Non-invasive monitoring of physiological stress in an Afrotropical arid-zone passerine bird, the southern pied babbler

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Highlights

• Faeces are a suitable matrix for monitoring stress in the southern pied babbler.

• Captive babblers have much higher stress levels than wild individuals
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• Dominant male babblers are more stressed than subordinate males.

• There is no difference in stress levels between dominant and subordinate females.

Abstract

Using faecal matter to monitor stress levels in animals non-invasively is a powerful technique for elucidating the effects of biotic and abiotic stressors on free-living animals. To validate the use of droppings for measuring stress in southern pied babblers (*Turdoides bicolor*) we performed an ACTH challenge on captive individuals and determined the effect of temporary separation from their social group on their faecal glucocorticoid metabolite (fGCM) concentration. Additionally, we compared fGCM concentrations of captive babblers to those of wild conspecifics and examined the effects of dominance rank on fGCM concentration. We found droppings to be a suitable matrix for measuring physiological stress in babblers and that individual separation from the group caused an increase in fGCM levels. In addition, babblers temporarily held in captivity had substantially higher fGCM concentrations than wild individuals, indicating that babblers kept in

captivity experience high levels of stress. In wild, free-living individuals, dominant males showed the highest levels of stress, suggesting that being the dominant male of a highly territorial social group is stressful. Non-invasive sampling allows field-based researchers to reduce disturbance related to monitoring adrenocortical function, thereby avoiding artificially increasing circulating corticosterone concentration as it is not necessary to physically restrain study animals.

Keywords

Corticosterone, faecal glucocorticoid metabolites, non-invasive hormone measurement, enzyme-immunoassay validation, ACTH challenge, avian physiology

1. Introduction

The stress response in vertebrates is physiologically mediated by the endocrine system, leading to hormonal and metabolic changes following a recognized threat to the body's homeostasis (Desborough, 2000; Moberg, 2000). The secretion of stress hormones occurs via the activation of one or both of the two main neuroendocrine stress responses (Hill et al., 2012). The first is the secretion of catecholamines from the activation of the sympathetic nervous system (Sheriff et al., 2011; Hill et al., 2012), which is a rapid response that supports hyperglycemia for fight-or-flight behaviour (Butcher and Lord, 2004). The second leads to the secretion of adrenocorticotropic hormone (ACTH) from the activation of the hypothalamic-pituitary-adrenal (HPA) axis, which in turn stimulates the release of glucocorticoids (GCs) into the blood and facilitates the maintenance of homeostasis (Touma and Palme, 2005; Sheriff et al., 2011; Hill et al., 2012). The predominant GC varies among taxa: cortisol in carnivores, ungulates and primates, and corticosterone in birds and reptiles (Palme et al., 2005; Touma and Palme, 2005). There is also within-taxon variation; for instance, some members of the mammalian order Rodentia predominantly secrete cortisol where others secrete corticosterone (Mateo and Cavigelli, 2005).

Stress can be divided into two broad categories manifested over different time scales. Acute stress involves the short-term release of these hormones during which concentrations of circulating GCs can be elevated several-fold above baseline (Beiko et al., 2004), supporting immune responses, mobilising energy reserves, and allowing individuals to escape potentially dangerous situations (Buchanan, 2000). Chronic stress, arising from prolonged periods of release of these hormones, negatively affects the well-being of animals and disrupts immune responses, behaviour, and cognition, leading to long-term damage (McEwen, 2004; Dhabhar, 2009; Lupien et al., 2009).

The concentration of circulating GCs can be quantified directly using blood plasma or non-invasively using GC metabolites in an alternate matrix e.g. faecal matter, urine, saliva, hair, or feathers (Sheriff et al., 2011). Non-invasive measurement of stress responses has important conservation applications and can be used to investigate the sub-lethal effects of social and/or ecological stressors (Wikelski and Cooke, 2006; Sheriff et al., 2011; Narayan, 2013, Dantzer et al., 2014) or to improve our understanding of the stress that may accompany translocation or reintroduction events (Teixeira et al., 2006). Faeces have been successfully used to quantify stress levels in a number of wildlife-related studies, including investigations of disease (Laver et al., 2012), injury (Ganswindt et al., 2010), and anthropogenic pressures (Creel et al., 2002). Metabolites of the biologically active hormone are measured because GCs are not present in, for example, faeces or droppings (a combination of faecal and urinary matter) (Palme et al., 2005; Hodges et al., 2010). Advantages of faeces sampling include the ease of collection and the potential to repeatedly collect samples from an individual without affecting its behaviour or disturbing other individuals (Touma and Palme, 2005; Goncalves et al., 2016). Additionally, faecal glucocorticoid metabolites (fGCMs) are unlikely to be affected by transient pulses of GC secretion, as they reflect circulating concentrations integrated over a longer period, typically 1 - 4 hours in small endotherms (Cyr and Romero, 2008; Palme, 2012).

Although increasing in popularity among researchers (Palme, 2019), these non-invasive methods have been used less frequently in avian studies, especially those involving free-ranging birds. However, measuring stress in wild birds is important for understanding the effects of natural stressors such as stochastic environmental events or predation risk, social stressors such as the balance between conflict and cooperation in group-living species, and anthropogenic stressors such as urbanization or pollution. The use of droppings to quantify stress in birds is usually much more feasible in captivity, permitting investigations of how potential stressors in the captive environment may be affecting these birds, or how the capture process itself may stress them (Wingfield et al., 1995; Dickens et al., 2009). These responses have been investigated in a variety of species including European starlings, *Sturnus vulgaris* (Rich and Romero, 2005) and the great cormorant, *Phalacrocorax carbo* (Dehnhard et al., 2003).

Species-specific validations confirming that changes in fGCM concentrations mirror circulating GC concentrations are an essential prerequisite for non-invasive approaches that rely on faeces sampling (Goymann, 2005; Sheriff et al., 2010; Palme, 2019). When quantifying fGCMs, knowledge of the physiology of the study species is important as there is variation both among

and within species, as well as between sexes, in terms of the nature and quantities of metabolites excreted (Palme, 2005; Palme et al., 2005; Hodges et al., 2010). Moreover, gut passage time can differ between species and/or sexes (Palme et al., 2005), leading to a delay in the excretion of fGCMs compared to the rapid increase in circulating GCs in the blood, and these differences need to be investigated in order to estimate the period reflected by excreted GC metabolites. Validation studies can be biological, where faecal samples are collected both before and after an assumed stressful event (Touma and Palme, 2005), or physiological, where the individuals are pharmacologically induced to produce elevated GC concentrations, often via injection of ACTH (Goymann, 2005; Sheriff et al., 2011).

This study focused on the southern pied babbler (*Turdoides bicolor*), a cooperatively breeding passerine endemic to the southern African arid zone. These babblers occur in groups varying from two to 12 adults (Ridley and Raihani, 2008; Engesser et al., 2016), each comprising a single dominant breeding pair and a number of subordinate helpers (Ridley and Raihani, 2008; Nelson-Flower et al., 2011). Group size is primarily determined by habitat size and quality (Thiele and Blaum, 2008) and can affect the number of offspring produced as all group members help raise the young produced by the dominant pair (Ridley and Raihani, 2008). In many species of cooperatively breeding vertebrates, subordinate individuals are reproductively suppressed (Young et al., 2006; Clutton-Brock, 1998). It has been argued that this is caused by high concentrations of GCs (Creel, 2001; Creel et al., 2013) due to increased aggression from dominant individuals, as seen in meerkats, *Suricata suricatta* (Young et al., 2006), and marmots, *Marmota marmota* (Hackländer et al., 2003). Often however, elevated GCs is a consequence of dominance rank, where dominant individuals experience high stress levels due to defending their position (Creel, 2001; Creel et al., 2013). Ultimately, the effect of dominance rank on stress levels varies between taxa and is also affected by the stability of the dominance hierarchy (Creel et al., 2013) with subordinate baboons showing higher stress levels when the hierarchy is secure and dominants reflecting higher stress levels when the hierarchy is unstable (Sapolsky, 1992). Thus, investigating the interactions between social dominance and stress could provide important information about the trade-offs and consequences of social status in cooperatively breeding species.

The aim of this study was to evaluate the suitability of five enzyme immunoassays (EIAs) for measuring fGCM concentrations to monitor adrenocortical activity in babblers. More specifically, we aimed to 1) investigate the effect of temporary separation from the group on fGCM

concentrations in this highly social species, as a form of biological validation, 2) determine stress-related physiological responses in babbler droppings by performing an ACTH stimulation test (ACTH challenge), and 3) compare fGCM concentrations of captive and wild individuals. Several studies have shown a relationship between dominance rank and stress levels, varying among taxa and affected by the stability of the dominance hierarchy in social species (Sapolsky, 1992; Clutton-Brock, 1998; Creel, 2001; Hacklander et al., 2003; Young et al., 2006; Creel et al., 2013). Babblers are a cooperatively breeding species with dominance hierarchy, therefore we also 4) investigate fGCM concentrations as a function of dominance rank in wild individuals under natural conditions, providing an example of an application of the non-invasive technique in the wild.

2. Materials and Methods

2.1 Separation test and ACTH challenge

The biological and physiological validations were performed on a population of babblers at Radnor farm (26°11 ‘E, 22°88 ‘S), a small settlement 1014 m above sea level located approximately 40 km from the village of Vorstershoop in South Africa’s Northwest province. It is a flat area with sandy substrate that supports semi-arid savannah vegetation dominated by grasses and various *Vachellia* (formerly *Acacia*) species (Mucina and Rutherford, 2006). Average annual rainfall is 332 ± 158 mm (Meyer et al., 2007) and temperatures fluctuate from sub-zero in winter to over 40 °C in summer (Smit and McKechnie, 2010).

Using mist nets, spring traps, and walk-in traps, ten wild babblers (five males and five females) from two social groups were captured on Radnor farm and ringed to facilitate individual identification. One group was comprised of six individuals (4 males and 2 females) and the second four individuals (1 male and 3 females). All individuals in each group were housed together in one of two aviaries each measuring 3.48 m long x 1.74 m wide x 1.74 m high. Aviaries were constructed from aluminium square tubing and wire mesh, with a layer of shade-cloth inside each mesh panel to prevent injuries and to provide shade for the birds. During the day, groups occupied their respective aviaries, while overnight, each group was transferred to a smaller cage (61 cm long x 43 cm high x 51 cm wide) and kept indoors to avoid harassment by nocturnal predators. The babblers were left to habituate to captive conditions for four days prior to the ACTH challenge. At all times, both when housed in the aviaries and during the ACTH challenge, birds were provided with food and water *ad libitum*. On day five at 07h00, all birds were moved into individual cages (61 cm long x 43 cm high x 51 cm wide) lined with wax paper, which was changed hourly to facilitate collection of droppings, and droppings were collected for 3 h prior to ACTH injection to

evaluate the effects of temporary separation from the social group (biological validation). The individual cages allowed the babblers to hear but not see one another. At 10h00, following the standard protocol to increase circulating corticosterone (Nakagawa et al., 2003; Lèche et al., 2009), each bird received an injection of 60 µl Synacthen® Depot (Novartis) (approx. dose 2 IU/kg) in a saline solution in the *pectoralis* muscle (IM) using a 27-gauge needle to initiate an increase in corticosterone production (physiological validation). Droppings were collected from each individual for a further 4 h post-injection. All samples were collected within 1 h post-defecation, immediately frozen, and stored at -20 °C to limit further alteration of fGCM composition. For males, the fGCM concentration at 1 h post-injection was used as the baseline concentration as this was when the lowest concentrations were recorded. There was insufficient sample material for two of the males and in this case hour zero, at the point of injection, was used for the baseline. For four of the five females, fGCM concentration measured at 1 h post-injection was used for the baseline concentration, and for the one female where there was insufficient sample material available, hour zero was used.

Blood samples were collected from all individuals during the ACTH challenge test to determine whether there was an increase in circulating corticosterone post-injection. A maximum of 200 μl was drawn from the brachial vein using a 26-gauge needle and non-heparinized capillary tubes and then transferred into Eppendorf tubules immediately prior to ACTH injection and again 30 min post-injection to determine the baseline and increase of plasma corticosterone concentrations, respectively. Blood samples were left to clot for 60 min and then centrifuged at 1500 G for 15 min. Blood serum was then pipetted into Eppendorf tubules and stored at −20 °C until analysis. At the end of the ACTH challenge (7 hours post-separation) the birds were returned to the aviaries with their group members. The birds were then transported to Pretoria for other research and after a total of three months in captivity were released back in their territories at Radnor farm.

The study was conducted with the approval of the University of Pretoria Animal Ethics Committee (protocol EC014-18) and Research Ethics and Scientific Committee of the National Zoological Garden (P18-27).

2.2 Stress monitoring in free-ranging babblers

To quantify fGCM concentrations in wild babblers, we collected droppings from a habituated population of free-living individuals resident at the Kuruman River Reserve (KRR; 26°58 ‘E, 21°49 ‘S) in the southern Kalahari Desert in the Northern Cape province, South Africa. Like Radnor,

KRR is flat with a sandy substrate supporting semi-arid savannah vegetation (Mucina and Rutherford, 2006) and it has a similar temperature range and an average annual rainfall of 197 mm (Kong et al., 2015). As part of an on-going behavioural study, 18 habituated groups (group size range 3-10) at KRR are observed on a rotational basis under natural conditions, where the birds are not interacted with and perform their daily activities with minimal human disturbance (Ridley, 2016). Individuals are marked as nestlings with a unique combination of metal and colour rings for identification. Droppings were collected from the ground following natural excretion only if there was a positive identification of which individual supplied the sample. The sampling protocol followed that of Bourne et al (2019; see Ridley and Raihani (2007) for information on the habituation process). Samples were frozen at -20 °C within four hours of collection and the date, time, identity of each bird, and time of freezing were recorded.

2.3 Analyses of circulating corticosterone and fGCM

2.3.1 Plasma corticosterone analysis

To determine plasma corticosterone concentrations; a Coat-A-Count© Corticosterone Radio-Immunoassay (Diagnostic Products Coat-A-Count Rat-Corticosterone) was used. In brief, 50 µL of the standards, controls, and plasma extracts, respectively, were transferred in duplicate into coated tubes. Corticosterone solution (1 ml ^{125}L) was then added to each tube and incubated for 120 m at room temperature. Subsequently, all liquid was removed; tubes were patted dry and immediately placed into a photometer (BioTek Instruments Elx 800 and Gen v5.00 software) to be counted. Additionally, to determine the sex of the birds in the captive study (sex was already known for all birds in the wild study), blood plasma was analysed using the three different primer sets described in Çakmak et al. (2017).

2.3.2 Faecal GCM analysis

Frozen faecal material was lyophilized, pulverized and sieved through a mesh strainer to remove undigested material before adding 1.5 ml of 80% ethanol in distilled water to 0.050 – 0.055 g of faecal powder and vortexing for 15 min to facilitate steroid extraction as described by Ganswindt el al. (2002). After centrifuging for 10 min at 1500 g, the supernatant was transferred into microcentrifuge tubes, and stored at −20 °C.

When comparing EIA performance, data for all droppings were pooled, and the number of individuals tested each hour reflected the number of droppings obtained. To determine the suitability of the EIAs for fGCM analysis, a subset of faecal steroid extracts (n = 27) from the four

captive individuals with the highest post-injection increases in plasma corticosterone concentrations were measured for fGCM concentrations using five different EIAs: (i) 11-oxoetiocholanolone I (detecting 11,17 dioxoandrostanes); (ii) 11-oxoetiocholanolone II (detecting fGCMs with a 5β-3α-ol-11-one structure); (iii) 5α-pregnane-3β,11β,21-triol-20-one (measuring 3β,11β-diol-cortisol metabolites), (iv) tetrahydrocorticosterone, and (v) corticosterone. Each of the five EIAs was tested for its suitability to detect fGCM concentrations in babbler droppings, using a set minimum increase of 100% post ACTH-injection, and for each of the EIAs, the respective diluted standard stocks were used as internal quality controls. Detailed assay characteristics, including full descriptions of the assay components and cross-reactivities, have been provided by Palme and Möstl (1997) for 11-oxoetiocholanolone I and corticosterone, by Quillfeldt and Möstl (2003) for tetrahydrocorticosterone, by Möstl et al. (2002) for 11-oxoetiocholanalone II, and by Touma et al. (2003) for 5α-pregnane-3β,11β,21-triol-20-one. The sensitivities of the EIAs used were 1.8 ng/g dry weight (DW) for corticosterone, 9.0 ng/g DW for tetrahydrocorticosterone, 0.6 ng/g DW for 11-oxoaetiocholanolone I and II and 2.4 ng/g DW for 5α-pregnane-3β,11β,21-triol-20-one. Intra-assay coefficients of variation, determined by repeated measurements of high- and low-value quality controls, were 4.15% and 5.41% (corticosterone), 6.33% and 6.64% (tetrahydrocorticosterone), 5.65% and 6.11% (11-oxoaetiocholanolone II), 2.21% and 2.15% (11-oxoaetiocholanolone I), and 6.62% and 6.70% (5α-pregnane-3β,11β,21-triol-20-one). Inter-assay coefficients of variation were determined by repeated measurements of high- and low-value quality controls for the top two performing EIAs (6.65% and 12.27% for the 11-oxoetiocholanolone II and 7.07% and 7.73% for the tetrahydrocorticosterone EIA), which were subsequently used to analyse all remaining faecal steroid extracts from captive and wild birds. All fGCM concentrations are given as μg/g faecal dry weight (DW). All EIAs were performed at the Endocrine Research Laboratory, University of Pretoria, as described by Ganswindt et al. (2002).

2.4 Statistical analysis

All analyses were conducted in R 3.4.3 (2016, R Foundation for Statistical Computing, Vienna, Austria) and SigmaPlot 13.1 (2015, Systat Software Inc, Chicago, USA). Data were tested for normality using a Shapiro-Wilk goodness of fit test. Wilcoxon rank-sum tests were used to determine the differences in circulating plasma corticosterone and fGCM concentrations pre- and post-ACTH injection as well as the effect of temporary separation on fGCM concentrations. In addition, a Wilcoxon rank-sum test was used to identify differences between fGCM concentrations in wild and captive individuals. For this comparison, the samples collected 1 h post-injection were used for the captive birds, indicative of baseline concentrations as this was when the lowest fGCM

concentrations were recorded. Pearson product moment correlations were used to test the relationship between fGCM concentration and the time required to freeze samples. In combination with t-tests, Wilcoxon rank-sum tests were used to compare fGCM concentrations of wild individuals in relation to dominance rank. Dominance rank was determined through behavioural and life history observations; courtship behaviours such as presenting sticks, mid-air chasing and display walks, nest-building behaviour, overnight incubation (only dominant females), and aggression towards subordinates (Raihani et al., 2010; Nelson-Flower et al., 2011; Wiley and Ridley, 2018).

3. Results

3.1 EIA selection

The corticosterone EIA showed an overall increase of 105% (114% for males and 72% for females) and the 5α-pregnane-3β,11β,21-triol-20-one EIA a 112% overall increase (122% for males and 87% for females). The 11-oxoetiocholanolone I EIA did not perform adequately for either sex, reporting concentrations that were below the sensitivity of the EIA. The tetrahydrocorticosterone and 11-oxoaetiocholanolone II EIAs performed best, showing overall increases of 159% (195% for males and 85% for females) and 168% (261% for males and 100% for females) respectively.

3.2 Separation test and ACTH challenge

Plasma corticosterone concentrations differed significantly ($Z = -1.58$, $p < 0.05$) between samples collected pre- (range 5.77 ng/ml – 21.46 ng/ml) and post-injection (range 16.11 ng/ml – 73.49 ng/ml; Figure 1), with no significant difference between sexes ($Z = 0.67$, $p = 0.69$).

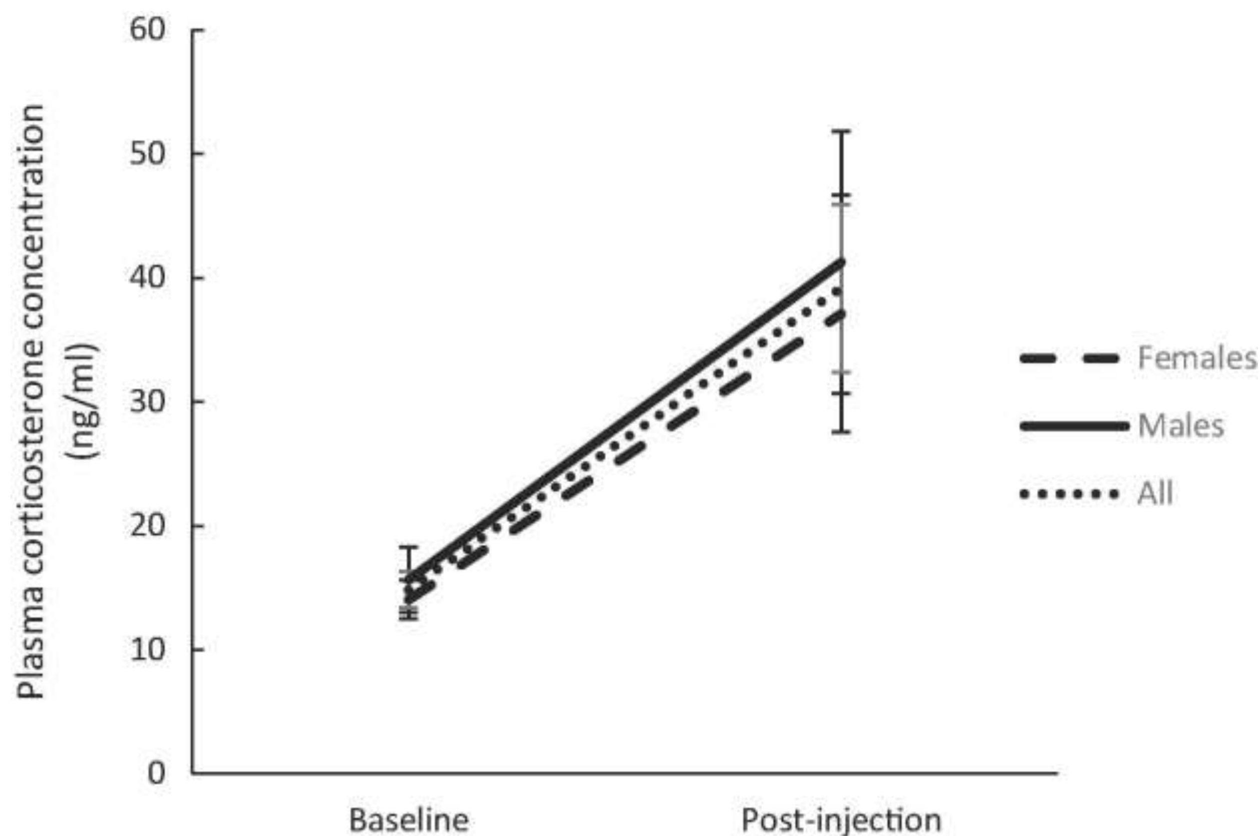

Fig. 1. Mean plasma corticosterone concentrations in southern pied babblers (*Turdoides bicolor*) for baseline and post-ACTH injection periods for all samples (i.e., both sexes), males only and females only. Mean (±SE).

The results of both EIAs followed the same approximate pattern, however, the 11-oxoaetiocholanolone II EIA yielded higher percent increases overall than the tetrahydrocorticosterone EIA (Figure 2). For both EIAs, fGCM concentrations peaked 2 h after separation, and 3 h after ACTH injection. The lowest fGCM concentration for both EIAs was measured one hour after the ACTH injection. For both the tetrahydrocorticosterone EIA ($Z = -3.52$, $p < 0.001$) and the 11-oxoaetiocholanolone EIA ($Z = -3.52$, $p < 0.001$) there were significant differences between the separation experiment peak (2 h after separation) and the baseline. Additionally, the peak from the ACTH challenge was significantly higher than the baseline for both the tetrahydrocorticosterone EIA ($Z = -3.62$, $p < 0.001$) and the 11-oxoaetiocholanolone II EIA ($Z = -3.62$, $p < 0.001$).

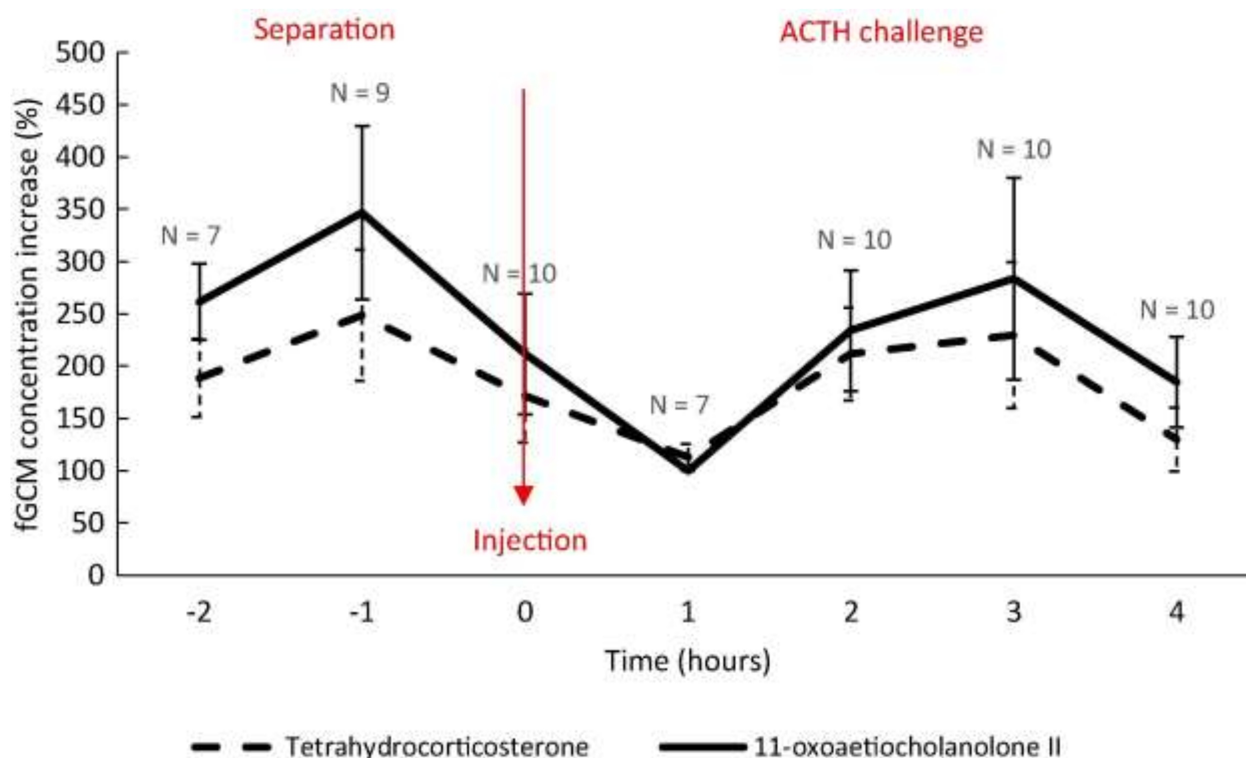

Fig. 2. Changes in mean faecal glucocorticoid metabolite (fGCM) concentrations (%) in southern pied babblers (*Turdoides bicolor*) over time during the periods of separation and pre- and post-ACTH injection when evaluated with the tetrahydrocorticosterone and 11-oxoaetiocholanolone II EIAs. 100% concentration measured at one hour post-injection was used as the baseline fGCM concentration from which % increase resulting from the ACTH challenge could be calculated. Changes in mean (±SE).

However, when samples were analysed separately according to sex, females (range tetrahydrocorticosterone: 1.68 μg/g – 3.95 μg/g; 11-oxoaetiocholanolone II: 1.76 μg/g – 4.25 μg/g) maintained overall much lower median fGCM concentrations than males (range tetrahydrocorticosterone: 1.25 μg/g – 4.34 μg/g ; 11-oxoaetiocholanolone II: 0.93 μg/g – 5.52 μg/g). In females, fGCM concentration peaks were measured 1 h post-separation and 2 h post-injection with the tetrahydrocorticosterone EIA and 2 h post-separation/ACTH injection with the 11-oxoaetiocholanolone II EIA. Further, the lowest fGCM concentrations were measured at the time of injection (tetrahydrocorticosterone) and 1 h post-injection (11-oxoaetiocholanolone II). Males on the other hand followed a similar but more distinct trend; very distinct peaks in fGCM concentrations were measured at 2 h post-separation and 3 h post-injection for both EIAs, and a very distinct trough was measured at 4 h post-separation/1 h post-injection for both EIAs (Figure 3). With both EIAs, both males and females showed significant differences between fGCM concentrations at their respective peaks and troughs (Wilcoxon rank-sum test, $p < 0.05$ for all).

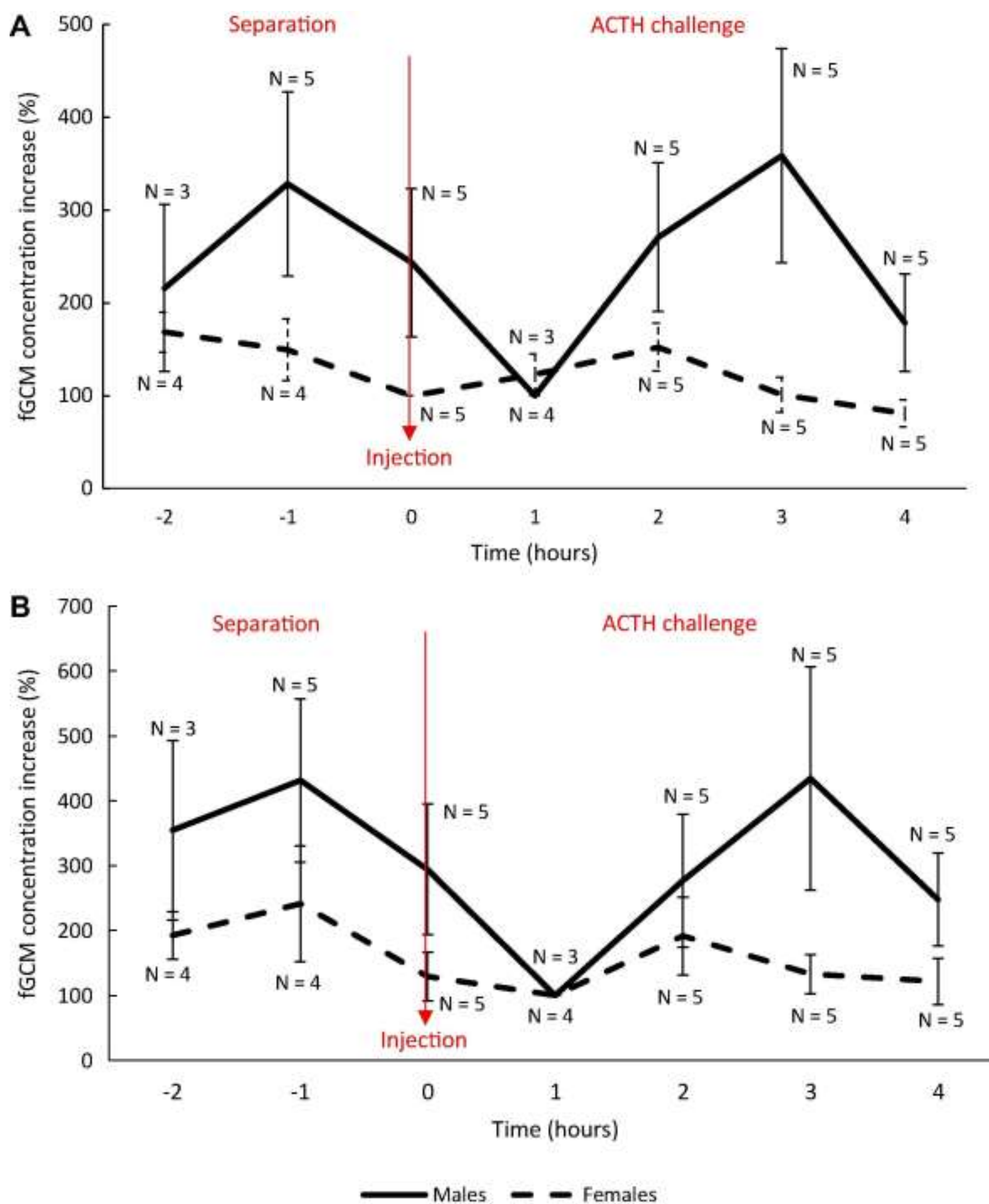

Fig. 3. Mean percent increase in faecal glucocorticoid metabolite (fGCM) concentrations over time for male and female southern pied babblers (*Turdoides bicolor*) when evaluated using the tetrahydrocorticosterone (A) and the 11-oxoaetiocholanolone (B) EIAs. For both sexes, 100% concentration measured at one hour post-injection (or hour 0 when insufficient sample material, see 2.4) was used as the baseline fGCM concentration from which % increase resulting from the ACTH challenge could be calculated. Changes in mean (±SE).

3.3 Stress monitoring in free-ranging babblers

There was no relationship between fGCM concentration and time required for freezing samples, with either the tetrahydrocorticosterone (r = 0.003) or the 11-oxoaetiocholanolone II EIA (r = 0.196). When both the tetrahydrocorticosterone and 11-oxoaetiocholanolone II EIAs were used to evaluate the differences in fGCM concentrations for wild and captive individuals; the wild birds had significantly lower concentrations (Z = -2.10, $p < 0.001$). In the captive birds, individual fGCM concentrations ranged between 0.90 μg/g – 8.57 μg/g (tetrahydrocorticosterone EIA) and 0.65 μg/g and 7.55 μg/g (11-oxoaetiocholanolone II EIA). Individual fGCM concentrations for wild birds ranged between 0.02 μg/g – 0.24 μg/g and 0.01 μg/g – 0.22 μg/g for the tetrahydrocorticosterone and 11-oxoaetiocholanolone II EIAs respectively (Figure 4). Thus, on average, the fGCM concentrations of captive birds were 32- and 43-fold higher than those of wild birds for the tetrahydrocorticosterone and 11-oxoaetiocholanolone II EIAs, respectively.

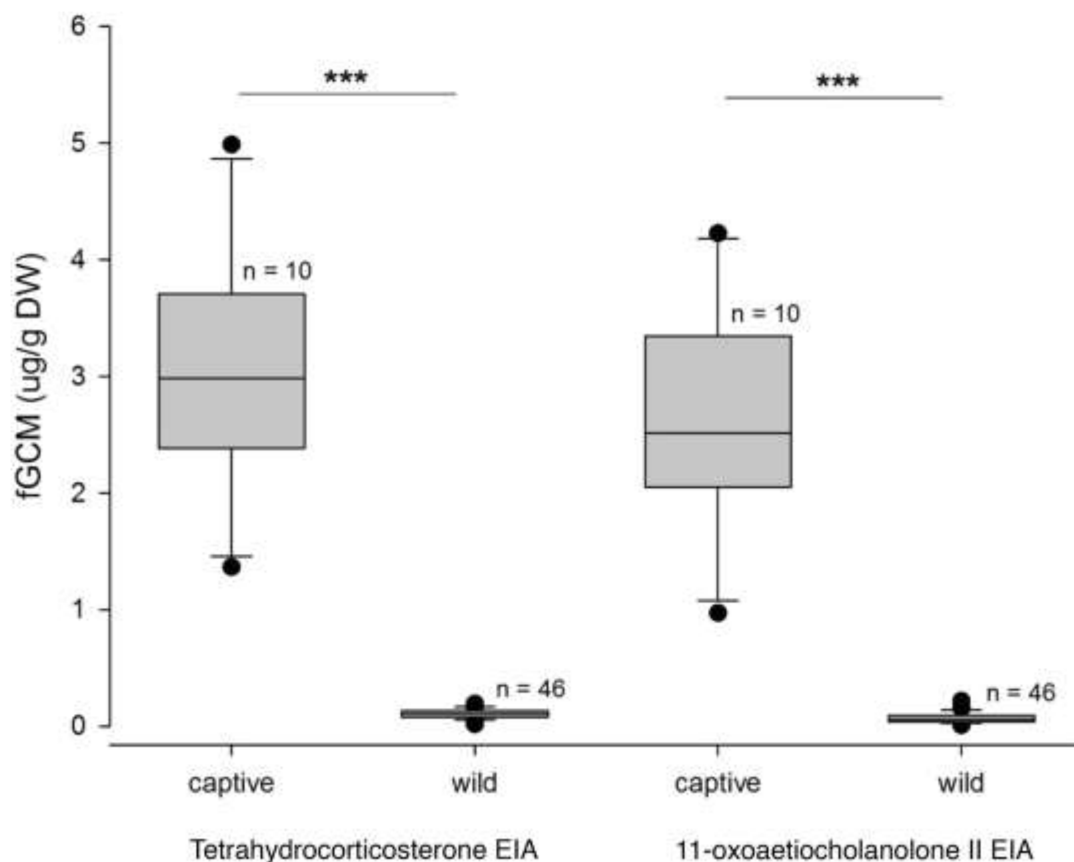

Fig. 4. Differences in baseline faecal glucocorticoid metabolite (fGCM) concentrations between captive and wild southern pied babblers (*Turdoides bicolor*) were significant when measured with the tetrahydrocorticosterone and the 11-oxoaetiocholanolone II EIAs. n = number of individual faecal samples. Median (IQR) for the tetrahydrocorticosterone EIA is 3.09 (1.85) and 0.10 (0.06) for the captive and wild babblers respectively. For the 11-oxoaetiocholanolone II EIA median (IQR) is 2.71 (1.83) and 0.05 (0.05) for the captive and wild babblers respectively.

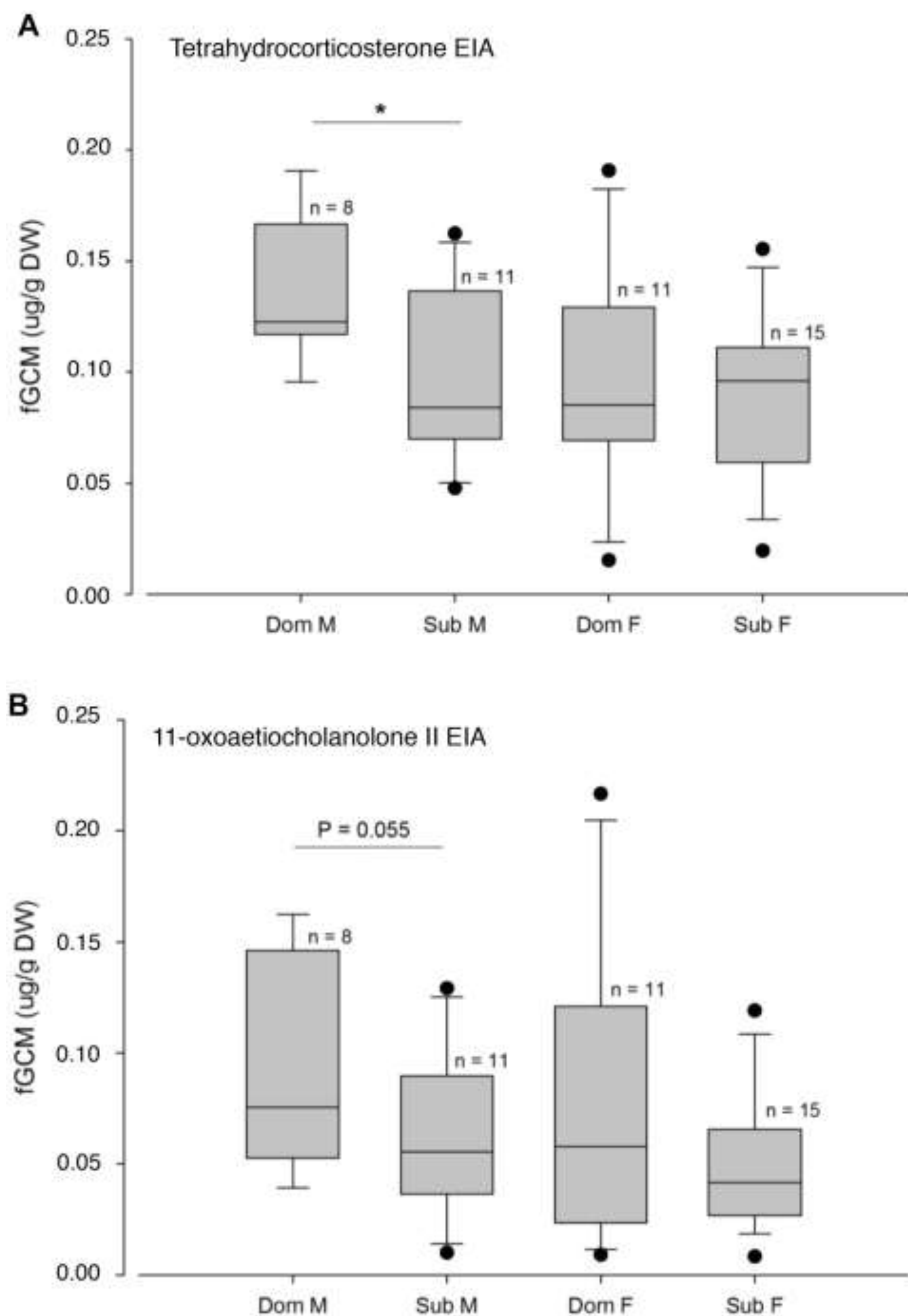

Fig. 5. Individual faecal glucocorticoid metabolite (fGCM) concentrations determined as a function of dominance in wild southern pied babblers (*Turdoides bicolor*). When analysed with the tetrahydrocorticosterone EIA differences in fGCM concentrations were found to be significant between dominant and subordinate males but not in the females. When analysed with the 11-oxoaetiocholanolone II EIA, there was a trend for higher fGCM concentrations in dominant males compared to subordinates, but no differences in the females. N = number of individual faecal samples. A) Median (IQR) is 0.12 (0.04) and 0.08 (0.05) for dominant and subordinate males respectively and 0.09 (0.06) and 0.09 (0.04) for dominant and subordinate females respectively. B) Median (IQR) is 0.08 (0.07) and 0.07 (0.04) for dominant and subordinate males respectively and 0.06 (0.07) and 0.04 (0.03) for dominant and subordinate females respectively.

In the free-living babblers, concentrations of fGCMs varied significantly according to dominance rank when assessed with the tetrahydrocorticosterone EIA. Dominant males showed significantly higher fGCM concentrations than subordinate males (t = 2.276, $p < 0.05$), while females showed no difference in fGCM concentrations according to dominance rank (t = 0.495, $p > 0.05$). Similarly, when dominance rank category was assessed in the same individuals using the 11-oxoaetiocholanolone II EIA, a trend showing higher fGCM concentration for dominant individuals was detected for males (t = 1.682, $p = 0.055$) but not females (Z = -0.68, $p = 0.38$; Figure 5).

4. Discussion

The increase in fGCM concentration in the droppings of captive babblers mirrored that of the circulating corticosterone in the blood, confirming that droppings are a suitable matrix for non-invasive monitoring of stress in this species. We found a strong effect of captivity on stress levels, with much higher fGCM concentrations in captive babblers compared to wild, free-ranging individuals. Our data also reveal that fGCM concentration varies with individual dominance rank for males, with dominant males having higher fGCM concentrations than their subordinate counterparts.

The magnitude of the increase in plasma corticosterone concentration following Synacthen injection is consistent with previous studies. Lèche et al. (2009), for instance, reported a 40-fold increase in the plasma corticosterone 60 min post-injection in greater rheas, *Rhea americana*, and Dehnhard et al. (2003) found a 16-fold increase after 1.5 h in chickens, *Gallus gallus domesticus*. Our finding that there are no differences in the stress response between the two sexes with regards to plasma corticosterone concentrations indicates that the magnitude of the stress response in both sexes is the same in captive conditions; a result consistent with those of Romero and Remage-Healey (2000) on European starlings and Lèche et al. (2009) on greater rheas.

Many previous studies that performed ACTH challenges on birds used radio-immunoassays for analyses (Astheimer et al., 1994; Cyr and Romero, 2008; Hayward et al., 2010). Among those that used EIAs, both a tetrahydrocorticosterone (Nakagawa et al., 2003) and an 11-oxoaetiocholanolone II (Koch et al., 2009; Kidawa et al., 2014) EIA were found to perform well. The peak in fGCM concentrations 2 h after separation in all babblers likely reflects the gut passage time, confirming the findings for this species by Bourne et al. (2019). Gut passage time differs greatly among species. Among European starlings (similar body mass to babblers) fed a diet of

insects, it takes around 1 h for 100% excretion (Levey and Karasov, 1994), European stonechats, *Saxicola rubicola*, excrete fGCMs after a period of around 3.7 h (Goymann et al., 2002), while the corresponding period in chickens is 2 - 5 h (Rettenbacher et al., 2004). In the ACTH challenge component of the present study, fGCM concentration peaked 3 h post-injection. This variation in lag time may be related to the intensity of the stressor or may reflect birds having more food in their gut during the ACTH challenge. Afik and Karasov (1995) showed that gut passage time differs with food type, with fewer nutrients in the passage shortening the transit time (Witmer, 1998). Interestingly, in our study, baseline fGCM concentrations were recorded 1 h after injection, suggesting the stress hormones secreted in response to this event had not been metabolized and excreted yet.

The difference in fGCM concentration between male and female babblers that emerged in both the tetrahydrocorticosterone and 11-oxoaetiocholanolone II EIAs is not surprising, since similar sex differences in hormone metabolism have been reported previously in other species (Touma et al., 2003, 2004). There are many records of sex differences in fGCM concentrations, with the magnitude and direction varying among species (Touma and Palme, 2005), and male babblers in captivity show the trend of having greater increases in fGCM concentration than females. However, the absence of data on sex differences in corticosterone metabolism pathways in babblers precludes any conclusions about variation in the magnitude of stress responses between sexes, although the lack of difference between sexes with regards to plasma corticosterone suggests that the stress response does not vary between sexes, it is simply the metabolism or metabolic pathways that create the difference. Additionally, the female babblers' gut passage time may differ from that of males; especially with regards to the tetrahydrocorticosterone EIA, where the two validation peaks as well as the baseline fGCM concentrations occurred one hour prior to that of the males. Sex-specific differences in the route and time it takes for the metabolites to be excreted have been reported in some species, as well as differences in the type and concentration of fGCM (Palme et al., 2005). Such differences have been empirically confirmed in chickens (Rettenbacher et al., 2004), European stonechats (Goymann et al., 2002) and black grouse, *Tetrao tetrix* (Baltic et al., 2005). Interestingly, while none of the male babbler peaks and troughs varied between the two assays, the females' separation experiment peak and baseline trough did, indicating that the females' results had less distinct fluctuations. More work needs to be done to investigate differences in how the sexes metabolise the hormones to explain intersexual differences in fGCM concentration.

Keeping wild babblers in captivity, even when supplied with sufficient resources, caused a sharp increase in corticosterone production, as evidenced by the extremely low fGCM concentrations of wild individuals compared to their captive counterparts. The uniformity between sexes of the fGCM concentrations of the wild birds, with the exception of the dominant males, suggests that these birds have very low baseline levels. These results are unsurprising and largely consistent with those of previous studies investigating capture stress in birds (Wingfield et al., 1995; O'Reilly and Wingfield, 2001). Increases in fGCM concentration in babblers were of similar magnitude to those seen in plasma corticosterone of wild-caught chukar, *Alectoris chukar* (Dickens et al., 2009), and Gambel's white-crowned sparrows, *Zonotrichia leucophrys gambelii* (Romero and Wingfield, 1999), indicating that in general, wild birds suffer stress when in captivity. Captivity-induced stress can lead to behaviours such as feather and skin plucking (Seibert, 2006; van Zeeland et al., 2009), and if the stress continues over long periods of time, becoming chronic, it can lower immune function (Matson et al., 2006). Chronic stress can also lower reproductive success and/or survival; Wingfield et al. (1998) found that high levels of stress in birds caused them to spend more time foraging or exhibiting escape behaviour and less time being aggressive or displaying reproductive or other social behaviour. A number of factors could contribute to the captivity-induced stress, including the inability to roam freely and forage at will (Dickens et al., 2009), harassment by predators (Clinchy et al., 2004) while in the aviaries, and separation from group members (Remage-Healey et al., 2003). Stocker et al. (2016) showed that in ravens, *Corvus corax*, the level of social integration of an individual is a factor in determining stress levels of these birds with highly integrated birds having low levels of stress while in the group and high levels when separated, and the opposite trend being seen in less integrated birds. Social integration has not been investigated to this extent in babblers but it would be interesting to see if they exhibit similar tendencies.

In Florida scrub jays, *Aphelocoma coerulescens*, Schoech et al. (1991) found that breeding (dominant) females were significantly more stressed than non-breeding (subordinate) females, but there was no corresponding difference in males. Rubenstein (2007) found that among superb starlings, *Lamprotornis superbus,* helpers had higher circulating corticosterone concentrations than breeders and non-breeders alike, and four other studies on cooperatively breeding birds reported no difference in corticosterone concentration between breeders and non-breeders (Mays et al., 1991; Schoech et al., 1991; Wingfield et al., 1991; Malueg et al., 2009). These examples do not support the hypothesis that subordinates are reproductively suppressed by high corticosterone concentrations in a phenomenon known as psychological castration (Brown, 1978;

Reyer et al., 1986). Our data instead support the idea that dominant individuals (males in the case of the babblers) have higher stress levels due to their need to defend their territory and position, behaviours which dominant male babblers display (Ridley, 2016). These results align with what previous studies have found in some species of cooperatively breeding animals, including African wild dogs, *Lycaon pictus* (Creel et al., 1997), grey wolves, *Canis lupus* (Sands and Creel, 2004), and African cichlid fish, *Neolamprologus pulcher* (Mileva et al., 2010). However, more work needs to be done to fully understand the role of dominance rank on stress levels in babblers.

5. Conclusions

Our study demonstrated that droppings are a suitable matrix through which stress levels in babblers can be monitored and that both the tetrahydrocorticosterone and 11-oxoaetiocholanolone II EIAs are satisfactory assays recommended for future use with babblers. Validation of a technique to non-invasively monitor fGCM concentration in a species opens up a multitude of possibilities for future research in this species. For example, with changing climate and rising temperatures already affecting the behaviour of bird species such as babblers (du Plessis et al., 2012; Wiley and Ridley, 2016), we now have a way to measure their stress responses to heatwaves and other climate-induced stressors, as well as a baseline to which to compare future measurements.

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