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Natural, *in ovo*, vertical transmission of the RNA viruses, *Chequa iflavirus* and Athtab bunyavirus, but not Cherax reovirus in redclaw crayfish (*Cherax quadricarinatus*)

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Abstract

The first molecular evidence for natural, *in ovo,* vertical transmission of *Chequa iflavirus* and Athtab bunyavirus from farmed female redclaw crayfish (*Cherax quadricarinatus*) cultured in northern Queensland, Australia is presented. Cherax reovirus was also examined, but evidence that this is spread via *in ovo* vertically transmission is very limited. Amongst 57 broodstock-derived pools of fertilized eggs, 38 (66.7%), 34 (59.6%) and 6 (10.5%) were positive for *Chequa*

iflavirus, Athtab bunyavirus and Cherax reovirus respectively with the respective average loads of 6.56×10^4, 2.33×10^3 and 1.03×10^1 copies/µl of tested samples. Grouping samples by farm origins, all viruses in most farms were statistically similar ($P>0.05$) for viral loads. As two viruses are transovarial transmitted, surface sterilizing practices would be ineffective and the load remains high as is seen in *Chequa iflavirus* and Athtab bunyavirus, but extremely low on surface-treated eggs as appears with Cherax reovirus. Our evidence, plus the literature, supports the general rule that if the virus is in the gut, most of the vertical transmission is faecal /oral and can be drastically reduced by efficient egg surface sterilization. If the virus is spread *in ovo,* then RT-qPCR testing coupled with therapeutic methods and selection of resistant crayfish families or detection of viral negative, geographically isolated populations of crayfish is the way forward for the removal of *Chequa iflavirus* and Athtab bunyavirus.

Keywords: vertical transmission, *Chequa iflavirus,* Athtab bunyavirus, Cherax reovirus, Redclaw crayfish, Australian *Cherax quadricarinatus, in ovo*

Running Title: *In ovo* vertical transmission of RNA viruses

1. Introduction

Many viruses including *Chequa iflavirus,* Athtab bunyavirus and Cherax reovirus have been associated with high mortalities linked to stress in redclaw crayfish (*Cherax quadricarinatus*) from farms in northern Queensland, Australia (Hayakijkosol and Owens, 2011; Sakuna et al., 2017, 2018b). These three RNA viruses are classified in different viral families. *Chequa iflavirus* is in the Order *Picornavirales*, Family *Iflaviridae,* genus *Iflavirus* (Sakuna et al., 2017), Athtab bunyavirus is in the Order *Bunyavirales* (Sakuna et al., 2018c) while Cherax reovirus is

classified in the Family *Reoviridae*, possibly the Genus *Aquareovirus* (Hayakijkosol and Owens, 2011).

Chequa iflavirus, Athtab bunyavirus and Cherax reovirus have been detected in the broodstock using quantitative PCR (qPCR) but vertical transmission of these viruses in redclaw crayfish has not been investigated.

The vertical transmission (adult to offspring) of Macrobrachium rosenbergii nodavirus (MrNV) and extra small virus (XSV) from brooders to progeny in *Macrobrachium rosenbergii* and *Artemia* has been reported (Sudhakaran et al., 2007). *Artemia* may be susceptible to MrNV and XSV and may transmit the virus from adult to hatchlings. In *Penaeus monodon*, *White spot syndrome virus* (WSSV) can be vertically transmitted from WSSV-positive spawners to offspring (Lo et al., 1997). Also, ponds stocked with low prevalence and WSSV-negative postlarvae had much higher survival rates of up to 80% (Pen et al., 2001). Therefore, brooders negative for viruses can significantly reduce the chances of a subsequent outbreak of viruses. With vertical transmission, the faecal/oral route is most common whilst the *in ovo* route is often not investigated effectively. Understanding the mode of transmission of viruses is one of the most important ways to interrupt the spread of viruses and this may prevent and control outbreaks in the broodstock.

Strong evidence for *in ovo* transmission of an unknown virus in crayfish has been accruing but the knowledge of what virus to test for and the ability to test for unknown viruses has been lacking. In an investigation of egg mortality in crayfish hatcheries in 2007, a necrotizing agent, probably a virus, was seen in embryonated eggs. Invasive chytrids and then saprolegnia attacked unfertilized eggs (Owens, unpublished report 2008). Later in 2014, during an investigation to

identify the cause of mortalities in juveniles and broodstock after transport stress, haemocytic infiltration into damaged muscle fibres was observed in juveniles and necrotic nerve cells and foci of infection were detected in craylings from day 1 onwards, before craylings began feeding or indeed had a patent gut (Sakuna et al., 2017). While two viruses (*Chequa iflavirus,* Athtab bunyavirus) were identified as likely candidates of broodstock mortalities (Sakuna et al., 2017, 2018b), a bacterial aetiology was initially investigated for the craylings (Hayakijkosol et al., 2017). Recently a study on sperm quality and fertilization has also been initiated. This current study was initiated on the strong suspicion that virus was in eggs and craylings which led to viral caused problems in egg hatcheries, mortality in craylings and stress-related deaths in juveniles and broodstock (Sakuna et al., 2017).

This study aims to investigate the *in ovo*, vertical transmission of *Chequa iflavirus,* Athtab bunyavirus and Cherax reovirus in redclaw crayfish because they are associated with significant losses in the crayfish industry in northern Queensland.

2. Materials and methods

2.1. Source of redclaw crayfish eggs

A total of 57 redclaw crayfish used in this study were randomly selected from 3 freshwater farms (Table 1) in the vicinity of the Atherton Tablelands, northern Queensland, Australia. All farms had been routinely used for the sourcing of broodstock for the commercial production of craylings. After spawning, eggs from each crayfish were frozen separately and transported to College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, Australia, for further study.

2.2. Total RNA template preparation

About 25 eggs (40 mg) from each crayfish was surface decontaminated according to the previous report (Edgerton and Owens, 1997) with minor modifications. Briefly, the eggs were soaked respectively in 0.5% formalin, 1% bleach, and 70% ethanol (1 min each), followed by a quick rinse in nuclease-free water. Then they were subjected to RNA extraction using Total RNA purification kit (Norgen, Canada) according to the manufacturer's instructions. The concentration of RNA extract of each egg pool was measured by spectrophotometer, and then adjusted to 100 ng/µl by nuclease-free water. Each RNA stock was kept at -20 °C until used.

2.3. RT-qPCR detection of Chequa iflavirus, Athtab bunyavirus and Cherax reovirus

Complementary DNA (cDNA) of each pooled egg-derived RNA extract (100 ng) was first produced using Tetro cDNA Synthesis Kit (Bioline, Australia) following the manufacturer's protocol. Briefly, a 9-µl cDNA premix containing 2 µl of 5x buffer, 0.5 µl each of random hexamers (50 ng/µl), dNTP mix (10 mg/µl), RNase inhibitor (200 units/µl), Tetro reverse transcriptase (200 units/µl), and 5 µl of nuclease-free water were prepared. After adding 1 µl of the RNA template, the reaction was incubated at 45 °C for 30 min. The resulting cDNA solution (1 µl) was further amplified by qPCR protocols developed to detect *Chequa iflavirus* (Sakuna et al., 2018a), Athtab bunyavirus (Sakuna et al., 2018c) and Cherax reovirus (Jaroenram et al., 2020). In each case, standard curves were made using serially diluted plasmids containing the relevant viral sequence as per Sakuna et al. (2018a), Sakuna et al. (2018b) and Jaroenram et al. (2020) respectively. For the first two viruses, the qPCR reactions were performed separately in 20 µl volumes contained 1× SensiFast SYBR No-Rox premix (Bioline, Australia), 0.4 µM each forward and reverse primer (F: 5'-CTCCTTCTGGGTGCGCTTTA-3'/R: 5'-

ATACTCTGGCGCATGCTCTC-3' for *Chequa iflavirus*, and F2: 5'-GATCCGGCAGAATACGAGGG-3'/R2: 5'-ACAACTGTCTGGCTACTGGC-3'for Athtab bunyavirus), nuclease-free water and the specified amount of cDNA. The thermal profile run on Rotor-Gene Q thermal cycler (QIAGEN, Germany) for both viruses was 95 °C for 10 min followed by 40 cycles of 95 °C 5s, 59 °C 10 s and 72 °C 10 s. with production of a melt curve after amplification (50-99°C, at 1°C/second). Template-free reactions were included as negative controls.

Cherax reovirus used the same PCR components as above except the primers which were 17Aus.F: 5'-GCGTAGACGGGACAGAGCCA-3' and 257.R: 5'-CGCATCGCCTAGTACTCGAGC-3' (0.1 µM each) (Jaroenram et al. 2020). The thermal profile consisted of initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s and 68 °C for 10 s with production of a melt curve after amplification (50-99°C, at 1°C/second). The PCR amplicons, 104 bp (*Chequa iflavirus*), 207 bp (Athtab bunyavirus), and 241 bp (Cherax reovirus), were visualized by 2% agarose gel electrophoresis (AGE) in order to confirm the melt curve results.

2.4. Viral load and distribution by statistical analysis

After the data acquisition and analysis were carried out with Rotor-Gene Q Series Software 2.3.1 (QIAGEN, Germany), viral load/sample of each virus was calculated and reported. Statistical analysis (ANOVA) was carried out using Excel software to understand the epidemiology across the farms of each virus. Overall test results and mean viral load/sample for each virus relative to the farm of origin were summarized (Table 1).

3. Results

The criteria to determine positive results for each virus were: 1) above-threshold amplification; 2), the correct corresponding melt curve peak; and 3), the correct size of qPCR amplicons on AGE.

3.1. Chequa iflavirus status in redclaw crayfish eggs by RT-qPCR

All 57 egg samples were tested for the presence or absence of *Chequa iflavirus* (Sakuna et al., 2018a). The expected melt temperature was 79.5 °C (Fig 1A). Clearly, the sample's melt peaks are not uniform as they shifted slightly around 79.5 °C. Examples of such variable melt curves were further elaborated (Fig. 1B), and proven to be true positives by their correct amplicons size of 104 bp (Fig. 1B, inset) on AGE. Peak variation may be due to single nucleotide polymorphisms (SNPs) of the virus as a result of a high mutation rate of RNA viruses (Duffy, 2018). Note that the mutation rate of RNA viruses, which is the product of the per-nucleotide site mutation rate and the genome size, is typically on the order of 10^{-8} to 10^{-6} substitutions/nucleotide site/cell infection (Peck and Lauring, 2018). The shifting phenomenon of melt curve peaks is also common in the presence of a very low amount of target RNA of *Chequa iflavirus* (Sakuna et al., 2018a), and Cherax reovirus (Jaroenram et al., 2020). This may occur due to slight variations in concentration of salt in the template solution as a result of inconsistent template preparation, or point mutation occurring during qPCR amplification as a result of *Taq* polymerase error (McInerney et al., 2014). The second melt peak at approximately 84 °C was observed in positive samples and the height of the peak varied inversely with that of the expected melt peak. These minor peaks were proven to be host DNA by sequencing (data not shown) of their PCR amplicons of 500 bp (Inset of Fig. 1B). While this means melt curves are required to

confirm the presence of virus, not just amplification, it allows for evidence of proper RNA extraction and RT-qPCR when the sample contains no virus. Overall, 38/57 (66.7% prevalence) samples harbored *Chequa iflavirus* (Table 1). Of those samples that tested positive, the number of copies was very high (6.56 $x10^4$/reaction, range $5.4x10^2$ – $2.28x10^5$/reaction) (Table 1).

3.2. Athtab bunyavirus *status in redclaw crayfish eggs by RT-qPCR*

Of 57 crayfish egg cDNA samples amplified by RT-qPCR (Sakuna et al., 2018c), 34 (59.7% prevalence) (Table 1) showed positive signals by both amplification plot (inset) and melt peak data (83.5 °C) (Fig. 2A). As expected, some samples had the peak shifting slightly with examples given in Fig. 2B. However, they were confirmed to be true positive according to their qPCR amplicon size of 207 bp (Fig. 2B, inset) on AGE. The factors driving such melt data non-uniformity resembled those for *Chequa iflavirus* mentioned above. The second melt peaks of approximately 89 °C were also observed in positive samples. They were not further investigated as their distance from the expected melt peak was far enough to not affect the interpretation of results. Of those samples that tested positive, the number of copies was high (2.33 $x10^3$/µl reaction, range $8.13x10^2$ – $4.02x10^3$/reaction) (Table 1).

3.3. Cherax reovirus *status in redclaw crayfish eggs by RT-qPCR*

Of 57 egg cDNA samples tested, 6 (10.5% prevalence) met all criteria above, in that they gave a specific melt curve (83.5 °C), corresponding to their amplification plots (Fig. 3 inset, top) and the correct DNA amplicon of 241 bp (Fig. 3 inset, bottom: lanes 1-6) on AGE, indicating that they were infected with Cherax reovirus (Fig. 3). The remaining samples (n = 51) showed flattened melt curves and amplification plots below the threshold (inset, top), without amplified

PCR product (N1, N2, N3 are the examples) (Fig. 3. inset, bottom). Of those samples that tested positive, the number of copies was low (1.03 $x10^1$, range $3.22x10^0$ – $2.13x10^2$) (Table 1) and typically at the end of the reliable detection limit of the RTqPCR (Jaroenram et al. 2020).

3.4. Viral load and demography by statistical analysis

The viral prevalence between farms was 40-100% for *Chequa iflavirus*; 40-89% for Athtab bunyavirus and 0-15% for Cherax reovirus (Table 1).

The viral loads were transformed by log10 +1 to homogenize the variation. As the sample size is relatively small, viral loads within farms were tested for significant differences to see if samples within farms could be pooled. There were no significant differences within farms (2K1 and 2K2, P>0.05), (3Ab and 3Ag, P>0.05), so the data was pooled for further analysis. There were no significant differences (P>0.05) in loads of *Chequa iflavirus* or Athtab bunyavirus between farms. The Cherax reovirus load between all farms was significantly different (P<0.05) but the positive results for the Cherax reovirus were limited by very few, low copy number positives so the number of samples positive for Cherax reovirus is considered too low to be a meaningful result. The Cherax reovirus qPCR positive results (1.44-3.22 copies) were at a level of virus at the end of the confident detection limit of the assay (10 copies), indicating other samples could also be positive at this low level, but not detected (Jaroenram et al. 2020).

4. Discussion

In this study, we present the first molecular evidence for natural, *in ovo,* vertical transmission of *Chequa iflavirus* and Athtab bunyavirus from farmed female redclaw crayfish (*Cherax*

quadricarinatus) cultured in northern Queensland, Australia. It is evident that these two viruses are present in spawned eggs as they are at high concentrations from egg samples which have been surface cleaned. However, the low prevalence and low number of copies of Cherax reovirus plus the epidemiology of outbreaks of reovirus suggest it is on the outside of the eggs (see below). Amongst 57 broodstock-derived pools of fertilized eggs, 38 (66.67%), 34 (59.56%) and 6 (10.53%) were positive for *Chequa iflavirus,* Athtab bunyavirus and Cherax reovirus with the respective average loads of, 6.56×10^4, 2.33×10^3 and 1.03×10^1/µl of tested samples. Grouping by farm origins of samples, all viral loads were statistically equal in most farms except Cherax reovirus in Farm 3Ab. Our finding clearly demonstrated the vertical transmission pathway of the viruses. If the viruses are associated with the egg surface only, it may be possible to disinfect or wash eggs to inactivate or reduce the contaminating viruses. This practice is routine in crayfish hatcheries tested here (Farm 3) as well as conducted by ourselves (see above), yet virus remained. If the viruses are transovarial transmitted, these practices would be ineffective and the load would be high as is seen in *Chequa iflavirus* and Athtab bunyavirus. If not transovarially transmitted, the load should be extremely low on surface-treated eggs, as appears with Cherax reovirus. Furthermore, previous testing of tissues has shown *Chequa iflavirus* (Sakuna et al., 2018a) and Athtab bunyavirus (Sakuna et al., 2018c) are systemic where crayfish haemolymph would move virus to the ovaries. Cherax reovirus has only been found in the hepatopancreas at measurable levels (Hayakijkosol et al., 2021), consistent with an adult faecal/crayling oral route of transmission. Furthermore, *Cherax reovirus* could not be detected by a standard RT-PCR in the haemolymph (Hayakijkosol et al., 2021) suggesting very low systemic circulation of the virus.

Metapenaeus ensis hosts an ovarian reovirus (Owens and Hall-Mendelin 1990, Hayakijkosol et al. 2021). Despite *M. ensis* and Cherax reovirus-infected *C. quadricarinatus* co-inhabiting the rivers of the Gulf of Carpentaria including the Flinders River, the evidence presented here suggests that Cherax reovirus is not found in the ovary or in systemic circulation and therefore because of differing tissue tropism, it is seems unlikely to be the same reovirus that is found in *M. ensis.* This also throws doubt on the hypothesis that feeding reovirus-infected *M. ensis* to broodstock crayfish as a maturation diet was the source of the reovirus in crayfish (see Hayakijkosol et al. 2021).

Our evidence, along with the literature supports the general rule that if the virus is in the gut, most of the vertical transmission is faecal/oral and it can be drastically reduced by efficient egg surface sterilization. Use of this technique in hatcheries has led to the dramatic removal of the enteric Cherax bacilliform virus (CBV), Cherax giardiavirus (CGV), and to a lesser extent, Cherax reovirus (CRV) from farmed crayfish populations. Evidence for this is the viral prevalence in studies of Edgerton et al. (1995) (CBV 6-88%; CGV 3-53%) and Gosh (2006) (CBV 0-27%; CGV 0-3%; CRV 7-41%) conducted before the crayfish industry developed hatchery technology compared to the post-hatchery sterilization studies of Hayakijkosol (2008, unpublished) (CBV 0%; CGV 0%; CRV 0%) and Rusaini and Powell (2012, unpublished) (CBV 0%; CGV 0%; CRV 0-53%). This may be the best way to control Cherax reovirus which uses the faecal/oral route. If the virus is spread *in ovo,* then RT-qPCR testing coupled with therapeutic methods (Sakuna et al., 2018b) and selection of resistant crayfish families or detection of viral negative, geographically isolated populations of crayfish is the way forward for the removal of *Chequa iflavirus* and Athtab bunyavirus.

5. Conclusions

Previous research reported the presence of *Chequa iflavirus* and Athtab bunyavirus in adult crayfish in a range of tissues (Sakuna et al., 2018a, 2018c) but modes of transmission of the viruses to these adult crayfish were unclear as individual craylings were not grown in isolation. While we cannot discount that some infections may have arisen through cannibalism or water-borne transmission during the limited grow-out, this study presents the first evidence of a natural, *in ovo,* vertical transmission pathway for *Chequa iflavirus* and Athtab bunyavirus. Better understanding of the mechanisms by which eggs, or later craylings, become infected following spawning will assist in the establishment of hatchery practices designed to limit vertical transmission of the viruses.

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Table 1. Origin, number of samples, % positive samples, and viral loads of, *Chequa iflavirus*, Athtab bunyavirus and Cherax reovirus in crayfish eggs. All samples were collected from crayfish farms in the vicinity of the Atherton Tablelands, northern Queensland, Australia, in 2019.

Virus			Farm (# 57 samples)					Average
			(1R)	(2K1)	(2K2)	(3Ab)	(3Ag)	
	# samples/farm:		20	9	8	10	10	
Chequa iflavirus	# positive samples	(%)	16 (80%)	4 (44%)	8 (100%)	4 (40%)	6 (60%)	38 (67%)
	Viral load[1]	Max:	2.08×10^{5}	8.38×10^{4}	7.25×10^{5}	2.03×10^{4}	1.22×10^{5}	2.28×10^{5}
		Mean[2]:	**1.98×10^{4}**	**2.36×10^{4}**	**2.49×10^{5}**	**8.43×10^{3}**	**2.70×10^{4}**	**6.56×10^{4}**
		Min:	3.34×10^{1}	4.58×10^{2}	2.97×10^{2}	1.28×10^{3}	6.29×10^{2}	5.40×10^{2}
Athtab bunyavirus	# positive samples	(%)	9 (45%)	8 (89%)	6 (75%)	4 (40%)	7 (70%)	34 (60%)
	Viral load[1]	Max:	3.82×10^{3}	2.72×10^{3}	5.57×10^{3}	1.92×10^{3}	6.11×10^{3}	4.03×10^{3}
		Mean[2]:	**2.08×10^{3}**	**1.22×10^{3}**	**3.00×10^{3}**	**1.88×10^{3}**	**3.48×10^{3}**	**2.33×10^{3}**
		Min:	5.87×10^{2}	1.09×10^{2}	1.47×10^{2}	1.81×10^{3}	1.61×10^{3}	8.13×10^{2}
Cherax reovirus	# positive samples	(%)	3 (15%)	1 (11%)	1 (13%)	0 (0%)	1 (10%)	6 (11%)
	Viral load[1]	Max:	1.05×10^{2}	1.44×10^{0}	1.91×10^{0}	0.00×10^{0}	1.09×10^{1}	2.13×10^{2}
		Mean[2]:	**3.73×10^{1}**	**1.44×10^{0}**	**1.91×10^{0}**	**0.00×10^{0}**	**1.09×10^{1}**	**1.03×10^{1}**
		Min:	1.85×10^{0}	1.44×10^{0}	1.91×10^{0}	0.00×10^{0}	1.09×10^{1}	3.22×10^{0}

[1]virus copies/µl where 40 mg of eggs (~25 eggs) was extracted into 50 µl.

[2]average load/positive samples only. Samples below detection limits (presumptive negatives) were excluded from the calculations.

Figure Captions

Fig. 1. Detection of *Chequa iflavirus* in redclaw crayfish eggs using RT-qPCR. (A) Melt data of 57 samples corresponding to its original linear amplification plot (inset, top). (B) Examples of result verification by AGE for unclear melt data. The melt curves shown here were selected from (A) to prove that they were a true iflavirus-positive, despite shifting slightly around the expected peak of 79.0 °C, as confirmed by the correct PCR amplicon size, 104 bp (inset). M, P and 1-7: molecular marker, 10^4 copies plasmid DNA (positive control), and examples of iflavirus-positive samples showing variable melt curves in correct amplicons size, respectively. The second melt peak centered around 84 °C were generated from the 500-bp nonspecific product (inset) which was proven to be host DNA (data not shown).

Fig. 2. Detection of Athtab bunyavirus in redclaw crayfish eggs using RT-qPCR. (A) Melt data of 57 samples corresponding to its original linear amplification plot (inset, top). (B) Examples of result verification by AGE for unclear melt data. The melt curves shown here were selected from (A) to prove that they were a true Athtab bunyavirus-positive, despite shifting slightly around the expected peak of 83.5 °C, as confirmed by the correct amplicon size of 207 bp (inset). M, P and 1-5: molecular marker, 10^4 copies plasmid DNA (positive control), and examples of Athtab bunyavirus-positive samples showing variable melt curves in correct amplicons size, respectively. The second melt peaks centered on 89 °C were generated from the 500-bp nonspecific product (inset).

Fig. 3. Detection of Cherax reovirus in redclaw crayfish eggs using RT-qPCR. Melt data corresponding to its original linear amplification plot (inset, top) was reported. The result was confirmed by AGE and shown in the bottom inset figure. M, P, 1-6, N1-N2 and N3: molecular marker, 10^4 copies plasmid DNA (positive control), Cherax reovirus-positive samples, Cherax reovirus-negative sample representatives, and nuclease-free water (negative control, N1-3), respectively. The expected Cherax reovirus amplicon is 241 bp.

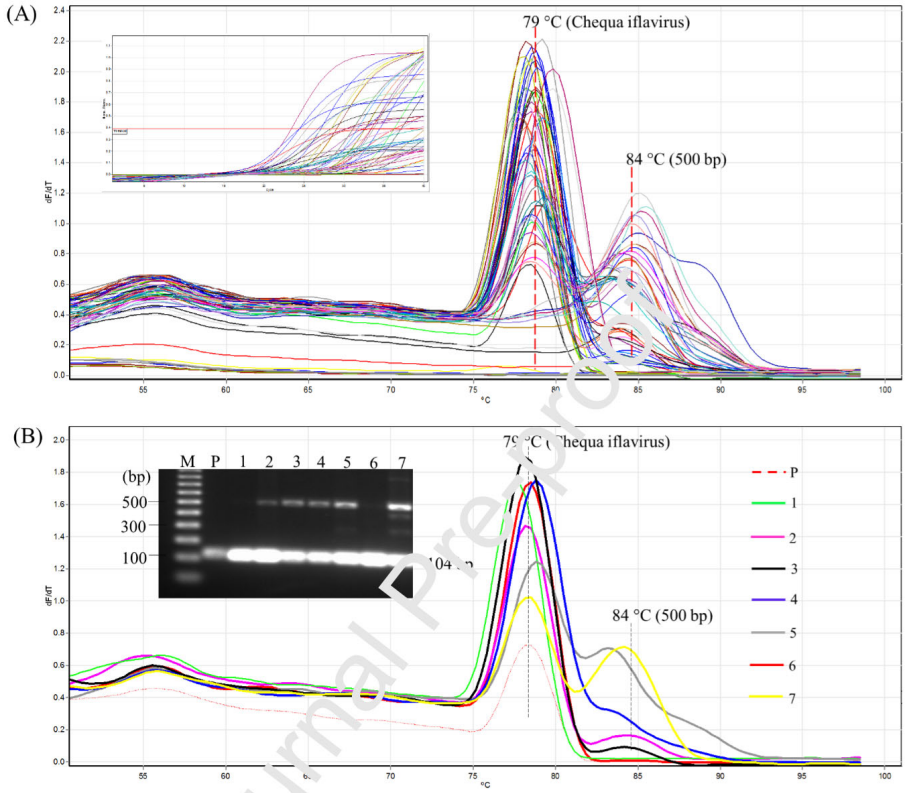

Figure 1.

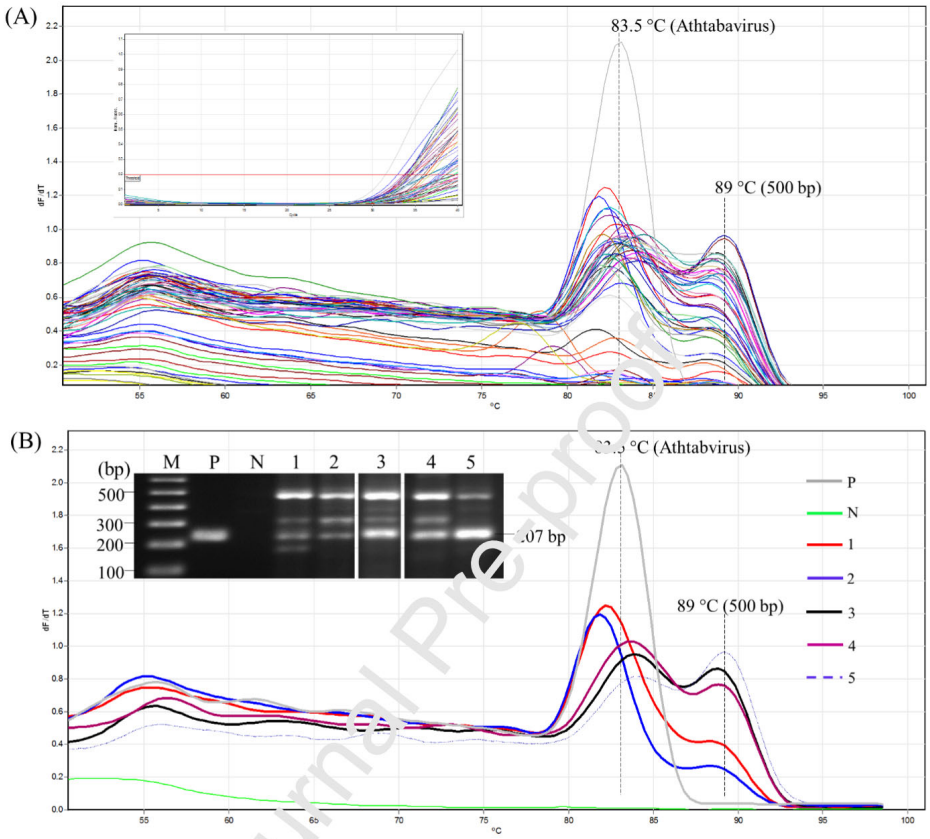

Figure 2.

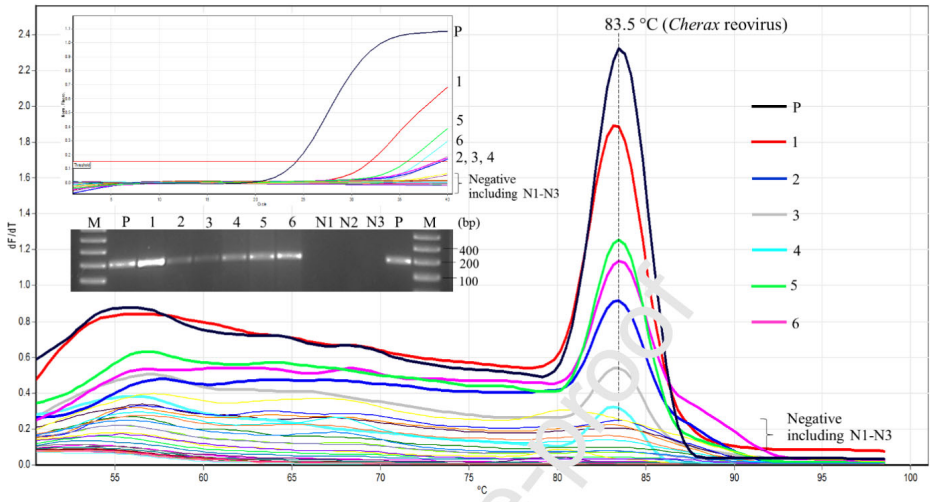

Figure 3.

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15 December 2020

Dear Sir

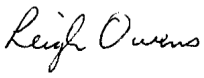
Please find attached the revised manuscript entitled "Natural, *in ovo*, vertical transmission of the RNA viruses, *Chequa iflavirus* and Atrato bunyavirus, but not Cherax reovirus in redclaw crayfish (*Cherax quadricarinatus*)" for publication in the journal Aquaculture. This is the first time RT-qPCR has been used to show unequivocally that RNA viruses are present in the crayfish eggs, not just contaminating the outside of the eggs. Thank you for the efforts and opinions of the reviewers.

We have undertaken the 3 changes requested by the reviewers:

In line 176 and 180: Fig 3 need to be changed to Fig. 3
In Line 177: lane 1-6 need to be changed to lanes 1-6

We declare we have no conflict of interests in any way whatsoever.

Yours Sincerely



Adjunct Professor Leigh Owens

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Dear Sir

We declare we, the authors, have no conflict of interests in any way whatsoever.

Yours Sincerely

Adjunct Professor Leigh Owens

Microbiology and Immunology

- Eggs of *C. quadricarinatus* were examined by RT-qPCR for transmission of RNA viruses
- *Cherax Iflavirus* was found to be *in ovo* in 67% of eggs at $6.6x10^4$ copies/µl
- Athtab bunyavirus was also found to be *in ovo* in 60% of eggs at $2.3x10^3$ copies/µl
- Cherax reovirus was only transmitted faecal/orally in 11% of eggs at 1 copies/µl
- There was no significant differences ($P>0.05$) in viral loads between 3 farms tested