



## Time-course analysis of peroxinectin mRNA in the shrimp *Litopenaeus vannamei* after challenge with *Vibrio campbellii*<sup>☆</sup>

Erin J. Burge<sup>a,b,\*</sup>, Louis E. Burnett<sup>a</sup>, Karen G. Burnett<sup>a</sup>

<sup>a</sup>Grice Marine Laboratory, College of Charleston and Hollings Marine Laboratory, 331 Fort Johnson Road, Charleston, SC 29412, USA

<sup>b</sup>Department of Marine Science, Coastal Carolina University, P.O. Box 261954, Conway, SC 29526, USA

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### ABSTRACT

Peroxinectin (Pox), which promotes cell adhesion and encapsulation of bacteria in crustaceans, is synthesized in granular and semigranular hemocytes. In this study, real-time PCR was used to quantify Pox transcripts in individual tissues of the Pacific white shrimp, *Litopenaeus vannamei*, over 48 h following injection of a sublethal dose of the shrimp pathogen *Vibrio campbellii*. The resulting data were used to infer the movements of hemocytes among the tissues in response to bacterial challenge. Over all times and treatments, Pox transcripts (ng total RNA)<sup>-1</sup> varied by orders of magnitude among individual tissues, such that circulating hemocytes » gills » heart » lymphoid organ » hepatopancreas ≈ muscle. Relatively low constitutive expression of Pox in the lymphoid organ compared to circulating hemocytes, gills, and heart supports a primary role for this organ in bacteriostasis and degradation, rather than encapsulation of invasive bacteria. Numbers of Pox transcripts increased significantly at the injection site within 4 h and remained significantly elevated for 48 h, consistent with a rapid and sustained recruitment of hemocytes to the site of injection. Transcripts increased significantly in the gill but not in other tissues over the time-course of this experiment. These expression data reinforce the role of the gill in trapping and encapsulating invasive bacteria as a primary strategic focus during the early phase of the crustacean immune response and, by comparison with earlier studies of lysozyme expression in the same tissues, suggest differential roles for various tissues in a successful immune response.

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### 1. Introduction

Global aquaculture production for the Pacific white (or white-leg) shrimp, *Litopenaeus vannamei*, was in excess of 2 million metric tons and valued at an estimated 7.8 billion USD in 2006 (FAO – Fisheries and Aquaculture Information and Statistics Service – 09/06/2008). At the recent rate of increase (2000–2006) aquaculture production for this species is projected to reach greater than 10 billion USD in 2008, among the most valuable aquaculture commodities globally. Threats to continued growth in this sector include numerous etiologic agents, such as viruses, especially white spot syndrome virus and Taura syndrome virus, and pathogenic *Vibrio* spp. Numerous *Vibrio* spp. are routinely isolated from moribund and healthy shrimp, and they appear to be normal members

of the marine bacterial flora [1]. Outbreaks of disease associated with *Vibrio* can include primary pathogens and opportunistic infections [reviewed in 2].

Arthropod immune surveillance and response is primarily effected through the cellular responses of hemocytes and humoral activities within the hemolymph. In decapod crustaceans, peroxinectin (Pox) is a multi-functional protein that mediates cellular adhesion to invading microorganisms and enhances the antimicrobial oxidative burst functions of hemocytes [3]. The protein acts as a secreted opsonin [4], regulates granule exocytosis [5], and promotes encapsulation [6]. Full-length peroxinectin gene sequences have been described from a number of decapod species, including the crayfish *Pacifastacus leniusculus* [7], giant freshwater prawn *Macrobrachium rosenbergii* [8], black tiger shrimp, *Penaeus monodon* [9], and *L. vannamei* [10]. Holmblad and Söderhäll [3] articulated a model of the function of peroxinectin within a suite of responses that characterize the hemocyte–pathogen interaction in crayfish. They proposed that peroxinectin mediates non-self recognition and subsequent integrin-binding as an initiator of phagocytosis or encapsulation by recruited hemocytes. In addition, peroxinectin was shown to bind with an extracellular superoxide

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\* Corresponding author. Department of Marine Science, Coastal Carolina University, P.O. Box 261954, Conway, SC 29526, USA. Tel.: +1 11 843 349 6491; fax: +1 11 843 349 2545.

E-mail address: [eburge@coastal.edu](mailto:eburge@coastal.edu) (E.J. Burge).

dismutase [11] expressed at the plasma membrane of hemocytes, where its peroxidase function uses reactive oxygen compounds to enhance antibacterial toxicity. Evidence from multiple crustaceans indicates that peroxinectin is a critical component of the hemocyte response to invading microorganisms, and as such its study provides a promising target for monitoring the movements of hemocytes within an infected animal.

Crustacean hemocyte subpopulations can be differentiated morphologically [12–14], biochemically [15,16] and using genomic and proteomic tools [17]. A recent study by Wu et al. [17] identified specific proteins and gene transcripts that corresponded to different lineages of hemocytes released from their progenitor tissue, the hematopoietic organ, and these markers were used to track hemocyte proliferation and differentiation into granular or semigranular hemocytes. Differential immune gene expression between hemocyte subtypes [18–20] also indicates that each subtype has specialized roles within the population of total cells. For peroxinectin, expression was noted in isolated granular cells, and in a population of cells comprised primarily of semigranular cells, with a few hyaline cells included. In situ hybridization of circulating hemocytes revealed that peroxinectin was only expressed in granular and semigranular cells [21]. In contrast, lysozyme, another gene implicated in hemocyte responses to bacteria, is expressed in all types of hemocytes, although the intensity of expression is higher in granular than hyaline cells [18]. Comparing the expression of these two genes by hemocytes allows inferences about hemocyte trafficking and the temporal changes that occur in hemocytes during an immune response.

Numerous studies have documented declines in circulating hemocytes associated with several factors, including LPS [22–24],  $\beta$ -1,3 glucans [9], and bacteria [18,20,25,26]. Other lines of evidence indicate that Pox transcripts numbers change over long time scales (weeks to months) and that they are related to changes in intrinsic factors, like molt stage, or treatments with health-promoting factors (diet augmentation, probiotics) or health-damaging ones (copper sulfate, trichlorfon) that also influence total circulating hemocytes numbers and parameters associated with immune competency [27–29].

Despite analysis of Pox expression under a variety of conditions (heat shock, bacterial and fungal antigens, diet augmentation, chemotherapeutants, and molt status), no authors have reported expression of the gene associated with whole tissues, despite the presence of circulating hemocytes within these tissues, nor has Pox expression as an indicator of hemocyte location within tissues been examined. Information regarding the degree and location of Pox expression is important toward understanding its role in immunity and functions in crustaceans. In this study, the range of tissues containing hemocyte-specific Pox was elucidated and quantification of peroxinectin transcripts was tested as an mRNA marker of hemocyte location within *L. vannamei*.

## 2. Materials and methods

### 2.1. Experimental design

Details on the source of *L. vannamei*, husbandry conditions, culture of *Vibrio campbellii* 90-69B3 strain and preparation of challenge doses, experimental design, tissue and hemolymph sampling, RNA extraction, and reverse transcription parameters were published previously [18]. Unless otherwise noted all reagents and chemicals were purchased from Sigma–Aldrich (St. Louis, MO).

Briefly, *L. vannamei* ( $n = 74$ ,  $19.6 \pm 2.5$  g, mean  $\pm$  SD) from a single pond stock were injected with  $2 \times 10^4$  viable *V. campbellii* g shrimp<sup>-1</sup> into the third abdominal segment. Injectate contained bacteria suspended in a sterile HEPES-buffered saline (2.5% NaCl,

10 mM HEPES, pH 7.5). Injection volumes for this experiment were  $0.5 \mu\text{L g}^{-1}$  shrimp of  $10^4$  CFU mL<sup>-1</sup>. Injection doses represented approximately 10% of the LD<sub>50</sub> dose in *L. vannamei* [30]. Shrimp injected with an equivalent volume of sterile HEPES-buffered saline served as controls.

Animals were injected and immediately transferred to 19 L holding tanks at a density of 2 animals tank<sup>-1</sup>. Replicate tanks consisted of control, saline-injected animals ( $n = 37$ , 4–6 animals time-point<sup>-1</sup>) and *Vibrio*-injected animals ( $n = 37$ , 4–6 animals time-point<sup>-1</sup>) that were housed separately. At 0.25, 1, 4, 12, 24, and 48 h post-injection, individual control and *Vibrio*-injected animals were removed from their respective tanks and dissected. No mortalities were noted for either group during the experiments.

At the predetermined time-points, shrimp were removed from their tanks and hemolymph was extracted into three volumes of ice-cold shrimp anticoagulant [31] (450 mM NaCl, 10 mM KCl, 10 mM Na<sub>2</sub>EDTA, 10 mM HEPES, pH 7.3, 850 mOsm/kg) by needle and syringe inserted into the hemolymph sinus at the base of each walking leg. Hemocytes were quickly separated from hemolymph by centrifugation and the pellet resuspended in 500  $\mu\text{L}$  RNAlater™ solution (Ambion, Inc., Austin, TX). Samples of gill, heart, hepatopancreas, muscle at the site of injection, and lymphoid organ were also dissected and placed into 1 mL RNAlater™ solution and stored until RNA extraction, reverse transcription and real-time PCR analysis.

### 2.2. RNA extraction and reverse transcription

Tissue and hemocyte samples were extracted for total RNA using the RNeasy® Mini Kit (Qiagen Inc., Valencia, CA) following the manufacturer's recommended protocol by homogenization in 600  $\mu\text{L}$  of lysis buffer using a Tissue-Tearor power homogenizer (Biospec Products, Inc., Bartlesville, OK), treated with RNase-free DNase on a silica-gel column, and eluted in 25–30  $\mu\text{L}$  RNase-free water. RNA sample concentrations and purity were measured spectrophotometrically at 260 nm and corrected for protein contamination at 280 nm.

Total RNA (100–2000 ng) was reverse transcribed using Omniscript® Reverse Transcription reagents (Qiagen Inc., Valencia, CA) and following the manufacturer's protocol for cDNA synthesis from animal cells for real-time RT-PCR. Reverse transcription reactions were primed with 1  $\mu\text{M}$  oligo dT<sub>16</sub> and 10  $\mu\text{M}$  random nonamers. Archival cDNA samples were prepared by tissue to minimize tissue-specific differences in reverse transcription. cDNA was stored at  $-20^\circ\text{C}$  prior to real-time PCR analysis.

### 2.3. Real-time PCR

Real-time PCR thermocycling and data collection was performed by an Applied Biosystems (Foster City, CA) 7500 Sequence Detection System (software version 1.2.3) using QuantiTect™ Probe PCR kits (Qiagen Inc., Valencia, CA). Forward primer Lv Pox 1890F (5'-CTG CCA ATC CAG AAA TTC G-3'), reverse primer Lv Pox 1989R (5'-TCA GAC TCA TCA GAT CCA TTC C-3') (Invitrogen, Carlsbad, CA), and dual-labelled hybridization probe Lv Pox 1937P (5'-CCA TCT GTT CCA GAC GCC CAG A), 5'-fluorophore-labelled with 6-carboxyfluorescein and 3'-quencher-labelled with Black Hole Quencher-1 (Integrated DNA Technologies, Coralville, IA), were used for all amplifications.

These primers were developed using Applied Biosystems Primer Express v. 2.0.0 software on the sequence for *L. vannamei* peroxinectin manually transcribed from Liu et al. [10]. The manually transcribed sequence has identity with 376/378 nucleotides from a partial *L. vannamei* peroxinectin mRNA sequence deposited by

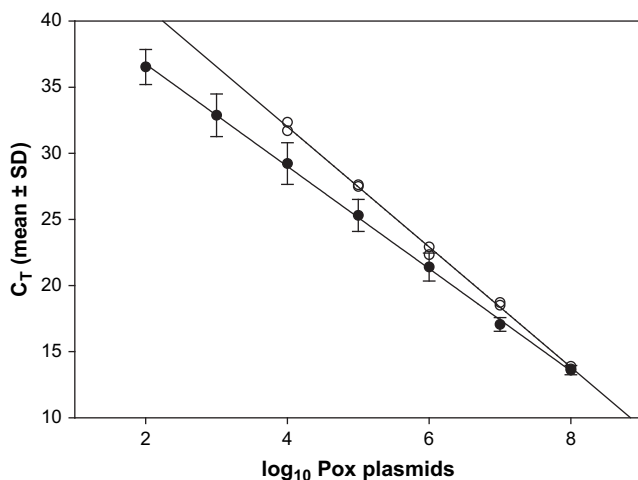
Wang, Y.-C. and Chang, P.-S. (Su-Zen College of Medicine and Management, Kaohsiung, Taiwan, unpublished; GenBank accession no. AY486425). Lv Pox 1890F and Lv Pox 1989R produced a 100 bp amplicon.

Quantitative real-time PCRs consisted of a final volume of 25  $\mu$ L with 1  $\mu$ L of 1:10 diluted sample cDNA, and final concentrations of 0.4  $\mu$ M per primer, 0.4  $\mu$ M dual-labelled hybridization probe, and 1 $\times$  QuantiTect™ Probe PCR kit HotStarTaq DNA polymerase with dNTPs in an appropriate buffer system. Each sample and standard was examined with duplicate reactions. Positive control reactions were analyzed in parallel with experimental samples and consisted of a standard curve of known concentrations ( $10^8$ – $10^2$  copies reaction<sup>-1</sup>) of a restriction endonuclease *Scal*-linearized pGEM®-T Easy plasmid (Promega Corporation, Madison WI) containing a 627 bp *L. vannamei* peroxinectin fragment [32]. PCR efficiencies were calculated for circular and *Scal*-linearized pGEM®-T Easy to determine the optimal standard curve configuration (Fig. 1). Individual standard curves were generated for each set of real-time PCR assays. Negative control reactions were run in parallel, omitting template cDNA.

Real-time PCR parameters were an initial 15 min denaturation and activation of HotStarTaq DNA polymerase at 95 °C. Amplification for 40 cycles consisted of 15 s denaturation at 94 °C with annealing and extension at 60 °C for 60 s. Fluorescence emission was monitored during the extension step using the 6-carboxy-fluorescein filter on the instrument.

#### 2.4. Data analysis

Absolute sample values of peroxinectin were derived by interpolation from the standard curve for each run and normalized to Pox transcripts ng<sup>-1</sup> total RNA using the RNA concentration of each sample. For each combination of treatment (saline-injected vs. *Vibrio*-injected), time-point (either 0.25, 1, 4, 12, 24 or 48 h post-injection), and tissue (circulating hemocytes, gill, heart, hepatopancreas, muscle at the site of injection, and lymphoid organ) the



**Fig. 1.** Peroxinectin standard curves. Linearized (closed circles ●, mean  $\pm$  SD,  $n = 22$  plasmid concentration<sup>-1</sup>) and circular plasmids (open circles ○,  $n = 2$  plasmid concentration<sup>-1</sup>) were tested for their relative efficiency in quantitative real-time PCR [32].  $\log_{10}$  Pox plasmids were serially diluted from a stock of  $1.0 \times 10^8$  copy number Pox plasmid solution.  $C_T$  was calculated by the real-time PCR software and represents the fractional cycle number at which the fluorescence surpasses background noise. Efficiency was closest to ideal ( $PCR_e = 2$ ) in linearized plasmids ( $PCR_e = 1.814$ ,  $R^2 = 0.9994$ ,  $p < 0.001$ ,  $y = -3.8667x + 44.4780$ ). Data for linearized plasmids include all sample tissue runs. No plasmid concentrations lower than  $10^4$  reaction<sup>-1</sup> were detected for circular plasmids ( $PCR_e = 1.660$ ,  $R^2 = 0.982$ ,  $p < 0.001$ ,  $y = -4.5435x + 50.182$ ).

arithmetic mean and standard error were calculated in SigmaStat 3.11 (Systat Software, Inc., Chicago, IL) and the results graphically displayed by SigmaPlot 9.01 (Systat Software, Inc., Chicago, IL).

Pox transcripts (ng total RNA<sup>-1</sup>) were  $\log_{10}$ -transformed and a three-factor, Model I analysis of variance (ANOVA,  $\alpha = 0.05$ ) in Minitab 15.1.1 (Minitab, Inc., State College, PA) was used to assess significant differences in the data. For the three-factor ANOVA the assumptions of normality and equal variance were violated, despite data transformation. Removal of the muscle from the three-factor analysis resulted in the equal variance assumption being satisfied. The nonparametric Kruskal–Wallis test on ranks for tissue and time-point, and the Mann–Whitney rank sum test on treatment support the findings of the three-factor analysis (data not shown). Despite this, ANOVA is robust with respect to deviations from both assumptions [33]. Where individual factors differed significantly ( $p \leq 0.05$ ) in the three-factor ANOVA, tissue, treatment and time-point effects were analyzed by two-factor ANOVA ( $\alpha = 0.05$ ) and multiple comparisons performed using the post hoc Holm–Sidak test in SigmaStat 3.11 (Systat Software, Inc., Chicago, IL). Regressions for standard curves and tests of normality and equal variance were conducted in SigmaStat 3.11.

### 3. Results

#### 3.1. Multi-tissue expression of peroxinectin

Peroxinectin mRNA expression was detected in all assayed tissues in all experimental animals and at all time-points ( $n = 357$ ). Pox transcripts (ng total RNA<sup>-1</sup>) varied significantly (three-factor ANOVA,  $p < 0.001$ ) and by orders of magnitude among individual tissues (Table 1), so the data were not normally distributed (Table 2), despite  $\log_{10}$  transformation. Pox transcripts also varied significantly with treatment ( $p = 0.006$ ) and time after injection ( $p < 0.001$ ) across the full data set. There was an inequality of variance between the treatments that was due to the large differences associated with injection treatment in the muscle (Table 2). Subsequent two-factor ANOVA and multiple comparisons within individual tissues and circulating hemocytes highlighted treatment and time-point specific differences.

Individual tissues differed in their overall magnitude of Pox expression, from a mean minimum value of 150 Pox transcripts (ng total RNA<sup>-1</sup>) in the hepatopancreas to 110 000 Pox transcripts (ng total RNA<sup>-1</sup>) in the circulating hemocytes (Table 1). In decreasing order of relative Pox expression circulating hemocytes » gills » heart » lymphoid organ » hepatopancreas  $\approx$  muscle. Every tissue was significantly different from every other tissue in the

**Table 1**  
Comparisons between tissues in the expression of peroxinectin.

Tissue	Pox transcripts	<i>n</i>	Tissue differences	<i>Vibrio</i> vs. control, <i>p</i>
Circulating hemocytes	112 451 $\pm$ 13 440	70	A	0.63
Gill	67 589 $\pm$ 6760	61	B	<b>0.03</b>
Heart	2629 $\pm$ 331	63	C	<b>0.04</b>
Lymphoid organ	1406 $\pm$ 180	65	D	0.87
Muscle (injection site)	451 $\pm$ 135	60	E	<b>0.00</b>
Hepatopancreas	151 $\pm$ 29	48	E	0.06

Normalized Pox transcripts (ng total RNA<sup>-1</sup>) (mean  $\pm$  SE) are presented in decreasing order. Sample sizes per tissue are indicated in the column,  $n = (\sum n = 367)$ . Different letters (A–E) indicate significant differences between tissues detected by Holm–Sidak multiple comparisons after the three-factor analysis of variance. For example, circulating hemocytes (A) are significantly different ( $p \leq 0.05$ ) than all other tissues in their expression of Pox. Two-factor analysis of variance was used to assess significant differences ( $p \leq 0.05$ ) within tissues between *Vibrio*- and saline-injected animals (also see Figs. 2–4).

**Table 2**  
Analytical statistics associated with peroxinectin expression.

Fixed factor	Levene's test, <i>p</i>	Bartlett's test, <i>p</i>
Tissue	0.000	0.000
Treatment	0.851	0.828
Time-point	0.933	0.983

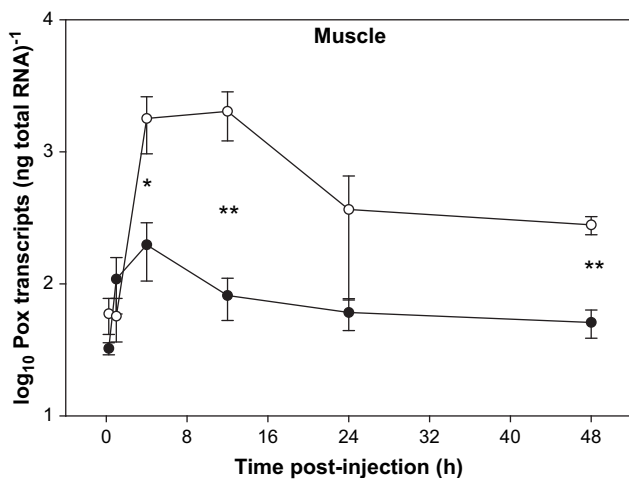
Preliminary analyses indicated that the experimental data had unequal variance (Levene's test) and a non-normal distribution (Bartlett's test), two of the underlying assumptions of the analysis of variance, despite  $\log_{10}$  transformation. Removal of Pox data from muscle tissue revealed that it was responsible for these violations. Nonparametric Kruskal–Wallis test on ranks for tissue and time-point, and the Mann–Whitney rank sum test on treatment support the findings of the three-factor analysis (data not shown), including the muscle tissue.

expression of Pox ( $p < 0.05$ ) with the exception of muscle at the site of injection and hepatopancreas ( $p = 0.568$ ).

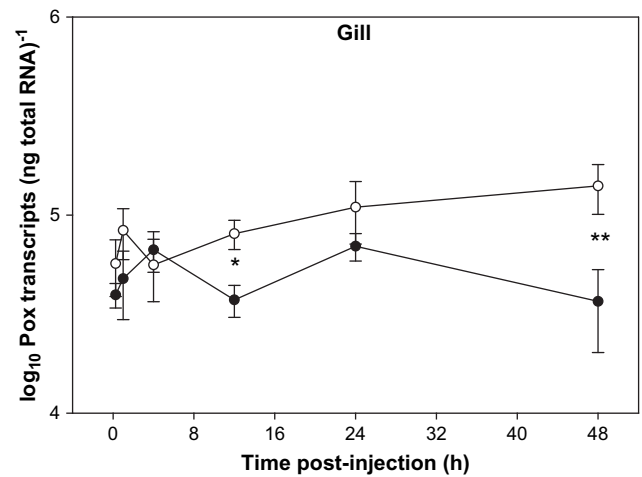
### 3.2. High peroxinectin expression occurs at the site of injection and in the gills of *Vibrio*-injected shrimp

Injection of bacteria into the tail muscle of *L. vannamei* induced a rapid increase in Pox mRNA at the site of injection (Fig. 2). Pox transcripts significantly increased within 4 h after injection of *V. campbellii* as compared to values at earlier time-points of 0.25 h ( $p = 0.011$ ) and 1 h ( $p = 0.003$ ) post-injection. Saline also produced a significant increase in Pox transcripts at the site of injection; however, the magnitude and duration of the response to saline was substantially lower than that to bacteria. By 12 h after injection Pox transcripts at the injection site were significantly higher in animals dosed with bacteria than in those treated with saline ( $p = 0.001$ ). This large difference in Pox transcripts at the injection site between *Vibrio*- and saline-injected animals persisted until the conclusion of the experiment at 48 h. Pox transcripts significantly increased in the gills of *Vibrio*-injected shrimp by 12 h compared to controls ( $p \leq 0.05$ ) and the difference persisted at 48 h after injection (Fig. 3).

In contrast, the expression of Pox in circulating hemocytes and lymphoid organ did not differ between saline- and *Vibrio*-injected animals and the relative numbers of Pox transcripts remained



**Fig. 2.** Time-course of peroxinectin (Pox) expression in tail muscle from the site of injection. Closed circles (●) represent shrimp that received a control injection (saline;  $n = 31$ , 4–6 animals per time-point) and open circles (○) represent *Vibrio*-injected animals ( $n = 29$ , 3–6 per time-point). Real-time PCR calculated  $\log_{10}$  Pox transcripts ( $\text{ng total RNA}^{-1} \pm \text{SE}$ ) are shown from 0.25 to 48 h after control (saline) or *Vibrio campbellii* injection with  $2 \times 10^4$  CFU ( $\text{g shrimp}^{-1}$ ). Transcripts rapidly accumulate at the site of injection and levels remain elevated in shrimp that received *Vibrio* injection. \* indicate  $p \leq 0.05$ , \*\* $p \leq 0.01$ .



**Fig. 3.** Time-course of peroxinectin (Pox) expression in gills. Real-time PCR calculated  $\log_{10}$  Pox transcripts ( $\text{ng total RNA}^{-1} \pm \text{SE}$ ) are shown from 0.25 to 48 h after control (saline) or *Vibrio campbellii* injection with  $2 \times 10^4$  CFU ( $\text{g shrimp}^{-1}$ ). Closed circles (●) represent shrimp that received a control injection (saline;  $n = 29$ , 4–6 animals per time-point) and open circles (○) represent *Vibrio*-injected animals ( $n = 32$ , 4–6 per time-point). Gills from *Vibrio*-injected shrimp had significantly higher Pox expression by 12 h post-infection, compared to control shrimp.

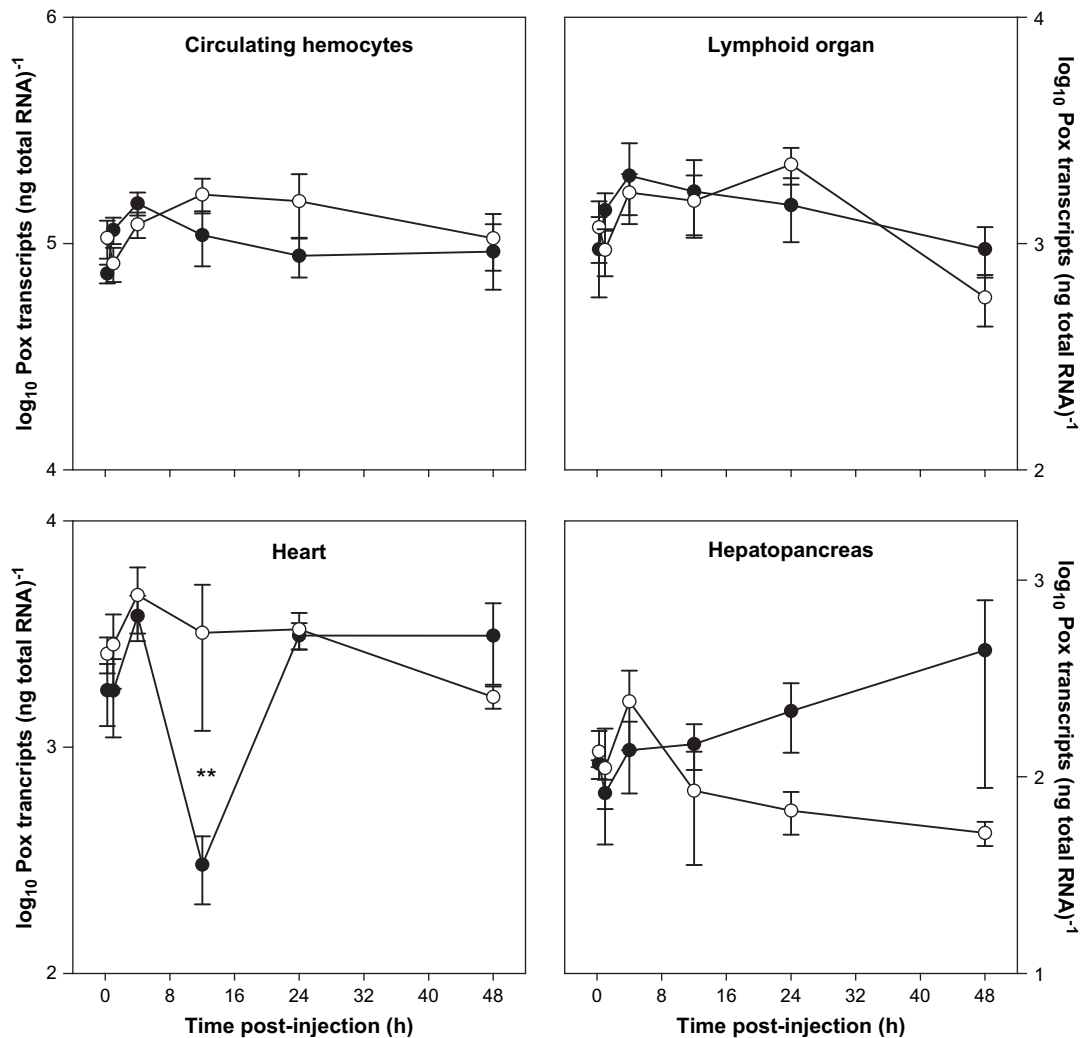
stable throughout the 48 h experiment (Fig. 4). A transitory decrease in Pox expression was noted in the heart at 12 h post-injection of bacteria. Pox transcripts in the hepatopancreas displayed a pattern unlike other tissues, although the changes were not significant. In general, animals that received *Vibrio* injections expressed higher numbers of Pox transcripts (Figs. 2–4), although the hepatopancreas does not fit this general trend.

As noted in Burge et al. [18], total circulating hemocytes in both saline- and *Vibrio*-injected animals significantly declined ( $p < 0.01$ ) during the time-course of the experiment, with animals experiencing a total decline of 23 and 63% respectively. Total Hemocyte Counts (THC)  $\text{mL}^{-1}$  in saline-injected shrimp reached its minimum value by 4 h after injection, and did not decline further. In contrast, THC in *Vibrio*-dosed animals continued to decline throughout the course of the exposure period.

## 4. Discussion

This study documents that peroxinectin mRNA can be quantified in many tissues of *L. vannamei* (Table 1) and reveals tissue-specific changes in Pox expression following a sublethal dose of pathogenic *V. campbellii* (Figs. 2–4). Building on the observation of Liu et al. [21], that Pox expression is confined to granular and semigranular hemocytes, the present work is important because it provides a method to infer the trafficking of hemocytes among individual organs and tissues. This approach is amenable across a broad array of gene transcripts providing that they are specifically expressed by hemocytes and important for the immune response. Together with our earlier report on changes in lysozyme expression in the same tissues [18], these present results support the idea of tissue-specific roles for encapsulation vs. phagocytosis/bacterial killing in the crustacean immune response.

In the current work, hemocyte infiltration to the injection site, as inferred from the increase in Pox transcripts, happened rapidly, within 1 h after injection in both saline- and *Vibrio*-injected animals, but subsequent declines in Pox transcripts of control animals (1–4 h post-injection) suggest that the presence of bacteria at the wound was necessary to retain these hemocytes at that site (Fig. 2). Lysozyme mRNA levels also significantly increased at the



**Fig. 4.** Time-course of peroxinectin (Pox) expression in other tissues and circulating hemocytes. Real-time PCR calculated  $\log_{10}$  Pox transcripts (ng total RNA)<sup>-1</sup>  $\pm$  SE are shown from 0.25 to 48 h after control (saline) or *Vibrio campbellii* injection with  $2 \times 10^4$  CFU (g shrimp<sup>-1</sup>). Closed circles (●) represent shrimp that received a control injection (saline; hemocytes  $n = 35$ , heart  $n = 32$ , lymphoid organ  $n = 34$ , hepatopancreas  $n = 25$ ; 3–7 animals per time-point and tissue) and open circles (○) represent *Vibrio*-injected animals (hemocytes  $n = 35$ , heart  $n = 31$ , lymphoid organ  $n = 31$ , hepatopancreas  $n = 23$ , 3–7 per time-point and tissue). Although circulating hemocytes express the highest absolute amount of Pox (Table 1) there was no effect on expression due to injection ( $p = 0.63$ ) of bacteria. \*\* $p \leq 0.01$ . Note the difference in scale between different tissues.

site of injection in shrimp, but were not significantly different between saline- and *Vibrio*-injected shrimp until 12 h post-injection [18]. Other authors [19,20,25], primarily using immunolocalization or in situ hybridization, have reported the accumulation of hemocytes in tissues in response to injury or infection, but these new data provide a quantitative portrait of the recruitment of circulating hemocytes through time and in multiple tissues simultaneously.

These expression data demonstrate that a rapid and persistent increase in Pox transcripts at the site of injection of bacteria is associated with rapid and sustained recruitment of hemocytes to that location. Once localized within tissues, circulating hemocytes undergo a series of changes that render them more phagocytic [26] and induce them to degranulate to release immune effectors, including peroxinectin [5]. Indeed, the presence of peroxinectin protein in the hemolymph enhances degranulation and exocytosis by hemocytes in crayfish and results in the release of similar proteins to those stimulated by treatment with the calcium ionophore A23187. Johansson et al. [7] had previously suggested that the release of peroxinectin may act as a local recruiter of hemocytes to sites of inflammation, infection or wounds in the crayfish. van de

Braak et al. [25] and Burgents et al. [34] found that encapsulation of bacteria at an intramuscular injection site was nearly immediate. The latter study reported that approximately 50% of the injection dose of *V. campbellii* delivered to the shrimp *L. vannamei* was sequestered at that site within 15 min. Intact bacteria persisted at the site of injection for longer than 4 h [34] and circulating hemocytes in the hemolymph were not associated with free bacteria or bacterial clumps for times between 5 min and 1 week after injection of live bacteria [25]. The recruitment of hemocytes to the presence of bacteria, and the rapid increase in Pox levels supports a recent report by Haine et al. [35] in insects that constitutive defences based on hemocyte mediated responses and regulated enzymatic cascades are critical “front-line” defences against bacteria.

Encapsulation, and subsequent elimination of bacteria and particles, also appears to be an important function of the crustacean gill [36–40], but different microbial challenges and species of crustaceans appear to differ in the degree to which the gill is important for bacterial capture or elimination [25,34]. Elimination of material begins with aggregation and encapsulation that is likely to be mediated, at least in part, by Pox protein exocytosed by

hemocytes within the gill. In fact, this aggregation and encapsulation begins within 10 min of injection of inert particles into several species of crustaceans [36]. The data in the present study indicate that levels of Pox mRNA are uniformly high in the gills of shrimp (Table 1) and that significantly higher numbers of transcripts are detected in the gills of shrimp that have been injected with bacteria (Fig. 3). In contrast, lysozyme transcripts in saline- or *Vibrio*-injected animals do not change significantly in the gills [18].

The lymphoid organ is important in penaeid shrimp for filtration of the hemolymph [25,26,41] and may be important for the accumulation (and/or inactivation) of intact, but nonculturable bacteria [34]. Given this role in immunity, it is perhaps surprising that Pox transcripts were few in number in this organ (Fig. 4), while lysozyme transcripts are detected at high levels ( $\sim 10^6$  transcripts ng total RNA<sup>-1</sup>) relative to other tissues ( $\sim 10^4$ – $10^5$  transcripts ng total RNA<sup>-1</sup>) [18]. Lysozymes function to break down cell walls of bacteria [42] and it is within the lymphoid organ of shrimp that the highest levels of bacteriostasis [34] and degraded bacterial antigens are found [25]. The different functional activities of the two proteins and their respective patterns of temporal expression suggest that encapsulation at the wound is of the highest priority (hence high Pox expression here) and that bacteria that evade encapsulation and/or become free in the hemolymph are subsequently taken up at the lymphoid organ, where high expression of lysozyme [18] likely enhances bacteriostasis [34], again supporting the contention that the arthropod immune response contains two phases, an initial constitutive defence based on hemocytes and regulated enzymatic cascades, and a secondary antimicrobial phase to completely clear infection [35]. Given the data presented here and in van de Braak et al. [25], encapsulation does not appear to be an important function that occurs in this tissue, and this is a likely explanation for the low levels of Pox expression in the lymphoid organ.

It has been previously shown that lysozyme transcription is present in all hemocyte subtypes [18], while that of Pox is confined to granular and semigranular cells [21]. It seems increasingly likely that peroxinectin plays a key role in the immediate sequestration by encapsulation of materials that enter the body, and that the detection of Pox transcript differences between tissues is a reflection of this role in immediate innate response.

Data in this study do not directly provide insight on the relative amounts of Pox message 'pre-packaged' and delivered by recruited hemocytes, compared to that synthesized *de novo* at the site of insult, however, data presented by others suggest that the messages delivered by hemocytes are the bulk of that measured in the present study. Sritunyalucksana et al. [9] determined that in *P. monodon* Pox is constitutively expressed in the hemocytes, but not in hepatopancreas, and those transcripts of Pox decrease in circulating hemocytes following systemic challenge with LPS or laminarin. They noted that the most likely explanation for this is a decline in the number of Pox-expressing cells associated with the treatments. In a similar study by Destoumieux et al. [19], penaeidin expression followed a similar pattern that was attributed to loss of hemocytes from circulation. Thus, it seems likely that the changes in Pox levels seen between *Vibrio*- and saline-injected shrimp are the result of the presence of hemocytes carrying their complement of transcripts, and not transcription of new Pox within minutes to hours of bacterial introduction. Measurement of transcripts in circulating hemocytes supports this view.

There was no significant difference in the numbers of transcripts (ng total RNA<sup>-1</sup>) detected in circulating hemocytes at any time-point that was associated with bacterial injection (Fig. 4), although at all time-points after 4 h, *V. campbellii* challenged shrimp have higher mean levels of Pox than do saline-injected ones. In a comparable study examining Pox expression in *L. vannamei*, Liu

et al. [21] report that injection of *Vibrio alginolyticus* into the ventral sinus of shrimp at 5000 CFU shrimp<sup>-1</sup> causes circulating hemocytes to produce more Pox transcripts with a difference of approximately 1–2 real-time PCR cycles between *Vibrio*- and saline-injected shrimp during the time-course (see Figs. 5 and 6 in [21]). The results of Liu et al. [21] and data from this study differs with regards to expression of Pox in circulating hemocytes of the hemolymph; however the dose, *Vibrio* species used, and location of challenge delivery are not the same between Liu et al. [21] and the results presented here.

In summary, the present study examined the distribution of peroxinectin transcripts by quantitative real-time PCR in the shrimp *L. vannamei* after injection with a pathogenic *Vibrio*. The results clearly indicate that hemocytes rapidly traffic to the site of an intramuscular injection. It is unclear, however, whether additional cells continue to arrive at that location over the 48 h time-course, or if present hemocytes upregulate transcription of Pox to continue responding to bacteria. From these data it can also be inferred that high levels of Pox detected in the gills of shrimp are important for encapsulation of bacteria that leads to their elimination. In contrast, low levels of Pox compared to lysozyme transcript in the lymphoid organ indicate the importance of this tissue in removal and bacteriostasis of invasive bacteria. Thus, the measurement of hemocyte-specific gene transcripts provides a method to quantitatively assess hemocyte trafficking during the course of a successful immune response to bacteria.

Work from a number of authors has indicated that Pox is transcribed by circulating hemocytes [9,10,27–29], but no previous reports have detected expression in whole tissues. It is a subtle but important distinction that all tissues, using a suitably sensitive assay, contain transcripts for Pox. It is highly likely that these transcripts represent hemocytes contained within those tissues at the time of detection and that a fuller understanding of the expression and function of this gene must include a battery of tissues in order to increase our understanding of the systemic immune response in crustaceans.

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