Mechanisms of host exploitation by a microsporidian parasite

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Abstract

Parasites are masters at exploiting their hosts. In doing so, they often affect the development of co-infecting parasites. Such is the case of microsporidia and *Plasmodium spp*., which have been shown to have their development within the mosquito repressed in the presence of the former. Therefore, microsporidia hold significant potential as biological control agents for malaria. However, the mechanisms behind this repression remain unclear. Here, we characterised the mechanisms underpinning the infection by using evolved lines of the microsporidian *Vavraia culicis* differing in their virulence upon infecting the mosquito *Anopheles gambiae*, the main malaria vector. Through the use of host metallomics and energy quantification throughout infection progression, we were able to identify important parasite mechanisms of host exploitation of general and virulence-specific interest, namely iron. Our findings advance the fundamental understanding of microsporidia infections and provide important insights into the design of effective malaria control strategies.

Keywords: microsporidia, virulence, iron, *Anopheles gambiae*, resource allocation, metallomics

Introduction

Since the beginning of life, parasites have infected and exploited their hosts (Carlsson and Råberg 2024). The negative cost a parasite has on its host, particularly its survival, is commonly referred to as virulence (Read 1994). Virulence is impacted by the contribution of both the host and parasite (Råberg and Stjernman 2012). While the host can modulate the cost of infection by controlling the damage or the parasite load (Kutzer and Armitage 2016; Råberg, Sim, and Read 2007; Silva 2021), the parasite can either use the host's resources to fuel its growth within the host (named host exploitation) or it can invest in toxins or virulence factors that cause additional damage, independently of its growth or load (referred to as per parasite pathogenicity or benevolence) (Acuña-Hidalgo et al. 2022; Råberg and Stjernman 2012; Wollein Waldetoft, Råberg, and Lood 2020).

Since parasites generally sequester resources from their hosts, many hosts have evolved ways to limit resource sequestration through metabolic or behavioural changes (Coop and Kyriazakis 1999). The mechanisms limiting exploitation have been heavily studied in recent years and have given rise to nutritional immunity (Núñez, Sakamoto, and Soares 2018; Hood and Skaar 2012; Murdoch and Skaar 2022; Sieksmeyer et al. 2022), where the main aim is to enhance host survival by manipulating the parasite's ability to exploit the host. The resources sequestered by the parasite are time- and spatial-dependent, as they depend on which stage of the host cycle and tissue they infect and have access to. Nevertheless, most parasites universally require a large portion of the resources. These can range from macromolecules, such as carbohydrates in the host diet, to minerals that can be forcefully acquired through parasite transporter proteins whose sole purpose is binding and stealing circulating molecules. Iron is perhaps the most well-studied mineral concerning infection (Cherayil 2011; Johnson and Wessling-Resnick 2012; Cassat and Skaar 2013; Iatsenko et al. 2020). The microbes' universal need for this mineral due to its structural and metabolic properties is easy to perceive when we glance over the number of taxa that have acquired or multiplied their number of genetic copies for the production of siderophores (Saha et al. 2013; Niehus et al. 2017; Franke, Ishida, and Hertweck 2014), i.e., iron-transporting molecules used to diverge iron from the host. Iron also acts as a vital signal for some parasites, namely *Plasmodium* (Ferrer et al. 2015; de Swart et al. 2023; Zhou et al. 2007; Maya-Maldonado et al. 2021). For instance, *Plasmodium falciparum* requires high iron concentrations for the fusion of gametocytes and its proper development within mosquitoes.

Despite the growing knowledge surrounding parasitic needs and ways they acquire their resources from the host, a lot is still unknown to us, particularly concerning intracellular parasites such as microsporidia. These are obligate intracellular single-celled fungi that can infect many hosts, from humans to dipteran insects (P. Keeling 2009; Wadi and Reinke 2020; Franzen 2008; Makhulu et al. 2024; Zeferino and Koella 2024; Han, Pan, and Weiss 2021). Despite their small genome (Khalaf,

Francis, and Blaxter 2024; Nakjang et al. 2013; P. J. Keeling et al. 2013), microsporidia possess a vast ecology, covering dry and aquatic environments (Becnel and Weiss 2014). Infection by microsporidia has been studied for over 150 years (Franzen 2008) , but it has recently attracted attention due to its negative impact on *Plasmodium spp.* when the two parasites co-infect an *Anopheles* mosquito (Bukhari, Pevsner, and Herren 2022; Koella, Lorenz, and Bargielowski 2009; Herren et al. 2020; Lorenz and Koella 2011; Bargielowski and Koella 2009). From this group, the microsporidian species *Vavraia culicis* and Microsporidia MB have been presented as possible malaria control agents (Bukhari, Pevsner, and Herren 2022; Koella, Saddler, and Karacs 2012), as demonstrated by experimental (Bargielowski and Koella 2009; Vyas-Patel 2023) and wild studies (Herren et al. 2020), respectively. Despite their potential use in malaria control, little is known about host exploitation and resource sequestration by this parasite. To our knowledge, only one study has partially characterised mosquito exploitation by *V. culicis* during the larval stage (Rivero et al. 2007), and one has described limited parasitic costs in proteins, carbohydrates and lipid content in adults (Zeferino, Silva, and Koella 2024).

Hence, in this study, we delved further into the mechanisms that allow the microsporidian parasite *V. culicis* to exploit one of its natural mosquito hosts, the main malaria vector, *Anopheles gambiae*. In particular, using lines of this parasite selected for early or late transmission within the host (Silva and Koella 2024b, 2024a; Silva 2024), and consequently varying in their virulence and host exploitation ability, we aimed to characterise: i) *Anopheles gambiae* exploitation by *V. culicis*; ii) understand which mechanisms can be enhanced upon selection for early or late transmission; iii) how changes in host exploitation relate to the different parasite lines' virulence. To accomplish this, we: a) quantified protein, carbohydrate and lipid content; b) performed metallomics; and c) knowing *V. culicis* load is conditioned by iron availability, we supplemented and blocked circulating iron within the mosquito to quantify the effect of iron on parasite growth. All the measurements were performed on days 5, 10, and 15 of adulthood after larval infection with the different parasite lines. We decided on these time points because they coincide with the beginning, the full and slow-down of the *V. culicis* growth phase. We believe the findings from this study are of considerable importance for microsporidia studies and disease control, as well as parasite evolution in general, as much of the machinery studied here is used and co-opted by other parasites.

Materials and Methods

Experimental model and mosquito rearing

We used 10 lines of the microsporidian parasite *Vavraia culicis floridensis* previously selected for seven generations in the mosquito *Anopheles gambiae* for early transmission and shorter time within the host, five lines selected for late transmission and longer time within the host and our unselected stock as a baseline reference (Silva and Koella 2024b). As a host, we used the Kisumu strain of the mosquito host

121 *Anopheles gambiae (s.s.)*. Further details on the selection experiment and its maintenance are described
122 in Silva and Koella (2024b). Experiments and rearing were conducted at standard laboratory conditions,
123 26 ± 1ºC, 70 ± 5% relative humidity and 12 h light/dark.

124 In each experiment, freshly hatched *A. gambiae* larvae were grown in groups of 50 per petri dish (120
125 mm diameter x 17 mm) containing 80 ml of distilled water. Larvae were fed daily with Tetramin Baby
126 fish food according to their age: 0.04, 0.06, 0.08, 0.16, 0.32 and 0.6 mg/larva for 0, 1, 2, 3, 4 and 5 day-
127 old or older larvae, respectively. Two-day-old larvae were exposed to 10,000 spores per larva of their
128 respective *V. culicis* treatment. Pupae were transferred to cages (21 x 21 x 21 cm) and left to emerge as
129 adults. For the entirety of their adult life, the mosquitoes were provided with a 6% sucrose solution up
130 until trait measurement. Males were removed as soon as they emerged to avoid mating-associated costs.

131 *Proteins, carbohydrates and lipids quantification*

132 Mosquitoes were collected 5, 10, or 15 days after emergence and frozen at -80 ºC. We quantified their
133 proteins, carbohydrates, and lipid content through colourimetry, which we adapted from (Foray et al.
134 2012). Each individual body was weighed and then submerged in a 100 µL PBS pH 7.4 solution before
135 homogenisation.

136 Proteins were quantified by centrifuging the samples at 1100 RPM at 4º C for four minutes, adding 200
137 µL Bradford reagent to each solution, incubating at room temperature with soft agitation for 20 min,
138 and reading the absorbance at 595 with a SpectraMax i3x plate reader. To quantify protein content, a
139 standard curve of seven concentrations ranging from 400 µg/ml to 10 µg/ml of albumin in extraction
140 buffer (100 mM monopotassium phosphate, 1 mM DTT, and 1 mM EDTA in Milli-Q water) was also
141 prepared.

142 Carbohydrates were measured by first adding 20 µL of sodium sulphate at 20% to the homogenate,
143 adding 5 µL of the extraction buffer and 1500 µL Chloroform-Methanol (1:2, v:v), vortexing, and
144 centrifuging at 1100 RPM, 4ºC for 15 minutes. The supernatant was then removed, of which 300 µL
145 was transferred to a 96-well borosilicate plate and left to evaporate under the hood for approximately
146 30 minutes at room temperature. 240 µL of anthrone reagent was added to each sample and incubated
147 at room temperature for 15 minutes. The plate was sealed and incubated in a water bath at 90ºC for 15
148 minutes before measuring absorbance at 625 nm. A standard curve of seven concentrations ranging
149 from 40 µg/ml to 1 µg/ml of glucose in Chloroform-Methanol (1:2, v:v) was also prepared to quantify
150 carbohydrate content.

151 Lipids were measured by adding 200 µL of the supernatant to a 96-well borosilicate plate, then heated
152 to 90 ºC for 30 minutes. 10 µL of 98% sulfuric acid was added, and the sample was sealed and incubated
153 for two minutes in a water bath at 90 ºC. The samples were transferred to ice for three minutes before

154 adding 190 µL of vanillin reagent. Samples were sealed and incubated at room temperature for 15
155 minutes with gentle agitation. Their absorbance was measured at 525 nm. A standard curve of seven
156 concentrations ranging from 200 µg/ml to 5 µg/ml of triolein in Chloroform-Methanol (1:2, v:v) was
157 also prepared.

158 To acquire individual resource content, the amount of resources measured was divided by the weight
159 of the mosquito.

160 *Metals quantification*

161 Five, 10 and 15 days after emergence, 30 alive females per parasite line were snap-frozen in a 2 ml
162 microcentrifuge tube at -80ºC. The samples were freeze-dried and submitted to a mineralisation
163 procedure using a Titan MPS microwave (Perkin Elmer, Waltham, MA). Each sample was placed into
164 a PTFE digestion vessel, and 2.5 mL HNO3 (Rotipuran Supra 69%, ROTH, Germany) and 2.5 mL
165 H2O2 (analytical reagent grade, Fisher Scientific, UK) were added. The vessels were heated following
166 a two-step digestion program consisting of a first step of 25 min until reaching 175°C and a second step
167 of 10 min at 175°C. After cooling, the digested sample solutions were transferred to 15 mL Falcon PP
168 tubes and stored in the fridge. A blank solution was prepared using the same procedure without the
169 sample. The elements iron, zinc, copper, calcium and manganese were simultaneously analysed using
170 inductively coupled plasma optical emission spectrometry (ICP-OES) (Maya-Maldonado et al. 2021).
171 An Avio 550 max equipped with a dual view and an S23 autosampler (PerkinElmer, Waltham, MA)
172 was used. The instrument was operated in axial view with the following conditions: RF power 1450 W,
173 cyclonic spray chamber with nebuliser nitrogen gas flow rate 0.7 mL/min, plasma argon gas flow rate
174 8 mL/min and auxiliary compressed air gas flow rate 0.2 mL/min. The sample was injected into the
175 plasma at 1.30 mL/min. All the calibration curves were prepared in 2% HNO3 MilliQ water (w/w) in
176 the concentration range of 0.1-10 mg/L and using SCP Science multi-element standards (SCP Science,
177 France). Yttrium (Atomic spectroscopy standard, PerkinElmer Pure, 2% HNO3, 1000 ppm) was used
178 as the internal standard and was added at a concentration of 1 mg/L in the blank standards and samples.
179 The elemental wavelengths were 238.204 nm for Fe, 206.200 nm for Zn, 327.393 nm for Cu, 317.933
180 nm for Ca, 257.610 nm for Mn and 371.029 nm for Y. Measurements were performed in triplicates.

181 *Iron metabolism assay*

182 In a separate experiment, mosquitoes were fed with daily prepared cotton pads with either: i) iron (1
183 mM $FeCl_3$ in 6% sucrose solution), ii) chelator (200 µM Bathophenanthroline disulfonic acid disodium
184 salt (BPS) in 6% sucrose solution) or iii) or sugar (6% sucrose solution) for five days after emergence.
185 From day six onwards, the mosquitoes were provided with cotton pads with only 6% sucrose solution.
186 Alive individual mosquitoes were collected for spore load quantification 5, 10, or 15 days after

emergence. They were placed individually into 2 ml microcentrifuge tubes with 1 ml of distilled water and a stainless-steel bead (Ø 5 mm, and homogenised with a Qiagen TissueLyser LT at a frequency of 30 Hz for one minute. The number of spores was counted in a haemocytometer under a phase-contrast microscope (400x magnification).

Statistical analysis

All the analyses were performed with R version 4.3.1. Statistical analysis and their diagnosis were conducted using: "DHARMa" (Hartig and Hartig 2017), "car" (Fox et al. 2012), "lme4" (Bates et al. 2009), "muhaz" (Gentleman and Winsemius 2015), "emmeans" (Lenth et al. 2019) and "multcomp" (Hothorn et al. 2016) .

First, we tested if the parasite treatment or the day of collection affected the mosquito's body weight. For this, we used a linear mixed model with log-transformed weight as a dependent variable and parasite treatment, time of collection and their interaction as explanatory variables and replicate as a random factor. Body weight was not affected by any of the factors (parasite treatment: $\chi^2 = 1.38$, $df = 2$, $p =$ 501; Time: $\chi^2 = 0.80$, $df = 1$, $p = 0.373$; Parasite treatment * Time: $\chi^2 = 1.04$, $df = 2$, $p = 0.60$).

Differences in resource content were assessed using a linear mixed model for each of the three resources (proteins, carbohydrates, and lipids), with parasite treatment and time of collection as explanatory variables and their interaction. Replicate was considered a random factor. Individual resource content was quantified by measuring the sample's quantity and adjusting it according to the respective individual weight. The square root of each resource content was calculated to fit the model best.

We ran a linear mixed model for each metal element to test for differences in the metallomes of differently infected or uninfected mosquitoes across time. The dependent variable was the log-transformed concentration of the element obtained by ICP-OES, while the parasite treatment and assay day were explanatory variables and their interaction. Replicate was included as a random factor.

We assessed the effect of the parasite treatment and diet on spore rate and growth for each time-point assayed. For each spore rate comparison, we ran a generalised linear mixed model with a binomial error structure, where parasite treatment and diet treatment were set as explanatory variables, their interaction, and the presence or absence of spores as a response variable. Replicate was included as a random factor. Concerning spore growth analyses, we only considered individuals with at least one spore. We then ran a linear mixed model with the same explanatory and random factors as the previous one.

Results

Resource and energy use in mosquitoes infected with lines of V. culicis

The protein content was affected by parasite treatment ($\chi^2 = 9.75$, $df = 2$, $p = 0.008$), time ($\chi^2 = 45.28$, $df = 1$, $p < 0.001$) and their interaction ($\chi^2 = 24.75$, $df = 2$, $p < 0.001$). In particular, protein content remained stable until day 10 of adulthood when it significantly increased for mosquitoes infected with late-selected spores (i.e., the most virulent ones) compared to the other parasite treatments.

The carbohydrate content increased with infection time ($\chi^2 = 139.65$, $df = 1$, $p < 0.001$). It was higher in mosquitoes infected by the most virulent parasites (i.e., late-selected ones) than in those infected by the unselected or the early-selected ones.

The lipid content decreased with infection time ($\chi^2 = 8.25$, $df = 1$, $p = 0.004$) irrespective of treatment, and the mosquitoes infected with selected spores (i.e., early or late) had higher lipid content than the ones infected with the unselected parasites ($\chi^2 = 6.33$, $df = 2$, $p = 0.042$).

Metallomics of mosquitoes infected with lines of V. culicis

All metals (iron, copper, zinc, manganese) and calcium concentrations in the mosquito increased with time (Fe: $\chi^2 = 18.88$, $df = 2$, $p < 0.001$; Cu: $\chi^2 = 11.93$, $df = 2$, $p = 0.003$; Zn: $\chi^2 = 18.62$, $df = 2$, $p = < 0.001$; Mn: ($\chi^2 = 17.91$, $df = 2$, $p < 0.001$; Ca: $\chi^2 = 34.08$, $df = 2$, $p < 0.001$). While iron, copper, and calcium concentrations were similar for parasite-exposed and unexposed mosquitoes and for all parasite lines (stats), the zinc concentrations were higher in infected mosquitoes, irrespective of parasite line (Zn: $\chi^2 = 13.97$, $df = 3$, $p = 0.003$) and manganese concentration was higher ($\chi^2 = 32.30$, $df = 3$, $p < 0.001$) (**Fig. 2b, e**).

Iron effect on parasite growth

In the iron supplementation or chelation experiment, all mosquitoes harboured spores by day 15. At days 5 and 10, the proportion of mosquitoes with spores was higher if the parasite had been late-seected than if it had been early-selected or not selected (day 5: $\chi^2 = 15.43$, $df = 2$, $p < 0.001$; day 10: $\chi^2 = 8.46$, $df = 2$, $p = 0.015$). On both days, iron supplementation increased while chelation decreased the spore rate (day 5: $\chi^2 = 15.43$, $df = 2$, $p < 0.001$; day 10: $\chi^2 = 8.46$, $df = 2$, $p = 0.015$). A similar pattern was observed for the spore load in individuals that harboured spores, with higher spore load after an iron-supplemented diet and lower spore load after a chelator-supplemented diet (day 5: $\chi^2 = 12.45$, $df = 2$, $p = 0.002$; day 10: $\chi^2 = 37.60$, $df = 2$, $p < 0.001$; day 15: $\chi^2 = 79.65$, $df = 2$, $p < 0.001$; **Fig. 3b**). Individuals infected with late-transmitted spores also showed the highest number of spores regardless of the time point or diet (day 5: $\chi^2 = 37.75$, $df = 2$, $p < 0.001$; day 10: $\chi^2 = 37.60$, $df = 2$, $p < 0.001$; day 15: $\chi^2 = 26.73$, $df = 2$, $p < 0.001$; **Fig. 3b**), except for time-point 15 days where infection by the unselected

parasite reached the same load as the late-transmitted spores for the iron-supplemented diet (interaction between parasite and diet treatments: $\chi^2 = 18.60$, $df = 4$, $p < 0.001$; **Fig. 3b**).

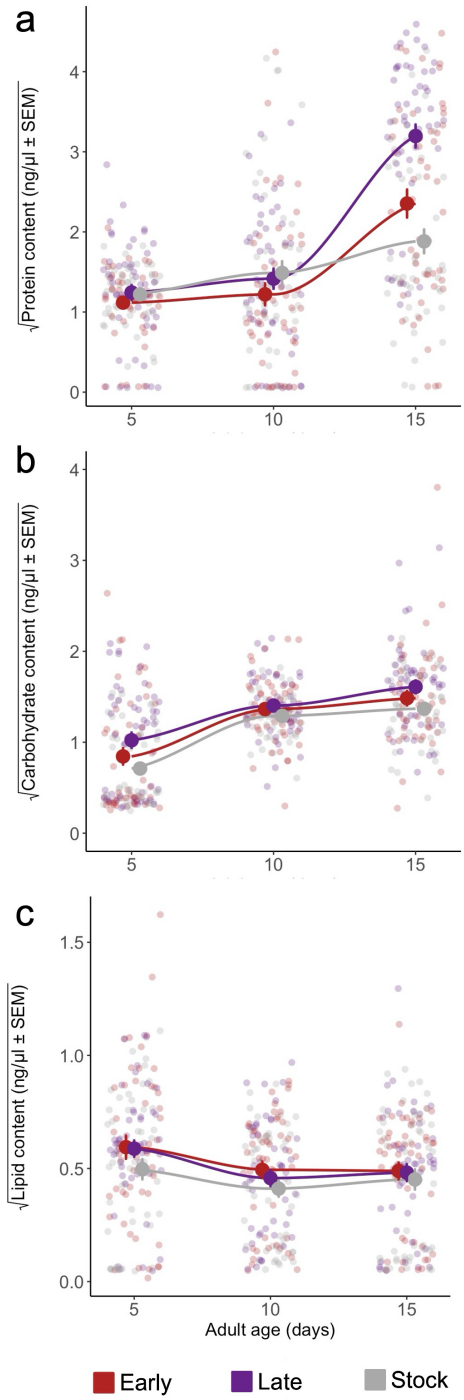
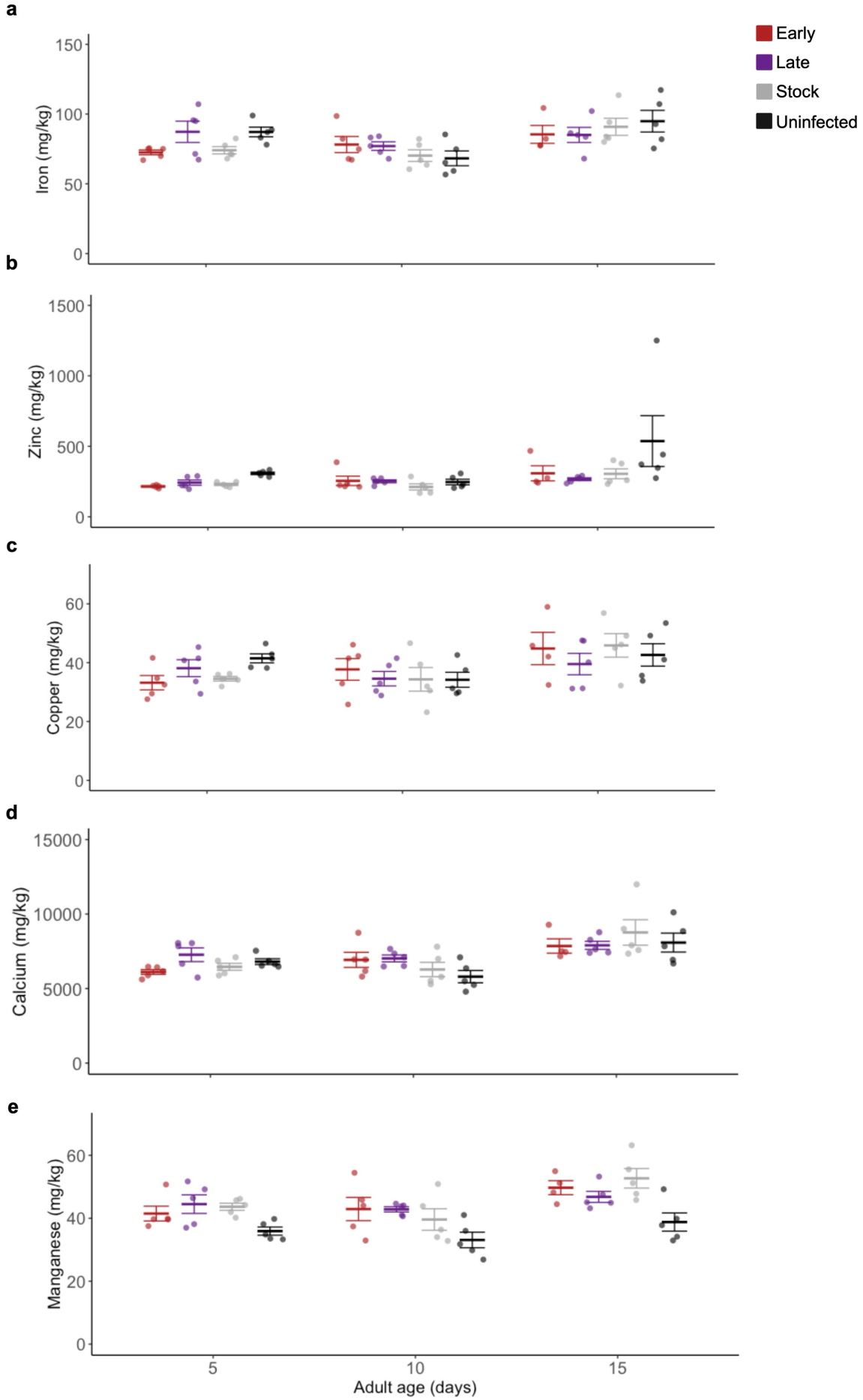


Figure 1. Mean protein, carbohydrate and lipid content through infection. Mean square root of the **(a)** protein, **(b)** carbohydrate and **(c)** lipid content at day 5, 10 and 15 of adulthood upon infection with early-, late-selected, or unselected stock parasite. Each time-point consists of individual resource content from 50 live females per parasite treatment. A smooth line with the "loess" function was added for ease of visualisation. For statistical details, see SI Appendix Table S1.


a
b
c
d
e
Early
Late
Stock
Uninfected
Iron (mg/kg)
Zinc (mg/kg)
Copper (mg/kg)
Calcium (mg/kg)
Manganese (mg/kg)
Adult age (days)
5
10
15

257

Figure 2. Metallomic profiles at adulthood. Metal concentrations determined by ICP-OES in uninfected *An. gambiae* and infected with one of three *V. culicis* parasite lines: early, late or unselected stock. Iron **(a)**, zinc **(b)**, copper **(c)**, calcium **(d),** and manganese **(e)** concentrations were quantified across adult age at days 5, 10 and 15 post emergences. For each time point, a pool of 30 females was used to assess metal concentration for each selection treatment x replicate combination (i.e., $n = 5$ data points per selection treatment). The mean and standard error of the mean were plotted. For further statistical details, see SI Appendix Table S2.

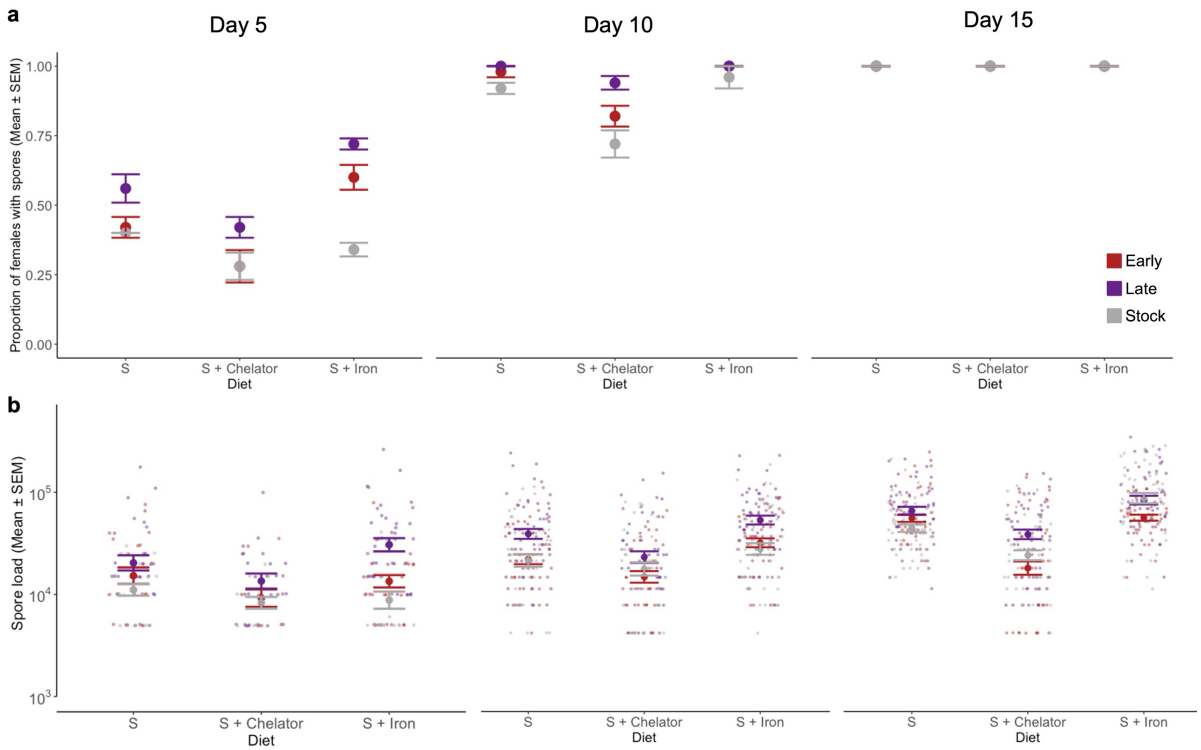


Figure 3. The effect of iron-supplementation and -chelation on parasite growth. The impact of a standard sugar diet (6% Sucrose), supplemented with an iron-chelator (6% Sucrose + 200 µl BPS) or iron itself (6% Sucrose + $FeCl_3$ 1mM) on the presence of detectable spores (i.e., the proportion of females with spores secreted) **(a)** and spore load **(b)** of differently selected, or unselected, *V. culicis* treatments. Each selection treatment has a sample size of 50 females split by five experimental replicates for each time point. For further statistical details, see SI Appendix Table S3.

Discussion

Parasites are masters at exploiting their hosts, sequestrating and manipulating the host resources for their benefit. This study shows how infection by the microsporidian parasite *V. culicis* affects host resource concentrations, particularly metals and energy markers. By using parasite lines differing in virulence, i.e., harm to the host, we could better link virulence and host exploitation. Hence, the findings

discussed below shed light on microsporidia-specific mechanisms of host exploitation that might be inherently present or selected upon certain conditions. Besides characterising these mechanisms, we also discuss them within the context in which they were differentially selected for and how that influences the role of microsporidia as biological agents in vector biology.

First, we observed that protein content in the mosquitoes increased with time, particularly between days 10 and 15 (**Fig. 1a**). To our knowledge, most immune effectors are protein-based, which might imply an immune change. Hence, if that is the case, these findings support the threshold hypothesis (Moreno-García et al. 2014), suggesting that an immune response is triggered once a certain level of parasite load is reached. This explains the delayed increase in proteins and potential antimicrobial immune effectors, such as antimicrobial peptides. Additionally, the extent of this increase varies with parasite virulence, with infection with the most virulent parasite (i.e., late-selected) showing higher host protein levels by day 15. This pattern has been noted in several studies (Acuña-Hidalgo et al. 2022; Duneau et al. 2017; Chambers et al. 2019) . It is likely related to the fact that the most virulent parasites, particularly late-selected spores, reach much higher burdens than the other treatments, as observed in our previous study (Silva and Koella 2024b). These results also underscore the ability of mosquito hosts to detect and adjust their life history investment in immunity or other fitness traits, such as fecundity, based on the virulence of the infecting parasite. For example, the late-selected spores used in this study have been shown to induce early reproduction in *An. gambiae* mosquitoes (Silva and Koella 2024b).

We measured two fundamental energetic reserves in mosquitoes: carbohydrates and lipids. Carbohydrates are typically more accessible and frequently used, whereas lipids are a last-resort energy source. We observed that lipid reserves decreased over time (**Fig. 1c**) while carbohydrate reserves increased **(Fig. 1b**). This pattern likely reflects our experimental conditions, where adult mosquitoes had ad libitum access to sucrose (a carbohydrate) as a substitute for nectar but no lipid-based diet was provided. Consequently, although both energy reserves are utilised during infection, as shown previously for this microsporidian species (Zeferino, Silva, and Koella 2024), carbohydrate reserves are replenished, unlike lipid reserves, which are steadily depleted or sequestered.

Moreover, parasite treatment affected the dynamics of these energy sources (**Fig. 1b**). Carbohydrates increased in response to the parasite's virulence. This is likely due to the host's increased energetic demand for immunity (Kay et al. 2014; Naclerio et al. 2017; Csata et al. 2024) and damage control (Mizock 1995). Additionally, *V. culicis* accesses the host's carbohydrate reserves for its own consumption early in mosquito adulthood (Zeferino, Silva, and Koella 2024). Therefore, a more virulent parasite elicits a stronger immune response from the host and produces more infective spores, demanding more energy. The proportional increase in carbohydrates with parasite virulence may reflect a host behavioural adaptation to fuel the investment in immunity.

311 Second, parasite treatment affects the baseline lipid reserves differently (**Fig. 1c**), with mosquitoes
312 infected by selected parasites exhibiting higher lipid levels. The reason for this is unclear, but e it may
313 reflect higher parasite growth in these treatments compared to the unselected stock parasite, leading to
314 greater lipid incorporation into spore production. Additionally, it is important to note that our previous
315 study demonstrated that lipids are heavily utilised by the parasite late in infection (Zeferino, Silva, and
316 Koella 2024) after day 22 of adulthood, which was beyond the scope of our current measurements.
317 Hence, lipid usage by the parasite during the time points assayed might not be strong enough yet to be
318 measurable.

319 We found some notable results when examining host metal content (**Fig. 2**), and its usage by the parasite
320 (**Fig. 3**). Among the metals measured, only zinc (**Fig. 2b**) and manganese (**Fig. 2e**) were affected by
321 the infection. Infection with any of the parasite treatments resulted in a decrease in zinc content. This
322 could be due to the heavy use of zinc by the microsporidian species or as a mechanism of immune
323 evasion (Silva 2024). Zinc is essential for parasite growth and proper metabolism (Núñez, Sakamoto,
324 and Soares 2018; P. J. Keeling et al. 2013) and is often associated with virulence factors (Vargas Rigo
325 et al. 2023; Wilson 2021; Ballou and Wilson 2016). Moreover, some fungal parasites can evade the
326 immune system by creating localised zinc deficits that impair the host's immune response (Ballou and
327 Wilson 2016; Hood and Skaar 2012; Vignesh et al. 2013; Staats et al. 2013). In any case, the selection
328 protocol did not affect any zinc-related processes, as no difference between parasite treatments was
329 observed. For manganese, we observed the opposite pattern: infections with any of the parasites led to
330 higher levels of this element (**Fig. 2e**). This might reflect a parasite response to the host immune system,
331 as many catalases in microsporidia, such as manganese superoxide dismutase, rely on manganese and
332 are crucial antioxidant enzymes for these taxa (Tamim El Jarkass and Reinke 2020; Xiang et al. 2010;
333 Wang et al. 2018). The increased manganese concentration was unrelated to the parasite's background
334 and, therefore, was not affected by its selection protocol.

335 Regarding temporal changes, all measured metals showed a slight increase over time. Although the
336 exact reason for this is unclear, metals may become more bioavailable as the infection progresses,
337 benefiting either the host or the parasite (**Fig. 2**). From the parasite's perspective, increased spore
338 production would require higher metal availability to be sequestered from the host. This sequestration
339 would increase over time, making it easier for our method to detect these metals at later stages. Further
340 studies are needed to understand and visualise metal mobilisation with a microsporidian parasite during
341 infection.

342 Our last set of results concerns iron usage for parasite growth within the host (**Fig. 3**). As previously
343 mentioned, iron plays a vital role as a nutrient for growth in several parasite species (Ferrer et al. 2015;
344 Cassat and Skaar 2013; Iatsenko et al. 2020; Maya-Maldonado et al. 2021; Bargielowski and Koella
345 2009) and is particularly abundant in mosquitoes (Whiten and Adelman 2018; Zhou et al. 2007) due to

346 their ecology. Therefore, we aimed to determine the effects of supplementing and chelating circulatory
347 iron in the mosquito's haemolymph on the growth rate of different *V. culicis* treatments (**Fig. 3**). The
348 five-day supplementation or chelation of iron resulted in an increase or decrease, respectively, of the
349 presence of infective spores in mosquitoes at day 5 and 10 (but not at day 15 when all mosquitoes
350 harboured spores (**Fig. 3a**). These observations were also reflected in the parasite burden under the two
351 diets (**Fig. 3b**). Overall, selected parasites demonstrated an enhanced ability to utilise circulatory iron
352 within mosquitoes compared to the unselected reference stock, leading to accelerated production of
353 infective spores. The differences observed in selected parasites can be attributed to an adaptation to
354 better sequester and parasite the mosquito's iron metabolism due to both selection protocols. However,
355 the extent of these differences was inversely related to parasite growth. This limitation may also explain
356 why unselected stock parasites attained higher parasite burden differences compared to selected ones
357 that already had high burdens without supplementation. This result was especially clear for day 15 (**Fig.**
358 **3**).

359 Altogether, the findings of this study complement recent research quantifying the resource and energetic
360 dynamics of the microsporidian parasite *V. culicis* upon infecting the mosquito *An. gambiae* (Zeferino,
361 Silva, and Koella 2024) while also highlighting the significance of host exploitation changes due to
362 parasite evolution. We demonstrated that iron is a crucial nutrient and that its sequestration is an easily
363 evolvable parasite trait important for its growth. Additionally, we identified general and virulence-
364 specific host responses to *V. culicis* infection, namely in protein expression and potential immune
365 effector production. Parasite-driven changes in host energy reserve usage were also observed. These
366 insights have important implications for the study of microsporidia but also for *Plasmodium spp.*
367 infection progression and control. As demonstrated, microsporidia can limit or block *Plasmodium*
368 infections in mosquitoes (Bargielowski and Koella 2009; Bukhari, Pevsner, and Herren 2022; Herren
369 et al. 2020; Koella, Lorenz, and Bargielowski 2009; Lorenz and Koella 2011), although the mechanisms
370 for this suppression remain unclear. Our results suggest this repression might occur through competition
371 for nutrients such as circulatory iron or host energetic reserves. Previous literature has shown that lipids
372 and iron are crucial for *Plasmodium* signalling, correct development and virulence upon malaria
373 infections within mammals (Maya-Maldonado et al. 2021; Costa et al. 2018; Ferrer et al. 2015;
374 Gordeuk, Thuma, and Brittenham 1994; Gwamaka et al. 2012; Portugal, Drakesmith, and Mota 2011).
375 Hence, our study significantly advances the understanding of microsporidia infections while raising
376 fundamental questions and hypotheses for correctly planning and designing malaria-control strategies.

377 **Author contributions**

LMS conceived the overall idea, JCK and LMS designed the experiments, LMS collected and analysed the data, AV performed the ICP-OES elemental analysis, and LMS wrote the first draft of the manuscript. All authors contributed critically to the drafts.

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572 # Supplementary Information

573 "Mechanisms of host exploitation by a microsporidian parasite"

574 by Luis M. Silva, Armelle Vallat and Jacob C. Koella

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576 **Table S1.** Protein, carbohydrate and lipid content through infection.

577 **Table S2.** Metallomic profiles at adulthood.

578 **Table S3.** Iron-supplementation and -chelation on parasite growth.

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Table S1. Protein, carbohydrate and lipid content through infection.

Tested effect			
Model 1a - Protein content	**χ2**	***df***	***p***
Parasite treatment	9.752	2	**0.008**
Time	45.283	1	**< 0.001**
Parasite treatment: Time	24.748	2	**< 0.001**
Model 1b - Carbohydrate content	**χ2**	***df***	***p***
Parasite treatment	16.558	2	**< 0.001**
Time	139.651	1	**< 0.001**
Parasite treatment: Time	0.644	2	0.725
Model 1c - Lipid content	**χ2**	***df***	***p***
Parasite treatment	6.333	2	**0.042**
Time	8.251	1	**0.004**
Parasite treatment: Time	0.924	2	0.630

Table S2. Metallomic profiles at adulthood.

Tested effect			
Model 2a - Iron (Fe)	**χ2**	***df***	***p***
Parasite treatment	2.258	3	0.521
Time	18.880	2	**< 0.001**
Parasite treatment: Time	10.555	6	0.103
Model 2b - Zinc (Zn)	**χ2**	***df***	***p***
Parasite treatment	13.970	3	**0.003**
Time	18.622	2	**< 0.001**
Parasite treatment: Time	8.840	6	0.183
Multiple comparisons for Model 2b	***t-ratio***	***df***	***p***
Early - Late	-0.038	43.3	1
Early - Stock	0.411	43.3	0.976
Early - Uninfected	-2.844	43.3	**0.033**
Late - Stock	0.460	43.0	0.967
Late - Uninfected	-2.868	43.0	**0.031**
Stock - Uninfected	-3.328	43.0	**0.009**
Model 2c - Copper (Cu)	**χ2**	***df***	***p***
Parasite treatment	0.663	3	0.882
Time	11.925	2	**0.003**
Parasite treatment: Time	6.333	6	0.387
Model 2d - Calcium (Ca)	**χ2**	***df***	***p***
Parasite treatment	3.190	3	0.363
Time	34.084	2	**< 0.001**
Parasite treatment: Time	10.268	6	0.114
Model 2e - Manganese (Mn)	**χ2**	***df***	***p***
Parasite treatment	32.303	3	**< 0.001**
Time	17.909	2	**< 0.001**
Parasite treatment: Time	4.655	6	0.589
Multiple comparisons for Model 2e	***t-ratio***	***df***	***p***
Early - Late	-0.127	43.3	0.999
Early - Stock	-0.205	43.3	0.997
Early - Uninfected	4.442	43.3	**< 0.001**
Late - Stock	-0.080	43.0	0.967
Late - Uninfected	4.671	43.0	**< 0.001**
Stock - Uninfected	4.752	43.0	**< 0.001**

655 **Table S3.** The effect of iron-supplementation and -chelation on parasite growth.

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Tested effect			657
Model 3a - Spore rate at day 5	**χ2**	***df***	***p***
Diet treatment	15.430	2	**< 0.001**
Parasite treatment	15.546	2	**< 0.001**
Diet treatment: Parasite treatment	4.986	4	0.289
Model 3b- Spore rate at day 10	**χ2**	***df***	***p***
Diet treatment	8.464	2	**0.015**
Parasite treatment	16.867	2	**< 0.001**
Diet treatment: Parasite treatment	0.504	4	0.973
Model 3d - Spore load at day 5	**χ2**	***df***	***p***
Diet treatment	37.751	2	**< 0.001**
Parasite treatment	12.451	2	**0.002**
Diet treatment: Parasite treatment	6.925	4	0.140
Model 3e - Spore load at day 10	**χ2**	***df***	***p***
Diet treatment	37.601	2	**< 0.001**
Parasite treatment	48.705	2	**< 0.001**
Diet treatment: Parasite treatment	3.038	4	0.552
Model 3f - Spore load at day 15	**χ2**	***df***	***p***
Diet treatment	7.271	2	**0.0264**
Parasite treatment	79.910	2	**< 0.001**
Diet treatment: Parasite treatment	18.102	4	**0.001**