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Pseudocapillaria tomentosa in laboratory zebrafish *Danio rerio*: patterns of infection and dose response

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ABSTRACT: Parasites in wild populations almost always exhibit aggregation (overdispersion), in which relatively few hosts are infected with high numbers of the parasites. This pattern of infection has also been observed in laboratory studies, where many of the sources of natural variation are removed. *Pseudocapillaria tomentosa* (Nematoda) is common in zebrafish (*Danio rerio*) facilities. We describe here patterns of infections in zebrafish experimentally infected with larvated *P. tomentosa* eggs in various trials with defined numbers of eggs. One trial with eggs delivered in a gelatin diet is also included. Fish were exposed at 25, 75, and 200 eggs fish^{-1}, and the minimal infectious dose was estimated to be 1.5 eggs fish^{-1}. The ID_{50} (50% infective dose) was calculated to be 17.5 eggs fish^{-1}. We also included data from a trial and 2 previously published experiments with undefined doses in which zebrafish were exposed to infectious water and detritus from a tank that previously contained infected fish. All doses resulted in a high prevalence of infection (>70%), except at the 25 eggs fish^{-1} dose, where the prevalence was 43–46%. Mean abundance of worms corresponded to dose, from 0.57 worms fish^{-1} at 25 eggs fish^{-1} to 7 worms fish^{-1} at 200 eggs fish^{-1}. Variance to mean ratios (V/M) and the *k* parameters showed aggregation across the 8 separate trials, including the gelatin diet. Aggregation increased with increased parasite abundance. Given the consistent observation of aggregation across our experiments, the zebrafish/*P. tomentosa* system provides a potentially robust, high-throughput model to investigate factors that influence differences in host susceptibility within defined populations.

KEY WORDS: Zebrafish · Nematoda · *Pseudocapillaria* · Parasitism · Experimental infection

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INTRODUCTION

Several species of capillarid nematodes are recognized as pathogens in fishes, particularly ornamental species (Moravec et al. 1999). *Pseudocapillaria tomentosa* has a broad host range, and infects many species of cyprinids as well as some fishes in other orders (Moravec 1987). This nematode is common in zebrafish *Danio rerio* used in research (Kent et al. 2002, Maley et al. 2013), and has been reported in about 10% of zebrafish laboratories based on data from the Zebrafish International Resource Center diagnostic service (https://zebrafish.org/health/index.php). It often causes high mortality associated with emaciation and severe inflammatory lesions in the intestine (Kent et al. 2002, Balla et al. 2010, Murray & Peterson 2015). Zebrafish are infected by ingestion of larvated *P. tomentosa* eggs in water (Kent et al. 2002, Collymore et al. 2014, Gaulke et al. 2016).

A well-recognized paradigm in parasitology is that macroparasites, such as nematodes, in naturally in-

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fected animals exhibit aggregated (overdispersion) distribution. This usually follows a negative binomial distribution, with relatively few hosts harboring many, if not most, of the parasites (Crofton 1971, Poulin 2013). This distribution has also been reported in laboratory transmission studies with nematodes in pigs (Roepstorff et al. 1997, Pedersen & Saeed 2002) and mice (Keymer & Hiorns 1986). This observation extends to laboratory studies with fish parasites: the fish copepod *Argulus coregoni* sp. (Bandilla et al. 2005), the trematode *Diplostomum spathaceum* (Karvonen et al. 2004), and *P. tomentosa* in zebrafish (Collymore et al. 2014).

Important factors responsible for parasite aggregation within a population are heterogeneity in host behavior and size, differences in effective immunity within the host population, spatial heterogeneity in the distribution of infective stages, and differences in parasite reproduction within the host (Anderson 1993, Shaw & Dobson 1995, Poulin 2013). Two other factors that may influence aggregation include occurrence of co-infections (Morrill et al. 2017) and parasite abundance (Kemmer & Hiorns 1986). The number of hosts examined will also influence the degree of aggregation that is observed (Poulin 1993).

Laboratory animals can serve as a valuable source in the effort to disentangle the specific and synergistic effects of these various factors on aggregation, given the control over experimental variables that such animal models offer. In particular, the zebrafish provides an excellent opportunity to define the relative contribution of such factors to aggregation, given the large sample sizes it affords as well as the diverse genetic tools (e.g. myriad knock-out lines and CRISPR/Cas) (Liu et al. 2017). Accordingly, we are developing the zebrafish/*P. tomentosa* system as a model to define the causes of aggregation and to discover anthelminthic drugs *in vivo*. To advance this model, it is imperative that we first measure the patterns of infection and dose-response indices to demonstrate the model's general consistency with other host systems as well as its repeatability across experiments. Given that overdispersion is essentially a law of parasitism (Poulin 2007), we attempted to further elucidate this phenomenon under defined laboratory conditions. To accomplish this, we measured parasite burden across 8 transmission studies, including experiments in which zebrafish were infected by exposure to infected tanks as well as varying defined doses of larvated eggs, and in 1 trial with eggs delivered in a gelatin diet. These 8 trials included data from 2 previous studies (Collymore et al. 2014, Gaulke et al. 2016 [preprint doi: 10.1101/076596]). Males in general tend to be more heavily parasitized than females (Poulin 1996, Schalk & Forbes 1997), and Chow et al. (2016) observed this phenomenon with *Pseudoloma neurophilia* in zebrafish. Hence, we also include evaluation of the influence of sex on worm abundance in these experiments.

MATERIALS AND METHODS

Experimental infection

Five separate trials were conducted by exposing zebrafish *Danio rerio* to defined concentrations of parasite *Pseudocapillaria tomentosa* eggs ranging from 25 to 200 eggs fish^{-1} (see Table 1). Three other trials, Trial 6, Collymore et al. (2014), and Gaulke et al. (2016), were conducted with undefined exposure doses (see Table 2). Parasite-free 5D line zebrafish were obtained from the Sinhubber Aquatic Resource Laboratory (SARL), Oregon State University, in which the nematode has never been observed following extensive examinations (Barton et al. 2016). This line of fish was established from outbred zebrafish from a tropical fish wholesale dealer in 2007 from about 10 adults (Kent et al. 2011). Donor fish were from a population of 5D fish in which we are maintaining the infection in our laboratory.

Our vivarium contains flowthrough water, derived from charcoal-filtered city water. Temperature was maintained at 27–28°C, with conductivity at 115–125 µS, and pH approximately 7.5. Light in the vivarium is provided for 14 h d^{-1}. Experimental conditions were standardized with each trial and all experiments were conducted in the same laboratory, hence environmental variation was minimized so that the only major variable was the infection dose.

For defined dose trials, eggs were collected and concentrated from debris from a tank containing infected zebrafish as described by Martins et al. (2017), whereby the final concentration step was not performed. In brief, *P. tomentosa*-infected zebrafish were placed into a 16 l tank for 72 h, the fish were removed, the water was allowed to settle for 4 h, and 1.25 l water was collected from the bottom of the tank. This was passed through 300 µm and then 200 µm nylon filters, and then concentrated by centrifugation at 1468 × *g* for 45 min. The pellet was then resuspended and held in 10 ml aquarium water with light aeration. The inoculum was held for 6–8 d at 26°C to allow *P. tomentosa* eggs to larvate and become infectious. Larvated eggs were evenly distrib-

Table 1. Defined dose trials. Prevalence and mean abundance of *Pseudocapillaria tomentosa* in zebrafish *Danio rerio* following exposure to larvated eggs for 24 h, at 10 fish per 2.8 l tank, except Trial 4 which contained 15 fish per 2.8 l tank. Lower numbers in certain tanks in Trial 1 was due to early mortality in which fish were not examined. Fish were examined at 26 d post-exposure (dpe), except Trial 3 at 31 dpe and Trial 5 at 50 dpe. Gel: eggs incorporated into a gelatin diet. V/M (variance to mean ratio) and *k* (mean^2/variance-mean) calculated for replicate tanks combined for each dose

Dose (eggs fish^{-1})	Replicate	No. of fish	Prevalence (%)	Abundance (worms fish^{-1}) Mean	Range	V/M	*k*
Trial 1							
25	A	4	50	0.75	0–2	0.92	–8.25
25	B	10	40	0.50	0–1		
75	A	4	100	6.00	3–11	3.83	1.32
75	B	7	57	3.44	0–13		
200	A	10	90	4.30	0–13	6.90	1.02
200	B	5	100	9.60	2–24		
Trial 2							
25	A	10	20	0.20	0–1	1.18	4.34
25	B	8	62.5	1.125	0–1		
25	C	10	60	0.9	0–2		
75	A	10	70	2.1	0–2	2.24	2.07
75	B	10	90	3.3	0–7		
75	C	10	60	1.9	0–7		
200	A	9	100	7.8	1–22	4.98	2.51
200	B	10	100	8.9	2–18		
200	C	10	100	13.8	4–24		
Trial 3							
200	A	8	100	3.25	1–8	3.97	1.30
200	B	8	87.5	5.75	0–18		
Trial 4							
200	A	15	93	4.1	0–13	2.47	2.87
200	Gel	15	71	2.4	0–11	3.13	3.60
Trial 5							
200	A	5	100	6.8	3–16	4.62	2.34
200	B	7	85.7	4.9	0–10		
200	C	8	100	7.6	3–14		

uted with a vortex mixture and then enumerated by placing 25 µl of sample under a 22 × 22 mm coverslip and counting the eggs at 100× magnification. Enumeration of eggs was conducted in triplicate and the fish were then exposed to the appropriate number of larvated eggs. There were 10 fish per 2.8 l tank, except Trial 4 which contained 15 fish per 2.8 l tank. Fish were held in either duplicate or triplicate tanks. The incoming water to the tank was turned off for 24 h while aeration was maintained, and then flow was returned at a low rate (50 ml min^{-1}).

Fish were evaluated for the prevalence and abundance of worms at 26 or 31 d post-exposure (dpe) for Trials 1–4, as this is a peak time of infection (Collymore et al. 2014, Gaulke et al. 2016). For Trial 5, fish were examined at 50 dpe to include abundance and prevalence later in the infection, as we previously observed infection in experimentally infected fish persisting for many months after exposure (Kent et al. 2002). To evaluate differences between ingestion of eggs directly in the diet rather than taken up more passively from the water during feeding, a group of fish were exposed to 200 eggs fish^{-1} incorporated into a gelatin diet in Trial 4. This diet (1 ml) was comprised of 1 ml of 7% gelatin, 10% Otohime A larval fish food (Marubeni Nisshin Feed Company), 5% cod liver oil, and 85% water. The gelatin diet contained 3000 eggs in the 1 ml diet which was fed to 15 fish (i.e. 200 eggs fish^{-1}). Eggs were added to the liquid, before it solidified, at 32°C. This resulted in 67 mg of gelatin feed per fish. Food was chopped fine and fed in 1 feeding in a static tank in which the water flow was turned off for 24 h, followed by a slow drip (50 ml min^{-1}) as was done for the waterborne exposures.

We also include data from 3 experiments in which fish were infected by exposure to tank water or aquarium detritus from tanks which contained infected zebrafish, and hence the egg exposure dose was undefined (see Table 2). One of these experi-

Table 2. Undefined dose exposure trials of *Pseudocapillaria tomentosa* infecting *Danio rerio*. Trial 6 and data from Gaulke et al. (2016) and Collymore et al. (2014). Trial 6: 15 fish $tank^{-1}$, half of fish examined at each timepoint. V/M (variance to mean ratio) and k ($mean^2$/variance-mean) calculated for replicate tanks combined for each dose. dpe: no. of days post-exposure; NA: no replicate

Timepoint (dpe)	Replicate	Fish $sample^{-1}$	Prevalence (%)	Abundance (worms $fish^{-1}$) Mean	Range	V/M	k
Trial 6							
27	A	7	100	10.86	1–24	7.90	1.87
27	B	8	100	14.63	2–42		
27	C	8	87.5	13.38	0–23		
32	A	8	100	10.4	4–18	3.62	3.50
32	B	7	85.7	7.7	0–18		
32	C	7	71.4	6.7	0–13		
Gaulke et al. (2016)							
6	NA	10	0	0	0	0	0
11	NA	10	100	50	15–112	13.70	4.10
18	NA	10	100	15.7	7–23	1.76	20.70
25	NA	10	100	11.6	2–27	4.57	3.20
32	NA	10	100	9.3	2–18	3.05	4.52
39	NA	10	90	5.5	0–19	4.69	1.49
Collymore et al. (2014)							
28	A	6	100	8.3	4–18	2.95	3.99
28	B	6	100	8.4	2–13		
28	C	6	100	6.67	3–10		
35	A	6	100	4.16	1–7	0.60	–11.14
35	B	6	100	4.2	3–7		
35	C	6	100	5.0	3–7		

ments was conducted in the present study (Trial 6), while data from the other 2 experiments were from 2 studies previously performed (Collymore et al. 2014, Gaulke et al. 2016) in which fish were exposed, and then maintained under the same husbandry conditions in our laboratory as in the present study. In all 3 experiments (henceforth referred to as undefined), fish were exposed to the infection by either placing the fish in a tank with infected fish (Gaulke et al. 2016) or by placing fish in a tank that previously contained infected fish. With Trial 6, all donor fish (approximately 25 infected fish) were removed from each of two 10 l tanks, and then 55 uninfected fish $tank^{-1}$ were exposed by holding them in the tank for 7 d. Exposed fish were removed from the exposure tank, then mixed together, and 45 fish were evenly distributed into three 2.8 l tanks. Then the fish (either 8 or 7 fish $tank^{-1}$ $timepoint^{-1}$) were examined at 27 or 32 dpe. With Collymore et al. (2014), 200 uninfected AB line zebrafish fish were placed in a 200 l circular tank that previously contained 100 infected fish. They were kept in the tank and exposed for 7 d. This study included groups treated with either ivermectin or emamectin, and hence only data from untreated control fish were evaluated here.

Analyses and patterns of infection

Prevalence and abundance (intensity of infection including uninfected fish) were evaluated as follows. The entire intestine of a fish was removed, placed on a glass slide, about 20 µl of water was added, and then a 60 × 20 mm coverslip overlaid. The preparation was then carefully examined with a compound microscope at 200×, and worms were enumerated from the entire intestine.

Here we present data on mean prevalence and abundance, and aggregation by combining data from each replicate using variance to mean (V/M) ratios and k values. V/M is also designated as 'D' (dispersion), and hence it is important to disambiguate this from D designating Discrepancy, a third method for calculating aggregation (Poulin 1993), which we did not measure here. In parasitology, the parameter k ($mean^2$/variance-mean) measures the overdispersion of counts across a population relative to a Poisson distribution. Consequently, lower positive values of k indicate overdispersion and parasitic aggregation (Anderson 1993, Poulin 2013). Negative values of k are possible and often represent relatively uniform distributions of parasite counts across individuals;

these values can tend to arise when low parasite abundances are observed across the population. We also present data graphically to demonstrate differences in aggregation and their overdispersion (see Figs. 1 & 3).

To determine the dose that yields a 50% infection rate (ID_{50}) and the minimum infectious dose (ID_{min}), we used a generalized linear model with a log-link function to fit the distribution of infection prevalence across experiments as a function of exposure dosage (see Fig. 2). The ID_{50} was measured as the dosage that corresponded to a 50% infection prevalence using this model. The ID_{min} was measured as the dosage that corresponded to 10% infection prevalence using this model. This analysis only included experiments for which the dose was defined and through which exposure occurred via the water. Our models included control samples (i.e. no exposure and no infection) as well as exposed samples that varied in infection burden. Control fish (n = 10 trial^{-1}) were included in Trials 1, 2, 5, and 6 (see Table 1), and these data were used in the calculations for ID_{min}.

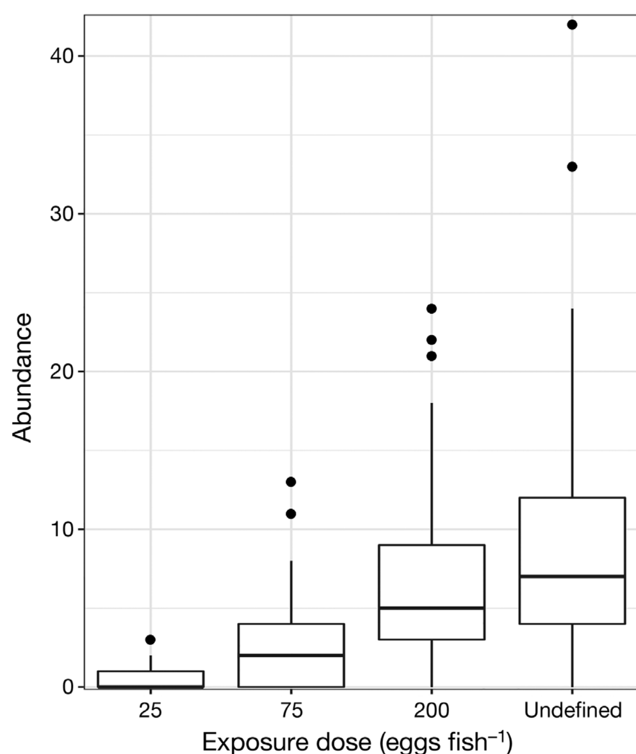

Fig. 1. Experimental *Pseudocapillaria tomentosa* infections in zebrafish *Danio rerio* at 25, 75, or 200 eggs fish^{-1} and undefined exposure show that abundance and dispersion are dose-dependent. Defined doses are from Trials 1–5 (see Table 1). Undefined exposure is combined from Collymore et al. (2014) and Trial 6. Boxes: interquartile range (IQR); points: observations >1.5 × IQR from the median (bar)

To evaluate the effect of sex on worm abundance, we first filtered the data to remove cases where sex was unknown. We then fit a negative binomial generalized linear model (NBGLM) to examine the relationship between sex, dose, and their interaction on parasite abundance. We also examined the impact of sex alone on abundance in the undefined exposure and 200 eggs fish^{-1} groups separately using NBGLMs.

RESULTS

Prevalence and abundance

All doses resulted in a high prevalence of infection, except at the lowest dose where the mean prevalence was 43 or 46% for Trials 1 and 2 (Table 1, Figs. 1 & 2). Fish exposed at 75 eggs fish^{-1} showed 72 or 73% mean prevalence, while prevalence values of the various trials at 200 eggs fish^{-1} or the undefined exposure were all >85%. Likewise, mean abundance corresponded to dose, ranging from 0.6 worms fish^{-1} at 25 eggs fish^{-1}, about 5 worms fish^{-1} at 75 eggs fish^{-1}, about 7 worms fish^{-1} at 200 eggs fish^{-1}, and 9 worms fish^{-1} for the undefined exposures when the various trials were evaluated together. This average for undefined exposure includes data from earlier studies (Collymore et al. 2014, Gaulke et al. 2016), but excludes the timepoints 11 and 15 dpe from Gaulke et al. (2016), as inflated abundance occurred at these timepoints due to intense larvae infections. All replicates in the present experiment (Trials 1–6) were initiated with 10 fish tank^{-1}, except in Trial 4 with 15 fish tank^{-1} and Trial 6 with 16 fish tank^{-1}. Lower numbers within the replicates represent mortalities that occurred before the examination. There was no indication of other prominent infectious disease in these fish based on clinical signs. These fish did not exhibit prominent emaciation, which is consistent with morbidity associated with severe infections by *Pseudocapillaria tomentosa*.

ID_{50} and ID_{min}

We found that the relationship between exposure dosage and infection prevalence follows a logarithmic relationship, such that logarithmic increases in dosage yield linear increases in the proportion of infected individuals (Fig. 2). The line of best fit through the observed distribution of infection prevalence across exposure doses provides an opportunity to quantify ID_{50} as well as ID_{min}, which is the dose

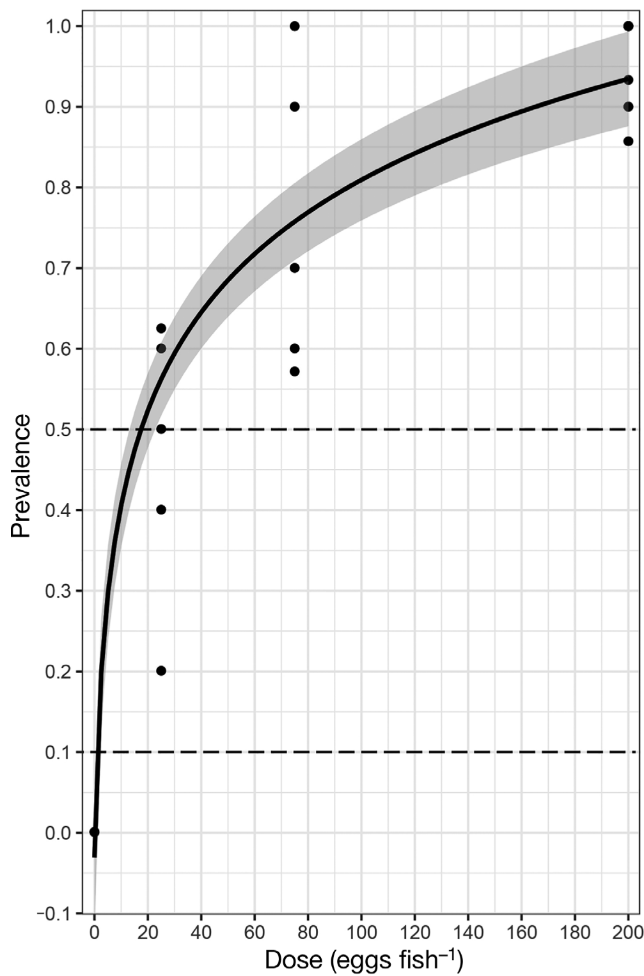

Fig. 2. Prevalence of infection (proportion) relating to dose from Trials 1–5 of *Pseudocapillaria tomentosa* infecting *Danio rerio*. Data for 0 eggs fish^{-1} (controls) were provided by 40 fish from Trials 1–3 and 6 (see Table 1). Estimates are minimum infectious dose (ID_{min}) and 50% infective dose (ID_{50}) indicated by dashed lines at 0.1 and 0.5, respectively. Points represent measures of infection prevalence for each of the defined exposure trials included in this study. Grey shading represents the standard error associated with the line of best fit (black line) through these points

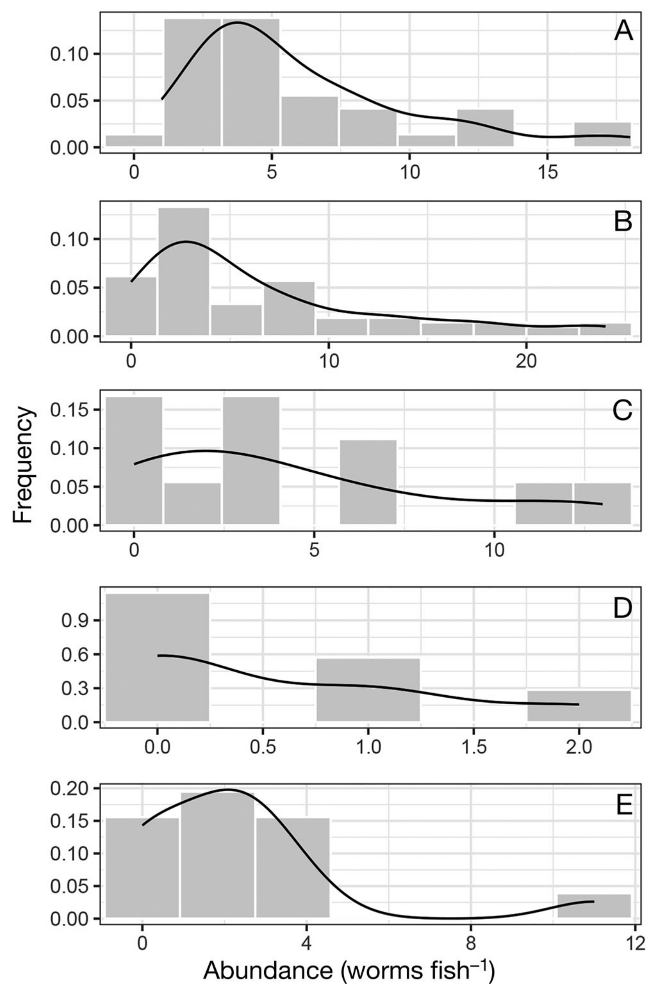

Fig. 3. Frequency distribution of abundance values illustrating the aggregation of *Pseudocapillaria tomentosa* in 5 groups of experimentally infected zebrafish *Danio rerio*. Frequency: proportion of fish with a specific number of parasites. (A) Undefined dose, (B) 200 eggs fish^{-1}, (C) 75 eggs fish^{-1}, (D) 25 eggs fish^{-1}, (E) exposure by gelatin diet. Note the different scales for the *x*- and *y*-axes

necessary to infect at least 10% of exposed individuals, corresponding to 1 in 10 individuals carrying an infection in our trials. Our analysis reveals an ID_{50} of 17.5 eggs fish^{-1} and an ID_{min} of 1.5 eggs fish^{-1}.

Aggregation

The V/M and *k* summary statistics consistently indicated aggregation across all exposure methods, trials, and the previously published studies (Tables 1 & 2). Corresponding with the lower levels of infections, lower doses showed less dispersion (Figs. 1 & 3). V/M ranged from 0.92 to 13.7, with an average of 4.3. The *k* values ranged from 1.02 to 20, with a mean of 3.8. Removal of the one k value of 20 (from a small, unreplicated group in Gaulke et al. 2016) resulted in a mean *k* value of 2.75. In both Trials 1 and 2, where all 3 defined doses were included, there is a linear relationship in respect to V/M with dose, with a Pearson's R^2 of 0.72 ($p = 0.021$).

Influence of time

Early exposure timepoints from the Gaulke et al. (2016) experiment with an undefined dose showed

inconsistent aggregation indices and it should be noted that fish were infected with numerous larvae at these times. As seen in the several defined dose trials and the undefined exposure experiments, both V/M and *k* scores demonstrating aggregation were not pronounced until after 3 wk post-exposure, a time at which larval worms decrease. One separate trial (Trial 5) exposing fish to 200 eggs fish^{-1} entailed examination of fish later in the infection (50 dpe). Here, fish showed similar abundance and aggregation as fish examined in other trials at earlier timepoints.

Gelatin diet

In Trial 4, fish were fed 200 eggs fish^{-1} in the gelatin diet, which they readily ate. They showed about half the abundance of infection compared to fish exposed at the same dose in the water. As with other trials, these fish exposed by diet also showed a trend to aggregation. Here, 14 of 15 fish showed 0–5 worms fish^{-1}, while 1 fish had 15 worms.

Sex

Mean abundance of the parasite based on sex of the host where sex data were available (n = 210) was as follows. For all doses, males (n = 95) had a mean abundance of 6.10 worms fish^{-1}, and females (n = 115) had 5.96 worms fish^{-1}; for the trials with undefined exposure (Trials 5 and 7), males (n = 39) showed mean abundance of 8.46 worms fish^{-1}, and females (n = 38) had 9.13 worms fish^{-1}; and for the dose of 200 eggs fish^{-1} (Trials 2, 4, and 6), males (n = 40) had a mean abundance of 8.15 worms fish^{-1}, while females (n = 25) had 6.88 worms fish^{-1}. We did not observe significant effects of sex on burden in the undefined (p = 0.68) or 200 eggs fish^{-1} (p = 0.41) groups. The interaction between dose and sex also was not significant.

DISCUSSION

Analyses of aggregation patterns based on review of hundreds of studies regarding differences between host/parasite systems and different studies showed that the most important factor affecting the extent of observed aggregation is the number of parasites per host, followed by the number of individual hosts that are evaluated, and then species-specific and study-specific effects reflecting idiosyncrasies of particular systems (Shaw & Dobson 1995, Poulin 2007, 2013). Infections by juvenile nematodes tended to show rather high V/M ratios (about 80), whereas adult nematodes showed lower values (about 15) (Poulin 2013). In the present analyses with *Pseudocapillaria tomentosa*, aside from larvae early in the infection (Gaulke et al. 2016), we could not differentiate between immature or mature worms, except for gravid females, and thus data were evaluated as total worms. V/M and *k* indices in the present study showed related results, indicating that the trends we highlight here are likely robust. Future investigations may consider implementing more complicated, but potentially more accurate, maximum likelihood measures of *k* (Pacala & Dobson 1988, Yakob et al. 2014) as an additional assessment of our study's robustness.

Laboratory studies provide the possibility to control or limit many of the factors affecting aggregation within studies. Two studies with pigs exposed to either *Ascaris suum* (Roepstorff et al. 1997) or *Trichuris suis* (Pedersen & Saeed 2002) are particularly noteworthy, as the pigs were orally infected with defined numbers of larvated eggs. The mode of infection, ingestion of larvated eggs, was the same as that in the present study with *P. tomentosa*. Experiments with laboratory mice experimentally infected with the trichostrongylid nematode *Heligmosoides polygyrus* (Keymer & Hiorns 1986) warrant inclusion in this discussion. Although the infectious stage is a larva rather than larvated egg, experimental infections were initiated by precise doses by oral gavage. In all 3 laboratory models, a high degree of aggregation was observed even when animals were given the same dose within groups by oral gavage, removing the influence of feeding behavior and environmental distribution of infective eggs. Moreover, whereas the pigs and mice were not isogenic, the genetic constitution was more uniform than in freely mating populations, and the laboratory animals were of similar ages, sizes, and strains within the treatment groups. Likewise, our fish were from the same parent population (either siblings or cousins), and were of similar age and size. Also, aside from Collymore et al. (2014), the fish were all from the 5D line.

Regarding variability of infectious dose, in contrast to the 2 pig and mouse studies, we did not directly infect individuals by gavage in either the controlled dose trials or the undefined dose trials. The gelatin diet trial provided some additional control of exposure, but we still could not confirm precise homogeneity between individuals within a tank, as some fish may have eaten more than others.

Nevertheless, the occurrence of 3 times more parasites in 1 fish than its tank mates would have required the fish to eat 3-fold more food than the others to achieve this difference if infectious dose was the only factor. Future experiments could include per os gavage with larvated eggs or single housing during feeding of a gelatin diet with a known egg concentration. The former has been accomplished with zebrafish (Collymore et al. 2013), but it can be very difficult to deliver a precise dose (Harriff et al. 2007), as zebrafish do not have a stomach and are quite small. Fish exposed to undefined numbers of eggs had about twice the abundance of infection compared to fish exposed to 200 eggs fish^{-1}. Collymore et al. (2014) used an undefined dose and the AB line rather than 5D, and that study showed less variability in abundance than 5D fish exposed to an undefined dose. The AB line was introduced to biomedical research about 40 yr ago and Balik-Meisner et al. (2018) showed that the 5D line is more genetically diverse than the AB line. Differences in abundance variability may therefore be related to fish lines, but as these were 2 separate experiments, we cannot verify this with the data presently available.

A high amount of variability in parasite abundance among individuals necessitates the examination of large numbers of hosts to obtain an accurate representation of aggregation (Anderson 1993). Gregory & Woolhouse (1993) showed that as sample sizes decrease below 100, means and variance are underestimated, and hence aggregation indices also decrease. As in other laboratory studies, our host sample sizes were smaller than most field studies. Nevertheless, even with the very small sample sizes in 2 trials (Trial 1 and Gaulke et al. 2016), we observed aggregation, particularly after early infection. Conflicting results occur with the first 2 timepoints from the Gaulke et al. (2016) data, where the sample sizes were 10 (Table 2). Combining all the fish together from these 2 points when larval stages were numerous resulted in a V/M of 21.8 and a k of 1.51, both indicating strong aggregation. Hence, laboratory studies, although well defined, probably represent some degree of underestimation of aggregation when host sample sizes are relatively small. Keymer & Hiorns (1986) showed that V/M increases with increasing exposure dose and adult worm burden in mice infected with *H. polygyrus* (Nematoda). Similarly, we observed this pattern in our trials, and using the data from the defined exposure dose trials included in this study, we found that V/M was strongly associated with exposure dose.

Poulin (2013) summarized from numerous studies that infections by juvenile nematodes tended to be more aggregated than those by adults. In contrast, in the defined laboratory experiment studies with *A. suum* in pigs and *H. polygyrus* in mice, the opposite was observed, and our evaluation of data from Gaulke et al. (2016) supported these experimental findings. All 3 studies entailed exposing the animals to the parasites with 1 dose (more precisely defined with the pig and mouse studies with oral gavage). These exposures were followed by infection by very large numbers of larval worms with little or no aggregation, and followed by a reduction in total burden and concurrent with increased aggregation as the infection progressed. At 3 different log doses, k values plummeted (i.e. aggregation increased) after 2 wk into the infection with *A. suum* in pigs (Roepstorff et al. 1997). These authors proposed that this pattern occurred because there was variable expulsion of worms amongst hosts.

As seen with other trichinelloid nematodes (e.g. whipworms), adult capillarid nematodes, including *P. tomentosa*, penetrate epithelial linings, and infections are long-lived (Kent et al. 2002, Murray & Peterson 2015). Multiple timepoints were assessed in the Gaulke et al. (2016) study, but none beyond 48 dpe. Fish were still infected at 55 dpe (Trial 5), and 100% (4/4) of fish were still infected at 23 wk post-exposure in an earlier study (Kent et al. 2002). The multiple trials here and previous papers suggest that prevalence remains high for many months, with a trend toward reduction in abundance. Gaulke et al. (2016) showed a dramatic increase in adult worms between 13 and 20 dpe. Then prevalence remains high with a moderate decline in abundance from between 4 and 5 or 6 wk (Collymore et al. 2014, Gaulke et al. 2016). This probably occurs with *P. tomentosa*, as the larval worm abundance at early timepoints far exceeded the abundance of other developmental stages as the infection progresses (Gaulke at al. 2016). An ongoing experiment in our laboratory includes a longitudinal sampling protocol from the same population of exposed fish to address the pattern of infection later in infection.

There are several studies using mice as model for *Trichuris* infections (Klementowicz et al. 2012, Hurst & Else 2013), and 40 eggs mouse^{-1} is considered a 'low dose' (Bancroft et al. 1994). With mice treated with cortisone, Michael & Bundy (1989) achieved infections at 10 eggs mouse^{-1}. Although our results must be cautiously interpreted given the limited number of dosages assessed in this study, our analysis reveals an ID_{50} of 17.5 eggs fish^{-1}. This

result was somewhat lower than our observed data, where fish exposed to 25 eggs fish^{-1} from 2 experiments showed a mean prevalence of 45%. ID_{min} was estimated to be 1.5 eggs fish^{-1}. Unfortunately, due to the relatively few trials conducted at low doses, this estimate carries low precision, and future investigations should seek to explicitly validate it. However, this estimate of 1.5 eggs fish^{-1} to establish an infection corresponds with our present studies, and with defined laboratory studies with pig and mice nematodes in that the maximum intensity of infection was seldom greater than 50% of the infectious dose (Keymer & Hiorns 1986, Michael & Bundy 1989, Roepstorff et al. 1997, Pedersen & Saeed 2002).

It should be noted that these estimates were based on eggs fish^{-1}, not a concentration of eggs in water. However, in the present study (Trials 1–5), the concentration of fish l^{-1} and all other husbandry parameters were the same across trials, except for Trial 4. Here, fish were exposed at 15 fish per 2.8 l tank, rather than 10, and thus the eggs l^{-1} was 50% greater in this trial than in the others at 200 eggs fish^{-1}. Interestingly, this group actually had slightly lower abundance than the other groups exposed at 200 eggs fish^{-1}. Nevertheless, it would be logical that fish exposed to the same number of eggs, but in a much more dilute environment, would show lighter infections. Likewise, the duration of undiluted exposure was about 24 h, and longer duration of exposure may result in increased abundance. Parasites often exhibit a threshold of abundance beyond which increased exposure does not ultimately result in higher established infections (Anderson 1993).

Zebrafish become sexually mature within a few months of hatching, and hence most studies with adult zebrafish are done with sexually mature fish. Sexual differentiation in zebrafish is complicated, as all fish are initially females, and then some become males. Hence, zebrafish can be categorized as juvenile hermaphrodites. Although zebrafish sex is primarily determined by genetics, a multitude of factors such as strain type, fish density, feeding, growth rate, oxygen levels, and temperature can profoundly influence sex (Liew & Orbán 2014). Hence there are often significant differences in sex ratios between populations of laboratory zebrafish (Lawrence et al. 2008). The influence of sex on the outcomes of laboratory experiments is now an important concern in biomedical research (Clayton & Collins 2014). Multiple studies have shown that males tend to be more heavily parasitized than females when examining mammal hosts (Schalk & Forbes 1997) or vertebrates in general (Poulin 1996). However, Michael & Bundy (1989) found no effect of host sex with experimental infections of *T. muris* in mice. Male fish are more prone to infection by the microsporidium *Pseudoloma neurophilia* (Chow et al. 2016), but in our nematode model, we saw no influence of sex on abundance. This is consistent with the review by Poulin (1996), in which collective analysis of several studies showed that while males of mammals and birds generally show a higher intensity of infection with nematodes than females, this phenomenon does not extend to fish.

In conclusion, as with other laboratory studies, we eliminated or significantly reduced many factors associated with variability in aggregation. As discussed above, we observed consistent patterns of infection and aggregation between trials. After other factors are addressed and accounted for, still about 65% of the aggregation variability between studies is accounted for by 'species-specific and study specific idiosyncrasies' (Poulin 2013). Regarding the latter, our various trials, including the earlier published studies, were conducted in the same laboratory, same water conditions, and with the same line of specific pathogen-free zebrafish. We saw a remarkable similarity of prevalence between trials at a given dose, but some variability in abundance and aggregations. This suggests that other unknown factors that may influence differences in host susceptibility within and between populations may be important.

Given the consistent observation of aggregation across our experiments, and the shared patterns observed between our studies and those conducted in other vertebrate hosts, the zebrafish/*P. tomentosa* model provides a potentially robust, high-throughput model to investigate the influence of differences among hosts within defined populations. Certainly differences in immune status may play a role, and this could be addressed with isogenic zebrafish or the mutant lines with specific immune gene alterations that are readily available. We are conducting various studies on the intestinal bacterial microbiome of zebrafish, and have found that the presence and abundance of *P. tomentosa* is significantly associated with differences in the bacterial microbiome (Gaulke et al. 2016). Other studies have reported alterations in the intestinal microbiome associated with intestinal helminths (Zaiss & Harris 2016). Germane to our model, the presence of intestinal bacteria are required for *T. muris* to hatch in mice (Hayes et al. 2010). The reliability of our zebrafish/*P. tomentosa* model may also provide a useful link between *in vitro*

and mouse assays for drug discovery, as the nematode in zebrafish is susceptible to traditional anthelminthic compounds and the cost ratio of husbandry compared with mice is 2 to 3 orders of magnitude lower (Goldsmith 2004).

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