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Helpful invaders: Can cane toads reduce the parasite burdens of native frogs?

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ABSTRACT

Many invading species have brought devastating parasites and diseases to their new homes, thereby imperiling native taxa. Potentially, though, invaders might have the opposite effect. If they take up parasites that otherwise would infect native taxa, but those parasites fail to develop in the invader, the introduced species might reduce parasite burdens of the native fauna. Similarly, earlier exposure to the other taxon's parasites might 'prime' an anuran's immune system such that it is then able to reject subsequent infection by its own parasite species. Field surveys suggest that lungworm counts in native Australian frogs decrease after the arrival of invasive cane toads (*Rhinella marina*), and laboratory studies confirm that native lungworm larvae enter, but do not survive in, the toads. In laboratory trials, we confirmed that the presence of anurans (either frogs or toads) in an experimental arena reduced uptake rates of lungworm larvae by anurans that were later added to the same arena. However, experimental exposure to lungworms from native frogs did not enhance a toad's ability to reject subsequent infection by its own lungworm species.

1. Introduction

Biological invasions can disrupt many aspects of ecosystem function. Much research has focused on mechanisms such as competition (e.g. Levine et al., 2003) and predation (e.g. Short et al., 2002) but disruption of host-parasite dynamics may be an important additional route of impact (Thieltges et al., 2009; Hartigan et al., 2011). Invaders often exhibit reduced parasite levels (the enemy release hypothesis: Marr et al., 2008), but novel pathogens brought by invaders can still devastate native taxa by directly reducing survival or by mediating the outcome of competition between native and invasive species (e.g. Settle and Wilson, 1990; Hudson and Greenman, 1998). The reverse scenario (the transfer of native pathogens to the invader) has similar effects (Dunn, 2009; Hartigan et al., 2011; Pizzatto and Shine, 2012).

Past studies have investigated cases where invaders act to increase parasitism of native fauna by introducing a new parasite or by acting as a reservoir for native parasites (Dobson and Foufopoulos, 2001; Mastitsky and Veres, 2010; Pizzatto and Shine, 2011; Hartigan et al., 2011). However, little research has been done on the alternative scenario — cases where invaders decrease parasite loads of native animals by acting as a 'sink' for native parasites (Kelly et al., 2009a; Lettoof et al., 2013). In this instance, native parasites are taken up by the invader but fail to complete their life cycle due to a lack of co-evolutionary history. When the parasite enters a foreign host it becomes disoriented or attacked by the immune system. Invasive species can therefore act as 'resistant targets', reducing the density of parasites in the environment and thus, lowering the risk of infection for native hosts (Heimpel et al., 2003; Kelly et al., 2009a).

Because free-living stages of parasites are time-limited and exposed to threats such as predation and desiccation (Johnson and Thieltges, 2010) they are under heavy selective pressure to rapidly infect an appropriate host. Finding a host becomes more of a challenge in assemblages with a number of host species that differ in susceptibility to the parasite, such as invasive systems (Keesing et al., 2006).

The 'sink' mechanism has been investigated in only a few invasive systems (Trejo, 1992; Telfer et al., 2005; Kopp and Jokela, 2007; Thieltges et al., 2009; Paterson et al., 2011, 2013a,b) but these studies are geographically and taxonomically diverse, meaning the crossover of native parasites to invaders is likely to be common (Dunn, 2009). Thus, it is important to explore the

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possibility of 'sink' mechanisms in other systems, especially involving parasite and host taxa from lineages that have not been the subjects of previous research in this respect.

Here, we examine the potential disruption of host-parasite interactions caused by the invasive cane toad, *Rhinella marina*, in Australia. Recent studies have shown that cane toads and native frogs have separate nematode lungworm fauna (Pizzatto et al., 2012). The cane toad lungworm, *Rhabdias pseudosphaerocephala*, arrived in Australia with the invasive species in 1935 when it was introduced into Queensland as a biological control agent for sugar cane pests (Dubey and Shine, 2008). In contrast, *Rhabdias hylae* is exclusive to native Australian frogs. These two parasite species are virtually indistinguishable in morphology, but genetic analysis shows that they are indeed separate species (Dubey and Shine, 2008), and experimental infections demonstrate that the lungworms perform differently in cane toad and native frog hosts (Pizzatto et al., 2010; Nelson et al., 2015a,b). *R. hylae* can penetrate cane toads but is killed by a strong immune response; it becomes 'lost' inside the novel host's body and never reaches the lungs, where it would normally mature, produce eggs and complete its life cycle (Nelson, 2014; Nelson et al., 2015b). *R. pseudosphaerocephala* readily penetrates native frogs but only reaches the lungs in a small number of species, for similar reasons (Pizzatto et al., 2010; Pizzatto and Shine, 2011).

These interactions (especially the ability of each parasite species to penetrate the 'wrong' host, but not survive) support fundamental assumptions underlying the 'sink' mechanism. The only field data to support this scenario come from a recent study conducted in northeastern New South Wales. Lettoof et al. (2013) found lower rates of infection with lungworms in native frogs living in areas with cane toads, than in the same frog species living in nearby areas that lack cane toads.

Two mechanisms could plausibly explain this result. Firstly, the cane toads could be acting as 'sinks' (removing parasite larvae from the environment, and dooming those parasites to an early death). Secondly, native frogs that are exposed to the cane toad parasite might thereby develop acquired immunity to their own parasite species (i.e., an initial exposure to toad *Rhabdias* spp. may instigate production of antibodies that are also effective targeting native *Rhabdias* spp.). A similar 'priming' of the cane toad's immune system by frog *Rhabdias* spp. against its more virulent native parasite would have substantial benefits for cane toads, and might enhance their invasion success.

Immunological 'priming' is the principle behind many vaccines, which exploit the capacity of the adaptive immune system to form 'memories' in response to inert parts of pathogens (Brunham and Coombs, 1998; Oettinger et al., 1999; Hooper et al., 2004). Within a few weeks of exposure, specific antibodies are generated to defend the body against attack. Upon re-infection, the immune response is more effective at stopping the spread of disease. Amphibians, like all vertebrates, have this capacity to encode a 'memory' of previously encountered pathogens and the acquired immune response has been shown to play a role in the improved response of amphibians towards infections (Richmond et al., 2009; Tinsley et al., 2012).

Here we test the plausibility of the 'sink' mechanism as it applies to cane toads soaking up native frog parasites, and the possibility that prior exposure to the native frog lungworm 'primes' the cane toad's immune system such that it is less vulnerable to infection by its own lungworm species.

2. Materials and methods

Descriptions and details of methods for breeding and husbandry of anurans, and collection and identification of lungworm larvae used in the following experiments, appear in the Supplementary Material.

2.1. Effect of precedence on rates of parasite uptake

To measure rates of *R. hylae* uptake by anurans we exposed each of 69 native frogs (30 *Cyclorana australis* and 39 *Limnodynastes convexiusculus*) and 31 cane toads (*R. marina*) to infective lungworm larvae. Feces containing free-living adult worms were collected from adult frogs between 4 and 18 days prior to infection and stored in petri dishes with untreated bore water. After 2–4 days in these petri dishes, the free-living adult worms had produced infective third stage larvae (L3) that we used for experimental infections. 30 larvae (L3) were collected using a glass pipette under a dissecting microscope and placed in a 3.5 cm-diameter petri dish with 2 mL of water. An anuran was then placed in each dish and held with infective larvae for 1 h. We then removed the anuran and placed the dish under a dissecting microscope to count the larvae remaining. A second anuran was then added to the petri dish for 1 h. After this second 1-h infection period, the second anuran was removed and the number of remaining larvae counted once more. The combination of anurans in each petri dish was as follows: (1) cane toad ($n = 13$) followed by *L. convexiusculus* ($n = 13$), (2) *L. convexiusculus* ($n = 13$) followed by *L. convexiusculus* ($n = 13$), (3) cane toad ($n = 10$) followed by *C. australis* ($n = 10$), (4) *C. australis* ($n = 10$) followed by *C. australis* ($n = 10$) and, (5) cane toad ($n = 4$) followed by cane toad ($n = 4$).

We measured parasite uptake as the difference in number of larvae between the beginning and end of each 1 h trial.

This assumes that any missing larvae had crawled onto the anuran host and been removed along with it at the end of the trial. Metamorphs varied by a maximum of only 1.62 g, but anuran body mass was still used as a covariate in the analyses. We analysed the data from this experiment using an ANOVA model that incorporated trial "type" (the precedence combination of species 1/species 2), order of exposure of each anuran (first vs second), body mass and the order*type interaction term as independent variables and the number of larvae taken up as the dependent variable.

We carried out histological examinations to verify that a 1 h exposure to 30 L3 was sufficient to allow successful larval penetration. Five days after the exposure trials a subsample of 17 anurans (5 cane toads, 6 *C. australis*, 6 *L. convexiusculus*) were euthanised by immersion in a solution of buffered tricaine methanesulfonate (MS-222). For histological examination, five to six 5-μm serial transverse sections were made encompassing the tissue from the head to the pelvis of each anuran and stained with hematoxylin and eosin (see Pizzatto et al., 2010 for detailed methods). Slides were examined for the presence of larvae and characteristic inflammatory foci associated with degenerating larvae (Nelson et al., 2015b).

2.2. Effect of exposure to R. hylae on the subsequent establishment of R. pseudosphaerocephala in the lungs of cane toads

As part of another study, we exposed 32 metamorph cane toads to 30 infective larvae of *R. hylae* for 24 h and then measured correlates of fitness over 45 days (Nelson et al., 2015a). After this experiment had concluded 45 days post-treatment (DPT), we exposed 7 of the cane toads that had been previously exposed to *R. hylae* as part of this experiment, and 7 control toads (with no prior exposure to *R. hylae*, but otherwise identical husbandry conditions) to 30 *R. pseudosphaerocephala* larvae. This was done by placing each metamorph separately in a 3.5 cm diameter petri dish with 2 mL of water (plus the parasite larvae) for 24 h. Toads were housed and fed for a subsequent 20 days and then euthanised by

immersion in MS-222 as above. These toads were then dissected on day 65 to determine the number of lungworms in each lung.

No cane toads out of the 25 that were dissected contained *R. hylae* in their lungs after 45 days (due to a severe immune response by toads and aberrant migration of larvae rather than a lack of penetration by larvae: Nelson et al., 2015a). Thus, any lungworms found in the re-exposed toad's lungs after 65 days were assumed to be *R. pseudosphaerocephala*, rather than *R. hylae*. We could therefore determine whether prior exposure to *R. hylae* influenced the number of *R. pseudosphaerocephala* that reached the lungs of the experimental toads.

We compared the presence versus absence of lungworms at the time of euthanasia between treatments (prior exposure to *R. hylae*, versus no prior exposure to *R. hylae*) using a nominal logistic regression with treatment as the independent variable. We compared the number of lungworms between treatments using nonparametric statistics (Wilcoxon test). Mean body mass was also compared between treatment groups using a Wilcoxon test.

This research was approved by the University of Sydney Animal Ethics Committee (AEC Protocol Number: 6042).

3. Results

3.1. Effect of precedence on parasite uptake rates

The number of *R. hylae* larvae taken up by an anuran was reduced by the prior presence of another anuran in the exposure chamber (the first anuran took up some of the available larvae, thus reducing the number available to infect the subsequently-available host). Secondarily-exposed anurans on average took up 42.4% fewer larvae than those exposed first ($F_{1,90} = 7.37$, $P = 0.008$). This effect was not dependent on body mass ($F_{1,90} = 0.05$, P = 0.83) or on which anuran species was exposed first versus second ($F_{4,90} = 0.60$, $P = 0.66$; Fig. 1a): that is, the 'second anuran's' reduction in parasite uptake was just as high if the 'first anuran' was a toad as if it was a frog. Anurans exposed first took up 8.68 larvae on average, whereas anurans exposed second took up 3.68 larvae on average. This pattern remained when all native frogs were pooled into one group and compared to toads (Order: $F_{1,92} = 8.11$, $P = 0.005$; mass: $F_{1,92} = 0.04$, $P = 0.84$, anuran combination: $F_{2,92} = 1.16$, $P = 0.32$; Fig. 1b).

Two of the 17 anurans examined histologically 5 days after infection, showed evidence of successful penetration by larvae. One *L. convexiusculus* had nematode cross-sections subcutaneously and in its coelom and one *C. australis* had nematode cross-sections in a lung.

3.2. Effect of exposure to R. hylae on the subsequent establishment of R. pseudosphaerocephala in the lungs of cane toads

We found relatively few lungworms (mean = 0.34 per toad) in the lungs of metamorphs when these animals were dissected after 65 days, with no significant difference in the absence or presence of lungworms between treatments (prior exposure to 30 *R. hylae* larvae vs. control: 42.9% vs 28.6% with larvae; $\chi^2 = 0.32$, $n = 14$, $P = 0.58$) or the number of lungworms between treatments (mean 0.7 larvae vs mean 0.6 larvae; Wilcoxon: $\chi^2 = 0.14$, $n = 14$, $P = 0.71$; Fig. 2). The average body mass was 0.72 g (range = 0.3 g). Body mass did not differ significantly between treatment groups (Wilcoxon: $\chi^2 = 0.50$, $n = 14$, $P = 0.48$).

4. Discussion

Our experiments support the plausibility of one putative mechanism by which toad invasion might reduce parasite numbers

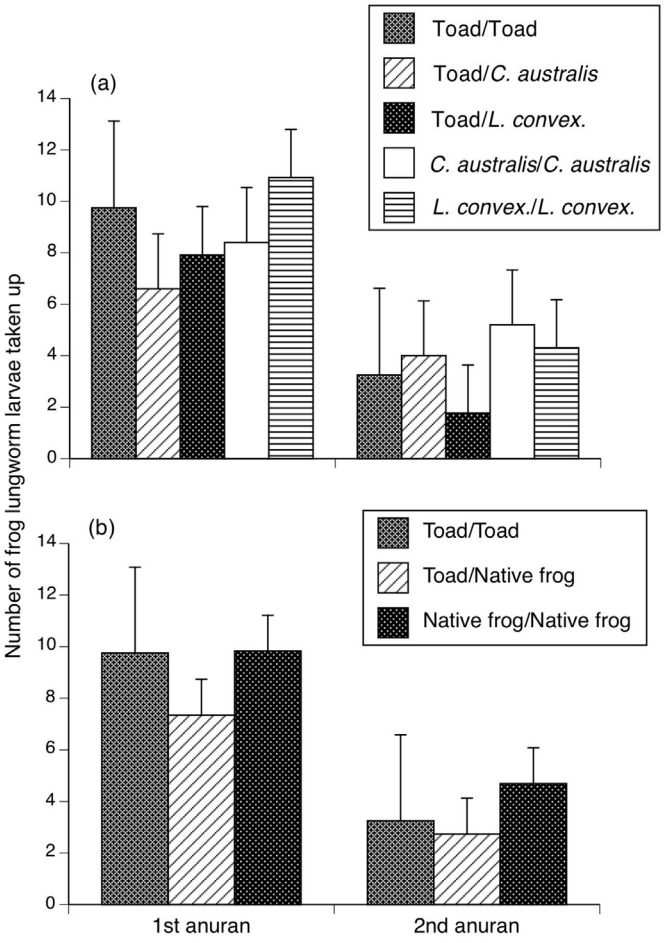

Fig. 1. Effect of order of exposure and type of anuran species (native frog versus cane toad) on the number of lungworm (*Rhabdias hylae*) larvae taken up in one hour in experimental arenas. Graph displays average values ±1 S.E.

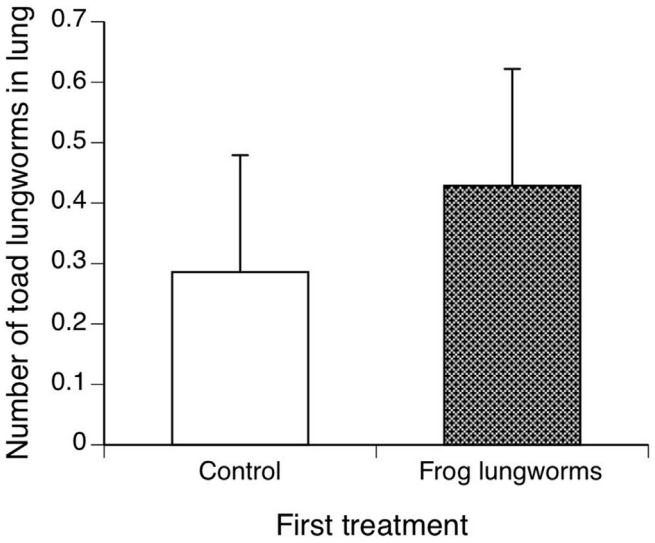

Fig. 2. Effect of prior exposure to *Rhabdias hylae* on the subsequent establishment of another lungworm species (*Rhabdias pseudosphaerocephala*) in the lungs of cane toad metamorphs. Graph displays average values ±1 S.E.

in native frogs. As predicted by the 'sink' hypothesis, the presence of a toad (or frog) in the experimental arena for an hour was enough to remove many of the parasite larvae in a defined area; and as a result, an anuran that was later placed in the same arena was infected at a lower rate than would otherwise have been the case. However, our data falsified the main prediction from the other hypothesis that we tested ('immunological priming'): prior infection with the lungworm from native frogs (*R. hylae*) did not render a cane toad more (or less) resistant to infection by the cane toad's own lungworm species (*R. pseudosphaerocephala*).

The 'sink' mechanism was tested here with a simplistic experimental design; the first anuran was forced into close contact with larvae in a small container and the subsequent anuran was placed in precisely the same location. In nature, the impact of prior residency of a frog or a cane toad will depend on infection dynamics, anuran densities and habitat overlap – as well as a wealth of other variables (Prenter et al., 2004; Paterson et al., 2013b). The three anuran species used in this experiment have very similar sizes as metamorphs but adult cane toads and *C. australis* are much larger than *L. convexiusculus*. Thus, the results of this experiment do not reflect the precise magnitude of any 'sink' effect in nature, as larger animals are likely to be penetrated by larvae at a higher rate. However, this experiment is a necessary first step in determining whether the 'sink' is a viable mechanism, as it confirms that a toad can have as much effect as a frog in soaking up infective larvae of the 'frog parasite'. Plausibly, toads might have had less effect than frogs – for example, toads might be less attractive to larvae, or the individual larvae that infect a toad might not be the same ones as are most likely to infect a frog.

That (at least some) *R. hylae* recognise cane toads as a potential host, and are capable of entering their bodies (Nelson et al., 2015b), is somewhat surprising. This nematode species would never have encountered a bufonid before 1935 (or, in the study area where we worked, before 2005). That the parasite recognises and manages to enter toads suggests that *R. hylae* may not be very discriminating in their choice of host. The same appears to be true for the toad's lungworm, *R. pseudosphaerocephala*, which readily enters Australian frogs (Pizzatto and Shine, 2011). Currently little is known about the specificity of signals used by *Rhabidas* spp. to locate hosts, although it is evident that these nematodes use a combination of chemotaxis and vibrations (Langford, 2010).

The lack of discrimination by *R. hylae* may be a disadvantage to this nematode when invasive cane toads arrive. Unless the nematode can quickly adapt to exploit the new host species, its natural life cycle might be interrupted by the abundance of 'decoy' toad hosts in which it cannot reproduce (Paterson et al., 2013b). Cane toads often attain very high densities, sometimes outnumbering native frogs (e.g., Freeland and Kerin, 1988), so that (all else being equal) a high proportion of *R. hylae* larvae may locate the 'accidental' toad host, where they are eliminated by the toad's immune defences (Nelson et al., 2015b). Whether or not this uptake by toads has a large-scale impact on prevalence and intensity of *R. hylae* infection in native frog populations in the wild (as suggested by the data of Lettoof et al., 2013) will depend on a number of factors. If larval output is high, then the larvae that cane toads extract from the system may have little impact in reducing the numbers still available to infect frogs. *Rhabdias* spp. have a high output (between 10 and 100 eggs a day per lungworm; personal observation), suggesting that the environment might be saturated by larvae (many of which will never reach a frog host, even without toads to contend with). That possibility would challenge the plausibility of the 'sink' mechanism in this system (e.g. Laracuente et al., 1979).

However, 'sink' mechanisms can operate under a wide array of circumstances, such as livestock 'decoys' reducing the spread of human diseases by vectors (Van Buskirk and Ostfeld, 1995; Miller and Huppert, 2013), invasive snails in New Zealand decreasing the transmission of a native trematode to native snails (Kopp and Jokela, 2007), and invasive molluscs decreasing parasite (trematode) burdens of native European mussels (Thieltges et al., 2009). Field surveys, experiments and population modelling have indicated that invasive salmonids in New Zealand act as a 'sink' for numerous native fish parasites (Kelly et al., 2009b; Paterson et al., 2011, 2013a,b).

In the only other amphibian host-parasite system in which such interactions have been studied, the presence of Grey tree frogs reduces the infection rates of toads (*Bufo americanus*) to *Ribeiroia ondatrae*, a trematode that causes limb deformities (Johnson et al., 2008). Importantly, some of these 'sink' effects have been documented not only in the laboratory, but also with field experiments in intertidal zones, outdoor mesocosms and even entire wetlands (e.g. Upatham, 1972; Upatham and Sturrock, 1973; Laracuente et al., 1979; Hopper et al., 2008; Johnson et al., 2009). It may often be true that increased biodiversity decreases disease risk in this way (Ostfeld and Keesing, 2000; Johnson et al., 2008; Ostfeld and Keesing, 2012; Vourc'h et al., 2012; Johnson et al., 2013).

These studies suggest that the 'sink' hypothesis may be important if we are to understand wildlife disease ecology in invasive systems. Nonetheless, the literature on ecological impacts of invasive species rarely considers the 'sink' mechanism. It may have been ignored because it is counter-intuitive; researchers may expect a detrimental impact from an invader, and hence focus their work on detecting such effects.

The toad-frog-lungworm interaction in Australia may offer an excellent model for further research on the 'sink' mechanism. For example, it would be logistically feasible to repeat our studies in small containers with different densities of the two hosts or in large outdoor enclosures under more natural conditions. It would also be interesting to test the reciprocal scenario (native frogs infected with the cane toad lungworm, *R. pseudosphaerocephala*) to see if native species act as a 'sink' for cane toad lungworms, or tend to 'spillback' the parasite to cane toad populations.

The absence of histological evidence of larval penetration in all the anurans examined may be attributable to several factors. Some larvae taken up from the infection arena may have been unable to penetrate the skin, being weakened or killed by antimicrobial peptides in the host's skin secretions (Bowie and Tyler, 2006). For instance, only approx. 70–75% of *Rhabdias* spp. larvae that reach the host are able to penetrate the skin (Gendron et al., 2003; Kelehear et al., 2012). The serial sections cut from each anuran were 2 mm apart and could have missed intersecting the larvae, which are only 1000 μm long and 50 μm in diameter. Our purpose in conducting histological examinations was not to quantify rates of larval penetration in each individual, but only to verify that the experimental conditions enabled successful infection.

In contrast to our experiments on the 'sink' hypothesis, our attempt to test the 'priming' mechanism showed no effect. There are many cases in immunology where initial exposure to a pathogen enables an animal to later recognise and reject a similar but not identical pathogen (this is the principle behind vaccines: Brunham and Coombs, 1998; Oettinger et al., 1999; Hooper et al., 2004). However, our experiments suggest that prior exposure to *R. hylae* has no effect on cane toad resistance towards *R. pseudosphaerocephala*. Histological studies (Nelson et al., 2015b) demonstrate that cane toads do mount an immune response to *R. hylae* larvae but the current experiment indicates that this activation of the toad's immune system does not immunise toads against subsequent attack by larvae of its co-evolved parasite, *R. pseudosphaerocephala*.

Although amphibians possess adaptive immune systems broadly similar to those seen in avian/mammalian species, some

components are lacking (Fournier et al., 2005) and therefore the efficacy of the system is in question (Hsu, 1998). However, acquired immunity in amphibians can reduce the intensity and impact of subsequent re-infections; frogs with previous exposure to chytridiomycosis are better able to survive re-infection than are immunologically naïve frogs (Richmond et al., 2009). Similarly, acquired immunity in *Xenopus laevis* due to prior infection with a monogenean (*Protopolystoma xenopodis*) increased resistance to re-infection (Tinsley et al., 2012). Given the long time frame in our experiment (45 days), we expected the first exposure to prime the toad's adaptive immune system to *Rhabdias* spp chemicals. This immunological memory of the parasite might then translate into efficacy against a similar invader. Contrary to this expectation, the initial exposure to *R. hylae* had no impact on the cane toad's resistance to secondary infection by a congeneric lungworm.

In summary, cane toads have the potential to act as 'sinks' for *R. hylae* and do not acquire immunity to their native lungworms through prior exposure to frog lungworms. This would be good news for native frogs if the advantage of reduced parasitism outweighed the stresses imposed by toads. However, the advantages of reduced parasitism may be low: Nelson et al. (2015a) showed that native frogs suffer few ill effects from infection by *R. hylae*. Also, cane toad invasion typically does not have major overall effects on the abundance or viability of frog populations (Shine, 2014). Thus, the influence of cane toads on native frogs populations via the disruption of host-parasite interactions (via 'sink' mechanism) may be minimal.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijppaw.2015.05.004.

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