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Laid-back invaders: Cane toads (*Rhinella marina*) down-regulate their stress responses as they colonize a harsh climateGeorgia K. Kosmala<sup>a</sup>, Gregory P. Brown<sup>a, b</sup>, Richard Shine<sup>a, b, \*</sup><sup>a</sup> School of Life and Environmental Sciences, University of Sydney, NSW, 2006, Australia<sup>b</sup> Department of Biological Sciences, Macquarie University, NSW, 2109, Australia

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## ABSTRACT

In response to encountering abiotic extremes, many organisms exhibit stress responses as measured by levels of corticosterone and heat-shock protein (e.g., HSP70) in the blood. Such responses can enhance organismal viability. How quickly can those responses shift if the organisms encounter novel challenges, as occurs with climate change, or a species' invasion into a new area? We found elevated levels of corticosterone and HSP70 in the blood of cane toads (*Rhinella marina*) that were desiccated, especially at low temperatures, a response that might jeopardize survival by increasing rates of water loss. Importantly, toads from the climatically equable native range in Brazil showed twofold higher levels of these stress hormones than did toads from the climatically harsh invaded range in Australia. Thus, the toads' invasion of abiotically extreme habitats has been accompanied by a substantial down-regulation of the acute stress response.

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## 1. Introduction

In any organism, an intricately co-adapted suite of physiological processes functions to support life by maintaining homeostasis of traits such as temperature regulation, blood flow and hormonal balance (Hill et al., 2004; Vitt and Caldwell, 2013). But when extreme external conditions threaten organismal survival, more dramatic responses are elicited. Such physiological responses to stressors include the perception of stressor magnitude, secretion of stress hormones, modulation of heart rate and behavioral responses (Hill et al., 2004; Vitt and Caldwell, 2013; Narayan, 2016).

Stress responses enable organisms to cope with unpredictable environmental variation. Several hormones and proteins are activated by stress, either as a direct response to the stressor itself or as a mechanism to reduce the impacts of the stress response. For example, corticosterone and heat-shock protein 70 (HSP70) are up-regulated by exposure to extreme climatic conditions (Chapovetsky and Katz, 2005; Angilletta, 2009; Narayan, 2016). Corticosterone, one of the primary endocrine mediators associated with the stress response, is highly responsive to a range of stressful conditions (Zimmermann and Critchlow, 1967; Denver, 1997; Belden et al., 2005; Crespi and Denver, 2005; Chambers, 2011; Dupoué et al., 2013, 2014; Narayan and Hero, 2014a, Narayan and Hero, 2014b; de Assis et al., 2015) as well as being affected by reproductive behavior (de Assis et al., 2012), development (Denver et al., 2002; Chambers et al., 2011) and balance of water and salts in the kidney

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(Brewer et al., 1980; Arad and Skadhauge, 1984; Vitt and Caldwell, 2013). HSP70 belongs to a class of chaperones intimately related to the thermal stress response, and is up-regulated as a means of cellular protection following exposure to thermal or hydric extremes (Krebs and Feder, 1997; Feder and Hoffmann, 1999; Chapovetsky and Katz, 2005; Schulte et al., 2011).

Climate change has exposed many organisms to abiotic conditions (temperature, humidity) that are more extreme than were previously the norm within the species' range. Thus, understanding how an organism's stress responses are modified by encountering such conditions can help us to predict the impacts of climate challenges. Invasive species provide excellent models for studies on this topic. As is the case with a species confronted by climate change, invaders encounter novel (and potentially extreme) abiotic conditions as they colonize hitherto-unoccupied areas (Carlton, 2000; Deutsch et al., 2008; Somero, 2010; Narayan et al., 2012; Rollins-Smith, 2017). If the invader can modify its stress responses to cope with those novel challenges (either through adaptation or plasticity), then we expect that stress responses of populations in the native range should differ from those in invaded regions (Sorte and Hoffmann, 2004; Somero, 2005, 2010; Zerebecki and Sorte, 2011; Liebl and Martin, 2012; Hernández et al., 2016; Martin et al., 2017).

One of the most intensively studied biological invasions is that of the cane toad (*Rhinella marina*, formerly *Bufo marinus*), a large anuran that has been translocated from South America to many other regions across the world (Freeland and Martin, 1985; Lever, 2001; Shine, 2018). Invasion may affect corticosterone responses in this species, with toads from recently-invaded sites in Florida exhibiting a lower increase in corticosterone levels in response to a novel stressor (1 h restraint) than did toads from long-colonized sites (Assis et al., 2020). In Australia, cane toads have penetrated into areas that are substantially hotter and drier, at least seasonally, than any that occur within the species' native range (Tingley et al., 2014). Jessop et al. (2013) recorded high levels of corticosterone in the blood of cane toads from these arid areas, presumably the result of thermal and hydric stress, and experimentally demonstrated that elevated corticosterone levels increased rates of water loss and thus, lowered survival under harsh conditions.

Cane toads thus provide an ideal species with which to investigate the ways in which exposure to abiotic extremes modifies the acute stress response. Based on the study of Jessop et al. (2013), we predicted that the responses of cane toads to thermal and hydric stresses (as measured by up-regulation of HSP70 and corticosterone) would have shifted as the anurans moved from the native range (where such stresses are rare) to Australia (where such stresses are common). More specifically, we predicted that cane toads in Australia would show a less dramatic elevation of the levels of corticosterone and HSP70 in response to thermal and hydric extremes, than would conspecifics from the native range. To test that prediction, we studied cane toads from five populations spanning both the ancestral and the invaded range, to see if they have adjusted their stress responses to local conditions.

## 2. Materials and methods

### 2.1. Study species

Cane toads are large "true toads" (Bufonidae) native to Latin America and southern North America. The toads were translocated via Puerto Rico and Hawai'i to Australia, with 101 toads brought to northeastern Australia in 1935 (Zug and Zug, 1979; Freeland and Martin, 1985; Lever, 2001). Within Australia, cane toads have colonized diverse environments, and pose a serious threat to some components of the Australian native fauna (Phillips et al., 2006; Alford et al., 2009; Shine, 2010, 2018). Previous research has revealed somewhat contradictory results with respect to geographic variation in corticosterone responses within Australian populations of cane toads. Brown et al. (2015) reported a higher acute stress response in free-ranging individuals from the range-edge than the range-core, whereas Selechnik et al., 2017a reported no significant geographic variation in stress responses among captive-raised offspring from different populations.

### 2.2. Collection of toads

Toads from the native range were collected 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 in November 2014, Kununurra in October 2015), two sites in the Northern Territory (NT: Katherine, Leaning Tree Lagoon, both in August 2015), two sites in Queensland (QLD: Townsville, Charters Towers, both in September 2015), and two sites in New South Wales (NSW: Brooms Head and Tabbimoble, both in October 2015). All toads were collected by hand, when the animals were active at night. Climatic characteristics of these locations are described in detail by Kosmala et al. (2017); briefly, the Brazilian sites offer relatively aseasonal and humid regimes whereas the Australian sites experience seasonally dry and hot conditions (Table 1).

### 2.3. Laboratory methods

Methods for the collection and husbandry of toads, for thermal and hydric exposure experiments, for the collection and treatment of blood samples, and the analytical approaches, were the same for studies in Brazil and Australia, and were performed by the same person (GK). Toads collected in Brazil were housed and tested in São Paulo, in facilities at Universidade de São Paulo – Instituto de Biociências. Toads collected in Australia were transported to facilities at Charles Darwin University – College of Engineering, IT & Environment. Those locations provided standardized conditions for both housing and

**Table 1**

Locations of collection, number of toads and mean mass ( $\pm$ standard deviation) of toads sampled in the study. Climatic data (from [Climate-data.org](https://climate-data.org)) show mean annual values for rainfall and ambient temperature, with mean monthly extremes (i.e., driest to wettest month; coolest to warmest month) in parentheses below. NSW = New South Wales, NT = Northern Territory, QLD = Queensland, WA = Western Australia, AM = Manaus, Amazonas, PA = Alter do Chão, Pará.

Country	Population Site	Latitude/ Longitude	Sex	N	Mass (g)	Mean annual rainfall (mm)	Mean annual temperature (°C)	
Australia	NSW	Brooms Head	Female	8	121.06 $\pm$ 22.03	1471	19.2 °C	
			Male	4	116.23 $\pm$ 19.15	(49–188)	(13.8–23.6)	
		Tabbimoble		Female	8	114.84 $\pm$ 32.86	1558	19.4 °C
				Male	4	96.53 $\pm$ 14.44	(52–193)	(14.0–23.6)
	NT	Katherine		Female	11	157.68 $\pm$ 71.91	1009	27.5 °C
				Male	7	94.17 $\pm$ 22.77	(0–250)	(22.1–31.6)
		Leaning Tree Lagoon		Female	10	168.61 $\pm$ 38.79	1500	27.2 °C
				Male	10	142.29 $\pm$ 22.71	(1–364)	(23.9–29.4)
	QLD	Charters Towers		Female	10	123.35 $\pm$ 32.78	692	23.2 °C
				Male	5	104.78 $\pm$ 13.15	(8–142)	(17.3–27.4)
		Townsville		Female	12	95.85 $\pm$ 13.97	1111	24.1 °C
				Male	7	78.24 $\pm$ 9.62	(9–275)	(19.0–27.6)
WA	Kununurra		Female	10	84.74 $\pm$ 18.54	720	28.8 °C	
			Male	7	91.73 $\pm$ 16.29	(0–186)	(23.3–32.6)	
	Oombulgurri		Female	17	132.84 $\pm$ 17.96	718	29.4 °C	
			Male	2	125.40 $\pm$ 64.06	(0–181)	(24.3–32.9)	
Brazil	AM	Manaus		Female	7	255.37 $\pm$ 146.19	2145	27.4 °C
				Male	11	75.08 $\pm$ 22.49	(56–295)	(26.9–28.2)
	PA	Alter do Chão		NA	2	93.29 $\pm$ 48.88		
				Male	20	112.52 $\pm$ 21.96	1991	25.9 °C
							(34–346)	(25.1–26.9)

experimentation. Toads were transported from field to experimental locations by car whenever possible, or by airline if not, using the most direct route available. In both scenarios, toads were placed in individual damp cloth bags, inside a Styrofoam carrier with cooling blocks. Once in their laboratory housing location, toads were allowed to acclimate for a week in order to recuperate from any stress caused by travelling. They were housed in 20 L individual plastic containers, with meshed lids for ventilation, a hiding spot and *ad libitum* access to water, in temperature-controlled rooms set to  $25 \pm 0.5$  °C. Toads were fed daily with crickets, cockroaches and mealworms, until the day before the beginning of the experiments. Food was then withheld (so as not to change toad mass), but the animals were allowed *ad libitum* access to food and water as soon as the experiments had concluded.

Toads were allowed to hydrate fully in individual containers with ~1 cm depth of filtered water and were kept at 25 °C for 2 h before we took baseline blood samples. We sampled 0.5 mL of blood via cardiac puncture using a heparinized 25-gauge needle and syringe, within 3 min of opening the toads' enclosures (a timespan that should prevent any stress response from affecting stress-hormone levels: [Wingfield and Romero, 2001](#); [Romero and Reed, 2005](#)). The blood sample was ~1% of the average mass of toads in our study and its removal did not affect the animal's health. We recorded mass for each toad, after gently squeezing the abdomen to empty the bladder. We then assigned toads randomly to treatments of a combination of temperature (15, 25 or 35 °C) and dehydration (100 and 70% of initial mass) such that each toad was only sampled twice – once for baseline and once post-treatment.

All samples were taken in a temperature-controlled room set to the test temperature. Toads assigned to the dehydration treatment were allowed to dehydrate slowly while exposed to a ventilated room with no access to water (over 3–4 days) until they reached 70% of their original body mass (bladder empty), and then blood-sampled again.

We attempted to obtain equal numbers of males and females from each population. All toads were subjected to all treatments, except that blood was taken only from toads whose body condition allowed for sampling without causing further stress. Thus, sample sizes were higher for the initial treatment than for all other groups, as any toads that appeared to be unwell were excluded on animal-ethics grounds (final sample sizes for corticosterone - N = 25 for 15 °C/70% hydration, N = 23 for 15 °C/100% hydration, N = 14 for 25 °C/70% hydration, N = 10 for 25 °C/100% hydration, N = 17 for 35 °C/70% hydration, N = 25 for 35 °C/100% hydration; HSP70 - N = 24 for 15 °C/70% hydration, N = 34 for 15 °C/100% hydration, N = 16 for 25 °C/70% hydration, N = 159 for 25 °C/100% hydration, N = 17 for 35 °C/70% hydration, N = 29 for 35 °C/100% hydration).

We centrifuged the blood samples at ~3000 rpm for 4–5 min, removed plasma to individually identified tubes and froze them until processing. First, we performed a corticosterone extraction by adding 3 mL of ether to 10  $\mu$ L of sample, vortexing for 30 s and centrifuging for 9 min at 1800 rpm and 4 °C. We allowed the samples to decant in –80 °C freezer for 7 min and transferred the liquid phase to a new tube, that was kept in a laminar flow hood at room temperature ( $20 \pm 2$  °C) until all ether evaporated overnight ([Mendonça et al., 1996](#)). We resuspended the samples in EIA buffer and assayed corticosterone using EIA kits (number 500655; Cayman Chemicals, Ann Arbor, MI), validated for the toad *Rhinella icterica* ([de Assis et al., 2015](#)). Samples were run in duplicate for each dilution, with dilutions starting at 1:50 and ending at 1:150, adjusted to fit the kit's standard range. Final concentrations were averaged from duplicated samples and adjusted for dilution factors. HSP70 levels

were measured from raw plasma samples with EIA kit specific for toads (number MBS108988; MyBiosource, San Diego, CA) as instructed by the manufacturer.

#### 2.4. Statistical analyses

We performed mixed models analyses using the restricted maximum likelihood method to determine the impact of experimental temperature and hydration levels on corticosterone and HSP70. The analyses included three independent variables: location of origin (Brazil, WA, NT, QLD, NSW), hydration level (100% or 70%) and temperature (15, 25 and 35 °C) and their interactions. We included sex as a fixed main effect in the models and toad ID was included as a random effect. Measurements were log-transformed prior to statistical analysis to satisfy assumptions of normality and variance homogeneity. Mixed models were performed using Proc Mixed in SAS 9.4 (SAS Institute, Cary NC). We performed a Tukey's post-hoc test to determine which groups differed significantly from each other.

### 3. Results

#### 3.1. Corticosterone and HSP70 levels

The intra and inter-assay coefficients of variation were 9.3% and 14.4% for corticosterone assays and 3.7% and 7.8% for HSP70 assays. Corticosterone levels differed among populations and were affected by the interaction between temperature and hydration (Table 2). Toads from Brazil, NT and QLD exhibited higher overall levels of corticosterone than did toads from WA and NSW (Fig. 1a). Dehydration dramatically increased corticosterone levels (Fig. 1b). Among fully hydrated toads, corticosterone levels increased as temperature increased, but among dehydrated toads, corticosterone was elevated at lower temperatures (Fig. 1b).

HSP70 levels also differed among populations, with a significant interaction between population and hydration (Table 3). Brazilian toads exhibited higher levels of HSP70 than did conspecifics from Australian populations (Fig. 2). In most populations HSP70 levels were relatively unaffected by hydration level. The effect of hydration level on HSP70 level varied among populations. Dehydration tended to increase HSP70 levels in Brazilian toads, but to reduce HSP70 levels in toads from NT and QLD populations (Fig. 2).

Overall, corticosterone and HSP70 levels were positively but non-significantly correlated (Pearson's correlation test,  $r = 0.14$ ,  $P = 0.06$ ). Neither sex nor body mass of individuals had significant effects on levels of corticosterone or HSP70.

### 4. Discussion

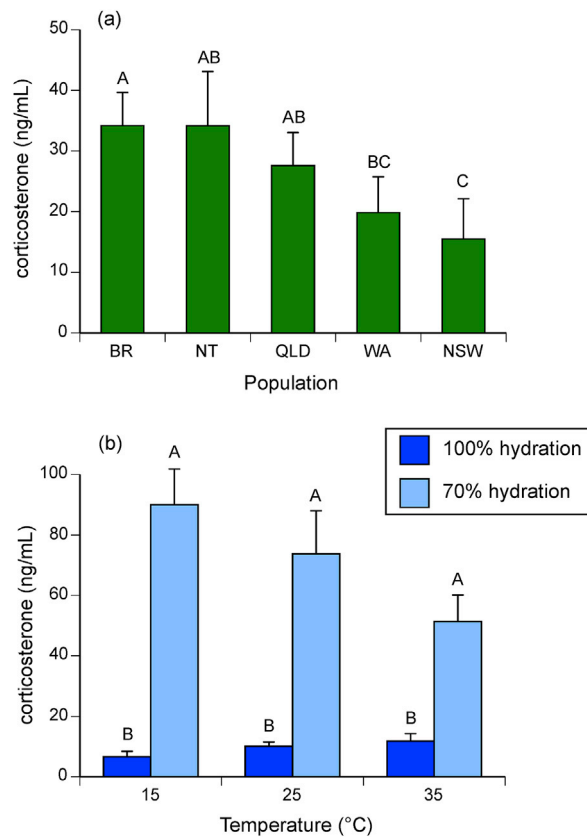
The translocation of cane toads to Australia has exposed them to more variable and extreme abiotic conditions than they experience within the native range (Tingley et al., 2014; Kosmala et al., 2017). One way in which toads respond to abiotic challenges is to up-regulate stress responses, including levels of corticosterone and heat-shock protein (Cao et al., 2009; Jessop et al., 2013; Brown et al., 2015; Assis et al., 2020). However, that up-regulation comes at a cost: elevated corticosterone levels increase a toad's rate of desiccation, and thus lower its survival under harsh conditions (Jessop et al., 2013). Hence, we predicted that the translocation of toads from equable climates to more extreme ones should have resulted in a down-regulation of the stress response within the invaded range; and indeed, levels of both corticosterone and HSP70 were lower within Australia than within the toad's native range in Brazil.

First, why was corticosterone elevated at lower test temperatures and after desiccation? The likely answer is that both cold and dehydration are stressful to a cane toad. The danger posed by desiccation is clear: excessive loss of water is fatal (see Webb et al., 2014). Desiccation also impairs some aspects of innate immune function in cane toads (Brusch et al., 2019). Low temperatures are unlikely to endanger survival per se, but they reduce locomotor ability (Kosmala et al., 2017) and so affect a toad's ability to move and seek out resources within the environment. Thus, the combination of desiccation and low temperatures is potentially life-threatening; a dehydrated toad needs to leave its shelter site to locate water, but is incapable of

**Table 2**

Results of mixed model analysis on the effects of sex, test conditions (temperature, hydration) and population of origin on levels of corticosterone in the blood of cane toads. Individual ID was included in the model as a random effect. Bold font indicates significant results.

Source	df	F value	P value
Sex	1, 90	2.52	0.1160
Temperature	2, 177	0.41	0.6669
Hydration	1, 176	232.10	< <b>0.0001</b>
Population	4, 104	14.50	< <b>0.0001</b>
Temperature* Hydration	2, 172	3.84	<b>0.0234</b>
Temperature* Population	8, 172	1.00	0.4382
Hydration* Population	4, 175	2.16	0.0755
Temperature*Hydration*Population	7, 173	0.33	0.9396



**Fig. 1.** Levels of corticosterone among cane toads (*Rhinella marina*) were significantly affected by a) the population from which they originated ( $P < 0.0001$ , Table 2) and b) a significant interaction between temperature and hydration level ( $P = 0.0234$ ; Table 2). Bars indicate mean values and errors indicate standard errors. Letters above bars indicate Tukey groupings, based on analysis of ln-transformed data. Bars with the same letter are not significantly different.

rapid locomotion. The observed pattern (maximal corticosterone levels at the combination of cold and dry) may reflect that challenge.

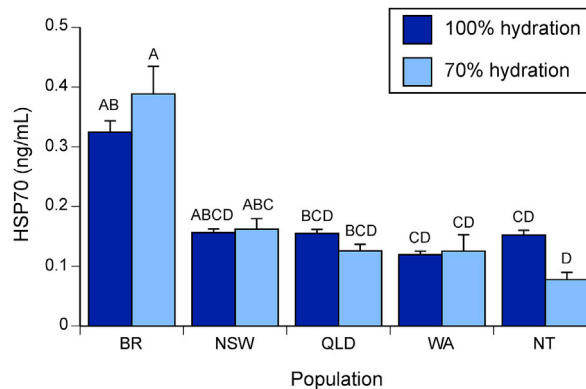
Second, why did toads from invasive populations in Australia (especially WA and NSW) tend to show a lower elevation of corticosterone levels in response to abiotic stressors than did conspecifics from Brazil? Corticosterone is involved in many pathways of water balance and salt concentration in the kidneys, as well as in the acute stress response (Zimmermann and Critchlow, 1967; Brewer et al., 1980; Arad and Skadhauge, 1984; Denver, 1997; Belden et al., 2005; Crespi and Denver, 2005; Chambers, 2011; Dupoué et al., 2013, 2014; Vitt and Caldwell, 2013). The role of corticosterone in water balance is poorly explored, but may relate to its ability to bind to mineralocorticoid receptors (MR) (Norris and Carr, 2013). MRs also bind aldosterone, which plays a central role in osmoregulation. Thus, high concentrations of corticosterone could affect water balance by chemically interfering with the role of aldosterone. That multiple set of functions may explain why experimental manipulation of the acute stress response lowers survival as well as increases water loss (Jessop et al., 2013). Up-regulation of the stress response to abiotic challenges may well be adaptive in the native range, but not in Australia where toads encounter abiotic conditions so extreme that the ancestral stress response would lower their survival (Jessop et al., 2013; Tingley et al., 2014). Corticosterone levels can adapt to thermal environments, so that species exposed to lower temperatures can tolerate higher corticosterone levels without damage (Telemeco and Addis, 2014). Considering the potential for rapid evolution of cane toads (including, for the thermal and hydric sensitivity of locomotor ability: Kosmala et al., 2017), the wider range of temperatures encountered in Australia (in comparison to the native climate) appears to have driven toads to adapt their corticosterone response to fit the new climatic challenges.

HSP70 shows similar geographical patterns as corticosterone, and probably for similar reasons. In a study on Chilean snails, Gaitán-Espitia et al. (2013) showed that snails from a hotter, drier population exhibited higher levels of HSP70 than did snails from a colder, more humid population. In tardigrades – creatures remarkable for their resistance to desiccation – HSP70 is elevated by heat and rehydration, but is lowered by desiccation, suggesting that its role is connected to repair processes (Jönsson and Schill, 2007). Our data on levels of HSP70 in toads from climatically different populations suggest that native-range toads increase HSP70 levels as a protective and repairing tool in response to (rarely-encountered) extremes of temperature and desiccation – but that after they were translocated to Australia, where such challenges are commonplace, the HSP70 response was down-regulated.

**Table 3**

Results of mixed model analysis on the effects of sex, test conditions (temperature, hydration) and population of origin on levels of HSP70 in the blood of cane toads. Individual ID was included in the model as a random effect. Bold font indicates significant results.

Source	df	F value	P value
Sex	1, 168	0.01	0.9313
Temperature	2, 215	0.81	0.4457
Hydration	1, 212	0.87	0.3515
Population	4, 223	41.97	<b>&lt; 0.0001</b>
Temperature* Hydration	2, 210	0.87	0.4213
Temperature* Population	8, 206	0.78	0.6168
Hydration* Population	4, 205	4.64	<b>0.0013</b>
Temperature*Hydration* Population	6, 206	0.69	0.6562



**Fig. 2.** Levels of heat-shock protein (HSP70) among cane toads (*Rhinella marina*) were significantly affected by the population from which they originated ( $P < 0.0001$ , Table 3) and a significant interaction between population and hydration level ( $P < 0.002$ , Table 3). Bars indicate mean values and errors indicate standard errors. Letters above bars indicate Tukey groupings, based on analysis of ln-transformed data. Bars with the same letter are not significantly different.

Alternatively, the geographic divergences that we have documented might be explained as non-adaptive shifts (founder effects during translocations and range expansion), or as adaptive responses to selective forces other than thermal and hydric extremes. We doubt the former (non-adaptive) explanation, given the magnitude of the divergences and the important consequences of stress responses for organismal viability (Jessop et al., 2013). It is more difficult to evaluate the second hypothesis, because a wide range of factors undoubtedly differ between the native range and the invaded range (e.g., enemy release due to loss of native-range parasites during translocation: Selechnik et al., 2017b). However, our experimental design specifically manipulated two factors (temperature and desiccation level) that differ between the two countries, and that have clear and direct impacts on the viability of an anuran (Tingley et al., 2014). That design thus increases (but does not guarantee) the likelihood that the responses we documented are related to geographic variation in the traits that we manipulated.

Future work could usefully explore the degree to which the differences that we have documented between Brazilian and Australian cane toads is due to phenotypic plasticity versus to genetically-based factors. Previous studies on a wide range of traits (including thermal and hydric effects on locomotor performance) have shown that both adaptation and plasticity have contributed to geographic divergence in cane toad phenotypes (Hudson et al., 2016a, 2017, 2018; Gruber et al., 2017a; McCann et al., 2018; Stuart et al., 2019). Thus, both processes may influence stress responses as well. Studies on captive-reared offspring from different populations could clarify this issue, as they have for other traits within this species (e.g., Brown et al., 2015; Hudson et al., 2016b, 2017; Gruber et al., 2017b).

Regardless of the proximate mechanisms responsible, our data show striking divergences that have accumulated in <85 years. Cane toads in Australia exhibit stress responses very different from those seen in conspecifics from South America. Clearly, then, the toads have been able to rapidly modify their stress responses in ways that plausibly assist them in dealing with novel abiotic challenges. This is an encouraging result in a world where many species are now encountering thermal and hydric conditions outside those they have experienced over recent centuries. If they are as flexible as cane toads, they may be able to deal with environmental change. On the other hand, successful invaders like the cane toad may exhibit higher levels of flexibility than most other species (Hudson et al., 2016a, 2017; Gruber et al., 2017a; Kosmala et al., 2017; McCann et al., 2018; Shine, 2018) and thus, we need studies on a much wider range of taxa before we can predict general levels of flexibility in the stress response.

## Research data

Data will be deposited in Dryad Digital Repository upon manuscript acceptance.



## Declaration of competing interest

The authors declare that they have no conflict of interest with any issues associated with this manuscript.

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