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Host–parasite interactions during a biological invasion: The fate of lungworms (*Rhabdias* spp.) inside native and novel anuran hosts

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ABSTRACT

The cane toad invasion in Australia provides a robust opportunity to clarify the infection process in co-evolved versus de novo host–parasite interactions. We investigated these infection dynamics through histological examination following experimental infections of metamorphs of native frogs (*Cyclorana australis*) and cane toads (*Rhinella marina*) with *Rhabdias hylae* (the lungworm found in native frogs) and *Rhabdias pseudosphaerocephala* (the lungworm found in cane toads). Cane toads reared under continuous exposure to infective larvae of the frog lungworm were examined after periods of 2, 6, 10 and 15 days. Additionally, both toads and frogs were exposed for 24 h to larvae of either the toad or the frog lungworm, and examined 2, 5, 10 and 20 days post-treatment. *R. hylae* (frog) lungworms entered cane toads and migrated through the body but were not found in the target tissue, the lungs. Larvae of both lungworm species induced inflammation in both types of hosts, although the immune response (relative numbers of different cell types) differed between hosts and between parasite species. Co-evolution has modified the immune response elicited by infection and (perhaps for that reason) has enhanced the parasite's ability to survive and to reach the host's lungs.

1. Introduction

Parasites differ in virulence, with effects strongly dependent on host phenotype and species (Poulin, 2007). Many parasites are highly host-specific, unable to infect even closely related species (Roberts and Janovy, 2009). Evolutionary theory attributes that host-specificity to co-evolutionary 'arms races' through time – whereby parasites constantly evolve to keep pace with the evolution of host defences, and hosts evolve to keep pace with the evolution of new strategies by the parasite (Anderson and May, 1982; May and Anderson, 1983; Ebert and Hamilton, 1996). Often, a stable equilibrium may arise whereby the relatively minor effects of a parasite on its host mean it is not worth the host allocating more energy to detect or fight the parasite; and the parasite benefits by their host remaining viable, and living long enough to facilitate parasite reproduction (Ebert and Hamilton, 1996; Combes, 1997).

Although this co-evolutionary hypothesis is intuitively reasonable, it is difficult to test empirically if all we can study is the finished product – a host and parasite that are mutually co-adapted. To look directly at the traits that change after a host and parasite first begin to interact with each other, we need to see those initial stages of the interaction before effects are blunted by co-evolution. Biological invasions give us that opportunity. If an invader brings a new parasite with it, then native taxa may encounter that parasite taxon for the first time (Prenter et al., 2004). Similarly, the invader may encounter novel parasites from native taxa within the invaded range (Kelly et al., 2009). In such a system, we may be able to observe the initial stages of two sets of host–parasite interactions, in both the invasive host and the native host (Trejo, 1992).

Our understanding of host–parasite interactions is often based on a "black box" approach, whereby data are based on what goes into a host and what comes out of it, but not on what happens inside the host's body. Although valuable, such data provide only a limited view of host–parasite interactions. Histology provides a more direct way to look at the environments encountered by larval parasites, and their migration through the host's body. That perspective enables us to answer questions such as whether the failure to set up mature infections in a novel host is due to the parasite's inability to migrate to appropriate places, and/or the ability of the host's immune system to destroy the parasite.

The invasion of cane toads (*Rhinella marina*) through Australia has created an opportunity to investigate co-evolutionary processes in this way. Cane toads were introduced in Queensland in

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1935 from Hawaii to control an agricultural pest, the cane beetle, *Saccharum officinarum* (Turvey, 2013). The toads carried with them a native lungworm, *Rhabdias pseudosphaerocephala* (Nematoda), and encountered a congeneric parasite, *Rhabdias hylae*, which is widespread in native frogs (Dubey and Shine, 2008). Previous research has investigated the possibility of crossover of the cane toad lungworm to native frogs (Pizzatto et al., 2010; Pizzatto and Shine, 2011a, 2011b) but has ignored the opposite scenario, the potential crossover of the native frog lungworm to cane toads.

Although cane toads can be penetrated by larvae of the frog lungworm species (Nelson et al., 2015), field surveys have not found any crossover in lungworm fauna between native frogs and cane toads (Pizzatto et al., 2012). *R. pseudosphaerocephala* can reduce survival, food intake, speed and endurance in metamorphs of its natural host, the cane toad (Kelehear et al., 2009). Laboratory experiments indicate that Australian frogs are penetrated by the cane toad lungworm, but that these lungworms generally do not survive (Pizzatto et al., 2010; Pizzatto and Shine, 2011a, 2011b). In most Australian frog species studied to date, morbidity and mortality of the host are unaffected by exposure to either the toad lungworm (Pizzatto et al., 2010; Pizzatto and Shine, 2011a, 2011b) or the native frog lungworm (Nelson et al., 2015). In laboratory trials, the toad lungworm was less competent at locating the target tissue (the lungs) in non-co-evolved hosts (native frogs) than in the co-evolved host (the cane toad), and was more effectively destroyed by the non-co-evolved host's immune system (Pizzatto et al., 2010).

To assess whether the interaction between cane toads versus frog lungworms was similar to that documented by Pizzatto et al. (2010) for frogs versus toad lungworms, we examined the infection dynamics of the frog lungworm both in a co-evolved host (the native frog) and in a novel host (the cane toad). We also conducted further studies on the toad lungworm for comparison. By exploring host–parasite biology at the time of first encounter as well as in long-term co-evolved systems, we can wind back the clock and observe how hosts and parasites interact with each other before selection pressures on both participants have modified those relationships.

2. Materials and methods

2.1. Host–parasite system

Nematode lungworms of the genus *Rhabdias* have a direct life cycle; infective larvae (L3) in the soil penetrate the host's skin and migrate through the body to the lungs where they develop into protandrous hermaphroditic adults. These adult worms attach to the internal lining of the lung and feed on blood from the capillary network. Eggs laid into the lung lumen are carried up through the trachea into the mouth, then swallowed into the digestive system where they hatch. The larval nematodes develop as they pass down the gastrointestinal tract and soon after being released into the environment in faeces, they become free-living adult males and females. These free-living adults mate within 4–7 days and give rise to infective larvae that subsequently break out through their mother's cuticle and move about actively to infect a new host (Baker, 1979).

2.2. General methods

In November 2013, we captured adult anurans (5 *Cyclorana australis*, 8 *Limnodynastes convexiusculus*, 20 *Litoria nasuta* and 4 *Rhinella marina*) within 10 km of the Tropical Ecology Research Facility (TERF) on the Adelaide River floodplain in the wet-dry tropics of Australia (12°34'42.1"S, 131°18'50.5"E). These animals were injected with the gonadotropin-releasing hormone (GnRH) agonist leuprorelin acetate diluted at 1:20 with amphibian Ringer's solution to induce spawning. After the resultant eggs hatched, we transferred 100 tadpoles of each species to large plastic containers (107 cm in diameter, 42 cm tall) and added algae as food. Emerging metamorphs were then maintained individually in tilted plastic containers ($7.5 \times 17 \times 12$ cm) half-filled with untreated bore water.

To obtain *R. hylae* larvae, we collected 148 adult frogs of 11 species near TERF in October 2013 and housed them as above. The frogs' faeces were examined in water under a dissecting microscope to look for lungworm larvae (*Rhabdias* spp.). Only one frog species (*Lit. nasuta*) was found to be infected; and of 96 *Lit. nasuta*, only 45 carried mature *Rhabdias* spp. in their lungs (as indicated by free-living nematodes in faeces). We collected faeces from infected frogs every second day, and placed them in a Petri dish with untreated bore water. After 2–4 days, the free-living adult worms had produced infective third stage larva (L3) that we used for experimental infections. We obtained *R. pseudosphaerocephala* larvae from locally caught *R. marina* in the same way.

2.3. Experiment 1: Infecting cane toads with Rhabdias hylae (sustained exposure to infective larvae)

This experimental treatment was designed to mimic potential exposure levels experienced by anurans sharing diurnal retreat sites. Suitable moist daytime refugia may be rare during the dry season in our tropical study area (Bleach et al., 2014). Furthermore, local native anuran species do not avoid sharing such shelters with toads (Bleach et al., 2014). Sharing a small diurnal shelter with even one infected native anuran is likely to expose toads to an environment containing a large number of infective larvae. If this shelter is used daily on an ongoing basis, exposure to high densities of larvae over a prolonged period will result. Thus, in November 2013, we placed 21 captive-bred cane toad metamorphs in a $17 \times 22 \times 7$ cm plastic box lined with a 1 cm layer of sand (disinfected through boiling), untreated bore water and 800 *R. hylae* larvae (counted and extracted from Petri dish cultures using a glass pipette). *R. hylae* larvae were extracted from the faeces of *Lit. nasuta* collected from the vicinity of Middle Point, Northern Territory TERF (DNA analysis confirmed the species of nematode used in the experiment: Nelson et al., 2015). We fed metamorphs with termites ad libitum over 15 days (excluding a 24-hour fasting period before euthanasia). The experiment was long enough for the larvae to reach the lungs of the cane toads. As larvae survive up to 7 days in these conditions, the cane toads may have been exposed to larvae for this period (Pizzatto et al., 2010). Five toads were chosen at random and euthanised at 2, 6, 10 and 15 days post-treatment (DPT). Toads were euthanised by overdose in an anaesthetic bath of tricaine methanesulfonate (MS-222; 5 mg/ml, buffered with sodium bicarbonate). All cane toads were then fixed in 10% neutral buffered formalin for histological examination.

2.4. Experiment 2: Infecting cane toads and native frogs with Rhabdias hylae and R. pseudosphaerocephala (short-term exposure to infective larvae)

To infect toads and frogs with *R. hylae* and *R. pseudosphaerocephala*, we placed treatment metamorphs in a 3.5 cm diameter Petri dish with 2 ml water and 30 L3 *Rhabdias* spp. larvae (collected from adult frogs or toads between 4 and 18 days previously). Control metamorphs were placed in Petri dishes with 2 ml of water. All Petri dishes were kept on a tray tilted to ensure that frogs could sit halfway out of the water. Twenty cane toads and 20 *C. australis* metamorphs (captive-produced: see above) were placed in Petri dishes containing 30 *R. hylae* larvae in 2 ml of water. A further 20 cane toad and 20 *C. australis* metamorphs were placed in Petri dishes containing 30 *R. pseudosphaerocephala* larvae in 2 ml of water. Eight additional cane toad metamorphs were placed in Petri dishes containing only 2 ml of water for 24 h to act as controls (we had too few *C. australis* metamorphs available to form a control group for that species). All anurans were kept under these conditions for

24 h, then removed from the Petri dishes and thereafter housed individually in tilted plastic containers (7.5 × 17 × 12 cm) half-filled with untreated bore water. The young anurans were fed termites every two days. To eliminate cross-contamination between *Rhabdias* species, collections of each lungworm species were kept in separate rooms on trays lined with disinfectant-soaked paper towel. Individuals of each species and treatment were randomly selected for euthanasia each at 2, 5, 10 and 20 DPT. Anurans were euthanised by overdose in an anaesthetic bath of tricaine methanesulfonate (MS-222; 5 mg/ml, buffered with sodium bicarbonate). All anurans were then fixed in 10% neutral buffered formalin for histological examination.

2.5. Methods for producing histological slides of infected anurans

For histological examination, 5–6 approx. 1–2 mm wide serial transverse sections were made encompassing the tissue from the head to the pelvis of each anuran. These sections were placed into a single cassette for each anuran, processed in standard histological fashion, and 5 μm sections stained with haematoxylin and eosin (Fig. 1; see Pizzatto et al., 2010 for detailed methods) and examined by a single observer (CS) blind to the treatment, species and DPT. Adult worms in the lungs were determined by their large size and presence of eggs, and each lungworm that was visible on a slide was counted individually (because it was impossible to determine the exact number of lungworms per slide due to their size and coiling). This method of counting may have overestimated the numbers of lungworms in the lung, but provided a standardised way to compare lungworm loads among individual anurans. Larvae migrating through the anuran's body were also counted. Multiple sections of larvae in close proximity, surrounded by the same inflammatory foci, were assumed to be the same nematode and were counted only once. Larvae encapsulated in an egg (either in the lung or intestine) were not counted, because they represent a subsequent generation, produced by adults in the lungs and en route to the external environment. A few larvae/adults undoubtedly were missed because they did not happen to fall in the histological section examined. However, the effort made to detect larvae/lungworms was the same for each individual anuran; equal numbers of transverse slides were made through the anuran body at approximately the same locations, and a recut was made if there were no lung sections in the slides. In most of the anurans, it was possible to see both lungs in at least one of the transverse sections. The location of each larva within the anuran was assigned to one of five categories based on the part of the body and type of tissue it was in. The categories were: (1) skin or muscle (skeletal muscle, fascia or subcutaneous tissue of the thorax, abdomen, pelvis or proximal limbs, but excluding the head); (2) head (any tissue in the head, except the eye); (3) eye (the eye or periocular tissue); (4) coelom (larvae free in the coelomic lining, mesenteries, or serosae of viscera other than the lung); and (5) lung (within the lung lumen).

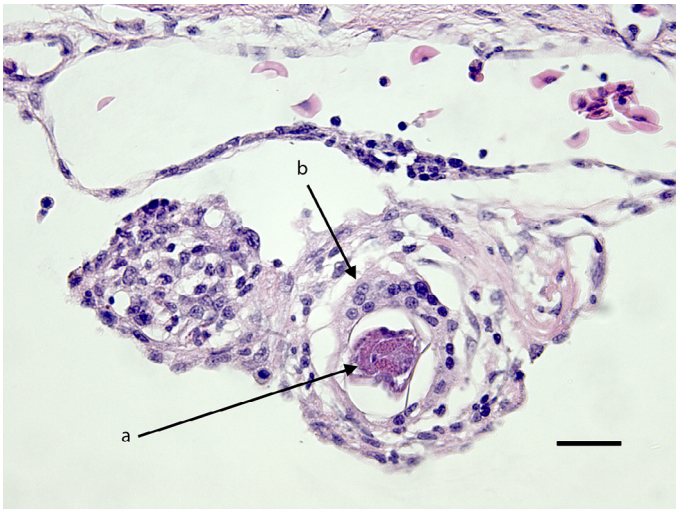

Fig. 1. Histological image depicting a transverse section of (a) *R. hylae* larva in the connective tissue of the head of a cane toad and (b) the inflammatory response composed primarily of macrophages and multinucleated giant cells surrounding the parasite. Haematoxylin and eosin stain, 400× magnification, scale bar equals 30 μm.

The condition of each larva was scored as 'intact' if it retained structural integrity, or 'degenerate' if it was disintegrating and invaded by immune cells. The extent of inflammation surrounding invading larvae was categorised based on how many layers of reactive cells surrounded the larvae: (1) none (no inflammation observed); (2) mild (1–2 layers of inflammatory cells); (3) moderate (3–4 layers of inflammatory cells); or (4) severe (more than 4 layers of inflammatory cells).

Areas of inflammation that did not contain visible traces of larva were recorded as 'foci' of inflammation and the extent of inflammation scored as above, with 'severity' of inflammation being a measure of the size of the inflammatory focus. Where inflammation was present (either in association with larvae or foci), the constituency of recruited immune cells was estimated as the proportion of: (1) neutrophils (cells with segmented nuclei and light-coloured eosinophilic non-granulated cytoplasm); (2) eosinophils (the same size as neutrophils with a round or slightly segmented nucleus and orange-stained granules); (3) lymphocytes (small cells with little cytoplasm and chromatin clumped in dark nuclei); (4) macrophages (larger than granulocytes containing light-coloured eosinophilic cytoplasm and a round to oval or bean-shaped nucleus); or (5) multinucleated giant cells (macrophages merged together to form a large cell) (Pizzatto et al., 2010).

Because samples sizes were small and data distributions uncertain, we used nonparametric tests to assess relationships between variables. We used Spearman correlations to compare pairs of continuous variables (e.g. number of larvae vs time) and χ^2 approximations of Wilcoxon tests to compare continuous variables between categories (e.g. number of inflammatory cells vs exposure to *Rhabdias* spp.). We used nominal logistic regression to assess whether three categories of infection level (larvae present/foci only/not infected) were affected by anuran species (cane toad vs *C. australis*), lungworm species (*R. pseudosphaerocephala* vs *R. hylae*), or the interaction between anuran and lungworm species. Data for this logistic analysis were pooled across all time periods for both species. All analyses were carried out using JMP 9.0 (SAS Institute, Cary, NC).

3. Results

3.1. Experiment 1: Infecting cane toads with Rhabdias hylae (sustained exposure to infective larvae)

3.1.1. Intensity of infection

R. hylae larvae located and penetrated 18 out of 21 cane toad metamorphs. The number of larvae per toad ranged from 0 to 11. As is common in parasite infections, the distribution of intensity followed a Poisson rather than normal distribution (3 toads had 0 larva, 9 had 1 larvae, 6 had 2 larvae, 2 had 3 larvae and 1 individual had 11 larvae).

3.1.2. Location of larvae within the host's body

The location of larvae within the toads changed with time since exposure (logistic regression: $\chi^2 = 10.43$, $P = 0.02$; Fig. 2). Two days after exposure began, most infecting larvae were located subcutaneously. Over time, larvae were found deeper in the body and, by

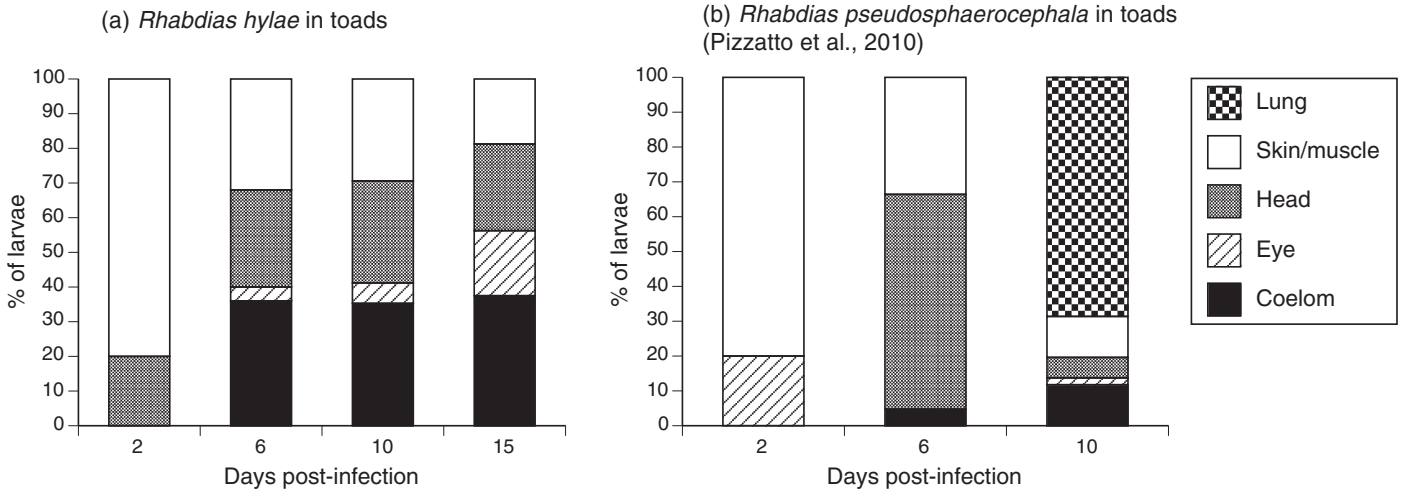

Fig. 2. The distribution of lungworm larvae in cane toad metamorphs. (a) Toad metamorphs infected with *Rhabdias hylae* (native frog lungworm) and (b) toad metamorphs infected with *Rhabdias pseudosphaerocephala* (cane toad lungworm). Data in panel (b) are from Pizzatto et al. (2010), with permission. LUNG refers to adult lungworms found within the lung, SKIN/MUSCLE refers to larvae found in the skeletal muscle or subcutaneous tissue, HEAD refers to larvae detected in the head or neck region (excluding those found in eye tissue), EYE indicates larvae found in the eye or periocular tissue, and COELOM denotes larvae within the coelom or coelomic membranes.

Day 15, all larvae were found within the coelom. Importantly, no *R. hylae* were found to have reached the lungs of any of the 21 cane toads within 15 days.

3.1.3. Severity and type of inflammation response

Larvae were less intact or visible with increasing DPT; that is, records of larval presence shifted from intact larva through to degenerating ones, through to inflammatory foci with no clear evidence of larval presence ($\chi^2 = 21.66$, $n = 64$, $P < 0.001$; Fig. 3). Although the number of visible larvae found in toads decreased through time since exposure, high variation meant that the change was not statistically significant (Spearman's $r = -0.15$, $P = 0.53$; Fig. 4a). However, the number of foci (areas of inflammation with no visible larvae) increased over time (Spearman's $r = 0.74$, $P < 0.0001$; Fig. 4b). The severity of inflammation around larvae also increased over the 15-day period (Spearman's $r = 0.60$, $n = 38$, $P < 0.0001$; Fig. 5), whereas severity (size) of inflammatory foci did not (Spearman's $r = 0.22$, $n = 25$, $P = 0.30$; Fig. 5).

Changes in the average severity of the inflammatory foci or response around the larvae were associated with changes in the relative proportions of neutrophils and lymphocytes. As the inflammation severity score increased, the proportion of neutrophils decreased (Spearman's $r = -0.46$, $n = 20$, $P = 0.043$) and the proportion of lymphocytes increased (Spearman's $r = 0.49$, $n = 20$, $P = 0.032$). The proportions of macrophages and eosinophils remained relatively constant (48% and 18% respectively) through all levels of inflammation. Temporal changes in neutrophils and lymphocytes reflected a trend for inflammation severity to increase through time, especially around visible larvae (see above). Hence, the proportion of neutrophils around inflammation sites decreased over time (Spearman's $r = -0.59$, $n = 20$, $P = 0.007$; Fig. 6a) whereas the proportion of lymphocytes increased (non-significantly) (Spearman's $r = 0.39$, $n = 20$, $P = 0.092$; Fig. 6b). Multinucleated giant cells were observed in three toads (two at 10 DPT and one at 15 DPT).

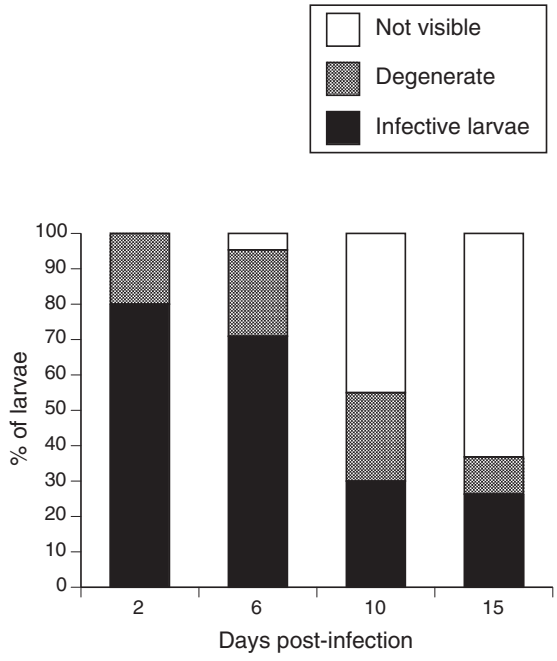

Fig. 3. The state of *Rhabdias hylae* larvae in cane toads as a function of days-post treatment. The graph shows larval numbers as the percentage of total larvae that were seen at each time period.

3.2. Experiment 2: Infecting cane toads and native frogs with Rhabdias hylae *and* R. pseudosphaerocephala *(short-term exposure to infective larvae)*

3.2.1. Intensity of infection

Of the 64 anurans exposed to *Rhabdias* larvae for 24-h and examined histologically, 15 (24%) were infected with larvae and/or adults, eight (13%) contained only inflammatory foci (i.e. areas of inflammation similar to those associated with larva (see above) but where no actual larval traces were seen in the section), and 41 (64%) showed no signs of infection.

Nominal logistic regression on the factors affecting level of infection indicated a significant interaction between anuran species and *Rhabdias* spp. ($\chi^2 = 11.97$, $n = 71$, $P = 0.002$; Fig. 7). Toads were less likely than *C. australis* to show evidence of infection by either *Rhabdias* type. Although *C. australis* was commonly infected by both types of *Rhabdias*, the incidence was higher after exposure to *R. hylae* (75%) than *R. pseudosphaerocephala* (50%; Fig. 7).

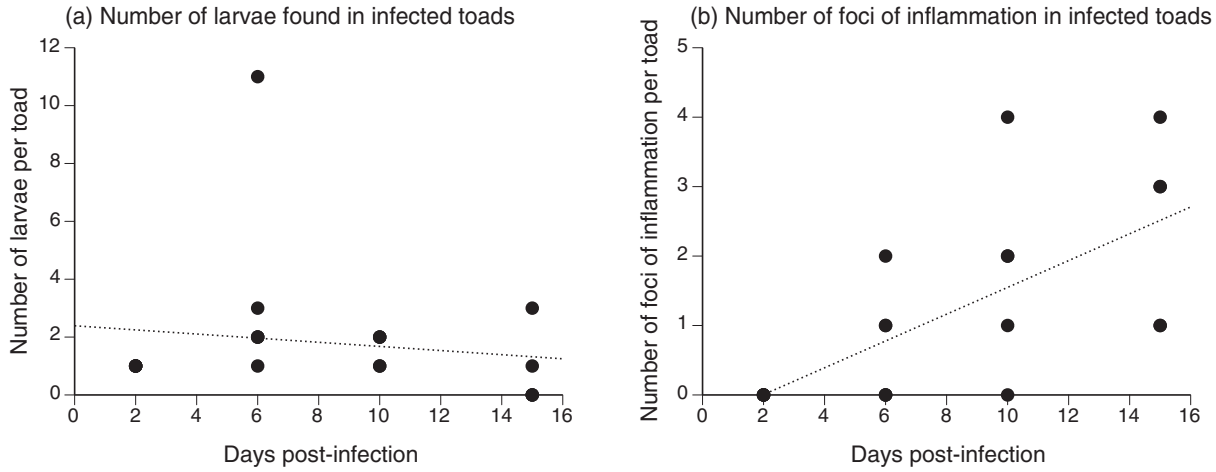

Fig. 4. Effect of time since exposure to *Rhabdias hylae* larvae on cane toad metamorphs: (a) shows the number of larvae found in toads and (b) shows the number of foci (areas of inflammation with no visible larvae) in toads, as determined by histological methods.

3.2.2. Location of larvae within the host's body

Six larvae were detected histologically from the 64 exposed anurans. These six larvae were contained in four *C. australis*, three of which had been exposed to *R. hylae* and one to *R. pseudosphaerocephala*. Three *R. hylae* larvae were located in the head, one *R. hylae* larva was located in the skin/muscle and one *R. hylae* larva and one *R. pseudosphaerocephala* larva were located in the coelom. None of these six larvae were surrounded by discernable inflammation. Six adult *R. hylae* and four adult *R. pseudosphaerocephala* were detected in the lungs of *C. australis*.

Reflecting their greater vulnerability to penetration by lungworm larvae, nine of 15 *C. australis* exposed to *R. hylae* were found to contain larvae or lungworms. After Day 10, five of eight frogs infected with *R. hylae* had lungworms in their lungs. *R. pseudosphaerocephala* also managed to penetrate *C. australis* and after Day 10, six out of eight native frogs had lungworms in their lungs. For toads, however, no larvae of either *Rhabdias* spp. were observed migrating through the body, and *R. hylae* larvae were never recorded in the body, or adult lungworms in the lungs, of toads. However, two of 10 toads had *R. pseudosphaerocephala* in their lungs after Day 10 (Fig. 8).

A larger number of inflammatory foci were detected than larvae ($n = 14$, distributed among 12 individual anurans; 4 toads, 8 frogs). Given that, by definition, no larvae were appreciable histologically in these inflammatory foci, can we conclude that they arise from the presence of *Rhabdias* larvae? Three lines of evidence support this inference. First, a sample of eight toads, which acted as procedural controls (i.e. were kept in Petri dishes for 24 h in the absence of *Rhabdias* larvae), did not contain any foci of infection. Second, the incidence of foci declined with time since exposure ($\chi^2 = 4.35$, df = 1, $P = 0.037$; Fig. 9), suggesting that their origin was contemporaneous with the start of the experiment. Finally, the foci were more likely to be located subcutaneously rather than in other tissues ($\chi^2 = 7.14$, df = 1, $P = 0.008$).

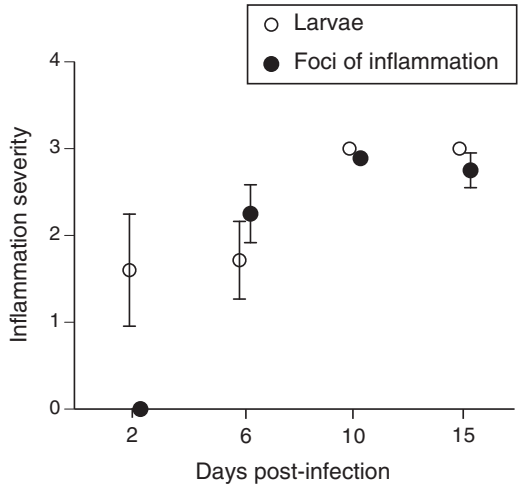

Fig. 5. Average inflammation severity surrounding *Rhabdias hylae* larvae and foci (probable larvae being broken down by the host's immune system) within infected cane toads at different numbers of days post-infection. Graph shows average values ±1 S.E.M.

3.2.3. Severity and type of inflammation response

If we assume that inflammatory foci are formed in response to the presence of *Rhabdias* larvae, we can assess whether novel parasites elicit different immune responses than do ancestral (coevolved) ones. Among cane toads, overall levels of inflammation at foci did not differ between individuals exposed to *R. hylae* and those exposed to *R. pseudosphaerocephala* ($\chi^2 = 0.33$, $P = 0.56$). Although toads produced inflammatory foci with somewhat higher levels of neutrophils and lower levels of lymphocytes when exposed to *R. pseudosphaerocephala* than when exposed to *R. hylae* (Fig. 10a), the differences were not significant (both $\chi^2 = 2.0$, $P = 0.16$). The levels of macrophages and eosinophils at foci in cane toads were also similar between individuals exposed to the two types of *Rhabdias* spp. (both $\chi^2 < 1.0$, $P > 0.32$; Fig. 10a).

Among *C. australis*, there was no overall difference in the severity of inflammatory foci between individuals exposed to *R. pseudosphaerocephala* and those exposed to *R. hylae* ($\chi^2 = 0.30$, $P = 0.58$). However, exposure to *R. pseudosphaerocephala* produced foci with higher levels of lymphocytes and lower levels of macrophages than was the case after exposure to *R. hylae* (both $\chi^2 = 4.2$, $P = 0.040$). Relative numbers of all other cell types in *C. australis* were similar after exposure to the different *Rhabdias* species (both $\chi^2 < 0.76$, $P > 0.38$; Fig. 10b).

There were two instances of inflammation associated with lungworms in the lungs of *C. australis*; one with *R. pseudosphaerocephala* and one with *R. hylae*.

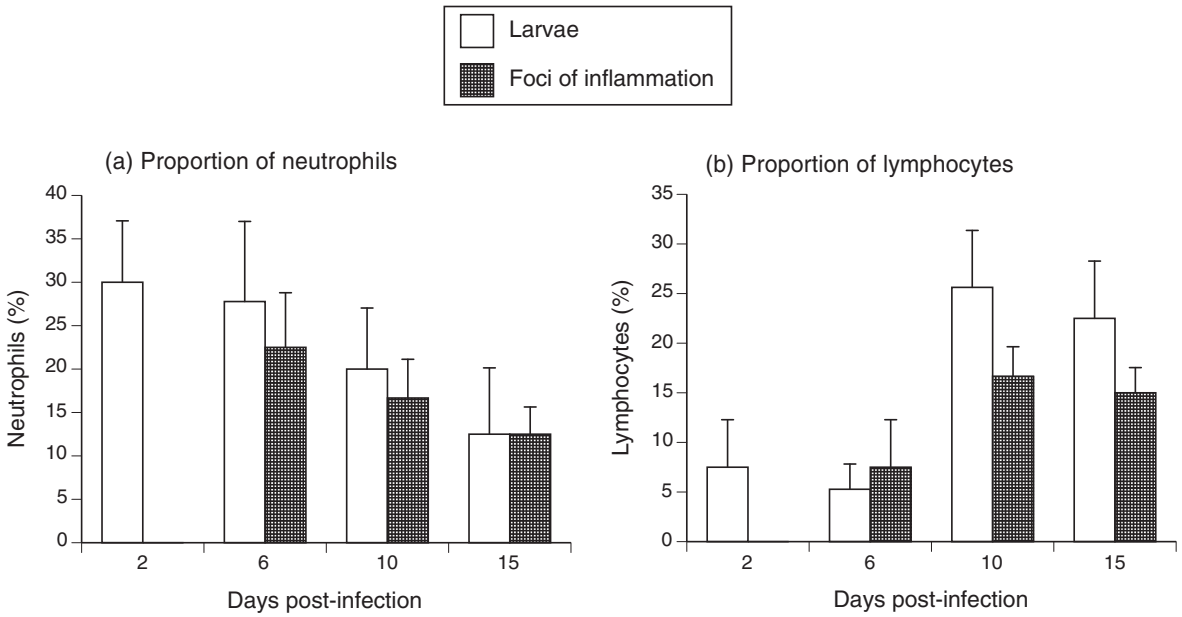

Fig. 6. Effects of *Rhabdias hylae* infection on cane toad metamorphs: (a) the average percentage of neutrophils and (b) lymphocytes around inflammation sites over time in cane toads infected with *Rhabdias hylae*. Graphs show average values ±1 S.E.M.

4. Discussion

Interactions between hosts and parasites change over time due to co-evolution. The relationship can become an 'arms race' where the host must increase its defences against an increasingly aggressive parasite (the Red Queen hypothesis: Dawkins and Krebs, 1979; Soler and Møller, 1990; Blanford et al., 2003); or the host may evolve to tolerate a parasite of ever-decreasing virulence (Lenski and May, 1994; Miller et al., 2006). Sometimes these two evolutionary forces reach a 'stalemate' situation where the virulence of the parasite stabilises at a level that optimises both the reproduction of the parasite and the host (Combes, 1997; Bull and Ebert, 2008) or fluctuates between periods of high and low virulence over time (Anderson and May, 1982). Biological invasions offer an opportunity to observe host–parasite interactions before natural selection stabilises, dulls or amplifies the effect of the parasite on the host. Here we address the initial contact between cane toads and the native frog parasite, *Rhabdias hylae*, and investigate how this differs with the relationship between *Rhabdias pseudosphaerocephala* and its ancestral host, the cane toad, as well as the opposite scenario (native frog exposed to *R. hylae* and native frog exposed to *R. pseudosphaerocephala).*

In our first experiment, cane toads were penetrated by the novel parasite (*R. hylae*) but the parasite was apparently unable to reach the lungs (larvae were not observed inside toads in the second experiment). Histology revealed two possible reasons for this failure: (1) an immune response of increasing severity prevented the larvae from proceeding to the lungs, and (2) larvae did not locate the lungs successfully and became lodged in other tissues. Larvae were observed migrating through subcutaneous tissue and skeletal muscle through to the coelom, and after 15 days larvae were found lodged in numerous tissues in different parts of the body (skin/muscle body, head, eye, coelom). Larvae came under increasing attack by the immune system over time and were less likely to be intact or visible after 15 days. The severity of inflammation around larvae and the

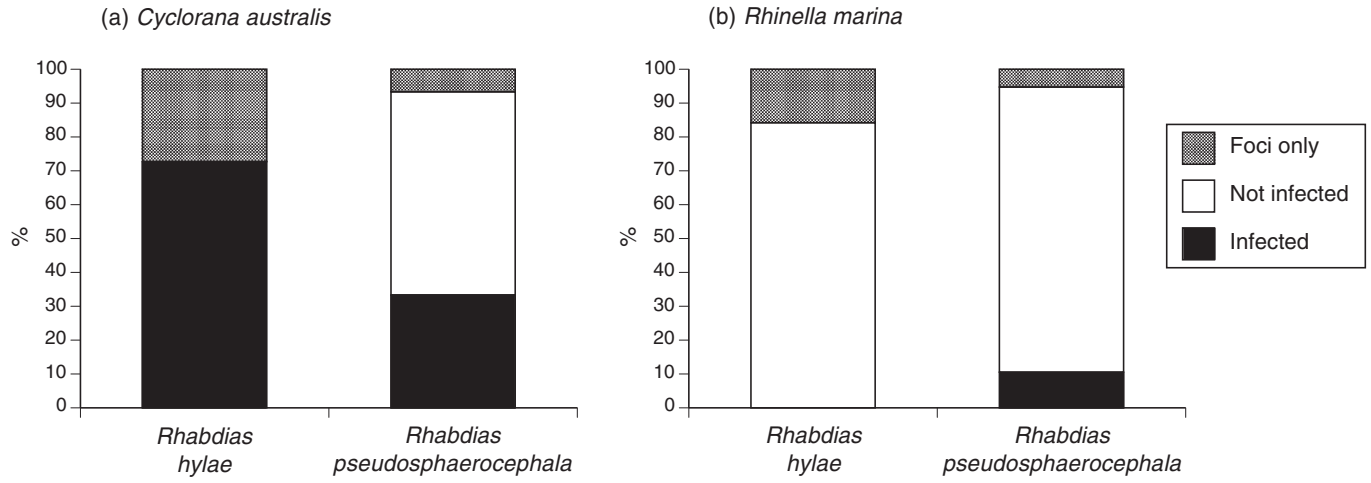

Fig. 7. Histological investigation of lungworm infection in anurans. Graphs show the proportion of (a) metamorph native frogs (*Cyclorana australis*) and (b) metamorph cane toads (*Rhinella marina*) infected with lungworms, not infected with lungworms, or with inflammatory 'foci' (probable cases of a lungworm larva penetrating the anuran's body but failing to survive).

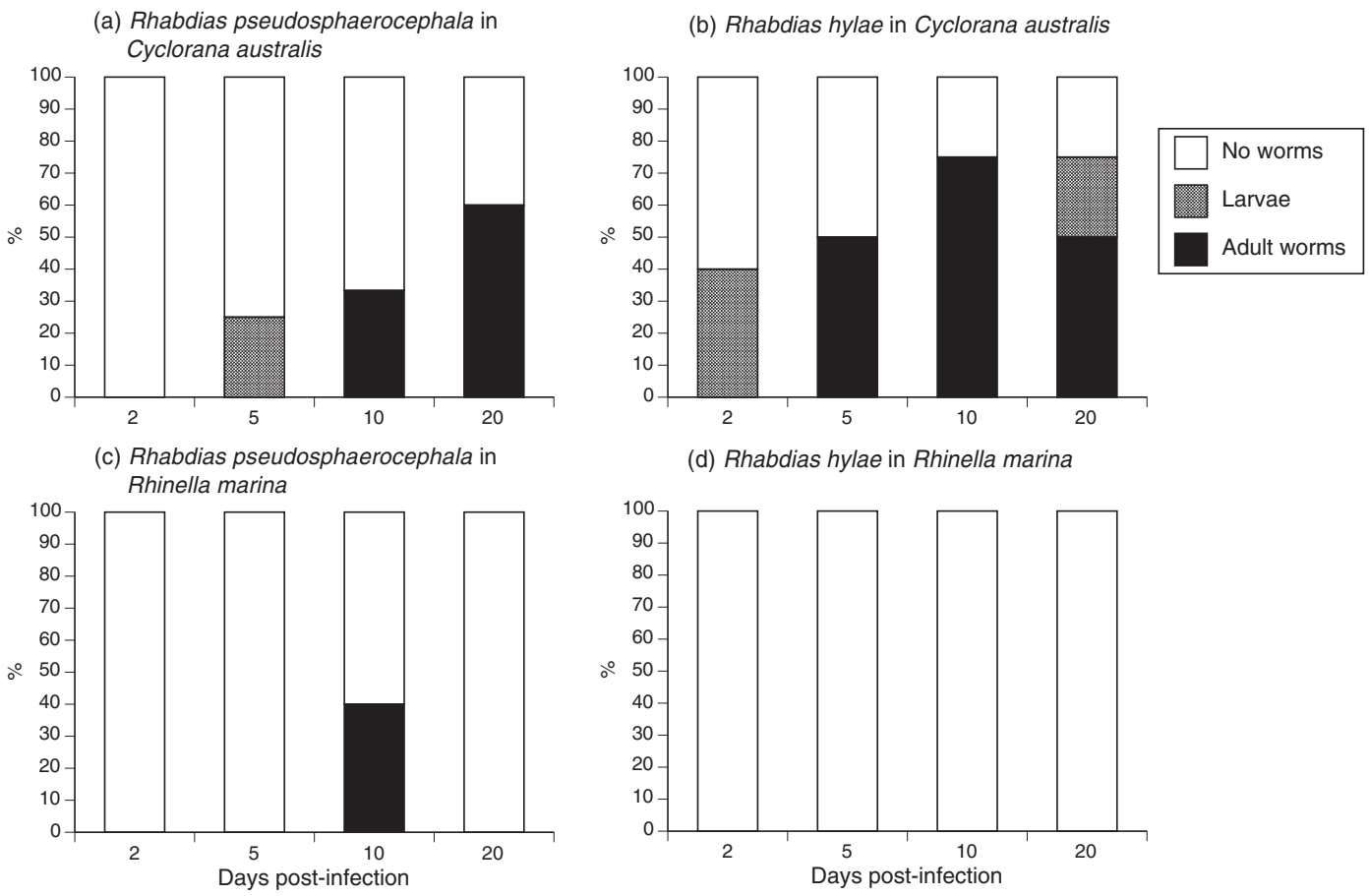

Fig. 8. Changes through time (days post-infection) on the relative numbers of anurans that were infected with lungworms, and that contained adult versus juvenile stages of the parasites involved. Data are shown for two lungworm species (*Rhabdias hylae* from native frogs, and *Rhabdias pseudosphaerocephala* from invasive cane toads) and for two types of host: the native frog, *Cyclorana australis*, and the cane toad, *Rhinella marina*. The panels show data for (a) *C. australis* infected with *R. pseudosphaerocephala*, (b) *C. australis* infected with *R. hylae*, (c) cane toads infected with *R. pseudosphaerocephala* and (d) cane toads infected with *R. hylae*.

number of inflammatory foci increased over time. Cane toads successfully repelled the invading non-co-evolved parasite, which was unsuccessful at navigating the new host's body.

These results mirror those of Pizzatto et al. (2010), who found that *R. pseudosphaerocephala* (the toad lungworm) became 'lost' inside the bodies of most but not all native frog species and migrated to the lungs

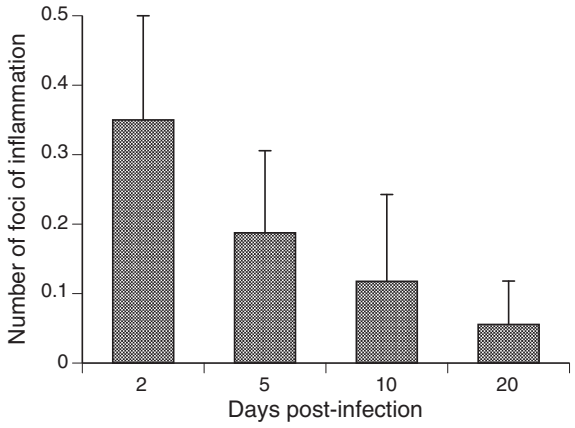

Fig. 9. Change in the average number of inflammatory foci (probable cases of larval parasites breaking down) observed in all anurans over time. Graph shows average values ± 1 S.E.M.

successfully in the co-evolved host (the cane toad) (Fig. 2b). This contrasts with our first experiment, which showed *R. hylae* becoming 'lost' in numerous body tissues inside the cane toad and never finding the lungs. The host specificity of parasites relies on evolutionary exposure to a range of host species and the taxonomic relatedness of new hosts to ancestral hosts (Poulin, 2007), so the disparity between the infection success of the Australian frog parasite and the cane toad parasite (originally from South America) in non-co-evolved hosts is unsurprising, given that both parasites evolved in entirely separate contexts.

In the second experiment, *C. australis* metamorphs were more susceptible to infection by both *R. hylae* and *R. pseudosphaerocephala* than were cane toads. Some native frog species, *Litoria caerulea*, *Cyclorana longipes*, *Litoria splendida* (Pizzatto and Shine, 2011b) and *Litoria dahlii* (Pizzatto et al., 2010) are vulnerable to infection by the cane toad parasite, although others, *Litoria nasuta*, *Opisthodon ornatus*, *Litoria rothii* and *Limnodynastes convexiusculus*, are not (Pizzatto et al., 2010; Pizzatto and Shine, 2011a). This result suggests asymmetry in the resistance of cane toads and native frogs to non-co-evolved rhabdiasid lungworms. Cane toads successfully resist native frog parasites, whereas at least some native frog species cannot resist toad parasites.

Cane toads and native frogs (excluding *Litoria splendida*) experience no decline in major indicators of viability (survival, feeding rates, growth rates, speed or endurance) when exposed to (or hosting) a non-co-evolved rhabdiasid lungworm (Pizzatto and Shine, 2011a, 2011b; Nelson et al., 2015). However, cane toads do suffer

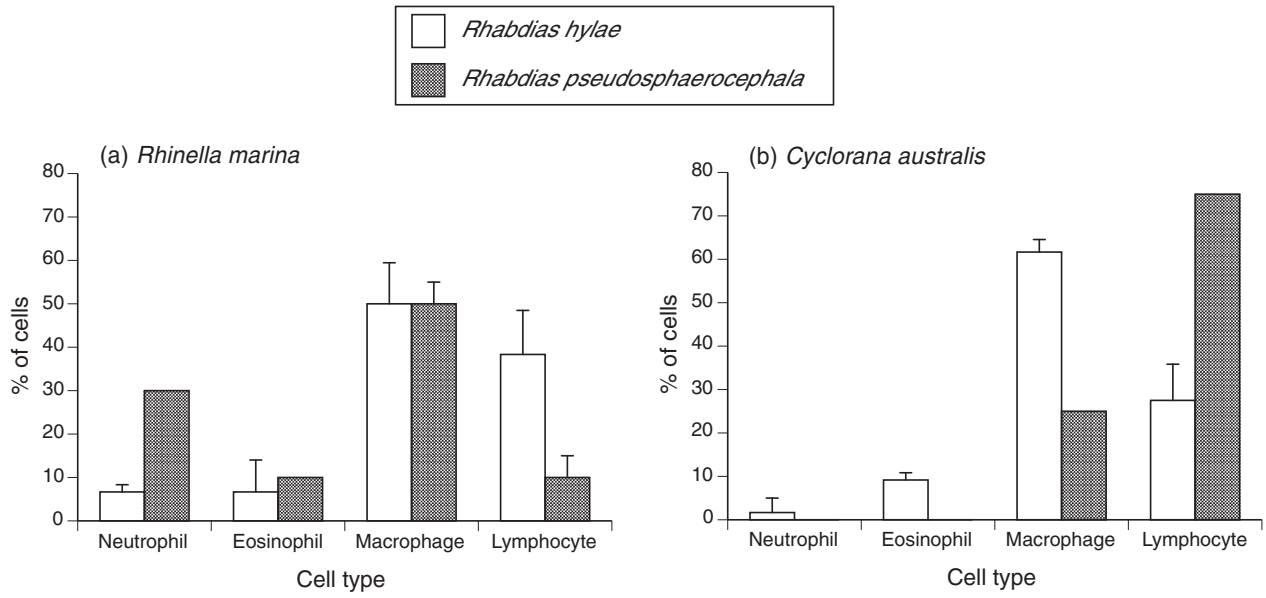

Fig. 10. Comparisons of inflammatory cells recruited to inflammatory foci in cane toads, *Rhinella marina* (a) and native frogs, *Cyclorana australis* (b). Each anuran species was exposed to infective larvae of *Rhabdias hylae* (white bars) and *Rhabdias pseudosphaerocephala* (grey bars). Graphs show average values ± 1 S.E.M.

deleterious effects from their native parasite, *R. pseudosphaerocephala* (Kelehear et al., 2009, 2011), whereas native frogs apparently suffer no ill effects from their co-evolved lungworm, *R. hylae* (Nelson et al., 2015). Again, this asymmetry implies a divergence in host–parasite evolutionary history. Because toad lungworms reduce host fitness, cane toads appear to invest in high resistance against their ancestral rhabdiasid lungworms. This investment may coincidentally protect them against attack from congeneric foreign lungworms, and explain their strong resistance to *R. hylae.* Alternatively, the lack of co-evolution may explain a more severe reaction by toads towards a novel threat. Prior infection with *R. hylae* fails to make toads more resistant to their own lungworm, *R. pseudosphaerocephala* (Nelson, 2014), which implies that there is a significant difference between the immune response towards the two nematode species. Cane toad lungworms appear to be able to evade detection through co-evolved mechanisms in cane toads, whereas frog lungworms cannot. The immune response to native frog stomach nematodes (Order Spirurida) by frogs and toads showed a similar effect; toads exhibited a severe immune response to the invading nematodes, whereas native frogs had minimal reaction to the parasites (Kelehear and Jones, 2010).

Amphibians use both adaptive and innate immune systems to recognise and react to *Rhabdias* infections (Rowley, 1988; Richmond et al., 2009; Rollins-Smith, 2011). We conducted experimental infections on recently metamorphosed amphibians to ensure that they were immunologically naïve to nematodes, without an arsenal of specific antibodies against them. In our experiments, the innate immune system, along with behaviour, was the metamorphs' chief defence against penetrating larvae.

Despite the small number of inflammatory foci found in anurans in the second experiment, we detected significant differences in the types of immune cells recruited to these foci between anurans exposed to the two different species of larva. Cane toads had more neutrophils surrounding *R. hylae* than *R. pseudosphaerocephala,* whereas *C. australis* had more lymphocytes and fewer macrophages surrounding *R. pseudosphaerocephala* than the co-evolved lungworm, *R. hylae.* These proportions were consistent throughout time. This result differed from those observed during the first experiment, in which the proportion of neutrophils decreased and the proportion of lymphocytes increased through time (along with the severity of inflammation) for cane toads infected with *R. hylae*.

The proportions of macrophages and eosinophils did not change over time post-infection in both experiments, but recruitment rates for eosinophils were lower than for macrophages across both species of anurans and both species of lungworm. Histological observations cannot identify the functional causes or consequences of different proportions of immune cells recruited to areas of inflammation. However, a general explanation may be that because the ability of nematodes to evade the immune systems of their hosts is partly based on disrupting or confusing several of the chemical messaging pathways involved in immune surveillance (Maizels et al., 2004; De Veer et al., 2007), and that this ability is honed and developed through long co-evolution with the host, then a novel (but related) parasite may disrupt the host immune system in a subtly different manner.

The early stages of inflammation around larvae generally contain a high proportion of granulocytes (mainly neutrophils), characteristic of an innate immune response. Over time, the proportion of neutrophils decreases and the proportion of lymphocytes increases (Rollins-Smith, 2011). Lymphocytes are predominantly associated with adaptive immune responses but are observed in growing numbers in the initial immune response acting as "natural killer" cells, which dissolve foreign bodies with cytolytic molecules, and as "scouting" T-cells and B-cells (Horton et al., 1998; Richmond et al., 2009; Rollins-Smith, 2011). This typical pattern of changing immune cell recruitment was seen in the first experiment; lymphocytes increased and neutrophils decreased significantly over time. In the second experiment, changes over time were difficult to see due to low numbers of larvae/ inflammatory foci.

The role of eosinophils in responding to helminth infections remains unclear (Meeusen and Balic, 2000; Klion and Nutman, 2004) but they are often implicated in effective clearance of metazoan parasites (Mitchell, 1982; Rowley, 1988; Shutler et al., 2009). Eosinophils made up between 14.5% and 25% of immune cells surrounding larvae in the first experiment and 16% in inflammatory foci in the second experiment. Macrophages have diverse roles in nonspecific early responses to antigens as well as chronic inflammatory reactions

(Pizzatto et al., 2010), and remained consistently high over all DPT across all experiments.

Successful helminth parasites are able to evade or modulate their hosts' immune system (Maizels et al., 2004; Shutler et al., 2009), particularly after the parasite has matured and its thickened cuticle acts as a barrier to detection and attack (Tinsley et al., 2012). Across both of our experiments, little inflammation was associated with lungworms in the lung (seen in only two frogs, in the second experiment), suggesting that an acquired immune response is ineffective at removing parasites once they have matured.

There were large differences between the results of our two experiments, in terms of the number of larvae seen in histological sections as well as the associated levels of inflammation. Different conditions of exposure in the two experiments presumably affected levels of infection and inflammation. Unsurprisingly, cane toads that were chronically exposed to high concentrations of *R. hylae* over 15 days showed greater evidence of larval penetration than did toads exposed to only 30 larvae for 24 hours. The lack of inflammation associated with any of the larvae seen in toads (and frogs) under the latter conditions is, however, surprising.

What does a lack of inflammation around larvae mean? It cannot be due to insufficient time having elapsed to produce an immune response, because our samples were taken at least two days post-exposure. A lack of inflammation suggests that the larva is alive and successfully cloaking itself from the host's immune system whereas severe inflammation suggests that the larva has been detected and recognised by the immune system and is being killed or removed by effector cells and chemicals.

Why were there more larvae dead and dying in the second experiment than the first? Larvae in the first experiment may have been in poor condition before they invaded a host and, once inside the host, they may have been incapable of avoiding the toad's immune system in their weakened state. The larvae in the first experiment were in a large enclosure and the prolonged exposure period may have resulted in larvae being weakened/aged before they found an appropriate host. Nematodes evade the host's immune system through active production of compounds that compromise the activation or deployment of host immune products (Maizels et al., 2001). Once a larva dies and its production of these 'cloaking agents' ceases, it becomes a large helpless foreign body, which the immune system can easily target for disintegration and clearance (Rollins-Smith, 2011). An inflammatory reaction to dying nematodes can cause pathologic conditions in humans and other animals, such as ocular toxocariasis (Holland and Smith, 2005) and eosinophilic meningitis (Kliks and Palumbo, 1992), and are a potential adverse effect of anti-helmintic treatment (Nielsen et al., 2013).

Alternatively, the toad's immune system, being under a constant barrage of larvae for several days in the first experiment, may have become fully activated (De Veer et al., 2007). If so, many larvae that did manage to penetrate a host would have been met with up-regulated immune surveillance and were thus more likely to be detected and responded to rapidly. In the second experiment, in contrast, briefer (24-h) exposure to infective larvae may not have provoked as much immune activation and larvae penetrating hosts in the second experiment may have encountered a relatively naïve response.

In conclusion, the separate lungworm faunas of cane toads and native Australian frogs induced different inflammatory responses, and had different levels of success in co-evolved and non-co-evolved hosts. These patterns accord with the hypothesis that hosts and parasites become adapted to each other over evolutionary time, and that host switching can incur fitness costs on parasites. Cane toads were a hostile environment for the native frog lungworm, whereas *C. australis* tolerated infections by the toad parasite, *R. pseudosphaerocephala,* as well as by its native parasite, *R. hylae*. Nonetheless, toad lungworms penetrated and reached the lungs of frogs less often than did the native parasite species. Invasive species offer unique opportunities to study co-evolution and the costs of host switching; further research could usefully exploit that potential to ask more detailed questions of the complex interactions that occur when a parasite and host first encounter each other.

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Conflict of interest

The authors declared that there is no conflict of interest.

References

Anderson, R.M., May, R.M., 1982. Coevolution of hosts and parasites. Parasitology 85, 411–426.
Baker, M.R., 1979. The free-living and parasitic development of *Rhabdias* spp. (Nematoda: Rhabdiasidae) in amphibians. Can. J. Zool. 57, 161–178.
Blanford, S., Thomas, M.B., Pugh, C., Pell, J.K., 2003. Temperature checks the Red Queen? Resistance and virulence in a fluctuating environment. Ecol. Lett. 6, 2–5.
Bleach, I., Beckmann, C., Brown, G.P., Shine, R., 2014. Effects of an invasive species on refuge-site selection by native fauna: the impact of cane toads on native frogs in the Australian tropics. Austral Ecol. 39, 50–59.
Bull, J.J., Ebert, D., 2008. Invasion thresholds and the evolution of nonequilibrium virulence. Evol. Appl. 1, 172–182.
Combes, C., 1997. Fitness of parasites: pathology and selection. Int. J. Parasitol. 27, 1–10.
Dawkins, R., Krebs, J.R., 1979. Arms races between and within species. Proc. R. Soc. Lond. B Biol. Sci. 205, 489–511.
De Veer, M.J., Kemp, J.M., Meeusen, E.N.T., 2007. The innate host defence against nematode parasites. Parasite Immunol. 29, 1–9.
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.
Ebert, D., Hamilton, W.D., 1996. Sex against virulence: the coevolution of parasitic diseases. Trends Ecol. Evol. 11, 79–82.
Holland, C.V., Smith, H.V., 2005. Toxocara: The Enigmatic Parasite. CABI Publishing, Wallingford, UK.
Horton, T.L., Ritchie, P., Watson, M.D., Horton, J.D., 1998. Natural cytotoxicity towards allogeneic tumour targets in *Xenopus* mediated by diverse splenocyte populations. Dev. Comp. Immunol. 22, 217–230.
Kelehear, C., Jones, H.I., 2010. Nematode larvae (Order Spirurida) in gastric tissues of Australian anurans: a comparison between the introduced cane toad and sympatric native frogs. J. Wildl. Dis. 46, 1126–1140.
Kelehear, C., Webb, J.K., Shine, R., 2009. *Rhabdias pseudosphaerocephala* infection in *Bufo marinus*: lung nematodes reduce viability of metamorph 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.
Kelly, D.W., Paterson, R.A., Townsend, C.R., Poulin, R., Tompkins, D.M., 2009. Parasite spillback: a neglected concept in invasion ecology? Ecology 90, 2047–2056.
Kliks, M.M., Palumbo, N.E., 1992. Eosinophilic meningitis beyond the Pacific Basin: the global dispersal of a peridomestic zoonosis caused by *Angiostrongylus cantonensis*, the nematode lungworm of rats. Soc. Sci. Med. 34, 199–212.
Klion, A.D., Nutman, T.B., 2004. The role of eosinophils in host defense against helminth parasites. J. Allergy Clin. Immunol. 113, 30–37.
Lenski, R.E., May, R.M., 1994. The evolution of virulence in parasites and pathogens: reconciliation between two competing hypotheses. J. Theor. Biol. 169, 253–265.
Maizels, R.M., Gomez-Escobar, N., Gregory, W.F., Murray, J., Zang, X., 2001. Immune evasion genes from filarial nematodes. Int. J. Parasitol. 31, 889–898.
Maizels, R.M., Balic, A., Gomez-Escobar, N., Nair, M., Taylor, M.D., Allen, J.E., 2004. Helminth parasites – masters of regulation. Immunol. Rev. 201, 89–116.
May, R.M., Anderson, R.M., 1983. Epidemiology and genetics in the coevolution of parasites and hosts. Proc. R. Soc. Lond. B Biol. Sci. 219, 281–313.
Meeusen, E.N.T., Balic, A., 2000. Do eosinophils have a role in the killing of helminth parasites? Parasitol. Today 16, 95–101.
Miller, M.R., White, A., Boots, M., 2006. The evolution of parasites in response to tolerance in their hosts: the good, the bad, and apparent commensalism. Evolution 60, 945–956.

Mitchell, J.B., 1982. The effect of host age on *Rana temporaria-Gorgoderina vitelliloba* interactions. Int. J. Parasitol. 12, 601–604.
Nelson, F., 2014. Parasites lost: the impact of invaders on native host-parasite systems. Honours thesis, University of Sydney, Sydney, NSW, Australia.
Nelson, F.B.L., Brown, G., Dubey, S., Shine, R., 2015. The effects of a nematode lungworm (*Rhabdias hylae*) on its natural and invasive anuran hosts. J. Parasitol. in press.
Nielsen, M.K., Betancourt, A., Lyons, E.T., Horohov, D.W., Jacobsen, S., 2013. Characterization of the inflammatory response to anthelmintic treatment of ponies with cyathostominosis. Vet. J. 198, 457–462.
Pizzatto, L., Shine, R., 2011a. Ecological impacts of invading species: do parasites of the cane toad imperil Australian frogs? Austral Ecol. 36, 954–963.
Pizzatto, L., Shine, R., 2011b. The effects of experimentally infecting Australian tree frogs with lungworms (*Rhabdias pseudosphaerocephala*) from invasive cane toads. Int. J. Parasitol. 41, 943–949.
Pizzatto, L., Shilton, C.M., Shine, R., 2010. Infection dynamics of the lungworm *Rhabdias pseudosphaerocephala* in its natural host, the cane toad (*Bufo marinus*), and in novel hosts (native Australian frogs). J. Wildl. Dis. 46, 1152–1164.
Pizzatto, L., Kelehear, C., Dubey, S., Barton, D., Shine, R., 2012. Host-parasite relationships during a biologic invasion: 75 years postinvasion, cane toads and sympatric Australian frogs retain separate lungworm faunas. J. Wildl. Dis. 48, 951–961.
Poulin, R., 2007. Evolutionary Ecology of Parasites, second ed. Princeton University Press, Princeton, NJ.
Prenter, J., Macneil, C., Dick, J.T.A., Dunn, A.M., 2004. Roles of parasites in animal invasions. Trends Ecol. Evol. 19, 385–390.
Richmond, J.Q., Savage, A.E., Zamudio, K.R., Rosenblum, E.B., 2009. Toward immunogenetic studies of amphibian chytridiomycosis: linking innate and acquired immunity. Bioscience 59, 311–320.
Roberts, L.S., Janovy, J.J., 2009. Foundations of Parasitology. McGraw-Hill, New York.
Rollins-Smith, W., 2011. Amphibian Immunity: Staying in Tune with the Environment in Ecoimmunology. Oxford University Press, New York.
Rowley, R., 1988. Vertebrate Blood Cells. Cambridge University Press, Cambridge, UK.
Shutler, D., Smith, T.G., Robinson, S.R., 2009. Relationships between leukocytes and *Hepatozoon* spp. in green frogs, *Rana clamitans*. J. Wildl. Dis. 45, 67–72.
Soler, M., Møller, A.P., 1990. Duration of sympatry and coevolution between the great spotted cuckoo and its magpie host. Nature 343, 748–750.
Tinsley, R., Stott, L., York, J., Everard, A., Chapple, S., Jackson, J., et al., 2012. Acquired immunity protects against helminth infection in a natural host population: long-term field and laboratory evidence. Int. J. Parasitol. 42, 931–938.
Trejo, A., 1992. A comparative study of the host-parasite relationship of *Pomphorhynchus patagonicus* (Acanthocephala) in two species of fish from Lake Rosario (Chubut, Argentina). J. Parasitol. 78, 711–715.
Turvey, N., 2013. Cane Toads: A Tale of Sugar, Politics and Flawed Science. Sydney University Press, Sydney, NSW.