

Virulence, parasite mode of transmission, and host fluctuating asymmetry

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SUMMARY

Horizontally transmitted parasites are broadly predicted to be more virulent, or costly to host fitness, than those with vertical transmission. This is mainly because vertical transmission, from host parent to offspring, explicitly links the reproductive interests of both parties. Underlying this prediction is a general assumption that parasite transmission success is positively correlated with its virulence. We report results where infection of larval yellow fever mosquitoes *Aedes aegypti* with the microsporidian *Edhazardia aedis* was experimentally manipulated. The parasite's complex life cycle allowed comparisons between estimates of horizontal and vertical transmission on host fitness. Our measure of virulence was the fluctuating asymmetry (FA) of adult female wings. Hosts harbouring spores showed higher FAs than controls. Horizontally transmitting spores were associated with higher FAs than vertically transmitting spores. Furthermore, within hosts FA correlated positively with the number of horizontally transmitting spores, while no relation was seen with the number of vertically transmitting spores. A developmental mechanism uncoupling the relationship between vertical transmission and virulence is proposed.

1. INTRODUCTION

Virulence, or cost to host fitness, can be viewed as a composite of traits contributing towards a parasite's overall fitness. The individual traits may have positive, neutral, or negative fitness effects on the parasite (reviewed in Bull 1994). The direction and strength of selection acting on individual traits is predicted to depend on their contribution towards overall fitness and on their correlation with other traits (Ebert & Herre 1996). Of particular interest are correlated traits which act in an antagonistic manner, i.e. where a response to selection in one trait reduces a correlated trait's contribution towards fitness. A widely used assumption is the balance required between a parasite's increased transmission success and additional costs to its host (reviewed by Anderson & May 1991). Consequently, selection on the parasite is often predicted to produce intermediate virulence, which has support from medical, field, and laboratory studies (reviewed by Anderson & May 1991; Bull 1994; Ewald 1994).

Comparative studies of virulence and parasite mode of transmission have been particularly illuminating in this evolutionary scenario. Various aspects of host and environmental ecology act to determine upper limits that virulence can be maintained at. Parasites transmitting purely vertically from parent to offspring have their reproductive success explicitly linked to that of their hosts. Any cost to host reproductive success affects

the parasite's own success and theoretically will drive the parasite to extinction. Therefore, such parasites are often benign (see Ewald 1994). Further support for this notion comes from parasites that are uniparentally inherited. These parasites show adaptations to directly enhance their own fitness, or that of the transmitting sex, by exploiting the non-transmitting sex (see Hurst 1993). Horizontal transmission among hosts is less constrained by host condition: increased parasite fitness can evolve at additional costs to the host. Comparative studies of horizontal transmission, e.g. vector-borne and direct contact, follow these predictions (Ewald 1994). Additionally, horizontally transmitting ectoparasites of rock doves are more virulent than vertically transmitting counterparts (Clayton & Tompkins 1994). An alternative approach shows that avian host's immunocompetence increases with nesting ecologies more exposed to horizontally transmitting parasites (Møller & Erritzøe 1996).

Direct testing of the transmission mode–virulence hypothesis is difficult if parasites have only one mode of transmission or different modes of transmission in different host species. A few studies have avoided this problem by studying parasites that use both vertical and horizontal transmission in the same host species. Phages selected for vertical transmission in their bacterial hosts developed a more benign relationship with their hosts than those from the original population selected for horizontal transmission (Bull *et al.* 1991). A comparative study of nematodes parasitizing fig wasps showed that as vertical transmission becomes more

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important in a particular host–parasite relationship the less virulent the nematodes are (Herre 1993). However, most comparative studies of transmission and virulence are not phylogenetically controlled (Harvey & Pagel 1991). This leads to the possibility that differences in virulence arose from different evolutionary histories rather than due to observed patterns of transmission. Herre's (1993) work suffers least from this potential problem by only considering closely related species. A further problem is that selection for vertical transmission necessarily acts on both host and parasite. Any response to selection could be due to the parasite, the host, or both. For example, Sweeney *et al.* (1989) selected for increased vertical transmission success of the microsporidian *Amblyospora dyxenooides* in its mosquito host *Culex annulirostris*, but were unable to attribute this to either host or parasite. Bull *et al.* (1991) largely controlled for this possibility and demonstrated that the response to selection was predominantly due to the parasite.

The host–parasite relationship between the yellow fever mosquito *Aedes aegypti* and the microsporidian *Edhazardia aedis* (Becnel *et al.* 1989) is suited for testing the mode of transmission–virulence hypothesis. The parasite uses both vertical and horizontal transmission at different stages in its complex life cycle (see Materials and Methods) and is host-species specific (Andreadis 1994). This avoids the problems stated above. Following horizontal infection of host larvae, the parasite's subsequent mode of transmission may be either vertical or horizontal. Each mode of transmission has its own distinct type of spore. Vertical transmission of the parasite requires the reproductive success as adults of females infected as larvae. In contrast, horizontally infected larvae can only transmit the parasite horizontally to other larvae if they themselves die as larvae or pupae. Death in this manner is induced by spore production of the parasite. This alone supports predictions of the transmission–virulence hypothesis. However, this binary measure of virulence (hosts alive vs dead hosts) related to transmission (vertical vs horizontal) is a coarse description of the correlations involved in this host–parasite relationship. Greater insight can be taken from comparison between adult females. Many of these females harbour spores capable of vertical transmission. Also, some females will have survived an attempt at horizontal transmission by the parasite, and harbour spores specific for that purpose. Theories on the evolution of virulence predict that the latter females should have experienced greater costs during their development than the former females. As these costs are experienced by hosts during development, we predict that the fluctuating asymmetry (FA) of adult characteristics should differ between females based on the mode of transmission pursued by the parasite. Some individuals harbour both spore types, thus permitting within-host analysis of their effects on FA. Only females were considered as only they provide a potential route for vertical transmission (Hembree & Ryan 1982), and the sexes may provide different developmental cues for the parasite.

Fluctuating asymmetries are small random deviations from an otherwise bilaterally symmetrical

trait (Van Valen 1962). They are believed to arise from some form of stress the individual is unable to buffer during development of the trait. These stresses may arise from a variety of sources (Møller & Swaddle 1996), including parasites (Møller 1996). In some cases, FAs themselves may have direct negative effects on components of fitness, e.g. avian flight performance (Balmford *et al.* 1993). Stabilizing selection on such traits is predicted to reduce developmental flux by canalization (Møller & Pomiankowski 1993). More often FA is cited as an indirect measure of fitness; numerous studies report increased FAs to correlate negatively with other fitness traits (reviewed Møller & Swaddle 1996).

We experimentally tested the mode of transmission–virulence hypothesis with *Ae. aegypti* and *E. aedis*. Our predictions were that the FA of adult female mosquito wings would correlate positively with the degree of stress hosts experienced during their development. The degree of stress was predicted to depend on whether the parasite pursued either vertical or horizontal transmission following larval infection.

2. MATERIALS AND METHODS

(a) *The host and its parasite*

Aedes aegypti, the yellow fever mosquito, has been extensively studied due to its role as a disease vector and its suitability for laboratory maintenance (Christophers 1960). The Rockefeller strain obtained from the Swiss Tropical Institute (Basel) was used. Mosquitoes were maintained at $28(\pm 0.5)$ °C and $85(\pm 5)$ % humidity with a 12 h–12 h light–dark cycle.

Edhazardia aedis is an obligate intracellular microsporidian parasite with a complex life cycle (Becnel *et al.* 1989). Host larvae ingest uninucleate spores from the environment (figure 1). In the mid-gut, polar tubes extend from the spores to

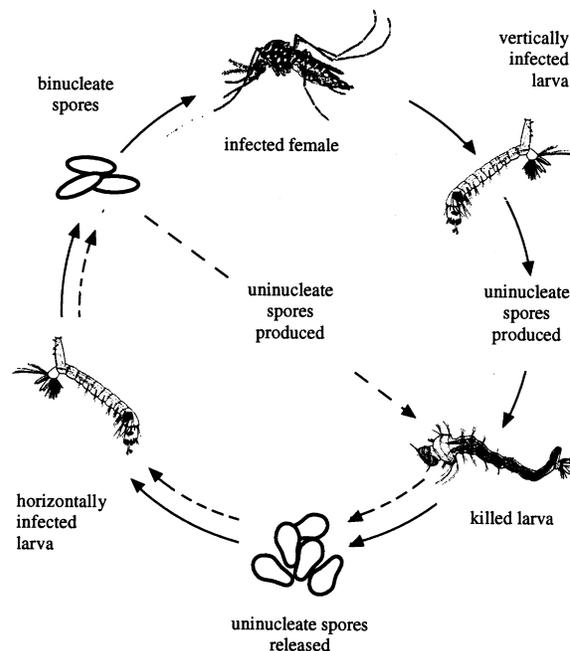


Figure 1. Alternative life cycles of *Edhazardia aedis* infecting *Aedes aegypti*. Solid lines indicate the horizontal–vertical transmission sequence. Dashed lines indicate the horizontal–horizontal transmission sequence (see text for details).

puncture epithelial cells and initiate the infection. Further tissues become infected by some unknown mechanism as the parasite progresses through several developmental stages. Binucleate spores are specifically produced within host oenocyte cells. These spores, carried in the haemocoel, are solely responsible for vertical transmission by infection of a female's developing eggs. Further development by the parasite is prevented until the eggs hatch and larvae develop. Infection is predominantly seen in fat body tissue where uninucleate spores are produced. Vertically infected larvae rarely survive beyond pupation. The proliferation of uninucleate spores results in their death. These spores are not shed during the host's life, only being released into the environment following the rupture of the host cuticle.

Alternatively, binucleate spores produced in horizontally infected larvae may germinate and infect tissues other than developing eggs. In this case, the parasite continues with development that otherwise would be seen in vertically infected larvae. This culminates in the production of uninucleate spores in the fat body. If enough spores are produced to induce the host's death before its emergence from the larval site, then uninucleate spores will be released and available for horizontal transmission: vertical transmission will have been bypassed. However, if insufficient numbers of uninucleate spores are produced, then hosts may emerge harbouring uninucleate spores. The two spore types are specific to their mode of transmission: uninucleate spores are incapable of vertically transmitting the parasite via a female's eggs.

Edhazardia aedis were obtained from stock maintained by Dr J. J. Becnel at the United States Department of Agriculture, Gainesville.

(b) Experimental protocol and data collection

Two experiments were designed to produce emerging adult female *Ae. aegypti* harbouring infections of *E. aedis* pursuing vertical transmission or having failed to achieve horizontal transmission from infected larvae or pupae. We manipulated dose \times food conditions in one experiment and dose \times age at infection in the other experiment to expose the parasite's potential patterns of transmission.

In both experiments uninfected mosquito eggs were synchronously hatched. Within 6 h of hatching larvae were separated into rearing pans measuring 10 cm \times 10 cm \times 10 cm and containing 300 ml of tap water. Dead larvae and pupae were removed daily. Live pupae from within treatments were transferred on a daily basis to small beakers of water kept under inverted mesh-bottomed beakers. Two days after emergence, adults were killed by freezing, and stored individually in 1.5 ml vials at -20°C until further investigation.

Experiment I consisted of 20 treatments that initially contained 200 larvae each. The treatments consisted of four larval food availabilities combined with exposure to five concentrations of uninucleate spores. Larval diet was manipulated as 25%, 50%, 75% and 100% of a standardized ration of TetraminTM fish food. Rations per treatment were adjusted daily allowing for mortality and pupation. Rations per larva on a 100% diet were: day 1, 0.06 mg; day 2, 0.08 mg; day 3, 0.16 mg; day 4, 0.32 mg; even-numbered days thereafter, 0.64 mg. Seventy-two hours after hatching, larvae were transferred to 100 ml beakers of water and exposed to either 0, 10, 100, 1000 or 10000 uninucleate spores ml⁻¹ for 24 h. The spores had been harvested from vertically infected larvae. The day's food ration was also added to the beakers. After exposure, larvae were rinsed in tap water and returned to their original rearing trays.

Experiment II consisted of nine treatments initially containing 100 larvae each. Treatments involved three larval ages of infection (24, 72 and 120 h post-hatch) and three concentrations of uninucleate spores (10, 100 and 1000 uninucleate spores ml⁻¹). Daily maintenance and infection were otherwise as in Experiment I.

Adult female wings were later removed and stuck to glass microscope slides. Length, from the distal end of the alula to the peripheral tip of vein R3, was measured with image analysis software (NIH Image 1995) to an accuracy of 0.01 mm via a video camera linked to a dissecting microscope. Signed wing asymmetries in each experiment were normally distributed (Shapiro-Wilk W test: Experiment I, $W = 0.946$, $p > 0.05$; Experiment II, $W = 0.986$, $p > 0.05$) with means not significantly different from zero (Experiment I, $t_{1098} = -0.073$, $p > 0.9$; Experiment II, $t_{129} = 1.235$, $p > 0.2$). Some mosquitoes were measured twice, from different video images. Their asymmetries were replicable (intra-class correlation coefficients, left $r = 0.99$, right $r = 0.98$, $F_{79,160} = 5.274$, $p < 0.0001$).

(c) Statistical analyses

The parasite's life cycle gives temporal correlations between the two spore types. This correlation cannot be deduced from spores found in individuals collected as adults. In neither experiment did the numbers of the two spore types significantly correlate (two-tailed linear regressions of log₁₀ (uninucleate spores) on log₁₀ (binucleate spores), Experiment I $F_{1,72} = 0.009$, $p > 0.9$, Experiment II $F_{1,23} = 0.659$, $p > 0.4$). Therefore, we treated them as independent variables in subsequent analyses.

Our *a priori* interest was in the relationship between the mode of transmission pursued by the parasite and virulence. The parameters chosen to assay this were the number and type of spores on host FA. Different experimental treatments were used to generate the ranges of spore production observed. However, there may have been a consistent relationship between treatment, production of spores, and the resulting asymmetry, as well as direct effects on asymmetry. Therefore, multiple linear regressions removed treatment effects from the log₁₀-transformed (number of spores) and square root-transformed (absolute asymmetry) data. In no case did the treatments or their interactions have significant effects ($p < 0.1$). The effects of residual spore numbers on the residual asymmetries were tested by multiple linear regression; these analyses were not sensitive to the ordering of terms entered into the models.

It was not possible to subtract treatment influence from the presence of spore. Instead, we show the effect of the treatments on binucleate and uninucleate spore presence and on asymmetry itself. Nominal logistic regressions were used for treatment effects on spore presence. Dosages were log₁₀-transformed with no exposure to uninucleate spores coded as zero. A non-parametric two-way ANOVA tested for absolute asymmetry's response to treatment conditions (Sokal & Rohlf 1995).

A one-tailed Wilcoxon ranked sums test on the data from Experiment I compared absolute asymmetries of controls vs individuals harbouring either type of spore. The same analysis, in each experiment, compared absolute asymmetries of individuals harbouring binucleate or uninucleate spores. Each experiment's result was combined for a probability of significance test. Our predictions were that FA would be higher in the presence of spores and with uninucleate spores. Non-parametric analyses were used when residuals of parametric analyses failed to give normal distributions, even after data transformations. The statistical package JMP (SAS Institute Inc. 1994) performed all the analyses.

3. RESULTS

Increased dosages of uninucleate spore concentration significantly increased the presence of both binucleate and uninucleate spores in adult females of Experiment I (table 1*a, b*). Higher food availabilities

Table 1. *Experiment I. Analyses of treatments on the presence of both spore types and on absolute asymmetry*

(a) Nominal logistic regression of treatments on binucleate spore presence

source	d.f.	χ^2	p
food	1	2.019	0.155
dose	1	8.957	0.003
interaction	1	5.927	0.015
$r^2 = 0.31, n = 1204$			

(b) Nominal logistic regression of treatments on uninucleate spore presence

source	d.f.	χ^2	p
food	1	2.997	0.0830
dose	1	13.792	0.0002
interaction	1	0.650	0.4201
$r^2 = 0.31, n = 1204$			

(c) Non-parametric two-way ANOVA of treatments on absolute asymmetry

source	d.f.	m.s.	H	p
food	3	65263	6.415	n.s.
dose	2	32766	3.221	n.s.
interaction	10	55909	5.495	n.s.
error	1080	95748		

Table 2. *Experiment II. Analyses of treatments on the presence of both spore types and on absolute asymmetry*

(a) Nominal logistic regression of treatments on binucleate spore presence

source	d.f.	χ^2	p
age	1	10.787	0.001
dose	1	0.171	0.679
interaction	1	1.633	0.201
$r^2 = 0.26, n = 161$			

(b) Nominal logistic regression of treatments on uninucleate spore presence

source	d.f.	χ^2	p
age	1	0.031	0.861
dose	1	0.658	0.417
interaction	1	0.856	0.347
$r^2 = 0.16, n = 161$			

(c) Non-parametric two-way ANOVA of treatments on absolute asymmetry

source	d.f.	m.s.	H	p
age	2	414	0.306	n.s.
dose	2	416	0.307	n.s.
interaction	4	1477	1.090	n.s.
error	120	1382		

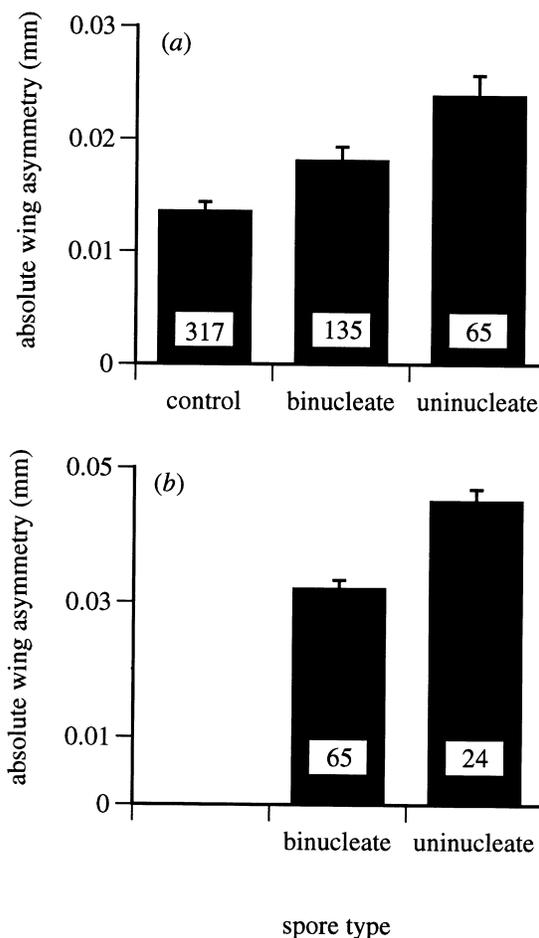


Figure 2. Mean (+s.e.) absolute asymmetries of female mosquito wings grouped by the type of spores that individuals harboured. (a) Experiment I. Control *vs* spore presence on absolute asymmetry; Wilcoxon ranked sums: $\chi^2_{[1]} = 9.741$, $p = 0.002$. Binucleate *vs* uninucleate presence on absolute asymmetry; Wilcoxon ranked sums: $\chi^2_{[1]} = 3.272$, $p = 0.071$. (b) Experiment II. Binucleate *vs* uninucleate presence on absolute asymmetry. Wilcoxon ranked sums: $\chi^2_{[1]} = 3.238$, $p = 0.072$. Combined probability test of binucleate and uninucleate presence on absolute asymmetry from Experiments I and II: $\chi^2_{[4]} = 10.569$, $p < 0.05$.

interacted with higher dosages to increase binucleate spore presence. Lower food availability contributed to a lower presence of uninucleate spores. In Experiment II, where larvae experienced high food conditions, increasing age at infection significantly increased the presence of binucleate spores (table 2*a*). The lower range of dosages had no significant effect on either spore type's presence (table 2*a, b*). In neither experiment did treatment conditions affect asymmetry (table 1*c, 2c*).

Within both experiments, individuals harbouring uninucleate spores tended to show more asymmetry than those with binucleate spores (figure 2*a, b*), and over both experiments this difference was significant.

Once treatment effects were removed from the number of each type of spore and on asymmetry there were significant and positive correlations between uninucleate spore number and asymmetry in both experiments (figure 3*a*, table 3*a, b*). In contrast, neither experiment showed a relationship between

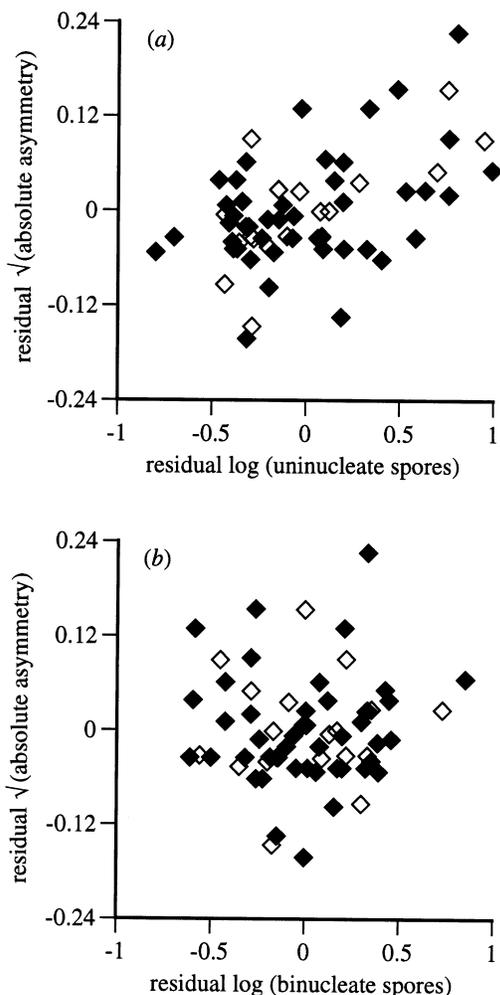


Figure 3. Correlations between residual \log_{10} (spore number) and residual $\sqrt{(\text{absolute asymmetry})}$ once the effects of the other spore type are removed. (a) Binucleate spores. (b) Uninucleate spores. Experiment I filled diamonds, Experiment II open diamonds.

Table 3. Multiple regressions of residual spore numbers on residual $\sqrt{(\text{absolute asymmetry})}$

(a) Experiment I

source	d.f.	s.s.	<i>F</i>	<i>p</i>
binucleate	1	0.0003	0.058	0.811
uninucleate	1	0.0328	7.457	0.009
interaction	1	0.0030	0.672	0.417
error	41	0.1806		

$r^2 = 0.20, n = 45. W = 0.973, p > 0.5$

(b) Experiment II

source	d.f.	s.s.	<i>F</i>	<i>p</i>
binucleate	1	0.0006	0.290	0.598
uninucleate	1	0.0377	17.757	0.001
interaction	1	0.0002	0.099	0.757
error	16	0.0340		

$r^2 = 0.60, n = 20. W = 0.926, p > 0.1$

binucleate spore number and asymmetry, nor any interaction between the spore types (figure 3*b*, table 3*a*, *b*).

4. DISCUSSION

Adult female mosquitoes from Experiment I harbouring either type of spore showed higher wing FA than control mosquitoes. Treatment conditions had effects on the presence of both spore types but not directly on FAs, but could still have had some effect via correlations with spore production. However, these relationships are likely to be context-dependent as the correlation of dosage with spore presence in the two experiments showed. The presence of uninucleate spores in both experiments was associated with higher FAs than that of binucleate spores. Thus, FA increased in the presence of either spore type, but was consistently higher in association with horizontally transmitting uninucleate spores than vertically transmitting binucleate spores.

All developmental stages of the infection should impose some metabolic cost to their host as microsporidia have no mitochondria (Sprague *et al.* 1992). Spore production in particular should be costly to hosts. A classic symptom of microsporidiosis is white patches of hypertrophied cells following their rupture after intracellular spore production. Other costs may involve direct competition between host and parasite for protein. This is often a limiting resource in aquatic environments, especially for non-carnivorous species, and is strongly implied in the evolution of adult female mosquito feeding ecology. Protein is a key constituent in the multi-layered walls of both spore types (Vávra & Maddox 1976). Beyond their structural role, proteins are vital to almost all metabolic processes, especially during the digestion and formation of tissues at metamorphosis (Chapman 1969).

Interpretation of these results relies on our assumption that FA is a measure of cost to the host. Aside from its wide use as a measure correlating negatively with fitness traits, we have no direct evidence that wing FA is costly to the host. Wing symmetry, however, is a trait likely to have experienced stabilizing selection for a long period of time leading to canalization and reduction of developmental flux (Møller & Pomiankowski 1993). The flat relationship between wing size and FA in both experiments supports this notion (linear regressions: Experiment I, $F_{1,1096} = 0.421, p > 0.5$; Experiment II, $F_{1,129} = 0.325, p > 0.5$). Biomechanical insect models show that the lift generated per wing is proportional to the square of wing length (Brodsky 1994). Therefore, even small asymmetries will require correction to maintain steady flight, with a cost to flight performance or energetic efficiency. Wing and tail asymmetries are reported as detrimental to avian flight performance (Balmford *et al.* 1993).

Further exploration of correlations between virulence and mode of transmission can be taken from within hosts. For parasites using vertical and horizontal transmission the correlations between virulence and associated traits can pose acute problems. Selection acting on traits favouring one mode of transmission may produce a correlated response in virulence that detrimentally affects the parasite's success in the other mode of transmission (Bull 1994). Spore production by

E. aedis is an example of this problem. Horizontal transmission success is known to increase with uninucleate spore densities in the aquatic environment (Hembree 1982; Hembree & Ryan 1982). A direct correlation between binucleate spore density and vertical transmission success has not been demonstrated directly. However, they are not motile and rely on being carried around in the host's haemocoel to the oocytes, suggesting that increased densities will lead to earlier and greater probabilities of transmission success. Therefore, we predict there is a positive selection pressure for increased spore production common to both modes of transmission. Against this, increased spore production should also entail greater costs to the host. Selection in the two modes of transmission for cost to the host are opposite; high virulence is required to kill the host for horizontal transmission, while it should be minimized for vertical transmission.

Where no conflict occurs between selective forces acting on spore production in horizontal transmission, there were positive and significant correlations between the number of uninucleate spores and FA in both experiments. However, conflict occurs between transmission success and virulence of vertical transmission. No significant correlations were found between FA and binucleate spore number in either experiment. These analyses were performed on residuals once treatment effects were removed from spore numbers and asymmetry. Additionally, the results are not explainable by volumetric differences in the two spore types or numerical arguments in total spore number, as interactions between the spore types were not significant.

We propose that the differential spore costs experienced by the host lie within an adaptive developmental mechanism of the parasite. Uninucleate spores are only produced in the fat body cells (Becnel *et al.* 1989). These cells perform numerous functions but are particularly active at the time of pupation and have a major role in protein metabolism (Christophers 1960). Disruption of this cell population probably has several developmental consequences for the fitness of those individuals surviving to adulthood. This suggests a possible causal link between 'stress' and FA, as the cuticle is generally between 50–75% protein (Chapman 1969). In contrast, binucleate spores are only produced in oenocytes (Becnel *et al.* 1989). These secretory cells are predominantly involved in the timing of larval developmental events (Christophers 1960). Disruption of these cells seems most likely to alter larval rates of development, but has little direct effect on adult characteristics.

Previous results have reported reduced fecundity and longevity of *E. aedis*-infected adult *Ae. aegypti* females (Becnel *et al.* 1995; Hembree 1982). Oocyte damage during binucleate spore germination was interpreted as a possible cause (Becnel *et al.* 1995). However, female blood-feeding behaviour declines sharply as uninucleate spore number increases, but is unaffected by binucleate spore number (Koella & Agnew 1997). This can account for both reduced fecundity and longevity. Uninucleate spore presence in adults represents *E. aedis* caught in transition between

its mutually incompatible modes of transmission. We suggest that reductions in a range of adult fitness characteristics arise from a suite of effects resulting in the damage caused to host fat body tissue by uninucleate spore production, and that *E. aedis* shows adaptation to specifically avoid such effects when pursuing vertical transmission.

In summary, we present relationships between a parasite's mode of transmission and its effects on host developmental stability. Horizontal transmission is associated with higher virulence than vertical transmission. Where transmission and virulence are not in conflict there are positive correlations between spore number and cost to the host. When conflict occurs, with vertical transmission, the parasite appears to maintain its fitness by partially uncoupling the correlation between spore number and cost. The mechanism for uncoupling this relationship may lie in specific developmental events of the parasite in host tissues of different functional roles.

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