Within-host transmission strategies of transovarial, feminizing parasites of *Gammarus duebeni*

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SUMMARY

The amphipod *Gammarus duebeni* harbours several species of vertically transmitted, feminizing microsporidian parasites. *G. duebeni* were collected from 3 localities in the UK. Animals from Budle Bay, Northumberland, were infected with *Octosporea effeminans*, and those from Millport, Isle of Cumbrae and Fenham Flats, Northumberland were infected with microsporidia of the genus *Nosema*. We derived expected distributions of parasites per host embryonic cell by modelling parasite transmission as a multitype, Galton–Watson branching process. Parasite prevalence (proportion of females infected) was significantly heterogeneous among localities. Parasite burden in zygotes was much higher for females infected with *Nosema* than in animals infected with *O. effeminans*. There was no significant difference between localities in the number of *Nosema* in the zygotes. Comparison of models and data from 64-cell host embryos showed that the distributions of parasites per cell were consistent with the hypothesis that sorting of parasites into daughter cells is biased for at least 1 cell lineage. Host embryos infected with *O. effeminans* could expect to contain a growing number of parasites in each cell generation within such biased cell lineages; similar estimates for *Nosema* predict a decline in the number of parasites per cell within a biased lineage. We discuss the possibility that the 2 species of parasite may be employing different strategies in order to ensure transmission to the next host generation.

Key words: branching process, *Gammarus duebeni*, microsporidian, *Nosema*, *Octosporea effeminans*, vertical transmission.

INTRODUCTION

Vertical transmission, from parent to offspring via the gametes, is used by a number of microparasites including viruses, bacteria, and protozoa (Dunn *et al*. 1995). In many cases, infection through vertical transmission leads to host death and subsequent horizontal transmission (e.g. Sweeney, Hazard & Graham, 1985; Agnew & Koella, 1996). However, for other parasites, vertical transmission from generation to generation appears to maintain the parasite within host populations (Dunn, Adams & Smith, 1993; Ni, Backus & Maddox, 1997; Terry, Dunn & Smith, 1997). Transmission efficiency is a key factor in this particular host–parasite relationship and is important for the evolutionary impact of these parasites on their hosts (Werren, 1987; Hatcher & Dunn, 1995).

Vertically transmitted parasites must overcome 2 bottlenecks in order to ensure their survival through successive host generations. First, the parasite must be present in the egg when laid; secondly, the parasite must find its way to the transmitting, gonadal tissue of the adult host. If the parasite cannot cross cell boundaries in mature or developing hosts, then it must find the germ line or gonad during host development in order to ensure transmission (Hatcher, Dunn & Tofts, 1996).

In a previous study of a transovarially transmitted microsporidian, *Octosporea effeminans* infecting *Gammarus duebeni*, we found that parasite numbers were very low during host development and that there was no evidence for parasite movement between host cells. The pattern of parasite distribution suggested a possible bias of parasite segregation into a cell lineage during host embryogenesis (Dunn *et al*. 1995). In this study, we investigated strategies of within-host transmission of 2 transovarially transmitted, feminizing microsporidia: *O. effeminans* and a recently described parasite of the genus *Nosema*, morphologically distinct from *O. effeminans* and present in relatively high numbers in adult and embryonic hosts (Terry, Dunn & Smith, 1997).

To investigate hypotheses of the mechanism by which the parasites are able to infect target host tissues, we developed mathematical models of various within-host transmission strategies, and used the models to generate expected distributions of the numbers of parasites within host cells. The results suggest several mechanisms for within-host transmission, including the possibility that the 2 species of microsporidia may employ different strategies in order to ensure transmission to the next host generation.

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MATERIALS AND METHODS

Collection of data

Adult *Gammarus duebeni* were collected from 3 field sites in the UK: Budle Bay, Northumberland, Fenham Flats, Northumberland, and Millport, Isle of Cumbrae. In the first population, females are infected with the microsporidian *Octosporea effeminans* (Bulnheim & Vavra, 1968; Dunn *et al*. 1993); at Millport females are infected with *Nosema* (Terry *et al*. 1997); and at Fenham Flats, females harbour a parasite also provisionally identified as *Nosema* (Terry, unpublished). Each of the parasites is vertically transmitted and feminizes the host. In the laboratory, pairs of animals were placed in individual containers of brackish water (salinity 6·5‰, equivalent to field salinity). Female *G. duebeni* lay their eggs into a brood pouch, where they are fertilized and brooded for 3–4 weeks. Pairs were examined daily for the presence of fertilized eggs. Early stage embryos (1–128 cells) were removed from the brood pouch of anaesthetized females, permeated with 5 M HCl, rinsed in distilled water, fixed in acetone at −20 °C, squashed on a microscope slide, and stained with DAPI (4,6-diamidino-2-phenyl-indole diluted 1/500 in 0·2 M NaH_2PO_4 plus 8 % glycerol, pH 7·5), a fluorescent dye for DNA. The embryos were screened for parasites using a Zeiss Axioplan fluorescent microscope. The developmental stage (number of host cells), total parasite count, and distribution of parasites within cells were recorded for all infected embryos.

Derivation of models

We consider the transmission of parasites through the successive cleavage stages of the host embryo as a branching process (Harris, 1963; Athreya & Ney, 1971; for general explanation and applications to parasitology see Mackinnon, 1997; Taneyhill, Dunn & Hatcher, 1998). A branching process describes the stochastic evolution of the numbers of descendants arising from 1 or more initial ancestors. The simplest type of branching process is known as the Galton–Watson process, where it is assumed that the initial ancestor and all descendants reproduce independently according to a fixed probability function that yields the chance of each individual producing k offspring, where k is a non-negative integer. Some familiar examples of offspring distributions are the binomial and Poisson distributions. The techniques used to analyse a branching process allow one to obtain the expected distribution of the number of offspring in each generation, the expected mean and variance of total offspring, and the probability that the process will eventually go extinct.

For our particular problem, the transmission of parasites through the developing embryo, consider first the number of parasites in a single, randomly chosen cell lineage. During development the parasites themselves may replicate via binary fission, and when embryonic cells divide the parasites are sorted into the resulting 2 daughter cells. Starting with the zygote, the number of parasites that each cell in the lineage contains will thus be determined by the transmitted fraction from the mother cell and the number of transmitted parasites that undergo binary fission. Let each parasite have a constant probability p of reproducing by fission each cell generation, and denote the probability of being transmitted to the lineage of interest by q. If there is no bias in transmission (each of the 2 daughter cells is equally likely to receive each parasite), then $q = 1/2$. If the mother cell contains i parasites, the probability that a chosen daughter cell contains j parasites, $P_i(j)$, is given by

$$P_i(j) = \sum_{k=i}^{2\times i} \binom{i}{k-i} p^{k-i}(1-p)^{2i-k} \binom{k}{j} q^j(1-q)^{k-j}. \quad (1)$$

To find the probability that a cell in generation n contains j parasites, we first convert equation (1) to a more convenient form called the probability generating function (p.g.f.). We denote this function by $G_i(s)$, where s is a dummy variable representing a real number in the interval $0 \leqslant s \leqslant 1$. The function $G_i(s)$ is defined as

$$G_i(s) = \sum_{j=0}^{2\times i} P_i(j)\, s^j, \quad (2)$$

which, from equation (1), defines $G_i(s)$ as

$$G_i(s) = (1-q+q\,s)^i\,(1-pq+pq\,s)^i. \quad (3)$$

The probabilities $P_i(j)$, along with the mean, variance, and other moments, may now be found from $G_i(s)$ by standard methods using probability generating functions (Karlin & Taylor, 1975). The branching nature of the processes of parasite fission and host cell division is shown schematically in Fig. 1.

The simple Galton–Watson branching process methods allow us to describe the evolution of parasite number in 1 chosen cell lineage. In order to examine the number of parasites in the entire embryo, we describe host development as a multitype branching process. In a multitype branching process, the particles under consideration may produce new particles of various types (for an application to cell proliferation see Stivers, Kimmel & Axelrod, 1996). In our particular case, we define the type of an embryonic cell as the number of parasites that it contains. During cleavage, the cell will produce exactly 2 offspring, each of which may possibly be of any type from zero (no parasites sorted into that daughter cell) to twice the number of parasites in the mother cell (if all parasites underwent binary fission and were sorted into the same daughter cell).

To analyse a multitype process, we construct a matrix $\boldsymbol{M}$ whose entries in the ith row and jth

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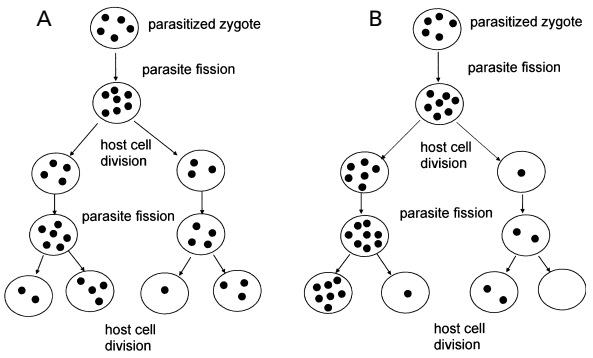

Fig. 1. Schematic illustration of parasite transmission within the developing host embryo, under 2 hypothetical models of parasite transmission strategy. (A) The parasites are confined to cell lines, and the probability of a parasite being transmitted to a particular daughter cell at cleavage is unbiased i.e. equal to $\frac{1}{2}$. (B) The transmission probability for 1 cell lineage is biased, so that 1 embryonic cell line (far left in the illustration) is expected to contain a greater number of parasites than other cell lines. The illustrations correspond to (A) Model 2 and (B) Model 3 as explained in the text.

column, (m_{ij}), are the average numbers of cells containing j parasites produced by a parent cell containing i parasites. Using standard methods from multitype branching processes (Athreya & Ney, 1971; Seneta & Tavaré, 1983), it may be shown that $m_{ij} = 2P_i(j)$.

The number of parasites present initially in the zygote will be denoted as Z_0. The zygote itself is represented, in the multitype process, as a vector Z_0 of the form $(0, 0, \ldots, 1, 0, \ldots, 0)$, having a 1 at the position corresponding to the number of parasites in the zygote, and zero everywhere else. Note that we ignore the zero class (i.e. empty host cells) because we wish to deal exclusively with the proportions of infected cells, meaning those containing 1 or more parasites. The reason for this is that since p, the probability of parasite replication, will be less than 1, the parasites will eventually become sparse within the embryo, so that the zero class would dominate the distributions for all models of parasite transmission. The expected distribution vector of parasites per cell at cell generation n is given by

$$(\hat{f}_n(1), \hat{f}_n(2), \ldots, \hat{f}_n(k))' = \frac{Z_0' M^n}{(Z_0' M^n)\,1}, \qquad (4)$$

where $1 = (1, 1, \ldots, 1)'$.

We used the above techniques to construct expected distributions of the number of parasites per cell for the following 4 models, each based on specific hypotheses of parasite replication and transmission.

Model 1. Free movement of parasites within the host embryo. In this null model, we assume that the parasites may cross cell boundaries i.e. they are not confined to specific cell lineages. Each parasite thus has an equal chance of being found in each embryonic cell. The expected proportions of cells containing j parasites may then be found from the successive terms of a binomial distribution with parameters α and N, where N is the total number of parasites observed in the embryo and $\alpha = 1/2^n$, n being the number of host cell divisions. The expected proportions for infected cells $\hat{f}(j)$ are

$$\hat{f}(j) = \frac{1}{1-\hat{f}(0)} \binom{N}{j} \alpha^j (1-\alpha)^{N-j},$$

with $\hat{f}(0)$, the expected frequency of empty cells, equal to $(1-\alpha)^N$.

Model 2. Confinement of parasites to cell lineages, unbiased sorting of parasites into daughter cells. In this model we assume that when cells divide there is no bias in the sorting of parasites into the 2 daughter cells, making q from equation (1) always equal to $\frac{1}{2}$ for all cell lineages. We assume that the probability of parasite fission p is constant each cell generation. Model 2 thus corresponds to the illustration in Fig. 1 A. Under this model, the mean number of parasites in a randomly chosen cell line after n generations is, from equations (1) and (2), equal to $Z_0[(1+p)/2]^n$. Compared to Model 1, the mean number of parasites per infected cell will be higher, since once a cell is cleared of parasites, all the cells descending from that cell will also contain no parasites.

Model 3. Confinement of parasites to cell lineages, biased sorting for one cell lineage. In this model we assume that there is 1 cell lineage for which sorting of parasites from the mother cell is biased toward that lineage, so that q from equation (1) is greater than $\frac{1}{2}$. All other cell lineages are assumed to have $q = \frac{1}{2}$. Model 3 corresponds to the illustration in Fig. 1 B. Compared to Model 2, this assumption of 1 lineage with biased sorting will increase the mean number of parasites per infected cell (for example as q approaches 1, more and more of the total parasites are contained in a single cell lineage). Model 3 thus consists of 2 branching processes, a simple Galton–Watson branching process for 1 cell lineage as described by equations (1) and (3), and a multitype process for all other cell lineages in the embryo, as described by equation (4). We note that mathematically, the pattern of distribution generated by these assumptions could also be produced by an unbiased probability of sorting ($q = 1/2$) combined with a biased probability of fission p for 1 cell lineage. However, since we can only observe the overall rate of parasite growth from measurements of entire embryos, it is more convenient to consider a biased lineage as the product of non-random sorting, since we have an *a priori* expectation for the value of q in the non-biased lineages i.e. $q = \frac{1}{2}$.

Model 4. Non-constant probability of parasite fission. Examination of the data sets suggested that the rate of parasite division might not have been constant

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each generation, at least for parasites from some localities. For example, the majority of parasites may replicate only once every other host cell generation. The resulting model will have several values of p depending on the cell generation. For Model 4 we assumed 2 alternating values of p, p_1 and p_2. The limit case of Model 4 is a lockstep form of parasite division, in which all the parasites divide with probability 1 during alternating cell generations, making $p_1 = 1$ and $p_2 = 0$ (see Dunn *et al.* 1995). Estimating 2 values of p keeps the number of free parameters estimated constant, because once p_1 is estimated, p_2 is constrained by the relation $(1+p_1)(1+p_2) = (1+p)^2$, where p is again the constant estimate from Models 2 and 3. While more values of p at various cell generations could be estimated from the data, setting the maximum number at 2 serves to keep the total number of estimated parameters constant for Models 3 and 4.

Estimation of parameters

Each of the above mathematical models may be completely parameterized by specifying the initial number of parasites in the zygote (Z_0), the probability that a parasite will undergo binary fission each cell generation (p), and the probability that a parasite in a mother cell is sorted into a chosen daughter cell (q). In the case of Model 4, there are 2 alternating fission probabilities p_1 and p_2.

Since each embryo was observed only at its present number of cells, we do not know with certainty how many parasites were present in the zygote stage. Therefore we obtained estimates $\hat{Z}_0$ of initial parasite loads via the observed mean parasite numbers for the various cell stages in the entire data set (Dunn *et al.* 1995). Each embryo was thus assigned an initial estimated number of parasites by back-calculating from the observed number, using the global mean change in parasite load. For example, 64-cell embryos from Millport contained approximately 4 times as many parasites on average as did zygotes, so each 64-cell stage embryo in the data set was assigned one-fourth its total parasite load as $\hat{Z}_0$. To estimate p, the mean numbers of parasites in 64-cell embryos (denoted Z_6, for 6 cell divisions) and the average numbers of parasites in the zygotes (Z_0) were used to estimate p as

$$\hat{p} = \exp\ \frac{\ln(Z_6/Z_0)}{6}\ -1.$$

Because of the very low numbers of early (1–4 cell stage) embryos sampled from Budle Bay, p was estimated from the changes in parasite numbers between the 16 and 32 cell stage and 32 and 64 cell stage embryos.

To estimate q, we assumed that the cell containing the maximum observed number of parasites in each embryo was the product of the biased division lineage. From equation (2), the expected number of parasites in the biased lineage at generation n equals $Z_0[(1+p)\,q]^n$, so the estimate $\hat{a}$ is given by

$$\hat{q} = \sqrt[n]{\frac{\max}{(1+p)^n Z_0}},$$

where the maximum is the average maximum for the embryos in that particular Z_0 class.

Because this method of obtaining q is indirect, we also investigated the hypothesis of non-random assortment of parasites into daughter cells by examining the numbers of parasites in 2-cell embryos. Counts of parasites in each 2-cell embryo were tested against the hypothesis of a 1:1 ratio via replicated log-likelihood test for goodness of fit. Tests were carried out only for data from Fenham Flats and Millport, due to the paucity of 2-cell embryos from the Budle Bay population.

For the data obtained from Millport and Fenham Flats, counts of the numbers of parasites in cells containing the maximum number per embryo often exceeded 50 parasites; such counts may be biased toward underestimates due to the difficulty of accurately counting such large numbers of parasites in 1 cell. To take the possible underestimation into account, models for both localities were fitted with 3 values of q equal to 0·6, 0·65 and 0·7; these values covered the range of values that would have been estimated from what we considered to be lower and upper bounds on the number of parasites in those heavily parasitized cells.

Analysis of data

Prevalences of the parasite (proportion of infected females) were tested for heterogeneity among the 3 localities by casting the data into a 3×2 contingency table, followed by a log-likelihood test of independence (Sokal & Rohlf, 1981).

To compare frequency distributions to predictions from the models, each embryo from each locality was first classified according to cell stage, from 1 to 64 cells, and according to initial parasite load. Since the preparations usually did not reveal all cells present, each embryo was assigned to cell stage by rounding upward to the next highest power of 2 (for example, an embryo with 45 visible cells was assigned as a 64-cell stage embryo). The number of parasites that the whole embryo contained was then estimated by multiplying the observed number of parasites by the ratio of the assigned cell stage to the observed cell number; this keeps the average number of parasites per cell constant. For each class of initial parasite load in each locality, the frequencies of cells containing various numbers of parasites were compared to the expected proportions from the 4 models detailed above, using log-likelihood goodness of fit tests (Sokal & Rohlf, 1981). For the data from Budle Bay, we also used t-tests to compare the mean

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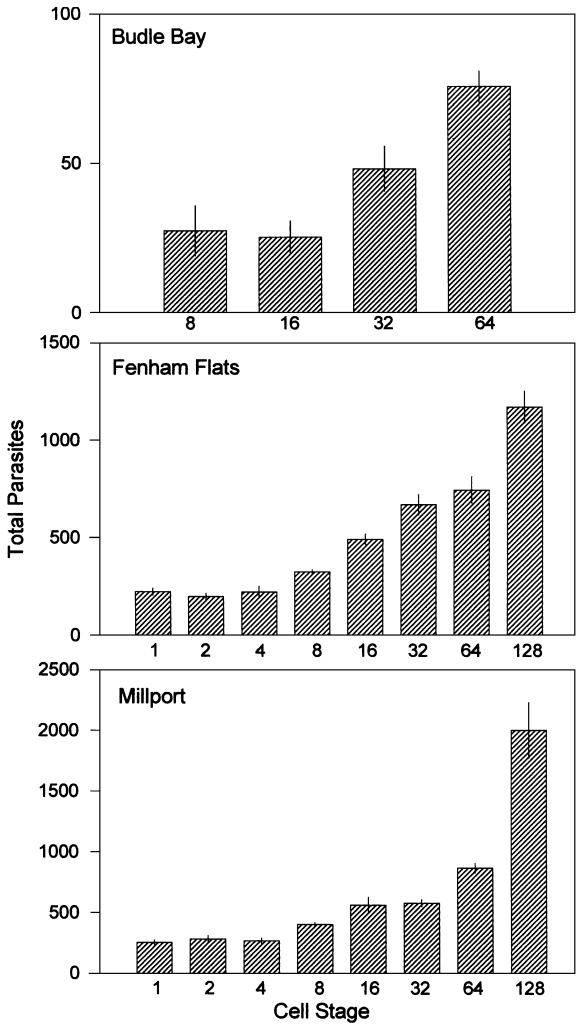

Fig. 2. Mean total parasites per embryo in sampled eggs of *Gammarus duebeni* from 3 localities. Cell stage denotes the total number of cells present in the sampled embryo. Mean values shown ± 1 S.E. Values for 1 to 4-cell embryos from Budle Bay are not presented due to the very low frequencies of such embryos in samples from that locality.

number of parasites per infected cell to the expected parametric means from the 4 models; since parasite load was relatively low from that area, most of the cells in the embryo are expected to be empty, making it difficult to differentiate between the models only on the basis of the expected frequency distributions. All results reported here are for embryos in the 64-cell stage. Only initial parasite load classes that had a minimum of 100 observed cells were used in the analysis; for the Millport and Fenham Flats data we pooled embryos within ± 5 parasites of the estimated value of Z_0.

RESULTS

Mean parasite load and parasite prevalence

The overall mean numbers of parasites in embryos from 1–128 cell stages are shown in Fig. 2. Early stage (1–4 cells) and 128-cell stage data for Budle Bay are not shown due to the paucity of embryos at those stages sampled from that locality; the few zygotes sampled had parasite densities consistent with the numbers estimated from the counts at later stages and the estimated parasite growth rate i.e. from 3 to 20 parasites in the zygote. This was much less than the total initial parasite loads of *Nosema* in the Fenham Flats and Millport zygotes; respective means were $223 \pm 21{\cdot}3$ and $255 \pm 27{\cdot}4$ parasites in zygotes (standard errors). The mean numbers of parasites per zygote were not significantly different between the Fenham Flats and Millport sites ($t = 1{\cdot}25$, 27 D.F., $0{\cdot}2 < P < 0{\cdot}25$, count data transformed to logarithms).

Parasite prevalence (proportion of females infected) differed among field sites. At Budle Bay 30 % of females ($n = 70$) were infected with *O. effeminans* and transmitted the parasite to offspring; 53 % ($n = 88$) of females from Fenham Flats and 46 % ($n = 173$) from Millport were infected with *Nosema*. These proportions were significantly heterogeneous (log-likelihood statistic $G = 9{\cdot}2$, 2 D.F., $P < 0{\cdot}01$).

Comparison of models to data

Budle Bay. The *O. effeminans*-infected embryos were grouped into 10 categories according to estimated initial parasite load, ranging from 3 to 14 estimated parasites in the zygote. Model 1 (unrestricted movement of parasites within the embryo) was strongly rejected for all 10 groups (Table 1). Model 2 (confinement of parasites to cell lineages, unbiased sorting to daughter cells) was rejected for 8 of 10 groupings (all groups with $Z_0 > 4$). Distributions were not significantly different from those expected under Model 3 (biased sorting for 1 cell lineage) for 5 of 10 groupings; Model 3 also provided the best overall fit to the data for 9 of 10 groups.

The test of mean parasite load per infected cell helped resolve the overall match between best-fit models; Model 3 invariably produced the best estimate of the mean (Table 1), and was not significantly different from the observed mean for 8 of 10 groups.

The global estimate of p was 0·5. For the individual groups based on initial parasite load, the estimates of q were very homogeneous, ranging only from 0·71 to 0·75 for the 10 groups. Combined with the results from the fits to the expected frequency distributions and comparisons with expected mean parasite load per infected cell, the results suggest that the probability of parasite division is relatively constant, and that there is strong evidence for at least 1 cell lineage for which there is biased sorting of parasites into daughter cells.

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Table 1. Analysis of data from *Gammarus duebeni* 64-cell embryos from females collected at Budle Bay, Northumberland

(Comparisons of the frequencies of embryonic cells containing various numbers of parasites with the expected frequencies under 4 models of parasite transmission, as detailed in the text. Analyses are grouped according to Z_0, the estimated initial parasite load. The observed mean numbers of parasites per infected cell (Obs.) are shown below each group; each observed mean is compared to those expected under the 4 models (μ). Significance levels for log-likelihood tests for goodness of fit (G) and t-tests for the fit to the expected mean (t): * = $P < 0{\cdot}05$; ** = $P < 0{\cdot}01$; *** = $P < 0{\cdot}001$.)

Z_0	N embryos	N cells	Model	G	μ	t
3	12	123	1	85·5***	1·28	4·38**
			2	4·2 N.S.	1·80	1·32 N.S.
			3	4·72 N.S.	2·06	0·212 N.S.
			4	2·4 N.S.	2·06	0·212 N.S.
			(Obs.)		(2·02)	
4	10	115	1	96·7***	1·56	4·52**
			2	5·31 N.S.	1·90	2·38*
			3	3·8 N.S.	2·17	0·54 N.S.
			4	4·55 N.S.	2·15	0·68 N.S.
			(Obs.)		(2·25)	
5	9	119	1	104·3***	1·50	6·68***
			2	24·2**	2·01	4·19**
			3	15·7**	2·39	2·34*
			4	15·8**	2·35	2·56*
			(Obs.)		(2·72)	
6	8	125	1	80·2***	1·62	5·18**
			2	29·8***	2·01	3·28*
			3	12·3*	2·45	1·25 N.S.
			4	18·7***	2·40	1·48 N.S.
			(Obs.)		(2·72)	
7	7	126	1	66·6***	1·75	5·22**
			2	20·9***	2·23	2·90*
			3	12·5*	2·52	1·49 N.S.
			4	16·7***	2·46	1·79*
			(Obs.)		(2·83)	
8	5	106	1	19·3***	1·87	3·87**
			2	7·96*	2·34	1·89*
			3	2·36 N.S.	2·69	0·42 N.S.
			4	4·85 N.S.	2·60	0·80 N.S.
			(Obs.)		(2·79)	
9	5	116	1	39·5***	2·01	4·34**
			2	11·97**	2·46	2·15*
			3	3·25 N.S.	2·78	0·59 N.S.
			4	8·37*	2·68	1·07 N.S.
			(Obs.)		(2·90)	
10	9	177	1	94·5***	2·14	5·2**
			2	32·7***	2·58	3·73*
			3	12·0*	3·11	1·96*
			4	17·8**	2·96	2·46*
			(Obs.)		(3·70)	
12	6	139	1	98·8***	2·48	4·86**
			2	29·6***	2·96	3·27**
			3	13·5*	3·33	2·06*
			4	18·8**	3·15	2·66*
			(Obs.)		(3·96)	
14	5	127	1	56·6***	2·85	3·81**
			2	18·4**	3·23	2·49*
			3	7·4 N.S.	3·79	0·55 N.S.
			4	11·1*	3·58	1·28 N.S.
			(Obs.)		(3·95)	

N.S., Not significant.

Fenham Flats. We obtained enough data from 64-cell stage embryos to analyse 3 classes of initial parasite load (110, 142 and 225 estimated initial parasites in the zygote). As for Budle Bay, Model 1 was very strongly rejected for each group (Table 2); Model 2 was also rejected for each group. Model 3 again gave the best fit to data for each group. The parasite division rate was slower than that estimated

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Table 2. Analysis of data from *Gammarus duebeni* 64-cell embryos, from females collected at Fenham Flats, Northumberland

(Comparisons of the frequencies of embryonic cells containing various numbers of parasites with the expected frequencies predicted by 4 models of parasite transmission, as detailed in the text. Analyses are grouped according to Z_0, the estimated initial parasite load in the zygote. Significance levels for log-likelihood tests for goodness of fit (G): * = $P < 0{\cdot}05$; ** = $P < 0{\cdot}01$; *** = $P < 0{\cdot}001$.)

Z_0	N embryos	N cells	Model	G
110	5	221	1	17·0***
			2	98·5***
			3	5·5 N.S.
			4	28·8***
150	7	284	1	102·9***
			2	128·4***
			3	8·9 N.S.
			4	41·75***
225	4	176	1	131·5***
			2	104·76***
			3	24·7**
			4	45·34***

N.S., Not significant.

from Budle Bay, with $\hat{p} = 0{\cdot}246$, meaning that the parasite numbers approximately tripled in 6 host cell generations. The estimates of q were uniformly lower than those from Budle Bay, ranging from 0·6 to 0·65.

Analysis of 2-cell embryos showed that sorting of parasites from the zygote stage to the 2-cell stage was generally non-random, with the degree of bias averaging at 0·61, in agreement from the estimates of q described above. Of 17 two-cell embryos, 13 were significantly different from a 1:1 ratio of parasites (total log likelihood $G_T = 224{\cdot}9$, 17 D.F., $P < 0{\cdot}001$), and the estimates were significantly heterogeneous (log-likelihood statistic for heterogeneity $G_H = 47{\cdot}9$, 16 D.F., $P < 0{\cdot}001$).

Millport. Embryos at the 64-cell stage were placed into 7 classes according to initial parasite load, ranging from 112 to 285 estimated initial parasites in the zygotes. Models 1, 2 and 4 were rejected for all 7 groups (Table 3). The observed frequencies were not significantly different from those predicted by Model 3 in 4 of 7 groups. The parasite growth rate was similar to that from Fenham Flats ($\hat{p} = 0{\cdot}26$); estimates of q ranged from 0·6 to 0·7.

For the analysis of 2-cell embryos, the overall occurrence of significant bias from a 1:1 parasite ratio was weaker than in the data from Fenham Flats; less than half of the sampled embryos showed significant bias (23 of 48 embryos; $G_T = 664{\cdot}7$, 48 D.F., $P < 0{\cdot}001$), and the proportions were significantly heterogeneous ($G_H = 395{\cdot}2$, 47 D.F., $P < 0{\cdot}001$).

Table 3. Analysis of data from *Gammarus duebeni* 64-cell embryos, from females collected at Millport, Isle of Cumbrae

(Comparisons of the frequencies of embryonic cells containing various numbers of parasites with expected frequencies predicted by 4 models of parasite transmission as detailed in the text. Analyses are grouped according to Z_0, the estimated initial parasite load. Significance levels for log-likelihood tests for goodness of fit (G): * = $P < 0{\cdot}05$; ** = $P < 0{\cdot}01$; *** = $P < 0{\cdot}001$.)

Z_0	N embryos	N cells	Model	G
112	4	156	1	50·31***
			2	44·2***
			3	7·4 N.S.
			4	41·5***
142	9	357	1	187·0***
			2	41·5**
			3	1·82 N.S.
			4	130·8***
155	8	344	1	271·1***
			2	166·3***
			3	4·88 N.S.
			4	160·4***
180	13	609	1	298·2***
			2	209·0***
			3	4·38 N.S.
			4	94·1***
200	5	238	1	140·2***
			2	23·4**
			3	4·88 N.S.
			4	28·8**
240	5	203	1	149·8***
			2	43·3**
			3	31·6**
			4	36·15***
285	5	157	1	141·45***
			2	37·74***
			3	30·11***
			4	26·03***

DISCUSSION

The comparisons of intracellular parasite distributions with those generated by theoretical models provide strong support for the hypothesis of non-random sorting of parasites in at least 1 cell lineage, possibly beginning as early as the zygote stage, for both species of parasite (Fig 3). The reason for the better fit by Model 3 in each case may be seen by examining the tails of the distributions; the unbiased sorting assumed under Model 2 underestimates the upper tail and either underestimates or overestimates frequencies of the lower tail, depending on the initial parasite load. As noted in the description of Model 3, the observed pattern could alternatively have been produced by non-biased sorting into daughter cells and a biased fission rate for the parasite in 1 cell lineage. The groups for which none of the models fit well tended to be those with the highest numbers of estimated initial parasites in the Fenham Flats and Millport data sets; this might be expected to be due

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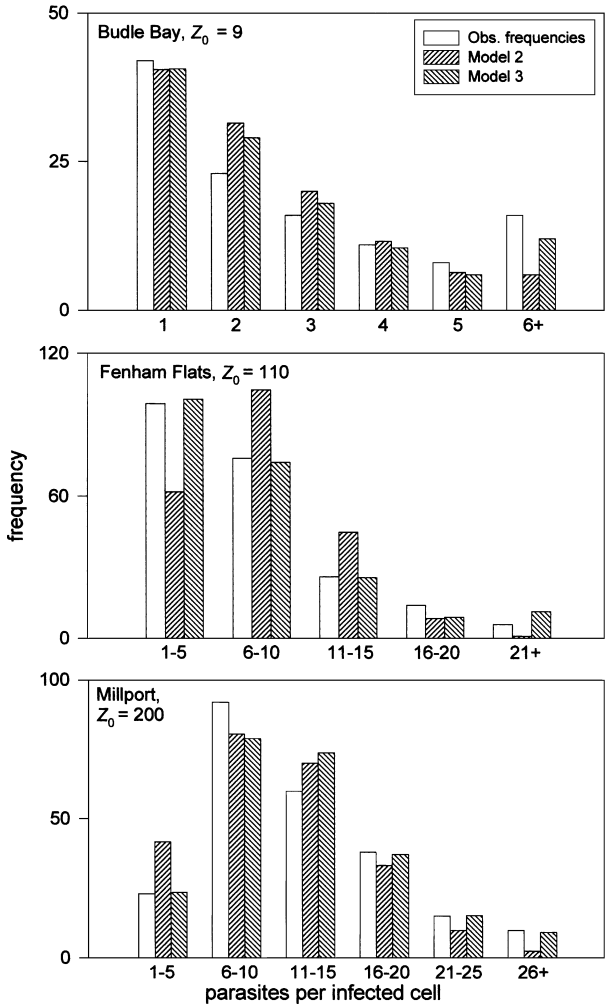

Fig. 3. Representative frequency distributions of the numbers of parasites within cells of 64-cell host embryos. The observed distributions are compared to expected distributions under Model 2 (confinement of parasites to cell lineages, unbiased sorting of parasites into daughter cells) and Model 3 (confinement of parasites to cell lineages, biased sorting into daughter cells of 1 host cell lineage). Observed distributions were not significantly different from those predicted under Model 3 (log-likelihood tests for goodness of fit; see Tables 1–3 for P values). Model 3 provides better fit to the data compared to Model 2 due to its predictions of frequencies in the lowest and highest categories of parasite number.

to larger expected bias in the estimates of Z_0 (embryos with higher parasite counts will have a larger variance in the number of parasites in the unobserved cells).

There are 2 classes of explanations for the observed pattern. The cause may be *passive*, in that the parasite itself does nothing to produce the non-random distribution. For example, the spatial distribution of parasites within the cells of the host embryo might generally be asymmetric with respect to the axis of cell division, so that when host embryo cells undergo cleavage, the majority of parasites are sorted into 1 daughter cell (Dunn *et al.* 1995). Alternatively, the pattern may be *active*, in that the parasite influences its chance of being transmitted to a particular daughter cell, or is able to regulate its rate of fission in the proper cell lineage. Recent studies of *Nosema* in *G. duebeni* have demonstrated an association between parasites and host microtubules during host mitosis, which may provide a mechanism for differential segregation (R. Terry, unpublished observations). A similar association with host microtubules has been reported for vertically transmitted bacteria infecting *Drosophila* (Callaini, Riparbelli & Dallai, 1994).

The most interesting hypothesis is that the parasites might be able to recognize and 'track' the cell line destined to become the proper host target tissue. The strong rejection of Model 1 for all data sets suggests that the parasites do not cross cell boundaries during early embryogenesis. Thus some parasites must be present in the appropriate cell lineage if the parasite is to find the appropriate host tissue, whether that is the germ line or another tissue from which the germ line is infected prior to host reproduction. It is not known precisely when in the development of *Gammarus* the germ line is determined, although several studies have suggested that the germ cells may arise during gastrulation (Weygoldt, 1958; Fioroni, 1981; Dixon, 1994). In well-studied model systems such as *Drosophila* and *Caenorhabditis elegans* (e.g. Strome *et al.* 1994), areas of cytoplasm within the zygote that are destined to be present in germ line cells have been identified; evidence from several systems suggests that bacterial parasites may be able to identify and segregate to these regions of the zygote (Ehrman & Daniels, 1975; Breeuwer & Werren, 1990; Stouthamer & Werren, 1993). For *Nosema* infecting *G. duebeni*, ultrastructural examination of juveniles (Terry *et al.* 1997) suggests that subcuticular cells may be the target tissue from which invasion of the gonad proceeds.

Our results suggest the possibility that the 2 species of parasite employ different strategies for ensuring transmission to the next host generation. The Fenham Flats and Millport data show that the mean number of *Nosema* per cell declines sharply during embryonic cleavage, even considering just the cells containing the maximum number of parasites per embryo. If there is biased sorting of parasites into 1 cell lineage, then from Model 3 we know that the expected number of parasites in that cell after n cell divisions is

$$Z_0[(1+p)\,q]^n$$

and so if the product $[(1+p)\,q] < 1$, the number of parasites in the cell line will decline geometrically. Estimates of this number from both the Millport and Fenham Flats data were below 1, thus even the embryonic cell with the maximum number of parasites contained only $\frac{1}{5}$ to $\frac{1}{10}$ the estimated initial

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number in the zygote. In contrast, the Budle Bay embryos infected with *O. effeminans* usually contained at least 1 cell with more parasites than were estimated to have been in the zygote. Given the observed average numbers of parasites in the zygotes from Budle Bay and Millport/Fenham Flats, and the respective estimates of p and q, it may be shown that the mean parasite loads in the cells containing the maximum number should converge at about the 512-cell stage, after which the number of parasites in the cell containing the maximum would be higher for animals infected with *O. effeminans*. However, embryos from females infected with *Nosema* actually contain many more parasites than do those from females infected with *O. effeminans*. *Nosema* might thus be employing more of a simple 'brute force' strategy, where a high initial parasite load ensures a good chance of getting enough parasites to the target tissue; once there, the parasite may then have enough time to rebuild its numbers during maturation of the female host up to the time of host reproduction. In contrast, the low initial density of *O. effeminans* in the zygotes may make it imperative for that species to be able to efficiently track the proper cell lineage.

The possibility that separate transmission strategies are being used by the parasites has some interesting implications. The observed lower division rate of *Nosema* may reflect a biological constraint, or it may reflect selection for reduced virulence. Vertically transmitted parasites rely on host reproduction for their own transmission, and it has been suggested that selection should favour reduced virulence (Ewald, 1987; Smith & Dunn, 1991; see Bull, Molineux & Rice, 1991 for empirical evidence). Nonetheless, parasite transmission within and between hosts is dependent on parasite burden (Dunn & Hatcher, 1997), so the observed pattern of parasite growth and transmission may reflect a trade-off between conflicting selective pressures. For *Nosema*, with its high density in the zygote, it might be best to employ a 'sit-and-wait' strategy, reproducing slowly enough in the early developmental stages to avoid harming the host, but rapidly enough to ensure that a sufficient number of parasites find the host target tissue.

The most important next question for this system is to determine the fate of the apparent biased cell lineages that we observed. Our overall goal will be to eventually connect the mechanism of within-host transmission to the patterns of parasitism observed in host populations.

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