The Physiology and Pathology of Heat Stress in Australian Desert Birds

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Abstract

Arid environments pose a unique set of challenges to organisms. These challenges include the lack of basic resources such as food and water, as well as extremes in temperatures. Birds are small flying endotherms that maintain high body temperatures close to lethal limits, which is advantageous in hot climates. However, as global temperatures increase, birds need to be able to respond to weather changes quickly and appropriately for their own well-being and survival. The inability to respond appropriately to heatwaves can be fatal to individual birds and translate into large-scale mortality events.

As there were gaps in the current knowledge regarding the physiological stress responses and behavioural adaptations of Australian desert birds to high ambient temperatures, I designed and conducted separate studies to investigate these responses. I found that the corticosterone (CORT) and heterophil:lymphocyte (H:L) ratio responses of budgerigars (*Melopsittacus undulatus*), zebra finches (*Taeniopygia guttata*) and diamond doves (*Geopelia cuneata*) to heat exposures were different. These species differences may reflect their ability to detect and adapt to high temperatures. There was also no significant correlation found between the change in CORT and H:L ratios, which may reflect differences in the timescales of these responses.

Based on the species differences in CORT response, I hypothesized that there would also be differences in the behavioural response to high ambient temperatures, as CORT has effects on the behaviour of birds. Observation of eight species of birds at the Adelaide Zoo confirmed my hypothesis. Psittaciform birds spent less time feeding and more time resting in cooler microsites during hot periods. Columbiform birds continued feeding and spent more time in the sun during hot periods rather than resting in cooler microsites. White-Browed Woodswallows, the only passerine species assessed, spent a significantly lower proportion of

time on stationary behaviours and higher proportion of time feeding compared to the other species. The smallest birds in the study, they also utilised wing venting more than other species of birds, possibly because it is more important for them to conserve water. These results suggest that columbiform birds may have an advantage during heatwaves as they can continue feeding through high ambient temperatures, as long as there is adequate access to food and water.

When physiological and behavioural adaptations are unable to prevent birds from maintaining their body temperatures below lethal limits, pathological changes are expected. There is currently very little documentation of these pathological changes in the literature, which makes diagnosis of heat injuries in birds difficult. I examined the histopathological changes in the organs of the birds from the CORT and H:L ratio study and found that the main changes were in the lungs and liver, albeit to different degrees and frequencies in different species. There were also changes in the hearts, kidneys and gastrointestinal tracts of some birds, but these were less frequently observed.

Having established there are species differences in how birds respond physiologically, behaviourally and pathologically when exposed to heat, I then sought to further characterize these responses and adaptations at a molecular level. I selected genes of interest and measured the mRNA expression of these genes in the organs of the birds from the CORT/H:L study. The results revealed that acute exposure of native Australian birds to high temperatures (45°C) would result in upregulation heat shock protein (hsp) genes, but there was no significant upregulation of other genes with protective effects against cell damage (BCL-2 and VEGFA) nor genes associated with inflammation (interleukins). There was also no downregulation of the genes involved in the coagulation pathway (fibrinogen) in these birds. The gastrointestinal tracts of all 3 bird species had the highest number of hsp genes upregulated, possibly indicating that this is the organ that requires the most protection to

continue its function. Diamond dove organs also had the highest number of hsp genes upregulated, possibly a reflection of their ability to protect their cells better during high temperatures.

The findings from my thesis have filled in gaps in the current knowledge regarding the physiological stress and behavioural responses of Australian desert birds to high ambient temperatures. I have also found clinical and histopathological changes in birds exposed to varying degrees of heat, which can be used to help diagnosis of heat injuries in birds. These findings have also revealed that there are important differences in the ways different species of birds respond to heat and that there is no single strategy that can be applied to help all birds survive the effects of climate change. Instead, it is important to identify the challenges each species may face and apply the correct strategy to the species in question in order to maximize the benefit of any intervention. For example, given the low heat tolerance and reliance on the availability of cool microsites for refuge of psittacine birds, the most important conservation strategy for them may be the conservation of microsite refuges in the desert. Furthermore, birds that can tolerate very high environmental temperatures, e.g. columbiform birds, may hold secrets that can be uncovered with further research. These may include deep sequencing molecular genetic techniques such as RNAseq, with a focus on heat shock proteins. Further understanding of the genetic adaptations required to confer high tolerances to heat will also allow better identification of vulnerable species of birds, so that appropriate resources can be allocated to helping them survive the effects of climate change proactively rather retrospectively.

Declaration of originality

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Chapter 1 Introduction

Arid environments pose a unique set of challenges to organisms. These challenges include the lack of basic resources such as food and water, as well as extremes in temperatures. Scientists have traditionally thought that birds are intrinsically equipped with the physiology to cope with the challenges of living in arid environments without any physiological and anatomical specializations (Maclean 1996, Williams and Tieleman 2001). Williams and Tieleman (2001) reviewed and summarized papers reflecting this traditional thought more than 15 years ago, and attempted to dispel it by showing that some desert birds have lower basal metabolic rates, field metabolic rates and rates of evaporative water loss compared to non-desert birds. This opened up a new field of thought where the evolutionary and ecological physiology of desert birds is unique and deserves further investigation.

One of the important features of arid environments is extreme heat, especially during summer. Other than having to cope with chronic high environmental temperatures coupled with a lack of food and water, desert birds also have to cope with the occasional and unpredictable, but slowly becoming more frequent, heat waves. Sterl et al. (2008) predicted that maximum temperatures may reach 48°C across the American mid-west, 54°C in South America and 50°C in Australia and India by the year 2100. Based on scientific (Serventy 1971, Finlayson 1932, McKechnie et al. 2012, Low 2011, Saunders et al. 2011) and anecdotal news reports (McKechnie and Wolf 2010, McKechnie et al. 2012), heat waves have already been reported to have killed large numbers of birds in Australia, including endangered species (Saunders et al. 2011). It appears that even if desert birds have evolved physiological specializations to cope with the extreme heat in arid environments, there are still limits to their ability to survive during prolonged periods of high temperatures. It has also been shown

that avian abundance and species richness usually decline during and after heat waves, in the same year as well as the subsequent year, in the United States (Albright et al. 2011). With heat waves occurring more frequently, this restructuring of avian communities may have lasting impacts for endangered avian species.

As temperatures continue to rise with climate change, sensitive taxa of birds will be challenged (McKechnie et al. 2012, Foden et al. 2013, Garnett and Franklin 2014). Garnett & Franklin (2014) analysed the effects of climate change on birds in Australia, but acknowledged that they were unable to factor in sensitivities of threatened bird species to extreme climate events such as heat waves, even though these events were a threat to taxon survival. Also, the physiology of Australian birds in general has not been well-studied, as reflected by a low number of studies and low number of species studied (Astheimer and Buttemer 2002). This is likely a result of the limited number of Australian ecophysiologists interested in endotherms, as well as the draw of studying Australian mammals due to their uniqueness and ease of capture relative to birds (Cooper 2017). Consequently, there is little known about the endocrinology, osmoregulation and thermoregulation of Australian desert birds. Responses to heat waves depend on the functional traits of different avian species (Albright et al. 2011). Therefore, different orders and/or species of birds may have different physiological and behavioural flexibilities that determine whether or not they survive during a heat wave.

One of the keys to survival of highly exposed bird taxa to climate change is the availability of climatic refugia within the landscape and identification of such refugia is important so that they can be secured (McKechnie et al. 2012, Garnett and Franklin 2014). However, management of climatic refugia is one of the more expensive measures that may eventually be required to protect exposed bird taxa from climate change (Garnett and

Franklin 2014). Further research identifying the sensitivities of various bird species to the effects of increased temperatures could therefore help focus and prioritize such efforts to the more sensitive bird species.

This review aims to summarize the current literature describing the adaptations of birds that bear on their ability to tolerate heat: differences in heat tolerance limits amongst avian orders, physiological stress responses of birds to heat, the features of heat illnesses in animals, and the roles of the neuroendocrine system and heat shock proteins in helping birds cope with the extreme heat experienced in arid environments. The physiological and behavioural adaptations of desert birds have been discussed in detail in Williams and Tieleman's (2001) review, but the main points will still be briefly discussed in this review.

General physiology of avian heat tolerance

Overview

Most birds are small flying endotherms. They maintain high body temperatures close to lethal limits due to high mass-specific metabolic rates and high surface area to volume ratios that tightly couple their environmental temperatures to their body temperatures. These physiological traits result in high rates of evaporative water loss (Dawson 1954). These are advantages that help to lower their risk of heat stress in hot climates but must be traded off against the need to maintain water balance. Most birds, being diurnal, usually reduce activity and retreat to shaded microsites during periods of high environmental temperatures. A combination of decreased physical activity and decreased exposure to solar radiation minimizes rates of water loss, but also limit rates of water intake. Therefore, small birds can still lose more than 5% of body mass per hour via evaporative water loss during periods of

high environmental temperatures even if they rest in completely shaded microsite (Wolf and Walsberg 1996a, Wolf and Walsberg 1996b). The fundamental physiological conflict between thermoregulation via evaporative water loss and water balance regulation is a major challenge faced by desert birds (Bartholomew and Cade 1963, Dawson and Schmidt-Nielsen 1964, Webster 1991, Tieleman and Williams 1999, Wolf 2000).

Energy, Water and Thermoregulation

Desert birds frequently struggle to meet their daily energy requirements due to their high rates of metabolism (as endotherms) and relatively low food availability in the desert (Dawson and Schmidt-Nielsen 1964, Dawson and Bennett 1973, Williams and Tieleman 2001). A relatively lower metabolic rate and the consequently lower endogenous heat production in desert birds relative to non-desert birds may be beneficial because less food for energy and less water for evaporative cooling are required (Williams and Tieleman 2001, Dawson and Bennett 1973). The lower basal metabolic, field metabolic and evaporative water loss rates described by Williams and Tieleman (2001) in desert birds relative to non-desert birds may therefore provide part of the mechanism with which desert birds cope with extreme temperatures.

It has been found that small avian species wintering in environments with unpredictable winter weather events do not adjust their thermogenic capacities and basal metabolic rates at the same time, suggesting that these parameters are regulated separately (Petit et al. 2013). It is possible that the thermogenic capacity of the pectoral muscles change early in winter in response to the dropping temperatures, but basal metabolic rates change later in winter as a result of changes in the type and amount of food consumed by birds (Petit

et al. 2013). It is likely that the converse of this is true for desert birds trying to cope with heat waves – the energy, water and thermo-regulatory mechanisms may be regulated separately during the season.

Anatomical and Physiological Adaptations of Desert Birds

There are several anatomical and physiological adaptations that desert birds have that help them cope with the challenge of living in hot, arid environments. These adaptations are summarized according to organ systems below.

Kidney

Avian kidneys are elongated, flattened and closely fitted into the bony concavity formed by the synsacrum (Braun and Dantzler 1972). They contain both reptilian-type and mammalian-type nephrons, with species differences in the relative proportions of each nephron type (Braun and Dantzler 1972). The mammalian-type nephrons can be further differentiated into long loop and short loop mammalian-type nephrons (Braun and Dantzler 1972). When desert birds are subjected to severe dehydration or salt loads, they need to prevent the rise of plasma osmolality by secreting ions extra-renally, producing concentrated urine, reducing the glomerular filtration rate or any combinations of these (Braun and Dantzler 1972). Desert quails (*Lophortyx*) can also decrease the overall glomerular filtration rate by reducing the number of functioning reptilian-type nephrons to increase water conservation at the expense of waste excretion, whilst maintaining the functions of the mammalian-type nephrons to increase the urine concentrating capability of the kidney (Braun and Dantzler 1972). In contrast to many other birds, chickens increase tubular absorption in the kidneys to conserve water, as mammals do (Stallone and Braun 1985).

A series of studies comparing the arid-occupying stubble quail (*Cortunix pectoralis*) and mesic king quail (*Cortunix chinensis*) revealed that although they had similar metabolic rates and thermoregulatory abilities (Roberts and Baudinette 1986), as well as similar physiological changes in body temperature, heart rate and respiratory rate in response to heat exposure (Roberts and Baudinette 1988), stubble quail are able to maintain normal plasma osmolarity despite significant electrolyte changes (Roberts and Baudinette 1984, Roberts et al. 1985). Stubble quail also had greater renal concentrating ability corresponding to a greater proportion of medullary tissue in their kidneys compared to king quail (Roberts and Baudinette 1984).

Desert mammals were found to have a larger medullary mass relative to total kidney mass compared to mesic mammals (Schmidt-Nielsen and O'Dell 1961, Heisinger and Breitenbach 1969). The vasa recta, loops of Henle and collecting ducts in birds are encapsulated in the medullary cone, making the division between cortex and medulla less clear in bird kidneys (Williams and Tieleman 2001). Therefore, similar studies in birds to identify differences in kidney structure between desert and mesic birds will depend on measurements of relative medullary cone length instead of relative medullary mass. One such study by Goldstein and Braun (1989) found that there was no association between relative medullary cone length and maximal ureteral urine concentration. Desert birds were also not found to have superior urine concentrating abilities relative to non-desert birds (Goldstein and Braun 1989), but arid populations of some species have been found to have greater renal concentrating abilities compared to mesic populations (Ambrose and Bradshaw 1988a). Williams and Tieleman (2001) commented that these results could be due to the small sample size, and the fact that two of the seven species studied were seabirds with salt glands and larger kidneys. Goldstein and Braun (1989) also suggested that the smaller birds have better

urine concentrating abilities regardless of habitat affinity and that there was a negative correlation between the maximal ureteral urine concentration and the loop of Henle length.

There were also very few differences found between nephron ultrastructures of mesic and arid zone honeyeaters (Casotti and Richardson 1993) and sparrows (Casotti and Braun 2000). In the latter study, it was found that the savannah sparrow (*Passerculus sandwichensis*) has the renal morphology to produce more concentrated urine than the mesic house sparrow (*Passer domesticus*), but this was not reflected in its physiology (Casotti and Richardson 1993). However, it was found that white-browed scrubwren (*Sericornis frontalis*) in arid regions had both increased renal concentrating ability and corresponding increase in proportion of medullary tissue in their kidneys compared to the same species in semi-arid and mesic regions (Ambrose and Bradshaw 1988a).

It has been suggested that the maximal ureteral urine concentration may correlate with the mass-adjusted field metabolic rate of a species because a lower metabolic rate means there is less metabolic waste for the kidneys to eliminate (Williams and Tieleman 2001). This could therefore partly explain the significance of a lower field metabolic rate in desert birds compared to non-desert birds (Williams and Tieleman 2001). For example, white-browed scrubwren have been shown to decrease their metabolic rates in summer compared to winter, and arid zone populations have a markedly lower metabolic rate compared to semi-arid and mesic populations (Ambrose and Bradshaw 1988b).

Gastro-intestinal tract

Birds are able to move urine from the cloaca by anti-peristalsis into the rectum (Brummermann and Braun 1995), where water is passively reabsorbed to reduce water loss in urine (Anderson and Braun 1985), likely explaining the lower water content found in the lumen of the lower gastrointestinal tract of desert birds (Amanova 1984). However, more

studies are needed to evaluate this hypothesis (Williams and Tieleman 2001). The avian gastrointestinal tract has a very important role in the regulation of overall fluid and electrolyte balance, and despite a lower renal urine concentrating ability compared to mammals (Braun 1997), allow them to be as good as mammals in conserving water during periods of high environmental temperature (Braun 2003, Laverty and Skadhauge 1999, McWhorter et al. 2009).

Some birds can also control cloacal evaporation as a means of thermoregulation at high ambient temperatures. This has been demonstrated in Inca doves, whose cloacal evaporation was negligible at 30, 35 and 40°C, but increased to 21.2% of total evaporative water loss at 42°C (Hoffman et al. 2007).

Skin

The avian skin has a well-vascularized dermal layer (Lucas and Stettenheim 1972) and increased cutaneous water loss can be achieved by vasodilating the dermal capillary bed (Peltonen et al. 2000). Conversely, the avian epidermis has the ability to secrete lipid-enriched organelles known as multigranular bodies during times of water deficit for rapid water-proofing (Menon and Menon 2000). The balance between losing enough water to decrease body temperature and maintaining sufficient hydration is the key to survival in times of heat stress (Williams and Tieleman 2001).

The skin, rather than the feathers, has been shown to be the primary barrier to cutaneous water loss in birds (Webster et al. 1985). Therefore, alterations of the composition of lipids in the skin and/or hydration of the stratum corneum can decrease skin vapour resistance to increase the efficiency of cutaneous water loss to maintain body temperature below lethal limits (Webster et al. 1985). Comparisons between the lipid composition of the epidermis of adult desert house sparrows and mesic house sparrows revealed that desert birds

had a higher amount of ceramides and cerebrosides and a lower percentage of cholesterol within the stratum corneum (Muñoz-Garcia and Williams 2005). A follow-up study also revealed that desert house sparrow nestlings showed a greater degree of plasticity in cutaneous water loss and lipid composition of the stratum corneum compared to mesic nestlings, possibly due to the increased exposure to environmental stressors (Muñoz-Garcia and Williams 2008). Similar differences were found between desert and mesic larks (Haugen et al. 2003). Desert sparrows also had lower cutaneous water loss, which can possibly be attributed to modifications in chain length and polarity of the sphingolipids that determine interactions among lipid molecules within the stratum corneum (Muñoz-Garcia and Williams 2005). Four southern hemisphere columbiform birds (Namaqua doves, laughing doves, Cape turtle doves and crested pigeons) have been shown to be able to dissipate heat very efficiently using cutaneous evaporation (McKechnie et al. 2016).

Respiratory tract

It has been suggested that desert animals could have more complex nasal turbinates to allow greater cooling of exhaled air, and therefore a larger reduction in respiratory evaporative water loss than non-desert animals (Schmidt-Nielsen et al. 1981). Some birds are able to reduce respiratory evaporative water loss by recovery of water in the nasal passages at moderate to low ambient temperatures, but this ability becomes insignificant at high ambient temperatures e.g. 45°C for crested larks (Tieleman et al. 1999). However, the results of that study did not support the hypothesis that desert birds have greater ability to recover water in the nasal passages compared to non-desert birds, because desert larks were found to not have the ability to reduce respiratory evaporative water loss by recovery of water in the nose (Tieleman et al. 1999). More studies directly comparing phylogenetically similar desert and non-desert birds are needed to confirm this finding.

Small passerine birds have high mass-specific metabolic rates and produce heat internally at a high rate (Dawson 1982). Coupled with their high respiratory rate, this results in a high rate of pulmonary water loss (Dawson 1982). Many other avian species including verdins (Wolf and Walsberg 1996a) and nightjars (O'Connor et al. 2017), have also been shown to rely heavily on respiratory evaporative water loss to maintain body temperature when exposed to heat. While this helps to prevent their body temperature from rising to lethal levels, it also increases the need for water. Maintaining hydration thus becomes a problem during extended periods of high environmental temperature (O'Connor et al. 2017) . Parrots can utilise gular fluttering in conjunction with panting to increase the efficiency of heat loss while panting (Bucher 1981, Weathers and Schoenbaechler 1976, Weathers and Caccamise 1975, McWhorter et al. 2018).

Differences in Heat Tolerance Limits Amongst Different Avian Orders

As seen from the examples above, different avian species, or different populations of the same species, can have different anatomical and physiological adaptations that result in differences in osmoregulation and thermoregulatory capacity. These differences can account for their ability to cope with high ambient temperatures in the desert, i.e. heat tolerance limit.

The heat tolerances of desert birds have been studied historically. For example, the spinifex pigeon (*Geophaps* plumifera) has been found to become hyperthermic when ambient temperature exceeds 40 °C and the species can dissipate more than the metabolic heat produced via evaporative heat loss to prevent further hyperthermia (Withers and Williams 1990). Studies on psittacines at high environmental temperatures also revealed their physiological responses to prevent hyperthermia (Weathers and Caccamise 1975, Weathers and Schoenbaechler 1976, Dawson 1982, Greenwald et al. 1967). However, these historical

studies did not establish the ambient temperature at which each species lose the ability to maintain body temperature below lethal limits, i.e. the heat tolerance limit. A recent series of studies performed in deserts across the globe using the same methodology revealed differences in heat tolerance limits, and therefore inferences about function and adaptation, amongst different taxonomic orders of birds (McWhorter et al. 2018).

Body mass (McKechnie and Wolf 2010) and the relative contribution of respiratory and cutaneous evaporative water loss (Whitfield et al. 2015) appear to be important factors determining the upper thermoregulatory limits in desert birds. In general, Passeriformes (Wolf and Walsberg 1996a, Whitfield et al. 2015, McKechnie et al. 2017) and Psittaciformes (McWhorter et al. 2018) rely on panting, Caprimulgiformes on gular fluttering (Talbot et al. 2017, O'Connor et al. 2017) and Columbiformes on cutaneous evaporative loss (Marder and Arieli 1988, McKechnie and Wolf 2004, Withers and Williams 1990). It is interesting that the heat tolerance limits for each avian order corresponds to the dominant method of evaporative water loss, i.e. studies performed on Australian desert birds revealed that the two orders reliant on panting have the lowest heat tolerance limits [T_a = 44 – 55°C for Psittaciformes (McWhorter et al. 2018), T_a = 46 – 54°C (McKechnie et al. 2017) for Passeriformes], whereas the two orders reliant on gular fluttering or cutaneous evaporative water loss have higher heat tolerance limits [T_a = 52 – 62°C for Caprimulgiformes (O'Connor et al. 2017, Talbot et al. 2017), T_a = 56 – 62°C for Columbiformes (McKechnie et al. 2016)]. However, this is not surprising as panting has the higher energetic costs compared to gular fluttering and cutaneous water loss (Calder and Schmidt-Nielsen 1968, Dawson 1982). Psittaciformes can also combine lingual fluttering with panting (Bucher 1981, Bucher 1985), but no studies have been performed to determine if this increases the efficiency of respiratory evaporative loss (Smit et al. 2018).

The phylogenetic distribution of the reliance on different modes of evaporative water loss, and therefore the functional significance and environmental selective pressures, is currently poorly understood (Smit et al. 2018). There is some evidence that the environmental selective pressure may play a part in the evolutionary development of high heat tolerance limits. For example, Australian desert Passeriformes had a markedly lower heat tolerance than African desert Passeriformes, which corresponded with a historically higher mean maximum in the Kalahari Desert compared to the Gluepot Reserve in Australia (McKechnie et al. 2017). Also, these differences in heat tolerance limits may result in differences in the behaviour and microsite selection of desert birds (McWhorter et al. 2018), but this has not been studied in Australian desert birds before.

Heat Illnesses

An inability of the body to cope with extreme temperatures will eventually lead to heat illness. Heat illnesses are most well-studied in humans, and are classified as heat cramps, heat exhaustion and heat stroke according to the increasing severity of symptoms (Leon and Kenefick 2012). While the exact pathophysiology of heat stroke in birds is not as well-studied, Figure 1 summarizes the pathophysiology of heat stroke in humans. Whether the same cascade of events occur in birds has not yet been determined, but given the effects of heat stress on intestinal integrity in chickens (Alhenaky et al. 2017, Cronjé 2005), as well as the beneficial effects gut protectants in alleviating the effects of heat stress in chickens (Deng et al. 2012, Abdelqader et al. 2017), it is likely that endotoxins have an important role in the long-term pathophysiological effects of heat illnesses. In desert birds, however, by the time they are found dead they have presumably gone through the cascade and suffered the effects of heat stroke. Studying naturally occurring avian heat illnesses is difficult because mass die-

off events of wild birds may not be discovered by humans until sometime later, and tissue autolysis is usually advanced at the time of discovery.

Studies of avian heat illnesses in the laboratory under controlled conditions have not been performed to date, although mammalian studies have been done. One study in baboons suffering from induced heat stroke demonstrated damage to multiple organs including the jejunum, liver, spleen, lung and kidney; and this manifested as vascular congestion, haemorrhage, thrombosis, increased inflammatory cells, and disruption of normal cell and tissue architecture (Roberts et al. 2008). In another report, the pathological changes of a bird that was suspected to have suffered heat stroke under general anaesthesia revealed mild multifocal acute degeneration and contraction band necrosis of the biceps femoris muscle, as well as diffuse moderate acute congestion of the lungs (Hofmeister 2005). There was also a diffuse moderate chronic left ventricular hypertrophy, but that was suspected to be a coincidental finding that contributed to the bird's death rather than a result of the presumed heat stroke (Hofmeister 2005). Further studies into the pathophysiology of heat stroke in birds and more pathology reports from such cases will help enhance our understanding of the syndrome.

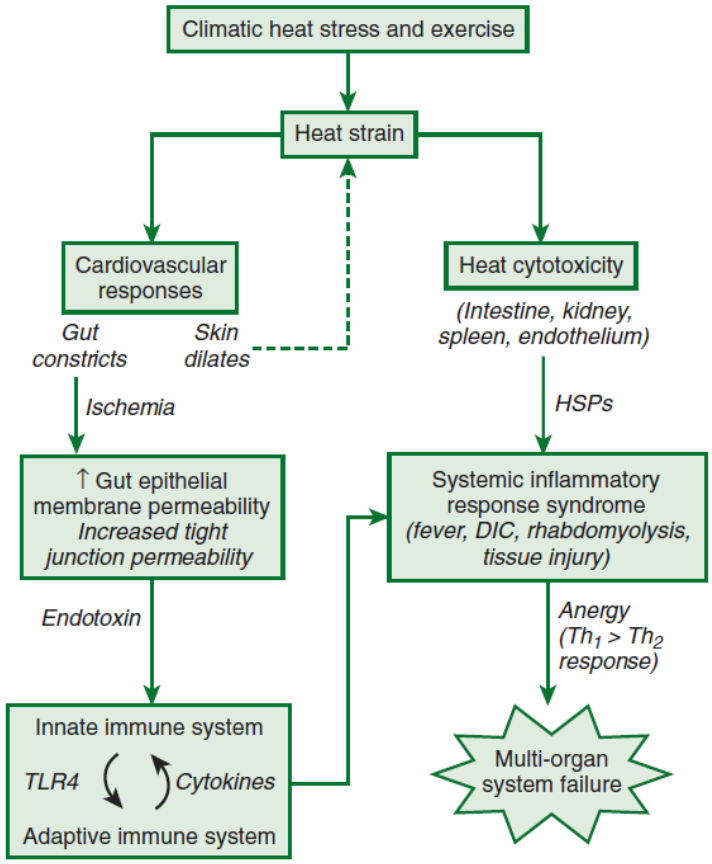

Figure 1. The pathophysiology of heat stroke in humans. During heat stress, cutaneous blood flow increases while blood flow to internal organs such as the gastrointestinal tract decreases to increase heat dissipation to the environment. The resultant gut ischemia increases permeability and leakage of endotoxin into systemic circulation. Endotoxin stimulate proinflammatory and antiinflammatory cytokine production via toll-like receptors (TLR4). Heat also stimulates the secretion of heat shock proteins (HSPs) that interact with cytokines and other proteins to mediate the systemic inflammatory response syndrome (Leon and Kenefick, 2012). Proinflammatory (Th_1 type) and antiinflammatory (Th_2 type) cytokines stimulate immune cells during progression of SIRS. Anergy results when there is inadequate Th2 cytokine production late in SIRS. Increased human patient mortality from peritonitis is associated with an inadequate Th2 cytokine response (Hatada et al. 2005).

Heat stress in poultry

It is perhaps not surprising that most of the research regarding heat stress in birds has been done in the poultry industry given its implications for production. The effects of heat stress in poultry include haematological changes such as decreased haematocrit, increased heterophil to lymphocyte ratio and basophil proportion (Altan et al. 2003, Mashaly et al. 2004); biochemical changes such as decreased calcium and phosphorus; decrease in egg quality and quantity; increased mortality rate; and adverse effects on semen characteristics and sperm function (Ayo et al. 2011). Acute heat stress has been shown to cause oxidative damage to cells (Altan et al. 2003, Mujahid et al. 2007), and influence expression of avian uncoupling protein (Mujahid et al. 2007). Heat stress also results in increased fearfulness and tonic immobility (Altan et al. 2003). Chronic heat stress has also been shown to cause pathological organ changes in broilers including right atrial and ventricular hypertrophy, myofibrillar degeneration and haemorrhage, diffused myocarditis and general fatty degeneration of myofibres of the heart; pulmonary congestion, oedema and hyperemia; yellow and pale livers with fatty degeneration of hepatocytes and sinusoidal dilation; oedema and haemorrhage in the subrenal capsule with glomerular damage and fatty degeneration of the kidneys (Aengwanich and Simaraks 2004). Some of these changes appear to be similar to those found in the previously mentioned acute heat stroke case. Although the studies done in poultry are helpful in aiding our understanding of the effects of heat stress in birds, more work needs to be done specific to desert birds because most of the poultry studies set the upper limit of temperature exposure at less than 35°C, which is representative of the summer temperatures poultry may be exposed to on a farm (Mashaly et al. 2004, Puthpongsiriporn et al. 2001), and do not include temperatures above 40°C, which desert birds frequently experience. Moreover, poultry species belong to the order Galliformes, whereas most desert

birds belong to other orders. Poultry species are also highly selected and potentially inbred to perform very specific functions such as rapid muscle growth and egg laying.

Role of the neuroendocrine system

Overview

Thermoregulation is controlled by the central nervous system after integration of thermal inputs via afferent neural pathways from thermoreceptors in the skin, tongue and beak, as well as deep body thermoreceptors (Baarendse et al. 2007). The central nervous system then sends signals via effector pathways to cause autonomic, behavioural and hormonal changes to regulate body temperature (Baarendse et al. 2007).

These changes also affect energy and water regulation by changing activity level, metabolic rate and food and water intake. A major component of these changes appears to be the hormone corticosterone, which has physiological (Sapolsky et al. 2000, Ingle 1952, Munck and Náray-Fejes-Tóth 1992) as well as behavioural (Buttemer et al. 1991, Lohmus et al. 2006, Pravosudov 2003, Hodgson et al. 2007) effects that are discussed in further detail below. The role of thyroid hormones, on the other hand, appears to be less significant in the physiology of thermoregulation (McNabb and Fox 2003, McNabb 2006) with relation to heat stress and will be only briefly explored.

The relationships between the central nervous system, endocrine system and thermoregulation are summarized in Figure 2.

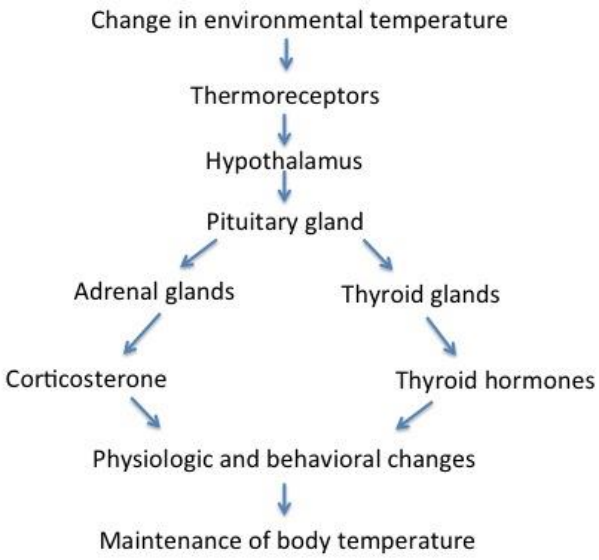

Figure 2. The relationships between nervous system, endocrine system and thermoregulation.

Neural control of thermoregulation

A stable body temperature is maintained by a thermoregulatory system consisting of thermo-, osmo- and baro-receptors that make up the sensory afferent and the neurological and endocrine responses that make up the efferent part (Yahav 2015). The integrating thermoregulatory centre is the hypothalamus which integrates signals from the spinal cord, brain, peripheral thermoreceptors and deep body thermoreceptors and acts as the control centre for thermoregulation by directing the appropriate physiological responses to temperature changes (Baarendse et al. 2007, Yahav 2015). The entire thermoregulatory process is summarized by Figure 3. The thermoregulatory set-point of the hypothalamus is therefore important in determining an individual's response to high ambient temperatures, e.g. determined by incubation temperatures.

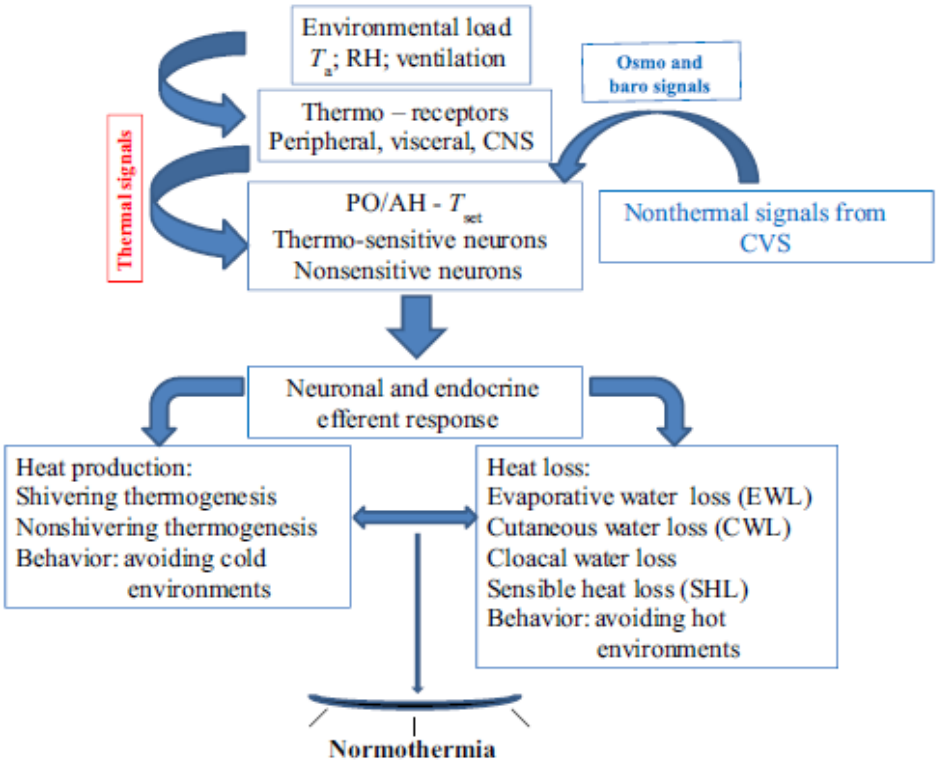

Figure 3. Flow chart summarizing peripheral inputs resulting in neuronal and endocrine responses to maintain normothermia (Yahav 2015). RH: relative humidity, CNS: central nervous system, CVS: cardiovascular system, PO/AH: preoptical area of the anterior hypothalamus.

Pre-natal high temperatures have been shown to decrease the proportion of warm-sensitive neurons and increase cold-sensitive neurons within the preoptical area of the anterior hypothalamus (PO/AH) region (Tzschentke and Basta 2002). The thermoregulatory set point also increases after pre-natal exposure to high temperatures, which is reflected by the strong influence of chronic changes in incubation temperatures at the end of embryonic development on the change in body temperatures of chicken and Muscovy duck embryos (Tzschentke 2007). The change in thermoregulatory set point may be caused by the development of synaptic contacts that would have otherwise not developed in the absence of

heat exposure (Tzschentke 2007). Heat stress during embryonic development also increased c-fos, an indirect marker of neuronal activity, expression in the pre-optical area of the anterior hypothalamus, but this increase was lower in temperature-experienced embryos (Tzschentke 2007), indicating that the response to heat stress is influenced by exposure to higher temperatures even at embryonic stages.

It was proposed that epigenetic factors, such as hormones, neuropeptides and cytokines, can possibly transmit information about the environment to the genome to cause changes in the genetic condition of the individual bird as summarized by Figure 4 (Tzschentke 2007, Tzschentke and Basta 2002). A study in zebra finches revealed that adult reproductive success and thermal preferences were influenced by exposure to acoustic signals emitted by parent birds during the developmental stage of embryos (Mariette and Buchanan 2016). These parent birds emitted specific acoustic cues when ambient temperatures were above 26°C and the offspring subsequently had better reproductive success and preferred breeding nests of higher temperature (Mariette and Buchanan 2016).

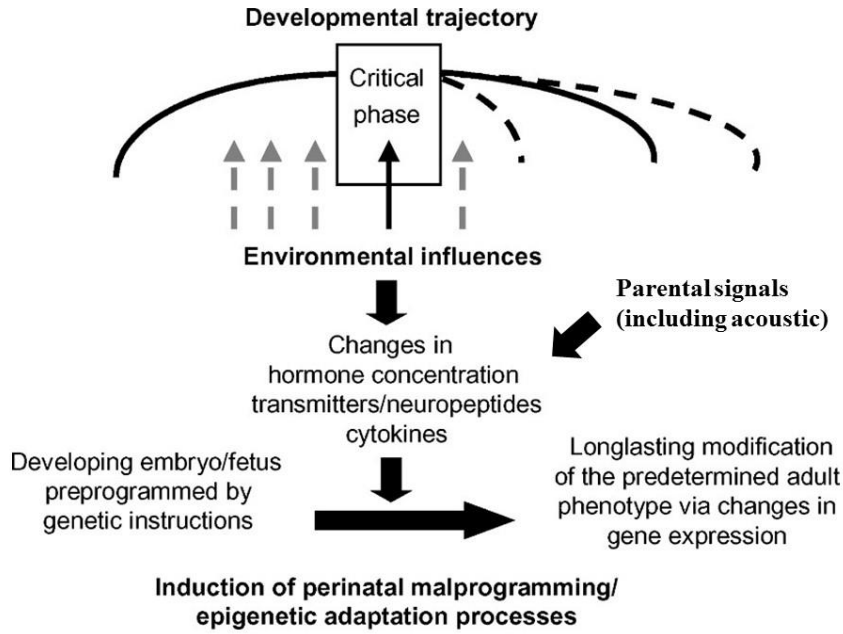

Figure 4. Induction of epigenetic adaptation processes such as epigenetic temperature adaptation by environmental factors during the critical period of early development (adapted from Tzschentke and Plagemann, 2006).

Once the thermoregulatory set point has been fixed, it will affect the thermoregulation of the birds later in life (Baarendse et al. 2007). Therefore, desert species may have a higher thermoregulatory set point that has been established very early in life, and what may be considered hyperthermia to a non-desert species may not be a problem for them.

Corticosterone

The Physiology of Stress

The 'stress' component of heat stress is an important part of the overall physiological response. Stress responses are neuroendocrine responses that occur after the sympathetic nervous system and hypothalamo-pituitary-adrenal (HPA) axis are activated and

glucocorticoids are secreted (Cockrem 2007, Romero and Wingfield 2016). Corticosterone is the main glucocorticoid hormone in birds, and has physiological and behavioural effects (Cockrem 2007, Romero and Wingfield 2016). The physiological effects of stress may not be restricted to the effects of glucocorticoids alone, and they include:

1) diversion of energy to muscle via mobilization of stored energy, gluconeogenesis and inhibition of energy storage

2) increased substrate delivery to muscle via increased cardiovascular tone

3) immune function alteration

4) inhibition of reproductive functions and behaviour

5) decreased appetite and food intake

6) improved cognition, cerebral perfusion rates and local cerebral glucose utilization

7) water retention through both renal and vascular mechanisms if there is fluid loss (Sapolsky et al. 2000).

However, because heat stress tends to last for more than ten minutes, glucocorticoids are likely to influence the physiology of heat stress more than catecholamines which are more involved during the acute phase (first few seconds) of the stress response (Sapolsky et al. 2000, Romero and Wingfield 2016). Chickens exposed to a high temperature environment for 2.5 hours had a rapid increase in plasma corticosteroid levels, peaking at slightly more than 1 hour of exposure before decreasing, a pattern similar to chickens injected with adrenocorticotropic hormone (Siegel 1980).

The effects of glucocorticoids can be classified as mediating, suppressive or preparative depending on the physiological endpoint in question (Sapolsky et al. 2000). Permissive actions are caused by glucocorticoids present before the stressor is encountered

and prepares the organism for a stress response; suppressive actions are a result of the rise in glucocorticoid levels induced by a stressor, usually more than an hour after onset of the stressor, and they prevent over-reactions to the stressor; stimulating actions are also a result of the rise in glucocorticoid levels post-stressor but they enhance the initial reactions to the stressor (Sapolsky et al. 2000, Munck and Náray-Fejes-Tóth 1992, Romero and Wingfield 2016). Animals with a basal level of glucocorticoid is more likely to survive than animals with no glucocorticoids, indicating that permissive glucocorticoid actions are important in priming some of the body's homeostatic defence mechanisms (Ingle 1952, Sapolsky et al. 2000). In the case of heat stress, permissive glucocorticoid actions could be important in priming the body's thermo-, energy and water regulatory functions.

Not all stimuli have to be stressors, even if they still illicit a sympathetic nervous system and behavioural response, but once the HPA axis is activated, a stress response can be expected (Figure 5) (Cockrem 2007). Therefore, a high temperature that does not activate the HPA axis in a desert bird may merely be a stimulus and not a stressor. To date, there have not been any studies investigating the temperatures at which desert birds start secreting corticosterone, i.e. initiate a stress response to heat. Stressors under similar physiological conditions within a species should produce similar magnitudes of glucocorticoid secretion, but physiological factors, such as the degree of control over the noxious stimulus (in the case of heat stress, it may be the animal's ability to thermoregulate via physiological or behavioural means), may alter glucocorticoid secretion (Romero et al. 1998, Romero and Wingfield 2016). Corticosteroid release induced by stress appears to be resistant to negative feedback effects (Romero et al. 1998, Romero and Wingfield 2016).

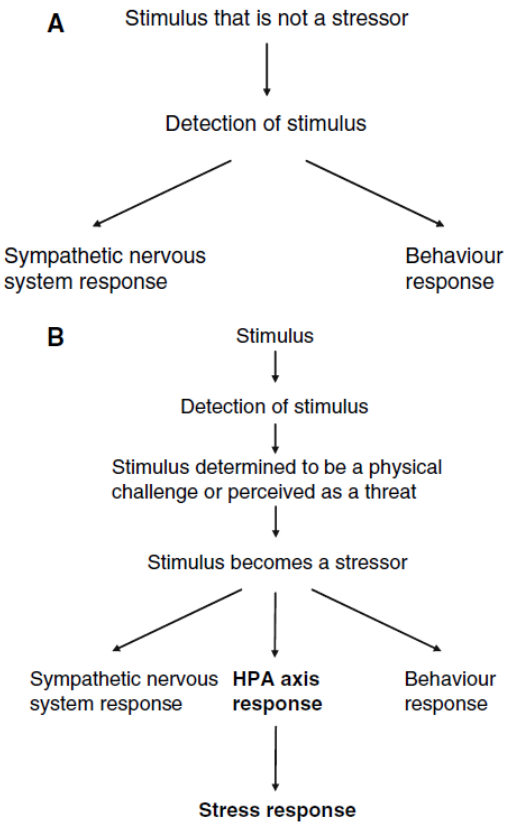

Figure 5. The difference between a bird's response to stimuli (A) and stressors (B) (Cockrem 2007).

The Relationship between Weather and Corticosterone Response

Birds of the Sonoran Desert in North America have been shown to be able to decrease their normal adrenocortical responses to stress so that they can breed during summer, when access to water is restricted (Wingfield et al. 1992). The stress response then returns to normal in winter (Wingfield et al. 1992). This ability to adjust the adrenocortical response to stress, however, varies with species (Wingfield et al. 1992, Romero and Wingfield 2016). The change in response was most pronounced in black-throated sparrows (*Amphispiza bilineata*), cactus wrens (*Campylorhynchus brunneicapillus*) and curve-billed thrashers (*Toxostoma curvirostre*), and less in Abert's towhees (*Pipilo aberti*) and Inca doves

(*Columbina inca*) (Wingfield et al. 1992). The latter two species are found in riparian habitats with water and shade always accessible, whereas the former three species are usually found in the open desert with restricted access to water (Wingfield et al. 1992).

In contrast, there were no such seasonal difference in snow buntings (*Plectrophenax nivalis*) in Barrow, Alaska (Smith et al. 1994). However, in this study, the adrenocortical responses were found to be lower in magnitude in general for snow buntings and Lapland longspurs (*Calcarius lapponicus*) compared to other species of birds and higher fat deposits were also correlated with a decreased responsiveness to stress (Smith et al. 1994). The correlation between fat scores and responsiveness to stress was not replicated by Romero et. al. (1998), so its importance and relevance remains to be determined. Cold climate species also appear to have a higher corticosterone response during adverse weather events e.g. storms when they are moulting, but not during the breeding season (Romero et al. 2000). Minor, rapid decreases in ambient temperature were also demonstrated to illicit a stress response in European starlings (*Sturnus vulgaris*) (de Bruijn and Romero 2011). Rock doves (*Columba livia*) exposed to both cold and heat for 90 minutes also demonstrated significant increases in serum corticosterone (Pilo et al. 1985).

Given the evidence that some birds appear to be able to alter their adrenocortical response to stress in accordance with the weather and season, and with the relationship between corticosterone and behaviour as demonstrated in other sections of this paper, desert species may be have different adrenocortical responses to stress during different weather conditions which may regulate their behaviour to achieve the best balance between thermoregulation, energy metabolism and water metabolism to aid their survival during the harshest of conditions.

The Effects of Corticosterone on Behaviour

Corticosterone appears to have no direct effect on the resting metabolic rates of Gambel's white-crowned sparrows (*Zonotrichia leucophrys*) and pine siskins (*Spinus pinus*), both small passerine birds, but can reduce the behavioural response of these birds to stimuli, promoting restfulness at night (Buttemer et al. 1991).

Corticosterone has effects on other behaviours of birds, and these effects could hold the key to explaining how corticosterone has a role to play in helping desert birds survive the heat. Even seemingly innocuous behaviours such as yawning have been shown to increase in budgerigars when ambient temperature is increased, although evaporative cooling behaviours such as panting and wing venting appear to be more important for thermoregulation (Gallup et al. 2010).

Corticosterone has been shown to increase foraging intensity (Lohmus et al. 2006), food intake and caching behaviour, as well as increase spatial memory performance (Pravosudov 2003) in adult avian species. These changes in behaviour may serve as an adaptation to unpredictable environments such as the desert (Pravosudov 2003). The exact behavioural changes associated with corticosterone may vary with species. The increased spatial memory associated with increased corticosterone in mountain chickadees (*Poecile gambeli*) (Pravosudov 2003) was in contrast to an impairment of spatial ability in zebra finches with acute high corticosterone responses to stress (Hodgson et al. 2007). Mineralocorticoid receptor mRNA expression in the hippocampus was also reduced in these zebra finches (*Taeniopygia guttata*) which may protect neurons from the detrimental effects of stress (Hodgson et al. 2007), although there were no deleterious effects associated with prolonged moderate elevation of corticosterone on hippocampal anatomy and neurogenesis in mountain chickadees (Pravosudov and Omanska 2005). Wingfield et. al. (1992) found that

some birds activate stress responses, albeit to a reduced level than in winter, in summer during unusually intense heat and raised the possibility that a more chronic episode of intense heat, e.g. during a heat wave, could result in a larger stress response.

Developing birds also alter their behaviour according to corticosterone levels. Seabird chicks with high corticosterone levels during development displayed increased food intake, foraging behaviour and aggression, which may offset the low growth efficiency and possible compromised cognitive abilities later in life associated with corticosterone (Kitaysky et al. 2003, Bebus et al. 2016). Male zebra finches exposed to corticosterone during the nestling stage also displayed a decreased tendency to approach a novel object and had less success when competing for perches (Spencer and Verhulst 2007). Maternal corticosterone has also been demonstrated to be transferable to the yolk and results in a decreased growth rate (Hayward and Wingfield 2004), as well as increased stress response of the offspring (Hayward and Wingfield 2004, Spencer et al. 2009, Weber et al. 2018).

Therefore, it appears that adult phenotypes may be shaped by the conditions the bird was exposed to during development (Spencer and Verhulst 2007, Schoech et al. 2011). Birds, such as zebra finches, also appear to be able to respond evolutionarily to different environments by modifying the response of their physiological system to these environments, and corticosterone may play a role in this mechanism (Evans et al. 2006). Female zebra finches, one of the species found in Australian deserts, have been shown to favour mating with males from low corticosterone lines with the lowest corticosterone titers (Roberts et al. 2007). The desert environment may be exerting a selection pressure for individuals with lower adrenocortical response to stress because the behaviour of these individuals are more suited for maintaining the balance between energy, water and thermoregulation that is essential for survival.

The Link between Corticosterone and Survival During Heatwaves

It is apparent that corticosterone has an important role in the ability to cope with stressors in birds (Cockrem 2007), and heat stress is no exception. With its ability to trigger specific physiological (Sapolsky et al. 2000, Ingle 1952, Munck and Náray-Fejes-Tóth 1992) and behavioural (Buttemer et al. 1991, Lohmus et al. 2006, Pravosudov 2003, Hodgson et al. 2007) responses that can contribute to maintaining the balance between energy, water and thermoregulation, corticosterone could be one of the important determinants of a bird's ability to survive in times of heat stress. For example, the high heat tolerance limit of Columbiformes (McKechnie et al. 2016) may enable them to continue foraging during a heat wave, but the role of corticosterone in driving this foraging behaviour is unknown.

One of the few studies investigating corticosterone responses of birds in response to heat was done in fully feathered pigeons (*Columba livia*), which found an increase in serum corticosterone levels after exposure to 45°C for 90 minutes but no significant increase in serum corticosterone levels for pigeons that had feathers removed from their dorsum and breast (Pilo et al. 1985). However, the focus of this study was liver Na^+K^+-ATPase activity and the effects of handling on corticosterone were not taken into account. Other than that, the corticosterone responses of different species of birds to high temperatures have not been previously studied to establish a link between the heat tolerance limits and corticosterone responses of different bird species.

Exogenous corticosterone (Astheimer et al. 1992) and chronic elevation of endogenous corticosterone (Cyr et al. 2008) have also been suggested to decrease metabolism. An increase in corticosterone during a heatwave can therefore still offer species of birds with a lower heat tolerance, e.g. psittacine and passeriform birds, an advantage in

survival, when their ability to forage for food and water is restricted by the need to avoid hyperthermia.

Role and significance of heat shock proteins

Overview

Heat shock proteins (hsps) are a group of proteins synthesized by almost all organisms when exposed to heat or other stressors (Lindquist and Craig 1988). They are named according to their relative molecular masses (Lindquist and Craig 1988, Etches et al. 2008), e.g. hsp70 and hsp90 have relative molecular masses of 70000 and 90000 kilodaltons respectively. The major families of hsps are hsp100, hsp90, hsp70, hsp40, small hsps and chaperonins (De Maio and Vazquez 2013).

Proteins encoded by the hsp70 and hsp90 gene families are highly conserved, as is the genetic system itself (Lindquist and Craig 1988). Hsps can be constitutively expressed and present at normal temperatures with important roles in normal cell function (Lindquist and Craig 1988, Etches et al. 2008). Other heat shock proteins are inducible and are synthesized in response to a heat shock (Lindquist and Craig 1988, Etches et al. 2008). However, the constitutive and inducible nature of hsps could be species and tissue specific, as the normally constitutive hsp73 (also known as hsc70) was induced in camel lymphocytes after heat shock, but the normally inducible hsp72 failed to be expressed under the same conditions (Ulmasov et al. 1993). Subsequently, hsp72 was found to exist in the camel genome and can be activated in fibroblasts found in skin cells (Ulmasov et al. 1993). Hsp72 was also detected in the plasma of Holstein-Fresian dairy cattle under seemingly un-stressful conditions (Kristensen et al. 2004), and the authors concluded that even apparently healthy individuals may experience extrinsic and/or intrinsic stress. However, it may be possible that the

inducible vs constitutive nature of hsp72 in Holstein-Fresian cattle is not the same as in other species. In a study on Japanese Quail, increased expression of both constitutive and inducible forms of hsp70 as a response to stress was found only in myocardial tissues but not in lung, liver, kidney and gonads (Hoekstra et al. 1998).

Hsps act as molecular chaperones, interacting with other proteins to minimize the probability that other proteins will interact inappropriately with one another (Feder and Hofmann 1999). The rapid and intense nature of the induction of hsps indicates that it is an emergency response (Lindquist and Craig 1988). Optimal synthesis of hsps in chicken blood cells were obtained after incubation at 45^0C (Miller and Qureshi 1992, Morimoto and Fodor 1984) and Burdon (1986) concluded that the optimum temperature range of induction is about 40-50˚C for birds and mammals, similar to the body temperatures at which desert birds are expected to start experiencing problems physiologically (McKechnie and Wolf 2010).

The functions of hsps at the molecular and cellular level are well studied in the laboratory, and are beyond the context of this review. Whole bird studies usually involve poultry species. Chicken embryos exposed to higher incubation temperatures increase hsps 27, 60, 70, 90α, 90β genes and protein expressions (Vinoth et al. 2015). Chicken exposed to heat stress have also been shown to increase expression of hsps in their organs, including the testes (Wang et al. 2013), brain (Tamzil et al. 2013, Tu et al. 2016, Zhang et al. 2014), gastrointesintal tract (Varasteh et al. 2015, Gu et al. 2012, Hao et al. 2012, Liew et al. 2003), liver (Zhang et al. 2014, Mahmoud et al. 2004, Zhen et al. 2006, Lei et al. 2009, Yu and Bao 2008, Yan et al. 2009), muscle (Zhen et al. 2006, Zhang et al. 2014, Xie et al. 2014), kidney (Lei et al. 2009, Yu and Bao 2008) and heart (Yu and Bao 2008, Lei et al. 2009). Studies similar to the ones in domestic chicken have not been performed on other species of birds, particularly Australian desert birds.

HSP70

Hsp70 has a wide variety of functions at cellular, tissue, organ and organismal levels. It has several functions including increasing tolerance to hyperthermia and endotoxins, as well as reduction of protein denaturation from heat exposure in mammalian cells (Feder and Hofmann 1999). Hsp70 also has a role in modulating glucocorticoid receptor responses, but to a lesser extent than hsp90 (Grad and Picard 2007). They are thought to disrupt established interactions between proteins, possibly via ATP dependent mechanisms or by facilitating the establishment of alternative interactions between these proteins (Lindquist and Craig 1988). In these ways, they are able to protect heat-sensitive proteins from degradation or prevent damaged proteins from precipitating immediately after heat shock and affecting cell viability permanently (Etches et al. 2008).

Hsp70 was shown to have several protective effects in poultry exposed to heat stress. Feed restriction and heat conditioning at an early age in male broiler chickens resulted in higher hsp70 expression, as well as better heat tolerance and disease resistance (Liew et al. 2003). Hsp70 was induced in the gastric mucosal and epithelial cells of the jejunum and ileum in broilers exposed to acute heat stress (Hao et al. 2012). This induction of hsp70 was positively correlated with increases in amylase, lipase, trypsin and alkaline phosphatase activities, possibly helping with intestinal digestion and absorption during acute heat stress (Hao et al. 2012). Lactic dehydrogenase, a sensitive indicator of cell damage, increases in the jejunal mucosa during heat stress, but its level is decreased by hsp70 (Gu et al. 2012). Hsp70 was also positively correlated to antioxidant levels and negatively correlated to corticosterone levels in the jejunal mucosa after acute heat stress (Gu et al. 2012). It is possible that hsp70 could have similar protective effects in in desert birds exposed to heat stress.

As mentioned earlier, expression of hsp70 in myocardial tissues was increased in Japanese Quail (*Coturnix japonica*) in response to stress, but there was no such increase in all tissues of hsp30, hsp60 and hsp90 (Hoekstra et al. 1998). This may be an indication that hsp70 has the most promise as an alternative to corticosterone as a measure of stress in birds.

A study in lizards revealed a positive correlation between the temperature range of hsp68 (a member of the hsp70 family) synthesis induction and the average temperature of an ecological niche inhabited by the species (Ulmasov et al. 1992). The same study also found that temperature elevation induced the synthesis of major hsps, reaching a maximum at critical temperatures when hsps became the major proteins synthesized in the cell while the synthesis of normal cellular proteins drops (Ulmasov et al. 1992). Any further increase in the temperature resulted in a coma-like state from which the lizards did not recover (Ulmasov et al. 1992). No such studies have been done in birds and the results could be interesting. Evgen'ev et al. (2007) went on to postulate that xeric lizard species synthesize constitutive hsps under normal physiological conditions because of the presence of activated HSF in the cells, while northern lizard species that are exposed to high temperatures less frequently do not normally synthesize hsps, but, instead, contain a high concentration of HSF in their cells enabling them to rapidly switch on the battery of the hsp genes in response to slight temperature fluctuations.

HSP90

Hsp90 is involved in the tolerance of hyperthermia and glucocorticoid receptor function amongst other functions in mammalian cells (Feder and Hofmann 1999). It appears to keep steroid receptor complexes inactive until the proper signal for activation is received (Lindquist and Craig 1988). It plays crucial roles in glucocorticoid receptor folding, hormone

binding to glucocorticoid receptors, transport of glucocorticoid receptors to the nucleus, activation of transcription in the nucleus, nuclear retention of glucocorticoid receptors and degradation of glucocorticoid receptors after their functions have been served (Grad and Picard 2007). Hsp70 may have more major roles in glucocorticoid receptor folding and degradation, but hsp90 is the major molecular chaperone for the other processes (Grad and Picard 2007).

Vertebrates should have both α and β forms of hsp90, and due to the fact that hsp90β was not induced by heat in chickens, it was suggested that hsp90α is both constitutive and inducible, but hsp90β is strictly constitutive in chickens (Meng et al. 1993). This is in contrast to other vertebrate species where both forms of hsp90s are inducible by heat shock (Meng et al. 1993) but it is unknown if this would apply to all avian species.

In studies of hsp levels in rat tissues after parasitization by *Trichinella spiralis,* hsp90 levels remained the same in brain and muscle, but decreased in the liver and spleen (Martinez et al. 1999b, Martinez et al. 1999a). In contrast, hsp25, hsp60 and hsp70 levels increased in the various rat tissues depending on the infection cycle (Martinez et al. 1999b, Martinez et al. 1999a). The significance of this difference is unknown, but given hsp90's key roles in the functions of glucocorticoid receptors, it may be possible that it is regulating the response of individual organs to increased levels of circulating glucocorticoids in response to parasitization. It would not be far-fetched to hypothesize that hsp90 could have a similar role in the face of increased corticosterone release during a heat stress event in birds.

Genetic regulation of heat shock proteins

Zatsepina et. al. (2000) did not find any significant differences in hsp70 gene copy numbers in lizard species with different levels of thermotolerance. Therefore, it is likely that

the difference in hsp70 responses to temperature changes is attributable to phenotypic and epi-genetic factors. It has been postulated that the hsp gene promoters represent areas for transposition of mobile elements that may create significant and distinctive variations in hsp genes and, therefore, play an important role in the evolution of this system (Walser et al. 2006).

Switching off expression of one or two members of the hsp70 family may in some cases be adaptively advantageous because the expression level of hsps in each species and geographical population is a balance between the benefits and negative impact of hsps overexpression on growth, fertility and other characteristics (Sørensen et al. 2003). This has been documented in *Drosophila* (Zatsepina et al. 2001), and studies in Pied Flycatchers also suggested that increased synthesis of hsps decreases humoral and cell-mediated immune responses (Morales et al. 2006).

HSPs in Wild Birds

Hsps have been gathering the interest of ecophysiologists as another measure of stress in wild birds (Herring and Gawlik 2007). Hsp70 in particular have been used as a stress indicator in different species of birds exposed to different stressors, e.g. lead exposure in great tits (*Parus major*) (Ruuskanen et al. 2015), increased brood size and ectoparasite infection in great tits (Wegmann et al. 2015) and salinity and temperature in red knots (*Calidris canutus*) (Gutiérrez et al. 2015). Interestingly, hsp70 was not found to be affected by these conditions in these species of birds. The effects of high environmental temperatures on hsps in desert birds have not been studied to date.

Conclusions

Desert birds are faced with the daily challenges of energy and water regulation in a resource-poor environment. When ambient temperatures increase beyond a certain point, maintaining body temperature below lethal levels becomes part of the physiological challenge. If desert birds are unable to maintain the balance among energy, water and thermoregulation, they will suffer from heat illnesses, culminating in heat stroke and death. A recent study by Iknayan and Beissinger (2018) found that the collapse of a desert bird community over the past century in the Mohave Desert of the western United States was driven by climate change. These authors found that the decline in precipitation (and thus the availability of surface water) was the most important driver of bird persistence. Current literature reveals differences in heat tolerance limits among birds belonging to different orders (McKechnie et al. 2016, McKechnie et al. 2017, Talbot et al. 2017, McWhorter et al. 2018), but the differences in stress responses, behavioural adaptations, pathological changes and genetic adaptations among bird species in response to heat exposure are not well characterised. This thesis aimed to investigate these differences, so that groups of birds that are more vulnerable to increasing temperatures as a result of climate change can be more easily identified (Garnett and Franklin 2014) and conservation efforts focused on them.

The neuroendocrine system plays an important role in the development of a thermoregulatory set-point early in a desert bird's life, and continues to play a part throughout its life cycle. In particular, the response of a desert bird to stressors, including heat, plays an important role in determining how it behaves to maintain the balance among energy, water and thermoregulation. Further research is required to investigate the contribution of genetic, phenotypic and epigenetic factors in a bird's adrenocortical response to stress. In particular, the corticosterone response of desert birds to high temperatures has not been well studied, even though corticosterone is likely to have important roles in regulating

the physiological and behavioural changes that enable desert birds to survive during heatwaves. Wingfield et al. (1992) measured corticosterone levels in birds in the Sonoran Desert, including samples collected during a heatwave, but these responses were only measured in a few species at limited time points. Stress responses to heat exposure in wild birds have not been characterised under controlled laboratory conditions. There may be important differences in corticosterone responses amongst desert bird species or taxonomic orders that determines whether physiological or behavioural changes are their primary method of surviving heatwaves. Chapter 2 of this thesis characterises the stress mediator (corticosterone and leukocyte) responses of three major orders of Australian desert birds using budgerigars, zebra finches and diamond doves as representatives of Psittaciformes, Passeriformes and Columbiformes birds, respectively (Xie et al. 2017a). This study was performed under controlled laboratory conditions, comparing the stress mediator responses of these bird species when acutely exposed to temperatures simulating a heatwave with that when exposed to temperatures simulating a normal summer day.

Given the effects of corticosterone on behaviour (Astheimer et al. 1992, Pravosudov 2003, Hodgson et al. 2007, Lohmus et al. 2006) and different heat tolerance limits amongst bird species (McKechnie et al. 2016, McKechnie et al. 2017, Talbot et al. 2017, McWhorter et al. 2018), there may also be interesting differences in the way bird species or orders modify their behaviour when exposed to heat. Studies on behavioural adaptations to heat have not previously been undertaken on native Australian bird species in zoo captivity. As temperatures continue to increase with climate change, bird species with higher behavioural plasticity may be able to better adjust their time budgets and microsite selections to avoid exposure to lethal temperatures. On the other hand, bird species that do not have the ability to adjust their behaviour in accordance with rising temperatures may have to rely more on physiological thermoregulatory mechanisms. Chapter 3 investigated the temperature

dependency of time budgets, microsite selections and thermoregulatory behaviours in captive Australian desert birds by quantifying the amount of time they spent on different behaviours, including thermoregulatory ones, and in different microsites (Xie et al. 2017b). Eight species of birds representing the orders Psittaciformes, Passeriformes and Columbiformes various sizes and housed within four different outdoor aviaries of Adelaide Zoo were included in this study.

Heat illnesses in birds are not well studied nor documented. Species differences in the pathology that results from heat illnesses, particularly amongst desert birds with different mechanisms of thermoregulation, are currently unknown. Chapter 4 describes the organ and haematological changes of the same bird species under the same conditions of mild and moderate acute heat exposure as in Chapter 2, as a representation of possible antemortem and post-mortem signs to look for in birds exposed to mild heat. Chapter 5 takes this a step further to describe physiological, organ and biochemical changes in galahs and rock doves when heatstroke was induced by exposure to more extreme heat under general anaesthesia.

Current literature also confirms that there is a role for heat shock proteins (hsp) in protecting a bird against the damaging effects of heat stress and possibly regulating the corticosterone response (Feder and Hofmann 1999, Lindquist and Craig 1988). The upregulation of hsp70 and hsp90 in particular may confer adaptive advantages to a bird living in the desert (Ulmasov et al. 1992). Although hsps have been studied in detail under different contexts (Martinez et al. 1999b, Martinez et al. 1999a, Merino et al. 2002, Morales et al. 2006, Merino et al. 2006, Moreno et al. 2008), there are no studies correlating hsps with the ability of desert birds to survive under high environmental temperatures. Further investigation of the contribution of genetic, phenotypic and epigenetic factors in upregulating these hsps may give us further insight into the evolutionary journey of desert birds in acquiring physiological specializations enabling to survive in the resource-poor environment. Different

species of desert birds may also express hsps differently in different organs, depending on which organs are more prone to damage during heat stress. The genetic adaptations in different organs, including expression of hsps, of Australian desert birds that may confer protection against exposure to high temperatures are identified in Chapter 6 (Xie et al. in press) using high-throughput qPCR to measure mRNA expression in the liver, lung, kidney and gastrointestinal tract of the same bird species under the same acute heat exposureconditions as Chapter 2.

Chapter 7 then concludes these findings to guide future research and provide applicable outcomes from the studies.

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Chapter 2 Stress responses to heat

Corticosterone (CORT) is the main stress hormone in birds, and influences both the physiology and behaviour of birds when exposed to stressors. The following original research article, published in the journal *Physiological and Biochemical Zoology*, presents the CORT and heterophil:lymphocyte ratio responses in 3 species of Australian desert birds and highlights the differences in these species that may be important in explaining their different physiological and behavioural adaptations.

Xie, S., L.M. Romero, Z.W. Htut and T.J. McWhorter. 2017. Stress responses to heat exposure in three species of Australian desert birds. *Physiological and Biochemical Zoology* 90(3):348-358 (doi:10.1086/690484).

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Contribution to the Paper	Performed CORT analysis on all samples, verified H:L ratio data, interpreted data, wrote manuscript and acted as corresponding author.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	L. Michael Romero		
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Contribution to the Paper	Supervised development of work, helped in data interpretation and manuscript evaluation.
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Stress responses to heat exposure in three species of Australian desert birds

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Abstract

Birds need to respond to weather changes quickly and appropriately for their own well-being and survival. The inability to respond appropriately to heatwaves can be fatal to individual birds and translate into large-scale mortality events. Corticosterone (CORT) and heterophil:lymphocyte (H:L) ratio responses of budgerigars (*Melopsittacus undulatus*), zebra finches (*Taeniopygia guttata*) and diamond doves (*Geopelia cuneata*) to heat exposures were investigated. The birds were exposed to a temperature similar to what they experience during a typical summer day (35 ^{0}C) and a higher temperature (45 ^{0}C) similar that experienced during a heat wave. There were no significant increases between the CORT concentrations before and after heat exposure in zebra finches and budgerigars at 35 ^{0}C and 45 ^{0}C, but there was a significant increase in CORT concentrations in diamond doves after exposure to 45 ^{0}C. The H:L ratios increased significantly after heat exposure in budgerigars at 35 ^{0}C and 45 ^{0}C, and diamond doves at 35 ^{0}C. No significant correlation was found between the change in CORT and H:L ratios. The data suggest that there are species differences in birds' stress responses to heat exposure that may reflect their ability to detect and adapt to high temperatures. There appears to be differences between the two types of stress measurements which may reflect differences in the timescales of these responses.

Keywords

Stress response; corticosterone; heat stress; climate change; heterophil lymphocyte ratio

Introduction

Birds live in a constantly changing environment where the availability of resources such as food and water, the presence of predators and weather conditions vary from one moment to the next. There can be some predictability to some of these changes, e.g. seasonal availability of food and water, but others such as inclement weather can change quickly and unpredictably. Birds are particularly vulnerable to the latter (Newton 1998), and desert birds in particular are faced with the prospect of heatwaves that are becoming more frequent and lasting longer as a result of climate change (Perkins et al. 2012).

Birds need to respond to these environmental threats quickly and appropriately for their own well-being and survival (de Bruijn and Romero 2011). The inability to respond appropriately to additional challenges, such as heatwaves, can be fatal to individual birds and this can translate into large-scale mortality events such as those reported in the popular press in Australia (PerthNow 2009a, b, 2010), South Africa (Eramus 2010) and India (news.oneindia.in 2010; newzstreet 2010). Historically, Finalyson et al. (as cited in Wyndham, 1981) also estimated that in 1932, 60 000 budgerigars died near a dam during a heatwave. However, Wyndham (1981) did not observe any deaths of budgerigars himself during a heatwave in 1971, during which the maximum temperatures exceeded 40°C for five consecutive days and the highest temperature recorded was 45°C. The response can include either or both of behavioural and physiological adjustments. Desert birds already struggle to meet their daily energy requirements under normal desert conditions due to their high metabolic rate and relative low food availability(Dawson and Schmidt-Nielsen 1964; Dawson and Bennett 1973; Williams and Tieleman 2001).

Physiological responses to stressors such as heat include the stress response consisting of two pathways (Romero and Wingfield 2016; Sapolsky et al. 2000): the faster fight-or-flight

response, regulated by the sympathetic-adrenal-medulla (SAM) axis that reacts within seconds of the stressor (Cannon 1914), and the slower hypothalamic-pituitary-adrenal (HPA) axis that has latent effects that take 30 to 60 minutes (Haller et al. 2008) to manifest themselves and last up to hours after encounter with the stressor (Rivier and Vale 1983). The HPA axis exerts its effects through the release of glucocorticoids (Rivier and Vale 1983) and corticosterone (CORT) is the major glucocorticoid in birds (Holmes and Phillips 1976). However, Lohmus et al. (2006) found that CORT treatment increased foraging intensity (Lohmus et al. 2006) in red-eyed vireos (*Vireo olivaceus*) , while Pravosudov (2003) found that long-term moderate elevation of CORT increased food intake, and food caching behaviour in mountain chickadees (*Poecile gambeli*) , so there may be species differences in the behavioural effects of glucocorticoids. Behaviour and CORT changes can occur independently of each other, as determined by the context of the stressor (Nephew and Romero 2003).

In general, stressors will activate the HPA axis (Cockrem 2007). A high temperature that does not activate the HPA axis in a desert bird could still activate the SAM. There has been extensive work done on the endocrinology of desert birds (Deviche et al. 2014; Deviche et al. 2012), but little work on responses to temperature. Although the ability of some desert bird species to regulate their stress response to continue breeding through severe heat has been studied (Wingfield et al. 1992), to date, there have not been any studies investigating the temperatures at which different species of desert birds start secreting corticosterone, i.e. initiate a stress response to heat. One of the few studies investigating CORT responses of birds in response to heat was done in fully feathered pigeons (*Columba livia*) found an increase in serum CORT levels after exposure to 45 ^{0}C for 90 minutes but no significant increase in serum CORT levels for pigeons that had feathers removed from their dorsum and

breast (Pilo et al. 1985). However, the focus of the study was liver Na^+K^+-ATPase activity and the effects of handling on CORT were not taken into account.

Prolonged activation of the HPA axis can result in chronic stress, which is considered maladaptive as opposed to acute stress (Sapolsky et al. 2000). Stress causes an increase in the number of heterophils, i.e. heterophilia, and a decrease in the number of lymphocytes, i.e. lymphopaenia, resulting in an increase in heterophil:lymphocyte (H:L) ratios (Gross and Siegel 1983; Harvey et al. 1984). H:L ratios have been shown to increase in 35-36 day old broiler chickens exposed to an ambient temperature of 38± 1^0C for three hours (Altan et al. 2003) and also in 31 week old laying hens exposed to constant 35^0C for 5 weeks (Altan et al. 2003; Mashaly et al. 2004). H:L ratios have also been shown to increase in response to a wide variety of stressors in wild birds, such as migration, transport, parasitic infection (Fokidis et al. 2008) and radioactive contamination (Davis et al. 2008), but there have been no studies specifically assessing the effects of heat exposure on H:L ratios of Australian desert birds.

The effect of handling time on H:L response is less well-understood than that for CORT response. CORT levels begin to increase 3 minutes after a disturbance (Romero and Reed 2005), and return to baseline levels 2 hours after the removal of the disturbance (Dickens et al. 2009; Nephew and Romero 2003). However, the time-scale of H:L responses to disturbances appear to vary. House finches (*Carpodacus mexicanus*) were not affected by routine handling times lasting less than 60 minutes (Davis 2005), whereas Great Tits (*Parus major*) showed an increase in H:L ratios 60 minutes and 120 minutes after they were captured (Cīrule et al. 2012). Cīrule et al. (2012) also found that the increase in H:L ratio due to handling stress was due to a combination of increased heterophil counts, which occurred between 30 and 60 minutes after capture, and decreased lymphocyte counts, which occurred between 60 and 120 minutes after capture. There have been no previous studies investigating

the correlation of the H:L response to the CORT response of birds after an acute heat exposure.

In this study, the CORT and H:L ratio responses to heat exposure were investigated. It was hypothesized that exposing native Australian birds whose natural habitat includes arid environments to a temperature similar to what they experience during a typical summer day (35 ^{0}C) would not result in increases in CORT and H:L ratios; whereas exposure to a higher temperature (45 ^{0}C) similar that experienced during a heat wave would result in increases in CORT and H:L ratios. It was also hypothesized that given the upper critical limit of thermoneutrality of passerine and psittacine birds are lower compared to columbiform birds (McKechnie et al. 2016; Smith et al. 2015; Whitfield et al. 2015; Wolf 2015), zebra finches and budgerigars would have a larger increase in CORT and H:L ratio than diamond doves when exposed to 45 ^{0}C.

Materials and methods

Animals

Eight each of captive-bred budgerigars (*Melopsittacus undulatus*) (2 male and 6 females), zebra finches (*Taeniopygia guttata*) (3 male and 5 females) and diamond doves (*Geopelia cuneata*) (5 males and 3 females) were used as model species for the bird orders Psittaciformes, Passeriformes and Columbiformes respectively. These species were chosen because their wild geographic ranges include the Australian desert, and were also widely bred in captivity for the pet industry.

The birds were obtained from private bird breeders in Adelaide during the austral summer of 2014-2015. The exact rearing conditions of the birds were not known, but they were all from

captive bred lines of each species, and raised in outdoor aviaries in metropolitan Adelaide, thereby being subjected to the same environmental conditions before being transferred to outdoor aviaries at the University of Adelaide Roseworthy Campus (Roseworthy, South Australia, latitude -34.53397°, longtitude 138.75023°). The birds had commercial bird seed mix consisting of white French millet, panorama millet, panicum, sorghum, canola seed, canary seed, dehulled oats, Japanese millet, Shirohie millet, wheat, red millet, linseed and shell grit; and water available *ad libitum* in the aviaries. They were allowed to acclimatize in the outdoor aviaries for at least 2 weeks before being subjected to the experimental protocol to allow their hypothalamic-pituitary-adrenal (HPA) axis to normalize (Dickens et al. 2009). All experiments were conducted according to the Australian Code for the Care and Use of Animals for Scientific Purposes and approved by the University of Adelaide Animal Ethics Committee (Approval Number S-2013-202).

Experimental setup

Experiments were conducted in an isolated surgery room with room temperature maintained at 25 ^{0}C with a built-in air-conditioner unit. Two commercial egg incubators (IM 504 egg digital incubator; Incubators & More Pty. Ltd., Australia), each measuring 130cm × 46cm × 47cm, were placed 2.5 m apart in the room facing away from each other so that birds in each incubator could be approached without the other one being disturbed. During the experiments, each bird was placed in a cage measuring 60cm × 40cm × 40cm with water and food available *ad libitum* and the entire cage was placed in the incubator. A digital video camera was placed inside each incubator so that each bird could be monitored from another room without disturbance. A total of three temperature data loggers (Thermochron iButton DS1922L; Maxim Integrated, USA) were placed in the experimental room – one in each

incubator and one at the end of the room opposite the air conditioner. The resolution of the iButtons were 0.5°C, rollover disabled and the sample rate was set at 120 seconds. There was no practical way of measuring the body temperature of the birds without it being a potential source of stress and therefore change in CORT and H:L ratios.

Experimental protocol

Each bird was moved into the exposure chamber set at 25°C at least 18 hours before the baseline blood collection. Between 0900 to 0915 on each day, baseline blood was collected from the bird by brachial venepuncture within three minutes of capture from the cage to ensure CORT levels were unaffected by the disturbance (Romero and Reed 2005). The bird was then returned to the incubator with access to food and water for 2 hours before any change in temperature was initiated by turning the incubator to the set temperature of 35°C or 45°C. This ensured that the blood collected contained baseline CORT levels and any increase in CORT levels resulting from capture returned to baseline before exposure to each temperature. Previous studies indicate that two hours is sufficient for CORT to return to baseline levels (Nephew and Romero 2003).

The incubator exposed the bird to heat up to a maximum temperature of 34.42±0.12°C when the incubator was set to 35°C and up to a maximum temperature of 43.18±0.12°C when it was set to 45°C, as illustrated by Figure 1. The incubator also took 86 minutes to reach maximum when it was set to 45°C, compared to 40 minutes when it was set to 35°C. This meant that the birds were exposed to a longer period of increasing temperature when the incubator was set to 45°C and a shorter period at the maximum temperature.

The bird was monitored using digital video camera during each temperature exposure and criteria for removal from the experiment included constant escape activity lasting more than 10 minutes, behavioural signs of distress (e.g. closed eyes, fluffed feathers and inactivity) and loss of righting reflex. However, none of these behaviours were observed throughout the experiment.

Each bird was randomly assigned to the first exposure temperature using a random number generator (Urbaniak and Plous 2013). The bird was then exposed to the assigned temperature as previously described and a post-exposure blood sample collected at the end of the 60-min exposure period within three minutes of capture from the cage. Less than 10% of total blood volume was collected on any experimental day, and the packed cell volume (PCV) was monitored each time blood was collected. There were no clinically significant decreases in PCV observed throughout the experiment. Following each venepuncture, blood was collected in a heparinized capillary tube. Blood samples were centrifuged at 400 *g* for 5 min so that plasma could be extracted and frozen at -80°C until analysis for corticosterone (CORT). For budgerigars and diamond doves, two blood smears were also made from each venepuncture. Due to the smaller size of zebra finches, the animal ethics committee considered it potentially dangerous to them if blood for both blood smears and CORT measurements were collected. The H:L ratios of zebra finches were therefore not measured. Water consumption in the four hour period between venepunctures was measured by marking the watering tube before the bird was replaced in the incubator after the first venepuncture and then measuring the decrease in water level after the second venepuncture. The water consumption measurements were corrected for evaporation by subtracting the average amount of water lost from watering tubes subjected to the same environmental conditions within the chambers when there were no birds present.

The same bird was then exposed to the other temperature after a recovery period of at least one week to allow its red blood cells to regenerate. At the end of exposure to this temperature, euthanasia was performed using 100mg/kg of sodium pentobarbitone intraperitoneal. The bird's gastrointestinal tract, liver, lungs and kidneys were collected for tissue histopathology and genetic analysis, the results of which will be published in separate papers.

CORT measurement

The CORT level in each plasma sample was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Corticosterone ELISA Kit ADI-900-097, Enzo Life Sciences, USA), following the manufacturer's protocol for small volume serum/plasma samples. 10µl of 1:100 steroid displacement reagent (SDR) was added to 10µl of each sample. The mixture was vortexed and allowed to stand for 5 minutes before 380µl of ELISA assay buffer was added to make a final 1:40 dilution of the sample. All samples were assayed in duplicate and the average of the duplicates used to calculate the final CORT concentration in each sample. The samples were randomly assigned to and assayed across 3 assay plates. Standard solutions of CORT in 32 pg/ml, 160 pg/ml, 800 pg/ml, 4000pg/ml and 20 000 pg/ml were used to calculate the inter-assay coefficients of variability. The intra and inter-assay coefficients of variability were 2.45% and 8.38% respectively. The assay sensitivity was 27pg/ml.

Heterophil:lymphocyte ratio

The blood smears were air-dried, fixed in absolute methanol and then stained with Wright-Giemsa using a Siemens Hema-Tek automatic stainer. The slides were examined by one person who was blind to the sequence of temperature exposures. The first one hundred leukocytes per slide were identified and counted as lymphocytes, heterophils, basophils, monocytes or eosinophils. The heterophil:lymphocyte ratio was then calculated. A subsample of smears (n = 15) were examined twice, and repeatability analyses performed.

Statistics

A paired samples t-test was used to analyze the changes in CORT,H:L ratio and water consumption(SPSS 21.0, IBM Corp., Armonk, NY). These values will be reported as mean +/- SD. Linear regression was used to analyze correlation between ΔCORT levels and ΔH:L ratios (SPSS 21.0, IBM Corp., Armonk, NY).

Results

CORT response

There were no significant differences between the CORT levels before (3.78 ± 2.63 ng/ml) and after heat exposure (4.87 ± 2.70 ng/ml; $t_7 = -1.64$, $P = 0.145$) in zebra finches at 35 ^{0}C and before (4.93 ± 3.46 ng/ml) and after (6.65 ± 5.82 ng/ml; $t_7 = -1.20$, $P = 0.270$) heat exposure at 45 ^{0}C.

In budgerigars, the CORT level before exposure to 35°C (4.96 ± 3.35 ng/ml) was significantly higher than the CORT level after exposure to 35°C (2.07 ± 1.33 ng/ml; $t_7 = 3.29$,

P = 0.013). However, there were no significant differences between the CORT levels before (3.78 ± 2.63 ng/ml) and after heat exposure (4.87 ± 2.70 ng/ml; t_7 = -1.64, *P* = 0.145) in budgerigars at 45 ^{0}C.

In diamond doves, there were no significant differences between the CORT levels before (1.92 ± 2.84 ng/ml) and after (1.04 ± 0.61 ng/ml) exposure to 35°C (t_7 = 0.84, *P* = 0.431). However, the CORT level before exposure (0.79 ± 0.33 ng/ml) was significant lower than the CORT level after exposure (2.61 ± 1.39 ng/ml) to 45°C in diamond doves (t_7 = -4.30, *P* = 0.004).

H:L ratio

No significant differences were found between the two sets of H:L ratio counts performed for the selected subset of samples when repeatability analyses were performed ($F_{1,14}$ = 0.46, *P* = 0.51).

In budgerigars, the H:L ratio before exposure (2.49 ± 2.77) was significantly lower than the H:L level after exposure to 35°C (4.64 ± 2.71; t_7 = -3.45, *P* = 0.011). The H:L ratio before exposure (1.34 ± 0.52) was also significantly lower than the H:L level after exposure to 45°C (2.60 ± 1.26; t_6 = -3.69, *P* = 0.010).

In diamond doves, the H:L ratio before exposure (0.54 ± 0.21) was significantly lower than the H:L level after exposure to 35°C (1.37 ± 0.92; t_7 = -2.63, *P* = 0.034). There was no significant difference in H:L ratios in diamond doves before (0.54 ± 0.31) and after exposure to 45 ^{0}C (1.17 ± 1.02; t_7 = -1.96, *P* = 0.091) (Figure 3).

After analysis of the H:L ratio data, one of the budgerigars had very high baseline H:L ratio due to a large amount of cell lysis that resulted in a low number of intact and countable white

blood cells on the blood smear. The H:L ratio data for this budgerigar was thus excluded from analysis for budgerigars exposed to 45 ^{0}C.

Correlation between CORT and H:L ratio

No significant correlation was found between ΔCORT and ΔH:L ratios (R^2=0.0442, $F_{1,62}$ = 1.342, *P* =0.256) (Figure 4).

Water consumption

There were no significant differences in water consumption at 35°C and 45°C for zebra finches (35°C: 0.005 ± 0.005µl/g*h, 45°C: 0.012 ± 0.018 µl/g*h; t_7 = -1.07, *P* = 0.313), budgerigars (35°C: 0.002 ± 0.002µl/g*h, 45°C: 0.005 ± 0.008 µl/g*h; t_7 = -1.15, *P* = 0.283) and diamond doves (35°C: 0.001 ± 0.002µl/g*h, 45°C: 0.000 ± 0.000 µl/g*h; t_7 = 1.09, *P* = 0.313) (Figure 5).

Discussion

The experimental design is strictly speaking not a common garden experiment as the birds were all raised in similar environmental conditions, and then moved to an environment that is not dissimilar to the one they were raised in. The only time they were subject to different environmental conditions was during the experiment itself. Even though thermal biology and thermoregulation of bird populations have been shown to vary amongst populations (Garland and Adolph 1991), the birds included in this study have been captive bred under similar

environmental conditions, and this should therefore not be a factor in the differences observed.

CORT

The CORT data supports the interpretation that heat exposure to 45°C for 2 hours was a stressor in diamond doves, but not in zebra finches and budgerigars. This was unexpected as other studies has shown a stress response in European starlings (de Bruijn and Romero 2011) and Greylag geese (Frigerio et al. 2004) to moderate changes (albeit decreases) in ambient temperatures.

The CORT data suggested that zebra finches and budgerigars did not perceive the temperatures they were exposed to during the experiment as stressors. This may be because they have constant access to *ad libitum* food and water in the experimental cages. In free-living desert birds, a combination of the decreased physical activity and exposure to solar radiation minimizes rates of water loss, but also limit rates of water intake. Small birds can still lose more than 5% of body mass per hour via evaporative water loss during periods of high environmental temperatures even if they rest in completely shaded microsites (Wolf and Walsberg 1996a; Wolf and Walsberg 1996b). The constant availability of water for the experimental birds allows them to replace the water lost through evaporation and therefore eliminates a potential source of stress. As there was no significant difference between water consumption at both temperatures, it may be possible that the amount of evaporative water loss required by the birds were not significantly different at the higher temperature. However, as there was no good way to check the water level just before increasing the incubator temperature without also disturbing the bird and potentially causing a rise in CORT that was not associated with the temperature increase, the total water consumption of each bird was

measured over the full 4 hours, i.e. 2 hours at 25°C and 2 hours at the higher temperature. Therefore, water consumption during the 2 hrs that birds were exposed to 25°C may have masked any small effects of elevated temperature on water consumption. It is also unclear why diamond doves exposed to 45C did not increase water consumption.

The temperatures and length of exposure were also lower and shorter respectively than that experienced during a heatwave in the Australian desert. However, the birds in this study did not have access to areas with different microclimates, i.e. microsites that free-living birds do in the desert which may allow them to avoid the high temperatures. Free-living desert birds can seek shaded microsites which have a lower temperature due to factors such as proximity to living plants, wind direction etc (McKechnie et al. 2012). A combination of a lack of food and water, higher temperatures and longer duration of heat may have resulted in a significant CORT increase in the experimental birds. Captive-breeding may also have altered the physiology and behaviour of these birds, so their responses to heat exposure may not be directly relevant to wild desert birds. Further studies using wild-caught individuals of the same bird species will allow us to understand these differences better. Moreover, some of the significant results were due to the drop of the baseline CORT, rather than increase CORT post-heat exposure, and some of the non-significant results were due to the large variation. These may affect the interpretation of these results, and it is possible that a larger sample size may yield different results.

Columbiform birds generally have a higher Critical Thermal Maximum (CTM) compared to Passeriform and Psittaciform birds (Gerson 2015; Wolf 2015). Acclimated rock pigeons have also been shown to be able to tolerate ambient temperatures of up to 60°C (Marder and Arieli 1988; Marder and Gavrieli-Levin 1986, 1987). In a captive situation, diamond doves have also been found to not alter their behaviour in response to heat, whereas budgerigars increased the proportion of time spent stationary and decreased the proportion of time spent

feeding in response to heat (Xie S. et al., unpublished data). Given that increases in CORT has been shown to be important for the regulation of feeding behaviour (Astheimer et al. 1992) and general activity levels (Breuner et al. 1998), it is possible that the CORT response of the diamond dove drives its feeding behaviour during high temperatures, which is in turn made possible by its high heat tolerance. This may confer a survival advantage to Columbiform birds during periods of high temperature when they are driven and able to continue foraging in a landscape that is scare in food and water, whereas other birds such as psittacines are forced to cease foraging so that they do not die of hyperthermia.

Exogenous CORT (Astheimer et al. 1992) and chronic elevation of endogenous CORT (Cyr et al. 2008) have also been suggested to decrease metabolism. An increase in CORT can therefore still offer species of birds with a lower heat tolerance, e.g. psittacine and passeriform birds, an advantage in surviving during a heatwave, when their ability to forage for food and water is restricted by the need to avoid hyperthermia. However, the lack of a CORT response in zebra finches and budgerigars would suggest that they are at a further disadvantage when compared to diamond doves in the face of surviving during a heatwave.

The difference in CORT response among different species of birds reflected by the data may be a factor in the restructuring of bird communities as a result of the increased frequency and intensity of heatwaves predicted as part of climate change (Perkins et al. 2012). Desert Columbiforms such as diamond doves are already capable of foraging in the desert during the hottest time of the day when predators and food competitors are absent (Schleucher 1993). They are in direct competition for resources with small desert passerines such as zebra finches (Schleucher 1993) and psittacines (Morton and Davies 1983). If the lack of CORT responses in pisttacine and passeriforme birds continue during exposure to heatwaves of higher temperatures and longer durations, then the survival disadvantage compared to

Columbiform birds may result in Columbiforms becoming the more dominant order within bird communities in areas of Australia where heatwaves are becoming more common.

Sterl et. al. (2008) predict that maximum temperatures may reach 48°C across the American mid-west, 54°C in South America and 50°C in Australia and India by the year 2100. Even though many Australian bird species are highly mobile and nomadic, particularly the three species included in the study which have the highest nomad status according to both the Schodde and Reid classifications (Allen and Saunders 2002; Schodde 1981), they may soon not be able to avoid environments with extremely high ambient temperatures. Future studies should investigate the CORT responses after exposure to higher temperatures of 50°C and above and longer duration to more closely simulate the conditions of a heatwave in the desert.

H:L ratio

H:L ratio is another potential indicator of the stress response in birds. However, the H:L data in our study appears to be the opposite of the CORT data. The H:L ratio in budgerigars showed a significant increase from pre to post-treatment at both 35 and 45 ^{0}C. This is consistent with findings from Altan *et al.* (2003).

The H:L ratio of diamond doves, however, showed a significant increase at 35°C but not at 45°C.

It is possible that the small sample size precluded the detection of a statistically significant effect at 45°C, and a larger sample size might have resulted in a different finding. Alternatively, the difference between the findings in budgerigars and diamond doves may suggest that there is a species variation in the H:L ratio to heat exposure. Previous studies have indicated that there are breed and species differences in the H:L ratio responses of birds to stressors. A study

of fear and stress levels in five Spanish breeds of chickens found that the H:L ratio of Castellana breed was significantly less than other breeds (Campo et al. 2001). Similarly, it was found that nestling blue tits (*Cyanistes caeruleus*)had higher average H:L ratio compared to nestling great tits (*Parus major*)when exposed to environmental stressors (Banbura et al. 2013).

Although the H:L ratio may be a reliable measure for physiological stress in birds, there are some limitations when interpreting the results independent of the CORT data. The leucocyte metric is very much dependent upon the age of the animal. Heterophils are part of the innate immunity while lymphocytes are part of the adaptive immunity, and these two branches of immune system develop at different periods of growth (Banbura et al. 2013). Therefore age of the birds should be taken into consideration when analysing the H:L ratio data. However, as the birds in this study were all adults from broods hatched at the same time period, this should not be a factor in this situation.

The experimental protocol in the present study was designed to minimize the effects of handling stress on CORT responses of the birds. If handling stress resulted in an increase in H:L ratios in the 2 species of birds in our study 60-120 minutes post-capture (Cīrule et al. 2012), then the increase in H:L ratios post-temperature exposure could also be attributed to the handling and blood sampling from the collection of the baseline sample. Similarly, the H:L increase in response to heat exposure may precede CORT increase, and remain for a longer duration post-heat exposure, resulting in the differences to the CORT data in our study.

Correlation between CORT and H:L ratio

Previous ecological studies in birds have found weak to no correlation between H:L ratios and baseline CORT levels (Clinchy et al. 2004; Ilmonen et al. 2003; Müller et al. 2011; Vleck et al. 2000). No correlation was found between H:L ratio and corticosterone levels in pied

flycatchers (*Ficedula hypoleuca*) subjected to brood size manipulation (Ilmonen et al. 2003), in song sparrows (*Melospiza melodia*) subjected to different levels of predator pressure (Clinchy et al. 2004) and in Eurasian kestrel (*Falco tinnunculus*) nestlings in relation to body condition and other measurable environmental stressors (Müller et al. 2011). Vleck et al. (2000) found moderate correlation between H:L ratios and corticosterone levels in Adelie Penguins (*Pygoscelis adeliae*) that were repeatedly sampled and those that were fasting, but no correlation in injured, fighting or nesting birds. These studies also indicated that the degree of change in H:L ratios varies with the type of stressor (Clinchy et al. 2004; Davis et al. 2008; Ilmonen et al. 2003; Vleck et al. 2000).

The data from our study supports the findings that there is weak to no correlation between H:L ratios and CORT levels (Figure 4). In particular, the correlation between the change in the CORT levels and change in H:L ratios before and after heat exposure was analyzed. This was different to the previous studies that analyzed the correlation between CORT levels and H:L ratios in single blood samples. This was an important difference because the change in CORT levels is more likely to be the physiologically relevant metric (Romero and Wingfield 2016). Budgerigars displayed a significant increase in H:L ratio at both temperatures, but diamond doves only displayed such a response at the lower temperature. Moreover, the lack of a significant H:L ratio response in diamond doves at the higher temperature did not correspond with the observation that this was the only species with a significant CORT response at the same temperature.

Conclusion

In conclusion, high temperatures pose significant threats to birds, and appropriate behavioural and physiological responses are important in determining the survival of an individual when exposed to such a threat. This is particularly important for desert birds given that the

availability of food and water is usually low in their habitat (Williams and Tieleman 2001). A major driver of such responses is CORT, and this study shows that there are species differences in the CORT response, as well as other stress responses. This reflects the need to study different species and/or orders of birds separately, instead of trying to extrapolate findings from one species/order to another, in order to be able to draw valid conclusions about their vulnerability to heat as a group. Birds that appear to be particularly vulnerable to heat, as demonstrated by mass mortality events e.g. psittacine birds, or birds that are already under threat from other processes should be targeted as priority for further studies.

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Figures

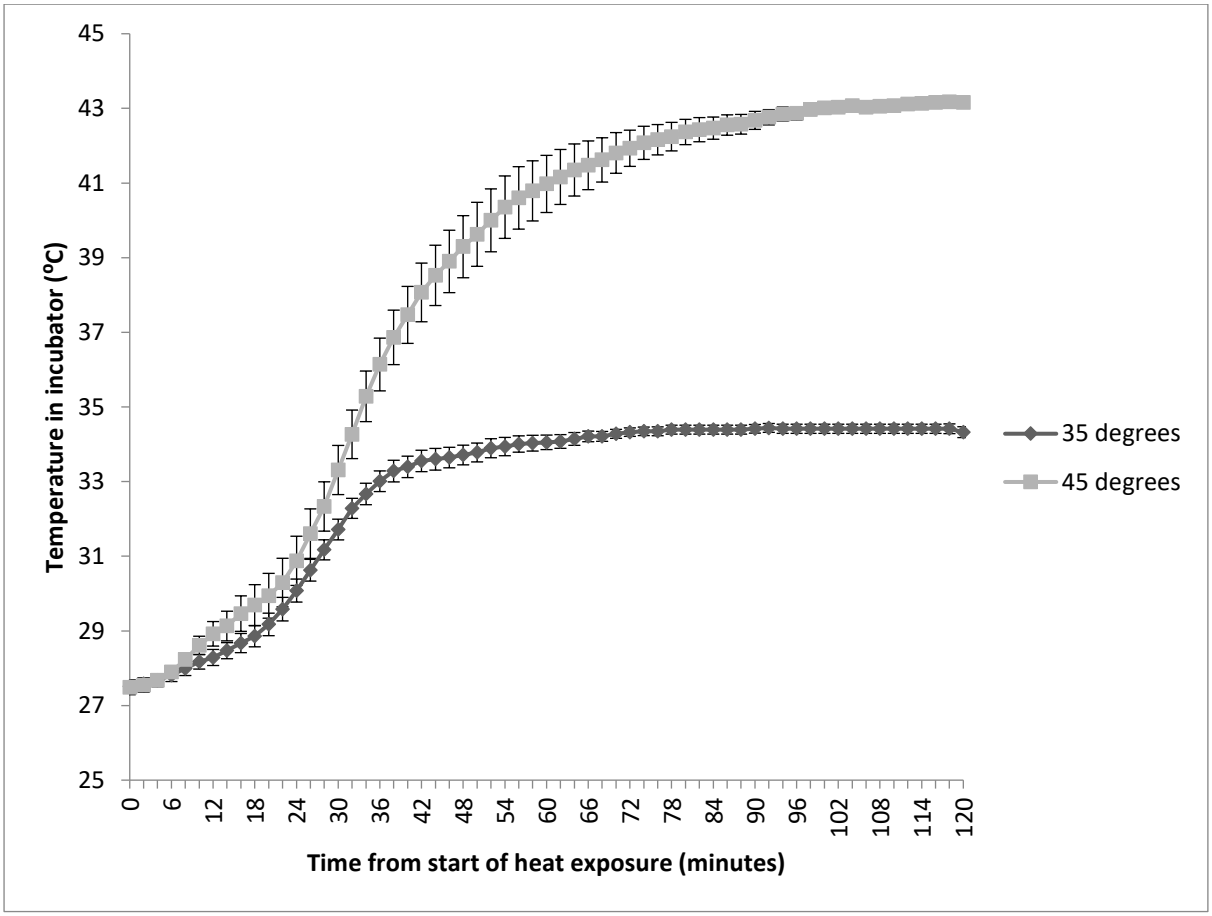

Figure 1. Temperature increase within the incubator when it was set at 35^0C and 45^0C.

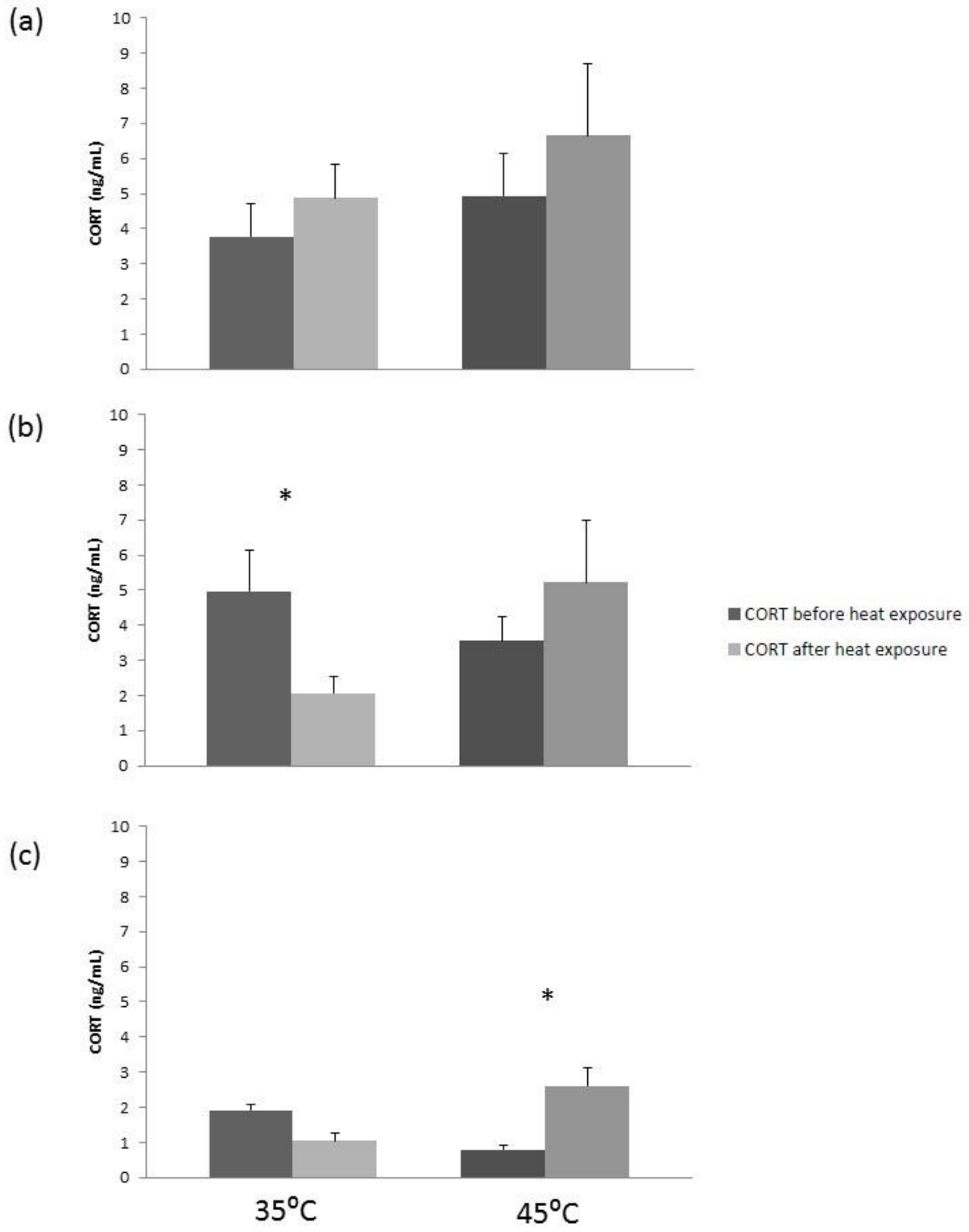

Figure 2. Corticosterone (CORT) responses of (a) zebra finches, (b) budgerigars, (c) diamond doves before and after heat exposure at 35°C and 45°C. * $p<0.05$.

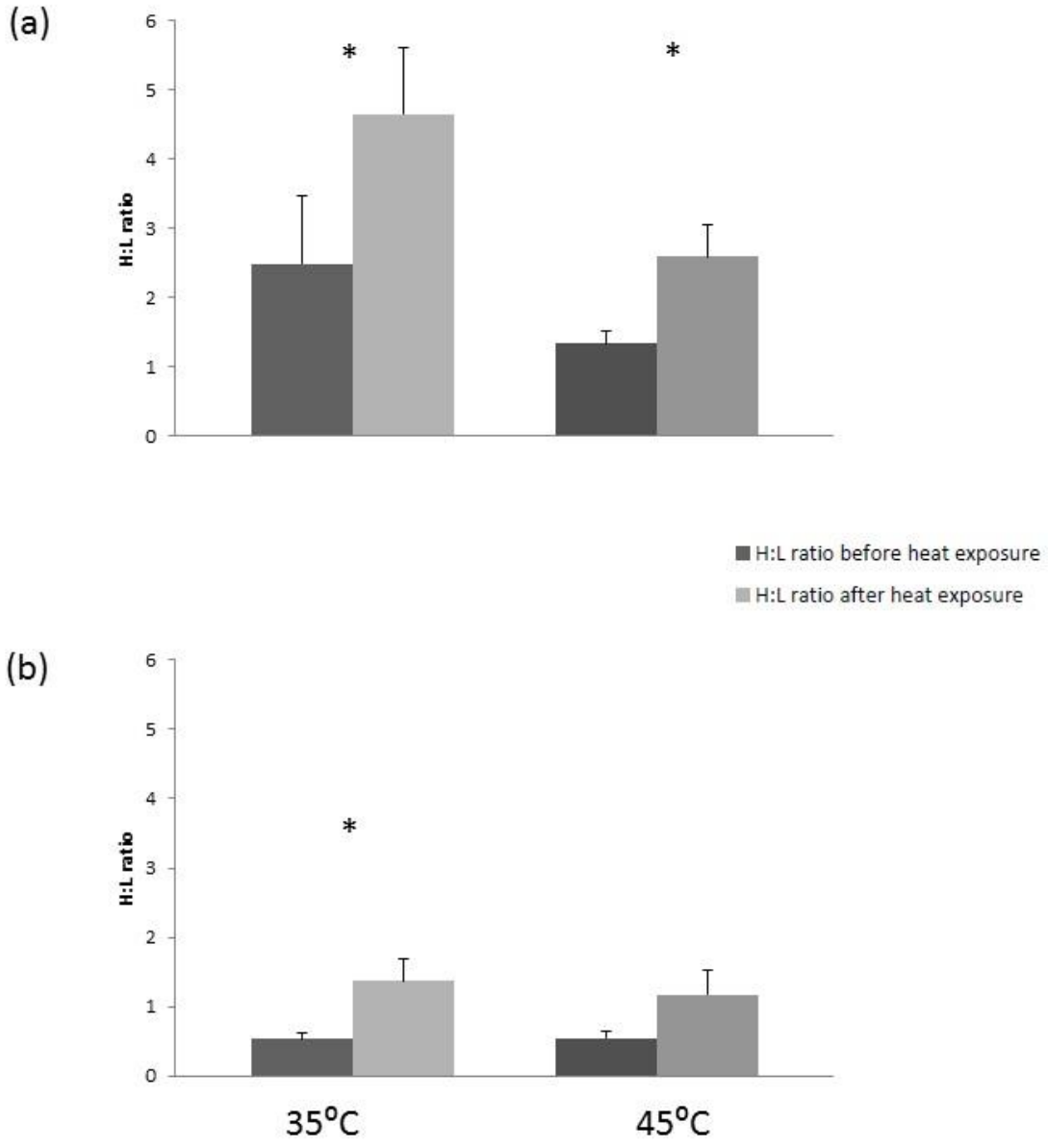

Figure 3. Heterophil:lymphocyte (H:L) ratio responses of (a) budgerigars, (b) diamond doves before and after heat exposure at 35^0C and 45^0C. * $p<0.05$.

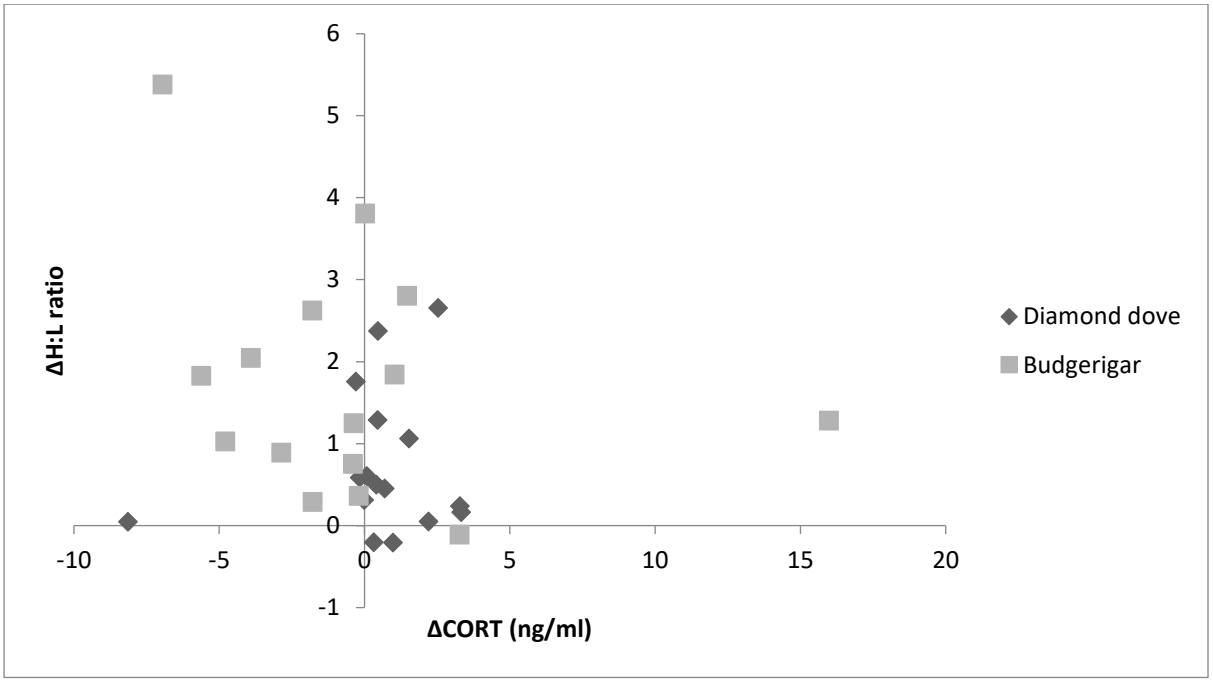

Figure 4. Relationship between the change in corticosterone (CORT) and change in heterophil:lymphocyte (H:L) ratios (combined data from budgerigars and diamond doves).

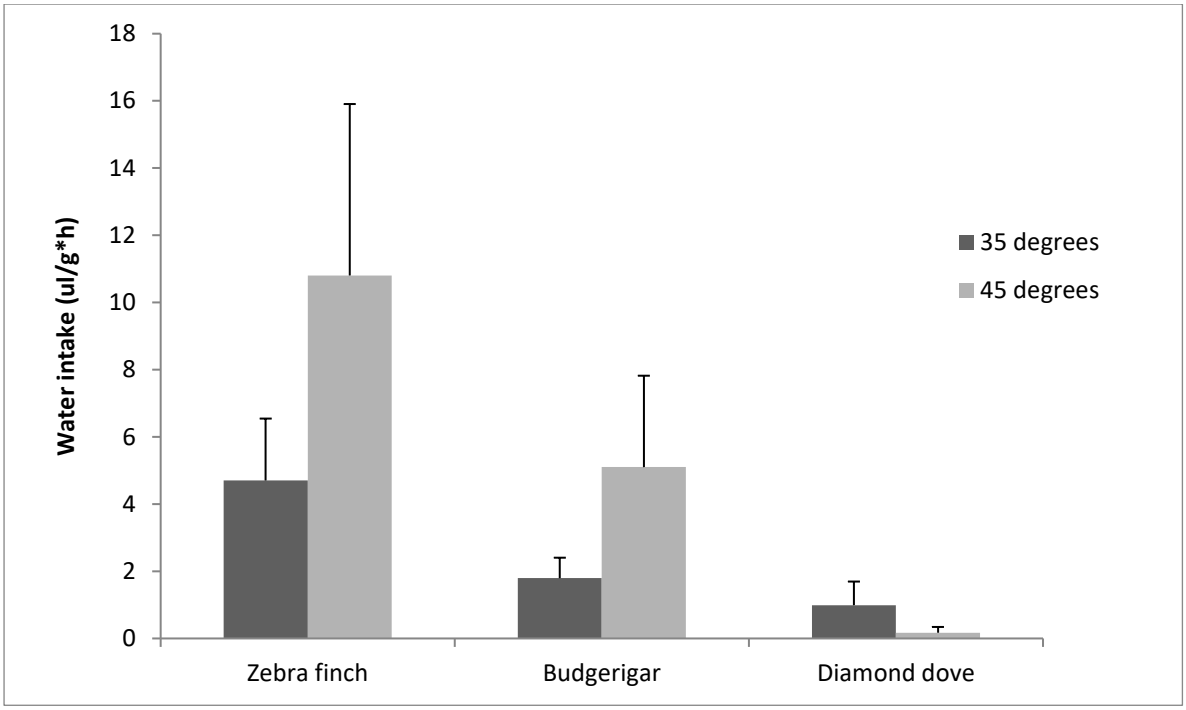

Figure 5. Water consumption of each species at each heat exposure temperature.

Chapter 3 Behavioural adaptations of captive birds to high environmental temperatures

As described in the previous chapter, the CORT and heterophil:lymphocyte ratio responses differ amongst species of Australian desert birds. CORT in particular has effects on the behaviour of birds. This original research article, published in *Emu – Austral Ornithology*, describes the differences in the behavioural adaptations of captive birds belonging to different orders. These differences indicate that different species of birds may rely on behavioural adaptations to combat the pathological effects of high environmental temperatures to different extents, and their susceptibility to these effects may therefore be dependent on their ability to adopt these behavioural changes. This publication was selected as part of the Editor's Choice 2017 Collection for *Emu – Austral Ornithology*.

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Contribution to the Paper	Collected part of data, interpreted data, wrote manuscript and acted as corresponding author.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	19/3/2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Erin Turrell		
Contribution to the Paper	Collected part of data, interpreted data.		
Signature		Date	3/4/2017

Name of Co-Author	Todd McWhorter		
Contribution to the Paper	Supervised development of work, helped in data interpretation and manuscript evaluation.		
Signature		Date	28/4/2017

Behavioural responses to heat in captive native Australian birds

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Abstract

Australian birds are under threat from climate change and heat waves. We investigated whether captive native Australian birds adjusted their time budgets, microsite selections or used thermoregulatory behaviours on typical or extremely hot summer days. Eight species of birds at the Adelaide Zoo were observed and the data revealed that the proportion of time spent on stationary behaviours, feeding, in the sun and on the ground differed amongst species of birds, between mornings and afternoons and day type. Wing-venting was used more frequently during the hottest observation periods. The smallest birds in the study utilised wing venting more than other species of birds, possibly because it is more important for them to conserve water. Psittaciform birds spent less time feeding and more time resting in cooler microsites during hot periods. Columbiform birds continue feeding and spent more time in the sun rather than resting in cooler microsites. White-Browed Woodswallows spent a significantly lower proportion of time on stationary behaviours and higher proportion of time feeding compared to the other species. Our results suggest that columbiform birds may have an advantage during heatwaves as they can continue feeding through high ambient temperatures, as long as there is adequate access to food and water.

Keywords

thermal stress; behavioural ecology; climate change; captive management; conservation; environment

Introduction

Australian birds are under increasing threat from the effects of climate change and extreme climate events like heat waves, both in the wild and when held in captivity outdoors. Models of global warming predict an increase in annual average temperatures of 0.4 to 2.0°C by the year 2030 and an increase of 1.0 to 6.0°C by the year 2070 (Whetton 2001). An increase in surface air temperature of 0.6°C has been recorded worldwide (Houghton, Ding *et al.* 2001) with a 0.7°C increase in Australia's average continental temperature (Nicholls 2003). 2013 has also been the hottest year on record for Australia, with average maximum temperatures 0.17°C higher than the previous record set in 2005 (CSIRO and Meteorology 2016; King, Karoly *et al.* 2014). The record for spring seasonal maximum temperature was also +2.32°C above the 1961–1990 mean, surpassing the record-breaking spring of 2013 (Hope, Reid *et al.* 2015). Present-day frequency, duration and intensity of heatwaves and warm spells have increased (CSIRO and Meteorology 2016), with recent data suggesting that annual warms spells may be having a greater impact on increased annual temperature trends than summer heat waves (Perkins, Alexander *et al.* 2012). Winter and spring warming has increased to a greater degree than summer or autumn warming (Perkins, Alexander *et al.* 2012) and night-time warm spell trends have also been shown to be increasing faster than those during day-time (Lenten and Moosa 2003; Perkins, Alexander *et al.* 2012). These patterns of warming and increased frequency and intensity of extreme events are expected to continue for the foreseeable future in Australia (CSIRO and Meteorology 2016; Reisinger, Kitching *et al.* 2014).

Birds inhabiting arid-zone desert habitats may be the most likely to be affected by an increase in both annual temperature as well as the occurrence of heatwaves: the avian upper lethal body temperature limit resides at 45-46°C, just 4 to 5°C above resting body temperature (Dawson and Schmidt-Nielsen 1964; Prinzinger, Preβmar *et al.* 1991).

However, arid-zone birds have evolved physiological adaptions to deal with excess heat-loading from higher surface air temperatures, which have been studied in great detail (Williams and Tieleman 2001). In contrast to the detailed understanding of the anatomical and physiological adaptations of birds to arid environments, less is known about the time budget adjustments, microsite selection and thermoregulatory behaviours that arid-zone birds may utilise to avoid heat stress.

Behavioural plasticity is thought to be one of the main mechanisms that wild animals use to cope with environmental challenges such as heatwaves (Sol, Lapiedra *et al.* 2013) and captivity (Mason, Burn *et al.* 2013). Sol et al. (2013) describe the options a foraging animal has when exposed to a potential predator as an example of 'activational' behavioural plasticity (Snell-Rood 2013). These options include: 1) a non-response, 2) a reduction in foraging activity to look for refuge, 3) adjustment of foraging activity based on the level of perceived threat (Sol, Lapiedra *et al.* 2013). Similarly, arid-zone birds are presented with options during periods of high air temperatures: 1) behaving in the same way (non-response), 2) reducing activity to reduce energy and water use, and 3) seeking cooler microsites or restricting activity to cooler parts of the day. Microsite selection as a mechanism to reduce direct heat loading from solar radiation is one of the better studied behavioural adaptation that birds, especially arid-zone passerines, utilise to reduce their heat loads (Wolf, Wooden *et al.* 1996). These small passerine birds have high mass-specific metabolic rates and produce heat internally at a high rate (Dawson 1982). Coupled with their high respiratory rate, this results in a high rate of pulmonary water loss (Dawson 1982). While this helps to prevent their body temperature from rising to lethal levels, it also increases their need for water in a habitat that is scarce in this resource.

Birds may also use specific cooling or thermoregulatory behaviours in order to prevent their body temperatures from rising towards lethal levels. These behaviours include

wing venting, panting and gular fluttering (lingual fluttering in psittacines). Birds wing vent by dropping their wings away from their bodies, allow convection to cool the torso (Gallup, Miller *et al.* 2010a; Gallup, Miller *et al.* 2010b) and facilitating convective heat loss from the blood vessels located on the underside of the wings (Tucker 1968). Panting and gular fluttering both increase evaporative heat loss via the respiratory tract (Bartholomew, Lasiewski *et al.* 1968; Serventy 1971), but panting can also occur when birds are under other stresses such as handling, in addition to being a thermoregulatory response (Geist 2000). Parrots can utilise lingual fluttering in conjunction with panting to increase the efficiency of heat loss while panting (Bucher 1981; Weathers and Schoenbaechler 1976; Weathers and Caccamise 1975).

To our knowledge, research on behavioural adaptations to heat has not previously been undertaken on native Australian bird species in zoo captivity. In addition to being forced to live in an urban environment that they might usually avoid (Sol, Lapiedra *et al.* 2013), captive zoo birds are also exposed to the rising annual temperatures and heat waves. As more bird species become endangered, the importance of keeping these species as conservation initiatives in zoos increases. For example, the Adelaide Zoo is actively involved in the breeding of endangered orange-bellied parrots. Captive zoo birds are unable to avoid hot environmental conditions and their behavioural plasticity and availability of microsites are restricted by their enclosures. As temperatures continue to increase with climate change, bird species with higher behavioural plasticity may be able to better adjust their time budgets and microsite selections to avoid exposure to lethal temperatures. On the other hand, bird species that do not have the ability to adjust their behaviour in accordance with rising temperatures may have to rely on physiological thermoregulatory mechanisms. Birds that can adjust both behaviourally and physiologically will have the highest chances of surviving heat waves and higher temperatures.

In this study, the temperature dependency of time budgets, microsite selections and thermoregulatory behaviours in captive Australian desert birds was investigated by quantifying the amount of time they spent on different behaviours, including thermoregulatory ones, and in different microsites. It was hypothesized that birds in general would employ the following mechanisms to reduce their heat loads when experiencing higher ambient temperatures: 1) increase the proportion of time spent on stationary behaviours (i.e. resting and preening); 2) decrease the proportion of time spent feeding; 3) select shaded microsites and avoid spending time on the ground; and 4) utilise wing venting. It was also hypothesized that birds that are able to tolerate higher temperatures, i.e. taxa with a higher critical thermal maximum (CTM), e.g. columbiform birds (McKechnie, Whitfield *et al.* 2016; Whitfield, Smit *et al.* 2015), would use these behavioural adjustments less than psittaciform and passeriform (Whitfield, Smit *et al.* 2015) birds, as they rely more on cutaneous evaporative cooling, which is less visible than respiratory mechanisms.

Methods

Location

All observations of birds were performed at Zoos South Australia's Adelaide Zoo (latitude: -34.9143, longitude: 138.6049, altitude: 50m above sea level).

Subjects

Eight species of birds housed within four different outdoor aviaries of various sizes were selected for this study (Table 1). Wild habitat ranges varied amongst the birds selected with some ranging throughout Australia, such as Budgerigars and Cockatiels, and others having more localised habitat ranges, such as Red-Collared Lorikeets on Australia's northern tropical

coasts or Regent Parrots with localised populations in the southwestern corner of Western Australia or Murray Mallee regions in South Australia (Pizzey and Knight 2012).

Experimental design

Fifteen minute focal observations were conducted on individual birds. Ten observations were performed for each species in each day type (see below for temperature ranges of each day type) and were repeated in the morning and afternoon of the same day. Birds were sampled randomly from an aviary with replacement, and because birds were not individually marked time of day and day type measurements were not paired for individuals. Observations were made between December 20, 2013 and March 20, 2014. Morning observations (AM) were conducted between 7:30 am and 10:30 am while afternoon observations (PM) were conducted between 12 pm and 5 pm of the same day. These observations were conducted during extreme summer weather with a maximum daily predicted air temperature above 35°C and normal summer weather with a maximum daily predicted air temperature between 25°C and 35°C. All predicted maximum daily air temperatures were for the city of Adelaide and were obtained from the Australian Bureau of Meteorology (BOM) website (http://www.bom.gov.au/sa/forecasts/adelaide.shtml). At the conclusion of each observation day, ambient temperature (T_a) data were downloaded for the Kent Town weather station (BOM station number: 023090; latitude: -34.9211, longitude: 138.6216, station height: 48.0 m; located 1500 m to the southeast of Adelaide Zoo) (Australian Bureau of Meteorology 2014). The T_a value recorded closest to the median time of each observation period for each individual bird was used in all analyses where T_a was treated as a continuous variable or covariate.

Observations were recorded using the Animal Behaviour Pro application (Newton-Fisher 2012) which allows for the input of coding and focal observational details including species, behaviours and microsite location. The behaviours recorded included resting, grooming, hopping/walking, flying, feeding, drinking, social interaction and other. These behaviours were considered mutually exclusive. The microsites recorded consisted of the location of the bird, including whether or not the bird was on the ground, on a perch, on cage wire, in vegetation or under a sprinkler, and the amount of shade the bird was in, including full shade, partial shade or in full sun.

Experimental protocol

At the start of each observation, an individual bird was selected at random within the aviary. Care was taken to not select birds from the same particular microsite and performing a particular behaviour for each new observation, but there is a certain degree of pseudo-replication which could not be eliminated. The behaviour of the bird at this point in time was selected on the Animal Behaviour Pro application, which started the timer, followed by the selection of microsite containing variables for both location and shade level. When the bird started to exhibit a different behaviour, the new behaviour and the relevant microsite were selected, which automatically ended the previous behaviour's time within the application and started the timer for the new behaviour. This process was repeated as the bird changed behaviours and microsites for the entire 15 minute observation period. Wing-venting, a thermoregulatory behaviour, was also recorded. Other more subtle thermoregulatory behaviours, such as gular fluttering, were not observed during the experiment, possibly due to the distance from which the observations were made (2-3 meters away from bird, from outside of the aviary).

Inter-observer variability was assessed because observations were performed by two observers. Simultaneous focal observations of the same individual bird by both observers were performed for seven species (one bird per species) and the proportion of time allocated to each behaviour and microsite by each observer for each bird compared.

Data processing

The time spent on each behaviour (excluding thermoregulatory behaviours) and in each microsite was converted into a percentage of the total 15-minute observation time. The proportion of time spent by the bird on stationary behaviours, i.e. sitting stationary on a perch resting or preening, was calculated by summing the total amount of time spent stationary and dividing by the total observation time. The mean proportion of time spent on stationary behaviours by all the birds for all day types was 66.79 ± 28.43 %. Similarly, the proportion of time spent by the bird feeding, i.e. observed handling and ingesting food, was calculated by dividing the total amount of time spent feeding by the total observation time. The mean proportion of time spent feeding by all the birds for all day types was 33.63 ± 26.78%.

Statistical analyses

All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 22.0. (IBM Corp. Released 2013. Armonk, NY: IBM Corp). Linear mixed model analysis was used to test for differences in T_a recorded by the BOM weather station between the day type and morning or afternoon (AM/PM). The Wilcoxon signed-rank test for related samples was used to assess inter-observer variability. Generalized linear models with binomial distributions and logit link functions were used to assess the effects of day type (extreme summer or normal summer), morning or afternoon (AM/PM) and aviary (for Budgerigars and

White-Browed Woodswallows only) on the proportion of time spent stationary, feeding, in sun and on the ground and to test for covariance of these parameters with T_a. A generalized linear model with a Poisson distribution and log link function was used to assess the effects of species, day type, morning or afternoon and aviary on the mean number of wing-venting episodes observed per 15 minutes observation period and to test for covariance of these behaviours with T_a. Wald statistics were used to assess the model fit and statistical significance was assessed against $\alpha = 0.05$. Graphical representations of data contain error bars consisting of the standard error of the mean (S.E.M.).

Ethical note

This project was approved under the University of Adelaide animal ethics protocol number S-2013-151A and by the Research Committee of Zoos South Australia.

Results

Inter-observer variability

No significant differences were found between the two observers for stationary behaviour (resting and preening combined, $P = 0.345$), flying ($P = 0.173$), hopping ($P = 0.463$), time spent in shade on perch ($P = 0.893$) or time spent on the ground ($P = 0.285$). Observations from both observers were therefore combined for all analyses.

Ambient temperature (T_a)

Ambient temperature during all observation categories (i.e. normal summer AM and PM, extreme summer AM and PM) was significantly different from all others ($P < 0.0001$) except that normal summer PM observations were not significantly different from extreme summer

AM observations (P = 0.350). T_a during observation periods were as follows (mean ± 95% confidence interval): normal summer AM = 19.91 ± 2.00°C, normal summer PM = 27.76 ± 2.00°C, extreme summer AM = 31.03 ± 2.00°C, extreme summer PM = 39.15 ± 2.00°C. These mean values are reported here as indicative of the ambient temperatures experienced by birds in each observation category only. When used as a continuous covariate in analyses of behaviour, the actual T_a value closest to the median time of any individual observation period obtained from the Bureau of Meteorology website (see Methods) was used.

Time spent on stationary behaviour and feeding

The results for the proportion of time spent on stationary behaviour and feeding by each species are summarized in Table 2 and Table 3, respectively. The differences between the proportion of time spent on stationary behaviour in the morning and afternoon of normal and extreme summer days by each species are summarized in Figure 1 and that for the proportion of time spent feeding in Figure 2. The White-Browed Woodswallows and four out of five the psittacine bird species included in this study, i.e. Budgerigars, Scarlet-Chested Parrots, Red-Collared Lorikeets and Regent Parrots, spent more time on low energy stationary behaviours and less time feeding during the hottest observation periods, i.e. in the afternoon, on both normal and extreme summer days.

Microsite selection

The results for the proportion of time spent in the sun and on the ground by each species are summarized in Table 4 and Table 5, respectively. The differences between the proportion of time spent in the sun in the morning and afternoon of normal and extreme summer days by

each species are summarized in Figure 3 and that for the proportion of time spent on the ground in Figure 4. Budgerigars spent less time in the sun and on the ground on extreme summer days, particularly in the afternoon. The columbiform birds included in this study spent more time in the hotter microsites, i.e. in the sun and/or on the ground, during the hottest observation periods. The White-Browed Woodswallows spent significantly less time in the sun on extreme summer days, particularly the afternoons, but significantly more time on the ground on the afternoons of extreme summer days.

Thermoregulatory behaviours

There were significant effects of species (Wald $\chi^2_{6,400}$=142.52, P<0.0001), day type (Wald $\chi^2_{1,400}$=25.21, P<0.0001) and time of the day (Wald $\chi^2_{2,400}$=94.35, P<0.0001) on the number of wing venting episodes observed. The ambient temperature was also a significant co-variant (Wald $\chi^2_{1,400}$=26.06, P<0.0001). There was no significant effect of aviary (Wald $\chi^2_{1,400}$=2.90, P=0.089). The number of wing venting episodes per observation period was highest during extreme summer (Mean=0.0052±0.00136), which was significantly higher (Wald $\chi^2_{1,400}$=25.51, P<0.0001) than normal summer (Mean=0.0003±0.00019). Wing venting in the afternoon on both extreme summer days (Wald $\chi^2_{1,400}$=90.87, P<0.0001) and normal summer days (Wald $\chi^2_{1,400}$=4.18, P=0.041) was significantly greater than in the morning . The species differences are summarized in Table 6. The mean number of wing-venting episodes observed increased significantly during the afternoon and on extremely hot summer days.

Discussion

Species differences in thermoregulatory capacity and feeding behaviour

There are species differences in the way birds utilise different strategies to decrease their heat load during periods of high ambient temperatures. However, as the statistical analyses were not controlled for phylogenetic relatedness, only absolute differences can be inferred from our data. It appears that Budgerigars, Cockatiels, Scarlet-Chested Parrots, Red-Collared Lorikeets and Regent Parrots increase the proportion of time spent on low energy stationary behaviours, select cooler microsites and utilised wing venting during periods of high ambient temperature. Cockatiels appeared to adjust their behaviours differently in response to high ambient temperatures compared to the other psittacines in this study, spending more time on low energy stationary behaviours during the afternoon on normal summer days and more time feeding overall on extreme summer days. This suggests that Cockatiels may have to obtain higher levels of nutrition and dietary water during extreme summer days to support energetically costly methods of thermoregulation such as panting and gular fluttering (Gallup, Miller *et al.* 2010b), even though these behaviours were not specifically observed in this study. Small birds can lose more than 5% of body mass per hour via evaporative water loss during periods of high environmental temperatures even if they rest in completely shaded microsites (Wolf and Walsberg 1996a; Wolf and Walsberg 1996b). Not being able to replace this water lost can cause heat stroke when their body temperatures exceed the lethal limit and dehydration when the loss of body water exceeds the limits required for homeostasis, resulting in mortality. The ability of Cockatiels to continue eating and drinking during periods of high temperatures may allow them to survive when food and water are plentiful, but the lack of these resources in the wild may become a limiting factor when temperatures are high.

The ambient temperature was a significant covariate of the proportion of time spent on low energy stationary behaviours only for the Scarlet-Chested Parrots and Regent Parrots, whereas the period of the day had a significant effect for all species, suggesting that the time budget adjustments in the afternoon was a habitual response rather than driven by higher ambient temperatures for Budgerigars, Cockatiels and Red-Collared Lorikeets. However, the ambient temperature was a significant covariate of the proportion of time spent feeding for all the psittacine birds except for Cockatiels. This suggests that high ambient temperature is a strong driving force for adjustments of feeding behaviour in these species.

The type of day (normal vs. extreme summer) was a significant factor for the proportion of time spent on low energy stationary behaviours for all the psittacine species except for Red-Collared Lorikeets, which usually inhabits tropical habitats. One possibility is that Red-Collared Lorikeets may not shift their behaviours to low energy stationary behaviours during the extreme summer days because they are not usually exposed to such high temperatures in the tropics. In general, it is predicted that a higher the degree of behavioural plasticity is to be expected when a species lives in an highly variable environment (Carroll and Corneli 1995; Komers 1997). However, one study found that hand-reared individuals of tropical birds such as Blue and Crimson-Backed Tanagers (*Thraupis cana and Ramphocelus dimidiatus*), and the Bananaquit (*Coereba flaveola*) showed a higher degree of behavioural plasticity than expected (Klopfer 1967). No studies have been performed to investigate if this is true of tropical birds born in the wild.

In contrast, the columbiform birds included in this study, Diamond Doves and Peaceful Doves, spent significantly less time on low energy stationary behaviours, i.e. were more active, during the afternoons of extreme summer days. In particular, Peaceful Doves spent more time feeding in the afternoons of extreme summer days compared to Diamond Doves, suggesting a high heat tolerance and ability to satisfy high metabolic costs even at

high ambient temperatures. It appears that columbiform birds do not support our hypothesis. Columbiform birds have a higher critical thermal maximum (CTM), i.e. the ambient temperature at which an animal loses its ability to regulate its body temperature, compared to birds belonging to other orders such as psittaciform and passeriform birds (McKechnie, Whitfield *et al.* 2016; Smith, O'Neill *et al.* 2015; Whitfield, Smit *et al.* 2015; Wolf 2015). Arid-zone columbiform birds can also utilise highly efficient cutaneous evaporative water loss during heat stress to remove heat produced or gained (Marder and Gavrieli-Levin 1987). Wild pigeons living in arid habitats are able to withstand temperatures up to 60°C for long periods without showing signs of stress (Marder 1983; Marder and Arieli 1988; Marder and Gavrieli-Levin 1987). Cutaneous evaporation is considered a more effective method of water loss than respiratory evaporative mechanisms as it prevents excess heat production from the increased respiratory muscle movement while also preventing the influx of hot ambient air into the lungs of the bird (Marder and Gavrieli-Levin 1987; McKechnie, Whitfield *et al.* 2016). The levels of cutaneous evaporation within pigeons changes to accommodate changing body temperatures. The doves observed within our study were not behaviourally affected by the extreme summer temperatures experienced and spent more time in the sun than all other species regardless of the season or time of the day. In fact, many of the doves were seen to bask under full sun for several minutes at a time during the hottest observation periods. There may therefore be fewer restrictions on other activities important to their survival in the desert, e.g. drinking, foraging and mating, during heatwaves, giving them an advantage over other birds (Walsberg 1993). However, it is difficult to make comparisons across bird orders solely based on our results, as there were small sample sizes with five psittaciform species and two columbiform species, but only one passeriform species, included in the study.

White-Browed Woodswallows spent more time on low energy stationary behaviours and less time feeding on extreme summer days, thereby supporting our hypothesis. Studies on black-faced woodswallows (close relatives of White-Browed Woodswallows) in Kakadu National Park showed that the air was their primary foraging site (75% of observations in wet season and 67% of observations in dry season) (Brooker, Braithwaite *et al.* 1990). White-Browed Woodswallows observed in this study exhibited similar behaviour, continuously searching for moths or other flying insects in the air or on substrates during all observational seasons. This preference probably has mixed effects on thermoregulation. On one hand, flight in birds is considered a highly energetic behaviour and can cost aerial foraging birds 2.7 to 5.7 times their resting energy expenditure (Flint and Nagy 1984; Masman and Klaassen 1987) while also producing high levels of heat within the body. On the other hand, the action of flight may also provide for cooling mechanisms. During flight, the undersides of wings are exposed to moving air, facilitating heat loss from the small veins via convection (Tucker 1968) while also allowing for an increase in blood circulation rate (Berger, Hart *et al.* 1970; Hart and Roy 1966). As ambient temperatures increase, the overall metabolic heat gain will exceed the convective heat loss from flight, and flight eventually becomes a disadvantage in thermoregulation.

Microsite selection

It was hypothesized that birds would select shaded microsites and avoid spending time on the ground during periods of high ambient temperature because open ground exposed to the sun is hotter than other microsites such as shaded ground or perches in shade (Ricklefs and Hainsworth 1968). The difference between the operative temperature can also be up to 18 ^{0}C higher on perches in sun compared to perches in shade (Bakken 1989). Shaded microsites also provide sources of cooling by conduction and convection (Wolf, Wooden *et al.* 1996).

Budgerigars did spend less time in the sun and on the ground on extreme summer days, particularly in the afternoon, thereby supporting our hypothesis. The other psittacine birds spent very little time in the sun overall, and this made it difficult to test our hypothesis statistically. The amount of time these birds spent on the ground also did not have a consistent pattern, possibly because there are factors other than their need to avoid high temperatures, such as individual preferences and availability of resources such as food and water, influencing their choice of microsite.

The columbiform birds included in this study did not support our hypothesis on microsite selection. The Peaceful Doves, spent significantly more time in the sun and on the ground during the afternoons compared to the mornings of extreme summer days. However, the Diamond Doves did not do this, possibly reflecting the differences in heat tolerance between the two columbiform species included in this study.

The White-Browed Woodswallows spent significantly less time in the sun on extreme summer days, particularly the afternoons, but significantly more time on the ground on the afternoons of extreme summer days, thereby only partially supporting our hypothesis.

Thermoregulatory behaviours

The smallest birds in the study, i.e. Budgerigars (compared to all other species) and White-Browed Woodswallows (compared to Scarlet-Chested Parrots, Red-Collared Lorikeets and Diamond Dove), utilised wing venting significantly more than other species of birds. This is possibly because smaller birds are in increased danger of heat stress as they have less water storage in the body for evaporative cooling, and gain heat more rapidly when the ambient temperature is higher than their body temperature due to high surface to volume ratios (Dawson 1982). It may therefore be important for smaller birds to conserve water by first

employing behaviours that help them lose body heat without losing excessive water through evaporation, i.e. wing venting rather than panting.

Other factors affecting behaviour in captive birds

Ad libitum food access, especially during seasons when wild conspecifics are experiencing limited resource availability, may allow captive birds to reduce energy expenditure and heat gain. A study into wild Arabian hoopoe-larks, a species of the Passeriformes order, indicated that birds provided with supplemented food sources during summer, and therefore allowing for *ad libitum* feeding periods, had decreased occurrences of foraging behaviours compared to un-supplemented birds (81% to 95% respectively) while increasing resting and grooming behaviours (Tieleman and Williams 2002). Early morning and afternoon, considered the optimal foraging periods, produced the greatest overall decreases in foraging compared to un-supplemented birds, around 20-40% (Tieleman and Williams 2002). This scenario may be reflected in captivity when food sources are *ad libitum* for the birds throughout the day and therefore birds may have lower energy behaviours during these morning and afternoon periods. Because food availability is consistent across all the species in our experiment, it is unlikely to be a factor in the differences observed. However, the possible effect of *ad libitum* feeding on the results of studies on captive animals must be considered when extrapolating to possible behavioural changes in wild birds exposed to heat. This particular limitation of the study design therefore only allows extrapolation to wild birds during seasons where there is relatively abundant food and water available in the desert when the birds are exposed to heatwaves.

The type of aviary that the Budgerigars and White-Browed Woodswallows were in also had a significant effect on their time budgets and microsite selections. White-Browed Woodswallows in the Mallee Aviary spent more time feeding and less time resting than

White-Browed Woodswallows in the Round-Top Aviary. This may simply be due to the fact that the Mallee Aviary has a smaller volume, and they can get from one feeding site to the next very easily. Similarly, Budgerigars in the Flat-Top Aviary spent more time feeding than the Budgerigars in the Natives Aviary that has a larger volume. The differences between the time that these two species spent in different microsites in different aviaries is also likely to be merely a reflection of the differences in the locations of the feeding trays within the aviaries.

Importance of conserving microsites in the face of climate change

Our results and those of other studies (Smit, Harding *et al.* 2013) suggest that some groups of birds, e.g. psittacine and passerine birds, start restricting their activities to shaded and cooler microsites at high temperatures, especially during the early afternoon when solar radiation levels are high. The loss of these microsites in the wild, as predicted with climate change (Garnett, Franklin *et al.* 2013), will therefore affect these groups of birds more than birds, e.g. columbiform birds, that are less reliant on these microsites in order to continue displaying their normal behaviours during heatwaves. Without shaded microsites, birds cannot seek shelter from the heat during heatwaves, thus decreasing the amount of time that birds can survive without water at high temperatures (Wolf 2000). This will in turn decrease the amount of time that birds have available to rest and "wait-out" a heatwave before they have to take the risk of flying to seek out a water source.

Habitat, and consequently microhabitat, conservation is therefore more important in ensuring the survival of these bird species as temperatures continue to rise, but unfortunately, this is also the most expensive aspect of conservation compared with other strategies such as captive breeding (Garnett, Franklin *et al.* 2013). Failure to conserve thermal refuges for these more sensitive bird species will likely lead to a restructuring of the bird populations in arid

environments, which can have unpredictable ecological effects on the rest of the ecosystem (McKechnie, Hockey *et al.* 2012; Wolf 2000). The importance of thermal refuges differs amongst species of birds (Martin, Cunningham *et al.* 2015), and the loss of these refuges has varying impacts on different species of birds, which can contribute to the restructuring of bird communities as a result of climate change.

Conclusions

This study confirmed that captive birds perform time-budget and microsite selection adjustments to varying degrees to avoid heat stress, possibly in relation to their physiological abilities to cope with high temperatures. Future studies should concentrate on studying wild counterparts of these bird species to see if this is true of wild birds living in the desert, and also the importance of physiological vs behavioural adaptations in determining the vulnerability of specific species or orders of birds to high temperatures.

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Species	Estimated average body mass (grams)	Foraging mode	Attack methods	Aviary	Volume (m^3)	Floor area (m^2)	Sample size
Diamond dove (*Geopelia cuneata*)	31.6	Granivore		Flat-top	147	70	8
Peaceful dove (*Geopelia placida*)	54.0	Granivore		Mallee	234	94	22
Budgerigar (*Melopsittacus undulates*)	26.74	Granivore	Surface and subsurface manoeuvres with no substrate manipulation	Natives	1378	300	6
				Flat-top	147	70	3
Cockatiel (*Nymphicus hollandicus*)	87.25	Granivore		Flat-top	147	70	6
Red-collared lorikeet (*Trichoglossus rubritorquis*)	124.56	Nectarivore/ Frugivore		Flat-top	147	70	11
Regent parrot (*Polytelis anthopeplus*)	177.02	Granivore		Mallee	234	94	4
Scarlet-chested parrot (*Neophema splendida*)	37.3	Granivore		Mallee	234	94	5
White-browed woodswallow (*Artamus superciliosus*)	37.0	Insectivore	Surface and wing-powered manoeuvres	Mallee	234	94	5
				Round-top	383	80	3

Table 1. Study species, estimated average body mass (HANZAB, 1999), sample sizes, foraging modes and aviary specifications.

	Significant effects of				Significantly more time on		Normal summer day		Extreme summer day	
	Day type	Period of the day	Ambient temperature	Aviary	Normal summer day	Extreme summer day	Significantly more time in		Significantly more time in	
							AM	PM	AM	PM
Budgerigar	**Wald $\chi^2_{1,80}$=36.70, *P*<0.0001**	**Wald $\chi^2_{2,80}$=287.86, *P*<0.0001**	Wald $\chi^2_{1,80}$=0.63, *P*=0.427	Wald $\chi^2_{1,80}$=3.53, *P*=0.060		**Wald $\chi^2_{1,80}$=36.70, *P*<0.0001**		**Wald $\chi^2_{1,80}$=24.20, *P*<0.0001**		**Wald $\chi^2_{1,80}$=263.66, *P*<0.0001**
Cockatiel	**Wald $\chi^2_{1,40}$=49.97, *P*<0.0001**	**Wald $\chi^2_{2,40}$=34.83, *P*<0.0001**	Wald $\chi^2_{1,40}$=1.20, *P*=0.273		**Wald $\chi^2_{1,40}$=49.97, *P*<0.0001**			**Wald $\chi^2_{1,40}$=33.33, *P*<0.0001**	No significant difference: Wald $\chi^2_{1,40}$=1.51, *P*=0.219	
Scarlet-chested parrot	**Wald $\chi^2_{1,40}$=40.56, *P*<0.0001**	**Wald $\chi^2_{2,40}$=318.77, *P*<0.0001**	**Wald $\chi^2_{1,40}$=85.98, *P*<0.0001**	Not applicable because these species were not housed in different aviaries.	**Wald $\chi^2_{1,40}$=40.56, *P*<0.0001**			**Wald $\chi^2_{1,40}$=252.40, *P*<0.0001**		**Wald $\chi^2_{1,40}$=114.90, *P*<0.0001**
Red-collared lorikeet	Wald $\chi^2_{1,40}$=0.07, *P*=0.794	**Wald $\chi^2_{2,40}$=289.30, *P*<0.0001**	Wald $\chi^2_{1,40}$=0.75, *P*=0.785		No significant difference: Wald $\chi^2_{1,40}$=0.07, *P*=0.794			**Wald $\chi^2_{1,40}$=215.22, *P*<0.0001**		**Wald $\chi^2_{1,40}$=74.08, *P*<0.0001**
Regent parrot	**Wald $\chi^2_{1,40}$=32.54, *P*<0.0001**	**Wald $\chi^2_{2,40}$=381.07, *P*<0.0001**	**Wald $\chi^2_{1,40}$=19.54, *P*<0.001**			**Wald $\chi^2_{1,40}$=32.54, *P*<0.0001**		**Wald $\chi^2_{1,40}$=222.90, *P*<0.0001**		**Wald $\chi^2_{1,40}$=237.04, *P*<0.0001**
Diamond dove	Wald $\chi^2_{1,40}$=0.57, *P*=0.449	**Wald $\chi^2_{2,40}$=11.47, *P*=0.003**	**Wald $\chi^2_{1,40}$=16.48, *P*<0.0001**		No significant difference: Wald $\chi^2_{1,40}$=0.574, *P*=0.449		No significant difference: Wald $\chi^2_{1,40}$=0.02, *P*=0.884		**Wald $\chi^2_{1,40}$=10.41, *P*=0.0001**	
Peaceful dove	**Wald $\chi^2_{1,40}$=4.35, *P*=0.037**	**Wald $\chi^2_{2,40}$=27.57, *P*<0.0001**	**Wald $\chi^2_{1,40}$=58.85, *P*<0.0001**			**Wald $\chi^2_{1,40}$=4.35, *P*=0.037**	No significant difference: Wald $\chi^2_{1,40}$=2.31, *P*=0.129		**Wald $\chi^2_{1,40}$=18.19, *P*<0.0001**	
White-browed woodswallow	**Wald $\chi^2_{1,80}$=256.07, *P*<0.0001**	**Wald $\chi^2_{2,80}$=141.87, *P*<0.0001**	**Wald $\chi^2_{1,80}$=122.61, *P*<0.0001**	**Wald $\chi^2_{1,80}$=16.31, *P*<0.0001**		**Wald $\chi^2_{1,80}$=256.07, *P*<0.0001**	**Mallee: Wald $\chi^2_{1,40}$=26.89, *P*<0.0001**			Round-top: Wald $\chi^2_{1,40}$=0.88, *P*=0.349
							Mallee: Wald $\chi^2_{1,40}$=26.89, *P*<0.0001			**Mallee: Wald $\chi^2_{1,40}$=109.89, *P*<0.0001**

Table 2. Summary of generalized linear models with binomial distributions and logit link functions analysis of the proportion of time spent on stationary behaviours by each bird species. The Wald χ^2 and P values are in bold where the results were statistically significant.

	Significant effects of				Significantly more time on		Normal summer day		Extreme summer day	
	Day type	Period of the day	Ambient temperature	Aviary	Normal summer day	Extreme summer day	Significantly more time in AM	Significantly more time in PM	Significantly more time in AM	Significantly more time in PM
Budgerigar	Wald $\chi^2_{1,80}$=2.78, *P*=0.095	**Wald $\chi^2_{2,80}$=133.33, *P*<0.0001**	**Wald $\chi^2_{1,80}$=3.69, *P*=0.055**	**Wald $\chi^2_{1,80}$=38.21, *P*<0.0001**	No significant difference: Wald $\chi^2_{1,80}$=2.78, *P*=0.095		**Flat-top: Wald $\chi^2_{1,40}$=4.13, *P*=0.042** Natives: No significant difference Wald $\chi^2_{1,40}$=2.21, *P*=0.137		**Flat-top: Wald $\chi^2_{1,40}$=32.66, *P*<0.0001**	**Natives: Wald $\chi^2_{1,40}$=62.71, *P*<0.0001**
Cockatiel	**Wald $\chi^2_{1,40}$=91.44, *P*<0.0001**	**Wald $\chi^2_{2,40}$=20.73, *P*<0.0001**	Wald $\chi^2_{1,40}$=2.70, *P*=0.100			**Wald $\chi^2_{1,40}$=91.44, *P*<0.0001**	**Wald $\chi^2_{1,40}$=19.16, *P*<0.0001**		No significant difference: Wald $\chi^2_{1,40}$=1.57, *P*=0.211	
Scarlet-chested parrot	**Wald $\chi^2_{1,40}$=28.87, *P*<0.0001**	**Wald $\chi^2_{2,40}$=232.42, *P*<0.0001**	**Wald $\chi^2_{1,40}$=57.75, *P*<0.0001**			**Wald $\chi^2_{1,40}$=28.87, *P*<0.0001**	**Wald $\chi^2_{1,40}$=178.96, *P*<0.0001**		**Wald $\chi^2_{1,40}$=90.03, *P*<0.0001**	
Red-collared lorikeet	**Wald $\chi^2_{1,40}$=8.87, *P*=0.003**	**Wald $\chi^2_{2,40}$=255.53, *P*<0.0001**	**Wald $\chi^2_{1,40}$=15.03, *P*<0.0001**	Not applicable because these species were not housed in different aviaries.	**Wald $\chi^2_{1,40}$=8.87, *P*=0.003**		**Wald $\chi^2_{1,40}$=163.20, *P*<0.0001**		**Wald $\chi^2_{1,40}$=128.10, *P*<0.0001**	
Regent parrot	**Wald $\chi^2_{1,40}$=29.49, *P*<0.0001**	**Wald $\chi^2_{2,40}$=282.87, *P*<0.0001**	**Wald $\chi^2_{1,40}$=16.06, *P*<0.0001**		**Wald $\chi^2_{1,40}$=29.49, *P*<0.0001**		**Wald $\chi^2_{1,40}$=133.75, *P*<0.0001**		**Wald $\chi^2_{1,40}$=199.27, *P*<0.0001**	
Diamond dove	**Wald $\chi^2_{1,40}$=4.18, *P*=0.041**	**Wald $\chi^2_{2,40}$=6.81, *P*<0.0001**	**Wald $\chi^2_{1,40}$=35.96, *P*<0.0001**		**Wald $\chi^2_{1,40}$=4.18, *P*=0.041**			**Wald $\chi^2_{1,40}$=4.44, *P*=0.035**	No significant difference: Wald $\chi^2_{1,40}$=0.89, *P*=0.344	
Peaceful dove	Wald $\chi^2_{1,40}$=2.24, *P*=0.134	**Wald $\chi^2_{2,40}$=26.16, *P*<0.0001**	**Wald $\chi^2_{1,40}$=33.14, *P*<0.0001**		No significant difference: Wald $\chi^2_{1,40}$=2.24, *P*=0.134		No significant difference: Wald $\chi^2_{1,40}$=2.81, *P*=0.094			**Wald $\chi^2_{1,40}$=26.15, *P*<0.0001**
White-browed woodswallow	**Wald $\chi^2_{1,80}$=285.52, *P*<0.0001**	**Wald $\chi^2_{2,80}$=145.90, *P*<0.0001**	**Wald $\chi^2_{1,80}$=148.26, *P*<0.0001**	**Wald $\chi^2_{1,80}$=10.35, *P*=0.0001**	**Wald $\chi^2_{1,80}$=285.52, *P*<0.0001**			**Round-top: Wald $\chi^2_{1,40}$=22.67, *P*<0.0001** **Mallee: Wald $\chi^2_{1,40}$=206.93, *P*<0.0001**	Round-top: Wald $\chi^2_{1,40}$=0.88, *P*=0.349	**Mallee: Wald$\chi^2_{1,40}$=109.89, *P*<0.0001**

Table 3. Summary of generalized linear models with binomial distributions and logit link functions analysis of the proportion of time spent feeding by each bird species. The Wald χ^2 and P values are in bold where the results were statistically significant.

	Significant effects of				Significantly more time on		Normal summer day		Extreme summer day	
	Day type	Period of the day	Ambient temperature	Aviary	Normal summer day	Extreme summer day	Significantly more time in		Significantly more time in	
							AM	PM	AM	PM
Budgerigar	**Wald $\chi^2_{1,80}$=557.83, *P*<0.0001**	**Wald $\chi^2_{2,80}$=13.30, *P*<0.0001**	**Wald $\chi^2_{1,80}$=119.94, *P*<0.0001**	**Wald $\chi^2_{1,80}$=4.40, *P*=0.036**	**Wald $\chi^2_{1,80}$=557.83, *P*<0.0001**		Flat-top: No significant difference Wald $\chi^2_{1,40}$=0.000, *P*=1.000 **Natives: Wald $\chi^2_{1,40}$=9.68, *P*=0.002**		Flat-top: Wald $\chi^2_{1,40}$=0.000, *P*=1.000 Natives: Could not calculate because there were too many zero values.	
Cockatiel	Wald $\chi^2_{1,40}$=0.00, *P*=1.000	**Wald $\chi^2_{2,40}$=25.05, *P*<0.0001**	**Wald $\chi^2_{1,40}$=20.25, *P*<0.0001**		No significant difference: Wald $\chi^2_{1,40}$=0.00, *P*=1.000		**Wald $\chi^2_{1,40}$=25.05, *P*<0.0001**		No significant difference: Wald $\chi^2_{1,40}$=0.00, *P*=1.000	
Scarlet-chested parrot	Wald $\chi^2_{1,40}$=0.29, *P*=0.590	**Wald $\chi^2_{2,40}$=10.42, *P*=0.005**	Wald $\chi^2_{1,40}$=0.83, *P*=0.363		No significant difference: Wald $\chi^2_{1,40}$=0.29, *P*=0.590			**Wald $\chi^2_{1,40}$=7.65, *P*=0.006**	No significant difference: Wald $\chi^2_{1,40}$=2.76, *P*=0.097	
Red-collared lorikeet	Wald $\chi^2_{1,40}$=0.00, *P*=1.000	Wald $\chi^2_{1,40}$=3.30 *P*=0.069	Wald $\chi^2_{1,40}$=3.11, *P*=0.078	Not applicable because these species were not housed in different aviaries.	No significant difference: Wald $\chi^2_{1,40}$=0.00, *P*=1.000		No significant difference: Wald $\chi^2_{1,40}$=3.30 *P*=0.069		NA	
Regent parrot	Wald $\chi^2_{1,40}$=0.00, *P*=1.000	Wald $\chi^2_{1,40}$=0.00, *P*=1.000	Wald $\chi^2_{1,40}$=3.04, *P*=0.081		No significant difference: Wald $\chi^2_{1,40}$=0.00, *P*=1.000		No significant difference: Wald $\chi^2_{1,40}$=0.00, *P*=1.000		NA	
Diamond dove	Wald $\chi^2_{1,40}$=2.46, *P*=0.117	**Wald $\chi^2_{2,40}$=133.75, *P*<0.001**	**Wald $\chi^2_{1,40}$=33.80, *P*<0.0001**		No significant difference: Wald $\chi^2_{1,40}$=0.00, *P*=0.96			**Wald $\chi^2_{1,40}$=38.74, *P*<0.0001**	**Wald $\chi^2_{1,40}$=67.81, *P*<0.0001**	
Peaceful dove	**Wald $\chi^2_{1,40}$=22.77, *P*<0.0001**	**Wald $\chi^2_{2,40}$=186.46, *P*<0.0001**	**Wald $\chi^2_{1,40}$=34.66, *P*<0.0001**			**Wald $\chi^2_{1,40}$=22.77, *P*<0.0001**		**Wald $\chi^2_{1,40}$=161.68, *P*<0.0001**		**Wald $\chi^2_{1,40}$=58.53, *P*<0.0001**
White-browed woodswallow	**Wald $\chi^2_{1,80}$=16.65, *P*<0.0001**	**Wald $\chi^2_{2,80}$=72.76, *P*<0.0001**	**Wald $\chi^2_{1,80}$=4.32, *P*=0.038**	**Wald $\chi^2_{1,80}$=32.15, *P*<0.0001**	**Wald $\chi^2_{1,80}$=16.65, *P*<0.0001**		**Round-top: Wald $\chi^2_{1,40}$=42.25, *P*<0.0001** **Mallee: Wald $\chi^2_{1,40}$=32.03, *P*<0.0001**		**Round-top: Wald $\chi^2_{1,40}$=5.48, *P*=0.019** Mallee: Wald $\chi^2_{1,40}$=0.60, *P*=0.438	

Table 4. Summary of generalized linear models with binomial distributions and logit link functions analysis of the proportion of time spent in the sun by each bird species. The Wald χ^2 and P values are in bold where the results were statistically significant. The values for budgerigars on normal summer days and red-collared lorikeets and regent parrots on extreme summer days could not be calculated because there were too many zero values.

	Significant effects of				Significantly more time on		Normal summer day		Extreme summer day	
	Day type	Period of the day	Ambient temperature	Aviary	Normal summer day	Extreme summer day	Significantly more time in		Significantly more time in	
							AM	PM	AM	PM
Budgerigar	**Wald $\chi^2_{1,80}$=4.86, *P*=0.028**	**Wald $\chi^2_{2,80}$=8.08, *P*=0.018**	**Wald $\chi^2_{1,80}$=4.83, *P*=0.028**	**Wald $\chi^2_{1,80}$=15.56, *P*<0.0001**	**Wald $\chi^2_{1,80}$=4.86, *P*=0.028**		Flat-top: Could not calculate because there were too many zero values.		Flat-top: Wald $\chi^2_{1,40}$=0.00, *P*=1.000	
								Natives: Wald $\chi^2_{1,40}$=0.00, *P*=1.000	Natives: Wald $\chi^2_{1,40}$=0.00, *P*=1.000	
Cockatiel	Wald $\chi^2_{1,40}$=0.00, *P*=1.000	**Wald $\chi^2_{2,40}$=21.08, *P*<0.0001**	**Wald $\chi^2_{1,40}$=4.51, *P*=0.034**		No significant difference: Wald $\chi^2_{1,40}$=0.00, *P*=1.000		No significant difference: Wald $\chi^2_{1,40}$=336906.67, *P*=1.000		**Wald $\chi^2_{1,40}$=21.08, *P*<0.0001**	
Scarlet-chested parrot	**Wald $\chi^2_{1,40}$=718.66, *P*<0.0001**	**Wald $\chi^2_{2,40}$=367.36, *P*<0.0001**	Wald $\chi^2_{1,40}$=1.58, *P*=0.208	Not applicable because these species were not housed in different aviaries.		**Wald $\chi^2_{1,40}$=718.66, *P*<0.0001**	**Wald $\chi^2_{1,40}$=327.04, *P*<0.0001**			**Wald $\chi^2_{1,40}$=40.33, *P*<0.0001**
Red-collared lorikeet	**Wald $\chi^2_{1,40}$=13515.81, *P*<0.0001**	**Wald $\chi^2_{2,40}$=13.73, *P*<0.0001**	Wald $\chi^2_{1,40}$=2.23, *P*=0.135			**Wald $\chi^2_{1,40}$=13515.81, *P*<0.0001**		**Wald $\chi^2_{1,40}$=13.73, *P*<0.0001**	NA	
Regent parrot	Wald $\chi^2_{1,40}$=3.48, *P*=0.062	**Wald $\chi^2_{2,40}$=152.33, *P*<0.0001**	**Wald $\chi^2_{1,40}$=162.39, *P*<0.0001**		No significant difference: Wald $\chi^2_{1,40}$=3.48, *P*=0.062		**Wald $\chi^2_{1,40}$=134.58, *P*<0.0001**			**Wald $\chi^2_{1,40}$=9.45, *P*=0.002**
Diamond dove	**Wald $\chi^2_{1,40}$=9.55, *P*=0.002**	**Wald $\chi^2_{2,40}$=63.79, *P*<0.0001**	**Wald $\chi^2_{1,40}$=12.80, *P*<0.0001**		**Wald $\chi^2_{1,40}$=9.55, *P*=0.002**		No significant difference: Wald $\chi^2_{1,40}$=1.58, *P*=0.21			**Wald $\chi^2_{1,40}$=62.96, *P*<0.0001**
Peaceful dove	**Wald $\chi^2_{1,40}$=160.98, *P*<0.0001**	**Wald $\chi^2_{2,40}$=233.17, *P*<0.0001**	Wald $\chi^2_{1,40}$=2.68, *P*=0.102			**Wald $\chi^2_{1,40}$=160.98, *P*<0.0001**		**Wald $\chi^2_{1,40}$=96.36, *P*<0.0001**		**Wald $\chi^2_{1,40}$=136.81, *P*<0.0001**
White-browed woodswallow	**Wald $\chi^2_{1,80}$=8.87, *P*=0.003**	**Wald $\chi^2_{2,80}$=69.95, *P*<0.0001**	**Wald $\chi^2_{1,80}$=9.40, *P*=0.002**	**Wald $\chi^2_{1,80}$=52.59, *P*<0.0001**		**Wald $\chi^2_{1,80}$=8.87, *P*=0.003**	**Round-top: Wald $\chi^2_{1,40}$=22.50, *P*<0.0001 Mallee: Wald $\chi^2_{1,40}$=21.53, *P*<0.0001**			**Round-top: Wald $\chi^2_{1,40}$=8.24, *P*=0.004** Mallee: Wald $\chi^2_{1,40}$=1.52, *P*=0.218

Table 5. Summary of generalized linear models with binomial distributions and logit link functions analysis of the proportion of time spent on the ground by each bird species. The Wald χ^2 and P values are in bold where the results were statistically significant. The values for red-collared lorikeets on extreme summer days could not be calculated because there were too many zero values.

	Budgerigar	Cockatiel	Scarlet-chested parrot	Red-collared lorikeet	Regent parrot	Diamond dove	Peaceful dove	White-browed woodswallow
Budgerigar (Mean=0.4634 ±0.12603)	NA	**Mean difference=0.293, P=0.032**	**Mean difference=0.461, P=0.007**	**Mean difference=0.453, *P*=0.007**	**Mean difference=0.343, *P*=0.016**	**Mean difference=0.463, *P*=0.007**	**Mean difference=0.403, *P*=0.010**	**Mean difference=0.319, *P*=0.016**
Cockatiel (Mean=0.1702 ±0.05416)	**Mean difference= -0.293, *P*=0.032**	NA	Mean difference=0.168, *P*=0.051	Mean difference=0.160, *P*=0.066	Mean difference=0.049, *P*=1.000	**Mean difference=0.170, *P*=0.047**	Mean difference=0.110, *P*=0.475	Mean difference=0.026, *P*=1.000
Scarlet-chested parrot (Mean=0.0023 ±0.00336)	**Mean difference= -0.461, *P*=0.007**	Mean difference= -0.168, *P*=0.051	NA	Mean difference= -0.008, *P*=1.000	Mean difference= -0.119, *P*=0.094	Mean difference=0.002, *P*=1.000	Mean difference=-0.058, *P*=0.427	**Mean difference= -0.142, *P*=0.021**
Red-collared lorikeet (Mean=0.0106 ±0.00799)	**Mean difference= -0.453, *P*=0.007**	Mean difference= -0.160, *P*=0.066	Mean difference=0.008, *P*=1.000	NA	Mean difference= -0.110, *P*=0.142	Mean difference=0.011, *P*=1.000	Mean difference= -0.050, *P*=0.975	**Mean difference= -0.134, *P*=0.029**
Regent parrot (Mean=0.1209 ±0.04074)	**Mean difference= -0.343, *P*=0.016**	Mean difference= -0.049, *P*=1.000	Mean difference=0.119, *P*=0.094	Mean difference= 0.110, *P*=0.142	NA	Mean difference=0.121, *P*=0.084	Mean difference=0.061, *P*=1.000	Mean difference= -0.234, *P*=1.000
Diamond dove (Mean=0.000± 0.00000)	**Mean difference= -0.463, *P*=0.007**	**Mean difference= -0.170, *P*=0.047**	Mean difference= -0.002, *P*=1.000	Mean difference= -0.011, *P*=1.000	Mean difference= -0.121, *P*=0.084	NA	Mean difference= -0.060, *P*=0.341	**Mean difference= -0.144, *P*=0.019**
Peaceful dove (Mean=0.0604 ±0.02409)	**Mean difference= -0.403, *P*=0.010**	Mean difference= -0.110, *P*=0.475	Mean difference=0.058, *P*=0.427	Mean difference= 0.050, *P*=0.975	Mean difference= -0.061, *P*=1.000	Mean difference= 0.060, *P*=0.341	NA	Mean difference= -0.084, *P*=0.431
White-browed woodswallow (Mean=0.1443 ±0.04253)	**Mean difference= -0.319, *P*=0.016**	Mean difference= -0.026, *P*=1.000	**Mean difference=0.142, *P*=0.021**	**Mean difference= 0.134, *P*=0.029**	Mean difference= 0.234, *P*=1.000	**Mean difference= 0.144, *P*=0.019**	Mean difference= 0.084, *P*=0.431	NA

Table 6. Summary of generalized linear models with Poisson distribution and logit link functions analysis of the mean number of wing venting events per observation period by each bird species. The mean difference and P values are in bold where the results were statistically significant.

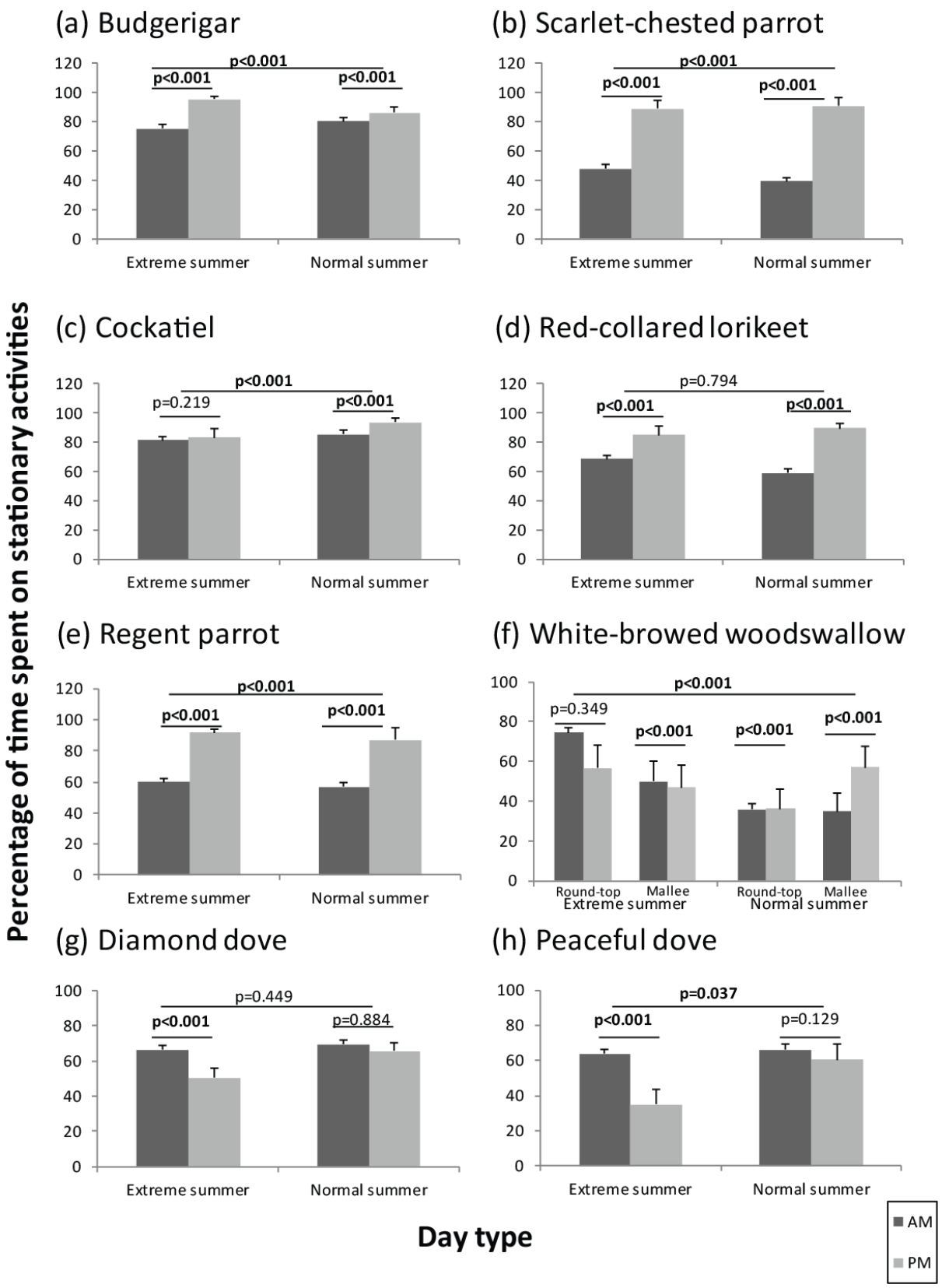
(a) Budgerigar
p<0.001
p<0.001
p<0.001
(b) Scarlet-chested parrot
p<0.001
p<0.001
p<0.001
(c) Cockatiel
p=0.219
p<0.001
p<0.001
(d) Red-collared lorikeet
p=0.794
p<0.001
p<0.001
(e) Regent parrot
p<0.001
p<0.001
p<0.001
(f) White-browed woodswallow
p=0.349
p<0.001
p<0.001
p<0.001
Round-top
Mallee
Round-top
Mallee
Extreme summer
Normal summer
(g) Diamond dove
p=0.449
p<0.001
p=0.884
(h) Peaceful dove
p=0.037
p<0.001
p=0.129
Extreme summer
Normal summer
Percentage of time spent on stationary activities
Day type
AM
PM

Figure 1. Percentage of time spent on stationary behaviour (see Methods) varied amongst species, day type and with time of day. Horizontal bars represent comparisons between seasons, and bold p-values indicate a significant difference between morning (AM) and afternoon (PM) observations. (a) budgerigar, (b) scarlet-chested parrot, (c) cockatiel, (d) red-collared lorikeet, (e) regent parrot, (f) white-browed woodswallow, (g) diamond dove, and (h) peaceful dove.

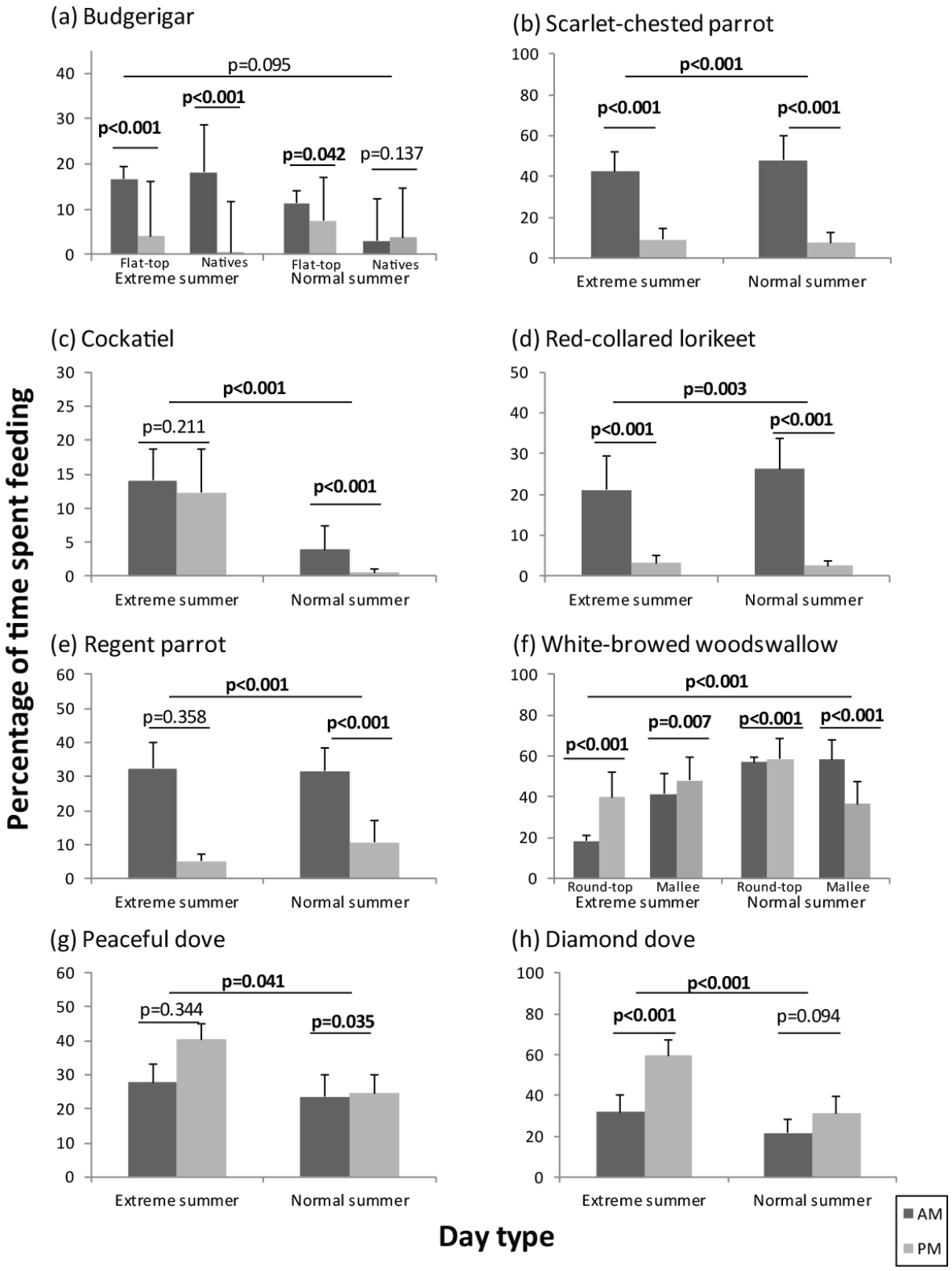
(a) Budgerigar
p=0.095
p<0.001
p<0.001
p=0.042
p=0.137
Flat-top
Natives
Flat-top
Natives
Extreme summer
Normal summer
(b) Scarlet-chested parrot
p<0.001
p<0.001
p<0.001
Extreme summer
Normal summer
(c) Cockatiel
p<0.001
p=0.211
p<0.001
Extreme summer
Normal summer
(d) Red-collared lorikeet
p=0.003
p<0.001
p<0.001
Extreme summer
Normal summer
(e) Regent parrot
p<0.001
p=0.358
p<0.001
Extreme summer
Normal summer
(f) White-browed woodswallow
p<0.001
p<0.001
p=0.007
p<0.001
p<0.001
Round-top
Mallee
Round-top
Mallee
Extreme summer
Normal summer
(g) Peaceful dove
p=0.041
p=0.344
p=0.035
Extreme summer
Normal summer
(h) Diamond dove
p<0.001
p<0.001
p=0.094
Extreme summer
Normal summer
Percentage of time spent feeding
Day type
AM
PM

Figure 2. Percentage of time spent feeding varied amongst species, day type and with time of day. Horizontal bars represent comparisons between seasons, and bold p-values indicate a significant difference between morning (AM) and afternoon (PM) observations. (a) budgerigar, (b) scarlet-chested parrot, (c) cockatiel, (d) red-collared lorikeet, (e) regent parrot, (f) white-browed woodswallow, (g) diamond dove, and (h) peaceful dove.

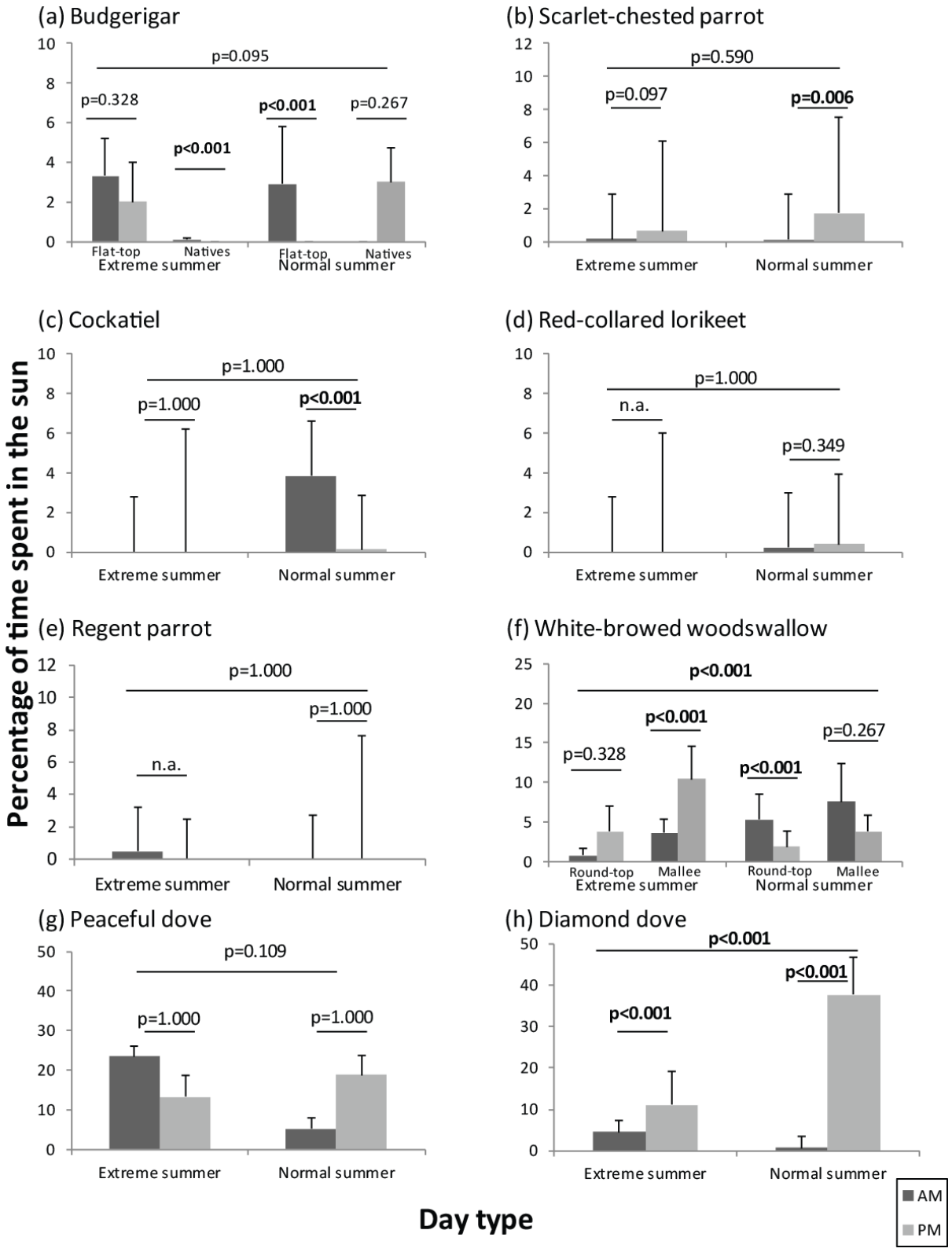
(a) Budgerigar
p=0.095
p=0.328
p<0.001
p<0.001
p=0.267
Flat-top
Natives
Flat-top
Natives
Extreme summer
Normal summer
(b) Scarlet-chested parrot
p=0.590
p=0.097
p=0.006
Extreme summer
Normal summer
(c) Cockatiel
p=1.000
p=1.000
p<0.001
Extreme summer
Normal summer
(d) Red-collared lorikeet
p=1.000
n.a.
p=0.349
Extreme summer
Normal summer
(e) Regent parrot
p=1.000
p=1.000
n.a.
Extreme summer
Normal summer
(f) White-browed woodswallow
p<0.001
p<0.001
p=0.328
p<0.001
p=0.267
Round-top
Mallee
Round-top
Mallee
Extreme summer
Normal summer
(g) Peaceful dove
p=0.109
p=1.000
p=1.000
Extreme summer
Normal summer
(h) Diamond dove
p<0.001
p<0.001
p<0.001
Extreme summer
Normal summer
Percentage of time spent in the sun
Day type
AM
PM

Figure 3. Percentage of time spent in the sun varied amongst species, day type and with time of day. Horizontal bars represent comparisons between seasons, and bold p-values indicate a significant difference between morning (AM) and afternoon (PM) observations. (a) budgerigar, (b) scarlet-chested parrot, (c) cockatiel, (d) red-collared lorikeet, (e) regent parrot, (f) white-browed woodswallow, (g) diamond dove, and (h) peaceful dove.

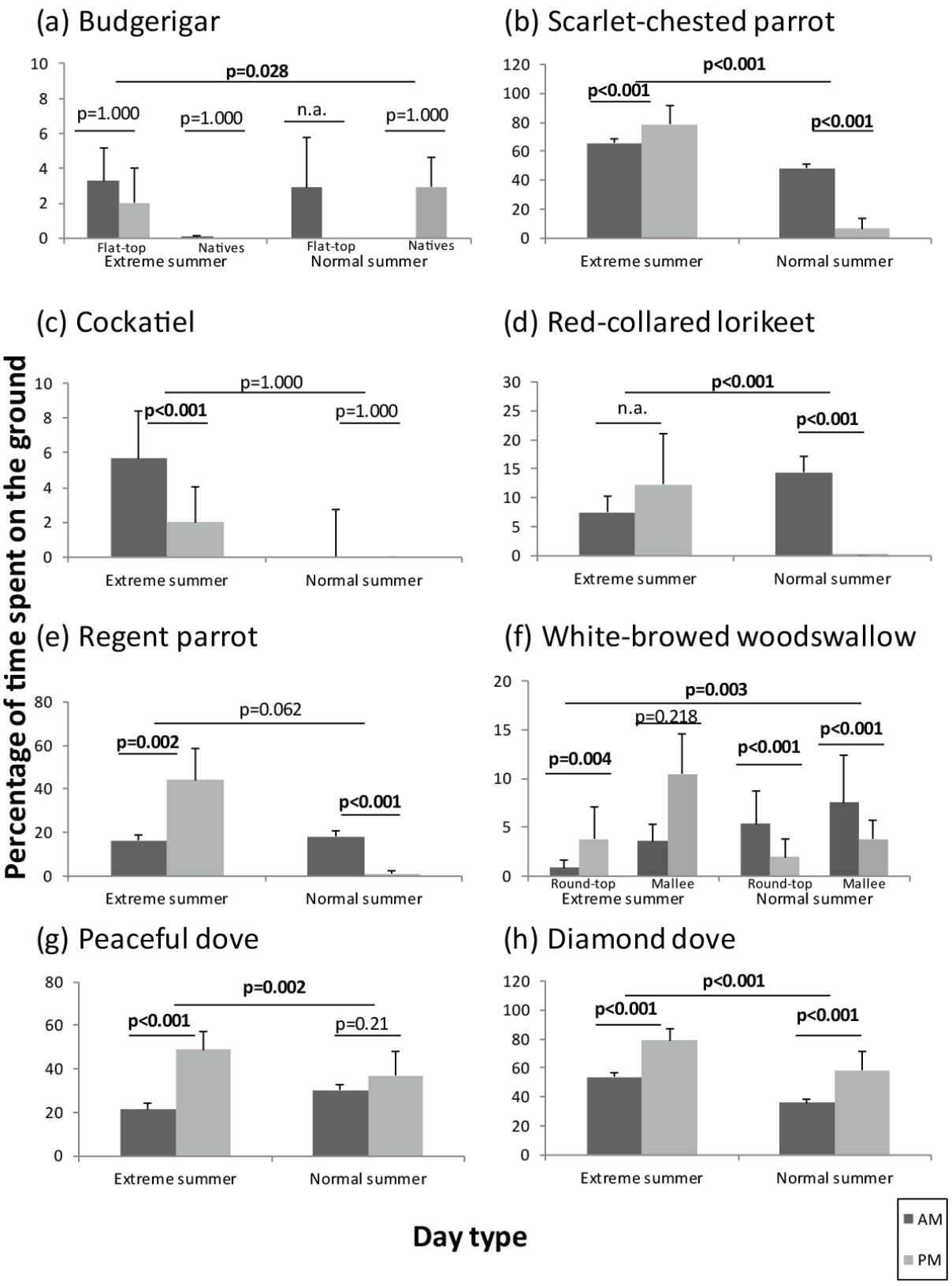
(a) Budgerigar
p=0.028
p=1.000
p=1.000
n.a.
p=1.000
Flat-top
Natives
Flat-top
Natives
Extreme summer
Normal summer
(b) Scarlet-chested parrot
p<0.001
p<0.001
p<0.001
Extreme summer
Normal summer
(c) Cockatiel
p=1.000
p<0.001
p=1.000
Extreme summer
Normal summer
(d) Red-collared lorikeet
p<0.001
n.a.
p<0.001
Extreme summer
Normal summer
Percentage of time spent on the ground
(e) Regent parrot
p=0.062
p=0.002
p<0.001
Extreme summer
Normal summer
(f) White-browed woodswallow
p=0.003
p=0.218
p<0.001
p=0.004
p<0.001
Round-top
Mallee
Round-top
Mallee
Extreme summer
Normal summer
(g) Peaceful dove
p=0.002
p<0.001
p=0.21
Extreme summer
Normal summer
(h) Diamond dove
p<0.001
p<0.001
p<0.001
Extreme summer
Normal summer
Day type
AM
PM

Figure 4. Percentage of time spent on the ground varied amongst species, day type and with time of day. Horizontal bars represent comparisons between seasons, and bold p-values indicate a significant difference between morning (AM) and afternoon (PM) observations. (a) budgerigar, (b) scarlet-chested parrot, (c) cockatiel, (d) red-collared lorikeet, (e) regent parrot, (f) white-browed woodswallow, (g) diamond dove, and (h) peaceful dove.

Chapter 4 Organ histopathology and haematological changes associated with heat exposure in Australian desert birds

Pathology is expected in birds when mechanisms to cope with high environmental temperatures, such as the ones discussed in chapters 2 and 3, break down. However, there has been very little documentation of the histopathological and haematological changes in birds exposed to high temperatures. Most of the current published data relate to poultry, which has very little application to Australian desert birds. The following original research article, accepted with minor revisions for publication to the *Journal of Avian Medicine and Surgery*, demonstrates the histopathological changes in the same Australian desert birds from chapter 3. There were again interesting interspecific differences.

Xie, S., L. Woolford and T.J. McWhorter. In press. Organ histopathology and haematological changes associated with heat exposure in three species of Australian desert birds. *Journal of Avian Medicine and Surgery*. **Accepted with minor revisions (Manuscript number 2017-263R2), awaiting final acceptance**.

Statement of Authorship

Title of Paper	Organ histopathology and haematological changes associated with heat exposure in Australian desert birds
Publication Status	☐ Published ☑ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Xie, S.,Woolford L., & McWhorter, T. J. (2018) accepted with minor revisions by the Journal of Avian Medicine and Surgery, revision submitted and currently awaiting final acceptance.

Principal Author

Name of Principal Author (Candidate)	Shangzhe Xie		
Contribution to the Paper	Collected, trimmed and processed all samples. Performed histopathological analysis on all samples, interpreted data, wrote manuscript and acted as corresponding author.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	10/8/18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Lucy Woolford		
Contribution to the Paper	Helped to verify histopathological findings and edit the manuscript.		
Signature		Date	30/08/2018

Name of Co-Author	Todd McWhorter		
Contribution to the Paper	Supervised development of work, helped in data interpretation and manuscript evaluation.		
Signature		Date	30/8/2018

Organ histopathology and haematological changes associated with heat exposure in Australian desert birds

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Abstract

An inability of the body to cope with extreme temperatures will eventually lead to heat illness. Mild heat exposure to a temperature above the zone of thermoneutrality can induce subclinical heat stress, which may be a precursor to heat illnesses. The ability to identify subtle changes that may be associated with subclinical heat stress can be important in early diagnosis and treatment of heat stress in birds. Organ changes post- heat exposure were histopathologically examined in 13 budgerigars, 15 zebra finches and 8 diamond doves as model species for the bird orders Psittaciformes, Passeriformes and Columbiformes respectively. There was mild to moderate congestion of the lungs of 28/36 birds examined, including all of the budgerigars and diamond doves. 8/15 zebra finches had no significant lung congestion. Interstitial and pulmonary haemorrhage was in observed in one diamond dove. The most common hepatic change found was micro- and macro-vesicular hepatocellular vacuolation (4/15 zebra finches, 5/13 budgerigars and 8/8 diamond doves). There were mild to moderate congestion in the kidneys of 1/15 zebra finch, 2/13 budgerigars and 4/8 diamond doves, as well as the gastrointestinal tract of 1/15 zebra finch and 7/8 budgerigars. Budgerigars showed a decrease in haematocrit and a significant change in heterophil and lymphocyte proportions after heat exposure. There were no changes in basophil proportions in both budgerigars and diamond doves. These findings indicate species differences in the organ and haematological changes post-heat exposure amongst birds.

Keywords

avian; heat stress; heat injury; desert birds

Introduction

Extreme environmental temperatures exceeding a bird's ability to thermoregulate will eventually lead to heat illness. Heat illnesses are most well-studied in humans, and are classified as a cascade of heat cramps, heat exhaustion and heat stroke according to the increasing severity of symptoms [1]. Based on scientific [2-6] and anecdotal news reports [4, 7], heat waves have been associated with mass mortality events in desert dwelling birds. However, by the time they are found dead they have presumably gone through the heat injury cascade [1] and suffered the effects of heat stroke. In order to maintain body temperatures within the normal range and prevent organal damage to their tissues, birds need to thermoregulate. They can achieve this by utilizing a combination of strategies, including thermoregulatory behaviours such as wing venting, panting and gular (lingual) fluttering [8], as well as cutaneous evaporation by vasodilating the dermal capillary bed [9].

Pathological findings in animals that have died from heat illness are most often manifested in vascular and coagulation aberrations, such a congestion, haemorrhage and thrombosis [10]. Histopathological reports of birds dying from heat stroke are uncommon, likely because the quick onset of autolysis under high temperatures precludes any useful post-mortem examination unless the carcasses were collected and examined immediately or stored appropriately until examination could be carried out. Overstreet & Rehak (1980) reported that chicks of the Least tern (*Sterna albifrons*) showed microscopic evidence of early ischaemic focal necrosis in the brain, characterised by neuronal degeneration, leukocytic infiltration and degeneration of the neuropil surrounding some cortical peripheral blood vessels. The splenic sinuses were also packed with red blood cells which may be an indication of circulatory collapse [11]. This change can also be an indication of active inflammation, for example during septicaemia. Additionally, there were small areas of degeneration in the liver, kidneys, and intestine [11]. Unfortunately, the lungs, heart, and other tissues were not examined in this report.

In another report, the pathological changes of a sun conure that was suspected to have suffered heat stroke under general anaesthesia revealed mild multifocal acute degeneration and contraction band necrosis of the biceps femoris muscle, as well as diffuse moderate acute congestion of the lungs. There was also a moderate left ventricular hypertrophy, but that was suspected to be a coincidental finding that likely contributed to the bird's death rather than a result of the presumed heat stroke [12].

In the present study, we describe the pathological findings in the gastrointestinal tract, liver, lungs, kidneys, adrenal glands, gonads, brain and eyes, as well as haematological changes, of zebra finches (*Taeniopygia guttata*, order Passeriformes), budgerigars (*Melopsittacus undulates*, order Psittaciformes) and diamond doves (*Geopelia cuneata*, order Columbiformes) after heat exposure under standardized conditions above the zone of thermoneutrality in these species that should result in subclinical heat stress.

Materials and methods

Animals. Thirteen budgerigars, 15 zebra finches and 8 diamond doves were used as model species for the bird orders Psittaciformes, Passeriformes and Columbiformes respectively. These species were chosen because they are found naturally in the Australian desert, and are also widely bred in captivity for the pet industry. After they were obtained from private bird breeders in Adelaide from 2014 to 2015, the birds were kept in outdoor aviaries at the University of Adelaide Roseworthy Campus (Roseworthy, South Australia). The birds had food and water available ad libitum in the aviaries. They were allowed to acclimatize in the outdoor aviaries for at least 2 weeks before being subjected to the experimental protocol to allow their hypothalamic-pituitary-adrenal (HPA) axis to normalize [13]. All experiments were

conducted according to the Australian code for the care and use of animals for scientific purposes and approved by the University of Adelaide Animal Ethics Committee.

Experimental setup. Experiments were conducted in a room with temperature maintained at 25°C with a built-in air-conditioner unit. Two egg incubators (IM 504 egg digital incubator; Incubators & More Pty. Ltd., Australia), each measuring 130cm × 46cm × 47cm, were placed 2.5 m in the room facing away from each other so that birds in each incubator could be approached without birds in the other one being affected. During the experiments, each bird was placed in a cage measuring 60cm × 40cm × 40cm with water and food available ad libitum and the entire birdcage was placed in the incubator. A digital video camera was placed inside of each incubator so that each bird could be monitored remotely from another room. A total of three temperature data loggers (Thermochron iButton DS1922L; Maxim Integrated, USA) were placed in the experimental room – one in each incubator and one in the room. Temperature measurements were taken every two minutes throughout the protocol.

Experimental protocol. Each bird was moved into the exposure chamber set at 25°C at least 18 hours before blood was collected between 0900 and 0915. The bird was then returned to the incubator with access to food and water for 2 hours before any change in temperature was instigated by turning the incubator to the set temperature of 35°C, a temperature similar to what they experience during a typical summer day (control group), or 45°C, similar to that experienced during a heat wave (experimental group). The incubator exposed the bird to heat up to a maximum temperature of 34.42±0.12°C when the incubator was set to 35°C and up to a maximum temperature of 43.18±0.12°C when it was set to 45°C. The control group was therefore exposed to a temperature that was within the zone of thermal neutrality for all 3

species, i.e. 29 to 41°C for budgerigars [14], 29.5 to 40°C for zebra finches [15] and 34 to 40°C for diamond doves [16] (which was a lower upper critical temperature compared to 45°C from an older study on the same species [17]). On the other hand, the experimental group was exposed to a temperature above the zone of thermal neutrality for all 3 species.

Each bird was randomly assigned to the first exposure temperature using a random number generator [18]. The bird was then exposed to the predetermined temperature for two hours and a second blood sample collected. Less than 10% of total blood volume was collected on any experimental day.

The bird was also monitored using digital video camera during each temperature exposure. The criteria for removal from the experiment included constant escape activity lasting more than 10 minutes, behavioural signs of distress (e.g. closed eyes, fluffed feathers and inactivity) and loss of righting reflex. However, none of these behaviours were observed throughout the experiment.

The same bird was then exposed to the other temperature at least one week later. At the end of exposure to this temperature, euthanasia was performed using 100mg/kg of sodium pentobarbitone intraperitoneal. The bird's gastrointestinal tract, liver, lungs and kidneys were collected and stored in 10 % neutral buffered formalin. 6 budgerigars, 7 zebra finches and 4 diamond doves were exposed to 35°C before euthanasia; 7 budgerigars, 8 zebra finches and 4 diamond doves were exposed to 45°C before euthanasia.

Two zebra finches, one budgerigar and six diamond doves also had their heads collected, in addition to the above organs, and stored in formalin to allow histopathological assessment of the brain and eyes. The skulls and periorbital tissues were cut to allow formalin to penetrate and fix the brain and eyes sufficiently before the tissues were trimmed.

Histopathology. Formalin-fixed samples of the organs collected were trimmed and embedded in paraffin blocks, sections cut at 3-4 μm, and then mounted and stained with haematoxylin & eosin for routine histopathological examination. The slides were examined at the Veterinary Diagnostic Laboratory, University of Adelaide, Roseworthy, South Australia using a BX53 microscope (Olympus, Japan), and photomicrographs were captured using Labsens (Olympus, Japan). The spleen of 1 budgerigar, pancreas of 4 budgerigars, the ovaries of 1 zebra finch and the adrenal glands of 1 zebra finch were included in the tissues collected. These additional organs were also examined. The organs of 1 zebra finch, 1 budgerigar and 1 diamond dove were also stained with Periodic acid–Schiff (PAS) and examined.

Haematology. Sufficient blood was collected from 8 budgerigars and 8 diamond doves for haematological analyses. The serum from these blood samples were used for corticosterone analyses that were published in a separate paper[19], and there was insufficient serum to perform either total protein or biochemistry analyses. The packed cell volume (PCV) was measured for each blood sample collected by spinning the blood samples within haematocrit tube a centrifuge at 10 000 rpm for 5 minutes and measuring using a haematocrit reading device. Blood smears were air-dried, fixed in absolute methanol and then stained with Wright-Giemsa using a Siemens Hema-Tek automatic stainer. The slides were examined by one person who was blind to the sequence of temperature exposures and whether samples were from control or treatment birds. The first one hundred leukocytes per slide were identified and counted as lymphocytes, heterophils, basophils, monocytes or eosinophils. The total white blood cell number was estimated by adding up the total number of white blood

cells counted from 10 different microscopic fields at 40X and multiplying the total by 200 [20]. A subsample of smears (n = 15) were examined twice, and repeatability analyses performed.

Statistics. The incidence ratio for each bird species and pathological finding was calculated (Table 1). A paired samples t-test was used to analyze the changes PCV and each white blood cell type (SPSS 21.0, IBM Corp., Armonk, NY). These values were reported as mean +/- SD (Table 2).

Results

Histopathology. Seven budgerigars, 8 zebra finches and 4 diamond doves were exposed to 45°C before euthanasia (experimental group) and 6 budgerigars, 7 zebra finches and 4 diamond doves were exposed to 35°C before euthanasia (control group) The significant histopathological findings are summarized in Table 1. Heart, lung, liver, kidney and gastrointestinal tract were examined in all birds.

There was mild to moderate congestion of the lungs (Figure 1) of 28 of the 36 birds examined, including all the budgerigars and diamond doves. Eight of 15 zebra finches had no significant lung congestion. Interstitial and airway haemorrhage was in observed in one diamond dove (Figure 2). The most common finding in the livers was micro- and macro-vesicular hepatocellular vacuolation (Figure 3); observed in in four zebra finches, five budgerigars and all eight diamond doves. Hepatic lipidosis was considered likely due to the size and appearance of vacuoles, including the displacement of nuclei and other features were more consistent with lipid rather than glycogen. The PAS stains, although only performed on a subset of the samples, were also negative for glycogen. However, special stains for the

detection of lipid, such as oil-red-O stain, were not performed to confirm that hepatic lipidosis was the cause of the hepatocellular vacuolation. Mild to moderate hepatic sinusoidal congestion was also seen in two zebra finches and a budgerigar (Figure 4). There were no significant findings in the kidneys except for mild to moderate congestion (Figure 5) in one zebra finch, two budgerigars and four diamond doves. Similarly, the gastrointestinal tract appeared normal except for mild to moderate congestion of submucosal vessels in a zebra finch and seven budgerigars. Mild lymphoid or haematopoietic aggregates found in some of the lung, liver, kidney and gastrointestinal samples, but these were within normal limits and considered non-significant. All hearts appeared microscopically unremarkable except for one bird that had focal infiltration of adipocytes within the myocardium and mild myocardial vacuolation. The latter may be intracellular lipid reflective of acute myocardial vacuolar degeneration that may be an age-related change but can also occur with acute ischaemic injury, albeit unlikely. There were no abnormalities found in the brain (n = 9), eyes (n = 9), spleen (n = 1), pancreas (n = 4), gonads (n = 1) and adrenal glands (n= 1) for the subset of birds in which these organs were examined.

Haematology. After exposure to 35°C, there was a significant increase in total white blood cells and heterophil proportion, as well as a significant decrease in PCV and monocyte proportion in budgerigars. After exposure to 45°C, there was a significant increase in heterophil proportion, as well as a significant decrease in PCV, lymphocyte and monocyte proportion in budgerigars. There was a significant decrease in eosinophil proportion after exposure to 35°C in diamond doves. The haematology results are summarized in Table 2.

Discussion

Mild heat exposure to a temperature above the zone of thermoneutrality can induce subclinical heat stress, which may be a precursor to heat illnesses. The ability to identify subtle changes that may be associated with subclinical heat stress can be important in early diagnosis and treatment of heat stress in birds. The paucity of reports and studies of heat stress and illnesses in birds means that our understanding of the syndrome is based on mammalian studies. Heatstroke itself is relatively well-studied and understood in species such as baboons [21], rats [22], guinea pigs [23] and rabbits [24, 25] serving as experimental models for heat stroke, as well as in dogs [26, 27] due to its relatively common occurrence in veterinary practice [28]. One study in baboons suffering from induced heat stroke demonstrated damage to multiple organs including the jejunum, liver, spleen, lung and kidney manifesting as vascular congestion, haemorrhage, thrombosis, increased inflammatory cells, and disruption of normal cell and tissue architecture [10].

It is perhaps not surprising that most of the research regarding heat stress in birds has been in the poultry industry given its implications for productivity. The effects of heat stress in poultry include haematological changes such as decreased haematocrit, increased heterophil to lymphocyte ratio and basophil proportion [29, 30], biochemical changes such as decreased calcium and phosphorus, decrease in egg quality and quantity, increased mortality rate and adverse effects on semen characteristics and sperm function [31]. In our study, only budgerigars showed a decrease in haematocrit after heat exposure. There was also only a significant change in heterophil and lymphocyte proportions in budgerigars. There were no changes in basophil proportions in both budgerigars and diamond doves. These differences indicate that the haematological changes associated with heat exposure may vary according to the avian species.

Acute heat stress has been shown to cause oxidative damage to cells [29, 32]. This was not specifically investigated in our study, but there were no signs of apoptosis observed, such as hypersegmented apoptotic heterophils nor an increase in the fraction of lysed leukocytes as indicated by basket cells.

Chronic heat stress has been shown to cause pathological organ changes in broilers including right atrial and ventricular hypertrophy, myofibrillar degeneration and haemorrhage, diffuse myocarditis and general vacuolar degeneration of myofibres of the heart; pulmonary congestion, oedema and hyperemia; yellow and pale livers with vacuolar degeneration of hepatocytes and sinusoidal dilation; oedema and haemorrhage in the subrenal capsule with glomerular damage and vacuolar degeneration of the kidneys [33]. Some of these changes appear to be similar to those found in the previously mentioned acute heat stroke case.

Although these poultry studies are helpful in aiding our understanding of the effects of heat stress in birds, they do not include temperatures above 40°C, which desert birds frequently experience. Moreover, poultry species belong to the order Galliformes, whereas desert birds include other orders. Poultry species are also highly selected and potentially inbred to perform very specific functions such as muscle growth and egg-laying. The focus of the effects of heat stress on poultry is therefore on the decreased growth and egg-laying, rather than mortality. However, reports of large scale bird mortalities associated with heatwaves suggest that death can result quickly from exposure to heat, and yet the basic understanding of the pathogenicity of heat exposure is unclear. The results from our study would therefore begin to demonstrate some of the changes in the organs of birds exposed to heat that may contribute to their eventual demise if the ambient temperature continued to increase.

Vascular congestion, haemorrhage, thrombosis, increased inflammatory cells, architecture disruption and cellular apoptosis of the jejunum, liver, spleen, lung and kidneys were found in baboons suffering from heatstroke [10]. Hyperaemia, oedema, haemorrhage and necrosis of the skin, lungs, heart, intestines, spleen, kidneys, liver and brain were also found in dogs suffering from heatstroke [26, 34-37]. Compared to mammals, birds have a few physiological advantages for surviving heatwaves. They maintain high body temperatures close to lethal limits due to high mass-specific metabolic rates and high surface area to volume ratios that tightly couple their environmental temperatures to their body temperatures [38]. These physiological traits result in high rates of evaporative water loss that is advantageous in hot climates [38], but that still needs to be balanced with water conservation in the desert [39]. Small birds can lose more than 5% of body mass per hour via evaporative water loss during periods of high environmental temperatures even when resting in completely shaded microsites [40, 41], which can quickly lead to dehydration and other pathological changes. There was no significant increase in PCV after heat exposure in the budgerigars and diamond doves in our study. On the contrary, there were significant decreases in PCV in the budgerigar after heat exposure. However, future studies should also measure total protein as a better measure of dehydration.

Some groups of birds also have evolved thermoregulatory adaptations. Small passerine birds have high mass-specific metabolic rates and produce heat internally at a high rate [42]. Coupled with their high respiratory rate, this results in a high rate of pulmonary water loss [42]. While this helps to prevent their body temperature from rising to lethal levels, it also increases the water requirements. Parrots can utilise gular fluttering in conjunction with panting to increase the efficiency of heat loss while panting [14, 43, 44]. Arid columbiform birds can also utilise highly efficient cutaneous evaporative water loss during heat stress to remove heat [45]. Wild pigeons living in arid habitats are able to withstand temperatures up to 60°C for

long periods without showing signs of stress [45-47]. Cutaneous evaporation is considered a more effective method of water loss than respiratory evaporative mechanisms as it prevents excess heat production from the increased respiratory muscle movement while also preventing the influx of hot ambient air into the lungs of the bird [45]. Inca doves (*Columbina inca*) have also been shown to be able to control cloacal evaporation as a means of thermoregulation at high ambient temperatures [48].

Given that different organs are involved in the different thermoregulatory strategies employed by different species of birds, it is expected that there will be different histopathological changes when they are exposed to heat. It would appear that the birds subjected to our experimental protocol were starting to develop microscopic changes consistent with heat exposure, albeit to different extents in different organs. A lower percentage of zebra finches (46.7%) developed congestion in the lungs compared to budgerigars and diamond doves (100%). A higher percentage of budgerigars developed congestion in the intestinal tract (53.8%) compared to zebra finches (6.7%) and diamond doves (0%). A higher percentage of diamond doves (50%) developed congestion in the kidneys compared to zebra finches (6.7%) and budgerigars (15.4%). These differences are interesting because they may reflect variation in organs that respond to heat related vasodilation and subsequent pooling of blood in the different bird species. This may be similar to the concept of 'shock organs' in mammals suffering from anaphylactic shock. For example, the lungs are considered the classic shock organ of the guinea pig, rabbit [49], cats [50] and goats [51] during anaphylaxis, whereas the liver and gastrointestinal tract appears to be more affected in dogs [52, 53] and rats [54]. This hypothesis could be further supported if budgerigars presented more commonly with gastrointestinal signs, e.g. diarrhoea and vomiting, and diamond doves presented more commonly with signs of respiratory distress

when exposed to heat, but such data are currently unavailable. The birds in this study were not showing any clinical signs at the time of euthanasia.

Alternatively, variation in organ congestion after heat exposure between these three species may reflect differences in efficiency of thermoregulation and the primary organ for heat loss. If this were the case, the kidneys of zebra finches (passerine), lungs of budgerigars (psittaciform) and gastrointestinal tracts of diamond doves (columbiform) would be the organs most likely to exhibit histopathological changes. However, our data reflects that zebra finches had the least kidney changes and none of the diamond doves displayed any gastrointestinal changes, which does not support this hypothesis.

There were limitations to our study design. Intracoelomic injection of sodium pentobarbitone may result in histological artefacts caused by as caustic burns to the serosae and subserosal tissue of coelomic organs, as well as congestive changes in the organs associated both with the action of the drug [55]. However, the congestive changes observed and reported in this study were throughout the organs, and were not uniformly reported in all birds, nor in the same organs across the three species, despite all birds receiving the same method of euthanasia. Therefore it is plausible to attribute congestive changes, if not in entirety, to the physiological consequences of heat exposure. Also, only a small subset of birds had their brains, eyes, spleen, pancreas, ovaries and adrenals examined, so even though no obvious histopathological changes were observed in these organs, the sample size was small and limited the inferences that can be made from the lack of changes. Future studies should investigate biochemical changes associated with heat exposure, and also investigate if there may be more significant histopathological changes in birds when exposed to higher temperatures, i.e. those that induce heat stroke.

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Figures

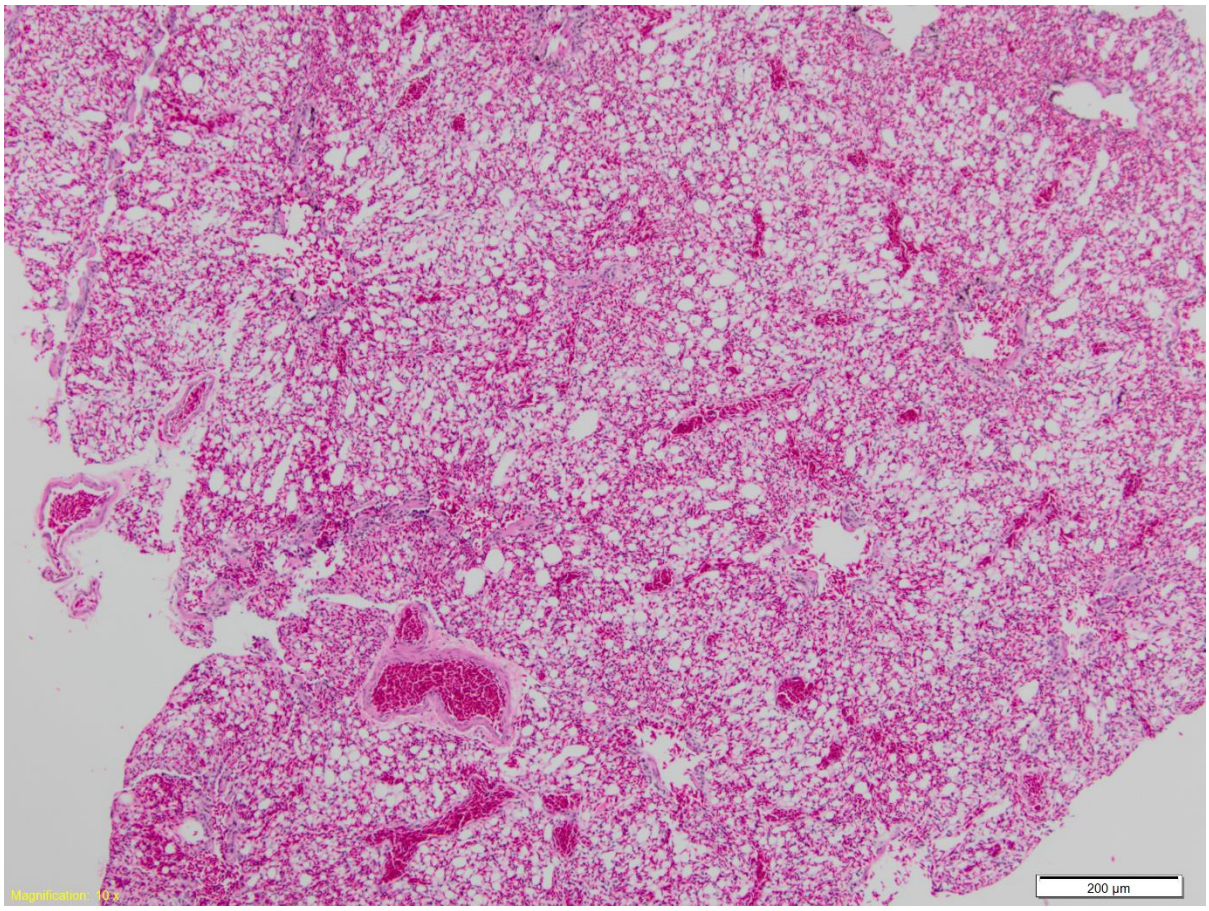

Figure 1. *Histological sections from the lung of a budgerigar with moderate diffuse congestion. Haematoxylin and eosin, 100 × magnification.*

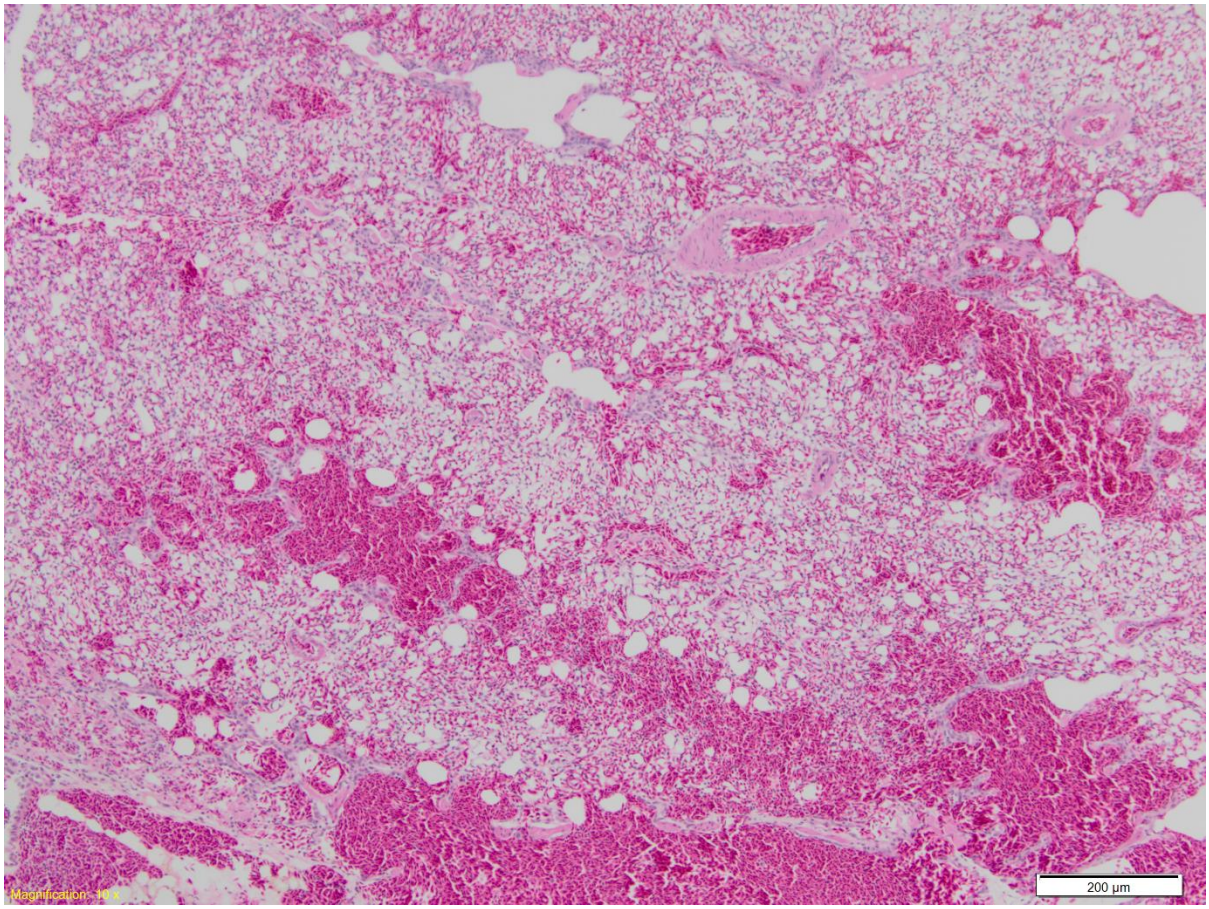

Figure 2. *Histological sections from the lung of a diamond dove with moderate diffuse congestion and parabronchi/air capillary haemorrhage. Haematoxylin and eosin, 100 × magnification.*

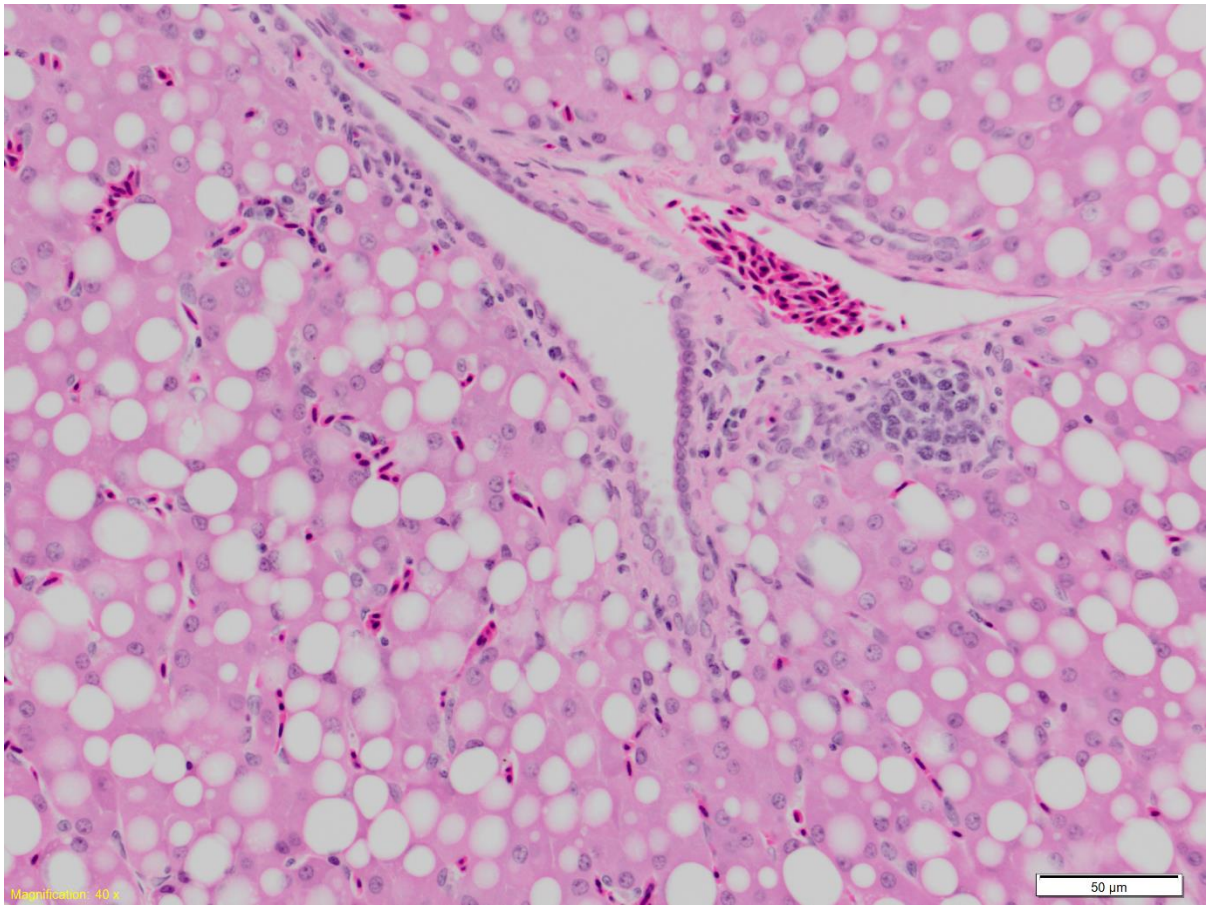

Figure 3. *Histological sections from the liver of a diamond dove with micro- and macro-vesicular hepatocellular vacuolation. Haematoxylin and eosin, 100 × magnification.*

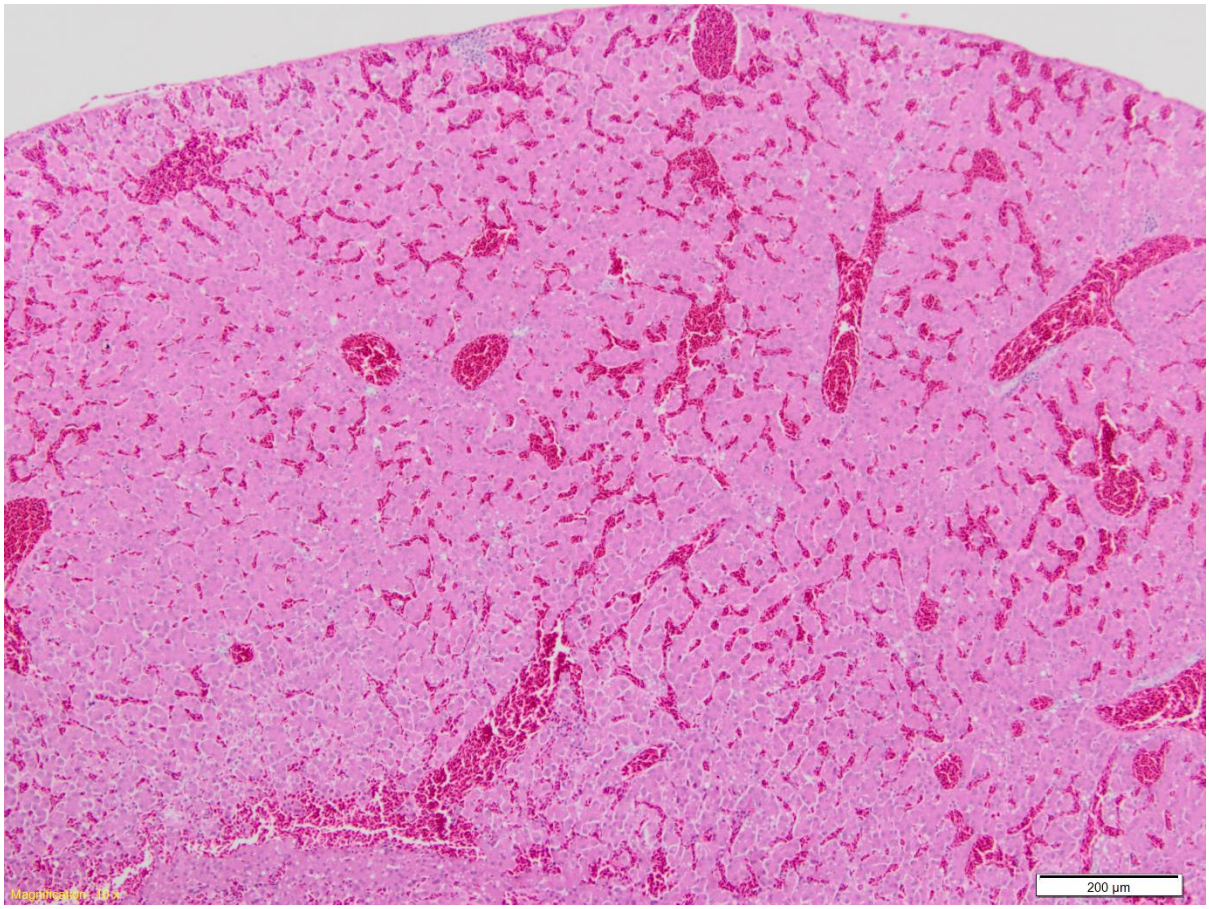

Figure 4. *Histological sections from the liver of a budgerigar with moderate diffuse congestion. Haematoxylin and eosin, 100 × magnification.*

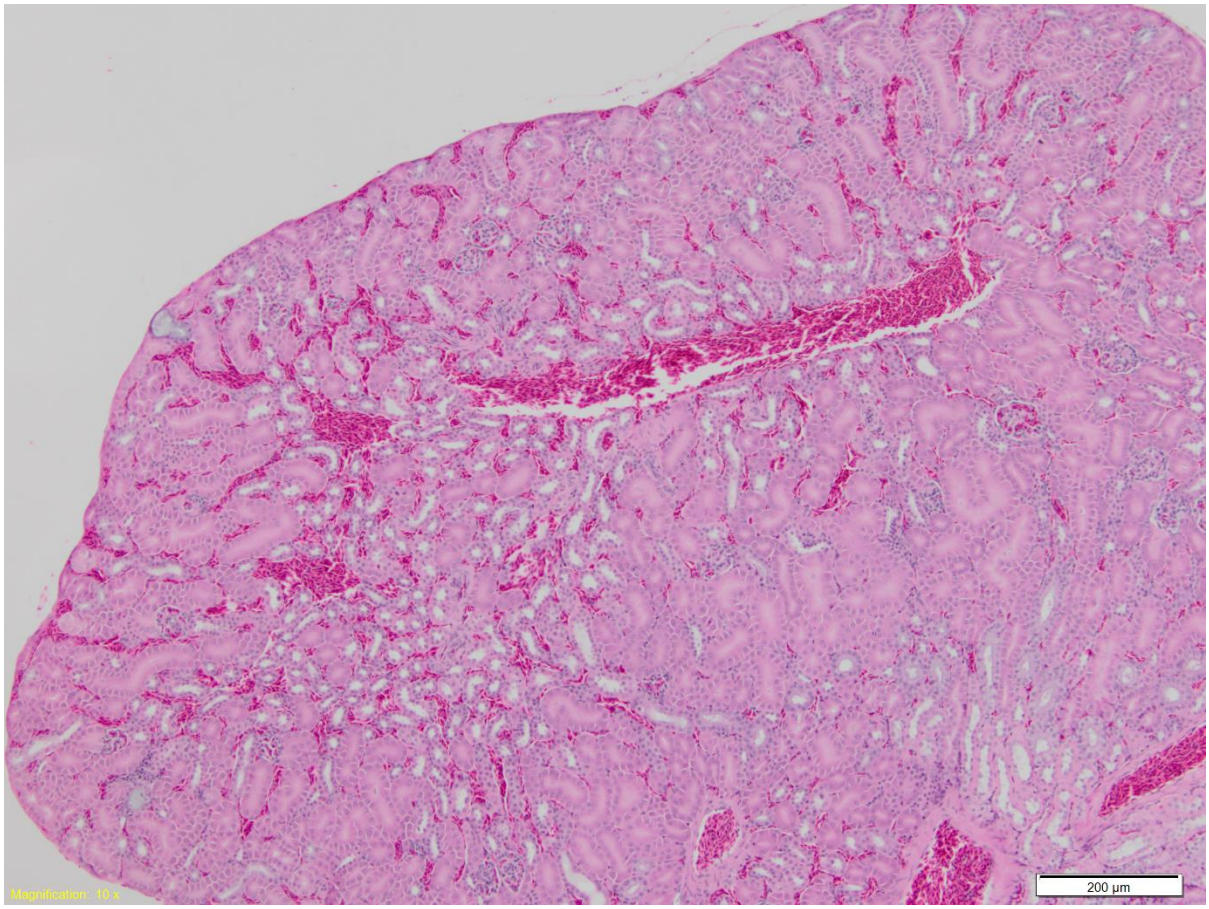

Figure 5. *Histological sections from the kidney of a diamond dove with moderate diffuse congestion. Haematoxylin and eosin, 100 × magnification.*

Species		Organ									
	Heart	Lung			Liver			Kidney		Intestinal tract	
	Focal myocardiocyte vacuolation and adipocyte infiltration	Congestion	Interstitial lymphoid/ haematopoietic aggregates	Airway haemorrhage	Mild micro-/macrovesicular vacuolation of hepatocytes	Congestion	Interstitial lymphoid/ haematopoietic aggregates	Congestion	Interstitial lymphoid/ haematopoietic aggregates	Submucosal congestion	Submucosal lymphoid/ haematopoietic aggregates
Zebra finch (n=15)	0 (0%)	7 (46.7%)	2 (13.3%)	0 (0%)	4 (26.7%)	2 (13.3%)	10 (66.7%)	1 (6.7%)	2 (13.3%)	1 (6.7%)	1 (6.7%)
Incidence ratio at 35°C	0.00	0.56	0.00	0.00	0.60	1.21	1.21	0.61	0.97	0.00	2.43
Incidence ratio at 45°C	0.00	0.79	4.75	0.00	0.53	2.38	1.43	0.00	0.00	0.59	0.00
Budgerigar (n=13)	0 (0%)	13 (100%)	1 (7.7%)	0 (0%)	5 (38.5%)	1 (7.7%)	3 (23.1%)	2 (15.4%)	3 (23.1%)	7 (53.8%)	1 (7.7%)
Incidence ratio at 35°C	0.00	1.31	1.42	0.00	0.70	1.42	0.35	0.71	1.13	2.83	0.00
Incidence ratio at 45°C	0.00	1.58	0.00	0.00	0.90	0.00	0.54	2.71	2.71	2.04	2.71
Diamond dove (n=8)	1 (12.5%)	8 (100%)	1 (12.5%)	1 (12.5%)	8 (100%)	0 (0%)	5 (62.5%)	4 (50%)	2 (25%)	0 (0%)	0 (0%)
Incidence ratio at 35°C	0.00	1.31	2.13	2.13	2.13	0.00	1.59	2.13	0.85	0.00	0.00
Incidence ratio at 45°C	4.75	1.58	0.00	0.00	2.11	0.00	0.95	0.00	0.00	0.00	0.00

Table 1. Summary of histopathological findings according to number, percentage (), and incidence ratios at each temperature exposure for each species.

	WBC	Heterophils	Lymphocytes	Monocytes	Basophils	Eosinophils	PCV
Budgerigar (n = 8)							
Before exposure to 35°C	36 ± 15.60*	44.125 ± 18.43*	32.625 ± 11.62	17.625 ± 9.75*	4.875 ± 2.75	0.75 ± 0.71	54.5 ± 3.38*
After exposure to 35°C	46.875 ± 40.67*	63.5 ± 16.54*	17.75 ± 8.88	14.25 ± 7.36*	4.875 ± 2.70	0.875 ± 1.46	54.25 ± 4.71*
Before exposure to 45°C	27.625 ± 9.74	48.25 ± 12.28*	33 ± 11.19*	12.25 ± 6.09*	5.625 ± 3.02	0.875 ± 0.99	56.125 ± 3.44*
After exposure to 45°C	30.125 ± 9.61	59.875 ± 15.38*	22.125 ± 11.47*	12.125 ± 5.99*	3.875 ± 2.36	0.75 ± 1.04	52.5 ± 4.87*
Diamond dove (n = 8)							
Before exposure to 35°C	50 ± 18.30	29.75 ± 8.92	58.375 ± 9.75	8.25 ± 4.80	1.25 ± 1.04	2.25 ± 2.49*	56.75 ± 2.25
After exposure to 35°C	53.625 ± 13.93	49.5 ± 15.15	44.125 ± 14.08	4.625 ± 2.33	0.875 ± 0.99	1.25 ± 1.83*	52.375 ± 2.97
Before exposure to 45°C	39 ± 12.05	31.125 ± 10.41	58.625 ± 8.94	6.625 ± 4.34	2.625 ± 2.26	1 ± 1.60	56.25 ± 3.11
After exposure to 45°C	44.375 ± 17.22	44.5 ± 12.34	47.625 ± 13.05	5.125 ± 3.80	1.625 ± 1.19	1.125 ± 1.36	52.375 ± 4.17

Table 2. Summary of haematology findings according to mean and standard deviation of each parameter. * difference between the values before and after heat exposure was significant ($p < 0.05$).

Chapter 5 Physiological, histopathological and biochemistry changes in birds exposed to heatstroke

Continuing from chapter 4, there has also been very little documentation of the physiological, histopathological and biochemistry changes associated with birds after the onset of heatstroke. The following original research article, submitted for publication to the journal *Avian Pathology*, demonstrates these changes in galahs and rock doves. The differences between these two species indicate that findings from one bird species may not necessarily be the same for other bird species, and further investigation is warranted in taxa that may be the most vulnerable to heatstroke.

Xie, S., A. Nicholson, L. Woolford and T.J. McWhorter. Physiological, biochemical and histopathological changes associated with heatstroke in the Galah (*Eolophus roseicapilla*) and Rock Dove (*Columba livia*). **Submitted to** *Avian Pathology*.

Statement of Authorship

Title of Paper	Physiological, biochemical and histopathological changes associated with heat stroke in the Galah (*Eolophus roseicapilla*) and Rock Dove (*Columba livia*)
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Xie, S., Nicholson, A., Woolford L., & McWhorter, T. J. (2018) submitted to Avian Pathology.

Principal Author

Name of Principal Author (Candidate)	Shangzhe Xie		
Contribution to the Paper	Conducted experiments according to the protocol. Collected, trimmed and processed all samples. Performed biochemical and histopathological analysis on all samples, interpreted data, wrote manuscript and acted as corresponding author.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	25/8/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Anthony Nicholson		
Contribution to the Paper	Helped to monitor anaesthesia, analyse the data and edit the manuscript.		
Signature		Date	Aug 27, 2018

Name of Co-Author	Lucy Woolford		
Contribution to the Paper	Helped to verify histopathological findings and edit the manuscript.		
Signature		Date	24/8/18

Name of Co-Author	Todd McWhorter
Contribution to the Paper	Supervised development of work, helped in data interpretation and manuscript evaluation.
Signature	Date 29/8/2018

Physiological, biochemical and histopathological changes associated with heatstroke in the Galah (*Eolophus roseicapilla*) and Rock Dove (*Columba livia*)

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Abstract

The pathophysiology of heat illnesses in birds has not been well characterised. In this study, the changes in heart rate, respiratory rate, blood biochemistry and histopathological findings in galahs and rock doves after heat exposure under standardized conditions designed to induce heatstroke were described. Birds in the heat exposed group were exposed to environmental heat stress and compared to control birds. Both groups of birds were under general anaesthesia throughout the experiment and serial blood collections were performed for biochemical analyses, while organs were collected at the end of the experiment for histopathology. No electromyography traces consistent with the onset of heat cramps were observed in any of the birds. Biochemical changes suggestive of skeletal muscle and hepatocellular injury, including hyperkalaemia and increased serum muscle and hepatic enzyme activities, were often observed in heat exposed galahs and rock doves at the onset of heatstroke. Histopathological analyses did not reveal any significant cardiac changes, although some lungs had signs of acute congestion. Some heat exposed rock doves had indications of necrosis in the pectoral muscle. There were significant hepatic changes in some heat exposed galahs, but not in rock doves. This suggests that there may be species differences amongst birds in the organs most affected by heatstroke. The observed species differences in the physiological, biochemical and histopathological changes indicate that bird species should be studied separately for clinical syndromes such as heatstroke.

Keywords

heat illnesses; avian; histopathology; physiology; clinical pathology

Introduction

An inability of the body to cope with extremely high temperatures will eventually lead to heat illness. Climatic heat waves have been associated with mass mortality events in desert dwelling birds (McKechnie *et al.*, 2012; McKechnie & Wolf, 2010), including endangered species (Saunders *et al.*, 2011). By the time the birds are found dead after a heatwave, they have presumably gone through the cascade of symptoms associated with, and suffered the effects of, heatstroke. It is therefore difficult to identify pathognomonic changes in wild birds suffering from heat illnesses during climatic heatwaves. Personal communications with avian veterinarians indicate that heat illnesses are extremely rare in clinical avian practice.

While the exact pathophysiology of heat illnesses in birds has not been well characterized, it is better understood in humans. Heat illnesses can be classified as heat syncope, heat cramps, heat exhaustion and heatstroke (Bricknell, 1995). Heatstroke in humans was traditionally defined as hot, dry skin accompanied by central nervous abnormalities associated with core body temperatures above 40°C (Bouchama & Knochel, 2002). An alternative definition classifying it as a form of hyperthermia associated with systemic inflammatory response resulting in multi-organ dysfunction predominated by encephalopathy has also been proposed (Bouchama & Knochel, 2002). Heat cramps, muscle spasms that result from exposure to heat, and heat exhaustion, the intermediate heat illness before heatstroke, have not been documented in animals to date. The paucity of reports and studies of heat illnesses in birds means that our understanding of the syndrome is largely based on mammalian studies. Heatstroke itself is relatively well-studied and understood in species such as baboons (Bouchama *et al.*, 2005), rats (Chen *et al.*, 2006), guinea pigs (Dechesne *et al.*, 1992) and rabbits (Abdelatif & Modawi, 1994; Lin & Lin, 1992) serving as experimental models for human heatstroke, and in dogs (Bruchim *et al.*, 2009; Oglesbee *et al.*, 1999) where it is a relatively common occurrence in veterinary practice (Johnson *et al.*, 2006).

One study of baboons suffering from induced heatstroke demonstrated damage to multiple organs including the jejunum, liver, spleen, lung and kidney; this manifested as vascular congestion, haemorrhage, thrombosis, increased inflammatory cells, and disruption of normal cell and tissue architecture (Roberts *et al.*, 2008). Histopathological reports from birds dying of heatstroke are uncommon, likely because the quick onset of autolysis under high temperatures precludes any useful post-mortem examination unless the carcasses were collected and examined immediately or stored appropriately until examination could be carried out. Overstreet & Rehak (1982) reported that chicks of the least tern (*Sterna albifrons*) suspected to be suffering from heatstroke showed microscopic evidence of early ischaemic focal necrosis in the brain, characterised by neuronal degeneration, infiltration of leukocytes and degeneration of the neuropil surrounding some cortical peripheral blood vessels. The splenic sinuses were packed with red blood cells, an indicator of circulatory collapse while small areas of degeneration were found in the liver, kidneys, and intestine (Overstreet & Rehak, 1982). However the lungs, heart, and other tissues were not examined. In another report, the pathological changes of a sun conure (*Aratinga solstitialis*) that was suspected to have suffered heatstroke under general anaesthesia revealed mild multifocal acute degeneration and contraction band necrosis of the biceps femoris muscle, as well as diffuse moderate acute congestion of the lungs (Hofmeister, 2005).

In the present study, the changes in heart rate, respiratory rate, blood biochemistry and histopathological findings in galahs (*Eolophus roseicapilla*) and rock doves (*Columba livia*) after heat exposure under general anesthesia in standardized conditions designed to induce heatstroke were described.

Materials and methods

Birds. 8 galahs and 8 rock doves were obtained from private bird breeders in Adelaide from 2016 to 2017 and immediately transported to the University of Adelaide Roseworthy Campus (Roseworthy, South Australia) to be subjected to the experimental protocol. All experiments were conducted according to the Australian code for the care and use of animals for scientific purposes and approved by the University of Adelaide Animal Ethics Committee (S-2015-189).

Experimental protocol. Each bird was randomly assigned to the heat exposed or control group, such that there were 4 birds of each species in each group. The birds were anaesthetised using isoflurane in 100% oxygen delivered via facemask connected to a non-rebreathing circuit, and anaesthesia was maintained at surgical depth this way throughout each procedure. Birds in the heat exposed group were exposed to environmental heat stress using three Thermotex Infrared Heating Mats (Thermotex Therapy Systems Ltd, Calgary, Alberta, Canada) each set at the maximum 55°C. Birds in the control group were placed on the same heat mats, but with each set at 45°C to maintain a constant normal T_b throughout general anaesthesia.

After each bird was anaesthetised, blood was collected via the jugular vein using an insulin syringe (Time 1). Birds in the heat exposed group also had blood collected when their body temperatures reached 41.5°C (Time 2), 43.0°C (Time 3) and after heatstroke has been induced (Time 4). Heatstroke was deemed to have occurred when the bird exhibited an exponential increase in body temperature with a concurrent increase in heart rate followed by a rapid decrease in heart rate. Birds in the control group had blood collected at 20 minutes (Time 2), 35 minutes (Time 3) and 60 minutes (Time 4) after the first blood collection. 0.2 ml

of blood was collected at each time point and all blood samples were collected into lithium heparin blood tubes. The blood samples were then centrifuged at 7000 g for 10 minutes, and the plasma subsequently collected and stored at -80°C until analysis.

Birds in the heat exposed group were euthanized once heatstroke had been induced whereas birds in the control group were euthanized after collection of the last blood sample; all birds were euthanized with 100mg/kg of sodium pentobarbitone administered intracoelomically. Each bird's brain, eyes, pectoral muscle, heart, gastrointestinal tract, liver, lungs and kidneys were collected and stored in 10 % neutral buffered formalin.

Physiological parameters. The heart rate, electromyograph (EMG), electrocardiograph (ECG) and cloacal temperature were monitored and recorded throughout the experimental protocol using PowerLab with LabChart 8 (AD Instruments, Colorado Springs, USA). The pectoral muscle of one of the heat exposed rock doves was stimulated using a nerve stimulator to simulate EMG traces that might be expected during a heat cramp. This was done to produce waveforms that could be used to identify muscle cramps associated with heat exposure for the rest of the birds in the experiment. The heart rate was obtained using the ECG function, and the respiration rate using the cyclic measurement rate detection function with smoothing at 500 milliseconds on the EMG data in LabChart 8. Heart and respiration rate data were averaged in 10 second blocks and then ranked by average cloacal temperature over the same 10 second block. The summarized heart rate and respiration rate data were then grouped into 0.25°C cloacal temperature increments (bins) and the average plotted against the median cloacal temperature. Data are presented as mean ± SEM.

Biochemistry. A biochemistry panel including sodium, potassium, chloride, calcium, phosphorus, total protein, albumin, globulin, uric acid, aspartate aminotransferase (AST), glutamate dehydrogenase (GLDH), creatinine kinase (CK), glucose and cholesterol was performed on each blood sample using a Beckman-Coulter AU480 Chemistry Analyzer (Beckman-Coulter Diagnostics, Lane Cove, NSW, Australia). All samples were diluted 1:4 to achieve sufficient volumes to run the full panel. Despite the dilution, the blood samples from 2 galahs and 2 rock doves (1 control and 1 heat exposed for each species) were still not of sufficient volume to include GLDH, albumin and globulin in the analyses.

Histopathology. Formalin-fixed samples of the organs collected were trimmed and embedded in paraffin blocks, sectioned at 3-4 μm, mounted and stained with haematoxylin & eosin for routine histopathological examination. The slides were examined at the Veterinary Diagnostic Laboratory, University of Adelaide, Roseworthy, South Australia using a BX53 microscope (Olympus, Japan), and photomicrographs were captured using Labsens (Olympus, Japan).

Statistics. For both species and all parameters included in the biochemical analyses, species baseline 95% reference intervals (RI) and 90% confidence intervals (CI) specific to this study population were calculated using the results of all blood samples in the control groups and only Time 1 and Time 2 blood samples from birds in heat exposed groups. Analyses were conducted using the Reference Value Advisor (RefVal) version 2.1 add-in for Microsoft Excel (Geffré *et al.*, 2011). RI and CI were determined for each biochemical analyte following guidelines for generation of *de novo* reference intervals as published by the American Society of Veterinary Clinical Pathology (Friedrichs *et al.*, 2012). All analytes, except GLDH, had

sample sizes $20 \leq n < 40$ and had Gaussian distributions. Outlier values for all analytes were identified by RefVal using Dixon's and Tukey's range tests, and manually removed if attributable to determinable reasons such as poor sample quality or analytic error. CI for the reference limits were calculated using nonparametric bootstrap methods for all analytes (except GLDH). Because n=10 for GLDH, a table of ascending values, mean, median values and a histogram were reported, but RIs were not determined due to the uncertainty of limits based on so few samples (Friedrichs, et al., 2012).

Broken-stick linear regression models fitted in the R package segmented (Muggeo, 2008) were used to identify inflection points for the relationships between the heart rate and T_b, as well as respiration rate and T_b, for individuals of each species in the heat exposed group, using the ranked 10 second block averaged data.

Results

Cloacal temperature. The cloacal temperature in heat exposed galahs increased at a rate of 0.0025 ± 0.0004°C min^{-1}, and lethal body temperature (T_b) was reached in 63.92 ± 30.25 minutes. The cloacal temperature in control galahs decreased at a rate of 0.0002 ± 0.0006°C min^{-1}. The cloacal temperature in heat exposed rock doves increased at a rate of 0.0018 ± 0.0006°C min^{-1}, and lethal T_b was reached in 92.38 ± 45.45 minutes. The cloacal temperature in control rock doves decreased at a rate of 0.0009 ± 0.0009°C min^{-1}.

Heart rate. The heart rate of control galahs remained between 206.92 and 340.54 beats per minute (BPM) throughout the experiment (Figure 1) and control rock doves remained between 111.08 and 228.51 BPM (Figure 2).

The mean T_b inflection point at which the heart rate of heat exposed galahs started increasing above the range of the control galahs was 43.63 ± 0.71°C. Past this point, heart rate increased with T_b until a maximum of 655.62 ± 45.23 BPM. The heart rate began to decrease after the second mean T_b inflection point of 46.09 ± 0.16°C (Figure 1). The analysis for T_b inflection points could only be performed for 2 heat exposed galahs as the other 2 died just as their T_b was reaching the lower inflection point. The peak heart rates for the two heat exposed galahs that showed a rapid increase and then decrease in heart rate occurred at 46.13°C and 45.88°C.

The mean T_b inflection point at which the heart rate of heat exposed rock doves started increasing above the range of the control rock doves was 44.59 ± 0.93°C. Past this point, heart rate increased with T_b until a maximum of 520.04 BPM. The heart rate began to decrease after the second T_b inflection point of 45.16 ± 0.91°C (Figure 2). The same pattern of increased and then decreased heart rate was observed in all the rock doves in the heat exposed group. The peak heart rates for individual heat exposed rock doves occurred at 47.38°C, 44.88°C, 42.88°C and 45.63°C.

Respiration rate. The respiration rate of control galahs remained between 13.26 and 29.89 breaths per minute (BPM) throughout the experiment (Figure 3) and for control rock doves remained between 11.80 and 28.81 BPM (Figure 4).

The mean T_b inflection point at which the respiration rate of heat exposed galahs started increasing above the range of the control galahs was 41.34 ± 0.02°C. Past this point, respiration rate increased with T_b until a maximum of 124.48 ± 45.11 BPM. The respiration rate began to decrease after the second mean T_b inflection point of 44.70 ± 1.05°C (Figure 3).

The mean T_b inflection point at which the respiration rate of heat exposed rock doves started increasing above the range of the control rock doves was 41.94 ± 0.68°C. Past this point, respiration rate increased with T_b until a maximum of 71.33 ± 22.26 BPM. The respiration rate began to decrease after the second mean T_b inflection point of 44.33 ± 0.63°C (Figure 4).

EMG. There was no evidence of heat cramps in the muscles, i.e. no EMG traces consistent with traces induced by muscle stimulation were observed, in both species in both control and heat exposed groups.

Biochemistry. The reference ranges of the measured biochemical parameters for control galahs and rock doves in this study are summarized in Tables 1 and 2 respectively, except for GLDH, which is summarized in Table 3 as the sample size was insufficient to determine reference ranges for this parameter. The blood biochemistry results are summarized in Figuress 4 and 5. At the final time point and compared to galahs in the control group, galahs in the heat exposed group had elevated potassium, phosphorus, total protein, AST and GLDH; whereas chloride and calcium were decreased. However, no such differences were found in rock doves in the control and heat exposed groups. Rock doves in both control and heat exposed groups had elevated potassium, AST and CK at the final time point.

None of the blood samples analysed were affected by significant haemolysis. There were obvious differences between galahs and rock doves in the biochemistry changes associated with the onset of heatstroke. 4/4 (100%) of the galahs in the heat exposed group developed hyperkalaemia and hyperphosphatemia and 2/4 (50%) had elevated AST and CK. 2/2 (100%) had elevated GLDH, along with elevations in AST. 3/4 (75%) had hypocalcaemia

and elevated total protein and individual birds were also identified with hyponatraemia or hypernatraemia, hypochloraemia, hypoglycaemia and elevated cholesterol.

Similar to galahs, many of the rock doves in the heat exposed group [3/4 (75%)] developed hyperkalaemia and elevated CK, and 2/4 (50%) developed elevated AST, 1/2 (50%) had elevated GLDH, while 1/4 (25%) had elevated uric acid.

One control galah had elevations in GLDH and AST and hepatocellular degeneration was observed histologically. These values were therefore excluded from generation of reference intervals.

Gross post-mortem findings. There were no obvious macroscopic lesions found on post-mortem of all the birds**.**

Histopathology. There was moderate diffuse congestion and intra-airway haemorrhage with endothelial hypertrophy of the lungs in 2 out of the 4 heat exposed galahs. There was also congestion of the brain, heart and small intestines of these 2 galahs. In the liver of galah 5 (heat exposed group), there was also congestion, mild endothelial hypertrophy, and scattered single cell necrosis and random foci of hepatocellular degeneration.

Histopathological changes were observed in one heat exposed rock dove; these included diffuse congestion and mild haemorrhage of the lungs, and scattered rare myofibre swelling with loss of striations, indicative of acute degeneration in the pectoral muscle. Rock dove 8 (control group) had mild to moderate periportal non-suppurative hepatitis and occasional multifocal perivascular infiltrates of mononuclear cells (interpreted as a pre-

existing non-specific change) surrounding congested vessels lined by plump endothelia indicative of focal non-suppurative encephalitis.

Discussion

Physiological changes

The changes in cloacal (body) temperature, heart rate and respiration rate in the present study were similar to those observed in anaesthetised experimental dog models for heatstroke (Bynum *et al.*, 1977). The slight decrease in the cloacal temperature with time in control birds was not unexpected, as maintaining the body temperature of birds under general anaesthesia is difficult due to their large surface area to volume ratio (Boedeker *et al.*, 2005; Phalen *et al.*, 1996; Rembert *et al.*, 2001). The increases in heart and respiration rates and subsequent decreases associated with heatstroke occurred at higher T_b in rock doves than in galahs. This was to be expected given the higher heat tolerance recently demonstrated in columbiform birds (McKechnie *et al.*, 2016; Smith *et al.*, 2015; Whitfield *et al.*, 2015; Wolf, 2015), relative to psittaciform birds (McWhorter et al. 2018).

The mean T_b inflection point for heart rate increase in galahs (43.63 ± 0.71°C) was lower than in rock doves (44.59 ± 0.93°C). However, the mean T_b inflection point for heart rate decrease in rock doves (45.16 ± 0.91°C) was lower than in galahs (46.09 ± 0.16°C). Therefore, a rock dove experiencing heat stress under anaesthesia has a very narrow window between showing measurable signs of heat stress and experiencing irreversible heat stroke. Intervention by active cooling should therefore be instituted at T_b well below that of the lower inflection point.

The mean T_b inflection points for the respiration rate of both galahs and rock doves in the present study were similar, i.e. increasing just after T_b exceeded 41°C and decreasing just

after T_b exceeded 44°C. McWhorter et al. (2018) found that the ambient temperature (T_a) at the onset of panting for galahs was 42.7 ± 2.5°C, similar to the T_b of the galahs in this study when panting started. Similarly, the T_a at the onset of gular fluttering for four southern hemisphere columbiform birds was found to range from 40.8 to 55.1°C , with the species closest in size to rock doves in that study, crested pigeons (*Ocyphaps lophotes*), at 48.0 ± 5.7°C (McKechnie, et al., 2016). The birds in these other studies were not under general anaesthesia, as the main purpose of these studies was to measure metabolic parameters to determine the heat tolerance limits and other physiological changes of birds when exposed to heat. The similarities in temperature of onset of panting in both conscious and anaesthetized birds indicate that general anaesthesia may not have significant effects on the ability of galahs and rock doves to utilize respiratory mechanisms of heat loss, even though the high inhalant oxygen concentration provided during inhalational anaesthesia may reduce the sensitivity of oxygen chemoreceptors in avian lungs, which could reduce respiratory drive and interfere with respiratory evaporative cooling. The humidity of the inspired gases was also not measured in our study, which may have been useful for determining the thermoregulatory efficiency of evaporative water loss, but those have already been studied in similar species (McKechnie, et al., 2016; McWhorter, et al., 2018).

The lack of obvious and consistent EMG indications of cramps suggest that heat cramps are not a common phenomenon in galahs and rock doves, unlike in humans, where heat cramps are common in early stages of heat illnesses (Leon & Kenefick, 2012). It is possible that birds may experience heat cramps with more chronic heat exposure, but that was not tested in our study.

Biochemistry changes

Biochemical changes suggestive of skeletal muscle and hepatocellular injury were often observed in heat exposed galahs and rock doves after heatstroke was induced, however other biochemical changes were more variable. In both galahs and rock doves, the hyperkalaemia, hyperphosphatemia (galahs only) and elevated AST and CK were suggestive of skeletal muscle degeneration and/or necrosis at the onset of heatstroke, however transcellular shifts in electrolytes associated with acid-base disturbances and effects of haemoconcentration must also be considered. Galah 7 (control group) had elevated AST, GLDH and CK at the final blood collection before euthanasia. These values from this control galah were left out for generation of reference intervals for the blood results. This control galah was suspected to have underlying disease that predisposed it to developing these hepatic changes just through the stress of handling and anaesthesia.

In humans with heatstroke, hypokalaemia usually occurs first (Bouchama & Knochel, 2002), either due to elevated catecholamine levels, respiratory alkalosis secondary to panting or loss in sweat and through the kidneys secondary to hyperaldosteronism (Grogan & Hopkins, 2002). Hypokalaemia as a result of loss in sweat and via kidneys is more commonly are observed with exertional heat illnesses in humans (Grogan & Hopkins, 2002),. However, prolonged hyperthermia, hypoxia and hypoperfusion of several hours will lead to failure of the Mg^{2+}-dependent Na^{+}/K^{+}-ATPase pump, causing K^{+} to leak extracellularly, resulting in hyperkalaemia (Grogan & Hopkins, 2002). Hyperkalaemia was the most common electrolyte abnormality in both bird species studied, which was possibly a sequela to hypoxia and hypoperfusion following the onset of heatstroke, but these were not specifically measured in our study.

Hypocalcaemia was observed in the majority of heat exposed galahs, but not in the rock doves. Hypercalcaemia and hyperproteinaemia commonly result from dehydration and

haemoconcentration associated with heatstroke (Bouchama & Knochel, 2002). However, hypocalcaemia associated with rhabdomyolysis and acute renal failure is more commonly observed in exertional heatstroke (Shieh *et al.*, 1992). Profound hypercalcaemia (8.96 mmol/L and 4.40 mmol/L compared with the control reference interval of 0.626 – 2.152 mmol/L) occurred in 2 heat exposed rock doves which could not be explained by physiological or pathological reasons. Therefore, analytical error was considered the most likely cause and they were excluded from analyses.

Hypophosphataemia has also been commonly observed in humans suffering from heatstroke (Bouchama & Knochel, 2002) and has been suggested to result from the increased glucose phosphorylation seen in acute alkalotic conditions (Grogan & Hopkins, 2002). Hyperuricaemia was observed in one heat exposed rock dove. Hyperuricaemia develops due to the release of purines from injured muscle (Grogan & Hopkins, 2002). The elevations in GLDH, AST and CK in rock doves indicate they may be likely to develop hepatocellular injury and rhabdomyolysis, respectively, from heatstroke. Exertional heatstroke tends to result in higher levels of CK elevation associated with rhabdomyolysis (Hsu *et al.*, 1997), but even heatstroke patients with no history of exercise exhibit CK elevation to a certain extent (Dematte *et al.*, 1998). However, severe liver damage is more common in exertional heatstroke due to direct cell damage and hypoxia (Giercksky *et al.*, 1999; Saissy, 1996).

Dogs with heatstroke are considered less useful as a model for human heatstroke studies as they display different changes (Damanhouri & Tayeb, 1992), but heatstroke in dogs is relatively better understood compared to other species in veterinary medicine due to their popularity as pets in all parts of the world, including those that experience high environmental temperatures. The most common biochemical changes observed in dogs with heatstroke are elevated CK, ALT, AST, ALP and creatinine (Bruchim *et al.*, 2006). Some of

these changes were found in the birds in our study. Hypoglycaemia is also frequently observed, and the possible association with heatstroke includes increased glucose utilization due to high body temperature, sepsis and liver failure (Bruchim, et al., 2006). Disseminated intravascular coagulation (DIC) is also a common sequelae of heatstroke (Bruchim, et al., 2006). The birds in our study were not kept alive long enough post-heatstroke to determine if DIC is a feature of avian heatstroke, and future studies could examine this.

Histopathological changes

Histopathological examination did not reveal any significant cardiac changes, but acute congestion in the lungs was consistent with those in a case report of a sun conure that was suspected to have suffered heatstroke under general anaesthesia (Hofmeister, 2005). In this sun conure, there was also mild multifocal acute degeneration and contraction band necrosis of the biceps femoris muscle, as well as diffuse moderate acute congestion of the lungs (Hofmeister, 2005). Some of our heat exposed rock doves also had indications of necrosis in the pectoral muscle, similar to that of the sun conure,which were consistent with the elevated blood CK levels observed. Significant hepatic changes were also observed in some heat exposed galahs, but not in any of the rock doves. For example, in galah 5, the histopathological findings of congestion, mild endothelial hypertrophy, and scattered single cell necrosis and random foci of hepatocellular degeneration in the liver corresponded with its biochemical abnormalities. There may therefore be species differences in the organs most affected by heatstroke. The nonsuppurative encephalitis observed in rock dove 8 (control group) were chronic and likely due underlying conditions that were unrelated to the study.

Histopathological reports of birds dying from heatstroke are uncommon because the quick onset of autolysis under high ambient and body temperatures precludes any useful post-

mortem examination unless the carcasses were collected and examined immediately or stored appropriately until examination could be carried out. Overstreet & Rehak reported that chicks of the least tern (*Sterna albifrons*) suffering from heatstroke after being exposed to high environmental temperatures at a narrow beach nesting area in Mississippi, USA had microscopic evidence of early ischaemic focal necrosis in the brain, characterised by neuronal degeneration, leukocytic infiltration and degeneration of the neuropil surrounding some cortical peripheral blood vessels (Overstreet & Rehak, 1982). Also the splenic sinuses were packed with red blood cells, an indicator of circulatory collapse (Overstreet & Rehak, 1982). There were small areas of degeneration in the liver, kidneys, and intestine (Overstreet & Rehak, 1982), however, the lungs, heart, and other tissues were not examined. None of these changes were identified in our study, which is likely to be because the birds were euthanized promptly after the onset of heatstroke. It is possible that the birds in our study may have developed similar histopathological changes if they were kept alive after the onset of heatstroke, but they were not due to ethical and welfare considerations.

In a baboon heatstroke model, severely affected animals had multifocal injury to the liver with intrasinusoidal and central vein accumulation of erythrocytes and neutrophils (Bouchama, et al., 2005). There was also injury to the jejunal villi with tissue loss, desquamation and exposure of the lamina propria (Bouchama, et al., 2005). Central nervous system (CNS) changes included cytoplasmic eosinophilia and nuclear pyknosis in the scattered neurons of the hippocampus, pallidum and cerebellar Purkinje cells (Bouchama, et al., 2005). All these changes were much more pronounced in baboons with severe heatstroke (heat exposed until hypotension occurred) and minimal in those with moderate heatstroke (heat exposed until T_b was 42.5°C). If similar changes were to be expected in birds, then the lack of histopathological changes in our study would indicate that these birds only suffered

moderate heatstroke, and a longer heat exposure or longer delay between the onset of heatstroke and euthanasia may be required to illicit similar histopathological changes.

In dogs that presented to university teaching veterinary hospitals with heatstroke and subsequently died, post-mortem examinations revealed that the most consistent histopathological changes were hyperaemia and diffuse oedema in the skin, lungs, brain and bone marrow (Bruchim, et al., 2009). There was also congestion of the splenic pulp and hepatic sinusoids, as well as necrosis in the small intestinal mucosa, large intestinal mucosa, renal tubular epithelium, hepatic parenchyma and brain neural tissue (Bruchim, et al., 2009). However, in dogs where heatstroke was induced as an experimental model, congestion of the liver, kidney and lung, as well as karyorrhexis of lymphocytes in the spleen and mesenteric lymph nodes were the only consistent findings (Bynum, et al., 1977). The difference in histopathological findings between these two studies may be due to differences in T_b reached, extent and duration at the exposed temperature and how soon after heating they were examined. Further studies in birds will need to be performed by exposing them to different durations and degrees of heat exposure to determine if this is the case. The birds in our study may have developed additional histopathological changes if maintained alive, under general anaesthesia, for a period of time post heat exposure.

Conclusion

The physiological, biochemical and histopathological changes associated with heatstroke have not been previously documented in galahs and rock doves. Our study has provided a baseline for further studies, as well as indications for diagnosing heatstroke in clinical cases presenting with a history of heat exposure. The study also provides T_b data to indicate when intervention in necessary under general anaesthesia to prevent overheating.

Rock doves especially require intervention early on as they are overcome by irreversible heatstroke very quickly once T_b exceeds the critical point. There were also significant species differences in the physiological, biochemistry and histopathological changes, which indicate that bird species should be studied separately for clinical syndromes such as heatstroke. Further studies should concentrate on bird species more vulnerable to heatstroke, e.g. psittaciform birds with lower tolerance for heat (McWhorter, et al., 2018), as well as investigate if chronic exposure to heat may result in different clinical and histopathological changes.

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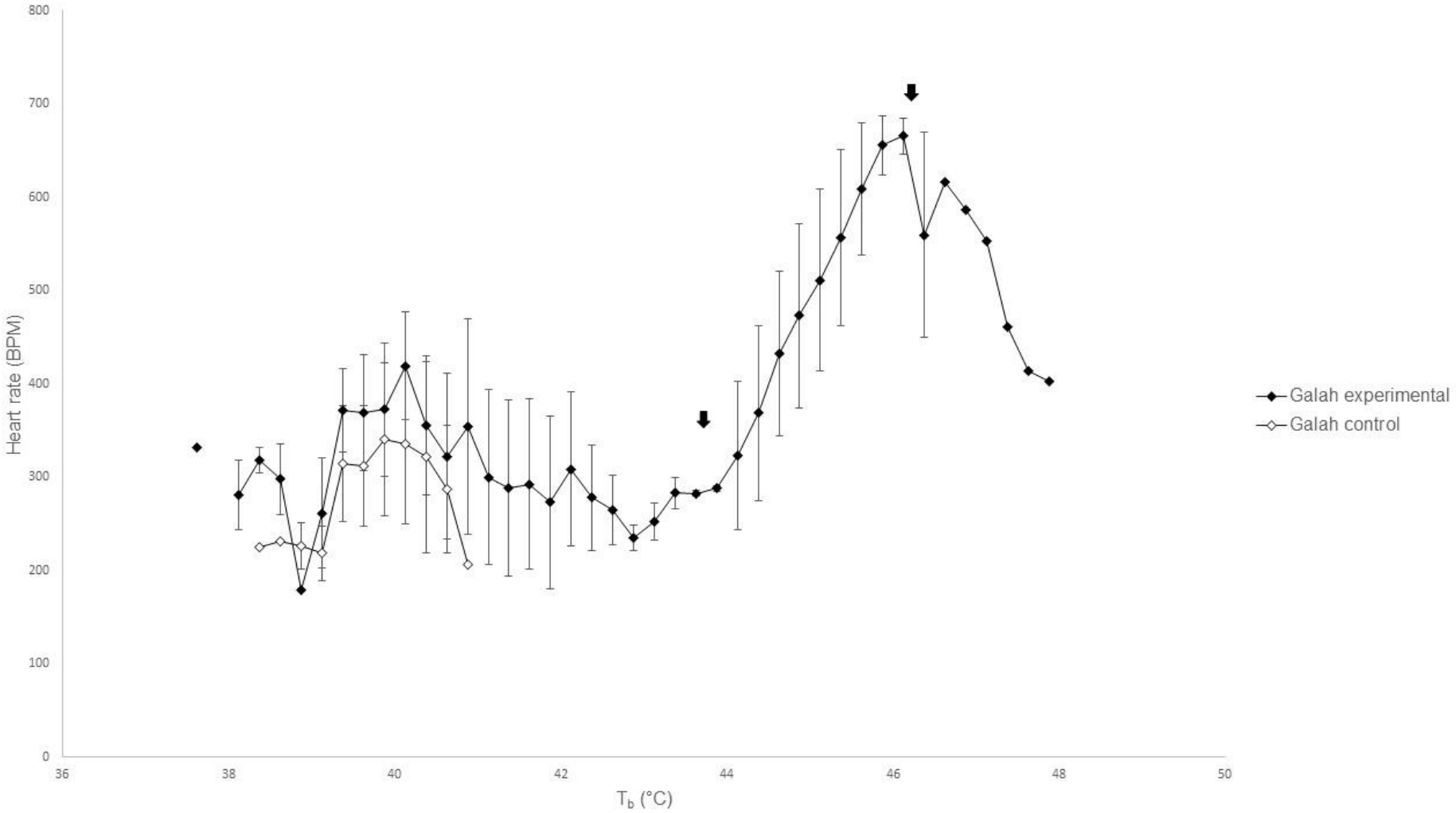


Figure 1. *Heart rate in control galahs remained stable with increasing cloacal temperatures, but increased in heat exposed galahs at higher cloacal temperatures initially before decreasing rapidly. The mean inflection points of these changes are indicated by the black arrows.*

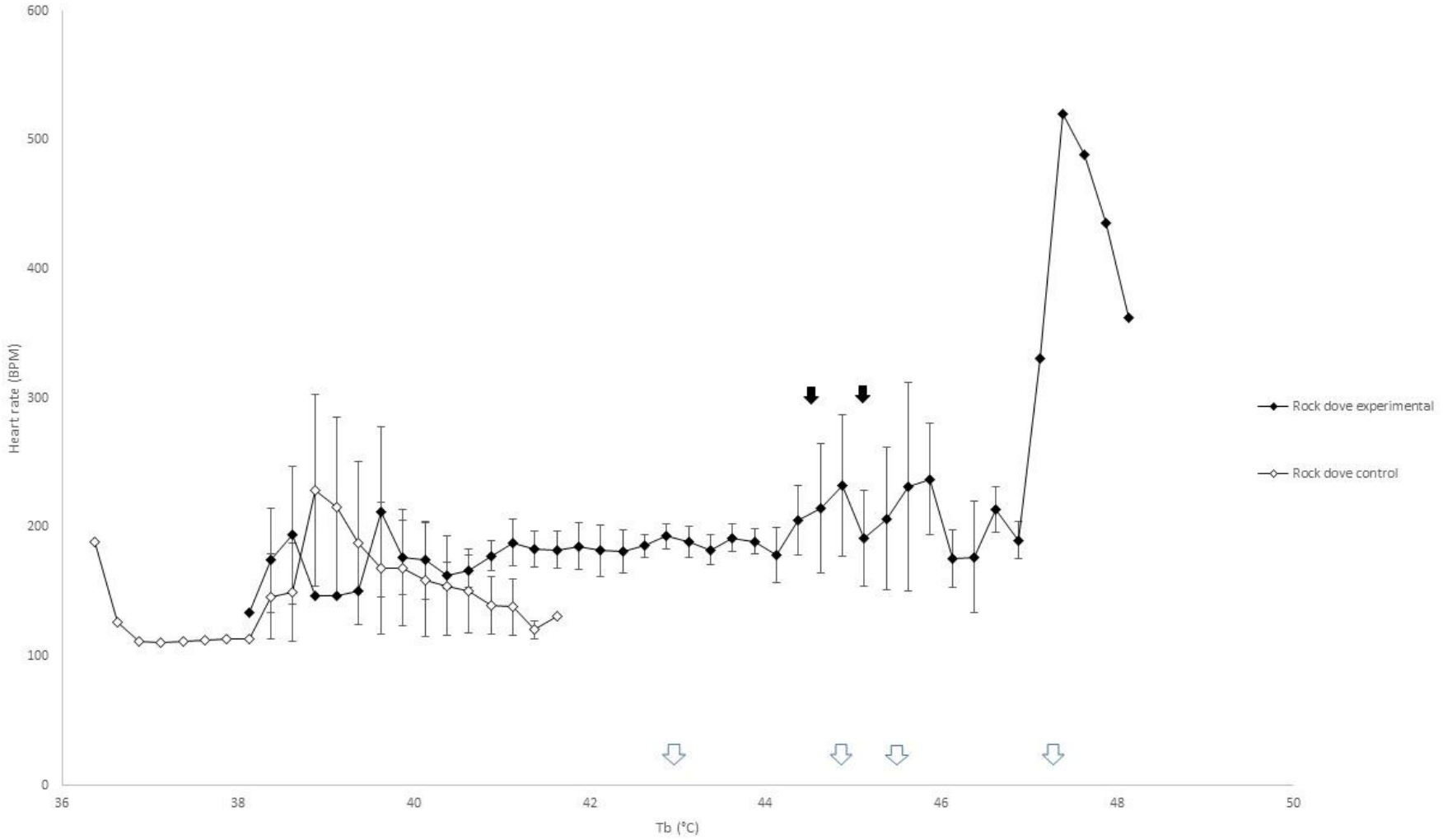


Figure 2. *Heart rate in control rock doves remained stable with increasing cloacal temperatures, but increased in heat exposed rock doves at higher cloacal temperatures initially before decreasing rapidly. The mean inflection points of these changes are indicated by the black arrows. The white arrows along the x-axis indicate the cloacal temperature at which peak heart rate was reached for each individual heat exposed rock dove.*

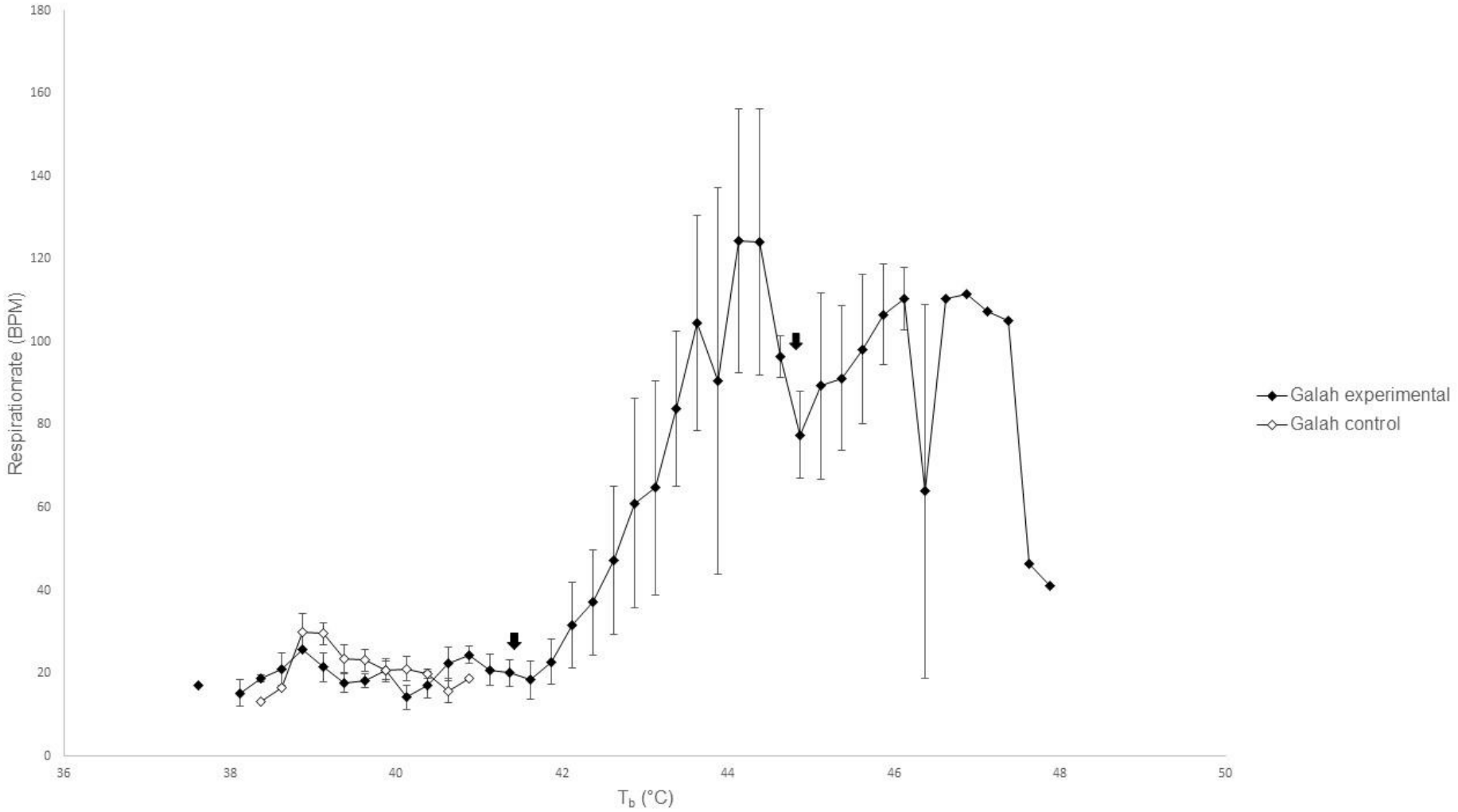


Figure 3. *Respiration rate in control galahs remained stable with increasing cloacal temperatures, but increased in heat exposed galahs at higher cloacal temperatures initially before decreasing. The mean inflection points of these changes are indicated by the black arrows.*

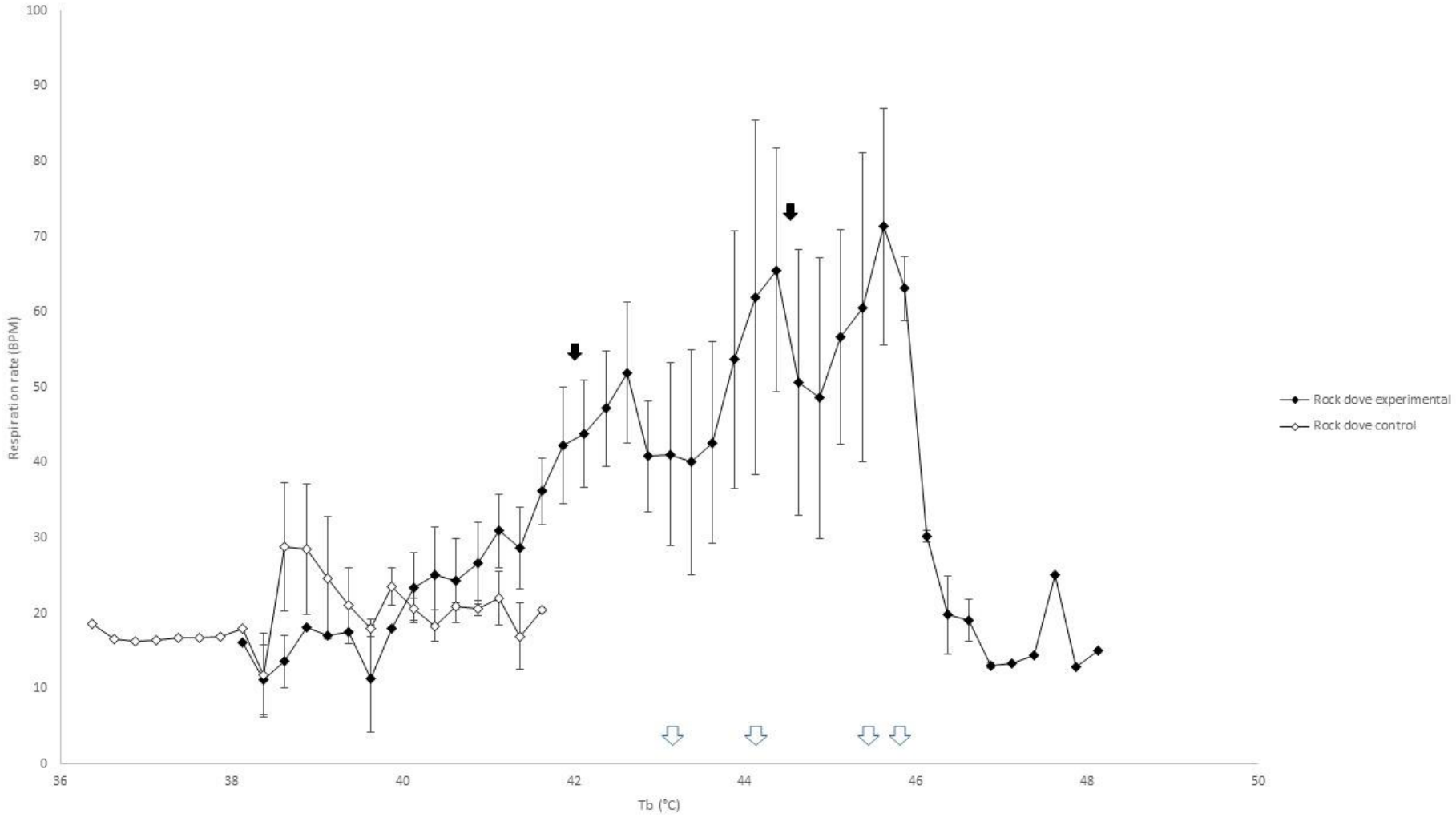


Figure 4. *Respiration rate in control rock doves remained stable with increasing cloacal temperatures, but increased in heat exposed rock doves at higher cloacal temperatures initially before decreasing. The mean inflection points of these changes are indicated by the black arrows. The white arrows along the x-axis indicate the cloacal temperature at which peak respiration rate was reached for each individual heat exposed rock dove.*

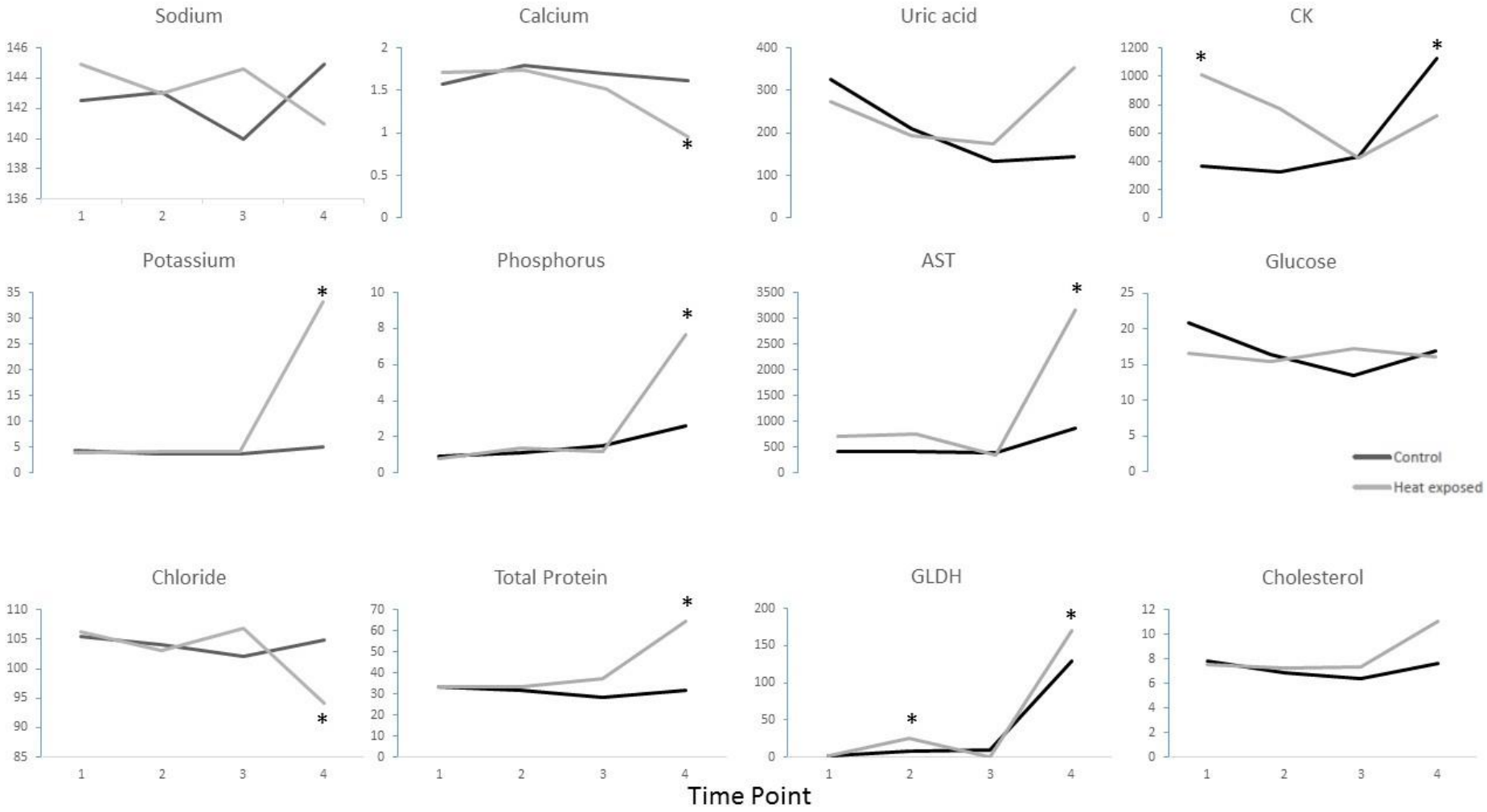


Figure 5. Summary of the blood results of galahs, with each biochemistry parameter plotted against time points 1 to 4. The units for sodium, potassium, chloride, calcium, phosphorus, glucose and cholesterol were mmol/L; total protein g/L; uric acid µmol/L; AST and CK IU/L; and AST IU/L. The SD values for each parameter were included in Table 4 of the supplemental materials section. Values outside of the reference intervals are indicated by *.

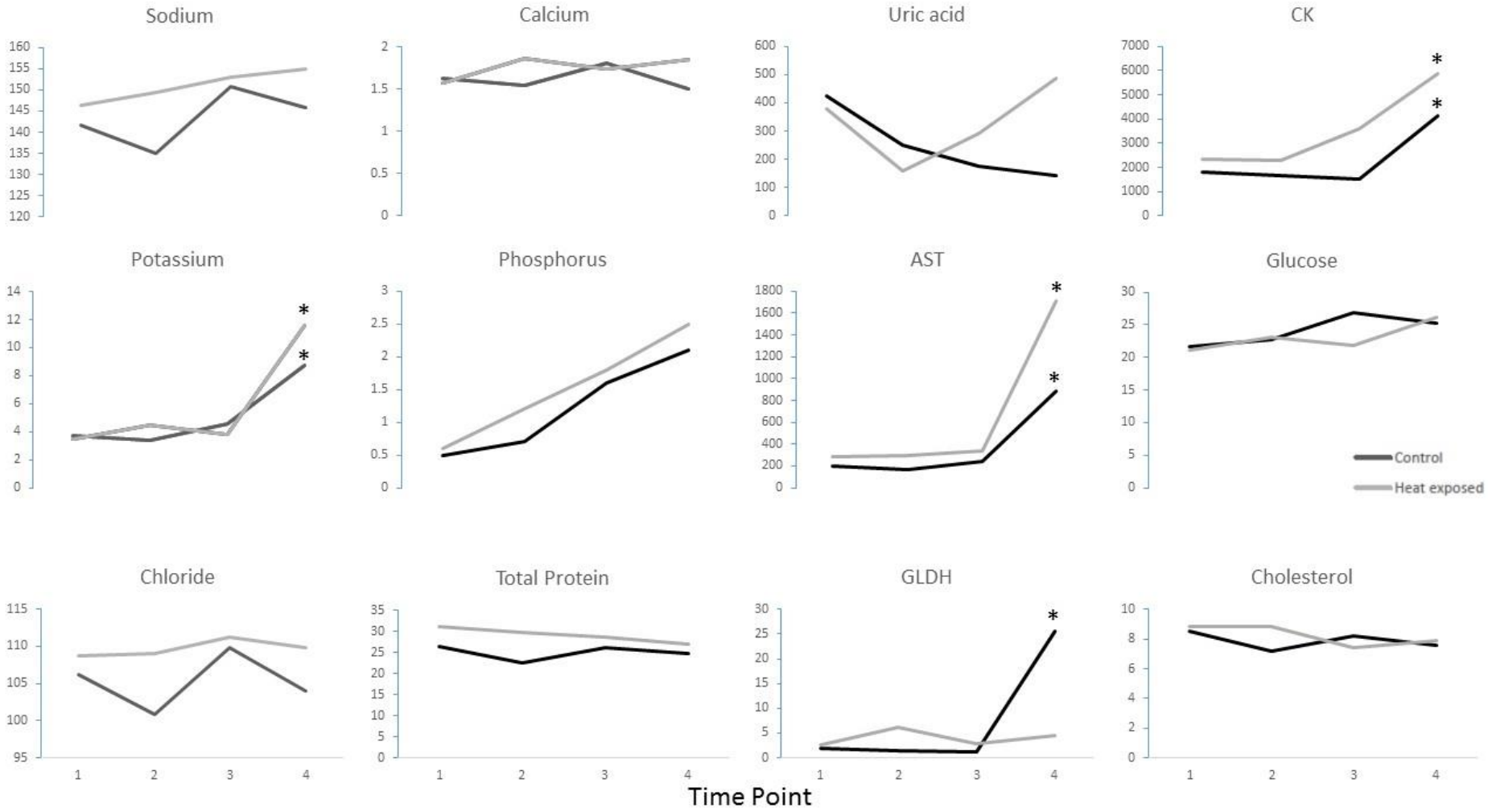
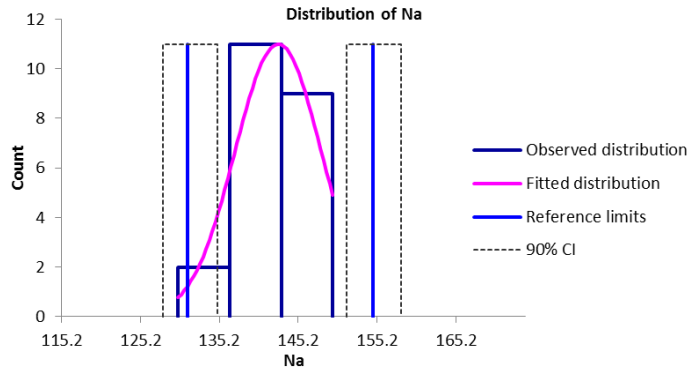
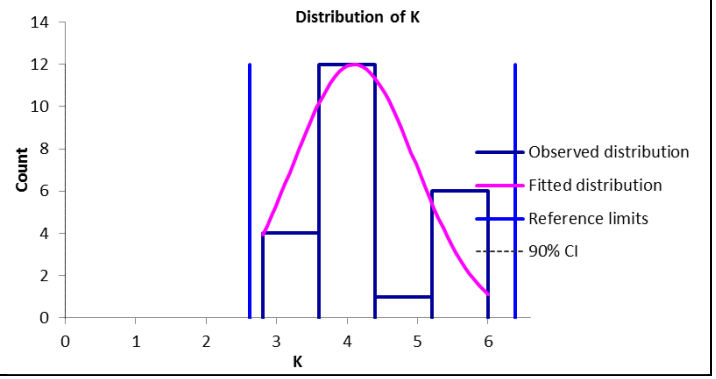


Figure 6. Summary of the blood results of rock doves, with each biochemistry parameter plotted against time points 1 to 4. The units for sodium, potassium, chloride, calcium, phosphorus, glucose and cholesterol were mmol/L; total protein g/L; uric acid µmol/L; AST and CK IU/L; and AST IU/L. The SD values for each parameter were included in Table 5 of the supplemental materials section. Values outside of the reference intervals are indicated by *.

Table 1. Descriptive statistics and reference intervals for galahs. Total sample size (n) was 24 samples, including all samples from the galahs in the control group, and samples from Times 1 and 2 for the galahs in the heat exposed group. However, some values were determined to be suspect after analysis and therefore left out of the calculation of the final reference intervals. Reference intervals were calculated using Box-cox transformed data with robust method.

Variable	*n*	Median	Mean	SD	Min	Max	LRI	URI	90% LCI	90% UCI	Histogram
Sodium (mmol/L)	22	142.00	142.65	5.47	130	149.6	131.20	154.71	128.06 – 134.95	151.33 – 158.26	
Potassium (mmol/L)	23	4.00	4.10	0.87	2.8	6	2.62	6.39	2.44 – 2.96	5.60 – 7.20	

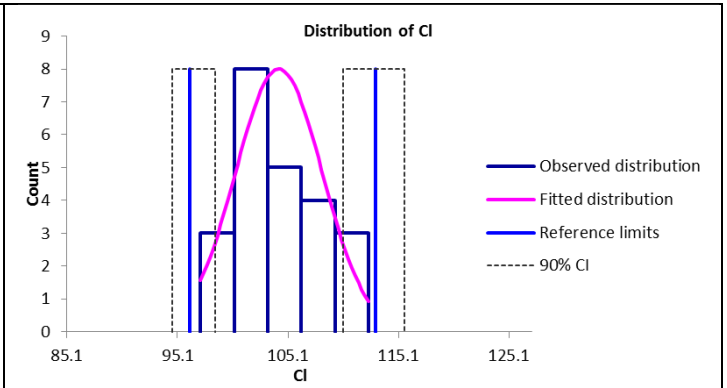
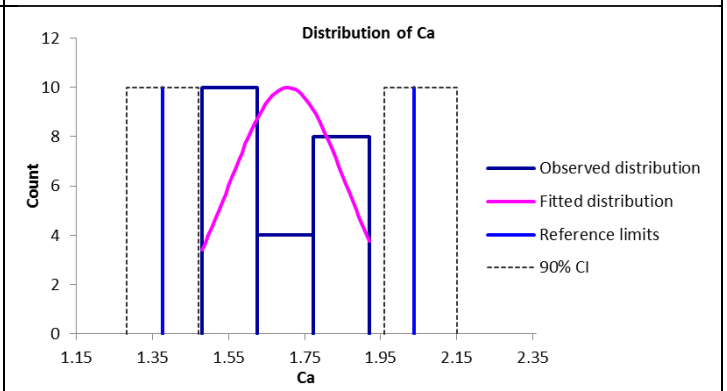
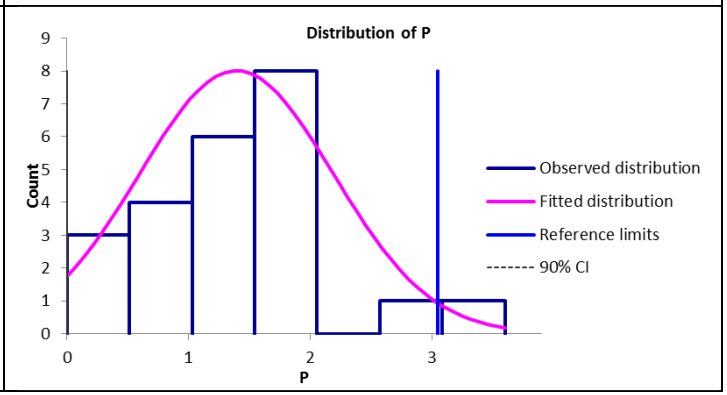
Chloride (mmol/L)	23	103.60	104.28	3.92	97.2	112.4	96.20	113.06	94.60 – 98.47	110.09 – 115.66	
Calcium (mmol/L)	22	1.720	1.705	0.153	1.48	1.92	1.377	2.038	1.284 – 1.471	1.960 – 2.150	
Phosphorus (mmol/L)	23	1.20	1.39	0.80	0	3.6	0	3.05	-0.67 – 0.24	2.59 – 3.67	

Table 1 continued

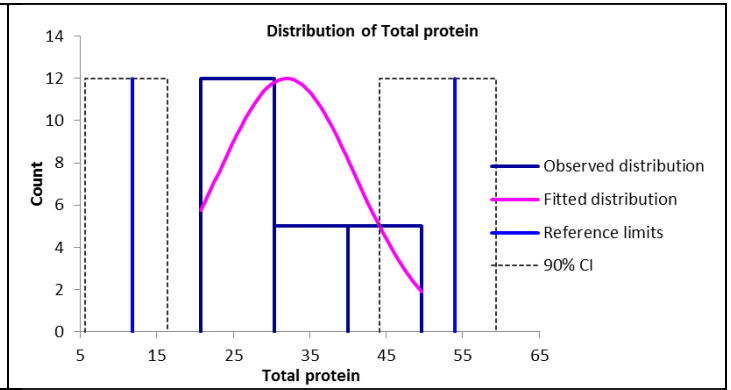
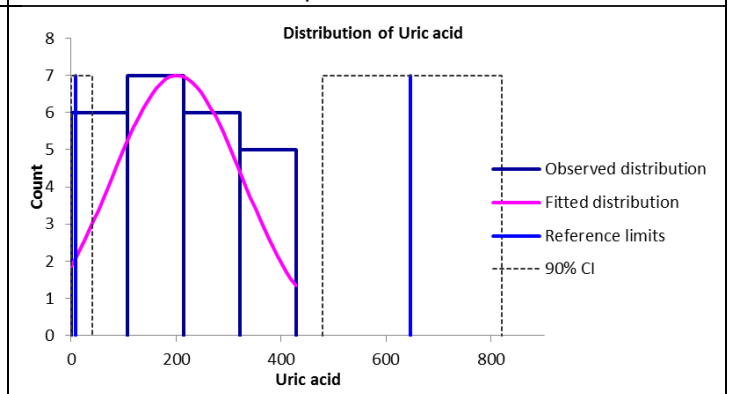
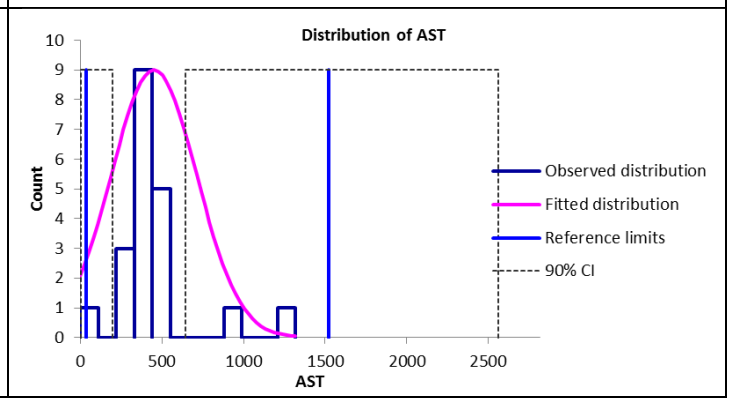
Total protein (g/L)	22	28.00	31.95	9.19	20.8	49.6	11.81	53.99	5.60 – 16.40	44.15 – 59.36	
Uric acid (µmol/L)	24	194.0	202.0	124.3	0	428	8.4	646.3	0 – 40.2	478.7 – 820.1	
AST (IU/L)	20	374.0	450.6	262.7	0	1320	34.4	1524.7	2.3 – 199.0	642.4 – 2561.1	

Table 1 continued

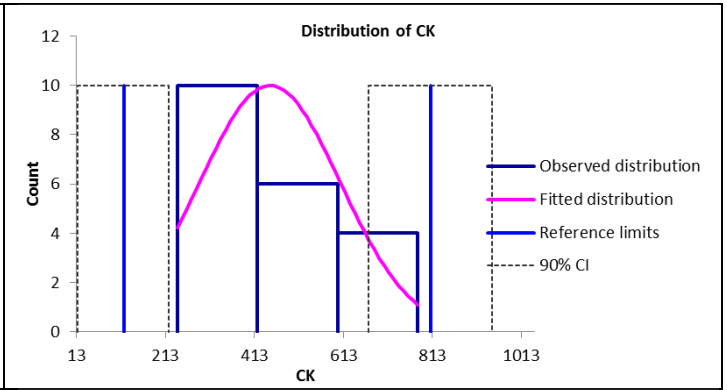
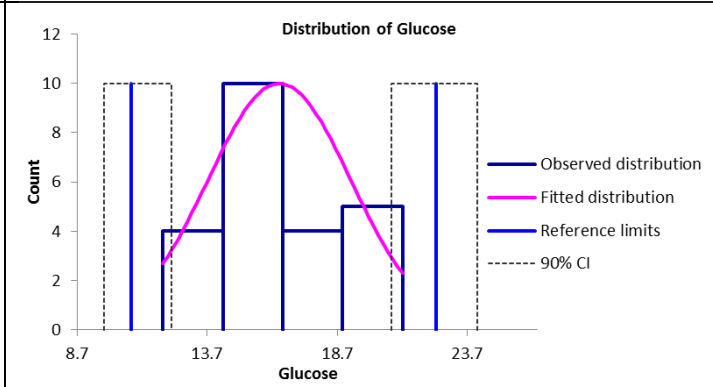
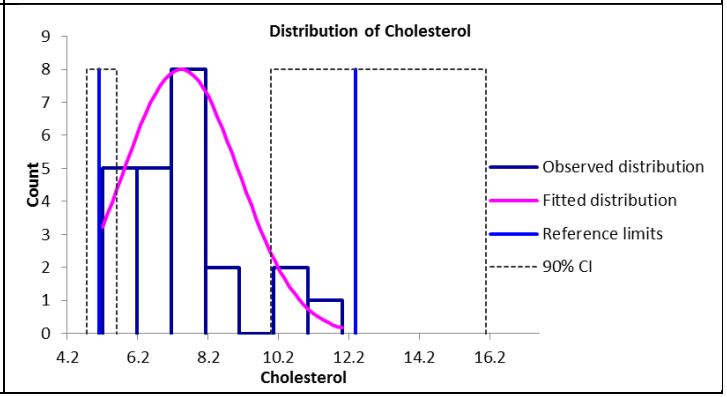
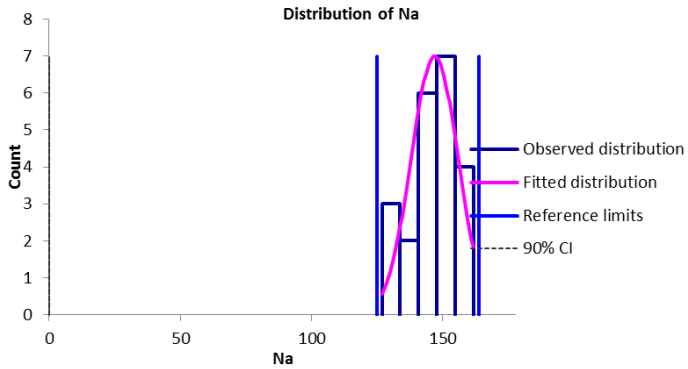
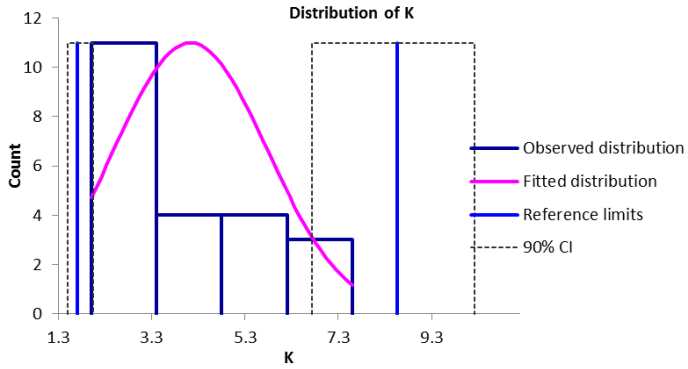
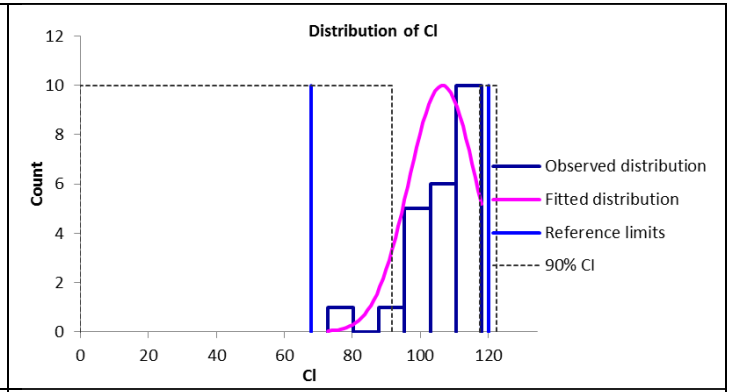
CK (IU/L)	20	416.0	448.0	158.1	240	780	121.1	808.7	15.3 – 220.2	669.3 – 947.3	
Glucose (mmol/L)	23	16.4	16.47	2.75	12	21.2	10.78	22.51	9.74 – 12.32	20.78 – 24.08	
Cholesterol (mmol/L)	23	7.20	7.43	1.65	5.2	12	5.11	12.38	4.76 – 5.60	9.97 – 16.09	

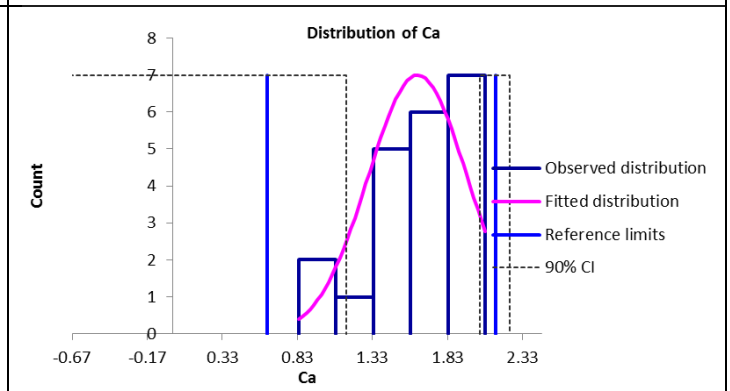
Table 1 continued

Table 2. Descriptive statistics and reference intervals for rock doves. Total sample size (n) was 23 samples, including all samples from the rock doves in the control group, and samples from Times 1 and 2 for the rock doves in the heat exposed group. However, some values were determined to be suspect after analysis and therefore left out of the calculation of the final reference intervals. Reference intervals were calculated using Box-cox transformed data with robust method.

Variable	*n*	Median	Mean	SD	Min	Max	LRI	URI	90% LCI	90% UCI	Histogram
Sodium (mmol/L)	22	148.00	146.88	8.96	126.8	161.6	124.97	163.65	115.46 – 132.97	159.17 – 167.69	
Potassium (mmol/L)	22	3.60	4.13	1.64	2	7.6	1.70	8.56	1.50 – 2.04	6.74 – 10.22	

Chloride (mmol/L)	23	108.80	106.56	9.97	72.8	118	68.00	120.03	ND – 91.71	117.44 – 122.35
Calcium (mmol/L)	21	1.680	1.630	0.330	0.84	2.08	0.626	2.152	-0.738 – 1.155	2.044 – 2.244
Phosphorus (mmol/L)	21	1.20	1.18	0.91	0.4	3.2	0.16	7.83	0.15 – 0.27	3.35 – 9.84





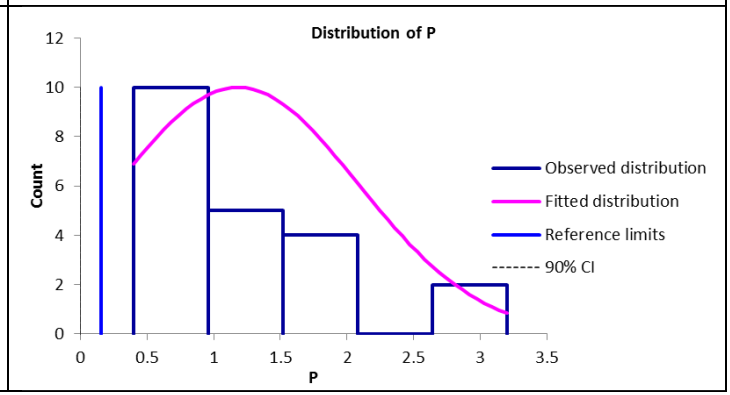


Table 2 continued

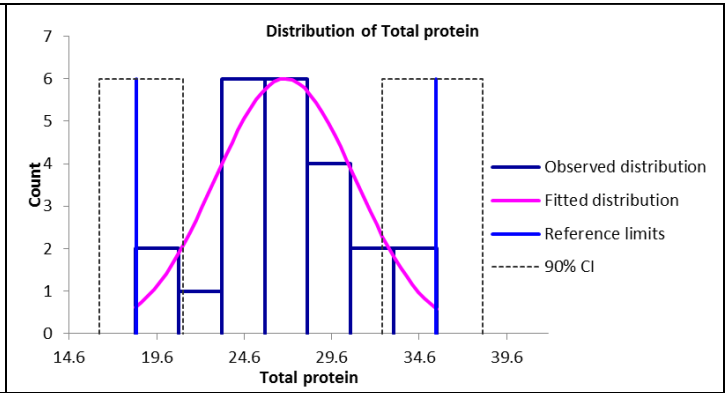
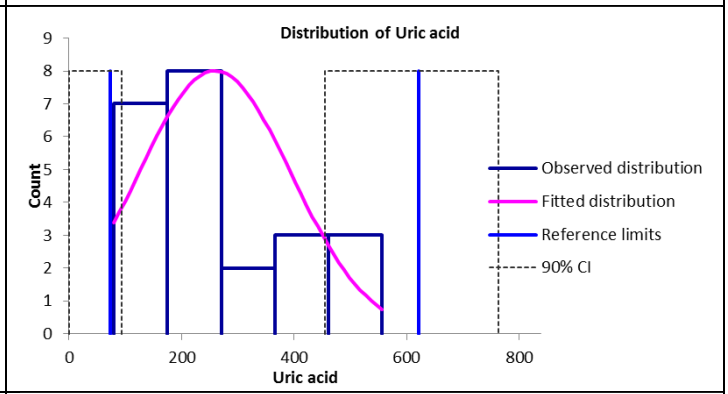
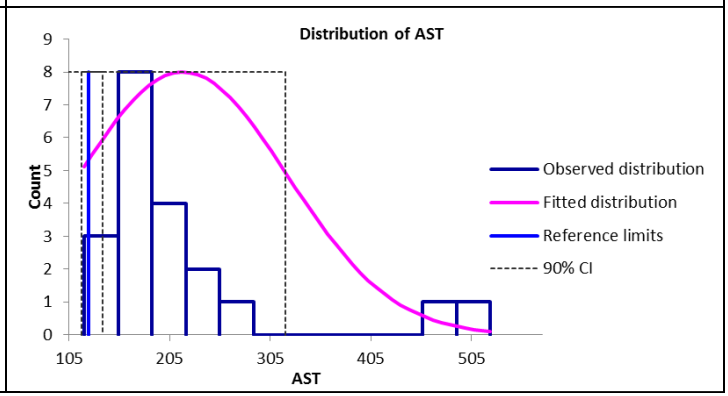
Total protein (g/L)	23	26.40	26.94	4.00	18.4	35.6	18.44	35.54	16.33 – 21.10	32.48 – 38.23	
Uric acid (µmol/L)	23	200.0	259.1	136.5	80	556	74.1	621.2	ND – 93.0	455.4 – 763.5	
AST (IU/L)	20	178.0	218.2	104.0	120	524	124.5	772.6	116.9 – 138.6	320.5 – ND	

Table 2 continued

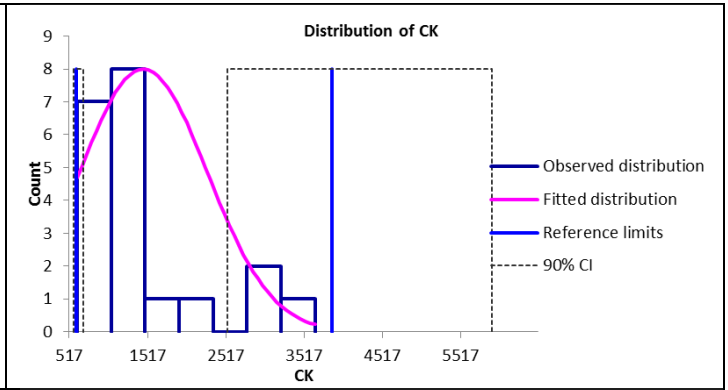
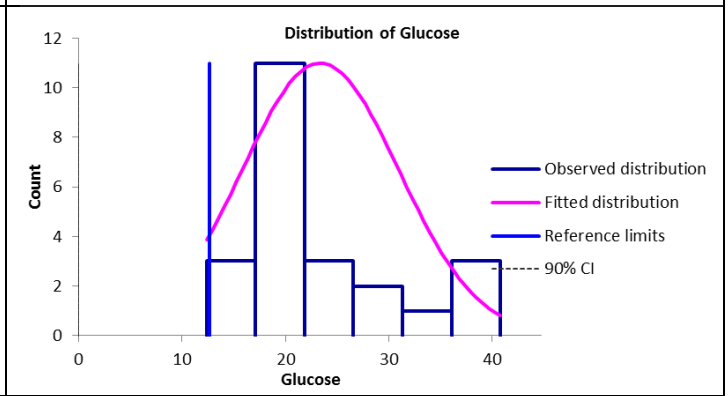
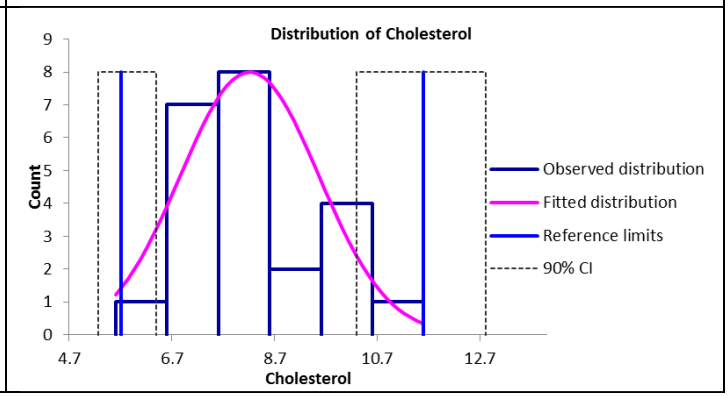
CK (IU/L)	20	1182.0	1464.0	817.5	620	3656	606.8	3870.3	574.9 – 695.1	2535.7 – 5912.3	
Glucose (mmol/L)	23	20.80	23.39	7.59	12.4	40.8	12.67	45.04	11.63 – 15.02	36.84 – 61.64	
Cholesterol (mmol/L)	23	8.40	8.21	1.35	5.6	11.6	5.71	11.59	5.26 – 6.40	10.30 – 12.81	

Table 2 continued

Table 3. Descriptive statistics and table of ascending values for GLDH in galahs and rock doves.

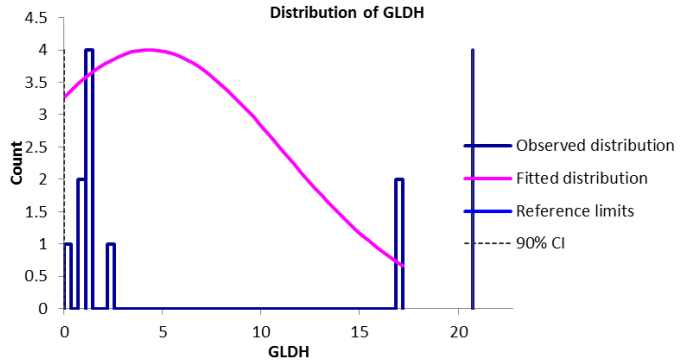
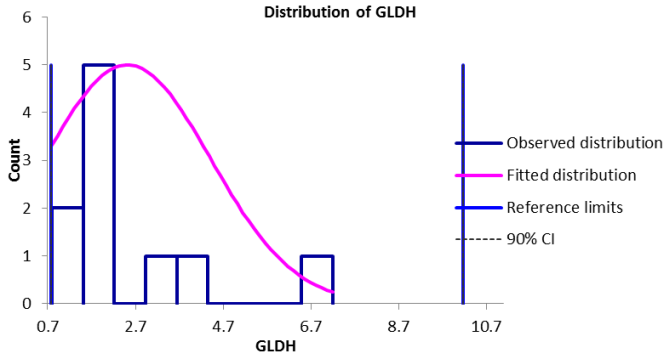
	Galah (U/L)	Rock dove (U/L)
	0	0.8
	0.8	1.2
	0.8	1.6
	1.2	1.6
	1.2	1.6
	1.2	2
	1.2	2
	2.4	3.2
	17.2	4
	17.2	7.2
Median	1.20	1.80
Mean	4.32	2.52
Histogram		

Table 4. Summary of blood results in galahs. Values outside of the reference intervals are in bold.

	Control								Experimental							
	Time 1		Time 2		Time 3		Time 4		Time 1		Time 2		Time 3		Time 4 / End point	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Sodium (mmol/L)	142.5	4.21	143.1	5.68	140.0	9.30	144.9	11.66	144.9	4.08	143	4.83	144.6	5.37	141	52.94
Potassium (mmol/L)	4.4	0.73	3.6	0.40	3.6	0.40	5.1	0.82	3.9	1.00	4.2	1.06	4.2	0.28	**33.3**	18.17
Chloride (mmol/L)	105.4	3.21	104.1	3.89	102.1	4.64	104.8	5.41	106.2	3.72	103.1	4.68	106.8	3.39	**94.2**	11.73
Calcium (mmol/L)	1.57	0.26	1.8	0.15	1.7	0.14	1.62	0.15	1.71	0.22	1.74	0.22	1.52	0.00	**0.95**	0.67
Phosphorus (mmol/L)	0.9	0.60	1.1	0.61	1.5	0.61	2.6	0.77	0.8	0.33	1.4	0.40	1.2	0.00	**7.7**	6.62
Total protein (g/L)	33.7	13.33	31.6	7.88	28.5	7.72	31.9	10.55	33.2	9.88	33.5	12.33	37.4	3.11	**64.7**	33.12
Uric acid (µmol/L)	326	85.95	209.3	129.26	134.7	71.14	145	79.56	273	105.04	194	116.96	174	76.37	354	241.63
AST (IU/L)	422	69.24	417.3	107.43	394.7	95.44	878	960.55	705	397.93	743	462.02	338	36.77	**3160**	3802.42

GLDH (U/L)	1	0.28	8.6	12.16	9.2	11.31	129	180.74	1.8	0.85	**25.8**	35.36	NA	NA	**170.4**	NA
CK (IU/L)	370	154.04	324.0	72.33	434.7	148.02	**1124**	1350.11	**1011**	676.80	769	461.41	424	356.38	720	694.70
Glucose (mmol/L)	20.9	5.14	16.4	4.00	13.5	2.54	17	3.79	16.6	1.15	15.5	0.76	17.2	1.70	16.1	6.46
Cholesterol (mmol/L)	7.8	1.74	6.9	0.83	6.4	0.80	7.6	3.12	7.5	0.89	7.3	1.10	7.4	0.28	11.1	5.28

Table 4 continued

Table 5. Summary of blood results in rock doves. Values outside of the reference intervals are in bold.

	Control								Experimental							
	Time 1		Time 2		Time 3		Time 4		Time 1		Time 2		Time 3		Time 4 / End point	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Sodium (mmol/L)	141.6	6.26	134.9	25.03	150.8	4.55	145.8	7.55	146.4	9.07	149.5	16.33	153	8.20	154.8	15.96
Potassium (mmol/L)	3.7	0.82	3.4	1.55	4.6	1.86	**8.7**	4.84	3.5	1.71	4.5	1.83	3.8	1.98	**11.6**	5.69
Chloride (mmol/L)	106.2	5.17	100.9	19.67	109.8	6.74	104.0	7.11	108.8	5.03	109	11.52	111.2	8.49	109.9	3.95
Calcium (mmol/L)	1.63	0.22	1.54	0.50	1.8	0.20	1.5	0.44	1.57	0.38	1.86	0.03	1.74	0.20	1.85	0.52
Phosphorus (mmol/L)	0.5	0.50	0.7	0.89	1.6	1.26	2.1	1.01	0.6	0.40	1.2	0.65	1.8	0.28	2.5	1.10
Total protein (g/L)	26.4	1.96	22.7	4.41	26.1	1.89	24.9	1.51	31.3	3.30	29.7	3.67	28.8	6.22	26.9	6.39
Uric acid (µmol/L)	426	123.70	249	101.32	174	27.23	141.3	53.72	377	112.49	158	52.71	290	274.36	487	285.01
AST (IU/L)	198	48.94	166	14.79	244	160.70	**882.7**	895.84	287	197.51	291	158.40	342	121.62	**1712**	1613.70
GLDH (U/L)	1.8	0.28	1.4	0.28	1.2	0.57	**25.6**	26.02	2.6	0.85	6.2	3.11	2.8	NA	4.4	1.13

CK (IU/L)	1827	1253.12	1675	959.79	1521	1037.21	**4132.0**	4662.85	2351	2605.87	2285	2477.15	3600	2460.73	**5860**	5580.35
Glucose (mmol/L)	21.7	7.63	22.8	11.70	26.8	9.74	25.2	10.87	21.2	3.75	23.1	3.21	21.8	3.68	26.1	4.43
Cholesterol (mmol/L)	8.5	1.36	7.2	1.35	8.2	1.33	7.6	1.06	8.8	1.93	8.8	0.80	7.4	0.85	7.9	1.54

Table 5 continued

Chapter 6 Heat shock protein expression is upregulated after acute heat exposure in three species of Australian desert birds

In the chapters thus far, I have demonstrated that there are major differences in the way that different species of birds cope with high environmental temperatures, as well as differences in the way their organs respond to heat physiologically and pathologically. The following original research article, accepted for publication in the journal *Avian Biology Research*, investigates the genes that may be involved in these physiological and pathological responses, in order to guide future research into this topic.

Xie, S., R. Tearle and T.J. McWhorter. In press. Heat shock protein expression is upregulated after acute heat exposure in three species of Australian desert birds. *Avian Biology Research*. **Accepted 11 Jun 2018 (ABR1800878).**

Statement of Authorship

Title of Paper	Heat shock protein expression is upregulated after acute heat exposure in three species of Australian desert birds
Publication Status	☐ Published ☑ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Xie, S., Tearle R., & McWhorter, T. J. (2018) accepted by Avian Biology Research (manuscript number ABR1800878) and currently awaiting PDF proofs.

Principal Author

Name of Principal Author (Candidate)	Shangzhe Xie		
Contribution to the Paper	Collected, trimmed and processed all samples. Performed mRNA extraction on all samples, interpreted data, wrote manuscript and acted as corresponding author.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	9/8/18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Rick Tearle		
Contribution to the Paper	Performed all statistical analyses on the data and evaluated manuscript.		
Signature		Date	9/8/18

Name of Co-Author	Todd McWhorter		
Contribution to the Paper	Supervised development of work, helped in data interpretation and manuscript evaluation.		
Signature		Date	9/8/18

Heat shock protein expression is upregulated after acute heat exposure in three species of Australian desert birds

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Abstract

Desert birds must cope with occasional and unpredictable heatwaves, which are slowly becoming more frequent with climate change. Different orders of birds have different physiological and behavioural capacities that may aid survival during a heat wave. To date, the expression of genes related to heat exposure have not been studied across different bird orders. It was hypothesized that acutely exposing native Australian birds whose natural habitat include arid environments to a high temperature (45°C), similar to during a heat wave, would result in the upregulation of genes with protective effects against cell damage (BCL-2, VEGFA and heat shock proteins) and inflammation (interleukins), as well as the downregulation of genes involved in the coagulation pathway (fibrinogen). Eight each of captive-bred budgerigars (*Melopsittacus undulatus*), zebra finches (*Taeniopygia guttata*) and diamond doves (*Geopelia cuneata*) were used. Four birds of each species were exposed to a temperature that was within the zone of thermal neutrality (35°C), while the other 4 birds were exposed to a higher temperature (45°C). The mRNA expression of selected genes were then measured using high-throughput qPCR platform (Fluidigm®, BioMark™). The results supported the hypothesis that acute exposure to a high temperature would result in the upregulation of heat shock protein (HSP) genes, but there was no significant upregulation of other genes with protective effects against cell damage nor genes associated with inflammation. The results also do not support the hypothesis that acute heat exposure would result in downregulation of the genes involved in the coagulation pathway in these birds. Among all the tissues that were analysed, the gastrointestinal tissue had the highest number of upregulated HSP genes, possibly indicating that this tissue requires the most protection to continue functioning. Diamond dove organs also had the highest number of HSP genes upregulated, possibly a reflection of their ability to better protect their cells at high temperatures.

Keywords

Avian heat tolerance; heat shock proteins; climate change adaptability

Introduction

Arid environments pose a unique set of challenges to organisms. These challenges include the lack of basic resources such as food and water, as well as extremes in temperatures. One of the most important features of arid environments is the extreme heat, especially during summer. In addition to coping with chronic, high environmental temperatures coupled with a lack of food and water, desert birds also have to cope with unpredictable heat waves that are slowly becoming more frequent. Sterl et. al. (2008) predicted that maximum temperatures may reach 48°C across the American Midwest, 54°C in South America and 50°C in Australia and India by the year 2100. Based on scientific (Serventy 1971, Finlayson 1932, McKechnie et al. 2012, Low 2011, Saunders et al. 2011) and anecdotal news reports (PerthNow 2009a, PerthNow 2010, PerthNow 2009b), heat waves have already been reported to have killed large numbers of birds in Australia. It appears that even if desert birds have evolved physiological specializations to cope with the extreme heat in arid environments, there may still be a limit to their ability to survive during prolonged periods of high temperatures.

As temperatures continue to rise with climate change, sensitive taxa of birds will be challenged (McKechnie et al. 2012, Foden et al. 2013, Garnett and Franklin 2014). Although extreme clime events such as heat waves threaten the survival of certain taxa of birds, Garnett et al. (2014) acknowledged that they were unable to factor in the sensitivities of threatened bird species to extreme climate events. Responses to heat waves depend on the functional traits of different avian species (Albright et al. 2011). Different orders and/or species of birds have different physiological and behavioural flexibilities that determine whether or not they survive during a heat wave. Columbiform birds generally have higher heat tolerance limits compared to passeriform and psittaciform birds (McKechnie et al. 2016, Whitfield et al. 2015,

McKechnie et al. 2017, Smith et al. 2017, Smith et al. 2015). In a captive situation, diamond doves (Columbiformes) have also been found to not alter their behaviour in response to heat, whereas budgerigars (Psittaciformes) and white-browed woodswallows (Passeriformes) increased the proportion of time spent stationary and decreased the proportion of time spent feeding in response to heat (Xie et al. 2017b). Zebra finches (Passeriformes) and budgerigars also lack a corticosterone response when exposed to high temperatures, suggesting that they may be at a further disadvantage when compared to diamond doves in the face of surviving during a heatwave (Xie et al. 2017a). These studies seem to indicate that psittaciform and passeriform birds have a lower capacity to cope with exposure to high temperatures than columbiform birds, but it is unknown if psittaciform or passeriform birds may have other mechanisms that confer survival advantages during heatwaves.

To date, the expression of genes related to heat exposure have not been studied across different orders of birds. A few studies have investigated the effects of heat on the transcriptome of chicken tissues (Coble et al. 2014, Li et al. 2011, Wang et al. 2013, Sun et al. 2015a, Sun et al. 2015b). Although these poultry studies are helpful in aiding our understanding of the genetic effects of heat exposure in birds, they do not include higher temperatures that desert birds frequently experience, and do not include bird species outside of the order Galliformes. However, despite the limitations in extrapolating the results from poultry studies to desert birds, they provide a good basis for selecting genes that may be expected to be differentially expressed under heat stress.

The objective of our study was to quantify the effects of heat exposure on the expression of genes in the lung, liver, kidney and gastrointestinal tract of three Australian desert avian species using high-throughput qPCR. It was hypothesized that acutely exposing native Australian birds to high temperatures above their thermoneutral zone would result in the upregulation of genes with protective effects against cell damage (BCL-2, VEGFA and

heat shock proteins) and inflammation (interleukins), as well as the downregulation of genes involved in the coagulation pathway (fibrinogen). Furthermore it was hypothesized that all the species would experience an upregulation in the expression of genes with protective effects against cellular damage as they should be adapted to living in the desert environment. This mechanism would be similar to the way in which indigenous chickens from harsh environments have strong selection signals for genes conferring protection from oxidative stress (Fleming et al. 2016). Based on histopathology changes in the organs of the same species of birds exposed to the same conditions (S. Xie, L. Woolford, T. J. McWhorter, unpublished data), it was also hypothesized that zebra finches would experience an upregulation in the expression of genes with protective effects against cell damage in kidneys more than other organs, whereas diamond doves would do so in the gastrointestinal tract more than in other organs.

Materials and methods

Animals

Eight each of captive-bred budgerigars [*Melopsittacus undulatus* (2 male and 6 females)], zebra finches [*Taeniopygia guttata* (3 male and 5 females)] and diamond doves [*Geopelia cuneata* (5 males and 3 females)] were used as model species for the bird orders Psittaciformes, Passeriformes and Columbiformes respectively. These species were chosen because their wild geographic ranges include the Australian desert, and were also widely bred in captivity for the pet industry.

The birds were obtained from private bird breeders in Adelaide during the austral summer of 2014-2015. Although the exact rearing conditions of the birds were not known,

they were all from captive bred lines. They were raised in outdoor aviaries in metropolitan Adelaide, thereby ensuring that they all were subjected to the same environmental conditions before being transferred to outdoor aviaries at the University of Adelaide Roseworthy Campus (Roseworthy, South Australia, latitude -34.53397°, longitude 138.75023°). The birds were fed a commercial bird seed mix consisting of white French millet, panorama millet, panicum, sorghum, canola seed, canary seed, dehulled oats, Japanese millet, Shirohie millet, wheat, red millet, linseed and shell grit; and water available *ad libitum* in the aviaries. They were allowed to acclimatize in the outdoor aviaries for at least 2 weeks before being subjected to the experimental protocol to allow their hypothalamic-pituitary-adrenal (HPA) axis to normalize (Dickens et al. 2009). All experiments were conducted according to the Australian Code for the Care and Use of Animals for Scientific Purposes and approved by the University of Adelaide Animal Ethics Committee (Approval Number S-2013-202).

Experimental setup

Experiments were conducted in an isolated surgery room with room temperature maintained at 25°C with a built-in air-conditioner unit. Two commercial egg incubators (IM 504 egg digital incubator; Incubators & More Pty. Ltd., Australia), each measuring 130cm × 46cm × 47cm, were placed 2.5 m apart in the room facing away from each other so that birds in each incubator could be approached without the other one being disturbed. During the experiments, each bird was placed in a cage measuring 60cm × 40cm × 40cm with water and food available *ad libitum*. The entire cage was subsequently placed in the incubator. A digital video camera was placed inside each incubator so that each bird could be monitored from another room without disturbance. A total of three temperature data loggers (Thermochron iButton DS1922L; Maxim Integrated, USA) were placed in the experimental room – one in

each incubator and one at the end of the room opposite the air conditioner. The resolution of the iButtons was 0.5°C, rollover was disabled and the sample rate was set at 120 seconds.

Experimental protocol

Each individual bird was moved into the exposure chamber set at 25°C at least 18 hours before the heat exposure. Each bird was randomly assigned to an exposure temperature of 35°C (control) or 45°C (experimental) using a random number generator (Urbaniak and Plous 2013). The incubator exposed the bird to a maximum temperature of 34.42±0.12°C when the incubator was set to 35°C and a maximum temperature of 43.18±0.12°C when it was set to 45°C (Xie et al. 2017a). The incubator also took 86 minutes to reach the maximum temperature when it was set to 45°C, compared to 40 minutes when it was set to 35°C. This meant that the birds were exposed to a longer period of increasing temperature when the incubator was set to 45°C and a shorter period at the maximum temperature. The control group was therefore exposed to a temperature that was within the zone of thermal neutrality for all 3 species, i.e. 29 to 41°C for budgerigars (Weathers and Schoenbaechler 1976), 29.5 to 40°C for zebra finches (Calder 1964) and 34 to 40°C for diamond doves (Schleucher 1999) [which was a lower upper critical temperature compared to the temperature used in an older study (45°C) on the same species (Schleucher et al. 1991)]. On the other hand, the experimental group was exposed to a temperature that is both above the zone of thermal neutrality for all 3 species, and also similar to temperatures during a heat wave.

Each bird was monitored using digital video camera during each temperature exposure and criteria for removal from the experiment included constant escape activity lasting more than 10 minutes, behavioural signs of distress (e.g. closed eyes, fluffed feathers

and inactivity) and loss of righting reflex. However, none of these behaviours were observed throughout the experiment.

At the end of heat exposure, euthanasia was performed using 100mg/kg of sodium pentobarbitone intraperitoneal. The bird's duodenum, liver, lungs and kidneys were collected and stored in RNAlater (Thermo Fisher Scientific) for genetic analysis. The duodenum samples were full thickness with the lumens washed in sterile 0.9% normal saline solution after collection.

Primer design

The full mRNA sequences of each gene were obtained from NCBI for all species. The sequences of the same gene for the 3 species were then entered into Clustal Omega (Sievers et al. 2011) to generate alignments. The FASTA output file for the aligned genes were then loaded in Gemi v1.5.0 (Sobhy and Colson 2012) to generate primers containing up to 2 degenerate bases that resulted in a product size of 100 base pairs with a melting temperature of 55°C. Primers were chosen to avoid 3' Gs and Cs. NCBI Primer-blast was then used to check each primer for specificity. As it was difficult to design primers that both worked across all three species and spanned exon junctions, non-reverse transcriptase controls were included to assess potential DNA contamination. The primers for the genes are summarized in Table 1.

cDNA synthesis and mRNA amplification

The frozen tissues were homogenized in 1.5 ml Eppendorf Safe-lock tubes using 1.5 mm stainless steel beads (30-40 beads per tube) at 50 Hz in two 180 second rounds of bead

beating. Total RNA was isolated using Qiagen RNeasy kits from each organ sample. The RNA was then treated with Ambion DNA-free to remove contaminating genomic DNA. 1µl of each RNA sample was quality checked using the Perkin Elmer LabChip GX. 8µl of RNA (~650ng) was converted to cDNA using SuperScript II FSSS (Invitrogen, 18080-05) and Random Hexamers cDNA was RNase H treated. 1.25µl cDNA was used as input into the Fluidigm Pre-amplification reaction with 14 cycles of PCR and a pool of all primers. Pre-amplified products were diluted 1:10 and used as template in the Fluidigm Gene Expression analysis using EvaGreen on the BioMark. The 48.48 Dynamic Array was primed in the Integrated Fluidic Circuits (IFC) controller (Fluidigm) prior to loading of sample and primer. The following cycle conditions were used: 1 minute at 95°C, followed by 30 cycles of PCR with denaturation for 5 seconds at 96°C and annealing/elongation for 20 seconds at 60°C. Melting curves were generated after each run to confirm a single PCR product (from 60°C to 95°C, increasing 1°C/3 s). Reactions were performed in duplicate (cDNA replicates) and non-template controls were included to indicate potential problems with non-specific amplification or sample contamination.

Data Analysis

qPCR amplification protocols typically report a Ct value i.e. the number of cycles to reach the exponential phase of amplification. The lower the amount of starting template, the higher the Ct value. For each tissue sample at each temperature, two qPCR Ct values were calculated for each gene. When both amplifications succeeded, the mean of the Ct values was taken. Where only 1 succeeded, it was taken. Where both failed, the Ct value was set to NA.

There were 4 birds in each experimental group (species and temperature) so to assist visual inspection of the data, the mean Ct and standard deviation (SD) for each gene was

calculated, for each tissue of each experimental group. As the Ct values are already reported on a log scale, the gene expression of the experimental group was compared to that of the control group by subtracting the mean gene values at 35°C from the mean gene values at 45°C for each species and tissue (creating a Delta Ct value). The SD of the Delta Ct value was also calculated.

The TFRC gene is frequently chosen to be a housekeeping gene in avian gene expression studies, and was included in our study for this purpose. From visual inspection of Delta Ct values, the TFRC gene was seen to be successfully assayed in all species, tissues and temperatures, and showed the least variation, so was confirmed as a suitable control.

Each gene was analysed for changes in gene expression due to temperature, by comparing the 45°C and 35°C value, using the control gene as the baseline for each temperature, utilising the function lme of the R package nlme. This function partitions the variance for the gene against the control gene, at the 2 temperatures, and reports the probability (P) that the difference in Ct value between 45°C and 35°C is 0. In cases where there were less than 2 data points for a gene in a species/tissue/temperature, the variance could not be calculated and P is reported as NA.

After correction for multiple testing (Holm), P values less than 0.05 were regarded as significant and genes with these P values reported as differentially expressed.

Results

The overall results are summarized in Table 2, Figure 1 and Figure 2. Figure 1 is a heat map indicating the direction and significance of fold changes in the expression of each

gene for each organ and species. Figure 2 shows the fold change in Ct value (± standard deviation) at 45°C relative to 35°C for each gene in each organ and species.

Discussion

The results supported the hypothesis that acute exposure of native Australian birds to high temperatures which are above the upper limits of their thermoneutral zones (45°C) would result in the upregulation of heat shock protein genes. However, there was no significant upregulation of other genes with protective effects against cell damage (BCL-2 and VEGFA) nor genes associated with inflammation (interleukins). The results also do not support the hypothesis that acute heat exposure would result in the downregulation of the genes involved in the coagulation pathway (fibrinogen).

The results also support the hypothesis that all species would experience an upregulation in the expression of genes with protective effects against cell damage as they are adapted to living in the desert environment, albeit to varying degrees. Diamond dove organs also had the highest number of HSP genes that were upregulated, possibly reflecting their ability to protect their cells better during high temperatures.

However, the results also do not support the hypothesis that zebra finches will experience an upregulation in the expression of genes with protective effects against cell damage in kidneys more than other organs. Diamond doves experience this response in gastrointestinal tissue more than any other tissue. Zebra finches had an equal number of upregulated HSPs in the kidney and liver. Diamond doves appeared to experience an upregulation in the expression of heat shock proteins equally in all organs. The

gastrointestinal tracts of all bird species had the highest number of upregulated HSP genes, possibly indicating that this tissue requires the most protection to continue functioning.

Heat shock proteins

Heat shock proteins (HSP) act as molecular chaperones, interacting with other proteins to minimize the probability that they will inappropriately interact with one another (Feder and Hofmann 1999, Kregel 2002). Several genes for HSPs belonging to the HSP70 family, i.e. HSP72 (inducible), HSP73 (constitutive) and HSP75 (tumour necrosis factor receptor associated protein 1) were included in the study. HSP70 has a wide variety of functions at cellular, tissue, organ and organismal levels (Kregel 2002). It has several functions including increasing tolerance to hyperthermia and endotoxins, as well as reduction of protein denaturation from heat exposure in mammalian cells (Feder and Hofmann 1999). In our study, the gene for the inducible HSP72, HSP70-P2, was upregulated in all the organs for all the species except in the zebra finch lung. This was to be expected given the inducible nature of the HSP and the need for its protective effects in all the organs when exposed to heat. The gene for the constitutive HSP73, HSCA8, was upregulated in only the budgerigar gastrointestinal tract, kidney and liver. This is possibly an indication that budgerigars have a higher capacity to produce cell protective HSPs after heat exposure, and therefore less susceptible to the effects of high temperatures. HSP70 also has a role in modulating glucocorticoid receptor responses, but to a lesser extent than HSP90 (Grad and Picard 2007). They are thought to disrupt established interactions between proteins, possibly via ATP dependent mechanisms or by facilitating the establishment of alternative interactions between these proteins (Lindquist and Craig 1988). In these ways, they are able to protect heat-sensitive

proteins from degradation or prevent damaged proteins from precipitating immediately after heat shock and affecting cell viability permanently (Etches et al. 2008).

HSP70 was shown to have several protective effects in poultry exposed to heat stress. Feed restriction and heat conditioning at an early age in male broiler chickens resulted in higher HSP70 expression, as well as better heat tolerance and disease resistance (Liew et al. 2003). HSP70 was induced in the gastric mucosal and epithelial cells of the jejunum and ileum in broilers exposed to acute heat stress (Hao et al. 2012). The results of our study indicate that desert bird species also had the ability to upregulate inducible HSP70 in their gastrointestinal tract. In poultry, this induction of HSP70 was positively correlated with increases in amylase, lipase, trypsin and alkaline phosphatase activities, possibly helping with intestinal digestion and absorption during acute heat stress (Hao et al. 2012). Lactic dehydrogenase, a sensitive indicator of cell damage, increases in the jejunal mucosa during heat stress, but its level is decreased by HSP70 (Gu et al. 2012). HSP70 was also positively correlated to antioxidant levels and negatively correlated to corticosterone levels in the jejunal mucosa after acute heat stress (Gu et al. 2012). It is possible that HSP70 could have similar protective effects in in the species of desert birds included in this study when exposed to heat stress. Expression of HSP70 in myocardial tissues was increased in Japanese Quail in response to stress, but there was no such increase in all tissues of HSP30, HSP60 and HSP90 (Hoekstra et al. 1998). The lack of HSP75 response in all the organs and species in this study indicates that HSP75 is unlikely to be an important HSP in the responses of these birds to heat exposure, even though it may have a role in regulating cellular stress responses (Zhang et al. 2015, Boles et al. 2015).

Other avian studies have found increases in HSP60 in response to stresses (Merino et al. 2002, Morales et al. 2006, Moreno et al. 2008, Merino et al. 2006), so HSP60 was also

included in this study. HSP60 is also proapoptotic (Kregel 2002). HSP60 was upregulated in all diamond dove organs and also in budgerigar and zebra finch gastrointestinal tracts in the present study, indicating that it is potentially an important HSP for its cell protective effects in gastrointestinal tracts and diamond doves. These results may also suggest that the gastrointestinal cells may be the first to undergo apoptosis if the temperatures continued to rise.

Both α and β forms of HSP90 were included in this study. HSP90 is involved in the tolerance of hyperthermia and glucocorticoid receptor function amongst other functions in mammalian cells (Feder and Hofmann 1999). It appears to keep steroid receptor complexes inactive until the proper signal for activation is received (Lindquist and Craig 1988). It plays crucial roles in glucocorticoid receptor folding, hormone binding to glucocorticoid receptors, transport of glucocorticoid receptors to the nucleus, activation of transcription in the nucleus, nuclear retention of glucocorticoid receptors and degradation of glucocorticoid receptors after their functions have been served (Grad and Picard 2007). HSP70 may have a role in glucocorticoid receptor folding and degradation, but HSP90 is the major molecular chaperone for the other processes (Grad and Picard 2007). Vertebrates should have both α and β forms of HSP90, and due to the fact that HSP90β was not induced by heat in chickens, it was suggested that HSP90α is both constitutive and inducible, but HSP90β is strictly constitutive in chickens (Meng et al. 1993). This is in contrast to other vertebrate species where both forms of HSP90s are inducible by heat shock (Meng et al. 1993) but it is unknown if this would apply to all avian species. In this study, the gene for HSP90α, HSP90AA1, was upregulated in all organs of all the species, whereas the gene for HSP90β, HSP90B1, was upregulated in all organs other than the liver in diamond doves, but only the kidney of budgerigars. This may indicate that HSP90α is inducible in budgerigars, diamond doves and zebra finches, but HSP90β is likely inducible in diamond doves only. The only species shown to have a

corticosterone response to this heat exposure was the diamond dove (Xie et al. 2017a), and it is interesting that it is the only species in which HSP90β was upregulated in all organs. This potentially reflects the diamond doves' need to regulate glucocorticoid function in the face of a glucocorticoid response to the heat exposure.

Another HSP that has a role in thermotolerance is HSP27 (Landry et al. 1989, Ciocca et al. 1993). HSP27 protects against apoptosis by interfering with the mitochondrial pathway of caspase-dependent cell death (Bruey et al. 2000), which may be important in protecting cells against the effects of high temperatures. Therefore, proteins 1, 2, 3 and 7 of HSP27 were included in this study. There was no clear pattern in the upregulation of the expression of the gene for HSP27. HSP27 protein 3 in the budgerigar liver. Protein 7 was upregulated in the budgerigar lung only. The lack of HSP27 upregulation is possibly an indication that the extent of heat exposure in this experiment was insufficient to induce cellular apoptosis, and the anti-apoptotic effects of HSP27 were less important.

Overall, the HSP genes of zebra finch organs were the least upregulated under the experimental conditions. This may be an indication that zebra finches may be more susceptible to the effects of high temperatures as fewer protective proteins are expressed and therefore less likely to survive during heatwaves. This expression pattern may also suggest that zebra finches have high levels of constitutive HSPs, therefore do not require strong HSP inductions after heat exposure. The latter has been shown in other desert species (Zatsepina et al. 2000, Arad et al. 2010). Conversely, diamond doves expressed more protective proteins compared to zebra finches and budgerigars, which may explain their higher predicted heat tolerance limits (McKechnie et al. 2016, Whitfield et al. 2015, McKechnie et al. 2017, Smith et al. 2017, Smith et al. 2015, Gerson 2015, Wolf 2015).

Other genes

While the exact pathophysiology of heat stroke in birds is not as well-studied, one study in baboons suffering from induced heat stroke demonstrated damage to multiple organs including the jejunum, liver, spleen, lung and kidney; and this manifested as vascular congestion, haemorrhage, thrombosis, increased inflammatory cells, and disruption of normal cell and tissue architecture (Roberts et al. 2008). In another report, the pathological changes of a bird that was suspected to have suffered heat stroke under general anaesthesia revealed mild multifocal acute degeneration and contraction band necrosis of the biceps femoris muscle, as well as diffuse moderate acute congestion of the lungs (Hofmeister 2005). There was also a diffuse moderate chronic left ventricular hypertrophy, but that was suspected to be a coincidental finding that contributed to the bird's death rather than a result of the presumed heat stroke (Hofmeister 2005). In humans, coagulopathy associated with a decrease in plasma fibrinogen, amongst other coagulation factors, was one of the findings in heat stroke patients (Al-Mashhadani et al. 1994).

The genes for fibrinogen A, B and G were generally unchanged in this study. The genes for fibrinogen A and G were downregulated in the diamond dove gastrointestinal tract and diamond dove lung. There was no change in the expression of the fibrinogen genes in any of the other tissues and species. The general lack of downregulation across most tissue types indicates that the birds were not close to suffering from clinical heat stroke under the heat exposure conditions in this study.

Interleukin 6 was only upregulated in the budgerigar liver, while interleukin 8 was upregulated in the diamond dove liver and lung. Interleukin 6 is correlated with the severity of heat stroke in primates (Bouchama et al. 2005). The results may indicate that these particular organs in budgerigars and diamond doves were more susceptible to inflammation from the

effects of heat exposure, but the general lack of upregulation in both interleukins suggest that the birds were not close to suffering heat stroke under these experimental conditions.

Other non-HSP genes included in this study were albumin, B cell lymphoma 2 protein (bcl-2) and vascular endothelial growth factor A (VEGFA). Albumin is involved in the binding of heat shock factor protein 1 to the heat shock element in the promoter to activate other HSPs such as HSP70 and HSP90 (Yiangou et al. 1998). Bcl-2 (Balcer-Kubiczek et al. 2000) and VEGFA (Gerber et al. 1998) has anti-apoptotic effects that can be important in protecting against cell death during heat exposure. Similar to the genes for HSP27, there was generally little up- or down-regulation of these genes, with no clear pattern to their regulation. Albumin was upregulated only in the zebra finch gastrointestinal tract. Bcl-2 was only upregulated in the diamond dove liver. VEGFA was unchanged in all organs and species. This is likely an indication that the extent of heat exposure in this experiment was insufficient to induce cell apoptosis, and the anti-apoptotic effects of these proteins were less important.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and transferrin receptor protein 1 (TFRC) were chosen as housekeeping genes for this study (Olias et al. 2014). TFRC proved to be a gene that was suitable as the control gene in this study, whereas GAPDH was not. Another gene that was previously found to be a suitable housekeeping gene in chickens, ubiquitin B (Fan et al. 2012), was also found to be upregulated by heat (Sonna et al. 2002). The gene for ubiquitin B was unchanged in diamond doves and zebra finches, but because it could not be assessed in budgerigars, its suitability as a housekeeping gene is unknown for this study.

Conclusion

This is the first reported study to use high-throughput qPCR to study the effects of acute heat exposure on differential gene expression of desert avian species belonging to different orders, thus providing novel insight into the relationship between gene expression and physiological performance in these taxa. This information provides a basis for future investigations into the expression of genes, possibly using next generation molecular genetic techniques such as RNA-seq analysis of transcriptomes of organs relevant to the survival of desert birds during heat waves.

The results of this study indicated that protection against protein degradation, as reflected by upregulation of HSP72, and HSP90α, was the most important gene expression when budgerigars, diamond doves and zebra finches were exposed to the experimental conditions of the study. Anti-apoptotic effects were not as important as the genes for proteins that confer protection against apoptosis were not regulated with a clear pattern. The genes for fibrinogen and interleukins were also unaffected in general, indicating that the temperatures the birds were exposed to were not high enough to induce coagulopathy nor significant inflammation associated with clinical heat stroke. Overall, the mRNA responses of the species in this study were consistent with their predicted heat tolerance limits (McKechnie et al. 2016, Whitfield et al. 2015, McKechnie et al. 2017, Smith et al. 2017, Smith et al. 2015, Gerson 2015, Wolf 2015), i.e. the higher the predicted heat tolerance limits, the larger the number of protective proteins expressed.

There were several limitations to our study. Future studies should build upon the results achieved in this study using only the experimental approach (qPCR) and investigate the effects of heat exposure on the transcriptome of these species, like was done for chicken tissues in Coble (2014) to reveal novel genetic responses to heat exposure. Also, for the genes

that did not have significant changes, it could not be determined if it meant that there were no changes in gene expression at 45°C, that the expression had not increased yet and might have increased with a longer exposure duration, or that expression was already declining due to failure of the birds' physiology or acclimation to exposure conditions. Further studies involving exposure of the same species to more intermediate and also higher temperatures for a longer duration are needed to differentiate between the three possible explanations for lack of changes in gene expression, as well as identify the genes that are important in protecting the cells against apoptosis. The inclusion of other genes and/or species will also help determine if there are particular genes that confer advantages to survival during heatwaves in particular bird species. Future studies can also include immunohistochemistry for the key HSPs to enable visualization of their expression in individual tissues.

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Figure 1. Heat map indicating the direction and significance of fold changes of each gene.

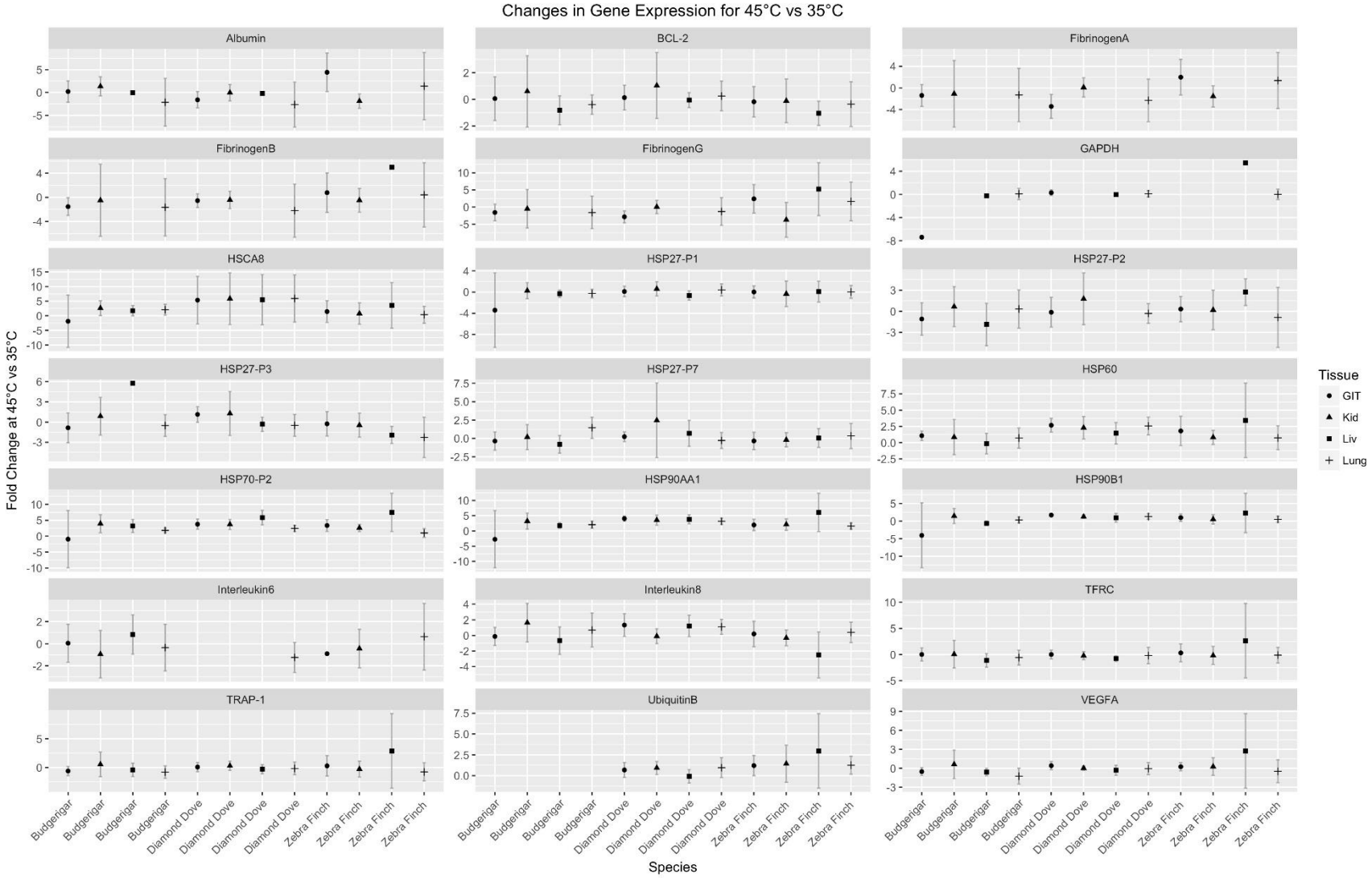

Figure 2. Difference in Ct values and standard deviation at each exposure temperature for each gene, organ and species.

Gene Heat shock protein expression	Forward primer	Reverse primer
HSP27 Protein 1	5’ CCCTTCACCTTCCTGCGCA 3’	5’ TGGGGCATCCCGAAGGA 3’
HSP27 Protein 2	5’ AGCAAGATYTAYGACCAGAAC 3’	5’ GATCCGRGGCCKGATGTAGTAG 3’
HSP27 Protein 3	5’ ACTTTCGAAGGCTGGCTCCTGA 3’	5’ GTAATTTGTATTGTCTGGTRAAG 3’
HSP27 Protein 7	5’ GAGGACATCATCGTCACC 3’	5’ TGTGGGTGAAGGTGTTCATGAC 3’
HSP60	5’ AATGTGGAAGATATTCAGCCT 3’	5’ CTTTTCACCCTTCCCCTTH 3’
HSPA8/HSC70	5’ TCYAAGAACTCCCTGGAGT 3’	5’ CTGCTTGTCMTCATCAGAGAT 3’
HSP72	5’ GACATYGACCGTATGGTGC 3’	5’ TAGGACTCRAGGGAGTTCTT 3’
HSP75 (TRAP1)	5’ TGATCAAGTTCTTCATTGACC 3’	5’ ACAATCCCCTCTCTCATGAA 3’
HSP90AA1	5’ TRCGCAGAGTTTTCATCATGG 3’	5’ TTCAGAGGTAAATCCTCAGART 3’
HSP90B	5’ GTGTGATAAAGAGAAGAACATG 3’	5’ AGACTTTGCAATGGTACCCA 3’
Ubiquitin B	5’ GATCCAGGACAARGAAGGC 3’	5’ GATGTTGTAGTCRGACAGG 3’
GAPDH	5’ TGGACCTGACCTGCCGT 3’	5’ GCCAGGATGCCCTTCAG 3’
TFRC	5’ GGACATGCCCACCTTGG 3’	5’ CCAGAAGATTCYACTGGTG 3’
Fibrinogen A	5’ CACCCAGAGCTTCAAACAA 3’	5’ CTGGCAGAGGCTTATGCTT 3’
Fibrinogen B	5’ CAGAGACAATGATGGATGG 3’	5’ GCTGAGTGGCAGCGGTT 3’
Fibrinogen G	5’ GACAATGGCATTATCTGGG 3’	5’ CATCTACTGACAGTCTGTTGA 3’
Interleukin 6	5’ GTCTGCSRGAAYAGCATGG 3’	5’ CCTCATYRAAGCCGGYGAR 3’

Heat shock protein expression

Interleukin 8	5’ CCATTCAAGAYGTGAAGCTGA 3’	5’ TCCAAGCACACCTCTCTG 3’
Apoptosis inhibitor BCL-2	5’ TGGATCCAGGACAACGGAGG 3’	5’ TAGTCTTCAGAGAGATCCAGGAG 3’
Albumin	5’ ATTCYTGTCAGAGTTTGTTTATG 3’	5’ CAGGAGTGYTTCATATCCCTTAG 3’

Table 1. List of forward and reverse primers.

Species Heat shock protein expression	Organ	Genes upregulated	Genes downregulated
Budgerigar	Gastrointestinal tract	HSP60 ($p < 0.001$) HSP70-P2 ($p = 0.02$) HSCA8 ($p < 0.001$) HSP90AA1 ($p = 0.01$)	None
	Kidney	HSCA8 ($p = 0.02$) HSP70-P2 ($p < 0.001$) HSP90AA1 ($p < 0.001$) HSP90B1 ($p < 0.001$)	None
	Liver	GAPDH ($p < 0.001$) HSCA8 ($p = 0.01$) HSP27-P3 ($p < 0.001$) HSP70-P2 ($p < 0.001$) HSP90AA1 ($p < 0.001$) Interleukin6 ($p < 0.001$)	None
	Lung	HSP27-P7 ($p = 0.02$) HSP70-P2 ($p < 0.001$) HSP90AA1 ($p < 0.001$)	None
Diamond dove	Gastrointestinal tract	HSP60 ($p < 0.001$) HSP70-P2 ($p < 0.001$) HSP90AA1 ($p < 0.001$) HSP90B1 ($p < 0.001$)	Fibrinogen A ($p < 0.001$) Fibrinogen G ($p < 0.001$)
	Kidney	HSP60 ($p < 0.001$) HSP70-P2 ($p < 0.001$) HSP90AA1 ($p < 0.001$) HSP90B1 ($p < 0.001$)	None
	Liver	BCL-2 ($p = 0.03$) GAPDH ($p < 0.001$) HSP60 ($p < 0.001$) HSP70-P2 ($p < 0.001$) HSP90AA1 ($p < 0.001$) Interleukin8 ($p = 0.01$)	None
	Lung	HSP60 ($p < 0.001$) HSP70-P2 ($p < 0.001$) HSP90AA1 ($p < 0.001$) HSP90B1 ($p = 0.01$) Interleukin8 ($p = 0.01$)	None

Zebra finch	Gastrointestinal tract	Albumin ($p = 0.04$) HSP60 ($p < 0.001$) HSP70-P2 ($p < 0.001$) HSP90AA1 ($p < 0.001$)	None
	Kidney	HSP70-P2 ($p < 0.001$) HSP90AA1 ($p < 0.001$)	None
	Liver	HSP70-P2 ($p < 0.001$) HSP90AA1 ($p < 0.001$)	None
	Lung	HSP90AA1 ($p < 0.001$)	None

Table 2. Summary of differential gene expression in each organ of each species.

Chapter 7 Discussion and conclusion

Australian desert birds face the challenge of maintaining energy and water balance in a resource-poor environment. Heatwaves and increasing environmental temperatures associated with climate change increase thermoregulation costs and add to the physiological challenges of desert environments. When the balance among energy, water and thermoregulation cannot be maintained, birds can suffer from heat illnesses, culminating in heat stroke and death. A recent study by Iknayan and Beissinger (2018) found that the collapse of a desert bird community over the past century in the Mohave Desert of the western United States was driven by climate change. These authors found that the decline in precipitation (and thus the availability of surface water) was the most important driver of bird persistence. Recent studies have revealed differences in heat tolerance limits among birds belonging to different orders (McKechnie et al. 2016, McKechnie et al. 2017, Talbot et al. 2017, McWhorter et al. 2018), but the differences in stress responses, behavioural adaptations, pathological changes and genetic adaptations amongst bird species in response to heat exposure are not well characterised. This thesis aimed to investigate these differences, so that groups of birds that are more vulnerable to increasing temperatures as a result of climate change can be more easily identified (Garnett and Franklin 2014) and conservation efforts focused on them. The three most common orders of birds in the Australian desert were focused on, i.e. Psittaciformes (parrots), Passeriformes (songbirds) and Columbiformes (pigeons/doves).

The chapters presented in this thesis demonstrate that there are fundamental differences in the way birds from different orders adjust their physiology and behaviour during times of high environmental temperatures, i.e. during heatwaves. Similarly, there are differences in the way birds from different orders develop clinical and histopathological

changes when exposed to high environmental temperatures of sufficient magnitude and/or duration.

Recent studies have demonstrated differences in heat tolerance limits, metabolic rate and evaporative water loss capacity related to high environmental temperatures among bird orders (McKechnie et al. 2016, McKechnie et al. 2017, Talbot et al. 2017, McWhorter et al. 2018). However, physiological stress responses and behavioural adaptations (Fisher et al. 1972) to heat were not well-characterised in Australian desert birds. This thesis confirms these differences and lays the foundation for more specific studies into each avian order. It also demonstrates that the findings from each avian order may not be easily extrapolated to other orders that inhabit Australian deserts, so similar studies should be performed for each avian order/species that is predicted to come under threat from rising temperatures associated with climate change, e.g. small-island birds such as the Lord Howe woodhen (*Gallirallus sylvestris*, Order Gruiformes) and Southern boobook (*Ninox novaeseelandiae undulata*, Order Strigiformes) (Garnett and Franklin 2014). There are currently no good physiological data on which conservation managers can rely on to make good predictions for how different bird species will adapt to rising global temperatures, and therefore no way to make good decisions regarding where to concentrate conservation efforts (Garnett and Franklin 2014).

The differences in clinical and histopathological changes in bird species when exposed to high environmental temperatures demonstrated in this thesis provide a guide for clinicians to make ante- and post-mortem diagnoses for birds presenting with a history of acute heat exposure. The data collected were not pathognomonic for heat exposure, but provides a basis for future studies to target specific groups of birds and/or organ systems for further investigation.

Differences in physiological stress responses among birds from different orders when exposed to high environmental temperatures

In Chapter 2, the physiological stress responses of Australian desert birds to heat exposure were investigated. The corticosterone (CORT) and heterophil:lymphocyte (H:L) ratio responses of budgerigars (*Melopsittacus undulatus*), zebra finches (*Taeniopygia guttata*) and diamond doves (*Geopelia cuneata*), as representatives of the orders Psittaciformes, Passeriformes and Columbiformes respectively, were measured. The birds were exposed to a temperature similar to what they experience during a typical summer day (35°C) and a higher temperature (45°C) similar that experienced during a heat wave. There were no significant increases between the CORT concentrations before and after heat exposure in zebra finches and budgerigars at 35°C and 45°C, but there was a significant increase in CORT concentrations in diamond doves after exposure to 45°C (Xie et al. 2017a). The H:L ratios increased significantly after heat exposure in budgerigars at 35°C and 45°C, and diamond doves at 35°C (Xie et al. 2017a). No significant correlation was found between the change in CORT and H:L ratios (Xie et al. 2017a). These data suggest that there are species differences in birds' stress responses to heat exposure that may reflect their ability to detect and adapt to high temperatures. Performing a stress study while minimizing potential confounding factors was a tricky endeavour. Factors such as social disruption of flock birds in order to expose each of them to the stressor, in this case high temperatures, could not be prevented. It was also difficult to completely eliminate the stress of moving each bird from an outdoor aviary into a small cage within an incubator. However, given that each bird was moved into the exposure chamber at least 18 h before the baseline blood collection, exposed to both temperatures and the CORT levels were compared in the same bird before and after each heat exposure, each acted as its own control, and the differences in CORT were most likely a result of the different exposure temperatures.

The resting metabolic rate (RMR) of Namaqua doves (*Oena capensis*), a Columbiform of similar size to the diamond dove, increased significantly with ambient temperature (T_a) above 35.3°C, whereas the evaporative water loss (EWL) increased significantly when T_a was above 40.9°C (McKechnie et al. 2016). The upper critical limit of thermoneutrality (T_{uc}) of yellow-plumed honeyeaters (*Ptilotula ornata*), a Passeriform of similar size to the zebra finch was 33.87°C and the EWL increased significantly when T_a was above 37.55°C(McKechnie et al. 2017). The T_{uc} for budgerigars was reported to be 41°C (Weathers and Schoenbaechler 1976), but the inflection points for RMR and EWL were not reported in this study. As the likely inflection points for both RMR and EWL were higher in diamond doves then zebra finches, and only diamond doves demonstrated a CORT response, it is possible that CORT may have a role in regulating RMR and EWL. Further studies are needed to establish this correlation better, starting with a repeat of the RMR and EWL measurements in diamond doves, zebra finches and budgerigars. Further extrapolation of the results from Chapter 2 to that of wild birds during a heatwave is limited as the experimental protocol involves far more rapid changes in air temperature than birds would experience during a heatwave. It is hard to predict how birds will cope with a more gradual, but more prolonged, increase in ambient temperature. However, the experimental setup from Chapter 2 could be modified for future studies to examine how different types of ambient temperature changes affect CORT levels in different bird species.

There also appears to be differences between the two types of stress measurements, i.e. CORT and H:L ratios, which may reflect differences in the timescales of these responses. H:L ratios are not considered to be a good clinical indication of disease by veterinarians (Latimer and Bienzle 2000), but are frequently used by physiologists, ecologists and conservation biologists as an indicator of stress integrated over a longer time period (Davis 2005, Davis et al. 2008, Cīrule et al. 2012, Müller et al. 2011). This reflects the differences in

opinions between the two disciplines. The data presented in Chapter 2 were the first to present both CORT and H:L ratios measured simultaneously in response to heat exposure. These data indicate that H:L ratios may be a useful indicator of stress as a result of heat exposure, but the time course of leukocyte responses in these birds remain unclear and the data should not be extrapolated to clinical situations without further study. Full blood cell counts of the same blood samples, which may be more useful for clinical pathologists and veterinarians, were presented in Chapter 4. However, as the cell counts in both Chapters 2 and 4 were estimated from blood film and not quantified, e.g. via uno-pipette, the white blood cell counts were an estimate only and subject to variation in slides, inherent variability in operator technique etc. Future studies could resolve the differences in the timescales of CORT and H:L ratio responses to stressors by measuring both parameters from the blood samples collected at multiple time points after exposure to a stressor to show how the time frames of induction for each parameter vary.

It would have been useful to measure the body temperatures of the birds in Chapter 2 in real time. This can be challenging but techniques such as thermometry, surgically implanted loggers or transmitters, gastrointestinal or non-surgically placed devices may be used to measure internal temperatures. Less invasive approaches measure peripheral temperature with subcutaneous stressors, none of these were suitable for this study. Anything that may have induced pain or discomfort would have affected the stress mediator responses in the test subjects, and any devices that emit light or sound may also contribute as additional stressors. As technology advances and the size and remote sensing capability of temperature recorders continue to improve, they may be more useful in future studies of similar nature.

Differences in behavioural adaptations among birds from different orders when exposed to high environmental temperatures

In Chapter 3, it was determined that differences also existed in behavioural adaptations of captive birds belonging to different orders during periods of high environmental temperature. Observations of eight species of Australian arid zone birds representing the same orders as in Chapter 2, i.e. Psittaciformes, Passeriformes and Columbiformes, at the Adelaide Zoo housed within four different outdoor aviaries of various sizes revealed that the proportion of time spent on stationary behaviours, feeding, in the sun and on the ground differed amongst species of birds, between mornings and afternoons and day type. Morning observations (AM) were conducted between 7:30 am and 10:30 am while afternoon observations (PM) were conducted between 12 pm and 5 pm of the same day. These observations were conducted during extreme summer weather with a maximum daily predicted air temperature above 35°C and normal summer weather with a maximum daily predicted air temperature between 25°C and 35°C. Wing-venting, a behaviour where birds hold their wings away from their body to help reduce their body temperature, was used more frequently during the hottest observation periods (Xie et al. 2017b). The smallest birds in the study utilised wing venting more than other species of birds, possibly because it is more important for them to conserve water (Xie et al. 2017b). Psittaciform birds spent less time feeding and more time resting in cooler microsites during hot periods (Xie et al. 2017b). Columbiform birds continue feeding and spent more time in the sun rather than resting in cooler microsites (Xie et al. 2017b). White-Browed woodswallows (*Artamus superciliosus*) spent a significantly lower proportion of time on stationary behaviours and higher proportion of time feeding compared to the other species (Xie et al. 2017b). These results suggest that columbiform birds may have an advantage during heatwaves as they can continue feeding through high ambient temperatures, as long as there is adequate access to food and water.

This also corresponds with the findings in Chapter 2, possibly indicating a link between increased CORT responses in columbiform birds (Xie et al. 2017a) to heat and their drive to continue foraging (Lohmus et al. 2006) during periods of high environmental temperature. This link should be future explored in future studies to better establish correlation. The heat tolerance limit for Columbiforms (56 – 62°C) (McKechnie et al. 2016) is also higher in general compared to Psittaciforms (44 – 55°C) (McWhorter et al. 2018) and Passeriforms (46 – 52°C) (McKechnie et al. 2017), but it is difficult to determine if their ability to forage through high environmental temperatures is a result of their high heat tolerance limit, or the reason they needed to evolve a high heat tolerance limit in the first place.

Interspecific and intraspecific social interactions were very rarely observed in this study. This was most likely because the stocking density of each aviary was low, and there were multiple food and water points, resulting in minimal foraging competition. This may not be reflective of the desert environment during a drought and heatwaves, so future studies should explore social factors on the time-budget of desert birds and how that might affect their behavioural plasticity.

The operative environmental temperatures were initially planned to be measured using temperature loggers encased in hollow spheres of different sizes to match the sizes of the birds within the aviary (Walsberg and Weathers 1986). However, using these operative temperature mounts may have affected the behaviours of the birds in the aviary, and there was a risk of the spheres being destroyed by the birds. Similar future studies should consider using infrared thermal sensors as an alternative measure of microsite temperatures (Carlson et al. 1994). Other factors, such as stocking density, inter-species interaction, competition and water/food station locations, which may have affected the behaviour of the birds in Chapter 3 were also not quantified and accounted for. These factors could not be manipulated or controlled as they were part of normal zoo husbandry and collection management practices.

Anecdotally, there were adequate water and food stations with a good distribution among available microsites, so the inter-species interaction and competition that would affect the behaviours of the different bird species within the mixed species exhibits were minimal. However, the *ad libitum* availability of water and food also limits the extrapolation of the data from Chapter 3 to wild populations of desert birds. Desert birds under heatwave conditions will have to balance the need to forage for food and water (du Plessis et al. 2012) against other factors such as the need to seek shelter from the high temperatures (Wolf and Walsberg 1996), or predation risk at foraging sites and waterholes (Tieleman et al. 2008). Future studies should consider studying the behaviour of different birds under standardized conditions with temperature being the only variable.

Differences in heat illnesses in different bird species

When both physiological and behavioural strategies to counter high environmental temperatures fail, birds can suffer heat illnesses and eventually die from them. Studying naturally occurring avian heat illnesses is difficult because mass die-off events of wild birds may not be discovered by humans until sometime later, and tissue autolysis is usually advanced at the time of discovery. Studies of avian heat illnesses in the laboratory under controlled conditions have not been performed to date. In Chapter 4, this gap in knowledge was filled by describing the histopathological and haematological changes in the birds used in Chapter 2 (exposed to mild and moderate heat acutely), as well as those of additional birds exposed to the same experimental conditions in a separate pilot study.

A total of 13 budgerigars, 15 zebra finches and 8 diamond doves were examined as model species for the bird orders Psittaciformes, Passeriformes and Columbiformes respectively. In the control group, 6 budgerigars, 7 zebra finches and 4 diamond doves were

exposed to 35°C before euthanasia. This temperature was within the zone of thermal neutrality for all 3 species, i.e. 29 to 41°C for budgerigars (Weathers and Schoenbaechler 1976), 29.5 to 40°C for zebra finches (Calder 1964) and 34 to 40°C for diamond doves (Schleucher 1999). In the experimental group, 7 budgerigars, 8 zebra finches and 4 diamond doves were exposed to 45°C before euthanasia. This temperature was above the zone of thermal neutrality for all 3 species.

There was mild to moderate congestion of the lungs of 28/36 birds examined, including all of the budgerigars and diamond doves. 8/15 zebra finches had no significant lung congestion. Interstitial and pulmonary haemorrhage was in observed in one diamond dove. The most common hepatic change found was micro- and macro-vesicular hepatocellular vacuolation (4/15 zebra finches, 5/13 budgerigars and 8/8 diamond doves). There were mild to moderate congestion in the kidneys of 1/15 zebra finch, 2/13 budgerigars and 4/8 diamond doves, as well as the gastrointestinal tract of 1/15 zebra finch and 7/8 budgerigars. Budgerigars showed a decrease in haematocrit and a significant change in heterophil and lymphocyte proportions after heat exposure. There were no changes in basophil proportions in both budgerigars and diamond doves.

Mild heat exposure to a temperature above the zone of thermoneutrality can induce subclinical heat stress, which may be a precursor to heat illnesses. The ability to identify subtle changes that may be associated with subclinical heat stress can be important in early diagnosis and treatment of heat stress in birds. These findings indicate species differences in the organ and haematological changes post-heat exposure amongst birds. In particular, there were interspecific differences for the organs that congestion occurred in most frequently. The incidence ratios for congestion in the lungs were higher for all species when exposed to 45°C compared to 35°C. The incidence ratios for congestion in the kidney and intestinal tract were higher for budgerigars when exposed to 45°C compared to 35°C. However, the incidence

ratios for congestion in the liver was only higher for zebra finches when exposed to 45°C compared to 35°C. Furthermore, a lower percentage of zebra finches (46.7%) developed congestion in the lungs compared to budgerigars and diamond doves (100%). A higher percentage of budgerigars developed congestion in the intestinal tract (53.8%) compared to zebra finches (6.7%) and diamond doves (0%). A higher percentage of diamond doves (50%) developed congestion in the kidneys compared to zebra finches (6.7%) and budgerigars (15.4%). These differences are interesting because they may reflect variation in organs that respond to heat related vasodilation and subsequent pooling of blood in the different bird species. This may be similar to the concept of 'shock organs' in mammals suffering from anaphylactic shock. For example, the lungs are considered the classic shock organ of the guinea pig, rabbit (Melli et al. 1963), cats (Aitken and McCusker 1969) and goats (Qureshi et al. 2006) during anaphylaxis, whereas the liver and gastrointestinal tract appears to be more affected in dogs (Booth et al. 1970, Kitoh et al. 1994) and rats (Church 1975).

Climatic heat waves have been associated with mass mortality events in desert dwelling birds (McKechnie and Wolf 2010, McKechnie et al. 2012), including endangered species (Saunders et al. 2011). By the time the birds are found dead after a heatwave, they have presumably gone through the cascade of symptoms associated with, and suffered the effects of heatstroke. It is therefore difficult to identify pathognomonic changes in wild birds suffering from heat illnesses during climatic heatwaves. In Chapter 5, galahs (*Eolophus roseicapilla*) and rock doves (*Columba livia*) were exposed to more extreme heat under general anaesthesia so that heatstroke was induced. This allowed us to describe the changes in heart rate, respiratory rate, blood biochemistry and histopathological findings.

Birds in the experimental group were exposed to environmental heat stress using Thermotex Infrared Heating Mats set at the maximum 55°C. Birds in the control group were placed on the same heat mats, but with each set at 45°C. Both groups of birds were under

general anaesthesia throughout the experiment and serial blood collections were performed for biochemical analyses, while organs were collected at the end of the experiment for histopathology. The heart rate, electromyograph (EMG), electrocardiograph (ECG) and cloacal temperature were monitored and recorded throughout the experimental protocol. Heatstroke was deemed to have occurred when the bird exhibited an exponential increase in body temperature with a concurrent increase in heart rate followed by a rapid decrease in heart rate.

No EMG traces consistent with the onset of heat cramp were observed in any of the birds. The mean T_b inflection points for the respiration rate changes in both galahs and rock doves in the present study were similar, i.e. respiration rate increased just after T_b exceeded 41°C and decreased just after T_b exceeded 44°C. These temperatures were also similar to temperatures of onset of respiratory cooling mechanisms such as panting/gular fluttering found in previous studies in wild, non-anaesthetized Psittaciforms (McWhorter et al. 2018) and Columbiforms (McKechnie et al. 2016).

The mean T_b inflection point for heart rate increase in galahs (43.63 ± 0.71°C) was lower than in rock doves (44.59 ± 0.93°C). However, the T_b inflection point for heart rate decrease in rock doves (45.16 ± 0.91°C) was lower than in galahs (46.09 ± 0.16°C). Therefore, a pigeon experiencing heat stress under anaesthesia has a very narrow window between showing measurable signs of heat stress and experiencing irreversible heat stroke.

Biochemical changes suggestive of skeletal muscle and hepatocellular injury, including hyperkalaemia, were often observed in experimental galahs and rock doves at the onset of heatstroke. Histopathological analyses did not reveal any significant cardiac changes, although some lungs had signs of acute congestion. Some experimental rock doves had indications of necrosis in the pectoral muscle. There were significant hepatic changes in some

experimental galahs, but not in rock doves. Our study has provided a baseline for further studies, as well as indications for diagnosing heatstroke in clinical cases presenting with a history of heat exposure. The study also provides physiological data to aid prevention of overheating in birds under general anaesthesia, where parameters such as cloaca temperature, heart rate and respiration rate are often monitored closely. Rock doves especially require intervention early on as they descend into irreversible heatstroke very quickly once T_b exceeds the critical point. There were also significant interspecific differences in the physiological, biochemistry and histopathological changes, which indicate that bird species should be studied separately for clinical syndromes such as heatstroke. Further studies should concentrate on bird species more vulnerable to heatstroke, e.g. psittaciform birds with lower tolerance for heat (McWhorter et al. 2018).

Differences in gene expression among birds from different orders when exposed to high environmental temperatures

In Chapter 6, the mRNA expression of 20 genes in the organs collected from the birds after the second heat exposure (randomly assigned 35°C or 45°C) in the Chapter 2 stress response study (Xie et al. 2017a) was studied. The results indicated that acute exposure of native Australian birds to high temperatures (45°C) results in upregulation heat shock protein (hsp) genes, in particular hsp60, 70 and 90, but there was no significant upregulation of other genes with protective effects against cell damage (BCL-2 and VEGFA) nor genes associated with inflammation (interleukins). There was also no downregulation of the genes involved in the coagulation pathway (fibrinogen) in these birds.

The genes for fibrinogen A, B and G were generally unchanged in this study. The genes for fibrinogen A and G were downregulated in the diamond dove gastrointestinal tract

and diamond dove lung. There was no change in the expression of the fibrinogen genes in any of the other tissues and species. The general lack of downregulation across most tissue types indicates that the birds were not close to suffering from heat stroke under the heat exposure conditions in this study.

Interleukin 6 was only upregulated in the budgerigar liver, while in the diamond dove Interleukin 8 was upregulated in both the liver and the lung. This is a possible indication that these particular organs in budgerigars and diamond doves were more susceptible to inflammation from the effects of heat exposure, and the immune-regulatory effects of interleukins 6 and 8 were important in protecting their organs.

Other non-hsp genes included in this study were albumin, B cell lymphoma 2 protein (bcl-2) and vascular endothelial growth factor A (VEGFA). Albumin is involved in the binding of heat shock factor protein 1 to the heat shock element in the promoter to activate other hsps such as hsp70 and hsp90 (Yiangou et al. 1998). Bcl-2 (Balcer-Kubiczek et al. 2000) and VEGFA (Gerber et al. 1998) has anti-apoptosis effects that can be important in protecting against cell death during heat exposure. Similar to the genes for hsp27, there was generally little up- or down-regulation of these genes, with no clear pattern to their regulation. Albumin was upregulated only in the zebra finch gastrointestinal tract. Bcl-2 was only upregulated in the diamond dove liver. VEGFA was unchanged in all organs and species. This is likely an indication that the extent of heat exposure in this experiment was insufficient to induce cell apoptosis, and the anti-apoptotic effects of these proteins were less important. Cytological evaluation of blood smears is a well-established means of assessing peripheral apoptosis of leukocytes (Squier et al. 1995; Shidham and Swami 2000), and future studies should measure expression of anti-apoptotic genes in birds where leukocyte apoptosis had occurred due to heat exposure.

The gastrointestinal tracts of all 3 bird species had the highest number of hsp genes upregulated, possibly indicating that this is the organ that requires the most protection to continue its function. Diamond dove organs also had the highest number of hsp genes upregulated, possibly a reflection of their ability to protect their cells better during high temperatures. The last finding was consistent with the findings in Chapters 2 and 3, where columbiform birds were different from passeriform and psittaciform birds in terms of CORT and behavioural responses to high environmental temperatures.

Future directions

The focus of this thesis was on captive populations of desert bird species. Future physiological studies should focus on wild populations of desert birds, with experimental conditions better simulating that of natural heatwaves. The experimental set-up described in Chapter 2 can be easily modified to investigate different durations of heat on the CORT levels of wild birds, as well as other variables that are of interest. The flaws of the set-up have been discussed previously, and these should also be improved in future studies.

Further experiments to study the pathological changes in birds exposed to high temperatures can also be conducted with other desert bird species and under different experimental conditions, but the animal welfare impact of such studies should be balanced against the likelihood of pathological changes being significantly different under these different conditions. The list of genes investigated in Chapter 6 should also be evaluated after exposure to higher temperatures or heat exposure over longer time periods, to determine whether protection against apoptosis is a more important factor under these conditions. More advanced molecular genetic modalities, such as RNAseq, should be considered as they can provide transcription data on all the genes in an organ/species. However, a highly annotated

reference genome is required for proper analysis of these type of data. Such studies have been performed in chicken (Coble et al. 2014), and as more avian genomes become annotated, its applicability to other bird species will increase.

All the experiments conducted in this thesis have also been on healthy populations of birds. The effects of disease, parasite loads, or drought-associated malnutrition on the parameters measured in these experiments, as well as other parameters such as the upper limit of heat tolerance, is unknown. It would be interesting to study the effects of avian diseases such as avian circovirus, gastrointestinal parasites and Chlamydia psittaci on these parameters. If disease has a significant effect on the heat tolerance of birds, then perhaps the desert bird species most at risk of mortalities during heatwaves are the bird species that already carry or are particularly susceptible to these diseases.

Conclusions

The results of this thesis indicate that there are a variety of adaptations which birds can utilize to prevent themselves from succumbing to the effects of high environmental temperatures. In this thesis, it was shown that some groups of birds, e.g. psittacine and passerine birds, start restricting their activities to shaded and cooler microsites at high temperatures, especially during the early afternoon when solar radiation levels are high. The loss of these microsites in the wild, as predicted with climate change (Garnett and Franklin 2014), will therefore affect these groups of birds more than birds, e.g. columbiform birds, that are less reliant on these microsites in order to continue displaying their normal behaviours during heatwaves. Without shaded microsites, birds cannot seek shelter from the heat during heatwaves, thus decreasing the amount of time that birds can survive without water at high temperatures (Wolf 2000). This will in turn decrease the amount of time that birds have

available to rest and “wait-out” a heatwave before they have to take the risk of flying to seek out a water source.

Habitat, and consequently microhabitat, conservation is therefore more important in ensuring the survival of these bird species as temperatures continue to rise, but unfortunately, this is also the most expensive aspect of conservation compared with other strategies such as captive breeding (Garnett and Franklin 2014). Failure to conserve thermal refuges for these more sensitive bird species will likely lead to a restructuring of the bird populations in arid environments, which can have unpredictable ecological effects on the rest of the ecosystem (Wolf 2000, McKechnie et al. 2012). The importance of thermal refuges differs amongst species of birds (Martin et al. 2015), and the loss of these refuges has varying impacts on different species of birds, which can contribute to the restructuring of bird communities as a result of climate change. Further understanding of the genetic adaptations required to confer high tolerances to heat will also allow better identification of vulnerable species of birds so that appropriate resources can be allocated to helping them survive the effects of climate change proactively rather retrospectively.

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Appendix 1 Supporting publications – Conference presentations

The following supporting publications include conference presentations of work undertaken during the PhD. These include the 2013 Unusual Pet and Avian Veterinarians (UPAV) Special Interest Group of the Australian Veterinary Association (AVA)/Association of Avian Veterinarians Australasian Committee (AAVAC) Combined Conference in Singapore, the 2015 ExoticsCon in San Antonio, USA and the 2015 UPAV/AAVAC Combined Conference in Sydney.

The Physiology of Heat Stress in Desert Birds

AAVAC-UPAV 2013

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Introduction

Arid environments pose a unique set of challenges to living organisms. These challenges include the lack of basic resources such as food and water, as well as extremes in temperatures. Recent studies have shown that desert birds have physiological specializations that allow them to survive in arid environments.

Anatomical and Physiological Adaptations of Birds to Life in the Desert

Kidney

Avian kidneys are elongated, flattened and closely fitted into the bony concavity formed by the synsacrum (Braun, Dantzler 1972). They contain both reptilian-type and mammalian-type nephrons (Braun, Dantzler 1972). The mammalian-type nephrons can be further differentiated into long loop and short loop mammalian-type nephrons (Braun, Dantzler 1972). When desert birds are subjected to severe dehydration or salt loads, a rise in plasma osmolality must be prevented by secreting ions extrarenally, producing concentrated urine, or reducing the glomerular filtration rate, or any combination of these (Braun, Dantzler 1972). Desert quails can also decrease the overall glomerular filtration rate by reducing the number of functioning reptilian-type nephrons to increase water conservation at the expense of waste excretion, whilst maintaining the functions of the mammalian-type nephrons to increase the urine-concentrating capability of the kidney (Braun, Dantzler 1972). In contrast to many other birds, chickens, as do mammals, increase tubular absorption in the kidneys to conserve water (Stallone, Braun 1985).

Desert mammals were found to have a larger medullary mass relative to total kidney mass compared to those adapted to a moist environment (mesic mammals) (Schmidt-Nielsen, O'Dell 1961; Heisinger, Breitenbach 1969). The vasa recta, loops of Henle and collecting ducts in birds are encapsulated in the medullary cone, making the division between cortex and medulla less clear in bird kidneys (Williams, Tieleman 2001). Therefore, similar studies in birds to identify differences in kidney structure between desert and mesic birds depend on measurements of relative medullary cone length instead of relative medullary mass. One such study by Goldstein and Braun (1989) found that there was no association between relative medullary cone length and maximal ureteral urine concentration. Desert birds were also not found to have superior urine-concentrating abilities to non-desert birds (Goldstein, Braun 1989). Williams and Tieleman (2001) commented that these results might be due to the small sample size and that two of the seven species studied were seabirds with salt glands and larger kidneys. Goldstein and Braun (1989) also suggested that smaller birds had better urine-concentrating abilities regardless of habitat affinity and that there was a negative correlation between the maximal ureteral urine concentration and the length of the loop of Henle.

It has been suggested that the maximal ureteral urine concentration may correlate with the mass-adjusted field metabolic rate of a species because a lower metabolic rate means there is less metabolic waste for the kidneys to eliminate (Williams, Tieleman 2001). This could therefore partly explain the significance of a lower field metabolic rate in desert birds compared to non-desert birds (Williams, Tieleman 2001).

Gastrointestinal Tract

Birds are able to move urine from the cloaca by anti-peristalsis into the rectum (Brummerman, Braun 1995), where water is passively reabsorbed to reduce water loss via urine (Anderson, Braun 1985).

Desert birds were found to have 15% lower water content in the lumen contents of their lower gastrointestinal tract compared to non-desert birds, suggesting that they might better reabsorb water from the gastrointestinal tract (Amanova 1984). However, more studies are needed to evaluate this hypothesis (Williams, Tieleman 2001).

Some birds can also control cloacal evaporation as a means of thermoregulation at high ambient temperatures. This has been demonstrated in Inca doves (*Columbia inca*), whose cloacal evaporation was negligible at 30, 35 and 40°C, but increased to 21.2% of total evaporative water loss at 42°C (Hoffman *et al.* 2007).

Skin

The skin of birds consists of an epidermis and a well-vascularized dermal layer (Lucas, Stettenheim 1972). The epidermis can secrete lipid-enriched organelles or multigranular bodies during times of water deficit for rapid water-proofing (Menon, Menon 2000). Birds can also increase cutaneous water loss by vasodilating the dermal capillary bed (Peltonen *et al.* 1998). The balance between losing enough water to decrease body temperature and maintaining sufficient hydration is the key to survival in times of heat stress (Williams, Tieleman 2001). Analysis done comparing lipid composition of the stratum corneum of adult desert house sparrows (*Passer domesticus*) and mesic house sparrows revealed that desert sparrows had a higher amount of ceramides and cerebrosides and a lower percentage of cholesterol within the stratum corneum (Munoz-Garcia, Williams 2005). A follow-up study also revealed that desert house sparrow nestlings showed a greater degree of plasticity in cutaneous water loss and lipid composition of the stratum corneum compared to mesic nestlings, possibly due to the increased exposure to environmental stressors (Munoz-Garcia, Williams 2008). Similar differences were found between desert and mesic larks (Haugen *et al.* 2003). Desert sparrows also had lower cutaneous water loss, which can possibly be attributed to modifications in chain length and polarity of the sphingolipids that determine interactions among lipid molecules within the stratum corneum (Munoz-Garcia *et al.* 2008).

Nasal Passages

It has been suggested that desert animals might have more complex nasal turbinates that allow greater cooling of exhaled air, and therefore a larger reduction in respiratory evaporative water loss than non-desert animals (Schmidt-Nielsen *et al.* 1981). Some birds are able to reduce respiratory evaporative water loss by recovery of water from the nasal area at moderate-to-low ambient temperatures, but this ability becomes insignificant at high ambient temperatures (e.g., 45°C for crested larks) (Tieleman *et al.* 1999). However, the results of that study did not support the hypothesis that desert birds had a greater ability to recover water from the nasal passages compared to non-desert birds, because desert larks could not reduce respiratory evaporative water loss from the nasal passages (Tieleman *et al.* 1999). More studies directly comparing phylogenetically similar desert and non-desert birds are needed to confirm this finding.

Energy, Water and Thermoregulation

Desert birds constantly struggle to meet their daily energy requirements due to their high rates of metabolism (as endotherms) and relatively low food availability in the desert (Williams, Tieleman 2001). A relatively lower metabolic rate and the consequently lower endogenous heat production in desert birds relative to non-desert birds may be beneficial because less food for energy; and less water for evaporative cooling are required (Williams, Tieleman 2001). The lower basal metabolic, field metabolic, and evaporative water loss rates described by Williams and Tieleman (2001) in desert birds relative to non-desert birds may therefore provide part of the mechanism by which desert birds cope with extreme temperatures.

Behavioural Adaptations

Opportunistic feeding and omnivory appear to be the best dietary strategy to survive in the desert (Williams, Tieleman 2001). During heat waves, microsite selection becomes an important strategy for survival. Hiding in shaded areas will minimize evaporative water

loss, and if these shaded areas are large enough to allow foraging, then the birds' energy requirements can be fulfilled as well (Williams, Tieleman 2001). However, if the body temperature needs to be kept low by seeking deep shade and pressing the body against cooler substrates, then the ability to forage is limited (Williams, Tieleman 2001). Desert birds will therefore have to take necessary risks to maintain the balance of water, energy, and thermoregulation.

The Neuroendocrine System

Corticosterone has an important role in the ability to cope with stressors in birds, and heat stress is no exception. With its ability to trigger specific physiological and behavioral responses that are crucial in maintaining the balance between energy, water, and thermoregulation, corticosterone might be one of the most important determinants of a bird's ability to survive in times of heat stress.

Thyroid hormones may play a role in the development of physiological adaptations such as basal metabolic rate and thermoregulation in juvenile desert birds (McNabb, Fox 2003), but have less adaptive plasticity in adult birds and are therefore less important than corticosterone in the physiology of heat stress in adult desert birds.

Heat-Shock Proteins

Heat-shock proteins (hsps) are a group of proteins synthesized by almost all organisms when exposed to heat or other stressors (Lindquist, Craig 1988). They are named according to their relative molecular masses (Lindquist, Craig 1988) (e.g., hsp70 and hsp90 have relative molecular masses of 70000 and 90000 kilodaltons respectively).

Heat-shock proteins act as molecular chaperones, interacting with other proteins to minimize the probability that other proteins will interact inappropriately with one another (Feder, Hofmann 1999). The rapid and intense nature of the induction of heat-shock proteins indicates that it is an emergency response (Lindquist, Craig 1988).

The differences in heat-shock protein responses to high temperatures in desert birds may hold the key to their ability to survive in these harsh conditions.

Conclusion

The implications of heat stress in avian species are important in the face of increasing global temperatures related to climate change. A better understanding of the physiology of heat stress and identification of the sensitivity of different bird species to increasing global temperatures will help wildlife managers to direct conservation efforts better by providing risk assessments for species and regions. This presentation will also demonstrate the links between climate change, as a result of human activities, and the survivability of desert birds, exemplifying the principles of the 'One Health' initiative.

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Speaker Information

(click the speaker's name to view other papers and abstracts submitted by this speaker)

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Beating the Heat – the Australian Way

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Abstract: Desert birds have adaptations that allow them to survive in arid environments, but these are being challenged as temperatures continue to rise. Our research revealed differences amongst Australian birds belonging to the orders Psittaciformes, Passeriformes and Columbiformes in their physiologic and behavioural adaptations to heat. These results can help us to better predict how individual avian species will respond to heat and prioritize conservation efforts in the face of climate change.

Australian birds are under increasing threat from the effects of climate change and extreme events like heat waves. As temperatures continue to rise with climate change, sensitive taxa of birds will be challenged.[1]

In our first physiologic study, we measured the critica thermal maxima (CTM) of different species of Australian desert birds using metabolic chambers. The birds belonging to the order Psittaciformes (n = 27) in the study had CTMs between 50-54°C; Passeriformes (n = 81) between 46-50°C; Columbiformes (n = 39) between 58-60°C; and Caprimulgiformes (n = 23) between 50-52°C.

In another study, Australian desert birds representing the orders Psittaciformes (budgerigars), Passeriformes (zebra finches) and Columbiformes (diamond doves), were exposed to temperatures of 33°C and 42°C for 2 hours sequentially in a random order 1-2 weeks apart to measure their stress responses. Interestingly, diamond doves had a higher corticosterone response, even though they have a higher CTM.

Our behavioural observations of birds at the Adelaide Zoo also revealed that columbiforme birds did not modify their behaviours (eg, spend more time at rest or in shade) as much as psittacine birds and passerine birds. Focal observations of these birds were performed 15 minutes at a time and recorded using an iPhone app, "Animal Behaviour Pro."

Understanding the different adaptations birds utilize to beat the heat will allow us to better predict how individual species will respond (both on the individual level, i.e. susceptibility to heat stress, and at the species distribution and biodiversity level), and help us prioritize conservation efforts.

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Clinical Revelations from a Research Project

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Introduction

Some research projects may not have immediate clinical applications at first sight and even when such projects aim to have a clinically relevant outcome, they can take a long time to eventuate (Contopoulos-Ioannidis et al., 2008). This is particularly obvious in translational studies where animal models are used to investigate human diseases (Pober et al., 2001; Contopoulos-Ioannidis et al., 2008; Jucker, 2010). However, the same difficulties are present for ecological projects involving species that are commonly presented for treatment at veterinary practices, e.g., zebra finches and budgerigars.

My PhD project investigating the physiology of heat stress in Australian desert birds is one such example. For one aspect of my project, zebra finches, budgerigars and diamond doves were exposed to two temperatures (35°C and 45°C) for two hours, and had blood samples collected before and after heat exposure for measurement of various haematological stress indications, e.g. serum corticosterone levels, blood cell heat shock protein 70 levels and heterophil:lymphocyte ratios. Organs (e.g., heart, lung, kidney, liver and gastrointestinal tract) were also collected at the end of the experiment for histopathological examination. I have learnt some useful lessons for my clinical work from the research project, even though the project did not aim to have clinically relevant outcomes. I have summarized these revelations below.

Smaller is not always better

27G needles are better for bleeding from the brachial vein than 30G needles even in small birds such as zebra finches. I had initially used 30G needles to bleed the first six zebra finches and budgerigars, but found that there were high incidences of haematoma formation and requirements for multiple punctures to be made for blood droplets to be of a sufficient size for collection. All these problems were alleviated simply by switching to 27G needles for the rest of the project, likely because 27G needles made a puncture hole of a sufficient size in the skin for the blood droplets to not be trapped subcutaneously to form a haematoma

Alcohol (swabs) is not always good

Swabbing the skin and feathers with alcohol over the brachial vein resulted in blood spreading out over the feathers rather than forming droplets that could be collected into haematocrit tubes via capillary action. This was likely due to the loss of down powder on the feathers which removed their waterproofing properties, allowing the blood to soak into them. This was alleviated by using water rather than alcohol for swabbing.

How hot is hot?

Budgerigars are less stressed (as indicated by blood corticosterone levels) after incubation at 35°C for two hours than before, even though this is a higher temperature than usually used in incubators for sick birds in practice. Interestingly, diamond doves appear to have a significant increase in blood corticosterone levels after exposure 45°C for two hours, even though they should have the highest physiological tolerance for heat compared to zebra finches and budgerigars (Wolf, 2015). Incubator temperatures may therefore need to be varied for different species of birds, possibly depending on the order to which they belong, but more avian species will need to be studied before more specific recommendations can be made.

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Appendix 2 Popular media

There was considerable interest from the general public and popular media regarding this project. The supporting publications from popular media include an article from the University of Adelaide E-Science magazine (July 2015 Issue 14, page 23-24), as well as an interview with ABC Radio.

ABC NEWS

00:00 00:00

Climate change heatwaves killing off Australian birds

Updated Tue 17 Feb 2015, 12:44pm

With climate change set to increase temperatures in Australia there is concern about how the heat will affect native birds. A researcher at the University of Adelaide says increasingly large numbers of birds are dying during heatwaves.

Natalie Whiting

Source: The World Today | Duration: 4min 36sec

Topics: birds, climate-change, australia

Hide transcript

ELEANOR HALL: A researcher at the University of Adelaide is warning about the impact of climate change on native birds.

Recent studies have shown that large numbers of birds are already dying during heatwaves.

And the university is now taking part in an international study to look at the sorts of temperature increases different birds can tolerate and how they change their behaviour in extreme heat.

Natalie Whiting spoke to researcher Dr Shangzhe Xie.

SHANGZHE XIE: There have been reports, most recently in Carnarvan Western Australia a few years ago where large numbers of budgies had died during a heatwave but that, you know, it's hard to confirm that's why they died and we don't really know how much heat they can tolerate.

NATALIE WHITING: Is there a concern with climate changes to increase heat, that is that is the course we could see more birds die?

SHANGZHE XIE: Yes I mean, from our first part of the project we have found that birds can actually tolerate quite a bit of heat, going up to 50 degrees in parrots.

With climate change I think the temperatures in the desert is pushing their limits.

NATALIE WHITING: So it could be that that could see more birds if it gets beyond heats they can tolerate.

SHANGZHE XIE: That's right.

NATALIE WHITING: So what's the next part of your research?

SHANGZHE XIE: There are a few different parts we have yet to do. We've done some behaviour studies that at Adelaide Zoo which show difference in behaviour as well, between parrots, song birds and pigeons and that correlates quite well with how much heat they can actually tolerate.

So the next part we want to extrapolate the behaviour differences into birds in the wild but they're quite difficult to observe the same way as we did the birds at Adelaide Zoo.

So we're using two different ways, we're putting a thermal meter logger on the some of the birds in the desert and that actually records the behaviour for us.

We are also using citizen finds and getting people involved in helping us observe these birds.

NATALIE WHITING: So how will the people be involved in the research?

SHANGZHE XIE: We're telling volunteers who are interested in bird watching and getting them to go out on days of different temperatures and actually recording which birds are out and what they're actually doing on those days.

NATALIE WHITING: And so that would give you an idea of which birds are still managing to be flying and whatnot in certain temperatures, is that the thought?

SHANGZHE XIE: Yes that's right, because what we found at the zoo was the pigeons, they don't seem to be affected, they continue doing their normal feeding and behaviour during really hot days, whereas the parrots tend to hide in the shade and just not do very much.

NATALIE WHITING: And is this part of a broader research base?

SHANGZHE XIE: Yes the research was actually kicked off by a group in America, the University of New Mexico. We were one of the places that they were doing part of their research at, so the part where we were measuring the limits of heat tolerance in birds, that's actually part of the bigger project and then we've extended that to the behaviour studies that we've done.

Plus we're also looking at measuring stress, hormones and proteins in most of the birds here.

NATALIE WHITING: If your research shows that birds are adjusting their behaviour to suit the temperatures, is it thought we'll be able to use some of this information to try and help the birds, what do you see as the possible outcomes of this?

SHANGZHE XIE: There's two aspects to it, the easier logical aspect of it is that we will have a large enough database combined with that information the American group has to be able to predict the tolerance of birds.

So that's one way to identify the species of birds that are more vulnerable to heat increase.

The other aspect is once we know the behaviour changes, for example because we know that parrots tend to hide during the heat, then we know that we need to be able to provide them with habitats that allow them to display this behaviour so that when there is heat there's actually somewhere for them to hide from it.

One of the most expensive parts of conversation is actually preserving the habitats. So this just reinforces the point that it's very important to make sure that there's appropriate vegetation and microsites for these birds.

ELEANOR HALL: That's scientists Dr Shangzhe Xie speaking to Natalie Whiting.

Our research has looked at increasing piglet viability through treating the sow while still pregnant using two different supplements. The first supplement was melatonin, a hormone that occurs naturally in our bodies to help regulate sleep and a powerful antioxidant able to fight off the effects caused by hypoxia. The second supplement was caffeine. Caffeine is regularly used in hospitals to treat premature babies as it helps stimulate breathing and also fights the effects of hypoxia.

By treating our sows with either melatonin or caffeine before birth (also known as 'farrowing') the piglets are treated before hypoxia can occur, allowing them to have a way of fighting hypoxia as it happens rather than trying to treat it after the damage has already occurred. Our study found this approach to be a positive one as more piglets from treated sows were born alive and were more viable during the critical first three days of life than those from untreated sows.

Our research is ongoing, but this result is potentially great news for pig producers who now have a relatively easy way of increasing the number of happy and healthy piglets born.

This research was performed by Brooke Dearlove, Karen Kind and William van Wettere from the School of Animal and Veterinary Sciences at the University of Adelaide. This research was funded by Australian Pork Limited (APL).

Helping Australia's birds to beat the heat

Jim Gerdon

Understanding how birds cope with the heat is important for conserving species in the face of climate change.

Averaging around 40°C, birds have a higher normal body temperature than mammals. This is one of the many adaptations that help them survive in hot places such as the Australian desert. However, as it gets increasingly hotter throughout the world due to climate change, birds are exposed to more extreme temperatures that are pushing their limits of survival. Combined with the general lack of food and water in the Australian desert, this extra challenge may push some over the edge, resulting in decreases in populations of certain bird species. There have already been reports of bird deaths during and after heat waves and, if we are to conserve our bird species in the face of climate change, we need to understand which are most susceptible.

We are studying the differences between three different groups of birds that commonly live in the Australian desert: parrots, songbirds and pigeons. We want to discover the different ways they cope with the heat, in order to identify the groups of birds that are more likely to be affected and die during heat waves.

We measured several parameters in these bird species in different temperatures, including their evaporative water loss and metabolic rates. Our results from these tests so far indicate that parrots can tolerate the least amount of heat and pigeons the most. We also observed bird behaviour and found parrots tended to seek shade and decrease their activity during periods of heat, whereas pigeons continued to carry out their normal activities despite the heat.

Interestingly, in another of our studies, pigeons also showed a greater increase in the stress hormone corticosterone compared to parrots and songbirds when exposed to heat. It is possible that this stress hormone response is associated with their higher heat tolerance and ability to continue activities important to their survival, such as looking for food and water, during heat waves.

We are conducting further studies to continue to unearth the secrets that pigeons and other birds hold in beating the heat. With more information about these strategies, we can identify the birds that need help during heat waves and intervene before bird extinctions caused by climate change become a reality.

This research was performed by Shangzhe Xie, Todd McWhorter and Erin Turrell from the School of Animal and Veterinary Sciences at the University of Adelaide in collaboration with Blair Wolf's research group from the University of New Mexico. Studies were performed at the Adelaide Zoo and BirdLife Australia's Gluepot Reserve. Funding support was provided by the Holsworth Wildlife Research Endowment and BirdLife Australia's Stuart Leslie Bird Research Award.