

Why does vulnerability to toxic invasive cane toads vary among populations of Australian freshwater crocodiles?

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Abstract

The ecological impact of an invasive species can be heterogeneous through space and time. One such case in Australia involves native freshwater crocodiles *Crocodylus johnstoni*, which are highly sensitive to invasive cane toads *Rhinella marina* in some areas, whereas other populations experience little or no mortality from ingestion of the toxic toads. We studied the impact of toad invasion on three crocodile populations: one crashed, one showed a minor decrease and one appeared unaffected. We tested three hypotheses for the cause of this spatial variation in impact: differences among populations in toad–crocodile encounter rates (proximity of toads to crocodiles during spotlight surveys), differences in crocodile feeding responses (trials of prey preference in the laboratory) and differences in crocodile physiology (reduction of swim speed after receiving a dose of toad toxin). We found little divergence among populations in any of these traits: crocodiles from the three populations all encountered cane toads in the wild, and exhibited similar feeding responses and toxin tolerances. Thus, we cannot confidently identify causation for the impact heterogeneity. Reliance on alternative food resources and an ability to rapidly learn taste aversion may have allowed crocodiles to deal with toad arrival in Lake Argyle and the Daly River. Future work could usefully evaluate potential explanations for the failure of these adaptive mechanisms in the severely affected (Victoria River) population. We suggest that spatial variation in the availability of alternative prey (and thus the willingness of crocodiles to attack a novel toxic prey item) may have contributed to that variation in impact.

Introduction

Biological invasions provide excellent opportunities to understand the ways in which species deal with novel challenges. Even when the nature of the invasion is well understood, however, the impacts of invaders on native ecosystems may be difficult to predict because of substantial spatial and temporal heterogeneity, and the potential for complex indirect effects. For example, invader impacts can vary temporally or spatially due to variation in climate, habitat structure (Melbourne *et al.*, 2007; Rayner, Hauber & Imber, 2007), the composition of local ecological assemblages (Roemer, Donlan & Courchamp, 2002; Rayner *et al.*, 2007; Letnic *et al.*, 2009), and physiological and behavioural traits of the native fauna (Phillips & Shine, 2006).

The invasion of the cane toad *Rhinella marina* in Australia provides an excellent opportunity to investigate how native species adapt to an alien invader. The cane toad was introduced to north-eastern Australia in 1935 and has since

spread through more than a million square kilometres of northern Australia (Urban *et al.*, 2007). Cane toads contain highly toxic bufadienolides, which differ greatly from the toxins found in native Australian frogs (Daly & Witkop, 1971). Direct poisoning of predators is the major mechanism by which cane toads impact the Australian native fauna (Shine, 2010). Many Australian reptilian and mammalian predators lack physiological resistance to toad toxins and die after ingesting or attacking toads (Covacevich & Archer, 1975; Webb, Shine & Christian, 2005; Smith & Phillips, 2006). The level of impact on different groups of animals varies from high and consistent (e.g. Northern quolls, Oakwood, 2003; O'Donnell, 2009), to minimal (e.g. birds, Beckmann & Shine, 2009), possibly reflecting evolved resistance to bufotoxins in groups that have coevolved with Asian bufonid anurans (Letnic, Webb & Shine, 2008; Llewelyn *et al.*, 2010).

Freshwater crocodiles *Crocodylus johnstoni* are highly susceptible to mortality after ingesting cane toads. Laboratory studies have documented low physiological resistance

Table 1 Climatic and environmental variables for Lake Argyle, Daly River and Victoria River

	Lake Argyle	Daly River	Victoria River
Mean annual rainfall (mm)	837	1171	737
Mean annual relative humidity (%) at 0900 h	47	70	57
Avg. days > 35°C each year	191	133	188
Water body type	Permanent, lentic	Seasonal, lotic	Seasonal, lotic
Surrounding vegetation	Semi-arid landscape dominated by tropical savannah and eucalypt woodland	Monsoon rainforest and tropical savannah	Semi-arid landscape dominated by sparse eucalypt forest
Toad invasion history	Matilda Creek flowing into Carl Bay in March 2009 and spread over 25 km into the lake on both banks by August 2010	2004/2005 (Doody <i>et al.</i> , 2006)	Victoria River Gorge in late 2005 and Victoria–Wickham junction in 2005–2006 (Letnic <i>et al.</i> , 2008)
Population level impact	None (Somaweera & Shine, 2012)	Low (Doody <i>et al.</i> , 2009)	High (Letnic <i>et al.</i> , 2008)

Climatic variables were recorded at Kimberley Research Station (15.65° S, 128.71° E), Douglas River (13.83° S, 131.19° E) and Kidman Springs (16.12° S, 131.96° E) situated near the Lake Argyle, Daly and Victoria River study areas, respectively (Source: Australian Bureau of Meteorology, 2011).

to cane toad toxins (Smith & Phillips, 2006), and more than 60% of *C. johnstoni* found dead in the Victoria River in the Northern Territory from 2007 to 2010 contained cane toads in their stomach ($n = 80$: Letnic *et al.* unpubl. data). Despite this apparent susceptibility, population-level responses of *C. johnstoni* to the cane toad invasion across their range in northern Australia vary enormously, from substantial population declines in some areas (77% reduction over a year in some sites, Victoria River, Northern Territory, Letnic *et al.*, 2008; mass mortality, Boodjamulla National Park, north-western Queensland, Whitehead *et al.*, 2008), moderate in some (~30% decline during the first year, Katherine River, Northern Territory, C. Manolis, pers. comm.), to little or no measurable impact in other areas (Roper and MacArthur Rivers, Catling *et al.*, 1999; Daly River Catchment, Freeland, 2004; Daly River, Doody *et al.*, 2009; Lake Argyle, Somaweera & Shine, 2012).

The mechanisms behind this spatial variability in the response of *C. johnstoni* populations to the cane toad invasion might reflect features of the toads, of the ecological context in which they interact with crocodiles, or of the crocodiles themselves. The first of these possibilities (differences in toad toxicity) is unlikely because the high mobility of these invaders reduces the possibility of substantial genetic differences among populations (Estoup *et al.*, 2004). The second possibility (environmental effects) could operate via differences in the rate of encounters between *C. johnstoni* and cane toads in different environments. The third possibility (spatial differences in crocodile vulnerability) might be due either to variation in the prey preferences of *C. johnstoni* or their toxin resistance, or a combination of both these factors. These mechanisms are not mutually exclusive, and any of them might be enough to allow a crocodile population to persist with toads. Therefore, we tested these possible causes of heterogeneity in the impact of cane toads on *C. johnstoni* using crocodiles sourced from three geographically discrete areas where cane toads have been documented to have no impact, low impact and high impact on crocodile populations.

Methods

Study areas

We worked at Lake Argyle in Western Australia, and the Daly and Victoria Rivers of the Northern Territory, Australia (Fig. 1). All three areas lie within monsoonal northern Australia and experience three main seasons: the wet season from November/December to April; the early dry season from May to July/August and the build-up or the late dry season from August to November (Webb, 1991). The three areas differ in mean annual rainfall, and their humidity and temperature profiles (Table 1).

Field studies to estimate impacts of toad invasion on crocodile populations

Data on our encounter rates with crocodiles (based on nocturnal spotlight counts, Bayliss, 1987) before and after the arrival of cane toads provide the most direct evidence of cane toad impact. We obtained such data before and after the arrival of cane toads for two sections of Lake Argyle (one section without toads at the time of study, for comparison), two sections of the Daly River and two sections of the Victoria River. Pre-toad data for the Daly River (1997) were obtained from the Parks and Wildlife Service of the Northern Territory, while those for Lake Argyle (2008) and Victoria River (2005) were from our own surveys. The sections surveyed were as follows: (1) Lake Argyle: Carl Bay (section length = 14.6 km) and BBQ Island Bay (section length = 8.9 km); (2) Daly River: Beeboom (section length = 18.9 km) and Ooloo Crossing upstream (section length = 17.1 km); and (3) Victoria River: Victoria River Gorge (section length = 12.6 km) and Wickham River/Victoria River Junction (section length = 6.7 km).

Due to annual fluctuations in water level heights, and thus the length of the shoreline of each survey section, we compared the density of crocodiles in each survey section, rather

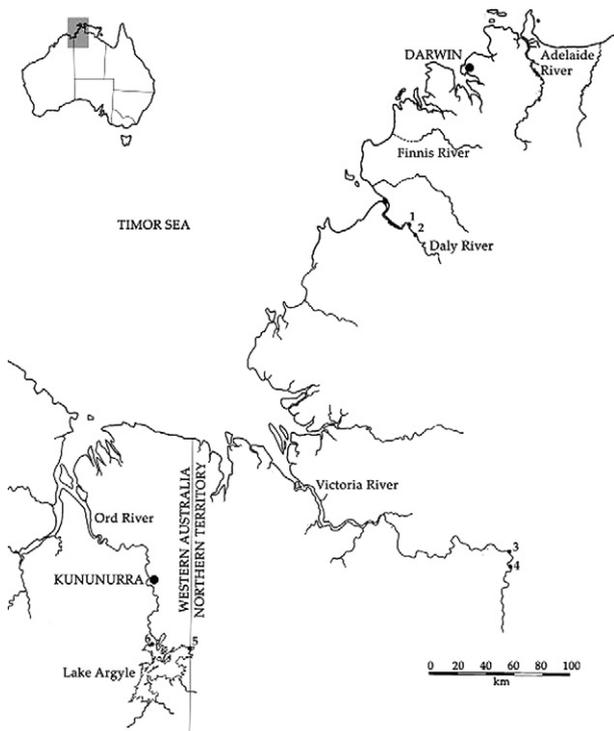


Figure 1 The distribution of the water bodies where field studies were conducted and hatchlings were collected for laboratory studies. Field study sites include (1) Beeboom, (2) Oolloo Crossing upstream, (3) Victoria River Gorge, (4) Wickham River/Victoria River junction, (5) Carl Bay and (6) BBQ Island Bay.

than the absolute number of crocodiles (Letnic & Connors, 2006). Because the density of hatchling crocodiles varies from year to year depending on flooding and breeding success (Webb, Buckworth & Manolis, 1983), we only analyzed non-hatchling (> 0.6 m total length) densities. Densities of non-hatchlings were calculated as the number of crocodiles divided by the length of shoreline surveyed (crocodiles km⁻¹). Pre- and post-toad surveys were conducted during the same months of the dry season in respective years.

Our analyses were based on density estimates available for years before and immediately (1–2 years) after toad arrival. Accordingly, survey data from 2009/2010, 1997/2005 and 2005/2007 were used for calculations for Lake Argyle, Daly River and Victoria River, respectively. Data for the Victoria River have been adapted from Letnic *et al.* (2008).

Source and husbandry of crocodiles used in laboratory studies

The animals used for laboratory work belonged to two experimental groups: captive-reared and wild-caught (Table 2). The eggs from captive-reared crocodiles from the Adelaide River were collected for incubation in November 2008. Hatchling crocodiles from the Daly and Victoria River were captured within a week post-hatching, and then reared in captivity until the trials were conducted. Several

Table 2 The composition of freshwater crocodiles *Crocodylus johnstoni* hatchlings used for prey preference and toxin resistance studies to determine reasons for population-level differences in cane toad *Rhinella marina* impact

Total collected		
Captive-reared		Wild-caught
Daly River – 20		Daly River – 5
Victoria River – 18		Victoria River – 11
Adelaide River – 8		Finnis River – 2
		Lake Argyle – 36
↗ ↘		
Prey preference studies (n = 59)		Toxin resistance studies (n = 86)
Captive-reared		Captive-reared and wild-caught
Daly River – 20		Victoria River – 11
Victoria River – 18		Finnis River – 2
Adelaide River – 8		Lake Argyle – 36

hatchling crocodiles still had yolks attached and had probably hatched less than 1 day prior to capture. The age of the crocodiles at hatching was determined by the width of the umbilical scar (Webb & Smith, 1984). The wild animals for the trials were collected in April 2009, 25–30 days prior to the trials. The captive-reared hatchlings were never exposed to cane toads (and thus were toad-naïve), whereas the wild-caught ones may well have encountered cane toads. All hatchlings were 4–5 months old when the trials commenced. Animals from different sources were maintained separately in plastic containers (1 × 1 × 1 m) and fed lean beef supplemented with calcium and multivitamins.

Hypothesis 1: Encounter rates between crocodiles and cane toads differ among populations

An encounter between two agents is the first step in any interaction between them, so the rate of encounters between toads and crocodiles may determine the likelihood of crocodiles ingesting toads (and hence dying from the encounter). Encounter rates may vary spatially as a result of local hydric conditions. Because toads are extremely susceptible to dehydration (Florance *et al.*, 2011), drier landscapes may force toads to rehydrate at water bodies (and thus encounter crocodiles) more frequently than would be the case in more mesic landscapes (Letnic *et al.*, 2008).

In April 2010, we quantified encounter rates between cane toads and crocodiles in Lake Argyle (Carl Bay, 14.6 km), Victoria River (Victoria River gorge, 12.6 km; Wickham River/Victoria River junction, 6.7 km) and the Daly River (upstream of Oolloo Crossing, 17.4 km) using a handheld spotlight from a boat (an all-terrain vehicle at Carl Bay, Somaweera & Shine, 2012) driven parallel to the shoreline. Based on our observations, anurans within 2 m of the water's edge were potentially accessible to a feeding crocodile. For each crocodile detected on the water's edge or on the bank, we thus recorded if cane toads were present in a 2 × 2 m quadrat in front of the crocodile. The encounter rate between crocodiles and cane toads for each location was

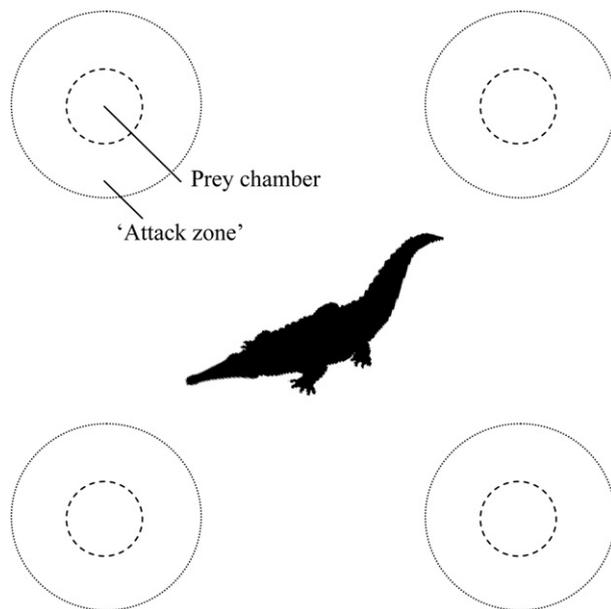


Figure 2 Arena used for the trials of prey selection by hatchling freshwater crocodiles. Prey items were placed inside the four 'prey chambers', and the responses of the hatchling crocodile in the 'attack zone' were scored.

calculated as the proportion of crocodiles sighted that were within 2 m of a cane toad when seen.

Hypothesis 2: Prey preferences of crocodiles differ among populations

Geographic variation in crocodile prey preferences (i.e. whether or not crocodiles will attack a toad as prey) might influence the impacts of toad invasion. If crocodiles rarely attack toads, then the probability of a crocodile dying from lethal toxic ingestion is low. Variation in feeding responses might be due either to intrinsic factors or to aversion learning. We used a total of 59 animals collected from the Victoria River, Daly River and northern flood plains in the Darwin region (Adelaide River and Finnis River) to look for possible spatial variation in prey preference between toad-exposed and toad-naïve crocodiles. The animals belonged to two experimental groups: toad-naïve (captive-reared) individuals and wild-caught individuals that were 4–5 month old at collection, and thus are likely to have been exposed to cane toads prior to capture (Table 2).

The test arena consisted of a large plastic container (1 × 1 × 1 m) filled with 3-cm deep water, and containing four circular prey chambers (6 cm diameter) comprising mesh domes fixed to the bottom at each corner of the arena. Within each dome, the prey item was placed on a floating piece of wood (3 cm long, 6 cm diameter) to keep it at the water level. A 10-cm radius circle around each prey chamber was designated as the 'attack zone' (Fig. 2). The study animals ranged in body lengths from 293 to 406 mm, so the enclosure provided sufficient space that the location of the

crocodile when it was placed in the enclosure would have had negligible impact on distance to the prey. Water temperature was maintained at $28 \pm 0.5^\circ\text{C}$ and air temperature at $29 \pm 2^\circ\text{C}$ using an infrared heat lamp (Hagen-150W).

Crocodiles were not fed for 2 days prior to testing. We introduced each animal to its experimental chamber and allowed it to acclimatize for 15 min before the trial. The prey chambers were empty during this period. Then, we introduced a single prey item (juvenile cane toad, sub-adult native frog *Cyclorana australis* or cockroach *Panesthia australis* to each chamber. Prey body sizes were similar (~40 mm Snout–Urostyle Length for anurans and ~40 mm total length for cockroaches). We randomly assigned prey types to prey chambers for each trial; the control chamber did not contain any prey. We videotaped the trials for 15 min to record: (1) time taken for the crocodile to orient towards the first prey type (or chamber); (2) the time the crocodile spent within the 'attack zone' of the particular prey chamber, while oriented to the chamber; and (3) whether or not the crocodile attacked the prey. We only included open-mouth attacks toward the prey chamber; crocodiles that did not attack were excluded from analysis. The last two variables (the time spent and whether or not there was an attack response) were recorded for the first three prey chambers explored by each crocodile.

Hypothesis 3: Toxin resistance of crocodiles differs among populations

Among-population differences in physiological resistance of crocodiles to toad toxins might explain spatial variation in mortality rates after toad arrival. We tested 86 *C. johnstoni* hatchlings for their susceptibility to toad toxin (Table 2), including some of the animals used for the prey-preference trials (above). All hatchlings were 3–5 months old when the trials commenced. Animals from different sources were maintained separately, as described earlier.

To quantify the resistance of individual crocodiles to one of the major components of cane toad toxins (Bufalin), we measured decrements in their swimming speeds following a dose of toxin (methodology from Brodie & Brodie, 1990, modified by Phillips, Brown & Shine, 2003; Phillips & Shine, 2006; Smith & Phillips, 2006). Before trials, we measured the mass, snout–vent length, total length and the gape width of each crocodile. One day prior to dosing, we subjected each crocodile to two swimming trials 1 h apart. Each crocodile was encouraged to swim around a circular pool 3 m in diameter, after a 10-min acclimation period (in separate 40 × 25 cm mesh cages in the middle of the pool). If a crocodile stopped for over 1 s, we gently tapped the tail tip to encourage the animal to continue. Water temperature was maintained at $30.5 \pm 0.5^\circ\text{C}$, and depth at 10 cm. Swimming speeds are relatively constant over the range of 23°C and 33°C in hatchling *C. porosus* (Elsworth, Seebacher & Franklin, 2003), so minor differences in temperature ($< 1^\circ\text{C}$) are unlikely to have affected our trial results. We used an electronic stopwatch to record the time taken for each crocodile to swim two separate laps around the pool, and averaged the

results to yield an estimate of speed before dosing (*b*). We allowed each animal to swim undisturbed unless it stopped for over 1 s (at which time we gently tapped the tail tip). If the animal became fatigued (repeated stops) during pre-dosing trials, it was removed and rested for at least 2 h before the next trial.

Dosing trials

We used Bufalin (3 β ,14-Dihydroxy-5 β ,20(22)-bufadienolide; Sigma-Aldrich) to assess the susceptibility of *C. johnstoni* to toad toxin. We gave each individual a specific dose of Bufalin by intraperitoneal injection. A total of 68 randomly selected animals were each given 0.001 mg of Bufalin per g body mass, a dosage based on pilot studies. The solution was prepared by dissolving 10 mg of Bufalin in 6 ml of 50% ethanol, and diluting it with 18 ml of saline to result in a 0.4167 mg ml⁻¹ solution of Bufalin (in 12.5% ethanol). The other 24 animals were used as controls which received 0.2 ml (average volume given to treatments) of 12.5% ethanol in saline. Each crocodile was tested once only. The animals were acclimated to pool temperature as above, and swimming trials commenced 1 h after dosing. Maximum speed was calculated as before to yield an estimate of maximum swimming speed after dosing (*a*).

To examine the duration of the effect of Bufalin on locomotion, we tested speeds of the 18 animals (nine treatments and nine controls) from Lake Argyle (set 1) 1 h, 2 h and 4 h after dosing. To assess the possibility of acquired resistance, we retested the same group of animals (again with post-dosing trials after 1 h, 2 h and 4 h) a week later. At the same time in the second week, we tested another group of 18 animals (nine treatments and nine controls) from Lake Argyle (set 2) for comparison. The % reduction in speed following Bufalin injection was calculated for each animal using the following formula (modified from Smith & Phillips, 2006):

$$\% \text{ reduction} = 100 \times (1 - b/a),$$

where *b* is the average speed before dosing, and *a* is the average speed after dosing. A larger reduction (%redn) in swimming speed indicates a lower resistance to toxin. Previous work with this method has confirmed that reduction in swimming speed is due to the toad toxin (Bufalin in the present study) and not the carrier fluid (Phillips *et al.*, 2003; Smith & Phillips, 2006).

Analyses

Distributional assumptions of the statistical tests were checked by examining residual plots. Data were analyzed using JMP 5.01 software (SAS Institute, Cary, NC, USA). We used two-factor analysis of variance (ANOVA) with location, and before versus after as factors to compare crocodile densities immediately before and after the toad invasion at each water body. In the laboratory studies, we used contingency tests to examine the effects of source population on prey selection by hatchling crocodiles, and

the effects of prior exposure to toads (crocodiles collected in November vs. April) on prey selection by crocodiles. Analyses were conducted on data from the first trial (three exposures of crocodiles to prey), with behavioural responses classified into four categories: attacked both toads and frogs, attacked toad only, attacked frog only, attacked insect only. No crocodiles attacked the control container, so this response category was excluded from the analysis.

We used two-factor ANOVA to test the hypotheses that Bufalin would reduce swim speed (two dosage levels, 0 and 0.001 units) and that the responses of crocodiles would differ between sites with different toad-impact histories. A repeated-measures ANOVA was used to compare the responses of the crocodiles in set 1 during their treatments during week one and two (each treatment had three post-dosing trials after 1 h, 2 h and 4 h). An interaction between the first and second weeks and time since injection (1 h, 2 h and 4 h) would suggest a physiological acclimation of Bufalin resistance. We also compared the responses of animals in set 1 during the second week to those of set 2 with repeated-measures ANOVA. Probability values for time-related *F*-tests in ANOVA were adjusted for correlations between observations using the Huynh-Feldt epsilon (Green, 1993).

Results

Changes in crocodile population densities following cane toad invasion

We have recorded only 18 dead crocodiles (average total length 157.4 cm) from Carl Bay at Lake Argyle, where toads invaded 2 years ago (early 2009) and are now abundant (Somaweera & Shine, 2012). None of the fresh carcasses had cane toads in their stomach contents, but neither did they show signs of entanglement in fishing nests (dead crocodiles obtained from fish nets often had deep cuts on the snout and around the neck) nor signs of fighting with other crocodiles (bite marks, missing body parts, rake marks). We did not find any dead crocodiles in areas of Lake Argyle without cane toads, suggesting that at least some of the crocodiles at Carl's Bay may have died from mouthing rather than ingesting cane toads, or regurgitated the toad before dying (e.g. Begg, Walden & Rovis-Hermann, 2000). In contrast, dead *C. johnstoni* have been found with cane toads in their stomachs in the Daly River (Letnic & Ward, 2005; Molloy & Henderson, 2006; Doody *et al.*, 2009) and the Victoria River (Letnic *et al.*, 2008; 30 of 59 dead crocodiles with total lengths ranging from 60 to 120 cm contained toads). The mortality rate in the Daly River was too low to have affected crocodile population densities (e.g. Doody *et al.*, 2009), whereas crocodile numbers have fallen substantially on the Victoria River. Our spotlight survey data show that the arrival of toads had different effects in the three water bodies (ANOVA water body \times before/after crocodile densities, $F_{2,6} = 5.14$, $P = 0.05$; Fig. 3). Surveyed densities of non-hatchling crocodiles did not differ significantly before versus

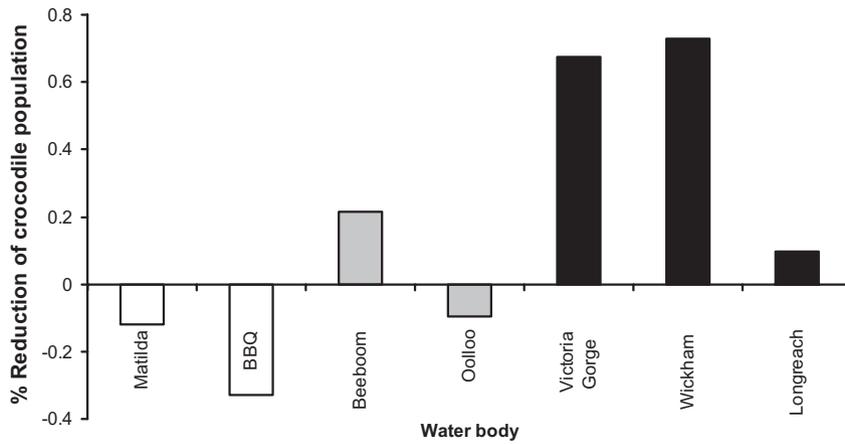


Figure 3 Percentage reduction in densities of non-hatchling crocodiles after the cane toad invasion in sites at Lake Argyle (white bars), Daly River (grey bars) and Victoria River (black bars). BBQ site at Lake Argyle is a control site (without toads at the time of study).

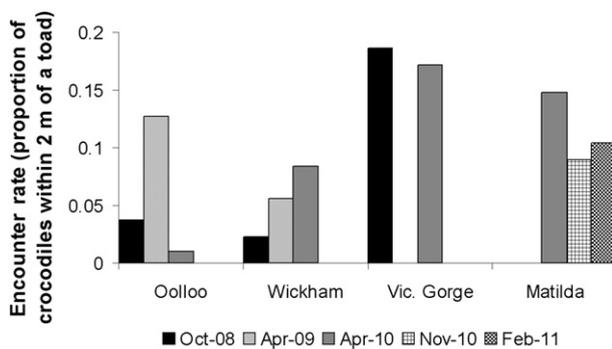


Figure 4 Encounter rates between freshwater crocodiles and invasive cane toads, as scored by the proportion of crocodiles within 2 m of a toad when first sighted. Data are shown from surveys at Oolloo Crossing on the Daly River, two water holes (Wickham and Victoria Gorge) in the Victoria River and at Matilda Creek in Carl Bay of Lake Argyle. Victoria Gorge was not surveyed in April 2009.

after the arrival of cane toads at Lake Argyle ($t_{2,3} = 0.98$, $P = 0.43$) or at Daly River ($t_{2,3} = 0.2$, $P = 0.69$). In contrast, the arrival of cane toads at the Victoria River was associated with dramatic reductions in crocodile densities ($t_{1,4} = 9.89$, $P = 0.04$).

Hypothesis 1: Encounter rates between crocodiles and cane toads differ among populations

In all three water bodies, crocodiles encountered cane toads (Fig. 4). All sites were surveyed at the same time of year, but we did not compare the encounter rates among sites statistically because the encounter studies were conducted in different years (reflecting different arrival times of toads). The percentage of edge-feeding crocodiles that were within 2 m of a toad when sighted was higher at Lake Argyle and Victoria River (0.11) than at the Daly River (0.05), but the important result is that cane toads and crocodiles were found in close proximity at all three sites.

Hypothesis 2: Prey preferences of crocodiles differ among populations

Animals showed attack responses towards the toad, frog and the insect, but not the empty container. In general, captive-born animals showed more attack responses than the wild-caught ones (Fig. 5a), and those from low-impact sites showed more responses than those from high-impact sites (Fig. 5b). However, of the 26 crocodiles that attacked prey in these trials, toad-naïve animals from high-impact sites were no more likely to attack toads than were crocodiles from low-impact sites ($\chi^2 = 6.2$, d.f. = 3, $P = 0.10$; Fig. 5b). Toad-naïve crocodiles from the Daly and Victoria Rivers were more likely to attack cane toads than were toad-exposed (wild-captured) crocodiles from the same water bodies ($\chi^2 = 9.4$, d.f. = 3, $P = 0.02$; Fig. 5a).

Hypothesis 3: Toxin resistance of crocodiles differs among populations

Comparison between water bodies

Buflin injection caused a significant reduction in the swim speed of crocodiles (ANOVA, $F_{1,86} = 13.2$, $P < 0.001$; Fig. 6a) from all populations (no significant population effect – $F_{1,86} = 0.7$, $P = 0.5$). The effect of Buflin on the swim speed of crocodiles did not differ between rivers with different levels of toad impact (interaction $F_{2,86} = 1.7$, $P = 0.2$; Fig. 6b).

Comparison between treatments

Buflin injection reduced crocodile swimming speeds by more than 20%, but this effect decreased rapidly after 2 h of injection (Fig. 7). The animals of the treatment group from Lake Argyle that were retested a week later showed no difference in swim speed reduction between the 2 weeks [multivariate analysis of variance (MANOVA) week \times time since injection $F_{2,15} = 2.25$, $P = 0.14$] and did not differ from the set of new animals subjected to the treatment only on week two (MANOVA set \times time since injection $F_{2,15} = 0.35$, $P = 0.71$). Animals in the control group showed the same

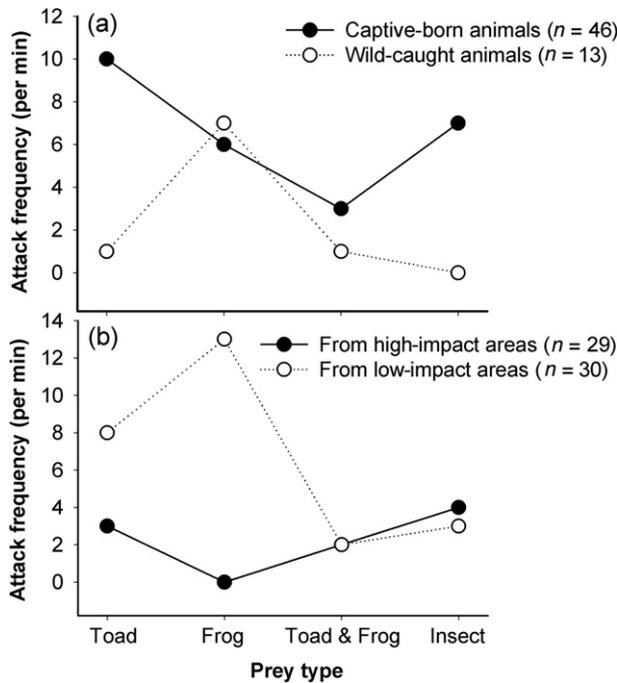


Figure 5 A comparison of attack frequencies towards different prey types by (a) captive-born versus wild-collected crocodiles from Daly River, Victoria River and northern flood plains in Darwin, and (b) from sites where crocodile populations were strongly impacted by cane toad *Rhinella marina* invasion (Victoria River) and where such impacts were much less marked (Daly River and northern flood plains in Darwin).

pattern with no difference between weeks ($F_{2,15} = 0.92$, $P = 0.42$) or between sets in week two ($F_{2,15} = 1.94$, $P = 0.17$). However, the overall speed reduction of animals in the treatment category was significantly different from those in the control category ($F_{1,52} = 16.28$, $P < 0.001$).

Discussion

Our data show significant spatial heterogeneity in the impact of invasive cane toads on populations of freshwater crocodiles, but the causal basis for that variation remains elusive. The impact of toad invasion on freshwater crocodiles was severe at the Victoria River but not at the nearby Daly River, as previously reported by Letnic *et al.* (2008) and Doody *et al.* (2009). Letnic *et al.* (2008) suggested that differences in aridity of the riparian habitat could explain the impact differential: in the more arid Victoria River site, the need for frequent rehydration in the river in an otherwise arid landscape (Florance *et al.*, 2011) would increase the rate at which cane toads would encounter crocodiles. Our data support one assumption of this hypothesis in that encounters between toads and crocodiles were about twice as common in the Victoria River than the Daly River (Fig. 4). However, high-encounter rates were observed also in the mosaic riparian habitats at Lake Argyle (Fig. 4). Moreover, the proportional

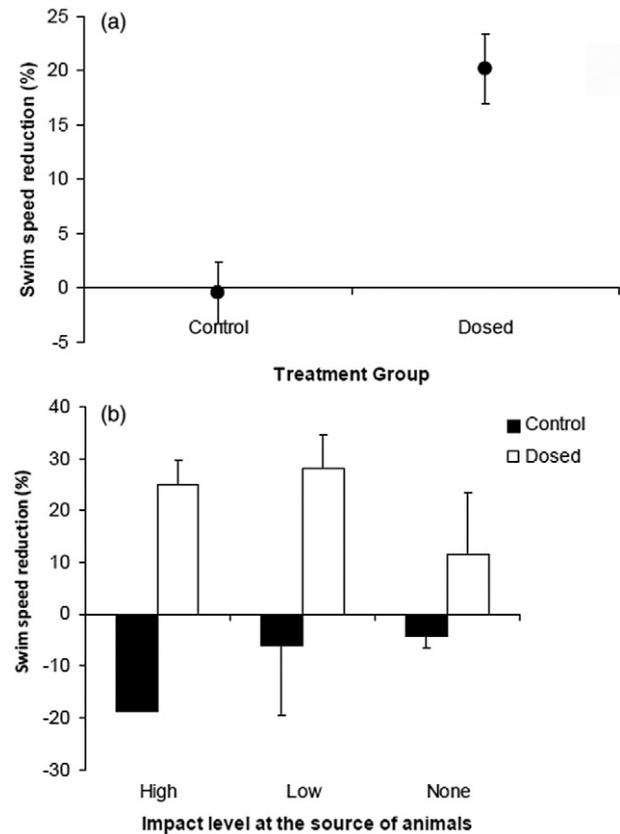


Figure 6 Effect of Bufalin (3 β ,14-Dihydroxy-5 β ,20(22)-bufadienolide) on the swimming speed of hatchling freshwater crocodiles *Crocodylus johnstoni*. The upper panel (a) shows the effect on controls versus dosed animals from pooled data; the lower panel (b) shows the effect on animals from regions with different levels of toad impacts (high = Victoria River, low = Daly River, none = Lake Argyle). All graphs show mean values with standard errors.

difference in encounter rates between sites was much less than the proportional difference in the density reduction of crocodiles (Fig. 3). Thus, we doubt that aridity-driven differences in encounter rates between cane toads and crocodiles can explain the much higher mortality rates of this native predator at some sites than at others.

How reliable is the inference of a difference in magnitude of toad impact on crocodiles in these three areas? One potential weakness involves methodology: although we used the same methods (spotlight surveys) to estimate crocodile densities before versus after toad arrival, different observers were involved in the pre-toad versus post-toad surveys of the Daly River sections. Although inter-observer differences in counts made by reliable spotters are trivial, estimates of crocodile sizes can be significant in such surveys (Webb *et al.*, 1989), potentially weakening the comparison. However, the modest response of crocodile populations to the arrival of cane toads observed both in this study and by Doody *et al.* (2009), in combination with the scarcity of direct observations of crocodile mortality (this study; Doody

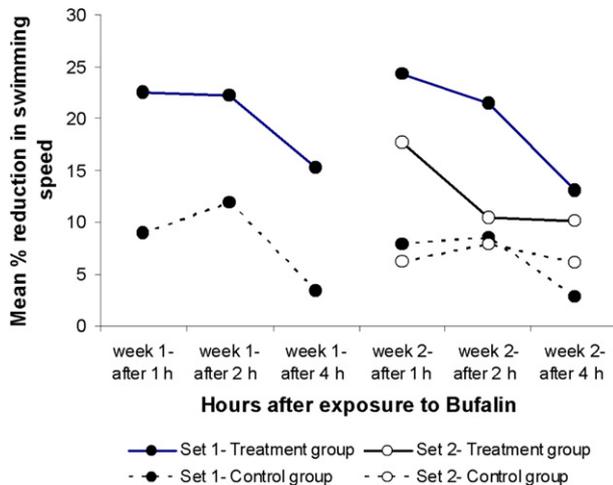


Figure 7 The mean percentage reduction in swimming speeds of 18 hatchling freshwater crocodiles *Crocodylus johnstoni* from Lake Argyle injected with Bufalin in successive trials conducted a week apart (set 1 – solid circles). The treatment animals (solid lines) showed no physiological acclimation of Bufalin resistance during the second week and no significant difference from another set of hatchlings (set 2 – open circles) tested only during the second week. The controls showed a similar pattern. To examine the duration of the effect of Bufalin, the swimming speed of crocodiles was measured 1, 2 and 4 h after dosing for each trial, respectively. The swim speed increased 2 h after exposure.

et al., 2009; WMI Pty Ltd, 2009, 2010; Somaweera & Shine, 2012), strongly suggest that cane toads have had much less impact on *C. johnstoni* populations either at Lake Argyle or on the Daly River, than has been the case on the Victoria River. This is true even though counts in a lake (where some crocodiles may be inaccessible to observers) may entail different constraints than counts in a river (where most or all crocodiles are observable). Below, we review potential explanations for this spatial heterogeneity in impact.

Hypothesis 1: Encounter rates between crocodiles and cane toads differ among populations

Our ‘encounter rate’ data show that crocodiles in all three water bodies encountered cane toads. However, to directly address interaction rates between the two species, we would need extensive behavioural and ecological data that are difficult to gather in a system such as ours (Wootton, 1997; Wootton & Emmerson, 2005). Relationships between prey density and prey encounter rate can be nonlinear (Mols *et al.*, 2004; Travis & Pal, 2005), and a predator may be more likely to attack novel (unfamiliar) prey when prey densities are low (Ioannou, Ruxton & Krause, 2008). We have observed crocodiles holding cane toads in their mouths, in all three water bodies. On most occasions, the crocodile eventually swallowed the toad, but in some cases the toad was released (possibly due to our presence).

However, due to logistic difficulties in continuously monitoring these crocodiles, the end result of these encounters are not known. Other data confirm that cane toads are eaten by freshwater crocodiles (Tucker *et al.*, 1996). Four of 60 stomach content samples collected from live crocodiles (snout–vent lengths 396–740 mm) from Carl Bay in Lake Argyle contained cane toads (Somaweera, unpubl. data), as did one of 34 yearling crocodiles (181–205 mm) from northern Lake Argyle (Somaweera *et al.*, 2011). None of these five crocodiles showed signs of illness. Freeland (2004) reported that freshwater crocodiles force-fed an adult cane toad died on average 1.5 h later, but it is difficult to relate this to natural behaviour. In summary, our data show spatial heterogeneity in encounter rates between cane toads and freshwater crocodiles, but suggest that such heterogeneity may not be great enough to explain the major divergence in crocodile mortality rates among sites following toad invasion.

Hypothesis 2: Prey preferences of crocodiles differ among populations

Our laboratory trials showed that crocodiles from low-impact populations were just as willing to attack and consume cane toads as were conspecifics from high-impact populations. However, toad-naïve crocodiles were more likely to attack cane toads than were crocodiles captured in the wild from toad-invaded areas (i.e. that likely had had opportunities to encounter toads). Some other Australian predators have an intrinsic preference for native frogs over cane toads (e.g. Llewelyn *et al.*, 2010), or rapidly learn taste aversion when they encounter toads (Webb *et al.*, 2008; Greenlees, Phillips & Shine, 2010). In at least one species (the red-bellied black snake *Pseudechis porphyriacus*), snakes from toad-infested areas have evolved not to consume toads (Phillips & Shine, 2006). However, the timescale of that adaptive response involves decades, whereas the crocodile populations that we studied have been exposed to cane toads for a few years at most.

Thus, aversion learning seems more likely than genetically based adaptation as a proximate cause for a refusal by field-collected crocodiles to consume toads. In support of this interpretation, hatchling freshwater crocodiles from toad-free areas learn to avoid cane toads as prey after one or two encounters in captivity (Somaweera *et al.*, 2011). Compared with toad-naïve conspecifics, ‘toad-smart’ crocodiles inflicted fewer bites on toads, held toads in their mouths for shorter time periods and were more likely to reject toads as prey in subsequent encounters. Although frogs and toads were seen in approximately equal numbers in the field, crocodiles consumed frogs more often than toads (Somaweera *et al.*, 2011). In summary, our data do not support the idea of pre-existing spatial heterogeneity in crocodile feeding responses to cane toads; instead, our results suggest, at least under captive conditions, that freshwater crocodiles from all populations will readily seize toads but soon learn to avoid them.

Hypothesis 3: Toxin resistance of crocodiles differs among populations

It is difficult to derive an *a priori* prediction about expected divergences in toad-toxin tolerance between impacted and non-impacted crocodile populations. On one hand, we might expect more sensitive animals to be more easily killed by ingesting toads (i.e. high-impact populations should exhibit greater sensitivity to toad toxins), but on the other hand, adaptive change [as in Phillips & Shine's (2006) black snakes] might rapidly reverse this situation, such that impacted populations rapidly evolve higher toxin resistance because of differential mortality of the most vulnerable animals. Our data did not support either of these scenarios; instead, physiological resistance to toad toxins was similar among the three crocodile populations we studied, and we found no evidence of physiological acclimation of Bufalin resistance. We also noticed an increase in the swim speed of the control animals, possibly due to alcohol that was used as the solvent. This dose of alcohol used, however, did not affect the locomotor speed of snakes in a previous study (Phillips *et al.*, 2010).

Future work could usefully examine toad-toxin tolerance in freshwater crocodiles from across a wider range of localities, encompassing areas where toads arrived decades ago. If adaptive shifts have occurred in toxin tolerance, such shifts should be evident at that larger spatial and temporal scale. Given a generational turnover period of *c.* 50 years (Smith & Webb, 1985), however, the 75-year period of toad occupancy in Australia may have been too brief for such adaptive changes to occur in crocodiles. In summary, we found no evidence of differential toxin resistance in crocodiles from our three study populations.

Overview and future research

Our field data on crocodile abundance confirm earlier reports of substantial spatial heterogeneity in the magnitude of cane toad impact on freshwater crocodile populations, but none of the three hypotheses we erected to explain that heterogeneity were supported by our data. Crocodiles from the three populations all encounter cane toads in the wild, and exhibit similar feeding responses and toxin tolerances. Why, then, did the Victoria River population crash following toad arrival, whereas the other two populations did not? We cannot answer this question but can point to some possibilities. Most obviously, the availability of alternative prey to crocodiles likely differs among the Daly River, Victoria River and Lake Argyle study sites, potentially influencing predator vulnerability during the toad invasion (Webb *et al.*, 2005). Lakes often contain more food (especially fish) than tropical rivers (Da Silveira & Magnusson, 1999), and fish are the main prey of adult *C. johnstoni* (Webb, Manolis & Buckworth, 1982; Tucker *et al.*, 1996). Both freshwater and saltwater crocodiles attacked cane toads readily after lengthy periods without feeding, but refused them when fully fed (Covacevich & Archer, 1975; Freeland, 2004). Spatial and temporal variation in the abundance of alterna-

tive prey (and thus the willingness of crocodiles to attack a novel prey type like the cane toad) might explain at least part of the spatial variation in the magnitude of toad impact. More detailed studies on the rates of encounter between cane toads and crocodiles, and the factors influencing these encounters, may help resolve this continuing puzzle.

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