Trypanosome co-infections increase in a declining marsupial population

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\begin{abstract}
Understanding the impacts of parasites on wildlife is growing in importance as diseases pose a threat to wildlife populations. Woylie (syn. brush-tailed bettong, \textit{Bettongia penicillata}) populations have undergone enigmatic declines in south-western Western Australia over the past decade. Trypanosomes have been suggested as a possible factor contributing towards these declines because of their high prevalence in the declining population. We asked whether temporal patterns of infection with \textit{Trypanosoma} spp. were associated with the decline patterns of the host, or if other factors (host sex, body condition, co-infection or rainfall) were more influential in predicting infection patterns. Species-specific nested PCRs were used to detect the two most common \textit{Trypanosoma} species (\textit{T. copemani} and \textit{T. vegrandis}) from 444 woylie blood samples collected between 2006 and 2012. Time relative to the decline (year) and an interaction with co-infection by the other trypanosome best explained patterns of infection for both trypanosomes. The prevalence of single species infections for both \textit{T. copemani} and \textit{T. vegrandis} was lower after the population crash, however, the occurrence of co-infections increased after the crash compared to before the crash. Our results suggest an interaction between the two parasites with the decline of their host, leading to a higher level of co-infection after the decline. We discuss the possible mechanisms that may have led to a higher level of co-infection after the population crash, and highlight the importance of considering co-infection when investigating the role of parasites in species declines.
\end{abstract}

\section{Introduction}
Parasites are recognized as having an important role in the regulation of host populations (Anderson & May 1978; Hudson et al., 1998; Watson, 2013). However, when the host is exposed to additional pressures, the impacts of parasites may be exacerbated, leading to population crashes without recovery (Holmes, 1996; Pedersen et al., 2007; Smith et al., 2009). Parasitic diseases can pose a threat to the conservation of wildlife populations, but we still lack a fundamental understanding of how parasites contribute towards many species declines, and how to detect when this is occurring (Preece et al., 2017).

Parasites can act directly on host mortality or reproductive rates to drive a species decline. Often in these cases, the role of disease in the decline of a species can be detected through the observation of disease-induced mortality as a wave of infection passes through the host population (e.g. Martin et al., 2017). However, more often parasites have sub-lethal effects on hosts, and overt disease may not always be present (Scott, 1988), making the cause(s) of the decline more difficult to diagnose. Instead, parasitic infections are more likely to interact with other population pressures (stress, malnutrition or predation, e.g. Pedersen and Grieves (2008)), or co-infecting parasites to exacerbate population decline rates.

Detecting the impacts of parasites on host population dynamics can be difficult without experimental manipulation (Tompkins et al., 2011), yet this is rarely practical in the case of endangered wildlife species. Instead, examining relationships between infection dynamics, decline dynamics and the health of hosts may provide clues to the involvement of a parasitic infection in the regulation of a wildlife host population (Winteritz et al., 2013). We examined this in a declining and critically endangered marsupial, the woylie (also known as the brush-tailed bettong, \textit{Bettongia penicillata}) in the south-west of Western Australia.

Historically, the woylie was an abundant marsupial, distributed over the southern half of Australia. However, declines caused by a range of factors (e.g. introduced predators and habitat destruction) left only three indigenous populations in the south-west of Western Australia by the 1970’s (Groom, 2010). Intensive conservation management restored populations to the point where they were delisted from the endangered species list in 1996 (Groom, 2010), however...
contemporary declines in south-west Western Australia and South
Australia have since resulted in a reduction in total population size by
90% between 1999 and 2006 (Wayne et al., 2013). The declines were
greatest in the largest woylie population, in the Upper Warren of south-
west Western Australia (Fig. 1) where there was a decline of 95% or c.
160,000 woylies between 2002 and 2010 (Wayne et al., 2015). In-

troduced predators (cats, Felis catus and red foxes, Vulpes vulpes) play a
significant role in suppressing woylie populations, and in indigenous
woylie populations where sustained fox-baiting has been carried out,
cats have replaced foxes as the dominant predator of woylies (Wayne
et al., 2011; Marlow et al., 2015). The replacement of foxes with a more
efficient and active predator has been hypothesized as a main driver of
recent woylie population declines (Marlow et al., 2015; Wayne et al.,
2015). However, other features of the woylie decline, namely a spati-
temporal pattern of decline with an apparent density threshold, associa-
tions with skin conditions, lymphocytosis and some parasite asso-
ciations, have indicated that a disease process may also be involved
(Wayne et al., 2015).

Three trypanosome species have been identified from woylies;
Trypanosoma copemani, T. vegrans (Botero et al., 2013) and T. noyesi
(Botero et al., 2016a), although only T. copemani and T. vegrans occur
commonly in woylies. Previous work has shown that the prevalence of
trypanosomes (and especially T. copemani) was higher (> 80% infected by
T. copemani) in a declining population in the Upper Warren region of
south-western Australia (where our present study is located), compared
with a stable, fenced and isolated population (< 10% infected by T.
copemani at Karakamia Sanctuary, about 300 km north of the Upper
Warren) (Smith et al., 2008; Botero et al., 2013). Trypanosoma copemani
has also been associated with pathological lesions in dead woylies, and
has been shown to invade cells in vitro (Botero et al., 2013, 2016b). It
has also been found in other critically endangered and vulnerable
Australian marsupials such as koala (Phascolarctos cinereus, McInnes
et al., 2011a), Gilbert’s potoroo (Potorous gilbertii) and quokkas (Setonix
brachyurus, Austen et al., 2009). Thus, among the trypanosomes in-
festing woylies, T. copemani has the most potential as a pathogen and its
higher prevalence in a declining woylie population might suggest a
causative role in the decline. However, the stable population is also
protected from predators, and this is likely to be the main reason for its
stability. Differences in parasitism between these two populations could
be coincidental and caused by ecological and environmental differ-
ences. To better understand the role of trypanosomes in the decline of
woylies we need a better understanding of the drivers and dynamics of
infection within the decline region itself.

The aim of this current study was to understand what factors were
associated with the temporal dynamics of trypanosome infection with
respect to the decline of the woylie. We expected that trypanosome
infection prevalence would vary with the decline of the woylie, either
because of density-dependent transmission processes, or because of
parasite-induced mortality leading to a reduction in the proportion of
infected hosts. Alternatively, we asked if other factors were more in-
fluential than host decline in shaping infection patterns (such as host
sex, or seasonality in environmental conditions). Because of the ob-
servational nature of our study, we cannot infer causality of the drivers
of the patterns, but we discuss the possible mechanisms generating the
patterns we observed.

2. Materials and methods

2.1. Sample collection

Sampling was carried out in Keninup (Fig. 1) in the Upper Warren
region as a part of the Woylie Conservation Research Project between
2006 and 2012, which spanned the decline of this population (Table 1).
Population monitoring in Keninup began in 2005, which revealed the
population had been increasing in abundance in the period preceding
the population declines (Wayne et al., 2015). Keninup was one of the
last populations in the Upper Warren Region to decline (declines
commenced in 2008 (Wayne et al., 2015)), and thus we have samples
pre-decline (2006–2007), during the decline (2008–2009), and post-
decline when the population had reset to a lower density (2010–2012)
(Fig. 2).

Table 1
A summary of the sampling across years, presented by month to indicate the
seasonal spread of sampling. Numbers refer to distinct captures (where samples
were collected), not individual woylies. Mean rainfall for each month (pre-

dented as an average of the rainfall for that month over the whole study period
for the purpose of summarizing this information) is also presented. No sampling
was carried out in the months of January or July.

| Month      | Mean Rainfall (mm) | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | 2012 |
|------------|--------------------|------|------|------|------|------|------|------|
| February   | 16.4               | 0    | 0    | 25   | 0    | 13   | 0    | 0    |
| March      | 24.4               | 0    | 20   | 29   | 9    | 0    | 0    | 0    |
| April      | 28.1               | 0    | 0    | 23   | 0    | 11   | 8    | 0    |
| May        | 111.9              | 0    | 6    | 37   | 15   | 0    | 0    | 0    |
| June       | 184.8              | 0    | 0    | 13   | 0    | 0    | 0    | 0    |
| August     | 46.2               | 0    | 0    | 54   | 0    | 0    | 0    | 0    |
| September  | 104.4              | 0    | 0    | 31   | 0    | 0    | 0    | 0    |
| October    | 65.4               | 16   | 0    | 31   | 0    | 2    | 9    | 0    |
| November   | 41.8               | 16   | 14   | 21   | 0    | 8    | 0    | 0    |
| December   | 39.3               | 8    | 0    | 25   | 0    | 0    | 0    | 0    |
| Total      |                   | 40   | 40   | 228  | 85   | 10   | 33   | 8    |
Fifty wire cage (Sheffield) traps (20 × 20 × 45 cm) baited with universal bait (peanut butter, rolled oats and sardines) were set at fixed locations 200 m apart along a single transect (composed of a series of connected vehicle tracks) as a part of a population monitoring program (see Wayne et al., 2015 for more details). The transect covered a 5 × 5 km area located within a larger, continuous population of woylies. Traps were opened before dusk, and checked within three hours of sunrise the next day. Woylies were marked with two ear tags to enable individual identification, weighed (g) and measured for head-length (cm), gender and age, and a blood sample (200–400 μl) was collected from the lateral caudal vein using a 25G × 5/8" needle and 1 ml syringe, into a 1 ml EDTA tube (Thompson et al., 2014). After blood collection, woylies were immediately released at the point of capture. Blood samples were frozen at −20 °C until DNA extraction was possible. Trapping was carried out across 23 discrete sampling trips between 2006 and 2012 (Table 1), with each sampling trip conducted over four consecutive nights. Recaptured woylies were released without sampling, so that individual woylies were not resampled in the same trapping session, but may have been sampled again in different trapping sessions.

2.2. DNA extraction and Trypanosoma spp. detection by PCR

DNA was extracted from frozen whole blood samples using the Wizard® Genomic DNA Purification Kit (Cat# A1125) as per the protocol for whole blood extraction and animal tissue (Promega, Wisconsin USA). DNA was eluted in 60 μl of DNA Rehydration Solution and stored at −20 °C prior to use. A negative control, containing neither blood nor tissue, was included in each batch of DNA extractions. A general Trypanosoma PCR was carried out first to screen samples (Maslov et al., 1996), and for samples that were positive, two separate clade-specific nested PCR protocols were used to amplify the trypanosome 18S rDNA region. Trypanosoma vegransis and T. copemani species-specific PCR primers (McInnes et al., 2011a; Botero et al., 2013) and PCR reactions were used as previously described by Botero et al. (2013). Four controls were used in both internal and external PCR reactions; a pre- and post-PCR negative control, a negative control using dH2O as a PCR template, and a PCR positive control, using either T. copemani or T. vegrandsis DNA. A negative control from the DNA extraction was also run in each PCR. Each control was monitored to ensure reliability of results. PCR products were run on a 2% agarose gel using SYBR Safe Gel Stain (Invitrogen, California USA) and visualized by illumination with LED light.

2.3. Statistical analyses

First, to understand how the prevalence of each parasite varied in context of the population decline of the host, we estimated the prevalence (using Jeffrey's confidence intervals) of infection among
individuals in each sampling trip, and graphed this against time, and the capture rates (percentage of traps occupied in a session) of woylies for each sampling trip. Capture rate estimates were taken from Wayne et al. (2015) who derived capture rate as the number of independent captures (which may have included individuals previously trapped within the same session) as a proportion of the total number of traps set over the sampling trip. Capture rates are known to closely correspond to abundance estimates (Wayne et al., 2013). It is possible that multiple captures of the same individual could influence capture rates, but the influence of trap-happy individuals should be minor and constant across the study period.

We then used an Akaike Information Criteria (AIC) model selection approach to test alternative hypotheses about the factors influencing the temporal variation in trypanosoma prevalence. We modeled each of the two parasites (T. copemani and T. vegrandis) separately, with infection status (present or absent) modeled as a binomial response variable in a generalized linear mixed model. Individual identity and sampling trip were included as random effects to model the influence of repeated measures of individuals through time. We considered predictor variables that were potentially influential in shaping infection patterns. We included predictor variables that measured information about the host (sex, body condition and co-infection with the other trypanosome), environmental variation, and time relative to the decline of the host population. Body condition index (BCI) was derived from the residuals of a linear regression between log-transformed weight and log-transformed head length (separately for each sex; males = 2.95 + 0.96*log (head length), females = 2.23 + 1.13*log (head length)), and each regression was checked for goodness of fit and outliers (R² was 0.138 and 0.204 for male and female regressions, respectively). Co-infection with the other trypanosome (e.g. for T. copemani, co-infection with T. vegrandis) was modeled as presence/absence. To represent temporal fluctuation (relating to seasonal changes) in environmental conditions, monthly total rainfall (mm) was obtained from Bureau of Meteorology records from a nearby station (Manjimup). We considered that changes in rainfall could influence infection patterns via its potential impacts on vector population abundance. Year was included as a continuous covariate to represent the time relative to the decline. Year and capture rates (calculated at yearly intervals) were strongly correlated (r = −0.907), thus year may also reflect the density of the population. These variables were considered both on their own, and in combinations with each other representing alternative hypotheses about the factors that explain infection prevalence. A full set of models considered is presented in Supporting Information Table 1. All explanatory variables were checked for collinearity, both visually and using variance inflation factors; none of the included variables had a variance inflation factor greater than two. Continuous predictors were scaled (to 0.5 SD) and centered prior to analysis so that effect sizes were on comparable scales (Gruber et al., 2011).

The fit of each model was measured using AIC values, corrected for small sample size (AICc), with smaller AIC values indicating a more parsimonious model (Burnham and Anderson, 2002). Models were ranked according to their AICc (from smallest to largest) and ΔAICc was calculated as the difference in AICc between the top model (smallest AICc) and all subsequent models. We calculated Akaike weights (ωi) that measure the likelihood that a particular model is the best explanation of the dataset, relative to the other models that were considered. Models ranked in the top 95% of model weights are presented. Because the top models for both parasites were ranked strongly (ωi > 0.5), we present model coefficients with 95% confidence intervals derived from likelihood profiles for each of these top models. To aid model interpretation, we also present evidence ratios (ER) which are calculated as the weight (ωi) of the best model (smallest AICc) divided by the weight (ωi) of the model it is being compared to. Modeling and model selection were carried out in R (R Core Team, 2015), using the packages lme4 (Bates et al., 2014), car (Fox and Weisberg, 2011), and AICmodavg (Mazerolle, 2016).

3. Results

In total, 444 samples were collected from 178 individual woylies between 2006 and 2012, with each individual woylie sampled an average of 2.5 times (range = 1–10 times) (Table 1). Overall, the percentage of individual woylies with a detectable infection of T. copemani over the duration of the study was high (85.9% (80.2–90.4%, 95% CI)), but prevalence varied across sampling periods (Fig. 2a). Trypanosoma copemani displayed an overall decline in prevalence with time, although when the population was undergoing its decline, prevalence was increasing steadily. By contrast, the percentage of individual woylies detected with T. vegrandis infections over the duration of the study was lower (46% (38.8–53.4%, 95% CI), and again, this varied among sampling periods (Fig. 2b). Trypanosoma vegrandis also tended to increase in prevalence as the population declined, but there was more variation in prevalence between adjacent sampling sessions than for T. copemani.

3.1. Model selection

Among the models considered to explain the patterns of Trypanosoma infection, a model including year, co-infection (with the other trypanosome) and an interaction between these effects was the
Table 3
Coefficients and 95% profile confidence intervals of standardized and centered model coefficients from the best (smallest AICc) models from generalized linear mixed effects models examining factors influencing Trypanosoma sp. infection patterns.

| Model parameters | Coefficient | 95% CI     |
|------------------|-------------|------------|
| (a) Trypanosoma copemani |             |            |
| Year             | −1.31       | −2.88 − (−) 0.33 |
| Co-infection     | 1.67        | 0.05–3.80  |
| Year*Co-infection| 1.33        | −0.18–3.04 |
| (b) Trypanosoma vegrandis |       |            |
| Year             | −0.53       | −1.50–0.25 |
| Co-infection     | 1.62        | 0.73–2.69  |
| Year*Co-infection| 0.99        | 0.21–1.96  |

The top ranked model for both parasites (Table 2). While the model for T. copemani was moderately weighted ($\omega_i = 0.514$), it had low R² values (marginal < 0.001, conditional = 0.063). Year was the only model coefficient that had confidence intervals that did not overlap zero (Table 3), but the model including an interaction between year and co-infection was more likely than models with co-infection (ER = 7.79) or year (ER = 9.17) only. The proportion of hosts infected by T. copemani decreased over time for individuals that were not concurrently infected with T. vegrandis, but not for individuals that were concurrently infected (Fig. 3a).

The model for T. vegrandis was strongly weighted ($\omega_i = 0.846$), with moderate R² values (marginal = 0.126, conditional = 0.350). Both co-infection and the interaction between year and co-infection had model coefficients with confidence intervals that did not overlap zero (Table 3). The proportion of hosts infected by T. vegrandis was much higher among individuals concurrently infected by T. copemani, and concurrent infection influenced how the prevalence of T. vegrandis changed over time. The proportion of individuals infected with T. vegrandis decreased over years among individuals that did not have a concurrent T. copemani infection (Fig. 3b). On the other hand, the proportion of individuals infected with T. vegrandis increased over years for individuals that were concurrently infected by T. copemani (Fig. 3b).

4. Discussion

Both Trypanosoma species showed strong temporal changes in prevalence as their host population declined, but the pattern depended on whether the host was concurrently infected by the other trypanosome. The prevalence of single infections decreased from prior to the declines to post-decline, however, during this same time period, the proportion of animals with co-infections increased for both parasite species.

The reduction in overall prevalence observed for both parasites is
consistent with what we would expect if transmission were density-dependent. The reduction in the density of available hosts reduces transmission opportunities, and could explain why both trypanosomes became less common as the host population declined. Alternatively, the reduction in prevalence observed could occur if infected hosts were lost more frequently than uninfected hosts. It is difficult to disentangle these two explanations. However, in either situation, we would expect the rate of co-infection to also decrease, as both parasites become less common in the host population. Yet, we observed the opposite pattern, where the percentage of co-infected hosts remained high (T. copemani) and even increased (T. vegrandsis), while that of singly infected hosts decreased. This suggests different processes underlie the infection patterns in these concurrently infected hosts.

It is possible that competitive interactions between parasites may increase the survival of co-infected hosts. In another trypanosome, T. brucei, experimental co-infection with a less virulent strain unexpectably increased the survival of the host (Balmer et al., 2009). The less virulent strain suppressed the density of the more virulent strain in the host, reducing the negative impacts of the more virulent strain on the host (Balmer et al., 2009). Previously, Thompson et al. (2014) found a negative interaction between the two Trypanosoma species in woylies, where infection with T. vegrandsis would preclude later infection with T. copemani. Thus, it is possible that infection by T. vegrandsis improves the outcomes of infection for individuals already infected by T. copemani, leading to co-infected hosts being more likely to persist throughout the decline, than those infected only with T. copemani. This hypothesis remains to be tested, but the pattern of infection observed in our study is consistent with this explanation.

Alternatively, infection with one parasite may reactivate parasitaemia of the other parasite, leading to a higher rate of detection of co-infections. Because T. vegrandsis is hypothesized to persist mainly in the peripheral blood (Thompson et al., 2014), we expect it to be present (and detectable) while the host is infected. However, T. copemani is hypothesized to alternate between the peripheral blood and tissues (Botero et al., 2016b), so it may remain undetectable in the peripheral blood until a stressor (possibly co-infection with T. vegrandsis) activates its cycle in the peripheral blood, leading to a higher parasitaemia. The PCR used in this study is sensitive to the number of parasites in the blood stream (Dunlop et al., 2014), thus it is possible that co-infections may be more easily detected if they result in a higher parasitaemia. If this were the case, we would expect a consistent difference in the detection of either trypanosome between concurrently infected hosts and those lacking a concurrent infection. However, the rate of coinfection changed as the population declined. Thus, we consider this explanation insufficient to explain the patterns we observed.

Other explanations of our results may be possible, but do not readily fit the patterns we observed. For example, if infected hosts are selectively removed from the population, the resulting host population should consist of a small number of more resistant hosts. This could result in a similar pattern in overall host numbers and detectable trypanosome infections to that observed, but it doesn’t explain why co-infected hosts became relatively more common after the decline. Alternatively, uninfected hosts may have been experiencing selective mortality for some other reason, although, again, this does not explain the relative difference in coinfection status. Notably, we did not find any other models or variables that provided better explanations for the infection patterns observed, even though we tested other factors relating to the host (sex and body condition) and environmental variation (rainfall). The top model for T. copemani had a relatively low explanatory power, suggesting that another (unmeasured) factor influenced infection patterns of T. copemani. When we considered the fine scale temporal patterns of T. copemani prevalence, we observed a very strong linear increase in infection prevalence during the period when sampling (and the rate of population decline) was most intense. It is possible that this striking temporal pattern in prevalence was unable to be captured by any of our fixed or random effects.

While we cannot identify if the trypanosomes are driving the decline in some way, or if trypanosomes became less common because of the decline, our study raises some important issues around understanding the ecology of parasites in endangered wildlife. Trypanosomes are not commonly associated with population declines in wildlife, and are more often implicated in human diseases. The Christmas Island rat (Rattus macleari) is the only species proposed to have been exterminated by a trypanosome, after the introduction of black rats (Rattus rattus) carrying an exotic Trypanosoma sp. to Christmas Island (Wyatt et al., 2008). Even if virulence is normally low, native trypanosomes may adversely impact their hosts under stressful conditions. For example, in koalas admitted to a veterinary hospital, another native Australian trypanosome (T. gilletti) was associated with lower packed-cell volumes and body condition among koalas that were carrying concomitant infections or conditions (McInnes et al., 2011b). It is becoming increasingly important to understand the impacts of stressors on disease dynamics as wildlife are exposed to a growing range and severity of stressors (Hing et al., 2016).

Rarely in wildlife declines do we have the opportunity to track the patterns of parasitic infection, so we have little understanding about how infection dynamics change as a population declines. In this study, the integration of health and disease data into a threatened species monitoring program has yielded critical insights into the ecology of infectious agents during a species decline. Importantly, at the time sampling was carried out, no clear decline agent had been identified. Retrospective analysis of the remarkably large database collected during routine monitoring programs may have enabled the identification of a significant factor in the critical declines of a species at risk of extinction. Our study reinforces the importance of long-term pathogen surveillance of native fauna that has been emphasized by others (e.g. Smith et al., 2009). Our results also highlight the importance of recognizing multiple infections in understanding host-parasite interactions, since complex interactions between parasites can have unexpected outcomes for both the hosts and the parasites (Telfer et al., 2010).

Conflicts of interest

None.

Acknowledgements

We thank the Australian Research Council, the Department of Parks and Wildlife (WA) [now Department of Biodiversity, Conservation and Attractions], WA Government’s ‘Saving Our Species’ a biodiversity conservation initiative and Natural Resource Management programs, and the Federal Government’s Caring for Our Country program in collaboration with the Warren Catchment Council for funding parts of this research. We thank two anonymous reviewers for constructive feedback on the manuscript. Samples were collected in the field with the assistance of a large number of people including DPAW staff Marika Maxwell, Colin Ward and Chris Vellios, Murdoch staff and students and volunteers. Wildlife sampling was carried out under Murdoch University animal ethics approval permit numbers S1182-06, W2172-08, W2350-10, and RW2659-14, and Department of Parks and Wildlife (WA) (formerly Department of Environment and Conservation (DEC)) animal ethics approval permit numbers 08/2006, 52/2009 and 57/2012.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijppaw.2018.06.002.
