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Uncovering the adaptive function of group-living in a facultatively social rodent, the highland tuco-tuco (Ctenomys opimus)

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Uncovering the adaptive function of group-living in a facultatively social rodent, the highland tuco-tuco (*Ctenomys opimus*)

By

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Professor Eileen A. Lacey, Chair
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Abstract

Uncovering the adaptive function of group-living in a facultatively social rodent, the highland tuco-tuco (*Ctenomys opimus*)

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Doctor of Philosophy in Integrative Biology

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An individual's social environment can profoundly affect many aspects of their biology, including their behavior, reproductive success, physiology, and survival. Facultatively social species—those in which some individuals live in social groups while other individuals live solitarily—provide an important opportunity to explore the impact of variable social environments on conspecifics that experience similar ecological conditions. Uncovering the relative biological differences between individuals across alternative social environments is critical to the advancement of our understanding of the adaptive benefits and evolution of sociality.

Tuco-tucos (Rodentia:Ctenomyidae) are subterranean rodents endemic to South America, ranging from southern Peru to southern Argentina. There are over 50 described species within the genus *Ctenomys*. Of the species whose behavior has been described, the majority are thought to be solitary meaning that a single adult occupies its own underground burrow, which is spatially distinct from other such burrows. However, recent field surveys are revealing greater complexity of social structure than previously realized, warranting targeted research on the social behaviors of these species. For my dissertation, I aimed to describe the behavior of a population of a previously undescribed tuco-tuco from the highlands in northwestern Argentina (*Ctenomys opimus*), commonly referred to as the highland tuco-tuco. I targeted this species due to anecdotal reports that suggested this population of highland tuco-tucos may be social.

For my first chapter, I used visual observations and radiotelemetry to quantify the spatial movements and consequent social structure of adult highland tuco-tucos located at Laguna de los Pozuelos, Jujuy Province, Argentina (hereafter referred to as Pozuelos). This study revealed that this population consisted of both lone and group-living individuals, and that the number of individuals per group as well as the sex ratio within groups varied markedly. Further, I compared the spatial and social structure of individuals across ecological contexts (i.e., above- versus below-ground) as well as during the daytime and nighttime. I found that social relationships were robust regarding ecological context (above- versus below-ground), but that some groups identified

during the daytime fissioned during the nighttime. Collectively, the findings from this chapter suggested that this population may be facultatively social.

For my second chapter, I aimed to confirm the possibility that the population of highland tuco-tucos located at Pozuelos are indeed facultatively social using spatial data collected over five consecutive years. From these data, I sought to (1) confirm the regular occurrence of both lone and group-living individuals within the population and (2) characterize the temporal consistency of individual social relationships. I found that while the study population consistently contained a mixture of both lone and group-living animals, individual spatial and social relationships varied markedly across time. Specifically, the extent to which individuals remained resident in the same location across years varied, as did the number of conspecifics with which an animal lived, with an overall tendency for individuals to live in larger groups over successive years. Collectively, this chapter indicated that population-level patterns of behavior in this population of *C. opimus* are consistent with facultative sociality but that this variation does not arise due to persistent differences in individual behavior.

For my third chapter, I sought to provide the first characterization of the glucocorticoid physiology in *C. opimus* and investigate how the observed variation in social behavior within the population of *C. opimus* at Pozuelos may impact individual glucocorticoid physiology. Earlier work in another known social tuco-tuco (*Ctenomys sociabilis*) found that yearling females that dispersed from their natal burrow to live alone had higher baseline glucocorticoid levels relative to females that remained in their natal burrow with conspecifics. Thus, for my third chapter, I aimed to determine if a similar pattern was also found in the population of highland tuco-tucos at Pozuelos. I collected fecal samples from all individuals captured on the field site during two consecutive years to assess the relationship between baseline glucocorticoid levels and multiple metrics of social behavior (i.e., group size, sex ratio of group, and metrics measured via social network analysis). Additionally, I conducted a biochemical validation study to confirm that fecal glucocorticoid metabolites provide robust measures of glucocorticoid levels in *C. opimus.* The results from the enzyme-linked immunosorbent assays revealed that corticosterone is the primary glucocorticoid metabolite produced by *C. opimus*. Despite marked variability in social relationships among the animals sampled, differences in social behavior did not appear to predict variation in fecal glucocorticoid metabolites. Rather, individual variability in fecal glucocorticoid metabolites was best explained by sex, with males having higher corticosterone levels than females. This pattern was also observed for individuals in the biochemical validation study. Collectively, this chapter underscores the importance of intrinsic factors (i.e., sex) in shaping glucocorticoid variation in wild populations of mammals.

For my fourth chapter, I sought to provide the first characterization of the gut microbiome in *C. opimus* and investigate how the observed variation in social behavior within the population of *C. opimus* at Pozuelos may impact diversity of gut microbiome composition both within and between individuals. Studies in other mammalian taxa have shown a strong link between gut microbiome diversity and sociality, such that individuals connected with more conspecifics had great microbial diversity. Additionally, these studies have shown that individuals within groups tend to have more similar gut microbiome compositions that individuals between groups, further demonstrating the

effect of sociality on the gut microbiome. Thus, for my final chapter, I aimed to determine if a similar pattern was also found in the highland tuco-tucos at Pozuelos. I collected fecal samples from all individuals captured on the field site during two consecutive years to assess the relationship between gut microbiome diversity and multiple metrics of social behavior (i.e., group size, sex ratio of group, and metrics measured via social network analysis). I found that gut microbiome alpha diversity (diversity within an individual) was best predicted by eigenvector centrality and clustering coefficient, relative to other social network metrics. Further, I found that while gut microbiome beta diversity (similarity between individuals) was not correlated with social network metrics, it was correlated with degree of home range overlap between individuals, highlighting the importance of contact between conspecifics outside of an individual's immediate social group. Additionally, I found that beta diversity clustered by year, likely due to differing preservation methods between field seasons. Sex did not explain variation in gut microbiome alpha or beta diversity. Collectively, this chapter provides the first description of the gut microbiome in highland tuco-tucos and suggests that horizontal transmission plays an important role in maintaining gut microbiome diversity in *C. opimus*.

Dedication

I dedicate this dissertation to Dr. Joseph Cook from the University of New Mexico. I got this far because you believed in me. Thank you.

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Introduction

Tuco-tucos (Rodentia:Ctenomyidae) are subterranean rodents endemic to South America, ranging from southern Peru to southern Argentina (Reig *et al.* 1990; Wilson and Reeder 2005). There are over 60 described species within the genus *Ctenomys* (Parada *et al*., 2011; Lessa and Cook 1998). Of the species whose behavior has been described, the majority are thought to be solitary (e.g., *Ctenomys opimus*—Pearson 1959; *C. australis*—Contreras and Reig 1965; *C. maulius*—Pearson and Christie 1985; C*. mendocinus*—Puig *et al.* 1992) meaning that a single adult occupies its own underground burrow, which is spatially distinct from other such burrows. However, recent field surveys are revealing greater complexity of social structure than previously realized, warranting targeted research on the social behaviors of these species. For my dissertation, I aimed to describe the behavior of a population of a previously undescribed tuco-tuco from the highlands in northwestern Argentina (*Ctenomys opimus*), commonly referred to as the highland tuco-tuco. I targeted this species due to anecdotal reports that suggested this population of highland tuco-tucos may be social.

Because subterranean animals are limited in their ability to move across the landscape, measuring patterns of space use and spatial overlap between individuals can serve as a reliable indicator of an individual's degree of sociality. Spatial data are used to create 95% minimum convex polygons (5% most extreme data points are excluded) of space use for each individual and then percentage of spatial overlap between individuals is calculated. Continuous measures of sociality (e.g., number of overlaps with conspecifics) are used to determine individual degree of sociality. Individuals that overlap spatially with many conspecifics are considered to be comparatively more social than individuals that overlap spatially with few or no conspecifics. Describing this variation in social environment across the population sets up a natural experiment, which allows for a deeper examination of the adaptive function of social behavior.

An individual's social environment can profoundly affect many aspects of their biology, including their behavior, reproductive success, physiology, and survival. Uncovering the relative biological differences between individuals across alternative social environments is critical to the advancement of our understanding of the adaptive benefits and evolution of sociality. Glucocorticoids such as cortisol and corticosterone (colloquially known as stress hormones) are one such measure that can be greatly influenced by an individual's biotic and abiotic environment. Even seemingly small-scale differences between individuals, such as the decision to live alone or in a group, can affect the challenges that an animal experiences as well as how those challenges are perceived (Rogovin et al. 2003; Goymann and Wingfield 2004; Raouf et al. 2006; Creel et al. 2013; Woodruff et al. 2013; Fürtbauer et al. 2014). Consequently, baseline glucocorticoid concentrations can vary greatly within a population. Glucocorticoids play a central role in multiple physiological processes related to allostasis and homeostasis (McMahon et al. 1988; Bartolomucci 2007; Vegiopoulos and Herzig 2007; de Guia et al. 2014; Cain and Cidlowski 2017), and thus socially mediated changes in glucocorticoid concentrations have the potential to profoundly impact individual health and survival.

Similarly, beneficial microorganisms with the gut serve as a key regulator of host health and fitness (Sekirov et al. 2010, Suzuki 2017) and play a major role in diverse host functions (Claus et al. 2008, Wikoff et al. 2009, Cho et al. 2012, Cox et al. 2014, Carlson et al. 2018, Desselberger 2018). In mammals, the acquisition of these microorganisms within the gut, collectively called the gut microbiome, begins at birth via vertical transmission from mother to offspring (Spor et al. 2011, Bonder et al. 2016, Asnicar et al. 2017, Ge et al. 2021) and continues throughout the lifetime of the host due to environmental factors such as horizontal transmission between hosts (Moeller et al. 2018). Sociality—the degree to which an individual host interacts with other, conspecific hosts—is thought to be a key factor that facilitates the horizontal transmission of gut microbiota. (Sarkar et al. 2020). Thus, as individuals come into contact, particularly in group-living species, microbiota are likely to transfer between hosts (Archie and Tung 2015). Therefore, the degree of sociality (or lack thereof) of an individual can directly impact their gut microbiome composition, which in turn may have health and fitness consequences for the individual.

Chapter 1: Facultative sociality in a subterranean rodent, the highland tuco-tuco (*Ctenomys opimus*)

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Introduction

The social environment in which an animal lives can have profound effects on multiple aspects of its biology, including access to mates and other resources (Le Boeuf & Peterson, 1969; Farentinos, 1972; Monaghan, 1985; Creel & Creel, 1995), exposure to predators and pathogens (Griffin, 2004; Prado *et al.*, 2009; Habig *et al*., 2018), and response to environmental challenges (Madison *et al.*, 1984; Madison & McShea, 1987; Schradin *et al.*, 2006; Rabosky *et al.*, 2012). Accordingly, intraspecific variation in social behavior may have significant effects on survival and fitness (Lott, 1991). A fundamental component of the social environment is the number of conspecifics with which an individual interacts on a regular basis. While studies of social structure have typically focused on characterizing a species as solitary or social, the number and frequency of social relationships can vary markedly among conspecifics (Chapman *et al.*, 1995; Creel & Winnie Jr., 2005). Facultatively social species – those in which solitary and group-living animals co-occur in a population and individuals display predictable variation in the extent to which they interact with conspecfics – provide an important opportunity to assess the consequences of differences in the nature or magnitude of social interactions. Potential effects of such variation include but are not limited to differences in stress physiology (Creel *et al*., 2013; Woodruff *et al*., 2013), gut microbial diversity (Tung *et al*., 2015; Moeller *et al*., 2016; Raulo *et al*., 2018), and overall health and immune function (Bartolomucci, 2007; Kappeler *et al*., 2015), indicating that facultative differences in social environment may play a significant role in determining the fitness consequences of interactions with conspecifics.

Because direct observations of social interactions are not possible for all species, numerous studies have employed spatial associations among individuals as a proxy for social relationships (Radespiel, 2000; Blundell *et al.*, 2002; Lusseau *et al.*, 2006; Hinze *et al.*, 2013; Scillitani *et al.*, 2013; Farine & Whitehead, 2015; Lacey *et al.*, 2019). Patterns of space use can generate critical insights into patterns of social behavior. For example, by determining which animals overlap spatially, such analyses can reveal the potential for interactions among specific individuals. Analyses of the temporal patterning of spatial overlap can generate additional insights; social interactions are expected to differ depending on whether individuals use the same portion of the habitat simultaneously (e.g. savanna baboons: Stammbach, 1987) versus at different points during the 24-hr cycle (e.g. coyotes: Atwood & Weeks, 2003). Further, in some taxa, spatial relationships may vary with ecological context (e.g. above versus below ground activity in ground squirrels: Smith *et al.*, 2018), with associated implications for social interactions. As a result, for many species, characterizing variability in spatial relationships among members of a population may reveal the extent to which social relationships vary.

To assess potential variability in social relationships among highland tuco-tucos (*Ctenomys opimus*), we examined patterns of space use within a population of this species from Jujuy Province, Argentina. Like other members of the rodent family Ctenomyidae, highland tuco-tucos are subterranean, meaning that individuals spend much of their time in below-ground burrows (Nevo, 1979; Lacey *et al*., 2000). While most of the > 60 known species of tuco-tucos (Parada *et al*., 2011) have not been characterized with respect to social structure, those that have been studied have generally been found to be solitary, with each adult occupying its own burrow system and displaying minimal if any spatial overlap with other adults (e.g. *C. australis:* Zenuto & Busch, 1998; *C. haigi:* Lacey *et al*., 1998; *C. talarum*: Cutrera *et al*., 2006). A notable exception to this solitary lifestyle is the colonial tuco-tuco (*C. sociabilis*), burrow systems of which are routinely occupied by multiple adult females and, in many cases, a single adult male (Lacey *et al*., 1997; Lacey & Wieczorek, 2004). This interspecific variation in social structure, including pronounced differences between species that occupy the same general habitat (*C. sociabilis* and *C. haigi*; Lacey & Wieczorek, 2003), makes the genus *Ctenomys* an important comparative system for exploring the causes and consequences of variation in social behavior.

Although the highland tuco-tuco has been described as solitary based on the capture of no more than one adult per burrow system in southern Peru (Pearson, 1959), our anecdotal observations of populations of *C. opimus* in northern Argentina suggest that these animals engage in some degree of burrow sharing. Highland tuco-tucos from the latter region are unusual in that they emerge completely from their burrows to forage, with the result that they are visible above ground for extended periods of time. Direct visual observations indicate that multiple adults may use the same burrow entrance when foraging but that individuals vary with regard to the number of conspecifics with which they interact. To quantify the social structure of *C. opimus* and to assess individual variation in the frequency of social interactions, we used a combination of visual observations and radio telemetry to document spatial and social relationships among members of this species. Specifically, we sought to confirm that adults in our study population engage in burrow sharing (a criterion for sociality in subterranean species: Lacey, 2000) and determine whether patterns of social interaction vary with temporal (daytime versus nighttime) or ecological (above versus belowground) context. Our analyses suggest that highland tuco-tucos from northern Argentina are characterized by an intermediate form of social structure not previously described in *Ctenomys*. Further, the animals display marked inter-individual variation in social behavior that provides a foundation for future studies aimed at exploring the adaptive function of potential facultative sociality in these animals.

Methods

Study site. The population of highland tuco-tucos (*Ctenomys opimus*) studied was located in Monumento Nacional Laguna de los Pozuelos (hereafter referred to as Pozuelos), Jujuy Province, Argentina (22°34’ S, 66°01’ W; elevation: 3,600 m). Pozuelos is located in a high Andean valley containing a mosaic of tola (*Parastrephia* sp.) shrubland and more open areas dominated by salt grass (*Distichlis* sp). The study site consisted of an approximately 1.5 ha area of salt grass habitat bordered to the east by the Río Cincel. The site was bounded to the west by tola habitat

and to the north and south by the remnants of adobe walls used historically to contain livestock. Annual rainfall at the site was $\leq$ 200 mm, with most precipitation occurring between December and March (Mascitti, 2001). Data for this study were collected between 24 December 2009 and 9 January 2010.

Animal capture and marking. All procedures were approved by the Animal Care and Use Committee at the University of California, Berkeley, and were consistent with guidelines established by the American Society of Mammalogists for the use of wild mammals in research (Sikes *et al*., 2016) as well as the guidelines of the Association for the Study of Animal Behaviour for the treatment of animals in behavioral research (Buchanan *et al*., 2012). Members of the study population were captured using tomahawk-style live traps baited with carrots. Open traps were placed at active burrow entrances, as identified by the presence of recently excavated soil and fresh fecal pellets as well as observations of animals using those entrances. Trapping was conducted during daylight hours; open traps were monitored continuously, and animals were retrieved immediately upon capture. The location of each capture was recorded using a hand-held GPS unit (accuracy ~ 6 m). Additionally, we recorded each capture locality using a Cartesian coordinate system (8 m x 8 m grid cells) that had been established on the study site prior to the start of trapping. This grid was also used to record the locations of animals during radio telemetry studies (see below) and thus documenting capture localities with the same coordinate system allowed us to more accurately relate captures to home ranges estimated from telemetry data.

Upon first capture, each animal was marked for permanent identification with a uniquely coded PIT tag (IMI-1000, Bio Medic Data Systems, Inc., Seaford, DE) that was inserted beneath the skin at the nape of the neck. PIT tags were read using a hand-held scanner (DAS 4000 Pocket Scanner, Bio Medic Data Systems Inc., Seaforth, DE). For visual identification, each animal was also marked by applying human hair dye to the fur in a unique combination of color patches; dye marks typically lasted 2-3 weeks before needing to be redone. Each time that an animal was captured, its sex and body weight were recorded. Data on body weight were used to determine the apparent age (juvenile versus adult) of each individual. For adult females, reproductive status was assessed based on the appearance of the external genitalia (sexually receptive), the ability to palpate fetuses (pregnant), or the presence of enlarged mammae (lactating). In contrast, because the testes of male tuco-tucos do not descend externally (Zenuto, 1999), the reproductive status of adult males in the study population could not be determined based on visual examination.

Radiotracking of study animals. All adults captured were fitted with radio transmitters (G3-1V transmitters, AVM Instrument Company, Colfax, CA) that were affixed using plastic cable ties as collars. The weight of the transmitter and collar together (~ 7 g) represented < 5% of the body weight of each individual (males: 364.0 $\pm$ 47.8 g, N = 10; females: 309.4 $\pm$ 39.1 g, N = 16), as recommended for studies of small mammals (Sikes *et al*., 2016). Collared animals were released at the point of capture, after which their locations were determined using R1000 receivers (Communications Specialists, Inc., Orange, CA) and 3-element hand-held Yagi antennas (AVM Instrument Company, Colfax, CA). Radio fixes were collected multiple times per day, with a minimum of 1 hour between successive recordings. For each fix, the location of an individual was recorded to the nearest half meter using the 8 m x 8 m grid system established on the study site.

Analyses of telemetry data for transmitters placed at known locations revealed this procedure to be accurate within 0.5 m; these analyses also confirmed the consistency of spatial data collected by different researchers (N = 5). Because these assessments were made under ideal conditions (e.g. daylight, immobile object), we used a more conservative error estimate when analyzing our telemetry data; all fixes occurring within a 1 m radius of each other were treated as the same location. Radio fixes recorded between sunrise and sunset (0700-2000 hrs) were categorized as daytime data points, while fixes recorded from sunset to sunrise (2000-0700 hrs) were designated as nighttime points. During daylight hours, if a collared individual was sighted aboveground at the time that a telemetry fix was made, that datum was noted as a visual sighting of the animal and the location at which the animal was observed was recorded. Although we did not detect evidence of above-ground activity during the night, we were not confident of the accuracy of visual observations conducted in the dark and thus we restricted comparisons of visual versus telemetry data to localities recorded during daylight. At the end of all data collection, individuals were recaptured, and their radio collars were removed.

Spatial relationships. Patterns of space use were analyzed using 95% minimum convex polygons (MCPs) generated with the adehabitatHR package in R (Calenge, 2015). To determine the number of telemetry fixes required to generate robust estimates of individual home ranges, we examined the relationship between number of fixes analyzed and MCP size for a random subset of 6 animals from our study population; this sample size is comparable to other studies that have examined space use in relation to the number of data points per individual (Santos & Lacey, 2011; Lacey *et al.*, 2019). To explore the temporal consistency of individual home ranges, we generated distinct daily MCPs (daytime radio fixes only; N = 5 successive days) and then quantified the percent overlap for MCPs for the same individual; this comparison was conducted for a subset of 6 animals for which we had $\geq$ 10 fixes per day for at least 5 successive days.

To determine if patterns of space use differed when animals were above versus below ground, separate MCPs were constructed for above-ground sightings versus telemetry fixes (animals not visible above ground) for the same individual. Because visual observations were only possible during daylight, the radio fixes used in this comparison were also restricted to those collected during the daytime. Only data from individuals for which $\geq$ 10 visual observations had been obtained were included in this analysis. The sizes of MCPs constructed from visual versus telemetry data from the same individual were then compared and the percent overlap between these MCPs was calculated. Distinct pairwise estimates of spatial overlap between different individuals were generated for both MCPs based on visual observations and those based on telemetry fixes. Because overlap between pairs of animals may not have been symmetric, estimates of percent overlap of MCPs were calculated from the perspective of each individual.

To characterize circadian patterns of activity within the study population and to determine if spatial relationships among individuals differed between day and night, radio fixes were collected hourly for a period of 5 days and nights (120 consecutive hours). Separate MCPs were then constructed for daytime and nighttime fixes for each individual; to avoid potential biases resulting from differences in data collection methods, only telemetry data used for these analyses. The sizes of daytime and nighttime MCPs for the same animal were compared and the percent

overlap between these MCPs was calculated. Based on evidence (see results) that members of the study population are diurnal, the nest site for each individual was identified as the most frequently recorded (modal) x and y coordinates obtained during nighttime telemetry fixes (Urrejola *et al*., 2005). The percentage of fixes that an animal spent at its putative nest site was calculated using the standard 1-m error distance described above. To account for the unknown sizes of nests (i.e. the potential for animals to change locations while remaining in the nest), the percentage of fixes falling within 5 m of the modal x and y coordinates for each animal was also calculated and this value compared to the percentage of fixes assigned to the nest using the more conservative 1 m error distance.

Social network analyses. To identify spatially distinct groups of animals and to assess potential variation in social relationships among members of the study population, we used social network analyses (Wey *et al.*, 2008; Krause, Lusseau, & James, 2009) to identify the number of significant social interactants per individual. Specifically, pairwise measures of percent overlap between MCPs for different animals were used to generate association matrices that were then analyzed with SOCPROG (Whitehead, 2009) to identify hierarchical spatial clusters of individuals. The fit between association matrices and the resulting clusters was assessed using the cophenetic correlation coefficient, with values ≥ 0.8 considered indicative of a strong correspondence between these data sets (Bridge, 1993). Social groups were identified using the maximum modularity criterion, which provides a measure of the degree to which the study population was divided into distinct spatial units (Newman, 2006; Whitehead, 2008). Cut-off values for significant spatial associations among individuals were generated by SOCPROG for each data set examined. Graphical depictions of networks among spatially clustered individuals were generated using the R package igraph (Csardi & Nepusz, 2006). To compare relationships during the day versus the night, separate network analyses were conducted for each temporal period. To compare relationships when animals were above versus below ground, separate analyses were conducted using daytime spatial data collected visually versus via telemetry; only individuals with ≥ 10 visual observations were included in these analyses.

Statistical analyses. Normality of the data was assessed using Shapiro-Wilks tests, after which parametric or non-parametric statistics were used as appropriate. Statistical analyses were performed using R v. 3.5.0 (R Core Team, 2013). All means are reported $\pm$ 1 SD.

Results

A total of 26 adults (10 males, 16 females) were monitored via telemetry over a period of 17 days. The mean number of days per animal on which telemetry data were collected was 8.9 ± 3.9 (range = 3 – 15) for males and 8.2 ± 3.9 (range = 2 – 16) for females. The number of animals under study increased over successive days as more individuals were captured and marked and thus our data set included multiple days in which all 26 adults were monitored concurrently. An additional 8 adults observed on the study site were not captured (N = 4) or were captured too late in the field season to generate substantial telemetry data (N = 4). Thus, overall, telemetry data were obtained from 76.5% of adults in the study population. The individuals that were not monitored were scattered throughout the study site suggesting that any impact of these animals

on our analyses should have been evenly distributed among the spatial clusters of individuals detected (see below). Further, comparisons of capture localities and localities at which unmarked animals were typically sighted suggested that these unmonitored individuals were unlikely to have overlapped spatially with the apparently solitary individuals identified by our social network analyses (see below). For animals monitored via telemetry, the mean number of daytime radio fixes recorded per individual was 62.9 ± 30.7 (range = 16 – 123); the mean number of visual sightings per individual was 12.9 ± 7.2 (range = 0 – 24). Analyses of daytime telemetry data from a randomly selected subset of individuals (N = 6) revealed that estimated home range size stabilized after ~ 30 radio fixes (Supplementary Figure 1). Radio collars for 4 individuals (2 males, 2 females) ceased functioning before nighttime telemetry data could be collected. As a result, data regarding nighttime spatial relationships were available for only 22 individuals, with 29-30 nighttime telemetry fixes recorded for each of these animals.

Visual observations versus telemetry. Analyses of the subset of 12 individuals for which both visual and telemetry data were available revealed no significant tendency for home range sizes based on telemetry data to differ from those based on direct visual observations (Wilcoxon Signed Rank Test, N = 12, V = 60, p = 0.1, Supplementary Figure 2).

Consistency of space use. Analyses of daytime telemetry data collected across 5 successive days (N = 6 individuals with $\geq$ 10 fixes per day) revealed that the mean overlap for MCPs for the same individual ranged from 33.0% to 52.5%, with a mean coefficient of variation of 0.56 among the animals sampled (Figure 1). Mean pairwise overlap between MCPs for the different individuals in this sample ranged from 18.5% to 45.8% per day (Supplementary Figure 3).

Daytime versus nighttime home ranges. Twenty-two animals were monitored via telemetry for 5 consecutive days and nights. Paired comparisons of daytime and nighttime MCPs revealed a significant tendency for the sizes of nighttime home ranges (90.8 ± 95.6 m^2) to be less than those for daytime home ranges (399.3 ± 334.9 m^2) (Wilcoxon Signed Rank Test, N = 22, V = 250, $p < 0.001$).

For each animal monitored, telemetry fixes revealed a single location at which that individual spent a large proportion of time; this location was the same for both daytime and nighttime fixes for the same animal. During the daytime, the mean percentage of fixes recorded within a 1-m radius of an animal's most frequently used (modal) location was 8.4 ± 7.3% (N = 22 individuals). When these analyses were repeated using a less restrictive 5-m radius around an animal's modal location, this value increased to 27.4 ± 21.4%. For nighttime data, the mean percentages of fixes recorded at an animal's modal locality (N = 22 individuals) were 49.6 ± 20.9% (1-m radius) and 78.9 ± 16.1% (5-m radius). The tendency for individuals to spend a greater percentage of fixes at a single, modal location during the night was not significant for the 1-m radius around the putative nest (Wilcoxon Signed Rank Test, N = 22, V = 110.5, p = 0.87), but was significant for the 5-m radius (Wilcoxon Signed Rank Test, N = 22, V = 210, $p < 0.001$). Analyses of the maximum distance at which each animal was detected from its modal location indicated that individuals traveled significantly further from their putative nests during the daytime (60.9 ± 46.1 m) than during the night (15.3 ± 9.49 m; Wilcoxon Signed Rank Test, N = 22, V = 231, $p < 0.001$).

Of the 22 individuals followed via telemetry during the nighttime, 18 (81.8%) used a single modal locality during all 5 nights of data collection. In contrast, the remaining 4 (18.2%) animals (3 males, 1 female) each used 2 nest localities; for each of these individuals, the most commonly used nest site was shared with conspecifics while the less commonly used nest site was not. The mean percentage of fixes at these animals' primary and secondary locations were 67.5 ± 9.95% and 31.75 ± 9.53%, respectively. Nest use by these latter 4 animals was dynamic, which these individuals switching between their primary and secondary nests both within and between nights.

Male versus female home ranges. When all individuals for which daytime telemetry data were available were considered (N = 26), mean home range size for males (773.1 ± 462.4 m^2; N = 10) was greater than that for females (355.5 ± 248.15 m^2; N = 16); this difference was significant (Mann-Whitney U, W = 114, p = 0.01). For the subset of individuals (N = 12) for which both visual and daytime telemetry data were available, there was no significant difference in mean home range size for males versus females for either data collection method (visual: Mann-Whitney U, N = 4, 8, W = 15, p = 0.93; telemetry: Mann-Whitney U, N = 4, 8, W = 16, p = 0.49). MCPs constructed from nighttime telemetry fixes revealed no significant difference between mean home range size for males (76.9 ± 76.4 m^2; N = 8) versus females (98.7 ± 106.9 m^2; N = 14) (Mann-Whitney U, W = 53, p = 0.80). Maximum distance traveled from the putative nest during the daytime did not differ between males (69.3 ± 28.3 m; N =8) and females (56.1 ± 54.0 m, N = 14; Mann-Whitney U, W = 74, p = 0.23). Similarly, there was no difference in the maximum distance traveled at night by males (16.8 ± 11.5 m, N = 8) versus females (15.3 ± 8.1 m, N = 14; Mann-Whitney U, W = 54, p = 0.91).

Overlap of home ranges. Mean percent overlap of home ranges among individuals for which both daytime and nighttime telemetry data were available (N = 22) was greater during the day (41.9 ± 30.6%) than during the night (26.5 ± 26.6%); this tendency was significant (Wilcoxon Signed Rank Test, V = 604.5, p = 0.009). Mean home range overlap among individuals for which both daytime visual and telemetry data were available (N = 12) was 28.8 ± 28.4% when animals were aboveground (visual data) and 42.6 ± 31.7% when they were belowground (telemetry data); the apparent tendency for overlap to be greater below-ground was not significant (Wilcoxon Signed Rank Test, V = 145, p = 0.07).

Evidence for spatially distinct groups. Analyses of association indices based on overlap of daytime MCPs (telemetry data only) revealed that members of the study population were spatially associated with a mean of 3.7 ± 2.1 conspecifics. Network analyses of the 26 individuals examined generated a cophenetic correlation coefficient of 0.89, indicating a strong correspondence between the association index and patterns of home range overlap. Maximum modularity was 0.71. Based on an association index cutoff of 0.08, these analyses identified 5 distinct clusters of animals plus 1 solitary individual (no significant spatial association with conspecifics detected). Mean overlap of daytime home ranges among individuals assigned to the same cluster was 46.1 ± 31.5% versus 23.6 ± 23.4% among individuals assigned to different clusters; this difference in mean percent overlap was significant (Mann-Whitney U, W = 1129.5, p = 0.003).

Temporal differences in spatial associations. To allow for more direct assessment of potential temporal differences in spatial and social relationships, analyses of daytime spatial

associations were repeated using the subset of 22 individuals for which both daytime and nighttime telemetry data were available. Analyses of this more restricted dataset generated a cophenetic correlation coefficient of 0.90. Maximum modularity was 0.72 and the association index cutoff was 0.1. These analyses revealed the same 5 spatially distinct clusters of individuals described above (Figure 2A); the 4 individuals excluded from these analyses due to the absence of nighttime data included the 1 solitary individual identified from analyses of all radio-collared animals (N = 26; see above). Mean overlap of home ranges among individuals assigned to the same cluster was 46.7 ± 30.0% versus 24.1 ± 24.7% among individuals assigned to different clusters; this difference in mean percent overlap was significant (Mann-Whitney U, W = 485, p = 0.03).

In contrast, analyses of nighttime telemetry data revealed 7 spatially distinct clusters of animals plus 2 solitary individuals (no significant spatial associations with conspecifics detected; Figure 2B). All individuals that were spatially associated at night were also spatially associated during the day; the greater number of nighttime clusters as well as the presence of the 2 apparently solitary animals was due to the subdivision of daytime clusters; all individuals that were spatially associated at night were also spatially associated during the daytime (Figure 2A-B). The cophenetic correlation coefficient for analyses of nighttime data was 0.97. Maximum modularity was 0.82 and the association index cutoff was 0.05. Although the mean number of individuals per nighttime cluster (2.4 $\pm$ 1.2, N = 7 clusters) was less than that for daytime clusters (4.3 $\pm$ 2.6, N = 5 clusters), this difference was not significant (Mann-Whitney U, W = 39.5, p = 0.14). Clusters containing more than 1 adult were typically female-biased (daytime: 3.2 females per male; nighttime: 1.8 females per male), although there were also daytime (N = 2) and nighttime (N = 1) clusters containing multiple adult males. Mean overlap of home ranges for individuals assigned to the same nighttime cluster was 25.3 ± 26.6% versus 4.9 ± 1.2% among individuals assigned to different clusters; this difference in mean percent overlap was significant (Mann-Whitney U, W = 151.5, p = 0.009).

Above- versus below-ground associations. Spatial associations based on MCPs constructed from direct visual sightings (animals located above ground) versus daytime telemetry fixes (animals located below ground) were completed for the subset of 12 individuals for which $\geq$ 10 visual sightings were obtained. The cophenetic correlation coefficient for visual data was 0.96 and maximum modularity was 0.60. Based on an association cutoff of 1.5, 4 spatially distinct clusters of animals as well as 2 solitary individuals were detected (Figure 2C). Analyses of daytime telemetry fixes for this subset of individuals revealed 3 clusters of individuals plus the same 2 solitary animals detected from visual observations (Figure 2D). The cophenetic correlation for the telemetry data was 0.90, with a maximum modularity of 0.51 and an association cutoff of 0.07. The smaller number of clusters detected via telemetry was due to the merger of two distinct clusters revealed by analyses of visual data.

Nest sharing. Comparisons of the modal nighttime location(s) identified for each individual revealed that multiple animals shared the same putative nest site during each night of data collection. Of the 22 individuals monitored during nighttime, only 2 (9.0%) were never detected at the same putative nest as other conspecifics (Figure 3). In contrast, 16 (72.7%) individuals were consistently found at the same putative nest site with one or more conspecifics. The remaining 4

(18.3%) animals (3 males, 1 female) had 2 nest localities each: for each of these animals, the most commonly used nest site was shared with conspecifics while the less commonly used site was not. With one exception (Figure 3c), all individuals that shared nighttime nests belonged to the same spatial cluster, as identified from daytime telemetry fixes.

Discussion

Our analyses of spatial relationships indicate that the population of *C. opimus* at Pozuelos is group living. Individual home ranges were larger during the day than at night, but the location at which each animal was most frequently detected (i.e. its putative nest site) was consistent across both time periods. Spatial relationships among individuals did not differ with ecological context, specifically whether individuals were observed above-ground or detected below-ground via telemetry. Although spatial clusters of animals were generally consistent throughout the 24-hour cycle, two daytime clusters appeared to fission at night, with the result that individuals in these groups tended to be associated with fewer conspecifics during the nighttime. All individuals that shared a nighttime nest site were assigned to the same daytime spatial cluster. In contrast, some animals that were spatially associated during the day occupied different nest sites at night. As a result, while social relationships tended to be linked to occupancy of a shared nocturnal nest site, this was not always the case, indicating that nest site alone was not a reliable predictor of spatial relationships among individuals.

In addition to spatially distinct clusters of individuals, our analyses revealed the presence of several animals that were apparently not associated with conspecifics. Because not all adults in the study population were fitted with radio collars, we cannot exclude the possibility that the "solitary" animals detected were in fact associated with individuals that were not monitored via telemetry. Visual observations, however, revealed that the animals for which telemetry data were lacking were scattered throughout the study site and did not occur in close proximity to apparently solitary individuals, suggesting that our identification of the latter was correct. More importantly, even if all adults in the study population had been followed via telemetry, variation in the number of individuals per spatial cluster would still have been evident, as would the tendency for some spatial clusters to fission during the night. Thus, while our data may not have captured the full composition of all spatial clusters of individuals, we believe that the general patterns revealed by our analyses are robust and provide a reasonable reflection of spatial and social structure in the study population.

Effect of ecological context: above- versus belowground relationships. Individuals at our study site were often sighted foraging and sunning aboveground during daylight hours; this behavior seemed to be influenced by weather conditions, with animals being most visible on sunny days with little wind. At all other times, individuals were below-ground and their locations could only be detected via telemetry. This variability in surface activity allowed us to assess above- versus below-ground spatial relationships independently of circadian patterns of activity. Our analyses revealed the same clusters of individuals for both above- and below-ground data sets, suggesting that spatial relationships were stable across these ecological contexts. Similar results have been reported for California ground squirrels (*Otospermophilus beecheyi*: Smith *et al*., 2018), in which

social network connections observed when individuals were above-ground were generally the same as those detected when the animals were below-ground. This consistency in spatial relationships has important implications for understanding the adaptive benefits of group living members of the study population. More specifically, differences in spatial relationships were not detected when individuals were above- versus below-ground, suggesting that the selective pressures favoring group living in this population do not differ significantly according to whether the animals are in their burrows or active on the soil surface.

Temporal variation in relationships. Home ranges were smaller, maximum distances traveled from the nest were shorter, and percentages of fixes at putative nest sites were greater during the night than during the day, suggesting that members of the study population are diurnal. Although most spatial clusters of animals persisted throughout the 24-hour cycle, 2 daytime clusters appeared to fission at night. As noted above, these nighttime clusters were subsets of larger, daytime clusters; in no case did an individual spend the night with animals with which it was not associated during the day. Similar variation in daytime versus nighttime patterns of spatial relationships have been described in degus (*Octodon degus*: Ebensperger *et al*., 2004) and cururos (*S. cyanus*: Lacey *et al*., 2019). While this temporal difference in behavior may increase the complexity of assigning individuals to social groups based solely on patterns of daytime space use, spatial overlap among members of our study population that were assigned to the same daytime cluster was significantly greater than that among individuals assigned to different clusters, suggesting that group membership in *C. opimus* can be reliably determined based on daytime spatial relationships. Nevertheless, comparing diurnal and nocturnal patterns of space use is important because circadian differences in spatial relationships may reflect biologically important differences in activity (e.g. foraging during daylight) that shape interpretations of the adaptive bases for social relationships among individuals. Because the data considered were collected during a single, limited portion of the year, future studies will benefit by assessing spatial and social relationships – including potential circadian differences in these parameters – across multiple seasons and portions fo the animals' annual reproductive cycles.

Evidence for group living. Spatial relationships among individuals were consistent with the 2 criteria typically used to identify sociality in subterranean rodents (Lacey *et al*., 2000). First, members of the study population displayed extensive belowground spatial overlap, providing evidence that these animals meet the criterion that multiple adults share the same burrow system. Second, most individuals shared their nest site(s) with conspecifics, thereby fulfilling the second criterion for sociality in subterranean species. Because members of the study population were less active at night, sharing of nest sites during this portion of the 24-hour cycle may be particularly informative regarding social relationships among individuals (Lacey *et al*., 2019). Burrow and nest sharing have been used to identify group living in other subterranean species, including colonial tuco-tucos (*C. sociabilis*: Lacey *et al*., 1997), naked mole rats (*Heterocephalus glaber*: Bennett & Faulkes, 2000), Damaraland mole-rats (*Fukomys damarensis*: Faulkes and Bennett, 2007), and cururos (*S. cyanus*: Lacey *et al*., 2019) and our data provide compelling evidence that the population of *C. opimus* at Pozuelos is also social.

To date, telemetry data have been used to characterize spatial and social relationships for only 7 of the $\geq$ 60 recognized species of ctenomyids (Figure 4). Of these, 4 species have been classified as solitary, meaning that each adult occupies its own burrow system (*C. australis:* Cutrera *et al*., 2010; *C. haigi:* Lacey *et al*., 1998; *C. minutus:* Kubiak *et al*., 2017; *C. talarum:* Cutrera *et al*., 2006; Cutrera *et al*., 2010). Although occasional spatial overlap among adults has been reported for *C. rionegrensis*, individuals do not appear to routinely share burrow systems and do not share nest sites (Tassino *et al*., 2011, Estevan *et al*., 2016) and thus, we have included this species with the solitary taxa shown in Figure 4. In contrast, *C. sociabilis* is clearly social (i.e. group living) based on the criteria outlined above, with multiple adults regularly sharing the same burrow system and nest site (Lacey *et al*., 1997; Lacey & Wieczorek, 2004; Izquierdo & Lacey, 2008). In comparison, our data suggest that *C. opimus* displays a form of sociality in which individuals share burrow systems and nests but group structure is somewhat more fluid than that in *C. sociabilis*, in which, social groups are clearly distinct (i.e. no overlap between animals from different spatial clusters) and there are no differences in the daytime versus nighttime compositions of spatial groups (Lacey *et al*., 1997; Lacey & Wieczorek, 2004). In contrast, although home range overlap in *C. opimus* was greater for individuals assigned to the same spatial cluster, individuals assigned to adjacent clusters did overlap with one another. Further, the composition of some clusters differed between daytime and nighttime, providing evidence of a temporal variability in behavior not observed in *C. sociabilis*. Collectively, these contrasts lead us to suggest that the population of *C. opimus* at Pozuelos is characterized by an intermediate form of spatial and social structure not previously reported for ctenomyids.

Individual variation in spatial and social relationships. The term *facultative sociality* has been used to describe the behavior of populations or species in which individuals vary in their degree of spatial and social interaction with conspecifics. Vertebrate species that have been characterized as facultatively social include European badgers (*Meles meles*: Newman *et al*., 2011), California ground squirrels (*Otospermophilus beecheyi*: Smith *et al*., 2016), yellow-bellied marmots (*Marmota flaviventer*: Blumstein, 2013) Amazon red squirrels (*Sciurus spadiceus*: Eason, 2010), yellow mongooses (*Cynictis penicillata*: Balmforth, 2004), and eider ducks (*Somateria mollissima*: Öst *et al*., 2015). This term has also been used to describe multiple invertebrates, notably some species of carpenter bees (*Ceratina australensis*: Rehan *et al*., 2010; *C. calcarata*: Shell & Rehan, 2017) and sweat bees (*Megalopta genalis*: Wcislo, 1997; Smith *et al.*, 2018). Our analyses have revealed a similar pattern of spatial and social variation in *C. opimus*, suggesting that this species – at least the population at Pozuelos – may also be facultatively social.

Identifying examples of facultatively sociality, however, may be more challenging than this discussion suggests. Definitions of this term differ and include individual- as well as population- and species-level variation in social behavior. We suggest that facultative sociality should refer to systems in which members of a population display consistent, predictable, and adaptive variation in spatial and social relationships. Differences in the degree to which animals are spatially and socially connected should not result solely from stochastic factors (e.g. lone animals arising due to mortality of social partners) but should instead reflect adaptive variation in individual responses to intrinsic (genotypic, phenotypic) and extrinsic (ecological, environmental) factors. To determine if apparent differences in degree of social connectedness among highland tuco-tucos meet this

more restrictive definition of facultative sociality, future studies of these animals will (1) examine the consistency of individual differences in behavior over longer timescales, (2) assess the fitness consequences of these differences, and (3) relate individual variation in spatial and social relationships to phenotypic and environmental parameters. These analyses should generate important insights into the factors associated with individual-level differences in social connectedness reported here. More generally, studies of *C. opimus* – in conjunction with analyses of other rodents characterized as facultatively social – should improve our understanding of how behavioral differences among individuals intersect with ecological and demographic factors to produce population-level patterns of social structure.

Figures

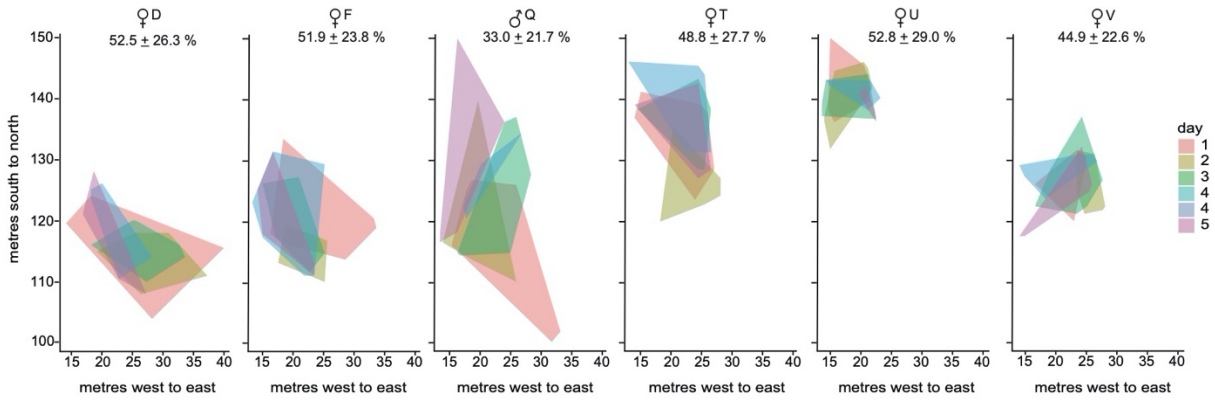

Figure 1. Minimum convex polygons (MCPs) depicting the daytime home ranges of 6 adult *C. opimus* (1 male, 5 females) monitored via telemetry for 5 consecutive days. The x and y axes denote the location of each MCP on the study site. For each individual, a separate MCP was constructed for each day of data collection; colors shown at right indicate the day corresponding to a given MCP. Percent overlap of MCPs for the same individual over the 5 days examined ranged from 3.8% to 98.8%; individual means are given below the animal ID in each panel of the figure.

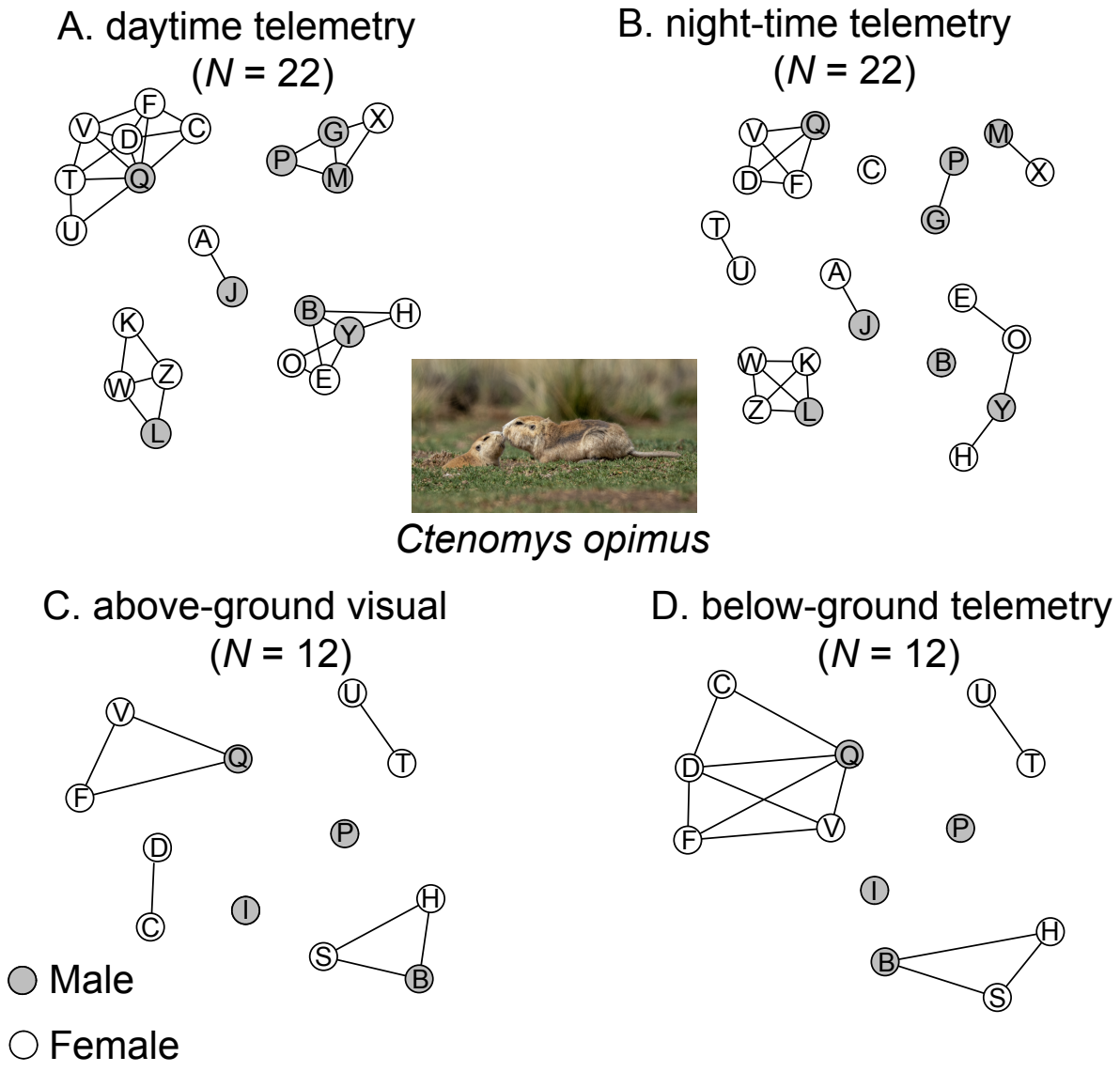

Figure 2. Undirected and unweighted social networks constructed for members of the study population. Networks based on telemetry data from 22 individuals were compared for (A) daytime and (B) nighttime radio fixes. Additionally, daytime networks were compared for 12 animals that were (C) sighted above-ground and (D) detected below ground via telemetry.

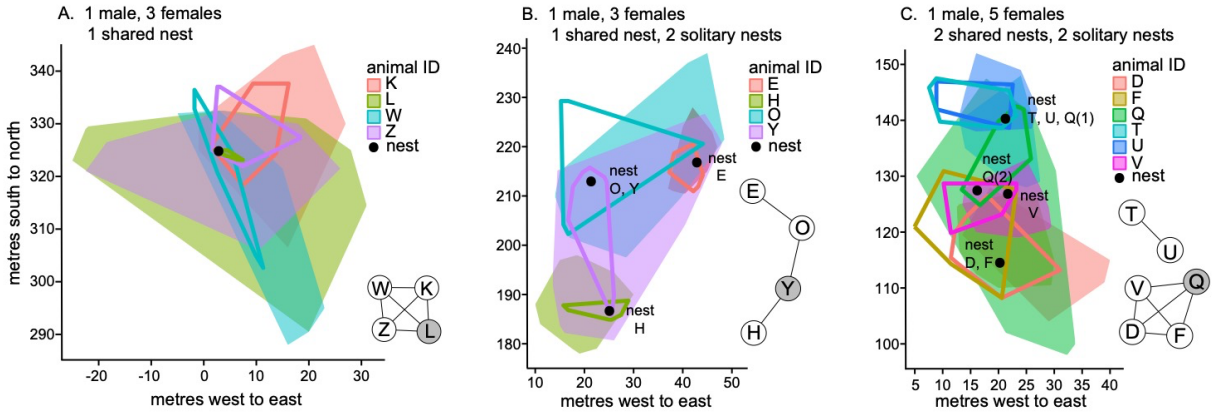

Figure 3. Patterns of nest use versus home range overlap in *C. opimus*. The 95% minimum convex polygons (MCPs) shown depict home ranges for a subset of adults in the study population. The x and y axes denote the location of each MCP on the study site. Each panel depicts home ranges for individuals assigned to the same spatial cluster based on analyses of daytime telemetry fixes. Each colored polygon indicates the daytime home range for one of the animals monitored; the polygon outlined in the same color indicates the nighttime home range for that individual. The identities of each animal are shown in each panel. Solid black circles denote nest sites. Social networks from Figure 2 are included for comparison; individuals in gray are males while individuals in white are females. In (A), all individuals (1 male, 3 females) shared a single nest site. In (B), 4 individuals used 3 distinct nest sites (1 male-female pair and 2 solitary females). In (C), 6 individuals occupied 4 nest sites (2 female-female pairs, 1 solitary female, and 1 male with 2 nest sites). In this last group, the nest most frequently used by male Q "Q(1)" was shared with females T and U. The less frequently used nest for this male, "Q(2)," was distinct from the nests used by the females in this spatial cluster.

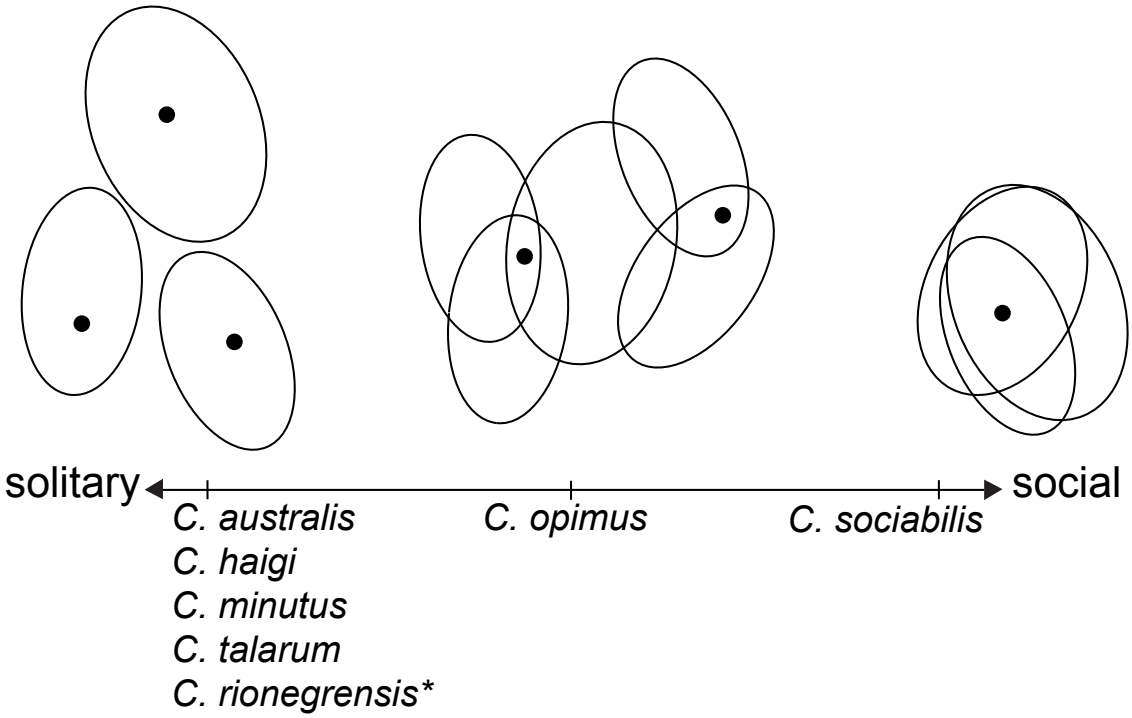

Figure 4. Schematic comparing spatial relationships reported for members of 7 species of *Ctenomys* for which telemetry data are available. Each oval represents the home range of one individual; black circles depict the distribution of nests relative to individual home ranges. Apparent social structures range from solitary (no overlap among individuals) to highly social (consistent, almost complete overlap among multiple adults). *C. opimus* at Pozuelos is the first ctenomyid reported to have an intermediate pattern of spatial and social structure, in which individuals overlap extensively but not completely. Figure adapted from Lacey (2000). Citations are as follows: ***C. australis*** (Cutrera *et al*., 2010), ***C. haigi*** (Lacey *et al*., 1998), ***C. minutus*** (Kubiak *et al*., 2017), ***C. talarum*** (Cutrera *et al*., 2006; Cutrera *et al*., 2010), ***C. rionegrensis*** (Tassino *et al*., 2011; Estevan *et al*., 2016), and ***C. sociabilis*** (Lacey *et al*., 1997; Lacey and Wieczorek, 2004; Izquierdo and Lacey, 2008). *There is minimal evidence that members of C. rionegrensis may engage in occasional spatial overlap (Tassino *et al*., 2011).

In Chapter 1, I used visual observations and radiotelemetry to quantify the spatial movements and consequent social structure of adult highland tuco-tucos located at Pozuelos. This study revealed that this population consisted of both lone and group-living individuals, and that the number of individuals per group as well as the sex ratio within groups varied markedly. Further, I compared the spatial and social structure of individuals across ecological contexts (i.e., above- versus below-ground) as well as during the daytime and nighttime. I found that social relationships were robust regarding ecological context (above- versus below-ground), but that some groups identified during the daytime fissioned during the nighttime. Collectively, the findings from this chapter suggested that this population may be facultatively social.

However, multiple years of data must be examined to confirm that facultative sociality is a consistent feature of the highland tuco-tuco population at Pozuelos and not merely a singular occurrence arising from stochastic factors. Thus, in Chapter 2, I used spatial and social data collected from this population over 5 consecutive years. From these data, I sought to (1) confirm the regular occurrence of both lone and group-living individuals within the population and (2) characterize the consistency of individual social relationships over time.

Chapter 2: Multi-year assessment of variability in spatial and social relationships in a subterranean rodent, the highland tuco-tuco (*Ctenomys opimus*)

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Introduction

Understanding the adaptive bases for differences in social relationships is a fundamental goal of behavioral research. In some species, these differences include the occurrence of both lone and group-living individuals within a population. Such variation – often referred to as facultative sociality – has been reported for numerous taxa, including mammals (Le Roux et al. 2009; Eason 2010; Blumstein 2013; Smith et al. 2016), birds (Öst et al. 2015), reptiles (Riley et al. 2018), fish (Soria et al. 2007), and insects (May-Itzá et al. 2014; Shell and Rehan 2017; Smith et al. 2018). Despite widespread use of this term, the definition of facultative sociality remains unclear. For example, facultative sociality has been used to describe adaptive variation in current social organization (Rabosky et al. 2012; Öst et al. 2015) as well as to imply an evolutionary progression from solitary to group life (Rehan et al. 2010; Shell and Rehan 2018). As part of distinguishing between these fundamentally different interpretations, it is necessary to understand the nature of intraspecific differences in social behavior. In terms of current adaptive function, facultative variation in social relationships should be temporally persistent, meaning that this variation occurs across multiple seasons or years as individuals respond to omnipresent short-term fluctuations in ecological or demographic conditions. Accordingly, a critical step in characterizing a population as facultatively social is to demonstrate that it regularly contains a mixture of solitary and group-living individuals.

Population level variation in social relationships results from the behavior of individuals. In facultatively social populations, lone versus group-living animals may arise for several reasons (Cahan et al. 1999). For example, individuals may vary in their tendency to associate with conspecifics (Lott 1984, 1991), resulting in some animals that consistently live alone while others consistently live in groups, regardless of ecological or demographic conditions. Variation in social relationships may also occur if individuals alter their behavior to better capitalize on the relative fitness benefits of different social options (Rehan et al. 2014; Ortiz et al. 2019). Finally, variation in social relationships may reflect stochastic demographic factors such as recruitment or mortality, each of which may influence the number of conspecifics with which an individual lives (Blumstein 2013; Hatchwell et al. 2013). Although these scenarios are not mutually exclusive, they generate distinct expectations regarding the temporal patterning of social relationships. Specifically, the first predicts that an individual's behavior should remain consistent over time. In contrast, the second predicts that individual behavior will change; if one social option (e.g., living in a group) consistently yields higher fitness benefits (Hayes and Solomon 2004; Lacey 2004; Blumstein et al. 2018) then such changes may be directional, reflecting the tendency for all individuals to move toward the same best fitness outcome. The third scenario predicts that changes in social

environment will not display any consistent directionality. Although facultative sociality likely reflects the complex interplay of one or more of these sources of behavioral variation, these predictions provide a useful framework for exploring relationships between individual and population level patterns of behavior.

One mammal that has been described as facultatively social is the highland tuco-tuco (*Ctenomys opimus*). This subterranean species of rodent occurs in high elevation habitats in Argentina, Bolivia, and Peru (Patton et al. 2015). To date, the only behavioral studies of *C. opimus* that have been conducted were completed in northern Argentina, where these diurnal animals inhabit open grassland areas on valley floors and along waterways. Unlike most other tuco-tucos, *C. opimus* spends a substantial proportion of time foraging above ground, with the result that individuals are fully visible while feeding on salt grass (genus *Distichlis*) and other high Andean vegetation. Both direct observations and analyses of radiotelemetry data indicate that members of the population of *C. opimus* at Laguna de los Pozuelos, Jujuy Province, Argentina are group living, with multiple adults (mean = 3.7 $\pm$ 2.1 SD; range = 1-7) of both sexes sharing burrow systems and subterranean nests (O'Brien et al. 2020). A social group may occupy several nest sites, resulting in variable combinations of group mates that share a nest on a given night (O'Brien et al. 2020). Importantly, these analyses have also revealed the presence of solitary individuals within this population, raising the possibility that *C. opimus* is facultatively social.

These findings were based on only a single season of field work and thus O'Brien et al. (2020) were unable to determine if the observed variation in social behavior is temporally persistent, with a mixture of solitary and group-living individuals present in the population in multiple years. For the same reason, these authors could not evaluate how individual patterns of behavior contribute to population-level differences in social relationships. To explore these aspects of the social organization of *C. opimus* and to determine if the behavior of these animals is consistent with definitions of facultative sociality based on current adaptive function, we documented spatial and social relationships among members of the population at Laguna de los Pozuelos across five consecutive years. Specifically, we sought to determine if (1) both lone and group living animals were present during each year of the study and (2) individual patterns of behavior (e.g., lone versus group living) remained consistent across years. While these analyses focus on the temporal consistency of individual behavior, they provide a critical foundation for future studies aimed at exploring the roles of ecological and demographic correlates of living alone versus within a group. In addition to providing the first longitudinal assessment of the social organization of *C. opimus*, our data generate important insights into interactions between individual- and population-level variability in social relationships in free-living animals.

Methods

Study site. The population of highland tuco-tucos (*Ctenomys opimus*) studied was located in Monumento Natural Laguna de los Pozuelos, Jujuy Province, Argentina (22°34' S, 66°01' W; elevation: 3,600 m); this is the same population of *C. opimus* studied by O'Brien et al. (2020). The ca. 3 ha study site was located near the park entrance, along the western bank of the Río Cincel. Data were collected between November and January during each year from 2010 to 2014. The

mean duration of each annual field effort was 14.6 ± 4.8 SD days (N = 5 years). All field work was conducted during the late austral spring, which corresponds to the primary breeding season for the study population.

Animal capture and marking. Members of the study population were captured using tomahawk-style live traps baited with carrots (O'Brien et al. 2020). Open traps were placed at active burrow entrances, as identified by the presence of recently excavated soil and fresh fecal pellets as well as observations of animals using those entrances. Trapping was conducted during daylight hours; open traps were monitored continuously and animals were retrieved immediately upon capture. Alternatively, trap-averse individuals were captured by hand using a soft, elastic noose that had been placed around an active burrow entrance; this procedure is described in detail in Lacey et al. (1997). The location of each capture was recorded using a hand-held GPS unit (accuracy ~ 6 m). Additionally, we recorded each capture locality using a Cartesian coordinate system (8 m x 8 m grid cells) that was (re)established on the study site each year prior to the start of trapping.

Upon first capture, each animal was marked for permanent identification with a uniquely coded PIT tag (IMI-1000, Bio Medic Data Systems, Inc., Seaford, DE) that was inserted beneath the skin at the nape of the neck. PIT tags were read using a hand-held scanner (DAS 4000 Pocket Scanner, Bio Medic Data Systems Inc., Seaforth, DE). Each time that an animal was captured, its sex and body weight were recorded. Data on body weight and reproductive status were used to determine the age-class (subadult or adult) of each individual during each field season. The reproductive status of adult females was assessed based on the appearance of the external genitalia (sexually receptive), the ability to palpate fetuses (pregnant), or the presence of enlarged mammae (lactating). Body weights for non-reproductive females were significantly less than those of reproductive individuals and thus non-reproductive females were classified as subadults (EAL et al., unpubl. data). In contrast, because the testes of males in the study population never descend externally, the reproductive status of these animals could not be determined based on external appearance. Instead, based on analyses of the distribution of male body weights within the population, individuals weighing less than 300 g were classified as subadults (EAL et al., unpubl. data). To facilitate visual observations of the study animals, human hair dyes (e.g., Manic Panic semi-permanent hair color cream) were used to mark the fur of each individual with a unique combination of colored patches, after which the animal was released at the location at which it had been captured.

Scan sampling of animal locations. Previous analyses of the study population revealed no significant differences between spatial and social relationships identified based on analyses of radio-telemetry data versus direct visual observations of animal locations (O'Brien et al. 2020). For simplicity, only visual observations were recorded during this study. A scan sampling protocol (Altmann 1974) was used to record the localities of all animals visible on the study site. Typically, the study site was divided into three sub-sections, each of which was monitored by a different observer stationed at a fixed location. Scans of each sub-section of the site were conducted simultaneously, with each observer visually searching their portion of the site following a standard pattern. It was not possible to record data blind because our study involved focal animals in the

field. The locality of each animal detected was recorded to the nearest half meter using the 8 m x 8 m grid system established on the study site (O'Brien et al. 2020); estimates of the locations of objects placed at known locations revealed this procedure to be accurate to within < 1 m. Scans (~ 10 min each) were completed multiple times per day with a minimum of 1 hour between successive scans. Scan sampling was conducted during daylight hours (0700-2000 hrs) on most days of each field season.

Spatial relationships. Patterns of space use were analyzed using 95% minimum convex polygons (MCPs) generated with the adehabitatHR package in R (Calenge 2015). MCPs are a commonly used method for visualizing the areas occupied by free-living animals (Harris et al. 1990). Although MCPs may overestimate home range size, exclusion of the 5% of data points that are most distant from an individual's centroid of activity (95% MCPs) reduces this tendency and provides a generally robust procedure for determining if the areas used by different animals overlap, as expected in group-living species (Ebensperger et al. 2004; Sobrero et al. 2014). The minimum number of observations allowed per individual was 6, which exceeds the minimum number of data points required by adehabitatHR to construct a home range (Calenge 2015); during each year of the study, most (> 90%) of the individuals for which 95% MCPs were constructed were characterized by > 10 data points (Table 1). Given that home range sizes tended to increase until ca 30 data points per individual were examined (O'Brien et al. 2020), use of fewer localities to characterize spatial relationships should have been conservative with respect to the size of the area used by an individual and thus the potential for spatial overlap with conspecifics. For animals captured during two or more years of the study, we examined the temporal consistency of patterns of space use by comparing estimates of home range size in successive years.

To characterize spatial relationships among members of the study population, we generated pairwise estimates of percent overlap between 95% MCPs. Because overlap between pairs of animals may not have been symmetric, estimates were calculated from the perspective of each individual in a pair. Within years, percent overlap was calculated for all pairwise combinations of individuals for which 95% MCPs were available; these data formed the basis for social network analyses aimed at identifying distinct social units within the study population (see below). Between years, the consistency of home range locations was assessed by calculating pairwise estimates of percent overlap of an individual with itself; these estimates were generated for all animals present on the study site during two or more successive field seasons.

Social network analyses. To characterize social relationships among members of the study population, we used social network analyses (Wey et al. 2008; Krause et al. 2009) to identify the number of conspecifics with which each individual was associated during each year of the study. Specifically, pairwise measures of percent overlap between 95% MCPs were used to generate association matrices that were then analyzed in SOCPROG (Whitehead 2009) to identify hierarchical spatial clusters of individuals. The fit between association matrices and the resulting clusters was assessed using the cophenetic correlation coefficient, with values ≥ 0.8 considered indicative of a strong correspondence between these data sets (Bridge 1993). Significant clusters of individuals were identified using the maximum modularity criterion, which provides a measure

of the degree to which a population is divided into distinct spatial units; values > 0.3 are generally interpreted as evidence of significant spatial clustering (Newman 2006; Whitehead 2008). To describe the results of these analyses, we use the term "social unit" to refer to any spatially distinct subset of animals identified by SOCPROG, including both lone and group-living individuals.

For individuals captured during two or more years of the study, we evaluated the temporal consistency of social relationships by examining the number of animals with which each individual was spatially associated (i.e., social unit size) during each year that they were present in the study population. We also examined annual changes in several of the social network metrics generated by SOCPROG (Whitehead 2009). The metrics examined were network strength (a measure of the sum of an individual's associations), eigenvector centrality (a measure of how well an individual is associated plus how well their associates are associated), affinity (a measure of the weighted average strength of an individual's associations), reach (a measure of how well an individual is indirectly connected to other individuals in the population), and the clustering coefficient for the network (a measure of how well an individual's associates are associated). Detailed descriptions of these parameters are provided in Whitehead (2009). To assess the consistency of social relationships across years, for all animals captured in two or more successive field seasons we compared the identities of the animals with which they were associated (i.e., the other members of the social unit to which they were assigned) in one year to the identities of the animals with which they were associated in the following year.

Statistical analyses. All statistical tests were performed in R v. 3.5.0 (R Core Team 2013). For two sample tests, normality of the data was assessed using Shapiro-Wilks tests, after which parametric or non-parametric statistics were employed as appropriate. For animals monitored during two or more field seasons, we used linear models to identify predictors of home range size, social unit size, and the extent to which each individual overlapped spatially with itself in successive years. For each of these response variables, Q-Q plots were used to determine the underlying distribution that best fit the data obtained. Based on these analyses, models were constructed as follows:

(1) *Home range size*. Linear mixed models based on a Gaussian distribution were used to identify predictors of home range size, with sex, age-class (adult or subadult), and number of years (1, 2, or 3) onsite as fixed effects and animal ID and year of data collection as random effects. Models were run with and without all possible interactions between predictor variables.

(2) *Social unit size*. Generalized linear mixed models based on a Poisson distribution were used to examine predictors of social unit size. As with analyses of home range size, sex, age-class (adult or subadult), and number of years (1, 2, or 3) onsite were included as fixed effects and animal ID and year of data collection were included as random effects. Models were run with and without all possible interactions between predictor variables.

(3) *Overlap with self*. Linear regressions based on a Gaussian distribution were conducted with sex and age-class (adult or subadult) included as fixed effects. Overlap with self was determined based on comparisons of home ranges in either years 1 and 2 or years 2 and 3 that an individual

was present on the study site. The model was run with and without all possible interactions among predictor variables.

The Akaike information criterion (AIC) was used to identify the best fit model for each response variable. When appropriate based on model outcomes, we used post-hoc Tukey's honest significant differences (HSD) tests to determine if variation in our response variables was influenced by the number of years that an animal was onsite. Kruskal-Wallis tests and Dunn's post-hoc tests were used to examine differences in social network metrics relative to the number of years that an animal was present on the study site. Throughout the text, means are reported $\pm$ 1 SD.

Results

A total of 208 (84 males, 124 females) highland tuco-tucos was captured during the course of this study. Review of trapping records and field notes indicated that the number of uncaught animals ranged from 1 to 4 per year, representing a mean of 7.6 $\pm$ 5.5 % of the individuals present on the site during each year of the study. Of the animals captured, 184 (88.5%; 71 males, 113 females) were observed a sufficient number of times for analyses of spatial and social relationships. The number of animals for which sufficient data could not be obtained ranged from 0 to 5 per year, representing a mean of 4.5 $\pm$ 3.6% of the individuals captured during each year of the study. Of the 184 animals for which home ranges were constructed, 27 (14.7%; 20 males, 7 females) were subadults at the time of first capture. Flooding of the study area during December 2012 resulted in a marked reduction in the number of animals resident on the site during the 2013 field season; although this event reduced the sample sizes for some analyses, it did not preclude efforts to characterize variation in social relationships within or between years. For each year of the study, the dates of data collection, the number of animals monitored, the mean number of days per individual on which data were collected, and the mean number of visual fixes recorded per individual are given in Table 1.

Annual variation in social unit size. Social network analyses generated cophenetic correlation coefficients > 0.8 (range = 0.84-0.97) for all years of the study (Supplementary Fig. 1), indicating a strong correspondence between overlap of 95% MCPs and the association indices generated by SOCPROG. Maximum modularity was > 0.43 (range = 0.43-0.76) in all years, suggesting significant spatial clustering of individuals within the study population. Based on the clusters of animals identified by these analyses (Supplementary Fig. 1), the study population contained a mix of lone and group living animals in four of the five years monitored; the sole exception was the 2012 field season, when only group-living individuals were detected (Table 2). In all cases, lone individuals were adults; 4 (66.7%) of the 6 lone individuals identified were females (Supplementary Fig. 1). Comparisons of home ranges for these animals with field notes and localities recorded for individuals for which home ranges could not be constructed indicated that in no case did putatively lone animals overlap with individuals not included in analyses of social unit size. The number of social units composed of $\geq$ 2 animals ranged from 3 to 9 per year (mean = 5.2 $\pm$ 1.8, N = 5 years). Social unit size (i.e., the number of individuals per social unit) varied significantly across years (one-way ANOVA, $F = 7.46$, $df = 4$, $p < 0.001$). Post-hoc comparisons

revealed that this difference was due to the large sizes of social units during 2012 (Tukey HSD, $p < 0.05$ for all pairwise comparisons including 2012; $p > 0.05$ for all other pairwise comparisons). All social units consisted of adults or a mix of adults and subadults; no social units consisting solely of subadults were detected.

Recaptures of marked animals across years. A total of 39 individuals (12 males, 27 females) were captured during two or more years of the study (Fig. 1). At first capture, 7 (17.9%) of these individuals (4 males, 3 females) were subadults; the remaining 32 individuals were adults when first caught (Fig. 1). With one exception, all individuals were recaptured in consecutive years; the exception was a female that was originally captured in 2010, not recaptured in 2011, but then recaptured in 2012. Most (61.5%, N = 9 males, 15 females) of the animals recaptured were trapped during two consecutive field seasons; the remaining individuals (38.5%, N = 3 males, 12 females) were captured during 3 successive field seasons. Of the 15 individuals captured during 3 successive field seasons, 3 (20%, N = 2 males, 1 female) were subadults at first capture.

Spatial consistency of individuals across years. Of the 39 individuals captured during two or more field seasons, 31 (79.5%, N = 7 males, 24 females) had sufficient spatial data to characterize their home ranges (95% MCPs) for each year in which they were present in the study population; 20 of these animals (64.5%, N = 6 males, 14 females) were captured during 2 different field seasons while the remaining 11 (35.5%, N = 1 male, 10 females) were captured during 3 different field seasons (Supplementary Table 1). Comparisons of AIC values revealed that the best fit model for individual home range size included the interaction between sex, age-class, and number of years onsite as predictor variables (AIC = 1159.69, df = 9; Supplementary Table 2). Number of years onsite was a significant predictor of changes in home range size between years one and two (Tukey HSD, $p < 0.01$), with size increasing significantly between an individual's first (721.5 ± 720.8 m^2) and second ($1806.6 + 1573.6$ m^2) years on the study site (Wilcoxon Signed Rank, $V = 42$, $N = 31$, $p < 0.001$). In contrast, number of years onsite was not a significant predictor of changes in home range size between years one and three (Tukey HSD, $p = 0.08$) or between years two and three (Tukey HSD, $p = 0.91$). Accordingly, there were no significant differences in home range size detected between an individual's first and third years or second and third years on the site (Wilcoxon Signed Rank, both $p > 0.05$).

Recaptured animals varied markedly with regard to spatial consistency across field seasons, ranging from individuals that displayed no overlap with themselves in successive years (N = 1 male, 9 females) to individuals whose home range during their first year was overlapped completely by their home range during their second year (N = 2 males, 2 females; Fig. 2; Supplementary Fig. 2). Comparisons of home ranges from successive years revealed that the mean percent overlap of an individual with itself from year 1 to year 2 was $25.5 \pm 24.3\%$ (N = 31). In contrast, mean overlap with other conspecifics in year 2 was $34.2 \pm 30.7\%$ (N = 31); this tendency to overlap more with other conspecifics was significant (Wilcoxon Signed Rank, $V = 150$, $p = 0.05$).

For animals captured during three different field seasons (N = 11), mean percent overlap of an individual with itself (year 2 to year 3) was $42.1 \pm 22.1\%$ versus a mean of $34.9 \pm 29.8\%$ overlap with other conspecifics (year 3); this difference in overlap was not significant (Wilcoxon

Signed Rank, V = 45, two-tailed p = 0.32). For individuals captured during three successive field seasons (N = 11), there was a significant tendency for mean percent overlap of an animal with itself from year 1 to year 2 (21.6 $\pm$ 23.5%) to be less than that from year 2 to year 3 (42.1 $\pm$ 22.1%; Wilcoxon Signed Rank, V = 4, p = 0.01). Comparison of AIC values revealed that the best fit model for overlap of an individual with itself included sex and age-class as predictor variables (AIC = 284.15, df = 4; Supplementary Table 2). Age-class was a significant predictor of overlap with self (t = 2.15, p = 0.04), with individuals first captured as subadults displaying greater overlap (52.5 $\pm$ 13.6%; N = 6) than individuals first captured as adults (19.0 $\pm$ 21.8%; N = 25); because all individuals were adults in year 2, overlap between years 2 and 3 was not affected by differences in age class.

Social consistency of individuals across years. Comparisons of social unit sizes for animals captured in successive field seasons revealed that no individuals were solitary for more than one year. Of the 6 animals identified as solitary during this study, only 2 (33.3%) were present in the study population for a second year; both of these individuals were assigned to social units containing multiple conspecifics during their second year. No individuals identified as social during their first year were solitary in subsequent years. More generally, of the 31 individuals captured in $\geq$ 2 years, most (58.1%, N = 18) lived in larger social units during their second year; in contrast, 9 animals (29.0%) lived in smaller social units and 4 animals (12.9%) experienced no change in social unit size from their first to their second year (Supplementary Table 3). This distribution differed significantly from that expected if each of these outcomes (increase, decrease, no change in social unit size) was equally likely (X^2 = 9.86, df = 2, two-tailed p = 0.0072). Of the 11 individuals captured during a third year, almost all (90.9%, N = 10) experienced an increase in social unit size from year 2 to 3; social unit size for the eleventh animal did not change.

Comparisons of AIC values revealed that the best fit model for social unit size included sex, age-class, and number of years onsite as predictor variables (AIC = 427.87, df= 6; Supplementary Table 3). Number of years onsite was a significant predictor of differences in social unit size between an individual's first and second (Tukey HSD, p < 0.01) and first and third (Tukey HSD, p < 0.01) years on the study site; in contrast, number of years onsite did not predict differences in social unit size between an animal's second and third years on the site (Tukey HSD, p = 0.21). Social unit size increased significantly from year one to year two (6.3 $\pm$ 3.1 versus 11.0 $\pm$ 7.7 animals/group; Wilcoxon Signed Rank, V = 77.5, N = 31 individuals, p = 0.007) and from year one to year three (7.0 $\pm$ 3.4 versus 14.6 $\pm$ 7.2 animals/group, Wilcoxon Signed Rank, V = 4.5, N = 11 individuals, p = 0.02).

Analyses of social network metrics indicated that values for Eigenvector centrality (Kruskal-Wallis, X^2 = 7.99, df = 2, p = 0.01), network strength (Kruskal-Wallis, X^2 = 19.94, df = 2, p < 0.001), reach (Kruskal-Wallis, X^2 = 21.14, df = 2, p < 0.001), and affinity (Kruskal-Wallis, X^2 = 21.37, df = 2, p < 0.001) varied significantly with the number of years that an animal was present in the study population (Fig. 3, Supplementary Table 4). In contrast, values for clustering coefficients did not differ with the number of years that an individual was present (Kruskal-Wallis, X^2 = 1.84, df = 2, p = 0.40, Fig. 3, Supplementary Table 4). For each of the four metrics that varied, post-hoc Dunn's tests revealed that values were significantly greater for animals during their second year relative to their first year onsite (Table 3). Values for network strength and reach were also significantly

greater for animals in their third year relative to their first year onsite (Table 3). All pairwise comparisons of measures of affinity were significant, with values of this metric increasing with each additional year that an animal was present in the population (Table 3).

Over the course of the study, we identified 9 instances in which > 2 animals resident in the same social unit in a given year were recaptured in the following year (N = 37 recaptures for 30 animals in 9 social units; Table 4; Supplementary Table 5). In 3 (33.3%) cases, all animals (N = 9) assigned to the same social unit in year 1 were also assigned to that social unit in year 2. In the remaining 6 (66.6%) instances, not all animals were resident in the same social unit in year 2; in these cases, an average of 60.8 $\pm$ 31.6% (range = 0.0 – 85.7%; N = 28 animals) of individuals assigned to the same social unit in year 1 were still resident in the same social unit in year 2. A total of 9 individuals (1 male, 8 females) changed social units between years. These changes occurred even though at least one other individual from an animal's social unit in year 1 was still present in the study population in year 2, indicating that these changes were not due to the loss of all other members of an individual's initial social unit.

Discussion

Our analyses revealed intriguing variation in social relationships among members of the study population. Both lone and group-living animals were detected in most years of this study, a pattern that is consistent with species described as facultatively social (Öst et al. 2015; Blumstein et al. 2018; Smith et al. 2018,). However, no animals lived alone for more than one field season and no group-living individuals were later detected living alone. Although there was an overall tendency for social unit size to be smaller during an animal's first year on the study site, the magnitude and the direction of between-year changes in social unit size varied considerably. Further, while most animals remained in the same social unit (i.e., with the same group mates) in successive years, between-year changes in social unit membership were detected despite the continued presence of at least some of an animal's group mates from the previous field season. Members of the study population also displayed marked variation in individual patterns of space use, notably the tendency to overlap spatially with themselves in successive years. Collectively, these findings suggest that the variation in social relationships reported here is not due to persistent differences in individual behavior but instead may reflect short-term responses to variation in factors such as ecological or demographic conditions.

Variation in spatial relationships. Our analyses of social relationships were based on spatial data and thus examining patterns of space use by members of the study population may generate insights into the variability in social behavior reported here. Among animals captured in successive years, the tendency to remain resident at the same location varied, with between-year spatial overlap of an individual with itself ranging from none to almost complete congruence of annual home ranges. Overall, overlap tended to be greater between an individual's second and third years on the study site, suggesting that animals became more spatially consistent over time. Individuals first captured as subadults overlapped more with themselves than did animals first captured as adults, indicating that age may contribute to individual patterns of space use (Rayor and Armitage 1991; Salvioni and Lidicker 1995; Ortiz *et al*. 2019). Variation in between-year overlap may also

reflect differences in dispersal history (Murray 1982; Nelson and Mech 1984; Costello 2010). For example, it is possible that animals first captured as subadults were individuals that had been born on the study site after the previous field season; in contrast, animals first captured as adults may have immigrated to the site. These differences in age and/or dispersal history may have contributed to variation in the location, size, or quality of individual home ranges during an animal's first year in the study population (Dahle et al. 2006; Saïd et al. 2009) and this variation may, in turn, have affected the tendency for an individual to shift its location over time. Dispersal patterns in *C. opimus* are not well understood and additional studies that monitor individuals throughout the year are required to evaluate the potential effects of age and dispersal history on temporal patterns of space use within the study population.

Variation in social relationships. Social relationships – as measured by social unit size – varied at both the population and individual levels. Within years, social units ranged from one to up to two dozen individuals. This variation was evident during four of the five years of this study, indicating that a mix of lone and group-living animals was a persistent feature of the study population. Although sample size was limited, no individuals lived alone for more than one field season; this observation, in conjunction with the overall spatial and social variability detected, suggests that living alone was not a consistent behavioral tendency among some members of the study population. At present, however, phenotypic or other predictors of living alone remain unknown. All lone animals were adults, providing no evidence that age contributed to the occurrence of this social outcome. Each of these individuals was living alone during the first field season in which it was captured, raising the possibility that lone animals were immigrants to the study population. However, other animals captured for the first time were group-living, making it difficult to evaluate the effects of demographic history on an individual's social environment. As noted above, future studies that provide more detailed information regarding individual patterns of movement should help to clarify the factors underlying variability in social relationships.

Among animals captured in successive years, annual changes in social unit size varied markedly, although there was an overall tendency for social unit size to increase with time. More specifically, number of years on the site was a significant predictor of social unit size, with number of group mates in year 1 being significantly less than in years 2 or 3. Consistent with this, animals that initially lived alone were group-living during their second year on the site. Further, values for most social network metrics examined were significantly greater for animals present in the study population for two or three years, suggesting that the strength of social associations increased over time. Although number of years on the site was not a direct measure of age, these outcomes suggest that individuals tended to associate with more conspecifics as they grew older. In general, individuals were assigned to the same social unit in successive years, raising the possibility of enduring relationships among specific members of the study population. For individuals that changed social units, the factors contributing to those changes remain unknown. Future studies that explore interactions between social unit size and composition in greater detail should help to clarify the reasons for the variability in social relationships reported here (Ebensperger et al. 2009).

Characterizing facultative sociality. The persistent occurrence of lone and group-living animals in the study population suggests that *C. opimus* can be described as facultatively social, as

originally proposed by O'Brien et al. (2020) based on data from a single season of research at Pozuelos. Intraspecific variability in spatial and social relationships can generate important insights into the adaptive bases for these aspects of behavior and comparative analyses of facultatively social taxa should facilitate such efforts (Rubenstein and Abbot 2017). Such comparisons are challenging, however, due to the lack of a consistent definition for facultative sociality. While some authors view the co-occurrence of lone and group-living conspecifics as part of an evolutionary transition toward obligate sociality (Rehan et al. 2010; Shell and Rehan 2018), others interpret such variation as differences in adaptive responses to current environmental conditions (Rabosky et al. 2012; Ortiz et al. 2019,). The latter perspective assumes that individuals adjust their behavior to reflect the fitness consequences of living alone versus within a group (Lacey 2004; Ebensperger et al. 2012); this assumption is critical to distinguishing adaptive variation in behavior from differences that arise due to more stochastic factors such as mortality of group mates. Relative fitness has not yet been assessed for lone versus group-living *C. opimus* and thus we cannot exclude the possibility that the observed variation in social unit size reflects random changes within the study population. However, the pronounced between-year differences in behavior detected for some individuals (e.g., no overlap of annual home ranges) as well as the tendency for some animals to change social units despite the continued presence of previous group mates suggest that temporal variation in spatial and social relationships is not simply a consequence of stochastic changes in the composition of the study population.

Implications for social organization. In facultatively social populations, variation in social behavior may arise due to persistent differences in individual behavior that lead some animals to consistently live alone while others consistently occur in groups (Krause et al. 2010; Wilson et al. 2013). Alternatively, animals may live alone versus in groups due to variability in the fitness consequences associated with these behavioral options (McGuire et al. 2002; Silk 2007; Woodruff et al. 2013). Distinguishing between these sources of behavioral variation is critical to evaluating the adaptive bases for population-level differences in social relationships. The fitness outcomes of behavior are influenced by current ecological and demographic conditions (Silk 2007; Rehan et al. 2011; Blumstein 2013) as well as by differences in individual phenotypes (Öst et al. 2015; Ferree et al. 2018). Each of these parameters may change over time, resulting in a dynamic suite of variables that can impact the adaptive bases for living alone versus within a group and, hence, the social organization of the population. As a result, understanding how ecological, demographic, and phenotypic differences interact to shape the behavior of individuals can generate critical insights into larger patterns of social behavior. We found no evidence that the tendency for members of our study population to live alone versus in groups occurred due to persistent differences in individual behavior. Instead, we suggest that the observed variability in spatial and social relationships reflects differences in adaptive responses to immediate ecological and demographic conditions. To test this hypothesis, we recommend that future studies of *C. opimus* include more detailed information regarding individual demographic histories as well as quantitative assessments of critical ecological parameters such as food resources and population density. These data, in conjunction with long-term monitoring of individual behavior, should substantially improve our understanding of the adaptive bases for facultative differences in social organization in this and other group-living species of animals.

Figures

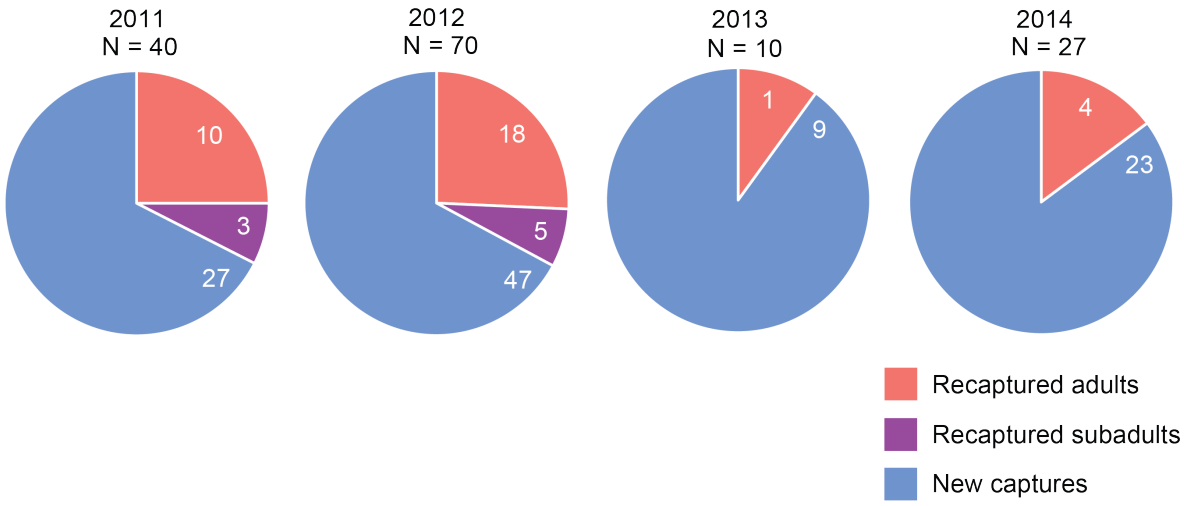

Figure 1. Proportion of animals in the study population that were recaptured from the previous field season. For each year of the study, the proportion of recaptured animals that had been adults during the previous field season is shown, as is the proportion of recaptured animals that had been subadults during the previous season. Values of N represent the total number of animals captured each year; the number of individuals corresponding to each capture category is shown within the associated pie chart. Animals captured in 2010 are described in O'Brien *et al*. (2020).

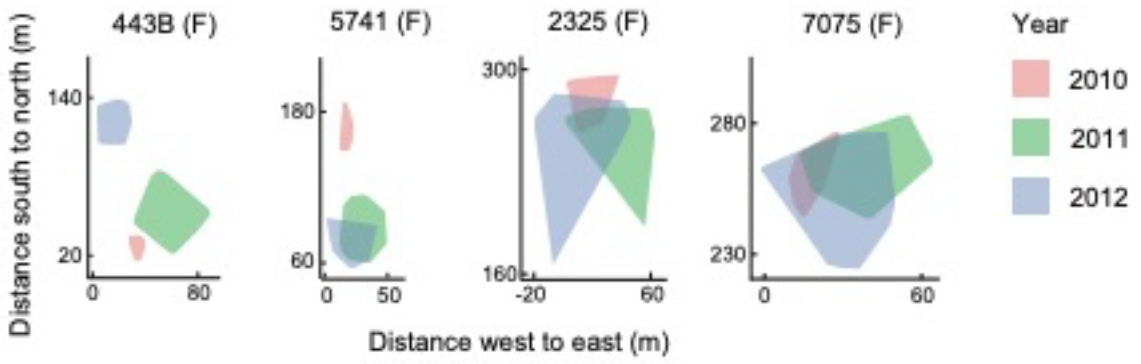

Figure 2. Patterns of home range overlap across years for a subset of individuals (N = 4 females) captured during 3 different field seasons. Animal identity is given above each panel. Colored shapes represent annual home ranges based on 95% MCPs; year of data collection is indicated to the right. Axes depict distance in meters; spatial scale differs among the individuals shown. Home ranges for all individuals captured in multiple years are shown in Supplementary Figure 2.

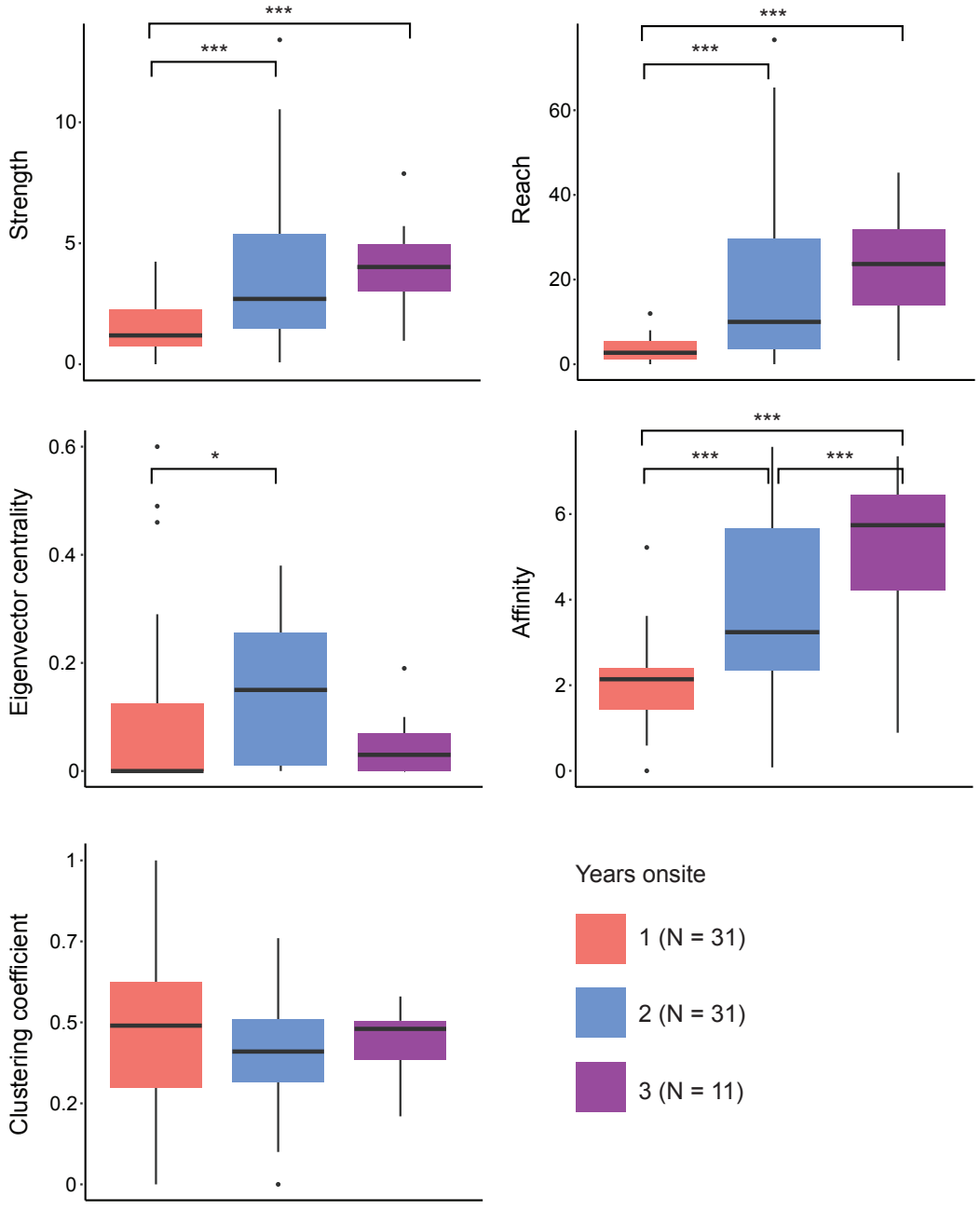

Figure 3. Social network metrics in relation to total number of years on the study site. Box and whisker plots depict minimum, maximum, median, quartile measures, and outliers of network strength, Eigenvector centrality, reach, affinity, and clustering coefficient, as calculated by SOCPROG (Whitehead 2009). For each metric, significant contrasts are indicated with asterisks (*). Measures of network metrics for each individual included in these analyses are presented in Supplementary Table 4.

Tables

Table 1. Summary of the data analyzed. For each year of the study, the number of animals of each sex is indicated, as are the dates of data collection, the mean (± 1 SD) number of days during which data were collected per individual, and the mean (± 1 SD) number of visual fixes recorded per individual. For means, the range of values is reported in parentheses.

Year	Field season	# of animals monitored	Mean # days observed	Mean # fixes recorded
2010	23 Dec – 9 Jan	8 M, 29 F	6.9 ± 4.0 (2 – 16)	30.2 ± 25.0 (7 – 120)
2011	29 Nov – 18 Dec	16 M, 24 F	9.4 ± 4.7 (3 – 19)	38.3 ± 25.3 (7 – 98)
2012	23 Nov – 9 Dec	27 M, 43 F	12.6 ± 3.8 (2 – 16)	48.8 ± 20.4 (6 – 112)
2013	20 Nov – 29 Nov	4 M, 6 F	6.6 ± 2.0 (4 – 9)	22.5 ± 12.4 (12 – 44)
2014	2 Dec – 18 Dec	16 M, 11 F	6.9 ± 2.3 (3 – 10)	24.9 ± 13.5 (6 – 46)

Table 2. Summary of spatial clustering of individuals within the study population. For each year of data collection, the number of lone animals, pairs (2 individuals), and groups (3+ individuals) revealed by social network analyses (Supplementary Fig. 1) are indicated, as is the total number of social units identified during that year. All social units included $\geq$ 1 adult; no social units were comprised only of subadults.

	Social unit size			
Year	**Lone**	**Pairs**	**Groups**	**Total # social units**
2010	2	0	4	**6**
2011	2	0	7	**9**
2012	0	0	5	**5**
2013	1	2	1	**4**
2014	1	5	4	**10**
Total	**6**	**7**	**21**	**34**

Table 3. Results of post-hoc Dunn's tests for all pairwise comparisons across years for measures of strength, Eigenvector centrality, reach, affinity, and clustering coefficient. Significant results are bolded.

Years	Strength	Eigenvector centrality	Reach	Affinity	Clustering coefficient
1-2	**Z = -3.62** **p < 0.001**	**Z = -2.60** **p = 0.02**	**Z = -3.48** **p = 0.001**	**Z = -3.36** **p < 0.001**	Z = 1.35 p = 0.53
1-3	**Z = -3.75** **p < 0.001**	Z = 0.09 p = 0.92	**Z = -4.06** **p < 0.001**	**Z = -4.17** **p < 0.001**	Z = 0.34 p = 0.73
2-3	Z = -1.14 p = 0.26	Z = 1.98 p = 0.10	Z = -1.54 p = 0.12	**Z = 1.75** **p < 0.001**	Z = -0.63 p = 1.00

Table 4. Proportion of animals remaining in the same social unit in successive years. For each entry, the numerator indicates the number of animals assigned to the same social unit in years 1 and 2; the denominator indicates the number of animals captured in year 1 (members of the same social unit) that were recaptured in year 2. Data for 9 distinct social units are shown. No animals were recaptured together from 2012 to 2013 and thus that pair of years is marked as "NA." The identities of specific pairs of individuals captured together in successive years are given in Supplementary Table 5.

	Successive years captured		
2010-2011	**2011-2012**	**2012-2013**	**2013-2014**
4/4	3/3	N/A	0/3
2/2	3/5		
2/3	6/7		
	6/7		
	2/3		

In chapter 2, I aimed to confirm the possibility that the population of highland tuco-tucos located at Pozuelos are indeed facultatively social using spatial data collected over five consecutive years. From these data, I sought to (1) confirm the regular occurrence of both lone and group-living individuals within the population and (2) characterize the temporal consistency of individual social relationships. I found that while the study population consistently contained a mixture of both lone and group-living animals, individual spatial and social relationships varied markedly across time. Specifically, the extent to which individuals remained resident in the same location across years varied, as did the number of conspecifics with which an animal lived, with an overall tendency for individuals to live in larger groups over successive years. Collectively, this chapter indicated that population-level patterns of behavior in this population of *C. opimus* are consistent with facultative sociality but that this variation does not arise due to persistent differences in individual behavior.

Understanding the consequences of this variation in sociality may provide insight regarding the adaptive function of sociality in this population. Thus, for chapter 3, I sought to investigate how the observed variation in social behavior within the population of *C. opimus* at Pozuelos may impact individual glucocorticoid physiology, a key regulator of allostasis and homeostasis. Earlier work in another known social tuco-tuco (*Ctenomys sociabilis*) found that yearling females that dispersed from their natal burrow to live alone had higher baseline glucocorticoid levels relative to females that remained in their natal burrow with conspecifics. Thus, for my third chapter, I aimed to determine if a similar pattern was also found in the population of highland tuco-tucos at Pozuelos. I collected fecal samples from all individuals captured on the field site during two consecutive years to assess the relationship between baseline glucocorticoid levels and multiple metrics of social behavior (i.e., group size, sex ratio of group, and metrics measured via social network analysis).

Chapter 3: Sex, not social behavior, predicts fecal glucocorticoid metabolite concentrations in a facultatively social rodent, the highland tuco-tuco (*Ctenomys opimus*)

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Introduction

Glucocorticoid hormones play a central role in multiple physiological processes (McMahon et al. 1988; Bartolomucci 2007; Vegiopoulos and Herzig 2007; de Guia et al. 2014; Cain and Cidlowski 2017). Glucocorticoid hormone concentrations in wild animals vary over multiple time scales and in response to multiple factors, including both intrinsic properties of individuals (e.g., circadian biology, sex, age: Touma and Palme 2005; Sopinka et al. 2015) and the extrinsic conditions that they experience (e.g., photoperiod, food availability, weather conditions: de Bruijn and Romero 2018). Interactions with conspecifics are expected to have significant effects on circulating glucocorticoids (Goymann and Wingfield 2004; Creel et al. 2013), particularly in group-living species in which social contact is frequent but often varies with respect to the nature and function of specific encounters (Broom et al. 2009; Kutsukake 2009). Consistent with this, relationships between social structure and glucocorticoid physiology vary, with greater social contact being associated with increased baseline glucocorticoid concentrations in some species (Rogovin et al. 2003; Raouf et al. 2006) but decreased baseline concentrations in others (Woodruff et al. 2013; Fürtbauer et al. 2014). These outcomes suggest that interactions between social behavior and baseline measures of glucocorticoids are complex and likely reflect variation in individual phenotypes as well as differences in the social environments in which conspecifics occur. As a result, efforts to understand relationships between social behavior and glucocorticoid physiology require detailed consideration of multiple intrinsic and extrinsic factors (Bonier et al. 2009).

Use of social network analyses to characterize interactions among conspecifics has revealed considerable and sometimes unexpected complexity in social relationships – particularly in species in which groups lack clear dominance hierarchies or other conspicuous forms of social structure (Kappeler et al. 2019; Smith and Pinter-Wollman 2021; Sosa et al. 2021). Aspects of relationships that have been examined using network analyses include the centrality of an individual within its social group as well as the extent to which each animal is directly and indirectly connected to conspecifics (Wey et al. 2008; Krause et al. 2009; Whitehead 2009). Collectively, these metrics provide a more comprehensive and nuanced description of an individual's social environment than do singular measures such as group size or composition. Despite increasing use of network metrics to characterize variability in social relationships, few studies have examined the effects of this variability on baseline glucocorticoid concentrations. Greater understanding of the effects of social behavior on glucocorticoid hormones is critical to elucidating the effects of social environment on homeostasis and allostasis in free-living animals.

Highland tuco-tucos (*Ctenomys opimus*) are subterranean rodents that are endemic to high elevation Puna habitats in Argentina, Bolivia, and Peru (Patton et al. 2015). Unlike most species of *Ctenomys* studied to date, highland tuco-tucos are social, meaning that multiple adults share a burrow system and subterranean nest site (Lacey 2000; O'Brien et al. 2020). Studies of a population of *C. opimus* at Laguna de los Pozuelos, Jujuy Province, Argentina, have revealed considerable variability in individual social relationships. In particular, while some members of this population live in groups, others are solitary (O'Brien et al. 2020, 2021). This variation does not appear to reflect persistent individual level differences in behavior; instead, social relationships vary markedly over time, with a general tendency for individuals to live in larger social groups in successive years (O'Brien et al. 2021). Given this behavioral variability, studies of highland tuco-tucos provide an ideal opportunity to examine the role of the social environment in shaping glucocorticoid responses in a natural population of mammals.

As part of ongoing studies of the behavioral ecology of *C. opimus*, we quantified baseline glucocorticoid concentrations in relation to multiple aspects of the social behavior of the population of this species at Pozuelos. Based on studies of the congeneric, group-living colonial tuco-tuco (*C. sociabilis*: Woodruff et al. 2010, 2013), we predicted that more social members of our study population would display lower baseline concentrations of circulating glucocorticoids. To test this hypothesis, we combined field observations of group size and composition with both network analyses of social behavior and enzyme-linked immunosorbent assays (ELISAs) of glucocorticoid metabolites in fecal samples collected from the same individuals for which social relationships were characterized. As part of these efforts, we also conducted a biochemical validation study (Touma and Palme 2005) to confirm that fecal metabolites provide robust measures of circulating glucocorticoid concentrations in highland tuco-tucos. In addition to providing the first characterization of the glucocorticoid physiology of *C. opimus*, our analyses generate insights into the effects of social relationships on differences in glucocorticoid hormone concentrations.

Material and Methods

Study site. The population of highland tuco-tucos (*Ctenomys opimus*) studied was located in Monumento Natural Laguna de los Pozuelos, Jujuy Province, Argentina (-22.469347, -65.994279, WGS 84; elevation: 3,600 m); this is the same population of *C. opimus* studied by O'Brien et al. (2020, 2021). The ~ 3 ha study site was located along the western bank of the Río Cincel in open, high elevation Puna habitat that was dominated by saltgrass (*Distichlis* sp.) and needlegrass (*Stipa* sp). The population of *C. opimus* at this location had been monitored annually from 2009 to 2014 and again from 2017 to 2019. Data for this study were collected from 23 December 2017 to 9 January 2018 (2017 field season) and from 21 December 2018 to 5 January 2019 (2018 field season). This corresponds to the early austral summer, which is the primary breeding season for members of the study population.

Animal capture and handling. All procedures involving live tuco-tucos were consistent with the guidelines of the American Society of Mammalogists for the use of wild mammals in research (Sikes et al. 2016) and had been approved by the Animal Care and Use Committee at the University

of California, Berkeley. Live-trapping was conducted on the primary study site and in surrounding portions of the habitat. Individuals were captured using Tomahawk-style live traps baited with carrots (O'Brien et al. 2020, 2021). Open traps were placed on the soil surface near active-burrow entrances, as identified based on recently excavated mounds of dirt or direct visual observations of tuco-tucos using a given entrance. All trapping was conducted during daylight hours. Traps were monitored continuously and animals were retrieved immediately upon capture. Capture locations were recorded using a hand-held GPS unit (accuracy ~ 6 m). In addition, capture localities for tuco-tucos trapped on the primary study site were recorded using a Cartesian coordinate system (8 m x 8 m grid cells) established on the site each year prior to the start of data collection. The same grid was used to record the localities of individuals during radio-telemetric monitoring of spatial relationships among members of the study population (see below).

Upon first capture, each animal was permanently marked with a PIT-tag (IMI-1000, Bio Medic Data Systems, Inc., Seaford, DE) inserted subcutaneously at the nape of the neck. PIT-tags were read using a hand-held scanner (DAS 4000 Pocket Scanner, Bio Medic Data Systems Inc., Seaforth, DE). Sex and body mass were recorded for each individual captured. Data on body mass and pelage coloration were used to determine the age-class (subadult or adult) of each animal (Lacey et al., in prep). For females, reproductive status was assessed based on the appearance of the external genitalia (sexually receptive), the ability to palpate fetuses (pregnant), or the presence of enlarged mammae (lactating). In contrast, because testes of males in the study population never descend externally, the reproductive status of members of this sex could not be determined based on external appearance.

The study species is unusual within *Ctenomys* in that individuals spend considerable time above ground while foraging, making it possible to observe the animals directly (O'Brien et al. 2020). To facilitate visual identification of individuals, human hair dyes (e.g., Manic Panic semi-permanent hair color) were used to mark the fur of each animal captured with a unique combination of colored patches. In addition, all adults captured on the primary study site were fitted with radio transmitters (G3-1V transmitters, AVM Instrument Company, Colfax, CA) that were affixed using plastic cable ties as collars. The weight of the transmitter and collar together (~ 7 g) represented < 5% of the body weight of adults in the study population (Sikes et al. 2016; O'Brien et al. 2020). Telemetric monitoring of individuals was used to characterize spatial and social relationships among members of the study population (see below).

Field collection of fecal samples. Captured tuco-tucos typically defecated during routine handling and marking procedures, providing a convenient means of collecting fecal samples directly from known individuals. To facilitate collection of fecal pellets, captured animals were transferred from traps to cloth bags that served to restrain individuals while also allowing us to gather pellets released during handling. All pellets from the same individual were placed in a cryogenic vial and frozen in liquid nitrogen until samples could be transferred to a -80° C freezer. To allow characterization of circadian patterns of fecal glucocorticoid metabolite (fGCm) production by members of the study population (Touma and Palme 2005), the time of collection was recorded for each sample. Tuco-tucos that did not defecate during handling were placed in plastic rodent cages (one animal per cage), the bottoms of which were lined with dry grass. Cages

were checked regularly until fecal pellets were produced (typically < 30 min), after which pellets were stored as described above. Cages used to collect fecal pellets were emptied of grass and wiped clean between uses.

Once animal marking and fecal sample collection procedures had been completed, individuals were released at the point of capture. During the 2018 field season, a subset of 12 adults (6 males, 6 females) captured outside of the primary study site was retained in captivity for use in validation studies of glucocorticoid response (see *ACTH challenge*, below). These individuals were chosen based on visual confirmation that they occupied burrow systems outside of the primary study site; as a result, temporary removal of these animals from the population should not have affected social network relationships among individuals on the primary study site. Fecal samples were collected from most captive-housed tuco-tucos at the time of capture; the few individuals that did not defecate during initial handling were checked every 30 min for the first few hours following capture, until fecal samples were obtained. Fecal pellets collected at or shortly after capture were used to evaluate potential differences in fGCm concentrations among captive tuco-tucos prior to the start of our validation study.

Characterizing social environments of free-living tuco-tucos. The number of conspecifics with which an individual lives is a key component of its social environment. To quantify social unit size (the number of individuals comprising a spatially distinct group: O'Brien et al. 2021), we used radiotelemetry to document spatial relationships among members of the study population. Locations of radio-collared tuco-tucos were determined using R1000 receivers (Communications Specialists, Inc., Orange, CA) and 3-element hand-held Yagi antennas (AVM Instrument Company, Colfax, CA). Radio fixes were collected multiple times per day, typically between 0700-2000 hrs, with a minimum of 1 hour between successive recordings. For each fix, we recorded the location of each collared individual to the nearest 0.5 m using the 8 m x 8 m grid system established on the study site. Analyses of data obtained for objects placed at known locations revealed this procedure to be accurate to within 0.5 m (O'Brien et al. 2020). At the end of each field season, individuals were recaptured, and their radio collars were removed.

Spatial relationships were quantified using 95% minimum convex polygons (MCPs) generated in the R package adehabitatHR (Calenge 2015). Percent overlap between 95% MCPs was estimated for all pairs of individuals captured on the study site during the same field season. The resulting association matrix was analyzed in SOCPROG (Whitehead 2009) to identify spatially distinct clusters of tuco-tucos. The fit between association matrices and the resulting clusters of individuals was assessed using the cophenetic correlation coefficient, with values ≥ 0.8 considered indicative of a strong correspondence between these data sets (Bridge 1993). Clusters (i.e., social units) were identified using the maximum modularity criterion, which provides a measure of the degree to which a population is divided into spatially distinct subsets; values > 0.3 are generally interpreted as evidence of significant spatial clustering (Newman 2006; Whitehead 2008). Clusters identified by these analyses were used to determine the size of the social unit to which each member of the study population belonged.

To quantify potential variability in relationships among members of the same social unit, we also examined five metrics of social network structure generated by SOCPROG. The specific metrics considered were network strength (the sum of an individual's associations), eigenvector centrality (how well an individual is associated, as well as how well their associates are associated), affinity (the weighted average strength of an individual's associations), reach (how well an individual is connected indirectly to other members of the population), and the clustering coefficient for the network (how well an individual's associates are associated). Detailed descriptions of these parameters are provided in Whitehead (2009). Evaluation of these metrics allowed us to examine quantitatively the effects of variable social relationships within social units on differences in baseline glucocorticoid concentrations (see below).

Captive housing. The 12 tuco-tucos (6 males, 6 females) retained for use in our adrenocorticotropic hormone (ACTH) challenge study (see below) were housed in captivity for 12-17 days (mean = 13.6 $\pm$ 1.9 days per animal) in a secure, weatherproof building located near the study site. All of these individuals were adults; all females were reproductively active (perforate vaginae) but none were detectably pregnant or lactating. In captivity, these animals were housed either singly (3 males, 3 females) or in pairs (3 male-female pairs). Pairs were identified based primarily on proximity of capture localities (mean distance between captures = 7.9 $\pm$ 7.7 m), such that pairs were likely to be members of the same social unit. Tuco-tucos housed alone were placed in ventilated plastic enclosures (33 x 25 x 20 cm), the bottoms of which were lined with shredded paper. Enclosures used to house pairs were roughly double in size (58 x 40 x 18 cm) but included a mesh partition that divided containers into two sections, each of which was lined with shredded paper. Partitions allowed members of a pair to interact (see and smell each other, huddle together against the mesh divider) while keeping them physically separated for collection of fecal samples. All enclosures used to house tuco-tucos included a short (16 cm) section of PVC pipe that served as a refuge. Enclosures were cleaned daily; dirty bedding was removed and the containers were wiped down with a 1:10 bleach solution, after which clean bedding was added. The animals were fed twice daily with *ad libitum* quantities of salt grass (*Distichlis* sp.) that had been collected near the study site, supplemented with carrots and corn. Individuals were weighed daily to detect potential changes in body condition associated with captive housing conditions.

ACTH challenge. To validate use of enzyme immunoassay (EIA) protocols for quantifying fGCm concentrations in highland tuco-tucos, an ACTH challenge study was conducted (Touma and Palme 2005; Woodruff et al. 2010). To allow acclimation to captive housing conditions, the study subjects were held in captivity for a minimum of 1 week (mean = 8.6 $\pm$ 1.9 days) prior to injection with synthetic ACTH; the duration of the acclimation period varied due to differences in the dates on which individuals were captured. Once the acclimation period had ended, fecal pellets were collected from all captive tuco-tucos every 6 hours (06:00, 12:00, 18:00, 24:00 hrs) for 48 hours; these samples were used to examine circadian variation in fGCm production (Dickmeis 2009; Reppert and Weaver 2002). At the end of this initial sampling period, 8 individuals (4 males, 4 females) were each given an intramuscular injection of 12 IU/kg body mass of Cortrosyn (Amphastar Pharmaceuticals Inc., Rancho Cucamonga, CA); doses of Cortrosyn were determined based on protocols used in similar studies of wild rodents (Woodruff et al. 2010; Hammond et al. 2015). The remaining 4 tuco-tucos (2 males, 2 females) received an equivalent volume (based on

individual body mass, Table 1) of 0.9% saline as a control. All animals were injected within a 30-minute period between 0700 and 0730 on 29 December 2018. Treatment versus control tuco-tucos were balanced across housing conditions (Table 1). After injection, fecal samples were collected from all individuals at 6-hour intervals (see above) for 72 hours. The pellets collected were placed in cryogenic vials and frozen in liquid nitrogen until they could be transferred to a -80° C freezer.

Steroid extractions and glucocorticoid assays. Following the methods of Mateo and Cavigelli (2005) as modified by Woodruff et al. (2010), frozen fecal samples were thawed and then dried in an oven at 95° C for 4 hours. After drying, samples were crushed using a mortar and pestle. For each sample, a 0.2 g aliquot of the resulting powder was transferred to a microcentrifuge tube, to which 1.5 mL of 100% ethanol was added. Tubes were vortexed and then centrifuged at 3,000 g for 45 minutes. The supernatant was collected from each sample, transferred to a clean microcentrifuge tube, and then frozen at -20° C until it was assayed.

Commercially available ELISA kits (Cayman Chemical Co., Ann Arbor, MI) were used to quantify fGCm concentrations. Because fGCm concentrations had not previously been characterized for highland tuco-tucos, initial analyses of a randomly selected subset of 24 samples were conducted using assay kits for both cortisol and corticosterone. Based on these preliminary analyses (see results), remaining samples were assayed only for corticosterone. Parallelism of fecal extracts with kit standards was determined using pooled samples from the pre-ACTH injection period (N = 8 individuals) as well as the post-ACTH injection period (N = 8 individuals). Pooled samples were serially diluted from 1:2 to 1:256, after which samples were assayed in triplicate. The resulting relationships between fGCm concentrations and antibody binding were compared to those for kit standards to confirm detection of corticosterone. These preliminary analyses indicated that a 1:16 dilution (sample:kit buffer) was within the binding range (20-80%) recommended by the kit manufacturer. Replicate samples for which the coefficient of variation exceeded 20% were reanalyzed (Woodruff et al. 2013).

Statistical analyses. Throughout the text, means are reported $\pm$ 1 SD. All statistical tests were performed in R v. 4.0.4 (R Core Team, 2017). For standard two-sample tests, normality of the data was assessed using Shapiro-Wilks tests, after which parametric or non-parametric analyses were used, as appropriate. When sample sizes were unequal, effect sizes were calculated using Cohen's d or Hedges' g. Parallelism between fGCm concentrations for serially diluted samples and kit standards was assessed using ANCOVAs. To examine circadian variation in fGCm production, mean fecal corticosterone concentration was calculated for each 6-hour sampling interval during the 48 hours prior to injection of captive tuco-tucos with Cortrosyn. Not all individuals defecated during each 6-hour sampling interval, with the result that sample sizes varied among the time points examined. As a result, a Skillings-Mack test was used to compare fGCm concentrations across sampling intervals; this test is a modification of Friedman's ANOVA that is robust to variation in sample sizes (Chatfield and Mander 2009), making it appropriate for our data regarding fGCm concentrations in captive highland tuco-tucos. Analyses were conducted using the 'Skillings.Mack' package in R (Srisuradetchai 2015).

As with analyses of circadian patterns of fGCm production, not all individuals in our ACTH challenge study defecated during each 6-hour sampling period, resulting in variable sample sizes across the time points examined. For comparisons of control versus Cortrosyn-injected tuco-tucos, fGCm concentrations were assessed by binning data from samples collected 0-6 hours post capture as well as those collected 0-12, 12-24, 24-36, 36-54, and 54-72 hours post-injection. No fecal samples were detected at 42- or 66-hours post-injection, resulting in larger time intervals (18 hours) for the final two temporal periods examined. Further parsing the data to examine the effects of sex and housing on response to ACTH challenge resulted in smaller sample sizes (number of individuals) per treatment combination, which increased the impact of time points for which fecal samples were not available. As a result, for the ACTH challenge study, data regarding fGCm concentrations were binned into larger temporal intervals. The intervals examined were 0-6 hours post-capture plus 0-24, 24-48, and 48-72 hours post-injection; these intervals were chosen because each represents one 24-hr period of data collection. Again, a Skillings-Mack test was used to compare fGCm concentrations for control versus Cortrosyn-injected tuco-tucos across all sampling intervals. In contrast, Mann-Whitney U tests were used to compare concentrations for these treatment groups during individual sampling intervals.

To explore relationships among fGCm concentrations and social behavior within the free-living population, we constructed linear mixed-effect models using the R package 'lme4' (Bates et al. 2007). Aspects of sociality examined included data on social unit composition (number of adults, number of adult males, number of adult females) as well as the five metrics obtained from social network analyses (strength, Eigenvector centrality, reach, clustering coefficient, affinity). Prior to model construction, a Q-Q plot was used to determine the underlying distribution of data regarding fGCm concentrations. Separate models were constructed for each metric of sociality examined. Each model included sex as a fixed effect, with animal ID and time of fecal sample collection included as random effects; because estimates of social network metrics were based on group-specific attributes, social group ID was not included in our models. All models were run with and without interactions between the predictor variables. The best-fit model for each set of predictor variables was identified using the Akaike information criterion (AIC). Post-hoc type III Wald Chi-square tests were then used to determine which explanatory variables in the best-fit model were significant predictors of fGCm concentrations; these post hoc tests were completed using the R package 'car' (Fox et al. 2007).

Results

Cortisol versus corticosterone. Analyses of fGCm concentrations from a randomly selected subset of 24 samples (10 males, 14 females) revealed concentrations that were above the manufacturer's reported limit of detection for corticosterone and cortisol (30 and 35 pg/mL at 80% binding, respectively). Sensitivity of the assay at 50% binding was 269 pg/mL for corticosterone and 85 pg/mL for cortisol. Paired comparisons of fGCm concentrations for the 24 tuco-tucos sampled revealed a significant tendency for corticosterone concentrations to be greater than those for cortisol (Wilcoxon signed-rank test, $V = 0$, $N = 24$, $P < 0.001$, Cohen's $d = 1.79$; Figure 1a). Accordingly, all subsequent analyses examined corticosterone metabolites only.

Intra- and inter-assay coefficients of variation for fecal corticosterone metabolites were 10.45% and 13.74 % (N = 12 plates), respectively.

Biochemical validation. The logit-transformed slopes for serial dilutions of pooled fecal samples did not differ from those for kit standards for either pre- or post-ACTH injection samples (ANCOVA, $F_{2,18} = 2.70$, P = 0.09, $\eta_p^2 = 0.23$; Figure 1b), providing no evidence that detection of glucocorticoids differed between kit standards and fecal samples collected from the highland tuco-tucos.

Circadian variation. Among the 12 tuco-tucos housed in captivity, fGCm concentrations were generally lowest in the morning and increased over the course of the day (Figure 2); this tendency was significant (Skillings-Mack test, $X^2 = 25.77$, df = 11, P = 0.007). In contrast, among free-living tuco-tucos captured on the primary study site, there was no correlation between fGCm concentrations and time of fecal sample collection (Kendall's rank correlation, Z = -0.46, Tau = -0.05, P = 0.65).

ACTH challenge. Analyses of fecal samples collected within the first 6 hours that tuco-tucos were housed in captivity revealed no significant differences in fGCm concentrations between individuals later assigned to saline versus Cortrosyn treatment groups (Mann-Whitney U test, W = 34, P = 0.92, Hedges' g = 0.31; Figure 3a). Similarly, there were no differences between initial fGCm concentrations for males versus females (Mann-Whitney U test, W = 57, P = 0.16, Cohen's d = 0.82) or between concentrations for individuals subsequently assigned to solitary versus paired housing (Mann-Whitney U test, W = 52, P = 0.31, Hedges' g = 0.29).

Post injection, there was significant variation in fGCm concentrations among Cortrosyn-treated but not among control individuals (Skillings-Mack tests, Cortrosyn: $X^2 = 33.24$, df = 14, P = 0.002, control: $X^2 = 3.09$, df = 7, P = 0.88). Comparisons of data from Cortrosyn-treated versus control individuals revealed significant differences between these groups for samples collected 0-12 hours post-injection (Mann-Whitney U test, W = 8, P = 0.03, Hedges' g = 0.85; Figure 3a), with Cortrosyn-treated individuals having higher mean fGCm concentrations (3681.24 $\pm$ 2648.02 pg/g feces) than control individuals (1631.05 $\pm$ 709.00 pg/g feces). None of the other post-injection time intervals examined revealed significant differences between treatment groups (Mann-Whitney U tests; 12-24 hours post-injection: W = 20, P = 0.25, Hedges' g = 0.73; 24-36 hours post-injection: W = 37, P = 0.15, Hedges' g = 0.75; 36-54 hours post-injection: W = 16, P = 0.31, Hedges' g = 0.68; 54-72 hours post-injection: W = 29, P = 0.31, Hedges' g = 0.56).

When these data were re-analyzed using larger (24-hour) temporal bins, no significant variation in fGCm concentrations was detected for either Cortrosyn-treated or control animals (Skillings-Mack tests, Cortrosyn: $X^2 = 30.72$, df = 23, P = 0.13, control: $X^2 = 12.39$, df = 10, P = 0.26). Despite this, significant differences between treatment groups were evident for samples collected 0-24 hours post-injection (Mann-Whitney U test, W = 63, P = 0.01, Hedges' g = 0.74; Figure 3b), with Cortrosyn-treated individuals having higher mean fGCm concentrations (3768.37 $\pm$ 2561.43 pg/g feces) than control individuals (2119.91 $\pm$ 1008.62 pg/g feces). Neither of the other sample collection intervals examined revealed significant differences between treatment groups (24-48

hours post-injection: Mann-Whitney U test, W = 43, P = 0.16, Hedges' g = 0.69; 48-72 hours post-injection: Mann-Whitney U test, W = 81, P = 0.13, Hedges' g = 0.66).

When these larger temporal bins were used to analyze fGCm concentrations as a function of captive housing conditions, no significant differences were detected between solitary versus pair-housed members of the control group for any of the time intervals examined (Mann-Whitney U tests, 0-24 hours post-injection: W = 24, P = 0.13, Hedges' g = 1.08; 24-48 hours: W = 6, P = 0.69, Cohen's d = 0.30; 48-72 hours: W = 18, P = 0.31, Cohen's d = 0.78; Figure 3c). In contrast, among individuals injected with Cortrosyn, mean fGCm concentrations were significantly higher for solitary (3497.13 $\pm$ 1833.77 pg/g feces) versus pair-housed tuco-tucos (1892.78 $\pm$ 902.61 pg/g feces) for samples collected 48-72 hours post-injection (Mann-Whitney U test, W = 15, P = 0.03, Hedges' g = 1.03; Figure 3c). No differences between solitary versus pair-housed Cortrosyn-treated individuals were detected for the other time intervals examined (Mann-Whitney U tests; 0-24 hours post-injection: W = 66, P = 0.78, Hedges' g = 0.38; 24-48 hours post-injection: Mann-Whitney U test, W = 25, P = 0.32, Hedges' g = 0.45).

When the same larger temporal bins were used to examine fGCm concentrations as a function of sex, no significant differences were detected between control males and females for any of the time intervals considered (Mann-Whitney U tests, 0-24 hours post-injection: W = 22, P = 0.25, Hedges' g = 0.91; 24-48 hours post-injection: W = 10, P = 0.69, Cohen's d = 0.03; 48-72 hours post-injection: W = 24, P = 0.13, Hedges' g = 0.95; Figure 3d). In contrast, among tuco-tucos injected with Cortrosyn, fGCm concentrations for males (5243.97 $\pm$ 2938.18 pg/g feces) were significantly greater than those for females (2292.77 $\pm$ 578.52 pg/g feces) in samples collected 0-24 hours post-injection (Mann-Whitney U test, W = 129, P = 0.0005, Cohen's d = 1.39; Figure 3d). No differences between Cortrosyn-treated males and females were detected for any of the other sampling intervals examined (Mann-Whitney U tests; 24-48 hours post-injection: W = 53, P = 0.08, Hedges' g = 0.90; 48-72 hours post-injection W = 87, P = 0.08, Hedges' g = 0.88).

Social relationships among free-living tuco-tucos. A total of 33 individuals (10 adult males, 20 adult females, 2 subadult males, 1 subadult female) were captured on the primary study site during the 2017 field season; a total of 17 individuals (4 adult males, 10 adult females, 3 subadult males, 0 subadult females) were captured on the primary site during the 2018 field season. Subsequent observations revealed no evidence of unmarked tuco-tucos on the primary site, suggesting that all animals resident in this area had been caught and identified with respect to sex, age, and (for females) reproductive status. As a result, our analyses should have captured information regarding all spatial relationships in which these individuals engaged. Sufficient spatial data for social network analyses plus fecal samples were available for a subset of 23 individuals (6 adult males, 14 adult females, 2 subadult males, 1 subadult female) from the 2017 field season and 14 individuals (4 adult males, 10 adult females, 0 subadult males, 0 subadult females) from the 2018 field season. A summary of the tuco-tucos captured as well as the spatial data and fecal samples obtained during each field season is provided in Supplementary Table 1. The number of subadults captured (N = 3) was too small to allow statistical evaluation of the effects of age. However, fGCm concentrations for these individuals (1114.14 – 2054.34 pg/g feces) fell within the

range of values recorded for adults (387.86 – 3008.29 pg/g feces) and thus age was not considered a factor in subsequent analyses of social network metrics or glucocorticoid concentrations.

Social network analyses of association matrices based on 95% minimum convex polygons revealed the presence of both lone and group living adults in both years of the study (Supplementary Figure 1). Cophenetic correlation coefficients for these analyses were > 0.93, indicating a strong correspondence between the association matrix and the degree of overlap of individual home ranges. Maximum modularity was > 0.56 based on an association matrix cut-off value of 0.08. Multiple distinct clusters of tuco-tucos were identified for each year of the study. Although mean social unit size did not differ significantly between years (2017: 5.0 $\pm$ 4.7 individuals per social unit, N = 5 units; 2018: 2.0 $\pm$ 1.2 individuals per social unit, N = 7 units; Mann Whitney U test, W = 22, P = 0.447, Hedges' g = 0.96), the range of social unit sizes was markedly greater in 2017 (N = 1-11 individuals) compared to 2018 (N = 1-4 individuals), which may have affected within-group social interactions. During the 2017 field season, all multi-animal social units (N = 3) contained adults of both sexes. In contrast, one of four multi-animal social units identified during the 2018 field season contained only adult females (Supplementary Figure 1). Five of the individuals (1 adult male, 4 adult females) included in our analyses were captured in both field seasons; between-year comparisons of social unit composition revealed that none of these animals lived with the same conspecifics in both years of the study (Figure 4) and thus data collected from these individuals in successive years were treated as independent for analyses of relationships between social behavior and fGCm concentrations.

Mean values for four (strength, reach, clustering coefficient, affinity) of the five social network metrics examined (Supplementary Table 2) differed significantly between years of the study (Mann-Whitney U tests, all P < 0.03; Supplementary Table 3); the sole exception was eigenvector centrality, mean values for which did not differ between 2017 and 2018 (Mann-Whitney U test, W = 181.5, N = 23, 14, P = 0.46, Hedges' g = 0.06). In contrast, no differences between mean values for males versus females were detected for any of the network metrics considered, either within years or when data from both years were pooled (Mann-Whitney U tests, all P > 0.08; Supplementary Table 3).

Effects of social relationships on glucocorticoids. When all data from free-living tuco-tucos were considered, mean fGCm concentrations did not differ between years of the study (2017: 1352.43 $\pm$ 597.89 pg/g feces, range = 387.86 – 3008.29, N = 23; 2018: 1316.64 $\pm$ 694.06 pg/g feces, range = 525.75 – 2818.99, N = 14; Mann-Whitney U test, W = 173, P = 0.72, Hedges' g = 0.06; Figure 4a). Within years, mean fGCm concentrations for males and females did not differ significantly (Mann-Whitney U tests, both P > 0.08, 2017 Hedges' g = 0.62, 2018 Hedges' g = 1.09). However, when data for both years were pooled, the mean fGCm concentration for males (1662.72 $\pm$ 564.73 pg/g feces, N = 12) was significantly greater than that for females (1183.44 $\pm$ 604.41 pg/g feces, N = 25: Mann-Whitney U test, W = 75, P = 0.01, Hedges' g = 0.81; Figure 4b). Among females, fGCm concentrations did not differ with reproductive status (i.e., pregnant, lactating, or neither; Kruskal-Wallis test, X^2 = 4.50, df = 2, P = 0.10). Based on these outcomes, glucocorticoid data from 2017 and 2018 were pooled for subsequent analyses and sex was

included as a factor in linear models exploring the effects of social behavior on fGCm concentrations.

In both years of the study, fGCm concentrations varied within and among the social units identified by our social network analyses (Figure 5). Use of linear mixed-effects models to explore relationships between measures of social unit size (number of adults), composition (number of adult males, number of adult females) and individual fGCm concentrations revealed that the best fit model included the interaction between sex and the number of adult males per social unit as predictor variables (AIC = 542.0275, df = 7; Table 2). Post-hoc tests indicated that sex was a significant explanatory variable in this model (Wald Chi-square type III, F = 11.09, df = 1, p = 0.0009). In contrast, neither overall social unit size nor the number of adult females per social unit appeared to affect individual fGCm concentrations.

Analyses of relationships between social network metrics and glucocorticoid metabolites revealed two models that, based on AIC values, were equally predictive of individual variation in fGCm concentrations (Table 2). One of these models included the interaction between sex and Eigenvector centrality as explanatory variables (AIC = 533.6091, df = 7; Table 2); the other included the interaction between sex and the clustering coefficient as explanatory variables (AIC = 535.3422, df = 7; Table 2). Models including the remaining three network metrics examined (strength, reach, affinity) received considerably less support (Table 2). Post-hoc tests of the two best-fit models revealed that sex was a significant explanatory variable in both (Wald Chi-square type III tests, F = 7.18, df = 1, p = 0.007; F = 14.86, df = 1, p = 0.0001). In contrast, neither Eigenvector centrality nor the clustering coefficient appeared to affect individual fGCm concentrations.

Discussion

Our analyses of the population of highland tuco-tucos at Laguna de los Pozuelos indicate that sex is an important determinant of fecal glucocorticoid metabolites in these animals, with males having higher baseline fGCm concentrations than females. In contrast, neither social unit composition (size, sex ratio) nor the social network metrics examined were significant predictors of differences in individual fGCm concentrations. Experimental treatment of a subset of individuals with Cortrosyn confirmed that measures of fGCm concentrations were responsive to exogenous ACTH, thereby validating use of these data to quantify baseline glucocorticoid levels in the study population. Among Cortrosyn-treated tuco-tucos, peak fGCm concentrations for males were significantly greater than those for females, again revealing an effect of sex on glucocorticoid response. Collectively, these findings suggest that sex may be more important than social environment in shaping hypothalamic-pituitary-adrenal axis (HPA) activity in highland tuco-tucos. In addition to providing the first information regarding glucocorticoid physiology in this species, our analyses underscore the importance of considering both intrinsic and extrinsic factors when evaluating the factors affecting HPA response in free-living mammals.

It is possible that the number of free-living tuco-tucos sampled during this study was not sufficient to detect relationships between social network metrics and variation in fGCm

concentrations. As is common in studies of wild mammals, our sample sizes were determined by the number of animals present on the study site each year and by our ability to collect the required behavioral and endocrine data from each of these individuals. Sample sizes for our analyses are comparable to those for other studies of relationships between social behavior and fGCm concentrations in wild populations of small mammals (e.g., Ebensperger et al. 2011; Woodruff et al. 2013). While larger sample sizes would potentially have increased our ability to detect effects of social network metrics on glucocorticoid metabolites, it seems unlikely that such expanded analyses would have altered the finding that sex was more important than social behavior in predicting differences in fGCm concentrations among members of our free-living study population.

Response to exogenous ACTH. Corticosterone was the predominant glucocorticoid metabolite in our study subjects, with peak response to exogenous ACTH occurring within 12 hours of injection. Corticosterone is also the predominant baseline metabolite in the colonial tuco-tuco (*C. sociabilis*), although peak response in this species is slower, not occurring until 16-24 hours after injection (Woodruff et al. 2010). Multiple factors may affect the time to peak response, including diet (White et al. 2015; Shively et al. 2020), life history stage (Lattin et al. 2012; Ensminger et al. 2014), and environmental conditions at the time of injection (Reeder and Kramer 2005). In *C. sociabilis*, response to injection with Cortrosyn was assessed using captive-reared tuco-tucos that were fed the same diet on which they were typically maintained in the lab (Woodruff et al. 2010). In contrast, our analyses of highland tuco-tucos were based on individuals that had been housed in captivity for only ~ 2 weeks prior to injection, where they were fed a mixture of natural and recently introduced food items. These environmental changes may have influenced both digestive physiology (Karasov and Diamond 1988; Hilton et al. 2000) and response to stressors (Romero 2004; Dantzer et al. 2010), with associated impacts on the timing of maximum fecal glucocorticoid metabolite production. Nevertheless, peak response time for our study subjects was within the range reported for other rodent species (Montiglio et al. 2012; Sheriff et al. 2012), thereby underscoring the suitability of fecal metabolites as a source of information regarding baseline glucocorticoid concentrations in *C. opimus*.

Experimental administration of ACTH revealed potentially important differences in response as a function of sex and social environment. Among Cortrosyn-injected highland tuco-tucos, peak response was significantly greater for males than for females. No comparable difference was evident among control (saline injected) individuals, suggesting a possible interaction between sex and response to physiological challenge. Similarly, only Cortrosyn-injected highland tuco-tucos demonstrated an effect of housing, with solitary individuals displaying significantly greater fGCm concentrations than pair-housed individuals during our final sample collection interval (54-72 hrs post injection). This corresponds to the timeline for return to baseline fGCm concentrations in other rodent species (Dantzer et al. 2010; Woodruff et al. 2010; Hammond et al. 2015), suggesting that highland tuco-tucos housed in pairs returned to pre-manipulation glucocorticoid concentrations more quickly than conspecifics that were housed alone. More generally, the results of our ACTH manipulation suggest that both intrinsic (e.g., sex) and extrinsic (e.g., housing environment) factors may contribute to individual variation in fGCm concentrations in *C. opimus*.

Social relationships and glucocorticoids. The social environments experienced by members of our free-living study population varied with respect to multiple parameters. For example, the number of highland tuco-tucos per social unit ranged from one to eleven, indicating the presence of both lone and group-living individuals in the population (O'Brien et al. 2020, 2021). Within social units comprised of multiple adults, the ratio of males to females differed. Finally, most of the social network metrics examined varied among groups, presumably reflecting differences in the range of social unit sizes present during each year of the study (Naug 2009). Despite this variation, none of these measures of social environment emerged as significant predictors of fGCm concentrations in our free-living study population. This is in apparent contrast to data from our captive, Cortrosyn-injected study animals, among which fGCm concentrations were greater for lone versus pair-housed individuals. These distinct outcomes may reflect differences in the number or saliency of challenges experienced in each setting. For example, natural environments likely present a greater array of challenges than captive settings, with the result that effects of social environment on free-living animals may be more difficult to detect because they occur in concert with responses to other stimuli (Reeder and Kramer 2005). In contrast, at least for our study subjects, the novelty of the captive environment may have rendered contact with conspecifics more important than it would be among free-living individuals (DeVries et al. 2003), resulting in a particularly pronounced effect of housing condition during our ACTH challenge study. Studies of multiple species have revealed different patterns of glucocorticoid response in captive versus free-living individuals (Calisi and Bentley 2009), thereby underscoring both the importance of environmental conditions on glucocorticoid physiology and the need for additional analyses that compare data from captive and free-living conspecifics.

Our finding that social environment was not a significant predictor of fGCm concentrations in free-living highland tuco-tucos contrasts with data from *C. sociabilis*, for which studies of captive and free-living animals indicate that fGCm concentrations are significantly higher for lone versus group-living individuals (Woodruff et al. 2010, 2013). Although both *C. sociabilis* and *C. opimus* have been characterized as group-living (Lacey et al. 1997; O'Brien et al. 2020), the social organizations of these species differ in several potentially important ways, including the extent to which lone individuals are isolated from conspecifics. In particular, while lone *C. sociabilis* do not overlap spatially with other adults (Lacey et al. 1997; Lacey and Wieczorek 2004), home ranges for lone *C. opimus* may overlap with those of multiple conspecifics (O'Brien et al. 2020), suggesting that the distinction between lone and group-living tuco-tucos is less extreme in the latter species. This difference may in turn contribute to interspecific differences in relationships between social environment and glucocorticoid regulation (Schoepf and Schradin 2013; Hill et al. 2021). Future studies that combine analyses of naturally occurring variation in social behavior with experimental manipulation of specific behavioral parameters should help to clarify the role of social relationships in shaping baseline glucocorticoid concentrations in highland and other species of tuco-tucos.

Sex and glucocorticoids. The primary predictor of differences in fGCm concentrations among members of our study population was sex, with males having higher baseline corticosterone metabolite concentrations than females. This difference was evident in our

analyses of free-living highland tuco-tucos as well as in the results of our ACTH challenge experiment. Sex-based differences in baseline glucocorticoids have been reported for several other species of rodents, including Syrian hamsters (Chelini et al. 2010), Siberian hamsters (Bilbo and Nelson 2003), yellow-bellied marmots (Smith et al. 2012), and spiny mice (Nováková et al. 2008). In contrast, no intersexual differences were detected for degus (Soto-Gamboa et al. 2009) or arctic lemmings (Fauteux et al. 2017). Among those species for which sex-based differences are evident, the directionality of these relationships differs, with glucocorticoid concentrations being higher for males in some species but higher for females in others (Tilbrook et al. 2000; Touma and Palme 2005). Thus, interactions between sex and glucocorticoid physiology likely reflect the effects of multiple factors, including environmental as well as phenotypic parameters (von der Ohe and Servheen 2002).

Among the factors that may contribute to contrasting glucocorticoid levels in males versus females are intersexual differences in reproductive behavior. Our studies of free-living highland tuco-tucos were conducted during the spring breeding season for this species, raising questions regarding the role of male versus female behavior in shaping baseline fGCm concentrations in these animals. For example, in species in which males compete aggressively to gain access to females, the energetic demands of competition combined with the potential for injuries and the need to mount an associated immune response may render reproduction more challenging for males (Berger et al. 2005; Ancona et al. 2010), leading to the expectation that baseline glucocorticoids should be higher among members of this sex (Girard-Buttoz 2014; Hudson et al. 2019). Potentially consistent with this, home ranges for males in our study population are larger than those for females (O'Brien et al. 2020, 2021) and aggressive interactions between males but not females are observed at home range boundaries (Lacey et al., unpublished data). Relationships between these differences in behavior and baseline fGCm concentrations, however, may not be straightforward; studies of other seasonally breeding mammals indicate that while males have higher baseline glucocorticoid concentrations during reproduction in some species (Lynch et al. 2002; Fichtel et al. 2007), in others it is females with higher baseline concentrations (Schradin et al. 2008; Dantzer et al. 2010). Because our analyses were limited to data collected during the breeding season, we were unable to assess seasonal variation in fGCm concentrations or to examine intersexual differences in these levels during other portions of the year. Studies that examine temporal changes in baseline glucocorticoid concentrations within and between the sexes are needed to understand how these factors interact to shape the differences in fGCm concentrations reported here. Additionally, studies that examine the relationship between fGCm concentrations and sex steroids may also prove useful, particularly regarding how variation in concentrations of these hormones influence group dynamics (Dakin et al. 2021).

Implications for GC physiology. Although we had expected that differences in social environment – including differences in both social unit composition and social network metrics – would be important contributors to baseline glucocorticoid concentrations in our study population, none of the behavioral parameters examined emerged as significant predictors of fGCm concentrations. Instead, the only significant predictor of fGCm concentrations in our study animals was sex. Because analyses of fGCm concentrations in *C. sociabilis* did not include males (Woodruff et al. 2010, 2013), the relative contributions of sex versus social behavior have not been

assessed for this species of tuco-tuco. More generally, few studies of free-living, non-primate populations have attempted to distinguish the effects of intrinsic factors such as sex from those of extrinsic variation in social environment. Those efforts that have considered social behavior have typically focused on specific forms of interactions such as position in a dominance hierarchy (Gesquiere et al. 2011; van Kesteren et al. 2012; reviewed in Creel 2001 and Creel et al. 2013), rather than more general variation in social environment. An exception to this pattern is a study of common degus that, in keeping with our findings, revealed reproductive status to be more important than social context in predicting baseline fGCm concentrations in a free-living population of this burrow dwelling rodent (Ebensperger et al. 2011). Collectively, available data suggest that the effects of social environment on glucocorticoid physiology are complex and are likely to vary situationally as well as among species. Future studies that combine experimental manipulation of extrinsic conditions with analyses of fGCm variation among free-living animals should help to clarify the roles of intrinsic phenotypic differences versus social environment in shaping glucocorticoid physiology.

Conclusion

Our analyses of free-living highland tuco-tucos revealed that sex was a significant predictor of individual differences in fGCm concentrations. In contrast, although multiple aspects of an individual's social environment varied within our study population, none of the behavioral metrics examined were associated with variation in fGCm concentrations. Treatment of captive highland tuco-tucos with synthetic ACTH indicated that corticosterone is the dominant glucocorticoid metabolite in this species and confirmed that fGCm concentrations are responsive to external conditions, suggesting that the absence of relationships between behavioral parameters and glucocorticoid metabolites was not due to limitations of the EIA procedures used. Although we expect that larger samples sizes would have increased our ability to detect such relationships, it seems unlikely that this difference in outcomes would have altered the relative importance of sex versus social behavior in determining individual fGCm concentrations. Our findings differ from those for the only other social species of tuco-tuco (*C. sociabilis*) for which glucocorticoid data are available, in which social environment is associated with significant differences in fGCm concentrations (Woodruff et al. 2013). Thus, in addition to providing the first characterization of glucocorticoid physiology in highland tuco-tucos, our results – particularly when compared to those for *C. sociabilis* -- underscore both the complexity of the factors affecting HPA function and the importance of considering both intrinsic and extrinsic variables when exploring glucocorticoid variation in free-living mammals.

Figures

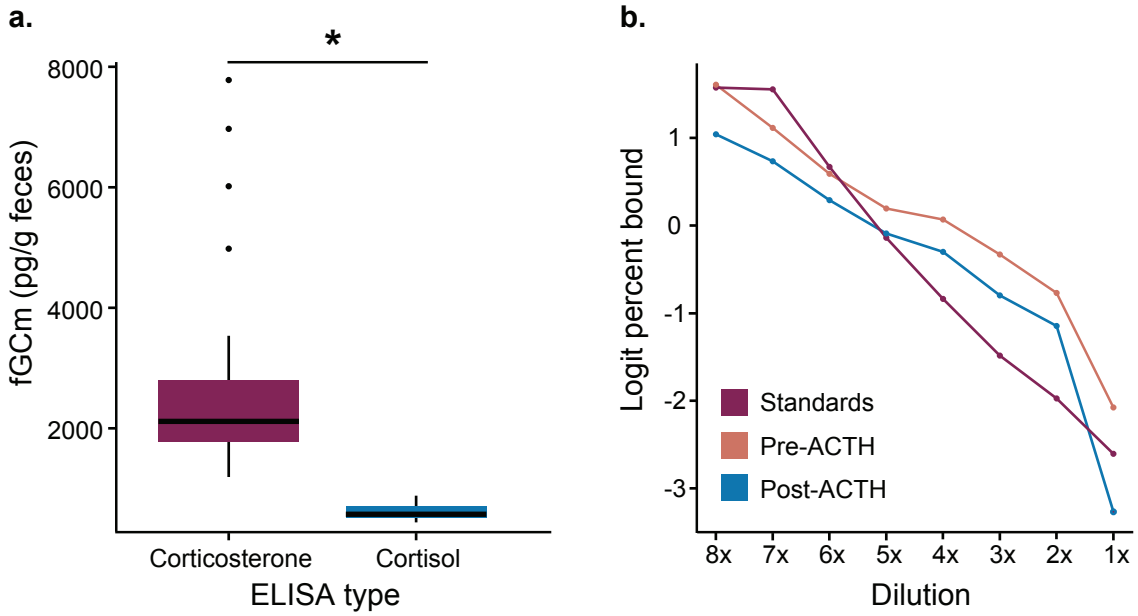

Figure 1. Biochemical validation of assays for fecal glucocorticoid metabolite (fGCm) concentrations in highland tuco-tucos. In (a), concentrations of corticosterone versus cortisol metabolites are compared for a subset of 24 randomly selected members of the study population. In (b), logit-transformed slopes are compared for 8 pooled, serially diluted samples collected pre- and post-injection with ACTH and kit standards. Significant contrasts based on Wilcoxon Signed Rank tests are denoted with an asterisk (*).

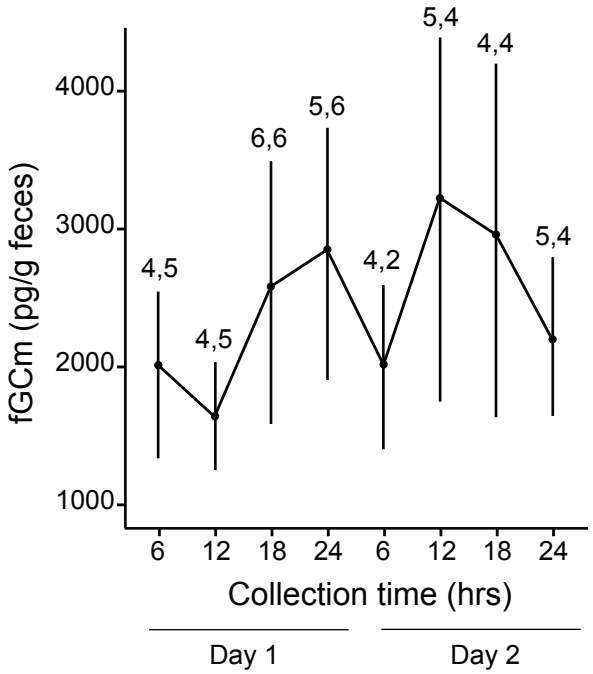

Figure 2. Patterns of circadian variation in fGCm concentrations. Mean (± SD) concentrations are shown for 12 captive highland tuco-tucos from which samples were collected every 6 hours for a total of 48 hours. Sample sizes are indicated above each time point; the number of males sampled is given first, followed by the number of females sampled.

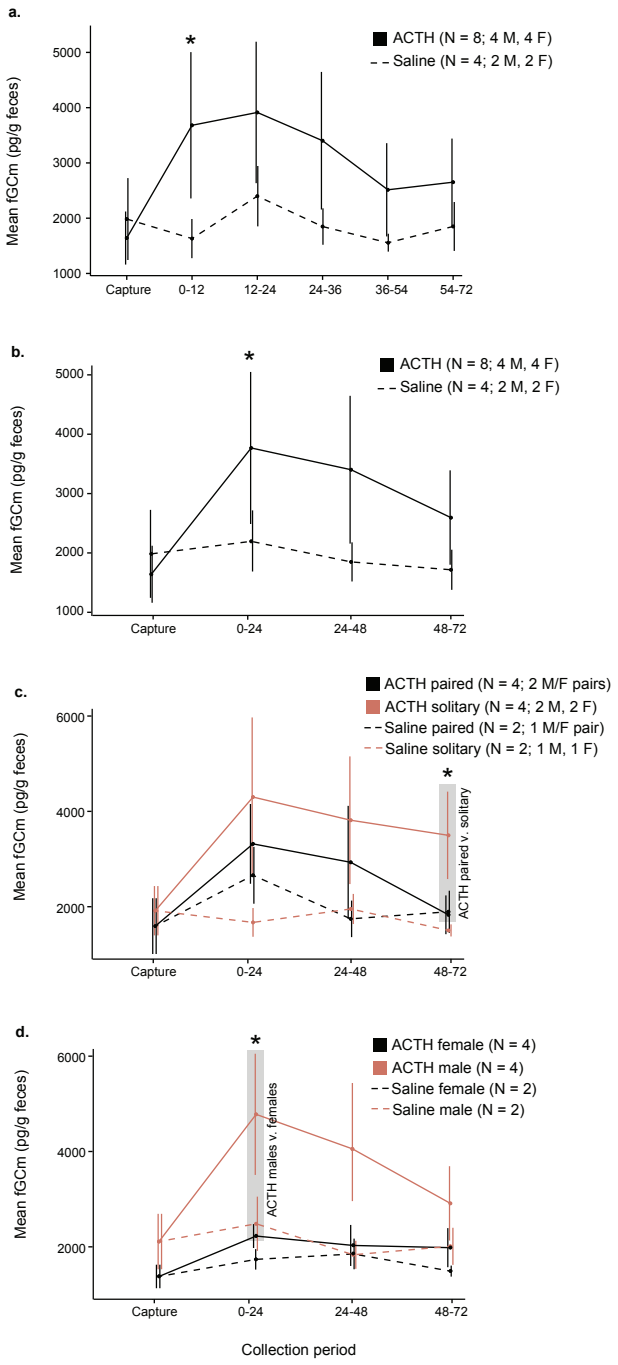

Figure 3. Results of ACTH challenge study. In (a), comparisons of fGCm concentrations are shown for ACTH- and saline-treated highland tuco-tucos at capture and at the specified time intervals continuing until 72 hrs after injection. In (b), the same data are shown but with samples pooled over the larger time periods used to assess the effects of housing and sex on fGCm concentrations. In (c), these data are presented as a function of the housing condition (solitary or paired) under which individuals were held in captivity. In (d), these data are shown as a function of sex. For each panel, sample sizes are denoted in the upper right. Significant contrasts are denoted with an asterisk (*); details of the statistical analyses are given in the text.

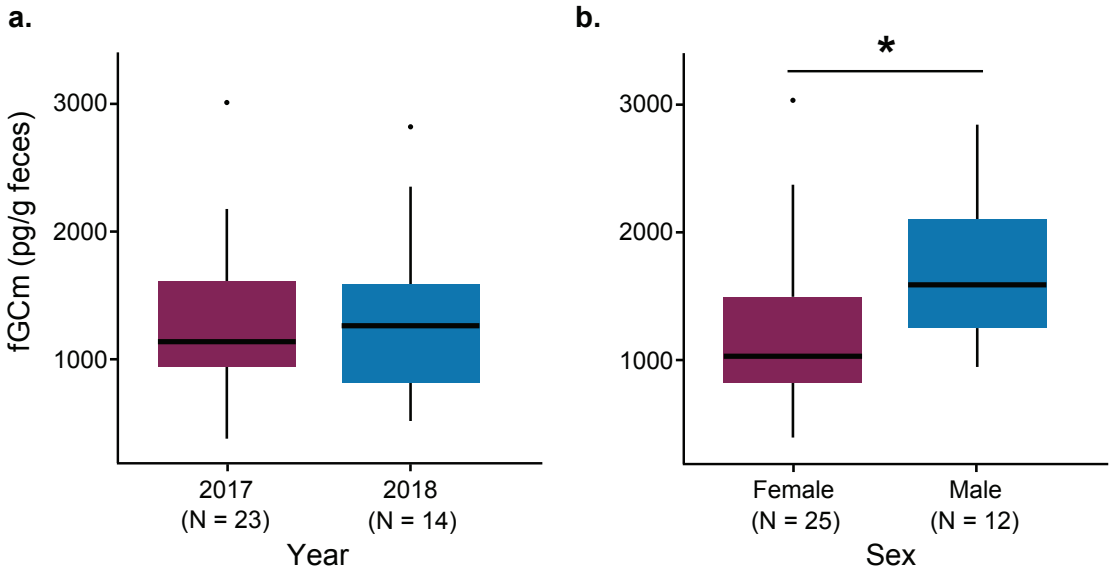

Figure 4. Comparisons of fGCm concentrations for free-living highland tuco-tucos as a function of (a) year and (b) sex. Mean and quartile values are depicted for each subset of individuals examined; sample sizes for each comparison are shown. Significant contrasts (Mann-Whitney U tests) are indicated with asterisks (*).

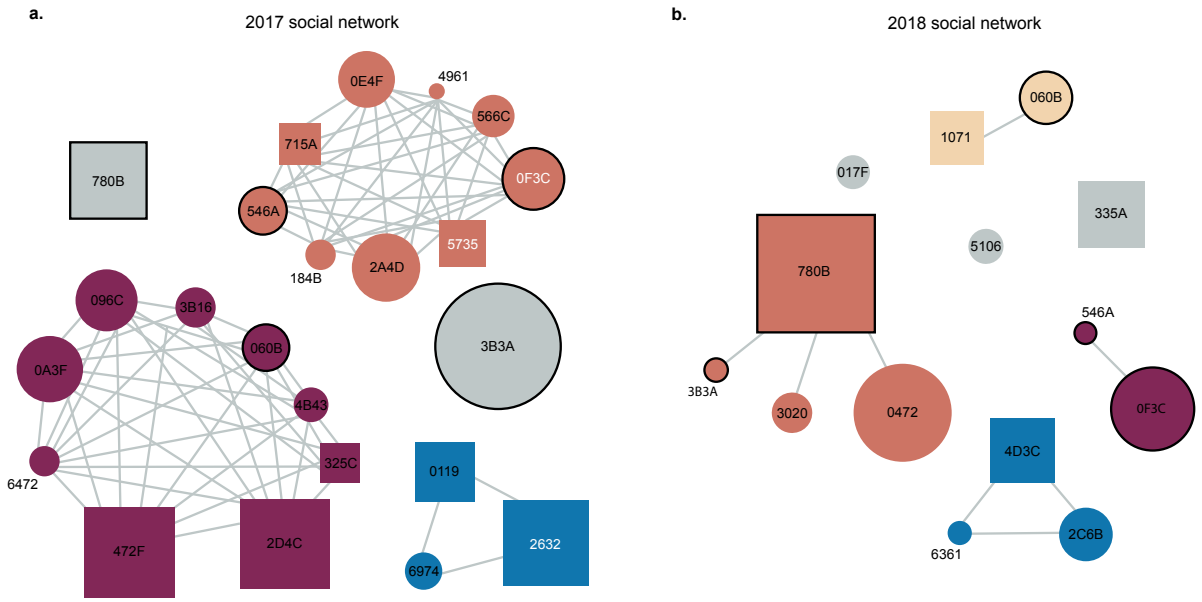

Figure 5. Relative fGCm concentrations within social units of free-living highland tuco-tucos. Data are from individuals monitored during (a) 2017 and (b) 2018; colors are the same as those used in Figure S1 to depict distinct social units. Males are indicated by squares and females are indicated with circles. Symbols used to denote individuals are sized proportionately to the fGCm concentration for each animal, with larger symbols indicating higher concentrations of these metabolites. The alphanumeric code associated with each symbol denotes the animal ID; black text indicates adults (N = 34) while white text denotes subadults (N = 3). Symbols for individuals captured in both years (N = 3) are outlined in black.

Tables

Table 1. Summary of phenotypic attributes and experimental conditions experienced by highland tuco-tucos used in the ACTH challenge experiment. For each treatment (injection with Cortrosyn versus injection with saline control), the number of adult males and females examined is indicated, as is the housing condition (single or paired), body mass, and injection volume for each individual.

Treatment	Housing	Sex	Mass (g)	Injection Volume (mg)
Cortrosyn	Single	M	325	0.16
Cortrosyn	Single	M	360	0.17
Cortrosyn	Single	F	265	0.13
Cortrosyn	Single	F	240	0.12
Cortrosyn	Pair 1	M	355	0.17
Cortrosyn	Pair 1	F	265	0.13
Cortrosyn	Pair 2	M	235	0.11
Cortrosyn	Pair 2	F	235	0.11
Saline	Single	M	225	0.11
Saline	Single	F	200	0.10
Saline	Pair 3	M	385	0.18
Saline	Pair 3	F	170	0.08

Table 2. Summary of the linear mixed-effect models used to examine variation in fGCm concentrations among members of the free-living population of highland tuco-tucos studied. Models based on measures of social unit size and composition are shown, as are models based on estimates of social network metrics. For each model considered, the predictor variables included are indicated, as are the associated degrees of freedom (DF) and Akaike Information Criterion (AIC). Best-fit models based on AIC values are indicated in bold. Models with differences in AIC values of ≤ 2 were interpreted as equally good at predicting variation in fGCm concentrations.

Type of explanatory variable	Equation	DF	AIC
Social unit size and composition	fGCm ~ sex + social unit size + (1|collection time) + (1|ID)	6	554.2386
	fGCm ~ sex + adult males + (1|collection time) + (1|ID)	6	551.9521
	fGCm ~ sex + adult females + (1|collection time) + (1|ID)	6	553.4476
	fGCm ~ sex * social unit size + (1|collection time) + (1|ID)	7	546.0504
	fGCm ~ sex * adult males + (1|collection time) + (1|ID)	**7**	**542.0275**
	fGCm ~ sex * adult females + (1|collection time) + (1|ID)	7	544.5902
Social network statistics	fGCm ~ sex + strength + (1|collection time) + (1|ID)	6	550.6
	fGCm ~ sex + eigenvector + (1|collection time) + (1|ID)	6	547.5827
	fGCm ~ sex + reach + (1|collection time) + (1|ID)	6	554.5715
	fGCm ~ sex + clustering + (1|collection time) + (1|ID)	6	548.0356
	fGCm ~ sex + affinity + (1|collection time) + (1|ID)	6	549.9214
	fGCm ~ sex * strength + (1|collection time) + (1|ID)	7	539.9269
	fGCm ~ sex * eigenvector + (1|collection time) + (1|ID)	**7**	**533.6091**
	fGCm ~ sex * reach + (1|collection time) + (1|ID)	7	547.6048
	fGCm ~ sex * clustering + (1|collection time) + (1|ID)	**7**	**535.3422**
	fGCm ~ sex * affinity + (1|collection time) + (1|ID)	7	536.1339

In chapter 3, I sought to investigate how the observed variation in social behavior within the population of *C. opimus* at Pozuelos may impact individual glucocorticoid physiology, a key regulator of allostasis and homeostasis. I collected fecal samples from all individuals captured on the field site during two consecutive years to assess the relationship between baseline glucocorticoid levels and multiple metrics of social behavior (i.e., group size, sex ratio of group, and metrics measured via social network analysis). I found that, despite marked variability in social relationships among the free-living tuco-tucos sampled, none of the measures of social behavior examined were significant predictors of variation in glucocorticoid concentrations. In contrast, individual variation in glucocorticoid metabolites was best explained by sex, with males having higher concentrations than females. These analyses provide the first characterization of the glucocorticoid physiology of highland tuco-tucos and underscore the potential importance of intrinsic phenotypic factors (e.g., sex) in shaping glucocorticoid variation in free-living mammals.

To build on these analyses examining the potential consequences of variation in sociality, I sought to study the role sociality plays in gut microbiome diversity within this population. Using fecal samples collected from the same individuals identified in chapter 3, I examined the relationship between gut microbiome alpha (within an individual) and beta (between individuals) diversity and multiple metrics of social behavior (i.e., group size, sex ratio of group, and metrics measured via social network analysis).

Chapter 4: Effects of spatial and social relationships on gut microbial diversity in a facultatively social rodent, the highland tuco-tuco (*Ctenomys opimus*)

Shannon L. O'Brien

Introduction

Beneficial microorganisms within the gut serve as key regulators of health and fitness (Sekirov et al. 2010, Suzuki 2017) that contribute to multiple critical host functions, including metabolic regulation (Claus et al. 2008, Wikoff et al. 2009), immune response (Desselberger 2018), and processes of ontogenetic change (Cho et al. 2012, Cox et al. 2014, Carlson et al. 2018). In mammals, acquisition of these organisms – collectively referred to as the gut microbiome – begins at birth with vertical transmission of microbes from mother to offspring (Spor et al. 2011, Bonder et al. 2016, Asnicar et al. 2017, Ge et al. 2021). Acquisition then continues throughout the lifetime of the host due to a combination of environmental exposure (e.g., diet; Muegge et al. 2011, Wu et al. 2022) and horizontal transfer of microbes from conspecifics (Moeller et al. 2018). Accordingly, the composition of an individual's gut microbiome reflects various sources, suggesting that analyses of gut microbial diversity can generate important insights into multiple aspects of a host's life history.

Social behavior, particularly the number of conspecifics with which an individual interacts and the frequency of those interactions, is thought to be a key factor contributing to the horizontal transmission of gut microbiota (Sarkar et al. 2020). For example, social contact associated with mating, grooming, playing, and fighting can result in transfer of microbes between hosts (Archie and Tung 2015). In general, such contact is expected to be greatest in species in which individuals routinely live in groups, members of which often spend extensive time in close physical proximity. The exclusivity of groups, however, varies markedly among taxa (Ebensperger 2001, Smith et al. 2017). In some species, groups are clearly spatially distinct, and members of different groups rarely interact (Asher et al. 2004, Ebensperger et al. 2004). In other species, however, groups are less discrete, and conspecifics may engage in regular contact with non-group mates (Silk and Kappeler 2017, Smith et al. 2017). Relationships between social behavior and gut microbial diversity have been examined for a limited number of wild mammals (Antwis et al. 2018,), notably several free-living populations of primates (Tung et al. 2015, Moeller et al. 2016, Perofsky et al. 2017, Wikberg et al. 2020). These analyses have emphasized taxa characterized by spatially distinct groups; in comparison, relationships between social contact and gut microbial diversity in species with more fluid group structures have received little attention (Raulo et al. 2021), despite an increasing number of studies that report flexible patterns of social organization in wild mammals (Schradin et al. 2012, Kappeler et al. 2013, Smith et al. 2017).

The highland tuco- tuco (*Ctenomys opimus*) is a subterranean rodent that is endemic to high elevation Puna habitats in northwestern Argentina (Lacey et al. 2022). Unlike most members of the genus *Ctenomys*, this species is social, meaning that multiple adults share the same burrow system and underground nest site (O'Brien et al. 2020). Studies of *C. opimus* from northern Jujuy Province, Argentina, have revealed that these animals are facultatively social, with both lone and

group-living individuals regularly co-occurring within the same population (O'Brien et al. 2020, 2021). Although group membership can be determined quantitatively using social network analyses, partial overlap of home ranges for individuals assigned to different social groups is common (O'Brien et al. 2020, 2021) and creates regular opportunities for interactions with non-group members. Overall, this variability in spatial and social relationships suggests that studies of *C. opimus* provide an ideal opportunity to examine the effects of group membership versus other forms of social contact on horizontal transmission of gut microbes in free-living mammals.

To explore the role of social behavior in shaping the gut microbiome, we compared multiple metrics of spatio-social relationships in *C. opimus* to measures of gut microbial diversity based on bacterial DNA extracted from fecal samples from these animals. If the number of distinct social contacts is important, then we predict that the diversity of gut microbes within individuals (i.e., alpha diversity) will increase as a function of one or more of the following: (1) number of home ranges overlapped, (2) social group size, and (3) social connectedness, as quantified using social network analyses. At the same time, if interactions with group mates are critical, we expect measures of gut microbial differentiation (i.e., beta diversity) to be associated with membership in the same social group. These analyses, which provide the first characterization of gut microbial diversity in highland tuco-tucos, generate important insights into the complex interplay of spatial and social relationships that contribute to gut microbial diversity in free-living populations of mammals.

Methods

Study site. The highland tuco-tucos (*Ctenomys opimus*) examined here were members of the same population studied by O'Brien et al. (2020, 2021, 2022) at Monumento Natural Laguna de los Pozuelos, Jujuy Province, Argentina (-22.469347, -65.994279, WGS 84; elevation: 3,600 m). The study site was located along the western bank of the Río Cincel and consisted of ca. 3 ha of open, high elevation Puna habitat dominated by saltgrass (*Distichlis* sp.) and needlegrass (*Stipa* sp). Data included in this study were collected during two field seasons: 23 December 2017 to 9 January 2018 (2017 field season) and 21 December 2018 to 5 January 2019 (2018 field season). These dates correspond to the primary breeding season for members of the study population.

Animal capture and handling. All procedures involving live animals were consistent with the guidelines of the American Society of Mammalogists for the use of wild mammals in research (Sikes et al. 2016) and had been approved by the Animal Care and Use Committee at the University of California, Berkeley. To capture individuals, Tomahawk-style live traps baited with carrots were placed at active burrow entrances, which were identified based on the presence of recently excavated mounds of dirt or direct visual observations of animals using a given entrance (O'Brien et al. 2020, 2021, 2022). Trapping occurred during daylight hours; traps were monitored continuously while open, allowing animals to be retrieved immediately upon capture. A hand-held GPS unit (accuracy ~ 6 m) was used to record the location of each capture. Additionally, capture localities were recorded using a Cartesian coordinate system (8 m x 8 m grid cells) established on the site each year prior to the start of data collection. This grid was also used to record the

localities of individuals during radio-telemetric monitoring of spatial relationships among members of the study population (see below).

At first capture, each animal was injected with a PIT-tag (IMI-1000, Bio Medic Data Systems, Inc., Seaford, DE); tags were inserted subcutaneously at the nape of the neck. PIT-tags were read using a hand-held scanner (DAS 4000 Pocket Scanner, Bio Medic Data Systems Inc., Seaforth, DE) and provided a means of permanently identifying each member of the study population. Sex and body mass were recorded each time that an individual was captured. For females, reproductive status was also assessed at the time of each capture based on the appearance of the external genitalia (sexually receptive), the ability to palpate fetuses (pregnant), or the presence of enlarged mammae (lactating). In contrast, the reproductive status of males could not be determined based on external appearance because the testes of males in the study population never descend externally.

Radiotracking of study animals. To quantify spatial relationships among members of the study population, all adults captured on the study site were fitted with radio transmitters (G3-1V transmitters, AVM Instrument Company, Colfax, CA) that were affixed using plastic cable ties as collars. The weight of the transmitter and collar together (~ 7 g) represented < 5% of the body weight of adults in the study population (Sikes et al. 2016; O'Brien et al. 2020). Following their release, collared animals were located multiple times per day and their positions on the study site recorded following the procedure in O'Brien et al. (2020). The resulting data were used to construct a home range for each animal monitored (see below).

Collection of fecal samples. Gut microbial diversity was assessed based analyses of fecal samples collected from members of the study population. Upon capture, each individual was transferred to a cloth bag that served to restrain the animal and to gather fecal pellets released during handling. All pellets from the same individual were placed in a cryogenic vial and frozen in liquid nitrogen until samples could be transferred to a -80° C freezer. During the 2017 field season, each vial was pre-filled with 0.5 ml of RNAlater, such that fecal pellets were submerged in this preservative prior to freezing; RNAlater was not used during the 2018 field season. Once all marking and handling procedures had been completed and fecal samples had been obtained, each individual was released at the point of capture.

Characterizing spatial and social relationships. Individuals were assigned to social units (i.e., spatially distinct clusters of animals) and social network metrics were assessed using the methods described in O'Brien et al. (2022). In brief, telemetry data were used to construct a 95% minimum convex polygon (MCP) for each radiocollared animal; MCPs were generated in the R package adehabitatHR (Calenge 2015). Based on these polygons, the number of conspecifics with which each individual overlapped spatially was determined. Percent overlap between 95% MCPs was estimated for all pairwise combinations of radiocollared individuals captured on the study site during the same field season. The resulting association matrix was analyzed in SOCPROG (Whitehead 2009) to identify spatially distinct clusters of animals. Five additional metrics of social network structure generated by SOCPROG (strength, eigenvector centrality, affinity, reach, and

clustering coefficient) were also examined; detailed descriptions of these parameters are provided in Whitehead (2009).

Bacterial DNA extractions and 16S rRNA sequencing. Bacterial DNA was extracted from fecal samples using the MoBio DNeasy PowerSoil kit (Qiagen, Hilden, Germany). Purity of fecal bacterial DNA was assessed via spectrophotometry (Nanodrop, ThermoScientific, Waltham, MA), after which extracts were stored at -80° C until analysis. Sequencing of the v4 region of the bacterial 16S rRNA gene was conducted on an Illumina Miseq platform by Microbiome Insights Inc. (British Columbia, Canada). Miseq-generated Fastq files were quality-filtered and high quality reads were clustered into 97% similarity operational taxonomic units (OTUs) using the mothur software package (Schloss and Westcott 2011); OTUs were then assigned to specific microbial taxa using the reference database in Greengenes v. 13_8. Downstream quality filtering as well as clustering and OTU assignment were done by Microbiome Insights Inc. (British Columbia, Canada).

Gut microbial diversity and composition. Due to the different fecal sample preservation methods used in 2017 versus 2018, data from each year of the study were analyzed separately. The diversity of microbial taxa detected within each individual (i.e., alpha diversity) was quantified using the Shannon index. To identify potential social predictors of this diversity, linear mixed-effect models were constructed with a Gaussian distribution using the R package 'lme4' (Bates et al. 2007). Predictor variables examined were number of conspecifics with which an individual overlapped spatially, social unit size, and the five social network metrics identified above. To avoid overfitting of the data set and to avoid potential confounds due to correlations among the different behavioral variables considered, a separate model set was constructed for each predictor variable. All models contained sex as a fixed effect. Year was included as a random effect in all models to account for potential variation. Models were run with and without an interaction between sex and the focal predictor variable. The Akaike information criterion (AIC) was used to identify the best-fit model for alpha diversity, after which post-hoc type III Wald Chi-square tests were used to determine which explanatory variables in the best-fit model were significant predictors of alpha diversity; post hoc tests were completed using the R package 'car' (Fox et al. 2007).

To examine patterns of gut microbial composition (i.e., beta diversity) in relation to social behavior, principal coordinates analyses was used to reduce data regarding the relative abundances of microbial OTUs within individuals into Bray-Curtis dissimilarity values. Permutational multivariate regression analysis (Permanova) was then used to test for associations between Bray-Curtis dissimilarities and each of the following predictor variables: social unit membership, sex, and year of data collection using R package 'vegan' (Oksanen et al. 2013). Mantel tests were used to assess the relationship between Bray-Curtis dissimilarities and degree of overlap between individual home ranges, calculated as percent overlap of 95% MCPs (see above).

Statistical analyses. Throughout the text, means are reported $\pm$ 1 SD. Parametric tests were used unless the data required that non-parametric tests were used. All statistical analyses were performed in R v. 4.0.4 (R Core Team, 2017).

Results

Thirty-three animals (10 adult males, 20 adult females, 3 subadults) were captured on the study site during the 2017 field season and 17 animals (4 adult males, 10 adult females, 3 subadults) were captured during the 2018 field season. Of these, sufficient spatial data for social network analyses were obtained for 25 animals (7 adult males, 15 adult females, 3 subadults) in 2017 and 14 animals (4 adult males, 10 adult females, 0 subadults) during 2018. Four adults (1 male, 3 females) were captured in both 2017 and 2018; none of these individuals lived with the same conspecifics in successive years and thus data for these animals collected during each field season were treated as independent (Figure 1; see also O'Brien et al. 2022). Social network analyses of home range overlap revealed the presence of 5 social units in 2017 (mean social unit size = 5.0 $\pm$ 4.7 individuals, range = 1-11) and 7 social units in 2018 (mean social unit size = 2.0 $\pm$ 1.2 individuals, range = 1-4; Figure 1); mean social unit size did not differ significantly between years (Mann-Whitney U test, W = 22, P = 0.447). Values for the social network metrics calculated for these animals (strength, eigenvector centrality, affinity, reach, and clustering coefficient) are summarized in Figure 2 and Supplementary Table 1. As reported by O'Brien et al. (2022), mean values for four of these metrics (strength, reach, clustering coefficient, affinity) differed significantly between years; no differences in mean values were found between the sexes.

Sequencing of bacterial DNA. Of the animals included in our social network analyses, fecal samples were available for 15 adults (5 males, 10 females) from 2017 and 14 adults (4 males, 10 females) from 2018. Bacterial DNA extracted from these individuals was sequenced to a mean depth of 23,558 $\pm$ 14,831 reads per animal. Across both years of the study, total of 13 bacterial phyla identified; two of these (*Deferribacteres, Elusimicrobia*) were only present in samples collected in 2017 (Supplementary Figure 1). In both years, some sequences could not be assigned to a known bacterial phylum and were thus categorized as "unclassified."

Among those sequences identified to phylum, samples from both years were dominated by *Bacterioidetes* and *Firmicutes* (Supplementary Figure 1), although proportions of these bacteria differed between field seasons (Mann-Whitney U tests; *Bacterioidetes*: W = 210, P < 0.001; *Firmicutes*: W = 210, P = P < 0.001). Relative abundances of *Bacterioidetes* were higher in 2018 (0.93 $\pm$ 0.03) than in 2017 (0.60 $\pm$ 0.07), whereas relative abundances of *Firmicutes* were higher in 2017 (0.15 $\pm$ 0.05) than in 2018 (0.02 $\pm$ 0.01). On average, no other phylum accounted for more than 10% of the sequences identified per individual.

Predictors of alpha diversity. Mean Shannon index values were 4.48 $\pm$ 0.46 in 2017 and 2.81 $\pm$ 0.44 in 2018, these differences were significant (Mann-Whitney U test; V = 120, P < 0.001). Preliminary analyses revealed significant positive correlations between Shannon index values and both social unit size (R = 0.63, t = 4.24, df = 27, P = 0.0002) and the number of conspecifics with which an individual overlapped spatially (R = 0.68, t = 4.83, df = 27, P < 0.0001). Similarly, significant positive correlations were detected between Shannon index values and four of the social network metrics examined (strength, affinity, reach, clustering coefficient; all P $\leq$ 0.01; Supplementary Table 2). The final network metric (Eigenvector centrality) was also positively correlated with Shannon index values; this relationship was not significant (R = 0.16, t = 0.82, df = 27, P = 0.42).

Given the overall tendency for these behavioral variables to be significantly correlated with Shannon index values, social unit size, number of conspecifics overlapped, and all five social network metrics were retained in subsequent analyses of predictors of alpha diversity.

Based on AIC values, four linear models were equally predictive of alpha diversity of gut microbes (Table 1). Two of these best-fit models included eigenvector centrality and two included the clustering coefficient. In contrast, models including social unit size, number of conspecifics overlapped, and the three remaining social network metrics examined received significantly less support (Δ AIC > 2.0; Table 1). Based on AIC values, the best-fit models were not improved by including the interaction between sex and the relevant network metric (Table 1). Post-hoc Wald chi-square tests revealed that none of the variables in the four best-fit models were significant predictors of Shannon index values ($P > 0.2$ for all variables). Thus, overall, linear models failed to reveal any significant behavioral predictors of alpha diversity of gut microbes in our study population.

Predictors of beta diversity. Analyses of Bray-Curtis dissimilarities revealed that the gut microbial composition of individuals clustered by year (Permanova $F = 69.6$, $P = 0.001$) but not by sex (Permanova $F = 1.4$, $P = 0.24$; Table 2, Supplementary Figure 2). When only social unit membership was considered, gut microbial composition appeared to cluster as a function of this variable (Permanova $F = 6.1$, $P < 0.001$), although inclusion of both social unit membership and year in these analyses provided evidence for an effect of year only (Permanova $F = 60.6$, $P = 0.001$; Table 2, Supplementary Figure 2). Values for Bray-Curtis dissimilarities increased as a function of the pairwise percentage overlap of individual home ranges (95% MCPs); this tendency was significant (Mantel test, $R = 0.52$, $P < 0.001$, permutations = 9999; Figure 3).

Discussion

Our analyses indicate that none of the behavioral metrics examined were significant predictors of gut microbial diversity within individual highland tuco-tucos. Although the best-fit models for this diversity included eigenvector centrality and clustering coefficient, post-hoc tests revealed that neither of these social network metrics predicted alpha diversity in our study population. In contrast, diversity among members of the study population (i.e., beta diversity) was predicted by year and, within years, by the degree of spatial overlap among individuals. More specifically, in both 2017 and 2018, greater overlap of individual home ranges was associated with greater similarity in gut microbial composition. These findings suggest that although variation in relationships within social units does not affect gut microbial diversity in our study animals, spatial relationships more generally are important determinants of the gut microbiota of highland tuco-tucos. This implies that while horizontal transmission of bacteria is important in this species, social unit boundaries are not critical determinants of that transmission.

Effect of preservation method. One factor that was associated with differences in alpha diversity of gut microbes was year of sample collection. Alpha diversity within the study population may have varied across years of the study for multiple reasons, including changes in diet or changes in composition of the study population (David et al. 2014, Maurice et al. 2015, Morrison

et al. 2020). At the same time, between-year differences in how fecal samples were preserved may have contributed to this outcome (Ma et al. 2020). Diversity was significantly higher in 2017, when RNAlater was used as a preservative, suggesting that the use of RNAlater prior to freezing is more effective at preserving gut microbiome diversity than freezing alone.

Implications for transmission of gut microbes. Gut microbial diversity in mammals is typically assumed to reflect a mixture of vertical and horizontal transmission (Moeller et al. 2018, Sarkar et al. 2020). Although we did not explicitly examine vertical (i.e., mother to offspring) transmission in our study animals, several lines of evidence suggest that this mode of transmission may be of limited importance in the study population. First, all the individuals included in our analyses were adults and none were known to represent mother-offspring pairs. Second, long-term studies of the social organization of this population indicate that individuals do not tend to remain in the same social unit across years (O'Brien et al. 2021), thereby decreasing the probability that individuals reside in strongly kin-structured groups such as those found in mammals such as elephants (Archie et al. 2006) yellow-bellied marmots (Wey and Blumstein 2010) and European badgers (Benton et al. 2016). Finally, studies of several populations of wild mammals for which kin relationships were known (e.g., wild mice: Raulo et al. 2021; several species of group-living primates: Amato et al. 2014, Tung et al. 2015, Perofsky et al. 2017, Wikberg et al. 2020) have failed to reveal a significant effect of kinship on gut microbial diversity, suggesting that transmission from mothers to offspring is not a primary determinant of microbial diversity in these species. Genetic analyses of parentage and kinship in our study population are currently in progress; these data will allow preliminary evaluation of the role of vertical transmission in shaping gut microbial diversity in *C. opimus* at Pozuelos. This information will, in turn, allow a more robust determination of the relative important of vertical and horizontal transmission of microbes in these animals.

Tuco-tucos are subterranean rodents, meaning that individuals spend a significant portion of their lives in underground burrows (Nevo 1979; Lacey et al. 2000; de Freitas et al. 2021). Although the extent to which individuals are active on the surface varies among species (pers. obs.), all members of the genus *Ctenomys* occupy underground nests and spend most of their time in a network of underground tunnels that are expected to constrain patterns of movement, at least when compared to movements by many surface-dwelling species (Lacey et al. 1997, Lacey et al. 1998, Luna and Antinuchi 2007). This aspect of tuco-tuco natural history may have implications for horizontal transfer of gut microbes. For example, although feeding and defecation may occur primarily at distinct locations, movement of food into and movement of waste out of burrow systems are likely accomplished via the same tunnels (Hickman 1985, Camin et al. 1995) and it is not uncommon to find fresh feces mixed with bits of freshly cropped vegetation around and just inside of active burrow entrances (pers. obs.). As a result, transfer of gut microbes may occur between individuals that use the same tunnels, even if those animals do not engage in direct, physical contact with one another. Indirect forms of microbiome transfer have been observed in humans (Neckovic et al. 2020), but this method of horizontal transmission is relatively unexplored in animal populations. Indirect horizontal transmission seems most applicable to populations like that at Pozuelos in which spatial overlap of individuals is common and habitat use is constrained by physical features such as burrows that increase the potential for indirect contact between animals.

Spatial versus social relationships among individuals. Our analyses of social behavior were based on data regarding spatial relationships among individuals. These included analyses of home range overlap and social unit membership as well as analyses of social network metrics. Use of spatial data to infer social relationships is common among studies of wild mammals (Wey et al. 2008, Pinter-Wollman 2014), particularly in subterranean mammals (Tassino et al. 2011, Lacey et al. 2019). While such data offer critical insights into social organization, the information that they capture is unlikely to be as detailed as direct observations of social interactions among conspecifics (Sterling et al. 2000, Gelardi et al. 2020). This lack of specificity may have contributed to our failure to detect relationships between most of the behavioral variables examined and diversity of gut microbes. In particular, spatial data may have failed to fully capture variability in the frequency or nature of social interactions among conspecifics whose home ranges overlapped, particularly individuals assigned to the same social unit. *C. opimus* is unusual among tuco-tucos in that individuals spend considerable time above ground, where they can be observed directly (O'Brien et al. 2020, 2021). When above ground, however, the animals spend most of their time foraging and interactions among conspecifics are relatively rare (unpubl. data) and thus spatial data remain the primary means of characterizing potential social relationships among members of the study population. Spatial data have been used to reveal striking variation in social network metrics (Smith et al. 2018, Smith and Pinter-Wollman 2021), indicating that such information can be used to explore individual variation in behavior.

Importance of group membership. Our analyses revealed that although similarity of gut microbial composition increased with the degree of spatial overlap among individuals, membership in the same social unit was not a significant predictor of beta diversity in gut microbial composition. This finding differs from those of several previous studies that have identified group membership as important to patterns of beta diversity (Tung et al. 2015, Moeller et al. 2016, Perofsky et al. 2017, Antwis et al. 2018, Wikberg et al. 2020). These analyses have generally focused on social species of mammals, members of which live in clearly discrete, often hierarchically structured groups. In contrast, members of our study population display a much more flexible social organization. Although quantitatively distinct social units can be identified based on spatial data (O'Brien et al. 2020), spatial overlap among members of different units is not uncommon and individuals often change social units between years (O'Brien et al. 2021). Collectively, these observations suggest that social interactions likely are not restricted to members of the same social unit, thereby potentially increasing the number of conspecifics with which horizontal transmission of gut microbes may occur and concomitantly decreasing the importance of social unit composition as a predictor of beta diversity of the gut microbiome in *C. opimus*.

The social organization of our study population appears to differ markedly from those of the other species of *Ctenomys* studied to date (O'Brien et al. 2020). In particular, the flexible spatial and social relationships that characterize highland tuco-tucos at Pozuelos differ from the distinct spatial boundaries between groups that occur in the colonial tuco-tuco (*C. sociabilis*), which is only other species of *Ctenomys* that is known to be group-living (Lacey et al. 1997). Several other species that have been examined tend to be more solitary (e.g., *C. haigi*: Lacey et al. 1998; *C.*

talarum: Cutrera et al. 2006; *C. australis*: Cutrera et al. 2010), with interactions among adults thought to be restricted largely to periodic reproductive encounters. Collectively, these studies suggest that comparative analyses within this genus may prove informative regarding the relative importance of different forms of spatial and social interaction in shaping gut microbial diversity in this clade of mammals.

Conclusions. Our analyses of the population of highland tuco-tucos at Pozuelos indicate that although spatial and putative social relationships among individuals are important determinants of beta diversity in gut microbes, membership within the same social unit does not predict diversity of gut microbes in these animals. Although this outcome contrasts with those of other studies of gut microbial diversity in group-living mammals, it is perhaps not surprising given the variability in spatial relationships that characterizes our study population. More specifically, regular spatial overlap with extra-group individuals may result in a greater degree of horizontal transmission relative to species in which little contact occurs among members of different social groups. This potential for transmission of microbial taxa may be enhanced by the subterranean lifestyle of our study animals, which may constraint opportunities for movement within the habitat in ways that increase the potential for contact with excrete from conspecifics. Future studies that compare patterns of gut microbial diversity in *C. opimus* with those in behaviorally distinct congeners should help to clarify the roles of vertical and horizontal transmission – including contactless horizontal exchange of microbes – in free living populations of mammals.

Figures

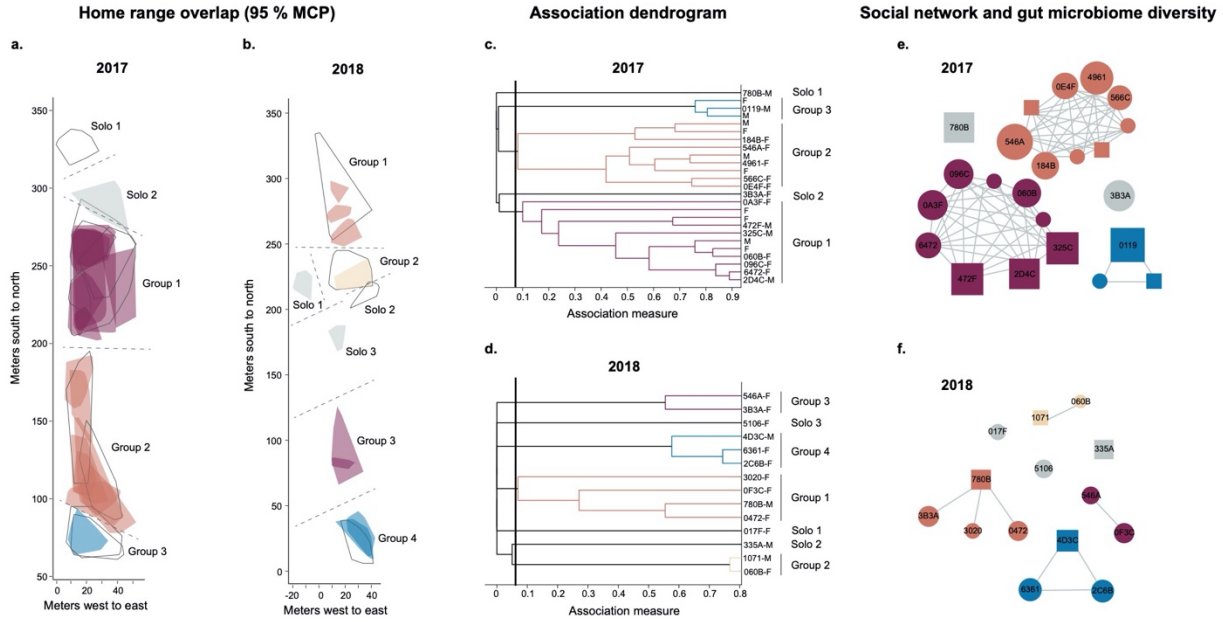

Figure 1. Summary of spatial and social relationships among members of the study population. In (a) and (b), home ranges (95% MCPs) are shown for all individuals included in this study. Males are represented with unfilled polygons while females are represented by colored polygons; distinct social units identified from social network analyses are indicated. Panels (c) and (d) present the dendrograms from social network analyses used to assign individuals to social units. Individual ID and sex are shown for animals that were included in our analyses of gut microbiome diversity; individuals that were included in social network analyses but for which microbial data were not obtained are identified by only by sex (M or F). Panels (e) and (f) depict the same information in network form. Each individual is shown as a square (male) or circle (female); nodes depicting individuals included in microbial analyses indicate the ID for each animal. Sizes of individual nodes are proportional to Shannon index values, which were used to quantify alpha diversity of the gut microbiome; larger nodes indicate larger index values and greater microbial diversity. Unlabeled nodes (individuals for which microbial data were not available) are all the same size. Within each year, the same color is used across all panels to indicate data from the same social unit.

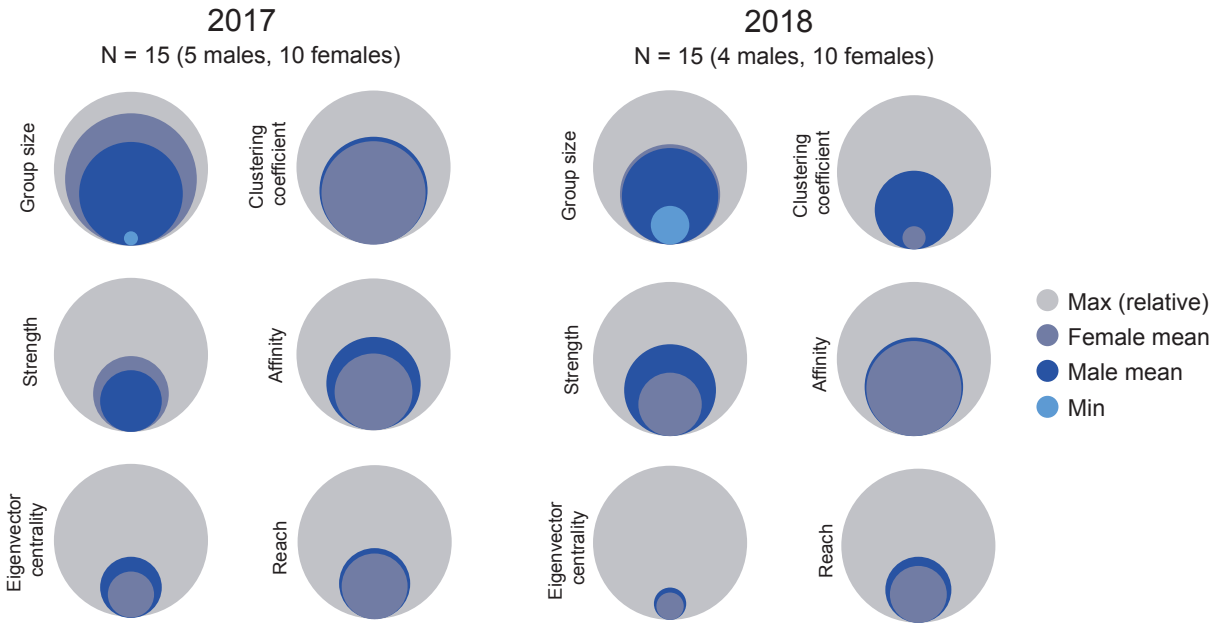

Figure 2. Summary of values for group size and the five social network metrics examined in this study. Data for 2017 and 2018 are shown separately. For each variable, colored circles denote minimum values, as well as mean values for males and females. The maximum values are a reference point for which the other values can be compared; it does not represent the true maximum value. Circles for minimum values are not included for variables for which the minimum value was zero. For each variable, the mean, standard deviation, and range of values per sex and year are provided in Supplementary Table 1. More detailed descriptions of each social network metric are given in O'Brien et al. (2022).

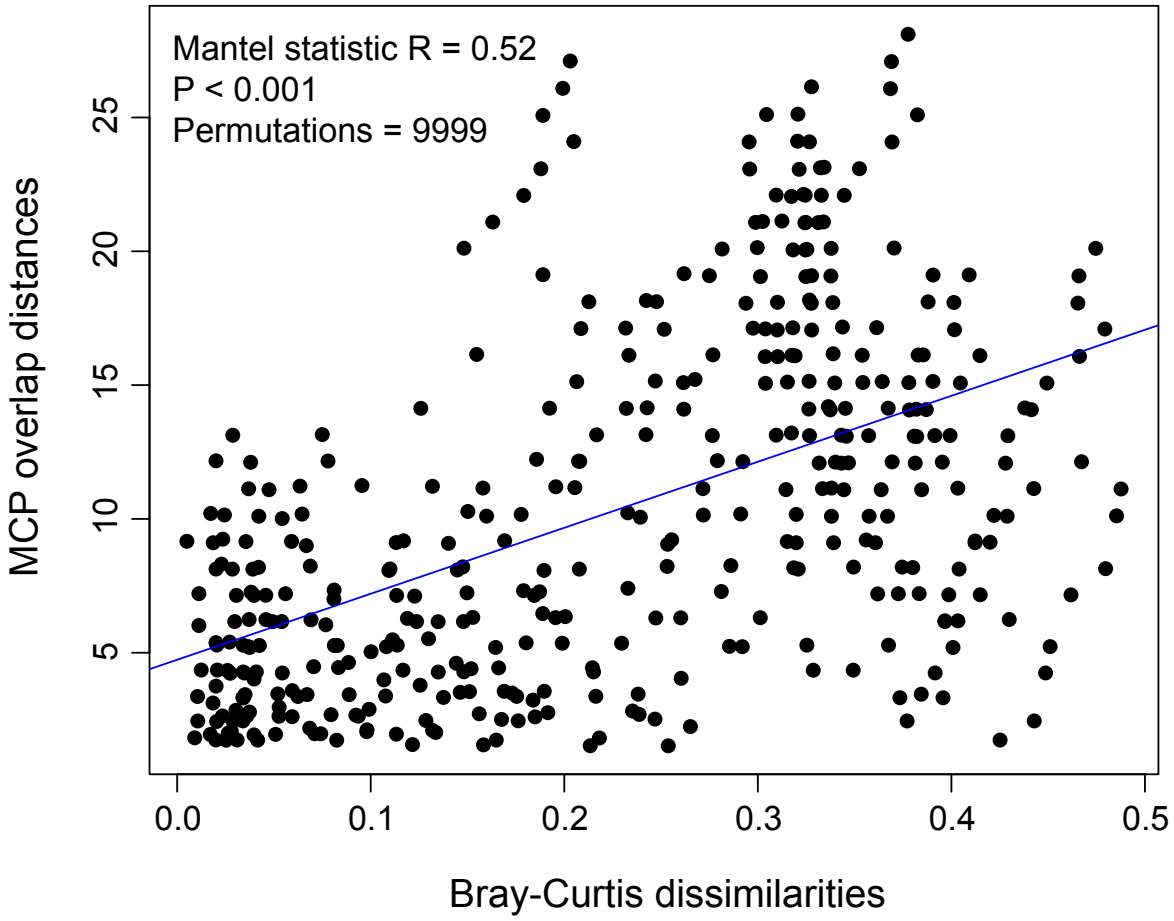

Figure 3. Results of a non-parametric (Spearman) Mantel test relating spatial overlap between pairs of individuals to beta diversity of gut microbes. Spatial overlap was measured as pairwise percent overlap on individual home ranges (95% MCPs); estimates of beta diversity are based on Bray-Curtis dissimilarities. Test results are shown in the figure.

Tables

Table 1. Summary of the linear mixed-effect models used to examine variation in gut microbiome alpha diversity. For each model considered, the predictor variables included are indicated, as are the associated degrees of freedom (DF) and Akaike Information Criterion (AIC). In the models, Eigen refers to Eigenvector centrality and Clust refers to clustering coefficient. Best-fit models based on AIC values are indicated in bold. Models with differences in AIC values of $\leq$ 2 were interpreted as equally good at predicting variation in fGCm concentrations.

Model	DF	AIC
lmer (Alpha_Diversity ~ Sex + Group_Size + (1|Year))	5	82.16
lmer (Alpha_Diversity ~ Sex * Group_Size + (1|Year))	6	87.48
lmer (Alpha_Diversity ~ Sex + Strength + (1|Year))	5	80.70
lmer (Alpha_Diversity ~ Sex * Strength + (1|Year))	6	83.86
lmer (Alpha_Diversity ~ Sex + Eigen + (1|Year))	**5**	**76.45**
lmer (Alpha_Diversity ~ Sex * Eigen + (1|Year))	**6**	**75.85**
lmer (Alpha_Diversity ~ Sex + Reach + (1|Year))	5	83.64
lmer (Alpha_Diversity ~ Sex * Reach + (1|Year))	6	89.28
lmer (Alpha_Diversity ~ Sex + Clust + (1|Year))	**5**	**76.71**
lmer (Alpha_Diversity ~ Sex * Clust + (1|Year))	**6**	**77.38**
lmer (Alpha_Diversity ~ Sex + Affinity + (1|Year))	5	79.60
lmer (Alpha_Diversity ~ Sex * Affinity + (1|Year))	6	83.48
lmer (Alpha_Diversity ~ Sex + Overlap + (1|Year))	5	57.57
lmer (Alpha_Diversity ~ Sex * Overlap + (1|Year))	6	62.55

Table 2. Permanova results based on Bray-Curtis dissimilarities revealed that samples clustered by year, but not by sex or social group. Df: degrees of freedom; Sum Sq: sum of squares; Pseudo-F: F value by permutation. Bolded P-values indicate significance; P-values based on 999 permutations.

	Df	Sum Sq	R2	Pseudo-F	P
Year	1	1.77	0.72	69.56	**0.001**
Residual	27	0.69	0.27		
Total	28	2.45	1		
Sex	1	0.12	0.05	1.41	0.24
Residual	27	2.33	0.95		
Total	28	2.45	1		
Social group	11	1.96	0.80	6.10	**0.001**
Residual	17	0.50	0.20		
Total	28	2.45	1		
Year x social group					
Year	1	1.77	0.72	60.60	**0.001**
Social group	10	0.19	0.08	0.65	0.82
Residual	17	0.50	0.20		
Total	28	2.45	1		

Conclusions

In summation, highland tuco-tucos (*Ctenomys opimus*) from Laguna de los Pozuelos, Jujuy Province, Argentina are facultatively social, consisting of both lone and group-living individuals. Groups may range from 2 – 24 individuals and typically contain a mixture of both adult males and females. However, groups consisting of only males and groups consisting of only females were also observed. Daytime and nighttime groups consistently contained the same individuals, however groups tended to fission at night such that larger daytime groups were broken into smaller social units during the night. These smaller nighttime social units appeared to consistently use one nest site, however there were a few observations of individuals of both sexes switching nests across consecutive nights. Although, nest partners always nested with individuals they associated with during the daytime.

Of the individuals that were recaptured across successive years, none were solitary for more than one year. In general, individuals became more social across successive years (i.e., their group size increased) and no individuals were observed to revert from group-living to solitary-living. In general, this suggests that while facultative sociality is a consistent feature of this population, the observed variation in social structure is not due to persistent features of individuals (i.e., individuals are not consistently solitary or consistently social). In fact, social groups were not consistent over time, rather individuals were observed in unique social groups each year despite groupmates from former years still residing on the field site.

The marked variation in sociality observed in this population was not associated with variation in fecal glucocorticoid metabolites. Rather, sex was the best predictor of variation in fecal glucocorticoid metabolites, such than males had higher corticosterone concentrations than females in both the captive and free-living populations. Interestingly, within the captive population, individuals that were housed with a partner recovered from injection of adrenocorticotropic hormone (i.e., returned to pre-injection corticosterone levels) more quickly than individuals that were housed alone. This suggests that corticosterone levels may be socially mediated in this population, such that group-living individuals can recover from stressors more quickly than solitary individuals. However, additional research is needed to confirm this possibility.

Metrics of sociality were associated with gut microbiome diversity. However, associations with primary group members seemed to play less of a role in gut microbiome diversity than initially predicted. In fact, there was evidence that horizontal transmission of gut microbiota is more likely occurring between individuals outside of primary social groups. It is likely that the high level of social flexibility in this population contributed to the observed patterns. Future research that incorporates data on kinship will be able to better elucidate the role that sociality plays in maintaining gut microbiome diversity within this population.

The findings from this dissertation serve as the first quantified description of the social behavior, glucocorticoid physiology, and gut microbiome diversity of *C. opimus* and add to a growing body of literature on the behavior of Ctenomyid rodents. The cause of the marked variation in sociality observed in this population and the subsequent consequences of this

variation remain to be explored. Ongoing research beyond this dissertation indicate that individuals remain social regardless of population density (i.e., social groups occur even when population density is extremely low) and suggest that individuals may congregate around their preferred food source (saltgrass: *Distichlis* spp.), such that sociality is merely a consequence of individuals congregating around the same area.

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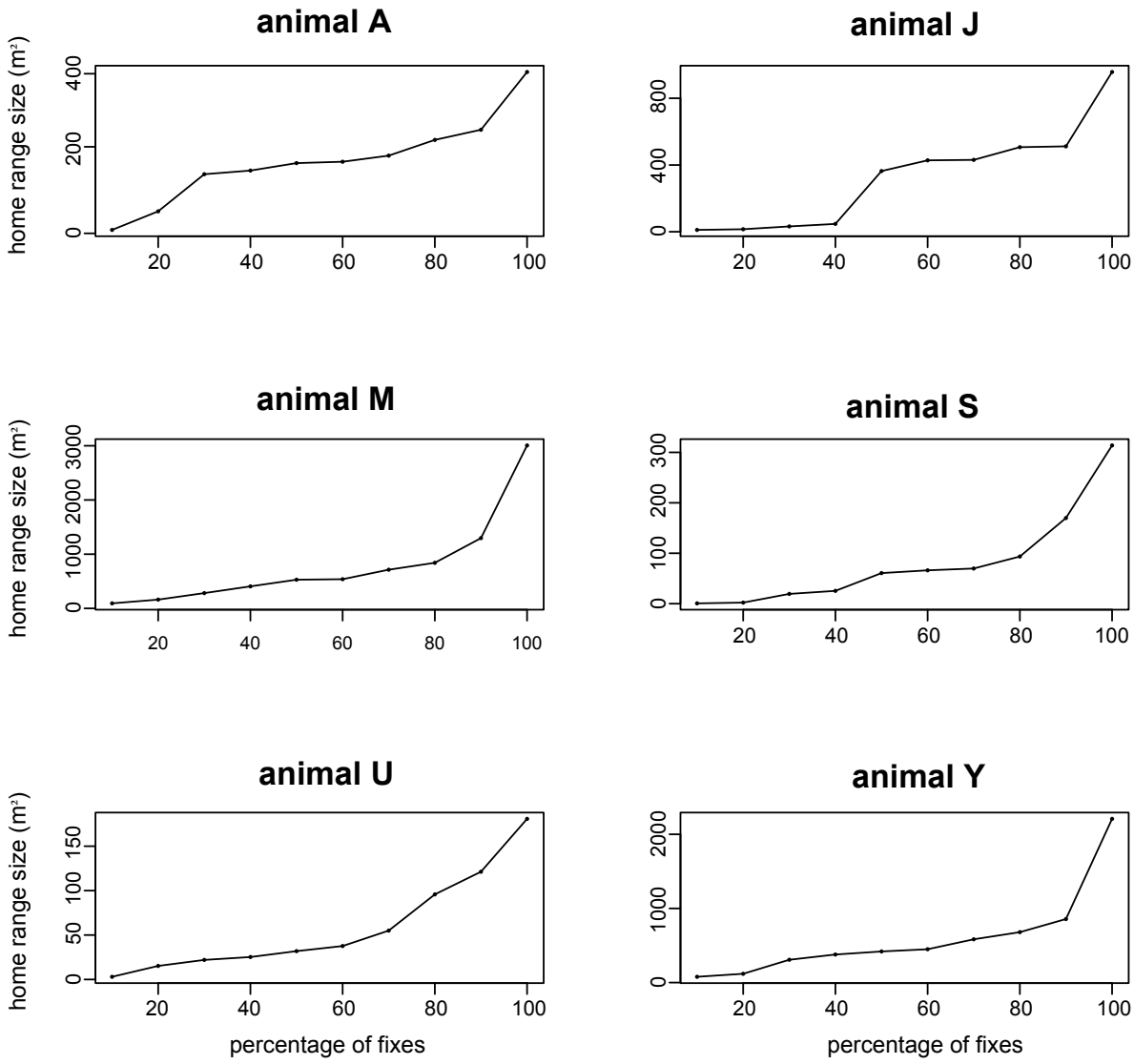
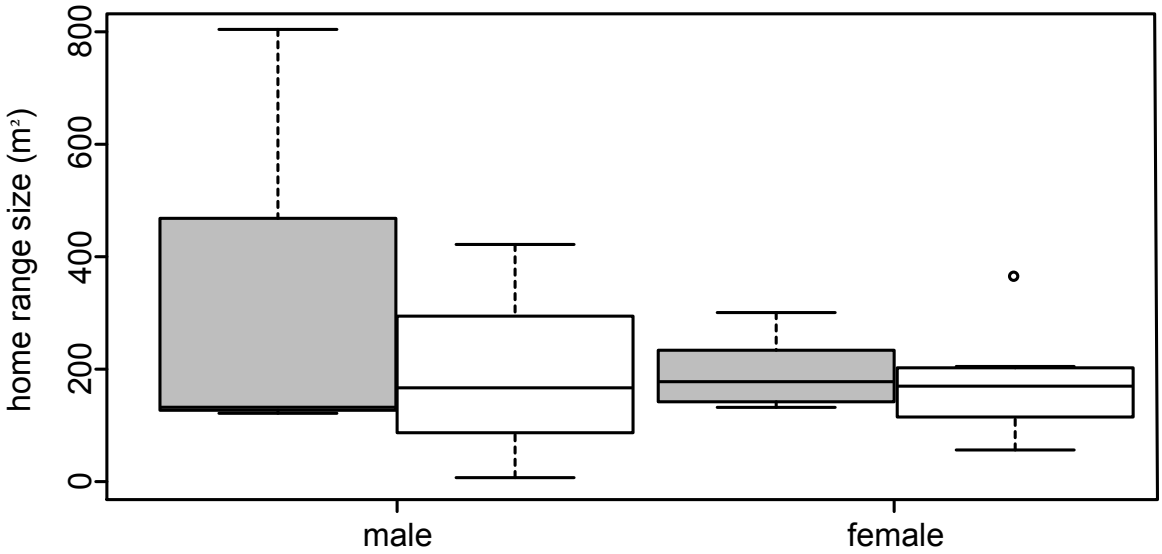
Appendices

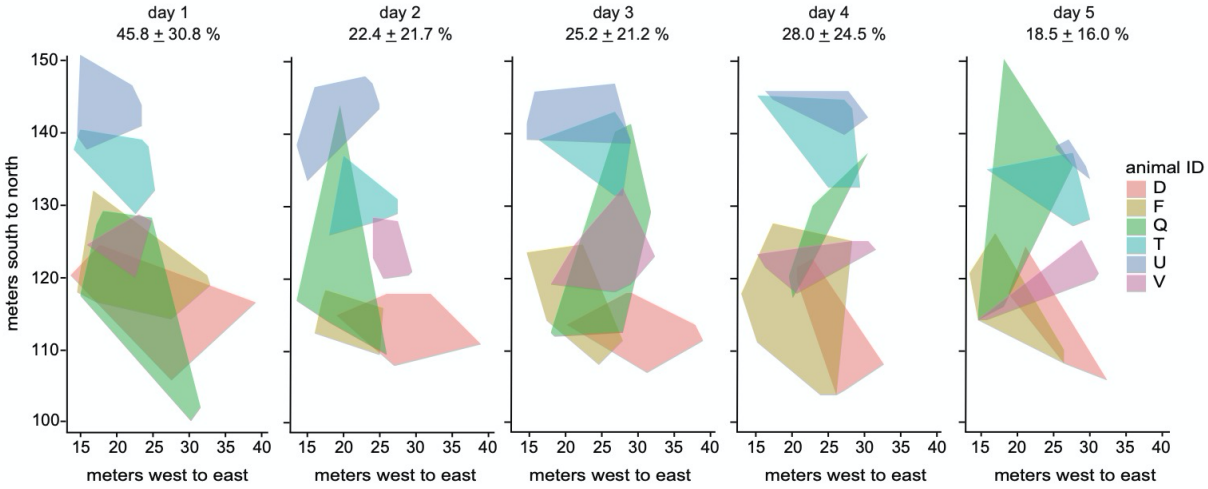
Chapter 1 Appendix



Supplementary Figure 1. Home range size versus number of telemetry fixes analyzed for a randomly selected subset of 6 individuals. Estimates of home range size are based on 95% minimum convex polygons (MCPs). In general, home range size tended to stabilize at ~ 50% of the total number of fixes for an animal, which corresponded to 29.1 ± 17.4 fixes per individual.



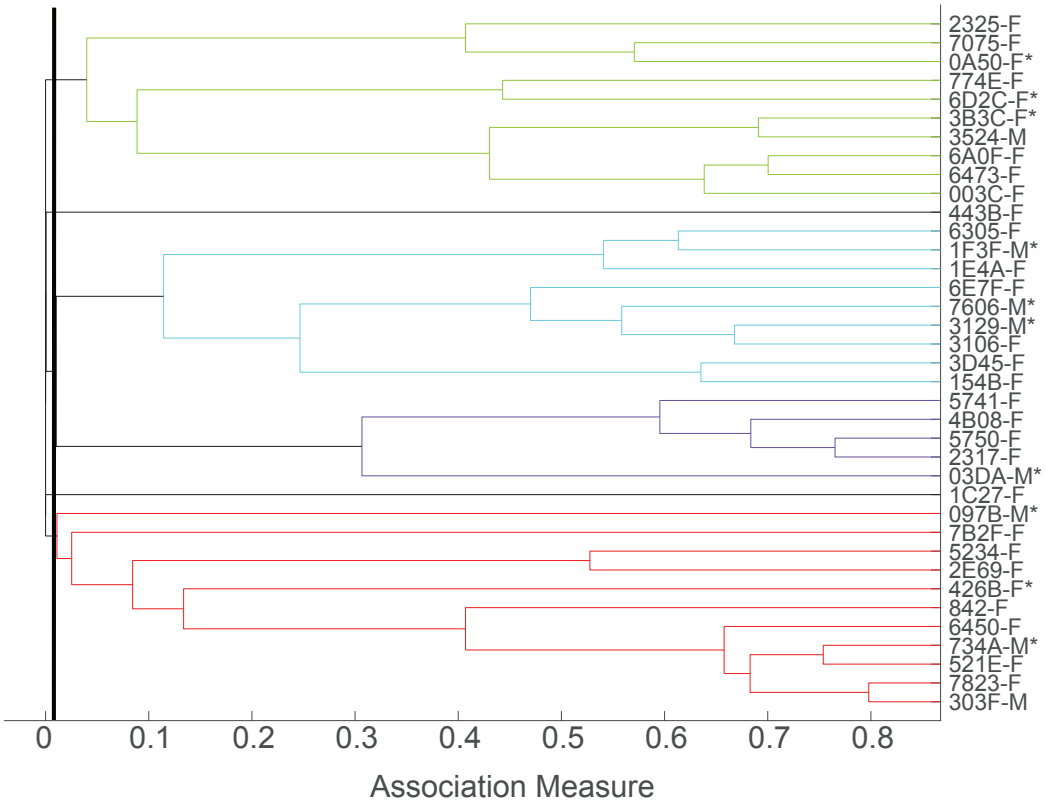
Supplementary Figure 2. Estimates of home range size (m^2) based on 95% minimum convex polygons (MCPs) constructed for a subset of 12 *C. opimus* (4 males, 8 females) for which both telemetry and visual data were available. Paired comparisons revealed no significant tendency for individual home range sizes to differ between estimates based on telemetry data (gray bars) versus visual data (white bars) (Wilcoxon Signed Rank Test, N = 12, V = 60, p = 0.1). Further, there were no significant differences in estimated home range sizes for males versus females for analyses based on either telemetry data (Mann-Whitney U, N = 12, W = 16, p = 0.49) or visual data (Mann-Whitney U, N = 12, W = 15, p = 0.93).



Supplementary Figure 3. Minimum convex polygons (95% MCPs) depicting the daytime home ranges of 6 adult *C. opimus* (1 male, 5 females) monitored via telemetry for 5 consecutive days. The x and y axes denote the location of each MCP on the study site. For each individual, a separate MCP was constructed for each day of data collection. All MCPs for the same day are shown together; colors at right indicate which individual corresponds to a given MCP. Mean daily pairwise percent overlap of MCPs ranged from 18.5% to 45.8% per day; daily means are shown in each panel.

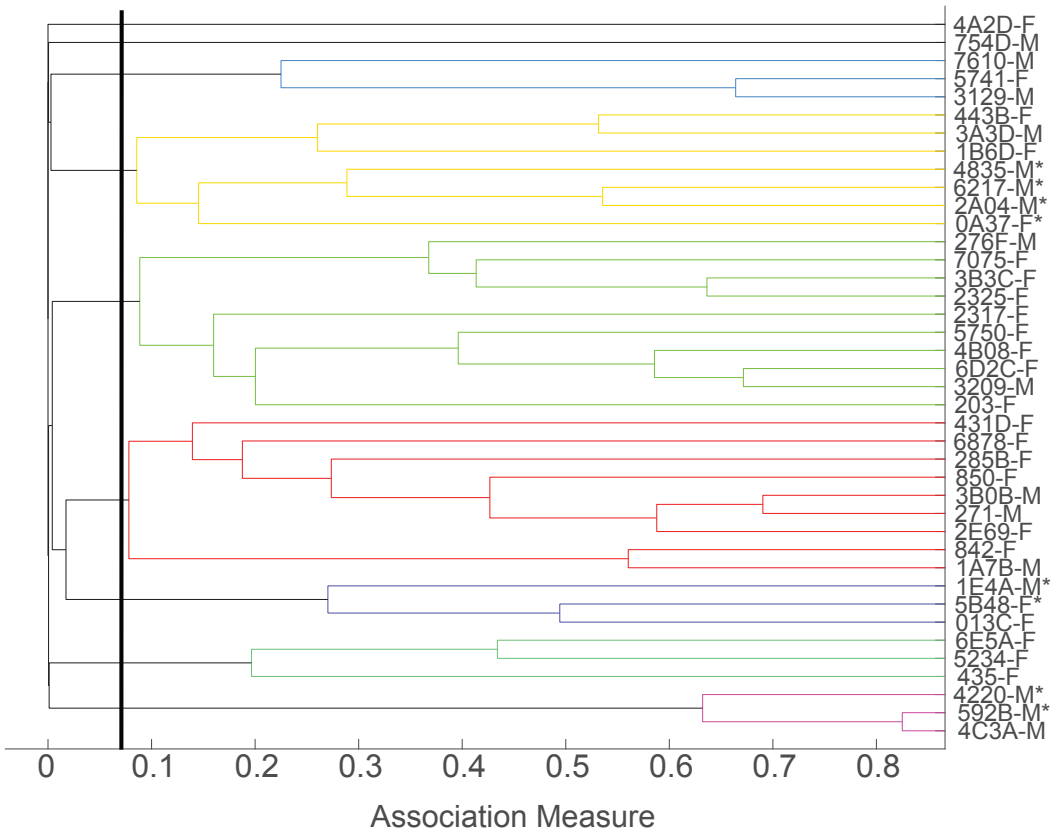
Chapter 2 Appendix

2010 Association Matrix



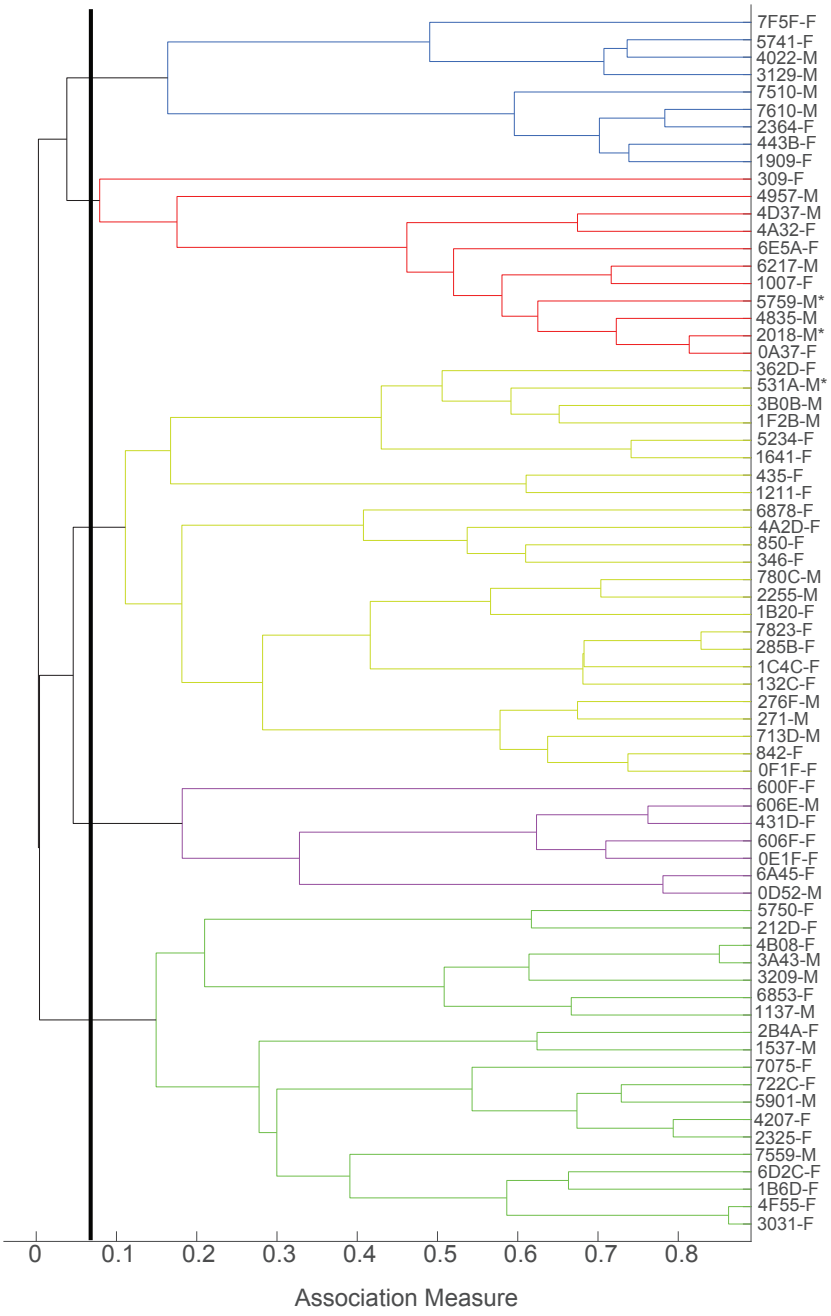
Cophenetic correlation: 0.94
Maximum modularity: 0.76
2 adult M, 25 adult F
6 subadult M, 4 subadult F

2011 Association Matrix



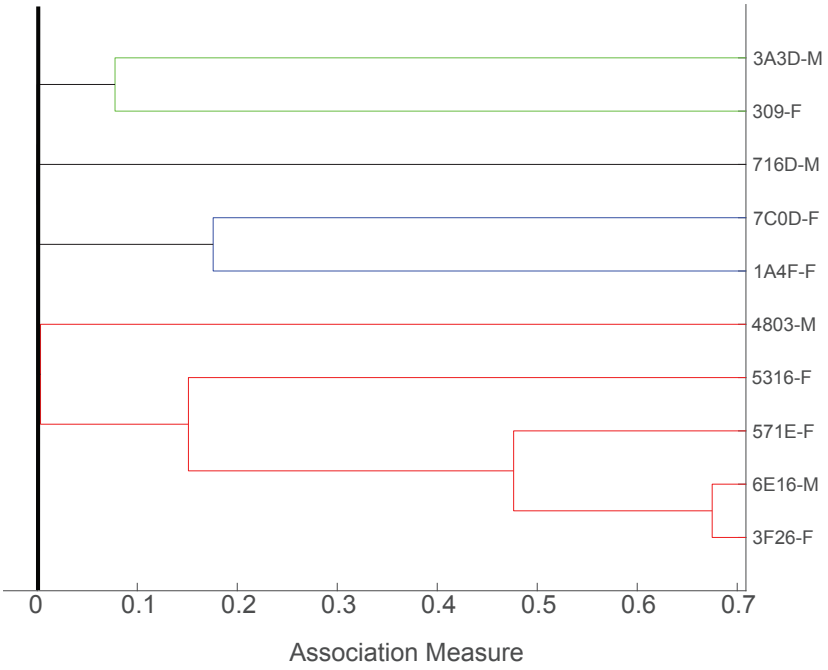
Cophenetic correlation: 0.86
Maximum modularity: 0.74
10 adult M, 22 adult F
6 subadult M, 2 subadult F

2012 Association Matrix

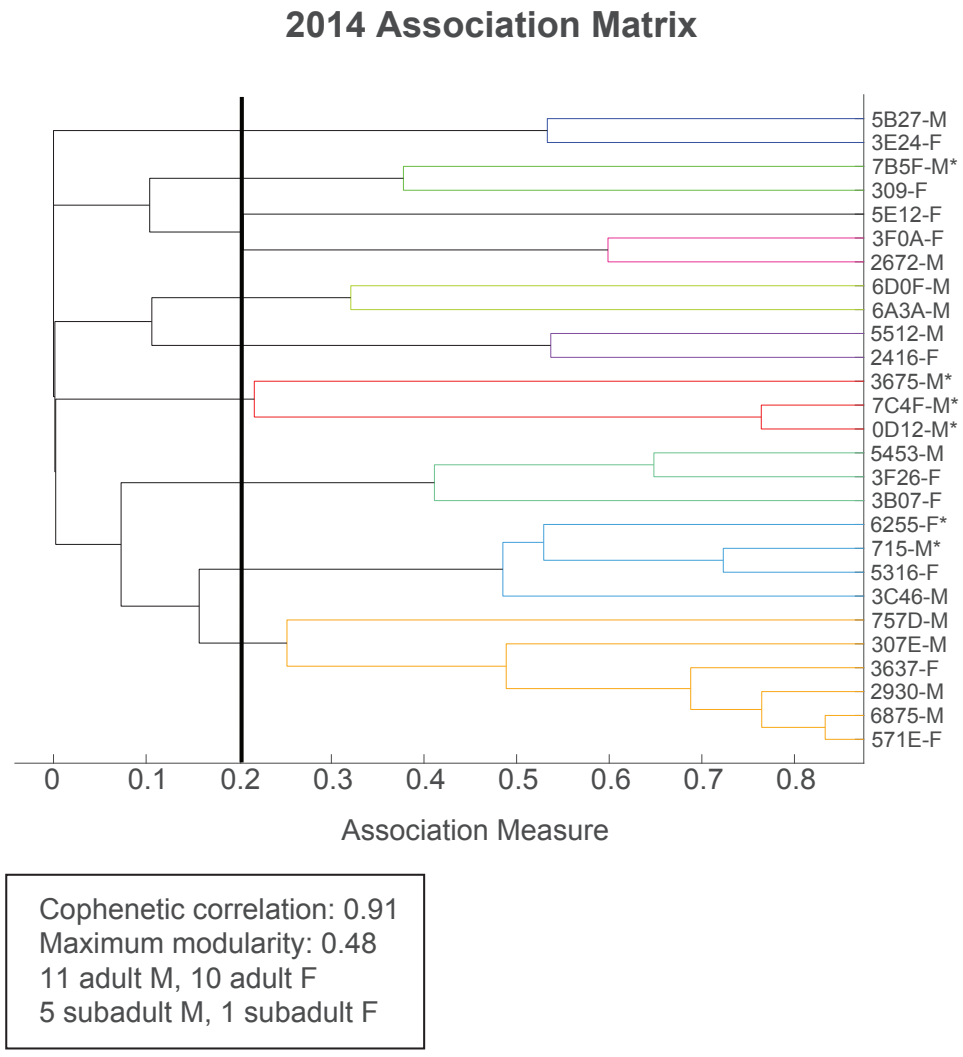


Cophenetic correlation: 0.84
Maximum modularity: 0.64
24 adult M, 43 adult F
3 subadult M, 0 subadult F

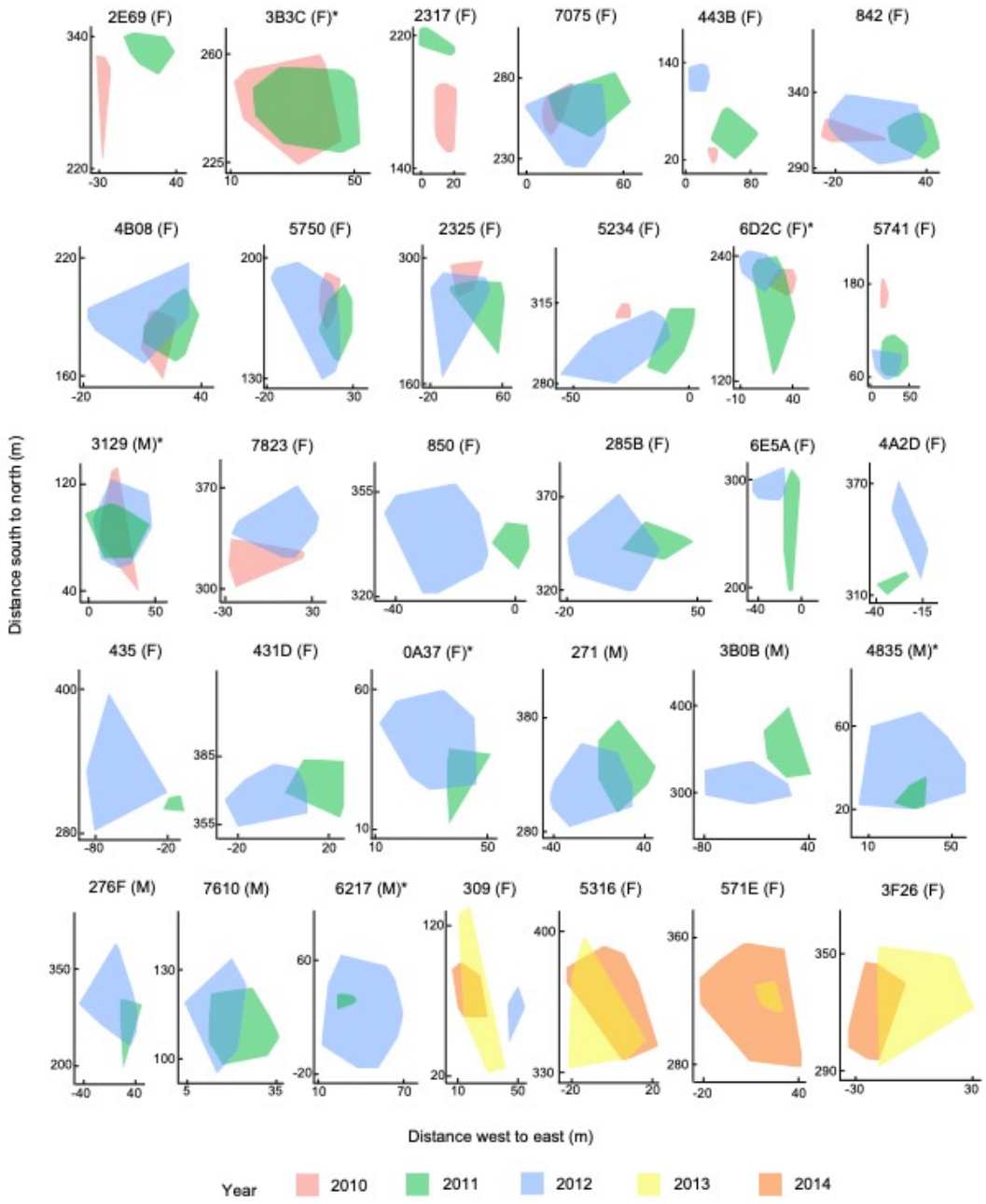
2013 Association Matrix



Cophenetic correlation: 0.97
Maximum modularity: 0.43
4 adult M, 6 adult F
0 subadult M, 0 subadult F



Supplementary Figure 1. Social units of *C. opimus* identified in the study population during each year from 2010 to 2014. Individuals were assigned to social units based on analyses of overlap of 95% MCPs constructed for animals present on the study site during each year. The association index cutoff (bolded black line) for each year was generated by SOCPROG; nodes to the right of this line denote significant spatial associations that were used to assign individuals to social units. Membership in the same social unit is indicated by the color of the lines used to connect individuals. Alphanumeric codes to the right of each dendrogram denote animal ID and sex (M or F). Asterisks denote subadults. In all years, cophenetic correlation coefficients were > 0.8, indicating a strong correspondence between association indices and the spatial clusters identified by SOCPROG (Bridge 1993). All annual values of maximum modularity were > 0.3, indicating significant spatial clustering of members of the study population (Newman 2006).



Supplementary Figure 2. Between-year overlap of home ranges for individuals (N = 31) captured in two or more consecutive field seasons. Annual home ranges are based on 95% minimum convex polygons (MCP); home ranges are color coded by year, as indicated. Axes (meters) vary among individuals. Animal ID and sex (M or F) are indicated above each panel; asterisks denote individuals that were subadults when first captured. Between years, percent overlap ranged from 0% or 73%; 7 animals (1 male, 6 females) displayed no spatial overlap with themselves across years.

Supplementary Table 1. Annual changes in home range size for animals (N = 31) captured in two or more successive field seasons from 2010 to 2014. The ID and sex (M or F) for each animal are shown; asterisks denote individuals that were subadults at first capture. For each animal, the years in which that individual was monitored are indicated, as is home range size during each year. The percent change in home range size (% Δ) between years ranged from -62.72 to 2975.88%. The percent coefficient of variation (CV %) of individual home range sizes across years ranged from 0.72 to 132.52%.

ID (Sex)	Year captured	Home range (m^2)	% Δ	Mean ± SD	CV %
7075 (F)	2010	353		1132.0 ± 727.2	64.2
	2011	1250	254.1		
	2012	1793	43.4		
443B (F)	2010	204		1068.3 ± 1015.0	95.0
	2011	2186	971.6		
	2012	815	-62.7		
7823 (F)	2010	1024.5		1407.8 ± 542.0	38.5
	2012	1791	74.8		
842 (F)	2010	390.5		1103.0 ± 952.6	86.4
	2011	733.5	87.8		
	2012	2185	197.9		
5234 (F)	2010	39		399.8 ± 389.6	97.5
	2011	347.5	791.0		
	2012	813	134.0		
2317 (F)	2010	511.5		374.3 ± 194.1	51.9
	2011	237	-53.7		
5750 (F)	2010	291		820.0 ± 685.6	83.6
	2011	574.5	97.4		
	2012	1594.5	177.6		
2325 (F)	2010	959.5		2698.3 ± 1696.0	62.9
	2011	2787.5	190.5		
	2012	4348	56.0		
4B08 (F)	2010	393.75		863.1 ± 590.1	68.4
	2011	670	70.2		
	2012	1525.5	127.7		
5741 (F)	2010	334.5		1033.0 ± 656.8	63.6
	2011	1638	389.7		
	2012	1126.5	-31.2		
2E69 (F)	2010	706.5		916.0 ± 296.3	32.3
	2011	1125.5	59.3		

4A2D (F)	2011	97		259.0 ± 229.1	88.5
	2012	421	334.0		
850 (F)	2011	126		503.5 ± 533.9	106.0
	2012	881	599.2		
431D (F)	2011	475.5		591.8 ± 164.4	27.8
	2012	708	48.9		
285B (F)	2011	443		1037.3 ± 840.4	81.0
	2012	1631.5	268.3		
435 (F)	2011	168		2093.0 ± 2722.4	130.1
	2012	4018	2291.7		
6E5A (F)	2011	1251.5		987.3 ± 373.7	37.9
	2012	723	-42.2		
309 (F)	2012	234		826.5 ± 724.3	87.6
	2013	1634	598.3		
	2014	611.5	-62.6		
5316 (F)	2013	1461.5		1469.0 ± 10.6	0.7
	2014	1476.5	1.0		
571E (F)	2013	235.5		1904.3 ± 2360.0	123.9
	2014	3573	1417.2		
3F26 (F)	2013	2028.5		1494.5 ± 755.2	50.5
	2014	960.5	-52.7		
6D2C (F)	2010*	491		1423.8 ± 1176.3	82.6
	2011	2745.25	459.1		
	2012	1035	-62.3		
3B3C (F)	2010*	834		808.8 ± 35.7	4.4
	2011	783.5	-6.1		
0A37 (F)	2011*	219.5		563.3 ± 486.1	86.3
	2012	907	313.2		
271 (M)	2011	2525		3146.0 ± 878.2	27.9
	2012	3767	49.2		
3B0B (M)	2011	2638		3120.8 ± 682.7	21.9
	2012	3603.5	36.6		
276F (M)	2011	1927		4830.8 ± 4106.5	85.0
	2012	7734.5	301.4		
7610 (M)	2011	467.5		463.5 ± 5.7	1.2
	2012	459.5	-1.7		
3129 (M)	2010*	1278.5		1575.8 ± 468.6	29.7
	2011	1333	4.3		
	2012	2116	58.7		
4835 (M)	2011*	146		987.8 ± 1190.4	120.5

	2012	1829.5	1153.1		
6217 (M)	2011*	113		1794.4 ± 2377.8	132.5
	2012	3475.75	2975.9		

Supplementary Table 2. Summary of linear models, including the degrees of freedom (DF) and Akaike Information Criterion (AIC) values for each model. Best fit models based on AIC values are in bold. For models with AIC differences $\leq$ 2, interaction terms were checked for significance to determine the best fitting model; in all cases the interaction terms were non-significant ($p > 0.05$), so the simplest model was chosen.

Response variable	Model	Distribution	Equation	DF	AIC
Home range	Linear mixed	Gaussian	**Home range ~ Sex * Age * Years Onsite + (1|Year) + (1|ID)**	**9**	**1159.69**
			Home range ~ Sex * Age + Years Onsite + (1|Year) + (1|ID)	8	1172.27
			Home range ~ Sex + Age + Years Onsite + (1|Year) + (1|ID)	7	1188.03
			Home range ~ Sex + Age * Years Onsite + (1|Year) + (1|ID)	7	1188.03
Social unit size	Generalized linear mixed	Poisson	**Social unit ~ Sex + Age + Years Onsite + (1|Year) + (1|ID)**	**6**	**427.871**
			Social unit ~ Sex + Age * Years Onsite + (1|Year) + (1|ID)	6	427.871
			Social unit ~ Sex * Age + Years Onsite + (1|Year) + (1|ID)	7	429.356
			Social unit ~ Sex * Age * Years Onsite + (1|Year) + (1|ID)	8	430.757
Overlap with self	Linear	Gaussian	**Overlap w/ self ~ Sex + Age**	**4**	**284.149**
			Overlap w/ self ~ Sex * Age	5	286.147

Supplementary Table 3. Summary of social unit composition and annual changes in social unit size for animals (N = 31) captured in two or more successive field seasons from 2010 to 2014. The ID and sex (M or F) for each animal are shown; asterisks denote individuals that were subadults at first capture. For each animal, the years in which that individual was monitored are indicated, as is social unit size and composition during each year. The percent change in social unit size (% Δ) ranged from -90 to 1050%.

ID (Sex)	Year Captured	Count conspecifics in social unit	% Δ	Adult male	Adult female	Subadult male	Subadult female
7075 (F)	2010	9		1	5	0	3
	2011	9	0	2	7	0	0
	2012	18	100	6	12	0	0
443B (F)	2010	0		0	0	0	0
	2011	6	-	1	1	3	1
	2012	8	33.3	4	4	0	0
7823 (F)	2010	10		1	6	2	1
	2012	23	130	7	15	1	0
842 (F)	2010	10		1	6	2	1
	2011	8	-20	3	5	0	0
	2012	23	187.5	7	15	1	0
5234 (F)	2010	10		1	6	2	1
	2011	3	-70	0	3	0	0
	2012	23	666.7	7	15	1	0
2317 (F)	2010	4		0	3	1	0
	2011	9	125	2	7	0	0
5750 (F)	2010	4		0	3	1	0
	2011	9	125	2	7	0	0
	2012	18	100	6	12	0	0
2325 (F)	2010	9		1	5	0	3
	2011	9	0	2	7	0	0
	2012	18	100	6	12	0	0
4B08 (F)	2010	4		0	3	1	0
	2011	9	125	2	7	0	0
	2012	18	100	6	12	0	0
5741 (F)	2010	4		0	3	1	0
	2011	2	-50	2	0	0	0
	2012	8	300	4	4	0	0
2E69 (F)	2010	10		1	6	2	1
	2011	8	-20	3	5	0	0

4A2D (F)	2011	0		0	0	0	0
	2012	23	-	7	15	1	0
850 (F)	2011	8		3	5	0	0
	2012	23	187.5	7	15	1	0
431D (F)	2011	8		3	5	0	0
	2012	6	-25	2	4	0	0
285B (F)	2011	8		3	5	0	0
	2012	23	187.5	7	15	1	0
435 (F)	2011	2		0	2	0	0
	2012	23	1050	7	15	1	0
6E5A (F)	2011	2		0	2	0	0
	2012	10	400	4	4	2	0
309 (F)	2012	10		4	4	2	0
	2013	1	-90	1	0	0	0
	2014	1	0	0	0	1	0
5316 (F)	2013	4		2	2	0	0
	2014	3	-25	1	0	1	1
571E (F)	2013	4		2	2	0	0
	2014	5	25	4	1	0	0
3F26 (F)	2013	4		2	2	0	0
	2014	2	-50	1	1	0	0
6D2C (F)	2010*	9		1	6	0	2
	2011	9	0	2	7	0	0
	2012	18	100	6	12	0	0
3B3C (F)	2010*	9		1	6	0	2
	2011	9	0	2	7	0	0
0A37 (F)	2011*	6		1	2	3	0
	2012	10	66.7	4	4	2	0
271 (M)	2011	8		2	6	0	0
	2012	23	187.5	6	16	1	0
3B0B (M)	2011	8		2	6	0	0
	2012	23	187.5	6	16	1	0
276F (M)	2011	9		1	8	0	0
	2012	23	155.6	6	16	1	0
7610 (M)	2011	2		1	1	0	0
	2012	8	300	3	5	0	0
3129 (M)	2010*	8		0	6	2	0

	2011	2	-75	1	1	0	0
	2012	8	300	3	5	0	0
4835 (M)	2011*	6		1	2	2	1
	2012	10	66.7	3	5	2	0
6217 (M)	2011*	6		1	2	2	1
	2012	10	66.7	3	5	2	0

Supplementary Table 4. Annual social network statistics for animals (N = 31) captured during two or more successive field seasons from 2010 to 2014. The ID and sex (M or F) for each animal are shown; asterisks denote individuals that were subadults at first capture. For each animal, the years in which that individual was monitored are indicated, as are network strength, Eigenvector centrality, reach, clustering coefficient, and affinity. Social network statistics were generated in SOCPROG; detailed definitions for the social network statistics used are given in Whitehead (2009).

ID (Sex)	Year Captured	Strength	Eigenvector centrality	Reach	Clustering coefficient	Affinity
7075 (F)	2010	1.46	0	2.5	0.3	1.7
	2011	1.47	0.22	3.63	0.52	2.46
	2012	4.14	0.06	29.51	0.48	7.13
443B (F)	2010	0.01	0	0.01	0	1.82
	2011	1.73	0	1.89	0.14	1.09
	2012	3.32	0	12.66	0.58	3.81
7823 (F)	2010	4.24	0.46	11.97	0.49	2.83
	2012	6.86	0.23	47.99	0.43	7
842 (F)	2010	2.57	0.29	8.01	0.56	3.12
	2011	1.35	0.08	3.09	0.33	2.29
	2012	5.55	0.19	40.77	0.46	7.35
5234 (F)	2010	1.06	0.08	2.28	0.31	2.15
	2011	0.67	0	0.36	0.19	0.55
	2012	3.79	0.1	26.13	0.51	6.89
2317 (F)	2010	2.5	0	5.71	0.59	2.29
	2011	0.82	0.16	2.66	0.73	3.23
5750 (F)	2010	2.48	0	5.51	0.61	2.22
	2011	1.19	0.21	3.46	0.76	2.91
	2012	2.76	0.02	15.23	0.42	5.52
2325 (F)	2010	0.92	0	1.5	0.58	1.62
	2011	2.45	0.34	5.21	0.33	2.13
	2012	7.88	0.08	45.26	0.35	5.74
4B08 (F)	2010	2.41	0	5.42	0.6	2.25
	2011	1.46	0.26	4.27	0.67	2.92
	2012	4.02	0.03	23.67	0.5	5.89
5741 (F)	2010	2.14	0	5.39	0.64	2.52
	2011	1.07	0	0.77	0.1	0.72
	2012	2.64	0	12.17	0.53	4.62
2E69 (F)	2010	1.24	0.09	2.71	0.31	2.19
	2011	2.24	0.13	5.6	0.39	2.5

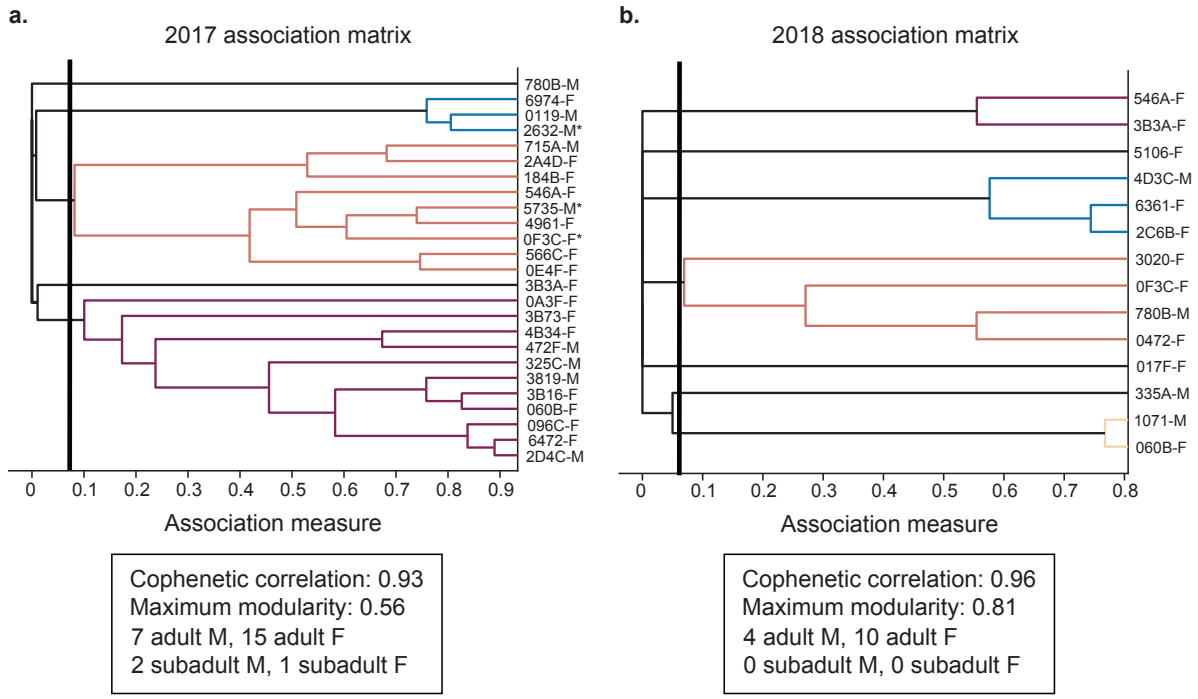
4A2D (F)	2011	0	0	0	0	0
	2012	4.16	0.15	31.52	0.5	7.57
850 (F)	2011	1.3	0.09	4.02	0.7	3.08
	2012	4.46	0.16	32.88	0.48	7.38
431D (F)	2011	0.86	0.06	3.13	0.76	3.62
	2012	4.47	0.1	24.99	0.37	5.59
285B (F)	2011	1.09	0.08	3.82	0.81	3.5
	2012	7.42	0.25	52.04	0.41	7.02
435 (F)	2011	0.39	0	0.25	0.53	0.64
	2012	2.01	0.05	12.14	0.47	6.05
6E5A (F)	2011	0.6	0	0.36	0.27	0.59
	2012	6.09	0.04	30.59	0.34	5.02
309 (F)	2012	0.79	0	4.13	0.64	5.22
	2013	0.08	0	0.01	0	0.08
	2014	0.97	0	0.87	0.21	0.89
5316 (F)	2013	0.45	0.23	0.64	1	1.41
	2014	2.89	0.28	10	0.52	3.46
571E (F)	2013	0.95	0.49	1.32	1	1.38
	2014	4.1	0.38	13.3	0.41	3.24
3F26 (F)	2013	1.45	0.6	1.47	0.41	1.02
	2014	2.19	0.12	5.24	0.26	2.39
6D2C (F)	2010*	1.19	0	2.56	0.28	2.14
	2011	2.7	0.36	5.31	0.29	1.97
	2012	5.71	0.05	34.31	0.48	6.01
3B3C (F)	2010*	1.71	0	4.74	0.61	2.77
	2011	1.8	0.3	5.03	0.49	2.79
0A37 (F)	2011*	0.56	0	0.93	0.39	1.64
	2012	5.64	0.01	28.83	0.55	5.11
271 (M)	2011	3.37	0.17	6.45	0.26	1.91
	2012	10.54	0.31	65.37	0.3	6.2
3B0B (M)	2011	3.87	0.16	6.29	0.18	1.62
	2012	6.18	0.17	36.93	0.33	5.98
276F (M)	2011	2.03	0.26	4.37	0.28	2.15
	2012	13.41	0.33	76.63	0.19	5.71
7610 (M)	2011	0.45	0	0.45	0.8	1
	2012	3.51	0	13.03	0.56	3.71
3129 (M)	2010*	3.03	0	6.87	0.34	2.27
	2011	0.77	0	0.76	0.42	0.99
	2012	4.39	0	16.32	0.33	3.72
4835 (M)	2011*	0.69	0	0.85	0.43	1.22

	2012	5.79	0.01	28.2	0.5	4.87
6217 (M)	2011*	0.88	0	1.31	0.3	1.48
	2012	5.11	0.01	24.87	0.52	4.87

Supplementary Table 5. Co-occurrence of animals recaptured during two or more successive field seasons from 2010 to 2014. Each pair of animals assigned to the same social unit during two successive years is listed by ID and sex (M or F). X's are used to denote the years in which each pair occurred in the same social unit.

		Years in same social group				
ID_1 (Sex)	**ID_2 (Sex)**	**2010**	**2011**	**2012**	**2013**	**2014**
7075 (F)	2325 (F)	X	X	X		
7075 (F)	6D2C (F)	X	X	X		
7075 (F)	3B3C (F)	X	X			
7075 (F)	5750 (F)		X	X		
7075 (F)	4B08 (F)		X	X		
7823 (F)	5234 (F)	X		X		
7823 (F)	842 (F)	X		X		
842 (F)	2E69 (F)	X	X			
2317 (F)	5750 (F)	X	X			
2317 (F)	4B08 (F)	X	X			
5750 (F)	2325 (F)		X	X		
5750 (F)	4B08 (F)		X	X		
5750 (F)	6D2C (F)		X	X		
2325 (F)	6D2C (F)	X	X	X		
2325 (F)	3B3C (F)	X	X			
2325 (F)	4B08 (F)		X	X		
4B08 (F)	6D2C (F)		X	X		
5741 (F)	7610 (M)		X	X		
5741 (F)	3129 (M)		X	X		
850 (F)	285B (F)		X	X		
850 (F)	3B0B (M)		X	X		
850 (F)	271 (M)		X	X		
850 (F)	842 (F)		X	X		
285B (F)	3B0B (M)		X	X		
285B (F)	271 (M)		X	X		
285B (F)	842 (F)		X	X		
435 (F)	5234 (F)		X	X		
6D2C (F)	3B3C (F)	X	X			
0A37 (F)	4835 (M)		X	X		
0A37 (F)	6217 (M)		X	X		
271 (M)	3B0B (M)		X	X		
271 (M)	842 (F)		X	X		
3B0B (M)	842 (F)		X	X		
7610 (M)	3129 (M)		X	X		

4835 (M)	6217 (M)	X	X



Supplementary Figure 1. Association matrices for free-living animals monitored via radiotelemetry during the (a) 2017 and (b) 2018 field seasons. Analyses are based on overlap of 95% MCPs constructed for these individuals. The association index cutoff (vertical black line) for each field season was generated by SOCPROG (Whitehead 2009); nodes to the right of this line denote significant spatial associations that were used to assign individuals to social units. Membership in the same social unit is indicated by the color of the lines used to connect individuals; alphanumeric codes to the right of each dendrogram denote animal ID and sex (M or F). Asterisks (*) denote subadult individuals. In both field seasons, cophenetic correlation coefficients were > 0.8, indicating a strong correspondence between association indices and the spatial clusters of animals identified by SOCPROG (Bridge 1993). Maximum modularity for both field seasons were > 0.3, indicating significant spatial clustering of members of the study population (Newman 2006). No fGCm data were available for two individuals (male 3819 and female 3B73) monitored during the 2017 field season; these animals are included here to provide a complete characterization of the social units in which they occurred.

Supplementary Table 1. Summary of the highland tuco-tucos included in this study. **All captures** represent the total number of individuals captured, including captures on the main study site as well as in surrounding areas. **Captures onsite** represents the subset of tuco-tucos captured on the primary study site. Of these, the number of individuals with sufficient spatial data for social network analyses (w/ spatial data) is indicated; the subset of these individuals for which fecal samples were collected (w/ fecal samples) is also given. For all columns, numbers shown are for 2017 and 2018 respectively, with data for each year separated by a comma.

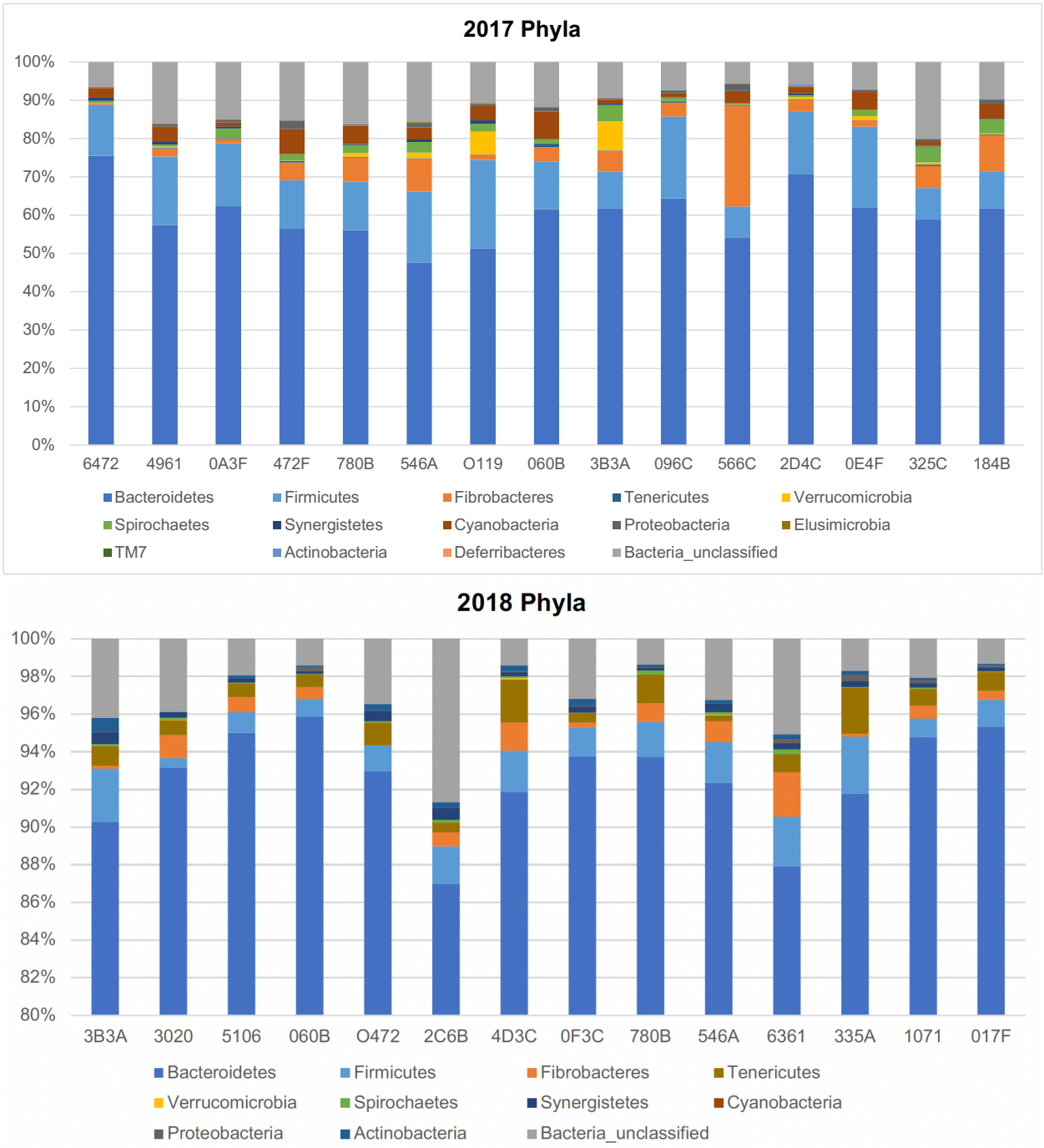
	Adult M	Adult F	Subadult M	Subadult F	Total
All captures	13, 4	24, 15	2, 7	1, 0	40, 26
Captures onsite	10, 4	20, 10	2, 3	1, 0	33, 17
w/ spatial data	7, 4	15, 10	2, 0	1, 0	25, 14
w/ fecal samples	6, 4	14, 10	2, 0	1, 0	23, 14

Supplementary Table 2. Comparisons of social network metrics calculated for members of the study population of highland tuco-tucos. For each network metric examined, mean (+ 1 SD) values are shown as a function of sex and year of data collection. Sample sizes for each subset of individuals are indicated.

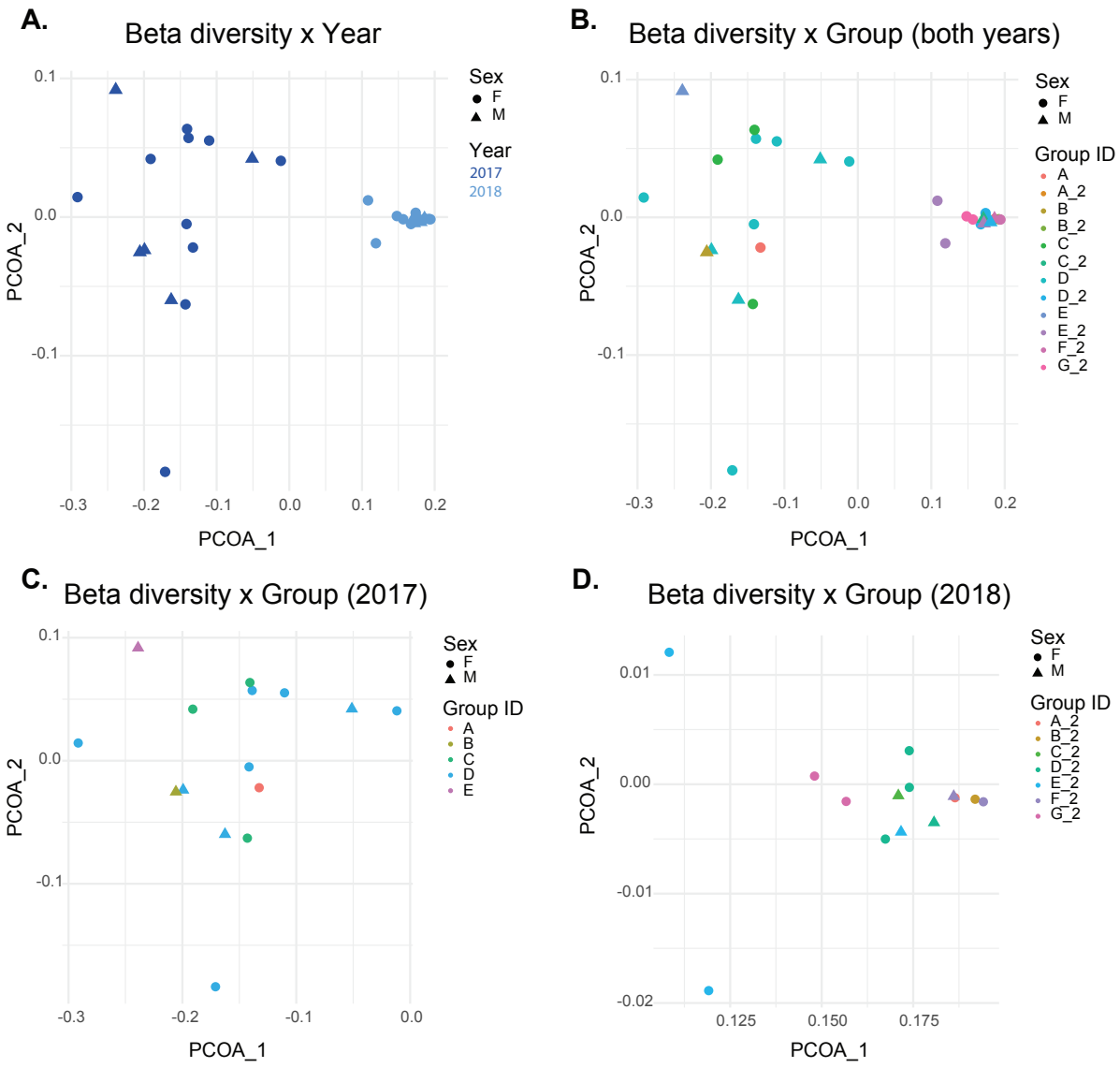
Year	Sex	Strength	Eigenvector centrality	Reach	Clustering coefficient	Affinity
2017	M and F (N = 23)	2.53 ± 1.40	0.11 ± 0.15	8.75 ± 6.43	0.54 ± 0.23	3.21 ± 1.42
	M (N = 8)	2.25 ± 1.16	0.10 ± 0.14	7.64 ± 6.05	0.52 ± 0.27	2.74 ± 1.69
	F (N = 15)	2.68 ± 1.53	0.12 ± 0.17	9.35 ± 6.75	0.55 ± 0. 21	3.46 ± 1.25
2018	M and F (N = 14)	0.66 ± 0.48	0.12 ± 0.25	0.65 ± 0.58	0.26 ± 0.41	0.84 ± 0.47
	M (N = 4)	0.84 ± 0.53	0.14 ± 0.27	0.73 ± 0.60	0.51 ± 0.55	0.86 ± 0.35
	F (N = 10)	0.63 ± 0.47	0.13 ± 0.25	0.66 ± 0.60	0.17 ± 0.32	0.78 ± 0.53

Supplementary Table 3. Summary of Mann-Whitney U tests comparing social network metrics for highland tuco-tucos between years and between the sexes. Significant P-values are indicated in bold. Effect sizes are listed as Hedges' g.

Mann-Whitney U tests	**Strength**	**Eigenvector centrality**	**Reach**	**Clustering coefficient**	**Affinity**
2017 v 2018 (sexes pooled)	W = 289, **P < .001,** g = 1.62	W = 181.5, P = 0.46, g = 0.06	W = 305.5, **P < .001,** g = 1.56	W = 227, **P = 0.03,** g = 0.93	W = 309, **P < .001,** g = 2.04
M v F (years pooled)	W = 143.5, P = 0.84, g = 0.04	W = 155.5, P = 0.84, g = 0.04	W = 153, P =0.93, g = 0.08	W = 111.5, P = 0.21, g = 0.37	W = 162.5, P = 0.69, g = 0.18
M v F (2017)	W = 52.5, P = 0.65, g = 0.30	W = 53.5, P = 0.67, g = 0.13	W = 53, P = 0.68, g = 0.26	W = 63, P = 0.87, g = 0.13	W = 44.5, P = 0.33, g = 0.51
M v F (2018)	W = 26, P = 0.44, g = 0.52	W = 20, P = 1, g = 0.06	W = 22, P = 0.83, g = 0.17	W = 31.5, P = 0.08, g = 0.90	W = 22, P = 0.83, g = 0.05



Supplementary Figure 1. Gut microbiota composition for individual adult highland tuco-tucos (C. opimus) included in this study. Data from 2017 and 2018 are shown separately as relative abundances of different bacterial phyla. The gut microbiota of members of the study population was dominated by the phyla *Bacterioidetes* and *Firmicutes*, although the relative prevalence of these taxa differed between field seasons. Note that scales for the y-axis differ between years due to the lower overall abundances of bacterial taxa in 2018.



Supplementary Figure 2. Results of principle coordinates analyses (PCoA) of Bray-Curtis dissimilarities (beta diversity) using phylum-level operational taxonomic units (OTUs). Samples clustered by year (a), but not by sex (b) or group ID (c, d). In all panels, triangles denote males while circles denote females. Statistical results for each analysis are given in the associated panel in the figure.

Supplementary Table 1. Summary of the social network statistics used in this study. Data are listed as mean ± SD with range provided in parentheses. In the table headings, Eigen refers to Eigenvector centrality and Clust refers to clustering coefficient. Detailed results of all metrics in the table are provided in O'Brien et al. 2022.

Year	Sex	Group size	Strength	Eigen	Reach	Clust	Affinity
2017	M	7.40 ± 4.98 (1 – 11)	2.20 ± 1.46 (0 – 3.82)	0.16 ± 0.15 (0 – 0.34)	9.55 ± 6.84 (0.72 – 20.77)	0.54 ± 0.23 (0 – 0.77)	3.72 ± 1.28 (1.98 – 6.08)
	F	9.40 ± 3.10 (1 – 11)	2.67 ± 1.63 (0.12 – 5.49)	0.12 ± 0.17 (0 – 0.40)	8.99 ± 7.36 (0 – 17.44)	0.52 ± 0.30 (0 – 0.71)	3.07 ± 2.12 (0 – 4.64)
2018	M	2.50 ± 1.29 (1 – 4)	0.84 ± 0.53 (0.10 – 1.30)	0.13 ± 0.27 (0 – 0.54)	0.72 ± 0.60 (0.08 – 1.54)	0.51 ± 0.55 (0 – 1.00)	0.85 ± 0.35 (0.49 – 1.33)
	F	2.60 ± 1.17 (1 – 4)	0.58 ± 0.47 (0 – 1.41)	0.11 ± 0.25 (0 – 0.62)	0.62 ± 0.60 (0 – 1.68)	0.15 ± 0.32 (0 – 0.87)	0.82 ± 0.53 (0 – 1.31)

Supplementary Table 2. Results of preliminary Pearson's correlations between alpha diversity and each of our predictor variables (group size, the number of conspecifics with which an individual overlapped spatially, strength, Eigenvector centrality, reach, clustering coefficient, and affinity. In the table headings, Eigen refers to Eigenvector centrality and Clust refers to clustering coefficient. Bolded P-values indicate significance.

	R	t	df	P
Group size	0.63	4.24	27	0.000231
Overlap count	0.68	4.83	27	< 0.0001
Strength	0.55	3.45	27	0.00185
Eigen	0.16	0.82	27	0.4194
Reach	0.56	3.52	27	0.001547
Clust	0.46	2.7	27	0.01184
Affinity	0.69	4.94	27	< 0.0001