1.0 Background

1.1 Societies provide increased opportunities for disease transmission.

Living in societies affects how diseases transmit. We need to understand the role of group size, group complexity and connectedness in driving infectious disease transmission so that we can reduce the heavy burden that infectious diseases impose. Many studies are examining this in humans, looking at correlations between our patterns of activity [1], or variation in our built environment [2], and how disease spreads in order to identify the potential drivers of transmission, such as highly connected individuals [3] and immunization efforts [4]. With the rise of the internet and mobile technology we can use additional tools such as satellite imagery or online activity to supplement traditional contact pattern studies [5, 6]. As informative as these approaches are, they do not lend themselves to hypothesis driven experimentation. We cannot build a school, populate it with kids, infect them, and then randomly remove walls or children to ask how disease transmits in such altered conditions. *It would be ideal therefore if we did have a complex, dense, society which we could repeatedly alter to determine what the drivers of disease transmission are within groups. We would like to have an experimental system*.

Ants present an ideal system to study the emergent properties of transmission in groups because they are amenable to experimental manipulation- of size, structure, and complexity. Our expertise in behavioral studies of ant societies will allow high-resolution quantification of contacts and transmission simultaneously helping us to understand what factors promote disease transmission. In addition, because ant societies have evolved ways to mitigate the spread of infectious disease, there are important gains to be had by studying how natural selection has resulted in reduced disease in such dense groups of relatives.

1.2 The ants as an experimental model to study the socio-ecological determinants of transmission

In contrast to human, domestic animal or wild animal studies (like wolf packs, prairie dogs, bottlenose dolphins or desert tortoises), several key ingredients for the successful analysis of the socio-ecology of transmission come together in ant systems: 1) Ants live at very high densities, 2) They live in nests and these can be architecturally very complex and variable, 3) Resource transmission (e.g. sugar/protein) and disease transmission can be measured exactly 4) The individual behavior and network position of each member of the group can be known exactly and 5) Unlike all other systems where disease transmission is of interest, ants can be experimentally perturbed. These attributes offer the possibility for an experimental approach where we can manipulate crowding conditions, network connections, resources and parasite pressure to understand the socio-ecological drivers of infectious diseases. We can then explore, with models, what factors are important for effective transmission in societies. With insights from modeling we can return to our experimental set up to test how disruption or changes in the network affects disease dynamics.

Disease spread in social insects (i.e. ants, bees, wasps and termites) is of general significance because these group living organisms have apparently solved an important problem: how to maintain a supply chain for food resources entering the colony while preventing the outbreak of pathogens that come with the food [7-10]. The general answer, provided by social insect researchers, is that social insects have **social immunity:** the ability of multiple individuals to act collectively, preventing the establishment of infectious diseases [11-14]. Many studies have experimentally shown how the colony can rapidly and effectively respond to pathogen incursion to prevent its transmission [12, 15-20], but our understanding of the mechanisms behind social immunity is limited. Studying infectious disease in social insects could provide general insights into the mechanisms behind social immunity in a broad range of species. *We will study the transmission of co-evolved and general pathogens and the food resources necessary for colony life to ask what factors function to allow resources, but not infectious diseases, to move through the group. Our ultimate goal is to*

discover, using a combination of experimental manipulation and mathematical models, general principles relevant to the socio-ecological drivers of infectious disease in groups.

2.0 Objectives and Overall Approach

Ant colonies are experimentally tractable systems. A mature colony of the common black carpenter ant (*Camponotus pennsylvanicus*, which nests in wood) might have 10,000 workers, a queen and thousands of brood [21]. It is possible to divide such a colony into 100 x 100 workers (or some other division), label each ant and record individual behavior as disease or resources are introduced. We can record contact rates and how these lead to transmission for a range or agents. We can analyze the behavior of individuals and their entire network over both space and time. Importantly, we can also experimentally perturb the system to test what factors influence transmission.

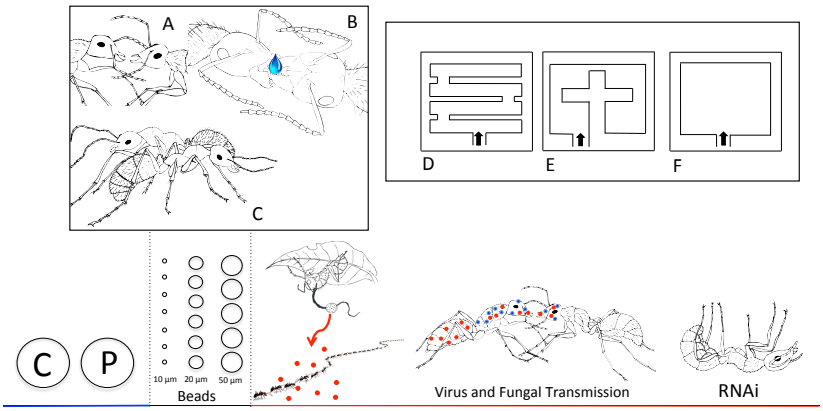

Figure 1 *A) Antennation, B) Trophallaxis, C) Proximity. D-F) Equal volume nests with different architecture. Below are the transmission agents. (l-r) =carbohydrates, liquids. P=Protein, prey/insects. Beads. Fungal spores that foraging ants encounter. Worker to worker transmission of spores, via touch and virus via liquid exchange.*

2.1 Testing the socio-ecology of disease transmission

Ant societies offer considerable scope for experimentation. We can record all behaviors relevant for disease spread: antennation (two ants touching each other with their antennae, Figure 1a) trophallaxis (exchange of liquids mouth to mouth, Figure 1b), and lateral contact (two ants side by side, not involving antennation or trophallaxis, Figure 1c). We can house ants in naturalistic, wooden nests (Figure 1e) and we can modify those spaces to test how architectural changes (e.g. 1d,f) impacts transmission. We can compare the transmission of different agents that range from beneficial (food), to agnostic (beads), to virulent (fungi/virus/RNAi). This allows us to study transmission as an emergent property of societies, independent of virulence. With this tractability we are motivated to ask the following questions:

Q1: How does transmission scale with colony size?
Q2: Does heterogeneity within the colony drive transmission, and how does social and spatial variation contribute to this?
Q3: How does the environment affect transmission in groups?
Q4: Can models and high-resolution data motivate knockout experiments?

----------Q1: How does transmission scale with colony size?----------

Rationale: A central question in disease dynamics is how the force of infection scales with community size- this question lies at the heart of the debate over the extent to which transmission depends on the density or frequency of infection in a population [22, 23]. These two caricatures for the scaling of transmission are extremes on a spectrum of patterns that may emerge as a consequence of the contact structure of the host population and the transmission mechanism of the parasite [24]. We have learnt a great deal about the scaling of transmission from both experimental manipulations [25-29] and mathematical models fit to

natural systems [26, 30, 31]. However, the experimental settings have been necessarily simplistic and the evidence from natural systems has been equivocal. The ant system we propose to study can improve on the prior work because **A)** we can generate and replicate ant colonies across a range of sizes (5-3,000) that are consistent with natural settings, and **B)** we can observe both the networks of potentially infectious contacts as well as the network of realized transmission. In addition, we can identify whether there are social mechanisms that specifically limit the spread of deleterious elements by contrasting the scaling of transmission of beneficial elements (e.g. food) with that of parasites.

We propose to study the transmission of 7 transmissible agents that range from beneficial (food), to agnostic (beads), to virulent (fungi/virus/RNAi), see Figure 1. Further, we propose to study virulent elements that have a long evolutionary history with these ant species (fungi and viruses) and those that are novel (RNAi). If social immunity is a specific response to virulent transmissible elements that have been experienced over evolutionary time, then we would expect to see different transmission patterns for the novel elements (RNAi) for which the ants have no evolutionary history. We will test the following hypotheses:

a) The rate of transmission of all elements will increase, but saturate as colony size increases.
b) The rate of transmission of deleterious transmissible elements will increase slower with colony size than that of beneficial transmissible elements.
c) The rate of transmission of novel deleterious transmissible elements (RNAi) will increase faster with colony size than that of deleterious elements for which the ants have an evolutionary history.

Methodology: Initially for a colony with 1,000 workers we will expose foragers outside the nest to each of the 7 agents and measure what proportion of non-foragers (susceptibles) become 'infected'. We have built wooden nest areas (units of 63cm^3) that hold approximately 100 ants (Figure 1e). We link these together (in this case 10) and the whole nest is linked to a foraging area that is 4m away (we do this so we can determine who is a forager and who is not). All transmission agents will be introduced in the foraging arena. We will mark each ant with a number (printed on plastic and glued on). Colonies are set up and given 1 week to acclimatize and after that time we find 15-30% are active foragers (the majority of workers remain inside the nest [32-35]). We will expose the colony to the infectious agents (sugar and protein at the same time and for each colony; fungi, virus, beads and RNAi separately). Each colony will always have sugar/protein. Virus/beads/RNAi will be in the sugar and no alternative sugar given. Fungal exposure will be via spores immersed in oil inside the foraging arena for the generalist fungi (we have two species we use *Metarhizium brunneum* and *Beauveria bassiana*). The other fungus is the specialist mind manipulator, *Ophiocordyceps unilateralis* [36-38] which we inject into foragers [39, 40].

Once exposed we will check infection status after 6, 18 and 24 hours and then 2 and 5 days post-exposure by sampling 10% of non-foraging workers at each time point (because they are marked we will know if they are non-forgers). We know from extensive studies with sugar (stable carbon isotope ratios) and protein (labeled Nitrogen studies) the way these resources are transmitted through the colony [33, 41, 42], as well as studies using beads [7, 43] and our own studies on generalist fungi [39] that these are the suitable sampling points. We will check for infection using microscopy, mass spectrometry and PCR (details below).

This will provide our baseline data. We will then repeat the exposure and measure transmission across a range of colony sizes (5 Workers/1Q, 10W/1Q, 50W/1Q, 100W/1Q, 500W/1Q, 1000W/1Q, 3000W/1Q with brood in each nest, n=1/5th number of workers). This is with the black Carpenter ant *Camponotus pennsylvanicus*. For each size we will measure transmission 2 and 5 days post-exposure by sampling 10% of non-foraging workers at each time point. We will repeat this experiment 10 times so that we can record the variation between experiments. We have previously studied Fire ants, *Solenopsis invicta,* which have

multiple queens and we will use these if varying queen number suggests itself to be of interest.

Expected outcomes and significance: Understanding the scaling of transmission with population size and density is critical to being able to predict epidemic spread in novel settings and has been a major area of debate in the epidemiological modeling literature [22, 24]. Larger communities can lead to larger absolute numbers of infected individuals, decreasing the probability of stochastic fadeout of infection, as well as larger numbers of susceptible individuals for each individual to contact and thus potentially transmit (Figure 2). Constraints on the number of interactions with others per unit time may limit the number of potentially infectious contacts as populations grow large [44, 45]. Social insects present an interesting test-case for studying the scaling of transmission as they require mechanisms that facilitate the transmission of beneficial transmissible elements; i.e. food. Food collected by foraging ants must be transmitted inward to feed the queen, the workers who don't themselves forage (these non-foragers are the majority of all workers) and the larvae [33]. This implies that a network of contacts exists such that deleterious transmissible agents (parasites and toxins) might easily traverse this network. The social immunity hypothesis suggests, however, that behavioral mechanisms limit the transmission of deleterious agents [13].

The proposed experiments will be the first in disease ecology to study the scaling of transmission as a generic process (i.e. independent of pathogens) and link that transmission to the spread of both beneficial and deleterious elements.

Pitfalls: The sample sizes we decide upon and the number of replications may need to be revised and we can do that easily. Our preliminary data (below) shows how we can conduct large-scale experiments so that part will not be a limiting step. We will be mindful of the biological interactions and alter the design as needed

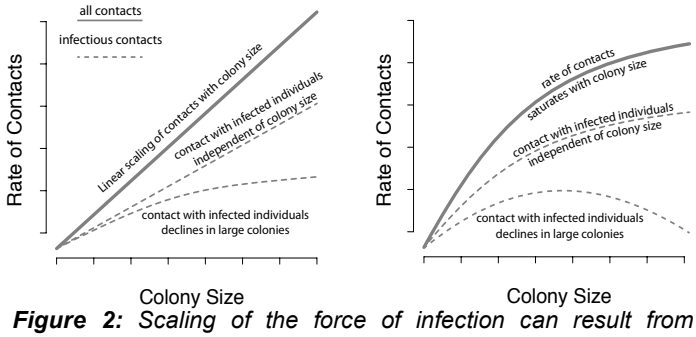

Figure 2: *Scaling of the force of infection can result from scaling of contact rate with population size, or the behavioral response to infected individuals that scales with population size*

-------Q2: Does heterogeneity within the colony drive transmission, and how does social and spatial variation contribute to this?-------

Rationale: A wide-angle view of transmission within a group is important to understand how community size drives transmission (Q1). But communities are collections of individuals and individual heterogeneity likely affects the rate at which both resources and pathogens travel. We know through some recent work on the important role of 'super-spreaders' that heterogeneity affects dynamics [3]. This heterogeneity is both in the contact rate of individuals as well as their pattern of spatial movement and the duration of time they spend with each contact. In social insects there is a strong heterogeneity [35, 46-48]. For example, only a small fraction of the colony leaves to forage [34]. This prompted an original experiment where exposure to small, inert beads, which were placed in sugar solution of different concentration created differences in contact rates between individuals (bees in this case) leading to marked differences in transmission of the 'pathogen' [7, 43].

Multiple recent technological advances have increased our ability to study group behavior in ants. The first is in digital cameras and data storage. We can (and the Hughes Lab does) record ant behavior in all nest and foraging areas, in complete darkness (under infra-red lights), 24 hours a day for months. The second advance is that we can use computer-tracking software to record millions of interactions (preliminary data below). Note that while a number of prominent publications in ants have shown the utility of such tracking

[46] it is our experience that it must be done with human observers because only then can we distinguish between three critically different interaction types: antennation (two ants touching each other with their antennae, Figure 1a) trophallaxis (exchange of liquids mouth to mouth, see Figure 1b), and lateral contact (two ants side by side, not involving antennation or trophallaxis, see Figure, 1c). (We discuss in the Outreach activities how our need for a large workforce is a great teaching tool, as it provides valuable opportunities for undergraduate students to participate in cutting-edge scientific research). Finally, advances in network and spatial analysis mean the very large datasets (thousands of individually labeled ants having millions of interactions recorded 24 hours day over months) can be analyzed. We propose to track in time and space thousand of ants to test the following hypotheses:

a) Contact rate is not uniform and some individuals are highly connected
b) Highly connected individuals occupy large spaces and move faster through groups
c) The social immune system triggers adaptive changes in network structure if diseases are introduced and contact rate and movement decrease to limit disease spread

Methodology: Using the same common black carpenter ant (*Camponotus pennsylvanicus*) we will use record individual behavior within the same sized nest chambers ($63cm^3$) using modified Go-Pro cameras (Hero 3 model) that have specially modified lenses (by www.RageCams.com) with increased sensitivity to infra-red (we find these are far superior to CMOS chips available on high-end Canon cameras). Each Go-Pro has a 128GB SD card that records 32 hours of continual data (we change them every 24hrs). Data is stored on 4TB disks that hold 20 days of continual data (24hrs/day). Each Go-Pro camera is positioned to record the entire nest ($63cm^3$) and each ant is uniquely identifiable at all times. This level of observation in their natural conditions of within wood, in complete darkness, has never before been achieved. We will link chambers together to form large colonies, again linked to an outside foraging arena (which also has a camera).

We will repeat the experiment above with the same range of colony sizes (5 Workers/1Q, 10W/1Q, 50W/1Q, 100W/1Q, 500W/1Q, 1000W/1Q, 3000W/1Q with brood in each nest, $n=1/5^{th}$ number of workers). We will measure behavior for 12 days. Because we will have recorded transmission the goal here is to measure if behavioral differences among workers are related to the rate of transmission. Also, we will ask if, following the introduction of pathogens does the behavior of both individuals and the group change. We will record the three behavioral measurements antennation, trophallaxis and lateral contact, Figure 1). We will also record the movement of each ant using a custom designed tracking program we developed that allows a human observer to rapidly track individuals but also to stop and record fine-scale observations when two individuals interact (see preliminary data below).

Expected Outcomes and Significance: We expect to measure every interaction for a complex society under different densities and disease exposure to answer the question of how heterogeneity (network and space) drives transmission. We will also ask how the adaptive flexibility of ant societies (their social immune system) changes upon exposure to a range of diseases of varying virulence and patterns of association. We expect that fungal spores of the generalist *Metarhizium brunneum* and *Beauveria bassiana* are recognizable and thus precipitate sudden shifts in the social network, but non-detectable killers like RNAi do not result in adaptive changes.

Pitfalls: The major challenge here is identifying which contact type is correlated to transmission of a given agent. The ability to measure the variety of contacts in addition to observing transmission will allow us to make the crucial link between contact and transmission. In addition, another challenge is accounting for variable sample sizes because not all ants are in the nest at the same time. This makes comparison of network metrics over time or between treatments difficult. One of the aims of the current proposal is to develop a method to detect statistically significant topological changes in contact networks. We outline

our approach in 3.4. The ability to model network changes over time and to measure the effect of changes on networks will significantly advance our understanding of disease transmission through social groups.

----------Q3: How does the environment affect transmission in groups?----------
Rationale: In Q1 and Q2 we address how group properties affect transmission. But the environment also plays an important role by presenting physical heterogeneity. For many social living species, variations in the built environment (e.g. burrows in prairie dogs, schools in humans) impact transmission. In addition to physical barriers that affect animal movement, the distribution of infectious particles is not uniform, with patches of disease being common. Such patchiness can lead to higher infection rates, or to the opportunity to avoid infection completely [49]. Ants provide an ideal experimental system where we can construct novel spaces and vary disease patches. We therefore wish to test the following hypotheses:

a) Increased architectural complexity reduces group size, contact rate and network heterogeneities, thereby reducing disease spread
b) Clumped patches of disease outside the nest results in reduced encounter rate and transmission

Methodology: Our basic nest design is a 4-chambered 63cm^3 which was constructed based on excavations of hundreds of ant colonies from trees in Pennsylvania (these ants mostly nest in dead wood). In nature these ants excavate chambers that are linked by tunnels. In the lab we can control the number of walls and rooms, while maintaining the same volume, as illustrated in Figure 1 d-e. From the work in Q1 and Q2 we will know the rate of transmission of different agents and the role of heterogeneity driving transmission. Building on from this we will ask how the three deigns (4 chambered, single chambered and galleries, Figure 1d-f) affects network dynamics, movement and, importantly, disease transmission. We will use colonies with 5 Workers/1Q, 10W/1Q, 50W/1Q, 100W/1Q in a single chamber (63cm^3). Using continual behavioral recording for 12 days, we will measure their contact rates, construct three social networks (antennation, trophallaxis and proximity) and measure their spatial arrangement. (See preliminary date below and Figure 3). We will then introduce the infectious agents and measure transmission (as in Q1).

We will also consider variation in the outside nest environment. Under natural conditions, in the forest, these ants maintain trails between the colony and food patches (notably aphids and other plant feeding bugs from which they collect honeydew). Some ants walk alone, off trails, collecting protein that is patchily distributed. In previous work our infectious agents were beside the food (fungal spores) or within the food (beads, virus, RNAi). What we want to do is test how heterogeneity in the location of disease affects transmission. We will only use the generalist fungi *Metarhizium brunneum* and *Beauveria bassiana* because we can exactly control exposure levels outside the nest. Spores are formulated in a mixture of mineral oils (20% Ondina: 80% Isopar M) and the concentration counted with an Improved Neubauer Hemocytometer. The volume of each formulation was then adjusted to a final concentration of 1 x 10^9 spores ml^{-1}. Each formulation will be sprayed using an artist's airbrush sprayer [50]. Briefly, each formulation is sprayed onto 100mm circles of copy paper in a biological hood at a rate of 20ml/m^2. Treated substrates are left to dry in fume hood overnight. We then place these outside the nest. For this work we will keep ants in 20m^2 benches in greenhouses at Penn State. Ants forage from a central nest to food placed across the benches. The paper with spores are then placed either near or far from the food source. We will quantify exposure by sampling foragers just before they enter the nest and quantifying spore load using qPCR [51]. Since we know that secondary infections can occur as foragers touch within nest ants, we will measure mortality. We will test this for colonies with 5 Workers/1Q, 10W/1Q, 50W/1Q, 100W/1Q, 500W/1Q, 1000W/1Q, 3000W/1Q with brood in each nest, n=1/5th number of workers. Each colony will be exposed for 48

hours (which we know is sufficient time for transfer). We will repeat the experiment 5 times per colony size.

Expected Outcomes and Significance: We hope to experimentally alter conditions such as walls and disease patches, to ask how they impact the spread of disease. This is not possible in any other group living species, but we can do this with ants repeatedly and across many different permutations.

Pitfalls: We have already experimented with wall removal and the effect that it has on the social network (Figure 3), so we are confident this is possible. Although we do have data on spore exposure and decline over time (below) it is possible in our large arenas ($20m^2$) ants just effectively avoid patches. Many insects can detect and avoid fungal spores [49]. If this happens we will search for spores that ants cannot recognize (e.g. a non-pathogenic *Trichoderma*) or we can use inert markers like talc powder or sticky beads.

------- Q4: Can models and high-resolution data motivate knockout experiments?---------

Rationale: We will model the spread of infection through a society based on continuous data recording across three orders of magnitude, with thousands of ants and millions of observations. In addition to studying scaling we will leverage continuous behavioral recording to identify if superspreaders occur and what role they play. Finally, we will experimentally modify the internal and external environment. This sets up an exciting opportunity to perturb complex systems.

In our final aim we wish to ask if the insights we gained from state of the art modeling of our experimental data allow us to predictably perturb a complex system leading to its collapse. In studies of many complex systems (such as genes in a critical pathway or neurons in the brain) the integration of massive data and complex models have allowed researchers to validate the importance of key parts through knockout experiments, leading to the loss of function. Our question therefore is if societies can be understood sufficiently well that certain components can be identified as important and knocked out. Of course, in single queen societies it is already known that the key individual is the queen, but it is not known which workers can be removed without the system collapsing, and which ones are so vital that the system collapses if removed.

Methodology. We will integrate compartmental, network and spatially-explicit movement models with the data collected in Questions 1-3 to identify what components of the system (which workers) can be removed leading to collapse. We will then remove them and test the resulting effect on the transfer of resources, the network patterns and the transmission of diseases. We cannot at this stage suggest sample sizes since that information is likely to arise from what we learn when the diverse modeling approaches are used together.

Expected Outcomes and Significance: This is an exciting aim because it integrates across modeling approaches and uses the extensive data provided in Q1-3 to ask if complex societies, like gene networks or neuronal arrangements, can be understood sufficiently well that they can be predictably perturbed. We know from extensive data of human [52], wildlife [53] and pollinator systems [54] that complex societies can collapse. Understanding why societies collapse is a critical question in the study of infectious diseases.

Pitfalls: This is our last aim and designed to maximize the integration of the three distinct modeling approaches with the behavioral and experimental observations. As such, there are many more pitfalls here than in Q1-3, but the unprecedented scope of the continuous behavioral observations we can obtain on ant systems provides a unique opportunity to integrate multiple modeling approaches to identify key individuals and mechanisms for disease spread and social immunity. Results from multiple approaches could provide conflicting results, but this is true in any complex system, and only helps us better understand the complexities of disease transmission. If results between models are equivocal or contradictory, this itself is informative and would no doubt stimulate further work.

3.0 Team, our approach & preliminary data with ant behavior and model fitting

3.1 A model system for transmission requires diverse expertise

We are a multi-disciplinary team with extensive and diverse knowledge of behavioral, computational, mathematical, and statistical ecology. Three of us work in the same building as members of the Center for Infectious Disease Dynamics (CIDD). Bansal used to work at CIDD before joining Georgetown University, a mere 3.5 hours drive away.

Hughes: The PI, Hughes, is a behavioral ecologist who has studied social insects and their diseases in 11 countries on 5 continents. He has worked with diverse diseases as well as the behavior of healthy and infected ants under field (rain- and temperate forests) and laboratory conditions. He will oversee all of the experiments and behavioral studies and integrate the three modeling co-PIs with the respective projects of students and post-docs.

Matt Ferrari: Co-PI Ferrari is a computational epidemiologist and statistician who has worked extensively on the analysis of time-series surveillance data to predict epidemic dynamics and evaluate management interventions. In this project, Ferrari will oversee the analytical and modeling components of student and post-doc projects relating to Q1, the scaling of transmission with colony size.

Shweta Bansal: Co-PI Bansal is a network epidemiologist and has worked extensively on the effects of immunity on network structure and disease dynamics. She is studying infectious disease-related network structure in several wildlife populations including Australian bottlenose dolphins and Mojave Desert tortoises. In this project she will oversee the components of student and post-doc projects relating to network analysis and SIR models in networks in Q2/Q3.

Ephraim Hanks: Co-PI Hanks is a spatial statistician and has worked extensively in the modeling of animal movement and connectivity. He has studied the spatial spread of disease in black spruce and mule deer, and the spatial properties of random walk models on networks. In this project he will oversee the components of student and post-doc projects relating to movement and spatial analysis in Q2/Q3.

Each of us will integrate to work on Q4 (how societies collapse).

3.2 Additional transmission agents

The purpose of our proposal is to measure transmission in a dense society that we can experimentally perturb and ask what models explain transmission. Because our system is experimental and undergoes adaptive responses when challenged with disease (social immunity) we can get at the question of what socio-ecological factors drive transmission. We will use seven different transmissions agents ranging from good (sugar/protein) to agnostic (beads) to deadly fungal/viral/RNAi (Figure 1a). The deadly agents differ in how they transmit and the extent to which ants have a co-evolutionary history and thus are expected to prevent their transmission. All agents are being studied in the Hughes lab. Measuring sugar/protein flow in ant societies is routine, has been done for decades and capitalizes on the ability to measure carbon isotope ratios and labeled nitrogen [42]. Using inert beads in social insects as models of disease spread was first pioneered by Dhruba Naug [43] and has also been used in ants [55]. The Hughes Lab uses the co-evolved fungus, *Ophiocordyceps unilateralis* [36, 37, 56-59] and have developed an artificial infection system [40]. We have also used the generalist fungal pathogen *Metarhizium brunneum* and *Beauveria bassiana* [39] and this is very commonly used in social immunity studies [15, 60, 61]. We do not need to develop any new techniques to study these agents.

We recently adopted a virus model, Acute Paralysis Virus *Burenella dimorpha,* which normally infects bees [62] but we have found that providing it in sugar water to ants not only kills ants but it can be transmitted to other ants in the same nest (Gracia et al, unpublished).

Finally, through a Gates Foundation funded project using RNAi to kill queens and worker ants the Hughes Lab has extensive experience working with RNAi that affected the Vitellogenin Receptor gene in ants [63] (sterilizing queens) and another RNAi construct which has been developed [64] which targeted the PBAN/Pyrokinin gene *via* feeding dsRNA

to workers and larvae. PBAN/Pyrokinin belongs to the pheromone-biosynthesis-activating neuropeptide (PBAN)/pyrokinin gene family, a family of neurohoromones. Not only can we provide ants with each of these different agents, we can also measure them as they pass (or don't pass) from ant to ant. The fungi, beads, RNAi are all measured with florescence microscopy allowing for accurate quantification. The fungi, virus, RNAi can also be measured with qPCR (which we have also done). The sugar and protein sources can be traced using both labeled carbohydrate and protein sources and this has been a standard approach for decades.

3.3 Scaling of transmission with population size

In Q1 we measure transmission of different agents across 3 orders of magnitude in group size. Following from the formulation of Begon et al [22], the force of infection felt by each susceptible can be characterized as $F=cpv$, where c is the rate of contacts, p is the probability that a contact is with an infectious host, and v is the probability that contact between an infectious and susceptible host leads to transmission. Much of the classic discussion in the literature regarding the frequency and density dependent scaling continuum has focused on how this aggregate force of infection scales with either population size or density [22, 24]. The classic mean field models often assume p to be the prevalence of infection I/N, and the scaling of transmission is determined by the relationship between the contact rate c and population size or density. Here, because of the tractability of the ant system, we can explicitly study the scaling of all three components of the force of infection with colony size. We can quantify both how the rate of contacts scales with colony size as well as whether the rate of contacts with infected individuals scales linearly (as is classically assumed) with the prevalence of infection; preliminary analyses by Ephraim Hanks (co-PI, Figure 4) indicate that infected individuals are not randomly mixed in the colony, which would indicate a non-linear scaling for p. Thus, saturating scaling relationships (i.e. on the frequency-dependent side of the scaling continuum) could result either from sub-linear scaling of contacts with population size, or from linear scaling of contacts and reduced probability of contact with infectious individuals in larger colonies due to emergent social behaviors (Figure 2). The combination of experimental manipulation over a large range of colony sizes and the ability to quantify both contact and infection events will allow us to disentangle these processes in a way not possible in most systems.

3.4 Within-colony social and spatial network dynamics

From the data to be collected in Q1-3, contact networks can be defined, with nodes representing individual ants, and edges representing each of the interaction types listed above (trophallaxis, antennation and lateral contact, Figure 1). We will build separate networks based on antennation, trophallaxis and proximity (Figure 3). Network edges will also encapsulate further attributes of the interaction such as interaction distance, duration, or direction as edge weights, allowing us to disentangle the drivers of these interaction processes (e.g. thickened arrows, Figure 3 showing the duration of interactions). These models will allow us to characterize the structural organization of contact between ants. As outlined in Q2 above we will then conduct experiments with transmission agents, and measure contact network structure with the spreading agent of uninfected and infected individuals. Co-PI Bansal has experience inferring such contact networks from data in empirical systems ranging from cattle to bottlenose dolphins. We also aim to integrate the network methodology with the spatial methodology (Section 3.6) to consider how space drives contact dynamics by studying if dynamic movement models (below) can predict contacts, and how this varies by contact type. These data and models will then provide us with the unique opportunity to compare the observed disease network dynamics to model-based disease predictions (on the baseline network). We will use a stochastic SIR framework to model infection dynamics on the baseline network of each contact type and compare this

to the spreading dynamics of the various transmission agents, assuming agent-specific parameters for transmission rate, β, and recovery rate, γ.

Preliminary data: We placed 75 worker ants (*C. pennsylvanicus*) with one queen and brood in a 4-chambered nest with a total area = 63 cm^2. In Figure 3a we present a static ant contact network recorded from one night (visualized using Gephi). In Figure 3b we present the average degree centrality of the ants, averaged over 8 consecutive nights of observation. In a surprising result that seems counter-productive to social immunity, the active foragers who have come into the nest from outside where disease is prevalent have significantly more connections than do nest worker ants who never foraged (Figure 3b, $p<0.01$ ANOVA, post-hoc Tukey). This result was not evident from examining the static network (Figure 3a).

A key mathematical challenge, therefore, and one we will address in this proposal is how to compare networks at different time points rigorously, and detect changes to the network. This will be particularly necessary to characterize social immunity (i.e. how the colony adaptively responds to diseases, reducing their transmission), as our preliminary data suggest that static networks alone will not be sufficient. Change detection in networks is a problem that has been considered [65, 66] but not robustly developed in network science. Developing a method to detect statistically significant topological changes in contact networks will allow us to: a) identify mechanistically the network changes related to "social immunity" and b) identify when these changes may occur after the introduction of a pathogen. For a change detection method, we propose adapting and further developing a novel class of latent process models for time-dependent network data [67]. In this formulation, a network is observed over time, with subgroups of nodes exhibiting a change in behavior at some (unknown) time points. These changes may take the form of a change in the overall probability of connections within or between subgroups, or a change in the distribution of edge attributes. The probability of edges with various attributes at a given time is then modeled using a latent-space stochastic process associated with each node (i.e. individual) such that the probability of nodes *i* and *j* having contact at time *t* is $P(G(i,j,t)=1)=X_i(t)\,X_j(t)$, where $X_i(t)$ is the K-dimensional latent position of node. These state of the art latent space models allow for the rigorous comparison of networks by identifying change points at which there is an identifiable shift between homogeneity and heterogeneity (and vice versa) in the network. While the methodology incorporates changing edges as well as edge data (e.g. edge weights), we must extend the current methodology to allow for networks of changing size (i.e. changing number of nodes, as is observed in the laboratory ant data) as well as to allow node-level attributes. The current framework makes this feasible.

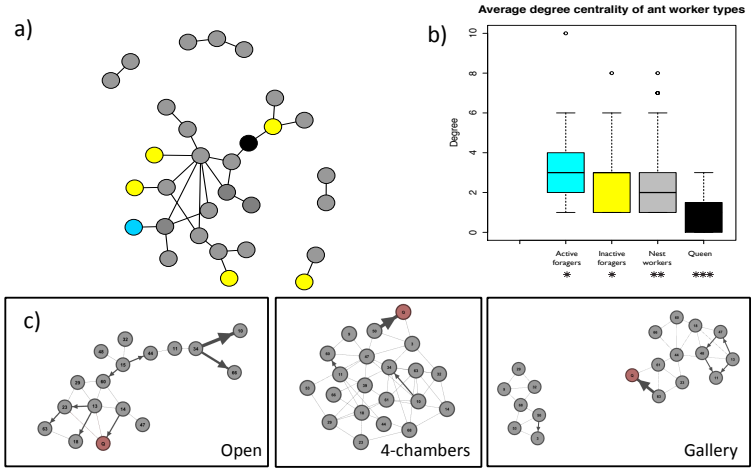

***Figure 3**: Ant networks. A) Static network (20min, 1 evening) with Queen (Black), active forager (blue), inactive (i.e. previously foraged but not during this observation, yellow), and nest workers (grey, never foraged). B) Average degree centrality for 4 groups, over 8 evenings. C) Network within 3 architecturally distinct nests* (Queen=red).

Co-PI Bansal has developed a mechanistic framework in the past to model the structural changes in a contact network, specifically due to immunity [68]. However, the proposed statistical methodology will produce a flexible and general change detection framework, which will also be valuable for questions of Part 2 to compare changes in

network structure and transmission when we perturb the system, such as changing the number of walls within the nest or location of disease patches outside (Q3). To demonstrate how we can change the walls and see different networks, we generated preliminary data (see discussed below section 3.7 and Figure 3c). This preliminary experiment indicates clearly that space influences social networks, and changing spatial environments will lead to changes in disease transmission.

3.5 Point Process Models of Spatial Segregation and Social Immunity

In our experiments we will expose individual ants to disease agents that will likely affect their behavior. We expect to see negative effects, such as mortality and leaving the nest, as well as positive effects such as uninfected individuals avoiding either sources of infection in the environment or infected siblings (who even if not infectious are draining the colony's resources). To illustrate our approach to individual behavior and to outline the modeling directions that we consider fruitful, we generated preliminary data.

Preliminary data: We infected ants with the behaviorally manipulating parasite, *Ohiocordyceps unilateralis,* which infects workers when foraging, grows inside their body and then manipulates them to die outside the nest [37]. An infected ant inside the nest is not infectious but does take resources from the colony, so it is a parasite at the colony level. We followed the individual behavior of 10 ants infected with the fungus, *Ophiocordyceps,* 10 that were sham treated and 10 that were untouched controls. We monitored the behaviors of each of these 30 ants for 24 hours/day for 18 days (using Go-Pro cameras, mentioned above). We measured the total number of food exchanges between individually identified ants (with exact identity). So far we have determined that infected ants do receive at normal rates (in prep.). They are not apparently recognized.

Perhaps the change is subtle; we set out to ask if infected individuals occupy the same or different space as controls. To do this we used computer-tracking software we developed, together with many undergraduate students (see Outreach) to measure the distance (in Euclidean space) between ants in each treatment (infected, sham, control) and all other ants in the colony. As an exploratory analysis, we have analyzed a subset of the available data by comparing the difference between the pairwise distances from healthy ants to healthy ants, and the corresponding pairwise distances from healthy ants to infected ants. The K-function [69] is a summary statistic for spatial point data that captures the expected number of individuals within a certain radius of an individual. The difference between the K-function from healthy to healthy ants and the K-function from healthy to infected ants (Figure 4a,b) indicates that there may be some spatial segregation, as infected ants are more likely to be found farther from healthy ants than are other healthy ants.

We will test this apparent spatial segregation and seek to understand the mechanisms driving it. We will employ spatial point process models known as Gibbs processes with pairwise interactions and model the likelihood of an ant being found in a

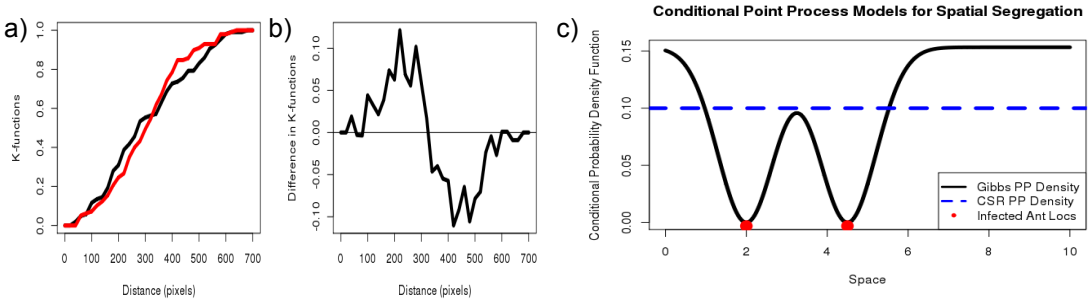

Figure 4: *(a) K-cross functions from healthy ants to other healthy ants (black) and to infected ants (red). (b) Difference in these K-functions, indicating that infected ants may be more likely to be found farther from healthy ants than other healthy ants. (c) Point process likelihoods for testing for spatial segregation. Message: we can test for evidence of spatial segregation as a mechanism in ants for social immunity.*

particular location conditionally on the locations of all other nearby ants. We will parameterize the conditional point process intensity function to allow for infected ants to potentially repulse or attract healthy ants and fit these Gibbs spatial point process models (Figure 4c) using Markov chain Monte Carlo methods. Kolmogorov-Smirnov permutation tests will be used to test for significant repulsion or attraction versus the null completely spatial random (CSR) point process model.

3.6 Models of Movement Behavior and Interaction

The preliminary point process analysis described above suggests that ants may use spatial segregation when in close proximity to infected individuals as a mechanism to induce social immunity. In fact, spatial segregation between foragers, non-foraging ants, and the queen may occur at multiple spatial scales, and across time as ants potentially use nest space asynchronously. Asynchronous use of space and spatial segregation are not behaviors unique to ants, but are common in humans and other group living species. Analyzing ant behavior will provide novel understanding of the ant system, and potential mechanisms for social immunity across species. We collected preliminary data on ant movement and space use behavior, and have used this data to motivate movement models that account for disproportional use of nest space by different classes of ants, changing behavior over time, and interactions with other ants.

Preliminary data: Using the same experimental set up described above (75 ants in 4-chambered nest with a total area = 63 cm^2) the inside-nest interactions and spatial locations were video-recorded for 8 consecutive nights (Figure 5). The spatial location (resolution of 1cm^2) of 5 focal foragers, 5 focal nest workers, and the queen was recorded for 20 minutes per night for each of the 8 nights (11 ants x 20 minutes x 8 nights = 1,760 minutes of observation). We considered discrete space (gridded) models for ant movement and space use [70, 71]. The observations were continuous in time, and we thus consider continuous-time, discrete space (CTDS) approaches [71, 72] to modeling space use and movement behavior. Under this approach, the i-th ant's movement path is a sequence of spatial grid cells $g = (G_1, G_2, \dots, G_T)$ together with the ant's residence times $\tau = (\tau_1, \tau_2, \dots, \tau_T)$ in each grid cell. Under a continuous-time Markov random walk model for movement, the likelihood of remaining in the spatial grid cell G_i for τ minutes and then transitioning to grid cell G_k is $\alpha_{ik} exp\{-\tau\alpha_i\}$, where α_{ik} is the average rate of transition from the i -th to the j -th cell and α_i is the total rate of transition from the i -th cell to any adjacent cell. The transition rates: α_{ik} can be parameterized to test varied hypotheses about ant movement behavior and the resulting contact networks which are central to our modeling approach of the spread of infectious disease through the colony.

We will consider three distinct approaches to modeling movement behavior. (1) The first approach models movement behavior that is *static in time* by specifying the transition rates α_{ik} between cells as a function of nest location (the *i*-th and *k*-th grid cells) and class of ant (e.g., forager). After fitting this model to the continuous ant observations, the stationary distribution of the random walk model for each class of ant reveals long-run average use of space within the nest (Figure 5a-c). (2) We will then model movement behavior that is *dynamic in time* by allowing each class of ants to have transition rates $\alpha_{ik}(t)$ that vary over time. This will allow us to examine whether non-uniform use of nest space over time by different ants could be a mechanism for social immunity. (3) Finally, we will consider movement behavior that is *dynamic in time with pairwise interactions*. Motivated by our

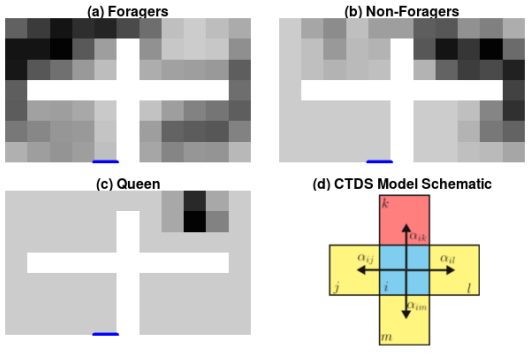

Figure 5: *(a)-(c) Heatmaps of space used by different classes of ants in our preliminary data. (d) Schematic of the CTDS modeling approach.*

preliminary analysis of spatial point process models of ant locations (Figure 4), we will consider models of movement behavior that are conditional on the locations of all other ants in the nest. Recent work by Co-PI Hanks [71, 73] has made modeling such movement behavior tractable. Movement models that are *dynamic in time* often use an approach based on potential functions which specify movement gradients at points in space [73-75]. Hanks et al. [71] integrate potential function approaches with movement models that allow for *pairwise interactions* by considering each ant as a potential attracting or repelling point. Inference on movement parameters $\alpha_{ik}(t)$ can then be made within a computationally-efficient Poisson GLM framework, allowing for inference on group behavior for huge datasets (millions of data points as we generate here). As we will be considering multiple potential drivers of ant movement behavior, we will use a LASSO approach to regularization that allows for selection of important drivers of movement and reduces the effects of potential multicollinearity between hypothesized movement mechanisms. We will employ a Bayesian approach to fit the CTDS movement model with LASSO penalty [76], and will test for significant differences in movement behavior using parametric bootstrap permutation tests. Pairing this state-of-the-art approach to modeling movement behavior and social interactions with the wealth of data to be collected as part of this project provides a singular opportunity to understand how fine-scale movement behavior contributes to social immunity.

3.7 Experimental changing the environment

While much could be learned about social contact networks, asynchronous use of space and interactions solely from the continuous observations of the ant system, modeling these behaviors allows for a principled approach to prediction of ant behavior under modification. We can thus use simulations and model-based predictions of ant behavior to generate hypotheses about the effects of different modifications and then verify (or falsify) these hypotheses by conducting the modifications in the lab. We have conducted some preliminary experiments in which we altered the internal environment by removing walls and changing the exposure to pathogens outside the nest.

Preliminary Data: We housed 24 workers ants (plus 1 Queen, plus brood) in one of three 63cm^3 chambers that had either a) 4 chambers, b) one chamber or c) 4 galleries (Figure 1). We recorded the individual behavior of each ant to build a social network (Figure 3c). Although we measured behavior for just for one evening (and in the real study it would for 12 days) it is already clear that changing the structure of the nest has a strong effect on the network dynamics.

To test environmental heterogeneity on exposure rate, we exposed 15 worker ants to spores of three fungal strains. We then measured the number of spores on the ant right after exposure and 12 hours later. To count the spores we placed ants in 15ml centrifuge tubes to which 3.0 ml of dichloromethane (DCM) was added and vortexed for 30s. This wash step was repeated once and the rinsate collected into clean tubes. An equal volume of ethanol (99.99% pure) was added to each tube to aid in the precipitation of spores. Tubes were then centrifuged at 4,000 rpm for 30 minutes. The supernatant was immediately removed and the pellets were allowed to dry in a biological hood at room temperature (~ 25°C) for 48 hours. Pelleted spores were then resuspended in 0.05% Tween solution and the concentration of spores was counted with a Hemocytometer. Ants picked up similar numbers of spores, regardless of isolate ($F_{1,16}$ = 0.839, p = 0.373). However, time significantly predicted the number of spores retained by ants ($F_{1,16}$ = 18.48, p = 0.001) as a significant loss of spores occurred for each isolate just 12 hours after exposure, but spore loss was consistent between ants regardless of isolate ($F_{1,16}$ = 1.369, p = 0.259).

4.0 Significance and originality

Formally connecting studies of individual contact patterns with the emergent properties of disease transmission have been limited by logistical constraints. Often we are limited to studying either the contacts of a limited subset of a population (ignoring contacts with

unmeasured individuals), some proxy for the relevant contacts that is easier to measure (i.e. online social networks or friendship networks), or we are limited only to the realized transmission network of some pathogen in the absence of the full network of potential paths. Here we propose a model system that will allow both formal quantification of contacts and transmission simultaneously. As a consequence, this proposed work will be the most comprehensive study of transmission as an emergent property of societies. Our unique approach to study the transmission of elements that range from beneficial to virulent will allow us to first establish baseline patterns for how the process of transmission scales as a function of colony size and extrinsic conditions (i.e. physical structure) and then quantify the change in these emergent, population-level patterns as a consequence of the costs imposed by parasitism. By studying both the beneficial and deleterious consequences of transmission in societies, we expect to shed new light on the role of infectious processes in structuring societies and novel insights into how to manipulate the process of transmission to manage populations.

5.0 Broader Impacts and potential societal gains

There are number of ways our project may impact the public. The first is by providing insights into the spread of disease in groups gained from experimental worked merged with multiple modeling approaches. Some of our proposed experiments have relevance for controlling experiments in high-density human settings such as schools and workplaces. By removing walls and charting the changes in both social networks and transmission our work could provide important insights into how building and city design influence disease transmission in humans. Also, within the human arena our focus on complex systems and how they remain stable has potential for a number of areas from telecommunications to the web [77]. Ants have long been used as models to understand the adaptive behavior of complex systems [78, 79]. Finally, ants are themselves pests in both farms and cities. One species alone, the Red Imported Fire ant, costs the US economy $6bn annually and ants are serious pests on small-holder farms across the world, notably in Africa where the spread fungal pathogens and increase viral load in plants as they protect and transport plant feeding bugs that vector such diseases. Insights into how ant societies maximize the transport of food but reduce the breakout of disease could be very important in designing effective control strategies. Indeed, through support from the Gates Foundation, the PI is currently working on a project in which RNAi is used to kill queens in pest ants in Cassava fields in Africa.

Education: We enjoy science dissemination and constantly seek ways to innovate. The PI's work on zombie ants has gained a lot of media attention and has been featured in CNN, New York Times, MSNBC, BBC, National Geographic, Discovery, WIRED and a wide range of blogs and other media in various languages. One approach he adopts is making videos that accompany papers [37] which combines narration, figures from the papers, and interviews carried out in the field to bring the excitement of the research findings out to the public. At the time of writing (Nov 2013) one of these videos has been viewed over 380,000 times on YouTube (http://tinyurl.com/7j2gp4h). Hughes has also participated in 4 feature length documentaries in 2013 (BBC/CBC/Discovery/Through the Wormhole) which will/have reach(ed) millions of viewers in the US, Canada and the UK as well as through the web. He also made two documentaries for SONY PlayStation on zoonotic diseases and Paramount Pictures on collective behavior (for Last of Us Game and WWZ, which he consulted on). For this proposal we want to go beyond telegraphing the cool biology of ants and parasites and instead use novel approaches to teach diverse audiences about math, biology and diseases.

K-12/University/MOOC/Public: For different ages and abilities we will make available ant videos on the web, instructions for recording behavior, spread sheets, R-packages and easy to run network building tools (using an open source network tool, Gephi). This can be very simple for K-6 and more complicated for older students. The named PhD student, Lauren Quevillon, is a former STEM High School teacher and perfectly knows the content teachers require, or knows how to discuss their needs with them (in ways a PI might not). Our goal is

to enable individuals and whole classes to study ant behavior as part of their lessons, measure behavior and construct a social network. We have already tested this in October 2013 in Hughes' *Sociobiology and Infectious Disease* 400 level class with 100 undergraduates at Penn State. The students observed data (each student observing two ants). This data was inputted and a network graph created. We aim to do this for 100/ undergraduates/year to involve 500 undergraduates directly in research over the course of the grant. For an additional 500 *freshman* undergraduates, we will take part in a new scheme at Penn State to involve the incoming class in research. Using teaching labs with plenty of space, students will work in groups of 5 to record and analyze behavior over the course of a semester, alongside graduate students and post-docs. Currently the PI has 12 undergraduates working on this very time-consuming data recording so we already know what is required. We have found that students greatly enjoy tracking ants and learning about the models, software and biology behind this work (we provide lots of tutoring).

For each group (K-12, University students) we will also provide information and links to on-going network studies (e.g. Framingham Heart study [80]) so teachers can place this in a wider context. Once students have the data, we will allow them to virtually control their generated networks and introduce outbreaks. This will be accomplished using Vax which is an exciting new open-source tool developed by researchers at Penn State http://vax.herokuapp.com/, to accompany our massive, open, online Epidemic Course https://www.coursera.org/course/epidemics) that eight faculty at Penn State are teaching (Fall 2013). In its current form, Vax allows users to prevent an outbreak by using vaccines and facilitates learning about epidemics and immunity. We will extend Vax to include social immunity. What we therefore propose is an integrated teaching tool from videos to interactive games where students of all ages can learn about social immunity, ants, networks and mathematical biology.

Ask Us Anything: One very successful feature of the MOOC: Epidemics course has been the "Ask Us Anything" sessions: weekly videos where MOOC participants sent in questions which the faculty answered (e.g. http://tinyurl.com/llo8hzn). This was a very well received format and a great way for a very diverse audience around the web to engage with experts in the field. We wish to do that here for our grant. We will have a special page on www.HughesLab.com where people can view the videos and lessons we will produce as part of this project and can ask questions. Every month during the entire grant the 4 PI's and post-docs/graduate students will gather to discuss the topics of our grant and answer questions. We can do this with Bansal at Georgetown (e.g. through Google Hangout). This is a minimal amount or work but such videos are really helpful to the community of learners.

6.0 Previous support

Hughes/Hanks: none **Ferrari:** NSF-NIH EEID R01 GM105247-01, Linking models and policy: Using active adaptive management for optimal control of disease outbreaks, 2012-16. This project seeks to develop methods to integrate real-time model projections and surveillance with optimal control for disease outbreaks; specifically foot-and-mouth disease. NSF-DEB 1145697 (Isabella Cattadori, PI) Host tolerance and resistance: applying ecological concepts to the dynamics of parasite-host infections. 2012-15. This project is to study the relative contribution of immune-mediated control and competitive interactions in driving selection for life-history traits in helminth co-infections. **Bansal:** NSF EEID DEB-1216054: Invasion and Infection: Translocation and Transmission: An Experimental Study with Mycoplasma in Desert Tortoises, 2012-17. This project aims to predict the spread of infection through a population using a population of resident and translocated desert tortoises and respiratory pneumonia. This project was funded one year ago and in that time, we have carried out field experiments, developed a theoretical framework to consider the impact of metapopulation structure in contact networks, and written a review of the impact of wildlife translocation on epidemiological threats, with the desert tortoise as a case study.