# Identifying factors that influence stress physiology of the woylie, a critically endangered marsupial

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**Short title**

Factors influencing the stress physiology of woylies

**Keywords**

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## Abstract

Faecal glucocorticoid metabolites are minimally invasive stress physiology indices that can be used to understand how animals respond to physical and/or psychological challenges (stressors) and inform how to optimise conservation management in view of these stressors. We investigated contextual biological, environmental and parasitological factors influencing variation in baseline faecal cortisol metabolite (FCM) concentration in a critically endangered marsupial, the woylie (syn. brush-tailed bettong, *Bettongia penicillata*). Woylies have undergone a rapid and significant population decline, with environmental stressors exacerbating disease suggested to contribute to these ongoing declines. We conducted a longitudinal field study of 15 adult woylies (9 females, 6 males) in a captive, naturalistic facility. FCM concentration in faecal samples (n=269) collected monthly over 20 months was quantified by enzyme immunoassay in parallel with measures of body condition, sex, season, female reproductive status and the presence of endoparasites and ectoparasites. Linear mixed effect modelling revealed a significant effect of season, sex, body condition index and nematode parasite status on FCM. Overall, mean FCM was lowest in summer and highest in autumn and winter, and females had higher mean FCM than males. There was a significant but weak negative association between body condition and FCM. When woylies were shedding oxyurid nematode eggs they had higher mean FCM compared to when they were not shedding. In future, knowledge of factors that influence FCM fluctuations in woylies may be considered when carrying out potentially stressful conservation interventions that may influence the future survival of this unique and threatened species.

## Introduction

Global biodiversity is threatened by a growing intensity and range of challenges or stressors. Stress physiology can provide insights into how animals respond to stressors (Cooke *et al.*, 2013). An essential part of the stress response involves the hypothalamic pituitary adrenal (HPA) axis, which aims to maintain homeostasis by modulating physiological and behavioural responses to stressors (Landys, Ramenofsky & Wingfield, 2006; Busch & Hayward, 2009; Parry-Jones, Webster & Divljan, 2016). Measuring glucocorticoids and their metabolites provide a means to monitor the HPA axis, the underlying neuroendocrine mechanism that determines how an organism functions under changing conditions (Wikelski & Cooke, 2006). Faecal glucocorticoid metabolites (of either cortisol or corticosterone), measured using minimally invasive methods, are commonly used in wildlife (Keay *et al.*, 2006) and are particularly practical when blood sample collection immediately following capture is not possible (Romero & Reed, 2005).

Interpretation of faecal glucocorticoid metabolite results is aided by knowledge of factors that influence baseline values. A variety of factors have been shown to correlate with faecal glucocorticoid metabolite values in wildlife species, ranging from sex to season (Millspaugh & Washburn, 2004). Parasites, including endoparasites (Clough, Heistermann & Kappeler, 2010) and ectoparasites (St Juliana *et al.*, 2014), have also been associated with alterations in faecal glucocorticoid metabolite concentration in wildlife hosts. In part, links between infection patterns and host stress physiology may be due to the effects of glucocorticoids on immune function (Biondi & Zannino, 1997; Sapolsky, Romero & Munck, 2000). Stress associated immunosuppression and exacerbation of infectious disease could be a significant threat to wildlife (Beldomenico & Begon, 2010) and understanding the relationships between host stress physiology and parasitism is important for wildlife research and conservation.

Stress exacerbating the impact of parasitic infections has been suggested to contribute to the ongoing decline of the woylie (syn. brush-tailed bettong, *Bettongia penicillata*), a critically endangered Australian marsupial (Botero *et al.*, 2013; Thompson *et al.*, 2014). Woylies were once abundant and widespread across much of mainland Australia but became locally extinct across most of their range by the 1970s. Remnant populations are now confined to Western Australia (Wayne et al., 2013a). Recent studies suggest that the distribution and abundance of woylies is related to stressors including habitat fragmentation, proximity to agriculture and invasive predators (Wayne *et al.*, 2013b; Yeatman *et al.*, 2016). In addition, more virulent trypanosomes (protozoan hemoparasites) have been found more commonly in declining woylie populations (Botero *et al.*, 2013; Thompson *et al.*, 2014), and white blood cell counts in declining populations are suggestive of "immunological stressors" (Pacioni *et al.*, 2013). Hence, stress-induced immunosuppression has been hypothesised to exacerbate the impacts of parasite (especially trypanosome) infections, contributing to declines (Botero *et al.*, 2013). Associations between faecal cortisol metabolites (FCM), immune cell (phagocyte) function and trypanosomes have since been found, which support the stress-induced immunosuppression hypothesis (Hing *et al.*, 2016). Thus, understanding how parasite infection may influence long-term variation in FCM in woylies is pertinent.

In this study, we asked what factors such as season, sex, body condition index, female reproductive status and the presence of parasites influenced variation in FCM in woylies, in a longitudinal study of a captive population.

## Materials and methods

*Trapping and sample collection*

We studied a captive population of woylies (15 adults; 6 male, 9 female), housed in four adjacent outdoor naturalistic enclosures (3 to 4 adult woylies per 35m x 55m pen) at Native Animal Rescue in Malaga, Western Australia. Woylies had access to underground fungi, native forage (bulbs, seeds etc.), insects and a year round supplementary ration of fruit and vegetables. We trapped all individuals monthly from September (austral-spring) in 2013 to June (austral-winter) in 2015. Galvanized wire Sheffield traps (220 x 220 x 550mm) (Sheffield Wire Products, Western Australia), baited with a mixture of peanut butter and oats, were set just prior to sunset and checked before sunrise (maximum total duration in the trap was 8 to 10 hours). We pooled the faeces deposited by each individual woylie at the bottom of their trap but time since defaecation could not be determined. We acknowledge that changes in FCM concentration can occur over time, and this could have influenced our results (Laver *et al.*, 2012). Woylies were individually identified by a unique microchip code. Animals were weighed, females were checked for the presence or absence of pouch young (pouch status), and the size of pouch young (mm) was estimated by palpation of the pouch.

Faecal samples were stored frozen at -20°C until they were prepared for FCM assays. All faecal samples were extracted within sixteen months of collection. A blood sample (400 to 1000µl) was collected from the lateral caudal vein into an EDTA MiniCollect tube (Greiner Bio-One, Germany) to enable DNA extraction and trypanosome PCR. EDTA blood samples were stored at -20°C and processed within six months of collection. In some cases, all assays could not be completed for every sample (e.g. due to insufficient sample volume) but a total of 269 faecal samples and 208 blood samples were analysed.

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*Faecal cortisol metabolite (FCM) enzyme immunoassay (EIA)*

FCM were analysed by an enzyme immunoassay (EIA) previously used for woylies (Hing *et al.*, 2016). In summary, faecal samples (0.2g dry weight) were lyophilised (freeze-dried) and extraction carried out using 90% ethanol and heat treatment (80$^{o}$C for 10min). Extracts were assayed for FCM by EIA using a polyclonal anti-cortisol antiserum R4866 protocol (Narayan *et al.*, 2012; Hing *et al.*, 2016). The R4866 anti-cortisol antiserum has been reported to cross react 100% with cortisol metabolites and less than 10% with other steroids (Webster, Narayan & de Vos, In Press). Results were expressed as FCM concentration (pg/g) on a dry weight basis.

*Parasitology analyses*

We performed microscopic and molecular parasitology analyses to determine what endoparasites were present. To detect gastrointestinal parasites (nematodes and protozoans), one gram wet weight of faeces was floated for 10 minutes using a concentrated sodium nitrate ($NaNO_3$) solution with centrifugation (Dryden *et al.*, 2005). The area under the coverslip was observed systematically under a BX51 microscope (Olympus, Japan) at 20x objective and eggs were classified as strongyle or oxyurid types (as these were the two major groups observed).

To detect trypanosome blood parasites, DNA extraction and *Trypanosoma* PCR amplification from blood samples were carried out using previously described protocols validated for woylies (Botero *et al.*, 2013; Hing *et al.*, 2016). Presence or absence of

*Trypanosoma* species in peripheral circulation as indicated by PCR positive or negative results was recorded. The ears of woylies were also visually inspected for the presence or absence of ticks.

*Statistical analyses*

We used linear mixed effect models to investigate which factors influenced FCM fluctuations in woylies. To fulfil model assumptions of data conforming to a normal distribution, FCM (the dependent variable) was log-transformed. Fixed effects included in our model were: season (summer/autumn/winter/spring), sex (male/female), body condition index, presence or absence of oxyurid eggs on faecal flotation, presence or absence of strongyle eggs on faecal flotation, PCR positive or negative for trypanosomes and the presence or absence of ticks. Two-way interactions between these effects were also included. Woylie ID nested within pen was included as a random effect in all models to account for repeated measures from the same individuals. Body condition index was derived from the residuals of a regression of hindfoot (pes) length to weight, calculated separately for males ($p<0.001$, co-efficient=28.5, $R^2=0.2114$) and females ($p<0.001$, co-efficient=21.6, $R^2=0.0806$) and adjusted for pouch young size in females by including pouch young size as a covariate. We were also interested in the effects of female reproductive activity on FCM, so we re-ran the models described above for females only (n=169) and included pouch status (0 = empty or 1 = pouch young present) as a fixed effect.

To ensure there was no strong multicollinearity between explanatory variables, we calculated the variance inflation factor (VIF) for all explanatory variables included in the maximal model, and ensured no variables had a VIF higher than 3 prior to modelling. To determine the minimal adequate models, we undertook model simplification by stepwise

reduction, removing non-significant terms from the maximal model until further model reductions resulted in significant changes in model deviances ($p<0.05$) (Crawley, 2007). Significance ($p\leq0.05$) was tested in a likelihood ratio test ($\chi^2$). Models were run using R 3.1.0 and the packages 'lme4' (Bates *et al.*, 2015) and 'car' (Fox & Weisberg, 2011).

**Results**

Overall, FCM concentration ranged widely from 0.03 to 457.40 pg/g. Host and environmental factors that significantly affected FCM in woylies included season, body condition index and sex ($p<0.05$) (Table 1). Modelling revealed that mean log-transformed FCM was lowest in summer (2.5 ± 0.2 SE, n=108 samples), moderate in spring (2.9 ± 0.1, n=50) and highest in the cooler months of autumn (3.3 ± 0.1 SE, n=71) and winter (3.3 ± 0.2 SE, n=40) (Figure 1a). Overall, there was a significant but weak relationship between body condition index and FCM (Table 1). The relationship between body condition index and FCM differed between the sexes with a more marked negative relationship in males compared to females (Figure 1b). Females (3.1±0.1 SE) had a higher mean FCM compared to males (2.7±0.1 SE) (Figure 1c). In female woylies, the presence or absence of pouch young overall did not have a significant effect on FCM (coefficient = 0.03, SE=0.40, df=1, $\chi^2$=1.7, p=0.193). However, there was a significant interaction between pouch status and season (coefficient = -0.79, SE = 0.72, df=1, $\chi^2$=8.15, p=0.04), with the greatest difference noted in winter when mean FCM in females with pouch young was higher (3.86 ± 0.20, n=119) than females without pouch young (2.46 ± 0.59, n=52).

Oxyurid pinworm eggs were present in 17% of 269 faecal samples, and were significantly associated with FCM (Table 1). When woylies were shedding oxyurid eggs, they had higher mean FCM than when oxyurid eggs were not detected (Figure 2a). An interaction between body condition and oxyurids also influenced FCM. When woylies

were shedding oxyurid eggs, there was a weak positive relationship between body condition index and FCM (Table 1). A weak negative relationship between body condition and FCM was observed when oxyurid eggs were not detected (Figure 2b). Woylies were also infected by trypanosomes (57% of 208 blood samples positive), strongyles (25% of 269 faecal samples positive) and ticks (44% infested of 260 inspections) in this study. Interactions between trypanosome and strongyle status, and between strongyle and tick status also influenced FCM. FCM was lowest when neither trypanosomes nor strongyles were detected (Figure 2c) and when neither ticks nor strongyles were detected) (Figure 2d).

**Discussion**

We identified several host, environmental and parasitological factors that influenced baseline FCM levels of captive woylies housed in semi-natural enclosures. These fluctuations in FCM may represent natural variations in HPA axis activity essential to survival (Crespi *et al.*, 2013) but they may also represent the physiological response of woylies to environmental and biological stressors (such as parasites and climate) which are of importance to conservation science.

Glucocorticoids are essential to the physiological and behavioural responses that allow animals to adapt to changing conditions (Jessop, Woodford & Symonds, 2013). Therefore it is not unexpected that we noted significant seasonal variation in FCM in woylies as in other wildlife studies (including marsupials) of faecal glucocorticoid metabolites (Romero, 2002). For example, winter peaks in FCM in koala (*Phascolarctos cinereus*) were hypothesised to be due to low winter temperatures and rainfall and associated resource limitations and metabolic demands (Davies *et al.*, 2014).

The important question in understanding seasonal variation is what proximate stressors are driving ultimate seasonal changes? Seasonal reproduction and changing resource quality and quantity are often cited as proximate causes of seasonal fluctuations in faecal glucocorticoid metabolites (Romero, 2002). However, our study provides a rather unique perspective as these woylies are continuous breeders and were supplementary fed throughout the study. Levels of anthropogenic noise and human interaction (including handling), which have been reported to constitute stressors in other marsupials (Narayan *et al.*, 2013) are also unlikely explanations as these factors remained relatively constant across the study period. Alternatively, peak FCM in woylies during the cooler seasons may be associated with circadian rhythms (Lane, 2006), shortened day length (Steinmetz *et al.*, 2006), or increased metabolic demands such as energy mobilisation for thermoregulation (Steffen & Musacchia, 1985). Seasonal variation in woylie stress physiology should be considered in the timing of management interventions as it may influence their response to interventions.

Woylie body condition remained within a healthy range throughout the study and body condition was only weakly negatively associated with FCM. This is consistent with other wildlife studies that show broader relationships between stress and body condition (Mumby *et al.*, 2015) that may be due to regulation of metabolism by glucocorticoids (Sapolsky *et al.*, 2000). The presence of only a weak effect in our study may be due to the protection of the captive study population from acute stressors such as limited food resources. In a study of free-ranging woylies during a translocation program, a significant and more pronounced negative association between body condition index and FCM after translocation was found (Hing *et al.*, In Review). The negative relationship between body condition index and FCM in our current study was more marked in males compared to females. This may be associated with sex differences in endocrine regulation of

metabolism and body weight (Shi & Clegg, 2009), but these interactions are currently uncharacterised in woylies. Longer-term monitoring of FCM and body condition in free-ranging populations that are exposed to other potential stressors is required to investigate the potential ramifications for woylie health and conservation.

Females had higher mean FCM than males, a pattern also reported in other Australian marsupials including the bilby (*Macrotis lagotis*) (Narayan *et al.*, 2012), koala (Narayan *et al.*, 2013) and southern brown bandicoot (*Isoodon obesulus*) (Dowle, Webster & Deane, 2012). These results may be a reflection of sex differences in glucocorticoid metabolism (Lane, 2006) and could suggest that male and female woylies have different physiological sensitivities to stressors, a pattern that has been observed in other species (Handa *et al.*, 1994).

Females woylies are capable of caring for young 96% of the time (Thompson *et al.*, 2015) as they are continuous breeders and can undertake embryonic diapause (Smith, 1989). Consequently, the majority of samples collected from female woylies during this study (n=171 in total) were collected when they were carrying pouch young (n=119). We found no association between female pouch status and FCM, which is consistent with previous suggestions that stress-induced reproductive inhibition is not a major concern for woylie conservation (Wayne et al., 2013a). Nevertheless, the greatest difference between females with versus without pouch young was noted in winter which may suggest greater physiological demands on mothers during this time.

When woylies were shedding oxyurid pinworm eggs, they had significantly higher FCM concentration compared to when oxyurid eggs were not detected. This may reflect parasite induced stress or exacerbation of infection by stress. Itching associated with the perianal deposition of oxyurid eggs has been found to cause mild chronic stress in rats (Silveira *et al.*, 2003). In addition, experiments have shown that stressor exposure

increases the shedding of oxyurid eggs in captive ground squirrels (*Citellus armatus*) (Noble, 1966). We also found that the relationship between body condition index and FCM was influenced by the presence or absence of oxyurid eggs. However the relationship was weak, so the biological significance of this effect is unclear.

We sought to investigate the impact of parasite co-infection on host stress physiology because animal hosts are commonly infected by multiple endoparasite and ectoparasite types with potential effects on host immunity and health (Ezenwa *et al.*, 2010). While we found FCM was lowest when neither trypanosomes nor strongyles were detected and when neither ticks nor strongyles were detected, other results found in this study suggest that the relationship between stress physiology and parasite co-infection in woylies is complex. For example, in trypanosome positive woylies mean FCM was higher when strongyle eggs were not detected compared to when strongyle eggs were detected. This is consistent with findings from studies of free-ranging woylies. When free-ranging woylies were trypanosome positive, strongyle egg counts decreased as FCM increased (potentially making eggs less likely to be detected if counts dropped below the detectable threshold) (Hing *et al.*, 2016). It is possible that these interactions reflect the influence of stress physiology on different arms of the immune system (such as T helper 1 and T helper 2 responses) responsible for defence against micro-parasites (such as trypanosomes) and macro-parasites (such as strongyles) (Padgett & Glaser, 2003; Hing *et al.*, 2016). The immune response to parasites and the potential coordinating role of glucocorticoids, while widely explored in other species (Sapolsky *et al.*, 2000), remain areas for further exploration in woylies.

Future projects that use FCM in woylies should be aware of the strengths and limitations of our approach. In general, given how woylies must be trapped and handled, FCM are more practical physiology metrics in this species compared to other measures

like plasma glucocorticoids. However, as the application of FCM in woylie research and management remains in its infancy, further optimisation would strengthen our ability to interpret results of the EIA used in our study. High performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS), though costly and resource intensive, would allow identification of hormone metabolite constituents in woylie faeces, which would be valuable for example to pinpoint sex-related differences in glucocorticoid metabolism (Monfort, 2003). Of most importance is the biological relevance of the hormonal titre, that is, does the FCM data relate to biological events of interest? Adrenocorticotropic hormone (ACTH) challenge for assay validation is not feasible for woylies housed in large enclosures because it is not practical nor ethically permissible to trap and collect faecal samples three times a day for several days as previously performed in a woylie (n=1) in a zoo study (Fanson *et al.*, 2015). However, we have demonstrated that the R4866 FGM EIA can detect variation in FCM concentration related to translocation (Hing *et al.*, In Review) and in association with differences in immune function (Hing *et al.*, 2016). Thus the protocol we employed provides physiologically relevant information by monitoring FCM in relation to environmental and management factors.

## 5. Conclusions

This longitudinal study revealed insights into factors influencing stress physiology indices in a captive population of woylies. Sex and seasonal factors had the greatest influence on FCM fluctuations and parasite parameters showed some interesting and complex interactions that are yet to be fully understood. This study provides a baseline for understanding what factors should be considered when carrying out potentially stressful conservation interventions or other anthropogenic activities that may influence the future

survival of this unique and critically endangered species.

## Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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## Tables and figures

**Table 1.** Coefficients for the minimum adequate linear mixed effect model of factors affecting faecal cortisol metabolites (FCM log-transformed). Significant factors in bold (n=202 readings from 15 individuals).

| Fixed effects | Coefficient | SE | Df | $\chi^2$ | p-value |
|---|---|---|---|---|---|
| **Body condition index** | **-0.001** | **0.001** | **1** | **3.905** | **0.048** |
| **Season** | **1.047** | **0.205** | **1** | **30.561** | **<0.001** |
| **Sex** | **-0.447** | **0.168** | **1** | **6.577** | **0.010** |
| Trypanosomes | 0.418 | 0.190 | 1 | 0.987 | 0.321 |
| **Oxyurid pinworm eggs** | **0.522** | **0.221** | **1** | **5.134** | **0.024** |
| Strongyle nematode eggs | 1.166 | 0.375 | 1 | 0.059 | 0.808 |
| Ticks | 0.288 | 0.213 | 1 | 0.129 | 0.720 |
| **Body condition index:sex** | **-0.447** | **0.002** | **1** | **4.522** | **0.034** |
| **Body condition index:pinworm eggs** | **0.005** | **0.003** | **1** | **3.970** | **0.046** |
| **Trypanosomes:strongyle eggs** | **-1.166** | **0.416** | **1** | **7.838** | **0.005** |
| **Strongyle eggs:ticks** | **-0.835** | **0.391** | **1** | **4.563** | **0.033** |

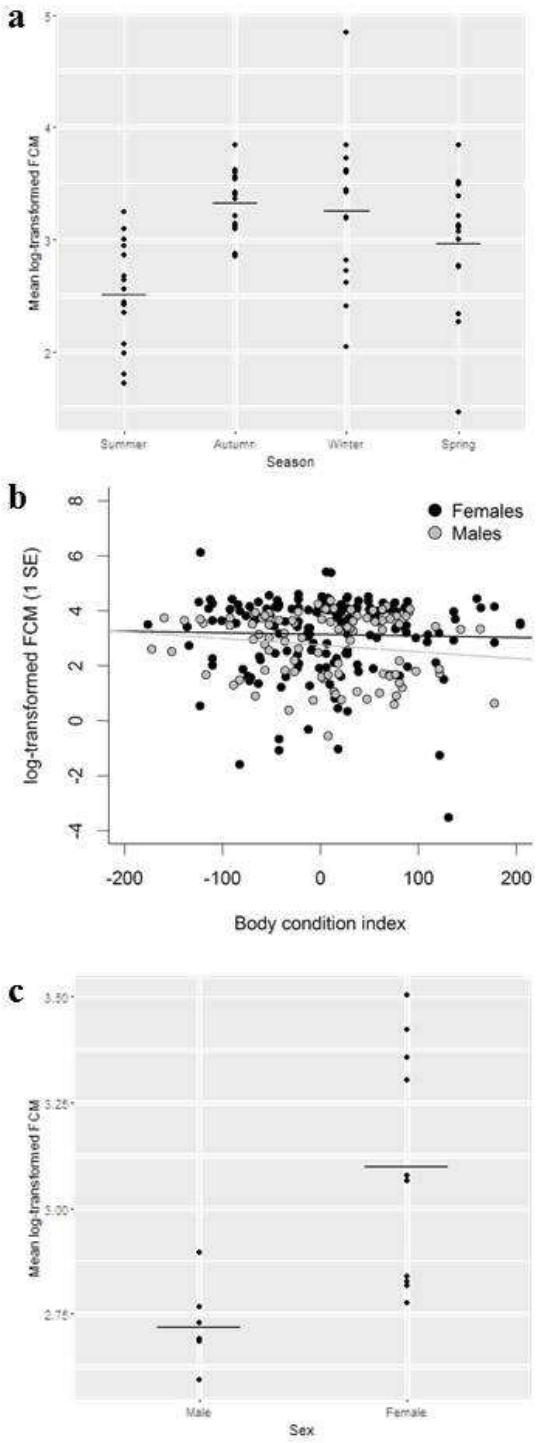



**Figure 1. Host and environment factors which influence faecal cortisol metabolites in woylies: (a)** Season**, (b)** Interactive effect of body condition and sex, **(c)** Sex. In (a) and (c), dots represent (averaged) FCM concentration of individual woylies and horizontal lines mark the overall mean.

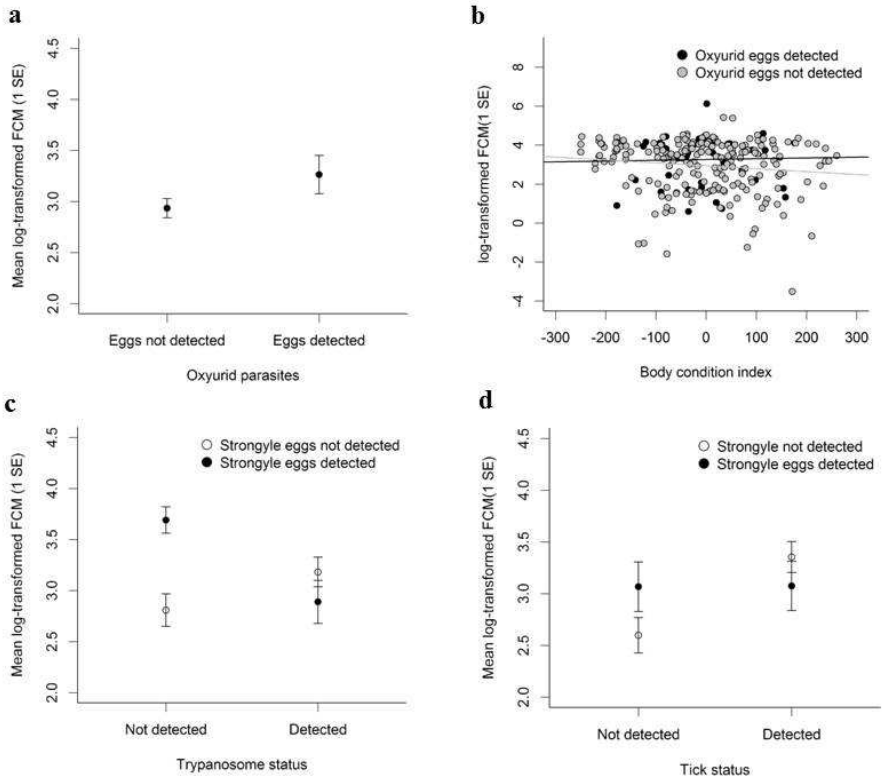
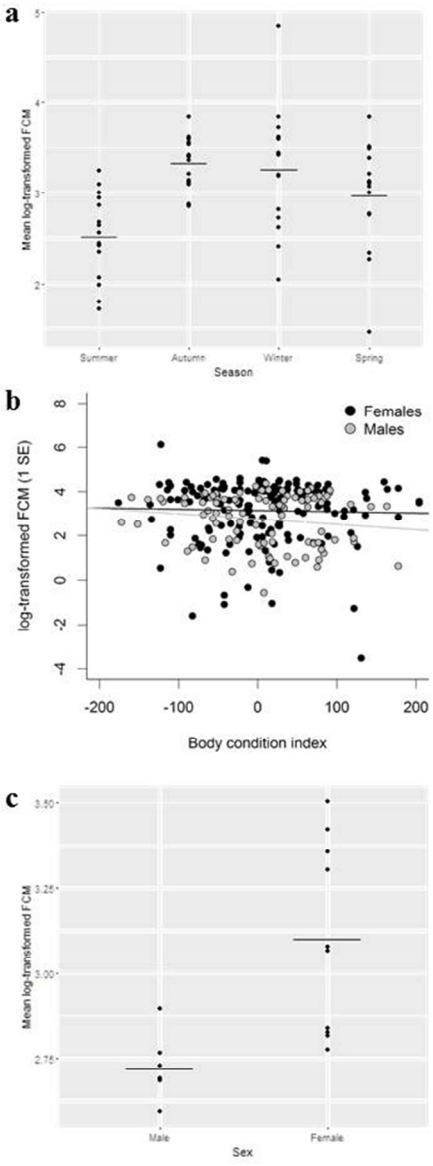



**Figure 2. Parasite factors which influence faecal cortisol metabolites in woylies**: **(a)** Oxyurid pinworm status (eggs not detected n=215, eggs detected n=44) **(b)** Interactive effect of body condition index and oxyurid status, **(c)** Interactive effect of trypanosome (not detected n=90, detected n=118) and strongyle status (not detected n=195, detected n=64), **(c)** Interactive effect of strongyle and tick status (not detected n=145, detected n=115).

**- END-**

| Fixed effects | Coefficient | SE | Df | $\chi^2$ | p-value |
|---|---|---|---|---|---|
| **Body condition index** | **-0.001** | **0.001** | **1** | **3.905** | **0.048** |
| **Season** | **1.047** | **0.205** | **1** | **30.561** | **<0.001** |
| **Sex** | **-0.447** | **0.168** | **1** | **6.577** | **0.010** |
| Trypanosomes | 0.418 | 0.190 | 1 | 0.987 | 0.321 |
| **Oxyurid pinworm eggs** | **0.522** | **0.221** | **1** | **5.134** | **0.024** |
| Strongyle nematode eggs | 1.166 | 0.375 | 1 | 0.059 | 0.808 |
| Ticks | 0.288 | 0.213 | 1 | 0.129 | 0.720 |
| **Body condition index:sex** | **-0.447** | **0.002** | **1** | **4.522** | **0.034** |
| **Body condition index:pinworm eggs** | **0.005** | **0.003** | **1** | **3.970** | **0.046** |
| **Trypanosomes:strongyle eggs** | **-1.166** | **0.416** | **1** | **7.838** | **0.005** |
| **Strongyle eggs:ticks** | **-0.835** | **0.391** | **1** | **4.563** | **0.033** |

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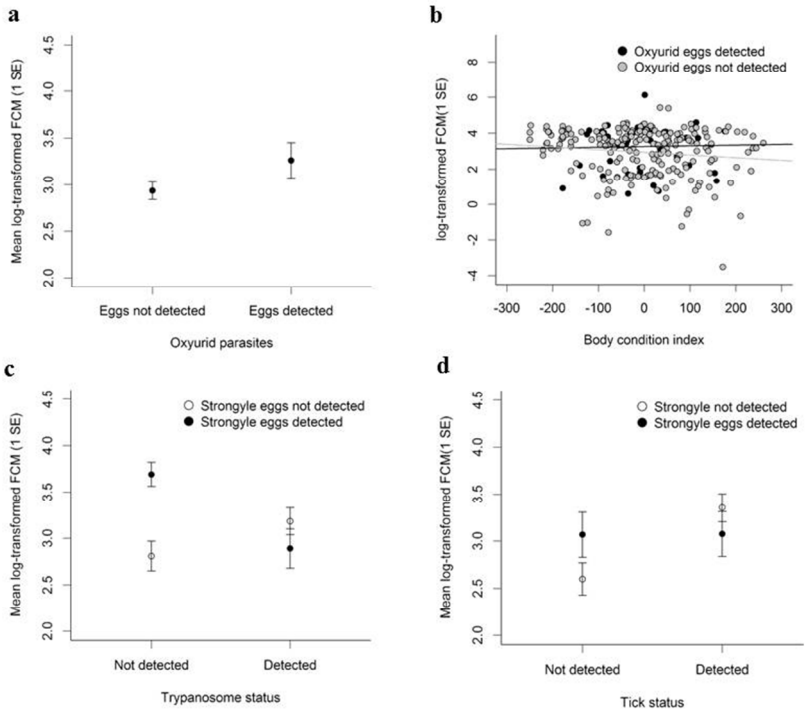


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