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Heterogeneous hosts: how variation in host size, behaviour and immunity affects parasite aggregation

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Summary

1. Infection heterogeneity is one of the most fundamental patterns in disease ecology, yet surprisingly few studies have experimentally explored its underlying drivers. Here, we used large-scale field assessments to evaluate the degree of parasite aggregation within amphibian host populations followed by a novel experimental approach to assess the potential influence of host size, behaviour and immunity in reproducing such heterogeneity.

2. Among 227 wetlands, 2468 hosts and seven parasite species, infections were consistently aggregated among host individuals within populations of the Pacific chorus frog (*Pseudacris regilla*). For each parasite species, the relationship between the log-mean and log-variance of infection load was strongly linear (R^2: 0·91–0·98) with a slope between 1·37 and 1·67, indicative of aggregation relative to the expected Poisson slope of unity.

3. In laboratory trials with *P. regilla* and the most virulent trematode (*Ribeiroia ondatrae*), experimental reductions in either host immunity (through corticosterone exposure) or antiparasite behaviours (through anaesthesia exposure) increased parasite infection loads in isolated hosts by 62–102% relative to unmanipulated individuals. In a second experiment designed to test how variation in host immunity, behaviour and body size affected variation in infection load within small groups (dyads), a reduction in immune function or behaviour of one host significantly amplified infection heterogeneity within the group, effectively doubling the variance-to-mean ratio. However, immunity affected aggregation only in the absence of behavioural manipulation, and changing the size distribution of hosts did not appreciably affect aggregation.

4. Using Taylor's Power Law to integrate field and laboratory data, we found that only treatments involving behavioural reductions achieved aggregation levels comparable to natural host populations. Thus, despite their short duration, our treatments generated heterogeneity in infection loads similar to natural observations.

5. These results emphasize how, alongside extrinsic variation in parasite exposure risk, individual host attributes generally and behaviour in particular have the potential to influence infection success and parasite aggregation. Continued integration of infection heterogeneity research across space, among host species, and over time has important implications for understanding and managing human and wildlife diseases.

Key-words: host behaviour, host heterogeneity, immunosuppression, parasite aggregation, Taylor's Power Law, superspreaders

Introduction

Perhaps the most fundamental and recurrent pattern in disease ecology involves the aggregation of parasites among hosts, such that a small fraction of hosts often supports a large percentage of the parasites (Crofton 1971; Lester 2012). Statistically, this translates into a mean infection that is less than the variance (overdispersion) (Crofton 1971; Wilson *et al.* 2001). Among >250 host–parasite data sets, Shaw and Dobson (1995) found

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that parasites were almost universally overdispersed within their hosts, leading Poulin (2007) to refer to aggregation as one of the few 'general laws' in parasite ecology. Mechanistically, heterogeneity in infection load results from variation in host exposure and host susceptibility (Wilson *et al.* 2001). These drivers are often further divided into intrinsic and extrinsic factors. For instance, intrinsic variation in host behaviour, diet, immunity, exposure history, sex, genotype and age can each influence both the likelihood of exposure and a host's susceptibility to infection (Hudson & Dobson 1995; Poulin 1996; Brzek & Konarzewski 2007; Frost, Ebert & Smith 2008). Concurrently, this individual-level variation is often accompanied by marked spatial and temporal variation in infection risk from the extrinsic environment (Scott 1987; Brunner & Ostfeld 2008). A chance encounter with an aggregated cluster of nymphal ticks ('tick bomb'), for example, can lead to a high infection burden on an 'unlucky' rodent host (Calabrese, Brunner & Ostfeld 2011). Finally, infection history and co-infection also have the potential to affect patterns of burden and aggregation by altering host immunity, behaviour or mortality (Boag *et al.* 2001; Rohani *et al.* 2003; Ezenwa *et al.* 2010), collectively underscoring the broad suite of factors that contribute to infection heterogeneity.

Despite a large number of comparative studies establishing the ubiquity of parasite aggregation within-host populations (Shaw & Dobson 1995) and of theoretical studies illustrating its importance for host–parasite dynamics (Anderson & May 1978; Dobson & Hudson 1992), surprisingly few studies have experimentally tested the hypothesized drivers of aggregation. Most studies are field based, for which it can be challenging to disentangle the relative contributions of proposed factors and how they interact (Duerr, Dietz & Eichner 2003). For instance, while male-biased infections have been reported in a number of host–parasite systems (e.g. Poulin 1996), it is often unclear whether such patterns emerge from differences in sex-specific behaviours, testosterone-induced immune suppression or a combination of the two (Barriga & Alkhalidi 1991; Eloi-Santos *et al.* 1992; Bojalil *et al.* 1993). This shortage of experimental work has hindered efforts to understand the relative importance of aggregation drivers and how they interact (see Wilson *et al.* 2001). In one of the few experimental studies of parasite aggregation, Keymer and Anderson (1979) showed that spatial variation in the distribution of tapeworm eggs within arenas enhanced infection heterogeneity among roaming flour beetles. However, even among treatments without spatial heterogeneity, overdispersion was high, highlighting the potential importance of individual variation in host physiology, immunity and behaviour (see also Galvani 2003).

Part of the reason why mechanistic studies of parasite aggregation remain relatively rare is because they pose significant logistical challenges. Sampling hosts non-randomly (e.g. opportunistic collections), with insufficient sample size or representation of sexes and age classes, or with indirect survey techniques (e.g. egg counts) can generate biased infection estimates (Gregory & Woolhouse 1993; Wilson *et al.* 2001). There is also no single method of measuring aggregation. Commonly employed metrics include the variance-to-mean ratio (variance divided by the mean), for which values significantly >1 indicate overdispersion, and the negative binomial parameter k (see Wilson *et al.* 2001), which is an inverse measure of aggregation. Yet both measures are sensitive to host sample size and mean infection, limiting their utility for comparing aggregation among populations (Scott 1987; Gregory & Woolhouse 1993; Wilson *et al.* 2001). Some researchers therefore advocate using the slope from Taylor's power law, which relates mean abundance and variance across sampled host populations (Taylor & Taylor 1977). The resulting log–log relationship is often strongly linear, and an estimated slope close to unity indicates a random (Poisson) distribution of parasites, whereas slopes >1 are suggestive of aggregation (Shaw & Dobson 1995; Boag *et al.* 2001; Morand & Krasnov 2008; Lester 2012). Because Taylor's power law depends on a regression-based approach, it is functionally less sensitive to the number of host replicates per site (as opposed to the number of sites) (see Cottingham, Lennon & Brown 2005) and thus offers a robust method of estimating parasite aggregation among host populations (Morand & Krasnov 2008).

Larval amphibians and their macroparasite infections offer a tractable study system in which to examine the potential drivers of parasite aggregation (e.g. Raffel *et al.* 2011; Koprivnikar *et al.* 2012). These parasites can be quantified as hosts approach metamorphosis, offering a standardized stage of comparison that minimizes age-intensity infection patterns as well as changes in behaviour or physiology associated with sexual development (Wilson *et al.* 2001). The absence of parasite reproduction within intermediate amphibian hosts ensures that measured infection represents the product of exposure and host defences only, rather than subsequent replication. Because many infections are acquired via penetration of free-living infectious stages (e.g. trematode cercariae) from the surrounding aquatic medium, patterns of infection can be affected by individual host-level attributes, alongside any spatial or temporal heterogeneity in exposure. For instance, variation in larval amphibian size, immune function and anti-parasite behaviours (i.e. vigorous swimming to avoid or dislodge colonizing infectious stages) has each been shown to influence infection load (Belden & Kiesecker 2005; Daly & Johnson 2011; LaFonte & Johnson 2013). Importantly, these traits can differ substantially among hosts in association with variation in both natural (e.g. competition and predation) and anthropogenic (e.g. contaminants) stressors, even within a single wetland (Kiesecker 2002; Werner *et al.* 2007; Rohr *et al.* 2008a, b).

Here, we used comparative field assessments to assess the degree of infection heterogeneity within amphibian host populations followed by mechanistic laboratory

experiments to evaluate the roles of host immunity, body size and behaviour in driving parasite aggregation. We collected data from 2468 recently metamorphosed Pacific chorus frogs (*Pseudacris regilla*) to quantify aggregation in infection load for seven macroparasite species within 227 wetlands. We then manipulated attributes of larval *P. regilla* to evaluate their effects on infection heterogeneity by the most virulent parasite (*Ribeiroia ondatrae*). We did this first for hosts maintained individually to ensure that experimental treatments functioned to increase infection load and lower variance among replicates. Then, we manipulated the characteristics of one host within an experimental dyad to compare the individual and combined effects of behaviour, immunity and size in controlling infection heterogeneity within a replicate. We expected that alteration of individual host attributes would enhance infection heterogeneity within experimental groups relative to isolated hosts or control conditions. Specifically, we predicted that reductions in host immunity or antiparasite behaviours would increase infection load in the manipulated host while drawing parasites away from the unmanipulated host, thereby enhancing variance; similarly, increases in host size should increase infection by making individuals a larger target for questing parasites (Taylor, Oseen & Wassersug 2004; Belden & Kiesecker 2005; Koprivnikar, Gibson & Redfern 2012; LaFonte & Johnson 2013). Finally, to better integrate field and experimental results and evaluate which treatments best approximated the heterogeneity observed in natural host populations, we used field-derived estimates for the slope of Taylor's power law to compare the variance generated through experimental manipulations with the expected variance.

Materials and methods

FIELD SAMPLING

Between June 2009 and August 2011, we sampled 2468 metamorphosing Pacific chorus frogs (*P. regilla*) from 227 wetlands in the East Bay region of California, USA. We focused on small ponds (<2 ha in surface area) that supported rams horn snails (*Helisoma trivolvis*), which function as first intermediate hosts for many species of digenetic trematodes. Larval amphibians become infected by free-swimming infectious stages (cercariae) released by infected snails, and the parasites typically complete their life cycle after an infected amphibian is consumed by the appropriate definitive host. Wetlands can vary in both parasite richness and the infection load of a given parasite due to variation in the presence and abundance of definitive hosts, which can include birds, mammals, reptiles and other amphibians depending on the parasite species. At each site, we hand-captured *c.* 100 metamorphosing frogs while walking the perimeter and randomly selected *c.* 10 (average = 10·9 ± 0·17 hosts per site; range 8–28 hosts) from which to quantify the numbers and types of each larval trematode, using a stereo-dissecting microscope to thoroughly examine the skin, major organs and digestive tract of each host (see Hartson *et al.* 2011). In these small systems, examination of a relatively small number of hosts provides a reasonable estimate of parasite richness and abundance (Hartson *et al.* 2011; Johnson & Hoverman 2012; Johnson *et al.* 2013). We focused on *P. regilla* because it is one of the most common amphibians in the region and has been previously studied with respect to the effects of immunity and behaviour on larval trematode infection (Daly & Johnson 2011; LaFonte & Johnson 2013). Also, unlike slower developing amphibian species for which seasonal or interannual variation in exposure can amplify heterogeneity (Raffel *et al.* 2011), *P. regilla* completes its development within a single season and has a very slow rate of *Ribeiroia* clearance (LaFonte & Johnson 2013).

To examine parasite aggregation, we plotted the log–log relationship between the average infection load of each parasite and its corresponding variance from each site where it occurred. We define parasite load as the number of larval trematodes per host; average load represents the mean infection abundance for a given parasite species, including any animals without infection. Taylor's power law involves the equation $s^2 = a \times m^b$, in which s^2 is variance, m is mean infection, and a and b are constants (turning into $\log(s^2) = \log(a) + b^*\log(m)$ with transformation). The slope between log-mean and log-variance (i.e. 'b') offers a measure of aggregation strength (Morand & Krasnov 2008) and enables comparisons among populations that differ in mean infection load (Morand & Krasnov 2008). For parasite count data, the null expectation is that parasites are randomly distributed following a Poisson distribution in which the variance equals the mean (i.e. slope of unity), whereas larger slopes indicate aggregation (Shaw & Dobson 1995). Once plotted, the log–log relationship also yields a robust method for deriving an expected level of variance for any particular value of mean infection load, which was used to compare our field and experimental results (see below).

INDIVIDUAL EXPOSURES

To characterize the influence of immunity and behaviour on infection load within isolated hosts, we conducted a 2 × 2 factorial experiment manipulating host behaviour (unmanipulated vs. anesthetized) and host immunity (unmanipulated vs. corticosterone treated). Egg masses of *P. regilla* were field collected and allowed to hatch in the laboratory. As larvae reached stage 28 (Gosner 1960), 10 individuals were randomly assigned to each treatment with an additional 15 animals in the control condition (55 total experimental units). To alter immunity, we exposed larvae to a 0·1 μM corticosterone solution for 8 days (0·234 mg dissolved in 0·42 mL of 80% ethanol), which has been shown effective as a broad-spectrum immunosuppressant in larval amphibians (see Belden, Wingfield & Kiesecker 2004; Belden & Kiesecker 2005; LaFonte & Johnson 2013). Previous studies have demonstrated that similar exposures elevate amphibian corticosterone levels by *c.* 35%, which is within the natural physiological range of tadpoles and is not associated with any long-term changes in behaviour (Glennemeier & Denver 2002; Middlemis Maher, Werner & Denver 2013). We replaced the solution daily. To manipulate amphibian behaviour and reduce the ability of hosts to avoid or dislodge trematode cercariae, we exposed tadpoles to a 0·125% solution of neutrally buffered MS-222 (tricaine methanesulfonate) for 2 min, which induced inactivity for <30 min, after which hosts regained full activity (Daly & Johnson 2011; Koprivnikar, Gibson & Redfern 2012). This was considered an ecologically relevant manipulation given observations that larval amphibians become inactive following exposure to cues

from naturally occurring predators (Relyea 2001a; Benard 2006), sometimes for periods of up to several hours (Van Buskirk & McCollum 2000).

Tadpoles were exposed individually to 30 *Ribeiroia* cercariae within 500 mL of water for 30 min. Cercariae were obtained from field-collected snails, pooled among snails and administered to amphibians within 4 h of release (see methods in Johnson *et al.* 2012). Following exposure, hosts were rinsed, transferred to new containers and necropsied after 36 h. This approach assumes that anaesthesia exposure does not affect short-term immune responses and that corticosterone did not alter host behaviour, which was supported by the lack of a statistical interaction between these treatments in determining infection after 36 h. Moreover, previous research has found no effects of either MS-222 on tadpole resistance to trematode infection (Sears, Snyder & Rohr 2013) or corticosterone exposure on tadpole behaviour (Glennemeier & Denver 2002).

PAIRED EXPOSURES

To evaluate how experimental condition affected the distribution of parasites between co-occurring hosts (i.e. aggregation), we conducted a second experiment in which we manipulated the behaviour, immunity and size of one host within experimental dyads (Fig. 1). Unlike in the individual exposure study above, here, we expected manipulation of one host to amplify the variation in infection load between paired hosts, and our goal was to compare the relative and interactive effects of each experimental condition on within-replicate infection variance. Treatments were replicated 12 times except for the control, for which we had 18 replicates (total of 102 units and 204 hosts) (Fig. 1). Host behaviour and immunity were manipulated as described previously. In this experiment, we introduced variation in host size, such that one host was *c.* 50% larger in body length (mean difference in snout-vent length = 57·4 ± 2·6%). Although the same age (days post-hatching) as smaller tadpoles, larger hosts were raised for 6 days at 26 °C to achieve the desired size difference (although we cannot rule out the possibility that this affected immune development as well). Each pair of hosts was exposed to 40 *Ribeiroia* cercariae within 2·0 L of water. After 36 h, all hosts were necropsied by observers who were blind to experimental condition. We then calculated the mean infection load and its variance for each pair of hosts within a replicate (see Fig. 1).

Because this design required us to track the manipulated host (hereafter, the 'focal host'), we used a novel biomarker involving infection by a second trematode (*Alaria* sp. 2) (Locke *et al.* 2012). Unlike *Ribeiroia*, *Alaria* causes no detectable pathology in amphibian hosts (Johnson & Hoverman 2012), even at high infection loads, but is nonetheless easy to detect during necropsy. This approach was preferable to elastomer tags or tail clipping methods that cause more significant trauma and could alter host behaviour (Skelly & Richardson 2009), particularly given the small size of the animals used. One-week prior to the experiment, we batch-exposed half of hosts to *Alaria* cercariae and half of these became 'focal hosts', whereas the other half was considered non-focal hosts. Although these parasites can interact over longer time-scales (e.g. when hosts develop to metamorphosis, see Johnson and Hoverman 2012), these effects were expected to be weak over 36 h (see Hoverman, Hoye & Johnson 2013). Moreover, we verified that our use of a biomarker did not affect the results by testing whether the *Alaria* tag had a significant effect on infection load or its variance in our analyses (all effects were non-significant).

ANALYSIS

To analyse the effects of behaviour, immune function and their interaction on *Ribeiroia* infection load within isolated hosts, we used a generalized linear model (GLM) with a negative binomial distribution (glm.nb in package MASS, R Development Core Team

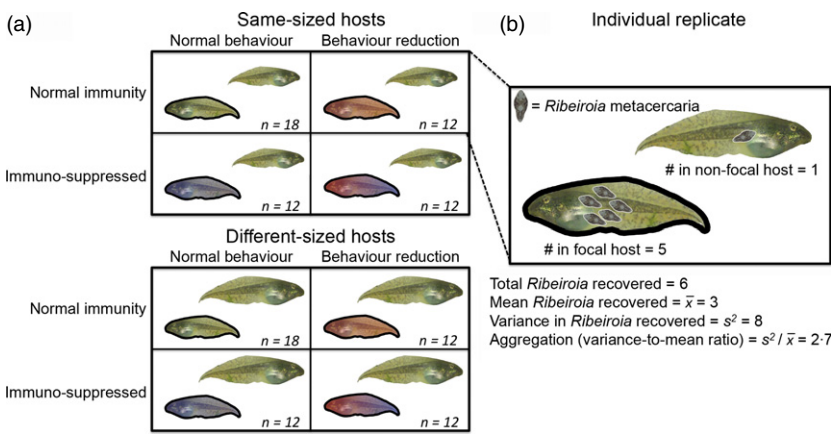

Fig. 1. (a) Schematic diagram of the paired-exposure experiment. The experiment consisted of a 2 × 2 × 2 factorial manipulation of the size, behaviour and immune function of one host within an experimental dyad prior to exposure to *Ribeiroia* infection. For each pair, the focal host is indicated by an outline in the diagram with manipulations indicated by different colours. Behaviour (i.e. activity level) of the focal host was reduced by brief exposure to anaesthesia, while immune function was suppressed through exposure to corticosterone prior to the experiment. Size was manipulated by pairing the focal host together with a tadpole that was either the same size or *c.* 50% larger than the focal host. The sample size for each treatment is shown in the bottom right corner of each square (102 experimental units and 204 total hosts). (b) Hypothetical illustration of a single replicate within the experiment. Thirty-six hours following exposure to 40 *Ribeiroia* cercariae, the focal and non-focal hosts were necropsied to determine the number of metacercariae (infection load). We calculated mean infection load, variance in infection load and the variance-to-mean ratio (a measure of aggregation) for each replicate.

2008). The negative binomial distribution often provides a reasonable approximation of overdispersed parasite data (e.g. Shaw & Dobson 1995), which we verified by comparing goodness-of-fit of the data to both the Poisson and the negative binomial distributions. For the paired-host experiment, we first used a GLM with a negative binomial distribution to test how treatment affected total *Ribeiroia* load summed between both hosts within a replicate. Secondly, to examine how infection load in the focal host changed relative to the non-focal host as a function of experimental treatment, we used a GLM with a binomial distribution after using the 'cbind' function to combine data columns for each host group. This analysis thus tested whether each experimental condition increased or decreased infection in the focal host relative to the non-focal host within the same replicate. Manipulation of behaviour and immunity always involved the focal host, whereas in the size treatment, the larger individual was always the non-focal host. In controls, designation of the 'focal host' was made randomly. For each analysis, we also included a binomial fixed effect to identify whether the focal host was tagged with the biomarker (0 vs. 1); this variable was always non-significant and was subsequently removed.

Finally, to compare experimental and field data, we used the field-derived slope (b) for *Ribeiroia* from Taylor's power law to back-calculate the expected variance for a given mean infection in the paired tadpole experiment (see also Lester 2012). Thus, for each replicate dyad, we used mean infection load (m, averaged between the two co-occurring tadpoles) to calculate the expected variance (s^2) based on the empirically observed log–log relationship from the field sites [for *Ribeiroia*, this was log (s^2) = 0·389 + 1·371*log(m)]. We then tested how each experimental treatment affected the difference between the field-expected vs. experimentally observed variance values (after back-transforming to non-log values) using ANOVA. This analysis evaluated which of the treatments (and their interactions) caused the difference between the observed and expected variance terms to deviate significantly from zero. As a null hypothesis, we also compared the variance observed in the experiment with the expected values from a Poisson distribution using the same approach (note that for a Poisson distribution, the variance is equivalent to the mean, such that the expected variance was simply the observed mean infection load). If a manipulation was effective at generating heterogeneity in infection load similar to the field, it should reduce the difference in variance between experimental animals and the expected variance based on field data while amplifying the difference from the Poisson. We emphasize, however, that while this analysis aimed to determine which experimental treatments generated variance similar to natural systems, it did not identify the role of each factor in causing parasite aggregation in the field.

Results

FIELD DATA

Results from the field sampling revealed consistent evidence of parasite aggregation. Among the seven larval trematodes, the log-mean and log-variance of infection load exhibited a strong, linear relationship (slope = 1·37–1·67), and the log-mean accounted for 91% to 98% of the variation in log-variance (Fig. 2). Combining among parasite species to examine total infection load per host, which varied from 0 to 13 541, yielded an R^2 of 0·91 and a slope of 1·64, which was substantially greater than the slope of unity expected under a random (Poisson)

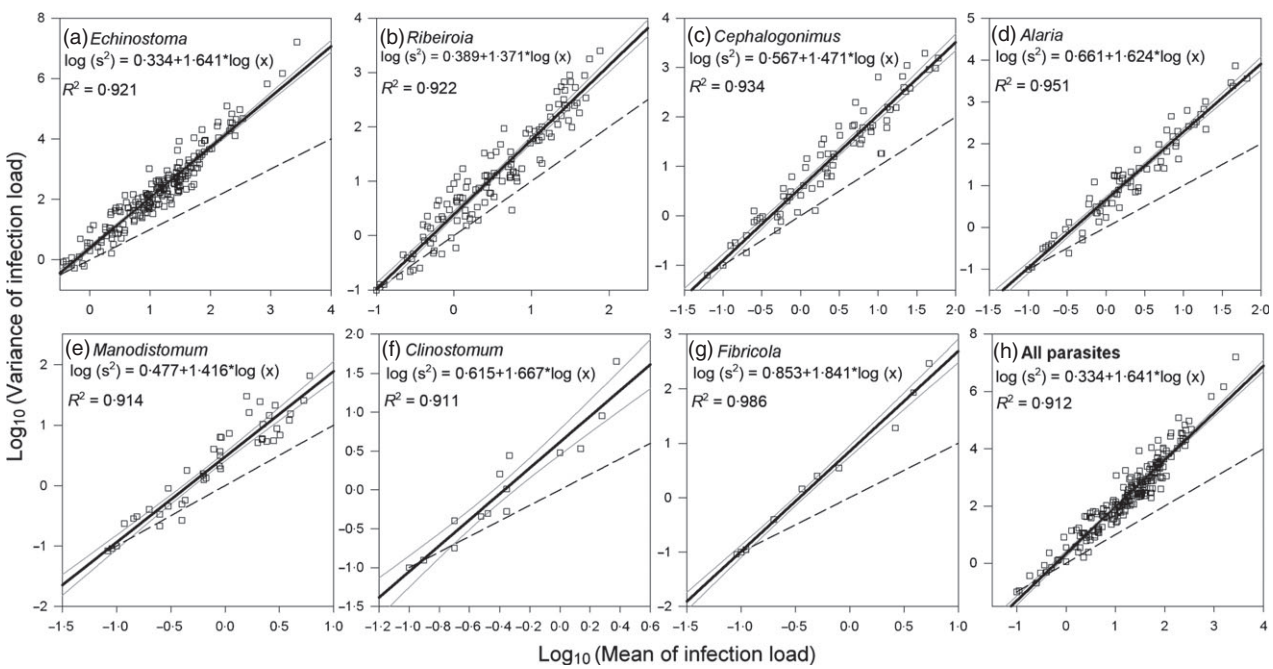

Fig. 2. Log–log relationship between mean infection and variance for each of the seven larval trematode species and total infection load (summed among all parasites). Data were derived from necropsies of amphibian hosts (*Pseudacris regilla*) from 227 sampled wetlands. *Echinostoma trivolvis* was the most common parasite group (detected at 211 sites), followed by *Ribeiroia ondatrae* (n = 135), *Cephalogonimus americanus* (n = 79), *Alaria* sp. 2 (n = 75), *Manodistomum syntomentera* (n = 53), *Clinostomum attenuatum* (n = 17) and *Fibricola* sp. (n = 14).

distribution. *Echinostoma* was the most common parasite group (detected at 211 sites), followed by *Ribeiroia* ($n = 135$), *Cephalogonimus* ($n = 79$), *Alaria* ($n = 75$), *Manodistomum* ($n = 53$), *Clinostomum* ($n = 17$) and *Fibricola* ($n = 14$). For *Ribeiroia*, per-host infection load ranged from 1 to 179 with a site-level average of 0·1–76·6 parasites per host.

EXPERIMENTS

Among isolated hosts, infection load in the absence of manipulations averaged ± 1 SE 4·8 ± 0·7 metacercariae (i.e. encysted parasites) per host (range: 1–15). Experimental reductions in either behaviour or immunity led to an approximate doubling of *Ribeiroia* infection load (behaviour $Z = 2{\cdot}65$, $P = 0{\cdot}0008$; immunity $Z = 3{\cdot}58$, $P = 0{\cdot}0003$) (Fig. 3). The combined effects of inhibiting host immunity and behaviour were additive (i.e. there was no significant interaction between treatments). Similarly, in the experiment involving host dyads in which treatments were applied to one of the two individuals within a replicate, manipulation of behaviour or immunity significantly increased total infection load (behaviour $Z = 2{\cdot}368$, $P = 0{\cdot}0178$; immunity $Z = 3{\cdot}089$, $P = 0{\cdot}002$). There was also a significant interaction between the size and immunity treatments ($Z = -2{\cdot}5$, $P = 0{\cdot}012$), such that immune suppression increased infection load only when hosts were comparably sized (Fig. S1, Supporting information).

Importantly, however, reductions in host behaviour, immunity and the behaviour-by-immunity interaction enhanced heterogeneity in infection load between hosts in experimental dyads (GLM with binomial distribution, behaviour $Z = 7{\cdot}65$, $P < 0{\cdot}00001$; immunity $Z = 5{\cdot}32$, $P < 0{\cdot}00001$; behaviour*immunity $Z = -5{\cdot}09$, $P < 0{\cdot}00001$). Depression of host immunity or behaviour caused infection load in the focal host to increase by 91% and 102%, respectively, relative to the non-focal host (Fig. 4a, although immunity effects were significant only when behaviour was not also manipulated). Correspondingly, the variance-to-mean ratio within replicates increased from an average ± 1 SE of 2·11 ± 0·49 in the control (same-sized individuals without any manipulations) to 3·43 ± 1·31 with immune suppression and 8·98 ± 2·75 with behavioural reduction (Fig. 4b). Increasing variation in host size had no significant effect on focal host infection load or aggregation between paired hosts nor did use of the biomarker parasite, *Alaria* ($P > 0{\cdot}43$). For instance, the average *Ribeiroia* infection in tagged and untagged hosts was 13·11 ± 0·61 and 13·0 ± 0·63, respectively (t-ratio = 0·101, two-tailed $P = 0{\cdot}91$).

Using the slope from the field-derived relationship between the log-mean and log-variance in infection load for *Ribeiroia*, we found that only treatments involving behavioural manipulation within the experimental dyads had observed variances within the expected range.

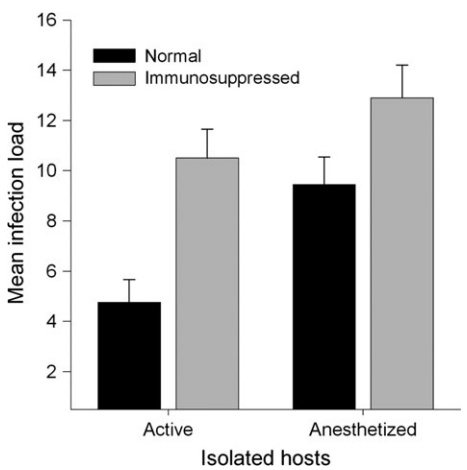

Fig. 3. Effects of experimental treatment on mean ± 1 SE *Ribeiroia* infection for larval amphibian hosts (*Pseudacris regilla*) exposed to cercariae in isolation. Hosts were exposed to 30 cercariae for 30 min in factorial treatments involving reduction in behaviour and/or immunity.

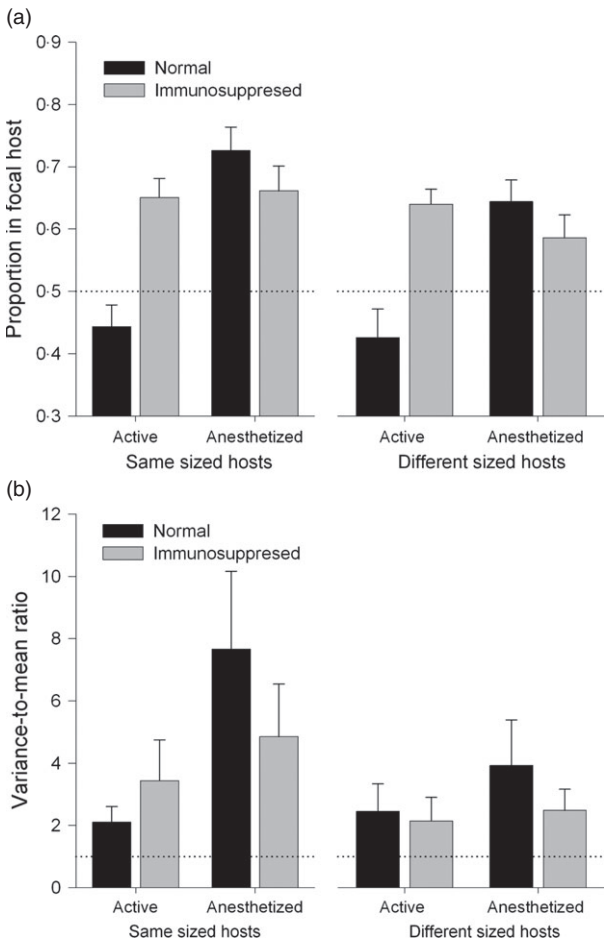

Fig. 4. Effects of experimental variation in host behaviour, immunity and size on *Ribeiroia* infection within pairs of amphibian hosts (*Pseudacris regilla*). (a) Proportion of total parasites found in the focal host, with the dashed line indicating the expected proportion of 0·5; (b) Variance-to-mean ratio as a function of treatment; here, the dashed line at 1 indicates the null expectation for a random (Poisson) distribution.

Behavioural manipulation significantly reduced the difference between the observed variance for a given replicate within the experiment and the field-expected variance (behaviour $P = 0{\cdot}048$; no other effects or interactions) while increasing the difference relative to the Poisson variance ($P = 0{\cdot}008$). Treatments involving immune suppression or variation in host size did not significantly influence the difference between observed and expected variance values, nor were there any interactions among treatments (all $P > 0{\cdot}05$). Similarly, among same-sized host treatments with one individual anesthetized, the distribution of values for the observed minus field-expected variance did not differ from zero ($P = 0{\cdot}356$), whereas it was significantly less than zero ($P < 0{\cdot}0001$) when behaviour was unmanipulated (Fig. 5). Simply stated, this indicates that a reduction in the behaviour of one host within an experimental dyad led to variance in infection load comparable to what would be expected in nature. In treatments with different-sized hosts, however, the observed variance was always significantly less than the field-expected variance ($P < 0{\cdot}001$), regardless of whether behaviour was manipulated.

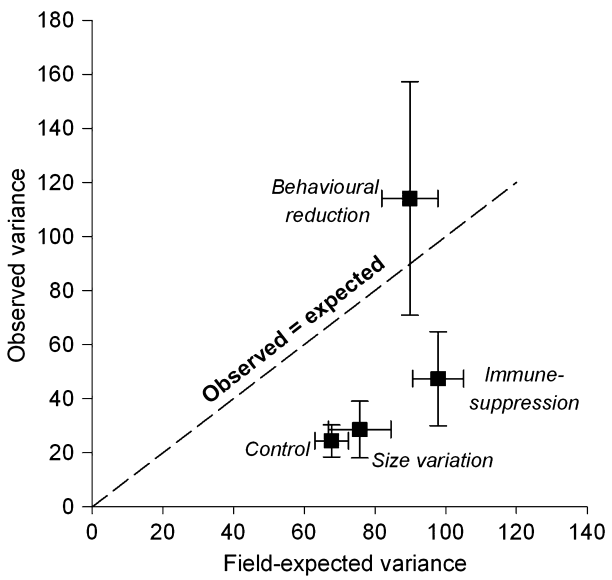

Fig. 5. Influence of experimental treatment on differences between the observed variance and the expected variance as determined from field data. Mean infection within each experimental dyad was used in conjunction with the field-derived regression between log-mean and log-variance (see Fig. 1) to back-calculate the field-expected variance (presented here ± 1 SE). Treatments that fall on or close to the 1 : 1 line reflect congruence between the observed and expected variance, whereas those substantially above or below the 1 : 1 line had more or less variance, respectively, than would be expected based on field observations. Only values from the behaviour manipulations involving same-sized hosts did not differ significantly from the expected variance (interactions not shown). Because no interactions were significant, only univariate effects are shown here (i.e. 'Behavioural reduction' was calculated using replicates for which that was the only manipulation). Here, 'Control' indicates the treatment in which both hosts were similarly sized and neither was manipulated for activity or immunity.

Discussion

Despite the ubiquity of parasite aggregation and its importance for stabilizing host–parasite dynamics (Shaw & Dobson 1995; Wilson *et al.* 2001), surprisingly few studies have experimentally explored the underlying drivers of infection heterogeneity. Comparative and theoretical efforts have thus far outpaced mechanistic experiments, in part owing to the challenges of testing proposed factors in most systems (Wilson *et al.* 2001). By combining field assessments of parasite distributions in amphibian host populations with controlled experiments, we explored how manipulations of host size, behaviour and immunity – individually and combined – affected patterns of parasite aggregation and whether such treatments could generate heterogeneity similar to field observations. Our results indicated that, within this system and for these specific manipulations, only reductions in host behaviour generated levels of aggregation and infection heterogeneity comparable to patterns observed in nature. While this does not mean that variation in behaviour was, in fact, the cause of aggregation observed in field sites, for which spatial and temporal variation in parasite exposure is also likely to contribute, it does emphasize the potential for even transient alterations in host behaviour to recreate similar amounts of heterogeneity to what is observed in naturally occurring populations. This study is also among the first to link field-based measurements of parasite distributions with experimental efforts to evaluate the potential contributions of multiple host attributes on aggregation.

We found strong evidence of parasite aggregation within naturally occurring amphibian host populations. Among 227 wetlands that varied naturally in infection load, the slope of the log–log relationship between mean infection load and variance ranged between 1·37 and 1·67 for the seven parasite species, broadly indicative of aggregated infections (Wilson *et al.* 2001). In all cases, the log of mean infection explained more than 90% of the scatter in log-variance, the latter of which spanned nearly eight orders of magnitude (Fig. 1). For count data such as parasites within hosts, the null expectation from a Poisson distribution would be a slope of unity, in which the variance equals the mean, whereas slopes >1 reflect overdispersion. By comparison, Shaw and Dobson (1995) reported a slope of 1·55 and an R^2 of 0·87 in their comparison of 263 host–parasite populations. On average, *c.* 87% of the parasites at a given site were recovered from the uppermost-infected half of infected hosts when individuals were ordered by infection load. While the degree of aggregation noted for these amphibian trematodes is somewhat lower than that reported in some other studies of macroparasites, this may owe to the high infection prevalence of the parasites studied here, the active

nature of cercariae transmission and greater mixing associated with the aquatic medium, all of which could reduce aggregation relative to rare infections acquired passively from other hosts or the terrestrial environment (Scott 1987; Shaw & Dobson 1995).

Through manipulations of host behaviour, immunity and body size, our experimental treatments compared the contributions of each factor in influencing parasite aggregation under laboratory conditions. Within isolated hosts, reductions in either behaviour or immunity increased infection load. Consequently, when these treatments were applied to hosts within experimental dyads, they increased the variance and heterogeneity in infection load between co-occurring hosts. Among control treatments, the variance-to-mean ratio averaged *c*. 2. However, when immunity or behaviour was reduced in one of the two hosts, this ratio increased to an average of 3·4 and 8·9, respectively, while the proportion of parasites in the focal host increased from 44% in the control to 65% in the immune-suppression condition and to 72% in the behavioural reduction treatment. By deriving expected values of variance from the strong empirical relationship between log-infection and log-variance in natural host populations, we found that only treatments involving changes in host behaviour generated infection heterogeneity within the range of field observations. Thus, despite the transient nature of our manipulations (e.g. behaviour was altered for <30 min) and the brief duration of the experiment (36 h), changes to individual host attributes led to comparable patterns of aggregation as observed in the field.

These findings emphasize the importance of host behaviour in determining infection load (Hart 1994; Hawley & Altizer 2010). In some cases, behaviour can enhance infection; for instance, more active hosts with larger home ranges often develop higher and more diverse parasite burdens, while hosts with a larger contact network can be disproportionately likely to acquire and spread infections (Craft *et al*. 2011). Alternatively, antiparasite behaviours such as grooming, migration and escape manoeuvres can reduce parasite encounter or establishment (e.g. Hart 1994), especially for actively colonizing parasites such as trematode cercariae. Erratic movements by tadpole hosts can prevent trematode colonization or encystment (Taylor, Oseen & Wassersug 2004; Daly & Johnson 2011), and individual variation in tadpole activity and responses to novel stimuli are broadly predictive of infection risk (Koprivnikar, Gibson & Redfern 2012). Additionally, the behaviour of larval amphibians is highly sensitive to natural stressors such as competition and predation (Relyea 2004). For example, many amphibian species become inactive (i.e. remain motionless for several hours) in the presence of predators to reduce the risk of detection (Van Buskirk & McCollum 2000; Relyea 2001a,b; Benard 2006). Given that infection often induces lethargy and lowered activity during the process of tissue repair (e.g. sickness behaviours; Hawley & Altizer 2010), small initial differences in infection (or co-infection) could also become amplified over time through parasite-induced suppression of host behavioural defenses, further contributing to infection heterogeneity among individuals (i.e. a 'snowball effect').

In the light of the short-term nature of these trials and our focus on macroparasite infections, it is perhaps unsurprising that the influence of behaviour, which affects host exposure, exceeded that of immunity, which limits susceptibility to infection and subsequent persistence. Because immunosuppression reduces the ability of hosts to clear infections over time (Belden & Kiesecker 2005), longer-term experiments might be expected to enhance infection heterogeneities as parasite loads accumulate in immunosuppressed hosts. Similarly, parallel experiments involving microparasites might very well emphasize the influence of host immunity, given the importance of host defences in limiting within-host replication of microparasitic infections. Counter to our expectations, however, increased variation in host size – which should also enhance differences in host behaviour and immunity – had no effects on aggregation. This could stem from the conflicting effects of host size on infection; while bigger hosts are larger targets for questing cercariae, they also have more vigorous antiparasite behaviours and a greater capacity for immune resistance (Flajnik *et al*. 1987; Holland *et al*. 2007; Raffel *et al*. 2011) that may collectively neutralize increases in colonization. Indeed, Daly and Johnson (2011) found that host size was a positive predictor of experimental infections only when larval amphibian hosts were anesthetized; among active hosts, size had neutral effects on trematode load. Because our experiments only manipulated the smaller host in the mixed-size treatments, further work is needed to explicitly evaluate concurrent changes in behavioural and immune defences with host size, preferably including a gradient in each manipulation rather than a single treatment.

Continued study of heterogeneity in infection would benefit from enhanced integration across scales and subdisciplines. Alongside patterns of aggregation, which have a long history in parasitology (e.g. Crofton 1971), infection heterogeneities have become an increasing focus of transmission research (e.g. 'superspreading events', Perkins *et al*. 2003; Lloyd-Smith, Schreiber & Getz 2005; Hudson, Perkins & Cattadori 2008), of multi-host communities (e.g. dilution and amplification hosts, LoGiudice *et al*. 2003) and of spatial epidemiology (e.g. hotspots, Paull *et al*. 2012). A better understanding of how aggregated infections among hosts translate into dynamic variation in transmission, for instance, could help link pattern with process. Aggregated infection burdens, which were the focus of our study, are but one component influencing transmission, which will also be affected by variation in the shedding of infectious propagules, the suitability of a host for parasite replication, duration of infection and contact frequency (Streicker, Fenton & Pedersen 2013). Concurrently, disentangling the relative influence of

host-level attributes (intrinsic factors) from environmental and temporal variation (extrinsic factors) in driving infection heterogeneity has important implications for the design of control programmes (Ferrari *et al.* 2004; Hudson, Perkins & Cattadori 2008; Stein 2011; Paull *et al.* 2012). While our experiments aimed to minimize extrinsic variation in infection, thus focusing on the effects of individual host characteristics, in other systems such spatial or temporal variation in infection risk may be the primary driver of heterogeneity (Brunner & Ostfeld 2008; Calabrese, Brunner & Ostfeld 2011; Raffel *et al.* 2011). Thus, from a management perspective, targeted treatment of highly infected hosts (and potential superspreaders) will be effective only if such individuals can be linked to particular traits that can be identified *a priori*, as opposed to representing chance events associated with environmental or temporal stochasticity in infection risk (Brunner & Ostfeld 2008; Hudson, Perkins & Cattadori 2008).

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Supporting Information

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

Fig. S1. Effects of experimental treatments on the total number of *Ribeiroia* metacercariae detected within paired-host treatments.