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Host stress physiology and *Trypanosoma* haemoparasite infection influence innate immunity in the woylie (*Bettongia penicillata*)

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Abstract

Understanding immune function is critical to conserving wildlife in view of infectious disease threats, particularly in threatened species vulnerable to stress, immunocompromise and infection. However, few studies examine stress, immune function and infection in wildlife. We used a flow cytometry protocol developed for human infants to assess phagocytosis, a key component of innate immunity, in a critically endangered marsupial, the woylie (*Bettongia penicillata*). The effects of stress physiology and *Trypanosoma* infection on phagocytosis were investigated. Blood and faecal samples were collected from woylies in a captive facility over three months. *Trypanosoma* status was determined using PCR. Faecal cortisol metabolites (FCM) were quantified by enzyme-immunoassay. Mean phagocytosis measured was >90%. An interaction between sex and FCM influenced the percentage of phagocytosing leukocytes, possibly reflecting the influence of sex hormones and glucocorticoids. An interaction between *Trypanosoma* status and FCM influenced phagocytosis index, suggesting that stress physiology and infection status influence innate immunity.

Keywords

immunity, infection, parasite, phagocytosis, stress, wildlife, woylie

Define abbreviations

EIA enzyme immunoassay

FCM faecal cortisol metabolites

1. Introduction

Globally, wildlife populations are under increasing pressure from infectious diseases [1]. Host immunity is a key factor determining the impact of disease on animal populations [2]. Therefore, investigating immune function in wildlife species can inform population management and conservation [3]. However, significant knowledge gaps remain in wildlife immunology including lack of basic species-specific data and outstanding questions surrounding factors that influence immune responses and infection patterns [2,4]. The relationship between immune function and infection has rarely been examined in wildlife despite suggestions that these processes play a part in health, population decline and extinction [5–7]. The need to address these outstanding questions in wildlife immunology is especially urgent for small populations of endangered species because they may be particularly vulnerable to the effects of infectious disease due to concurrent stressors and their influence on immune function [8]. Addressing knowledge gaps in wildlife immunology in part depends on translating progress made in research focusing on health in human populations to wildlife [9].

Marsupials, including many of Australia's most threatened species, have long been singled out as having an "extra-ordinary susceptibility to certain infections" [10], which may influence population health and survival [11]. The woylie (or brush-tailed bettong, *Bettongia penicillata*) is an ecologically important, and critically endangered marsupial, which has undergone over 90% declines in population size since 1999 [12]. There are now only two remnant populations of woylie in south-west Western Australia. Although the cause of the recent woylie decline is unknown, it has been suggested that stress, immunosuppression and infectious disease may have exacerbated the impact of introduced predators [13,14]. Trypanosomes (protozoan haemoparasites, *Trypanosoma spp.*) have been identified as possible disease candidates due to their association with the decline of the woylie [15,16].

Trypanosoma species are endemic in Australian mammals and occur in free-ranging woylie populations [17]. Woylie are host to multiple species of trypanosomes, which fall into several clades [15,18] . Botero et al. (2013) found that Clade A, including *Trypanosoma copemani*, appears more virulent and prevalent in a declining woylie population (Upper Warren, Western Australia) compared to a higher prevalence of less virulent clades in a stable population (Karakamia Wildlife Sanctuary, Western Australia) [15] . This observation has been further supported by a temporal association between decline patterns in the Upper Warren and *T. copemani* prevalence [16]. It has been suggested that woylies in the declining population may be less efficient at responding to more virulent genotypes of this endemic parasite [15]. In addition, a haematological study found variation in differential leukocyte counts between stable woylie populations and those sampled during or soon after decline [19]. In the majority of populations sampled (including stable populations in sanctuaries), neutrophils were the predominant leukocyte type [19]. In the declining populations of the Upper Warren region of Western Australia, lymphocyte and neutrophil counts were similar, which the authors suggested was consistent with involvement of immunologic challenges in the declining population [19]. Hence, understanding leukocyte function and *Trypanosoma* infection in woylies addresses outstanding questions in the conservation of this species.

A key component of innate cell-mediated immunity and the host immune response to *Trypanosoma* haemoparasites is phagocytosis, the engulfment and destruction of foreign particles by phagocytic leukocytes [20]. Deviations from normal phagocytic function such as that which might occur with stress [21] can render animals more susceptible to disease [22]. Phagocytosis has been described as a particularly difficult immunological process to measure [23]. However, different methods have been used to assess phagocyte function ranging from microscopy [24] to flow cytometry [25,26]. Phagocytosis flow cytometry is a high throughput, quantitative assay, which assesses leukocytes' capacity to phagocytose

fluorescently labelled bacteria [25,27]. It has numerous advantages for use in comparative immunology and has proven to be a rapid, reliable tool to evaluate phagocytosis in different species ranging from teleosts [28], mice and macaques [27] to salamanders [29], seals [30], camels [31], sea turtles [22,32], beluga whales [33] and Tasmanian devils [34]. The *ex vivo* nature of the phagocytosis flow cytometry assay also has advantages over *in vivo* immunologic tests such as allograft rejection, which may be ethically challenging to carry out in critically endangered species. The phagocytosis flow cytometry protocol adapted for this study [35] was particularly well suited for use in the woylie as it requires small blood volumes (<25µl) and, with some modifications, did not require species-specific markers or reagents.

We aimed to identify the factors that affect phagocytosis in the woylie, including host stress physiology and investigated the influence of phagocyte function on *Trypanosoma* haemoparasite infection status. We adapted a small blood volume phagocytosis flow cytometry protocol developed for very preterm human infants [35] and validated in newborn lambs [26], to perform the first study of immune function of the woylie. We then investigated factors, which influence host immunity and host-parasite interactions to determine their impact, if any, on phagocytosis. We hypothesised that woylie exhibit deficits in phagocyte function, these deficits are associated with parasite status and together, these factors may in part have contributed to the species' dramatic decline.

2. Materials and methods

2.1 Animal trapping and sample collection

We trapped woylies three times during the winter and spring of 2014 (June, July and November) in four adjacent naturalistic enclosures (35m x 55m each) at Native Animal Rescue in Western Australia. Fifteen adult woylies (9 females, 6 males) were housed (3 to 4 adult woylies per pen). In each trapping session, 32 galvanized wire Sheffield traps (220 x 220 x 550mm) (Sheffield Wire Products, Western Australia) were set just prior to sunset and checked before sunrise. All 15 woylies were re-captured each month. At each capture, woylies were individually identified by a unique microchip code, examined for ticks and females were inspected for the presence or absence of pouch young (pouch status). Faecal samples (deposited overnight) were collected (from underneath the trap), and a blood sample was collected from the lateral caudal vein (400 to 1000µl). At each time point, half the blood volume from each animal was collected in an EDTA MiniCollect tube (Greiner Bio-One, Germany) for DNA extraction and *Trypanosoma* PCR. The other half of the blood sample was collected in a lithium heparin MiniCollect tube (Greiner Bio-One, Germany) for phagocytosis flow cytometry. EDTA and faecal samples were stored at -20°C and processed within ten months of collection. Lithium heparin samples were kept at room temperature and processed within 24 hours of collection, as the phagocytosis flow cytometry assay required live viable cells. In some cases, all assays could not be completed for every sample (e.g., due to insufficient sample volume) but a total of 41 faecal samples, 40 lithium heparin samples and 39 EDTA samples were analysed. Valid samples were collected for each individual for at least 2 time points. This research was carried out under a Western Australian Department of Parks and Wildlife Regulation 17 License to Take Fauna for Scientific Purposes (SF009623) and Murdoch University Animal Ethics Permit (RW2611/13).

2.2 Phagocytosis flow cytometry assay

We investigated phagocytosis in woylie by adapting a flow cytometry protocol developed for very pre-term human infants [35]. As this was the first time that flow cytometry has been carried out to assess phagocytosis in the woylie, the amount of fluorescently labelled bacteria added to whole blood was first titrated. Titration was performed by adding phRodo® Red *Staphylococcus aureus* Bioparticles® Conjugate for Phagocytosis prepared as per the manufacturer's instructions (hereby referred to as phRodo®) (Gibco Life Technologies, Mulgrave Australia), to whole blood samples from two individuals opportunistically sampled as part of a pilot study. Aliquots of phRodo® ranging from 0 to 50ul were added and plotted against the percent of phagocytosing leukocytes. The proportion of phagocytosing leukocytes was maximal above 10ul of phRodo labelled *S. aureus* and a clear separation in fluorescence of positive cells above that of negative cells was evident at 10 ul. Hence, 10ul of phRodo® was added to treated samples for the assay.

Samples were prepared for flow cytometry as described by Prosser et al. (2013) with some modifications [35]. In brief, as per the woylie titration, 10ul of phRodo® was added. Heparinsed whole blood (25µl) was incubated at 37°C, 5% CO_2 in the dark for 60 minutes with 5% foetal calf serum media (80µl). Cells were washed twice with cold PBS (1ml) (Gibco Life Technologies, Mulgrave Australia) and the supernatant was removed by vacuum suction. Red blood cells were lysed with FACSLyse (1ml) (BD, North Ryde, Australia) and the remaining cell pellet resuspended in Stabilising Fixative (200ml) (BD). Analysis was performed on a BD FACSCanto flow cytometer (BD). Detection of phRodo® fluorescence was by a 450nm violet long-pass filter. No compensation was required for this single colour panel. We aimed to record twenty thousand events for each sample. Data was processed in FlowJo v.10. Single cells were gated and doublets excluded based on side-scatter height (SSC-H) and side-scatter width (SSC-W). Phagocytes were subsequently identified during

FACS analysis as those with intermediate to high forward-scatter (FSC) and side-scatter (SSC) properties, whereas lymphocytes have low SSC and FSC. Untreated whole blood samples served to determine the cut-off for fluorescence for phagocytosis in each donor.

2.3 DNA extraction and PCR amplification for *Trypanosoma* species

We used an established and validated PCR protocol to detect *Trypanosoma* parasites in the blood stream of woylies [15,16,36]. DNA extraction and *Trypanosoma* PCR amplification were carried out as per previously described protocols [15,16,36]. Whole genomic DNA was extracted from frozen whole blood samples stored in EDTA using a DNA Blood Kit following the manufacturer's guidelines (Qiagen, Hilden, Germany). Species-specific primers for *Trypanosoma* species were used in a nested PCR: *T. vegrandis* (TVEF, TVER, TVIF and TVIR), *T. copemani* (S825F, SLIR, WoF and WoR) and H25 (H25EF, H25ER, H25IF and H25IR) [15]. Negative and positive controls were used for each PCR, with the positive control derived from a known stock and the negative control containing neither blood nor tissue. Presence or absence of *Trypanosoma* species in peripheral circulation as indicated by PCR positive or negative results was recorded as trypanosome status (+ or -). We did not quantify the number of parasites in the blood stream because qPCR has not been developed for this host-parasite system.

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

Faecal samples were prepared for faecal cortisol metabolite (FCM) enzyme immunoassay (EIA) by lyophilising (freeze-drying) to remove water while inhibiting glucocorticoid metabolite breakdown by micro-organisms [37,38]. Extraction was carried out according to protocols previously described using 90% ethanol and heat treatment [37,38].

Extracts were assayed for FCM by enzyme immunoassay (EIA) using a polyclonal anti-cortisol antibody R4866 protocol [39] . We demonstrated parallelism (between dilutions of pooled faecal extracts and the cortisol standard curve), recovery of exogenous cortisol added to extracts (y=1.1026x – 0.8897, R^2 = 0.9929), sensitivity of the assay (2.04 ± 0.39pg/well, n=15) and degree of intra-assay variation (CV 4% for high binding internal control and CV 6% for low binding internal control) and inter-assay variation (CV 2% for high binding internal control, CV 12% for low binding internal control). Dilution factors were 1:8 for male woylie and 1:16 for female woylie based on the 50% binding point on the parallelism curve. Results were expressed as FCM concentration (pg/g) on a dry weight basis.

2.8 Statistical analysis

We used linear mixed effect models (lmer) in R 3.1.0 [40] using the packages 'lme4' [41] and 'car' [42] to investigate the host and environmental factors that may influence percent of phagocytosing leukocytes and phagocytosis index. The study had a nested design with repeated samples from individual woylies in their respective pens. Percent of phagocytosing leukocytes and phagocytosis index of each woylie were the dependent variables. To fulfil model assumptions of data conforming to a normal distribution, the percent of phagocytosing leukocytes was arc-sine square root transformed, phagocytosis index was scaled by z-transformation (using the function 'scale' in R) [43] and FCM was log-transformed. Sex (male/female), season (winter/spring) and FCM were included as fixed effects. 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 in their respective pens. 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 deviance [44]. Significance ($p \leq 0.05$) was tested in a likelihood ratio test (χ^2).

We were also interested in the effects of female reproductive activity on immune function, so models were re-run for females only (n=26 samples from 9 females). In the same way described above, we used lmer to investigate the effect of season, pouch status (0 = empty or 1 = pouch young present) and FCM on percent of phagocytosing leukocytes and phagocytosis index.

To investigate if immune function influenced the likelihood of trypanosome infection, we used a generalised linear mixed effect model (glmer) [40] to explore the influence of FCM, percent of phagocytosing leukocytes, phagocytosis index, season, and tick status (0=absent, 1=present) on trypanosome status. Trypanosome status (0 = PCR negative or 1 = PCR positive) was the dependent variable with a binomial error distribution. Model simplification was carried out in the same way as the lmer models, described above.

3. Results

We demonstrated phagocytosis in woylie leukocytes by flow cytometry (Figure 1). As expected, phagocytosis (higher mean fluorescence in the presence compared to in the absence of phRodo®) was negligible in lymphocytes (Figure 1c), but was detected in phagocytes (Figure 1d). The median fluorescence (*x*-axis: PE-A pHRodo) was an indirect indicator of phagocyte functional efficiency or *Staphylococcus aureus* particle uptake (phagocytic index). The ratio of phRodo® positive cells indicated the percentage of the phagocyte population that phagocytosed the phRodo® labelled *S.aureus* particles (percent phagocytosing leukocytes). Woylies had competent phagocytes with a mean percent phagocytosis of 91% ± 2.6 and mean phagocytosis index of 4730 ± 388.

3.1 Factors influencing phagocytosis parameters

There was a significant positive association between phagocytosis index and the percent of phagocytosing leukocytes (Table 1 and 2, Figure 2). We also found a significant interaction between sex and FCM on the percent of phagocytosing leukocytes (Table 1). In females, lower FCM was associated with higher percent of phagocytosing leukocytes, but the opposite relationship was detected in males (Figure 3). In female woylies, the presence or absence of pouch young did not significantly affect phagocytosis index or the percent of phagocytosing leukocytes ($p>0.05$).

3.2 Relationships between phagocytosis parameters and trypanosome infection

Overall *Trypanosoma* prevalence was 52% over the course of the study, and all cases were identified as *T. copemani.* The trypanosome status of five of the fifteen individuals changed from month to month during this study (Table 3). We found a significant interaction between *Trypanosoma* status and FCM on phagocytosis index (Table 2). When woylies were trypanosome positive, higher FCM was associated with lower phagocytosis index, but when woylies were trypanosome negative, higher FCM was associated with higher phagocytosis index (Figure 4). Sex, season, FCM, the presence or absence of ticks and phagocytosis parameters did not have a significant association with *Trypanosoma* status ($p>0.05$).

4. Discussion

In this first investigation into woylie immune function, we adapted a phagocytosis flow cytometry protocol developed in very pre-term human infants [35] to characterise factors that affect phagocytosis in the woylie. Using this protocol, uniquely combined with minimally invasive physiology and parasitology techniques in individually-identified woylies sampled

repeatedly over time, we determined that host stress physiology and *Trypanosoma* infection status influence phagocytosis.

We found a significant positive relationship between the percent of phagocytosing leukocytes and phagocytosis index. This differed from the findings of Prosser et al. (2013), where preterm infants where found to have fewer phagocytes capable of engulfing bacteria than term infants, but overall had a similar phagocytic index due an increased compensatory uptake of the amount of bacteria in the cells that were phagocytosing [35]. When woylies had a lower percent of phagocytosing leukocytes, they also had a lower phagocytosis index and in these instances, they may be vulnerable to disease. Though none of the animals in this study showed observable signs of clinical disease, these were captive animals that were protected from some of the stressors (eg. nutritional stress) and the full spectrum of pathogens that might be found in the wild. Deficiencies in phagocytosis have also been shown to be associated with a range of infectious conditions in rodent [20] and human subjects [45]. Alternatively, woylies may employ alternative immunological mechanisms to compensate when phagocytosis index and percent of phagocytosing leukocytes are both low in comparison to the population mean.

The effect of an interaction between sex and FCM on percent of phagocytosing leukocytes is likely to reflect the three-way relationship between sex hormones, glucocorticoids and immune function [46,47]. Our results appear to be consistent with comprehensive reviews of this relationship in laboratory rodents and humans, which highlight that oestrogens exert their effect on immune function in part due to an oestrogen augmented glucocorticoid response [47]. In general, females are considered more ‘immunoreactive’ than males to the immunosuppressive effects of glucocorticoids because oestrogens potentiate glucocorticoids [47]; this leaves females more vulnerable to inflammatory stimuli and autoimmune diseases [46]. Hence, our results may suggest that female woylies, similar to

females of other species, may also be at higher risk of these conditions. In males, testosterone is described as immunosuppressive but the relationship between testosterone hormones, glucocorticoids and immune function is not well understood [48] and the positive relationship between FCM and percent of phagocytosing leukocytes in male woylie requires further exploration. Development of sex hormone assays for woylies would allow for further investigation of the relationship between sex hormones, immunity and infection in the woylie.

Given the influence of sex hormones on immunity [49], we expected female woylies phagocytosis parameters would vary with reproductive state. In studies on humans, aspects of innate immunity such as phagocytosis have been found to be upregulated in pregnant women to compensate for weakened adaptive immunity [50]. However, pouch status did not significantly affect phagocytosis index or percent of phagocytosing leukocytes in female woylies. As continuous breeders with no defined breeding season, woylies may have developed mechanisms to maintain a steady state system through different phases of the female reproductive cycle to protect dam and young from erratic fluctuations in maternal immune function. However, generalisations are drawn with caution given the small number of data points where females did not have pouch young (n=6) (Figure 3). Indeed, female woylies are rarely without young and this may be another factor contributing to unique endocrine and immunological patterns throughout the reproductive cycle. Similar to other marsupials, embryonic diapause enables female woylies to simultaneously gestate a foetus, lactate to support pouch young and nurture young at heel [51], which may translate to a distinct relationship between female reproductive status and immune function.

The only relationship we found between infection state and phagocytosis parameters involved an interaction with stress hormones (FCM). The negative relationship between FCM levels and phagocytosis index in trypanosome positive woylie may indicate that during

periods of *Trypanosoma* parasitaemia, woylies are more vulnerable to the immunosuppressive effects of glucocorticoids [52]. Alternatively, a combination of host stress physiology and infection status may affect the efficiency of leukocyte function [53]. Laboratory rodent studies focused on elucidating the pathogenesis of Chagas disease (*Trypanosoma cruzi*) in humans have highlighted the important role of stress-related immunocompromise in the course of trypanosomiasis [54]. Our results are consistent with the hypothesis that stress-related immunosuppression may also be associated with the course of trypanosomiasis in the woylie. These findings may have relevance for woylie health and conservation, as they suggest that woylies experiencing concurrent stressors and active infection may be more likely to have compromised immune function. However, we acknowledge that PCR, while indicative of the presence or absence of the parasites in peripheral circulation at a given point in time, may not be an indicator of true infection prevalence; as parasitaemia fluctuates, trypanosome concentration can drop below detectable levels, particularly in chronic infections [36]. Consequently, trypanosome status can change from month to month (Table 3) and intermittent low grade parasitaemia makes simple light microscopy of woylie peripheral blood smears an unreliable means to quantify trypomastigotes (life cycle stage in mammalian host bloodstream). If logistically feasible, cytometry could be used to quantify trypomastigotes in fresh blood immediately following collection. However, higher volumes (several millilitres) of blood and species specific antibodies are required [55], neither of which were available at the time of this study.

We did not detect single factor associations between phagocytosis parameters and FCM or trypanosome status (Table 1). The study population, which was well-provisioned and protected from terrestrial predators, may not have been stressed to an extent (allostatic overload) where immune function and infection dynamics were affected by host stress physiology. Physiological stress and glucocorticoids have been associated with changes in

phagocytosis in laboratory rodents [56] but our ability to detect a relationship between stress and phagocytosis may be less than in controlled laboratory studies where relatively severe experimental stressors are imposed. In the future, using a bench top flow cytometer in the field, it would be valuable to assess phagocytosis of the last two remaining wild woylie populations who may encounter additional stressors, such as introduced predators, from which the animals in our study were protected.

Phagocytosis parameters did not appear to have a significant effect on trypanosome presence. However, the immune response to trypanosomes is complex [57], and we were only able to test a single component of immune defense. Nevertheless, the phagocytosis flow cytometry protocol used in this study may be applied to further our understanding of the pathogenesis of trypanosomiasis in varied host species. While *Trypanosoma* infection status was not found to be affected by FCM or phagocytosis parameters, the degree of parasitaemia or pathology could be affected. Future research could consider quantitatively assessing degree of parasitaemia by, for example, using concentration techniques [58] or real-time quantitative PCR [59]. We also found no association between the presence or absence of ticks and trypanosomes despite suggestions that ticks act as vectors for trypanosomes in woylie [16]. Previous studies indicate that the woylies included in the present study are chronically infected with trypanosomes [18]. Hence, even if ticks are vectors for trypanosomes in woylie, detecting ticks on an individual is not a pre-requisite for these individuals to be PCR positive for trypanosomes.

Seasonal changes in host immunity, including phagocytosis, have been noted in animals including humans [60]. However, we did not find an effect of season on phagocytosis parameters in woylie. This may indicate that phagocytosis index and percent of phagocytosing leukocytes in woylie are not subject to seasonal fluctuations, which may otherwise render woylie particularly vulnerable to infectious disease at certain times of the

year. However, the study only considered samples collected over two 'seasons' (winter and spring), so it is possible that an effect was not detected looking only at these two seasons. Seasonal changes in immune function are thought to be associated with resource quality and availability or according to the 'seasonal stimulus hypothesis', with UV exposure and vitamin D [61]. Hence alternative explanations for absence of detected seasonal differences in phagocytosis parameters in these woylie may be associated with their protection from seasonal fluctuations such as year round supplementary feeding in this population and the species' nocturnal habits.

Here, we focused on only one aspect of the innate immune system, so studies into other aspects of the woylie immune system may reveal other patterns. We have avoided making generalisations about overall immune function in woylie because immunocompetence encompasses a complex multivariate response to many different kinds of parasite and pathogens. A variety of other tests such as lymphocyte proliferation with mitogens, mixed lymphocyte reactions and genomics could be used in the future to assess different components of the woylie immune system. There are limitations to the interpretation of our flow cytometry results. For example, we were unable to determine specific phagocyte populations and absolute numbers of monocytes or neutrophils. Further improvement of the method will be possible with the development of species-specific reagents, such as those being developed for other marsupial species, which allow for more precise identification of cell types [62]. An in vitro bacterial killing assay and microscopy would also provide complementary measurements of immune function [34,63].

We have conducted the first study on immune function in the critically endangered woylie. Key results indicate that host immunity is likely to have ramifications for woylie conservation. If indeed infectious disease played a role in the species' decline, it may have occurred when percent of phagocytosing leukocytes and phagocytosis index were both low

leaving woylies more vulnerable to infectious disease. Most notably, our findings indicate that an interaction between host stress physiology and *Trypanosoma* haemoparasites are associated with the functional efficiency of woylie phagocytes. This is consistent with the hypothesis that stress-related immunosuppression may also be associated with infection status in the woylie. To manage wildlife species that may be threatened by multiple stressors and infectious disease challenges, we need to consider how these processes influence immune function because these interactions have the potential to influence health and conservation outcomes.

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Table 1 Summary of the minimal adequate linear mixed effect model of factors influencing the percent of phagocytosing leukocytes (arc-sine square root transformed). FCM was log transformed and phagocytosis index was z-transformed.

Fixed effects	Coefficient	Df	χ2	p-value
FCM	-0.0853	1	2.382	0.123
Phagocytosis index	**-0.064**	**1**	**21.878**	**<0.001**
Sex	-0.482	1	0.027	0.870
Season	-0.120	1	0.000	0.983
FCM:phagocytosis index	0.054	1	3.048	0.081
FCM*sex	**0.0156**	**1**	**5.090**	**0.024**
Season:trypanosome status	0.2896	1	3.284	0.070

Table 2. Summary of the minimal adequate linear mixed effect model of factors influencing phagocytosis index (z-transformed). FCM was log transformed and percent of phagocytosing leukocytes was arc-sine square root transformed.

Fixed effects	Coefficient	Df	χ2	p-value
FCM	1.386	1	0.363	0.547
Percent of phagocytosing leukocytes	**5.354**	**1**	**15.843**	**<0.001**
Trypanosome status	1.570	1	0.991	0.320
FCM:percent of phagocytosing leukocytes	-0.679	1	1.806	0.179
FCM*trypanosome status	**-0.591**	**1**	**4.191**	**0.041**

Table 3. Fluctuating trypanosome parasitaemia detected by PCR

Animal ID	June	July	August
WC2842	-	+	-
WC2920	+	+	+
K1642-1680	+	+	+
YH-W2930-18	+	+	+
K1305-1268	+	+	+
K1282-1289	-	+	-
WC2807	-	-	-
WC2830	+	+	-
K1483-1610	+	+	NA
K1294-1291	+	+	-
WC2844	+	-	+
WC2930	+	+	NA
WC2841	NA	+	+
K1639-1265	-	+	NA
K1677-1635	-	NA	-

Highlights

- The first woylie immune function study explored factors influencing phagocytosis
- Flow cytometry can be used to assess phagocytosis in woylies
- Interaction between sex and cortisol affected percent of phagocytosing leukocytes
- Interaction between cortisol and trypanosome status influenced phagocytosis index

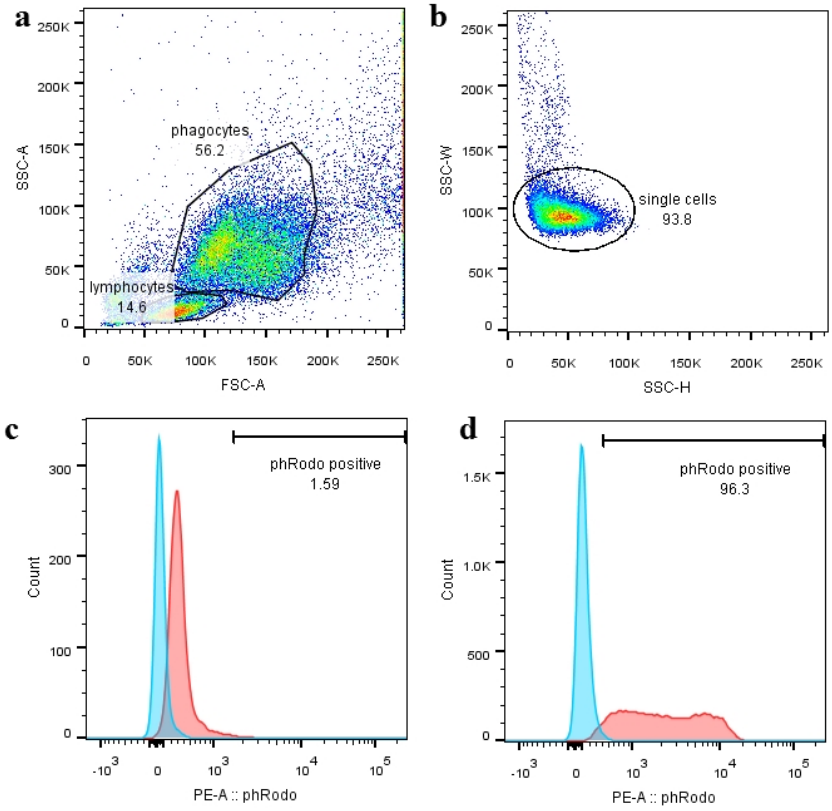

Figure 1. Representative plots from a peripheral blood sample from a woylie showing analysis of phagocytosis using flow cytometry with pHrodo-labelled bacteria. The top two panels indicate inclusion gates for: **a)** phagocytes and lymphocytes, based on forward-scatter area (FSC-A) and side-scatter area (SSC-A); and **b)** single cells, based on side-scatter height (SSC-H) and side-scatter width (SSC-W). The bottom two panels show histograms of pHrodo fluorescence (PE-A:phRodo) and cell count of **c)** lymphocytes and **d)** phagocytes in the presence (pink) and absence (blue) of pHRodo® labelled bacteria.

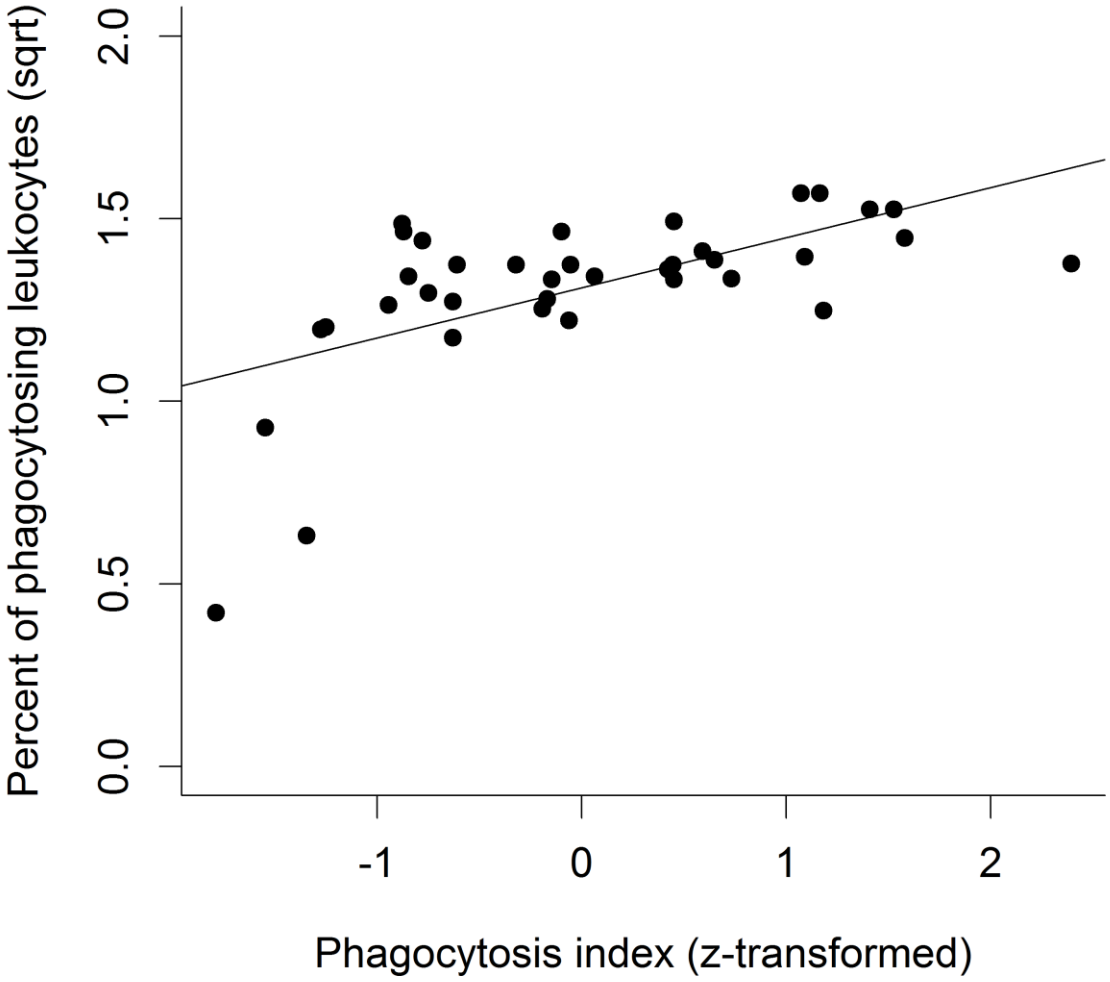

Figure 2. A scatter plot of the relationship between the percent of phagocytosing leukocytes (arc-sine square-root transformed) and phagocytosis index.

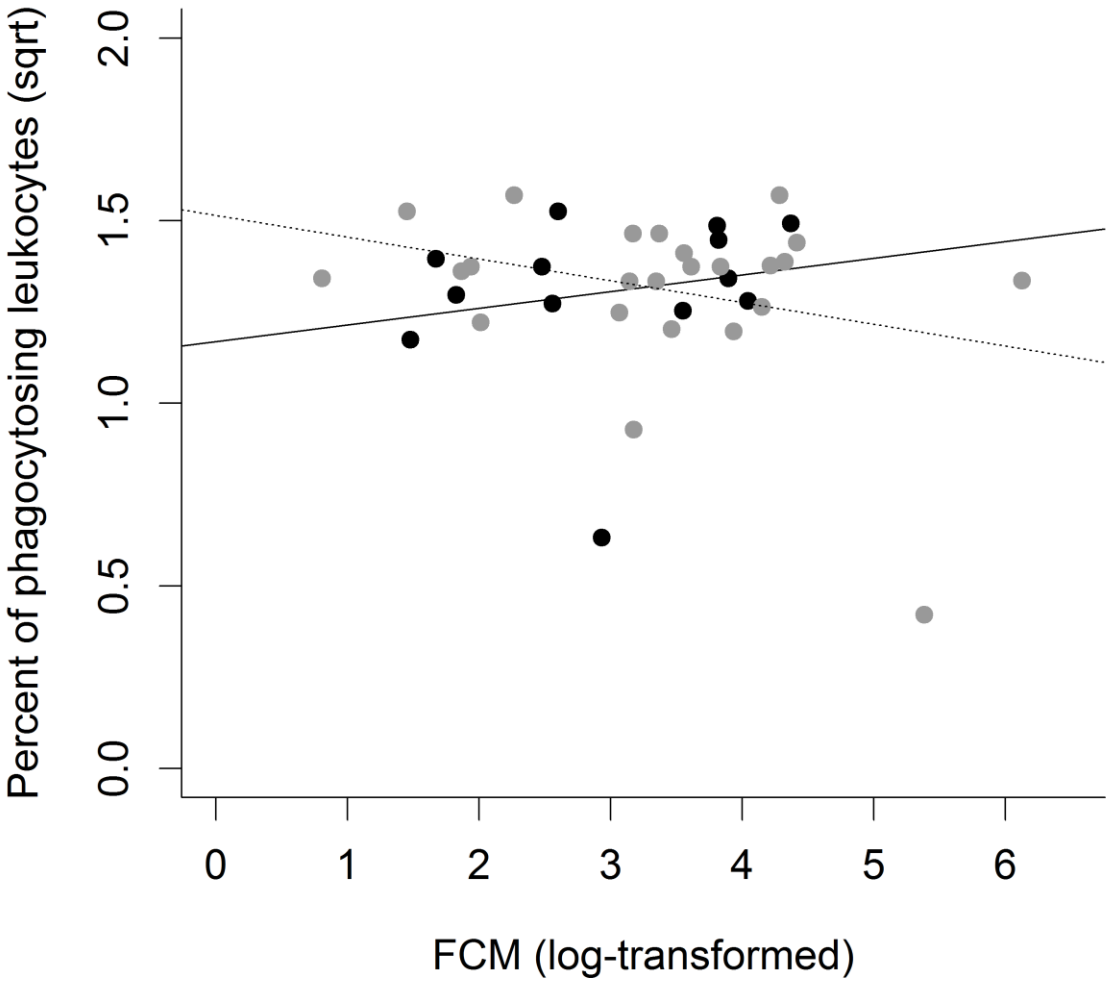

Figure 3. A scatter plot of the relationship between the percent of phagocytosing leukocytes (arc-sine square-root transformed) and faecal cortisol metabolite (FCM log-transformed) concentration for males (black symbols, solid line, percent= 0.05FCM+1.16) and females (grey symbols and dotted line, y= -0.06FCM + 1.51).

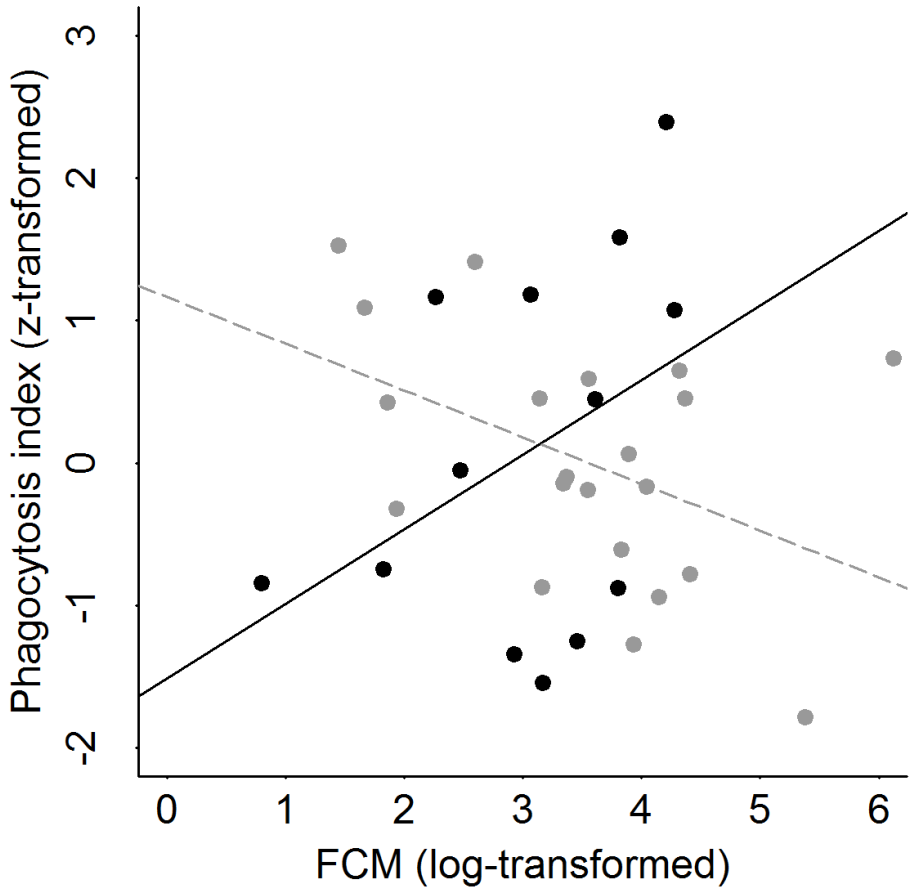

Figure 4. A scatter plot of the relationship between phagocytosis index (z-transformed) and faecal cortisol metabolite (FCM log-transformed) concentration for woylies detected as positive for *Trypanosoma copemani* (grey symbol, grey dashed line, index= -0.33FCM+1.17) and negative (black symbol, black solid line, index= 0.524FCM-1.51).