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Host contact and shedding patterns clarify variation in pathogen exposure and transmission in threatened tortoise *Gopherus agassizii*: implications for disease modelling and management

Christina M. Aiello[1,2]*, Kenneth E. Nussear[3], Todd C. Esque[2], Patrick G. Emblidge[1], Pratha Sah[4], Shweta Bansal[4,5] and Peter J. Hudson[1]

[1]*Department of Biology, Pennsylvania State University, University Park, PA, USA;* [2]*U.S. Geological Survey, Western Ecological Research Center, Las Vegas Field Station, 160 N. Stephanie St., Henderson, NV 89074, USA;* [3]*Department of Geography, University of Nevada Reno, Reno, NV, USA;* [4]*Department of Biology, Georgetown University, Washington, DC, USA; and* [5]*Fogarty International Center, National Institutes of Health, Bethesda, MD, USA*

Summary

1. Most directly transmitted infections require some form of close contact between infectious and susceptible hosts to spread. Often disease models assume contacts are equal and use mean field estimates of transmission probability for all interactions with infectious hosts.

2. Such methods may inaccurately describe transmission when interactions differ substantially in their ability to cause infection. Understanding this variation in transmission risk may be critical to properly model and manage some infectious diseases. In this study, we investigate how varying exposure and transmission may be key to understanding disease dynamics in the threatened desert tortoise *Gopherus agassizii*.

3. We created heterogeneity in *Mycoplasma agassizii* exposure (the putative bacterial agent of a respiratory disease) by varying the duration of interactions between naturally infected and uninfected captive desert tortoises. Using qPCR, we identified new infections and compared models of transmission probability as a function of contact duration and pathogen load. We then examined the contact patterns of a wild tortoise population using proximity loggers to identify heterogeneity in contact duration.

4. The top-ranked model predicting *M. agassizii* transmission included a dose term defined as the product of the number of days in proximity to an infected host and the infection level of that host. Models predicted low transmission probability for short interactions, unless the infectious host had a high load of *M. agassizii*: such hosts were predicted to transmit infection at higher rates with any amount of contact. We observed predominantly short-lived interactions in a free-ranging tortoise population and thus, expect transmission patterns in this population to vary considerably with the frequency and duration of high infection levels.

5. Mean field models may misrepresent natural transmission patterns in this and other populations depending on the distribution of high-risk contact and shedding events. Rapid outbreaks in generally solitary species may result from changes to their naturally low-risk contact patterns or due to increases in the frequency of severe infections or super-shedding events – population characteristics that should be further investigated to develop effective management strategies.

Key-words: contact heterogeneity, detecting infection, infectiousness, per-contact transmission probability, social behaviour, upper respiratory tract disease, wildlife disease ecology

*Correspondence author. E-mail: caiello@usgs.gov

Introduction

Infectious diseases play a significant role in the ecology and conservation of wildlife, but deciding how to model pathogen transmission and its consequences effectively can be challenging (Deem, Karesh & Weisman 2001; Hudson *et al.* 2002). Many microparasites depend on close contact between their hosts to spread, which can vary in frequency, type and duration (Bansal, Grenfell & Meyers 2007; Clay *et al.* 2009; Madden *et al.* 2009). Certain behaviours between hosts or a greater period of interaction time may result in more effective pathogen transmission (Vittinghoff *et al.* 1999; Drewe 2010). Similarly, the infectiousness of the transmitting host can vary, and certain individuals may cause new infections more frequently with each contact (Matthews *et al.* 2006; Stein 2011). Modelling transmission without considering this variability can produce inaccurate predictions (often overestimation of transmission rates) if some hosts or contacts play lesser/greater roles in pathogen transmission (Keeling & Grenfell 2000).

In epidemiological models, many complexities of pathogen transmission are encompassed in the effective contact rate, β – the product of population contact rate and per-contact infection risk (Anderson & May 1991). Using empirical data from the host-pathogen system of interest to estimate model parameters such as β can produce predictions that better reflect natural disease patterns (Hosseini, Dhondt & Dobson 2004; Morgan *et al.* 2006; Tompkins *et al.* 2011). While a number of recent studies have focused on estimating heterogeneity in host contact rate and its effect on transmission patterns, few studies explore heterogeneity in the second component of β: per-contact transmission risk. Combining data on contact patterns in wild populations with study-based estimates of per-contact transmission risk will likely improve our understanding of transmission in natural systems and our ability to predict the risk of rapid outbreaks. We used this approach to investigate the effect of contact variation on transmission of an infectious pathogen (*Mycoplasma agassizii*) that infects the desert tortoise (*Gopherus agassizii*): a threatened species and focus of concerted conservation effort in the Mojave Desert of the southwestern United States.

HOST-PATHOGEN SYSTEM

The Mojave population of *G. agassizii* received threatened status following range-wide declines attributed to threats such as habitat loss and degradation, drought and disease (USFWS 1990). Extreme local mortality events attributed to an upper respiratory tract disease (URTD) prompted research that identified *M. agassizii* as an important causal organism (reviewed in Jacobson *et al.* 2014). URTD appears similar to other mycoplasmal infections: following colonization the host develops clinical disease in association with antibody production, reducing the pathogen load but progressing into chronic disease with intermittent clinical signs. Disease-caused mortality seems generally low, but may increase with environmental stress or at later stages of infection (Brown *et al.* 2002). Little detailed data exist on URTD patterns in wild tortoise populations apart from a few seroprevalence surveys, leading managers to wonder how to best prevent future severe outbreaks (Lederle *et al.* 1997; Sandmeier *et al.* 2013).

URTD TRANSMISSION AND INFECTION DYNAMICS

Understanding transmission may be key to managing this disease, but most data for this pathogen come from direct inoculations with few studies of contact-caused transmission (Brown *et al.* 1994). When inoculated intranasally, transmission occurs readily (even at low doses) in *G. agassizii* and *G. polyphemus* (Brown *et al.* 1994, 1999). Clinical signs can develop as early as 2 weeks post-inoculation with antibody production often detected by 8 weeks (Brown *et al.* 2002). A typical social encounter between tortoises may not replicate the conditions of an intranasal inoculation, thus natural transmission may follow different patterns than controlled transmission studies. Indeed, one contact-based *M. agassizii* transmission study recorded delays in seroconversion of 18 months following the appearance of clinical signs and pens of all seronegative animals produced new infections (Maloney 2011). The results highlight both the potential discrepancy between inoculation and natural-caused transmission and the unreliability of seroconversion as an indicator of early infection in species with slow-responding immune systems (Zimmerman, Vogel & Bowden 2010).

Researchers often cite direct transmission as the method of spread for this pathogen based on the sensitivity of *M. agassizii* to its environment and lack of evidence for indirect transmission (McLaughlin 1997; Brown *et al.* 2002). Tortoises directly interact during short periods of above-ground activity, e.g. during courtship, mating and fighting, but will also share a burrow or den for hours to months during inactive periods (Ruby & Niblick 1994; Guyer, Hermann & Johnson 2014). These variable interactions, coupled with sporadic periods of clinical disease, indicate that some contacts may be more likely to result in transmission than others.

STUDY GOALS

Natural transmission events are difficult to observe in the wild due to the mostly solitary nature of tortoises, so we used a captive population to document host-to-host transmission of *M. agassizii*. We staged a series of interactions that reflected the natural range of contact duration between infected and uninfected captive desert tortoises and documented transmission using qPCR: a sensitive diagnostic tool that can often detect pathogen colonization of hosts in the early stages of infection, i.e. prior to development of antibodies or clinical signs (Feberwee

et al. 2005; Suarez *et al*. 2005; Mouchantat *et al*. 2014). Using the results of these introductions, we modelled transmission probability in relation to contact duration and host characteristics to determine which conditions carried a higher probability of transmitting *M. agassizii*. Subsequently, we examined data on contact rates of a wild tortoise population and asked what the frequency of high-risk contacts in wild populations might suggest about natural transmission rates.

Materials and methods

EXPOSURE AND TRANSMISSION

We conducted all transmission studies at the Desert Tortoise Conservation Center (DTCC) in Las Vegas, Nevada, USA. This outdoor captive facility maintained a population of *G. agassizii* formerly kept as pets or removed from development sites in wild habitat. The origin of animals was often unknown so captive bred and formerly wild tortoises were indistinguishable. Tortoises lived in pens constructed in native creosote-bursage open desert scrub that contained one or more artificial burrows. Fencing with flashing that extended below-ground divided adjacent pens to prevent contamination. We used aseptic techniques when entering and exiting pens and handling tortoises (USFWS 2011).

Selecting study animals

We only considered individuals with a midline carapace length larger than 180 mm (size generally associated with adult class) and determined sex based on dimorphic characteristics of the gular, plastron and tail (Woodbury & Hardy 1948). From April to August 2013, we conducted one or two health assessments at least 1 month apart on potential study animals ($n = 231$) that included a visual assessment of condition [further described in USFWS 2011], oral swab, nasal lavage and blood draw. To perform an oral swab, we held two sterile polyester swabs side-by-side and spun each individual swab while slowly moving both spinning swab tips across the surfaces of the mouth in one full rotation. Each swab tip was stored in a separate vial and stored on ice while in the field. A nasal lavage consisted of a flush of 2·5 mL of 0·09% sterile saline solution into each naris using a sterile syringe while collecting the fluid exiting the opposite naris in a sterile conical vial. After swirling the collected fluid, we used a sterile pipette to transfer 1 mL of flush to a vial containing 200 μL RNA*later* RNA stabilizing reagent (Qiagen, Hilden, Germany), which was then stored on ice. We used a sterile needle and syringe to collect 0·5 mL whole blood from the subcarapacial sinus (Hernandez-Divers, Hernandez-Divers & Wyneken 2002). Blood samples were transferred to lithium heparin microtainers and stored on ice for no longer than 5 h. Plasma was separated using centrifugation with a centrifugal force of 1318 × *g* and a 50 μL plasma aliquot was stored separately for analysis.

All samples (oral swabs, nasal lavage fluid, blood plasma and red blood cells) were temporarily stored in a freezer until transferred to an ultra-cold freezer for extended storage (−70 °C). Oral swabs (1 per assessment) and nasal lavage samples were shipped on dry ice to the San Diego Zoo Amphibian Disease Lab (Escondido, CA, USA) for quantitative Polymerase Chain Reaction (qPCR) to detect and estimate abundance of *M. agassizii* (Braun *et al*. 2014). Results for each qPCR test could be negative, positive or equivocal (inconclusive). A positive result also included three estimates of *M. agassizii* abundance – we present the mean of these three values in results (Tables 1–3; Dataset S3, Supporting information). Blood plasma was shipped to the Dr. Mary Brown Lab at University of Florida (Gainesville, FL,

Table 1. *Mycoplasma agassizii* qPCR results for naive group 1 before and after discrete exposure to an infected host. Animals were quarantined following exposure and oral swabs, nasal lavage and blood samples were collected at intervals. Values presented are *M. agassizii* abundance estimates for a single oral swab sample based on qPCR. Two results are shown (oral result/nasal result) if results did not agree. Eq = equivocal or inconclusive results. All ELISA results from plasma collected during this period were negative, except those denoted otherwise

					Quarantine 1 qPCR					
Naive ID, sex	Initial qPCR	Infected ID, sex	Infected qPCR	Contact dur (h) Jun	Jul	Aug	Sep	Oct	Mar	Apr
22149, F	0,0	18985, M	120595	0·5	0	0	0	0	0	–
17477, F	0,0	18985, M	120595	1·5	0	0	0	0	0	–
17195, F	0,0	18985, M	120595	4·0	0	0	0	0/Eq	0	–
19220, M	0,0	18985, M	120595	>4·0*	0	0	0	0	Eq	0
22292, F	0,0	19415, M	10690	0·5	0	0	0	0	0	–
22059, F	0,0	19415, M	10690	1·5	0	0	0	0/Eq	0	–
21347, M	0,0^{S}	19415, M	10690	4·0	0	0	0	0	467	–
16846, M	0,49/0	21190, M	24890	0·5	0	0	0	0	Eq	0
16656, M	0,0	21190, M	24890	1·5	0	0	0	0	0	–
19344, F	0,0	21190, M	24890	4·0	0	0	Eq/148	0	Eq	0
22267, F	0,0/Eq^{S}	6070, M	43373	0·5	0	0	0	0	0	–
19340, F	0,0	6070, M	43373	1·5	0	0	0	0/Eq	0	–
21499, M	0,0	6070, M	43373	4·0	0	0	0	0	0	–
22159, F**	0,0	6349, M	11788	0·5	–	–	–	–	–	–
22083, F	0,Eq/0	6349, M	11788	1·5	0	0	0	0	0	–
22181, M	0,0	6349, M	11788	4·0	0	0	0	0	0	–

*Additional exposure day due to fence breach, total contact time unknown; **died soon after exposure due to unrelated trauma; ^{S}Suspect ELISA.

Table 2. *Mycoplasma agassizii* qPCR results for naive group 1 after a second discrete exposure to an infected host. After an initial exposure resulted in no new detectable infections, *Gopherus agassizii* (n = 14) were re-exposed to an infected host for a pre-determined exposure time. Tortoises were quarantined following each exposure and oral swabs were collected at intervals (first two assessments included a blood draw). Values presented are *M. agassizii* abundance estimates for a single oral swab sample based on qPCR. Eq = equivocal or inconclusive results. All ELISA results for blood collected during this time were negative

			Exposure 2 Apr-14		Quarantine 2 qPCR		
Naive ID, sex	Infected ID, sex	Infected qPCR	Days in pen	Contact dur (h)	May	Jun	Jul
21347, M*	–	–	–	–	0	0	0
19344, F	18985, M	557053	3	15·8	0	0	0
22059, F	19415, M	76493	4	0·9	0	0	0
16656, M	19415, M	76493	5	12·2	1777585	4435776	424238
21499, M	20304, M	891	1	0·4	0	0	0
17477, F	20304, M	891	2	7·0	0	Eq	0
17195, F	21190, M	26160	2	2·2	0	0	0
19220, M	21190, M	26160	9	5·5	0	0	0
22181, M	21897, M	140316	1	1·4	0	0	0
22267, F	21897, M	140316	3	25·0	0	0	Eq
19340, F	6070, M	272473	1	2·9	0	0	Eq
22083, F	6070, M	272473	1	10·7	469	0	0
16846, M	6070, M	272473	2	17·0	0	0	0
22149, F	6349, M	126470	6	0·2	0	398	0
22292, F	6349, M	126470	4	4·1	4984260	5110088	33453

*Not exposed second time due to suspect status at end of quarantine 1.

Table 3. *Mycoplasma agassizii* qPCR results before, during and after continuous exposure to an infected host. Individuals were health assessed at intervals and oral swabs, nasal lavages (2013 only) and blood samples (not collected Apr 2014) were collected to assess infection status. Values presented are *M. agassizii* abundance estimates for a single oral swab sample. Two results are shown (oral result/nasal result) if results conflicted. Eq = equivocal or inconclusive results. ELISA results from plasma collected during this time were negative unless denoted otherwise

				Exposure 19 Aug – 16 Nov 2013 qPCR			Quarantine 2014 qPCR		
Naive ID, sex	Initial qPCR	Infected ID, sex	Infected initial qPCR	3–5 Sep	1–2 Oct	31 Oct–1 Nov	25–26 Mar	4 Apr	11 Apr
21804, M	0,0	18985, M	22911	0	62/Eq	1491289	1357889	4647186	4769004
22340, F	0,0	18985, M	22911	0	1293648	394167	2332249	3483092	2326168
14839, M	0,0	19415, M	153686	0	107/0	7382743	2739180	5864166	13496163
22404, F	0**	19415, M	153686	0	Eq/0	0	Eq	Eq	0
22409, M	0**	20304, M	41174	183261	452014^{P}	42772^{P}	37543^{P}	254434	236247
22417, F	0**	20304, M	41174	0	5498851	898364	2585251^{S}	4893490	2404840
22003, M	0,0	21190, M	244466	639190	318254	411312	1626054	2747628	510998
22419, F	0**	21190, M	244466	0	3110700	614525	2951001	6048124	10249199
15780, M	0,0	21894, M	733143	0/204	3409210	2721137	911677	8220049	4040862
22335, F	0,0	21894, M	733143	Eq/462	141369	2993780	1676746	2069216	6393441
22286, F*	0,0	21897, M	522835	0	0	0	0	–	–
22390, M	0,0	21897, M	522835	0/317	558126	1088161	7368059	2830298	6191128
22211, F	0,0	6070, M	591811	3445/Eq	3869277	1121742	3897770	7398174	2160592
22314, M	0,0	6070, M	591811	2851673	3740471	333991	4488860	3572943	1945467
22301, F	0,0	6349, M	39400	1415283	4006279	899054	5008616^{S}	16065426	6013114
22399, M	0,0	6349, M	39400	0	1805458	245780	2525409	1441838	1348844

*Acquired injury at start of study that may affect transmission- removed from data set, no nasal lavages due to injury; **Only one health assessment performed prior to exposure; ^{P}Positive ELISA; ^{S}Suspect ELISA.

USA) for an Enzyme-Linked Immunosorbent Assay (ELISA) to detect antibodies to *M. agassizii* (Wendland *et al.* 2007). Results from ELISA tests were reported as negative (antibody titre <32), suspect (antibody titre ≥32 & <64) or positive (antibody titre ≥64). We similarly tested for *M. testudineum* infection, which can also cause respiratory disease in tortoises, but was not the primary focus of this study. Only individuals testing negative for *M. testudineum* were selected.

We selected eight adult males considered infected with *M. agassizii* based on positive results for all qPCR and ELISA tests, and observed nasal discharge – a clinical sign of URTD. We chose male tortoises as transmitting hosts due to their likelihood to interact

with both male and female tortoises. Females tend to infrequently associate with other females, thus their use as transmitting hosts may have compromised our need for extended contact between some study pairs (Ruby & Niblick 1994). For uninfected animals, we sought tortoises housed individually or in pens where all tortoises met our criteria for uninfected status: negative results for all qPCR and ELISA results and no observed nasal discharge or other moderate to severe clinical signs of URTD. All male (n = 12) and female (n = 16) adult tortoises fitting this definition were used in the study. Four additional tortoises were used as uninfected hosts but did not meet all criteria: one female returned an inconclusive result on a single qPCR test; one female had a single suspect ELISA result and a single inconclusive qPCR; one male returned a single suspect ELISA result and one male tortoise returned a single positive qPCR result with all other results negative (Table 1; Dataset S3, Supporting information). The positive bacterial abundance estimate was so low, we considered the result unreliable and possibly due to contamination. In total, 14 males and 18 females (n = 32) were chosen to expose to infected hosts.

Control group

Concomitant to these assessments, collaborating researchers used the same procedures to locate nine healthy, uninfected adult tortoises (n = 5 males, n = 4 females) among the same captive cohort for an unrelated study. Prior to their study, these tortoises were housed at the DTCC in the same conditions as our study animals without any exposure to *M. agassizii* infected tortoises. We considered this an opportunity to further validate the methods used to select naïve tortoises, as we cannot guarantee 100% detection probability of *M. agassizii* infection with one or even two health assessments. If our selection procedures for naive tortoises were adequate, we would expect a group of naive tortoises unexposed to infection to continue to test negative for both presence of *M. agassizii* and antibodies to *M. agassizii*. We performed four health assessments and collected oral swabs from these tortoises during April-August, October and November 2013 and March 2014. The initial assessment and October assessment also included a nasal lavage and blood draw.

Naive group 1 – first exposure

Sixteen tortoises (n = 6 males, n = 10 females) considered uninfected were quarantined in individual pens and will be referred to as group 1. To test the transmission potential of brief contacts typically experienced during the tortoise active season, we selected host contact times that reflected interactions observed in the wild (C. M. Aiello, personal observation). Naive tortoises were randomly assigned to one of five infected male tortoises (randomly chosen from our pool of eight infected males) and one of three contact times: 0·5 h (n = 5), 1·5 h (n = 5), or 4 h (n = 5), so that each of the five infected males was exposed to a unique naive tortoise for each contact time (Fig. 1).

Prior to introductions, we reassessed the health of infected males and collected oral swabs to determine *M. agassizii* infection load using qPCR (Table 1). On June 3rd – 5th 2013, we introduced each pair within a shaded, trough-shaped enclosure that kept the tortoises face-to-face and within touching distance (approximately 10 cm apart). An observer kept time, recorded behaviour and ensured tortoises remained face-to-face throughout the interaction. Interactions occurred in early morning during typical tortoise activity periods and preferred temperatures: 25–35 °C (Zimmerman *et al.* 1994).

At the end of the interaction, we returned tortoises to their individual pens. One uninfected tortoise exposed for 4 h had

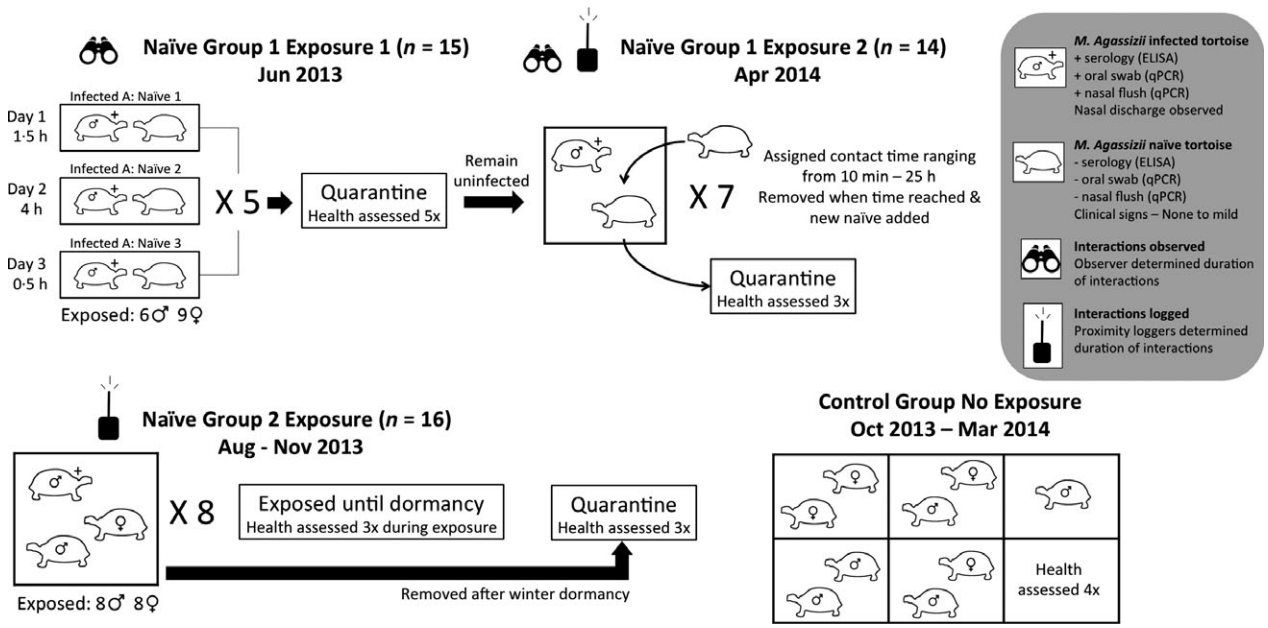

Fig. 1. Criteria for selecting study animals and design of exposure trials. Introductions between *Mycoplasma agassizii* infected and uninfected hosts (*Gopherus agassizii*) were either observed by researchers or logged by proximity loggers. Exposure to an infected tortoise either lasted a set amount of time (discrete) or occurred sporadically as animals shared a pen over multiple months (continuous). Tortoises in naive group 1 remaining uninfected after a single exposure event and 10 months in quarantine were re-exposed and retested for infection.

additional contact opportunities following a fence breach so total contact time was unknown. This exposure time is not included in analysis but this tortoise was used in subsequent exposures so all sample results are shown in Table 1. The 16th uninfected tortoise from group 1 was then used to conduct another 4-h exposure to replace the discrepant replicate. One female tortoise exposed for 0·5 h died soon after exposure due to unrelated trauma and so was removed from the study. From July to October 2013, we performed health assessments each month on exposed tortoises in the same manner used to select study animals. We reassessed tortoise health in March 2014 following a period of winter dormancy and again in April if results were inconclusive.

Naive group 1 – second exposure

After spring assessments, we considered any exposed tortoises returning consistent negative qPCR and ELISA results for *M. agassizii* and showing no new clinical signs of respiratory disease to be uninfected ($n = 14$). One of the 15 surviving tortoises returned one positive qPCR result immediately prior to the second exposure and so was not re-exposed as a precaution, but all subsequent results were negative. We assigned unique contact times to each remaining uninfected tortoise for a second exposure to one of seven infected males. Times ranged from 10 min up to 25 h to include times more typically experienced during overnight or multi-day burrow sharing (Table 2).

We attached proximity loggers (Encounternet LLC, Portland, OR, USA) to the anterior carapace of each tortoise set to document another logger when pairs were approximately ≤10 cm from each other (settings based on trial tests with model and live tortoises). We selected a 10 cm distance to capture interactions where physical contact was most likely and thus able to facilitate direct transmission of bacteria. Each logger recorded a start time when another logger was detected and an end time of the interaction when tortoises were >10 cm apart for longer than 1 min. Absolute spatial precision for contact loggers can vary, however, so we observed initial contacts between pairs to compare to logger data following the event to evaluate accuracy (Boyland *et al.* 2013). We noted some loggers failed to record observed interactions and either replaced the logger or observed all interactions and manually recorded contact time for that tortoise pair ($n = 6$).

Prior to introductions, we reassessed the health of infected males and collected oral swabs to determine *M. agassizii* infection load using qPCR (Table 2). On April 14th 2014, we placed the first seven (of 14) uninfected tortoises in one of seven pens housing an infected male. We monitored all pairs and logged contact times, documented observed behaviours every 30–60 min, and removed the naive tortoise from the pen when contact totals neared their assigned time (Fig. 1). Seven new tortoises were introduced after the first seven were removed. All exposed tortoises were quarantined and health assessed at 2–3 weeks, 6–7 weeks and 12–13 weeks after exposure (these assessments did not include nasal lavage and the final quarantine assessment did not include a blood draw).

Naive group 2 – continuous exposure

The remaining 16 uninfected tortoises ($n = 8$ males, $n = 8$ females) selected for the study were quarantined in individual pens and are referred to as group 2. We randomly assigned one male and one female naive tortoise to one of eight pens housing the eight infected males. Prior to introductions, we reassessed the health of the eight infected males and collected oral swabs to determine *M. agassizii* infection load using qPCR (Table 3). All tortoises were fitted with proximity loggers at the start of the study and were set to record interactions as described for group 1, with logger accuracy validated by video recordings and observed interactions throughout the study. On August 19th, we placed the two naive tortoises into their assigned pen with the infected male and performed health assessments on all tortoises at day 16–18, 44–45 and 74–75 of exposure. Tortoises then hibernated in separate burrows and were quarantined in spring. We performed weekly health assessments after quarantine in 2014 and present results up to 11 April (2014 assessments did not include nasal lavage and only March assessments included a blood draw).

Final animal disposition

Fifteen tortoises from naive group 2 were retained for additional research. Remaining tortoises were transferred to the DTCC veterinary staff at the end of this study for antibiotic treatment and care.

EXPOSURE MODELS

This study design resulted in each tortoise being exposed to a hazard (an infected host) on a continuous time scale, but sampling for the event of interest (infection) occurred at discrete time points. The exact timing of the infection, therefore, could only be narrowed down to the interval between sampling periods (also known as interval-censored, or grouped time data). One approach used for a binary response (infected = 1, uninfected = 0) occurring at an unknown point within a discrete interval is a generalized linear model (glm) with complementary log-log link: $\text{cloglog}(x) = \log[-\log(1-x)]$ (Singer & Willett 2003). If we assume a constant risk of infection λ for any unit of time t, then a glm with cloglog link and an offset (a predictor variable with coefficient = 1) for exposure acts as a proportional hazards model (Vittinghoff *et al.* 2005). To estimate the probability of acquiring infection $P(\text{Inf})$ over an exposure period $E = \Delta t$, an offset of $\log E$ was added to any other covariates (X_t) included in the model so that at time t:

$$P(\text{Inf}) = 1 - \exp[-\exp(\beta_0 + X_t\beta_1 + \log E)]$$

It can be shown that $1-\exp[-\exp(\beta_0)]$ in this model is equivalent to the infection risk λ for a single unit of exposure (Vittinghoff *et al.* 2005). We used this model structure and the results of our exposure trials to estimate *M. agassizii* infection probability over increasing exposure, defined in various ways described below, and determined whether other covariates improved model fit.

Response variable

We determined the value of the response variable (1 = infected, 0 = uninfected) for each exposure event in our data set based on two definitions of infection: (i) we first considered a naive tortoise to be infected with *M. agassizii* at the end of a period of exposure if more than one oral swab or nasal lavage sample tested

qPCR positive following the exposure period; (ii) we then tested a stricter assumption that considered a naive tortoise infected if any single qPCR result following exposure was positive. While we believe definition 1 more accurately describes the infection process in this system, we cannot ignore the possibility that low level infections may have developed but were missed by our sampling methods. We found the results were not sensitive to our assumptions (further discussed in results) and so we present the results of all models using infection definition 1 heretofore, and the results from infection definition 2 are provided in a supplementary document (Appendix S1, Supporting information).

Cumulative exposure

We first estimated infection probability using cumulative exposure time of naive tortoises up to the point of infection or censor (omitting the first exposure of naive group 1), resulting in a single datum for each tortoise (analysis1: Data set S2, Supporting information). The first model used the number of days spent in a pen with the infected host (cum.days) as the exposure variable E. We added the covariates sex and grp (which naive group the individual belongs) to this model and performed a Chi square analysis of deviance test to determine if addition of either covariate significantly improved model deviance, with $P < 0{\cdot}05$ considered significant (Singer & Willett 2003). The second model used cumulative duration of close contact (cum.dur) either logged or observed as the exposure variable, using a subset of data from tortoises for which this data were available (analysis2: Dataset S2, Supporting information). We added the covariate logmethod (whether contact duration was logged by proximity loggers or determine by observers) sex and grp, and similarly performed a Chi square analysis of deviance to test whether addition of either covariate improved model deviance.

Discrete exposure

We next considered each interval between health assessments as a discrete exposure event and used data from each exposure interval up to the point of infection or censor. This approach resulted in a 'period-person' data format with multiple exposures for some individuals, an infection result for each interval, and interval-specific covariates (Singer & Willett 2003). Using this format, we were able to include the qPCR-determined infection load of the transmitting tortoise (inf.pcr) as a covariate, which varied over time. We used two subsets of the full data to run a set of models defining the exposure term E as either number of days in the interval (int.days) or the total contact duration during the interval (int.dur), and incorporating the inf.pcr term as either a covariate or as a component of the exposure term (dose1 = int.days*inf.pcr; dose2 = int.dur*inf.pcr; Table 4).

The first subset of data included all intervals for which contact data were available, regardless of logmethod, and only included intervals where the focal tortoise was exposed to one infected host (i.e. in group 2 tortoises, when one naive tortoise became infected in an interval earlier than the other naive tortoise, we excluded subsequent interval data due to uncertainty regarding the infectiousness of the newly infected host; analysis3: Dataset S2, Supporting information). The second subset of data included the same conditions as subset one, but only for intervals with contact data logged by proximity loggers (analysis4: Dataset S2, Supporting information). Observers may over-estimate contact time compared to loggers because interactions within burrows could not be observed, and tortoises may be spaced further apart than the logger detection threshold. We compared model fits for the set of models estimated from the same data subset with normalized Akaike weights (w_i) and corrected AIC (AICc) for small sample sizes (Burnham & Anderson 2002). We added covariates (sex, grp) to the top-ranking models and performed a Chi square analysis of deviance to determine if the addition significantly improved model deviance. All model estimates and test statistics were calculated using program R (R Development Core Team 2014).

NATURAL INTERACTION PATTERNS

Natural interactions were studied at Fort Irwin National Training Center near Barstow, California, USA. We selected 25 tortoises ($n = 14$ males, $n = 11$ females) from past research with overlapping or adjacent home-ranges over a 900 ha area. We

Table 4. Models of transmission probability ranked by normalized Akaike weights (w_i, highest ranked model in bold). All models were run using data from observed and logged interaction and again on a subset of data from interactions logged by proximity loggers only

Observed & logged data				Logged data only			
Model	AICc	dAICc	w_i	Model	AICc	dAICc	w_i
offset(log(dose1))	**23·77**	**0·00**	**0·49**	**offset(log(dose1))**	**17·45**	**0·00**	**0·35**
offset(log(int.days)) + inf.pcr	25·14	1·37	0·25	offset(log(dose2))	18·50	1·05	0·21
offset(log(int.days))	26·06	2·29	0·16	offset(log(int.dur))	19·05	1·60	0·16
offset(log(int.dur))	27·67	3·90	0·07	offset(log(int.days)) + inf.pcr	19·92	2·47	0·10
offset(log(int.dur)) + inf.pcr	29·32	5·55	0·03	offset(log(int.days))	20·31	2·86	0·08
offset(log(dose2))	31·31	7·54	0·01	offset(log(int.dur)) + inf.pcr	20·50	3·05	0·08
log(inf.pcr)	36·48	12·71	0·00	log(inf.pcr)	24·86	7·42	0·01
Null	38·82	15·05	0·00	Null	25·16	7·72	0·01

int.days: number of days in pen with infected host during exposure interval.
int.dur: duration (h) of close contact with infected host during exposure interval.
inf.pcr: estimated *M. agassizii* infection load of transmitting host from qPCR of oral swab taken before introductions.
dose1: product of days in pen with an infected host and that host's estimated infection load for the exposure interval.
dose2: product of duration (h) of contact with an infected host and that host's estimated infection load for the exposure interval.

attached radio transmitters (RI-2B, Holohil Ltd, Carp, ON, Canada) and proximity loggers (Sirtrack Ltd, Hawkes Bay, New Zealand) to the first costal scutes and calibrated loggers to record contacts of tortoise pairs spaced approximately ≤10 cm. Loggers documented the start time of an interaction when another logger came in range and an end time when the logger was not detected for 1 min. Data from each logger in an interacting pair were combined to create a single record for each logged encounter (Data set S4, Supporting information). Twenty-four loggers were attached and active from 1 June 2012 to 26 October 2012 (one logger was attached 19 June 2012), a period of the year encompassing extensive burrow use in summer months, and surface activity and mating in fall (Rostal, McCoy & Mushinsky 2014).

To ensure proximity loggers accurately recorded contact events of interest, we compared logger data to observer recorded interactions at this site and three additional sites with sample tortoises fitted with proximity loggers ($n = 51$) in the same manner for additional studies. Observers located all tortoises at a site at least twice monthly during the study period and recorded surface interactions and burrow sharing events to compare to logger data.

For comparison to interaction conditions in exposure trials, we aggregated contacts into 'contact events' by combining data from contacts occurring over a period of consecutive days. For each contact event, we calculated the number of consecutive days each pair interacted and the cumulative duration of interactions over this period based on logger data. Using the highest-ranking exposure models from our captive trials (E = dose1; E = dose2), we predicted the transmission probability of contact events between wild tortoises for an average infection load (mean[inf.pcr]) based on the number of consecutive days an event lasted and the total contact time logged over the contact event. We created two weighed contact networks in the R package igraph, where each contact event between tortoises was given a weight equal to the transmission probability predicted by the model (Csardi & Nepusz 2006).

Results

EXPOSURE AND TRANSMISSION

Naïve group 1

During 0·5, 1·5, or 4-h introductions, tortoise pairs frequently engaged in physical contact and displayed typical behaviours of aggression or courtship. After quarantine, no group 1 tortoises returned consistent positive qPCR results over a 10-month period of sampling (Table 1). We observed no moderate to severe clinical signs of respiratory disease during health assessments.

Following the second exposure, oral swabs from two tortoises exposed for 4·1 and 12·2 h tested positive for *M. agassizii* and continued to test positive in subsequent samples (Table 2). Both tortoises developed moderate clinical signs of respiratory disease. Two additional tortoises tested qPCR positive on one occasion, but negative for all others and lacked signs of disease in 2014. No tortoises in this group tested positive for antibodies to *M. agassizii* throughout the study (Data set S3, Supporting information).

Naïve group 2

During the first 2 weeks of cohabitation, some proximity loggers fell off and were not reattached for a period of several days. Other loggers appeared to record interactions inaccurately as determined by review of video footage taken during the study. As a result, we recorded incomplete interaction data for nine exposed tortoises and complete interaction data for seven tortoises. Incomplete or suspect logger data for an exposure interval were removed from analyses (Data set S2, Supporting information).

Four tortoises tested qPCR positive for *M. agassizii* in both oral and nasal samples and three tested positive only in nasal samples at the first health assessment following 2 weeks of exposure. By day 75 of exposure, 14 of 16 tortoises were positive for both samples (Table 3). Tortoises remained positive in all subsequent tests and while in quarantine the following spring. One qPCR positive tortoise tested positive for antibodies in October 2013 and two additional tortoises were suspect for antibodies in March 2014 (Table 3). Of the 14 new infections, 11 developed moderate to severe clinical signs of respiratory disease including nasal discharge, one developed mild signs with rare nasal discharge and two showed mild signs but lacked nasal discharge during all assessments in 2013 & 2014. Two tortoises remained negative or equivocal for all tests throughout the study and showed no clinical signs of disease, one of which acquired an injury to the face early in the introduction that resulted in scarring of the nares. Due to this potentially confounding injury, the data for this tortoise were removed from analysis.

Controls

Of four oral swabs collected for each of nine tortoises considered uninfected and unexposed, all returned negative ($n = 33$) or equivocal ($n = 3$) results for *M. agassizii*. Of two nasal lavages and plasma samples collected for each control tortoise, all returned negative qPCR (nasal $n = 18$) and negative ELISA (plasma $n = 17$; one sample was lost) results for *M. agassizii* and antibodies to *M. agassizii* (Data set S3, Supporting information).

EXPOSURE MODELS AND PREDICTORS OF INFECTION PROBABILITY

Cumulative exposure

The model using cum.days as the exposure term was estimated to be: $P(\text{Inf}) = 1 - \exp[-\exp(-3{\cdot}314894 + \log(\text{cum.days})]$, intercept SE = 0·2747 (Fig. 2a). This model predicts a daily infection risk of 0·036 (±1 SE: 0·027–0·047). The exposure time estimated to reach a 0·5 probability was 19·1 days (±1 SE: 14·5–25·1 days). The addition of a sex or grp covariate did not significantly

improve the model (sex: $P = 0{\cdot}069$, grp: $P = 0{\cdot}63$). The model using cum.dur as the exposure term was estimated to be: $P(\text{Inf}) = 1 - \exp[-\exp(-4{\cdot}193494 + \log(\text{cum.dur})]$, intercept SE = 0·4461 (Fig. 2b). This model predicts an hourly infection risk of 0·015 (±1 SE: 0·0096–0·023). The exposure time estimated to reach a 0·5 probability was 45·9 h of contact (±1 SE: 29·4–71·7 h). The addition of covariates sex, grp or logmethod did not significantly improve the model (sex: $P = 0{\cdot}73$, grp: $P = 0{\cdot}61$, logmethod: $P = 0{\cdot}97$).

Discrete exposure

The model using the dose1 variable as the exposure term best fit the data for both data subsets (Table 4). The logger-only data set produced similar results, with dose1 as the best fit (Table 4), however, the dose2 model ranked higher in this model set and had similar AICc and model weight to the dose1 model. In both analyses, models including information about either interaction time (int.days, int.dur) or *M. agassizii* load of the transmitting host (inf.pcr) out-performed an intercept-only null model.

The model with the highest weight for data subset 1 was estimated to be: $P(\text{Inf}) = 1 - \exp[-\exp(-14{\cdot}5707 + \log(\text{dose1})]$, intercept SE = 0·4548. This model suggest daily infection risk depends on the bacterial load of the transmitting host, predicting that the same exposure times will have a higher transmission probability as infection load increases (Fig. 3a). The second best model in data subset 2 includes a similar relationship between contact duration and infection load. The dose2 model was estimated to be: $\text{P(Inf)} = 1 - \exp[-\exp(-14{\cdot}8374 + \log(\text{dose2})]$, intercept SE = 0·5261. This model similarly predicts a naive host exposed to a tortoise with a greater load of *M. agassizii* will require less interaction time for infection (Fig. 3b). Neither model was significantly improved by the addition of covariates sex or grp (data subset 1, sex: $P = 0{\cdot}17$, grp: $P = 0{\cdot}53$; data subset 2, sex: $P = 0{\cdot}097$, grp: $P = 0{\cdot}83$)

Model sensitivity

Model estimates and predictions (±SE) using an alternative infection definition to determine the response variable for our data (any single positive qPCR result after an exposure event) overlapped with results using infection definition 1 (more than 1 positive qPCR result after an exposure; Appendix S1, Supporting information). The primary difference between the two approaches was reduced support for using contact duration in the exposure term to predict transmission probability when compared to other models and a slightly higher transmission risk for all exposure scenarios.

NATURAL INTERACTION PATTERNS

All loggers remained attached throughout the study and 39 tortoise pairings out of 325 possible pairings were logged by proximity loggers and were assumed to have interacted (Dataset S4, Supporting information). Approximately half of the interactions occurred within one day's time (54/105, Fig. 4a) and total close interaction time over 1 day or consecutive days was often less than 1 h (81/105, Fig. 4b). Some pairings did have contact over several consecutive days and the most extensive pair interaction recorded totalled 268 h over 20 consecutive days. Observers recorded 57 burrow sharing events during radio-telemetry relocations at all sites with proximity loggers: 35/57 events were recorded by loggers at the time of the observation; 22/57 events were not recorded at the time of observation but loggers recorded contacts at earlier or later times on the day of the observation. Observers also recorded 13 surface interactions that all corresponded to a logger-recorded contact at the same date and time. Additionally, we observed burrow sharing after loggers were removed in four pairs that lasted at least 5, 6, 105 and 109 days.

The transmission probabilities predicted for contact events were low overall. The model based on the number of days the event lasted and mean infection load (dose1) predicted transmission probabilities of: <0·1 for 51% of

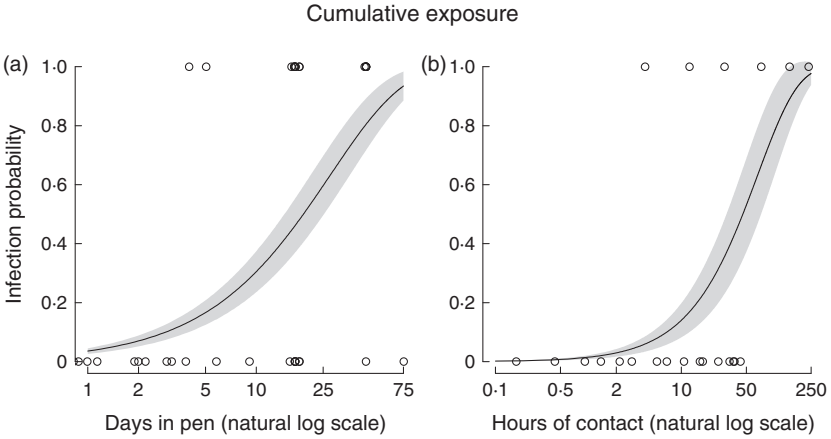

Fig. 2. Transmission results and predicted infection risk for cumulative exposure time to an *Mycoplasma agassizii* infected tortoise. Points represent transmission results (0 = uninfected, 1 = infected) of each *Gopherus agassizii* following an exposure event (jittered on × axis). Infection probability (line ± SE) was predicted using generalized linear models with complementary log—log link function. Results are calculated based on number of days in a shared enclosure (a) and total duration of close contact (b).

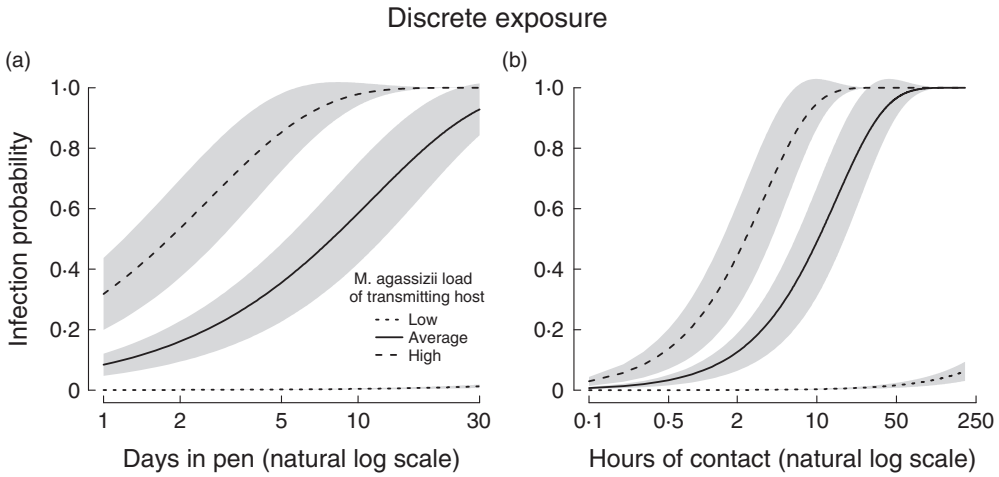

Fig. 3. Predicted infection probability (±SE) within an exposure interval for different infection levels of a transmitting host using the two best-supported models from transmission experiments. Both models had a single predictor, (a) dose1: the product of days spent in a pen with an infected tortoise and the estimated infection load of the transmitting host, and (b) dose2: the product of hours spent in contact with an infected host and that host's infection load. Infection probability was predicted for three levels of transmitting host infection load: the minimum (891), mean (220 354) and maximum (1 239 158) values for hosts used in this study.

Fig. 4. Frequency of contact events between pairs of wild *Gopherus agassizii* occurring in a single day or over consecutive days (a) and the cumulative duration of interactions over consecutive days (b). Distributions are based on a sample population of 25 adult tortoises fitted with proximity loggers calibrated to detect close contact (≤10 cm) between individuals from 1 June 2012 to 26 October 2012.

contact events (54/105), 0·1–0·3 for 38% of events (40/105), 0·3–0·5 for 8·6% of events (9/105) and ≥0·5 for 1·9% of events (2/105; Fig. 5a). The model using total duration of contact and mean infection load (dose2) predicted transmission probabilities of: <0·1 for 79% of contact events (83/105), 0·1–0·3 for 11% of events (12/105), 0·3–0·5 for 3·8% of events (4/105) and ≥0·5 for 5·7% of events (6/105; Fig. 5b).

Discussion

Directly transmitted pathogens require close contact between hosts to spread, but only a small proportion of total contacts may facilitate transmission. We created variation in exposure to an infected host by initiating interactions between captive desert tortoises uninfected and infected with the pathogen *M. agassizii* and terminating interactions after varying amounts of time and contact. We reassessed infection status after each exposure and used the results of these trials to estimate transmission risk over a range of exposures, accounting for unequal infection loads among the transmitting hosts. Our study provides evidence of a dose—response effect for *M. agassizii* transmission in desert tortoises where the exposure time and pathogen dose may determine effective pathogen transmission. The data imply that in most situations, high transmission rates are limited to extensive contacts between hosts over multiple days. When we examined the duration of contact events in a wild tortoise population using proximity loggers, such extensive contacts were rare events during the study period, suggesting low transmission risk under these conditions. However, our transmission models show less contact may be needed for transmission if an infection is particularly high, thus identifying patterns of both these key host contacts and shedding events will be critical to understanding transmission risk in tortoise populations.

DETECTING INFECTION

Detecting infection in animals poses several challenges and can affect interpretation of results if assumptions are not met. We used multiple sampling techniques and repeated diagnostic tests to reduce the likelihood of false negatives or positives affecting results. While most results were consistent, some exposed tortoises returned single and low-value positive qPCR results among a series of negative results. These solitary positives could indicate low infection levels that avoid detection (Shin *et al.* 2014), but we suspect such isolated positives in our study

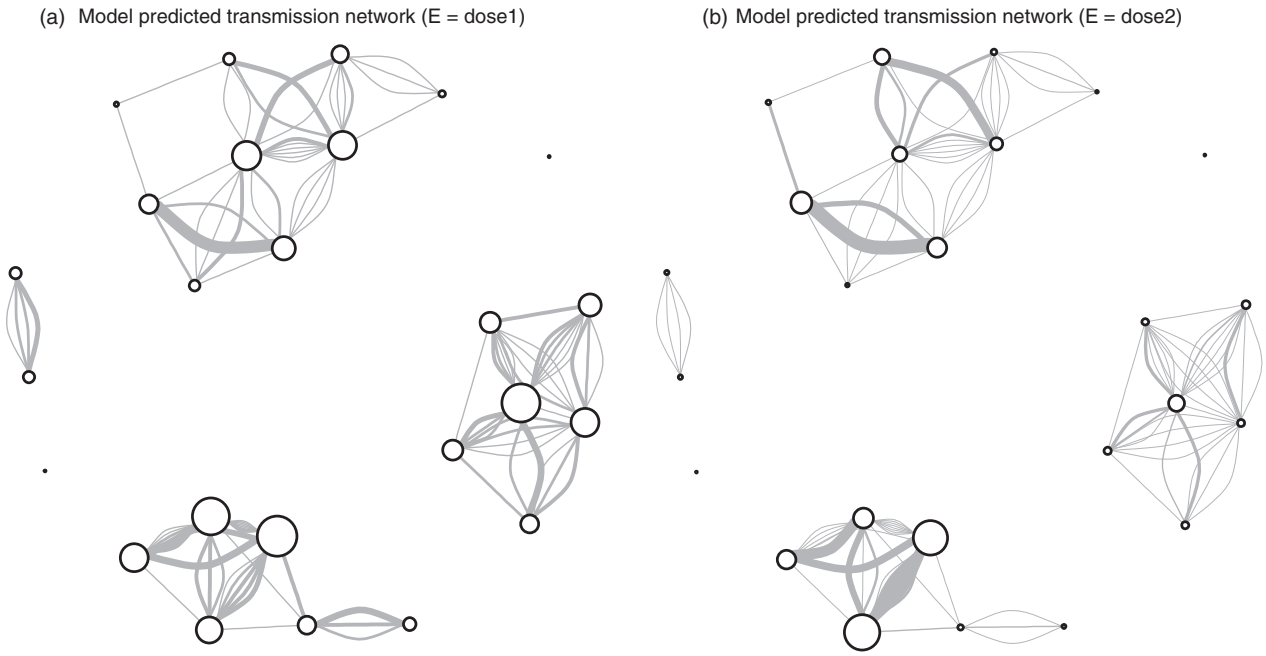

Fig. 5. Two representations of a contact network of 25 wild adult *Gopherus agassizii,* weighed by transmission model predictions. Each point represents a tortoise and lines connecting points represent contact events as recorded by proximity loggers. Line width is scaled by the predicted transmission probability of the contact event, calculated using the highest-ranking exposure models from captive transmission studies. The dose1 and dose2 term used in the models were the product of mean infection load for transmitting hosts in the study and exposure time based on the number of days the contact event lasted (a) or close contact duration logged during the event (b). Point size is scaled by the summed probability of all contact events involving that tortoise.

are not indicative of an established infection. False-positives can occur in single PCR tests, but are less likely when repeated sampling methods are used to diagnose infection (Cuenca-Estrella *et al.* 2009). To ensure our results were robust even if this assumption was not met, we conducted our analyses using a second, more conservative approach to account for potential missed infections. Many of the patterns, model parameter estimates and predictions were conserved using this alternative method. To ensure confidence in conclusions, we encourage the use of multiple methods where possible and repeated sampling when investigating disease processes in wild animals.

CONTACT HETEROGENEITY AND TRANSMISSION RISK

The use of contact network analyses in disease studies demonstrates the growing awareness of the role contact heterogeneity plays in transmission (Keeling & Eames 2005; Bansal, Grenfell & Meyers 2007; Craft 2015). However, the correct use of this tool is important, as the full contact network rarely reflects the transmission network (Craft 2015). Our results support this notion and we show how empirically derived estimates of transmission likelihood can be used to create network models that reflect variation in per-contact transmission risk. Our results suggest short interactions between *M. agassizii* infected and uninfected desert tortoises (<3 days, <5 h) have low infection probabilities, but may be the most frequent type of interaction in some free-ranging tortoise populations. Thus, transmission would likely be over-estimated if all contacts were treated equally in these network models. Other animal host—parasite systems similarly show that contact network models using more detailed information about the type or duration of interactions occurring between hosts better predict new infections or parasite abundance and diversity (Clay *et al.* 2009; Drewe 2010; Rimbach *et al.* 2015).

Similar to contact heterogeneity, including variation in shedding rate in transmission models may improve prediction. Our results suggest variation observed in qPCR data from infected hosts may reflect host infectiousness: hosts with a higher qPCR-estimated bacterial load were predicted to transmit infection more effectively with less contact. Thus, the pattern of infectiousness in hosts may determine which contacts and what time periods contribute significantly to transmission. Other studies similarly show that the timing and severity of infectiousness can greatly change transmission patterns in structured populations (Cross *et al.* 2005; Lloyd-Smith *et al.* 2005; Grear, Luong & Hudson 2013). The occurrence of a few 'super-shedders' or 'super-shedding' events can have a disproportional impact on the outcome of disease outbreaks (Stein 2011).

A key next step will be to identify *M. agassizii* shedding patterns in infected wild hosts, as this will inform interpretation of contact networks and their effective use

in transmission models. Oral swabbing may prove a useful method for this task, but additional tests and alternative methods for assessing infectiousness should still be explored. We standardized our swabbing technique to improve repeatability, but variation in qPCR results may be caused by sampling error and should be further validated (Miller *et al.* 2012). We encourage further investigation into the detection, distribution and timing of highly infectious hosts or shedding events that likely promote rapid pathogen spread in *G. agassizii* and other species.

The relationships we observed between transmission risk and exposure can help managers interpret variation in disease patterns in tortoise populations and identify potentially high-risk scenarios. Little data exist on *M. agassizii* transmission in wild desert tortoises, but infection risk in many cases appears low compared to captive populations (Johnson, Morafka & Jacobson 2006; Ozgul *et al.* 2009; Sandmeier *et al.* 2013). If the apparent majority of contacts in the wild have low transmission probability as we observed, low infection prevalence would be expected compared to a captive situation where contact rates and durations are amplified. The conditions of captivity may also increase infection severity and shedding rates, as has been documented with *Salmonella* in Australian reptiles (Scheelings, Lightfoot & Holz 2011), which would further increase transmission rates according to our predictions. Similarly, seasonal, regional or stress-driven variation in host behaviour and infection severity in wild populations could affect transmission rates and account for differences in infection prevalence (Altizer *et al.* 2006; Sandmeier *et al.* 2013; VanderWaal *et al.* 2013). Further research documenting natural & disturbance-caused variation in contact and pathogen shedding patterns could shed light on the circumstances leading to more rapid and severe outbreaks in desert tortoise populations.

Collecting data on such small-scale processes may seem unreasonable, but many host patterns can be linked to general environmental or population characteristics (e.g. seasonally driven patterns of contact and infectiousness; Altizer *et al.* 2006; Morgan *et al.* 2006). In our model comparisons, pathogen exposure estimated by the number of days in shared housing with an infected host out-performed exposure estimated by hours of close interaction. This may reflect inaccuracies in documenting close interaction or suggest a coarse description of pair proximity may be adequate for describing contact heterogeneity. While technology such as proximity loggers present new opportunities for research, their current cost and variable performance limit use in large-scale studies (Boyland *et al.* 2013). Such devices may not be necessary if more general indices of pair interaction, e.g. shared space and burrow-use data, can sufficiently estimate transmission dynamics in *G. agassizii* and other generally solitary species (Godfrey *et al.* 2009; Leu, Kappeler & Bull 2010).

CONCLUSION

Our results provide clear data on how variation in contact duration and the infectiousness of the transmitting host influence the likelihood of transmission and show that incorporating host contact and shedding data could improve the predictive power of epidemiological models. Future work should focus not only on identifying the distribution of individual and population level host characteristics leading to high transmission risk, but also on potential environmental drivers associated with such characteristics. A clearer understanding of the conditions surrounding pathogen transmission may inform strategies to prevent or disrupt outbreaks.

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Data accessibility

Data used in analyses can be found in Supporting Information accompanying this paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Appendix S1. Additional results. An alternative way to define a successful transmission event was used to estimate all models and results were compared to those presented in the manuscript (see methods).

Dataset S2. Exposure data used in glms. Full data set used in generalized linear models. Status 1 and 2 correspond to infection definitions 1, 2 (see methods). Analysis columns 1:4 can be used to subset data for each analysis as described in the methods.

Dataset S3. qPCR and ELISA results. Raw estimates of *M. agassizii* abundance (3 estimates per sample) for all swab and lavage samples analysed by San Diego Zoo Amphibian Disease Lab and ELISA result (Positive, Negative, Suspect) provided by University of Florida Mary Brown Lab.

Dataset S4. Proximity logger contacts in wild population.