region Temperature (T_{eye})

	Fixed Effects	Estimate±95% CI	t-value	p-value	*r*
a.	Intercept	26.44±1.05	49.24	<0.0001	
	Condition Index	0.69±0.66	2.05	0.05	0.37
	Air Temperature	0.29±0.13	4.45	0.0001	0.65
	Relative Humidity	-0.02±0.02	1.61	0.12	0.30
	Model adjusted R^2 = 0.47				
b.	Intercept	25.87±1.39	36.52	<0.0001	
	Condition Index	0.86±0.69	2.43	0.028	0.09
	Air Temperature	0.23±0.09	4.76	<0.0001	0.17
	Relative Humidity	-0.02±0.02	1.14	0.26	0.04
	Event Type (Out)	1.01±0.18	11.19	<0.0001	
	Event Type (No entry/exit)	1.24±0.78	3.12	0.002	
	Random Effect	**Variance**	**SD**	**p-value**	
	Bird ID	0.77	0.88	<0.0001	
	Model adjusted R^2 = 0.35				

Table 4-1 Summary of statistical models relating body condition index, air temperature and humidity with T_{eye} in (a) winter (GLM model), and (b) breeding season (GLMM model); *r* is the parameter effect size. In the breeding season whether birds were entering or leaving the nest box was also considered.

Response Variable: Baseline Eye Region Temperature (T_{eye})

	Fixed Effects	Estimate±95% CI	t-value	p-value	*r*
a.	Intercept	30.70±2.79	21.50	<0.0001	
	Baseline free CORT	-4.99 ± 3.84	2.55	0.017	0.44
	Air temperature	0.19±0.13	2.90	0.007	0.49
	Relative Humidity	-0.034±0.022	3.09	0.005	0.51
	Model adjusted R^2 = 0.56				
b.	Intercept	28.71±0.41	135.86	<0.0001	
	Baseline total CORT	0.028±0.098	0.55	0.586	0.11
	Air Temperature	0.24±0.14	3.25	0.003	0.53
	Relative Humidity	-0.025±0.022	2.18	0.008	0.34
	Model adjusted R^2 = 0.48				

Table 4-2 Summary of GLM models relating air temperature, humidity and (a) baseline free CORT or (b) baseline total CORT with T_{eye} in winter; *r* is the parameter effect size.

Discussion

Baseline eye region surface temperature (T_{eye}) was related both to body condition and baseline glucocorticoid levels in undisturbed wild blue tits, suggesting that individual differences in T_b can reflect physiological state. Variation in metabolic demand, and/or stress-related vasoconstriction could have brought about variation in T_{eye}.

Variation in T_{eye} was similar in extent to that previously reported for other measures of T_b (Nord et al. 2015). T_{eye} was influenced by ambient conditions, with T_a and relative humidity explaining a significant proportion of variation. The positive relationship between T_{eye} and T_a is consistent with previous studies showing surface temperature is sensitive to environmental conditions (Nilsson et al. 2016). Despite this, the repeatability of T_{eye} can be high (>0.7) for periods up to 40 min (Nord et al. 2015). Breeding birds entering the nest box had lower T_{eye} than when leaving, possibly due to its warmer microclimate. We sampled T_{eye} during two relatively short periods of the annual cycle and found that T_{eye} was consistently related to body condition at both stages (with differences in intercepts most likely resulting from seasonal differences in T_a). This relationship may indicate a reduced metabolic rate resulting in lower heat production in individuals experiencing poor feeding conditions (MacLeod et al. 1993). Moreover, lowering body temperature to reduce the ambient temperature gradient can offset thermoregulatory costs (Nilsson et al. 2016), providing energy savings for birds in poorer condition.

Alternatively, environmental challenges can reduce individual body condition, and may lead to greater physiological stress, and changes in HPA activity (Johnstone et al. 2012). We found that after accounting for ambient conditions, T_{eye} also related to free, but not total baseline CORT levels, both of which were within the range found elsewhere (Müller et al. 2007; Fokidis et al. 2009). As sampling latency was unrelated to CORT levels, our values likely represent true baselines, and were not overestimated due to the acute stress of trapping and handling. T_{eye} also responds to acute stress, but on a shorter time scale than CORT (Jerem et al. 2015). Although baseline T_{eye} could be affected by the acute stressor of entering the trap, this is unlikely as the birds were habituated over time. Short term acute factors that would

affect the measure of T_{eye} but not CORT would be expected to confound the relationship between T_{eye} and glucocorticoids, suggesting the association is conservative in this respect. Bird T_b can also be affected by social defeat >24 h after the event (Carere et al. 2001) demonstrating that longer term stress-related T_b changes also occur.

Debate remains whether free or total CORT concentrations play a greater biological role (Romero & Wingfield 2015). If only free CORT is active, and T_{eye} is linked to HPA axis activity via stress-induced hyperthermia, then only a relationship between T_{eye} and free CORT would be expected, as suggested by the Free Hormone Hypothesis (Mendel 1992).

Variation in T_{eye} mediated by body condition in both non-breeding and breeding birds could be either a metabolic effect of poor food intake or reflect stress-related vasoconstriction. The latter hypothesis is supported by the association we found between T_{eye} and glucocorticoid levels. However, future studies also need to account for food mediated changes in T_b that may influence physiological state. The results suggest remotely measured surface T_b could be a novel way to track ecologically relevant annual variation in physiological state of free-living organisms.

5. An experimental test of the relationship between baseline plasma glucocorticoids and surface temperatures measured from free-moving birds

Abstract

Plasma glucocorticoid levels are the most frequently used physiological indicator of stress. Nonetheless, assessment of glucocorticoid levels in wild animals is often difficult, as it usually requires trapping and invasive blood sampling. In emergency situations, the sympathetic-adrenal-medullary (SAM) system is also triggered, increasing blood flow to the core. The sympathetically mediated redirection of blood flow increases core body temperature, and lowers body surface temperature (T_s). T_s measured using thermal imaging has been related to plasma glucocorticoids during responses to acute stressors in hens (*Gallus gallus*). Also, previous work on wild blue tits (*Cyanistes caeruleus*) provided correlative evidence that baseline T_s and plasma free corticosterone (CORT) levels may be linked. Therefore, it may be possible to estimate chronic stress-related baseline glucocorticoid levels from non-invasive measurements of T_s. To test this hypothesis, we compared baseline plasma total and free CORT levels, and baseline T_s in captive zebra finches exposed to a reduced enrichment environment intended to induce chronic stress. Neither baseline measures of total plasma CORT, CBG binding capacity, free plasma CORT or T_s differed between groups of birds housed in either low or high enrichment environments. This suggests that enrichment removal did not act as a stressor. As the treatment did not induce physiological stress, it was not possible to experimentally test whether T_{eye} and plasma glucocorticoid concentrations are related.

Introduction

Vertebrates exhibit a generalised response to a wide range of stressors (Sapolsky 2002). Glucocorticoids are secreted in response to a stressor as a result of activation of the hypothalamic-pituitary-adrenal (HPA) axis (Sapolsky 2002). Glucocorticoid secretion is the most studied process taking place during the stress response (Romero & Wingfield 2015), and plasma glucocorticoid levels are the most frequently used physiological indicator of stress (Sapolsky et al. 2000). Nonetheless, there are a number of issues associated with the use of such measures. First, blood sampling can be a stressful event in itself, and may cause a confounding stress response (Stewart et al. 2005). Secondly, in wild animals, acquiring baseline measures can be difficult. This is not only due to the complications presented by safely trapping, handling, and extracting blood in natural environments. It is also necessary to sample blood within 2 minutes of capture, before glucocorticoids released as a result of trapping reach the bloodstream, distorting baseline values (Romero & Reed 2005). Consequently, the development of alternative measures that circumvent the need for logistically challenging and invasive sampling would represent an important advance, especially for researchers studying free-living populations.

The HPA axis is not the only physiological process associated with the generalised response to stress. In emergency situations, the sympathetic-adrenal-medullary (SAM) system is also triggered (Sapolsky et al. 2000), with both systems having been shown to respond proportionally to stressor intensity (Hennessy & Levine 1978; Kant et al. 1983; Van der Heyden et al. 1997; Fenner et al. 2016). Activation of the SAM system results in a fight or flight response, where among other physiological changes, blood flow to the core is increased to meet the heightened demands for oxygen and nutrients of involved organs (Sapolsky 2002). This sympathetically mediated redirection of blood flow increases core body temperature (Briese & Cabanac 1991), and lowers body surface temperature (Herborn et al. 2015). Stress induced hyperthermia (SIH) of core body temperature (Briese & Cabanac 1991), as the process is known, has been reported in a wide variety of species (Bouwknecht et al. 2007), and appears closely related to HPA axis activation

(Groenink et al. 1994) (although the HPA axis is not thought to be directly involved in SIH mediation (Carrasco & Van de Kar 2003)). Therefore, body temperature and glucocorticoid dynamics are expected to be related, opening up the possibility of inferring glucocorticoid levels non-invasively via thermal imaging of body surface temperature (T_s).

Associations between T_s and levels of plasma glucocorticoids have been reported during responses to acute stressors in hens (Herborn et al. 2015). Also, our previous work on wild blue tits (*Cyanistes caeruleus*) provided correlative evidence that baseline T_s and plasma free CORT levels may be linked (Chapter 4). Therefore, the aim of this study was, to investigate the relationship between baseline T_s and plasma glucocorticoid levels experimentally, in particular exploring for the first time whether it might be possible to estimate chronic stress-related baseline glucocorticoid levels from T_s. Instead of continuing to work with wild blue tits, we chose to use captive bred zebra finches (*Taeniopygia guttata*) in this study. Importantly, using captive bred birds avoided potentially confounding effects of captivity-related stress associated with bringing wild animals into controlled environments (Dickens et al. 2009). Working with captive birds also allowed a greater level of control over the various potential confounding variables (e.g. air temperature, humidity, abiotic and biotic stressors) than would be possible when studying free-living birds.

Environmental enrichment is expected to alter physiological functioning in captive animals (Fox et al. 2006). For example, both short and long-term enrichment have been shown to affect feather corticosterone levels in wild-caught captive Clark's nutcrackers (Nucifraga columbiana), with stress being proposed as the underlying process (Fairhurst et al. 2011). Also, pilot work (R. Nager, unpublished data) found that mean baseline eye region temperatures (measured weekly over one month via thermal imaging) in captive zebra finches were approximately 0.5 ºC lower in birds housed in cages with minimal enrichments, when compared to those housed in cages with multiple enrichments. Thus, the level of environmental enrichment seems likely to affect both HPA activity and body temperature. Consequently, we used changes in

the amount of environmental enrichment available to birds to assess relationships between stress-related baseline plasma glucocorticoid levels and baseline Ts. We hypothesised that both Ts and glucocorticoid levels would exhibit changes in relation to reduced environmental enrichment. If such changes were found to be related, this would indicate that it may be possible to estimate baseline glucocorticoid levels from non-invasive measurements of Ts.

Methods

Subjects, housing and experimental treatments

Adult zebra finches were acquired from a number of breeders through a commercial supplier, and randomly allocated to same-sex pairs. Birds were housed two per cage (60 cm x 50 cm x 50 cm) to avoid isolation stress (Perez et al. 2012a) while allowing identification of individuals in thermal footage from opposite placement of leg rings. The room in which the birds were kept was maintained at 21.6±0.1 ºC (mean±SEM), with a 14:10 light:dark photoperiod. Food (Foreign Finch Mixture, John E Haith Ltd., Grimsby, UK), water, and cuttlebone were provided ad-libitum. For an initial acclimation period of 7 days (Figure 5-1), all birds were kept on a 'high enrichment' regime (Table 5-1). Subsequently, for a further 7 days, half of the birds were allocated to a 'low enrichment' regime (Table 5-1), meaning that the majority of enrichments were removed from their cages, whilst half remained on the 'high enrichment' regime (Figure 5-1). Physiological measurements were made on the final day of the second week (see below). The experiment was run in two consecutive replicates, each consisting of 36 birds in 18 cages. It was not possible logistically to blood sample more than six birds per day, therefore, start dates were staggered by one day by groups of three cages, meaning that on the first day of the experiment (Day 7 Figure 5-1), three cages were allocated to their enrichment regime, with a further three allocated the next day, and so on. The staggered timetable required that some birds experienced longer habituation (Replicate 1), or pre-habituation (Replicate 2) stages however neither had any effect on glucocorticoid measures (Replicate 1: total CORT $F_{1,25} = 0.12$, $p = 0.74$, CBG binding capacity $F_{1,24} = 0.04$, $p = 0.84$, free CORT $F_{1,24} = 0.09$, $p = 0.77$, Replicate 2: total CORT $F_{1,29} = 0.0008$, $p = 0.98$, CBG binding capacity $F_{1,26} = 0.07$, $p = 0.79$, free CORT $F_{1,26} = 0.06$, $p = 0.8$), or surface temperatures (linear mixed models with bird identity as a random effect, Replicate 1: $t = 1.47$, $p = 0.15$, Replicate 2: $t = 0.78$, $p = 0.44$).

	Enrichment Regime	
Enrichment	**High**	**Low**
Card sheet on cage floor	✓	✓
Auboise hemp bedding on cage floor	✓	
Solid dowel perch	✓	✓
Bamboo perch with leaves	✓	
Millet spray	✓	
Vegetable treats (alternate days)	✓	
Weekly water bath	✓	

Table 5-1 Enrichments included in cages allocated to either high or low enrichment regimes. Vegetable treats consisted of coarsely chopped carrot and broccoli, frozen into ice cube trays. One 'vegetable cube' was provided to each pair every other day.

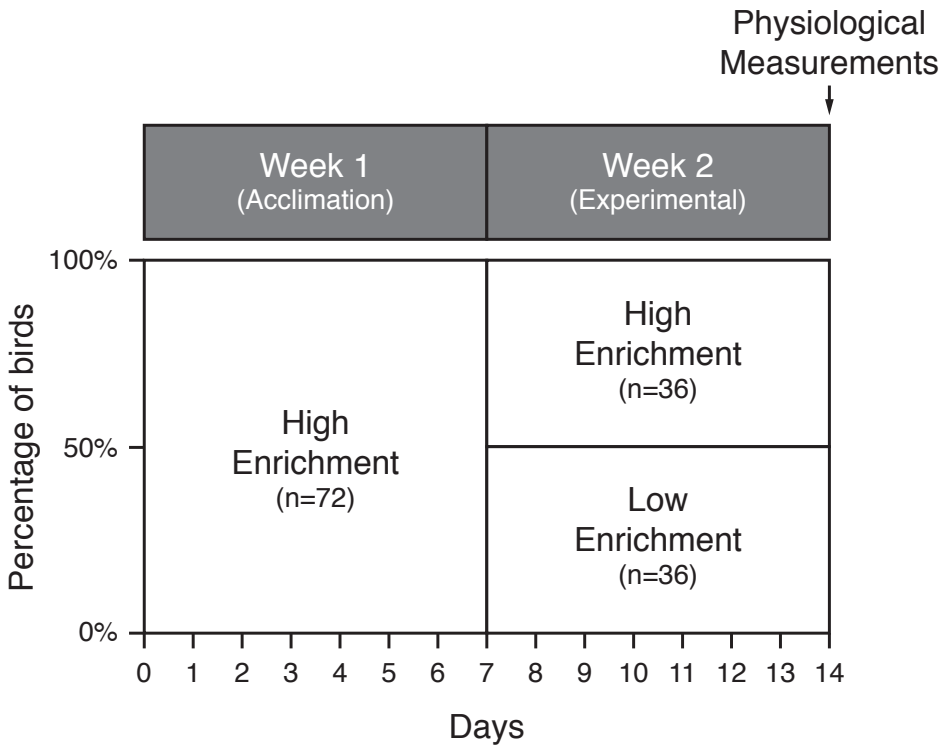

Figure 5-1 Diagram of experimental design illustrating the splitting of the birds into two housing treatment groups (low enrichment and high enrichment) on Day 7. Physiological measures were taken on Day 14 (see text).

Measurement of plasma CORT concentrations

To assess baseline plasma CORT concentrations, blood samples (~75 µl) were taken from the birds' brachial vein by venipuncture at approximately 16:00 on the final day of Week 2 (Day 14 Figure 5-1). Blood was successfully extracted from 69 birds, within 69.6±4.3 s (range = 32 – 188 s) of the investigator entering the room. Samples were placed on ice immediately, then separated into red blood cells and plasma by centrifugation (10 min at 2000 rpm), and stored at -80 ºC until analysed. Sampling latency was related to both total and free plasma CORT concentrations (total CORT $R^2 = 0.07$, $p = 0.02$, free CORT $R^2 = 0.14$, $p = 0.002$). Therefore, to correct for the confounding effect of these relationships, sampling latency was included as a covariate in the analyses (see below).

Total plasma CORT concentrations were determined using an enzyme immunoassay (Munro & Stabenfeldt 1984; Munro & Lasley 1988), with samples run in triplicate. CORT from 5 µl of plasma in 195 µl of water was extracted using 4 ml dichlormethane, re-dissolved in phosphate buffer. The anti-corticosterone antibody (Chemicon, Merck Millipore, Darmstadt, Germany; cross-reactivity: 11-dehydrocorticosterone 0.35%, Progesterone 0.004%, 18-OH-DOC 0.01%, Cortisol 0.12%, 18-OH-B 0.02% and Aldosterone 0.06%) was diluted to 1:8000. ABTS was used as the substrate, while HRP (dilution 1:400,000) linked to corticosterone was used as the enzyme label. Standard curves run in duplicate on each plate were used to calculate the concentration of CORT in the plasma samples. Pooled chicken plasma of two differing concentrations were also included on each plate, as internal controls. Three readings were below the limit of detection, and so were assigned a value equal to the lowest detectable concentration (0.11 ng/ml). One reading was substantially more than two standard deviations above the mean, and so was removed from analyses as being likely due to experimental error. Also, the volume of 6 blood samples was too small to assay, meaning total CORT was assessed for 62 of the 69 individuals from whom blood was extracted. Inter assay variation was 2.29-5.24%, and intra assay variation was 3.27-14.61%.

Corticosteroid binding globulin (CBG) affinity and capacity were assessed using a radioligand-binding assay with tritiated corticosterone, after Breuner et al. (2003). For point sample analysis, endogenous steroids were stripped from 5µl of plasma using two parts dextran-coated charcoal solution (0.1% dextran, 1% Norit A charcoal in 50mM Tris) for 30 min at room temperature. The binding assay was carried out for 1 h in 50mM Tris buffer at 4ºC. Final assay dilution was 1:1500. All assays contained 50 µl plasma preparation, 50 µl [^{3}H] corticosterone (radioligand), and 50 µl unlabelled corticosterone (nonspecific binding) (1 µM) or buffer (total binding). Specific binding was calculated by subtraction of unlabelled corticosterone. All samples were run in triplicate, and plasma was maintained at 4ºC throughout the procedure. Free and bound radioligands were separated using rapid vacuum filtration (Brandel Harvester, Alpha Biotech, Killearn, Glasgow). Prior to filtering, glass fibre filters (Whatman, GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire) were soaked in 25 nM Tris with 0.3% polyethyleneimine for 40 min. Filters were rinsed rapidly three times using 3 ml ice-cold 25 mM Tris post filtration. Radioactivity bound to filters was subsequently measured by liquid scintillation spectroscopy (Tri-Carb® 2910TR, Perkin Elmer, Waltham Massachusetts). Saturation analysis was carried out on pooled samples. 0.25–12 nM [^{3}H] corticosterone was incubated with pooled plasma in the presence or absence of 1 µM unlabeled corticosterone. For individual birds, CBG capacity was estimated using 20 nM [^{3}H] corticosterone. Iterative, least-squares curve-fitting (GraphPad Prism, San Diego, CA) of untransformed data to a single site binding hyperbola ($y=Bmax*x/(Kd+x)$ was used to calculate maximal site binding capacity (*Bmax)*, and the disassociation constant (*Kd)*. *Bmax* was 787.5 nM, whilst *Kd* was 2.98 nM (Figure 5-2, Figure 5-3) The intra-assay coefficient of variation was 7.43%; The inter-assay coefficient of variation was 9.33%. The equation of (Barsano & Baumann 1989) was used to estimate free CORT titres from total CORT concentrations and CBG binding parameters (*Bmax* and *Kd*). Due to limited volumes of blood collected from some individuals, CBG binding capacity and free CORT titres were calculated for 57 (out of 69) individuals. Two values of CBG binding capacity and one value of free CORT were substantially more than two standard deviations above the mean, and so were removed from analyses as being likely due to experimental error.

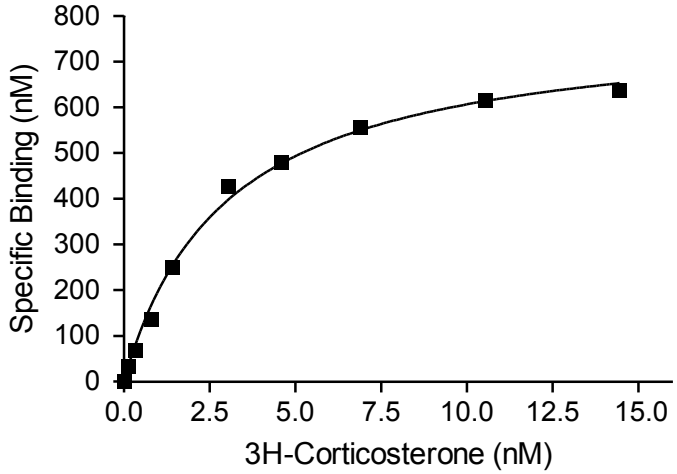

Figure 5-2 Specific binding of [3H] corticosterone to blue tit plasma in relation to increasing concentrations of radiolabeled corticosterone. Solid squares represent means ± standard error.

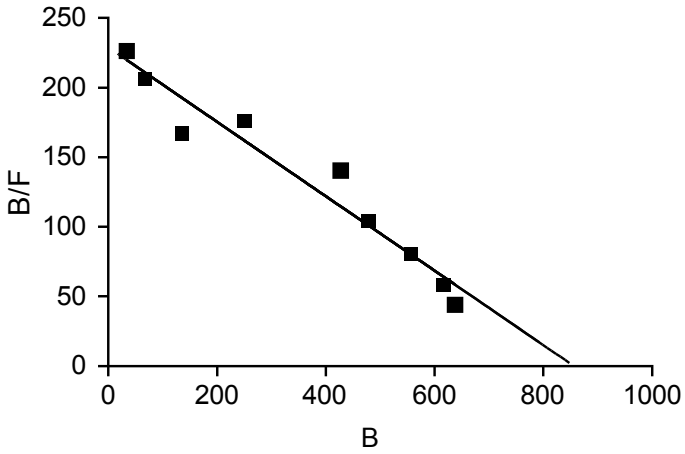

Figure 5-3 Scatchard-Rosenthal re-plot of Figure 5-2, where F = free, and B = bound [3H] corticosterone fraction.

Measurement of surface temperature

Surface temperatures of the birds were measured using thermal imaging, according to the principles set out in Jerem et al. (2015). To capture baseline eye region temperatures (T_{eye}) from undisturbed birds, a thermal imaging camera (A65, FLIR Systems, Wilsonville, Oregon, US) was mounted onto a custom built radio-controlled motorised dolly. The dolly allowed the camera to be moved along and between cages (filming the birds within) at approximately 5 cm s^{-1}, whilst being controlled from an ante-room by an operator who was not visible to the birds. The dolly ran along a rail which was positioned so that birds on the dowel or bamboo perches were in the camera's zone of focus. To habituate the birds to the presence and movement of the camera, mock filming was carried out on non-filming days at approximately the same time and for a similar duration as the actual filming. Because of the staggered timing of the experimental protocol (see above), all birds were exposed to an approximately equivalent duration of either mock or actual filming each day throughout the experiment. Actual filming took place between 11:30-13:30 on the final day of Week 2 (day 14, Figure 1-1). The camera was set to record at ~7.5 frames per second, and the dolly moved backwards and forwards constantly in front of each cage. Filming sessions only captured footage from a single cage, and lasted for approximately three minutes. Filming of each cage continued until at least three sessions had been completed, and when an estimated 45 measures per bird had been captured (the maximum number of filming sessions for any one cage was seven (4.15±0.16). Prior to the start of each filming session, the camera was set to perform a non-uniformity correction using the controlling software (ResearchIR v3.4, FLIR Systems, Wilsonville, Oregon, US). To calibrate the temperatures measured by the thermal imaging camera, filming sessions always started from a position where a temperature logger probe (Tinytag Talk 2, Gemini Data Loggers, Chichester, West Sussex, UK), mounted in between the cages, was in the field of view. The difference between the mean temperature of the logger probe measured via thermal imaging, and the actual temperature recorded by the logger probe was used to calibrate all thermal images from that filming session (Jerem et al. 2015). As the cage bars partially obscured the view of the target bird, a single measure was defined as a

sweep of the moving camera across the subject, that revealed a clearly focussed image of the eye region between the cage bars. Single values of T_{eye} were extracted from the resulting thermal videos as the highest temperature measured from the eye region during each sweep of the camera across the subject, with the five highest measurements of T_{eye} being recorded for each filming session. Only individuals with 15 or more measurements of T_{eye} across three filming sessions were included in the analyses (no. of measurements 22.15±0.66, range = 15-35). Individuals were identified in the footage from the position of metal leg rings (either right or left).

Other measures

A number of factors can potentially affect the measures relating to glucocorticoids, and T_{eye}. Where possible, these potential confounding variables were controlled for through the experimental design. Where this was not feasible, the variable was assessed or measured, enabling statistical control in the analyses. Firstly, as the birds from the two replicates were sourced from different breeders, differences in prior housing conditions may have resulted in variation in plasma CORT levels between replicates. Therefore, replicate was included as a potential explanatory variable in all analyses. As both CORT and body temperature exhibit a circadian rhythm (Aschoff 1979; Breuner et al. 1999), sampling was restricted to the same time of day for all birds (CORT – 16:00, T_{eye} – 11:30-13:30). Additionally, HPA activity can differ between sexes (Bonier et al. 2007), and may be linked to preening behaviour (Delius 1988), locomotor activity (Lynn et al. 2003), and body condition (Müller et al. 2011). As we predicted stress physiology to relate to T_{eye}, sex, body condition, preening behaviour and locomotor activity were all recorded and included as potential explanatory variables in all analyses. As zebra finches are dimorphic (Zann 1996), sex was identified by plumage colouration. Individual body condition index was calculated as the residual value of a linear regression between body mass and wing chord length^3 (Schulte-Hostedde et al. 2005). Body mass for the condition index calculation was measured at the same time as blood was sampled (Day 14, Figure 1-1). Condition index could not be calculated for three birds which had damaged primaries, therefore these birds were removed from analyses in which condition

index was a covariate. Preening behaviour (categorised as none / preening / allopreening given / allopreening received) at the point of each measurement of T_{eye} was noted and included in the T_{eye} analysis as a covariate. Also, relationships between preening, T_{eye} and the HPA axis were expected to be integrated over longer periods of time. Therefore, a mean preening score for each individual over all filming sessions was calculated and used to account for preening in all analyses. As only the highest five measures of T_{eye} were recorded for each filming session, preening score consisted of the total number of measures (max = 5) where a given preening behaviour was taking place. These preening scores were then averaged across all filming sessions for each individual to give the mean preening score.
A similar approach was taken to account for activity. Whilst long tem activity levels may relate to HPA axis activation and body temperature, the most pronounced effect of activity on T_{eye} is likely to be negative error associated with motion blur, (Jerem et al. 2015), with values of T_{eye} tending to be higher for less active subjects. Therefore, activity (defined as a change in position of both feet) in the second prior to each measurement of T_{eye} was recorded and included as a potential explanatory variable in the T_{eye} analysis. Mean activity score, calculated in the same way as the mean preening score, was included as a potential explanatory variable in all analyses.
Three further factors were expected to influence body temperature, and so were also recorded and included as potential explanatory variables in the T_{eye} analysis; air temperature, proximity to other birds, and lateralisation. Body temperature is modulated by air temperature (Aschoff 1979), which was recorded by the temperature logger used to calibrate the thermal images (see above). Proximity between birds during measurement of T_{eye} is expected to influence thermoregulation (Gilbert et al. 2010). For this reason, the presence or absence of a 'thermal social interaction' (i.e. bodily contact between sitting birds) at the point of measurement of recorded. Finally, stress-related body temperature changes may exhibit lateralisation, with different effect magnitudes on different sides of the body (Mazzotti & Boere 2009; Magnani et al. 2011). Therefore, the eye (either left or right) from which each T_{eye} measurement was made, was also noted.

Statistical Analyses

All analyses were performed in R v3.3.0 (R Core Team 2016). Univariate and multivariate and linear models (R function: *lm*) were used to assess relationships between glucocorticoid related measures and the treatment groups, controlling for confounding factors where necessary. To account for repeated measures of T_{eye}, linear mixed effect (LMM) models, (R function: *lme*, R package: nlme 3.1-127, Pinheiro et al. 2014) were used for the T_{eye} analysis, with bird ID included as a random effect. The significance of explanatory variables (critical two-tailed p = 0.05) was determined by backwards-stepwise (from most to least complex), pairwise model selection (R function: *drop1*). All models met with assumptions. The intra class correlation coefficient of T_{eye} was calculated using R function: ICCest (R package: ICC 2.3.0, Wolak et al. 2012). Model plots presented in the Results section were created using the 'visreg' R package (Breheny & Burchett 2016).

As the number of potential explanatory variables exceeded 10% of the sample size, explanatory variables (up to a maximum of six, plus housing treatment group, the variable of interest) were included in the final multivariate analyses, only if they were significantly associated with the response variable in preliminary univariate tests (critical p = 0.1). We predicted a relationship between the glucocorticoid measures and T_{eye}. Therefore, absolute maximum T_{eye} (chosen as representing the single most accurate measure (Jerem et al. 2015)), was added to the statistical models explaining variation in the glucocorticoid measures. Accordingly, Total CORT, CBG binding capacity, and Free CORT were added to the list of potential explanatories in the T_{eye} analysis.

For the total CORT analysis, only sampling latency and mean preening score had *p*-values < 0.1 (sampling latency: $F_{1,59} = 5.59$, $p = 0.02$; mean preening score: $F_{1,56} = 3.72$, $p = 0.06$). Consequently, housing treatment group, sampling latency and mean preening score were specified as explanatory variables, with total CORT as the response variable (log transformed to correct for a non-normal distribution). Neither the potential confounding variables, nor sampling latency were found to have

p-values < 0.1 in univariate tests with CBG binding capacity. Thus, CBG binding capacity was analysed using a univariate linear model, with housing treatment group as the only explanatory variable. Similarly, as none of the potential confounding variables had *p*-values < 0.1 in univariate tests with free CORT, only housing treatment group and sampling latency were included as explanatory variables in the free CORT analysis. Free CORT was also log transformed to correct for a non-normal distribution. Inspection of diagnostic plots showed that two data points had substantially failed to meet the assumption of normal of residual distribution in the free CORT model, and so were removed from the free CORT analysis. In univariate tests with T_{eye}, air temperature, activity, mean activity score, the presence/absence of thermal social interactions, and preening behaviour were all found to have *p*-values < 0.1 (linear mixed models with bird identity as a random effect; air temperature $t = 8{,}28$, $p < 0.0001$; activity $t = 13.32$, $p < 0.0001$; mean activity score $t = 5.49$, $p < 0.0001$; thermal social interaction $t = 13.95$, $p < 0.0001$; preening $t = 8.26$, $p < 0.0001$), and so were all included as explanatory variables in the final multivariate analysis, with T_{eye} specified as the response variable.
To illustrate the relationships between T_{eye} and total CORT, free CORT and body condition index, residual T_{eye} from the multivariate T_{eye} model described above (i.e. T_{eye} corrected for all confounding variables) was specified as the response variable, with total CORT, free CORT or body condition index specified as explanatory variables in univariate linear models.

Results

Effect of enrichment removal on plasma glucocorticoids

Total CORT ranged from 0.11-7.07 ng/ml (2.51±0.23 ng/ml, n = 58), whereas CBG binding capacity varied from 364.8-999.5 nM (715.3±18.1 nM, n=54), and free CORT from 0.0023-0.0222 ng/ml (0.0108±0.0009 ng/ml, n=53). Neither total CORT, CBG binding capacity, or free CORT differed between housing treatment groups (Figure 5-4, Table 5-2).

a.

Response Variable: Total CORT

Fixed Effects	Estimate	t-value	p-value
Treatment (High Enrichment)	-0.16±0.30	0.66	0.51
Sampling Latency	0.009±0.007	2.57	0.01
Mean Preening Score	-0.39±0.32	1.90	0.06

b.

Response Variable: CBG Binding Capacity (B_{max})

Fixed Effects	Estimate	t-value	p-value
Treatment (High Enrichment)	-2.83±73.88	0.08	0.94

c.

Response Variable: Free CORT

Fixed Effects	Estimate	t-value	p-value
Treatment (High Enrichment)	-0.16±0.33	0.96	0.34
Sampling Latency	0.01±0.005	3.20	0.002

Table 5-2 Parameter estimates (±95% confidence intervals), t-values and p-values for fixed effects included in linear models relating housing treatment group to total plasma CORT (a), CBG binding capacity (b), or free plasma CORT (c) concentrations.

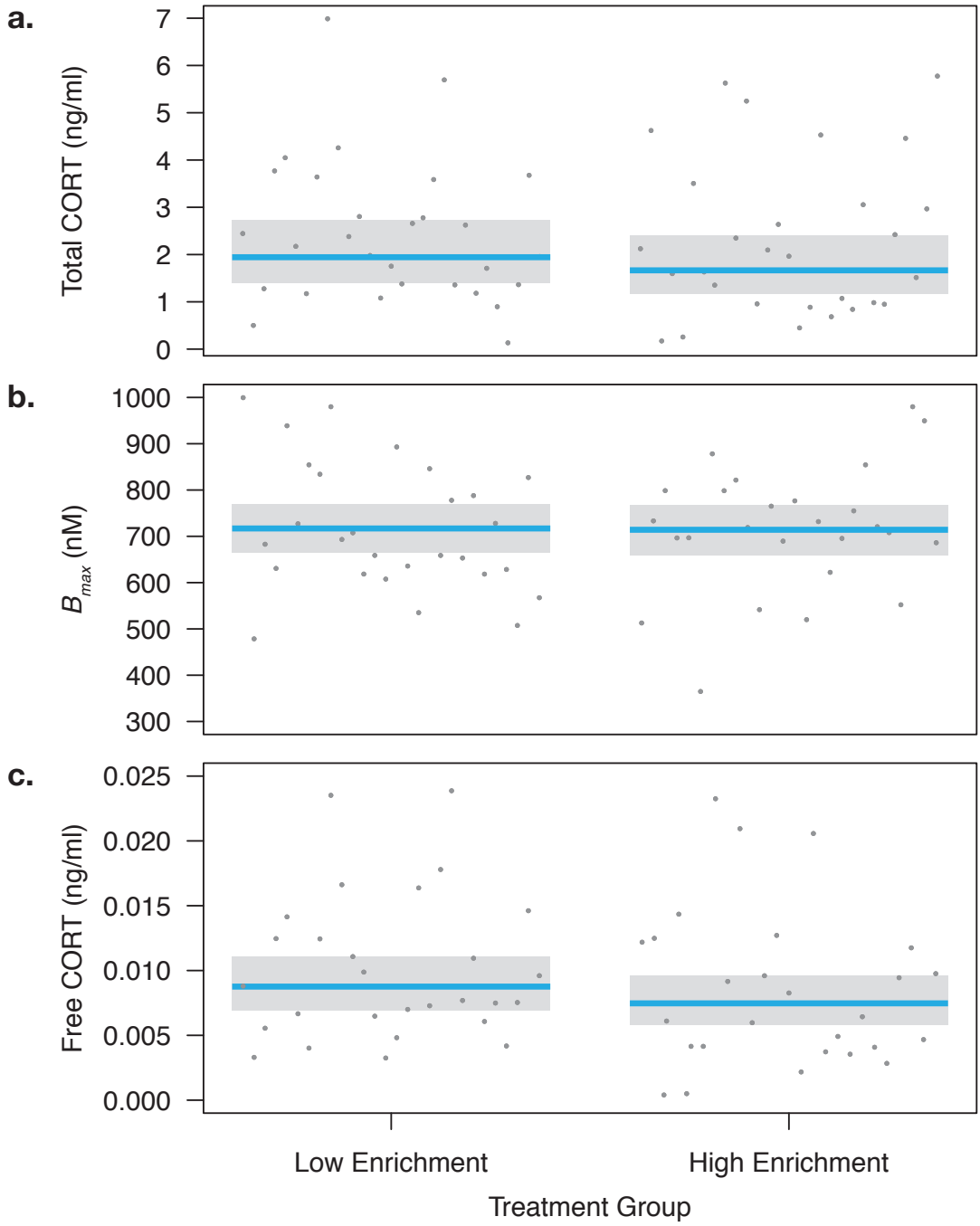

Figure 5-4 Model predictions (horizontal lines) of baseline total plasma CORT concentration (a), CBG binding capacity (b), and free plasma CORT concentration (c) for each housing treatment group (total CORT; low enrichment n=29, high enrichment, n=29, CBG binding capacity; low enrichment n=28, high enrichment, n=26, free CORT; low enrichment n=28, high enrichment, n=25) . Shaded areas represent 95% confidence intervals, and data points are partial residuals (conditional on the other variables in the models being set to their median values).

Effect of enrichment removal on surface temperature

T_{eye} (33.3±0.02 ºC, range = 31.2-36.1 ºC, n = 1440 measurements, from 65 individuals) did not differ between housing treatment groups (Figure 5-5, Table 5-3). Neither total plasma CORT, free plasma CORT, or body condition index were related to T_{eye} (corrected for all confounding variables in the model presented in Table 5-3) (total plasma CORT; $\chi^2 = 0.0001$, df = 1, p = 0.98, free plasma CORT; $\chi^2 = 0.0005$, df = 1, p = 0.96, body condition index: $\chi^2 = 0.0002$, df = 1, p = 0.97, Figure 5-6). The intra class correlation coefficient of T_{eye} was 0.53±0.10 (ICC±95% confidence interval).

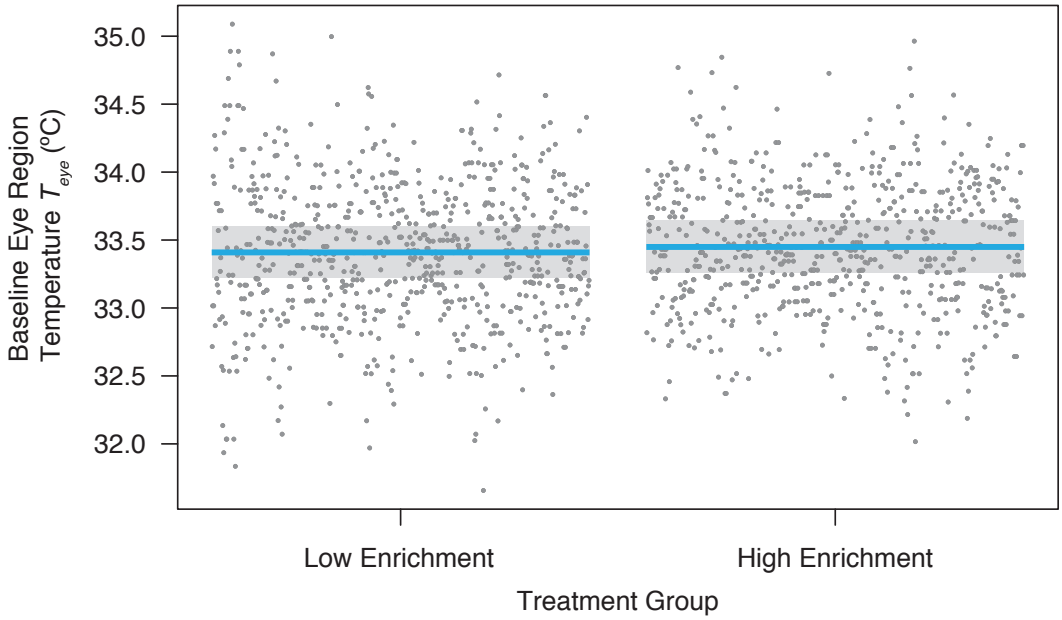

Figure 5-5 Model predictions of baseline eye region temperature (T_{eye}), for each housing treatment group. Shaded areas represent 95% confidence intervals, and dark circles are partial residuals (conditional on other variables in the model being set to their median values).

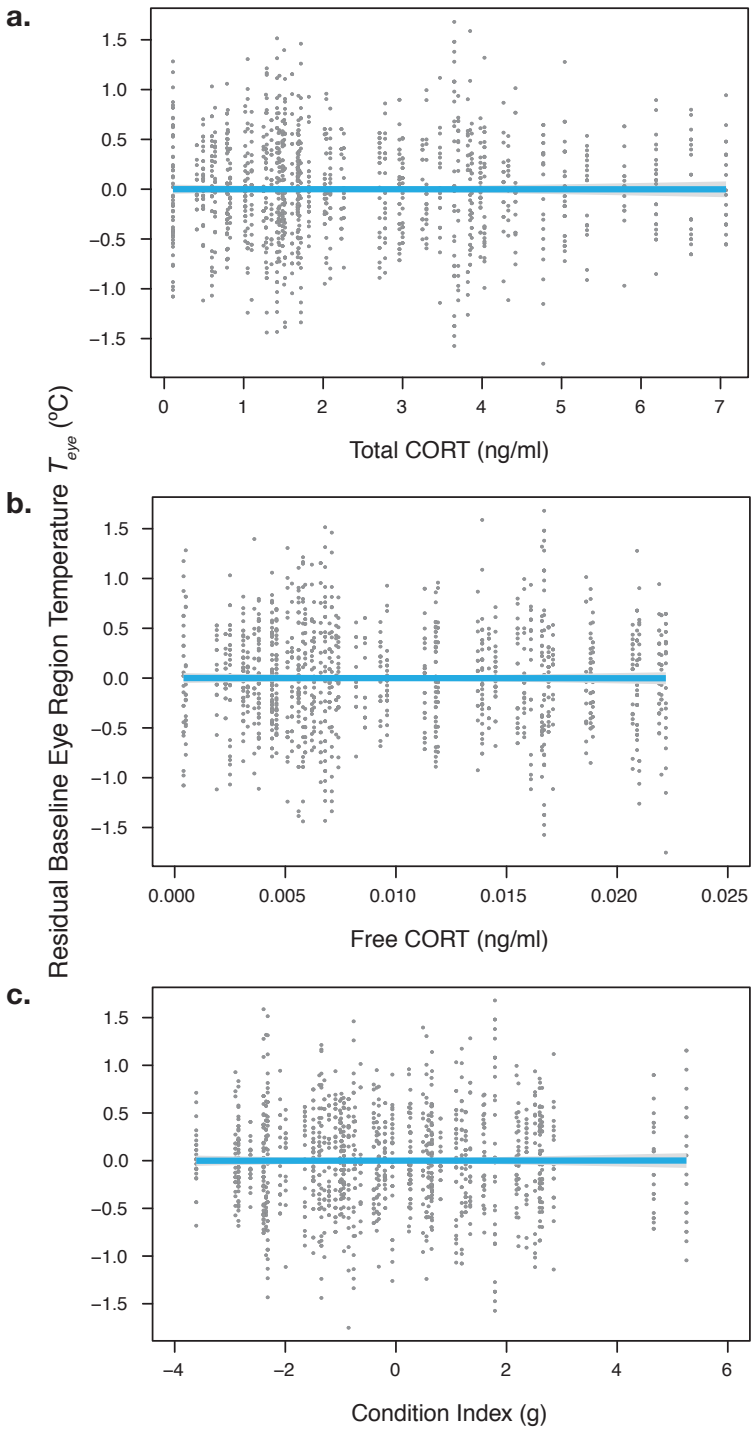

Figure 5-6 Model predictions (blue lines) of the relationships between residual baseline eye region temperature T_{eye} (from the model presented in Table 5-3) and total plasma CORT concentration (a), free plasma CORT concentration (b), and body condition index (c). Shaded areas represent 95% confidence intervals. For condition index, zero represents mean condition, with positive and negative values indicating mass above or below average for a given wing length. For residual T_{eye}, zero represents the expected T_{eye} for associated values of confounding variables, with positive and negative values indicating temperatures above or below that expected for values of associated confounding variables.

Response Variable: Baseline Maximum Eye Region Temperature (T_{eye})

Fixed Effects	**Estimate**	**t-value**	**p-value**
Treatment (High Enrichment)	0.04±0.27	0.30	0.77
Air Temperature (ºC)	0.48±0.21	4.47	<0.0001
Activity (Active)	-0.35±0.07	9.90	<0.0001
Mean Activity Score	-0.18±0.12	2.98	0.004
Thermal Social Interaction (Yes)	0.57±0.13	8.61	<0.0001
Preening (Preening)	0.35±0.10	6.50	<0.0001
Preening (Allopreening Given)	0.23±0.18	2.54	0.01
Preening (Allopreening Received)	0.25±0.21	2.40	0.02
Random Effect	**Variance**	**SD**	**p-value**
Bird ID	0.26	0.51	<0.0001

Table 5-3 Parameter estimates (±95% confidence intervals), t-values and p-values for fixed effects included in a linear mixed model relating housing treatment group to baseline maximum eye region temperature (T_{eye}), controlling for the effects of air temperature, mean activity score, thermal social interactions, and activity/preening in the second prior to the measurement. Also, variance, standard deviation and p-values for the random effect included in the model.

Discussion

Neither baseline measures of total plasma CORT, CBG binding capacity, free plasma CORT, or eye region temperature (T_{eye}) differed between groups of birds housed in either low or high enrichment environments. This suggests that the removal of enrichments did not act as a stressor. As the treatment did not induce physiological stress, it was not possible to experimentally test whether T_{eye} and plasma glucocorticoid concentrations are related. Additionally, no relationship was found between free plasma CORT and T_{eye}, or between body condition index and T_{eye}, in contrast to data from wild blue tits (Chapters 4 and 6).

Comparison of absolute measures relating to glucocorticoids between studies should be made with caution due to differences between assay methodologies. However, baseline total plasma CORT concentrations here were similar to those measured from a separate population housed in the same facility (in conditions similar to the high enrichment group in this study) (Marasco et al. (2015), 2.32±0.02 ng/ml), and from three other studies conducted elsewhere; (Breuner et al. (2006) 2.29±0.23 ng/ml; Wada et al. (2008) 2.09±0.42 ng/ml); Lynn et al. (2010) 2.15±0.57 ng/ml), but markedly higher than in one further study (Banerjee & Adkins-Regan (2011) Trial 1 0.69±0.06 ng/ml). Corticosterone binding globulin (CBG) binding capacity in our study was also substantially higher than previously reported, (Breuner et al. (2006) ~298 nM; Lynn et al. (2010), 376.98±20.6 nM), although birds were housed singly in the case of Lynn et al. (2010), which is known to act as a stressor in zebra finches (Perez et al. 2012b). Accordingly, baseline free plasma CORT concentrations observed here were considerably below those reported in Breuner et al. (2006) (0.01±0.001 ng/ml), and also lower than those observed by Wada et al. (2008) (0.03±0.00 ng/ml), where CBG binding capacity was not reported.

If only the free portion of plasma CORT is physiologically active (Mendel 1992), the comparatively low levels reported here would suggest that none of the birds involved in this study were exhibiting a physiological stress response. In any case, an absence of physiological stress seems the most likely reason for the lack of difference in the

various glucocorticoid related measures between birds in the low and high enrichment groups. Fairhurst et al. (2011) found that feather CORT in wild-caught Clark's nutcrackers increased on removal of enrichments. However, interspecies variation, sourcing differences (i.e. wild-caught vs captive bred), and differences in experimental design (enrichment removal followed 92 days of exposure to high enrichment, compared to 7 days here) may all have played a role in the contrasting results. It is also possible that the low enrichment housing regime is still comparatively benign, given the *ad libitum* food and water, and the presence of a conspecific cage-mate. Indeed, other birds housed in the same facility, in conditions similar to the high enrichment group here, exhibited only a relatively small increase in total plasma CORT concentrations when being subjected to repeated food restriction (one third of daylight hours, four days per week for two weeks) (Marasco et al. 2015). Even then, the CORT response was only detectable on days where restriction had taken place, and not on the following day (V. Marasco, pers. comm.).

The lack of a glucocorticoid response to removal of enrichment may also relate to the specific stress-physiology of the zebra finch. Zebra finches are popular as experimental models, predominantly due to the ease with which they can be maintained and bred in captivity (Schmidt 2010). At least part of the suite of characteristics contributing to this state of affairs is likely to relate to stress tolerance, which has been shown to increase in captive bred birds. For example, captive bred psittacines exhibit a much shorter CORT response to capture and handling than wild caught individuals (Cabezas et al. 2013). Increased negative feedback efficacy of this kind could dampen CORT responses to all stressors, both acute and chronic. Additionally, where a downregulated stress response may be a sign of inability to cope in wild animals (Romero 2004), it may be that predictable, favourable captive conditions are so benign that downregulation of the stress response is energetically advantageous, and therefore a likely evolutionary outcome. Finally, there may be one further alternative explanation for the lack of differences between the high and low enrichment groups. Zebra finches have been shown to match physiological state (increased plasma CORT) via vocal communication (Perez et al. 2015). As both the low and high enrichment birds in this study were housed in the same room (albeit not

visible to one another), it remains possible that vocal communication resulted in physiological 'state matching' between the two groups, 'cancelling out' likely weak effects of contrasts in environmental enrichment on HPA axis activation.

Values of T_{eye} measured in this study were approximately 1 ºC lower than those recorded during a pilot study also conducted with zebra finches, and in similar conditions (R. Nager, unpublished data). This relatively small difference is likely to relate to a combination of factors, possibly including variation in air temperature, and methodological differences (T_{eye} was not calibrated against a temperature logger probe in the earlier study). Given the lack of difference in glucocorticoid related measures between groups, it is not surprising to find a similar lack of variation in T_{eye} between groups. If neither of the groups were under physiological stress, then we would not expect T_{eye} to differ. However, our results do call into question those of the pilot study upon which this work was based, that found T_{eye} to be approximately 0.5 ºC lower in birds housed with minimal enrichments. One potential reason for the difference between studies is that enrichments were added to barren cages at the beginning of the measurement period in the pilot study, but were removed from highly enriched cages here. It would be expected that removal of enrichments would be more stressful than adding them, but this hypothesis is not supported by the results of this study, where enrichment removal failed to induce stress. A further possible explanation may relate to the effect of activity on $T_{eye,}$ which was not accounted for in the previous study. However, this would have required a difference in activity level between low and high enrichment groups, which was not apparent here ($F_{1,63} = 0.37$, $p = 0.55$). Regardless, the effects of both activity and preening behaviour on $T_{eye,}$ reported here will allow refined re-analysis of data where the effect of activity was not quantified (Chapters 2, 3, 4 and 6), and usefully inform the design of future studies.

The lack of relationships between free plasma CORT and T_{eye}, and between body condition index and T_{eye} found here (in contrast to previous data from wild blue tits) seems most likely to relate to the environmental and/or species differences between the studies. Studies investigating endocrinology seem especially prone to effects of

the environment in which they are performed (Calisi & Bentley 2009), and differences in responses to stressors can be surprisingly pronounced, despite close taxonomic proximity (Mason 2010). Comparison of the distributions of plasma free CORT concentrations between this study and those observed previously in free-living blue tits reveal that free CORT levels in the zebra finches were generally lower (Appendix 2, Figure 10-1), suggesting differences in stress state. Also, variation in blue tit free CORT concentrations was substantially greater than that found in this study, meaning that there was less variation with which T_{eye} could relate in the zebra finches. In contrast, the zebra finches in this study exhibited far greater between-individual variation in body condition index than was found in blue tits either in late winter (Chapter 4), or during the breeding season (Chapter 6) (Appendix 2, Figure 10-2). As there was clearly sufficient variation in condition index for T_{eye} to relate to in the zebra finches, the absence of a relationship here is more likely to relate either to species or environmental differences.

6. Human disturbance versus Predation risk: Do fitness consequences differ?

Abstract

Human disturbance often has negative effects on both individuals and populations, and is inevitably increasing with human population growth. However, while human disturbance related changes in behaviour, abundance and distribution have frequently been investigated, effects on fitness have not received equivalent attention. From an evolutionary point of view, the consequences of human disturbance are hypothesized to be equivalent to those of predation risk. This is principally because responses to both disturbance stimuli and predation risk divert time/energy from fitness enhancing activities. Yet, despite an apparent consensus, this hypothesis does not seem to have been tested. To test this hypothesis, we compared acute behavioural responses, physiological state, provisioning rates and measures of breeding success, in free-living breeding blue tits (*Cyanistes caeruleus*) subjected to repeated human disturbance and predation risk. We found that while behavioural responses to human disturbance and predation risk differ, the apparent physiological costs are similar. However, the human disturbance and predation risk treatments failed to induce differences in breeding success with the control group, most likely because the environment was too favourable. As such, the question of whether the fitness consequences of human disturbance and predation risk are analogous remains open.

Introduction

Human disturbance of wildlife may be defined as nonlethal stimuli caused by humans, which lead to deviations in behaviour from patterns occurring without human influence (Frid & Dill 2002). Human disturbance often has negative effects on both individuals and populations (Boyle 1985; Carney & Sydeman 1999), and is inevitably increasing with human population growth. However, while human disturbance related changes in behaviour, abundance and distribution have frequently been investigated (reviewed in Blumstein et al. 2005; Fahrig & Rytwinski 2009), effects on fitness have not received equivalent attention (Leblond et al. 2013). From an evolutionary point of view, the consequences of human disturbance are hypothesized to be equivalent to those of predation risk (Frid & Dill 2002; Beale & Monaghan 2004; Blumstein et al. 2005). This is principally because responses to both disturbance stimuli (Blumstein 2006) and predation risk (Lima & Dill 1990; Lima 1998) divert time/energy from fitness enhancing activities (e.g. foraging or parental care). Yet, despite an apparent consensus, this hypothesis does not seem to have been tested. Human disturbance has been found to affect fitness metrics (Kight & Swaddle 2007; Strasser & Heath 2013; Watson et al. 2014), and there is evidence that behavioural responses to predators differ from responses to humans (Tilgar et al. 2011; Clinchy et al. 2016). Clinchy et al. (2016) reported that badgers restricted feeding behaviour more in response to playback of human voices than when exposed to recordings of current or extinct predators. The study was only short term, restricted to three weeks in autumn, and did not link behavioural effects with breeding. Tilgar et al. (2011) found that provisioning rates decreased markedly when pied flycatcher (*Ficedula hypoleuca*) parents were exposed to a predator model placed near the nest, but not when human investigators visited the nest. Effects on breeding success were hypothesized, but not reported, therefore fitness effects remain uncertain. To our knowledge, no study has experimentally compared fitness outcomes between predation risk and human disturbance.

There are three key components in the processes that transform disturbances or predation risk into a selective outcome; acute behavioural responses, physiological

state, and breeding success. Immediate behavioural responses can not only give an indication of whether a potential disturbance is interpreted as such (i.e. through interruption of 'normal' behaviour, and/or increased vigilance/alarm behaviours), but may also reveal information regarding the nature and intensity of perceived risk (Curio et al. 1983; Curio & Regelmann 1985; Evans et al. 1993; De Villiers et al. 2005; Geist et al. 2005; Templeton et al. 2005; Soard & Ritchison 2009). Contrastingly, measures of physiological state can usefully characterise effects of disturbance or predation risk on energetic and stress status. Most vertebrates exhibit a generalised physiological response to homeostatic challenge (Sapolsky 2002), which is thought to induce long term behavioural adjustments intended to maintain allostasis (McEwen & Wingfield 2003; Romero et al. 2009). Ultimately it is the underlying physiological changes made to cope with challenges that are presumed to provide the mechanism by which fitness is affected, through the preferential allocation of limited resources to either survival or reproduction (Zera & Harshman 2007). This implies that challenges perceived as being more threatening will result in greater resources being diverted away from reproduction.

From previous work (Frid & Dill 2002; Beale & Monaghan 2004; Blumstein et al. 2005), it would be predicted that the distinct threats posed by predators and humans should trigger a broadly analogous response. Immediate behavioural changes might be expected to vary with the precise nature of threat posed. For example, in birds, exposure to a potential nest predator would be expected to elicit behavioural responses that would protect the brood (e.g. concealing the location of the nest, or distracting attention away from it (Byrkjedal 1987)), whereas a potential predator of the parents should induce behaviours that reduce risk of direct mortality (e.g. increased alarm calling, or mobbing behaviour (Curio et al. 1983; Curio & Regelmann 1985)). However, longer term physiological and behavioural changes, and fitness consequences should be identical for both human disturbance and predation risk. To test this hypothesis, we compared acute behavioural responses, physiological state, provisioning rates and measures of breeding success, in free-living breeding blue tits (*Cyanistes caeruleus*) subjected to repeated human disturbance and predation risk. Blue tits are particularly well suited to studies

of breeding behaviour and fitness, as they are not only abundant, but also readily use artificial nest boxes, allowing easy access for assessment of provisioning rates and breeding success. If fitness metrics associated with human disturbance differ from those associated with predation risk, this would suggest selective pressures resulting from human or predator disturbance may differ and will need further consideration.

Methods

Field site, study species & experimental protocols

The study was carried out during April-June 2015, on a breeding population of blue tits that nest in Schwegler 1B nest boxes (32mm entrance hole, Schwegler, Schorndorf, Germany) installed in the predominantly oak woodland around the Scottish Centre for Ecology and the Natural Environment (56.13ºN, 4.13ºW). Nest boxes were checked weekly from April 1st to determine occupancy, laying date and clutch size. To determine hatch date, daily checks were made starting 10 days after clutch completion (the date of clutch completion was calculated from clutch sizes, based on a single egg being laid per day). Once laying had commenced, individual nests were randomly allocated to either a control group, or one of two treatment groups - human disturbance, or predation risk. Neither clutch size or timing of breeding differed between groups (clutch size; $F_{2,43} = 0.69$, $p = 0.51$, hatch date; $F_{2,43} = 0.83$, $p = 0.44$). An annual programme of trapping during winter months meant that a proportion of the population were already marked using a combination of metal and colour leg rings, and a leg mounted RFID tag (125 kHz 2.3mm EM4102 Bird Tag, IB Technology Glenfield, Leicestershire). The RFID tags are detectable by nest box mounted loggers, which were used to identify individuals as they entered and left their nest box, and in doing so measure their respective provisioning rates (see below). 14 unringed Parents were trapped at the nest on days 8-9 after hatching (day of hatching = day 0), and fitted with metal/colour rings and an RFID tag. The proportion of nests at which birds were trapped did not differ between groups (Pearson's Chi-squared test, $\chi^2 = 0.57$, df = 2, p = 0.75).

In the human disturbance group (n = 15 nests), it was assumed that any effect of human disturbance on fitness would result from 'unusual interruptions' to provisioning. Historically, humans have rarely posed a direct threat to adult blue tits, therefore the effect of exposure to human disturbance was expected to relate to protection of the brood, rather than parental survival strategies (Tilgar et al. 2011). To create an 'unusual interruption' during the human disturbance protocols, ensuring

that both parents missed at least one provisioning opportunity, an experimenter stood underneath the nest box for approximately twice the length of the parent's longest expected absence from the nest during provisioning (20 min, R. MacLeod, unpublished data). During human disturbance protocols, experimenters alternated wearing orange and yellow high visibility tabards to inhibit habituation. In the predation risk group (n = 17 nests), any effect of increased predation risk was expected to relate to longer term behavioural/physiological changes made to reduce the risk of direct mortality (Cresswell 2008; Thomson et al. 2010). As sparrowhawks (*Accipter nisus*) are a typical adult blue tit predator (Perrins 1979), nests in the predation risk group were exposed to one of two sparrowhawk models (1x male and 1x female, alternated to avoid habituation (Montgomerie & Weatherhead 1988)) to increase perceived predation risk. The model was placed adjacent to the nest box for a little more than the length of the parent's longest expected absence from the nest during provisioning (10 min, R. MacLeod, unpublished data), during which the nest box was observed from an inconspicuous location >20 m away. The duration was chosen as the shortest time necessary to ensure the exposure of both parents to the model, while minimising opportunities for habituation (Montgomerie & Weatherhead 1988). In the control group (n = 14 nests), an experimenter observed the nest box from an inconspicuous location >20 m away for 10 min. Each nest was repeatedly subjected to its allotted protocol throughout chick rearing (on days 2, 4, 6, 10 and 12 after hatching). Experimental protocols were carried out at a total of 46 nests.

Behaviour during experimental protocols

Three aspects of the birds' behaviour were recorded throughout each experimental protocol – alarm calls, perch changing rates and the distance of the individual's closest approach to the nest box (closest approach distance). Alarm calls are a frequent response to threatening situations in birds, and can function both to alert conspecifics to the presence of a predator, and/or communicate to the predator that the prey is aware of it (Klump & Shalter 1984). Blue tits make distinct scolding or churring alarm calls in response to nearby threats (Perrins 1979). To assess this

indicator of perceived threat, the observer recorded whether or not each individual made any of these alarm calls during each minute of each experimental protocol. Previously, perch changing rates have been found to vary in relation to bird eating raptor abundance (Tellería et al. 2001), and with corticosterone levels (Astheimer et al. 1992; Lynn et al. 2003). Therefore, the observer also recorded whether or not an individual made any perch changes (defined as a jump or flight between distinct perches) during each minute of each experimental protocol. For both alarm calls and perch changing, we calculated their frequency per 10 min. A further gauge of the threat perceived by nesting birds during chick rearing was gained by assessing their readiness to approach a threat situated close to the nest. This was achieved by estimating individual closest approach distances, as the minimum distance (in metres) between the individual and the nest box attained during each protocol. This data was also supplemented by separately recording whether individuals entered the nest box during protocolss (as a closest approach distance of zero would only indicate that the individual had landed on the box). Such behaviour would be likely to indicate that the perceived threat was not sufficient to interrupt normal behaviour. To control for times when an individual might be away from the nest box, and so unaware of the treatment, the number of minutes during the treatment where an individual was visible was recorded. Individuals were identified by their colour ring combinations or distinguishing features. Behavioural data was not recorded for 29 of the potential 230 protocols due to weather conditions. During periods of heavy rain, birds were not generally observed, presumably having taken shelter out of sight of the observer.

Physiological State

Earlier work carried out on the study population investigated here found effects of acute physiological stress on body surface temperatures (Chapters 2 and 3), and evidence of links between baseline body surface temperatures (taken from undisturbed birds) and longer term physiological processes (Chapter 4). Additionally, repeated exposure to stressors, and chronic stress exposure have previously been shown to lead to long term changes in body temperature (Keeney

et al. 2001). Therefore, to assess potential differences in physiological state between experimental groups, body surface temperatures were measured from individuals as they entered and left their nest box using a thermal imaging camera (A65, FLIR Systems, Wilsonville, Oregon). The camera was mounted so that individuals could be recorded continuously at 7.5 frames per second, passing through the camera's field of view (and within the camera's zone of focus), as they entered and left the nest box (Figure 6-1). Body surface temperatures were measured in the resulting thermal videos from the ring of exposed skin around the eye. As the exposed skin of the periopthalmic ring is the least insulated area of the integument, this area provides the most representative (least modulated) measure of surface temperature available from a free-living blue tit. Maximum eye region temperatures (T_{eye}) were extracted from the thermal video following the principles described by Jerem et al. (2015), where the highest temperature measured from the eye region is assumed to be the most accurate. Baseline T_{eye} was defined here as the maximum temperature measured from the eye region of an undisturbed individual during either entry or exit from the nest box, or during the period where the bird was in the camera's field of view, if

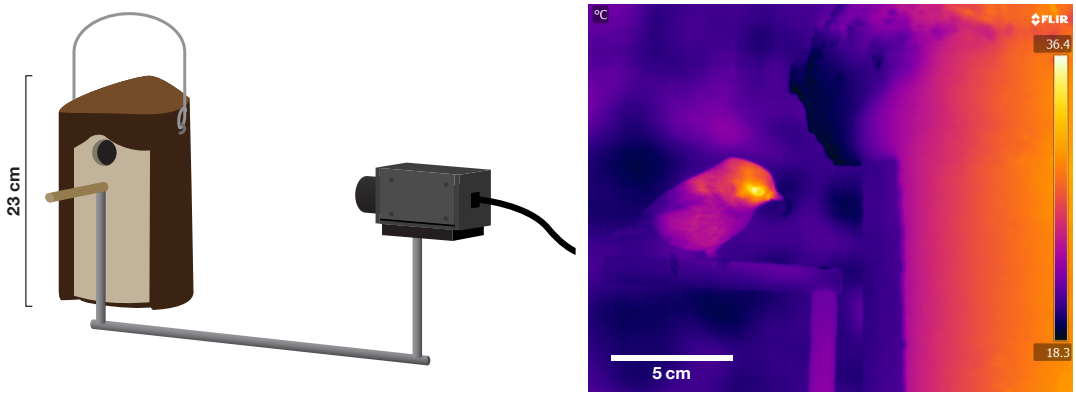

Figure 6-1 Diagram of thermal imaging set up designed to measure eye region temperatures of blue tits entering and leaving their nest box (left), and typical thermal image captured showing the eye region as the warmest area of the bird's surface (right).

it did not enter or leave the nest box. As it was not logistically possible to use the thermal imaging camera at all nests, surface temperatures were measured at a randomly selected subset of 29 nests (control n = 9, human disturbance n = 9, predation risk n = 11). Individuals were identified in the thermal videos using a

combination of the records of the RFID tag logger mounted in the nest box entrance, and distinguishing features such as leg ring combinations. Data from nest boxes where identification of individuals proved impossible (e.g. due to logger failure) were excluded from the analysis. To enable the use of a single camera and temperature logger between multiple nests, whilst habituating the birds to their presence, dummy cameras and temperature loggers were installed at each focal nest ≥ 7 days prior to filming. The dummy camera and temperature logger were replaced with the actual camera and temperature logger immediately prior to filming, which took place on day 13 after hatching (the day after the final experimental protocol). Once the camera and temperature logger were installed, thermal video was recorded for 45 minutes. To avoid acute stress related changes in T_{eye} (resulting from disturbance associated with installation) confounding baseline measurements, only T_{eye} measures from video recorded after the initial 15 minutes were included in the analyses.

Accurate absolute temperatures can be extracted from thermal images by the inclusion of an object of known temperature and emissivity within the field of view, against which the temperature measured from the bird's surface by the thermal imaging camera can be calibrated. To achieve this, a thermistor probe coated in black insulation tape (Tesa UK, Milton Keynes, Buckinghamshire) was installed onto the perch support, and connected to a Tinytag Talk 2 Temperature Logger (Gemini Data Loggers UK, Chichester, West Sussex). The logger was set to record at 1 s intervals, and the resulting temperature data used to calibrate the individual frames from which T_{eye} was extracted.

Body temperature exhibits a circadian rhythm modulated by air temperature (T_a) (Aschoff 1979), and humidity (Lin et al. 2005), and may also be affected by solar radiation (Lustick et al. 1970, 1979). Therefore, time of day, T_a, humidity, and the presence or absence of solar radiation were considered as potential confounding explanatory variables. T_a was recorded by the perch mounted temperature logger/ probe, whilst relative humidity data was obtained from the MIDAS MET Office weather station at Bishopton (approximately 25 km from the study site; 55.91ºN,

-4.53ºW). The presence or absence of solar radiation was recorded by the camera operator as a single categorical measure, depending on whether direct solar radiation fell on the perch and/or nest box entrance at any point during any given recording. Visits to the nest box were defined as 'in' when an individual entered the nest box, 'out' when an individual left the nest box, and 'no entry/exit' when an individual entered the field of view of the camera, but did not enter or exit. In preliminary analyses, the type of event from which T_{eye} was measured also appeared to influence the value of T_{eye}, with 'out' and 'no entry/exit' type visits being associated with higher values of T_{eye} than 'in' type visits. Consequently, event type was also recorded and included as a covariate in the analysis (see 'Statistical analyses' below).

Finally, one further aspect of physiological state that has previously been shown to relate to T_{eye} is body condition (Chapter 4). Calculation of individual body condition involves correction of body mass for body size, and as such requires trapping and handling of individuals. While it was not logistically possible to trap all individuals and measure the relevant biometrics, body mass and wing chord length were measured from the 14 birds trapped on day 8-9 to fit them with RFID tags (see Field site, study species & experimental protocols section above). Body condition index was calculated for these individuals as the residual value of a linear regression between body mass and wing chord length^3 (Christe et al. 1998).

Provisioning rates

Provisioning rates were measured using four types of RFID tag loggers (Nature Counters, Maidstone, Kent; IB Technology Glenfield, Leicestershire; Francis Scientific Instruments, Ltd., Huntingdon, Cambridgeshire; University of Glasgow Bioelectronics Dept., Glasgow). Loggers were connected to nest box door mounted antennae, which detected leg mounted RFID tags as parents passed through the entrance hole. Provisioning rates were measured during daylight hours on days 2-12 after hatching (the period during which protocols took place), and were calculated as the mean number of visits per hour for each individual (equal to the no. of detections per hr / 2, to account for entry and exit of the nest box, which are

recorded as two separate events). Detection rates (calculated by comparing visits recorded on the thermal video recordings with the RFID logger data) were used to correct visit rates where necessary. Only data from loggers with detection rate ≥ 0.5 were included in the analyses (25 of the 29 loggers had detection rates >0.9).

Fitness measures

Two offspring fitness measures were assessed – chick mass and fledging success. Chick mass was measured as it can predict survival to recruitment age (reviewed in Schwagmeyer & Mock 2008). Individual chicks were weighed on day 13 after hatching, and mean chick mass calculated across the whole brood. Chick mass is likely to be affected by chick age and brood size (Nadav 1984; Wiggins 1990; Blondel et al. 1998), therefore both variables were included as covariates in the chick mass analysis. Brood size was defined as the number of chicks that successfully fledged (determined by examining nests post fledging for unhatched eggs and remains of dead chicks, and deducting these from clutch size). Fledging success was defined as the proportion of the brood which fledged (clutch size divided by number of chicks fledged).

Statistical analyses

All analyses were carried out using R v3.3.0 (R Core Team 2016). A combination of multivariate linear and linear mixed effect models was employed to assess potential relationships between the various measures and the experimental groups, controlling for confounding factors. Significance of explanatory variables (critical two-tailed $p = 0.05$) was determined by backwards-stepwise (from most to least complex), pairwise model selection (R function: *drop1*). P-values for fixed/random effects in the mixed effect models were calculated using *lmerTest/rand* (R package: lmerTest 1.0, Kuznetzova et al. 2016). For the mixed effect models, the most appropriate random structure was determined prior to fixed effect selection by comparing AIC values of candidate models following Zuur et al. (2009). Random structures for each analysis were selected from two alternatives, with either bird ID, or bird ID nested within nest box as random effects. All models met with assumptions, except where

indicated. Interactions were tested for, but only reported where significant. Model plots presented in the Results section were all created using the visreg R package (Breheny & Burchett 2016).

Generalised linear mixed effect models (GLMM, R function: *glmer*, R package: nlme4 1.1-12, Bates et al. 2014) with binomial error distributions were used to compare alarm calling or perch changing behaviour between groups. In each case, the proportion of minutes during each experimental protocol where the behaviour (either alarm calling or perch changing) occurred was used as the response variable. The difference in distance of the closest approach of individuals to the nest box between groups was assessed using a linear mixed effect model (LMM, R function: *lmer*, R package: nlme4 1.1-12, Bates et al. 2014), with the square root of the closest approach distance as the response variable (to correct for a skewed distribution). In all three behavioural analyses, group was included as a fixed effect, being the variable of interest. As habituation, sensitization, or changes in brood value (Barash 1975; Andersson et al. 1980) were hypothesised as being likely to affect disturbance related behaviours, chick age and an interaction between chick age and group were included as covariates in all behavioural models. The interaction term was added contingent upon any effect of chick age differing between experimental groups. Additionally, the number of minutes where an individual was visible was added as a covariate in the closest approach distance analysis, to control for presence/absence during the protocols. It was not possible control for presence/absence in the alarm calling and perch changing models, as the number of minutes where an individual was visible was collinear with both responses variables (and so would have resulted in rank deficiency if included as a covariate). For the alarm calling and perch changing models, a random structure with bird ID only was preferred, whereas bird ID nested within nest box was preferred for the closest approach distance model. An observation level random effect (OLRE) was also added to the alarm calling and perch changing models to account for overdispersion (Harrison 2014).

The relationships between T_{eye} and group, T_{eye} and condition index, and provisioning rates and group were all examined using separate LMMs. For the T_{eye} vs group

model, T_{eye} was specified as the response variable, with group, T_a, relative humidity, the presence or absence of direct sunlight, and visit type all being included as fixed effects. As time of day and T_a were highly correlated, only one of the two variables could be included in the analyses. The largest changes in body temperature with time are expected between the active and resting phases (i.e. day and night) (e.g. Rashotte et al. 1995). Given that measurements of T_{eye} in this study only took place during the active phase (range = 10:23-20:15, mean±SEM = 13:57±00:34), T_a was expected to have greater influence over T_{eye}. Therefore, T_a rather than time of day, was included in our analyses. For the T_{eye} vs condition index model, all significant confounding variables from the T_{eye} vs group model (T_a, relative humidity, and visit type), and experimental group were also included as fixed effects. Provisioning rates were expected to be affected by brood size and chick age and timing of breeding (due to changes in caterpillar abundance (Tremblay et al. 2005; García-Navas & Sanz 2011)). Therefore, for the provisioning rate model, mean visits per individual parent, per hour, per day was the response variable, and group, brood size, chick age and hatch date were included as fixed effects. The preferred random structure for both the T_{eye} vs experimental group and provisioning rate models included Bird ID nested within nest box as a random effect, whilst only Bird ID was preferred for the T_{eye} vs condition index model. A total of 372 measurements of T_{eye} (from 14 individuals with condition index data) were analysed in the T_{eye} vs condition index model. As such, the number of predictors (total=18; 4 fixed effects + 14 random effects) was considered within the appropriate range, being approximately 5% of the total number of observations.

In terms of fitness effects, the relationship of experimental group with mean chick mass was analysed using a linear model (R Function: lm) with mean chick mass (across the brood) as the response variable, and group, weighing age, and brood size as explanatory variables. Also, a GLM (R Function: glm) with binomial error distribution was used to compare fledging success between experimental groups. The proportion of chicks fledged was specified as the response variable, with group and clutch size included as explanatory variables. Finally, the relationship between condition index and experimental group was investigated using a linear model

(R Function: lm) with condition index as the response variable, and experimental group as the explanatory variable.

Results

Behaviour during experimental protocols

The proportion of time during which alarm calls were made during protocols varied from zero to one (0.55±0.22, n = 201 observations of 81 individuals; control n = 20; human disturbance n = 33; predation risk = 28, from 41 nests; control n = 10; human disturbance n = 14; predation risk = 17). The probability of an individual parent alarm calling was lowest in the control group and highest in the human disturbance and predation risk groups, (Figure 6-2 a., Table 6-1). Although the probability of alarm calling appeared higher in the predation risk group than in the human disturbance group, the difference was not significant. Alarm calling also increased at a similar rate in all groups over the course of the experiment (i.e. with chick age). The proportion of time during which perch changing occurred also varied from zero to one (0.57±0.22, n = 201 protocols). However, the probability of individuals changing perch during protocols did not differ between experimental groups (Figure 6-2 b., Table 6-2), and while perch changing appeared to increase with chick age, the effect was not significant. Closest approaches to the nest box during the protocols ranged from 0-30m (4.59±0.37 m, n = 201 protocols), with birds in the human disturbance group consistently staying further away from the nest box than those in the other groups (Figure 6-2 c., Table 5-3). Whilst the closest approach distance for the human disturbance group was independent of chick age, both the predation risk and control groups gradually stayed further away from the nest box as the chicks grew older. Also, the nest box was entered by one or both of the nesting parents 25 times during control protocols, and 8 times during predation risk protocols, however no birds entered the nest box during human disturbance protocols

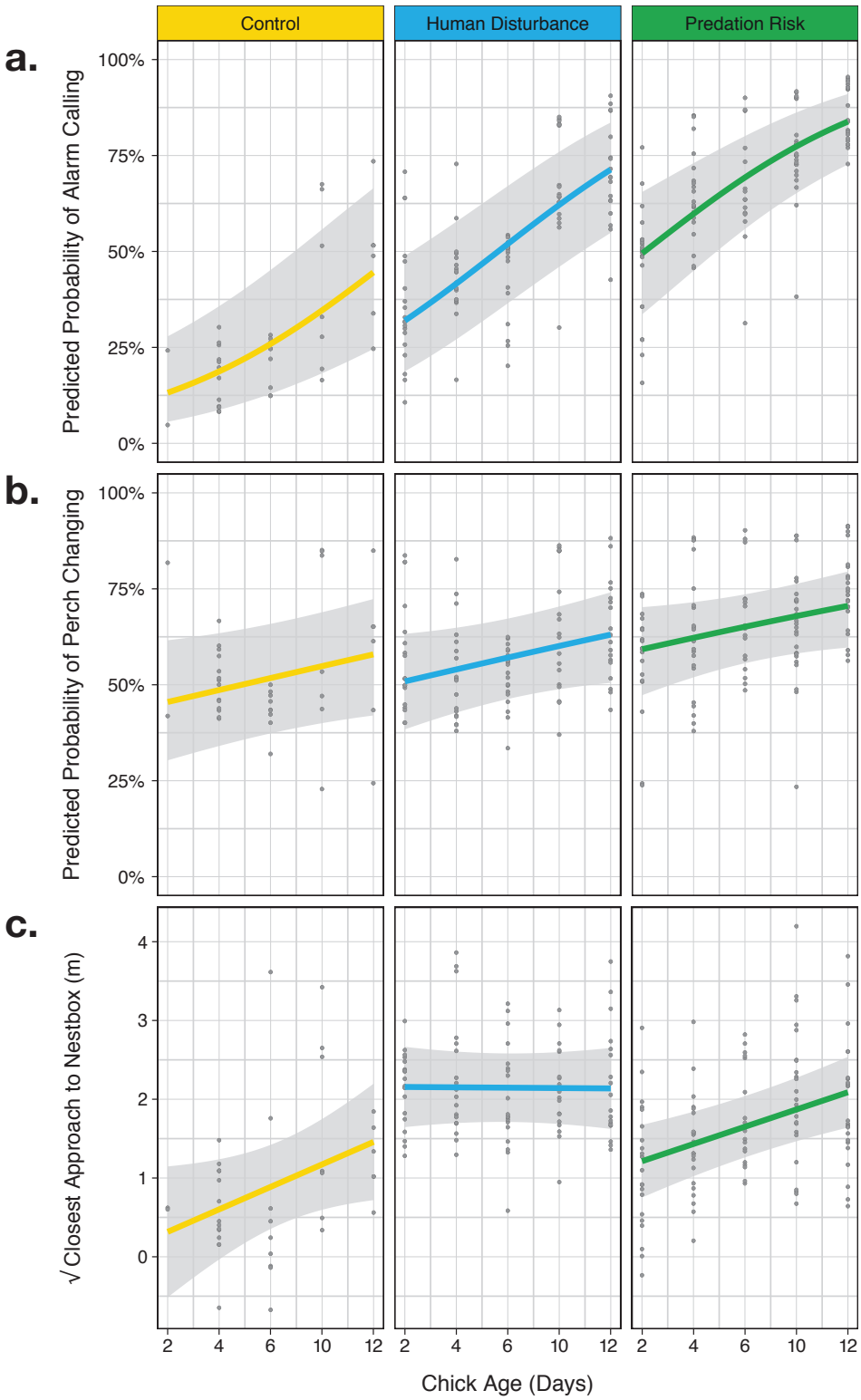

Figure 6-2 Model predictions (coloured lines) of the probability of alarm calling (a), and perch changing (b) in a given minute, and closest approach by individuals to the nest box (c) during experimental protocols, for each experimental group (control, human disturbance and predation risk). Shaded areas represent 95% confidence intervals, and dark circles are partial residuals (conditional on the random intercept, with other variables in the models being set to their median values).

Response Variable: Probability of Alarm Calling

Fixed Effects	Estimate	z-value	p-value
Treatment Groups Combined vs Control	1.55±1.01	3.11	0.002
Group (Human Disturbance vs Control)	1.13±1.10	2.08	0.04
Group (Predation Risk vs Control)	1.86±1.06	3.56	0.0004
Group (Human Disturbance vs Predation Risk)	0.74±0.86	1.68	0.09
Chick Age (Days)	0.42±0.17	4.96	<0.0001
Group (Human Disturbance) * Chick Age	-0.25±0.61	0.80	0.43
Group (Predation Risk) * Chick Age	-0.18±0.60	0.58	0.56
Random Effects	**Variance**	**SD**	**p-value**
Bird ID	2.02	1.42	<0.0001
Observation level random effect (OLRE)	1.70	1.31	<0.0001

Table 6-1 Parameter estimates (±95% confidence intervals), z-values and p-values for fixed effects included in a generalised linear mixed model relating experimental group to the probability of alarm calling, controlling for chick age, and a possible interaction between group and chick age. Also, variance, standard deviation and p-values for random effects included in the model (see methods). The overall effect of human disturbance and predation risk was estimated by combining both treatment groups into a single level of the group factor. The estimate, z-value and p-value for the non-significant interaction were obtained prior to its removal during model selection (see methods).

Response Variable: Probability of Perch Changing

Fixed Effects	Estimate	z-value	p-value
Treatment Groups Combined vs Control	0.40±0.67	1.184	0.24
Group (Human Disturbance vs Control)	0.22±0.74	0.58	0.56
Group (Predation Risk vs Control)	0.55±0.71	1.54	0.12
Group (Human Disturbance vs Predation Risk)	0.33±0.60	1.13	0.26
Chick Age (Days)	0.12±0.14	1.78	0.07
Group (Human Disturbance) * Chick Age	0.07±0.48	0.27	0.78
Group (Predation Risk) * Chick Age	0.19±0.66	0.79	0.43
Random Effects	**Variance**	**SD**	**p-value**
Bird ID	0.82	0.90	<0.0001
Observation level random effect (OLRE)	1.30	1.14	<0.0001

Table 6-2 Parameter estimates (±95% confidence intervals), z-values and p-values for fixed effects included in a generalised linear mixed model relating experimental group to the probability of perch changing, controlling for chick age, and a possible interaction between group and chick age. Also, variance, standard deviation and p-values for random effects included in the model (see methods). The overall effect of human disturbance and predation risk was estimated by combining both treatment groups into a single level of the group factor. The estimate, z-value and p-value for the non-significant interaction were obtained prior to its removal during model selection (see methods).

Response Variable: Closest Approach Distance

Fixed Effects	**Estimate**	**t-value**	**p-value**
Treatment Groups Combined vs Control	1.46±1.16	1.59	0.11
Group (Human Disturbance vs Control)	2.13±1.22	3.42	0.001
Group (Predation Risk vs Control)	0.96±1.18	1.59	0.11
Group (Human Disturbance vs Predation Risk)	-1.17±0.78	2.879	0.005
Chick Age (Days)	0.29±0.29	1.94	0.05
Minutes Visible	-0.11±0.05	4.50	<0.0001
Group (Human Disturbance) * Chick Age	-0.29±0.31	1.79	0.07
Group (Predation Risk) * Chick Age	-0.07±0.30	-0.42	0.68
Random Effects	**Variance**	**SD**	**p-value**
Bird ID:Nestbox	0.30	0.64	<0.0001

Table 6-3 Parameter estimates (±95% confidence intervals), t-values and p-values for fixed effects included in a linear mixed model relating experimental group to the (square root transformed – see methods) closest approach of individuals to the nest box, controlling for chick age, presence/absence (minutes visible), and a possible interaction between group and chick age. Also, variance, standard deviation and p-values for random effects included in the model (see methods). The overall effect of human disturbance and predation risk was estimated by combining both treatment groups into a single level of the group factor.

Physiological State

The mean T_{eye} recorded was 31.5±0.3 ºC (range = 28.9-36.1 ºC, n = 1008 measurements, from 45 individuals; control n = 16; human disturbance n = 13; predation risk n =16, from 26 nests; control n = 9; human disturbance n = 8; predation risk n =9) and did not differ between groups (Figure 6-3, Table 6-4). However, T_{eye} increased with air temperature, decreased with relative humidity, and was lower when birds entered the nest box (compared to when they left, or appeared in the field of view without entering or leaving the nestbox). For those parents trapped on days 8-9 after hatching, T_{eye} was lower in individuals in poorer condition (14 individuals; control n = 3; human disturbance n = 8; predation risk n = 3, from 12 nests; control n = 3; human disturbance n = 7; predation risk n = 2) (Figure 6-4, Table 6-5). Also, among these birds, individuals in the human disturbance and predation risk groups were in poorer condition than those in the control group (Figure 6-5).

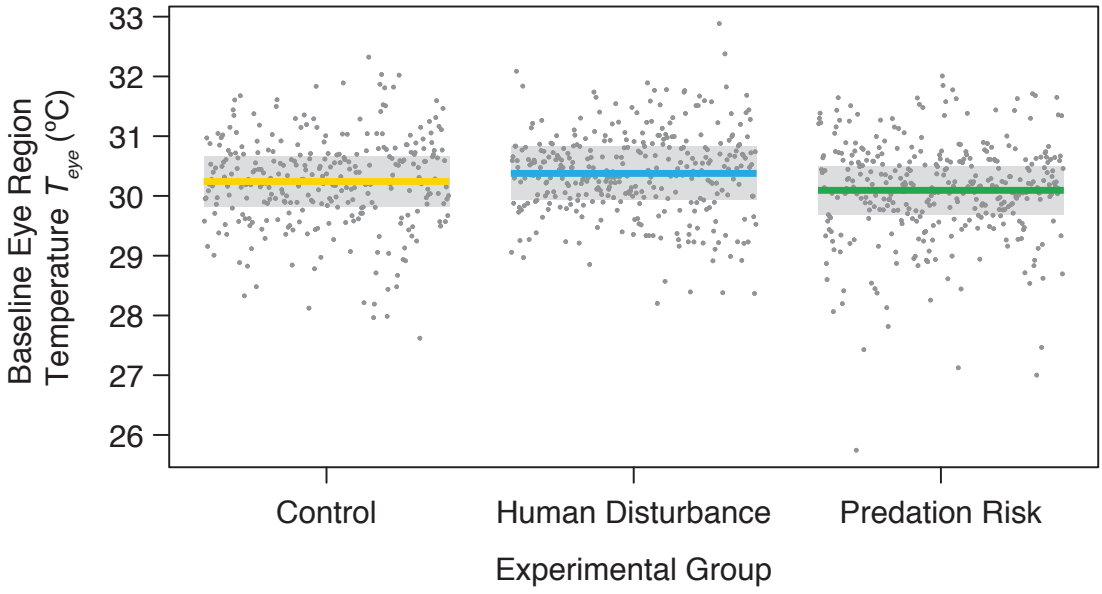

Figure 6-3 Model predictions (coloured horizontal lines) of baseline eye region temperature (T_{eye}), for each experimental group (control, human disturbance, and predation risk). Shaded areas represent 95% confidence intervals, and dark circles are partial residuals (conditional on other variables in the model being set to their median values).

Response Variable: Baseline Eye Region Temperature (T_{eye})

Fixed Effects	**Estimate**	**t-value**	**p-value**
Treatment Groups Combined	-0.03±0.55	0.09	0.92
Group (Human Disturbance)	0.15±0.69	0.43	0.67
Group (Predation Risk)	-0.17±0.65	0.50	0.62
Air Temperature (ºC)	0.20±0.04	8.15	<0.0001
Relative Humidity (%)	-0.03±0.02	2.56	0.014
Direct Sunlight (Yes)	0.31±0.71	0.84	0.41
Visit Type (Out)	1.33±0.11	4.16	<0.0001
Visit Type (No Entry/Exit)	1.11±0.52	23.60	<0.0001
Random Effects	**Variance**	**SD**	**p-value**
Bird ID: Nestbox	0.29	0.53	<0.0001

Table 6-4 Parameter estimates (±95% confidence intervals), t-values and p-values for fixed effects included in a linear model relating experimental group to the baseline eye region temperature (T_{eye}), controlling for air temperature, relative humidity, the presence or absence of direct sunlight, and visit type. The overall effect of human disturbance and predation risk was estimated by combining both treatment groups into a single level of the group factor. Estimates, t-values and p-values for non-significant terms other than experimental group (which remained in the final model) were obtained prior to their removal during model selection (see methods).

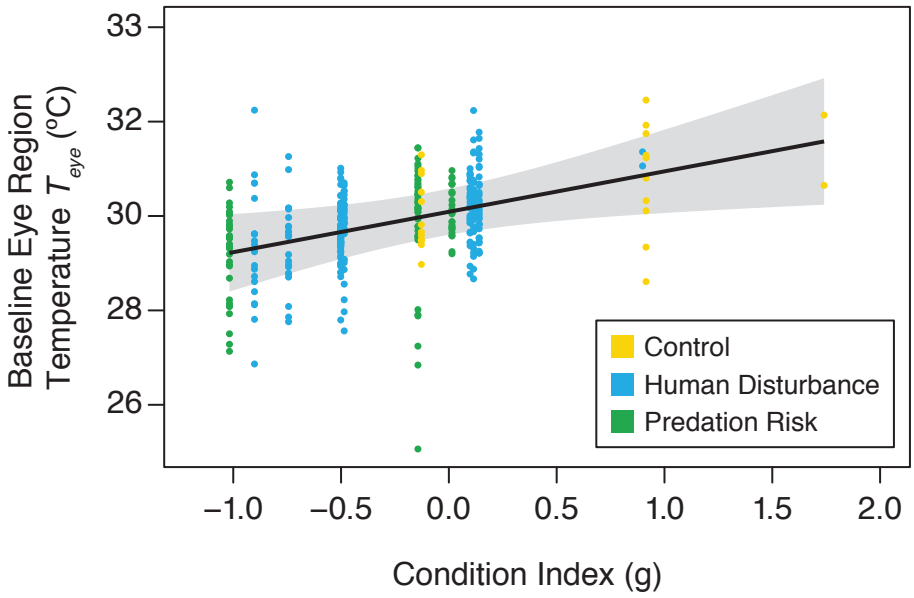

Figure 6-4 Model prediction (black line) of the relationship between baseline eye region temperature and body condition index. The shaded area represents the 95% confidence interval, and the circles are partial residuals (conditional on other variables in the model being set to their median values), coloured by experimental group. For condition index, zero represents mean condition, with positive and negative values indicating mass above or below average for a given wing length.

Response Variable: Condition Index

Fixed Effects	**Estimate**	**t-value**	**p-value**
Condition Index	0.86±0.84	2.54	0.028
Group (Human Disturbance)	1.01±1.47	1.35	0.21
Group (Predation Risk)	0.33±1.74	0.37	0.72
Air Temperature (ºC)	0.23±0.09	4.74	<0.0001
Relative Humidity (%)	-0.02±0.04	1.23	0.23
Visit Type (Out)	1.01±0.18	11.21	<0.0001
Visit Type (No Entry/Exit)	1.23±0.78	3.11	0.002
Random Effect	**Variance**	**SD**	**p-value**
Bird ID	0.79	0.88	<0.0001

Table 6-5 Parameter estimates (±95% confidence intervals), t-values and p-values for fixed effects included in a linear model relating condition index to maximum eye region temperature (T_{eye}), controlling for experimental group, air temperature, relative humidity, and visit type. Estimates, t-values and p-values for the non-significant relative humidity term was obtained prior to its removal during model selection (see methods).

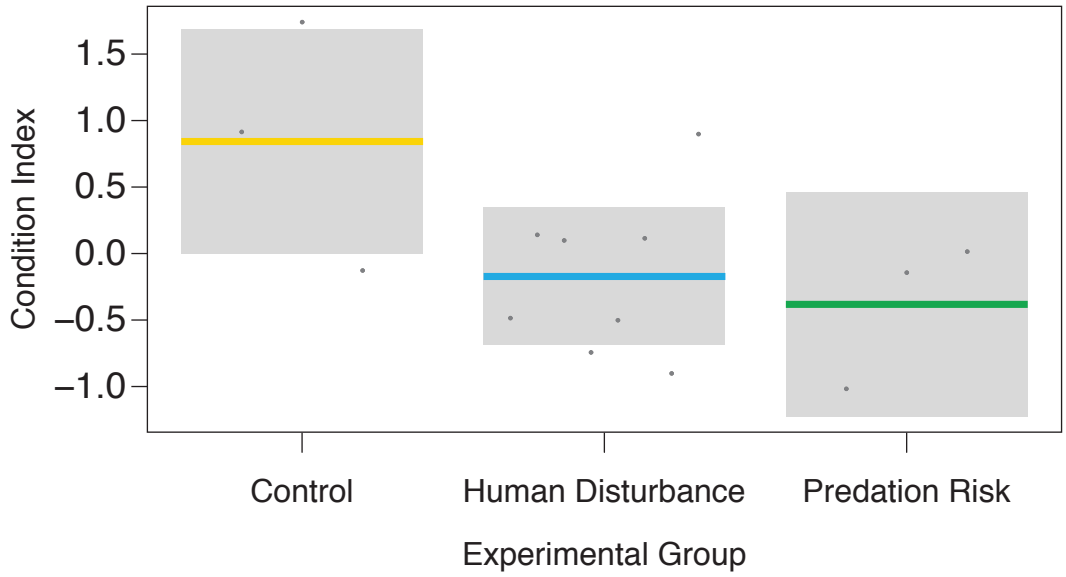

Figure 6-5 Model predictions (coloured horizontal lines) of condition index, for each experimental group (control n=3, human disturbance n=8, and predation risk n=3). Shaded areas represent 95% confidence intervals, and dark circles are partial residuals (conditional on other variables in the model being set to their median values). Individual condition index was lower in the human disturbance and predation risk groups, than in the control group ($\chi 2 = 6.41$, df = 2, p = 0.04, human disturbance; t = 2.261, p = 0.045, predation risk; t = 2.262, p = 0.045). For condition index, zero represents mean condition, with positive and negative values indicating mass above or below average for a given wing length.

Provisioning rates

Mean hourly provisioning rates per individual parent, per day (25.2±1.0 visits, range = 5.1-84.5 visits, n=209 days, from 26 individuals; control n = 4; human disturbance n = 11; predation risk n = 11, from 17 nests; control n = 3; human disturbance n = 7; predation risk n = 7) increased with total number of fledged chicks (Table 6-6), but did not differ between experimental groups (Figure 6-6).

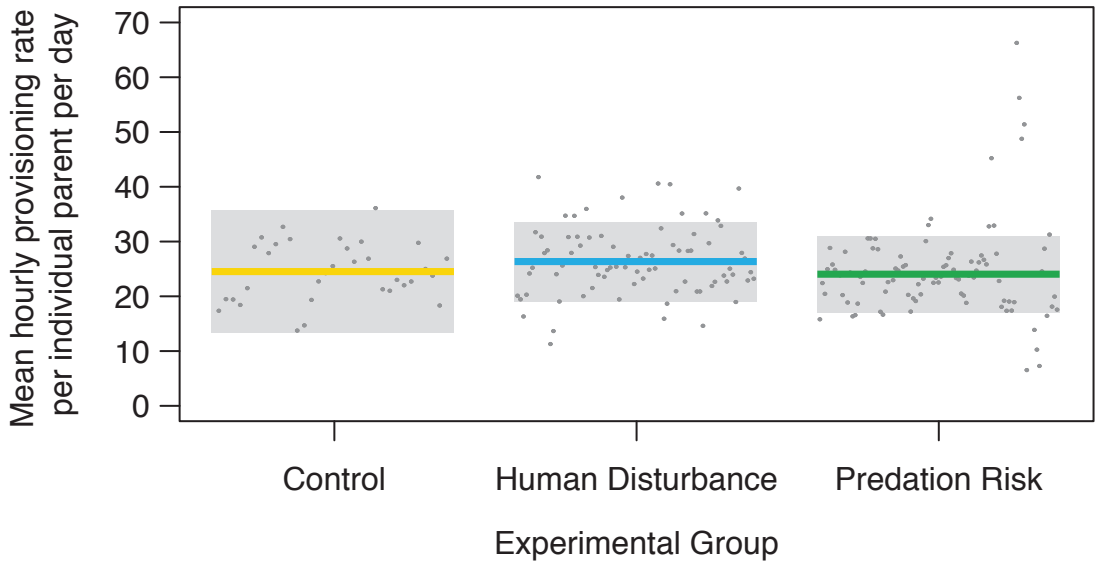

Figure 6-6 Model predictions (coloured horizontal lines) of mean hourly provisioning rate per individual parent between days 2-12 after first hatching, for each experimental group. Shaded areas represent 95% confidence intervals, and dark circles are partial residuals (conditional on the random intercept where appropriate, and other variables in the models being set to their median values).

Response Variable: Provisioning Rate

Fixed Effects	**Estimate**	**t-value**	**p-value**
Treatment Groups Combined	0.77±12.60	0.12	0.91
Group (Human Disturbance)	2.96±13.88	0.42	0.68
Group (Predation Risk)	-1.09±13.56	0.16	0.88
Total No. of Fledged Chicks	3.66±3.24	2.22	0.05
Hatch Date	1.00±1.29	1.52	0.09
Chick Age (Days)	-0.09±0.41	0.41	0.68
Random Effects	**Variance**	**SD**	**p-value**
Bird ID:Nestbox	26.31	5.129	<0.0001

Table 6-6 Parameter estimates (±95% confidence intervals), t-values and p-values for fixed effects included in a linear mixed model relating experimental group to provisioning rates, controlling for total no. of fledged chicks, hatch date, and chick age. Also, variance, standard deviation and p-values for random effects included in the model. The overall effect of human disturbance and predation risk was estimated by combining both treatment groups into a single level of the group factor. Estimates, t-values and p-values for non-significant terms other than experimental group (which remained in the final model) were obtained prior to their removal during model selection (see methods).

Fitness measures

Neither mean chick mass (11.14±0.12 g, range = 8.37-12.33 g, n = 47 nests – control n = 14; human disturbance n = 15; predation risk = 17) or fledging success (0.77±0.03, range = 0.11-1.00, n = 47 nests – control n = 14; human disturbance n = 15; predation risk = 17) differed between experimental groups (Table 6-7, Table 6-8, Figure 6-7 a. & b.).

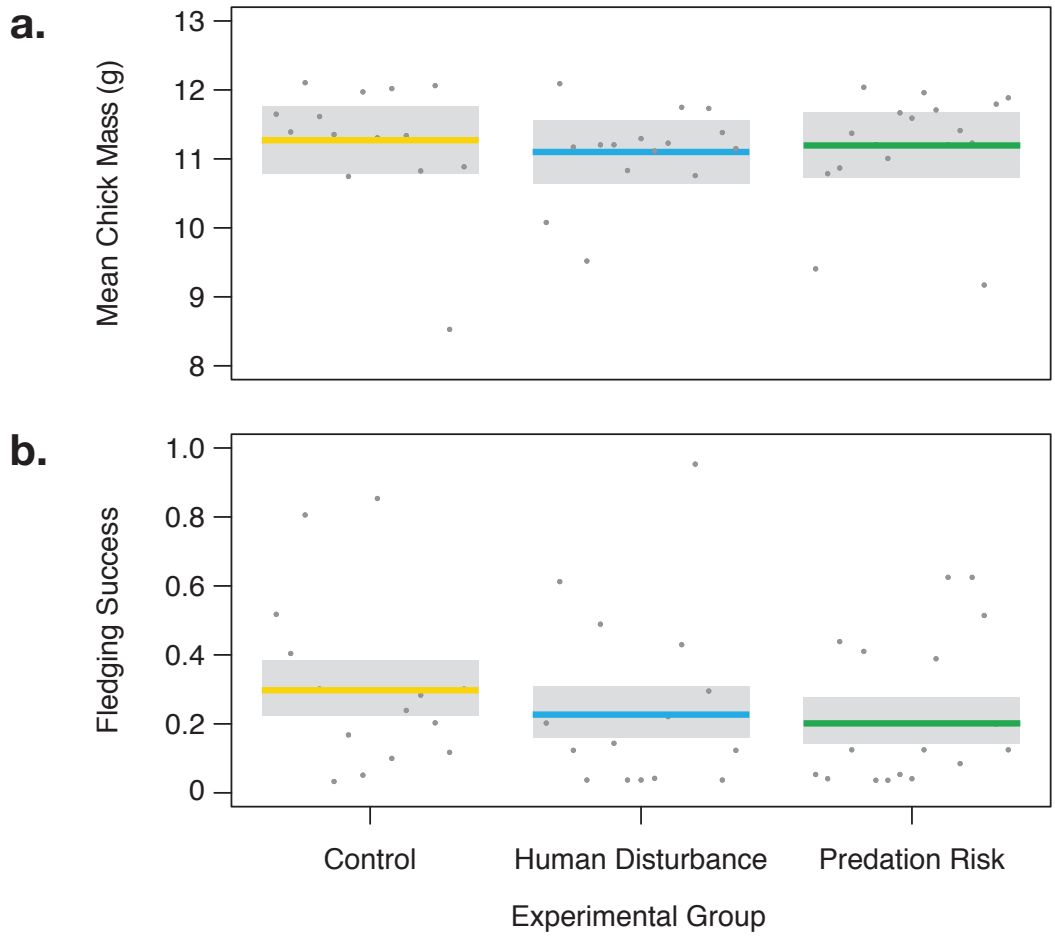

Figure 6-7 Model predictions (coloured horizontal lines) of mean chick mass (a), and fledging success (b) for each experimental group (control, human disturbance and predation risk). Shaded areas represent 95% confidence intervals, and dark circles are partial residuals (conditional on variables in the models being set to their median values).

Response Variable: Mean Chick Mass

Fixed Effects	Estimate	t-value	p-value
Treatment Groups Combined	-0.12±0.51	0.47	0.64
Group (Human Disturbance)	-0.17±0.59	0.57	0.57
Group (Predation Risk)	-0.08±0.58	0.26	0.80
Weighing Age (Days)	0.16±0.32	1.02	0.30
Total No. of Fledged Chicks	-0.06±0.13	0.86	0.41

Table 6-7 Parameter estimates (±95% confidence intervals), t-values and p-values for fixed effects included in a linear model relating experimental group to mean chick mass, controlling for weighing age, and total no. of fledged chicks. The overall effect of human disturbance and predation risk was estimated by combining both treatment groups into a single level of the group factor. Estimates, t-values and p-values for non-significant terms other than experimental group (which remained in the final model) were obtained prior to their removal during model selection (see methods).

Response Variable: Fledging Success

Fixed Effects	Estimate	z-value	p-value
Treatments Groups Combined	-0.45±0.49	1.78	0.07
Group (Human Disturbance)	-0.37±0.58	1.24	0.21
Group (Predation Risk)	-0.52±0.57	1.79	0.07

Table 6-8 Parameter estimates (±95% confidence intervals), t-values and p-values for fixed effects included in a linear model relating experimental group to fledging success, controlling for clutch size. The overall effect of human disturbance and predation risk was estimated by combining both treatment groups into a single level of the group factor. Estimates, t-values and p-values for non-significant terms other than experimental group (which remained in the final model) were obtained prior to their removal during model selection (see methods).

Discussion

Acute behavioural responses during the experimental protocols differed between human disturbance and predation risk groups. Birds exposed to human disturbance consistently stayed furthest away from the nest box. Despite this difference in behaviour, baseline eye region temperature (T_{eye}) was similar across all experimental groups, suggesting no between group differences in those aspects of physiological state linked with body temperature. However, in a subsample of the birds, body condition was higher in the control group, and lower in the human disturbance and predation risk groups, indicating a physiological cost of the treatment protocols. Nonetheless, provisioning rates, chick mass and fledging success did not differ between experimental groups, indicating no effect of the treatment protocols on breeding success.

Given the differing nature of threats posed by humans and sparrowhawks, it was not surprising that immediate behavioural responses differed during the experimental treatments. Humans are thought to be viewed as potential nest predators by small cavity nesting birds such as blue tits (Müller et al. 2006). In contrast, a sparrowhawk is not threatening to offspring (as it cannot enter and is unlikely to be able to retrieve chicks through the nest opening), but represents a clear danger to the parents. The higher alarm calling in both the human disturbance and predation risk groups suggests that alarm calling was being used to alert the other parent, and possibly the offspring to perceived threats (Klump & Shalter 1984). The persistent large distance between the bird and the nest box during the human disturbance group protocols seems consistent with concealment of the nest location (Müller et al. 2006). Perch changing has previously been shown to vary with predator abundance, during normal foraging behaviour in winter, outside of the breeding season (Tellería et al. 2001). Although perch changing is considered to be an antipredator behaviour (Tellería et al. 2001), the lack of difference between experimental groups reported here may be a result of differing season. The adrenocortical response is often implicated in the regulation of such behaviours (McEwen & Wingfield 2003; Romero et al. 2009), with increases in both endogenous and exogenous corticosterone having being

associated with increased perch changing in captive birds (Astheimer et al. 1992; Lynn et al. 2003). As the adrenocortical response may be modulated during the breeding season (Wingfield et al. 1992), it is possible that related behavioural changes, apparent in winter, might be suppressed during the breeding season.

The increases in alarm calling across all groups, and in closest approach distances in the control and predation risk groups, over the course of the experiment may result from a combination of possible factors. The responses may have changed in relation to sensitisation, although habituation could be possible in the 8 cases where birds entered the nest box despite the presence of the predator model. Alternatively, the increased fitness value of the growing chicks (Barash 1975; Andersson et al. 1980), or an increase in the potential for harm resulting from feeding interruptions may have affected responses (Dale et al. 1996). Additionally, the increases in alarm calling and closest approach distance in the control group indicate that the birds were most likely aware of the observer during control protocols. This also suggests that our inability to statistically control for times when an individual might be away from the nest box (and so be unaware of the treatment) was unlikely to have been problematic. The relatively small size of blue tit territories (~0.5 ha, Dhondt et al. (1982)) mean it is already doubtful that individuals would not easily detect intruders within their territory. As experimenters appeared to be consistently detected, even during the control group protocols (i.e. despite efforts to make observations from inconspicuous vantage points ≥ 20 m from the nest box), it seems most likely that the birds were always aware of the treatments.

The mean value of T_{eye} reported in this study is slightly higher than found previously in the same population (Chapter 4). Given the positive relationship between air temperature and T_{eye} observed both here and in Chapter 4 this is most likely to result from the earlier studies having taken place during winter (and hence in colder air temperatures). The similar values of T_{eye} found across all experimental groups suggests that aspects of physiological state which affect body temperature were also similar in all groups. The previously reported association between higher baseline plasma free CORT concentrations and lower T_{eye} in the same population (Chapter 4)

led us to predict that repeatedly challenged individuals in the human disturbance and predation risk experimental groups would exhibit lower T_{eye}. There are a number of possible explanations why this was not the case. Firstly, even if the relationship between free CORT and T_{eye} did hold in this case (which we cannot know, as we did not measure CORT), increased baseline free CORT levels (and so lower T_{eye}) may not result from repeated stressor application in blue tits (Dickens & Romero 2013). Alternatively, it may also be the case that the repeated challenges were not sufficient to induce long-term changes in physiological state. T_{eye} was measured on the day after the final protocol, therefore it is conceivable that any effect of the protocols on stress physiology had dissipated by this point. Equally, it may be possible that despite the efforts of observers to conceal themselves during the control protocols, that all birds were equally challenged by all protocols, and so exhibit similar values of T_{eye}. However, the evidence of poorer body condition in the human disturbance/predation risk groups does imply that those birds were more physiologically stressed than those in the control group. Differences in T_{eye} between event types (in/out/no entry or exit) are most likely a composite of behavioural differences, and the warming effect of having spent time in the nest box microclimate. Where birds entering the nest box were rarely stationary, birds leaving the nest box often paused (presumably to check for potential hazards) before exiting. Similarly, birds which entered the field of view of the camera, but did not enter or exit also tended to be more stationary than birds entering the nest box. As much of the measurement error associated with thermal imaging of small passerines results from motion blur (Jerem et al. 2015), values of T_{eye} tend to increase with the inactivity of the subject.

The relationship observed between T_{eye} and body condition in the subset of birds trapped on days 8-9 was similar to that previously reported in overwintering birds (Estimates±95% Confidence Intervals; This study = 0.86±0.69, Overwintering birds = 0.97±0.83 - see Chapter 4) .The reproducibility of this phenomenon across markedly different life history stages highlights the considerable potential value of thermal imaging of surface temperature in estimating this element of physiological state non-invasively. Given that body condition has been linked not only to survival,

reproduction and behaviour, but also to the impact of human activities (reviewed in Labocha & Hayes 2012), the opportunities presented are numerous. The mechanisms underlying this relationship are not known, but may result from non-mutually exclusive factors including differences in the thermal properties of individuals with varying levels of fat mass and/or feathering, and/or differences in metabolism or activity levels, as well as any potential effect of stress physiology on circulation. While caution must be exercised due to the small subsample size (n = 14), with only three individuals in the control group, the poorer body condition among birds in the human disturbance and predation risk groups is noteworthy for two reasons. Firstly, because both human disturbance and predation risk appeared to induce a similar physiological cost. Secondly, because this experimental evidence suggests the existence of a causal link between T_{eye} and longer term physiological processes, such as those contributing to body condition, adding to the correlative evidence presented here, and in Chapter 4. Furthermore, as condition index was calculated from measurements taken on days 8-9, the implication is that effects of the three human disturbance/predation risk protocols which took place between days 2-6 were still detectable (both in terms of body condition and T_{eye}) two or three days later. Nevertheless, this finding appears to contradict the lack of observed differences in T_{eye} between the experimental groups. If birds in poorer condition exhibit lower T_{eye}, and the birds in the human disturbance and predation risk groups were in poorer condition, then the birds in these groups should have exhibited lower T_{eye}, than birds in the control group. It is possible that the values of T_{eye} for the subset of control birds with condition data were unusually high, or that T_{eye} was unusually low in the subset of birds with condition data in the human disturbance and predation risk groups. If this was the case, then the reported relationship between condition index and eye temperature could be an artefact of the small subsample size. However, there were no differences in T_{eye} between birds with condition data, and those without, in any of the experimental groups (linear mixed models, with bird ID as a random effect, control; $t = 0.73$, $p = 0.48$, human disturbance; $t = 0.39$, $p = 0.71$, predation risk; $t = 0.42$, $p = 0.18$). Rather, this suggests that the measure of T_{eye} used in this study was not sensitive enough to pick up condition related differences in T_{eye} between experimental groups. Consequently, more precise validations of T_{eye} against

experimental manipulations of body condition would be a desirable next step in developing thermal imaging of surface temperature as a technique for assessing body condition.

Provisioning rates reported here are similar in range to those measured elsewhere from blue tits (e.g. Tremblay et al. 2005; Nour et al. 2008). The observed increase in provisioning rate with brood size is also in agreement with previous investigations (e.g. Stauss et al. 2005; García-Navas & Sanz 2010). However, additional studies have found an effect of timing of breeding (i.e. hatch date) on provisioning rates (Tremblay et al. 2005; García-Navas & Sanz 2011), which was not observed here. Most researchers investigating the effect of human disturbance or predation risk on provisioning rates have only measured over limited periods, typically 1-3 hrs (reviewed in Pagani-Núñez & Senar 2013). While it has been demonstrated that increased perceived predation risk (but not human disturbance) affects provisioning rates in the short term (Tilgar et al. 2011; Ghalambor et al. 2013), longer-term effects are generally only presumed, based on evidence that hourly rates correlate with mean or total measurements made over the whole day (García-Navas & Sanz 2012; Pagani-Núñez & Senar 2013). Yet, our substantially longer term measurement of provisioning rates (individual mean hourly visits per day, throughout days 2-12 after hatching) indicate that the human disturbance and predation risk groups were no different from the control group in this respect. Provisioning was totally suspended during the majority (85%) of protocols, suggesting that fluctuations relating to the repeated protocols were compensated for, and that the protocols did not evoke any longer-term adjustments. Such compensations have been seen in other species (Wheelwright & Dorsey 1991), without apparent effect on breeding success, although compensatory growth after prolonged resource deprivation may incur longevity costs for offspring later in life (Mangel & Munch 2005). Additionally, if compensations prove costly for the parents, trade-offs between reproduction and subsequent moult may affect their own future fitness/survival (Nilsson & Svensson 1996). While short term changes in provisioning rate are beyond the scope of this study, it would clearly be of interest to re-examine our data at a finer scale to gain a

better understanding of exactly how plasticity of provisioning rates may have been used to compensate for treatment related interruptions/fluctuations.

Similar fitness measures across all groups confirm that despite generating differences in acute behavioural responses and physiological state, the human disturbance and predation risk treatments did not compromise breeding success. Blue tit chick mass has previously been reported to be lower in the presence of sparrowhawks (Adriaensen et al. 1998). Also, human disturbance has been found to reduce fledging success in other cavity nesting species (Watson et al. 2014). Therefore, the lack of between-group differences found here suggests that parents were able to buffer their offspring from the effects of the treatments. Annual blue tit mortality is generally high (Snow 1956), with adult birds likely to survive long enough for only one attempt at breeding. Given this life history imperative, it may be expected that blue tit parents would sacrifice their own state in order to increase breeding success (as is indeed suggested by the lower body condition in the human disturbance and predation risk groups), and so be quite capable of coping with adverse situations. How environmental circumstances (e.g. food availability and/or weather conditions) may affect ability to cope with human disturbance or predation risk would be an area worthy of further investigation. One further alternative explanation for the lack of between group differences in fitness is that the birds in the control group suffered similar fitness costs to those in the human disturbance and predation risk groups. There is evidence from their behavioural responses that the birds in the control group were aware of the observers, and so could have been affected by that. However, the apparent lack of physiological cost suggested by higher body condition in control birds suggests that the human disturbance and predation risk groups were subject to a costlier challenge, but that all groups were able to compensate and maintain similar breeding success.

In conclusion, the results of this novel study demonstrate that while behavioural responses to human disturbance and predation risk differ, the apparent physiological costs are similar. However, the experimental treatments failed to induce differences in breeding success from the control group, most likely because the environment was

too favourable. As such, the question of whether the fitness consequences of human disturbance and predation risk are analogous remains open.

7. General Discussion

In this thesis, my initial aims were to develop a method of measuring body surface temperatures from free-living blue tits using thermal imaging, in order to non-invasively assess physiological state. I then aimed to use this method to contrast surface temperature dynamics between different acute stressors in overwintering birds. I also aimed to explore correlations between baseline plasma glucocorticoid levels, body condition, and body surface temperature in free-living overwintering blue tits, and the response to experimentally manipulated chronic stress in captive zebra finches. Finally, I aimed to compare effects of experimentally manipulated human disturbance and predation risk on behaviour, body surface temperature, and breeding success in free-living breeding blue tits. All of these aims were achieved, with the exception of the experimental aviary study, where the putative stressor failed to induce a detectable physiological response. Despite this, useful information was generated by the experiment, such as quantifying the effect of activity levels on surface temperatures recorded from moving birds.

Key Findings

The thermal imaging techniques first developed in Chapter 2 established that body surface temperatures can be reliably measured non-invasively from free living birds in a variety of contexts, permitting detailed characterisation of physiological responses in real time. This finding is supported by the successful acquisition of body surface temperatures from overwintering blue tits visiting food-baited traps in Chapters 2, 3 and 4, and from breeding blue tits entering and leaving their nests in Chapter 6. Using this data, it was shown (in Chapters 2 and 3), that body surface temperature exhibits a characteristic response to acute stress, which differs with stressor type. While the effects of environmental conditions, and the mechanisms underlying these differences require further investigation, it is evident that a considerable amount of potentially useful information is made available by measuring body surface temperature dynamics during acute stress.

Having demonstrated that immediate changes in physiological state can be detected from body surface temperature, links between body surface temperature and longer

term physiological process were then also sought. In Chapter 4 near identical correlations between body surface temperature and body condition were observed across differing seasons and life history stages (Figure 4-4). The direction of the correlations (birds in poorer condition had lower body surface temperatures) agree with the predictions of stress-induced hyperthermia, in that individuals under physiological stress would be expected to exhibit lower surface temperatures (Busnardo et al. 2010). While it remains possible that differences in body surface temperature with condition are also related to insulative/thermoregulatory state, metabolic rate, or immune status, the correlation reported in Chapter 4 between baseline free corticosterone and body surface temperature raises the possibility of a link with the hypothalamic-pituitary-adrenal axis. Additionally, the evidence that repeated acute stressors (predation risk and human disturbance) had a chronic effect on body condition breeding blue tits (Chapter 6), and that surface temperature in those birds was linked to body condition suggests that long term surface temperature changes do take place in response to environmental challenges. If confirmed, this would be particularly interesting in a conservation physiology context, not least as it suggests that it may be possible to non-invasively detect a signal of persistent physiological effect(s) relating to human disturbance.

Limitations and caveats

Probably the most substantial practical limitation of the thermal imaging techniques described in this thesis is that measurement of body surface temperature is only possible from animals in a restricted, static area (i.e. the zone of focus within the field of view of a fixed focus camera). While this is undoubtedly restrictive, it is encouraging to refer to the vast archives of wildlife photography and film footage that have accumulated (Bousé 2000; Kidman Cox 2014), remembering that analogous limitations also applied to them. Photographers and filmmakers typically employ two key approaches; either individuals are encouraged to appear in front of the camera (e.g. by providing food or water), or the camera is placed where individuals can be predicted to appear (e.g. during site-specific breeding) (Tipling 2011). Behavioural knowledge, and creative use of these simple strategies have

together yielded tremendous rewards for photographers and documentary makers, and so could reasonably be expected do the same for physiological ecologists. However, it should of course be noted that such rewards can be somewhat hard-earned, as is frequently demonstrated during the 'making of' sections that are now a regular feature of BBC wildlife documentaries, and that the technical demands of thermal imaging are somewhat more exacting than photography. A related additional practical limitation of thermal imaging is that difficulties in obtaining footage are often necessarily followed by a laborious process of data extraction. Even with the largely automated extraction process developed in Chapter 1, substantial effort was still required in identifying regions of interest, and eliminating the various sources of error. In subsequent chapters, where multiple birds were filmed simultaneously, the effort required was even greater, as automated identification of individuals is not yet possible. That the data extraction stage of thermal imaging can be demanding is not a new observation, having been noted previously by other authors (e.g. Tattersall 2016). However, the level of work required is far from prohibitive, and probably little different from that involved in the laboratory analysis of tissue or blood samples. Also, thermal imaging captures real-time data, whereas sampling tissues or blood rarely provides more than a snapshot in time. As such, there will undoubtedly be many circumstances where the advantages of collecting physiological data non-invasively, and at high frequencies from free-living animals outweighs the costs incurred in extracting data from thermal images. Nonetheless, the development of more powerful automation solutions would be particularly advantageous.

The negative effect of activity on body surface temperatures quantified in Chapter 5 (presumably relating to motion blur) suggests that all analyses of thermal images of moving animals would benefit from the inclusion of activity as a covariate. Accordingly, the data presented in Chapters 2, 3 and 4, collected from active birds where activity levels were not taken into account, might benefit from re-analysis in this respect. However, the reported dynamics and relationships would not be expected to change qualitatively. In the case of the surface temperature responses to acute stressors, similar surface temperature dynamics were also reported by Herborn et al. (2015) from chickens subjected to handling stress (and subsequently released),

where activity was accounted for. Additionally, Busnardo et al. (2010) observed analogous patterns in tail surface temperatures while mice were subjected to restraint (i.e. activity was prevented). In terms of the baseline measures of body surface temperature presented in Chapter 4, all birds were filmed undisturbed while performing the same behaviour (feeding, within the trap). Consequently, only limited variability in activity would be expected. Therefore, adding activity to these analyses would only be expected to remove a limited amount of noise, and so provide minimal extra clarity. The considerable effort required to revisit the footage and quantify activity probably outweighs such marginal gains. However, re-analysis of the 'trapping only' data presented in Chapter 2 might be the most likely to yield useful insights. Subsequent to an initial drop, body surface temperatures recovered, but remained slightly below the baseline value for the remainder of the test (Chapter 2, Figure 2-4). Unlike the birds in the 'trapping and handling tests' reported in Chapter 3, these birds were free to move within the trap throughout the test, and generally remained highly active, making repeated escape attempts. Therefore, it remains possible that surface temperatures did return to baseline, but this was obscured by the effect of consistently increased activity after baseline measurement.

It should also be noted that the effects of convective heat loss were not included in the presented analyses. The importance of convective heat loss to the environment is expected to increase in windy environments (McCafferty et al. 2011). Accordingly, it may be necessary to account for wind speed when investigating body surface temperatures. However, during the studies involving use of walk-in box traps (Chapters 2, 3 and 4), participating birds were either confined within the shelter of the trap, or shielded from wind by the experimenter's body. Therefore, the consequences of variation in wind speed were considered to be minimal, and convective heat loss expected to be relatively constant. Also, given the very low movement of air within the aviary, convective heat loss was not expected to vary substantially during the experiment presented in Chapter 5. Contrastingly, the birds filmed entering and leaving their nest boxes (Chapter 6) were not sheltered from wind. Consequently, changes in convective heat loss due to variation in wind speed may have contributed noise to these measurements of body surface temperature,

leading to their hypothesized lack of sensitivity (see p148). However, if wind was having a significant biological effect, there should have been a substantial difference in variance between body surface temperatures measured from sheltered individuals during winter and those measured from unsheltered individuals during the breeding season. This was not the case (Levene's Test for Homogeneity of Variance; $F=0.1024$, $p=0.75$), suggesting that variation in convective heat loss did not substantially affect the sensitivity of body surface temperature measurements made during the breeding season.

Future Directions

As the experimental investigation of potential relationships between plasma glucocorticoids, body surface temperatures, and chronic stress (Chapter 5) failed to induce chronic stress, revisiting this test would seem to be the most appropriate immediate next step. Marasco et al. (2015) successfully used food restriction to induce elevations in plasma glucocorticoid concentrations in the same facilities with the same species (albeit with a different population). Therefore, this approach might usefully replace removal of environmental enrichments. However, that study used what might be described as close to the maximum amount of ethically responsible food restriction possible (1/3 daylight hrs, 4x per week). This induced only a minimal glucocorticoid response, and even then restricted to the days when food restriction took place (unpublished data), so it is doubtful whether biologically relevant chronic stress was, or could be induced in this way in captive zebra finches. Instead, it may prove more advantageous to bring wild blue tits into aviaries. Firstly because the correlation between free CORT and body surface temperature was observed in that species, and secondly because captivity alone would be likely to chronically alter stress physiology (Dickens et al. 2009), without extra effort. Similarly, it would be desirable to re-assess the relationships between body surface temperature and human disturbance/predation risk reported in Chapter 6. The analysis of the unbiased subsample of birds with condition index data showed that both surface temperatures and body condition were lower in the human disturbance/predation risk groups within those birds. This suggests that there was

also an effect of human disturbance/predation risk in the larger group of birds for which we did not have body condition data, but that it was masked by a lack of precision in the measurement of surface temperature. As activity was shown to have considerable influence on surface temperatures in Chapter 5, it may prove useful to more rigorously quantify activity levels from the thermal videos, and re-analyse the surface temperature data with activity as a distinct covariate. This should go some way to reducing the level of noise apparent in the surface temperature measurements. Alternatively, it may also be productive to experimentally manipulate body condition in a larger sample of birds, comparing body condition with surface temperature. Body condition has previously been altered in free-living passerines by manipulating food availability in overwintering birds (Brown & Sherry 2006), and by manipulating brood size in breeding birds (Chastel & Kersten 2002). Consequently, it should be feasible to test the relationship at both of the life-history stages where correlations between body surface temperature and body condition have already been observed.

The potential applications of the relationships found between acute stress and body surface temperatures would be greatly enhanced if it could be shown that features of body temperature changes (e.g. magnitude) relate to the intensity of acute stress. There is evidence from lab rodents, that both the degree of stress-induced hyperthermia, and its duration are related to the intensity of a given stressor (Van der Heyden *et al.* 1997). Additionally, the body surface temperature response to acute stress has been demonstrated to vary with stressor intensity in chickens (Herborn et al. 2015). However only two levels of intensity were investigated; more levels would be required to comprehensively characterise the relationship. Given the different outcomes of studies of wild and domesticated birds in this thesis, it would also be prudent to explore the effects of differing levels of acute stress intensity in wild birds. Plasma glucocorticoids vary linearly with acute stress intensity (Hennessy & Levine 1978; Kant et al. 1983). As such, comparison of body surface temperature responses with plasma glucocorticoid levels would be a useful addition to such an experiment in confirming manipulated differences in stressor intensity (Herborn et al. 2015).

Given the potential for integrated and repeated measurement of the entire body surface temperature response to acute stress (Chapters 2 and 3), further exploration of a number of features of the response would be of particular interest. Characterising short and long-term repeatability, inter-individual variability, responses to diverse stressors such as social interactions and predator attack, and variation relating to environmental conditions would all be useful in characterising how generalised the response is. Additionally, response magnitude and duration should give an indication of coping ability. Particularly extreme (very large or small) physiological responses to stressors are expected to indicate inability to cope, and would be predicted to reduce fitness (Romero et al. 2009; Wingfield et al. 2011). Accordingly, measurement of body surface temperatures during acute stressors may prove to be of considerable use in predicting which individuals and populations are likely to prosper (or otherwise), in the face of environmental challenges – an urgent goal of conservation (Wingfield 2008; Fefferman & Romero 2013).

Finally, the precise physiological mechanisms underlying the correlations and empirically determined relationships reported in this thesis remain unclear. If non-invasive measures of body surface temperature are to become credible indicators of physiological state, then it is critical that the physiological mechanisms contributing to body surface temperature and its dynamics are well understood. Given the difficulties involved in obtaining physiological samples from free-living species, it would be particularly challenging to address this situation in the field. Therefore, it seems likely that targeted laboratory studies would be more appropriate in filling these knowledge gaps.

Conclusions

In conclusion, the results contained in this thesis suggest that body surface temperatures measured using thermal imaging are highly likely to prove useful in determining aspects of physiological state non-invasively from free-living animals. While further investigation and validations are necessary, this work has laid the foundations for an exciting new methodology that could help solve many questions that remain unanswerable using current techniques. The use of thermal imaging to

infer physiological state opens the way to a better fundamental understanding of why organisms behave as they do, and how they interact with each other and their environment. Additionally, there are many potential uses of the methods described in applied settings, most notably conservation physiology. Physiological biomarkers are of particular interest to conservation biologists wishing to determine, predict and mitigate threats to wildlife, or assess management impact (Bradshaw & Brook 2010). However, the cross-sectional physiological data typically collected by conservation biologists (due to the oft-mentioned difficulties in their acquisition), is not sufficient to determine responses to environmental change (Madliger & Love 2016). As such, the ability to acquire longitudinal data is a prerequisite for useful application/ interpretation of physiological biomarkers in conservation. As a technique, non-invasive thermal imaging of body temperatures is uniquely well-suited to fulfilling this requirement.

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9. Appendix 1:
Chapter 2 Supplementary Materials

a) R Code

```
############################
### Peak Search Function ###
############################

peaks <- function (series, span = 3, ties.method = "first")
{
  if ((span <- as.integer(span))%%2 != 1)
    stop("'span' must be odd")
  z <- embed(series, span)
  s <- span%/%2
  v <- max.col(z, ties.method = ties.method) == 1 + s
  pad <- rep(FALSE, s)
  result <- c(pad, v, pad)
  result
}

#### Peak Search Function Usage

# Create an extra column in the dataframe 'df', called 'peaks'
# containing either 'TRUE' or 'FALSE' depending on whether the value
# on any given row of 'temp'is a peak (based on a span of 3)

df$peaks <- peaks(df$temp, 3)

# Extract all rows where the value of 'temp' was a peak to a new
# dataframe 'dfpeaks'

dfpeaks <- subset(df, peaks=='TRUE')


############################
### Linear Interpolation ###
############################

# This code assumes that 'df' is a dataframe containing 2 columns,
# 'time' & 'temp'.'time' is assumed to be standardised to 'time
# since trap shut'.'temp' is assumed to be standardised to
# temperature difference from baseline.

# Load Required Packages
# (Packages need to be installed beforehand)

require("zoo")
require(ggplot2)
require(Hmisc)

# Set Up Required Zoo Object to Data Frame Conversion Function

zoo.to.data.frame <- function(x, index.name="datetime") {
  stopifnot(is.zoo(x))
  xn <- if(is.null(dim(x))) deparse(substitute(x)) else colnames(x)
  setNames(data.frame(index(x), x, row.names=NULL),
c(index.name,xn))
}
```

```
# Convert Dataframe to Zoo Object

dfzoo <- zoo(df)

# Make Relative Time Column Index for na.approx

index(dfzoo) <- dfzoo[,1]

# Interpolate Sequence of Temps for 0-120 sec, giving a single value
# for each second

dfzoo.interpolated <- na.approx(object=bleeding1.zoo.for.interp,
                                xout=seq(from=0, to=120, by=1))

# Convert Zoo Object back to Dataframe

dfzoo.interpolated.df <- zoo.to.data.frame(dfzoo.interpolated)


#################
### Mean Plot ###
#################

# This code assumes that all interpolated dataframes have been
# joined into a single dataframe 'df' containing 3 columns, 'time',
# 'temp', and 'test' (with 'test' indicating the identity of each
# individual temperature response curve)

ggplot(df,aes(x=time,y=temp)) +
  stat_summary(fun.data ="mean_cl_boot", mult=1, geom = "smooth",
colour="red"
```

b) Baseline Temperature Statistics

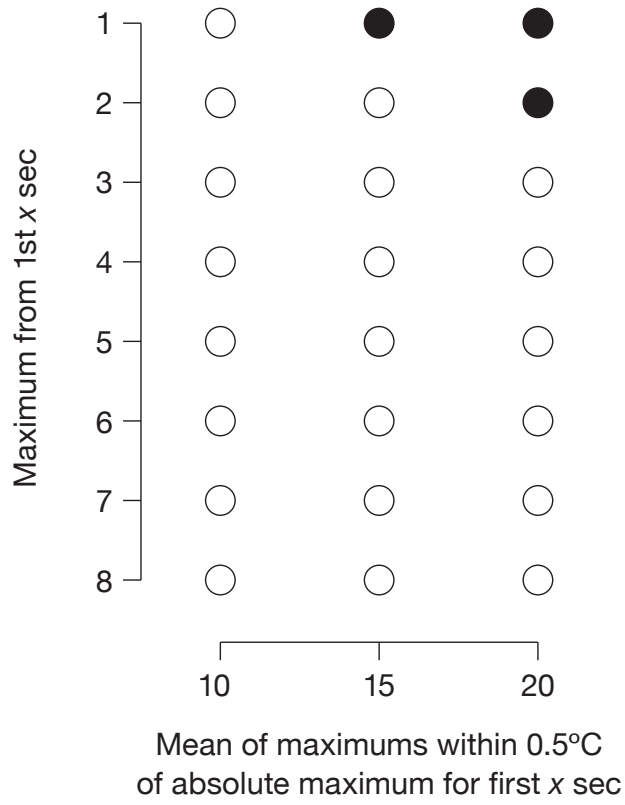

Figure 9-1 Graphic representation of results of paired t-tests (see

Table 9-1) comparing maximum temperature recorded in the first 1-8 s of a clip with the 'mean of the maxima' within 0.5º C of the highest value recorded for the first 10, 15 and 20 s of the clip. Closed circles represent a significant difference, open circles represent no significant difference (critical p=0.05).

Maximum from first *x* seconds	Mean of maximums within 0.5ºC of absolute maximum for first *x* seconds					
	10 s		15 s		20 s	
	t	p	t	p	t	p
1	3.058	0.216	4.184	0.024	4.556	0.024
2	1.974	1.000	3.178	0.168	3.953	0.048
3	1.077	1.000	2.216	1.000	2.863	0.312
4	0.644	1.000	1.931	1.000	2.711	0.432
5	0.176	1.000	1.685	1.000	2.506	0.624
6	0.397	1.000	1.521	1.000	2.336	0.864
7	1.323	1.000	0.950	1.000	1.696	1.000
8	3.620	0.072	0.631	1.000	1.415	1.000

Table 9-1 Results of paired t-tests (critical p=0.05 with Bonferroni Correction) comparing maximum temperature recorded in the first 1-8 s of a clip with the 'mean of the maxima' within 0.5º C of the highest value recorded for the first 10, 15 and 20 s of the clip. Degrees of freedom = 13 for all tests.

10. Appendix 2:
Chapter 5 Supplementary Materials

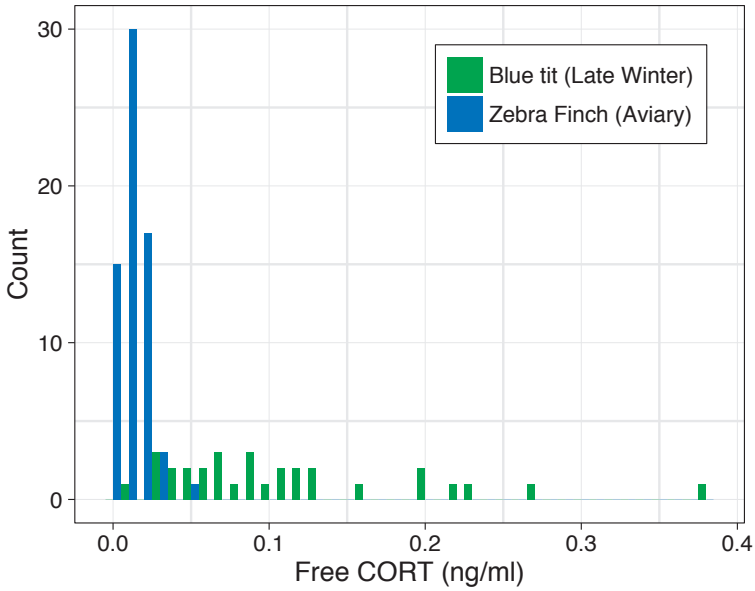

Figure 10-1 Distributions of free plasma corticosterone concentrations measured from the captive zebra finches used in this study (blue bars), and in wild blue tits reported previously (green bars) (Chapter 4).

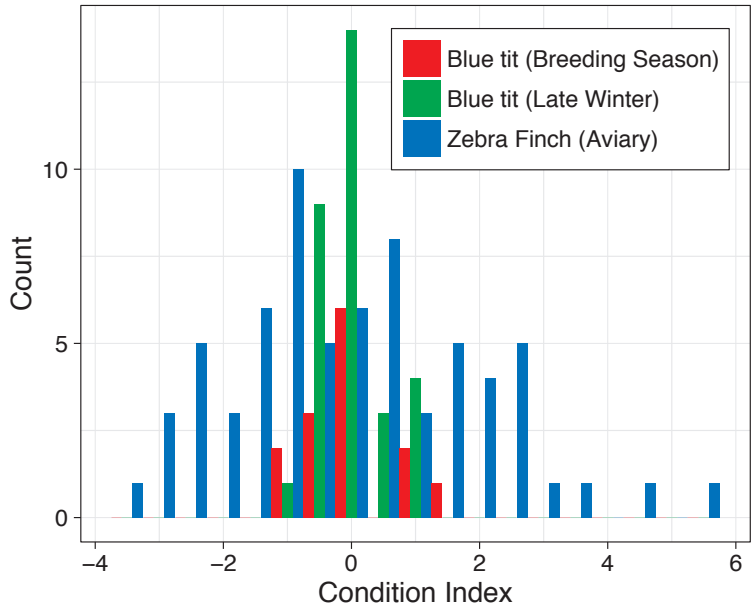

Figure 10-2 Distributions of body condition index measured from the captive zebra finches used in this study (blue bars), and in wild blue tits reported previously (red/green bars) (Chapter 4).