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Localization and bacteriostasis of *Vibrio* introduced into the Pacific white shrimp, *Litopenaeus vannamei*

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Abstract

Although numerous mechanisms of immune defense have been described in crustaceans, the tissue distribution and fate of live bacteria introduced into the host remain unclear. In the present study, *Litopenaeus vannamei* were injected with a sub-lethal dose of kanamycin-resistant *Vibrio campbellii* expressing green fluorescent protein**.** Accumulation of intact bacteria was quantified by real-time PCR, while bacteriostasis was quantified as the percentage of intact bacteria that could not be recovered by selective plating. Over the 240 min examined, the lymphoid organ contained the greatest number of intact *V. campbellii* per gram tissue as well as the lowest percentage of culturable *V. campbellii* compared to other tissues, including the hemolymph. In contrast, the gills and hepatopancreas accumulated intact bacteria, but contained a significantly greater percentage of culturable bacteria than the hemolymph after 240 min. These data suggest that the lymphoid organ plays a major role in bacterial uptake and bacteriostasis in penaeid shrimp.
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1. Introduction

The economic importance of decapod crustaceans and the deleterious effects that diseases have on shrimp aquaculture have stimulated a number of studies on the immune systems of these invertebrates. Several different mechanisms of immune defense against microbial pathogens have been identified in decapod crustaceans. Humoral defenses include clotting factors, antimicrobial peptides, and the prophenoloxidase cascade [1–3]. Hemocytes, which comprise the main cellular defense, mediate phagocytosis, the production of reactive oxygen species, and the formation of hemocyte-bacterial nodules [4–6]. It remains unclear how these individual mechanisms of immune defense are integrated to form an effective response against invading bacterial pathogens to kill and eliminate bacteria from the animal.

Live, culturable bacteria injected into penaeid shrimp are rapidly removed from the hemolymph, as measured by the recovery of bacterial colony-forming units [7,8]. A number of studies with decapod crustaceans have suggested the gills to be the main site of subsequent accumulation of live and heat-killed

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bacteria or their degradation products [7,9–12]. Martin et al. [7] detected 55% of the ^{14}C-radiolabeled bacteria *Bacillus subtilis* in the gills of the penaeid shrimp *Sicyonia ingentis* 15 min after injection into the hemolymph. This accounted for more than 75% of the total recoverable radioactivity. Martin et al. [13] proposed that the gills, which have not been shown to contain fixed phagocytes, act as a passive filter, trapping hemocyte-bacterial nodules when they reach sufficient size to become lodged in the small branchial blood vessels. Limited evidence also suggests that the presence of both cell-bound lectins and nephrocytes in the gills facilitates the recognition and uptake of intact and/or degraded products of Gram-positive [10,13,14] and Gram-negative bacteria [12].

In contrast to the above results, van de Braak et al. [8] reported that the live, pathogenic bacterium, *Vibrio anguillarum*, does not accumulate in the gills of *Penaeus monodon* and proposed instead that the lymphoid organ is a major site of bacterial uptake. These investigators reported that cells in the lymphoid organ duct and lymphoid organ contribute to a significant portion of the uptake of bacteria from the circulation [8]. The lymphoid organ, first described in *Penaeus orientalis* by Oka [15], was originally thought to function in shrimp as a hematopoetic organ [16,17], however, the limited number of cells in this tissue that undergo mitosis does not support this function [18]. Although van de Braak et al. [8] demonstrated that the lymphoid organ was a site of bacterial uptake and phagocytosis by hemocytes, their histological study was not able to quantify the contribution of this tissue to the accumulation and killing of bacteria as compared to other possible sites of bacterial clearance.

In addition to accumulation in the gills and the lymphoid organ, bacteria accumulate in a variety of other tissues, including the hepatopancreas, the heart, the gut, and the antennal gland [7,11,12,19]. In fact, White and Ratcliffe [11] found the hepatopancreas to be the major site of accumulation of heat-killed, ^{14}C-radiolabeled *B. subtilis* injected into the shore crab, *Carcinus maenas.* Furthermore, both the hepatopancreas and the heart contain fixed cells capable of phagocytosis [20–22]. It is likely that the relative contribution of each of these tissues to the removal of bacteria from the circulation differs among species, and also depends on the strain, dose, and route of entry of the pathogen.

The goal of the current study was to assess the relative contribution of the lymphoid organ, the gills, the hepatopancreas, and the heart to the active removal of a bacterial pathogen introduced into the penaeid shrimp *Litopenaeus vannamei*. Shrimp were injected with a pathogenic strain of *Vibrio campbellii* expressing green fluorescent protein (GFP) and resistance to the antibiotic kanamycin (kan). *V. campbellii* were quantified in each of these tissues and in the hemolymph, at 15, 60, and 240 min after injection using both selective plating on growth medium containing kan and real-time PCR to detect the kanamycin resistance gene (*kanR*). Selective plating was used to quantify the number of the bacteria that were culturable, while real-time PCR was used to quantify the number of intact *V. campbellii,* defined as the number of *V. campbellii* that retained plasmid DNA. Using both methods of quantification allowed us to determine not only the major site(s) of accumulation of intact bacteria (real-time PCR) but also to estimate the in vivo bacteriostatic activity at each site, by determining the percentage of intact bacteria that were culturable. To our knowledge this represents the first study aimed at quantifying the tissue distribution and bacteriostasis of injected live pathogenic bacteria in penaeid shrimp.

2. Materials and methods

2.1. Animals

L. vannamei post-larvae (Kona Specific Pathogen Free Stock, Oceanic Institute, Kona, HI) were grown to approximately 5 g juvenile stage animals at the Waddell Mariculture Center, Bluffton, SC. Approximately 200 juveniles were transported to the Grice Marine Laboratory, Charleston, SC, and placed in recirculating seawater at 30 ppt salinity, 23–25 °C and pH 7.8–8.1. They were fed daily with commercial shrimp pellets (Rangen Inc., Buhl, ID). Shrimp (5–9 g) were held for 2 weeks before use in experiments.

2.2. Vibrio campbellii

Vibrio campbellii 90-69B3 was isolated from diseased shrimp by D. Lightner and L. Mahone, University of Arizona. A 1475-bp fragment of

the 90-69B3 16S rDNA sequence was PCR amplified using primers 1492R [23] and 8F, the latter of which hybridizes to the same sequence as primer fD1 [24] but lacks the 5′ linker extension of fD1. Analysis of the amplicon sequence (Genbank AY738129) places it in the *Vibrio parahaemolyticus/V. harveyi* family with greatest sequence identity (99%) to *V. campbellii*. Genes encoding resistance to kanamycin (*kanR*) and green fluorescent protein (GFP) were conjugally introduced into 90-69B3 on pMSB6, a *Vibrio*-derived plasmid, as previously described [25]. This multi-copy plasmid was highly stable in *V. campbellii,* being lost at a rate of approximately 10^{-4} per generation when grown in the absence of kanamycin. Introducing these genetic markers on a multi-copy plasmid rather than inserting single-copy genes in the bacterial chromosome ensured that GFP would be brightly and uniformly expressed in *V. campbellii* colonies, facilitating detection by fluorescence microscopy. In addition, the presence of multiple copies of marker genes in each bacterium was expected to enhance the sensitivity of the PCR-based detection system in tissues with low numbers of *V. campbellii* (see Section 2.4 below).

2.3. Bacterial challenge

For each injection, *V. campbellii* from a single working stock were streaked onto a Tryptic Soy Agar (TSA) plate supplemented with 2.5% NaCl and 100 μg mL^{-1} kanamycin A (Sigma-Aldrich) and grown overnight at 25 °C. To prepare the injection dose, bacteria from the plates were suspended in buffered saline (2.5% NaCl, 10 mM HEPES, pH 7.5), quantified by optical density at 540 nm, as described by Mikulski et al. [26], then serially diluted to 1×10^7 CFU mL^{-1}. *L. vannamei* were injected in the third abdominal segment with 2 μL g^{-1} body weight of 1×10^7 CFU mL^{-1} *V. campbellii* for a final injection dose of approximately 2×10^4 CFU g^{-1} shrimp. This dose is 10 times less then the LD_{50} for *L. vannamei* and was used in order to profile a successful defense of the shrimp immune system against a bacterial pathogen [26]. At 15, 60, or 240 min after injection hemolymph was sampled from the base of the third walking leg using a 23 gauge needle. The gills, the heart, the lymphoid organ, the hepatopancreas, and the injection site (third muscle segment) were removed and the tissues were weighed. The hemolymph sample was also weighed. The tissue and hemolymph samples were homogenized individually in 5 mL of sterile HEPES buffered saline (2.5% NaCl, 10 mM HEPES, pH 7.5). Aliquots of the homogenates were stored at −70 °C until use for real-time PCR while the remainder of the homogenates were diluted and plated on selective plates.

2.4. Real-time PCR

Real-time PCR was used to quantify the total number of intact *V. campbellii* in each tissue. Homogenates were thawed, then precipitated by centrifugation for 10 min at 5000*g*. Precipitated material, which included shrimp cells/tissue and bacteria, was resuspended in 1/5 volume DI water. Samples were incubated at 95 °C in order to lyse the bacteria and to denature proteases or nucleases that might interfere with the PCR. The samples were then precipitated by centrifugation for 5 min at 18,000*g* and the resultant supernatant analyzed by quantitative real-time PCR. A 129-bp fragment of *kanR* was amplified using 200 nM forward primer RTKnF 5′-TGATGCGCTGGCAGTGTT-3′, 200 nM reverse primer RTKnR 5′-CTCGCATCAACCAAACCGTTA-3′, and 200 nM Taqman probe 5′-TGCGCCGGTTGCATTCGATTCCTGT-3′, 5′ labeled with 6-carboxyfluorescein (FAM) and 3′ labeled with Black Hole Quencher-1 (Integrated DNA Technologies, Inc., Coralville, IA). Reaction mixtures (25 μL) were prepared using the QIAGEN QuantiTect Probe PCR Kit (catalog # 204343). The amplification was monitored using Applied Biosystem's 7000 Sequence Detection System and consisted of a 15 min incubation at 95 °C, in order to activate the hot-start polymerase, followed by 50 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 45 s, and elongation at 72 °C for 30 s.

Since bacterial cells in the tissue samples were sedimented by centrifugation and the supernatants discarded, the quantification using real-time PCR was limited to plasmids associated with intact *V. campbellii.* Detection of plasmids released from lysed or degraded bacteria was not expected due to the short residence time of nucleic acids in the nuclease-rich shrimp tissue homogenate. A standard curve was generated from dilutions of known numbers of culturable *V. campbellii*, as determined by selective

plating. Consequently, the number of injected bacteria would be, by definition, the same regardless of whether they were enumerated by plating or PCR, which allowed us to compare the values obtained by real-time PCR and selective plating. Water samples as well as tissues sampled from shrimp injected with sterile HEPES saline were used as negative controls. Tissue samples from shrimp injected with saline were also spiked with a known number of *V. campbellii* to ensure that the PCR was not inhibited in any of the tissue homogenates.

Using standard curves generated from known numbers of bacterial CFU, we established that the real-time PCR could be used to quantify as few as one *V. campbellii* per 20 μL reaction volume. Threshold cycles of replicate samples differed by no more than one cycle and the efficiency of all reactions was greater than 1.80, with most above 1.90. No amplification was detected in tissues from saline-injected control shrimp nor was any inhibition of the PCR detected based on the amplification of tissue homogenates spiked with a known number of *V. campbellii*.

2.5. Selective plating

Selective plating was used to quantify the number of culturable *V. campbellii* in each tissue. Homogenates of shrimp tissues were plated in marine agar supplemented with 1% NaCl and 100 μg mL^{-1} kan and overlaid on thiosulfate–citrate–bile–sucrose (TCBS) agar supplemented with 2% NaCl and 100 μg mL^{-1} kan. Plates were incubated at 25 °C and colony forming units (CFU) counted 24 h after plating. Fluorescence microscopy was used to confirm the identity of colonies growing on selective plates as *V. campbellii* expressing GFP. For each shrimp, the injection dose was plated as a positive control. *V. campbellii* not containing *kanR* was used as a negative control.

Water samples were plated to ensure that culturable kan-resistant bacteria were not present in the water at the start of injection experiments. Tissue homogenates from control shrimp injected with sterile HEPES saline and their holding water were also plated as negative controls. At the 15 min time point, *V. campbellii* (2–11 CFU mL^{-1}) could be cultured from 1 mL samples of the water in which bacteria-injected shrimp were held, suggesting that a substantial portion of the dose escaped from the injection site during the first 15 min. *V. campbellii* in the holding water was not expected to interfere with the quantification of bacteria in the shrimp since, with the exception of the gills, all of the tested tissues are internal to the animal. In addition, if the entire bacterial dose for a shrimp of average weight (7g) were released into the holding water, the maximum density of *V. campbellii* would be less than 10 CFU mL^{-1}. If this water was diluted to the same extent as shrimp tissues are diluted during homogenization, the resulting number of bacteria in the small volumes (0.1 mL or less) used for tissue assays would fall below the limit of detection for both the CFU and real-time PCR assays.

2.6. Statistical analyses

The effects of time after injection and tissue type were tested on the following variables using a series of mixed model analyses of variance (ANOVA): intact *V. campbellii* g^{-1} tissue, intact *V. campbellii* g^{-1} shrimp, culturable *V. campbellii* g^{-1} shrimp, and the percentage culturable *V. campbellii*. Time after injection and tissue were treated as fixed effects while individual shrimp were treated as a random effect in order to account for the variance among animals. The tests were modeled using R, a language for statistical computing [27]. The use of R, and 'lme' within R, which differs from the normal least squares approach, allowed a more accurate estimate of the parameters and significance of this unbalanced, mixed-model. *V. campbellii* g^{-1} tissue and *V. campbellii* g^{-1} shrimp data were log-transformed and percentage culturable as a percentage of intact *V. campbelllii* were arc sin square root-transformed in order to conform to the assumptions of equal variance and normality of the parametric tests. SigmaStat 3.0 software was used to perform pairwise multiple comparisons on significant effects and interactions using the Holm–Sidak method.

3. Results

3.1. Recovery of V. campbellii

At 15 min after injection, 38.7 ± 2.1% (mean ± SE) of the injected dose could be recovered using

Table 1
Recovery of intact and culturable bacteria from all examined tissues and from the injection site of *Litopenaeus vannamei* injected in the third abdominal segment with 2.0×10^4 *Vibrio campbellii* g^{-1} shrimp, at 15, 60 and 240 min after injection

Time postinjection (min)	Total recovered bacteria[a]		Bacteria recovered from injection site[b]	
	Intact bacteria[b]	Culturable bacteria	Intact bacteria[b]	Culturable bacteria
	Injection dose (%)	Injection dose (%)	Injection dose (%)	Injection dose (%)
15	38.7±2.1	28.0±2.2	24.4±2.8	23.6±2.2
60	21.2±3.3	13.2±2.0	12.3±2.1	11.8±1.9
240	14.7±2.0	10.9±2.0	9.7±1.9	10.7±2.0

[a] Bacteria recovered from injection site, hemolymph, heart, lymphoid organ, hepatopancreas and gill.
[b] Bacteria that remain associated with the *kanR* plasmid, as detected by quantitative PCR.

real-time PCR in the tested tissues, including the injection site, hemolymph, gills, hepatopancreas, lymphoid organ and heart, and assuming a hemolymph volume equal to 25% of shrimp weight [28]. By 240 min only 14.7±2.0% (mean±SE) of the injected dose could be detected as intact bacteria. This number of intact *V. campbellii*, as quantified using real-time PCR, included both culturable bacteria as well as those rendered non-culturable but that still retained the plasmid. Approximately 72% of the intact bacteria recovered from all tissues at 15 min could be cultured. By 240 min after injection, 74% of intact bacteria were culturable (Table 1).

The site of injection retained 24.4±2.8% (mean±SE) of injected bacteria at 15 min, as quantified by real-time PCR, representing approximately 63% of all intact bacteria that were recovered at this time point. The number of culturable *V. campbellii* at the injection site did not differ significantly from the number of intact bacteria at any of the time points used (Table 1). The hemolymph contained approximately 27% of the intact *V. campbellii* recovered using real-time PCR 15 min after injection, while the remaining 10% of the recoverable intact *V. campbellii* were located in the gills, hepatopancreas, lymphoid organ and heart.

3.2. Tissue specific concentration of V. campbellii

The hemolymph contained approximately 6700±1200 (mean±SE) intact *V. campbellii* g^{-1} hemolymph at 15 min after injection. By 240 min after injection the number of intact *V. campbellii* g^{-1} hemolymph had significantly decreased to approximately 2100±600 (mean±SE) as determined using real-time PCR. (Fig. 1).

The tissue specific concentration of *V. campbellii* (intact bacteria g^{-1} tissue) significantly decreased between 15 and 240 min (two-way ANOVA, $p<0.05$, df=107) in heart, gills, and hepatopancreas, but not in the lymphoid organ. Of the four tissues examined, the lymphoid organ contained the greatest number of *V. campbellii* per unit tissue weight, followed by the heart, the gills, and the hepatopancreas (two-way ANOVA, $p<0.05$, df=229). All four tissues examined contained significantly more intact *V. campbellii* per unit tissue weight compared to the hemolymph 15 min after injection (Fig. 1). The lymphoid organ was the only tissue in which the number of *V. campbellii* per unit tissue weight did not decrease significantly from 15 to 60 min after injection (Fig. 1). By 60 min after injection the number of intact *V. campbellii* per unit tissue weight in the lymphoid organ, the heart, and the gills remained significantly higher than in the hemolymph, but the number of intact *V. campbellii* in the hepatopancreas decreased so that it was no longer significantly different from the hemolymph (Fig. 1). By 240 min after injection, the mean number of *V. campbellii* g^{-1} lymphoid organ (61,200±15,500 SE), was over five times greater than in the heart (10,400±3400 SE), and 30 times greater than in the hemolymph (2100±600 SE). The heart was the only other tissue besides the lymphoid organ to contain a significantly greater number of *V. campbellii* per unit tissue weight compared to the hemolymph at 240 min after injection (Fig. 1).

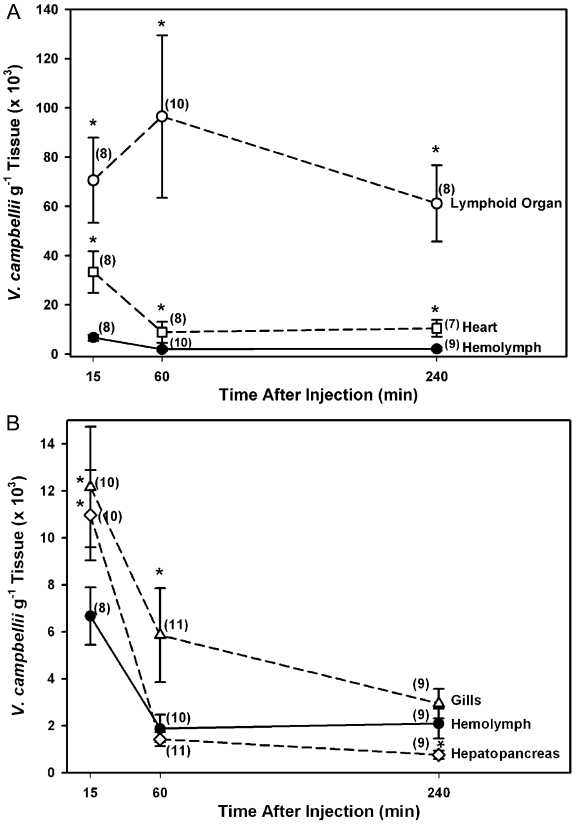

Fig. 1. Mean number of intact *V. campbellii* g^{-1} tissue ($\pm$ SE) in (A) the lymphoid organ, the heart, and the hemolymph, and (B) the gills, the hepatopancreas, and the hemolymph at 15, 60, and 240 min after injection of 2×10^4 *V. campbellii* g^{-1} shrimp. Significant differences in the number of intact *V. campbellii* g^{-1} tissue were analyzed on log-transformed data using a mixed-model ANOVA with tissue type and time after injection as fixed effects and individual shrimp as a random effect. The mean number of intact *V. campbellii* g^{-1} tissue in the four tissues and the hemolymph were all significantly different from each other ($p<0.01$, df = 135). The number of intact *V. campbellii* g^{-1} tissue in the heart ($p<0.001$, df = 22), the gills ($p=0.02$, df = 29), the hepatopancreas ($p<0.001$, df = 29), and the hemolymph ($p=0.03$, df = 26) decreased over time, while the number of intact *V. campbellii* g^{-1} tissue in the lymphoid organ did not decrease ($p=0.64$, df = 25). Asterisks were used to designate significant differences in the number of intact *V. campbellii* in the tissues compared to the hemolymph. The number of shrimp used (n) to calculate each mean is in parentheses.

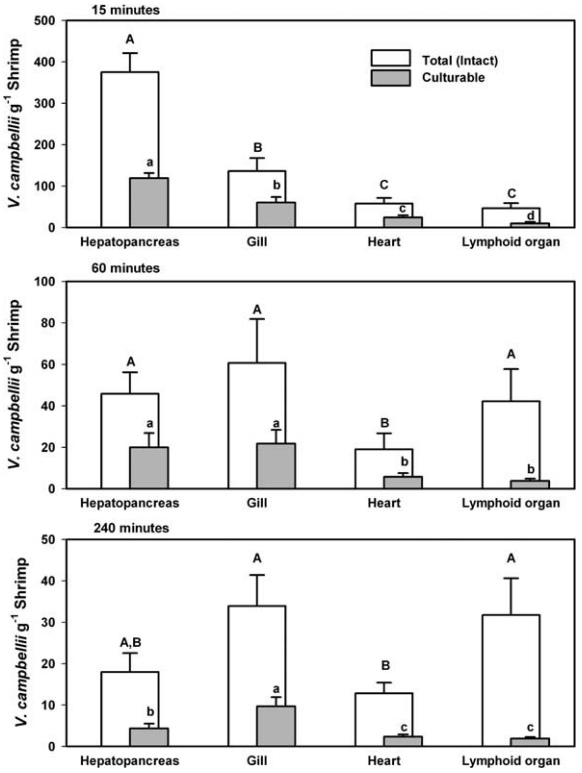

Fig. 2. Mean number of *V. campbellii* g^{-1} shrimp ($\pm$ SE) in the hepatopancreas, the gills, the heart, and the lymphoid organ at 15, 60, and 240 min after injection of 2×10^4 *V. campbellii* g^{-1} shrimp. Open bars represent the mean number of intact *V. campbellii* g^{-1} shrimp recovered using real-time PCR while the shaded bars represent the mean number of culturable *V. campbellii* g^{-1} shrimp recovered using selective plating. Note that the scale of the *y*-axis changes over the three panels. The complete data set was analyzed on log-transformed data using a mixed model ANOVA with tissue and time after injection as fixed effects and individual shrimp as a random effect. The effects of tissue, time after injection, and the interaction between tissue and time after injection were all significant on both the number of intact ($p<0.05$, df = 119) and culturable ($p<0.05$, df = 108) *V. campbellii*. Means marked with different letters contained significantly different numbers of intact (capital) or culturable (lower case) *V. campbellii* within each time-point after injection.

3.3. Total distribution of V. campbellii

Although the lymphoid organ contained the greatest number of intact *V. campbellii* per unit tissue weight at 15 min after injection (Fig. 1), as a whole tissue it contained fewer intact *V. campbellii* than the gills or the hepatopancreas (Fig. 2). The hepatopancreas, due to its large size, contained the greatest number of total *V. campbellii*, followed by the gills (Fig. 2). From 15 to 60 min after injection the total number of intact *V. campbellii* decreased in each of the tissues except the lymphoid organ (Fig. 1) so that

by 60 and 240 min after injection there was no longer a significant difference in the total number of intact *V. campbellii* in the lymphoid organ and the hepatopancreas or the gills (Fig. 2). Despite having similar numbers of intact bacteria in these three tissues, fewer culturable *V. campbellii* were recovered from the lymphoid organ compared to the gills and the hepatopancreas (Fig. 2).

3.4. Percentage of culturable V. campbellii

The majority of intact *V. campbellii* recovered by real-time PCR from all of the tissues, including the hemolymph, were not culturable. There was no significant difference in the percentage of intact *V. campbellii* that could be cultured from the four tissues and the hemolymph at 15 min after injection (Fig. 3). At 60 min after injection, the lymphoid organ contained the lowest percentage of culturable *V. campbellii* (7.7±2.3% SE) compared to the other tissues (32–54%) and the hemolymph (34±8% SE) (Fig. 3). The percentage of culturable *V. campbellii* in the hemolymph decreased from 43±11.7% SE at 15 min to less than 1% at 240 min, comparable to the lymphoid organ but significantly lower than that found in any of the other tissues (Fig. 3). At the same time point, the percentages of culturable *V. campbellii* in the gills and the hepatopancreas were significantly higher than the percentages in both the lymphoid organ and the heart (two-way ANOVA, $p<0.01$, df=137).

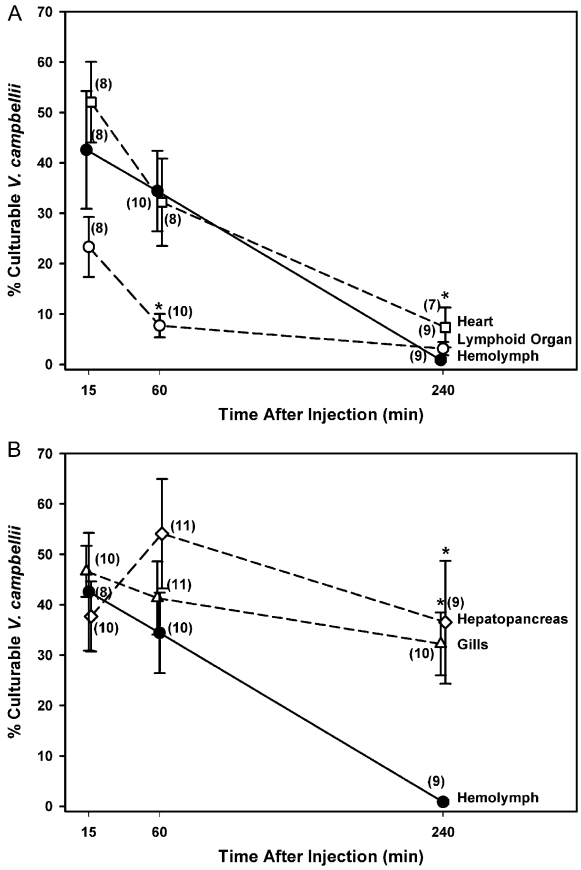

Fig. 3. Mean percentage of intact *V. campbellii* using real-time PCR (± SE) in (A) the heart, the lymphoid organ, and the hemolymph, and (B) the gills, the hepatopancreas, and the hemolymph that were culturable using selective plates at 15, 60, and 240 min after injection of 2×10^4 *V. campbellii* g^{-1} shrimp. Significant differences in the percentage of culturable *V. campbellii* were analyzed on arc sin square root-transformed data using a mixed-model ANOVA with tissue type and time after injection as fixed effects and individual shrimp as a random effect. The effects of tissue ($p<0.01$) and time after injection ($p<0.01$) on the percentage of culturable bacteria were significant. Asterisks were used to designate significant differences in the percentage of culturable *V. campbellii* in the tissues compared to the hemolymph. The number of shrimp used (n) to calculate each mean is in parentheses.

4. Discussion

The current study was unique in that both the number of intact and culturable *V. campbellii* were quantified in selected tissues following whole-animal injection of a sub-lethal dose of bacteria. Determining the number of intact bacteria allowed us to measure the overall removal of bacteria from the hemolymph and subsequent accumulation in the tissues. Determining the number, or percentage, of intact bacteria that remained culturable in the hemolymph, lymphoid organ, heart, gills, and hepatopancreas provided insights as to the roles of each tissue in bacteriostasis. Bacteriostasis was observed in the hemolymph, and the rapid removal of bacteria from the hemolymph that has been reported by other investigators appears to be due in part to the loss of culturability of the bacteria and not solely a result of the physical removal of bacteria from the hemolymph. The lymphoid organ appears to be the main site of bacterial accumulation as well as the main site of bacteriostasis. The lymphoid organ was the only tissue examined in

which the bacteriostatic activity was greater than that observed in the hemolymph. The gills and the hepatopancreas were major sites of accumulation of live bacteria, with little or no bacteriostatic activity observed in these tissues over the 240 min examined.

The majority of *V. campbellii* recovered using both real-time PCR and selective plating were located at the site of injection and remained there for the entire 240 min after injection. Although the current study could not determine whether this apparent sequestration was the result of a specific immune response or simply the result of decreased circulation through this region, it is interesting that the number of culturable *V. campbellii* at the injection site did not decrease over the entire 240 min examined. Histological studies have shown that hemocytes migrate to the site of injection where they are believed to participate in wound sealing and bacterial degradation [3,8,29]. Fontaine and Lightner [29] found that although injected carmine particles formed tightly packed extracellular masses at the site of injection after 1 h, they were not observed inside hemocytes until 18 h. Along with the current data these observations suggest two separate stages of defense, the first involved in the sequestration of bacteria at the site of injection and the latter involved in the eventual phagocytosis and killing of bacteria.

In contrast to what happened at the site of injection, the number of culturable *V. campbellii* in the hemolymph decreased with time. Nearly 100% of the bacteria recovered from the hemolymph were not culturable after 4 h. The rapid disappearance of live bacteria from the hemolymph has been demonstrated in a number of decapod crustaceans, including penaeid shrimp [7,8,30,31]. In the present study, real-time PCR detected approximately 2×10^3 intact *V. campbellii* per g hemolymph 240 after injection, suggesting that many bacterial cells remain in the hemolymph after the loss of culturability. This bacteriostatic activity has not been demonstrated previously in vivo, and may be mediated by hemolymph proteins that bind to *V. campbellii*. Pattern recognition proteins, including lipopolysaccharide- and glucan- binding protein (BGBP), are located in the hemolymph and are an essential first response in the removal of potential pathogens from the circulation [32]. The binding of these proteins to *V. campbellii* may have prevented the bacteria from being cultured. In vitro studies also have demonstrated that antimicrobial peptides (AMP), specifically the penaeidin *Litvan*-Pen3-1, may act as opsonins and agglutinins of *Vibrio* in the hemolymph of *L. vannamei* [33]. AMP are believed to be extremely important effectors of the innate immune system, accounting for 8–17% of all sequences isolated from *L. vannamei* hemocyte cDNA libraries [34]. In penaeid shrimp, AMP are produced and stored in hemocytes and released following microbial challenge. In the current study, the greatest decrease in culturable *V. campbellii* was observed between 60 and 240 min after injection, concurrent with maximum reported levels of penaeidins [35] and hemocyanin-derived AMP [36] in the hemolymph of *L. vannamei* following bacterial challenge. This suggests that the loss of culturable bacteria observed in the present study could be a result of the bacteriostatic activity of AMP.

Of the tissues examined, the heart and the lymphoid organ were the only tissues that contained a significantly greater number of *V. campbellii* per unit tissue weight compared to the hemolymph over the entire 240 min after injection. These results are consistent with an active accumulation of bacteria in these tissues. Both the heart and the lymphoid organ contain fixed cells capable of engulfing bacteria, although, as mentioned above, it is possible that the fixed cells originate from circulating hemocytes after injection [22]. The accumulation of bacteria in the heart is minimal due to its small size. Similarly, the heart appeared to play a small role in removal of radiolabeled bacteria after injection in the shore crab, *Carcinus maenas* [11], and the penaeid shrimp *S. ingentis* [7].

On the other hand, the present study found that the number of intact *V. campbellii* per unit tissue weight in the lymphoid organ was large enough to contribute significantly to the total accumulation of injected bacteria compared to the other tissues examined. The total number of intact *V. campbellii* recovered from the lymphoid organ was not significantly different from the number recovered from the gills and the hepatopancreas, which are approximately 20 and 60 times larger, respectively, than the lymphoid organ. The lymphoid organ was also the only tissue in which the number of intact *V. campbellii* did not decrease over the 240 min examined. Although researchers

have reported an accumulation of injected bacteria in the lymphoid organ, this study demonstrates the relative importance of this accumulation to the overall localization and bacteriostasis of bacteria. After injection, hemocytes migrate to the lymphoid organ where they are believed to participate in the active uptake and phagocytosis of invading bacteria [8]. Phagocytosis is rarely observed in vitro in non-adherent cells and in vivo data suggest that phagocytosis by hemocytes does not occur free in the hemolymph [11,22]. Bacterial cells may remain intact for some time after being engulfed by phagocytes, but at some point these cells will lose culturablilty and eventually be degraded. Once degraded, having released their plasmids containing *kanR*, these bacteria would no longer be detected using the real-time PCR. The absence of a decrease in the number of intact *V. campbellii* in the lymphoid organ, along with the assumption that bacteria are being phagocytosed in this tissue, suggest that *V. campbellii* continue to accumulate in the lymphoid organ over the entire 240 min examined.

The lymphoid organ was also the only tissue examined that contained a significantly lower percentage of culturable bacteria than the hemolymph. It is also possible, as described above, that the observed decrease in the percentage of culturable *V. campbellii* is due to the recognition of bacteria and the resultant bacteriostatic activity of AMP. The observed temporal difference in the percentage of culturable bacteria in the lymphoid organ and the hemolymph suggests a specialized function of the lymphoid organ in the recognition and killing of invading bacteria. It is likely that the concentrations of AMP are greater within the lymphoid organ compared to the general circulation.

Most studies examining the fate of bacteria injected into decapod crustaceans have identified the gills as the main site of accumulation of bacteria and/or bacterial degradation products [7,9–12]. In the present study, although intact *V. campbellii* initially accumulated in the gills (15 min after injection), a difference could not be detected between the number of intact *V. campbellii* per unit tissue weight in the gills and the hemolymph by 240 min after injection. This apparent absence of accumulation at 240 min after injection is consistent with the findings of van de Braak et al. [8] who did not find bacteria associated with the gills or with hemocytes in the gills of *Penaeus monodon* following injection of live *V. anguillarum*. They proposed that the accumulation in the gills reported by other researchers was an artifact of the large hemolymph volume of the gills. Although the current study could not detect a significant difference in the number of intact bacteria per unit tissue weight in the gills and the hemolymph 240 min after injection, the gills did contain a significantly greater percentage of culturable bacteria than the hemolymph. In contrast to van de Braak et al. [8], the present study shows that bacteria do in fact accumulate in the gills, but these bacteria remained culturable over the entire 240 min examined.

The mechanisms responsible for bacterial accumulation in the gills are unclear. The gills of decapod crustaceans do not appear to contain fixed cells capable of engulfing bacteria by phagocytosis [22]. Nephrocytes, or branchial podocytes, specific to the gills [37] were originally believed to be phagocytic, but recent data suggest that their activity is limited to pinocytosis of soluble particles and small viruses [22]. Martin et al. [13] observed hemocyte-bacterial nodules in the gills of the penaeid shrimp *S. ingentis* and proposed that these nodules form in the hemolymph, becoming trapped in the gills due to the small size of the branchial vessels. It is possible that the culturable *V. campbellii* recovered from the gills in the current study are contained in these nodules, but the decrease in the total number of intact *V. campbellii* recovered from the gills over time would suggest that this is not a major route for eliminating bacteria from the hemolymph. The removal of injected bacteria from the hemolymph and subsequent accumulation in the gills observed by Martin et al. [7] was not affected by decreased levels of circulating hemocytes, suggesting a clearance mechanism independent of circulating hemocytes. In addition, vibriosis has been observed in the gills of penaeid shrimp [38]. Based on these observations, the most plausible sequence of events leading to bacterial accumulation in shrimp gills is that the bacteria attach to the gills first, and at a later time hemocytes attach to the bacteria, possibly forming nodules and phagocytosing the bacteria.

The present study also suggests that the hepatopancreas is an important site of bacterial accumulation. As a whole tissue, the hepatopancreas contained the greatest

number of intact *V. campbellii* compared to the other tissues examined. These data are in contrast to those of Martin et al. [7] who found the gills to contain 75% of the carbon 14-labeled *B. subtilis* injected into the penaeid shrimp *S. ingentis*, with the hepatopancreas, despite its large size and hemolymph volume, accounting for less than 3% of the total recoverable radioactivity. Also, the percentage of culturable bacteria in the hepatopancreas was greater than in the hemolymph and not significantly different from that in the gills at 240 min after injection. Similar to the gills, the hepatopancreas is a known site of *Vibrio* infection [38]. Unlike the gills, there is evidence that the hepatopancreas of crustaceans contains fixed cells capable of phagocytosis, although it is unclear whether these cells are distinct from hemocytes [22] and the location of these fixed cells in penaeid shrimp is unknown. It is possible that the significant decrease in the total number of intact *V. campbellii* recovered from the hepatopancreas is a result of the degradation of bacteria by these phagocytic cells. Since the hepatopancreas contained a high percentage of culturable *V. campbellii*, it is more likely that the decrease in the number of intact *V. campbellii* recovered from the hepatopancreas over time is a result of the physical removal of bacteria and not bacteriostatic or killing activity. Lectins and other pattern recognition molecules, including β-1,3-D-glucan-binding protein, appear to be expressed in the hepatopancreas of *L. vannamei* and not in the hemocytes [34]. Although these immune recognition molecules are found in the general circulation, it is possible that they are more concentrated in the hemocoel space of the hepatopancreas. Therefore, it is possible that the *V. campbellii* are marked by these proteins in the hepatopancreas and then transferred to sites of active uptake.

Although determining the mechanisms of bacterial uptake and killing were beyond the scope of the present study, it is clear that the lymphoid organ is a major site of localization and bacteriostasis of *V. campbellii* introduced into *L. vannamei*. The gills and the hepatopancreas appear to be sites of accumulation of live bacteria and not bacteriostasis over the 240 min examined. It is interesting to note that most decapod crustaceans do not contain lymphoid organs. Instead, these crustaceans are believed to contain a homologous group of fixed phagocytes in the hemolymph vessels of the hepatopancreas that engulf and degrade bacteria [22,39]. This anatomical difference is a good example of the heterogeneity that exists among decapod crustaceans. Such species-specific differences are often overlooked in studies of immune function in crustaceans and invertebrates as a whole. The results of the present study illustrate that caution must be taken when inferring the mechanisms and workings of one specific immune system from another.

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