

## RESEARCH REPORT

***Anopheles coluzzii* infection by the microsporidian, *Vavraia culicis*: the effect of host age****Nayna Vyas-Patel***Imperial College, London, Silwood Park Campus, Buckhurst Road, Berkshire SL5 7PY, United Kingdom**This is an open access article published under the CC BY license**Accepted November 13, 2024***Abstract**

Host age at infection has important implications for disease development. In mosquitoes, infections with microsporidia and later concurrent infections with malaria parasites, leads to a suppression in the development of malaria parasites. Host age at infection with microsporidia could have implications for disease outcomes when infection occurs subsequently with malaria parasites. Mosquito larvae can take between five to seven days or more depending on the temperature to reach the adult stage, giving the microsporidian *Vavraia culicis*, a theoretical head start in establishing and developing within larvae and possibly resulting in different levels of infection in emergent adult mosquitoes. To determine the effects of early or late infection with *V. culicis*, equal numbers of *Anopheles coluzzii* larvae were infected individually with a high or low dose of *V. culicis*, at different ages post hatching. Significantly fewer spores were produced from mosquitoes infected later, than ones infected earlier with microsporidia and there was an initial delay in the production of spores from later infected mosquitoes. In early infected larvae, there was no such initial delay and spore production took off unchecked. The infectious dose of *V. culicis* did not affect the total spore count per mosquito. Male mosquitoes produced fewer spores than females. Daily mosquito longevity and pupation was not affected significantly by infection, the infectious dose of *V. culicis* given or by the sex of the mosquito. Considering hourly deaths, early infected hosts died 17 to 18 hours earlier than later infected larvae. The number of *V. culicis* spores rose with increasing duration of infection. When equal duration of infection was considered, the findings remained the same. Host age at infection influences disease outcomes and virulence.

**Key Words:** *Vavraia culicis*; *Anopheles coluzzii*; *Plasmodium berghei*; host age; infectious dose; double infections**Introduction**

*Vavraia culicis* (Weiser, 1947,1977; Vávra and Becnel, 2007), a microsporidian parasite with a wide host range, naturally parasitizes a number of mosquito genera and species (Vávra and Becnel, 1977). This obligate and horizontally transmitted parasite has been the subject of numerous studies, not merely to explore its potential as a biological control agent (Reynolds, 1970; Kelly *et al.*, 1981; Agnew *et al.*, 1999), but also for its capacity to interfere with the subsequent infection and establishment of *Plasmodium* species (malaria parasites) in the adult mosquito (Bano, 1958; Bargielowski and Koella, 2009; Lorenz and Koella, 2011).

Mosquitoes have life stages in two different mediums - larvae develop to pupation in water and emerge as flying adults into the terrestrial world.

Each stage (larval and adult) has its own developmental age and challenge. Mosquito larvae first encounter the infective spores of the microsporidian *V. culicis* in the aquatic environment, however, nature is not always uniform and at any given time in a tropical environment, a typical mosquito breeding pool generally harbours mosquito larvae of varying ages and species (Vyas-Patel, 1988; Karuitha, 2019), some of which could have been infected with parasites if present and others which had yet to be infected. A mix of larval ages and indeed species could be present in a mosquito breeding pool; seldom will a pool contain larvae of uniform age as it would continuously be used by different adult mosquito species to lay eggs at different times. The distribution of spores in water in the field is not known, it might be patchy (clumped) or evenly (uniformly) distributed in water affecting spore uptake by mosquito larvae. Little is known about the effect of larval host age on the development of the parasite, especially as mosquito larvae are infected horizontally with *V. culicis* in water (spores are ingested) and this can happen at any larval age.

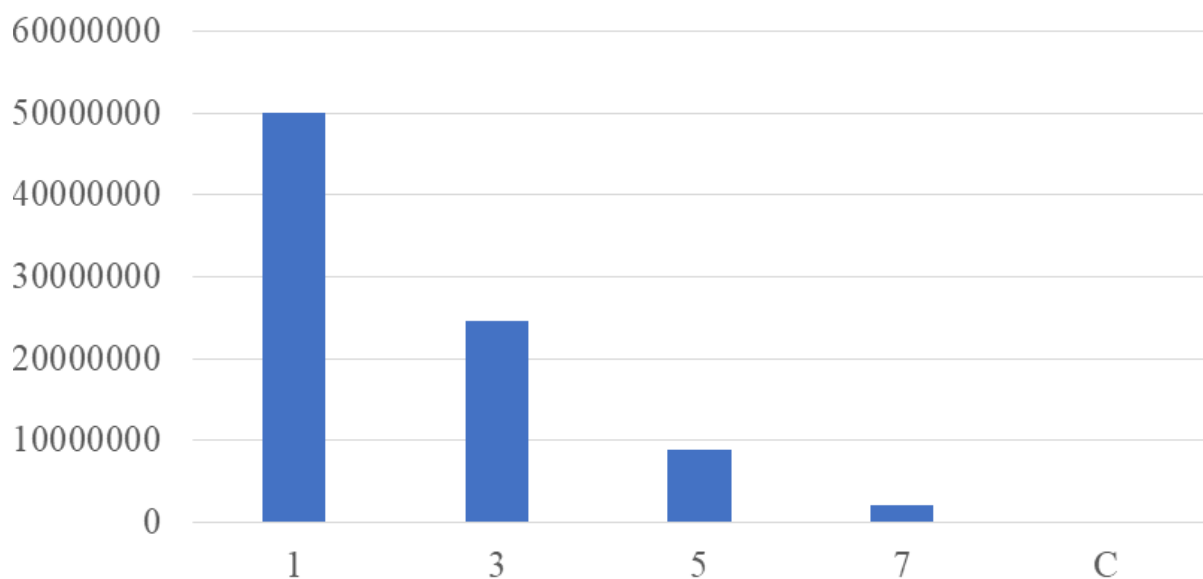
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**Table 1** No significant interactions between the explanatory variables - i.e. between day infected, sex of the mosquito, female wing length and infection level

Coefficients	Estimate	Std. Error	T value	Pr(> t )
(intercept) ***	14.0054	0.1449	96.643	< 2e-16
<b>Infection.level</b>	<b>0.08129</b>	<b>0.16221</b>	<b>0.50</b>	<b>0.616578</b>
<b>Day.Infected</b>	<b>-0.4689</b>	<b>0.0496</b>	<b>-9.454</b>	<b>&lt; 2e-16</b>
***				
<b>Sex M</b>	<b>-0.5743</b>	<b>0.1736</b>	<b>-3.309</b>	<b>0.00103 **</b>

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1  
 (Dispersion parameter for quasipoisson family taken to be 560912.5)

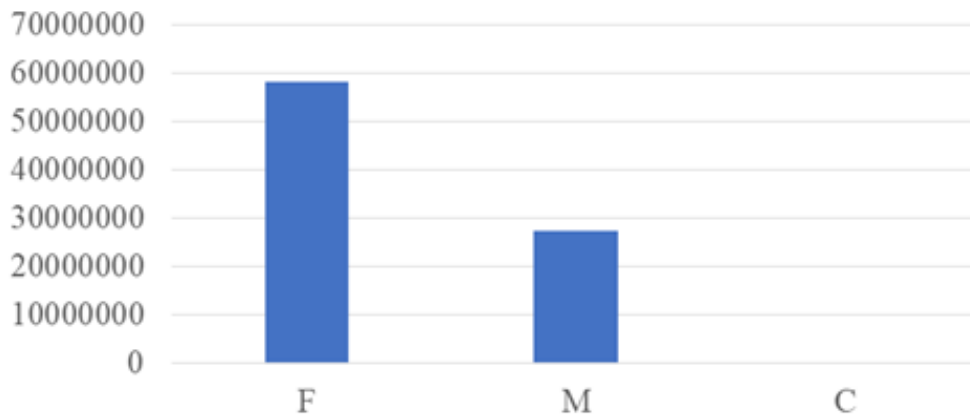


**Fig. 1** Sum of Spore count (y axis) by Day infected (x axis). Mosquitoes infected later (older larvae) resulted in fewer spores in the adults compared to early infected larvae

The virulence of *V. culicis* infection at different host ages was also of interest. Virulence being the degree of pathogenicity of a parasite and determined by its ability to invade and multiply within a host. Does pathogenicity, hence parasite virulence, increase with increasing host age at infection in *V. culicis*? Futuyma (2001) stated that the larger the parasite load, the higher the virulence of the parasite and the greater its potential to cause harm and consequently, host fatalities. Therefore, it was postulated that the larger the numbers of *V. culicis*, the greater its virulence and capability to cause harm to the host, hence the numbers of *V. culicis* spores at the end of a mosquito's life was a key indicator of its virulence. If these numbers differed, it would give some indication of *V. culicis* virulence within younger and older infected mosquitoes.

The trade-off between parasite virulence and its survival and the concept of optimal virulence has

been elucidated for many host parasite interactions. A number of factors affect the virulence of *V. culicis* in mosquitoes. Intraspecific competition, host parasite interactions and host condition were explored by Michalakis *et al.*, (2008) as factors affecting *V. culicis* virulence. Using four different isolates of *V. culicis*, Bargielowski and Koella (2009) found that three of the isolates were more virulent than others and gave rise to higher spore counts at the end of a mosquito's life, hence virulence can change between different isolates of microsporidians. Bedhomme *et al.*, (2004) demonstrated that consuming host resources is another factor that influences a parasite's virulence and that the nutritional status of the host was an important factor. As older hosts provide larger reserves of food (being larger in size), it would be natural to conclude that infections of older hosts should result in larger numbers of spores, a factor explored here.



**Fig. 2** Sum of Spore count by sex. Females (F) produced larger numbers of spores in total than Males (M). C = controls

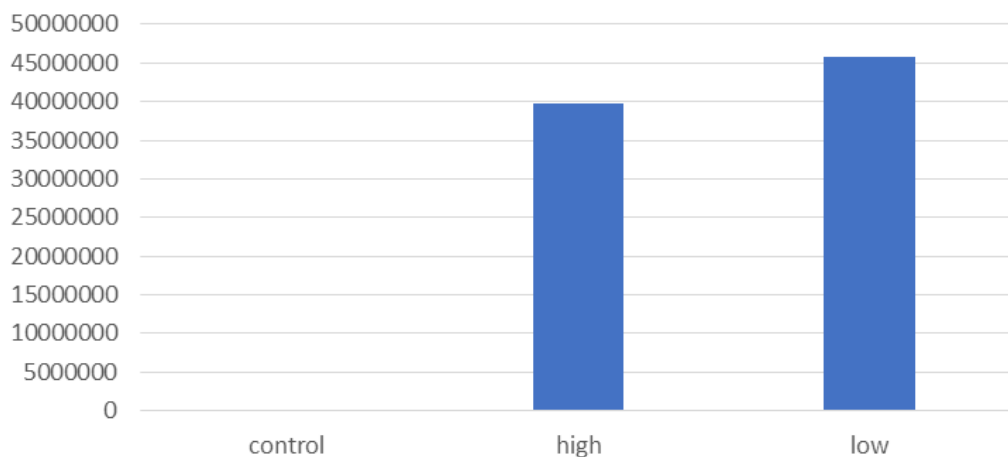
Studies have examined the effect of host age on parasitism by microsporidia in different insect species. In the red flour beetle *Tribolium castaneum*, investigators noted that virulence to the host and the success of its microsporidian parasite *Nosema whitei* was mainly determined by host age at infection. Furthermore, it was found that infection was only possible in young larvae and that the disease prolonged the life span of the larval stages and prevented them from developing to pupation (Blaser and Schmid-Hempel P, 2005). Similarly, different workers investigating *N. whitei* in *T. castaneum*, concluded that age structure, spore concentration, and timing of infection (i.e. host age when infected) were important factors to consider in the reduction of infestation of flour by the beetle (Onstad and Maddox, 1990). In Monarch butterflies, early instars were more susceptible to a higher spore dose of their microsporidian parasite, *Ophryocystis elektroscirrha* than when the later, older larvae were infected, although all larval stages tolerated the parasite at the lower doses. At the highest spore dose, the early instars decreased survival to eclosion (Altizer and Oberhauser, 1999). Vijendravarma *et al.*, (2008), found that the susceptibility of *Drosophila melanogaster* by the microsporidian *Tubulinosema kingi*, decreased with larval age, only the early stage larvae were highly susceptible and that the microsporidian only proliferated to a small degree in the larval stages and increased replication greatly within the adult stages. Considering larval age in mosquitoes when infected with parasites other than microsporidia, Umphlett and Huang, (1973) found that early instars of *Aedes aegypti* infected at a lower dose with the fungus *Lagenidium*, were highly susceptible to the fungus compared to older larvae. Similarly, when larvae of *Ae. aegypti* were infected with the mermithid nematode, *Romanomermis culicivorax* early instars were more susceptible than when later, fourth instars or pupae were infected (Vyas-Patel, 1983). These studies indicate that larval age at the time of infection by microsporidia and by other parasites is a factor that influences outcomes for both the parasite and host. *V. culicis* is considered

a useful agent in the suppression of *Plasmodium* species within adult mosquitoes (Bargielowski and Koella, 2009), it is essential therefore, to ascertain any impact of larval host infections at different ages on adult parasite load. Clearly, if the microsporidian spore load is low or high in an emergent adult due to differential larval infection, this could affect subsequent infection and interference with later infections of the adult with malaria parasites.

To examine the effect of larval age, two groups of *Anopheles coluzzii* larvae were infected with *V. culicis* spores on day one, then day three, five and seven after hatching. Group one larvae were infected at a low dose of 5,000 spores per individual mosquito and group two infected at the higher dose of 50,000 spores per mosquito. All groups including controls, received the optimum daily food dose and they were reared under ideal insectary conditions. The day of pupation and death was noted for every mosquito. As only females carry and transmit the malaria parasite, wings were removed and measured from the experimental and control female mosquitoes; wing lengths being highly correlated with mosquito weight (Lounibos *et al.*, 1995). Spores were counted from every mosquito infected with *V. culicis*, after the death of the adult. All of the mosquitoes were given the same nutrients and conditions and were allowed to die naturally, without any intervention at any stage.

## Materials and Method

*Anopheles gambiae* N'gesso strain, reared at Imperial College London (ICL) and subsequently at the Silwood Park, Ascot, campus, were used. Maintained over many years at ICL, this colony known to be from Yaoundé, Cameroon was reclassified as *An. coluzzii* (previously *An. gambiae* 'M' form) following molecular characterisation (Habtewold T *et al.*, 2016). Note that previous publications (prior to 2016) referring to *An. gambiae* from Yaoundé Cameroon and reared at ICL, are more than likely to be the *An. gambiae* 'M' form strain, i.e. *An. coluzzii*.



**Fig. 3** Sum of Spore count (y axis) by Infection level (y axis) by Infection level (x axis). The original level of infection in larvae did not affect the spore count in adult mosquitoes at death. Infection with *V. culicis* does not affect the age at death of the mosquitoes ( $t = 0.77$ ,  $d.f. = 598$ ,  $p\text{-value} = 0.44$ ), Table 2

Mosquito larvae were infected at different ages; day one, three, five and seven days post hatching, with *V. culicis* and compared to uninfected mosquito larvae. Two different infection levels were used, group one was infected with the lower dose of 5,000 spores per mosquito larva and group two with the higher dose of 50,000 spores per larva. All groups and controls received optimum nutrition and rearing conditions. The day of pupation was noted for every larva as was the day of adult death. All of the female mosquito wings were measured and a spore count carried out for every infected mosquito used in the experiment.

#### Experimental Design

A total of 1,800 newly hatched mosquito larvae were individually placed in a 12 well plate; one larva per well, in 2 ml of deionised water per well. Falcon Multiwell™ 12 well plates, (Becton Dickinson) were used.

The first row of four wells in a plate was allocated to group one larvae (low infections), the second row was allocated to group two larvae (high infections) and the final four rows were allocated to the control larvae which were not infected with *V. culicis*. For subsequent plates, treatment rows were allocated randomly. This ensured that all the treatments were included in every individual plate and the rows were allocated randomly. A fully random allocation of treatments (with every well selected at random for treatment) could have been used, but was avoided; as such an attempt had previously resulted in pipetting errors. This way, accuracy could be maintained along the rows; without compromising random allocation of treatments.

#### Larval Food Regime

TetraMin<sup>R</sup> fish food was weighed and made up to 300 ml in a beaker, so that when 2 µl of the food was pipetted into the wells, the larva in each well received 0.05 mg of food on day one, then 0.06 mg

on day two, 0.1 mg on day three, 0.16 mg on day five, 0.32 mg on day six, and 0.6 mg on subsequent days. This gentle increase in food dissemination ensured that mosquito larvae received the optimum food levels for their size, without the attendant growth in bacteria that would occur if too much food was placed into the wells. The food was freshly prepared daily and the beaker placed on a magnetic stirrer to ensure even distribution of the food particles, whilst 2 µl aliquots of the food solution were applied to each well.

#### Mosquito Rearing

*An. coluzzii*, originating from Yaoundé Cameroon and maintained over many generations in the laboratory at Imperial College London (ICL), was used for the experiment. Mosquitoes were reared in a sealed insectary, kept at a temperature of 26 °C (+/- 1), with a 12:12 hour light: dark cycle and a humidity of 70% (+/-5). Adults were blood fed on a human arm introduced into the rearing cage and allowed access to sugar in the form of a sugar cube placed inside the cage. Eggs were collected from a shallow container, lined with filter paper around the sides, half filled with water and placed inside the cage.

#### Experimental Mosquitoes

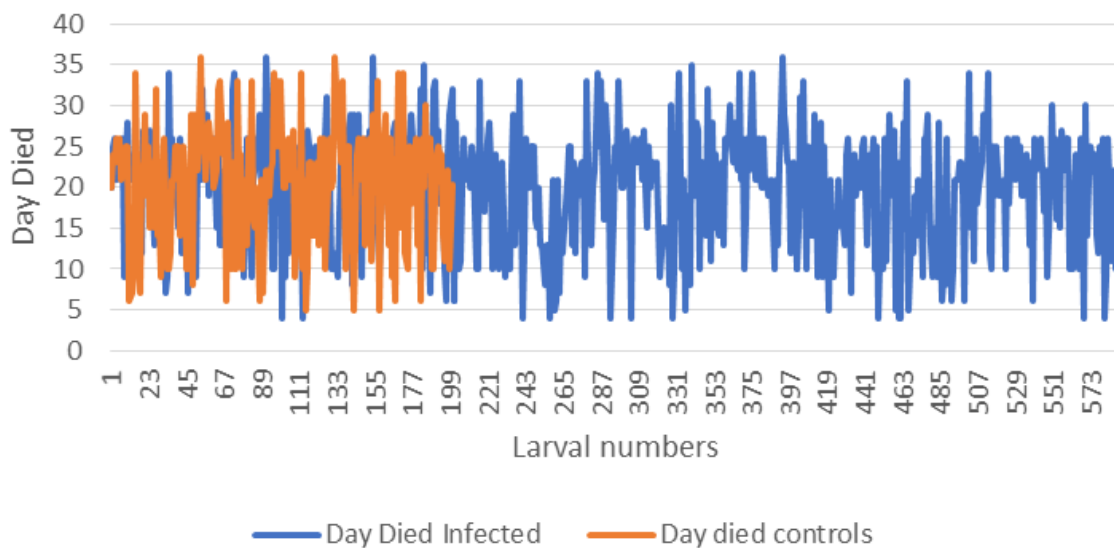
Once an experimental larva had pupated in a well, the day of pupation was noted and the pupa transferred to an Eppendorf tube, placed lid open, in a 10 ml Falcon tube. Mosquito netting was secured to the open end of the Falcon tube and cotton wool soaked with a 10% sucrose solution placed on top of the netting. This ensured that when adults emerged (usually within 24 hours), they had unlimited access to sucrose. Thus, each mosquito was reared individually from larva to adult.

Falcon tubes were examined daily for adult death and fresh sucrose supplied. The day of death was noted and the mosquito was transferred to a

**Table 2** Infection does not affect the age at death of adult mosquitoes

Coefficients	Estimate	Std. Error	t value	Pr(> t )
(Intercept)	20.2950	0.4959	40.924	<2e-16***
<b>Infected</b>	<b>-0.4700</b>	<b>0.6074</b>	<b>-0.774</b>	<b>0.439</b>

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1. Residual standard error: 7.013 on 598 degrees of freedom. Multiple R-squared: 0.001, Adjusted R-squared: -0.0006702; F-statistic: 0.5988 on 1 and 598 DF, p-value: 0.4393.



**Fig. 4** Infection does not affect the age at death of adults. In order that the same time frame available for spore production could be considered in both early (younger larvae) and late (older larvae) infected hosts, the duration of infection was considered. The day of infection was taken away from the day of mosquito death and the spore count for the same duration was considered. When duration of infection was considered (day died – day infected = duration of infection), it did not affect the results. Once again it was seen that the original level of infection, i.e. whether larvae were infected at a high or low dose, did not affect the number of spores at death ( $t=0.12$ , d.f. = 1,  $p = 0.9$ ). As expected, the number of spores rose with the duration of infection ( $t=8.9$ , d.f. =1,  $p<0.001$ ). Furthermore, when duration of infection was considered, the age of the mosquito at infection affected the spore count at death, as older mosquitoes not only produced fewer spores, but were initially able to delay the onset of spore production ( $t=5.9$ , d.f. = 1,  $p<0.001$ ), Table 3 and Figure 5

labelled Eppendorf tube and stored in the fridge. The sex of each adult was noted. Female wings were removed for measurement from every adult female (whether infected or not). Finally, all the infected adults were individually crushed in their Eppendorf tube with a 1.5 ml plastic pestle and the spores counted using a haemocytometer.

#### *Microsporidian Cultures*

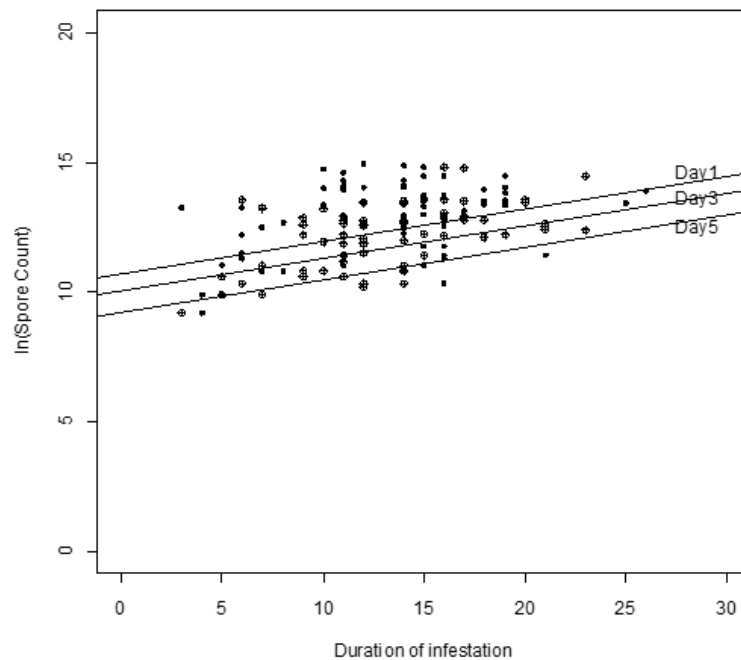
*V. culicis floridensis* spores, initially obtained from a stock culture maintained by J.J. Becnel (USDA Gainesville, USA) were cultured by infecting batches of 40, two day old mosquito larvae in Petri dishes with a dose of 40,000 spores per 40 larvae. The spores were counted using a haemocytometer, under a phase contrast microscope. The infected larvae were

reared to pupae in the Petri dishes and on pupation, were transferred to cages for adult emergence. Adults were collected after two weeks, placed in a fridge and collections of three or four were crushed in 1 ml of deionised water in an Eppendorf tube with a 1.5 ml plastic pestle to release the spores. The culture was pooled and aliquots of it counted and made up to the required dilutions, as and when required for experiments. For this experiment, a large stock culture was counted and diluted into two flasks to ensure that 250  $\mu$ l aliquots from one of the flasks would contain 5,000 spores and 250  $\mu$ l aliquots from the second flask would contain 50,000 spores. It was ensured that the volume of the dilution in each flask was enough to apply to all 600 larvae in both sets.

**Table 3** Age at infection and spore counts at death (considering during of infection)

Coefficients:	Estimate	Std. Error	t value	Pr(> t )
(Intercept)				
DayF1	10.74193	0.31375	34.237	< 2e-16 ***
DayF3	-0.66002	0.17198	-3.838	0.000155 ***
DayF5	-1.50327	0.25849	-5.816	1.56e-08 ***
<b>Duration</b>	<b>0.12487</b>	<b>0.01394</b>	<b>8.960</b>	<b>&lt; 2e-16 ***</b>
<b>Infection.level</b>	<b>0.01863</b>	<b>0.15392</b>	<b>0.121</b>	<b>0.903748</b>
<b>Day.Infected</b>	<b>-0.33075</b>	<b>0.05595</b>	<b>-5.912</b>	<b>9.23e-09 ***</b>

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1  
 (Dispersion parameter for quasipoisson family taken to be 486432.7)



**Fig. 5** Indicating the rate of growth of *V.culicis* infected at different host ages, showing the initial suppression in spore production in older infected mosquitoes (lower intercepts). Figure 5 indicates that the rate of growth of *V. culicis* does not differ with the age at infection (the slope is the same), but the numbers of spores were depressed initially with later infection (the intercepts are lower with greater age at infection). Between days 10 and 20, spore production appeared to reach a maximum plateau in all cases. As before, the longer the duration of infection (early infected larvae), the higher the spore count. The age at pupation was not affected by the initial dose of *V. culicis*, or by the sex of the mosquito, however there was a small delay in the onset of pupation with later infection, Table 4

#### Wing Length Measurements

After female mosquitoes had died, both wings were detached with forceps and placed on a drop of water on a microscope slide and covered with transparent Sellotape. A row of labelled wings was prepared on a slide. The slides were scanned alongside a ruler on a scanner attached to a computer. The scanned images of the wings were measured using the program Image J, (<http://rsb.info.nih.gov/ij/>).

#### Statistical Analysis

Statistical analysis was performed using R (<http://www.r-project.org/>) and Windows Excel. A maximal quasibinomial Generalised Linear Model (GLM) was fitted to the data; this contained all factors, interactions and covariates that might be of any interest (maximum likelihood estimation). This would indicate the minimal adequate model to describe the data. Spore count was the response variable; day infected, sex of the mosquito, infection

**Table 4** Pupation was not affected by the age at infection, dose of *V. culicis* or mosquito gender

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Infection.level	1	0.459	0.45868	1.8225	0.177888
<b>Day.Infected</b>	<b>1</b>	<b>1.908</b>	<b>1.90845</b>	<b>7.5829</b>	<b>0.006198 **</b>
Sex	1	0.511	0.51090	2.0300	0.155112
Residuals		352	88.591	0.25168	

The above Anova indicates that there was an effect on pupation. To resolve the delay, a linear model was fitted with the only significant term in it (day infected). This indicated that early infected mosquitoes started to pupate on day 7 (Table 4), but that the onset of this pupation was delayed by 0.03 days (about an hour and a half) for every day later they are infected (Table 5). Whilst this is statistically significant, given that pupation was noted only once a day, it is not meaningful to infer that pupation was affected in later infected hosts, Table 5

level were the explanatory variables. Model simplification was carried out by stepwise elimination of non-significant factors and interactions.

## Results

The table of results (Tables 1 to 9) from 'R' statistical analysis and the Figures 1 to 5 arising are presented.

A maximal model was fitted to the data and the significance of terms was tested by stepwise deletion. The data were over dispersed and the appropriate model was a quasipoisson Generalised Linear Model (GLM). Spore count was the response variable, day infected, sex, infection level were the explanatory variables.

It was found that there were no significant interactions between the explanatory variables (i.e. between day infected, sex of the mosquito, female wing length and infection level). The day infected affected spore production; later infections produced fewer spores ( $t=9.45$ , d.f. =1,  $p<0.001$ ) (Table 1, Figure 1). Male mosquitoes produced fewer spores than did females ( $t=3.31$ , d.f.=1,  $p<0.01$ ), Figure 2. The original level of infection did not affect the spore count at the end of the mosquito's life ( $t=0.501$ , d.f. =1,  $p=0.62$ ), i.e. the infectious dose did not affect spore count (Figure 3).

Infection with *V. culicis* does not affect the age at death of the mosquitoes ( $t = 0.77$ , d.f. = 598,  $p$ -value = 0.44), Table 2, Figure 4.

Considering the same time frame (duration) of infection, it was seen that this did not affect the results – the initial infection level (high or low) did not affect the spore count for the same time frame. The numbers of spores rose when the same time frame was considered. As before, in older hosts, there was an initial delay in spore production with older host infections producing fewer total spore counts that early infected, younger hosts. Table 3, Figure 5.

The onset of pupation was delayed in older infected hosts Table 4. Further examination revealed this delay to be an hour and a half which was not meaningful as larvae were only examined for pupation once a day, Table 5.

Regardless of the level of infection (high or low), the only important effect was the time of infection, that is if the larvae were infected at an early age compared to older larval infections, Table 6.

Early infected larvae (younger age infections) died slightly earlier than older infected larvae. Older infected larvae lived around 18 hours longer, Table 7.

The initial dose of infection (whether higher or lower numbers of spores administered), nor the sex of the host, affected how long the adults lived in both groups (younger or older host infections), Table 8.

Examining the adult survival rate, in keeping with the results seen in Table 7, hosts infected at an earlier age died slightly earlier (around half a day) than older infected hosts, Table 9.

**Table 5** Pupation data, examined further

Min	IQ	Median	3Q	Max
-1.2436	-0.2436	-0.1850	-0.1263	1.8150
Coefficients:	Estimate	Std. Error	t value	Pr(> t )
(Intercept)	7.09701	0.05351	132.63	<2e-16 ***
<b>Day.Infected</b>	<b>0.02932</b>	<b>0.01150</b>	<b>2.55</b>	<b>0.0112 *</b>

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Examining the age at which mosquitoes died, the only important effect was the time of infection (larval age). The interaction between the level of infection and the day they were infected did not appear to matter, Table 6

**Table 6** Time and level of infection, age at death

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
	1	14.2	14.18	0.4815	0.4882
Day.infected	1	625.5	625.50	21.2401	5.68e-06***
Sex	1	2.0	1.98	0.0673	0.7954
Infection.level:Sex	1	8.7	8.65	0.2938	0.5881
<b>Infection.level: Day.Infected</b>	<b>1</b>	<b>1.6</b>	<b>1.59</b>	<b>0.0542</b>	<b>0.8161</b>
Residuals		351	10336.7	29.45	

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

The minimal adequate model only has the day infected in it and a linear model fit suggests that early infected mosquitoes died earlier than later infected mosquitoes (about ¼ of a day (17-18 hours) of extra life for every day later infected), Table 7

## Discussion

A difference exists in spore production in larvae that were infected at a younger, compared to an older age. Later infected, older larvae, produced significantly lower numbers of spores compared to early infected, younger larvae (Table 1, Figure 1). This effect persisted even when the duration of infection was considered. There was an initial delay in spore production from older infected hosts (Figure 5). This initial delay may account for the lower spore counts from older, compared to younger infected larvae.

It is known that the aquatic environment presents a greater challenge in terms of the density and diversity of microorganisms that can infect mosquito larvae compared to the adult aerial stage, League *et al.*, (2017). Unlike the study by Brown *et al.*, (2019), where larvae were found to be more proficient than adults in killing bacteria, here, spore production rose unchecked from younger larvae, indicating little resistance to the infection and that the larvae were overwhelmed. Examples from other microsporidian infections (Milner, 1973; Maddox, 1990, 2000; Altizer and Oberhauser, 1999; Blaser and Schmid-Hempel 2005; Vijendravarma *et al.*, 2008) and of fungal and nematode pathogens of mosquito larvae (Umphlett and Huang, 1973; Vyas-Patel, 1983) similarly indicated that younger, rather than older mosquito larvae were more susceptible to

infection by parasites and pathogens in their environment.

This study demonstrated a difference in the immune response from younger compared to older larvae. The indications are that larvae are not born with a fully developed immune system, otherwise none of the spores ingested by one day and three day old larvae could develop; to the contrary, the opposite was found here, spore production rose unchecked from younger larvae. There may be other explanations, but a study by Biron (2005) also concluded that older larvae reacted most strongly to infection. The decrease in susceptibility to infections due to greater/better, host defences, with an increase in larval age is also in agreement with previous studies in other insect-microsporidian models (Milner, 1973; Maddox *et al.*, 1990, 2000; Altizer and Oberhauser 1999; Blaser and Schmid-Hempel, 2005; Vijendravarma *et al.*, 2008) as well as in infections of larvae with bacterial and nematode pathogens (Umphlett and Huang, 1973; Vyas-Patel, 1983). There was a delay in the production of spores from older infected larvae here and it is probably the initial, early, immune response that may be of more importance. So that no matter how many spores a larva ingests, the initial immune response may decimate viable spore numbers within the host to a similar level whether high or low numbers of spores have been ingested.

**Table 7** Difference in age at death between early and later infected larvae

Coefficients	Estimate	Std. Error	t value	Pr(> t )
<b>(Intercept)</b>	<b>16.7570</b>	<b>0.6738</b>	<b>24.869</b>	<b>&lt; 2e-16 ***</b>
Day.infected	0.7670	0.1470	5.126	2.94e-07 **

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 6.576 on 398 degrees of freedom. Multiple R-squared: 0.06399, Adjusted R-squared: 0.06164, F-statistic: 27.21 on 1 and 398 DF, p-value: 2.941e-07

Examining adult longevity of the mosquitoes, it was found that neither the sex of the mosquito, nor the initial dose of *V. culicis*, mattered, Table 8



**Table 8** Age at death, mosquito gender and infection dose.

Analysis of Variance Table

Model 1: Ad ~ Infection.level \* Day.Infected \* Sex

Model 2: Ad ~ Infection.level + Day.Infected + Sex

> summary(ad2)

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
<b>Infection.level</b>	<b>1</b>	<b>9.6</b>	<b>9.63</b>	<b>0.3271</b>	<b>0.5677</b>
Day.Infected	1	559.0	558.98	18.9949	1.722e-05 ***
<b>Sex</b>	<b>1</b>	<b>0.5</b>	<b>0.54</b>	<b>0.0184</b>	<b>0.8921</b>
Residuals		352	10358.6	29.43	

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Using adult longevity data, once again the minimally adequate model indicated that the earlier mosquitoes are infected, the earlier they die (by about ½ a day) for each delay in infection, Table 9. This is similar to the results seen in Table 7

Infection with *V. culicis* did not affect the daily age at death between the mosquito groups, Table 2 and Figure 4. Closer scrutiny revealed that early infected larvae died 17 to 18 hours earlier than later infected mosquitoes. Although this was just under a day earlier (Table 7), this difference could be greater in the field as the mosquitoes were reared individually under optimal conditions here. A difference in the time of death would be enough to alter malaria transmission in cases of subsequent infection of the host with malaria parasites, killing the mosquito before the average nine to ten days that malaria parasites require to mature and transmit the disease in the field.

Physical traits such as gut volume and content could also be factors of influence in spore production in larvae infected at an earlier age which were more susceptible to infection and proliferation by microsporidia and produced greater numbers of spores. It may be easier for microsporidia to multiply rapidly in the relatively immature cellular structure proffered by younger larvae compared to the more mature and densely packed body of older larvae. Gut volume (Weiser, 1969), gut content and histological reorganisation in preparation for pupation (Milner, 1973), may present contributing factors that affect spore production in older larvae.

Infection with *V. culicis* depletes the food resources of infected mosquitoes. A lack of host nutrients is a limiting factor for spore development (Bedhomme *et al.*, 2004; Rivero *et al.*, 2007), hence the smaller the food store, the fewer the spores. The wings of the males tested here were smaller than those of females (Vyas-Patel, 2019); wing size correlates with adult mosquito size (Thornhill and Alcock, 1983). Here, spore counts were lower from males (Tables 4, 5 and 8), probably due to their smaller size limiting nutrient storage space, as also indicated by Agnew *et al.*, (1999). In the present study, female *An. coluzzii* wing lengths were not affected by any of the factors tested, indicating a degree of tolerance to microsporidian infection as found by other workers (Andreadis, 2007) and an inability to clear the infection.

Neither the sex of the mosquito nor a higher infectious dose affected the longevity of mosquitoes. The spore count on death was not affected by the infectious dose of spores used. Improved spore storage conditions over time needs to be investigated for *V. culicis* as this might improve spore viability. In general, all microsporidian spores deteriorate in UV light (Li *et al.*, 2003) hence the type of lights used in rearing rooms may affect the viability of spores.

Studies of *N. whitei* in *T. castaneum* have similarly noted that spore count at death correlated with host body size (Blaser and Schmid-Hempel, 2005). Here, it could also be that as male mosquitoes do not have to reserve or divert their energy stores for the production of eggs, they may be able to invest more energy in trying to ward off the infection, thus leading to a lower spore count. It is also possible that the quality of the nutritional status (lipids and carbohydrates) presented by blood feeding females is different and more suitable for spore proliferation than that found in males, but this remains to be investigated.

Infection with *V. culicis* did not affect the daily age the mosquitoes died, or the time they pupated compared to uninfected mosquitoes (Table 2, Figure 4). There was an hourly difference where infected mosquitoes died 17 to 18 hours earlier and the importance of this can only be speculated. This study was of interest because infection with the microsporidian, *V. culicis* hindered the subsequent development of *Plasmodium* species (Bargielowski and Koella, 2009). It is possible to select for *V. culicis* strains in the laboratory that lead to earlier host death and culture such strains to achieve earlier death of the mosquito. How useful would it be for *V. culicis* or any other microsporidian to have a double effect, i.e. achieve early host death as well as impede malaria parasite development? If *V. culicis* or any other microsporidian species is highly efficient at halting the production of malaria parasites, regardless of when the larva was infected, then the timing of adult mosquito death would be inconsequential. If the suppressive effects did not result in the absolute halting of the secondary parasite infection, then

**Table 9** Time of death and age/time of infection

Coefficients	Estimate	Std. Error	T value	Pr(> t )
<b>(Intercept)</b>	<b>11.4401</b>	<b>0.6339</b>	<b>18.049</b>	<b>&lt; 2e-16 ***</b>
Day.Infected	0.4855	0.1362	3.565	0.000411 ***

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.879 on 376 degrees of freedom

Analysing the wing measurement data, it was found that there were no significant interactions between wing length and spore count, dose of infection, or mosquito age at infection

achieving earlier host deaths would be a highly desirable microsporidian trait.

It is clear that the age at which mosquitoes first encounter parasites has important implications for the development of both the parasite and the mosquito host. Host age at the time of infection is an important factor to consider in any host/parasite interaction, especially if virulence and later infections with malaria parasites is a consideration.

## Conclusions

1. The age of mosquito larvae at infection with *V. culicis* affects the spore count on death. Larvae infected later (older hosts) produced a net total of fewer spores compared to early infected, younger larvae, indicating a more robust immune response from older larvae. There was an initial delay in spore production in later infected older hosts not seen from younger infected larvae.
2. Early infected mosquitoes died slightly earlier than later infected ones. Later infected mosquitoes having between 17 to 18 hours of extra life, for every day later that they were infected.
3. The level of infection of *V. culicis*, i.e. the higher (50,000) and lower (5,000) numbers of spores supplied, did not affect the age at death of the mosquito. There were no significant interactions between infection levels (dose), sex of the mosquito and day infected. Hence the longevity of infected mosquitoes was not affected significantly by either the initial dose of *V. culicis* given or by the sex of the mosquito. This suggests a level of tolerance to the parasite by the host.
4. The level of infection with *V. culicis* (spore numbers supplied) did not affect the total spore count.
5. The age at death of the mosquitoes was not significantly affected by infection with *V. culicis*.
6. The age at pupation was not affected by the initial dose of *V. culicis* given, or by the sex of the mosquito.
7. Male mosquitoes produced fewer spores than females.
8. The number of spores from infected adults rose with increasing duration of infection.
9. These findings remained the same even when equal duration of infection was considered in both younger and older infected larvae.

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