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Ambient temperature and dietary supplementation interact to shape mosquito vector competence for malaria

Courtney C. Murdock [a,*], Simon Blanford [a], Shirley Luckhart [b], Matthew B. Thomas [a]

[a] Center for Infectious Disease Dynamics, Department of Entomology, Pennsylvania State University, Merkle Lab, Orchard Road, University Park, PA 16802, United States
[b] Department of Medical Microbiology and Immunology, School of Medicine, University of California Davis, Davis, CA 95616, United States

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ABSTRACT

The extent to which environmental factors influence the ability of *Anopheles* mosquitoes to transmit malaria parasites remains poorly explored. Environmental variation, such as change in ambient temperature, will not necessarily influence the rates of host and parasite processes equivalently, potentially resulting in complex effects on infection outcomes. As proof of principle, we used *Anopheles stephensi* and the rodent malaria parasite, *Plasmodium yoelii*, to examine the effects of a range of constant temperatures on one aspect of host defense (detected as alterations in expression of nitric oxide synthase gene – *NOS*) to parasite infection. We experimentally boosted mosquito midgut immunity to infection through dietary supplementation with the essential amino acid L-Arginine (L-Arg), which increases midgut nitric oxide (NO) levels by infection-induced NOS catalysis in *A. stephensi*. At intermediate temperatures, supplementation reduced oocyst prevalence, oocyst intensity, and sporozoite prevalence suggesting that the outcome of parasite infection was potentially dependent upon the rate of *NOS*-mediated midgut immunity. At low and high temperature extremes, however, infection was severely constrained irrespective of supplementation. The effects of L-Arg appeared to be mediated by NO-dependent negative feedback on *NOS* expression, as evidenced by depressed *NOS* expression in L-Arg treated groups at temperatures where supplementation decreased parasite infection. These results suggest the need to consider the direct (e.g. effects of mosquito body temperature on parasite physiology) and indirect effects (e.g. mediated through changes in mosquito physiology/immunity) of environmental factors on mosquito-malaria interactions in order to understand natural variation in vector competence.

1. Background

Transmission of malaria involves an intimate interaction between the mosquito vectors and the malaria parasites. When mosquitoes feed on a malaria parasite-infected vertebrate host, gametocytes localize to the posterior midgut and within minutes become gametes. Male gametes will then fertilize female gametes to form zygotes. Under standard laboratory conditions, zygotes become ookinetes within the first 12–24 h post-infection and traverse both the peritrophic matrix and the midgut epithelium from 24–32 h post-infection to establish as oocysts under the basal lamina (Vaughan, 2007). Established oocysts mature by approximately 14 days post-infection, releasing sporozoites into the hemolymph. These sporozoites eventually invade the salivary glands and are transmitted through the bite of an infectious mosquito to the next vertebrate host (Vaughan, 2007). Throughout this process, mosquitoes mount coordinated midgut (e.g. Oliveira et al., 2012; Peterson et al., 2007; Luckhart and et al., 1998; Kumar and Barillas-Mury, 2005; Price et al., 2013; Horton and et al., 2011; Surachetpong and et al., 2009) and hemolymph-mediated immune responses (e.g. Oliveira et al., 2012; Garver et al., 2009; Mitri and et al., 2009; Dong and et al., 2006) involving midgut enzymes including peroxidases, oxidases, and nitric oxide synthase (NOS), as well as activation of immune pathways (Toll, IMD, MAPK) and the mosquito complement-like effector molecule thioester binding protein 1 (TEP1).

Current understanding of these physiological and molecular interactions between mosquitoes and malaria parasites derives largely from studies conducted under standardized laboratory settings. However, malaria transmission occurs across diverse environments (Blanford and et al., 2013; Cator and et al., 2013) and we expect the net outcome of insect-parasite interactions to depend on both genetic and environmental factors (Schulenburg and et al., 2009; Thomas and Blanford, 2003). Specifically, the

* Corresponding author. Tel.: +1 814 865 1024.
E-mail addresses: ccm15@psu.edu (C.C. Murdock), stb13@psu.edu (S. Blanford), sluckhart@ucdavis.edu (S. Luckhart), mbt13@psu.edu (M.B. Thomas).

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effectiveness of mosquito immune responses toward malaria parasites will be dependent upon the rates of host enzymes involved in nitration and lysis of *Plasmodium* spp. parasites as well as the rates of *Plasmodium* ookinete formation and migration through the midgut epithelium (Oliveira et al., 2012). Given the influence of temperature on enzyme kinetics and ectotherm physiology in general, changes in ambient temperature are expected to affect the rates of these mosquito and parasite processes. The net effect on overall mosquito vector competence will depend on the relative thermal sensitivity of both host and parasite traits (Murdock and et al., 2012).

Here we investigated how temperature and dietary supplementation influenced the outcome of the mosquito-malaria interaction. To do this we assessed how metrics of parasite fitness and one aspect of midgut-mediated immunity, the production of nitric oxide (NO) by the enzyme nitric oxide synthase (NOS), were affected by varying mean ambient temperature and providing a subset of mosquitoes with access to an essential amino acid, L-Arginine (L-Arg). NO is a free radical gas that can react with a variety of oxidants to generate cytotoxic reactive nitrogen species RNS (Colasanti and et al., 2001, 2002). RNS are toxic to a wide diversity of pathogens (James, 1995) and are key to the mosquito midgut immune response toward *Plasmodium* parasites (Oliveira et al., 2012; Peterson et al., 2007; Luckhart and et al., 1998; Ali and et al., 2010). NO-mediated defenses may also limit the number of sporozoites that successfully invade the salivary glands because NO can be produced and secreted into the hemolymph by circulating hemocytes (Hillyer and Estevez-Lao, 2010; Hillyer et al., 2007). Further, NO production has been implicated in the response of other *Anopheles* species to infection with *Plasmodium* (Oliveira et al., 2012; Dimopoulos and et al., 1998; Herrera-Ortiz and et al., 2011; Vijay and et al., 2011), in the response of *Rhodnius prolixus* to infection with *Trypanosma rangeli* (Whitten and et al., 2001; Ascenzi and Gradoni, 2002), and of *Drosophila melanogaster* to infection with *Asobara rabid* (Kraaijeveld et al., 2011).

Both temperature and L-Arg supplementation have been shown to influence components of NOS-mediated immunity (Luckhart and et al., 1998; Kraaijeveld et al., 2011; Murdock et al., 2013; Murdock and et al., 2012). The expression of NOS increases with warming temperatures and is maximal between 30 °C and 34 °C (Murdock et al., 2013; Murdock and et al., 2012), suggesting that higher NOS activity could enhance NO-mediated immunity at warmer temperatures. L-Arg is an essential amino acid used in mosquito reproduction (Uchida, 1993), which can only be obtained through diet (Vrzal et al., 2010). Malaria infection induces severe hypoargininemia in the vertebrate host (Chau and et al., 2013), resulting in low L-Arg levels obtained by the mosquito in the bloodmeal. L-Arg is further limited because it is also utilized in midgut-mediated immune defenses against the parasite (Luckhart and et al., 1998) and potentially in parasite development (Vincendeau and et al., 2003; Rivero, 2006). Previous studies have shown that L-Arg supplementation results in enhanced production of NO, increasing both NO-mediated immunity and reducing *Plasmodium* prevalence and intensity (Luckhart and et al., 1998).

Due to established effects of environmental temperature on *Plasmodium yoelii* development (Paaijmans and et al., 2012) and boosted immunity and resistance in response to L-Arg supplementation in *Anopheles stephensi* (Luckhart and et al., 1998) and in *D. melanogaster* (Kraaijeveld et al., 2011), we make the following predictions: (1) overall, parasite establishment (oocyst prevalence) should begin declining at warmer than optimal temperatures for the parasite (26 °C and 28 °C); (2) oocyst intensities and sporozoite prevalence should be highest around the thermal optimum for parasite development (24 °C); (3) in general, *NOS* expression and activity should increase with temperature (Murdock and et al., 2012); and (4) overall, L-Arg supplementation should decrease oocyst prevalence, oocyst intensities, and sporozoite prevalence except at cool temperatures were *NOS* expression and NOS activity are lowest. We demonstrate that NO-dependent host immunity and parasite transmission potential are affected by changes in ambient temperature. Further, while the effects of supplementation were apparent at intermediate temperatures, high and low temperature extremes relieved the effects of supplementation on parasite infection and on negative feedback regulation of *NOS* expression, indicating that the upper and lower bounds for parasite transmission were set irrespective of boosted immune function.

2. Methods

2.1. Mosquito rearing and handling

We reared *A. stephensi* (Liston) under standard insectary conditions at 27 ± 1 °C, 80% humidity, and a 12 h light: 12 h dark photo-period. We placed mosquito eggs into plastic trays (25 cm × 25 cm × 7 cm) filled with 1.5 L of water. To minimize any potential variation in emerging adult mosquito body size, we divided recently hatched larvae to ensure a density of 400 individuals per tray. Larvae were fed Liquifry for the first five days post-hatching, and then were fed Tetrafin fish flakes for the duration of the larval period. Pupae were collected from larval trays and placed into experimental cages approximately two weeks after egg hatch. Upon emergence, 2000 adult females each were divided into two cages of 1000 mosquitoes supplemented with either sugar cubes and water alone, or sugar cubes and the NOS enzyme substrate L-Arg (0.002%) in water Luckhart and et al., 1998; this dietary supplement regime was continued throughout the entire course of the experiment (Supplementary information).

2.2. Malaria parasite infections

Forty mice (female C57Bl/6 laboratory mice, Charles River Laboratories) were inoculated with 10^5 *P. yoelii* parasites (clone 17XNL, from the WHO Registry of Standard Malaria Parasites, University of Edinburgh, UK). Four days after inoculation, each cage of 1000 mosquitoes (3 days post-emergence) were fed on 20 anesthetized, infectious mice at 24 ± 0.5 °C and 80 ± 5% relative humidity for 30 min. Two hours post-infection, mosquitoes that had taken a full blood-meal from each dietary treatment were randomly allocated to one of five reach-in incubators set at the following temperatures: 20 °C, 22 °C, 24 °C, 26 °C, and 28 ± 0.5 °C with relative humidity 80 ± 5% and 12 L: 12 D cycle photoperiod. Two and three days later mosquitoes were provided with egg laying bowls. This experiment was replicated two times through time, with each temperature replicated in different incubators (Supplementary information).

To estimate infection prevalence and intensity, we dissected both midguts and salivary glands under a standard dissecting microscope, with 25 mosquitoes randomly selected from each replicate per temperature treatment per dissecting time interval. We recorded the number of midguts with oocysts, oocysts per midgut, and salivary glands with sporozoites to estimate the prevalence of infected and infectious mosquitoes, as well as the intensity of infection, for each temperature treatment and replicate. Because the rate of parasite development is affected by ambient temperature (Paaijmans and et al., 2012), we dissected midguts from a small sample of mosquitoes to determine when parasites had reached a specific developmental stage in each temperature treatment (mature oocysts and no salivary gland sporozoites versus oocysts with salivary gland invasion). We began recording daily mosquito mortality post-infection and terminated counts after

sporozoite dissections, which ran from day 15 to day 30 depending on the temperature treatment (Supplementary information).

2.3. Gene expression assays: RNA collection, cDNA synthesis, and quantitative PCR

To monitor *NOS* expression patterns with temperature and L-Arg supplementation throughout the course of infection, we removed 10 mosquitoes at 12 h, 24 h, 48 h, as well as 1–2 days post sporozoite release from each experimental group ($n = 500$ total). After removal, mosquitoes were killed rapidly using a 5–10 s exposure to chloroform and immediately stored in RNA*later* RNA stabilization reagent at −20 °C until termination of the experiment (25 days). Messenger RNA was extracted using the Qiagen RNeasy Mini Kit for animal tissues (as per the manufacturer's protocol) with the optional DNase step included. Mosquitoes were isolated individually in β-Mercaptoethanol and RLT lysis buffer. Standards for quantitative PCR (qPCR) were prepared by extracting mRNA from a pool of four mosquitoes. The concentration of mRNA in each sample was quantified with a Nanodrop and stored at −80 °C, and RNA integrity from a subset of individuals was assessed using an Agilent 2100 bioanalyzer (RNA integrity scores ranged from 7 to 9). 100 ng of RNA was converted to cDNA with a high-capacity cDNA reverse transcription kit (10 μL of mRNA suspended in water into a 10 μL reaction mix) as per the manufacturer's protocol (Applied Biosystems) on a Mastercycler Gradient thermal cycler (Eppendorf) under the following reaction conditions: 10 min at 25 °C, 120 min at 37 °C, 5 min at 85 °C, and held at 4 °C until storage at −80 °C.

We quantified our diluted cDNA (1:10 dilution of neat cDNA) from our experimental samples by comparing their threshold cycle numbers against a standard curve generated from 1:10 serial dilutions of our standard sample (cDNA from a pool of four mosquitoes) (Murdock and et al., 2012). Three replicates of each cDNA standard spanning six orders of magnitude were included in each qPCR run. We quantified cDNA for our target gene (*NOS*) and a standard reference gene *ribosomal protein S7* (*rpS7*) from individual mosquitoes using the standard curve for each assay. We normalized our target gene expression (see Section 2.4) against a single reference gene because *rpS7* was not influenced in this study by experimental conditions (Supplementary information), its abundance is strongly correlated with total amounts of mRNA (Supplementary information), and it is used in wide diversity of expression studies on *Anopheles* as an internal control (e.g. Oliveira et al., 2012; Garver et al., 2009; Arrighi and et al., 2009; Coggins et al., 2012; Dong and et al., 2011). Within and between plate replicates of samples were incorporated in each plate to confirm an absence of significant variation between and within assays. DNA contamination was confirmed to be undetectable using qPCR on RNA samples. All real-time quantification was performed using an Applied Biosystems 7500 Fast Real-Time PCR System and Sequence Detection software (version 1.4) with an initial denaturation of 95 °C for 20 s followed by 40 cycles of denaturation at 95 °C for 3 s and annealing/extension at 60 °C for 30 s. Two microliters of working concentration cDNA were included in each 25 μL volume PCR with the following components: 1.5 μL each of forward and reverse primer (final concentration of 300 nM), 12.5 μL of 2 × PerfeCTa™ qPCR FastMix™, Low Rox, 1 μL of MGB probe (final concentration of 200 nM), and 6.5 μL of sterile water. Primers and probes for *NOS* and *rpS7* were designed using Primer Express 3.0 (Applied Biosystems) off of the following GenBank sequences for *A. stephensi* and *Anopheles gambiae*: *NOS* (Accession number: AY583529) and *rpS7* (Accession number: AF539918).

Primers used in the quantification process were *NOS* forward 5′-GGTTCCCATCCGAAGCATT-3′, *NOS* reverse 5′-GCAACACAGGG CAGGTTACAT-3′, *rpS7* forward 5′-CGTGAGGTCGAGTTCAACAACA-3′, and *rpS7* reverse 5′-CGTGCTTGCCGGAGAACTT-3′. Probes used in quantification were *NOS* 5′ FAM-CCCCATTCGTCCTTG-MGB 3′ and *rpS7* 5′ FAM-CGATCATCATCTACGTGCC-MGB 3′ resulting in amplicon lengths of 133 and 127 base pairs for *NOS* and *rpS7*, respectively. We performed BLAST analysis to ensure our primers and probes targeted sequences from the genes of interest, and assay efficiencies were confirmed to be not significantly different at 96% for both *NOS* and *rpS7* (Independent Samples *t*-test: $n = 16$, $t_{1,14} = 0.211$, $p = 0.836$). In addition to efficiency, we also calculated the average slopes, *y*-intercepts, and R^2 values from eight standard/calibration curves for each assay: *NOS* slope = −3.42, *y*-intercept = 42.82, $R^2 = 0.996$; *rpS7* slope = −3.43, *y*-intercept = 40.89, $R^2 = 0.997$). We did not determine a limit of detection (LOD) for our experimental samples because all of our positive samples fell well within ($C_q < 33$) the linear dynamic range for each assay (*NOS1*: range of C_q's = 25–35; *rpS7* range of C_q's = 24–34).

2.4. Statistical analyses

Statistical analyses were performed with IBM SPSS Statistics 20.0 (IBM Corporation). Full factorial models from generalized linear model (GZLM) analysis were reduced through backward elimination of non-significant interactions. We assessed goodness of fit of the final models through model deviance, log likelihood values, and model residuals. Covariates included in GZLMs were centered on their grand mean, and the significance of pair-wise interactions was assessed with Bonferroni-adjusted post-hoc tests.

2.4.1. Malaria parasite infections

We used a binary logistic GZLM (logit function) to estimate how changes in ambient temperature and L-Arg supplementation influenced the mean probability of a mosquito being infected (presence of oocysts). To compare how the intensity of infection (number of oocysts per midgut) varied with treatment, we used GZLM analysis assuming a negative binomial distribution (log link function). Finally, to compare how experimental treatment affected the number of infectious mosquitoes (mosquitoes with sporozoites in their salivary glands), we performed a GZLM analysis on transformed data assuming a linear distribution. Full factorial analyses were performed for each response variable with temperature (20 °C, 22 °C, 24 °C, 26 °C, and 28 °C), supplementation treatment (*water* or *L-arginine*), and replicate included as fixed factors.

2.4.2. NOS expression

To compare differences in *NOS* expression, we used GZLM analysis assuming a gamma distribution (log link function) to estimate how mean *NOS* cDNA varied with experimental treatment. We performed a full factorial analysis with ambient temperature (20 °C, 22 °C, 24 °C, 26 °C, and 28 °C), supplementation treatment (*water* or *L-Arg*), sampling time point (12 h, 24 h, 48 h *post-infection*, or *sporozoite invasion*), and replicate as fixed factors. To normalize our target gene expression for any inter-sample variation introduced through sampling, RNA extraction, or reverse transcription, we included *rpS7* cDNA counts of each sample as a covariate in all models (Murdock and et al., 2012) to adjust the means predicted by the model for *NOS* expression by *rpS7* expression. This normalization approach controls for inter-sample variation, is more conservative because it does not introduce artificial skew to the response variable as ratio data, and allows for parametric (more powerful) statistical analysis.

2.4.3. Mosquito survival

To assess the effects of temperature and L-Arg supplementation on mosquito mortality, we used an interval censored survival GZLM analysis (binomial distribution, complementary log–log function) to compare how the average probability of mosquito

death varied with experimental treatment. Temperature (20 °C, 22 °C, 24 °C, 26 °C, or 28 °C), supplementation treatment (*water* or L-*Arg*), and replicate were included as fixed factors, while number of days post-infection was included as a covariate in the model. We also used a Kaplan–Meier survival analysis (log rank, Mantel–Cox test) to determine if temperature, supplementation treatment, and replicate affected daily cumulative mosquito survival.

3. Results

3.1. Malaria parasite infections

Changes in average ambient temperature and L-Arg supplementation significantly affected both the mean prevalence of mosquitoes infected with *P. yoelii* oocysts and sporozoites, as well as the mean number of oocysts per midgut (Table 1). L-Arg supplementation significantly reduced oocyst and sporozoite prevalence, and the intensity of oocysts per midgut (Fig. 1). Overall, the probability of oocyst establishment in mosquito midguts was relatively unaffected by changes in ambient temperature, with significant reductions occurring only in mosquitoes maintained at 28 °C; this effect of temperature was consistent whether mosquitoes received water or L-Arg supplementation (Fig. 1A, both treatment groups: 20 °C vs. 28 °C, $p < 0.0001$; 22 °C vs. 28 °C, $p = 0.001$; 24 °C vs. 28 °C, $p < 0.0001$; and 26 °C vs. 28 °C, $p < 0.0001$).

Mean ambient temperature significantly influenced the number of oocysts that successfully established (Table 1); however, the effects of temperature were qualitatively different when mosquitoes were supplemented with water compared with mosquitoes supplemented with L-Arg, as indicated by the significant two-way interaction between temperature and supplementation treatment (*temperature* × *supplementation*, Fig. 1B). For example, oocyst intensities were highest in control mosquitoes housed at 22 °C and 24 °C, while there is no significant increase in oocyst intensities in L-Arg supplemented mosquitoes housed at these temperatures. Oocyst intensities were significantly lower at 28 °C relative to cooler temperatures in both water (20 °C vs. 28 °C, $p < 0.001$; 22 °C vs. 28 °C, $p < 0.0001$; 24 °C vs. 28 °C, $p < 0.0001$; 26 °C vs. 28 °C, $p < 0.0001$) and L-Arg supplemented mosquitoes (20 °C vs. 28 °C, $p < 0.001$; 22 °C vs. 28 °C, $p < 0.0001$; 24 °C vs. 28 °C, $p < 0.0001$; 26 °C vs. 28 °C, $p < 0.0001$; Fig. 1B).

We also observed a similar effect of temperature on the number of mosquitoes with sporozoites in their salivary glands (Fig. 1C). In both L-Arg- and the control treatment group, there were significantly more infectious mosquitoes reared at 24 °C relative to warmer and cooler temperatures (Fig. 1C, both treatment groups: 20 °C vs. 24 °C, $p < 0.0001$; 22 °C vs. 24 °C, $p < 0.0001$; 24 °C vs. 26 °C, $p < 0.0001$; 24 °C vs. 28 °C, $p < 0.0001$). L-Arg supplementation, in general, decreased the number of infectious mosquitoes (except for those maintained at a cold and warm temperature extremes) compared with mosquitoes provided with water only (Fig. 1C).

We did observe a significant effect of experimental replicate on mean oocyst intensities, with mosquitoes experiencing on average 23 oocysts per midgut (SE ± 3.23) vs. 32 oocysts per midgut (SE ± 3.84) in the first and second experimental replicate, respectively. However, we did not observe any interactions between experimental treatment and replicate, demonstrating that there were no significant qualitative differences in temperature and L-Arg treatment on oocyst intensities across both experimental replicates.

3.2. NOS expression

GZLM analysis suggested that *NOS* expression was significantly affected by sampling time point, L-Arg supplementation, and

Table 1
Results from GZLM model analysis demonstrate significant effects of temperature and L-arginine supplementation on measures of vector competence and on the expression of *nitric oxide synthase (NOS)*. Dashes indicate factors or interaction terms that were either backward eliminated from full models or were not included in the overall experimental design.

Factors	Oocyst prevalence ($n = 500$)			Oocyst intensity ($n = 497$)			Mosquitoes with sporozoites ($n = 476$)			*NOS* expression ($n = 485$)		
	Wald X^2	d.f.	*p*-Value	Wald X^2	d.f.	*p*-Value	Wald X^2	d.f.	*p*-Value	Wald X^2	d.f.	*p*-Value
Intercept	15.54	1	<0.0001	4145.89	1	<0.0001	1122.16	1	<0.0001	27312.86	1	<0.0001
Temperature	29.40	4	<0.0001	324.98	4	<0.0001	1509.75	4	<0.0001	18.52	4	0.001
Supplementation	18.05	1	<0.0001	10.69	1	0.001	294.39	1	<0.0001	12.27	1	<0.0001
Sampling time point	–	–	–	–	–	–	–	–	–	580.67	3	<0.0001
Replicate	0.92	1	0.338	30.02	1	<0.0001	2.16	1	0.141	0.84	1	0.360
Centred *rpS7* cDNA counts	–	–	–	–	–	–	–	–	–	12.59	1	<0.0001
Temperature × supplementation	1.71	4	0.789	17.15	4	0.002	403.74	4	<0.0001	–	–	–
Supplementation × sampling time point	–	–	–	–	–	–	–	–	–	35.92	3	<0.0001
Temperature × sampling time point	–	–	–	–	–	–	–	–	–	33.32	12	0.001

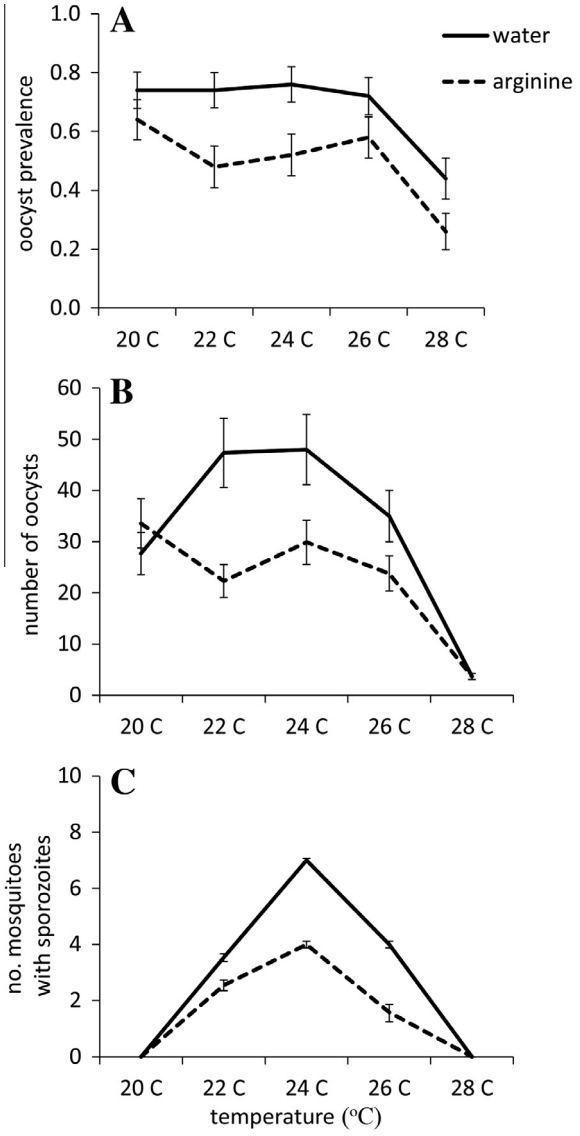

Fig. 1. Both mean ambient temperature and supplementation treatment (water, solid lines; L-arginine, dotted lines) significantly affected the probability that a malaria infection will establish (mean oocyst prevalence, A), the intensity of malaria infection (the mean number of oocysts per midgut, B), and the probability of becoming infectious (mean no. of mosquitoes with sporozoites adjusted by mosquitoes sampled, C). Vertical bars represent standard errors of the mean.

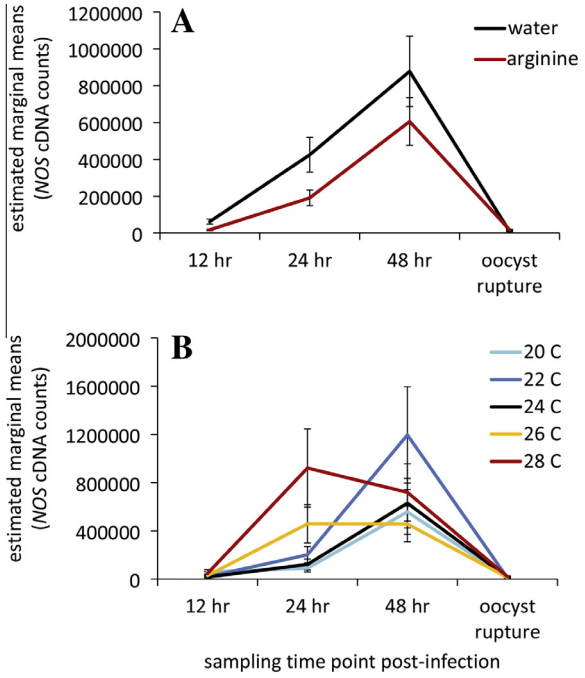

Fig. 2. Supplementation treatment (A) and changes in mean ambient temperature (B) both significantly influenced the expression dynamics of *nitric oxide synthase* (*NOS*) throughout the course of malaria infection. (A) In both supplemenation treatment groups, *NOS* expression on average experienced a peak in expression at 48 h post-infection; yet, this peak was significantly lower in L-arginine-fed mosquitoes (red line) relative to mosquitoes supplemented with water (black line). (B) Further, this peak in *NOS* expression at 48 h post-infection is being driven by mosquitoes housed at 20 °C (light blue line), 22 °C (dark blue line), and 24 °C (black line). Mosquitoes housed in warmer thermal environments (26 °C, orange line and 28 °C, red line) experienced relatively similar amounts of *NOS* expression at 24 h as they did at 48 h post-infection. Vertical bars represent standard errors of the mean.

temperature (Table 1). In general, *NOS* expression peaked 48 h post-infection in both L-Arg and water supplemented individuals (6 h vs. 48 h, $p < 0.0001$; 12 h vs. 48 h, $p < 0.0001$; 48 h vs. oocyst rupture, $p < 0.0001$), was on average lower in L-Arg supplemented mosquitoes, and significantly increased at warmer temperatures (28 °C) relative to cooler temperatures (20 °C vs. 28 °C, $p = 0.002$; 24 °C vs. 28 °C, $p = 0.001$), which is consistent with previous work (Murdock et al., 2013; Murdock and et al., 2012).

Intriguingly, *NOS* expression was also significantly influenced by two, two-way interactions (Table 1) between sampling time point and supplementation (*supplementation x sampling time point*, Fig. 2A) and sampling time point and temperature (*temperature × sampling time point*, Fig. 2B). Both L-Arg and the control treatment group exhibited greater mean *NOS* expression at 24 h relative to 12 h post-infection, a period that spans ookinete maturation (12–20 h) and transit across the midgut epithelium (20–24 h) Luckhart and et al., 2013 (water: 12 h vs. 24 h, $p = 0.003$; L-Arg: 12 h vs. 24 h, $p = 0.002$); however, this effect was greater in mosquitoes supplemented with water only (Fig. 2A). Across both supplementation treatment groups *NOS* reached peak expression levels at 48 h post-infection, a time consistent with early oocyst development for *P. yoelii* (water: 12 h vs. 48 h, $p < 0.0001$; L-Arg: 12 h vs. 48 h, $p < 0.001$ and 24 h vs. 48 h, $p = 0.003$), with substantial declines in *NOS* expression at later stages of oocyst development (both treatment groups: 48 h vs. sporozoite invasion, $p < 0.0001$). The peak in *NOS* expression at 48 h post-infection was greatest in mosquitoes maintained at 22 °C (Fig. 2B). Further, *NOS* expression increased earlier (24 h post malaria infection) in mosquitoes maintained at warmer temperatures (24 h: 26 °C vs. 20 °C, $p = 0.005$; 26 °C vs. 24 °C, $p = 0.045$; 28 °C vs. 20 °C, $p = 0.011$; 28 °C vs. 22 °C, $p = 0.029$; 28 °C vs. 24 °C, $p = 0.016$).

3.3. Mosquito mortality

GZLM analysis (deviance/d.f. = 4.238; Likelihood Ratio $X^2_{1,7} = 194.09$, $p < 0.0001$) revealed that mean ambient temperature significantly affected the probability of mosquito death of both L-Arg- and water-supplemented mosquitoes (Wald $X^2_{1,4} = 13.52$, $n = 1227$, $p = 0.009$). There was no significant effect of supplementation treatment on the probability of mosquito mortality throughout the experiment (Wald $X^2_{1,4} = 0.288$, $n = 1227$, $p = 0.591$). Survival curves generated from Kaplan–Meier survival analysis illustrate that cumulative daily mosquito survival decreased, in general, for mosquitoes housed in standard laboratory conditions and warmer ambient temperatures relative to those mosquitoes housed at cooler temperatures (Fig. 3, Table 2).

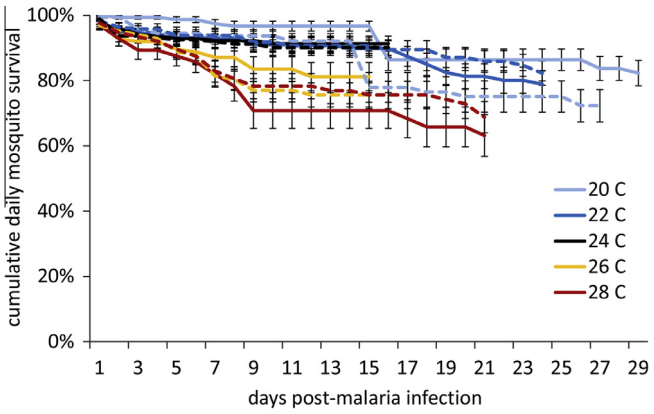

Fig. 3. Mosquito survival curves were generated from Kaplan–Meier estimates of mosquito cumulative survival in each treatment group (water supplementation, solid lines; L-arginine supplementation, dashed lines). Temperature, but not L-arginine supplementation treatment, significantly influenced cumulative daily mosquito survival. Mosquitoes housed in warmer ambient temperatures (26 °C, orange lines and 28 °C, red lines) died significantly faster than mosquitoes housed at cooler temperatures (20 °C, light blue lines; 22 °C, dark blue lines; and 24 °C, black lines).

4. Discussion

Both L-Arg supplementation and environmental temperature significantly influenced the probability of parasite establishment, the number of parasites establishing, transmission potential (number of mosquitoes with sporozoites in the salivary glands), and the expression of *NOS*. The main effects of temperature variation on *P. yoelii* infection, *NOS* expression (thermal maxima between 22 °C and 24 °C and around 30 °C, respectively), and mosquito mortality closely matched previous observations (Murdock and et al., 2012; Paaijmans and et al., 2012). We demonstrated additionally that L-Arg supplementation significantly reduced all aspects of *P. yoelii* infection except at the upper and lower temperature extremes, where immunity was either not boosted, or the effects of L-Arg supplementation appeared to have no immunological effect.

To maintain experimental feasibility across a large temperature range, we did not include supplementation groups often found in experiments that have explored the effects of L-Arg supplementation on insect resistance: for example, L-NAME, a non-oxidizable L-Arg analog that competes with L-Arg for the substrate binding site of NOS enzyme; D-NAME, the non-oxidizable enantiomer to L-Arg; and citrulline, produced along with NO from L-Arg oxidation). Thus, we cannot entirely rule out the possibility that the negative effects of L-Arg on parasite prevalence and intensity resulted from supplementing the host with additional nutrients. Further, L-Arg supplementation might also increase constitutively expressed levels of NO involved in cellular signaling; thus, supplementation with L-Arg could potentially modulate chemosensory signaling (Muller, 1997) and induction of humoral and cellular immune responses (Foley and O'Farrell, 2003; Nappi and et al., 2000).

Previous studies on *A. stephensi* found that infection with *Plasmodium* significantly increased NOS production, activity and NO levels in the midgut (Luckhart and et al., 1998). When mosquitoes were supplemented with L-Arg, both *Plasmodium berghei* and *Plasmodium falciparum* oocyst intensities were reduced due to a direct interaction between L-Arg and the NOS enzyme (both citrulline and D-NAME controls were equivalent to unsupplemented individuals, while L-NAME enhanced parasite infection (Luckhart and et al. (1998), and also see Kraaijeveld et al. (2011). Finally, catalytic activity of NOS and, hence, NO feedback to repress *NOS* mRNA expression in the *A. stephensi* midgut, was consistent with reductions in *NOS* expression seen here at temperatures associated with L-Arg-dependent inhibition of parasite development. It is likely, therefore, that L-Arg supplementation in our studies resulted in enhanced levels of NO that were toxic to developing parasites and that repressed *NOS* mRNA expression in a negative feedback loop. Repression of *NOS* mRNA levels would ultimately reduce NOS protein levels and, therefore, contribute to endogenous protection of mosquito cells from self-induced damage (Peterson and Luckhart, 2006), which might explain why there is no effect of L-Arg supplementation on mosquito mortality. This reduction in NOS mRNA via negative feedback combined with endogenous protective mechanisms against nitrosative stress might represent a balance between parasite killing and NO-mediated reductions in survivorship. However, even at reduced NOS levels, NO synthesis would likely persist for hours, based on observed half-lives of approximately 2 h for inducible NOS protein in a variety of mammalian cell types (Kolodziejski et al., 2004) and high catalytic activity of inducible NOS (Alderton et al., 2001; Bredt and Snyder, 1994).

Our observations of increased mosquito resistance with L-Arg supplementation are likely due to increased NO levels during the first three days of infection when parasites are invading and estab-

Table 2
Pairwise comparisons generated from Kaplan–Meier survival analyses comparing the effects of mean ambient temperature and supplementation treatment on cumulative daily mosquito survival. Significant differences in daily cumulative survival are highlighted in bold.

	Log rank (Mantel–Cox) pairwise comparisons (N = 1778)									
	20 °C		22 °C		24 °C		26 °C		28 °C	
	X^2	p-Value	X^2	p-Value	X^2	p-Value	X^2	p-Value	X^2	p-Value
Water										
20 °C	–	–	0.488	0.485	0.911	0.34	**13.33**	**<0.0001**	3.809	0.051
22 °C	0.488	0.485	–	–	0.058	0.81	**11.985**	**0.001**	**10.935**	**0.001**
24 °C	0.911	0.34	0.058	0.81	–	–	**9.139**	**0.003**	**7.336**	**0.007**
26 °C	**13.33**	**<0.0001**	**11.985**	**0.001**	**9.139**	**0.003**	–	–	0.003	0.955
28 °C	3.809	0.051	**10.935**	**0.001**	**7.336**	**0.007**	0.003	0.955	–	–
Arginine										
20 °C	–	–	3.281	0.07	0.012	0.914	**16.033**	**<0.0001**	**23.74**	**<0.0001**
22 °C	3.281	0.07	–	–	0.079	0.778	3.215	0.073	**9.488**	**0.002**
24 °C	0.012	0.914	0.079	0.778	–	–	**3.913**	**0.048**	**12.474**	**<0.0001**
26 °C	**16.033**	**<0.0001**	3.215	0.073	**3.913**	**0.048**	–	–	1.935	0.164
28 °C	**23.74**	**<0.0001**	**9.488**	**0.002**	**12.474**	**<0.0001**	1.935	0.164	–	–

lishing as early oocysts. In particular, mature ookinetes in transit from the lumen to the outer surface of the midgut could become nitrated or damaged, consistent with previous observations of NO-mediated apoptosis of midgut ookinetes (Hurd et al., 2005; Al-Olayan et al., 2003), resulting in fewer robust oocysts that produce fewer sporozoites. Alternatively, it is also possible that sporozoites could be rapidly deactivated upon entering the hemolmyph by circulating hemocytes that also produce NO (Hillyer and Estevez-Lao, 2010; Hillyer et al., 2007; Herrera-Ortiz and et al., 2011; Kraaijeveld et al., 2011).

While L-Arg supplementation tended to reduce malaria parasite infection, the effects were restricted to intermediate temperatures. At the highest temperature (28 °C), *P. yoelii* infection was severely constrained irrespective of L-Arg supplementation. Whether this is an effect of temperature on the parasite, a result of earlier induction of *NOS* resulting in higher expression levels of *NOS* irrespective of L-Arg treatment (Murdock and et al., 2012), or the involvement of other immune mechanisms at 28 °C is unclear. However, given that other *Plasmodium* species (notably the human malaria parasites) can be transmitted successfully at temperatures > 30 °C, it seems likely that the reduced parasite performance we observed at 28 °C was a consequence of direct thermal sensitivity of *P. yoelii* or increased efficiency of rodent malaria specific immune responses (TOLL activation, TEP1/APL1-C/LRIM-1 activity). At the lowest temperature (20 °C), *P. yoelii* was able to establish following a blood meal and the lack of a supplementation effect could be because of limited *NOS* expression and/or inefficient conversion of L-Arg by the NOS enzyme at sub-optimal temperatures (6–7 °C below standard rearing temperatures for *A. stephensi*). That said, failure of the parasite to produce sporozoites suggests again the potential for some direct negative effects of cooler temperatures on late stage parasite development that are independent of immunity. These results are consistent with a range of studies demonstrating thermally-induced shifts in the outcome of infection due to differences in the thermal performance of the invertebrate host and the parasite (e.g. Vale et al., 2008; Mitchell and et al., 2005; Lazzaro and et al., 2008; Stacey and et al., 2003).

Overall we have shown that mosquito susceptibility to malaria parasite infection is affected by temperature and L-Arg supplementation. Given that the vast majority of studies examining mosquito-parasite interactions ignore any such environmental variation, this is an important insight. Further, we found that over a certain range of temperatures, addition of a key nutritional supplement to boost immunity reduced infection prevalence and intensity, while at higher and lower temperature extremes, boosting immunity had no effect on the parasite. These results indicate that susceptibility is modulated in part via host effects. Exact temperature responses depend on the specifics of the mosquito-parasite pairing. We used a rodent malaria parasite as a model, and the temperature dependencies of rodent malarias do not map directly to temperature dependencies of the human malarias (Sato and et al., 1996; Noden et al., 1995; Okech and et al., 2004), thus extrapolating to field environments is difficult. However, as it currently stands, we have very little understanding of the thermal performance curves for the four major human malaria species or the 20 or so key malaria vectors responsible for transmission worldwide, and whether or not the thermal performance of vector and parasite traits are affected by adaptation to local conditions (Sternberg and Thomas, 2014). Nonetheless, we would expect that human malaria parasites would exhibit similar qualitative responses, with extrinsic factors shaping the outcome of parasite infection success via direct and indirect effects. These insights are relevant for understanding natural variation in mosquito vector competence and for extending insights derived under abstracted laboratory environments to more realistic transmission conditions.

5. Competing interests

The authors declare that they have no competing interests.

6. Authors' contributions

C.C.M. conceived and designed this research, acquired the data, ran the gene expression assays, analyzed the data, and wrote/revised the manuscript.
S.B. participated in the design and execution of this experiment.
S.L. participated in the design of this experiment and in the writing/revising of the manuscript.
M.B.T. participated in the design of this experiment and in the writing/revising of the manuscript.
All authors have read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2014.05.020.

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