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Michele A. Johnson

Trinity University, mjohnso9@trinity.edu

Maria Veronica Lopez

Trinity University, mlopez6@trinity.edu

Tara K. Whittle

Trinity University, twhittle@trinity.edu

Bonnie K. Kircher

Trinity University, kircherbk@gmail.com

A.K. Dill

Trinity University

See next page for additional authors

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Authors

Michele A. Johnson, Maria Veronica Lopez, Tara K. Whittle, Bonnie K. Kircher, A K. Dill, Divina Varghese, and J. Wade

The evolution of copulation frequency and the mechanisms of reproduction in male *Anolis* lizards

Michele A. JOHNSON^{1*}, Maria Veronica LOPEZ¹, Tara K. WHITTLE¹,
Bonnie K. KIRCHER¹, Alisa K. DILL¹, Divina VARGHESE¹, Juli WADE²

¹ Trinity University, Department of Biology, One Trinity Place, San Antonio, Texas 78212, USA

² Michigan State University, Departments of Psychology and Zoology, East Lansing, Michigan 48824, USA

Abstract The evolution of many morphological structures is associated with the behavioral context of their use, particularly for structures involved in copulation. Yet, few studies have considered evolutionary relationships among the integrated suite of structures associated with male reproduction. In this study, we examined nine species of lizards in the genus *Anolis* to determine whether larger copulatory morphologies and higher potential for copulatory muscle performance evolved in association with higher copulation rates. In 10–12 adult males of each species, we measured the size of the hemipenes and related muscles, the seminiferous tubules in the testes, and the renal sex segments in the kidneys, and we assessed the fiber type composition of the muscles associated with copulation. In a series of phylogenetically-informed analyses, we used field behavioral data to determine whether observed rates of copulation were associated with these morphologies. We found that species with larger hemipenes had larger fibers in the RPM (the retractor penis magnus, a muscle that controls hemipenis movement), and that the evolution of larger hemipenes and RPM fibers is associated with the evolution of higher rates of copulatory behavior. However, the sizes of the seminiferous tubules and renal sex segments, and the muscle fiber composition of the RPM, were not associated with copulation rates. Further, body size was not associated with the size of any of the reproductive structures investigated. The results of this study suggest that peripheral morphologies involved in the transfer of ejaculate may be more evolutionarily labile than internal structures involved in ejaculate production [*Current Zoology* 60 (6): 768–777, 2014].

Keywords *Anolis*, Copulation, Hemipenes, Lizards, Reproduction, Reptiles

The behavioral context in which a morphological structure is used can determine the selective pressures that drive its evolutionary trajectory. This relationship is well known in the context of copulation, in which variation in morphologies that facilitate mating behaviors is often strongly associated with the evolution of mating systems. In particular, species in which males experience strong sexual selection, and/or those that copulate frequently, often evolve enhanced copulatory structures. For example, testis size is associated with mating strategy in taxa as diverse as primates (Harcourt et al., 1995), bats (Pitnick et al., 2006), birds (Birkhead and Møller, 1992), frogs (Byrne et al., 2002), and butterflies (Gage, 1994); males of species who experience greater sperm competition generally have larger testes (reviewed in Lupold et al., 2014). In addition, interspecific variation in penis size and shape are associated with mating system across many invertebrate and mammalian taxa (reviewed in Hosken and Stockley, 2004), and variation in male phallus length in waterfowl is likely due to varia-

tion in the strength intersexual selection across species (Brennan et al., 2007). Successful copulation requires the integration of multiple structures that serve diverse physiological and behavioral functions, and thus selection likely acts concurrently on these structures. However, the multiple components underlying copulation are rarely evaluated in a single study. Here, we examined the morphology and physiology of the suite of structures that underlie ejaculation in a group of *Anolis* lizard species to determine if these traits evolved in association with copulation behavior.

Detailed descriptions of reptilian reproductive structures have revealed that they are often highly variable among species (e.g., Dowling and Savage, 1960; Arnold, 1986), yet studies of this variation in relation to mating system or copulation behaviors remain relatively rare. Reptiles provide an excellent taxonomic group in which to study relationships between copulatory morphologies and behaviors, as their reproductive behaviors are easily observed in their natural environments, mating strate-

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* Corresponding author. E-mail: mjohnso9@trinity.edu

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gies vary among species (e.g., Stamps, 1983; Tokarz, 1995), and the relevant structures are well described (e.g., Wade, 2005). Other taxa present more challenges to these types of investigations. For example, mammal and insect copulatory structures have been frequently studied, but it is often extremely difficult to observe reproductive behaviors in the wild. In contrast, while the behaviors of some fishes and particularly birds can be more readily monitored, these groups of organisms generally do not have penes, so comparisons involving copulatory organ structure are not feasible.

Although the gross anatomy of the male reproductive system of reptiles is similar to other amniotes, several important distinctions exist between reptilian reproductive morphology and that of other vertebrate taxa (reviewed in detail in Gist, 2011; Kumar et al., 2011; Fig. 1). In brief, sperm is produced in the seminiferous tubules of the two testes, where it empties into bilateral efferent ductules that lead to the epididymides (Jones, 1998), the main locations of male sperm storage in reptiles. As in mammals, each epididymis is a highly coiled tube adjacent to a testis. The caudal end of the epididymis becomes the ductus deferens (or, vas deferens), which leads to the penile groove of one of the two paired copulatory organs called hemipenes. From this groove, the sperm is transferred to a female during copulation (Gist, 2011).

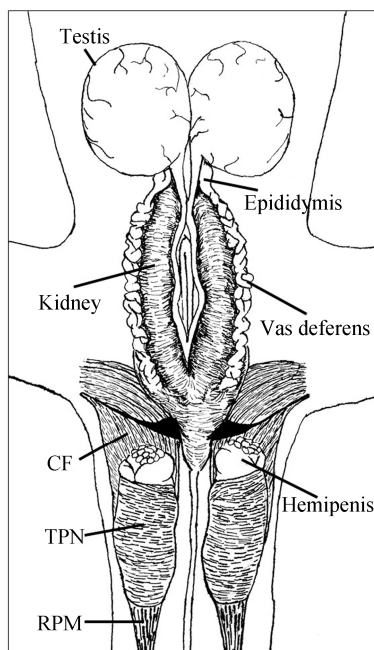


Fig. 1 Morphological structures involved in lizard copulation

CF = caudofemoralis, RPM = retractor penis magnus, TPN = transversus penis magnus. When not everted, the hemipenis lies largely under the TPN. Line drawing by Terrin N. Blackmon.

In contrast to mammals, however, there are no accessory sex glands in male reptiles except the renal sex segments of the kidneys, structures found only in lizards and snakes (Gist, 2011; Kumar et al., 2011). Secretions from the renal sex segments are thus the major component of male semen. These structures are responsive to androgens (e.g., Prasad and Reddy, 1972; Crews, 1980; Neal and Wade, 2007a), and increase to their maximum size during the period of sperm production (Holmes and Wade, 2004; Sever and Hopkins, 2005).

Copulation in lizards and snakes occurs when a male mounts a female, positions his pelvis under hers, and everts one of his two bilateral hemipenes into her cloacal vent (Crews, 1978; Shine et al., 2000). Movement of the independently-controlled hemipenes is directed by a pair of ipsilateral muscles in the rostral region of the tail (Fig. 1). Eversion through the cloacal vent is caused by contraction of the transversus penis (TPN) muscles, and after copulation, retraction of the hemipenes back into the tail occurs via contraction of the retractor penis magnus (RPM; Arnold, 1984).

Few studies to date have investigated the evolution of the mechanistic traits underlying copulatory behaviors of reptiles in general, and lizards in particular (but see Gredler et al. 2014 for a recent review of genital development in reptiles). Yet, studies examining variation in these traits *within* single species (a literature comprehensively reviewed in Norris and Lopez 2011) provide a wealth of data from which to base evolutionary hypotheses, as morphological and physiological traits that vary among individuals with differing copulatory behaviors may be those most likely to vary across species with different mating systems. For example, the structures that support male copulation are commonly absent or reduced in size in females: female renal sex segments in lizards are dramatically smaller than those in males, and females of many species lack hemipenes and the muscles that move them altogether (e.g., Raynaud and Pieau, 1985; Ruiz and Wade, 2002; Kumar et al., 2011; but see Holmes et al., 2005). Further, many lizards breed seasonally, with cyclical transitions in morphology and behavior that are primarily activated by increased circulating sex steroid hormones. However, while some copulatory structures change in size with season (most notably, testes and renal sex segments; e.g., Holmes et al., 2005; Neal and Wade, 2007a), comparisons between intact (non-gonadectomized) males in the breeding and nonbreeding seasons have generally shown few differences in other copulatory morphologies (e.g., Holmes and Wade, 2004, 2005). In addition,

tests of breeding vs. nonbreeding males have revealed changes in the fiber type composition of the muscle that controls extension of the anole dewlap (a throat fan involved in courtship and aggressive behavior), demonstrating seasonal variation in the physiological capacity of this muscle (Holmes et al., 2007; although this study did not find a seasonal effect on fiber type composition within the RPM). Finally, Neal and Wade (2007b) considered the behavioral implications of morphological variation among breeding season male lizards and found that breeding male green anoles *Anolis carolinensis* that copulated more frequently displayed enhanced renal sex segments, larger fibers in the muscle that moves the dewlap (but not the RPM muscle), and larger somata in the amygdala, a brain region involved in the display of sexual behaviors.

The goal of this study was to determine the evolutionary relationships between the frequency of copulation behavior (an important component of a species' mating system) and the morphology and physiology of a group of structures that support copulation. In this study, we used nine species of *Anolis* lizards that exhibit substantial variation in mating behavior. We used phylogenetically-informed statistical analyses to test the following evolutionary predictions, extending from the traits identified in the single-species studies described above: 1) species with larger copulatory morphologies have evolved higher copulation rates, and 2) species with the potential for higher performance in copulatory muscles, as measured by muscle fiber type, have evolved more frequent use of the copulatory muscles. We also examined relationships among the copulatory morphologies themselves to determine whether these traits evolved together in this group.

1 Materials and Methods

1.1 Morphological measurements

Adult male lizards were captured by hand or noose during the summer breeding season (May–July) from the localities listed in Johnson and Wade (2010), with *Anolis brevirostris*, *A. coelestinus*, *A. cybotes*, *A. olssoni* collected near Baoruco, Dominican Republic; *A. bahorucoensis* collected near Polo, Dominican Republic; *A. grahami*, *A. lineatopus*, and *A. valencienni* collected on the north shore of Jamaica; and *A. carolinensis* collected in southern Louisiana, USA. These lizards were the same individuals from which tissues were collected for the study described in Johnson and Wade (2010). Each animal was kept in an air-filled plastic bag until measurements were taken. Following transportation to a

field laboratory near each collection site, we measured each lizard's snout-vent length (SVL) using a ruler, and mass using a Pesola spring scale. Each animal was then rapidly decapitated (the average time from field capture to decapitation was 82 min). After confirming that each male had large, vascularized testes (indicating that it was in breeding condition), we immediately collected the testes, kidneys, and a portion of the tail that included the hemipenes and associated muscles, and all tissues were frozen on dry ice. Tissues were transported on dry ice to Michigan State University, where they were stored at -80°C until further processing.

Frozen tissues were sectioned at $20\ \mu\text{m}$ and stained using hemotoxylin and eosin. All subsequent measurements were performed with the observer blind to the species of each tissue sample. Using ImageJ (NIH) software, for each individual we measured the cross-sectional area of 10 renal sex segments and 30 seminiferous tubules. Because the seminiferous tubules are coiled in the testes, we only measured tubules that were round and symmetrical in a given section, to ensure that our measures of cross-sectional area of these structures were comparable among individuals and species. On each side of the tail, we also measured 25 arbitrarily-selected RPM muscle fibers at the proximal end of the muscle, and 25 arbitrarily-selected caudofemoralis (CF) muscle fibers in the same section of tissue, following Ruiz and Wade (2002) and Holmes and Wade (2004). Measures of the CF were used as a procedural control, as this muscle occurs in the same cross-sections of tissue as the RPM, but is not involved in movement of the hemipenis during copulation. Because this muscle regulates thigh movement (Snyder, 1954), there was no reason to expect the muscle fiber size to vary with respect to copulation behavior. Finally, we measured the cross-sectional area of the hemipenes in 4 or 5 sections of tissue at approximately $300\ \mu\text{m}$ intervals (Four sections were measured in species for which the length of the hemipenes did not exceed approximately $1,000\ \mu\text{m}$; for all other species, 5 sections were measured). Measures of all morphological traits were averaged within each individual for use in statistical analysis.

1.2 Muscle fiber typing

To obtain measures of fiber type in the RPM muscle, we used histological stains for myosin ATPase (an indication of contraction speed; Guth and Samaha, 1969) and succinate dehydrogenase (SDH, an indication of metabolic capacity), following Rosen et al. (2004) and Holmes et al. (2007). For both stains, we thawed alternate sections of muscle tissue for each individual, and

air-dried slides at room temperature for 30 min.

To stain for myosin ATPase, we placed slides in 2% buffered paraformaldehyde (0.1 M sodium cacodylate, 0.18 M CaCl_2 , 0.34 M sucrose, pH 7.6) for 5 min, and then in alkaline preincubation solution (18 mM of CaCl_2 in 100 mM of alkaline buffer solution (Sigma), pH 10.3) for 15 min. Slides were then incubated in incubation solution (2.7 mM ATP, 50 mM KCl, 18 mM CaCl_2 in 100 mM alkaline buffer solution (Sigma), pH 9.4) for 60 min at 37°C. Between each step, slides were rinsed twice in Tris buffer (100 mM Tris, 18 mM CaCl_2 , pH 7.8). After the incubation period, we rinsed the slides three times in 1% CaCl_2 , incubated for 3 min in 2% CoCl_2 , rinsed in distilled water, and then incubated for 3 min in 1% ammonium sulfide. Then, the slides were rinsed in distilled water, and held under running deionized water for 5 min. Finally, we coverslipped the slides with Aquamount (Fisher Scientific).

To stain for SDH, after thawed sections of muscle tissues were dry, we incubated slides for 37 min at 37°C in 130 mM Tris buffer (pH 7.4) with 0.2 mM nitrobluetetrazolium (Sigma) and 60 mM sodium succinate. Slides were then rinsed under running deionized water for 1 min, dehydrated in a series of ethanol solutions, cleared in xylene, and coverslipped with DPX (Fisher Scientific).

Because RPM muscle fibers are generally homogeneously stained throughout the muscle of an individual anole lizard (Holmes et al., 2007), we did not differentiate among fiber types within each muscle. In all species, RPM fibers were scored as slow-oxidative fibers (all fibers stained light with the myosin ATPase stain, and intermediate with the SDH stain), consistent with Holmes et al. (2007). However, for both stains, increased staining intensity indicates higher enzyme activity, and so we calculated the average stain intensity (measured as relative optical density, OD) for each lizard following Holmes et al. (2007). We calculated this relative OD by measuring the OD in 20 arbitrarily selected RPM fibers using ImageJ (NIH). We then measured OD in 10 lightly-stained fibers in the ischiocaudalis (IC), a muscle that stains light and dark fibers for both enzyme stains and is visible in the same tissue sections as the RPM. Our calculation of relative OD for each lizard was the ratio of the OD of RPM fibers to the OD of IC fibers, a measure that controls for variation in staining intensity among individuals, across species, and among staining runs.

1.3 Behavioral data collection

To determine whether male copulatory morphologies

are associated with copulation rates, we used previously-collected field behavioral data from each species. Each species was observed for a minimum of 40 hours during the summer (June–July) breeding season, in the same location that lizards were collected for the morphological measurements described above. All observations occurred between 0600 and 1900, and never during inclement weather (i.e., rain).

Behavioral data collection for *A. bahorucoensis*, *A. coelestinus*, *A. cybotes*, and *A. olssoni* from the Dominican Republic and *A. grahami*, *A. lineatopus*, and *A. valencienni* from Jamaica is described in Johnson et al. (2010). In brief, for each of these 7 species, 1–2 approximately 500 m² plots were established, and each lizard captured within the plot was given a unique mark. Focal observations of marked individuals generally lasted for 20 min, but for the more cryptic species *A. bahorucoensis* and *A. valencienni*, observations lasted for a maximum of 3 hours. Each lizard was observed for a maximum of five periods, or 5 hours, and data from multiple observations of a given lizard were averaged for use in statistical analyses. During each focal observation, all observed behaviors were recorded, including copulations. Observations of *A. brevirostris* in the Dominican Republic (described in Johnson and Wade, 2010; Cook et al., 2013) and *A. carolinensis* in Louisiana (described in Johnson et al., 2011) were similar, except that unmarked lizards were observed. A copulation rate (number of copulations per hour) was calculated for each individual, and averaged for each species for use in subsequent analyses.

1.4 Statistical analyses

We conducted a series of ANOVAs, followed by Tukey's HSD post hoc tests, to determine whether species differed in each of the morphological traits: SVL; mass; cross-sectional areas of seminiferous tubules, renal sex segments, hemipenes, and RPM muscle fibers; and relative OD from myosin ATPase and SDH stains (A multivariate analysis was not appropriate in this case, because due to histological artifacts, not all measures were available for all lizards). We also conducted a series of ANCOVAs, with SVL as the covariate, to determine whether species differed in these traits when controlling for variation in body size.

To determine whether morphological traits evolved in association with each other and/or with copulation rates, we used a series of phylogenetically-informed statistical analyses, performed using the phylogeny of *Anolis* lizards in Rabosky and Glor (2010) and pruned to only include the species in this study (Fig. 2). We

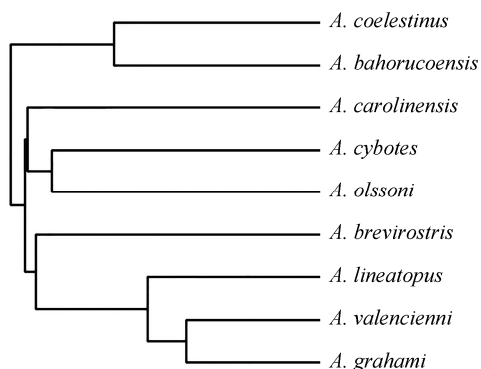


Fig. 2 Phylogeny of nine *Anolis* lizard species used in this study, pruned from the anole phylogeny in Rabosky and Glor (2010)

calculated phylogenetic independent contrasts (Felsenstein, 1985) on species' averages for all morphological traits and copulation rate using the *ape* package (Paradis et al., 2004) in the statistical program R (R Core Team, 2013).

To determine relationships among morphological traits, we used contrast data for each pair of traits to calculate uncentered correlations (analogous to forcing a regression through the origin; Garland et al., 1992). We determined the p-value for each correlation by forcing the regression of each pair of traits through the origin, using the statistical program SPSS. Further, to determine whether copulation rate was associated with morphological traits across species, we performed a stepwise multiple regression, forced through the origin, where contrasts for copulation rate was the dependent variable and contrasts of the copulatory morphologies (listed above) were the independent variables. We also conducted a similar, non-phylogenetic stepwise regression using species averages for each trait.

To compare the strength of the relationships between copulation rate and two highly correlated traits (hemipenis size and RPM fiber size), we performed an Akaike information criteria (AIC) model selection test using the *MuMIn* package (Bartoń, 2012) in R. We generated the AIC analyses from a multiple linear regression with both hemipenis size contrasts and RPM size contrasts as independent variables and copulation rate contrasts as the dependent variable. We evaluated models based on their ΔAICc values, used to compare the strength of each model to the highest-ranked model, and used Akaike weights (w) to evaluate the likelihood that a given model was the strongest predictive model.

Because SVL exhibited non-significant but moderate correlations with hemipenis and RPM fiber sizes (see Results), we also conducted regression analyses testing

the relationship between contrasts of each of these traits and copulation rates, including SVL contrasts as a covariate.

2 Results

2.1 Differences among species in copulatory morphologies

The nine species of anoles displayed significant differences in body size (SVL and mass) and most of the copulatory morphologies, including sizes of the semiferous tubules, hemipenes, and RPM fibers, and the relative OD of SDH stains (Tables 1 and 2). However, three traits did not differ among species: the size of the renal sex segments in the kidneys, the muscle fiber size in the CF (the procedural control measure), and the relative OD of myosin ATPase stains, for which there were marginally significant differences among species (Tables 1 and 2). When controlling for differences among species in body size, SVL was a significant covariate for each trait (all $P < 0.02$), and all morphological traits significantly differed among species (all $P < 0.04$) except CF fiber size ($P = 0.37$).

2.2 Evolutionary relationships among copulatory morphologies

Correlation analyses among copulatory morphologies revealed a strong positive relationship between hemipenis size and the fiber size of the RPM, a muscle that moves the hemipenes (Table 3; Fig. 3). Further, SVL and mass were positively correlated with one another and with the size of CF muscle fibers, and SVL was correlated with the density of SDH staining (Table 3). Other measures of copulatory morphology were not correlated with one another, and most were not correlated with body size (Table 3).

2.3 Relationships between copulatory morphologies and copulation rates

To determine the relationship between copulation rates and copulatory morphologies, we conducted a stepwise multiple regression using all morphological traits. Hemipenis size was revealed to be the strongest predictor of the evolution of copulation rates ($F_{1,7} = 7.88$, $P = 0.026$, $R^2 = 0.53$), with all other morphological traits excluded in this analysis, such that lineages with larger hemipenes copulated with higher frequency. However, a nonphylogenetic analysis indicated that RPM fiber size was the strongest predictor of copulation rate ($F_{1,7} = 12.8$, $P = 0.009$, $R^2 = 0.65$). Because contrasts of hemipenis size and RPM fiber size were strongly correlated (Table 3), we also conducted a regression with RPM contrasts as the only independent

Table 1 Morphological trait means (and standard error) for nine *Anolis* lizard species

	SVL	Mass (g)	Seminiferous Tubules (mm ²)	Renal Sex Segments (mm ²)	Hemipenes (mm ²)
<i>A. bahorucoensis</i>	41.3 ^a (0.75)	1.3 ^a (0.08)	0.134 ^a (0.008)	0.0194 ^a (0.0018)	0.81 ^a (0.16)
<i>A. brevirostris</i>	44.0 ^b (0.54)	2.5 ^a (0.08)	0.183 ^{abc} (0.007)	0.0150 ^b (0.0026)	1.89 ^{abc} (0.14)
<i>A. carolinensis</i>	62.8 ^{bc} (0.87)	5.3 ^{bcd} (0.12)	0.140 ^a (0.014)	0.0184 ^a (0.0015)	2.79 ^{cd} (0.28)
<i>A. coelestinus</i>	64.0 ^c (1.38)	6.2 ^{cd} (0.40)	0.140 ^a (0.008)	0.0149 ^a (0.0011)	1.72 ^{abc} (0.20)
<i>A. cybotes</i>	61.3 ^{bc} (1.79)	6.7 ^d (0.60)	0.227 ^c (0.009)	0.0230 ^a (0.0019)	2.31 ^{bc} (0.30)
<i>A. grahami</i>	60.2 ^{bc} (1.41)	5.9 ^{cd} (0.48)	0.166 ^{bc} (0.014)	0.0179 ^a (0.0021)	2.20 ^{bc} (0.44)
<i>A. lineatopus</i>	58.3 ^b (1.00)	4.8 ^{bc} (0.23)	0.217 ^{bc} (0.015)	0.0174 ^a (0.0011)	1.00 ^{ab} (0.14)
<i>A. olssoni</i>	42.9 ^a (1.35)	1.2 ^a (0.31)	0.143 ^a (0.011)	0.0188 ^a (0.0016)	0.80 ^a (0.14)
<i>A. valencienni</i>	62.5 ^{bc} (0.58)	4.2 ^b (0.18)	0.202 ^{bc} (0.003)	0.0186 ^a (0.0016)	3.81 ^d (0.17)

	RPM Fibers (μm ²)	CF Fibers (μm ²)	Myosin ATPase (Relative OD)	SDH (Relative OD)	Copulation/h
<i>A. bahorucoensis</i>	550 ^a (70)	2783 ^a (193)	0.93 ^a (0.05)	0.97 ^{ab} (0.008)	0.00†
<i>A. brevirostris</i>	993 ^{abcd} (58)	2906 ^a (252)	1.01 ^a (0.07)	0.95 ^a (0.006)	0.16
<i>A. carolinensis</i>	759 ^{abc} (143)	3191 ^a (236)	1.11 ^a (0.07)	1.00 ^b (0.010)	0.10
<i>A. coelestinus</i>	651 ^{ab} (90)	3386 ^a (222)	1.10 ^a (0.04)	1.01 ^b (0.004)	0.03
<i>A. cybotes</i>	1148 ^{bcd} (201)	3436 ^a (341)	0.89 ^a (0.05)	0.99 ^{ab} (0.012)	0.05
<i>A. grahami</i>	1294 ^d (143)	3709 ^a (374)	0.82 ^a (0.09)	0.98 ^{ab} (0.005)	0.23
<i>A. lineatopus</i>	769 ^{abc} (85)	3529 ^a (392)	0.98 ^a (0.03)	1.00 ^b (0.005)	0.14
<i>A. olssoni</i>	485 ^a (137)	2493 ^a (308)	0.96 ^a (0.04)	0.98 ^{ab} (0.005)	0.00†
<i>A. valencienni</i>	1232 ^{cd} (65)	3107 ^a (223)	0.97 ^a (0.04)	1.00 ^b (0.006)	0.28

Values with different superscripts are statistically different ($P < 0.05$) in Tukey's HSD post hoc tests. † indicates species for which no copulations were observed during focal observation periods (although copulations in these species were observed at other times). CF = caudofemoralis, OD = optical density, RPM = retractor penis magnus, SDH = succinate dehydrogenase, SVL = snout-vent length.

Table 2 ANOVAs comparing morphological traits across nine *Anolis* species. Each trait was analyzed in a separate ANOVA

	df	F	P
SVL	8, 106	74.7	< 0.001
Mass	8, 106	43.2	< 0.001
Seminiferous Tubules	8, 106	10.4	< 0.001
Renal Sex Segments	8, 106	1.69	0.109
Hemipenes	8, 106	9.89	< 0.001
RPM Fibers	8, 106	6.54	< 0.001
CF Fibers	8, 106	1.76	0.094
Myosin ATPase	8, 106	2.01	0.059
SDH	8, 106	4.31	< 0.001

CF = caudofemoralis, RPM = retractor penis magnus, SDH = succinate dehydrogenase, SVL = snout-vent length.

variable, and found that RPM fiber size is also a significant predictor of the evolution of copulation rate ($F_{1,7} = 7.39$, $P = 0.030$, $R^2 = 0.51$). AIC analyses comparing independent contrasts of both predictor variables yielded a best model with hemipene size only ($\Delta AICc =$

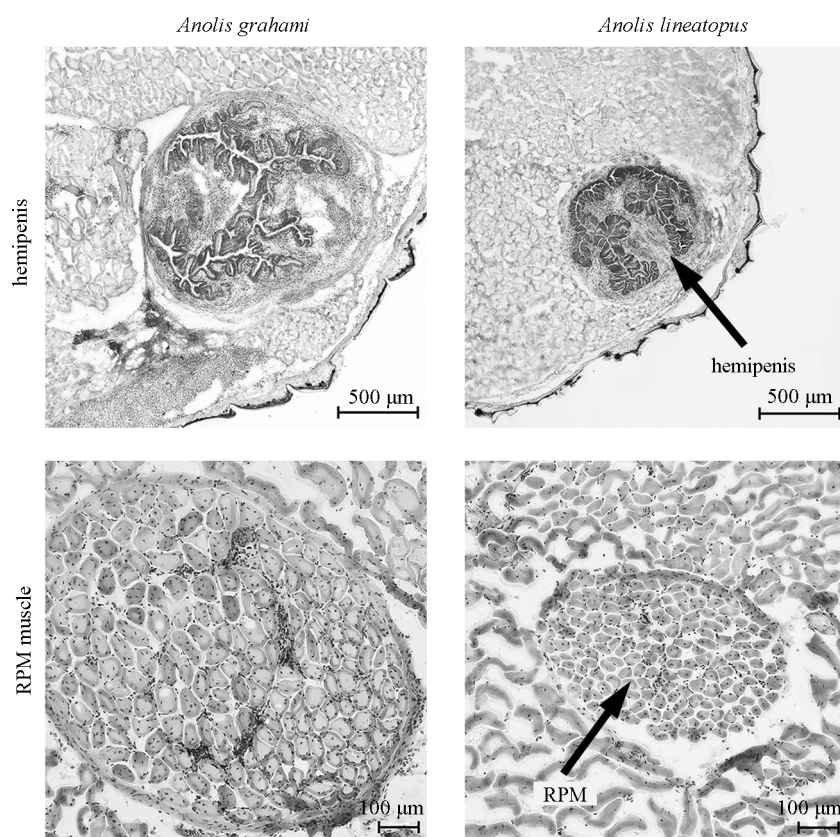
-52.7). This model was supported with an Akaike's weight (w) of 0.474, indicating that there is a probability of 47.4% that this is the best model. The next best model included RPM size only ($\Delta AICc = -52.6$); this model had approximately the same support as the hemipene model with a w of 0.453. The model including both RPM size and hemipenis size contrasts had the lowest support ($\Delta AICc = -49.0$, $w = 0.072$).

Although hemipenis and RPM fiber sizes were not significantly correlated with SVL in this dataset (Table 3), the r values for these correlations were moderate (greater than 0.4). Therefore, to confirm that differences in body size were not driving the results of our analyses, we repeated the regressions between contrasts of these morphologies and copulation rate including SVL contrasts as a covariate. The loss of statistical power in these analyses, resulting from the loss of a degree of freedom from the covariate, caused the results to be marginally significant, but the relative effect sizes allow us to assess the potential impact of SVL on the copulation morphologies. In the analysis with hemipenis size and SVL ($F_{2,6} = 3.49$, $R^2 = 0.54$, $P = 0.099$), hemipenis

Table 3 Uncentered correlations among copulatory morphologies in nine species of *Anolis* lizards

	Mass	Seminiferous Tubules	Renal Sex Segments	Hemipenes	RPM Fibers	CF Fibers	Myosin ATPase	SDH
SVL	0.901**	0.278	-0.006	0.582	0.433	0.706*	0.309	0.798*
Mass		0.290	0.005	0.345	0.501	0.899**	0.069	0.557
Seminiferous Tubules			0.333	0.222	0.415	0.269	-0.071	0.118
Renal Sex Segments				0.122	0.206	-0.056	-0.509	-0.008
Hemipenes					0.667*	0.062	0.244	0.302
RPM Fibers						0.465	-0.405	-0.101
CF Fibers							-0.172	0.372
Myosin ATPase								-0.481

** indicates $P < 0.001$, * indicates $P < 0.05$. SVL = snout-vent length, RPM = retractor penis magnus, CF = caudofemoralis, SDH = succinate dehydrogenase.

**Fig. 3** Representative hemipenis and RPM fiber sizes of two species similar in body size, but differing in copulation rates

Anolis grahami exhibits high copulation rates and has large hemipenes and fibers in the RPM, a muscle that moves the hemipenes. *Anolis lineatopus* exhibits low copulation rates, and has small hemipenes and RPM muscle fibers.

size had a stronger relationship with copulation rate (standardized β coefficient = 0.66, $P = 0.100$) than SVL (standardized β coefficient = 0.11, $P = 0.75$). Likewise, in the analysis with RPM ($F_{2,6} = 3.49$, $R^2 = 0.56$, $P = 0.099$), RPM size also had a stronger relationship with copulation rate (standardized β coefficient = 0.62, $P = 0.087$) than SVL (standardized β coefficient = 0.23, $P = 0.47$).

3 Discussion

The findings of this study indicate that, independent-

ly of body size, species that copulate more frequently evolved larger male copulatory organs (i.e., hemipenes) and larger fibers in the muscles that control the movement of those organs. However, the performance capacity of the hemipene-associated RPM muscle, as measured by fiber type composition, did not vary with the frequency of copulation behavior. In addition, we found no evidence that the two primary structures involved in production of the ejaculate (seminiferous tubules in the testes and renal sex segments in the kidneys) evolved in

association with copulation frequency.

The relationship between hemipenis size and RPM muscle fiber size in our data is an intuitively appealing one, as a larger structure logically requires larger muscle fibers to control its movement. This finding is supported by the results of intraspecific studies of diverse taxa, in which larger structures are used more frequently and supported by larger muscles (e.g., clawed frogs: Sassoon and Kelley, 1986; midshipmen fish: Bass, 1990; zebra finches: Arnold, 1997; Wade, 2001). Further, our finding that larger hemipenis sizes have evolved in association with greater copulation frequency across species is consistent with a general intraspecific pattern in invertebrate and mammalian taxa that larger penes are associated with increased male fitness (reviewed in Hosken and Stockley, 2004). However, the tight collinearity of hemipenis and RPM fiber sizes makes it difficult to determine whether either one is the primary mechanism driving the evolution of copulation frequency, or if copulation frequency is primarily the result of the behavioral integration of the functions of these two traits.

In addition, the present study cannot directly distinguish whether more frequent behavioral use of the hemipenes results in the growth of larger hemipenes and associated musculature, or whether the larger size of the structures in some species has evolved to facilitate their more frequent use. With regard to muscle fiber size and use, "training effects" – in which the use of a muscle results in its growth – are common in mammalian taxa, but there remains no evidence in squamate reptiles of an increase in fiber size directly caused by muscle use on the order of the interspecific differences in fiber size reported here (Eme et al., 2009, but see Husak et al. in review). Thus, larger muscle fibers may be more likely to support increased behavioral use of the hemipenes in frequently copulating species, rather than their frequent use causing the growth of larger fibers.

Surprisingly, while the species in this study differed in the sizes of the seminiferous tubules, this trait was not associated with copulation frequency. Perhaps variation in sperm morphology, number, or swimming speed (reviewed in Simmons and Fitzpatrick, 2012) is more closely associated with copulation rate among anoles than the size of the seminiferous tubules. In addition, a relationship between overall testis size and mating system has been reported in many animal taxa, including mammals, birds, reptiles, amphibians, and invertebrates (reviewed in Calhim and Birkhead, 2007), and testis size, while not measured here, may be a

stronger predictor of copulation rate than seminiferous tubule size. Further, we found that the size of the renal sex segments, the sole accessory glands contributing to male semen in reptiles, did not differ across anole species. This suggests that a seasonal threshold effect might occur, in which the increased testosterone associated with the onset of the summer breeding season causes the renal sex segments to increase to the size needed to produce the fluids in the ejaculate (e.g., Prasad and Reddy, 1972; Crews, 1980; Neal and Wade, 2007a), but once that size is attained, further growth has no benefit.

Additionally, we found no association between RPM fiber type composition and copulation rates across anole species. This finding is consistent with the results of Holmes et al. (2007), who found no variation within green anole *Anolis carolinensis* RPM fiber type across season or testosterone treatments. Together, these results indicate that fiber type variation is not associated with intra- or interspecific variation in copulatory behaviors.

In sum, the results of this study propose that the peripheral reproductive morphologies (hemipenes and associated musculature) are more evolutionarily labile than internal copulatory structures. While the documented variation in reptile hemipenis morphology (e.g., Dowling and Savage, 1960; Arnold, 1986) indicates that this trait is particularly labile, our study suggests that at least one aspect of this variation (size) is directly associated with copulation behaviors. In addition, our finding that the fiber size of a muscle that controls hemipenis movement is positively associated with the size of the hemipenes and the frequency of their use contrasts with the results from a similar study of the dewlap and associated musculature in the same group of species, in which muscle fiber size was not related to dewlap size or behavioral use (Johnson and Wade, 2010). Thus, similar morphological traits may evolve differently with regard to the specific behaviors they support. By simultaneously considering multiple components of the reproductive system, we can begin to determine how morphological variation in these traits is associated with the evolution of the copulation behaviors that determine mating strategies.

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