Using experimental de-worming to measure the immunological and pathological impacts of lungworm infection in cane toads

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1. Introduction

Host–parasite biology has been studied for many years, but the ecological impacts of parasites on their hosts have become a major focus only recently (Thompson et al., 2010; Gómez and Nichols, 2013; Jenkins et al., 2015; Polley and Thompson, 2015). Correlations between parasite infections and host biology, although relatively straightforward to document, provide only a weak basis for inferences about causation (Brown et al., 2015a). For example, animals with heavy parasite infections may also be in a weakened condition. But from this observation it is not possible to ascertain if the parasites cause the weakness, or if some other factor causes weakness and the weakened animals become more prone to parasite infections. A more powerful method is to experimentally manipulate infection status and monitor the results, ideally in free-ranging hosts in order to document effects under ecologically relevant conditions (Kelehear et al., 2011; Heise-Pavlov et al., 2014).

One means of experimentally manipulating parasite levels is by exposing naïve hosts to infective larvae. This method is ideal for studying responses to the initial establishment stages of infection (Pizzatto et al., 2010; Kelehear et al., 2011; Nelson et al., 2015) but may be less feasible in studying longer-term effects of chronic infection, especially if the parasite has an extended lifespan. Experimentally eradicating parasites from hosts using parasite-specific drugs developed for use in domestic animals (Stien et al., 2002; Pedersen and Antonovics, 2013) is an alternative approach that selectively removes adult parasites from a subset of infected hosts. This experimental removal of parasites may also overcome ethical and logistical difficulties associated with deliberately infecting animals. Experimental de-worming has been used with great success in several wildlife studies. For example, de-worming increased the time spent moving and foraging by wild Grant’s gazelles, Nanger granti (Worsley-Tonks and Ezenwa, 2015), increased host body mass and fecundity in wild reindeer, Rangifer tarandus (Stien et al., 2002), enhanced breeding success in free-ranging pheasants, Phasianus colchicus (Draycott et al., 2006), increased survival in free-ranging African buffalo (Ezenwa and Jolles, 2015),
and altered the parasitic community in free-ranging whitetailed and deer mice (Pedersen and Antonovics, 2013).

If we are to use anthelmintic drugs to quantify the impacts of parasitism on host behavior and performance, we need to understand the consequences of those drugs on host physiology. For example, are impacts of de-worming on the host mediated by shifts in immune system functioning, and/or by the inflammation induced by decomposing parasites within the host’s body? Our current understanding of parasite-host interactions is focused on the parasite and the resultant changes in host behavior, performance, reproductive output, and demographic traits (Bakker et al., 1997; Fenner and Bull, 2008; Kelehear et al., 2011). Understanding the pathological and immunological consequences of parasitism on a host can clarify the processes by which parasites induce these specific changes.

As part of a study to quantify the behavioral and ecological effects of removing lungworms (Rhabdias pseudosphaerocephala) from cane toads (Rhinella marina), we quantified aspects of immune system responses and morphological changes associated with (i) injection of the anthelmintic drug Ivermectin, (ii) decomposition of parasites in the host’s lungs, and (iii) long-term variation in parasitic infection intensity. We assessed the effects of de-worming over two time periods:

1. Short-term (<1-week) effects of experimental de-worming on infected versus non-infected toads, to separate effects of the anthelmintic drug itself versus the effect of the drug plus decomposing worms on host immunological responses (concentrations of blood cells and bactericidal ability), and the pathological effects of R. pseudosphaerocephala infection on lung tissue (Kucik et al., 2004; Turner et al., 2010);
2. Long-term (>2-month) effects of experimental de-worming (which generated variation in parasitic infection intensity among hosts) on organ mass, colonic tissues (the site of larvae shed by those adult worms), and immune responses (concentrations of blood cells and bactericidal ability) of free-ranging and captive cane toads. Do histological, physiological and pathological responses differ between toads of varied infection intensities several months after hosts are subjected to ‘de-worming’?

2. Materials and methods

2.1. Host–parasite system

Cane toads (Rhinella marina, formerly Bufo marinus) are large (up to 500 g) toxic bufonid anurans native to Central and South America. Since being introduced into Australia in 1935, these toads have caused the decline of many populations of endemic predators that lack physiological resistance to the toad’s powerful bufotoxins (Smith and Phillips, 2006; Jolly et al., 2015).

The lung nematode Rhabdias pseudosphaerocephala is found throughout most of the cane toad’s Australian range (Dubey and Shine, 2008), but is absent from the expanding invasion front (Phillips et al., 2010). Rhabdias nematodes have a direct life cycle (Baker, 1979). Briefly, hermaphroditic adults inside the toad’s lungs lay eggs that pass into the toad’s digestive tract and hatch into first-stage male and female forms which are free-living in the soil once they are defecated by the toad. These larvae mate to produce infective third-stage larvae (L3) which develop inside free-living females. After 3–4 days the larvae break free from the mother and are released into the soil (Baker, 1979). When an L3 locates an anuran host it pierces through the skin, alimentary tract or membrane behind the eye and burrows through tissue to reach the lungs of the toad where it feeds on blood (Pizzatto et al., 2010). After they reach the host’s lungs the parasites mature and begin producing eggs in as little as 5 days (Kelehear et al., 2012). Although infection dynamics can vary climatically and seasonally (Barton, 1998; Pizzatto et al., 2013), up to 80% of cane toads are infected in populations in far north Queensland (Barton, 1998), with infection intensity reaching up to 282 adult worms per host (Pizzatto et al., 2013).

2.2. Study site

Our study took place between August and December 2016 at Leaning Tree Lagoon (12.7°S, 131.4°W) and nearby areas in the Adelaide River floodplain, Northern Territory, Australia. Leaning Tree Lagoon is a 6-ha billabong situated 80 km south-east of Darwin. The area experiences a tropical climate that is dry for about half of the year and wet for the other half, with monsoonal rainfall between November and April (Shine and Brown, 2008). Our study took place primarily over the dry-season (May–November). Average maximum temperature surpassed 35°C each month and the mean monthly minimum temperature between August and November was 21°C (BOM, 2016). Cane toads appeared in the area late in 2005, and lungworms were first recorded in toads in the area in 2008 (Phillips et al., 2010).

2.3. Short-term effects of de-worming on immune responses of adult toads

We collected 16 toads from Leaning Tree Lagoon on the night of 5th December 2016. Toads were housed individually in 300 × 200 × 200 mm plastic boxes and fed four large adult crickets daily. We examined at least three fecal samples (obtained on different days) per individual for the presence of R. pseudosphaerocephala larvae to assay their infection status. The identification of Rhabdias (L3) larvae as R. pseudosphaerocephala was confirmed under a dissecting microscope based on their shape, size and movement patterns. No other known cane toad parasites in Australia resemble R. pseudosphaerocephala larvae, or have been recorded in cane toad feces. At initial capture, 10 of the 16 animals were infected with Rhabdias and six were not. We injected toads with Ivermectin (0.02 mg/100 g toad; Ivomec®, Merial, Duluth, USA) over a range of times prior to euthanasia. Five toads (3 infected, 2 non-infected) were injected 7 days prior to euthanasia, four toads (3 infected, 1 non-infected) 4 days prior to euthanasia, three toads (2 infected, 1 non-infected) 2 days prior to euthanasia, two toads (1 infected, 1 non-infected) 1 day prior to euthanasia, and two toads (1 infected, 1 non-infected) were euthanized but not injected with de-wormer. Thus, at the time of euthanasia, toads had been treated with Ivermectin 1, 2, 4 or 7 days previously, and two toads had not been treated at all. Blood samples were immediately collected from euthanized toads for use in immune assays (see below).

2.4. Histological analysis of the short-term effects of de-worming adult toads

Lungs of the 16 captive toads were removed at the main stem bronchus and injected with 10% phosphate-buffered formalin prior to being placed in a jar of the same fixative. After fixation, lungs were incised lengthwise and worms floating free in the lungs were removed, counted and retained. For histological processing, one
lung from each toad was sectioned transversely four times, at equal distances along its length. These four transverse sections were processed in a standard fashion (see below) for histological examination, and 5 μm sections stained with hematoxylin and eosin were cut, resulting in one slide for each of the four transverse sections from one toad. Lung slides were examined by a pathologist (C.S.) who was blind to the treatment group of the toad. Lung sections on each slide were screened at 100× magnification and areas of inflammation were categorized based on the predominant cell types involved in those inflammatory reactions. Two types of areas of inflammation were identified:

(i) lymphocytic inflammation — focal interstitial aggregates of 10–20 small mononuclear cells; mostly lymphocytes, with a few admixed macrophages.

(ii) neutrophilic inflammation — focal intraluminal loose aggregates of 5–20 neutrophils, often mixed with red blood cells and macrophages (Santos et al., 2016).

For histological processing, the tiny size of the nematodes necessitated embedding them in agar before cutting slides. Larvae were fixed in 10% buffered formalin for 24 h and then embedded in agarose agar (0.5 g of agarose mixed with 50 mL hot water) in a small rectangular (2 × 3 cm) shallow (0.5 cm deep) mold. Once set, the agar containing the larvae was removed from the mold using a scalpel and placed in a beaker of methanol for 2 h to harden. The resultant piece of agar containing embedded larvae was then placed in a histology cassette, processed in standard fashion (see below) for histology, embedded in paraffin, sectioned and stained. *Rhabdias* were examined, blind to the toad treatment group, and viability scored as follows: 0 = non-viable (indistinct staining, generally pale, eosinophilic, cells with indistinct boundaries, poorly delineated nuclei, blurred cuticular layers, and hypodermal boundaries); 1 = intermediate (transitional between features from categories 0 and 2); and 2 = viable (cells were well-delineated and well-stained, with distinct nuclei, cuticle layers distinct, with a sharp boundary between the hypodermal cells and underlying coelom).

### 2.5. Long-term effects of de-worming on immune responses of adult toads

#### 2.5.1. Captive cane toads

We captured 49 toads over three nights from multiple sites on the Adelaide River floodplain (12.6°S, 131.3°W) in late August 2017. Toads were taken to the laboratory where they were weighed, measured (snout-urostyle length [SUL], head width and right tibia length) and given a unique toe-clip. Toads were individually housed in plastic containers (300 × 200 × 200 mm) lined with newspaper. Water was provided in a shallow earthenware dish and toads were fed four large crickets every second day. We determined whether this could be caused by colonic irritation (possibly in response to the presence of motile *Rhabdias* larvae), we also collected colon samples from these 11 free-ranging toads for histological examination.

#### 2.5.2. Free-ranging cane toads

Over a 12-week mark-recapture study at Leaning Tree Lagoon between August and November 2016, we determined the infection status of 454 toads (Finnerty et al., 2017). At initial capture, the infection status of each toad was assessed by inspecting fecal samples after which each toad was randomly assigned to receive either an injection of anthelmintic Ivermectin (0.02 mg/100 g toad) or a control injection of Amphibian Ringer’s solution (Wright and Whitaker, 2001). This resulted in the same four treatment groups as above in Section 2.5.1. At recapture, each toad was re-dosed with the same treatment it received at its initial capture. Individual toads were recaptured and re-dosed up to four times. At the end of the 12-week mark-recapture study, six IC (i.e. not de-wormed) toads and five ID (i.e. de-wormed) toads were fitted with radio-transmitters and tracked for 5 days. These 11 toads were then recaptured and taken back to the laboratory, euthanized, blood samples were taken for use in immune assays, and all 11 toads were dissected (see below in Section 2.7). Elsewhere (Finnerty, 2017), we describe the propensity for toads infected with *Rhabdias* to produce feces with a higher water content than that of non-infected toads. To determine whether this could be caused by colonic irritation (possibly in response to the presence of motile *Rhabdias* larvae), we also collected colon samples from these 11 free-ranging toads for histological examination.

#### 2.6. Dissection and histological methodology

Post euthanasia and after the removal of blood samples from both captive and free-ranging toads, toads were dissected via a mid-ventral incision and we recorded carcass mass, liver mass (±0.001 g) and infection intensity (number of lungworms in lungs). Livers were dabbed dry on paper towel to remove excess fluid before being weighed. Samples of colonic wall were fixed in formalin, sectioned and stained for histological examination (see below). Sections of the colon were inspected for epithelial differentiation and the presence of necrotic cells. Lymphoplasmacytic and granulocytic infiltrations of the mucosa were scored (1 = mild, 2 = moderate, 3 = marked).

Toad tissues and lungworm larvae were processed for histological examination using standard techniques. To make histology slides, formalin-fixed toad tissues were appropriately trimmed, placed in histology cassettes and processed in standard fashion. Processing involves a series of dehydrating steps accomplished by passing the tissues through increasingly concentrated ethanol solutions, followed by clearing using limonene, and impregnation with paraffin wax. Processed tissues were then embedded in paraffin wax blocks and 5 μm sections cut using a microtome, placed on glass slides, stained with hematoxylin and eosin and a cover slip fixed in place using permanent mounting medium.

#### 2.7. Euthanasia, blood sampling and immune assays

We euthanized toads with an overdose of pentobarbital sodium (50% Lethabarb diluted in water: Pizzatto et al., 2013) and then removed 0.3 mL blood samples via cardiac puncture. Each sample was refrigerated immediately and immune assays began as soon as the blood sample was collected from the last toad. Blood samples
were used to (1) measure numbers of each leukocyte cell type (i.e. basophil, eosinophil, monocyte, lymphocyte, neutrophil), (2) assay whole blood phagocytic activity, (3) measure concentrations of white and red blood cells, and (4) assay bacteria-killing ability of plasma. This combination of measures reflects the configuration of standing constitutive lines of immune defense (relative leukocyte concentrations) as well as host ability to react to experimentally induced immune challenges (phagocytosis and bacteria-killing assays). Methods are explained in detail elsewhere (Brown and Shine, 2014; Brown et al., 2015b, 2016). Briefly, the methodology used was as follows:

1. Blood smears were prepared and stained using modified Wright’s stain. Slides were examined under 1000× magnification to identify leucocytes, which were classified as either lymphocytes or neutrophils (Brown et al., 2015b).

2. We measured light emitted from the chemical reactions that occur when blood cells (mainly neutrophils) phagocytize pathogen particles. The pathogen particles used were Zymosan (Sigma Z4250, from yeast cells). We added Luminol (Sigma A051) to amplify the photons produced by the reaction to detectable levels. Using sterile Amphibian Ringer’s solution, whole blood was diluted 1:20 and 240 μL was added to the wells of a 96-well assay plate. 10 μL of a 20-mg/mL solution of Zymosan (in phosphate-buffered saline [PBS]) and 30 μL of a 10-mM solution of Luminol (in 0.2 M sodium borate buffer) was then added to each well. For each sample prepared we added a negative control well by substituting 10 μL PBS in place of Zymosan. The plate was placed in a luminometer (BMG Labtech, Ortenberg, Germany) where light emissions were recorded over 160 min. From the light emission time series we calculated average emission over 160 min, maximum emission, and time of maximum emission (Brown et al., 2015b) for statistical analysis. However, for the short-term study a power failure truncated this assay after only 95 min. It was not possible to determine the time at which the maximum level of luminescence would have occurred for these toads. Therefore, only the mean and maximum levels of luminescence during the 95-min assay period could be calculated for each sample.

3. Hemocytometry was used to quantify concentrations of red and white cells in toad blood samples. 0.05 mL of whole blood was diluted 1:200 in Natt-Herrick’s stain. 0.1 mL of the solution was placed into a hemocytometry chamber and inspected under 400× magnification. Red and white blood cells were counted to estimate cell concentrations (Wright and Whitaker, 2001).

4. We measured bactericidal capacity of soluble immune products in blood plasma (Brown et al., 2015b). After blood samples were centrifuged for 4 min, we removed the plasma portion and diluted it 1:20 in CO2 independent medium. In distilled water, we rehydrated a lyophilized pellet of Escherichia coli (ATCC 8739, Microbiologics, USA) so a 10-μL sample contained approximately 600 colony-forming units (CFUs). As an immune ‘challenge’, we added 10 μL of the E. coli suspension to 140 μL of each diluted plasma sample. We then spread 50 μL of the plasma—bacteria mixture onto a tryptone soya agar plate. We incubated the remaining plasma—bacteria mixture for 1 h at 25°C. Then spread a 50-μL aliquot onto a second agar plate. Using samples of CO2 independent medium (without plasma) after 0 min and incubating with 600 CFUs of E. coli, control plates were prepared in a similar manner. All plates were then incubated for 24 h at 37°C. The total number of E. coli colonies on each plate was counted, and the % change in colonies after 60 min of incubation with plasma was calculated as:

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\frac{(\text{60mincount} - \text{0mincount})}{(\text{0mincount})} \times 100
\]

2.8. Statistical analyses

2.8.1. Histological analysis of short-term effects of de-worming

Because of small sample sizes and non-normal distribution of data we used non-parametric Spearman’s correlations to explore relationships between histological measures and infection intensity.

2.8.2. Immune assay analysis

To assess the short-term effects of Ivermectin injection on immune and bactericidal responses we used initial infection status (infected vs. non-infected), number of days since injection, and their interaction as factors in the analyses. We used linear mixed effects models fit by restricted maximum likelihood estimation (REML) to investigate the main effects of de-worming on immune and bactericidal responses over both the short-term and the long-term.

Mean and maximum luminescence values and counts of Rhabdias in the lungs were log-transformed prior to analysis to normalize their distributions. Data from the 19 captive and 11 free-ranging wild toads were pooled into a common analysis to assess the effects of parasite infection on blood cell parameters and immune assay responses. The source of the toads (captive vs. free-ranging) was included as an independent variable in the analysis. We used actual counts of Rhabdias infection intensity as a second independent variable in the analysis, along with its interaction with toad source. Thus we used variation in infection intensity caused by anthelmintic treatment rather than ‘treatment’ per se as an independent variable in the analyses.

2.8.3. Dissection analysis

To investigate the effect of R. pseudophaeocephala infection on relative liver mass of toads, we used an ANCOVA with ‘infection group’ (de-wormed vs. not de-wormed) as a fixed factor and carcass mass as a covariate. To explore the possibility that relative liver mass was affected by the number of lungworms (rather than simply presence/absence of these parasites), we repeated these analyses with infection intensity as the independent variable. Toads without any lungworms were excluded from this latter analysis.

In all cases, we inspected residuals from analyses for violations of test assumptions. Where necessary, data were log-transformed to better meet assumptions of normality and homogeneity of variance. For all analyses where group had a significant effect in the model, we conducted Tukey HSD post-hoc tests to locate significant differences (indicated by alphabetical superscript in figures). All analyses were performed with JMP Pro 11 (SAS Institute, Cary, NC).

3. Results

3.1. Short-term effects of de-worming on immune response

Infection status at time of treatment (infected vs. uninfected) did not influence blood cell measures in the days following anthelmintic injection (Table 1). However, the maximum level of phagocytosis increased with time since Ivermectin injection (Table 1), in both infected and uninfected toads. Bacteria-killing ability was affected by an interaction between infection status
and the time since Ivermectin injection. Bactericidal ability increased with time since injection in infected toads, but decreased in non-infected toads (Table 1, Fig. 1).

### 3.2. Histological analysis of short-term effects of de-worming

#### 3.2.1. Evidence of autolysis of lungworms

Worm viability scores decreased with time since Ivermectin injection ($n = 8$, Spearman $r = -0.78$, $p = 0.02$; Fig. 2). Deterioration of worms was evident within 48 h of Ivermectin administration and increased up to the end of the experiment (7 days).

#### 3.2.2. Lung pathology and infection intensity

*Rhabdias* infection induced subtle but significant pathological signs. Areas of inflammation associated with lymphocytes and neutrophils both increased with heavier *Rhabdias* infections (non-infected toads had intensity $= 0$; $n = 15$, Spearman $r = 0.51$, $p < 0.01$; Fig. 3A) and were associated with more areas of regional septal fibrosis ($n = 15$, Spearman $r = 0.61$, $p = 0.02$; Fig. 3B). Although only two melanomacrophages were observed, they both occurred in toads with heavy parasite infections, generating a barely significant correlation with infection level ($n = 15$, Spearman

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**Table 1**

Effects of lungworm infection status at time of anthelmintic treatment (present vs. absent), and time since anthelmintic treatment on concentrations of blood cells and phagocytic and bactericidal ability of the blood in adult cane toads (*Rhinella marina*). Significant values ($p < 0.05$) are shown in boldface font.

| Trait                        | Effect                                | F1,12 | p    |
|------------------------------|---------------------------------------|-------|------|
| **Blood cell density (per ml.)** |                                       |       |      |
| Erythrocytes                 | Infection status                       | 0.93  | 0.35 |
|                              | Days post injection                    | 0.75  | 0.40 |
|                              | Infection status $\times$ days post injection | 0.04  | 0.85 |
| In-leukocytes                | Infection status                       | 0.01  | 0.92 |
|                              | Days post injection                    | 0.17  | 0.69 |
|                              | Infection status $\times$ days post injection | 0.21  | 0.65 |
| In-neutrophils               | Infection status                       | 1.74  | 0.21 |
|                              | Days post injection                    | 0.13  | 0.72 |
|                              | Infection status $\times$ days post injection | 0.10  | 0.76 |
| In-lymphocytes               | Infection status                       | 1.29  | 0.28 |
|                              | Days post injection                    | 0.05  | 0.83 |
|                              | Infection status $\times$ days post injection | 0.00  | 0.97 |
| Neutrophil:lymphocyte ratio | Infection status                       | 1.67  | 0.22 |
|                              | Days post injection                    | 0.03  | 0.87 |
|                              | Infection status $\times$ days post injection | 0.12  | 0.73 |
| **Immune assay measure**     |                                       |       |      |
| log10 mean luminescence      | Infection status                       | 1.91  | 0.19 |
|                              | Days post injection                    | 3.64  | 0.08 |
|                              | Infection status $\times$ days post injection | 0.07  | 0.80 |
| log10 max luminescence       | Infection status                       | 3.06  | 0.10 |
|                              | Days post injection                    | 5.00  | **0.04** |
|                              | Infection status $\times$ days post injection | 0.13  | 0.72 |
| Bactericidal ability         | Infection status                       | 0.00  | 0.99 |
|                              | Days post injection                    | 0.23  | 0.64 |
|                              | Infection status $\times$ days post injection | 4.90  | **0.04** |

‘Days post injection’ refers to the number of days (1, 2, 4 or 7 days, see Section 2.3) between the time when toads had been treated with anthelmintic and the time of euthanasia and blood sampling. Two toads had not been treated with anthelmintic when euthanized and were given scores of 0 days post injection.
3.3. Long-term effects of de-worming on immune response

The five free-ranging and 11 captive toads treated with anthelmintic drugs had significantly fewer *Rhabdias* than the six free-ranging and eight captive control toads (1.28 vs. 27.45; $F_{1,29} = 84.53$, $p < 0.01$). We assessed the effects of this resultant variation in infection intensity on immune measures. Concentrations of leukocytes responded to parasite infection intensity differently in captive vs. free-ranging toads (Table 2, Fig. 4). Captive toads exhibited high leukocyte concentrations that were independent of the level of *Rhabdias* infection. In contrast, leukocyte concentrations were low in non-infected free-ranging toads, but increased with *Rhabdias* infection level (Fig. 4).

Neutrophil numbers did not vary significantly with toad status or with infection level (Table 2). Lymphocyte concentration was higher in captive toads than in free-ranging toads but was not significantly affected by parasite infection level (Table 2). These contrasting patterns in neutrophil (N) and lymphocyte (L) concentration resulted in a significantly lower N:L ratio in captive toads than in free-ranging ones (Table 2).

The whole blood of free-ranging toads had higher mean and maximum phagocytosis capacity than did the blood of captive toads (Table 2, Fig. 5A and B). Similarly, the blood of toads with heavier *Rhabdias* infections had higher phagocytotic ability, and reached peak levels sooner (Table 2, Fig. 5C). Bactericidal capacity of plasma was not significantly related to parasite infection level, but free-ranging toads had marginally higher scores ($p = 0.051$) than did captive toads (Table 2).

There was no evidence that *Rhabdias* infection caused histological changes in the colon. The number of parasites infecting toads was unrelated to levels of lymphoplasmacytic ($n = 11$, Spearman $r = 0.31$, $p = 0.35$) or granulocytic ($n = 11$, Spearman $r = 0.11$, $p = 0.74$) infiltration of their colon mucosa.

3.4. Liver mass

Among the 11 free-ranging toads, de-wormed individuals had significantly larger relative liver masses than did toads that were not de-wormed ($F_{1,10} = 64.28$, $p < 0.01$; Fig. 6A). Similarly, among captive toads the group of infected individuals that were not de-wormed (IC) had significantly smaller livers than toads in the other three treatment groups ($F_{1,18} = 66.10$, $p < 0.01$; Fig. 6B).

Among infected hosts, higher rates of infection were associated with a reduction in liver mass both in the free-ranging toads ($F_{1,5} = 9.96$, $p < 0.01$; Fig. 6C) and in captive toads ($F_{1,12} = 11.21$, $p < 0.01$; Fig. 6D).

4. Discussion

Previous studies on the effects of *Rhabdias* on cane toads have not reported any effects of the parasite on the host’s immune configuration or response (Graham et al., 2012; Brown et al., 2016). However, those studies relied on correlational data from naturally infected individuals, or experimental infection protocols. Our study, utilizing experimental de-worming, detected significant effects of parasite infection level on host immune responses. These contrasting results suggest (i) that a host’s immune configuration correlates with the level of infection, and may indicate either an influence on or a response to infection, and (ii) that the toads’ physiology is perturbed by the chronic presence of adult *Rhabdias* in the lungs, not simply by larval migration through body tissues soon after the larva enters the host’s body (as has been inferred by earlier studies: Kelehear et al., 2009; Pizzatto et al., 2010).

Over both the short-term and the long-term, anthelmintic treatment generated variation in lungworm infection intensity in both captive and free-ranging hosts, and induced multiple physiological and pathological changes. Some of these effects can be attributed to the removal of parasites, whereas others may relate to
Rhabdias pseudosphaerocephala abundance on leucocyte concentrations in cane toads. Captive toads (n = 19) exhibited high leucocyte concentrations that were independent of the level of Rhabdias infection. In contrast, leucocyte concentrations in free-ranging toads (n = 11) increased with time since Rhabdias infection level.

Pathological effects of infection on the cane toad host appeared relatively minor. Histopathological differences in lung tissue were detected between treatment groups, but the cellular changes were subtle. Similarly mild pathological consequences of infection were recorded in a South American population of cane toads parasitized by Rhabdias parensis, with infection also resulting in detectable but minor tissue damage and inflammation (Santos et al., 2016). In keeping with the inference that pathological consequences of infection by Rhabdias in bufonid hosts often may be minor, R. pseudosphaerocephala infection caused no inflammation of the colonic epithelium tissue of toads. Thus, a tendency for lungworm infection to induce increased fecal water content and defecation rate in their toad hosts (Finnerty, 2017) may represent a compensatory response to the increase in phagocytic ability following anthelmintic injection. In the absence of a counter-acting stimulus (like dead worms), a trade-off between phagocytic and bactericidal capacities may occur (Brown and Shine, 2014; Brown et al., 2015a).

In the short-term (<1 week after anthelmintic injection), de-worming drugs had only minor immunological consequences on the host; the only significant immunological change caused by the de-worming of infected hosts was a shift in the bacterial killing ability (BKA) of host whole blood over time. Given the gradual decomposition of lungworm bodies inside the lungs of infected toads following de-worming, an increase in BKA may protect against bacteria-induced pneumonia. In addition, the decomposition of dead lungworms may release antigenic compounds into the blood stream and act as a trigger to up-regulate immune responses. The decrease in bactericidal ability seen in uninfected toads may be a compensatory response to the increase in phagocytic ability following anthelmintic injection. In the absence of a counter-acting stimulus (like dead worms), a trade-off between phagocytic and bactericidal capacities may occur (Brown and Shine, 2014; Brown et al., 2015a).

Table 2
Effects of lungworm infection intensity (number of lungworms at dissection) and source (captive vs. free-ranging) on concentrations of blood cells and phagocytic and bactericidal ability of blood in adult cane toads (Rhinella marina). Significant values (p < 0.05) are shown in bold.

| Trait                        | Effect                        | F<sub>1,26</sub> | p   |
|------------------------------|-------------------------------|-----------------|-----|
| Blood cell concentration per mL | Free-ranging/captive            | 0.05            | 0.83|
|                              | ln-lungworm count             | 0.19            | 0.67|
|                              | Free-ranging/captive × ln-lungworm count | 0.44 | 0.51|
|                              | ln-leukocytes                 | 19.3            | -0.01|
|                              | ln-lungworm count             | 5.37            | 0.03|
|                              | Free-ranging/captive × ln-lungworm count | 6.14 | 0.02|
|                              | ln-neutrophils                | 3.09            | 0.09|
|                              | ln-lungworm count             | 3.32            | 0.08|
|                              | Free-ranging/captive × ln-lungworm count | 0.87 | 0.36|
|                              | ln-lymphocytes                | 49.64           | -0.01|
|                              | ln-lungworm count             | 2.15            | 0.15|
|                              | Free-ranging/captive × ln-lungworm count | 3.54 | 0.07|
| Neutrophil:lymphocyte ratio  | Free-ranging/captive           | 14.9            | -0.01|
|                              | ln-lungworm count             | 2.27            | 0.14|
|                              | Free-ranging/captive × ln-lungworm count | 1.75 | 0.2 |

Immune assay measure

| Trait                       | Effect                        | F<sub>1,26</sub> | p   |
|-----------------------------|-------------------------------|-----------------|-----|
| Mean luminescence           | Free-ranging/captive           | 21.22           | <0.01|
|                              | ln-lungworm count             | 6.73            | 0.02|
|                              | Free-ranging/captive × ln-lungworm count | 0.66 | 0.42|
| Maximum luminescence        | Free-ranging/captive           | 19.29           | <0.01|
|                              | ln-lungworm count             | 6.71            | 0.02|
|                              | Free-ranging/captive × ln-lungworm count | 0.68 | 0.42|
| Time at maximum luminescence | Free-ranging/captive           | 1.68            | 0.21|
|                              | ln-lungworm count             | 6.13            | 0.02|
|                              | Free-ranging/captive × ln-lungworm count | 3.55 | 0.07|

Fig. 4. The effects of lungworm (Rhabdias pseudosphaerocephala) abundance on leucocyte concentrations in cane toads. Captive toads (n = 19) exhibited high leucocyte concentrations that were independent of the level of Rhabdias infection. In contrast, leucocyte concentrations in free-ranging toads (n = 11) increased with Rhabdias infection level.
In contrast to these impacts over the short-term, longer-term (>2-month) monitoring of free-ranging hosts post injection revealed significant shifts in immunological components that differed between hosts of varying infection intensity. Leukocyte (white blood cell) concentrations increased with parasite burden and the concentration of phagocytic neutrophils, although

Fig. 5. The effects of lungworm (*Rhabdias pseudospherocephala*) abundance on the phagocytic ability of the blood of 19 captive and 11 free-ranging cane toads. (A) mean luminescence over 160 min, (B) maximum luminescence over 160 min, and (C) time of maximum luminescence. RLU = relative luminescence units.

Fig. 6. (A) The effects of anthelmintic treatment on relative liver mass of 5 de-wormed (open bars) vs. 6 not de-wormed (grey bars) free-ranging toads. (B) Effect of experimental treatment on relative liver mass of 49 captive cane toads. ID = infected, de-wormed (n = 11), IC = infected, control (n = 13), ND = non-infected, de-wormed
Brown, G.P., Kelehear, C., Shilton, C.M., Phillips, B.L., Shine, R., 2015a. Stress and immunity at the invasion front: a comparison across cane toad (Rhinella marina) populations. Biol. J. Linn. Soc. 116, 748–760.

Brown, G.P., Phillips, B.L., Dubey, S., Shine, R., 2015b. Invader invasion: invasion history alters immune system function in cane toads (Rhinella marina) in tropical Australia. Ecol. Lett. 18, 57–65.

Brown, G.P., Shine, R., 2014. Pathogenic and non-pathogenic responses vary with rate of dispersal in invasive cane toads (Rhinella marina). Plos One 9, e99734.

Draycott, R.A.H., Woodburn, M.L.A., Ling, D.E., Sage, R.B., 2006. The effect of an indirect anthelmintic treatment on parasites and breeding success of free-living pheasants (Phasianus colchicus). J. Helminthol. 80, 413–415.

Dubey, S., Shine, R., 2008. Origin of the parasites of an invading species, the Australian cane toad (Bufo marinus): are the lungworms Australian or American? Mol. Ecol. 17, 4418–4424.

Ezemena, V.O., Jolles, A.E., 2015. Opposite effects of anthelmintic treatment on microbial infection at individual versus population scales. Science 347, 175–177.

Fenner, A.L., Bull, C.M., 2008. The impact of nematode parasites on the behaviour of an Australian lizard, the gidgee skink Egermia stokesi. Ecol. Res. 23, 897–903.

Finnerty, P.B., 2017. The Pathological, Physiological, Ecological and Behavioural Effects of Lungworm Infection on Free-ranging Cane Toads. Honours dissertation, School of Life and Environmental Sciences, University of Sydney, Sydney, Australia.

Finnerty, P.B., Shine, R., Brown, G.P., 2017. The costs of parasite infection: removing lungworms improves performance, growth and survival of cane toads. Funct. Ecol. in press.

Gomez, A., Nichols, E., 2013. Neglected wild life: parasitic biodiversity as a conservation target. IJPPA-W 2, 222–227.

Grant, S.P., Kelehear, C., Brown, G.P., Shine, R., 2012. Corticosterone-immune interactions during captive stress in invading Australian cane toads (Rhinella marina). Brain, Behav. Ecol. 23, 43–51.

Heise-Pavlov, S.R., Paleologo, K., Glenny, W., 2014. Effect of Rhabdias pseudohaemorchestops on prey consumption of free-ranging cane toad (Rhinella marina) during Australian tropical wet seasons. J. Pest Sci. 87, 89–97.

Isehunwa, G.O., Yusuf, I.O., Alada, A.R.A., 2016. Effects of petrol exposure on glucose, liver and muscle glycogen levels in the common African toad Bufo regulus. Niger. J. Physiol. Sci. 31, 139–145.

Jenkins, E.J., Simon, A., Bachand, N., Stephen, C., 2015. Wildlife parasites in a one health world. Trends Parasitol. 31, 174–180.

Jolly, C.J., Shine, R., Greenlees, M.J., 2015. The impact of invasive cane toads on native wildlife in southern Australia. Ecol. Evol. 5, 3879–3894.

Kelehear, C., Webb, J.K., Shine, R., 2009. Rhabdias pseudohaemorchestops infection in Bufo marinus: lung nematodes reduce viability of metamorphic cane toads. Parasitology 136, 919–927.

Kelehear, C., Brown, G.P., Shine, R., 2011. Influence of lung parasites on the growth rates of free-ranging and captive adult cane toads. Oecologia 165, 585–592.

Kelehear, C., Brown, G.P., Shine, R., 2012. Rapid evolution of parasite life history traits on an expanding range-edge. Ecol. Lett. 15, 329–337.

Kuck, C.J., Martin, G.L., Sortor, B.V., 2004. Common intestinal parasites. Am. Fam. Physician 69, 1161–1168.

Morton, M.L., 1981. Seasonal changes in total body lipid and liver weight in the Yosemite toad. Copeia 1981, 234–238.

Nelson, F.B.L., Brown, G.P., Shilton, C., Shine, R., 2015. Host–parasite interactions during a biological invasion: the fate of lungworms (Rhabdias spp.) inside native and novel anuran hosts. IJPPA-W 4, 206–215.

Pedersen, A.B., Antonovics, J., 2013. Anthelmintic treatment alters the parasite community in a wild mouse host. Biol. Lett. 9, 20130205.

Phillips, B.L., Kelehear, C., Pizzotto, L., Brown, G.P., Barton, D., Shine, R., 2010. Parasites and pathogens in the rear of their host during periods of host range advance. Ecology 91, 872–881.

Pizzotto, L., Kelehear, C., Shine, R., 2013. Seasonal dynamics of the lungworm, Rhabdias pseudohaemorchestops, in recently colonised cane toad (Rhinella marina) populations in tropical Australia. Int. J. Parasitol. 43, 753–761.

Pizzotto, L., Shilton, C.M., Shine, R., 2010. Infection dynamics of the lungworm Rhabdias pseudohaemorchestops in its natural host, the cane toad (Bufo marinus), and in novel hosts (native Australian frogs). J. Wildl. Dis. 46, 1152–1164.

Polley, L., Thompson, A., 2015. Parasites and wildlife in a changing world. Trends Parasitol. 31, 123–124.

Santos, J.N., da Silva, D.C., Feitosa, L.A., Furtado, A.P., Giese, E.G., de Vasconcelos Melo, F.T., 2016. Rhabdias pseudosphaerocephala versus Rhabdias pseudosphaerocephala (Nematoda: Rhabdiasidae); expanding the view on a natural infection. J. Parasitol. 102, 349–355.

Scapin, S., Di Giuseppe, G., 1994. Seasonal variations of glycogen synthase and phosphorylase activities in the liver of the frog Rana esculenta. Comp. Biochem. Physiol. B 107, 189–195.

Schmid-Hempel, P., 2011. Evolutionary Parasitology. The Integrated Study of Infections, Immunology, Ecology, and Genetics. Oxford University Press, Oxford, UK.

Silverstein, R., Shine, R., Oldroyd, B., Greenlees, M., 2017. The ecological impact of commercial beehives on invasive cane toads (Rhinella marina) in eastern Australia. Biol. Invasions 19, 1097–1106.

Shine, R., Brown, G.P., 2008. Adapting to the unpredictable: reproductive biology of vertebrates in the Australian wet–dry tropics. Phil. Trans. R. Soc. B 363, 363–373.

Smith, C., 1950. Seasonal changes in blood sugar, fat body, liver glycogen, and goslings in the common frog, Rana temporaria. J. Exp. Biol. 26, 412–425.
Smith, J.G., Phillips, B.L., 2006. Toxic tucker: the potential impact of cane toads on Australian reptiles. Pac. Conserv. Biol. 12, 40–49.

Stien, A., Irvine, R.J., Ropstad, E., Halvorsen, O., Langvad, R., Albon, S.D., 2002. The impact of gastrointestinal nematodes on wild reindeer: experimental and cross-sectional studies. J. Anim. Ecol. 71, 937–945.

Thompson, R., Lymbery, A., Smith, A., 2010. Parasites, emerging disease and wildlife conservation. Int. J. Parasitol. 40, 1163–1170.

Turner, W.C., Cizauskas, C., Getz, W., 2010. Variation in faecal water content may confound estimates of gastro-intestinal parasite intensity in wild African herbivores. J. Helminthol. 84, 99–105.

Worsley-Tonks, K.E.L., Ezenwa, V.O., 2015. Antihelmintic treatment affects behavioural time allocation in a free-ranging ungulate. Anim. Behav. 108, 47–54.

Wright, K.M., Whitaker, B.R., 2001. Amphibian Medicine and Captive Husbandry. Krieger Publishing Company, Malabar, FL.