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Research

Whether larval amphibians school does not affect the parasite aggregation rule: testing the effects of host spatial heterogeneity in field and experimental studies

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Almost all macroparasites show over-dispersed infections within natural host populations such that most parasites are distributed among a few heavily-infected individuals. Despite the importance of parasite aggregation for understanding system stability, the potential for population regulation, and super-spreading events, many questions persist about its underlying drivers. Theoretically, aggregation results from heterogeneity in host exposure, resistance, and tolerance. However, few studies have examined how host spatial arrangement – which likely affects both parasite encounter and density-dependent interactions – influences infection and dispersion, representing a critical gap in our current knowledge regarding the possible drivers of parasite aggregation. Using field data from over 165 ponds and 8000 hosts, we evaluated how the spatial clustering of amphibian larvae within ponds 1) varied among different amphibian species, and 2), affected the distribution of parasites within the host population using Taylor's power law. A complementary mesocosm experiment used field-guided manipulations of the spatial arrangement of larval amphibians to create a gradient in host clustering while controlling host density, thereby testing for spatial effects on both infection success and aggregation by three different trematode species. Our field data indicated that larval amphibians exhibited significant spatial clustering that was well captured by Taylor's power law (R^2 0.92 to 0.97 for different host species), but the residual variation only weakly correlated with observed patterns of trematode parasite over-dispersion. Correspondingly, experimental manipulation of host clustering had no effects on parasite infection success or the degree of parasite aggregation among cages or mesocosms. Given the importance of parasite over-dispersion for host populations and disease dynamics, we advocate for further investigations of host and parasite spatial aggregation, particularly studies that incorporate and/or control for heterogeneity in exposure and susceptibility.

Introduction

The aggregated distribution of macroparasites among hosts is a near-universal pattern (reviewed by Shaw and Dobson 1995, Shaw et al. 1998, Poulin 2007, Krasnov et al. 2010, Sanchez et al. 2011). First detailed by Crofton (1971a, b), the tendency of

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most hosts to have few parasites while a small number support high infection loads has been described as a 'fundamental law' of parasitology (Poulin 2007). Consequently, the variance in the number of parasites per host tends to exceed its mean value ('over-dispersion'), and the magnitude of the variance-to-mean ratio has historically been one approach to characterize the degree of aggregation (Wilson et al. 2002). Another is to use the aggregation parameter (k) from the negative binomial distribution (Shaw et al. 1998), for which the majority of host–macroparasite systems have $k < 1$, indicating greater aggregation (Shaw and Dobson 1995). However, these metrics are less suited for comparing the degree of parasite aggregation across multiple host populations with varying infection loads because they change with the mean value of infection. As an alternative measure of aggregation, Taylor's power law describes the relationship between population density and its spatial or temporal variance. The resulting pattern is typically linear in logarithmic space, for which the magnitude of the slope parameter, b, offers an estimate of aggregation across a range of mean infection values (Wilson et al. 2002, Morand and Krasnov 2008).

Such approaches for characterizing and quantifying parasite aggregation lay the foundation both for testing underlying mechanistic hypotheses and understanding their implications for parasite transmission, host–parasite population dynamics, and the ecology and evolution of host–parasite interactions. Parasites are more likely to regulate their hosts through density-dependent processes and exert selection pressure when infections are over-dispersed (Anderson and May 1978, May and Anderson 1978, Anderson 1981, Anderson and Gordon 1982, Poulin 1993, Wilson et al. 2002). If over-dispersion results in a disproportionate infection burden among fewer individuals, these may be more likely to die or exhibit changes in reproductive output. Aggregation can also reduce macroparasite fecundity and survival by promoting within-host competition, thus affecting parasite population dynamics without host death (May and Woolhouse 1993). The net effect of parasitism on a given host population thus depends not just on mean macroparasite burden, but also on its variability among individuals (Boulinier et al. 1996). Intermediate values of over-dispersion may help to maximize parasite fitness when prevalence is low by increasing within-host co-occurrence of males and females while limiting the risk of parasite-induced host mortality (May and Woolhouse 1993, Jaenike 1996). However, the ubiquity of aggregated infections even among larval or non-reproductive parasite stages suggests broader underlying drivers, and recent simulation models show that parasite fitness is increased when their infectious stages attack hosts as aggregations rather than singly, especially when hosts invest heavily in defence (Morrill and Forbes 2016).

Mechanistically, parasite aggregation results from any processes that promote heterogeneity either in hosts' exposure to parasites or in their susceptibility to infection (Poulin 2013). Variation in susceptibility is often associated with differences in host genetics, body condition, sex ratio, prior infection history, or coinfection, while heterogeneity in exposure could stem from host behavior, habitat use, body size distribution, and phenology (Anderson and Gordon 1982, Shaw et al. 1998, Poulin 2013). Disaggregating forces often include demographic processes related to changes in host or parasite reproduction and/or death, such as parasite-induced mortality that eliminates heavily infected hosts from the population, or reduction of parasite fitness (Anderson and Gordon 1982, May and Woolhouse 1993). However, while the role of susceptibility in macroparasite aggregation has been extensively examined (Lysne and Skorping 2002, Galvani 2003, Bandilla et al. 2005, Johnson and Hoverman 2014), the influence of spatial and temporal heterogeneity in exposure has thus far proven more challenging to address.

The field of 'spatial epidemiology' particularly focuses on how ecological processes can result in spatially structured patterns of infection risk and incidence owing to changes in the occurrence of pathogens, hosts, vectors, and their interactions (Ostfeld et al. 2005, Killilea et al. 2008, Acevedo et al. 2015, Cohen et al. 2016). Although some components of exposure to parasites may be reliably linked to host characteristics, stochastic encounters between susceptible hosts and patches of parasites can also contribute significantly to observed aggregation patterns (Calabrese et al. 2011, Gourbière et al. 2015). Pioneering laboratory experiments by Keymer and Anderson (1979) demonstrated how the spatial arrangement of parasite infectious stages can affect aggregation as more clumped distributions of tapeworm eggs led to the greatest over-dispersion of infection among flour beetle hosts, even though the average infection intensity was consistent. More recent theoretical models and fieldwork support that spatial or temporal clustering of parasite infectious stages is sufficient to generate over-dispersion among identical hosts (Leung 1998, Hansen et al. 2004). The highly-aggregated spatial distribution of tick vectors on host mice is one such example, with important implications for human exposure risk to the Lyme disease-causing bacterium. However, the relative influences of host exposure and susceptibility in generating tick clustering are not clear. This might be the simple bad luck of hosts inhabiting areas with high vector densities, i.e. heterogeneity in exposure (Calabrese et al. 2011), while others suggest a greater role for host susceptibility to tick infestation (Devevey and Brisson 2012). That investigators using the same system have arrived at contrasting conclusions illustrates the need to untangle how spatially-related processes may drive parasite over-dispersion – a fundamental step towards understanding natural variation in parasite aggregation (Woolhouse et al. 1997, Lloyd-Smith et al. 2005).

Relative to parasites and vectors, the role of host spatial clustering is less studied. Many animals create aggregations in the form of herds, schools, and flocks owing to benefits such greater foraging efficiency, higher reproductive success, and protection from predation (Hamilton 1971, Parrish and Edelstein-Keshet 1999, Lehtonen and Jaatinen 2016). There is evidence for both increased and decreased parasitism associated with host clusters (Blower and Roughgarden 1988, 1989, McCarthy 1990, Grosholz 1994): herding

mammals experience a decreased risk of attack from biting flies (Hamilton 1971) while shoaling in aquatic animals mitigates against parasites that actively seek out their hosts (Wisenden et al. 2009, Stumbo et al. 2012, Hockley et al. 2014). However, this could lead to greater parasitism if groups are more conspicuous, facilitate between-host transmission, or attract predators (Parrish and Edelstein-Keshet 1999, Rieucau et al. 2015, Lehtonen and Jaatinen 2016), and models often assume that host aggregation increases infection owing to greater searching efficiency by parasites that use chemical or visual location cues (Hassell and May 1973). For instance, spatially aggregated insect hosts experience higher parasitoid attack (Walde and Murdoch 1988, but see Voinovich et al. 1999), with important implications for models of biological control (Walde and Murdoch 1988). Not only have few studies considered host spatial distribution, these primarily focused on infection prevalence or intensity as outcomes rather than considering parasite aggregation, representing a critical gap in our current knowledge regarding the possible drivers of over-dispersion.

Larval amphibians and their trematode (flatworm) parasites represent a useful system to investigate the influence of host spatial distribution. Encysted larval trematodes (metacercariae) in tadpoles do not replicate and are thus ideal for manipulative and field studies (Koprivnikar et al. 2012), while the use of amphibians approaching metamorphosis allows for a standardized comparison that can minimize variation in intrinsic host factors such as age, immunity, sexual maturity, and developmental stage which can affect macroparasite aggregation (Wilson et al. 2002). Notably, tadpoles of many species have clumped distributions within sites driven by microhabitat preferences and interspecific competition (Alford 1986, Smith et al. 2003), but also form transient aggregations in response to predation cues, often in sibling-preferred schools (Waldman 1982, Venesky et al. 2011, reviewed by Wassersug 1973). Such tadpole aggregations have proven important for the transmission of fungal pathogens, whereby host clustering leads to a higher prevalence of the pathogenic *Batrachochytrium dendrobatidis* (Venesky et al. 2011). Whether tadpole spatial clustering affects infection by trematode parasites is currently unknown, but these infections have been shown to frequently exhibit aggregation within their hosts, such that the variance-to-mean ratio consistently exceeds unity, but varies among sites and parasite species (Johnson and Hoverman 2014). Related laboratory experiments found that individual host attributes, including body size, immunity, and particularly behavior, affected trematode infection success and aggregation (Johnson and Hoverman 2014).

In the current study, we used a combination of field surveys and experimental approaches to evaluate how spatial variation in the distribution of amphibian hosts affected the aggregation of their trematode parasites in pond ecosystems. Using field data from over 165 ponds in California, we first tested how spatial variance in the abundance of larval amphibians within a pond changed with mean abundance and host species identity. We then used the residuals from this relationship as an explanatory variable to account for aggregation of larval trematodes among host individuals, between host species, and across ponds. We complemented the field-based analysis with an outdoor mesocosm experiment in which we manipulated the spatial arrangement of larval amphibians while controlling for host density to test its effects on both infection success and aggregation by three different trematode species. Observed levels of parasite aggregation in each experimental condition were then compared with the expected values at similar levels of infection using field data. Taken together, this combination of a large-scale field survey with a mechanistic experiment offers an in-depth assessment of the link between the spatial arrangement of hosts and their parasites in aquatic environments.

Material and methods

Field sampling

As part of an ongoing sampling program in the East Bay region of California (Alameda, Contra Costa and Santa Clara counties), we visited 186 ponds between 2009 and 2015 to examine patterns of host spatial distribution and larval trematode infection. For full field sampling methods see Johnson et al. (2013). In brief, each pond was visited twice; in early summer, we conducted standardized, 1-m long dipnet sweeps every 15 m around the perimeter to quantify the abundance of larval amphibians. These data allowed us to calculate the mean abundance of each species per sweep and its variance. In late summer, we collected 10 to 15 late-stage larvae or recent metamorphs of each amphibian species, measured their snout–vent lengths using digital calipers, and quantified their larval trematodes. Recently metamorphosed amphibians offer a standardized stage in which to assess patterns of infection by parasites transmitted during the aquatic phase, such as larval trematodes. We focused on the four most common amphibian host species: Pacific chorus frogs *Pseudacris regilla*, western toads *Anaxyrus boreas*, American bullfrogs *Lithobates catesbeianus* and California newts *Taricha torosa*. Although three other species occur in these ponds, they are either protected (e.g. California red-legged frog *Rana draytonii* and California tiger salamander *Ambystoma californiense*), or geographically restricted in distribution (i.e. rough-skinned newt *Taricha granulosa*).

Mesocosm experiment

To investigate the effects of host spatial distribution on parasite aggregation, we conducted a mesocosm experiment in which we used cages to control the arrangement of amphibian hosts. Ten days prior to initiating the experiment, we obtained green frog *Lithobates clamitan* tadpoles (Gosner (1960) stage 27 to 29) from Charles D. Sullivan Biological Supply Company (Nashville, Tennessee) and acclimated them to

local well water within indoor aquaria (40-l). Because larval amphibians show relatively little variation in susceptibility to *Ribeiroia ondatrae* after Gosner stage 29 (Schotthoefer et al. 2003, Johnson et al. 2011), this allowed us to reduce variation in intrinsic host factors that can affect macroparasite aggregation (Wilson et al. 2002). Although green frogs do not occur within the field study area, previous research has established that they are susceptible to cercariae of *R. ondatrae* and other trematodes (Holland et al. 2007, Johnson and McKenzie 2009, Koprivnikar et al. unpubl.); patterns of trematode aggregation in *L. clamitans* are also similar to those observed in California amphibians. For instance, among 1252 larval and metamorphic green frogs collected from 17 states in the US, 28% were infected with *R. ondatrae* (4.57 metacercariae per infected host), 14% were infected with *Manodistomum* sp. (3.99 metacercariae per infected host), and 38% were infected with *Cephalogonimus* sp. (39.60 metacercariae per infected host) – the three trematode taxa used in this experiment. Estimates of the slope of Taylor's power law slope for these parasites were 1.76 ± 0.073, $p < 0.00001$, $R^2 = 0.925$, $n = 48$; 1.51 ± 0.066, $p < 0.00001$, $R^2 = 0.868$, $n = 79$; 1.633 ± 0.055, $p < 0.00001$, $R^2 = 0.896$, $n = 102$, respectively.

Tadpoles were fed ad libitum a diet of TetraMin and we necropsied a subset ($n = 11$) to verify they were free of larval trematodes. We filled polyethylene mesocosms (dimensions $175.0 \times 160.0 \times 63.5$ cm) with 750 l of conditioned well water and assigned tanks randomly to one of three treatments based on the spatial arrangement of cages containing amphibian hosts: no aggregation (cages evenly spaced into each of the four quadrants), low aggregation (two cages placed in two diagonal quadrants), and high aggregation (all four cages in a single quadrant) (Fig. 1). Each treatment was replicated 4 times. Groups of three tadpoles were selected at random and placed into small (15.2×14 cm) mesh cages (0.32 cm mesh size to allow cercariae through) suspended 5 cm from the surface and 3.4 cm above the bottom of each mesocosm. We included four cages in each mesocosm for a total of 12 tadpoles per tank. To avoid any unintended positional effects, we randomly varied which quadrant contained cages. For instance, in the high aggregation treatment, we used a random number generator to select which quadrants (north, south, east or west) received the four cages.

To obtain trematode stages (cercariae) infectious to tadpoles, we collected snails (the first intermediate hosts) from pond ecosystems in the East Bay region of California, individually isolated them into 50 ml centrifuge tubes, and examined the water for cercariae over a 24-h period both in the dark and in the light following a natural light: dark cycle for identification. We focused on three trematode taxa: *R. ondatrae* (Family Echinostomatidae) and *Cephalogonimus americanus* (Family Cephalogonimidae) – both of which use rams horn snails *Helisoma trivolvis* as their first intermediate host but birds and mammals or adult amphibians as final hosts, respectively, and *Manodistomum syntomentera* (Family Plagiorchiidae), which infects physid snails *Physa* spp. as first intermediate hosts and garter snakes as final hosts. Over 12 days, we added cercariae daily to each

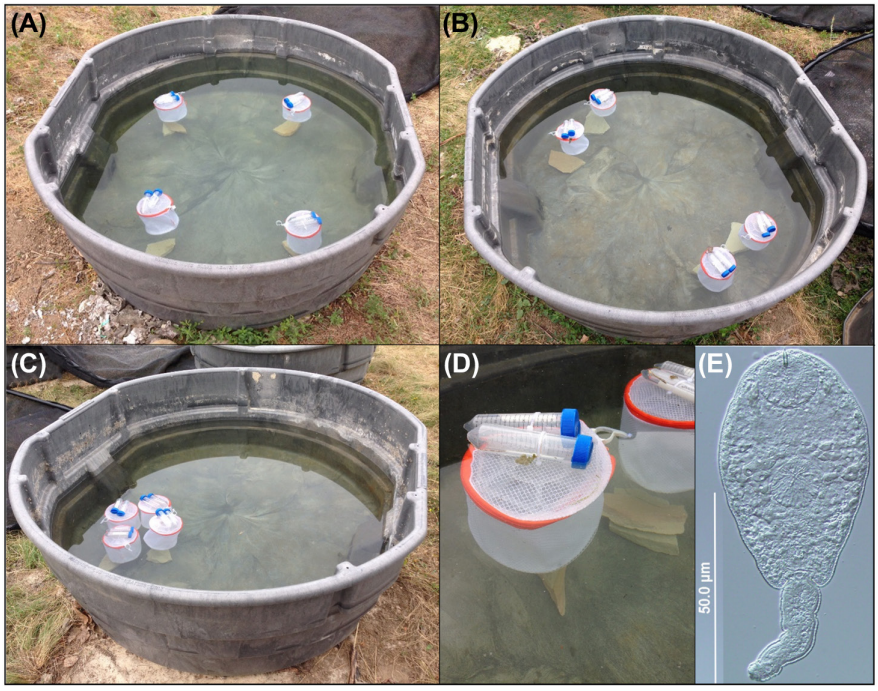

Figure 1. Spatial configuration of amphibians within mesocosms corresponding to three treatments: (A) no aggregation (cages evenly spaced into each of the four quadrants), (B) low aggregation (two cages placed in two diagonal quadrants), and (C) high aggregation (all four cages in a single quadrant), with (D) and (E) illustrating cages used to hold tadpoles and one of the three trematode species used for experimental infections (*Cephalogonimus americanus*), respectively.

mesocosm by allowing infected snails to release cercariae into 50 ml centrifuge tubes filled with treated water for ~6 to 8 h. Water containing cercariae from each snail was pooled into a larger container (volume 20 l) and we used a 40 ml aliquot procedure to transfer subsamples into 500 ml Nalgene bottles. Bottles were assigned randomly to mesocosms, floated for 30–60 min to equalize any temperature differences, and then poured slowly into the center of each mesocosm, taking care to avoid any directional currents. Bottles were rinsed and re-emptied three times using mesocosm water. Two additional Nalgene bottles were used to quantify the number of cercariae per addition and its variance by counting parasites under a stereo-dissecting microscope after refrigeration. This process was repeated individually for each of the three parasite species. For *R. ondatrae*, which emerges at night, snails were shed from ~21:00 to 05:00 h; for *M. syntomentera* and *C. americanus*, snails were shed from ~07:00 to 14:00 h. Thus, parasite additions were performed twice daily.

Tadpoles were fed daily throughout the experiment by adding pre-measured food blocks (pre-rinsed green lettuce and/or TetraMin fish flakes mixed with agar) into each cage and added well water to offset any evaporation. Mesocosms did not contain any sediment, zooplankton, or vegetation as we specifically sought to focus on the effects of host spatial position while minimizing other sources of variation (e.g. stochastic variation in zooplankton could alter transmission through consumption of cercariae). Forty-eight hours after the final parasite addition, we removed each tadpole, recorded its Gosner stage and snout–vent length, and quantified the number of metacercariae for each trematode species. Seven tadpoles died over the course of the experiment prior to removal; because these were distributed among treatments with no more than two individual deaths per mesocosm, we assumed they were unlikely to affect the analyses.

Analysis

For each of the four most common host species at California field sites (*P. regilla*, *A. boreas*, *L. catesbeianus* and *T. torosa*), we used a linear mixed effects model with random effects for site and year to examine the degree of spatial clustering within ponds by the larval stage. Specifically, we regressed the $\log_{10}$mean density (larvae per netsweep within a pond) against $\log_{10}$variance in larvae per sweep and assessed whether the relationship was significant and identified the slope (i.e. how spatially clustered larvae tended to be within ponds). This was done only for sites with at least five dipnet sweeps performed. We extracted the residuals from this regression as a measure of the 'extra' spatial clustering by hosts after accounting for the expected influence of density. We then included the residuals as a covariate to test whether they helped account for the aggregation of parasites within hosts, with the hypothesis that higher clustering of amphibian larvae could amplify the amount of parasite aggregation. Thus, we evaluated if the residuals significantly influenced the $\log_{10}$variance in trematode infection, summed across larval trematode taxa and averaged among hosts of the same species within a site, either as a main effect or through its interaction with the $\log_{10}$mean infection value. We only included sites with at least 10 dissected hosts and mean infection values of 0.1 or greater, owing to problems that can arise with lower values in logarithmic space. As a result, not all 186 ponds were included in certain analyses (minimum of 165). Building from Taylor's power law, we expected that, if the spatial distribution of hosts significantly influences parasite aggregation, residuals from the logmean–logvariance relationship between amphibian abundance per dipnet sweep would help account for variation in the logvariance of trematode abundance per host, either as a main effect or as an interaction term (i.e. a change in the intercept or slope, respectively).

To analyze how aggregation treatment (none, low, or high) affected parasite infection success within the mesocosm experiment, we used a generalized linear mixed model in which the number of metacercariae per tadpole (either per parasite species or summed in total) was modeled as a negative binomial response using the package glmmADMB in R. Host body size and treatment were included as fixed effects, and both cage and mesocosm identities were included as random intercept terms. An offset term was included to account for differences in the number of cercariae added per parasite species. We compared among alternative models with Gaussian and Poisson distributions using AIC values. To further evaluate changes in parasite aggregation among host individuals, we calculated the $\log_{10}$-transformed values of variance in infection load 1) among hosts in the same cage, 2) among cages in the same mesocosm, and 3) among all individuals in the same mesocosm, independent of cage. We used a linear mixed effects model to test how these measures of variance changed in response to experimental treatment. Depending on the model, we included mesocosm as a random intercept term to account for the nested structure of multiple cages within the same mesocosm. Mean infection was always calculated at the same scale as variance (i.e. among individuals in the same cage, among cages in the same mesocosm, or among all hosts together). As an alternative metric of parasite aggregation we also tested the effects of treatment on the variance-to-mean ratio of infection, either for each parasite species individually or for their summed infection (all $\log_{10}$-transformed). Finally, we used data from field sites to estimate the expected value of infection variance in natural systems by capitalizing on the consistent logmean-logvariance relationship observed in California ponds (Johnson and Hoverman 2014). To be conservative, this was done using the relationship among all host and trematode parasite species from the field. After the appropriate back-transformation, we evaluated whether observed values of parasite variance from the experiment differed from the expected values in the field using paired-tests with parasite species as a fixed effect.

Data deposition

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.tk5cq> (Koprivnikar et al. 2017).

Results

Field surveys

Over six years, we sampled 402 site-by-year combinations on 174 unique ponds (average of 2.3 visits per site). For each of the four sampled amphibian species, the mean number of larvae captured per sweep positively predicted the variance in individuals per sweep (both $\log_{10}$-transformed) (LME with site and year as random effects; Fig. 2). The slope of this relationship, as measured by the regression coefficient, varied from 1.39 to 1.71 with R^2-values from 0.92 to 0.98, indicating that larvae were aggregated at ponds but to varying degrees among species. Toads *Anaxyrus boreas* exhibited the highest levels of log-variance for a given log-density, suggesting that they had the greatest amount of spatial clustering (Fig. 2). While much lower than toads, aggregation by bullfrogs *Lithobates catesbeianus* was also moderately higher than that observed in chorus frogs *Pseudoacris regilla* and newts *Taricha torosa*, which were themselves more similar in magnitude.

As expected, the logmean of infection was a strong, linear predictor of the logvariance in trematode infection among amphibian hosts (LME with site and year as random

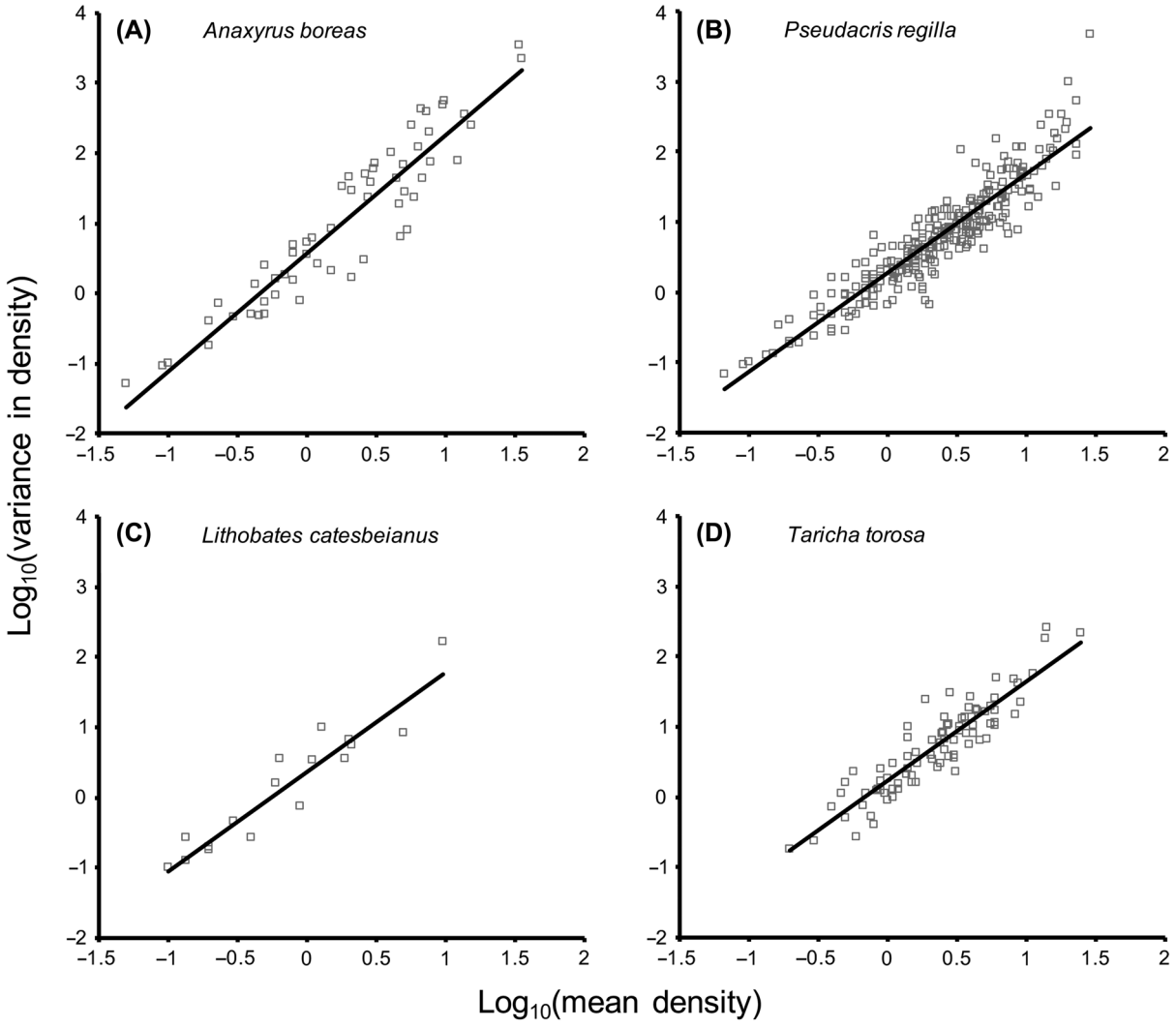

Figure 2. Spatial distribution of larval amphibian hosts within ponds. Presented is the relationship between the number of larvae per dipnet sweep within a pond and its variance, both of which are $\log_{10}$-transformed. For each of the four amphibian species, mean density is a positive, linear predictor of the variance in density based on a linear mixed effects model with pond and year as random effects (*P. regilla*: coefficient = 1.4039 ± 0.0262, t = 53.34, $p < 0.00001$; conditional $R^2 = 0.94$; n = 353; *A. boreas*: coefficient = 1.715 ± 0.0629, t = 27.28, $p < 0.00001$; conditional $R^2 = 0.968$; n = 77; *L. catesbeianus*: coefficient = 1.472 ± 0.0901, t = 16.337, $p < 0.00001$; conditional $R^2 = 0.977$; n = 24; *T. torosa*: coefficient = 1.390 ± 0.050, t = 27.77, $p < 0.00001$; conditional $R^2 = 0.921$; n = 116).

effects, logmean coefficient = 1.699 ± 0.026, t = 64.627, $p < 0.00001$ conditional $R^2 = 0.9253$; Fig. 3), consistent with application of Taylor's power law to parasite infection. Nonetheless, neither the main effect of host spatial heterogeneity (as measured by the residuals from the logmean–logvariance relationship for host density), nor its interaction with the logmean of infection, related significantly to logvariance of infection for any of the host species (*P. regilla*: density residuals = 0.094 ± 0.0618, t = 1.52; p = 0.128; *A. boreas*: density residuals = 0.0112 ± 0.132, t = 0.085; p = 0.932; *L. catesbeianus*: density residuals = –0.696 ± 0.4971, t = –1.40; p = 0.174; *T. torosa*: density residuals = 0.079 ± 0.1395, t = 0.567; p = 0.571). This indicates that the amount of host spatial clustering over-and-above that expected due to mean density did not significantly affect either the intercept or the slope of the relationship between mean parasites per host and its variance.

Mesocosm experiment

In total, we added 603, 101, and 1541 cercariae of *Ribeiroia ondatrae*, *Manudistomum syntomentera* and *Cephalogonimus americanus*, respectively, to each mesocosm over 12 days.

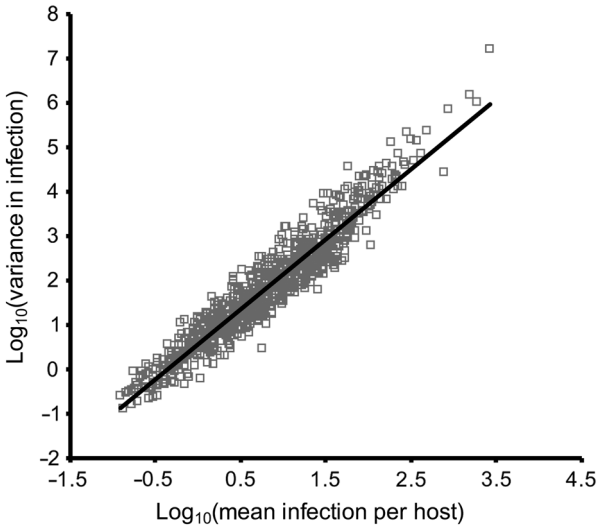

Figure 3. Parasite aggregation within amphibian hosts. In accordance with Taylor's power law, the mean number of parasites per host (log_{10}-transformed) positively predicts the variance in infection among hosts from the same pond (log_{10}-transformed). The slope of this relationship is frequently interpreted as a measure of the degree of aggregation. The mean number of larval trematodes per amphibian host was calculated by averaging the sum of all larval trematodes among all dissected hosts from a given pond-year sampling event. Only populations with at least 10 sampled hosts are included, and pond identity and sample year are included in the model as random intercept terms. LME with site and year as random effects, logmean coefficient = 1.699 ± 0.026, t = 64.627, $p < 0.00001$ conditional $R^2 = 0.9253$; n = 414 site-years [note that the sample size for this analysis differs slightly from the host population examination because here the n = 10 cutoff applies to the sum of all examined host individuals, regardless of species identity].

Counts of subsamples collected on each addition date indicated that the aliquot method was effective in minimizing variance in the number of cercariae added. Across dates, the coefficient of variation (or the ratio of the standard deviation to the mean) values ± 1 SE between the two mean counted samples ranged from 1.38 ± 0.22% to 4.43 ± 1.13% (i.e. all replicates were within 4% of one another). All examined hosts were infected with at least one parasite (n = 134), with overall prevalence values of 18.7%, 88.6%, and 98.5% alongside mean parasite abundances of 1.2 ± 2.4, 4.84 ± 0.34, and 13.81 ± 0.75 for *R. ondatrae*, *M. syntomentera* and *C. americanus*, respectively. However, the spatial arrangement of hosts within mesocosms had no effect on parasite infection success (Fig. 4a). Based on the negative binomial GLMM, which nested hosts within cages and mesocosms, neither experimental treatment (none, low or high spatial clustering), host body size (SVL), nor the interaction between treatment and parasite species affected parasite load per host (treatment coefficient = -0.000286 ± 0.1143; $p > 0.9$; SVL coefficient = 0.042921 ± 0.0464; $p > 0.3$). There was a main effect of parasite species, such that load increased from *R. ondatrae*, *M. syntomentera* to *C. americanus*, even after accounting for differences in the number of cercariae introduced per mesocosm with the offset term (i.e. although more *R. ondatrae* were added than were *M. syntomentera*, the latter had three times higher average load per host).

Manipulation of the spatial distribution of hosts also had no consistent effects on parasite aggregation, regardless of whether data were analyzed among individual hosts or across cages within a mesocosm (Fig. 4b). For each of the parasite species individually as well as the summed total, neither the variance in infection among tadpoles nor the variance-to-mean ratio responded to increases in the spatial clustering of host cages (LM, all $p > 0.05$). However, variation in host body size (i.e. the variance-to-mean ratio of tadpole SVL within a mesocosm or cage) tended to enhance parasite aggregation. While there were no overall differences in mean host size among cages (LME with mesocosm as a random effect, treatment coefficient = –0.13 ± 0.093, t = –1.412; p = 0.165), the variance-to-mean ratio in host body size within a cage positively correlated with variance in infection for *M. syntomentera* (p = 0.10), *C. americanus* (p = 0.014), and all parasites combined (p = 0.0154) (LMEs with mesocosm as a random effect). These effects were even stronger when infection variance-to-mean ratio was used as the measure of parasite aggregation (coefficients for host size variance-to-mean on infection variance-to-mean ratio for *M. syntomentera* = 0.0643 ± 0.0376, t = 1.707, p = 0.09; *C. americanus* = 0.1176 ± 0.0398, t = 2.953, p = 0.0049; total infection = 0.116 ± 0.043, t = 2.707, p = 0.0095; n = 48 cages in 12 mesocosms). Thus, greater amounts of body size variation within cages generally enhanced parasite aggregation, albeit by variable magnitudes. These analyses were less informative for *R. ondatrae* owing to the number of cages in which all tadpoles were uninfected (28 of 48), which precluded estimates of within-cage variance.

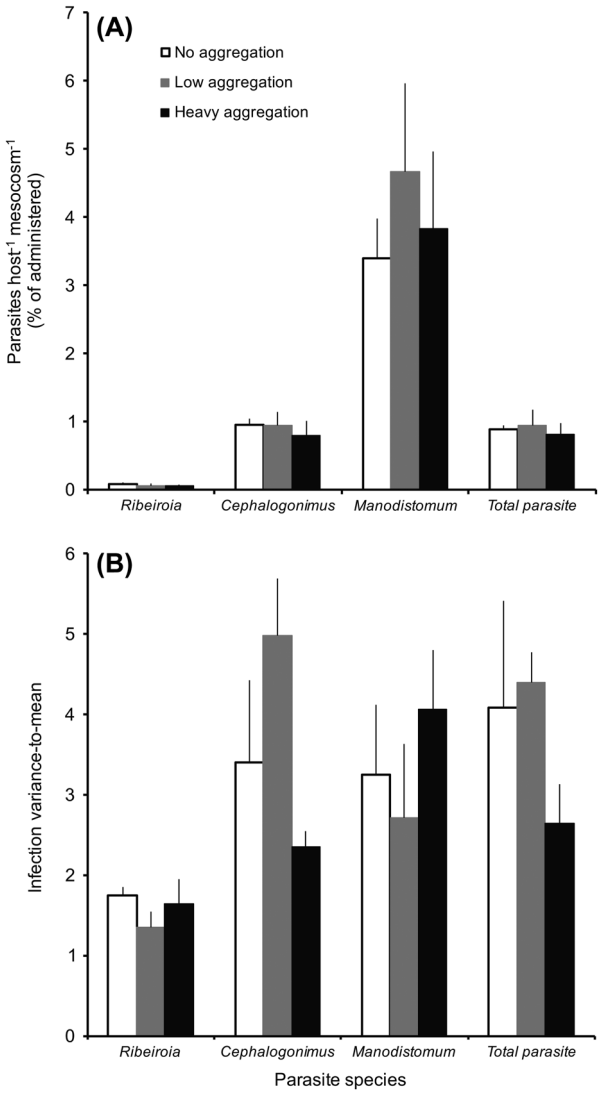

Figure 4. The effect of host spatial arrangement within experimental mesocosms in relation to (A) parasite infection per host (percentage relative to the total number of cercariae added for that trematode species), and (B) the variance-to-mean ratio of infection within each mesocosm. For each of the three added parasite species, presented is the average number of established metacercariae per host at the end of the experiment + 1SE. The variance-to-mean ratio, or the variance in infection among hosts divided by the mean infection, is an indicator of over-dispersion.

Importantly, however, parasite aggregation within experimental hosts was consistently and substantially lower than the expected levels of aggregation derived from field data (Fig. 5). By using the strongly linear relationship between the logmean of infection and the logvariance from 402 pond-by-year combinations in California (Fig. 3), the mean values of infection from the experiment (either per tank or per cage) were used to calculate the expected variance in naturally-occurring amphibian populations with the appropriate back-transformation. Based on paired comparisons, the expected variance averaged 45 times higher than observed variances

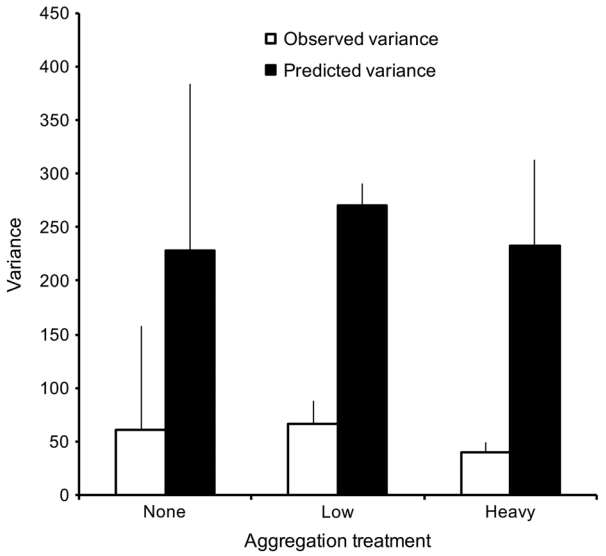

Figure 5. A comparison of the observed and expected variance in parasite infection among hosts. Observed variance was calculated as the variance in total infection (sum of all three trematodes) per host within a mesocosm, averaged among mesocosms in the same treatment. The expected variance was derived from the field-observed relationship between the mean and variance in trematode infection among ponds. We then predicted the expected variance for the observed mean value of infection in each mesocosm using the relationship between the number of larvae per dipnet sweep within a pond and its variance (Fig. 2).

from the experiment (mean difference between log[predicted variance] and log[observed variance] = 0.974 ± 0.1003, $t = 9.71$, $p < 0.0001$). This effect also varied by parasite species (among-pairs $F = 152.27$, $p < 0.0001$), such that the discrepancy increased from *R. ondatrae* to *M. syntomentera* to *C. americanus*. The magnitude of the difference correlated positively with mean infection across the three parasite species (Fig. 4a). Results were similar when cages rather than individual hosts were used as the scale of analysis.

Discussion

Although heterogeneity in host exposure is postulated to play a key role in parasite over-dispersion (Keymer and Anderson 1979, Wilson et al. 2002), there have been few tests as to how host spatial distributions influence the aggregation of parasites within hosts, either experimentally or in natural systems. Our coupled field and experimental efforts represent an integrated approach to investigate this possible driver and allowed us to evaluate the degree to which the manipulative component of the study captured natural patterns of aggregation. The field data clearly indicated that trematode parasites were over-dispersed in their amphibian hosts. Using Taylor's power law, we found an overall slope of 1.69, consistent with other studies of macroparasite infections in hosts; Shaw and Dobson (1995) reported a slope of 1.55 in an analysis of 263 host–parasite datasets. Similarly,

amphibian hosts demonstrated spatial clustering within ponds – a pattern that varied by host species and was especially strong among larval toads, which are known to form aggregations (Waldman 1982, Watt et al. 1997). However, despite the strength of these two patterns independently (R^2-values between 0.90 and 0.99), the degree of spatial clustering by amphibians explained little appreciable variance in parasite aggregation among individual hosts. Correspondingly, our mesocosm manipulation of larval amphibian spatial distribution found no effect of host clustering on infection success or aggregation for the three tested trematodes. As such, both the experimental and field results suggest that clustering of hosts in space was not influential in driving parasite dispersion within this system.

While the spatial distribution of parasite infective stages or vectors can affect aggregation in mobile hosts (Keymer and Anderson 1979, Calabrese et al. 2011), previous studies of host clustering have largely focused on infection prevalence or intensity as outcomes rather than parasite aggregation (Walde and Murdoch 1988, Blower and Roughgarden 1989, Boulinier et al. 1996, Voinovich et al. 1999). Studies with larval trematodes also found aggregation of relatively sessile hosts to be influential on these measures. Increased aggregation of bivalve hosts in a field experiment resulted in fewer individuals harboring cysts (Grosholz 1994). In contrast, infection intensity and prevalence were greatest in caged aquatic snails with a random rather than aggregated distribution (even though the latter had the highest rate of transmission), and may have been driven by cercariae use of chemosensory cues to locate hosts (McCarthy 1990). However, we found no effect of tadpole spatial distribution on parasite infection success for any of the three trematode species used in our mesocosm experiment. Even though we controlled for host density in our mesocosm study, as did most previous investigations of host spatial aggregation (Blower and Roughgarden 1989, McCarthy 1990, Grosholz 1994, Fong 2016), there is a general need for studies that disentangle these two parameters (Blower and Roughgarden 1988). Group size is generally critical for infectious disease dynamics (Côté and Poulin 1995, Rifkin et al. 2012), although this is influenced by the mode of parasite transmission, especially whether it is direct or indirect (Altizer et al. 2003, Patterson and Ruckstuhl 2013). As such, more experiments specifically examining host spatial distribution while controlling for overall density are required to elucidate this aspect of host encounter for parasite over-dispersion.

Variation in host body size tended to positively affect parasite aggregation within the mesocosm experiment. Thus, the greater the variation in tadpole body size either within cages or among cages, the higher the corresponding amount of parasite aggregation among hosts. Nonetheless, the magnitude of these effects were relatively small, and comparisons to the field patterns indicated that parasite aggregation within the mesocosms was much less. Johnson and Hoverman (2014) reported that manipulations of host behavior – but not body size or immunity – were sufficient to generate aggregation patterns similar to those in nature, at least within laboratory manipulations. Raffel et al. (2011) suggested that aggregated distributions of trematodes within larval amphibians was largely explained by heterogeneity in exposure, and primarily driven by seasonality, whereas host mass, age, and previous trematode infection were not significant in predicting over-dispersion. Because cercariae of many species emerge in discrete temporal waves of different periodicities in nature (Shostak and Esch 1990, Vignoles et al. 2006), temporal heterogeneity in host exposure may play a role. The mesocosm study was designed to allow for some of this variation given that exposures were done over 12 days, with strong variance in how many parasites were added per day. Conversely, continuous availability of infectious stages could theoretically result in 100% prevalence within hosts, along with high parasite numbers and low among-host aggregation. Simulations with infective stages introduced in single large waves resulted in a low mean and prevalence of infection, along with highly-aggregated parasite populations (Janovy and Kutish 1988). This is one potential explanation for the stronger parasite aggregation observed in the field-collected tadpoles relative to those in the mesocosm study, along with possible processes controlling parasite over-dispersion in natural settings that operate at a higher spatial scale than a single host aggregation (Boulinier et al. 1996).

The often highly-clustered distribution of snails (source of infective cercariae) within ponds (Sapp and Esch 1994) may also account for some of the trematode over-dispersion from tadpoles in natural environments (Raffel et al. 2011). For instance, Sapp and Esch (1994) suggested that microhabitat-level differences within ponds likely affected trematode transmission dynamics, resulting in distinct aggregations of infected snails. In addition, cercariae are likely to display spatial clustering along with temporal heterogeneity. Many trematode cercariae orient themselves in general ways (e.g. phototaxis) to maximize their chances of host encounter, and others use host-generated cues to actively search (Haas 2003, Sukhdeo and Sukhdeo 2004) – both general strategies could result in spatial aggregations. If over-dispersion benefits some macroparasites (Jaenike 1996, May and Woolhouse 1993), one would further expect motile infective stages to show aggregating behavior in the absence of external stimuli (Morrill and Forbes 2016). Although high degrees of aggregation have been observed with entomopathogenic nematodes, there is an overall lack of study regarding the expected distributions of parasite infective stages in the environment (Morrill and Forbes 2016). As such, the possible contributions of temporal and spatial heterogeneity in the distributions of infected snails and cercariae require further study.

For all three trematode species used in the mesocosm experiment, the spatial clustering of host cages had little effect on the variance in infection among tadpoles, nor the variance-to-mean ratio. However, infection success as a whole showed interspecific differences. Even though almost six times as many *Ribeiroia ondatrae* cercariae were added to mesocosms than were *Manodistomum syntomentera*, the mean infection intensity of the latter was three times higher. The reason for this is not apparent, but could be driven by

differences in the behavior (particularly host-seeking strategies) and/or longevity of cercariae. Although *M. syntomentera* achieved almost 5% transmission success, it is difficult to determine whether this reflects natural rates. Differences among species might be due to the physical arrangement of cages in the mesocosms where tadpoles were located approximately at mid-depth in the water column – a possible mismatch if any cercariae show photo- or geotaxis to facilitate host encounter. Possible low transmission success, and the resulting low mean infection load, has important implications because most of the variation in parasite aggregation (> 80%) is determined by mean infection intensity (Poulin 2013). As such, low values for the latter might reduce over-dispersion so that it is not pronounced enough to be influenced by experimental manipulations of host spatial distribution. Notably, mean infection intensities for field-collected *Lithobates clamitans* tadpoles were higher than for those in the mesocosms (e.g. mean of 39.60 *Cephalogonimus* sp. metacercariae per infected host in the field versus 13.81 in mesocosms). It is possible that use of a host species more susceptible to infection than *L. clamitans* may resulted in a different outcome in our mesocosm study, and such interspecific variation should be considered in future manipulative experiments

Results from the field sampling clearly indicate that multiple species of tadpole in ponds show heterogeneous distributions within sites, supporting previous observations (reviewed by Wassersug 1973). However, this varied among larval amphibian species: spatial clustering was highest in the western toad *Anaxyrus boreas* and lowest in the California newt *Taricha torosa*. This corresponds with the 'schooling behavior' reported for other bufonids (Waldman 1982, Watt et al. 1997), but the highly-competitive (and sometimes cannibalistic) interactions among California newts (Elliott et al. 1993) likely explains why individuals are not typically found in close proximity to one another. If tadpole aggregations facilitate feeding for herbivorous species (Eterovick 2000), this may not benefit predatory larval amphibians either.

Further work will be needed to critically evaluate the likely biological and statistical drivers of parasite aggregation within aquatic hosts. While the experiment performed here represents a simplification relative to natural environments, the integrated approach of testing for linkages between host spatial distributions and parasite over-dispersion in both natural ponds and mesocosms increases the potential inferences possible from this study. By including a large number of ponds, multiple years of study, and site-specific information on tadpole density and its variance, our field data provide a rigorous examination of host spatial aggression with consistent age classes and reproductive states – a key consideration for studies of parasite aggregation in natural systems (Wilson et al. 2002). Similarly, while mesocosms cannot capture all possible factors found in field sites, they represent a semi-realistic approximation of interactions between larval trematodes and amphibian hosts in pond environments, many of which have a relatively small volume and short hydroperiod. Relative to laboratory exposures, mesocosms included a large enough volume such that not all or even most parasites were likely to be successful, allowing for heterogeneity in infection success and host exposure as a function of experimental treatments. We also used multiple parasite species in our manipulative experiment and administered parasites over an extended duration with multiple waves of parasite exposure to better approximate temporal variation encountered in ponds.

It is important to understand the biological and statistical drivers behind parasite over-dispersion for many reasons, including the relative roles of host encounter versus parasite success (i.e. extrinsic or intrinsic factors). This is essential for predicting infectious disease transmission and burden, and also the development of control strategies, as has been attempted for Lyme disease (Calabrese et al. 2011, Devevey and Brisson 2012). In addition, host species that show strong within-site parasite aggregation can also display significant among-site aggregation (Haukisalmi and Henttonen 1999), which has significant implications for larger-level dynamics, such as infection hot-spots and local adaptations. Given the importance of this fundamental 'first law of parasitism' (Poulin 2007), further investigations of both host and parasite spatial aggregation will be critical to identify the drivers of this near-universal phenomenon, especially experiments that incorporate and/or control for heterogeneity in exposure and susceptibility which may operate in natural settings. Such investigations should also carefully consider inherent constraints imposed by the data, which can influence selection of the appropriate null model and subsequent efforts to detect biological process (Xiao et al. 2015).

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