International Journal of Biological Macromolecules 338 (2026) 149596

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Antimicrobial peptide SCY2 with its interacting proteins Scyreprocin mediate the innate immune defense of *Scylla paramamosain* against *Pseudomonas putida* infection

Hanxiao Li [a,1], Ying Wang [a,1], Yuqi Bai [a], Fangyi Chen [a,b,c,*], Ke-Jian Wang [a,b,c,*]

[a] State Key Laboratory of Marine Environmental Science, College of Ocean & Earth Sciences, Xiamen University, Xiamen, 361102, China
[b] State-Province Joint Engineering Laboratory of Marine Bioproducts and Technology, College of Ocean & Earth Sciences, Xiamen University, Xiamen, 361102, China
[c] Marine Biological Antimicrobial Peptides Industry Research Institute, Fujian Ocean Innovation Center, Xiamen, 361102, China

ARTICLE INFO

Keywords:
Antimicrobial peptides
Reproductive immunity
rSCY2
rScyreprocin
Pseudomonas putida

ABSTRACT

Pseudomonas putida was identified in the testis of *Scylla paramamosain,* where it induced elevated expression of two interacting antimicrobial peptides (AMPs), SCY2 and Scyreprocin, in the gonad, indicating their potential role in reproductive immunity. In this study, we demonstrate that both peptides exerted systemic immune protection in males against this pathogen, which colonizes multiple host tissues and causes substantial mortality. Furthermore, they also mediate reproductive-specific immune functions in females. Administration of recombinant SCY2 or Scyreprocin (8 μg per crab, average weight 250 g ± 10 g) *in vivo* increased the survival of *P. putida*-infected males by 40–50 %. In addition, both peptides promoted bacterial clearance in key immune-related tissues, including the hepatopancreas, gills, hemocytes, testis, and ejaculatory duct, and modulated the expression of immune-related genes. Subsequently, the peptides' function in mated females was examined through *in vivo* blockade, with the efficacy validated by immunofluorescence and flow cytometry. As expected, the *in vivo* blockade of SCY2 and Scyreprocin in female crabs resulted in increased bacterial colonization and a significant reduction in survival rates, which declined to only 5–10 % within 96 h post-reinfection. Taken together, this study reveals a dual function for the male-derived AMPs SCY2 and Scyreprocin: they not only mediate systemic innate immunity in males but are also transferred to females to establish reproductive immunity. Consequently, this establishes a direct correlation between male immune competence and the reproductive success of the species.

1. Introduction

The mud crab *Scylla paramamosain* is a commercially important crustacean widely distributed across the Indo-Pacific. Highly prized as a culinary delicacy, it is especially valued in Asian countries such as China, Thailand, and Malaysia, where it underpins major coastal aquaculture and capture fisheries [1]. Its high market value, attributed to its size and flavor, underscores its considerable economic and cultural significance. Consequently, extensive research has been devoted to its biology, management, and sustainable aquaculture practices to meet the rising consumer demand [2–4]. In China, *S. paramamosain* cultivation is concentrated primarily along the southeastern coast. By 2022, the mariculture area dedicated to this species had reached approximately 24,170 ha, yielding an annual production of over 154,000 tons [5]. The life cycle of *S. paramamosain* is complex, consisting of five developmental stages from juvenile to adult, with reproductive behavior playing a critical role. During mating, sperm stored in the male ejaculatory duct is transferred to the female spermathecae for temporary storage [6]. However, with increased culture density, *S. paramamosain* is increasingly vulnerable to disease outbreaks across various developmental stages [7]. The rapid replication rate of bacterial pathogens often causes acute mortality in cultured crabs, leading to substantial economic losses. Current disease control strategies primarily rely on antibiotics and chemical agents, which can lead to the emergence of antibiotic-resistant

* Corresponding authors at: State Key Laboratory of Marine Environmental Science, College of Ocean & Earth Sciences, Xiamen University, Xiamen, 361102, China.
E-mail addresses: chenfangyi@xmu.edu.cn (F. Chen), wkjian@xmu.edu.cn (K.-J. Wang).
[1] The co-authors contributed equally to this paper

https://doi.org/10.1016/j.ijbiomac.2025.149596
Received 18 September 2025; Received in revised form 25 October 2025; Accepted 8 December 2025
Available online 21 December 2025

bacteria, environmental contamination, and the development of novel pathogens [8–10]. Although biological alternatives such as vaccines are promising, their development in aquaculture remains insufficient to meet the urgent demand for disease control [11,12].

Pseudomonas putida is a facultative pathogen with high environmental tolerance, commonly found in soil, aquaculture water, and various aquatic animals [13,14]. Under stress conditions, such as abrupt climate change or compromised host immunity, *P. putida* can rapidly proliferate and cause infection. It causes diseases in multiple aquatic species, such as tilapia ulcer syndrome [15,16], ulcerative lesions in golden-headed snapper [17], and skin ulceration in rainbow trout [18]. Moreover, *P. putida* is zoonotic. Reported human cases include bacteremia, chronic diarrhea, and secondary skin infections [19–21]. Some strains also exhibit antibiotic resistance and biofilm-forming capacity [22], which complicates treatment and increases persistence in the host and environment [23,24]. Our laboratory previously isolated a virulent *P. putida* strain from the testis of *S. paramamosain*, which was found to cause severe damage to sperm cells, representing the first report of *P. putida* colonization in the reproductive system of this species [25].

Antimicrobial peptides (AMPs) are small, bioactive polypeptides that function as key effectors of innate immunity in invertebrates. They exert direct antimicrobial activity against bacteria, viruses, and parasites, and also play immunomodulatory roles within the host [7,26–28]. This role in combating bacterial challenges is well-documented in fish, where *P. putida* infection alters AMP expression profiles [29]. In particular, AMPs derived from the reproductive system have gained increasing attention [30]. Besides sperm, seminal plasma contains a variety of bioactive molecules critical for sperm development, motility, and transfer during mating [31,32]. Our previous research has identified several reproductive AMPs in *S. paramamosain* with diverse *in vitro* activities [33–35]. Among them, SCY2 is an anionic AMP specifically and highly expressed in the male gonads, whose expression can be upregulated by progesterone and transferred to females during mating [36]. Scyreprocin, a peptide identified *via* yeast two-hybrid screening as an SCY2-interacting protein, also exhibits broad-spectrum antimicrobial activity [37]. Previous *in vitro* studies have characterized their distinct antimicrobial profiles, revealing that Scyreprocin possesses potent activity while SCY2's activity is comparatively weaker [36,37]. Their *in vitro* combined antimicrobial effects have also been previously tested without significant combined effects [37]. This contrasts with their established cooperative (non-antimicrobial) function, where both peptides are known to be essential, responding to calcium and progesterone, to facilitate the sperm acrosome reaction [25]. Despite these findings, the *in vivo* synergistic immune roles of SCY2 and Scyreprocin—especially in reproductive immunity and pathogen resistance—remain largely unexplored.

The present work aims to comprehensively elucidate the roles of SCY2 and Scyreprocin in the immune defense of *S. paramamosain* against *P. putida*. First, we characterized the systemic pathogenesis of *P. putida* in male crabs using an integrated approach combining bacterial quantification and histopathological analysis. We then examined the *in vivo* expression patterns of SCY2 and Scyreprocin under infectious conditions by quantifying their gene and protein levels using qPCR and western-blot. We determined their tissue distribution using immunofluorescence staining. Subsequently, the immunoprotective efficacy of recombinant SCY2 and Scyreprocin was assessed in infected male crabs by evaluating survival rates, bacterial clearance, antioxidative activity, and immune gene expression. Finally, to elucidate their role in mating-induced cooperative reproductive, *in vivo* peptide blockade experiments were performed in mated females. The blockade efficacy was verified *via* immunofluorescence and flow cytometry, followed by bacterial challenge to verify the essential contribution of these male-transferred peptides to female immune defense. Our findings highlight that male-specific antimicrobial peptides act as direct immunoprotective factors in females post-mating, laying the groundwork for deciphering the interplay between reproduction and immunity in crustaceans.

2. Materials and methods

2.1. Animal collection and acclimatization

Healthy adult *S. paramamosain* (250 g ± 10 g) specimens were obtained from coastal fisheries in Zhangzhou, Fujian Province, China. Prior to experimentation, crabs were acclimated for 2–3 days in cement tanks filled with aerated saltwater (12 ‰ salinity) maintained at 25–27 °C. To minimize handling stress and ensure humane treatment, crabs were anesthetized in an ice bath for 1 min before sampling.

2.2. Bacterial strain preparation and challenge experiments

The *P. putida* strain (ATCC 12633) used for infection was sourced from the China Center for Industrial Microbiology (CICC) and cultured on *Pseudomonas* CFC selective medium (Hope Bio-Technology Co., Ltd.) at 28 °C for 48 h. Inocula for injection were prepared by suspending colonies from second-generation CFC plates in 0.85 % saline. Optical density at 600 nm (OD_{600}) was measured to estimate bacterial concentrations, and colony-forming units (CFU) were quantified by serial dilution and plating. A range of bacterial concentrations was injected into crabs ($n = 10$) to determine the median lethal dose (LD_{50}) following EUCAST guidelines (https://www.eucast.org). All infection experiments were conducted in accordance with institutional biosafety protocols at Xiamen University.

2.3. Bacterial load assay

Male crabs were injected at the base of the third walking leg with a lethal dose of *P. putida* (2×10^7 CFU per crab). Control animals were injected with saline. Crabs were dissected at 3, 6, 12, 24, 48 h time points post-infection to collect the hepatopancreas, thoracic ganglion, midgut, heart, hemocytes, gills, testis, and ejaculatory duct. Hemocytes were isolated using pre-cooled anticoagulant buffer [34]. centrifuged at 500 ×*g* for 5 min at 4 °C, and resuspended. Solid tissues (0.1 g) were homogenized in 1 mL PBS. Tenfold serial dilutions of hemocyte suspensions and tissue homogenates were plated on CFC medium. Colonies were enumerated after 24 h of incubation at 28 °C, and bacterial identity was confirmed *via* 16S rRNA gene amplification and sequencing (Sangon Biotech, China). Colony counts between 20 and 200 CFU per plate were considered valid.

2.4. Histopathological examination

Tissues from *P. putida*-infected male crabs ($n = 10$) and healthy controls ($n = 3$) were fixed overnight in 4 % paraformaldehyde, dehydrated, embedded in paraffin, and sectioned into 10 μm slices. Sections were mounted on positively charged glass slides, stained with hematoxylin and eosin (H&E), and analyzed using the AxioScan.Z1 slide scanner (Carl Zeiss AG, Germany).

2.5. Expression and purification of recombinant proteins and antibody production

Recombinant SCY2 (rSCY2) and Scyreprocin (rScyreprocin) were expressed and purified following optimized protocols based on previous [37,38]. The recombinant proteins were purified using HisTrap FF crude columns on an ÄKTA Pure™ system (GE Healthcare Life Sciences, USA). Polyclonal antibodies against SCY2 and Scyreprocin were produced in rabbits and mice at the Laboratory Animal Center of Xiamen University. Antibody titers were assessed by ELISA, and protein concentrations were measured using the BCA assay (Beyotime, China).

2.6. qPCR and Western blotting analysis

Total RNA was extracted from male crab tissues using TRIzol (AG,

China), followed by cDNA synthesis according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed using SYBR Green Master Mix (Vazyme, China) on the CFX Opus 384 system (Bio-Rad, USA). Primer sequences are listed in Supplementary Tables S1–S3. *spGAPDH* was used as a reference gene for SCY2 and Scyreprocin expression, while *spef1α* was used for immune-related genes. Expression levels were calculated using the 2ˆ–ΔΔCt method [39].

For Western blotting, testis and ejaculatory duct tissues from male crabs were dissected under cryogenic conditions and homogenized in pre-chilled RIPA lysis buffer (Solarbio, China). Lysates were centrifuged at 12,000 ×*g* for 15 min at 4 °C, and the supernatant was collected for protein quantification using a BCA Protein Assay Kit (Beyotime, China). Equal amounts of protein (20 μg per lane) were separated by SDS–PAGE and transferred onto PVDF membranes. Membranes were incubated with mouse polyclonal anti-SCY2 or rabbit polyclonal anti-Scyreprocin antibodies (1:5000) as primary antibodies, followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H + L) or goat anti-rabbit IgG (H + L) secondary antibodies (1:10000; Thermo Fisher Scientific, USA). β-actin (anti-β-actin mouse monoclonal antibody from Transgen Biotech, China) was used as the loading control. Protein bands were visualized using a chemiluminescence imaging system (Tanon, China) and analyzed with the accompanying software.

2.7. Detection of oxidative and antioxidative indicators

Hepatopancreas tissues were homogenized and processed according to kit instructions. Commercial assay kits (Jiancheng Bioengineering Institute, Nanjing, China) were used to measure the activity of peroxidase (POD), superoxide dismutase (T-SOD), malondialdehyde (MDA), alkaline phosphatase (AKP), and acid phosphatase (ACP).

2.8. Immunofluorescence detection of SCY2 and Scyreprocin

To investigate the spatial expression patterns of SCY2 and Scyreprocin following *P. putida* infection, testis and ejaculatory duct tissues from male crabs were collected, cryosectioned, and subjected to immunofluorescence analysis following protocols described in previous research [25,40], with appropriate modifications. Briefly, tissue sections were fixed in pre-cooled methanol at −20 °C for 10 min. After washing with PBS, sections were blocked with 7 % BSA (Macklin, China) for 30 min at room temperature and incubated overnight at 4 °C in a humid chamber with primary antibodies against SCY2 and Scyreprocin (1:800 dilution). Negative control sections were treated with pre-immune rabbit and mouse serum (1:100 dilution) under the same conditions. After three washes with TBS containing 0.025 % Triton X-100, sections were incubated for 1 h with fluorescently labeled secondary antibodies in a dark, humidity chamber: goat anti-mouse IgG conjugated with Dylight® 488 and goat anti-rabbit IgG conjugated with Alexa Fluor® 647 (1:1000 dilution; Thermo Fisher, USA). Nuclei were counterstained with DAPI-containing mounting medium (Solarbio, China). Stained sections were sealed with coverslips and imaged using a FLUOVIEW FV4000 confocal laser scanning microscope (Olympus, Japan). Spermathecae tissues from mated female crabs were also subjected to the same staining procedure to assess the blockade effects of SCY2 and Scyreprocin following mating.

2.9. In vivo antibody blockade in female crabs

The reproductive aperture (genital opening) of female *S. paramamosain* is located on the ventral side of the carapace, at the base of the third walking leg, with one opening on each side. For *in vivo* blockade, polyclonal antibodies against SCY2 or Scyreprocin were diluted in sterile Dulbecco's PBS (DPBS) to a final concentration of 80 μg per crab. The antibody solutions were kept on ice prior to injection. The post-mating female crabs were briefly anesthetized in ice water. To perform the injection, each crab was positioned ventral side up to expose the genital openings. A 1 mL syringe fitted with a No. 6 stainless-steel gavage needle was vertically inserted into the genital pore to a depth of approximately 1–2 cm. The diluted antibodies were slowly injected into the spermathecal region. After a 24-h interval, a second injection was administered using the same method. Crabs in the negative control group received equivalent volumes of pre-immune rabbit serum and mouse serum diluted in DPBS. All procedures were performed under sterile conditions, and animal handling was standardized across all treatment groups to ensure consistency.

2.10. Isolation of sperm from spermathecae

Following antibody treatment, female crabs were dissected, and spermathecae were excised and minced in 10 mL of calcium-free artificial seawater (CaFASW). The suspension was gently rotated at 4 °C for 20 min, filtered through 150-mesh strainers, and centrifuged at 2,000 ×*g*. The pellet was washed, resuspended, and digested with 0.25 % trypsin (Solarbio, China) for 30 min at room temperature. Digestion was terminated by adding 10 % FBS and DNase I in an equal volume. After filtration and centrifugation, sperm cells were washed with PBS (without Ca^{2+} and Mg^{2+}) and stored at 4 °C for use within 24 h.

2.11. Acrosome reaction induction and flow cytometric analysis

Sperm suspensions were adjusted to OD_{600} = 1 and treated with artificial seawater (ASW), ASW + progesterone (50 μg/mL; MCE, USA), or CaFASW. After rotational incubation at room temperature overnight, cells were washed and blocked with 5 % BSA for 30 min at 4 °C. Primary antibodies against SCY2 and Scyreprocin (1:200) were applied for 1 h, followed by secondary antibodies (1:500). Sperm cells were directly stained with PNA-FITC (FITC-labeled Peanut Agglutinin) and PI in the dark [41–43], filtered (300-mesh), and analyzed using a BD Fortessa X20 flow cytometer.

2.12. Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics (v26) and GraphPad Prism (v10.2). One-way ANOVA followed by LSD post-hoc tests was used for comparisons among multiple groups. Different lowercase letters (*e.g., a, b, c, d*) above the bars indicate statistically significant differences between groups at $p < 0.05$, with groups sharing the same letter not significantly different. Kaplan–Meier survival curves were analyzed with the log-rank (Mantel–Cox) test. Western blot grayscale values were quantified using ImageJ. Significance levels were defined as $p < 0.05$ (*), < 0.01 (**), and < 0.001 (***).

3. Results

3.1. Localization, distribution, and histopathology of P. putida in male crabs

To determine the pathogenicity of *P. putida* in *S. paramamosain*, bacterial growth curves were used to establish the relationship between optical density (OD_{600}) and colony-forming units (CFU). Infected crabs displayed reduced mobility and aggression compared to healthy controls. Within seven days post-infection, mortality rates reached 100 %, 70 %, 30 %, and 10 % for bacterial doses of 4×10^8, 8×10^7, 1×10^7, and 3×10^6 CFU per crab, respectively. The median lethal dose (LD_{50}) was calculated as 2×10^7 CFU per crab using the log-rank (Mantel–Cox) test (Fig. 1A).

Dissection of infected individuals revealed pathological signs, including skin lesions, dark and malodorous peritoneal fluid, weak myocardial contractions, and gill blackening (Fig. S1). Tissue homogenates were cultured on CFC selective medium, and colonies were identified by their pyocyanin fluorescence under 365 nm UV light (Fig. S2).

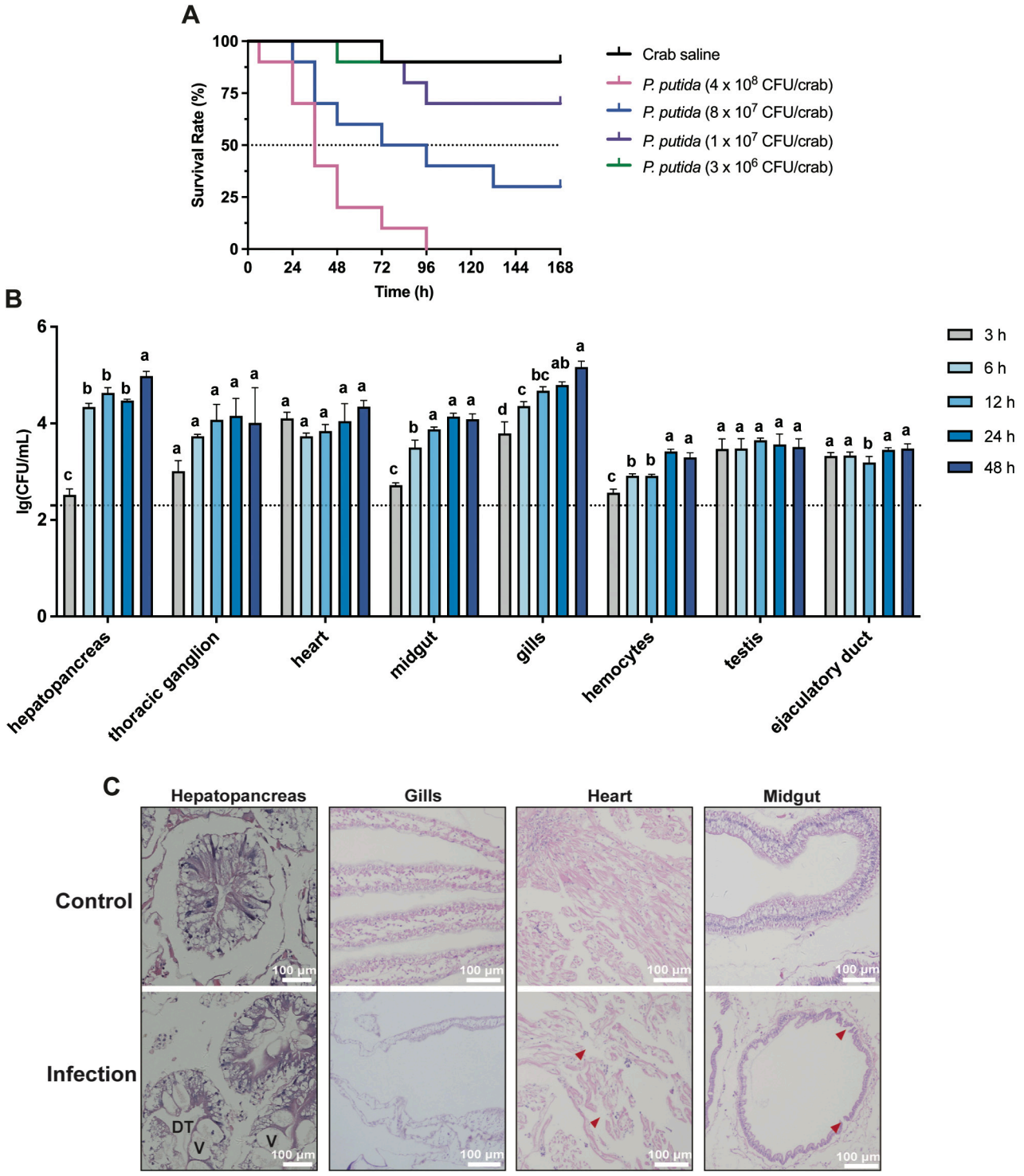


Fig. 1. Lethal dose, tissue colonization, and pathological effects of *P. putida* infection in male crabs
(A) Survival curves of *S. paramamosain* following *P. putida* infection at different doses ($n = 10$ per group). Statistical analysis was conducted using the log-rank (Mantel–Cox) test. (B) Bacterial load in tissues at 12 h post-infection. The dashed line indicates the minimum countable bacterial load. Tissues analyzed include hepatopancreas, thoracic ganglion, heart, midgut, gills, hemocytes, testis, and ejaculatory duct ($n = 3$). Statistical comparisons were performed using One-way ANOVA followed by LSD post-hoc tests; different letters denote significant differences ($p < 0.05$). (C) Representative H&*E*-stained tissue sections from infected male crabs. Arrows indicate key pathologies: disorganization and fragmentation of myocardial fibers in the heart; disruption of the epithelial layer in the midgut. DT: destroyed hepatic tubules; V: vacuolization. Scale bar: 100 μm.

Quantitative bacterial analysis considered counts within 20–200 CFU as valid (Fig. 1B). Notably, bacterial colonization levels in the hepatopancreas, thoracic ganglion, midgut, gills, and hemocytes increased progressively over time. In contrast, bacterial loads in the heart, testis, and ejaculatory duct remained relatively stable across all time points. However, they consistently remained above the detection threshold (>20 CFU).

Histological analysis of infected crabs revealed significant tissue

damage in infected crabs, including vacuolization of hepatic tubules, disruption of basement membranes in the hepatopancreas and midgut, and structural disorganization of gill and myocardial tissues (Fig. 1C). These findings confirm that *P. putida* invades and colonizes multiple organs, causing systemic pathology. Notably, bacterial counts in hemocytes were relatively low when compared to the high levels of colonization observed in the primary immune organs such as the hepatopancreas and gills. This is consistent with the crab's semi-open circulatory system, where pathogens are largely sequestered by fixed tissues rather than circulating hemocytes.

3.2. Differential immune functions of SCY2 and Scyreprocin in male crabs

Previous studies have shown that *P. putida* infection in *S. paramamosain* can lead to structural damage to the spermatophore and induce the expression of male-specific AMPs [25]. To further investigate their functional roles in innate immunity, we analyzed their transcriptional and translational dynamics in the male gonads following bacterial infection.

In the testis, Scyreprocin mRNA levels were significantly upregulated as early as 3 h post-infection and remained elevated through 24 h (Fig. 2A). At the protein level, Scyreprocin expression showed a sustained increase, with marked upregulation observed at 12-, 24-, and 48-h post-infection (Fig. 2C), indicating both rapid transcriptional activation and continued protein accumulation.

In contrast, SCY2 transcripts in the ejaculatory duct exhibited a consistent downregulation trend over the 48-h infection period (Fig. 2B). However, Western blot analysis revealed that SCY2 protein levels initially decreased before returning to baseline by 24 h post-infection (Fig. 2D). This suggests the involvement of post-transcriptional regulatory mechanisms or protein stabilization effects.

Immunofluorescence analysis demonstrated distinct spatial and temporal expression patterns for the two peptides (Fig. 2E). In the testis, Scyreprocin (red) was the dominant peptide, and its fluorescence intensity significantly increased after infection, a finding consistent with our Western blot results. In contrast, SCY2 signals (green) were predominantly expressed in the ejaculatory duct, and its signal remained stable post-infection, also aligning with the Western blot data. These findings suggest that SCY2 and Scyreprocin respond differentially to pathogen challenge, underscoring their specialized functions in male reproductive immunity.

3.3. Recombinant SCY2 and Scyreprocin enhance survival and bacterial clearance in male crabs

Recombinant SCY2 (rSCY2) and Scyreprocin (rScyreprocin) were successfully produced using an optimized eukaryotic expression system (Fig. S3). To evaluate their protective effects, male crabs were infected with *P. putida* and then injected with different doses of recombinant peptides, as illustrated in the experimental timeline (Fig. 3A).

Crabs injected with 1, 4, or 8 μg of rScyreprocin showed a consistent survival rate of 80 %, compared to 40 % in the infection-only group, indicating strong protective efficacy across all tested concentrations. For rSCY2, the highest dose (8 μg) resulted in a survival rate of 90 %, whereas 1 μg and 4 μg provided partial protection, with survival rates of 60 % and 40 %, respectively (Fig. 3B, C). These findings suggest a dose-dependent immune protective effect of rSCY2, and a robust effect of rScyreprocin even at lower concentrations.

To further assess antimicrobial activity *in vivo*, tissue bacterial loads were measured at multiple time points. Both rSCY2 and rScyreprocin significantly reduced bacterial colonization in the hepatopancreas, gills, hemocytes, testis, and ejaculatory duct from 12 to 96 h post-injection (Fig. 4A–E), demonstrating broad tissue clearance capacity and strong immune-enhancing effects. Additionally, a preliminary co-injection of rSCY2 and rScyreprocin (4 μg each) was tested, showing no significant synergistic effect on survival or bacterial clearance compared to the 8 μg individual treatments (Data not shown).

3.4. SCY2 and Scyreprocin regulate immune gene expression and oxidative stress responses

To further explore the regulatory role of AMPs, the expression of immune-related genes expression and oxidative indicators were examined. Upon infection, genes associated with the Toll-Dorsal (*spPelle, spTRAF6, spDorsal*), Imd-Relish (*spImd, spRelish*), and JAK/STAT (*spSTAT*) pathways—as well as AMPs (*spArasin, spCrustin5, spCrustin7*), were significantly upregulated at 3 h, but downregulated after 12 h, indicating a transient immune activation (Fig. 5A–I).

Meanwhile, the enzymatic activities of oxidative stress markers (POD, T-SOD, MDA, AKP, ACP) were dysregulated upon infection, but returned to normal following peptide treatment, indicating that SCY2 and Scyreprocin restored immune homeostasis (Fig. 6A–E).

3.5. Blockade of SCY2 and Scyreprocin leads to immune imbalance in female crabs

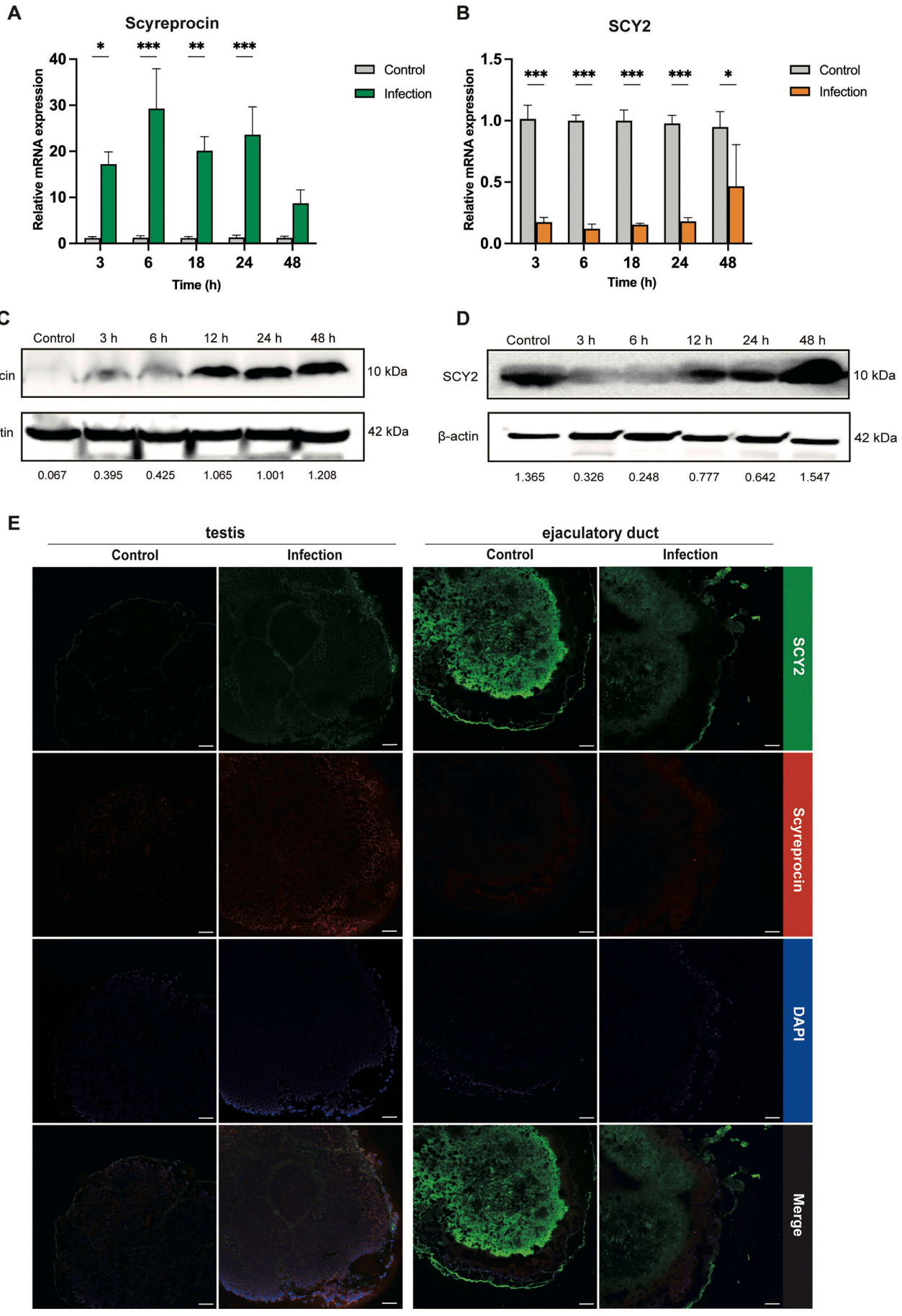
During sperm capacitation in the spermathecae of female crabs, sperm undergo the acrosome reaction (AR) in response to calcium ions, progesterone, and other physiological signals, ultimately achieving fertilization [25,44]. To investigate the immune function of SCY2 and Scyreprocin in females, we designed an experimental workflow combining *in vivo* antibody blockade, acrosome reaction induction, and flow cytometry (Fig. 7A; Fig. S4).

Immunofluorescence staining results showed that both SCY2 and Scyreprocin were both localized within the lumen of the spermathecae. Given that these peptides are not expressed in female tissues [25], this observation provides direct evidence of their successful transfer from the male during mating. In contrast, the fluorescence intensity was markedly reduced in the peptide-blockade groups, confirming the efficacy of *in vivo* inhibition (Fig. 7B; Fig. S5).

Flow cytometry analysis, using PNA-FITC and PI staining, assessed the acrosome reaction (AR) in live spermatozoa. The results showed that the number of AR-positive sperm was significantly higher in the ASW and ASW + PG treatment groups compared to the FASW group. The presence of calcium ions significantly enhanced AR, with progesterone providing additional stimulation. However, antibody-mediated blockade of SCY2 or Scyreprocin resulted in a significant decrease in AR-positive sperm, indicating that these peptides are essential for optimal acrosome function (Fig. 8A–F; Fig. S6).

To further investigate the physiological effects of peptide inhibition, female crabs were challenged with *P. putida* after antibody blockade. At 96 h post-infection, survival rates dropped significantly to 5 % in the anti-SCY2 group and 10 % in the anti-Scyreprocin group compared to 50 % in the negative control group (Fig. 9A). Moreover, bacterial loads in the spermathecae were significantly elevated in the antibody-treated groups, confirming a compromised bacterial clearance capacity (Fig. 9B).

The expression levels of immune-related genes, including *spToll2*, *spPelle*, *spDorsal*, and *spRelish*, were significantly upregulated in the antibody-treated groups (Fig. 9C–F). This indicates an overactivated or dysregulated immune response. Antioxidant enzyme activity analysis showed a significant reduction in T-SOD and an increase in MDA levels in the antibody-treated groups (Fig. 9G–H), suggesting heightened oxidative stress and immune imbalance. These findings collectively indicate that after being transferred to the female reproductive tract during mating, SCY2 and Scyreprocin help maintain local immune homeostasis and enhance resistance to pathogenic invasion. This mechanism thereby supports the reproductive success and survival of the female.



(caption on next page)

Fig. 2. Transcriptional and translational dynamics of SCY2 and Scyreprocin after *P. putida* infection.
(A–B) qPCR analysis of Scyreprocin and SCY2 mRNA expression in the testis and ejaculatory duct, respectively. Statistical analysis was performed using Two-way ANOVA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (C—D) Western blot detection of SCY2 and Scyreprocin protein expression in corresponding tissues. Numbers represent the ratios of gray values. (E) Immunofluorescence staining of Scyreprocin (red) and SCY2 (green) in testis and ejaculatory duct sections before and after infection. Scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

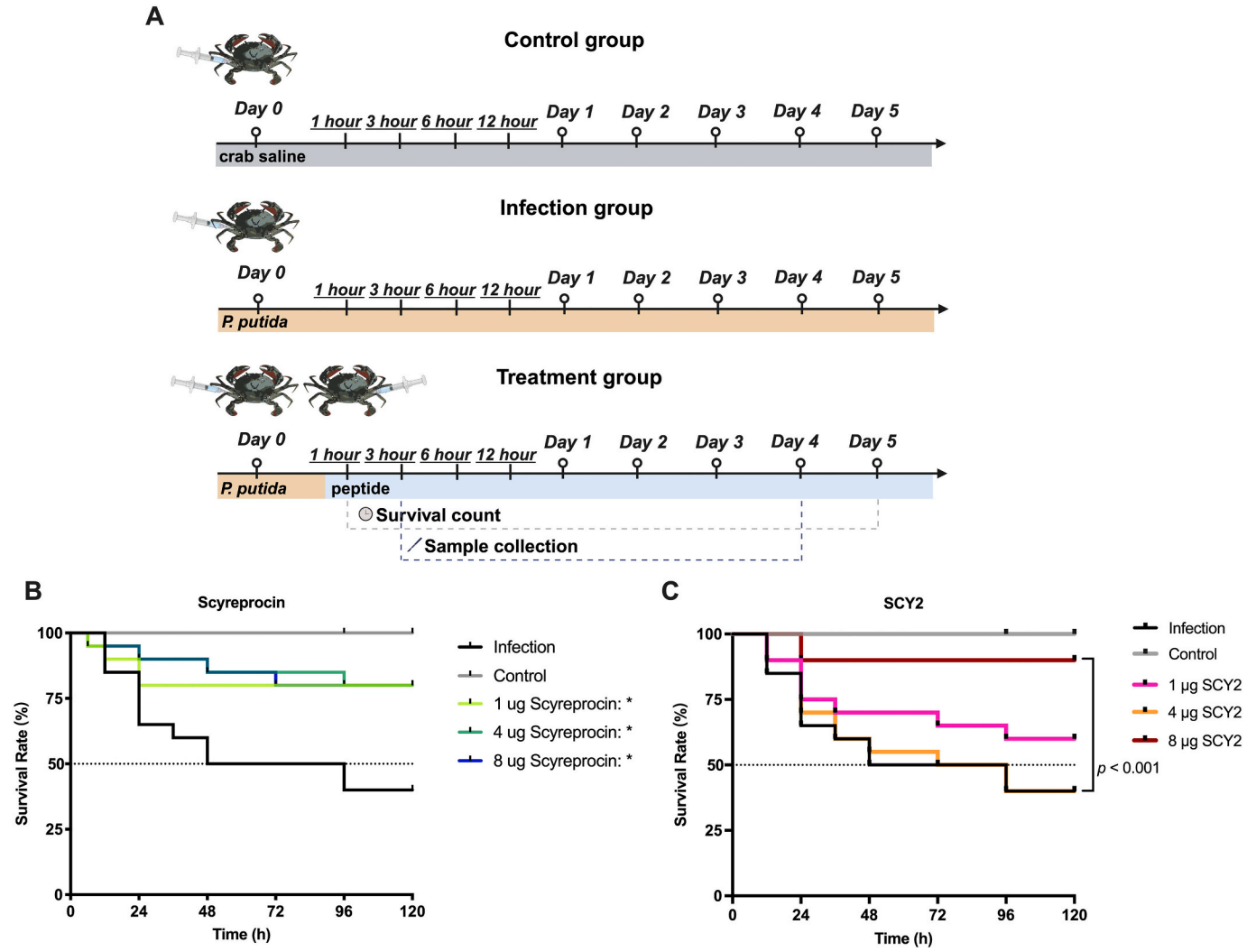


Fig. 3. Effect of rSCY2 and rScyreprocin on survival of male crabs challenged with *P. putida*.
(A) Experimental workflow: crabs were infected with *P. putida* on day 0, followed by peptide injection 1 h later. Survival was monitored over 120 h ($n = 20$ per group).
(B—C) Survival curves for male crabs treated with different doses of rSCY2 and rScyreprocin. Statistical significance was determined using the log-rank (Mantel–Cox) test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

4. Discussion

Bacterial infections represent a serious risk in intensive *S. paramamosain* aquaculture [45], where high stocking densities and environmental stresses often trigger disease outbreaks [8,9]. Pathogens, especially *Vibrio* species such as *Vibrio parahaemolyticus* and *Vibrio harveyi*, are known to cause mass mortality events [8,46–48]. The pathogen used in this study, *P. putida*, is also a recognized opportunistic pathogen [13,14], known to cause acute, high-mortality outbreaks in other stressed aquatic species [15,18]. Its significance is particularly relevant to our model, as our laboratory previously isolated a virulent strain directly from the testis of *S. paramamosain* [25]. The LD_{50} challenge dose (2×10^7 CFU/crab) was selected to model an acute, severe infection. This dose is higher than those used for highly virulent pathogens like *V. parahaemolyticus* (effective at 10^4–10^6 CFU/crab [49,50]), which is consistent with the higher infectious threshold expected for an opportunistic pathogen to overcome basal host immunity. Importantly, this dose elicits severe pathology and high mortality (Fig. 1A, C), and establishes a high bacterial load state that mimics the critical phase of natural epizootics. It has helped us provide a valid system to assess the protective efficacy of immune effectors. This specific challenge, from an environmental pathogen that colonizes the gonads [25], is inherently connected to the crab's unique reproductive strategy.

After mating, sperm are stored in the female's spermathecae for extended periods (ranging from one to six months) before oviposition and fertilization occur [51]. This prolonged storage presents a significant immunological challenge: females must tolerate allogeneic sperm while simultaneously defending against microbial pathogens introduced

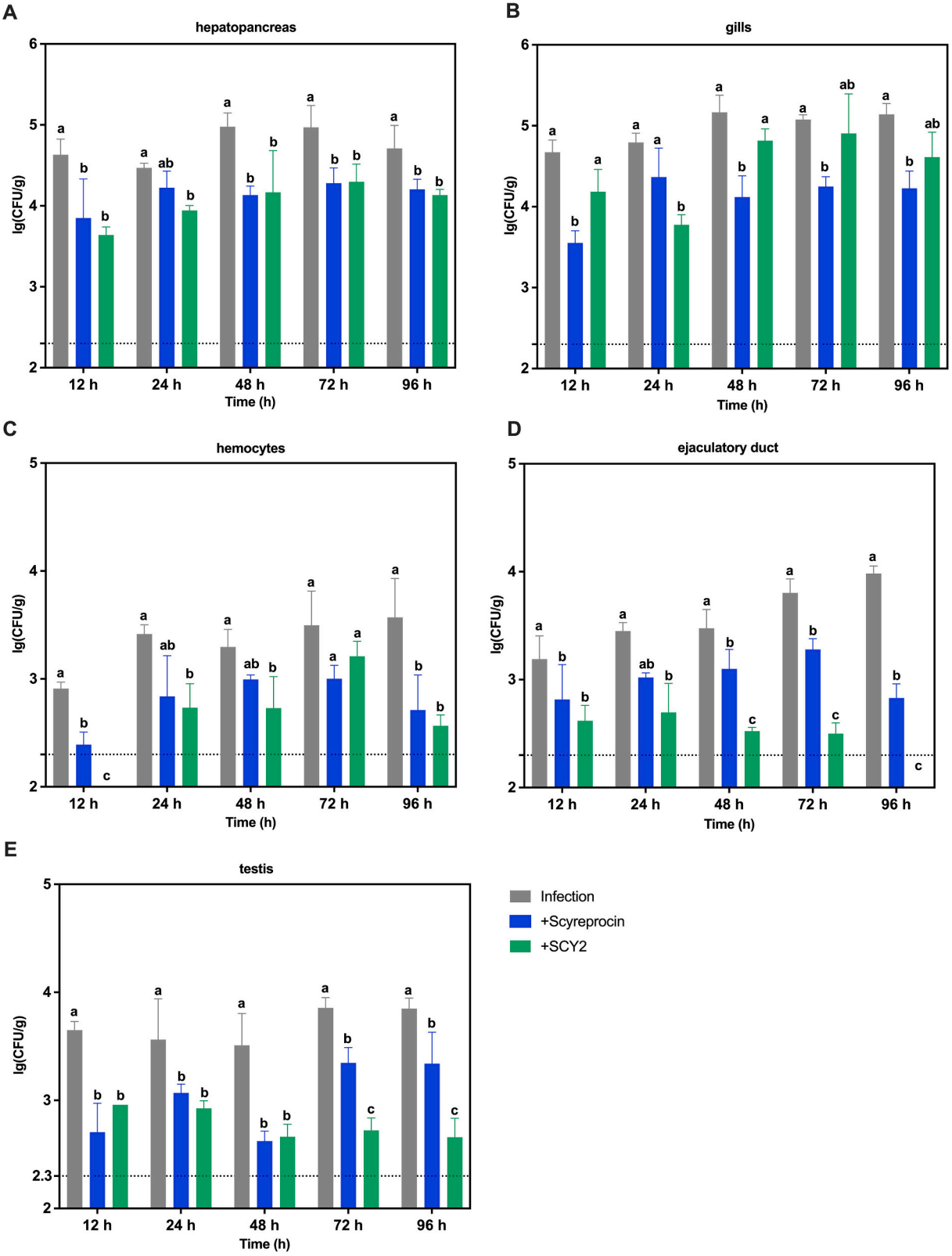


Fig. 4. Tissue-specific bacterial clearance in male crabs following peptide treatment.
(A–E) Quantification of bacterial loads in hepatopancreas, gills, hemocytes, testis, and ejaculatory duct ($n = 3$). The dashed line indicates the detection limit. One-way ANOVA followed by LSD post-hoc test was used; different letters indicate significant differences ($p < 0.05$).

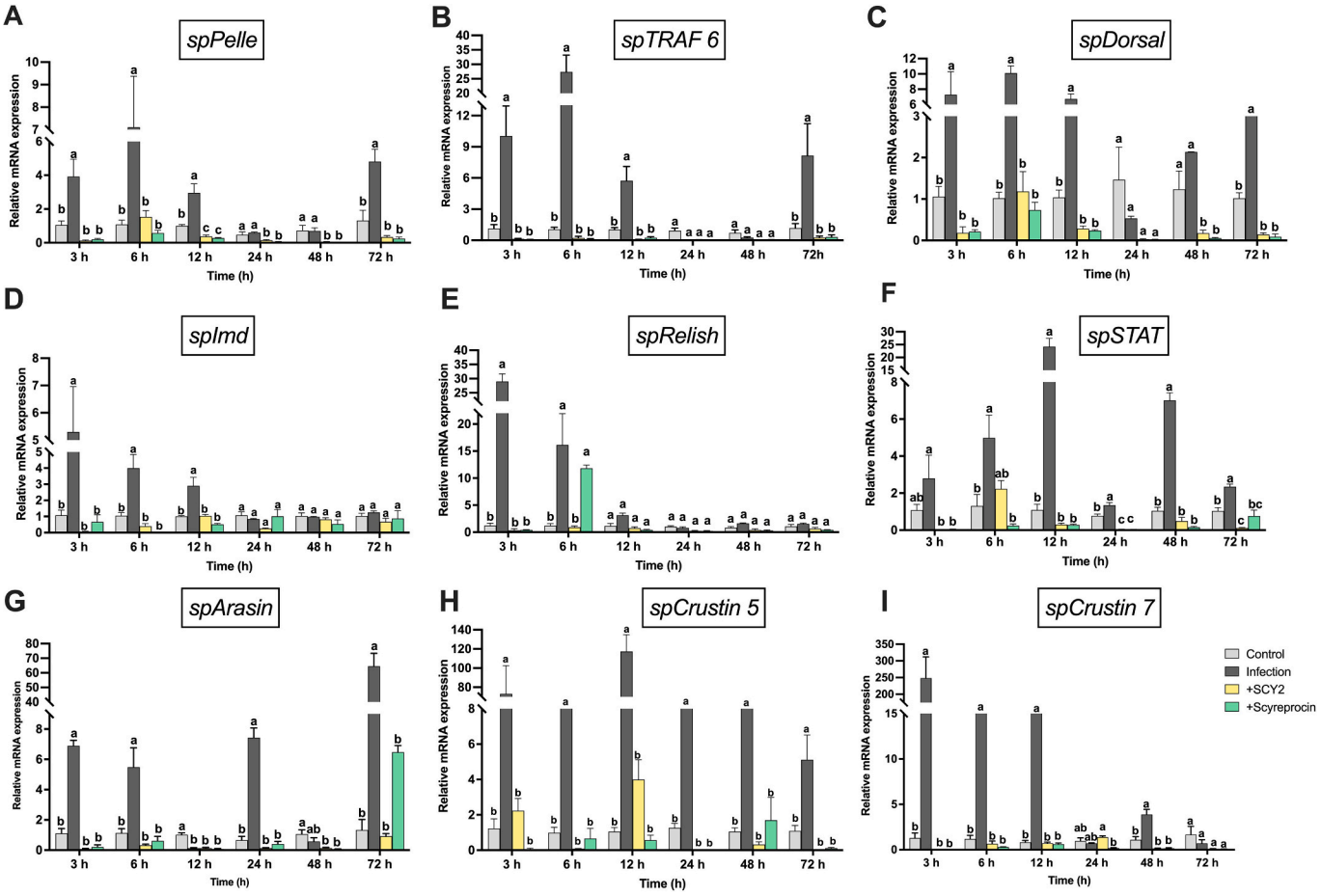


Fig. 5. Modulation of immune-related gene expression by rSCY2 and rScyreprocin in male crabs.
(A–I) Expression levels of *spPelle, spTRAF6, spDorsal, spImd, spRelish, spSTAT, spArasin, spCrustin5*, and *spCrustin7* (n = 3–4). One-way ANOVA with LSD post-hoc test; different letters denote significant differences ($p < 0.05$).

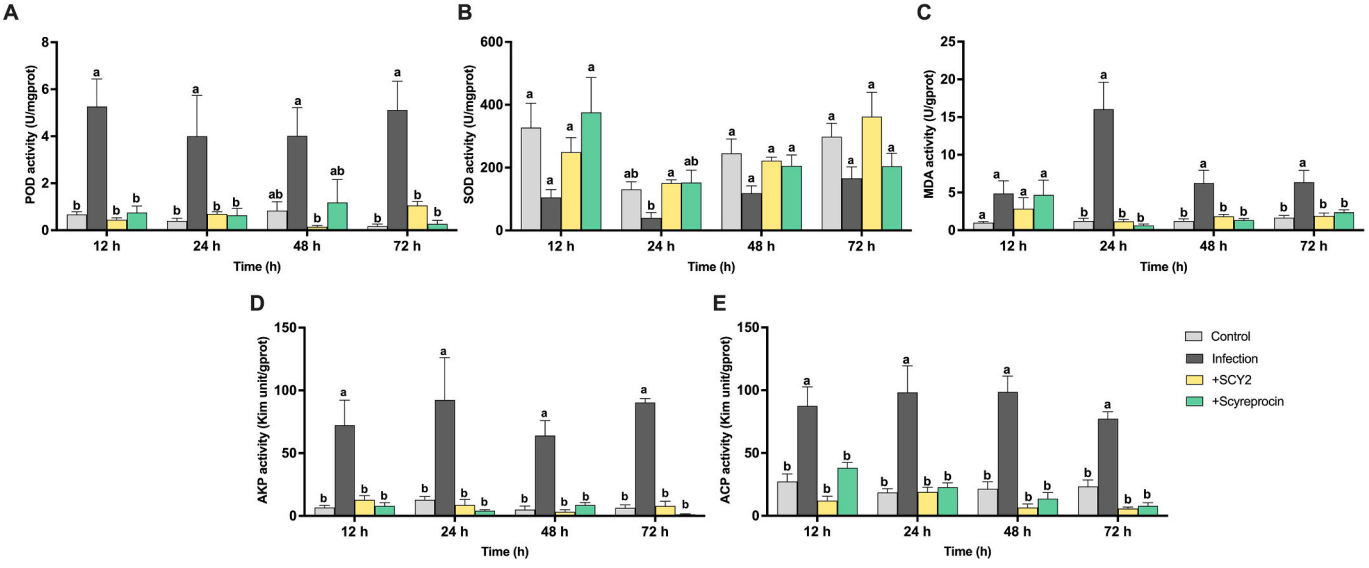


Fig. 6. Effect of rSCY2 and rScyreprocin on oxidative stress biomarkers in male crabs
(A–E) Enzymatic activity of POD, T-SOD, MDA, AKP, and ACP in hepatopancreas tissue (n = 3–5). Statistical analysis was performed using One-way ANOVA followed by LSD post-hoc test; different letters indicate significant differences ($p < 0.05$).

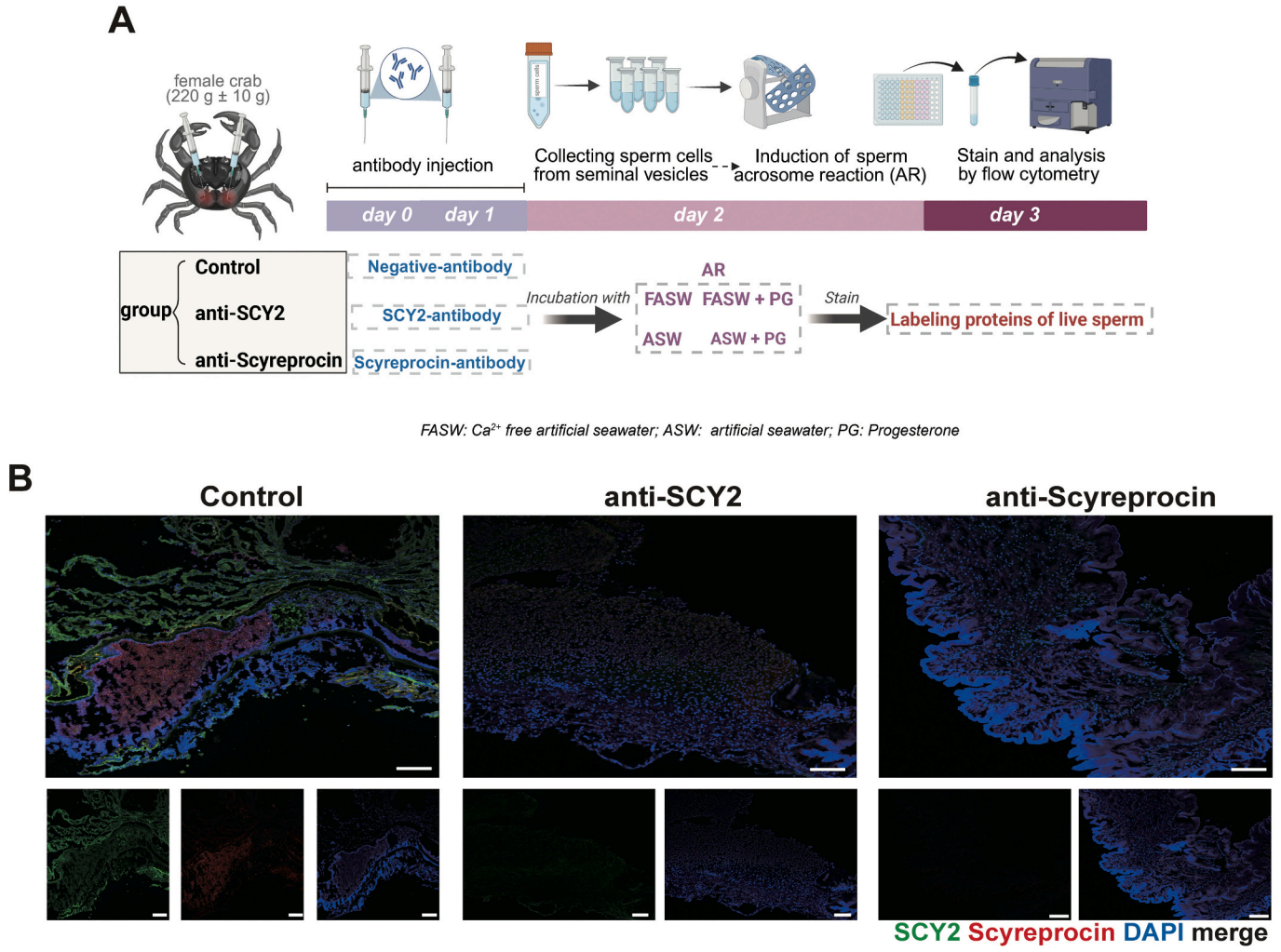


Fig. 7. Validation of SCY2 and Scyreprocin blockade in female crabs.
(A) Schematic of the *in vivo* antibody blockade experiment. Polyclonal antibodies targeting SCY2 or Scyreprocin were injected into the genital pore of female crabs on days 0 and 1. On day 2, tissues were harvested for cryosectioning, and sperm cells were simultaneously isolated from spermathecae. The AR was then induced using calcium ion and progesterone treatments, followed by flow cytometric analysis on day 3. (B) Representative immunofluorescence images of SCY2- and Scyreprocin-blocked spermathecae. Reduced fluorescence signal indicates successful *in vivo* inhibition. Scale bar: 100 μm.

during mating. Seminal plasma delivered by males not only ensures sperm viability but also serves as a carrier for a complex array of bioactive molecules [52–54]. However, in *S. paramamosain*, the immunoprotective and anti-infective mechanisms conferred by these seminal components remain to be elucidated.

Our previous studies demonstrated that the antimicrobial peptides SCY1 and SCY2 are transferred from males to females during mating [36]. Interestingly, their expression is not induced by immune stimulants such as LPS, but is markedly upregulated by progesterone, a key hormone during mating, suggesting a role in the reproductive process. In addition, Scyreprocin, identified as a novel SCY2-interacting protein, exhibits similar functions to SCY1 and SCY2 and can likewise be transferred to females during copulation [25]. Collectively, those findings point to a dual role for these male-derived AMPs, which bear protecting their own gametes and conferring immune benefits to females post-mating.

A key finding of this study is the distinct spatial localization and differential regulation of SCY2 and Scyreprocin within the male reproductive tract. Our results clearly show that Scyreprocin acts as a primary, inducible immune effector in the testis, with its expression rapidly upregulated following a pathogen challenge. This suggests its role as a frontline defense mechanism to protect the crucial site of spermatogenesis. In stark contrast, SCY2 expression is primarily confined to the ejaculatory duct, where it is maintained at a high constitutive protein level. This pattern of a male reproductive tract-specific AMP is a known strategy in arthropods, first described for Andropin in the *Drosophila* ejaculatory duct [55]. Instead of an immediate transcriptional response to infection, the stable, pre-existing pool of SCY2 likely serves as a persistent barrier to safeguard mature sperm. This storage-based mechanism has been documented in other crustaceans, such as shrimp, where AMPs are produced and stored in granulocytes, ready for rapid release [56]. This dual-component system—where one peptide is inducible at the production site and its binding partner is constitutive at the storage site—highlights a sophisticated and compartmentalized immune strategy [30], essential for ensuring reproductive success in a pathogen-rich environment.

Then we confirmed the potent *in vivo* immune-protective roles of SCY2 and Scyreprocin in male crabs. As a marine invertebrate, *S. paramamosain* lacks antibody-mediated adaptive immunity and relies exclusively on innate immune defenses. The bacterium *P. putida* was first detected during our preliminary sperm cell culture experiments using spermathecae samples collected from mud crabs. Repeated culture contaminations prompted us to investigate the source, which was most likely the spermathecae tissue itself. Subsequent isolation identified

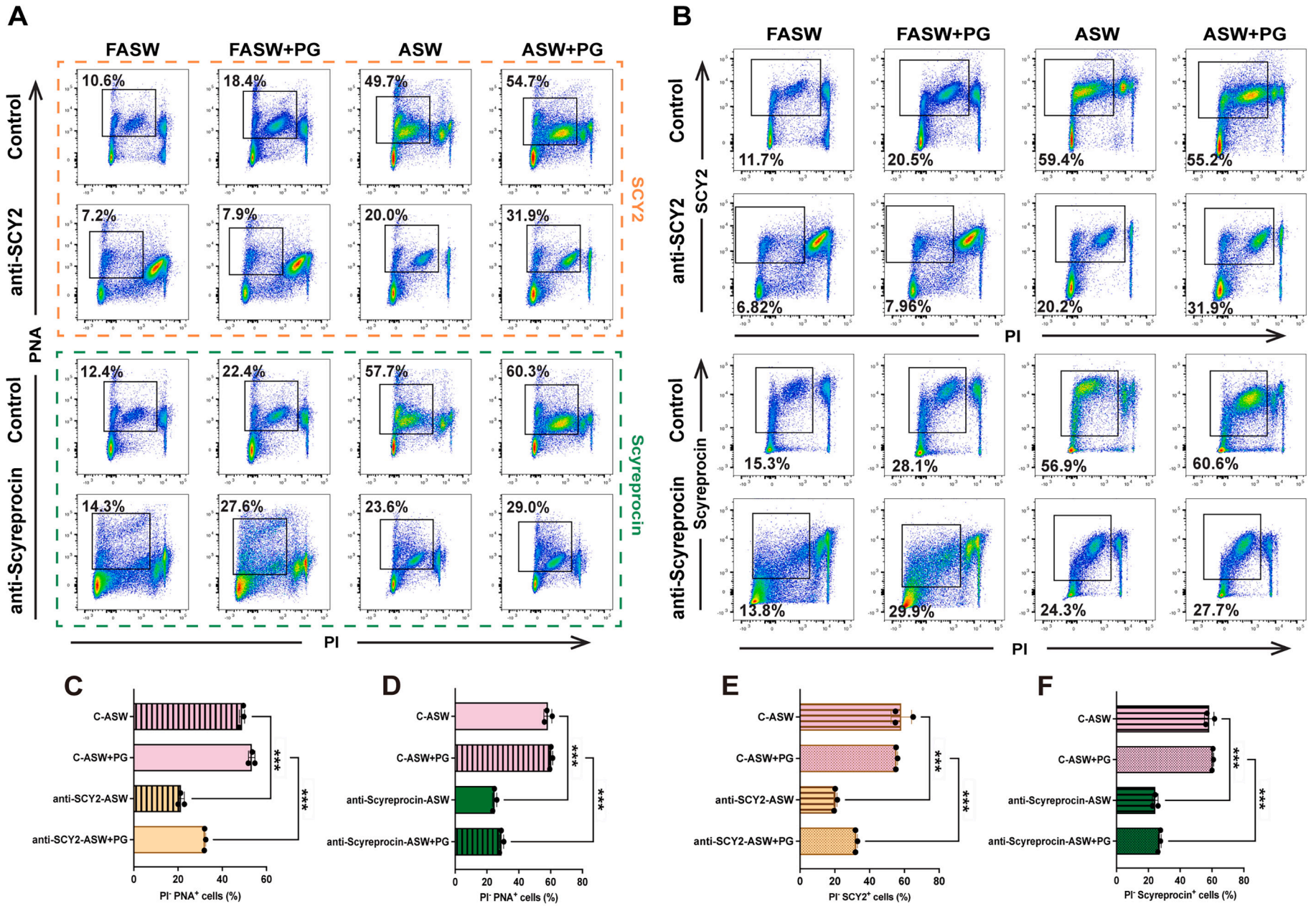


Fig. 8. Flow cytometry of the blockade effect of SCY2 and Scyreprocin in female crabs

A-B: Representative flow cytometry diagrams showing the proportions of live sperm cells with AR (PNA^+, PI^-), and live sperm cells stained with SCY2/Scyreprocin ($SCY2^+$/$Scyreprocin^+$, PI^-); C—F. Quantitative analysis of the percentage of PNA^+, $SCY2^+$, or $Scyreprocin^+$ sperm cells in different groups (n = 3). Statistical analysis was performed using One-way ANOVA test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

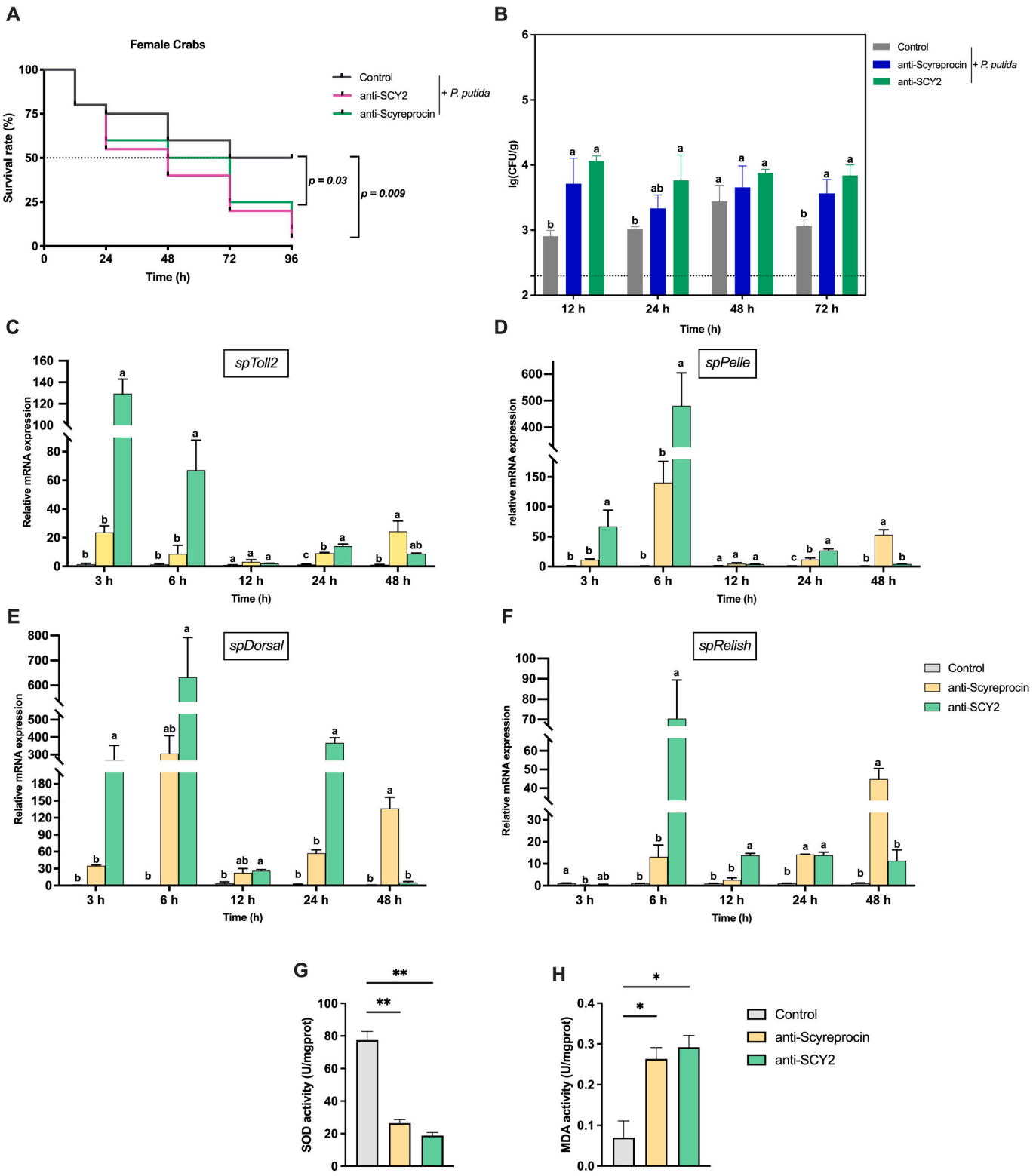


Fig. 9. Immune and oxidative effects of SCY2 and Scyreprocin blockade in female crabs.
(A) Survival curves of female crabs' post-infection with *P. putida* following peptide blockade (n = 20 per group). Statistical analysis used log-rank (Mantel–Cox) test. (B) Bacterial load in spermathecae tissues (n = 3). (C—F) qPCR analysis of immune-related gene expression (*spToll2*, *spPelle*, *spDorsal*, *spRelish*) (n = 3–4). One-way ANOVA with LSD post-hoc test was applied; different letters indicate significant differences ($p < 0.05$). (G–H) Enzymatic activity of T-SOD and MDA in spermathecae tissue ($n = 4$). Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

P. putida; a bacterium not commonly encountered as a laboratory cell culture contaminant. To evaluate its pathogenicity, we conducted challenge experiments. We found that high-dose infection caused 50 % mortality within 48 h, with bacterial colonization observed in the hepatopancreas, gills, heart, and gonads, resulting in severe tissue damage. A recent study in *Oreochromis niloticus* similarly demonstrated the contagious nature of *P. putida*, reporting clinical symptoms such as skin darkening and ulceration, and mortality rates of up to 56 % in infected fish. In response to this pathogenic threat, both SCY2 and Scyreprocin exhibited strong protective efficacy. Administration of the recombinant peptides increased crab survival by 40–50 % and significantly reduced systemic bacterial loads.

To elucidate the mechanisms underlying this protection, we examined the modulation of key immune signaling pathways. The Toll and IMD pathways form the core of the crab's innate immune system, responsible for pathogen recognition and the induction of AMP synthesis [57]. The Toll pathway, defined initially in *Drosophila*, is activated upon recognition of pathogen-associated molecular patterns (PAMPs). This recognition triggers a signaling cascade that involves the dissociation of the TRAF6–IRAK1 complex, Pelle-mediated degradation of the inhibitor Cactus, which subsequently allows the nuclear translocation of the NF-κB-like transcription factor Dorsal. This process ultimately drives the transcription of AMPs such as ALFs and Crustins [58,59]. Complementing this, the IMD pathway is primarily responsible for detecting Gram-negative bacteria through recognition of peptidoglycan (PGN) by PGRP receptors. Ligand binding initiates recruitment of the IMD protein and activation of a caspase cascade involving FADD and Dredd, leading to cleavage of the NF-κB transcription factor Relish. The active Relish fragment then translocates into the nucleus and induces expression of AMPs such as Diptericins and Cecropins [60]. In our study, following exposure to *P. putida*, transcripts of key genes in the Toll signaling pathway (*spPelle*, *spTRAF6*, and *spDorsal*), the IMD pathway (*spImd*, *spRelish*, *spSTAT*), and several AMP genes (*spArasin*, *spCrustin5*, and *spCrustin7*) were all significantly upregulated within 3 h. The significant upregulation of AMPs, including Arasin, Crustin5, and Crustin7, confirmed the activation of these critical pathways during *P. putida* infection. These results are consistent with previous reports. For example, the transcription of *spIMD*, *spRelish*, and *spDorsal* in *S. paramamosain* within 3 h of *V. parahaemolyticus* stimulation [61]. Similarly, *Arasin* mRNA expression increases in *Scylla serrata* 3 h after an LPS challenge [62]. Interestingly, the expression of genes like *spPelle*, *spSTAT,* and *spArasin* showed dynamic fluctuations over the subsequent 24–72 h. This suggests that their regulation is not merely transient but is a sustained process, likely to prevent an over-activation of the immune system.

Notably, treatment with SCY2 and Scyreprocin restored immune gene expression to near-baseline levels as early as 3 h post-infection. This rapid modulatory effect suggests a mechanism that operates faster than a full *de novo* transcriptional response. We propose that this is linked to the unique biological context of these peptides. SCY2 and Scyreprocin are induced by reproductive hormones and stored as pre-synthesized proteins in the testis and spermathecae [25,36]. The rapid release of such pre-existing AMPs, followed by a secondary transcriptional response, represents a hallmark of biphasic innate immunity in arthropods [60]. Accordingly, we hypothesize that stored SCY2 and Scyreprocin act as a fast-acting, first-line defense, immediately modulating the immune milieu upon infection. This initial protein-level response likely stabilizes the system while the host's transcriptional machinery initiates a more sustained, secondary wave of AMP synthesis *via* the Toll and IMD pathways. Such a dual-action model provides a plausible explanation for their potent regulatory role in maintaining immune homeostasis.

Our analysis of immune signaling pathways revealed that *P. putida* infection rapidly activates both the Toll and IMD pathways, leading to a pronounced upregulation of downstream AMP genes. While this swift immune response is critical for pathogen clearance, it often comes at a substantial physiological cost, a phenomenon referred to as immunopathology [63]. A major driver of this collateral damage is oxidative stress, resulting from the excessive production of reactive oxygen species (ROS) by activated immune cells. Although ROS are essential for pathogen elimination, uncontrolled accumulation can severely damage host tissues [64]. The hepatopancreas, a central metabolic and immune organ in crustaceans, is particularly susceptible to such infection-induced stress and therefore provides a key site for evaluating host responses.

The disruption was evident as a clear state of oxidative stress. We evaluated SOD and POD, the two primary antioxidant enzymes that constitute the first line of defense against ROS. SOD catalyzes the dismutation of the superoxide radical (O^{2-}) into oxygen (O_2) and hydrogen peroxide (H_2O_2), while POD subsequently neutralizes H_2O_2. Measuring their activity is essential, as decreases often indicate that the antioxidant system has been overwhelmed by excessive ROS production during infection [65]. In parallel, we measured MDA, a product of lipid peroxidation and a reliable marker of oxidative damage to cell membranes. Elevated MDA levels directly reflect cellular injury caused by ROS that escape enzymatic defenses [66]. Beyond oxidative damage, infection also elicited a strong innate immune response, as indicated by changes in ACP and AKP. These hydrolytic enzymes, which play roles in metabolic processes such as phagocytosis, are widely recognized as indicators of non-specific immune status in crustaceans. Increased ACP and AKP activity typically reflect an active immune response directed at eliminating invading pathogens [67]. These findings are consistent with other stress models. For example, alkalinity stress in crabs elevates MDA and reduces SOD activity, while exposure to toxins significantly increases ACP and AKP levels.

Crucially, our study demonstrates that the administration of SCY2 and Scyreprocin effectively counteracted this physiological disruption. Antioxidant capacity in the hepatopancreas was restored, as evidenced by the recovery of SOD and POD activities and a significant reduction in the lipid peroxidation marker MDA. These results suggest that the peptides function not only as direct bactericidal agents but also as modulators of host responses, mitigating oxidative damage. In addition, normalization of immune-related enzymes ACP and AKP indicates a transition from a hyperactivated immune state toward homeostasis, thereby preventing chronic stress and conserving energy. Collectively, these findings highlight that the protective role of SCY2 and Scyreprocin extends beyond antimicrobial activity to supporting the host's ability to maintain physiological homeostasis during infection.

Despite these advances, “reproductive immunity” remains an underexplored aspect of invertebrate defense, particularly in crustaceans. The concept was first described in *Drosophila melanogaster*, where the male-specific AMP Andropin is upregulated post-mating and localized to the ejaculatory duct [55]. In other species, seminal plasma contains immune-active molecules such as lactoferrin [68,69], Lysozyme [70] and Andropin [71], which are involved in spermatogenesis, sperm motility, and pathogen defense. More recently, studies in *D. melanogaster* revealed that activation of the immune-related cGAS–STING pathway can also be transferred from males to females during mating, thereby influencing female physiology and behavior [72]. In mammals, epididymal AMPs such as β-defensin 15 are essential for sperm maturation and immune protection [73]. While SCY2 and Scyreprocin were previously shown to be transferred to females and regulated by reproductive hormones [25,36], their roles in female immunity were speculative. Here, we used a novel *in vivo* antibody-blocking strategy in post-mated females, verifying blockade efficacy *via* immunofluorescence and flow cytometry with peanut agglutinin (PNA) staining. To our knowledge, this is the first demonstration that SCY2 and Scyreprocin confer reproductive immunity in *S. paramamosain*. This finding may help explain how successful mating and fertilization can occur in the complex and pathogen-rich marine environment.

The use of PNA staining is particularly valuable for assessing sperm

function in the mud crab. Unlike mammalian sperm, which are flagellated and motile, decapod crustacean sperm—including those of *S. paramamosain*—are non-flagellated, non-motile, and structurally complex, featuring a prominent acrosomal vesicle. For these sperm, the AR—an exocytotic event that releases enzymes necessary for penetrating the egg's outer layers—is an essential prerequisite for fertilization. This process is typically triggered by species-specific molecular cues; for instance, in mammals, sperm–egg recognition *via* surface glycoproteins induces the AR [74,75]. PNA, a lectin derived from peanuts, is widely used as a specific marker for the acrosome. Its binding is mediated by high affinity for the β-galactosyl(1–3)-*N*-acetylgalactosamine (Galβ1-3GalNAc) oligosaccharide motif. This motif becomes exposed on the inner acrosomal membrane only after the AR has occurred [41]. This principle is well-established in mammals [76]. Moreover, the specificity of PNA binding to internal acrosomal structures has also been confirmed in marine invertebrates such as sea squirts and sea urchins [43].

Previous research in the mud crab demonstrated that the AR can be induced by calcium ions or progesterone from the female reproductive tract and is closely associated with SCY2 and Scyreprocin. A study further showed that blocking these two peptides on the sperm surface prevented progesterone-induced AR [25]. Building on this, our *in vivo* experiments confirmed that blockade of SCY2 and Scyreprocin significantly reduced the proportion of live sperm undergoing calcium-induced AR. To our knowledge, this represents the first application of PNA-based flow cytometry to validate the AR in mud crab sperm, establishing it as a reliable and informative method for functional assessment.

Having confirmed that peptide blockade compromised sperm function, we next examined the immunological consequences for females. Neutralization of SCY2 and Scyreprocin markedly increased female susceptibility to *P. putida* challenge, leading to reduced survival, impaired bacterial clearance, and disruption of immune homeostasis. These findings demonstrate that male-derived AMPs are not passive byproducts of mating, but active and indispensable contributors to female immune defense during sperm storage.

Taken together, this study demonstrates the dual functionality of SCY2 and Scyreprocin: serving as potent effectors of systemic immunity in males and as essential, transferable mediators of reproductive immunity in females. In males, these peptides enhance resistance to bacterial infection by reducing pathogen load, modulating immune factor expression, and improving survival rates. Their transfer to females during mating extends this protective role, as shown by antibody-mediated blockade, which markedly increased female susceptibility to infection, impaired survival, and disrupted immune homeostasis. These findings provide novel insights into the defensive roles of reproductive-system-specific AMPs, underscoring their significance in both systemic and reproductive immunity. Future investigations should dissect the downstream molecular pathways activated by these peptides in both sexes, potentially involving Toll, IMD, or cAMP/PKA cascades, with approaches such as proteomics and chromatin immunoprecipitation (ChIP) offering powerful tools to construct a comprehensive network of reproductive immune regulation.

CRediT authorship contribution statement

Hanxiao Li: Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ying Wang:** Validation, Software, Methodology, Investigation. **Yuqi Bai:** Writing – review & editing. **Fangyi Chen:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Ke-Jian Wang:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Funding sources

This work was supported by several grants, including the Youth Innovation Project of Fujian Province's Natural Science Foundation of China (2021 J05008), the National Natural Science Foundation of China (42376089), Fujian Ocean and Fisheries Bureau (FJHYF-L-2025-02-004, FJHY-YYKJ-2024-2-3), Fujian Ocean Synergy Alliance (FOCAL2023–0207), and Xiamen Ocean Development Bureau (22CZ002HJ08).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank laboratory engineers Huiyun Chen, Zhiyong Lin, Hui Peng, and Ming Xiong for their technical assistance. We are grateful for Ying Yang's support in this research. We also appreciate the support and services provided by the Laboratory Animal Center of Xiamen University, the Center of Major Equipment and Technology Platform of the School of Ocean and Earth Sciences, and the Core Facility of Biomedical Sciences of Xiamen University.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2025.149596.

Data availability

Data will be made available on request.

References

[1] D.M. Naim, S.A.M. Nor, S. Mahboob, Reassessment of species distribution and occurrence of mud crab (*Scylla spp., Portunidae*) in Malaysia through morphological and molecular identification, Saudi J. Biol. Sci. 27 (2) (2020) 643–652.
[2] E. Apine, P. Ramappa, R. Bhatta, L.M. Turner, L.D. Rodwell, Challenges and opportunities in achieving sustainable mud crab aquaculture in tropical coastal regions, Ocean Coast. Manag. 242 (2023).
[3] M.N. Syafaat, M.N. Azra, K. Waiho, H. Fazhan, A.B. Abol-Munafi, S.D. Ishak, M. Syahnon, A. Ghazali, H. Ma, M. Ikhwanuddin, A review of the nursery culture of mud crabs, *Genus Scylla*: current progress and future directions, Animals (Basel) 11 (7) (2021).
[4] Y. Yu, M.Q. Zhang, D.H. Wang, Z.F. Xiang, Z.L. Zhao, W.X. Cui, S.P. Ye, H. Fazhan, K. Waiho, M. Ikhwanuddin, H.Y. Ma, Whole transcriptome RNA sequencing provides novel insights into the molecular dynamics of ovarian development in mud crab, *Scylla paramamosain* after mating, Comp. Biochem. Physiol. Part D Genomics Proteomics 51 (2024).
[5] F.a.F.A.B. Ministry of Agriculture and Rural Affairs, China Fisheries Statistical Yearbook, China Agricultural Press2024, 2023.
[6] J.S. Li, W.L. Qiu, H. Hao, F.Y. Chen, K.J. Wang, Morphology of the complete embryonic and larval development of commercially important mud crab, Aquac. Res. 53 (6) (2022) 2298–2316.
[7] F. Chen, K. Wang, Characterization of the innate immunity in the mud crab *Scylla paramamosain*, Fish Shellfish Immunol. 93 (2019) 436–448.
[8] D. Hou, T. Lian, G. Guo, H. Gong, C. Wu, P. Han, S. Weng, J. He, Integration of microbiome and Koch's postulates to reveal multiple bacterial pathogens of whitish muscle syndrome in mud crab, *Scylla paramamosain*, Microbiome 11 (1) (2023) 155.
[9] Z. Xintong, X. Zaozhe, X. Sijia, T. Shaolin, J. Zhang, First report of *Ameson portunus* (*microsporidia*) infection in earthen pond-cultured mud crab, *Scylla paramamosain* (*Decapoda: Portunidae*) in China, causing mass morbidity, J. Fish Dis. 46 (6) (2023) 715–721.
[10] Y.G. Zhu, Y. Zhao, B. Li, C.L. Huang, S.Y. Zhang, S. Yu, Y.S. Chen, T. Zhang, M. R. Gillings, J.Q. Su, Continental-scale pollution of estuaries with antibiotic resistance genes, Nat. Microbiol. 2 (2017) 16270.
[11] A.A. Irshath, A.P. Rajan, S. Vimal, V.S. Prabhakaran, R. Ganesan, Bacterial pathogenesis in various fish diseases: recent advances and specific challenges in vaccine development, Vaccines (Basel) 11 (2) (2023).

[12] T.A.J. Priya, S. Kappalli, Modern biotechnological strategies for vaccine development in aquaculture-prospects and challenges, Vaccine 40 (41) (2022) 5873–5881.
[13] J.G. Hughes, X. Zhang, J.V. Parales, J.L. Ditty, R.E. Parales, *Pseudomonas putida* F1 uses energy taxis to sense hydroxycinnamic acids, Microbiology 163 (10) (2017) 1490–1501.
[14] M. Kivisaar, Narrative of a versatile and adept species *Pseudomonas putida*, J. Med. Microbiol. 69 (3) (2020) 324–338.
[15] A.N.A. Rahman, H.H. Mahboub, G.E. Elshopakey, M.I.M. Darwish, H.S.A. Gharib, M. Shaalan, E.M. Fahmy, H.M. Abdel-Ghany, S.H. Ismail, H.M. Elsheshtawy, *Pseudomonas putida* infection induces immune-antioxidant, hepato-renal, ethological, and histopathological/immunohistochemical disruptions in Oreochromis niloticus: the palliative role of titanium dioxide nanogel, BMC Vet. Res. 20 (1) (2024) 127.
[16] O.M. Alzahrani, P. Elumalai, H.S. Nada, S.A.A. Ahmed, A.W. Zaglool, S.M. Shawky, M. Alkafafy, H.H. Mahboub, *Pseudomonas putida: sensitivity to various antibiotics, genetic diversity, virulence, and role of formic acid to modulate the immune-antioxidant status of the challenged compared to Carvacrol oil*, Fishes-Basel 8 (1) (2023).
[17] I.M. Aboyadak, M. Abdel-Tawwab, N.G. Ali, Identification and florfenicol-treatment of *pseudomonas putida* infection in gilthead seabream (*Sparus aurata*) fed on tilapia-trash-feed, BMC Vet. Res. 20 (1) (2024) 156.
[18] I. Altinok, S. Kayis, E. Capkin, *Pseudomonas putida* infection in rainbow trout, Aquaculture 261 (3) (2006) 850–855.
[19] D. Rowe, S. Patel, V. Lakhmani, H. Patel, A rare presentation of *Pseudomonas putida* bacteremia secondary to skin and soft tissue infection, J. Community Hosp. Intern. Med. Perspect. 15 (1) (2025).
[20] Y. Chen, K. Dai, B. Ruan, H. Wang, G. Zhou, Y. Jiang, Mild encephalitis/encephalopathy with reversible splenial lesion (MERS) associated with respiratory syncytial virus and *Pseudomonas putida* infection: A case report, Heliyon 10 (20) (2024) e39685.
[21] Q. Wang, S. Salman, Complete genomic sequencing of a *Pseudomonas putida* isolate derived from a patient suffering from chronic diarrheal infection in Hangzhou, China, Microbiol. Resour. Announc. 13 (1) (2024) e0088523.
[22] G. Tan, Y. Xi, P. Yuan, Z. Sun, D. Yang, Risk factors and antimicrobial resistance profiles of *Pseudomonas putida* infection in Central China, 2010-2017, Medicine (Baltimore) 98 (44) (2019) e17812.
[23] H. Liu, S. Li, X. Xie, Q. Shi, *Pseudomonas putida* actively forms biofilms to protect the population under antibiotic stress, Environ. Pollut. 270 (2021) 116261.
[24] F. Fanelli, L. Caputo, L. Quintieri, Phenotypic and genomic characterization of *Pseudomonas putida* ITEM 17297 spoiler of fresh vegetables: focus on biofilm and antibiotic resistance interaction, Curr. Res. Food Sci. 4 (2021) 74–82.
[25] Y. Yang, F. Chen, K. Qiao, H. Zhang, H.Y. Chen, K.J. Wang, Two male-specific antimicrobial peptides SCY2 and Scyreprocin as crucial molecules participated in the sperm acrosome reaction of mud crab *Scylla paramamosain*, Int. J. Mol. Sci. 23 (6) (2022).
[26] J. Xia, C. Ge, H. Yao, Antimicrobial peptides: An alternative to antibiotic for mitigating the risks of antibiotic resistance in aquaculture, Environ. Res. 251 (Pt 1) (2024) 118619.
[27] Y.Q. Bai, W.B. Zhang, W.B. Zheng, X.Z. Meng, Y.Y. Duan, C. Zhang, F.Y. Chen, K. J. Wang, A 14-amino acid cationic peptide Bolespleein$_{334\text{-}347}$ from the marine fish mudskipper *Boleophthalmus pectinirostris* exhibiting potent antimicrobial activity and therapeutic potential, Biochem. Pharmacol. 226 (2024).
[28] T. Rodrigues, F.A. Guardiola, D. Almeida, A. Antunes, Aquatic invertebrate antimicrobial peptides in the fight against aquaculture pathogens, Microorganisms 13 (1) (2025).
[29] H.H. Mahboub, M. Yousefi, H.A. Abdelgawad, A.A. Abdelwarith, E.M. Younis, E. Sakr, T. Khamis, S.H. Ismail, A.N. Abdel Rahman, Expression profiling of antimicrobial peptides and immune-related genes in Nile tilapia following *Pseudomonas putida* infection and nano-titanium dioxide gel exposure, Fish Shellfish Immunol. 156 (2025) 110037.
[30] V.L. Yarbrough, S. Winkle, M.M. Herbst-Kralovetz, Antimicrobial peptides in the female reproductive tract: a critical component of the mucosal immune barrier with physiological and clinical implications, Hum. Reprod. Update 21 (3) (2015) 353–377.
[31] M. Camargo, P. Intasqui, R.P. Bertolla, Understanding the seminal plasma proteome and its role in male fertility, Basic Clin Androl 28 (2018).
[32] C. Yang, W.B. Guo, W.S. Zhang, J. Bian, J.K. Yang, Q.Z. Zhou, M.K. Chen, W. Peng, T. Qi, C.Y. Wang, C.D. Liu, Comprehensive proteomics analysis of exosomes derived from human seminal plasma, Andrology 5 (5) (2017) 1007–1015.
[33] W.B. Zhang, Z. An, Y.Q. Bai, Y. Zhou, F.Y. Chen, K.J. Wang, A novel antimicrobial peptide Scyreptin1-30 from exhibiting potential therapy of early infection in a mouse burn wound model, Biochem. Pharmacol. 218 (2023).
[34] X. Zhu, F. Chen, S. Li, H. Peng, K.J. Wang, A novel antimicrobial peptide Sparanegtin identified in *Scylla paramamosain* showing antimicrobial activity and immunoprotective role *in vitro* and *vivo*, Int. J. Mol. Sci. 23 (1) (2021).
[35] X.Z. Meng, Y.Y. Duan, Y.Q. Bai, W.B. Zhang, C. Zhang, F.Y. Chen, K.J. Wang, The antimicrobial peptide Sparamosin26-54 exhibits antiviral activity against three aquatic enveloped viruses through lipid-binding-mediated virus lysis, Aquaculture 592 (2024).
[36] K. Qiao, W.F. Xu, H.Y. Chen, H. Peng, Y.Q. Zhang, W.S. Huang, S.P. Wang, Z. An, Z. G. Shan, F.Y. Chen, K.J. Wang, A new antimicrobial peptide SCY2 identified in *Scylla Paramamosain* exerting a potential role of reproductive immunity, Fish Shellfish Immunol. 51 (2016) 251–262.
[37] Y. Yang, F. Chen, H.Y. Chen, H. Peng, H. Hao, K.J. Wang, A novel antimicrobial peptide Scyreprocin from mud crab *Scylla paramamosain* showing potent antifungal and anti-biofilm activity, Front. Microbiol. 11 (2020) 1589.
[38] H. Peng, H.P. Liu, B. Chen, H. Hao, K.J. Wang, Optimized production of scygonadin in Pichia pastoris and analysis of its antimicrobial and antiviral activities, Protein Expr. Purif. 82 (1) (2012) 37–44.
[39] A. Arocho, B.Y. Chen, M. Ladanyi, Q.L. Pan, Validation of the 2 calculation as an alternate method of data analysis for quantitative PCR of BCR-ABL P210 transcripts, Diagn. Mol. Pathol. 15 (1) (2006) 56–61.
[40] S. Long, F.Y. Chen, K.J. Wang, Characterization of two new Scygonadin homologous SCY4 and SCY5 in *Scylla paramamosain*, Aquaculture 559 (2022).
[41] F. Pellestor, B. Andréo, K. Taneja, B. Williams, PNA on human sperm: a new approach for aneuploidy estimation, Eur. J. Hum. Genet. 11 (4) (2003) 337–341.
[42] M.S. Yousef, A.I. Lopez-Lorente, M. Diaz-Jimenez, C. Consuegra, J. Dorado, B. Pereira, I. Ortiz, S. Cardenas, M. Hidalgo, Nano-depletion of acrosome-damaged donkey sperm by using lectin peanut agglutinin (PNA)-magnetic nanoparticles, Theriogenology 151 (2020) 103–111.
[43] S. Nakazawa, M. Shirae-Kurabayashi, H. Sawada, Peanut agglutinin specifically binds to a sperm region between the nucleus and mitochondria in tunicates and sea urchins, Mol. Reprod. Dev. 85 (6) (2018) 464–477.
[44] H. Kruevaisayawan, R. Vanichviriyakit, W. Weerachatyanukul, S. Iamsaard, B. Withyachumnarnkul, A. Basak, N. Tanphaichitr, P. Sobhon, Induction of the acrosome reaction in black tiger shrimp (Penaeus monodon) requires sperm trypsin-like enzyme activity, Biol. Reprod. 79 (1) (2008) 134–141.
[45] W. Wang, Bacterial diseases of crabs: a review, J. Invertebr. Pathol. 106 (1) (2011) 18–26.
[46] Z. Zhang, S.K. Li, C.P. Xie, L.Z. Zhou, C.B. Li, W.H. Liu, X.B. Wen, Innate immune response and gene expression of *Scylla paramamosain* under *Vibrio parahaemolyticus* infection, Aquac. Res. 46 (2) (2015) 462–471.
[47] C.H. Cheng, H.L. Ma, Y.Q. Deng, J. Feng, Y.K. Jie, Z.X. Guo, Effects of Vibrio parahaemolyticus infection on physiological response, histopathology and transcriptome changes in the mud crab (Scylla paramamosain), Fish Shellfish Immunol. 106 (2020) 197–204.
[48] C. de Souza Valente, A.H.L. Wan, Vibrio and major commercially important vibriosis diseases in decapod crustaceans, J. Invertebr. Pathol. 181 (2021) 107527.
[49] J. Xie, H. Mei, S. Jin, L. Bu, X. Wang, C. Wang, Q. Zhao, R. Ma, S. Zhou, Outbreak of vibriosis associated with *Vibrio parahaemolyticus* in the mud crab *Scylla paramamosain* cultured in China, Dis. Aquat. Org. 144 (2021) 187–196.
[50] J. Wang, W. Hong, F. Zhu, The role of Astakine in Scylla paramamosain against Vibrio alginolyticus and white spot syndrome virus infection, Fish Shellfish Immunol. 98 (2020) 236–244.
[51] C.L. McLay, L.S.L. Greco, A hypothesis about the origin of sperm storage in the *Eubrachyura*, the effects of seminal receptacle structure on mating strategies and the evolution of crab diversity: how did a race to be first become a race to be last? Zool. Anz. 250 (4) (2011) 378–406.
[52] P. Franchimont, S. Chari, A.M. Schellen, A. Demoulin, Relationship between gonadotrophins, spermatogenesis and seminal plasma, J. Steroid Biochem. 6 (6) (1975) 1037–1041.
[53] J. Vitku, L. Kolatorova, R. Hampl, Occurrence and reproductive roles of hormones in seminal plasma, Basic Clin. Androl. 27 (2017).
[54] J. Leyria, L.L. Fruttero, P.A. Paglione, L.E. Canavoso, How insects balance reproductive output and immune investment, Insects 16 (3) (2025).
[55] C. Samakovlis, P. Kylsten, D.A. Kimbrell, A. Engstrom, D. Hultmark, The andropin gene and its product, a male-specific antibacterial peptide in *Drosophila melanogaster*, EMBO J. 10 (1) (1991) 163–169.
[56] D. Destoumieux, M. Munoz, C. Cosseau, J. Rodriguez, P. Bulet, M. Comps, E. Bachere, Penaeidins, antimicrobial peptides with chitin-binding activity, are produced and stored in shrimp granulocytes and released after microbial challenge, J. Cell Sci. 113 (Pt 3) (2000) 461–469.
[57] L.W. Bai, K.M. Zhou, H. Li, Y.K. Qin, Q. Wang, W.W. Li, Bacteria-induced IMD-relish-AMPs pathway activation in Chinese mitten crab, Fish Shellfish Immun. 106 (2020) 866–875.
[58] M.P. Belvin, K.V. Anderson, A conserved signaling pathway: the *Drosophila* toll-dorsal pathway, Annu. Rev. Cell Dev. Bi 12 (1996) 393–416.
[59] W.W. Sun, X.X. Zhang, W.S. Wan, S.Q. Wang, X.B. Wen, H.P. Zheng, Y.L. Zhang, S. K. Li, Tumor necrosis factor receptor-associated factor 6 (TRAF6) participates in anti-lipopolysaccharide factors (ALFs) gene expression in mud crab, Dev. Comp. Immunol. 67 (2017) 361–376.
[60] B. Lemaitre, J. Hoffmann, The host defense of *Drosophila melanogaster*, Annu. Rev. Immunol. 25 (2007) 697–743.
[61] Y.L. Zhou, L.Z. Wang, W.B. Gu, C. Wang, Q.H. Zhu, Z.P. Liu, Y.Y. Chen, M.A. Shu, Identification and functional analysis of (IMD) from : the first evidence of IMD signaling pathway involved in immune defense against bacterial infection in crab species, Fish Shellfish Immunol. 81 (2018) 150–160.
[62] A. Anju, C.K. Smitha, K. Preetha, R. Boobal, P. Rosamma, Molecular characterization, recombinant expression and bioactivity profile of an antimicrobial peptide, Ss-arasin from the Indian mud crab, *Scylla serrata*, Fish Shellfish Immunol. 88 (2019) 352–358.
[63] D.R. Ardia, J.E. Gantz, B.C. Schneider, S. Strebel, Costs of immunity in insects: an induced immune response increases metabolic rate and decreases antimicrobial activity, Funct. Ecol. 26 (3) (2012) 732–739.
[64] S.Y. Han, M.Q. Wang, B.J. Wang, M. Liu, K.Y. Jiang, L. Wang, A comparative study on oxidative stress response in the hepatopancreas and midgut of the white shrimp *Litopenaeus vannamei* under gradual changes to low or high pH environment, Fish Shellfish Immunol. 76 (2018) 27–34.
[65] M. Castex, P. Lemaire, N. Wabete, L. Chim, Effect of probiotic *Pediococcus acidilactici* on antioxidant defences and oxidative stress of *Litopenaeus stylirostris* under *Vibrio nigripulchritudo* challenge, Fish Shellfish Immunol. 28 (4) (2010) 622–631.

[66] D. Tsikas, Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: analytical and biological challenges, Anal. Biochem. 524 (2017) 13–30.
[67] X.C. Zhu, M. Nie, N. Sun, Y.Z. Zhang, M.X. Sun, C.L. Li, Q. Jiang, H. Wei, Y.D. Li, Q. B. Hu, Y.Y. Zhao, X.D. Li, Comparative analysis of crab growth performance, enzyme activity, and microbiota between rice-crab coculture and pond farming systems, Front. Vet. Sci. 12 (2025).
[68] D.K. Walmer, M.A. Wrona, C.L. Hughes, K.G. Nelson, Lactoferrin expression in the mouse reproductive-tract during the natural estrous-cycle - correlation with circulating estradiol and progesterone, Endocrinology 131 (3) (1992) 1458–1466.
[69] C.T. Teng, C. Beard, W. Gladwell, Differential expression and estrogen response of *lactoferrin* gene in the female reproductive tract of mouse, rat, and hamster, Biol. Reprod. 67 (5) (2002) 1439–1449.
[70] K.X. Zhang, R. Gao, H.X. Zhang, X. Cai, C.H. Shen, C.Q. Wu, S.Y. Zhao, L. Yu, Molecular cloning and characterization of three novel lysozyme-like genes, predominantly expressed in the male reproductive system of humans, belonging to the c-type lysozyme/alpha-lactalbumin family, Biol. Reprod. 73 (5) (2005) 1064–1071.
[71] C. Samakovlis, D.A. Kimbrell, P. Kylsten, A. Engstrom, D. Hultmark, The immune response in *Drosophila*: pattern of cecropin expression and biological activity, EMBO J. 9 (9) (1990) 2969–2976.
[72] X. Ai, H. Deng, X. Li, Z. Wei, Y. Chen, T. Yin, J. Zhang, J. Huang, H. Li, X. Lin, L. Tan, D. Chen, X. Zhang, X. Zhang, C. Meignin, J.L. Imler, H. Cai, cGAS-like receptors drive a systemic STING-dependent host response in *Drosophila*, Cell Rep. 43 (12) (2024) 115081.
[73] Y. Zhao, H. Diao, Z. Ni, S. Hu, H. Yu, Y. Zhang, The epididymis-specific antimicrobial peptide beta-defensin 15 is required for sperm motility and male fertility in the rat (*Rattus norvegicus*), Cell. Mol. Life Sci. 68 (4) (2011) 697–708.
[74] N. Hirohashi, N. Kamei, H. Kubo, H. Sawada, M. Matsumoto, M. Hoshi, Egg and sperm recognition systems during fertilization, Develop. Growth Differ. 50 (2008) S221–S238.
[75] H. Breitbart, Signaling pathways in sperm capacitation and acrosome reaction, Cell. Mol. Biol. 49 (3) (2003) 321–327.
[76] H. Nakata, T. Wakayama, T. Asano, T. Nishiuchi, S. Iseki, Identification of sperm equatorial segment protein 1 in the acrosome as the primary binding target of peanut agglutinin (PNA) in the mouse testis, Histochem. Cell Biol. 147 (1) (2017) 27–38.