



Social group size influences pathogen transmission in salamanders

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Abstract

Individuals within animal societies are expected to mitigate the costs and enhance the benefits associated with group living. For example, sociality can facilitate the sharing of beneficial microbes among individuals, but can also increase transmission of pathogens, representing a major cost of group living. We examined the costs of sociality in the California slender salamander (*Batrachoseps attenuatus*), a terrestrial salamander which naturally forms close social aggregations. We investigated whether innate sociality (e.g., skin-to-skin contact) increases an individual's transmission risk of *Batrachochytrium dendrobatidis* (*Bd*), a fungal pathogen that emerged throughout the salamander's range over the last 50 years and has decimated hundreds of amphibian species globally. We found that in captivity, *B. attenuatus* exhibit random mixing within social groups, resulting in high contact rates and high potential for *Bd* transmission. Our experimental infection trials resulted in 50% mortality after 1 month in moist conditions. In order to test how group size affects pathogen transmission, we manipulated social group size and found a marked effect on the spread *Bd* among individuals; a single, uninfected individual contracted *Bd* much more rapidly in larger groups of infected individuals. Surprisingly, this did not translate into a more rapid death rate or higher pathogen infection loads. Our results show that the innate behavior of group formation represents a per-individual risk of socially acquired pathogens, with direct transmission being magnified in larger social groups. This study highlights one important cost of sociality in terrestrial salamanders and underscores the general susceptibility of social animals to novel invasive pathogens.

Significance statement

Social behaviors typically evolve due to the benefits of associating with others, but they can also present risks such as disease transmission. The California slender salamander is highly social, with individuals forming close aggregations underneath cover items. Populations of this species have recently been discovered to suffer from the widespread and deadly fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) which is transmitted through aquatic zoospores. Because this salamander host species is fully terrestrial, we set out to determine if close aggregations (leading to skin-to-skin contact) provide opportunities for direct transmission of *Bd*. Infection trials in larger social groups revealed a more rapid spread of *Bd*; however, we did not witness more rapid death rates or ultimately higher pathogen infection loads. Our results show that the social behavior of these salamanders leads to a higher probability of acquiring *Bd*, highlighting the complex effects that emergent pathogens may have on social species.

Keywords Amphibians · Chytridiomycosis · Disease · Social behavior · Salamanders · *Batrachochytrium dendrobatidis*

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Introduction

The evolution of animal societies via natural selection represents a balance between the fitness benefits associated with group living and the costs of such behaviors. While group living can increase direct fitness through mechanisms such as social foraging or predator defense, costs of living in groups include conflict over resources as well as higher risk of parasites (Patterson and Ruckstuhl 2013; Schmid-Hempel 2017).

Symbiotic microbes, such as bacteria and fungi, represent both a cost and a benefit of group living. For example, sociality can facilitate the transmission of beneficial microbes among individuals, stabilizing altruistic associations (Lombardo 2008; Lewin-Epstein et al. 2017). Conversely, socially transmitted microbial pathogens represent a major cost of group living and have the potential to disrupt the stability of animal societies (Kappeler et al. 2015; Ezenwa et al. 2016a, b). As a result, it has been recognized that social animals may be particularly vulnerable to emerging wildlife diseases, which are now considered a major cause of recent biodiversity loss (Wake and Vredenburg 2008; Vredenburg et al. 2010; Barnosky et al. 2011; McCallum 2015).

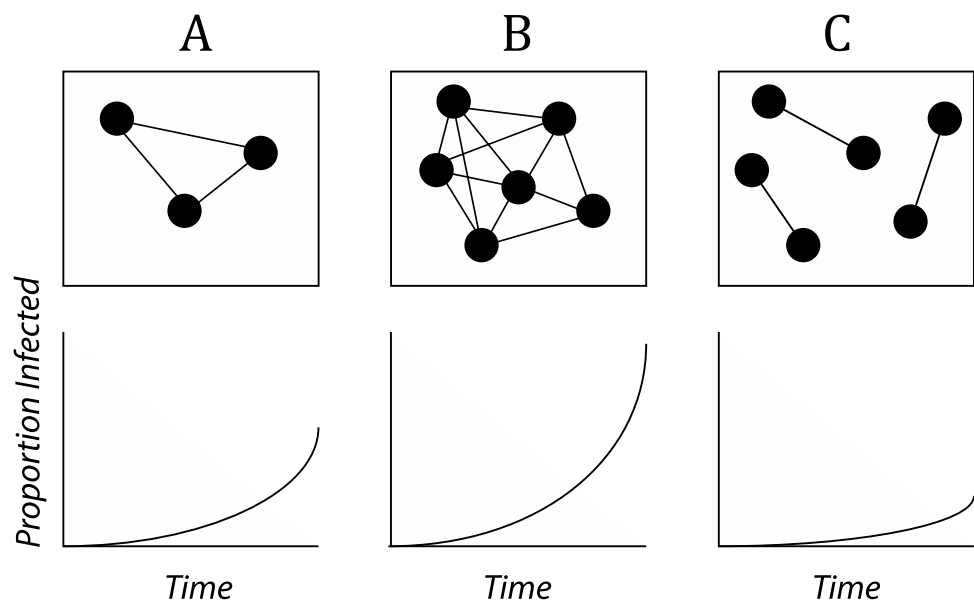
While disease-driven population declines have been witnessed in a broad range of social animals, the specific mechanisms influencing disease dynamics are varied (Kim and Harvell 2004; Cameron et al. 2011; Hewson et al. 2014). For example, it is often the specific network of interactions that most influences the initial spread of a disease (Dolan et al. 2014; White et al. 2017, 2018). While the size of social groups is usually positively associated with rates of disease transmission, the degree to which individuals interact on a more local (versus global) scale is also an important factor (Côté and Poulin 1995; Ezenwa 2004; Rowley and Alford 2007). For example, greater population structure (localized interactions) leads to more contained disease outbreaks relative to random widespread mixing (Boots and Meador 2007; Miller 2009). This underscores the importance of understanding rates of social mixing in the context of behaviors such as mating, food sharing, nesting, and parental care (Naug 2008; Hamede et al. 2009; Wendland et al. 2010; Langwig et al. 2012).

Tasmanian devils, for example, exhibit localized social networks that restrict facial tumor disease from spreading

among all individuals (Hamede et al. 2009). Social behavior in gopher tortoise populations is also highly structured, leading to low transmission rates of upper respiratory tract disease between adults and juveniles (Wendland et al. 2010). In contrast, little brown bats display more uniform social mixing which facilitates transmission of white-nose syndrome; as a result, disease prevalence is strongly correlated with larger aggregations at roosting sites (Blehert et al. 2009; Langwig et al. 2012; Eskew and Todd 2013). In summary, it is the network types of host species (highly networked, loosely networked, or uniformly mixing) that directly impact disease dynamics (Gear et al. 2013; Nunn et al. 2015; Sah et al. 2018). Figure 1 depicts a simple graphic of these relative influences of group size with and without local networked interactions.

The fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) has now been found in hundreds of amphibian species, causing the disease chytridiomycosis (Lips et al. 2006; Skerratt et al. 2007; Wake and Vredenburg 2008; Cheng et al. 2011; Martel et al. 2013; James et al. 2015). The infective stage of *Bd*, flagellated zoospores, encyst in the skin of their amphibian hosts and disrupt osmotic balance as the infection spreads across the skin, eventually leading to cardiac arrest and death (Longcore et al. 1999; Berger et al. 2005; Voyles et al. 2009, 2012). While *Bd* is currently the focus of much research worldwide, it is not understood why amphibian species vary in their susceptibility to *Bd* (Lloyd-Smith et al. 2005a; Briggs et al. 2010; Bancroft et al. 2011; Reeder et al. 2012; Searle et al. 2011; Voyles et al. 2014; Chaukulkar et al. 2018). Given the broad range of social behaviors in amphibians, the *Bd*-host system presents an opportunity to understand the relationship between host sociality and pathogen spread. In particular,

Fig. 1 Connectivity (localized interactions) and density of hosts affect disease dynamics over time. A randomly mixing smaller group (A) is expected to have lower pathogen transmission than a larger randomly mixed group (B), but even a large group can have lower transmission if individuals are spatially connected (C). In group C, transmission events are isolated in small subsets of the population



because *Bd* is an aquatic fungus spread by swimming zoospores, fully terrestrial amphibians are most likely acquire *Bd* socially via skin-to-skin contact (Piotrowski et al. 2004; Kolby et al. 2015; Sette et al. 2015).

The fully terrestrial California slender salamander, *Batrachoseps attenuatus* (Family: Plethodontidae), provides an opportunity to study the transmission of *Bd* via social interactions. Highly abundant throughout their range, these salamanders are extremely sedentary, with individuals having a home territory estimated at less than two meters (Hendrickson 1954); this has presumably led to impressive levels of geographic isolation and speciation throughout the genus *Batrachoseps* in California (Jockusch and Wake 2002; Jockusch et al. 2020). Sensitive to desiccation and heat, all life stages of *B. attenuatus* live underground or on the soil surface under cover items (Sette et al. 2015). Like other plethodontid salamanders, this species is often found in large aggregations of individuals (Hendrickson 1954; Jaeger and Forester 1993) and exhibits joint nesting (Harris et al. 1995; Jockusch and Mahoney 1997).

Salamanders of the Family Plethodontidae (such as *B. attenuatus*) lack lungs and breathe through their skin. Hyperkeratosis, a thickening of the skin, is one of the symptoms of chytridiomycosis, and suggests that these lungless amphibians may be particularly susceptible to *Bd* (Longcore et al. 1999). Field-acquired *Bd* infections have been documented in *B. attenuatus* and symptomatic individuals that are brought into the lab often die within weeks (Maiorana 1977; Weinstein 2009). In addition, experimental infections of individually housed *B. attenuatus* in moist conditions resulted in over 50% mortality (Weinstein 2009). The high level of sociality in this species, combined with susceptibility to a horizontally transmitted disease, provides a unique opportunity to investigate how behavior, social group size, and proportion of infected hosts all interact to influence disease transmission and survival. In this study, we document rates of mixing in salamander groups and show they conform to a random mixing pattern that theory predicts should be associated with high levels of disease transmission (Lloyd-Smith et al. 2005a, b). In the lab, we follow individually marked salamanders and manipulate group size, in order to test the effects of group size and proportion of infected hosts on *Bd* transmission. We hypothesize that *Bd* transmission occurs faster in larger host groups and when the ratio of infected to uninfected hosts is higher within a given group. Results of this study are directly relevant to the costs of group aggregations for *B. attenuatus* and other plethodontid salamanders, which informs the conservation of these social species as well as our general understanding of the relationships between pathogen transmission and social behavior for wildlife diseases.

Methods

Animal collection

Prior to experiments, 181 individuals of *B. attenuatus* were hand-collected from wild populations in Contra Costa, Marin, Napa, and Sonoma counties in California between April and June of 2014. Each individual was put into a temporary, separately marked container upon collection, placed into a cooler and transported back to the laboratory within 8 h of capture. A skin swab was taken for each individual in the field at time of collection to verify their initial *Bd* status. We used this same standard skin swabbing technique throughout all subsequent experiments for assessing *Bd* status (Boyle et al. 2004; Hyatt et al. 2007). All experiments used new sterile swabs and gloves for every individual sample. Swabs were analyzed using a qPCR *Bd* assay that tests for the presence of infection and indicates infection intensity in terms of estimated number of zoospores (Boyle et al. 2004; Hyatt et al. 2007). Only ten of the 181 salamanders we collected tested *Bd*-positive from the initial swab taken at the time of collection; all ten individuals were excluded from experiments leaving 171 salamanders.

All salamanders were initially housed individually in separate cages in the SFSU Animal Care Facility and were cared for in accordance with SFSU IACUC (#A15-05 Zink and Ritchie) approved protocols (which included daily monitoring). During this initial housing period, and also during all subsequent experiments, salamanders were fed ad libitum twice a week with pinhead crickets or wingless *Drosophila*. Salamanders were housed in a temperature-controlled room that ranged from 16 to 20 °C in standard mouse cages with lids (25.5 × 18 × 15 cm) for two experiments (1 and 3) and in a larger standard rat cage (48 × 46 × 27 cm) for experiment 2. All behavioral observations and skin swabs were conducted during lighted hours, and all cages were exposed to a 12-h:12-h light to dark cycle throughout all experiments. All cage bottoms were lined with moistened, sterile paper towels, with one towel loosely balled together in the center of the cage to provide the salamanders with both substrate and cover. Towels were re-moistened as necessary and replaced with new ones during cage cleaning which occurred 1–2 times per week. To minimize observer bias, blinded methods were used whenever possible for collecting and analyzing data. The behaviors of each salamander were monitored using the unique and distinct color patterns that naturally vary across individuals.

Experiment 1: Establishing baseline survival and social mixing in uninfected individuals

We randomly chose 104 individuals from the 171 uninfected healthy field-collected individuals, placing them in groups of one (“single host”) or three (“trio host”) individuals; this

resulted in a total of 26 replicate cages per treatment. We observed these salamanders over a period of 60 days in order to document baseline patterns, social behavior, and survival of individuals. We used the survival rates of uninfected individuals as controls in a comparison with survival rates of infected individuals in experiment 3 (described in more detail below). Individuals in the groups of three were tracked using their unique (natural) dorsal markings to determine if individuals randomly associate or form exclusive pairings. We chose a group size of three salamanders because this is the average group size of *B. attenuatus* in field studies (Sette et al. 2015); given our cage size this resulted in one salamander per 150 square cm which was well within field-measured densities under cover items (Sette et al. 2015). The infection status of individuals was also monitored using qPCR from a skin swab every 6 days throughout the entire experiment.

Salamanders were given 2 days to acclimate to their new cages before behavioral observations began on July 16, 2014. After this acclimatization period, we recorded any pairing behaviors (defined as skin-to-skin contact between two specific individuals) every 2 days, for a total of 60 days (30 total observations per salamander). When two individuals were observed touching, we visually approximated the percentage of body overlap between individuals. Photographs were taken to further verify data collected. Natural variation in individual markings, coloration, and size (snout-to-vent length) allowed us to keep track of individual location, orientation, and pairings with other similarly identified individuals within each group of three salamanders.

Statistical tests that were performed included a survival analysis for individuals in groups of one and three using R (more details in the methods for experiment 3). We also calculated the percentage of groups (of three) displaying aggregation behavior by at least two individuals on each separate day and then took the average percentage across all 30 days of observation. We used a linear regression model to determine the effect of observation date on the frequency of aggregation behavior across all the trios. We calculated the proportion of days a specific individual spent touching another individual and used a Shapiro–Wilk *W* test to determine if the number of contact days fit a normal distribution. We also analyzed the percentage of body contact in all pairings to determine the average overlap between individuals. Finally, for each of the 26 trios, we identified the most common pairing (among the three possibilities) and used a Fisher's exact test to determine if this pair was more common across all 30 observations in that trio relative to the two other pairing alternatives. Only one pair across all 26 trios exhibited a non-random association of repeated pairings, but this was not significant after a Bonferroni correction. The assumptions of the above statistical tests were also checked and verified.

Experiment 2: Pathogen transmission in large social groups with low initial infection prevalence

In this experiment, we inoculated seven salamanders from the initially *Bd*-free field collection with *Bd* lab strain CJB57-(4)-p6 (collected in the field at Marmot Lake, Sierra Nevada, CA, in 2011). An additional 77 uninfected field-collected salamanders that had been taken from experiment one and housed individually were randomly assigned to each of three separate cages measuring 48 × 46 × 27 cm; this density was approximately twice that of experiment one but still within range of natural field densities under cover items (Sette et al. 2015). The *Bd* status of all 77 uninfected individuals was verified at the beginning of the experiment using the qPCR assay. The seven lab-infected individuals (with initial *z*-swab levels averaging 91.8 and ranging from 22–223) were split among the three cages, resulting in a total of 28 salamanders in each cage. The first two of these cages began the experiment with two *Bd*-infected individuals and the other cage (cage 3) began with three *Bd*-infected individuals (initial prevalence of 7% and 11% respectively). We took care to distribute these seven infected individuals such that the initial average *z*-swab score was similar across cages. Starting on October 6, 2014 (exactly 3 days after groups were formed), we collected skin swabs from all salamanders in each of the three cages twice per week for 3 weeks. At the terminal timepoint of 3 three weeks, all salamanders were swabbed for *Bd* and subsequently placed back into individual cages.

We calculated the proportion of individuals infected with *Bd* in each of the 3 groups across the 3-week experiment duration. After accounting for the initial infection frequency, we used the proportion infected at each (semi-weekly) time step to estimate the maximum R_0 (for a given time step) in each of the three cages (conforming to outbreak conditions for smaller populations; Lloyd-Smith et al. 2005b). We also calculated the average *z*-swab infection intensities across the infected individuals within each of the three cages for each time step. An outlier individual from cage R3 at day 4 was excluded from analyses due to a *z*-swab score of over 19 million which was considered biologically unrealistic for a single swab sample.

Experiment 3: Risk of infection in social groups with high initial infection prevalence

This experiment began immediately after experiment 2 (October 27, 2014) and used 64 randomly chosen infected individuals from that experiment. We established 16 cages which each received one uninfected (focal) individual with unique markings that had not been exposed to any other salamanders (i.e., had been housed individually) and tested negative for *Bd* (i.e., had never been *Bd*-positive during their entire time in captivity

or at time of collection). The 64 infected individuals were then randomly allocated in two treatments groups: a “pair group” where one infected joined one uninfected focal, and a “large group” where seven infected joined one uninfected focal. Each of the two treatments was therefore replicated 8 times (16 cages). In wild populations, California slender salamanders are commonly found under cover items (e.g., logs) in pairs or trios, but they can also be found in larger groups of more than ten individuals under a single cover item (Sette et al. 2015).

Throughout the experiment all focal individuals ($n = 16$) remained identifiable using photographs and notes on coloration and markings. We collected a single swab from each individual once a week for 7 weeks. In the case of a mortality event for focal or non-focal individuals, we did not replace them but continued to monitor remaining individuals in the group. Seven weeks after the initiation of the experiment, all remaining salamanders were euthanized using standard MS-222 protocols approved by the SFSU IACUC (#A15-05 Zink and Ritchie) and deposited as specimens to the permanent collection of the Museum of Vertebrate Zoology, University of California at Berkeley. An explicitly paired control (non-*Bd*) was not run alongside this experiment due to small numbers of salamanders available; however, we used the uninfected individuals (housed singly and in groups of three) from experiment 1 as controls since they experienced similar experimental conditions.

Statistical tests that were performed included a Fisher’s Exact test for the proportion of initially uninfected focal individuals (in each treatment) that became infected in week 1 versus week 2 (virtually all individuals were infected by week 2). We calculated the mean z -swab for infected focal individuals (in both pairs and groups of 8) each week, as well as the mean z -swab for infected non-focal individuals. Multiple tests comparing these z -swab values across the entire experiment did not reveal any differences between pairs versus groups, so we focused our attention on the initial 3-week period when individuals were acquiring their infections. In this case, we calculated mean z -swab and maximum mean z -swab for infected focal individuals across this period, using t -tests to compare these z -swab values. To determine if there was a difference in the survival rate of focal individuals in pairs versus groups of 8, we performed a survival analysis in R using `servfit` function. We then compared these survival data to the survival of a subset of uninfected individuals from experiment 1 that had neither contracted nor been exposed to *Bd* (half of all “single host” cages and half of all “trio host” cages), using the same survival analysis. The assumptions of the above statistical tests were also checked and verified.

Results

Experiment 1: Establishing baseline survival and social mixing in uninfected individuals

We found that on any given day, approximately one-third (mean = 7.8, SD = 2.7) of the 26 “trio host” replicates displayed aggregation behavior (skin-to-skin contact) during the 30 days of observation. While the total number of days trio hosts were found aggregating across all 30 observations varied, the average number of aggregation days (for any given trio) was approximately one-third of the observation days (mean = 9.0, SD = 2.9). These instances of aggregation were clearly a behavioral choice by individuals in the group, as the amount of available surface area in the cages easily allowed individuals the choice of remaining solitary. Often individuals were completely “aligned” in terms of entire torsos touching; the degree of overlap for any specific touching event ranged from 5 to 100% body contact across all instances observed, with an average of 49% overlap. Individual contact did not always occur in the paper towel; on average 29% (SD 22%) of the observations for a given individual were in the towel.

In each of the 26 trios monitored, we identified the most common pairing (two among the three) and tested if this pair was more common across all 30 observations in that trio relative to the two other pairing alternatives. Only one pair (across all 26 cages) exhibited a non-random association of repeated pairings (Fisher’s exact test, $p = 0.004$), but this was not significant after a Bonferroni correction for multiple tests among all trios tested. Our conclusion was that the individuals within all trios were pairing at a moderate frequency (one-third of the time on average) and doing so completely at random and without preference among the two other individuals in their cage.

While some individuals were more social than others (higher proportion of days spent touching another individual) the number of contact days for individuals fit a normal distribution (Shapiro–Wilk W test, $W = 0.97$, $n = 78$, $p = 0.06$). This suggests that there are not discrete alternative tactics of social versus asocial individuals. In addition, we found no effect of day (observation) number on the frequency of aggregation behavior across all the trios using a linear model ($R^2 = 0.01$, $n = 30$, $p = 0.70$). The survival of solitary salamanders was not different than that of individuals within trios using a survival analysis ($X^2 = 0.3$, $df = 1$, $p = 0.56$), showing that (with no disease present) there was no intrinsic short-term survival cost of being in a social group versus being solitary. This indicates that being grouped randomly in a laboratory setting does not have any detrimental effects on survival due to intrinsic factors (e.g., unmeasured pathogens, aggression). Taken

together, these results indicate that, within the timeframe that is required to spread *Bd* in these salamander groups, a model using assumptions of random mixing and constant interaction rates among group members is warranted for further experiments using this species.

Experiment 2: Pathogen transmission in large social groups with low initial infection prevalence

Within a 3-week period, the average proportion of individuals infected with *Bd* across the 3 groups of 28 salamanders went from 8.3% to 91% (Fig. 2a). Thus, *Bd* transmission through direct contact with conspecifics occurs rapidly in large groups of *B. attenuatus*, even when initial *Bd* prevalence is very low. After accounting for the initial infection frequency, we used the proportion infected at each (semi-weekly) time step to estimate the maximum R_0 (for a given time step) in the three cages as 5, 4.5, and 1.4 respectively (conforming to outbreak conditions for smaller populations; Lloyd-Smith et al. 2005b). In addition, we found a corresponding increase in the average z -swab infection intensities across the infected individuals within each of these three cages over time (Fig. 2b). An outlier individual from cage R3 at day 4 was excluded from analyses due to a z -swab score of over 19 million which was considered biologically unrealistic for a single swab sample. Over the 3-week time frame of this experiment, one individual died in cage 2, one in cage 3, and six in cage 1.

Experiment 3: Risk of infection in social groups with high initial infection prevalence

Group size affected transmission rate of *Bd* as there was a significant difference in the time that it took focal individuals to become infected; uninfected focal individuals in the “large group” treatments became infected more rapidly than in the “pair group” treatments within the first two weeks (Fisher’s exact test, $df=2$, $p=0.007$; Fig. 3a). Specifically, all focal individuals in “large group” treatments (100%) became infected within the first week of the experiment, whereas only 2 out of 8 in the “pair group” treatment (25%) became infected during the same time period. All focal individuals became infected after the first three weeks with the exception of one individual from the pair group (Fig. 3a).

We calculated the mean z -swab across all infected focal individuals in both groups relative to the z -swab average of the non-focal individuals in their group (single or average of 7) during the course of the experiment (Fig. 3b). We found that, once infected, the focal individual’s infection intensity progressed similarly independent of group size (Fig. 3b). Over the entire first 3-week period, while individuals were first acquiring infection, there was no significant difference in either the average zoospore

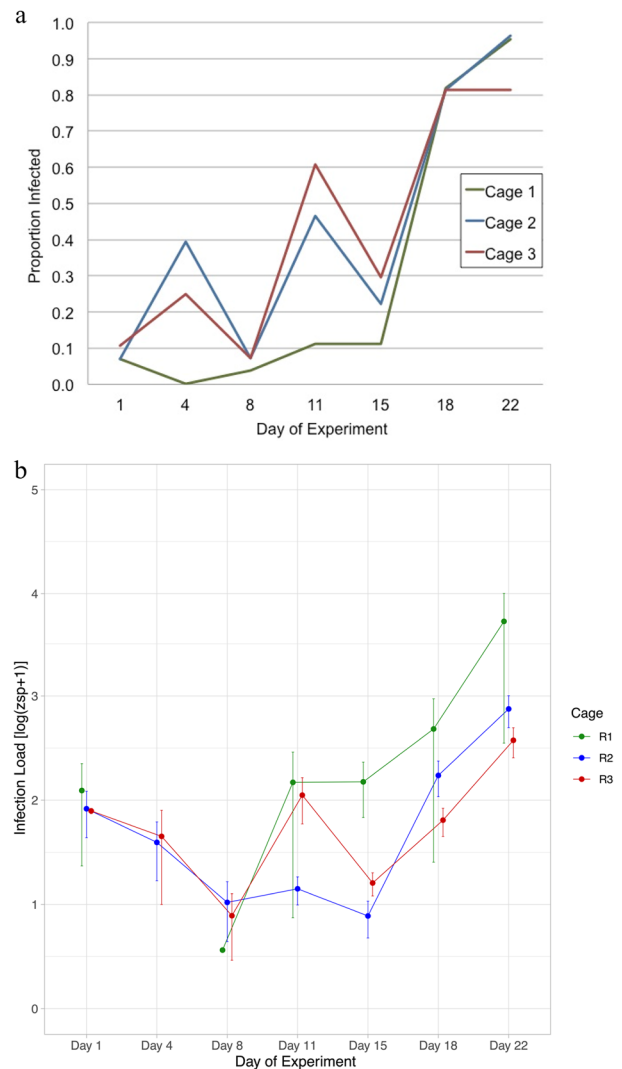


Fig. 2 **a** The change in proportion of *Bd*-infected individuals in three aggregations of California slender salamanders through time in experiment 2. All cages started with 28 individuals. Cages 1 and 2 started with two infected individuals while cage 3 started with three infected individuals. At the end of the experiment, cage 1 had 22 individuals, and both cages 2 and 3 had 27 individuals (due to mortality). **b** The average z -swab data for just the infected individuals in each of the three cages in experiment 2 over each time period, including standard errors around the mean. All three cages were sampled on the same days (indicated on the x -axis) but are offset in the figure for clarity. Note that there is no data for cage 1, day 4 as no individuals had detectable infections

infection intensity (load) or the maximum zoospore *Bd* infection intensity between infected focal individuals from the “pair groups” or the “large groups” ($t=1.16$, $df=14$, $p=0.27$; $t=1.71$, $df=14$, $p=0.26$, respectively). This pattern continued for the remaining weeks, during which we also found that non-focal individuals in both group sizes maintained a similar *Bd* infection intensity (load) over time (Fig. 3b).

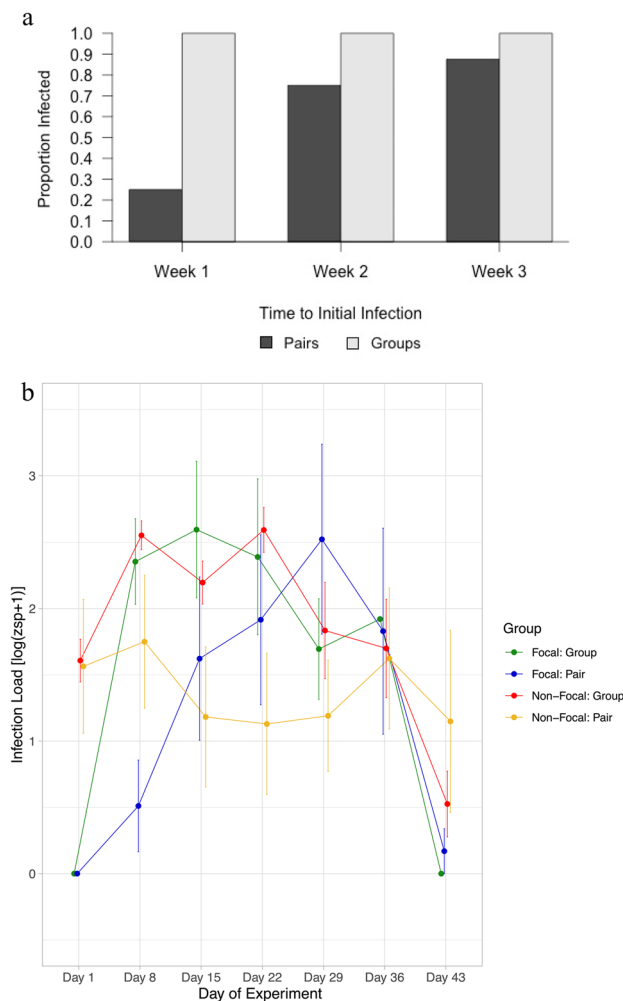


Fig. 3 **a** Time to initial infection for focal individuals who were uninfected at week 0 in experiment 3. Comparisons of these focal individuals in pairs (i.e., housed with one infected individual only) versus in groups of 8 (i.e., housed with seven infected individuals) show that it took significantly longer for focal individuals in pairs to become infected when compared to focal individuals in groups of 8 (Fisher's exact test, $p < 0.01$). Proportion of individuals infected over time is out of the eight (initially uninfected) focal individuals per treatment. **b** Average z-swab data (infection load) in experiment 3 for just infected individuals in pairs versus groups of eight, including standard errors around the means. Note that focal individuals (one per group, initially uninfected on day 1) are averaged at each time step across the paired or large group treatments respectively. The initially infected (non-focal) individuals in paired groups were also averaged at each time step. Because there were up to seven non-focal individuals in large groups that could not be followed individually, we used the average of these non-focals within each group and the graph represents the mean of those values across all 8 groups. Swabs were all collected on the same day (once per week) but data points are offset for clarity. Mortality over the 43 days led to decreased sample size at each time point with only four focal individuals surviving to day 43

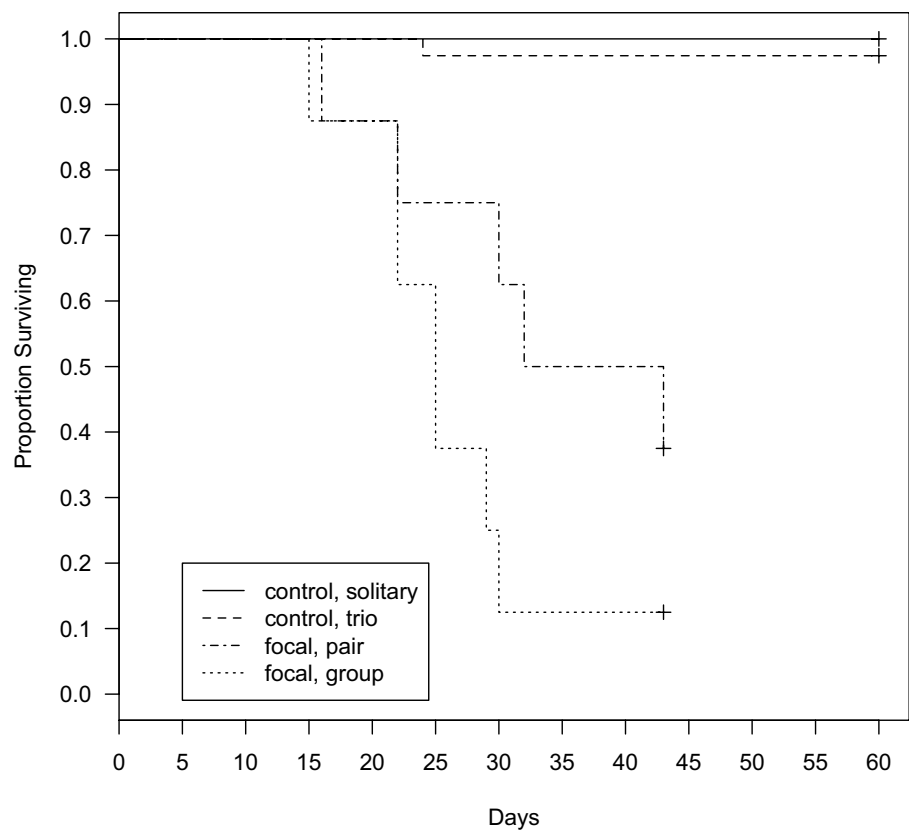
A survival analysis for experiment 3 showed an equally high risk of mortality among focal individuals in both treatments during the seven-week period ($X^2 = 2.4$, $df = 1$,

$p = 0.12$; Fig. 4). However, when we compared survival between focal individuals of both group types (experiment 3) and uninfected individuals from experiment 1, there was a clear effect of *Bd* infection on survival across the same time intervals (survival analysis in R, $X^2 = 60.7$, $df = 3$, $p < 0.0001$; Fig. 4). We also found that average rates of contact for focal individuals over all timepoints in experiment 3 were similar to that of trios in the experiment 1, and there was no difference between overall rates of contact by focal individuals in the group sizes of 2 and 8 (two-tailed t -test, $t = 0.72$, $df = 14$, $p = 0.48$). However, one obvious difference was that in “pair groups” contact occurred between the same individuals, versus contact among new individuals in “large groups”.

Discussion

For many amphibian species it is well established that sociality confers direct benefits to group members; examples include thermoregulation, social foraging, predator avoidance, and even the transmission of mutually beneficial bacteria (Bradford 1984; Jaeger and Forester 1993; Harris et al. 2006; Blaustein et al. 2011). In the current study, we found evidence that the innate behavior of social grouping by the California slender salamander also confers significant costs. In particular, we found an increased rate of lethal pathogen transmission to previously uninfected individuals when they were housed with greater numbers of infected individuals in a laboratory setting. A previous study had found that the California slender salamander is highly susceptible to dying from chytridiomycosis in the lab (Weinstein 2009). We also witnessed high mortality of our study species when individuals were exposed to the fungal pathogen *Batrachochytrium dendrobatidis* and housed in moist conditions. Interestingly, the previous study also found that experimentally infected individuals were less likely to die when housed in dry conditions, but it was unable to compare infected individuals directly to uninfected controls due to small sample size (Weinstein 2009). Our experiment helps clarify the effects of *Bd* on California slender salamanders (under laboratory conditions) by confirming that the *Bd*-associated mortality in field-collected *B. attenuatus* observed by Weinstein (2009) was likely due to *Bd* alone, and not to other correlated factors. It is interesting to note that, while this species occurs in microclimates across its range that are described as moist (Jockusch and Wake 2002; Jockusch et al. 2020), the prevalence of *Bd* in field populations remains quite low (approximately 15%; Sette et al. 2015). Individuals with later stages of chytridiomycosis are rarely observed in the field, despite high susceptibility in the lab, likely due to the fact that these salamanders are more cryptic than other amphibians such as frogs.

Fig. 4 Survival analysis for uninfected control individuals (no *Bd*) housed in solitary and trio groups) from experiment 1 revealed that there was no mortality attributed to effects of sociality (with no *Bd*) or captivity (with no *Bd*). Initially uninfected “focal” individuals in experiment 3 did experience mortality when placed with one infected individual (termed “pair”) or placed with 7 infected individuals (termed “group”). All of these focal individuals in experiment 3 eventually became infected with *Bd* and suffered increased mortality risk as a result



Our results indicated that uninfected focal individuals acquired *Bd* more rapidly when in larger groups relative to small groups (“pair groups”); however, this did not necessarily translate into differences in mortality during the time-frame of our experiment (Fig. 4). It is important to note that, due to limited numbers of individuals available for collection as a result of the 2014 California drought, our experimental design did not separate the relative effects of group size and proportion of individuals infected. Future experiments will examine the relative influence of (and potential interaction between) these two factors. Nevertheless, while larger group size appears to increase transmission and risk of acquiring *Bd*, group size alone may not impact survival. Rather, it is the continuum of sociality versus asociality that is most important as well as the duration and extent of skin-to-skin contact within social species (e.g., in our experiment touching events involved half of an individual’s full body length, on average, in contact with another). Individuals that are more solitary may eventually avoid infection altogether, raising the question of why these salamanders are so highly social in nature. Social behavior is likely to prevent desiccation, aid in thermoregulation, and may even provide protective mechanisms to protect against pathogens (e.g., anti-*Bd* bacteria) under some circumstances (Bradford 1984; Harris et al. 2006; Vazquez et al. 2009; Vredenburg et al. 2011).

We cannot rule out that social grouping of *B. attenuatus* spreads beneficial microbes that suppress *Bd* zoospore development in the field as seen in other amphibian species (Woodhams et al. 2007; Walke et al. 2011; Walke and Belden 2016). Indeed, in other animal systems, mutualistic species can increase shared benefits among social group members in ways that stabilize sociality (Frank 1994; Morales 2000; Zink 2015). However, given the results of our experiments, such a situation would require that anti-*Bd* mutualistic bacteria were unable to survive in our lab environment or were not present in the populations of salamanders where we collected individuals for these experiments. It is more likely that *B. attenuatus* evolved their grouping behavior for other reasons and this behavior has more recently become an evolutionary liability by increasing transmission of novel parasites or diseases (Altizer et al. 2003; Frick et al. 2010). The fact that *Bd* appears to have only recently invaded the west coast of North America also suggests that the costs of sociality due to this deadly pathogen are only recently imposing natural selection for more asocial behaviors in *B. attenuatus* hosts (Sette et al. 2015, 2020).

We regularly witnessed skin-to-skin contact in our groups of salamanders (both infected and uninfected) despite large arenas, suggesting active aggregation by individuals, and this behavior is similar to what has been observed in nature

(Sette et al. 2015). Our results (experiment 1) clearly show that these salamanders do not exhibit individual preferences within aggregations and that contact occurs randomly within social groups. Future work could further verify this through video recordings covering longer time periods of observation rather than using photographic evidence taken every 2 days. It is important to note that while this contact is likely to be a main mechanism for transferring *Bd* zoospores, it is also possible that zoospores were shed on the moist paper towel and picked up by other individuals without direct contact. It is also possible that we might have seen more social structure (e.g., preferred pairings) if we had housed individuals together who had a familiar history, rather than randomly associating them. In addition, we were unable to choose individuals based on sex in the formation of our groups because sexual identification is not possible in most instances. While our experiments did not occur during the winter mating season, future work should consider the role of mating behavior in *Bd* transmission.

Is it possible that *Bd* infection status shifts the behavior of *B. attenuatus* individuals to behave in a way that is more or less social, which can cause important feedbacks on disease dynamics (Ezenwa et al. 2016a, b; Hoverman and Searle 2016). Increased activity by infected individuals could increase transmission rates and spread of disease (Venesky et al. 2011). We could not address this issue directly in our experiments; however, studies of larval and adult amphibians have found mixed results in terms *Bd* infection status and the propensity to aggregate (Han et al. 2008; Venesky et al. 2011; Koprivnikar et al. 2012). Unfortunately, in experiment 3, we were unable to observe more than a few occasions of specific contact within the initial two weeks (totaling 2 days of behavioral observations) prior to the rapid infection, making it impossible to assess contact rate as a predictor of *Bd* acquisition in focal individuals. This also made it impossible to ascertain if *Bd* acquisition causes individuals to become more or less social without sufficient pre-infection data with which to compare. Future work on *Bd* in terrestrial amphibian populations should consider whether infection status decreases the sociality of individuals and whether this change in behavior affects transmission rate (Dolan et al. 2014; Han et al. 2015; Araujo et al. 2016).

Repeated exposure to a deadly transmissible pathogen over time could shift the social behavior within host populations as a result of natural selection. In fact, previous work revealed that *B. attenuatus* populations with a longer history of *Bd* are less likely to aggregate relative to populations that are only recently infected (Sette et al. 2015). Similar results were found in bat populations which exhibited less roosting behavior after a fungal pathogen had swept through the population (Langwig

et al. 2012). Rather than short-term changes in behavior due to *Bd*, these data suggest that there may be long-term natural selection for more asocial behavior in response to socially transmitted outbreaks of *Bd*. Future work will address the complementary effects of short-term *Bd* avoidance behaviors (e.g., via learning) and long-term shifts in social behavior of populations due to natural selection via *Bd*-caused mortality (as suggested by Sette et al. 2015). Our study is the first step in experimentally revealing an effect of varying group size on *Bd* transmission in fully terrestrial amphibians; as such, it adds to the broader evidence for rates of parasitism and disease being highly influenced by group size across a range of social species (Côté and Poulin 1995; Brown and Brown 1996; Ezenwa 2004; Rifkin et al. 2012). Our results suggest that, unlike aquatic amphibians which can contract *Bd* via swimming zoospores in the water column, terrestrial amphibians may be most susceptible to contracting *Bd* when they exhibit group living and skin-to-skin contact.

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Author contributions KR, AZ, and VV conceived and designed study, performed statistical analyses, and wrote the manuscript. SC and HB assisted throughout study with behavioral observations, data collection, qPCR assays, and manuscript revisions. All authors gave final approval before publication.

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Data availability All data are on Dryad (<https://doi.org/10.5061/dryad.9cnp5hqj5>).

Code availability R code is available in Dryad (<https://doi.org/10.5061/dryad.9cnp5hqj5>).

Declarations

Ethics approval All procedures were carried out under the approval of the San Francisco State University IACUC committee (#A15-05 Zink and Ritchie). All applicable international, national, and institutional guidelines for the use of animals were followed.

Consent for publication All authors have read and approve of final manuscript version and have agreed to be included as authors.

Conflict of interest The authors have no competing interests.

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