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Original Article

Differences in neophobia between cane toads from introduced and native populations

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For species moving into new environments, locating and using unfamiliar resources is crucial for survival. The cane toad (*Rhinella marina*) has been successful in many countries worldwide, persisting in both urban and rural landscapes. Given that animals exploiting urban habitats are confronted with novel feeding opportunities, individuals in those areas are expected to exhibit reduced neophobic tendencies compared with individuals in rural populations. Additionally, individuals persisting in introduced populations are expected to be less neophobic than individuals in native populations, taking advantage of novelty as their range expands. To investigate such predictions, we examined the response to novel prey and a novel object in native toads in Panama and introduced populations (urban and rural) in Florida. Toads were tested in an arena with novel or familiar prey and later with a novel object next to familiar prey. We found differences in response to novelty between cane toads in different ranges but not in different habitats. Most introduced individuals from both urban and rural habitats consumed novel prey with no difference in latency to eat between prey types. Few native toads, however, consumed any prey during trials, spending most of their time moving about the arena. When familiar prey was presented near a novel object, more than half of the introduced toads ate, but no native individuals ate. This study emphasizes the importance of behavior as a mechanism used by invasive species to exploit novel resources and successfully colonize new environments.

Key words: *Bufo marinus*, invasive, marine toad, neophobic, novelty, *Rhinella marina*.

INTRODUCTION

Introduced species are increasingly common worldwide and recognized as threats to biodiversity and a source of economic concern (reviewed in Pimentel et al. 2005). Although the exact reasons explaining a species' success in novel environments are often difficult to determine or remain largely unknown, several hypotheses have been proposed about mechanisms promoting invasive ability (Lodge 1993). Release from selective pressures from predators, competitors, or diseases, for instance, increase survival within the introduced ranges of multiple taxa (e.g., Phillips et al. 2003; Torchin et al. 2003; Callaway and Ridenour 2004). Specific characteristics, such as recent disturbance, can make habitats more likely to be invaded by a given species (reviewed in Lodge 1993). In addition, particular life-history traits including high fecundity and rapid developmental rates are considered critical characteristics that determine the outcome of an invasive species (Lodge 1993; Sakai et al. 2001; Rouget and Richardson 2003; Beard and Pitt 2005). Recently, behavioral traits have been recognized as important mechanisms for successful invasions in many taxa (e.g.,

mammals: Thorsen et al. 2000; birds: Sol et al. 2002; invertebrates: Pintor et al. 2009; lizards: Chapple et al. 2011; fish: Rehage and Sih 2004; Cote et al. 2011).

One behavioral trait receiving increased attention in species confronted with new environments is neophobia, or avoidance of novelty (Greenberg and Mettke-Hoffmann 2001). Neophobia was traditionally considered one end of an approach-avoidance continuum conflict of possible responses to novelty with neophilia, exploratory interest in novelty, at the other end (Montgomery 1955). These behaviors, however, are now recognized to correspond to different motivations (Wood-Gush and Vestergaard 1993; Mettke-Hofmann et al. 2002) and personality axes (Gosling and John 1999). Neophilia and neophobia may also be linked to different genes (Powell et al. 2003; Weissstaub et al. 2006). Because introduced species expand their range across new habitats, they are under strong selection to exploit novel resources (Sol and Lefebvre 2000). Reduced neophobia, along with neophilia, is expected to enable individuals to locate food and safe areas within their new range (Sol et al. 2011). Studies investigating neophobia in mammals and birds are consistent with such predictions. Introduced Norway rats (*Rattus norvegicus*), for instance, are less neophobic than individuals from native populations (Thorsen et al. 2000). Similarly,

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invading house sparrows (*Passer domesticus*) are less neophobic than individuals from resident populations (Martin and Fitzgerald 2005). Although multiple studies have examined neophobia in many species (e.g., rats, Barnett 1957; warblers, Greenberg 1983; sparrows, Greenberg 1992; convict cichlids, Brown et al. 2014), few studies directly compare native and invasive individuals of the same species (Parker et al. 2013) or populations of individuals inhabiting different environments (Bókony et al. 2012).

Within the invasive range, different habitats are expected to impose different selective pressures. Urban and rural habitats, for example, pose varying challenges on individuals resulting in different successful strategies (Kark et al. 2007). One important reason for such differences may be availability of novel resources. Urban areas present more novelty and fewer vertebrate predators than rural areas (Faeth et al. 2005). Hence, urban individuals that frequently encounter novel food under lower predation risk are hypothesized to exhibit lower neophobia. Despite predictions that invasive and urban populations should respond better to novelty challenges, there is no agreement across the few species examined to date. Although invasive common mynas (*Acridotheres tristis*) in urban habitats are more exploratory than those in rural habitats (Sol et al. 2011), house sparrows from populations in differently urbanized areas exhibit no difference in latency to eat novel food (Bókony et al. 2012). In this study, we investigate neophobia in populations from different habitats in a widespread introduced species, the cane toad (*Rhinella marina*, formerly *Bufo marinus*), to investigate the role of behavior in the success of introduced species.

Cane toads are an ideal species to study behavioral changes associated with the invasion process (Child et al. 2008). This species is one of the most widespread anuran species (Pitt et al. 2005) and has been introduced from its natural range in Central and South America to more than 20 countries worldwide (Lever 2001; Kraus 2009). Although the history of cane toad introductions has been well-documented (reviewed in Lever 2001), little is known about introductions to areas other than Australia. The success of introduced cane toads has been attributed to many factors including their life-history strategy (Freeland and Martin 1985; Van Bocxlaer et al. 2010) and ability to outcompete natives (Phillips and Shine 2004). The role of behavior in their success, however, has received little attention. Here we investigate the hypothesis that reduced neophobia promotes invasive ability allowing cane toads to better cope with novelty. We test the responses of cane toads to novel prey and a novel object in their native and introduced ranges. This species also inhabits both urban and rural habitats (Zug and Zug 1979). To examine the role of urbanization on cane toad behavior, we investigate neophobia in urban and rural populations within the introduced range. We predict that introduced individuals from urban and rural populations will be less neophobic than native individuals. Within the introduced range, urban individuals are expected to exhibit reduced neophobia compared with rural individuals because opportunities for novelty are reduced in rural environments. To our knowledge, this is the first study to investigate anuran neophobic tendencies and how they relate to invasive potential.

METHODS

Study species

Cane toads are large, terrestrial anurans native to the Neotropics. Their natural foraging behavior is well-documented (Zug and Zug 1979) and their diet has been reported across their natural

(1981; Lever 2001) and invasive ranges (Zug et al. 1975; González-Bernal et al. 2011). Cane toads are generalists that consume a variety of insects in their native range, but also include small mammals and conspecifics in their invasive range (Easteal 1981; Pizzatto et al. 2008). Anecdotal evidence suggests cane toads also exploit novel food sources such as trashcans and pet food bowls (Alexander 1964).

Study areas

To investigate neophobia, juvenile cane toads were collected in populations from their introduced and native ranges. Following Zug and Zug (1979), toads less than 9 cm in length and with multi-coloration pattern were considered juveniles. Individuals were checked for nuptial pads to confirm they were not sexually active. Two populations in the introduced range and one population in the native range were sampled.

Twenty cane toads were collected each from an urban and rural population in their introduced range in Florida. Rural toads were collected near the Everglades Research and Education Center in Belle Glade (26°40'15"N, 80°38'17"W). This area is largely agricultural consisting mostly of sugar cane and rice fields. Urban toads were collected behind a shopping center in Wellington (26°40'57"N, 80°12'15"W). These two sampling populations are approximately 65 km apart and, therefore, are likely distinct populations (Zug et al. 1975).

Introduced individuals were killed with an overdose of the anesthetic MS-222, tricaine methanesulfonate, after the completion of experimental trials. Toads were immersed in a solution of 0.50 g/L of MS-222 (0.05%) (Fellers et al. 1994; Cecala et al. 2007) and died without any struggle within 20–30 min.

In their native range, 22 cane toads were collected in Gamboa, Panama (9° 7'0.46"N, 79°42'2.79"W) around the facilities of the Smithsonian Tropical Research Institute. Gamboa is a small town surrounded by mature forest of the Soberania National Park. All toads were found near buildings in town with the exception of a single individual found on a road. Following experimental trials, individuals were marked by toe-clipping to avoid retesting and released at the site of collection. The Guidelines for the Use of Live Amphibians and Reptiles in Field Research compiled by the American Society of Ichthyologists and Herpetologists (ASIH), the Herpetologists' League (HL), and the Society for the Study of Amphibians and Reptiles (SSAR) were followed when toe-clipping toads. A maximum of two toes were clipped from each individual and no thumbs on the forefeet were clipped given their importance in amplexus.

Upon capture, all individuals from all populations were weighed using an Adam® HCB302 Highland 300 g × 0.01 g portable scale. Snout-vent length (SVL) was measured with an ROK 150 mm digital caliper.

Experimental set-up

Toads were housed individually in plastic containers (introduced: 38 cm × 30.5 cm × 15 cm; native: 33 cm × 40.5 cm × 16.5 cm) covered with clear plastic. Each individual was given a blue water bowl and blue food bowl. During the first night of captivity, each individual was given two mealworms (*Tenebrio molitor*) inside their home container. The following day, home containers were checked for presence of mealworms. Toads that did not eat overnight were given fresh mealworms. All toads ate both mealworms within three days and no toads were excluded from experiments. Toads were considered to be familiar with the mealworms after they ate both worms. Food was

then withheld for three nights. Trials were run on the fourth night to mimic natural feeding behaviors in the wild (Zug and Zug 1979).

To investigate neophobia, each toad was individually placed in a test arena (91.5 cm × 61 cm × 61 cm; Figure 1). A food bowl identical to that in the home tank was placed at one end of the arena. A red light was placed above the arena to simulate natural feeding conditions that usually occur under low-light intensity (Zug and Zug 1979). A Sony HDR-UX20 camera set on night shot function was used to record trials. To eliminate subjective bias, an observer unaware of the population of the toads later scored the video recordings.

Novel prey

Toads were presented with familiar prey, mealworms, or novel prey, yellow-colored waxworms (*Galleria mellonella*). Although waxworms have a broad distribution, they live exclusively in beehives (Paddock 1918), making them inaccessible to toads in the wild. Waxworms are also considered novel prey because they differ from mealworms in their general shape and locomotion pattern. Preliminary

studies indicated that yellow-colored worms elicited stronger neophobic responses in toads (e.g. long latency to eat) than worms of other colors (e.g. pink). Although cane toad color vision has not been investigated, toads respond to blue and yellow visual components (Reuter and Virtanen 1976) and differentiate blue moving prey on gray backgrounds (Dietz 1972). The blue food bowls coupled with yellow waxworms likely increased conspicuousness of this novel prey to the toads.

At each population, half of the toads were presented with familiar prey first and the other half with novel prey first. The prey was placed in the bowl first. Then, the toad was placed into the center of the arena facing the food bowl, approximately 30 cm away (Figure 1a). Once the first prey item was eaten, the second was placed in the bowl and the toad was returned to its starting position. Videos were analyzed for time to prey detection, time spent moving, time spent along edges of the arena (15 cm out from wall), escape attempts, latency to eat, and reaction to prey items, i.e. leaning in and examining prey versus striking from a distance. Latency to eat was measured as time from detection of prey to striking at or eating the prey. A toad was considered to have detected the prey

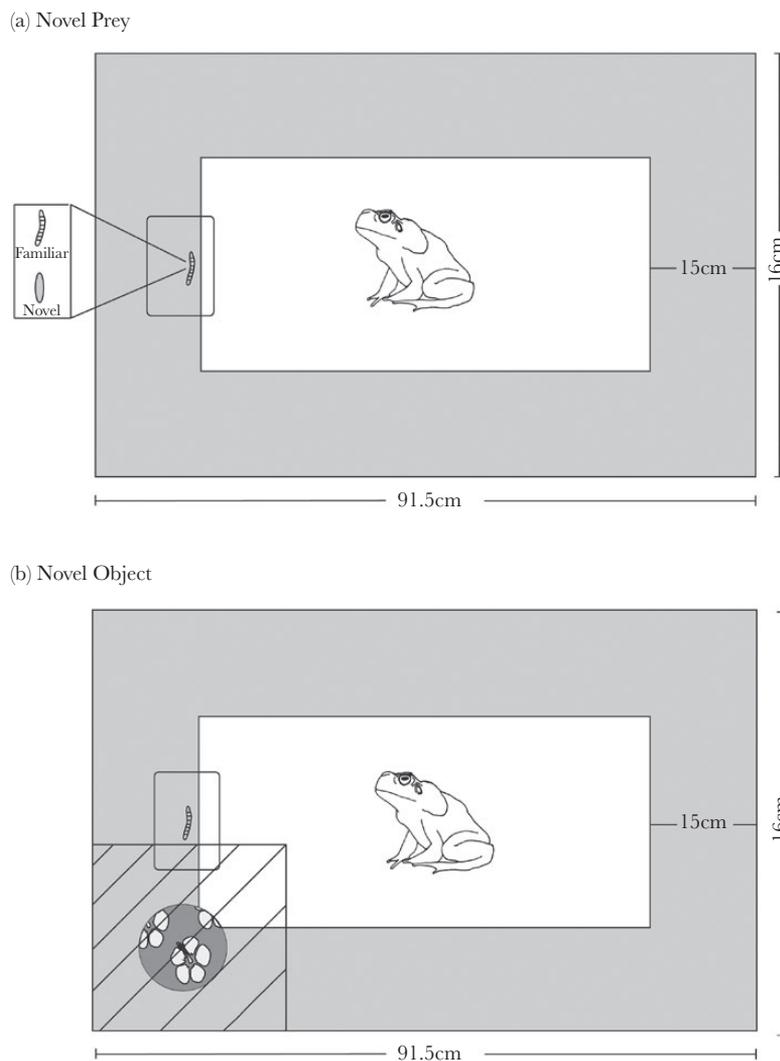


Figure 1

Diagram of experimental set-up used to investigate neophobia in cane toads. Individuals were released in the experimental arena at the middle (indicated by the toad) and presented with novel prey (a) or a novel object by familiar prey (b). The arena was divided into different areas to quantify the behavior of the toads: edge (dark gray area), center (white area) and, in the novel object experiments, near the object (striped area).

when it looked toward the prey and oriented to it. Escape attempts were defined as a rapid jump or set of connected jumps toward the arena's wall as the toad attempted to go above or beyond it.

Novel object

To further test the hypothesis that introduced and urban individuals are less neophobic, toads were presented with familiar prey by a novel object following previous studies (Webster and Lefebvre 2001; Mettke-Hofmann et al. 2002; Martin and Fitzgerald 2005). Feeding near unfamiliar objects is risky in many contexts and, therefore, is considered a bold behavior.

Toads were allowed to rest one night between the novel prey and novel object experiments. During this time, individuals were not handled or given food. Using the same arena as the novel prey experiments, a mealworm was placed in a food bowl directly next to a novel object (Figure 1b). We used a blue ball (15 cm) patterned with white flowers as a novel object following previous studies (Martin and Fitzgerald 2005; Lendvai et al. 2011). A blue object was chosen because, as mentioned above, toads respond to blue dummy prey on gray backgrounds (Dietz 1972). Videos were analyzed to measure latency to eat the mealworm, time to prey detection, time spent moving, time spent near the walls of the arena, time spent near the novel object (10 cm out from ball), time spent touching the novel object, and escape attempts.

Statistical analyses

A one-way Anova was performed to compare the size of the toads across populations. We used Fisher's exact tests to compare the proportions of individuals that ate as well as the proportions of each type of prey eaten between populations. The fiducial level of significance, α , was set at 0.025 to control for error rate in pairwise comparisons. A Mann-Whitney test was used to compare latency to eat the different prey between populations following previous neophobia studies (Greenberg 1992; Kelly and Marples 2004; Martin and Fitzgerald 2005). Finally, we performed independent analyses of variance (Anova) to examine how behavior compared among populations (Zar 2010). Analyses were performed for time spent moving, in the center of the arena, near the novel object, and on the novel object. To examine whether movement in the arena was associated with trying to escape, we performed Pearson correlations between time spent moving and number of escape attempts.

RESULTS

Juvenile cane toads from native and both introduced populations tested in this study were similar in size. There was no difference in the snout-vent length or weight of the toads from different populations (SVL: $F_{1,112} = 5.771$, $P = 0.115$; Weight: $F_{1,112} = 23.016$, $P = 0.848$).

Novel prey

Introduced cane toads were more likely to eat in the arena than native cane toads (Fisher's exact test, $P = 0.021$; Figure 2). Native individuals visually examined both prey items, but few consumed either prey. To examine whether this was due to lack of hunger, native individuals were given mealworms in their home container following testing. All individuals ate mealworms when presented in this context. Although there was no difference between urban and rural populations in the proportion of individuals that ate (Fisher's exact test, $P = 0.2$, $n_{\text{rural}} = 20$, $n_{\text{urban}} = 20$), both populations were significantly different than native toads (Fisher's exact test, urban

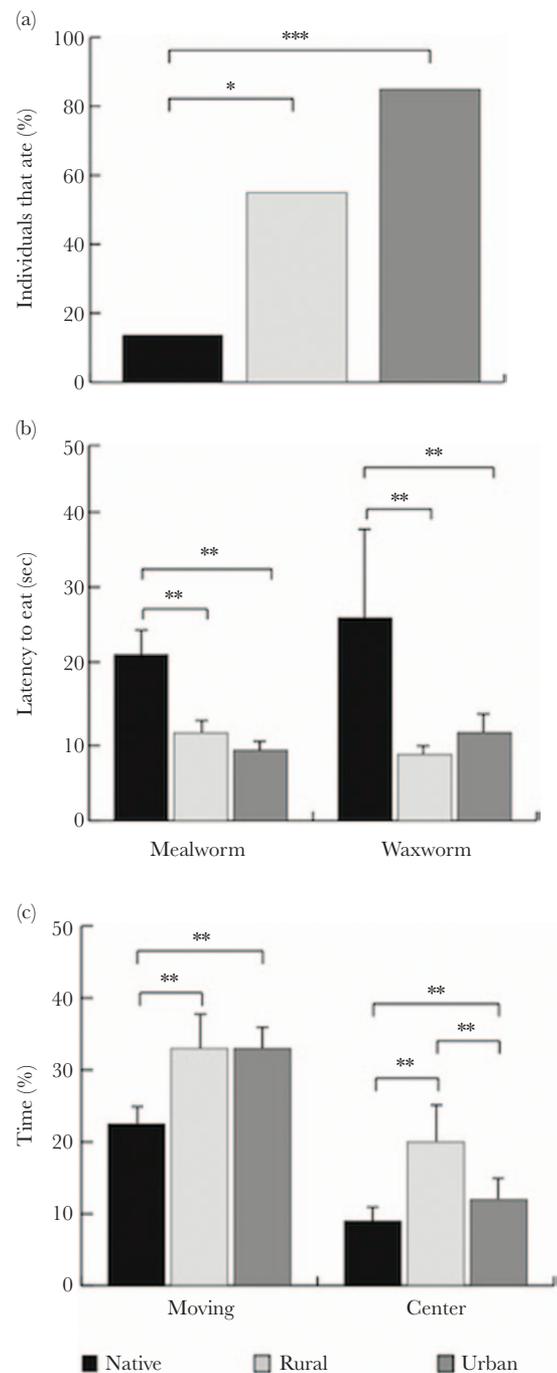


Figure 2

Behavior of cane toads in response to novel and familiar prey in individuals from native (black bars) and introduced populations (rural: light gray bars; urban: dark gray bars). (a) Proportion of individuals from each population that ate prey items; (b) Latency to eat prey items (mean \pm SE of individuals that chose to eat prey); and (c) proportion of time during each trial toads from each population spent moving and in the center of the arena. * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$.

versus native: $P = 0.04$; rural versus native: $P = 0.00038$; $n_{\text{native}} = 22$). Of the individuals that did not eat in the arena, 90.9% of native toads detected the prey while 32.5% of introduced individuals detected it. In addition, introduced toads did not preferentially eat either prey item (Fisher's exact test, rural: $P = 0.527$, $n_{\text{rural}} = 11$;

urban: $P = 0.716$, $n_{\text{urban}} = 17$). Neophobia is expected to result in longer latency to eat. Consistent with our predictions, introduced toads showed no signs of neophobia to novel prey. Among introduced cane toads that consumed both prey items, mealworms were eaten no quicker than waxworms (Mann–Whitney test, $U = 358$, $P = 0.6$; $n_{\text{rural}} = 11$, $n_{\text{urban}} = 17$; Figure 2b). In contrast, the few native individuals that ate showed signs of neophobia, eating slower than introduced individuals (urban vs. native: $U = 289$, $P = 0.000032$; rural vs. native: $U = 671$, $P = 0.0015$).

All populations differed in their movement in the arena (Figure 2c). Individuals from the urban population spent less time in the center of the arena compared with rural individuals ($F_{1,112} = 31.395$, $P < 0.001$). However, there was no difference in time spent moving between urban and rural toads ($F_{1,45} = 0.007$, $P = 0.938$). The native population moved less (rural: $F_{1,112} = 109.77$; urban: $F_{1,112} = 69.286$) and spent more time along the walls (rural: $F_{1,112} = 15.148$; urban: $F_{1,112} = 10.521$) than the introduced populations ($P < 0.001$ for all cases). Time spent moving was correlated to escape attempts (Pearson correlation; $r = 0.468$, $P = 0.01$). Overall, the movement in the arena of toads from both introduced populations was more similar to one another (pairwise comparisons; Hotelling's t -square = 2.662, $P = 0.277$) than to the native population (urban vs. native: Hotelling's t -square = 12.742, $P = 0.003$; rural vs. native: Hotelling's t -square = 6.998, $P = 0.036$).

Novel object

There were differences in consumption of familiar prey by a novel object between native and introduced individuals (Fisher's exact test, $P < 0.001$; Figure 3a). Although native individuals did not consume mealworms next to the novel object (0 out of 22 toads), introduced individuals did (urban: 12 out of 20 toads; rural: 10 out of 20 toads). There was, however, no difference in latency to consume mealworms next to the novel object among urban and rural toads (Mann–Whitney test, $U = 52$, $P = 0.6277$; Figure 3b). Of the individuals that did not eat, 32.5% of native individuals detected the prey while 9.4% of introduced individuals detected the prey.

Introduced populations differed in how individuals used their time and moved around the arena. Urban toads moved more than rural toads ($F_{1,45} = 19.259$, $P < 0.001$; Figure 4a); however, rural toads spent significantly more time near the novel object ($F_{1,45} = 10.309$, $P = 0.002$; Figure 4b) and in the center of the arena ($F_{1,45} = 4.838$, $P = 0.033$; Figure 4a) than urban toads. Urban and rural individuals did not differ in time spent touching (on) the novel object ($F_{1,45} = 0.004$, $P = 0.949$; Figure 4b).

Introduced and native populations also differed in their movement (Figure 4). Toads from both introduced populations spent less of their time moving (urban vs. native: $F_{1,45} = 133.163$, $P < 0.001$; rural vs. native: $F_{1,45} = 35.485$, $P < 0.001$; Figure 4a) and more time in the center of the arena than native individuals (urban vs. native: $F_{1,45} = 26.135$, $P < 0.001$; rural vs. native: $F_{1,45} = 9.427$, $P = 0.004$). Similar to the novel prey experiment, time spent moving during novel object trials was correlated to escape attempts (Pearson correlation: $r = 0.427$, $P = 0.021$). Individuals from native and introduced populations did not differ in time spent on the object (urban vs. native: $F_{1,45} = 0.121$, $P = 0.729$; rural vs. native: $F_{1,45} = 0.004$, $P = 0.947$; Figure 4b). Toads from native populations spent the same amount of time near the novel object as urban toads ($F_{1,45} = 0.168$, $P = 0.068$) but less time than rural toads ($F_{1,45} = 14.854$, $P < 0.001$; Figure 4b).

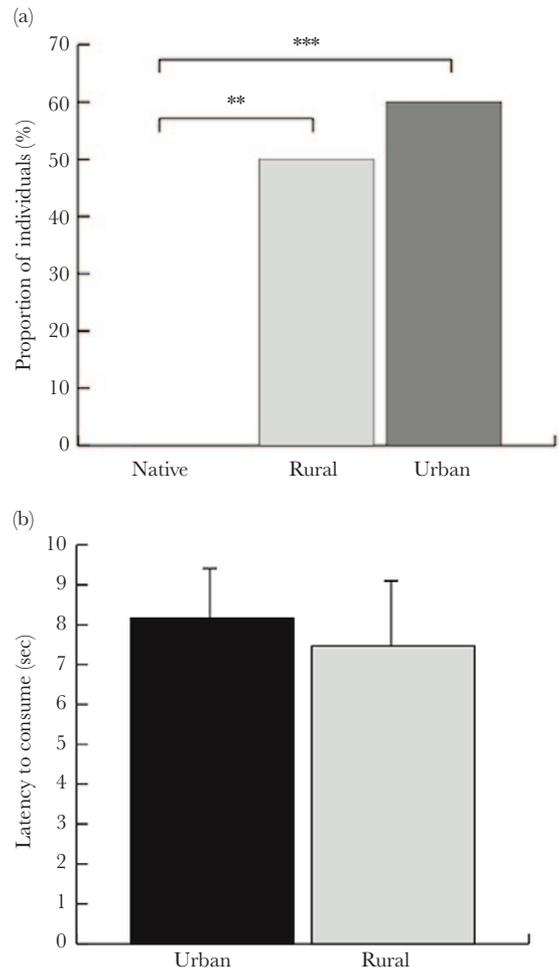


Figure 3

Behavior of cane toads in response to familiar prey by a novel object in individuals from native (black bars) and introduced populations (rural: light gray bars; urban: dark gray bars). (a) Proportion of individuals from each population that ate familiar prey when presented next to a novel object; and (b) latency to eat mealworms when presented next to a novel object (mean \pm SE).

DISCUSSION

We found that, in a novel arena, introduced juvenile cane toads readily ate novel and familiar prey while native cane toads were less likely to eat either prey. Native individuals showed signs of increased neophobia and anxiety-like behaviors when confronted with a novel environment; they spent more time by the arena walls and were less likely to approach a novel object. These behaviors match those expected in habitats where encounters with unfamiliar resources are rare while predation risk is high (Greenberg and Mettke-Hoffmann 2001; Sol et al. 2011). Previous studies in birds and mammals support the hypothesis that introduced individuals are less neophobic than native individuals (Thorsen et al. 2000; Sol et al. 2002; Martin and Fitzgerald 2005; Sol et al. 2011). Accordingly, we found that cane toads from Florida have a higher propensity to eat novel prey than native toads from Panama. Such reduced aversion to novelty, perhaps coupled with neophilia, may have facilitated the establishment of invasive cane toad populations due to increased ability to cope with and exploit novelty.

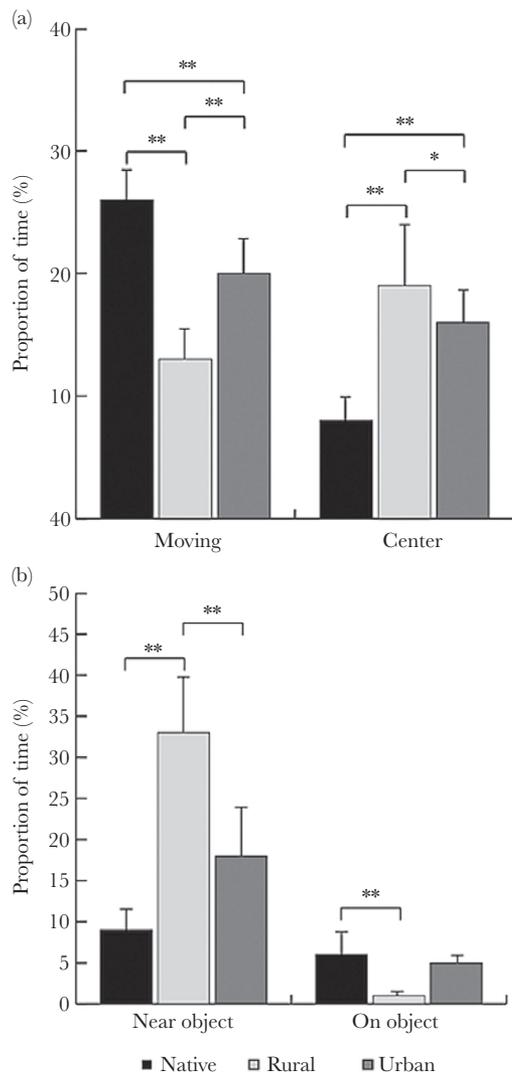


Figure 4

Behavior of cane toads in response to a novel object by familiar prey in individuals from native (black bars) and introduced populations (rural: light gray bars; urban: dark gray bars). (a) Proportion of time toads from each population spent moving and in the center of the arena during novel object trials and (b) proportion of time toads from each population spent near and on the novel object. * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$.

The amount of movement and areas visited in the arena by native toads differed from introduced toads. Native toads spent most of their time by the walls, away from the center of the arena. Equivalent behaviors are associated with high levels of anxiety in rodents (Bailey and Crawley 2009). The proportion of time moving differed between native and introduced individuals during novel prey and novel object experiments. Although native toads spent less time moving than introduced toads in the novel prey experiment, they moved more than introduced toads during the novel object experiment. This change in movement, however, is due to the decreased movement and reduced escape attempts by introduced toads during the novel object experiment. Similar behavior in both experiments may reflect lack of habituation of native toads to the arena.

Although not eating novel prey indicates neophobia, long latency to eat is also a sign of increased neophobia. Although cane toads

from both introduced populations ate novel food and familiar food by the novel object, subtle differences in their response such as latency to eat could be expected. Contrary to expectations, we found no differences in latency to eat between urban and rural populations. It is possible that the level of novelty perceived by the toads was not enough to reveal subtle differences between introduced populations in their response to unfamiliar prey. In addition, urban toads showed more anxiety-like behaviors than rural toads, spending more time moving around the arena edges. These results contrast with previous studies (i.e., Echevarría and Vassallo 2008). In birds, the ability to exploit novel resources is recognized as an important trait favoring individuals and species in urban habitats (Kark et al. 2007; Møller 2009; Sol et al. 2011). Urban habitats provide more novelty challenges, confronting animals with human-derived resources absent in their natural habitat. Individuals able to exploit novel resources are expected to succeed and become established (Webster and Lefebvre 2001; Liker and Bókony 2009). Although urban birds are more likely to exploit new resources in some contexts, there are exceptions to this pattern. For example, house sparrows from habitats of varying levels of urbanization do not differ in their feeding behavior when confronted with novel food or novel feeding situations (Bókony et al. 2012). To our knowledge, no studies have examined the neophobic behavior of anuran species in urban and rural habitats. It is thus unclear whether lack of decreased neophobic behavior in urban cane toads is representative of the strategies in this taxonomic group.

In addition to habitat, experience may play a role in behavioral plasticity of neophobic responses (Kendal et al. 2005; Mettke-Hofmann et al. 2006; Brown et al. 2014). Differences in exploratory behavior and neophobic tendencies may be expected between juveniles and adults. For instance, juvenile chimango caracaras (*Milvago chimango*) are less neophobic toward novel objects than adults (Biondi et al. 2010). In contrast, adult vervet monkeys (*Chlorocebus pygerythrus*) are less neophobic because of a greater variety of experiences (Hauser 1988). How neophobia changes over development is likely affected by whether individuals have positive or negative experiences with novelty throughout their life. Juvenile cane toads may be more exploratory, or neophilic, and less neophobic than adults. Because cane toads disperse during the early stages of life (Zug and Zug 1979), juveniles must exploit novelty when moving across new habitats. Examining neophobia over a broad age range in cane toads is necessary to determine the role of experience in such behaviors and whether differences between rural and urban populations are accentuated over ontogeny.

Overall, we found that introduced toads are less neophobic than native toads. In addition, bolder, potentially neophilic individuals that readily approach novel prey/objects are present in all cane toad populations studied here. Through increased exploration and decreased avoidance of novel prey, introduced cane toads are likely able to exploit novel resources. Successful establishment of introduced species relies on multiple factors (reviewed in Lodge 1993). Cane toads are thought to be successful for several reasons: they lay large numbers of eggs that develop quickly, eat a wide variety of prey, and possess toxins that release them from predation in some of their introduced ranges (Zug and Zug 1979). Recently, the role of behavior in successful invasive species has received increased attention (Sol and Lefebvre 2000; Sol et al. 2002; Martin and Fitzgerald 2005; Kark et al. 2007). To our knowledge, this is the first study investigating neophobia in anurans and examining the role of such behaviors in invasive potential in this taxonomic group. In light of current worldwide population decline of anurans (Stuart

et al. 2004), this work provides valuable insights of how behavior influences the fate of introduced anuran species.

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