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RESEARCH ARTICLE

Senescence of humoral antimicrobial immunity occurs in infected mosquitoes when the temperature is higher

Lindsay E. Martin, Monzerrat Ruiz and Julián F. Hillyer*

ABSTRACT

Mosquitoes cannot use metabolism to regulate their body temperature and therefore climate warming is altering their physiology. Mosquitoes also experience a physiological decline with aging, a phenomenon called senescence. Because both high temperature and aging are detrimental to mosquitoes, we hypothesized that high temperatures accelerate senescence. Here, we investigated how temperature and aging, independently and interactively, shape the antimicrobial immune response of the mosquito *Anopheles gambiae*. Using a zone-of-inhibition assay that measures the antimicrobial activity of hemolymph, we found that antimicrobial activity increases following infection. Moreover, in infected mosquitoes, antimicrobial activity weakens as the temperature rises to 32°C, and antimicrobial activity increases from 1 to 5 days of age and stabilizes with further aging. Importantly, in *E. coli*-infected mosquitoes, higher temperature causes an aging-dependent decline in antimicrobial activity. Altogether, this study demonstrates that higher temperature can accelerate immune senescence in infected mosquitoes, thereby interactively shaping their ability to fight an infection.

KEY WORDS: Mosquitoes, Immune response, Aging, Temperature, Antimicrobial, Senescence

INTRODUCTION

The ability of mosquitoes to transmit diseases to humans and animals largely depends on the robustness of their immune response (Bartholomay and Michel, 2018). Upon infection, mosquitoes mount a multifaceted innate immune response. The cellular branch of the immune response includes phagocytosis by hemocytes, whereas the humoral branch includes melanization, antimicrobial peptides (AMPs), lysozymes and other lytic factors (Bartholomay and Michel, 2018; Hillyer, 2016). Mosquitoes, like most insects, maintain a basal level of antimicrobial effectors so they can quickly respond to an infection (Zhang et al., 2021). Once a pathogen is detected, effector pathways, such as the Toll and immune deficiency (IMD) signaling cascades, are activated, and a subsequent outcome is the production of AMPs such as cecropins and defensins (Bartholomay and Michel, 2018; Hillyer, 2016; Zhang et al., 2021). These effector molecules are released into the hemolymph to damage and kill pathogens.

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The immune response of mosquitoes is altered by environmental factors such as temperature and humidity (Brown et al., 2023; Murdock et al., 2012). Most insects, including mosquitoes, are both poikilotherms and ectotherms, so their body temperature is equivalent to the environmental temperature. As environmental temperatures are predicted to rise by >1.5°C between 2030 and 2052 (Pörtner et al., 2022), the ability of mosquitoes – or indeed any insect – to respond to an infection will change. Higher temperature, for example, reduces the ability of hemocytes to phagocytose (Murdock et al., 2012), weakens melanization (Murdock et al., 2012; Suwanchaichinda and Paskewitz, 1998; Ferreira et al., 2020; Simões et al., 2022; Martin and Hillyer, 2024) and alters the expression of immune genes (Ferreira et al., 2020; Wimalasiri-Yapa et al., 2021; Murdock et al., 2012).

In addition to temperature, aging alters mosquito immunity (Müller et al., 2013). Like most organisms, as mosquitoes age, they experience a gradual, irreversible deterioration of physiological function in a process known as senescence (Müller et al., 2013; Styer et al., 2007). This includes a weakening of immune proficiency, increasing infection intensity and a reduction in survival (Hillyer et al., 2005; Christensen et al., 1986; Barr et al., 2024). For example, senescence weakens phenoloxidase-based melanization (Martin and Hillyer, 2024; Chun et al., 1995; Christensen et al., 1986) and decreases the number of immune cells (hemocytes), that circulate with the hemolymph (Hillyer et al., 2005; Christensen et al., 1986; King and Hillyer, 2013; Castillo et al., 2006).

Both temperature and age affect mosquito immunity, but how they interact to shape senescence is poorly understood. We hypothesize that higher temperature accelerates immune senescence, thereby uncoupling chronological age (measured in terms of time) and physiological age (measured in terms of performance). In support of this hypothesis, higher temperature and aging interact to alter mosquito survival and shape the mosquito's body composition by accelerating the aging-dependent decline in protein content (Barr et al., 2023, 2024). Moreover, higher temperature accelerates the aging-dependent increase in infection intensity and the decline in the melanization immune response (Martin and Hillyer, 2024; Barr et al., 2024). Here, we tested how higher temperature and aging interact to shape the humoral immune response of the African malaria mosquito *Anopheles gambiae*. We found that infection increases the antimicrobial activity of hemolymph. Moreover, in infected mosquitoes, antimicrobial activity decreases with higher temperature and increases from 1 to 5 days of age, stabilizing with further aging. Importantly, in *E. coli*-infected mosquitoes, higher temperature causes an aging-dependent decline in antimicrobial activity, and therefore, higher temperature can accelerate the senescence of the humoral immune response.

MATERIALS AND METHODS

Mosquito rearing, treatments, and experimental overview

Mosquitoes were reared as described (Martin and Hillyer, 2024). A colony of *Anopheles gambiae* Giles *sensu stricto* (G3 strain;

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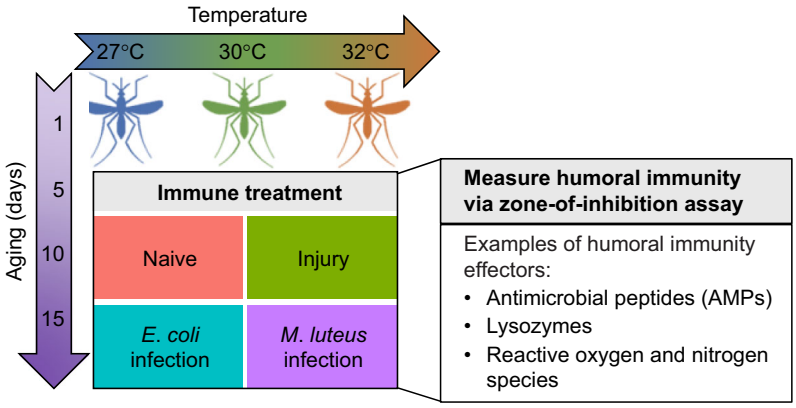

Fig. 1. Experimental overview for investigating the effects of higher temperature, aging and their interaction on mosquito antimicrobial activity.

Diptera: Culicidae) was reared at 27°C, 75% relative humidity, and a 12 h:12 h light:dark photoperiod. Mosquito eggs were collected from this colony and hatched in environmental chambers held at 27°C, 30°C or 32°C, and reared to adulthood (Fig. 1). Larvae were fed a mixture of 2.8:1 koi food:baker's yeast daily, and pupae were separated daily. Upon eclosion, adults were fed 10% sucrose solution and maintained in ∼2.5 liter (80 oz) plastic buckets with a mesh top. These temperatures were selected because they are experienced by *A. gambiae* in nature, and they represent temperatures rising with climate change but are within the thermal physiological limits of this mosquito (Sinka et al., 2010; Lindsay et al., 1998; Pörtner et al., 2022).

For each temperature, immune function was assessed in female mosquitoes at 1, 5, 10 and 15 days of adulthood (Fig. 1). Mosquitoes experience immunosenescence over the first 15 days of age, and these ages are also milestones for *Plasmodium* sp. development (Hillyer et al., 2005; Phillips et al., 2017). The antimicrobial activity of hemolymph was measured following four immune treatments: (i) naïve (unmanipulated) mosquitoes, (ii) injured mosquitoes, (iii) mosquitoes infected with GFP-expressing tetracycline-resistant *Escherichia coli* (Gram-negative bacteria; modified DH5α) or (iv) mosquitoes infected with *Micrococcus luteus* (Gram-positive bacteria; ATCC 4698). Bacterial cultures were grown overnight in a shaking incubator (New Brunswick Scientific, Edison, NJ, USA) in Luria–Bertani (LB) broth at 37°C, and then normalized to OD_{600}=2. To infect mosquitoes, a capillary glass needle was inserted into the thoracic anepisternal cleft, and 69 nl of sterile LB (injured mosquitoes) or a bacterial culture (infected mosquitoes) was injected into the hemocoel using a Nanoject III Programmable Nanoliter Injector (Drummond Scientific Company, Broomall, PA, USA). After infections, cultures were diluted and plated on LB agar plates, and the absolute infection dose was calculated. The average infection dose for *E. coli* was 12,010 and for *M. luteus* was 7431. In the text and figures, the age of the mosquito is when the immune treatment was initiated.

Quantification of antimicrobial activity of hemolymph

For each condition, hemolymph was extracted from 18–25 mosquitoes at 24 h after the immune treatment as previously described (Martin and Hillyer, 2024). Briefly, mosquitoes were cold-anesthetized and holes were punctured in the lateral thorax using 0.2 mm diameter minutien pins. Mosquitoes were then transferred to a 0.6 ml microfuge tube that had an incision at the bottom, and this tube was nested inside a 1.5 ml tube. Together, these tubes were centrifuged at 5000 RCF at 4°C for 5 min, resulting in the collection of ∼1–2 µl of hemolymph in the bottom of the 1.5 ml tube for each sample. Hemolymph was preserved at −20°C until use. The 24 h timepoint was selected because antimicrobial activity of hemolymph is greatest at this time (Morejon and Michel, 2023).

To quantify the antimicrobial activity of hemolymph or the ability of hemolymph to kill pathogens using antimicrobial compounds such as AMPs, we used a zone-of-inhibition assay (Fig. 2) as previously described (League et al., 2017). To make *M. luteus*-seeded plates, cultures were grown overnight and normalized to OD_{600}=10. Then, sterile, liquid 1% LB agar was made, cooled to ∼55°C, and the *M. luteus* culture was added to create a ratio of 1 part *M. luteus* culture to 11 parts LB agar liquid.

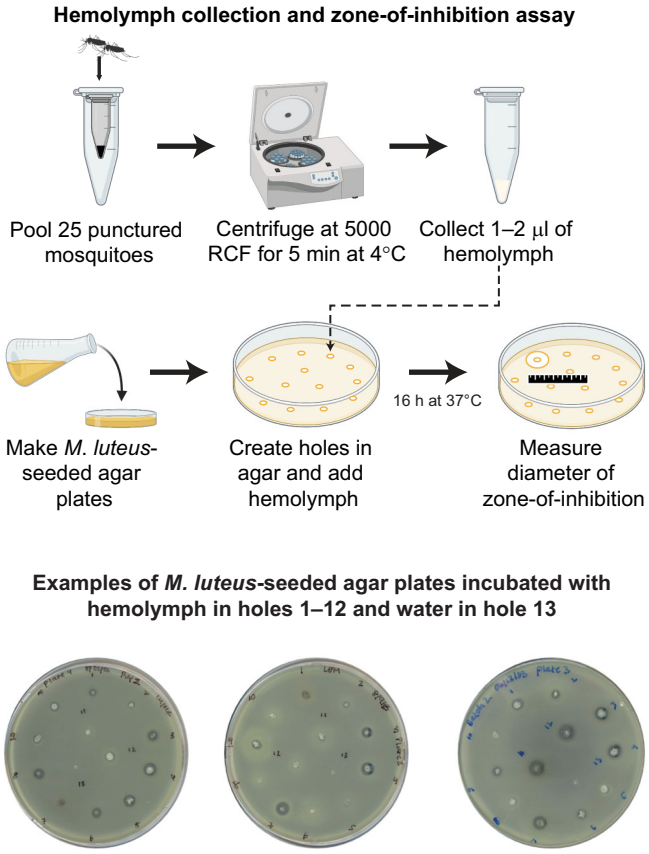

Fig. 2. Workflow of hemolymph collection and subsequent zone-of-inhibition assays to measure antimicrobial activity of hemolymph. Graphics depicting methods were created with Biorender.com.

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The solution was immediately mixed, 6 ml of the solution was pipetted into 9 cm diameter Petri dish plates, and the plates were allowed to solidify at room temperature. Next, 13 equidistant holes of 1 mm in diameter were made in the plates by poking the agar with sterile 1000 µl pipette tips. Each plate contained approximately 1.4×10^8 *M. luteus*.

To measure the zone of inhibition, 1 µl of pure hemolymph was pipetted into each of the first 12 holes on each plate. As a negative control, 1 µl of distilled, sterile water was pipetted into the 13th hole. The plates were incubated at room temperature for 10 min to allow the hemolymph to absorb into the agar, and the plates were then inverted and incubated at 37°C for 16 h. After incubation, plates were scanned alongside a ruler with the Epson Perfection V600 Photo scanner (Epson America, Inc., Long Beach, CA, USA) at 46.8 pixels per mm, and the images were analyzed using ImageJ software (Schneider et al., 2012). For each hole, the diameter of the zone of bacterial growth inhibition was measured in triplicate, and the average diameter was used to calculate the area of the zone-of-inhibition.

Each temperature-age-immune treatment group was analyzed over an average of 4 independent biological trials. For each group, each trial was composed of 1 hemolymph sample, which was collected from a pool of 18–25 mosquitoes. The volume of each hemolymph sample was 1–3 µl, and because only 1 µl of hemolymph was necessary for the zone-of-inhibition assay, when the volume of hemolymph was ≥2, we conducted a second zone-of-inhibition assay with the same sample as a technical replicate. Approximately 33% of the samples were ≥2 µl, so the antimicrobial activity of these samples was assayed twice, and the technical replicates were included as a random effect in the analysis when significant. In total, 229 hemolymph samples were assayed, derived from approximately 5000 mosquitoes. The number of hemolymph samples for each temperature-age-treatment group, including the number of biological replicates and technical replicates, are included in Table S1 (‘Sample size’ sheet).

Statistical analysis

Statistical analyses were completed using R Statistical Software, v.4.3.0 (r-project.org). The data for each temperature–age–immune treatment combination were tested for normality using the Shapiro–Wilk test, and because the data were non-normal, the data were zero-adjusted and log-transformed to achieve normality.

The entire dataset was first analyzed using a linear mixed-effects model, fit by restricted maximum likelihood, to identify the relationship between antimicrobial activity and the main linear effects of immune treatment, age, and temperature, as well as the non-linear effects of age and temperature (polynomial=2), using the ‘lme4’ package (Bates et al., 2015). Two-way interactions among each of the main effects (temperature×immune treatment; polynomial temperature×age) and the random effects of the plate number and the seeded-plate batch were included as predictors in the model. Other interactions and random effects (e.g. age×immune treatment; technical replicate) did not meaningfully contribute to antimicrobial activity, so they were excluded from the model.

Because the immune treatment had a disproportionally large effect on antimicrobial activity in the initial analysis, the data were separated by immune treatment and analyzed by linear mixed-effects models, fit by restricted maximum likelihood, to identify the effects of temperature, aging, and their interaction. For the uninfected mosquitoes (naïve and injury groups), the effects of temperature and age were linear (polynomial=1), and the seeded-plate batch was included as a random effect. Additionally, the interaction between temperature and age did not contribute to antimicrobial activity and was excluded from the models-of-best-fit (naïve interaction: χ^2_1=1.3, P=0.250; Injury interaction: χ^2_1=2.2, P=0.140). For the infected mosquitoes (*E. coli* and *M. luteus* groups), the effects of temperature and age were non-linear (polynomial=2). For *E. coli*-infected mosquitoes, the interaction between temperature and age (polynomial=2) was included in the model-of-best-fit (*E. coli* polynomial interaction: χ^2_2=11.0, P=0.004), but for *M. luteus*-infected mosquitoes this interaction did not meaningfully contribute to antimicrobial activity (*M. luteus* polynomial interaction: χ^2_2=1.3, P=0.512), so it was excluded from the model. For infected mosquitoes, technical replicate and seeded-plate batch meaningfully contributed to antimicrobial activity and were therefore included as random effects with *E. coli* infection, whereas seeded-plate number (within batches of plates) was similarly included as a random effect with *M. luteus* infection. Other random effects did not meaningfully contribute to antimicrobial activity and were therefore excluded. All final models were determined by a stepwise, multidirectional selection method, comparing model residuals, log-likelihood ratios and Akaike information criterion (AIC).

For each model, the dependent variables of temperature and age were normalized by centering and scaling prior to model fitting. Statistical significance of main and interactive effects was assessed using a type-II ANOVA with Kenward–Roger approximation of degrees of freedom using the ‘lmerTest’ package (https://CRAN.R-project.org/package=lmerTest; Kuznetsova et al., 2017), and estimated marginal means were calculated using the ‘emmeans’ package (https://CRAN.R-project.org/package=emmeans; Searle et al., 1980). Tukey-adjusted (for overall differences in treatment) or Šidák-adjusted (for each immune treatment model) *post hoc* comparisons of estimated marginal means were performed using the ‘multcomp’ package (https://CRAN.R-project.org/package=multcomp; Hothorn et al., 2008). Estimated marginal means, ANOVA P-values, partial effect sizes and effect size confidence intervals are presented in the main figures. Observed means are presented in Figs S1 and S2. Estimated marginal means from the full model fitted to the entire dataset are presented in Fig. S3.

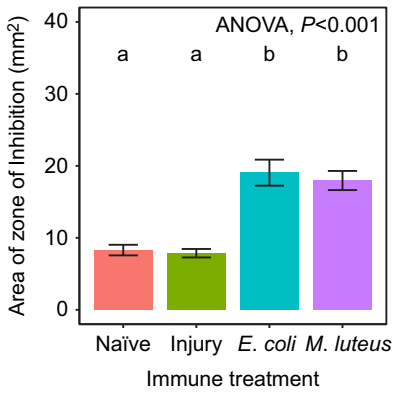

Fig. 3. Antimicrobial activity is a constitutive immune response that increases with infection. Area of zone-of-inhibition of hemolymph from naïve, injured and infected mosquitoes, irrespective of temperature or age. The column heights mark the estimated marginal means and whiskers denote s.e.m. Data were analyzed by a type-II ANOVA with Kenward–Roger approximation of degrees of freedom followed by Tukey-adjusted *post hoc* multiple comparisons of means. Pairwise, *post hoc* comparisons are indicated by letters; columns sharing the same letter are not significantly different.

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Because infectious doses were normalized using the optical density of the bacterial culture, doses contained natural variation. Therefore, the correlation between the initial infectious dose (calculated after infection by plating the inoculum cultures) and the area of the zone of inhibition was assessed. The infectious doses and the area of zone of inhibition were non-normal using the Shapiro–Wilk normality test, so the Spearman's rank correlation was used to test for a relationship between variables. Additional information in the supplement includes the raw data, code, observed means, estimated marginal means, model coefficients and full ANOVA tables (supplementary Materials and Methods; Table S1). Graphs depicting data were created with R and assembled into figures using Adobe Illustrator.

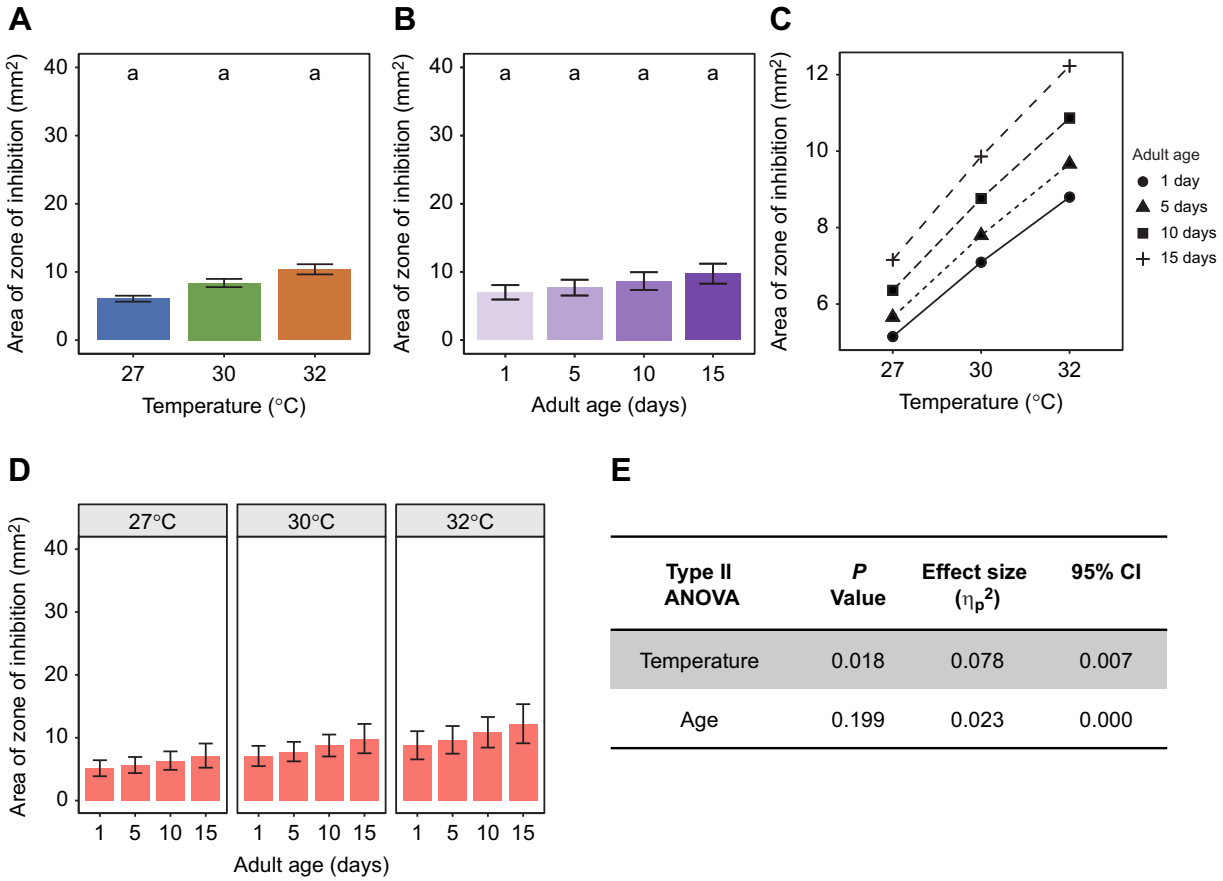
RESULTS

Antimicrobial activity is a constitutive immune response that significantly increases after infection, regardless of temperature or age

To test the effects of higher temperature, aging and their interaction on the overall antimicrobial activity of mosquitoes, we isolated hemolymph, incubated it in bacterially seeded plates, and measured the zone of inhibition, which is indicative of the ability of the hemolymph to inhibit microbial growth (Figs 1 and 2). We began by assessing the antimicrobial activity of naïve, injured and infected (*E. coli* and *M. luteus*) mosquitoes, irrespective of the rearing temperature or the mosquito age. We found that the hemolymph from all mosquitoes inhibited microbial growth whereas water did not (Fig. 3 and Fig. S1). Although all hemolymph had antimicrobial activity, infection significantly increased antimicrobial activity (Fig. 3). Specifically, naïve and injured mosquitoes had a similar, constitutive level of antimicrobial activity, whereas *E. coli* and *M. luteus*-infected mosquitoes had antimicrobial activity that was ∼2.3-times higher than that of uninfected mosquitoes. Therefore, antimicrobial activity is strongly shaped by immune treatment, which accounts for 32% of the variation (Table S1). In summary, infecting a mosquito more than doubles the antimicrobial activity of hemolymph. Because of the large effect of immune treatment, we conducted subsequent analyses separately for each immune treatment.

In uninfected mosquitoes, temperature and aging do not strongly shape antimicrobial activity of hemolymph

We next analyzed how higher temperature, aging and their interaction alter antimicrobial activity in uninfected mosquitoes. Irrespective of age, antimicrobial activity in both naïve and injured mosquitoes marginally increased as the temperature increased from



Type II ANOVA	*P* Value	Effect size (η_p^2)	95% CI
Temperature	0.018	0.078	0.007
Age	0.199	0.023	0.000

Fig. 4. Antimicrobial activity in naïve mosquitoes is low but marginally increases with higher temperature and aging. (A) Antimicrobial activity in naïve mosquitoes reared at different temperatures, regardless of age. (B) Antimicrobial activity in naïve mosquitoes at different ages, regardless of temperature. (C) Interaction plot for antimicrobial activity. (D) Antimicrobial activity in naïve mosquitoes at different ages, reared at different temperatures. (E) Results from a linear mixed-effects model followed by a type-II ANOVA with Kenward–Roger approximation of degrees of freedom followed by Šidák-adjusted *post hoc* multiple comparisons of means. The same measurements are shown in A–D, but grouped or arranged differently. Main effects of temperature (irrespective of age) and age (irrespective of temperature) are shown in A and B, respectively, and unaggregated data (values for each temperature-age combination) are shown in C and D. Bars show the estimated marginal means, and whiskers indicate the s.e.m. Pairwise, *post hoc* comparisons in A and B are indicated by letters; columns sharing the same letter are not significantly different.

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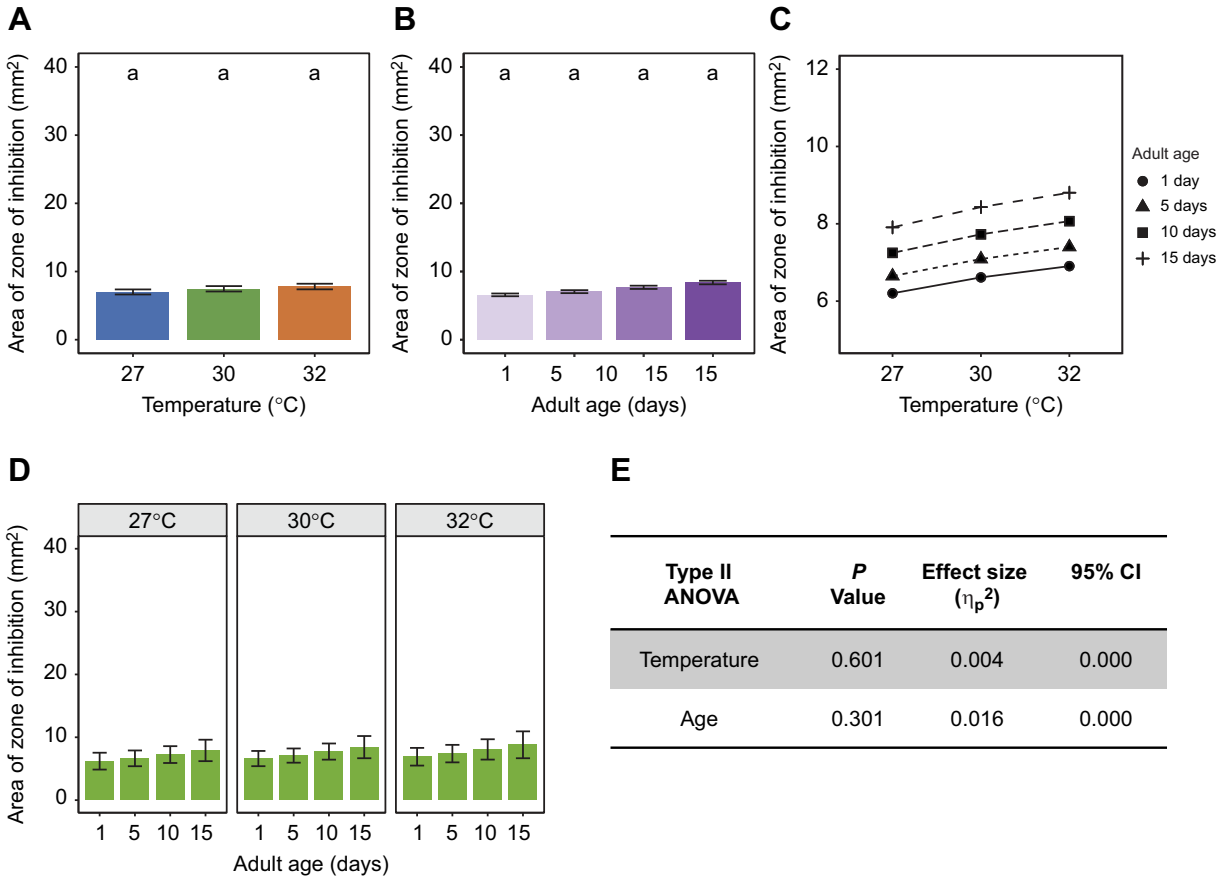

Type II ANOVA	*P* Value	Effect size (η_p^2)	95% CI
Temperature	0.601	0.004	0.000
Age	0.301	0.016	0.000

Fig. 5. Antimicrobial activity in injured mosquitoes is low but marginally increases with temperature and aging. (A) Antimicrobial activity in injured mosquitoes reared at different temperatures, regardless of age. (B) Antimicrobial activity in injured mosquitoes at different ages, regardless of temperature. (C) Interaction plot for antimicrobial activity. (D) Antimicrobial activity in injured mosquitoes at different ages, reared at different temperatures. (E) Results from a linear mixed-effects model followed by a type-II ANOVA with Kenward–Roger approximation of degrees of freedom followed by Šidák-adjusted *post hoc* multiple comparisons of means. The same measurements are shown in A–D, but grouped or arranged differently.

27°C to 32°C. That is, in naïve mosquitoes, antimicrobial activity was 38% and 71% greater at 30°C and 32°C than at 27°C, respectively (Fig. 4A). In injured mosquitoes, antimicrobial activity was 7% and 11% greater at 30°C and 32°C than at 27°C, respectively (Fig. 5A). However, the temperature groups were not significantly different from one another because the magnitudes of activity are small (File S5). Therefore, temperature only had a marginal effect on antimicrobial activity in naïve and injured mosquitoes, accounting for 8% and <1% of the variation, respectively (Figs 4E and 5E).

Irrespective of temperature, antimicrobial activity in both naïve and injured mosquitoes also marginally increased as mosquitoes aged. That is, in naïve mosquitoes, antimicrobial activity in 5-, 10- and 15-day-old mosquitoes was 10%, 24% and 39% greater than in 1-day-old mosquitoes, respectively (Fig. 4B). In injured mosquitoes, antimicrobial activity in 5-, 10- and 15-day-old mosquitoes was 7%, 17% and 27% greater than in 1-day-old mosquitoes, respectively (Fig. 5B). However, the age groups were not significantly different from one another (File S5). Therefore, aging only has a marginal effect on antimicrobial activity in naïve and injured mosquitoes, accounting for 2% and <1% of the variation, respectively (Figs 4E and 5E).

For naïve and injured mosquitoes, temperature and age did not interact to shape antimicrobial activity (naïve interaction: χ^2_1=1.3, P=0.250; injury interaction: χ^2_1=2.2, P=0.140), meaning that the effects of temperature are not modified by aging or vice versa (Figs 4C,D, 5C,D; File S5). In summary, antimicrobial activity in the hemolymph of uninfected mosquitoes did not meaningfully change with either higher temperature or aging. This is probably because without infection, immune signaling is mostly inactive and antimicrobial activity is inherently low.

In infected mosquitoes, antimicrobial activity decreases at the highest temperature and increases from 1 to 5 days of age, stabilizing with further aging

We then assessed antimicrobial activity in *E. coli*-infected and *M. luteus*-infected mosquitoes. Irrespective of age, antimicrobial activity in both *E. coli*- and *M. luteus*-infected mosquitoes marginally increased as the temperature increased from 27°C to 30°C and then significantly decreased when the temperature further increased to 32°C. That is, in *E. coli*-infected mosquitoes, the antimicrobial activity of hemolymph increased 3% between 27°C and 30°C, and then decreased 60% between 30°C and 32°C (Fig. 6A). Similarly, in *M. luteus*-infected mosquitoes, the antimicrobial activity of hemolymph increased 26% between 27°C and 30°C and then decreased 40% between 30°C and 32°C (Fig. 7A). Overall, temperature accounted for 27% and 25% of the variation in *E. coli* and *M. luteus*-infected mosquitoes, respectively (Figs 6E and 7E).

Irrespective of temperature, antimicrobial activity in both *E. coli*- and *M. luteus*-infected mosquitoes increased from 1 to 5 days of age, stabilized between 5 and 10 days of age, and then marginally

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decreased as mosquitoes aged further to 15 days. That is, in *E. coli*-infected mosquitoes, the antimicrobial activity of hemolymph increased 16% between 1 and 5 days old, but then decreased by 7% between 5 and 10 days old and by 25% between 10 and 15 days old (Fig. 6B). In *M. luteus*-infected mosquitoes, the antimicrobial activity of hemolymph increased 38% between 1 and 5 days old and by 20% between 5 and 10 days old, but then decreased by 6% between 10 and 15 days old (Fig. 7B). Overall, age accounts for 5% and 25% of the variation in *E. coli*- and *M. luteus*-infected mosquitoes, respectively (Figs 6E and 7E).

For both *E. coli*- and *M. luteus*-infected mosquitoes, the strength of the antimicrobial activity correlates with the presence of infection, but not pathogen load. That is, irrespective of age or temperature, antimicrobial activity in infected mosquitoes was not meaningfully shaped by the initial infectious dose of bacteria (Fig. 8).

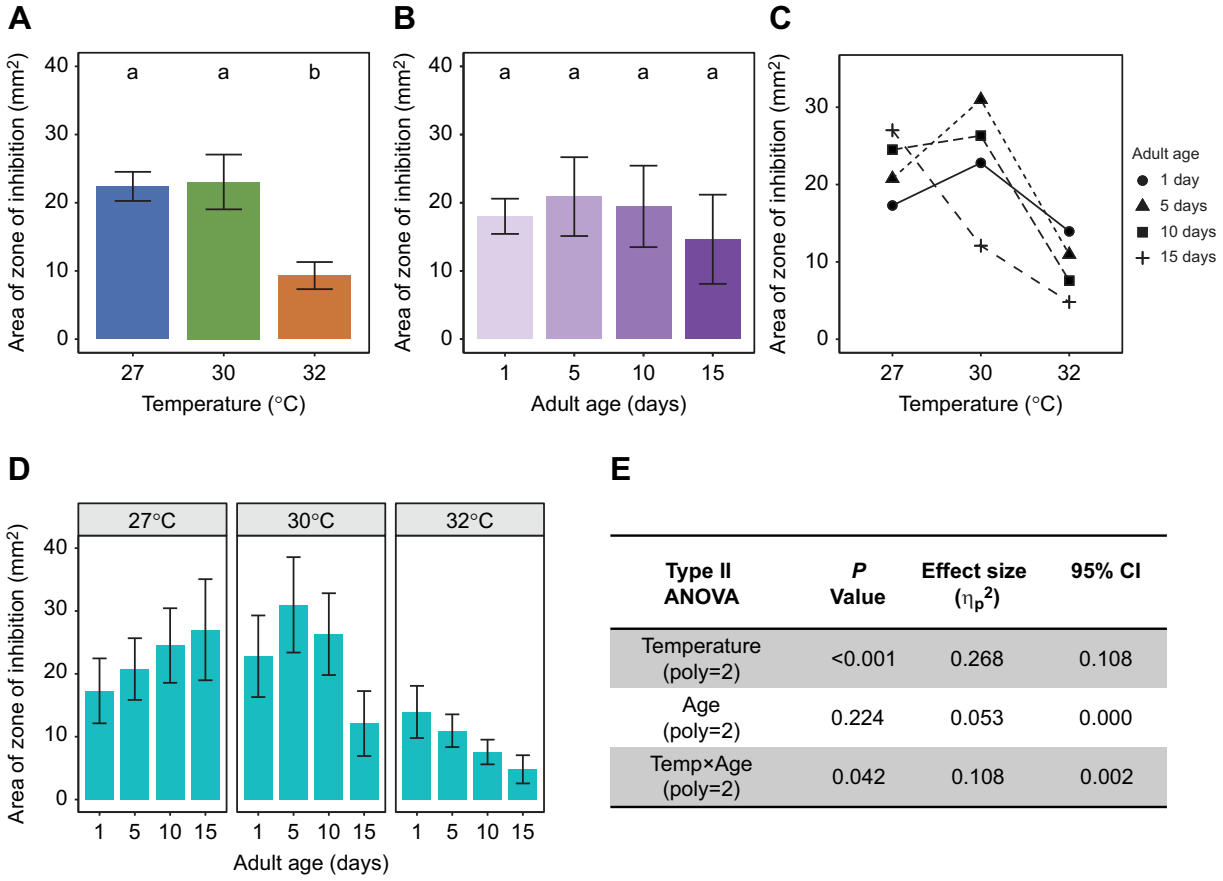
In summary, antimicrobial activity in infected mosquitoes decreases at the highest temperature of 32°C and increases after 1 day of age, stabilizing with further aging. Therefore, antimicrobial activity strengthens with infection and aging, but the ability to respond to infection weakens at higher temperature.

In *E. coli*-infected mosquitoes, higher temperature causes an aging-dependent decline in antimicrobial activity

Given that higher temperature and aging individually shape antimicrobial activity in infected mosquitoes, we next investigated whether the effects of temperature are modified by aging and vice versa. We discovered that when mosquitoes were infected with *E. coli*, temperature and age significantly interacted, with higher temperature causing an aging-dependent decline in antimicrobial activity (χ^2_2=11.0, P=0.004; Fig. 6C and D).

There are two ways to visualize the interaction between temperature and aging in *E. coli*-infected mosquitoes. First, aging has effects that depend on the temperature (Fig. 6C,D). At 27°C, antimicrobial activity increased linearly with aging, whereas at 30°C, antimicrobial activity increased between 1 and 5 days of age and then decreased with further aging. At 32°C, antimicrobial activity was highest in 1-day-old mosquitoes and progressively decreased with aging. In other words, the age of maximum antimicrobial activity occurs earlier in the mosquito's life when the temperature is warmer, shifting from 15 days old at 27°C, to 5 days old at 30°C and to 1 day old at 32°C. Importantly, at the highest temperature of 32°C, antimicrobial activity in 1-day-old mosquitoes was 19% and 39% lower than the antimicrobial activity of same-aged mosquitoes reared at 27°C and 30°C, respectively. This means that not only does the age of maximum activity occur earlier in the mosquito's life when the temperature is warmer, thereby accelerating an aging-dependent weakening, but antimicrobial activity is lower when the temperature is warmer.

Second, temperature had effects that depend on aging (Fig. 6C and D). When *E. coli*-infected mosquitoes were 1, 5, or



Type II ANOVA	*P* Value	Effect size (η_p^2)	95% CI
Temperature (poly=2)	<0.001	0.268	0.108
Age (poly=2)	0.224	0.053	0.000
Temp×Age (poly=2)	0.042	0.108	0.002

Fig. 6. In *E. coli*-infected mosquitoes, antimicrobial activity decreases at the highest temperature and stabilizes with age, and higher temperature causes an aging-dependent decline of antimicrobial activity. (A) Antimicrobial activity in *E. coli*-infected mosquitoes reared at different temperatures, regardless of age. (B) Antimicrobial activity in *E. coli*-infected mosquitoes at different ages, regardless of temperature. (C) Interaction plot for antimicrobial activity. (D) Antimicrobial activity in *E. coli*-infected mosquitoes at different ages, reared at different temperatures. (E) Results from a linear mixed-effects model followed by a type-II ANOVA with Kenward–Roger approximation of degrees of freedom followed by Šidák-adjusted *post hoc* multiple comparisons of means. The same measurements are shown in A–D, but grouped or arranged differently.

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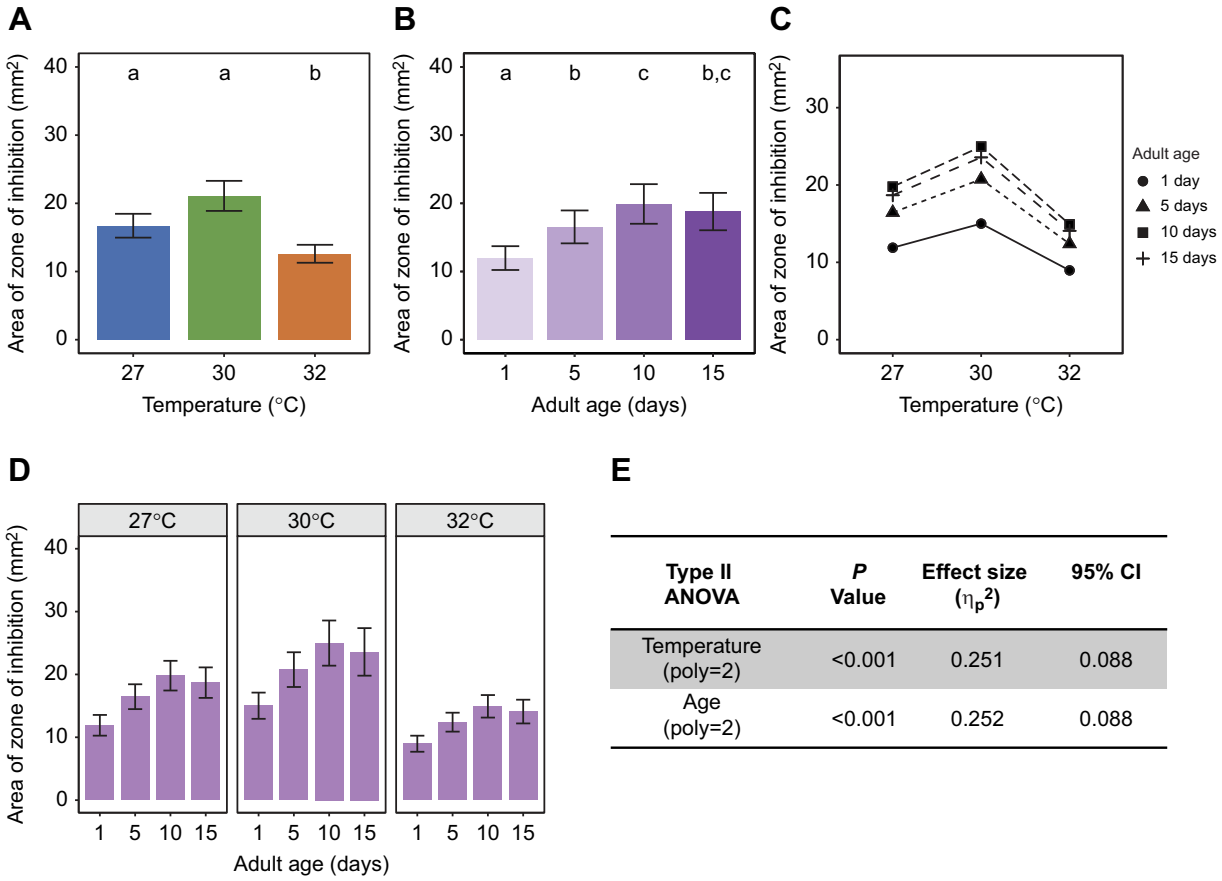

E

Type II ANOVA	*P* Value	Effect size (η_p^2)	95% CI
Temperature (poly=2)	<0.001	0.251	0.088
Age (poly=2)	<0.001	0.252	0.088

Fig. 7. In *M. luteus*-infected mosquitoes, antimicrobial activity decreases at the highest temperature and stabilizes with age, but higher temperature does not alter the effects of aging. (A) Antimicrobial activity in *M. luteus*-infected mosquitoes reared at different temperatures, regardless of age. (B) Antimicrobial activity in *M. luteus*-infected mosquitoes at different ages, regardless of temperature. (C) Interaction plot for antimicrobial activity. (D) Antimicrobial activity in *M. luteus*-infected mosquitoes at different ages, reared at different temperatures. (E) Results from a linear mixed-effects model followed by a type-II ANOVA with Kenward–Roger approximation of degrees of freedom followed by Šidák-adjusted *post hoc* multiple comparisons of means. The same measurements are shown in A–D, but grouped or arranged differently.

10 days old, antimicrobial activity was highest at 30°C and lowest at 32°C. However, at 15 days of age, antimicrobial activity was highest at 27°C and is markedly lower at 30°C and 32°C. Therefore, antimicrobial activity is highest at 30°C except at the oldest age, when the maximum antimicrobial activity shifts to 27°C. Overall, the interaction of higher temperature and aging on antimicrobial activity in *E. coli*-infected mosquitoes accounts for 11% of the variation (Fig. 6E).

When mosquitoes are infected with *M. luteus*, however, the interaction between temperature and aging did not meaningfully shape antimicrobial activity (χ^2_2=1.3, *P*=0.512; Fig. 7C,D; Table S1). This indicates that, for *M. luteus*-infected mosquitoes, the effects of temperature are not modified by aging or vice versa. In summary, when mosquitoes are infected with *E. coli* (but not *M. luteus*), antimicrobial activity is shaped by the interaction between temperature and aging. More specifically, higher temperature accelerates immune senescence, weakening the antimicrobial response.

DISCUSSION

The robustness of the mosquito's immune response depends on many factors. Here, we show that higher temperature and aging, individually and interactively, shape the mosquito's humoral immune response to infection (Fig. 9). We found that antimicrobial activity in infected mosquitoes decreases at the highest temperature of 32°C and increases beyond 1 day of age, stabilizing with further aging. Importantly, we discovered that higher temperature causes an aging-dependent weakening of antimicrobial activity when mosquitoes are infected with *E. coli*, dampening their ability to fight an infection.

The highest temperature of 32°C weakens antimicrobial activity in infected mosquitoes. Similarly, the strength of melanization decreases when the temperature warms (Murdock et al., 2012; Martin and Hillyer, 2024). This agrees with other costs associated with high temperature. That is, temperatures above the insect's thermal optimum exert costly physiological stress, and thermodynamic conditions constrain organismal growth and fitness (Deutsch et al., 2008; Wojda, 2017). High temperature also increases insect metabolism, alters endocrine and nervous system regulation, and increases oxidative stress and desiccation risk (Catalán et al., 2012; Ma et al., 2021). Therefore, high temperatures are costly; there are fewer resources available to respond to infection, and the effectors that are produced may function suboptimally.

Antimicrobial activity increases as infected mosquitoes age from 1 to 5 days old and then stabilizes with further aging. This increase in antimicrobial activity with aging is supported by an expansion of the types of AMPs expressed and an increase in their expression

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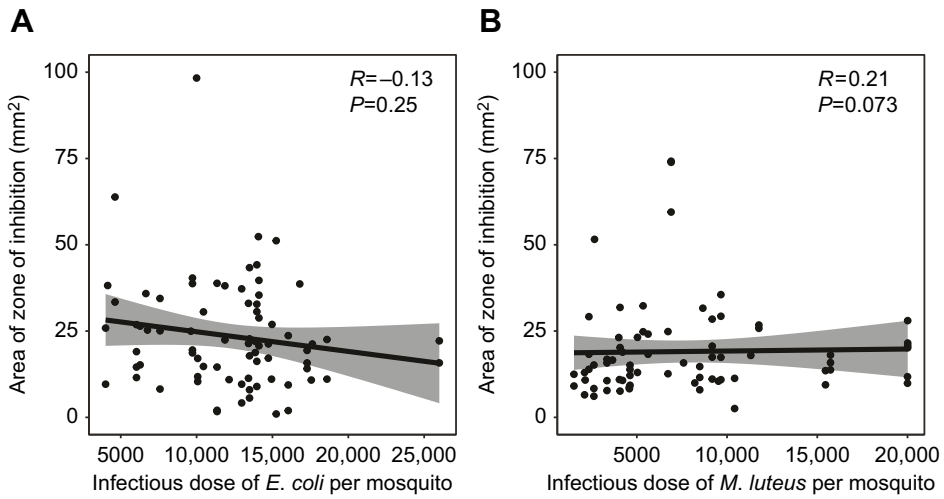

Fig. 8. The initial dose of bacteria used to infect mosquitoes does not meaningfully affect antimicrobial activity. (A) Correlation between *E. coli* infectious dose and antimicrobial activity. (B) Correlation between *M. luteus* infectious dose and antimicrobial activity. Data were analyzed using Spearman's rank correlation. Gray shading indicates 95% confidence intervals.

with aging (Shit et al., 2022; Zerofsky et al., 2005). Although at first glance this may indicate a more robust immune response, greater AMP expression in aging flies results in neurodegeneration, cytotoxicity and apoptosis, and shorter lifespans, serving as a hallmark of senescence (Landis et al., 2004; Badinloo et al., 2018; Nayak and Mishra, 2022). Thus, an aging-associated increase or stabilization in antimicrobial activity may aid in the fight against infection, but it is associated with other costs of aging.

In uninfected mosquitoes, antimicrobial activity marginally increases with aging, whereas in infected mosquitoes, antimicrobial activity marginally decreases as mosquitoes age from 10 to 15 days. In aging flies, the fat body, where AMPs are primarily produced (Eleftherianos et al., 2021), undergoes the loss of structural component lamin B, which results in increased secretion of peptidoglycan recognition proteins (PGRPs), increased IMD pathway signaling and cytotoxicity (Chen et al., 2014; Badinloo et al., 2018). Given that the fat body regulates nutrient sensing and metabolism in flies (Maruzs et al., 2019; Tatar et al., 2001), and the body composition of mosquitoes is shaped by aging (Barr et al., 2023), we suspect that the beginning decline of antimicrobial activity at 15 days of age is likely driven by aging-dependent dysregulation of the fat body.

Importantly, we discovered that higher temperature and aging interact to shape antimicrobial activity, with higher temperature causing an aging-dependent weakening of antimicrobial activity in *E. coli*-infected mosquitoes. High temperature accelerates the aging-dependent decline in both body condition and the melanization immune response, altering mosquito survival and increasing infection intensity in *A. gambiae* (Martin and Hillyer, 2024; Barr et al., 2023, 2024). The present study addresses humoral immunity more broadly, measuring the additive activity of antimicrobial effectors that synergistically kill pathogens, rather than a single effector molecule or pathway. It is likely that higher temperature and aging upregulate some effectors while downregulating others (Murdock et al., 2012), and the relative importance of each effector may depend on the type of infection, the temperature or the age (Morejon and Michel, 2023; Murdock et al., 2014). In addition, gene expression levels may not directly correlate with effector protein abundance and protein abundance may not directly correlate with antimicrobial activity (Jovanovic et al., 2015). Regardless, the overall effect is a temperature-dependent acceleration of immunosenescence, with this weakening resulting from the deterioration of a suite of humoral immune factors that typically work together to fight infection.

Here, we used live bacteria to measure the outcome of infection at increasing temperatures and ages to determine how infection would occur in a natural setting. Unsurprisingly, infection increased the antimicrobial activity in the hemolymph. Similarly to our findings, infection with *E. coli*, *M. luteus*, *Enterobacter* sp. and *Staphylococcus aureus* also increases the antimicrobial activity in the hemolymph (League et al., 2017; Morejon and Michel, 2023). While we found that *E. coli* and *M. luteus* infection resulted in similar levels of antimicrobial activity and were both individually shaped by temperature and aging, higher temperature caused an aging-dependent weakening of antimicrobial activity after *E. coli* infection but not *M. luteus* infection. However, the warming-based acceleration of senescence of melanization is more pronounced in mosquitoes infected with *M. luteus* than with *E. coli* (Martin and Hillyer, 2024). The activation of different immune pathways by different types of pathogens may explain these differences (Stączek et al., 2023; Hillyer et al., 2003a,b, 2004).

In conclusion, our study demonstrates that higher temperature weakens antimicrobial activity in response to infection. Importantly, when mosquitoes are infected with *E. coli*, higher temperature uncouples chronological aging from physiological aging by accelerating senescence (Fig. 9). Future studies must continue to holistically evaluate how multiple abiotic and biotic stressors, such as higher temperature and aging, interact to impact infection outcomes. This is increasingly important because mosquitoes, along with other poikilothermic and ectothermic insects, face rising

Humoral immunity

Does higher temperature reduce antimicrobial activity?	**Yes** (with infection)
Does aging reduce antimicrobial activity?	**No**
Does higher temperature accelerate senescence?	**Yes** (in *E. coli*-infected mosquitoes)

Fig. 9. Summary of the effects of higher temperature, aging and their interaction on the antimicrobial humoral immune response.

global temperatures, which may alter their ability to transmit diseases, serve as pollinators or populate different geographic regions.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: L.E.M., J.F.H.; Methodology: L.E.M., M.R., J.F.H.; Software: L.E.M.; Validation: L.E.M., J.F.H.; Formal analysis: L.E.M.; Investigation: L.E.M., M.R.; Resources: J.F.H.; Data curation: L.E.M., J.F.H.; Writing - original draft: L.E.M., J.F.H.; Writing - review & editing: L.E.M., M.R., J.F.H.; Visualization: L.E.M., J.F.H.; Supervision: J.F.H.; Project administration: L.E.M., J.F.H.; Funding acquisition: L.E.M., J.F.H.

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Data availability
All relevant data and code can be found within the article and its supplementary information.

ECR Spotlight
This article has an associated ECR Spotlight interview with Lindsay Martin.

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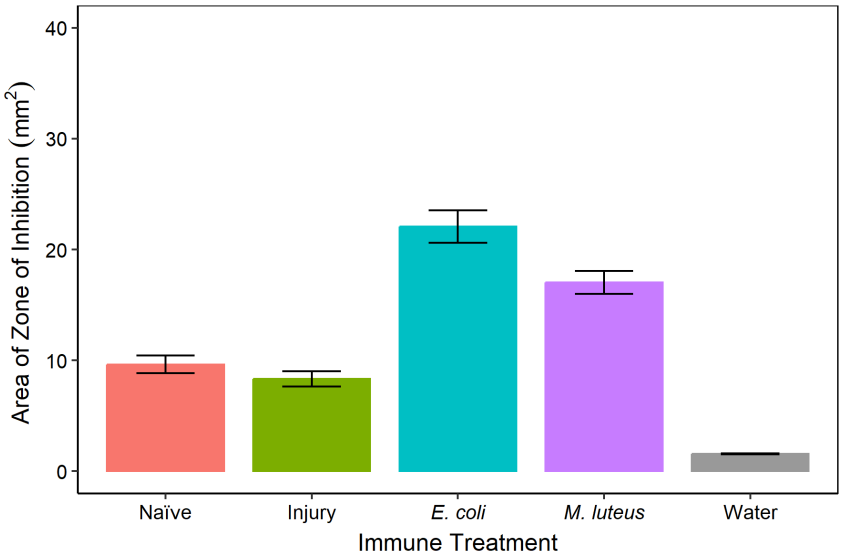

Fig. S1. Observed means of antimicrobial activity: all mosquitoes have some level of antimicrobial activity, regardless of infection. Area of zone-of-inhibition of hemolymph from naïve, injured and infected mosquitoes, irrespective of temperature or age, as well as water. The column heights mark the observed means, and whiskers indicate the S.E.M. The same measurements are plotted in Figs S1 and S2, but grouped or arranged differently, with aggregated data shown in this figure. The estimated marginal means of these data, resulting from the linear mixed model fitted to the entire data set, are presented in Figs 3 and S3.

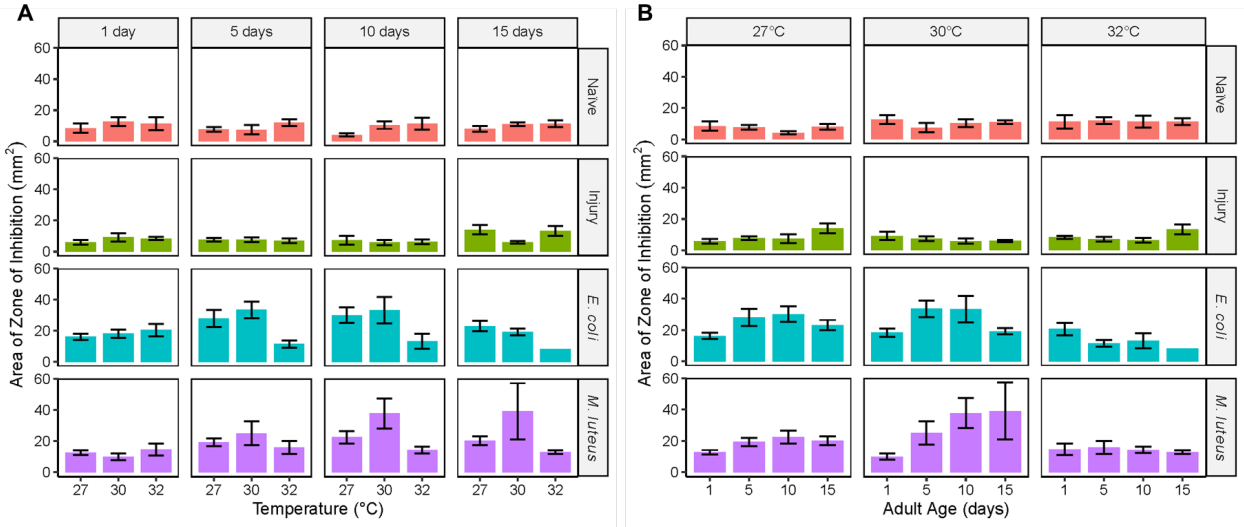

Fig. S2. Observed means of antimicrobial activity: area of zone-of-inhibition of hemolymph from naïve, injured and infected mosquitoes, stratified by temperature and age. **A.** Antimicrobial activity, showing the effects of temperature within each age. **B.** Antimicrobial activity, showing the effects of age within each temperature. The column heights mark the observed means, and whiskers indicate the S.E.M. The same measurements are plotted in A and B and in Fig S1, but grouped or arranged differently. The estimated marginal means of these data, resulting from linear mixed models on the full data set and on data stratified by immune treatement, are presented in Figs 3-7 and S3.

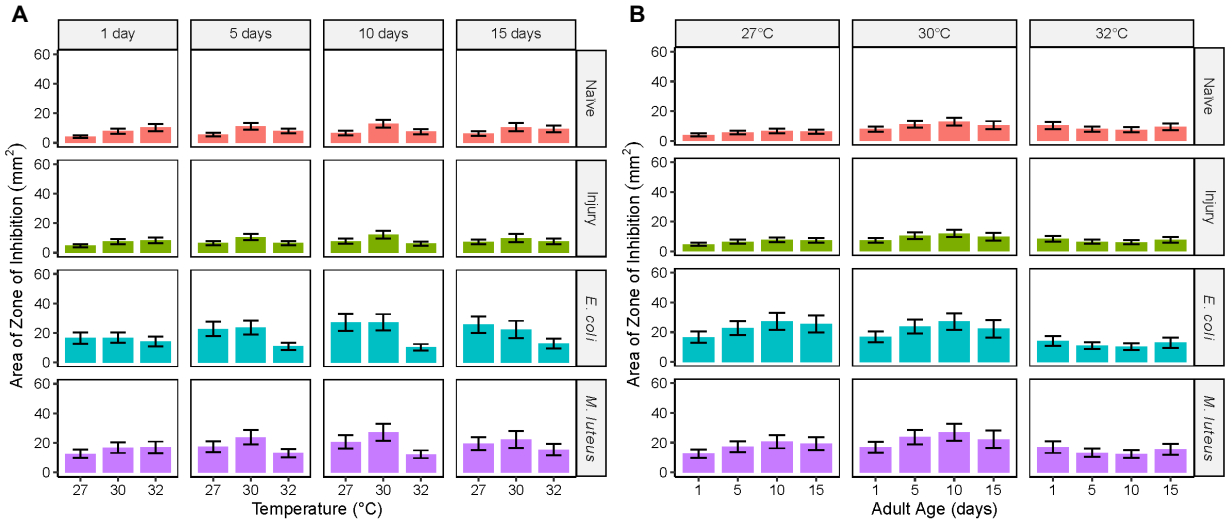

Fig. S3. Estimated marginal means of antimicrobial activity: area of zone-of-inhibition of hemolymph from naïve, injured and infected mosquitoes, stratified by temperature and age. **A.** Antimicrobial activity, showing the effects of temperature within each age. **B.** Antimicrobial activity, showing the effects of age within each temperature. The column heights mark the estimated marginal means, and whiskers indicate the S.E.M. The same measurements are plotted in A and B, but grouped or arranged differently. The estimated marginal means of these data result from the full linear mixed model fitted to the entire data set, including all immune treatments, and are also presented as aggregated data in Fig 3.

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