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ARTICLE

Decline in symbiont-dependent host detoxification metabolism contributes to increased insecticide susceptibility of insects under high temperature

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The interactions between insects and their bacterial symbionts are shaped by a variety of abiotic factors, including temperature. As global temperatures continue to break high records, a great deal of uncertainty surrounds how agriculturally important insect pests and their symbionts may be affected by elevated temperatures, and its implications for future pest management. In this study, we examine the role of bacterial symbionts in the brown planthopper *Nilaparvata lugens* response to insecticide (imidacloprid) under different temperature scenarios. Our results reveal that the bacterial symbionts orchestrate host detoxification metabolism via the *CncC* pathway to promote host insecticide resistance, whereby the symbiont-inducible *CncC* pathway acts as a signaling conduit between exogenous abiotic stimuli and host metabolism. However, this insect-bacterial partnership function is vulnerable to high temperature, which causes a significant decline in host-bacterial content. In particular, we have identified the temperature-sensitive *Wolbachia* as a candidate player in *N. lugens* detoxification metabolism. *Wolbachia*-dependent insecticide resistance was confirmed through a series of insecticide assays and experiments comparing *Wolbachia*-free and *Wolbachia*-infected *N. lugens* and also *Drosophila melanogaster*. Together, our research reveals elevated temperatures negatively impact insect-bacterial symbiosis, triggering adverse consequences on host response to insecticide (imidacloprid) and potentially other xenobiotics.

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INTRODUCTION

The world is experiencing unprecedented challenges due to global climate changes, with rising temperatures becoming the driver of biodiversity loss and habitat deterioration [1]. Elevated temperatures also severely impact organism phenology, development time, lifespan, behavior, and physiology [2–4]. In the context of the agroecosystem, it has been predicted that a warmer climate will alter many characteristics of insect pests, which could lead to more severe crop losses. These changes include elevated food consumption due to increased metabolic rates, as well as larger pest populations as the pests grow faster under higher temperatures [5]. A plausible outcome of higher pest pressure is increased insecticide use [6]. Indeed, the use of insecticides and other agrochemicals is expected to increase under global warming, while insecticides will become more toxic to organisms (both pest and beneficial insects) at higher temperatures [6, 7].

In the face of global climate change, an important research priority is to better understand how insect pests respond and adapt to rising temperatures in conjunction with changing pest management practices [8, 9]. Studies on various insects have shown that elevated temperatures can increase insect sensitivity to many insecticides (organophosphates, carbamates, and neonicotinoids) by downregulating the detoxifying metabolic enzymes, including the cytochrome P450s (P450s) and glutathione *S*-transferases (GSTs) [10–13]. However, the precise mechanisms by which elevated temperatures mediate changes in insect detoxification metabolism remain poorly understood.

A key driver of evolution and extended phenotype of insects is their symbioses with microbes [14]. Particularly, symbionts have been shown to affect insect resistance to insecticides through direct and indirect routes. For instance, symbionts *Burkholderia* in the bean bug *Riptortus pedestris* and *Citrobacter* in the oriental fruit fly *Bactrocera dorsalis* encode insecticide-degradation enzymes that can directly break down toxins for the hosts, while the symbionts *Lactobacillus* in *Drosophila melanogaster* and *Arsenophonus* in *Nilaparvata lugens* have been shown to influence host detoxification gene expression [15–18].

Changes in temperature can have profound impacts on the mutualistic interactions between insects and their symbionts [19, 20]. Thermal stress has been shown to disrupt symbiosis in several insects in the orders Hymenoptera, Hemiptera, Coleoptera, and Psocoptera, leading to deregulation of

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metabolism and reduced fitness [19, 21–25]. Based on these observations, we speculate that symbionts could contribute to the temperature-dependent response to insecticides of their insect hosts, which may account for increased insecticide susceptibility at higher temperatures as have been shown in several insects [12, 26–28].

In this study, we examine how bacterial symbionts respond to high temperature and its relationship with host detoxification metabolism and insecticide susceptibility, in an important agricultural pest, the brown planthopper *N. lugens*. *N. lugens* is a major pest of rice in Asia and has serious metabolic resistance to multiple insecticides [29]. Our results reveal that the decline of bacterial symbionts, especially *Wolbachia*, contributes to increased insecticide susceptibility and decreased detoxification metabolism in *N. lugens* in response to the high-temperature treatment. The role of *Wolbachia* in temperature-dependent insecticide response in insects was validated by comparative insecticide assays and gene expression analysis between *Wolbachia*-free (WU) and *Wolbachia*-infected (WI) in both *N. lugens* and *D. melanogaster*. Our finding supports that host-bacterial symbiosis is a crucial component of insect detoxification metabolism and that this partnership function is vulnerable to elevated temperature.

MATERIALS AND METHODS

Insects

A field population of brown planthopper (*N. lugens*) was originally collected from rice paddy field in XinYang (31.58°N, 115.24°E), China, in 2017. WI and WU *N. lugens* were kindly provided by Prof. Xiaoyue Hong (Nanjing Agricultural University, Nanjing, China), WI and WU *N. lugens* were isolated from natural populations with ~50% infection rate of *Wolbachia*. Their nuclear genetic background was homogenized by backcrossing for at least seven generations, and their symbiont content has characterized. Both WI or WU insects were *Arsenophonus*-free [30]. The presence or absence of *Wolbachia* infection was verified by PCR using primers (*wsp*-F: 5′-TGGTCCAATA AGTGATGAAGAAACT-3′, *wsp*-R: 5′-AAAAATTAAACGCTACTCCAGCTTC-3′) that targeted the *Wolbachia wsp* gene (Fig. S1a). *N. lugens* have been maintained on rice seedlings at 28 °C under 70–80% relative humidity and a 16 h light/8 h dark photoperiod [31].

WI (Dmel *w*Mel) and WU (Dmel *w*Mel T) *Drosophila melanogaster* (Brisbane nuclear background with introgressed Dmel *w*Mel from YW, namely, Dmel *w*Mel) were kindly provided by Prof. Yufeng Wang (Central China Normal University, Wuhan, China). Dmel *w*Mel T was generated from Dmel *w*Mel by tetracycline treatment. Male Dmel *w*Mel T flies were then backcrossed with Dmel *w*Mel females for three generations to allow recolonization of gut microbiota [32]. The presence or absence of *Wolbachia* infection was verified by PCR using primers (*wsp*-F: 5′-TGGTCCAATAAGTGATGAAGAA-3′, *wsp*-R: 5′-AAAAATTAAACGCTACTC-CAAC-3′) that targeted the *Wolbachia wsp* gene (Fig. S1b). All laboratory lines were reared on cornmeal-yeast-agar medium (per 400 mL medium contains 350 mL water, 7.5% corn flour, 7.5% brown sugar, 0.5% yeast, and 0.5% agar) at 25 °C with a photoperiod of 14 h light/10 h dark conditions (200 ± 10 eggs per 50 mL vial of medium in a 150 mL conical flask) [33].

Temperature treatment

Healthy fifth instar nymphs of *N. lugens* or 3–10-day-old female *D. melanogaster* adults were exposed to different constant temperatures (indicated in the result figures) for 24 h in an artificial climate chamber under 70–80% relative humidity (Ruihua, China), and the treated insects were subsequently tested in the different assays.

Antibiotic treatment

The antibiotic treatment of *N. lugens* was performed according to a previous study [34]. Briefly, rice seedling roots were dipped in the 1 g/L tetracycline, sulbactam, or ciprofloxacin solution for 1 day and then *N. lugens* adults were allowed to lay eggs on the antibiotics-treated rice seedlings for 3 days. The adults were then discarded while the rice seedlings containing eggs continued to be treated with the antibiotic solutions. Antibiotics were replaced daily until the nymphs grew to the fourth instar stage, before they were transferred onto new fresh rice seedlings (without antibiotics) and grew to the fifth instar. These fifth instar nymphs were used for future experiments. *N. lugens* fed on no-antibiotic (sterile water only) treated rice seedlings were set up in parallel as the control group.

Insecticide bioassays

Two different insecticide bioassays were used, the glass vial and topical application methods. For both methods, fifth instar nymphs of *N. lugens* (pretreated with/without high temperature or antibiotic) or 3–10-day-old female *D. melanogaster* adults were treated with technical grade imidacloprid (96%, Sigma-Aldrich, USA) [35]. The glass vial method was performed following previous protocol [35]. Briefly, glass vials with a total inner surface of 36.9 cm^2 (4.3 cm in height and 2.4 cm in diameter) were coated with 350 µL of an imidacloprid solution in acetone (200 mg/L for *N. lugens* or 1 g/L for *D. melanogaster*) or with acetone alone (control). The vials were then rotated on a mechanical roller (35 rpm) for 30 min at room temperature until the acetone evaporated completely to achieve a uniform insecticide coating on the inner surface. Twenty insects were placed into each vial, and five replicate vials were set up per treatment. The vials were held upright in a growth chamber (28 °C) with the caps kept loosely closed. The number of dead insects was recorded every 10 min or 3 h for *N. lugens* and *D. melanogaster*, respectively. For the topical application method, a droplet of 40 nL insecticide solution (containing 0.012 ng imidacloprid for *N. lugens* and 0.02 ng imidacloprid *D. melanogaster*) was applied to the pronotum or pleuronotum of each insect. Five replicates of twenty insects each were tested per treatment. The number of dead insects was recorded 24 h later.

Enzyme activity assay

The activities of detoxifying enzymes (cytochrome P450s [P450s], GSTs, and esterases (ESTs)) were assayed following our previous protocol [12]. Fifth instar nymphs of *N. lugens* or 3–10-day-old female *D. melanogaster* adults were used. Briefly, 0.2, 0.02, and 0.05 g of insects were homogenized in 0.1 M sodium phosphate buffer for the P450s, ESTs, and GSTs activity assays, respectively. The substrates used for the assays were 7-ethoxycoumarin-O-deethylase for P450s, *α*-naphthyl acetate for ESTs, and 1-chloro-2, 4-dinitrobenzene for GSTs. For P450s, the absorbance of reactions was measured by the Spark 10 M Multimode Microplate Reader (Tecan, Switzerland) at the optical density (OD) excitation wavelength of 358 nm and an emission wavelength of 456 nm. For ESTs and GSTs, the OD was measured at 600 and 340 nm using a NP80 nanophotometer (Implen, Germany), respectively.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Thirty healthy fifth instar nymphs of *N. lugens* or 3–10-day-old female *D. melanogaster* adults (snap frozen in liquid nitrogen before storage at −80 °C) were used for total RNA extraction, using the RNAiso plus kit (TAKARA, DaLian, China) following the manufacturer's protocol. Samples were homogenized in 1 mL RNAiso Plus for the extraction. RNA (1 µg) was used to synthesize cDNA using the RevertAid First Strand cDNA Synthesis Kit (TAKARA, DaLian, China). Subsequently, to detect the P450, GST, and *N. lugens CncC* (*NlCncC*) gene expression levels, the cDNA was used as the template for qRT-PCR with 20 µL reactions containing 10 µL of the SoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) and 100 nM of the primers. Housekeeping genes *GAPDH* and *β-actin* were employed as the double references to normalize gene expression levels for *N. lugens* [36], and *Dmrp49* was employed as the reference for *D. melanogaster* (Table S1). Details of all primers are shown in Table S1. PCR was run in an iQ2 Optical System (Bio-Rad) at the following thermal cycle: initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 10 s. After the thermal cycles, a melt curve analysis was conducted from 55 to 95 °C. The relative expression levels were calculated based on the $2^{-\Delta\Delta CT}$ method [37].

Relative and absolute quantification of insect bacterial load

Total genomic DNA was extracted from the whole bodies of *N. lugens* using the FastDNA SPIN Kit (MP, Biomedicals, California, USA) for soil following the manufacturer's protocol. The relative total and individuals bacterial load was measured via qRT-PCR as described above using specific primers

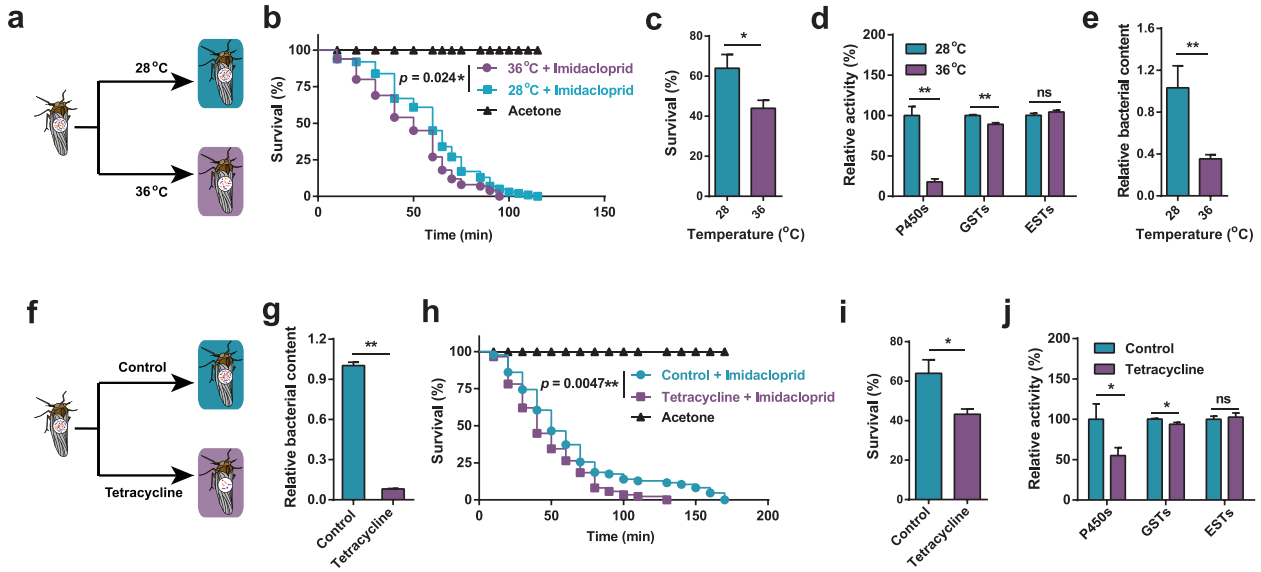

Fig. 1 ***Nilaparvata lugens*** **responses to insecticide are negatively impacted when they are pre-exposed to elevated temperature or antibiotics. a** Temperature treatments. Fifth instar nymphs of *N. lugens* were treated at two different temperatures (28 and 36 °C) for 24 h, followed by assays for their susceptibility to insecticide, detoxification metabolism, and bacterial content. **b** Survival curves of *N. lugens* to imidacloprid using the glass vial method after pre-exposure to 28 or 36 °C ($n = 100$ per treatment). Statistical comparison was based on the log-rank (Mantel–Cox) test, and the level of significance for results was set at $*p < 0.05$ and $**p < 0.01$, respectively. **c** Survival of *N. lugens* to imidacloprid using the topical application method after pre-exposure to 28 or 36 °C ($n = 100$ per treatment). **d** The impact of temperature on the detoxifying enzyme activities (cytochrome P450s: P450s; glutathione *S*-transferases: GSTs; esterases: ESTs) of *N. lugens*. **e** The impact of temperature on the bacterial load of *N. lugens* ($n = 3$). **f** Antibiotic treatment. *N. lugens* were treated with tetracycline for 1 generation, followed by assays for their susceptibility to insecticide, detoxification metabolism, and bacterial content. **g** Effect of tetracycline on the bacterial load of *N. lugens* ($n = 3$). **h** Survival curves of tetracycline-treated and control *N. lugens* to imidacloprid using the glass vial method ($n = 100$ per treatment). Statistical comparisons were based on the log-rank (Mantel–Cox) test, and the level of significance for results was set at $**p < 0.01$. **i** Survival of tetracycline-treated and control *N. lugens* to imidacloprid using the topical application method ($n = 100$ per treatment). **j** The impact of antibiotic treatment on the detoxifying enzyme activities (P450s, GSTs, and ESTs) of *N. lugens*. Bar graphs reflect the mean ± standard error of the mean (SEM), and statistical comparisons were based on *t*-tests. The level of significance for the results was set at $*p < 0.05$ and $**p < 0.01$. ns no significant difference.

(Table S2). To quantify absolute bacterial abundance, the full sequence of the 16S rRNA gene fragments was cloned into the pClone 007 blunt vector (Tisngke, Beijing, China), and the recombinant vector was used as a standard sample for qRT-PCR. The absolute total insect bacterial load was calculated based on standard curves from amplification of the cloned target sequence in recombinant vector, and absolute copies of each genus 16S rRNA were calculated via the ratio of each genus 16S rRNA to total bacterial 16S rRNA gene amplicons. All the above operations were carried out on a clean bench [38].

High-throughput sequencing of the 16S rRNA gene amplicons

DNA samples comprised four biological replicates of 30 fifth-instar nymphs of *N. lugens* for each treatment. The insects were surface-washed with 75% ethanol for 90 s, rinsed three times with sterilized deionized water, then homogenized with 1 mL sterile deionized water and frozen at −80 °C before DNA extraction. DNA extraction was performed as described above. The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified with the primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGAC-TACHVGGGTWTCTAAT-3′) in a thermocycler PCR system (GeneAmp 9700, ABI, USA). The PCRs were conducted using the following thermal cycle: 95 °C for 3 min, 27 cycles of 30 s at 95 °C, at 55 °C for 30 s, and 72 °C for 45 s, and a final extension at 72 °C for 10 min. PCRs were performed in triplicate in a 20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of the template DNA. PCR products were loaded on 2% agarose gel in gel electrophoresis, further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor-ST kit (Promega, USA). Purified amplicons were pooled in equimolar amounts and paired-end sequenced (2 × 300) on a MiSeq platform (Illumina, San Diego, USA) according to the standard protocols of Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

Amplicon sequence analysis

Raw fastq files were demultiplexed, quality-filtered by QIIME2 (version 2020.11) according to the official tutorials (https://docs.qiime2.org/2020.11/tutorials/) with slight modifications. Briefly, raw sequence data were demultiplexed using the demux plugin following by primers cutting with the cutadapt plugin [39]. Sequence data were processed with DADA2 plugin to quality filter, denoise, merge and remove chimera [40]. Non-singleton amplicon sequence variants (ASVs) were aligned with mafft [41], Alpha diversity and beta diversity were analyzed by diversity plugin with the samples rarefied. Taxonomy was assigned to ASVs using the classify-sklearn naïve Bayes taxonomy classifier in feature-classifier plugin [42], against the SILVA 138. Principle Coordinate Analysis (PCoA) was done by Bray–curtis scaling of *β*-diversity distance matrices using the cmdscale function in R.

RNA interference (RNAi)

The primers for the target sequences of *NlCncC* and *GFP* are provided in Table S3. The PCR products were used as templates for dsRNA synthesis using the T7 Ribo MAX Express RNAi System (Promega, Madison, WI, USA). After synthesis, the dsRNA was precipitated with isopropanol and resuspended in DEPC water. The quantity of dsRNA was determined by agarose gel electrophoresis (1%) and an NP80 NanoPhotometer. DsRNA was kept at −80 °C until use. The dsRNA injection was conducted on the fourth instar nymphs, in which 50 nL (5 μg/μL) of purified dsRNA was injected at a slow speed at the conjunction between the prothorax and mesothorax as described previously [43]. Injected insects were placed in empty pots to recover, then randomly selected for checking interference efficiency and the bioassays. The relative expression of *NlCncC* in dsRNA injected *N. lugens* was detected by qRT-PCR as described.

Statistical analyses

The PCoA plots were compared to one another using the procrustes analyses in the vegan version 2.5–7 package, and the significance

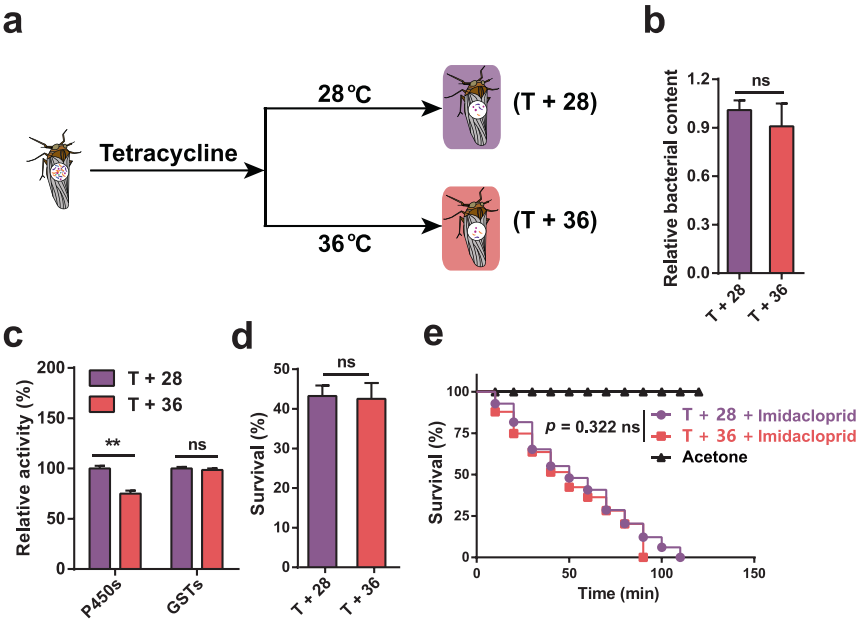

Fig. 2 Temperature-dependent effects on *Nilaparvata lugens* insecticide response is abolished by antibiotic pretreatment. a Experimental design. Tetracycline-treated *N. lugens* were exposed to the two different temperatures (28 and 36 °C) for 24 h (named T + 28 and T + 36), then subjected to the different bioassays. **b** Bacterial symbiont content of T + 28 and T + 36 *N. lugens* ($n = 3$). **c** Relative activity of cytochrome P450s and glutathione *S*-transferases under T + 28 and T + 36. Bar graphs reflect the SEM, and statistical comparisons were based on *t*-tests. **d** Survival of T + 28 and T + 36 *N. lugens* to imidacloprid using the topical application method ($n = 100$ per treatment). **e** Survival curves of T + 28 and T + 36 *N. lugens* to imidacloprid using the glass vial method ($n = 100$ per treatment). The level of significance for the results was set at $^*p < 0.05$ and $^{**}p < 0.01$. ns no significant difference.

parameters were calculated with the PROTEST function, which is a permutation test [44]. Statistical analyses of differentially abundant bacterial symbionts and differentially expressed genes were performed using the DESeq2 version 1.28.1 package in R. Network diagram was made by Gephi 0.9.2. GraphPad Prism version 7.0 was used for statistical analyses and data plotting. Significant differences in microbial diversity were analyzed by one-way analysis of variance with means compared by applying Fisher's least significant difference test. Unpaired *t*-tests were used to analyze significant differences in mortality and qRT-PCR, and the survival curve was analyzed by the log-rank (Mantel–Cox) test. The level of significance was set at $^*p < 0.05$ and $^{**}p < 0.01$.

RESULTS

Pre-exposure to high temperature reduces *N. lugens* detoxification enzyme activities and exacerbates their susceptibility to imidacloprid

To investigate the effect of temperature on *N. lugens* response to insecticide, fifth instar nymphs were pre-exposed to two different temperatures (28 and 36 °C) for 24 h, and subsequently, the treated with imidacloprid (Fig. 1a). The results indicate that *N. lugens* pre-exposed to the high temperature became more susceptible to imidacloprid, as they died at a faster rate in the glass vial method (Fig. 1b) and had higher mortality in the topical application method (Fig. 1c). In addition, the activities of key detoxification enzymes cytochrome P450s (P450s) and GSTs, but not ESTs, were significantly reduced in the high-temperature treatment ($p < 0.0001$, $p = 0.002$, and $p = 0.285$, respectively) (Fig. 1d).

The temperature-dependent responses to insecticide are linked to bacterial abundance in *N. lugens*

Through qRT-PCR, we learned that the total bacterial content was substantially reduced in *N. lugens* exposed to 36 °C as compared to 28 °C, by 64.6% (Fig. 1e). This leads us to hypothesize that *N. lugens* susceptibility to insecticide is regulated by their bacterial symbionts.

To verify this relationship, tetracycline was employed to disrupt the bacterial symbionts of *N. lugens* (Fig. 1f), resulting in over 90% reduction in their bacterial content (Fig. 1g). Similar to high-temperature-treated *N. lugens*, tetracycline-treated *N. lugens* also became more susceptible to imidacloprid, as demonstrated by both the glass vial (Fig. 1h) and topical application (Fig. 1i) methods. Similarly, the activities of cytochrome P450s and GSTs, but not ESTs, were also significantly reduced in tetracycline-treated *N. lugens* ($p = 0.049$, 0.047, and 0.689, respectively) (Fig. 1j).

Both high temperature and antibiotic treatment rendered *N. lugens* more susceptible to the insecticide, in conjunction with reduced bacterial content and reduced expression of specific detoxification enzymes. To test whether the effect on insecticide response involves additional non-redundant mechanisms, tetracycline-pretreated *N. lugens* were exposed to the two different temperatures (28 and 36 °C), labeled T + 28 and T + 36 (Fig. 2a). Total bacterial content was not different between T + 36 and T + 28 (Fig. 2b). Importantly, while the P450s activity decreased in T + 36 as compared to T + 28 (Fig. 2c), the susceptibility to imidacloprid did not differ between T + 36 and T + 28 *N. lugens* (Fig. 2d, e), suggesting that the high-temperature effect on *N. lugens*'s insecticide susceptibility was attributed to reduced bacterial content.

Bacterial symbionts regulate detoxifying gene transcriptional responses to temperature

The enzymatic activities of cytochrome P450s and GSTs are controlled by large families of genes. To investigate the genetic mechanism by which high temperature reduced bacteria-dependent detoxification, the expression levels of 53 P450 genes and 11 GST genes involved in detoxification metabolism were examined by qRT-PCR (Fig. 3a). Treatment with tetracycline and high temperature resulted in significant ($p < 0.05$) downregulation of 30 and 20 detoxifying genes, respectively. Among them, eleven genes were downregulated in both tetracycline- and high-temperature-treated *N. lugens* (*NlGSTm2*, *NlCYP4CE1*, *NlCYP6AY1*,

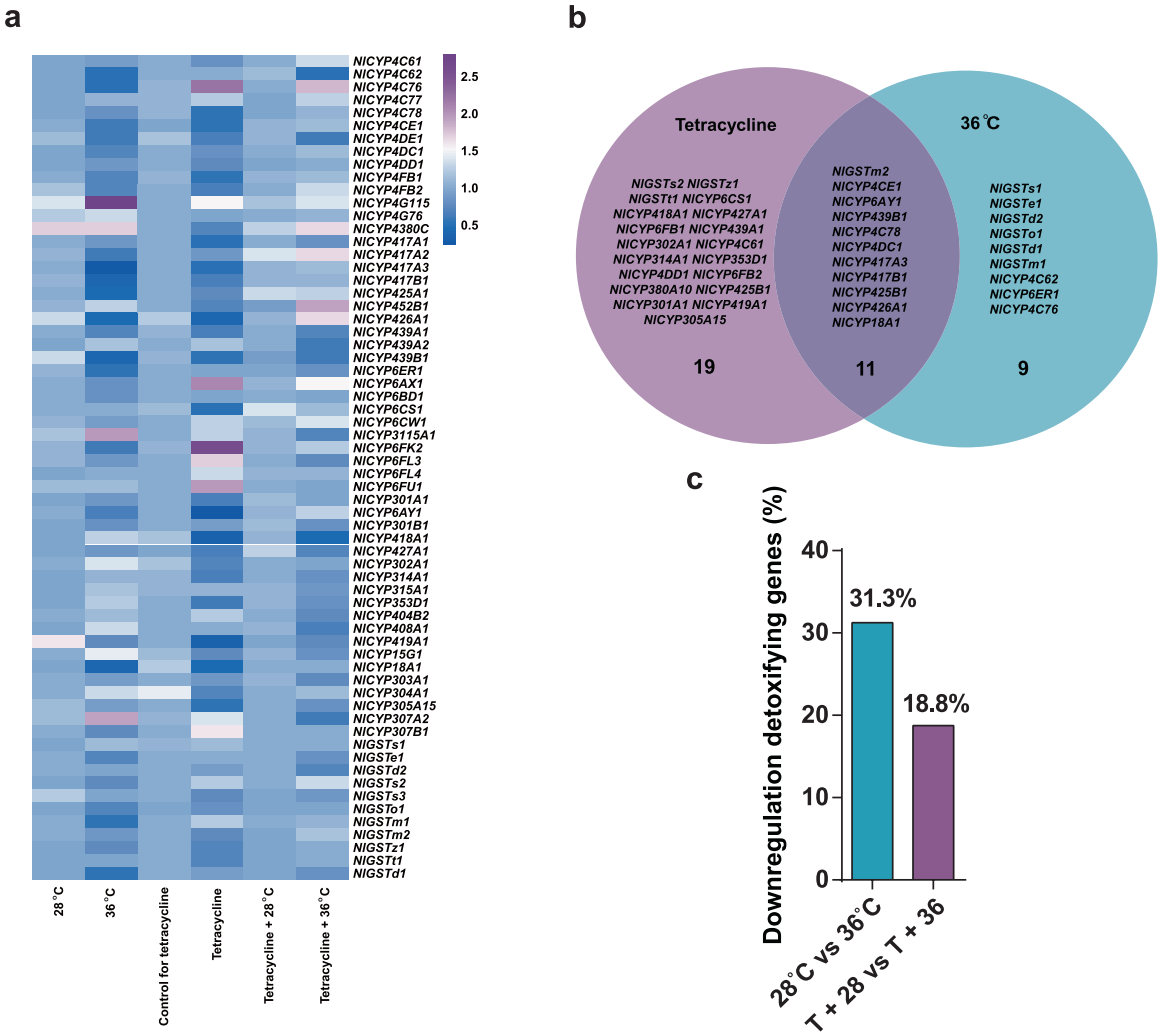

Fig. 3 Transcriptional responses of detoxifying genes in *Nilaparvata lugens* exposed to the different temperatures and/or antibiotics. a A heatmap describing the majority of detoxifying genes were downregulated by exposure to high-temperature, tetracycline, and tetracycline-treated *N. lugens* exposing to the high temperatures (36 °C) for 24 h (T + 36). **b** A Venn diagram showing the core and distinct sets of detoxification genes being downregulated by the high temperature and tetracycline. **c** Percentage of downregulated *N. lugens* detoxifying genes by the 36 °C treatment alone as compared to T + 36.

NlCYP439B1, *NlCYP4C78*, *NlCYP4DC1*, *NlCYP417A3*, *NlCYP417B1*, *NlCYP425B1*, *NlCYP426A1*, and *NlCYP18A1*), and their expression were not different between the T + 36 and T + 28 treatments (Fig. 3b and Table S4), which suggests these genes play a role in symbiont-dependent insecticide detoxification sensitive to high temperature. Furthermore, 31.3% of detoxifying genes were significantly downregulated in 36 °C compared to 28 °C group, while only 18.8% were downregulated in T + 36 compared to T + 28 (Fig. 3a, c and Table S4), implying that the impact of temperature on detoxification gene transcription independent of bacterial symbiosis is less pronounced.

Bacterial symbionts modulated detoxifying genes via the Cap 'n' Collar C (CncC) pathway

A previous study revealed that the *D. melanogaster* gut bacterial symbiont *Lactobacillus* mediated paraquat detoxification through upregulating the CncC pathway (also known as the Nrf2, a regulator of cellular responses to oxidative stress across metazoan) [16]. We hypothesize that bacterial symbionts in *N. lugens* also regulate host detoxification metabolism via this highly conserved pathway. To this end, the expression profile of *NlCncC* was analyzed by qRT-PCR. The results indicated that the expression level of *NlCncC* was significantly downregulated in bacteria-depleted *N. lugens* after exposure to high temperature or tetracycline treatment (Fig. 4a). To confirm the role of *NlCncC* in detoxification metabolism in *N. lugens, dsNlCncC* was injected into the insect, resulting in 67–44% reduction in gene expression of *NlCncC* at 24–72 h (Fig. 4b). The successful knockdown of *NlCncC* by RNAi resulted in repressed P450 and GST gene expression (Fig. S2 and Table S4) and also P450s and GSTs activities (Fig. 4c), leading to increased susceptibility of *N. lugens* to imidacloprid (Fig. 4d). In addition, the imidacloprid susceptibility of *dsNlCncC*-injected *N. lugens* was not further compromised by the high-temperature treatment even though the bacterial symbionts were reduced by the high temperature (Fig. 4e–g). Together, these findings suggested that the bacterial symbionts modulated *N. lugens* detoxication to insecticide via the *CncC* pathway.

Wolbachia contributes to temperature-dependent host detoxification metabolism

Our high-throughput sequencing data show that the *N. lugens* bacteriome is dominated by 3–5 genera. Specifically, *Arsenophonus* had the highest relative abundance among all symbionts, accounting for 40–75% of the reads. *Wolbachia* accounted for 5–45%, and the other genera accounted for

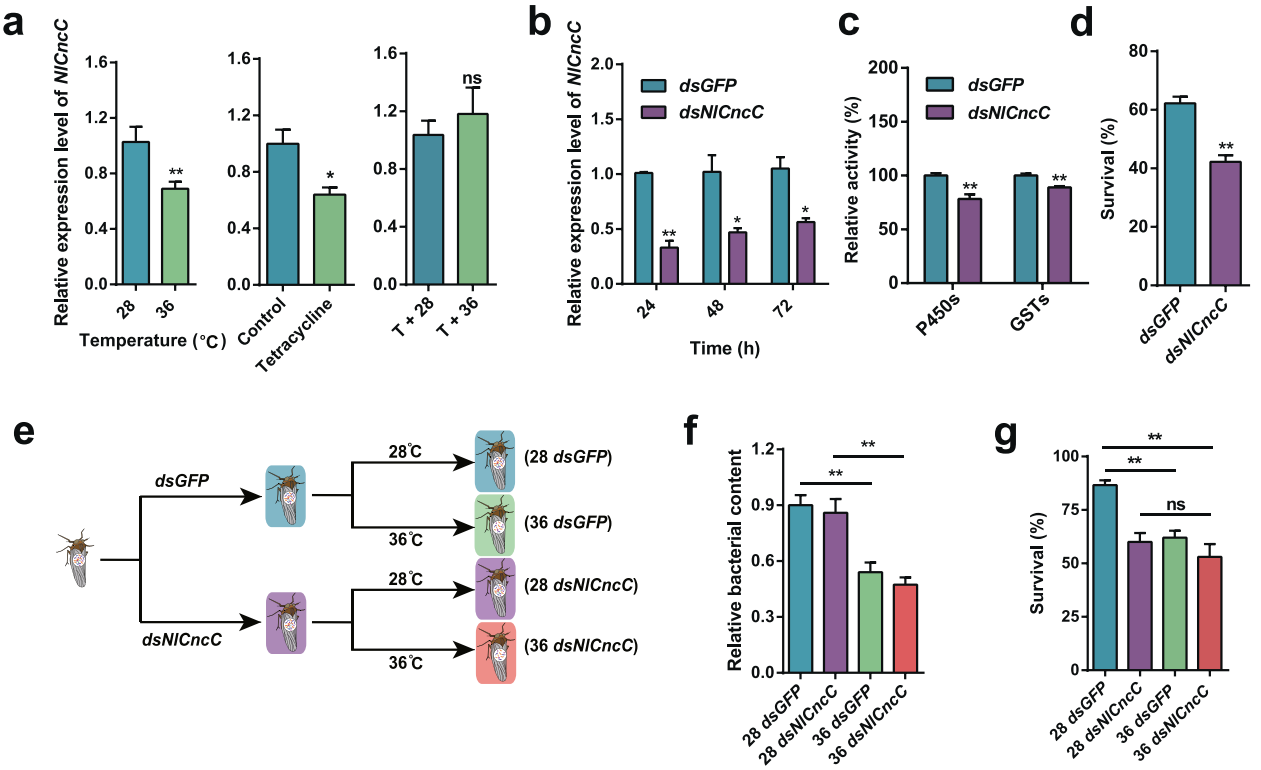

Fig. 4 Cap 'n' Collar C (*NlCncC*) is involved in temperature-mediated detoxification metabolism via bacterial symbionts in *Nilaparvata lugens*. **a** The expression level of *NlCncC* in *N. lugens* subjected to the different treatments. Control- and tetracycline-treated *N. lugens* were exposed to the two different temperatures (28 and 36 °C) for 24 h, then the expression levels of *NlCncC* were detected in three group treatments (28 and 36 °C; control and tetracycline; T + 28 and T + 36). The relative expression levels of *NlCncC* were normalized with respective control treatment. **b** Interference efficiency of *NlCncC* at 24, 48, and 72 h after injection with *dsNlCncC* ($n = 3$). **c** The impacts of *NlCncC* knockdown on the cytochrome P450s and glutathione *S*-transferases activities in *N. lugens*. **d** The impacts of *NlCncC* knockdown on the survival of *N. lugens* in response to imidacloprid. **e** Design of the experiment to confirm the role of *NlCncC* in regulating the temperature-dependent effect on insecticide resistance and detoxification in *N. lugens*. **f** Total bacterial content of *dsNlCncC* or *dsGFP*-injected *N. lugens* following exposure to the different temperatures. ($n = 3$). **g** Survival of *dsNlCncC*- and *dsGFP*-injected *N. lugens* exposed to imidacloprid following exposure to the different temperatures using the topical application method ($n = 100$ per treatment). Bar graphs reflect the SEM, and statistical comparisons were based on *t*-tests. The level of significance for the results was set at $^{*}p < 0.05$ and $^{**}p < 0.01$.

5–25% of the reads (Fig. S3a, b). Tetracycline but not high temperature changed the diversity of bacterial symbionts of *N. lugens* (Fig. S4a, b). Whereas there is no difference in α diversity index after exposure in either tetracycline or high temperature (Fig. S4c, d). Nonetheless, both high temperature and antibiotic treatments drastically reduced the absolute abundances of many genera, especially, the abundances of the dominant *Arsenophonus*, *Wolbachia*, and *Acinetobacter* (Fig. S3c). To pinpoint the relative contribution of these different bacteria to host detoxification against the insecticide, we first conducted an antibiotic susceptibility test on the *N. lugens* using three different antibiotics, tetracycline, ciprofloxacin, and sulbactam (Fig. 5a). The results indicated that *Wolbachia* was selectively perturbed by tetracycline; while *Arsenophonus* could be reduced by both ciprofloxacin and tetracycline, and all three antibiotics could significantly reduce the *Acinetobacter* load (Fig. 5b, c, and S5, and Table S5).

Subsequently, the expression profile of detoxifying genes under each antibiotic treatment was measured by qRT-PCR. The PCoA plots demonstrated that the detoxifying gene expression patterns were differentially affected by the different antibiotic treatments (Fig. 5d). Notably, 15 and 18 genes were upregulated and downregulated, respectively, in the tetracycline-treated *N. lugens* (Fig. S6a, d, e). Seventeen and eighteen genes were upregulated and downregulated after ciprofloxacin treatment, respectively (Fig. S6b, d, e). However, in the sulbactam group, 15 and 14 genes were upregulated and downregulated, respectively (Fig. S6c, d, e). In tetracycline-treated *N. lugens*, eight genes (seven downregulated and one upregulated) were specific changed different from the other two antibiotics, whereas ciprofloxacin and sulbactam only have three (one downregulated and two upregulated, respectively) (Fig. S6f). The expression profiles of 13 genes were changed across all three antibiotics treatments (Fig. S6f). When we examined the relationship between changes in bacterial community structure and detoxifying gene expression pattern in response to the antibiotic treatments by the Procrustes analysis, a significant degree of congruence was found between the tetracycline-molded symbiont structure and detoxifying gene expression ($p = 0.001$), while the other two antibiotics that did not show such significant congruence (Fig. 5e–h). The Venn diagram further shows the abundances of four bacteria that were specifically affected by the tetracycline relative to the other three treatments (no-antibiotic control, ciprofloxacin, and sulbactam), and *Wolbachia* dominated the abundance changes (Fig. 5i, Tables S5–8). These results suggested that *Wolbachia* plays a leading role in the detoxification metabolism of *N. lugens*.

To test the role of *Wolbachia* in host insecticide susceptibility in relation to detoxification metabolism, we employed WI and WU *N. lugens* (Fig. 6d). The WU *N. lugens* were more susceptible to imidacloprid compared to WI *N. lugens* (Fig. 6a) and had a lower P450s activity as well as P450 and *NlCncC* gene expressions (Fig. 6b, c). In addition, the negative impact of high-temperature

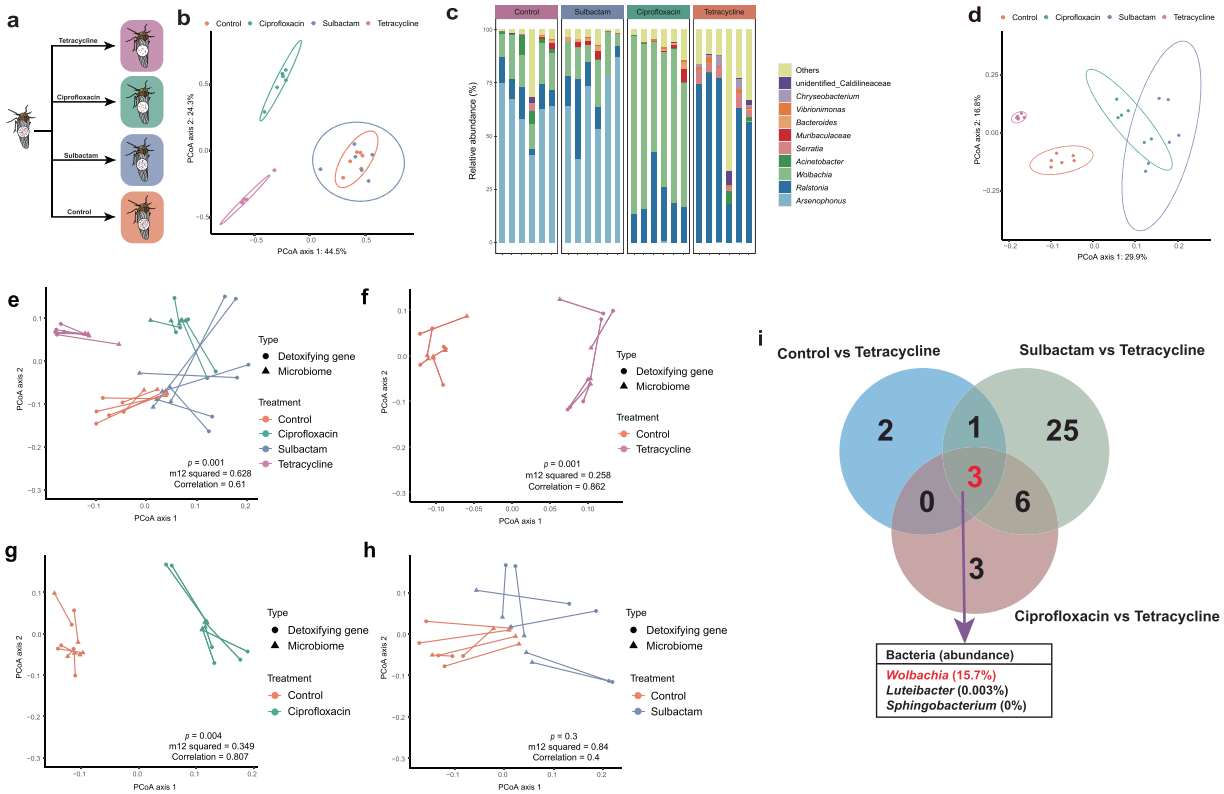

Fig. 5 The structural molding of a microbiome with respect to the detoxifying gene expression profile diversity in *Nilaparvata lugens*. **a** Experimental design. *N. lugens* were treated using 1 g/L of different antibiotics (tetracycline, ciprofloxacin, and sulbactam) for one generation, then subjected to microbiome and expression of detoxifying genes analyzed. ($n = 6$). **b** The principal coordinate analysis (PCoA) plots of the Bray–Curtis distances for the sample bacterial communities from each point corresponds to a different sample and are colored by treatment. The ellipse showed confidence intervals of 95%. **c** Relative abundance of bacterial sequence read counts as classified at the taxonomic genus level. **d** The PCoA plots of the Bray–Curtis distances for the relative expressions sample detoxifying genes from each point corresponds to a different sample, as colored by treatment. The ellipse showed confidence intervals of 95%. Procrustes analysis under all treatments (**e**), procrustes analysis under group control and tetracycline (**f**), ciprofloxacin (**g**), and sulbactam (**h**) treatments, the color of each dot/triangle represents the different antibiotics. Satisfying conditions were significant ($m12^2$ values of <0.45, correlation values of >0.775, and significance values of ≤0.001). **i** A Venn diagram showing the significantly difference genera based on DESeq2 test between tetracycline treatment and control, ciprofloxacin, or sulbactam, *Wolbachia* was the dominant genera in all comparison group' genera.

pre-exposure *N. lugens* survival against insecticide was only noticeable in WI strains but not WU strains (Fig. 6d–f). Importantly, we also find that the effect of *Wolbachia* on host detoxification metabolism and insecticide susceptibility was not restricted to the brown planthoppers when we conducted the same set of assays on WI (Dmel *w*Mel) and WU *D. melanogaster* (Dmel *w*Mel T) (Fig. 7d). Like the *N. lugens*, the Dmel *w*Mel T flies were more susceptible to imidacloprid compared to Dmel *w*Mel flies (Fig. 7a), had a reduced detoxification metabolism in the context of P450s activity (Fig. 7b) and detoxifying gene expression (Fig. 7c).

The *Wolbachia* content in Dmel *w*Mel flies was significantly reduced, by over 68% after exposure to high temperature (Fig. 7e), which accounted for the increased susceptibility to imidacloprid (Fig. 7f), whereas the high temperature did not affect the susceptibility of Dmel *w*Mel T flies. Together, our results support that *Wolbachia* was the key symbiont underlying temperature-dependent host detoxification of, and susceptibility to, imidacloprid.

DISCUSSION

Temperature is one of the most important abiotic factors that impacts the development, physiology, and behavior of organisms at both individual and population scales [5, 45]. As the global temperature continues to rise, many ecological interactions between organisms will be affected, including the partnerships between arthropods and their symbiotic microbes [46–48]. In this study, we reveal a significant consequence of insect-bacterial symbiosis breakdown due to elevated temperature on host response to imidacloprid, the most widely used insecticide that belongs to the neonicotinoid class. Our results demonstrate that bacterial symbionts in the planthoppers function as a mediator of host detoxification metabolism. This partnership function is disrupted under high temperature due to the decline of bacterial symbionts, causing the host to become more imidacloprid-susceptible.

Mechanistically, the bacterial symbionts affect *N. lugens* imidacloprid susceptibility indirectly by modulating host detoxification metabolism. We further establish that the bacterial symbionts orchestrate host detoxification enzyme activities and gene expression through the *CncC*-dependent pathways in *N. lugens*. Our RNAi experiments confirm *NlCncC* expression has a direct impact on P450s activity, a result consistent with previous studies [49, 50]. The involvement of *CncC* (also named Nrf2 in mammals) in insecticide resistance has been documented in other insects [50–52], but our study is the first that links the regulation of this pathway to insect-bacterial symbiosis.

Our work also points to a potential role of *Wolbachia* in regulating host insecticide resistance in response to environmental temperature changes. A previous study demonstrated that displacing N-type *Arsenophonus* with S-type through experimental injection reduced *N. lugens* P450 *CYP6AY1* gene expression and

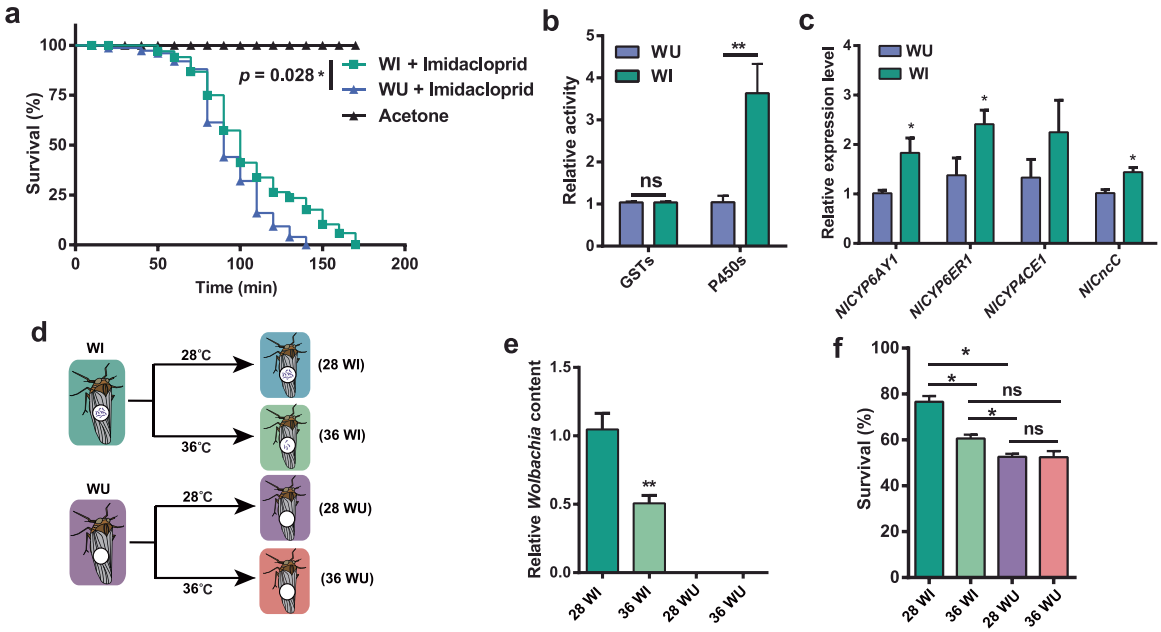

Fig. 6 The effects of high temperature on insecticide susceptibility and detoxification metabolism of *Wolbachia*-infected (WI) and *Wolbachia*-free (WU) *Nilaparvata lugens*. **a** Survival curves of WI and WU *N. lugens* exposed to imidacloprid ($n = 100$ per treatment). Statistical comparisons were based on the log-rank (Mantel–Cox) test, and the level of significance for results was set at $^*p < 0.05$. **b** The enzymatic activities of cytochrome P450s and glutathione *S*-transferases in WI and WU *N. lugens* (without any treatment). **c** The expression profile of P450 genes in WI and WU *N. lugens* ($n = 3$). **d** The experimental design. WI or WU *N. lugens* were exposed to two different temperatures (28 and 36 °C) for 24 h, followed by assays for their susceptibility to insecticide, and *Wolbachia* abundance. **e** The impact of temperature on the *Wolbachia* abundance in WI and WU *N. lugens* ($n = 3$). **f** Survival of WI and WU *N. lugens* exposed to imidacloprid following the different temperature pre-treatments using the topical application method ($n = 100$ per treatment). Bar graphs were expressed as the mean ± standard error of the mean (SEM), and statistical comparison was based on *t*-tests. The level of significance for results was set at $^*p < 0.05$ and $^{**}p < 0.01$. ns no significant difference.

increased insecticide susceptibility [18]. *CYP6AY1* and three other P450 genes (*CYP6ER1*, *CYP4CE1*, and *CYP6CW1*) have been shown to mediate imidacloprid resistance of *N. lugens* in the previous study [36], and these genes are regulated by transcription factors, including *NlCncC*, adipokinetic hormone receptor and inhibition of hepatocyte nuclear factor 4 [53, 54]. However, the relationship between *Wolbachia* and insecticide resistance in *N. lugens* has not been considered. In our *N. lugens* microbiome, *Wolbachia* was the second most abundant genera after *Arsenophonus*. Significant decline of *Wolbachia* alongside *Arsenophonus* by the high temperature resulted in increased imidacloprid susceptibility and reduced host expression of detoxification genes, rendering the hosts more imidacloprid-susceptible. By comparing the imidacloprid responses between WI and WU host pre-exposed to the different temperatures in both *N. lugens* and *Drosophila*, we reveal that hosts bearing *Wolbachia* are more resistant to the imidacloprid, while *Wolbachia* decline leads to increased imidacloprid susceptibility associated with reduced detoxification metabolism. The findings are consistent with studies that supported *Wolbachia*-dependent insecticide resistance in *Laodelphax striatellus* [55] and *Culex pipiens* [56]. Strikingly, host gene expression was steered via *Wolbachia* in both previous studies and in the present study [57, 58]. The mutualistic facet of *Wolbachia* in protecting host against insecticide or other xenobiotics may promote the host survival and thus the stability of symbiosis [59].

One aspect we did not definitively test in this study is whether *Wolbachia* directly activates the *CncC* pathway to enhance host detoxification as was indicated in *lactobacillus*, a gut symbiont of *D. melanogaster* [16]. It is possible that the highly conserved *CncC* pathway is a universal regulator of insect detoxification that senses microbial signals coming from different symbionts in different insects. In *Drosophila*, *CncC* is elicited by *lactobacillus*-induced enzymatic generation of reactive oxygen species (ROS) in the gut [16]. ROS induction by *Wolbachia* has also been shown in the mosquitoes [60]. Together, *Wolbachia* may direct host detoxification using ROS as a transducer of bacterial signals into host gene regulatory networks in the planthoppers, which provides a guide for future investigations.

It is worth noting that depletion of both *Wolbachia* and *Arsenophonus* was found to correlate with insecticide susceptibility [18]. The relative contribution of *Wolbachia* and *Arsenophonus* to *N. lugens* susceptibility to imidacloprid warrants future investigation. It will be important to clarify whether the two symbionts exert additive or synergistic effects, or if they are functionally redundant, in supporting host detoxification. Gnotobiotic *N. lugens* with selective *Wolbachia-Arsenophonus* profiles can be generated to address this question, whereas the symbionts might also be introduced to the insect at different life stages or ages by transinfection to elucidate any developmental effects [30, 61]. Understanding the impact of temperature on symbiont gene expression (metatranscriptome) in a community context will also help inform their interactive effects on host detoxification metabolism.

While our results indicate that bacterial symbionts participate in host detoxification of imidacloprid, we acknowledge there may be other host detoxification processes independent of symbiosis, especially given other pathways besides *NlCncC* have been shown to regulate *N. lugens* imidacloprid resistance [53, 54, 62].

Our findings glimpsed a possible scenario in which global warming may impact insecticide usage in a positive way, on the basis that elevated temperatures may make insects more insecticide-susceptible by disrupting their symbioses with microbes, particularly *Wolbachia* that is wide spread among arthropods [63]. It is, however possible that symbionts could adapt to gradual rises in temperature. A previous study on fish (tropical tilapias) suggested that host selection for cold tolerance is

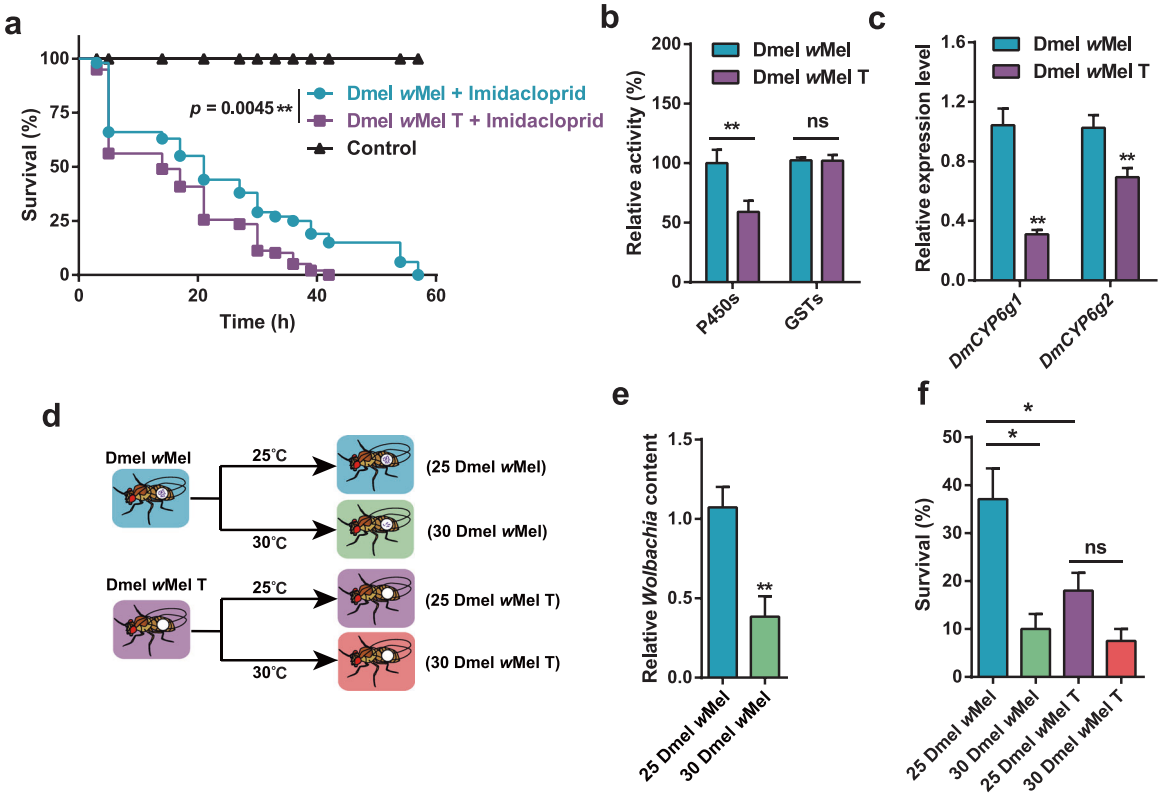

Fig. 7 The effects of high temperature on insecticide susceptibility and detoxification metabolism of *Wolbachia*-infected (Dmel *w*Mel) and *Wolbachia*-free (Dmel wMel T) *Drosophila melanogaster*. **a** Survival curves of Dmel *w*Mel and Dmel *w*Mel T exposed to imidacloprid ($n = 100$ per treatment). Statistical comparisons were based on the log-rank (Mantel–Cox) test, and the level of significance for results was set at $^{*}p < 0.05$ and $^{**}p < 0.01$. **b** The enzymatic activities of cytochrome P450s and glutathione *S*-transferases in Dmel *w*Mel and Dmel *w*Mel T (without any treatment). **c** The expression profile of P450 genes in Dmel *w*Mel and Dmel *w*Mel T ($n = 3$). **d** The experimental design. Dmel *w*Mel and Dmel *w*Mel T were exposed to two different temperatures (25 and 30 °C) for 24 h, followed by assays for their susceptibility to insecticide, detoxification metabolism, and *Wolbachia* abundance. **e** The impact of temperature on the *Wolbachia* abundance in Dmel *w*Mel ($n = 3$). **f** Survival of Dmel *w*Mel and Dmel *w*Mel T exposed to imidacloprid following the different temperature pre-treatments using the topical application method ($n = 100$ per treatment). Bar graphs were expressed as the mean ± standard error of the mean (SEM), and statistical comparison was based on *t*-tests. The level of significance for results was set at $^{*}p < 0.05$ and $^{**}p < 0.01$, ns no significant difference.

associated with changes in the gut microbiome, and the gut microbiomes of cold-resistant fish also had higher resilience to temperature changes [64]. From this point of view, the decline of insect-bacterial symbiosis by elevated temperature may become less severe due to symbiont adaptation.

In summary, our study offers new insights into considering the consequences of elevated temperatures on insect-microbe symbiosis that ultimately alter insect response to xenobiotics, including imidacloprid. Additional work will clarify the microbial signals that act on *CncC* pathway of *N. lugens*, and test the relationship between insect-bacterial symbiosis and detoxifications in other insects.

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AUTHOR CONTRIBUTIONS

H.W. and Y.Z. designed the experiments. Y.Z., T.C., Z.R., Y.L., M.Y., and Y.C. performed the experiments. Y.Z., C.Y., S.H., and J.L. analyzed the data and generated figures. Y.Z., A.W., and H.W. wrote the paper. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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