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Quantifying microbe transmission networks for wild and domestic ungulates in Kenya

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ABSTRACT

Multi-host wildlife pathogens are an increasing concern for both wildlife conservation and livestock husbandry. Here, we combined social network theory with microbial genetics to assess patterns of interspecific pathogen transmission among ten species of wild and domestic ungulates in Kenya. If two individuals shared the same genetic subtype of a genetically diverse microbe, *Escherichia coli,* then we inferred that these individuals were part of the same transmission chain. Individuals in the same transmission chain were interlinked to create a transmission network. Given interspecific variation in physiology and behavior, some species may function as "super-spreaders" if individuals of that species are consistently central in the transmission network. Pathogen management strategies targeted at key super-spreader species are theoretically more effective at limiting pathogen spread than conventional strategies, and our approach provides a means to identify candidate super-spreaders in wild populations. We found that Grant's gazelle (*Gazella granti*) typically occupied central network positions and were connected to a large number of other individuals in the network. Zebra (*Equus burchelli*), in contrast, seemed to function as bridges between regions of the network that would otherwise be poorly connected, and interventions targeted at zebra significantly increased the level of fragmentation in the network. Although not usually pathogenic, *E. coli* transmission pathways provide insight into transmission dynamics by demonstrating where contact between species is sufficient for transmission to occur and identifying species that are potential super-spreaders.

1. Introduction

Understanding the dynamics of pathogen transmission is important for predicting the potential impact of wildlife diseases and developing disease control strategies. Approximately 77% of livestock pathogens are multi-host (Cleaveland et al., 2001), and pathogens shared among livestock and wild ungulates may have adverse effects on both populations. Spillover of pathogens from domestic animals to wildlife can cause population declines and even local extirpation (Delahay et al., 2009; Jessup et al., 1991; Leendertz et al., 2006; Pederson et al., 2007; RoelkeParker et al., 1996; Thorne and Williams, 1988). Indeed, most endangered species at risk from disease acquire their pathogens from domestic populations (Altizer et al., 2003). Pathogen transmission is of particular concern in sub-Saharan Africa because of the close proximity of wildlife to livestock and the high prevalence and diversity of pathogens (Cleaveland et al., 2005; Wambwa, 2005). Better data on the dynamics of interspecific transmission and the risks associated with wildlife-livestock pathogen transmission are urgently needed (Osofsky, 2005).

Critical questions concerning pathogen transmission have remained relatively unexplored because the field is limited by available methodology. It is difficult to study transmission pathways in wild populations using current methods, such as commonly-used serological techniques, because data on who transmitted an infection to whom is almost impossible to obtain (Caley et al., 2009). However, such data can be obtained by assessing the genetics of the microbe itself: if two individuals share similar genetic subtypes of a microbe, then transmission can be inferred (Archie et al., 2008; Criscione et al., 2005; Goldberg et al., 2007; Jay et al., 2007; Metzker et al., 2002). Here, we use genetic data to infer transmission pat-

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terns in a community of African herbivores, and use these data to construct interspecific transmission networks to further our understanding of how microbes spread through multi-species communities.

Theoretical epidemiological models often assume that the probability of contact is equal for every pair of individuals in the population, even though spatial and social structure create heterogeneity in transmission patterns (Bansal et al., 2007; Craft and Caillaud, 2011; Keeling and Eames, 2005; Otterstatter and Thomson, 2007; Perkins et al., 2008). One mechanism to account for heterogeneity in contact patterns is to incorporate contact networks into epidemiological models (Bansal et al., 2007; Craft and Caillaud, 2011; Keeling, 2005; May, 2006). As compared to traditional mass-action models, these models tend to result in reductions in the early growth rate, number of secondary infections for each infected individual, and final size of an epidemic (Keeling and Eames, 2005; Turner et al., 2008). Thus, failing to account for heterogeneity reduces our ability to understand and predict the spread of infectious diseases (Ames et al., 2011; Keeling, 1999; Keeling and Eames, 2005). Despite the potential utility of network models in epidemiology, the structure of transmission networks in wildlife is relatively unknown and difficult to quantify. Empirical transmission networks in wildlife are often constructed based on interactions between individuals, which may be quantified through direct observation, proximity-logging collars, or records of shared space use (Corner et al., 2003; Drewe, 2009; Godfrey et al., 2009; Hamede et al., 2009; Otterstatter and Thomson, 2007; Porphyre et al., 2011; VanderWaal et al., 2013a). Because of limitations in detecting the occurrence of transmission, conclusions about pathogen spread are often based on the possibility that transmission *might* occur between interacting individuals (Böhm et al., 2009; Corner et al., 2003; Craft et al., 2009; Drewe, 2009; Godfrey et al., 2009; Grear et al., 2009; Hamede et al., 2009; Otterstatter and Thomson, 2007; Perkins et al., 2009, 2008). In contrast, we reveal where transmission has *already* occurred using the genetics of a diverse microbe, *Escherichia coli*, allowing us to construct a transmission network based on quantifiable transmission events. If two individuals share genetically similar subtypes of *E. coli*, then we infer that they are part of the same chain of transmission, either through direct transmission via interaction or indirect transmission due to exposure to a common environmental source. Individuals are interlinked in the transmission network based upon patterns of *E. coli* subtype sharing (VanderWaal et al., 2013b).

While not usually pathogenic, *E. coli* is a useful proxy for pathogen transmission because it allows us to study transmission pathways without waiting for a true epidemic or making posthoc conclusions after an epidemic has occurred. It should be noted, however, that commensal *E. coli* is not expected to alter an infected host's behavior, so caution must be exercised in applying transmission patterns of *E. coli* to other pathogens. Because of its immense genetic diversity, subtyping is a common method for tracing *E. coli* to an environmental source (Simpson et al., 2002). *E. coli* subtype sharing has been used to demonstrate that transmission regularly occurs between humans and their pets, and between humans, livestock, and wild primates (Damborg et al., 2009; Goldberg et al., 2008; Johnson et al., 2008), but such data have rarely been combined with a network approach (Bull et al., 2012; VanderWaal et al., 2013b). Recently, the emergence of highly virulent forms of *E. coli* have become a significant public health concern (Beutin, 2006). For these reasons, *E. coli* is an excellent model organism for examining enteric pathogen transmission networks in wildlife.

The epidemiological term "super-spreader" refers to individuals who are disproportionately involved in transmission due to either high pathogen shedding rates or high levels of sociality (Lloyd-Smith et al., 2005). Given interspecific variation in physiology and behavior, some species may function as super-spreaders if individuals of that species are consistently central in the transmission network. Although control strategies targeted at super-spreaders are potentially a very effective tool for managing disease (Craft and Caillaud, 2011; Woolhouse et al., 1997), such strategies are not currently feasible in wildlife because logistical and diagnostic limitations make it difficult to identify super-spreaders (Cross et al., 2009). Here, we combine microbial genetics with network analyses to examine heterogeneity in transmission patterns in a community of East African ungulates. We first examine which species-level attributes contribute to sharing *E. coli* subtypes. We then explore how network connectivity varies across species and discuss possible implications for pathogen control and management.

2. Methods

2.1. Study site and species

This study was conducted in Ol Pejeta Conservancy (OPC), a 364-km^2 wildlife reserve that integrates commercial cattle ranching with wildlife conservation in central Kenya. OPC is a fenced, semi-arid savanna ecosystem located on the equator (0° N, 36°56′ E). The reserve is an *Acacia*-woodland/grassland mosaic and receives on average 900 mm of rainfall per year (Birkett, 2002). Species included in this study were the African buffalo (*Syncerus caffer*), eland (*Taurotragus oryx*), Grant's gazelle (*Gazella granti*), Thomson's gazelle (*Gazella thomsonii,* status: Near threatened), reticulated giraffe (*Giraffa camelopardalis reticulata*, subspecies status: Lower risk – conservation dependent), Jackson's hartebeest (*Alcelaphus buselaphus jacksonii*, sub-species status: Endangered), impala (*Aepyceros melampus*), black rhinoceros (*Diceros bicornis,* status: Critically endangered), plains zebra (*Equus burchelli*), and domestic cattle (*Bos indicus*). These species account for 99% of ungulate biomass and 97% of the ungulate population in OPC (OPC Ecological Monitoring department, unpublished data).

Because dietary niche is likely to affect both pathogen exposure and the micro-environment within the gut (Apio et al., 2006; Dehority and Odenyo, 2003), study species included three browsers, four grazers, and three mixed feeders whose diets consist of a combination of browse and grass (Table 1). Species whose diets consist of >70% grass were defined as grazers, 30–70% grass as mixed feeders, and <30% grass as browsers. Values for diet composition were taken from the literature (Cerling et al., 2003; Codron et al., 2007; Copeland et al., 2009; Gagnon and Chew, 2000; Hoffmann, 1989; Spinage et al., 1980; Sponheimer et al., 2003; Watson and Owen-Smith, 2000). Digestive physiology may also have an effect on the types of *E. coli* fostered in the gut, and studies of *E. coli* population genetics demonstrate that host order is among the most important factors differentiating *E. coli* (Souza et al., 1999). This set of species includes eight ruminants (Order Artiodactyla) and two hindgut fermenters (Order Perissodactyla, Table 1). The inclusion of buffalo is especially relevant to livestock owners because they are closely related to cattle and are considered a major reservoir for both Foot and Mouth Disease and bovine tuberculosis (Kock, 2005). The black rhinoceros is endangered and vulnerable to disease-related population declines (Emslie and Brooks, 1999).

E. coli is generally transmitted through the ingestion of fecal contaminated forage and water. It can persist in natural water sources for months, but densities rapidly drop off in the first two weeks (Avery et al., 2008; Medema et al., 1997). Inactivation by sunlight further reduces populations (Sinton et al., 2007). In soil, *E. coli* can persist eight to 25 weeks, but survival is reduced by low moisture content and warm temperatures (Habteselassie et al., 2008).

Table 1
Sample size, population size, home range size (HR), and other attributes of study species.

	N	Pop. size	Order	Family	Sub-family	Digestion	Foraging Niche	HR (km^2)
Buffalo	29	1200	Artiodactyla	Bovidae	Bovinae	Rumen	Grazer	60[a]
Cattle	30	6500	Artiodactyla	Bovidae	Bovinae	Rumen	Grazer	6[b]
Eland	31	400	Artiodactyla	Bovidae	Bovinae	Rumen	Browser	38[a]
Grant's gazelle	17	900	Artiodactyla	Bovidae	Antilopinae	Rumen	Mixed	6[c]
Thomson's gazelle	32	1400	Artiodactyla	Bovidae	Antilopinae	Rumen	Mixed	3[d]
Hartebeest	31	140	Artiodactyla	Bovidae	Alcelephinae	Rumen	Grazer	5[e]
Impala	32	3600	Artiodactyla	Bovidae	Aepycerotinae	Rumen	Mixed	2[a]
Giraffe	32	200	Artiodactyla	Giraffidae	n/a	Rumen	Browser	73[f]
Black rhino	14	90	Perissodactyla	Rhinocerotidae	n/a	Hindgut	Browser	12[e]
Plains Zebra	31	4500	Perissodactyla	Equidae	n/a	Hindgut	Grazer	120[a]

[a] Jones et al. (2009).
[b] OPC Cattle Department, personal communication.
[c] Vanessa Ezenwa, unpublished data.
[d] Walther (1973).
[e] OPC Ecological Monitoring Department, unpublished data.
[f] VanderWaal et al. (2013c).

2.2. Interspecific associations

To quantify the extent to which each species aggregated with other species, we recorded the proximity of each species to others while driving pre-determined road transects trough OPC. Routes were approximately 100 km in length, covered approximately 115 km^2 each, and traversed all habitat types. Transects were designed so the majority of OPC was surveyed once every three days. Observations of interspecific association patterns were recorded between March 17 and August 2, 2011 ($N = 2143$ observations). Association was defined as the percentage of observations that two species were observed within 50 m of each other relative to the total number of times those species were observed together or apart.

2.3. Sample collection, genetic analysis and network construction

We collected a total of 279 fecal samples (Table 1). Sample collection was stratified across ten spatial blocks in the study area. The Ewaso Ngiro River bisected OPC into eastern and western halves. Three spatial blocks were located within the eastern half, while seven were located within the western half. For species with small population sizes, caution was exercised to ensure that individuals were not sampled multiple times: each giraffe and black rhinoceros was individually identifiable as a result of ongoing population monitoring (giraffe, VanderWaal et al., 2013c; rhinoceros, OPC Wildlife Department). Because there may be significant monthly turnover of *E. coli* subtypes in the gut (Anderson et al., 2006), fecal samples were collected during the brief period between August 28, 2011 and October 7, 2011 to ensure comparability. A few giraffe samples were collected as early as August 14. Black rhinoceros samples were collected from the rectum during routine immobilizations conducted by the Kenya Wildlife Service. Fecal samples for other species were collected from the ground immediately after defecation was observed and transported on ice to the field laboratory. Samples were then diluted in sterilized water, streaked onto CHROMagar EC agar plates (CHROMagar, Paris France), and incubated overnight at 37° C. CHROMagar is a selective chromagenic agar that exhibits high specificity for *E. coli*. After incubation, four randomly selected *E. coli* colony isolates were cultured and then frozen.

Samples were shipped on dry ice to the UC Davis School of Veterinary Medicine. DNA was extracted from cultured cells using QIAGEN DNeasy Blood and Tissue kits (QIAGEN, Valencia, CA) and genetic subtypes were determined using BOX-PCR and gel electrophoresis, which is a well-established method for discriminating between genetically similar *E. coli* subtypes (Cesaris et al., 2007; Goldberg et al., 2006; Mohapatra and Mazumder, 2008). Similarity of each isolate to all others was determined through pairwise comparisons of the densitometric curves generated for each isolate by gel electrophoresis. Isolates were considered to be matching subtypes if their densitometric curves were >90% similar (Pearson's correlation coefficient). Based on a reproducibility analysis conducted in our lab, this cutoff value minimizes Type I errors in matching while limiting the Type II error rate to <5%. Detailed laboratory methods are described elsewhere (VanderWaal et al., 2013b).

A transmission network was constructed from patterns of *E. coli* subtype sharing. Sampled individuals were represented as nodes and nodes were linked if they shared at least one *E. coli* subtype. Links in the network were undirected and unweighted, meaning that we do not know in which direction transmission occurred nor do we attach more weight to a link if the pair shared multiple subtypes. All network analysis was performed in R (R Development Core Team, 2012).

2.4. Statistical analysis

2.4.1. Dyad-level analysis: What factors influence the likelihood of two individuals sharing E. coli subtypes?

To determine which factors influenced the likelihood of a transmission link occurring between dyads, we performed multiple regression quadratic assignment procedures (MR-QAP), a method of matrix regression developed for network data (Dekker et al., 2007; Krackhardt, 1988). Essentially, MR-QAP coerces matrices into vectors, and then performs a standard logistic regression on the log-odds of an edge occurring in the transmission network given dyad-level attributes. MR-QAP uses a Monte Carlo method, in which rows and columns are randomly permuted within matrices, to determine the significance of regression coefficients because traditional p-value estimates are potentially biased due to the inherent interdependencies of relational data (Dekker et al., 2007). Using MR-QAP with double Dekker semi-partialling and 1000 permutations (Dekker et al., 2007), we investigated the effect of dyad-level attributes on the log-odds of a tie in the transmission network.

Dyad-level covariates included whether the individuals in the dyad were the same species, sub-family, or family, whether they shared the same foraging niche (browser, mixed feeder, or grazer) or digestive system (rumination or hindgut fermentation), whether they were sampled from the same spatial block or same side of the river that bisects OPC (east or west), and the frequency with which

those two species were found associating together in multi-species aggregations. Taxonomic order was not considered because it covaried perfectly with digestive system. Because the use of goodness-of-fit statistics, such as AIC, is controversial in MR-QAP, we used a stepwise approach to construct multivariate statistical models. Non-significant terms were dropped from the full model in a stepwise manner to leave a minimal multivariate model. Univariate models are also reported. Analyses were performed using the 'sna' package of R (Butts, 2010).

2.4.2. Species-level comparisons

We calculated four measures of connectivity for each individual: degree, information centrality, betweenness, and cutpoint potential (Wasserman and Faust, 1994). "Degree" is the number of individuals, or neighbors, to which the focal node was connected. "Information centrality" essentially measures the distance of the focal node to all others, providing a measure of the extent to which an animal is located at the center of the network. It is calculated by taking the harmonic mean of all possible paths in the network that originate from the focal node (Wasserman and Faust, 1994).

We calculated an individuals' "betweenness" using Newman's (2005) random-walk definition. Betweenness is defined as the number of paths that pass through the focal node if random paths between every other pair of individuals are traced (Newman, 2005). Individuals can have high betweenness (i.e., many paths pass through them) either because (a) they are connected to a large number of other individuals, or (b) because they lie on paths that are bottlenecks for flow. The former leads to high correlations between betweenness and degree ($r = 0.57$). The latter is more relevant for disease management because these individuals are potential "cutpoints" in the network; removal of individuals that serve as bottlenecks may divide the network into multiple disconnected sub-networks. We developed a measure to disentangle betweenness from its correlations with degree. A regression line was fitted that related betweenness to degree. From this regression equation, we calculated each individual's expected betweenness given its degree. A residual was then calculated by subtracting observed betweenness from expected betweenness. We refer to this residual as an individual's "cutpoint potential." Positive cutpoint potentials indicate individuals that are candidate bottlenecks for pathogen flow in the network. Degree and information centrality were calculated using R's "sna" package (Butts, 2010), and random-walk betweenness was calculated using NetMiner (NetMiner 2.6, Cyram Corporation, Seoul, Korea).

Connectivity measures (degree, information centrality, betweenness, cutpoint potential) were compared across species using Kruskal–Wallis tests followed by pairwise comparisons with a Bonferroni correction. We hypothesized that cutpoint potential would be related to species-typical home range size because animals with large home ranges may connect different regions in the study area. Therefore, we regressed cutpoint potential (ranked) on species-typical home range size (km^2), and used permutation methods to calculate p-values. This method first calculates the slope for the regression using ordinary least squares, then recalculates the slope for 5000 iterations in which y is randomly permuted relative to x. P-values were defined as the proportion of random permutations with slopes more extreme than the observed value (Good, 2000).

To assess whether differences in species connectivity were an artifact of the fact that we sampled the same number of individuals (~30) from species of vastly different population sizes (e.g. 85 rhinos verses 6500 cattle), we constructed five large random Bernoulli networks (n = 4700 nodes per network) with the same density as the observed transmission network and species represented proportionally to their abundance in the ungulate community. From each of these networks, nodes were randomly selected with each species' sample size proportional to our real-life sampling strategy. We constructed a sub-network using these randomly sampled individuals. For each random graph, we performed 200 sampling iterations for a total of 1000 sub-networks. These sub-networks were considered the null expectations for connectivity patterns produced from a random network. Connectivity patterns (degree, betweenness, and information centrality) in the random sub-networks were compared to the observed network. Connectivity values in the random networks did not differ across species, regardless of how well sampled species were relative to their population size.

We also analyzed the effect of the removal of certain species on the overall connectivity of the transmission network. Removals were analytical only and were intended to simulate the effect of removing animals from transmission chains through either vaccination or treatment. We summarized overall network connectivity using two metrics, density and transitivity, that are theoretically highly important in determining pathogen spread (Ames et al., 2011; Badham and Stocker, 2010; Keeling, 1999; Newman, 2003; Turner et al., 2008; Wu and Liu, 2008). Density is the proportion of ties that occur in the network relative to the total possible number of ties. Transitivity is defined as the number of triangles in the network (A is linked to B, B is linked to C, and C is also linked to A) relative to the number of triplets (e.g. A is linked to B, B is linked to C, but C is not linked to A). Theoretical models predict that pathogen spread is slower in networks with lower density or higher transitivity (Ames et al., 2011; Keeling, 2005). We calculated the change in density (Δ-density) and transitivity (Δ-transitivity) produced by removing individuals of a given species. The observed Δ-values were compared to permuted distributions of Δ-values. These distributions were generated by removing an equal number of random individuals and calculating the resulting Δ-values. P-values were calculated as the percentage of permuted Δ-values that were more extreme than the observed Δ-value. A p-value of <0.05 indicated that the removal of a given species produced significantly greater change in network connectivity than removing an equal number of random individuals. All statistical analyses were performed using R (R Development Core Team, 2012).

2.4.3. Sampling effort and network robustness

We also examined how robust the species-level comparisons were relative to sampling effort. To assess the robustness of our results if only 90% of the network had been sampled, we randomly deleted 10% of individuals in each species. We then re-calculated two of the node-level measures (betweenness and degree) on the resulting networks and re-ranked each species according to their mean connectivity. We calculated the extent to which the rankings in the sub-sample correlated with the rankings in the full network (Spearman's rank correlation). We repeated this sub-sampling 100 times to generate a distribution of Spearman's rank correlations produced by a 90% sampling effort. This process was repeated at 80%, 70%, 60%, 50%, and 40% sampling effort.

3. Results

3.1. What factors influence the likelihood of two individuals sharing E. coli subtypes?

The density of the transmission network was 0.14, indicating that 14% of possible links existed in the network. The likelihood of a transmission link forming between any pair of individuals was highly dependent on host relatedness, with conspecific links being approximately 1.99 times more likely to occur than heterospecific links (Table 2). Family and sub-family were not considered in multivariate models due to the nested nature of the taxonomic

Table 2
MR-QAP models on the likelihood that two individuals are linked in the transmission network. The best multivariate model was determined by stepwise selection of covariates. Coefficients are reported as odds ratios. *P*-values are given in parentheses. (*) indicate relationships that were significant at $p < 0.05$. Family and subfamily were not considered in multivariate models.

Covariate	Best multivariate model	Univariate models
Same species	1.99 (<0.001)*	1.51 (<0.001)*
Same digestion	1.28 (0.089)	1.21 (0.197)
Same side of river	1.18 (0.016)*	1.19 (0.009)*
Freq. of interspecific association (%)	1.07 (<0.001)*	1.06 (<0.001)*
Same foraging niche		1.10 (0.039)*
Same spatial block		1.07 (0.267)
Same family		1.11 (0.448)
Same subfamily		1.08 (0.362)

covariates; dyads that are the same species are always same subfamily, which are always in the same family. Although digestive system is equivalent to host order, digestive system was retained in the multivariate models because it espoused an important physiological as well as taxonomic difference between hosts. Pairs of individuals with the same digestive physiology were approximately 1.3 times more likely to share *E. coli* subtypes than pairs with different physiologies, although the effect was not quite significant in multivariate models. The effect of sharing a foraging niche had a small positive effect on subtype sharing in univariate models, but it did not remain significant in multivariate models (Table 2). In addition, individuals were more likely to be linked if they were sampled from the same side of the river, but not from the same spatial block. Individuals of species that associated more frequently were more likely to share *E. coli* subtypes (Table 2, Fig. 1).

3.2. How does network connectivity vary across species?

There were significant differences among species for degree, information centrality, and betweenness (Kruskal–Wallis tests, $p < 0.05$), and near significant differences for cutpoint potential ($p = 0.088$). Pairwise comparisons revealed that Grant's gazelles were consistently the most well-connected in all measures except cutpoint potential (Fig. 2). Grant's gazelle information centrality and degree were significantly higher than all species except hartebeest. Buffalo and cattle were consistently among the least connected in all measures except cutpoint potential. These differences between species were not an artifact of the fact that we sampled the same number of individuals from species of different population sizes; when observed connectivity patterns were compared to the randomized sub-networks, species were not predicted to differ in their connectivity in the random networks, regardless of how well sampled they were relative to their population size. Cutpoint potential was positively correlated with species-typical home range size ($\beta = 0.36$, $p < 0.01$), while other network metrics were unrelated to home range.

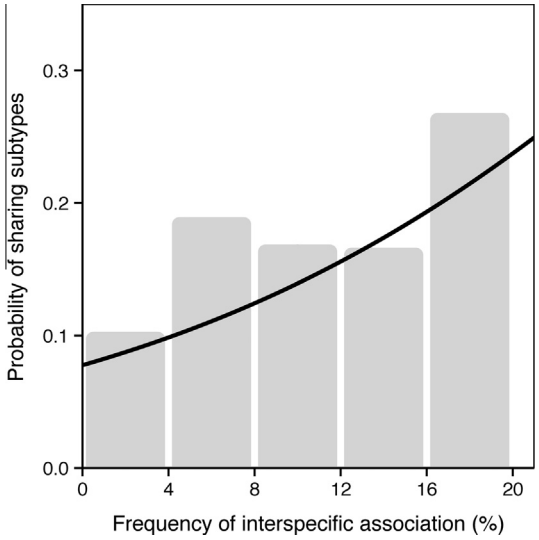

Fig. 1. Relationship between the frequency of interspecific association and the probability of sharing genetic subtypes of *E. coli*. Bars indicate the proportion of dyads that share subtypes at varying levels of association, while the trendline shows the fitted relationship between the two variables in the multivariate model.

3.3. Implications of "targeted interventions" on network structure

Targeting highly connected species during disease interventions may be very effective because individuals of those species are on average better connected in the network. Thus, removing these species from transmission chains has greater consequences for reducing the connectivity of the network than choosing individuals at random. In practice, removals could be achieved via targeted treatment or vaccination. The only species whose removal altered network density was the Grant's gazelle (Δ-density = −0.01, $p = 0.02$) and hartebeest (Δ-density = −0.02, $p < 0.01$). Their removal reduced network density significantly more than the removal of an equal number of random animals. These species were also the two highest ranked species for degree (Fig. 2). To illustrate the implications of removing certain species, we constructed a transmission network that removed the two species ranking the most highly in degree (Fig. 3a–b).

We also constructed a network that removed the two species with the highest cutpoint potential (zebra and buffalo, Fig. 3c). Because betweenness is highly dependent on tracing paths through the network, betweenness values may be drastically altered after the removal of one species. Therefore, we first removed the species with the highest cutpoint potential (zebra) and then re-calculated betweenness and cutpoint potential on the resulting network. After the removal of zebra, buffalo exhibited the highest cutpoint potential. The removal of these two species together produced networks that were significantly more clustered (higher transitivity) than the removal of an equal number of random individuals (Δ-transitivity = 0.034, $p < 0.01$). The removal of the two highest-ranked species without the re-calculation step (zebra and eland) did not produce more transitive networks, highlighting the importance of re-calculating path-based measures after the removal of the first species.

3.4. Sampling effort and network robustness

Species rank-order was relatively conserved when sampling effort was reduced. Degree was more robust than betweenness. Even with sampling efforts as low as 60%, sub-networks still maintained high correlations in species rank-order when compared to the full network (Spearman's rank correlation, $r > 0.9$). Spearman's rank correlations for betweenness, in contrast, fell much more rapidly with reduced sampling effort. Sampling effort of >70% allowed correlations to remain above 0.8, but even 90% sampling effort failed to produce correlations of greater than 0.9. However, if our aim is to identify the most connected species, rank-order switches may not be that relevant as long as the top few ranks are unaffected. More than 90% of sub-samples had the same two species ranked the highest for betweenness when sampling effort was set at 90%.

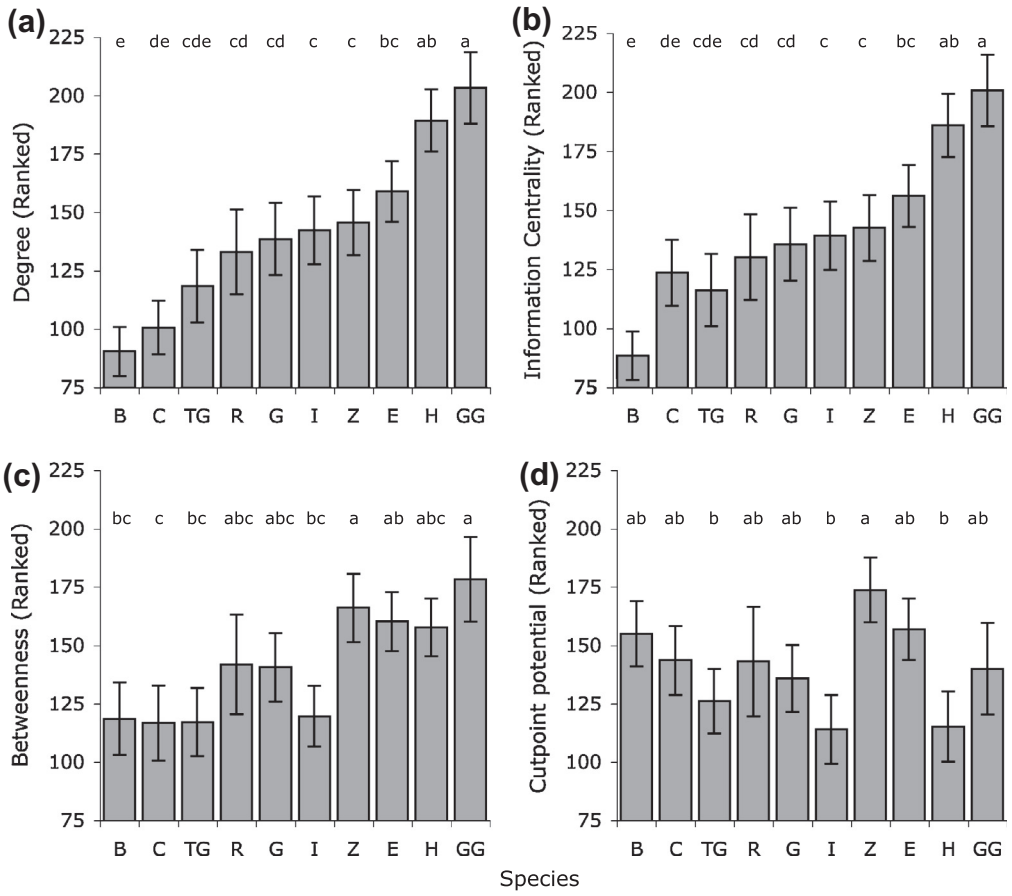

Fig. 2. Bar graphs summarizing species connectivity values for (a) degree, (b) information centrality, (c) betweenness, and (d) cutpoint potential. Ranked values are represented and used in tests because of the highly non-normal distributions produced by some connectivity values. Axis labels indicate species: B-Buffalo, C-Cattle, TG-Thomson's gazelle, R-Black rhino, G-Giraffe, I-Impala, Z-Zebra, E-Eland, H-Hartebeest, and GG-Grant's gazelle. Species in the same letter group (lower-case) were not significantly different in pairwise comparisons.

4. Discussion

4.1. Factors influencing the likelihood of two individuals sharing E. coli subtypes

Our results suggest that *E. coli* transmission patterns are influenced by a combination of spatial, behavioral, and physiological factors. Individuals from the same side of the river were more likely to be connected in the transmission network, indicating that transmission patterns are spatially structured and that the river may pose a substantial barrier to the spread of microbes. Individuals were also approximately twice as likely to be linked to conspecifics than heterospecifics, a pattern that could arise through either social interactions or similar physiology (Table 2).

The fact that interspecific association positively influenced the likelihood of subtype sharing indicates that behavioral factors, such as shared space use or tendency to aggregate, may play a considerable role in determining transmission patterns. Animals that utilize the same habitats are more likely to be in proximity to one another other, creating opportunities for transmission. Perhaps more importantly, they are likely exposed to the same environmental sources of *E. coli.* It is also possible for flies to function as mechanical vectors and transmit *E. coli* when they move from one animal to another (Ahmad et al., 2007; Förster et al., 2007).

Physiological similarity also played an important role in determining patterns of subtype sharing, as shown by the fact that individuals sharing the same digestive physiology (or host taxonomic order) were more likely to share *E. coli* subtypes. In this ecosystem, digestive physiology did not covary with behavioral factors, such as foraging niche (Table 1), nor did species with the same digestive physiology associate more frequently (Table 3). Other studies have shown that host order and digestive physiology are among the most important factors in genetically differentiating *E. coli* (Souza et al., 1999). Digestive physiology, taxonomic grouping, and host diet are among the primary factors influencing the internal environment and growth substrate (sugars) present within the gut. Subtypes that are well adapted for one host species may not be competitive in hosts offering different internal environments. Part of the reason for this is that different *E. coli* strains vary in the numbers and kinds of sugars they utilize (Souza et al., 1999). Thus, even if species of different orders ingest the same *E. coli* subtypes from the environment, not all subtypes are equally likely to establish themselves in the herbivore's gut. In addition to digestive physiology, we found that dyads sharing the same foraging niche were slightly more likely to share subtypes. This could arise through either similarity in exposure or selective establishment of *E. coli* subtypes based on similar internal environments.

4.2. Species-level variation in transmission network connectivity

Our transmission network quantifies patterns of interspecific transmission for a fecal-orally transmitted microbe in wildlife. One potential drawback of using a commensal bacterium as a model pathogen is that host behaviors, and consequently contact patterns among hosts, are not altered by infection with *E. coli* as they might be for a true pathogen (Hawley et al., 2011). Thus, caution

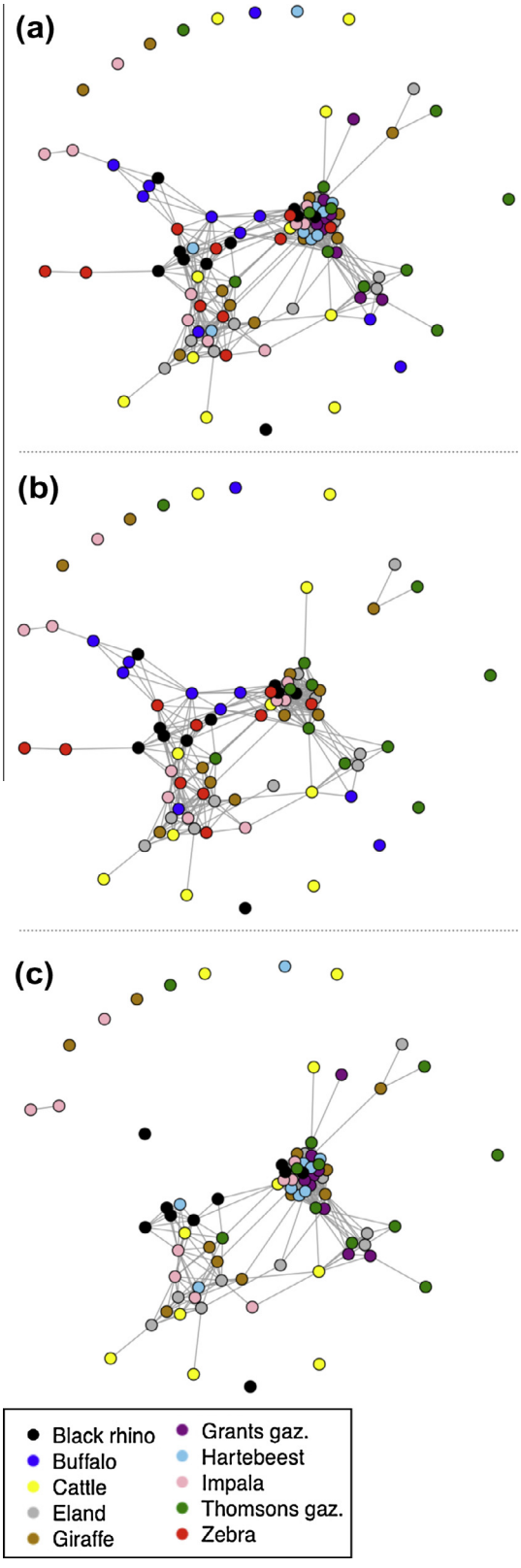

Fig. 3. Transmission network (a) inclusive of all species (density = 0.14, transitivity = 0.73). Transmission network with (b) the two species with the highest degree removed (Grant's gazelle and hartebeest, density = 0.11, transitivity = 0.67) and (c) the two species with the highest cutpoint potential removed (zebra and buffalo, density = 0.16, transitivity = 0.77). For visualization purposes, only 100 randomly selected nodes are shown.

should be exercised in applying *E. coli* transmission patterns to other pathogens. Nonetheless, *E. coli* transmission routes give insight into transmission dynamics by demonstrating where contact between species is sufficient for transmission to occur and which species are potential super-spreaders. There were significant differences among species in their connectivity in the transmission network. While interspecific differences are unsurprising, we previously lacked tools to quantify this variation despite the theoretical importance of such heterogeneity in pathogen spread.

The Grant's gazelle is one candidate super-spreader species. Individuals of this species not only shared transmission links with a large number of others (high degree), but also tended to occupy central positions in the network with high flow (high information centrality and betweenness). They were also among the species most frequently found in multi-species aggregations (Table 3). Ezenwa (2003) found that as compared to sympatric ungulates, Grant's gazelles tended to have higher prevalence of strongyle nematodes, another generalist fecal-oral microbe. Furthermore, their shedding intensities (eggs per gram of feces) were well more than double that of nearly all other species (Ezenwa, 2003). We are unable to determine directionality in transmission using our methods and thus cannot distinguish whether Grant's gazelles were frequent transmitters or recipients of *E. coli*. Longitudinal studies of known individuals would be necessary to infer directionality in transmission. Nonetheless, our results indicate that highly connected species, such as the Grant's gazelle, may play an integral role in fecal-oral transmission chains.

The plains zebra ranked the highest in cutpoint potential and second highest in betweenness, though not significantly. Betweenness, put simply, quantifies the extent to which an individual serves as a conduit for pathogen flow through a network (Borgatti, 1995; Salathé and Jones, 2010; Wey et al., 2008). Because paths through the network are more likely to pass through nodes with high degree, betweenness does not allow us to differentiate between individuals that have high betweenness because they have high degree, such as the case for Grant's gazelle, or because they lie along paths that are bottlenecks for flow. In contrast, cutpoint potential quantifies the extent to which individuals are potential bottlenecks. Individuals that lay on a larger number of paths than expected given their degree were scored more highly for this measure, and the removal of such individuals had higher potential for fragmenting the transmission network. Here, we find that cutpoint potential was significantly correlated with species-typical home range size. Zebra may have high cutpoint potential because they are the most widely ranging species, connecting geographically separated clusters of animals. The literature-reported home range size for zebras is 120 km^2 (Jones et al., 2009). This is 60% larger than the second widest ranging species, giraffe, and 100% larger than the third widest ranging species (Table 1). Hartebeest and impala had the smallest cutpoint potential and also have among the smallest home ranges (5 and 2 km^2 for hartebeest and impala, respectively). Therefore, zebras may serve as "bridges" between regions of the network that would otherwise be relatively separated. Animals such as impala and hartebeest, whose home ranges are much more localized, tend not to function as bridges.

Interestingly, livestock were among the least well-connected species (Fig. 2). Although our results indicate that cattle were not particularly important disseminators of fecal-oral microbes within this ecosystem, cattle remain an important factor in the emergence of wildlife diseases due to their role in introducing novel diseases into ecosystems and transporting diseases between geographically distinct wildlife populations (Osofsky, 2005).

4.3. Implications for management

While we recognize that commensal *E. coli* is not a microbe that needs management, our main intent in this section is to show the potential utility of an approach combining network theory with

Table 3
Percent of observations each pair of species was observed <50 m of each other. Species below the dotted line were not actively surveyed and were only recorded when observed with the study species.

	Black Rhino (n=56)	Buffalo (n=109)	Cattle (n=113)	Eland (n=218)	Giraffe (n=262)	Gr. Gazelle (n=650)	Harte-beest (n=184)	Impala (n=688)	Th. Gazelle (n=532)	Zebra (n=739)
Black rhino										
Buffalo	2.5%									Key
Cattle	0.6%	0.5%								0-2%
Eland	1.9%	2.8%	0.9%							2-5%
Giraffe	2.6%	2.5%	1.1%	5.5%						5-8%
Gr. gazelle	0.6%	1.7%	1.7%	5.2%	4.1%					8-12%
Hartebeest	0.4%	1.0%	0.3%	1.8%	3.0%	6.5%				>12%
Impala	1.4%	2.2%	0.6%	7.9%	6.4%	18.3%	6.9%			
Th. gazelle	1.6%	2.9%	1.4%	6.4%	4.3%	15.0%	9.6%	10.8%		
Zebra	1.0%	3.2%	2.4%	14.2%	6.6%	13.4%	7.5%	13.4%	7.7%	
Elephant	0.0%	0.0%	0.7%	1.2%	0.0%	0.9%	0.0%	0.6%	0.2%	0.3%
Gerenuk	0.0%	0.0%	0.0%	0.0%	0.0%	0.2%	0.0%	0.0%	0.0%	0.0%
Grevy's zebra	0.0%	0.0%	0.0%	0.4%	0.0%	0.2%	1.0%	0.0%	0.6%	0.7%
Oryx	0.0%	2.6%	0.6%	0.0%	1.6%	3.9%	5.5%	2.8%	4.7%	2.6%
Sheep	0.0%	0.0%	4.4%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Warthog	1.5%	4.7%	1.0%	8.7%	2.6%	6.4%	7.4%	5.1%	5.7%	5.2%
Waterbuck	1.1%	0.4%	0.4%	5.6%	1.3%	1.6%	5.2%	2.7%	1.1%	1.7%
White rhino	1.2%	3.0%	0.0%	0.0%	0.3%	1.0%	0.5%	0.7%	1.6%	0.8%

microbial genetics and to outline possible courses of analysis that could be beneficial to disease management. This approach could be readily applied to other microbes to quantify transmission patterns. Models suggest that control strategies targeted at super-spreaders have the potential to limit disease spread much more effectively than conventional methods, such as mass vaccination (Lloyd-Smith et al., 2005), but the implementation of targeted interventions in wildlife is limited by our inability to identify super-spreaders (Dalahay et al., 2009). The development of methods to quantify heterogeneity, such as the one demonstrated here, is the first step in making targeted intervention strategies feasible. The advantage of focusing on species-level rather than individual-level differences in network connectivity is that it is not necessary to quantify each unique individual's network position in every new population, which is often nearly impossible due to high turnover of individuals, large population sizes, and the intensity of monitoring necessary to acquire such data. Rather, knowledge of the characteristic role that individuals of a species play in the transmission network may allow results to be more generalizable across populations and over time.

We examined the effect of topologically "removing" key species from the transmission network to demonstrate the importance of targeted interventions. We do not mean that these animals would be culled, but rather that they could be removed from transmission chains through some measure of intervention (vaccination, treatment, etc.). Control strategies aimed at high degree individuals have been discussed at length in the literature as an effective way to limit the size of disease outbreaks in both humans and wildlife (Craft and Caillaud, 2011; Hudson et al., 2008; Lloyd-Smith et al., 2005; Woolhouse et al., 1997). In our network, targeted removal of the species with the highest degree (Grant's gazelle and hartebeest) reduced the density of the transmission network. Transmission is more rapid through denser networks, so control strategies that reduce network density may have practical implications for slowing the spread of a pathogen (Ames et al., 2011; Otterstatter and Thomson, 2007). However, removing high degree species did little to fragment the network, primarily because their removal merely eliminates individuals within dense clusters without affecting many between-cluster paths (Fig. 3b).

In contrast, the removal of the species with the highest cutpoint potential (zebra and buffalo) increased the overall level of transitivity in the network (Fig. 3c). Transitivity can be interpreted as the extent to which a network is clustered, and high levels of clustering reduce the ability of a pathogen to spread in a population (Ames et al., 2011; Badham and Stocker, 2010; Keeling, 1999; Newman, 2003; Turner et al., 2008; VanderWaal et al., 2013a; Wu and Liu, 2008). Low density and high transtivity are both associated with slower rates of pathogen spread (Ames et al., 2011; Otterstatter and Thomson, 2007), although the differences in density and transitivity observed here were not large. Targeting bottleneck species, such as the zebra and buffalo, is similar to the low-coverage vaccination strategy proposed by Haydon et al. (2006) for Ethiopian wolves (*Canis simensis*). Epidemiological models demonstrated that vaccinating wolf packs occupying habitat corridors connecting subpopulations reduced extinction risk and the size of rabies outbreaks in wolves by reducing the ability of a rabies epidemic to spread between metapopulations (Haydon et al., 2006). In our system, animals functioned as bottlenecks not by occupying certain spatial regions, such as habitat corridors, but because their long-distance ranging behavior allowed them to function as bridges between different regions of the study area.

These examples highlight the potentially different implications of targeting high degree versus high cutpoint potential species for modifying network structure. For example, if our intent is to control pathogen outbreaks by increasing network clustering, one species we might target for their high cutpoint potential is buffalo. However, buffalo have the lowest average degree and thus its removal would do little to change network density. Thus, targeted control strategies may not be able to simultaneously reduce densities and increase transitivity. Combining transmission networks with quantitative epidemiological models would help determine

the relative merits of reducing density versus increasing transitivity for disease control. This would be a fruitful next step in translating these sorts of data into management strategies.

Transmission dynamics vary seasonally because of changes in host behavior, host contact rates, and the ability of pathogens to persist in the environment (Altizer et al., 2006). Because shifts in precipitation modify the distribution of water and forage in semi-arid ecosystems, animals seasonally change the way they use the landscape and associate with other species (Altizer et al., 2006; Hirst, 1975). During the dry season, transmission opportunities between species may decline because ungulates generally decrease diet and habitat overlap due to increased competition (Fritz et al., 1996; Sinclair, 1985; Voeten and Prins, 1999), yet limited water availability may increase contact rates among species (Artois et al., 2009; Muoria et al., 2007; Ward et al., 2009; Waret-Szkuta et al., 2011). These changes in behavior have the potential to alter the dynamics of pathogen transmission through an ecosystem (Altizer et al., 2006). How transmission network connectivity and structure change with season remains an open question, and this would be a productive area for future research.

Transmission networks provide different insights than other genetic approaches. Population genetic studies tend to focus on gene flow between pathogen metapopulations found in different host species/populations (Brown et al., 2008; Rwego et al., 2008). Phylogenetic approaches study the evolutionary relationships among genetic lineages, inferring historical host-switching events or evolutionary relationships on large geographic scales (Liu et al., 2010; Wallace et al., 2007). Haplotype networks, for example, focus on the evolutionary relationships between subtypes as measured by mutations (e.g. Archie and Ezenwa, 2011; Beja-Pereira et al., 2009), whereas our approach focuses on connectivity between individuals as measured by shared subtypes. We do not make any assumptions on the evolutionary relatedness of various *E. coli* subtypes. It may be possible to use relatedness data to weight network edges. To implement this, however, additional assumptions must be made about the rate at which mutations occur. Evolutionary changes may not be occurring on a time scale that is epidemiological relevant for inter-individual transmission chains.

Our methods can be employed to demonstrate possible routes of transmission through an ecosystem and are broadly applicable across studies of both intra- and inter-specific routes of transmission. Even though transmission routes demonstrated by commensal *E. coli* are likely only applicable to fecal-oral microbes that are epidemiologically similar (pathogenic *E. coli*, *Cryptosporidium spp.*, and *Clostridia spp.*, some helminthes, etc.), this approach allows us to quantify heterogeneity in transmission patterns. Transmission networks of other pathogens could be examined in a similar way, although consideration must be taken to select microbes with suitable amounts of genetic variation. Too much variation may lead to a disconnected network, while too little will lead to all individuals being connected to all others. Also, it would be difficult to construct networks for microbes with low prevalence due to the fact that many sampled individuals would not be infected and thus would not yield any new information about transmission network structure. In conclusion, this study demonstrates the utility of an approach that combines genetics and network theory both for quantifying interspecific heterogeneity in transmission patterns and as a first step in making targeted control strategies feasible for the management of infectious diseases.

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