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Consistent Differences in Sperm Morphology and Testis Size between Native and Introduced Populations of Three *Anolis* Lizard Species

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Abstract.—**Sperm morphology can be highly variable among individuals and across species, but less is known about its variation among populations. Within the past 20–80 yr, several species of *Anolis* lizards have been introduced to Miami, Florida, USA from different source islands in the Caribbean, thereby permitting comparisons of sperm morphology between native and introduced populations of multiple species. We collected sperm samples from native populations of *Anolis sagrei* (Bahamas), *Anolis distichus* (Dominican Republic), and *Anolis cristatellus* (Puerto Rico) and compared them to samples from introduced populations of each species that are now sympatric in Miami. In each of these three species, lizards from introduced populations had sperm with shorter tails and larger midpieces relative to lizards from native populations. We also measured testis size in *A. distichus* and *A. cristatellus* and found that introduced populations of each species had smaller testes for a given body size relative to their native counterparts. The consistency of these differences across species argues against random genetic drift as an explanation, suggesting instead that sperm morphology and testis size may exhibit predictable phenotypic plasticity or genetic adaptation in response to the process of introduction and/or the shared local environment in Florida. Though these population differences in male reproductive physiology and morphology may be repeatable, their underlying causes require further study.**

Sperm is the most morphologically diverse cell type among animals, showing high variation both within and among species and ranging several orders of magnitude in size (Pitnick et al., 2009). Much of the variation among species can be attributed to differences in the strength of postcopulatory sexual selection because of cryptic female choice and sperm competition (Immler et al., 2008; Tourmente et al., 2009; Higginson et al., 2012). Variation in sperm morphology within species can be influenced by an individual's genes (Simmons and Moore, 2008), social environment (Immler et al., 2010; Johnson et al., 2012), and diet or condition (Merrells et al., 2009; Rahman et al., 2013; Kahrl and Cox, 2015; Kaldun and Otti, 2016). Collectively, these studies indicate that variation in sperm morphology arises from a combination of genetic divergence (most evident at the interspecific level) and phenotypic plasticity (most evident at the individual level within species).

Studies that have compared sperm morphology among populations of a species have found that variation in sperm morphology can be influenced by genetic drift (Stewart et al., 2016) and the strength of selection (Pitnick et al., 2003; Manier and Palumbi, 2008; Elgee, et al. 2010; Laskemoen et al., 2013). Sperm morphology can evolve relatively quickly, resulting in divergence between populations after only a few generations of selection (Landry et al., 2003; Pitnick et al., 2009; Hogner et al., 2013). Though several studies have documented population-level variation in sperm morphology (Pitnick et al., 2003; Hettyey and Roberts, 2005; Lüpold et al., 2011; Stewart et al., 2016), none have explored how the process of introduction into a novel environment may structure this variation. Introduction into a novel environment can drive rapid phenotypic changes via adaptive evolution (Novak, 2007), phenotypic plasticity in response to novel environmental conditions (Davidson et al., 2011), or random divergence because of population bottlenecks (i.e., founder effects and/or genetic drift) (Prentis et al., 2008). One way to discern between these possibilities is to test whether differences in sperm morphology between native and introduced populations are consistent across species, which is predicted in the case of adaptive genetic change or phenotypic plasticity but not in the case of genetic drift or founder effects. Genetic drift or founder effects may cause shifts in sperm morphology between populations, but it is unlikely that drift or founder effects would cause these shifts to be the same direction across several species.

To address this question, we sampled native and introduced populations of three species of anoles (*A. sagrei*, *A. distichus*, and *A. cristatellus*) to test whether sperm morphology and testis size differ consistently between geographically disparate populations in the native range of each species versus introduced populations that are now sympatric in Miami, Florida, USA (Fig. 1). These species are native to different islands in the Caribbean and were separately introduced to Miami within the last 20–80 yr (Lee, 1985; Schwartz and Henderson, 1991; Bartlett and Bartlett, 1999). Sperm morphology and testis size are highly variable both within (Kahrl and Cox, 2015) and among species of lizards (Uller et al., 2010), but the extent of variation among populations is unknown. We predicted that, if either the process of introduction or the local environment in Miami favors similar reproductive phenotypes, then any differences between native and introduced populations within each species would be broadly consistent across species. We also tested for the condition-dependence of sperm morphology in these populations as a signal of phenotypic plasticity.

Materials and Methods

We collected a total of 111 male lizards of *Anolis sagrei* (=*Norops sagrei*) (Duméril and Bibron, 1837), *Anolis cristatellus* (=*Ctenonotus cristatellus*) (Duméril and Bibron, 1837), and *Anolis distichus* (=*Ctenonotus distichus*) (Cope, 1861) from their native range in the Bahamas, Puerto Rico, and the Dominican Republic, respectively, as well as introduced populations of all three species from Miami, Florida, USA (Fig. 1). Collections occurred during the middle of their breeding seasons (between 15 May and 30 June) in 2013–2015 (collection times and localities in Table 1). We captured individual lizards using nooses or by

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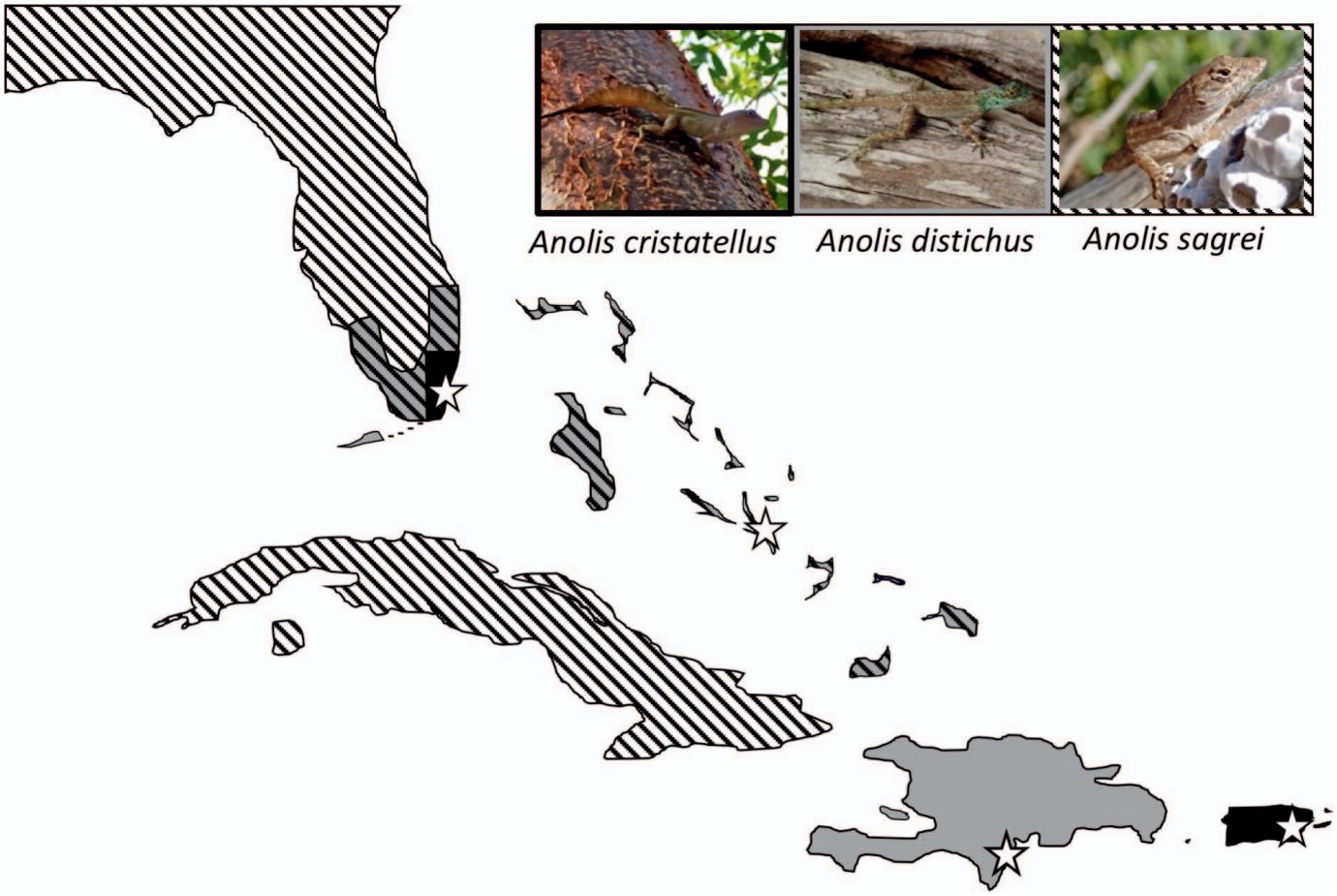

FIG. 1. Species range maps and the locations of our four sampling sites of *Anolis* lizards. See Table 1 for detailed locality coordinates.

hand and measured their snout–vent length (SVL, to the nearest mm) and mass (to the nearest 0.01 g). We sampled sperm from each male by applying pressure to the abdomen and collecting the ejaculate into a microcapillary tube inserted partially into the cloaca, and then transferred this sample to 500 μL of phosphate-buffered saline (PBS) with 4% paraformaldehyde (PFA) to fix the cells. We centrifuged the sample and resuspended it in water, then dried the cells on a microscope slide and stained them with SpermBlue™ (Microptic SL, Barcelona, Spain). We imaged the cells with an Olympus Magnafire Camera (Olympus America; Melville, New York, USA) at × 100 magnification using differential interference contrast microscopy. We then measured the length of the sperm head, midpiece, and tail of 15 cells per male using ImageJ (Schneider et al., 2012) and calculated the mean length of each part of the cell for each individual. We measured the length and width (to the nearest 0.1 mm) of the right testis from all individuals of *A. cristatellus* and *A. distichus* by dissection after euthanasia but did not collect these data for *A. sagrei*. We calculated the volume of the testis using the equation for an ellipsoid, $(4/3\pi a^2 b)$, where a is the radius of the width of the testis and b is the radius of its length.

We tested for differences in sperm morphology using a generalized linear model (GLM) with the mean sperm phenotype for each individual as the dependent variable and effects of species, population (native or introduced), and their interaction as independent variables. We conducted post hoc comparisons using Tukey's honest significant difference (HSD) test to determine which populations were significantly different. We then pooled data across species and populations and calculated restricted maximum likelihood (REML) variance component estimates to partition the total variance in sperm morphology into species-level, population-level, and residual (individual-level) variation.

Previous work indicated correlations between body condition and sperm morphology within a native population of *A. sagrei* (Kahrl and Cox, 2015). To test for condition-dependence of sperm morphology within native and introduced populations of each species in this study, we calculated body condition for each male by using the residuals from a regression of $\log_{10}$ mass on $\log_{10}$ SVL, conducted separately for each species. Log_{10} transformation was used to remove dimensionality of mass and SVL and linearize the regression between these two variables. We then tested for correlations between these residual measures of condition and the mean measures of sperm morphology for each male using ordinary least-squares regression for each species. We also tested for differences in condition between native and introduced populations of each species using an analysis of covariance (ANCOVA) with $\log_{10}$ mass as the dependent variable, population (native or introduced) as the independent variable, and $\log_{10}$ SVL as a covariate, after confirming homogeneity of slopes. We then tested for a correlation between body condition and residual testis size (calculated from a regression of $\log_{10}$ testis volume on $\log_{10}$ SVL) for each species. We also tested for differences in testis size between native and introduced populations of *A. cristatellus* and *A. distichus* using separate ANCOVAs with SVL as a covariate, after first confirming homogeneity of slopes between popula-

TABLE 1. Samples size, locality information, sperm morphology mean ± SD (min–max), and collection date for six populations of three species of *Anolis* lizards. Population abbreviations: N = Native, I = Introduced, GPS = global positioning system.

Species	Locality	Pop.	*n*	Head length	Midpiece length	Tail length	Date collected	GPS data
A. cristatellus	El Yunque, Puerto Rico	N	19	15.11 ± 0.39 (14.44–15.81)	3.24 ± 0.08 (3.08–3.42)	82.36 ± 1.49 (78.8–84.6)	June 2014	18°20′32.28″N 65°49′33.72″W
A. cristatellus	Miami, Florida	I	18	15.16 ± 0.34 (14.66–15.83)	3.26 ± 0.12 (3.05–3.46)	80.44 ± 1.14 (78.68–82.37)	May 2014	25°42′28.51″N 80°09′27.75″W
A. distichus	Bani, Dominican Republic	N	20	16.37 ± 0.34 (15.75–16.80)	3.17 ± 0.15 (2.91–3.43)	96.76 ± 1.71 (94.69–101.67)	June 2015	18°13′50.80″N 70°20′44.15″W
A. distichus	Miami, Florida	I	15	16.26 ± 0.72 (14.75–17.55)	3.38 ± 0.15 (3.16–3.72)	94.50 ± 2.47 (91.45 –99.26)	May 2014	25°42′28.51″N 80°09′27.75″W
A. sagrei	Great Exuma, The Bahamas	N	20	14.11 ± 0.38 (13.39–14.89)	2.46 ± 0.09 (2.24–2.65)	80.88 ± 2.39 (78.06–86.58)	May 2013	23°30′23.17″N 75°45′57.53″W
A. sagrei	Miami, Florida	I	19	13.50 ± 0.16 (13.32–13.97)	2.62 ± 0.11 (2.44–2.76)	73.24 ± 0.99 (71.18–74.98)	May 2014	25°42′28.51″N 80°09′27.75″W

tions within each species. Finally, we used *t*-tests to test for differences in SVL and mass between native and introduced populations of each species. All statistical analyses were performed using JMP v.9 (SAS Institute Inc., Cary, North Carolina, USA) and evaluated with $\alpha = 0.05$.

RESULTS

Across three *Anolis* species, we found significant overall effects of species and population (native vs. introduced) on sperm morphology (head, midpiece, and tail length) (Table 2). We also found a significant interaction between species and population for each aspect of sperm morphology, indicating that the extent to which native and introduced populations differed in sperm morphology was variable across species, though the direction of change from native to introduced was nearly always consistent across species (Fig. 2, Table 2). Tukey's HSD post hoc test revealed significant differences between populations of *A. sagrei* (Fig. 2D) vs. nonsignificant differences between populations of *A. cristatellus* (Fig. 2B) and *A. distichus* (Fig. 2C). The significant interaction for midpiece length was driven by the combination of significant differences between populations of *A. distichus* (Fig. 2F) and *A. sagrei* (Fig. 2G) vs. nonsignificant differences between populations of *A. cristatellus* (Fig. 2E). Finally, the interaction for tail length was driven by variation in the magnitude of significant differences in tail length between populations of all three species: *A. cristatellus* (Fig. 2H), *A. distichus* (Fig. 2I), and *A. sagrei* (Fig. 2J). In general, introduced populations had sperm with shorter tails and longer midpieces relative to native populations (Fig. 2). When we partitioned the total variation in sperm morphology, we found that the majority of the total phenotypic variance occurred among species (mean = 87% across the three sperm traits) whereas a moderate amount of variation occurred between populations of a species (6.5%) and among individuals within a population (6.5%, Table 3).

Based on ANCOVA with $\log_{10}$ body mass as the dependent variable and $\log_{10}$ SVL as a covariate, we found no differences in body condition between native and introduced populations of *A. cristatellus* (population: $F_{2,36} = 0.01$, $P = 0.934$; SVL: $F_{2,36} = 192.73$, $P < 0.001$) or *A. distichus* (population: $F_{2,25} = 3.39$, $P = 0.078$; SVL: $F_{2,25} = 43.04$, $P = < 0.001$), but the introduced population of *A. sagrei* had higher body condition than the native population ($F_{2,38} = 12.60$, $P = 0.001$; SVL: $F_{2,38} = 229.81$, $P = < 0.001$). We did not find a correlation between body condition (residuals from the regression of $\log_{10}$ mass on $\log_{10}$ SVL) and sperm morphology in any population (all $R^2 < 0.02$, $P > 0.5$) except the native population of *A. sagrei*, which had a weak negative correlation between condition and midpiece size ($R^2 = 0.209$, $t_{20} = -2.18$, $P = 0.043$). We also found no correlation between condition and relative testis size in either *A. cristatellus* ($R^2 < 0.001$, $t_{35} = -0.12$, $P = 0.907$) or *A. distichus* ($R^2 = 0.029$, $t_{33} = -0.86$, $P = 0.401$), the two species for which we measured testis size.

We found significant differences in SVL and body mass between native and introduced populations of *A. sagrei* (SVL: $t_{38} = -3.33$, $P = 0.002$, mass: $t_{38} = -4.85$, $P < 0.001$) and *A. cristatellus* (SVL: $t_{36} = -2.64$, $P = 0.012$, mass: $t_{36} = -2.26$, $P = 0.030$), where individuals from the introduced populations were significantly longer and more massive. By contrast, we found no difference in SVL or mass between the native and introduced populations of *A. distichus* (SVL: $t_{35} = -0.64$, $P = 0.525$; mass: $t_{25} = 0.23$, $P = 0.820$). Individuals from native populations also had

TABLE 2. Results from a generalized linear model testing for effects of species, population (native or introduced), and their interaction on sperm morphology in three species of *Anolis* lizard.

		Head length		Midpiece length		Tail length	
Effect	df	*F*	*P*	*F*	*P*	*F*	*P*
Species	2,110	344.13	<0.0001	470.33	<0.0001	1,078.46	<0.0001
Population	1,110	7.72	0.0065	30.88	<0.0001	140.55	<0.0001
Species * Population	2,110	6.34	0.0025	6.62	0.0020	32.69	<0.0001

larger testes, relative to their SVL, than did individuals from introduced populations in *A. distichus* (population: $F_{2,32} = 25.30$, $P < 0.001$; SVL: $F_{2,32} = 11.14$, $P = 0.002$) and, to a lesser extent, in *A. cristatellus* (population: $F_{2,35} = 3.29$, $P = 0.078$; SVL: $F_{2,35} = 5.31$, $P = 0.027$).

DISCUSSION

We found significant differences in sperm morphology and testis size between native and introduced populations of three species of *Anolis* lizards. Sperm morphology is known to vary within and among individuals (Kahrl and Cox, 2015) and across lizards species (Uller and Olsson, 2008), but this is the first study to demonstrate significant differences in sperm morphology between native and introduced populations. Additionally, we found the direction of change in sperm morphology and testis size from native to introduced populations was consistent across all three species, with introduced populations characterized by larger sperm midpieces, shorter sperm tails, and smaller testes relative to native conspecifics (Fig. 2). The midpiece (the area of the cell containing the mitochondria) and the tail (which influences sperm velocity in other lizards [Blengini et al., 2014]) are likely targets of selection because of these links to function and performance (Lüpold et al., 2009; Firman and Simmons, 2010). These consistent changes in sperm morphology and testis size argue against a role of random factors such as genetic drift and founder effects, instead suggesting that either the process of introduction or the local environment in Miami has consistently favored the same adaptive changes or induced the same plastic responses in each of these three species.

Published measurements of sperm morphology in lizards are scattered across the phylogeny and show that head length of lizard sperm ranges from 5.50–23.5 μm (mean ± SD = 18.75 ± 4.27 μm), midpiece length ranges from 1.82–11.50 μm (4.18 ± 1.80 μm), and tail length ranges from 40.10–85.56 μm (60.17 ± 13.47 μm) (Uller et al., 2010). *Anolis* sperm morphology falls

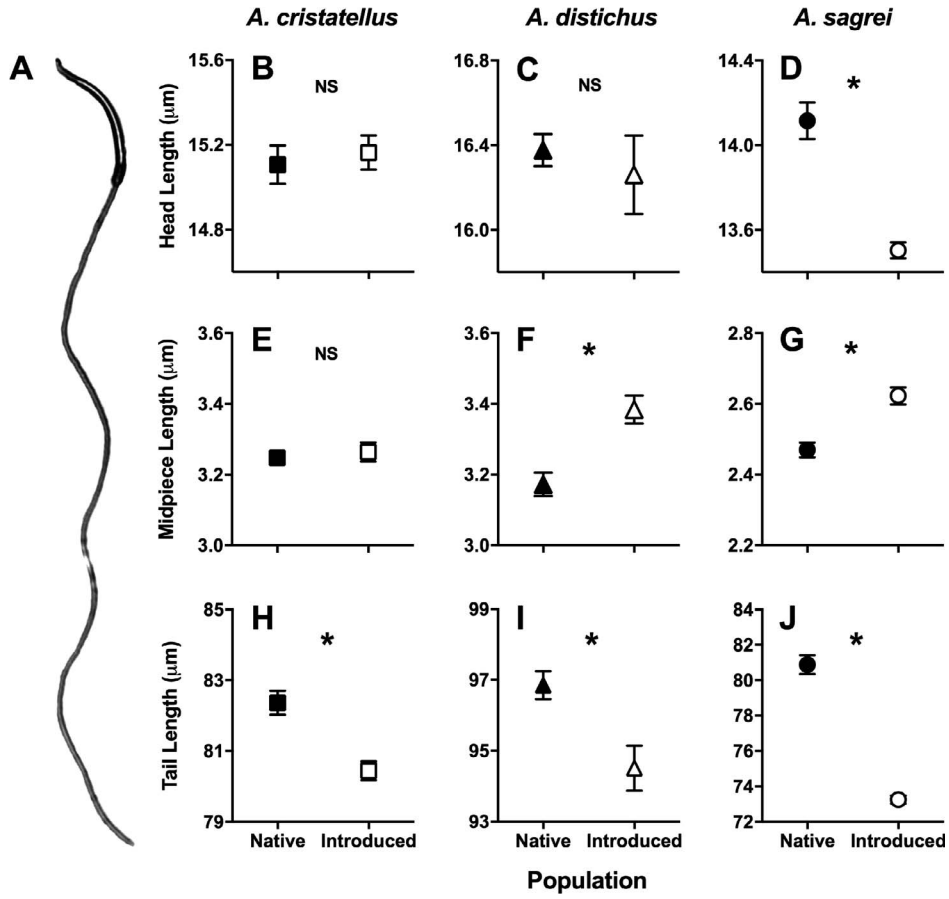

FIG. 2. *Anolis* spermatozoa (A). Population means ± SE calculated from individual mean values (across 15 cells per male) for length of the sperm head, midpiece, and tail in native (black symbols) and introduced (white symbols) populations of three species of *Anolis* lizards. Significant differences between populations ($P < 0.05$) were determined using Tukey's HSD test and are noted with an asterisk.

TABLE 3. Restricted maximum likelihood variance component estimates partitioning the variation in sperm morphology between native and introduced populations of *Anolis* lizard. Estimates for sperm morphology were made with all six populations while testis size estimates (here measured as residual testis size from a regression with body mass) were calculated from only the populations *of A. cristatellus* and *A. distichus*.

Phenotype	Species	Population	Individual
Sperm head length	0.873	0.032	0.094
Sperm midpiece length	0.872	0.056	0.072
Sperm tail length	0.862	0.107	0.030
Testis size	0.112	0.270	0.617

near these interspecific means for the group as a whole, with the exception of sperm tail length, which is longer in anoles than in most other lizard lineages. In particular, *A. distichus* has the longest sperm tail and the longest total sperm length reported for any lizard. Though we know little about the relationships between sperm morphology and function in *Anolis* lizards, sperm velocity increases with sperm tail length in *Tupinambis* lizards (Blengini et al., 2014).

Variation in the local environment can affect the expression of sexually selected traits via both phenotypic plasticity (Griffith et al., 1999; Harris and Moore, 2004; Karubian et al., 2011; Somjee et al., 2015) and genetic adaptation (Boughman, 2001; Hettyey and Roberts, 2005). As an example of the former, native *Anolis sagrei* males vary in sperm count and sperm morphology as a function of their body condition, and this variance can also be induced by dietary manipulation (Kahrl and Cox, 2015), suggesting that population differences in prey availability or local environmental quality could generate intraspecific variation in sperm phenotypes. We found no differences in body condition between native and introduced populations of *A. cristatellus* and *A. distichus*, however, nor did we find significant correlations between sperm morphology and body condition in any population but the native population of *A. sagrei*. This suggests that, although sperm morphology may be condition-dependent in some contexts, in this study it is unlikely plasticity is driving the differences between populations.

Sperm morphology, velocity, and count are under sexual selection in a variety of species (Hettyey and Roberts, 2005; Manier and Palumbi, 2008; Álvarez et al., 2013), and the strength and direction of this selection can vary among populations because of differences in sex ratio (Sasson and Brockmann, 2016), predator abundance (Elgee et al., 2010), or latitude (Pitcher and Stutchbury, 1998; Lüpold et al., 2011). Given that sperm phenotypes often are heritable and evolve rapidly in response to selection in other species (Landry et al., 2003; Pitnick et al., 2009; Hogner et al., 2013), adaptive differences between native and introduced *Anolis* populations could emerge over the relatively short timescales (20–80 generations) since introductions first occurred in Miami (Kolbe et al., 2004, 2007a,b). Although such evolutionary change could also result from random sampling because of population bottlenecks and genetic drift (Stewart et al., 2016), these factors would be unlikely to result in parallel responses (i.e., larger midpieces, shorter tails, smaller testes) across species. Therefore, although we do not know the causes of the observed differences in sperm morphology and testis size between native and introduced populations, our data suggest these differences are more consistent with adaptive genetic change or phenotypic plasticity than with random divergence because of drift.

The three species of *Anolis* in our study are widely distributed across islands in the Greater Antilles and have been introduced multiple times to the Miami area over the past 80 yr (Kolbe et al., 2004, 2007a,b). Genetic analysis has established that the introduced population of *A. sagrei* that we sampled in Miami is likely descended from multiple native source populations in Cuba (Kolbe et al., 2007b), the introduced population of *A. cristatellus* is an admixture from San Juan and Aguas Claras, Puerto Rico, and the introduced population of *A. distichus* is an admixture from the Dominican Republic and the Bahamas (Kolbe et al., 2007a). Though our sampling locations for the native populations of *A. cristatellus* and *A. distichus* are close to the source locations for our introduced populations, the introduced populations are admixed and therefore our native populations should not be viewed as true genetic source populations. Likewise, our native population of *A. sagrei* in the Bahamas is genetically distinct from Cuban populations (the major genetic source for the introduced population that we sampled in Miami), which may explain why population differences in sperm morphology were most pronounced in this species (Fig. 2). Despite this caveat, the consistency in the direction of observed changes in sperm morphology and testis size across these three species suggests that some combination of convergent adaptation or phenotypic plasticity may be driving this population-level variation. The extent to which this convergent adaptation and/or plasticity are because of the process of introduction per se, or simply the local environment in Miami, is currently unknown. Therefore, further studies characterizing differences local in environments, assessing phenotypic plasticity, and quantifying selection on sperm morphology are needed to rigorously test these alternatives.

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