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

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ABSTRACT

The immunological and pathological consequences of parasite infection can be more rigorously assessed from experimental manipulation than from correlational studies of natural infections. We used anthelmintic treatment to experimentally decrease intensities of lungworm infection in captive and free-ranging wild cane toads to assess parasite impacts on host immune responses. First, we administered the anthelmintic drug Ivermectin to both infected and uninfected toads, to distinguish drug effects *per se* from the impacts of killing lungworms. Worms began dying and decomposing <48 h after injection. The only immunological variables that were affected by anthelmintic treatment were bactericidal capacity of the blood which increased in parasitized toads (presumably triggered by decomposing worms in the lungs), and the phagocytic capacity of blood (which increased in both infected and uninfected toads); the latter effect presumably was caused by the injection of Ivermectin *per se* rather than removal of parasites. Second, we looked at correlates of variation in the infection intensity induced by de-worming (in both captive and free-ranging toads) over an eight-week period. Heavier lungworm infection was associated with increased phagocytic ability of the host's blood, and a reduction in the host's liver mass (and hence, energy stores). Experimental de-worming thus revealed pathological and immunological costs of the presence of lungworms, and of their removal by anthelmintic injection.

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),

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and altered the parasitic community in free-ranging whited footed 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 through 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