

Phenotypic shifts in urban areas in the tropical lizard *Anolis cristatellus*

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Urbanization is an increasingly important dimension of global change, and urban areas likely impose significant natural selection on the species that reside within them. Although many species of plants and animals can survive in urban areas, so far relatively little research has investigated whether such populations have adapted (in an evolutionary sense) to their newfound milieu. Even less of this work has taken place in tropical regions, many of which have experienced dramatic growth and intensification of urbanization in recent decades. In the present study, we focus on the neotropical lizard, *Anolis cristatellus*. We tested whether lizard ecology and morphology differ between urban and natural areas in three of the most populous municipalities on the island of Puerto Rico. We found that environmental conditions including temperature, humidity, and substrate availability differ dramatically between neighboring urban and natural areas. We also found that lizards in urban areas use artificial substrates a large proportion of the time, and that these substrates tend to be broader than substrates in natural forest. Finally, our morphological data showed that lizards in urban areas have longer limbs relative to their body size, as well as more subdigital scales called lamellae, when compared to lizards from nearby forested habitats. This shift in phenotype is exactly in the direction predicted based on habitat differences between our urban and natural study sites, combined with our results on how substrates are being used by lizards in these areas. Findings from a common-garden rearing experiment using individuals from one of our three pairs of populations provide evidence that trait differences between urban and natural sites may be genetically based. Taken together, our data suggest that anoles in urban areas are under significant differential natural selection and may be evolutionarily adapting to their human-modified environments.

KEY WORDS: Adaptation, urbanization, rapid evolution, Puerto Rico, anole.

Urbanization is increasing rapidly worldwide in both spatial extent and density (United Nations 2012). Nearly two-thirds of the world's terrestrial area now supports human populations, and associated land-use changes have resulted in the destruction of natural areas, along with the creation of new habitats composed

of human structures, impervious surfaces, nonnative and managed vegetation, unstable microclimates, and isolated natural remnants (Bradley 1995; McKinney 2002; Millennium Ecosystem Assessment 2005). Many species cannot withstand such dramatic changes to their environments; however, other species persist and even thrive in urban habitats (Mallery et al. 2003; Ditchkoff et al. 2006). This leads to patterns of reduced species diversity but increased abundance of urban-tolerant species (Shochat et al. 2006).

Unique ND2 sequences deposited in GenBank (KX173468 – KX173775).
Data Archived: Dryad: 10.5061/dryad.h234n

Novel selection pressures may arise due to environmental differences between natural and urban areas. These have the potential to drastically alter communities and ecological interactions (Klein 1989; Eggleston et al. 2005; Foley et al. 2005; Laeser et al. 2005; Ackley et al. 2009). Moreover, rapid contemporary adaptation is often associated with anthropogenic disturbance in terms of novel food resources, altered biophysical conditions, or shifts in predators or competitors (Reznick and Ghalambor 2001). Urban habitats provide subsidized food resources in the form of anthropogenic waste (as well the insects that are attracted to waste), and artificial light sources that can increase localized food availability during night hours (Henderson and Powell 2001; Perry et al. 2008). Changes in microhabitat conditions such as light, humidity, and temperature profiles can all have significant impacts on urban species (Shochat et al. 2006; Marnocha et al. 2011). Altered predation pressures due to increases in generalist predators can also lead to shifts in behavior and increased mortality (Ditchkoff et al. 2006).

A small number of prior studies have documented phenotypic differences between urban and natural populations (Ditchkoff et al. 2006), such as bird and frog species adjusting calls to be heard over city noise (Slabbekoorn and Peet 2003; Lengagne 2008; Hoskin and Goosem 2010; Potvin et al. 2011), and increased heat tolerance in urban-persistent ants (Angilletta et al. 2007). Unique adaptive landscapes in urban habitats have the potential to lead to divergent phenotypes and rapid evolutionary change at rates exceeding those observed in more natural habitats (Hendry et al. 2006, 2008; Smith and Bernatchez 2008). These shifts may have a heritable genetic basis (Ashley et al. 2003), although even plastic responses can have significant evolutionary implications if they permit species to temporarily persist under novel selective conditions while natural selection simultaneously acts on heritable phenotypic attributes (Price et al. 2003; West-Eberhard 2003).

Lizards in the genus *Anolis* (“anoles”) provide an ideal system to investigate the role of adaptation in urban persistence. A large number and range of ecomorphological and evolutionary studies in natural environments have characterized adaptive trait–environment relationships for behavior, morphology, and physiology (Losos 2009; Gunderson and Leal 2012; Kolbe et al. 2012b). Other research has shown rapid adaptation in these characteristics under certain circumstances (Losos et al. 1997; Kolbe et al. 2012a,b). Finally, many anole species persist in urban habitats where they use human resources as refugia and for basking, foraging, interacting, nesting, and sleeping (Henderson and Powell 2001; Perry et al. 2008; Meshaka 2011). The few studies so far that have investigated the responses of anoles to urbanization have found evidence for differences in behavior and morphology between urban and natural populations (Irschick et al. 2005; Marnocha et al. 2011).

The Puerto Rican Crested Anole, *Anolis cristatellus*, is a small (SVL 50–75 mm in adult males) arboreal lizard. Among the ecologically and morphologically convergent anole faunas in the Greater Antilles (reviewed in Losos 2009), *A. cristatellus* is categorized as a “trunk-ground” specialist. As such, it has relatively long limbs, a stocky build, and tends to be found on broad surfaces such as the ground or the trunks of trees. The species is native to Puerto Rico, where it has an island-wide distribution (Rivero 1998), and is nearly ubiquitous in urban areas.

Since European colonization, Puerto Rico has been heavily impacted by human activities. Throughout the nineteenth and early twentieth centuries the island was utilized intensively for agricultural cash crops such as sugar cane (*Saccharum officinarum*), bananas (*Musa × paradisiaca*), tobacco (*Nicotiana tobacum*), and coffee (*Coffea arabica*), which led to drastic declines in forest cover and native plant and animal species (Miller and Lugo 2009). By the 1940s the island had only 6% of its original forest cover remaining (Koenig 1953). The vast majority of Puerto Rico at that time was rural, although densely populated cities also existed. Also around this time, Puerto Rico began to undergo substantial industrialization, resulting in widespread abandonment of agriculture, recovery of forest, and intensification of development in and around urban regions (López et al. 2001; Helmer 2004; Gould et al. 2012). Although many of the formerly agricultural areas of the island are undergoing significant forest regeneration, the trend toward increasing urbanization continues to this day (Miller and Lugo 2009). Urban areas are now estimated to cover 11% of the island (Martinuzzi et al. 2007), and 94% of Puerto Rico’s approximately 3.7 million citizens are now reported to reside in urban areas (U.S. Census Bureau 2012).

In this study, we asked if populations of *A. cristatellus* are phenotypically adapting to urbanization. Specifically, based on expected habitat differences and relationships between morphology and habitat use, we predicted that urban lizards would have longer limbs and more subdigital lamellae. These a priori predictions were based on hypothesized habitat use: that urban lizards utilize the broader and smoother substrates common to urban areas. To investigate these hypotheses, we compared urban and natural populations of lizards in and around three of the largest urban centers in Puerto Rico (Mayagüez, Ponce, and San Juan). We measured various functionally relevant morphological traits, collected environmental data, and made behavioral observations of substrate use. To assess the genetic context of divergence, we also collected mitochondrial DNA sequence data from urban and natural areas in each municipality. Finally, to determine if observed phenotypic differences were genetically based, we conducted a common-garden rearing experiment with offspring from adult pairs obtained from one urban and one natural population in the municipality of Mayagüez.

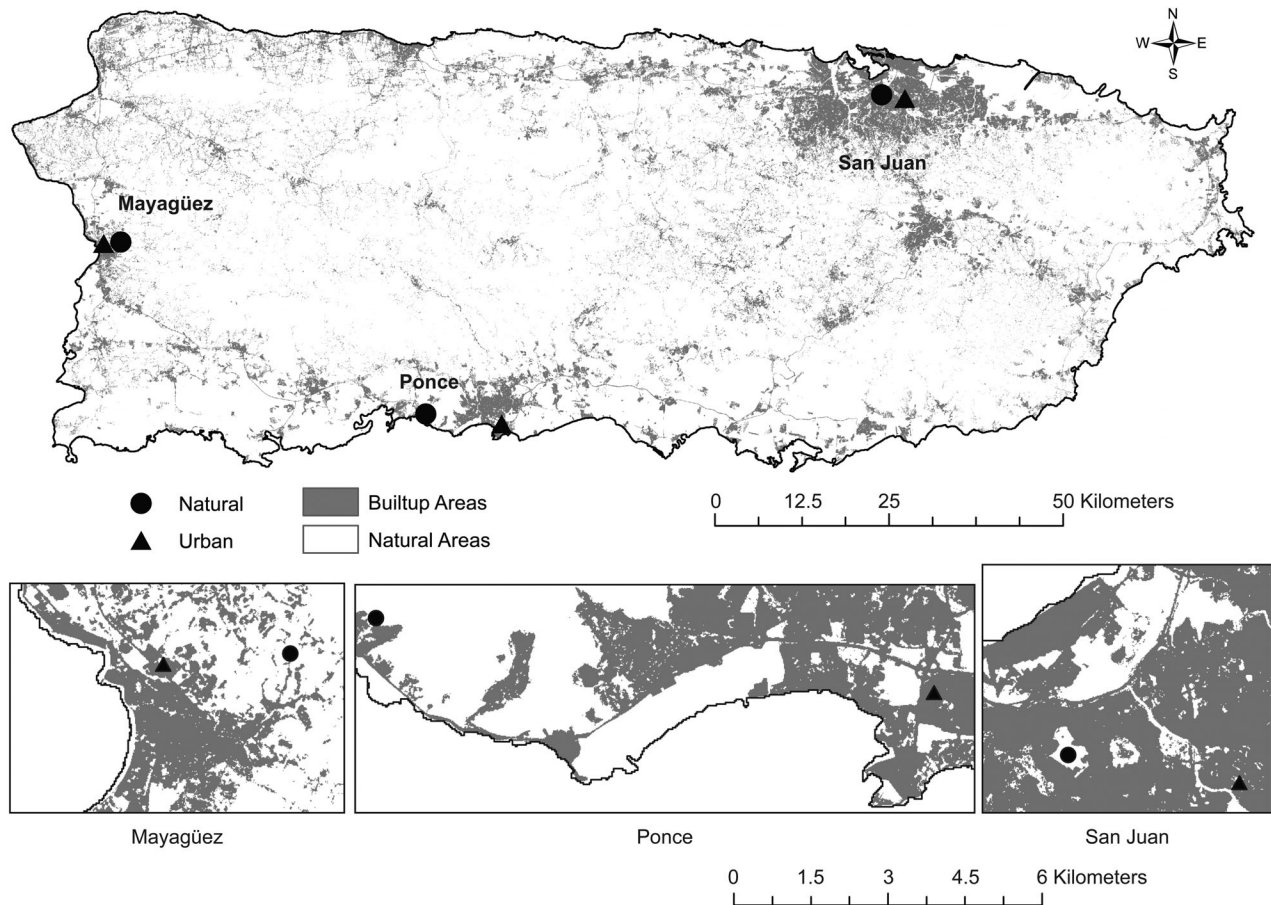


Figure 1. Map of Puerto Rico showing paired study sites in Mayagüez, Ponce, and San Juan as well as extent of built-up areas (gray) as quantified in Gould et al. (2008).

Methods

Between June 12 and June 30, 2012 we sampled *A. cristatellus* from paired natural and urban sites in three of the largest Puerto Rican municipalities: Mayagüez, Ponce, and San Juan (Fig. 1). All three urban sites were high-density residential areas dominated by impervious surfaces, sparse tree cover, and an abundance of anthropogenic substrates such as metal fences and concrete walls (See Appendix S1 for additional details). Our natural sites in San Juan and Mayagüez consisted of subtropical moist forest and receive approximately 1800 mm and 2100 mm of rain per year, respectively. By contrast, our natural site in Ponce consists of tropical dry forest and receives total annual precipitation less than 1000 mm (National Weather Service 2010). All three natural sites were dominated by a diverse suite of native and nonnative plant species. We selected these populations for the study based on the availability of high quality natural forests (which can be scarce around some urban areas of Puerto Rico) as well as logistical concerns, such as the personal safety of investigators.

We sampled adult male *A. cristatellus* (≥ 50 mm SVL) for 1–3 days at each site by noosing. We did not specifically target individuals on artificial or natural substrates in either urban or

natural areas, thus while ascertainment bias (in which we somehow systematically sampled lizards from one habitat or the other with a consistently different mean phenotype) is nearly impossible to test, we believe that our sampling method was minimally biased with respect to the ecological and phenotypic attributes that we measured for this study. We sampled only adult males for several reasons. First, a large proportion of ecomorphological studies in *Anolis* focus only on males, so collecting data from males permits more direct comparison to prior work. Second, this species is highly sexually dimorphic in morphology, behavior, and probably ecology, meaning that data from the adults of different sexes could not be easily pooled. Finally, prior work has shown that morphological differences in anoles tend to be more pronounced in males (Losos 1990b), leading us to suspect that males might provide us with the best prospect for detecting ecomorphological adaptation to urban areas.

Immediately following the capture of each individual, we measured its body (cloacal) temperature using a custom type K thermocouple and probe connected to a digital handheld thermometer (Omega HH12B). We also measured several habitat variables: perch height and diameter, substrate type, perch

temperature, ambient temperature, and ambient relative humidity (Extech Hygro-thermometer + Infrared Thermometer RH101). We recorded the time and location of capture using a handheld GPS (Garmin eTrex 20) then transported all captured lizards to a common field lab in each municipality. In the field lab, we immobilized each animal using aerial isoflurane to obtain high-resolution digital *x*-ray images (custom designed Kodex portable digital *x*-ray system) and digital toepad scans using a standard flatbed scanner (2100 dpi, Epson V300). We also measured the mass, total size (snout-vent length, SVL), and head height of each lizard. Finally, we manually removed a 3 mm portion of the tail tip for subsequent DNA analyses. We placed all tissues in 95% EtOH and transported them to the University of Massachusetts Boston where they were stored at -80°C for whole DNA extraction and analysis. We returned all lizards to their point of capture within 24 hours after marking them semipermanently using nontoxic ink to prevent inadvertent recapture of the same animal on a subsequent survey.

To test for habitat differences between urban and natural sites, we first compared our data for ambient relative humidity and temperature between sites. We fit an ANCOVA model with municipality (Mayagüez, Ponce, San Juan), site type (urban, natural), and their interactions as model factors, and time of day as a covariate (to account for temperature changes during the day). We also quantified impervious surface and canopy cover using GIS (ArcMAP 10.0, ESRI 2011) and NLCD layers at $30 \times 30 \text{ m}^2$ grid cell resolution (NLCD 2001 v. 1.0, Homer et al. 2007), set to a common datum and projection (NAD 1983; State Plane Puerto Rico FIPS 5201). To assess the validity of the impervious surface data, we compared our sampled areas to the “built-up surfaces” identified by Gould et al. (2008), which is a fine-scale (15 m^2) intensive assessment of urbanization in Puerto Rico that utilized multiple data sources to identify urban, developed, and nonvegetated areas that result from human activities.

To compare habitat use between urban and natural populations, we compared perch temperature, height, diameter, and type (natural vs. man-made) between sites. We compared perch temperatures between contexts using an ANCOVA with ambient temperature and municipality as covariates. We compared perch heights among contexts using an ANCOVA with municipality as a covariate; and we compared perch types among sites using a contingency test. Because diameter cannot be sensibly calculated for completely flat perches such as walls, we binned perch diameters into 17 categories from 0–8 cm on a 0.5 cm interval, in which perch diameters greater than 8 cm are assumed to be functionally equivalent for small lizards (Cartmill 1985; following Irschick and Losos 1999). We performed a two sample Kolmogorov–Smirnov test (KS-test; a nonparametric method to compare means) for perch diameters in pooled urban and natural sites. We excluded lizards captured on the ground ($n = 4$) from habitat use analyses

as males of this species typically utilize elevated perches in a sit-and-wait foraging strategy (Losos 2009), and thus we assumed that any lizard captured on the ground was most likely disturbed by the investigators or in transit to another substrate.

We processed all digital *x*-rays using the software tpsDIG2 (Rohlf 2013) to mark bone endpoints. We then exported landmarks from tpsDIG2 as plain text and processed these files using custom scripts in R (R Development Core Team 2015). From each *x*-ray image we obtained linear measures of the following bones or morphological structures in the fore- and hindlimbs of each animal: the 3rd metacarpal, ulna, and radius (forelimb); and the 4th metatarsal, 4th phalanx, femur, and tibia (hindlimb). We measured 3rd and 4th digits of the fore- and hindfeet, respectively, because these normally correspond with the longest digit of each foot. To get total fore- and hindlimb lengths, we added the lengths of each bone. For all animals with fully intact left and right fore- or hindlimbs, we averaged the right and left sides of the animal for bone lengths. We manually counted the number of subdigital lamellae (expanded subdigital scales used for clinging) on the 3rd digit on both forelimbs and the 4th digit on both hindlimbs from high resolution digital scans. To ensure repeatability, we counted subdigital lamellae for each individual three times, each time randomizing the order of the individuals in the dataset. After three counts we chose the two counts that agreed (if no two counts agreed exactly, then all three measurements were averaged). Right and left lamellae counts were averaged for front and rear digits separately.

We compared all morphological traits among sites using ANCOVAs with municipality and context as factors. Because some of the attributes we are studying in this project are strongly correlated with overall body size, we also included SVL as a covariate in this analysis. We analyzed body temperature differences between sites in a similar way, but used ambient temperature as a covariate. We natural logarithm transformed both linear measurements and lamellae numbers. We performed all statistical analyses using R 3.2.2 (R Development Core Team 2015).

DNA ANALYSES

We collected DNA data in this study to verify that none of our paired urban and natural populations had been sampled across some heretofore unknown deep genetic break or some previously unidentified barrier to gene flow unrelated to contemporary urbanization; not to assess contemporary gene flow between populations (which would require multilocus genetic data and might be the subject of future research). Consequently, we used polymerase chain reaction (PCR) to amplify the NADH dehydrogenase subunit 2 region of the mitochondrial genome (*ND2*; Macey et al. 1997; primers and conditions in Revell et al. 2007) using an Eppendorf Mastercycler Pro. We visualized PCR products using gel electrophoresis, and then purified and sequenced

PCR products on an automated sequencer (ABI 3730XL) at Massachusetts General Hospital DNA Core Facility, Cambridge, MA. We assembled contigs and manually verified ambiguous base calls using SEQUENCHER 5.0 (Gene Codes Corporation 2011). We then aligned sequences using the CLUSTALW 2.1 algorithm (Larkin et al. 2007) implemented in MESQUITE 2.75 (Maddison and Maddison 2011) using reference sequences. We have deposited all unique *ND2* sequences obtained in this study in GenBank (KX173468 – KX173775).

We estimated a single, Maximum Likelihood (ML), mtDNA gene tree for all of our samples, as well as for 508 additional *ND2* samples obtained from GenBank (accessions in Appendix S2) and from Strickland et al. (in review). We also included samples from *Anolis monensis* and *A. cooki* to root the estimated tree using the outgroup method. To estimate the tree, we used the software RAXML (Stamatakis 2014) executed in the R environment using the package phyloch (Heibl 2008). We used the general time reversible (GTR) nucleotide substitution model with Γ -distributed rate heterogeneity among sites (Tavaré 1986; Yang 1994). We estimated the tree using all samples, but then we manually removed all tips from GenBank samples containing redundant information about phylogeography (for instance, closely related haplotypes from the same municipality). We also pruned the outgroup sequences (*A. monensis* and *A. cooki*) from the tree. This left us with a gene tree consisting of 433 tips. To visualize the distribution of *ND2* haplotypes across municipalities and between urban and natural areas, we plotted the gene tree using the R packages ape (Paradis et al. 2004) and phytools (Revell 2012), labeling clades by municipality and coloring tips by site type: urban or natural.

In addition, we estimated Φ_{ST} (an analogous measure to F_{ST} but for mitochondrial genetic sequence data; Excoffier et al. 1992) between all populations measured in this study. We used the gene tree to identify a small number of instances in which samples appeared to represent individuals translocated between regions. We pruned these individuals from the dataset and estimated population pairwise Φ_{ST} using ARLEQUIN 3.5.1.3 (Excoffier and Lischer 2010), and we determined the significance of Φ_{ST} values using the maximum number of permutations in ARLEQUIN 3.5.

COMMON GARDEN

To determine if phenotypes observed in natural and urban populations have a genetic basis, we conducted a common-garden study from August 2013–May 2015 (IACUC Protocol #2012001). We collected 100 adult *A. cristatellus*: 25 males and 25 females from each of two populations (urban and natural). Both parental populations were from the municipality of Mayagüez and had trait differences similar to those observed in the wild populations we sampled (Appendix S3). The urban population was the same population sampled above, whereas the natural population (for

logistical reasons) was a mature secondary forest 2.75 km from the previously sampled natural forest in Mayagüez. We transported adults from Puerto Rico to the animal care facility at the University of Massachusetts Boston. We paired each female with a single male from the same population and collected eggs twice weekly from August 2013 to April 2014.

We removed eggs from cages within 4 days of being laid and placed them in hydrated vermiculite in an incubator at 28° C until hatching. Although we do not know the incubation conditions of *A. cristatellus* in the wild for urban and natural sites in our study region, these conditions are within the range for nest sites used by other Caribbean lizard species (e.g., Du et al. 2010; Robbins and Warner 2010) and are similar to conditions used in other anole rearing studies (e.g., Warner et al. 2012; Stuart et al. 2014). Conditions were constant for all incubated eggs and small variations in thermal and hydric incubation environment are expected to have no effect on size-independent morphological traits (Warner et al. 2012).

Immediately upon hatching, we transported hatchling lizards to individual cages in the animal-care facility where they were reared in identical conditions (Stuart et al. 2014). The animal-care facility is a climate-controlled facility with average temperatures during the experiment ranging from 19.9° C (at night; SD = 2.5) to 27.6° C (during the day; SD = 1.7). Average humidity of the room is approximately 45%, but with twice daily misting individual cage humidity is maintained at approximately 80%. Full spectrum UV light (ReptiSun 10.0 UVB, ZooMed Inc.) was provided directly over all cages on a 12-hour daylight cycle, similar to what is experienced in the wild. Both parents and offspring were cared for following methods in Sanger et al. (2008) and Stuart et al. (2014). All cages contained a single plant (*Callisia repens*), a 3/4 inch diameter wood dowel for perching, and ZooMed reptile carpet cage line. We fed lizards live crickets dusted in calcium and vitamins twice a week, every 3–4 days. We regularly rearranged cages to avoid localized differences in temperature and humidity in the animal care room. At approximately one year of age, we measured limb lengths and toepad lamellae number in the same manner as described previously for field-caught adult lizards. We analyzed log-transformed trait values (lamellae numbers and limb lengths) using ANCOVAs with context, sex, and log-transformed body size.

Results

Habitat in urban sites differed from natural ones in several different measured attributes (Fig. 2). Ambient temperature differed significantly by context, with urban areas being warmer on average compared to natural areas (ANCOVA; $+0.87^{\circ}\text{C} \pm 0.28^{\circ}\text{C}$, $t = 3.11$, $P = 0.002$), although the urban San Juan site was cooler than the sampled natural site ($-0.29^{\circ}\text{C} \pm 0.41^{\circ}\text{C}$, $t = -4.48$,

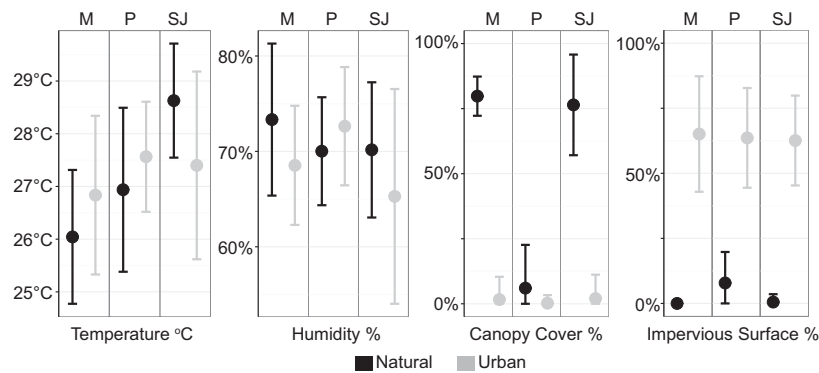


Figure 2. Mean and standard deviation for habitat characteristics (temperature, humidity, canopy cover, impervious surface cover) of the three paired study sites sampled (Mayagüez, M.; Ponce, P.; and San Juan, S.J.). Temperature and humidity are standardized by the time of day the sample was taken. Canopy and impervious surface cover are measured as the percentage of 900 m² grid cells from GIS data.

$P < 0.001$). Relative humidity differed by context but this effect was also mediated by municipality. Humidity was lower in urban sites for mesic Mayagüez and San Juan, but slightly higher in xeric Ponce. In general, urban sites were less humid than the nearby natural areas (ANCOVA; $-5.70\% \pm 1.45\%$, $t = -3.92$, $P = 0.0001$).

GIS analyses confirmed that urban habitats were dominated by large amounts of impervious surfaces and sparse canopy cover (per 900 m²) while natural areas were the opposite (Fig. 2). Ponce was an exception to this pattern, with GIS analyses showing areas of low canopy cover and some impervious surface categorized in the natural area. Typical of dry forests in the region, this site had a less dense canopy and spanned a dry riverbed, resulting in large natural areas of sparse tree cover. Comparison of the impervious surface data to the built-up surfaces identified by Gould et al. (2008) confirms that there was no anthropogenic land cover within our forested study sites in Mayagüez and San Juan, although a portion (18%) of the natural site in Ponce was categorized as anthropogenic cover.

HABITAT USE AND MORPHOLOGY

We analyzed habitat use at time of capture and morphological data from 319 adult male lizards from three paired urban and natural sites (mean 53.2 individuals per site, range 44–55). Habitat use differed between urban and natural contexts for perch type, perch temperature, and perch diameter. Lizards perching on artificial surfaces comprised 46% of urban captures whereas lizards in natural populations used natural substrates exclusively ($\chi^2 = 89.71$, d.f. = 1, $P < 0.001$; Fig. 3A). Lizards captured in urban areas were also found on warmer perches (ANCOVA; $+1.27^\circ\text{C} \pm 0.20^\circ\text{C}$, $t = 6.53$, $P < 0.001$). We found no difference in perch height between contexts, but perch diameter differed highly significantly between urban and natural sites (K–S test;

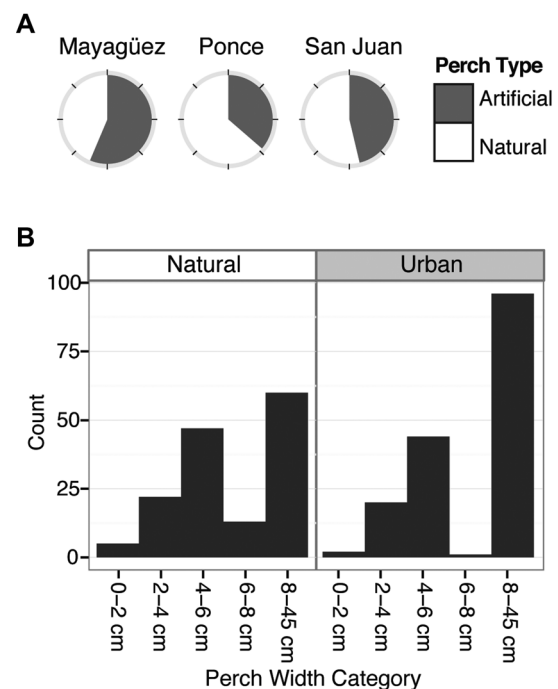


Figure 3. Habitat use differences between natural and urban populations: (A) In urban habitats, lizards were encountered on manmade surfaces at high frequencies. (B) Lizards were more frequently encountered on wider perches in urban habitats compared to natural habitats.

$D = 0.208$, $P = 0.002$) with urban populations utilizing wider perches on average than their forest counterparts (Fig. 3B).

Of the eight morphological and physiological characters that we measured in this study, six differed between urban and natural sites. Hindlimb and forelimb lengths were both longer in urban areas after accounting for body size and municipality (ANCOVA; hindlimb: $+2.16\% \pm 0.59\%$, $t = 3.66$, $P < 0.001$; forelimb: $+2.00\% \pm 0.58\%$, $t = 3.42$, $P < 0.001$; Fig. 4A). Head height

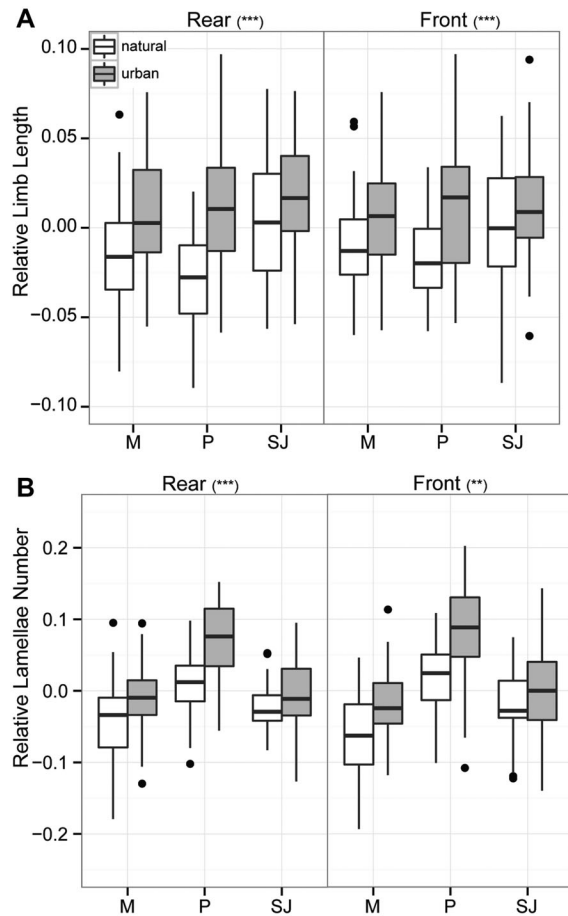


Figure 4. Urban and natural populations differed in key morphological variables: (A) Subdigital lamellae number and (B) limb length for the three municipalities sampled (Mayagüez, M.; Ponce, P.; and San Juan, S.J.). Significance levels are indicated next to each variable for urban versus natural groups: $***P < 0.001$, $**P < 0.01$. Relative trait values shown here are the residual values of log-transformed variables after regression against log-transformed size (SVL). Because the vertical axis is on the log scale, differences are proportional changes (i.e., a difference of 0.01 is equivalent to 1% change).

was different in urban areas but was mediated by the municipality. Specifically, lizards in Mayagüez and Ponce had smaller head heights in urban areas (ANCOVA; $-0.38 \text{ mm} \pm 0.09 \text{ mm}$, $t = -4.48$, $P < 0.001$), whereas lizards in urban San Juan had larger head heights (ANCOVA; $0.74 \text{ mm} \pm 0.12 \text{ mm}$, $t = 6.04$, $P < 0.001$). Neither snout-vent length nor mass consistently differed between urban and natural habitats. Subdigital lamellae number was greater in urban habitats for both hindlimbs and forelimbs (ANCOVA; hindlimb: $+3.51\% \pm 0.90\%$, $t = 3.89$, $P < 0.001$; forelimb: $+3.18\% \pm 1.04\%$ lamellae, $t = 3.07$, $P = 0.001$; Fig. 4B). Lastly, body temperature was higher for lizards captured in urban areas after accounting for the effect of

ambient air temperature (ANCOVA; $+0.54 \pm 0.12^\circ\text{C}$, $t = 4.63$, $P < 0.001$).

DNA ANALYSES AND ESTIMATES OF GENE FLOW

We obtained a total of 978 base pairs (bp) (partial cds) of mtDNA sequence data from each of 308 individual *A. cristatellus* from six populations, with an average of 51.3 individuals per population (range 42–55 individuals). While we found significant differentiation among municipalities, pairwise comparisons (Φ_{ST}) between paired urban and natural sites revealed little differentiation in paired sites within municipalities (Table 1). In addition, the gene tree for all haplotypes shows that in two out of the three municipalities, urban and natural haplotypes are completely interdigitated on the tree. In the third municipality (Ponce) there is a single monophyletic mitochondrial clade in our natural site nested within an urban clade, resulting in a significant value of Φ_{ST} between urban and natural sites in this municipality. However, even in this case the genetic divergence between urban and natural areas is low. In San Juan, though *ND2* haplotypes come from two divergent mitochondrial clades (Appendix S2) there is no tendency for urban or natural samples to be represented in one clade or the other. Finally, we found evidence of translocation of individuals between municipalities. For instance, we found at least one individual in our Mayagüez urban site with a mitochondrial *ND2* haplotype that is very clearly affiliated with GenBank sequences from the southeastern municipality of Cabo Rojo (Appendix S2), an area that we did not sample for this study and have not studied in the past (thus ruling out the possibility of contamination).

COMMON-GARDEN ANALYSIS

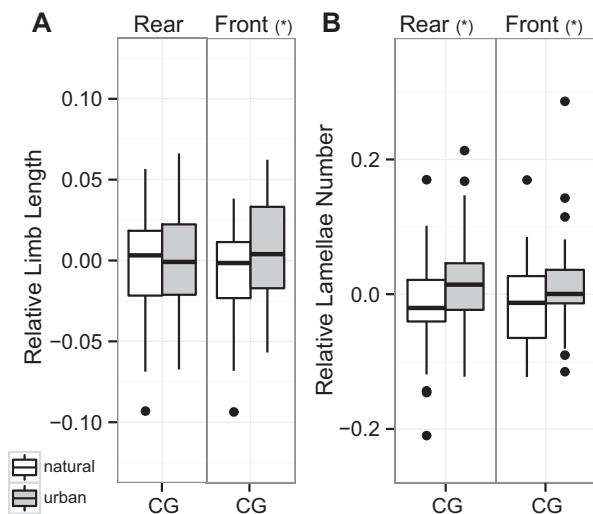
We reared a total of 117 offspring from 41 of 50 wild-caught male–female pairs: 20 pairs from the urban population yielded a total of 64 offspring (mean 3.2 offspring per female); and 21 pairs from the natural population yielded 53 offspring (mean 2.5 offspring per female). Differences between urban and natural wild populations were maintained in the captive-reared offspring. Specifically, offspring from urban populations had more lamellae and longer limbs for front and rear limbs (Fig. 5). Differences between offspring groups were statistically significant for lamellae number (ANCOVA; forelimb: $+2.96\% \pm 1.23\%$, $t = 2.40$, $P = 0.018$; hindlimb: $+3.08\% \pm 1.25\%$, $P = 0.015$) and forelimb length (ANCOVA; $+1.36\% \pm 0.56\%$, $t = 2.43$, $P = 0.017$), and nonsignificant for hindlimb length (ANCOVA; $+0.67\% \pm 0.59\%$, $t = 1.14$, $P = 0.258$).

Discussion

In spite of the considerable evolutionary and ecological interest in Caribbean *Anolis* species and intense urbanization pressure

Table 1. Pairwise Φ_{ST} between sites for the mitochondrial locus *ND2*.

	Mayagüez natural	Mayagüez urban	San Juan natural	San Juan urban	Ponce natural
Mayagüez urban	0.0059				
San Juan natural	0.84*	0.84*			
San Juan urban	0.82*	0.82*	0.0002		
Ponce natural	0.95*	0.95*	0.89*	0.88*	
Ponce urban	0.96*	0.96*	0.90*	0.90*	0.53*

* $P < 0.001$.Note that Φ_{ST} is low between sites, within regions except for Ponce, and high between regions.**Figure 5.** Captive-reared offspring from wild-caught lizards from an urban and natural population in Mayagüez raised in common-garden conditions. Offspring showed the same trend as wild-caught populations of more subdigital lamellae and longer limbs in urban individuals. Significance levels are indicated next to each variable for urban versus natural groups: * $P < 0.05$. Relative trait values shown here are the residual values of log-transformed variables after regression against sex and log-transformed size (SVL). As in Figure 4, as the vertical axis is on a log scale, differences reflect proportional change.

present on Caribbean islands, the ecology, and adaptation of anoles to urbanization has been little studied (but see Rodriguez-Schettino 1999; Germano et al. 2003; Mallery et al. 2003; Ruiz-Jaen and Aide 2006; Ackley et al. 2009; Marnocha et al. 2011). In this project, we conducted the first study of the urban ecology and evolution of a widespread and widely studied Caribbean anole: *Anolis cristatellus*, the Puerto Rican crested anole. Our primary goals in this study were, firstly, to quantify the urban habitat use of this species; and, secondly, to investigate the possibility that populations of *A. cristatellus* in urban areas of the island may be evolutionarily adapting to the intensely modified habitat resulting from urbanization.

URBAN HABITAT USE IN *A. cristatellus*

Urban habitats differed from natural habitats as expected. In general, urban sites sampled were warmer, drier, and had less canopy cover and more impervious surfaces. Despite nonzero values of anthropogenic surface cover for the natural site in Ponce, we did not observe any man-made groundcover at this site. Aberrantly large cell values for impervious surface cover in forested areas are not necessarily indicative of manmade land cover but rather a known source of error in remote sensing data, which may inaccurately categorize rock or similar bare surfaces, particularly at the rural-urban interface (Elvidge et al. 2007; Nowak and Greenfield 2010; Lu et al. 2011). We believe this result to be an artifact of the resolution of the remote sensing data as this site abuts urban development on two sides.

Given the unique land-use history of Puerto Rico and the extensive historical deforestation, the possibility exists that presently natural areas were formerly urban. This calls into question the directionality of phenotypic shifts that we observed in this study. However, the majority of reforestation in Puerto Rico in the past century has been of formerly agricultural lands and not of urban areas. In two of our three sites, Mayagüez and Ponce, it is extremely unlikely that they were ever “urban,” although evidence exists of prior agricultural land use of our Mayagüez study area (unmanaged relict shade coffee and cacao plants still persist in the area). In our third natural locality, Bosque San Patricio in San Juan, our sampling area was in regenerated forest in the site of a former housing complex in which the built structures were removed in the 1970s. Consequently, it is possible that differences we uncovered in this study between urban and natural sites in San Juan actually reflect readaptation to natural habitats from an urban-adapted lizard that now finds itself in a forested milieu, rather than the converse. However, even in this case the natural site is immediately adjacent to a forested mogote (karst “hay-stack hill”) that, so far as we know, has never been completely deforested or built upon. (In aerial imagery from 1930, a time of peak deforestation in Puerto Rico, the mogote clearly appears to be fully covered by forest). Even so, phenotypic responses to ecological change can occur extremely rapidly in anoles (e.g., Losos et al. 2004, 2006) and it is possible that the trait shifts we observed have occurred in

both directions (adaptation and readaptation) multiple times over the course of Puerto Rico's modern history.

We found that *A. cristatellus*, which is nearly ubiquitous on the island, is highly abundant in urban areas and that these lizards commonly use artificial substrates as perches. Across all sampled municipalities in this study, the fraction of lizards captured on perches of human construction (walls, fences, posts, etc.) approached 50% in urban areas. In other more intensely urbanized areas where vegetation is very scarce, lizards are also common (authors' personal obs.) and likely use artificial substrates almost exclusively.

We found not only that perch usage differed between habitat types, but that the physical attributes of the perches themselves differed measurably as well. Specifically, perches in urban areas are broader (on average) than perches in natural areas. Other studies have similarly found that anoles in disturbed habitats utilize broad perches at high frequencies (Irschick et al. 2005; Marnocha et al. 2011). In our study, higher average perch breadth in urban areas is largely due to the use of flat perches (especially walls and the sides of buildings, which are used frequently by urban *A. cristatellus*). In addition, we found that natural perches in urban areas also tend to be broader, likely because large ornamental trees, such as junipers, cypress, *Ficus sp.*, and flowering trees (e.g., flamboyán, *Delonix regia*), dominate urban vegetation but are much rarer in natural sites.

We also found that urban and natural areas differed in temperature, with urban sites being hotter on average than natural sites. Although we did not sample urban and natural sites on the same days, we sampled temperature across both urban and natural areas in this study over 15 days in noncontiguous blocks. Thus, differences in temperature likely reflect actual patterns and not random differences between sites on different days. This is consistent with predictions based on the urban heat island effect that urban areas are often warmer on average than surrounding natural habitats (Arnfield 2003). Furthermore, controlling for ambient conditions, body temperatures of anoles in urban areas were also higher than those of their counterparts in natural forests. This result is intriguing because of the importance of temperature to growth, reproduction, thermoregulation, and performance in ectotherms (Huey and Kingsolver 1993; Angilletta 2009). Additionally, lizards that experience higher ambient temperatures tend to have higher optimal temperatures for sprinting and these differences do not appear to be attributable to acclimatization alone (Huey and Kingsolver 1993; McMillan et al. 2011). Because we did not measure operative temperature (the passive body temperature of a lizard model in the absence of thermoregulation) these data should be considered preliminary with respect to lizard physiology; however, we nonetheless propose that this is an observation that should be investigated by further research.

MORPHOLOGICAL DIFFERENCES BETWEEN URBAN AND NATURAL AREAS

We found consistent, highly significant differences in phenotype between urban and natural populations of *A. cristatellus* in the municipalities of Mayagüez, Ponce, and San Juan. Specifically, we found that lizards in urban areas had slightly longer limbs and more subdigital lamellae than their counterparts in natural forests. Our investigations did not reveal any difference in overall body size (SVL) between urban and natural sites; nor did we find any difference in size-controlled mass. Some differences in head-depth were revealed between sites; however the direction of this difference was inconsistent among the three municipalities of this study.

Head shape in anoles tends to correlate with habitat use such that species that use broad perches have greater head depths and species that use narrow surfaces have flatter head shapes (Losos 2009). Our results do not support this pattern between urban and natural populations of *A. cristatellus*: ANOVA indicated that head depth was not associated with perch diameter. Head depth could be under selection in adult male lizards because it is correlated with bite force, which may be important for consuming different food sources or fighting other males or predators (Herrel et al. 2001; Verwaijen et al. 2002; Losos 2009). Previous studies have demonstrated the importance of lizard head depth and bite force for obtaining quality territories and winning male–male combat (Hews 1990; Perry et al. 2004). Thus, differences in male head depth could result from local differences in food availability or changes in male density in urban habitats, although we did not measure these variables and so cannot test this hypothesis at the present time.

The changes in limb length and lamellae number between urban and natural areas in this study are also intriguing. Extensive comparative and functional ecomorphological studies in anoles have shown that longer limb lengths in anoles increase locomotory performance, particularly on broader substrates (Losos 1990a,b; Larson and Losos 1996; Macrini and Irschick 1998; Calsbeek and Irschick 2007), and that individuals with longer limbs tend to use broader substrates (Losos 1994). Indeed, our data suggest that urban *A. cristatellus* have longer limbs and more often use broader substrates as perches than do their conspecifics in natural areas. The openness of urban habitats (few and sparsely distributed perches) may also be relevant. In general, urban areas are more open, with significantly lower canopy cover (urban mean canopy cover 1.15%; natural 64.67%) and greater distances to nearest perches. In other urban and natural habitats that we have sampled in Puerto Rico, distances to nearest perches in urban areas have been as great as 18 m whereas the greatest distances to nearest perches in natural areas was invariably less than 2 m (Winchell unpublished data). Longer limbs enable faster sprint speeds in anoles (Losos 1990a; Vanhooydonck et al. 2006), which would

enable quicker transitions between perches across open habitats. Consequently, we suspect that longer fore- and hindlimbs improve locomotory performance both on perches and on the ground in urban areas, a similar conclusion to that reached by Marnocha et al. (2011).

It is also possible that selection is working in ways that we have not yet considered. For example, differences in territory size, patchiness of habitat, perch availability, community composition, and density of animals may all impact male–male competition, possibly leading to selection on morphology. For example, Husak et al. (2006) found longer limbed and faster sprinting collared lizards (*Crotaphytus collaris*) had greater mating success, likely because they were better able to defend their territories. However, if male–male competition was elevated in either urban or natural areas in the present study, we would also expect to observe differences in size and head height between natural and urban areas, as both are associated with male dominance, yet we did not. Nonetheless, this is an intriguing possibility that should be investigated by additional research.

We also discovered that urban anoles had more lamellae than conspecifics in nearby natural sites. Among and within other anole species, lamellae number is closely linked to clinging performance. In particular, lizards with more lamellae are better able to cling to smooth surfaces (Zani 2000). Consequently, we might reasonably expect that lizards in urban areas with more lamellae should be better equipped to adhere to hard and smooth urban substrates where claws are ineffective. Man-made substrates (such as painted walls, glass windows, and metal fence posts) commonly used as perches by *A. cristatellus* in urban areas are generally smoother than natural perches such as tree bark. This is corroborated by anecdotal observations that lizards using smooth walls often fall from their perches, something that is seldom if ever observed in natural forest. Such falls have severe energetic costs to the lizard and pose serious threats of injury and death (Elstrott and Irschick 2004). We would expect that use of smooth artificial substrates would impose selection on the lamellae, which are used for adhesion in anoles and other lizards, especially since the claws (also used for vertical climbing on rough surfaces) would be rendered useless on such substrates. Irschick et al. (2005) came to similar conclusions regarding larger toepads in a population of *A. carolinensis* utilizing smoother natural surfaces (palmetto leaves, *Aspidistra elatior*) found in high frequency in a human-modified environment. We initially did not measure perch smoothness in this study; however in Appendix S4 we report smoothness data from the material science literature affirming that the artificial substrates we observed in our study are indeed smoother, in quantitative terms, than natural substrates found in the environment. In addition, we report preliminary results from macro-photography analysis conducted with the software ImageJ (V1.48, Rasband 2014) for samples col-

lected from natural and synthetic perches found in the research sites of this project (Appendix S4, Fig. S4). These results corroborate the literature numbers we report and support our contention that artificial perches are, as a generality, smoother than natural substrates.

It is plausible that the differences that we report between populations could be influenced by genetics, environment, or both. In related species, lamellae number is thought to be fixed at hatching and does not change through life (Hecht 1952; Collette 1961). Additionally, studies of other closely related species have demonstrated that differences in lamellae number between populations persist in common-garden rearing experiments, suggesting that differences among populations in this trait can be genetically based (Thorpe et al. 2005; Stuart et al. 2014). With regard to plasticity in limb length, prior studies have both found (Losos et al. 2000, 2001; Kolbe and Losos 2005) and failed to find (Thorpe et al. 2005) evidence for significant developmental plasticity in limb length in anoles. Due to high heritability values (Kolbe and Losos 2005; Calsbeek and Bonneaud 2008; Stuart et al. 2014) and evidence of natural selection (Losos et al. 2004; Thorpe et al. 2005) for both lamellae number and limb length, there is at least the potential for the rapid genetically based evolution of these traits in urban populations.

In the present study, we show evidence that the differences between urban and natural populations in at least one municipality (Mayagüez) persist in a common-garden rearing experiment. Our results show a highly consistent difference between urban and natural population in the four traits (fore and rear lamellae number and limb lengths), and are significant for three traits (both lamellae counts, and forelimb length). Unfortunately, because we only reared animals for a single generation, we cannot definitively rule out a maternal effect—although no prior study with anoles has demonstrated a maternal effect for these traits. We believe, thusly, that our results provide fairly strong support that the phenotypic differences we have found between urban and natural populations in the wild may indeed have a genetic basis and are not merely the result of phenotypic plasticity, although this should be the subject of future study.

POPULATION GENETIC ANALYSIS

Our *ND2* mtDNA gene tree showed that no pair of urban-natural populations was reciprocally monophyletic, and, in general, that the divergence between pairs of populations was low (Appendix S2). We also found that Φ_{ST} measured between urban and natural populations tended to be quite low, whereas Φ_{ST} measured between populations in different municipalities was high. We found some evidence for translocation of individuals between populations. For instance, two individuals in the urban area of Ponce possessed haplotypes, which, though distinct from all others in our sample, were closely related to haplotypes from

San Juan and GenBank sequences reportedly obtained from Río Grande and Humacao. In addition, three haplotypes sampled from Mayagüez were closely related to GenBank sequences from Cabo Rojo, Utuado, and Isabela—sites that were not sampled in this study or previously by our lab (thus ruling out the possibility of contamination or sample mislabeling). The effect of translocation on genetic variability within and among populations, as well as gene flow between populations, should be the subject of future, more involved genetic research on this species.

We caution that the relative lack of genetic structure between urban and natural populations in each municipality should not be taken as an indication that contemporary urbanization will not result in genetic fragmentation of species distributed across both habitats. Researchers interested specifically in the effects of urbanization on contemporary gene exchange between populations will need to use many more markers distributed across the entirety of the genome (such as might be provided by next generation sequencing approaches) to characterize the effect of urbanization on contemporary levels of gene flow across the landscape. In the present study, however, we focused only on characterizing the historical genetic context of divergence between urban and natural populations in Puerto Rico to ensure that we did not sample across a deep genetic divide or unknown barrier to gene flow. In that regard, we believe our results are fairly clear. The phenotypic differences that we have uncovered between urban and natural populations in Puerto Rico most likely arose recently or in the context of ongoing gene flow between populations—possibilities that we cannot presently distinguish from our data.

Conclusions

In recent years the lizard genus *Anolis*, particularly in the Caribbean, has become a model system in evolutionary ecology. The Caribbean region is recognized as a “biodiversity hotspot” (Myers et al. 2000), and has been subject to centuries of intense human pressure, including significant urbanization. Nonetheless, relatively few researchers have studied anoles specifically in the context of urbanization in the Caribbean. In the present study, we measured habitat use across urban and natural areas in the nearly ubiquitous Puerto Rican anole, *A. cristatellus*. We found that lizards used man-made substrates extensively in urban areas and that urban substrates are broader than substrates of natural forests. We also collected phenotypic data from urban and natural sites and found that urban lizards have longer limbs and more subdigital lamellae. This phenotypic shift is predicted by ecomorphological considerations if urban lizards use broader, smoother substrates; and our habitat use data show that urban lizards indeed use broader and smoother substrates for perching than do animals in nearby natural sites. Results from a common-garden rearing experiment suggest that these differences are probably

genetically based. Our population genetic findings suggest that observed phenotypic differences likely arose recently or in the presence of high levels of gene flow between populations. This suggests that strong differences in natural selection may exist between urban and natural habitat types.

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DATA ARCHIVING

Data archived: Dryad: 10.5061/dryad.h234n Unique ND2 sequences deposited in GenBank (KX173468 – KX173775)

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

- Supplementary Appendix S1:** Study Sites.
Supplementary Appendix S2: Additional results from genetic analyses.
Supplementary Appendix S3: Parental Trait Values.
Supplementary Appendix S4: Summary of Surface Roughness.
Supplementary Appendix S5: Random Effects Models for Common Garden Result.