



# Manipulation of the shore crab *Metopograpsus thukuhar* by the parasitic barnacle *Polyascus planus* (Crustacea: Cirripedia: Rhizocephala): Effects of the parasitism on hemolymph parameters and rootlet-infiltrated tissues of the host

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## ABSTRACT

The present study assessed the impact of parasitism of *Polyascus planus* on the hemolymph parameters and rootlet-infiltrated tissues of the shore crab *Metopograpsus thukuhar*. First, hemolymph parameters were compared between hosts harboring internal infection (Hit) and non-parasitized host crabs (Hnp). The percentage of Hit with culturable bacteria in the hemolymph was significantly higher than that of Hnp when samples were cultured on Luria-Bertani (LB) agar, but the difference between Hit and Hnp was statistically insignificant when samples were cultured on Vibrio-selective X-VP agar. In addition, hemolymph bacterial load was quantified, revealing that bacterial load of Hit was significantly higher than that of Hnp when samples were cultured on LB agar, but was not on X-VP agar. Moreover, total hemocyte count was significantly lower in Hit than in Hnp. Histological examinations suggested that no cell-mediated immune responses, specifically melanization or encapsulation, were directed towards infiltrating rootlets in the examined tissues. However, large numbers of dead hemocytes exhibiting cytological features of oncotic necrosis accumulated in the rootlet-infiltrated hepatopancreas and hematopoietic tissue, where they were phagocytized by the fixed phagocytes. The mitotic index of hematopoietic stem cells did not differ significantly between Hnp and Hit. In rootlet-infiltrated ovaries, functional follicles were either disrupted (detachment of the follicular envelop) or failed to form. The combined results indicate that internally parasitized *M. thukuhar* was immunologically suppressed, with oncotic necrosis of hemocytes being a contributing cause of the weakened immunity. Moreover, the significance of the tissue-specific effects on host tissues is discussed in the context of host manipulation by the parasitic barnacle.

## 1. Introduction

Parasitic manipulation is a phenomenon in which parasites exert control over their hosts by altering or influencing host physiology, behavior, or morphology to increase parasite's fitness (Thomas et al., 2005; Poulin, 2011). Intriguing examples observed across taxa of hosts and parasites provide models for the study of mechanism of host control by the parasite, offering insights into adaptation to parasitism (Thomas et al., 2010; Biron and Loxdale, 2013; Lafferty and Shaw, 2013; Poulin

and Randhawa, 2015).

The order Rhizocephala comprises a group of highly evolved and specialized parasitic barnacles, which mainly parasitize decapod crustaceans (Høeg et al., 2020). After colonization of the host by the infective larva, the rhizocephalan parasite develops a ramifying root system (interna) that permeates the hemocoel and infiltrates multiple tissues of the hosts during its internal growth phase. The internal phase of growth is followed by the emergence of a reproductive sac (externa) of the parasite, which is connected to the interna by a stalk. The root system of

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rhizocephalan parasites is not only a trophic organ for absorbing and processing nutrients from the host, but also vital for establishing and maintaining the control rhizocephalans exert over the host (see for reviews Høeg, 1995; Høeg and Lützen, 1995; Bresciani and Høeg, 2001).

Rhizocephalans are often described as body-snatching parasites (Lafferty and Kuris, 2009; Tobias et al., 2021). Although the extent of host control varies considerably both among different parasite species and among different host species infected with the same parasite, there are species that exert profound and extensive control over their hosts (Høeg, 1995). Thus, the interaction between rhizocephalans and their hosts represents an excellent model for the study of parasitic manipulation. Fascinating observations regarding host control by rhizocephalans have long been documented, i.e., those known as parasitic anecidysis, feminization, and sterilization (see for reviews Reinhard, 1956; Hartnoll, 1967; O'Brien and Van Wyk, 1985; Høeg, 1995; Høeg and Lützen, 1995, and for more recent studies, Isaeva et al., 2005; Kristensen et al., 2012; Waiho et al., 2017; Mouritsen et al., 2018; Fazhan et al., 2020; Barón et al., 2012; Chen et al., 2022; Feng et al., 2023; Toyota et al., 2023; Yoshioka et al., 2023). Moreover, other aspects of the host control have also been described, including those influencing metabolism (Shirley et al., 1986; Powell and Rowley, 2008; Hsiao et al., 2016; Yoshioka et al., 2023) and immunity (Bresciani and Høeg, 2001; Goddard et al., 2005; Bortolini and Alvarez, 2008; Powell and Rowley, 2008; Rowley et al., 2020) of the parasitized hosts. Recent studies of invasive rootlets that penetrate the nervous ganglia of the host are considered important sites of host-parasite interaction where the parasites may alter the activity of the host's nervous system via humoral factors (Miroliubov et al., 2020; Lianguzova et al., 2021, 2023).

While earlier studies have suggested that the host control induced by rhizocephalans is achieved through parasite-induced alterations in various host tissues (e.g., endocrine glands, central nervous system), the detailed mechanisms underlying these control processes remain largely uncharacterized (see Høeg, 1995). Recent transcriptome and proteome studies of host tissues have revealed in the parasitized hosts differential expression levels of effectors known to be involved in immune defense, reproduction, metabolism, and molt regulation, thereby highlighting the potential molecular targets whose expression levels may be altered by rhizocephalan parasitism to achieve successful host control (Waiho et al., 2020; Tobias et al., 2021; Yang et al., 2022; Feng et al., 2023; Zatylny-Gaudin et al., 2023; Zhao et al., 2023).

Based on available evidence, the crustacean immune system, similar to other invertebrates, lacks vertebrate-type adaptive immunity. However, while traditionally considered strictly innate, crustacean immunity exhibits characteristics typically associated with adaptive immunity, such as immune memory (Ng et al., 2014; Armitage et al., 2015; Milutinović and Kurtz, 2016). Extensive research over the past decades has firmly established that the crustacean immune system is versatile and efficient, equipped with a wide range of highly interrelated cell-mediated and humoral immune arsenal, such as phagocytosis, nodulation and encapsulation, melanization, blood coagulation, synthesis and release of cytotoxic molecules, for wound healing and defense against foreign invaders (Johansson et al., 2000; Jiravanichpaisal et al., 2006; Vazquez et al., 2009). Circulating hemocytes are vital effector cells carrying out and mediating immune reactions (Jiravanichpaisal et al., 2006; Söderhäll, 2016). These immune effector cells are produced continuously by a hemocyte-forming tissue, the hematopoietic tissue, to sustain hemocyte homeostasis and replenish the hemocytes consumed during immune reactions (Söderhäll, 2016; Söderhäll and Söderhäll, 2022).

With respect to the effects of rhizocephalan parasitism on host immunity, previous studies of the European green crabs *Carcinus maenas* parasitized by *Sacculina carcini* showed that the number of circulating hemocytes was significantly lower in parasitized hosts (Powell and Rowley, 2008), although a later study found that the number was not significantly different from that of non-parasitized hosts (Rowley et al.,

2020). The discrepancy may be because the parasitized hosts analyzed in the two studies were of different status of parasitization. One of the studies further reported that the number of bacteria in the hemolymph of the parasitized hosts was significantly higher, compared to that of non-parasitized hosts, suggesting a weakened immunity of the parasitized hosts (Rowley et al., 2020). Importantly, observations derived from studies of various species of rhizocephalans, including both kentrogonids and akentrogonids, showed that host tissue-infiltrating rootlets of the parasite did not elicit significant immunological responses from the host (Payen et al., 1979, 1981; Goddard et al., 2005; Bortolini and Alvarez, 2008; Powell and Rowley, 2008; Rowley et al., 2020; also see Bresciani and Høeg, 2001 for review).

Our previous studies showed that parasitism of *Polyascus planus* (Boschma, 1933) altered the metabolic profile of the shore crab *Metopograpsus thukuhar* (Owen, 1839) (Hsiao et al., 2016), feminized its males (as evidenced by the induction of vitellogenin synthesis), and exerted a host-sex specific effect on host's gonadal (ovarian) development (Chen et al., 2022). These studies also suggested that the sex-specific effects on the gonadal development of the parasitized hosts depend on rootlet infiltration. Hence, the ovary, where heavy rootlet infiltration occurred, was developmentally arrested, whereas the testis, in which no rootlet was present, developed normally (Chen et al., 2022).

The present study aimed to assess the effects of parasitism of *P. planus* on hemolymph parameters and rootlet-infiltrated tissues of *M. thukuhar* during the internal phase of the infection. The specific objectives of the present study were to quantify total hemocyte count and hemolymph bacterial load, and examine histopathological responses of host tissues (the hepatopancreas, ovary, and hematopoietic tissue) to rootlet infiltration.

## 2. Materials and methods

### 2.1. Collection of animals and determination of sex and parasitization status

Host animals (*Metopograpsus thukuhar* Owen, 1839) were collected and reared as previously described (Hsiao et al., 2016). Briefly, animals used in the present study were sampled during five trips spanning from March to June 2022. They were manually collected from intertidal zones of the Da'an estuary (24.36° N, 120.58° E), Taichung, Taiwan, and transported to the National Changhua University of Education, where they were reared in seawater tanks with a salinity of 25‰ at 25 ± 2 °C and constant aeration. Sex of the animals was determined by the sexually dimorphic pattern of the pleopods, 4 pairs for females and 2 for males (Davie et al., 2015.), which is not modified in *M. thukuhar* by parasitism (unpublished observations). Parasitization status of host crabs was determined based on whether externa was present on the host's abdomen, external characteristics of the externa as described in detail by Chen et al. (2022, 2025), and whether the parasite *Polyascus planus* (Boschma, 1933) 18S rRNA gene was detected in the thoracic ganglia of the hosts by a polymerase chain reaction (PCR) (Hsiao et al., 2016). The external characteristics of the externa refer to the color change of the externa from creamy, orange, yellow to brown during its development and maturation. For the 18S rRNA PCR, a pair of primers, Sac18srRNA-F1 (5'-CGG ATT GCT GGT TGA CAT CGT T-3') and Sac18s rRNA-R1 (5'-GCG GTT AAA AAGCTC GTA GTT GG-3'), was designed based on an 18S rRNA gene sequence (GenBank accession number AY265368) of *P. planus* (formerly *P. plan*; Glenner et al., 2003). PCR control reactions containing double distilled water (negative controls) or genomic DNA extracted from externa (positive controls), instead of genomic DNA extracted from tested samples (the thoracic ganglia), were concurrently assayed with sample reactions in each run of PCR assay. The expected size of the amplicon is 486 bp. Auto-sequencing of amplified PCR products revealed that the sequence of the amplified products is identical to the stretch of sequence of the *P. planus* 18S rRNA gene bracketed by the primers (Hsiao et al., 2016).

In the present study, only adult animals (with a carapace width larger than 2 cm) that were either non-parasitized (Hnp) or internally parasitized animals (Hit) were used. Hnp and Hit animals are those not carrying any externa but distinguished by the result of the parasite 18S rRNA PCR, with the former giving a negative result and the latter a positive result.

## 2.2. Hemolymph and tissues sampling for bacterial loads and histology

A set of animals, including 39 crabs (21 females and 18 males) for the Hnp group and 17 crabs (10 females and 7 males) for the Hit group, was sacrificed for sampling of hemolymph and tissue. Before experimental procedures and sampling, crabs were anesthetized with ice-cold seawater (Tsai et al., 2008) until they became immobilized (usually within 2 min) and the surface of the animals disinfected using 75% ethanol. Hemolymph and tissues harvested from animals of the desired parasitization status (Hnp or Hit) were used for various types of analysis. Briefly, hemolymph was withdrawn from anesthetized animals through the arthroal membrane at the base of the chelae using syringes coupled to a 27-gauge needle. Hemolymph samples were used for the experiments of hemolymph bacterial loads (see 2.3.). Tissues, including the ovary and hepatopancreas, were promptly dissected out under stereoscopes (E24, Leica) using fine iris scissors and forceps (Fine Science Tools Inc.) and fixed in 10% neutral formalin at room temperature for subsequent histology analysis (see 2.4.). The thoracic ganglia were also harvested for genomic DNA extraction to be used for the parasite 18S rRNA PCR assay mentioned above.

## 2.3. Bacterial loads in hemolymph

Hemolymph bacterial load was determined as an indicator to evaluate the overall immune-competence of the animals (e. g., Fagutao et al., 2009; Wang et al., 2014). Hemolymph samples (see 2.2.) were immediately diluted 1:1 (V:V) with sterilized 3% NaCl. Two media were used for determination of hemolymph bacterial loads, i.e., Luria-Bertani (LB) agar, which is commonly used to culture bacteria of crustacean hemolymph for the general purpose of bacterial loads or microbial composition (see Wang and Wang, 2015), and X-VP agar, which selectively grows *Vibrio* spp., including several pathogenic species (Teramura et al., 2011). For culturing on the LB agar, a 100- $\mu$ l aliquot of the diluted samples was spread on the agar plates and the plates incubated at 25 °C for 48 h; for culturing on the X-VP agar, a 50- $\mu$ l aliquot of the diluted samples was spread on the agar plates and the plates incubated at 37 °C for 24 h. After incubation, the number of bacterial colonies on the plates was counted and corrected for dilution factor. The colony-forming unit (CFU) is reported as CFU/ml hemolymph. The percentage of the host animals having culturable bacteria in the hemolymph was also calculated.

## 2.4. Tissue histology

Tissue fixed in 10% neutral formalin (see Section 2.2) were processed, sectioned, and hematoxylin/eosin stained for histological observations as described previously (Chen et al., 2022). Histological observations and measurements were done and images taken under light microscopes (DM 500, Leica) with a software platform Application Suite X (Leica). Observations of the hematoxylin/eosin-stained sections reported in the present study were based on examination of serial tissue sections of the hepatopancreas (8 Hnp, including 5 females and 3 males; 8 Hit, including 6 females and 2 males) and ovary (5 Hnp and 6 Hit).

## 2.5. Total hemocyte count

A second set of animals, including 7 crabs (4 females and 3 males) for the Hnp group and 6 crabs (3 females and 3 males) for the Hit group, was used. Animals were treated and their hemolymph samples withdrawn as

described above (see Section 2.2). Hemolymph samples were diluted 1:3 (V:V) with an anticoagulant solution (0.01 M EDTA in Pantin's crab saline, pH 7.6, Chen et al., 2022). A 10- $\mu$ l aliquot of the diluted samples was observed under microscopes (DM 500, Leica) with the aid of an improved Neubauer hemocytometer (Marienfeld, Germany) for hemocyte counting. For each individual sample, three aliquots were separately analyzed and the mean value calculated. Data were corrected for dilution factor and reported as the total hemocyte count (total number of cells/ml hemolymph).

## 2.6. Mitotic index in hematopoietic tissue

A third set of animals, including 10 crabs (6 females and 4 males) for the Hnp group and 10 crabs for the Hit group, was used for the determination of mitotic index. Before experimental procedures and sampling, animals were anesthetized and the surface of the animals disinfected as described above (see Section 2.2). Animals received an injection of 50 mM 5-bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich, B5002) at the dosage of 10  $\mu$ l/g body weight, according to Lin et al. (2008), through the base of the chelae. After injection, animals were returned back to individual seawater tanks. Three hours after injection, hematopoietic tissues were carefully dissected out from animals anesthetized and disinfected as described. The hematopoietic tissue is a thin sheet of tissue covering the dorsal side of the stomach and surrounded by connective tissue (Lin and Söderhäll, 2011). Hematopoietic tissues were processed for tissue sectioning as previously described (Chen et al., 2022). Specific BrdU binding was probed using a rabbit anti-BrdU IgG (500 $\times$  dilution; GTX128091, GeneTex) followed by amplification of the binding signal using the Polink-2 Plus HRP Rabbit DAB Detection Kit (D39, OriGene) according to the manufacture's protocol. Tissue sections were counterstained with hematoxylin (HT). Sections serving as a negative control were treated as above with the exception that a normal rabbit IgG was used in place of the rabbit anti-BrdU IgG. Histological observations were done and images taken under light microscopy. BrdU-negative (HT-stained) hematopoietic stem cells (HSCs) and BrdU-positive HSCs were identified and separately counted on the microscopic images with an image processing software (Image J, v.1.53 t). For tissue sections derived from each individual animal, a total of at least 500 randomly selected HSCs were included for analysis. Proliferation activity of HSCs, i.e., the mitotic index, was calculated as the percentage of the BrdU-positive cells in the total (BrdU-positive and BrdU-negative) HSCs counted.

## 2.7. Statistical analysis

Data analysis was performed using SigmaPlot (10.0, SYSTAT Software). Effects of parasitization on the percentage of the hosts with bacteria-contaminated hemolymph and on the mitotic index of hematopoietic stem cells were analyzed using z-test, effects on colony-forming unit using Mann-Whitney U test, and effects on total hemocyte count using Student's t-test.

# 3. Results

## 3.1. Hemolymph parameters

The percentage of animals having bacteria-contaminated hemolymph was significantly higher in internally parasitized hosts (Hit) (64.7%) than in non-parasitized hosts (Hnp) (20.5%), when hemolymph samples were cultured on the Luria-Bertani (LB) agar (Table 1). Moreover, bacterial loads, as quantified by the colony-forming unit (CFU), in the hemolymph of Hnp and Hit were compared. The five-number summary for the CFU datasets of Hnp and Hit is presented in Table 2. Median CFU for Hnp and Hit was 0 and 20, respectively (Table 2). The interquartile range (i.e., the difference between the third and first quartiles of the data set) for Hnp and Hit were 0 and 2270 CFU, respectively, and

**Table 1**

Effect of parasitization by *Polyascus planus* on the percentage of the host animals *Metopograpsus thukuhar* having bacteria-contaminated hemolymph.

| Parasitization status | Sample size | Culture medium | Percentage of animals having bacteria in hemolymph (%) |
|-----------------------|-------------|----------------|--------------------------------------------------------|
| Hnp <sup>a</sup>      | 39          | LB             | 20.5                                                   |
| Hit                   | 17          | LB             | 64.7                                                   |
| Hnp <sup>b</sup>      | 21          | X-VP           | 0                                                      |
| Hit                   | 15          | X-VP           | 6.7                                                    |

<sup>a,b</sup> The percentage of Hit was significantly higher than that of Hnp ( $z = 2.752$ ,  $P = 0.006$ ) when samples were cultured on LB agar, but not ( $z = 0.177$ ;  $P = 0.860$ ) on X-VP agar. Hit: internally parasitized host, Hnp: non-parasitized host, LB: Luria-Bertani.

there were two outlying data (860, 6220 CFU) in the Hnp and one (31,000 CFU) in the Hit. Data distributions in the two groups differed significantly (Table 2). On the other hand, when hemolymph samples were cultured on the X-VP agar, a medium that selectively grows *Vibrio* spp. (Teramura et al., 2011), the percentage of animals having bacteria-contaminated hemolymph was not significantly different between the Hnp (0%) and Hit (6.7%) (Table 1). The five-number summary for the CFU dataset of the Hnp and Hit cultured on the X-VP agar is also presented in Table 2. Median CFU, as well as the interquartile range, was 0 for both the Hnp and Hit (Table 2). There was one outlying data (2 CFU) in the Hit. Data distribution in the two groups was not significantly different (Table 2).

In addition, analysis of hemolymph samples showed that total hemocyte count (THC) of the Hit ( $1.8 \times 10^6 \pm 0.9 \times 10^6$  cells/ml) was significantly lower than that of the Hnp ( $3.4 \times 10^6 \pm 1.1 \times 10^6$  cells/ml), representing a more than 50% reduction of hemocyte count in the Hit (Fig. 1).

### 3.2. Tissues responses to rootlet infiltration

First, the ovary and hepatopancreas, which are known to be infiltrated by rootlets (Chen et al., 2022), were examined histologically. In the hepatopancreas of Hnp, circulating hemocytes and fixed phagocytes were present in the interstitium between the hepatopancreas tubules; fixed phagocytes, typically forming rosette-like groups, were found attached to arteriolar walls (Fig. 2A, B). In the hepatopancreas of Hit, a notable observation was the presence of large numbers of necrotic hemocytes (Fig. 2C). These necrotic cells presented various cytological features typical of oncotic necrosis — cell swelling, a uniformly eosin-stained cytoplasm, karyolysis, cytoplasmic vacuoles, etc. (Fig. 2D–F). Moreover, fixed phagocytes were found closely attached to necrotic hemocytes (Fig. 2E and F). Fixed phagocytes also formed pseudopod-like cellular projections that were engulfing necrotic hemocytes (Fig. 3A and B). Cell debris of necrotic hemocytes were also observed inside the fixed phagocytes (Fig. 3C and D). No apparent reaction taken by fixed phagocytes towards nearby rootlets could be observed, despite these host's immune cells were often found in close contact with the infiltrating rootlets (Fig. 3E and F).

**Table 2**

Effect of parasitization by *Polyascus planus* on the colony-forming unit in the hemolymph of the host animals *Metopograpsus thukuhar*.

| Parasitization status | Sample size | Culture medium | Colony-forming unit (CFU/ml) <sup>a</sup> |              |        |              |        |
|-----------------------|-------------|----------------|-------------------------------------------|--------------|--------|--------------|--------|
|                       |             |                | Min.                                      | 1st quartile | Median | 3rd quartile | Max.   |
| Hnp <sup>b</sup>      | 39          | LB             | 0                                         | 0            | 0      | 0            | 780    |
| Hit                   | 17          | LB             | 0                                         | 0            | 20     | 2270         | 21,020 |
| Hnp <sup>c</sup>      | 21          | X-VP           | 0                                         | 0            | 0      | 0            | 0      |
| Hit                   | 15          | X-VP           | 0                                         | 0            | 0      | 0            | 0      |

<sup>a</sup> Five-number summary for the CFU dataset includes the minimum (Min.), first quartile (1st quartile), median (Median), third quartile (3rd quartile), and maximum (Max).

<sup>b,c</sup> Data distribution of CFU was significantly different between the Hnp and Hit groups when samples were cultured on LB agar (Mann–Whitney  $U = 176.5$ ,  $P < 0.001$ ), but not on X-VP agar (Mann–Whitney  $U = 147.0$ ,  $P = 0.260$ ). Hit: internally parasitized host, Hnp: non-parasitized host, LB: Luria-Bertani.

In the mature ovary of Hnp, mature oocytes were tightly enveloped by a thin layer of follicle cells—the follicular envelope; pre-vitellogenic oocytes with a hematoxylin-stained cytoplasm were also present, many of which were in the process of being enveloped by follicle cells (Fig. 4A and B). In the mature ovary of Hit, in which infiltration by rootlets was in early phase, rootlets were found penetrating the periphery of the tissue (Fig. 4C). In these ovaries, rootlets were found adjacent to mature oocytes, with the thin follicular envelopes beginning to detach from the oocytes (Fig. 4D). Rootlets could also be seen intimately attached to the surface of these de-folliculated oocytes, the yolk bodies of which were clearly being resorbed (Fig. 4E and F). In the ovary of Hit, in which infiltration was in later phase and much heavier, the ovary contained only oocytes developmentally arrested at the pre-vitellogenic stage (Fig. 4G), which were kept at a distance from a ring of follicle cells (4H). Hemocytes were rarely found within the ovary of Hnp and no significant recruitment of hemocytes into the ovary of Hit was observed.

As total hemocyte count was significantly decreased in the parasitized hosts, effects of rootlet infiltration on the hematopoietic tissue were also examined. The hematopoietic tissue, a thin sheet of tissue covering the dorsal side of the stomach, are composed of lobules, inside which hematopoietic stem cells (HSCs) reside; young hemocytes are released from the ruptured end of lobules into the interstitial space among the connective tissues and nearby hemal lacunae (Fig. 5A). The hematopoietic tissue of Hit was infiltrated by rootlets; some of the lobules in the infiltrated tissue contained necrotic HSCs, which had lost the large, characteristically basophilic nucleus but instead stained intensely with eosin (Fig. 5B). In addition, many newly released hemocytes also became necrotic; these necrotic hemocytes were usually found in the interstitium between the spongy connective-tissue cells (Fig. 5C). Cells, morphologically similar to the fixed phagocytes observed in the

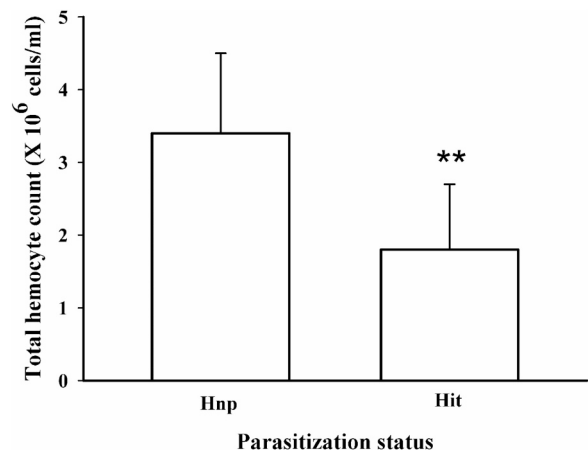


**Fig. 1.** Effect of parasitization by *Polyascus planus* on the total hemocyte count in the hemolymph of the host animals *Metopograpsus thukuhar*.  $n = 7$  and  $6$  for Hnp and Hit groups, respectively. \*\*: significant difference between Hnp and Hit,  $P = 0.016$ , Student's  $t$ -test.

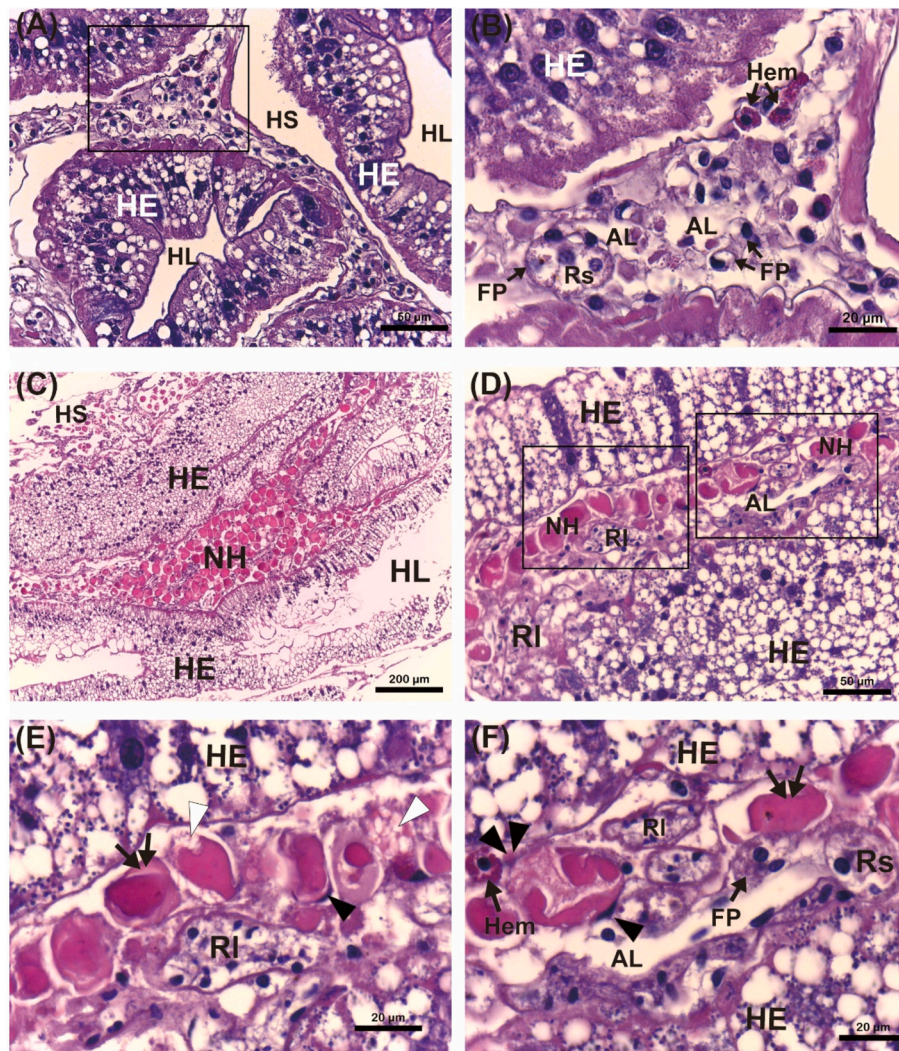


**Fig. 2.** Pathohistological responses in the hepatopancreas of the host animals *Metopograpsus thukuhar* parasitized by *Polyascus planus*. Representative images of the hepatopancreas of Hnp (A, B), showing in (A) the interstitium (enclosed area) between hepatopancreas tubules and (B: magnified view of the enclosed area in A), rosettes of fixed phagocytes attached to the outer surface of arteriolar wall and circulating hemocytes within the lumen of arterioles, and those of Hit (C–F), showing in (C) large number of necrotic hemocytes and (D) that necrotic hemocytes were swollen, having a homogeneously eosin-stained cytoplasm without a clearly delineated nucleus. Note in (E, F: magnified view of the enclosed areas in D) that necrotic hemocytes with nuclear dissolution (karyolysis) (double black arrows), lysed hemocyte (double black arrowheads), or cytoplasmic vacuoles (white arrowhead) were also present, and that individual fixed phagocytes (black arrowhead) were found attached to necrotic hemocytes. AL: lumen of arteriole; FP: fixed phagocyte, HE: epithelium of hepatopancreas tubule, Hem: hemocyte, HL: lumen of hepatopancreas tubule, HS: hemal space; NH: necrotic hemocyte, RI: rootlet, Rs: rosette.

hepatopancreas (see Fig. 2E and F), were seen in close contact with necrotic hemocytes (Fig. 5D and E).

### 3.3. Effects on hematopoiesis

Cellular proliferation activity of the hematopoietic tissue was evaluated based on the levels of in vivo incorporation of BrdU into newly synthesized DNAs. Cells that actively underwent mitosis (BrdU-positive cells) were restricted to the HSCs inside.

the lobule (Fig. 6). The mitotic index (% of HSCs that are BrdU-positive) in the hematopoietic tissue of the Hnp group was  $39.4 \pm 6.4\%$  and that of the Hit group  $23.2 \pm 3.7\%$ ; the difference between the two groups was not statistically significant (Table 3).

## 4. Discussion

In this study, we show that *Metopograpsus thukuhar* parasitized by the rhizocephalan barnacle *Polyascus planus* displays pronounced immunosuppression, with hemocyte necrosis contributing at least in part to this

suppressive effect. Notably, although host defenses against the parasite's tissue-infiltrating rootlets are suppressed, fixed phagocytes become activated and engaged in the clearance of necrotic hemocytes. Furthermore, pathohistological responses of host tissues to rootlet infiltration indicate that tightly regulated, specific actions of putative parasite-derived regulatory factors—likely released locally by infiltrating rootlets—are vital for achieving the parasite's control over its hosts.

### 4.1. Suppression of host immunity

Evidence of immunosuppression in parasitized *M. thukuhar* in general agrees with previous studies examining the effects of rhizocephalan parasitism on host immunity. Thus, despite heavy rootlet infiltration that occurred in all host tissues examined in the present study, cell-mediated immune responses—such as melanization, encapsulation, or nodule formation (Johansson et al., 2000; Jiravanichpaisal et al., 2006)—directed towards infiltrating rootlets were virtually absent, consistent with studies of several rhizocephalan species (Payen et al.,

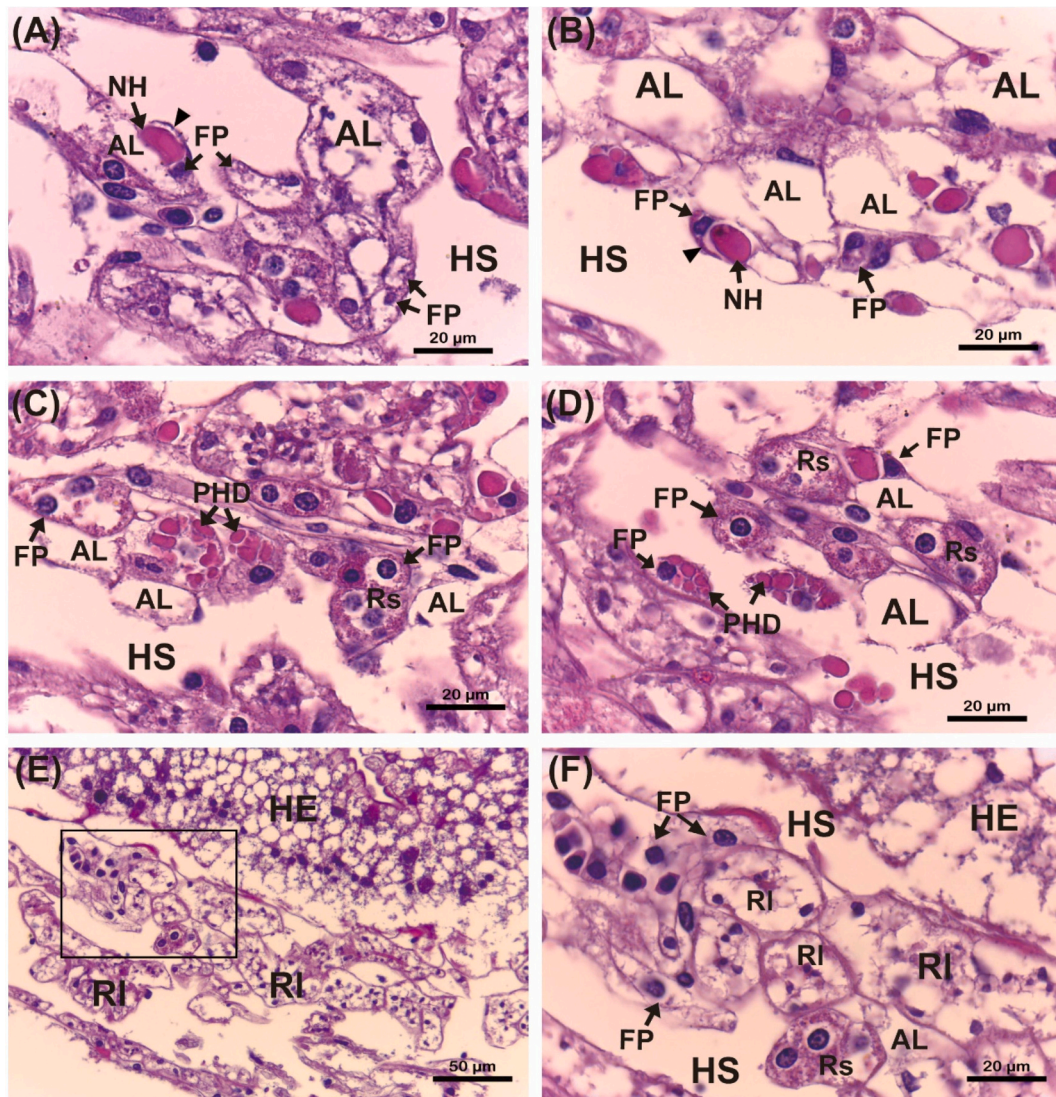


**Fig. 3.** Histopathological responses of the fixed phagocytes in the hepatopancreas of the host animals *Metopograpsus thukuhar* parasitized by *Polydora planus*. Representative images of the hepatopancreas of Hit showing in (A, B) fixed phagocytes enclosing necrotic hemocytes with cellular projections (arrowhead), (C, D) phagocytized cell debris of necrotic hemocytes inside fixed phagocytes, and (E and F, magnified view of the enclosed area in E) the lack of response of fixed phagocytes to adjacent rootlets. AL: lumen of arteriole; FP: fixed phagocyte, HE: epithelium of hepatopancreas tubule, HS: hemal space, NH: necrotic hemocyte, PHD: phagocytized hemocyte debris, Rl: rootlet, Rs: rosette.

1979, 1981; Bresciani and Høeg, 2001; Bortolini and Alvarez, 2008; Powell and Rowley, 2008). Although, in a large-scale study of the European shore crab *Carcinus maenas* parasitized by *Sacculina carcini*, a small proportion (7.5%) of the parasitized hosts showed encapsulation and melanization reactions towards tissue-infiltrating rootlets, it was concluded that most rootlets circumvented the attention of the host's immune system and that the host responses did not effectively hinder the progression of the infection (Rowley et al., 2020). Consistent with the observations that immune responses were suppressed, parasitized hosts exhibited significantly elevated bacterial loads in the hemolymph, lower survivorship, or both, compared to non-parasitized hosts (Alvarez et al., 1995; Goddard et al., 2005; Rowley et al., 2020; the present study).

Interestingly, in another study of *S. carcini* parasitism, melanized bodies (melanized developing rootlets) were frequently found in tissues of experimentally infected, non-host California crabs, but not in those of similarly experimentally infected, natural hosts (*C. maenas*) (Goddard et al., 2005). In non-hosts, the melanization response often failed to stop rootlet growth. Resulting abnormal interna development increased mortality of non-host species—parasitized *C. magister* (a non-host) died significantly earlier than parasitized *C. maenas* (Goddard et al., 2005).

The authors suggested that the fatal outcome caused by the immune responses elicited by *S. carcini* reflects the absence of an evolutionary history between the parasite and non-host California crabs (Goddard et al., 2005; Kuris et al., 2007), implying that suppression of immunity in natural hosts may be an adaptive host manipulation by rhizocephalans evolved through long-term host–parasite interaction.

A recent study by Tobias et al. (2021) examined the transcriptomic response of the white-fingered mud crab (*Rhithropanopeus harrisi*), a natural host of the parasite *L. panopaei*, to experimental infection of the parasite. The authors compared host populations from three regions with differing history of parasite exposure: a native range (long-term sympatry), an introduced range (~60 years of association), and an unexposed range (naïve population). Across all populations, infection triggered a core transcriptional response characterized by the widespread downregulation of immune-related transcripts. Moreover, the introduced-range population unexpectedly exhibited the most distinct transcriptomic profile among the three examined populations, showing a significant upregulation of numerous immune transcripts. While other explanations also exist, it was suggested by the authors that the elevated response observed in the population from the introduced range mostly

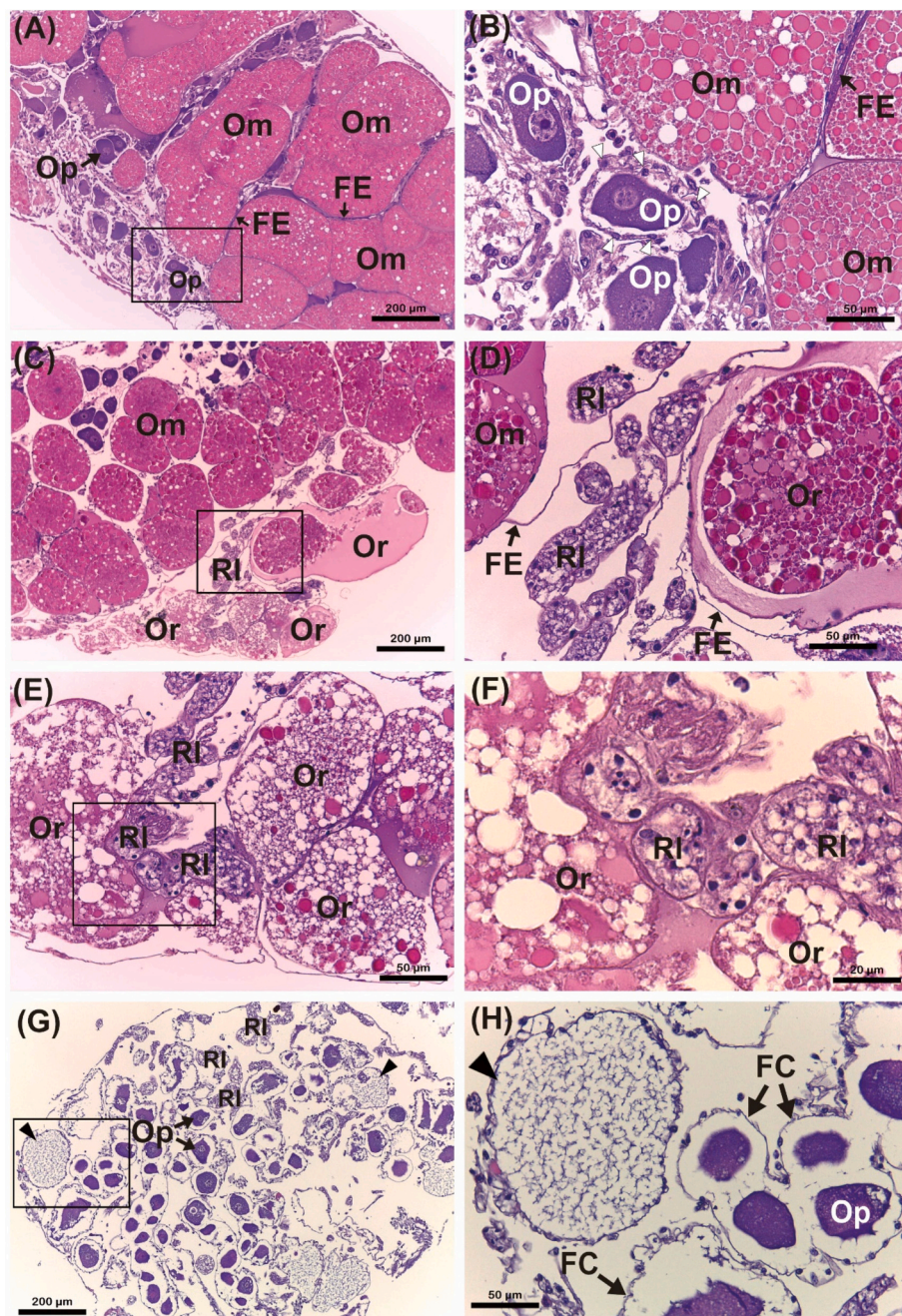


**Fig. 4.** Histopathological responses of the ovary of the host animals *Metopograpsus thukuhar* parasitized by *Polyascus planus*. Representative images of the ovary of Hnp (A, B) showing in (A) mature oocytes and pre-vitellogenic oocytes, (B, magnified view of the enclosed area in A) follicle cells (white arrowhead) enveloping pre-vitellogenic oocytes, and those of Hit (C–F) showing in (C) penetration of rootlets into ovary, (D, magnified view of the enclosed area in C) rootlets adjacent to a resorbed oocyte, from which follicular envelope was separating, and (E and F, magnified view of the enclosed area in E) rootlets attached to de-folliculated oocytes, the yolk bodies of which were being resorbed. Note in (G) where rootlet infiltration was very heavy, only developmentally arrested pre-vitellogenic oocytes and completely resorbed oocytes (black arrowhead) were present and in (H, magnified view of the enclosed area in G) developmentally arrested pre-vitellogenic oocytes were surrounded, at a distance, by a ring of follicle cells. FC: follicle cell, FE: follicular envelope; Om: mature oocyte, Op: pre-vitellogenic oocyte, Or: resorbed oocyte, Rl: rootlet.

likely represents an immune response potentiated by previous exposure (Tobias et al., 2021). Thus, their findings reinforce the abovementioned suggestion that rhizocephalan parasites induce host immunosuppression and highlight a unique scenario in which the host's immune system is most highly elicited in a population by repeated exposure to a parasite, possibly driven by an immune priming mechanism previously unreported in rhizocephalan host-parasite system.

#### 4.2. Mechanism underlying immunosuppression: hemocyte necrosis

Although substantial evidence indicates that hosts parasitized by rhizocephalans are immunosuppressed, the mechanisms underlying parasite-induced immune suppression remain largely uncharacterized. In the present study, the total hemocyte count in parasitized *M. thukuhar* was significantly lower than that in non-parasitized crabs, consistent with observations in *C. maenas* parasitized by *S. carcini* (Powell and Rowley, 2008). Importantly, our results revealed extensive hemocyte

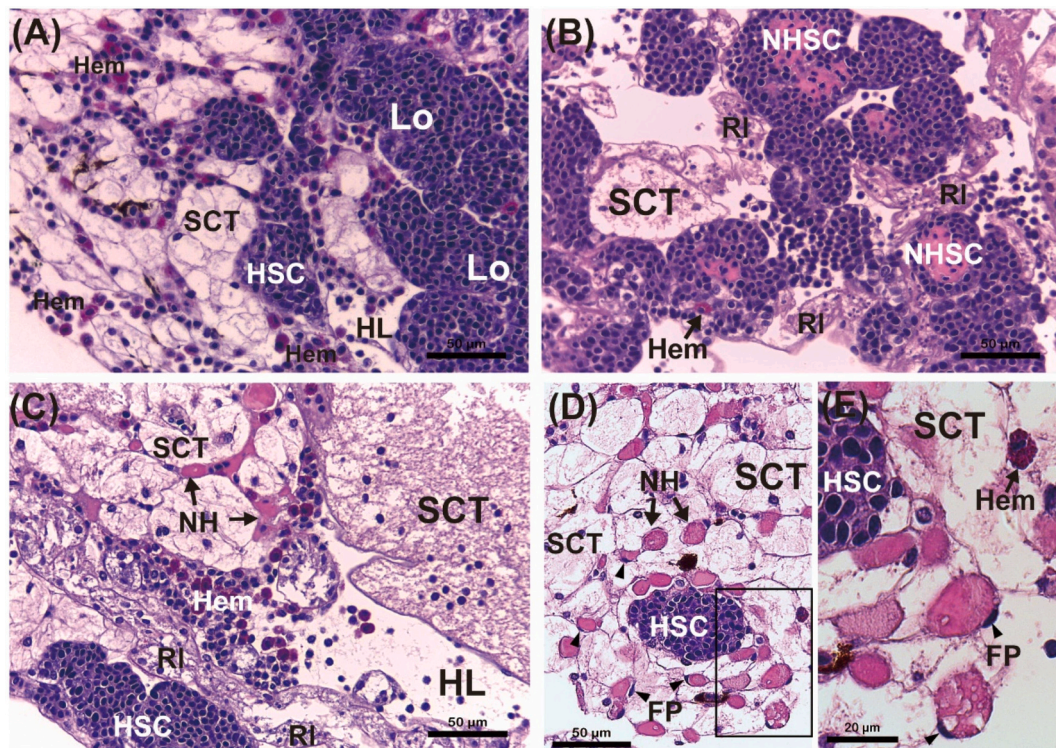


**Fig. 5.** Histopathological responses of the hematopoietic tissue of the host animals *Metopograpsus thukuhar* parasitized by *Polyascus planus*. Representative images of the hematopoietic tissue of Hnp (A) and Hit (B-E) were presented. Note in (A) hematopoietic stem cells residing within lobules and hemocytes released into the interstitial space among spongy connective tissue and hemal lacunae, (B) necrotic hematopoietic stem cells, (C, D) necrotic hemocytes between spongy connective-tissue cells, and (D and E, magnified view of the enclosed area in D) fixed phagocytes (black arrowhead) closely attached to necrotic hemocytes. FP: fixed phagocyte, Lo: lobule, Hem: hemocyte, HSC: hematopoietic stem cell, HL: hemal lacuna, NHSC: necrotic hematopoietic stem cell; NH: necrotic hemocyte, RI: rootlet, SCT: spongy connective tissue.

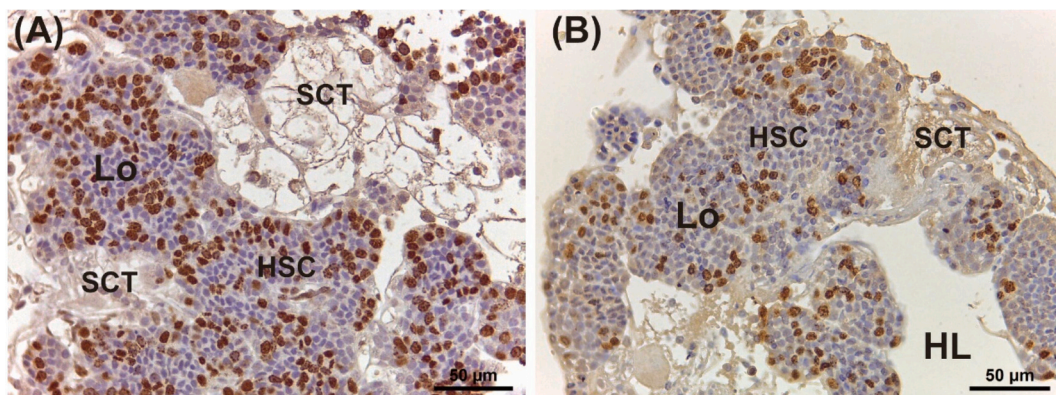


**Fig. 6.** Cellular proliferation activity in the hematopoietic tissue of the host animals *Metopograpsus thukuhar*. Cellular proliferation activity was evaluated based on in vivo incorporation of bromodeoxyuridine (BrdU) into newly synthesized DNAs (see Materials and Methods). Representative images of tissue section of the hematopoietic tissue of Hnp (A) and Hit (B) showing actively dividing (BrdU-incorporated) stem cells (brown staining) and non-dividing cells counterstained with hematoxylin (light blue staining) residing inside hematopoietic tubules. Lo: lobule, HSC: hematopoietic stem cell, HL: hemal lacuna, SCT: spongy connective tissue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

necrosis—specifically oncotic necrosis (Fink and Cookson, 2005; Elmore et al., 2016)—in the hepatopancreas and hematopoietic tissue of parasitized hosts, which likely contributed to the pronounced (>50%) reduction in the total hemocyte count.

In crustaceans, circulating hemocytes mediate a wide range of immune functions, and their continuous production and release from hematopoietic tissue are essential for maintaining immune homeostasis (Johansson et al., 2000; Jiravanichpaisal et al., 2006; Vazquez et al., 2009). Although oncotic necrosis of hematopoietic stem cells was also observed in parasitized *M. thukuhar*, it occurred at a relatively low

frequency. Accordingly, the proliferative activity of hematopoietic stem cells in parasitized *M. thukuhar* was not significantly reduced compared with that in non-parasitized crabs. Hematopoietic stem cells may be shielded from the necrotizing effect by the connective tissues that envelope the lobules in which these stem cells reside. Taken together, these findings strongly implicate necrosis of circulating hemocytes as a key contributing factor underlying the immunosuppression observed in parasitized hosts.

**Table 3**

Effect of parasitization by *Polyascus planus* on the mitotic index of the hematopoietic stem cells of the host animals *Metopograpsus thukuhar*.

| Parasitization status | Sample size | Mitotic index (%) <sup>a</sup> |
|-----------------------|-------------|--------------------------------|
| Hnp <sup>c</sup>      | 10          | 39.4 ± 6.4                     |
| Hit                   | 10          | 23.2 ± 3.7                     |

<sup>a</sup> Mitotic index = % of hematopoietic stem cells that were BrdU-incorporated.

<sup>b</sup> The mitotic index was not significantly different between the Hnp and Hit groups ( $z = 0.299$ ,  $P = 0.765$ ). Hit: internally parasitized host, Hnp: non-parasitized host.

#### 4.3. Preservation of immunity against potentially lethal pathogens

An interesting observation is that, although the immunity of the parasitized *M. thukuhar* was apparently suppressed, the immunity against certain pathogenic bacteria remained largely intact, as evidenced by the near-complete clearance of *Vibrio* spp. from the hemolymph of parasitized hosts to the extent that was comparable to that observed in non-parasitized crabs. *Vibrio* is a genus of Gram-negative bacteria that are ubiquitous members of estuarine and marine ecosystems (Baker-Austin et al., 2017). Several species of *Vibrio* are causative agents of severe or lethal diseases to many marine animals including crustaceans (Brock and Lightner, 1990). Interestingly, a microbiome study of the rhizocephalan parasite *L. panopaei* and its host *Eurypanopeus depressus* identified an unclassified genus of *Vibrionaceae* that has a strong and significant association with the parasite externa and larvae (Swanson et al., 2023), revealing the immediate proximity of these potential pathogens to the parasitized hosts. Hence, it would be critical for the host–parasite pair to strictly maintain those immune defenses targeting common yet life-threatening pathogens, even within an otherwise suppressed immune system. It is further suggested that this selective preservation of immune functions may be achieved by retaining the ability to selectively express the required immune effectors, for instance, antimicrobial peptides, which are known to display distinct target species-specific activity profiles (Cuthbertson et al., 2004, 2008; Schmitt et al., 2016; Barreto et al., 2022). Indeed, transcriptomic and proteomic analysis indicated that various effector molecules involved in immune defenses are differentially expressed in rhizocephalan-parasitized hosts (Yang et al., 2022; Zatylny-Gaudin et al., 2023; Zhao et al., 2023).

#### 4.4. Histopathological responses of host tissues to rootlet infiltration

It is informative to highlight and compare how host tissues responded histopathologically to rootlet infiltration as shown in the present study. The hepatopancreas, which is involved in several aspects of immune defense including synthesis of pathogen recognition proteins and clotting proteins, also harbors the fixed phagocytes that are known to be involved in the clearance of foreign particles (see Röszer, 2014; Vogt, 2019, 2020 for reviews). In the rootlet-infiltrated hepatopancreas of *M. thukuhar*, the most notable immune response was phagocytosis of necrotic hemocytes by these classic fixed phagocytes. The fixed phagocytes, typically forming rosette groups, adhere to the outer surface of arterioles in hemal space of the hepatopancreas and are thus exposed to the hemolymph circulating through the interstitium, trapping and phagocytizing foreign particulate matters, including microbial pathogens, injected ink (Johnson, 1987). In this regard, it should be mentioned that the phagocytosis of necrotic hemocytes as observed in the present study represents the first record of the fixed phagocytes engaging in the clearance of the debris of damaged host cells, suggesting their involvement in tissue repair. Phagocytosis of necrotic hemocytes also occurred in the rootlet-infiltrated hematopoietic tissue. In both the hepatopancreas and hematopoietic tissues, phagocytosis was activated only towards necrotic hemocytes, but not other types of host cells or infiltrating rootlets.

Activation of phagocytosis in these tissues where extensive hemocyte

necrosis takes place may represent an adaptive host manipulation. For multicellular organisms, as an initial step towards tissue repair, clearance of necrotic cell debris through phagocytosis is a critical inflammatory response to tissue injury; many cellular contents and pro-inflammatory mediators, once released from necrotic cells, act as the so-called damage-associated molecular patterns (DAMPs), which are capable of eliciting immune responses including recruiting and activating phagocytes, through activation of the pattern-recognition receptors (PRRs) (see Westman et al., 2020). Several types of hemocyte-derived DAMPs have been reported for crustaceans, including heat shock proteins, histones, high-mobility group box proteins, peroxiredoxin (Chen et al., 2014; Junprung et al., 2022; Wanvimonsuk et al., 2022); activation of crustacean PRRs by DAMPs has also been shown to be involved in activating various immune responses of the innate immunity (Farias et al., 2019; Junprung et al., 2022; Li et al., 2022; Wanvimonsuk et al., 2022). The mechanisms by which phagocytosis is activated in rootlet-infiltrated tissues and whether the rootlets contribute to this activation are intriguing questions that warrant further investigation.

Parenthetically, it is worth noting that phagocytes have never been reported in the hematopoietic tissue of any brachyuran species. Small-granule hemocytes with phagocytic activity residing within the hematopoietic tissue have been described in penaeid shrimps (Martin et al., 1996), which lack the classic fixed phagocytes in the hepatopancreas (Johnson, 1987; Martin et al., 1993). The phagocytes activated in the rootlet-infiltrated hematopoietic tissue of *M. thukuhar* are morphologically different from the penaeid phagocytes, but similar to the fixed phagocytes in the hepatopancreas of the same species, in the manner they interact with necrotic cells and in that both are anchored/fixed to tissue—to the spongy connective-tissue cells in the hematopoietic tissue and to the arterioles in the hepatopancreas (compare Fig. 2E and F2 and Fig. 5D and E). It may be that the fixed phagocytes in both tissues share a common lineage, e.g., derived from the hyaline cells as proposed by Johnson (1987).

The histopathological responses of the ovary of parasitized *M. thukuhar* to rootlet infiltration included arrested oocyte development and resorption of mature oocytes. Both responses were likely the results of preventing or disrupting the integration of the oocyte and follicle cells into a functional follicle, i.e., the process of folliculogenesis. It is well established for Malacostraca crustaceans that establishing the follicular layer that envelops the oocyte is critical for oocyte development, including uptake of vitellogenin of extra-ovarian origin during the so-called secondary vitellogenesis (Charniaux-Cotton, 1985; Meusy and Payen, 1988). Thus, the observation that in rootlet-infiltrated ovary the developmentally arrested pre-vitellogenic oocytes were deprived of a closely contacted follicular envelope (see Fig. 4H) indicates that preventing the formation of a functional follicle is responsible for the arrest. As for the mature oocytes, detachment of the follicular envelope from the oocyte, allowing rootlets to be able to establish close contacts with the de-folliculated oocyte (see Fig. 4D–F), presumably for absorption of oocyte materials, is likely to be the underlying mechanism of oocyte resorption observed in the infiltrated ovary. The host materials absorbed by rootlets could be transported to the externa for its development and maturation. Indeed, we demonstrated in a previous study that fragments of vitellogenin, the major yolk protein precursor, of host origin, were present in the parasite's egg yolk proteins (Chen et al., 2022). Various cell types of the rootlet, specialized for absorption, digestion, metabolism of nutrients from the host and for transport of these nutrients to the externa, have been described for several species of rhizocephalans (Bresciani and Høeg, 2001).

It is revealing that, notwithstanding the fact that the follicular envelope was disrupted or prevented from being formed in infiltrated ovaries of *M. thukuhar*, as discussed above, the constituent follicle cells remained intact (see Fig. 4D and H), highlighting the highly controlled and specific nature of the effects of the parasitism on host tissues. Ovarian follicle cells have been demonstrated to be the intra-ovarian site

of vitellogenin synthesis in decapods (e.g., Tsutsui et al., 2000; Jia et al., 2013). Like their non-parasitized counterparts, the parasitized *M. thukuhar* retained the capacity of ovarian synthesis of vitellogenin to be used, not for host's, but for parasite's vitellogenesis (Chen et al., 2022). Therefore, maintaining the structural and functional integrity of the host's follicle cells in infiltrated ovaries is critical for the ovarian maturation of the parasite.

#### 4.5. Conclusion

To conclude, the present study of *M. thukuhar* parasitized by *P. planus* illustrates the highly versatile and adaptive strategy the parasite employs for host control. The immune system of the parasitized *M. thukuhar* is apparently compromised, caused at least in part by reducing the number of circulating hemocytes through necrosis of the hemocytes, while the part of the immunity against certain potentially lethal pathogens is well preserved and phagocytosis of necrotic hemocytes activated. Furthermore, the pathohistological effects of parasitism on various host tissues imply that close contact between invading rootlets and the affected cells of the infiltrated tissue are required, suggesting that the tissue-specific effects of the parasitism rely on rootlet-derived, locally produced regulatory factors. They also indicate that host control of rhizocephalans involves tightly controlled, highly selective processes. Thus, it is tempting to suggest that tissue-infiltrating rootlets secrete various parasite-derived regulatory factors (PDRFs) to locally exert the control over these tissues. Recent studies of the rootlet-invaded tissues of several species of hosts provided ultrastructural evidence for potential host-parasite interaction sites where the parasites may alter the activity of host tissues via humoral factors (Miroljubov et al., 2020; Lianguzova et al., 2021, 2023; Nesterenko and Miroljubov, 2023). Identification of any of these PDRFs would greatly advance our understanding of the mechanisms underlying host manipulation by parasitic barnacles.

#### CRediT authorship contribution statement

**Hsiang-Yin Chen:** Visualization, Validation, Investigation, Data curation, Conceptualization. **Su-Ling Yang:** Investigation, Data curation. **Hung-Chang Liu:** Writing – review & editing, Resources. **Jean-Yves Toullec:** Writing – review & editing, Resources. **Benny K.K. Chan:** Writing – review & editing, Resources. **Chi-Ying Lee:** Writing – review & editing, Writing – original draft, Validation, Resources, Conceptualization.

#### Declaration of competing interest

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

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