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Stress and immunity at the invasion front: a comparison across cane toad (*Rhinella marina*) populations

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At an invasion front, energetic and physiological trade-offs may differ from those at the range-core as a result of selection for enhanced dispersal, combined with a low density of conspecifics (which reduces pathogen transmission and competition for food). We measured traits related to energy stores and immunity in wild cane toads (*Rhinella marina*) across a 750-km transect from their invasion front in tropical Australia, back into sites colonized 21 years earlier. Several traits were found to vary with population age; some linearly and others in a curvilinear manner. The relative size of spleens and fat bodies was highest in the oldest and newest populations, where rates of lungworm infection were lowest. Toads from older populations produced more corticosterone in response to a standardized stressor, and had higher lymphocyte counts (but lower basophil counts). The amount of skin swelling elicited by phytohaemagglutinin injection did not vary geographically, although recruitment of leukocytes to the injected tissue was higher in toads from long-colonized areas. Because this was a field-based study, we cannot differentiate the effects of population age, toad density or pathogen pressure on our measures of stress and immune responses, nor can we distinguish whether the causation involves hard-wired adaptive processes or phenotypically plastic responses. Nonetheless, our data demonstrate substantial variation in immune systems among toads at varying distances from an invasion front, showing that a biological invasion imposes strong pressures on physiological systems of the invader. © 2015 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2015, **00**, 000–000.

ADDITIONAL KEYWORDS: amphibian – *Bufo marinus* – fat bodies – immunocompetence – immune response – *Rhabdias pseudosphaerocephala* – spleen size.

INTRODUCTION

Eco-evolutionary conditions on invasion fronts differ dramatically from those experienced by non-expanding populations (Phillips, Brown & Shine, 2010a; Zalewski & Bartoszewicz, 2012; Therry *et al.*, 2014b). Perhaps most fundamentally, populations on the expanding range edge are (by definition) at a lower density than those in the core of the range. This low density has clear ecological implications. Because competition with conspecifics is lessened, individuals on the range edge have greater resources available to them and so may be fitter than counterparts in the range core (Brown, Kelehear & Shine, 2013). Second, range-front individuals are likely to suffer less parasitism than range-core individuals because of serial founder events at the invasion front (particularly if uninfected individuals are better dispersers) and also (possibly) lowered transmission rates at low host density (Knolle, 1989; Phillips *et al.*, 2010c; Kelehear, Brown & Shine, 2012). The strong density gradient on the invasion front also has evolutionary implications: invasion front populations will tend to be *r*-selected rather than *K*-selected (Phillips, Brown & Shine, 2010b), and several evolutionary forces will combine to create upward pressure on dispersal rates (Travis

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& Dytham, 2002; Duckworth & Badyaev, 2007; Shine, Brown & Phillips, 2011; Perkins *et al.*, 2013).

As we move from the invasion front through to the core of a species' range, we can imagine moving through three stages of colonization. First, we encounter the low-density front. Here, we should see highly dispersive individuals, largely free of parasites and competitors. Second, as we move further into the range, population density increases, competition becomes important, and parasites become prevalent. In this second stage, we might also expect to see the greatest mismatch between environment and phenotype. Behind us are populations adapted to conditions on the invasion front; in front of us are populations adapted to conditions in the core of the range; around us now, however, is a population in flux: it is largely adapted to the range-front conditions, although it is no longer experiencing them. Here, we might expect to see the greatest effects of population density on fitness as genotypes not equipped for parasitism and competition encounter precisely these agents of selection. Finally, as we move further back into the range, we eventually come to a third, 'equilibrium' stage. Here, we have equilibrium density and rates of parasitism; the invasion front is now sufficiently far away that individuals are adapted to these equilibrium conditions.

Because population density can influence the nature and strength of selection acting on a population, and also affect phenotypes directly (e.g. through competition and pathogen exposure), we would predict the three stages of colonization to manifest in different organism phenotypes. The immune system is an especially promising trait to detect this variation because it is largely shaped by the factors identified above; resource availability, pathogen exposure, and dispersal. First, food restriction arising at high conspecific density could reduce the amount of energy available for the production of immune cells and compounds (Martin *et al.*, 2007; Beldomenico & Begon, 2010; Neuman-Lee *et al.*, 2015). Second, the immune system is affected by the level of pathogen exposure an individual experiences (Vaclav, Calero-Torralbo & Valera, 2008). Rates of pathogen infection should be higher in areas where post-colonization host density is high (Knolle, 1989; Bull, 1994; Phillips *et al.*, 2010c). In addition to host density effects, individuals at the forefront of expanding ranges may be exposed to lowered pathogen levels if the arrival of parasites lags behind that of the host (Phillips *et al.*, 2010c). Individuals that are exposed to many conspecifics shedding pathogens are likely to develop different immune responses than individuals that rarely encounter pathogens (Horrocks, Matson & Tieleman, 2011). Third, the evolution of faster dispersal rates might favour reduced investment into energy-expensive immune processes that conflict with dispersal (Burton, Phillips & Travis, 2010; White & Perkins, 2012). Thus, range-edge individuals may exhibit modifications to the immune response relative to individuals from longer established sites where dispersal rates are lower (Brown *et al.*, 2015). Selection on the invasion front could also act to down-regulate immune function to decrease the risk of inappropriately severe responses to novel (and nondangerous) pathogens, or of autoimmune response to muscle damage arising from sustained physical activity (Lee & Klasing, 2004; Martin *et al.*, 2010; White & Perkins, 2012; Brown & Shine, 2014). Alternatively, immune investment might be increased among invasion front individuals if range-expansion results in exposure to dangerous novel pathogens (Therry *et al.*, 2014a).

Stress is another factor with known immunomodulatory effects (Graham *et al.*, 2012), which could vary with colonization time and animal density. Novel challenges imposed by range expansion (as a result of extreme physical activity, or encountering new predators, competitors and habitats) might also favour shifts in the invader's physiological response to stressors (Liebl & Martin, 2012; Therry *et al.*, 2014a, b). By analogy, animals from urban environments (where they frequently encounter anthropogenic stressors) alter the amount of corticosterone that they release in response to stress (Partecke, Schwabl & Gwinner, 2006).

Previous investigations into the effects of invasion on physiology, immunology and pathogens have used interspecific comparisons between invasive species and related non-invasive taxa (Lee, Martin & Wikelski, 2005; Martin *et al.*, 2010; Coon *et al.*, 2014) or compared invasive taxa in their native vs. introduced ranges (Torchin *et al.*, 2003; Roche *et al.*, 2010). To understand the dynamics playing out on invasion fronts, however, a more robust approach is to make intraspecific comparisons along gradients from the invasion front through successively older populations towards the range core (Coon *et al.*, 2014; Martin & Liebl, 2014; Martin *et al.*, 2014; Martin, Liebl & Kilvitis, 2015). In the present study, we use the latter approach to identify *in situ* spatial patterns and interactions among immune measures, energy stores, stress response, and pathogen exposure in cane toads (*Rhinella marina*) across their invasive range in tropical Australia.

MATERIAL AND METHODS

Study animals

Cane toads are large (typically with an adult mass in the range 100–300 g) (Fig. 1) bufonid anurans native

Figure 1. Cane toads *(Rhinella marina)* were introduced into eastern Australia in 1936.

to South and Central America (Lever, 2001). In 1935, the progeny of 101 adult toads (imported from the Caribbean, via Hawaii) were released in north-eastern Queensland in an attempt to control insect pests of commercial agriculture (Easteal, 1989). Cane toads have now spread westwards across Queensland and the Northern Territory and into Western Australia (a straightline distance of > 2000 km). The tropical (west-moving) invasion front has accelerated from approximately 15 km per annum in the decades post-release, to approximately 60 km per annum in recent years (Phillips *et al.*, 2006). That acceleration has been facilitated by shifts in dispersal behaviour (e.g. path straightness, time spent in dispersive mode: Brown, Phillips & Shine, 2014; Lindström *et al.*, 2013) and morphology (relative limb length; Phillips *et al.*, 2006). A significant heritability of overall dispersal rates and path straightness, combined with standardized dispersal trials of toads collected from different regions, indicates that the acceleration of toad dispersal rate is driven by evolutionary processes rather than phenotypic plasticity (Phillips *et al.*, 2008, 2010a; Brown *et al.*, 2014).

Study area

We worked in the wet–dry tropics of the Northern Territory, where the predominant habitat is savannah woodland. In this region, cane toads are inactive during the long annual dry-season (broadly, May to November) and disperse only when monsoonal rains provide moist soil (Brown, Kelehear & Shine, 2011a). At the time of our collections in early June 2009, none of the sites had experienced any rain for 2 months. During 6–11 June 2009, we collected and sampled cane toads from eight sites across the Northern Territory (Fig. 2). The collection transect ran approximately east–west and spanned a distance of 750 km. At the time of the collections, the toad population at the easternmost site, Borroloola, had been established for 21 years, whereas the westernmost site, Keep River, was at the front line of the invasion.

Data collection

Data from toads collected across the invasion transect were used to assess spatial patterns in morphology, physiology, and pathogens. Toads were captured by hand at night from areas such as drying riverbeds or watered lawns, where toads congregate to hydrate. We did not attempt to quantify density at our collection sites because many of them were intermittently 'toad-busted' by community groups attempting to stop the spread of toads by catching and killing them. Although toads were still abundant at all sites at the time of our collections, estimates of local densities might not have reflected broader densities. Data in the present study are based on 201 toads collected from the eight sites (four to 47 toads per site). After capture, toads were kept in damp linen bags, inside plastic bins in an air-conditioned room. The bags were cleaned and moistened daily. Three to 4 days after capture, all toads were killed by an overdose of pentobarbital, weighed, measured for snout-to-urostyle length and systematically dissected. Spleens and fat bodies were excised, patted dry and weighed. Lungs were removed, opened and inspected for the presence of parasitic rhabditid lungworm nematodes (*Rhabdias pseudosphaerocephala*; Kelehear *et al.*, 2012). The stomach and intestines were inspected and we counted the number of encysted spirurid nematode larvae visible in these tissues (Kelehear & Jones, 2010). For each type of parasite, we defined prevalence as the percentage of individual toads carrying at least one lungworm/spirurid cysts, and abundance as the total number of each parasite per toad (including both infected and uninfected individuals; Bush *et al.*, 1997). A summary of the measures from each site is provided in the Supporting information (Table S1). From a subsample of these 201 toads, we also collected data on immune-related measures, as described below.

White blood cell differentials and corticosterone stress response

At four sites (Keep River, Baines River, Victoria River, and Borroloola), we obtained blood samples immediately upon capture for nine or 10 toads per site. Approximately 0.1 mL of blood was obtained via

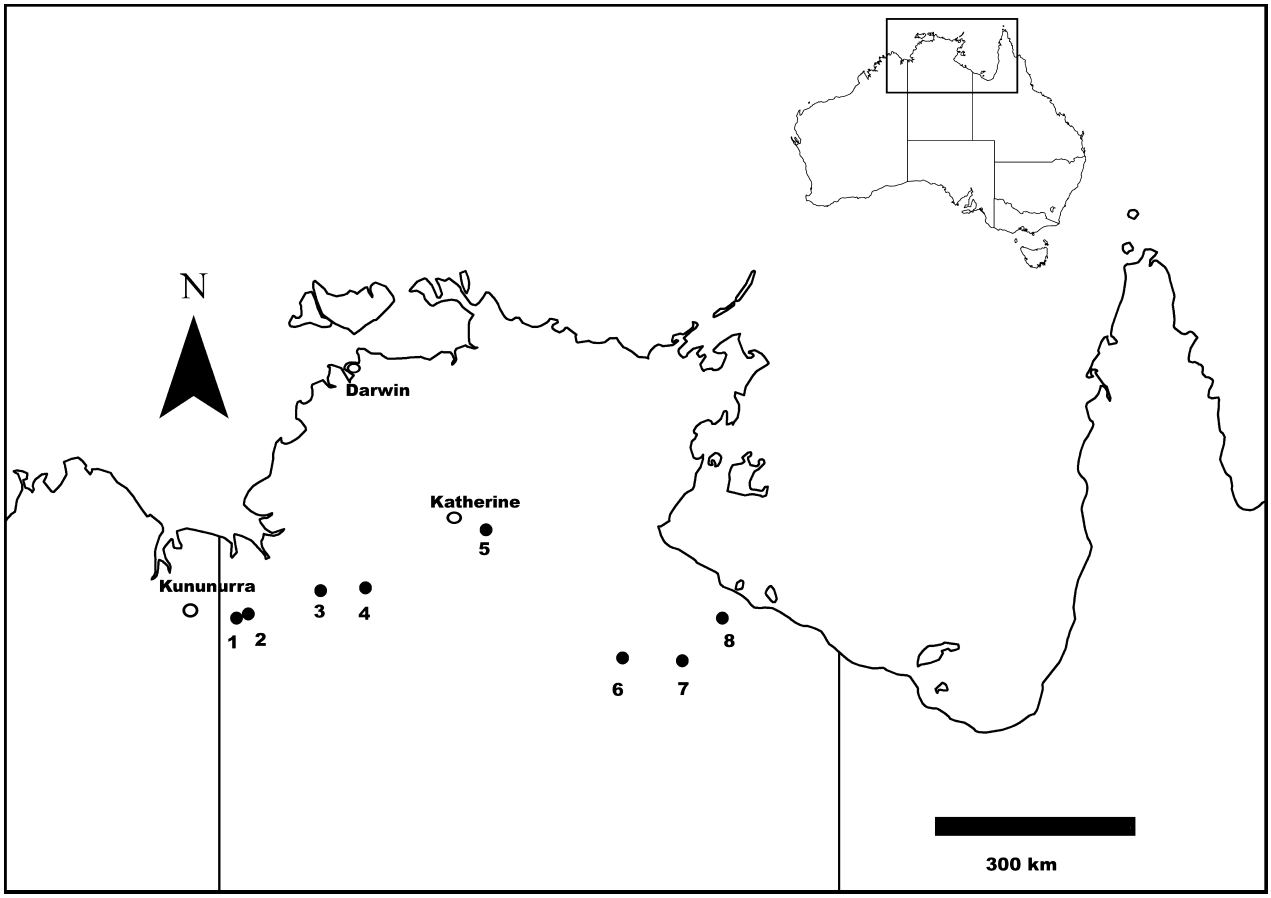

Figure 2. Map of collection sites across northern Australia. Location (and year of toad arrival): 1, Keep River (2009); 2, Baines River (2008); 3, Timber Creek (2006); 4, Victoria River (2005); 5, Mataranka (2000); 6, October Creek (1995); 7, Cape Crawford (1993); 8, Borroloola (1988).

cardiac puncture within 3 min of capture using syringes fitted with 27-gauge needles. A thin smear was prepared from each sample and the remaining blood placed into a 1-mL serum separator vial (Microtainer; Becton-Dickinson). Blood was allowed to clot for 30 min and then centrifuged at 1300 *g* for 10 min. Serum was collected and immediately frozen at −20 °C. Following capture, toads were held overnight in individual damp cloth bags. After 12 h, toads were removed from their bags and a second 0.1-mL blood sample was obtained, as described above (Graham *et al.*, 2012). Serum collected from the initial blood sample was used to measure baseline levels of corticosterone (CORT). Serum from the second sample was used to measure post-stress CORT levels, with the stressor being 12 h of captivity in a damp cloth bag. One week after the last collection, baseline and post-stress samples from 19 toads (four or five toads per site) were randomly selected to have CORT concentrations measured. The 38 samples were run in duplicate on a single plate of a commercial enzyme immunoassay kit (Corticosterone HS EIA; ImmunoDiagnostic Systems Ltd), which has been validated for use on cane toads (Jessop *et al.*, 2013). Because we only had access to a single assay kit, there was a limit to the number of samples we were able to run.

Thin smears prepared from the initial blood samples were air-dried, fixed with methanol and stained with Giemsa. They were fitted with cover slips and examined under × 1000 and 100 white blood cells (WBC) categorized as either basophil, eosinophil, monocyte, lymphocyte or neutrophil (Brown & Shine, 2014). Circulating WBC populations consist of numerous cell types and their relative proportions are not independent of each other. Therefore, we used principal component (PC) analyses to reduce the dimensionality of the five WBC cell types to a single variable. We then used PC1 as a dependent variable to formally assess the effects of population age on circulating WBC populations, and also carried out separate analyses on each cell type for illustrative purposes.

We also calculated the ratio of red blood cells (RBC) to WBCs on each smear. Accordingly, we

re-examined each slide and counted the number of red and white cells (not differentiated by type) in each field of view until at least 1000 RBCs had been counted. We then divided the WBC count by the RBC count to estimate WBCs per RBC.

Phytohaemagglutinin (PHA) skin-swelling assay and biopsies

PHA is a protein derived from kidney beans and acts as a mitogen on lymphocytes. Quantifying the swelling elicited by intradermal injection of PHA is a commonly-used technique for assaying T-cell mediated immune responses in animals (Vaclav *et al.*, 2008; Brown, Shilton & Shine, 2011b). We subjected a total of 70 toads (four to 12 individuals from each of the eight sites) to a PHA skin-swelling assay (Brown *et al.*, 2011b). None of the toads used for this assay had been blood-sampled (see above). After capture, toads were held overnight in damp cloth bags. The next morning, the thickness of the webbing between the second and third toes of each hind foot was measured using a dial thickness gauge (Peacock G1-A; Ozaki Manufacturing Ltd). Then, 0.05 mL of a 2 mg mL^{-1} solution of PHA (Sigma L8754), dissolved in sterile phosphate-buffered saline (PBS), was injected into the webbing of the right rear foot and 0.05 mL of sterile PBS was injected into the webbing of the left rear foot. Toads were returned to their cloth bags, and the thickness of the injected toe webs re-measured after 24, 48 and 72 h.

After the 72-h measurement, these toads were humanely killed and systematically dissected, as described above. Skin biopsies (diameter 3 mm) were taken from injected toe webs of interest (centred on points of injection) and fixed in 10% formalin. Biopsies were later sectioned and stained (for details, see Brown *et al.*, 2011b). Histological sections of toe webs were scanned blindly at × 400 and we counted the number and types (eosinophil, lymphocyte, macrophage, neutrophil) of all infiltrating WBCs observed in four to 10 adjacent fields. Cell counts were averaged across all fields of view for each biopsy and the mean proportion of each cell type was calculated. For each toad, values of averaged total WBC counts and mean proportions of each cell type in PHA biopsies were subtracted from the values from its PBS biopsy. These differences were used as dependent variables in further analyses on the effect of population age on WBC responses to PHA injection.

Statistical analysis

We ln-transformed body size, organ masses and parasite abundances prior to analyses to meet regression assumptions. Because the sudden arrival of cane toads at a location is both noticeable and notable, there are reliable records of arrival dates across the collection transect (Fig. 2). To assess the effect of invasion history on the traits of interest, we used the age of the population at each site (based on the date of toad arrival) as a continuous independent variable in our analyses.

For some dependent variables, visual inspection of scatterplots suggested curvilinear relationships with population age. In these cases, a quadratic term for population age was added to the appropriate logistic or least-squares regression models. In addition, morphological and immune traits often vary with sex and body size in cane toads (Brown *et al.*, 2011b; Brown & Shine, 2014), and so we included these two variables as covariates in all analyses to correct for trait variation with body size or between sexes. To confirm that curvilinear relationships were not a result of small sample sizes at intermediate sites, we repeated analyses after pooling the three contiguous sites with lowest sample size (Mataranka, October Creek, Cape Crawford) into a single age group with population age of 12.5 years. These results were qualitatively identical to the results obtained without pooling sites, and so we present only the results of analyses of without pooling.

We used nonparametric Spearman correlations to assess relationships among infection intensities of parasites (*Rhabdias* and spirurid cysts) and organ masses (fat body and spleen). Prior to this analysis, we corrected organ masses for body size by taking the residuals from regressions of ln-transformed organ mass on ln-transformed snout-to-urostyle length.

We used repeated-measures analysis to assess whether the swelling response elicited by PHA varied over 72 h as a function of population age. For each toad at each time period, we subtracted the thickness of the PBS-injected web from the thickness of the PHA-injected web and used this difference as the dependent variable in our repeated-measures analysis.

Other analyses were carried out using multiple regression. In all cases, residuals were inspected for violations of regression assumptions. Analyses were performed using JMP, version 9 (SAS Institute) with an alpha level of 0.05.

RESULTS

Dissection data

After correcting for sex and body size, the mass of fat bodies and spleens both showed concave trends over population age (Fig. 3, Table 1). The relative sizes of these organs were higher at the invasion

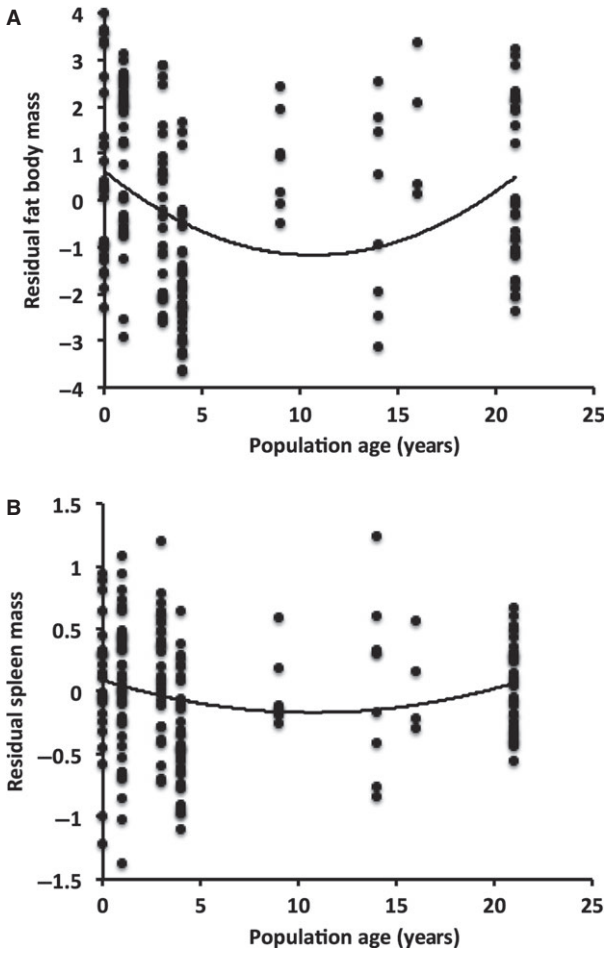

Figure 3. Curvilinear relationship between population age and (A) residual fatbody mass and (B) residual spleen mass of 201 cane toads collected along a 750-km transect.

front and in long-established populations, reaching minimum values at population age of approximately 10 years.

The prevalence and abundance of *Rhabdias* lungworms also varied in a curvilinear manner with population age, although in a manner opposite to fat body and spleen mass. *Rhabdias* was most common and most numerous in toads from populations aged approximately 9 years (Fig. 4, Table 2). The prevalence of spirurid larval cysts in toad viscera also varied with population age, although in a linear rather than curvilinear manner (Table 2). Infection with cysts was found less often in toads from near the invasion front than in older populations. The abundance of spirurid infections, however, was unrelated to population age (Table 2).

Spearman correlations relating parasite abundance to organ masses among individual toads indicated that relative spleen size was negatively related to the abundance of spirurid cysts ($N = 194$, $r = -0.17$, $P = 0.017$) but not related to the abundance of *Rhabdias* ($N = 194$, $r = -0.09$, $P = 0.19$). Relative fat body size was negatively correlated with *Rhabdias* abundance ($N = 183$, $r = -0.24$, $P = 0.001$) but unrelated to the abundance of spirurid cysts ($N = 183$, $r = -0.09$, $P = 0.22$). Relative masses of fat bodies and spleens were positively related to one another ($N = 183$, $r = 0.63$, $P < 0.0001$).

Table 1. Effects of sex, size, and population age on organ weights of cane toads collected along a transect through an invasion front

Dependent variable	Independent variable	Estimate	*F*	*P*
ln fat	Sex	0.043	0.09	0.7697
	ln SUL	5.900	53.02	**< 0.0001**
	Population age	−0.138	11.48	**0.0009**
	Population age*	0.016	14.80	**0.0002**
ln spleen	Sex	−0.058	2.36	0.1265
	ln SUL	3.559	294.38	**< 0.0001**
	Population age	−0.021	3.61	0.0591
	Population age*	0.002	4.04	**0.0459**

The mass of fat bodies and spleens were related to body size (larger toads had heavier organs). Values shown in bold indicate significance at $P < 0.05$.
*Denotes the quadratic term for population age.
SUL, snout-to-urostyle length.

Corticosterone stress response

Baseline CORT levels of the 19 sampled toads averaged 15.6 ng mL^{-1}, increasing to a mean of 37.9 ng mL^{-1} after 12 h in a damp bag. Neither baseline, nor post-stress CORT level was related to sex (both $F_{1,17} < 0.61$, $P > 0.45$) or body size (both $F_{1,17} < 3.0, P > 0.10$). Analysis of covariance with time (baseline vs. post-stress) as the factor and population age as the covariate produced a significant interaction ($F_{1,17} = 6.05$, $P = 0.025$) (Fig. 5). Post-hoc simple linear regressions indicated that, although baseline CORT levels were not related to population age ($F_{1,17} = 0.36$, $P = 0.56$), post-stress CORT levels increased with population age ($F_{1,17} = 5.17$, $P = 0.036$); toads from older populations produced more CORT in response to restraint in a damp bag.

Circulating WBC differentials

The 37 blood smears from toads collected from four sites contained (on average): 15% basophils, 8%

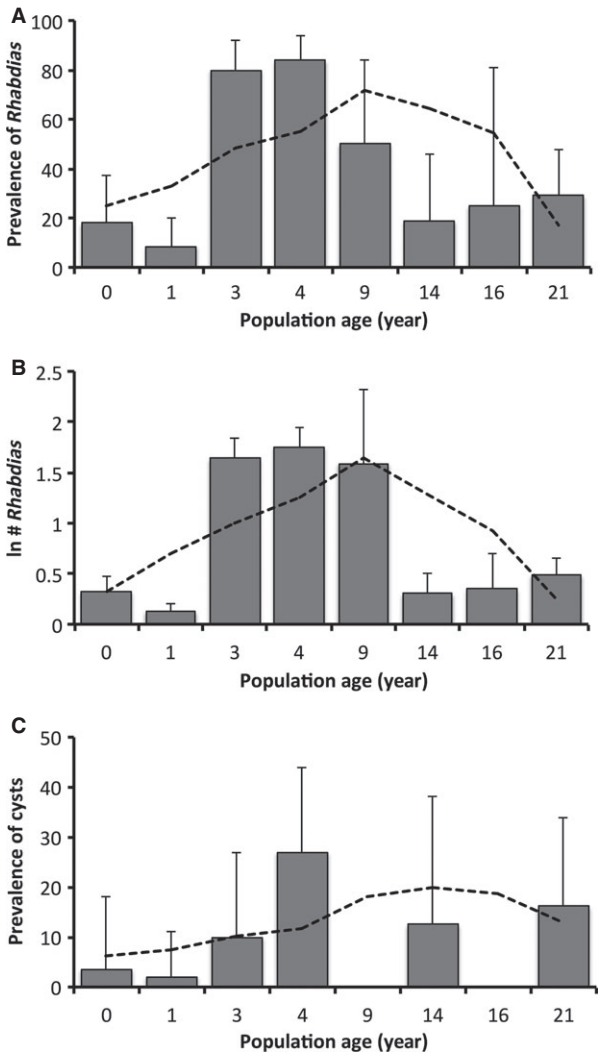

Figure 4. Variation in (A) prevalence and (B) abundance of lungworm (*Rhabdias pseudosphaerocephala*) infections, and (C) prevalence of spirurid cyst infections across a cane toad invasion transect. Dashed lines indicate fitted values from polynomial regressions for each trait, modelling population age as a continuous trait. Bars are for illustrative purposes and denote mean values at each site, with errors bars indicating upper 95% binomial confidence values for prevalence data and the SE for abundance data.

eosinophils, 1% monocytes, 55% lymphocytes, and 20% neutrophils. We used PC analysis to reduce dimensionality of these five WBC types, and the resulting PC1 explained 40% of the variation in differential counts. PC1 described blood with high proportions of basophils, eosinophils, and neutrophils (loadings of 0.67, 0.50, and 0.55, respectively) but a low proportion of lymphocytes (loading = −0.98).

Neither sex, nor body size affected WBC differentials and so these factors were removed from the final multiple regression model. The concentration of WBCs relative to RBCs, however, was a significant covariate in the model (Table 3), with lower values of PC1 associated with higher numbers of WBC per RBC. After correcting for WBC concentration, we found a significant decrease in PC1 with population age (Fig. 6, Table 3). Repeating the analysis on individual cell types confirmed the PC1 results; the proportion of lymphocytes increased with population age, whereas the proportion of basophils decreased (Fig. 6, Table 3).

Table 2. Effects of sex, size and population age on parasite prevalence and abundance in cane toads collected along a transect through an invasion front

Dependent variable	Independent variable	Test statistic and value	*P*
Prevalence			
Rhabdias	Sex	$\chi^2 = 0.07$	0.7866
	ln SUL	$\chi^2 = 13.13$	**0.0003**
	Population age	$\chi^2 = 13.02$	**0.0003**
	Population age*	$\chi^2 = 21.37$	**< 0.0001**
Spirurid	Sex	$\chi^2 = 0.15$	0.6984
cysts	ln SUL	$\chi^2 = 13.98$	**0.0002**
	Population age	$\chi^2 = 3.95$	**0.0468**
	Population age*	$\chi^2 = 2.38$	0.1225
Abundance			
ln	Sex	$F_{1,196} = 0.01$	0.9081
Rhabdias	ln SVL	$F_{1,196} = 9.29$	**0.0026**
	Population age	$F_{1,196} = 14.84$	**0.0002**
	Population age*	$F_{1,196} = 25.25$	**< 0.0001**
ln spirurid	Sex	$F_{1,196} = 0.13$	0.7195
cysts	ln SUL	$F_{1,196} = 8.87$	**0.0033**
	Population age	$F_{1,196} = 2.18$	0.1415
	Population age*	$F_{1,196} = 0.67$	0.4139

Values shown in bold are significant at $P < 0.05$.
SUL, snout-to-urostyle length.
*Quadratic term for population age.

PHA SKIN-SWELLING ASSAY

We used repeated-measures analysis to assess whether population age affected the relative amount of toe-web swelling elicited by injection of PHA (vs. that elicited by injection of PBS) measured at 24, 48, and 72 h post-injection. Covariates in the model included sex and body size. After 24 h, toe webs injected with PHA had increased in thickness by 15% on average, whereas toe webs injected with PBS had only increased in thickness by 1.8%. Swelling in PHA webs then subsided to 13.7% and 8.8% after 48 and 72 h. PBS webs after 48 and 72 h were 0.2% and 0.9% of their original thicknesses. However, the

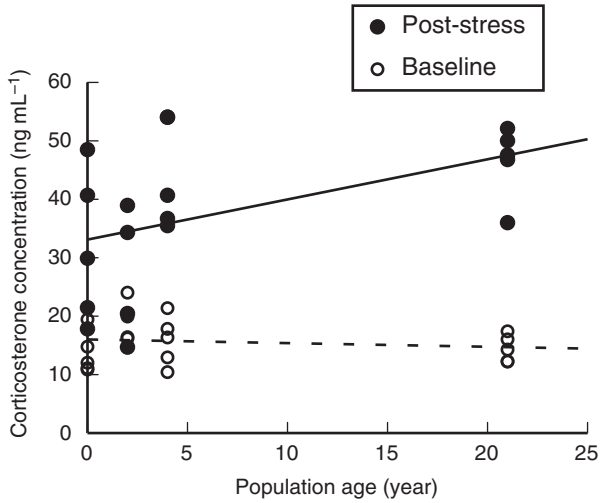

Figure 5. Relationships between baseline (dashed line, open symbols) and post-stress (solid line, closed symbols) corticosterone levels and population age. Baseline corticosterone levels were similar among sites, although post-stress levels were higher in longer-established populations.

Table 3. Effects of population age on circulating white blood cell (WBC) populations in cane toads

Dependent variable	Population age	WBC per 1000 RBC
PC1	$F_{1,34} = 4.96$	4.48
	$P =$ **0.0327**	**0.0417**
% Neutrophils	$F_{1,34} = 0.43$	3.96
	$P = 0.5160$	0.0546
% Monocytes	$F_{1,34} = 2.17$	2.08
	$P = 0.1503$	0.1586
% Lymphocytes	$F_{1,34} = 4.15$	6.89
	$P =$ **0.0494**	**0.0129**
% Eosinophils	$F_{1,34} = 0.35$	1.97
	$P = 0.5604$	0.1690
% Basophils	$F_{1,34} = 5.03$	5.80
	$P =$ **0.0316**	**0.0216**

The concentration of white blood cells, relative to red blood cells (RBC), was used as a covariate in the analyses. Values shown in bold are significant at $P < 0.05$. PC, principal component.

repeated measures analysis indicated no significant effect of population age on toe-web swelling, neither as a main effect ($F_{1,175} = 0.03$, $P = 0.87$), nor in interaction with time ($F_{3,198} = 1.55$, $P = 0.20$). The only significant effect in the model was an interaction between time and body size, with larger toads showing more swelling at later time periods ($F_{3,198} = 3.30$, $P = 0.022$).

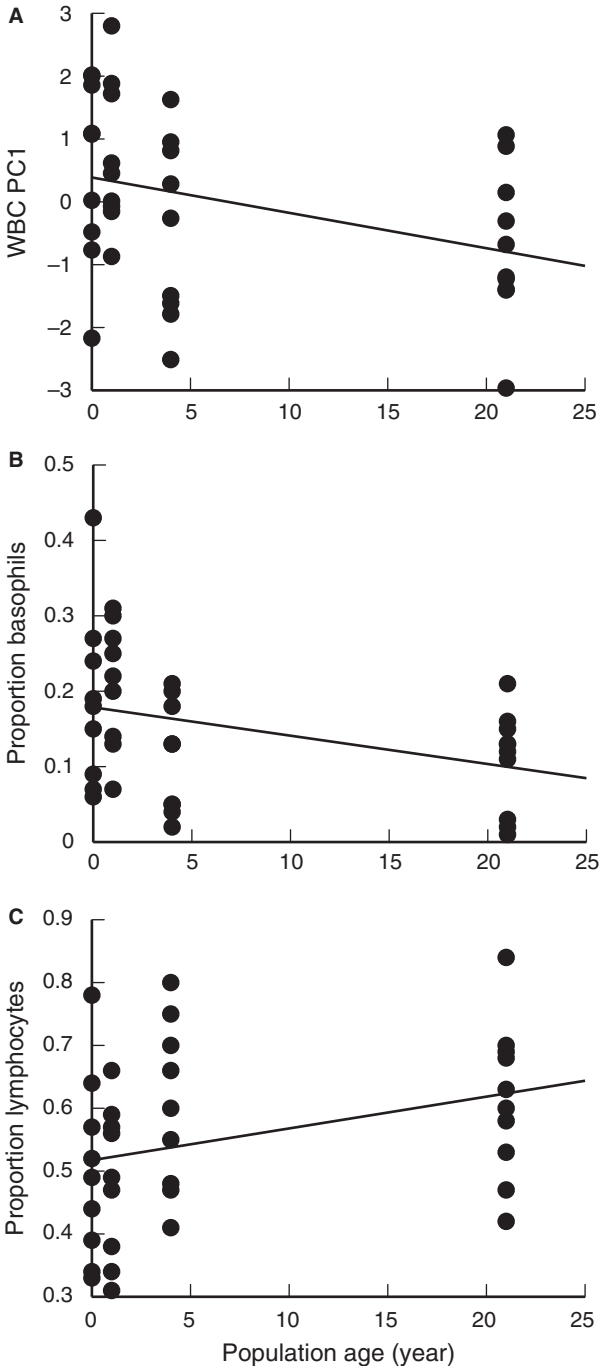

Figure 6. Changes in circulating white blood cells with population age. A, first principal component formed from proportions of five cell types. B, basophils. C, lymphocytes.

WBC differentials from toe-web biopsies

On average, biopsies of toe webs injected with PBS contained 74 WBCs, whereas those injected with PHA contained 311 WBCs. The difference in the total number of WBCs recruited to PHA vs. PBS

injected toe webs varied with population age but not with sex or body size (Table 4). At 72 h post-injection, toads from longer-established populations had recruited significantly more WBCs to toe webs injected with PHA (compared to webs injected with PBS) than had toads from closer to the invasion front (Table 4). However, the composition of the cell types recruited to injection sites did not vary with population age, nor with sex or body size (i.e. changes in the relative proportions of the different cell types between PBS and PHA webs were consistent across populations) (Table 4).

DISCUSSION

Our data support the a priori prediction of substantial variation in physiological traits among populations of an invading species as they transition between the three stages of colonization. More interestingly, the actual form of that variation differed among traits, and may have been influenced by a combination of evolutionary (genetically-based, canalized) shifts (Easteal, 1989; Shine *et al.*, 2011) and phenotypically plastic responses to local conditions (Brown *et al.*, 2013). Some traits showed curvilinear relationships with population age: the relative size of spleens and fat bodies was highest in old and new populations, where rates of lungworm infection were lowest. Other traits exhibited significant linear trends across the invasion front: toads from older populations produced more corticosterone in response to stress, had blood that contained more lymphocytes but fewer basophils, and recruited more WBCs to tissue injected with PHA. These linear trends appear in variables for which data from intermediate sites are sparse (Figs 5, 6), and additional data from these sites might reveal these linear relationships to be curvilinear.

Table 4. Effects of sex, body size, and population age on the difference in white blood cells (WBC) recruited to toe webs injected with phytohaemagglutinin vs. phosphate-buffered saline

Dependent variable	Independent variable	*F*	*P*
Difference in total WBCs	Sex	3.33	0.0703
	ln SUL	0.05	0.8169
	Population age	9.47	**0.0030**
Difference in percentage of eosinophils	Sex	0.62	0.4427
	ln SUL	0.24	0.5243
	Population age	1.91	0.1699
Difference in percentage of lymphocytes	Sex	0.51	0.4856
	ln SUL	0.09	0.7972
	Population age	0.00	0.9794
Difference in percentage of macrophages	Sex	1.12	0.3057
	ln SUL	2.85	0.0815
	Population age	2.12	0.1431
Difference in percentage of neutrophils	Sex	2.23	0.1458
	ln SUL	1.28	0.2566
	Population age	0.15	0.6905

Values shown in bold are significant at $P < 0.05$.
SUL, snout-to-urostyle length.

The curvilinear relationships observed in several traits likely reflect the mismatch between an invasion front phenotype and a post-colonization environment: a phenotype evolved for low density conditions where dispersal is the primary arbiter of fitness but which is now subject to high density conditions that cannot be escaped by dispersing. Although we have no data available on the absolute densities of toads along our transect, invasive species typically show a 'travelling wave' density pattern, with low density at the front, high density for several years post-colonization, and then a decrease (Simberloff & Gibbons, 2004; Hilker *et al.*, 2005). This pattern holds for toads also (Freeland, Delvinqueir & Bonnin, 1986; Brown *et al.*, 2013) and is to be expected given their extremely high reproductive rate. It is likely, therefore, that toad densities were lowest at sites closest to the invasion front and at the longest established sites, and highest at intervening sites.

Information on the pathogen pressure to which individuals are exposed can allow variation in immune measures to be put in context (Horrocks *et al.*, 2011). However, in field situations, pathogen density can co-vary with other factors that could influence immune configuration (Beldomenico & Begon, 2010). In the present study, the low occurrence of *Rhabdias* at the oldest and youngest sites is consistent with the expectation that high toad density facilitates parasite transmission (Freeland *et al.*, 1986; Kelehear *et al.*, 2012). In addition, the low incidence of *Rhabdias* at the youngest populations is perpetuated by the occurrence of a lag in their spread relative to the spread of their host (Phillips *et al.*, 2010c). The spatial pattern of spiruid infections (prevalence increases linearly with population age, no trend in abundance) is unlikely to reflect patterns of toad abundance because, unlike *Rhabdias*, these parasites are not toad-specific and toads are not the final host (Kelehear & Jones, 2010). Spirurids normally use an arthropod intermediate host to reach a final reptile, bird or mammal host. Toads or other amphibians may act as paratenic hosts after eating an infected arthropod, although many infecting larvae are destroyed by the toads' immune system (Kelehear & Jones, 2010). A spatial gradient

may exist in the abundance of spirurid parasites among their native hosts, as occurs in toads.

The opposing curvilinear relationships between population age and spleen mass on the one hand, and *Rhabdias* infection on the other, suggest an association between high parasite exposure and decreased immune products. This interpretation is backed up by the direct negative correlations between spirurid cyst abundance and relative spleen mass observed among the pooled sample of toads. Although these results suggest that pathogen presence causally affects spleen mass, energetics may also be a causative factor. Spleen size and fat body size were positively correlated and, at sites where toads had small spleens, they also had small fat stores. The negative relationship between toad density and energy stores arises through conspecific competition for food. Studies through time at a Northern Territory site showed higher rates of food intake and larger fat bodies in toads at the invasion front compared to the same site a few years later when density was higher (Brown *et al.*, 2013). The observed shift in spleen size could result either from a positive association with body condition or a negative association with parasite exposure (Vicente, Pérez-Rodríguez & Gortazar, 2007; Schulte-Hostedde, Bowman & Nituch, 2012). Although relative spleen size is often used as a gross measure of immunocompetence (Morand & Poulin, 2000; Corbin *et al.*, 2008; Koprivnikar & Leung, 2015), the causes and effects of mass variation are difficult to disentangle in an organ with such a multitude of functions (Smith & Hunt, 2004; Vicente *et al.*, 2007; Koprivnikar & Leung, 2015). Changes in relative spleen mass observed in the present study appear to be influenced by conspecific density, although experimental manipulations are required to determine whether the mechanism is energetic (e.g. decreased energy balance as a result of competition for food) or immunological (e.g. increased exposure to pathogens at high density: Horrocks *et al.*, 2011; Vicente *et al.*, 2007). Thus, we are unable to distinguish between the scenario that toads with relatively large spleens can better resist infection with spirurid cysts through some immune mechanism, or that spleen size decreases as a result of heavy spirurid infection (or even that spleen size and cyst number are functionally independent but linked through inverse correlations with a third factor such as density or body condition; Beldomenico & Begon, 2010).

Because of the immunosuppressive effects of CORT in cane toads (Graham *et al.*, 2012), variation in stress response is potentially linked to spatial patterns of parasite infection and immune measures. Toads at the invasion front disperse long distances (up to 2 km) almost daily, constantly encountering unfamiliar places and conditions (Brown *et al.*, 2006). Thus, this lifestyle could impose stressors that would not be experienced by individuals from long-established populations (Brown *et al.*, 2007; Therry *et al.*, 2014b) and be confirmed by higher CORT levels among frontal animals. For example, house sparrows (*Passer domesticus*) from range-edge populations exhibit greater CORT increases following a standard stressor than do individuals from longer established populations (Liebl & Martin, 2012; Martin & Liebl, 2014). This hyper-responsiveness of range-edge sparrows was interpreted as a means to enhance the identification of stressors and subsequently avoid them.

However, recent work has not supported our prediction that frontal toads have elevated stress levels or responses. First, we found no link between movement rate and baseline or post-stress CORT levels among radio-tracked toads (Brown & Shine, 2014). Second, a study on laboratory-reared (common-garden) offspring of toads, collected from across Australia, found no effect of parental origin (i.e. frontal vs. established populations) on baseline or post-stress CORT levels of the progeny (Brown *et al.*, 2015). Combined, these findings suggest, surprisingly, that baseline and post-stress CORT levels are affected neither by the physical act of rapid dispersal exhibited by frontal animals, nor by parental invasion history.

Why then did we observe stress response increasing with population age? Baseline CORT levels of invasive cane toads increase in response to stressful (i.e. hot, dry) environmental conditions (Jessop *et al.*, 2013). The consistency in baseline CORT levels across our sampling sites suggests that environmental conditions were similar among sites. Nonetheless, after spending 12 h in a damp bag, toads from older populations produced more CORT than did frontal toads. One possible explanation is that frontal toads may have dampened their CORT response to stress. Plausibly, this physiological tolerance of stressful situations (as opposed to stressful environments) is a result of the high rate at which the frontal toads encounter novel stressors because they are continuously exposed to unfamiliar habitats during dispersal. Similarly, dampened stress responses have been described in birds from urban settings compared to individuals from rural settings (Partecke *et al.*, 2006; Liebl & Martin, 2012). This pattern has been attributed to animals in urban settings habituating to frequent anthropogenic stressors and thus decreasing their response to ubiquitous disturbances. The dampened response may have a genetic basis in some cases (Partecke *et al.*, 2006) but, in others, it may reflect flexibility that allows individuals to adjust to variable environmental

conditions (Coppens, de Boer & Koolhaas, 2010). This latter scenario is likely in cane toads, with the dampened post-stress elevation in CORT in frontal toads being a phenotypically plastic response to their dispersive, nomadic lifestyle, and constant exposure to new habitat. However, if this is the case, the dampened response is not quickly transient because, at the time of our sampling, toads would not been dispersing and would have inhabited the collection area for several weeks.

Among invasive house sparrows, stress response, spleen size, and immune configuration all vary with population age (Liebl & Martin, 2013; Martin *et al.*, 2014, 2015;. Variation in traits such as these may arise commonly where ranges are expanding, either through selection or plasticity. The main changes observed in immune characteristics across our study populations involved numbers and relative proportions of WBCs, either in circulation or recruited to tissue injected with PHA. Do these changes tell us something about trade-offs among immune system components wrought by selective or proximate factors during the process of invasion? Or do they instead reflect the effects of population density on physiological mechanisms or pathogen exposure? The latter explanation appears more likely, given that spleen size varied in a curvilinear fashion across populations, in a pattern that mirrored density. Because one of the functions of the spleen is to produce and release WBCs (Hansen & Zapata, 1998), a decrease in its size may affect the levels and types of these cells found in blood smears and tissue biopsies and such changes might alter immune function and response. The observed correlations among spleen size, fat body size, and parasite abundances are intriguing but difficult to interpret in the absence of experimental manipulation. Although field studies are useful for quantifying trait variation across an invasive range, controlled experiments are needed to distinguish canalized evolutionary mechanisms from environmentally-induced plasticity (Phillips *et al.*, 2010a; Brown *et al.*, 2015).

In summary, our findings reveal substantial shifts in the immunophysiology of toads as we move through the three stages of colonization. Several traits changed significantly with population age. Although the curvilinear responses (*Rhabdias* prevalence and abundance, fat stores, spleen size) hint at mismatches between invasion phenotype and post-invasion conditions, other traits (spirurid prevalence, circulating and infiltrating WBCs, stress response) appear to show simple linear responses. Rather than evolutionary shifts in trait values over time subsequent to colonization (Shine *et al.*, 2011) or genetic drift (Easteal, 1989), simple variation in population density through the invasion front may generate (plastic) trait shifts. This density gradient is a consistent feature of the invasion process, along with differential selective forces and spatial sorting for dispersal-enhancing traits. Teasing apart plastic vs. evolved shifts through this complex transition of the invasion front is not a simple task, requiring datasets that capture patterns of variation among wild-caught individuals (as in the present study), as well as laboratory-based analyses that control for causal factors such as invader genetics, lifestyles, and pathogen load. Nonetheless, invasion fronts represent fascinating natural laboratories in which we can observe evolution in disparate environments (frontal and core), and the mismatch that results as populations rapidly transition between these environments (Therry *et al.*, 2014b). Invasion fronts have much to teach us about plasticity and trade-offs in organismal life history, and they hold particular promise for increasing our understanding of the factors, as well as the trade-offs, that govern immune configuration and response.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Summary of samples sizes, sex ratios, body size, organ mass, and parasite infection data from 201 cane toads (*Rhinella marina*) collected from eight sites across Australia's Northern Territory. Data are the mean (range).