The Effect of Dissolved O_2 Level on KHV Reactivation from Latency and Pathologic analysis during KHV Reactivation

by
Sammi Chen

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Microbiology
(Honors Scholar)

Presented May 31, 2016
Commencement June 2016

AN ABSTRACT OF THE THESIS OF

Sammi Chen for the degree of Honors Baccalaureate of Science in Microbiology presented on May 31, 2016. Title: The Effect of Dissolved O_2 Level on KHV Reactivation from Latency and Pathologic analysis during KHV Reactivation.

Abstract approved:_____

Ling Jin

Koi herpesvirus (KHV) is a new emerging viral pathogen of koi and common carps. It has been demonstrated that KHV becomes latent in the white blood cells in the recovered koi. The latent KHV can be reactivated under stressful conditions, such as heat and injury, and the reactivated viruses can cause diseases or death in the latently infected fish. Outbreaks of KHV often occur in the spring and summer when temperature suddenly increases. The dissolved oxygen level goes down when water temperature rises and it is unknown whether the change in dissolving oxygen has any effect on KHV reactivation. In this study, the dissolving oxygen level was investigated under temperature that could lead to KHV reactivation from latency. Our studies demonstrated that by maintaining the dissolved oxygen level at the non-stressing temperature level does not prevent KHV reactivation during temperature stress, but lowers the KHV shedding in feces. We also found KHV reactivation can occur as early as day 3 post-temperature stress or at 18˚C and peaks between 20˚C and 22˚C. Reactivated KHV can be detected in both gills and droppings. By day 15 post-temperature stress, both gross lesion and histopathology were present. There were inflammation and necrosis in multiple tissues,

especially in gills, skin, eye, intestine and kidney. KHV DNA can also be detected in multiple tissues in KHV latently infected koi at day 15 post-stress. The cortisol levels between day 3 and day 15 post-temperature stress are all above normal range, which suggests that KHV reactivation is a result of physiological stress.

Key Words: KHV, reactivation, latency, low O_2, post-temperature stress

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Honors Baccalaureate of Science in Microbiology project of Sammi Chen presented on May 31, 2016.

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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ACKNOWLEDGEMENTS

First, I would like to thank my mentor, Dr. Ling Jin for her tremendous support and guidance throughout the past two years of being in her lab. She has provided me with many opportunities to learn and gain hands-on experience. Also, I am very grateful for her flexibility and help on this project.

Next, I would like to thank my committee members, Dr. Dan Rockey and Dr. Linda Bruslind for their time, edits and feedback on this project.

I would also like to thank members of the Jin Lab, especially Lisa Lin and Tiffany Song who has assisted me throughout the study.

Finally, I would like to thank my family and friends for their support and encouragement throughout my undergraduate years at OSU.

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INTRODUCTION

Cyprinid herpesvirus 3 (CyHV-3), commonly known as koi herpesvirus (KHV), is a highly contagious virus that causes disease in koi and common carp (8, 11). KHV has been classified as a member of the *Alloherpesviridae* family within the order of *Herpesvirales* (18). Members of *Alloherpesviridae* affect mainly fish and frog (3). KHV infection is a newly emerged disease in koi and common carps in late 1997 and early 1998 in Israel (6). Later, cases of KHV infections have been reported across the world and now can be found in many areas, such as Europe, Asia and USA (1, 12).

KHV affects koi and common carp of most ages, but has a higher mortality rate in fry (8). The virus has an incubation period of 7 to 14 days and may cause between70% to 100% mortality, especially in fry (8). Clinical symptoms of KHV infections includes red or white mottling of gills, gill hemorrhage, sunken eyes, pale patches along with blisters on skin (6, 7). After the onset of clinical symptoms, death may occur within 24 to 48 hours in the KHV infected koi (10). Infected fish can shed the virus by means of feces, gill and skin mucus, which can lead to infection of naive fish (20).

One of the unique features of all herpesvirus infections is latency, which is characterized by the absence of infectious viral particles and lifelong maintenance of the viral genome in cells within the clinically recovered host (14, 17). Latently infected koi can live a normal life for over 15 years. KHV can reactivate under stressful condition, such as high temperature (15). Members of subfamilies of *Herpesviridae*, such as

Alphaherpesvirinae, Betaherpesvirinae and *Gammaherpesvirinae* can establish latent infection in the absence of productive infection (17). The site of latent infection varies between the different subfamilies of *Herpesviridae*. It has been found that the dorsal root ganglia and sensory ganglia, sites of the peripheral nervous system, are places where most alphaherpesviruses establish latent infections (13). Herpesviruses of the subfamily, *Betaherpesvirnae,* are found to become latent in mostly lymphoid tissue, bone marrow and kidney (2, 14). Splenic B cells are also common sites of latent infection in members of the subfamily, *Gammaherpesvirinae* (14). It has been reported that KHV becomes latent mostly in the B cells in peripheral blood (15, 16). During latent infection, viral DNA can be detected in the white blood cells from the latently infected koi (6, 8). It has also been shown that KHV can reactivate under temperature stress, when water temperature increases from 18˚C to 26˚C (15).

As temperature increases, the oxygen level decreases. This is a result of oxygen being less soluble in warmer water as opposed to in colder temperatures. Most fish do well when the level of dissolved oxygen is above five parts per million (ppm) or 5.0 mg/L (5). When dissolved oxygen level in water drop below the optimal level, aquatic life is stressed (5). Outbreaks of KHV infection occur mostly during spring and summer when water temperatures become warmer (18). During this time, the dissolving oxygen concentration in the water also decreases as a result of the temperature increase. The question is whether the change of oxygen level during temperature increase has any effect on KHV reactivation. To address this question, KHV reactivation is investigated by temperature stress under constant O_2 level in this study.

MATERIALS AND METHODS

Source of koi and sampling:

Koi from the pet store with a history of KHV infection in the past were obtained in the summer of 2015 in Corvallis, Oregon. All koi were quarantined for two weeks at 15°C in accordance with Animal Care and Use Committee guidelines before being used in the study. Three groups of koi with six per group were kept segregated and maintained at 15°C in 4-ft diameter tanks at OSU-SDL in accordance with Animal Care and Use Committee regulations. All blood samples were collected via caudal vein puncture after the koi were anesthetized with MS-222 (100 ppm). All koi were euthanized via MS-222 (500 ppm) overdose. Tissues including the brain, spleen, gills, heart, eye, intestine, kidney, liver and gonad were collected at necropsy.

To confirm whether the purchased koi were latently infected by KHV, blood samples from all koi were collected and examined by nested PCR as previously reported (19). The koi were anesthetized with equal volumes of MS-222 and sodium bicarbonate buffer before blood sample collection. Approximately 2 mL of blood were extracted from the caudal vein of each fish and stored in 0.2mL of the anti-coagulant, 3.5% sodium citrate. All samples were kept on ice before white blood cell separation.

The set-up of water tanks:

The water temperature of tank 1 (W1) was maintained at 15°C throughout the study as control. Water temperature of tank 2 (W2) and tank 3 (W3) were increased by 1°C each

day starting at 15°C to 22°C, and then maintained at 22°C for additional 7 days before dropping the temperature back down to 15°C (Fig. 1). During temperature increase, O_2 was supplemented to tank W3 to maintain the dissolving oxygen level at 15°C, using supplemented O_2, which was connected to an air stone diffuser placed in the tank. The oxygen levels were monitored daily by using an YSI (Yellow Springs Instrument) meter.

Monitoring KHV reactivation:

To monitor KHV reactivation in koi, gill and anal vent sites were swabbed on days 3, 6, 8, 10, and 15 post-temperature stress and stored at -80°C for later analysis. The swabs were placed in 0.5 mL of Improved MEM (1 X Richters with L- glutamine) with both penicillin (200 U/ml) and streptomycin (200µg/ml) (Sigma-Aldrich, Inc., S. Louis, MO). Both nested and real-time PCR were performed to detect the presence of KHV DNA. Viral DNA was tested by using 10 µl swab solution stored in SideStep Lysis and Stabilization Buffer (Agilent Technologies) (1:1 dilution).

In addition, blood samples were taken on days 0, 3, 10, 15 and day 50. On day 15, three fish from each tank with the most severe clinical signs of KHV reactivation were euthanized on day 15 post-stress. Tissues were collected, which includes brain, gill, spleen, anterior kidney, posterior kidney, liver, mid-intestine, heart, gonad and eye, via necropsy.

Total DNA isolation from WBC and Cortisol level:

Approximately 2 mL of whole blood with anti-coagulant was transferred to a 15 mL centrifuge tube and an equal volume of RPMI-1640 balanced salt solution was used to dilute the blood. After mixing gently, the diluted blood sample was carefully layered on top of 3 mL Ficoll-Paque PLUS (GE Healthcare) in a new 15 mL centrifuge tube and centrifuged at 400 X g for 40 minutes at 20°C. After centrifugation, the upper layer (containing plasma) was transferred to a 1.5 mL microcentrifuge tube to check the antibody titer and cortisol level. The lymphocytes layer (approximately 2mL) was then transferred to a new centrifuge tube followed by washing the cells twice in 6 mL of RPMI-1640 balanced salt solution and centrifuging at 3000 rpm at 20°C for 10 minutes. Following washing, the cell pellet was resuspended in 400 µl of hypotonic buffer (50 mM Tris-HCl [pH 8.0], 5mM EDTA, 2% Triton X-100) with 50 µg/ml RNAse and incubated overnight at 4°C. Cell nuclei were removed by centrifuging the sample at 10,000 rpm for 10 minutes at 4°C. The supernatant was then transferred to a DNA binding column and centrifuged at 12,000 X g for 1 minute. Viral DNA was isolated following the instructions of the Roche Diagnostics GmbH manual for High Pure PCR Template Preparation Kit. The total DNA from the WBC was eluted in 75 µl of TE buffer (10mM Tris-HCL [pH 8.0]-1 mM EDTA). The isolated DNA from 2 mL of blood range from 0.01 µg/µl to 0.1 µg/µl in concentration. Before running nested PCR or real-time PCR, the total DNA of all WBC samples were adjusted to 0.1 µg.

Total DNA extraction from tissue samples and fecal sample

Tissue samples collected from freshly euthanized fish were stored at -80°C. Approximately 100 to 200 mg of the tissues collected were disrupted with a homogenizer with 2.5-mm silica beads (Biospec Product) and incubated overnight at 55°C in 800 µl of 1 X tissue lysis buffer, containing 20 µl of proteinase K (20 µg/ml). Total DNA was isolated by using High Pure PCR Template Preparation Kit. Approximately 0.1 to 1.0 µg/µl of total DNA was isolated from the tissue lysates. About 0.5 µg tissue DNA was used in real-time PCR and nested PCR.

Total DNA of fecal samples obtained from each tank was isolated similarly as with the fish tissue. Approximately 100 to 200 mg of the samples were digested overnight in 400 µl of 1 X tissue lysis buffer and 20 µl of proteinase K (20 µg/ml) at 55°C. Total DNA was then extracted as described. The concentration of total DNA extracted from each sample ranges between 0.006 µg/µl and 0.1 µg/µl. Before running nested PCR or real-time PCR, the total DNA of all fecal samples were adjusted to 0.075 µg in the PCR reaction.

PCR primers:

Primers specific for KHV sequence amplification were selected based on the KHV genome sequence data available through Genbank (NC_009127). For nested PCR, F525 and R525 primers were used in the first PCR reaction and F368 and R368 primers were used in the second PCR reaction as described before (19).Together, the two sets of primers were designed to amplify the open reading frame 25-26 (ORF25-26) gene region

in KHV DNA (Table 1). For real-time PCR, KHV86F and KHV 163R primers and Taqman probe KHV 109P with FAM (6-carboxyfluorescein) as the reporter and TAM (6-carboxtetramethylrhodamine) as the quencher were used to quantify the number of copies of KHV DNA in each sample (Gilad et. Al, 2004).

Nested PCR and real-time PCR:

PCR amplification with nested set primers was carried out in a 25 µl reaction consisting of 4 µl H_2O, 15 µl Platinum PCR Supermix (Invitrogen, Carlsbad, CA), 0.5 µl of each primer at 20 µM and 5 µl DNA sample with a concentration between 0.01-0.1 µg/ µl or tissue lysates diluted 1:1 with Sidestep Lysis Stabilization buffer (Agilent technologies). The reaction was subjected to 94°C for 2 min followed by 35 cycles of amplification at 94°C for 30s, 55°C for 30s, 72°C for 45s, and lastly, a 5 min elongation step at 72°C after the final cycle. A 2 µl aliquot of the PCR product from the first reaction was used as a template for the second run of nested PCR amplification.

Real-time PCR for KHV DNA quantification was performed in a 25 µl reaction consisting of 6 µl H2O, 12.5 µl Platinum qPCR Supermix-UDG with ROX (Invirogen, Carlsbad, CA), 0.5 µl of each primer at 20 µM, Taqman probe at 10mM and 10 µl (approximately 100ng) of DNA template. The mixture was subjected to 50°C for 2 min, 95°C for 2 min and 40 cycles of 95°C for 15s and 60°C for 60s.

Standard curve of KHV DNA real-time PCR:

To quantitate the KHV DNA copy number, the PCR product of real-time PCR was cloned into a TOPO 2.1 PCR cloning vector (Invitrogen, Carlsbad, CA). Restriction

digestion and DNA sequencing were performed to confirm correct insert. The purified plasmid was used to make the standard curve for quantifying the copy numbers of viral DNA in tissue samples and swab solution using real-time PCR. Based on the cycle threshold (CT) values, the number of copies of viral DNA can be calculated from the equation given the standard curve, $Y= -1.267\ln(x) + 37.202$ (Fig. 2).

RESULTS:

KHV latent infection status in koi to be used for KHV reactivation study:

To confirm that koi are latently infected with KHV, total DNA of white blood cells (WBC) from all the experimental koi were collected and tested by nested PCR specific for ORF25-26. WBC total DNA was isolated by modified hypotonic buffer and Roche's method, respectively. KHV DNA was detected in the white blood cells from koi in all three tanks; detection of KHV genome was shown in Fig. 3A from selected koi from each tank. The detection of KHV using total DNA isolated by the modified hypotonic buffer is better than DNA extracted by the Roche's method (Fig. 3B). The koi obtained from the local merchant were mostly all confirmed to have KHV latent infection. They were healthy without any signs of illness before the start of the experiment. To determine if KHV reactivation occurred following transferring koi to our research facility, fecal samples were collected from the quarantine tank within the first two weeks upon their arrival. Total DNA was extracted from the fecal samples as reported before. As shown in Fig. 3C, KHV DNA can be detected in the fecal samples at the bottom of the fish tank when they first arrived the Salmon Disease Laboratory. These results also suggest that

these koi are latently infected by KHV. All koi were quarantined for two months until no KHV could be detected in the fecal samples.

Dissolved oxygen level changes between 15°C and 22°C:

To determine whether there is a change in dissolved oxygen level following temperature increases, oxygen level from all three tanks were measured and compared. The water temperature in W2 and W3 was increased by 1°C each day starting on day 1 (16°C) until the temperature reached 22°C on day 7. After the water temperature reached 22°C, it remained constant at 22°C for one week (Fig. 1). Dissolving oxygen level from all three tanks were measured daily throughout the experiment using an YSI meter. With water temperature from W1 staying constant at 15°C, the dissolving oxygen level measured in mg/L remained between 9.85 and 10.82 (Fig. 4). The oxygen level in W2 went down gradually when the water temperatures were elevated between day 1 and day 14. The dissolving O_2 went down to 7.29 mg/L at 22°C on day 7.

KHV reactivation during temperature stress:

To investigate the effect of dissolving O_2 on KHV reactivation, one group of koi (W3) was supplemented with O_2 to maintain the dissolving O_2 level at 15°C, while another group of koi (W2) was kept in the tank without O_2 supplementation during temperature stress. Another group of koi (W1) was kept at 15°C without temperature stress. To monitor the KHV reactivation, virus shedding in gills and vents was monitored by swabbing on days 0, 3, 6, 10, and 15, and the amount of KHV shed in the gills and vents were examined by real-time PCR. As shown in Fig. 5A, KHV DNA can be detected in vent swabs in 33% of koi in tanks W1 and W3, 66% of koi in tank 2, respectively, on day

3 post-temperature stress. By day 8 at 22°C for two days, 83% of koi and 66% of koi had KHV reactivation in group W2 and W3, respectively, while only 16% of koi had KHV reactivation in tank W1, which was maintained at 15°C. The rate of KHV reactivation slowly decreased in vent after day 8 post-temperature stress. When the amount of genome was compared, similar amount of viral genomes were detected in koi experiencing KHV reactivation and no significant difference was observed between groups (Fig. 6). It was noticed that a significant number of koi had KHV reactivation on day 3 post-temperature stress (Fig. 5B). However, no KHV reactivation was detected on day 6 post-temperature stress (Figs.5A and 5B). 83% of gill swabs collected from tank W1 tested positive on day 8 post-stress, while 83% and 100% of gill swabs collected from W2 and W3, respectively are KHV positive on day 10 post-stress. KHV reactivation went down after day 10 post-stress in the gills. No KHV genome was detected in either gill swab or vent swab when tank water temperature was brought back down to 15°C on day 50 post-stress. It was interesting to find that KHV shed in gill is significantly higher in tank W3 on day10 post-stress at 22°C.

The average number of KHV genome per vent swab was approximately 10^4 DNA copies in tank 1, and 10^3 DNA copies in both tank 2 and tank 3 on day 3 post-stress (Fig. 6A). On day 8 and day 10 post-temperature stress, an average of 10^3 DNA copies per swab were detected in koi from both tank 1 and tank 2, while only an average of 10^2 DNA copies per swab on day 8 and no detectable viral genome on day 10 from koi in tank 3. On day 15 post-temperature stress, the average copy number of KHV genome was similar (10^2 DNA copies) for both tank 1 and tank 3, while koi from tank 2 were detected KHV

negative. The amount of virus present in the gill samples collected were also quantitated by real-time PCR. As shown in Fig. 6B, the average number of KHV genome per gill swab detected on day 3 post-temperature stress, were similar (10^4 DNA copies) for koi from all tanks. On days 8, 10, and 15 post-temperature stress, the average amount of KHV DNA detected per gill swab was similar for all tanks (10^3 DNA copies) with the exception on day 10, where about 1 X 10^4 DNA copies per swab was detected in koi in tank 3.

KHV latency in the WBC during temperature stress:

To investigate the status of KHV genome within the latently infected white blood cells during temperature stress, total WBC were isolated on days 0, 3, 10, 15, and 50 post-stress. Total DNA of WBC was examined by KHV specific real-time PCR. As shown in Fig. 7, the number of KHV genome detected in the WBC were all low, under 1x 10^3 copy per 2 mL of total blood and does not change significantly during temperature stress. In addition, KHV genome was undetectable by real-time PCR in over 50% of koi from each group. However, KHV genome can be detected in all koi at day 50, over one month post-stress, when the temperature was dropped back to 15°C; the KHV DNA copy number detected from the white blood cells of the koi was slightly higher than total blood collected before stress. An average of 10^4 DNA copies per 2 mL of blood can be detected in koi from tank 1 and tank 2 while an average of 10^5 DNA copies per 2 mL of blood can be detected in koi from tank 3.

Pathology induced from temperature stress and sampling:

To monitor the KHV reactivation, the gross morphology was recorded on day 0, 8, 10 and 50 post-stress. As shown in Table 2, no gross lesion was observed in the three tanks before temperature stress. However, by days 8 post-temperature stress, 2 out of 6 koi from tank 1 showed white mottling of the gills, 5 out of 6 koi from tank 2 showed gill necrosis along with 4 out of 6 from tank 3. On day 10 post-temperature stress, 2 out of 6 koi from tank 1, all 6 koi from tank 2 and 5 out of 6 koi from tank 3 showed symptoms of gill necrosis. When the koi were no longer under temperature stress on day 50, only 1 out of 6 from tank 1, 2 of the 6 koi from tank 2 and 1 of the 6 koi from tank 3 had symptoms of gill necrosis. Hemorrhaging in the anal vent was another common symptom observed in many koi during temperature stress. Figure 8 shows the gills of koi on days before, during and after temperature stress. Depicted in Figure 9 is an example of hemorrhaging in the anal area from one of the koi in tank 3 during temperature stress.

KHV reactivation in the tissues on day 15 post-stress:

To determine whether or not there is difference in KHV reactivation in internal organs, three koi (50%) were euthanized from each group on day 15 post-stress. Tissues listed in table 3 were harvested and examined by KHV specific real-time PCR. Total DNA from each tissue was adjusted to 0.5 µg before testing. As shown in Fig. 10, KHV can be detected in all the collected tissues, suggesting an active infection was present. It is also noticed that koi tissue from tank 1 also had significant amount of KHV present in the collected tissue, which suggests there is KHV reactivation in koi from tank W1 also. Tissues of the gills, middle intestine and brain collected in koi from tank 2 had higher

KHV DNA copy numbers than those from tank 1 and tank 3. In general, tissues from tank 3 had lower copy numbers of KHV DNA, below 10^3 DNA copies, than those from tank 1 and tank 2. The spleen was found to have similar level of KHV in all three tanks with KHV DNA copy number between 274 to 1153 DNA copies.

Histopathological examination of koi tissue at day 15 post-temperature stress:

The above study demonstrated that there was an active KHV infection present in koi collected on day 15 post-stress. To determine whether there was any histopathology associated with KHV reactivation from temperature stress, individual tissues from each koi was harvested and examined by H&E staining. One of the unique histopathology signs is a lack of inclusion body detected in those tissues investigated. The intestine and eye were affected in all three groups. Koi kept in tank W1, which had no temperature stress, all three koi had mild hyperplasia of gill surface epithelium (Fig.11C), mild lymphocytic enteritis, two of the koi had moderate lymphocytic dermatitis and keratinocyte necrosis in the skin (Fig.11D), one of the koi has focal peritubular and periureteral histiocytic inflammation in the kidney, one had moderate lymphocytic and histiocytic myocarditis and epicarditis (Fig. 11A). Koi kept in tank W2, which were stressed without O_2 supplementation, all three koi had severe focal lymphocytic myelitis in the spinal cord (Fig.11B), moderate focal necrotizing oophoritis in ovary, mild hyperplasia of gill surface epithelium, one koi with moderate focal lymphocytic encephalitis in the brain, and moderate epidermal necrosis and lymphocytic inflammation in the skin. Koi kept in tank W3, which were stressed with O_2 supplementation. Histopathologies were similar to those seen in tank 2, and were characterized with mild

focal lymphocytic myocarditis in the heart, mild focal lymphocytic myelitis in the spinal cord, focal peritubular fibrosis and periureteral histiocytic inflammation. There was also mild hyperplasia of epithelium covering the tips of secondary lamellae in the gills. There is a mild lymphocytic infiltrates within the lamina propria and increased lymphocytes within the surface epithelium; severe lymphocytic inflammation in the surrounding adipose. The connective tissue is hypercellular with increased lymphocytes and macrophages (steatitis).

Cortisol level under Tm stress:

Another way to confirm whether the koi was stressed or not was to measure their cortisol levels throughout the study. As shown in Fig.12, the reference value used to indicate normal cortisol level in blood plasma of the koi was about 20 micrograms per deciliter (μg/dL) (9). Any value above the reference line indicates the koi in a stressed condition. Looking at Fig.12 below, the cortisol levels of the koi on day 0 from all three tanks were normal, indicating the absence of stress. Starting on day 3 post-temperature increase, the koi were stressed with cortisol levels slightly above the normal values. On days 10 and 15 where peak reactivation of KHV occurs, the cortisol level of the koi in W2 reaches approximately 50 μg/dL and 60 μg/dL, respectively. The cortisol level measured in the koi from W3 was slightly below 50 μg/dL on both days. For the koi in W1, the cortisol levels were consistent around 35 μg/dL on days 3, 10, and 15. By day 50 where the koi were back in non-stressful temperature, the cortisol levels of the koi from W1 and W3 were within the normal range, koi from W2 had a cortisol value of above normal range, 30 μg/dL.

DISCUSSION

Our studies demonstrate that there is a difference in dissolving oxygen level when temperature was increased from 15 ˚C to 22 ˚C. The dissolving oxygen level measured on day 0 at 15 ˚C for all three tanks was between 10.26 to 10.82 mg/L. As shown in Fig. 4, the oxygen level in tank 2 decreases from approximately 10 mg/L to 7 mg/L when temperature reaches 22 ˚C. However, our study finds that oxygen supplementation does not prevent KHV reactivation during temperature stress. Depicted in Fig. 5A, 83% of the koi in tank 2 shows KHV reactivation in the vent and 66% of the koi in tank 3 (with oxygen supplementation) shows reactivation from KHV latency on day 8. Using real-time PCR, the average KHV DNA copy number detected per vent swab in the koi from tank 2 on day 8 was approximately 10^3 DNA copies, while in tank 3 was about $9X10^2$ DNA copies. Although there was a higher reactivation and a slightly greater number of KHV genome found in koi from tank 2, koi from tank W3 maintained at constant dissolving O_2 still reactivated from latency. As shown in Fig. 5B, peak reactivation in the gills occurred on day 10 post-temperature stress. In tank 2, 83% of the koi shows reactivation with an average KHV DNA copy number of around 10^3 DNA copies per gill swab. In tank 3, there was 100 percent reactivation where all of the koi fish showed reactivation from latency with an average KHV DNA copy number of about 10^4 DNA copies per gill swab on day 10 post-stress. Our data suggest that oxygen supplementation does not prevent KHV-infected koi fish from reactivating. However, our results show that the amount of KHV shedding is lower in koi during temperature stress when oxygen is maintained at 15 ˚C level (Fig. 6).

The cortisol level measured for koi in tank 1 after day 3 suggested that these koi were stressed, which explained koi had KHV reactivation in tank 1. KHV reactivation may come from mechanical stress caused by nettling and handling of the koi. Prior to temperature stress, the gills of the koi from all three tanks appeared healthy and normal. The white mottling of the gills is a common sign of KHV infection. During temperature stress on days 8 and 10, most of the koi from tank 2 and tank 3 had symptoms of gill necrosis. Koi from tank 2 showed more severe symptoms of white mottling of the gills. Gill lesions disappeared by day 50 post-stress and 6 months.

When KHV reactivation was examined in the internal organs, there was a lower DNA copy number in the tissues of koi from tank W3 as compared to tissues from the first two tanks. Koi from tank 2 had a higher magnitude of KHV DNA detected in the liver whereas, koi from tank 1 had a higher KHV DNA copy number detected in the eye. As shown in Fig. 10, a higher magnitude of KHV DNA was detected in koi from tank 1 as compared to tank 3, suggesting that the koi from tank 1 also reactivated even when the water temperature was maintained at a non-stressful temperature of 15 ˚C. Again, this may be a result of stress from handling and netting of the koi throughout the study.

An interesting finding in our study was that during reactivation, KHV genome in the white blood cells is relatively low and often undetectable by real-time PCR, and only detectable by nested PCR. As shown in Fig. 7, KHV DNA copy number in the white blood cells was lower during reactivation phase between day 3 and day 15 post-temperature stress as compared to KHV DNA copy number detected by day 50 post-stress. On days 3, 10, and 15 post-stress, approximately 10^3 DNA copies of KHV DNA were detected in 2 mL of blood. However, on day 50, one month post-stress, (15˚C), an

average of 10^4 DNA copies were detected in tank 1 and tank 2 and greater than 10^5 DNA copies in tank 3. It is possible that more white blood cells are latently infected after temperature stress.

SUPPLEMENTARY TABLES

Table 1. Primer pairs used to detect KHV DNA in wild common carp and koi

PCR	Primer name	Gene Target	Primer sequences (5'-3')
nested PCR	F525	ORF-25-26	GGTGGTGCTCATCGTCATAA
	R525		TGGTGATGAACTTGGTGGTG
	F368		AGGCGCTGATCATCGTATTC
	R368		GCAGATGGTACGTGATGCTG

Table 2. Gill necrosis observed before the study, on days 8, 10 and after the study.

Day	Tank 1	Tank 2	Tank 3
Before study (D0)	0/0	0/0	0/0
D8	2/6	5/6	4/6
D10	2/6	6/6	5/6
After study (D50)	1/6	2/6	1/6

*Fraction indicates number of koi out of six in each tank with symptoms of gill necrosis.

Table 3: List of tissues collected from euthanized koi for detection of KHV reactivation using Real-time PCR

Day of Sample	Internal Organs
15	Anterior kidney
15	Posterior kidney
15	Liver
15	Gills
15	Brain
15	Eye
15	Gut
15	Spleen
15	Heart
15	Gonad

FIGURES

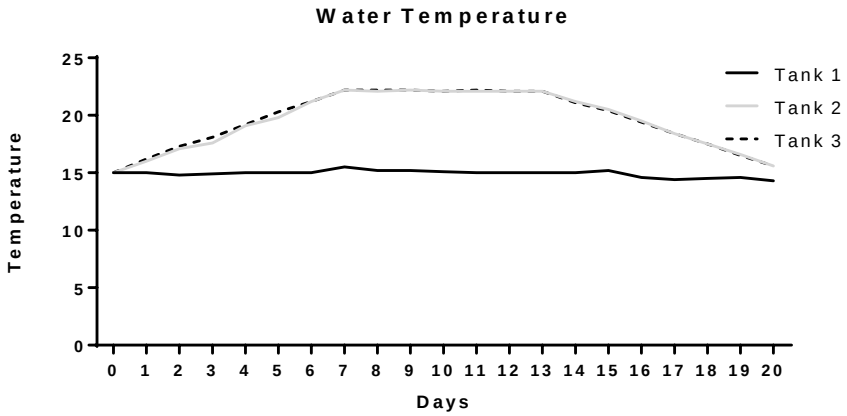


Figure 1: The set-up of tank water temperatures. The X-axis represents the day of study and the Y-axis represents temperature measured in Celsius (°C). The black line represents tank1, light gray line represents tank 2, and dash line represents tank 3. Tank 1(W1) water temperature stays constant at 15°C from day 0 to day 20. Tank 2 (W2) and tank 3 (W3) water temperature starts at 15°C on day 0, raises 1°C each day and reaches 22°C on day 7. Temperature stays at 22°C for seven days before dropping 1°C each day, back to 15°C.

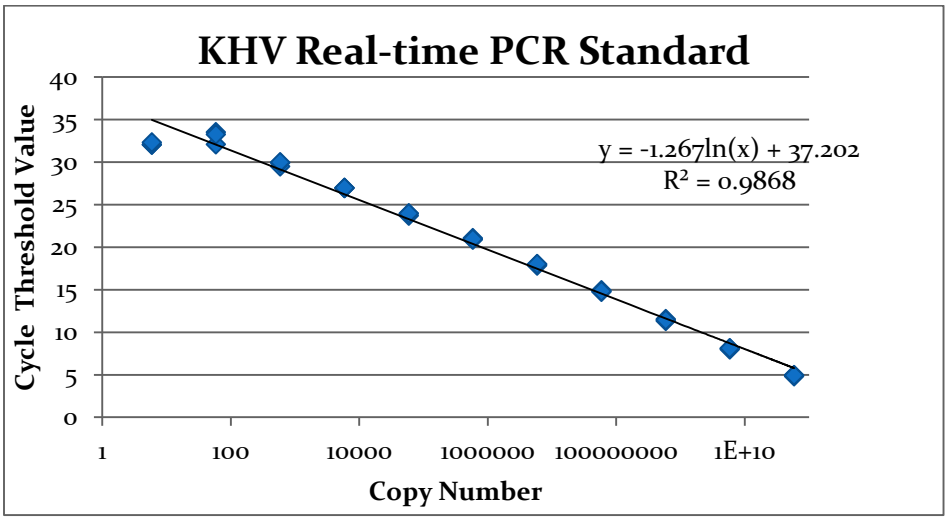


Figure 2: Standard curve using cycle threshold values to calculate KHV genome equivalents with the real-time TaqMan PCR. The X-axis represents the copy number of KHV DNA and Y-axis is the cycle threshold value. The assay detects from 10 to 10^{10} copies of a plasmid with the KHV target sequences.

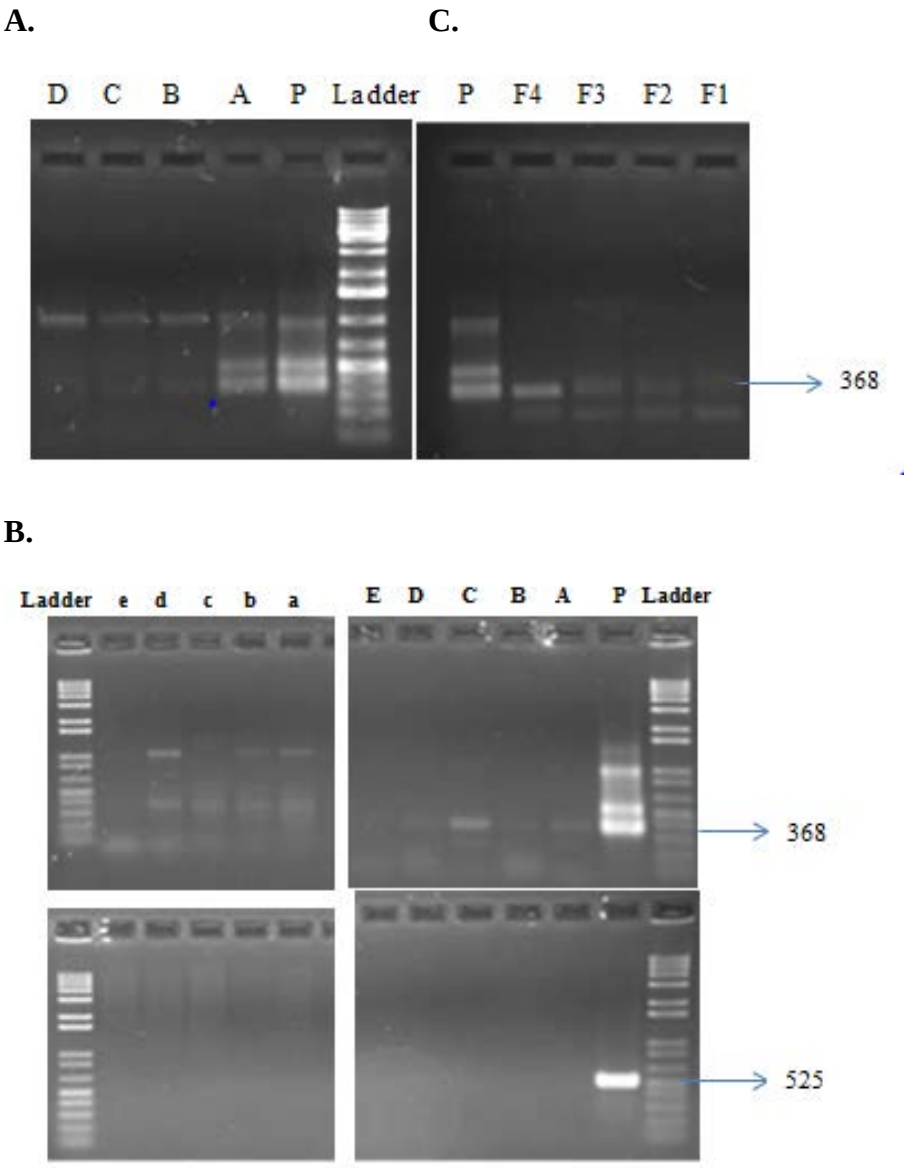


Figure 3: The nested PCR specific for ORF25-26 of KHV **(**A): Gel Electrophoresis of total DNA extracted from white blood cells in koi from all three tanks. The first lane after the ladder is control (P) followed by DNA extracted from white blood cells in tank 1 (A), tank 2 (B, C) and tank 3 (D). (B): Gel electrophoresis of DNA extracted from white blood cells using new extraction method for samples A, B, C, D, E on the right (Hypotonic buffer) and old extraction method for samples a, b, c, d, e on the left (Roche). Sample A represents DNA extracted from WBC of koi from tank 1; B is sample from tank 2; and C, D and E are samples from tank 3. Samples a and b are from tank 1; c is from tank 2; d and e is from tank 3. Nested PCR of DNA samples from all three tanks where P is the control. The top gel is the second run of nested PCR with primers 368F, R and the bottom gel is the first run using primers 525F,R; the labeled lanes for second run corresponds to the first one. (C): P is control. F1, F2, F3 and F4 are DNA extracted from fecal samples. Pictures A and C are picture of the second run of nested PCR with primers 368 F, R.

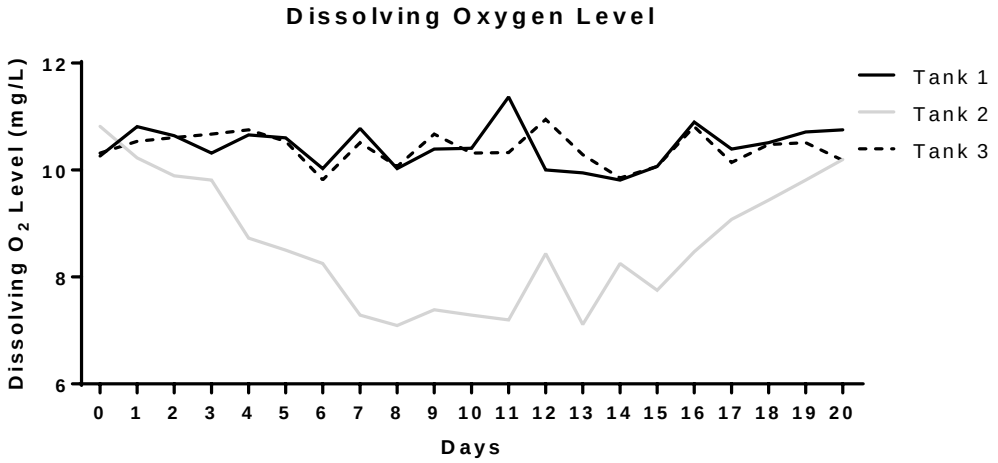
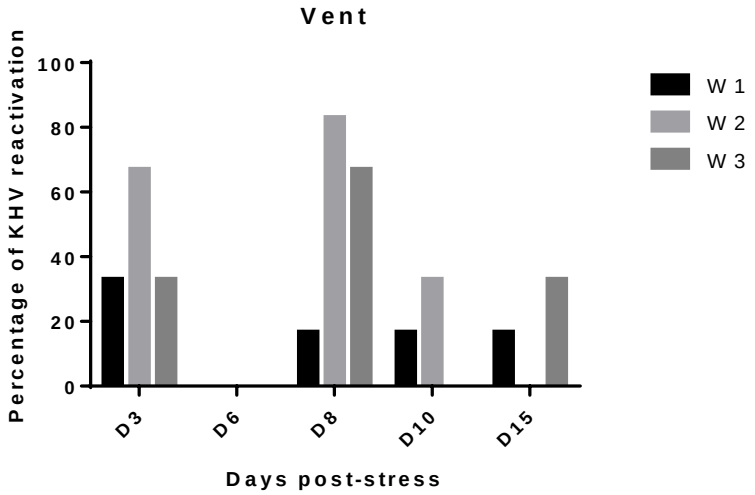


Figure 4: The record of dissolving oxygen level between 15°C and 22°C. The X-axis represents day of study and the Y-axis depicts the oxygen level measured in mg/L. The black line represents tank 1, light gray line represents tank 2, and dash line represents tank 3. No manipulation of oxygen level in tank 1 and tank 2. Oxygen level in tank 3 adjusted to oxygen level in tank 1 by supplementing oxygen and monitoring level with an YSI meter.

A.



B.

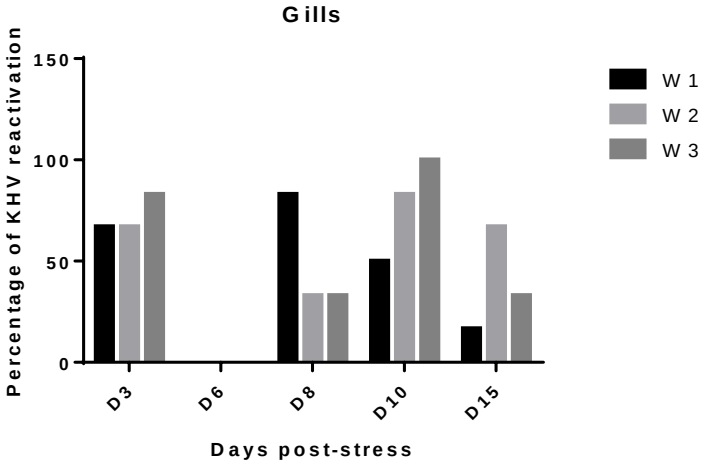
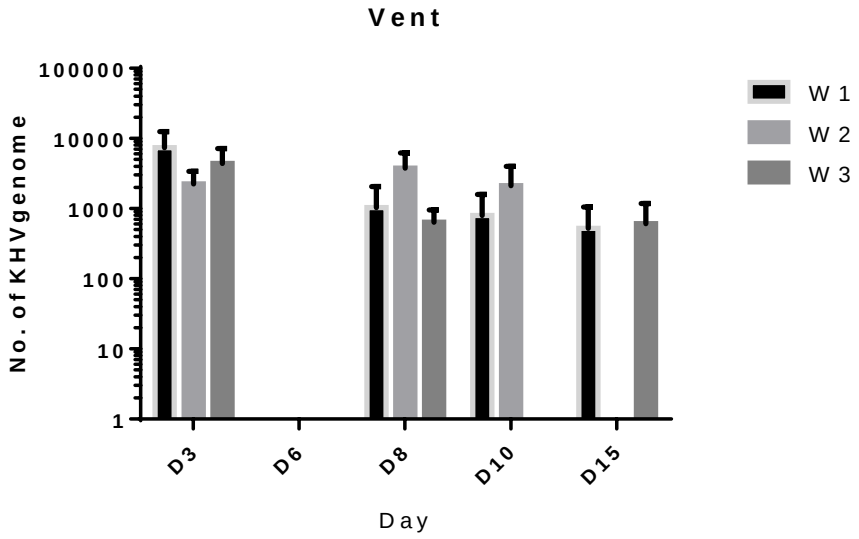


Figure 5: Percentage of KHV reactivation by real-time PCR. (A): Percentage of KHV reactivation in the vent swabs. (B): Percentage of KHV reactivation in the gill swabs. The X axis represents day of study and Y-axis is the percentage of KHV reactivation. The black bar represents koi from tank W1, lighter gray bar represents koi from tank W2 and darker gray bar represents koi from tank W3. Both vent swabs and gill swabs were collected from all six koi in tank 1, 2 and 3 on days 3, 6, 8, 10, and 15 post-temperature stress.

A.



B.

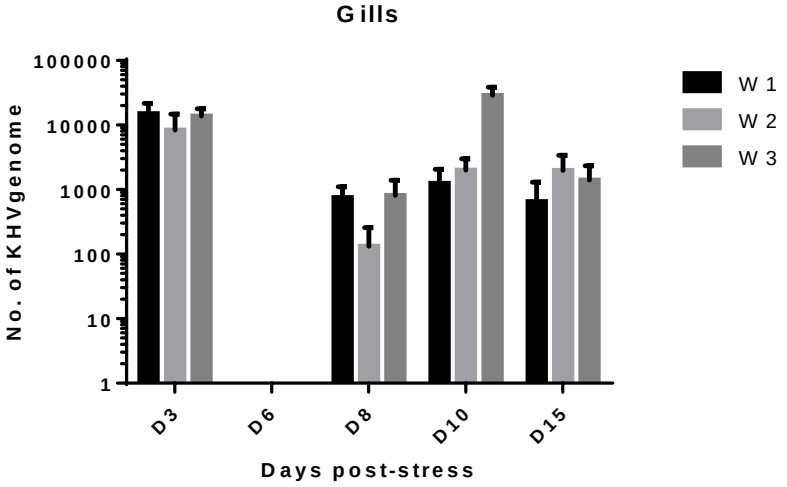


Figure 6: Real-time PCR of KHV PCR amplicons from swabs. (A): Average KHV DNA copy number per vent swab of all six koi in each tank. (B): Average KHV DNA copy number per gill swab of all six koi in each tank. The swabs were diluted 1:1 with Sidestep Lysis Stabilization buffer on days 3, 6, 8, 10, and 15 before running real-time PCR. The X-axis represents day of study and Y-axis is copy number of KHV genome detected. The black bar represents tank 1, lighter gray bar represents tank 2, and darker gray bar represents tank 3.

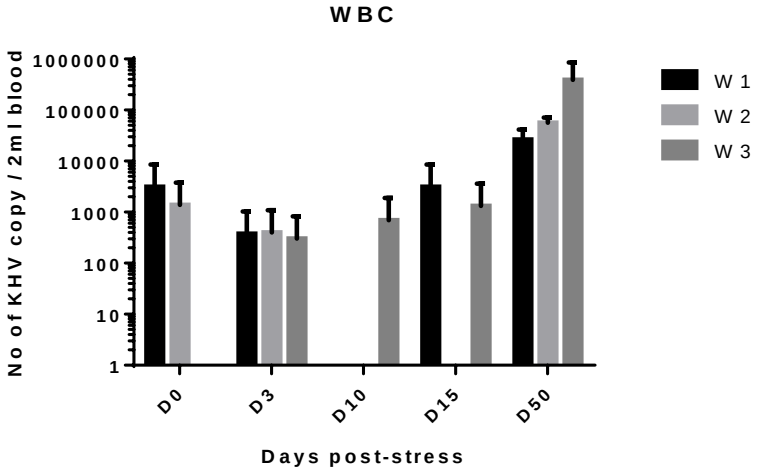


Figure 7: Average KHV DNA copy number in koi WBC isolated from 2 mL of blood on days 0, 3, 10, 15 and 50. The X-axis is the day of study and Y-axis represents average copy number of KHV genome per 2 ml of blood. The black bar represents tank 1, lighter gray bar represents tank 2, and dark gray bar represents tank 3. WBC DNA was adjusted to 0.1 microgram before running real-time PCR.

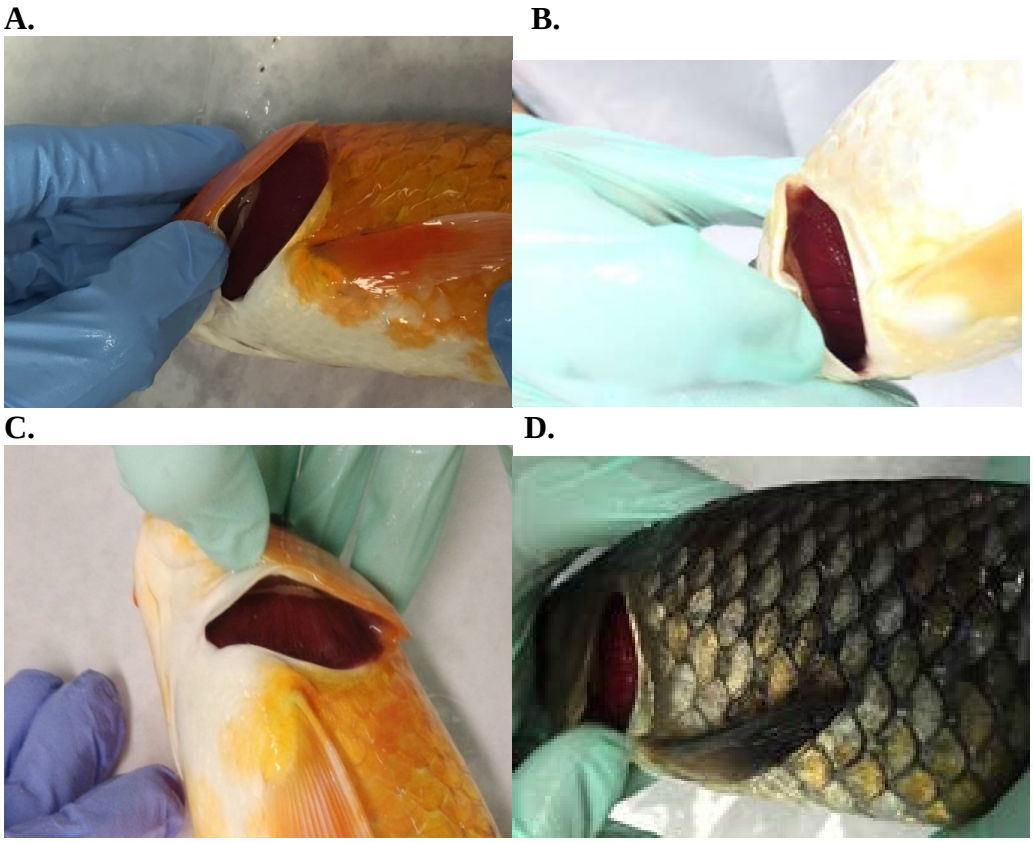


Figure 8: Gills of koi recorded on day 0, 8, 10, and 50 post-temperature stress (A): Picture of koi taken before temperature stress (D0). (B): White mottling of gills on day 8 post-temperature stress. (C): White mottling of gills on day 10 post-temperature stress. (D): Picture of koi after they are no longer under temperature stress (D50).

Figure 9: Hemorrhage in anal vent area of koi from W3 post-temperature stress

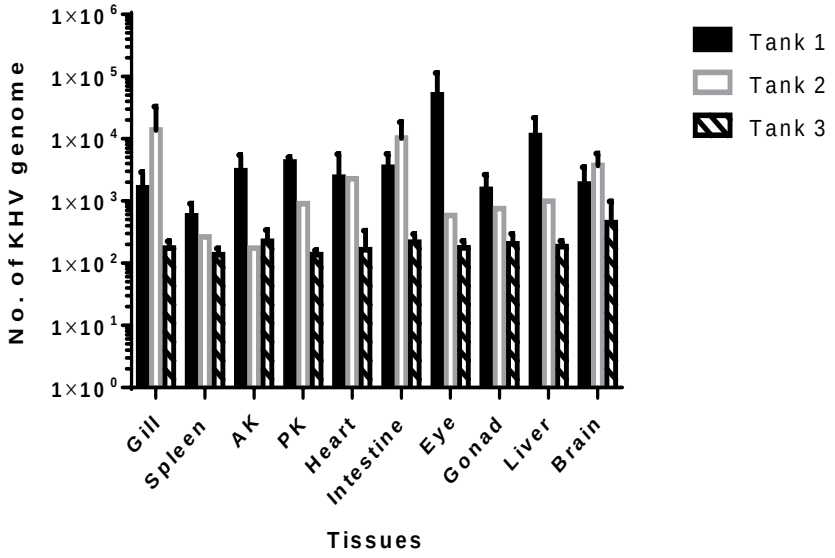


Figure 10: Real-time PCR of KHV DNA in tissues of 3 euthanized koi fish from each tank on day 15 post-stress. The X-axis represents the tissue type and Y-axis refers to copy number of KHV genome detected. The solid black bar represents samples from tank 1, white bar represents samples from tank 2 and stripe bar represents samples from tank 3. Tissue DNA was adjusted to 500 ng before running real-time PCR. KHV DNA copy number converted to per microgram of total DNA in tissues. KHV DNA detected in higher magnitudes in the gill, MI, eye, and liver.

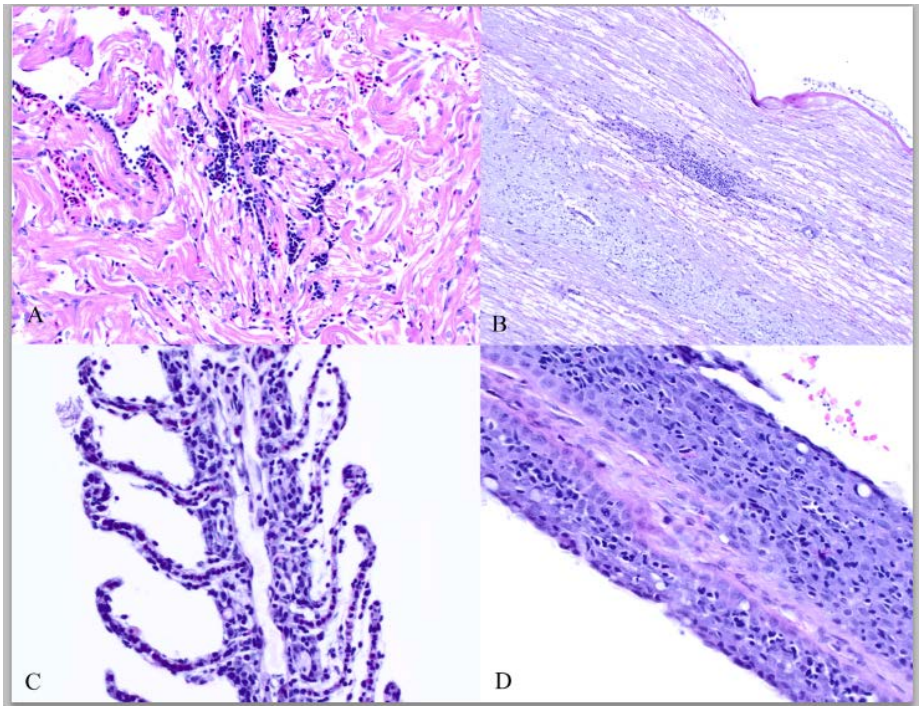


Figure 11: Histological sections of koi following KHV reactivation on day 15 post-stress, stained with hematoxylin and eosin. (A): Heart. Moderate lymphocytic endomyocarditis. (B): Spinal cord. Focal lymphocytic myelitis. (C): Gill. Mild hyperplasia of surface gill epithelium. (D): Fin. Moderate lymphocytic exocytosis and frequent karyorrhectic debris interpreted as necrosis.

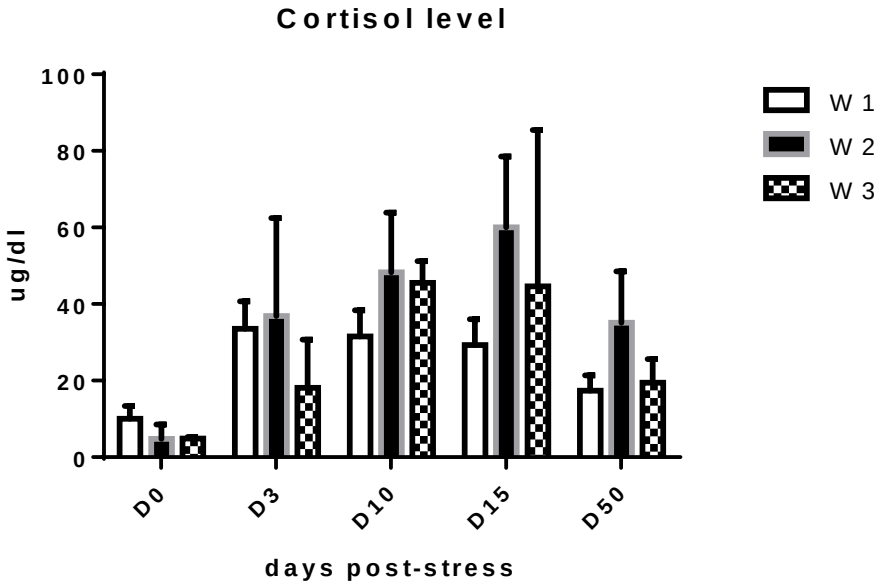


Figure 12: Average cortisol level in koi blood plasma, tested on days 0, 3, 10, 15, and 50. The X-axis is the day of study and Y-axis represents cortisol level measured in ug/dl. The white bar represents cortisol level of koi from tank 1, black bar represents cortisol level of koi from tank 2 and checkered pattern bar represents cortisol level of koi from tank 3. The normal range of cortisol levels for koi is about 20 ug/dl. On day 0, koi from all tanks had normal cortisol levels. The cortisol level after day 3 post-stress was above normal for all tanks. On day 50, the cortisol level for tank 1 and tank 3 are normal, while koi from tank 2 was still above normal.

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