RESEARCH **5**

2017-135

WILL CARP VIRUS BIOCONTROL BE EFFECTIVE?

NATIONAL CARP CONTROL PLAN

Essential studies on Cyprinid herpesvirus 3 (CyHV-3) prior to release of the virus in Australian waters

This suite of documents contains those listed below.

NCCP TECHNICAL PAPERS

1. Carp biocontrol background
2. Epidemiology and release strategies
3. Carp biocontrol and water quality
4. Carp virus species specificity
5. Potential socio-economic impacts of carp biocontrol
6. NCCP implementation
7. NCCP engagement report
8. NCCP Murray and Murrumbidgee case study
9. NCCP Lachlan case study

NCCP RESEARCH (peer reviewed)

Will carp virus biocontrol be effective?

1. 2016-153: Preparing for Cyprinid herpesvirus 3: A carp biomass estimate for eastern Australia
2. 2018-120: Population dynamics and carp biomass estimates for Australia
3. 2017-148: Exploring genetic biocontrol options that could work synergistically with the carp virus
4. 2016-170: Development of hydrological, ecological and epidemiological modelling
5. 2017-135: Essential studies on Cyprinid herpesvirus 3 (CyHV-3) prior to release of the virus in Australian waters
6. 2020-104: Evaluating the role of direct fish-to-fish contact on horizontal transmission of koi herpesvirus
7. 2019-163 Understanding the genetics and genomics of carp strains and susceptibility to CyHV-3
8. 2017-094: Review of carp control via commercial exploitation

What are the carp virus biocontrol risks and how can they be managed?

9. 2017-055 and 2017-056: Water-quality risk assessment of carp biocontrol for Australian waterways
10. 2016-183: Cyprinid herpesvirus 3 and its relevance to humans
11. 2017-127: Defining best practice for viral susceptibility testing of non-target species to Cyprinid herpesvirus 3
12. 2019-176: Determination of the susceptibility of Silver Perch, Murray Cod and Rainbow Trout to infection with CyHV-3
13. 2016-152 and 2018-189: The socio-economic impact assessment and stakeholder engagement
    Appendix 1: Getting the National Carp Control Plan right: Ensuring the plan addresses community and stakeholder needs, interests and concerns
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    Appendix 3: Socio-economic impact assessment — commercial carp fishers
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15. 2017-054: Social, economic and ecological risk assessment for use of Cyprinid herpesvirus 3 (CyHV-3) for carp biocontrol in Australia
    Volume 1: Review of the literature, outbreak scenarios, exposure pathways and case studies
    Volume 2: Assessment of risks to Matters of National Environmental Significance
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16. 2016-158: Development of strategies to optimise release and clean-up strategies
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NCCP PLANNING INVESTIGATIONS

1. 2018-112: Carp questionnaire survey and community mapping tool
2. 2018-190: Biosecurity strategy for the koi (*Cyprinus carpio*) industry
3. 2017-222: Engineering options for the NCCP
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FISHERIES RESEARCH & DEVELOPMENT CORPORATION

Essential studies on *Cyprinid herpesvirus 3* (CyHV-3) prior to release of the virus in Australian waters: Seasonality studies

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Abbreviations

ACDP	Australian Centre for Disease Preparedness
CPE	Cytopathic effect
C_T	Cycle threshold
CyHV-3	Cyprinid herpesvirus 3
MDB	Murray-Darling Basin
NCCP	National Carp Control Program
qPCR	Quantitative PCR
SAC	Scientific Advisory Group
$TCID_{50}$	50% tissue culture infective dose

Executive Summary

Background

In Australia, common carp (*Cyprinus carpio*) are an introduced environmental pest, and the virulence and apparent host-specificity of *Cyprinid herpesvirus 3* (CyHV-3) may offer an important means of biological control. Nevertheless, integrated ecological and epidemiological modelling work to determine how best to release the virus identified that there would be a considerable logistical challenge undertaking a field release in multiple sites across south-eastern Australia. This is because there is a relatively short period in the spring when behavioural aggregation would achieve sufficient onward transmission of the virus to susceptible carp. A theoretical possibility to overcome this might be to undertake inoculation of fish during either the late autumn or winter when temperatures are near or below the disease inducing threshold of ~16 ˚C, and accordingly the virus would enter into a dormant state. Following the rise in water temperature in the spring, if virus reactivation occurred in these inoculated fish, then this would provide an efficient method of release as these fish would transmit the disease during spring aggregation events. We termed this potential late autumn/winter release method 'Trojan carp'. To assess whether this is biologically possible, we conducted a formal laboratory experiment simulating both late autumn and winter conditions with respect to water temperature conditions. We also compared the effect of physical stress in both temperature simulations, to ascertain the potential impact of spring aggregation and mating on virus reactivation.

Aims

The aims of this project are to determine whether:

1) injection of carp with CyHV-3 at water temperature of 22°C and then decreased to 12°C – the late autumn simulation – results in sub-clinical infection,
2) injection of carp with CyHV-3 at water temperature of 12°C – the winter simulation – results in sub-clinical infection,
3) these carp sub-clinically infected with CyHV-3 at 12°C develop clinical disease when temperature is raised to the permissive temperature of 22°C and,
4) stress is required for clinical disease to develop at the permissive temperature of 22°C.

Methodology

The experiment had four treatment groups: inoculation of carp with CyHV-3 at two different water temperatures (22°C and 12°C), corresponding to a late autumn and winter release respectively; and two capture stress treatments, to simulate infection with and without stress during the subsequent spring. Each of these treatment groups was kept in a separate biosecure room in the ACDP secure Large Animal Facility and comprised six replicate tanks containing 16-21 carp per tank. There was also one negative control tank for each capture stress group. Two groups (Group 1 and 2) – the late autumn simulation – were exposed to CyHV-3 by intra peritoneal (IP) injection at 22°C, held at 22°C for 2 days before the water temperature decreased to 12°C over a further 5 days. An additional two groups (Group 3 and 4) were subjected to the winter simulation whereby they were inoculated with CyHV-3 by IP injection at 12°C. All four groups were held at 12°C for 14 days and then the water temperature was returned to 22°C over 10 days, which simulated the spring rise in temperature. Group 2 and Group 4 were subject to capture stress once the water temperature reached 22°C and this stress continued for 10 days until the experiment terminated. All fish were monitored twice daily during the non-disease period and three times per day during the disease period. Diseased fish with moderate clinical signs were deemed to have reached the humane endpoint for the experiment and were euthanised, and gills, kidney, and skin lesion samples (when observed) were collected and tested by real-time PCR (qPCR).

Results

Injection of carp with CyHV-3 at 12°C and kept at this non-permissive temperature for two weeks (Groups 3 and 4) – the winter release simulation – resulted in sub-clinical infection and reactivation only when water temperature was raised to a permissive temperature of 22°C. Reactivated virus led to expression of clinical signs of disease and morbidity of carp of 40.35% (Group 3 without stress) and 51.38% (Group 4 with stress).

Injection of carp with CyHV-3 at 22°C and lowering of the temperature to 12°C – the late autumn simulation – prevented the progression to clinical disease but in the non-stress treatment room after 4-5 days CyHV-3 induced morbidity occurred, being particularly high in two of the tanks (i.e., 61% and 89%). The appearance of disease in these tanks was unexpected but was possibly related to temporary increases in the water temperature to above the 16°C threshold during the period of daily changes in the tank water. Asides from the unexpected morbidity in the low water temperature treatment, the experiment was consistent with the literature in showing the re-appearance of clinical disease when the temperature was raised back to 22°C. These morbidities exclusive of those which occurred at the lower temperatures were comparable at 17% (12/69; Group 1 without stress) and 15% (15/101; Group 2 with stress).

In all four groups of inoculated carp, testing with the CyHV-3 qPCR confirmed that all fish showing clinical signs of disease, except for two which were negative, tested positive for CyHV-3 with low CT value (average 24.47), suggesting that the carp were indeed infected with high concentration of the virus. In contrast, all apparently healthy carp humanely killed at the end of the experiment were either qPCR positive with high CT value (average 34.04, suggesting low level of CyHV-3 infections) or qPCR negative. All carp in the negative control groups were also qPCR negative. No clinical signs of CyHV-3 disease were observed in the mock-infected negative control groups.

Conclusion

The winter simulations demonstrate that infection at a non-permissive low temperature could result in a persistent infection, with subsequent reactivation when the temperature was raised above the permissive threshold. It thus provides some biological evidence that a Trojan carp phenomenon occurs which might form the basis of a winter release.

The late autumn simulations confirmed previous work that inoculation of the CyHV-3 in the permissive water temperature range, followed soon after by a reduction of water temperature below this range, prevented disease if the low temperature was consistently maintained. However, it was indicated that transient temperature rises above the lower permissive range threshold might result in disease. Thus, a late autumn release might be less successful as virus excretion and mortalities might occur prior the fish aggregating in spring and therefore would not be able to effectively onward transmit the virus.

For the two groups exposed to the winter simulation, capture stress was shown to increase the proportion of fish expressing disease, but in the late autumn simulation the lack of any effect of stress is less certain due to the confounding effect of the disease during the period when the water temperature was nominally at 12°C. The unexpected appearance of disease in the low water temperature requires further investigation. As no other diagnostics were done on the diseased fish other than CyHV-3 qPCR testing, secondary infection can't be ruled out. Additional diagnostics such as next-generation sequencing may be helpful to explain the unexpected morbidity.

Implications

By demonstrating that infection of carp at low water temperature results in a persistent infection, which could then be reactivated when the temperature was raised above the permissive threshold,

this laboratory experiment has provided initial proof-of-concept for an alternative “Trojan carp” release strategy based on capture and inoculation of fish over the winter period. However, further experimentation is required to confirm that infective virus will persist in carp over a period longer than the 14-days used in our trial, and thus permit inoculation of fish throughout the winter and not just immediately before the spring temperature rise.

Keywords: Common carp, biological control, cyprinid herpesvirus 3, seasonality, temperature, stress, virus reactivation, release strategy.

Introduction

Common carp (*Cyprinus carpio*) are an introduced environmental pest in Australia (Davidson, 2002). The virulence (Sunarto et al., 2011) and apparent host-specificity (McColl et al., 2017) of koi herpesvirus (KHV), formally known as *Cyprinid herpesvirus 3* (CyHV-3), may offer an important means of control (McColl, Sheppard, & Barwick, 2017). CyHV-3 is a highly contagious virus which causes an acute viraemia in koi and common carp with resulting morbidity and mortality as high as 100% and 90%, respectively (Haenen et al., 2004; Hedrick et al., 2000; Perelberg et al., 2003). Those fish that survive an acute infection may develop a long-term infection (persistent or latent), with the capacity of the virus to reactivate under certain conditions, such as temperature stress (Eide et al., 2011). This allows successive virus transmission to the susceptible hosts, thereby sustaining its existence. The ability to establish life-long latent infection with sporadic virus reactivation to facilitate transmission is the hallmark of members of the order *Herpesvirales* including *Alloherpesviridae* (fish herpesvirus family which includes CyHV-3).

Water temperature is the principal environmental factor for induction of the disease (Gilad et al., 2003), as well as reactivation from persistent infection (St-Hilaire et al., 2005; Sunarto et al., 2014). The disease is temperature dependent, occurring at permissive temperatures between 16 and 28°C (Gilad et al., 2003; Haenen et al., 2004; Hedrick et al., 2000; OIE, 2021; Perelberg et al., 2003; Pikarsky et al., 2004; Sano et al., 2004). Under experimental conditions the disease has caused high mortality at 28°C but not at 30°C, nor at 13°C. Infectious virus was continuously shed from the infected carp for 34 days (7–40 days post infection, dpi) at 16°C, for 14 days (1–14 dpi) at 23°C and for 12 days (3–14 dpi) at 28°C (Yuasa, Ito, & Sano, 2008). Fish exposed to CyHV-3 at 13°C by bath immersion succumbed to disease after the water temperature was increased to 23°C at 30 dpi, but not at 64 dpi (Gilad et al., 2003). Although St-Hilaire et al. (2005) suggested that reactivation of CyHV-3 occurred up to 30 weeks after initial exposure to the virus at permissive temperatures, CyHV-3 was only reactivated in fish from 3 out of 5 experimental tanks. Stress such as heat stress, netting or handling has also been associated with CyHV-3 reactivation in koi and carp (Bergmann and Kempter, 2011; Eide et al., 2011; Lin et al., 2017). An increase of viral DNA was detected by qPCR as early as three days post-netting or temperature stress, but neither clinical signs of disease nor mortality of fish were reported (Bergmann and Kempter, 2011; Lin et al., 2017). All these data probably reflect the absence of a robust stress model for reactivation of CyHV-3 infection.

It is possible that carp in countries in which CyHV-3 is enzootic are exposed to CyHV-3 year-round, and yet, clinical signs of disease only appear to occur in autumn, spring, and summer when water temperatures are within the permissive range for virus replication. Therefore, several questions arise: Do carp become infected at non-permissive temperatures, but the virus fails to multiply? Is the virus then activated in these infections when the water reaches a permissive temperature? What role does stress play in virus reactivation resulting in outbreaks of disease? Nevertheless, integrated ecological and epidemiological modelling work to determine how best to release the virus identified that there would be a considerable logistical challenge undertaking a field release in multiple sites across south-eastern Australia (Durr et al., 2019). This is because there is a relatively short period in the spring when behavioural aggregation would achieve sufficient onward transmission of the virus to susceptible carp. A theoretical possibility to overcome this might be to undertake inoculation of fish during either the late autumn or winter when temperatures are near or below the disease inducing threshold of ~16 ˚C, and accordingly the virus would enter into a dormant state. Following the rise in water temperature in the spring, if virus reactivation occurred in these inoculated fish, then this would provide an efficient method of release as these fish would transmit the disease during spring aggregation events. We termed this potential late autumn/winter release method 'Trojan carp'. To assess whether this concept is possible, we conducted a formal laboratory experiment simulating both late autumn and winter conditions with respect to water temperature conditions. We also compared the effect of physical stress in both temperature simulations, to ascertain the potential impact of spring aggregation and mating on virus reactivation.

Objectives

The aims of this project are to determine whether:

1) injection of carp with CyHV-3 at water temperature of 22°C and then decreased to 12°C – the late autumn simulation – results in sub-clinical infection,
2) injection of carp with CyHV-3 at water temperature of 12°C – the winter simulation – results in sub-clinical infection,
3) these carp sub-clinically infected with CyHV-3 at 12°C develop clinical disease when temperature is raised to the permissive temperature of 22°C and,
4) stress is required for clinical disease to develop at the permissive temperature of 22°C.

Methods

The experimental design is adapted from one previously undertaken at ACDP (Sunarto et al., 2014) which corresponds to the conditions of treatment Group 1 (fish were infected at a permissive temperature of 22°C without capture stress), temperature was reduced to a non-permissive level and fish were held for 14 days then temperature was raised to permissive levels and reactivation of persistent virus was achieved. Similar temperature manipulation scheme for viral reactivation has also been used by other workers (Gilad et al., 2003; St-Hilaire et al., 2005; Eide et al., 2011).

Cell culture and virus isolate

The Indonesian CyHV-3 C07 isolate used in this experiment was isolated from common carp suffering mass mortalities in West Java, Indonesia in 2007 (Sunarto et al., 2011). The virus was cultured in the koi fin cell line (KF-1) (Hedrick et al., 2000) provided by Professor R. P. Hedrick (University of California, Davis, USA). The cells were maintained in Leibovitz L-15 medium (Life Technologies, USA) supplemented with 10% foetal bovine serum (FBS) (Thermo Trace, Australia) and incubated at 25°C. The cultures were observed daily for evidence of cytopathic effect (CPE) and the virus was harvested from the cultures exhibiting CPE with expected titre of 10^4 50% tissue culture infective dose per mL ($TCID_{50}$/mL). The titre of the virus was estimated by determining the $TCID_{50}$ (Reed & Muench, 1938) and after inoculation, the virus titre was confirmed via a back titration.

Experimental design

The experiment was approved by the CSIRO ACDP Animal Ethics Committee (AEC 1973). Four hundred seventy juvenile carp (mean length ± standard deviation: 146.1 ± 20.2 mm) were supplied by K & C Fisheries, Stratford, Victoria. Juvenile fish were used in this experiment because all age groups of carp, from juvenile upwards (but not carp larvae), appear to be susceptible to infection with CyHV-3 (Bretzinger, et al., 1999; Sano et al., 2004). These carp were wild caught and were held at K & C Fisheries facility for four months prior to transport to ACDP. On arrival at ACDP, it was noted that fish were infested with anchor worm (*Lernaea cyprinacea*) and were treated by physically removing the adult parasites, along with salt baths and diflubenzuron at a dose of 0.066mg/L before and during the experiment. The fish were held in 80 L tanks containing 16 to 21 fish per tank (total 26 tanks with average 18 fish per tank) on a 12 h/12 h day/night cycle, receiving 30% daily water changes and fed with commercial feed once per day throughout the experiment.

There were four treatment groups (infection at two water temperatures; with and without capture stress) and 6 replicates for each group of 18 (16 -21) fish per tank (Table 1). The detailed experimental plan is included as Appendix 1. Each treatment group were kept in a separate room. There was one negative

control for each capture stress group (18 fish per tank). The negative-control (mock-infected) groups are required to demonstrate if there are any adverse effects due to fish being initially held at 22°C, undergoing a water temperature reduction to 12°C then a temperature increase to 22°C and then being held at this temperature, with and without capture stress treatments.

Table 1. Experimental design

	Without Stress	**With Stress**
Virus injection at 22ºC	Group 1	Group 2
Virus injection at 12ºC	Group 3	Group 4
Mock injection	Negative control group	Negative control group

All fish were subjected to the same water temperature regime (Figure 1; Appendix 2) with carp infected at 22°C on Day 0 and carp infected at 12°C on Day 7. There were only two negative controls for the stress treatment: Tank 25 (without capture stress) and Tank 26 (with capture stress). Fish in the negative controls were treated identically to those of the virus-infected groups but received only IP injection of 100 µL of tissue culture medium without virus and were kept in a separate room from virus-infected groups.

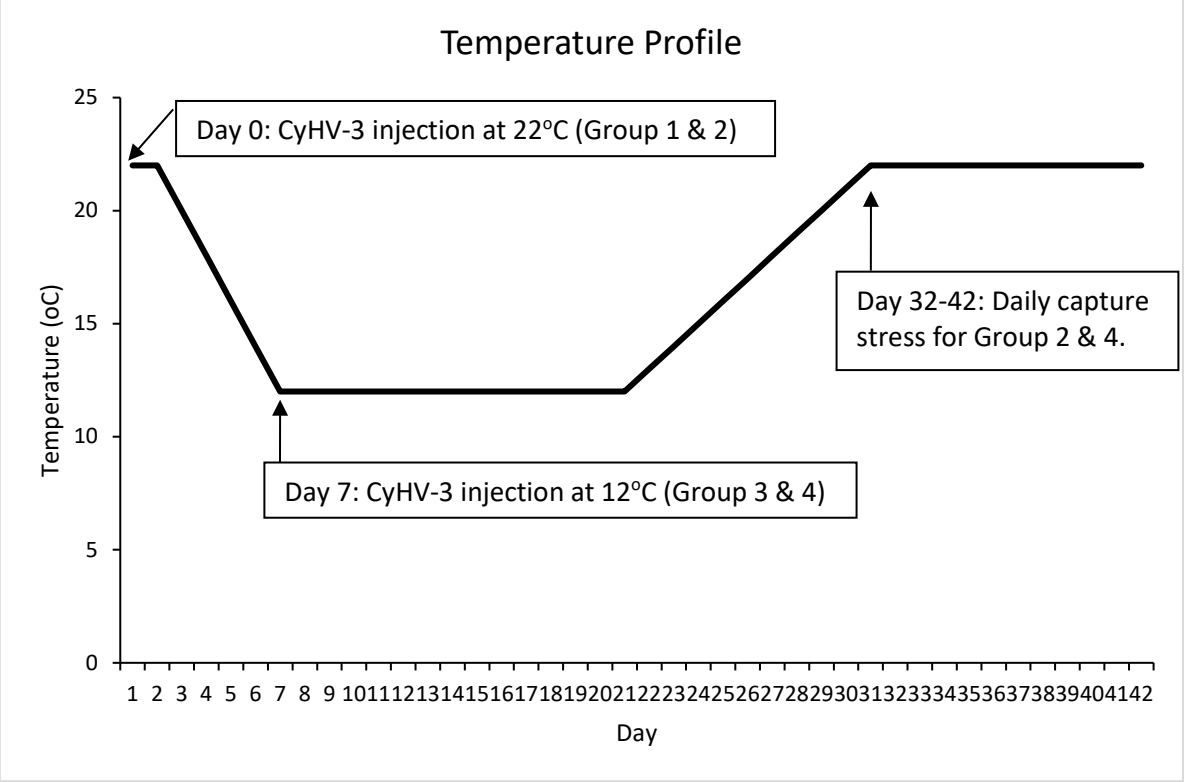

Figure 1. Water temperature profile, virus injection and capture stress schedules. All fish were subjected to the same water temperature regime but injected with CyHV-3 at different time points (Group 1 & 2 at day 0 at 22ºC; Group 3 & 4 at day 7 at 12ºC). Capture stress was applied to Group 2 & 4 from day 32 to 42 when water temperature reached 22ºC. Group 1 & 3 did not receive stress treatment throughout the course of the experiment, which was terminated at day 42.

Two hundred and twelve carp in 16 tanks across Groups 1 and 2 were injected with CyHV-3 by intraperitoneal (IP) injection of 100 µL containing $10^{2.9}$ $TCID_{50}$/fish at 22ºC. IP injection was used for this experiment because this exposure model is considered as the most feasible way of releasing the virus. For example, carp could be captured and injected with the virus before being released back into the waterway in which they were caught. In this experiment, all carp were initially kept at 22ºC for 2 days and then the water temperature was reduced to 12°C over 5 days (reduced by 2ºC per day). Fish were held at a water temperature of 12ºC for 14 days and then the water temperature was increased from 12 to 22ºC over a

period of 10 days (increased by 1°C per day). For Group 1 (Tanks 1-6), fish did not receive capture stress throughout the experiment. For Group 2 (Tanks 7-12), capture stress was commenced once water temperature reached 22°C for 10 days until the experiment terminated on day 42. Capture stress was performed once per day by capturing the fish with a net and transferring them to an aerated 30 L tank at 22°C, in which they were held for 5 minutes. Then fish were re-captured and transferred back to the original tank.

Two hundred and twenty-three carp across 16 tanks in Groups 3 and 4 were injected with CyHV-3 by IP injection of 100 µL at a dose of $10^{3.6}$ $TCID_{50}$/fish at 12°C at day. The fish were maintained at 12°C for 14 days and then the water temperature was increased to 22°C over 10 days (increased by 1°C per day). For Tanks 13 to 18, fish did not receive capture stress throughout the experiment (Group 3). For fish in Tanks 19 to 24 (Group 4), capture stress was commenced once water temperature reached 22°C in the same manner as those in Group 2.

All fish were monitored twice daily during the non-disease period (8am and 3pm) and with an additional observation period added when clinical signs of disease were observed (8am, 3pm and 11pm). The humane endpoint for this experiment was defined as any severe signs of clinical disease, or any moderate clinical signs observed over a 24-hour period. Fish were humanely killed immediately using AQUI-S® Aquatic Anaesthetic according to the manufacturer's protocols (AQUI-S New Zealand Ltd.) if "severe" clinical signs of disease were observed at any observation point. Severe signs include gasping constantly at the surface, nervous signs (shaking, uncoordinated movements, erratic swimming), gill damage (discoloured, swollen, and necrotic gill filaments with excess mucus), fin and tail rot, dorso-ventral recumbency on the bottom of tanks and lateral recumbency (i.e., lying on their side). Fish exhibiting moderate signs of disease were humanely killed within 24 hours of the observation. Moderate signs include small and round skin lesions, erosion/ ulceration of the skin, blistered skin, sunken eyes, and fish swimming lethargically at the water surface. Fish showing mild clinical signs of disease were monitored for their progression of disease. Mild signs, whilst rarely observed include skin discolorations (focal pale, darkened or reddened skin), excess mucus production and foci of dry skin which may lead to rough skin known as 'sandpaper' skin. Focal reddening of the skin particularly those occurring on ventral part of the body such as the base of pectoral and ventral fins are very small and requires netting out of individual fish to observe, which could confound results in this experiment. Netting fish out could potentially cause stress and reactivation of disease, and therefore observations of clinical signs of disease per tank were done without netting individual fish.

Sample collection

To confirm that dead or humanely killed fish were infected with CyHV-3, samples of gills, kidney and skin lesions (if present) were tested by real-time PCR (qPCR) specific for CyHV-3. All surviving fish and those from negative control tanks were humanely killed at the end of the experiment and tested for CyHV-3 by qPCR. Samples from each fish were collected into separate tubes containing 80% (v/v) ethanol and stored at 4°C until further use.

DNA extraction

To extract total nucleic acid, the samples were homogenised by bead beating with the FastPrep-96 (MP Biomedicals) platforms according to manufacturer's protocols. Briefly, bead beating of samples were performed at 1,800 rpm in 600 µl of lysis buffer for MagMAX-96 Viral RNA Isolation Kit (Applied Biosystems). Sample tubes were quenched in ice for 2 min prior to centrifugation at 14,000 rpm for 2 min. Sixty microlitres of the clarified supernatants were used for nucleic acid extraction using the MagMAX-96 Isolation Kit. A negative extraction control (NEC) was included in each run. This is an additional tube prepared in the same manner as for the test samples, but which has no sample added to the well and is processed with the test samples.

TaqMan qPCR

A housekeeping gene qPCR, using the 18S-F/R primers and an 18S-P probe for generic detection of the 18S ribosomal (r)RNA gene (Applied Biosystems), was used to confirm nucleic acid integrity. To detect CyHV-3 DNA, a qPCR targeting the ORF 89 using the CyHV-3-86f/163r primers and a CyHV-3-109p probe was used (Gilad et al., 2004). The reaction mixtures (25 µL) were set up in duplicate and comprised 12.5 µL TaqMan Universal PCR Master Mix (Applied Biosystems), 1.25 µL each primer (18 µM) and 5 µM probe, 2 µL template DNA and 6.75 µL DNase-free water. A 7500 Fast Real-time PCR System was used with the following profile: one cycle at 50°C for 2 min and at 95°C for 10 min; 45 cycles at 95°C for 15 s and 60°C for 1 min with the C_T value determined using a threshold of 0.1.

Statistical analysis

As the results on fish morbidity in the autumn simulations were complex and somewhat counter-intuitive, with the stress treatment of Group 2 having a lower percentage of morbidity than the non-stressed treatment of Group 1, we undertook statistical analyses to further explore this pattern. We first did a univariate analysis of the number of carp showing disease (or infection) in response to stress, using the Chi-square test for independence to determine if significant associations were present. Following confirmation of this for infection status, we then undertook more advanced statistical modelling, using fixed effects logistic regression with stress as the fixed effect. As this also showed a marginally significant effect of stress on infection status, we followed up with a mixed-effects multilevel logistic regression analysis, which allowed for the clustering of the fish in tanks, which was treated as a random effect. All analyses were undertaken in the R environment (version 3.6) using the "xtabs" function (from the "stats" package) for the cross-tabulation analysis, the "glm" function ("stats" package) for the fixed effects logistic regression and the "glmer" function ("lme4" package) for the mixed-effects modelling. Note that due to the lack of randomisation of the stress treatment between the two rooms, i.e., the two groups were in different rooms, all p-values should be interpreted as indicative only.

Results

Injection of carp with CyHV-3 at 12°C and kept at this non-permissive temperature for two weeks (Groups 3 and 4) – the winter release simulation – resulted in sub-clinical infection and reactivation when water temperature was raised to a permissive temperature of 22°C (Table 2; Figure 2; Appendix 4). Reactivated virus led to expression of clinical signs of disease and morbidity of carp of 40.35% (Group 3 without stress) and 51.38% (Group 4 with stress).

Injection of carp with CyHV-3 at 22°C and lowering of the temperature to 12°C (Groups 1 and 2) – the late autumn simulation – prevented the progression to clinical disease but in the non-stress treatment room after 4-5 days CyHV-3 induced morbidity occurred, being particularly high in two of the tanks, i.e., Tank 3 (55%) and Tank 4 (83%) (Table 2; Figure 2; Appendix 4). The appearance of disease in these tanks was unexpected but was possibly related to temporary increases in the water temperature to above the 16°C threshold during the period of daily changes in the tank water (Appendix 2). In Japan, it has been reported that daily temperature fluctuation of 3°C increased the susceptibility of carp to CyHV-3 infection (Takahara et al., 2014). However, other tanks did have water temperature fluctuations and morbidity wasn't observed in these tanks. The possible reasons for the water temperature spikes and the results will be further discussed in detail in the Discussion section. As no other diagnostics were done on the diseased fish other than CyHV-3 qPCR testing, secondary infection can't be ruled out. Additional diagnostics such as next-generation sequencing may be helpful to explain the unexpected morbidity. However, the clinical signs of diseased carp at 12°C were the same as those observed at 22°C with most diseased carp showing blistered skin, suggesting CyHV-3 infection. Furthermore, in all four groups of inoculated carp, testing with the CyHV-3 qPCR confirmed that all fish showing clinical signs of disease, except for two which were negative (fish from Tank 8 Fish 2 (T8F2) and T18F2), tested positive for CyHV-3 with low CT value (average 24.47), suggesting that the diseased carp were indeed infected with high concentration of the virus. Asides from

the unexpected morbidity in the low water temperature treatment, the experiment was consistent with the literature in showing the re-appearance of clinical disease when the temperature was raised back to 22°C. These morbidities exclusive of those which occurred at the lower temperatures were comparable at 18% (12/69; Group 1 without stress) and 15% (15/101; Group 2 with stress).

The effect of capture stress in Group 2 & 4, which commenced daily when water temperature reached 22°C and continued for 10 days, was complex and diverged between the autumn and winter simulations. For the winter simulation, the stressed fish in Group 4 had a higher overall percentage of morbidity than the unstressed Group 3 (51.38% vs. 40.35%) – see Table 2 – but for the autumn simulation the opposite effect was observed with stress apparently being protective (17.31% stressed vs. 47.22% unstressed). However, the high percentage of morbidity in the latter group was mainly the result of the very high CyHV-3 morbidity in two tanks (Tank 3: 61.11% and Tank 4: 88.89%), the impact of which was demonstrated by the exploratory statistical analysis (Table 3). Thus, without adjusting for the clustering of fish within tanks, whilst there was a marginal effect of stress on infection status (approximate p values < 0.05) when the tank effect was taken into account stress was no longer significant. The effect of stress on clinical signs between Groups 1 and 2 were all no significant.

Table 2. Morbidity of carp following CyHV-3 infection.

Group	Tank #	No. fish per tank	Cumulative morbidity at five different water-temperatures regimes in time-course manner as described in Figure 1						
			Day 0[a]-2 (22°C)	Day 3-7[b] (20-14°C)	Day 8-21 (12°C)	Day 22-31 (13-21°C)	Day 32-42[c] (22°C)	Total	%
1. Virus injection at 22°C, kept at 12°C for 2 weeks, returned to 22°C, without capture stress.	1	18	0	0	2	3	2	7	38.89
	2	18	0	0	3	1	1	5	27.78
	3	18	0	0	10	0	1	11	61.11
	4	18	0	0	15	0	1	16	88.89
	5	19	0	0	4	1	1	6	31.58
	6	17	0	0	5	1	0	6	35.29
	Total	**108**	**0**	**0**	**39**	**6**	**6**	**51**	**47.22**
2. Virus injection at 22°C, kept at 12°C for 2 weeks, returned to 22°C, with capture stress, which only occurred on day 32 onwards.	7	17	0	0	0	4	1	5	29.41
	8	18	0	0	0	0	2	2	11.11
	9	16	0	0	0	3	0	3	18.75
	10	18	0	0	0	0	2	2	11.11
	11	17	0	0	2	1	1	4	23.53
	12	18	0	0	1	1	0	2	11.11
	Total	**104**	**0**	**0**	**3**	**9**	**6**	**18**	**17.31**
3. Virus injection at 12°C, kept at 12°C for 2 weeks, increased to 22°C, without capture stress.	13	19			2	1	3	6	31.58
	14	19			0	1	5	6	31.58
	15	19			0	0	6	6	31.58
	16	18			0	1	7	7	38.89
	17	18			0	1	9	10	55.56
	18	21			2	1	7	10	47.62
	Total	**114**			**4**	**5**	**37**	**46**	**40.35**
4. Virus injection at 12°C, kept at 12°C for 2 weeks, increased to 22°C, with capture stress, which only occurred on day 32 onwards.	19	18			0	0	6	6	33.33
	20	18			0	0	7	7	38.89
	21	19			0	1	11	12	63.16
	22	18			0	0	13	13	72.22
	23	17			0	1	6	7	41.18
	24	19			0	1	10	11	57.89
	Total	**109**			**0**	**3**	**53**	**56**	**51.38**
Negative control group (mock-infected at 22°C, kept at 12°C for 2 weeks, increased to 22°C):									
Without capture stress	25	18*	0	0	0	0	0	0	0
With capture stress	26	17	0	0	0	0	0	0	0

Note: Cumulative morbidity of carp at five different water-temperature regimes in time-course manner as described in Figure 1. Day 0-2 (water temperature 22°C), Day 3-7 (22-14°C), Day 8-21 (12°C), Day 22-31 (13-21°C) and Day 32-42 (22°C). All fish were kept in the same water-temperature regimes but infected with the virus at different time points.

[a] Day 0: virus injection at 22°C for Group 1 and 2.

[b] Day 7: virus injection at 12°C for Group 3 and 4.

[c] Day 32: once water temperature reached 22°C, capture stress was commenced and continued for 10 days until the experiment terminated on day 42 (highlighted in yellow).

*One fish died at 3 dpi without clinical signs of disease and qPCR was negative.

Red font highlights Tanks 3 and 4, in which high morbidity occurred at water temperatures of 12°C.

Table 3. Results of the statistical tests to assess the effect of stress on clinical disease and infection for Group 1 and 2. For the mixed effects logistic regression, the random effect was the tank. Significant p-values (< 0.05) are shown in bold, but due to the lack of randomisation of treatments between rooms, all p values should be treated as indicative only.

Statistical model / test	**Response variable**	
	Clinical signs of disease	**Infection**
Chi square test for independence	0.1997	**0.0404**
Fixed effects logistic regression	0.2002	**0.0448**
Mixed effects logistic regression	0.250	0.0687

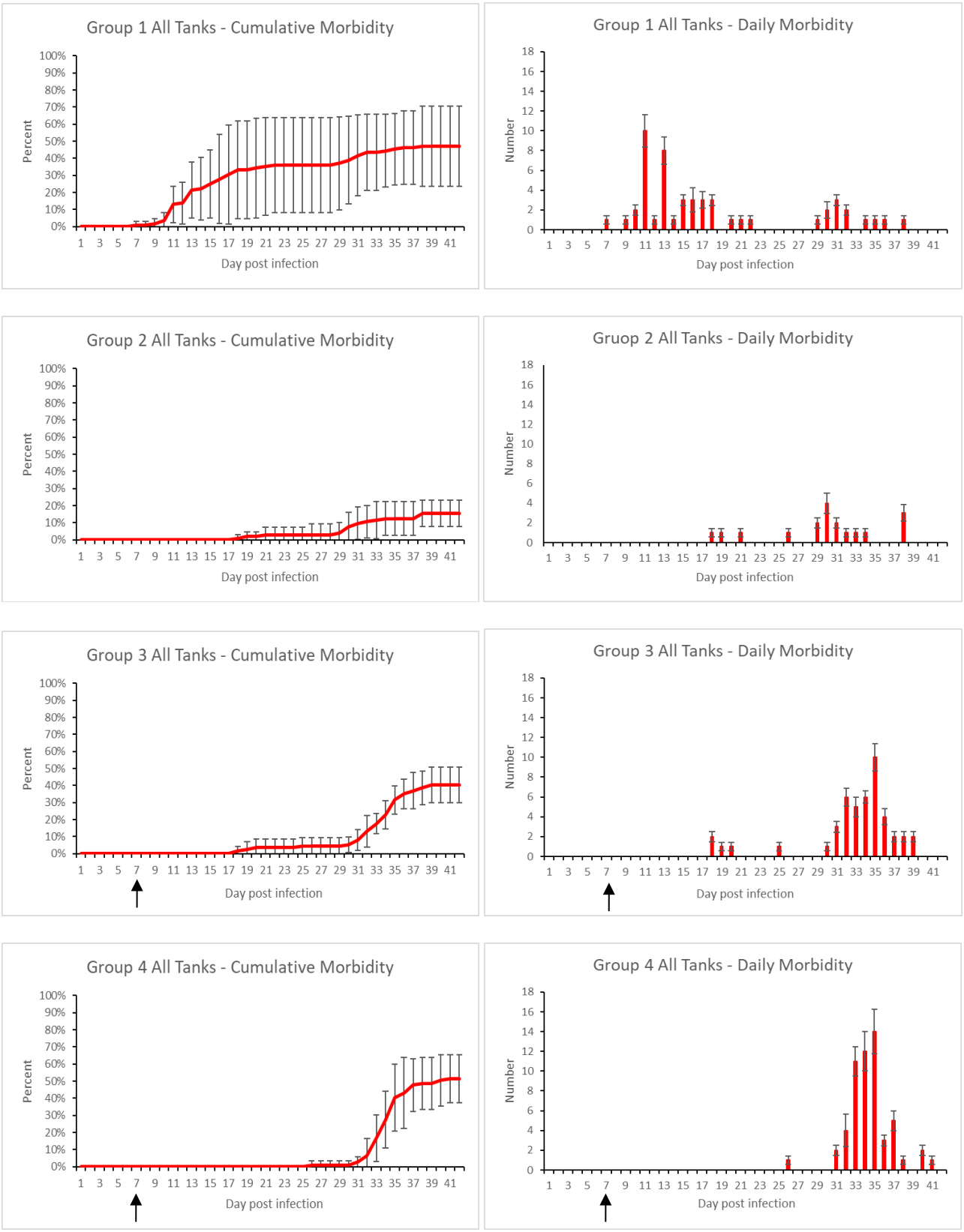

Figure 2. Cumulative and daily morbidity of carp following CyHV-3 infection. In Group 1 & 2, fish were injected with CyHV-3 at 22°C and held at 12°C for 14 days before the water temperature returned to 22°C. In Group 3 & 4, fish were injected with CyHV-3 at 12°C and held at this temperature for 14 days before the water temperature returned to 22°C. Arrow indicates virus injection at 12°C. Fish in Group 1 & 3 did not receive capture stress throughout the course of the experiment. Fish in Group 2 & 4 received capture stress which was commenced once water temperature reached 22°C for 10 remaining days until the experiment terminated on day 42. Error bars are standard deviations of six replicates for each group.

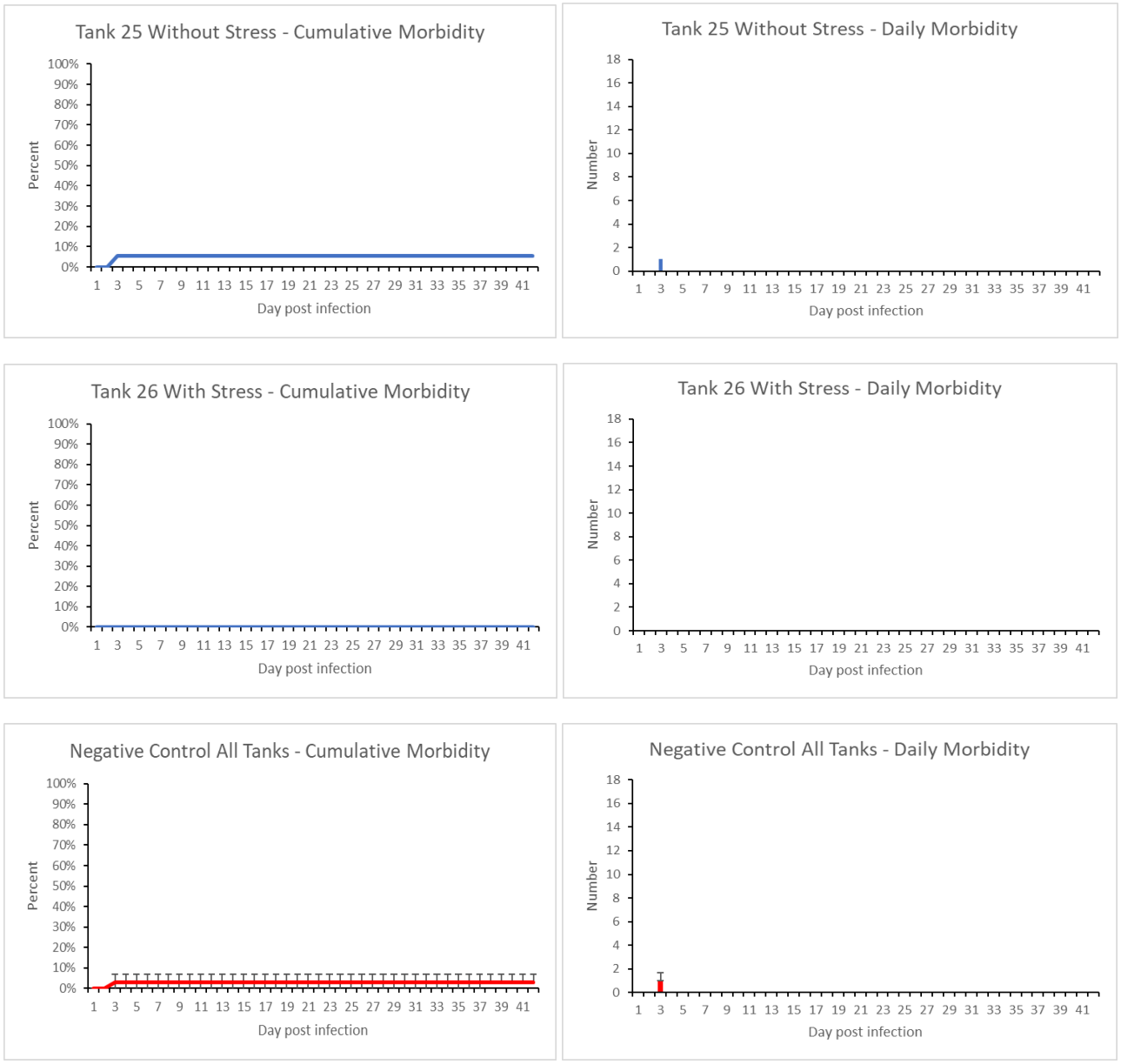

Figure 3. Cumulative and daily morbidity of carp in negative control group, in which fish were treated identically to those of the virus-infected groups at 22°C but received only tissue culture medium without virus and were kept in a separate room from virus-infected groups. Error bars are standard deviations of two duplicates for negative control group.

The clinical signs of diseased carp at 12°C were the same as those observed at 22°C with the majority of diseased carp showing blistered skin (Figure 4). Other clinical signs including reddened fins, darkened or sandpaper skin, gill damage, tail and fin rot were also observed. The same pattern of clinical signs was observed in all virus-infected groups regardless of the temperature and capture stress treatments. No clinical signs of CyHV-3 disease were observed in mock-infected negative control groups. One fish died at 3 dpi without clinical signs of disease (Table 2; Figure 3) and qPCR was negative (Appendix 3).

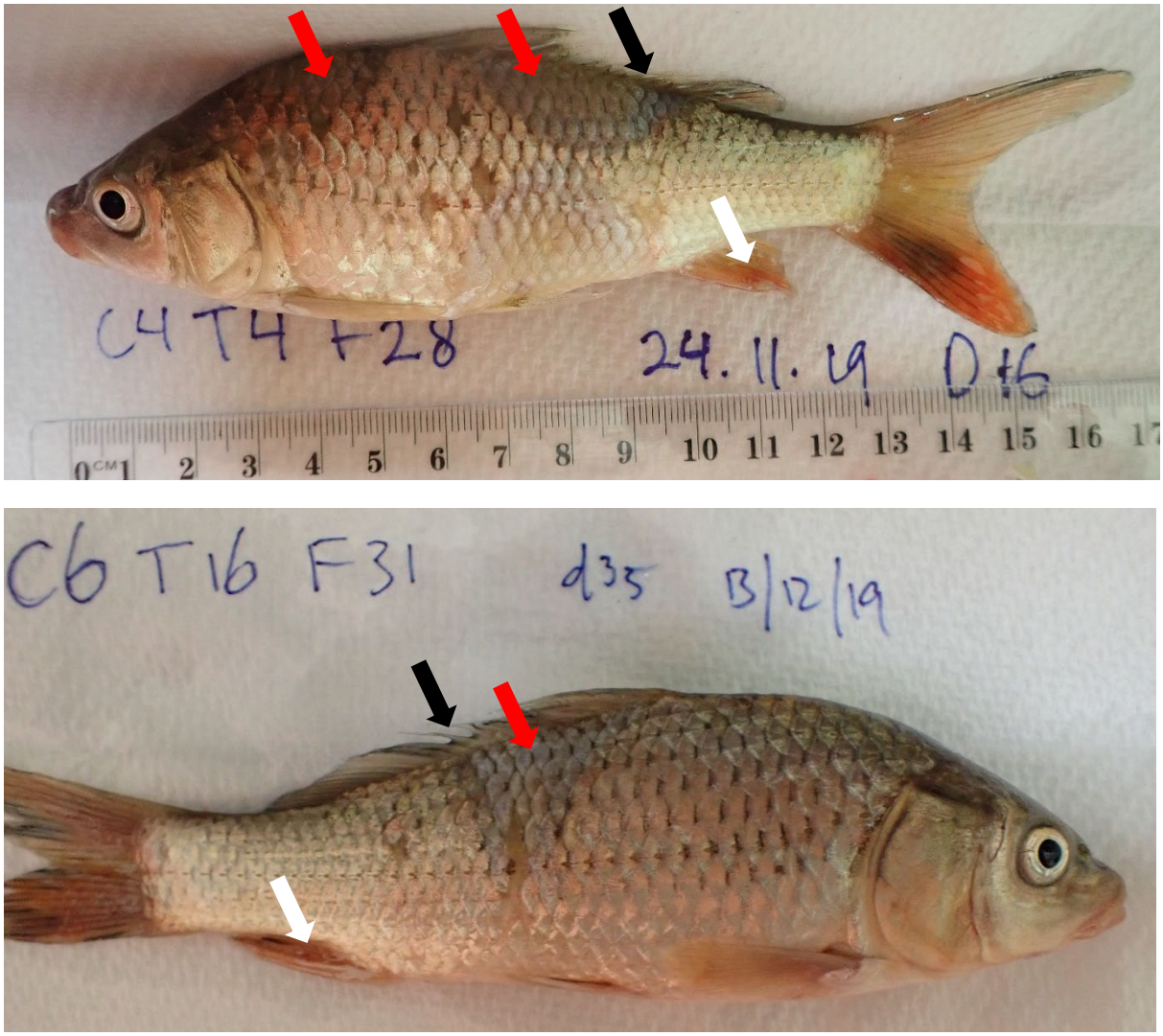

Figure 4. Clinical signs of CyHV-3 infected carp at 12°C (upper image) and 22°C (lower image). Note the reddened fin (white arrow) and blistered skin (red arrows), and fin rot (black arrow).

To confirm that carp were infected by the virus, sample of gill, kidney and skin lesion were collected for qPCR tests as they are major target tissues for CyHV-3 infection (Gilad et al., 2004). The results showed all diseased carp (dead or moribund), except carp from Tank 8 Fish 2 (T8F2) and T18F2, were qPCR positive, suggesting that the carp were indeed infected with the virus (Appendix 3). Carp in T8F2 (Group 2) showed exophthalmia (popeye) when euthanised for humane reason at 38 dpi and carp T18F2 (Group 3) showed dorsal fin rot and blistered skin when euthanised at 19 dpi. Carp euthanised when the experiment was terminated were either qPCR positive with high C_T value (average 34.04), suggesting low level of CyHV-3 infection, or qPCR negative (Appendix 3). In contrast, all diseased carp, except carp T8F2 and T18F2, were qPCR positive with low C_T value (average 24.47), suggesting high level of CyHV-3 infection. All gill and kidney samples collected from carp in the mock-infected negative control groups including those from fish T25F1 which died at 3dpi, without showing clinical signs of disease, were qPCR negative. Furthermore, the 18S qPCR results confirmed the integrity of the samples and the assays.

Comparative test results for detection of viral DNA and viral messenger RNA (mRNA) for diseased carp at water temperatures of 12°C from Group 1, in which morbidity occurred at low water temperatures, are provided in Table 4. Positive detections of mRNA by CyHV-3 mRNA RT-qPCR indicate the detection of viral mRNA, which is indicative of the presence of replicating infectious virus. The majority (33/37) of the carp in Table 4 were humanely killed due to the presence of moderate clinical signs of infection for 24 hours. Only samples from Group 1 were tested using the CyHV-3 mRNA RT-qPCR, as these samples were tested as part of the evaluation of this assay which was developed for FRDC 2019-176 "NCCP: Determination of the susceptibility of silver perch, Murray cod and rainbow trout to infection with CyHV-3" (Moody et al., 2021). Details of the CyHV-3 mRNA RT-qPCR are described in the Final Report for FRDC 2019-176.

Table 4. Comparative detection of CyHV-3 viral DNA (by qPCR) and CyHV-3 viral mRNA (by RT-qPCR) for diseased carp when water temperature was 12°C in Group 1. Numbers in the table are C_T values.

Day	Tissue	Tank 1		Tank 2		Tank 3		Tank 4		Tank 5		Tank 6	
		DNA	mRNA	DNA	mRNA	DNA	mRNA	DNA	mRNA	DNA	mRNA	DNA	mRNA
7	Gill	21.72	27.99										
	Kidney	19.31	25.30										
	Skin	22.50	31.35										
9	Gill							28.54	36.82				
	Kidney							26.46	33.22				
	Skin							30.90	ND				
10	Gill			29.07	35.70			34.73	ND				
	Kidney			24.91	29.43			28.96	35.24*				
	Skin			24.97	33.22			28.76	36.66				
11	Gill	26.38	31.01			32.50	ND	28.83	38.99*			22.57	27.42
	Kidney	24.07	29.71			27.37	33.95	27.62	32.16			20.97	26.25
	Skin	27.51	33.64			23.35	34.92	24.41	32.70			22.33	32.09
	Gill					28.30	35.12	23.23	28.37			19.65	27.43
	Kidney					24.55	31.34	21.34	26.71			15.92	23.37
	Skin					23.87	32.71	24.73	30.78			18.01	28.66
	Gill							31.57	ND			19.32	24.88
	Kidney							30.69	38.41*			14.26	20.65
	Skin							23.79	32.29			22.45	28.59
	Gill											20.79	25.85
	Kidney											17.40	22.96
	Skin											25.51	31.42
12	Gill							21.42	26.41				
	Kidney							14.49	24.59				
	Skin							21.56	ND				
13	Gill			27.60	ND	27.74	ND	29.85	ND	28.34	ND		
	Kidney			25.75	ND	28.40	ND	23.95	ND	23.82	ND		
	Skin			26.67	ND	25.88	ND	25.44	37.23	21.03	32.36		
	Gill					26.68	ND	31.14	ND				
	Kidney					27.08	ND	28.83	ND				
	Skin					25.42	ND	27.77	ND				
	Gill					33.25	ND	20.43	ND				
	Kidney					31.86	ND	21.21	34.13				
	Skin					27.11	ND	24.85	40.13				
14	Gill							28.15	ND				
	Kidney							26.43	ND				
	Skin							27.34	ND				
15	Gill					30.01	ND	20.31	ND	23.04	ND		
	Kidney					25.38	34.20	25.56	ND	21.69	ND		
	Skin					28.91	ND	31.36	ND	22.09	ND		

Table 4 (continued)

Day	Tissue	Tank 1		Tank 2		Tank 3		Tank 4		Tank 5		Tank 6	
		DNA	mRNA	DNA	mRNA	DNA	mRNA	DNA	mRNA	DNA	mRNA	DNA	mRNA
16	Gill							33.91	ND				
	Kidney							26.63	ND				
	Skin							28.06	ND				
	Gill							31.81	ND				
	Kidney							27.48	ND				
	Skin							30.26	ND				
	Gill							22.80	ND				
	Kidney							19.03	38.81				
	Skin							26.17	ND				
17	Gill					23.21	37.86	20.22	36.34				
	Kidney					23.60	ND	23.33	ND				
	Skin					26.80	ND	23.51	ND				
	Gill					22.04	ND						
	Kidney					17.27	34.10						
	Skin					27.71	ND						
18	Gill			29.81	ND	28.00	ND					27.22	ND
	Kidney			26.73	ND	26.15	ND					25.92	ND
	Skin			30.13	ND	25.85	ND					32.40	ND
20	Gill					29.07	ND						
	Kidney					25.05	ND						
	Skin					31.37	ND						
21	Gill									26.93	ND		
	Kidney									26.56	ND		
	Skin									24.01	ND		
	Gill									19.70	33.92		
	Kidney									20.46	39.43*		
	Skin									19.37	33.28		

Note: Day = day post injection; ND = not detected, samples tested with qPCR and RT-qPCR assays in duplicates, cycle threshold 0.1; * = C_T vale of single data. Red font highlights Tanks 3 and 4, in which high morbidity occurred at water temperatures of 12°C. Samples of gill, kidney, and skin from individual fish showing clinical signs of disease at water temperatures of 12°C from Group 1 were tested for viral DNA using the CyHV-3 qPCR (Gilad et al., 2004) and viral mRNA using the CyHV-3 mRNA RT-qPCR (Moody et al., 2021).

Discussion

In this study we sought to determine if there was experimental evidence to support an alternative release strategy for CyHV-3 through IP injection during the late autumn or the winter. This would take advantage of the absence of disease in infected carp when the water temperature was below the threshold of 16°C and allow outbreaks to occur when infected fish aggregate with non-infected ones during spring mating events when the temperature returned to the permissive range (16-28 °C). This would in turn facilitate rapid spread of the virus through direct skin-to-skin transmission and thus provide a more natural initiation of outbreaks than attempting to catch and infect fish during these spring aggregations.

As the research was highly exploratory - we are not aware of existing reports of low temperature IP injection of carp with CyHV-3 - we undertook the trial in relation to previous work we had performed wherein immersion was used to infect carp with CyHV-3 at 22°C and then the water temperature was lowered and subsequently raised (Sunarto et al., 2014). We also applied stress (via net capture) as a variable to assess if this might enhance the development of disease when the water temperature was in the permissive range of 16-28°C. Thus, we undertook four inter-related simultaneous experiments with water temperature conditions and changes which simulated late autumn and winter releases with and without spring stress.

Both of the winter release simulations, *viz*. IP injection at 12°C and then raising the temperature to 22°C after 14 days, resulted in a high rate of active infection, with 51.38% and 40.35% of fish displaying symptoms of CyHV-3 with and without capture the stress treatment respectively. Furthermore, these results agree with a previous experimental study in which five of six domestic Koi carp were infected by immersion at 13°C and subsequently developed disease when moved to tanks where the temperature was raised to 23°C (Gilad et al., 2003). Therefore, both experiments indicate that a winter release of CyHV-3 based on capturing carp, injecting them via the IP route with CyHV-3 and returning them to the water may be a practical method of initiating outbreaks in the subsequent spring.

Nevertheless, these results only established a proof-of-concept for a winter "Trojan carp" release, and it cannot be assumed that this will be effective in the field. This caution arises as our results only apply to the defined conditions of the experiment - specifically, the fact that: (a) the experiments used juvenile carp; and (b) the length of time the virus was in a dormant state at 12°C was only 14 days. To be an effective method of field release will require the injection of mature adults as only these will undertake the subsequent spring mating where enhanced transmission occurs via direct skin-to-skin contact. Likewise, to be cost-effective there needs to be an extended period in the winter when specialist teams can move throughout a catchment inoculating carp rather than an attempt to undertake this within a short 2-3 week window before the spring mating. It is actually this duration of activity of the IP infected virus that might be the most substantive impediment to the use of a Trojan carp release strategy, as in the experiments reported by Gilad et al. (2003), the Koi carp infected at 13°C and subsequently moved to the water temperature at 23°C developed disease when held for 30 days but not when held for 64 days post exposure. Both of these caveats suggest a logical follow-on study wherein adult carp are kept for 30, 60 and 90 days at 12°C following IP injection and an accompanying gradual rise in water temperature to 22°C. In this follow-on study it might also be useful to confirm that the inoculated carp also transmit the virus by undertaking co-habitation with susceptible carp when they are returned to the permissive range of water temperature.

Although the priority for follow-on research needs to be to establish that a Trojan carp release will be effective in the field, it would be beneficial to have a better understanding the actual mechanism of how the virus persists in carp following inoculation at low temperature. Currently, two mechanisms for persistence of CyHV-3 are proposed, *viz*. latency and dormancy. Latency refers to the capacity of viruses, particularly those infecting mammals and birds, to enter into a specific state within the nucleus of the infected cell wherein its genome forms a circular episome and very few viral genes and microRNAs are expressed (Cohen, 2020). Upon reactivation, the virus enters into a "lytic" state which results in a mature virus particle being produced and released from the cell. By contrast, dormancy refers to body

temperatures outside of the permissive range inducing in the virus a state wherein viral propagation is turned off and viral gene transcription is minimised allowing the virus to persist.

There is firmer evidence that dormancy occurs as a mechanism of CyHV-3 persistence than there is for latency. Using samples collected from the experiment undertaken by Sunarto et al. (2014) on the effect of cold water temperature on CyHV-3, Neave et al. (2017) undertook a RNA-seq analysis on the three phases, i.e. the "acute" phase following infection via immersion at 22°C, the "persistent" phase when they infected carp were held at 11°C and the "reactivation" phase when the water temperature was raised back to 22°C. During the persistent phase it was confirmed that gene expression continued at a low rate. By contrast although latency of CyHV-3 is often presumed to occur in CyHV-3, it is important to note that to date it has not been definitively proven. As McColl et al. (2018) notes much of the research which claims to demonstrate latency has relied on wild-caught carp that were collected from waters at non-permissive temperatures for CyHV-3, and then the fish were maintained at those non-permissive temperatures for experimental work. Thus, it is very likely that what was claimed to be latency might really have been dormancy. What is lacking therefore is a demonstration of latency occurring in carp at permissive temperature, ideally with demonstration of markers of latency such as nuclear episomes and expression of latency associated genes.

A further topic of potential research arising from our investigation relates to the unexpected finding of reactivation of the virus in some of the tanks which simulated the late autumn release. This reactivation was unexpected as this has not been reported in studies with comparable methodologies, *viz.* Gilad et al. (2003); St-Hilaire et al. (2005); Sunarto et al. (2014). As noted above, we hypothesise that this reactivation might have been caused by temporary increases in the water temperature when the electrical chillers were turned off to enable the daily changes in the tank water. What is unusual is that this temporary increase of water temperature above ~16°C possibly occurred in all tanks in the room of Group 1 where there were CyHV-3 detections during the cold-water phase and yet only two of the tanks were affected. Similarly, it is hard to explain why there were only morbidity during the cold-water treatment phase in only Group 1 and not in Group 2 which had the same water temperature treatments. It is important to note that due to the complexity of the overall study, the husbandry of the fish was carried out by team roster with 16 operators involved, and thus although both followed the same protocol, there might have been minor differences in its implementation, such as the duration of time the chillers were turned off during the daily water changes.

Potentially these unexplained cold-water reactivations pf CyHV-3 could be investigated by follow-on experiments in which the water temperature would be systematically varied around the 16°C threshold. However, whilst this might be of scientific value to further the understanding of the CyHV-3 reactivation process, it would be difficult to justify in terms of our overall objective of developing a pre-spring Trojan carp release strategy for the virus. Our series of four experiments indicate that the winter simulated release was considerably more efficient than the late autumn one in producing infected fish which can then potentially initiate local outbreaks during spring aggregations. For this reason, and because it more closely relates to the actual strategy, we recommend that only the winter inoculation design needs to progress to the next stage of determining the duration which the virus remains infective following intra-peritoneal injection.

Conclusion

The winter simulations demonstrate that infection at a non-permissive low temperature could result in a persistent infection, with subsequent reactivation when the temperature was raised above the permissive threshold. It thus provides some biological evidence that a Trojan carp phenomenon occurs which might form the basis of a winter release.

The late autumn simulations confirmed previous work that injection of the CyHV-3 in the permissive water temperature range, followed soon after by a reduction of water temperature below this range, prevented

disease if the low temperature was consistently maintained. However, it was indicated that transient temperature rises above the lower permissive range threshold might result in disease. Thus, a late autumn release might be less successful as virus excretion and mortalities might occur prior the fish aggregating in spring and therefore would not be able to effectively onward transmit the virus.

For the two groups exposed to the winter simulation, capture stress was shown to increase the proportion of fish expressing disease, but in the late autumn simulation the lack of any effect of stress is less certain due to the confounding effect of the disease during the period when the water temperature was nominally at 12°C. The unexpected appearance of disease in the low water temperature requires further investigation.

Implications

By demonstrating that infection of carp at low water temperature results in a persistent infection, which could then be reactivated when the temperature was raised above the permissive threshold, this laboratory experiment has provided initial proof-of-concept for an alternative "Trojan carp" release strategy based on capture and injection of fish over the winter period. However, further experimentation is required to confirm that infective virus will persist in carp over a period longer than the 14-days used in our trial, and thus permit injection of fish throughout the winter and not just immediately before the spring temperature rise.

Recommendations

Further research is recommended to confirm this initial proof-of-concept study.

Extension and Adoption

Consultation and communication have been restricted to discussions with the NCCP, both the leadership and the Scientific Advisory Group (SAG).

Project materials developed

Data generated in this project is being prepared for publication of a peer reviewed manuscript in a suitable journal for this field of study (to be determined).

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Appendices

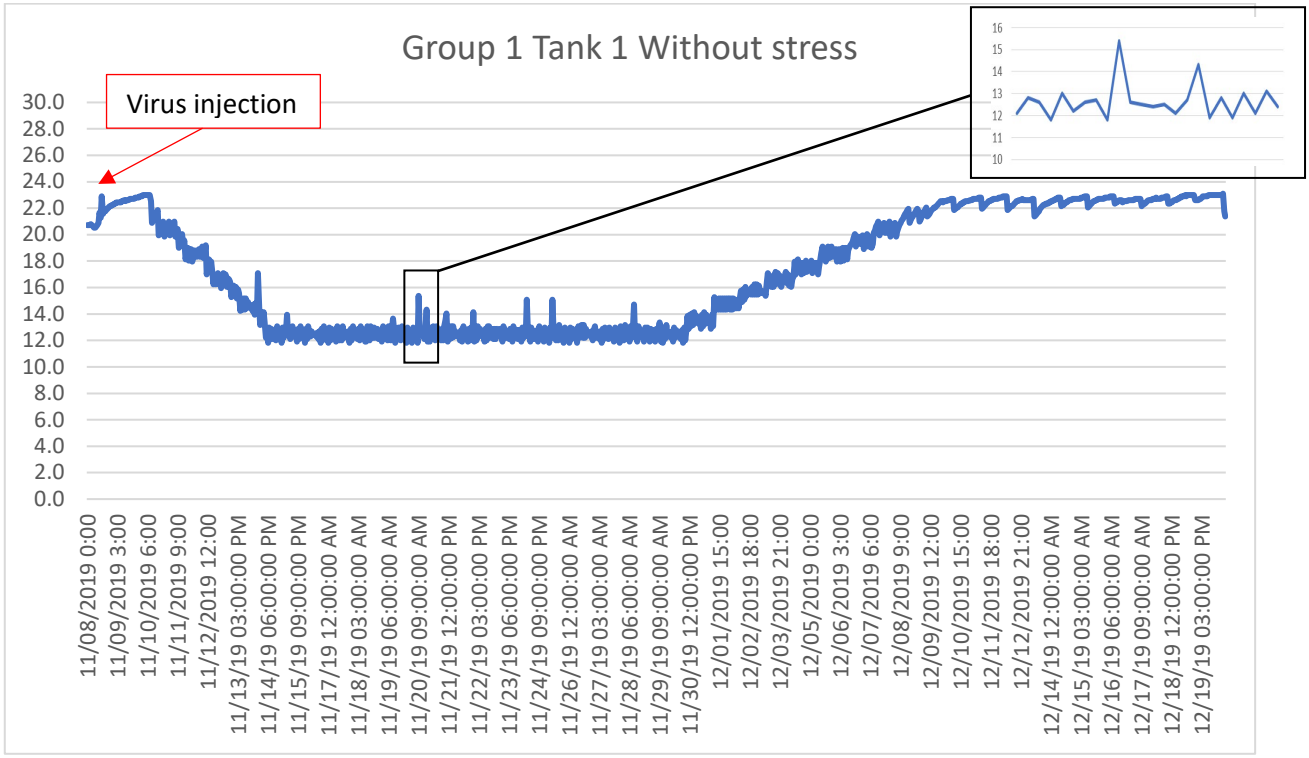
Appendix 1. Description of treatments and time series of events during the experimental trial

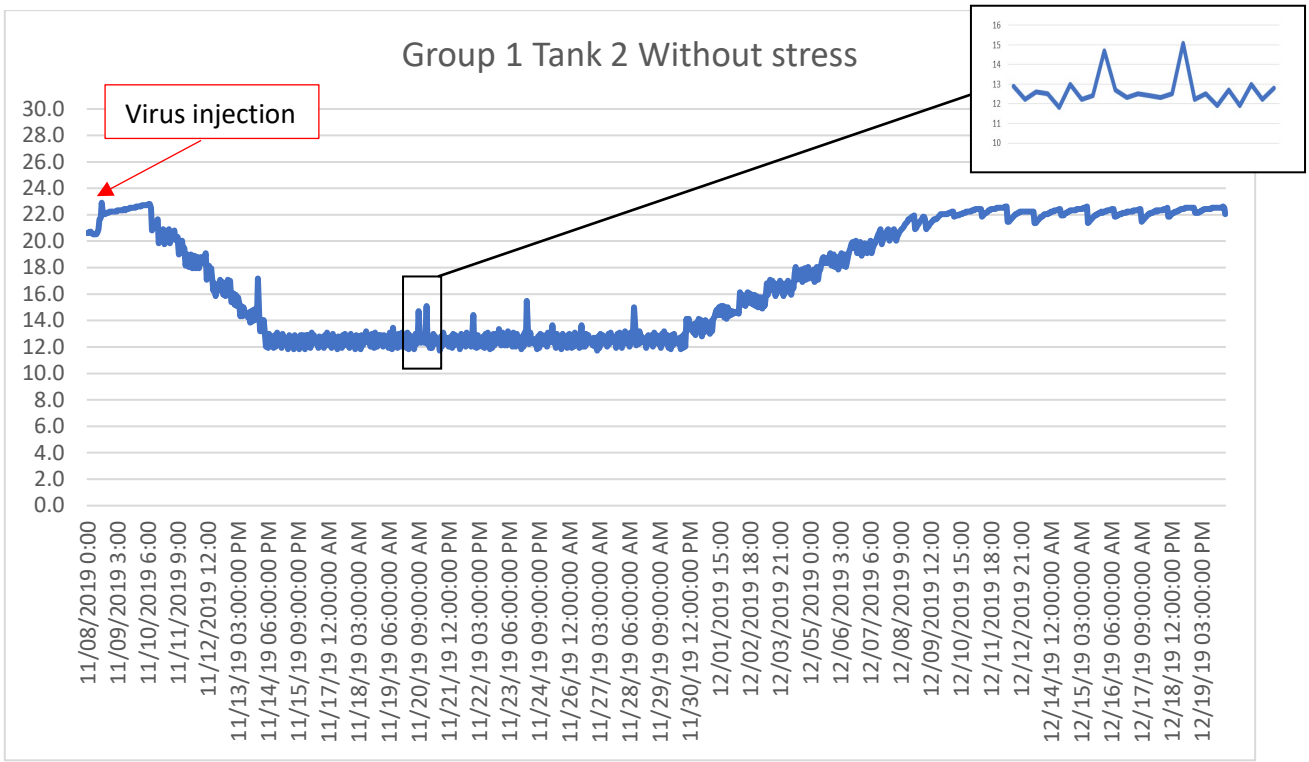
Day	Group 1 (Tank 1-6): Room C4	Group 2 (Tank 7-12): Room C5	Group 3 (Tank 13-18): Room C6	Group 4 (Tank 19-24): Room C7	Negative Controls (Tank 25 & 26): Room C8
Fish arrive (planned 24/10)	6 tanks with average 18 carp/tank	6 tanks with average 18 carp/tank	6 tanks with average 18 carp/tank	6 tanks with average 18 carp/tank	2 tanks with average 18 carp/tank
25/10/19	Tanks temperature set at 17°C based on advice from supplier				
26/10/19	Increase temperature by 2°C to 19°C				
27/10/19	Increase temperature by 2°C to 21°C				
28/10/19	Increase temperature by 2°C to 22°C				
29/10/19	Hold at 22°C				
30/10/19	Hold at 22°C				
31/10/19	Hold at 22°C				
Day 0	Intraperitoneally inject with CyHV-3	Intraperitoneally inject with CyHV-3	Nothing done	Nothing done	Intraperitoneally inject Tank 25 with media
Day 1	Hold at 22°C				
Day 2	Decrease temperature by 2°C to 20°C				
Day 3	Decrease temperature by 2°C to 18°C				
Day 4	Decrease temperature by 2°C to 16°C				
Day 5	Decrease temperature by 2°C to 14°C				
Day 6	Decrease temperature by 2°C to 12°C				
Day 7	Nothing done	Nothing done	Intraperitoneally inject with CyHV-3	Intraperitoneally inject with CyHV-3	Intraperitoneally inject Tank 26 with media
Day 8	Hold at 12°C				
Day 9	Hold at 12°C				
Day 10	Hold at 12°C				
Day 11	Hold at 12°C				
Day 12	Hold at 12°C				
Day 13	Hold at 12°C				
Day 14	Hold at 12°C				
Day 15	Hold at 12°C				
Day 16	Hold at 12°C				
Day 17	Hold at 12°C				
Day 18	Hold at 12°C				
Day 19	Hold at 12°C				
Day 20	Hold at 12°C				
Day 21	Hold at 12°C				
Day 22	Increase temperature by 1°C to 13°C				
Day 23	Increase temperature by 1°C to 14°C				

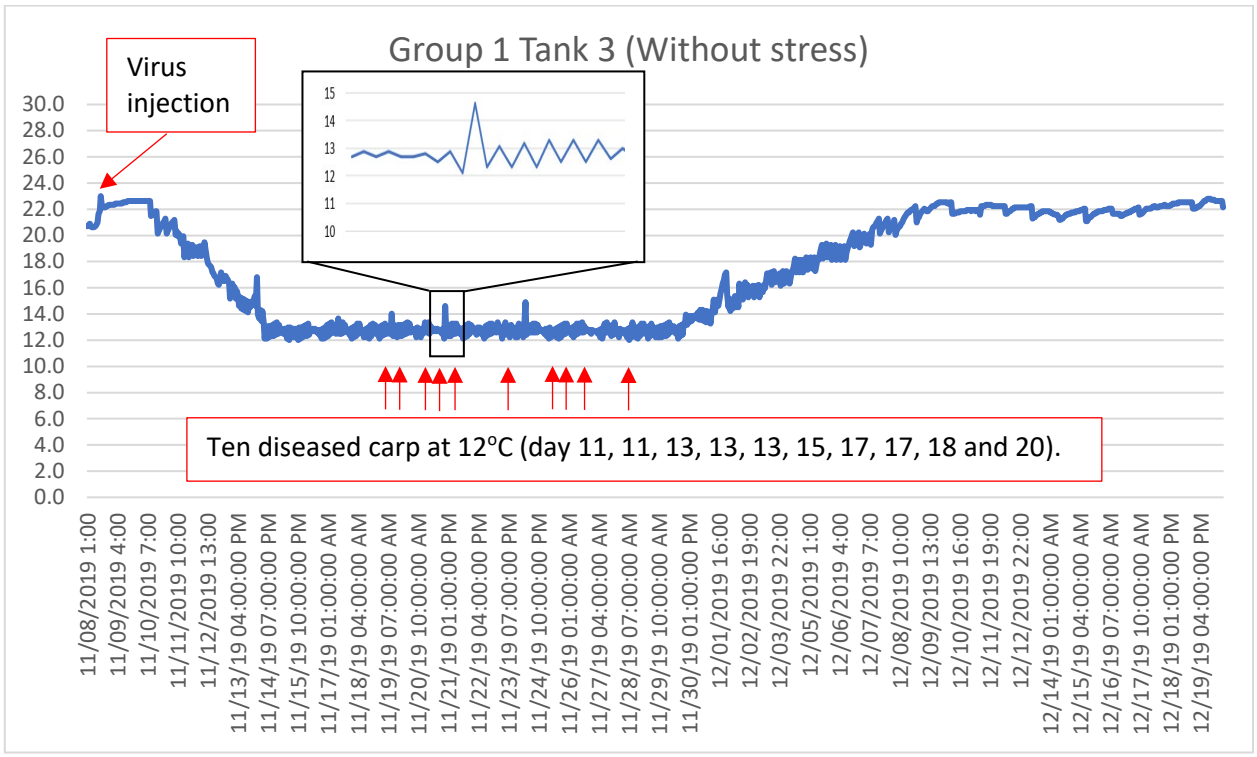
Day	Group 1 (Tank 1-6)	Group 2 (Tank 7-12)	Group 3 (Tank 13-18)	Group 4 (Tank 19-24)	Negative Controls (Tank 25 & 26)
Day 24	Increase temperature by 1°C to 15°C				
Day 25	Increase temperature by 1°C to 16°C				
Day 26	Increase temperature by 1°C to 17°C				
Day 27	Increase temperature by 1°C to 18°C				
Day 28	Increase temperature by 1°C to 19°C				
Day 29	Increase temperature by 1°C to 20°C				
Day 30	Increase temperature by 1°C to 21°C				
Day 31	Increase temperature by 1°C to 22°C				
Day 32	Hold at 22°C	Hold at 22°C Capture stress	Hold at 22°C	Hold at 22°C Capture stress	Hold at 22°C Capture stress in Tank 26
Day 33	Hold at 22°C	Hold at 22°C Capture stress	Hold at 22°C	Hold at 22°C Capture stress	Hold at 22°C Capture stress in Tank 26
Day 34	Hold at 22°C	Hold at 22°C Capture stress	Hold at 22°C	Hold at 22°C Capture stress	Hold at 22°C Capture stress in Tank 26
Day 35	Hold at 22°C	Hold at 22°C Capture stress	Hold at 22°C	Hold at 22°C Capture stress	Hold at 22°C Capture stress in Tank 26
Day 36	Hold at 22°C	Hold at 22°C Capture stress	Hold at 22°C	Hold at 22°C Capture stress	Hold at 22°C Capture stress in Tank 26
Day 37	Hold at 22°C	Hold at 22°C Capture stress	Hold at 22°C	Hold at 22°C Capture stress	Hold at 22°C Capture stress in Tank 26
Day 38	Hold at 22°C	Hold at 22°C Capture stress	Hold at 22°C	Hold at 22°C Capture stress	Hold at 22°C Capture stress in Tank 26
Day 39	Hold at 22°C	Hold at 22°C Capture stress	Hold at 22°C	Hold at 22°C Capture stress	Hold at 22°C Capture stress in Tank 26
Day 40	Hold at 22°C	Hold at 22°C Capture stress	Hold at 22°C	Hold at 22°C Capture stress	Hold at 22°C Capture stress in Tank 26
Day 41	Hold at 22°C	Hold at 22°C Capture stress	Hold at 22°C	Hold at 22°C Capture stress	Hold at 22°C Capture stress in Tank 26
Day 42	End of experiment	End of experiment	End of experiment	End of experiment	End of experiment

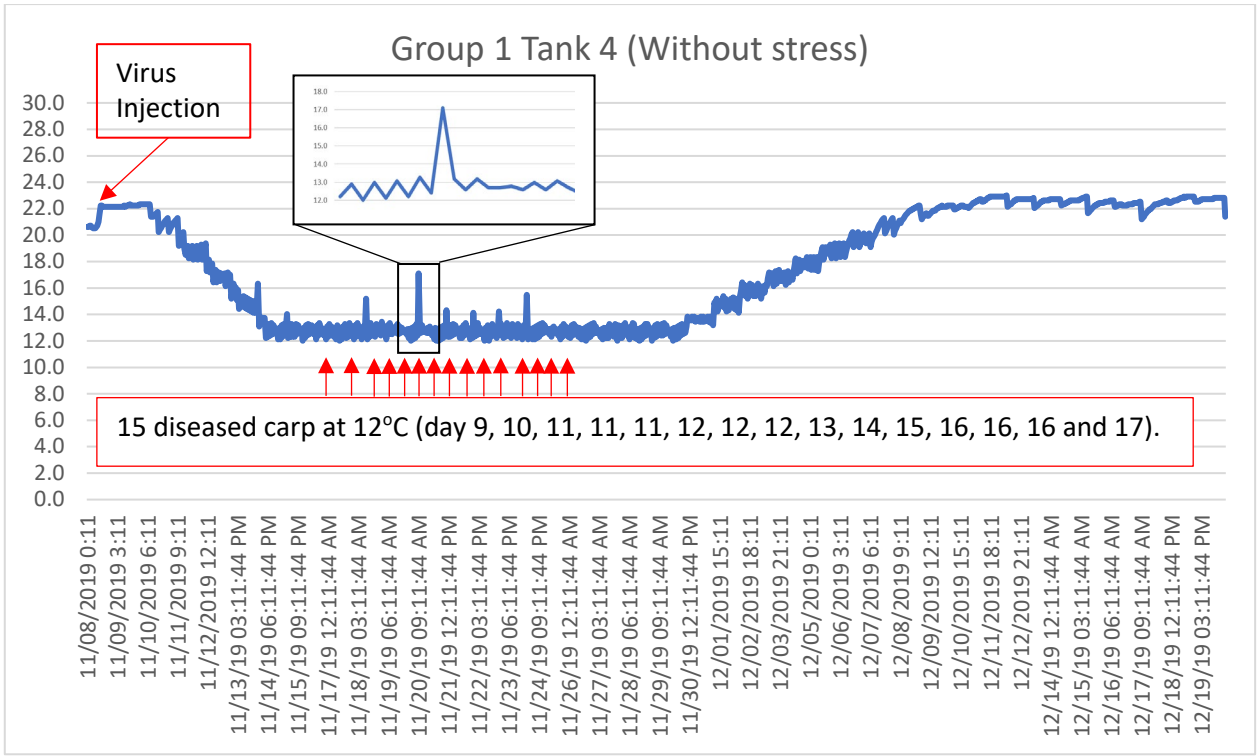
For all tanks in all rooms, husbandry and monitoring occur at 8am and 3pm, with an additional monitoring point occurring at 11pm when any clinical signs are observed.

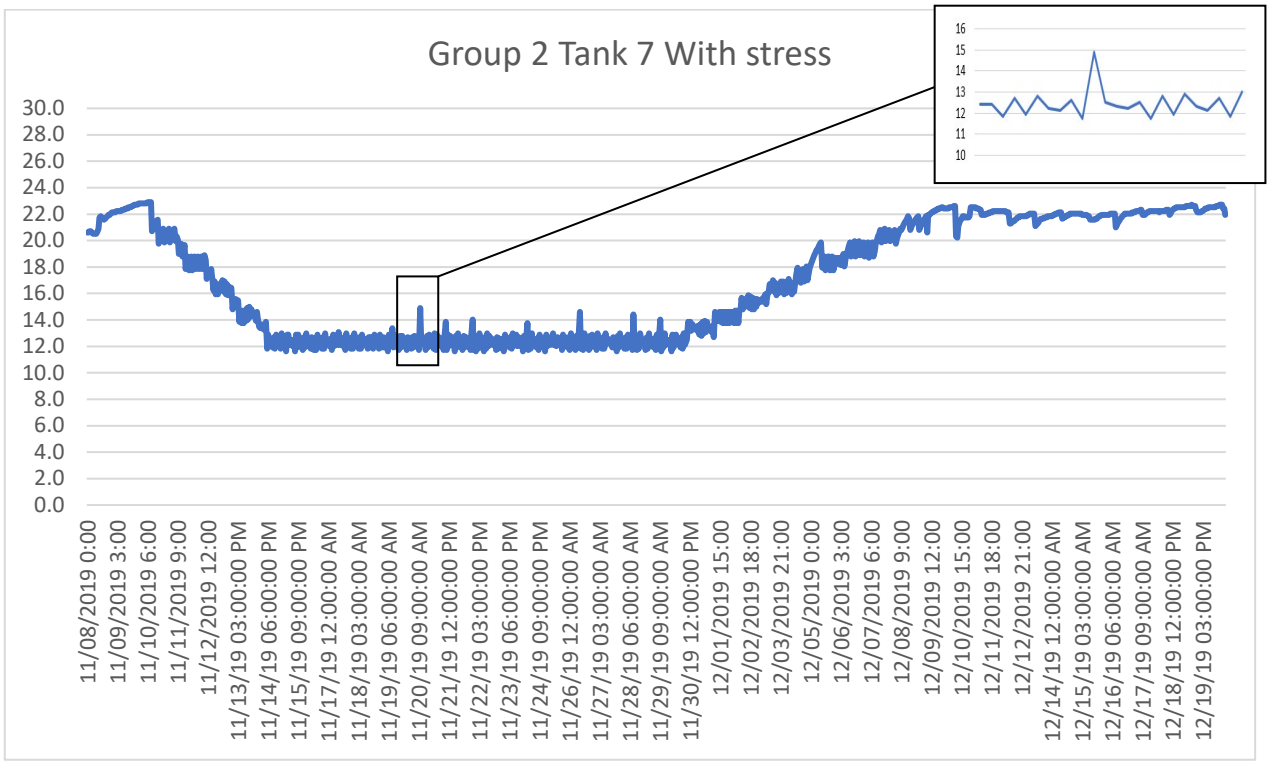
Appendix 2. Water temperature profiles. The insert window represents water temperature profile over 24 hours (00:00 – 24:00). Note the spikes at 08:00 and 15:00 during fish husbandry and observations.






Group 1 Tank 3 (Without stress)
Virus injection
Ten diseased carp at 12°C (day 11, 11, 13, 13, 13, 15, 17, 17, 18 and 20).


Group 1 Tank 4 (Without stress)
Virus Injection
15 diseased carp at 12°C (day 9, 10, 11, 11, 11, 12, 12, 12, 13, 14, 15, 16, 16, 16 and 17).


Group 2 Tank 7 With stress
30.0
28.0
26.0
24.0
22.0
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
11/08/2019 0:00
11/09/2019 3:00
11/10/2019 6:00
11/11/2019 9:00
11/12/2019 12:00
11/13/19 03:00:00 PM
11/14/19 06:00:00 PM
11/15/19 09:00:00 PM
11/17/19 12:00:00 AM
11/18/19 03:00:00 AM
11/19/19 06:00:00 AM
11/20/19 09:00:00 AM
11/21/19 12:00:00 PM
11/22/19 03:00:00 PM
11/23/19 06:00:00 PM
11/24/19 09:00:00 PM
11/26/19 12:00:00 AM
11/27/19 03:00:00 AM
11/28/19 06:00:00 AM
11/29/19 09:00:00 AM
11/30/19 12:00:00 PM
12/01/2019 15:00
12/02/2019 18:00
12/03/2019 21:00
12/05/2019 0:00
12/06/2019 3:00
12/07/2019 6:00
12/08/2019 9:00
12/09/2019 12:00
12/10/2019 15:00
12/11/2019 18:00
12/12/2019 21:00
12/14/19 12:00:00 AM
12/15/19 03:00:00 AM
12/16/19 06:00:00 AM
12/17/19 09:00:00 AM
12/18/19 12:00:00 PM
12/19/19 03:00:00 PM
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12
11
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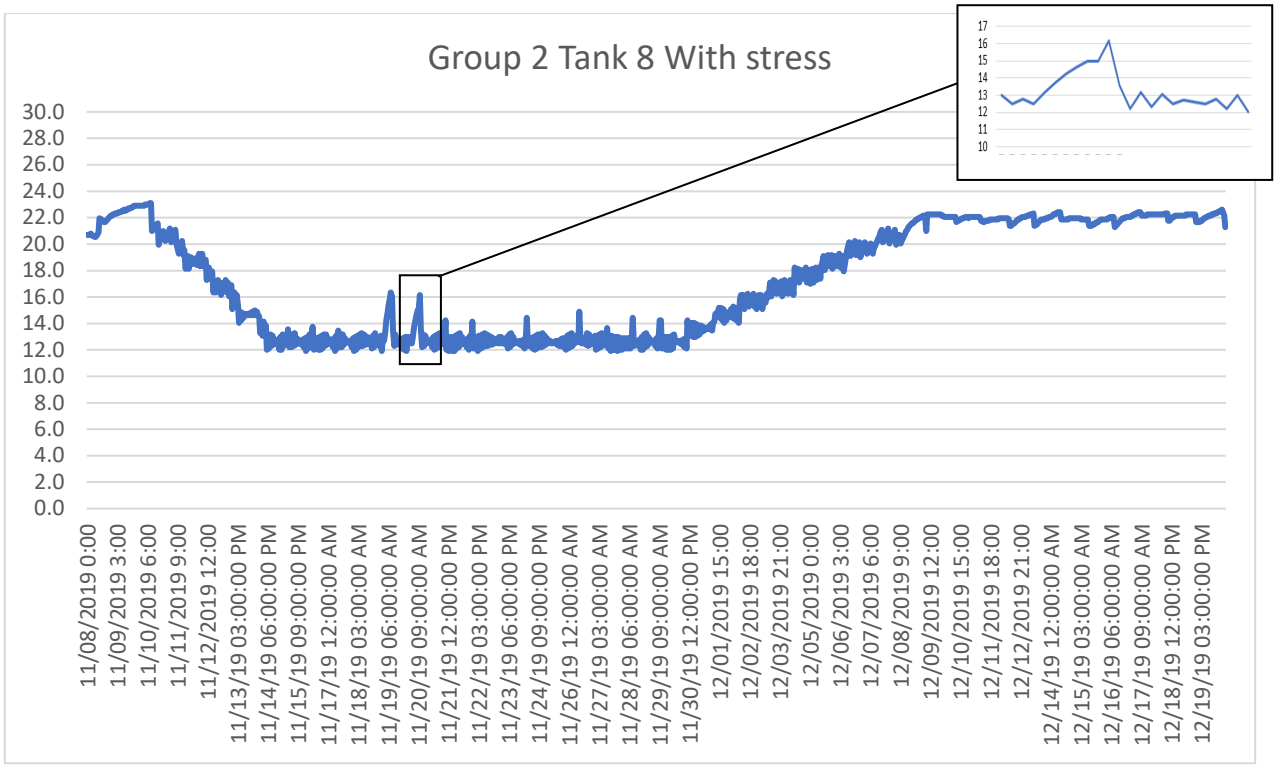
Group 2 Tank 8 With stress
30.0
28.0
26.0
24.0
22.0
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
11/08/2019 0:00
11/09/2019 3:00
11/10/2019 6:00
11/11/2019 9:00
11/12/2019 12:00
11/13/19 03:00:00 PM
11/14/19 06:00:00 PM
11/15/19 09:00:00 PM
11/17/19 12:00:00 AM
11/18/19 03:00:00 AM
11/19/19 06:00:00 AM
11/20/19 09:00:00 AM
11/21/19 12:00:00 PM
11/22/19 03:00:00 PM
11/23/19 06:00:00 PM
11/24/19 09:00:00 PM
11/26/19 12:00:00 AM
11/27/19 03:00:00 AM
11/28/19 06:00:00 AM
11/29/19 09:00:00 AM
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12/02/2019 18:00
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12/06/2019 3:00
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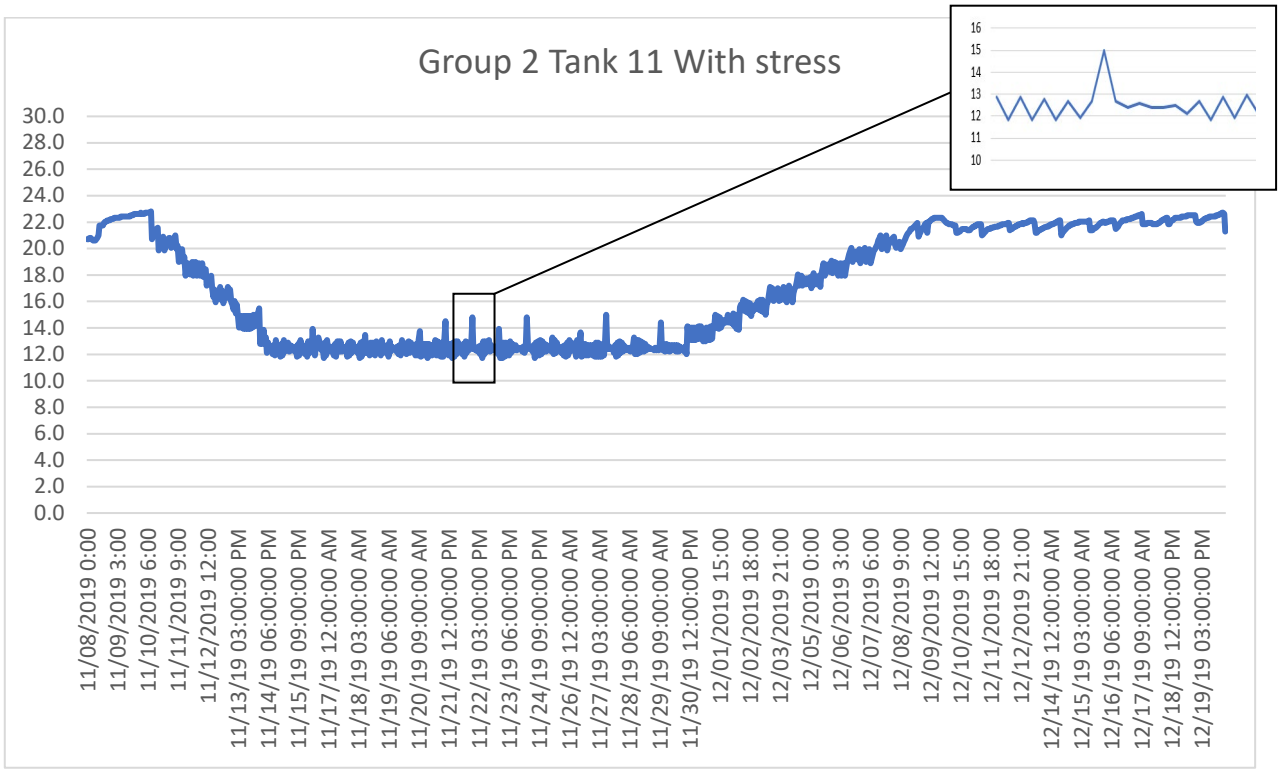
Group 2 Tank 11 With stress
30.0
28.0
26.0
24.0
22.0
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
11/08/2019 0:00
11/09/2019 3:00
11/10/2019 6:00
11/11/2019 9:00
11/12/2019 12:00
11/13/19 03:00:00 PM
11/14/19 06:00:00 PM
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11/19/19 06:00:00 AM
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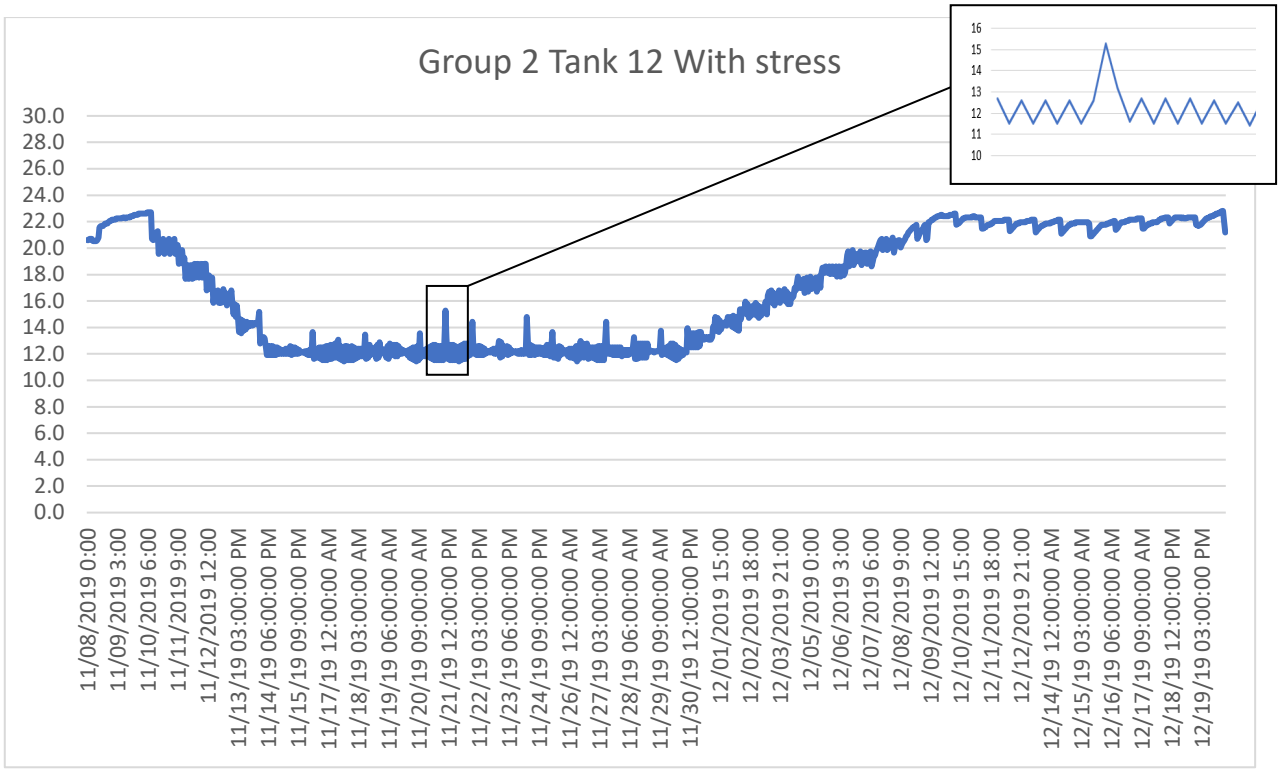
Group 2 Tank 12 With stress
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26.0
24.0
22.0
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
11/08/2019 0:00
11/09/2019 3:00
11/10/2019 6:00
11/11/2019 9:00
11/12/2019 12:00
11/13/19 03:00:00 PM
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11/15/19 09:00:00 PM
11/17/19 12:00:00 AM
11/18/19 03:00:00 AM
11/19/19 06:00:00 AM
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12/14/19 12:00:00 AM
12/15/19 03:00:00 AM
12/16/19 06:00:00 AM
12/17/19 09:00:00 AM
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Appendix 3. CyHV-3 qPCR results of gill, kidney and skin

Group 1										Fish #									
Tank 1	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Gill	21.72[a]	26.38	20.61	19.47	26.42	35.28	30.21	Und[c]	37.04[b]	37.50	34.45	35.63	32.85	35.18	29.03	29.24	35.18	26.27	
Kidney	19.31	24.07	16.41	22.93	23.31	28.26	25.94	Und	34.41	36.62	34.26	35.36	33.88	35.69	28.54	31.45	29.81	32.93	
Skin	22.50	27.51	19.20	24.42	25.58	28.93	29.17												
Average							**24.65[d]**	▲										**33.27[e]**	
Tank 2																			
Gill	29.07	27.60	29.81	14.65	27.18	24.60	33.56	38.32	Und	33.02	34.64	37.89	32.30	34.41	34.41	36.90	36.26	33.83	Und
Kidney	24.91	25.75	26.73	22.51	21.82	27.09	32.68	35.36	Und	35.83	37.06	37.20	36.64	33.36	32.12	35.52	37.20	31.41	37.19
Skin	24.97	26.67	30.13	21.55	30.14	28.51													
Average						**25.76**	▲												**34.13**
Tank 3																			
Gill	32.50	28.30	27.74	26.68	33.25	30.01	23.21	22.04	28.00	29.07	Und	35.80	Und	36.84	34.85	34.92			
Kidney	27.37	24.55	28.40	27.08	31.86	25.38	23.60	17.27	26.15	25.05	37.70	Und	36.26	34.96	32.60	34.58			
Skin	23.35	23.87	25.88	25.42	27.11	28.91	26.80	27.71	25.85	31.37	▲								
Average										**26.79**						**35.00**			
Tank 4																			
Gill	28.54	34.73	28.83	23.23	31.57	21.42	29.85	31.14	20.43	28.15	20.31	33.91	31.81	22.80	20.22	32.88	Und	Und	
Kidney	26.46	28.96	27.62	21.34	30.69	14.49	23.95	28.83	21.21	26.43	25.56	26.63	27.48	19.03	23.33	34.68	36.52	Und	
Skin	30.90	28.76	24.41	24.73	23.79	21.56	25.44	27.77	24.85	27.34	31.36	28.06	30.26	26.17	23.51	32.71			
Average															**26.17**		▲	**34.20**	
Tank 5																			
Gill	28.34	23.04	26.93	19.70	17.74	27.26	34.21	35.82	31.93	33.83	34.91	36.2	35.72	31.75	36.56	36.51	Und	35.43	34.19
Kidney	23.82	21.69	26.56	20.46	17.78	25.86	35.43	35.77	34.68	26.57	Und	37.24	36.72	24.99	34.57	Und	36.57	Und	31.02
Skin	21.03	22.09	24.01	19.37	20.90	27.02													
Average						**22.98**	▲												**34.12**
Tank 6																			
Gill	22.57	19.65	19.32	20.79	27.22	17.13	26.01	Und	35.62	Und	36.54	36.79	35.71	Und	36.54	35.91	37.36		
Kidney	20.97	15.92	14.26	17.40	25.92	16.99	33.00	Und	34.47	Und	37.33	36.38	36.67	Und	Und	33.99	36.97		
Skin	22.33	18.01	22.45	25.51	32.40	23.10	▲												
Average						**21.22**											**35.29**		

Group 2										Fish #								
Tank 7	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
Gill	16.20	16.54	21.67	16.07	25.70	31.82	Und	31.20	34.95	37.61	33.20	36.11	33.45	35.43	34.23	36.99	35.60	
Kidney	18.29	20.27	26.03	23.14	24.73	32.74	33.31	31.99	30.40	36.28	33.81	36.04	Und	33.70	32.49	Und	35.71	
Skin	20.73	23.48	27.14	23.63	23.45													
Average					**21.80**	▲											**34.15**	
Tank 8																		
Gill	29.01	Und[f]	Und	33.33	Und	Und	Und	33.80	35.62	Und	36.63	32.44	33.49	36.81	Und	Und	36.81	36.80
Kidney	26.20	Und	34.63	37.04	36.79	Und	Und	32.22	35.34	Und	37.28	32.29	33.57	Und	Und	35.99	Und	31.86
Skin	30.33																	
Average		**28.51**	▲															**34.88**
Tank 9																		
Gill	15.69	18.52	23.25	36.34	36.84	36.89	38.46	Und	Und	31.10	36.05	33.90	Und	Und	35.16	32.28		
Kidney	18.71	24.75	22.69	30.88	36.90	33.70	28.85	32.48	36.25	35.71	33.61	36.74	34.96	35.94	36.03	33.31		
Skin	17.83	25.56	28.27															
Average			**21.70**	▲												**34.65**		
Tank 10																		
Gill	19.32	37.10	Und	34.43	32.76	33.73	Und	36.62	Und	Und	Und	Und	Und	37.44	Und	34.86	Und	Und
Kidney	20.79	32.02	36.76	32.73	30.87	33.04	Und	36.03	32.28	33.48	Und	Und	34.10	Und	36.20	33.20	Und	36.70
Skin	25.38																	
Average	**21.83**	▲																**34.44**
Tank 11																		
Gill	32.27	32.18	26.59	33.93	35.25	36.64	31.46	Und	32.32	Und	Und	Und	Und	36.92	31.66	35.14	32.24	
Kidney	31.54	27.86	25.72	36.17	34.90	36.56	29.64	36.93	Und	Und	Und	34.97	35.80	Und	31.22	34.99	30.71	
Skin	30.58	27.74	29.86															
Average			**29.37**	▲													**34.08**	
Tank 12																		
Gill	21.93	20.54	30.55	34.80	35.58	34.60	36.28	Und	Und	36.46	36.42	Und	Und	35.39	Und	33.68	33.87	34.66
Kidney	19.81	26.36	28.76	31.66	35.73	32.87	35.84	37.42	35.76	Und	35.92	36.66	34.59	35.10	Und	28.90	33.44	29.66
Skin	23.76	24.73																
Average		**22.86**	▲															**34.18**

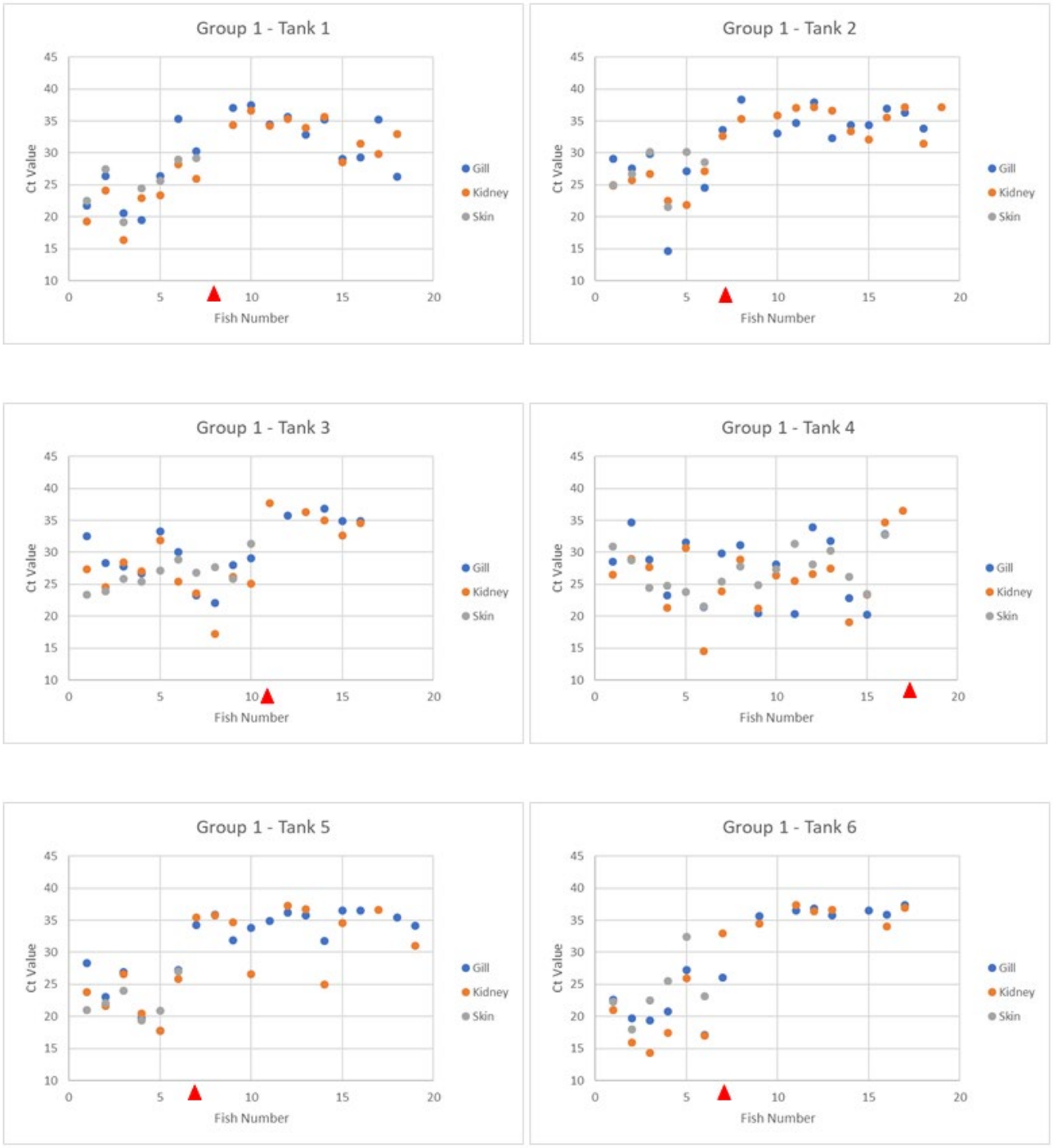
Group 3										Fish #											
Tank 13	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Gill	33.02	Und	18.44	19.78	18.55	21.39	30.92	33.71	34.97	34.65	35.38	Und	36.48	Und	32.76	Und	Und	36.30	32.11		
Kidney	26.24	34.40	18.24	23.55	25.14	18.86	29.45	29.87	34.13	25.63	31.99	28.35	30.71	36.82	27.89	37.83	Und	32.35	30.61		
Skin	25.85	32.80	23.82	28.26	27.87	25.18															
Average						**24.79**	▲												**32.52**		
Tank 14																					
Gill	31.42	15.61	16.67	20.49	20.21	25.93	34.98	36.21	35.62	37.35	36.40	32.72	30.92	29.76	32.85	35.55	36.37	33.39	Und		
Kidney	26.35	12.02	12.82	22.29	24.15	24.38	32.92	30.03	29.93	33.91	33.01	32.58	29.22	27.04	30.52	34.49	31.76	32.87	34.51		
Skin	22.00	17.70	19.27	25.15	21.49	24.32															
Average						**32.52**	▲												**33.00**		
Tank 15																					
Gill	20.55	17.74	23.04	20.97	32.53	27.81	34.74	37.04	35.20	29.44	35.14	36.02	29.99	36.44	Und	32.03	28.62	36.40	Und		
Kidney	17.69	18.16	28.05	25.06	25.38	29.36	35.09	36.94	29.92	31.90	35.11	30.60	33.76	36.38	Und	33.83	30.69	Und	Und		
Skin	19.31	21.55	28.24	24.20	32.88	28.04															
Average						**24.48**	▲												**33.59**		
Tank 16																					
Gill	33.22	19.06	16.85	28.24	26.63	27.49	20.12	22.55	33.30	36.58	34.71	36.34	34.05	34.85	33.03	Und	Und	32.19			
Kidney	31.10	15.77	15.56	20.62	19.87	23.09	24.28	22.99	Und	36.84	35.90	31.41	29.44	29.77	30.40	36.72	37.39	31.80			
Skin	35.91	23.24	20.16	23.68	27.07	26.15	23.37	28.44													
Average								**23.98**	▲									**33.81**			
Tank 17																					
Gill	13.95	16.59	20.41	24.33	22.75	21.72	22.04	17.06	29.30	32.76	37.30	34.43	35.40	34.57							
Kidney	12.68	13.78	18.99	25.41	19.56	19.69	19.76	24.24	23.67	31.73	35.58	30.78	35.59	36.37							
Skin	19.41	18.36	22.28	22.24	24.42	21.29	21.14	26.18	26.50	29.17											
Average										**22.05**	▲			**35.00**							
Tank 18																					
Gill	Und	Und[g]	26.39	16.18	17.30	18.43	17.28	27.65	27.88	27.97	Und	35.09	Und	31.27	35.94	30.19	Und	33.15	32.54	29.54	34.98
Kidney	35.64	Und	22.20	15.15	17.42	19.98	18.50	25.75	24.91	23.41	34.38	35.50	Und	32.40	34.20	27.28	Und	34.08	30.79	19.74	Und
Skin	36.85	Und	22.61	17.16	20.30	21.12	19.75	22.37	22.12	23.95											
Average										**22.63**	▲										**31.94**

Group 4										Fish #									
Tank 19	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Gill	27.16	19.43	27.83	28.10	23.57	27.43	Und	Und	36.59	35.90	36.69	Und	37.07	Und	Und	30.70	35.47	33.65	
Kidney	24.60	22.66	23.90	21.27	19.89	24.65	Und	36.59	Und	35.71	Und	36.52	30.40	36.53	Und	24.63	36.64	31.40	
Skin	24.82	23.82	22.52	24.47	20.55	28.23													
Average						**24.16**	▲											**34.30**	
Tank 20																			
Gill	19.27	22.42	24.17	23.98	27.17	28.53	29.15	37.57	Und	36.48	Und	33.95	33.06	35.61	Und	33.04	34.34	36.45	
Kidney	23.01	21.41	28.26	24.50	21.81	28.50	17.44	Und	35.16	Und	35.36	36.52	27.94	35.29	36.20	29.69	32.38	35.57	
Skin	25.70	22.57	26.15	23.95	22.17	32.89	25.88												
Average							**24.71**	▲										**34.39**	
Tank 21																			
Gill	18.36	24.71	21.49	23.85	21.61	26.30	21.35	26.05	22.02	19.35	23.81	22.83	Und	37.52	37.51	36.57	36.49	Und	Und
Kidney	16.57	25.42	19.84	23.61	23.93	24.89	25.02	23.54	29.19	23.12	26.38	26.99	Und	Und	Und	30.68	37.06	Und	36.59
Skin	23.69	22.03	21.45	21.72	23.91	21.25	29.36	24.56	24.26	21.10	22.91	26.03							
Average												**23.40**	▲						**36.06**
Tank 22																			
Gill	22.79	23.43	25.15	25.07	19.66	24.54	19.53	28.35	27.77	18.05	25.04	36.73	Und	Und	36.56	32.73	36.53	32.32	
Kidney	18.07	22.24	20.52	27.14	22.04	24.32	24.46	23.26	24.29	20.82	26.75	Und	Und	Und	33.30	31.50	Und	32.93	
Skin	23.25	24.65	24.47	24.96	21.70	21.99	29.02	25.35	27.24	22.76	25.58	35.91							
Average												**24.48**	▲					**33.70**	
Tank 23																			
Gill	15.46	19.60	18.35	24.68	27.58	19.81	21.34	Und	Und	31.56	Und	32.09	32.78	Und	34.98	31.91	29.91		
Kidney	13.32	22.30	16.65	25.94	26.31	24.87	21.72	Und	Und	32.51	Und	29.70	36.86	Und	Und	30.65	28.45		
Skin	15.36	18.72	17.58	21.58	22.57	27.47	26.78												
Average							**21.33**	▲									**31.94**		
Tank 24																			
Gill	29.00	21.83	22.27	26.65	20.93	16.06	24.47	23.64	31.79	30.16	29.07	34.81	36.11	31.20	31.18	35.57	34.84	34.38	32.40
Kidney	28.45	21.81	23.88	26.07	26.31	21.35	24.78	24.15	23.56	27.59	27.21	36.67	36.17	35.77	36.50	33.07	30.02	35.98	33.13
Skin	25.05	20.57	22.45	23.71	23.21	21.03	24.81	24.19	22.60	25.84	24.00								
Average											**24.50**	▲							**34.24**

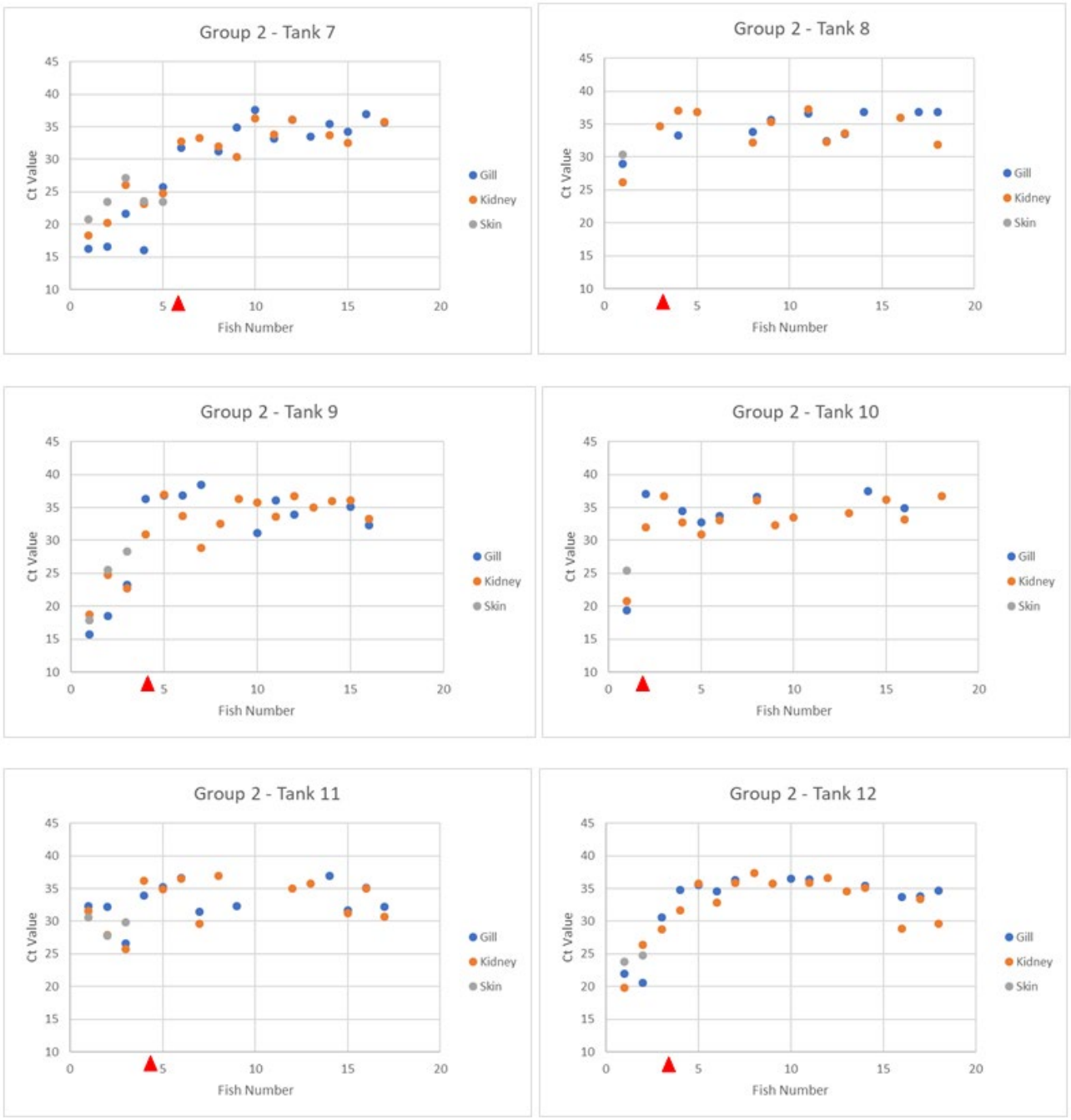
Negative Control Group

	Fish #																	
Tank 25	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
Gill	Und[h]	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und
Kidney	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und
		▲																
Tank 26																		
Gill	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und
Kidney	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und	Und
	▲																	

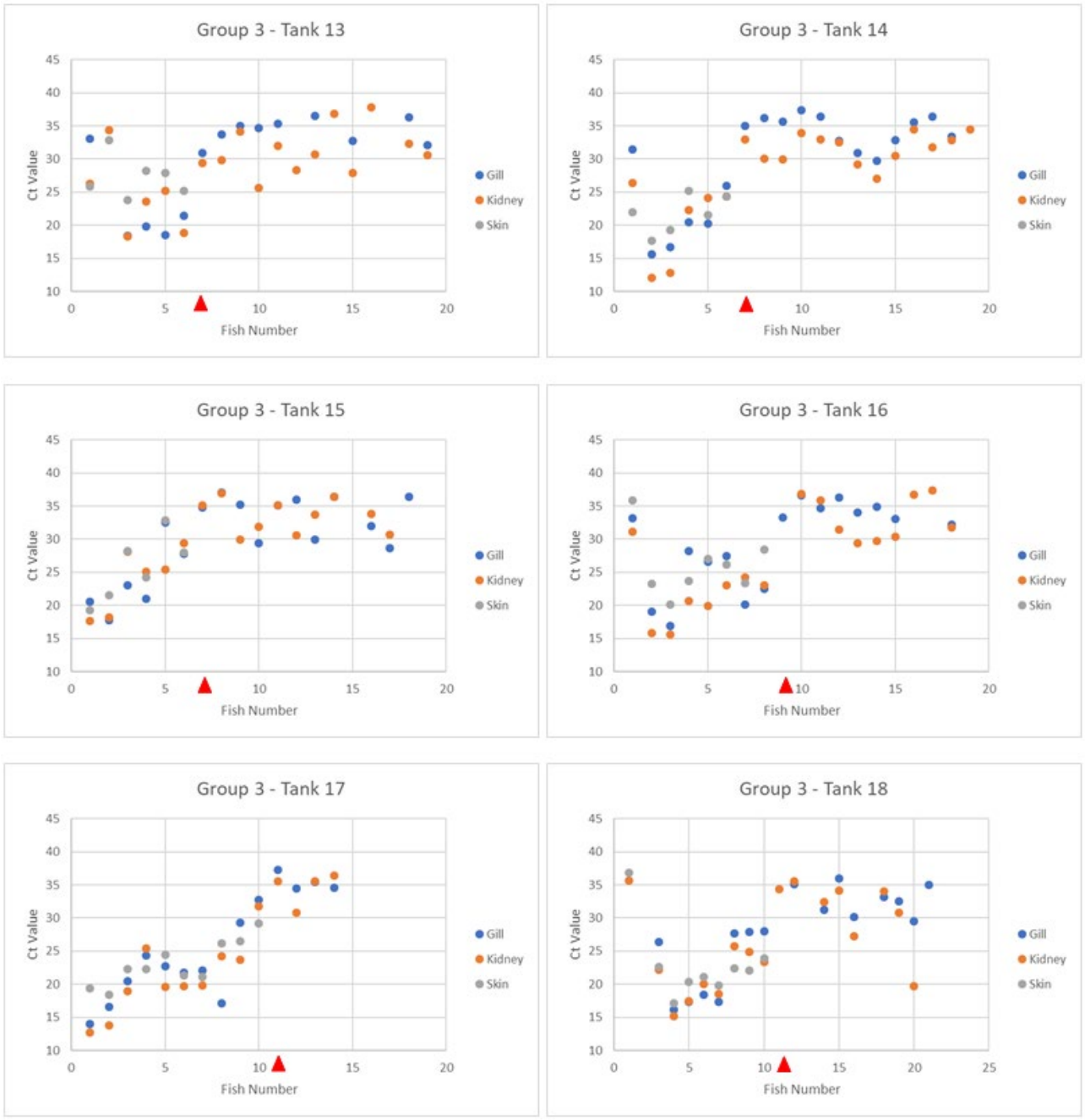
[a] Black font: C_T value mean of duplicates.
[b] Red font: C_T value of single data.
[c] Und: undetermined (not detected).
[d] C_T value average of diseased fish (dead and moribund).
[e] C_T value average of surviving fish euthanised in the end of the experiment in which no skin sample was collected.
[f] Carp T8F2 showed exophthalmia (popeye) when euthanised for humane reason at 38 dpi.
[g] Carp T18F2 showed dorsal fin rot and blistered skin when euthanised at 19 dpi.
[h] Carp T25F1 died at 3dpi without showing clinical signs of disease.
▲ Red triangle represents the termination of the experiment, in which all surviving fish were euthanised and tested.



Appendix 3 Figure A. Viral DNA load in gill, kidney and skin of CyHV-3-infected carp of Group 1. The data presented are the means of duplicate measurements of each sample. Red triangle represents the termination of the experiment, in which all surviving carp were euthanised. Note the lower the C_T value the greater the number of viral DNA copies and the higher likely concentration of the virus in the samples. Also note the carp euthanised at the termination of the experiment were either qPCR positive with high C_T value (average 34.04) or qPCR negative.



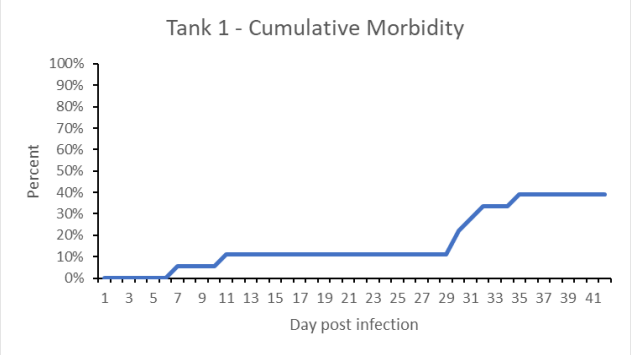
Appendix 3 Figure B. Viral DNA load in gill, kidney and skin of CyHV-3-infected carp of Group 2. The data presented are the means of duplicate measurements of each sample. Red triangle represents the termination of the experiment, in which all surviving carp were euthanised. Note the lower the CT value the higher the concentration of the virus in the samples, and the carp euthanised at the termination of the experiment were either qPCR positive with high CT value (average 34.04) or qPCR negative.

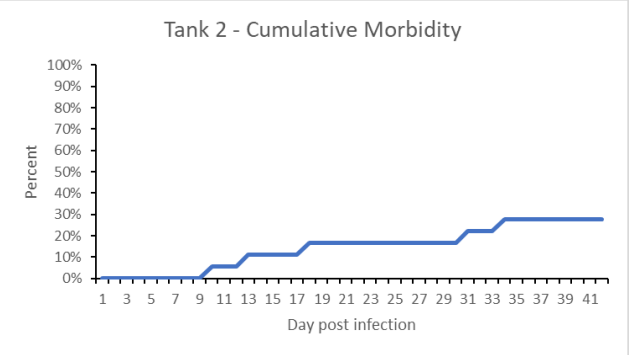


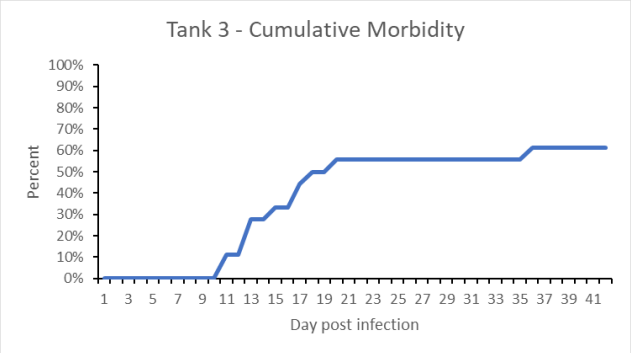
Appendix 3 Figure C. Viral DNA load in gill, kidney and skin of CyHV-3-infected carp of Group 3. The data presented are the means of duplicate measurements of each sample. Red triangle represents the termination of the experiment, in which all surviving carp were euthanised. Note the lower the CT value the higher the concentration of the virus in the samples, and the carp euthanised at the termination of the experiment were either qPCR positive with high CT value (average 34.04) or qPCR negative.

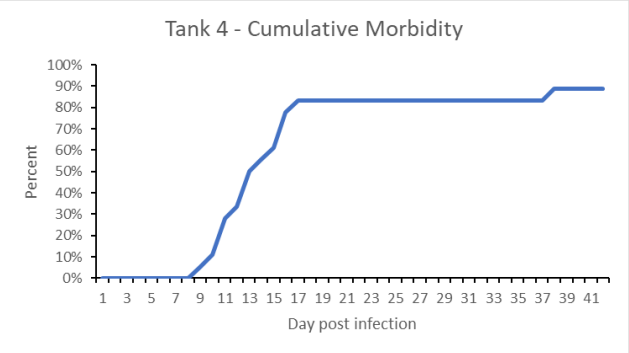
Appendix 3 Figure D. Viral DNA load in gill, kidney and skin of CyHV-3-infected carp of Group 4. The data presented are the means of duplicate measurements of each sample. Red triangle represents the termination of the experiment, in which all surviving carp were euthanised. Note the lower the CT value the higher the concentration of the virus in the samples, and the carp euthanised at the termination of the experiment were either qPCR positive with high CT value (average 34.04) or qPCR negative.

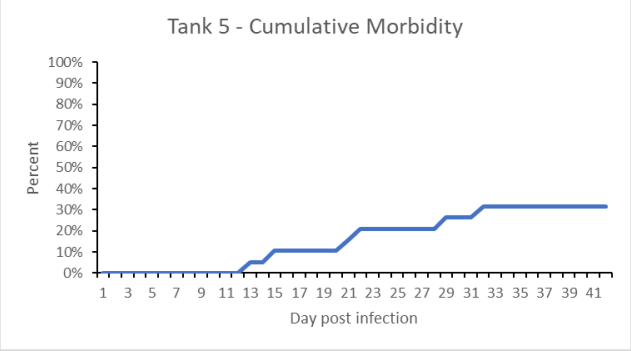
Appendix 4. Cumulative and daily morbidity of carp in individual tanks following CyHV-3 infection

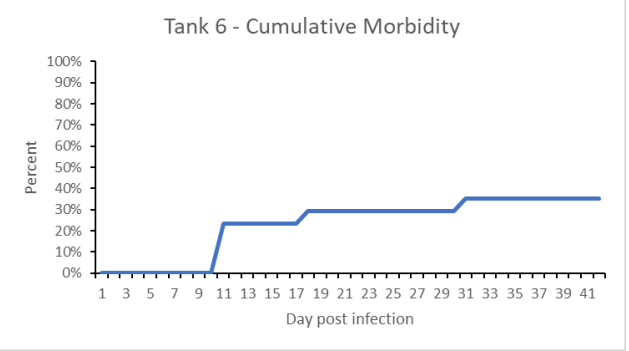




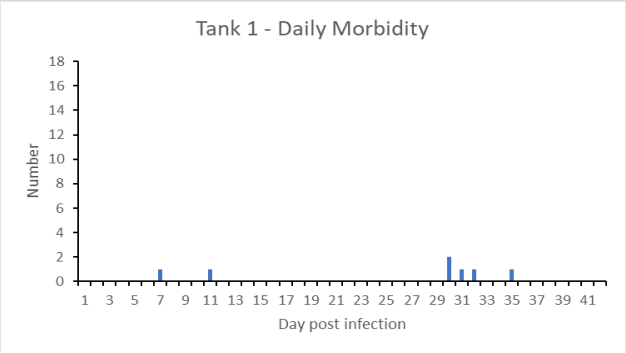


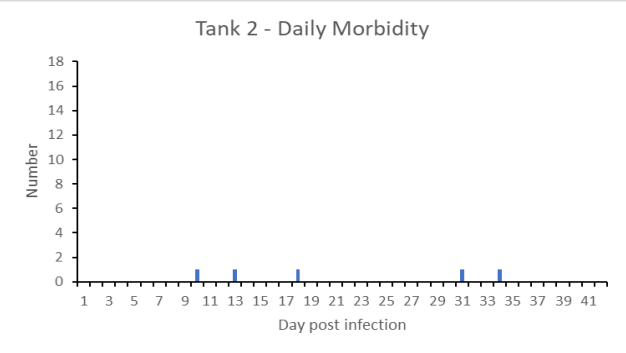


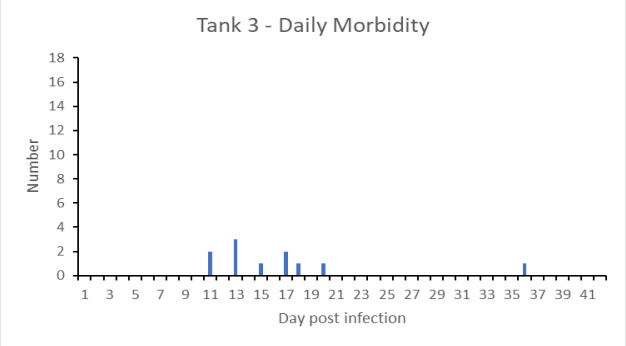


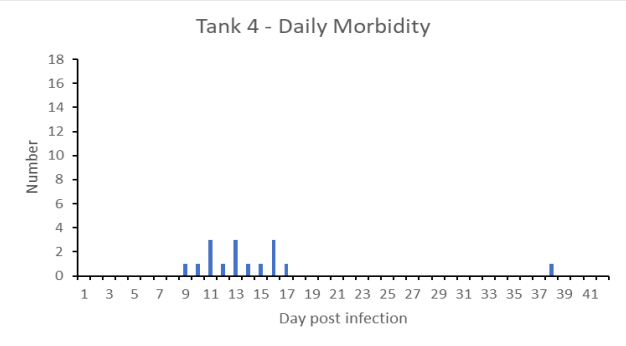


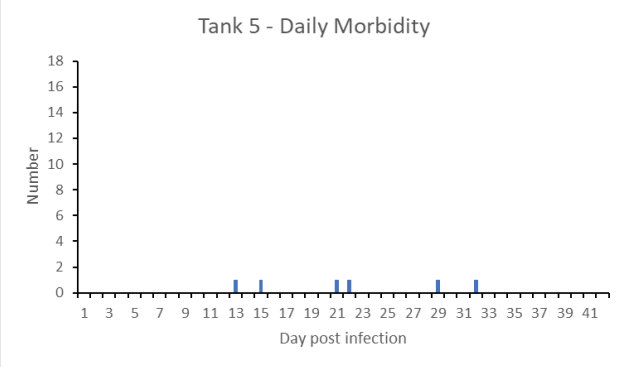
Cumulative morbidity of carp in Group 1, in which fish were injected with CyHV-3 at 22oC and held at 12oC for 14 days before the water temperature returned to 22oC. Fish in this group did not receive capture stress throughout the course of the experiment.

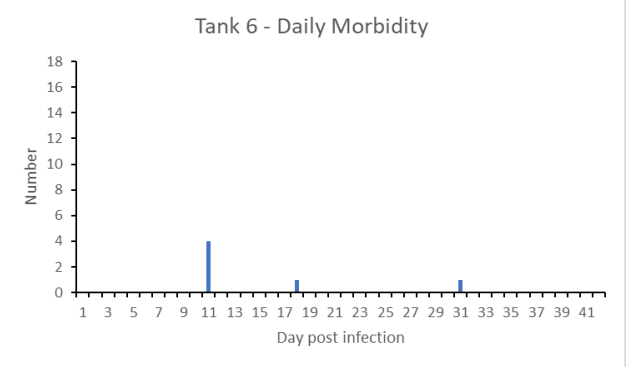




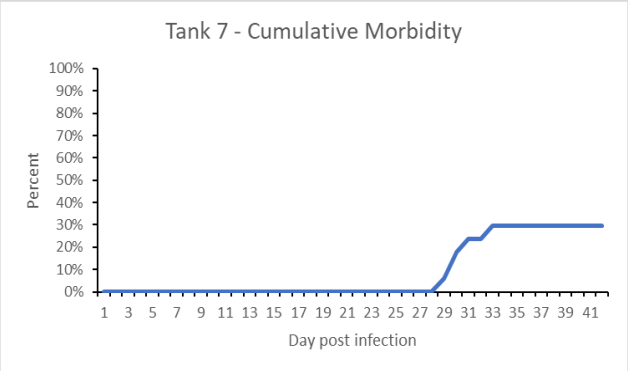


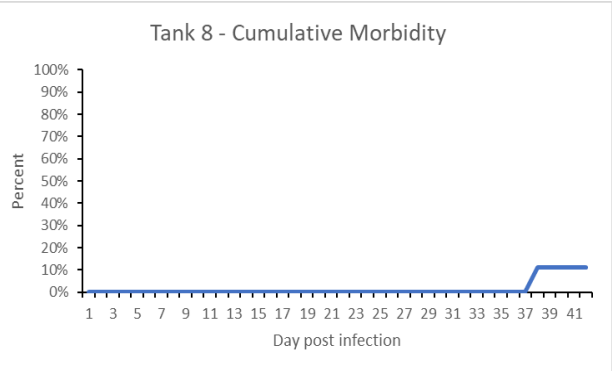


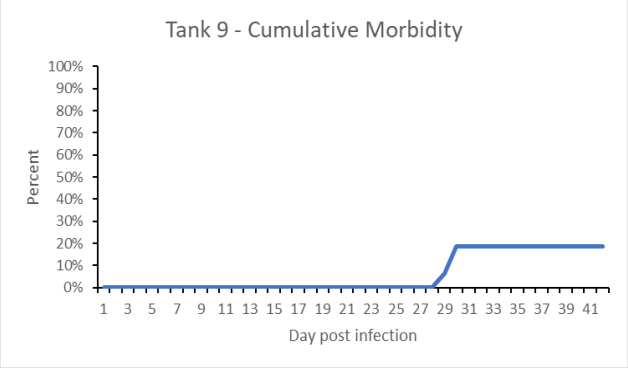


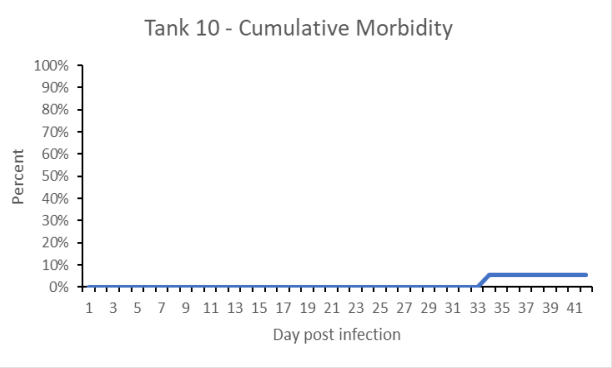


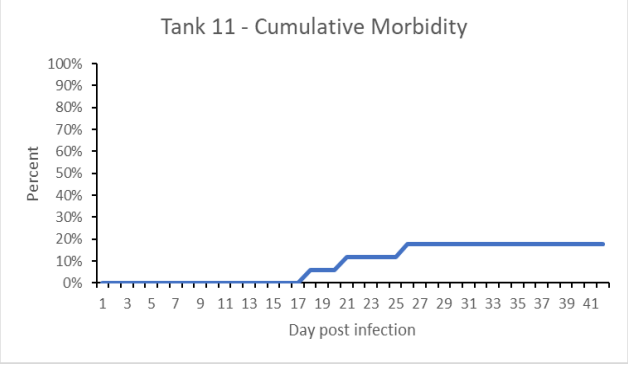
Daily morbidity of carp in Group 1, in which fish were injected with CyHV-3 at 22oC and held at 12oC for 14 days before the water temperature returned to 22oC. Fish in this group did not receive capture stress throughout the course of the experiment.

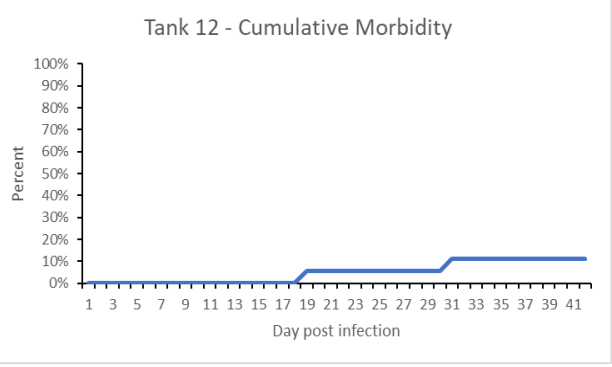




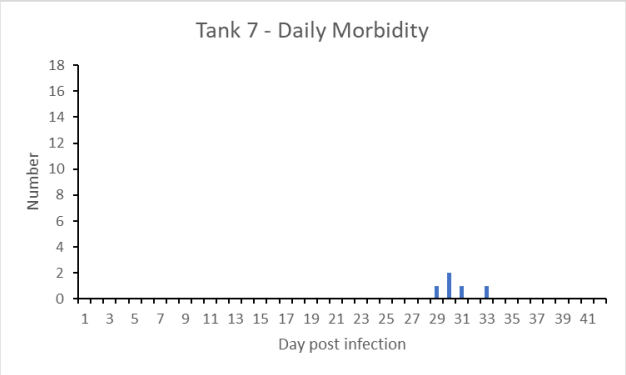


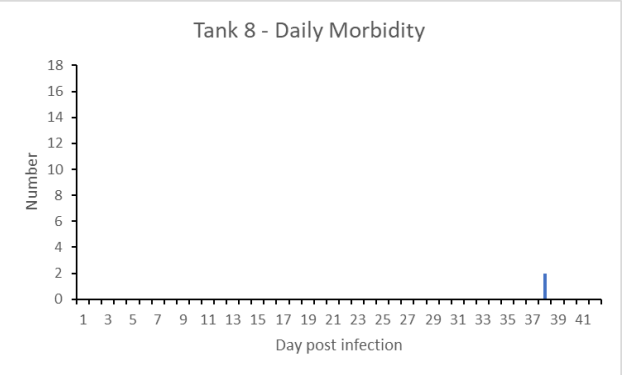


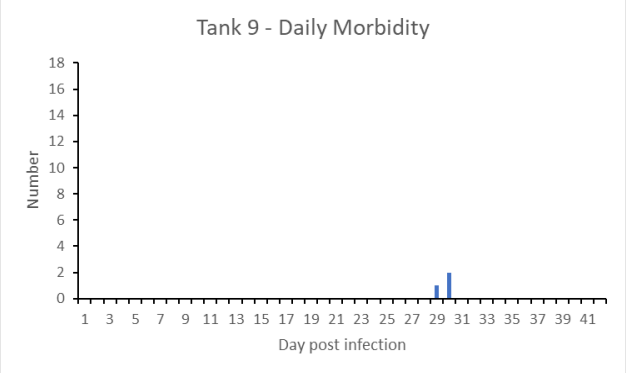


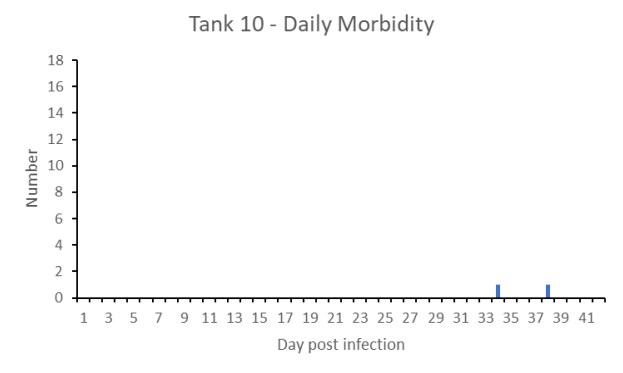


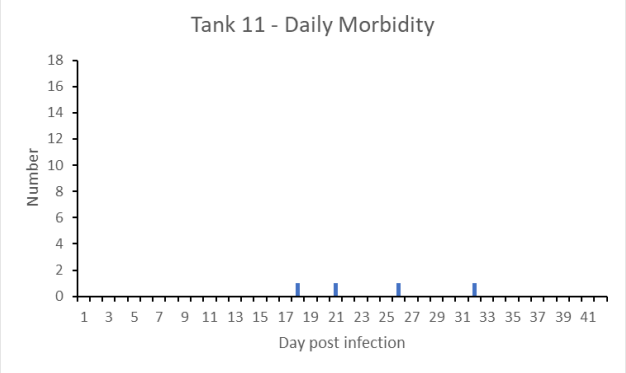
Cumulative morbidity of carp in Group 2, in which fish were injected with CyHV-3 at 22oC and held at 12oC for 14 days before the water temperature returned to 22oC. Capture stress was commenced once water temperature reached 22oC for 10 days until the experiment terminated on day 42.

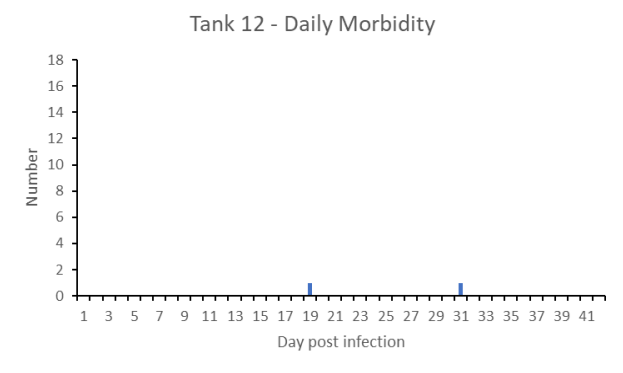




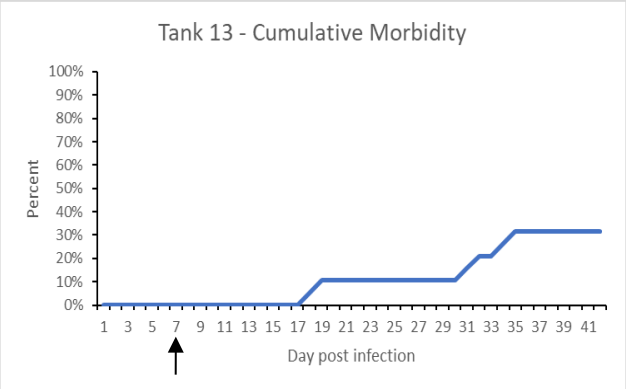


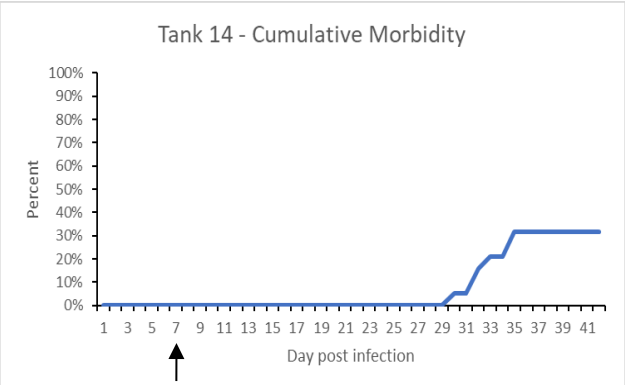


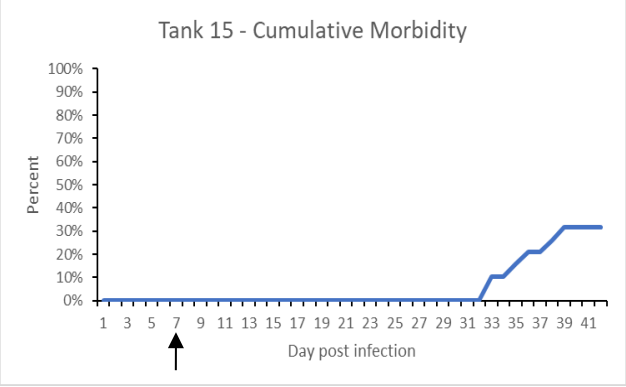


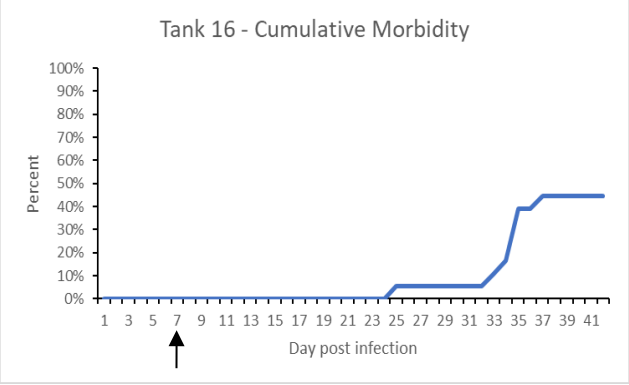


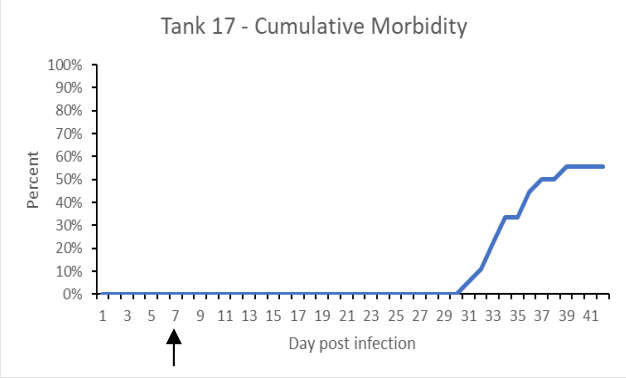
Daily morbidity of carp in Group 2, in which fish were injected with CyHV-3 at 22oC and held at 12oC for 14 days before the water temperature returned to 22oC. Capture stress was commenced once water temperature reached 22oC for 10 days until the experiment terminated on day 42.

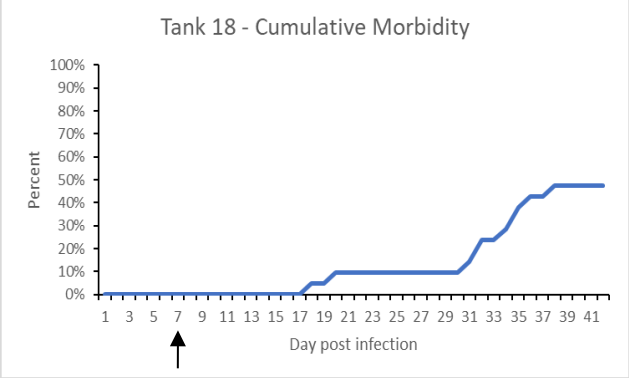




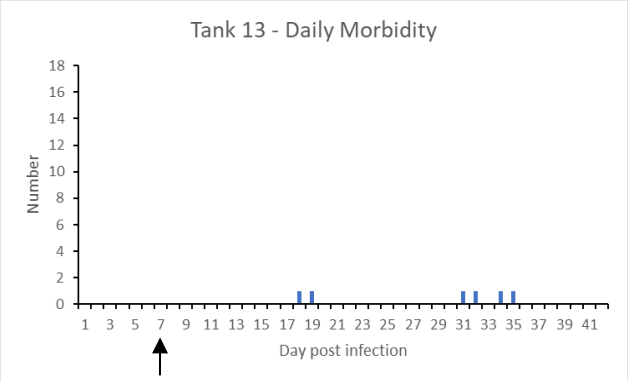


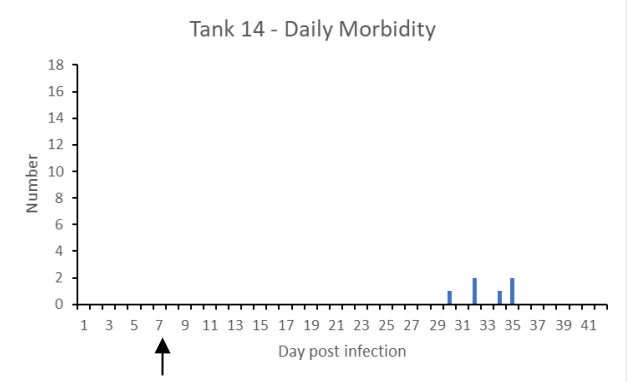


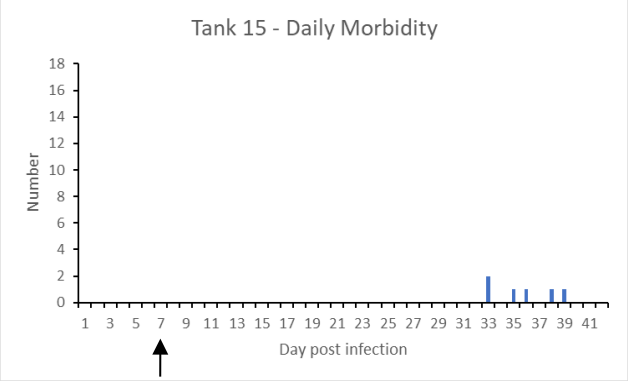


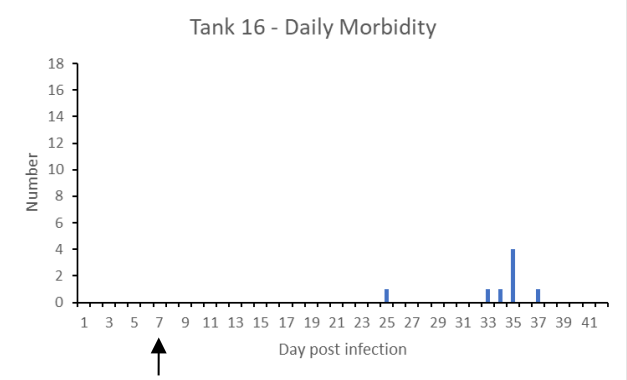


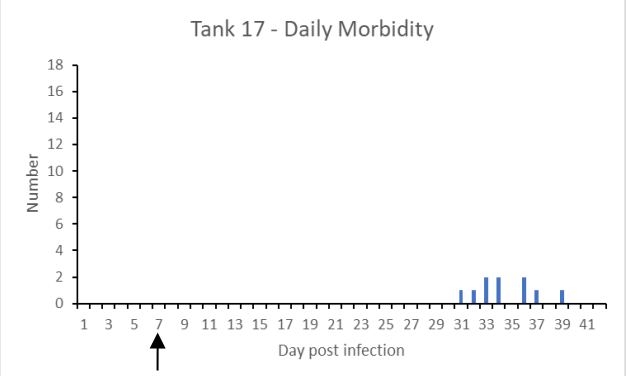
Cumulative morbidity of carp in Group 3, in which fish were injected with CyHV-3 at 12oC and held at this temperature for 14 days before the water temperature returned to 22oC. Arrow indicates day of inoculation. Fish in this group did not receive capture stress throughout the course of the experiment.

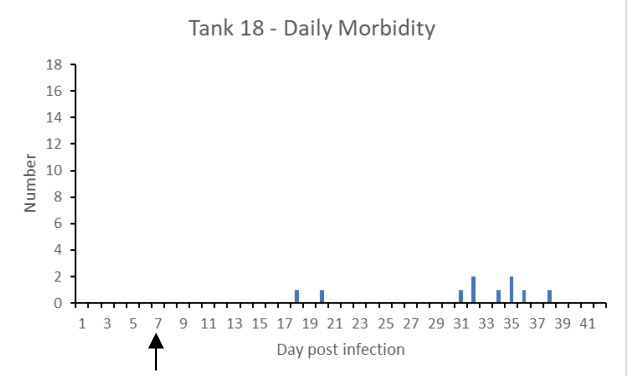




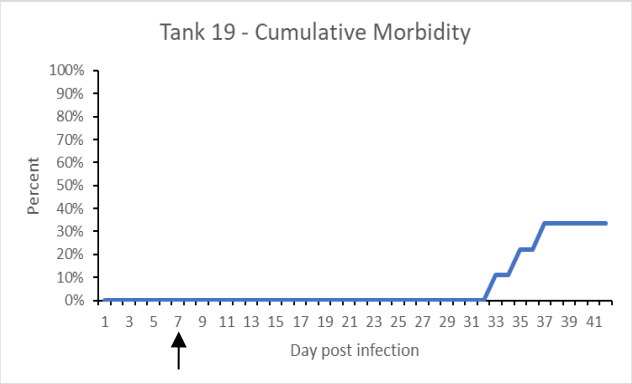


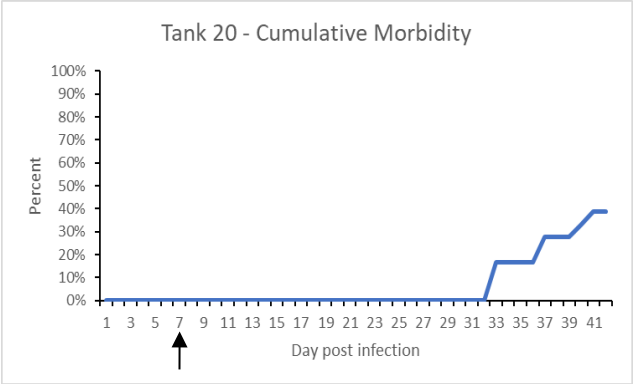


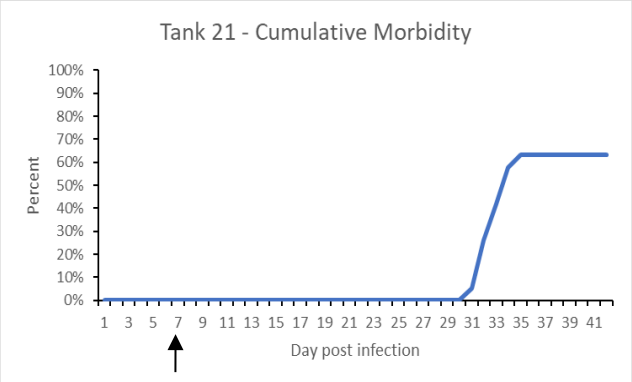


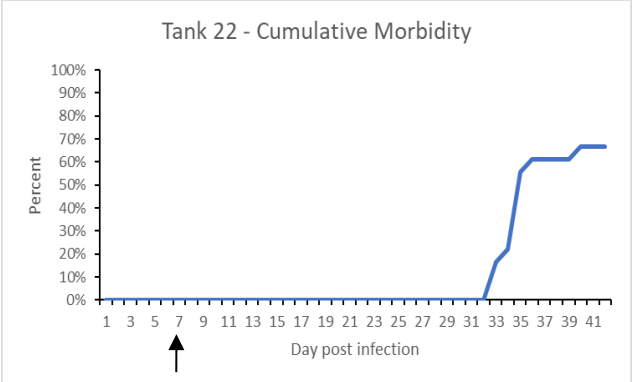


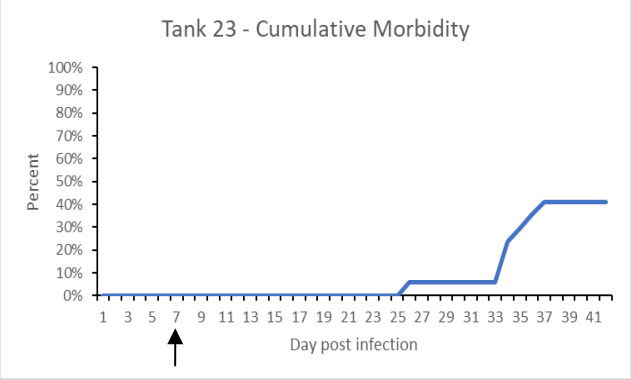
Daily morbidity of carp in Group 3, in which fish were injected with CyHV-3 at 12oC and held at this temperature for 14 days before the water temperature returned to 22oC. Arrow indicates day of inoculation. Fish in this group did not receive capture stress throughout the course of the experiment.

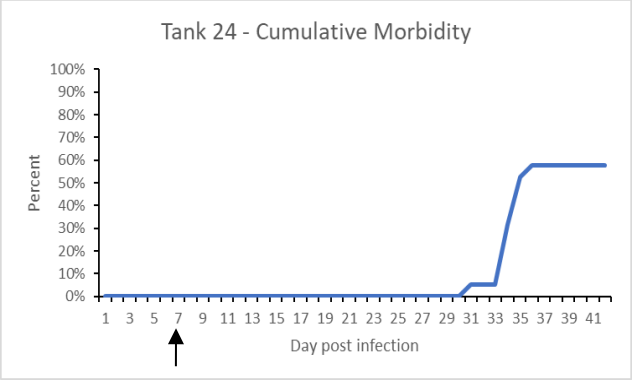




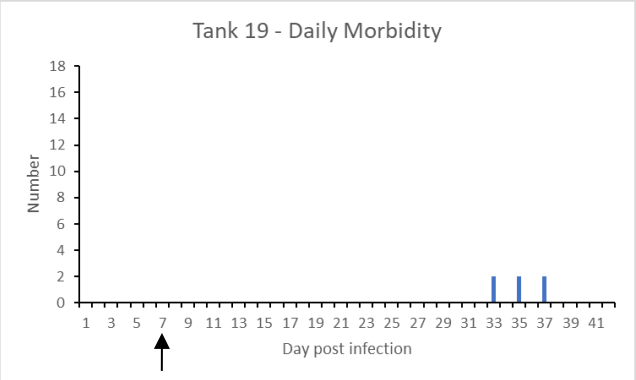


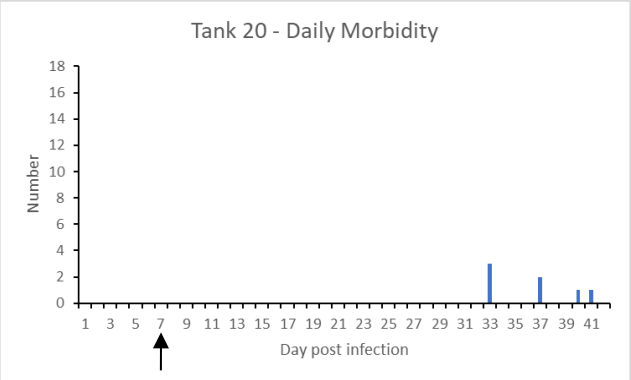


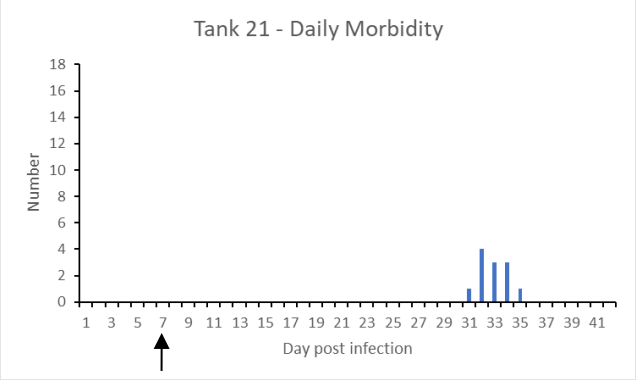


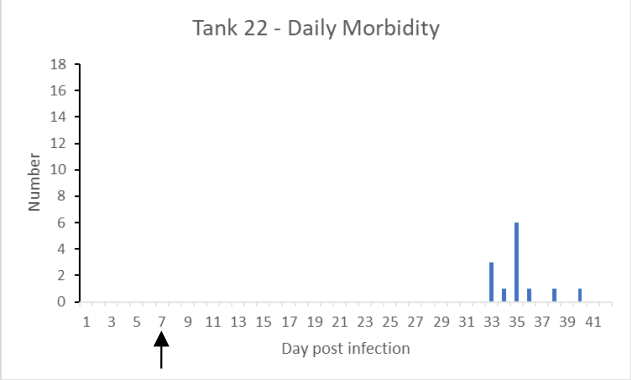


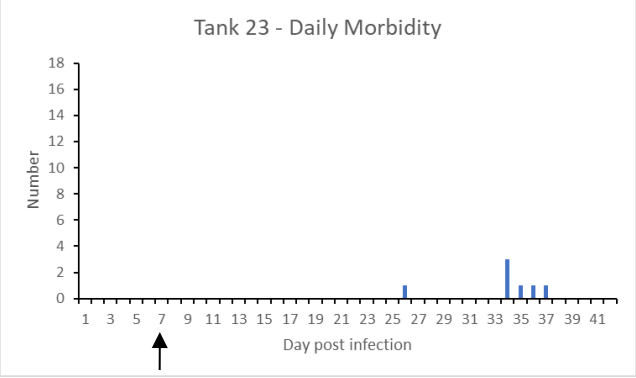
Cumulative morbidity of carp in Group 4, in which fish were injected with CyHV-3 at 12oC and held at this temperature for 14 days before the water temperature returned to 22oC. Arrow indicates virus injection at 12oC. Capture stress was commenced once water temperature reached 22oC for 10 remaining days until the experiment terminated on day 42.

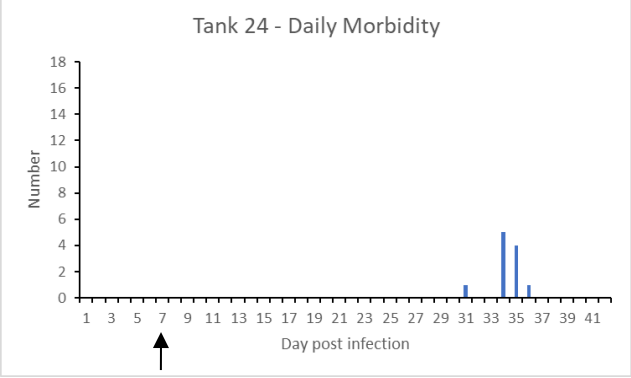




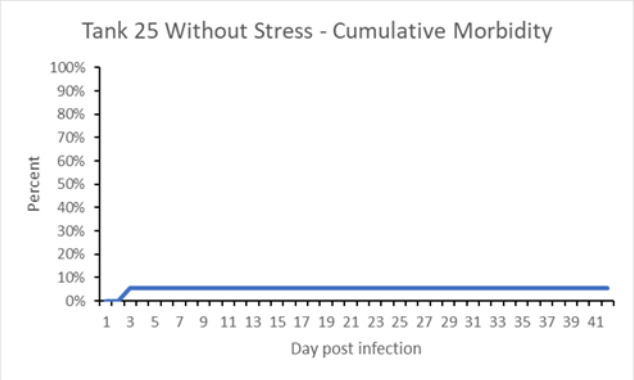


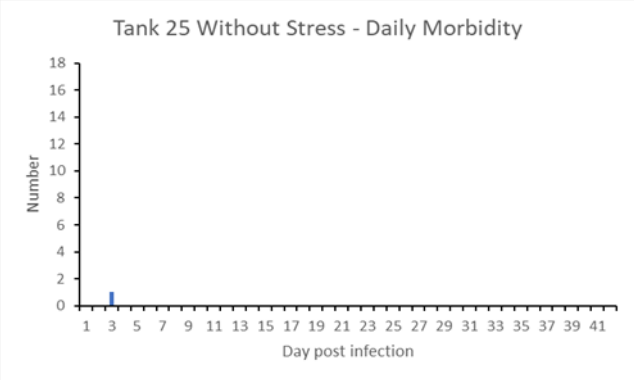


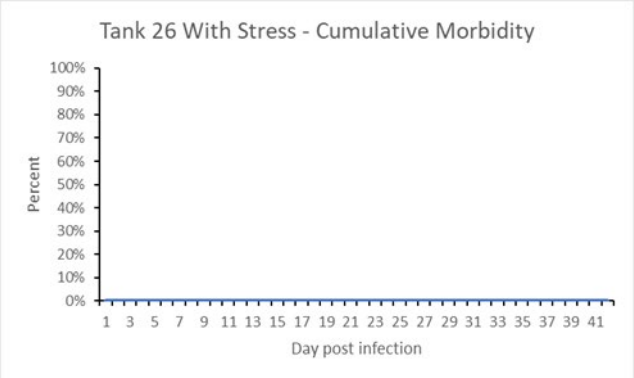


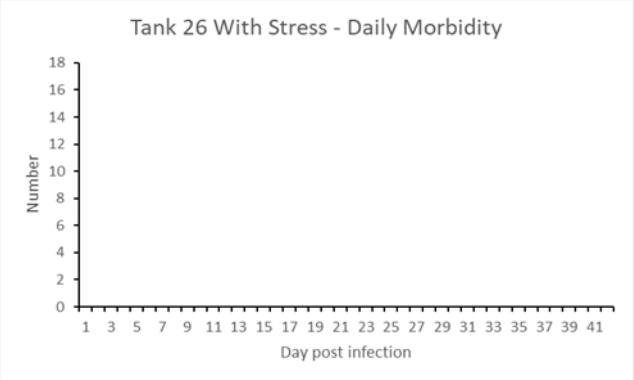


Daily morbidity of carp in Group 4, in which fish were injected with CyHV-3 at 12oC and held at this temperature for 14 days before the water temperature returned to 22oC. Arrow indicates virus injection at 12oC. Capture stress was commenced once water temperature reached 22oC for 10 remaining days until the experiment terminated on day 42.









Cumulative and daily morbidity of carp in negative control group, in which fish were treated identically to those of the virus-infected groups at 22oC but received only tissue culture medium without virus and were kept in a separate room from virus-infected groups.

Appendix 5. List of researchers and project staff

Agus Sunarto, Nick Moody, Peter Mohr, Joanne Slater, Nette Williams, John Hoad, Stacey Valdeter, Reuben Klein, David Cummins, Serge Corbeil, Sarah Riddell, Sheree Brown, Brenton Rowe, Nick Rye, Warren Michell, Jemma Bergfeld and Peter Durr.

FRDC FINAL REPORT CHECKLIST

Project Title:	Essential studies on cyprinid herpesvirus 3 (CyHV-3) prior to release of the virus in Australian waters – Seasonality studies.
Principal Investigator:	Agus Sunarto
Project Number:	2017-135
Description:	In Australia, common carp (*Cyprinus carpio*) are an introduced environmental pest, and the virulence and apparent host-specificity of *Cyprinid herpesvirus 3* (CyHV-3) may offer an important means of biological control. Nevertheless, integrated ecological and epidemiological modelling work to determine how best to release the virus identified that there would be a considerable logistical challenge undertaking a field release in multiple sites across south-eastern Australia. This is because there is a relatively short period in the spring when behavioural aggregation would achieve sufficient onward transmission of the virus to susceptible carp. A theoretical possibility to overcome this might be to undertake inoculation of fish during either the late autumn or winter when temperatures are near or below the disease inducing threshold of ~16 ˚C, and accordingly the virus would enter into a dormant state. Following the rise in water temperature in the spring, if virus reactivation occurred in these inoculated fish, then this would provide an efficient method of release as these fish would transmit the disease during spring aggregation events. We termed this potential late autumn/winter release method 'Trojan carp'. To assess whether this is biologically possible, we conducted a formal laboratory experiment simulating both late autumn and winter conditions with respect to water temperature conditions. We also compared the effect of physical stress in both temperature simulations, to ascertain the potential impact of spring aggregation and mating on virus reactivation. The winter simulations demonstrate that infection at a non-permissive low temperature could result in a persistent infection, with subsequent reactivation when the temperature was raised above the permissive threshold. It thus provides some biological evidence that a Trojan carp phenomenon occurs which might form the basis of a winter release. The late autumn simulations confirmed previous work that inoculation of the CyHV-3 in the permissive water temperature range, followed soon after by a reduction of water temperature below this range, prevented disease if the low temperature was consistently maintained. However, it was indicated that transient temperature rises above the lower permissive range threshold might result in disease. Thus, a late autumn release might be less successful as virus excretion and mortalities might occur prior the fish aggregating in spring and therefore would not be able to effectively onward transmit the virus. For the two groups exposed to the winter simulation, capture stress was shown to increase the proportion of fish expressing disease, but in the late autumn simulation the lack of any effect of stress is less certain due to the confounding effect of the disease during the period when the water temperature was nominally at 12°C. The unexpected appearance of disease in the low water temperature requires further investigation. By demonstrating that infection of carp at low water temperature results in a persistent infection, which could then be reactivated when the temperature was raised above the permissive threshold, this laboratory experiment has provided

	initial proof-of-concept for an alternative “Trojan carp” release strategy based on capture and inoculation of fish over the winter period. However, further experimentation is required to confirm that infective virus will persist in carp over a period longer than the 14-days used in our trial, and thus permit inoculation of fish throughout the winter and not just immediately before the spring temperature rise.		
Published Date:	18/10/2022	**Year:**	2022
ISBN:		**ISSN:**	
Key Words:	Common carp, biological control, *Cyprinid herpesvirus 3*, seasonality, temperature, stress, virus reactivation, release strategy.		

Please use this checklist to self-assess your report before submitting to FRDC. Checklist should accompany the report.

	Is it included (Y/N)	Comments
Foreword (optional)	N	
Acknowledgments	Y	
Abbreviations	Y	
Executive Summary		
– **What the report is about**	NA	
– **Background – why project was undertaken**	Y	
– **Aims/objectives – what you wanted to achieve at the beginning**	Y	
– **Methodology – outline how you did the project**	Y	
– **Results/key findings – this should outline what you found or key results**	Y	
– **Implications for relevant stakeholders**	Y	
– **Recommendations**	Y	
Introduction	Y	
Objectives	Y	
Methodology	Y	
Results	Y	
Discussion	Y	
Conclusion	Y	
Implications	Y	
Recommendations	Y	
Further development	Y	Combined with recommendations
Extension and Adoption	Y	
Project coverage	NA	
Glossary	NA	
Project materials developed	NA	
Appendices	Y	

END

NATIONAL CARP CONTROL PLAN

The National Carp Control Plan is managed by the
Fisheries Research and Development Corporation

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