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Lack of spermatogenic variation in a polymorphic lizard, *Sceloporus aeneus* (Squamata: Phrynosomatidae)

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Abstract
Although different mechanisms exist to explain the presence of polymorphism in lizards, one model suggests that multiple morphotypes display the same level of fitness. Three male morphs (grey, yellow and orange) coexist in *Sceloporus aeneus*, a Mexican endemic oviparous lizard. Using a histological perspective, we test the hypothesis that spermatogenic output does not vary across morphotypes of *S. aeneus* during its maximum testicular activity. Males of *S. aeneus* (five grey, five yellow and five orange) were collected in Calimaya, Estado de México, Mexico. Snout-vent length (SVL), testis mass, diameter and epithelial heights for the seminiferous tubules and epididymis, and the number of layers of germ cells did not vary among morphs; moreover, according to principal component analysis, a high overlap among lateral colour morphs exists. Our results suggest strongly that the lateral colour morphs in *S. aeneus* have the same spermatogenic output, and natural selection may be a stronger driving force than sexual selection within this species. Further studies into other lizard species with multiple morphotypes are required to determine whether the lack of variation in spermatogenic output observed in this endemic lizard is consistent across polymorphic species which will provide a greater understanding of the selective mechanisms acting on an individual's fitness.

KEYWORDS
histology, lizard, morphotypes, reproduction, testis

1 | INTRODUCTION

Morphological variation stemming from sexual selection is common within squamate lizards (Aguilar-Moreno et al., 2010) and plays a vital role in reproductive success involving all aspects of reproduction from mate choice to postcopulatory sperm competition (Olsson & Madsen, 1998). Many studies have shown that variation exists in sexual selective pressures interspecifically, and in recent years, variation intraspecifically has been noted (Bastiaans et al., 2014). For example, Rheubert et al. (2017) found sperm size varied between populations of two species of *Sceloporus* which corroborated recent findings concerning variation in sperm morphometrics at the intraspecific level (Blengini, Naretto, Cardozo, Giojalas, & Chiaraviglio, 2014). However, polymorphic populations have been understudied despite the level of complexity they present such as social hierarchy in *Uta stansburiana* in which three male morphotypes are present and social dominance resembles a game of "rock–paper–scissors" (Sinervo & Lively, 1996). Due to this, a large knowledge gap concerning variation exists within polymorphic populations in terms of the large realm of characters influenced by sexual selection.

Male polymorphisms have been reported in multiple genera within Phrynosomatidae including, but not limited to the following: *Uta* (Sinervo & Lively, 1996), *Urosaurus* (Taylor & Lattanzio, 2016) and *Sceloporus* (Rand, 1990). The *Sceloporus* genus is one of the most diverse genera of squamates (Wiens & Reeder, 1997) and exhibits a wide array of morphological variation, specifically sexually dimorphic characters (Jiménez-Arcos, Sanabria-Urbán, & Cueva del Castillo, 2017). Additionally, the genus exhibits extreme reproductive variation including oviparous and viviparous species as well as continuous and seasonal activity (Gribbins et al., 2011; Méndez-de la Cruz, Villagrán-Santa Cruz, & Andrews, 1998). Furthermore, the *Sceloporus* genus has numerous species in which multiple morphotypes exist within the same population (Bastiaans et al., 2014; Bustos Zagal, Castro-Franco, Manjarrez, & Fajardo Guadarrama, 2014; Jiménez-Arcos, 2013; Rand, 1990; Stephenson, 2010). This variation makes them excellent models for studies concerning reproductive characteristics and their interactions. However, there have been few comparisons among male morphs within *Sceloporus* including morphological (Bustos Zagal et al., 2014; Jiménez-Arcos, 2013; Stephenson, 2010), life history traits, performance (Jiménez-Arcos, 2013), frequency (Jiménez-Arcos, 2013; Rand, 1990), coloration intensity and its hormonal control (Calisi & Hews, 2007; Rand, 1990, 1992), behaviour (Bastiaans et al., 2014; Stephenson, 2010) and microhabitat use (Bustos Zagal et al., 2014).

Sceloporus aeneus is a Mexican endemic oviparous lizard with maximum testicular activity of spermiogenesis and spermiation occurring from March to May (Hernández-Gallegos et al., 2014). As colour polymorphism has been recorded in congeneric *Sceloporus* (Bustos Zagal et al., 2014), adult males of *S. aeneus* exhibit a lateral colour polymorphism including three morphs: grey, yellow and orange (Jiménez-Arcos, 2013). Numerous studies have shown that male sexual coloration is an important cue in female mate choice (Cooper & Greenberg, 1992). To our knowledge, few studies exist demonstrating the relationship between morphotype and fitness (Olsson, Schwartz, Uller, & Healey, 2009; Zamudio & Sinervo, 2000), despite hypotheses suggesting multiple morphotypes display the same level of fitness (Ryan, Pease, & Morris, 1992; Shuster & Wade, 1991); therefore, the purpose of this study was to test, using histological techniques, univariate and multivariate analyses, the hypothesis that spermatogenic activity does not vary across morphotypes within a single population of lizards exhibiting polymorphic coloration.

2 | MATERIAL AND METHODS

2.1 | Animal collection

During May 2015 (i.e., peak reproductive period, Hernández-Gallegos et al., 2014), 15 adult males (five grey, five yellow and five orange) of *S. aeneus* were collected (Scientific Collector Permit SEMARNAT FAUT 0186) at Calimaya, Estado de México, Mexico (19º10′50′′ N, 99º36′18.8′′ W; 2646 masl). Individuals were sacrificed using an intracoelomic injection of sodium pentobarbital (10%) following ethical guidelines of the Universidad Autónoma del Estado de México. Morphological measurements taken from each male included: SVL, and testis mass; moreover, gross reproductive conditions of testicular ducts were recorded. Males were fixed in 10% neutral-buffered formalin, preserved in ethanol 70% and deposited in the Laboratorio de Herpetología, Facultad de Ciencias, Universidad Autónoma del Estado de México.

2.2 | Histological procedures

In accordance with previous studies on spermatogenic cycles, conventional histological techniques were performed on testicular tissues included the following: dehydration via graded ethanol, clearing tissues in xylene and embedding tissues in paraffin (Granados-González et al., 2015; Hernández-Gallegos et al., 2014; Rheubert et al., 2014). Embedded samples were then cut at 5 μm using a rotatory microtome, placed on albuminized slides and stained with haematoxylin-eosin for general cytology. Samples were then viewed via a compound microscope. Germ cells (spermatogonia, primary and secondary spermatocytes and spermatids) were classified according to morphological descriptions presented by Gribbins (2011), and phase of spermatogenesis was classified according to previous spermatogenic studies in lizards (Gribbins & Gist, 2003; Hernández-Gallegos, Méndez-de la Cruz, Villagrán-Santa Cruz, & Andrews, 2002; Lowe & Goldberg, 1966; Rheubert et al., 2014; Villagrán-Santa Cruz, Méndez-de la Cruz, & Parra-Gámez, 1994). Relative abundance of germ cells was estimated by counting the number of layers of each germ cell class (spermatogonia, spermatocytes and spermatids) in 25 seminiferous tubules (Hernández-Gallegos et al., 2002). Additionally, tubule diameter and epithelial heights for the seminiferous tubules and epididymis were measured in 25 representative microscopic fields per male using an ocular micrometre.

2.3 | Statistical procedures

Snout-vent length, testis mass, diameters and epithelial heights of seminiferous tubules, and diameters and epithelial heights of epididymal ducts were tested for normality and for homoscedasticity. We used ANCOVA's (to exclude the effect of covariation of SVL with the measurements) to compare testis mass and histological traits, with the morph of male as the factor to determine whether morphs differed for each of the values. Moreover, to explore characters (histological measurements and germ cells) that could define morphs, a principal component analysis (PCA) was performed based on a correlation matrix. All analyses were performed in Statgraphics with α set at 0.05.

3 | RESULTS

The average SVLs were 51.5 mm (±1 mm, range 49-54, $n = 5$), 52.4 mm (±1 mm, range 51-56 mm, $n = 5$) and 51.8 mm (±0.7 mm, range 49-53 mm, $n = 5$) for the morphs grey (Figure 1a), yellow (Figure 1d) and orange (Figure 1g), respectively, and did not vary among morphs ($p = 0.8086$). The SVL, testis mass and the histological data were normally distributed (p values 0.6219–0.9813 for overall models, Kolmogorov–Smirnov's test), and met the assumption of homoscedasticity (p values 0.3593-0.9464 for overall models, Levene's test).

All males exhibited vascularized testes and convoluted epididymides (indicating high reproductive activity). All males in the three morphs were in peak reproductive of spermiogenesis and spermiation, with high relative concentration of mature sperm were found in both seminiferous tubules (Figure 1b,e,h, MS) and epididymal ducts (Figure 1c,f,i, MS), and abundant mature secretory granules were observed within the epithelia of the epididymides (Figure 1c,f,i,*).

Testis mass, diameters of seminiferous tubules, seminiferous epithelial heights, diameters of epididymal ducts, epididymal epithelial heights and the number of layers of spermatogonia (Sg), primary spermatocytes (1°sp), secondary spermatocytes (2°sp) and spermatids (Spt) did not vary among morphs (Figure 1b,e,h) or as a function of SVL (p values 0.0782-0.7916 for overall models; Table 1). The principal component analysis showed: a) no differences among morphs (PC1, $F = 0.37$, $df = 2,14$, $p = 0.6971$; PC2, $F = 0.11$, $df = 2,14$, $p = 0.8931$) and b) high degree overlap on principal components 1 and 2 among morphs (Figure 2). The first axis explained 32% of the variation and was loaded mainly on seminiferous epithelial heights, while the second axis explained 22% of the variation and was loaded mainly on diameters of epididymal ducts.

4 | DISCUSSION

Investigations of reproductive characteristics at the intraspecific level are limited, but have noted that variation does exist. Rheubert et al. (2017) noted that studies at the intraspecific level provide valuable information as they remove any potential phylogenetic signal/influence on the data and information provided at the intraspecific levels, which aids in the overall understanding of potential proximate causes of the observed variation. Furthermore, studies investigating the complexities

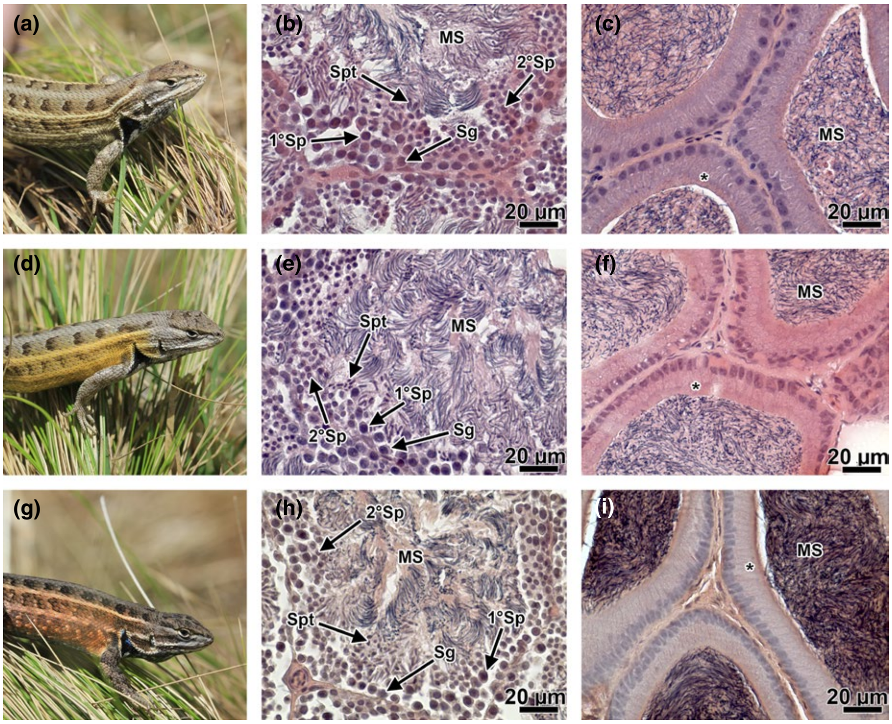

FIGURE 1 Lateral view and testicular and epididymal histology of grey (a, b, c), yellow (d, e, f) and orange (g, h, i) morphotypes in *Sceloporus aeneus* from Calimaya, Estado de México, Mexico. Spermatogonia, Sg; primary spermatocytes, 1°sp; secondary spermatocytes, 2°sp; spermatids, Spt; mature sperm, MS. The epididymal epithelial cells are filled with secretory material (*), and mature sperm (MS) are seen in the lumen [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 ANCOVA results for male reproductive characters in *Sceloporus aeneus* from Calimaya, Estado de México, Mexico

	Morphs			SVL		
Trait	***F***	***df***	***p*-value**	***F***	***df***	***p*-value**
Testis mass	0.77	2,11	0.4844	2.7	1,11	0.1286
Diameters of seminiferous tubules	1.18	2,11	0.3428	1.82	1,11	0.2046
Seminiferous epithelial heights	0.39	2,11	0.6882	0.56	1,11	0.4710
Diameters of epididymal ducts	0.94	2,11	0.4193	0.16	1,11	0.7010
Epididymal epithelial heights	1.22	2,11	0.3330	1.05	1,11	0.3278
Spermatogonia	3.24	2,11	0.0782	1.01	1,11	0.3360
Primary spermatocytes	0.85	2,11	0.4534	0.35	1,11	0.5680
Secondary spermatocytes	0.38	2,11	0.6911	2.16	1,11	0.1699
Spermatids	0.24	2,11	0.7916	0.20	1,11	0.6598

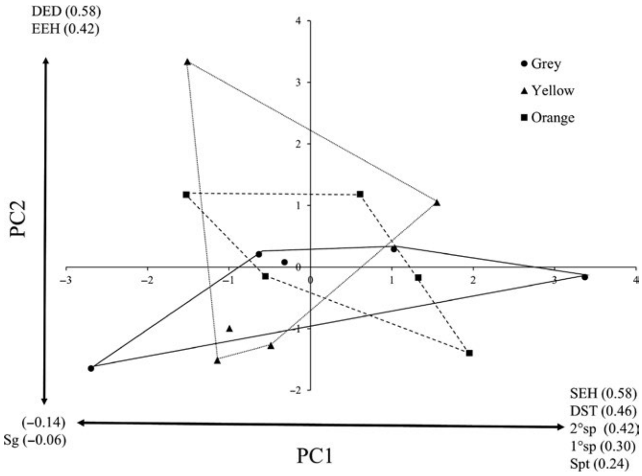

FIGURE 2 Lateral colour morphs of male *Sceloporus aeneus* from Calimaya, Estado de México, Mexico, using principal component analysis on histological measurements and germ cells. Notice the high overlap among lateral colour morphs. Variable loadings and direction on each principal component are shown. DST = diameters of seminiferous tubules; SEH = seminiferous epithelial heights; DED = diameters of epididymal ducts; EEH = epididymal epithelial heights; Sg = spermatogonia; 1°sp = primary spermatocytes; 2°sp = secondary spermatocytes; and Spt = spermatids

of reproductive characteristics and their interactions are lacking, despite the valuable information they may provide in terms of resource allocation and relative importance to fitness. Polymorphic species, both intersexual and intrasexual, present unique opportunities to investigate resource allocation hypotheses (Olsson, Stuart-Fox, & Ballen, 2013). Within lizards, the number and frequency of male morphs vary among populations and species (Bastiaans et al., 2014). However, a challenge to evolutionary theory arises when multiple morphs exist, specifically how and why polymorphisms evolve and are preserved within populations (Bastiaans et al., 2014; Olsson et al., 2013; Sinervo & Lively, 1996). In *U. stansburiana*, the morphs are maintained by frequency-dependent selection (Sinervo & Lively, 1996) and a similar situation was previously suggested for *S. aeneus* (Jiménez-Arcos, 2013) suggesting the underlying mechanisms of polymorphism preservation may be consistent across taxa (Skúlason & Smith, 1995).

In contrast with other populations of *S. aeneus*, where thermoregulation and predation vary across colour morphs (Jiménez-Arcos, 2013), in Calimaya the lateral colour morphs did not differ in body size, and according to both univariate and multivariate analysis, the morphs on average have the same spermatogenic output. Different hypotheses exist to explain the presence of polymorphism (Sinervo & Lively, 1996), including that the selection is responsible for its maintenance when fitness of each male morph is equal over time (Shuster & Wade, 1991). Similarly, our results strongly suggest that: 1) the lateral colour morphs in *S. aeneus* do not vary in spermatogenic output and thus do not exhibit differences in resource allocation in terms of spermatogenesis and 2) frequency-dependent selection may be a larger driving force than sexual selection within this species.

Although the number of spermatogenic cells did not differ between morphs from Calimaya, investment in sperm production may differ. For example, Rheubert et al. (2017) and Blengini et al. (2014) have shown that sperm size differs at the intraspecific level (although neither study investigated the intrapopulational variation, as various morphotypes are not present in their studied species). However, testis size nor SVL differed between morphs of *S. aeneus* in this study and previous data have documented the correlations between sperm size/number and paternity to testis size (Olsson et al., 2009). Nonetheless, future studies investigating sperm size should be conducted to verify our hypothesis that morphotype does not affect spermatogenic output or fitness.

In lizards, male morphs differ in several traits including behaviour (Stephenson, 2010; Taylor & Lattanzio, 2016;

Yewers, Pryke, & Stuart-Fox, 2016), morphology (Huyghe, Vanhooydonck, Herrel, Tadic, & Van Damme, 2007; Stephenson, 2010), territory (Zamudio & Sinervo, 2000), parasitic load (Megía-Palma et al., 2018) and physiology (LaDage, Riggs, Sinervo, & Pravosudov, 2009; Stephenson, 2010). However, different comparisons among morphs did not reveal any significant difference including morphometry (Meyers, Irschick, Vanhooydonck, & Herrel, 2006; Sacchi et al., 2007; Yewers, Jessop, & Stuart-Fox, 2017), total of number of offspring and their survivorship (Zamudio & Sinervo, 2000), performance (Huyghe et al., 2007; Meyers et al., 2006), behaviour, microhabitat use, thermoregulation (Huyghe et al., 2007) and/or spermatogenic output (this study). Currently, we lack relevant data concerning lateral coloration in *S. aeneus* from Calimaya, including whether males with different morphotypes are also: (i) genetically, (ii) behaviourally, (iii) physiologically and (iv) ecologically different. Species with complex reproductive characteristics provide unique opportunities to investigate reproductive allocation and selective pressures. Although data collected suggest reproductive output does not differ between morphotypes in *S. aeneus* and that the duration of each phase and its timing within the spermatogenic cycle are similar among morphotypes, there are multiple variables that should still be investigated such as social hierarchy, female mate choice, sperm size and sperm performance (i.e., velocity). These future studies will provide a holistic view of reproduction in a complex species and provide valuable insight concerning fitness pressures and resource allocation. Furthermore, investigations into other polymorphic species (i.e., *U. stansburiana* and *Urosaurus ornatus*) are needed to see whether the lack of variation in spermatogenic output that is observed here in *S. aeneus* is consistent across polymorphic taxa of lizards which will provide a greater understanding of the selective mechanisms acting on an individual's fitness.

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