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Does immune priming in *Galleria mellonella* reveal plastic mechanisms for survival?

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ABSTRACT

Immune priming enhances protection in invertebrates upon secondary exposure to specific pathogens. Despite significant advances in understanding this phenomenon, it remains unclear whether the elevated defense observed through priming arises from identical or distinct effector-mediated responses within the same species. To address this, we used the model species *Galleria mellonella* from two geographically distinct origins (Siberia and Mexico), both of which exhibited immune priming with enhanced survival. We measured five immune effectors in primed individuals to investigate whether the mechanisms behind immune priming were conserved. Remarkably, we identified distinct effector responses associated with immune priming between the two groups. Individuals of Siberian origin exhibited an increased total hemocyte count, and a higher number of live hemocytes in primed individuals. In contrast, individuals of Mexican origin demonstrated a higher lytic activity and a higher level of hydrogen peroxide production in the priming group compared with control. Phenoloxidase activity did not significantly differ across treatments in either group. Our findings suggest that *G. mellonella* from different origins achieve similar survival through different physiological effectors. These results highlight the diversity of immune priming mechanisms within a single species and support the idea that the immune priming mechanisms in invertebrates may be plastic within and across species.

1. Introduction

Recent studies have demonstrated that prior exposure to a parasitic challenge can lead to enhanced protection in invertebrates when confronted with a subsequent, specific challenge (Kurtz, 2005). This phenomenon, referred to as immune priming (Little and Kraaijeveld, 2004; Milutinović and Kurtz, 2016) or innate immune memory (Contreras-Garduño et al., 2016), highlights the capacity of invertebrate immune systems to "remember" previous infections and mount a more effective immune response upon re-exposure. The experimental design to test immune priming involves the initial exposure of a host to dead, attenuated, or low-dose (sublethal) microbes, microbe-derived molecules or novel immune challenge, which subsequently enhances the host's protection upon encountering a higher dose than the previous one with the same challenges compared with different subsequent immune challenges (Little et al., 2005; Contreras-Garduño et al., 2016; Lanz-Mendoza et al., 2024). One of the pioneering works that demonstrated immune memory was conducted by Kurtz and Franz (2003). They designed an experiment to investigate the specificity of immune memory in the copepod *Macrocyclops albidus* against its natural parasite *Schistocephalus solidus*. Each copepod was initially exposed to three parasite larvae, and on the third day, they were exposed to another three larvae,

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which were either siblings or non-siblings of the initial parasites. The results revealed that prior exposure to sibling parasites led to a significantly lower secondary infection rate compared to copepods exposed to non-sibling parasites (Kurtz and Franz, 2003). Furthermore, in *Musca domestica* exposed to *Candida albicans*, the immune priming group, when compared to the control group, demonstrated differential gene expression related to immune response pathways, including the Toll and Phagosome signaling pathways. Additionally, they reported increased expression of the pattern recognition receptor PGRP-SD-like precursor, lectin subunit alpha-like, and antimicrobial peptides such as phormicin, cecropin-A2-like, defensin-1, attacin-A-like, sarcotoxin-1C, and lysozyme 1-like (Li et al., 2022). Similarly, in the crab *Eriocheir sinensis* challenged with *Aeromonas hydrophila*, the priming group exhibited higher levels of total hemocyte count, phenoloxidase, pro-phenoloxidase, lysozyme activity, phagocytic activity, and the crustin anti-lipopolysaccharide factor, compared to the control group (Wang et al., 2019).

Over the past two decades, research has provided compelling evidence for the memory-like response of invertebrates' immune system (Lanz-Mendoza et al., 2024; Mendez-Lopez et al., 2024). Despite significant progress, empirical studies reveal a complex landscape of immune responses, largely due to variability in recorded outcomes (Lanz-Mendoza et al., 2024; Martin et al., 2021). In general, insect immune responses against invading microorganisms are initiated when pattern recognition receptors (PRRs) bind to microbe-associated molecular patterns (MAMPs; Wang et al., 2019; Lin et al., 2020). This recognition triggers downstream immune signaling pathways, activating both cellular and humoral responses. Cellular response, mediated by hemocytes, includes encapsulation, phagocytosis, and nodule formation (Jiravanichpaisal et al., 2006; Strand, 2008). Immune priming has been linked to changes in hemocyte numbers post-priming (Lanz-Mendoza et al., 2024). The humoral response involves phenoloxidase (PO), antimicrobial peptides (AMPs), and reactive oxygen or nitrogen species (ROS/RNS). PO plays a key role in melanization and cuticle sclerotization, providing defense against pathogens (Castillo et al., 2011; Eleftherianos et al., 2021). It is activated through the proPO pathway, while AMPs are regulated by the Toll and IMD pathways (Imler and Hoffmann, 2001). The Toll pathway primarily responds to fungi and Gram-positive bacteria, while the IMD pathway targets Gram-negative bacteria (Ali Mohammadie Kojour et al., 2020), though cross-talk between them exists. Additionally, lysozyme and AMPs exhibit direct lytic activity (Schneider, 1985; Schmid-Hempel, 2005), while the DUOX pathway generates ROS and pro-oxidants such as H_2O_2 to combat diverse pathogens (Herrera-Ortiz et al., 2011; Kuraishi et al., 2013; Kim and Lee, 2014). Together, these components form a highly coordinated immune defense system.

While many studies report the induction of these immune components following priming, immune markers do not consistently correlate with resistance: some increase after secondary challenges, others decrease, and some remain unchanged (Lanz-Mendoza et al., 2024). Even within a species, the immune responses vary. In *Anopheles albimanus* exposed to *Plasmodium berghei*, gambicin and cecropin increased in the midgut, while attacin decreased, and only cecropin increased in the fat body (Contreras-Garduño et al., 2015), highlighting tissue-specific immune responses. Similarly, *in Tribolium castaneum*, not all populations exhibited immune memory against *Bacillus thuringiensis* (Khan et al., 2016, 2019). These variations may result from life-history traits, genetic drift, pathogen susceptibility (Khan et al., 2016), or environmental factors like temperature (Lanz-Mendoza et al., 2024). This differences in immune resistance between populations suggest plasticity in immune priming. This hypothesis predicts that distinct populations may express different immune markers, but they may still derive the key benefit: enhanced survival.

In this study, we used *Galleria mellonella* to test this hypothesis, leveraging its well-established role in immune response in terms of reactive oxygen species, lytic activity, phenoloxidase and hemocyte number (Glupov et al., 2003; Mukherjee et al., 2010; Kavanagh and Fallon, 2010; Vogel et al., 2011; Wu et al., 2016; Tsai et al., 2016; Wojda, 2017a; Ménard et al., 2021). In *Galleria mellonella*, the phenomenon of immune priming has been demonstrated in response to *Bacillus thuringiensis* (Wu et al., 2022). In the immune priming group, which was challenged twice with a sublethal dose of *B. thuringiensis* followed by a lethal dose of the same bacteria, a higher expression of antimicrobial peptides (including cecropin, hemolin, gallerimycin, and lysozyme 1) and an increased phagocytic rate were observed compared to the control group, which received an injection of phosphate-buffered saline followed by a lethal dose of *B. thuringiensis* (Wu et al., 2022). In a separate study, *G. mellonella* was challenged with *Candida albicans*, and the results indicated that, 24 h after the subsequent challenge, the immune priming groups exhibited a stronger immune response compared to the control group (Vertyporokh et al., 2019). However, this study also noted that not all immune parameters were elevated in the primed group when compared to the control group; in some cases, values were lower, or no significant differences were observed. These findings are particularly interesting as they suggest the plasticity of immune priming (Lanz-Mendoza et al., 2024). Hence we examined immune the immune priming plasticity by measuring a combination of cellular and humoral immune effectors in individuals from two geographically distinct origins -Siberia and Mexico. To ensure a standardized immune challenge, we used lyophilized cells of *Micrococcus lysodeikticus* and hence, there is no chance of encountering this bacterium in the field infecting *G. mellonella*. This approach minimized population-specific adaptations to local pathogens, allowing us to attribute any observed differences in immune priming to inherent plasticity rather than prior pathogen exposure.

2. Material and methods

2.1. Study system

Insects from Siberia (Palearctic) or Mexico (Neotropical) were captured (about 1500–2000 insects) in nature and bred for five generations in the lab. Moths were fed *ad libitum* with a homogenized mix of equal proportions of honey, glycerol, beeswax, dried milk, wheat flour, dry yeast, and distilled water, as well as cornmeal (Krams et al., 2017a, 2017b). Insects were reared in 3-L plastic boxes at 30 ± 1 °C and relative humidity of 30 % at dark. For the experiments, each insect per population was kept individually in each of a 6 well-plate (Corning) inside an environmental chamber at 30 ± 1 °C and relative humidity of 30 % at dark (Lumistell). To minimize batch effects, physiological analyses were staggered across different experimental groups. This means that, for example, in a 96-well plate, we placed the sample of organism 1 from the control group in well 1 A, the sample of organism 1 from the priming group in well 2 A, the sample of individual 2 from the control group in well 3C, and the sample of individual 2 from the priming group in well 3D, and so on until all samples were placed. This approach allowed us to ensure that the readings were not influenced by the organization of organisms into separate batches, such as using one 96-well plate for the control organisms and another for the priming organisms. To account for larval stage-dependent immune responses, we controlled for larval size to 17–20 mm across all populations and treatments.

To assess immune priming, we employed a standardized protocol that involved comparing a group subjected to two exposures to the same immune challenge with a control group, which was only injected with PBS and subsequently challenged (Little et al., 2005; Contreras-Garduño et al., 2016). This same method has been implemented to test immune priming in *G. mellonella* (Vertyporokh et al., 2019; Wu et al., 2022). We established Control and Priming groups within each population (using same-aged and similarly sized larvae). To create a Control group, we injected (with 10 μL Hamilton syringes) larvae with 1 μL of phosphate buffer (sham-priming with PBS, 140 mM NaCl, 2.6 mM KCl, 1.5 mM KH2PO4, pH 7.4; Sigma Aldrich). In the Priming group, we injected lyophilized cells of *M. lysodeikticus* (Merck, ATCC No. 4698) dissolved in

PBS (1 mg/mL). Post five days of treatments, both groups were challenged with 1 μL of *Micrococcus lysodeikticus* dissolved in PBS at a concentration of 2 mg/mL. The syringes that we used for injecting the *M. lysodeikticus* or PBS were washed 2 times in ethanol and 4 in distilled water to avoid samples being mixed within and between treatments and among populations.

We selected these doses based on a pilot experiment, in which no significant differences in mortality were observed between 0.5 and 1 mg/mL, while a dose of 3 mg/mL resulted in insect mortality within 12 h. Additionally, we chose to allow a 5-day interval between the first and second challenges, as previous studies have indicated that immune priming occurs within 72 h (Vertyporokh et al., 2019; Wu et al., 2022). Thus, the 120-h time lapse employed in our study was deliberately conservative.

We compared survival and immune response at 24 h after the second challenge between the treatments Control (n = 50) and Priming (n = 50) and its origin (Siberia *vs* Mexico). We did not continue recording survival data, as many animals succumbed within 24 h. Furthermore, in this species, key immune response parameters such as antimicrobial activity, melanogenesis, and hemocyte count typically reach their peak activation within 24 h (Taszłow et al., 2017; Smith et al., 2022; Gallorini et al., 2024; Genç et al., 2024). During immune priming, a similar pattern has been observed in *G. mellonella* (Vertyporokh et al., 2019).

2.2. Hemolymph collection and protein concentration standardization

Hemolymph was obtained 24 h after injecting the second challenge by piercing the third segment of the abdomen to obtain between 10 and 15 μL of hemolymph. To extract hemolymph, we performed a puncture using an ACCU-CHECK Softclix lancet and, with a 20-μL micropipette (Eppendorf), collected the hemolymph drop that emerged from the puncture. This drop was immediately placed in pre-cooled Eppendorf tubes (1 mL) containing 500 μl of sterile PBS. The samples were stored at −70 °C until the immune response analyses were conducted.

The protein content was estimated before the immune analyses (Contreras-Garduño et al., 2007). A kit based on the bicinchoninic acid assay (BCA Protein Assay, Thermo Scientific) was used to calculate the amount of protein per sample. With this method, there is variation in color in response to different protein content. In short, we added 10 mL of sample to 40 mL of PBS and 150 mL of Pierce reagent (a mixture of the A and B reagents provided by the kit). As a standard curve, we used a known concentration of albumin provided in the kit. Given that protein concentration may vary between organisms and groups, we adjusted protein concentration to 40 μg/mL of total protein. This means that we used 40 μg/mL of total protein per sample to analyze phenoloxidase, H_2O_2 and lytic activity (Contreras-Garduño et al., 2007). This amount of protein per sample is used to control for any potential differences in the amount of protein between samples.

2.3. PO activity

PO activity was measured spectrophotometrically by recording the formation of dopachrome from L-dihydroxyphenylalanine (L-DOPA, Sigma; Contreras-Garduño et al., 2007). From each sample, a volume that had 40 μg/μL of protein was dose-titred until 150 μL of sample + PBS. These 150 μL were mixed with 50 μL of L-Dopa (4 mg/mL) to obtain the final volume of 200 μL. Samples were read in a microplate reader (Varioskan Flash Multimode Reader, Thermo Scientific) at 490 nm every 5 min for 1 h. Time points for measurements were standardized in pilot experiments based on previous studies including *G. mellonella* (Contreras-Garduño et al., 2007; Nicoletti et al., 2020; Trejo-Meléndez et al., 2023). We assessed the PO activity of each sample by calculating the slope of the kinetic curve during the linear phase of the reaction (Trejo-Meléndez et al., 2023).

2.4. Lytic activity

Each sample that had 40 μg/μL of protein was dose-titred with PBS until reaching 30 μL and this sample was mixed with 200 μL of a solution of *M. lysodeikticus* (Nicoletti et al., 2020). This solution was prepared by dissolving 9 mg/mL of *M. lysodeikticus* in 25 mL PBS. The mixture that reached 230 μL was incubated at room temperature and the change in absorbance was read at 540 nm every 5 min for 30 min (Contreras-Garduño et al., 2007). All reads were carried out in an ELISA reader (Varioskan Flash Multimode Reader, Thermo Scientific). Here, a lower absorbance value represents a more intense response. We assessed the lytic activity by calculating the slope of the kinetic curve during the linear phase of the reaction (Trejo-Meléndez et al., 2023).

2.5. Hydrogen peroxide

To record hydrogen peroxide (H_2O_2) in the hemolymph, we used the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit, following the indications of the manufacturer. This kit employs the reactive Amplex® Red (10-acetyl-3,7-dihydroxyphenoxazine) to detect hydrogen peroxide or peroxidase activity (Nicoletti et al., 2020). The reagent Amplex® Red mixed with peroxidase is used to detect the levels of H_2O_2 delivered by biological samples. We obtained 50 μL per sample that had 40 μg/μL of protein and was mixed with 50 μL of the reagent Amplex® Red/HRP for 30 min. Absorbance was read at 562 nm in an ELISA reader (Varioskan Flash Multimode Reader, Thermo scientific). Results are expressed as μM/μL.

2.6. Hemocytes

The numbers of total and live hemocytes were obtained with a cell counter (TC20™ Automated Cell Counter, Biorad). In each larva, a puncture was made in the third segment of the abdomen to obtain 5 μL of hemolymph. This hemolymph was mixed with 5 μL trypan blue (Biorad). These 10 μL were deposited inside the cell counter on a reading plate (Biorad). The cell counter TC20 equipment provides measurements of the total and life hemocytes immediately after reading the plate. We chose to measure both the total number of hemocytes in the hemolymph and an indirect estimate of the proportion of these cells that were actively engaged at the time of infection. We believe it is important to consider both the total count and the number of active cells, as this approach not only provides an overall understanding of the hemocyte population but also highlights those cells that may play a crucial role in the immune response during infection.

2.7. Statistical analysis

All data were analyzed using R statistical software (version 4.2.2). We used a Generalized Linear Mixed Model (GLMM) with a binomial distribution to analyze survival data. The fixed effects in the model were treatment groups (primed vs. control) and geographic origin (Mexico vs. Siberia), while replicate ID was included as a random effect to account for variability among independent experimental units. The outcome variable was survival (binary: alive or dead). Post hoc pairwise comparisons were performed using the “emmeans” package to calculate estimated marginal means and conduct pairwise comparisons with Tukey’s adjustment for multiple comparisons. To analyze different effectors of cellular and humoral response, we used a Generalized Linear Model (GLM) with the stats package, where the response variables included total hemocyte count, live hemocyte count, phenoloxidase (PO) activity, lytic activity, and H_2O_2 production. The categorical factors, geographic origin (Mexico vs. Siberia), and treatment groups (primed vs. control), were included as fixed effects in the model. All data were tested for normality using the Shapiro-Wilk test and for homogeneity of variances using Levene’s test.

3. Results

3.1. Immune priming increases survival

First, we confirmed that immune priming significantly enhanced survival in both groups (Fig. 1A, GLMM: Likelihood Ratio $\chi^2 = 65.26$, df = 3, $P < 0.001$), with survival probabilities differing across treatments. After priming, survival increased by an average of 54 % compared to the unprimed controls. More specifically, in the Siberian group, 24 h post-secondary challenge, survival was substantially higher in primed individuals (82 %; 50/34, GLMM: Estimated mean = 0.82, SE = 0.065, 95 % CI: [0.19–0.44]) compared to the control group (12 %; 50/6, GLMM: Estimated mean = 0.12, SE = 0.046, 95 % CI: [0.76–0.95]). In the Mexican group, primed individuals exhibited a survival rate of 80 % (50/40, GLMM: Estimated mean = 0.8, SE = 0.057, 95 % CI: [0.11–0.33)], significantly exceeding the control group's survival rate of

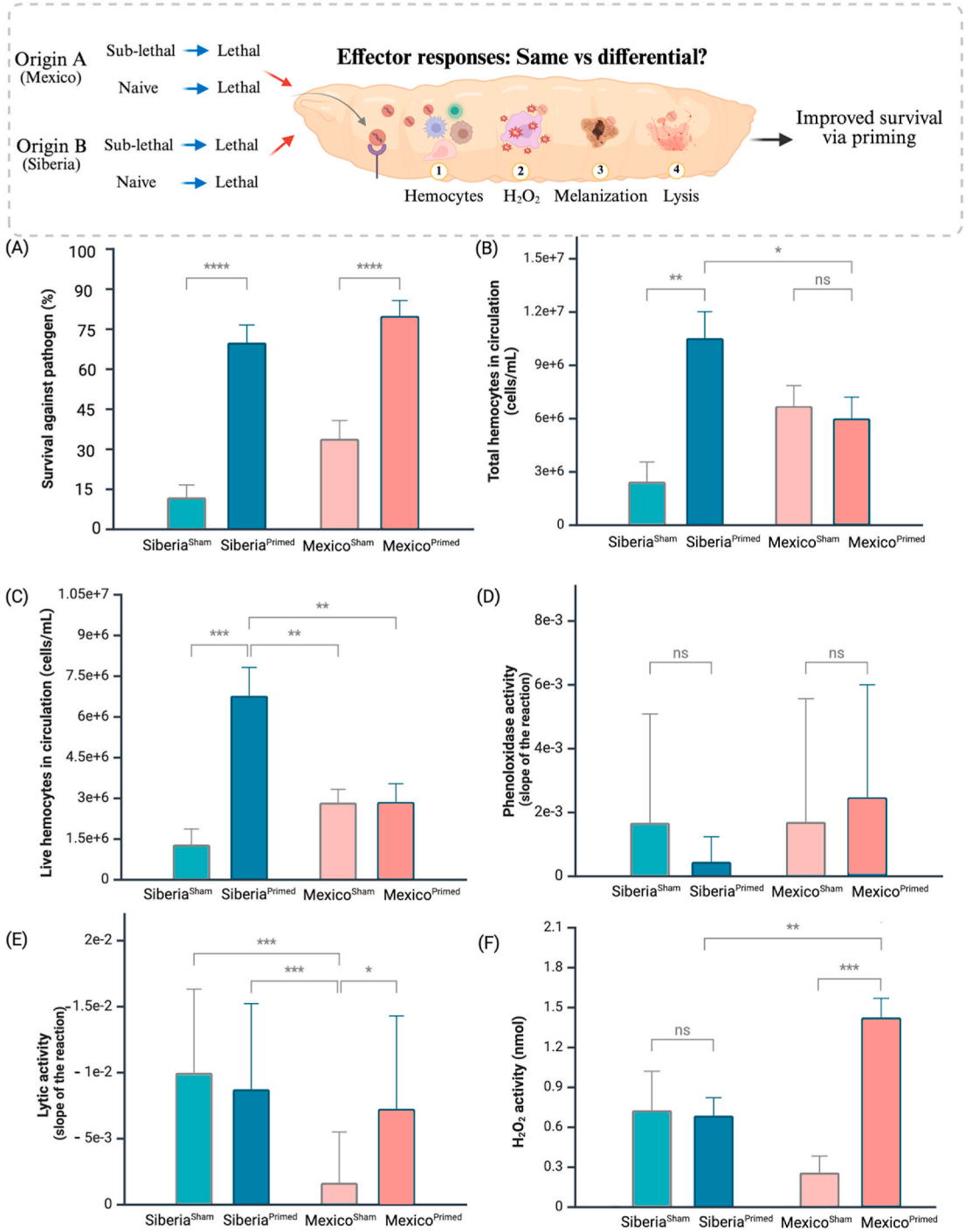

Fig. 1. Immune priming elicits distinct effector-mediated survival responses within a species. (A) Priming enhances survival following a homologous pathogen challenge in two groups of *G. mellonella* larvae, collected from Siberia and Mexico. Cellular response, assessed by the total number of circulating hemocytes (B) and live hemocytes (C), demonstrates differential induction following priming. The humoral immune response, measured by Phenoloxidase activity (D), showed no significant difference, while lytic activity (E) and hydrogen peroxide (H_2O_2) production (F) were differentially induced. Data are presented as mean ± standard error for each immune marker in *G. mellonella*. Significant differences were determined using a generalized linear mixed model or generalized linear model. Light blue: $Siberia^{Sham}$ and dark blue: $Siberia^{Primed}$, Pink: $Mexico^{Sham}$ and Red: $Mexico^{Primed}$, 'ns' represents statically not significant.

32 % (50/16, GLMM: Estimated mean = 0.32, SE = 0.057, 95 % CI: [0.52–0.78]).

3.2. Cellular immune response

The total number of hemocytes was significantly higher (~8 times) in the primed Siberian group compared to their sham-primed control counterparts (Fig. 1B, GLM: Likelihood Ratio $\chi^2 = 17.45$, df = 1, 3, $P < 0.01$). In contrast, no significant differences were observed between primed and sham-primed individuals in the Mexico group (GLM: SE = 1,209,969, 95 % CI: [8,644,413, 13,387,405]). When comparing the control groups, no significant differences in total hemocyte counts were found between Mexico and Siberia (GLM: $P > 0.05$). However, primed Siberian individuals exhibited a significantly higher induction of total hemocytes compared to primed individuals from Mexico (GLM: $P < 0.0001$). A similar pattern was observed for live hemocyte counts, with primed Siberian individuals showing at least an 8-fold increase in live hemocytes compared to controls (Fig. 1C, GLM: Likelihood Ratio $\chi^2 = 22.58$, df = 1, 3, $P < 0.0001$. Table 1).

3.3. Humoral immune response

We assessed three humoral immune effectors: phenoloxidase activity, lytic activity, and hydrogen peroxide (H_2O_2) production. Interestingly, no significant differences were observed between treatments (Control vs. Priming) or groups (Siberia vs. Mexico) for phenoloxidase activity (Fig. 1D, GLM: Likelihood Ratio $\chi^2 = 9.8$, df = 1, 3, P = 0.2. Table 1). In contrast, lytic activity was significantly affected by the priming treatment in Mexico, but not in Siberia (Fig. 1E, GLM: Likelihood Ratio $\chi^2 = 20.25$, df = 1, 3, P = 0.001. Table 1), with primed individuals from Mexico exhibiting approximately 7 times higher lytic activity compared to controls (GLM: $P < 0.0001$). However, no increase in lytic activity was observed in primed individuals from Siberia (GLM: $P > 0.05$). Regarding H_2O_2 production, primed individuals from Mexico showed a significant increase in activity compared to all other groups (Fig. 1F, GLM: Likelihood Ratio $\chi^2 = 27.84$, df = 1, 3, P = 0.0001. Table 1), while individuals from Siberia did not exhibit any priming-related effects on H_2O_2 activity (GLM: $P > 0.05$).

4. Discussion

Our study highlights how immune priming enhances survival in *G. mellonella*, yet the mechanisms underlying this protection vary depending on their geographic origin. Rather than a single conserved response, we find that in the Siberian-origin individuals, immune priming was linked to elevated total hemocyte counts, and a higher proportion of live hemocytes. In contrast, Mexican-origin individuals primarily relied on heightened lytic activity and increased hydrogen peroxide (H_2O_2) production. Finally, no differences were found in PO activity across treatments.

Table 1
Immune response parameters according to their population of origin. Values show the mean ± standard error.

Immune Marker	Siberia	Mexico
Lytic activity	Control: 0.0027 ± 0.0007 Priming: 0.008 ± 0.001	Control: 0.009 ± 0.001 Priming: 0.01 ± 0.0009
Hydrogen Peroxide	Control: 0.72 ± 0.2 Priming: 0.68 ± 0.13	Control: 0.38 ± 0.13 Priming: 1.42 ± 0.13
Phenoloxidase	Control: 0.001 ± 0.001 Priming: 0.002 ± 0.0006	Control: 0.001 ± 0.0008 Priming: 0.0004 ± 0.0001
Total hemocytes	Control: 7,365,000 ± 1,117,896 Priming: 12,755,847 ± 1,298,709	Control: 8,771,538 ± 887,674 Priming: 11,609,285 ± 692,264
Live hemocytes	Control: 3,902,500 ± 470,272 Priming: 8,292,222 ± 943,837	Control: 3, 723,077 ± 367,364 Priming: 5, 535,714 ± 777,584

In general, our findings provide support for the hypothesis that immune priming enhances survival and immune response in *G. mellonella* as indicated by increased antimicrobial activity and cellular response (Vertyporokh et al., 2019; Wu et al., 2022). Specifically, we show that the immune priming group exhibited elevated levels of cellular response, antimicrobial peptides, lytic enzymes, and oxidative stress compared to the control group. These results also align with the growing body of evidence suggesting that invertebrates, like vertebrates, possess immune memory (Lanz-Mendoza et al., 2024). Furthermore, our data is aligned with studies that suggest that phenoloxidase (PO), a key player in the melanization response, may not be a mechanism underlying immune priming (Milutinović and Kurtz, 2016; Contreras-Garduño et al., 2016). A novel aspect of our study is the observation of plasticity in the immune response between the two populations examined. While we acknowledge that our study does not provide a broad population comparison as seen in some previous studies with beetles (Khan et al., 2016, 2019; Prakash et al., 2022), our focus on two contrasting populations within a species known to exhibit immune priming offers insight into the potential plasticity of immune responses across populations.

We do not know the origin-dependent differences that favor the plasticity between Siberia and Mexico. They may be driven by environmental factors such as temperature, pathogens, and resource availability (Ardia et al., 2011; Cotter et al., 2011; Rauw, 2012), which favor the plasticity of immune response (Vilcinskas, 2013; Martin et al., 2021). Hemocyte-mediated defenses observed in the Siberian population may represent an adaptation to colder climates, where cellular immune responses are either less sensitive to temperature fluctuations or are adversely affected by low temperatures. For instance, *Pieris napi* individuals reared at higher temperatures (25 °C) exhibited a reduction in hemocytes number compared to those reared at lower temperatures (17 °C; Bauerfeind and Fischer, 2014). This pattern in *P. napi* aligns with our findings, as *G. mellonella* from the Siberian population exhibited higher hemocyte numbers than those from the Mexican population. Given that immune function can be influenced by hemolymph viscosity (which itself is affected by ambient temperature) future studies should investigate potential differences in hemolymph viscosity between these populations and assess their relationship with immune response (Kenny et al., 2018).

Conversely, relying on lytic activity in the Mexican population could reflect adaptations to warmer environments, where enzymatic activity is more effective or energetically favorable (Peterson et al., 2007; Wojda, 2017a). Exposure to elevated temperatures can activate the insect immune response by inducing the expression of molecular chaperones and upregulating heat shock proteins, a process observed following the injection of immune elicitors such as bacterial or fungal cell wall components (Wojda, 2017b). Additionally, in some insect species, such as *G. mellonella*, lysozyme plays a key role in immunity and is constitutively present in the hemolymph, although its concentration increases upon immune stimulation (Wojda, 2017b). In this context, the influence of temperature on metabolism, and in turn the influence of metabolism on immune function, should be investigated to better understand the costs associated with immune activation and the potential for population-level variation (Mendez-Lopez et al., 2024; Lanz-Mendoza et al., 2024). Nevertheless, caution is warranted, as the effect of temperature on the immune response may also vary depending on the specific parasite involved (Adamo and Lovett, 2011). Furthermore, other factors potentially contributing to population-specific variation in immune responses in *G. mellonella* warrant investigation, particularly the roles of diet and microbiota. For example, differences in dietary diversity and the presence or absence of microbiota have been shown to influence the expression of antimicrobial peptides such as Gallerimycin, Gloverin, 6-tox, Cecropin-D, and Galiomicin (Krams et al., 2017a, 2017b). Taken together, understanding the differences in immune

responses between the Siberian and Mexican populations of *G. mellonella* requires further research, with careful consideration given to the potential roles of temperature, pathogen type, diet, and microbiota.

Regardless of the underlying cause promoting plasticity in the immune response, we observed a higher number of circulating hemocytes in the hemolymph, as well as a greater proportion of viable hemocytes, in the Siberian population compared to the Mexican population. While some of the observed mortality may result from technical factors such as suboptimal hemolymph extraction or mechanical stress during slide preparation, increasing evidence suggests that hemocyte death, particularly apoptosis, is a biologically relevant outcome of immune activation. Apoptosis is now recognized as an integral component of insect immune responses, often involving hemocyte rupture and degranulation. These processes expose the extracellular matrix, which facilitates downstream defense mechanisms (Eleftherianos et al., 2021). Thus, apoptosis actively contributes to host immunity in insects (Eleftherianos et al., 2021; Cerenius and Söderhäll, 2021). Although apoptosis and autophagy are commonly associated with antiviral responses (Liu et al., 2013), in Drosophila, both apoptosis and necrosis occur because of crystal cell death following activation of the PPO pathway (Bidla et al., 2007; Dudzic et al., 2015). In Lepidoptera, crystal cells are referred to as granulocytes, which interestingly represent the predominant hemocyte type (Eleftherianos et al., 2021), and, in *Manduca sexta*, granulocyte degranulation has been shown to contribute to bacterial agglutination (Jearaphunt et al., 2014). In addition, the immune priming group from the Siberian population exhibited a higher rate of hemocyte death compared to both the immune priming group from the Mexican population and the control groups from both populations. This pattern suggests a possible association between increased cell death and immune priming. A similar observation has been reported in *Crassostrea gigas*, where exposure to Ostreid herpesvirus 1 resulted in differential expression of genes associated with apoptosis and autophagy specifically in the immune priming group (Lafont et al., 2020). Finally, we acknowledge that the distinction between live and dead hemocyte numbers remains challenging to interpret, even when considering mechanisms such as apoptosis and degranulation. However, in support of our findings, previous studies have reported similar trends. For example, in *G. mellonella* infected with *Candida albicans*, hemocyte viability decreased approximately twofold after 24 h, coinciding with a reduction in pathogen load (Sheehan and Kavanagh, 2018). A comparable decline in hemocyte viability after 24 h was observed in *G. mellonella* exposed to varying doses of *Escherichia coli* (Campbell et al., 2024). Additionally, it has been reported that hemocyte numbers in *G. mellonella* decrease depending on the parasite species or strain, as well as over time and according to the composition of the hemocyte population (i.e., healthy, necrotic, or apoptotic cells; Arteaga-Blanco et al., 2017). For instance, the proportion of healthy hemocytes declined from 77 % at 2 h post-infection to 0.63 % following infection with strain 1022 of *Actinobacillus pleuropneumoniae*, and from 75 % to 1.59 % with strain R8 (Arteaga-Blanco et al., 2017). These observations underscore the need for further investigation into the cellular mechanisms governing not only immune priming but also the dynamics of hemocyte viability in relation to the intensity of the immune response and parasite virulence.

Finally, our results may reveal a potential trade-off. Specifically, the Siberian population may prioritize investment in hemocytes at the expense of oxidative stress and lytic activity, whereas the opposite pattern may be observed in the Mexican population. Future research should investigate whether these immune responses exhibit plasticity over time, particularly in relation to pathogen growth dynamics (Tate et al., 2017), and whether interpopulation differences arise from immune reconfiguration or reflect underlying evolutionary trade-offs (Adamo et al., 2016).

Our findings contribute to the growing body of literature demonstrating that immune priming does not adhere to a universal model. Rather, populations and species may evolve distinct mechanisms to achieve similar outcomes, suggesting that different immune strategies can emerge within populations of a single species—and potentially across species—ultimately converging on the common goal of enhanced survival. A deeper understanding of these patterns will be crucial for uncovering broader principles that govern immune plasticity and host-pathogen dynamics in invertebrates. By identifying the underlying mechanisms that drive such variability, we can gain valuable insights into the adaptive nature of immune responses in diverse ecological contexts.

CRediT authorship contribution statement

Fátima Terán-Murillo: Writing – original draft, Methodology, Investigation. **Enakshi Ghosh:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Markus J. Rantala:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization. **Indrikis Krams:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Ronald Krams:** Writing – review & editing, Methodology, Investigation. **Jorge Contreras-Garduño:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Ethics statement

None.

Conflict of interest statement

The authors declare no conflicts of interest.

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Data availability

In DRYAD at https://doi.org/10.5061/dryad.qnk98sfv9.

References

Adamo, S.A., Davies, G., Easy, R., Kovalko, I., Turnbull, K., 2016. Reconfiguration of the immune system network during food shortages in the caterpillar *Manduca sexta*. J. Exp. Biol. 219, 706–718.

Adamo, S.A., Lovett, M.M., 2011. Some like it hot: the effects of climate change on reproduction, immune function and disease resistance in the cricket gryllus texensis. J. Exp. Biol. 214, 1997–2004.

Ali Mohammadie Kojour, M., Han, Y.S., Jo, Y.H., 2020. An overview of insect innate immunity. Entomol. Res. 50, 282–291.

Ardia, D.R., Parmentier, H.K., Vogel, L.A., 2011. The role of constraints and limitation in driving individual variation in immune response: constraints on immunity. Funct. Ecol. 25, 61–73.

Arteaga Blanco, L.A., Crispim, J.S., Fernandes, K.M., de Oliveira, L.L., Pereira, M.F., Bazzolli, D.M.S., Martins, G.F., 2017. Differential cellular immune response of *Galleria mellonella* to *actinobacillus pleuropneumoniae*. Cell Tissue Res. 370, 153–168.

Bauerfeind, S.S., Fischer, K., 2014. Integrating temperature and nutrition–environmental impacts on an insect immune system. J. Insect Physiol. 64, 14–20.

Bidla, G., Dushay, M.S., Theopold, U., 2007. Crystal cell rupture after injury in drosophila requires the JNK pathway, small GTPases and the TNF homolog Eiger. J. Cell Sci. 120, 1209–1215.
Campbell, J.S., Pearce, J.C., Bebes, A., Pradhan, A., Yuecel, R., Brown, A.J., Wakefield, J. G., 2024. Characterising phagocytes and measuring phagocytosis from live *Galleria mellonella* larvae. Virulence 15, 2313413.
Castillo, J.C., Reynolds, S.E., Eleftherianos, I., 2011. Insect immune responses to nematode parasites. Trends Parasitol. 27, 537–547.
Cerenius, L., Söderhäll, K., 2021. Immune properties of invertebrate phenoloxidases. Dev. Comp. Immunol. 122, 104098.
Contreras-Garduño, J., Lanz-Mendoza, H., Franco, B., Nava, A., Pedraza-Reyes, M., Canales-Lazcano, J., 2016. Insect immune priming: ecology and experimental evidences. Ecol. Entomol. 41, 351–366.
Contreras-Garduño, J., Rodríguez, M.C., Hernández-Martínez, S., Martínez-Barnetche, J., Alvarado-Delgado, A., Izquierdo, J., Rodríguez, M.H., Lanz-Mendoza, H., 2015. *Plasmodium berghei* induced priming in *anopheles albimanus* independently of bacterial co-infection. Dev. Comp. Immunol. 52, 172–181.
Contreras-Garduño, J., Lanz-Mendoza, H., Córdoba-Aguilar, A., 2007. The expression of a sexually selected trait correlates with different immune defense components and survival in males of the American rubyspot. J. Insect Physiol. 53, 612–621.
Cotter, S.C., Simpson, S.J., Raubenheimer, D., Wilson, K., 2011. Macronutrient balance mediates trade-offs between immune function and life history traits. Func. Ecol. 25, 186–198.
Dudzic, J.P., Kondo, S., Ueda, R., Bergman, C.M., Lemaitre, B., 2015. Drosophila innate immunity: regional and functional specialization of prophenoloxidases. BMC Biol. 13, 1–16.
Eleftherianos, I., Heryanto, C., Bassal, T., Zhang, W., Tettamanti, G., Mohamed, A., 2021. Haemocyte-mediated immunity in insects: cells, processes and associated components in the fight against pathogens and parasites. Immunology 164, 401–432.
Gallorini, M., Marinacci, B., Pellegrini, B., Cataldi, A., Dindo, M.L., Carradori, S., Grande, R., 2024. Immunophenotyping of hemocytes from infected *Galleria mellonella* larvae as an innovative tool for immune profiling, infection studies and drug screening. Sci. Rep. 14, 759.
Genç, T.T., Kaya, S., Günay, M., Çakaloğlu, Ç., 2024. Humoral immune response of *Galleria mellonella* after mono-and co-injection with *Hypericum perforatum* extract and *Candida albicans*. APMIS 132, 358–370.
Glupov, V.V., Slepneva, I.A., Serebrov, V.V., Khvoschevskay, M.F., Martem'yanov, V.V., Dubovskiy, I.M., Khramtsov, V.V., 2003. Influence of the fungal infection on the production of reactive oxygen metabolites and the antioxidant state of haemolymph of *Galleria mellonella* L. (Lepidoptera: pyralidae) larvae. Russ. Entomol. J. 12, 103–108.
Herrera-Ortiz, A., Martínez-Barnetche, J., Smit, N., Rodriguez, M.H., Lanz-Mendoza, H., 2011. The effect of nitric oxide and hydrogen peroxide in the activation of the systemic immune response of *Anopheles albimanus* infected with *Plasmodium berghei*. Dev. Comp. Immunol. 35, 44–50.
Imler, J.L., Hoffmann, J.A., 2001. Toll receptors in innate immunity. Trends Cell Biol. 11, 304–311.
Jearaphunt, M., Noonin, C., Jiravanichpaisal, P., Nakamura, S., Tassanakajon, A., Söderhäll, I., Söderhäll, K., 2014. Caspase-1-like regulation of the proPO-system and role of ppA and caspase-1-like cleaved peptides from proPO in innate immunity. PLoS Pathog. 10, e1004059.
Jiravanichpaisal, P., Lee, B.L., Söderhäll, K., 2006. Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. Immunobiology 211, 213–236.
Kavanagh, K., Fallon, J.P., 2010. *Galleria mellonella* larvae as models for studying fungal virulence. Fungal Biol. Rev. 24, 79–83.
Kenny, M.C., Giarra, M.N., Granata, E., Socha, J.J., 2018. How temperature influences the viscosity of hornworm hemolymph. J. Exp. Biol. 221, jeb186338.
Khan, I., Prakash, A., Agashe, D., 2019. Pathogen susceptibility and fitness costs explain variation in immune priming across natural populations of flour beetles. J. Anim. Ecol. 88, 1332–1342.
Khan, I., Prakash, A., Agashe, D., 2016. Divergent immune priming responses across flour beetle life stages and populations. Ecol. Evol. 6, 7847–7855.
Kim, S.H., Lee, W.J., 2014. Role of DUOX in gut inflammation: lessons from drosophila model of gut-microbiota interactions. Front. Cell. Infect. Microbiol. 3, 116.
Krams, I.A., Kecko, S., Jõers, P., Trakimas, G., Elferts, D., Krams, R., Luoto, S., Rantala, M.J., Inashkina, I., Gudrā, D., Fridmanis, D., Contreras-Garduño, J., Grantiņa-Ieviņa, L., Krama, T., 2017a. Microbiome symbionts and diet diversity incur costs on the immune system of insect larvae. J. Exp. Biol. 220, 4204–4212.
Krams, I., Kecko, S., Inashkina, I., Trakimas, G., Krams, R., Elferts, D., Vrublevska, J., Jõers, P., Rantala, M.J., Luoto, S., Contreras-Garduño, J., Jankevica, L., Meija, L., Krama, T., 2017b. Food quality affects the expression of antimicrobial peptide genes upon simulated parasite attack in the larvae of greater wax moth. Entomol. Exp. Appl. 165, 129–137.
Kuraishi, T., Binggeli, O., Opota, O., Buchon, N., Lemaitre, B., 2013. Genetic evidence for a protective role of the peritrophic matrix against intestinal bacterial infection in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. 108, 15966–15971.
Kurtz, J., 2005. Specific memory within innate immune systems. Trends Immunol. 26, 186–192.
Kurtz, J., Franz, K., 2003. Evidence for memory in invertebrate immunity. Nature 425, 37–38.
Lafont, M., Vergnes, A., Vidal-Dupiol, J., De Lorgeril, J., Gueguen, Y., Haffner, P., Montagnani, C., 2020. A sustained immune response supports long-term antiviral immune priming in the Pacific oyster, Crassostrea gigas. mBio 11, e02777, 19.
Lanz-Mendoza, H., Gálvez, D., Contreras-Garduño, J., 2024. The plasticity of immune memory in invertebrates. J. Exp. Biol. 227 (Suppl. l_1), jeb246158.
Li, Z., Jia, L., Jiao, Z., Guo, G., Zhang, Y., Xun, H., Shang, X., Huang, L., Wu, J., 2022. Immune priming with Candida albicans induces a shift in cellular immunity and gene expression of Musca domestica. Microb. Pathog. 168, 105597.
Lin, Z., Wang, J.L., Cheng, Y., Wang, J.X., Zou, Z., 2020. Pattern recognition receptors from lepidopteran insects and their biological functions. Dev. Comp. Immunol. 108, 103688.
Little, T.J., Kraaijeveld, A.R., 2004. Ecological and evolutionary implications of immunological priming in invertebrates. Trends Ecol. Evol. 19, 58–60.
Little, T.J., Hultmark, D., Read, A.F., 2005. Invertebrate immunity and the limits of mechanistic immunology. Nat. Immunol. 6, 651–654.
Liu, B., Behura, S.K., Clem, R.J., Schneemann, A., Becnel, J., Severson, D.W., Zhou, L., 2013. P53-mediated rapid induction of apoptosis conveys resistance to viral infection in *Drosophila melanogaster*. PLoS Pathog. 9, e1003137.
Martin, L.B., Hanson, H.E., Hauber, M.E., Ghalambor, C.K., 2021. Genes, environments, and phenotypic plasticity in immunology. Trends Immunol. 42, 198–208.
Ménard, G., Rouillon, A., Cattoir, V., Donnio, P.Y., 2021. *Galleria mellonella* as a suitable model of bacterial infection: past, present and future. Front. Cell. Infect. Microbiol. 11, 782733.
Mendez-Lopez, T.T., Carrero, J.C., Lanz-Mendoza, H., Ochoa-Zarzosa, A., Mukherjee, K., Contreras-Garduño, J., 2024. Metabolism and immune memory in invertebrates: are they dissociated? Front. Immunol. 15, 1379471.
Milutinović, B., Kurtz, J., 2016. Immune memory in invertebrates. Semin. Immunol. 28, 328–342.
Mukherjee, K., Altincicek, B., Hain, T., Domann, E., Vilcinskas, A., Chakraborty, T., 2010. *Galleria mellonella* as a model system for studying listeria pathogenesis. Appl. Environ. Microbiol. 76, 310–317.
Nicoletti, M., Gilles, F., Galicia-Mendoza, I., Rendón-Salinas, E., Alonso, A., Contreras-Garduño, J., 2020. Physiological costs in monarch butterflies due to forest cover and visitors. Ecol. Indic. 117, 106592.
Prakash, A., Agashe, D., Khan, I., 2022. The costs and benefits of basal infection resistance vs immune priming responses in an insect. Dev. Comp. Immunol. 126, 104261.
Peterson, M.E., Roy, M.D., Danson, M.J., Eisenthal, R., 2007. The dependence of enzyme activity on temperature: determination and validation of parameters. Biochem. J. 402, 331–337.
Rauw, W.M., 2012. Immune response from a resource allocation perspective. Front. Genet. 3, 267.
Schmid-Hempel, P., 2005. Evolutionary ecology of insect immune defenses. Annu. Rev. Entomol. 50, 529–551.
Schneider, P.M., 1985. Purification and properties of three lysozymes from hemolymph of the cricket, *gryllus bimaculatus* (De Geer). Insect Biochem. 15, 463–470.
Sheehan, G., Kavanagh, K., 2018. Analysis of the early cellular and humoral responses of *Galleria mellonella* larvae to infection by *Candida albicans*. Virulence 9, 163–172.
Smith, D.F., Dragotakes, Q., Kulkarni, M., Hardwick, J.M., Casadevall, A., 2022. *Galleria mellonella* immune melanization is fungicidal during infection. Commun. Biol. 5, 1364.
Strand, M.R., 2008. The insect cellular immune response. Insect Sci. 15, 1–14.
Taszłow, P., Vertyporokh, L., Wojda, I., 2017. Humoral immune response of Galleria mellonella after repeated infection with *Bacillus thuringiensis*. J. Invertebr. Pathol. 149, 87–96.
Tate, A.T., Andolfatto, P., Demuth, J.P., Graham, A.L., 2017. The within-host dynamics of infection in trans-generationally primed flour beetles. Mol. Ecol. 26, 3794–3807.
Trejo-Meléndez, V.J., Méndez-López, T.T., Contreras-Garduño, J., 2023. The coincidental evolution of virulence partially explains the virulence in a generalist entomopathogenic. Acta Parasitol. 68, 293–303.
Tsai, C.J.Y., Loh, J.M.S., Proft, T., 2016. *Galleria mellonella* infection models for the study of bacterial diseases and for antimicrobial drug testing. Virulence 7, 214–229.
Vertyporokh, L., Kordaczuk, J., Mak, P., Hułas-Stasiak, M., Wojda, I., 2019. Host-pathogen interactions upon the first and subsequent infection of Galleria mellonella with Candida albicans. J. Insect Physiol. 117, 103903.
Vilcinskas, A., 2013. Evolutionary plasticity of insect immunity. J. Insect Physiol. 59, 123–129.
Wang, X., Zhang, Y., Zhang, R., Zhang, J., 2019. The diversity of pattern recognition receptors (PRRs) involved with insect defense against pathogens. Curr. Opin. Insect Sci. 33, 105–110.
Vogel, H., Altincicek, B., Glöckner, G., Vilcinskas, A., 2011. A comprehensive transcriptome and immune-gene repertoire of the lepidopteran model host Galleria mellonella. BMC Genom. 12, 1–19.
Wang, J., Yang, B., Wang, W., Song, X., Jiang, Q., Qiu, L., Song, L., 2019. The enhanced immune protection in Chinese mitten crab Eriocheir sinensis against the second exposure to bacteria Aeromonas hydrophila. Front. Immunol. 10, 2041.
Wojda, I., 2017a. Immunity of the greater wax moth *Galleria* mellonella. Insect Sci. 24, 342–357.
Wojda, I., 2017b. Temperature stress and insect immunity. J. Therm. Biol. 68, 96–103.
Wu, G., Liu, Y., Ding, Y., Yi, Y., 2016. Ultrastructural and functional characterization of circulating hemocytes from *Galleria mellonella* larva: cell types and their role in the innate immunity. Tissue Cell 48, 297–304.
Wu, G., Liu, J., Li, M., Xiao, Y., Yi, Y., 2022. Prior infection of Galleria mellonella with sublethal dose of Bt elicits immune priming responses but incurs metabolic changes. J. Insect Physiol. 139, 104401.