Does greater thermal plasticity facilitate range expansion of an invasive terrestrial anuran into higher latitudes?

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Temperature has pervasive effects on physiological processes and is critical in setting species distribution limits. Since invading Australia, cane toads have spread rapidly across low latitudes, but slowly into higher latitudes. Low temperature is the likely factor limiting high-latitude advancement. Several previous attempts have been made to predict future cane toad distributions in Australia, but understanding the potential contribution of phenotypic plasticity and adaptation to future range expansion remains challenging. Previous research demonstrates the considerable thermal metabolic plasticity of the cane toad, but suggests limited thermal plasticity of locomotor performance. Additionally, the oxygen-limited thermal tolerance hypothesis predicts that reduced aerobic scope sets thermal limits for ectotherm performance. Metabolic plasticity, locomotor performance and aerobic scope are therefore predicted targets of natural selection as cane toads invade colder regions. We measured these traits at temperatures of 10, 15, 22.5 and 30°C in low- and high-latitude toads acclimated to 15 and 30°C, to test the hypothesis that cane toads have adapted to cooler temperatures. High-latitude toads show increased metabolic plasticity and higher resting metabolic rates at lower temperatures. Burst locomotor performance was worse for high-latitude toads. Other traits showed no regional differences. We conclude that increased metabolic plasticity may facilitate invasion into higher latitudes by maintaining critical physiological functions at lower temperatures.

Key words: Aerobic scope, cane toad, invasive species, metabolic rate, Rhinella marina, thermal plasticity

Introduction

Recent evidence indicates that biodiversity is declining on a global scale (Butchart et al., 2010). This is a major concern because of the largely negative impact that a reduction in species richness has on many ecosystem functions (Cardinale et al., 2012). Human activity is a major cause of this reduction in species richness, primarily through actions leading to habitat loss (Hanski, 2011). The transportation of alien species into new environments also has the potential to impact biodiversity significantly and is a factor that has become increasingly relevant as human activity has globalization (Mooney and Cleland, 2001). Invasive species may reduce or eliminate native populations through processes such as predation (Savidge, 1987; Case and Douglas, 1991; Marsh and Douglas, 1997; Burbidge and Manly, 2002; Blackburn et al., 2004), competition (Porter and Savignano, 1990; Kenward and Holm, 1993; Douglas et al., 1994; Petren and Case, 1996; Holway, 1999; Byers, 2000) and pathogenicity (Mamaev et al., 1995; Whittington et al., 1997; Wyatt et al., 2008). Understanding the factors that allow a...
given alien species to invade successfully, i.e. to colonize and flourish in a new environment, is critical for their management, and numerous studies have been designed to improve our understanding of what factors predict invasive success (Gallien et al., 2010). There are many hypotheses that attempt to explain determinants of successful invasion, implicating factors such as the traits of the invaders, size and frequency of introductions and the geography at the sites of introduction (reviewed by Catford et al., 2009), but no consensus regarding what creates a successful invader or even how best to address this question experimentally has been reached (van Kleunen et al., 2010). Instances of post-arrival adaptation of invaders have been demonstrated repeatedly, suggesting that local adaptation may be an important factor for invasive success yet this is often not considered in studies aiming to determine what makes some species superior colonists (Whitney and Gabler, 2008). The present study investigates the possibility of local adaptation among populations of the highly invasive cane toad (Rhinella marina) within Australia, where this species has had a significant negative ecological impact. The question of whether adaptation will facilitate invasion beyond current predictions is critical for informing management strategies.

The cane toad (R. marina) is a large anuran species native to Central and South America (Zug and Zug, 1979) that has been introduced to numerous countries around the world, including Australia, Puerto Rico, Fiji and the USA (Hawaii and Florida), as a method of agricultural pest control (Easteal, 1981). The cane toad is toxic (Smith and Phillips, 2006; Hayes et al., 2009), exhibits a flexible breeding cycle with high reproductive output (Lampo and Medinaldea, 1996; Shine, 2010), has proved to be adept at colonizing new environments (Lever, 2001) and is considered to be one of the 100 worst invasive alien species in the world (Lowe et al., 2000). In Australia, the cane toad has gained a significant foothold, spreading to >15% of the continent since its introduction in 1935 (Kolbe et al., 2010). After its release into tropical Queensland, the cane toad expanded its range predominantly across lower latitudes in northern Australia and is now present in northern Western Australia.

How far introduced cane toads will ultimately spread has been a question that has commanded considerable attention, and several predictive studies have been carried out in an attempt to provide an answer (reviewed by Phillips et al., 2008). The first of these suggested that cane toads had already exceeded their expected distribution limits in Australia (Sutherst et al., 1996), but more recent studies all predict cane toad distributions that extend significantly further at lower latitudes than the present distribution and predict comparatively minimal expansion into higher latitudes (Urban et al., 2007; Kearney et al., 2008; Kolbe et al., 2010). In addition, in all three of these recent studies the potential for adaptation to confound predictions of future distributions is discussed. This possibility deserves attention because species removed from their native environment may be afforded the opportunity to adapt in novel ways due to the addition and subtraction of various selection pressures (Ghalambor et al., 2007; Sexton et al., 2009).

Establishing evidence of local adaptation within populations is challenging. To demonstrate that phenotypic differences between populations have a genetic basis, multiple generations must be raised in common controlled conditions; the so-called ‘common garden experiment’ (Kawecki and Ebert, 2004). Such an experimental approach is necessary to account for the confounding effects of phenotypic and developmental plasticity (Wilson and Franklin, 2002). This approach has been used to demonstrate heritable differences in growth rate (Phillips, 2009) and dispersal rate (Phillips et al., 2010) between populations of cane toads along the rapidly expanding northwest invasion front and long-established populations near the point of introduction. Adaptation of other traits, including limb morphology, hopping speed, endurance and desiccation resistance (Phillips et al., 2006; Llewelyn et al., 2010; Jessop et al., 2013), has also been implicated as a mechanism facilitating the accelerating northwest push across low latitudes, but no study as yet has considered whether adaptation to cold conditions is facilitating the movement of cane toads into higher latitudes.

A recent theory that provides a mechanistic explanation for temperature limitation in ectotherms is the oxygen-limited thermal tolerance (OLTT) hypothesis (Zielinski and Pörtner, 1996; De Watchter et al., 1997). This hypothesis postulates that thermal limits (i.e. the minimal and maximal temperatures at which an organism can survive) in ectotherms are determined by the inability of the circulatory system to deliver oxygen above and beyond an organism’s basal metabolic requirements at low and high temperatures. This difference between maximal oxygen supply and the metabolic cost of basic physiological maintenance (aerobic scope) allows an organism to devote energy to additional functions that enhance its Darwinian fitness, such as locomotion and reproduction (Bennett and Ruben, 1979; Carey, 1979). Several studies have provided support for the OLTT hypothesis among aquatic vertebrates and invertebrates (Frederich and Pörtner, 2000; Mark et al., 2002; Peck et al., 2002; Lannig et al., 2004; Nilsson et al., 2009; Neuheimer et al., 2011; Verberk and Calosi, 2012), but relatively little attention has been given to its applicability for terrestrial species. Only two studies have tested OLTT on a terrestrial vertebrate ectotherm, both on the cane toad, but neither found any evidence for OLTT (Seebacher and Franklin, 2011; Overgaard et al., 2012).

An interesting observation to emerge from the studies by Seebacher and Franklin (2011) and Overgaard et al. (2012) was that cane toads acclimated to 20 and 30°C showed no difference in metabolic rate when measured at their respective acclimation temperatures, despite a 10°C difference in test temperature, demonstrating the significant thermal metabolic plasticity of the species (Seebacher and Franklin, 2011; Overgaard et al., 2012). Such a response suggests that maintaining metabolic rate above a minimal threshold may be an important strategy for coping with low temperatures; hence, metabolic plasticity may be key to expanding the cane toad’s thermal niche. While increased metabolic rate may be
Seebacher and Boratyński (2010) noted that the first population was sourced from a 150 km stretch of coast in northern Queensland in September and October 2012. The low-latitude cane toads were collected from two populations adjacent to the Gulf of Carpentaria, and the second was collected in Mareeba. These populations were chosen due to their proximity to Cairns, where cane toads were first introduced to Queensland. High-latitude cane toads were collected from two populations in Ballina and Yamba in northern New South Wales in December 2012 (Supplementary Fig. S1). New South Wales Parks and Wildlife officers specializing in invasive species management reported that these represent some of the most southern locations in Australia where cane toads are well established.

Once transported to The University of Queensland, 40 toads from each region (high and low latitude) were divided equally and randomly across two acclimation temperatures of 15 and 30°C. Toads were maintained in groups of five in plastic containers 65 cm long × 35 cm wide × 35 cm deep containing water and damp bark chips deep enough for burrowing. Containers were housed inside two temperature-controlled rooms, in which the acclimation temperatures were regulated to ±1.5°C, and maintained a 12 h light–12 h dark photoperiod. All toads were acclimated to these conditions for a minimum of 8 weeks and a maximum of 14 weeks before measurements. Animals were collected at different times during the year to reduce the time over which measurements were taken and to align with the later emergence of high-latitude toads, thus the collection and acclimation of high-latitude toads began as experimental procedures on low-latitude toads commenced. Toads maintained at 30°C were fed an average of four adult cockroaches (Nauphoeta cinerea) per week, while those at 15°C were fed two adult cockroaches per week. This was chosen based on the reported energy density of this cockroach (Secor and Faulkner, 2002) and cane toad resting rates of oxygen consumption determined in a previous study (Halsey and White, 2010).

**Physiological traits and test temperatures**

The physiological traits of \( V_{O2,rest} \), \( V_{O2,max} \), aerobic scope and locomotor performance were measured at the four test temperatures of 10, 15, 22.5 and 30°C. The \( V_{O2,rest} \) was measured during the inactive circadian phase (day). The \( V_{O2,max} \) and locomotor performance were measured during the active circadian phase (night). A stratified random-order regimen was used for all measurements.

**Resting rate of oxygen consumption**

The \( V_{O2,rest} \) (in millilitres of \( O_2 \) per hour) of an individual toad was measured using positive-pressure flow-through respirometry. Atmospheric air was drawn from outside using a pump (TR-SS3; Sable Systems, USA) and scrubbed of \( CO_2 \) using soda lime and water vapour using Drierite before passing through a mass flow controller (GFC17; Aalborg, Orangeburg, NY, USA) that regulated the flow rate to a nominal value of 50 ml min\(^{-1}\) at 10, 15 and 22.5°C and 100 ml min\(^{-1}\) at 30°C. Mass flow controllers were calibrated using a NIST-traceable bubble film flowmeter (1–10–500 ml; Bubble-O-Meter, Dublin, OH, USA). After the mass flow controller, air was humidified using a 1 litre gas-washing bottle (Schott, French’s Forest, NSW, 2013).

**Materials and methods**

**Animal collection and maintenance**

Low-latitude cane toads were collected from two populations in northern Queensland in September and October 2012. The first population was sourced from a 150 km stretch of coastline adjacent to the Gulf of Carpentaria, and the second was collected in Mareeba. These populations were chosen due to their proximity to Cairns, where cane toads were first introduced to Queensland. High-latitude cane toads were collected from two populations in Ballina and Yamba in northern New South Wales in December 2012 (Supplementary Fig. S1). New South Wales Parks and Wildlife officers specializing in invasive species management reported that these represent some of the most southern locations in Australia where cane toads are well established.

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Peak post-exercise rate of oxygen consumption

Logistical constraints dictated that the measurement regimen for VO\(_{2}\)\(_{\text{max}}\) (in millilitres of O\(_{2}\) per hour) differed between high- and low-latitude toads. Low-latitude toads were tested on consecutive nights after all VO\(_{2}\)\(_{\text{rest}}\) measurements were complete, with 20 toads measured per night at a single test temperature each night until all toads had been tested at all test temperatures. As with VO\(_{2}\)\(_{\text{rest}}\) measurements, all toads in a container were tested consecutively in a random order, with the order of containers from both acclimation treatments also randomized. For high-latitude toads, every 5 days following a block of VO\(_{2}\)\(_{\text{rest}}\) measurements on 20 toads at a given test temperature, those toads underwent the VO\(_{2}\)\(_{\text{max}}\) measurements the following evening at the same test temperature and in the same random order in which they had undergone VO\(_{2}\)\(_{\text{rest}}\) measurements. Both high- and low-latitude toads were placed in a temperature-controlled cabinet at the test temperature for the night prior to measurements in enclosures similar to those in which they normally lived.

To obtain estimates of VO\(_{2}\)\(_{\text{max}}\), toads were exercised in a swimming flume (SW10100; Loligo Systems, Tjele, Denmark) at a water velocity of 0.35 m s\(^{-1}\) until exhaustion, and then oxygen consumption was immediately measured using the same respirometry system described for VO\(_{2}\)\(_{\text{rest}}\) measurements. Similar methods have previously been shown to provide values of VO\(_{2}\) that approximate, if not exceed, VO\(_{2}\)\(_{\text{max}}\) during exhaustive exercise and are thus a good indication of maximal respiratory capacity (Reidy et al., 1995). Flow velocity was measured just below the surface of the water in the middle of the swim chamber (Hand-held unit HFA and 30 mm vane wheel probe, ZS30GFE-md20T/100/p10; Hontsch, Waiblingen, Germany). The test temperature was controlled by circulating water from a temperature-controlled water bath (CB 8-30E, Heto, Allerød; Denmark) through the heat exchanger of the flume. Toads were encouraged to swim continually by gentle prodding with a wooden spatula. When toads stopped swimming and fell back against the rear of the chamber, their heads were submerged and they were encouraged to continue swimming with gentle prods from behind. If this was done four times in quick succession and the toads still refused to swim, they were considered to be exhausted and were removed from the swim chamber. If toads felt limp in hand they were immediately placed within the respirometry chamber, but if they struggled against being handled then they were returned to the swim chamber and encouraged to keep swimming. Once exhausted, toads were immediately placed within the respirometry chamber to measure VO\(_{2}\)\(_{\text{max}}\). At 15, 22.5 and 30°C, toads were left within the respirometry chamber for 5 min. At 10°C, toads had a tendency to hold their breath upon placement inside the respirometry chamber, so were left within the chamber until oxygen consumption was observed to rise and then for a further 5 min. The respirometry chamber was submerged within the water of the buffer tank of the swimming flume to regulate temperature. The mass flow controller in the respirometry system regulated the flow rate to a nominal value of 500 ml min\(^{-1}\) at all test temperatures.

Instantaneous rates of oxygen consumption were calculated using the method of Bartholomew et al. (1981). The effective chamber volume (including the chamber, tubing and analysers) was measured using nitrogen washout tests, with dead toads of different sizes placed within the chamber. There was no effect of toad mass on the measured effective volume, so a mean value calculated for the different-sized toads was used. Opening the respirometry chamber caused a transient 16 s decrease in O\(_{2}\) concentration associated with the admittance of room air; therefore, 16 s of data were removed from the beginning of each measurement. The highest value for oxygen consumption occurring during the remainder of the 5 min period was then used as an estimate of VO\(_{2}\)\(_{\text{max}}\).

Locomotor performance

Measurements of both locomotor endurance and burst locomotor performance were made during the exercise phase that was used to obtain estimates of VO\(_{2}\)\(_{\text{max}}\). A camera (PS3eye; Sony Corporation, Japan) mounted above the swim...
chamber and connected to a PC was used to record each swimming session at 60 frames s⁻¹ using VirtualDub v1.9.11 (www.virtualdub.org). The time for toads to reach exhaustion was used as a measurement of locomotor endurance. The largest distance that they were able to propel themselves with a single jump from the back of the chamber against the water current was used as a measurement of burst locomotor performance. Jumps were considered for analysis if the toad was not prodded or touched, did not push off from the sides and did not leave the water. Jump distance was measured from the video footage with Kinovea v0.8.15 (www.kinovea.org).

**Aerobic scope**

Absolute aerobic scope was calculated by subtracting \( \dot{V}_O_{2}\text{rest} \) from \( \dot{V}_O_{2}\text{max} \). Absolute aerobic scope was used as opposed to factorial aerobic scope (ratio of \( \dot{V}_O_{2}\text{max} \) to \( \dot{V}_O_{2}\text{rest} \)) because factorial scope was not considered to be informative (Clark et al., 2013).

**Statistical analyses**

Data for each physiological trait were analysed with a linear mixed-effects model using maximum likelihood using the lme4 package v1.0-4 (Bates et al., 2013) of R v3.0.2 (R Core Team, 2013) in RStudio v0.97.551 (RStudio, 2013) with region (low latitude or high latitude), acclimation temperature (15 or 30°C), test temperature (10, 15, 22.5 or 30°C), body mass, time spent at the acclimation temperature and the full factorial combination of two- and three-way interactions among acclimation temperature, test temperature and region as fixed effects, with population (Gulf, Mareeba, Ballina and Yamba), container (nested within population) and individual identity (nested within container and population) as random effects. The significance of the random effects and the fixed effect of time spent at the acclimation temperature was examined first using likelihood ratio tests, and non-significant effects were removed from subsequent models. The significance of the remaining fixed effects within this minimum adequate model were then tested using likelihood ratio tests. Random effects were retained within the minimum adequate model if significant at \( \alpha = 0.25 \) according to the recommendations of Quinn and Keough (2002), and \( \alpha \) was set at 0.05 for tests of significance for the fixed effects. When the main effect(s) of region or acclimation temperature were significant or any interaction involving these effects was significant, post hoc pairwise comparisons were made among groups at each test temperature using linear models when the model included no random effects or likelihood ratio tests when the model did include random effects. For details of the results of tests involved in model simplification see the Supplementary material.

**Results**

**Resting rate of oxygen consumption**

There was a significant positive association between log(\( \dot{V}_O_{2}\text{rest} \)) and log(mass) (\( \chi^2 = 49.24, P < 0.001 \)), and there was a significant three-way interaction between test temperature, acclimation temperature and region (\( \chi^2 = 7.54, P = 0.006 \)), indicating that between high- and low-latitude regions the effect of acclimation temperature on \( \dot{V}_O_{2}\text{rest} \) varied with test temperature (Fig. 1). Among low-latitude toads, post hoc pairwise analyses showed significantly higher \( \dot{V}_O_{2}\text{rest} \) for cold-acclimated animals at 22.5 and 30°C, and no difference at 10 and 15°C (Fig. 1a). Among high-latitude toads, post hoc pairwise analyses showed significantly higher \( \dot{V}_O_{2}\text{rest} \) for cold-acclimated animals at 10, 15, 22.5 and 30°C (Fig. 1b). Among all cold-acclimated toads, post hoc pairwise analyses showed significantly higher values of \( \dot{V}_O_{2}\text{rest} \) for high-latitude animals than low-latitude counterparts at 10 and 15°C and no significant difference between 22.5 and 30°C (Fig. 1c). See Supplementary Table S1 for parameter estimates and test statistics for the minimum adequate model used.

**Peak post-exercise rate of oxygen uptake**

There was a significant positive association between \( \dot{V}_O_{2}\text{max} \) and log(mass) (\( \chi^2 = 19.4, P < 0.001 \)), and there was a significant two-way interaction between test temperature and acclimation temperature (\( \chi^2 = 6.26, P = 0.012 \)). There was no effect of region, indicating that high- and low-latitude toads exhibit the same response, which is an effect of acclimation temperature that varies with test temperature (Fig. 2). A post hoc pairwise analysis showed significantly higher \( \dot{V}_O_{2}\text{max} \) only at 10°C. See Supplementary Table S2 for parameter estimates and test statistics for the minimum adequate model used.

**Locomotor endurance**

For time to exhaustion, there was a significant interaction between test temperature and acclimation temperature (\( \chi^2 = 47.34, P < 0.001 \)), but no effect of log(mass) (\( \chi^2 = 4.24, P = 0.12 \)). Both high- and low-latitude toads showed very similar patterns of endurance, with cold-acclimated animals becoming exhausted in approximately half the time at 22.5 and 30°C (Supplementary Fig. S2). A post hoc pairwise analysis showed significantly longer time to fatigue at 22.5 and 30°C, while at 10 and 15°C there was no significant difference. See Supplementary Table S3 for parameter estimates and test statistics for the minimum adequate model used.

**Burst locomotor performance**

For jump distance, there was a significant positive association with log(mass) (\( \chi^2 = 32.6, P < 0.001 \)), and there was a significant two-way interaction between test temperature and region (\( \chi^2 = 8.35, P = 0.004 \)), indicating that there was no effect of acclimation temperature, but that the effect of test temperature on performance varied with region (Supplementary Fig. S3). Post hoc pairwise analyses showed no significant difference at any test temperature. See Supplementary Table S4 for parameter estimates and test statistics for the minimum adequate model used.
There was a significant positive association between absolute aerobic scope and log(mass) ($\chi^2 = 18.77$, $P < 0.001$) and a significant two-way interaction between test temperature and acclimation temperature ($\chi^2 = 6.87$, $P = 0.008$). The pattern seen here is almost identical to that of $V_{\text{O}_2}\text{max}$ because values of $V_{\text{O}_2}\text{rest}$ are so small compared with $V_{\text{O}_2}\text{max}$ that they make little difference when subtracted. A post hoc analysis showed significantly higher absolute aerobic scope for cold-acclimated toads at 10°C (Fig. 3). Analysis indicates a significant three-way interaction between region, test temperature and acclimation temperature (see Results section for details). For plotting, values of log $V_{\text{O}_2}\text{rest}$ have been adjusted for the scaling effect of log mass. *Statistically significant ($P < 0.05$) pairwise differences at individual temperatures. $n = 20$ for each data point, and error bars represent SEM.

**Aerobic scope**

There was a significant positive association between absolute aerobic scope and log(mass) ($\chi^2 = 18.77$, $P < 0.001$) and a significant two-way interaction between test temperature and acclimation temperature ($\chi^2 = 6.87$, $P = 0.008$). The pattern seen here is almost identical to that of $V_{\text{O}_2}\text{max}$ because values of $V_{\text{O}_2}\text{rest}$ are so small compared with $V_{\text{O}_2}\text{max}$ that they make little difference when subtracted. A post hoc analysis showed significantly higher absolute aerobic scope for cold-acclimated toads at 10°C (Fig. 3). See Supplementary Table S5 for parameter estimates and test statistics for the minimum adequate model used.

**Discussion**

In the present study, we investigated geographical variation among the physiological traits of $V_{\text{O}_2}\text{rest}$, $V_{\text{O}_2}\text{max}$, aerobic scope and locomotor performance. Of the measured traits, only $V_{\text{O}_2}\text{rest}$ and burst locomotor performance showed differences among high- and low-latitude populations. Our most significant finding was that, as hypothesized, cane toads from higher latitudes showed increased thermal metabolic plasticity at low temperature compared with low-latitude counterparts (Fig. 1a, b). As a consequence of this difference in thermal acclimation capacity, high-latitude cane toads have higher resting metabolic rates than low-latitude cane toads when acclimated to cold temperatures (Fig. 1c). This elevation of metabolic rate in low-temperature conditions is likely to benefit high-latitude cane toads through increased rates of ATP production that counteract the thermodynamic depression of critical physiological functions (Seebacher et al., 2009).

The second trait that showed a difference between regions was burst locomotor performance. Within high- and low-latitude populations there was no effect of acclimation...
temperature, while between regions the pattern observed was the opposite of that which would be expected for a beneficial acclimation response (i.e. where performance is maximized at acclimation temperatures; Supplementary Fig. S3). Other studies have also reported no effect or negative effects of cold acclimation on similar measures of jump performance in a variety of anurans (Miller and Zoghby, 1986; Whitehead et al., 1989; Knowles and Weigl, 1990). As discussed by Wilson and Franklin (2002), if the environmental variable to which the response is expected is in fact stressing or damaging to the animal then it may result in reduced performance. Such negative responses may be trait specific, occurring even as other traits respond favourably (Hoffmann and Hewakapuge, 2000). It is also possible that selection for this trait may vary between high- and low-latitude environments, or perhaps other traits that are more beneficial are selected for at the expense of burst locomotor performance. If two important traits share some common genetic underpinnings, stronger selection for one may have detrimental effects on the other (Leroi et al., 2005).

The theory of OLTT suggests that narrowing aerobic scope may be a limiting factor for ectotherms at the edge of their thermal tolerance (Zielinski and Pörtner, 1996; De Watchter et al., 1997). Contrary to the predictions of this theory, in cane toads scope is reduced at low temperatures yet the capacity for oxygen uptake following exhaustive exercise remains well in excess of resting metabolic demands (Fig. 3). Likewise, measures of locomotor performance (both sustained and burst) may fail to show an improvement at low temperatures for high-latitude toads because they are simply not important factors limiting the species distribution. While the depressive effects of temperature are certainly clear for all traits measured in this study, if this depression in colder climates does not ultimately impact fitness then there may be no selection pressure for an adaptive change to occur.

The question motivating the present study was whether the continued advance of cane toads in Australia into higher latitudes is the result of, or facilitated by, adaptive changes. The significant differences found in Vo2 rest between high- and low-latitude cane toads reported here (Fig. 1) provide compelling evidence suggestive of such a change, but the design of the present study is not sufficient to demonstrate that the observed differences have a genetic basis because it only eliminates the effects of relatively short-term acclimation of the order of months. Presumably, the animals collected had lived in their respective environments throughout their lives, and thus the differences detected may be the result of irreversible longer-term acclimation responses or the effect of early development in different thermal environments. The latter has been previously shown to affect acclimation limits in adult zebra fish that underwent embryonic development at different
temperatures (Scott and Johnston, 2012). To correct for any such developmental effects, several generations would need to be raised in controlled conditions to obtain test animals divorced from their ancestral environment.

Additionally, inherent in any assertion of adaptive change is the assumption that cane toads from high and low latitudes are migrants from the original introduction in Cairns. This may not be the case. Cane toads were separately introduced into Byron Bay (NSW, Australia) between 1964 and 1966 (van Beurden and Grigg, 1980; Seabrook, 1991). It is not known for certain whether the cane toads introduced into Byron Bay were sourced from Queensland, from their native range or from elsewhere. If high-latitude cane toads do have a different origin or if they are an admixture resulting from separate introductions then they may have genetic differences that were established elsewhere. However, if an origin is shared between regions then adaptation is certainly possible. One prerequisite for genetic differences to arise between populations is sufficient isolation to prevent gene flow from homogenizing genetic variation. Populations can be isolated by geographical or other barriers that physically prevent dispersal, but populations can also be isolated if dispersal and migration distances are sufficiently low that gene flow is not sufficient to prevent genetic differences from arising along a spatial gradient (isolation by distance; Wright, 1943). There is empirical evidence to support the assertion that isolation by distance can occur over relatively short time scales and facilitate local adaptation (Mimura and Aitken, 2007). In comparison to other terrestrial vertebrates, this process of genetic differentiation may occur more rapidly in amphibians because their reliance on standing water bodies limits dispersal and reduces interbreeding between populations over relatively short distances (Hillman et al., 2014). Estoup et al. (2004) tested for evidence of isolation by distance for cane toads in Australia by analysing 10 microsatellites in multiple populations along two transects. The first began in North Queensland and extended northwest for ∼900 km, and the second began in Byron Bay and extended southeast for ∼50 km. Along each transect there was evidence of genetic separation between populations, supporting a scenario of migration only between adjacent populations, with multiple founder effects occurring in a directional manner. Despite covering a considerably shorter distance, populations along the southern transect showed a higher degree of genetic separation, indicating successive reductions in genetic diversity (Estoup et al., 2004). Such a pattern could be the result of continual selection for individuals with enhanced survival and reproduction at high latitudes, i.e. selection pressures that favour higher thermal metabolic plasticity and metabolic rates at low temperatures.

The cane toad has already gained a considerable foothold within Australia. While the damage is already done in some regions of the continent, there are areas yet untouched by this species. Management decisions regarding the spread of this species depend critically upon whether it will invade pristine ecosystems further or halt at the currently understood limits of its environmental tolerance. The importance of assessing whether adaptation may facilitate the invasion of regions beyond what has been predicted previously is obvious. Here, we add to the growing body of evidence to suggest that the cane toad is in fact evolving rapidly since its introduction to Australia. If the cane toad exceeds invasion predictions by a significant and unexpected margin then not only will the current impacts upon biodiversity intensify, but new species, as yet unaffected, may be impacted by this uniquely successful invader as predator, prey or competitor. This is particularly important as the cane toad invades higher latitudes and different thermal environments, where it will increasingly coexist with a growing list of native inhabitants. These concerns apply not only to this situation but to all instances of invasive species worldwide and illustrate the importance of assessing adaptation of invaders as they move into novel environments. Such assessments will be valuable in ensuring that appropriate decisions can be made to manage this growing threat to global biodiversity.

Supplementary material

Supplementary material is available at Conservation Physiology online.

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References

Bartholomew GA, Vleck D, Vleck CM (1981) Instantaneous measurement of oxygen consumption during pre-flight warm-up and post-flight cooling in sphingid and saturniid moths. J Exp Biol 90: 17–32.
Bates D, Maechler M, Bolker B, Walker S (2013) lme4: linear mixed-effects models using eigen and S4, Ed R package version 1.0-4. http://CRAN.R-project.org/package=lme4.
Bennett AF, Ruben JA (1979) Endothermy and activity in vertebrates. Science 206: 649–654.
Blackburn TM, Cassey PC, Duncan RP, Evans KL, Gaston KJ (2004) Avian extinction and mammalian introductions on oceanic islands. Science 305: 1955–1958.
Boratyński Z, Koteja P (2010) Sexual and natural selection on body mass and metabolic rates in free-living bank voles. Funct Ecol 24: 1252–1261.
Boratyński Z, Koskela E, Mappes T, Oksanen TA (2010) Sex-specific selection on energy metabolism – selection coefficients for winter survival. J Evol Biol 23: 1969–1978.

Burbidge AAM, Manly BFI (2002) Mammal extinctions on Australian islands: causes and conservation implications. J Biogeogr 29: 465–473.

Butchart SH, Walpole M, Collen B, van Strien A, Scharlemann JP, Almond RE, Baillie JE, Bomhard B, Brown C, Bruno J et al. (2010) Global biodiversity: indicators of recent declines. Science 328: 1164–1168.

Byers JE (2000) Competition between two estuarine snails: implications for invasions of exotic species. Ecology 81: 1225–1239.

Cardinale BJ, Duffy JE, Gonzalez A, Hooper DU, Perrings C, Venail P, Narwani A, Mace GM, Collen B, van Strien A, Scharlemann JP, Almond RE, Baillie JE, Bomhard B, Brown C, Bruno J et al. (2012) Biodiversity loss and its impact on humanity. Nature 486: 59–67.

Carey C (1979) Aerobic and anaerobic energy expenditure during rest and activity in the montane Bufo b. boreas and Rana p. Cardinale BJ, Duffy JE, Gonzalez A, Hooper DU, Perrings C, Venail P, Narwani A, Mace GM, Collen B, van Strien A, Scharlemann JP, Almond RE, Baillie JE, Bomhard B, Brown C, Bruno J et al. (2012) Biodiversity loss and its impact on humanity. Nature 486: 59–67.

Case TJ, Douglas T (1991) The role of introduced species in shaping the ecology by integrating hypotheses into a single theoretical framework. Conserv Physiol 39: 213–228.

Catford JA, Jansson R, Nilsson C (2009) Reducing redundancy in invasion ecology by integrating hypotheses into a single theoretical framework. Divers Distrib 15: 22–40.

Clark TD, Sandblom E, Jutfelt F (2013) Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. J Exp Biol 216: 2771–2782.

De Watchter B, Sartoris F, Pörtner HO (1997) The anaerobic endproduct lactate has a behavioural and metabolic singalling function in the shore crab Carcinus maenas. J Exp Biol 200: 1015–1024.

Douglas ME, Marsh PC, Minckley WL (1994) Indigenous fishes of western North America and the hypothesis of competitive displacement: Meda fugida (Cyprinidae) as a case study. Copeia 1: 9–19.

Easteal S (1981) The history of Bufo marinus (Amphibia: Anura); a natural experiment in evolution. Biol J Linn Soc 16: 93–113.

Estoup A, Beaumont M, Sennedot F, Moritz C, Cornuet J-M (2004) Genetic analysis of complex demographic scenarios: spatially expanding populations of the cane toad, Bufo marinus. Evolution 58: 2021–2036.

Frederich M, Pörtner HO (2000) Oxygen limitation of thermal tolerance defined by cardiac and ventilatory performance in spider crab, Maja squinado. Am J Physiol Regul Integr Comp Physiol 279: R1531–R1538.

Gallien L, Münkemüller T, Albert CH, Boulangeat I, Thuiller W (2010) Predicting potential distributions of invasive species: where to go from here? Divers Distrib 16: 331–342.

Ghalambor CK, McKay JK, Carroll SP, Reznick DN (2007) Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. Funct Ecol 21: 394–407.

Halsey LG, White CR (2010) Measuring energetics and behaviour using accelerometry in cane toads Bufo marinus. PLoS ONE 5: e10170.

Hanski I (2011) Habitat loss, the dynamics of biodiversity, and a perspective on conservation. Ambio 40: 248–255.

Hayes RA, Crossland MR, Hagman M, Capon RJ, Shine R (2009) Ontogenetic variation in the chemical defenses of cane toads (Bufo marinus): toxin profiles and effects on predators. J Chem Ecol 35: 391–399.

Hillman SS, Drewes RC, Hedrick MS, Hancock TV (2014) Physiological vagility: correlations with dispersal and population genetic structure of amphibians. Physiol Biochem Zool 87: 105–112.

Hoffmann AA, Hewa-Kapuge S (2000) Acclimation for heat resistance in Trichogramma nr. brassicae: can it occur without costs? Funct Ecol 14: 55–60.

Hogstad O (1987) It is expensive to be dominant. Auk 104: 333–336.

Holway DA (1999) Competitive mechanisms underlying the displacement of native ants by the invasive Argentine ant. Ecology 80: 238–251.

Jackson DM, Trayhurn P, Speakman JR (2001) Associations between energetics and over-winter survival in the short-tailed field vole Microtus agrestis. J Anim Ecol 70: 633–640.

Jessop TS, Letnic M, Webb JK, Dempster T (2013) Adrenocortical stress responses influence an invasive vertebrate’s fitness in an extreme environment. Proc Biol Sci 280: 20131444.

Kawecki TJ, Ebert D (2004) Conceptual issues in local adaptation. Ecol Lett 7: 1225–1241.

Kearney M, Phillips BL, Tracy CR, Christian KA, Betts G, Porter WP (2008) Modelling species distributions without using species distributions: the cane toad in Australia under current and future climates. Ecography 31: 423–434.

Kenward REH, Holm JL (1993) On the replacement of the red squirrel in Britain. A phytotoxic explanation. Proc Biol Sci 251: 187–194.

Knowles TW, Weigl PD (1990) Thermal dependence of anuran burst locomotor performance. Copeia 1990: 796–802.

Kolbe JJ, Kearney M, Shine R (2010) Modeling the consequences of thermal trait variation for the cane toad invasion of Australia. Ecol Appl 20: 2273–2285.

Lampo M, Medialeona V (1996) Energy allocations patterns in Bufo marinus from two habitats in Venezuela. J Trop Ecol 12: 321–331.

Lannig G, Bock C, Sartoris FJ, Pörtner HO (2004) Oxygen limitation of thermal tolerance in cod, Gadus morhua L., studied by magnetic resonance imaging and on-line venous oxygen monitoring. Am J Physiol Regul Integr Comp Physiol 287: R902–R910.

Leroi AM, Bartke A, De Benedictis G, Franceschi C, Gartner A, Gonos ES, Fedele ME, Kivisild T, Lee S, Kartaf-Ozer N et al. (2005) What evidence is there for the existence of individual genes with antagonistic pleiotropic effects? Mech Ageing Dev 126: 421–429.

Lever C (2001) The Cane Toad: the History and Ecology of a Successful Colonist. Westbury Academic and Scientific Publishing, Otley.

Lighton JRB (2008) Measuring Metabolic Rates: a Manual for Scientists. Oxford University Press, Oxford.
Llewelyn J, Phillips BL, Alford RA, Schwarzkopf L, Shine R (2010) Locomotor performance in an invasive species: cane toads from the invasion front have greater endurance, but not speed, compared to conspecifics from a long-colonised area. Oecologia 162: 343–348.

Lowe S, Brown M, Boudjelas S, De Poorter M (2000) 100 of the World’s Worst Invasive Alien Species: a Selection from the Global Invasive Species Database. The Invasive Species Specialist Group (ISSG), a specialist group of the Species Survival Commission (SSC) of the World Conservation Union (IUCN), Auckland.

Mamaev LV, Denikina NN, Belikov SI, Volkhovk VE, Visser IKG, Fleming M, Kai C, Harder TC, Liess B, Osterhaus ADME et al. (1995) Characterisation of morbilliviruses isolated from Lake Bilak seals (Phoca sibirica). Vet Microbiol 44: 251–259.

Mark FC, Bock C, Pörtner HO (2002) Oxygen-limited thermal tolerance in Antarctic fish investigated by MRI and 31P-MRS. Am J Physiol Regul Integr Comp Physiol 283: R1254–R1262.

Marsh PC, Douglas ME (1997) Predation by introduced fishes on endangered humpback chub and other native species in the Little Colorado River, Arizona. Trans Am Fish Soc 126: 343–346.

Miller K, Zogby GM (1986) Thermal acclimation of locomotor performance in anuran amphibians. Can J Zool 64: 1956–1960.

Mimura M, Aitken SN (2007) Adaptive gradients and isolation-by-distance with postglacial migration in Picea sitchensis. Heredity 99: 224–232.

Mooney HA, Cleland EE (2001) The evolutionary impact of invasive species. Proc Natl Acad Sci USA 98: 5446–5451.

Neuheimer AB, Thresher RE, Lyle JM, Semmens JM (2011) Tolerance limit for fish growth exceeded by warming waters. Nat Climate Change 1: 110–113.

Nilsson GE, Crawley N, Lunde IG, Munday PL (2009) Elevated temperature reduces the respiratory scope of coral reef fishes. Glob Change Biol 15: 1405–1412.

Nilsson JA (2002) Metabolic consequences of hard work. Proc Biol Sci 269: 1735–1739.

Overgaard J, Andersen JL, Findsen A, Pederson PB, Hansen K, Ozolina K, Wang T (2012) Aerobic scope and cardiovascular oxygen transport is not compromised at high temperatures in the toad Rhinella marina. J Exp Biol 215: 3519–3526.

Peck LS, Pörtner H, Hardewig I (2002) Metabolic demand, oxygen supply, and critical temperatures in the Antarctic bivalve Laternula elliptica. Physiol Biochem Zool 75: 123–133.

Petren KC, Case TJ (1996) An experimental demonstration of exploitation competition in an ongoing invasion. Ecology 77: 118–132.

Phillips BL (2009) The evolution of growth rates on an expanding range edge. Biol Lett 5: 802–804.

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

Phillips BL, Chipperfield JD, Kearney MR (2008) The toad ahead: challenges of modelling the range and spread of an invasive species. Wildlife Res 35: 222–234.

Phillips BL, Brown GP, Shine R (2010) Evolutionarily accelerated invasions: the rate of dispersal evolves upwards during the range advance of cane toads. J Evol Biol 23: 2595–2601.

Porter SD, Savignano DA (1990) Invasion of polygyne fire ants decimates native ants and disrupts arthropod community. Ecology 71: 2095–2106.

Quinn GP, Keough MJ (2002) Experimental Design and Data Analysis for Biologists. Cambridge University Press, Cambridge.

R Core Team (2013) R: a Language and Environment for Statistical Computing, Ed 3.0.2. R Foundation for Statistical Computing, Vienna.

Reid D, Armstrong JD, Metcalfe NB (2011) Estimated standard metabolic rate interacts with territory quality and density to determine the growth rates of juvenile Atlantic salmon. Funct Ecol 25: 1360–1367.

Reidy SP, Nelson JA, Tang Y, Kerr SR (1995) Post-exercise metabolic rate in Atlantic cod and its dependence upon the method of exhaustion. J Fish Biol 47: 377–386.

Raskať E, Järv T, Bakken M, Bech C, Reinertsen RE (1986) The relationship between social status and resting metabolic rate in great tits (Parus major) and pied flycatchers (Ficedula hypoleuca). Anim Behav 34: 838–842.

RStudio (2013) RStudio: Integrated Development Environment for R, Ed 0.97.551. Boston. http://www.rstudio.org.

Savidge JA (1987) Extinction of an island forest avifauna by an introduced snake. Ecology 68: 660–668.

Scott GR, Johnston IA (2012) Temperature during embryonic development has persistent effects on thermal acclimation capacity in zebrafish. Proc Natl Acad Sci USA 109: 14247–14252.

Seabrook W (1991) Range expansion of the introduced cane toad Bufo marinus in New South Wales. Aust Zool 27: 58–62.

Secor SM, Faulkner AC (2002) Effects of meal size, meal type, body temperature, and body size on the specific dynamic action of the marine toad, Bufo marinus. Physiol Biochem Zool 75: 557–571.

Seebacher F, Franklin CE (2011) Physiology of invasion: cane toads are constrained by thermal effects on physiological mechanisms that support locomotor performance. J Exp Biol 214: 1437–1444.

Seebacher F, Murray SA, Else PL (2009) Thermal acclimation and regulation of metabolism in a reptile (Crocodylus porosus): the importance of transcriptional mechanisms and membrane composition. Physiol Biochem Zool 82: 766–775.

Sexton JP, McIntyre PJ, Angert AL, Rice KJ (2009) Evolution and ecology of species range limits. Annu Rev Ecol Evol Syst 40: 415–436.

Shine R (2010) The ecological impact of invasive cane toads (Bufo marinus) in Australia. Q Rev Biol 85: 253–291.

Smith JG, Phillips BL (2006) Toxic tucker: the potential impact of cane toads on Australian reptiles. Pac Conserv Biol 12: 40–49.

Sutherland RW, Floyd RB, Maywald GF (1996) The potential geographical distribution of the cane toad, Bufo marinus L. in Australia. Conserv Biol 10: 294–299.
Urban MC, Phillips BL, Skelly DK, Shine R (2007) The cane toad’s (Chaunus [Bufo] marinus) increasing ability to invade Australia is revealed by a dynamically updated range model. *Proc Biol Sci* 274: 1413–1419.

van Beurden EK, Grigg GC (1980) An isolated and expanding population of the introduced toad *Bufo marinus* in New South Wales. *Aust Wildl Res* 7: 305–310.

van Kleunen M, Dawson W, Schlaepfer D, Jeschke JM, Fischer M (2010) Are invaders different? A conceptual framework of comparative approaches for assessing determinants of invasiveness. *Ecol Lett* 13: 947–958.

Verberk WCEP, Calosi P (2012) Oxygen limits heat tolerance and drives heat hardening in the aquatic nymphs of the gill breathing damselfly *Calopteryx virgo* (Linnaeus, 1758). *J Therm Biol* 37: 224–229.

Whitehead PJ, Pickridge JT, Leigh CM, Seymour RS (1989) Effect of temperature on jump performance of the frog *Limnodynastes tasmaniensis*. *Physiol Zool* 62: 937–949.

Whitney KD, Gabler CA (2008) Rapid evolution in introduced species, ‘invasive traits’ and recipient communities: challenges for predicting invasive potential. *Divers Distrib* 14: 569–580.

Whittington RJJ, Jones JB, Hine PM, Hyatt AD (1997) Epizootic mortality in the pilchard *Sardinops sagax neopilchardus* in Australia and New Zealand in 1995. *I. Pathology and epizootiology. Dis Aquat Org* 28: 1–16.

Wilson RS, Franklin CE (2002) Testing the beneficial acclimation hypothesis. *Trends Ecol Evol* 17: 66–70.

Wright S (1943) Isolation by distance. *Genetics* 28: 114–138.

Wyatt KB, Campos PF, Gilbert MT, Kolokotronis SO, Hynes WH, DeSalle R, Ball SJ, Daszak P, MacPhee RD, Greenwood AD (2008) Historical mammal extinction on Christmas Island (Indian Ocean) correlates with introduced infectious disease. *PLoS One* 3: e3602.

Yamamoto T, Ueda H, Higashi S (1998) Correlation among dominance status, metabolic rate and otolith size in masu salmon. *J Fish Biol* 52: 281–290.

Zielinski S, Pörtner HO (1996) Energy metabolism and ATP free-energy change of the intertidal worm *Sipunculus nudus* below a critical temperature. *J Comp Physiol B Biochem Syst Environ Physiol* 166: 492–500.

Zug GR, Zug P (1979) The Marine Toad, *Bufo marinus*. A Natural History Resume of Native Populaions. Smithsonian Institution Press, Washington.