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Temperature-modulated host-pathogen interactions between *Hermetia illucens* L. (Diptera: Stratiomyidae) and *Pseudomonas protegens* Pf-5

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ABSTRACT

Temperature is an important abiotic factor influencing the survival and fitness of pathogens as well as their hosts. We investigated the effect of three temperatures (18 °C, 27 °C and 37 °C) on survival and performance of black soldier fly larvae (BSFL), *Hermetia illucens* L., upon infection by an entomopathogenic Gram-negative bacterium, *Pseudomonas protegens* Pf-5. The effect of different temperatures on pathogen fitness was investigated both *in vivo* and *in vitro*. Pathogen performance under exposure to the insect antimicrobial peptide cecropin was investigated at the three temperatures using radial-diffusion plate assays. Higher rearing temperatures resulted in higher larval survival, increased larval weight, and higher inhibitory activity of cecropin against *P. protegens* Pf-5. At higher temperature, bacterial growth, both *in vivo* and *in vitro*, was reduced, resulting in increased BSFL survival. These observations collectively indicate the important effect of rearing temperature on host-pathogen interactions and the possibility to apply temperature treatment in reducing entomopathogen effects in BSFL.

1. Introduction

Temperature is one of the most important abiotic factors influencing insect health. The temperature of their natural environment strongly influences the growth rate, development and immunity of insects (Dixon et al., 2009; Lazzaro et al., 2008). Ectothermic organisms like insects develop and exhibit optimal growth rates within a narrow thermal tolerance range, spanning ca. 20 °C (Dixon et al., 2009). However, insects are exposed to a wide range of temperatures in their natural environment that may not always be ideal for optimal growth and development. To mitigate and survive temperature stress, insects employ multiple strategies like behavioral fever, active navigation to a favorable temperature, and plastic regulation of molecular pathways (Chen et al., 2019; Elliot et al., 2002; Fedorka et al., 2016; Hunt et al., 2016; Kortsmit et al., 2023; Linder et al., 2008). Behavioral fever in insects is regulated by eicosanoids (20-carbon polyunsaturated fatty acids), allowing the insects to increase body temperature and fend-off pathogens and thereby improve survival (Boltana et al., 2013; Watson et al., 1993; Wojda, 2017). In contrast, insects like adult *Drosophila melanogaster* actively select for low temperature upon bacterial infection that not only improves survival but also upregulates their immune function (Fedorka et al., 2016; Linder et al., 2008).

Among the molecular mechanisms involved in adaptation to or tolerance of a temperature stress, the role of heat-shock proteins (HSPs) in insect immunity has been extensively studied (Lindquist and Craig, 1988; Wojda, 2017; Wronska and Bogus, 2020). HSPs are widely conserved across organisms (Lindquist and Craig, 1988). For example, hsp83 improves survival at low temperatures in *D. melanogaster* (Linder et al., 2008). Apart from heat-stress tolerance, hsp70 is also known to facilitate memory formation and consolidation in *D. melanogaster* (Zatsepina et al., 2021). Mild heat treatment (40 °C for 2 h) of *Sarcophaga crassipalpis* provides it with thermal pre-conditioning to survive for a period of up to 72 h after lethal thermal stress (45 °C for 90 min); this tolerance is regulated by hsp70 (Denlinger and Yoccum, 1998). A mild heat shock (i.e. 38 °C) upregulated hsp90 in *Galleria mellonella*, which improved survival upon infection with *Pseudomonas aeruginosa* (Wojda and Jakubowicz, 2007). The HSP BmHop (heat shock 70/90 organizing protein) is upregulated upon thermal stress in the fat body and haemocytes of *Bombyx mori* (Kausar et al., 2020). Molecular responses of insects to thermal stress are plastic and can be modulated to maximize survival and development as seen in e.g. *Ostrinia furnacalis* (Chen et al., 2019). Cold stress (8 °C) led to up-regulation of immune responses (e.g. PGRP- LB, antimicrobial peptides, lysozymes, etc.), while heat stress (40 °C) led to up-regulation of stress response genes (i.e. HSP and P450-pathway related genes).

The black soldier fly (BSF), *Hermetia illucens* L. (Diptera:

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Stratiomyidae), is a saprophagous species, native to the Neotropic regions. The larvae can be used as feed for poultry, pigs, and fish or for extraction of lipids (Chia et al., 2021; Dörper et al., 2021; Veldkamp and Vernooij, 2021). Despite being a tropical species, BSF is increasingly being mass-produced in temperate regions as well. The mass-production of insects is achieved either in open/outdoor or closed/indoor facilities. Outdoor insect production is vulnerable to seasonal temperature fluctuations while indoor systems allow to maintain a constant temperature (van Huis and Oonincx, 2017). The rearing temperature is known to significantly affect the growth and development of the black soldier fly *H. illucens* across all life stages (Harnden and Tomberlin, 2016; Tomberlin et al., 2009). Survival across life stages, development time and adult longevity in BSF differs considerably across a range of temperatures (Chia et al., 2018; Raimondi et al., 2020). The ideal temperature range for development of BSF larvae lies between 25 and 30 °C (Chia et al., 2018; Shumo et al., 2019). Exposure of *H. illucens* larvae to rearing temperatures at the lower (15 °C) and upper (40 °C) tolerance thresholds results in slower development and lower larval survival respectively (Chia et al., 2018).

BSF larvae may be fed with diverse range of organic residual streams that contain a wide spectrum of microorganisms, some of which could negatively affect larval health and growth (Joosten et al., 2020). Host-pathogen interactions are antagonistic, resulting in lethal or sub-lethal consequences for the host, while the host employs multiple cellular and humoral strategies to inhibit or kill the pathogen (Vogel et al., 2018; Vogel et al., 2022). The interaction requires the host to activate or increase its immune responses, often at the expense of the host's growth and/or development (Brace et al., 2017). Upon exposure to pathogens, BSF larvae upregulate the secretion of antimicrobial peptides that are produced primarily within the insect fat body and gut (Vogel et al., 2022). Temperature may influence the ability of the pathogen to multiply within the host. Virulence genes in microbial pathogens are most efficiently expressed within a defined temperature range (Johansson et al., 2002). Post-transcriptional processing of a transcriptional regulator, PrfA, in *Listeria monocytogenes* is strongly influenced by temperature, resulting in 5-fold higher PrfA levels at 37 °C compared to 30 °C, thereby influencing the expression of virulence genes. Expression of virulence genes in *Listeria monocytogenes* is optimal at 37 °C, leading to higher host mortality in *D. melanogaster* compared to hosts maintained at lower temperatures (Mansfield et al., 2003).

The presence of entomopathogens in an insect production system can have devastating effects, such as the colony collapse of the European house cricket, *Acheta domesticus* resulting from the presence of a densovirus (Bertola and Mutinelli, 2021). Although there are currently no known or reported pathogens of BSF (Joosten et al., 2020), studying host-pathogen interactions can provide insights into immunity-related mechanisms in the BSF.

Despite its commercial importance, information on the effect of temperature on interactions between *H. illucens* and entomopathogenic microorganisms has not been studied previously. In this study, we investigate the effects of different rearing temperatures on the interaction between *H. illucens* larvae and the bacterial entomopathogen *Pseudomonas protegens* Pf-5. First, we investigated host survival upon pathogen infection and pathogen effects on host weight at different temperatures. Then, we determined the effect of temperature and host on pathogen growth with both *in vitro* and *in vivo* growth assays. Next, we evaluated host immune responses through qPCR analysis of selected genes encoding antimicrobial peptides (cecropin, defensin, hsp70) that are known to be involved in immune responses to bacterial infections (Vogel et al., 2018). Finally, we assessed the effect of temperature on the bactericidal activity of the antimicrobial protein cecropin against *P. protegens* Pf-5. Collectively, these experiments allow us to determine if the rearing temperature regulates host-pathogen interactions between *H.illucens* and *P. protegens* Pf-5.

2. Materials and methods

2.1. Insect rearing

Insects used in the experiments were taken from the *Hermetia illucens* L. (Diptera: Stratiomyidae) colony maintained at the Laboratory of Entomology, Wageningen University (the Netherlands) in a climate room (27 ± 1 °C, 70 ± 10% R.H., L12:D12). Eggs laid within a period of six hours were collected from a cage harboring adult flies. Two egg clutches (~1500 eggs) were selected at random and placed on a chicken-feed diet (150 g, Kuikenopfokmeel 1; Kasper Faunafood, Woerden, the Netherlands) and mixed with 300 mL water within a plastic container (15.5 × 10.5 × 6 cm) on top of an inverted Petri dish. The container was closed with a plastic lid, having a rectangular ventilation hole (7 × 5.5 cm) that was covered with nylon of 1 mm mesh size.

2.2. Bacterial culture conditions

A stock of *Pseudomonas protegens* Pf-5 (kindly provided by Dr. Virginia Stockwell, Oregon State University, USA) was stored in 50% glycerol at −80 °C. A loopful of *P. protegens* Pf-5 cells was transferred on King's B (KB) agar plate (Merck Millipore, Germany) and placed in an incubator at 27 °C for 48 h. A single colony was transferred to 10 mL Luria-Bertani (LB) broth (LB broth (Miller); Merck Millipore, Darmstadt, Germany) and grown overnight at 24 °C and 180 rpm in a rotary shaker (Innova™ 40 Incubator, New Brunswick Scientific). One mL was drawn from the culture the following day, centrifuged at 3500 rpm for 3.5 min, washed once in sterile PBS buffer (Oxoid™ Phosphate Buffered Saline Tablets, Thermo Scientific™) and re-suspended in PBS buffer. OD_{600} was measured using a DS-11 FX + Spectrophotometer Fluorometer (DeNovix, Wilmington (DL), U.S.A.) and the culture was then serially diluted to the desired LD_{50} concentration (~2.5×10^3 cells/mL) of *P. protegens* Pf-5.

2.3. Survival assay

Five-day-old larvae (5DOL) of *H. illucens* were surface-sterilized by dipping for 5 s in 70% ethanol, followed by two consecutive washes in sterile distilled water for 5 s each. The surface-sterilized larvae were placed on a clean paper towel and allowed to air-dry for five minutes, following which they were transferred into a clean Petri dish. The larvae were picked randomly and injected with 1 μL of LD_{50} concentration of *P. protegens* Pf-5 or 1 μL PBS as control. The experiment was repeated in a second experimental block, on a different day with different sets of larvae and a new bacterial culture. Each experimental block involved four replicate containers (volume 550 mL, height 11 cm, top-width 10 cm, bottom width 8.5 cm; the lid (9.5 cm × 5 mm) had a circular vent of 5 cm diameter covered with nylon of 1 mm mesh size. Each container housed 20 larvae and was supplied with premixed chickenfeed (10 g) and distilled water (20 mL). Larval survival was monitored at multiple time points for up to 72 h post-infection. The wet weight of individual larvae from all treatments was measured at the end of experiment.

2.4. Pathogen quantification

Pseudomonas protegens Pf-5 was transferred from the glycerol stock onto KB agar plates and placed in a climate cabinet maintained at 27 °C for 48 h.

In vitro growth: Seed culture was prepared by transferring an isolated bacterial colony into 10 mL LB broth in 50 mL Fisherbrand™ Easy Reader™ plastic centrifuge tubes (Fisher Scientific), placed in rotary incubator (Innova™ 40 Incubator, New Brunswick Scientific) at 24 °C and 180 rpm. After overnight incubation, 1 mL of the culture was harvested and spun down at 3500 rpm for 3.5 min, pellet-washed once with PBS buffer and re-suspended in PBS. OD_{600} of the culture was determined using a DS-11 series spectrophotometer (DeNovix), and serially

diluted to obtain the LD_{50} concentration as described above. One-hundred µL was used to inoculate three biological replicates of experiment culture in 10 mL LB broth (in 50 mL flat cap tubes) and placed in rotary shakers maintained at 18 °C, 27 °C and 37 °C, respectively, at 180 rpm. Three biological replicates were sampled per temperature/treatment combination. One mL aliquots were harvested at three timepoints (6 h, 24 h and 48 h), pellet-washed once with PBS buffer at 3500 rpm for 3.5 min. Fifteen µL from the appropriate serial dilution was plated on Pseudomonas Isolation Agar (PIA; Merck Millipore, Germany). The Petri dishes were placed at 27 °C for 24 h after which colonies were counted. Enumerated colony-forming units (CFUs) were used as proxy to quantify *in vitro* growth of *P. protegens* Pf-5 at different temperatures.

In vivo growth: Isolated bacterial colonies were transferred to 10 mL LB media and placed in a rotary shaking incubator overnight at 24 °C and 180 rpm. The bacterial culture was centrifuged at 3500 rpm for 3.5 min, pellet-washed once in PBS buffer and re-suspended in PBS buffer. OD_{600} was determined and the suspension was diluted to $OD_{600} = 0.25$ ($\sim 5 \times 10^8$ cells/ml), followed by serial dilution to the LD_{50} dosage ($\sim 2.5 \times 10^3$ cells/ml or $\sim$ 3 cells injected per larva). 5DOL were harvested from the chickenfeed substrate, surface- sterilized in 70% ethanol and subsequently twice in sterile distilled water, air-dried and injected with 1 µL of LD_{50} concentration as described above. Larvae were placed in climate cabinets maintained at 18 °C, 27 °C and 37 °C. Larvae were collected at 6 h, 24 h and 48 h post-injection and cleaned again as described above. Individual larvae were placed in 1.5 mL Eppendorf tubes with two 5-mm glass beads and 1 mL PBS buffer and homogenized using a bead-mill homogenizer (TissueLyser II, Qiagen) at 30 oscillations/s for 5 min. The samples were serially diluted and appropriate dilutions were plated on PIA and placed at 37 °C for 24 h, following which viable bacterial cells were enumerated. The bacterial cell count is represented as the.

number of viable bacterial cells $= \ln(N_t/N_0)$,

where N_t = number of viable cells at time t, and N_0 = number of injected cells.

2.5. RNA extraction and qPCR analysis

Five-day-old larvae were surface-sterilized one time in 70% ethanol and then washed twice in sterile distilled water for 15 s each. The larvae were then dried on tissue paper and injected with 1 µL of PBS as negative control or 1 µL of LD_{50} concentration of *P. protegens* Pf-5. The injected larvae from the respective treatments (i.e. PBS and Pf-5) were placed separately on a chickenfeed: water (1:2 ratio) diet in different incubators maintained at 18 °C, 27 °C or 37 °C.

At multiple timepoints (2 h, 6 h, 16 h and 24 h), three biological samples (of two larvae each) were collected per treatment in 1.5 mL Eppendorf Safe-Lock Tubes (Eppendorf, Hamburg, Germany). 200 µL TRI-Reagent® (Sigma-Aldrich) and two 5-mm glass beads were added to each tube, and samples were homogenized using a bead-mill homogenizer (TissueLyser II, Qiagen) at 30 oscillations/s for 5 min. Potential DNA contamination was cleared using TURBO™ DNase (ThermoFisher Scientific) and samples were cleaned with phenol: chloroform: isoamyl alcohol (25:24:1; Sigma-Aldrich) and suspended in RNase-free water. The quality and quantity of extracted RNA was assessed using a DS-11 series spectrophotometer (DeNovix). RNA was stored at −80 °C until further use.

A total of 800 ng of RNA was converted to complementary DNA (cDNA) using qScript cDNA Supermix (Quantabio, Massachusetts, U.S.A.). To quantify gene expression, quantitative polymerase chain reaction (qPCR) was performed using SYBR Green (SensiFAST™, Bioline). Primers were designed for the genes of interest (i.e. cecropin, defensin, heat- shock protein 70) and housekeeping genes (tubulin and ribosomal protein L8 (rpL8)) (Table 1).

Primers were designed using an online primer design-tool (Primer3; https://primer3.ut.ee/) with default settings and synthesized by Eurofins (Wageningen, the Netherlands). Primer specificity and amplification efficiency were evaluated by generating a standard curve for each gene using two-fold serial dilution of the template. The amplification efficiency was calculated using the following formula:

% Primer efficiency = (fold dilution ^ (-1 / slope of intercept) − 1) * 100.

The efficiency values of all primers ranged between 95 and 105.4%, with regression coefficient values from 0.9956 to 0.9995. From a list of eight potential housekeeping genes (Gao et al., 2019), the optimal combination of reference genes was determined using GeNorm (Vandesompele et al., 2002) in qbase+ (Biogazelle, Ghent, Belgium).

The qPCR reaction system (10 µL) contained 5 µL SYBR Green, 3.7 µL RNase-free H2O, 0.15 µL of forward and reverse primers (10 µM) and 1 µL of first-strand cDNA. The PCR program included an initial denaturation step at 95 °C for 2 min, followed by 40 cycles at 95 °C for 5 s, 60 °C for 10 s and 72 °C for 20 s. Finally, a melting curve analysis from 65 °C to 95 °C was performed to determine specificity of PCR products. Three technical replicates were analyzed for each biological replicate. Relative gene expression, normalized to selected reference genes and accounting for primer efficiency, was calculated using the ΔCt method.

Table 1
Information of the genes used for qPCR analysis.

Gene name	Gene ID	Primer sequence (5′- 3′)
Tubulin	119655171	F: GTCTGGAGTTACCACCTGCC R: CTGGAACTGTGAGGGCTCTG
RpL8	119649398	F:ACAGTTCGTCTATTGCGGCA R:CGGCCTCGATCTCCTGTTTT
Cecropin	119656616	F:TTCGCTGTTGTCCTTGTTGC R:AATTGCGATTCCCTGAACCC
Defensin	119658450	F:AAAATGCGTGTGACCGTGTG R:AAGGGCTCAACAGGTCACAG
Heat-shock protein 70	119653206	F:ACAACTTGCTTGGCACCTTC R:ACATTGAGAATGCCGTTCGC

2.6. Cecropin plate assay

To determine the anti-microbial efficacy of pure protein against *P. protegens* Pf-5 at different temperatures, stock solutions of cecropin A and B (Sigma) were diluted to a concentration of 1.628 µM in 0.01% acetic acid prior to use. First, 5×10^8 cells/ml of *P. protegens* Pf-5 were diluted in 200 mL LB agar (0.8%) and 15 mL of this mixture was plated on each Petri dish as underlay gel and allowed to air-dry. A holes of 3 mm diameter was pierced and removed from the centre of the plate and 10 µL of cecropin A or B respectively were placed in the hole and allowed to diffuse for 15 min. A negative control (10 µL of 0.01% acetic acid) was included to determine the effects of acetic acid on bacterial growth. The plates were placed in climate cabinets maintained at 18 °C, 27 °C or 37 °C respectively for a period of 3 h, following which 15 mL of double-power LB agar (1%) was poured as overlay gel and allowed to air-dry for 15 min. The plates were returned to the climate cabinets maintained at 18 °C, 27 °C or 37 °C respectively. The zone of inhibition (in mm) produced at the respective temperatures was measured after 24 h of incubation. The experiment was performed with five technical replicates for each temperature.

2.7. Statistical analysis

Statistical analyses were performed using R 4.2.0 (R Project for Statistical Computing) within RStudio statistical software version 1.4.717 (R Core Team, 2022a) using the following packages: "survminer", "ggeepack" and "ggplot2".

Survival analysis: The survival curves were plotted as Kaplan-Meier lifespan plots and statistical significance was analyzed using Cox-regression model (coxph) using the following model:

Model 1 <- coxph (status, time) ~ temperature + treatment + temperature*treatment

Survival status at a given timepoint was considered as dependent variable, fixed explanatory variables included in the model were temperature, treatment (pathogen injection) and their interaction. The model assumptions were checked using graphical display of residuals against the model fitted values, and for each explanatory variable separately prior to further statistical analysis. Post-hoc analysis was performed with pairwise comparison using the log-rank (Mantel-Cox) test with Bonferroni adjustment. The models and graphs were built using packages 'survival' and 'coxme' in R (R Core Team, 2022b; Therneau, 2022a; b). Differences in survival were extracted using the ANOVA

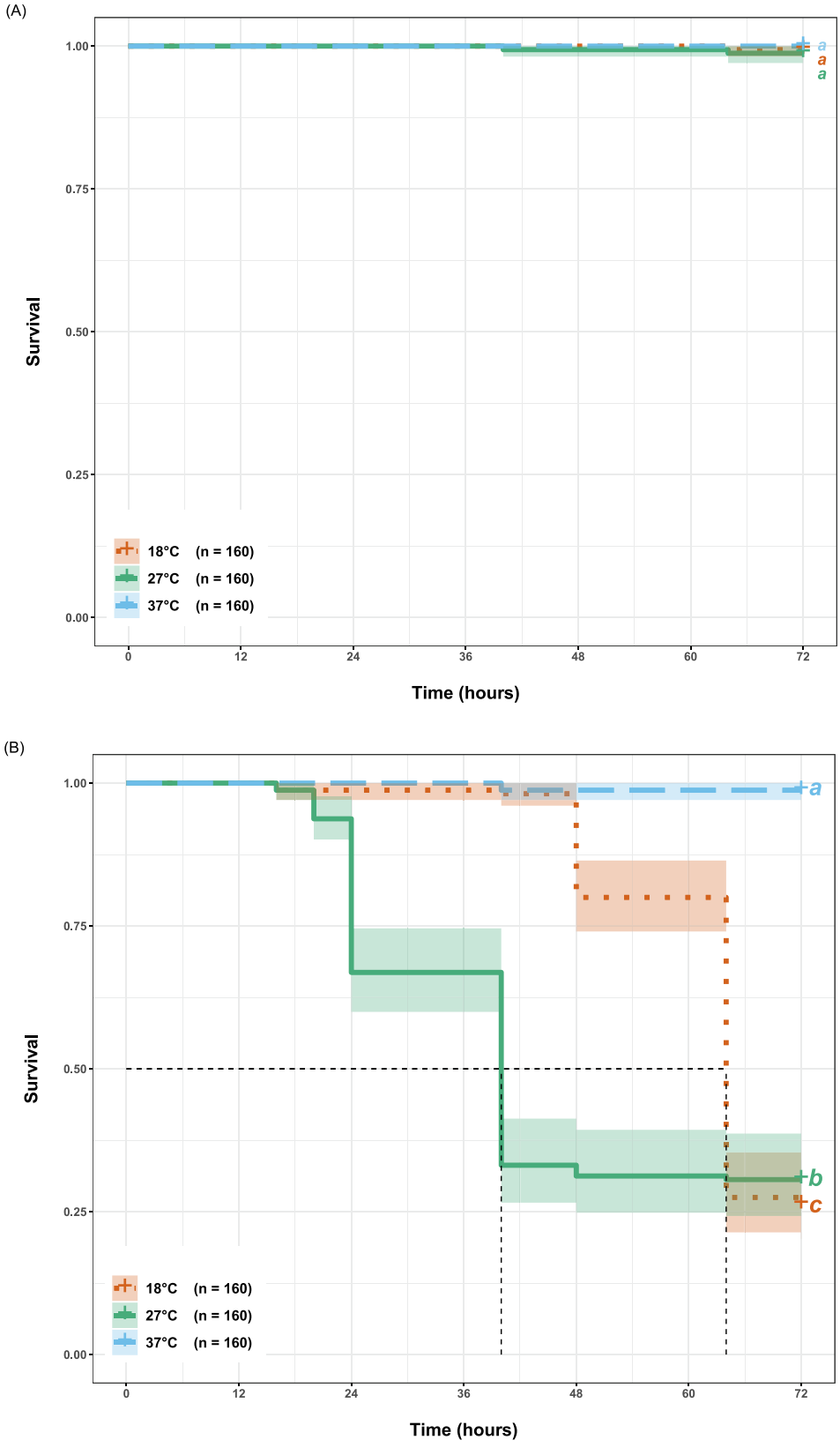

Fig. 1. Kaplan-Meier survival curves of 5-day-old BSF larvae injected with (A) 1 µL PBS; and (B) 1 µL of LD_{50} concentration (~2.5×10^3 cells/ml) of *P. protegens* Pf5, monitored for survival until 72 h post-injection. Results from two independent experimental blocks are combined for graphical representation of each survival curve (n=160 larvae per treatment). Significance of differences in larval survival across temperatures and treatments was analyzed by pairwise log-rank comparison ($p < 0.05$). Different letters next to the lines in the figure indicate statistically significant differences between treatments. Black dotted lines in panel B indicate LT_{50} for the respective treatments.

contrasts command from the car package (Fox and Weisberg, 2019). The figures were produced using the ggplot2 package (Wickham, 2016).

Larval weight: Differences in larval weight (A) at different temperatures (B) and treatments (C) were estimated with two-way ANOVA analysis. The model used was:

Model 2 <- aov(A ~ B * C)

Model assumptions were checked as described above and post-hoc comparisons were performed using Tukey's HSD ($p < 0.05$).

Bacterial growth: The bacterial growth at different temperatures was plotted as logarithm of relative population size [$y = \ln(N/N_0)$] against sampling timepoints. A Generalized Least Squares (GLS) model was used to analyze the effect of temperature (discrete variable) and sampling timepoints (continuous, as explanatory variables). For repeated measures GLS, temporal autocorrelation for each tube across time was modelled/accounted for using an autoregressive correlation structure of order 1 'AR1' (data points are correlated over 1 lag or time point) (Pinheiro and Bates, 2000). The model assumptions were checked using graphical display of residuals against the model fitted values for each explanatory variables separately. Post-hoc analysis was performed using Tukey's HSD ($p < 0.05$).

Gene expression analysis: Expression levels of genes of interest in bacteria-infected larvae were normalized against those of the control larvae (i.e. PBS-injected) subjected to different temperatures. Subsequently, expression levels of genes of interest were compared between temperatures for sampling timepoints using two-way ANOVA (gene expression ~ temperature × sampling timepoints) followed by Tukey's HSD post-hoc test.

Cecropin plate assay: Effect of temperature on the diameter of zone of inhibition was evaluated using one-way ANOVA, followed by post-hoc test (Tukey's HSD).

3. Results

3.1. Survival analysis

Control treatments resulted in 98% survival (Fig. 1A). Rearing temperature (Cox-PH: $\chi^2 = 204.56$, df = 2, $p < 0.001$) and pathogen treatment (Cox-PH regression: $\chi^2 = 435.65$, df = 1, $p < 0.001$) significantly influenced larval survival. The interaction effect is significant (temperature × treatment; Cox-PH regression: $\chi^2 = 640.3$, df = 5, $p < 0.001$). Although mortality of larvae injected with *P. protegens* Pf-5 at 72 h post-injection was similar for 18 °C and 27 °C (74% and 70% respectively), larval mortality at 27 °C occurred significantly faster compared to that at 18 °C (Fig. 1B, pairwise log-rank comparison, $p < 0.001$). The differences in larval mortality at 18 °C and 27 °C can be attributed to the difference in LT50 (i.e. time to 50% mortality), which was 40 h at 27 °C, and 64 h at 18 °C (Fig. 1B). At 37 °C, injection with *P. protegens* Pf-5 did not result in significant mortality compared to the control with PBS injection (Fig. 1B).

3.2. Larval weight

Temperature significantly affected final larval weight of 8-day-old larvae (Fig. 2), increasing with increase in rearing temperature (two-way ANOVA; F = 1171.990, df = 2, $p < 0.001$). Treatment (i.e. PBS or Pf-5) also influenced larval weight (two-way ANOVA; F = 10.379, df = 1, $p = 0.002$). Furthermore, the interaction between treatment and temperature was significant (F = 7.51, d = 2, $p < 0.001$). At 37 °C, bacterial injection led to significant reduction in larval weight compared to control larvae (Tukey's HSD, $p < 0.05$). No effects of bacterial injection on weight are observed for larvae reared at 18 °C and 27 °C.

3.3. Pathogen growth

In vitro bacterial growth (Fig. 3) was strongly influenced by temperature (GLS, F = 2182.23, df = 2, $p < 0.001$) and sampling time point (GLS; F = 21862, df = 2, $p < 0.001$). A significant interaction between temperature and sampling time point was observed (GLS; F = 305.4, $p < 0.001$). Although the bacterial growth at 37 °C is significantly higher than at 18 °C at the earliest time point (i.e. 6 h) (Tukey's HSD, $p < 0.05$), the bacterial growth at 18 °C is larger than at 37 °C at later time points (Tukey's HSD, $p < 0.05$). The bacterial growth was significantly larger at 27 °C compared to 18 °C (Tukey's HSD, $p < 0.05$). *In vitro* growth of bacteria was smaller at 37 °C compared to 27 °C and 18 °C after 24 and 48 h, indicating a negative effect of higher temperature on pathogen

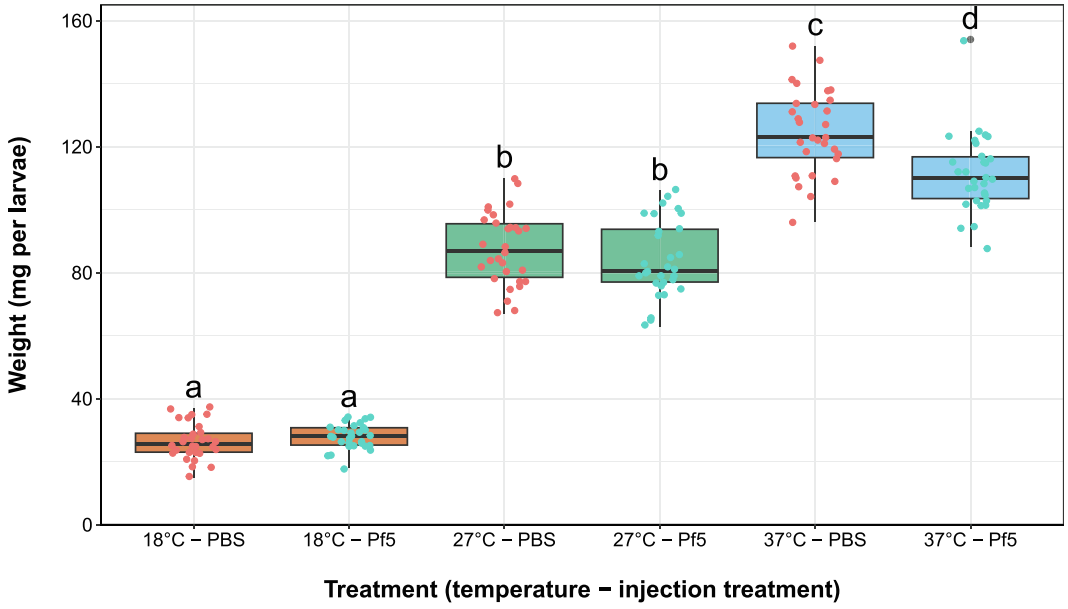

Fig. 2. Wet weight of 8-day-old larvae (i.e. 72 h post-injection) reared at three temperatures and injected with either PBS or with LD_{50} suspension (~2.5×10^3 cells/ml) of *Pseudomonas protegens* Pf-5. Starting weight of an individual larva on day 5 was 33 ± 2 mg. Weight data from two independent experimental blocks were pooled, n = 30 per treatment. Boxplots show median (horizontal bold line), first and third quartiles, and minimum and maximum. Two-way ANOVA with post-hoc Tukey's HSD: boxplots marked with different letters differ significantly ($p < 0.05$).

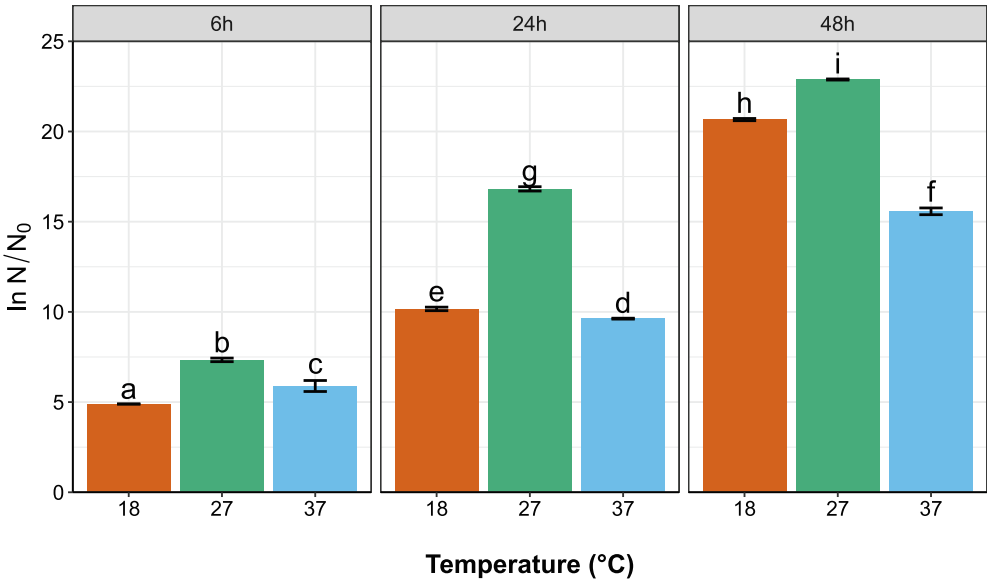

Fig. 3. *In vitro* growth of *P. protegens* Pf-5 at three temperatures (18 °C, 27 °C and 37 °C) at three timepoints (6 h, 24 h and 48 h). One mL of bacterial culture (from three biological replicates) was serially diluted and plated on Pseudomonas Isolation Agar to enumerate the number of isolated bacterial colonies; colony count is recorded as ln Nt / N_0 (log of timepoint x/ N_0; where x = 6 h, 24 h or 48 h; N_0 is the bacterial population at injection at t = 0 h). Error bars represent standard error of mean. Letters above bars indicate significant differences ($p < 0.05$) between different temperatures across all time points based on a non-adjusted Tukey's post-hoc analysis.

growth.

Bacterial growth *in vivo* (Fig. 4) was strongly influenced by temperature (two-way ANOVA; F-value = 47.743, df = 2, $p < 0.001$) and sampling time point (two-way ANOVA; F-value = 31.070, df = 2, $p < 0.001$). The interaction between temperature and sampling time point is not significant (two-way ANOVA; F-value = 2.381, df = 4, p = 0.0697), indicating that the temperature effect on bacterial growth is consistent across all three time points. Growth of *P. protegens* Pf-5 *In vitro* and *in vivo* displayed a similar pattern indicating the negative effect of high temperature on pathogen growth and corresponds to higher larval survival at 37 °C compared to 18 °C and 27 °C (Fig. 1).

3.4. qPCR gene-expression analysis

The expression levels of genes of interest were determined by normalizing the Ct values against Ct values of reference genes. Subsequently, expression levels of genes of interest are depicted relative to the control treatment (i.e. PBS-injected larvae, subjected to similar temperature conditions) (Fig. 5).

Neither temperature (two-way ANOVA, $p > 0.05$) nor sampling time

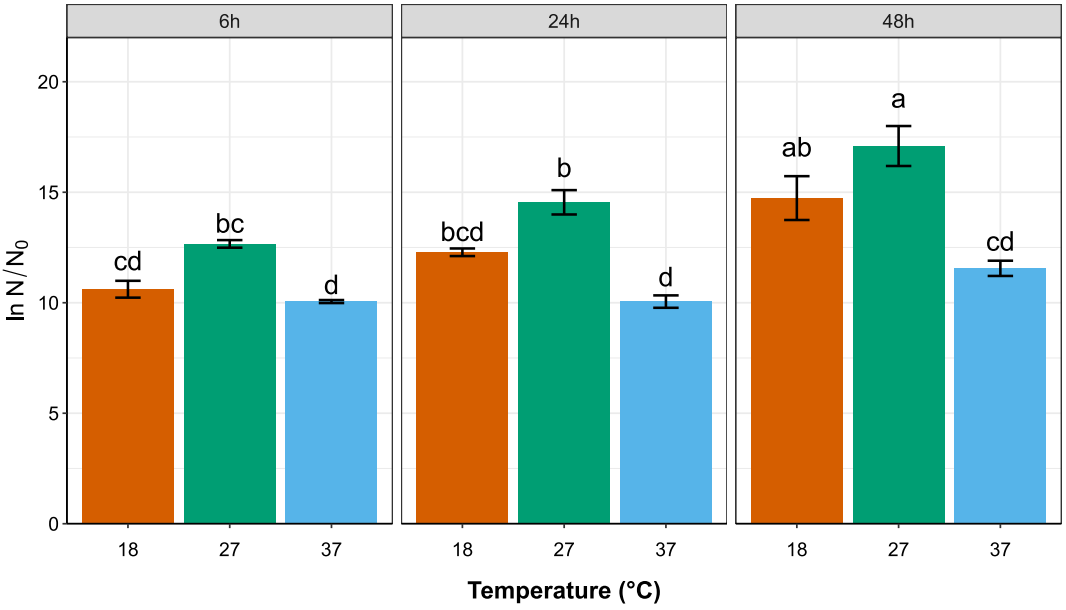

Fig. 4. *In vivo* growth of *P. protegens* Pf-5 at three temperatures (18 °C, 27 °C and 37 °C) at three timepoints (6 h, 24 h and 48 h). Larvae were homogenized, serially diluted and plated on Pseudomonas Isolation Agar to enumerate isolated bacterial colonies; colony count is plotted as ln Nt / N_0 (log of timepoint x/ N_0; where x = 6 h, 24 h or 48 h; N_0 is the population at t = 0 h). Error bars represent standard error of mean, N = ten biological replicates per treatment per time point. Letters above bars indicate significant differences ($p < 0.05$) between different temperatures across all time points based on a non-adjusted Tukey's post-hoc analysis.

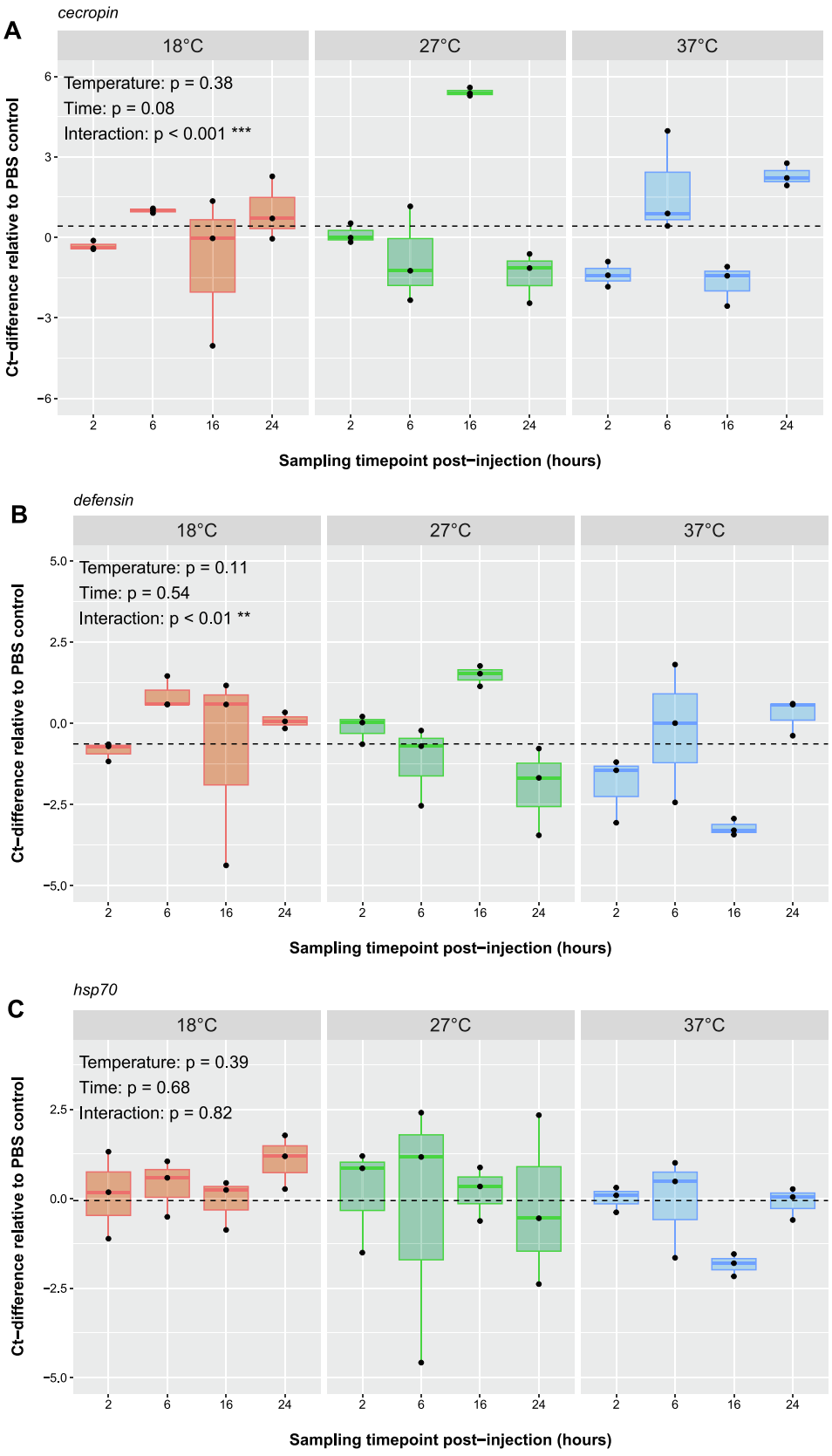

Fig. 5. Relative expression of cecropin, defensin and hsp70 in larvae injected with *P. protegens* Pf-5 relative to control treatment (i.e. PBS-injected larvae) at different temperatures (i.e. 18 °C, 27 °C and 37 °C; indicated at top of each panel) across multiple time points (2 h, 6 h, 16 h, 24 h) post-injection. Three biological replicates (of 2 pooled larvae each) per treatment per time point were analyzed. Dotted horizontal line indicates mean expression across temperature × time-points. Boxplots show median (horizontal bold line), first and third quartiles, and minimum and maximum. Two-way ANOVA analysis (expression ~ temperature × sampling time-point) was performed along-with Tukey's HSD.

point (two-way ANOVA, $p > 0.05$) influenced the expression of cecropin, defensin and hsp70 (Fig. 5) across all sampling time points, indicating the stability of gene expression upon bacterial infection across all temperatures.

3.5. Cecropin plate assay

Bactericidal activity of cecropin A and B against *P. protegens* Pf-5 is influenced by temperature (Fig. 6; ANOVA, df = 2, F = 2281.880, p <

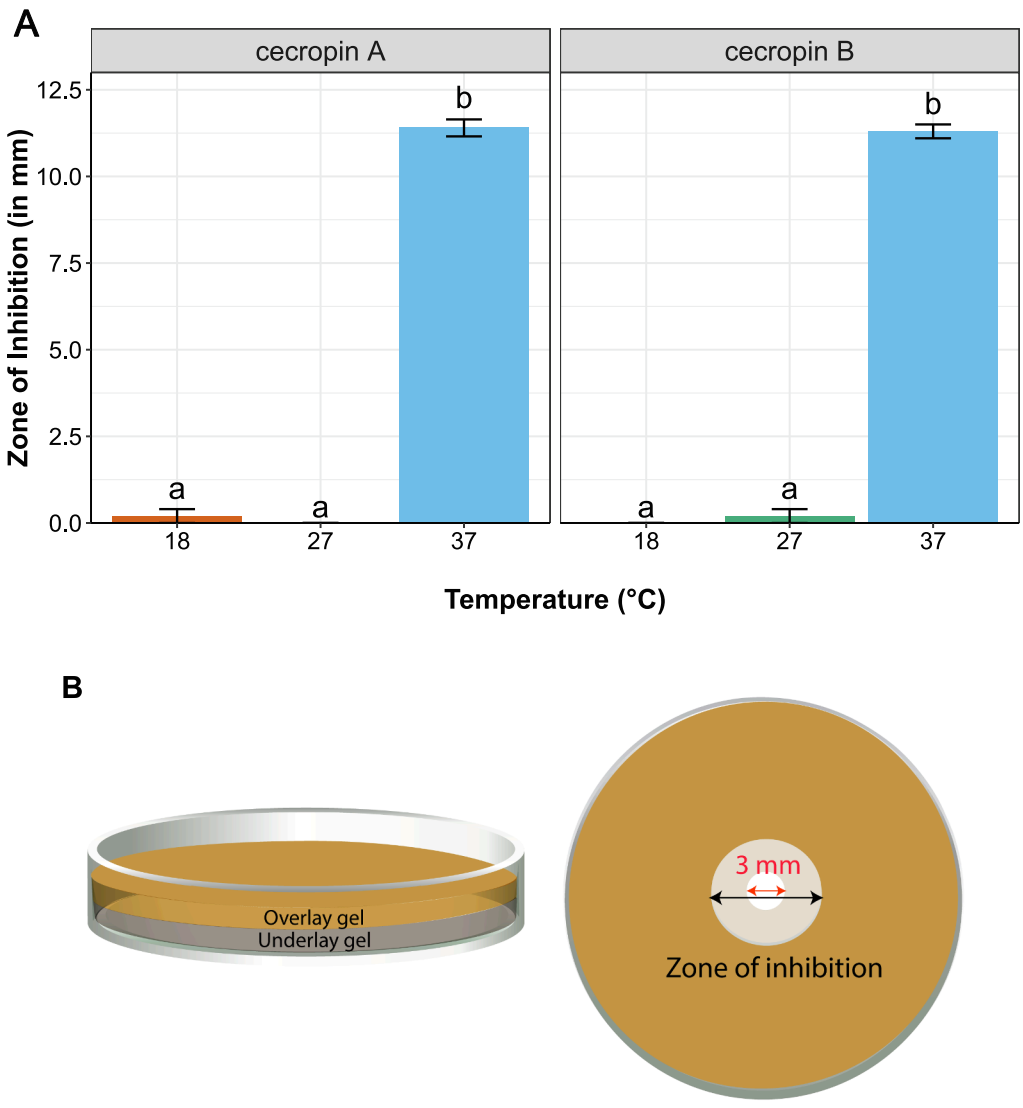

Fig. 6. Anti-bacterial activity of Cecropin A and Cecropin B against *P. protegens* Pf-5 at three temperatures (18 °C, 27 °C and 37 °C). Bar plots in Panel A indicate the zone of inhibition (diameter, in mm) observed. Panel B presents a schematic representation of the set-up, indicating an inhibition zone around a 3-mm hole into which 10 μL of cecropin A or B (1.628 μM in 0.01% acetic acid) was added, respectively. Five independent replicates were tested per temperature × treatment combination, error bars represent standard error of the mean. Letters above bars in panel A indicate significant differences ($p < 0.05$) between different temperatures based upon a non– adjusted Tukey's post-hoc analysis.

0.001). The negative control (0.01% acetic acid) did not display any zone of inhibition against *P. protegens* Pf-5. The activities of cecropin A and B do not significantly differ (ANOVA, df = 1, F = 0.0402, p = 0.53). The zone of inhibition (Fig. 6A) formed by bactericidal activity of cecropin A and B against *P. protegens* Pf-5 is significantly bigger (~11.5 mm) at 37 °C than at 18 °C and 37 °C (Tukey HSD, $p < 0.001$). The two AMPs did not generate any zone of inhibition at 18 °C and 27 °C, indicating lack of activity against *P. protegens* Pf-5 at these temperatures.

4. Discussion

Our data shows that the rearing temperature affects the outcome of host-pathogen interactions between *H. illucens* L. and *P. protegens* Pf-5. Although, the ideal temperature range for growth and development of *H. illucens* larvae lies between 25 and 30 °C (Chia et al., 2018; Harnden and Tomberlin, 2016; Shumo et al., 2019; Tomberlin et al., 2009), we observed that rearing temperatures of 18 °C and 27 °C confer negative consequences on larval survival upon infection with the pathogen whereas such negative consequences for survival were absent at the higher temperature 37 °C. Exposure to high temperature improves survival in, e.g., locusts and houseflies, upon infection by entomopathogenic fungi (Elliot et al., 2002; Watson et al., 1993). In contrast, in the fly *D. melanogaster* exposure to a low temperature (i.e. < 20 °C) after infection with the entomopathogen P. aeruginosa improves survival (Fedorka et al., 2016; Linder et al., 2008). Further, exposure of *D. melanogaster* to high temperature post-infection with P. aeruginosa, Lactococcus lactis and Providencia rettgeri resulted in enhanced mortality and shorter median time to mortality (Lazzaro et al., 2008; Linder et al., 2008). Thus, insect species differ in their ability to survive entomopathogen infection at different temperatures.

The rearing temperature also influences larval weight gain. Larvae reared at 37 °C displayed the highest weight gain amongst the tested temperatures, likely due to increased metabolic activity. Surprisingly, *H. illucens* larva weight in the control and bacterial treatments were significantly different at 37 °C, indicative of a trade-off between investment in growth versus immune responses contributing to surviving bacterial infection. However, the cost of a lower weight is compensated by higher survival post-infection at 37 °C.

High rearing temperature negatively affects the pathogen growth. The differences in larval LT50 at 18 °C and 27 °C can be explained by the differences in the rate of bacterial growth *in vitro* and *in vivo*, whereby the bacterial population increased faster at 27 °C compared to 18 °C. Results of *in vivo* and *in vitro* bacterial quantification indicate the inability of the entomopathogen to multiply rapidly within the host at 37 °C and are in agreement with the findings of Ng (2018).

The expression of genes encoding cecropin, defensin and hsp70 have been demonstrated to respond to temperature changes and/or bacterial infection in insect species such as *D. melanogaster* and *G. mellonella* (Carboni et al., 2022; Hanson et al., 2022; Lindquist and Craig, 1988; Wronska and Bogus, 2020; Zatsepina et al., 2021). Similarly, bacterial infection in BSF larvae also resulted in production of AMPs (Elhag et al., 2017; Ouyang et al., 2015; Park et al., 2015; Park and Yoe, 2017). However, in adult *H. illucens* females, the expression of hsp70 and hsp90 did not differ with age or different rearing temperatures (Malawey et al., 2021). Interestingly, age-related differences in gene expression of hsp70 and hsp90 were observed in adult *H. illucens* males, being particularly higher in older male individuals. In the current study, the expression of immunity-related genes (i.e. cecropin, defensin and hsp70) was not significantly different across the three rearing temperatures tested, and can therefore not be used to assess host-pathogen interactions at multiple temperatures.

Cecropin is broadly attributed to possess antibacterial activity, specifically against Gram negative bacteria (Carboni et al., 2022). Yet, no such differences were observed in gene expression across different temperature treatments. We hypothesized that there may be temperature effects on protein activity and therefore its bactericidal activity. Indeed, we observed that activity of the protein cecropin A and B against *P. protegens* Pf-5 is clearly affected by temperature. The proteins displayed strong bactericidal activity against *P. protegens* Pf-5 at 37 °C, and no bactericidal activity was observed at 18 °C and 27 °C. This clearly shows that temperature influences the effect of this AMP. Thus, not AMP gene expression but protein activity is relevant to understanding the ability of *H. illucens* to suppress the growth of *P. protegens* Pf-5 at 37 °C.

BSF is a commercially important species produced as feed for livestock. Therefore, it is important to manage pathogen risks and adhere to feed safety requirements. This study demonstrates the possibility of utilizing temperature-modulated pathogen remediation that improved larval survival. Larval exposure to *P. protegens* Pf-5 at 37 °C resulted in reduced weight gain relative to unexposed larvae, but the significant improvement in larval survival more than compensates for this. While high temperatures improve host survival upon bacterial infection, it comes with trade-offs during later developmental stages of BSF. We, therefore, conclude that the trade-off associated with utilizing high temperature to suppress pathogen multiplication should be taken into account by adjusting exposure time to meet production goals.

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.

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