COLLEGE OF AGRICULTURE & LIFE SCIENCES
Animal & Comparative Biomedical Sciences

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THE UNIVERSITY OF ARIZONA
AQUACULTURE PATHOLOGY
Tucson, Arizona

TO: [redacted]
Department of Agriculture, Water and the Environment (Canberra)

FROM: [redacted]
University of Arizona

DATE: 23 December 2021

RE: Results of UAZ Case 21-671, examination of the viability of EHP-infected tissue to cause infection in SPF shrimp after tissue has been frozen at -18 ^{0}C.

SUMMARY:

At the request of [redacted] of the Australian Department of Agriculture, Water, and the Environment, the University of Arizona (UAZ) fed tissue infected with *Enterocytozoon hepatopenaei* (EHP) to specific pathogen free (SPF) *Penaeus vannamei*. The infected tissue was fed to 4 groups of *P. vannamei*, each group with three replicate tanks. The HPs were excised and fed immediately or frozen at -18 ^{0}C for 24 hr, 7 days, and 14 days prior to feeding. The purpose of this study was to examine whether freezing of EHP-infected tissue at -18 ^{0}C would cause an infection when fed to SPF *P. vannamei*.

AQUARIA AND ANIMALS:

Prior to the start of the study, a total of 225 SPF *P. vannamei* (average weight 1 g) were stocked into fifteen 90 L tanks. The *P. vannamei* were obtained from a commercial producer and reared at UAZ. The 90 L challenge tanks were outfitted with pre-acclimated biological filters, aeration, and covered with a plastic sheeting to contain aerosols and prevent escape. The salinity of the challenge tanks ranged from 28-30 ppt. Tanks were backflushed and water quality parameters were recorded weekly. The temperature ranged from 26.0-28.0°C (avg daily temp of 27.1°C), the average ammonia value was 0.18 ppm and the average nitrite value was 0.0 ppm.

CHALLENGE METHOD:

The infected material utilized in this study derived from the EHP holding tank at The University of Arizona West Campus Agriculture Center. These tanks have been maintaining an active EHP infection since 2018 (UAZ Case 18-455) and were confirmed to be positive for EHP infection by PCR on 1 September 2021.

On day 0 of the challenge, 24 *Penaeus vannamei* were removed from the EHP holding tank. Eighteen were frozen at -18°C and the remaining six had their hepatopancreases (HPs) excised. These six HPs were each divided into six portions, with one portion per HP preserved in 95% ethanol for real-time PCR to determine EHP load. The remaining portions were randomized,

agglomerated, and fed to tanks 4-6 at 10 portions per tank.

After 24 hours, six of the infectious HPs frozen at -18°C were removed from the freezer. These HPs were also divided into six portions, with one portion per HP preserved in 95% ethanol for real-time PCR to determine EHP load. The remaining portions were agglomerated and randomized. The HP portions were then fed to tanks 7-9 at 10 portions per tank.

On day 7 of the study, six infectious HPs frozen at -18° C were removed from the freezer. These HPs were divided into six portions, with one portion per HP preserved in 95% ethanol for real-time PCR to determine EHP load. The remaining portions were randomized, agglomerated, and fed to tanks 10-12 at 10 portions per tank.

The final group in the study was infected on day 14 of the study in the same manner as the previous tanks. The final six HPs from the EHP holding tank which had been frozen at -18°C were removed from the freezer and divided into six portions, with one portion per HP preserved in 95% ethanol for real-time PCR to determine EHP load. The remaining portions were agglomerated, randomized, and fed to tanks 13-15 at 10 portions per tank.

SAMPLING AND OBSERVATION:

Prior the start of the study, two SPF *P. vannamei* were preserved in Davidson's AFA to document the health status of the population.

At the start of EHP challenge, every hepatopancreas used for infection had 1/6 of the HP collected, pooled, and preserved in 95% ethanol for real-time PCR prior to feeding each challenge group (see CHALLENGE METHOD). These pooled samples were analyzed for EHP load to verify potential infectivity of each group (see RESULTS).

After feeding EHP-infected tissue to SPF *P. vannamei*, animals in each EHP challenge group were observed for 21 days (see Table 1). The animals in the uninfected negative control group was also maintained in parallel for the duration of the study. At termination of each group, five survivors per tank were preserved in Davidson's AFA for histology and five HPs were excised for real-time PCR. The remainder of the survivors were counted and frozen at -80°C.

There were no mortalities noted in any tank for the duration of the study. Any losses in the tanks were assumed to be cannibalized and were unaccounted for. Additionally, there were no moribund shrimp observed during the course of the study. All samples collected were termination samples.

RESULTS:

<u>Negative Control Group:</u>

The negative control group was maintained for the full duration of the study (35 days). This group served as the negative environmental controls and were not fed EHP-infected tissue. There were no moribund nor mortalities noted in any tank in this group during the study. The final survival

was 93.3% for the group (see Table 3) at termination of the study.

At termination of the study, five shrimp were sampled for H&E examination and five shrimp were frozen for real-time PCR. Histology samples displayed no symptoms of EHP infection and all five PCR samples were negative for EHP. See Table 4 for real-time PCR results and Table 5 for histology results.

Positive Control Group:

The positive control group was fed EHP-infected hepatopancreas tissue which had been excised from *P. vannamei* housed in a tank known to be infected with *Enterocytozoon hepatopenaei*. The tissue was excised, samples were collected for real-time PCR (see CHALLENGE METHOD), and the remaining tissue was fed to the challenge tanks. The tissue fed to these tanks was quantified by PCR and were determined to have a Ct value ranging from 19.80 to 25.95 (avg value: 23.09).

The survival for this group was 88.8% at termination of these tanks on day 21 of the study. No moribund *P. vannamei* were noted during the study. Histological results for this group showed Grade 4 EHP infections in termination samples collected from each tank. Histological lesions characteristics of EHP infection were noted in 14 of 15 samples and ranged from Grade 1 to Grade 4.

Samples were pooled and determined to have Ct values of 15.38, 14.32, and 16.86 respectively for each replicate tank. These low Ct values represent a strong positive result by real-time PCR for detection of EHP.

24 Hour Group:

Tanks 7-9 were fed EHP-infected hepatopancreas tissue which had been frozen for 24 hours at -18° C. The tissue fed to these tanks had a Ct value ranging from 15.72 to 27.40 (avg value: 22.32). There were no mortalities collected nor moribund noted during the observation period of these tanks.

The final survival for this group at termination was 93.3% on day 22 of the study. Survivors at termination were quantified by PCR and tanks 7, 8, and 9 were shown to have Ct values of 39.76 (considered Not Detected since a Ct value of >37 is considered negative), 14.08, and 33.76 respectively. These results exhibit a wide range of EHP load, as a Ct value of 14.08 indicates a high amount of EHP present, while a value of 33.76 represents very low levels of EHP. See Table 4 for a summary of real-time PCR results.

Histological examination mirrored the results of real-time PCR. Animals from Tank 7, where EHP could not be detected by real-time PCR, did not show any histological lesions characteristic of EHP in any of the 5 samples examined. In both Tank 8 (Ct value 14.08) and Tank 9 (Ct value of 33.76) had some animals showing histological lesions of EHP infection. The histology grade of these samples ranged from G1 to G4 for animals in Tank 8, and G-trace for animals in Tank 9. See Table 6 for histology results.

7 Day Group:

The *P. vannamei* in tanks 10-12 were fed EHP tissue which had been frozen for 7 days. The EHP tissue was determined by real-time PCR to have a Ct values ranging from 19.55 to 30.66 (avg value: 25.07). prior to feeding on day 7. There were no mortalities collected nor moribund noted during the observation period for these tanks.

The final survival for this group was 97.8% (See Table 2). At termination of this group on day 28, survivors were preserved for histology and real-time PCR. Tanks 10 and 11 had Ct values of 39.01 and 38.05. These Ct values are above 38.00, designating these tanks as undetected for EHP (Table 4). Tank 12 had a Ct value of 33.51, designating this tank as positive for EHP.

Histology on the samples collected from these tanks at termination confirmed the low level of infection, as there were no signs of EHP in any shrimp from tanks 10 or 11. Tank 12, positive by real-time PCR, had 2 of 5 samples exhibit EHP histological lesions grades as G1. The remaining samples did not have detectable levels of histological lesions.

14 Day Group:

Tanks 13-15 were fed tissue which was determined by real-time PCR to have Ct levels ranging from 21.05 to 29.30 (avg value: 26.17). This was the highest average Ct value of all groups in this study. There were no mortalities or moribund *P. vannamei* noted during the observation period for these tanks. The final survival for this group was 88.8%. Tank 15 had the lowest survival of any tank in this study, as only 11 of 15 shrimp were noted at termination (see Table 3).

Histological analysis of survivors at termination showed low levels of EHP infection throughout all three replicates (see Table 5). Every tank in this group had 3 of 5 samples exhibit trace and/or G1 symptoms of EHP infection. Quantification of the EHP load in termination samples by real-time PCR resulted Ct values of 29.42 in tank 13 and 37.66 in tank 14. Tank 15 was undetectable.

PATHOLOGY:

Histological examination of the samples from the challenge was completed by [redacted] and Table 5 and Appendix 1 summarizes the histology findings from this case.

Sincerely,

[redacted]

Reviewed and Approved By,

[redacted]

Table 1. **Definition of tanks used in determining the effect of freezing *Enterocytozoon hepatopenaei* -infected tissue at -18^{0}C in determining the viability of the pathogen in causing infection in SPF *Penaeus vannamei***

Tank	No Stocked	Tank Designation	Tissue Fed (Stored at -18^{0}C)
1	15	Negative Control	None
2	15	Negative Control	None
3	15	Negative Control	None
4	15	Positive Control	Day 0 (Fresh Tissue)
5	15	Positive Control	Day 0 (Fresh Tissue)
6	15	Positive Control	Day 0 (Fresh Tissue)
7	15	EHP 24 hr	Day 1
8	15	EHP 24 hr	Day 1
9	15	EHP 24 hr	Day 1
10	15	EHP 7 days	Day 7
11	15	EHP 7 days	Day 7
12	15	EHP 7 days	Day 7
13	15	EHP 14 days	Day 14
14	15	EHP 14 days	Day 14
15	15	EHP 14 days	Day 14

Table 2. **Sampling schedule of *Penaeus vannamei* following an experimental challenge with *Enterocytozoon hepatopenaei* -infected tissue.**

	Day -1	Day 0	Day 1	Day7	Day 14	Day 21	Day 22	Day 28	Day 35	Total
Tank	SPF *P. vannamei* Holding	4-6	7-9	10-12	13-15	4-6	7-9	10-12	1-3 13-15	15
Sample Type	Health Status	Fresh EHP-infected Tissue	24 H EHP-infected Tissue	7 Day EHP-infected Tissue	14 Day EHP-infected Tissue	Termination	Termination	Termination	Termination	-
Histology	2					15	15	15	30	77
Real-time PCR		6	6	6	6	3	3	3	6	39

Table 3. **Survival of *Penaues vannamei* upon feeding *Enterocytozoon hepatopenaei* - infected tissue.**

Tank	Time of Tissue Feeding	No. Stocked (Day 0)	Survivors Collected	Survival	Group Survival
1	N/A	15	15	100%	93.3%
2		15	14	93.3%	
3		15	13	86.7%	
4	Day 0	15	14	93.3%	88.8%
5		15	13	86.7%	
6		15	13	86.7%	
7	24 hours	15	14	93.3%	93.3%
8		15	13	86.7%	
9		15	15	100%	
10	Day 7	15	15	100%	97.8%
11		15	15	100%	
12		15	14	93.3%	
13	Day 14	15	15	100%	88.8%
14		15	14	93.3%	
15		15	11	73.3%	

Table 4. **Detection and quantification of *Enterocytozoon hepatopenaei* by real-time PCR in experimentally challenged *Penaeus vannamei* shrimp. A Ct value of >37 is considered as "Not Detected".**

Tank Number	Group Designation	Sample Collection Point	EHP	Ct
1	Neg Control	Termination	NOT DETECTED	-
2	Neg Control	Termination	NOT DETECTED	-
3	Neg Control	Termination	NOT DETECTED	-
Tissue	Fresh	Tissue	POSITIVE	19.80
Tissue	Fresh	Tissue	POSITIVE	23.80
Tissue	Fresh	Tissue	POSITIVE	25.95
Tissue	Fresh	Tissue	POSITIVE	22.23
Tissue	Fresh	Tissue	POSITIVE	23.01
Tissue	Fresh	Tissue	POSITIVE	23.72
4	Pos Control	Termination	POSITIVE	15.38
5	Pos Control	Termination	POSITIVE	14.32
6	Pos Control	Termination	POSITIVE	16.86
Tissue	24 H Frozen	Tissue	POSITIVE	24.22
Tissue	24 H Frozen	Tissue	POSITIVE	15.72
Tissue	24 H Frozen	Tissue	POSITIVE	25.62
Tissue	24 H Frozen	Tissue	POSITIVE	18.98
Tissue	24 H Frozen	Tissue	POSITIVE	27.40
Tissue	24 H Frozen	Tissue	POSITIVE	21.98
7	24H Frozen	Termination	NOT DETECTED	39.76
8	24H Frozen	Termination	POSITIVE	14.08
9	24H Frozen	Termination	POSITIVE	33.76
Tissue	7 Day Frozen	Tissue	POSITIVE	19.55
Tissue	7 Day Frozen	Tissue	POSITIVE	19.70
Tissue	7 Day Frozen	Tissue	POSITIVE	29.71
Tissue	7 Day Frozen	Tissue	POSITIVE	21.97
Tissue	7 Day Frozen	Tissue	POSITIVE	28.84
Tissue	7 Day Frozen	Tissue	POSITIVE	30.66
10	7 Day Frozen	Termination	NOT DETECTED	39.01
11	7 Day Frozen	Termination	NOT DETECTED	38.05

12	7 Day Frozen	Termination	POSITIVE	33.51
Tissue	14 Day Frozen	Tissue	POSITIVE	21.05
Tissue	14 Day Frozen	Tissue	POSITIVE	28.27
Tissue	14 Day Frozen	Tissue	POSITIVE	29.30
Tissue	14 Day Frozen	Tissue	POSITIVE	26.41
Tissue	14 Day Frozen	Tissue	POSITIVE	28.62
Tissue	14 Day Frozen	Tissue	POSITIVE	23.34
13	14 Day Frozen	Termination	POSITIVE	29.42
14	14 Day Frozen	Termination	POSITIVE	37.66
15	14 Day Frozen	Termination	NOT DETECTED	-

Table 5. **Histopathology of *Enterocytozoon hepatopenaei*- challenged shrimp upon feeding fresh inoculum or inocula frozen at -18 ^{0}C for 1 day, 7 days and 14 days.**

Tank Number	Pathogen Challenged	Treatment	UAZ ID#	EHP[1]	Comments
1	Negative control	Health Status Check	21-671A/1	ND[2]	Pre-Study
1	Negative control	Health Status Check	21-671A/2	ND	Pre-Study
1	Negative control	Negative control	21-671A/3	ND	survivor
1	Negative control	Negative control	21-671A/4	ND	survivor
1	Negative control	Negative control	21-671A/5	ND	survivor
1	Negative control	Negative control	21-671A/6	ND	survivor
1	Negative control	Negative control	21-671A/7	ND	survivor
2	Negative control	Negative control	21-671B/1	ND	survivor
2	Negative control	Negative control	21-671B/2	ND	survivor
2	Negative control	Negative control	21-671B/3	ND	survivor
2	Negative control	Negative control	21-671B/4	ND	survivor
2	Negative control	Negative control	21-671B/5	ND	survivor
3	Negative control	Negative control	21-671C/1	ND	survivor
3	Negative control	Negative control	21-671C/2	ND	survivor
3	Negative control	Negative control	21-671C/3	ND	survivor
3	Negative control	Negative control	21-671C/4	ND	survivor
3	Negative control	Negative control	21-671C/5	ND	survivor
4	EHP	Positive Control	21-671D/1	G4[3]	survivor
4	EHP	Positive Control	21-671D/2	G2	survivor
4	EHP	Positive Control	21-671D/3	G4	survivor
4	EHP	Positive Control	21-671D/4	G3	survivor
4	EHP	Positive Control	21-671D/5	G2	survivor
5	EHP	Positive Control	21-671E/1	G4	survivor
5	EHP	Positive Control	21-671E/2	G1	survivor
5	EHP	Positive Control	21-671E/3	G3	survivor
5	EHP	Positive Control	21-671E/4	G2	survivor
5	EHP	Positive Control	21-671E/5	G3	survivor
6	EHP	Positive Control	21-671F/1	G1	survivor
6	EHP	Positive Control	21-671F/2	G4	survivor
6	EHP	Positive Control	21-671F/3	G1	survivor
6	EHP	Positive Control	21-671F/4	ND	survivor
6	EHP	Positive Control	21-671F/5	G3	survivor
7	EHP	24 Hour	21-671G/1	ND	survivor
7	EHP	24 Hour	21-671G/2	ND	survivor
7	EHP	24 Hour	21-671G/3	ND	survivor

7	EHP	24 Hour	21-671G/4	ND	survivor
7	EHP	24 Hour	21-671G/5	ND	survivor
8	EHP	24 Hour	21-671H/1	G1	survivor
8	EHP	24 Hour	21-671H/2	G4	survivor
8	EHP	24 Hour	21-671H/3	G4	survivor
8	EHP	24 Hour	21-671H/4	G3	survivor
8	EHP	24 Hour	21-671H/5	G2	survivor
9	EHP	24 Hour	21-671I/1	ND	survivor
9	EHP	24 Hour	21-671I/2	ND	survivor
9	EHP	24 Hour	21-671I/3	ND	survivor
9	EHP	24 Hour	21-671I/4	G-trace	survivor
9	EHP	24 Hour	21-671I/5	G-trace	survivor
10	EHP	7 Day	21-671J/1	ND	survivor
10	EHP	7 Day	21-671J/2	ND	survivor
10	EHP	7 Day	21-671J/3	ND	survivor
10	EHP	7 Day	21-671J/4	ND	survivor
10	EHP	7 Day	21-671J/5	ND	survivor
11	EHP	7 Day	21-671K/1	ND	survivor
11	EHP	7 Day	21-671K/2	ND	survivor
11	EHP	7 Day	21-671K/3	ND	survivor
11	EHP	7 Day	21-671K/4	ND	survivor
11	EHP	7 Day	21-671K/5	ND	survivor
12	EHP	7 Day	21-671L/1	G1	survivor
12	EHP	7 Day	21-671L/2	ND	survivor
12	EHP	7 Day	21-671L/3	G1	survivor
12	EHP	7 Day	21-671L/4	ND	survivor
12	EHP	7 Day	21-671L/5	ND	survivor
13	EHP	14 Day	21-671M/1	ND	survivor
13	EHP	14 Day	21-671M/2	G1	survivor
13	EHP	14 Day	21-671M/3	G-trace	survivor
13	EHP	14 Day	21-671M/4	G1	survivor
13	EHP	14 Day	21-671M/5	ND	survivor
14	EHP	14 Day	21-671N/1	G1	survivor
14	EHP	14 Day	21-671N/2	G-trace	survivor
14	EHP	14 Day	21-671N/3	ND	survivor
14	EHP	14 Day	21-671N/4	G1	survivor
14	EHP	14 Day	21-671N/5	ND	survivor
15	EHP	14 Day	21-671P/1	G-trace	survivor
15	EHP	14 Day	21-671P/2	ND	survivor
15	EHP	14 Day	21-671P/3	G-trace	survivor
15	EHP	14 Day	21-671P/4	ND	survivor
15	EHP	14 Day	21-671P/5	G-trace	survivor

Note:

1) EHP = Lesions diagnostic of *Enterocytozoon hepatopenaei*
2) ND = Not detected
3) G-trace to G4: Severity grade of infection/lesion, according to enclosed severity grade table. Numbers on the left side of the parentheses indicate number of affected shrimp.

APPENDIX 1

Severity Grade	Clinical Findings
0	> No signs of infection/infestation by pathogen, parasite, or epicommensal present.
trace	> Signs of infection/infestation by pathogen, parasite or epicommensal are present at just above diagnostic procedure minimum detection limits.
1	> Signs of infection/infestation by pathogen, parasite or epicommensal are present, but at levels that may be below those needed for clinical disease. > Agent detected may be in early stages of infection and represent preclinical disease.
2	> Moderate signs of infection/infestation as shown by low to moderate numbers of parasite or epicommensal, or by number and severity of pathogen caused lesions. > Prognosis is for possible production losses and/or slight increases in mortality if no treatment (if treatable) or management change is applied.
3	> Moderate to high signs of disease apparent as shown by relatively higher numbers of parasite or epicommensal, or by number and severity of pathogen caused lesions. > Potentially lethal prognosis if no treatment (if treatable) or management change is applied.
4	> High numbers of parasite or epicommensal present, or for pathogen caused infections the presence of severe lesions and advanced tissue destruction. > Lethal prognosis, especially under conditions conducive to disease development (i.e. with low oxygen, ecdysis, changes in salinity or temperature, etc.).

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The University of Arizona
Aquaculture Pathology
Tucson, Arizona

May 20, 2022

[redacted]
Department of Agriculture, Water and the Environment (Canberra)
GPO Box 858 Canberra ACT 2601 Australia
Australia.

[redacted]

Re: **Case 21-671 *In-situ* EHP Final report. Rev 1**

Dear [redacted]

We have revised this report to add additional information requested by [redacted] This included H&E and ISH pictures and an explanation of the results.
We have completed the EHP *In-situ* hybridization of your sample of seventy seven (77) Davidson's fixed shrimp *Penaeus (Litopenaeus) vannamei.* These specimens were processed according to conventional techniques for paraffin embedding and sectioning. The EHP 510 F (5'-GCC TGA GAG ATG GCT CCC ACG T-3') and EHP 510 R (5'-GCG TAC TAT CCC CAG AGC CCG A-3') primers were used for *in situ* hybridization (ISH). These primers were tailed at 3′-end with digoxigenin-11-dUTP (Integrated DNA Technologies®, San Diego, CA). Below you will find a summary of our results in the Table 1.

We hope this information is useful to you. [redacted]. Should you have any questions about this report, please do not hesitate to contact us.

UAZ Policy on certification: This report provides our findings on the samples submitted to our laboratory for examination, health status evaluation, disease diagnosis, or pathogen detection. It is our policy and intent to perform the most appropriate assay(s) for the determination of the health/pathogen status of all specimens submitted to our laboratory. However, this report in no way constitutes a stock or facility "certification" or a "certificate" of health/pathogen status for the sample(s) tested or for the stocks, or facility from which the sample(s) were derived.

Best regards,

[redacted]

[redacted]

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Table 1. Summary of EHP *In-situ* hybridization from Case 21-671 from Australia. Samples of juvenile *Penaeus* (*Litopenaeus*) *vannamei* infected with EHP.

Tank# / treatment	Pathogen challenged	Treatment	Sampling date mm/dd/yy	UAZ ID#	EHP *In-situ*	H&E Results	PCR
Tank #1 Case A	Negative control	Negative control	**9/13/21 Day 0**	**21-671A/1**	**ND**	**ND**	
Tank #1 Case A	Negative control	Negative control	**9/13/21 Day 0**	**21-671A/2**	**ND**	**ND**	
Tank #1 Case A	Negative control	Negative control	**10/6/21 Day 35**	**21-671A/3**	**ND**	**ND**	**ND**
Tank #1 Case A	Negative control	Negative control	**10/6/21 Day 35**	**21-671A/4**	**ND**	**ND**	
Tank #1 Case A	Negative control	Negative control	**10/6/21 Day 35**	**21-671A/5**	**ND**	**ND**	
Tank #1 Case A	Negative control	Negative control	**10/6/21 Day 35**	**21-671A/6**	**ND**	**ND**	
Tank #1 Case A	Negative control	Negative control	**10/6/21 Day 35**	**21-671A/7**	**ND**	**ND**	
Tank #2 Case B	Negative control	Negative control	**10/6/21 Day 35**	**21-671B/1**	**ND**	**ND**	**ND**
Tank #2 Case B	Negative control	Negative control	**10/6/21 Day 35**	**21-671B/2**	**ND**	**ND**	
Tank #2 Case B	Negative control	Negative control	**10/6/21 Day 35**	**21-671B/3**	**ND**	**ND**	
Tank #2 Case B	Negative control	Negative control	**10/6/21 Day 35**	**21-671B/4**	**ND**	**ND**	
Tank #2 Case B	Negative control	Negative control	**10/6/21 Day 35**	**21-671B/5**	**ND**	**ND**	
Tank #3 Case C	Negative control	Negative control	**10/6/21 Day 35**	**21-671C/1**	**ND**	**ND**	**ND**
Tank #3 Case C	Negative control	Negative control	**10/6/21 Day 35**	**21-671C/2**	**ND**	**ND**	
Tank #3 Case C	Negative control	Negative control	**10/6/21 Day 35**	**21-671C/3**	**ND**	**ND**	
Tank #3 Case C	Negative control	Negative control	**10/6/21 Day 35**	**21-671C/4**	**ND**	**ND**	
Tank #3 Case C	Negative control	Negative control	**10/6/21 Day 35**	**21-671C/5**	**ND**	**ND**	
Tank #4 Case D	EHP	Positive Control	**9/23/21 Day 22**	**21-671D/1**	**G4**	**G4**	**POSITIVE (Ct=15.38)**
Tank #4 Case D	EHP	Positive Control	**9/23/21 Day 22**	**21-671D/2**	**G4**	**G2**	
Tank #4 Case D	EHP	Positive Control	**9/23/21 Day 22**	**21-671D/3**	**G4**	**G4**	

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Tank# / treatment	Pathogen challenged	Treatment	Sampling date mm/dd/yy	UAZ ID#	EHP *In-situ*	H&E Results	PCR
Tank #4 Case D	EHP	Positive Control	**9/23/21 Day 22**	**21-671D/4**	**G4**	**G3**	
Tank #4 Case D	EHP	Positive Control	**9/23/21 Day 22**	**21-671D/5**	**G2**	**G2**	
Tank #5 Case E	EHP	Positive Control	**9/23/21 Day 22**	**21-671E/1**	**G4**	**G4**	**POSITIVE (Ct=14.32)**
Tank #5 Case E	EHP	Positive Control	**9/23/21 Day 22**	**21-671E/2**	**G3**	**G1**	
Tank #5 Case E	EHP	Positive Control	**9/23/21 Day 22**	**21-671E/3**	**G3**	**G3**	
Tank #5 Case E	EHP	Positive Control	**9/23/21 Day 22**	**21-671E/4**	**G3**	**G2**	
Tank #5 Case E	EHP	Positive Control	**9/23/21 Day 22**	**21-671E/5**	**G3**	**G3**	
Tank #6 Case F	EHP	Positive Control	**9/22/21 Day 21**	**21-671F/1**	**G3**	**G1**	**POSITIVE (Ct=16.86)**
Tank #6 Case F	EHP	Positive Control	**9/22/21 Day 21**	**21-671F/2**	**G4**	**G4**	
Tank #6 Case F	EHP	Positive Control	**9/22/21 Day 21**	**21-671F/3**	**G3**	**G1**	
Tank #6 Case F	EHP	Positive Control	**9/22/21 Day 21**	**21-671F/4**	**ND**	**ND**	
Tank #6 Case F	EHP	Positive Control	**9/22/21 Day 21**	**21-671F/5**	**G4**	**G3**	
Tank #7 Case G	EHP	24 Hour	**9/23/21 Day 22**	**21-671G/1**	**ND**	**ND**	**ND (Ct=39.76)**
Tank #7 Case G	EHP	24 Hour	**9/23/21 Day 22**	**21-671G/2**	**ND**	**ND**	
Tank #7 Case G	EHP	24 Hour	**9/23/21 Day 22**	**21-671G/3**	**ND**	**ND**	
Tank #7 Case G	EHP	24 Hour	**9/23/21 Day 22**	**21-671G/4**	**ND**	**ND**	
Tank #7 Case G	EHP	24 Hour	**9/23/21 Day 22**	**21-671G/5**	**ND**	**ND**	
Tank #8 Case H	EHP	24 Hour	**9/23/21 Day 22**	**21-671H/1**	**G4**	**G1**	**POSITIVE (Ct=14.08)**
Tank #8 Case H	EHP	24 Hour	**9/23/21 Day 22**	**21-671H/2**	**G4**	**G4**	
Tank #8 Case H	EHP	24 Hour	**9/23/21 Day 22**	**21-671H/3**	**G4**	**G4**	
Tank #8 Case H	EHP	24 Hour	**9/23/21 Day 22**	**21-671H/4**	**G4**	**G3**	
Tank #8 Case H	EHP	24 Hour	**9/23/21 Day 22**	**21-671H/5**	**G2**	**G2**	

COLLEGE OF AGRICULTURE & LIFE SCIENCES
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The University of Arizona Aquaculture Pathology Tucson, Arizona

Tank# / treatment	Pathogen challenged	Treatment	Sampling date mm/dd/yy	UAZ ID#	EHP *In-situ*	H&E Results	PCR
Tank #9 Case I	EHP	24 Hour	**9/23/21 Day 22**	**21-671I/1**	**ND**	**ND**	**POSITIVE (Ct=33.76)**
Tank #9 Case I	EHP	24 Hour	**9/23/21 Day 22**	**21-671I/2**	**ND**	**ND**	
Tank #9 Case I	EHP	24 Hour	**9/23/21 Day 22**	**21-671I/3**	**ND**	**ND**	
Tank #9 Case I	EHP	24 Hour	**9/23/21 Day 22**	**21-671I/4**	**ND**	**G-trace**	
Tank #9 Case I	EHP	24 Hour	**9/23/21 Day 22**	**21-671I/5**	**ND**	**G-trace**	
Tank #10 Case J	EHP	7 Day	**9/29/21 Day 28**	**21-671J/1**	**ND**	**ND**	**ND (Ct=39.01)**
Tank #10 Case J	EHP	7 Day	**9/29/21 Day 28**	**21-671J/2**	**ND**	**ND**	
Tank #10 Case J	EHP	7 Day	**9/29/21 Day 28**	**21-671J/3**	**ND**	**ND**	
Tank #10 Case J	EHP	7 Day	**9/29/21 Day 28**	**21-671J/4**	**ND**	**ND**	
Tank #10 Case J	EHP	7 Day	**9/29/21 Day 28**	**21-671J/5**	**ND**	**ND**	
Tank #11 Case K	EHP	7 Day	**9/29/21 Day 28**	**21-671K/1**	**ND**	**ND**	**ND (Ct=38.05)**
Tank #11 Case K	EHP	7 Day	**9/29/21 Day 28**	**21-671K/2**	**ND**	**ND**	
Tank #11 Case K	EHP	7 Day	**9/29/21 Day 28**	**21-671K/3**	**ND**	**ND**	
Tank #11 Case K	EHP	7 Day	**9/29/21 Day 28**	**21-671K/4**	**ND**	**ND**	
Tank #11 Case K	EHP	7 Day	**9/29/21 Day 28**	**21-671K/5**	**ND**	**ND**	
Tank #12 Case L	EHP	7 Day	**9/29/21 Day 28**	**21-671L/1**	**ND**	**G1**	**POSITIVE (Ct=33.51)**
Tank #12 Case L	EHP	7 Day	**9/29/21 Day 28**	**21-671L/2**	**ND**	**ND**	
Tank #12 Case L	EHP	7 Day	**9/29/21 Day 28**	**21-671L/3**	**ND**	**G1**	
Tank #12 Case L	EHP	7 Day	**9/29/21 Day 28**	**21-671L/4**	**ND**	**ND**	
Tank #12 Case L	EHP	7 Day	**9/29/21 Day 28**	**21-671L/5**	**ND**	**ND**	
Tank #13 Case M	EHP	14 Day	**10/06/21 Day 35**	**21-671M/1**	**ND**	**ND**	

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Tank #13 Case M	EHP	14 Day	**10/06/21 Day 35**	**21-671M/2**	**ND**	**G1**	**POSITIVE (Ct=29.42)**
Tank #13 Case M	EHP	14 Day	**10/06/21 Day 35**	**21-671M/3**	**ND**	**G-trace**	
Tank #13 Case M	EHP	14 Day	**10/06/21 Day 35**	**21-671M/4**	**ND**	**G1**	
Tank #13 Case M	EHP	14 Day	**10/06/21 Day 35**	**21-671M/5**	**ND**	**ND**	
Tank #14 Case N	EHP	14 Day	**10/06/21 Day 35**	**21-671N/1**	**ND**	**G1**	**ND (Ct=37.66)**
Tank #14 Case N	EHP	14 Day	**10/06/21 Day 35**	**21-671N/2**	**ND**	**G-trace**	
Tank #14 Case N	EHP	14 Day	**10/06/21 Day 35**	**21-671N/3**	**ND**	**ND**	
Tank #14 Case N	EHP	14 Day	**10/06/21 Day 35**	**21-671N/4**	**ND**	**G1**	
Tank #14 Case N	EHP	14 Day	**10/06/21 Day 35**	**21-671N/5**	**ND**	**ND**	
Tank #15 Case P	EHP	14 Day	**10/06/21 Day 35**	**21-671P/1**	**ND**	**G-trace**	**ND**
Tank #15 Case P	EHP	14 Day	**10/06/21 Day 35**	**21-671P/2**	**ND**	**ND**	
Tank #15 Case P	EHP	14 Day	**10/06/21 Day 35**	**21-671P/3**	**ND**	**G-trace**	
Tank #15 Case P	EHP	14 Day	**10/06/21 Day 35**	**21-671P/4**	**ND**	**ND**	
Tank #15 Case P	EHP	14 Day	**10/06/21 Day 35**	**21-671P/5**	**ND**	**G-trace**	

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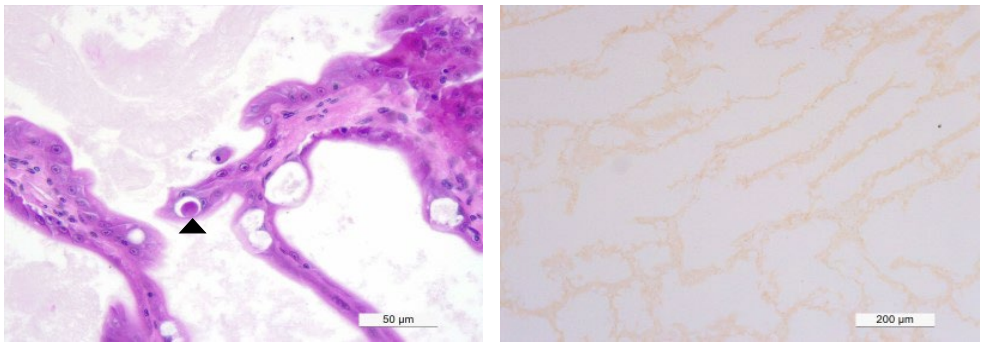
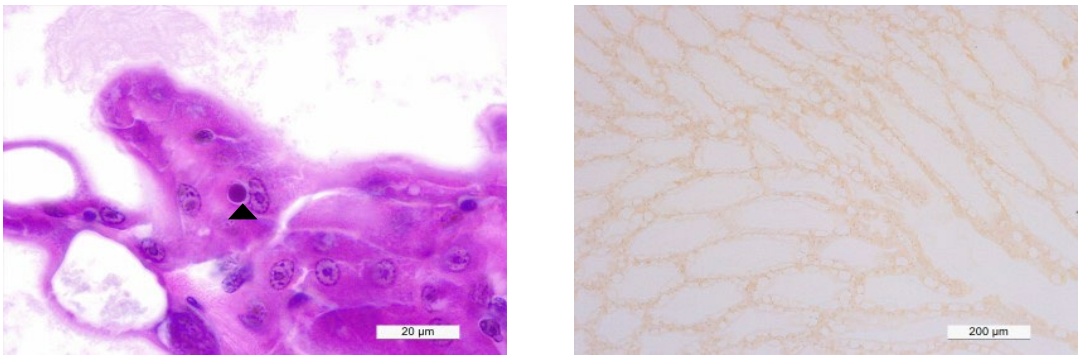
H&E EHP *In-situ* hybridization



▲ =Cytoplasmic inclusion body within HP epithelium

Fig 1. Case 21-671 / M2. Hepatopancreas section showing cytoplasmic inclusion bodies (Arrow head) by H&E (left) and negative reaction by EHP *in-situ hybridization* (right).

H&E EHP *In-situ* hybridization



▲ =Cytoplasmic inclusion body within HP epithelium

Fig 2. Case 21-671 / M3. Hepatopancreas section showing cytoplasmic inclusion bodies (Arrow head) by H&E (left) and negative reaction by EHP *in-situ hybridization* (right).

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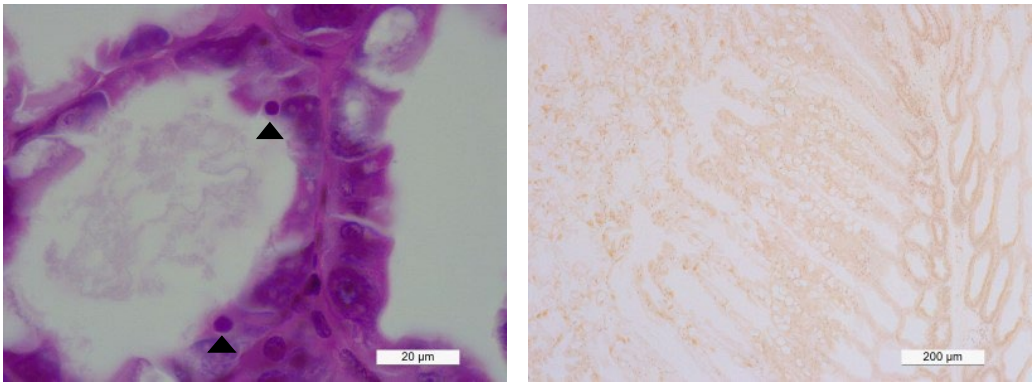
H&E

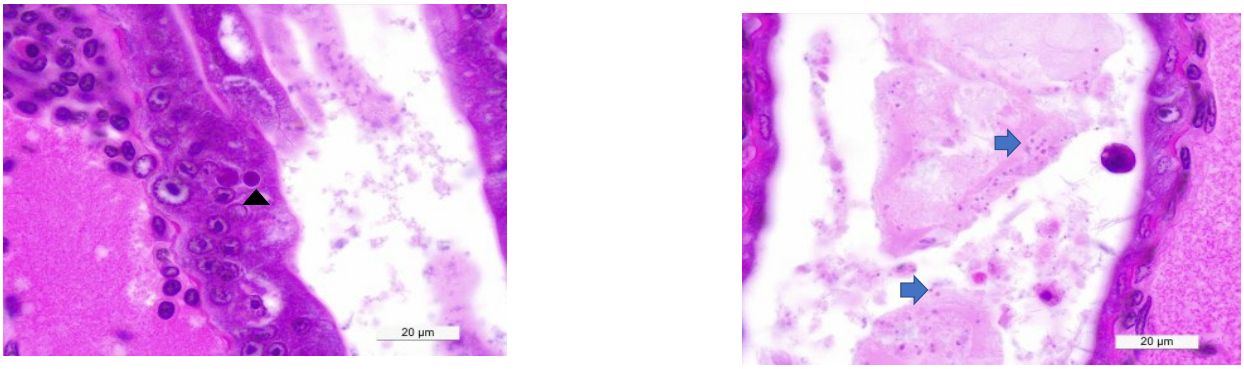
EHP *In-situ* hybridization



▲ =Cytoplasmic inclusion body within HP epithelium

Fig 3. Case 21-671 / N4. Hepatopancreas section showing cytoplasmic inclusion bodies (Arrow head) by H&E (left) and negative reaction by EHP *in-situ hybridization* (right).

H&E



▲ =Cytoplasmic inclusion body within HP epithelium; ➡ =spore within the HP tubule lumen

Fig 4. Case 21-671 / D1. Hepatopancreas section showing cytoplasmic inclusion bodies (arrow head) and suspected spores (blue arrow) typical of EHP by H&E

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EHP *In-situ* hybridization

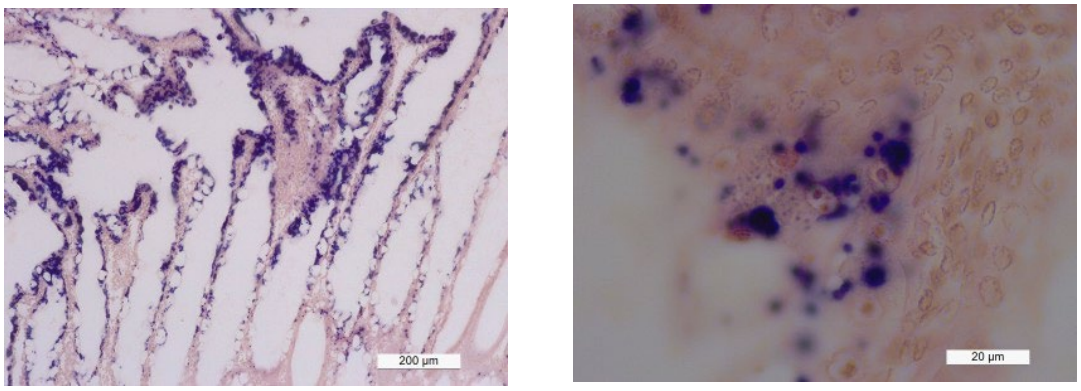

Fig 5. Case 21-671 /D1. Hepatopancreas section showing a positive reaction (dark color) for EHP by *in-situ hybridization*.

Comments:

- Results obtained by H&E histology, *In-situ* hybridization (ISH), and PCR for the positive and negative controls are comparable. Often time infection grade assessed by ISH was higher than the H&E grade for the corresponding sample. This is expected considering the sensitivity of ISH is more than H&E histology.
- Tank H (Tissue frozen for 24 hr): All five samples showed EHP infection by all three methods tested and the severity of infection was much higher than the samples obtained from Tanks G and I which represented two other replicate tanks of the same treatment.
- There are differences among H&E, ISH, and PCR results obtained for the samples representing Day 7 and Day 14 treatments, especially in Tanks L (Tissue frozen for 7 days) and M (Tissue frozen for 14 days). We proposed that inactive spores remain even after 21 days post-feeding. Also, it might be possible that inactive spores/ plasmodium are taken up by the epithelial cells and putative light eosinophilic to basophilic cytoplasmic inclusion bodies detected by the H&E histology may represent cellular response to limit the invading microbes. However, it is unknown how such a cellular response can be seen even after 21-days post-feeding.

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APPENDIX 1

Severity Grade	Clinical Findings
0	> No signs of infection/infestation by pathogen, parasite, or epicommensal present.
trace	> Signs of infection/infestation by pathogen, parasite or epicommensal are present at just above diagnostic procedure minimum detection limits.
1	> Signs of infection/infestation by pathogen, parasite or epicommensal are present, but at levels that may be below those needed for clinical disease. > Agent detected may be in early stages of infection and represent preclinical disease.
2	> Moderate signs of infection/infestation as shown by low to moderate numbers of parasite or epicommensal, or by number and severity of pathogen caused lesions. > Prognosis is for possible production losses and/or slight increases in mortality if no treatment (if treatable) or management change is applied.
3	> Moderate to high signs of disease apparent as shown by relatively higher numbers of parasite or epicommensal, or by number and severity of pathogen caused lesions. > Potentially lethal prognosis if no treatment (if treatable) or management change is applied.
4	> High numbers of parasite or epicommensal present, or for pathogen caused infections the presence of severe lesions and advanced tissue destruction. > Lethal prognosis, especially under conditions conducive to disease development (i.e. with low oxygen, ecdysis, changes in salinity or temperature, etc.).