Thin-skinned invaders: Geographic variation in the structure of the skin among populations of cane toads (*Rhinella marina*)

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Abstract

The structure of the skin may evolve rapidly during a biological invasion, for two reasons. First, novel abiotic challenges such as hydric conditions may modify selection on traits (such as skin thickness) that determine rates of evaporative water loss. Second, invaders might benefit from enhanced rates of dispersal, with locomotion possibly facilitated by thinner (and hence more flexible) skin. We quantified thickness of layers of the skin in cane toads (*Rhinella marina*) from the native range (Brazil), a stepping-stone population (Hawai'i), and the invaded range in Australia. Overall, the skin is thinner in cane toads in Australia than in the native range, consistent with selection on mobility. However, layers that regulate water exchange (epidermal *stratum corneum* and dermal Ground Substance layer) are thicker in Australia, retarding water loss in hot dry conditions. Within Australia, epidermal thickness increased as the toads colonised more arid regions, but then decreased in the arid Kimberley region. That curvilinearity might reflect spatial sorting, whereby mobile (thin-skinned) individuals dominate the invasion front; or the toads' restriction to moist sites in this arid landscape may reduce the importance of water-conservation. Further work is needed to clarify the roles of adaptation versus phenotypic plasticity in generating the strong geographic variation in skin structure among populations of cane toads.

ADDITIONAL KEYWORDS: *Bufo marinus* – integument – invasion – dispersal – skin stratum – water balance.

INTRODUCTION

An organism's integument is the envelope that maintains all of its vital internal processes, and serves multiple functions (Vitt & Caldwell, 2013). For example, the colour of the skin influences courtship and reproduction, communication, camouflage and protection against predation (Rudh & Qvarnström, 2013; Kim & Velando, 2014; Teyssier *et al*., 2015; Condez et al. 2020). Also, toxins are produced and stored in the skins of many species (e.g. Chen *et al*., 2017), and skin microbiota serve as an antimicrobial barrier (e.g. Weitzman *et al*., 2019). For amphibians, one of the most important functions of the skin is its role in hydroregulation (Brattstrom, 1979; Navas *et al*., 2007). The thickness of an individual's outer covering also might affect the animal's mobility, impeding flexion and thus, reducing both speed and endurance (Long et al. 1996; Irschik & Jayne, 1999; Losos *et al*., 2002).

From a physiological perspective, a living organism is a homeostatic island within a highly variable ocean (Heatwole & Barthalmus, 1994; Hill *et al*., 2004). As the interface between the organism and the external world, the skin facilitates homeostasis by affecting rates of exchange of heat, water and gas (Lillywhite & Licht, 1974; Lillywhite, 1975; Bentley & Yorio, 1979; Heatwole & Barthalmus, 1994; Hill *et al*., 2004; Anderson *et al*., 2017). Reflecting that importance, much interspecific variation in skin structure is linked to challenges posed by different kinds of habitats. For example, desiccating conditions may favour thicker (less permeable) skin (Bentley & Yorio, 1979; Toledo & Jared, 1993a; Angilletta *et al*., 2002), or adaptations for rapid water uptake (e.g., Navas *et al*., 2004), or "waterproofing" (McClanahan *et al*., 1978; Christian & Parry, 1997; Angilletta, 2009). Many types of organisms can rapidly adjust the structure of the skin in ways that enable them to deal with novel environmental challenges (e.g. fishes – Pickering & Richards, 1980; lizards – Kattan & Lillywhite, 1989; birds – Menon *et al*., 1996).

Invasive species provide ideal models with which to explore organismal responses to environmental challenges (Mooney & Hobbs, 2000; Sax *et al*., 2007). Many invaders encounter novel abiotic conditions (Hoffmann & Sgrò, 2011; McGaugh et al. 2020). Given the critical role of water balance for terrestrial amphibians, and the potential for high rates of evaporative water loss from their bodies (Bentley & Yorio, 1979; Jørgensen, 1997), the skin of an anuran may be under intense selection if the species is translocated from a mesic to an arid environment. The international diaspora of the cane toad (*Rhinella marina*, formerly *Bufo marinus*) provides an ideal example of such a transition; the native range of this species (in Latin America) is moister and more thermally equable than are sites recently colonised within Australia (Tingley *et al*., 2014; Kosmala *et al*., 2017). The toad's invasion across tropical Australia also involves acceleration in rates of dispersal, with individuals in the vanguard of the invasion travelling several times further per night than do conspecifics in range-core locations (e.g. Alford *et al*., 2009). The acceleration is associated with shifts in morphology (e.g. of limb lengths and cranial dimensions: Phillips *et al*., 2006; Hudson *et al*., 2016) and plausibly could impose selection on skin flexibility (and thus thickness) as well, because toad locomotion involves substantial flexing of the body in each leap and bound (e.g. Akella & Gillis, 2011; Reilly *et al*., 2015).

Shifts in the cane toad's phenotype across its invaded range involve physiology as well as morphology and behaviour (e.g. Brown *et al*., 2015; Gruber *et al*., 2017). For example, toads from Australia are better-able to maintain locomotion under a wide range of conditions (temperatures, hydration states) than are conspecifics from the native range (Kosmala *et al*., 2017); and the progeny of toads from different locations within Australia, even if raised under identical conditions, also exhibit divergent locomotor abilities (Kosmala *et al*., 2018). To evaluate whether the structure of the skin also varies geographically among populations of cane toads, we examined samples of skin from toads collected over this range.

METHODS

STUDY SPECIES

Rhinella marina is a large "true toad" (Bufonidae) native to South America (Bessa-Silva et al. 2020). These toads were translocated to Puerto Rico and Hawai'i, and thence to Australia, where 101 toads were brought to Queensland in 1935 (Zug & Zug, 1979; Lever, 2001). Genetic studies confirm that the toads brought to Australia belong to the east-of-Andes clade *R. marina* rather than the Central American clade *R. horribilis* (Slade and Moritz 1998). In Australia, cane toads have dispersed over a wide geographic range (Phillips *et al*., 2006; Alford *et al*., 2009; Shine, 2010, 2018).

STRUCTURE OF ANURAN SKIN

The skin of an anuran can be separated into a series of layers, with different positions and structures and putatively, different functions (Vitt & Caldwell, 2013; Fig. 1). The outermost layer is the *stratum corneum*, made of keratinised dead cells, a primary protection against desiccation and mechanical damage to the integument. Just below is the *stratum germinativum*, responsible for producing cells to replace the *stratum corneum* following ecdysis or injury (Elias & Shapiro, 1957; Vitt & Caldwell, 2013). These two layers form the epidermis, which is connected to the underlying dermis by a thin basement membrane (Elias & Shapiro, 1957; Vitt & Caldwell, 2013).

The top layer of the dermis is the *stratum spongiosum*, containing integumentary structures such as blood vessels, mucus and granular glands and chromatophores (Elias & Shapiro, 1957; Vitt & Caldwell, 2013). The lower portion of the *stratum spongiosum* consists of a calcified 'Ground Substance' (also known as *substantia amorpha*) composed of mucopolysaccharides, calcium salts and proteins (Toledo & Jared, 1993b). Ground Substance

is highly variable both intraspecifically and interspecifically, and often is not a well-defined layer (Sampson *et al*., 1987; Schwinger *et al*., 2001). The most basal layer of the dermis, the *stratum compactum*, maintains the basic structure and form of the skin. The dermis is connected to the muscles by the *tela subcutanea*, a thin membranous, grid-like layer of tissue (Elias & Shapiro, 1957; Vitt & Caldwell, 2013).

COLLECTION OF ANIMALS

Toads were collected from the native range in Manaus, Amazonas (AM) and Alter do Chão, Pará (PA) in Brazil (BR), during January and February 2015. In Australia, we collected toads from two sites in Western Australia (WA; Oombulgurri, Kununurra), two sites in the Northern Territory (NT; Katherine, Leaning Tree Lagoon), two sites in Queensland (QLD; Townsville, Charters Towers), and two sites in New South Wales (NSW; Brooms Head and Tabbimoble) from November 2014 to November 2015. Finally, we collected toads from two sites in Hawai'i (HI; Kailua-Kona and Hilo) in June and July 2015. Climates and general environmental characteristics of the locations are summarised in Table 1 (see Kosmala *et al*., [2017] for more detail). There was a lag period of ~45 days between collection and skin sampling, during which time the animals were maintained with *ad libitum* access to food and water.

COLLECTION OF TISSUES

Toads were euthanized in a bath of tricaine methanosulfate (MS222 – 1g/L solution). We used a round biopsy punch (4 mm diameter) to remove samples of skin from a central dorsal site and a central ventral site of each individual (i.e. two samples per individual). Samples were then preserved in 10% formalin.

STAINING WITH HEMATOXYLIN AND EOSIN

Fixed tissue was dehydrated through graded ethanol solutions, cleared in xylene and infiltrated with paraffin wax (performed on an automatic platform – Shandon, Excelsior, Thermo-Fisher Scientific). Samples were sectioned trnasversally at 4–7 μm and stained with haematoxylin–eosin (Bancroft & Gamble, 2008).

MEASUREMENTS OF SKIN

Using ImageJ (Rasband, 2014), we measured depth of the epidermis and dermis, and within those, the *stratum corneum*, *stratum granulosum* + *germinativum* (combined due to the poor separation), *stratum spongiosum*, layer of Ground Substance and *stratum compactum*. We calculated an average thickness per layer from 10 measurements per animal, and we assigned a score for the density of material within the Ground Substance layer; density scores ranged from 1 (sparse) to 3 (dense). We photographed slides containing the stained tissue sections, to identify the dimensions of layers within the dermis and epidermis using ImageJ. We took 10 measurements for each layer of each sample, equally spaced across the available tissue sample.

ANALYSIS OF DATA

To examine geographic variation in structure of the skin, we compared the thickness of skin layers to the geographic origin of toads (R Development Core Team, 2013). Mean body masses of toads did not vary significantly among regions ($F_{2,69} = 0.84$, $P = 0.44$) but skin-layer thicknesses were positively correlated with body mass (all $P < 0.0001$) and thus, we included body mass as a covariate in our analyses. Mean skin thickness did not differ significantly between male and female toads from any locality if we included body mass as a

covariate (i.e. any sex difference was apparently due to size dimorphism), so we did not include sex as a factor in our later analyses.

To look at overall patterns in skin thickness on the dorsal and ventral sides of the body, we first performed a multivariate analysis of variance (MANOVA) with region of origin as the factor, body mass as the covariate, and total skin thicknesses (from dorsal and ventral surfaces) as the repeated measure. This analysis revealed a significant interaction between region and dorsal/ventral skin thickness (i.e. toads from different regions differed in relative thickness of dorsal vs. ventral skin). Thus, we examined patterns in dorsal and ventral skin separately using separate ANCOVAs for each layer. These ANCOVAs used geographic origin as the factor, and a relevant size measurement (body mass/ total skin thickness/ thickness of the dermis or epidermis) as a covariate. The thickness of the specific layer was the dependent variable. We used post-hoc Tukey's tests to identify which groups were significantly different.

Our initial analyses focused on spatial comparisons across the toads' geographic range (Brazil vs. Hawai'i vs. Australia). We then focused on spatial patterns within Australia, comparing toads sampled from the four states across the continent (QLD, NSW, NT, WA). For both sets of analyses we performed separate ANCOVAs for each dorsal and ventral skin layer measure, with body mass as the covariate and geographic location (either region, or state within Australia) as the factor. We accepted significance at $\alpha = 0.05$ and inspected residuals from all analyses for violations of assumptions.

In a related study (Kosmala, 2018), we measured water-exchange rates across the skin for the same animals from which we measured skin morphology. To clarify relationships between skin structure and function, we calculated Pearson correlations between each measure of skin layer thickness and measures of (i) skin resistance and (ii) rehydration rates. To adjust for body size, we performed Pearson correlations on residuals from regressions of skin layer

thicknesses against total skin thickness, and from regressions of skin resistance and rehydration rate on body mass. Because skin resistance was measured from toads in water-conserving posture (WCP), where only the dorsal surface was exposed to the air (Christian *et al*., 2017; Kosmala, 2018), we correlated skin resistance residuals with residual measures of dorsal (not ventral) skin. Likewise, because toads rehydrated using pelvic skin patches, we correlated rehydration rate residuals with residual measures of ventral (not dorsal) skin. Finally, we performed non-parametric correlations (Spearman's correlation test) between each residual skin layer measure (from regressions against total thickness) and climate variables in order to clarify relationships between relative layer thickness and climatic conditions.

RESULTS

OVERALL THICKNESS OF THE SKIN

In general, Australian toads had thinner skin than did either Brazilian or Hawai'ian toads, on both the dorsal and ventral surface (Fig. 2a, Table 2; Supporting Information Tables S1 and S2). On the dorsal surface of the body, Hawai'ian toads had thicker skin than did Australian toads, with Brazilian toads intermediate in this respect. Differences in thickness of the ventral skin were more pronounced; Brazilian toads had the thickest ventral skin, and Australian toads the thinnest (all post-hoc comparisons have $P < 0.05$; Fig. 2a; Supporting Information Tables S1 and S2). Patterns in skin thickness were more complex within Australia (Table 2; Supporting Information Tables S3 and S4). Toads from the Northern Territory had the thickest skins, both dorsally and ventrally (Fig. 3a, Table 2; Supporting Information Table S3).

LAYERS WITHIN THE DORSAL SKIN

The thickness of individual layers generally followed the same pattern as seen for overall skin thickness (thinner in Australian toads than in those from other regions; Fig. 2, Table 2; Supporting Information Table S1). However, Hawai'ian toads exhibited thicker dorsal epidermis and dermis than did either Brazilian or Australian toads. Despite their thinner dorsal skin, Australian toads had relatively thick dorsal epidermis (8.7% of total dorsal skin thickness, vs. 7.6% in Brazil and 7.9 % in Hawai'i).

Within Australia, toads from the Northern Territory had the thickest dorsal skin (Fig. 3a; Supporting Information Table S3 and S4) but the lowest proportional thickness of the epidermis (ratio of epidermis to total dorsal skin = 7.5%, vs. 9.8%, 9.3% and 8.8% in WA, QLD and NSW respectively). Thus, the thicker skins of NT toads were primarily due to thickening of the dermis.

LAYERS WITHIN THE VENTRAL SKIN

Brazilian toads had thicker ventral skin than did conspecifics from either Hawai'i or Australia (Fig. 2a,b,e; Supporting Information Table S1), due almost entirely to thickening of the dermis rather than epidermis (ratio of epidermis thickness to total ventral skin thickness = 6.4%, vs. 11.8% in Australia). As for the dorsal skin, NT toads had the thickest ventral skin within Australia, again due to thickening of the dermis (Fig. 3a,b,e; Supporting Information Table S3).

CORRELATIONS BETWEEN DENSITY AND THICKNESS WITHIN GROUND SUBSTANCE

Within the *stratum spongiosum,* the thickness of the Ground Substance was not significantly correlated with its density score (Pearson's correlation test, dorsal $r = 0.05$, $t = 0.39$, $P = 0.70$, 95% confidence interval = -0.19 to 0.28; ventral $r = -0.05$, $t = -0.41$, $P = 0.70$, 95% confidence interval = -0.28 to 0.19). That is, thicker layers did not correspond to denser

layers. Australian toads had denser ventral Ground Substance than did Brazilian conspecifics (Supporting Information Table S2) but the mean density of the dorsal Ground Substance did not differ significantly among populations. In comparisons restricted to data from Australian populations, density and thickness of Ground Substance were also not significantly correlated (Pearson's correlation test, dorsal $r = 0.02$, $t = 0.16$, $P = 0.87$, 95% confidence interval = -0.26 to 0.39; ventral $r = -0.15$, t -1.032, $P = 0.31$, 95% confidence interval = -0.42 to 0.14). The dorsal Ground Substance was denser in toads from Queensland than from conspecifics from WA or NT, but not significantly different from that seen in NSW toads (Fig. 3g; Supporting Information Table S4). In the ventral Ground Substance, mean density was similar among the populations (Fig. 3g; Supporting Information Table S4).

Correlations between skin structure and rates of water exchange

Skin resistance to water evaporation (the latter as measured by Kosmala [2018]) was positively associated with thickness of the dorsal *stratum corneum* ($r = 0.30$, $n = 45$, $P < 0.05$; Supporting Information Table S5) and negatively correlated with thickness of the dorsal *stratum compactum* ($r = -0.32$, $n = 45$, $P < 0.05$). No other thicknesses were significantly correlated with skin resistance to water loss (all $P > 0.05$), nor with rates of rehydration (also from Kosmala [2018]; all $P > 0.05$; Supporting Information Table S5).

Correlations between skin structure and climate variables

The density score for the ventral Ground Substance was correlated with mean annual rainfall at the collection site (Spearman's $\rho = -0.3081$, $P = 0.023$). Density of the ventral Ground Substance and thickness of the ventral *stratum compactum* were both correlated with mean temperature ($\rho = -0.35$, $P = 0.0097$; $\rho = 0.289$, $P = 0.035$) and mean minimum temperature (ρ

= -0.36, $P = 0.008$; $\rho = 0.35$, $P = 0.009$, respectively). Mean maximum temperature was also correlated with density of the ventral Ground Substance ($\rho = -0.30$, $P = 0.027$).

DISCUSSION

We examined the structure of skin from *Rhinella marina* in populations across much of the species' global range, to test two predictions. First, based on the idea that overall skin thickness impairs mobility, we expect toads from invasive populations (where individuals are highly dispersive) to have thinner skin than do toads within the native range. As predicted, skins were thicker in the native range than in the invaded range. Second, because toads experience hotter drier conditions in Australia than within their native range, we predicted that specific layers of the skin (those that constrain water exchange) should exhibit the reverse pattern to that noted above: that is, those layers should be thicker in Australian toads. Again, that prediction was supported; Australian toads had thicker epidermis relative to dermis than did Brazilian and Hawai'ian toads.

Our predictions depend upon arguments that link thickness of the skin (and of layers within the skin) to performance traits such as mobility and rates of water loss. The evidence for that link remains largely unexplored for mobility, especially in anurans. Detailed biomechanical analyses of toad locomotion reveal considerable flexion of the body during each leap, especially along the ventral surface (e.g. Reilly *et al.*, 2015), so we regard this functional hypothesis as plausible but untested. Comparative studies to link locomotor traits to skin structure would be of great interest. Stronger evidence is available for the role of the epidermis (especially the keratinised *stratum corneum*) in protecting against dehydration (Alibardi, 2003; Vitt & Caldwell, 2013). Consistent with that inference, toads with a thicker *stratum corneum* in our study had higher resistance to water loss. Australian toads also had a thicker layer of Ground Substance relative to the rest of the dermis (29.3% of the thickness of

292 the dorsal dermis and 27.2% of the thickness of the ventral dermis, vs. 22.5% and 22.7% for
293 Brazilian toads). The Ground Substance also may protect against dehydration (Elkan, 1976;
294 Kobelt & Linsenmair, 1986) by binding water molecules (Rogers, 1961). In summary, then,
295 Australian toads have thinner skins overall (thereby enhancing mobility), but the layers that
296 resist water loss are thicker (thereby reducing rates of water loss).
297 Although these conflicting pressures on structure of the skin are consistent with broad
298 patterns in our data, the variation in skin thickness among populations of Australian toads is
299 not. We expected skin to be thicker in eastern Australia, where toads are sedentary, than in
300 western sites where the animals are mobile (Alford *et al*., 2009). No such pattern was evident.
301 Consistently thin skins of Australian cane toads might reflect the ubiquity of mobility even in
302 eastern Australia, perhaps in response to unpredictable environmental challenges (e.g.
303 Schwarzkopf & Alford, 2002; Pettit *et al*., 2017).
304 In Brazil, toads are found in habitats with high humidity and ready availability of water
305 (Tingley *et al*., 2014). Their thick ventral skin (mostly due to thickening of the dermis) fits
306 well with their high rates of rehydration (Kosmala, 2018). In the environments occupied by
307 cane toads within their native range, low skin resistance to water evaporation (Kosmala,
308 2018) likely enhances survival. The toads' translocation to more severe climatic conditions in
309 Australia has resulted in complex changes to that ancestral condition. Based on the transition
310 in skin thickness from Brazil to Australia, we expected a continuing reduction in skin
311 thickness within Australia as the toads colonised hotter more arid environments. Instead,
312 toads in an intermediate climatic zone (NT) had the thickest skin, both dorsally and ventrally.
313 Why did overall skin thickness increase during the toads' invasion from Queensland into
314 the Northern Territory, but then decrease again as the toads moved even further west into the
315 highly arid Kimberley region of Western Australia? We suggest two possible (and non-
316 contradictory) explanations. First, cane toads in highly arid regions spend most of their time

close to waterbodies, minimising the importance of physiological control over rates of desiccation (Tingley & Shine, 2011). Thus, behavioural shifts could buffer the selective pressures associated with a change in skin morphology, such that shifts in skin morphology enhance organismal fitness only when climatic divergence is too great to be buffered by behavioural modulation.

A second possibility is that non-adaptive evolutionary processes at the expanding range edge have resulted in rapid spatial separation of phenotypes. Traits that enhance rates of dispersal accumulate at an invasion front even if those traits confer no selective advantage ("spatial sorting": Shine *et al*., 2011). Under that process, thin-skinned toads may dominate the extreme western extent of the species' range simply because thinner skin enhances dispersal rate, without conferring any benefit to an individual's survival or reproductive success (Shine *et al*., 2011). That enhanced dispersal rate might reflect an impact of skin thickness on bodily flexibility (as above), or an allocation of limited resources away from a thicker skin into other traits such as overall growth, metabolic expenditure or locomotion.

In summary, the morphology of the skin in cane toads shows extensive geographic variation, both among regions and within Australia. We do not know how much of that variation is adaptive (genetically based) or a phenotypically plastic response to local climatic conditions (Pickering & Richards, 1980; Kattan & Lillywhite, 1989; Menon *et al*., 1996). Neither do we know the exact benefits and costs associated with variation in skin morphology. It is clear, however, that this highly successful invasive species has made substantial adjustments to its integument in the course of its international diaspora. Our results emphasise the ability of range-expanding organisms to undergo rapid shifts in phenotypic traits, in ways likely to enhance their colonising ability.

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REFERENCES

Akella T, Gillis GB. 2011. Hopping isn't always about the legs: forelimb muscle activity patterns during toad locomotion. *Journal of Experimental Zoology A* **15:** 1–11.

Alford RA, Brown GP, Schwarzkopf L, Phillips BL, Shine R. 2009. Comparisons through time and space suggest rapid evolution of dispersal behaviour in an invasive species. *Wildlife Research* **36:** 23–28.

Alibardi L. 2003. Adaptation to the land: the skin of reptiles in comparison to that of amphibians and endotherm amniotes. *Journal of Experimental Zoology B* **298:** 12–41.

Anderson RC, Bovo RP, Eismann CE, Menegario AA, Andrade DV. 2017. Not good, but not all bad: Dehydration effects on body fluids, organ masses, and water flux through the skin of *Rhinella schneideri* (Amphibia, Bufonidae). *Physiological and Biochemical Zoology* **90:** 313–320.

Angilletta MJ Jr, Niewiarowski PH, Navas CA. 2002. The evolution of thermal physiology in ectotherms. *Journal of Thermal Biology* **27:** 249–268.

Angilletta MJ. 2009. *Thermal Adaptation: A Theoretical and Empirical Synthesis*. Oxford, UK: Oxford University Press.

Bancroft JD, Gamble M. 2008. *Theory and Practice of Histological Techniques*. Philadelphia, USA: Elsevier Health Sciences.

Bentley PJ, Yorio T. 1979. Evaporative water loss in anuran amphibia: a comparative study. *Comparative Biochemistry and Physiology Part A* **62:** 1005–1009.

Bessa-Silva A, Vallinoto M, Sampaio I, Flores-Villela OA, Smith EN, Sequeira, F. 2020. The roles of vicariance and dispersal in the differentiation of two species of the *Rhinella marina* species complex. *Molecular Phylogenetics and Evolution* **145**:06723.

Brattstrom BH. 1979. Amphibian temperature regulation studies in the field and laboratory. *American Zoologist* **19:** 345–356.

Brown GP, Phillips BL, Dubey S, Shine R. 2015. Invader immunology: invasion history alters immune system function in cane toads (*Rhinella marina*) in tropical Australia. *Ecology Letters* **18:** 57–65.

Chen W, Hudson CM, DeVore JL, Shine R. 2017. Sex and weaponry: the distribution of toxin-storage glands on the bodies of male and female cane toads (*Rhinella marina*). *Ecology and Evolution* **7:** 8950–8957.

Christian KA, Tracy CR, Tracy CR. 2017. Physical calculations of resistance to water loss improve predictions of species range models: comment. *Ecology* **98:** 2962–2964.

Christian K, Parry D. 1997. Reduced rates of water loss and chemical properties of skin secretions of the frogs *Litoria caerulea* and *Cyclorana australis*. *Australian Journal of Zoology* **45:** 13–20.

Condez TH, Haddad CF, Zamudio KR. 2020. Historical biogeography and multi-trait evolution in miniature toadlets of the genus *Brachycephalus* (Anura: Brachycephalidae). *Biological Journal of the Linnean Society* **129**: 664-686.

Elias H, Shapiro J. 1957. Histology of the skin of some toads and frogs. *American Museum Novitates* **1819:** 1–27.

Elkan E. 1976. Ground substance: an anuran defence against desiccation. In: Lofts G (ed) *Physiology of the Amphibia. Volume 3.* London: Academic Press, 101–110.

Gruber J, Brown G, Whiting MJ, Shine R. 2017. Is the behavioural divergence between range-core and range-edge populations of cane toads (*Rhinella marina*) due to evolutionary change or developmental plasticity? *Royal Society Open Science* **4:** 170789.

Heatwole H, Barthalmus GT. 1994. *Amphibian Biology Volume 1: The Integument.* Chipping Norton, NSW: Surry Beatty and Sons.

Hill RW, Wyse GA, Anderson M. 2004. *Animal Physiology. Volume 2*. Sunderland, MA: Sinauer Associates.

Hoffmann AA, Sgrò CM. 2011. Climate change and evolutionary adaptation. *Nature* **470:** 479.

Hudson CM, McCurry MR, Lundgren P, McHenry CR, Shine R. 2016. Constructing an invasion machine: the rapid evolution of a dispersal-enhancing phenotype during the cane toad invasion of Australia. *PLoS One* **11:** e0156950.

Irschick DJ, Jayne BC. 1999. Comparative three-dimensional kinematics of the hindlimb for high-speed bipedal and quadrupedal locomotion of lizards. *Journal of Experimental Biology* **202:** 1047–1065.

Jørgensen CB. 1997. 200 years of amphibian water economy: from Robert Townson to the present. *Biological Reviews* **72:** 153–237.

Kattan GH, Lillywhite HB. 1989. Humidity acclimation and skin permeability in the lizard *Anolis carolinensis*. *Physiological Zoology* **62:** 593–606.

Kim SY, Velando A. 2014. Stickleback males increase red coloration and courtship behaviours in the presence of a competitive rival. *Ethology* **120:** 502–510.

Kobelt F, Linsenmair KE. 1986. Adaptations of the reed frog *Hyperolius viridiflavus* (Amphibia, Anura, Hyperoliidae) to its arid environment. *Oecologia* **68:** 533–541.

Kosmala G. 2018. Evolution of physiological traits in the invasive cane toad (*Rhinella marina*). PhD thesis, School of Biological Sciences, University of Sydney, NSW, Australia.

Kosmala G, Christian K, Brown G, Shine R. 2017. Locomotor performance of cane toads differs between native-range and invasive populations. *Royal Society Open Science* **4:** 170517.

Kosmala G, Brown GP, Christian K, Hudson CM, Shine R. 2018. The thermal dependency of locomotor performance evolves rapidly within an invasive species. *Ecology and Evolution* **8:** 4403–4408.

Lever C. 2001. *The Cane Toad. The History and Ecology of a Successful Colonist*. Otley, West Yorkshire: Westbury Academic and Scientific Publishing.

Lillywhite HB. 1975. Physiological correlates of basking in amphibians. *Comparative Biochemistry and Physiology Part A* **52:** 323–330.

Lillywhite HB, Licht P. 1975. A comparative study of integumentary mucous secretions in amphibians. *Comparative Biochemistry and Physiology A* **51:** 937–941.

Long J, Hale M, McHenry M, Westneat M. 1996. Functions of fish skin: flexural stiffness and steady swimming of longnose gar, Lepisosteus osseus. *Journal of Experimental Biology* **199**: 2139-2151.

Losos JB, Mouton PLFN, Bickel R, Cornelius I, Ruddock L. 2002. The effect of body armature on escape behaviour in cordylid lizards. *Animal Behaviour* **64:** 313–321.

McClanahan LL, Stinner JN, Shoemaker VH. 1978. Skin lipids, water loss, and energy metabolism in a South American tree frog (*Phyllomedusa sauvagei*). *Physiological Zoology* **51:** 179–187.

McGaugh SE, Weaver S, Gilbertson EN, Garrett B, Rudeen ML, Grieb S, Roberts J, Donny A, Marchetto P, Gluesenkamp AG. 2020. Evidence for rapid phenotypic and

behavioural shifts in a recently established cavefish population. *Biological Journal of the Linnean Society* **129**: 143-161.

Menon GK, Maderson PFA, Drewes RC, Baptista LF, Price LF, Elias PM. 1996. Ultrastructural organization of avian *stratum corneum* lipids as the basis for facultative cutaneous waterproofing. *Journal of Morphology* **227:** 1–13.

Mooney HA, Hobbs RJ. 2000. *Invasive Species in a Changing World*. Washington DC: Island Press.

Navas CA, Antoniazzi MM, Jared C. 2004. A preliminary assessment of anuran physiological and morphological adaptation to the Caatinga, a Brazilian semi-arid environment. *International Congress Series* **275:** 298–305.

Navas CA, Antoniazzi MM, Carvalho JE, Suzukwe H, Jared C. 2007. Physiological basis for diurnal activity in dispersing juvenile *Bufo granulosus* in the Caatinga, a Brazilian semi-arid environment. *Comparative Biochemistry and Physiology Part A* **147:** 647–657.

Pettit L, Greenlees M, Shine R. 2017. The impact of transportation and translocation on dispersal behaviour in the invasive cane toad. *Oecologia* **184:** 411–422.

Phillips BL, Brown GP, Webb JK, Shine R. 2006. Invasion and the evolution of speed in toads. *Nature* **439:** 803.

Pickering AD, Richards RH. 1980. Factors influencing the structure, function and biota of the salmonid epidermis. *Proceedings of the Royal Society of Edinburgh B* **79:** 93–104.

R Development Core Team. 2013. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. Available at http://www.R-project.org/ (accessed 2 April 2016).

Rasband WS. 2014. *ImageJ*. US National Institutes of Health; Bethesda, Maryland, USA (1997–2012).

Reilly SM, Montuelle SJ, Schmidt A, Naylor E, Jorgensen ME, Halsey LG, Essner RL. 2015. Conquering the world in leaps and bounds: hopping locomotion in toads is actually bounding. *Functional Ecology* **29:** 1308–1316.

Rogers HJ. 1961. The structure and function of hyaluronate. *Biochemical Society Symposium* **20:** 51–78.

Rudh A, Qvarnström A. 2013. Adaptive colouration in amphibians. *Seminars in Cell and Developmental Biology* **24:** 553–561.

Sampson HW, Canon MS, Davis RW. 1987. The calcified amorphous layer of the skin of *Bufo marinus* (Amphibia: Anura). *Journal of Zoology* **213:** 63–69.

Sax DF, Stachowicz JJ, Brown JH, Bruno JF, Dawson MN, Gaines SD, Grosberg RK, Hastings A, Holt RD, Mayfield MM, O'Connor MI, Rice WR. 2007. Ecological and evolutionary insights from species invasions. *Trends in Ecology & Evolution* **22:** 465–471.

Schwarzkopf L, Alford RA. 2002. Nomadic movement in tropical toads. *Oikos* **96:** 492–506.

Schwinger G, Zanger K, Greven H. 2001. Structural and mechanical aspects of the skin of *Bufo marinus* (Anura, Amphibia). *Tissue and Cell* **33:** 541–547.

Shine R. 2010. The ecological impact of invasive cane toads (*Bufo marinus*) in Australia. *Quarterly Review of Biology* **85:** 253–291.

Shine R. 2018. *Cane Toad Wars*. Berkeley, CA: University of California Press.

Shine R, Brown GP, Phillips BL. 2011. An evolutionary process that assembles phenotypes through space rather than through time. *Proceedings of the National Academy of Sciences USA* **108:** 5708–5711.

Slade RW, Moritz C. 1998. Phylogeography of *Bufo marinus* from its natural and introduced ranges. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **265:** 769-777.

Teyssier J, Saenko SV, Van Der Marel D, Milinkovitch MC. 2015. Photonic crystals cause active colour change in chameleons. *Nature Communications* **6:** 6368.

Tingley R, Shine R. 2011. Desiccation risk drives the spatial ecology of an invasive anuran (*Rhinella marina*) in the Australian semi-desert. *PLoS One* **6:** e25979.

Tingley R, Vallinoto M, Sequeira F, Kearney MR. 2014. Realized niche shift during a global biological invasion. *Proceedings of the National Academy of Sciences USA* **111:** 10233–10238.

Toledo RC, Jared C. 1993a. Cutaneous adaptations to water balance in amphibians. *Comparative Biochemistry and Physiology Part A* **105:** 593–608.

Toledo RC, Jared C. 1993b. The calcified dermal layer in anurans. *Comparative Biochemistry and Physiology Part A* **104:** 443–448.

Vitt LJ, Caldwell JP. 2013. *Herpetology: An Introductory Biology of Amphibians and Reptiles*. Cambridge, MA: Academic Press.

Weitzman C, Kaestli M, Gibb K, Brown GP, Shine R, Christian K. 2019. Disease exposure and anti-fungal bacteria on skin of invasive cane toads, Australia. *Emerging Infectious Diseases* **25:** 1770–1771.

Zug GR, Zug PB. 1979. The marine toad, *Bufo marinus*: a natural history resumé of native populations. *Smithsonian Contributions to Zoology* **284:** 1–58.

SUPPORTING INFORMATION

Table S1. Mean ± standard deviation of thickness of skin layers for toads from Australia, Brazil and United States (Hawai'i). Measurements in μm.

Table S2. Results table for ANCOVAs comparing thickness of skin layers between cane toads from three regions (Brazil, Hawai'i, Australia). Bold font indicates significant values.

Table S3. Mean ± standard deviation of thickness of skin layers for toads from Australian states (WA = Western Australian, NT = Northern Territory, QLD = Queensland, NSW = New South Wales). Measurements in μm.

Table S4. Results table for ANCOVAs of skin layers by Australian state comparing thickness of skin layers between cane toads from four locations (states) within Australia. Bold font indicates significant values.

Table S5. Correlation between skin resistance to water exchange and thickness of skin layers, based on 45 cane toads for which we assessed both water-exchange rates and skin morphology. The residual score refers to the residual from the linear regression of (1) resistance to water loss vs. toad body mass, and (2) skin-layer thickness against toad body mass.

Table 1. Locations of collection and general climatic characteristics of those sites. *Köppen and Geiger Climate Classification System. Am = equatorial monsoonal, Af = equatorial fully humid, Aw = equatorial winter dry, BSh = arid steppe hot arid, Cfa = warm temperate fully humid hot summer. **Values in parentheses indicate range of mean monthly values for rainfall and temperature. NSW = New South Wales, NT = Northern Territory, QLD = Queensland, WA = Western Australia, BR = Brazil, HI = Hawai'i.

Region	Population	Site	Climate Classification*	Mean Annual Rainfall (mm)**	Mean Annual Temperature (°C)**
Australia	NSW	Brooms Head	Cfa	1471 (49–188)	19.2°C (13.8–23.6°C)
		Tabbimoble	Cfa	1558 (52–193)	19.4°C (14.0–23.6°C)
	NT	Katherine	Aw	1009 (0–250)	27.5°C (22.1–31.6°C)
		Leaning Tree	Aw	1500 (1–364)	27.2°C (23.9–29.4°C)
	QLD	Charters Towers	BSh	692 (8–142)	23.2°C (17.3–27.4°C)
		Townsville	Aw	1111 (9–275)	24.1°C (19.0–27.6°C)
	WA	Kununurra	BSh	720 (0–186)	28.8°C (23.3–32.6°C)
		Oombulgurri	BSh	718 (0–181)	29.4°C (24.3–32.9°C)

Brazil	BR	Manaus	Am	2145	27.4°C
				(56–295)	(26.9–28.2°C)
		Alter do Chão	Am	1991	25.9°C
				(34–346)	(25.1–26.9°C)
Hawai'i	HI	Hilo	Af	3459	23.1°C
				(177–397)	(21.7–24.6°C)
		Kailua-Kona	Aw	862	23.5°C
				(55–88)	(22.0–24.9°C)

540

541

Table 2. Results of MANOVA comparing total thickness of dorsal and ventral skin between cane toads from different regions. The first part of the Table (a) compares toads from Brazil, Hawai'i, and Australia; the second part compares toads from four locations (states) within Australia. Bold font indicates significant values.

(a) Among countries

	Variable	DF	Sum Sq	Mean Sq	*F*-value	*P*-value
Dorsal skin thickness	Region	2,66	0.071	0.035	4.55	**0.014**
	Mass	1,66	0.10	0.104	13.35	**0.0005**
	Region*Mass	2,66	0.008	0.004	0.50	0.61
Ventral skin thickness	Region	2,66	0.02	0.012	2.02	0.14
	Mass	1,66	0.14	0.14	23.41	**8.20e-06**
	Region*Mass	2,66	0.006	0.003	0.52	0.59

(b) Within Australia

	Variable	DF	Sum Sq	Mean Sq	*F*-value	*P*-value
Dorsal skin thickness	State	3,40	0.03	0.011	1.51	0.23
	Mass	1,40	0.045	0.045	6.08	**0.018**
	State*Mass	3,40	0.034	0.011	1.56	0.21
Ventral skin thickness	State	3,40	0.057	0.019	3.40	**0.03**
	Mass	1,40	0.036	0.036	6.35	**0.016**
	State*Mass	3,40	0.23	0.003	0.51	0.68

Figure Legends

Figure 1. Dorsal skin of *Rhinella marina*, stained with eosin and haematoxylin, with 4x augmentation.

Figure 2. Thickness of layers of the skin of cane toads collected in Brazil, USA (Hawai'i) and Australia. Letters represent significantly different groups, obtained with post-hoc Tukey's test of the ANCOVAs. Regular-font letters represent post-hoc of dorsal skin/ layers, while italic letters represent post-hoc of ventral skin/ layers.

Figure 3. Thickness of layers of the skin of cane toads collected from four geographic locations (states) within Australia (NSW = New South Wales, QLD = Queensland, NT = Northern Territory, WA = Western Australia). Letters represent significantly different groups, obtained with post-hoc Tukey's test of the ANCOVAs. Regular-font letters represent post-hoc of dorsal skin/ layers, while italic letters represent post-hoc of ventral skin/ layers.