SYMPOSIUM

What Determines Paternity in Wild Lizards? A Spatiotemporal Analysis of Behavior and Morphology

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From the symposium "Physical mechanisms of behavior" presented at the virtual annual meeting of the Society for Integrative and Comparative Biology, January 3–7, 2021.

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Synopsis Mating behavior in animals can be understood as a sequence of events that begins with individuals encountering one another and ends with the production of offspring. Behavioral descriptions of animal interactions characterize early elements of this sequence, and genetic descriptions use offspring parentage to characterize the final outcome, with behavioral and physiological assessments of mates and mechanisms of copulation and fertilization comprising intermediate steps. However, behavioral and genetic descriptions of mating systems are often inconsistent with one another, complicating expectations for crucial aspects of mating biology, such as the presence of multiple mating. Here, we use behavioral and genetic data from a wild population of the lizard *Anolis cristatellus* to characterize female multiple mating and the potential for sexual selection through female mate choice in this species. We find that 48% of sampled females bore offspring sired by multiple males. Moreover, spatiotemporal proximity between males and females was associated with whether a male sired a female's offspring, and if yes, how many offspring he sired. Additionally, male body size, but not display behavior, was associated with reproductive outcomes for male–female pairs. While much remains to be learned about the mechanisms of mating and targets of sexual selection in *A. cristatellus*, it is clear that female multiple mating is a substantial component of this species' mating system in nature.

Introduction

Animal mating requires a complex sequence of behavioral, physiological, ecological, and biomechanical events. The opportunity to mate is first determined by the spatial and temporal proximity of potential mates (DuVal et al. 2018); those that encounter one another more often may be more likely to mate. Yet, in most animal taxa, many encounters do not directly lead to copulation (reviewed in Jennions and Petrie 1997; Johnstone 2000). Among those individuals who are capable of and receptive to mating, a wide range of traits can influence whether an encounter will lead to copulation, including the size,

social status, access to resources, or courtship displays of the potential mates (e.g., Cowlishaw and Dunbar 1991; Borgia 1995; Tokarz 1995; Martin and Martin 2007). These traits arise from both physiological and biomechanical features of the organism itself, as well as from their interactions with social and ecological environments (Fusani et al. 2014; Conley et al. 2021). Thus, predicting an individual's reproductive success, or the distribution of reproductive success among individuals in a natural population, is a complex and challenging task.

This complexity is often apparent in taxa with a disconnect between the social organization of a

population (a behavioral description of which individuals interact, in the context of space and time) and its mating system (a genetic description of whom reproduces with whom; Avise et al. 2002; Griffith et al. 2002; Uller and Olsson 2008; Boomsma et al. 2009). While the existence of such discrepancies has been acknowledged for decades (e.g., Burke and Bruford 1987; Gibbs et al. 1990), they often continue to challenge efforts to identify the traits under sexual selection in natural populations. This is because traits that we expect to be under sexual selection are often behavioral and morphological, and are thus more intuitively understood in the context of a species' social organization, whereas the strength of sexual selection is calculated based on genetic assessments of mating. Here, we suggest that adopting a sequential consideration of components of the mating process, from encounters between potential mates to offspring production, can help to reconcile discrepancies across social organization and genetic mating system, thus clarifying our understanding of sexual selection (Kokko and Mappes 2013; Kamath and Losos 2018).

Lizards in the widely-studied genus *Anolis* have long been described as territorial, with lizards of both sexes exhibiting some degree of site fidelity and behavioral defense of a (somewhat) exclusive space. Stamps (1983) argued that among territorial lizards, male territories are generally larger than female territories, such that they encompass multiple female territories, and that female territory location (in relation to the distribution of males) is determined by habitat quality, and not by the quality of male traits. Because females were not thought to leave their territories to mate, female choice was traditionally expected to play a minimal role, if any, in determining which males reproduce. However, authors from Rand (1967) to Tokarz (1998) to Johnson et al. (2010) have demonstrated that natural populations of anoles from multiple species exhibit turnover in territory occupancy and extensive spatial overlap in their home ranges, presumably providing ample opportunity for multiple mating encounters. Further, genetic studies of anole populations have revealed the prevalence of female multiple mating, and consequently suggest the potential for sexual selection to act through female mate choice (Passek 2002; Calsbeek et al. 2007; Harrison 2014; reviewed in Kamath and Losos 2017). Thus, anole lizards offer an opportunity to explicitly test hypotheses of sexual selection in nature by considering social organization and mating system concurrently.

In this study, we combine field-based behavioral observations and genetic assessments of paternity in the lizard *Anolis cristatellus* to determine what factors influence its mating system. First, we seek to understand the prevalence of multiple mating and the opportunity for female mate choice in *A. cristatellus*. Second, we consider the proximity of potential mates in space and time to characterize the relationship between encountering a potential mate and producing offspring with them. Specifically, in contrast to previous work that has primarily considered spatial proximity between observations of potential mates (using metrics such as home range overlap), we focus on additionally including temporal data in a combined metric of spatiotemporal proximity (following Lyons et al. 2013). Third, we consider if traits that potentially influence male competition or female mate choice (here, male body size and the frequency of male behavioral displays) can predict paternity and the number of offspring. We end by discussing the complexity of factors that may play a role in shaping social and reproductive behavior in this lizard.

Materials and methods

Focal species and study site

Anolis cristatellus Dumeril and Bibron, 1837 is a common and abundant lizard species that occurs in sunny, open areas of Puerto Rico and offshore islands on the Puerto Rican Bank (including the USA and British Virgin Islands; Schwartz and Henderson 1991; Rivero 1998). This species is in the "trunk-ground" ecomorph category (Williams 1983; Losos 1990), and typically perches on tree trunks less than 2 m above the ground, frequently descending to the ground to forage and interact with conspecifics (Rand 1964). *Anolis cristatellus* is a medium-sized, sexually dimorphic species; males (Fig. 1a) of the study population have an average snout–vent length (SVL) of 59 mm, while the average SVL of females (Fig. 1b) in this population is 45 mm. Males interact with male and female conspecifics using displays composed of pushups, headbobs, and extensions of a colorful throat fan called a dewlap (Fig. 1a).

We conducted the field component of this study in July and August 2004 near the El Verde Field Station's Stream House (18° 20.51′ N, 65° 49.49′ W) near Rio Grande, Puerto Rico. The study site was a plot of approximately 550 m² within a 33 m × 23 m rectangle. The plot was located beside the stream Espiritu Santo in a stand of *Syzygium jambos* (Myrtaceae), an introduced tree common in disturbed areas of Puerto Rico (J. Rauscher, personal
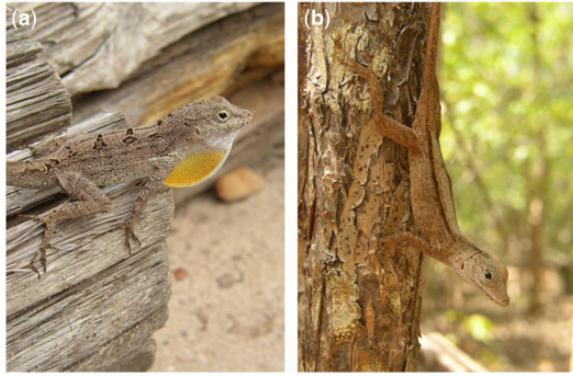
Fig. 1 Adult *A. cristatellus* (**a**) male, extending dewlap, and (**b**) female.

Fig. 2 Study plot beside the stream Espiritu Santo in a stand of *Syzygium jambos* (Myrtaceae).

communication). No spatial variation in habitat quality was apparent across the plot (Fig. 2).

Marking and observation of study animals

Within the study plot, we captured all adult *A. cristatellus* lizards encountered either using a loop of dental floss or by hand. We determined the sex of each individual, and measured its SVL with a clear plastic ruler. To perform undisturbed observations of individual lizards subsequent to their capture, we marked each lizard either by sewing distinct combinations of beads into the most proximal region of the tail (Fisher and Muth 1989) or by attaching queen bee marking tags in unique locations on the dorsum (Johnson 2005). We removed minimal tissue samples (∼10% of the lizard's tail) for molecular analyses and stored these samples in 95% ethanol at room temperature for approximately 1 month, after which they were stored at −80°C. After measurement, marking, and tissue collection, we released each lizard at its site of capture. To ensure that we collected tissue from a wide range of potential sires, we also captured and obtained tissue samples from all males observed within 15 m of the study plot, resulting in a total of 33 candidate sires (27 males within the plot and 6 males outside of it).

Over a period of 3 weeks, we conducted 231 focal observations on 56 marked lizards in the study plot, for a total of approximately 75 h. During these observations, we recorded all display behaviors performed by the individual, measuring the time spent displaying and counting the number of dewlap extensions, pushups, and headbobs. We opportunistically located undisturbed lizards using binoculars, observing each lizard for no more than once per 4 h interval in a day (morning or afternoon). We observed each lizard for three to five 20 min periods, and summed behaviors across observations for each individual for use in statistical analyses. We did not

conduct observations before 08:00 or after 18:00 h, or during inclement weather, as lizards often take refuge during these periods (Hertz et al. 1993). At each observation, we determined the lizard's location within the plot by measuring the distance and compass angle from the perch to one of several predetermined reference points located throughout the plot. Additional location data were opportunistically collected whenever lizards were sighted, but at a minimum of 4 h intervals. At the time of data collection, we were primarily concerned with collecting temporally independent location data, impacting the resolution of our spatial dataset. We measured a median of six locations per lizard (range: 1–13). These measures were converted to Cartesian coordinates for subsequent analysis.

Egg collection

Female *Anolis* lizards lay one egg approximately every 6–10 days, fertilized by sperm stored in sperm storage tubules (Fox 1963; Licht 1973; Conner and Crews 1980). Eggs are buried in the soil or leaf litter and then abandoned. It is therefore difficult to regularly locate eggs in the field and eggs found in this manner would have uncertain maternity. To circumvent these challenges, immediately after the 3-week observation period, we collected 36 of the 45 females from the plot and transferred them to the Animal Care Facility at Washington University in St. Louis, MO, USA. Of these, offspring were obtained from 27 females. As female collection occurred in early August (toward the end of the breeding season), all females had ample opportunity to copulate in the field prior to collection. We individually housed each female, following the standard anole care protocol described in Sanger et al. (2008). Each female cage contained a small plant, *Callisia repens*, where

she laid her eggs. These females from whom we collected offspring are referred to hereafter as "mothers."

We collected eggs 2–3 days each week by removing the plant from the cage and thoroughly sifting through the potting substrate. We then transferred each egg to a standard tissue-culture dish packed with a moist, coarse-grained vermiculite mix (1:1 vermiculite to de-ionized water by weight). Eggs were incubated at 27°C until hatching or egg death. Egg collection continued from mid-August until late December (to maximize the available data on egg paternity), at which point we euthanized all females, removed their livers for molecular analysis, and prepared them as specimens for deposition at the Museum of Comparative Zoology at Harvard University, Cambridge, MA, USA. We collected samples of tail tissue from each hatchling and dissected dead eggs for embryos. All samples were stored at −80°C.

Molecular analyses and paternity assignment

We extracted DNA from each sample using Blood and Tissue Genomic DNA Extraction kits from Viogene (Sunnyvale, CA). We genotyped all individuals at five microsatellite loci (c22, c92, c124, c128, and c136) developed for this species (Glor et al. 2007). These loci were amplified using polymerase chain reactions (PCRs) optimized via experimentation with non-commercial buffers designed at Washington University for use in microsatellite amplification (as described in Glor et al. 2007). Each buffer consisted of 20 mM Tris (pH ranging from 8.4 to 9.2), 50 mM KCl, and 2.5 mM MgCl$_2$. The PCR mix consisted of 2 μL genomic DNA, 2 μL of the preferred buffer, 0.2 μM of each primer, 0.2 mM of each dNTP, 0.25 U taq polymerase (Promega, Madison, WI), and 12 μL distilled H$_2$O, for a total reaction volume of 20 μL. The forward primer of each primer pair was labeled with a fluorescent dye (6-FAM, HEX, VIC, or NED), and the reverse primer was unlabeled. The amplification profile consisted of initial denaturation at 95° for 5 min followed by 30 cycles of 95° for 30 s, 58–63° for 30 s, and 72° for 60 s, and a final extension period at 72° for 10 min. We genotyped PCR products on an ABI 3130 using a 400HD ROX ladder, and alleles were scored using GeneMapper and inspected manually. All mothers and potential sires, and 178 of the 187 eggs laid (95%) were successfully genotyped.

Microsatellite loci c92 and c136 were in Hardy–Weinberg equilibrium in this population; however, null alleles existed for the other three loci. As these loci were highly polymorphic (all five loci had 9–11 alleles/locus), we included them in the paternity analysis nonetheless. Following LeBas (2001) and Husak et al. (2006), all apparently homozygous offspring were typed at only one allele for these loci to prevent offspring carrying their mother's null allele from mismatching with her genotype. Observed heterozygosity at the five loci ranged 0.49–0.83, and the total exclusionary power with these loci was 99.7%.

To determine the paternity of each offspring, we used the software package CERVUS (Marshall et al. 1998; Kalinowski et al. 2007) with the following simulation parameters: 10,000 cycles, 95% of candidate sires sampled, and a genotyping error of 1%. CERVUS assigns paternity to a single male based on the likelihoods of the male being the sire of the offspring, and the male and offspring being unrelated (Jones and Ardren 2003). To confirm that the males in our candidate sire group were not closely related, we used the program ML-RELATE (Kalinowski et al. 2006) to estimate the relatedness among all males in the study. Although null alleles can be problematic in assessments of relatedness (Wagner et al. 2006), null alleles are explicitly accommodated in ML-RELATE (Kalinowski et al. 2006).

Because mismatches between sire and offspring genotypes can also occur as the result of null alleles (known to be present in this dataset), we allowed a single mismatch between an assigned sire and offspring at no more than one locus. When two males were equally likely to have sired an offspring, we assigned paternity to the male whose home range overlapped the female's range, instead of the male whose home range did not overlap the female's range (following a similar approach in McEvoy et al. [2013] and references therein), where home range was estimated as described in Johnson et al. (2010). For example, two males in the study area had identical microsatellite genotypes, and their home ranges did not overlap. Offspring from females whose paternity was assigned to one of these two males, and whose home range overlapped only one of these two males, were assigned to the male with whom the mother overlapped. In another case, a female with nine offspring had seven of the offspring assigned to one likely sire (with whom her home range overlapped). For each of the other two offspring, they were equally likely to have been sired by that male, or by one of two other males, whose home ranges did not overlap the mother's. Thus, all nine of her offspring were assigned to the same male. These paternity assignment decisions were made in an attempt to be conservative in assigning multiple paternity to the offspring of a given female.

Spatiotemporal proximity metrics

Statistical analyses were performed in R v. 4.0.3 (R Core Team 2020). We used a metric of time-scaled distance (TSD; Lyons et al. 2013) to calculate proximity in both space and time for all male–female pairs in the dataset:

$$tsd = \sqrt{(\Delta x^2 + \Delta y^2 + (sv\Delta t)^2)},$$

where Δx, Δy, and Δt are the distance between two points along the x-axis, y-axis, and in time (measured here as day of sampling; two lizards observed on the same day have $\Delta t = 0$), respectively; v (or velocity) is the maximum, across all lizards in the dataset observed twice or more, of the distance between consecutively observed locations of a lizard divided by one plus the number of days elapsed between observations; and s is a scaling parameter. Larger values of s weight proximity in time more heavily, and the effect of s on TSD is greater for pairs that are closer in space.

We expected that male–female pairs that were closer in space and time would be more likely to mate and bear offspring, but had no *a priori* reason to select a particular value of s. Thus, we initially considered a range of values of s from 0 to 1, at an interval of 0.05. Setting the upper limit of this range as 1 implies an assumption that lizards do not move more than v meters per day, the observed maximum across all sampled lizards. For each value of s, we calculated TSD for all pairs of observations for each male–mother pair, and then calculated the minimum TSD for each pair (our best estimate of how close the two individuals were to one another, in space and time) for each value of s. To estimate the best-fit value of s, for each value of s we ran a logistic regression (generalized linear model with a binomial error distribution) with the response variable of offspring borne (a binary variable with a value of either "yes" or "no" for each pair) against the predictor variable of minimum TSD. Models were run using the *lme4* package (Bates et al. 2015). We calculated AIC scores of all logistic regression models to estimate the value of the scaling parameter s for which the AIC score was lowest (i.e., the best fit to the data; note that the number of variables in the logistic regression models does not differ between $s = 0$ and $s > 0$, because minimum TSD was calculated prior to running these models) and used the corresponding values of minimum TSD for subsequent analyses.

To quantify whether male body size and behavior predicted male reproductive success, we used a logistic regression to quantify the effects of the following variables on the binary variable of offspring borne (yes/no) by each male–female pair: minimum TSD, male SVL, and two metrics of male display behavior: proportion of observation time spent displaying and rate of display elements employed (measured as the total number of dewlap extensions, pushups, and headbobs per second of display time). We calculated P-values for each independent variable using a permutation approach wherein we randomized the order of the response variable 10,000 times and recalculated the parameter estimates. Based on previous evidence that larger males have higher reproductive success, we predicted that larger males would sire more offspring, and thus calculated a one-tailed P-value for this statistical test. One-tailed P-values were also reported for the effect of minimum TSD, because we expected individuals that were closer in space-time to be more likely to produce offspring together. Our expectations for the direction of the relationship between display behavior and reproductive success were less clear, and so we calculated two-tailed P-values for these tests.

We then restricted our dataset to those pairs that produced one or more offspring and considered two response variables: the total number of offspring produced by the pair and the proportion of the female's offspring sired by the male in the pair. The first is a measure of absolute fitness of a male within a pair, and is influenced by both male's siring probability and female fecundity. This metric is not a complete assessment of a male's absolute reproductive success because he may have mated with females not in our dataset. The second is a measure of a male's relative reproductive success compared with other males who sired a particular female's offspring, not relative to all males in the population; error in this measure is higher for those pairs where females produced fewer eggs. In short, neither measure is a perfect assessment of male reproductive success, but both allow us to ask about the trait variation between males that are more likely to sire offspring and those that are less likely to sire offspring.

We used generalized linear models to estimate the effects of minimum TSD, male SVL, and display behavior (proportion of time displaying and display rate) on the number and proportion of offspring produced by a male–female pair, using Poisson and Gaussian error distributions, respectively. P-values were again calculated using a permutation approach (one-tailed P-values for minimum TSD and body size, and two-tailed P-values for display behavior).
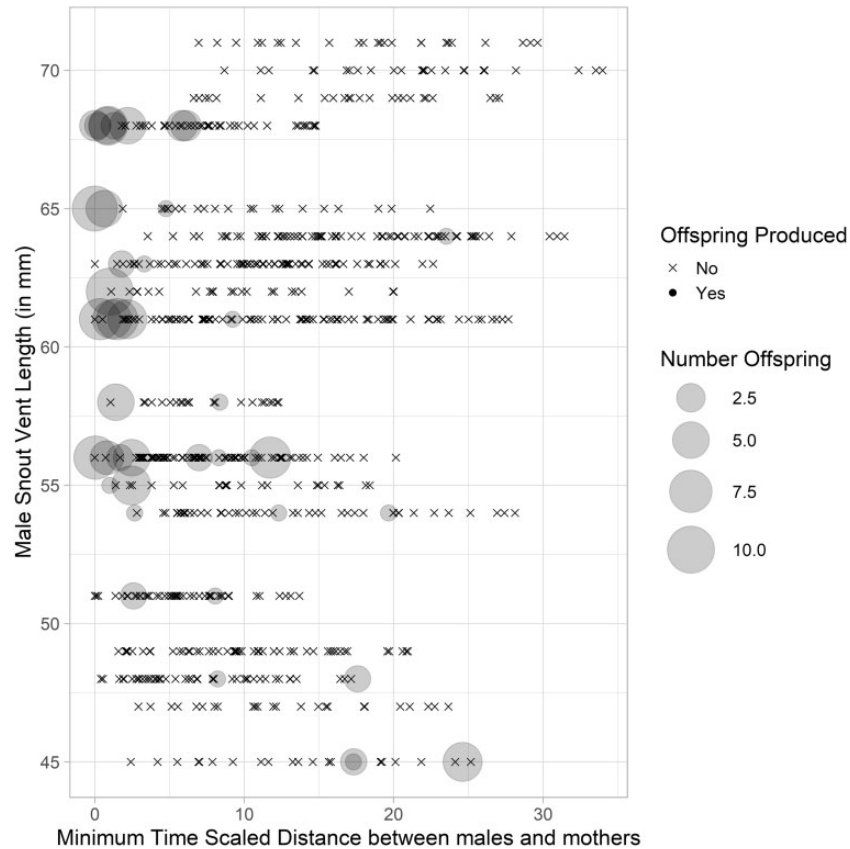
Fig. 3 Male reproductive success is predicted by both spatiotemporal proximity and male body size, but smaller males and farther males did sire offspring.

Results

We collected 178 offspring from 27 females (mean = 6.5 eggs/female), genotyped each individual at an average of 4.75 microsatellite loci, and successfully assigned paternity to 173 of the 178 offspring (97%). The average relatedness among males in the study was 0.069, indicating that close relationships among the candidate sires were not a general problem for paternity assignment in this dataset.

Females laid eggs sired by an average of 1.9 males (range: 1–5); 48% of females had multiple sires for their offspring. Of the females that bore offspring sampled for paternity (referred to as "mothers"), three (with a total of 26 offspring) did not have associated spatial location data and were not included in subsequent analyses.

Δt values (days elapsed between consecutive observations of a lizard) ranged from 0 to 12, and we calculated v to be 21.9 m/day. In calculating minimum time-scaled distance, we estimated that a value of $s = 0.1$ best explained whether a male sired a female's offspring as a function of their spatiotemporal proximity. However, AIC scores for models with values of s ranging from 0.025 to 0.770 were

within 2 of the best fit model, indicating that while including temporal separation between observations of a male and female lizard is important for predicting whether or not they bore offspring together (i.e., our confidence interval for s did not include $s = 0$), our data do not allow for a precise estimation of s. Note that a more sophisticated model that combined the estimation of minimum TSD with the logistic regression would have one fewer variable for $s = 0$ than for $s > 0$, but that is not the case for the analyses reported here.

Spatiotemporal proximity between a male and female was a significant predictor of whether a male sired a female's offspring (time-scaled distance, $\beta = -0.24$, $P < 0.0001$; Fig. 3). However, sires also included males observed far away in distance and/or time from the females who bore their offspring (i.e., those with high TSD; Fig. 3), indicating either a prior encounter (and subsequent sperm storage) or an undetected encounter, which in turn implies a long-distance movement by one or both lizards either before or during sampling. Male body size also predicted whether a male sired offspring ($\beta = 0.07$, one-tailed $P = 0.04$), but sires included some smaller males (Fig. 3). Proportion of time spent displaying

by the male ($\beta = 0.14$, $P = 0.17$), and male rate of display ($\beta = 0.63$, $P = 0.17$) did not predict whether a male sired a female's offspring.

Considering all pairs that bore one or more offspring together, we found that spatiotemporal proximity between a male and female again predicted both the number of offspring produced by the pair ($\beta = -0.09$, one-tailed $P = 0.02$) and proportion of a female's offspring sired by the male ($\beta = -0.03$, one-tailed $P = 0.01$). Neither the number of offspring nor proportion of offspring were associated with male body size ($\beta = 0.01$, one-tailed $P = 0.36$; $\beta = 0.02$, one-tailed $P = 0.09$), proportion of time spent displaying by the male ($\beta = -0.003$, $P = 0.99$; $\beta = 0.008$, $P = 0.87$), or male display rate ($\beta = -0.57$, $P = 0.36$; $\beta = -0.11$, $P = 0.65$).

Discussion

Whether or not females mate with multiple males determines the nature of sexual selection in a population. To assess the opportunity for multiple mating, we can begin by estimating potential mating encounters, the necessary first step of any mating decisions, using behavioral data that quantify individual locations in space and time. Genetic data then allow us to determine, of the potential mates a female encounters, which males successfully sire offspring, and whether those successful males exhibit a nonrandom suite of traits. In this study of a natural population of the crested anole lizard, *Anolis cristatellus*, we find evidence for substantial female multiple mating. Our spatiotemporal data (Fig. 3) reveal that females came within close proximity of multiple potential sires within the sampling duration, suggesting that the opportunity for multiple mating is widespread. Indeed, our genetic data showed that almost half of the females in the sampled population bore offspring sired by multiple males, implying that, at a minimum, almost half of the females mated with multiple males. This prevalence of female multiple mating should inform all future considerations of sexual selection in this species.

Mating pairs of individuals were observed on average within 4.9 m of one another (range 0–24.6 m) within 4.2 days (range 0–9), corresponding to an average TSD of 6.0 (range 0–24.6). Incorporating a temporal dimension into our consideration of proximity between potential mates significantly contributes to predicting paternity, though our methods have some statistical limitations. One could debate the labor costs of collecting temporal data (very low) and the statistical costs of including time as another explanatory variable (see the "Results" section), and it would be worthwhile for future work to further

develop statistical methods for estimating spatiotemporal proximity that can be applied to location data collected at lower temporal frequencies than those afforded by GPS tracking technology, which is at present limited in its applicability to smaller animals such as most *Anolis* lizards (Kays et al. 2015). Collecting the behavioral data necessary for parameterizing mechanistic models specifying individual movement behavior (e.g., Moorcroft et al. 2006) could also be a fruitful next step for quantifying spatiotemporal proximity between potential mates in populations of anoles. Ultimately, the bottom line is that mating requires two individuals to be in the same place at the same time; incorporating time is thus essential to make sense of dynamic space use behaviors and interactions among potential mates.

Yet, not all close encounters lead to the production of offspring. We examined whether two male traits potentially under sexual selection affected offspring production, and we found evidence that body size was a factor in paternity success, but display behavior was not. Male body size was positively associated with siring offspring, consistent with a substantial body of research on lizard mating systems (reviewed in Tokarz 1995; Cox and Kahrl 2014). This relationship could result from female mate choice, but also from male–male competition, age-related physiological changes, and other factors in the chain of events leading to offspring production (Bonduriansky 2003; Cox et al. 2007). But reproductive success was not restricted to larger males (Fig. 3), and surprisingly, among those males that sired offspring with a female, larger males did not sire more offspring than smaller males. Second, male display behavior did not predict paternity success (consistent with Tokarz et al. 2003). This may result from the multiple social contexts in which displays are employed, as anole dewlap, headbob, and pushup displays are used in courtship encounters that precede mating, aggressive encounters with rival males, advertisement displays without a specific target, and predator-pursuit deterrence (e.g., Leal and Rodriguez-Robles 1997; Jenssen and Nunez 1998). Males may exhibit more frequent displays to novel potential mates or rivals. But, particularly if displays are energetically costly or may attract the attention of a predator, they may be less likely to perform displays to familiar mates or rivals (the "dear enemy" hypothesis; Fisher 1954; Paterson and McMann 2004; McMann and Paterson 2012; Bush et al. 2016). Further, individuals may exhibit different behavioral tactics for achieving reproduction; some males may rely on large size to obtain matings without frequent displays, while others may display frequently to

compensate for smaller size. While we did not design this study to test that hypothesis, our data (Fig. 3) suggest it as a possibility. Thus, much remains to be learned about any phenotypic targets of sexual selection in this species, as well as the mechanisms by which such selection might act.

Finally, not all offspring were produced by pairs in close proximity (Fig. 3). Sperm storage across the breeding season (and maybe even from one season to the next) certainly facilitates females bearing offspring sired by males they were not observed as being close to (Tokarz 1998; Calsbeek et al. 2007). Future work needs to consider dynamic movement patterns and sperm storage across long breeding seasons, as well as the complex outcomes of displays and other interactions, to build a full picture of sexual selection in this taxon. We posit that a sequential approach to organizing such investigations will be illuminating.

Data availability statement

The data and code underlying this manuscript are available in github at https://github.com/ambika-math/cristatellusspaceuse.

Acknowledgments

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