

https://dspace.org/

Generation of specific immune memory by bacterial exposure in the major malaria vector Anopheles arabiensis Diptera Culicidae

Nashrin Patel; Shune Oliver

WILEY-BLACKWELL
https://hdl.handle.net/10539/41172
SCOPUS

Downloaded from a DSpace repository. https://dspace.org/

Current Research in Insect Science 5 (2024) 100085



Contents lists available at ScienceDirect

Current Research in Insect Science

journal homepage: www.elsevier.com/locate/cris





Generation of specific immune memory by bacterial exposure in the major malaria vector *Anopheles arabiensis* (Diptera: Culicidae)

Nashrin F. Patel [a,b], Shüné V. Oliver [a,b,*]

[a] Centre for Emerging Zoonotic and Parasitic Diseases, National Institute for Communicable Diseases of the National Health Laboratory Service, Johannesburg 2192, South Africa
[b] Wits Research Institute for Malaria, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg 2193, South Africa

ARTICLE INFO

Keywords:
Priming
Immune memory
Transgenerational
Insecticide resistance

ABSTRACT

There is a growing body of evidence that invertebrates can generate improved secondary responses after a primary challenge. This immunological memory can be primed by a range of pathogens, including bacteria. The generation of immunological memory has been demonstrated in mosquitoes, with the memory primed by a range of initial stimuli. This study aimed to examine whether insecticide resistance affects the capacity to generate immunological memory. The primary hypothesis was tested by examining the capacity of genetically related laboratory-reared *Anopheles arabiensis* strains that differ by insecticide resistant phenotype to generate immunological memory. The competing hypothesis tested was that the bacterial virulence was the key determinant in generating immunological memory. Immune memory was generated in F1 females but not males. Immunological memory was demonstrated in both laboratory strains, but the efficacy differed by the insecticide resistant phenotype of the strain. An initial oral challenge provided by a blood meal resulted generated better memory than an oral challenge by sugar. The efficacy of memory generation between the two bacterial strains differed between the two mosquito strains. Regardless of the challenge, the two strains differed in their capacity to generate memory. This study therefore demonstrated that insecticide resistant phenotype affected the capacity of the two strains to generate immunological memory. Although this study needs to be replicated with wild mosquitoes, it does suggest that a potential role for insecticide resistance in the functioning of the immune system and memory generation of *An. arabiensis*.

1. Introduction

The immune system is a form of defence against the challenge of pathogens. This system is broadly divided into the innate and adaptive immune system. Adaptive immunity is defined as only occurring in jawless fishes and later, and as such is considered a vertebrate feature (as reviewed in Ghosh et al. (2011). The innate immune system, however, is present in all multicellular organisms. The innate immune system is characterised as being a non-clonal system, capable of perfect self-recognition. This is due to the capacity to recognise pathogen associated molecular patterns (PAMPS) rather than specific antigens. Crucially, however, one of the characteristics ascribed to the innate immune system is the lack of memory (Medzhitov and Janeway, 1997).

Immunological memory is defined as the ability to "store and recall previously encountered characteristics" and as such, it results in "a faster and more powerful response to subsequent antigenic exposure (Kurtz, 2005). The adaptive immune system achieves this by rearrangement of cellular receptors. As the innate immune system is a non-clonal system without generalised receptor rearrangement, it was not believed to be capable of memory generation. This was thought to be particularly true for short-lived invertebrates, which were not believed to derive any benefits from immunological memory (Ottaviani, 2011). This view, however, has been challenged, particularly in recent times.

Enhanced innate resistance to infection in plants, which was referred to as systemic acquired resistance, was described as early as the 1930s (Netea *et al.*, 2011). Understanding innate immune memory in invertebrates requires specific definitions. To be considered analogous to adaptive memory, innate immune memory needs to conform to three attributes: specific resistance, long lasting protection and a biphasic response (as reviewed in Contreras-Garduño *et al.*, 2016).

There are a range of terms associated with the immune memory that are often used interchangeably. In this study, we will be using specific terminology. The term "immune priming" has been used as a proxy for memory. In this study we will use the term priming as the event leading

* Corresponding author.

https://doi.org/10.1016/j.cris.2024.100085
Received 7 May 2022; Received in revised form 7 May 2024; Accepted 8 May 2024
Available online 9 May 2024
2666-5158/© 2024 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC license (http://creativecommons.org/licenses/by-nc/4.0/).

to the induction of memory (Boraschi and Italiani, 2018). Priming can result in an increased response after a second exposure. Where the increased response is non-specific, the process is referred to as trained immunity (Netea *et al.*, 2011). By contrast, there have also been demonstrations of examples of a specific increased secondary response. Specificity is demonstrated by an increased response after a homologous challenge (a secondary challenge with the same pathogen as the priming challenge). An increase in the secondary response to a heterologous challenge (a different secondary challenge to that of the initial priming challenge) is a hallmark of trained immunity or immune enhancement rather than immune memory (Contreras-Garduño *et al.*, 2016).

When considering immunological memory for insects in particular, there are additional considerations when looking at innate immunity in vertebrates. For insects that undergo complete metamorphosis, there is often an uncoupling of the immune system of the immature and adult stages (Fellous and Lazzaro, 2011). Therefore, significant immunological memory would need to persist across the crucial metamorphosis stage. This was first demonstrated with the beetle *Tenebrio confusum* (Thomas and Rudolf, 2010). This study demonstrated that specific immunological memory in invertebrates was not only confined to long-lived invertebrates. A later meta-analysis demonstrated that species longevity did not affect the generation of immunity (Rutkowski *et al.*, 2023).

Even though lifespan did not affect the generation of memory, it has been suggested that "short-lived species exposed continuously to the same pathogen should favour the generation of immunological memory" (Contreras-Garduño *et al.*, 2016). *Anopheles* mosquitoes would be continuously exposed to *Plasmodium* parasites through blood meals. As such, there has been examples of *Plasmodium* inducing biphasic memory in *An. gambiae* (Rodrigues *et al.*, 2010;Ramirez *et al.*, 2015). This has also been demonstrated in A*n. albimanus* (Contreras-Garduño *et al.*, 2014; Contreras-Garduño *et al.*, 2015).

There are various examples of trained immunity to bacteria in mosquitoes rather than immunological memory. Larval treatment with *E. coli, Enterobacter*, and *Staphylococcus aureus* resulted in a significant decrease in the amount of *E. coli* present in the haemocoel (Brown *et al.*, 2019). A homologous challenge was provided through injection in *An. gambiae* and a biphasic response was demonstrated (the reduction in *E. coli* after secondary challenge was higher after a secondary challenge at 3 days rather than at 1 and 7 days) (Powers *et al.*, 2020). Specific biphasic immune memory was demonstrated in *An. albimanus* by priming with *P. berghei* (Contreras-Garduño *et al.*, 2015). It is worth noting that *P. falciparum* infection in *An. colluzzi* had transgenerational costs, with the offspring of infected mothers having lower immune resistance (Vantaux *et al.*, 2014). Therefore, mosquitoes have been shown to have specific and biphasic memory. There are still questions, however, of exactly how long-lasting this memory can be.

Although the generation of both trained immunity as well as specific memory has been generated in a range of invertebrates, the mechanisms are not well understood (Netea *et al.*, 2011). As the innate immune system would not generate memory by antibodies, how would the system deal with a secondary challenge? This could broadly be done by two routes. Firstly, memory could be generated due to an increased expulsion or reduction of the pathogen. This would be referred to as immunological resistance. This would ultimately result in a reduction of the prevalence of the pathogen in the individual. By contrast, memory could potentially also be generated by the system reducing the effect of the pathogen, rather than removing the challenge. This is known as immunological tolerance. Tolerance would result in the pathogen remaining in the population and would be measured population-wide rather than in individuals. Furthermore, tolerance would result in increased prevalence of the pathogen within the population, while resistance would result in reduced prevalence within the population (Råberg, 2014). Although memory has been demonstrated in mosquitoes, there is no evidence as to whether this is modulated by resistance or tolerance. This may differ in the case of bacteria and parasites in mosquitoes.

Due to their epidemiological importance, the focus of improved immunological responses in mosquitoes has focussed on the effect of various priming events on the capacity to transmit parasites. Homologous bacterial challenges are in the minority of a relatively small pool of studies. As such, there are still many questions about immunological memory in mosquitoes. This is particularly true for less examined malaria vectors.

Members of the *An. gambiae* complex are prevalent throughout sub-Saharan Africa, the continent most affected by malaria (Sinka *et al.*, 2010). Despite their close genetic relatedness, members of this complex differ in the capacity to transmit malaria. Over and above their behavioural variation, these differences are largely underpinned by differences in their immune system (Habtewold *et al.*, 2008;Habtewold *et al.*, 2017). As such, an improved understanding of immune memory in species other than the major malaria vector *An. gambiae* and *An. colluzzi* is needed. It has been demonstrated that the dynamics of closely related *Tenebrio* species are not identical (Dhinaut *et al.*, 2018). This further highlights the importance of the gathering of information on memory generation in this species that is comparatively poorly studied.

Anopheles arabiensis is a dominant vector species throughout Africa. Although not as efficient a malaria vector as *An. gambiae*, it does provide several unique challenges. As an outdoor biting and resting species, it is not well controlled by traditional vector control methods such as indoor residual spraying and long-lasting insecticide treated nets (Kitau *et al.*, 2012). Furthermore, like with most mosquito species, insecticide resistance is a rapidly spreading challenge that confounds vector control operations (Paine and Brooke, 2016).

Insecticide resistance is known to have a range of pleiotropic effects. Yet, despite the widespread prevalence of insecticide resistance, the effect of resistance profile on immunity is poorly understood. This is potentially due to the difference of the expression of immunity in laboratory and wild populations (Cornet *et al.*, 2013;Vézilier *et al.*, 2013). The immune system is one of the factors that is poorly represented in laboratory strains. The immune response is often exaggerated in laboratory strains (Cornet *et al.*, 2013). This may possibly be due to the absence of natural challenges allowing the greater allocation of resources to the immune system. Finding fully insecticide susceptible and resistant populations in the wild is challenging. As such, making comparisons between insecticide resistant and susceptible individuals difficult. Furthermore, it would be difficult to attribute changes observed solely to the insecticide resistant phenotype without a shared genetic background. This could potentially explain the paucity of information on the subject.

The primary hypothesis of this study is that immunological memory that is generated in *An. arabiensis* is affected by insecticide resistant phenotype. This hypothesis predicts that the generation of memory will not be generated equally well in insecticide resistant and susceptible individuals. As such, if this hypothesis is correct, the most consistent modulator of the generation of memory would be the resistant phenotype.

The competing hypothesis is that bacterial virulence affects the generation of immune memory. In this hypothesis, the generation of memory is not equal between different pathogens. In all the experiments, a representative Gram-positive and Gram-negative bacterium was used in this experiment. *Streptococcus pyogenes* (Gram-positive) and *E. coli* (Gram-negative) were tested as these bacterial species were not found as commensal bacteria in the laboratory strains used in this study (see (Singh *et al.*, 2022).

Therefore, in this study we will examine the generation of immune memory in two laboratory strains of *An. arabiensis* that differ in insecticide resistant phenotype. To determine whether the primary or competing hypothesis is correct, we will determine whether the memory generated differs more by insecticide resistant phenotype (primary hypothesis) or whether a specific bacterial strain induces memory more efficiently than the other (competing hypothesis).

2. Methods

2.1. Study hypothesis and summary of methods

2.2. Materials

Two laboratory strains of *An. arabiensis* were used in this study. The SENN strain was colonized from Gezira, Sudan in 1979 (Hemingway, 1983) . From this strain, the SENN-DDT strain was selected by continuous exposure to DDT. This DDT selection continues to the present day. The strain currently displays resistance to DDT, pyrethroids and malathion. The resistance is mediated by increased cytochrome P450, general esterase and Glutathione S-transferase activity as well as fixation of the L1014-F knock down resistance mutations (Oliver and Brooke, 2013;2017). Wild material was represented by the F1 progeny of wild isofemale lines.

The strains were housed in the Botha de Meillon insectary, Johannesburg, South Africa. Strains are reared as per (Hunt *et al.*, 2005). In brief, larvae were fed on a diet of Beano™ dog biscuits and yeast (3:1). Adults were housed at 25 °C (±2 °C) and 80 % humidity (±5 %). Adults were also kept at a 12:12 hour photoperiod with a 30-minute dusk/dawn cycle.

The bacterial species used were based on previous studies on bacteria and the strains used as per (Barnard *et al.*, 2019). In all cases bacterial exposure is done with *Streptococcus pyogenes* (Gram-positive: ATCC 19, 615) or *E. coli* (Gram-negative: ATCC 25,922). Exposure was only ever with a single bacterial species.

2.3. Generation of memory in F1 An. arabiensis

Wild samples of *An. arabiensis* were collected from KwaZulu-Natal were used to generate F1 offspring. The samples were collected between December 2019 and March 2020 from a site at S 27°23′50.5″; E 032° 12′20.1″. Upon confirmation of species identity, F1 *An. arabiensis* larvae were exposed to either *S. pyogenes* or *E. coli* first instar larvae (<24 h) in 1000 ml of water supplemented with an initial inoculum 2 ml of bacteria (either *S. pyogenes* or *E. coli*) per litre where the stock culture had a concentration of 6.0×10^7 CFU/ml. A control group consisted of 1000 first instar larvae reared in untreated water. The larvae were reared under standard larval conditions, with both cohorts fed equal amounts of food. Larvae were reared under standard insectary conditions, and adults were collected. Adults were split into three groups and challenged with either *S. pyogenes* or *E. coli* sugar. A 1:1 dilution of sugar and bacteria served as the bacterial challenge. This is based on a starting concentration of 6.0×10^7 CFU/ml. This served as a homologous challenge, with the bacterial challenge matching the bacteria they were reared in. This bacterial and sugar solution was provided as a sole carbon source, and the mixture was changed twice a week. Control adults were exposed to sugar only. Longevity was assessed daily until all adults were dead. Cadavers were removed daily. A total of 150 females and 158 males were exposed to *S. pyogenes* and 103 females and 110 males to *E. coli*.

2.4. Primary hypothesis

For this hypothesis, the effect of an initial immune challenge in the form larval exposure (larval priming) on the generation of immune memory on the two laboratory strains was tested. This was examined on a challenged first generation and an unchallenged second generation.

2.4.1. Establishment of transgenerational larval priming cohorts

A first-generation primed cohort of SENN and SENN-DDT adults were established by rearing 1000 first instar larvae (<24 h) in 1000 ml of water supplemented with an initial inoculum 2 ml of bacteria (either *S. pyogenes* or *E. coli*) per litre where the stock culture had a concentration of 6.0×10^7 CFU/ml. A control group consisted of 1000 first instar larvae reared in untreated water. The larvae were reared under standard larval conditions, with both cohorts fed equal amounts of food. Upon emergence, the adults were split into two groups, the first being used for subsequent first-generation experiments. The second group was used to generate larvae for the second-generation experiments. The second generation was generated by allowing ovipositioning after two Guinea pig-derived blood-meals at age 3 and 7 days. The second generation of both groups were reared in untreated water, and as such the second generation was not exposed to the bacterial stressors until adulthood.

2.4.2. The role of insecticide resistance in the capacity to reduce colonisation by a non-commensal bacterium

The capacity of first-generation adults primed by larval exposure and second-generation adults not challenged as larvae to eliminate exogenous bacteria was assessed. *E. coli* was chosen as the exogenous bacteria as it was not previously identified in the gut of either of these strains (e.g., Singh et al. (2022). Furthermore, a commercially available chromogenic agar (Diagnostic Media Products: South Africa) allowed simple discrimination of this species. Fifty SENN and SENN-DDT F1 and F2 females were provided with *E. coli* sugar treated water (1:1 bacterial stock: 10 % sucrose) upon emergence. Fifty F1 and F2 SENN and SENN-DDT adult females provided with untreated sugar constituted untreated primed controls. The experiment was replicated with unprimed mosquitoes, and this constituted the control cohort. After three days, the adult midguts were extracted using sterilised forceps under a stereomicroscope (Olympus SZ61:Wirsam Scientific) in replicates of six with five midguts per replicate. This accounted for a total of 25 mosquitoes per replicate and the study was replicated three times ($n = 75$). After extraction, the guts were stored in 500 μl of 0.1 M phosphate-buffered saline (PBS) pH 7.4. Two ml of lysogeny (LB) broth was added to each of these preparations and incubated overnight at 37 °C. The bacterial culture was diluted 10^6 times in PBS. From this dilution, 500 μl of the bacterial culture was plated on chromogenic UTI agar (Diagnostic Media Products, South Africa) plates in duplicate. These plates provide a distinction between the *E. coli* colonies and other bacterial colonies, as the *E. coli* colonies appear pink. *Enteroccoci* would appear as blue colonies, *Coliforms* as purple, *Proteus* as brown and *Staphylococci* as unpigmented. The number of *E. coli* colonies from the primed groups that grew after an overnight culture were counted. From this data, the colony forming units were calculated.

2.4.3. The role of insecticide resistance in the transgenerational capacity to survive continuous oral homologous challenge

F1 and F2 SENN and SENN-DDT adults were generated for both Gram-positive and Gram-negative challenge. For both the F1 and F2 cohorts of the SENN and SENN DDT strain, 100 females were collected upon emergence. These females were split into two groups, with first group exposed to a bacterial challenge, and the second to untreated sucrose only. The first group were exposed to the same bacteria as their F1 parents were initially primed with in a 1:1 bacterial stock: 10% sucrose solution. This bacterial challenge was presented as the sole carbon source, with bacterial sugar as well as the untreated changed twice a week. Longevity was monitored until all individuals were dead with cadavers removed daily. This experiment was replicated three times.

2.5. Competing hypothesis

The competing hypothesis is that bacterial virulence, rather than the insecticide resistant status of the mosquito is the major factor affecting the generation of memory. To test this hypothesis, the two bacterial strains were tested by provision of a homologous challenge through two different oral routes. If this hypothesis is correct, there should be a consistent induction of improved memory by one of the strains,

regardless of the type of challenge. This should be consistent in both mosquito strains.

2.5.1. Exposure by infected sugar meal

An initial pilot study demonstrated that for both bacterial strains, a 1:1 (50 %) dilution of bacteria to 10 % sucrose was a concentration that served as a good stressor. Undiluted bacteria resulted in complete death in under 72 h. A 1:4 (25 %) bacteria to sucrose dilution did not result in a significant change in longevity. As such the 1:1 dilution served as the bacterial challenge. This is based on a starting concentration of 6.0×10^7 CFU/ml. The initial culture was seeded by a single bacteria-impregnated bead in 250 ml of lysogeny broth (LB broth). This was cultured at 37 °C with 150 rpm shaking. The liquid culture was grown for 16 h, and the concentration was determined by preparing dilutions, quantified on LB agar. This data was used to standardise concentration.

To assess the effect of an early-life exposure of bacteria by oral exposure, SENN and SENN-DDT were collected upon emergence and provided with a 1:1 bacteria: 10 % sucrose dilution for 72 h. After 72 h, 50 males and 50 female survivors were kept subsisting on 10 % sucrose only until the mosquitoes reached 11 days of age. The age of 11 days was chosen as this age coincides with the age that a female anopheline that had taken an infected blood meal would start being infective. The immune response of an older mosquito is therefore of epidemiological relevance. It must be noted that survivors of the initial exposure were used as approximately 50 % of starting mosquitoes did not survive the initial exposure. Using the survivors means that the exposed mosquitoes have been primed for a second exposure with the same bacteria.

At 11 days, the mosquitoes were provided with a 1:1 bacteria: sucrose solution as their carbon source for the remainder of their life. This served as the immune challenge. Mosquitoes were monitored daily until all the individuals were dead. Cadavers were removed daily. Mosquitoes that were not initially exposed to bacteria constituted unprimed controls. For both primed and unprimed treatments, specimens exposed to sugar without bacteria constituted untreated controls. This experiment was replicated three times from three different egg batches arising from multiple females from the laboratory strains. For all experiments involving infected sugar as a bacterial challenge, when the challenge was happening to the experimental group, the untreated control was provided with 10 % sucrose prepared as 1:1 dilution with uninoculated LB broth. This was to ensure that the presence of the broth in the carbon source did not alter the longevity of the unchallenged individuals.

2.5.2. Exposure by infected blood meal

Adult male and female mosquitoes from the SENN and SENN DDT strains were collected upon emergence. The males and females were maintained together to allow mating. The mosquitoes were maintained on ad libitum access to 10 % sucrose for three days. Prior to feeding with the bacteria-infected blood, the 3-day-old mosquitoes were deprived of sucrose for three hours to stimulate subsequent blood feeding. After three hours, mosquitoes were provided with cow blood using a Haemotek™ feeder. The sterilised, defibrinated blood was supplemented with 5 ml of bacteria in 15 ml of blood, where the stock culture had a concentration of 6.0×10^7 CFU/ml. An equivalent group was fed untreated blood. After feeding, fully fed females were separated from unfed females to ensure that all samples used subsequently would have received an initial blood meal. At the age of 11 days, the group fed infected blood (the primed cohort) and the group fed uninfected blood (the unprimed cohort) were split into three cohorts consisting of fifty females. The first cohort was fed a meal of infected blood corresponding to the initial treatment at the same bacterial concentration. The second cohort was fed untreated blood. This was to control for the effect of blood on longevity, as blood feeding has previously been demonstrated to increase longevity in the two strains (Oliver and Brooke, 2016). The final cohort was not provided with a second blood meal, but sugar only. This was to establish baseline longevity. Blood feeding was continued until the females were fed to repletion, and any females that had not fed were removed from the cage. Within 72 h of the feeding, the females were allowed to oviposit. For the remainder of the females' lives, they were only allowed access to untreated 10 % sucrose, with cadavers removed.

2.6. Data analysis

The response to bacterial challenge was assessed as changes in longevity. Longevity was assessed using a Kaplan-Meier estimator with a Log-rank test as a measure of significance (Kaplan and Meier, 1958). Analysis of differences in means were first tested for normality by the Shapiro-Wilk test (Shapiro and Wilk, 1965). Non-parametric data was analysed using a Kruskal-Wallis one-way Analysis of Variance (ANOVA) (Kruskal and Wallis, 1952) or a Mann-Whitney test (Mann and Whitney, 1947). Parametric data was analysed using One-way ANOVA (Tukey, 1949). Bartlett's test was used to assess homogeneity of variance (Bartlett and Fowler, 1937). Data was analysed using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com).

3. Results

3.1. Larval bacterial exposure increased longevity with continuous homologous challenge in F1 An. arabiensis adults

For all the longevity data, day 0 is counted as the day of emergence, rather than age of exposure. Therefore, all figures reported are a representation of total lifespan of the mosquito.

The first result represents the generation of memory in wild populations of *An. arabiensis*. This was done by examining longevity with continuous homologous challenge in F1 adults eclosing from larvae reared in bacteria-supplemented water. *Streptococcus pyogenes* (Gram-positive) larval priming resulted in a significant increase in longevity when continuously exposed to the same bacteria compared to females that were unprimed. The exposed cohorts lived significantly shorter than their unchallenged counterparts, with the priming resulting in a significant decrease in longevity even when unchallenged (Log-rank test: $p < 0.01$, χ^2=131.5, df= 3) (Fig. 1A). This was not true for primed males. Although the primed and exposed males lived significantly shorter than untreated controls ($p < 0.01$, χ^2=109.8 df= 3), there was no significant difference between primed and unprimed males when challenged with *S. pyogenes* ($p = 0.06$, χ^2=3.42, df=1) (Fig. 1B). The same was true for *E. coli* (Gram-negative) primed females, with the primed females living significantly longer when exposed to bacteria ($p < 0.01$, χ^2=103.45, df= 3) (Fig. 1C). Similarly, untreated control males lived significantly longer than treated unprimed as well as primed males regardless of treatment ($p < 0.01$, χ^2=103.4, df= 3). However, there was no significant difference in longevity between primed and unprimed *E. coli* challenged males ($p = 0.09$, χ^2=3.12, df= 1) (Fig. 1D).

3.2. Primary hypothesis

3.2.1. Larval bacterial priming increases the capacity to reduces the amount of viable bacteria recoverable across generations after homologous challenge and is affected by insecticide resistance phenotype

Larvae reared in *E. coli*-supplemented laboratory strains constituted an F1 group. Their offspring that were not reared with bacteria and constituted the F2 group. Both of these groups were provided with an oral sugar secondary homologous challenge. The amount of viable *E. coli* CFUs recovered from both groups were determined. As with previous studies, no viable *E. coli* was not recoverable from either F1 strain without treatment (Singh *et al.*, 2022). In the SENN strain, there was a significant decrease in number of viable *E. coli* recovered in the primed compared to the unprimed treated cohorts in both the F1 and F2 generation (Kruskal Wallis: $p < 0.01$, χ^2= 13.83) (Fig. 2A). This was also true for the SENN-DDT strain ($p < 0.01$, χ^2= 23.08) (Fig. 2B). Without

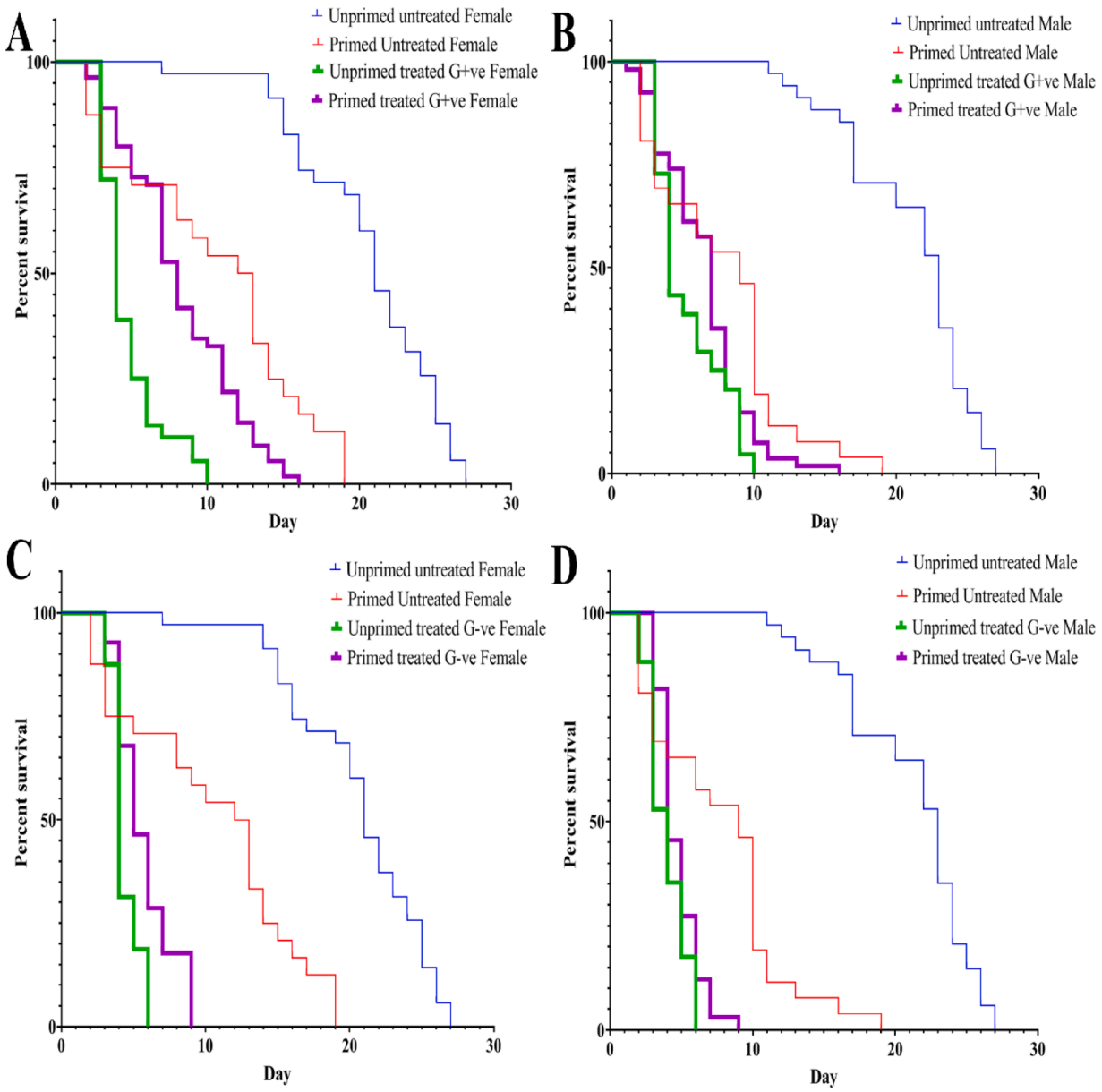


Fig. 1. The effect of larval priming on adult longevity in F1 *An. arabiensis* continuously exposed to bacteria in sugar. A: Longevity of F1 females exposed to *S. pyogenes*. B: Longevity of F1 males exposed to *S. pyogenes*. C: Longevity of F1 females exposed to *E. coli*. D: Longevity of F1 males exposed to *E. coli*. Thick purple lines represent longevity of primed individuals exposed to bacteria. Thick green lines represent longevity of unprimed individuals exposed to bacteria. Thick lines represent adults exposed to bacteria.

priming, treated SENN had more viable colonies recovered than SENN-DDT in the F1 group (Mann-Whitney test: $p = 0.02$, $U = 20$) as well as the F2 group ($p < 0.01$, $U = 10$). After priming, this difference was not evident in the F1 strains ($p = 0.82$, $U = 47.50$) or the F2 strains ($p = 0.95$, $U = 48.50$).

3.2.2. Larval exposure to bacteria results in increased longevity with continuous secondary homologous challenge in the first generation but not always the second generation

As with the previous experiment, an F1 and F2 laboratory group were established. However, this was for both Gram-postive and Gram-negative bacteria. The longevity of the adults were assessed with continuous oral homologous challenge. Larval exposure to bacteria increased the longevity in adults when the adults were exposed continuously to the same bacteria in the first generation. Primed SENN had increased longevity than the unprimed SENN when continuously exposed to Gram-positive challenge (Log-rank test: $p < 0.01$, $\chi^2 = 104.6$, df=3) (Fig. 3A). This was also true for Gram negative challenge ($p < 0.01$, $\chi^2 = 412.5$, df=3) (Fig. 3B).

These findings were also found in the SENN-DDT strain. Primed SENN-DDT had increased longevity than the unprimed SENN-DDT when continuously exposed to Gram-positive challenge ($p < 0.01$, $\chi^2 = 231.7$, df=3) (Fig. 3C). This was also true for Gram negative challenge ($p < 0.01$, $\chi^2 = 71.78$, df=3) (Fig. 3D).

When comparing the longevity of primed challenged Gram-positive F1 SENN and F1 SENN-DDT, the insecticide resistant strain lived longer than the susceptible strain ($p < 0.01$, $\chi^2 = 29.34$, df=1). This difference was not present in the Gram-negative challenge, where there was no difference in the longevity of treated primed F1 SENN and SENN-DDT ($p = 0.05$, $\chi^2 = 3.79$, df=1).

In the F2 generation where the larvae had not been exposed to bacteria, primed SENN lived longer when exposed to Gram-positive challenge compared to their unprimed counterparts ($p < 0.01$, $\chi^2 = 113.4$, df=3) (Fig. 3E). This was also true for Gram-negative challenge (p

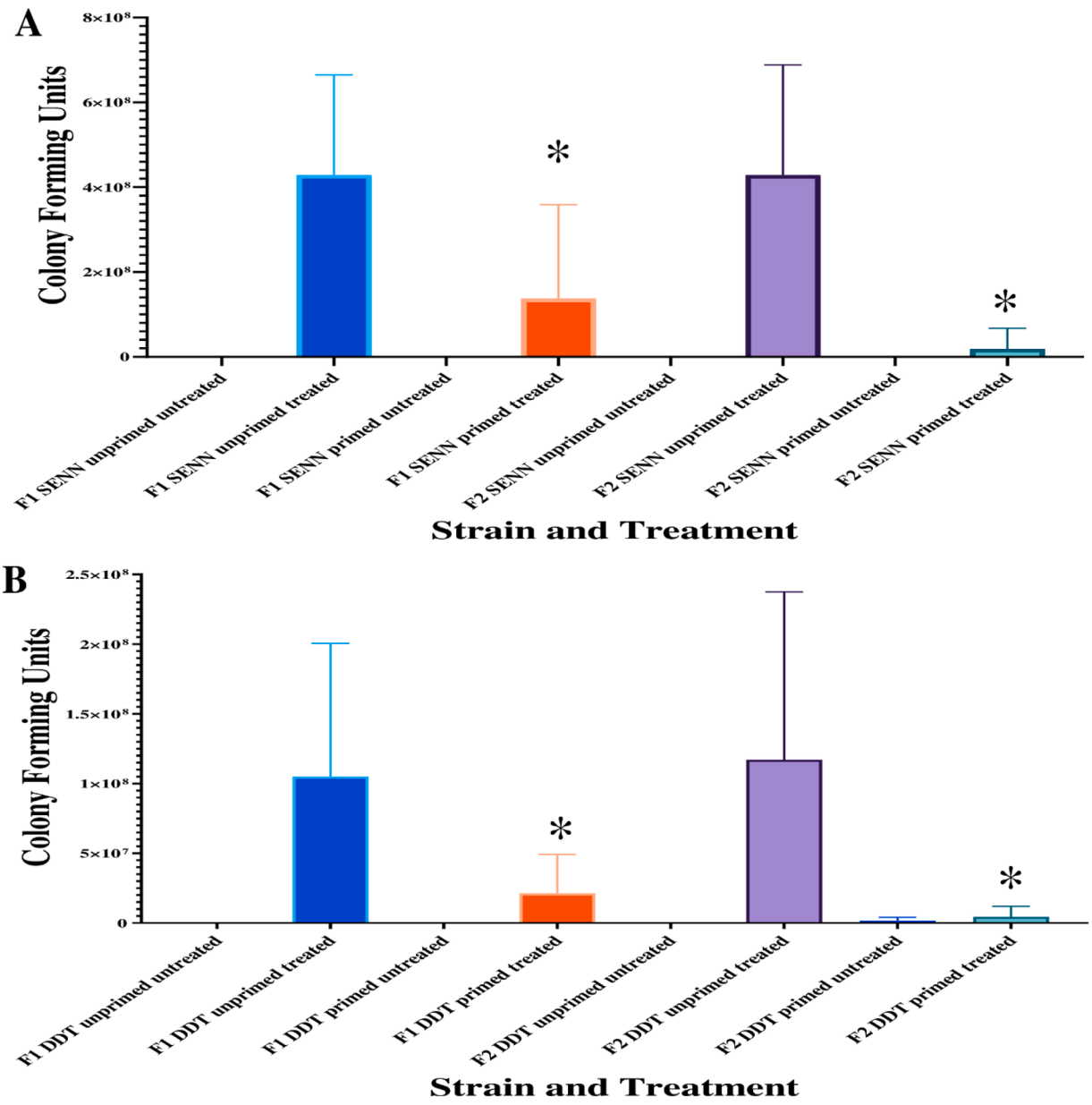


Fig. 2. Transgenerational effects of larval priming on the capacity to recover viable *E. coli*. A: Total colony forming units (CFU) of *E. coli* recovered from the guts of unprimed and primed SENN exposed using bacterial supplemented sugar as well as that of unprimed in the F1 and F2 generation. B: Total colony forming units (CFU) of *E. coli* recovered from the guts of unprimed and primed SENN-DDT exposed using bacterial supplemented sugar as well as that of unprimed in the F1 and F2 generation. Asterisks indicate significant differences from treated, unprimed controls.

< 0.01, $\chi^2 = 43.56$, df=3) (Fig. 3F).

By contrast F2 SENN-DDT that are the offspring of primed adults lived significantly shorter than their unprimed counterparts when continuously challenged by Gram-positive bacteria ($p < 0.01$, $\chi^2 = 167.5$, df=3) (Fig. 3G). This was also true for Gram-negative treatment ($p < 0.01$, $\chi^2 = 78.31$, df=3) (Fig. 3H).

3.3. Competing hypothesis

3.3.1. An oral challenge provided through sugar did not consistently induce memory

In this experiment, an initial 3-day bacterial sugar challenge served as the priming event. The secondary homologous challenge was provided from day 11, and longevity was measured. The initial bacterial priming did result in mortality, with up to 50 % of the starting population dying with the first 3-day exposure. If the initial exposure resulted in an over 50 % mortality, the experiment was not continued.

The SENN and SENN-DDT strains did not have the same longevity when continuously exposed to the same bacteria later in life. There was also a difference in response to Gram-positive and Gram-negative. For females, priming with an initial Gram-positive sugar challenge did not change subsequent tolerance to the bacterium in SENN (Log rank test: $p = 0.69$, χ^2=2.71, df=1). There was also no difference in longevity in unexposed adults in primed and unprimed females ($p = 0.10$, χ^2=0.22, df=1) (Fig. 4A).

For Gram-negative challenge, initial priming resulted in female SENN living significantly longer when continuously challenged by the same bacterium (Log rank test: $p < 0.01$, χ^2=17.47, df=1). Like with the Gram-negative exposure, there was no difference in the longevity of longevity of unexposed females, regardless of whether they were primed or unprimed ($p = 0.24$, χ^2=1.37, df=1) (Fig. 4B).

The subsequent adult longevity when continuously challenged by

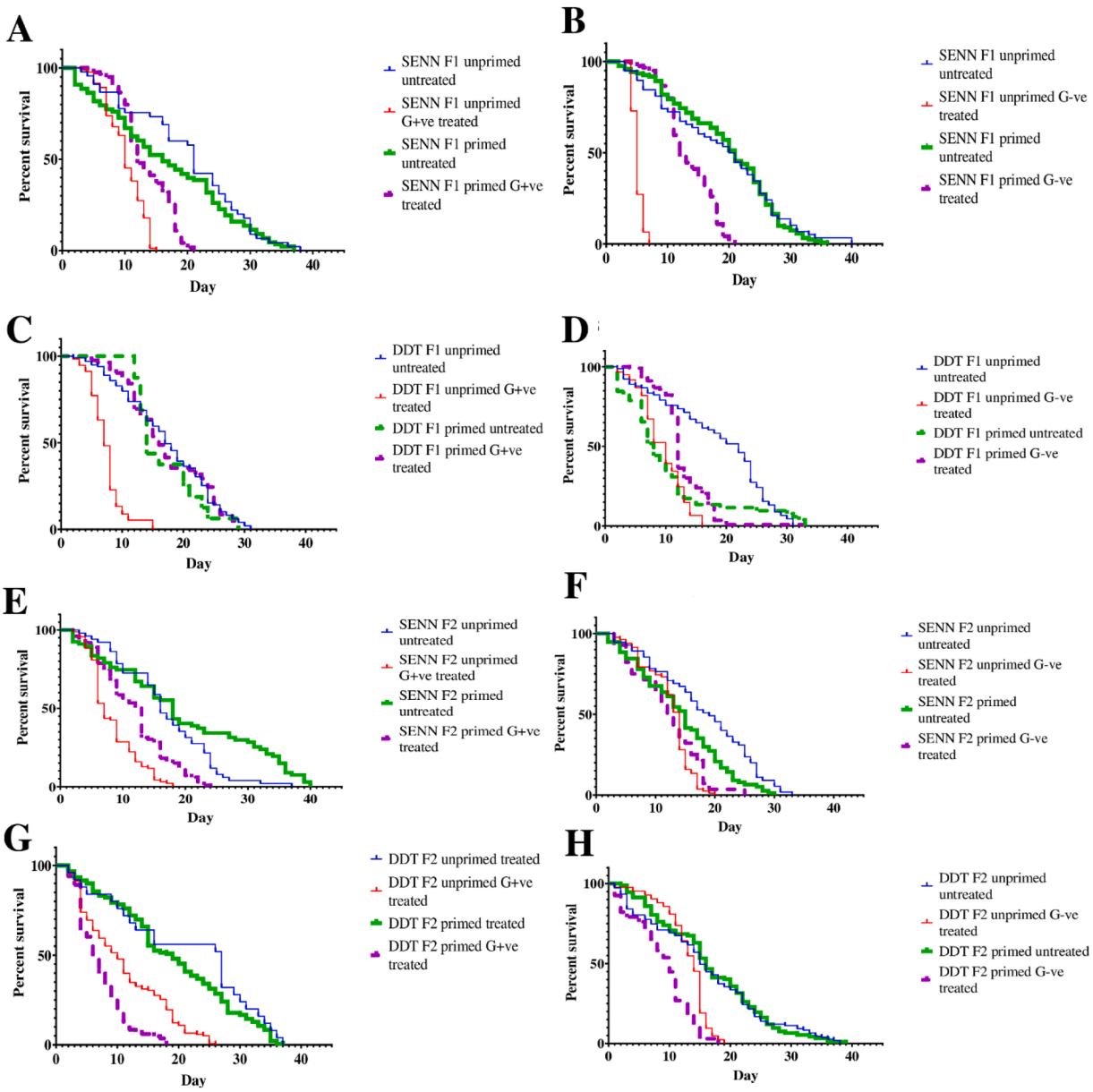


Fig. 3. The effect of larval priming on adult longevity of *An. arabiensis* continuously exposed to bacteria in sugar. A: Longevity of F1 SENN exposed to *S. pyogenes*. B: Longevity of F1 SENN exposed to *E. coli*. C: Longevity of F1 SENN-DDT exposed to *S. pyogenes*. D: Longevity of F1 SENN-DDT exposed to *E. coli*. E: Longevity of F2 SENN exposed to *S. pyogenes*. F: Longevity of F2 SENN exposed to *E. coli*. G: Longevity of F2 SENN-DDT exposed to *S. pyogenes*. H: Longevity of F2 SENN-DDT exposed to *E. coli*. Thin red lines represent unprimed challenged adults and thick purple lines represents primed challenged adults.

S. pyogenes (Gram-positive) was significantly longer in SENN-DDT females that had received an initial sugar priming compared to control females that had not been primed ($p < 0.01$, χ^2=27.76, df=1). This contrasted with the lack of difference found in SENN females. In a similar contrast, the sugar primed unexposed SENN-DDT female lived significantly longer than their unprimed, unexposed counterparts ($p < 0.01$, χ^2=37.56, df=1) (Fig. 4C).

The initial *E. coli* (Gram-negative) challenge increased the longevity of SENN-DDT females continuously exposed to the same bacterium compared to unprimed counterparts ($p < 0.01$, χ^2=30.39, df=1) (Fig. 4D). Like SENN females, there was no significant differences in the longevity of unexposed primed and unprimed SENN-DDT females ($p = 0.25$, χ^2=1.32, df=1).

3.3.2. An oral challenge provided through blood induced memory that was not consistent with bacterial virulence determining memory

In this experiment, the initial bacterial priming was provided through an artificial bloodmeal at day 3. A secondary homologous challenge was provided at day 11. Longevity after the secondary challenge was assessed. Although the initial sugar priming resulted in substantial death, this was not the case with blood priming. The number of dead adults three days after the infection compared to the unprimed did not differ significantly (Log rank test: $p = 0.89$, χ^2=0.17, df=11). An infected blood meal at 3 days of age resulted in protection against the effects of a second infected meal. This was observed for SENN challenged with Gram-negative bacteria ($p = 0.03$, χ^2=4.47, df=1) (Fig. 5A), as well Gram-positive challenged SENN-DDT ($p < 0.01$, χ^2=10.58, df=1), with the difference in SENN-DDT being more marked (Fig. 5B). The subsequent longevity was not due to blood feeding alone as untreated blood did not increase longevity in primed females compared to

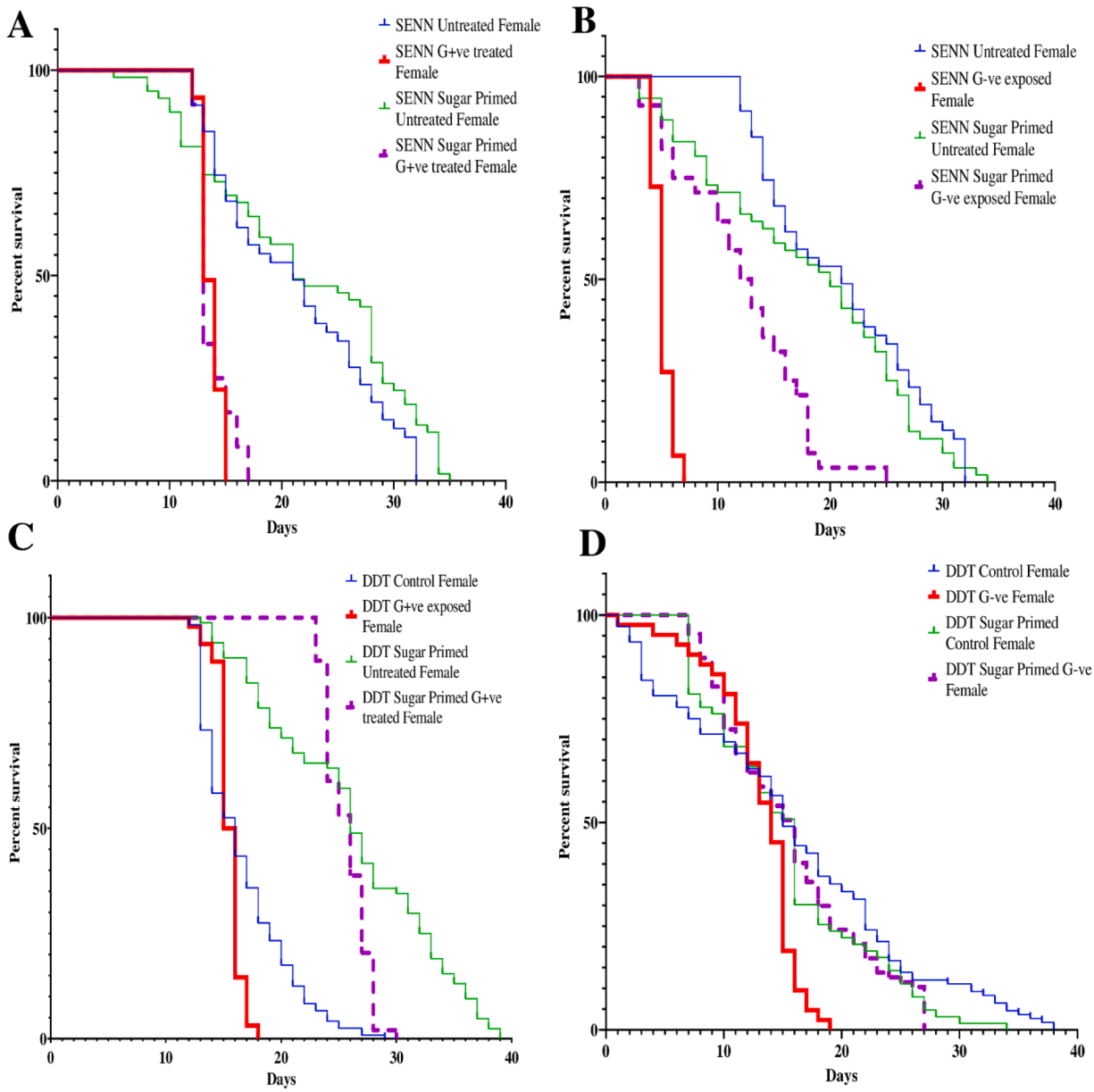


Fig. 4. The effect of an infected sugar meal early in life on subsequent longevity when exposed to the same bacterium later in life. Longevity was assessed with continuous exposure to bacteria supplemented in 10 % sucrose. A: Longevity of SENN females exposed to *S. pyogenes*. B: Longevity of SENN females exposed to *E. coli*. C: Longevity of SENN-DDT females exposed to *S. pyogenes* D: Longevity of SENN-DDT females exposed to *E. coli*. Thin blue lines represent unprimed, untreated controls. Thin green lines represent primed untreated controls. Thick red lines represent unprimed, exposed females. Thick dashed purple lines represent primed, exposed females. Longevity is reported from after the second exposure at 11 days.

unprimed females in SENN ($p = 0.56$, χ^2=0.33, df=1) or SENN-DDT ($p = 0.45$, χ^2=0.57, df=1). Priming did not affect generalised longevity in SENN ($p = 0.06$, χ^2=3.53, df=1). Priming did result in reduced longevity SENN-DDT as unprimed, unchallenged females that did not take a blood meal lived significantly longer than primed, unchallenged counterparts ($p < 0.01$, χ^2=13.04, df=1).

When challenged with Gram-negative infected blood, primed SENN lived longer after challenge compared to unprimed females ($p = 0.02$, χ^2=5.51, df=1) (Fig. 5C). This is similar to the finding in SENN-DDT, where primed females also lived longer than their unprimed counterparts ($p < 0.01$, χ^2=16.20, df=1) (Fig. 5D).

There was also species-specific difference in the controls in the Gram-negative treatment. For SENN, the primed and unprimed specimens did not have any differences in longevity when untreated ($p = 0.21$, χ^2=1.60, df=1). If the primed specimen was provided with an untreated blood meal, however, these females lived significantly longer ($p < 0.01$, χ^2=45.84 df=1). By contrast, blood treatment alone did not result in a significant difference between primed and unprimed SENN-DDT ($p = 0.06$, χ^2=3.41, df=1). Primed, untreated females lived significantly longer than their unprimed counterparts ($p < 0.01$, χ^2=78.76, df=1).

4. Discussion

The insect immune system is an essential part of the survival of the organism. For disease vectors, however, they are a critical determinant of whether an insect efficiently transmits a disease or not (Barillas-Mury and Kumar, 2005). *Anopheles quadriannulatus* is not considered a malaria vector, due to not being implicated in disease transmission in the wild (Sinka *et al.*, 2010). However, over and above its preference for cattle, another reason it is not a vector is because of its substantial immune

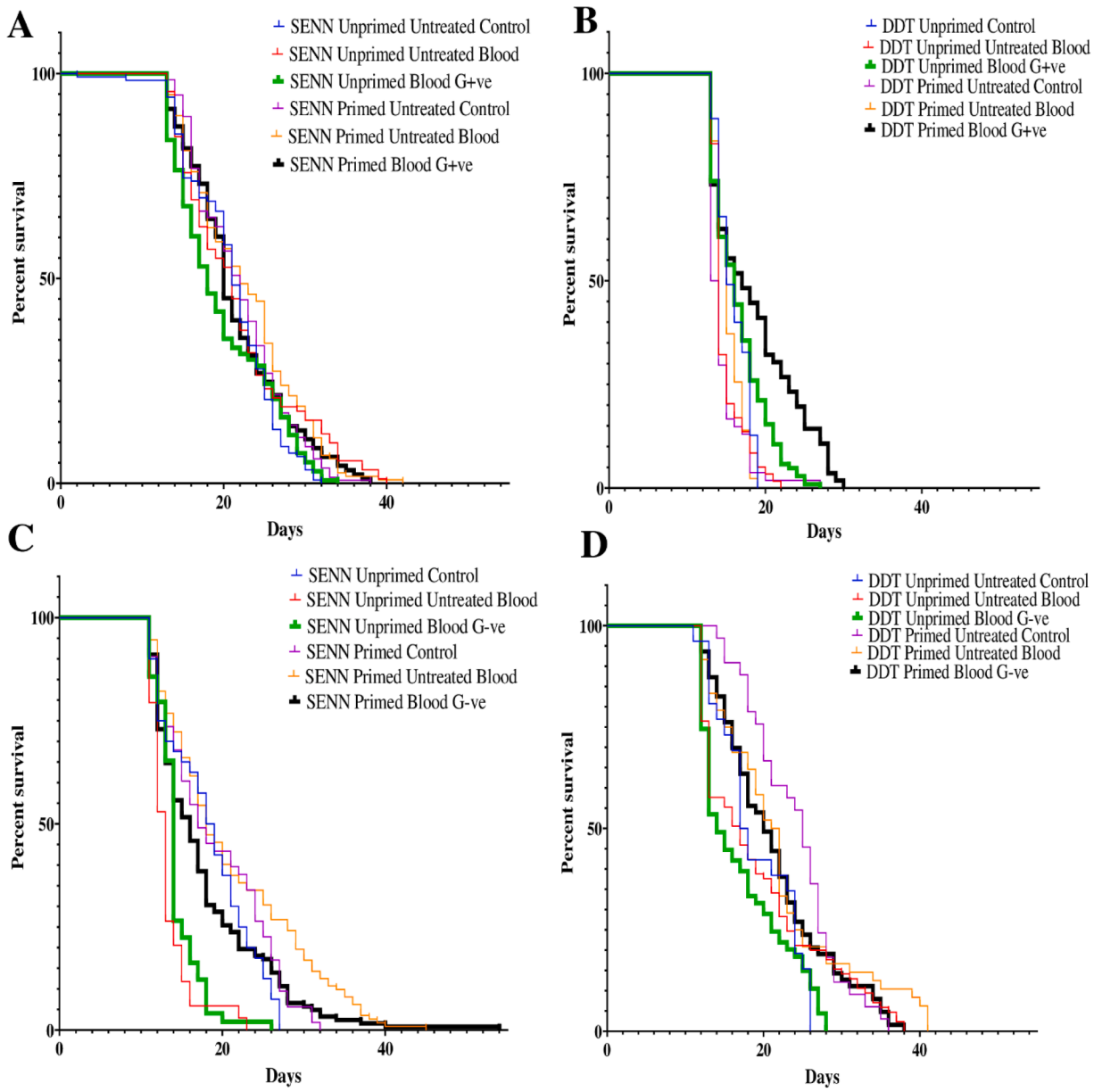


Fig. 5. The effect of an infected blood meal on the subsequent longevity after a second infected blood meal. Longevity was assessed after the second infected blood meal. Thick green lines represent the longevity of females that were exposed to a single infected blood meal only. Thick black lines represent the longevity of females that were primed with an initial infected blood meal. A: Longevity of SENN exposed to *S. pyogenes*-infected blood. B: Longevity of SENN exposed to *E. coli*-infected blood. C: Longevity of SENN-DDT exposed to *S. pyogenes*-infected blood. D: Longevity of SENN-DDT exposed to *E. coli*-infected blood. Thick lines represent bacteria-exposed females, with green representing unprimed females and black representing primed females.

response to the malaria parasite (Habtewold *et al.*, 2008;Habtewold *et al.*, 2017). As such, a thorough understanding of the immune system of mosquitoes would provide insight into what determines the construction of vector competence. Furthermore, understanding factors that modulate the immune system would therefore provide useful epidemiological information.

This study examined whether insecticide resistance could alter the generation of immunological memory in *An. arabiensis*. This was done by comparing the generation of memory in two genetically related laboratory strains; the insecticide susceptible SENN and the insecticide resistant SENN-DDT.

To demonstrate that memory could be generated in this species, F1 *An. arabiensis* larvae were reared in water polluted with either Gram-positive (*S. pyogenes*) or Gram-negative (*E. coli*) bacteria. Upon emergence, adults subjected to a homologous challenge to orally administered bacteria were found to live longer when continuously challenge than unprimed adults. Specific memory to both Gram-positive and Gram-negative bacteria was generated in females, but not males (Fig. 1).

The prevalence of viable *E. coli* that could be recovered in laboratory strains that were reared in *E. coli* (F1 adults) and their offspring that were not reared in polluted water (F2 adults) was assessed after oral homologous challenge. In both F1 and F2 adults, there were lower numbers of CFUs obtained where the F1 adults were reared in *E. coli* polluted water. This was true for both SENN and SENN-DDT (Fig. 2).

Where SENN and SENN-DDT were reared in bacterially polluted water, the F1 offspring lived longer when continuously exposed to a homologous oral sugar challenge. This was maintained in the F2 population not exposed to bacteria as larvae in SENN for both Gram-positive and Gram-negative challenge. This was not true for SENN-DDT. This

tested the primary hypothesis that the generation of memory wa s affected by insecticide resistant phenotype (Fig. 3).

The competing hypothesis was that the generation of memory was affected by bacterial virulence. This stated that Gram-positive and Gram-negative bacteria would not generate memory to the same degree, and that the more virulent bacterium would generate memory better than its less virulent counterpart. As such, one of the bacterial strains would always generate memory to a greater degree than the other.

Oral priming with a sugar challenge resulted in increased longevity with a continuous homologous challenge for Gram-negative bacteria in SENN but for both strains in SENN-DDT (Fig. 4). When provided a priming challenge through blood, both bacterial strains resulted in increase in longevity after a secondary homologous challenge.

The most marked factor about the induction of memory in the F1 adults is that there is a sexually dimorphic response in the generation of immune memory in the adult that eclosed from larvae exposed to bacteria. While memory was generated in females, it was absent in males. Sexual dimorphism in the generation of trained immunity was observed in laboratory strains of *An. gambiae*, with females having the greater advantage (Brown *et al.*, 2019). This effect is predicted by an extension of Bateman's principle. This principle postulates that males increase fitness through mating success, while females do so through longevity. This is due to the cost of investment in reproduction. As such, females would invest more in immunity than males would (Rolff, 2002). This is congruent with the immunological challenge that females would experience through blood feeding that males would not experience (Dana *et al.*, 2005). Although greater immunocompetence in females could be predictable, improved trained immunity was greater in male *T. molitor* (Dhinaut *et al.*, 2018). As such, although the improved memory demonstrated by F1 females rather than males is not unexpected, it is not a given. However, as immune memory was only generated in wild females, subsequent experiments in the study were primarily performed on females. Where the experiment was performed on laboratory males, the females generated memory more efficiently. Again, however, memory was generated more efficiently generated in SENN-DDT. This suggests that the sexual dimorphism in memory generation in may be a true hallmark of mosquito immune memory. This may be due to both Bateman's principle as well as the specific immunological needs of the haematophagous females.

Although these experiments need to be confirmed with wild mosquitoes, it is worth noting that the population used to test the generation of memory in the F1s have basal DDT and pyrethroid resistance (Munhenga *et al.*, 2022). Although there is not a wild comparison for the generation of memory in insecticide susceptible and resistant populations, the findings of this study still have value. Most studies on trained immunity and memory generation in mosquitoes did not consider insecticide resistant phenotype. Even though these experiments were only performed on laboratory strains, the consideration of the effect of the resistance on memory generation is important due to the prevalence of the phenotype in mosquitoes. The fact that a difference exists between the two phenotypes in the laboratory would still be of interest and potentially inform mechanistic insights on the generation of memory.

When looking at larval priming, which was tested on wild and laboratory strains, there is another difference that must be noted. For both males and females, the larval exposure to bacteria without a subsequent challenge resulted in reduced longevity when compared to unprimed, unexposed controls in the F1s. This was not the case for laboratory strains. With the exception of first-generation larval exposure to Gram-negative bacteria in SENN-DDT, there was no cost in terms of longevity to the larval priming. This is possibly due to the higher immune function in laboratory strains (Cornet *et al.*, 2013;Vézilier *et al.*, 2013). It is worth noting that memory generation in the wild is likely to come at a fitness cost, and this has been seen in both first generation as well as transgenerational memory (Contreras-GarduÑO *et al.*, 2016;Dhinaut *et al.*, 2018;Rutkowski *et al.*, 2023). This does suggest that memory generation in wild mosquitoes will not be as efficient as it could be in laboratory mosquitoes.

Regardless of comparison with the wild population, there is a strong argument for the fact that the generation of immune memory can be altered by insecticide resistant phenotype. The generation of memory in bacterially exposed first generation laboratory strains and their unprimed offspring differed between two strains. Larval exposure to bacteria was the most effective mechanism of generating memory, with all treatments generating memory in both strains. However, there is a marked difference between the two strains. In the insecticide susceptible SENN strain, there was a marked difference in the toxicity of Gram-positive and Gram-negative bacteria in unprimed control adults. The longevity of the primed adults was similar for Gram-positive and Gram-negative treatment. However, due to the toxicity of the treatment, the gain in longevity after priming was greater in the SENN strain. By contrast, there was not a significant difference in longevity of the unprimed SENN-DDT regardless of which bacterial strain they were challenged with. Crucially, in the subsequent generations, there is a marked difference in transgenerational memory. Although the SENN-DDT strain was generally more successful in generating memory after an initial priming, this was lost in the second unprimed generation. This contrasted with the SENN strain where memory was sustained. Combined with the finding that SENN-DDT had a markedly reduced number of CFUs after homologous challenge after a larval priming, there is an argument for the generation of memory differs between the two strains. As such, it is possible that insecticide resistant phenotype could affect the generation of memory.

The understanding of the interplay of immunity is poorly understood. What is known is that laboratory insecticide resistant mosquitoes had higher immune function compared to their susceptible counterparts. This, however, is not constitutive and comes at a fitness cost, like many other aspects of insecticide resistance. The mechanism for this is not well understood, and is believed to be part of the pleiotropic effects of resistance (Vézilier *et al.*, 2013). Mosquito immunity is generally examined in context of vector competence. As such, the majority of examination of insecticide resistance and immunity in *Anopheles* mosquitoes relate to the effect on malaria transmission. Although the effect of insecticide resistance on malaria transmission is mixed, there is evidence for increased parasite prevalence and intensity in insecticide resistant mosquitoes. This is true for both target-site resistance as well as metabolic resistance (Alout *et al.*, 2013;Ndo *et al.*, 2019). This effect is not consistent, however. To date, there is no conclusive answer to the question of whether insecticide resistant mosquitoes are better vectors as reviewed in (Suh *et al.*, 2023). Although bacterial priming occurs independently of parasite priming (Contreras-Garduño *et al.*, 2015), this study adds to the body of evidence that insecticide resistance can alter the functioning of the immune system. More significantly for the study the consistent variation of memory generation between the two strains, it is yet further evidence for the primary hypothesis that insecticide resistance phenotype shapes the generation of immunological memory.

The competing hypothesis involves the consideration of bacterial virulence. In a study on trained immunity in *T. molitor*, Gram-positive bacteria consistently generated trained immunity more effectively than Gram-negative bacteria (Dhinaut *et al.*, 2018). Bacterial strain-specific memory induction has been demonstrated in other invertebrate species (Little *et al.*, 2003;Milutinović *et al.*, 2014;Futo *et al.*, 2017). Variation in priming efficacy has been noted when larvae have been treated with the Gram-negative *Enterobacter* species and Gram-negative *Serratia fonticola* (Kulkarni *et al.*, 2021). A similar increased priming effect of Gram-negative *Pseudomonas entomophila* compared to Gram-positive *Lactococcus lactis* in *Drosophila melanogaster* (Kutzer *et al.*, 2019). Therefore, if this competing hypothesis is correct, one of the bacterial strains would consistently generate better immunological memory. This is not the case in this study. In the larval priming example, Gram-negative bacteria induced greater memory in SENN, but in SENN-DDT, Gram-positive bacteria generated better memory. This is

considered as the degree of increase in longevity after challenge when primed. In SENN, where larval priming induced transgenerational memory was better maintained for Gram-positive bacteria. Similarly, for oral sugar priming did not induce memory in SENN, but Gram-positive bacteria did. By contrast, although both bacterial strains induced memory in SENN-DDT, Gram-positive oral sugar priming induced greater memory in the strain than Gram-negative bacteria did.

Blood-borne priming results in immunological memory in both strains for both bacterial types. However, for the SENN strain, the Gram-negative bacteria induces better memory than Gram-positive bacteria. Unlike for the other type of priming in SENN-DDT, oral blood priming resulted in the generation of better when the initial challenge was Gram-negative. As such, there was no consistency in which bacterial strain generates better memory in both strains. Therefore, the differences are more between the mosquito strains rather than a function of bacterial strain. It is worth noting that in general, Gram-positive bacteria was better at generating memory in SENN-DDT. This was the same case for the wild population tested. The population tested does have low-level insecticide resistance (Munhenga *et al.*, 2022). Again, this suggests a role for insecticide resistance in the generation of memory.

As there is not a strong argument for the bacterial virulence being the most important factor of generating memory, are there any other potential factors that could be considered? Could the type of priming mechanism play a role? Oral sugar priming was the least effective mechanism of generating memory. It is worth noting that in this mechanism, the initial priming resulted in large scale mortality. As such, the survivors that underwent the secondary challenge could potentially be a generally fitter population which could be likely to be more survive the challenge rather than representing immunological memory. While this cannot be ruled out completely, a study in *T. confusum* suggests otherwise. When considering the same question, after adjusting for all factorsselective mortality alone could not account for the generation of long-lasting memory. It was not possible for the selection for fitter individuals did not fully explain the generation of memory (Thomas and Rudolf, 2010). As such, the potential selection for fitter adults through the oral sugar challenge is unlikely to account for the memory generation observed. The efficacy of larval priming may be due to the longer exposure, but the longer exposure in the in the oral sugar exposure was not advantageous. Coupled with the fact that the generation of immune memory was still more predictable by mosquito strain rather than by type of priming challenge, there is not a strong argument for the type of immune priming challenge being the most definitive factor in memory generation.

This study examined transgenerational immune memory. This was tested only of the offspring of adults that were exposed to bacteria as larvae. This is because this was the most effective mechanism of generating memory, and it was effective in wild mosquitoes. Transgenerational memory has been demonstrated in a range of invertebrates (as reviewed in Rutkowski et al. (2023). Immunological memory has demonstrated within mosquito generations, but the generation of specific memory across generations, to the best of our knowledge, has not been demonstrated in mosquitoes. A recent review examined the different factors that alter the efficacy of transgenerational immunity. It was found that transgenerational memory would be better generated in hemimetabolous insects and that the sex of the primed parent was not significant in generating memory. Pathogen specificity was also considered. Host-specific pathogens induced greater transgenerational memory than *E. coli*. In this study, *S. pyogenes* generated better transgenerational memory than *E. coli* in the SENN strain. This was unusual for SENN, where Gram-negative bacteria typically generated memory more consistently in the first generation. This induced transgenerational memory, as both bacterial strains were not natural mosquito pathogens, may be due to the fact that gut is dominated by Gram-negative bacteria. It is possible that the memory induction of memory across generations would not be as strong for Gram-negative bacteria would constitute the majority of symbionts.

This study does not provide mechanistic insights on memory generation. Some of the mechanisms previously suggested include the transfer of signals, messenger RNA or effectors as well as and epigenetic modification (as reviewed in (Rutkowski *et al.*, 2023). In SENN and SENN-DDT metal pollution differentially affected epigenetic modulation. This was specifically notable for histone modification (Jeanrenaud *et al.*, 2019). Similarly, bacterial challenge also differentially affected epigenetic modulation in both of the strains in this study. Again, this was particularly notable in histone modification (Patel and Oliver, manuscript in preparation), Therefore, epigenetic modification may possibly be one of the mechanisms underlying the differential generation of memory in these strains.

This study has a range of shortcomings. As mentioned previously, the study must be replicated in a wild population to confirm the effects of the insecticide resistance phenotype. Furthermore, this study only examined memory as a response to a secondary challenge usually measured as a sustained second exposure. The effect of the treatments on haemocytes populations and other antimicrobial effectors would need to be examined to assess whether the effects are due to resistance or tolerance. Tolerance is the capacity limit the health effects of an immunological challenge. To confirm tolerance, proof that pathogen is still present, and that there is an investment in defence in against the pathogen and its' effect. This may be difficult to assess, as tolerance needs to be assessed on a population rather than an individual level. Immunological memory based on tolerance would have a positive effect on the prevalence of the pathogen (Råberg, 2014), and to confirm this mechanism this effect would have to be confirmed. Finally, a full quantification of the amount of the introduced bacteria by a method such as quantification of the 16 s gene by quantitative real time PCR would also strengthen the argument. Further studies are required to understand the mechanisms of potentially variable generation of memory in wild insecticide and susceptible resistant populations. This is particularly true for transgenerational memory.

Regardless of the Gram status of the bacteria used and the type of immune priming, there was a difference in the generation of memory in the two strains. Coupled with the fact that baseline immunity differs between the strains, the evidence differs between the strains, the evidence supports the primary hypothesis that insecticide resistant phenotype is the most significant modulator of memory generation tested in this study.

In conclusion, although different initial challenges have variable capacity to induce capacity to induce immune memory, the insecticide resistant phenotype can alter the efficacy of the priming effect. The immune priming effect is also sexually dimorphic, with females capable of inducing memory more efficiently than males. Larval bacterial exposure is the most efficient mechanism of initial challenge. Gram-negative exposure induces a weaker immune memory than Gram-positive exposure. The insecticide resistant phenotype also modulated the capacity for induction of transgenerational memory. Although this needs to be confirmed in wild material, these data suggest a critical pleiotropic role for insecticide resistance in the construction of the immune response.

Ethical approval

This study was performed as per the ethics waiver from the University of the Witwatersrand to S Oliver: 03–01–2018.

CRediT authorship contribution statement

Nashrin F. Patel: Investigation, Formal analysis, Visualization. **Shüné V. Oliver:** Conceptualization, Methodology, Formal analysis, Resources, Data curation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data in this experiment is available as supplementary file as a spreadsheet.

Acknowledgements

The staff of the National Institute for Communicable Diseases, particularly Dr Givemore Munhenga, and National Department of Health are thanked for the provision of specimens from the field. Dr Rodney Hull is thanked for his valuable comments on the drafting of the manuscript. Two anonymous reviewers are thanked for their valuable comments.

Funding

This project was funded by a National Research Foundation Competitive Support for Unrated researchers grant [SRUG190313423259], National Research Foundation of South Africa (Grant National Research foundation support for Y-rated researchers [CSRP210405592233], National Research Foundation Competitive Support for Rated Researchers [SRUG2204133394], National Health Research Development Grants [2017–2DEV08SOL01 and 2017–2DEV08SOL02] to SVO. This work is based on the research supported in part by the Wits Chancellor's Female Academic Leaders Fellowship.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cris.2024.100085.

References

Alout, H., Ndam, N.T., Sandeu, M.M., Djégbe, I., Chandre, F., Dabiré, R.K., Djogbénou, L. S., Corbel, V., Cohuet, A., 2013. Insecticide resistance alleles affect vector competence of *Anopheles gambiae* s.s. for *Plasmodium falciparum* field isolates. PLoS. ONE 8, e63849.

Barillas-Mury, C., Kumar, S., 2005. *Plasmodium*-mosquito interactions: a tale of dangerous liaisons. Cell Microbiol 7, 1539–1545.

Barnard, K., Jeanrenaud, A., Brooke, B.D., Oliver, S.V., 2019. The contribution of gut bacteria to insecticide resistance and the life histories of the major malaria vector *Anopheles arabiensis* (Diptera: culicidae). Sci. Rep 9, 9117.

Bartlett, M.S., and Fowler, R.H. (1937). Properties of sufficiency and statistical tests. 160, 268–282.

Boraschi, D., Italiani, P., 2018. Innate immune memory: time for adopting a correct terminology. Front. Immunol 9, 799.

Brown, L.D., Shapiro, L.L.M., Thompson, G.A., Estévez-Lao, T.Y., Hillyer, J.F., 2019. Transstadial immune activation in a mosquito: adults that emerge from infected larvae have stronger antibacterial activity in their hemocoel yet increased susceptibility to malaria infection. Ecol. Evol 9, 6082–6095.

Contreras-Garduño, J., Lanz-Mendoza, H., Franco, B., Nava, A., Pedraza-Reyes, M., Canales-Lazcano, J., 2016. Insect immune priming: ecology and experimental evidences. . Ecol. Entomol 41, 351–366.

Contreras-Garduño, J., Rodríguez, M.C., Hernández-Martínez, S., Martínez-Barnetche, J., Alvarado-Delgado, A., Izquierdo, J., Herrera-Ortiz, A., Moreno-García, M., Velazquez-Meza, M.E., Valverde, V., Argotte-Ramos, R., Rodríguez, M.H., Lanz-Mendoza, H., 2015. *Plasmodium berghei* induced priming in *Anopheles albimanus* independently of bacterial co-infection. Dev. Comp. Immunol 52, 172–181.

Contreras-Garduño, J., Rodríguez, M.C., Rodríguez, M.H., Alvarado-Delgado, A., Lanz-Mendoza, H., 2014. Cost of immune priming within generations: trade-off between infection and reproduction. Microb. Infect 16, 261–267.

Cornet, S., Gandon, S., Rivero, A., 2013. Patterns of phenoloxidase activity in insecticide resistant and susceptible mosquitoes differ between laboratory-selected and wild-caught individuals. Parasit. Vectors 6, 315.

Dana, A.N., Hong, Y.S., Kern, M.K., Hillenmeyer, M.E., Harker, B.W., Lobo, N.F., Hogan, J.R., Romans, P., Collins, F.H., 2005. Gene expression patterns associated with blood-feeding in the malaria mosquito *Anopheles gambiae*. BMC. Genomics 6, 5.

Dhinaut, J., Chogne, M., Moret, Y., 2018. Immune priming specificity within and across generations reveals the range of pathogens affecting evolution of immunity in an insect. J. Anim. Ecol 87, 448–463.

Fellous, S., Lazzaro, B.P., 2011. Potential for evolutionary coupling and decoupling of larval and adult immune gene expression. Mol. Ecol 20, 1558–1567.

Futo, M., Sell, M.P., Kutzer, M.a.M., Kurtz, J., 2017. Specificity of oral immune priming in the red flour beetle *Tribolium castaneum*. Biol. Lett 13.

Ghosh, J., Lun, C.M., Majeske, A.J., Sacchi, S., Schrankel, C.S., Smith, L.C., 2011. Invertebrate immune diversity. Dev. Comp. Immunol 35, 959–974.

Habtewold, T., Groom, Z., Christophides, G.K., 2017. Immune resistance and tolerance strategies in malaria vector and non-vector mosquitoes. Parasit. Vectors 10, 186.

Habtewold, T., Povelones, M., Blagborough, A.M., Christophides, G.K., 2008. Transmission blocking immunity in the malaria non-vector mosquito *Anopheles quadriannulatus* species A. PLoS. Pathog 4, e1000070.

Hemingway, J., 1983. Biochemical studies on malathion resistance in *Anopheles arabiensis* from Sudan. Trans. R. Soc. Trop. Med. Hyg 77, 477–480.

Hunt, R.H., Brooke, B.D., Pillay, C., Koekemoer, L.L., Coetzee, M, 2005. Laboratory selection for and characteristics of pyrethroid resistance in the malaria vector *Anopheles funestus*. Med. Vet. Entomol 19, 271–275.

Jeanrenaud, A.C.S.N., Brooke, B.D., Oliver, S.V., 2019. The effects of larval organic fertiliser exposure on the larval development, adult longevity and insecticide tolerance of zoophilic members of the *Anopheles gambiae* complex (Diptera: culicidae). . PLoS. ONE 14, e0215552.

Kaplan, E.L., Meier, P., 1958. Nonparametric Estimation from Incomplete Observations. J. Am. Stat. Assoc 53, 457–481.

Kitau, J., Oxborough, R.M., Tungu, P.K., Matowo, J., Malima, R.C., Magesa, S.M., Bruce, J., Mosha, F.W., Rowland, M.W, 2012. Species shifts in the Anopheles gambiae complex: do LLINs successfully control *Anopheles arabiensis*? PLoS. ONE 7, e31481.

Kruskal, W.H., Wallis, W.A., 1952. Use of ranks in one-criterion variance analysis. J. Am. Stat. Assoc 47, 583–621.

Kulkarni, A., Pandey, A., Trainor, P., Carlisle, S., Chhilar, J.S., Yu, W., Moon, A., Xu, J., 2021. Trained immunity in *Anopheles gambiae*: antibacterial immunity is enhanced by priming via sugar meal supplemented with a single gut symbiotic bacterial strain. Front. Microbiol 12, 649213.

Kurtz, J., 2005. Specific memory within innate immune systems. Trends Immunol 26, 186–192.

Kutzer, M.a.M., Kurtz, J., Armitage, S.a.O., 2019. A multi-faceted approach testing the effects of previous bacterial exposure on resistance and tolerance. J. Anim. Ecol 88, 566–578.

Little, T.J., O'connor, B., Colegrave, N., Watt, K., Read, A.F., 2003. Maternal transfer of strain-specific immunity in an invertebrate. Curr. Biol 13, 489–492.

Mann, H.B., Whitney, D.R., 1947. On a test of whether one of two random variables is stochastically larger than the other. Annals Mathem. Statis 18, 50–60.

Medzhitov, R., Janeway Jr., C.A., 1997. Innate immunity: the virtues of a nonclonal system of recognition. Cell 91, 295–298.

Milutinović, B., Fritzlar, S., Kurtz, J., 2014. Increased survival in the red flour beetle after oral priming with bacteria-conditioned media. J. Innate. Immun 6, 306–314.

Munhenga, G., Oliver, S.V., Lobb, L.N., Mazarire, T.T., Sekgele, W., Mashatola, T., Mabaso, N., Dlamini, D.M., Zulu, M., Moletsane, F., Letinić, B.D., Zawada, J., Burke, A., Matamba, A., Brooke, B.D., 2022. Malaria risk and receptivity: continuing development of insecticide resistance in the major malaria vector *Anopheles arabiensis* in northern KwaZulu-Natal, South Africa. S. Afr. J. Sci. 118.

Ndo, C., Kopya, E., Irving, H., Wondji, C., 2019. Exploring the impact of glutathione S-transferase (GST)-based metabolic resistance to insecticide on vector competence of *Anopheles funestus* for *Plasmodium falciparum*. Wellcome Open Res 4, 52.

Netea, M.G., Quintin, J., Van Der Meer, J.W, 2011. Trained immunity: a memory for innate host defense. Cell Host Microbe 9, 355–361.

Oliver, S.V., Brooke, B.D., 2013. The effect of larval nutritional deprivation on the life history and DDT resistance phenotype in laboratory strains of the malaria vector *Anopheles arabiensis*. Malar. J 12, 44.

Oliver, S.V., Brooke, B.D., 2016. The role of oxidative stress in the longevity and insecticide resistance phenotype of the major malaria vectors *Anopheles arabiensis* and *Anopheles funestus*. . PLoS. ONE 11, e0151049.

Oliver, S.V., Brooke, B.D., 2017. The effect of elevated temperatures on the life history and insecticide resistance phenotype of the major malaria vector *Anopheles arabiensis* (Diptera: culicidae). Malar. J 16, 73.

Ottaviani, E., 2011. Is the distinction between innate and adaptive immunity in invertebrates still as clear-cut as thought? Ital. J. Zool 78, 274–278.

Paine, M.J.I., Brooke, B, 2016. Insecticide resistance and its impact on vector control. In: Horowitz, A.R., Ishaaya, I. (Eds.), Advances in Insect Control and Resistance Management. Springer International Publishing, Cham, pp. 287–312.

Powers, J.C., Turangan, R., Joosse, B.A., Hillyer, J.F., 2020. Adult mosquitoes infected with bacteria early in life have stronger antimicrobial responses and more hemocytes after reinfection later in life. Insects 11.

Råberg, L., 2014. How to live with the enemy: understanding tolerance to parasites. PLoS Biol 12, e1001989.

Ramirez, J.L., De Almeida Oliveira, G., Calvo, E., Dalli, J., Colas, R.A., Serhan, C.N., Ribeiro, J.M., Barillas-Mury, C., 2015. A mosquito lipoxin/lipocalin complex mediates innate immune priming in *Anopheles gambiae*. Nat. Commun 6, 7403.

Rodrigues, J., Brayner, F.A., Alves, L.C., Dixit, R., Barillas-Mury, C., 2010. Hemocyte differentiation mediates innate immune memory in *Anopheles gambiae* mosquitoes. Science 329, 1353–1355.

Rolff, J., 2002. Bateman's principle and immunity. Proc. Biol. Sci 269, 867–872.
Rutkowski, N.J., Mcnamara, K.B., Jones, T.M., Foo, Y.Z., 2023. Trans-generational immune priming is not mediated by the sex of the parent primed: a meta-analysis of invertebrate data. Biol. Rev. Camb. Philos. Soc 98, 1100–1117.
Shapiro, S.S., Wilk, M.B., 1965. An analysis of variance test for normality (complete samples). Biometrika 52, 591–611.
Singh, A., Allam, M., Kwenda, S., Khumalo, Z.T.H., Ismail, A., Oliver, S.V., 2022. The dynamic gut microbiota of zoophilic members of the Anopheles gambiae complex (Diptera: culicidae). Sci. Rep 12, 1495.
Sinka, M.E., Bangs, M.J., Manguin, S., Coetzee, M., Mbogo, C.M., Hemingway, J., Patil, A.P., Temperley, W.H., Gething, P.W., Kabaria, C.W., Okara, R.M., Van Boeckel, T., Godfray, H.C., Harbach, R.E., Hay, S.I., 2010. The dominant *Anopheles* vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic précis. Parasit. Vectors 3, 117.
Suh, P.F., Elanga-Ndille, E., Tchouakui, M., Sandeu, M.M., Tagne, D., Wondji, C., Ndo, C., 2023. Impact of insecticide resistance on malaria vector competence: a literature review. Malar. J 22, 19.
Thomas, A.M., and Rudolf, V.H.W. (2010). Challenges of metamorphosis in invertebrate hosts: maintaining parasite resistance across life-history stages. 35, 200–205.
Tukey, J.W., 1949. Comparing individual means in the analysis of variance. Biometrics 5, 99–114.
Vantaux, A., Dabiré, K.R., Cohuet, A., Lefèvre, T., 2014. A heavy legacy: offspring of malaria-infected mosquitoes show reduced disease resistance. Malar. J 13, 442.
Vézilier, J., Nicot, A., Lorgeril, J., Gandon, S., Rivero, A., 2013. The impact of insecticide resistance on *Culex pipiens* immunity. Evol. Appl 6, 497–509.