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RESEARCH REPORT

The suppression of *Plasmodium berghei* in *Anopheles coluzzii* infected later with *Vavraia culicis*

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Abstract

The suppression of malaria parasites in the presence of an existing microsporidian infection of *Vavraia culicis,* was examined using fluorescent *Plasmodium berghei*. Younger hosts infected with *V. culicis* had a greater suppressive effect on the subsequent development of *P. berghei* than older infected hosts. This effect was density dependant on the numbers of microsporidian spores present, consequently it was also dependant on the age at which mosquitoes were infected with microsporidia, as larvae infected later harboured comparatively fewer *V. culicis* spores. The timing of primary and secondary infections of larval and adult mosquitoes (host age) with different parasites affects parasite development and hence disease outcomes. The use of green, fluorescent *P. berghei* enabled easier and rapid visualisation and separation of malaria oocysts from *V. culicis* spores. Different degrees of melanisation of *V. culicis* spores were seen in a number of hosts and merits further investigation.

Key Words: microsporidia; mosquito; *Vavraia culicis*; *Anopheles coluzzii*; *Plasmodium berghei*; larval host age; spore count; double infection

Introduction

Infection with microsporidia renders the mosquito less hospitable to the development of later *Plasmodium* infections, (Garnham, 1956; Bano, 1958; Bray, 1958; Fox and Weiser, 1959; Hulls, 1971; Savage *et al*., 1972; Ward and Savage, 1972; Gajana *et al*., 1979; Schenker *et al*., 1992; Margos *et al*., 1992; Weiser and ŽIŽKA, 2004; Herren *et al*., 2020). The first recorded observation of this effect came from infections of laboratory colonies where it proved difficult to infect mosquitoes harbouring microsporidia, with malaria parasites (Garnham, 1956). It was noted that ‘‘In experimental malaria work, where microsporidian-infected mosquitoes are employed as the vector, there may be interference with the normal development of the oocyst and sporozoites’’ (Garnham, 1956). This was followed by numerous reports where infections of larval mosquitoes with microsporidians, then later with malaria parasites, inhibited the development of the second infection i.e., the malaria parasite, both in terms of numbers of oocysts produced and their size. These reports were largely from inadvertent infections of microsporidia in laboratory reared mosquito colonies. Infections of two-day old mosquito larvae with the microsporidian *Vavraia culicis* had a suppressive effect on the development of *Plasmodium berghei* (Bargielowski and Koella, 2009). The inhibitory effects of one organism on the development of another is not merely restricted to microsporidia, reports examining field collected anophelines indicated that the composition and diversity of the mosquito microbiome could also have a similar effect (Cirimotich *et al*., 2010; Tainchum *et al*., 2020). Another example is that of filarial nematode infections negatively impacting the development of plasmodium in mosquitoes, leading to the conclusion that treating the filarial nematode could result in the rise of malaria infections (Aliota *et al*., 2011).

A study examining the effects of host age on *V. culicis* indicated that infecting older larval mosquitoes led to the production of comparatively fewer spores (Vyas-Patel, 2024). It was queried (Vyas-Patel, 2024) whether infecting older larvae could also bring about the same suppressive effect on the development of the malaria parasite, as found by Bargielowski and Koella (2009) and Vyas-Patel (2024), from early infected larvae. Could the suppressive effect of microsporidia be dependent on the extent of infection i.e., the numbers of microsporidian spores present in a host? The suppressive effect of *V. culicis* on Plasmodium parasites has been stated to be the result of immune priming (Bargielowski and Koella, 2009), it could also be a consequence of the numbers of

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Table1 Numbers of hosts dissected daily, and percentages found positive for Oocysts for Experiment 1. V+2 = Mosquito larvae infected on day 2 post hatching with *V. culicis*

Day Post hatching	Numbers dissected Controls	Numbers with Oocysts, Controls	Percentage with Oocysts, Controls	Numbers dissected V+2	Numbers with Oocysts, V+2	Percentage with Oocysts, V+2
10	20	9	45%	10	0	0%
11	10	5	50%	10	0	0%
12	20	13	65%	10	0	0%
13	10	4	40%	10	0	0%
14	10	3	30%	10	0	0%
15	10	3	30%	10	0	0%

A statistical comparison between the controls (C) and the experimental values (V+2), indicated a highly significant difference. A two-sample t-test between the groups, resulted in a t(5) statistic of 7.98 and a $p<0.001$, demonstrating the highly significant difference. Similarly, a paired t-test also resulted in $p<0.001$. This is unsurprising as the experimental results (V+2) are entirely static with a mean and median of zero and there is zero variance (all the results are zero). Welch's two sample t-test resulted in a 't' value of 7.98 and a two-paired paired 'p' value of 0.0004 (well below 0.001). A Mann-Whitney U test was therefore conducted which gave $p<0.0027$ (U statistic 36.0), again, well below common significance levels of 0.05. The oocyst counts from the controls were demonstrably significantly higher than from the experimental hosts
Sporozoites were not seen in the experimental hosts. One out of the 10 control hosts (10%) were positive for sporozoites on day 15 (not so in the experimental group), this is within the normal time frame for sporozoite development. Speeding up of the developmental time of *P. berghei* was not seen

spores present, a physical, nutritional, or metabolic reaction as well as priming of the host's immune system. Or the result of a combination of factors. It has been reported that the presence of microsporidia could lead to faster moulting and development of infected hosts (Agnew et al., 1990); could microsporidian infection also speed up and affect any subsequent infections with *Plasmodia,* leading to missed observations of oocysts as they quickly developed into the sporozoites stage? It was known that infecting older mosquito larvae with *V. culicis* would present an altered host environment for the development of any subsequent *Plasmodium berghei*, compared to younger larval infections (Vyas-Patel, 2024). If older and younger larval mosquitoes were infected with *V. culicis*, then subsequently infected with *P. berghei* as adults on the same day, it would give some indication of the suppressive effects of microsporidia when they inhabit larvae at different ages as the numbers of *V. culicis* spores would comparatively be fewer in the older infected larvae (Vyas-Patel, 2024). Furthermore, the nutritional and immune status of the host would be different in larvae infected at an older age. A study was therefore undertaken to ascertain if older mosquito larval infections with *V. culicis* could still exert a suppressive effect on the development of subsequent infections with *P. berghei*.

Materials and method

Experiments 1 and 2

Mosquito larvae were infected with 20,000 spores per larva, on day 2 for Experiment 1 and day 2 or day 6 for Experiment 2, post hatching, with *V. culicis*, then subsequently infected with *P. berghei* as adults and compared with mosquitoes not infected with microsporidia, but only infected with *P. berghei* – the controls. Infections of adults with *P. berghei* was carried out on five-day old female adults. From day 10 until day 15 (Experiment 1) mosquito guts and salivary glands were dissected and examined for oocysts and sporozoites. For Experiment 2, mosquito guts were dissected until 23 days and photographed. The different experimental groups were kept in separate cages. The aim of Experiment 1 was to determine if prior infection with microsporidia could speed up the development of *P. berghei*, in which case sporozoites would be seen earlier, at least before day 15 post infection. In the second study, experiment 2, mosquito larvae were infected with *V. culicis* on day 2 and a separate group of mosquitoes on day 6 post hatching. Subsequently, both groups were infected with *P. berghei* as adults on day 5 post emergence, to determine if infections of older larvae with microsporidia had the same suppressive effect on the development of the malaria parasite, *P. berghei*.

Table 2 Numbers of hosts dissected daily, and percentages found positive for Oocysts for Experiment 2. Vc+6 and Vc+2= Mosquito larvae infected on day 6 and day 2 post hatching with *V. culicis*

Day	Numbers dissected, Controls	Numbers with Oocysts, Controls	Percentage with Oocysts, Controls	Numbers dissected V+6 & V+2	Numbers with Oocysts, V+6	Percentage with Oocysts, V+6	Percentage with Oocysts, V+2
10	20	5	25%	10	0	0%	0%
11	10	5	50%	10	0	0%	0%
12	10	6	60%	10	0	0%	0%
13	10	4	40%	10	0	0%	0%
14	10	4	40%	10	1	10%	0%
15	20	5	25%	10	2	20%	0%
16	10	2	20%	10	1	10%	0%
17	10	1	10%	10	0	0%	0%
18	10	4	40%	10	0	0%	0%
19	20	5	25%	10	0	0%	0%
20	20	5	25%	10	0	0%	0%
21	20	3	15%	20	3	15%	0%
22	10	1	10%	10	0	0%	0%
23	10	1	10%	10	0	0%	0%

The Control group had a mean of 28, median of 25, mode of 25 and standard deviation of 15.64. The experimental group Vc+6 had a mean of 3.93, mode and median of 0 and a standard deviation of 6.84. The experimental group Vc+2 had no hosts with oocysts, the results for the mean, median and mode were uniformly zero. Once again, the experimental groups were heavily skewed towards zero as in Experiment 1. Conducting Welch's Independent Samples t-test gave a p= 0.000048 (t=5.32), indicating a statistically significant difference between the control and experimental group Vc+6. A Mann-Whitney U test (a non-parametric test, as there were large numbers of zeros and the results were skewed); resulted in p=0.000043 indicating again, a significant difference between the groups (Controls and Vc+6). The control group had higher numbers of hosts positive for Oocysts than the experimental Vc+6 group and this was statistically significant

Mosquito rearing and microsporidian culture (V. culicis).

An. coluzzii mosquitoes originating from the Cameroons were reared as described by Vyas-Patel (2024). Cultures of *V. culicis floridensis*, originating from USDA Gainesville, USA, courtesy of JJ Becnel, were maintained as described in Vyas-Patel (2024).

Plasmodium berghei *ookinete culture*

P. berghei, a murine parasite first cultured from forest dwelling, African thicket rats (Natarajan et al, 2002) were employed. The *P. berghei* ookinetes used were from a transgenic cell line, generated to express green fluorescent protein (GFP) throughout the life cycle of the parasite and were produced using non-viral transfection technology, Nucleofactor^R as described by Janse *et al*., (2006). These fluorescent *P. berghei* cultures were maintained in the laboratory of Prof. R Sinden, Imperial College, London (ICL), using the methodology described by Sinden (2002).

P. berghei *infections*

Ookinetes were transported from Prof. Sinden's laboratory at Imperial College London (ICL) to the college campus at Silwood Park, Ascot. The ookinete rich blood was centrifuged at 3000 rpm, for 10 minutes and the supernatant removed. The precipitated ookinetes were then quantified using a haemocytometer under a phase contrast microscope. The remaining blood (precipitate containing ookinetes) was mixed with naive mouse blood and calibrated to deliver a concentration of 800 ookinetes/µl. Mosquitoes were introduced to the infected blood by means of membrane feeders specially produced by Glass Precision Engineering Ltd. (GPE Scientific, product code SP 3046). Using a syringe, 300µl of the prepared infected blood was introduced into the top of the feeder. The bottom of the feeder, which was covered with stretched Parafilm was gently placed on top of the mosquito cage containing experimental mosquitoes to be infected, so that the Parafilm only touched the outside of the mosquito netting. Experimental mosquitoes were allowed to feed for an hour, after which the membrane feeders were detached. Mosquitoes that had visibly fed and had distended abdomens were removed and reared in separate cages for each of the groups. They were allowed access to a sugar solution and dissected and examined for oocysts daily from day 10 to 15 (Experiment 1, to determine if there was a speeding

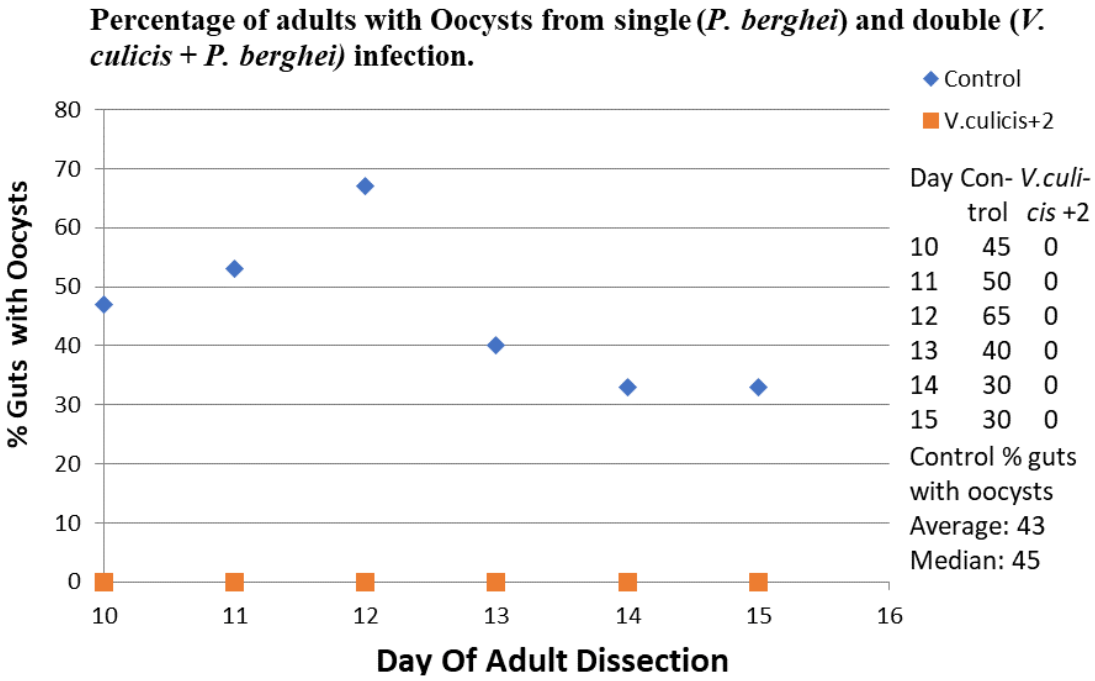

Fig. 1 The development of *P. berghei* in the presence and absence (control) of *V. culicis,* day 2 infected larvae. Control = Adults not infected with *V. culicis*; but singly infected with *P. berghei*. *V. culicis* + 2 = Mosquitoes infected as larvae, day 2 post hatching, with *V. culicis* and subsequently infected as adults with *P. berghei*

up of oocyst development) and day 10 to 23 (Experiment 2, to determine if age at infection with microsporidia affected oocyst development).

Experimental Design

One thousand five hundred, newly hatched mosquito larvae were individually placed in the well of 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. Row one of each plate was infected on day 2 post hatching with 20,000 spores of *V. culicis* as described in Vyas-Patel (2024). Row two of each plate was similarly infected, but on day 5 post hatching. The remaining row was left uninfected and was the larval control. The treatment rows were randomly allocated in subsequent plates. Mosquito larvae were fed with an increasing amount of food daily, as described in Vyas-Patel (2024). Larvae 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). On pupation, each pupa was placed individually in 3 ml of deionised water into a netting covered 50 ml Falcon tube and allowed to emerge into adults.

On emergence (usually the next day), each adult mosquito was sexed, males and females were placed separately into labelled cages, around 60 to 80 mosquitoes per cage and supplied with an unlimited amount of sucrose solution. On day 4 post emergence, the female adults were transferred to another rearing room maintained at 19 °C, 12:12 hour light: dark cycle, relative humidity of 70% and were deprived of sucrose for 24 hours to encourage feeding the next day (following the advice from ICL, S. Kensington). *P. berghei* maturation is triggered by the drop in temperature from the endothermic mouse body to the ambient temperature and thicket rodents being forest dwelling species, *P. berghei* develops best at the lower 19 °C temperature. On day 5 post emergence, adult female mosquitoes were infected with *P. berghei* ookinetes via a membrane blood feeder. Mosquitoes were examined to ensure that their abdomens were full or half full of blood. Unfed mosquitoes were removed from the cages and fed mosquitoes were allowed access to unlimited amounts of sucrose solution and extra humidity in the form of water-soaked filter paper placed inside the cage. Mosquitoes were dissected and examined for the presence of oocysts and sporozoites each day from day 10 until day 15 (Experiment 1).

Mosquito dissections

Mosquitoes to be dissected were transferred into cups and placed on ice to anaesthetise the adults. The salivary glands and guts were dissected as described by Service (1980) and examined initially under a phase contrast light microscope to determine that doubly infected mosquitoes did indeed show the presence of *V. culicis* spores from every doubly infected mosquito dissected. Also, to determine the absence of *V. culicis* from control mosquitoes, to rule out any kind of observational error. This was followed by examination under the fluorescence microscope to determine the presence/absence of oocysts from the gut. To discount the possibility that co-infections speeded

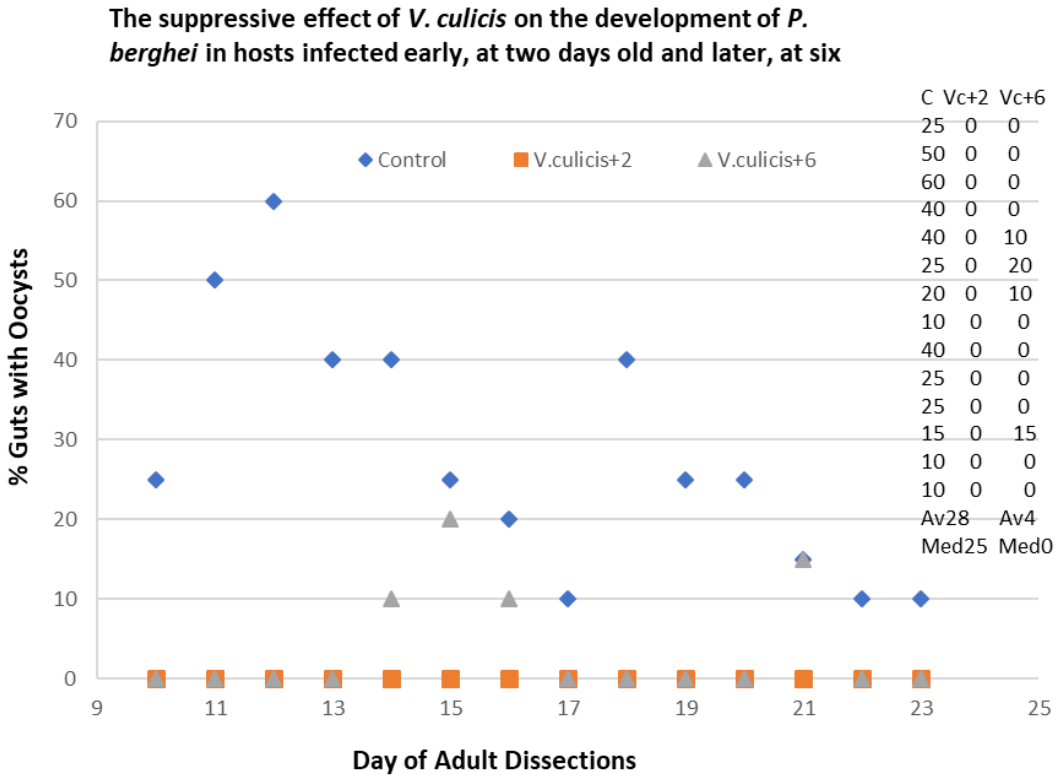

Fig. 2 The development of *P. berghei* in younger, day 2 infected larvae (Vc+2), compared with older larvae infected with *V. culicis* on day 6 post hatching (Vc+6)
C = Controls infected only with *P. berghei* (no *V. culicis*). *Vc* +2 & Vc+6 = Mosquitoes infected as larvae on day 2 and day 6 post hatching, with *V. culicis* and then infected as adults on day 5 post emergence, with *P. berghei*

up *P. berghei* development and hence the production of sporozoites, the salivary glands were also examined under the fluorescence microscope for the presence of any sporozoites from day 10 to 15.

The numbers of experimental mosquitoes in each group depended on the numbers of females that had fed on the *Plasmodium* ookinete rich blood culture. Fed mosquitoes were observed to have distended abdomens and were removed for each group. Care had to be taken to preserve numbers of experimental mosquitoes (each group was kept separately) until the last day of observation. The numbers of mosquitoes dissected daily from each group therefore varied from a minimum of ten to a maximum of twenty mosquitoes from each of the experimental groups; the results were presented as percentages. Tables 1 and 2 gives the numbers of mosquitoes dissected daily and the percentages of hosts found positive for oocysts, together with statistical analysis of the results.

Results

Experiment 1: Does infection with microsporidia speed up the development of malaria parasites?

Figure 1 indicates the presence or absence of oocysts from singly and doubly infected adults. Sporozoites were not seen. Here, there was complete absence of oocysts from doubly infected (with *V. culicis,* day 2 larvae infections + *P. berghei*) mosquitoes (0 values) and their presence from singly infected (with *P. berghei* alone) - the control mosquitoes.

The highest numbers of hosts with *P. berghei* from single infections (controls) were recorded on day 12; this is within the normal range for peak oocyst formation in single infections with *P. berghei*. The average percentage of mosquitoes infected with oocysts in controls over the 6 days was 43%, the median numbers of mosquitoes infected was also 43% compared to double infections, where it was zero. .A host was counted as being positive for oocysts regardless of the numbers of oocysts observed in a gut. Sporozoites were not seen in doubly infected mosquitoes as no oocysts were seen from which they could develop. Ten percent of the controls were positive for sporozoites on day 15. Statistical comparison between the controls (C) and the experimental values (V+2), indicated a highly significant difference, Table1, where a paired t-test resulted in $p<0.001$ and a Mann-Whitney U test an even lower value of $p<0.0027$, both indicating a highly significant result.

Experiment 2: Do later infections (of older larvae) with Microsporidia also suppress the development of Malaria parasites?

The second experiment compared the development of oocysts in double infections where

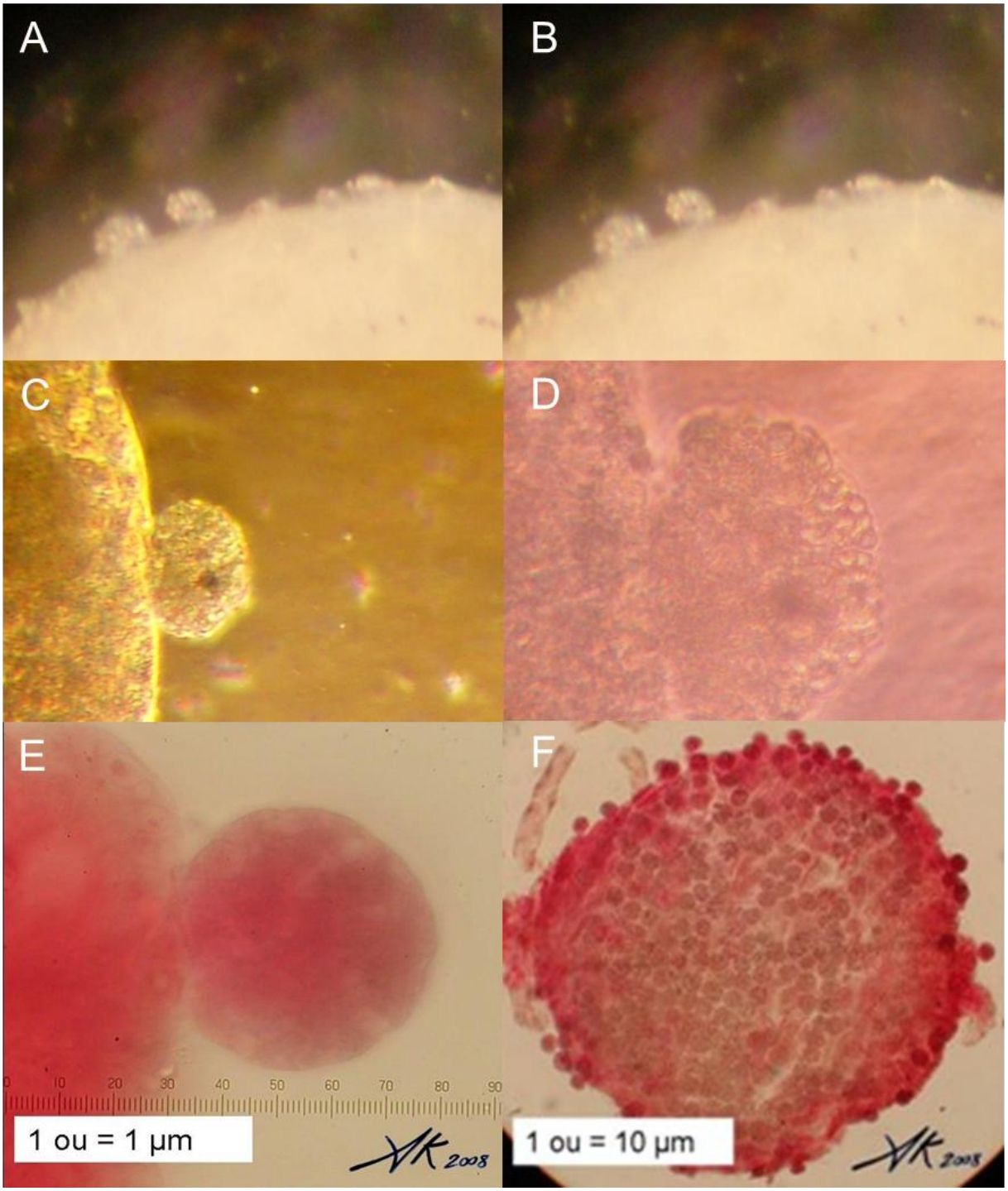

Fig. 3 Images of singly (either with *V. culicis* or *P. berghei*) infected mosquito guts using the dissection and phase contrast microscopes A) A mosquito gut heavily and singly infected with *V. culicis*, note the sporophorous vesicles on the outside of the gut B) Close up of sporophorous vesicles on the outside of the mosquito gut C) A sporophorous vesicle, outside of the mosquito gut, under the phase contrast microscope, X200 D) The close connection of the protruding sporophorous vesicle with the mosquito gut wall, X400 E) *Plasmodium* oocyst on the outside of a mosquito gut, courtesy of Alain Knipes, note the similarity with *V. culicis* sporophorous vesicles. The outline of *Plasmodium* is better defined than *V. culicis* sporophorous vesicles F) Stained *Plasmodium* oocysts on the mosquito gut, looking remarkably similar to the sporophorous vesicles of *V. culicis*, picture 3a & 3b, above. Photo courtesy of Alain Knipes

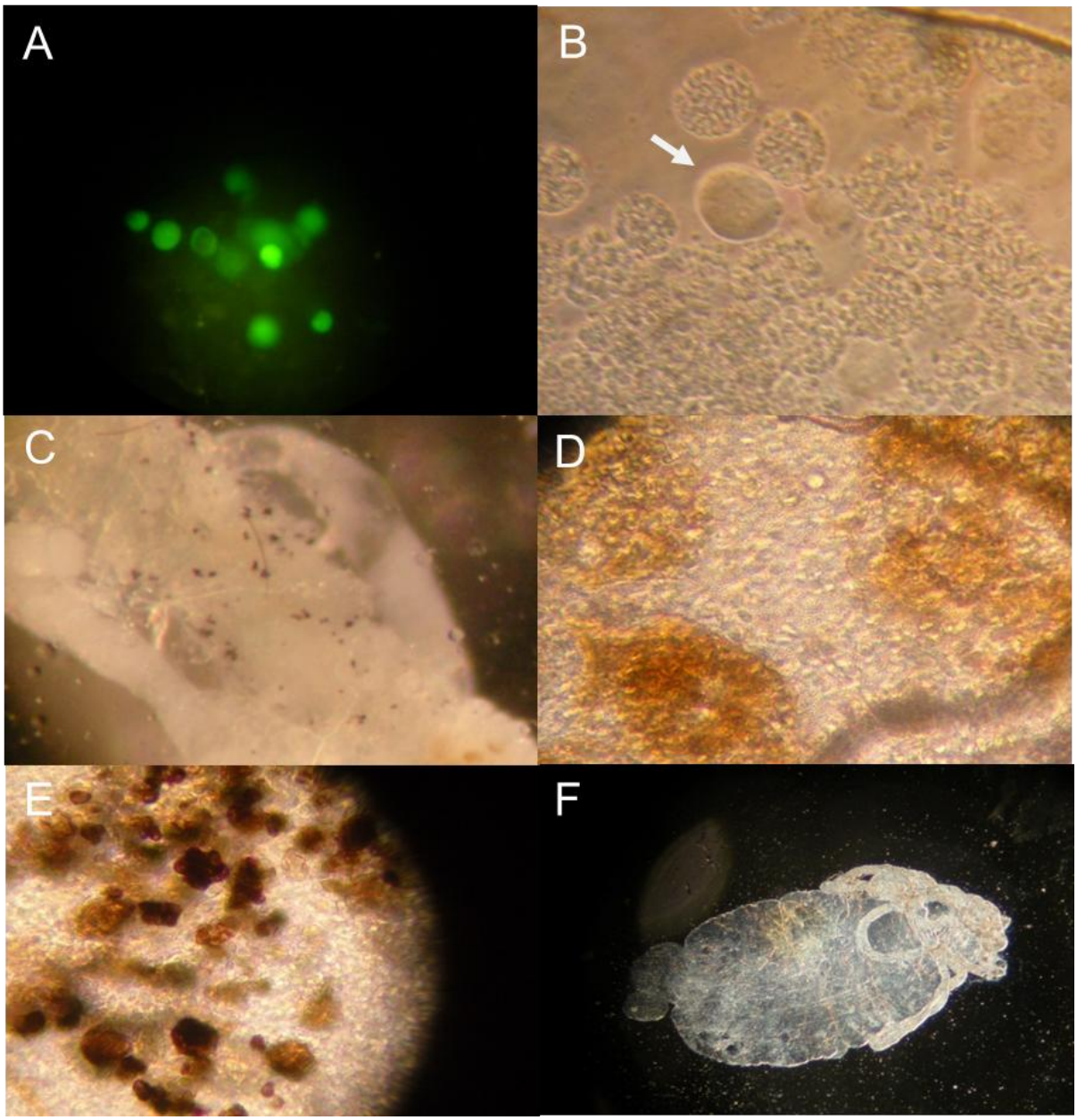

Fig. 4 Images of doubly infected mosquitoes (*V. culicis* + *P. berghei)* using fluorescence and phase contrast microscopes A) *P. berghei* oocysts, fluorescing; hence easily seen and counted, using the fluorescence microscope B) Doubly infected mosquito gut, phase contrast microscope, X100. Oocyst in the middle (arrow), surrounded by *V. Culicis*. *V. culicis* spores are between 1 to 4 µm wide and *P. berghei* oocysts are slightly larger at 7-8 µm in diameter C) Speckles of melanised spores seen in the mosquito gut under the dissection microscope D) Patches of light melanisation of *V. culicis* sporophorous vesicles in the mosquito gut E) Both intense and lighter melanisation of sporophorous vesicles F) Uninfected mosquito gut, no spores

the microsporidian infection was introduced early on (day 2 post hatching, younger hosts) and later on (day 6 post hatching, older hosts), with singly infected (only with *P. berghei*) controls. All three of the mosquito groups were infected with *P. berghei* on day 5 post adult emergence. The aim was to determine if the timing/delay of the initial microsporidian infection and hence host age at infection could have any impact on oocyst production in doubly infected mosquitoes. Figure 2 is a graphical representation of the results, indicating the presence/absence of oocysts from all three treatments.

The average percentage of hosts found positive for oocysts in controls was 28%. The median numbers of mosquitoes infected was 25. The average percentage of mosquitoes with oocysts in double infections, day 2 + *V. culicis* was 0, indicating as before, the complete suppression of oocyst development in day 2 infected mosquitoes.

The average percentage of mosquitoes with oocysts in double infections, day 6 +*V. culicis* was 4%, median value 0, indicating incomplete suppression of oocyst development from later infected hosts. Statistical analysis (Tables 1 and 2) indicated a significant difference between the control groups and the experimental groups, supporting the observation that there was complete suppression of *Plasmodium* development from early, day 2, *V. culicis* infected hosts and incomplete suppression from later, day 6 *V. culicis* infected hosts. The control group had higher numbers of hosts positive for Oocysts than the experimental Vc+6 group and this was statistically significant, with a p=0.000043 (Mann-Whitney U test, Table 2).

Figures 3 and 4 illustrates that under the dissection and light microscopes, a mosquito gut infected with microsporidia can look remarkably similar to one infected by *Plasmodium*. The developing sporophorous vesicles of *V. culicis* look similar to groups of oocysts when they protrude outwards from the gut. Microsporidian species vary in size from 1 to 4 µm; *Plasmodium* oocysts are generally 30 to 40 µm on day 9 post infection. Spores were seen to break out of the gut wall and float into the body cavity. Different degrees of *V. culicis* spore melanisation were seen.

Discussion

Being the first parasite to invade and colonise the mosquito host in the larval stage allows microsporidia to be at a distinct advantage over any later arrivals such as *Plasmodium* species. Not only has *V. culicis* been inside the mosquito host a good six to ten days before the arrival of any *P. berghei*, but it has also made the host's environment its own and created a habitat to suit its own abundant growth and development. By the time the second invader arrives, *V. culicis* has not only depleted the host's resources (Rivero *et al*., 2007), but changed the host's internal environment, which is taken over by the unchecked multiplication of *V. culicis* spores. It should be considered surprising that anything at all could possibly grow and develop in mosquitoes under the conditions presented by *V. culicis* multiplication. That day six, *V. culicis* infected hosts could harbour double infections, albeit at low levels (average 3.93% of hosts found with oocysts) and not early, day two infected hosts, where spore production would have gained considerable hold, suggests that the intensity of *V. culicis* infection plays a significant part in the suppression of later *P. berghei* infection.

The results are similar to the findings by Fox and Weiser (1959) noting suppression in the development of *Plasmodia* by the microsporidian *Nosema*, but slightly different to those obtained by Bargielowski and Koella (2009) where higher levels of experimental *P. berghei* (58.5%) were noted from day two infected larvae, compared to the 0% here, where different isolates of *V. culicis* and fluorescent *P. berghei* were used. In a study where the double infections were from two different species of microsporidia, both larval in nature and where both the parasites were introduced at the same time, increasing the parasite burden of *V. culicis* resulted in differential development of the second microsporidian parasite *Ascogregarina culicis* (Fellous and Koella, 2009).

Co-infection did not lead to a speeding up in the development of the *Plasmodium* parasite, as sporozoites were not seen to develop early (before 15 days post infection Table 1). Egress from oocysts is generally 11 to 12 days, observation in salivary glands is from day 14-15 post infection (Klug and Frischknecht, 2017). As oocysts from which sporozoites form, were also not seen from doubly infected experimental mosquitoes (Figure 1), the outcome was unsurprising. Ten percent of the controls were positive for sporozoites on day 15, (Experiment 1).

Competition for nutrition (Margos *et al*., 1992), interference by metabolites (Dussaubat *et al*., 2012) and the effect of pH (Tsang *et al*., 1982; Carter *et al*., 2007; Undeen 2007), may all play a role in the results seen here. Fox and Weiser (1959) suggested that invasion and disintegration of the gut wall by microsporidians (Tanada and Kaya, 1992; Becnel and Andreadis, 1999; Maddox *et al*., 2000; Vijendravarma, 2008) meant that the second parasite would not be able to lodge and develop well in the destroyed gut wall – a structural, physical and mechanical issue that is also seen from Figures 3 and 4. Both parasites have to cross the peritrophic matrix /membrane lining the gut wall, first formed in the larval stage (Wigglesworth, 1930; Terra, 2001) if they are to escape into the haemocoel. *Plasmodium* uses chitinase to do so (Shahabuddin *et al*., 1993), but the matrix is not required for *in vitro* development of *Plasmodium* (Carter *et al*., 2007). How microsporidians escape from the gut is less clear as chitinase would destroy the chitin cell wall of microsporidia (Tanada and Kaya, 1992; Becnel and Andreadis 1999; Maddox *et al*., 2000). The impact of the host's immune system on both parasites needs further study as conditions are different within the gut and outside of it, in the haemocoel (Whitten and Coates, 2017). In *Plasmodium* the peritrophic membrane protects it, by preventing mosquitoes from mounting an immune response (Kumar *et al*., 2010). Fox and Weiser (1959) reported that the peritrophic matrix was not found in mosquitoes infected with the microsporidian, Nosema. Hence, the presence or absence of the peritrophic matrix following microsporidian infection could impact *Plasmodium* species development in vivo.

Microsporidian infection results in a robust immune response (melanisation) from the host (Figures 3, 4). *Anopheles* species also melanise *Plasmodium*, but it is a relatively rare event in natural populations because the parasite has evolved strategies to evade this response. In *An. gambiae* (genetically distinct but closely related to the *An. coluzzii* used here), it was found that melanisation of *Plasmodium* was dependent on the intensity of infection (Simões *et al*., 2017).

Here, the robust immune response initiated against *V. culicis* in the form of melanisation was incomplete (Figures 3 and 4), it was inadequate to halt all spore production. Patches of both light and darker areas of melanisation was seen, as well as areas of unaffected spores (Figures 4d and 4e).

This may reflect an inability of the host to sustain melanisation or that spore production exceeded the capacity of haemocytes or other gut immune processes. Older larvae have a greater capacity to hinder spore production at the beginning of infections, compared to younger larvae (Vyas-Patel, 2024). In *Plasmodium,* Zeineddine *et al*., (2024) concluded that melanisation was by far the most potent anti-*Plasmodium* defence system in the mosquito, but *P. berghei* oocyst melanisation was not observed here, either in the controls or the experimental groups. The host, *An. coluzzii* was highly susceptible to *P. berghei,* it needs to be, as it, together with *An. stephensi* were the host species used to rear *P. berghei* cultures at ICL.

The observation that melanisation was seen in Microsporidian infection but not in *Plasmodium;* yet *Plasmodium* did not thrive in the presence of Microsporidia, indicates a suppressive effect in the presence of the microsporidian and requires further study.

As well as the initial, primary melanisation response, the antimicrobial defence system which produces a later, sustained response, is also known to regulate invaders and defend mosquitoes against not just bacterial, but also microsporidian, malarial and fungal parasites (Dong *et al*., 2006; Cirimotech, 2010; Jarkass and Reinke, 2020). Typically, melanisation is the immediate, fast acting immune response that generally comes first, this is followed by the synthesis of antimicrobial peptides (AMPs) such as TP1, which contributes a later, sustained attack (Tang *et al*., 2006; Zeineddine, 2024) and should be studied further. It may shed light on how the host melanises *V. culicis* in this study, but not *P. berghei* and if any later immune responses such as AMP plays a part in the suppression of *P. berghei* in double infections.

The biting capability of doubly infected mosquitoes also merits investigation, although ethical constraints would apply. Hulls (1971) reported that *P. berghei* sporozoites from *Nosema* infected mosquitoes were less infective compared to controls when inoculated into mice, but this does not address the natural biting capability of doubly infected mosquitoes. Microsporidian infections reduced the size of individual malaria parasites (Bano, 1958; Bray, 1958), but not enough is known about the capacity and transmission capability of smaller sized malaria parasites to cause disease. Until then, microsporidia-based control strategies should target early, newly hatched larvae to achieve sufficient infection intensity.

Reducing malaria parasites in the adult mosquito requires a critical level of microsporidian infection in the adult mosquito. *V. culicis* and *P. berghei* were used here, as cultures of both were established in the laboratory and could safely be used. As *V. culicis* is horizontally transmitted; maintaining high levels of infection in the field might require repeated microsporidian releases. Vertically transmitted microsporidia could avoid this need, once infection levels were assessed. High suppression has been observed in the vertically transmitted *Microsporidia MB* in *Anopheles arabiensis* (Herren *et al*., 2020), suggesting that the transmission mode may influence effectiveness. However, outcomes vary by system; for example, *Nematocida parisii* infection in *Caenorhabditis elegans* produced offspring with temporary, dose-dependent immunity lasting only one generation (Willis *et al*., 2021). Therefore, each host-parasite system needs to be individually studied and assessed.

An interaction exists between the two parasites with the sheer numbers of microsporidia exerting a strong negative impact on the numbers of *Plasmodium* that can develop in doubly infected hosts. This is evidenced by the presence of oocysts from a few mosquitoes infected with *V. culicis* later on, (day 6 post hatching) compared to early infected mosquitoes (day 2 post hatching), where *P. berghei* development was completely halted. The numbers of *V. culicis* spores, hence the intensity of microsporidian infection, plays a major part in the suppression of *P. berghei* development in doubly infected mosquitoes.

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