Female philopatry as evidence of kin selection in a solitary mammal (#104879)

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Female philopatry as evidence of kin selection in a solitary mammal

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Antipredatory behavior may be shaped by a number of evolutionary and ecological factors, including kin selection. Ground squirrels exhibit alarm vocalizations in the presence of predators; however, the degree to which kin selection shapes alarm calling behavior varies with species ecology and is not fully understood. We studied a solitary ground squirrel species that exhibits sex-biased calling propensity to determine if kin selection influences antipredatory behavior in this species.In this study, we used double digest restriction-site associated DNA sequencing (ddRADseq) to sample the genomes of *Ammospermophilus harrisii* to determine the relatedness between individuals and test whether genetic and geographic distance were correlated. We found that geographic distance had a positive relationship with genetic distance, and that this relationship was sex-dependent, suggesting male-biased dispersal. Our results provide supporting evidence that kin selection affectsantipredatory behavior in this species and may be responsible for higher calling propensity observed in female squirrels. Our findings add to a growing body of evidence that current hypotheses used to explain sociality in highly social animals can be extended to solitary animals.

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Abstract

Antipredatory behavior may be shaped by a number of evolutionary and ecological factors, including kin selection. Ground squirrels exhibit alarm vocalizations in the presence of predators; however, the degree to which kin selection shapes alarm calling behavior varies with species ecology and is not fully understood. We studied a solitary ground squirrel species that exhibits sex-biased calling propensity to determine if kin selection influences antipredatory behavior in this species. In this study, we used double digest restriction-site associated DNA sequencing (ddRADseq) to sample the genomes of *Ammospermophilus harrisii* to determine the relatedness between individuals and test whether genetic and geographic distance were correlated. We found that geographic distance had a positive relationship with genetic distance, and that this relationship was sex-dependent, suggesting male-biased dispersal. Our results provide supporting evidence that kin selection affects antipredatory behavior in this species and may be responsible for higher calling propensity observed in female squirrels. Our findings add to a growing body of evidence that current hypotheses used to explain social behaviors in highly social animals can be extended to solitary animals.

Introduction

Alarm signals are a widespread communication system used to mediate predator-prey interactions (Caro, 2005). Alarm signals may be olfactory (Chivers and Smith, 1998), visual (Blank, 2018; Woodland et al., 1980), or acoustic (Amorim and Dias, 2019), and some species may use electromagnetic alarm signals as well (Scheffel and Kramer, 2006; Dunlap, DiBenedictis & Banever 2010). The evolution of alarm signals has received much research interest due to their seemingly altruistic function. Alarm signals can serve multiple functions, sometimes simultaneously in some species (Zuberbühler et al., 1999; Zuberbüler, 2001; Digweed and Rendall, 2009; Schel et al., 2010). For example, Diana monkeys *(Cercopithecus diana*) emit alarm vocalizations that both deter leopards (Zuberbühler et al., 1999) and warn conspecifics (Zuberbühler, 2000).

Ground squirrels of the family Sciuridae emit alarm vocalizations in response to predators. However, alarm calling behavior and structure can vary substantially across species and between individuals, providing an excellent model system for understanding how ecological and evolutionary selection pressures influence antipredatory behavior (Blumstein and Armitage, 1997). Many ground squirrels live in close proximity to their relatives, and kin selection is thought to be a strong evolutionary driver of alarm vocalizations (Dunford, 1977; Sherman, 1977). Male ground squirrels often disperse away from natal burrows whereas females typically exhibit philopatry (e.g., Holekamp, 1984; Shriner and Stacey, 1991; Neuhaus, 2006). As a result, adult males may be unrelated to surrounding conspecifics, so alarm calling would not benefit adult males via kin selection. Thus, individuals emitting alarm vocalizations may be more likely to be female in species with male-biased dispersal (Dunford, 1977; Sherman, 1977).

The Harris's antelope squirrel (*Ammospermophilus harrisii)* is a solitary species that emits alarm calls throughout the year, regardless of juvenile presence, indicating that alarm calls may be directed toward predators as a deterrent (Burnett and Koprowski, 2020). Opportunistic observations of alarm vocalizations showed a higher proportion of female callers (Burnett and Koprowski, 2020), suggesting that alarm vocalizations may be subject to kin selection. Although adult *A. harrisii* live solitarily in large home ranges, neighboring home ranges overlap (Burnett and Koprowski, 2024) and individuals were occasionally seen sharing burrows (A. Burnett, 2018, unpublished data*)*. Further, *A. harrisii* alarm vocalizations can be high amplitude (*personal observation)* and are structured to carry over long distances (Bolles, 1988), potentially benefiting neighbors if individuals use alarm vocalizations as a warning or if predators are deterred from the area. However, whether neighboring *A. harrisii* are related and kin selection influences calling behavior is unknown, allowing an opportunity to test kin selection theory in a solitary mammal. To understand whether kin selection could influence calling propensity in *A. harrisii*, we sampled the genomes of neighboring individuals to analyze their relatedness and estimate any sex-dependency. If kin selection influences alarm calling behavior, we expect neighboring squirrels to be related. Given our observation of more female antelope squirrels alarm calling (Burnett and Koprowski, 2020), we further expect this relationship to be sex-dependent, showing relatedness between neighboring females but not males. Conversely, if neighboring individuals are not related or dispersal is not male-biased, higher call propensity in females may be maintained primarily through direct fitness or alternative selection pressures (e.g., Blumstein et al., 1997).

Materials & Methods

*Sample Collection and DNA Extraction*_

As part of a larger research study conducted in 2017 and 2018, *A. harrisii* were baited and captured with Tomahawk live traps (model No. 201, Tomahawk Live Trap, Hazelhurst, WI, U.S.A.) in the Santa Rita Experimental Range (SRER), located in the Sonoran Desert approximately 65 kilometers south of Tucson, Arizona (Fig. 1). Traps were checked frequently (once/hour) and shaded with vegetation and/or shade cloth to prevent heat stress and sun exposure. Once captured, we used a cloth handling cone (Koprowski 2002) to minimize stress during handling. We recorded weight, sex, life stage, and reproductive status for each animal captured collected tissue samples with an ear punch (Fisherbrand Animal Ear Punch, 1mm, Thermo Fisher Scientific, Waltham, MA, U.S.A). Squirrels were tagged with sterile passive integrated transponder (PIT) tags (HPT9, 8.4 x 1.4 mm, 0.02-0.04% body weight, Biomark, Inc., Boise, ID, U.S.A.) before being released (*n* = 47 individuals, 51 tissue samples prior to removal of duplicate individuals). Some adult individuals (>110g) were additionally fitted with a radio collar for VHF tracking (Wildlife Materials; <5% body weight; see Burnett and Koprowski 2024). We took no more than one tissue sample per ear from squirrels captured more than once. We received approval from University of Arizona Institutional Animal Care and Use Committee (16-169) and complied with the Animal Welfare Act for all procedures. We additionally followed ethical guidelines for trapping and handling small mammals published by the American Society of Mammalogists (Sikes and Animal Care and Use Committee, 2016). We obtained a scientific collecting permit from Arizona Game and Fish Department (SP501610). We did not give the animals anesthesia or analgesia because the effects of these agents are not well-studied in Harris's antelope squirrels. Animals were not chemically immobilized due to the nature of the procedures (i.e., momentary pain). Lack of chemical immobilization also limits time spent handling, risk of thermoregulatory distress, and additional stress caused by immobilization procedures (Sikes and Animal Care and Use Committee, 2016). The DNA of the collected tissue samples was extracted at the University of Arizona Conservation Genetic Laboratory using a Qiagen DNeasy blood and tissue extraction kit (Qiagen Inc. California, USA). We used a Qubit fluorometer (Invitrogen™, Thermo Fisher Scientific Inc. Massachusetts, USA) to quantify DNA products before sequencing.

Figure 1: **Sampling locations of Harris's antelope squirrels (*A. harrisii*) in the Santa Rita Experimental Range, AZ.** Green dots indicate sampling sites.

Library Preparation and Genomic Sequencing

DNA (1100 ng) from each sample was sent to Floragenex (Oregon, USA) for library preparation and double-digest restriction site associated DNA sequencing (ddRADseq). Library preparation was performed using the restriction enzymes *Pst*I and *M*seI, with a size selection range of 250-800 base pairs (bp). The final pooled library was sequenced on an Illumina HiSeq 3000 with 1x100 bp reads.

Bioinformatic pipeline

We used FASTQC v. 0.11.9 (Andrews 2010) to confirm presence of the enzyme cut sites and assess read quality. We used the program *process_radtags* in Stacks v. 2.60 (Catchen et al., 2013) to demultiplex the pool into individuals based on in-line adapter barcodes, quality filter reads (with a minimum Phred score of 30), and remove reads with missing RAD enzyme cut sites. Following the error clean-up, we used Stacks to assemble loci and call single nucleotide polymorphisms (SNPs).

No reference genomes exist for *A. harrisii*. Thus, we used the Stacks *denovo_map* pipeline with the output from *process_radtags* to *de novo* assemble loci with default parameters. We also applied the --*write_random_snp* flag to obtain one SNP per locus from the *populations* module. We sequentially filtered SNPs using PLINK v. 1.90 (Purcell et al., 2007), removing loci genotyped in less than 75% of individuals (--*geno 0.25*) and loci with a minor allele frequency (MAF) less than 5% (--*maf 0.05*). We further filtered SNPs to remove individuals with more than 50% missing data at the retained loci (--*mind 0.5*). No individuals were removed during this additional filtering step; however, we found that our sample set included duplicates of four individuals, which were subsequently removed.

Genetic Summary Statistics

With our unduplicated dataset containing one SNP per locus, we reran the *populations* module to generate population-level genetic summary statistics (observed and expected heterozygosity; H_O and H_E, respectively), nucleotide diversity (π, considering variant and invariant sites), and inbreeding coefficient (F_{IS}). We performed an additional run of *populations* using a population map file denoting males and females to compare results between sexes in downstream analyses. To estimate effective population size, we used the software NeEstimator v. 2.1 (Do et al. 2014) with the linkage disequilibrium method and a minor allele frequency cutoff of 0.05.

Genetic Structure Analyses

To better understand population structure, we visualized our data with the R package *pophelper* (Francis 2017) and ran a Principal Component Analysis (PCA) as well as a Discriminant Analysis of Principal Components (DAPC) using *adegenet* (Jombart 2008). We derived DAPC results based on the K value with the lowest Bayesian Information Criterion (BIC). Additionally, we used ADMIXTURE (Alexander et al. 2009) to analyze population substructure and to determine the most likely number of ancestral lineages, identifying the best-supported K value by the lowest cross validation error.

Relatedness

We used the *related* package in R (Pew et al., 2015) to estimate dyadic relatedness (the dyadml estimator) between individuals. After assessing the distribution of relatedness and finding it non-normal, we used a Kruskal-Wallis test followed by a Dunn post-hoc test with a Bonferroni correction using *FSA* (Ogle et al. 2023) to evaluate statistical differences in relatedness between and within sex.

Isolation by Distance

We tested whether genetic distance and geographic distance were significantly correlated (isolation by distance; IBD), by performing a Mantel test (Mantel 1967) with the *adegenet* package, using geographic Euclidean distance and the inverse proportion of shared alleles (D_{PS}) for each sample pair and 9,999 permutations to assess significance. We further ran the test separately for males (n= 18) and females (n=29) to determine if this relationship differed between sexes.

Results

Genetic Diversity Estimates

We retained 21,958 SNPs following quality filtering and the removal of four duplicate individuals (n=47 individuals). Genetic summary statistics indicated an inbreeding coefficient of 0.079 suggesting that a low level of inbreeding might be occurring within this population. Genetic diversity parameters showed an observed heterozygosity (±SE) of 0.269 ± 0.001 and expected heterozygosity of 0.286 ± 0.001. Furthermore, sampled individuals showed nucleotide diversity (pi) of 0.003 (considering variant and invariant sites). Effective population size based on NeEstimator calculations using a minor allele frequency cutoff of 0.05 was 105.7 (95% confidence interval 105.5, 105.9).

Population Structure

Our population structure results showed that all squirrels sampled fell under one panmictic population. Furthermore, PCA and DAPC results grouped samples under one cluster based on the lowest BIC value (Supplementary Materials Fig. S1 and S2), indicating that all individuals sampled belong to one population. Admixture results identified K=1 as the best supported number of clusters, having the lowest cross-validation error (0.55).

Relatedness

Average relatedness (±SE) between individuals was 0.014 ± 0.001 based on the dyadic likelihood estimator (*n* = 47 individuals). Female-female relatedness (r = 0.021 ± 0.003), female-male relatedness (r = 0.008 ± 0.002) and male-male relatedness (r = 0.015 ± 0.001) were significantly different from each other (Kruskal-Wallis test; $\chi^2 = 181.21$, df = 2, p < 2.2e -16). Results of Dunn post-hoc test showed that male-male relatedness differed from female-male (Dunn post-hoc; Z = -11.8, p = 1.11e -31) and female-female relatedness (Dunn post-hoc; Z = -13.13, p = 6.6e -39). Female-female relatedness and female-male relatedness also differed (Dunn post-hoc; Z = -2.42, p = 0.046).

Isolation by Distance

Genetic distance between squirrels showed a significant relationship with geographic location (Mantel test; R = 0.18, p = 0.001, n = 47), such that squirrels at closer distances were more related. When squirrels were separated by sex, correlation between genetic and geographic distance strengthened and significant patterns remained for females (Mantel test; R = 0.29, p = 1e

-4, n = 29; Fig. 2), but not males (Mantel test; R = 0.03, p = 0.39, n = 18) (Supplementary Materials Fig. S3 and S4).

Figure 2: **Isolation by distance of female Harris's antelope squirrels.** Scatterplot showing the relationship between geographic distance (spatial Euclidean in meters) and genetic distance (inverse proportion of alleles shared between individuals) of female Harris's antelope squirrels (n = 29). Colors represent the relative density of points: red showing higher density, yellow medium density, and blue lower density. Mantel test showed a significant relationship between geographic and genetic distance for females (R = 0.29, p < 0.001) but not males (R = 0.03, p = 0.39) .

Discussion

Harris's antelope squirrels in the SRER showed genetic diversity similar to that of northern and southern Idaho ground squirrels (Barbosa et al., 2021), reflective of small, isolated and fragmented populations (Garner et al., 2005). Our population may exhibit similar genetic diversity due to major physical barriers in all cardinal directions, with cities and major highways positioned to the north and west of the SRER and the Santa Rita Mountains extending from the southern boundary of the SRER to the northeastern boundary.

We found that geographic proximity is a significant predictor of relatedness in our population, particularly relatedness among females, suggesting that kin selection may be responsible for higher calling propensity in females. Relationships between geographic and genetic distance vary across ground squirrel species. Speckled ground squirrels (*Spermophilus suslicus*) show significant positive correlations between genetic and geographic distances (Matrosova et al., 2016), whereas northern and southern Idaho ground squirrels (*Urocitellus brunneus* and *U. endemicus,* respectively*;* Garner et al., 2005), round-tailed ground squirrels (*Xerospermophilus tereticaudus;* Munroe and Koprowski, 2014), and California ground squirrels (*Otospermophilus beecheyi;* Glover, 2018) do not exhibit positive relationships between genetic and geographic distances.

The sex-dependent relationship between geographic distance and genetic distance, as well as the higher relatedness observed between female-female pairs compared to female-male or male-male pairs provides evidence that *A. harrisii* exhibits male-biased dispersal, or female philopatry. Female philopatry is a widespread mammalian trait, particularly in species with polygynous mating systems (Lawson, Handley and Perrin, 2007; Mabry et al., 2013) like those found in ground squirrels. Male-biased dispersal is exhibited in a number of other ground squirrel species (Devillard et al., 2004), including Columbian ground squirrels (Waterman, 1992; Neuhaus, 2006), rock squirrels (*Otospermophilus variegatus;* Shriner and Stacey, 1991), and Belding's ground squirrels (*Urocitellus beldingi;* Holekamp, 1984). Male-biased dispersal in ground squirrels may help prevent inbreeding (Holekamp, 1984) or reduce exposure to female aggression (Neuhaus, 2006).

Our results support kin selection theory, in which solitary females that exhibit overlapping territories are expected to be related, such that tolerance of neighbors benefits females via indirect fitness (Hamilton, 1964; Clutton-Brock and Lukas 2021). Our study area exhibited a uniform distribution of resources at a low spatial density, and antelope squirrels in our study area maintain large, overlapping home ranges (Burnett and Koprowski, 2024). Other solitary species similarly benefit from female philopatry via territory acquisition (Lutermann et al., 2006; Goodrich et al., 2010; Payne et al., *in press*) or thermoregulation (Williams et al., 2013). Females in a number of solitary species, including bobcats (*Lynx rufus;* Janečka et al., 2007; Payne et al., *in press*), Amur tigers (*Panthera tigris altaica;* Goodrich et al., 2010), and brown bears (*Ursus arctos*; Støen et al., 2005), share home ranges with their daughters, resulting in kin-related spatial structure that could have important repercussions for indirect fitness and social relationships (Støen et al., 2005; de Oliveira et al., 2022).. Although female philopatry and kinship theory is useful for understanding the social relationships in some solitary mammals, other solitary species exhibit adaptive social strategies that are maintained by familiarity with neighbors (Siracusa et al., 2019) or reciprocity (Elbroch et al., 2017). For example, North American red squirrels (*Tamiasciurus hudsonicus*) are highly territorial but exhibit behavioral plasticity, reducing effort spent defending their territory (i.e., emitting territorial vocalizations) and increasing time spent in the nest as familiarity with their neighbors increases over time (Siracusa et al., 2019). Pumas (*Puma concolor*) cofeed at kill sites with unrelated individuals and maintain social networks via reciprocity (Elbroch et al., 2017). Thus, a number of ecological factors can select for social structure to evolve across the spectrum of sociality. Our findings add to a growing body of evidence that the principles used to explain these social structures in highly social mammals, such as kinship theory, may also be applicable to solitary mammals.

Our findings highlight the nuanced role that ecological patterns like female philopatry can play in species behavior. Kin selection resulting from high genetic relatedness between neighboring females may be partially responsible for sex differences in alarm calling behavior in *A. harrisii* (Burnett and Koprowski, 2020). Kin selection may have a strong evolutionary influence on antipredatory behavior in many ground squirrels, especially those that are highly social, in which alarm vocalizations likely serve as a warning to surrounding relatives and provide predator details (Ackers and Slobodchikoff, 1999; Owings 2010). However, *A. harrisii* is largely solitary and emits alarm vocalizations at a wide range of amplitudes, including very low amplitudes that do not travel across the landscape and would be difficult for neighboring squirrels to detect (*personal observation).* Additionally, sex bias in calling propensity is nonsignificant under high-risk contexts (e.g., when confined; Burnett and Koprowski, 2020), and thus predation pressure may still be primarily responsible for the maintenance of alarm vocalizations in this species. Although these results show alarm calling behavior in our population of antelope squirrels is likely subject to kin selection, whether alarm vocalizations serve multiple functions is still unclear. During our field observations over the course of two years, we did not observe a clear behavioral response to alarm calls from neighboring antelope squirrels; however, relatives may benefit from alarm vocalizations if predators leave the immediate area to hunt elsewhere (Blumstein et al., 1997). Therefore, kin selection does not necessarily inform the function vocalizations play, and whether vocalization may serve as warnings requires further study. To definitively identify the function alarm vocalizations play in antelope ground squirrels, behavioral observations and playback trials are needed to resolve how both conspecifics and predators respond to alarm vocalizations.

Conclusions

Our study aimed to determine whether kin selection is influencing alarm calling behavior in Harris's antelope squirrels. We hypothesized that relatedness between squirrels would be correlated with geographic distance and that females would be more closely related to neighboring squirrels than males, based on previous findings of greater calling propensity in female antelope squirrels. We found that genetic distance and geographic distance were positively correlated for female squirrels but not males, indicating that kin selection may be responsible for sex differences in calling behavior. Our results also show that dispersal in Harris's antelope squirrel is male-biased. Relatedness between neighboring females supports kin selection theory predicting that solitary females with overlapping home ranges are likely to be related. Low genetic diversity suggests that our population may be somewhat isolated from other populations due to topographical barriers. Further investigation into whether this population is genetically isolated may be warranted. Additional studies on how Harris's antelope squirrels respond to alarm vocalizations are also needed to determine the mechanism through which neighboring ground squirrels may benefit from alarm vocalizations.

Acknowledgements

We thank our field assistants for their help in the field, including A. Blair, M. Bethel, N. Bokanoski, H. Yomantas, and A. Blanche. We also thank K. Bennett, M. Merrick, and V. Greer for their assistance with training and logistics

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Figure 1

Sampling locations of Harris's antelope squirrels (*A. harrisii)* in the Santa Rita Experimental Range, AZ.

Green dots indicate sampling sites.

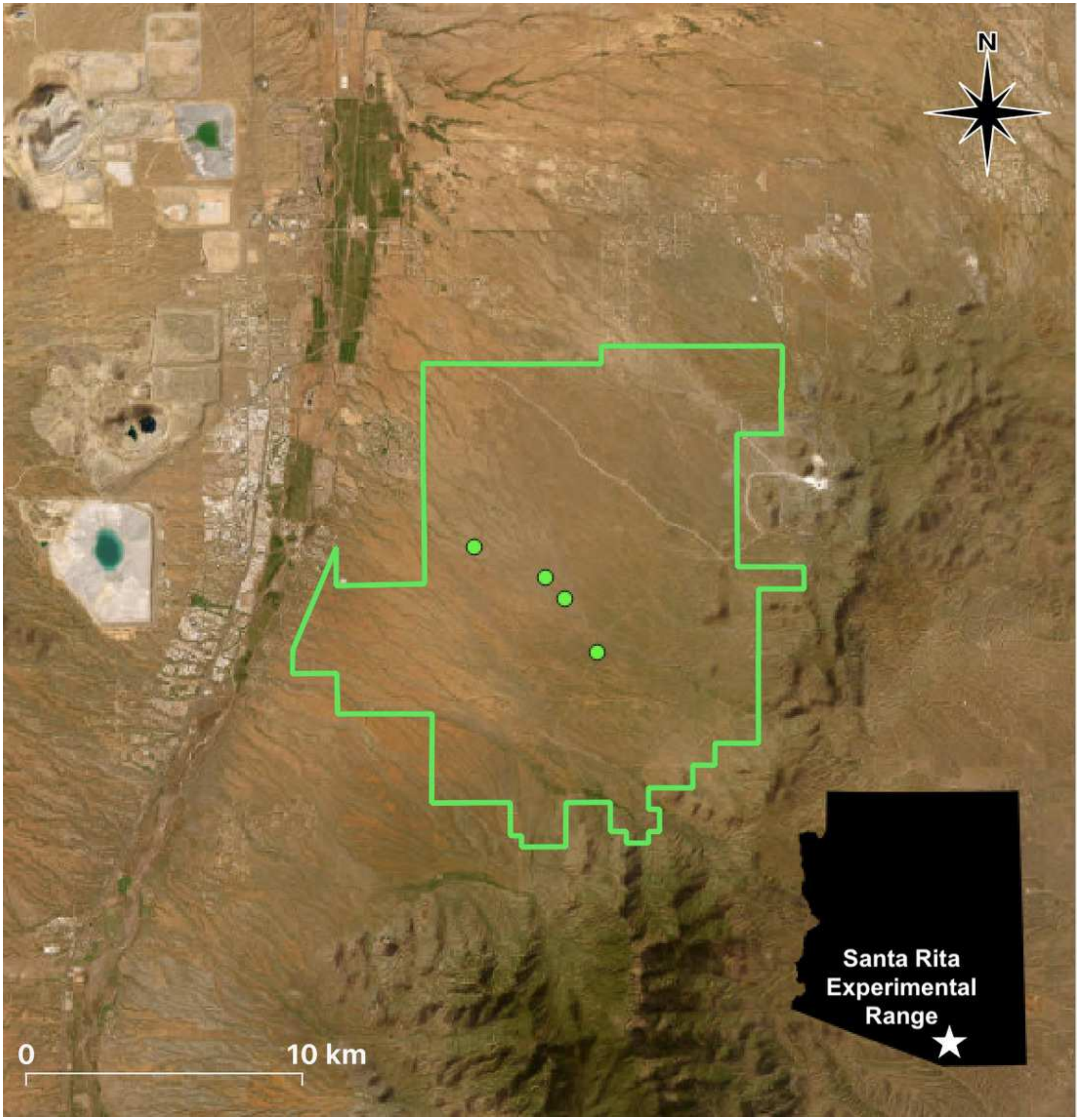
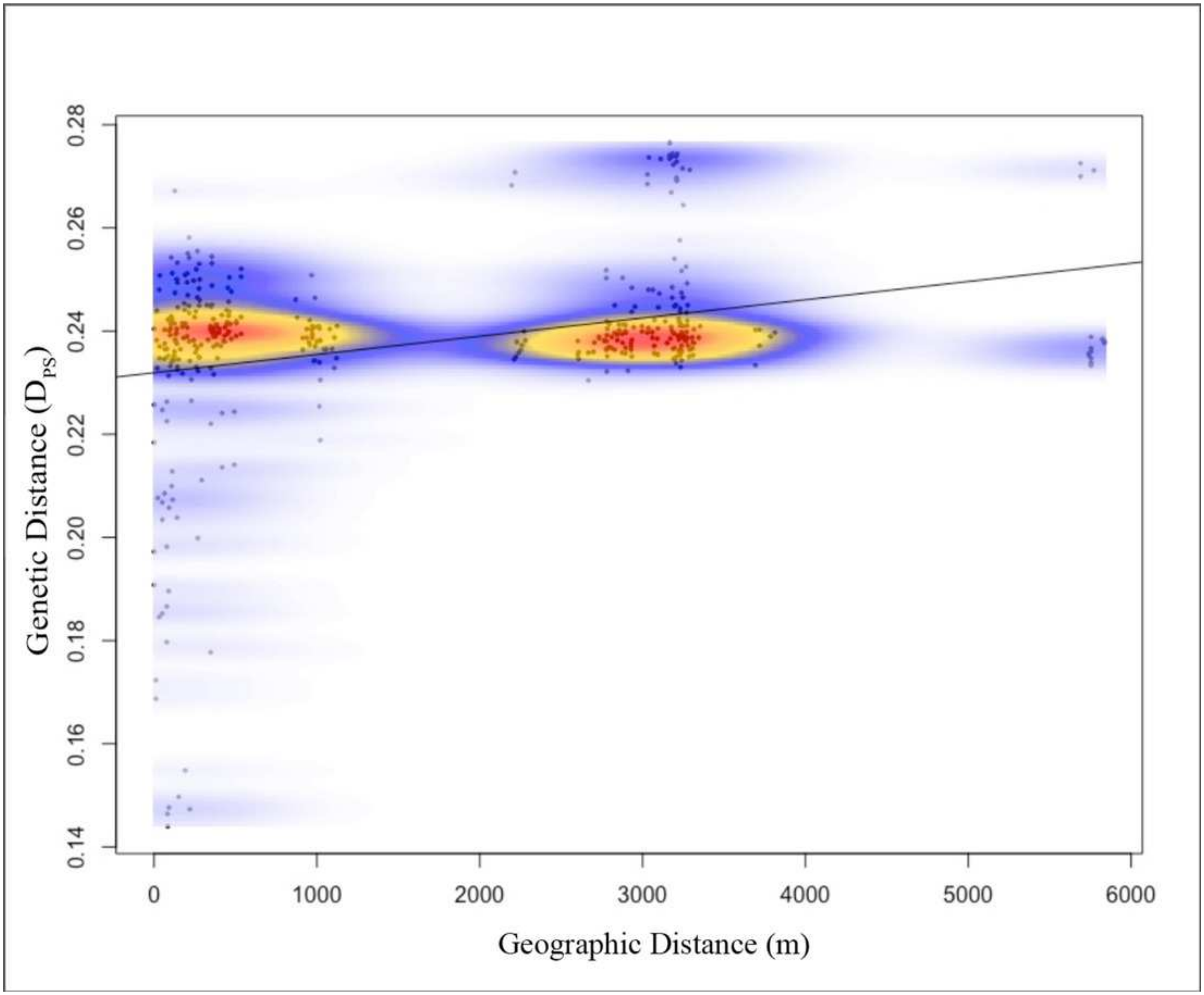

N
Santa Rita Experimental Range
0
10 km

Figure 2

Isolation by distance of female Harris’s antelope squirrels.

Scatterplot showing the relationship between geographic distance (spatial Euclidean in meters) and genetic distance (inverse proportion of alleles shared between individuals) of female Harris’ antelope squirrels (n = 29). Colors represent the relative density of points: red showing higher density, yellow medium density, and blue lower density. Mantel test showed a significant relationship between geographic and genetic distance for females ($R = 0.29$, $p < 0.001$) but not males ($R = 0.03$, $p = 0.39$) .


Genetic Distance (D_{PS})
0.14
0.16
0.18
0.20
0.22
0.24
0.26
0.28
0
1000
2000
3000
4000
5000
6000
Geographic Distance (m)