Elevated water temperature increases the levels of reo-like virus and selected innate immunity genes in hemocytes and hepatopancreas of adult female blue crab, *Callinectes sapidus*

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Abbreviations: CsRLV: *C. sapidus* reo-like virus, ecCuZnSOD-2: extracellular CuZn superoxide dismutase-2, TPS: trehalose 6-phosphate synthase; PPO: prophenoloxidase; LpR: lipoprotein receptor; GlyP: glycogen phosphorylase

Abstract

Seasonal changes in water temperature directly affect the aquatic ecosystem. The blue crab, *Callinectes sapidus*, inhabiting the Chesapeake Bay has been adapted to seasonal changes of the environmental conditions. In this, the animals halt their physiological process of the growth and reproduction during colder months while they resume these processes as water temperatures increase. We aimed to understand the effect of the elevated temperatures on a disease progression of reo-like virus (CsRLV) and innate immunity of adult female *C. sapidus*. Following a rise in water temperature from 10 to 23^{o}C, CsRLV levels in infected crabs rose significantly in hemocytes and multiple organs. However, in hemocytes, the elevated temperature had no effect on the levels of three innate immune genes: *Cas-ecCuZnSOD-2*, *CasPPO* and *CasLp*R three carbohydrate metabolic genes: *CasTPS*, *CasGlyP*; and *CasTreh* and the total hemocyte counts (THC). Interestingly, the hemocytes of CsRLV infected animals exposed to 23^{o}C for 10 days had significantly elevated levels of *Cas-ecCuZnSOD-2 and CasTPS*, compared to those of the uninfected ones also exposed to the same condition and compared to hatchery-raised females kept at 23^{o}C. Despite the lack of changes in THC, the types of hemocytes from the animals with high CsRLV levels differed from those of uninfected ones and from hatchery animals kept at 23^{o}C: CsRLV-infected crabs had hemocytes of smaller size with less cytosolic complexity than uninfected crabs. It therefore appears that the change in temperature influences rapid replication of CsRLV in all internal tissues examined. This implies that CsRLV may have broad tissue tropism. Interestingly, the digestive tract (mid- and hindgut) contains significantly higher levels of CsRLV than hemocytes while hepatopancreas and ovary have lower levels than hemocytes. Innate immune responses differ by tissue: midgut and hepatopancreas with upregulated *Cas-ecCuZnSOD-2* similar to that found in hemocytes. By contrast, hepatopancreas showed a down-regulated *CasTPS*, suggesting carbohydrate stress during infection.

1. Introduction

Season affects the physiology and behavior of most animals, including decapod crustaceans, via changes in environmental factors. The growth and reproduction of poikilothermic vertebrates and invertebrates usually depends most strongly on temperature. In the Chesapeake Bay, the blue crab *Callinectes sapidus* including adult females experiencing low water temperatures during the overwintering period responds to the elevated water temperature in the spring. These adult females at various ovarian development stages increase the levels of vitellogenin (VtG) expression in the hepatopancreas and ovary [1]. In the fall, decreasing water temperature slows the vitellogenic activities in these tissues. During overwintering, adult females show high winter mortality rates, indicating that they may be sensitive to lower temperature [2]. The health status of these dead animals is unknown but it is assumed that their reproductive activity is halted due to low water temperature.

The susceptibility to a disease(s) depends on the initial health status of an individual animal. Stress is intimately associated with predisposition toward infection. It has been well-established in decapod crustaceans that changes in environmental conditions including temperature, salinity and dissolved oxygen at extremes are often considered as stressors and are directly reflected in the changes in physiological and metabolic processes [3-9].

In the blue crab, a reo-like virus (CsRLV) has been described that infects mesodermally- and ectodermally-derived tissues, especially hemocytes and epidermal tissues [10, 11]. CsRLV is associated with the majority of mortality in captive molting crabs from shedding facilities, and CsRLV prevalence in wild intermolt crabs is reported to average 20% [12]. In decapod crustaceans, molting and reproduction are high energy demanding processes that are mutually antagonistic [13]. Therefore, although the mode of infection of this virus to blue crabs has not yet been defined, the inherent changes in physiology of the animals at molting or active ovarian development may be a predisposing factor to CsRLV infection.

We investigated how a shift from low to moderate temperature affects disease manifestation and progression, i.e., CsRLV in *C. sapidus* adult females that had been overwintered at water temperatures lower than 10^{o}C. Specifically we aimed to further understand how the animals responded to temperature changes by analyzing the levels of following gene transcripts that are known to be changed due to pathogenic challenges [14-17]:

prophenoloxidase=PPO; lipoprotein receptor= LpR; extracelluar CuZn superoxide dismutase-2= ecCuZnSOD-2, compared to the animals kept at constant temperatures. The genes involved in carbohydrate energy metabolism (*Glycogen phosphorylase =GlyP, Trehalose 6-phosphate synthase =TPS* and trehalase=*Treh*) were also examined, since temperature changes affect the energy metabolism of these animals [3]. Additionally, we examined levels of CsRLV in various internal tissues.

Here we report that elevated temperature is followed by increased levels of CsRLV in the infected crabs, as well as changes in the levels of immune-response and metabolic genes. Elevated temperature has no effect on the expression levels of these genes in the animals without CsRLV, as compared to those of the animals kept at constant temperatures.

2. Materials and Methods

2.1 Female brood stock collection

Adult vitellogenic female crabs (20 in total, carapace width (CW) 110-125 mm, 130 ± 12g) were obtained from the 2013-4 winter dredge survey (http://dnr.maryland.gov/fisheries/crab/dredge.asp) by the Virginia Institute of Marine Science (VIMS). The animals were kept in the indoor-tank at ~10^{o}C for two weeks. Each individual animal was wrapped in a wetted newspaper, and transported in a cooler.

2.2 Temperature exposure

The animals were kept individually in a pathogen-free quarantine room in the Aquaculture Research Center at the Institute of Marine and Environmental Technology (IMET, Baltimore, MD) in 30 ppt artificial seawater (ASW) (16L: 8D) at ambient room temperature (21-23˚C). Animals were fed a piece of squid (~10g) and monitored for daily food consumption and behavioral activity during the sampling period. Water was exchanged at least once a day.

Seven hatchery-raised adult females (110-130 mm CW, 118 ± 8g) that had not experienced low temperatures, were used as reference animals [18].

2.3 Flow cytometric analysis of the hemocytes of C. sapidus

Hemolymph samplings were carried out on the animals exposed to 23^{o}C for 10 days. All hemolymphs were collected between 3-5:00 pm, in order to avoid possible variation in the physiology. The hemolymph was withdrawn directly into an insulin syringe containing 50 µl of 4% paraformaldehyde (PFA, made in PBS) at a 1:1 ratio. The hemocytes were separated by

centrifugation at 800 g for 10 min at 4^{o}C and re-suspended in 100 µl of 4% PFA, and then stained with SYBR-Green I (Life Technologies) for 15 min at room temperature with rotation (10 rpm) in the darkness. Hemocytes were counted and analyzed based on their sizes (forward scatter; FSC) and cellular granularity (side scatter; SSC) using a BD Accuri™ C6 and CellQuest Program (BD Biosciences). The data for total hemocyte count (THC) are presented as THC/ ml hemolymph (n), where n is the number of animals.

2.4 A reo-like virus (CsRLV) test using qPCR assay

2.4.1. Hemocytes

At 23^{o}C exposure for 1, 10 and 15 days, the hemolymph sample (100 µl) was drawn from the arthrodial membrane, located between the first walking leg and the chela, directly into an insulin syringe containing 100 µl of anticoagulant (30mM trisodium citrate, 26mM citric acid, 0.1M glucose, 10mM EDTA, pH. 4.6 [19]). Sample tubes were placed immediately on dry-ice and kept at -80^{o}C until further processing. Total RNA was extracted from these hemolymph samples using Qiazol (Qiagen) as previously described [17, 20]. The quality and quantity of RNA was estimated on a Nanodrop spectrometer (FisherSci). The levels of CsRLV RNA present in the total RNA (=equivalent of 2.5 % of hemolymph) were assayed in triplicate using a one-step RT-qPCR assay with TaqMan® Fast Virus 1-Step Master Mix (Life Technologies) using the primers listed in Table 1 [11]. Standards for the CsRLV qPCR assay were prepared as described [12] ranging from 10 to 10^{6} copies. The data are presented as mean ± SE copies/100 µl hemolymph (n) where n is the number of females.

2.4.2 Tissues

To examine the levels of CsRLV in internal tissues, hemolymphs were collected as above before the following tissues (~50 -100 mg wet weight) were dissected on day 17 from the females that had been identified with an CsRLV infection on day 15, and from those that were raised in the hatchery: eyestalk, brain, thoracic ganglia complex, mandibular organ, midgut, hindgut, hepatopancreas, gill, abdominal muscle, antennal gland, ovary, spermathecae, hypodermis and heart. These tissue samples were frozen immediately on dry-ice and kept at -80^{o}C, until further processing. The procedure for total RNA extraction and its quantification was carried out as described [17, 20]. The infection levels of CsRLV were examined with total RNA (100 ng) using qPCR assays as above. All samples were assayed in triplicate. The data are shown as mean ± SE copies/µg total RNA (n), where n is the number of females.

2.5. Hematodinium sp. test using a qPCR assay

The procedures for DNA extraction using a FastDNA kit (Qbiogene) and qPCR analysis from all the hemolymph samples were as described [21]. In brief, PCR primers listed in Table 1 were designed targeting the reported sequence the *Hematodinium sp*. 18S rRNA: forward primer (5'-GGTAATCTTCTGAAAACGCATCGT-3'); reverse primer (5'-GTACAAAGGGCAGG GACGTAATC-3'). For qPCR assay, an end-labeled fluorescent probe (5'-6FAM-AATTCCTAGTAAGCGCGAGTCATCAGCTCG-3') was used using TaqMan Universal Master Mix (Applied Biosystems). All samples were assayed in triplicate.

2.6 Expression analysis using qPCR assays

One to two µg of total RNA (from hemocytes from the animals exposed to 23^{o}C for 10 days and other tissues obtained on day 17) was subjected to 1st strand cDNA synthesis using a Takara PrimeScript® Kit by following the manufacturer's instruction. Prior to qPCR assays for expression analysis, the expression levels of arginine kinase were estimated by an end-point RT-PCR assay using PCR conditions as reported [17, 20]. Each cDNA sample containing 25 ng equivalent of total RNA was evaluated in duplicate.

Levels of expression for the following genes were determined using qRT-PCR assays as described [14, 16, 17, 20]: *ecCuZnSOD-2*, *TPS*, *glycogen phosphorylase* (*GlyP*), trehalase (*Treh*), *prophenoloxidase* (*PPO*) and *lipoprotein receptor* (*LpR*). Standard curves for these assays were obtained as described [14, 16, 17, 20]. The data are presented as copies/µg total RNA (n), where n is the number of animals.

2.7 Statistical analysis

The data were subjected to the normality test using a Kolmogorov-Smirnov test and homogeneity of variances test with a Cochran C test. The data that did not meet the parametric analysis were analyzed with a non-parametric test. Kruskal-Wallis ANOVA and Median test were used to evaluate the statistical difference (InStat). Statistical significance of one way ANOVA was accepted at $P < 0.05$ and post-hoc Tukey's test was adopted to distinguish the source of variation. The data are presented as mean ± SE (n), where n is the number of animals.

3. Results

3.1 Levels of a CsRLV in hemolymph samples

CsRLV levels were estimated from hemolymph samples collected at 10^{o} C and after 10 and 15 days at 23^{o}C (Fig. 1). At day 1, the lowest levels of CsRLV are detected in the hemolymph of two animals with average values of 3.8 ± 0.16 x 10^{6} copies/ 100 µl hemolymph (n=2). At 23^{o}C for 10 days exposure, the highest levels of CsRLV are found with 4.7 ± 1.9 X 10^{8} copies/100 µl hemolymph (n=6). Prior to day 15 sampling, one of these animals died and the other was sacrificed. The remaining four females carried reduced amounts (*P*=0.125, Wilcoxson matched pairs test) of CsRLV in their hemolymph: 9.1 ± 5.4 X 10^{7} copies/ 100 µl hemolymph (n=4). The hatchery raise animals kept at 23^{o}C had no CsRLV in their hemocytes.

3.2 Levels of total hemocyte count

Because the highest levels of CsRLV were detected in the crabs held at 23^{o} C for 10 days, the total hemocyte counts (THC) were determined to see if these high levels of CsRLV might affect THC. Elevated temperature has no significant (*P*= 0.14, One Way, ANOVA, Krustal-Wallis test) effect on THC of the animals with high CsRLV (4.2 ± 1.4 X 10^{6}/ ml hemolymph, n=5) as compared to those without CsRLV (7.6 ± 0.8 X 10^{6}/ ml hemolymph, n=12) and hatchery-raised animals kept at constant 23^{o} C (6.6 ± 1.2 X 10^{6}/ ml hemolymph, n=7).

The size of hemocytes was also measured and was grouped into two size classes using flow cytometry (Figs. 2A-C and D, FSC-H): small for hyaline cells (closed arrow) and large for granulocytes and semigranulocytes (open arrow). CsRLV infection status had a significant effect on the size distribution of hemocytes, but temperature did not. Granulocytes and semigranulocytes are the majority in both the hatchery-raised crabs kept at constant 23^{o} C (Fig. 2A) and in animals without CsRLV shifted from 10˚ C to 23^{o} C for 10 days exposure (Fig. 2B), with 89.2 ± 0.9 % (n=7) and 83.6 ± 3.5 % (n=12) of the cell populations, respectively (Table 2). The animals with CsRLV at 23^{o} C for 10 days exposure, however, contained significantly fewer granulocytes and semigranulocytes than the other two groups (*P* <0.05), with a total of 64.1 ± 11.2 % (n=5). The hatchery-raised animals kept at constant 23^{o} C, and the animals without CsRLV shifted to 23^{o} C for 10 days carried 7.5 ± 0.6 % (n= 7) and 11.4 ± 2.4 % (n=12) of hyaline cells (Table 2). On the other hand, the animals with CsRLV at 23^{o} C for 10 days exposure had significantly higher portions of hyaline cells with 25.8 ± 8.2 % (n=5, *P* <0.05) (Fig. 2C and Table 2).

Elevated temperature had no effect of the SSC-H of the animals kept at constant at 23^{o}C and those elevated to 23^{o}C for 10 days, reflecting cytosolic complexity and granularity that differentiate three groups of cells in all three groups (Figs. 2A'-D'). Non-infected and control crabs share a similar pattern (Figs. 2A' and B' and D and D'), while a notably different pattern is found only with the animals carrying CsRLV at 23^{o} C for 10 days exposure (Figs. 2C', D and D'). Specifically, the hemocytes obtained from the latter group include higher numbers of cells with less internal complexity (Figs. 2C' and D').

3.3 Expression analyses of Cas-ecCuZnSOD-2, CasPPO, and CasLpR in hemocytes

The following three immune response genes in hemocytes were assessed using qRT-PCR assays: *Cas-ecCuZnSOD-2*, *CasPPO*, and *CasLpR*. These were chosen due to their known elevation in response to lipopolysaccharide (LPS) injection [16, 17, 20]. When comparing hatchery-raised crabs to non-infected wild crabs, elevated temperature did not influence the levels of *Cas-ecCuZnSOD-2*, *CasPPO*, or *CasLpR* in hemocytes. However animals carrying CsRLV did have higher expression levels of *Cas-ecCuZnSOD-2* in their hemocytes, as compared to the other two groups.

The levels of *Cas-ecCuZnSOD-2* in animals without CsRLV, and in hatchery-raised crabs, were: 6.5 ± 2.3 X 10^{6} copies/ µg hemocyte total RNA (n=12) and 9.0 ± 6.7 X10^{6} copies/ µg hemocyte total RNA (n=7), respectively (Fig. 3A). However, the animals carrying CsRLV at 23^{o}C for 10 days exposure contain levels10-20 times elevated in the hemocytes (1.7 ± 0.5 X10^{8} copies/ µg total RNA, n=5), as compared to the other two groups.

In regard to the levels of *CasPPO* (Fig. 3B), the levels of *CasPPO* of the animals with no CsRLV at 23^{o}C for 10 days exposure and those kept constant at 23^{o}C: 1.2 ± 0.3 X 10^{7} copies/ µg hemocyte total RNA (n=12) and 9.7 ± 6.7 X 10^{6} copies/ µg hemocyte total RNA (n=7), respectively. However, the animals carrying CsRLV at 23^{o}C for 10 days exposure contained slightly higher levels: 2.2 ± 0.5 X 10^{7} copies/ µg hemocyte total RNA (n=5), but these values do not differ significantly from those obtained from the other groups.

Elevate temperature or CsRLV had no effect on the levels of *CasLpR* expression in hemocytes (Fig. 3C). The animals without CsRLV at 23^{o}C for 10 days exposure are measured

with 2.5 ± 0.7 X 10^5 copies/ µg hemocyte total RNA (n=12), while those carrying CsRLV at the same condition with 6.9 ± 3.0 X 10^5 copies/ µg hemocyte total RNA (n=5). The animals kept constant at 23oC are determined with 4.1 ± 2.4 X 10^5 copies/ µg hemocyte total RNA (n=7).

3.4 Expression analyses of CasTPS, CasTreh and GlyP in the hemocytes

Expression of three genes that regulate carbohydrate metabolism in crustaceans were also measured in hemocytes: *CasTPS, CasTreh*, and *CasGlyP* (Figs. 4A-C). Elevated temperature had no effect on the levels of *CasTPS, CasTreh*, and *CasGlyP* in hemocytes. The animals carrying CsRLV contain higher expression levels of *CasTPS*, compared to the other two groups.

The levels of *CasTPS* of the animals without CsRLV at 23^{o}C for 10 days exposure and those kept constant at 23^{o}C was 2.8 ± 0.8 X 10^6 copies/ µg hemocyte total RNA (n=12) and 3.1 ± 1.0 X 10^6 copies/ µg hemocyte total RNA (n=7), respectively (Fig. 4A). However, the animals carrying CsRLV at 23^{o}C for 10 days exposure contained significantly elevated levels of *CasTPS* in the hemocytes (7.3 ± 1.6 X 10^6 copies/ µg total RNA, n=5), as compared to the other two groups.

Levels of *CasTreh* expression shown in Fig.4B do not differ in the animals with or without CsRLV at 23^o C for 10 days exposure and those kept constant at 23^{o}C. The hemocyte cDNAs of the animals without CsRLV at 23^{o}C for 10 days exposure contain 8.5 ± 3.0 X 10^5 copies/ µg hemocyte total RNA (n=12); those with CsRLV (1.0 ± 2.7 X 10^6 copies/ µg hemocyte total RNA, n=5); and those kept constant at 23^o C (1.5 ± 0.8 X 10^6 copies/ µg hemocyte total RNA, n=7).

For levels of *CasGlyP*, its expression patterns show similar to *CasLpR* shown as in Fig. 3C. Elevated temperature or CsRLV had no effect on the levels of *CasGlyP* expression in hemocytes (Fig. 4C). The animals without CsRLV at 23^{o}C for 10 days exposure are measured with 8.5 ± 3.7 X 10^5 copies/ µg hemocyte total RNA (n=12), while those carrying CsRLV at the same condition with 1.1 X 10^6 copies/ µg hemocyte total RNA (n=5). The animals kept constant at 23^{o}C are estimated with 1.5 ± 0.7 X 10^6 copies/ µg hemocyte total RNA (n=7).

3.5 Differential CsRLV levels in various internal tissues of adult female C. sapidus

High levels of CsRLV RNA in hemocytes suggest that other internal organs may be infected with CsRLV. Fourteen different internal organs were dissected on day 17, which represent five physiologically defined functions including the central nervous system (brain, eyestalk and thoracic ganglia complex) and one endocrine tissue; the mandibular organ; the digestive system (midgut, hindgut, and hepatopancreas); the excretory system (gill and antennal gland); the circulatory system (heart); and finally the reproductive system (ovary and spermathecae). The hypodermis and abdominal muscle were also included.

In agreement with previous work [10], there was broad tissue tropism, but surprisingly most tissues contained higher levels of CsRLV than in hemocytes (Fig. 5). As CsRLV infection is known to progress to the CNS, neurological tissues in this study (eyestalk and brain) as expected had higher levels of CsRLV than in hemocytes. Interestingly, the highest levels were observed in midgut with $4.1 \pm 2.6 \times 10^9$ copies/µg total RNA (n=4). Only two tissues showed lower levels of CsRLV: the hepatopancreas with $4.5 \pm 3.1 \times 10^7$ copies/µg total RNA and ovary with $3.6 \pm 2.1 \times 10^7$ copies/µg total RNA.

3.6 Expression analyses of Cas-ecCuZnSOD-2 and CasTPS in the digestive system and antennal gland of CsRLV-carrying adult female C. sapidus

3.6.1 Cas-ecCuZnSOD-2

Since the elevated temperature had no effect on hemocyte populations (Figs. 2A and B), the animals kept at constant 23°C were used as controls and data are compared to as CsRLV infected crabs. The cDNA samples derived from hypodermis, antennal gland and digestive system including midgut, hindgut, and hepatopancreas of CsRLV-positive animals maintained at 23°C for 17 days and CsRLV-negative controls were assessed for expression *of Cas-ecCuZnSOD-2*. As shown in Fig. 6A, hepatopancreas, which had lower levels of CsRLV than in hemocytes, contained levels of *Cas-ecCuZnSODec-2* significantly ($P = 0.002$) higher in CsRLV infected animals ($3.8 \pm 2.2 \times 10^6$ copies/µg total RNA, n=4) than in controls ($9.3 \pm 3.0 \times 10^4$ copies/µg total RNA, n=7). However, the hindgut of CsRLV infected animals also shows higher levels of *Cas-ecCuZnSOD-2* ($4.7 \pm 1.9 \times 10^6$ copies/µg total RNA, n=4) than controls ($4.3 \pm 0.9 \times 10^5$ copies/µg total RNA, n=7). In other tissues (midgut, hypodermis and antennal gland) there are no significant differences in the levels of *Cas-ecCuZnSOD-2* expression between CsRLV infected and controls.

3.6.2 CasTPS

As shown in Fig. 6B, among the tissues examined from hatchery-raised animals, the hypodermis has the lowest *CasTPS* expression: 1.9 ± 1.2 X 10^3 copies/ µg total RNA (n=7). The antennal gland, however, contains the highest *CasTPS* expression: 2.3 ± 1.0 X 10^5 copies/ µg total RNA (n=7). The hepatopancreas shows a significant difference in the levels of *CasTPS* between CsRLV infected and control females. CsRLV infected animals contain much lower *CasTPS* expression: 4.3 ± 1.5 X 10^3 copies/ µg total RNA (n=4) than hatchery-raised ones: 1.7 ± 0.4 X 10^4 copies/ µg total RNA (n=7). In the rest of tissues of CsRLV infected and control females, there were no differences in the levels of *CasTPS* expression.

4. Discussion

This study described progression of CsRLV infections and associated immune responses in adult female *C. sapidus* that have been shifted from 10˚C to 23˚C.

THC measured in this study was similar to that reported previously in juvenile *C. sapidus* [14]. The CsRLV-infected females exposed at 23^{o}C for 10 days, had slightly fewer hemocytes than non-infected wild and naive hatchery-raised adult female crabs kept constant at 23^{o}C, but the differences were not statistically significant (all three groups of the animals carry similar levels of THC ranging from about 6-8 million cells/ml hemolymph). In *P. monodon* and *P. indicus,* a significant reduction in THC is associated with WSSV infection, possibly due to apoptosis of hemocytes, and is closely related to the loss of hemolymph coagulation [22]. It remains to be further ascertained if infection by CsRLV affects THC and coagulation in *C. sapidus*.

The hemolymph of *C. sapidus,* like other decapod crustaceans, carries three types of hemocytes: granulocytes, semi-granulocytes, and hyaline cells [23-25]. Despite similar total hemocyte counts, there is an observed difference in morphology among groups. As expected, such a difference is not related with the elevated temperature but is noted in the infection of CsRLV. Granulocytes and semi-granulocytes (larger cells) seem to be the major types of hemocytes in both hatchery-raised and not-infected animals. Inclusions and cytoplasmic swelling in hemocytes, indicative of CsRLV infection, have been previously observed [12, 26]. However, it is still unknown if CsRLV targets a specific type of hemocyte as found with the

infection of WSSV, which specifically targets granulocytes and semi-granulocytes of *P. merguiensis* [27]. In this study, on the contrary, CsRLV infected animals show significantly higher portions of small sized and less granulated hemocytes, which may indicate an increase in hyaline cells. It remains to be further studied to define the functional significance of the increase in these cell populations.

The expression levels of genes in hemocytes are changed in the animals infected with CsRLV, while the exposure to elevated temperature from 10^{o}C to 23^{o}C for 10 days does not affect, compared to the control, hatchery-raised animals kept at constant 23^{o}C. *C. sapidus* respond to LPS or lipoteichoid acid (LTA) challenge by elevating the THC, as well as expression of *ecCasCuZnSOD-2*, *CasTPS* and *CasLpR* in hemocytes [16, 17, 20]. In addition, *CasPPO* levels were examined in this study, as its activation is often associated with crustacean disease responses to pathogens including WSSV [28]. *C. sapidus* utilizes 4 different types of SOD in various tissues [17, 29, 30], with the highest expression of *Cas-ecCuZnSOD-2* in hemocytes [17]. A significant increase in levels of *ecCasCuZnSOD-2* is seen with CsRLV infected animals, as compared to those prepared from non-infected, hatchery-raised adult females. Our finding is congruent with reports showing that WSSV infection increases the levels of ROS and subsequent SOD activity in shrimp [31, 32]. However, it contrasts with other findings that WSSV infection is associated with reduced SOD activity, which subsequently results in lipid peroxidation of the tissues in *P. monodon* [33] and in *C. quadricarinatus* [34].

It seems that there are different responses in *PPO* expression to viral infections in crustacean hemocytes. In the hemocytes of *F. chinensis*, the levels of *PPO* expression are down-regulated [35]. This result somewhat contrasts with the transcriptomic analysis of the whole body of *F. chinensis* with WSSV injection or infection. These shrimp, upon acute WSSV infection, upregulate immune responsive genes including the PPO activating system such as PPOs, their inhibitors (serpins), PPO-activating enzyme, and PO [36]. This suggests that *PPO* expressed in tissues other than hemocytes may be involved in an innate immune response to WSSV infection. The hemocytes obtained from the female *C. sapidus* with high levels of CsRLV did not show differences in *CasPPO* expression, indicating that *CasPPO* expression in the hemocytes may be irrelevant to CsRLV infection at later stages in blue crabs. However, this does not exclude the possibility that PPO may be involved in initial CsRLV infection to hemocytes.

Viral infection causes changes in metabolic pathways in host cells, particularly in energy metabolism. Particularly, viral infection induces the Warburg effect to support viral replication in endothelial cells and in hemocytes of shrimp [37, 38]. The hemocytes of CsRLV infected animals displayed significantly elevated *CasTPS* expression, similar to what is found with LPS injection [20]. Glucose mobilization via trehalose synthesis is plausible as CsRLV replication in hemocytes may be an energy dependent process. Trehalose is also reported to protect fly and mammalian cells from oxidative damage induced by hypoxia or anoxia [39]. Thus, elevated *CasTPS* expression for the synthesis of trehalose, as a non-reducing disaccharide, may protect *C. sapidus* cells from increased oxidative burst which is correlated with upregulation of *Cas-ecCuZnSOD-2* expression upon CsRLV infection.

The presence of CsRLV in all the examined tissues indicates its broad tissue tropism, although it still remains to be determined as to what the primary and secondary target tissue(s) of CsRLV are during a natural infection. Interestingly, midgut and hindgut contain much higher levels of CsRLV than hemocytes, whereas ovary and the hepatopancreas have lower amounts of CsRLV than hemocytes. Our results are in agreement with a previous finding in which CsRLV was found in multiple tissues including epidermis, gill, antennal gland, Y-organ and connective tissue [40]. It has been reported that upon experimental infection, via injection of viral particles, CsRLV is also found in gill and leg muscle [12]. Moreover, progressive infection by CsRLV has been reported in *C. sapidus*, with late-stages showing virus spreading into the nervous system including the brain and thoracic ganglia complex, resulting in sluggishness [40]. The levels of CsRLV in these tissues indicate that the primary target of infection may not be the hemocytes or hematopoietic tissue as suggested [41]. Hence, hemolymph sampling, although easy and convenient, may not be the most precise method for monitoring the progression of CsRLV infection.

The two genes that are upregulated in hemocytes by the infection of CsRLV, *Cas-ecCuZnSOD-2* and *CasTPS,* were examined in tissues that contain higher levels of CsRLV than hemocytes: midgut, hindgut, hypodermis and antennal gland, together with the hepatopancreas which showed lower levels of CsRLV. In regard to *Cas-ecCuZnSOD-2* expression, tissues show differential responses to a CsRLV infection, although the basal levels of *Cas-ecCuZnSOD-2* expression are constant. CsRLV infection elevates *Cas-ecCuZnSOD-2* expression in hindgut and hepatopancreas, similar to the effect in hemocytes.

In contrast to what was observed in hemocytes, none of the other tissues showed an elevation in *CasTPS* expression. The basal levels of *CasTPS* in the tissues varied significantly, and did not directly reflect the level of CsRLV in these tissues. No changes were found in the levels of *CasTPS* in any tissue examined except the hepatopancreas. In contrast to the hemocytes, where higher *CasTPS* expression was associated with CsRLV infection, the hepatopancreas in CsRLV infected animals had significantly reduced *CasTPS* expression. Levels of carbohydrate contents were not determined in this tissue. Trehalose is the major carbohydrate in hemolymph and hepatopancreas of *C. sapidus* [20, 42]. The reduced levels of *CasTPS* may be related to the nutritional condition of these crabs in that, in the later stages CsRLV infection, infected animals often do not feed or reduce their food intake (personal observation).

Sudden changes in the environmental conditions constitute stress and influence the metabolic and physiological status of animals. A high mortality of *C. sapidus* females has often been observed immediately after arrival to the IMET facility (personal observation). Water temperature at the spawning ground of *C. sapidus* in the Chesapeake Bay ranges from ~4º to 26º C. The animals were caught in February, 2014 at <4 º water temperature (http://tidesandcurrents.noaa.gov/physocean.html) and kept at ~10^{o}C (VIMS), prior to transfer. Particularly, elevation of water temperature from ~10^{o}C to 23^{o}C may affect both host and pathogen, as it could be stressful to the animals. On the other hand, it may provide a favorable condition for the replication of CsRLV resulting in the manifestation and the increase in the numbers of CsRLV at day 10, as seen with WSSV in crayfish [43].

The physiological and metabolic status of an individual animal plays a critical role in infection and disease progression [44]. However, little is known about the relationship between susceptibility to infections and physiological events such as molting and vitellogenesis: two energetically demanding processes. Particularly, *C. sapidus* adult females engage reproductive phases immediately after the pubertal-terminal molt and continue throughout their adulthood [45, 46]. In the Chesapeake Bay, the reproductive activity of these animals depends on season and is high during the months of warmer water temperatures [1]. Given that energy available for allocation to vitellogenesis competes with other physiological actions such as activity, homeostasis, maintenance, etc [47, 48], the cost of increased reproductive activity of those females in response to elevated water temperature in the spring may reduce their fitness as a trade-off [47, 48], resulting in being more susceptible to potential pathogens.

Like vertebrates, innate immune functions of invertebrates provide the first lines of defense in to an infectious non-pathogenic or pathogenic agent [49]. In challenge experiments with WSSV or bacteria, shrimp and other crustacean species show changes in the expression of genes in hemocytes and other tissues that are known to be involved in innate immunity [36, 50-55]. The hemocytes of *C. sapidus* respond to injections of LPS and LTA with elevated expression of *TPS* [20], *LpR* [16], and *SOD* [17]. Moreover, reduced feeding behavior and food consumption that are often influenced by the status of infections may further suppress the expression of immune-related genes, which subsequently increase susceptibility to other opportunistic pathogens. The degree of infections may also reflect the various metabolic costs of such immune reactions [56]. Moreover, the reproductively active adult females may have an increase in the susceptibility to infection, as we have observed in this study that all CsRLV infected females were at late vitellogenic stages. Life-stage dependent infection rate and trade-offs between reproduction and immune reactions upon an infection need to be further studied.

To date, it is not known what the exact mode of viral entry is in decapod crustaceans. It has been suggested that infection by WSSV may occur through an interaction between the host membrane transporter Glut 1 and the viral envelope protein, VP53A [57]. In vertebrate systems, reoviruses have been known to bind to the junctional adhesion molecule A (JAM-A), followed by activation of integrins [58]. At the organismal level, the mode of CsRLV infection may be through cannibalism or horizontal transmission via sharing water with infected crabs. If the infection indeed occurred in the holding tank, we suggest that it may be due to horizontal transmission as these females significantly reduce food intake at lower temperature. The fact that infected females were at late vitellogenic stages suggests the importance of physiological status of the animals in disease susceptibility. The precise mode and primary site of CsRLV infection need to be studied using hatchery-raised animals such that life stage and physiological states (i. e., molt and vitellogenic stages) are defined and tractable.

Acknowledgements

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no. xxxx of the University of Maryland Center for Environmental Science and contribution no. xx-xxxx of the Institute of Marine and Environmental Technology.

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Figure legends

Figure 1. The number of a CsRLV in the hemolymph of the infected female crabs exposed at 23^{o}C for 1, 10, and 15 days. The samples were assayed in triplicated using a CsRLV pPCR assay. Standards are ranged from 10-10^{6} copies/well. Data are presented as mean ± SE (n=2-6) CsRLV copies/100 µl hemolymph eqv.

Figure 2. Flow cytometric analysis of the hemocytes obtained from the experimental animals exposed at 23^{o}C for 10 days with CsRLV infected (C and C'), not-infected (B and B') and hatchery-raised adult females (A and A'). A-C) are obtained by FSH-H. Open arrow: larger sized cells and closed arrow: smaller sized cells; A'-C') are obtained by SSC-H. Arrows indicate three groups of cells. D and D') show the size and the granularity distribution of hemocytes of all three groups, respectively. CsRLV-I: CsRLV infected; N-I: not infected; and H-R: hatchery-raised.

Figure 3. Expression analyses of *Cas-ecCuZnSOD-2* (A), *CasPPO* (B), and *CasLpR* (C) in the hemocytes of the experimental animals exposed at 23^{o}C for 10 days: CsRLV not-infected (n=12) and infected with CsRLV (n=5), compared to the hatchery-raised adult animals kept constant at 23^{o}C (n=7). Data are presented as mean ± SE copies/µg total RNA. Letters are noted for statistical significance at $P < 0.05$ and N. D. = No difference

Figure 4. Expression analyses of *CasTPS* (A), *CasTreh* (B), and *CasGlyP* (C) in the hemocytes of the experimental animals exposed at 23^{o}C for 10 days: CsRLV not-infected (n=12) and infected with CsRLV (n=5), compared to the hatchery-raised adult animals kept constant at 23^{o}C (n=7). Data are presented as mean ± SE copies/µg total RNA. Letters are noted for statistical significance at $P < 0.05$ and N. D. = No difference

Figure 5. The number of a CsRLV in the various internal organs of the infected female crabs exposed at 23^{o}C for 17 days. The samples were assayed in triplicated using a CsRLV pPCR assay. Dotted line indicates the levels of CsRLV in hemocytes. Data are presented as mean ± SE copies of CsRLV/25 ng total tissue RNA.

Figure 6. Expression analyses of *Cas-ecCuZnSOD-2* (A) and *CasTPS* (B) in various tissue cDNAs of the infected female crabs exposed at 23^{o}C for 17 days (closed bar, n=5) and the hatchery-raised adult females kept constant at 23^{o}C (open bar, n=7). Data are presented as mean ± SE copies/µg total tissue RNA. Statistical significance is examined using one way ANOVA followed by a post hoc assay, Krustal-Wallis test for the different tissues in each group and Student's *t* test (nonparametric two tails) of a tissue obtained the CsRLV infected animals exposed to 23^{o}C for 17 days and hatchery raised animals kept constant at 23^{o}C. Significance is noted with different letters with * for $P<0.05$ and *** for $P<0.001$

1 Table 1. List of primers used for qPCR assays

Gene	Primer sequence (5'-3')	References
CsRLV	TGCGTTGGATGCGAAGTGACAAAG (F) GCGCCATACCGAGCAAGTTCAAAT (R)	Bowers et al., 2010 Flower et al, 2015
Hematodinium sp.	GGTAATCTTCTGAAAACGCATCGT (F) GTACAAAGGGCAGG GACGTAATC (R)	Pitula et al., 2012
TPS	ATGTTGGTGGAACACAATTCAAGGAC (QF) CTTTGTATAATCTAACCGATCCACTC (QR)	Chung, 2008
LpR	CATGACAGCAAGGAACAAGAGGTT (QF) CCTCACTTGCTCTCCATGAGTGAT(QR)	Tsutsui and Chung, 2012
ecCuZnSOD-2	TCAACAAGGAACCCTTGGTCTCGGA (QF) TGTACGTGGAATCCGTGCTTGCCCGG (QR)	Chung et al., 2012
Treh	GCAGAGAGTGGATGGGA (QF) CCCTGACAGCAGCAAGCCCTCA (QR)	Chung, 2008
PPO	CACCTCTTCATCCATCACAAACTC (QF) CAACCACACCCACAGAAGTTAAAG (QR)	Alvarez and Chung, 2013
GlyP	TATGAGTATGGTATTTTCGCCCAGAAGATCA (QF) AGGGATCATGTACTCAGGGCGGGCCTTC (QR)	

2

3

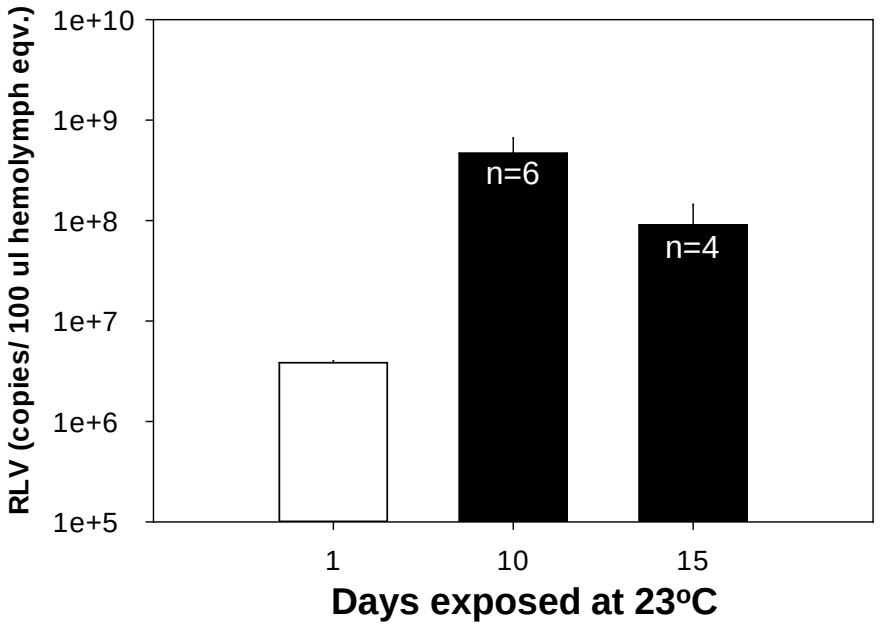
1 Table 2. Size distribution in the population of hemocytes present in the hemolymph of the
2 CsRLV infected and non-infected animals exposed at 23°C for 10 days and non-infected
3 hatchery raised kept constant at 23°C

4

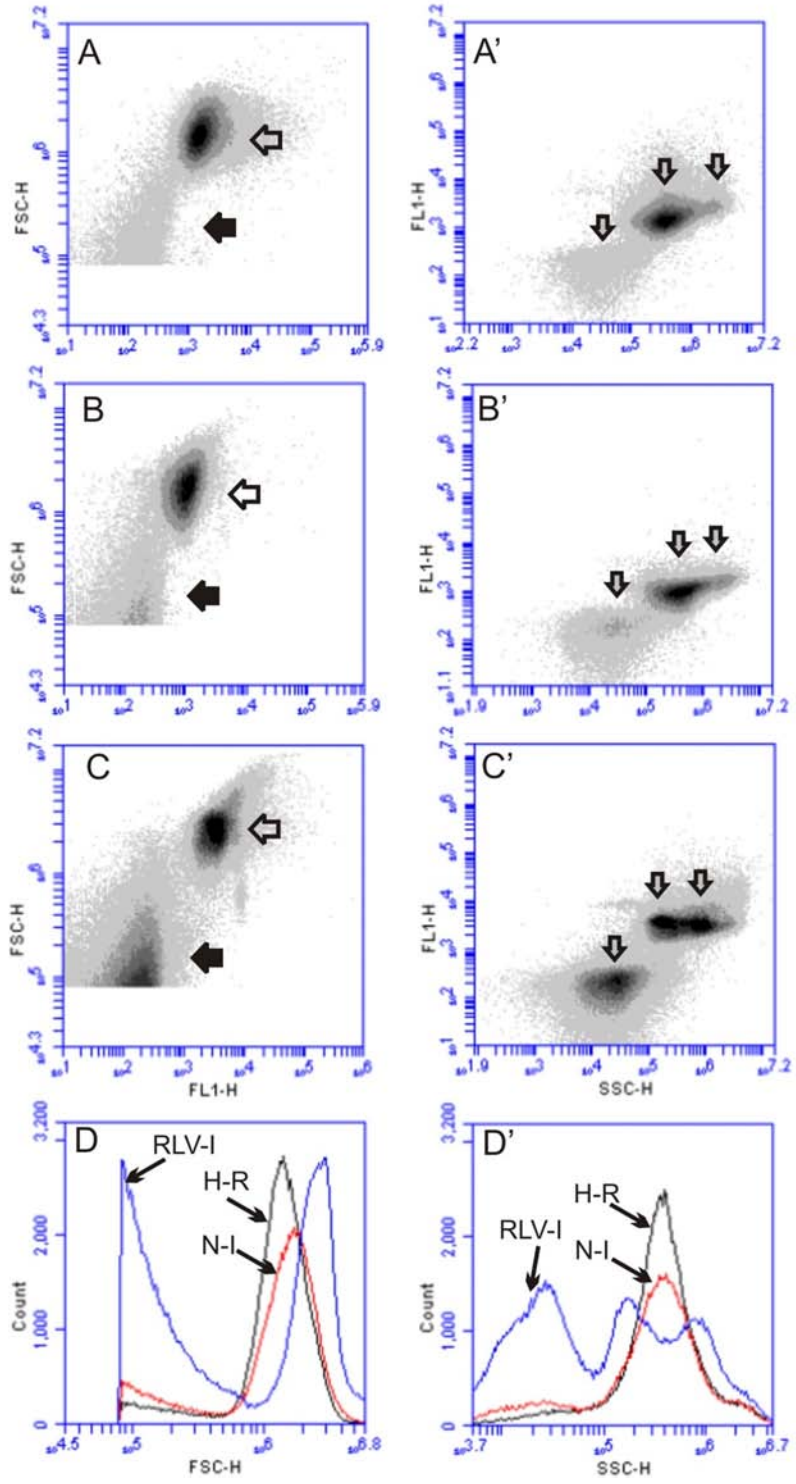
Group	Small size (%)		Large size (%)	
	Mean ± SE (n)	*P<0.05*	Mean ± SE (n)	*P<0.05*
Hatchery-raised	7.5 ± 0.6 (7)	a	89.2 ± 0.9 (7)	a
Not-infected	11.4 ± 2.4 (12)	a	83.6 ± 3.5(12)	a
CsRLV infected	25.8 ± 8.2 (5)	b	64.1 ± 11.2 (5)	b

5

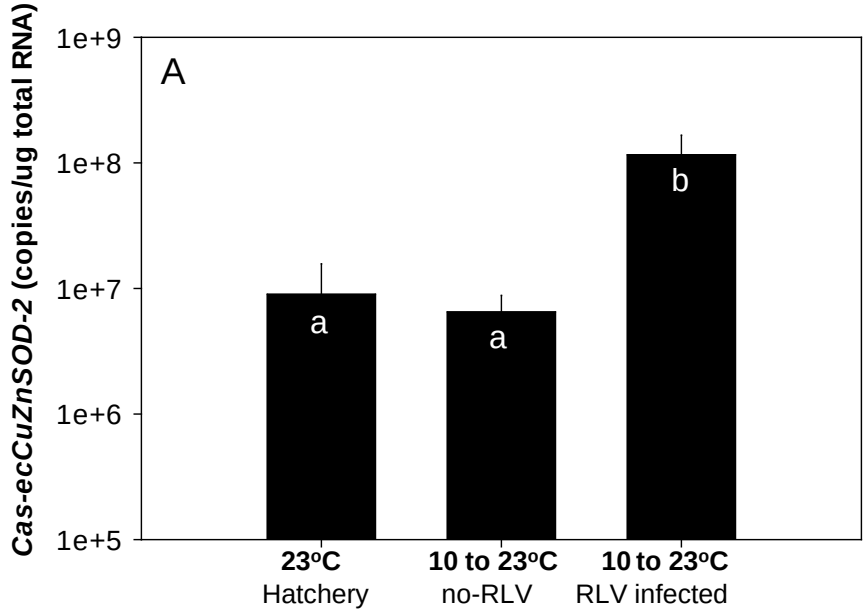
Chung et al., Figure 1

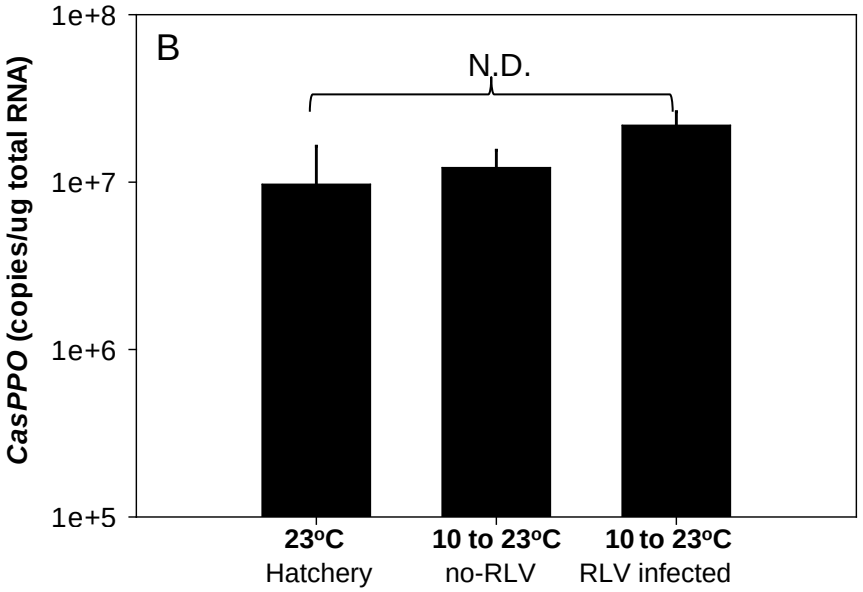


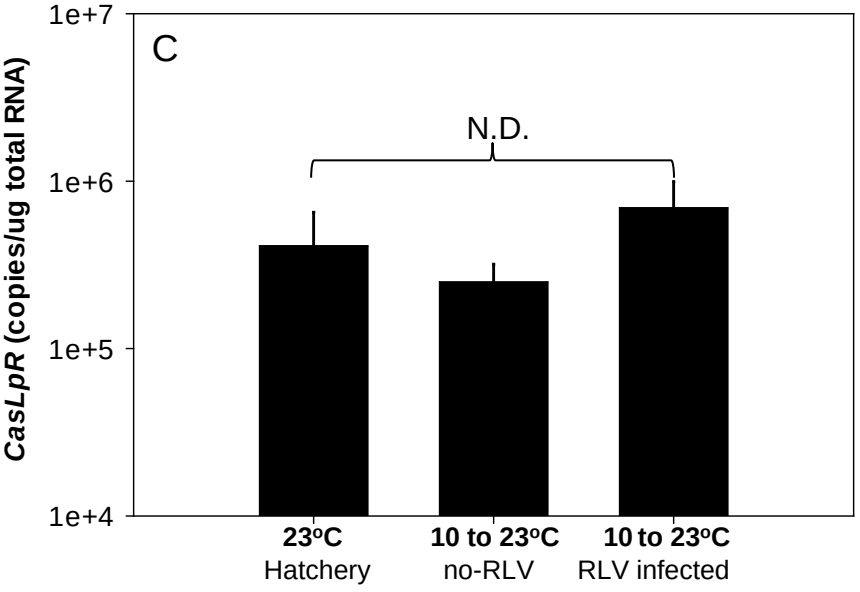
Chung et al., Figures 2A-D



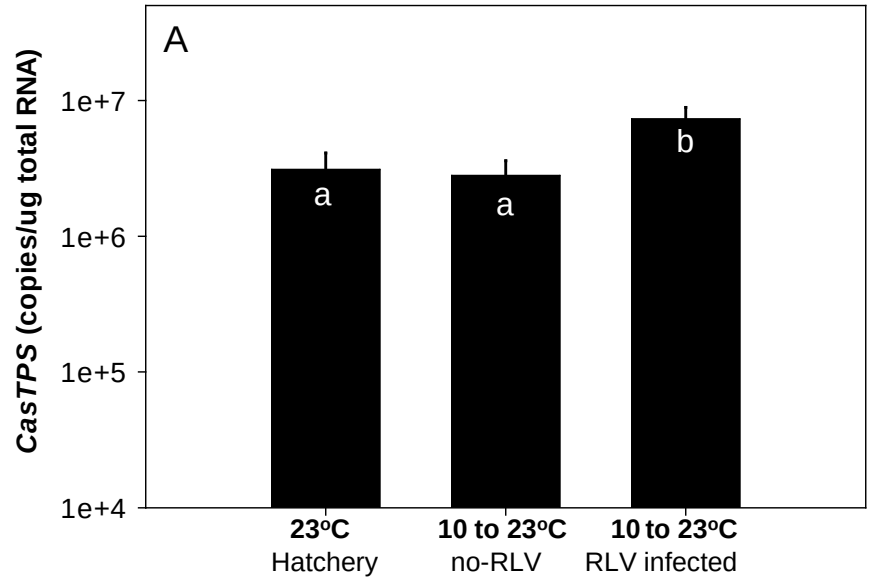
Chung et al., Figures 3A-C

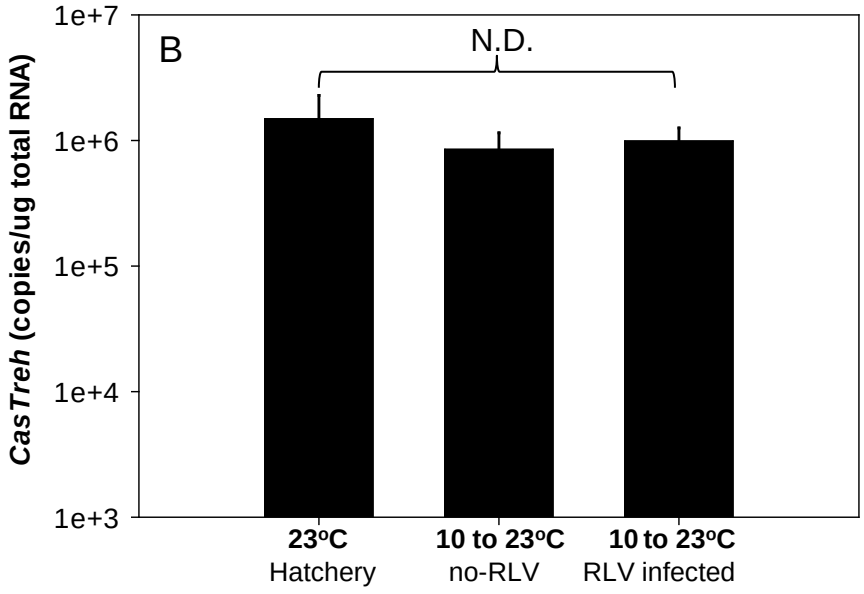


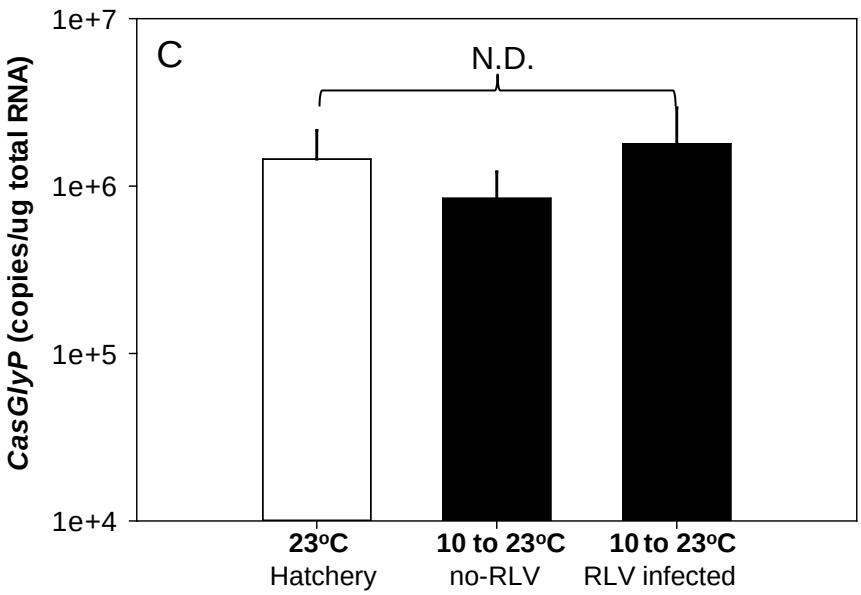




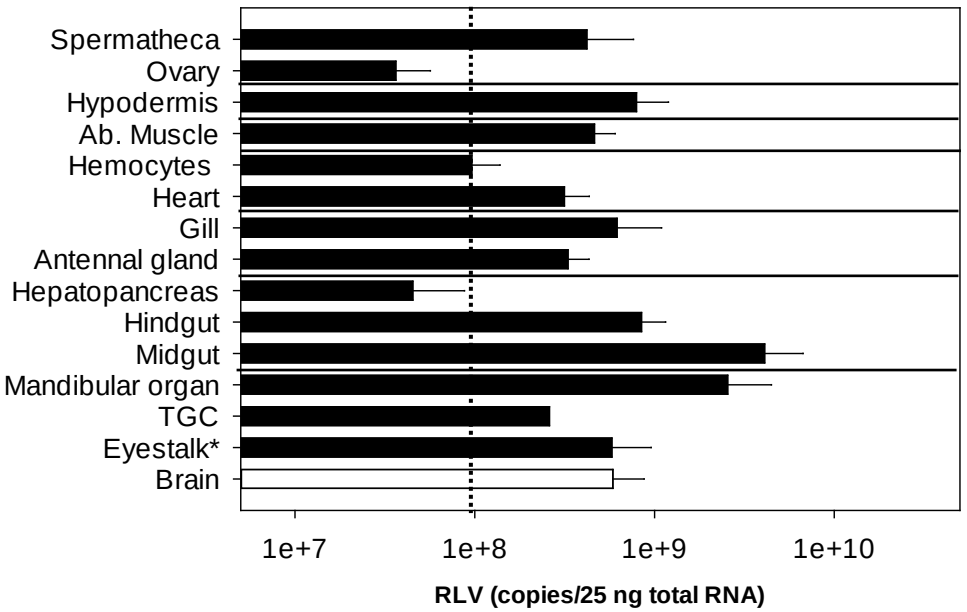
Chung et al., Figures 4A-C







Chung et al., Figure 5



Chung et al., Figures 6A and B