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ORIGINAL ARTICLE

Fortune favors the bold toad: urban-derived behavioral traits may provide advantages for invasive amphibian populations

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Abstract

Many biological invasions occur within and between urban areas. If native species adapted to anthropogenically altered habitats are subsequently moved from an urban area in their native range to one within a novel region, then their urban-specialized phenotypes may provide them an advantage via prior adaptation. Here we examine if urban-derived behavioral traits are present within native guttural toad, *Sclerophrys gutturalis*, populations (Durban, South Africa) and investigate whether these localized phenotypes persisted within their invasive populations in Mauritius and Réunion. In our study, we measured boldness and exploration in populations along the toad's invasion route and found that toads were significantly bolder in urban populations, within both native and invasive ranges. This suggests boldness increased when toads transitioned to urban living in their native range and these heightened levels of boldness were maintained within invaded urban areas. This provides evidence that a bolder phenotype was a prior adaptation that likely increased guttural toad's invasion success. Interestingly, toad boldness returned to pre-urbanization levels within invasive populations that spread into natural areas, replicated on both islands. Exploration, on the other hand, was not increased above pre-urbanization, or pre-invasion, levels for any of the populations, and was lower in toads from Mauritius. Overall, our findings suggest that increased boldness is favored in urban habitats and that urban-derived behavioral traits may provide individuals an advantage when invading new urban landscapes.

Significance statement

Species adapting to anthropogenic landscapes have the ability to increase their invasive potential if the altered phenotypes they accrue can provide them advantages once they are transported outside their native range. Our study examined differences in behavioral traits, boldness, and exploration, along the invasion route of guttural toads, *Sclerophrys gutturalis*, between natural and urban sites from their native origin populations around Durban, South Africa, to their invasive populations in Mauritius and Réunion. We determined that populations were bolder in urban areas in their native range and that this increased boldness persisted in the other anthropogenic habitats within their invasive ranges, but reverted back to natural-native levels within populations that had spread into natural areas on both islands. Our findings support the growing trend that anthropogenically altered landscapes favor bolder individuals, as well as the assertion that urban-derived traits may bolster a species' ability to establish and spread within novel landscapes.

Keywords AIAI hypothesis · Amphibian · Boldness · Exploration · Invasion biology · Urban ecology

Introduction

The modern era presents a host of human-related challenges to the world's ecosystems, with two of the most pervasive threats stemming from the growing urban footprint and the increasing spread of invasive species (Corlett 2015; Pelletier and Coltman 2018; Pyšek et al. 2020). Due to the nature of how invasive species are relocated outside their native ranges (e.g., transportation networks, pet trade, or as a

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This article is a contribution to the Topical Collection Using behavioral ecology to explore adaptive responses to anthropogenic change – Guest Editors: Jan Lindström, Constantino Macias Garcia, Caitlin Gabor

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Extended author information available on the last page of the article biocontrol for pests), many biological invasions are innately linked to human-dominated landscapes (Pyšek et al. 2020). On the surface, the connection between urbanization and biological invasion may simply reflect an increased probability (i.e., if a species lives near people, it may be more likely to be transported, deliberately or accidentally, and where it is moved to is more likely to be another human-dominated landscape). Yet, there is growing evidence to suggest that this relationship is more complex, with urban ecosystems potentially acting as biological filters which can promote urban-specialized phenotypes (i.e., urban evolution; see Johnson and Munshi-South 2017) that may secondarily increase a given species' invasive potential (Hufbauer et al. 2012; Rey et al. 2012; González-Bernal et al. 2016; Borden and Flory 2021). For example, the "anthropogenically induced adaptation to invade" (AIAI) hypothesis posits that species adapting to human-modified landscapes can inflate their invasive potential by (1) increasing the likelihood of being moved due to human proximity and (2) through the formation of adaptive phenotypes that provide advantages in anthropogenic habitats, which then can promote successful establishment and spread after translocation (Hufbauer et al. 2012). In part, this is due to the fact that many human-dominated landscapes, even when geographically distinct, share a lot of ecological and environmental characteristics (e.g., the similarities between cities may be more than between a given city and its closest natural areas). If the AIAI hypothesis is correct, urban areas could be viewed as "sorting grounds" for many of the world's species, selecting taxa flexible enough to adapt to urban landscapes and then phenotypically increasing their invasive potential via traits that bolster success in human and novel environments (Hufbauer et al. 2012). Research into how phenotypes adapted to human landscapes can promote invasion success have provided examples across a wide variety of taxa and biological traits, including adult plant size in weeds (Waselkov et al. 2020), locomotory performance in lizards (Battles et al. 2019), and changes in thermal tolerance for ants and birds (Rey et al. 2012; Jackson et al. 2015; Strubbe et al. 2015).

Behavior is an important aspect of how species overcome challenges from novel environments and changes in animal behavior can arise through behavioral flexibility or plasticity, but also through fixed traits that are heritable and subject to natural selection (Plotkin 1988; Slater and Halliday 1994; Lapiedra et al. 2017; Thompson et al. 2018). Differences in behavioral traits between origin and colonizing populations have been well documented in both urban ecology (Lowry et al. 2013) and invasion biology (Hudina et al. 2014), and there are several convergent behavioral traits associated with successful establishment and persistence of populations within novel habitats. For example, an increase in boldness (i.e., an individual's propensity to take risks) is favored in some urbanized songbirds (Evans et al. 2010; Holtmann et al. 2017) and lizards (Pellitteri-Rosa et al. 2017; Baxter-Gilbert et al. 2019), and so too promotes invasion success within populations of crayfish (Pintor et al. 2008), fish (Rehage and Sih 2004; Myles-Gonzalez et al. 2015), lizards (Short and Petren 2008; Damas-Moreira et al. 2019), and rodents (Malange et al. 2016). Other examples of shifts in specific behavioral traits, like increased activity level, aggression, exploration, and neophilia, have been similarly observed in species living in either urban (Evans et al. 2010; Kralj-Fišer and Schneider 2012; Thompson et al. 2018) or invasive populations (Rehage and Sih 2004; Myles-Gonzalez et al. 2015; Damas-Moreira et al. 2019). Of course, these changes in behavior do not always consistently trend in the same direction, with examples of decreased levels of boldness (Putman et al. 2020) and increased neophobia (Miranda et al. 2013) occurring in some urban populations, as well as instances where no differences are shown for certain phenotypes (e.g., increased boldness in urban populations, but no differences in exploration and neophilia between urban and rural populations; Baxter-Gilbert et al. 2019). Given that the colonization of novel habitats can drive behavioral change (Lowry et al. 2013; Hudina et al. 2014; Lapiedra et al. 2017), including the formation of innate and potentially heritable traits (Holtmann et al. 2017; Baxter-Gilbert et al. 2019) or increased levels of behavioral flexibility (Dammhahn et al. 2020), it stands to reason that native urban populations may promote phenotypes that could benefit individuals invading novel landscapes (i.e., prior adaptation; Hufbauer et al. 2012). This could effectively prime urbanized native taxa to become better invaders (Borden and Flory 2021).

To test the AIAI hypothesis, we compare measures of boldness and exploration between populations of guttural toad, *Sclerophrys gutturalis*, across an urban-natural/native-invasive gradient, following their invasion route (i.e., the geographic pathway propagules traveled between the source and invading populations; Estoup and Guillemaud 2010) from their native range in Durban, South Africa, to their invasive populations in Mauritius and Réunion (Telford et al. 2019). Previous research from these three locations has shown that the invasive island toad populations on both islands have reduced body sizes and disproportionately shorter hind limb lengths (Baxter-Gilbert et al. 2020), suggesting selective pressure from these colonization events and unique landscapes have driven physical phenotypic changes. Given the fact that these invasive populations are known to have undergone phenotypic divergence, as well as the potential for urban environments to be a driver, behavioral alterations may have also occurred and be related to prior adaptation through urban filters. As such, we studied toads from natural and urban populations in Durban, Mauritius, and Réunion to determine whether they (1) express different levels of boldness and exploration between natural or urban habitats within their native range, (2) maintained or increased these phenotypic differences within the urban, invasive habitats where they were first established, and (3) maintained or increased these phenotypic differences once they spread into natural habitats within the invasive range. If urban environments are selecting for bolder and more explorative toads, which in turn may also improve a toad's invasive potential (e.g., Damas-Moreira et al. 2019), then we expect similar, higher levels of boldness and exploration to be expressed by invasive toad populations within the urban habitats in which they were introduced, which may also be carried further into natural areas in their invaded ranges.

Methods

Study species and sites

Guttural toads, *Sclerophrys gutturalis*, are a large generalist bufonid (maximum snout-vent length (SVL) = 140 mm; du Preez et al. 2004) with female-biased sexual size dimorphism (Baxter-Gilbert et al. 2020) and a broad distribution in sub-Saharan Africa spanning from Angola in Central Africa to Kenya in East Africa and ranging south to eastern South Africa (see Telford et al. 2019). These toads are known for their proclivity for thriving in human-disturbed habitats (Vimercati et al. 2019) and are frequently-encountered urban residents. For almost a century, these toads have had invasive populations in Mauritius and Réunion, both a result of failed biocontrol attempts (Cheke and Hume 2010), and these alien populations express locally-specific reductions in adult body size (i.e., insular dwarfism; Baxter-Gilbert et al. 2020). Molecular research has confirmed that these invasions came from the same native source population that originated near Durban, South Africa (Telford et al. 2019). The molecular research also supports the historic accounts (Cheke and Hume 2010; Telford et al. 2019), which when combined with what we know about their anthropophilic behavior (Vimercati et al. 2019; JB-G pers obs.) suggests the invasion route for the guttural toads likely consisted of (1) *pre-urbanization*, toads existed in natural habitats in their native range; (2) *post-urbanization*, toads in the immediate vicinity of Durban started to become urbanized from 1850 onward, (3) *establishment in Mauritius*, in 1922 toads were collected from the Durban area, likely from around human-disturbed habitats, and shipped to a Mr. Regnard who was the dock manager in Port Louis, Mauritius (Cheke and Hume 2010), who released them around human settlements and agricultural areas to control pest insects; (4) *establishment in Réunion*, in 1927, toads were collected in Mauritius, likely from around human settlements, and shipped to a Mr. de Villèlle (Cheke and Hume 2010), whose family estate was in Saint-Gilles-les-Hauts, Réunion, where they were released to control pest insects; and (5) *invasive spread*, on both islands the toads numbers grew, populations spread, and the toads invaded from human settlements and disturbed areas to natural ecosystems on both islands.

To select our sampling sites, we examined a 1 km^2 area around each prospective sampling site on Google Earth® and used the polygon function to measure the percentage of land cover represented by a human footprint (e.g., hardscape, infrastructure, impervious surfaces, and/or residential areas). For the purposes of our study, natural areas needed to have $\leq 1\%$ human footprint and urban areas as those with a human footprint of $\geq 50\%$, which follows similar frameworks used by McKinney (2008), Larson et al. (2020), and Bókony et al. (2021). Our natural-native site was a reclaimed grassland located 110 km north of Durban on a private property which consisted of open grasslands, forest patches, and a wetland, with the overall land covered being comprised of $\leq 1\%$ human footprint (Fig. 1A). The urban-native site (i.e., the Durban Botanical Gardens; established in 1849) was within the city of Durban and was a heavily modified greenspace which has undergone human development and urban envelopment over the last 170 years and is represented by 81% human footprint (Fig. 1B). Our sampling in the invasive ranges, Mauritius and Réunion, targeted urban locations close to where we predicted the toads were originally introduced and the natural sites in these locations represent native greenspaces which the toads have spread into. Ecologically, both islands are similar in size, 2040 km^2 (Mauritius) and 2512 km^2 (Réunion) with tropical climates, and are both considered biodiversity hotspots (Myers et al. 2000; Telford et al. 2019). The urban-invasive site in Mauritius was located in the village of Norte Dame, 10 km from Port Louis, and the habitat consisted of backyards, roadsides, ditches with streams, and refuse piles, with 61% of the area being comprised of human footprint (Fig. 1D). Our natural-invasive site on Mauritius was in the Brise Fer forest of Black River Gorges National Park (Fig. 1C), located 40 km south of Port Louis, which is part of the last 4.4% of remaining natural forest on the island (Hammond et al., 2015) and had $< 0.1\%$ human footprint. Within Réunion, our urban-invasive site was in the village of Villèlle, 2 km from Saint-Gilles-les-Hauts, and the habitat consisted of backyards, roadsides, refuse piles, and a golf course with 58% of land cover representing human footprint (Fig. 1F). Our Réunion natural-invasive site was located in a natural greenspace consisting of treed and grassland habitat outside the village of Point Payet (Fig. 1E) which had $< 0.1\%$ of human footprint and was 41 km east of Saint-Gilles-les-Hauts and adjacent to the large, protected area of Grand Étang.

Data collection

Adult toads were hand-caught during opportunistic walking surveys during time periods where toads were locally active at each study site (Durban: February to March 2020;

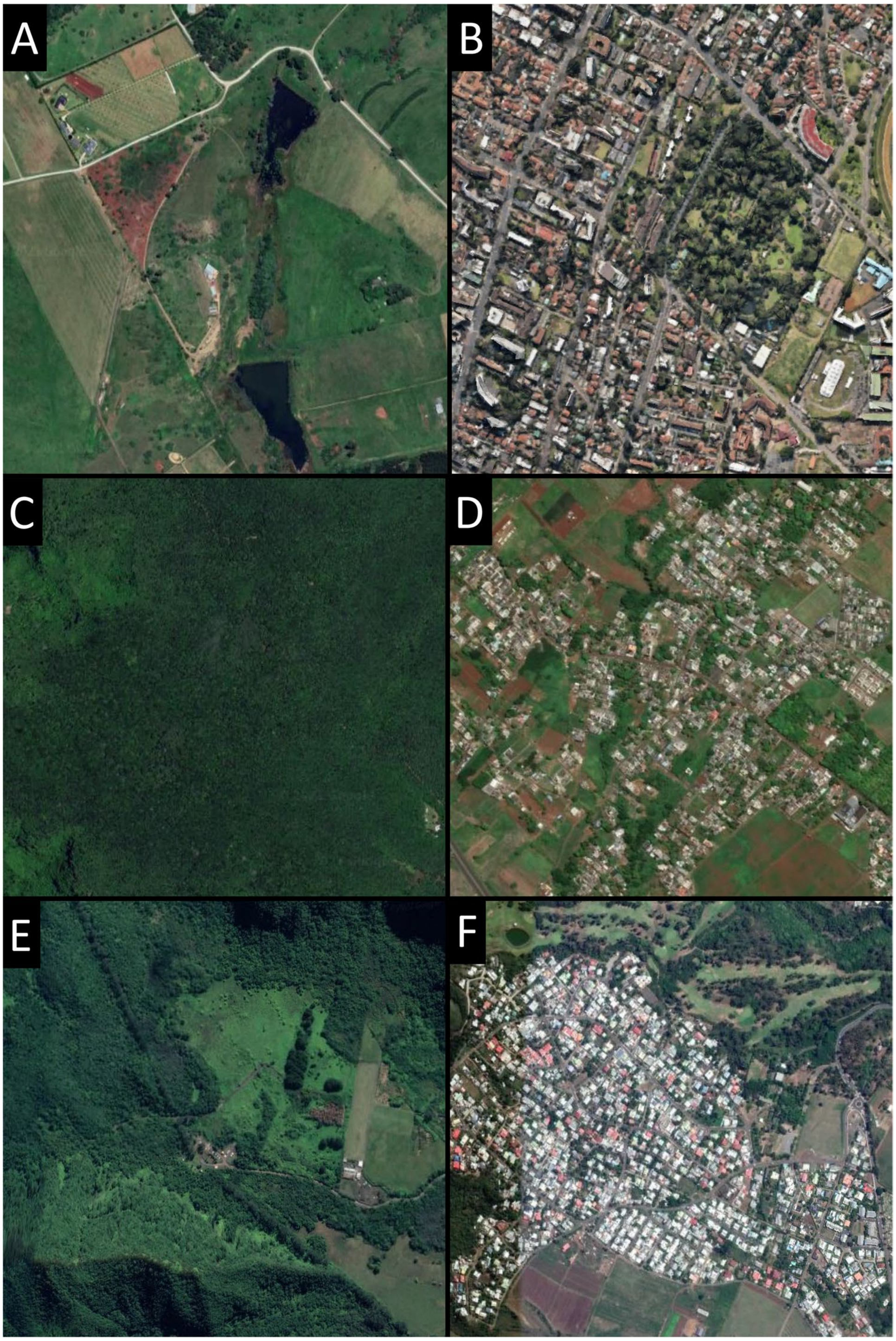

Fig. 1 Landscape images (approximately 1 km^2) showing the differences in human footprint (e.g., infrastructure, impervious surfaces, and residential areas) between natural and urban sites from South Africa (native range; **A** natural site with 1% footprint; **B** urban site with 81% footprint), Mauritius (invasive range, **C** natural site with < 0.1% footprint; **D** urban sites with 61% footprint), and Réunion (invasive range, **E** natural site with < 0.1% footprint; **F** urban site with 58% footprint). Images generated and human footprint area measured using Google Earth®

Mauritius: June to July 2019; and Réunion: July 2019). Adult size thresholds were locally specific (39 mm Mauritius, 36 mm Reunion, and 57 mm Durban; for details see Baxter-Gilbert et al. 2020). Once captured, toads were brought to temporary field stations at each of the locations. We housed the toads in two experimental groups ("A" and "B") of 16 toads each (for a total of 32 toads, with a 50:50 sex ratio, per site), in large circular (1.83-m diameter) plastic containers (i.e., collapsible children's swimming pools) outfitted with wetted sand, rocks, and dried leaves, allowing the toads to seek shelter, encourage normal burrowing behavior, and regulate their hydric conditions. Each toad was individually marked with a unique passive integrative transponder (PIT tag) and was given a minimum of 24 h post-capture to acclimate prior to behavioral testing.

All behavioral assays occurred between 1800 and 2300 h, aligning with our observations of wild activity periods, and room temperature (°C) was recorded for each behavioral assay. Behavioral assays were remotely recorded using a four-camera CCTV setup (SA Lucky ABC, IR Color CCD Camera, model: ABC-5504H-4) with recordings being stored in an internal DVR unit. This allowed the assays to occur without humans being present within the room and allowed for four toads to be independently assayed at the same time. For this reason, recording of the behavioral assays was done in batches (one to five in total) of four toads each on any given night, allowing for 16 toads to be tested per night. Additionally, the assays were conducted within a dark room, and researchers only used dimmed red lights within the experimental room during assay setup (detailed below) to minimize the impact artificial white light may have on the toad's behavior. Furthermore, one researcher (JLR) was responsible for carrying out the behavioral assays to ensure consistency of the methods throughout our study. At each of the six sites, the toads' explorative behavior was assayed first—taking two nights to complete, with one of the experimental groups (i.e., group "A" or "B") being tested each day—followed by boldness for the following two nights. Each toad was assayed once per behavioral trait.

Exploration assay

To begin the exploration assay, we first placed a toad under a 114 mm (D) × 81 mm (H) circular acclimation chamber within a 0.40 m (L) × 0.40 m (W) × 0.40 m (H) arena lined with a grid paper base. The arenas were also outfitted with four hides (identical to the acclimation chamber) each with one opening, to break up the blank space and allow for normal explorative behavior to occur, such as investigating potential refuges. After 5 min, the acclimation chamber was removed, which exposed the toad to the novel environment and the individual was left to explore for 30 min. From the video recordings, we scored the total area explored (cm^2; continuous variable) by the toad, which was calculated by counting the number of grid squares the animal crossed during the 30-min period including the spaces occupied by hides. Video scoring of both assays was limited to one researcher (JB-G) to avoid any inter-observer bias within this study and was done so using individual ID numbers so that the researcher was blind. We used this metric (i.e., total area explored) as our measure of exploration (i.e., a quantitative measure of an individual toad's propensity to investigate its novel surroundings).

Boldness assay

To begin our boldness assay, we exposed individuals to a standardized "mock predation" event, wherein the toad was flipped onto its back and allowed to right itself five consecutive times within the palm of the researcher (JLR). After this, the now "frightened" toad was placed within a single hide facing away from the opening that was located in the center of the same testing arena that was used in the "Exploration assay" section. The same individual grid paper that was used in the "Exploration assay" section lined the arena during this assay so that the individual had familiar smells within the testing environment. After the toad was placed in the hide, it was filmed for 30 min. From the video recordings, we scored whether the toad exited the hide (binary variable) and the time (i.e., latency) it took the toad to exit the hide (s; continuous variable). We used these metrics as our measures of boldness (i.e., a quantitative measure of how quickly an individual is willing to leave the safety of a hide after encountering a frightening situation).

Statistical analyses

All statistical tests were conducted in R version 4.0.4 (R Core Team 2021). Before starting analyses, we explored our data following a similar protocol as outlined in Zuur et al. (2010). We did not find any unexplainable outliers. There was a significant correlation between our study sites (Durban, Mauritius, and Réunion) and the room temperature documented during the behavioral assays (tested using a one-way ANOVA, using the "lm" and "anova" function in the R "stats" package; R Core Team 2021, for the exploration assay: $F_{2,\,185} = 1305.30$, $p < 0.01$; and the boldness assay: $F_{2,\,184} = 357.99$, $p < 0.01$). For the exploration and boldness assays, the average room temperature varied by 6.9 °C and 3.9 °C between study sites, respectively (exploration assay: Durban 30.5 °C ± 0.1 *SE*; Mauritius 23.6 °C ± 0.1 *SE*; Réunion 21.0 °C ± 0.1 *SE*; boldness assay: Durban 27.7 °C ± 0.2 *SE*, Mauritius 23.9 °C ± 0.1 *SE*, Réunion 22.0 °C ± 0.1 *SE*). Yet, the three response variables we selected to reflect exploration and boldness were not significantly correlated to room temperature (tested using the "lm" and "glm" function in the R "stats" package; R Core Team 2021): the arena area explored (cm^2; $\beta = 2.25$, $SE = 8.59$, $t_{1,\,186} = 0.26$, $p = 0.79$, $R^2 < 0.01$ as calculated using the "rsq" function from the "rsq" R package; Zhang 2020), whether or not a toad exited the hide ($\beta = 0.05$, $SE = 0.05$, $z = 0.92$, $p = 0.36$, $R^2 < 0.01$), and latency to exit the hide (s; $\beta = -40.63$, $SE = 20.48$, $t_{1,\,185} = -1.98$, $p = 0.06$, $R^2 < 0.02$). It is important to note that these localized temperatures reflect each populations' regional norms. Thus, we opted not to include room temperature in our models below, because study site and room temperature were confounded, and room temperature appears to have a minimal effect on these behavioral traits. Furthermore, we did not include morphological traits in our analyses because previous work has found these to be related to the study site (Baxter-Gilbert et al. 2020). For all models, prior to interpretation, we verified the assumptions of normality and homoscedasticity of residuals. Data are presented as predicted means ± standard error (*SE*) in the text, unless otherwise specified, and α was set at 0.05 for all models.

Exploration assay

We measured the exploration of 188 adult guttural toads (93 females and 95 males). Toads were located within natural sites in Durban, Mauritius, and Réunion (16 females and males per study site), as well as urban sites in each of these locations (Durban: 15 females and 16 males; Mauritius: 14 females and 15 males; Réunion: 16 males and 16 females). Sample sizes vary slightly between behavioral assays, because different numbers of video recordings were corrupted between them (see below).

We used a linear mixed-effect model (LMM) to examine differences in the area of the arena (cm^2) explored by the toad during the 30-min assays using the function "lmer" in the R package "lmerTest" (Kuznetsova et al. 2017). The LMM included the fixed effects of the study site (categorical with three levels: Durban, Mauritius, or Réunion), site type (categorical with two levels: natural or urban), and an interaction effect between these two factors to statistically test for the AIAI hypothesis. Additionally, the LMM also included the fixed effect of toad sex (categorical with two levels: female or male), as well as the random intercepts of experimental group and within-day batch to control for dependency among experimental groupings that occurred as an artifact of our sampling design.

After running the LMM and verifying its assumptions, we examined the significance of the interaction effect between study site and type using a post hoc test for multiple comparisons. This was run using the function "emmeans" from the "emmeans" R package, and the p-values generated for these comparisons were corrected using an "mvt" adjustment that uses a Monte Carlo method to produce "exact" Tukey corrections (Lenth 2020). If the interaction was not significant, then it was removed from the model and the model was re-run in order to allow interpretation of the main effects. In those cases, post hoc multiple comparisons between all study sites were tested using the "emmeans" R package using the same protocol as described above.

Boldness assay

We measured the boldness of 187 adult guttural toads (93 females and 94 males). These toads were located in natural (Durban and Réunion: 16 of both sexes; Mauritius: 15 of both sexes) and urban sites (Durban and Réunion: 16 of both sexes; Mauritius: 14 females and 15 males).

We used a binomial generalized linear mixed-effect model (GLMM) to examine differences whether a toad exited the hide or not (exited = 1, stayed inside = 0), during our 30-min boldness assay using the function "glmer" in the R package "lmerTest" (Kuznetsova et al. 2017). This GLMM included the fixed effects of the study site (categorical with three levels: Durban, Mauritius, or Réunion), site type (categorical with two levels: natural or urban), and an interaction effect between study site and site type to statistically test for the AIAI hypothesis. Additionally, the GLMM also included the fixed effect of toad sex (categorical with two levels: female or male), as well as the random intercepts of the experimental group and within-day experimental batch to control for dependency among experimental groupings that occurred as an artifact of our sampling design. We examined the significance of interaction effects and post hoc multiple comparisons between study sites post hoc using the same protocol as described above in regard to the LMM that analyzed toad exploration tendency. Further, we analyzed the latency for a toad to exit the hide (s) during our 30-min boldness assay using the same LMM approach as described above.

Results

Exploration assay

The amount of area (cm^2) explored by guttural toads significantly differed between study sites (Table 1) with toads in Mauritius being significantly less explorative than their counterparts in Durban or on Réunion (Fig. 2). From our models, and accounting for additional factors, we saw that toads from Mauritius (692.64 ± 6.24) explored an average of 212.03 cm^2 less of the arena than Durban toads (904.67 ± 6.72) and an average of 248.58 cm^2 less of the arena than toads from Réunion (941.23 ± 6.54). Guttural toad exploration propensity was not affected by toad sex, whether a site was in natural or urban habitats, nor an interaction between study site and site type (Table 1).

Boldness assay

Whether or not a toad exited the hide did not significantly differ between study sites (Tables 2 and 3, Fig. 3). Toads from urban areas were 20% more likely to exit the hide than toads from natural areas, which represents a bolder phenotype (Table 2, Fig. 3). Toads from urban areas also took an average of 277 s less to exit the hide (i.e., latency) than toads from natural areas (Tables 3 and 4). There was no significant interaction between the study site and site type. In addition, male toads were significantly more likely to exit the hide than females across all locations (Table 2).

Table 1 (a) Outcome of the linear mixed-effect model (LMM) examining differences in the arena area (cm^2) explored by a toad during our 30-min exploration assay. The interaction between study site and site type was not significant and so it was removed and the models re-run. Model estimates (β) of fixed effects are presented with their corresponding standard errors (*SE*), variance estimates (σ^2) are supplied for residuals and random effects, and all significant values ($p < 0.05$) are bolded. Reference levels for the categorical variable are given in brackets following the variable name. (b) We also present post hoc multiple comparisons of arena area explored (cm^2) between all study sites, and in this case, *p*-values (p_{corr}) were corrected using an "mvt" adjustment (Lenth 2020)

(a) Output from the linear mixed-effect model

Variable names				
Fixed effects	β	*SE*	*t*	*p*
Intercept (Durban, natural, female)	**950.89**	**83.43**	**11.40**	**< 0.01**
Study site (Mauritius)	**−212.08**	**85.53**	**−2.48**	**0.01**
Study site (Réunion)	35.49	84.38	0.42	0.67
Site type (urban)	−31.90	69.45	−0.46	0.65
Sex (male)	−61.20	69.40	−0.88	0.38
Random effects	σ^2			
Experimental group	4099.00			
Within-day batch	0.00			
Residuals	226,013.00			

(b) Multiple comparisons between study sites

Study sites	β	SE	*t*	p_{corr}
Durban vs. Mauritius	**212.10**	**85.70**	**2.47**	**0.05**
Durban vs. Réunion	−35.50	84.40	−0.42	0.92
Mauritius vs. Réunion	**−247.60**	**85.40**	**−2.90**	**0.02**

Discussion

Our findings provide support for the assertion that behavioral phenotypes arising from selection or being more commonly expressed through flexibility or plasticity, in urban populations may provide advantages for individuals colonizing anthropogenically altered habitats outside of their native range (i.e., AIAI hypothesis; Hufbauer et al. 2012) with respect to increased boldness. The same trend, however, was not observed in the toads' tendency to explore. The bolder phenotype of guttural toads was restricted to urban populations, with invasive toads that had expanded their range into natural ecosystems reverting to boldness levels comparable to that of the natural-native population (i.e., pre-urbanization). Taken together, these findings outline three aspects of how urban-derived behavior may contribute to the spread of invasive populations, including that (1) the urbanization of toads in Durban preceding their invasions likely provided them an advantage through prior adaptation, (2) all urban toad populations were significantly bolder than that of natural living conspecifics, and (3) that increased exploratory behavior does not appear to be currently favored in guttural toads above natural-native levels in any of the other five populations we studied and appears to have decreased in the Mauritian populations.

Across their invasion route, guttural toads were consistently bolder in urban areas—either through innate, fixed behavioral traits (Sprau and Dingemanse 2017; Baxter-Gilbert et al. 2019), increased behavioral flexibility (Dammhahn et al. 2020), or adaptive phenotypic plasticity (Yeh and Price 2004; Partecke 2013). Furthermore, guttural toads maintain this increased level of boldness as they established

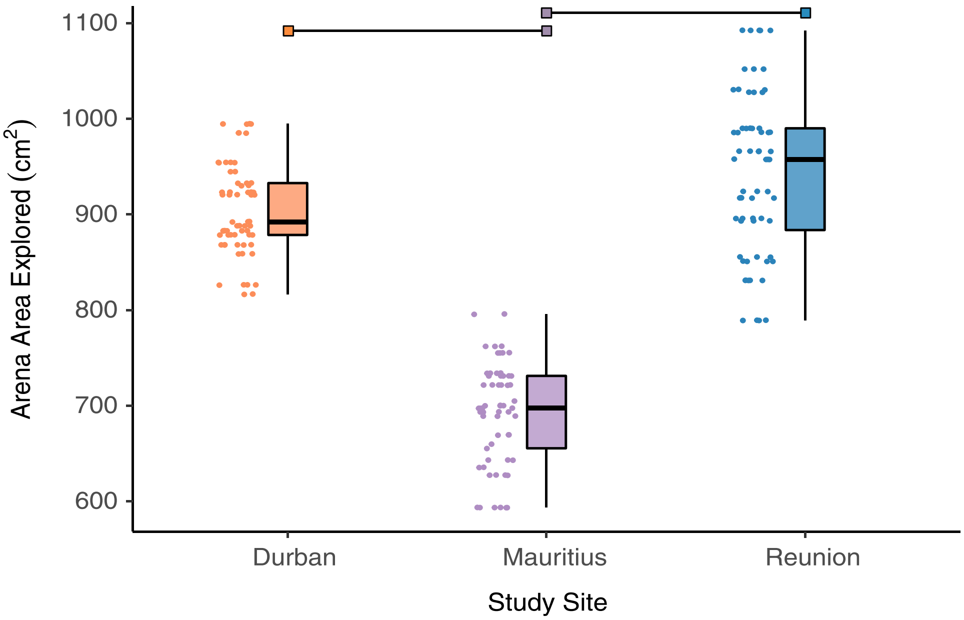

Fig. 2 The arena area explored (cm^2) by guttural toads during our 30-min exploration assay, as predicted from our linear mixed-effect model, for each of our study sites [Durban (native) = orange, Mauritius (invasive) = purple, and Réunion (invasive) = blue]. Significant differences are denoted using a black line with location-specific colors at the ends located above the boxplots. Predicted jittered data points are shown on the left with corresponding boxplots to the right. In the boxplots, the thick horizontal line represents the median, the boxes encompass the quartile ranges, and the whiskers represent the minimum and maximum of the data, excluding outliers (points that are 3/2 times the upper quartile)

Table 2 (a) Outcome of the generalized linear mixed-effect model (GLMM) examining differences in if a toad exited the hide during the 30-min boldness assay. The interaction between study site and site type was not significant, and so it was removed and the models re-run. Model estimates (β) of fixed effects are presented on the latent (*logit* link) scale with their corresponding standard errors (*SE*), variance estimates (σ^2) are supplied for residuals and random effects, and all significant values ($p < 0.05$) are bolded. Reference levels for the categorical variable are given in brackets following the variable name. (b) We also present post hoc multiple comparisons of the probability a toad exited the hide between all study sites, and in this case, *p*-values (p_{corr}) were corrected using an "mvt" adjustment (Lenth 2020). These values are on the response scale (i.e., back-transformed from *logit* link and the latent scale)

(a) Output from the linear mixed-effect model				
Variable names				
Fixed effects	β	*SE*	*t*	*p*
Intercept (Durban, natural, female)	−0.52	0.41	−1.27	0.21
Study site (Mauritius)	−0.38	0.38	−0.99	0.32
Study site (Réunion)	−0.14	0.37	−0.37	0.71
Site type (urban)	**0.85**	**0.31**	**2.76**	**< 0.01**
Sex (male)	**0.73**	**0.32**	**2.32**	**0.02**
Random effects	σ^2			
Experimental group	0.08			
Within-day batch	0.05			
Residuals	1.00			
(b) Multiple comparisons between study sites				
Study sites	β	SE	*t*	p_{corr}
Durban vs. Mauritius	1.46	0.56	0.99	0.58
Durban vs. Réunion	1.15	0.43	0.37	0.93
Mauritius vs. Réunion	0.77	0.30	−0.63	0.80

their urban-invasive populations. Irrespective of the mechanism that drove the expression of this phenotype, the toads likely experienced over 70 years (~ 35 generations; Vimercati et al. 2017) of selection within urban/anthropogenic areas within their native range before individuals were collected and relocated to Mauritius and subsequently Réunion (Cheke and Hume 2010). Thus, guttural toads were already primed for living in disturbed and anthropogenically altered habitats before they arrived (i.e., prior adaptation) which could have provided an advantage during establishment and localized spread in urban, invasive habitats (Hufbauer et al. 2012; Borden and Flory 2021). Our findings do not entirely conform to the AIAI hypothesis in full (Hufbauer et al. 2012), however, because the urban-invasive populations which spread into natural habitats, on both Mauritius and Réunion, reverted to boldness levels that mirror that of the natural-native population. This suggests that living within an urban area prior to their arrival in urban, invasive habitats may have altered their behavior in a beneficial way and that without this step the anthropogenically altered habitats they were released into on both islands may have proven more challenging. Our findings also point to the adaptive value of being able to shift behavioral traits, like boldness, higher or lower depending on their environment—bolstering species' persistence and invasive potential.

Boldness reflects an individual's propensity to take risks and, within an urban context, bolder individuals may be more active in novel landscapes and situations due to lessened perceived risk, which could increase their time spent foraging or mate searching (Réale et al. 2007; Sol et al. 2013; Sprau and Dingemanse 2017). For this reason, it is fitting that increased boldness appears to be a convergent and commonly noted phenotypic shift across a variety of taxa encountering urban landscapes (Lowry et al. 2013). An increase in boldness within urban-living individuals has been found across numerous vertebrates (e.g., birds, Sprau and Dingemanse 2017; fish, Rehage and Sih 2004; mammals, Dammhahn et al. 2020; reptiles, Baxter-Gilbert et al. 2019) and invertebrate taxa alike (e.g., insects, Schuett et al. 2018; isopods, Houghtaling and Kight 2006; spiders, Kralj-Fišer et al. 2017). Our findings not only support this growing trend, but further provide evidence that advantageous behavioral traits can be maintained in populations after being moved from one urban area to another, across entirely different regions of the world, and that once populations spillover into natural areas behavioral traits can shift again to match that of native, natural norms. Interestingly, if this was

Table 3 The boldness measured during this study summarized by study site (Durban, Mauritius, and Réunion) and site type (natural or urban). We summarized (a) the number of toads that exited the hide with the total number of toads we measured following and separated using a backslash, (b) the probability of toads exiting the hides as predicted from the generalized linear mixed-effect model (see Table 2a), and (c) the latency to leave the hide (s) as predicted from a linear mixed-effect model (see Table 4a). The latter two variables are displayed as mean ± standard error (*SE*). Significant differences between variables are shown using asterisks (*) and carets (^), respectively showing separate comparisons, following the pertinent means and standard errors

Boldness measure	Study site			Site type	
	Durban	Mauritius	Réunion	Natural	Urban
(a) Number of toads exited hide/total number measured	36/64	28/59	34/64	40/94	58/93
(b) Predicted probability of toads exiting hides	0.56 ± 0.02	0.47 ± 0.02	0.53 ± 0.02	0.42 ± 0.01*	0.62 ± 0.01*
(c) Predicted latency to exit the hide (s)	935.75 ± 25.04	1080.37 ± 25.78	1131.59 ± 23.39	1186.21 ± 15.89^	909.12 ± 17.19^

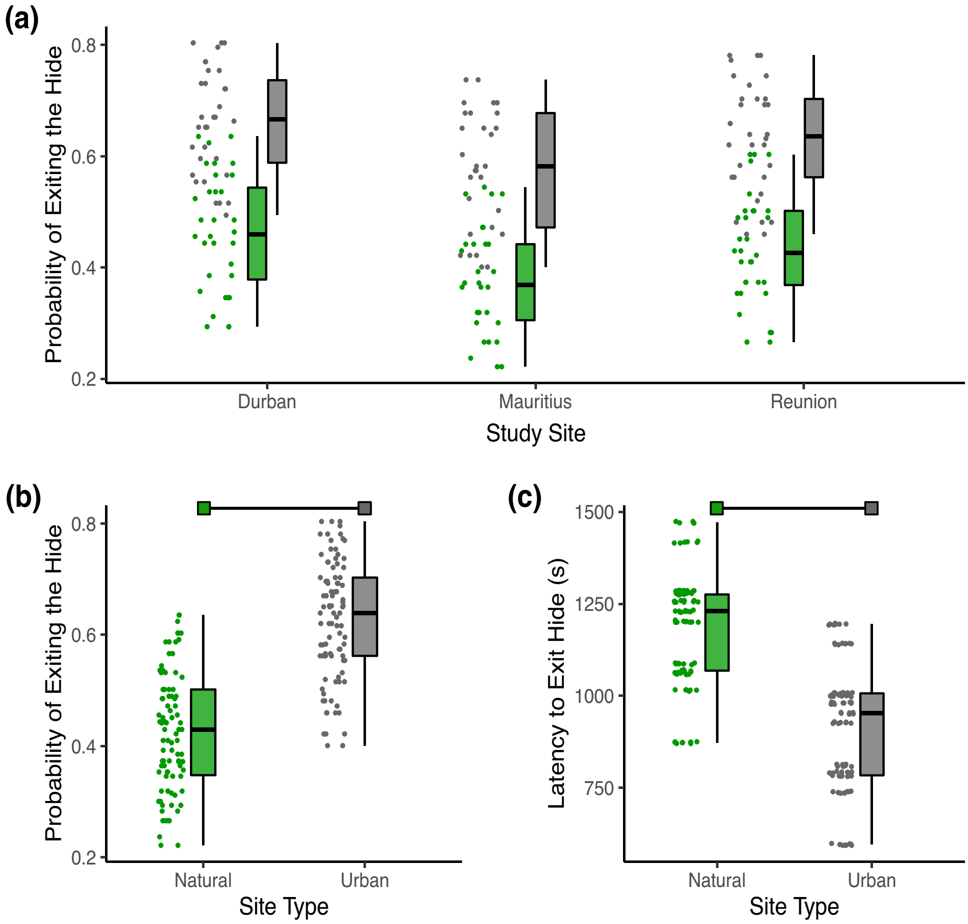

Fig. 3 (**a**) The probability of a guttural toad exiting the hide during our 30-min boldness assay, as predicted from our generalized linear mixed-effect model, for each of our study sites by site type (urban = grey, natural = green). (**b**) We also depict the predicted probability of exiting the hide and (**c**) the predicted latency to exit the hide for the main effect of site type. Significant differences are denoted using a black line with site-type-specific colors at the ends located above the boxplots. Predicted jittered data points are shown on the left with corresponding boxplots to the right. In the boxplots, the thick horizontal line represents the median, the boxes encompass the quartile ranges, and the whiskers represent the minimum and maximum of the data, excluding outliers (points that are 3/2 times the upper quartile)

to occur on a relatively short time scale, then one may presume this is a product of behavioral flexibility (Dammhahn et al. 2020) or adaptive phenotypic plasticity (Yeh and Price 2004). However, with over 70 years of urban/anthropogenic selective forces shaping toad behavior pre-invasion, and almost 100 years of selection acting on both natural- and urban-invasive toad populations post-invasion, the potential for these phenotypes to be adaptive and heritable certainly does exist. We suggest future investigations look to determine the evolutionary mechanisms driving the behavioral differences we observed between our study populations (i.e., fixed vs. plastic) using appropriately robust study designs to test for urban evolution (see Lambert et al. 2020).

Contrary to our predictions, explorative behavior did not differ between urban and natural habitats, nor in a consistent fashion along the toad's invasion route. Although increased levels of exploration are thought to be favored in populations colonizing new landscapes and urban habitats (Lapiedra et al. 2017; Damas-Moreira et al. 2019; Dammhahn et al. 2020). The advantages conferred from increased explorative behavior are most closely related to particular stages of the invasion process—transport, introduction, establishment, and spread (Chapple et al. 2012)—rather than within long-founded invasive populations. For example, differences between invasive and native lizards' explorative tendency were found in a 20-year-old invasive population of Italian Wall Lizards, *Podarcis sicula*, in Portugal (Damas-Moreira et al. 2019); however, this invasion is much more recent than the colonization by guttural toads on either island. Our findings instead seem to align with research on another invasive amphibian, the cane toad (*Rhinella marina*), whereby established island populations in Hawai'i express lower levels of explorative behavior compared to the expanding invasive populations in Australia, supporting the idea that once invasive population reaches saturation in a closed system (e.g., islands), the drive to maintain dispersive behavioral phenotypes is relaxed (Gruber et al. 2016; Gruber 2017). As such, it appears that, unlike boldness within urban landscapes, if guttural toad populations had increased their explorative behavior during their colonization of either the urban landscape in their native range or the ecosystems of Mauritius and Réunion, then it has not been maintained. Rather we see a significant reduction in explorative behavior for toads in Mauritius. Although not following what we would expect, based on studies on the

Table 4 (a) Outcome of the linear mixed-effect model (LMM) examining differences in the time (s) it took a toad to exit the hide during the 30-min boldness assay. The interaction between study site and site type was not significant, and so it was removed and the models rerun. Model estimates (β) of fixed effects are presented with their corresponding standard errors (*SE*), variance estimates (σ^2) are supplied for residuals and random effects, and all significant values ($p < 0.05$) are bolded. Reference levels for the categorical variable are given in brackets following the variable name. (b) We also present post hoc multiple comparisons of arena area explored (cm^2) between all study sites, and in this case, *p*-values (p_{corr}) were corrected using an "mvt" adjustment (Lenth 2020)

(a) Output from the linear mixed-effect model

Variable names				
Fixed effects	β	*SE*	*t*	*p*
Intercept (Durban, natural, female)	**1181.52**	**160.47**	**7.36**	**< 0.01**
Study site (Mauritius)	142.51	134.28	1.06	0.29
Study site (Réunion)	195.84	131.51	1.49	0.14
Site type (urban)	**− 276.62**	**108.81**	**− 2.54**	**0.01**
Sex (male)	**− 214.92**	**109.26**	**− 1.97**	**0.05**
Random effects	σ^2			
Experimental group	22,381.00			
Within-day batch	0.00			
Residuals	55,3429.00			

(b) Multiple comparisons between study sites

Study sites	β	*SE*	*t*	p_{corr}
Durban vs. Mauritius	− 142.50	134.00	− 1.06	0.54
Durban vs. Réunion	− 195.80	132.00	− 1.49	0.30
Mauritius vs. Réunion	− 53.30	134.00	− 0.40	0.92

explorative behavior of establishing or spreading invasive populations (Chapple et al. 2012), this curious finding does support the assertion that the reduced hind limb sizes in toads from both islands may be related to a "less dispersive" phenotype (see Baxter-Gilbert et al. 2020). Yet, the fact that reduced explorative behavior was only significant in Mauritius—and not Réunion—and similarities in boldness between urban populations of larger mainland toads and the smaller urban conspecifics (Baxter-Gilbert et al. 2020), this suggests that the relationship between changes in morphological and behavioral phenotypes may be more complex and will require further investigation. The absence of an increase in exploration, as well as a decrease in Mauritius, may be attributed to local factors favoring more sedentary behavior such as increased food availability (Lyons et al. 2017) or differences in predation levels (Huang et al. 2012); however, much more research into the ecological differences between locations and populations is needed (e.g., examining dietary and trophic changes along the invasion route). Overall, we are unable to determine whether increased toad exploration played a role in their success as they shifted from natural to urban, or native to invasive, habitats. All we are able to observe now, ~ 170 years after the process began, is that a highly explorative phenotype, above the natural-native norm, is not currently favored in any of the six populations we studied.

Our study highlights several key aspects of the relationship between behavior, urbanization, and biological invasions. Notably, we observed that toads from urban habitats were significantly bolder than natural living conspecifics, both in native and invasive ranges. If selection had favored this phenotype, as toads slowly adapted to urban living in their native range over generations, either through increased flexibility, adaptive plasticity, or through rapid localized adaptation, then these acquired phenotypes likely provided them a substantial advantage once they were introduced to anthropogenic habitats in both Mauritius and Réunion. This support for the AIAI hypothesis, albeit promising, raises several new questions, particularly regarding the evolutionary mechanisms driving these changes in behavior, but also if these behaviors are consistent within individuals (i.e., repeatability), present across life stages (i.e., tadpoles vs. adults), and how this bolder phenotype specifically benefits guttural toads in urban landscapes (i.e., urban behavioral ecology). We recommend future research on this promising study system to examine these questions, which should advance our understanding of how urban habitats may be priming native species to become better invaders and how behavioral shifts can increase a given taxa's invasive potential.

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Data availability The datasets and R code for this study are available from Open Source Framework (OSF, DOI: https://doi.org/10.17605/OSF.IO/54TAC), which can be found here: https://osf.io/54tac/

Declarations

Ethics approval Ethical approval for toad captures and our experimental protocols followed animal ethics guidelines set out and approved by the Stellenbosch University's Research Ethics Committee (Animal Care and Use: ACU-2020–10386). National guidelines for the use of animals in scientific research were followed (South Africa National Standard: The Care and Use of Animals for Scientific Purposes; SANS

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Conflict of interest The authors declare no competing interests.

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