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Proximate mechanisms underlying the rapid modification of phenotypic traits in cane toads (*Rhinella marina*) across their invasive range within Australia

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Biological invasions often involve rapid modification of phenotypic traits. This is presumably in response to the novel pressures to which an invader is exposed, but the proximate basis for those changes remains unclear. Phenotypic changes may be generated by environmental factors (E), genetic factors (G) or the interaction between these two processes (G×E). To explore this issue, we obtained eight clutches of the cane toad (*Rhinella marina*) from three regions across its range of invasion within Australia, and raised the offspring under standard conditions (diet and/or exercise level manipulations) to clarify the proximate underpinnings of geographical divergence in phenotypic traits. Our results demonstrate that phenotypic variation among Australian cane toad populations is affected by genetics and environment, and an interaction between these two processes. Some traits (e.g. sprint distance) differed among populations, suggesting a heritable basis. Other traits (e.g. relative heart mass) were affected by experimental treatments but not by population. Intriguingly, other traits (e.g. body length) were affected by interactions between population of origin and experimental treatments. The relative importance of G, E and G × E differs among traits, but all three mechanisms have contributed to the rapid phenotypic divergence observed across the Australian range of invasive cane toads.

ADDITIONAL KEYWORDS: adaptation – *Bufo marinus* – common-garden experiment – geographical variation – phenotypic plasticity – rate of invasion.

INTRODUCTION

Many species with broad geographical ranges display phenotypic divergence in morphology, physiology and behaviour among populations (Eckert *et al.*, 2008). Broadly, the proximate mechanisms that cause these divergences can be classified into three processes. On one end of the spectrum are divergences due solely to heritable variation, such that individuals display the characteristics of their population of origin regardless of the conditions under which they are reared (e.g. Uy *et al.*, 2009). Alternatively, divergence may be due solely to environmental influence, such that an individual's phenotype is determined by the conditions it experiences as it grows (e.g. Zhao *et al.*, 2015; Ortega *et al.*, 2017). Lastly, trait divergence may result from an interaction between these two processes, such that an individual's phenotype is affected not only by its genotype and by the local environment, but also by the interaction between those factors (e.g. Diamond *et al.*, 2017; Hudson *et al.*, 2018b). For example, populations may differ in their phenotypically plastic responses to environmental cues (Matesanz *et al.*, 2015).

However, determining the relative importance of each of those factors, and of their interaction, is hampered not only by the ability of the same genotype to produce multiple phenotypes, but also by the complex array of processes that can modify genotypes through time and space (e.g. natural selection, genetic drift, spatial sorting: Via & Lande, 1985; Shine *et al.*, 2011), and the multiple mechanisms by which traits can be passed from one generation to the next (e.g. epigenetic changes and maternal effects: Bateson, 2014; Newcombe *et al.*, 2015). Clarifying the determinants of geographically variable phenotypes is easiest if the populations to be compared have been separated for only a short period. A longer time span since common ancestry allows divergences to accumulate, and provides opportunities for confounding effects of hybridization, genetic drift and population bottlenecks (Putman & Carbone, 2014).

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The classic example of species in which populations have become isolated only recently, yet experience very different conditions, is the case of an invasive species. Invasive species often exhibit rapid phenotypic change in response to the novel abiotic and biotic conditions within their new environment (Prentis *et al.*, 2008) and/or to the process of range expansion itself (Shine *et al.*, 2011). Substantial phenotypic divergence within invasive species can occur rapidly (over as few as ten generations) (Hendry *et al.*, 2007). As a result, biological invasions are increasingly being used to examine the proximate mechanisms that underlie geographical variation in phenotypes.

The invasion of Australia by cane toads (*Rhinella marina*) provides a robust opportunity to examine these mechanisms. Genetic differences between cane toads from invasive populations in Australia versus the species' native range in Latin America are minor, reflecting the short duration of separation (80 years) (Leblois *et al.*, 2000). Nonetheless, cane toads within Australia show less genetic variation than populations within the native range (reflecting the small number of founders: Slade & Moritz, 1998). As a result, strong phenotypic divergences between long-established (range-core) populations and newly established (range-edge) populations within Australia (Table A1) have not been accompanied by a correspondingly high genetic divergence (Lillie *et al.*, 2014; Rollins *et al.*, 2015; Hudson *et al.*, 2016a). Some of the phenotypic traits that differ between populations of cane toads within Australia exhibit high heritability (e.g. dispersal distance and limb dimensions) whereas others do not (e.g. dispersal directionality: Phillips *et al.*, 2010; Brown *et al.*, 2015b; Hudson *et al.*, 2016a). This combination of low genetic diversity and variable heritability suggests that phenotypic divergence among populations may have been influenced by genotype–environment interactions, including phenotypic plasticity (Davidson *et al.*, 2011; Rollins *et al.*, 2015).

In the current study, we examined the degree to which phenotypic differences among cane toads from populations across three regions of Australia are driven by innate factors versus plastic responses to environmental conditions. We raised representatives from range-edge, range-core and an intermediary mid-range population to allow for a clear comparison between potentially differing traits across the invasion gradient (Díaz *et al.*, 2007; Phillips *et al.*, 2010). By raising toads from different populations along the invasion gradient under standardized environmental conditions, we sought to clarify the mechanisms responsible for the extensive geographical variation that occurs in phenotypic traits within this species. In particular, what roles are played by innate genetic differences, environmentally elicited responses, and genotype–environment interactions? Traits related to rate of dispersal were primarily examined as these are directly pertinent to understanding and modelling range expansion of invasive species (Urban *et al.*, 2007). To measure divergence in norms of reaction across the cane toad's range of Australian invasion, we experimentally manipulated two factors (exercise and diet) that plausibly differ among toads from different areas.

One treatment involved the level of exercise, chosen because adult toads are sedentary in range-core populations but highly dispersive in range-edge populations (Phillips *et al.*, 2006; Alford *et al.*, 2009). High levels of activity can affect morphological development (Monkkonen, 1995; Kelly *et al.*, 2006). We also manipulated diet quality, because food availability changes with stage of invasion, and geographical regions probably differ in types of prey (Freeland *et al.*, 1986; Brown *et al.*, 2015a). If so, the generalist diet of cane toads presumably results in spatial and temporal variation in the nutritional quality of their prey (Lever, 2001; Brown *et al.*, 2013). Calcium was chosen as the dietary manipulation because the morphological traits of interest were skeletal features, and calcium is integral to bone formation (Pough, 2007).

MATERIAL AND METHODS

Study animals and husbandry

The subjects for our study were juvenile cane toads from eight clutches produced by parents collected from sites spanning the toads' invaded range across tropical Australia. Parental toads from two Queensland populations (QLD: Innisfail, 17.52°S, 146.02°E; Townsville, 19.25°S, 146.81°E) represented Range-core, long-established populations (72–80 years since invasion). Parental toads from the Northern Territory (NT: Middle Point, 12.62°S, 131.3°E) represented intermediate-aged populations (Midrange: 11 years since invasion), and parental toads from two sites in Western Australia (WA: Kununurra, 15.77°S, 128.74°E; Oombulgurri, 15.18°S, 127.84°E) represented Range-edge, newly established toad populations (2–6 years since invasion). Clutches were obtained by inducing captive pairs to breed by injecting them with leuprorelin acetate (Lucrin; Abbott Australasia, Botany, NSW, Australia) diluted 1:20 with Amphibian Ringer's solution (Hudson *et al.*, 2015). Clutches were produced by two pairs of adult toads from each of three sites (Innisfail, Townsville and Middle Point) and single clutches were produced by parents from Kununurra and Oombulgurri.

Tadpoles hatched during early September 2016 and were reared in captivity at a field station in the Northern Territory (12.62°S, 131.3°E) under standardised conditions [70-L plastic bins, fed pulverized algae wafers (Hikari, Kyorin, Japan) with water changed

every 2 weeks]. As tadpoles metamorphosed into juvenile toads, they were individually toe-clipped for identification (toe-clipping is relatively non-stressful in this species, and it does not elevate plasma corticosterone levels: Fisher *et al.*, 2013). We retained the first 40 individuals to metamorphose from each clutch, as the experimental subjects. The young toads were then relocated into 400 × 300 × 250 mm plastic containers with soil substrate, water, rocks and shelter. The container lids were covered in mesh, and the containers were placed close to windows to provide diffuse natural light and, hence, exposure to UV radiation (important for calcium metabolism: Pough, 2007). Individuals from the same clutch were housed together prior to the experimental trials, and they were fed daily on termites.

When the experiment began we assigned individuals to one of four treatment groups differentiated by exercise regime and dietary supplementation: (1) Low Exercise and Control Diet, (2) Low Exercise and Supplemented Diet, (3) High Exercise and Control Diet, and (4) High Exercise and Supplemented Diet. Allocation to treatment groups was random within each clutch, such that near-equal numbers of siblings were included in all treatments. Individuals within each treatment were maintained in five replicate containers, with 12–13 toads per container. Eight weeks into the experiment, as disparities in body size developed within housing containers, we reassigned toads within the same treatment to new housing boxes depending on their snout–urostyle length (SUL). This reallocation ensured that larger toads did not outcompete or cannibalize smaller toads. We fed the toads on termites until they reached 25 mm SUL, after which we also offered larger prey items (crickets and mealworms). To minimize disparities in daily prey intake among toads, excess food was provided in each feeding session, and the remaining excess was removed a few hours later, to ensure all individuals had equal opportunity to eat until full.

Prey items given to the Supplemented Diet treatment group were dusted in calcium powder (JurassiCal, JurassiPet, Seachem Laboratories, Madison, GA, USA) once every 2 weeks. The calcium powder was applied in a ratio of 5 g of calcium to 200 g of prey (as recommended by the manufacturer). No calcium was added to prey items given to the Control Diet treatment. Thus, the caloric content of all treatment diets was equivalent (i.e. calories per gram of food was the same), but one treatment had supplemented calcium intake.

Individuals assigned to the High Exercise regime treatment were exercised for 1 min three times a week (every 2–3 days). For this procedure, the toad was placed on a linear racetrack (a 1.5-m length of flat-bottomed plastic PVC half-piping, 60 mm wide, with the base covered in damp paper towel; average ambient temperature 27.5 °C and relative humidity 67%), and we gently touched the urostyle with a plastic rod to encourage each toad to hop for 1 min. Individuals assigned to the Low Exercise treatments were placed on a similar linear track and left undisturbed for 1 min.

We imposed these treatments for 16 weeks, from mid-September 2016 to mid-January 2017. Any forced exercise of the toads or data collection occurred at least 6 h after the most recent feeding, to allow time for digestion.

Collection of data

Morphology

At the end of the experiment (week 18), we measured body length (SUL), leg length (tibia–fibula length), arm length (radius–ulna length) and head width, with digital callipers. All toads were then euthanized via an intracoelomic injection of pentobarbital (80 mg/kg). Following euthanasia we made a mid-ventral incision in each toad and fixed the specimen for 48 h in 10% phosphate-buffered formalin. The toads were then dissected, and their livers and hearts removed. The mass of the heart and liver was recorded using an electronic balance.

Anti-predator behaviour

We conducted trials during week 16 of the experiment, with individuals from different treatments spread evenly over the three nights of testing. Temperatures during the trials ranged from 27.8 to 29.5 °C, and relative humidity from 64 to 75%. When the toad was picked up from its container to be measured, we also recorded the number of kicks with its back legs until the toad remained still for 5 s (hereafter, 'Struggle Score'). 'Righting Time' was quantified by placing the toad on its back and scoring how long it took to right itself.

Locomotor performance

(a) Sprint Distance – We conducted Sprint Distance trials after the anti-predator behaviour trials during week 16. Toads were placed on the linear track and encouraged to run for 1 min (this constituted one of the three 'weekly running sessions' described above). We recorded the total distance covered by the toad during the 1-min trial.

During weeks 15 and 16, with individuals from each treatment evenly represented across days, we subjected toads to repeated trials of locomotor ability. Temperatures during the trials ranged from 26.9 to 29.7 °C, and relative humidity from 61 to 75%. To measure a toad's initial locomotor speed, we encouraged the individual to run along a linear 900-mm track, with

markings every 300 mm (track lined with damp paper towel to maintain moisture levels). We timed how long the toad took to travel between each 300-mm marking, resulting in three measurements of speed. The toad was then placed at the lower end of a 2-m-long track (lined with damp paper towel), set at a 15° gradient to increase the effort required to ascend it. We encouraged the toad to hop up the slanted track, and once it reached the top of the slope we relocated it to the bottom and repeated the sequence, for a total of eight consecutive ascents. We then placed the toad back onto the first linear track to remeasure the time it took to travel between each 300-mm marking. The toad was then placed under a small plastic container, left to rest undisturbed for 2 min and then again run along the linear track. The distances obtained for each 300-mm marking were averaged for each individual. We then divided the average distance moved by time to obtain estimates of speed pre-exhaustion, post-exhaustion and post-recovery.

(b) Stamina Score – To quantify the degree to which performance was reduced by exhaustion (i.e. stamina: how much slower an individual was after being exhausted), we regressed post-exhaustion speed against pre-exhaustion speed, and used the residual score from this linear regression as our measure of stamina. A lower Stamina Score indicated a more dramatic decrease in the toad's performance after sustained exercise.

(c) Exhaustion Score – To quantify how much an individual's speed diminished during extended exercise ('Exhaustion Score'), we measured the total time an individual took to complete the eight inclined track exhaustion lengths. A higher Exhaustion Score indicated an individual whose performance diminished dramatically by the end of the extended bout of exercise.

(d) Recovery Score - To quantify how much an individual's speed improved after 2 min of rest ('Recovery Score'), we regressed post-recovery speed against post-exhaustion speed, and used the residual score from this linear regression as our measure of rate of recovery. A higher Recovery Score indicated an individual whose performance improved dramatically after 2 min of rest.

Level of spontaneous activity

We conducted trials of spontaneous movement in an outdoor shed at night over three trial nights during week 14, with individuals from different treatments spread evenly over the three nights (temperature 27.8–32.4 °C, humidity 66–92%). Trials were conducted in large (1.2 m width and breadth, 500 mm depth) plastic arenas. A video camera was fixed above the centre of each arena (2 m from the ground) with a red lamp (at the same height) providing the only illumination. Red light is used to encourage natural behaviour during night-time behavioural observations, as cane toads are nocturnal (Candler & Bernal, 2015). We placed a single toad in the centre of the arena, with a small opaque bin over it to allow the toad to acclimatize for 2 min. We then removed the bin, and allowed the toad to roam freely for 30 min. The toad was then removed, and the arena was washed to remove chemical cues.

We analysed the videos using the 'manual tracking' plugin in ImageJ (Abramoff *et al.*, 2004), calibrated against a 300-mm scale bar marked on the arena floor. Exposures were taken 8 s apart, yielding 225 frames within the allotted 30 min. Based on the toad's position in every frame, we calculated the total distance moved by the toad, as well as distances between each successive position. The 95% quantile distance for each toad was used to quantify the level of spontaneous activity.

Dispersal rates in the field

We conducted radio-telemetry trials over seven nights during week 17, with individuals from each treatment spread evenly over the seven trial nights (temperature 28.2–33.1 °C, humidity 72–91%). Individuals that were ≥ 5 g in mass (so that the 0.5-g radio-transmitter did not exceed 10% of their body mass) were used, with equal numbers from each treatment group and Population of Origin. We attached the radio-transmitters to the toads using elastic around the waist of the toad. The toads were released just after sundown at a single location within the grounds of the research station, left overnight and retrieved the next morning, enabling us to calculate the overnight displacement distance and net direction of displacement of each toad over the 12-h radio-tracking period.

Analysis of data

Sample sizes varied slightly among traits and treatment groups due to mortality in the early weeks post-metamorphosis, as well as occasional acts of cannibalism during the experiment. By the end of the experiment, the average number of toads from each clutch in each treatment group was 3.8 (SE = 0.51). At week 16, we measured external morphology and behaviour from a total of 121 toads, and at week 18, we dissected 84 of these individuals. We obtained data for 121 individuals during the sprint trials, and 118 individuals for the locomotor trials (Stamina, Exhaustion and Recovery Score) and the spontaneous movement trials. During week 17 we radio-tracked a total of 30 individuals.

We used multiple regression models to determine if morphological and behavioural measures (body length,

limb length, head width, liver mass, heart mass, Struggle Score or Righting Time) or locomotor measures (Sprint Distance, Stamina Score, Exhaustion Score, Recovery Score, Spontaneous Movement and Radio-telemetry distance) differed significantly among treatment groups (Diet and Exercise) and/or Populations of Origin (Range-edge, Midrange and Range-core). Measurements were ln-transformed prior to analysis to normalize the data (Stamina, Exhaustion and Recovery scores were ln-transformed prior to regression). To standardize our measurements of morphology and behaviour to body size, SUL was included as a covariate in the model (except in the model with SUL as the dependent variable). Clutch of Origin (nested within Population of Origin) was included in the regression models to account for familial relationships among toads. All interactions (up to third order) among Diet, Exercise and Population of Origin were included in an initial full model. Non-significant three- and two-way interactions were sequentially removed, and the reduced models were rerun.

To verify the assumption that our measure of spontaneous movement reflects dispersal tendency in the wild, we regressed dispersal distance (obtained from radio-telemetry) against the distance travelled during the spontaneous activity trials.

With one exception (see below), we performed all statistical tests using JMP Pro 11 (SAS Institute, Cary, NC, USA) and all analyses satisfied assumptions of normality and variance homogeneity.

We used the R package 'circular' to conduct three separate circular ANOVAs to determine if the directionality of movement of radio-tracked toads varied among Diets, Exercise treatments or Populations of Origin.

RESULTS

Snout–urostyle length (SUL)

Mean body sizes of the young toads were affected by a three-way interaction among Exercise, Diet and Population of Origin (Table 1, Fig. 1). For two of our treatment groups (Range-edge populations under the Supplemented Diet, and Range-core populations under the Control Diet), the young toads grew more rapidly under the Low Exercise regime than under the High Exercise regime (Fig. 1A, B).

Level of spontaneous activity

Spontaneous activity was affected by the interaction between Exercise, Diet and Population of Origin (Table 2). Population of Origin had little effect on the level of spontaneous activity under the Control Diet

Table 1. Results of analyses showing the effects of Exercise (Low vs. High), Diet (Control vs. Calcium Supplemented), and Population (Range-edge, Midrange, and Range-core) on morphological and behavioural traits of juvenile cane toads (*Rhinella marina*). Clutch ID was included to incorporate family effects into the model. Snout-urostyle length (SUL) was included in relevant models to control for body size effects. Boldface font indicates effects with $P < 0.05$ (* $0.05 > P > 0.01$, ** $0.01 > P > 0.001$, *** $P < 0.001$). Non-significant interactions (indicated by a dash) were deleted, and the model recalculated

Effect	SUL	Leg Length	Arm Length	Head Width	Heart Mass	Liver Mass	Struggle Score	Righting Time
SUL	–	$F_{1,110}$ = **1860*****	$F_{1,110}$ = **104*****	$F_{1,110}$ = **1160*****	$F_{1,73}$ = **127*****	$F_{1,68}$ = **87*****	$F_{1,110}$ = 0.47	$F_{1,105}$ = 1.58
Clutch {Population}	$F_{5,104}$ = 1.77	$F_{5,110}$ = **3.87****	$F_{5,110}$ = 0.72	$F_{5,110}$ = 0.52	$F_{5,73}$ = 1.76	$F_{5,68}$ = 0.40	$F_{5,110}$ = 1.68	$F_{5,105}$ = 0.80
Population	$F_{2,104}$ = 0.41	$F_{2,110}$ = **4.58***	$F_{2,110}$ = 1.60	$F_{2,110}$ = 0.39	$F_{2,73}$ = 1.78	$F_{2,68}$ = 0.95	$F_{1,110}$ = 2.12	$F_{2,105}$ = 0.28
Exercise	$F_{1,104}$ = 4.20	$F_{1,110}$ = 0.10	$F_{1,110}$ = 1.27	$F_{1,110}$ = 0.50	$F_{1,73}$ = 0.03	$F_{1,68}$ = 0.64	$F_{1,110}$ = 0.00	$F_{1,105}$ = 0.00
Diet	$F_{1,104}$ = 0.69	$F_{1,110}$ = 1.05	$F_{1,110}$ = 0.67	$F_{1,110}$ = 0.20	$F_{1,73}$ = **10.14*****	$F_{1,68}$ = 0.15	$F_{1,110}$ = 1.03	$F_{1,105}$ = **6.46***
Population × Exercise	$F_{2,104}$ = 0.94	–	–	–	–	$F_{2,68}$ = 0.15	–	$F_{2,105}$ = **4.97****
Population × Diet	$F_{2,104}$ = 0.47	–	–	–	–	$F_{2,68}$ = **3.89***	–	$F_{2,105}$ = 2.43
Exercise × Diet	$F_{1,104}$ = 2.24	–	–	–	–	$F_{1,68}$ = 0.07	–	$F_{1,105}$ = 1.21
Population × Exercise × Diet	$F_{2,104}$ = **5.11****	–	–	–	–	–	–	–

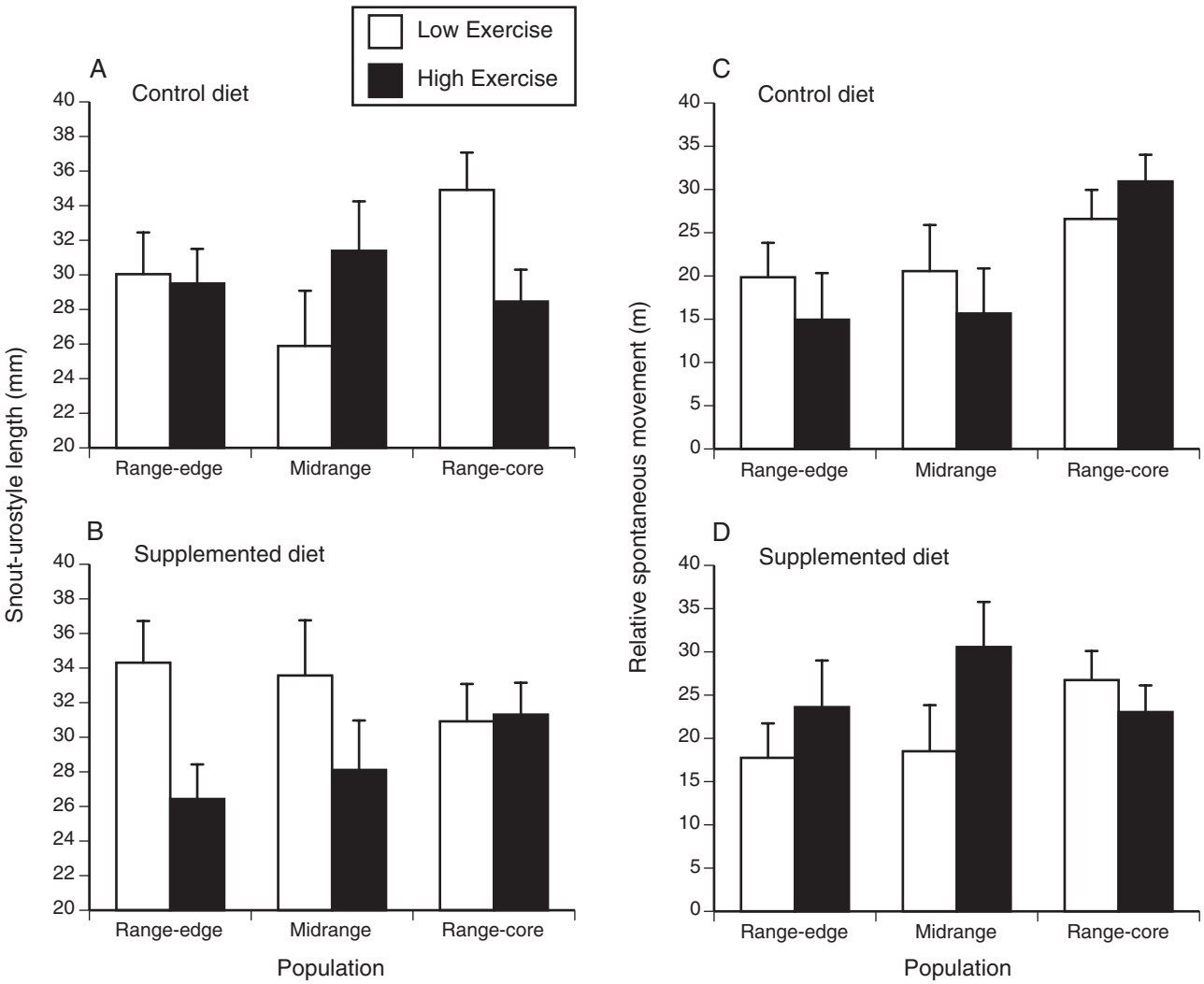

Figure 1. Mean snout–urostyle lengths and relative Spontaneous Movement (distances travelled during 30 min trial) of juvenile cane toads as a function of Diet (Control vs. Calcium Supplemented), Exercise (Low vs. High) and Population of Origin (Range-edge, Midrange and Range-core). Bars shows mean values and associated standard errors.

treatment, regardless of Exercise regime (Fig. 1C, D). In comparison, Exercise had substantial (and population-specific) effects on performance for individuals with Supplemented Diets (Fig. 1C, D).

Body shape

Relative leg length was affected by Population of Origin (Table 1). Relative to SUL, individuals from Range-core populations had longer legs compared with individuals from Midrange populations (mean ± SE: Range-edge = 11.29 ± 0.12 mm, Midrange = 11.04 ± 0.15 mm, Range-core = 11.50 ± 0.10 mm). However, neither relative forearm length nor relative head width were significantly affected by Population of Origin, Diet or Exercise, or interactions among these factors (Table 1).

Mass of the heart relative to SUL was affected by Diet (Table 1), with heavier hearts in toads given the Supplemented Diet (Control Diet = 23.67 ± 3.67 mg, Supplemented Diet = 26.02 ± 4.00 mg). Mass of the liver relative to SUL was affected by the two-way interaction between Population of Origin and Diet (Table 1). Within the Control Diet treatment, individuals from Range-edge populations (WA) had lighter livers than most other treatment groups (Fig. 2).

Anti-predator behaviour

Struggle Score was not significantly affected by Population of Origin, Diet, Exercise or any interactions (Table 1). However, Righting Time was affected by an interaction between Exercise and Population

Table 2. Results of analyses showing the effects of Exercise (Low vs. High), Diet (Control vs. Calcium Supplemented), and Population (Range-edge, Midrange, and Range-core) on dispersal-related traits of juvenile cane toads (*Rhinella marina*). Clutch ID was included to incorporate family effects into the model. Snout-urostyle length (SUL) was included in models to control for body size effects. Boldface font indicates effects with $P < 0.05$ (* $0.05 > P > 0.01$, ** $0.01 > P > 0.001$, *** $P < 0.001$). Non-significant interactions (indicated by a dash) were deleted, and the model recalculated

Effect	Sprint Distance	Stamina	Exhaustion	Recovery	Spontaneous movement	Radio-telemetry Distance
SUL	$F_{\mathbf{1,110}} = \mathbf{9.27}$**	$F_{\mathbf{1,107}} = \mathbf{46.5}$***	$F_{\mathbf{1,102}} = \mathbf{14.0}$**	$F_{\mathbf{1,102}} = \mathbf{6.06}$*	$F_{\mathbf{1,97}} = \mathbf{14.84}$**	$F_{\mathbf{1,22}} = \mathbf{12.31}$**
Clutch {Population}	$F_{\mathbf{5,110}} = \mathbf{3.98}$**	$F_{5,107} = 1.96$	$F_{5,102} = 2.06$	$F_{\mathbf{5,102}} = \mathbf{5.53}$***	$F_{5,97} = 1.18$	$F_{5,22} = 1.17$
Population	$F_{\mathbf{2,110}} = \mathbf{3.62}$*	$F_{\mathbf{2,107}} = \mathbf{5.65}$**	$F_{2,102} = 1.82$	$F_{\mathbf{1,102}} = \mathbf{6.13}$**	$F_{\mathbf{2,97}} = \mathbf{3.90}$*	$F_{2,22} = 2.65$
Exercise	$F_{1,110} = 0.03$	$F_{\mathbf{1,107}} = \mathbf{12.44}$***	$F_{1,102} = 2.09$	$F_{\mathbf{1,102}} = \mathbf{10.37}$**	$F_{1,97} = 1.07$	$F_{1,22} = 3.53$
Diet	$F_{\mathbf{1,110}} = \mathbf{7.03}$**	$F_{1,107} = 0.05$	$F_{\mathbf{1,102}} = \mathbf{5.80}$*	$F_{\mathbf{1,102}} = \mathbf{6.79}$**	$F_{1,97} = 0.91$	$F_{\mathbf{1,22}} = \mathbf{9.97}$**
Population × Exercise	–	–	$F_{2,102} = 1.03$	$F_{\mathbf{2,102}} = \mathbf{3.64}$*	$F_{2,97} = 1.12$	–
Population × Diet	–	–	$F_{2,102} = 0.00$	$F_{2,102} = 1.11$	$F_{2,97} = 0.09$	–
Exercise × Diet	–	–	$F_{\mathbf{1,102}} = \mathbf{6.82}$*	$F_{1,102} = 0.20$	$F_{1,97} = 0.60$	–
Population × Exercise × Diet	–	–	–	–	$F_{\mathbf{2,97}} = \mathbf{3.29}$*	–

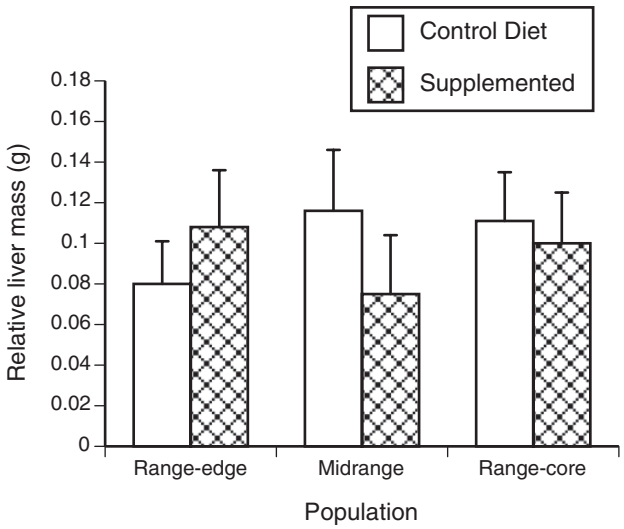

Figure 2. Differences in relative liver mass of juvenile cane toads as a function of Diet (Control and Calcium Supplemented) and Population of Origin (Range-edge, Midrange and Range-core). The graph shows mean values and associated standard errors.

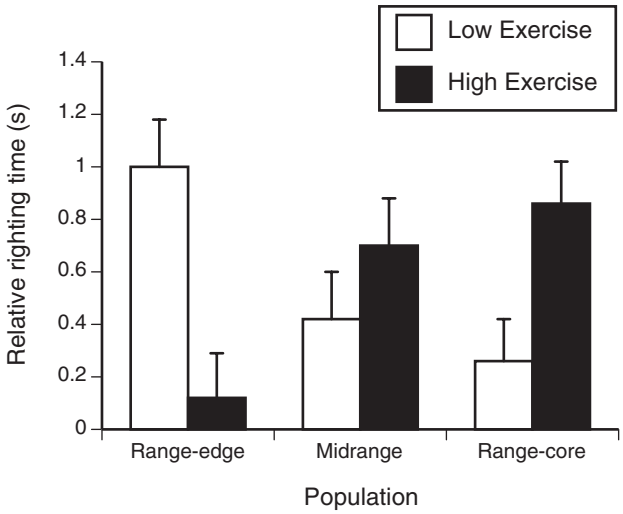

Figure 3. Relative Righting Times for juvenile cane toads as a function of Exercise (Low vs. High) and Population of Origin (Range-edge, Midrange and Range-core). The graph shows mean values and associated standard errors.

of Origin (Table 1). Individuals from Range-edge populations were slower to right themselves if kept under the Low Exercise treatment rather than the High Exercise treatment (Fig. 3). The reverse trend was seen in Range-core individuals, which exhibited longer righting times if kept under the High Exercise treatment. Righting time was also affected by Diet; Range-core individuals given the Supplemented Diet were slower in this task (Control Diet = 3.97 ± 1.70 s, Supplemented Diet = 9.57 ± 4.17 s).

Locomotor performance

(a) Sprint distance was significantly affected by Population of Origin and Diet (Table 2). Individuals from the Range-edge (WA) populations

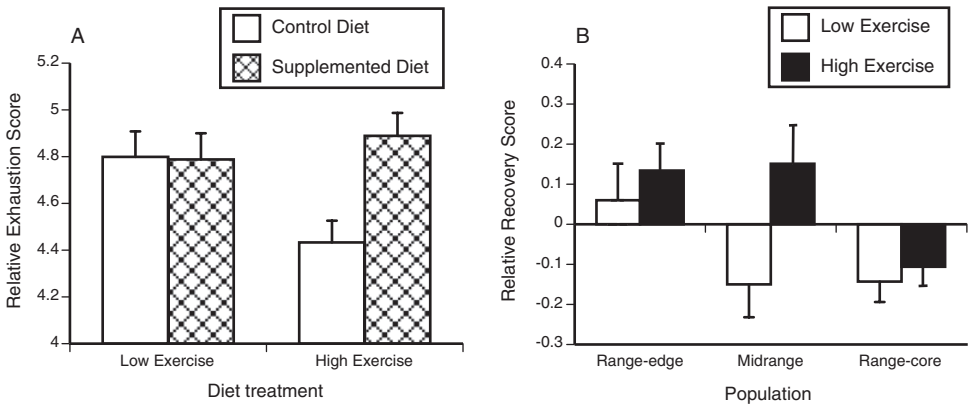

Figure 4. A, effects of Diet (Control and Calcium Supplemented) and Exercise (Low vs. High) on Exhaustion Score (rate that locomotor ability was reduced by a period of intensive exercise) of juvenile cane toads. B, effects of Exercise (Low vs. High) and Population of Origin (Range-edge, Midrange and Range-core) on Recovery Score (rate that locomotor ability recovered after intensive exercise) of juvenile cane toads. The graphs show mean values and associated standard errors. Higher Exhaustion scores indicate that speed decreased substantially during exercise. Higher Recovery scores indicate performance improved substantially after a 2-min rest period.

were slower than those from Range-core (QLD) and Midrange (NT) populations (Range-edge = 4.73 ± 0.34 m, Midrange = 5.73 ± 0.49 m, Range-core = 5.52 ± 0.29 m), and toads given the Control Diet travelled further than those given calcium supplements (Control Diet = 5.72 ± 0.27 m, Supplemented Diet = 4.97 ± 0.27 m).

(b) Stamina Score was affected by main effects of Population and Diet (Table 2). Range-core toads had a higher Stamina Score than Midrange or Range-edge toads (Range-edge = −0.08 ± 0.06, Midrange = −0.10 ± 0.08, Range-core = 0.14 ± 0.05). Individuals in the Low Exercise treatment had a higher Stamina Score than individuals in the High Exercise treatment (Low Exercise = 0.07 ± 0.05, High Exercise = −0.10 ± 0.04).

(c) Exhaustion Score was affected by a significant Diet*Exercise interaction (Table 2). Under the Control Diet, individuals in the Low Exercise treatment had higher Exhaustion Scores than did individuals in the High Exercise treatment (Fig. 4A). But on the Supplemented Diet, individuals in both Exercise treatments exhibited similar levels of exhaustion (Fig. 4A).

d) Recovery Score was affected by a Population × Exercise interaction as well as by a main effect of Diet (Table 2). Among Midrange (NT) toads, individuals in the High Exercise group recovered more quickly than toads in the Low Exercise group. Among toads from other populations, recovery rates were similar between Exercise groups (Fig. 4B). Individuals given the Control Diet took longer to recover than conspecifics given the Supplemented Diet (Control Diet = −0.07 ± 0.04, Supplemented Diet = 0.05 ± 0.04).

Dispersal rates in the field

Distances moved were significantly affected only by Diet (Table 2), with individuals given the Supplemented Diet treatment travelling further than those given the Control Diet (33.0± 5.2 and 15.5 ± 6.6 m, respectively). An individual's displacement during radio-telemetry trials was positively correlated with the mean distance it moved during trials to measure levels of spontaneous activity (R^2 = 0.16, $F_{1,28}$ = 5.37, P = 0.028). The directionality of toad movement was not significantly affected by a toad's Population of Origin ($\chi^2_{2,60}$ = 1.07), Exercise treatment ($\chi^2_{1,30}$ = 0.03) or Diet ($\chi^2_{1,30}$ = 0.45; all $P > 0.05$).

DISCUSSION

The phenotypes of our juvenile toads were influenced by a mixture of innate (presumably genetically controlled) differences, environmental responses, and genotype × environment interactions. For a few traits, phenotype was significantly influenced only by treatment (Diet or Exercise, or an interaction between the two), or by Population of Origin. However, many traits were affected by an interaction between Population of Origin and one or both of the experimental treatments. In other words, the manner in which treatment

affected a toad's traits depended on the population from which the toad's parents had been collected.

We detected geographical shifts in norms of reaction for SUL growth and Righting Time, with treatment having opposite effects in Range-edge (WA) populations than in Range-core (QLD) populations. These differences suggest evolved changes in reaction norms for these traits, with individuals from Range-edge (WA) populations benefiting from conditions that reduced performance in conspecifics from Range-core (QLD) populations, and vice versa.

These reaction norm shifts were not seen in all traits. For example, leg length relative to body length was influenced primarily by the geographical origin of the parents. Relative leg lengths exhibited a curvilinear rather than linear pattern with respect to population age (Midrange toads had shorter limbs than Range-edge or Range-core conspecifics). Similar curvilinear trends in phenotypic traits (including in relative limb lengths) have been reported in wild-caught adult cane toads also, across the same invasion transect (Hudson *et al.*, 2016a). These curvilinear trends thus appear to be due to heritable differences rather than to environmental factors that affect the translation of the genotype into the phenotype.

The curvilinearity seen in leg length may be a result of two interacting evolutionary forces – spatial sorting and natural selection (Brown *et al.*, 2015b). Spatial sorting results in the accumulation of dispersal-enhancing traits on invasion fronts, causing long-limbed individuals (the fastest dispersers) to dominate the expanding edge of the population (Phillips *et al.*, 2006; Shine *et al.*, 2011). Natural selection may also favour long-legged individuals within the Range-core, as individuals with these traits may be better at predator avoidance and male–male rivalry (Lee, 2001). In the Midrange area, in contrast, toads use a more energy-efficient mode of locomotion (bounding rather than leaping) to cover long distances, hence conferring a selective advantage to a (transitory) reduction in relative limb length (Hudson *et al.*, 2016a). The proximate basis of these 'innate' effects may involve differences in genotype, or in gene expression resulting from epigenetic or maternal effects (Rollins *et al.*, 2015). This interaction between spatial sorting and natural selection may also help to explain the geographical shifts in reaction norms for SUL and Righting Time.

Another key reaction norm shift reported by our data is that of Spontaneous Activity. Contrary to the situation in free-ranging adult toads (where Range-core toads move very little, Range-edge toads are highly mobile, and Midrange toads are intermediate: Urban *et al.*, 2007; Alford *et al.*, 2009), displacements during our Spontaneous Movement trials were lowest (and least sensitive to prior exercise) in Range-edge toads. Similar results were seen in analysis of Sprint Distance and Stamina Score. These result runs counter to the previously reported higher displacement rates of adult Range-edge toads in the field (e.g. Alford *et al.*, 2009; Lindström *et al.*, 2013) and the similarity in speeds of dispersal in adult toads from different populations (Llewelyn *et al.*, 2010). Possible explanations for the reduced dispersal-related performance in Range-edge populations is that individuals at the invasion vanguard may move in novel ways (by bounding vs. leaping), potentially facilitating sustained locomotion but at the cost of lower speeds over short distances (Hudson *et al.*, 2016b). Additionally, Range-core toads rarely encounter novel environments, and dramatically increase movements when they do so (Pettit *et al.*, 2016). Lastly, these differences may be a result of variance in willingness to sprint when stimulated during the Sprint Distance trails, indicating behavioural rather than physiological differences (Yap *et al.*, 2017). Interestingly, the Recovery Score was highest for Range-edge individuals, contrary to the results reported for Sprint Distance. This suggests that juveniles from the Range-edge can recover more rapidly after intense activity. Perhaps then, despite lower Sprint Distance, the Range-edge toads are physiologically better equipped for long-term movement (as seen at invasion frontlines).

Although our treatments on captive toads altered many traits, the experimental conditions were unlikely to have mimicked the experiences of wild juvenile toads. Climatic conditions, food quality, population density, competition, pathogens and other factors no doubt interact in complex ways to influence morphology, activity and behaviour of wild toads. Additionally, long-range dispersal is undertaken primarily by adult cane toads (due to decreased desiccation risk compared to the juveniles: Child *et al.*, 2009), and movement patterns of juvenile toads in the wild are not well understood. Hence the magnitudes, and even directions, of the effects observed in our study may not reflect patterns in the wild. The primary aim of our study was not to replicate natural patterns in detail, but simply to identify which traits (if any) were able to plastically respond to controlled levels of Exercise and Diet variation. It is important to note that Spontaneous Movement correlated strongly with Radio-telemetry Displacement Distance, suggesting that this laboratory-based measure can serve as an inexpensive and easy proxy for movement in the field.

We also report interesting, and in many cases non-intuitive, effects of calcium on the phenotypic traits of the young toads. One unexpected result was that extra calcium in the diet tended to reduce rather than improve a young toad's sprint distance. Calcium supplementation is associated with improved performance in

many animals, and such supplements are commonly recommended in amphibian husbandry (Gala *et al.*, 2001; Pough, 2007). Calcium uptake in vertebrates (including amphibians) is heavily reliant on UV levels and vitamin D3 (the precursor to calcitriol, the hormone responsible for calcium metabolism and by extension bone formation: Antwis & Browne, 2009). Excess vitamin D3 elevates calcium levels in the plasma, placing a burden on the usual means of calcium storage (e.g. in lymphatic sacs: Stiffler, 1993), and resulting in over-calcification of the skeleton (Antwis & Browne, 2009). However, the housing conditions used in the study are unlikely to have resulted in excessive vitamin D3 production in the juvenile toads, suggesting that the negative effects of the calcium were not due to over-calcification (Pough, 2007; Antwis & Browne, 2009). Instead, the extra calcium in the toads' diet may have reduced speed through changes to morphology or physiology (Govindappa *et al.*, 1977; Sampson *et al.*, 1987). For example, excess storage of calcium may burden a toad enough to reduce its ability to sprint (Sampson *et al.*, 1987). Alternatively, a calcium-rich diet may stimulate greater bone density, again compromising locomotor performance (Gala *et al.*, 2001). Future work could usefully examine bone densities and assay levels of calcium in tissues. Calcium supplementation also increased the mass of the heart, but we cannot unequivocally score such a change as a net positive or negative effect.

Calcium did interact with the other experimental treatments (Exercise and Population of Origin). For example, liver mass was increased by calcium supplementation in Range-edge (WA) individuals but not in Range-core (QLD) or Midrange (NT) toads. High liver mass has been linked to better health as the liver is used as a site of energy storage (Morton, 1981), but this organ does not play a direct role in metabolic recovery after exercise in anurans (Gleeson, 1991). The small liver masses of Range-edge (WA) toads in the Control Diet treatment suggest that these animals may prioritize immediate use of energy (i.e. activity that might translate to an increased rate of dispersal) rather than storage. Supplementation of calcium levels in the diet of Range-edge (WA) cane toads increased liver mass in these animals, unlike in the other two populations, hinting that calcium availability at the invasion front may constrain rates of dispersal.

Differences among toad populations in their responses to exercise and calcium supplementation are difficult to interpret, because we do not know how these aspects of the toads' ecology have shifted as the invader has moved from the wet tropics to the seasonally arid Kimberley (WA, Range-edge) region. Differences in the diet of cane toads along the invasion front have been documented, but calcium contents of the diets are unknown (Freeland *et al.*, 1986). A shift in prey types might interact with the enhanced dispersal rate; and interactions between Diet, Exercise and Population of Origin suggest that changes to diet and activity levels may influence the phenotypic traits of young cane toads. Assays looking at calcium levels in the bladder could clarify potential physiological differences in processing calcium between toads from different Populations of Origin (Stiffler, 1993). Additionally, further research into the role of calcium in development and performance in amphibians would also shed light on these intriguing results.

In summary, our data confirm previous reports of phenotypic divergences among populations of cane toads within Australia and show that the causal mechanisms underlying those divergences are complex. Importantly, the level of phenotypic plasticity differs among traits and among populations. In some cases, impacts of a specific treatment on a trait were reversed by the impacts of another treatment, and were manifested differently in toads from one Population of Origin than from another. Cane toads have undergone rapid evolution during their range expansion (e.g. Rollins *et al.*, 2015; Hudson *et al.*, 2016a), and our experiments confirm that those evolved changes include shifts in norms of reaction as well as in highly canalized traits. The rapid modification of norms of reaction has important implications for management strategies, and in particular for modelling rates of range expansion (Urban *et al.*, 2007). Previous attempts to predict the ranges of the invading population have underestimated the rate of range expansion for the invasive cane toad in Australia (Phillips *et al.*, 2010; Shine & Phillips, 2014). If we are to successfully predict invader spread, and develop new ways to combat the invader's impacts, we need to be aware of the cane toad's ability to modify its phenotype in response to local environments, in ways that we are only now beginning to understand.

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APPENDIX

Table A1. Summary of literature characterising differences in phenotypic traits across the Australian cane toad invasion. SUL = snout-urostyle length

Trait type	Trait	Trait gradient	Reference
Morphological	Size (SUL)	WA = NT = QLD	Llewelyn *et al.* (2010)
			Hudson *et al.* (2016b)
	Arm Length	WA >> NT < QLD	Hudson *et al.* (2016a)
	Leg Length	WA >> NT < QLD	Phillips *et al.* (2006)
			Hudson *et al.* (2016a)
	Head Width	WA < NT < QLD	Hudson *et al.* (2016b)
Behavioural	Escape response	WA < QLD	Hudson *et al.* (2018a)
Physiological	Dispersal distance	WA > NT > QLD	Phillips *et al.* (2010)
			Phillips *et al.* (2006)
	Endurance	NT > QLD	Llewelyn *et al.* (2010)
	Directionality	WA = NT = QLD	Brown *et al.* (2015b)