Journal of Invertebrate Pathology 190 (2022) 107739

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

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

High prevalence of CsRV2 in cultured *Callinectes danae*: Potential impacts on soft-shell crab production in Brazil

Camila Prestes dos Santos Tavares [a,b,*], Mingli Zhao [d], Éverton Lopes Vogt [c], Jorge Felipe Argenta Model [c], Anapaula Sommer Vinagre [c], Ubiratan de Assis Teixeira da Silva [b], Antonio Ostrensky [b], Eric James Schott [d]

[a] *Graduate Program in Zoology of the Federal University of Paraná, Curitiba, Paraná 80035-050, Brazil*
[b] *Integrated Group of Aquaculture and Environmental Studies, Federal University of Paraná, Curitiba, Paraná 80035-050, Brazil*
[c] *Department of Physiology, Federal University of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil*
[d] *Institute of Marine and Environmental Technology, University of Maryland Baltimore County, Baltimore, MD 21202, USA*

A R T I C L E I N F O

Keywords:
Reovirus
CsRV2
Blue crab
Glycogen
Total proteins
RAMP

A B S T R A C T

Crabs can be infected by a variety of pathogenic micro-organisms but the most damaging are viruses. Naturally-occurring Callinectes sapidus reovirus 1 (CsRV1) is thought to contribute to mortality of *Callinectes sapidus* in soft crab culture in the USA. In Brazil, soft crabs are frequently produced using *Callinectes danae*, which suffers a similar rate of mortality in culture as *C. sapidus*. This study investigated whether CsRV1 could be detected in healthy or dead *Callinectes danae* from Paraná, Brazil and kept in captivity, we also evaluated the relationship between viral infection, and biochemical and behavioral parameters. *C. danae* from Paranaguá Bay were kept in a recirculation system for 14 days and subjected to weekly biochemical analyses and a reflex action mortality predictors (RAMP) test. RT-qPCR assays for CsRV1 were negative for all samples. However, electrophoretic analysis of extracted RNA from some crabs showed a pattern of 12 dsRNA bands that indicated intense infection by a reovirus with a genome organization different from CsRV1. The banding pattern was indistinguishable from a putative novel reovirus detected in *C. sapidus* in Rio Grande do Sul, Brazil, provisionally called CsRV2. The prevalence of dsRNA of CsRV2 showed no significant difference between crabs that died and survived. Interestingly, the presence of CsRV2 dsRNA was correlated with a significant reduction in glycogen concentration in hepatopancreas and a decrease in reflex action. The results obtained in this study are an early glimpse of the occurrence of reoviruses in *C. danae* and their potential effects in soft-shell crab systems in Brazil.

1. Introduction

Portunid crabs (Decapoda, Crustacea) are distributed globally and are important fishery resources in many countries, with annual landings of around 2 million tonnes worldwide between 2010 and 2018 (FAO, 2020). Several portunids are also highly valued as aquaculture species, with a global value of crab aquaculture production estimated at $2.2 million in 2018 (FAO, 2020; Tobias-Quinitio et al., 2015). In addition to the culture of hard shell crabs, there is a significant and growing industry producing soft crabs, which are newly molted crabs still covered with a soft tegument, a characteristic that allows them to be eaten whole (Chang and Mykles, 2011; Freeman et al., 1987; The and Johns, 1965). Soft-shell crabs are considered a high-value gastronomic delicacy, appreciated around the world (Hungria et al., 2017).

Short-term soft-shell crab culture has been practiced for>100 years in the US and several Asian countries, and is now a multimillion dollar activity to increase the value of a fishery product (Ferdoushi et al., 2010; Kennedy and Cronin, 2007; Tavares et al., 2018). However, mortality rates of 25% or more occur in soft crab production facilities, representing a significant loss of fishery resource, and fisherman time and labor, which compromises the financial viability of the enterprise (Chaves and Eggleston, 2003; Spitznagel et al., 2019).

In the United States, *Callinectes sapidus* is the most cultivated species for soft-shell crab production, due to the abundance of animals from fishing and the simplicity of aquaculture techniques (Tavares et al., 2018). In Brazil, soft shell crab culture is conducted with *C. sapidus*, as well as with *C. danae* and *C. exasperatus*, which are also commonly harvested along the Brazilian coast. Aquaculture research in Brazil

* Corresponding author.
E-mail address: camilapstavares@gmail.com (C. Prestes dos Santos Tavares).

https://doi.org/10.1016/j.jip.2022.107739
Received 29 September 2021; Received in revised form 15 February 2022; Accepted 1 March 2022
Available online 3 March 2022

indicates that mortality rates of *C. danae* and *C. exasperatus* are also both approximately 25%, as observed for *C. sapidus* in the USA (Ostrensky et al., 2015).

The causes of mortality of crabs during the soft-shell crab production process include poor water quality in the aquaculture systems (Hochheimer, 1988; Lakshmi et al., 1984), stress caused by harvest and transport of animals (Chaves and Eggleston, 2003), and the physiological stresses associated with the molting process (deFur et al., 1988). It is also known that stresses can make crabs more susceptible to infection by pathogens or exacerbate the pathogenicity of infections (Kennedy and Cronin, 2007; Perazzolo et al., 2012; Shields and Overstreet, 2003), especially viruses (deFur et al., 1988; Johnson, 1977; Kennedy and Cronin, 2007; Perazzolo et al., 2012; Shields, 2003; Spitznagel et al., 2019).

The stress response of virus-infected invertebrates is still poorly understood (Fabbri and Moon, 2016; Gallo et al., 2016). Stress stimulates the secretion of catecholamines and crustacean hyperglycemic hormone (CHH) by the main neuroendocrine center, organ X (Aparicio-Simón et al., 2010; Chung, 2010; Model et al., 2019). This primary neuroendocrine response is followed by changes in hemolymph and tissue metabolites, such as glucose and lactate, which are considered the secondary stress response (Aparicio-Simón et al., 2010; Lorenzon et al., 2007; Mercier et al., 2006; Vinagre et al., 2020a). For example hyperglycemia and hyperlactatemia are induced by the white spot syndrome virus (WSSV) in *Cherax quadricarinatus* and *Litopenaeus vannamei* (Fan et al., 2016; Hernández-Palomares et al., 2018). Hyperglycemia and decreased glycogen in the Caribbean spiny lobster *Panulirus argus* infected with Panulirus argus virus 1 (PaV1) (Herrera-Salvatierra et al., 2019; Zamora-Briseño et al., 2020). PaV1 can also alter the biochemical pathways of neurotransmitter synthesis and, the hosts behavior (Herrera-Salvatierra et al., 2019; Zamora-Briseño et al., 2020).

The eight virus species reported in *Callinectes* spp. are all described in *C. sapidus*. Four are lethal, and two are found simultaneously with other viruses, with evidence of synergistic pathogenic effects (Kennedy and Cronin, 2007; Rogers et al., 2015). Callinectes sapidus reovirus 1 (CsRV1), also called CsRV or Reolike-virus (RLV) (Johnson, 1977; Tang et al., 2011), is closely associated with mortality of *C. sapidus* in soft crab aquaculture facilities in North America (Bowers et al., 2010; Flowers et al., 2018; Spitznagel et al., 2019). CsRV1 is a double-stranded RNA (dsRNA) reovirus, which infects tissues of mesodermal and ectodermal origin, especially hemocytes, and invades the brain and thoracic ganglia and causes tremors and paralysis in infected animals (Bowers et al., 2010; Johnson, 1977; Kennedy and Cronin, 2007). The genome of a second putative C. sapidus reovirus provisionally termed CsRV2 was recently detected and sequenced from a crab captured in Rio Grande do Sul, Brazil (Zhao et al., 2021a). However, virions of the putative virus have not been visualized or characterized within crab tissues, and no histopathological manifestations or consequences of possible infections are known. There are also no studies of its prevalence or pathogenicity in crabs of any other species of *Callinectes*.

There are records of high CsRV1 prevalence in wild *C. sapidus* in temperate estuaries of the Atlantic coast of the USA, and Rio Grande do Sul, Brazil (Flowers et al., 2016; Spitznagel et al., 2019; Zhao et al., 2020). Identification of CsRV1 has relied upon detection of the virus genome using RT-qPCR which is costly, time-consuming, and requires specialized facilities. Despite the potential utility, there are no reports of successful visual, biochemical, or behavioral screening methods to identify virus-infected crabs.

Given the association of CsRV1 with *C. sapidus* mortality in soft crab production, and the presence of the virus in *C. sapidus* in Brazil, it is important to investigate whether CsRV1 contributes to mortality of *C. danae* in Brazilian soft crab production. This study was designed to investigate whether CsRV1 or any other virus is associated with mortality of *C. danae* kept in a recirculating aquaculture system, and to evaluate whether virus-infected crabs would exhibit metabolic or behavioral changes that may be used to identify infected crabs.

2. Material and methods

2.1. Crab collection

Male and female *C. danae* were obtained by artisanal fishermen in the village of São Miguel, Paranaguá, Paraná, Brazil (25°25′48″ S/ 48°27′12″ W). The crabs were caught using traps covered by 20 mm mesh and using fish as bait. Immediately after capture, the animals were transferred to polyethylene boxes (70 L) containing seawater from the field site and kept under constant aeration. In each box, the crabs were individually protected by flexible plastic screens to minimize injuries and mortality during transport to the Marine Aquaculture and Restocking Center, belonging to the Integrated Group of Aquaculture and Environmental Studies (GIA) of the Federal University of Paraná (25°41′29.94″S, 48°27′57.09″W).

In the laboratory, the animals were identified up to the species level based on the manual developed by Melo (1996). Sex, molting stage, and possible injuries to the carapace or appendices were recorded by visual examination (Baptista et al., 2003; Freeman et al., 1987; Ostrensky et al., 2015). The carapace (measured between the base of the lateral spines) and live weight of each crab were measured, respectively, with the aid of a manual caliper and a precision digital scale (Mars AL 500c − 0.001 g, Brazil).

2.2. Experimental system

The crabs (n = 178), without distinction of sex, were placed in 70 L polyethylene boxes (n = 6 crabs per box), divided by perforated PVC barriers (height: 35 cm; width: 35.5 cm and length: 71 cm) and covered by plastic screens, to avoid cannibalism. The boxes (n = 31) were interconnected to a recirculation system that included mechanical and biological filters and provided aeration. The photoperiod was adjusted to 12:12 h (light: dark), controlled by an analog timer. Temperature was maintained at 25 ± 2 °C and salinity at 25 psu. The animals were monitored daily, dead animals were tallied, and dissected, following the methodology described in section 2.3. All crabs were fed daily with fish to satiety. One hour after feeding, the waste and feces were collected and disposed of to prevent water quality deterioration.

The following physical and chemical water parameters were analyzed daily during the experiment: salinity, measured by optical refractometer (Instrutemp, Brazil); pH, measured using a digital pH meter (AZ-86505, Taiwan); temperature (°C), dissolved oxygen ($mg.L^{-1}$) and percentage oxygen saturation (%), by digital oximeter (YSI, 550A, USA); concentration of total ammonia nitrogen ($mg.L^{-1}$ *N*-TA), obtained by the indophenol method (APHA, 1995) by reading the samples by spectrometry (Spectronic 20 Genesys, USA).

2.3. Hemolymph collection and crab dissection

Hemolymph collection was performed three times: upon arrival of the crabs at the laboratory, at the end of the first week, and the end of the experimental period (14 days). Hemolymph (~1 mL) was collected from randomly selected individuals (n = 111) from the arthrodial membrane located at the base of the fifth pereiopod, using insulin-like syringes containing 10% sodium citrate as an anticoagulant (Inohara et al., 2015). After the experimental period, surviving crabs were cryo-anesthetized for 15 min. for the last hemolymph harvest and dissected. All the animals that died during the experimental period were also dissected shortly after their death. During the dissection, samples of hepatopancreas, gonads and pereiopod muscle were collected. All hemolymph and tissue samples were immediately transferred to 2 mL microfuge tubes and stored at −20 °C for further analysis.

2.4. Total RNA extraction from the hemolymph

After collection, the hemolymph samples were transported in a styrofoam box containing dry ice to the GIA Genetics Laboratory, in Curitiba, Paraná, for RNA extraction. The hemolymph samples (~100 μL) were homogenized in 1 mL of an acid solution containing guanidinium thiocyanate, sodium acetate, and phenol, prepared according to the protocol developed by Rodríguez-Ezpeleta et al. (2009), with modifications. RNA extraction was performed following the protocol used by Spitznagel et al. (2019). The resulting RNA precipitate was dissolved in 1 mM ethylenediaminetetraacetic acid (EDTA) and stored at −20 °C. Negative control samples (distilled water) were extracted before and after each set of crab hemolymph samples to monitor for cross-contamination. The purity and concentration of RNA were evaluated by fluorimetry Qubit® 4.0 (Thermo Fisher Scientific, USA), performed according to the manufacturer's recommendations.

2.5. Total RNA extraction from the pereiopod muscle

Pereiopods of each crab were preserved in 95% ethanol and transported to the Institute of Marine and Environmental Technology (IMET), Maryland, USA, for the extraction of total RNA and subsequent determination of viral load of CsRV1. The pereiopods were dissected with sterile razor blades, and all handling surfaces were cleaned with a 10% sodium hypochlorite solution to avoid possible contamination.

Fragments of 25–100 mg of muscle were excised from a pereiopod and homogenized in 1.0 mL of acid extraction solution using ceramic beads in a Fastprep24 homogenizer (MP Biomedicals, USA) at a speed of 4.5 m/second (m/s) for 30 s. The remaining extraction process was performed according to the protocol described above. Negative control samples (frozen fish muscle) were extracted before and after the experimental sample extraction to test cross-contamination among the experimental samples. The purity and concentration of RNA were evaluated by Nanodrop spectrophotometry 2000™ (Thermo Fisher Scientific, USA), performed according to the manufacturer's recommendations.

2.6. Quantification of CsRV1 by RT-qPCR

CsRV1 infection was evaluated by measuring the number of copies of CsRV1 genomes present in crab muscle, using RT-qPCR methods adapted from Flowers et al., (2016) using primers designed to detect a 158 bp region of the 9th genomic segment of CsRV1 (GenBank entry KU311716): 5′-TGCGTTGGATGCGAAGTGACAAAG-3′ (RLVset1F) and 5′-GCGCCATACCGAGCAAGTTCAAAT-3′ (RLVset1R). The calibration curves of the CsRV1 genome for RT-qPCR were produced by purified and isolated viral dsRNA (prepared using the methods of Bowers et al., (2010) from crabs collected in the United States that presented intense infections by CsRV1, with more than 10^8 copies per mg of muscle. Viral genomic dsRNA was quantified, and then the standards were prepared from serial dilution ranging from 10 to 10^7 copies per microliter.

RT-qPCR reactions were performed with qScript™ One-Step SYBR® Green qRT-PCR Kit, Low ROX™ (Quanta Bio - Cat.no.:95089–050) in 10 μL reactions containing 0.5 μM of each CsRV1-specific primer. The primers were dissolved in 1 mM EDTA. To anneal PCR primers with dsRNA, the primers and extracted RNA were combined, heated to 95 °C for 5 min, and then cooled to 4 °C, before being added to the reaction mixture of reverse transcriptase and Taq polymerase. Reverse transcription and amplification reactions were conducted in an Applied Biosystems Fast 7500 thermocycler using an initial temperature of 50 °C for 5 min. (reverse transcription), followed by 5 min. to 95 °C (reverse transcriptase inactivation and template denaturation). Amplification was achieved through 40 cycles of 5 s at 95 °C (denaturation) and 30 s at 61 °C (annealing and elongation). The fluorescence signals were collected during annealing of each cycle. After amplification, a melting curve analysis monitoring SybrGreen fluorescence was performed to verify amplification of the expected product (Flowers et al., 2016). The reaction conditions of melting curve followed the default continuous melt curve setting of the thermocycler: 95 °C for 15 s, 60 °C for 60 s and continuous heating of the amplicon from 60 °C to 95 °C at a rate of 0.15 °C/s.

2.7. Identification and quantification of CsRV2 dsRNA

A total of 5 μL of the total RNA of each *C. danae* sample was subjected to 1% agarose gel electrophoresis (0.5X TBE buffer solution) and visualized by staining with ethidium bromide or, when available, GelRed™ dye (Biotium, CA, USA), and fluorometric detection. For each sample applied, 1 μL of Gel Loading Dye Purple (6X) dye was used (Cat.no.: B70255, New England Biolabs, USA). Each gel was also loaded with 1 μL a 1 kb ladder (Cat.no.:N32325, New England Biolabs, USA). Electrophoresis conditions (volts, amperage, time) were standardized to permit comparison of bands across different gels. To create a semi-quantitative measure of dsRNA band intensity, gels were photographed under ultraviolet light (UV) and documented (in tiff format) using a ChemiDoc™ gel documentation system (Bio-rad, Australia) to estimate the intensity of dsRNA bands for all the samples. All parameters and experimental conditions used were kept constant throughout the study. The intensity of the viral dsRNA bands was analyzed by densitometry from the digital images, using Image J version 1.8.0, and numerically expressed as relative density (Abràmoff et al., 2004). For pragmatic convenience, despite the uncertainties about the characterization of the second putative reovirus and its potential effects on the host, this report refers to that putative reovirus as CsRV2 without qualification, and to experimental crabs whose extracted RNAs showed the characteristic electrophoretic pattern of Zhao et al., (2021), as infected with CsRV2.

2.8. Behavioral assessment

The behavioral responses of crabs ($n = 100$) were analyzed using the RAMP (reflex action mortality predictor) test to assess whether CsRV2 viral infection was associated with behavioral changes in *C. danae* before each hemolymph collection (days 0, 7, and 14). This test was composed by six crustaceans reflexes (Table 1) described by Stoner et al., (2008). An index (score ranging from 0 to 6) was created from the results obtained, reflecting the total number of lost reflexes for each crab. When an individual reacted to all the stimuli, its score was 0 since no reflex was absent. The proportion of reflex impairment was calculated by summing the reflex score for each animal and dividing it by the maximum number of possible reflexes. The values of this score were used for statistical analysis and were correlated with viral infection.

2.9. Biochemical analysis

Samples of hemolymph (~0.9 mL), hepatopancreas, muscle, and gonads were sent, in styrofoam boxes with dry ice, to the Laboratory of Metabolism and Comparative Endocrinology (LAMEC), Department of Physiology, Federal University of Rio Grande do Sul (UFRGS), in Porto Alegre, Brazil, for biochemical analysis of metabolic changes resulting from the secondary stress response caused by the viral infection. The concentrations of glucose, triglycerides, and total proteins in the hemolymph samples were determined using Labtest commercial kits and analyzed in a spectrophotometer, as routinely used in clinical biochemistry (Antunes et al., 2010). The results were expressed in mg. dL^{-1}.

The extraction and determination of glycogen concentration in the samples were performed using the methodology described by Inohara et al., (2015). To determine triglyceride concentrations, hepatopancreas samples were homogenized in 0.9% saline (1: 10, w/v) and triglyceride concentrations were determined using the same colorimetric kit that used for the hemolymph assay (Model et al., 2019). The results were expressed in mg.g^{-1} of tissue.

Table 1
RAMP reflexes used in the behavioral assessment of *Callinectes danae*.

Order of observation	Reflex observed	Test description	Positive response	Negative response
1	Cheliped extension	The animal was lifted with the carapace up and was observed the extent of the chelipeds in a horizontal plane.	All chelipeds spread wide and high, near horizontal orientation.	Chelipeds droop below horizontal with no attempt to raise them
2	Cheliped retraction	While held as above, the chelipeds were pushed in the previous direction.	Strong retraction of appendages in the opposite direction.	No resistance to the manipulation.
3	Chela closure	Chela was touched with an object.	Manipulation results in immediate strong closure.	No motion in the chela under manipulation.
4	Eye retraction	The eyestalk was carefully touched with a pointed object.	Eyestalk retracts in the lateral direction below the carapace hood or shows resistance to lifting.	No motion or resistance to manipulation in the eyestalk.
5	Mouth closure	The third maxilliped was carefully pushed with a pointed object.	Third maxillipeds retract quickly and strongly to cover the smaller mouth parts.	No motion in the maxillipeds.
6	Kick	With the crab in ventrum-up position, a pointed object was used to lift the abdominal flap away from the body.	Immediate and robust agitation of the pereiopods and legs.	No or slow motion in the legs or chelipeds.

*The "test description" is the manipulation required to elicit a stereotypic positive response (modified from Stoner et al., (2008)).

2.10. Data analysis

A database was created and organized using Excel® and all data were analyzed using the statistical software Statsoft Statistica®, version 10 (StatSoft, USA). The Shapiro-Wilk and Kolmogorov-Smirnoff normality tests, followed by the homogeneity test of the Chochan C., Hartley & Bartlett variances, and the homoscedasticity test were used to analyze the aquaculture water quality and the biometric data of the crabs. The survival of crabs during the experiment was analyzed using Kaplan-Meier curves and compared using Mantel-Cox, Breslow, and Tarone-Ware (IBM SPSS® Statistical Package for Social Sciences, version 22). Pearson's chi-square test (χ^2) for a 2x2 contingency table was used to analyze dichotomous data on virus prevalence. The physiological, behavioral, and densitometric data were grouped into two treatments (infected and uninfected or dead and survivors), and the results, when parametric, were analyzed through ANOVA One way, followed by Tukey's post hoc test. Non-parametric data were analyzed through the Mann-Whitney or Kruskal-Wallis tests. Spearman's correlation coefficient (r_S) was used to test the association between the number of daily deaths and the physical and chemical water parameters in the aquaculture system, as well as between the proportion of reflex impairment and water quality parameters measured over the experimental period. The statistical significance accepted in all analyzes was $p \leq 0.05$, considering a significance level of 5% ($\alpha = 0.05$).

3. Results

3.1. Water quality

The physical and chemical water parameters in the aquaculture system remained relatively stable and within the recommended limits for cultivated crabs throughout the experimental period, except for dissolved nitrite and oxygen (Table 2). Although dissolved oxygen and nitrite concentrations were relatively outside the limits recommended by Malone and Burden (1988) and Hochheimer (1988), there was no significant correlation between the number of deaths on each day of the experiment and these water quality parameters (Spearman's correlation test, dissolved oxygen: $r_s = 0.31$, $p > 0.05$; nitrite: $r_s = -0.13$, $p > 0.05$).

3.2. Mortality

In total, 48.9% (n = 91) of crabs died during the experiment. The Kaplan-Meier survival curve analysis revealed that the deaths occurred relatively uniformly from the first to the fourteenth day of the experiment (Fig. 1). When the crabs arrived at the laboratory, 35.4% had lost one or two chelipeds and up to four pereiopods. A total of 58.7% of injured crabs died during the trial period. Using the Mantel-Cox, Breslow, and Tarone-Ware methods, we verified that injured and intact crabs' mortality was not significantly different ($p > 0.05$). In addition, there was no statistical difference ($p > 0.05$) in survival between crabs of different sexes (female: 44%, male: 53.2%) or molt stage (premolt: 47.3%, intermolt: 51.5%).

3.3. Prevalence of CsRV1 using RT-qPCR and discovery of a CsRV2-like genome by visualization of its dsRNA

Total RNA from muscle was analyzed using the RT-qPCR assay for CsRV1. Calibration curves typically had a slope close to −3.493 and coefficient of determination (R^2) between the genome's copy and the threshold cycle higher than 0.996. All the total RNA samples analyzed (n = 178) were negative for CsRV1 regardless of whether crabs lived to the end of the experiment. Therefore, in the search for evidence of possible presence of other viruses, total RNA of all the crabs was analyzed by agarose gel electrophoresis. The analysis showed that 12 bands of apparent dsRNA were evident in many of the tissue samples. However, it was observed that the migration of the dsRNA bands was different from the bands produced by the CsRV1 genome (Fig. 2). The 12 fragments' sizes were estimated between 3.6 and 0.7 kb, based on comparison with a marker of 1 kb ladder. No differences were observed in the migration of dsRNA bands when using the different staining methods (GelRed™ or ethidium bromide). The putative virus was here tentatively identified as "Callinectes sapidus reovirus 2", or CsRV2,

Table 2
Water quality in the experimental system: parameters, measured values, and recommended limits by Malone and Burden (1988) and Hochheimer (1988).

Physical and chemical variable	Mean ± standard deviation	Recommended values
Temperature (°C)	25.0 ± 0.2	22–28
Dissolved oxygen ($mg.L^{-1}$)	5.8 ± 0.6	> 7.0
Dissolved oxygen (%)	78.0 ± 5.7	Nd*
pH	8.0 ± 0.1	6.5–8.5
Total ammonia ($mg.L^{-1}$)	0.1 ± 0.1	< 1.0
Nitrite ($mg.L^{-1}$)	0.9 ± 0.2	< 0.5
Salinity (psu)	25.3 ± 0.5	5 psu from the collection site

* Not available.

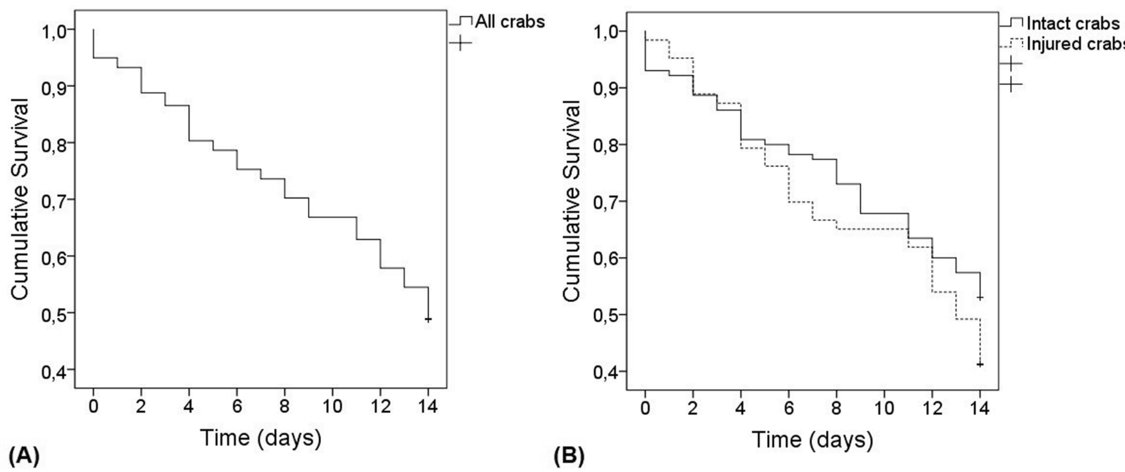

Fig. 1. Kaplan-Meier survival curves of *Callinectes danae* specimens kept under experimental conditions for 14 days: (A) survival curve of the entire set of tested crabs; (B) survival curves of crabs without visible injury, and of animals showing lesions or absence of pereiopods. No significant differences were found between intact and injured animals (Mantel-Cox, Breslow and Tarone-Ware tests).

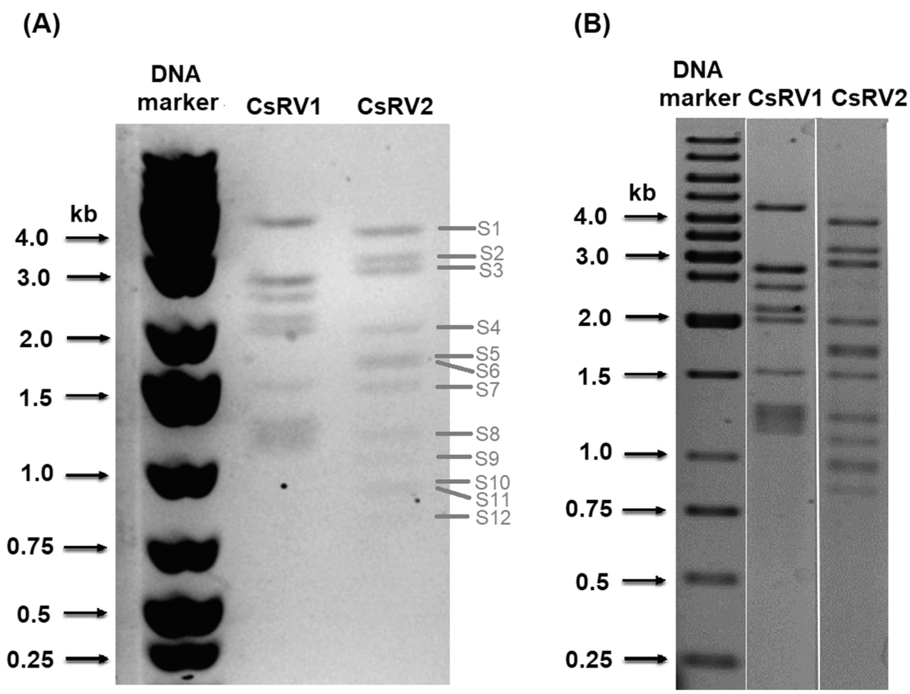

Fig. 2. (A) Comparison of electrophoretic mobility of the dsRNA genomes of a North American example of CsRV1 of *Callinectes sapidus* and dsRNA of CsRV2 of *C. danae* of the present study. (B) Electrophoresis of the dsRNA genome of CsRV1 and CsRV2 of *C. sapidus* identified and described by Zhao et al., (2021a). In both analyses, electrophoresis was performed in 1% agarose and visualized by ethidium bromide staining. The dsRNA band sizes were determined by comparison with a 1 kb ladder shown in the first line (Cat.no.: SM1331, Thermo Fisher Scientific, USA).

based on the similarity to the dsRNA pattern described by Zhao et al., (2021), and for brevity is hereafter referred to as CsRV2.

The putative CsRV2 genome was identified in the muscle RNA of 78% ($n = 139$) of the crabs analyzed at the end of the experiment. CsRV2 dsRNA was identified in the hemolymph of live crabs, with a 42% prevalence at the beginning, a significant increase to 97% after seven days (χ^2: 27.49; df = 1, $p < 0.001$), and remained at 88.5% until the end of the experiment (χ^2: 2.21; df = 1, $p > 0.05$). Pearson's chi-square test indicated no statistical difference in the prevalence of CsRV2 between hemolymph and muscle samples (Fig. 3A). Densitometry analysis for all samples showed similar patterns, with an increasing relative density of the putative CsRV2 genome bands throughout the experimental period (Fig. 3B). At the end of the experiment, despite the high prevalence of CsRV2 observed in *C. danae*, there was no significant difference in the CsRV2 genome's presence in the hemolymph of dead or surviving crabs. However, in muscle samples, we observed a higher prevalence and density of viral dsRNA bands in animals that survived compared to those that died during the experimental period (Table 3).

Using the chi-square method, no statistical difference was observed ($p > 0.05$) in the prevalence of CsRV2 between females ($n = 84$, 78.6% prevalence) and males ($n = 94$, 76.6% prevalence). In addition, there was no significant difference in the prevalence of CsRV2 between crabs in intermolt and premolt stages ($p > 0.05$) after 14 days of experiment. Still, also using the chi-square method, the prevalence of CsRV2 was significantly lower ($\chi^2 = 3.99$; df = 1, $p = 0.04$) in crabs that successfully molted than the prevalence of CsRV2 in premolt crabs (Table 4). Of the animals tested, 62.6% reached the premolt stage during the experimental period, but only 4% of the premolt crabs successfully molted, and only animals already collected in premolt stage were able to molt during the experimental period.

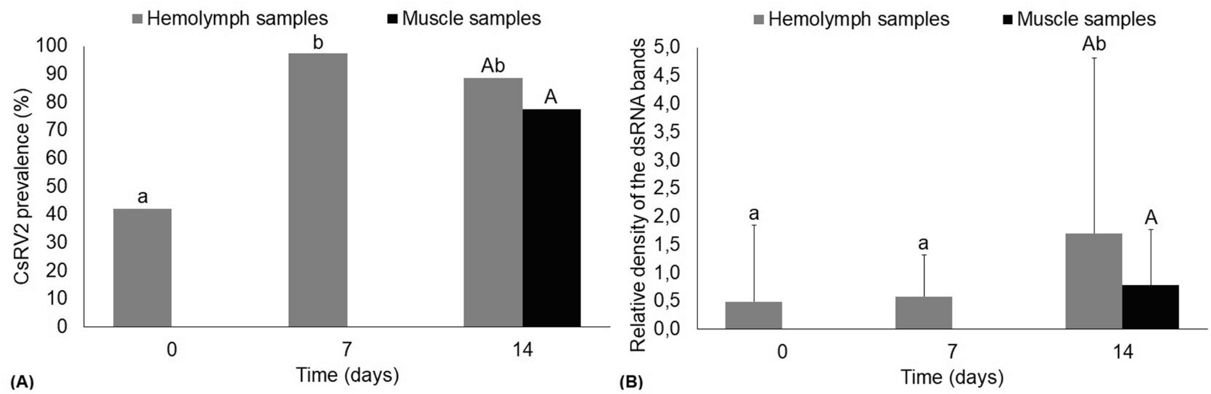

Fig. 3. Prevalence (A) and relative staining intensity of CsRV2-like dsRNA bands (B) of the RNA extracted from hemolymph (gray bar) and muscle (black bar) from *Callinectes danae* over 14 days of culture. Different letters indicate significant differences ($p < 0.05$), according to Pearson's chi-square test for prevalence data (%) and one way ANOVA, followed by Tukey's post hoc test for relative density of the dsRNA bands (Mean ± SD). Lowercase letters indicate significant differences among the crab's hemolymph samples over the experimental period. Uppercase letters indicate significant differences between hemolymph and muscle samples on the last (14th) day of the experiment.

Table 3
CsRV2-like dsRNA prevalence and relative density of dsRNA bands in blue crab *Callinectes danae* individuals that survived or died over the experimental period.

		Alive crabs			Dead crabs		
Sample origin	Time (days)	CsRV2 prevalence (%)	*n*	Relative density (mean ± SD)	CsRV2 prevalence (%)	*n*	Relative density (mean ± SD)
Hemolymph	0	12.5[a]	8	0.7 ± 0.0[a]	50.0[a]	45	0.5 ± 1.4[a]
	7	100.0[b]	10	0.5 ± 0.4[a]	96.4[b]	29	0.6 ± 0.7[b]
	14	86.6[b]	30	1.8 ± 3.3[b]	100.0[b]	5	1.1 ± 3.1[b]
Muscle	14	87.3[A]	87	1.0 ± 1.2[A]	68.1[B]	91	0.5 ± 0.5[B]

Different letters indicate a significant difference ($p < 0.05$) according to Pearson's Chi-square test, for prevalence data, and One-way ANOVA and Tukey's test, to compare relative density data sets. Lowercase letters indicate differences among the days of the experiment (vertical direction). Uppercase letters indicate differences between alive and dead crabs (horizontal direction).

Table 4
CsRV2 prevalence of *Callinectes danae* at different molting stages after 0 and 14 days in laboratory conditions.

Molt stages	Time (days)			
	0		14	
	n	CsRV2 prevalence (%)	*n*	CsRV2 prevalence (%)
Intermolt	171	44.4	63	79.4[a]
Premolt	7	0.0	115	76.5[ab]
Molt	0	0.0	5	40[b]

Different letters indicate significant differences ($p < 0.05$), according to Pearson's Chi-square test for prevalence data.

3.4. Metabolic evaluation

The levels of glucose, triglycerides, and total protein in *C. danae* hemolymph were not significantly different ($p > 0.05$) between CsRV2-infected and uninfected crabs (Table 5). In CsRV2 uninfected crabs, hemolymph metabolites remained constant ($p > 0.05$) throughout the experiment. Total protein levels in CsRV2-infected animals remained constant until the seventh day but were significantly reduced one week later (Kruskal-Wallis test: $H = 16.81$; $p = 0.0002$). In CsRV2 uninfected crabs, the total protein concentration decreased until the seventh day and remained the same until the last day of the experiment, but there was no statistical difference ($p > 0.05$) due to the reduced number of uninfected crabs sampled.

When the sexes were analyzed separately, there were no significant differences in glucose, triglyceride, and total protein concentrations in the hemolymph of infected versus uninfected males or females. The concentration of plasma glucose of infected males increased significantly over time (Kruskal-Wallis test: $H = 6.59$; $p = 0.03$), but this increase was not observed for infected and uninfected females ($p > 0.05$). It was not possible to analyze the metabolic changes in uninfected males, due to the lack of samples from uninfected males from the final week of the experiment. On the other hand, triglyceride concentrations in infected females' hemolymph decreased appproximately 50% (Kruskal-Wallis test: $H = 6.55$; $p = 0.03$) throughout the experiment, which did not happen in males ($p > 0.05$). Although not signifficant, a similar trend can be observed in males ($p > 0.05$). Total protein concentration declined significantly from day 7 to 14 in both infected females (Kruskal-Wallis test: $H = 15.11$; $p = 0.0005$) and infected males (Kruskal-Wallis test: $H = 6.48$; $p = 0.04$). Sample size ($n = 3$ and 1 for day 7 and 14, respectively) for uninfected crabs was very low.

Triglyceride concentrations in the gonads and hepatopancreas followed a similar pattern among CsRV2-infected and uninfected crabs. However, glycogen levels in hepatopancreas decreased (Mann-Whitney *U* test**:** $U = 1740$; $p = 0.04$) in infected compared to uninfected crabs (Table 6). The concentration of glycogen and triglycerides in the hepatopancreas and gonads was not significantly different when the sexes of infected and uninfected crabs when compared separately or between crabs in different molting stages.

3.5. Behavioral results

CsRV2 was detected in 74% ($n = 74$) of the crabs whose reflexes were evaluated. Among infected animals, 48.6% ($n = 36$) did not lose any of the six reflexes evaluated, and 51.4% ($n = 38$) showed some behavioral change. The proportion of reflex impairment considering all six reflexes differed significantly (Mann-Whitney *U* test: $U = 202$; $p = 0.009$) between CsRV2-infected and uninfected crabs only on the seventh day of the experiment (Table 7). In CsRV2-infected crabs, reflex impairment increased after 7 days and remained at this level until the end of the experiment. Of the six RAMP reflexes analyzed, the kick presented the

Table 5
Relationship of CsRV2-like dsRNA with hemolymph concentrations (in $mg.dL^{-1}$) of glucose, triglycerides, and total proteins in female and male *Callinectes danae* maintained under laboratory conditions. Values are expressed as median (min–max).

Plasma Metabolite	Time (days)	CsRV2 positive crabs				CsRV2 negative crabs			
		Median (min-max)			Total *n* (Male: Female)	Median (min-max)			Total *n* (Male: Female)
		All crabs	Male	Female		All crabs	Male	Female	
Glucose	0	9.3 (3.7–14.9)	7.7^{a} (1.7–9.8)	9.2 (5.7–19.5)	15 (5:10)	8.5 (1.7–19.5)	9.3 (3.7–14.5)	10.8 (4.5–14.9)	22 (14:8)
	7	8.9 (3.5–44.8)	9.7^{a} (6.6–16.2)	8.2 (3.5–44.8)	35 (14:21)	6.4 (6.4–6.4)	–	6.4 (6.4–6.4)	1 (0:1)
	14	7.9 (3.1–19.3)	10.6^{b} (8.2–19.3)	7.1 (3.1–19.2)	26 (9:17)	3.8 (0.5–12.9)	–	3.8 (0.5–12.9)	4 (0:4)
Triglycerides	0	14.9 (4.9–36.2)	9.4 (4.9–14.6)	20.6^{a} (7.2–36.2)	14 (5:9)	13.3 (6.6–79.5)	12.2 (6.6–79.5)	19.0 (7.5–63.1)	22 (14:8)
	7	11.0 (7.3–71.7)	9.3 (7.3–11.0)	13.8^{b} (8.3–71.7)	33 (14:19)	25.6 (25.6–25.6)	–	25.6 (25.6–25.6)	1 (0:1)
	14	10.2 (5.2–74.9)	5.7 (5.2–74.9)	9.3^{b} (8.1–9.7)	26 (11:15)	9.3 (8.1–9.7)	–	10.9 (6.5–25.4)	3 (0:3)
Total Protein	0	2.3^{a} (1.0–4.0)	2.2^{a} (1.0–3.8)	2.3^{a} (1.5–4.0)	15 (5:10)	2.6 (1.0–4.2)	2.3 (1.0–3.6)	2.7 (1.5–4.2)	21 (14:7)
	7	2.8^{a} (1.4–3.3)	2.3^{a} (1.4–3.3)	3.0^{a} (1.7–3.3)	32 (14:18)	1.8 (1.9–1.9)	–	1.9 (1.9–1.9)	1 (0:1)
	14	1.9^{b} (1.4–3.3)	1.8^{b} (1.4–2.4)	1.9^{b} (1.2–2.7)	28 (10:18)	1.9 (1.6–1.9)	–	1.9 (1.6–1.9)	3 (0:3)

Lowercase letters indicate a significant difference ($p < 0.05$), according to the Kruskal-Wallis test and Mann Whitney's *U* test, among the days of the experiment (vertical direction). There was no significant difference between CsRV2 infected and uninfected crabs for each day.

Table 6
Relationship of CsRV2-like dsRNA with the concentrations (in $mg.g^{-1}$) of glycogen in hepatopancreas and triglycerides in hepatopancreas and gonads of *Callinectes danae* maintained in laboratory conditions.

Tissue Metabolite	Time (days)	CsRV2 positive crabs				CsRV2 negative crabs			
		Median (min–max)			Total *n* (Male: Female)	Median (min–max)			Total *n* (Male: Female)
		All	Male	Female		All	Male	Female	
GT	14	57.4 (19.2–325.4)	230.7 (230.6-230.6)	57.3 (19.2-325.4)	50 (1:49)	44.3 (13.9–154.6)	–	44.3 (13.9-154.6)	12 (0:12)
HG	14	0.01^{A} (0.0–1.3)	0.01 (p.0-1.3)	0.02 (0.0-0.5)	124 (61:63)	0.02^{B} (0.0–1.0)	0.02 (0.0-1.0)	0.02 (0.0-0.1)	36 (19:17)
HT	14	42.6 (6.0–153.2)	40.6 (6.0-92.9)	45.2 (7.2-153.2)	94 (49:45)	36.3 (2.9–95.5)	39.69 (2.9-70.5)	31.3 (7.3-95.5)	27 (13:14)

GT: Gonad Triglycerides; HG: Hepatopancreas Glycogen; HT: Hepatopancreas Triglycerides. Uppercase letters indicate significant differences ($p < 0.05$), according to Mann Whitney's *U* test, between CsRV2 infected crabs and uninfected crabs.

Table 7
Relationship of CsRV2-like dsRNA with reflex impairment (RAMP test) in *Callinectes danae* maintained in laboratory conditions.

	Time (days)	Reflex impairment (RAMP) - Median (min–max)			
		CsRV2- crabs	*n*	CsRV2 + crabs	*n*
All reflexes tested	0	0.0 (0.0–0.17)	26	0.00^{a} (0.0–0.3)	74
	7	0.0^{A} (0.0–0.17)	13	0.17^{Bb} (0.0–0.8)	58
	14	0.0 (0.0–0.17)	3	0.17^{b} (0.0–0.7)	32

CsRV2-: CsRV2 negative crabs; CsRV2+: CsRV2 positive crabs. Lowercase letters indicate significant differences ($p < 0.05$), using the Kruskal-Wallis test among the days of the experiment. Uppercase letters indicate significant differences, according to the Mann Whitney *U* test, between infected and uninfected crabs for each day.

most significant reduction throughout the experiment: it declined 23% at the beginning, 44.1% after 7 days and, by 14 days, 69.4% of the animals no longer had this reflex (Fig. 4A). Meanwhile, cheliped retraction, ocular retraction, and mouth closure reflexes were more rarely lost. The Mann Whitney *U* test indicated a significant difference in the loss of kick reflex between infected and uninfected crabs on the seventh day of the experiment (Mann-Whitney *U* test: $U = 217$; $p = 0.01$), while the other reflexes did not present significant changes (Fig. 4B).

The Mann Whitney *U* test showed no significant difference ($p > 0.05$) in the concentrations of total proteins, triglycerides and glucose in the hemolymph between crabs that maintained the kick reflex and those that lost it over the experimental period. In addition, the Spearman correlation test showed that there was no correlation between the loss of kick reflex and the nitrite concentrations (Spearman's correlation test, $r_s = -0.04$, $p > 0.05$) measured on the behavioral assessment days. The test also showed a statistically significant but weak correlation (Spearman's correlation test, $r_s = 0.28$, $p < 0.05$) between the loss of kick reflex and dissolved oxygen concentrations.

4. Discussion

The present study provided the first quantification of the mortality of blue crab *C. danae* cultivated in a recirculation system. It was also the first study to investigate whether CsRV1, which is pathogenic to captive *C. sapidus*, was present in cultured *C. danae*. The investigation revealed that, while CsRV1 was not detected, a novel putative reovirus, provisionally called CsRV2, was highly prevalent. The prevalence of CsRV2 was not associated with mortality, although virus load increased over time among surviving crabs. The study investigated the correlation of CsRV2 with metabolic state and behavioral signs in *C. danae* captured in the wild and kept in captivity.

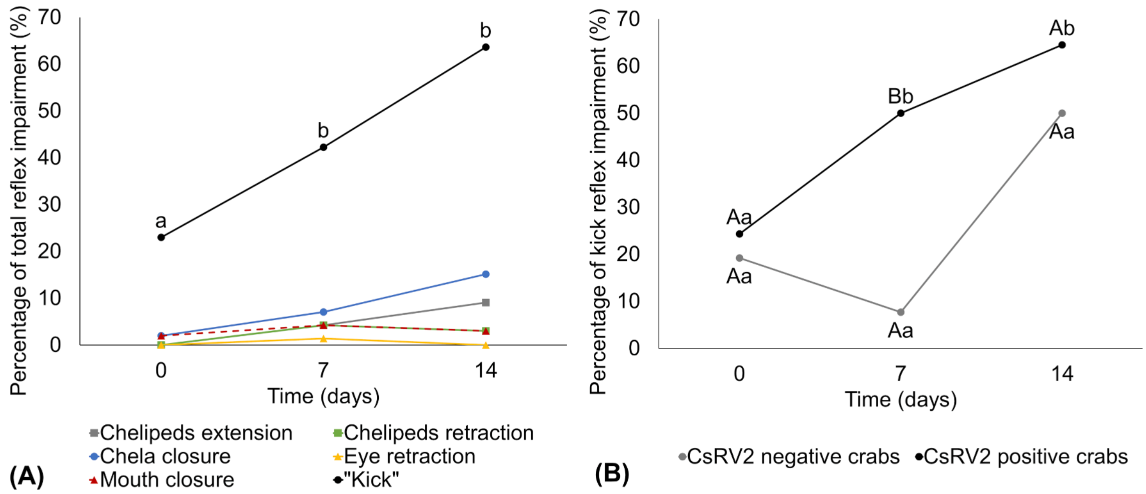

Fig. 4. (A) Percentage of *Callinectes danae* individuals with compromised reflexes during the experiment; (B) Percentage of infected and uninfected crabs that had compromised kick reflex. Lowercase letters represent significant differences in loss of reflexes between assessment days ($p < 0.05$; Kruskal-Wallis test). Uppercase letters represent significant differences between infected and uninfected animals over the experiment ($p < 0.05$; Mann Whitney *U* test).

4.1. Mortality

Information on *Callinectes* spp. aquaculture and mortality rates in commercial enterprises are still rare, both due to the small number of companies pursuing this activity and the commercial secrets usually involved. However, the mortality rate recorded in the present study was similar to that reported by Ostrensky et al., (2015), which aimed to identify the biological characteristics of *C. danae* and *C. exasperatus* for soft-shell crabs production in Brazil. In that case, the authors reported mortality rates in a continuous flow system ranging from 37.5% to 60% for *C. danae* and *C. exasperatus*, respectively.

The main pathogenic microorganisms reported to cause mortality in *Callinectes* spp. include the protozoan *Hematodinium perezi* (Messick and Shields, 2000; Stentiford and Shields, 2005), bacterium *Vibrio* spp. (Thibodeaux et al., 2009), microsporidian *Ameson michaelis* (Shields and Overstreet, 2003) and the CsRV1 reovirus (Spitznagel et al., 2019). The cause of the high mortality rate of *C. danae* in the present study has not been determined, but there is no evidence that most deaths were caused by bacterial or protistan infections. Mortality caused by bacteria occurs quickly, usually in the first three days of infection, as the bacteria are typically controlled by the cellular and humoral immune response of crustaceans (Johnson, 1976). Infections by *A. michaelis* in crabs are relatively rare, usually found in low prevalence and infected animals show severe muscle lysis that results in a condition known as “cotton crab”, a condition not identified in the animals analyzed (Kennedy and Cronin, 2007). *Hematodinium perezi* is a pathogenic dinoflagellate that causes a high mortality rate in *C. sapidus*, but has not been detected in *C. danae* in previous investigations using RT-qPCR (Tavares et al., unpublished data).

Viral pathogens are the main infectious agents limiting the survival of crabs in commercial scale aquaculture (Kennedy and Cronin, 2007; Perazzolo et al., 2012). Among the eight virus species reported in *Callinectes* spp. along the Atlantic coast, four are lethal, such as Bi-Facies herpes-like virus (BFV ou HLV) (Johnson, 1977), Chesapeake Bay picornavirus (CBV) (Johnson, 1977), Rhabdo-like virus (RhVA) (Jahromi, 1977) and *Callinectes sapidus* reovírus 1 (CsRV1) (Bowers et al., 2010). CsRV1 is the virus most associated with episodes of high and rapid mortality of *C. sapidus* in soft-shell crab facilities, with mortality of up to 100% in a few days after experimental injections (Bowers et al., 2010; Flowers et al., 2018; Spitznagel et al., 2019; Tang et al., 2011). The fact that this virus was undetected in any of the *C. danae* in this study suggests that either the pathogen is absent from the geographic region, or that *C. danae* is not a host for CsRV1. Zhao et al., (2020) analyzed the presence of CsRV1 in *C. sapidus* collected in the same region where *C. danae* was collected in the present study, the authors observed that all tested crabs were negative for CsRV1. However, it is unlikely that CsRV1 is not present, as Zhao et al., (2020) report CsRV1 across the entire geographic range of *C. sapidus*, including in Rio Grande do Sul with a prevalence of 33.3%. Furthermore, Zhao et al., (2020) observed that no other species of *Callinectes* were infected with CsRV1 during the survey, which further supports the hypothesis that CsRV1 is likely host-specific for *C. sapidus*.

No significant difference was observed in the prevalence of the putative reovirus in dead or surviving crabs in the present study, suggesting that the CsRV2 is not pathogenic to *C. danae* in these conditions. Potentially pathogenic viruses can occur at high prevalence in aquaculture settings and yet cause no disease or mortality until environmental or developmental stresses combine with infection to cause disease (Walker et al., 2011). A well-known example is the outbreak of white spot syndrome virus disease in shrimp farms induced by a rapid change in salinity or temperature (Granja et al., 2003). In crustaceans, disease development is also strongly associated with the characteristics of the host’s life history, such as molting state, physical condition, sex, and age (Bateman et al., 2011).

Although the association of mortality and infection by the putative CsRV2 was not evident in the present study, crustacean mortality is frequently reported in experimental infections with other reovirus species. Experimental injection of purified P reovirus initially caused tremors in 60% of *Macropipus depurator*, followed by paralysis and mortality of 70 to 80% of infected animals after 9 days (Bonami et al., 1976). The experimental infection of Mud Crab Reovirus (MCRV) in *S. serrata* by intramuscular injection, bath inoculation and oral inoculation also led to 100% mortality, while cohabitation caused 80% mortality (Weng et al., 2007). The experimental infection of purified EsRV905 and EsRV816 in *Eriocheir sinensis* caused 30% mortality, moreover, the mortality rate was increased in co-infections with bunyavirus-like viruses (Bonami and Zhang, 2011; Zhang et al., 2004).

Zhao et al., (2021), proposed that CsRV2 and EsRV905 belong to the same genus, *Cardoreovirus*, based on the nucleotides identity and similarity encoded proteins, as well as their electrophoretic genome organization. Due to the genomic similarity of CsRV2 with a fatal reovirus for crabs, understanding the potential for CsRV2 to cause disease will be crucial for assessing the risks associated with infection with this previously unknown virus for the soft-shell crab industry in Brazil.

Crab mortality can also be a result of the stress of capture, handling, transport, temperature, injury and poor water quality, especially in poorly-managed aquaculture systems (Johnson, 1976). To avoid excessive stress and the consequent mortality, we strictly followed the process of collecting and transporting recommended by Chaves and Eggleston, (2003). Although the sumulative mortality of captive crabs was > 50% in the current study, there was no pulse of mortality at the beginning of the culture period, suggesting that capture and transport were not the major causes of *C. danae* mortality. In addition, the absence of changes in metabolites in the hemolymph of uninfected crab corroborates the efficiency of the collection and transport method.

We also found little evidence that captive crab mortality was related to water quality parameters during the experiment. Among the water quality parameters suggested by Hochheimer (1988), and Malone and Burden (1988) to be ideal for crab aquaculture, only nitrite and dissolved oxygen strayed outside these limits. However, as outlined below, variation of nitrate and oxygen do not appear to have interfered with the results. According to Ary and Poirrier (1989), blue crabs have a relatively high tolerance to nitrite, the authors recorded a mortality rate of only 4.5% in premolt crabs after 96 h of exposure to 10 mg/L $N\text{-}NO_2$, representing a concentration 10 × higher than that found in the crab maintenance systems used in the present experiment. Regarding dissolved oxygen, Tomasetti et al., (2018) considered as "moderate hypoxia conditions" concentrations below 4 mg/L O_2. Brill et al., (2015) concluded that the critical levels of dissolved oxygen for *C. sapidus* are on the order of 45% saturation and that the metabolic rates of crabs are not compromised with dissolved oxygen above 50% saturation.

4.2. Reovirus prevalence

We tested the hypothesis that mortality of cultured *C. danae* would be be associated with CsRV1, similar to correlation of this virus with mortality in cultured *C. sapidus* (Bowers et al., 2010; Spitznagel et al., 2019). CsRV1 is present in 30–33% of *C. sapidus* collected in Lagoa do Tramandaí, Rio Grande do Sul, Brazil (Flowers et al., 2016) and 10% in Punta del Este, Uruguay (Zhao et al., 2020), which shows that this virus is endemic to South America. While the absence of CsRV1 in aquaculture crabs refutes the hypothesis, it does not mean that *C. danae* is can never be a host. Viruses have differential abilities to infect, transmit, and cause disease in specific host taxa, and this capacity in CsRV1 needs to be better understood by experimental infection studies involving the injection of CsRV1 into *C. danae* (Stentiford et al., 2012).

The discovery that > 70% of the *C. danae* in this study were infected by a novel reovirus was remarkable. The electrophoretic pattern of the dsRNA (3/4/5) is identical to that of a putative virus, CsRV2, described by Zhao et al., (2021) in *C. sapidus* from Southern Brazil. The electrophoretic pattern of the CsRV2 genome segments is very similar but not identical to that of Eriocheir sinensis reovirus 905 (Zhang et al., 2004). In contrast, the electrophoretic pattern of CsRV1 (1/5/6) is shared by *Carcinus mediterraneus* W2 virus (Mari and Bonami, 1988), *Macropipus depurator* P virus (Montanie et al., 1993) and *Scylla serrata* MCRV reovirus (Bonami and Zhang, 2011; Weng et al., 2007). To confirm whether the virus observed in this study is indeed CsRV2, some or all of the viral genome need to be sequenced, and a phylogenetic analysis needs to be conducted to discern its relationship to reoviruses already identified in brachyuran crabs.

Even in the absence of virus genome sequence, we developed a semi-quantitative method to measure CsRV2 dsRNA band intensity on agarose gels to estimate the viral load in infected animals. Similar semi-quantitative methods have been used in other studies, to quantify the intensity of Western Blot bands of shrimp and crab proteins and measure the abundance of PCR amplicons of the bacterium *Fusobacterium necrophorum* (Antiabong et al., 2016; Feng et al., 2020; Johnston et al., 2019; Lin et al., 2012). The semi-quantitative method revealed that CsRV2 prevalence increased 46.5% over the 14 days experiment and showed that the average apparent intensity of infection rose significantly in surviving crabs. Although the semi-quantitative method has proved to be useful for estimating the relative viral load of CsRV2, the method must be validated by RT-qPCR to determine its accuracy in determining the concentration of CsRV2 in future studies.

4.3. Biochemical changes in hemolymph and hepatopancreas

Fluctuations in temperature, salinity and dissolved oxygen are stressful to crustaceans and increase susceptibility to pathogen infections (Chung et al., 2015; Shields and Overstreet, 2003; Spitznagel et al., 2019; Vinagre et al., 2020b). The crustacean stress response starts with neuroendocrine alterations that trigger metabolic, physiological and behavioral adjustments, also known as the secondary stress response, in order to reestablish internal homeostasis (Aparicio-Simón et al., 2010; Inohara et al., 2015; Lorenzon et al., 2007; Model et al., 2019; Zamora-Briseño et al., 2020). Some of these biochemical and physiological adjustments are monitored as stress indicators/markers that may be useful in predicting mortalities (Aparicio-Simón et al., 2010; Model et al., 2019; Stoner, 2012).

Glucose levels in the hemolymph of crustaceans are mainly controlled by crustacean hyperglycemic hormone (CHH), as it plays a key role in the regulation of glucose synthesis and release from the hepatopancreas and muscle, stimulating glycogenolysis in these tissues (Chen et al., 2020; Verri et al., 2001; Vinagre et al., 2020). Besides CHH, glucose homeostasis in crustaceans can also be influenced by monoamines, such as serotonin, melatonin and cathecolamines, that can act either directly on metabolic organs or indirectly by stimulating CHH release from the sinus gland (Inohara et al., 2015; Maciel et al., 2014; Model et al., 2019). Furthermore, it was recently evidenced that both cathecolamines, norepinephrine and epinephrine hemolymphatic levels, can increase during stress conditions in crustaceans (Aparicio-Simón et al., 2010; Avramov et al., 2013).

Our investigation revealed that the presence of CsRV2 dsRNA was generally not associated with changes in metabolites when both sexes are grouped together, except for hepatopancreas glycogen. The levels of metabolites in uninfected crabs also did not change during the experiment suggesting that, despite the deaths recorded, the environmental conditions were appropriate. The concentrations of metabolites measured in the hemolymph and tissues are in agreement with concentrations recorded in other crabs such as *Ocypode quadrata* and *Neohelice granulata*, and validate these analyses as a tool to evaluate crab health (Vinagre et al., 2020a; Vinagre and Chung, 2016). However, when crabs were analyzed separately by sex, some differences in metabolites were seen, which suggests that the impact of CsRV2 infection on intermediary metabolism is different between males and females.

Hemolymph glucose was higher in CsRV2-infected males, but not females, after 14 days of culture. When decapod crustaceans undergo stressful conditions, such as infection by viral pathogens (Herrera-Salvatierra et al., 2019; Yoganandhan et al., 2003), glucose in hemolymph can increase via glycogenolysis in muscle or hepatopancreas, which can be associated with the release of CHH from the sinus gland into the hemolymph (Jimenez and Kinsey, 2015). In lobsters *Panulirus argus*, infection with the PaV1 virus caused an increase in glucose concentration in hemolymph and a drastic reduction in glycogen concentration in the hepatopancreas of infected lobsters compared to uninfected lobsters (Herrera-Salvatierra et al., 2019).

In the present study, it was also observed that glycogen concentrations were lower in the hepatopancreas of CsRV2-infected crabs, however, no statistically significant reduction in hepatopancreas glycogen concentration was observed specifically in CsRV2-infected males, which may indicate that the increase in circulating glucose in males can be due to glycogenolysis that occurs in other tissues or even by metabolic pathways such as gluconeogenesis (Chen et al., 2020; Jimenez and Kinsey, 2015; Vinagre et al., 2020b). Glycogen depletion can have negative consequences for CsRV2-infected crabs, such as impaired locomotion, respiration, and osmoregulation (Gabbott, 2009; Rainer and Brouwer, 1993; Vinagre et al., 2020). These alterations in carbohydrate metabolism and the expression of CHH should be investigated in future studies on the impact of CsRV2 in *C. danae*.

Triglyceride concentrations in the hemolymph of CsRV2-infected *C. danae* females were 50% lower than uninfected females. Although not significant, hemolymph triglycerides decreased by 40% in infected males. In PaV1-infected *P. argus*, hemolymph triglycerides increased and were associate with downregulation of triacylglycerol lipase, one of the main enzymes of lipolysis (Zamora-Briseño et al., 2020). Since the concentration of triglycerides in the gonads and hepatopancreas were not affected in *C. danae* of either sex, the decrease in female hemolymph triglycerides may be related to a sex-specific alteration in lipid metabolism and requires further investigation.

A significant decrease in total hemolymph protein in male and female *C. danae* infected by CsRV2 was recorded throughout the experimental period. Although not significant, a similar trend was observed in uninfected crabs. Therefore, it seems that CsRV2 infection exacerbates this protein loss. Pathological processes may explain the reduction in the concentration of hemolymph proteins in crabs (Findley et al., 1981; Pauley et al., 1975; Shields and Overstreet, 2003; Yoganandhan et al., 2003). According to Shields et al., (2003), the metabolic demands due the logarithmically proliferating pathogens in hemolymph of the host drains protein and carbohydrate constituents in infected animals. Crustaceans heavily infected by pathogens also become lethargic and reduce eating which can cause declines in total protein concentrations (Herrera-Salvatierra et al., 2019; Shields and Overstreet, 2003; Taylor et al., 1996). In the transcriptomic comparison between *P. argus* PaV1-infected and uninfected, many enzymes involved in amino acid and protein metabolism were down-regulated, and digestive proteases were reduced, which suggests that directly or indirectly, virus infection induced a general disruption of protein metabolism (Zamora-Briseño et al., 2020).

4.4. Behavioral changes

An animal's behavior may suffer alterations due to stress caused by pathogen infection. Behavioral changes include simple or complex actions such as evasion, aggression, even the loss of specific reflexes (Mcintyre et al., 1980). The Reflective Action Mortality Predictor (RAMP) is a method that relates reflex impairment to a mortality probability (Davis, 2007; Davis and Ottmar, 2006; Stoner, 2012). The RAMP test was initially developed to predict mortality of Alaskan crab *Chionoecetes opilio* bycatch (Stoner et al., 2008), and tested in other decapod crustaceans (Stoner, 2012; Yochum, 2016; Yochum et al., 2015). This study was the first to apply the RAMP test in *C. danae* and to relate the test results to the presence of a specific virus, such as CsRV2.

Of the six reflexes analyzed, eye retraction and mouth closure were rarely lost, similar to those reported by Yochum et al., (2015) for *Chionoecetes bairdi* and *C. opilio* collected and discarded from trawls. The kick reflex was most often lost in *C. danae* during the experiment, while in *C. bairdi* it was only the fourth most lost reflex in response to catching stress. Studies by Stoner (2009) and Stoner et al., (2008) reveal that when *C. bairdi* and *C. opilio* were subjected to thermal stress, the chela closure and kick were the reflexes usually lost. Stoner et al., (2008) also concluded that the kick reflex was one of the most sensitive indicators of stress in snow crabs. Although the collection, transport and maintenance in aquaculture facilities are stressful for crabs (Chaves and Eggleston, 2003), the secondary stress metabolites analyzed in the present study did not differ significantly between the normal crabs and those that lost the kick reflex.

The RAMP test revealed that *C. danae* infected with CsRV2 had a significant kick reflex deficit on the seventh day of experiment, which was a likely consequence of physiological exhaustion, as previously reported by Raby et al., (2012). Although ours is the first report of the RAMP test to evaluate viral infection, behavioral changes such as lethargy, paralysis and abnormal feeding have been reported in crabs infected by different species of reovirus (Zhao et al., 2021b). In addition, lobsters infected by PaV1 became lethargic in association with a deficient nutritional status (Herrera-Salvatierra et al., 2019). Thus, the kick reflex should be explored and tested as a tool at crab shedding facilities to indicate possible infection by CsRV2 or other pathogens that might predict the premature mortality of premolt crabs.

5. Conclusion

Callinectes danae is of great ecological, social, and economic importance as a fishery resource, and to the nascent soft-shell crab aquaculture industry in Brazil, which suffers from > 25% mortality. There is abundant literature showing that mortality in aquaculture is often a combination of stress and infectious pathogens. This investigation of possible virus pathogens related to *C. danae* mortality revealed that a well-known pathogen of *C. sapidus* was absent and that a novel putative virus, CsRV2, was highly prevalent in both live and dead *C. danae*. Moreover, the observation of this novel putative reovirus, and the description of metabolic and behavioral changes affecting the crabs infected with CsRV2, serves as an indication to the scientific community and to soft-shell crab farmers that there are many more pathogens to be discovered in *C. danae*. Although this study's results did not indicate a clear association between CsRV2 infection and an increase in mortality rates in *C. danae*, this hypothesis cannot yet be ruled out. Therefore, a more complete investigation of CsRV2 should be carried out, beginning with three priorities: 1) to assess the degree of virulence and pathogenicity CsRV2 using experimental infections, so that control or coexistence measures with the novel virus can be developed if necessary; 2) to understand the genetic diversity of CsRV2, which will require sequencing the virus genome and will permit the development of PCR-based detection methods; and 3) to evaluate the phylogenetic relationship with the reoviruses already identified.

Declaration of Competing Interest

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

Acknowledgement

The authors would like to acknowledge CNPq for the Research Productivity Grant to Dr. Antonio Ostrensky (process No 304633/2017-8; for the financing of the project (process No 381091/2014-7), and the grant of a doctoral scholarship to Camila P. S. Tavares (process No 141022/2017-5). To CAPES for granting the Sandwich Doctorate scholarship abroad to Camila P. S. Tavares (process No 88881.190487/2018-01). Award NSF Division of Ocean Sciences – Biological Oceanography award 1658466 to Dr. Eric J. Schott.

References

Abràmoff, M.D., Magalhães, P.J., Ram, S.J., 2004. Image processing with image. J. Biophotonics Int. 11, 36–41. https://doi.org/10.1201/9781420005615.ax4.

Antiabong, J.F., Ngoepe, M.G., Abechi, A.S., 2016. Semi-quantitative digital analysis of polymerase chain reactionelectrophoresis gel: Potential applications in low-income veterinary laboratories. Vet. World 9, 935–939. https://doi.org/10.14202/vetworld.2016.935-939.

Antunes, G.d.F., do Amaral, A.P.N., Ribarcki, F.P., Wiilland, E.d.F., Zancan, D.M., Vinagre, A.S., 2010. Seasonal variations in the biochemical composition and reproductive cycle of the ghost crab *Ocypode quadrata* (Fabricius, 1787) in Southern Brazil. J. Exp. Zool. 9999A, n/a–n/a.

Aparicio-Simón, B., Piñón, M., Racotta, R., Racotta, I.S., 2010. Neuroendocrine and metabolic responses of Pacific whiteleg shrimp *Litopenaeus vannamei* exposed to acute handling stress. Aquaculture 298 (3-4), 308–314. https://doi.org/10.1016/j.aquaculture.2009.10.016.

APHA, 1995. Association News: 1995 APHA Award Nomination Forms, American Journal of Public Health. American Public Health Association, Washington, DC, USA. https://doi.org/10.2105/ajph.85.1.127.

Ary, R.D., Poirrier, M.A., 1989. Acute toxicity of nitrite to the blue crab (*Callinectes sapidus*). Progress. Fish-Culturist 51, 69–72. https://doi.org/10.1577/1548-8640(1989)051<0069:ATONTT>2.3.CO;2.

Avramov, M., Rock, T.M., Pfister, G., Schramm, K.W., Schmidt, S.I., Griebler, C., 2013. Catecholamine levels in groundwater and stream amphipods and their response to temperature stress. Gen. Comp. Endocrinol. 194, 110–117. https://doi.org/10.1016/j.ygcen.2013.09.004.

Baptista, C., Pinheiro, M.A.A., Blankensteyn, A., Borzone, C.A., 2003. Estrutura populacional de *Callinectes ornatus* Ordway (Crustacea, Portunidae) no Balneário Shangri-Lá, Pontal do Paraná, Paraná. Brasil. Rev. Bras. Zool. 20 (4), 661–666.

Bateman, K.S., Hicks, R.J., Stentiford, G.D., 2011. Disease profiles differ between non-fished and fished populations of edible crab (*Cancer pagurus*) from a major commercial fishery. ICES J. Mar. Sci. 68, 2044–2052. https://doi.org/10.1093/icesjms/fsr148.

Bonami, J.-R., Comps, M., Veyrunes, J.C., 1976. Etude histopathologique et ultrastructurale de la paralysie virale du crabe *Macropipus depurator*. L. Rev. Trav. Inst. Pêches Marit. 40, 139–146.

Bonami, J.-R., Zhang, S., 2011. Viral diseases in commercially exploited crabs: A review. J. Invertebr. Pathol. 106 (1), 6–17. https://doi.org/10.1016/j.jip.2010.09.009.

Bowers, H.A., Messick, G.A., Hanif, A., Jagus, R., Carrion, L., Zmora, O., Schott, E.J., 2010. Physicochemical properties of double-stranded RNA used to discover a reo-like virus from blue crab *Callinectes sapidus*. Dis. Aquat. Organ. 93 (1), 17–29. https://doi.org/10.3354/dao02280.

Brill, R.W., Bushnell, P.G., Elton, T.A., Small, H.J., 2015. The ability of blue crab (*Callinectes sapidus*, Rathbun 1886) to sustain aerobic metabolism during hypoxia. J. Exp. Mar. Bio. Ecol. 471, 126–136. https://doi.org/10.1016/j.jembe.2015.06.003.

Chang, E.S., Mykles, D.L., 2011. Regulation of crustacean molting: A review and our perspectives. Gen. Comp. Endocrinol. 172 (3), 323–330. https://doi.org/10.1016/j.ygcen.2011.04.003.

Chaves, J.C., Eggleston, D.B., 2003. Blue crab mortality in the North Carolina soft-shell industry: Biological and operational effects. J. Shellfish Res. 22, 241–249.

Chen, H.Y., Toullec, J.Y., Lee, C.Y., 2020. The crustacean hyperglycemic hormone superfamily: progress made in the past decade. Front. Endocrinol. (Lausanne). https://doi.org/10.3389/fendo.2020.578958.

Chung, J.S., 2010. Hemolymph ecdysteroids during the last three molt cycles of the blue crab, *Callinectes sapidus*: Quantitative and qualitative analyses and regulation. Arch. Insect Biochem. Physiol. 73 (1), 1–13. https://doi.org/10.1002/arch.20327.

Chung, J.S., Pitula, J.S., Schott, E., Alvarez, J.V., Maurer, L., Lycett, K.A., 2015. 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*. Fish Shellfish Immunol. 47 (1), 511–520. https://doi.org/10.1016/j.fsi.2015.09.027.

Davis, M.W., 2007. Simulated fishing experiments for predicting delayed mortality rates using reflex impairment in restrained fish. ICES J. Mar. Sci. 64, 1535–1542. https://doi.org/10.1093/icesjms/fsm087.

Davis, M.W., Ottmar, M.L., 2006. Wounding and reflex impairment may be predictors for mortality in discarded or escaped fish. Fish. Res. 82 (1-3), 1–6. https://doi.org/10.1016/j.fishres.2006.09.004.

deFur, P.L., Nusbaumer, D., Lewis, R.J., 1988. Physiological aspects of molting in blue crabs from the tidal fresh-water Potomac River. Virginia. J. Crustac. Biol. 8 (1), 12. https://doi.org/10.2307/1548425.

Fabbri, E., Moon, T.W., 2016. Adrenergic signaling in teleost fish liver, a challenging path. Comp. Biochem. Physiol. Part - B Biochem. Mol. Biol. 199, 74–86. https://doi.org/10.1016/j.cbpb.2015.10.002.

Fan, W., Ye, Y., Chen, Z., Shao, Y., Xie, X., Zhang, W., Liu, H.P., Li, C., 2016. Metabolic product response profiles of *Cherax quadricarinatus* towards white spot syndrome virus infection. Dev. Comp. Immunol. 61, 236–241. https://doi.org/10.1016/j.dci.2016.04.006.

FAO, 2020. Global Capture and Aquaculture Production (FishStat) [WWW Document]. Rome, Italy. URL http://www.fao.org/figis/servlet/SQServlet?file=/work/FIGIS/prod/webapps/figis/temp/hqp_5215617422254205836.xml&outtype=html (accessed 6.27.20).

Feng, H., Dong, Y.-T., Liu, X., Qiu, G.-F., 2020. Cyclin B protein undergoes increased expression and nuclear relocation during oocyte meiotic maturation of the freshwater prawn *Macrobrachium rosenbergii* and the Chinese mitten crab *Eriocheir sinensis*. Gene 758, 144955. https://doi.org/10.1016/j.gene.2020.144955.

Ferdoushi, Z., XiangGuo, Z., Hasan, M.R., 2010. Mud crab (*Scylla* sp.) marketing system in Bangladesh. Asian J. Food Agro-Industry 3, 248–265.

Findley, A.M., Blakeney, E.W., Weidner, E.H., 1981. *Ameson Michaelis* (Microsporida) in the Blue Crab, *Callinectes sapidus*: parasite-induced alterations in the biochemical composition of host tissues. Biol. Bull. 161 (1), 115–125. https://doi.org/10.2307/1541112.

Flowers, E.M., Bachvaroff, T.R., Warg, J.V., Neill, J.D., Killian, M.L., Vinagre, A.S., Brown, S., Almeida, A.S.e., Schott, E.J., 2016. Genome sequence analysis of CsRV1: A pathogenic reovirus that infects the blue crab *Callinectes sapidus* across its trans-hemispheric range. Front. Microbiol. 7 https://doi.org/10.3389/fmicb.2016.00126.

Flowers, E.M., Johnson, A.F., Aguilar, R., Schott, E.J., 2018. Prevalence of the pathogenic crustacean virus Callinectes sapidus reovirus 1 near flow-through blue crab aquaculture in Chesapeake Bay. USA. Dis. Aquat. Organ. 129 (2), 135–144. https://doi.org/10.3354/dao03232.

Freeman, J.A., Kilgus, G., Laurendeau, D., Perry, H.M., 1987. Postmolt and intermolt molt cycle stages of *Callinectes sapidus*. Aquaculture 61 (3-4), 201–209. https://doi.org/10.1016/0044-8486(87)90149-9.

Gabbott, A.P., 2009. Energy metabolism. In: Bayne, B.L. (Ed.), Marine Mussels: Their Ecology and Physiology. Cambridge University Press, Cambridge; London; New York, pp. 293–356.

Gallo, V.P., Accordi, F., Chimenti, C., Civinini, A., Crivellato, E., 2016. Catecholaminergic system of invertebrates: comparative and evolutionary aspects in comparison with the octopaminergic system. Int. Rev. Cell Mol. Biol. 322, 363–394. https://doi.org/10.1016/bs.ircmb.2015.12.006.

Granja, C.B., Aranguren, L.F., Vidal, O.M., Aragón, L., Salazar, M., 2003. Does hyperthermia increase apoptosis in white spot syndrome virus (WSSV)-infected *Litopenaeus vannamei*? Dis. Aquat. Organ. 54, 73–78. https://doi.org/10.3354/dao054073.

Hernández-Palomares, M.L.E., Godoy-Lugo, J.A., Gómez-Jiménez, S., Gámez-Alejo, L.A., Ortiz, R.M., Muñoz-Valle, J.F., Peregrino-Uriarte, A.B., Yepiz-Plascencia, G., Rosas-Rodríguez, J.A., Soñanez-Organis, J.G., 2018. Regulation of lactate dehydrogenase in response to WSSV infection in the shrimp *Litopenaeus vannamei*. Fish Shellfish Immunol. 74, 401–409. https://doi.org/10.1016/j.fsi.2018.01.011.

Herrera-Salvatierra, N., Pascual-Jiménez, C., Huchin-Mian, J.P., Lozano-Alvarez, E., Montero-Muñoz, J., Briones-Fourzán, P., Rodríguez-Canul, R., 2019. Nutritional and immunological evaluation of juvenile spiny lobsters *Panulirus argus* (Latreille, 1804) (Decapoda: Achelata: Palinuridae) naturally infected with the PaV1 virus. J. Crustac. Biol. 39, 162–171. https://doi.org/10.1093/jcbiol/ruy108.

Hochheimer, J., 1988. Water quality in soft crab shedding, crab shedders workbook series. University of Maryland, Sea Grant Extension Program, College Park, MD, USA.

Hungria, D.B., dos Santos Tavares, C.P., Pereira, L.Â., de Assis Teixeira da Silva, U., Ostrensky, A., 2017. Global status of production and commercialization of soft-shell crabs. Aquac. Int. 25 (6), 2213–2226. https://doi.org/10.1007/s10499-017-0183-5.

Inohara, E.T.S., Pinto, C.B., Model, J.F.A., Trapp, M., Kucharski, L.C., Da Silva, R.S.M., Vinagre, A.S., 2015. Serotonin effects in the crab *Neohelice granulata*: Possible involvement of two types of receptors in peripheral tissues. Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol. 185, 80–90. https://doi.org/10.1016/j.cbpa.2015.03.012.

Jahromi, S.S., 1977. Occurrence of rhabdovirus like particles in the blue crab, *Callinectes sapidus*. J. Gen. Virol. 36 (3), 485–493. https://doi.org/10.1099/0022-1317-36-3-485.

Jimenez, A.G., Kinsey, S., 2015. Energetics and metabolic regulation, The Natural History of the Crustacea. Physiology. 391–419.

Johnson, P.T., 1977. A viral disease of the blue crab, *Callinectes sapidus*: Histopathology and differential diagnosis. J. Invertebr. Pathol. 29 (2), 201–209. https://doi.org/10.1016/0022-2011(77)90194-X.

Johnson, P.T., 1976. Bacterial infection in the blue crab, *Callinectes sapidus*: course of infection and histopathology. J. Invertebr. Pathol. 28 (1), 25–36. https://doi.org/10.1016/0022-2011(76)90067-7.

Johnston, E.B., Kamath, S.D., Iyer, S.P., Pratap, K., Karnaneedi, S., Taki, A.C., Nugraha, R., Schaeffer, P.M., Rolland, J.M., O'Hehir, R.E., Lopata, A.L., 2019. Defining specific allergens for improved component-resolved diagnosis of shrimp allergy in adults. Mol. Immunol. 112, 330–337. https://doi.org/10.1016/j.molimm.2019.05.006.

Kennedy, V.S., Cronin, L.E., 2007. The Blue crab: *Callinectes sapidus*, Choice Reviews Online. Maryland Sea Grant College, University of Maryland, College Park, Md. https://doi.org/10.5860/choice.45-1459.

Lakshmi, G.J., Trigg, C.M., Perry, H., Venkataramiah, A., 1984. The effect of ammonia accumulation on blue crab shedding success. Gulf Coast Research Laboratory, Ocean Springs, Miss.

Lin, S.-J., Hsia, H.-L., Liu, W.-J., Huang, J.-Y., Liu, K.-F., Chen, W.-Y., Yeh, Y.-C., Huang, Y.-T., Lo, C.-F., Kou, G.-H., Wang, H.-C., 2012. Spawning stress triggers WSSV replication in brooders via the activation of shrimp STAT. Dev. Comp. Immunol. 38 (1), 128–135. https://doi.org/10.1016/j.dci.2012.04.013.

Lorenzon, S., Giulianini, P.G., Martinis, M., Ferrero, E.A., 2007. Stress effect of different temperatures and air exposure during transport on physiological profiles in the American lobster *Homarus americanus*. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 147 (1), 94–102. https://doi.org/10.1016/j.cbpa.2006.11.028.

Maciel, F.E., Geihs, M.A., Cruz, B.P., Vargas, M.A., Allodi, S., Marins, L.F., Nery, L.E.M., 2014. Melatonin as a signaling molecule for metabolism regulation in response to hypoxia in the crab *Neohelice granulata*. Int. J. Mol. Sci. 15, 22405–22420. https://doi.org/10.3390/ijms151222405.

Malone, R.F., Burden, D.G., 1988. Design of recirculating soft crawfish sheddind systems. Lousiana Sea Grant College Program. Louisiana Sea Grant College Program, Center for Wetland Resources. Louisiana State University, Baton Rouge, LA, USA.

Mari, J., Bonami, J.-R., 1988. W2 virus infection of the crustacean *Carcinus mediterraneus*: a reovirus disease. J. Gen. Virol. 69 (3), 561–571.

Mcintyre, A.D., Pearce, J. b., Olla, B.L., Pearson, W.H., Studholme, A.L., 1980. Applicability of behavioral measures in environmental stress assessment [pollution, marine animals, migration, chemoreception, predator prey interactions, daily and seasonal cycles, review].

Melo, G.A.S., 1996. Manual de identificação dos Brachyura (caranguejos e siris) do litoral brasileiro/ Gustavo Augusto Schmidt de Melo; [ilustrações Jamil Tannus Neto]. Pleiade, FAPESP, São Paulo, SP, Brazil.

Mercier, L., Palacios, E., Campa-Córdova, Á.I., Tovar-Ramírez, D., Hernández-Herrera, R., Racotta, I.S., 2006. Metabolic and immune responses in Pacific whiteleg shrimp *Litopenaeus vannamei* exposed to a repeated handling stress. Aquaculture 258 (1-4), 633–640. https://doi.org/10.1016/j.aquaculture.2006.04.036.

Messick, G.A., Shields, J.D., 2000. Epizootiology of the parasitic dinoflagellate Hematodinium sp. in the American blue crab *Callinectes sapidus*. Dis. Aquat. Organ. 43, 139–152. https://doi.org/10.3354/dao043139.

Model, J.F.A., dos Santos, J.T., Da Silva, R.S.M., Vinagre, A.S., 2019. Metabolic effects of epinephrine on the crab *Neohelice granulata*. Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol. 231, 111–118. https://doi.org/10.1016/j.cbpa.2019.01.028.

Montanie, H., Bossy, J.-P., Bonami, J.-R., 1993. Morphological and genomic characterization of two reoviruses (P and W2) pathogenic for marine crustaceans; Do they constitute a novel genus of the *Reoviridae* family? Journal of General Virology 74 (8), 1555–1561. https://doi.org/10.1099/0022-1317-74-8-1555.

Ostrensky, A., Ventura, R., Corrêa, A.M.A., Santos, G.V., Castilho-Westphal, G.G., 2015. Improving production of soft-shelled swimming crabs: Macroscopic signs of molting as a tool for selection and management of *Callinectes danae* and *Callinectes exasperatus* (Portunidae, Decapoda, Crustacea). Arch. Vet. Sci. 20, 122–131. https://doi.org/10.5380/avs.v20i2.39855.

Pauley, G., Newman, M., Gould, E., 1975. Serum changes in the blue crab, *Callinectes sapidus*, associated with *Paramoeba perniciosa*, the causative agent of gray crab disease. Mar Fish Rev 37, 34–38.

Perazzolo, L.M., Rosa, R.D., Barracco, M.A., 2012. Defesas antivirais em crustáceos: Estado da Arte., in: Maringá (Ed.), Patologia e Sanidade de Organismos Aquáticos. Massoni, Maringá - PR, pp. 315–354.

Raby, G.D., Donaldson, M.R., Hinch, S.G., Patterson, D.A., Lotto, A.G., Robichaud, D., English, K.K., Willmore, W.G., Farrell, A.P., Davis, M.W., Cooke, S.J., 2012. Validation of reflex indicators for measuring vitality and predicting the delayed mortality of wild coho salmon bycatch released from fishing gears. J. Appl. Ecol. 49, 90–98. https://doi.org/10.1111/j.1365-2664.2011.02073.x.

Rainer, J., Brouwer, M., 1993. Hemocyanin synthesis in the blue crab *Callinectes sapidus*. Comp. Biochem. Physiol. – Part B Biochem. 104 (1), 69–73. https://doi.org/10.1016/0305-0491(93)90339-7.

Rodríguez-Ezpeleta, N., Teijeiro, S., Forget, L., Burger, G., Lang, B.F., 2009. Construction of cDNA libraries: Focus on protists and fungi. Methods Mol. Biol. 533, 33–47. https://doi.org/10.1007/978-1-60327-136-3_3.

Rogers, H.A., Taylor, S.S., Hawke, J.P., Schott, E.J., Anderson Lively, J.A., 2015. Disease, parasite, and commensal prevalences for blue crab *Callinectes sapidus* at shedding facilities in Louisiana. USA. Dis. Aquat. Organ. 112 (3), 207–217. https://doi.org/10.3354/dao02803.

Shields, J.D., 2003. Research priorities for diseases of the blue crab *Callinectes sapidus*. Bulletin of Marine Science. 505–517.

Shields, J.D., Overstreet, R.M., 2003. The blue crab: diseases, parasites and other symbionts, in: The Blue Crab: *Callinectes sapidus*. p. 426.

Shields, J.D., Scanlon, C., Volety, A., 2003. Aspects of the pathophysiology of blue crabs, *Callinectes sapidus*, infected with the parasitic dinoflagellate *Hematodinium perezi*. Bull. Mar. Sci. 72, 519–535.

Spitznagel, M.I., Small, H.J., Lively, J.A., Shields, J.D., Schott, E.J., 2019. Investigating risk factors for mortality and reovirus infection in aquaculture production of soft-shell blue crabs (*Callinectes sapidus*). Aquaculture 502, 289–295. https://doi.org/10.1016/j.aquaculture.2018.12.051.

Stentiford, G.D., Neil, D.M., Peeler, E.J., Shields, J.D., Small, H.J., Flegel, T.W., Vlak, J. M., Jones, B., Morado, F., Moss, S., Lotz, J., Bartholomay, L., Behringer, D.C., Hauton, C., Lightner, D.V., 2012. Disease will limit future food supply from the global crustacean fishery and aquaculture sectors. J. Invertebr. Pathol. 110 (2), 141–157. https://doi.org/10.1016/j.jip.2012.03.013.

Stentiford, G.D., Shields, J.D., 2005. A review of the parasitic dinoflagellates *Hematodinium* species and *Hematodinium*-like infections in marine crustaceans. Dis. Aquat. Organ. 66, 47–70. https://doi.org/10.3354/dao066047.

Stoner, A.W., 2012. Assessing stress and predicting mortality in economically significant crustaceans. Rev. Fish. Sci. 20 (3), 111–135. https://doi.org/10.1080/10641262.2012.689025.

Stoner, A.W., 2009. Prediction of discard mortality for Alaskan crabs after exposure to freezing temperatures, based on a reflex impairment index. Fish. Bull. 107, 451–463.

Stoner, A.W., Rose, C.S., Munk, J.E., Hammond, C.F., Davis, M.W., 2008. An assessment of discard mortality for two Alaskan crab species, Tanner crab (*Chionoecetes bairdi*) and snow crab (*C. opilio*), based on reflex impairment. Fish. Bull. 106, 337–347.

Tang, K.F.J., Messick, G.A., Pantoja, C.R., Redman, R.M., Lightner, D.V., 2011. Histopathological characterization and in situ detection of Callinectes sapidus reovirus. J. Invertebr. Pathol. 108 (3), 226–228. https://doi.org/10.1016/j.jip.2011.08.010.

Tavares, C.P.D.S., Silva, U.A.T., Pereira, L.A., Ostrensky, A., 2018. Systems and techniques used in the culture of soft-shell swimming crabs. Rev. Aquac. 10 (4), 913–923. https://doi.org/10.1111/raq.12207.

Taylor, A.C., Field, R.H., Parslow-Williams, P.J., 1996. The effects of *Hematodinium* sp.-infection on aspects of the respiratory physiology of the Norway lobster, *Nephrops norvegicus* (L.). J. Exp. Mar. Bio. Ecol. 207 (1-2), 217–228. https://doi.org/10.1016/S0022-0981(96)02649-4.

The, I.N., Johns, S.T., 1965. Biology of the blue crab, C*allinectes sapidus* Rathbun,·. Fish. Bull. 67, 17–33.

Thibodeaux, L.K., Burnett, K.G., Burnett, L.E., 2009. Energy metabolism and metabolic depression during exercise in *Callinectes sapidus*, the atlantic blue crab: Effects of the bacterial pathogen *Vibrio campbellii*. J. Exp. Biol. 212, 3428–3439. https://doi.org/10.1242/jeb.033431.

Tobias-Quinitio, E.J., Libunao, G.X.S., Parado-Estepa, F.D., Calpe, A.T., 2015. Soft-shell crab production using hatchery-reared mud crab. Southeast Asian Fisheries Development (SEAFDEC), Tigbauan, Iloilo, Philippines.

Tomasetti, S.J., Morrell, B.K., Merlo, L.R., Gobler, C.J., Vengatesen, T., 2018. Individual and combined effects of low dissolved oxygen and low pH on survival of early stage larval blue crabs. *Callinectes sapidus*. PLoS One 13 (12), e0208629. https://doi.org/10.1371/journal.pone.0208629.

Verri, T., Mandal, A., Zilli, L., Bossa, D., Mandal, P.K., Ingrosso, L., Zonno, V., Vilella, S., Ahearn, G.A., Storelli, C., 2001. d-Glucose transport in decapod crustacean hepatopancreas. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 130 (3), 585–606.

Vinagre, A.S., Chung, J.S., 2016. Effects of starvation on energy metabolism and crustacean hyperglycemic hormone (CHH) of the Atlantic ghost crab *Ocypode quadrata* (Fabricius, 1787). Mar. Biol. 163, 1–11. https://doi.org/10.1007/s00227-015-2797-3.

Vinagre, A.S., Model, J.F.A., Vogt, É.L., Manara, L.M., Trapp, M., Da Silva, R.S.M., Chung, J.S., 2020. Diet composition and long-term starvation do not affect crustacean hyperglycemic hormone (CHH) transcription in the burrowing crab *Neohelice granulata* (Dana, 1851). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 247, 110738. https://doi.org/10.1016/j.cbpa.2020.110738.

Vinagre, A.S., Trapp, M., Kucharski, L.C., Silva, R.S.M., 2020b. Metabolism, in: Publishing, C.S. (Ed.), *Neohelice Granulata* a model species for studies on crustaceans Volume II: Morphology and Physiology. p. 41.

Walker, P.J., Gudkovs, N., Pradeep, B., Stalin Raj, V., Sergeant, E., Chandra Mohan, A.B., Ravibabu, G., Umesh, N.R., Karunasagar, I., Santiago, T.C., Mohan, C.V., 2011. Longitudinal disease studies in small-holder black tiger shrimp (*Penaeus monodon*) farms in Andhra Pradesh, India. III. A complex dynamic of WSSV infection and WSSV genotype distribution in farmed shrimp and wild crustaceans. Aquaculture 319 (3-4), 319–327. https://doi.org/10.1016/j.aquaculture.2011.07.035.

Weng, S.-P., Guo, Z.-X., Sun, J.-J., Chan, S.-M., He, J.-G., 2007. A reovirus disease in cultured mud crab, *Scylla serrata*, in southern China. J. Fish Dis. 30 (3), 133–139. https://doi.org/10.1111/j.1365-2761.2007.00794.x.

Yochum, N., 2016. Evaluating the efficacy of predicting bycatch mortality using reflex impairment through an assessment of crab discards. Oregon State Univ, Oregon State University, Corvallis, Oregon.

Yochum, N., Rose, C.S., Hammond, C.F., 2015. Evaluating the flexibility of a reflex action mortality predictor to determine bycatch mortality rates: A case study of Tanner crab (*Chionoecetes bairdi*) bycaught in Alaska bottom trawls. Fish. Res. 161, 226–234. https://doi.org/10.1016/j.fishres.2014.07.012.

Yoganandhan, K., Thirupathi, S., Sahul Hameed, A.S., 2003. Biochemical, physiological and hematological changes in white spot syndrome virus-infected shrimp, *Penaeus indicus*. Aquaculture 221 (1-4), 1–11. https://doi.org/10.1016/S0044-8486(02)00220-X.

Zamora-Briseño, J.A., Ruiz-May, E., Elizalde-Contreras, J.M., Hernández-Velázquez, I.M., Hernández-Pérez, A., Fuentes-García, A.G., Herrera-Salvatierra, N., Briones-Fourzán, P., Pascual-Jiménez, C., Lozano-Álvarez, E., Rodríguez-Canul, R., 2020. iTRAQ-based proteomic profile analysis of the hepatopancreas of Caribbean Spiny Lobsters infected with Panulirus argus Virus 1: Metabolic and Physiological Implications. Front. Microbiol. 11 https://doi.org/10.3389/fmicb.2020.01084.

Zhang, S., Shi, Z., Zhang, J., Bonami, J.-R., 2004. Purification and characterization of a new reovirus from the Chinese mitten crab. *Eriocheir sinensis*. J. Fish Dis. 27 (12), 687–692. https://doi.org/10.1111/j.1365-2761.2004.00587.x.

Zhao, M., Behringer, D.C., Bojko, J., Kough, A.S., Plough, L., Tavares, C.P.S., Aguilar-Perera, A., Reynoso, O.S., Seepersad, G., Maharaj, O., Sanders, M.B., Carnales, D., Fabiano, G., Carnevia, D., Freeman, M.A., Atherley, N.A.M., Medero-Hernández, L. D., Schott, E.J., 2020. Climate and season are associated with prevalence and distribution of trans-hemispheric blue crab reovirus (Callinectes sapidus reovirus 1). Mar. Ecol. Prog. Ser. 647, 123–133. https://doi.org/10.3354/meps13405.

Zhao, M., Flowers, E.M., Schott, E.J., Matthijnssens, J., 2021a. Near-complete sequence of a highly divergent reovirus genome recovered from *Callinectes sapidus*. Microbiol. Resour. Announc. 10 (1).

Zhao, M., dos Santos Tavares, C.P., Schott, E.J., 2021b. Diversity and classification of reoviruses in crustaceans: A proposal. J. Invertebr. Pathol. 182, 107568. https://doi.org/10.1016/j.jip.2021.107568.