Mosquitoes self-medicate according to the dynamics of a microsporidian infection

Tiago G. Zeferino[1*],

Alfonso Rojas Mora[1],

Armelle Vallat[2]

Jacob C. Koella[1]

[1] Institute of Biology, University of Neuchâtel, Rue Emile-Argand 11, 2000 Neuchâtel, Switzerland

[2] Neuchâtel Platform of Analytical Chemistry, Institute of Chemistry, University of Neuchâtel, Avenue de Bellevaux 51, 2000 Neuchâtel, Switzerland

* Corresponding author

E-mail: tiago.goncalves@unine.ch (TGZ)

ORCID

TGZ: 0009-0004-0767-5200

Abstract

Immune responses protect against the impact of infectious diseases on behaviour and other traits underlying reproductive fitness. But these responses often come with a cost. In mosquitoes, for example, some immune responses induce oxidative stress, increasing the selective pressure to manage oxidative homeostasis. One way that mosquitoes could stimulate their immune system while maintaining oxidative homeostasis is by self-medicating with appropriate, biologically active substances like nectars. We therefore compared the dietary preferences of *Anopheles gambiae* mosquitoes that were uninfected or infected with the microsporidian parasite *Vavraia culicis*. To do so, we measured the proportions of 0, 4 and 8-day-old mosquitoes feeding on sugar and on sugar supplemented with hydrogen peroxide (a prooxidant) or vitamin C (an antioxidant), and we measured the impact of these diets on oxidative homeostasis and parasite load. Uninfected mosquitoes preferred to feed on sugar without either of the supplements. One reason could be that supplementing the sugar meal with the prooxidant for seven days increased their oxidative stress. In contrast, infected mosquitoes preferred to feed on sugar supplemented with the prooxidant when they were young and increased their preference for the antioxidant as they grew older. Consuming the prooxidant for seven days decreased the parasite load, and, while infection itself increased the oxidative stress, consuming the prooxidant lowered the oxidative stress of infected mosquitoes. Finally, feeding on the antioxidant had only little influence on the parasite load or on oxidative stress. These findings suggest that mosquitoes can self-medicate by consuming nectar with appropriate levels of prooxidants and antioxidants. Since mosquitoes, including *An. gambiae*, are vectors of many vector-borne infectious diseases, our results may have important implications for public health.

Introduction

Many animals change their behaviour when they that are infected by a parasite. While this is often interpreted as manipulation by the parasite in an attempt to increase its transmission, it can also be an adaptive response of the infected animals to increase their reproductive fitness. Anti-parasitic behaviour has indeed been observed in many animals, including arthropods [1] and primates [2]. One such behaviour is self-medication, that is, the use of secondary plant chemicals or other non-nutritive substances [3], either therapeutically as a response to parasitic infection [4] or prophylactically (often in anticipation of a high parasite risk [5–8]). However, the boundaries between nutrients, medicines and toxins are flexible and are often determined solely by the ingested dose of a chemical [9].

Self-medication was already described by Aristotle in his work “Historia animalium” (384-322 B.C.) [10]. It was initially thought to require observation, learning and conscious decision-making [2,11], and thus restricted to animals with high cognitive abilities [12]. A typical example is a chimpanzee seeking medicinal plants to treat its diseases [3,13]. However, self-medication has been observed in “less intelligent

mammals" [14], but also in insects (like moths [4,15], ants [5] and fruit flies [16]), indicating innate-based medication.

Mosquitoes are intriguing species to study self-medication. As vectors carrying parasites with significant implications for human health, mosquitoes feed on nectar [17] and thereby ingest many biologically active compounds, some of which possess anti-parasitic properties [18]. In addition, the compounds can have oxidative or antioxidative properties, thus affecting the production of reactive oxygen species (i.e., ROS, metabolic by products) and the development of oxidative stress (i.e., OS) in mosquitoes [19], both of which mediate life-history traits [20,21] and the immune response [22]. Therefore, the protective mechanisms that shield mosquitoes from parasites come at a cost in the form of OS, increasing the selective pressure for them to manage their oxidative homeostasis.

Finally, the plants that mosquitoes feed on have a strong influence on the outcome of their infection by, for example, malaria parasites [23], probably because of a combination of the toxic secondary metabolites and the nutritional quality of the nectar.

Here, we tested whether the mosquito *Anopheles gambiae* engages in therapeutic self-medication after infection with the microsporidian parasite *Vavraia culicis* [24], by feeding on appropriate sugar sources. Additionally, we examined whether the mosquitoes' choice was affected by the host's age (and, thus, by the parasite's developmental stage). In particular, we compared a standard sugar source with one supplemented with either a prooxidant or an antioxidant. Furthermore, we assessed whether this behaviour adhered to all the criteria for therapeutic medication previously reported [6,25]: **(i)** the behaviour must be initiated by a parasite infection, **(ii)** it must be avoided by uninfected individual because of the associated cost,, **(iii)** it must consist on the ingestion of a chemical compound, **(iv)** the compound must be relevant in the natural environment of the host, and **(v)** the behaviour must increase the reproductive success (that is, fecundity or longevity) of infected individuals, either by killing the parasite or increasing tolerance to infection. Finally, we discussed the potential widespread nature of this behaviour, which may be more common than previously anticipated. Notably, these dietary items can be easily found in wild, and our findings carry direct public health relevance, given the mosquito's role as a vector for several infectious diseases.

Materials and Methods

Experimental system

We used the Kisumu strain of *An. gambiae s.s* [26], which had been maintained at our standard laboratory conditions (about 600 individuals per cage, constant access to 6% sucrose solution, 26 ± 1ºC, 70 ± 5% relative humidity and 12 h light/dark) for many years before the experiments.

The microsporidian *V. culicis floridensis* was provided by J.J Becnel (USDA, Gainesville, FL, USA). Originally discovered in *Aedes albopictus*, this parasite was later found to parasitize several mosquito genera, including *Aedes*, *Culex*, *Anopheles*, *Culiseta*, *Ochlerotatus* and *Orthopodomyia* [27–29]. We maintained it by alternately infecting *Ae. aegypti* and *An. gambiae* to ensure its status as a generalist parasite, as described previously [24].

V. culicis is an obligatory, intracellular parasite. Mosquitoes are infected when, as larvae, they ingest the spores along with their food. After several rounds of replication, the parasite produces new infectious spores that spread to gut and fat body cells. The spores are released to larval sites when infected larvae die, when adults die on the surface of water, or when eggs covered with spores are laid onto the surface of water.

Mosquito rearing and maintenance

Freshly hatched (0-3 hours old) larvae were individually placed into 12-well culture plates, each containing 3 ml of deionized water. The larvae received Tetramin Baby® fish food every day according to their age (0.04, 0.06, 0.08, 0.16, 0.32 and 0.6 mg/larva respectively on days 0, 1, 2, 3, 4 and 5 or older [30]).

Two-day-old larvae were exposed to either 0 or 10,000 spores of *V. culicis*. Pupae were transferred to individual 50 ml falcon tubes with approximately 10 ml of deionized water [31]. Female adults were individually moved to 150 ml cups (5.5 cm Ø x 10 cm) covered with a net. The cups contained a 50 mm Ø petri dish (to prevent mosquitoes from drowning) floating on the surface of 50 ml deionized water, along with a 10 x 7 cm rectangular filter paper to maintain humidity. The mosquitoes were provided with uncoloured cotton balls soaked in a 10% sucrose solution, which were replaced daily up to the day before the assay.

Diets

We compared three diets: 10% sucrose (referred to as sugar diet), 10 % sucrose supplemented with 8 mM of hydrogen peroxide (prooxidant diet) and 10% sucrose supplemented with 1mg/mL of ascorbic acid (antioxidant diet). The doses used for the different treatments correspond to concentrations found in nectar [32–35], and had been tested before the experiment to ensure they did not kill the mosquitoes. Redox cycles induced by hydrogen peroxide and ascorbic acid have been suggested as the most likely defence mechanism against a broad spectrum of bacteria and fungi in nectar [36,37].

Preference

The preferences of infected and uninfected mosquitoes were tested 0, 4 and 8 days after their emergence as adults. The diets were compared pairwise, that is, mosquitoes were put individually into cups with two food choices: prooxidant and sugar, antioxidant and sugar, or prooxidant and antioxidant. The

solutions were coloured with pink or green food dye (Trawosa®). An equal number of mosquitoes were tested for each diet in pink and green to prevent a possible preference for the colour from biasing the results.

The mosquitoes were starved for 12 hours before the test and were given 30 minutes to choose. They were then collected in a 2 ml Eppendorf tube and frozen at -20º C to prevent any digestion, and their choices were assessed from the colour of their abdomen observed under a dissecting scope.

On the right wing of every mosquito we measured the distance from the axillary incision to the tip [38,39] with the software ImageJ v1.54 [40]. The remaining portion of the mosquito, together with a stainless-steel bead (Ø 5 mm), was put into a 2 ml Eppendorf tube containing 0.1 ml of deionised water, and homogenised with a Qiagen TissueLyser LT at a frequency of 30 Hz for two minutes. 0.1 µl of each sample was added to a haemocytometer and the spores were counted at 400x magnification. All of the exposed mosquitoes harboured spores.

Oxidative stress

Female adults received one of the diets (uncoloured cotton balls soaked in the sugar, the prooxidant or the antioxidant solution) up to seven days after emergence. The next day, alive individuals were collected in a 2 ml Eppendorf tube, weighed, snap-frozen and then stored at -80º C until we measured oxidative stress and spore load.

We assessed oxidative stress [41,42] with a combination of the reduced (GSH) and the oxidised (GSSH) form of glutathione using an HPLC-MS. GSH neutralizes reactive oxygen species with a chemical reaction that converts it into GSSG [43]. Our three measures of oxidative stress were therefore the amount of GSH, the amount of GSSG and, the proportion of GSSG over the total glutathione (i.e., tGSH = GSH + GSSG), which is a reliable indicator of cellular oxidative stress [44]. Note that an analysis of the total glutathione content (GSH + GSSG) would be biased by the reduced form, since the concentration of GSH is usually much higher than that of GSSG; in our experiment there was a 25-fold difference between the two.

To measure GSH and GSSG [45] we kept the samples and ice. We added a stainless-steel bead (Ø 5 mm) and 80 µl of PBS (pH = 7.4) to each sample, and homogenised it with a Qiagen TissueLyser LT at 45 Hz for four minutes. The homogenates were centrifuged at 10 000 RPM at 4º C, and 12 µl of the supernatant was transferred to a 2 ml Eppendorf tube; the rest of the homogenate was stored at -20º C for later spore load quantification, as described for the experiment on preference. We added 10 µl of formic acid (1%), 181 µl of mqH_2O and 5 µl of glutathione reduced ethyl ester (800ng/ml, which serves as the internal standard; Sigma-Aldrich®), and vortexed the samples for 10 seconds. After centrifuging the samples for 15 min at 13 000 RPM at 4º C, 180 µl of the extract was pipetted into a syringe with a filter, and the extract was gently blown into a 250 µl conical glass insert placed inside a vial.

The samples were then injected into a HPLC-MS system for quantification. The amounts of GSH (Sigma-Aldrich®) and GSSG (Sigma-Aldrich®) were based on linear regression from ten calibration points (0.5–500 ng/mL). The liquid chromatography-mass spectrometry analyses were performed on a QTRAP 6500+ (Sciex) coupled to Acquity UPLC® I-class (Waters). The column used was an Acquity UPLC® HSS T3 (1.8 µm, 2.1 x 100 mm) at a temperature of 25°C. The mobile phases were milliQ water-1mM ammonium formate-0.05% formic acid (A) and acetonitrile-0.05% formic acid (B), with the following gradient elution: 100% A to 95% A in 2 min, at 4.7 min 100%B until 6.5 min, and at 6.7 min 100% A, then re-equilibration to 100% A for 4.8 min for a total run time of 11.5 minutes. The flow rate was 0.4 mL/min, with the volume injected equal to 2 µL.

With these settings, the limit of detection (LOD) for GSH was 0.07 ng/ml while the limit of quantification (LOQ) was 0.25 ng/ml. The LOD for GSSG was 0.5 ng/ml, and the LOQ was 2 ng/ml. The retention times were for GSH 2.06 min, GSSG 2.83 min and IS (GSH-ee) 3.60 min. Detection in ESI positive mode and for the quantification MRM (Multiple Reaction Monitoring) mode was used.

Table 1. MRM Transitions, Declustering Potential, Collision Energy, and Collision Cell Exit Potential for GSH, GSSG, and IS detection and quantification.

Compound	MRM transition	DP (V)	CE (V)	CXP (V)	Dwell time (msec)
GSH Quanti	308.0 → 179.0	56	17	14	30
GSH Quali	308.0 → 162.0	66	33	14	30
GSSG Quanti	613.2 → 355.1	16	33	24	30
GSSG Quali	613.2 → 484.2	21	31	12	30
IS	336.0 → 190.0	71	21	14	30

DP, declustering potential. CE, collision energy. CXP, collision cell exit potential.

Source (ESI) parameters
Ion spray voltage: 5500 V
Curtain gas: 35
Ion source gas: GS1 40 and GS2 35
Temperature: 550 °C

Statistical analyses

All analyses were conducted with the R software [46] version 4.4.2, using the packages DHARMa [47], car [48], glmmTMB [49], emmeans [50] and multcomp [51]. Significance was assessed with the "Anova" function of the "car" package [48]. We used a type III ANOVA in the case of a significant interaction and a type II ANOVA otherwise. When relevant, we performed post-hoc multiple comparisons with the package "emmeans", using the default Tukey adjustment.

The preference for a diet of each mosquito was coded as a binary response. Preference in each choice test (prooxidant vs. sugar, antioxidant vs. sugar, prooxidant vs. antioxidant) was analysed with a generalised linear model with a binomial distribution of errors, where the explanatory variables were age, infection status and their interaction. In a preliminary analysis we had included wing length and colour of the diet, but omitted both factors from the results shown here, for they explained very little of the variance.

We also considered the impact of spore load on this preference by analysing only infected individuals with a generalised linear model with a binomial distribution of errors, in which infection status was replaced by spore load, and wing length was added as a covariable. Because spore load did not affect this preference, we omitted it from the analyses and results presented here.

The GSH content was analysed with a linear model with a Gaussian distribution of the errors, and the GSSG content was analysed with a generalised linear model with negative binomial distribution of errors, both with infection status, diet and their interaction as explanatory variables, and mosquito weight as a covariable.

The ratio of GSSG to total glutathione (tGSH) was analysed with a generalised linear model with Gamma distribution of errors, where infection status, diet and their interaction, were the explanatory variables and mosquito weight was a covariable.

The presence of spores was coded as a binary response (presence or absence of detectable spores), and analysed with a generalized linear model with a binomial distribution of errors; the spore load was analysed with a generalised linear model with a quasi-Poisson distribution of errors. In both analyses the explanatory variables were diet, GSH content, GSSG, diet and their interactions with each of these variables, and weight was included as a covariable. Because we counted the number of spores in a haemocytometer containing 0.1 µl of the sample (i.e. 1/1000 of the total volume), the detection threshold was estimated to be 1000 spores.

Results

Preference

Prooxidant vs Sugar: Overall, about 56 % of the mosquitoes chose the sugar diet and 44% the prooxidant diet. Infected individuals, however, were more likely to feed on the prooxidant diet than uninfected individuals (57% vs 31%, $\chi^2 = 23.85$, df = 1, $p < 0.001$). This preference was only observed 0 days (67%) and 4 days (70%) after emergence, and decreased to 33% 8 days after emergence ($\chi^2 = 18.33$, df = 2, $p < 0.001$, **Fig. 1a**).

Antioxidant vs Sugar: 62% of the mosquitoes chose the sugar diet and 38% the antioxidant diet. Infected individuals, however, were more likely to feed on the antioxidant diet than uninfected

individuals (47% vs 29%, χ^2 = 11.54, df = 1, p < 0.001), but this preference was only observed 8 days (65%) after emergence, and increased with the age of the mosquitoes (χ^2 = 7.69, df = 2, p = 0.021, **Fig. 1b**).

Prooxidant vs Antioxidant: 51% of the mosquitoes chose the prooxidant diet and 49% the antioxidant diet. Averaged across ages, infected individuals (54%) and uninfected individuals (49%) were as likely to feed on the prooxidant (χ^2 = 0.90, df = 1, p = 0.343). Younger individuals, however showed a stronger preference for the prooxidant and lost their preference as they got older (χ^2 = 5.35, df = 2, p = 0.068, **Fig. 1c**).

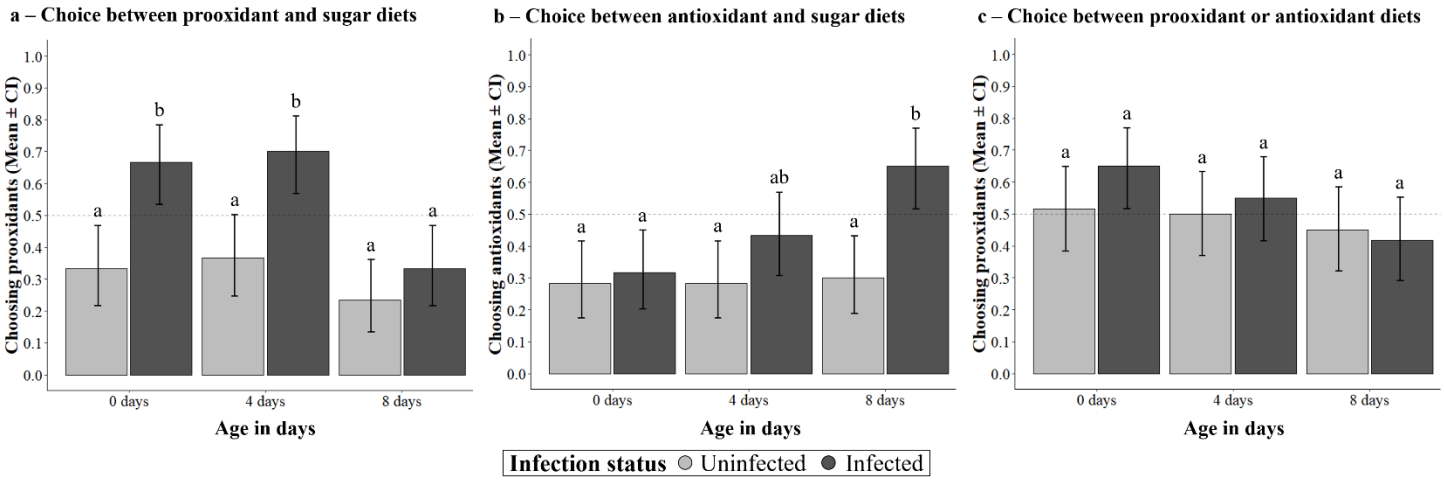

Figure 1. Food preferences of infected and uninfected mosquitoes across different age classes when given two food choices. The proportion of mosquitoes **(a)** choosing the prooxidant diet when offered a choice between prooxidant and sugar diets, **(b)** choosing the antioxidant diet when offered a choice between antioxidant and sugar diets, and, **(c)** choosing the prooxidant diet when offered a choice between prooxidant and antioxidant diets. The sample size underlying each bar was 60, and error bars show the 95% confidence intervals of the means. Letters indicate statistically significant differences from multiple comparisons within each choice.

Oxidative stress

Glutathione reduced (GSH): Infected individuals contained less GSH than uninfected individuals (1785 ng vs 1980 ng, χ^2 = 4.74, df = 1, p = 0.029), independently of the diet (χ^2 = 0.63, df = 2, p = 0.730) and the interaction between infection status and diet (χ^2 = 3.67, df = 2, p = 0.160, **Fig. 2a**).

Glutathione oxidised (GSSG): In contrast, the amount of GSSG was not affected by infection status (infected: 72 ng; uninfected: 73 ng, χ^2 = 0.32, df = 1, p = 0.570), but was greater if the diet was supplemented with either the prooxidant or the antioxidant (prooxidant: 92 ng; antioxidant: 89 ng; sugar: 46 ng; χ^2 = 101.05, df = 2, p < 0.001) and was affected by the combination of infections status and diet (χ^2 = 41.80, df = 2, p < 0.001, **Fig. 2b**). Thus, infected individuals contained more GSSG than uninfected

ones if they fed on the sugar diet (61 ng vs 36 ng), a similar GSSG if they fed on the antioxidant (93 ng vs 86 ng) and less GSSG content if they fed on the prooxidant (66 ng vs 127 ng).

GSSG/tGSH: Combining these results to consider a measure of oxidative stress - the ratio of GSSG over total glutathione – showed that oxidative stress was similar in infected an uninfected individuals (0.0458 vs 0.0364, $\chi^2 = 1.90$, df = 1, p = 0.168), that individuals taking the sugar diet had a lower level of oxidative stress than those feeding on either supplement (sugar: 0.0256; prooxidant: 0.0508; antioxidant: 0.0521; $\chi^2 = 82.53$, df = 2, $p < 0.001$) and that the effect of the diet was influenced by infection status ($\chi^2 = 35.05$, df = 2, $p < 0.001$, **Fig. 2c**). Infected individuals had a higher level of oxidative stress than uninfected individuals when consuming the sugar diet (0.0400 vs 0.0164), a similar level when consuming the antioxidant diet (0.0581 vs 0.0468), and a lower level when consuming the prooxidant diet (0.0414 vs 0.0625).

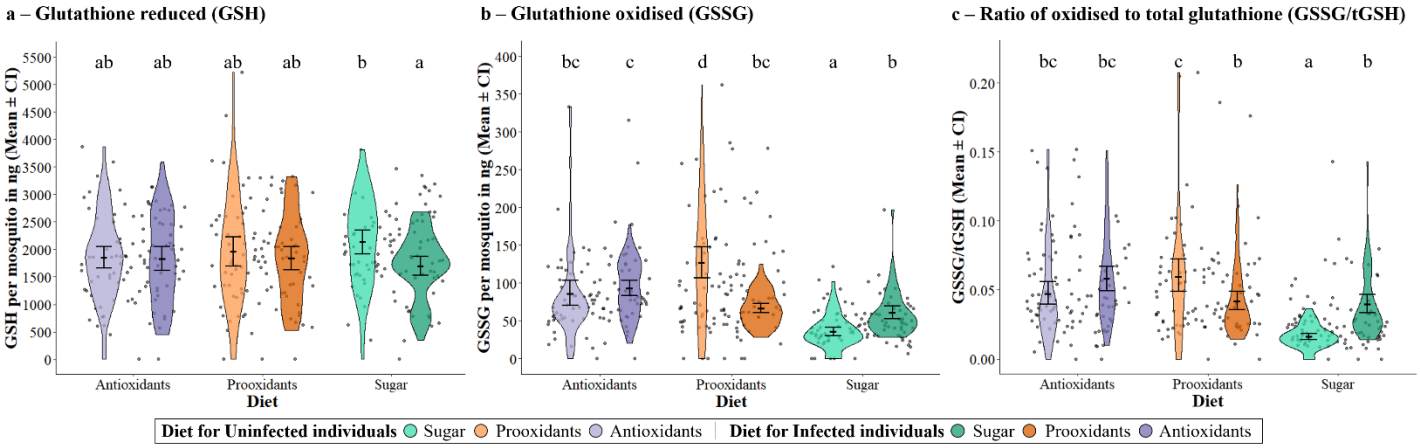

Figure 2. Oxidative stress of infected and uninfected mosquitoes after consuming a specific diet for 7 days. (a) GSH and **(b)** GSSG content in ng per mosquito. Note that the scales differ between GSH and GSSG content. The proportion of **(c)** oxidised glutathione per mosquito, calculated by dividing the GSSG content over the total glutathione (i.e., tGSH = GSH + GSSG). The sample sizes were 59, 48, 60, 50, 55 and 48. Error bars show the 95% confidence intervals of the means. Letters indicate statistically significant differences from multiple comparisons.

Spore load

All of the individuals feeding on sugar harboured spores, while only 82% were infected if they fed on the prooxidant and 87% if they fed on the antioxidant ($\chi^2 = 10.70$, df = 2, p = 0.005, **Fig. 3a**). The proportion of mosquitoes harbouring spores decreased with the amount of GSSG ($\chi^2 = 5.03$, df = 1, p = 0.025), but was not affected by the amount of GSH ($\chi^2 = 2.95$, df = 1, p = 0.086) or any of the interactions between diet, GSSG and GSH (diet * GSH content: $\chi^2 = 0.68$, df = 2, p = 0.712; diet * GSSG content: $\chi^2 = 0.06$, df = 2, p = 0.970; GSH content * GSSG content: $\chi^2 = 1.49$, df = 1, p = 0.221; diet * GSH content * GSSG content: $\chi^2 = 0.00$, df = 2, p = 0.998).

If the mosquitoes were infected the spore load was higher if they fed on sugar (8.2 x 10^4) than on either of the supplements (prooxidant: 4.5 x $10^{4;}$ antioxidant: 4.7 x 10^4; χ^2 = 8.11, df = 2, p = 0.017, **Fig. 3b**). Spore load decreased with GSH content if the mosquitoes fed on the prooxidant, but not on the other diets (diet * GSH content: χ^2 = 7.81, df = 2, p = 0.020, **Fig. 3c**), and it decreased with GSSG content if they had fed on either supplemented diet (diet * GSSG content: χ^2 = 5.78, df = 2, p = 0.050, **Fig. 3d**).

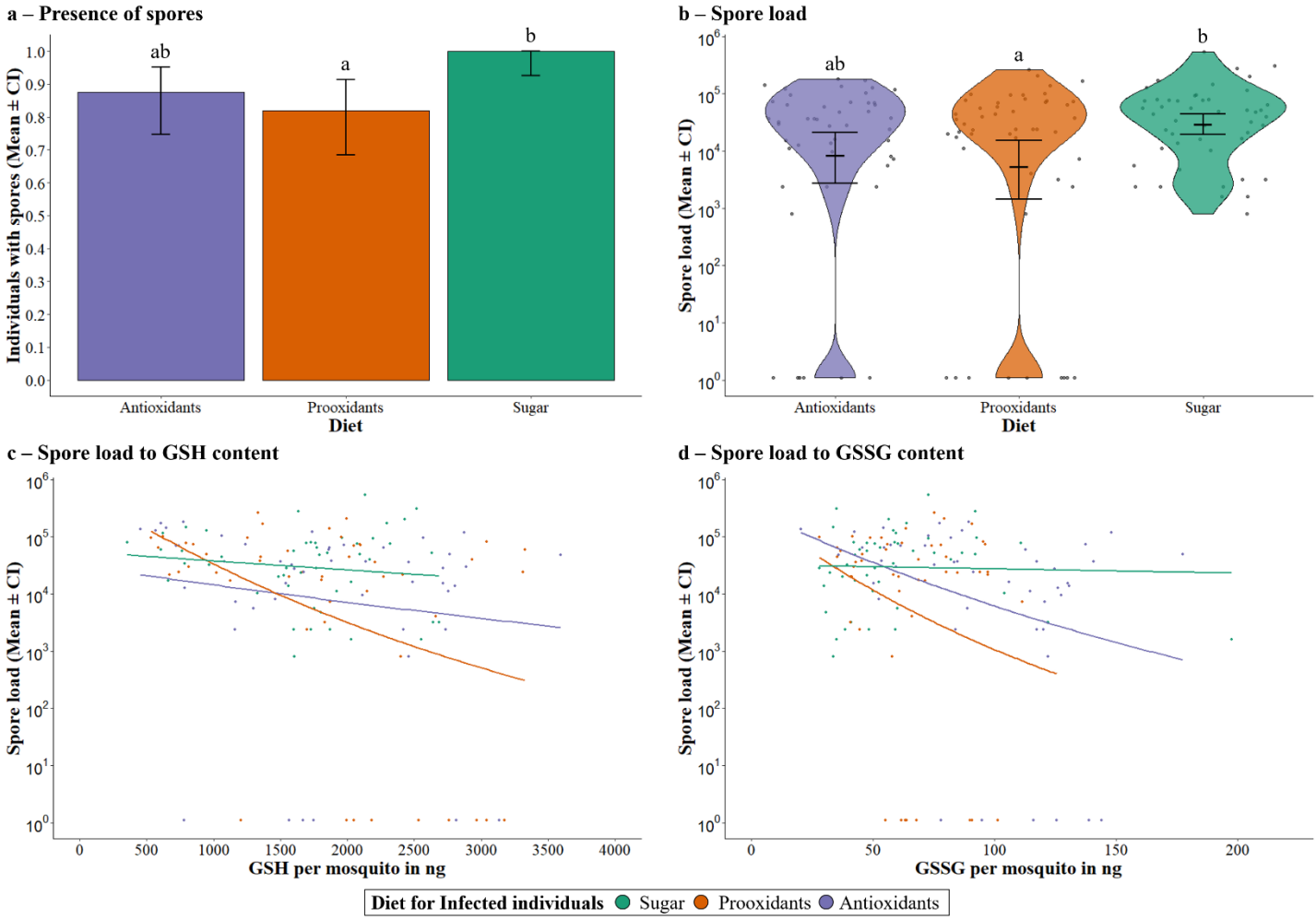

Figure 3. Parasite load of infected mosquitoes after consuming a specific diet for 7 days. The proportion of mosquitoes that had detectable spores for **(a)** each diet, and the spore load for **(b)** each diet, **(c)** GSH or **(d)** GSSG content in ng per mosquito. The sample sizes were 48, 50 and 48 and for ease of visualisation, a smooth line was drawn to show the evolution of the spore load with an increase of GSH or GSSG content, using the "glm" method, family = "quasipoisson" in ggplot2. Error bars show the 95% confidence intervals of the means. Letters indicate statistically significant differences from multiple comparisons.

Discussion

We demonstrated that infection of mosquitoes by *V. culicis* induced significant shifts in their dietary choices. These shifts disrupted the mosquitoes' oxidative homeostasis and delayed parasite

development, supporting the hypothesis of therapeutic self-medication. Importantly, uninfected mosquitoes actively avoided these diets, further reinforcing this interpretation. In the following sections, we delve deeper into our results, exploring the potential mechanisms by which these dietary changes influence infected and uninfected mosquitoes, emphasizing their effects on oxidative homeostasis. Our findings align with established criteria for therapeutic self-medication [6,52], providing strong evidence for this behaviour in *An. gambiae*. Additionally, we consider the broader epidemiological implications of this phenomenon, particularly its potential impact on disease transmission dynamics and host-parasite coevolution.

Uninfected mosquitoes preferred the sugar diet, actively avoiding both the prooxidant (**Fig. 1a**) and the antioxidant (**Fig. 1b**) diets, while displaying random preferences when the sugar diet was unavailable. This avoidance may stem from the oxidative stress induced by these diets, as our data indicate a three-fold increase in the oxidative stress marker (GSSG/tGSH ratio) with antioxidants and a four-fold increase with prooxidants (**Fig. 2c**).

Infection, however, altered mosquito's preference away from the sugar diet, with younger individuals preferring the prooxidant diet (**Fig. 1a**) but shifting this preference towards the antioxidant diet as they grew older (**Fig. 1b**). This shift could be seen as a response to the infection, as our oxidative markers highlight an infection-induced two-fold increase in oxidative stress (**Fig. 2c**). Notably, the oxidative stress levels in infected mosquitoes were comparable across all diets, suggesting that the prooxidant and antioxidant diets were no more burdensome than the sugar diet for infected individuals.

Crucially, both supplements—particularly the prooxidant one—reduced the proportion of individuals harbouring spores (**Fig. 3a**) and the parasite burden (**Fig. 3b**) by day 8, indicating a potential fitness benefit. This is significant because *V. culicis* infection is known to reduce *An. gambiae* fecundity [53]. It is also worth noting that the differences in parasite load might have been more pronounced if measured earlier, as nearly all individuals harbour spores by day 4 [24]. These findings support the hypothesis that infected mosquitoes engage in self-medication to mitigate infection impacts, although further fitness measures (e.g., longevity, fecundity, parasite load at earlier and later stages) are needed for confirmation.

Another intriguing avenue for exploration would be to investigate whether the F1 generation from infected parents might exhibit pre-emptive self-medication (prophylactic self-medication) even in the absence of infection, or if they would adopt counter-medication strategies (such as taking antioxidants if their parents used prooxidants). The latter scenario could provide insights into the possible reduction of transgenerational residual oxidative stress resulting from their parents' choices.

Hydrogen peroxide and ascorbic acid, naturally found in wild nectar [32–35], play critical roles as natural defences against a wide range of bacteria and fungi [36,37]. Here, we propose that their consumption could help mosquitoes to counteract the negative effects of parasite infection, similarly to their demonstrated

efficacy in mitigating stressors such as insecticides [54]. This behaviour may be more widespread than previously recognized and could serve as a strategy for mosquitoes to manage the oxidative imbalance caused by blood meal ingestion [55,56] and digestion [57,58], processes essential for reproduction. Consequently, mosquitoes might already be adapted to dealing with the respective oxidative imbalance.

This study highlights therapeutic self-medication as a dynamic behaviour that evolves with the progression of infection, reflecting the parasite's infection dynamics. To our knowledge, this is the first report of such adaptive dietary changes during the course of infection. Mosquitoes initially favour the prooxidant diet during the parasite's exponential growth phase (prior to day 8, [24]), potentially hindering parasite replication. Later, they switch to the antioxidant diet during the slower growth phase (after day 8, [24]), possibly enhancing their ability to tolerate the infection and mitigate its associated costs.

Interestingly, while infection altered dietary preference, the parasite burden did not influence these choices, suggesting that mosquitoes respond qualitatively rather than quantitatively to infection. When offered both diets simultaneously, we observed a gradual shift from the prooxidant diet to the antioxidant diet. This smooth transition, particularly evident in 4- and 8-day-old mosquitoes, implies that individual conditions may mediate how these diets influence tolerance [59]. Such findings underscore the complexity of dietary choices during infection, driven by both the parasite's life cycle and the mosquito's physiological state.

Our findings suggest that therapeutic self-medication in mosquitoes could diminish the efficacy of microsporidians, such as *V. culicis*, as agents of biological control. Although *V. culicis* has long been proposed as a method to slow [60] or impede [61,62] the transmission of malaria, our observations indicate that by self-medicating, mosquitoes could mitigate the fitness reduction associated with the parasite infection [24,62–64]. This adaptation could counteract the intended impact of *V. culicis* in reducing mosquito populations or impairing their ability to transmit diseases.

Moreover, this behaviour may not be specific to *V. culicis* infections. It is plausible that *An. gambiae* might exhibit similar self-medicating responses to other pathogens, such as *Plasmodium falciparum*. This potential adaptability could alter infection dynamics and significantly influence malaria transmission, with critical epidemiological consequences.

References

1. Deligne, J., Quennedey, A. & Blum, M. S. The enemies and defense mechanisms of termites. *Social insects* 2, 1–76 (1981).
2. Huffman, M. A. Self-Medicative Behavior in the African Great Apes: An Evolutionary Perspective into the Origins of Human Traditional Medicine: In addition to giving us a deeper understanding of our closest living relatives, the study of great ape self-medication provides a window into the origins of herbal medicine use by humans and promises to provide new insights into ways of treating parasite infections and other serious diseases. *Bioscience* 51, 651–661 (2001).

3. Huffman, M. A. Animal self-medication and ethno-medicine: exploration and exploitation of the medicinal properties of plants. *Proceedings of the Nutrition Society* 62, 371–381 (2003).
4. Singer, M. S., Mace, K. C. & Bernays, E. A. Self-medication as adaptive plasticity: increased ingestion of plant toxins by parasitized caterpillars. *PLoS One* 4, e4796 (2009).
5. Castella, G., Chapuisat, M. & Christe, P. Prophylaxis with resin in wood ants. *Anim Behav* 75, 1591–1596 (2008).
6. de Roode, J. C., Lefèvre, T. & Hunter, M. D. Self-medication in animals. *Science (1979)* 340, 150–151 (2013).
7. Abbott, J. Self-medication in insects: Current evidence and future perspectives. *Ecol Entomol* 39, 273–280 (2014).
8. Choisy, M. & de Roode, J. C. The ecology and evolution of animal medication: Genetically fixed response versus phenotypic plasticity. *American Naturalist* 184, (2014).
9. Raubenheimer, D., Simpson, S. J. & Mayntz, D. *Nutrition, Ecology and Nutritional Ecology: Toward an Integrated Framework*. *Ecology* vol. 23 (2009).
10. Álvaro, M. M., Luis, R. R., de Lollano, S. & Joaquin, P. The origins of zoopharmacognosy: how humans learned about self-medication from animals. *Int J Appl Res* 5, 73–79 (2019).
11. Huffman, M. A. Current evidence for self-medication in primates: a multidisciplinary perspective. *Yearb Phys Anthropol* 40, 171–200 (1997).
12. Lozano, G. A. Parasitic stress and self-medication in wild animals. *Advances in the Study of Behaviour* 27, 291–318 (1998).
13. Janzen, D. H. Complications in interpreting the chemical defenses of trees against tropical arboreal plant-eating vertebrates. *The ecology of arboreal folivores* (1978).
14. Huffman, M. A. Folklore, Animal Self-Medication, and Phytotherapy–Something Old, Something New, Something Borrowed, Some Things True. *Planta Med* (2021).
15. Singer, M. S. & Bernays, E. A. Specialized generalists: behavioral and evolutionary ecology of polyphagous woolly bear caterpillars. *Tiger moths and woolly bears: behavior, ecology, and evolution of the Arctiidae* 103–114 (2009).
16. Milan, N. F., Kacsoh, B. Z. & Schlenke, T. A. Alcohol consumption as self-medication against blood-borne parasites in the fruit fly. *Curr Biol* 22, 488–493 (2012).
17. Ode, P. J. Plant chemistry and natural enemy fitness: Effects on herbivore and natural enemy interactions. *Annual Review of Entomology* vol. 51 163–185 Preprint at https://doi.org/10.1146/annurev.ento.51.110104.151110 (2006).
18. Richardson, L. L. *et al.* Secondary metabolites in floral nectar reduce parasite infections in bumblebees. *Proceedings of the Royal Society B: Biological Sciences* 282, (2015).
19. Forrester, S. J., Kikuchi, D. S., Hernandes, M. S., Xu, Q. & Griendling, K. K. Reactive oxygen species in metabolic and inflammatory signaling. *Circ Res* 122, 877–902 (2018).
20. Dowling, D. K. & Simmons, L. W. Reactive oxygen species as universal constraints in life-history evolution. *Proceedings of the Royal Society B: Biological Sciences* 276, 1737–1745 (2009).
21. Metcalfe, N. B. & Alonso-Alvarez, C. Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. *Funct Ecol* 24, 984–996 (2010).
22. Pham-Huy, L. A., He, H. & Pham-Huy, C. Free radicals, antioxidants in disease and health. *Int J Biomed Sci* 4, 89–96 (2008).
23. Hien, D. F. d. S. *et al.* Plant-Mediated Effects on Mosquito Capacity to Transmit Human Malaria. *PLoS Pathog* 12, (2016).
24. Zeferino, T. G. & Koella, J. C. Host-specific effects of a generalist parasite of mosquitoes. *Sci Rep* 14, 18365 (2024).
25. de Roode, J. C. & Hunter, M. D. Self-medication in insects: when altered behaviors of infected insects are a defense instead of a parasite manipulation. *Current Opinion in Insect Science* vol. 33 1–6 Preprint at https://doi.org/10.1016/j.cois.2018.12.001 (2019).
26. Vulule, J. M. *et al.* Reduced susceptibility of Anopheles gambiae to permethrin associated with the use of permethrin-impregnated bednets and curtains in Kenya. *Med Vet Entomol* 8, 71–75 (1994).

27. Kelly, J. F., Anthony, D. W. & Dillard, C. R. A laboratory evaluation of the microsporidian Vavraia culicis as an agent for mosquito control. *J Invertebr Pathol* 37, 117–122 (1981).
28. Fukuda, T., Willis, O. R. & Barnard, D. R. Parasites of the Asian tiger mosquito and other container-inhabiting mosquitoes (Diptera: Culicidae) in northcentral Florida. *J Med Entomol* 34, 226–233 (1997).
29. Andreadis, T. G. Microsporidian parasites of mosquitoes. *J Am Mosq Control Assoc* 23, 3–29 (2007).
30. Kulma, K., Saddler, A. & Koella, J. C. Effects of age and larval nutrition on phenotypic expression of insecticide-resistance in Anopheles mosquitoes. *PLoS One* 8, e58322 (2013).
31. Dao, A. *et al.* Reproduction-longevity trade-off in Anopheles gambiae (Diptera: Culicidae). *J Med Entomol* 47, 769–777 (2010).
32. Santos, V. H. M. *et al.* Peptide composition, oxidative and insecticidal activities of nectar from flowers of Spathodea campanulata P. Beauv. *Ind Crops Prod* 97, 211–217 (2017).
33. Bartlett, L. J., Martinez-Mejia, C. & Delaplane, K. S. Honey Bees (Apis mellifera Hymenoptera: Apidae) Preferentially Avoid Sugar Solutions Supplemented with Field-Relevant Concentrations of Hydrogen Peroxide Despite High Tolerance Limits. *Journal of Insect Science* 22, (2022).
34. Herbert, E. W., Vanderslice, J. T. & Higgs, D. J. *Vitamin C Enhancement of Brood Rearing by Caged Honeybees Fed a Chemically Defined Diet*. *Archives of Insect Biochemistry and Physiology* vol. 2 (1985).
35. Herbert, E. W. & SHIMANUKI Bioenvironmental Bee Gaboratory, J. H. *CHEMICAL COMPOSITION AND NUTRITIVE VALUE OF BEE-COLLECTED AND BEE-STORED POLLEN*.
36. Schmitt, A., Roy, R. & Carter, C. J. Nectar antimicrobial compounds and their potential effects on pollinators. *Current opinion in insect science* vol. 44 55–63 Preprint at https://doi.org/10.1016/j.cois.2021.03.004 (2021).
37. Carter, C. & Thornburg, R. W. Is the nectar redox cycle a floral defense against microbial attack? *Trends Plant Sci* 9, 320–324 (2004).
38. Petersen, V. *et al.* Assessment of the correlation between wing size and body weight in captive Culex quinquefasciatus. *Rev Soc Bras Med Trop* 49, 508–511 (2016).
39. Koella, J. C. & Lyimo, E. O. Variability in the relationship between weight and wing length of Anopheles gambiae (Diptera: Culicidae). *J Med Entomol* 33, 261–264 (1996).
40. Abràmoff, M. D., Magalhães, P. J. & Ram, S. J. Image processing with ImageJ. *Biophotonics international* 11, 36–42 (2004).
41. Cohen, A. A. & McGraw, K. J. No simple measures for antioxidant status in birds: Complexity in inter- and intraspecific correlations among circulating antioxidant types. *Funct Ecol* 23, 310–320 (2009).
42. Monaghan, P., Metcalfe, N. B. & Torres, R. Oxidative stress as a mediator of life history trade-offs: Mechanisms, measurements and interpretation. *Ecology Letters* vol. 12 75–92 Preprint at https://doi.org/10.1111/j.1461-0248.2008.01258.x (2009).
43. Halliwell, B. & John M. C. Gutteridge. *Free Radicals in Biology and Medicine*. *Cell Biology International* (Oxford university press, USA, 2015). doi:10.1006/cbir.1999.0607.
44. Cnubben, N. H. P., Rietjens, I. M. C. M., Wortelboer, H., Van Zanden, J. & Van Bladeren, P. J. *The Interplay of Glutathione-Related Processes in Antioxidant Defense*. *Environmental Toxicology and Pharmacology* vol. 10 www.elsevier.com/locate/etap (2001).
45. Mora, A. R., Meniri, M., Glauser, G., Vallat, A. & Helfenstein, F. Badge size reflects sperm oxidative status within social groups in the house sparrow Passer domesticus. *Front Ecol Evol* 4, (2016).
46. Team, R. C. R: A language and environment for statistical com-puting. Preprint at (2017).
47. Hartig, F. DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models. Preprint at https://CRAN.R-project.org/package=DHARMa (2022).
48. Fox, J. & Weisberg, S. Using car and effects Functions in Other Functions. *Published online* 1–5 (2020).
49. Brooks, M. E. *et al.* glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *R Journal* 9, (2017).

50. Lenth, R. V. emmeans: Estimated Marginal Means, aka Least-Squares Means. Preprint at https://CRAN.R-project.org/package=emmeans (2023).
51. Hothorn, T., Bretz, F. & Westfall, P. Simultaneous inference in general parametric models. *Biom J* 50, 346–363 (2008).
52. Clayton, D. H. & Wolfe, N. D. The Adaptive Significance of Self-medication. *Trends Ecol Evol* 8, 60–63 (1993).
53. Lorenz, L. M. & Koella, J. C. The microsporidian parasite Vavraia culicis as a potential late life–acting control agent of malaria. *Evol Appl* 4, 783–790 (2011).
54. Carrasco, D. *et al.* Behavioural adaptations of mosquito vectors to insecticide control. *Current Opinion in Insect Science* vol. 34 48–54 Preprint at https://doi.org/10.1016/j.cois.2019.03.005 (2019).
55. Nouzova, M., Clifton, M. E. & Noriega, F. G. Mosquito adaptations to hematophagia impact pathogen transmission. *Current Opinion in Insect Science* vol. 34 21–26 Preprint at https://doi.org/10.1016/j.cois.2019.02.002 (2019).
56. Graça-Souza, A. V. *et al.* Adaptations against heme toxicity in blood-feeding arthropods. *Insect Biochemistry and Molecular Biology* vol. 36 322–335 Preprint at https://doi.org/10.1016/j.ibmb.2006.01.009 (2006).
57. Davenport, M. *et al.* Identification of the Aedes aegypti peritrophic matrix protein AeIMUCI as a heme-binding protein. *Biochemistry* 45, 9540–9549 (2006).
58. Oliveira, J. H. M. *et al.* Blood meal-derived heme decreases ROS levels in the midgut of Aedes aegypti and allows proliferation of intestinal microbiota. *PLoS Pathog* 7, (2011).
59. Blanchet, S., Rey, O. & Loot, G. Evidence for host variation in parasite tolerance in a wild fish population. *Evol Ecol* 24, 1129–1139 (2010).
60. UNICEF & others. *Fourth Meeting of the Scientific Working Group on Biological Control of Insect Vectors of Disease*. (1980).
61. Bargielowski, I. & Koella, J. C. A possible mechanism for the suppression of Plasmodium berghei development in the mosquito Anopheles gambiae by the microsporidian Vavraia culicis. *PLoS One* 4, e4676 (2009).
62. Koella, J. C., Lorenz, L. & Bargielowski, I. Microsporidians as evolution-proof agents of malaria control? *Adv Parasitol* 68, 315–327 (2009).
63. Bedhomme, S., Agnew, P., Sidobre, C. & Michalakis, Y. Virulence reaction norms across a food gradient. *Proc R Soc Lond B Biol Sci* 271, 739–744 (2004).
64. Michalakis, Y. *et al.* Virulence and resistance in a mosquito–microsporidium interaction. *Evol Appl* 1, 49–56 (2008).

Acknowledgements

We thank Luís M. Silva for his advice and technical support. We also thank three anonymous reviewers for their insightful and constructive comments. TGZ and the project were supported by SNF grant 310030_192786.

Author contributions

ARM conceived the overall idea. TGZ, ARM and JCK designed the experiments. TGZ collected, analysed, and interpreted the data and wrote the first draft of the manuscript. AV developed the method to analyse GSSG/tGSH ratio and analysed the samples. All authors contributed critically to the drafts.

Data availability

All data generated or analysed during this study are included as Supplementary Information files.

Additional Information

The authors declare no competing interests.