Kinetics of the Chronic Stress Response to Stocking Density and Impacts on Infectious Hematopoietic Necrosis Virus Mortality in Rainbow Trout

Jenna J Klug

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Committee:

Gael Kurath

James Winton

George E. Sanders

Piper M. Treuting

Program Authorized to Offer Degree:

Department of Comparative Medicine

University of Washington

Abstract

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Jenna J Klug

Chair of the Supervisory Committee:

Gael Kurath

Pathobiology Program, Department of Global Health

The goal of this study was to examine the effect of stocking density on the stress response and susceptibility to infectious hematopoietic necrosis virus (IHNV) induced mortality in fingerling rainbow trout (*Oncorhynchus mykiss*) housed in flow through systems at 15°C. To characterize the stress response to holding conditions, fish (1-2 grams) were sorted into one of two selected stocking densities (high density "HD", 20-40 kg/m^3), or (low density, "LD", 4-8 kg/m^3). Stress indices including cortisol levels in serum, cortisol levels in water, and neutrophil:lymphocyte ratios from blood smears were then measured at multiple time points over 21 days. Serum cortisol was increased at 1-2 hours compared to baseline, and again at 14 days, although

increases were only significant for one of the two density treatments at each peak. Water cortisol concentrations were significantly higher in LD tanks compared to HD tanks on day 14, and generally elevated in both groups on day 17 and 21. Neutrophil: lymphocyte ratios were significantly elevated in HD tanks on Day 14 compared to LD tanks and from baseline. In a virus challenge experiment, the effect of density on IHNV mortality with selected M genogroup isolate HG508 ($4x10^2$ pfu/ml) was compared between fish that were sorted into HD or LD conditions and acclimated for 14 days prior to virus challenge (HDa and LDa), and fish that were sorted into HD or LD conditions (HDna and LDna) without acclimation before being immediately challenged with virus at the same time as fish in the density acclimated tanks. Mortality was monitored for 21 days post challenge. No significant differences in cumulative percent mortality were found between HD and LD treatment groups, but overall fish held at higher density and/or fish not acclimated to holding conditions had slightly higher mortality than fish at low density and/or acclimated prior to challenge. Results indicate that: rainbow trout (1-2 grams) did not demonstrate significant differences in stress indices at the density conditions tested; were not significantly affected in the ability to adapt to densities of 4-26 kg/m^3 over a 21 day period, (with the exception of day 14); and that stocking densities up to 26 kg/m^3 did not have an effect on IHNV mortality.

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Dedication:

This work is dedicated to my mom and dad, who have always been and continue to be my inspiration.

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INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV) is a common virus of salmonid fish with significant clinical and economic consequences. It was first reported in fish hatcheries in Oregon and Washington state in the 1950's, and is endemic among many wild and farmed salmonids in the Pacific Northwest [1]. Clinically, infection is most common in young fish up to 2 months old, causing acute lethal disease associated with destruction and necrosis of the blood forming tissues in the kidney (hematopoietic) and spleen [2]. In farmed fish, the disease can have a major economic impact, with reported cumulative mortality rates in young rainbow trout and salmon reaching 90-95% [1].

While it has been widely recognized that fish density is likely a factor in the severity of disease outbreaks in various salmonids [3], relatively few studies have investigated the quantitative effect of density on IHNV transmission. One previously published paper demonstrated a density dependent effect of density on virus transmission in juvenile rainbow trout when challenged by cohabitation with a single IHNV-infected donor fish. In that study, increased rearing densities (0.16 – 8 fish/L) in 2.8-3.1 gram fish resulted in increased probability of transmission [3]; however, the maximum density tested, 8 fish/L is considered very low for commercial aquaculture settings in this size of fish [3, 4, Kyle Martin, geneticist, Troutlodge, Inc, pers. Comm]. In commercial farm settings, fish are often reared at high densities (>30 kg/m^3) due to the economic realities of large-scale production [5,6,7]. Currently, there are no regulations regarding densities at which fish can be farmed [6]. From a review of the effects of density on rainbow trout welfare, a wide range of densities from 15-60 kg/m^3 were reported to be used by commercial rainbow trout farmers in North America [6]. This is confirmed for the Pacific Northwest, where densitities commonly used in rainbow trout aquaculture were estimated to

range from 15-50 kg/m^3, with 25-50 kg/m^3 most common (Kyle Martin and Jim Parsons, Trout lodge Inc, personal communication).

Scientific investigation and recommendations for optimal density ranges in farm fish populations is challenging given the various definitions and measurements for the term density, and the species-specific preferences and tolerances [5, 8 and 9]. Regardless, if densities are inappropriately high for the species, fish may become stressed due to deteriorations in water quality (physiological requirements, "carrying capacity") and/or physical need for space (behavioral requirements, "density tolerance") [9]. Stress is known to increase the susceptibility of fish to infectious diseases through immune suppression and increased metabolic demand, notably over periods of continuous stress from which the fish cannot escape. In these cases, fish must acclimate, but at a reduced performance capacity, if they are to survive [10, 11].

Cortisol is the most frequently used indicator of stress in fish [12]. Its levels in the blood rise rapidly in response to stress, so the stress response can be measured by quantifying cortisol in the blood and tissues, or noninvasively through cortisol released into tank water [13, 14 and 15]. The secondary physiologic responses that occur in the blood and tissues as a result of cortisol release can also be measured and used as stress indices. For example, cortisol is known to induce alterations in leukocyte profiles (relative proportions of white blood cell type) [16]. In general, the response consists of an increase in the number of neutrophils (neutrophilia) and decrease in the number of lymphocytes (lymphopenia) [17]. Because the numbers of neutrophils and lymphocytes are affected in opposite directions, researchers have considered the ratio of neutrophils to lymphocytes, (the N:L ratio), read from blood smear differential leukocyte counts, to be a composite measure of the stress. The cortisol-induced lymphopenia is also accompanied

by a decrease in function (reduced antibody production and cell differentiation), which both contribute to immune suppression [18].

When assessing stress responses, the duration of stressors is important to consider because different effects will be seen depending on whether the stressor is acute or chronic [9, 10 and 11]. Acute stressors (minutes to few hours) are those in which the duration of the stress is shorter than the physiologic response. Examples in fish farm settings include handling, grading, and transport. In salmonids, blood cortisol levels are usually elevated within minutes of an acute stress and typically remain elevated for a period of several hours [12]. In general, neutrophils and lymphocytes in peripheral blood samples decrease within hours after acute stressors, and generally return to baseline in 1-2 days [12, 19]. Chronic stressors (days to months) are those in which the stress is continuous in nature. Examples in industry include crowding and water quality deterioration [11]. Blood cortisol levels in these situations can remain elevated for prolonged periods (days to months) before returning to pre-stress values. Lymphocyte numbers may remain reduced even though stress-induced blood cortisol elevations return to pre-stress levels [12]. In contrast to well-developed models of acute stressors in fish (e.g. handling), the response of fish to chronic stressors and if/how the stressors affect the ability of fish to survive are not as well characterized [10,16]. The purpose of the current work was to examine host population factors (stocking density and stress) that may influence IHN disease in fish farm settings, in a host and age (juvenile rainbow trout 1-3 grams) where mortality due to IHNV is most common. To achieve this goal, two separate studies were performed. The first study assessed the kinetics of the chronic stress response profile at two different density conditions over a 21-day period. Stocking densities were selected based on ranges reported in the literature and personal communication with professional staff at a major commercial rainbow trout

producer in Washington (Kyle Martin and Jim Parsons, Troutlodge, Inc). Stress indices included cortisol concentrations in water and serum, and differential white blood cell counts. The second study investigated the relationship between density and disease induced mortality using a standardized disease challenge model for IHN. To distinguish between the effects of density and potential of water quality deterioration, water quality parameters were measured and maintained at recommended safe levels for rainbow trout throughout the experiments.

MATERIALS AND METHODS

Animals

Fish were research-grade, juvenile, 1-4.4 gram rainbow trout, provided by Trout Lodge, Incoporated (Bonney Lake, WA, USA) (November, May and February lots) and Riverence, LLC (WA, USA) (1 lot, ponded February 2019). Before the experiments, each lot of fish was housed in 278 L circular tanks with flow-through freshwater that had been sand filtered or membrane filtered and then UV irradiated. All tanks were aerated, unless otherwise described, and temperature was kept constant at 15 ˚C. Stock tank fish were fed Skretting (Toole, UT, USA) diet feed size #1 (1mm sinking pellets) once a day, at 1% of their body weight. The fish were acclimated in stock tanks for a minimum 2 weeks prior to use in experiments. All procedures complied with and were approved by the University of Washington Institutional Animal Care and Use Committee.

1. Preliminary Studies

Pilot scale experiments were first conducted to generate/test high and low density conditions and euthanasia procedure.

1.1 Generating High and Low Density Conditions

Fish were randomly selected and sorted into the same size tanks to create high or low density conditions. All tanks were 5 L circular tanks, maintained in flow through conditions. Density conditions were created by adjusting the volume of water in the 5 L tanks. Specifically, as a low density (LD) treatment we used 20 fish in 5 L of water. This represents the standard density used for many years for virus infection studies at the Western Fisheries Research Center. This density is equivalent to 4-8 kg/m^3 (for 1-2 gram fish), and is considered very low for commercial aquaculture. For a high density (HD) treatment we used the same number of fish, but in a five-fold lower volume, meaning 20 fish in 1 L of water. This is equivalent to 20-40 kg/m^3, which falls close to or within the range for commercial trout farm production (15-50 kg/m^3, with 25-50 kg/m^3 most common) (Figure 1). Fish tanks were placed into wetlab tables with effluent collection tubes to direct outflows to trough drains for standard wetlab effluent disinfection. For ease of collecting water samples from tank effluent tubes, all HD tanks, with lower volumes of water, were placed onto supporting platforms (tupperware containers) to raise them approximately 4 inches above the level of the wetlab tables.

An initial pilot study was conducted to assess the feasibility of these experimental density conditions, with the goal of validating methods of water quality measurements and documenting fish survival at proposed densities. Rainbow trout (Trout Lodge, February lot, average 1.0 grams) were acclimated to stock tanks for five weeks and then randomly selected and allocated to single replicate tanks of high (HD) or low (LD) density treatment. Water quality and fish survival were assessed daily for a period of three weeks. Water quality parameters were measured daily from the outflow of experimental tanks using Tetra ® test strips (Blacksburg, VA, USA), Total Gas Pressure (TGP) meter (Pentair, Aquatic Eco-Systems, Point Four Tracker, Apopka, FL, USA) and Hach ® Aquaculture test kit (Fish Farmer's Water Quality Test Kit,

Model FF-1A, Loveland, CO, USA). Flow rates were determined through timed measurements with a graduated cylinder from the tank inflow, over 6 seconds, and rates were calculated in l/min. We planned to adjust flow rates as necessary to maintain water quality parameters at recommended safe levels for rainbow trout throughout the experiment. Criteria for acceptable water quality included dissolved oxygen (DO) above 6 mg/L and un-ionized ammonia (NH_3) below 0.02 mg/L [9].

For subsequent experiments, the same density conditions were used, and water quality parameters were measured after experimental time point collections for cortisol in future studies (at minimum once a week) to minimize disturbances to tanks. All parameters were maintained within acceptable levels recommended for rainbow trout throughout the experiments. Experimental fish were fed 1% body weight every other day.

1.2 Euthanasia

For accurate cortisol measurement in fish serum as an indicator of fish stress it is imperative that euthanasia be rapid, in order to avoid artefactual stress from the euthanasia process itself [17, 20, 21]. Based on published recommendations our goal was to identify the dose of buffered MS-222 that resulted in rapid, consistent euthanasia within one minute, as defined by cessation of all opercular motion within one minute of immersion in buffered MS-222 and non-responsiveness to tactile stimulation, deep pain (e.g. tail pinch). Fish were tested in groups of four at successively increasing concentrations of MS-222 (buffered to pH 7.0-7.5, prepared fresh each day), starting at the lab standard euthanasia concentration of 240 mg/L, and increasing until consistent euthanasia occurred for all fish within 1 minute.

2. Design of Experiment 1: Characterizing the chronic stress response to holding at high and low density conditions for 21 days

Juvenile rainbow trout (Trout Lodge, November 2017 lot) were acclimated to a stock tank for 12 days at 15 ˚C. The 278 L stock tank was supplied an average inflow of 3.23 L/min, and contained 220 fish, with an average weight of 0.84 grams per fish. Therefore the stock tank biomass was 185 grams, stocking density 0.66 kg/m^3, and the biomass loading rate 0.6 kg/Lmin [9]. To initiate the experiment, Time 0 (T_0) samples were taken from the stock tank, consisting of 3 stock tank fish euthanized for serum samples, and one 500 ml water sample. A subset of fish were then randomly selected from the stock tank and sorted into triplicate tanks of 20 fish at low or high density conditions. At 1 hour in HD tanks and 2 hours in LD tanks, a sample of 3 fish were euthanized (one fish per replicate) for blood collection (1 serum cortisol and differential leukocyte count) and one 700 ml water sample was collected from the outflow of each HD and LD tank. The difference of 1 and 2 hour time points between HD and LD groups was due to time needed to process samples, and was determined in statistical tests not to have an effect on stress indices between densities measured at that time point, and the time point is referred to as 1 hr in the rest of the document. Subsequently, the same samples were taken on days 1, 3, 8, 14 and 21. At each time point water samples were always collected across both density conditions before fish samples to avoid acute response to neting of fish. Additional water samples (without matched serum samples) were collected on day 6, 11, and 17. At the paired (water and blood collection) sample time points (1 hr, day 1, 3, 8, 14, 21) two 700 ml effluent water samples were taken per replicate tank and one fish was sacrificed from each replicate tank in the HD and LD groups. One water sample was processed and the other saved. At the water-only collection time points (day 6, 11, 17) one water sample was collected

per replicate tank in the HD and LD groups. All samples were collected between 12:00-18:00 to avoid diurnal effects [26].

2.1 Blood collection and hematologic analysis.

Within 5 minutes of euthanasia, blood was collected from each fish by severing the caudal peduncle with a scalpel blade a few millimeters cranial to the caudal fin. The first two drops of fresh whole blood were placed onto slides (1 drop per slide) and a blood smear was prepared. The blood smear was then left to dry while the remainder of the blood from the fish was collected by centrifugal separation for serum cortisol analysis as described in section 2.2 below. Blood smear slides were stained with Leishman Giemsa the same day of collection. Slides were examined and leukocyte differentials were performed under 40x magnification using a battlement edge count method [22, 23]. Readouts consisted of 200 white blood cell counts, unless the cell population was limited, in which case 100 white blood cells were counted. Specific cell type quantified were: monocyte, lymphocyte, and neutrophils.

In addition to experimental samples, control sample reference ranges were defined by conducting differential white blood cell counts in experimentally naive stock fish from two different lots of Trout Lodge fish, May 2018 (n=9 fish) and November 2017 (n=10 fish), which were housed in the stock tanks for 21 days and 33 days, respectively, prior to euthanasia and blood smear collection. Average individual fish weights and stocking densities were similar, at an average of 1.27 grams, 0.34 kg/m^3 for May fish and 2.13 grams and 0.44 kg/m^3 for November fish.

2.2 Serum collection and cortisol measurement

Serum collection was modified from a technique described previously [24]. Briefly, fish with severed caudal peduncles were placed in a fenestrated 5 ml tube (Falcon Round-Bottom Polypropylene Tubes, Corning, NY, USA), which was then placed inside a 15 ml conical

centrifuge tube (Falcon 15 ml Conical Centrifuge Tube, Corning, NY, USA) for centrifugation at 400 x *g* for 5 minutes at room temperature. The resulting blood sample in the 15 ml centrifuge tube was then collected, and transfered to a 0.6 ml microfuge Eppendorf tube, and centrifuged at 13,000 x *g* for 15 minutes at 4°C. Supernatant (serum) was collected, and stored at -80°C until analysis. Serum cortisol concentrations measured using a cortisol ELISA kit (Salimetrics, State College, PA, USA) following the manufacturer's instructions. Absorbances were measured at 450 nm in a Biotech model ELx808 automated plate reader (Winooski, VT, USA). Raw data optical densities obtained from the plate reader were converted to cortisol concentrations using the Salivary Cortisol (μg/dL) Four Parameter Logistic Fit software (Salivary Cortisol μg/dL, MyAssays Ltd, Brighton, East Sussex, UK) [25]). Data was reported in units of μg cortisol per dL of fish serum. Final data was converted to units of ng/ml. In each assay, samples were measured in duplicate. On the first ELISA run, all serum samples were diluted 1:25 with kit Assay Buffer. Samples that were out of range of the standard curve were tested again at 1:5 dilutions, which generated readouts within the standard curve. The sensitivity of the assay was 0.007 μg/dL (Salimetrics, State College, PA, USA).

2.3 Water cortisol sampling and measurement

Tank water samples of 700 ml were collected from tank outflows into 1 gallon ziplock freezer bags (Hefty, Reynolds Consumer Products, Lake Forest, IL, USA) that were positioned into 1 L tri-corner beakers. Water samples were collected from outflow tubes to avoid stress to fish, and collected prior to serum samples, in the same order (LD replicates before HD replicates). Collected samples were frozen at -20°C within 2 hours of sample collection and stored until cortisol extraction. To assess background cortisol levels in wetlab water, water samples were collected from the inflow line connected to the experimental tanks (negative control). To assess

the recovery rate for our water cortisol protocol, positive controls were made by spiking inflow water samples with a known amount of steroid from the cortisol ELISA kit to achieve a calculated concentration of [10 ng/L].

The protocol for processing water samples was similar to previously described methods [13, 27, 28, 29, 30]. In summary, samples were thawed overnight for up to 18 hours at room temperature [13]. Once thawed, 500 ml of each sample was measured with a graduated cylinder, and passed through a filter (Nalgene Rapid Flow Sterile Disposable Filter Units with PES membrane, 0.45 µm pore size, ThermoScientific, #295-454, Waltham, MA, USA) into a receiver, which was either a 500 ml polypropylene receiver unit, (Nalgene, Thermo Scientific, #2105-0016, Waltham, MA, USA) or a 1000 ml pyrex glass reusable media storage bottle (Waltham, MA, USA). The water sample was then passed through a Sep Pak Vac C18 cartridge (Waters#WAT043395, Milford, MA, USA) attached to a vacuum manifold (Millipore #WP6111560, Jaffrey, NH, USA) that applied vacuum to 3 samples simultaneously. Each cartridge was conditioned prior to receiving the water sample by flushing 5 ml of 100% methanol and 5 ml of distilled water through the cartridge, at a rate of 5-10 ml/min. Each water sample was then loaded onto a cartridge at a constantly monitored rate of 2-10 ml/min. Thus, each set of 3 water samples was processed through the sep pak cartridge in approximately 60-120 minutes. Tygon tubing was used to connect samples to cartridges (Tygon #57629, Akron, OH, USA). After loading, cartridges were removed from the manifold, the ends were covered with parafilm, and the samples were stored at -20°C until eluted. For elution, cartridges were thawed at room temperature for at least 30 minutes, then steroid hormones were eluted from the cartridges with 5 ml of ethyl acetate (Sigma Aldrich, #319902, Saint Louis, MO, USA) via positive displacement with a 10 ml luer lock syringe at a rate of 2-10 ml/min, followed by a 4 ml purge of air. Elutions

were collected into a 15 ml conical centrifuge tube (Falcon 15 ml Conical Centrifuge Tube). The amount of eluent in each 15 ml conical tube was recorded, and each roughly 5 ml volume of eluent per sample was then further divided into five 1.7 ml eppendorf tubes containing 1 ml of eluent per sample. These eluted samples were then immediately dried in a vacuum concentrator (SpeedVac, OligoPrep OP120 Savant, Holbrook, NY, USA) for 6 hours. The resulting dried steroid pellet was stored at -20°C until resuspension.

A variety of resuspension solvent options were tested due to methodological challenges of resuspending the dried pellets in the kit assay buffer. The solvent options tested were based on literature review of solvents that have been used for cortisol extraction from tissues and water [28-32]. Solvents tested included absolute ethanol (5% and 10%), Bovine Serum Albumin (BSA; 0.2% and 2%), Phosphate Buffered Saline with 1% Gelatin (PBSG) and 0.2% BSA with 5% ethanol or 10% ethanol. Of these, 0.2% BSA with 10% ethanol was selected as the best option, upon confirming it did not change the final concentration of known cortisol standards from the ELISA kit.

The dried samples were subsequently resuspended in a multi-step process that involved adding resuspension solvents sequentially, intermixed with manual titration and rack shaking. Specifically, the steps were: absolute ethanol (10% total volume, 30 μL) added directly onto the bottom of the 1.7 ml eppendorf tube, then the tubes were shaken on a rack shaker (Melrose Park, IL, USA) at ~1100 rpm for 2 hour. The resuspension volume in each tube was then brought up to 300 μL with 0.2% BSA, mixed by manual pipette titration, and then placed on a rack shaker at the same settings (1100 rpm) for another hour. The samples were stored at -20°C, and thawed within the next 1-2 days and shaken again at ~1100 rpm for 2 hours. After this last shaking step, the 5 samples that were split into 1.7 ml tubes were recombined into one 2 ml tube (for a total

resuspension volume of 1.5 ml/sample), and stored at -20°C for 1 week or less on average until the ELISA assay. The difficulty in resuspending the dried cortisol pellets was unanticipated, and constituted a major labor cost to the determination of cortisol concentration in water samples.

Water cortisol concentrations were measured using a cortisol ELISA kit (Salimetrics, State College, PA, USA) following the manufacture's instructions. In each assay, samples were quantified in duplicate reactions. On the first ELISA run, all water samples were diluted 1:10 with kit Assay Buffer. Samples that were out of range of the standard curve were tested again at 1:5 dilutions, which generated readouts within the standard curve. The sensitivity of the assay was less than 0.007 ug/dL. Absorbances were measured at 450 nm in a Biotech model ELx808 automated plate reader (Winooski, VT, USA). Raw data optical densities obtained from the plate reader were converted to cortisol concentrations using the Salivary Cortisol (*ug*/dL) Four Parameter Logistic Fit software (Salivary Cortisol ug/dl, MyAssays Ltd, Waltham, MA, USA [25]). Final cortisol concentrations (in ng/g/hr) were calculated based on previously derived equations for flow through systems [13, 14], in which the cortisol concentration of the water sample (ng/L) is multiplied by flow rate (L/hour) and divided by tank biomass of fish (g). Thus final data is reported in units of ng cortisol released per gram of fish per hour, taking into account total numers of fish and volume of water in each tank.

3. Experiment 2: Impacts of acclimation and holding densities on mortality due to virus exposure

3.1 Virus and Host

For IHNV exposures, we tested a single M genogroup IHNV isolate, HG508, which was originally isolated in our laboratory by G. Kurath from farmed rainbow trout in the Hageman Valley of Idaho in 2014. A stock of the HG508 virus strain was used at a single dose of ($4x10^2$ pfu/ml) to expose replicate groups of juvenile fish using a standard one hour static immersion

challenge [33]. This dose was selected to target approximately 50% mortality in LD tanks, using results from pilot studies.

The fish tested in the virus challenge experiment were juvenile rainbow trout from Riverence LLC, and weighed an average of 1.3 grams.

3.2 Virus Challenge Experiment

To assess the effect on IHNV mortality of chronic stress following prior exposure of fish to high density conditions, 160 fish were randomly sorted into HD or LD tank density conditions, with 20 fish per tank in 4 replicate tanks each, and held for 14 days prior to virus challenge. These are referred to as acclimation groups HDa (high density acclimation) and LDa (low density acclimation) respectively.

To control for the effect of fish density and acute stress during challenge with IHNV, additional subsets of 20 fish were randomly selected from the stock tank and sorted into HD or LD tank density conditions, 4 replicate tanks each, and then immediately challenged with virus at the same time as the acclimated groups were challenged with virus. HD and LD tanks that were sorted and challenged immediately with virus are referred to as non-acclimation groups HDna (high density no acclimation) and LDna (low density no acclimation) respectively.

For the 4 replicates in each of the four acclimation and density condition tested, 3 tanks were challenged with virus, and 1 was mock challenged with media that had no virus. Fish were challenged by batch immersion in static conditions with 4×10^2 pfu/ml of virus for one hour, in the same volume and tanks that they were housed in throughout the experiment, except that the tanks were converted to static conditions for the 1 hour virus challenge. After the 1 hour of static virus exposure, water flow was turned back on, and fish were monitored daily until study endpoint 21 days post challenge. The number of daily mortalities in each tank was recorded, and

fish that died were removed and were saved at -80C. At 21 days post challenge, all survivers were euthanized and counted to terminate the experiment.

Statistical analyses:

Data from the first experiment (stress kinetics at high and low density) were analyzed in Prism (GraphPad Software, La Jolla, CA, USA). Statistical significance was set at a P value of less than 0.05. Stress indices (serum cortisol, water cortisol, and neutrophil and lymphocyte ratios from white blood cell counts) were analyzed using a 2 way ANOVA, with density and time as the two factors. A Tukey post-hoc test was performed for pairwise comparisons. Data from the viral challenge experiment was assessed by survival analyses using R studio (Version 1.2.1335, Boston, M, USA). Statistical significance was set at a P value of less than 0.05. Differences in survival kinetics were assessed using Wilcoxon test. Final cumulative mortality data was analyzed using chi square test, using P value of less than 0.05.

RESULTS

1.1 Pilot test of water quality and fish survival in HD and LD holding conditions.

The experimental outputs of the first pilot study consisted of daily water quality measurements, flow rates, temperature, and fish survival. A summary of the outputs from each single tank replicate (mean, median, minimum maximum) is represented in Table 1.

The results of the measurements indicated that experimental tank conditions were sufficient and stable for use in experiments. No fish morbidity or mortality occurred over the three week duration, and all water quality parameters measured were within normal limits. No adjustments were needed in flow to maintain DO or NH_3 in either density tank condition, and there was very little fluctuation between parameters of tanks each day. Hach ® parameters matched or approximated test strip parameters, notably for DO, carbon dioxide (CO_2) and alkalinity (KH).

1.2 Pilot test of rapid euthanasia for accurate serum cortisol measurements

MS-222 doses were tested in the range of 240-800 mg/L. A dose of 800 mg/L, buffered to a pH of 7.0-7.5, resulted in rapid euthanasia meeting the desired criteria of within 1 minute. This dose was therefore selected as the dose to be used for all subsequent experiments.

2. Experiment 1: Define chronic stress response to high and low density conditions

The general goals of this experiment were to characterize the magnitude and kinetics of the cortisol stress response in rainbow trout over a chronic duration of 21 days to high and low density treatments, as measured by stress indices of N:L ratios (obtained from differential leukocyte counts), serum cortisol and water cortisol.

2.1 Differential white blood cell counts

Results of differential blood white cell counts obtained from experimentally naive rainbow trout (Trout Lodge, May 2018 and November 2017 stock) provided reference levels that corresponded to differential white blood cell count ranges reported in the literature for juvenile rainbow trout (Table 2, [34,35]). Lymphocytes encompassed the majority of leukocytes counted (over 90%), followed by neutrophils (4.1% - 5.5%) and monocytes (1.8-2.7%). Eosinophils were rare to absent on blood films and not included in analyses.

For data from experiment 1, the relative proportion of neutrophils to lymphocytes was compared as a ratio (N:L ratio) to assess for stress induced changes in leukocyte profiles over a time course of 21 days [17], (Table 3). The average baseline N:L ratio was 0.03 (range 0.02 – 0.04). In general, both density conditions showed a trend of mild elevations in N:L ratios at 1 hr post sorting through Day 3 (up to 0.16 in HD groups and 0.11 in LD groups), followed by a return to baseline values on Day 8. The N:L ratios in HD groups increased significantly on day 14 to approximately 20x baseline levels (average 0.62, $P<0.0001$), and compared to LD groups

(average 0.07, P<0.0001), Figure 2. On day 21, N:L ratios were similar in both HD (average 0.16) and LD (average 0.14) conditions, with a trend of slightly increased values from baseline.

There was a significant interaction between density and time accounted for 46.23% of total variation ($F_{10,34}$ = 11.20, P<0.0001). Density accounted for 15.10% of the total variance ($F_{2, 34}$ =18.29, P<0.0001), and time accounted for 24.13% of the total variance ($F_{5, 34}$=11.70, P<0.0001).

2.2 Serum Cortisol Response

The average baseline serum cortisol concentration was 1.57 ng/ml (range 0.98 – 2.42 ng/ml). The cortisol response in both densities demonstrated similar patterns of changes over time, characterized by a rise at 1 hour, to an average of 15 ng/ml in HD groups and 19 ng/ml in LD groups, followed by a decline over time to levels below baseline on Day 8. On day 14, serum cortisol levels were elevated again in both groups (average HD 16 ng/ml, average LD 6 ng/ml). On day 21, levels in both groups had declined relative to day 14 measurements, and were near baseline ranges.

Compared to values at baseline (time zero), serum cortisol levels were significantly elevated in LD groups at 1 hour post sorting (P=0.01). and in HD groups on Day 14 (P=0.05), Figure 3. There were no significant differences between density group groups at any time points. Overall, time was considered to significantly affect the results, accounting for 22.95% of the total variation ($F_{5,36}$=3.20, P=0.02). A trend of density accounting for total variance was noted ($F_{2,36}$ =2.93, P=0.07).

2.3 Water Cortisol levels

The baseline measurement of cortisol in the stock tank at Time 0 was 2.5 ng/g/hr. The general pattern of water cortisol release rates in both HD and LD groups decreased to levels below baseline on Day 1, and gradually increased over time to a peak on day 14 in LD groups (average

9 ng/g/hr), and day 17 in HD groups (8.8 ng/g/hr). Levels in both groups then declined on day 21 to average cortisol release rates of 5.3 and 6 ng/g/hr in HD groups and LD groups respectively.

Water cortisol values in LD groups were significantly elevated compared to HD groups at day 14 (P=0.03), Figure 4. There were no significant differences in either density group compared to baseline over time. The average recovery rate for spiked samples of 10 ng/L was 79% (n=2). Blank samples from headbox inflow were lower than test samples (tanks that contained fish). The pattern of changes in water reflected the pattern of changes in serum in both LD and HD conditions over the 21 day sample period, as has been previously validated for this species [13, 14], which is described further in the following section 2.4.

2.4 Comparison of Serum and Water Cortisol Profiles

In general, water cortisol was observed to be reflective of serum cortisol concentrations over the 21 day period, but with some indications of temporal delay between peaks in serum and peaks in water (Figure 5). In serum, cortisol concentrations rise to a peak in both density conditions at the 1 hour sample time point, and decrease at Day 1. The 1 hour peak is not reflected in water cortisol concentrations, but HD and LD groups decrease through Day 1.

The pattern of changes in LD water samples generally follows the pattern of changes in LD serum samples, in that both LD water and serum increase from Day 1-3. Serum cortisol between time point 3 and 8 decreases, which is reflected in the water only measurement of time point 6 for LD conditions. The decreased levels in the LD serum at day 8 are delayed temporally in the water until Day 11. Then, both follow the sample pattern of changes from Day 14-21, which is a peak at Day 14, followed by a decrease through Day 21.

The pattern of changes in cortisol levels in HD water samples generally follows a temporal delay from HD serum samples. Serum cortisol concentrations in HD groups decrease from Day 1-8,

but are generally higher than LD levels at Day 1. The water cortisol levels in HD tanks gradually increase after Day 1 measurements, through day 6. The successive decrease from Day 3-8 in the HD serum is followed by a similar successive decrease in HD water cortisol levels from Day 6-11. HD serum cortisol increases to a peak at Day 14 – the corresponding peak in HD water samples is mirrored at Day 17, and both water and cortisol samples show decreased levels on Day 21.

3. Experiment 2: Impacts of acclimation and holding densities on mortality due to virus

The relationship between density and disease induced mortality was tested using a standardized disease challenge model for IHN in subgroups of fish that had been acclimated to density condition prior to challenge (HD and LD) and groups that had not been acclimated prior to challenge (Figure 6).

3.1 Selection of optimal virus challenge dose

A series of pilot studies (Table 4) were conducted to determine the optimal virus challenge dose with our selected M group isolate, HG508. In the experiment designated SAD, for "Stress and Disease" fish were challenged with virus in triplicate groups n20 fish/tank and there was a single tank of n20 fish/group for mock challenge. Four treatment groups tested were high density acclimated (HDa), low density acclimated (LDa), high density no acclimation (HDna), low density no acclimation (LDna). Acclimation period for holding fish at the densities prior to challenge was 10 days**.** Final cumulative mortality rates at 21 days were 80-100% in LD/HD viral challenge tanks regardless of preexposure.

A second pilot study designated SADR, for "Stress and Disease Repeat" In the experiment designated SADR, fish were challenged with virus in duplicate groups of n11 fish/tank, and mock challenge groups were a single tank of n20 fish held at HD condition, with a single mock

tank of n20 fish held in HD conditions. There were no acclimated groups in SADR. The experiment was ended at 21 days. Final cumulative percent mortality was 45% in both LD replicates and ranged from 82-91% in HD replicates.

The experiment TMI was performed in by others in the laboratory using fish from a different source rainbow trout (Riverence, February 2019, average 0.3 grams) triplicate groups of n20 fish/tank, with a single tank of n20 fish/group for mock challenge ad a single dose of 4×10^2 pfu/ml. There were no acclimated groups in TMI. Final cumulative percent mortality ranged from 50-60% at 14 days.

Based on these pilot studies, the dose 4×10^2 pfu/ml was selected to be used in experiment 2.

3.2 Mortality in HD and LD treatment groups

Mortality curves were generated for each treatment group by showing the average cumulative percent mortality of replicate tanks in HDa, LDa, HDna, LDa (n=3 per group) as well as pooled mocks (n=4 total) (Figure 7). The mortality curve for virus treated tanks was typical for IHNV kinetics in terms of onset, duration, and mortality plateau. In general, the first mortalities started to occur in tanks on Day 3, with the bulk of mortality occurring between days 5-14, and starting to plateau at day 15.

In addition, the clinical signs noted were typical for IHNV, including one or more of the following in at least some fish from each treatment group: exophthalmia, periocular and fin hemorrhage and skin darkening. Survival analysis found no significant differences between virus exposed groups. However, a trend of greater mortality in non-acclimated groups, compared to acclimated groups was observed, wilcoxin (P=0.07) (Figure 7 and 8). The average final cumulative percent mortalities compared between acclimated and nonacclimated groups were: HDna (82%) versus HDa (74%), and LDna (74%) LDa (65%). Thus, at each density the non-

acclimated treatment groups had an average of 8-9% higher mortality. Within acclimated groups, those at high density also had 8-9% higher mortality than the low density groups.

DISCUSSION

The results of this study did not support our hypothesis that the high density condition tested (20-40 kg/m^3) would be consistently more stressful than the low density condition (4-8 kg/m^3) over a 21 day period. Density also did not affect IHNV mortality.

The stress response kinetics to density in the first experiment demonstrated two main findings. First, there was evidence of an acute stress response at the 2 hour time point, which was significantly higher than baseline in LD fish serum, and showed a trend for significance at the 1 hour time point in HD fish serum (P=0.07). The second main finding was on day 14, where multiple stress indices from serum samples indicated a significant peak in HD tanks with increased serum cortisol levels and N:L ratios compared to baseline, while water samples showed higher cortisol levels in LD tanks. Collectively, the baseline values and acute peak values of cortisol concentrations in water and serum cortisol were consistent with ranges reported for rainbow trout in the literature [11-14, 19, 36, 37, 38]. Subsequent rises or prolonged elevations in serum and water cortisol concentrations after initial acute stressors have also been reported in the literature with temporal measurements of stress response in rainbow trout, usually under conditions associated with chronic or sequential stressors (confinement, crowding, acclimation post transport) [7, 19, 37, 38]. One potential explanation for the second peak was due to capture (netting) and sorting into stock tanks over time. Similar stress kinetics of plasma cortisol have been noted to occur in fingerling rainbow trout exposed to multiple stressors [37]. In that study, a similar rise in blood cortisol was noted post capture, followed by subsequent

decreases that returned to baseline levels by day 8, and started to increase again at day 11-14 [37].

Experiment 1 also demonstrated that water cortisol concentrations in our experimental setup approximated serum cortisol concentrations in both density conditions over time. Measurement of steroid hormone from water was based on initial studies that demonstrated periovulatory sex steroid pheromones released by female goldfish were detectable in the water, and that the pattern of release of the steroid approximated its secretion into the plasma [14]. The concept was adapted for other steroids, including cortisol, followed by validation of water cortisol measurements in several fish species, including rainbow trout (adults) housed in flow through systems [13]. We validated the measurement in our experimental setup and life stage (fingerling) of rainbow trout, demonstrating the pattern of changes of cortisol in water samples followed the changes in serum samples obtained at the same time points over the 21 day period. In our study the water cortisol data indicated a significant difference at day 14 between HD and LD conditions, with LD tanks having higher water cortisol than HD tanks. Water cortisol measurements are considered to represent integrated responses of the population in the tank, which may explain why the difference was detected in water, but not serum, at that time point. In addition to representing differences in individual versus population responses, water cortisol measurements also represent a longer snap shot in time, as steroid levels need to accumulate in the water over time, and generally have a minimum time lag to detection of 30 minutes in static systems [14]. In recirculating systems, a time lag of several hours is possible [13, 14]. Considering the results of our study, it is possible the peak of the acute stress response occurred after the time points we selected for water measurements, taking time to accumulate in the flow through system at some time point between 2 – 24 hours. Similarly, this may explain the trend

between water samples and serum samples between Day 14-Day 17, where the significant increase in serum cortisol in HD tanks from Day 14 compared to baseline is followed by an increase in water concentrations at Day 17 time point, and the trend of lower average serum cortisol concentration in LD tanks at Day 14 is followed by a decrease in water cortisol at day 17.

Differential leukocyte counts performed on naive experimental fish in our study were consistent with ranges reported for rainbow trout in the literature [34, 35]. Samples obtained from stock fish at baseline time 0 of our experiment were also generally within these ranges, within the exception of one fish with slightly high monocytes (7%), which was only mildly higher than our reference ranges or those in the literature, and may likely represent individual variation. Neutrophil:lymphocyte ratios were increased significantly on day 14 in HD tanks, both compared to baseline measurements and also compared to LD tanks at the same time point. Changes in differential white blood cell parameters have been suggested in some case to be considered more reliable indicators of chronic stress than blood cortisol levels [17]. For example, previous studies in rainbow trout have shown that crowding produced a transient increase in plasma cortisol levels which acclimated after 10 days, but a prolonged reduction in circulating lymphocytes (21 days), in adult rainbow trout and brown trout [19]. Although there are other mechanisms that may cause a prolonged lymphopenia other than stress, the authors suggest use of differential lymphocyte counts and assessment of N:L ratios may be beneficial to use in conjunction with other stress indices, notably over periods of chronic stress. Inter-renal production of cortisol may be inhibited by continuous stimulation of the hypothalamic pituitary axis, resulting in decreased cortisol levels [38]. In these cases the fish can appear to have compensated, but may be still be experiencing a disruption of homeostasis due to a stressor,

manifested in other parts of the pathway. To the author's knowledge, few studies to date have measured simultaneous stress indices of serum, water and N:L ratios in rainbow trout or other fish species.

The viral challenge experiments also did not demonstrate an impact of density on IHNV mortality. The mortality curve (onset, duration, and plateau) and clinical signs were consistent with those expected with IHNV in rainbow trout. The density ranges tested represent the lower end of stocking density used for this size of fish in commercial trout farm settings (Kyle Martin, geneticist, Troutlodge, Inc, pers. Comm). It is possible that the high density treatment used in this experiment was too low to produce the expected effects. Ogut et al [3] demonstrated a density dependent effect of IHNV transmission by cohabitation. Based on the size of fish at the time of the study and tank volume, the range of densities tested was 0.03-24.8 kg/m^3, and IHNV transmission was demonstrated to occur at 0.45-24.8 kg/m^3. The authors note that the highest density tested was much lower than densities reported to be used in the field. In our study, fish were challenged in batch by immersion, and based on the weight of the fish, the high density conditions were roughly equivalent to the highest density tested by Oget et al [3], approximately 26 kg/m^3 at the time of challenge and prior to onset of mortalities. It is possible a density dependent effect would have been observed at higher densities. The densities selected in this case were selected based on the shape, size and volume of tanks as well as the size of fish in them. Considering fish size in relation to volume of water in the tanks, if the challenge was to be repeated at a higher density in the same tanks, then the method of obtaining densities would likely have to be switched to holding fish at the same volume of water (5L), but adjusting the numbers in the volume of water to create desired HD and LD conditions.

Tanks of fish provided with 14 days in which to acclimate prior to challenge with IHNV showed a limited effect on mortality post challenge, with tanks that were not acclimated having higher mortalities than those that were not acclimated. Nonacclimated fish experienced multiple potential acute stressors on the day of challenge (netting, transfer challenge), as opposed to acclimated fish. Therefore, future studies could employ measures of stress indices to help characterize changes in acclimated versus non-acclimated fish at challenge as well as the effects of even higher densities that might approach the upper end of densities found in commercial farms.

The results of the study collectively did not indicate significant differences in stress indices or on IHNV mortality at the density conditions tested (4-26 kg/m^3). For applications and conclusions, it is important to consider that the fish in this case were domesticated. Compared to domesticated hatchery-reared fish, wild rainbow trout can exhibit more extreme stress responses when exposed to stressors encountered with common management practices. For example, stress induced responses measured by primary indices of plasma cortisol and secondary stress indices of glucose, have shown to be significantly higher in wild rainbow trout than hatchery reared counterparts when exposed to common management practices of confinement in a net and electroshock [39]. The domesticated fish used in our experiments are likely more resistant to stress, and thus the densities used here could still adveresely affect wild populations.

Lastly, for management implications, the collective data obtained from these studies indicate that at the densities and flows tested, there was no effect on water quality, stress or other measures of fish welfare, and thus these densities should likely be considered to be within an acceptable range for the species.

Tables

Table 1: Water quality and fish survival from pilot experiment testing the HD and LD conditions

	Acceptable	Average		Median		Range		Hach test
		HD	LD	HD	LD	HD	LD	
NH3/NH4	< 1 mg/L	0	0	0	0	0	0	NH3 - 0
KH	> 20 mg/L	32	33	30	30	25-40	30-40	34.2
pH	6.5-9	7	7	7	7	7	7	7.5
CO2 (calculated)	< 10-15 mg/L	7.6	7.7	7	7	5.8-9.3	7-9.3	
CO2 (Hach)		8.5	8	10	10	5-10	5-10	
DO mg/L (meter)	>6 mg/L	9.5	9.6	9.6	9.6	9-9.8	8.8-9.8	9
Other Measurements								
flow rate (L/min)		0.32	0.3	0.33	0.31	0.24-0.37	0.07-0.65	
temperature	10-15 C	15	15	15	15	14.2-15.9	14.2-15.9	
morbidity/mortality	0	0	0	0	0	0	0	

Density conditions were single replicates of a low density (LD) tank, which contained 20 fish in 5 L of water, equivalent to 4-8 kg/m^3 (for 1-2 gram fish), or a high density (HD) tank of 20 fish in 1 L of water, equivalent to 20-40 kg/m^3, which falls close to or within the range for commercial trout farm production (15-50 kg/m^3, with 25-50 kg/m^3 most common). Water samples were measured daily over 21 days.

Table 2: Reference ranges for differential white blood cell counts in juvenile rainbow trout

	May Lot			**November Lot**			**Reference Intervals[a]**
	Mean%	SEM	Range%	Mean%	SEM	Range%	
Lymphocytes	91.8	1.4	85.5-97.5	94.2	0.7	90 - 97	0 - 5
Neutrophils	5.5	1.2	0.5 - 10.5	4.1	0.5	2.5 - 8	89 - 98
Monocytes	2.7	0.4	0.5 - 4.5	1.8	0.4	0.5 - 4	1 – 9

[a] References intervals for differential white blood cell count intervals measured in juvenile rainbow trout [34, 35].

Table 3. Differential WBC Counts and N:L ratios for fish samples in Experiment 1

		% Monocytes			% Lymphocytes			% Neutrophils			N:L Ratio		
Time		**Mean**	**SEM**	**Range**	**Mean**	**SEM**	**Range**	**Mean**	**SEM**	**Range**	**Mean**	**SEM**	**Range**
Baseline	**Stock**	4.3	1.6	1.6 - 2	93	1.6	90.5 - 96.0	2.7	0.7	2.0 - 4.0	0.03	0.01	0.02- 0.04
Hr	**HD**	4.2	1.4	2.5 - 7	90.7	3.6	83.5 - 94.5	5.2	2.2	3.0-9.5	0.06	0.03	0.03-0.11
	LD	2.3	1.3	1.0 - 3.5	94	2	92.0 - 96.0	3.8	0.8	3.0 - 4.5	0.04	0.01	0.03- 0.05
Day 1	**HD**	2.5	0.8	1 - 3.5	90.3	3.8	83.0 - 95.5	7.2	3.2	3.5 - 13.5	0.08	0.04	0.04 - 0.16
	LD	1	0	1	91	1.8	89.0 - 94.5	8	1.8	4.5 - 10	0.09	0.02	0.05-0.11
Day 3	**HD**	0.3	0.2	0 - 0.5	96.2	1.4	93.5 - 98.0	3.5	1.3	1.5 - 6.0	0.04	0.01	0.02 - 0.06
	LD	0.3	0.3	0 - 0.5	92.3	1.3	91.0 - 93.5	7.5	1.5	6.0 - 9.0	0.08	0.02	0.06-0.10
Day 8	**HD**	1.3	0.9	0 - 3.0	96.7	1.4	94.0 - 98.5	2	0.8	0.5 - 3.0	0.02	0.01	0.01 - 0.03
	LD	0.5	0.3	0 - 1.0	96.5	1.3	94.0 - 98.0	3	1.3	1.5 - 5.5	0.03	0.01	0.02 - 0.06
Day 14	**HD**	1.7	0.7	0.5 - 3.0	61.5	5.1	51.5 - 68.0	36.8	5.3	29.0 - 47.0	0.62	0.15	0.43 - 0.91
	LD	0.5	0.3	0 - 1.0	93.3	1.2	91.5 - 95.5	6.2	1.4	3.5 - 8.0	0.07	0.02	0.04 - 0.09
Day 21	**HD**	1	0.6	0 - 2.0	85.2	0.7	84.0 - 86.5	13.8	1.3	11.5 - 16.0	0.16	0.02	0.13 - 0.19
	LD	0.7	0.2	0.5 - 1.0	87.3	3.2	81.0 - 90.5	12	3	9.0 - 18.0	0.14	0.04	0.10 - 0.22

Table 4. Preliminary Experiments Used to Determine Optimal Virus Challenge Dose

In the experiment designated SAD, fish were challenged with virus in triplicate groups n20 fish/tank and there was a single tank of n20 fish/group for mock challenge. Four treatment groups tested were high density acclimated (HDa), low density acclimated (LDa), high density no acclimation (HDna), low density no acclimation (LDna). Acclimation period for holding fish at the densities prior to challenge was 10 days**.** In the experiment desingated SADR, fish were challenged with virus in duplicate groups of n11 fish/tank, and mock challenge groups were a single tank of n20 fish held at HD condition, with a single mock tank of n20 fish held in HD conditions. There were no acclimated groups in SADR. The experiment TMI was performed in by others in the laboratory using fish from a different source, triplicate groups of n20 fish/tank, with a single tank of n20 fish/group for mock challenge.

Study Title	**Challenge Dose of IHN strain Hg508**	**Rainbow Trout Source, Lot**	**Average Fish Weight**	**Virus-challenged Treatment Groups[a]**	**Observation Period Post Challenge**	**Final Mortality LD virus**	**Final Mortality HD virus**
Pilot SADa	5x103 pfu/ml	Trout Lodge, May 2018 lot	1.27 grams	HDa LDa HDna LDna	21 days	80-100%	80-100%
Pilot SADR[a]	$4x10^2$ pful/ml	Trout Lodge, May 2018 lot	4.4 grams	HDna, LDna	28 days	45%	82-91%
Pilot TMI[a]	$4 x10^2$ pfu/ml	Riverence, February 2019	0.3 grams	LDna	14 days	50-60%	NA

[a] HD = high density, LD = low density, a = acclimated, na = no acclimation; Pilot SAD is for "Stress and Disease", SADR is for Stess and Disesase Repeat; TMI was conducted by others in the laboratory

Figures

Figure 1: Photo of High and Low Density Conditions

Density conditions were created by adjusting the volume of water in the 5 L tanks. Specifically, as a low density (LD) treatment we used 20 fish in 5 L of water. This represents the standard density used for many years for virus infection studies at the Western Fisheries Research Center. This density is equivalent to 4-8 kg/m^3 (for 1-2 gram fish), and is considered very low for commercial aquaculture. For a high density (HD) treatment we used the same number of fish, but in a five-fold lower volume, meaning 20 fish in 1 L of water. This is equivalent to 20-40 kg/m^3, which falls close to or within the range for commercial trout farm production (15-50 kg/m^3, with 25-50 kg/m^3 most common).

Figure 2. Neutrophil: Lymphocyte Ratios in serum of fish held in HD versus LD conditions

Relative ratios of neutrophils to lymphocytes generated from differential leukocyte counts of serum from rainbow trout held in high and low density conditions and smapled at specified time points over a 21 day period in rainbow trout. Data shown as mean +/- SEM of pooled data from 3 fish/time point (1 from each of 3 replicate tanks of each density condition per time point). **** indicate a significant difference of P<0.0001 compared to baseline. #### indicate a significant difference of P <0.0001 between the two density conditions (2-way ANOVA).

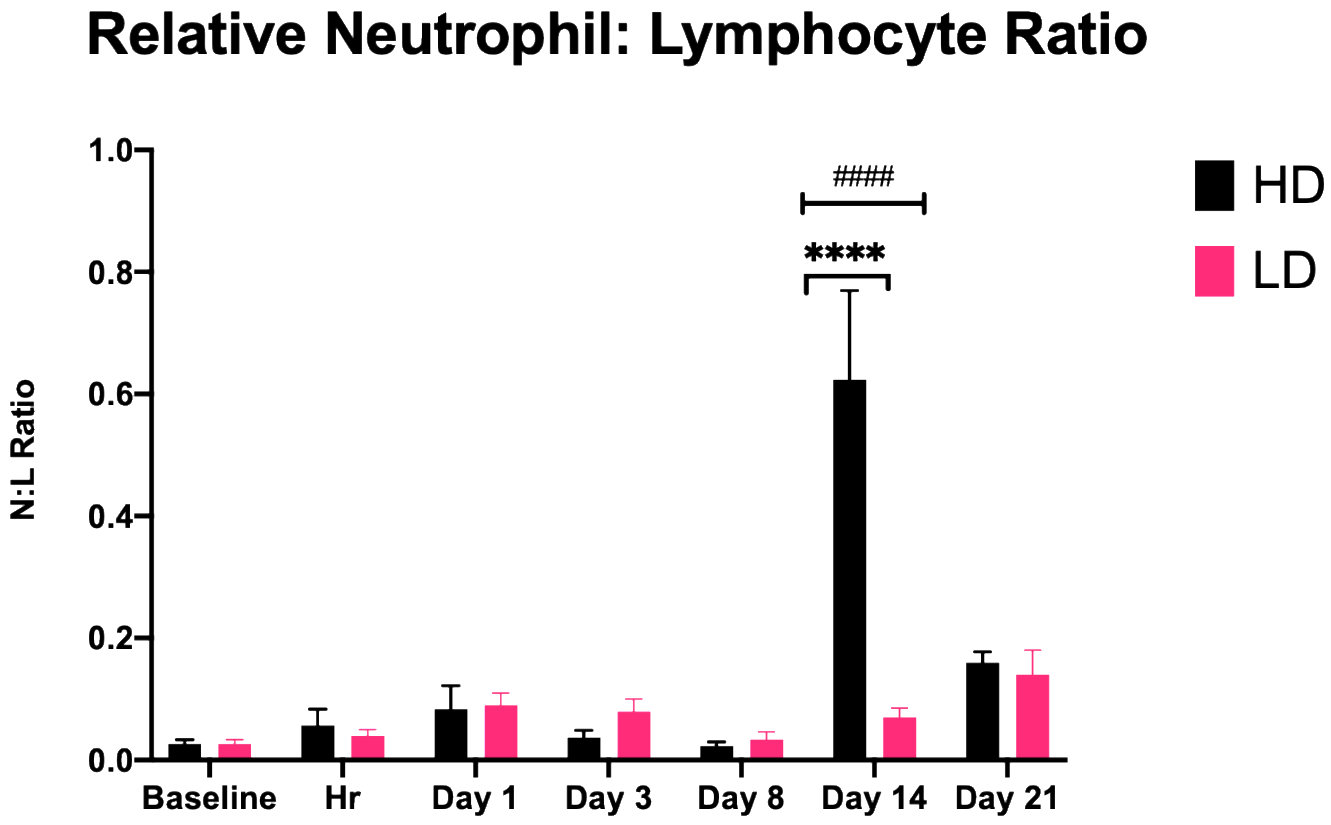


Figure 3. Serum cortisol concentrations in ng/ml for fish held in HD versus LD conditions

Serum cortisol levels in water at high and low density conditions for a 21 day period in rainbow trout. Data shown as mean +/- SEM of pooled data from n3 fish/time point (1 from each replicate tank, 3 replicate tanks of each density condition per time point). Data shown as mean +/- SEM of pooled data from 3 fish/time point (1 from each replicate tank, 3 replicate tanks of each density condition per time point). Hr corresponds to a 1 hr timepoint measurement in HD tanks and 2 hr time measurent in LD tanks. *indicate significance ($p<0.05$) of the tank density condition compared to baseline measurement (2-way ANOVA).

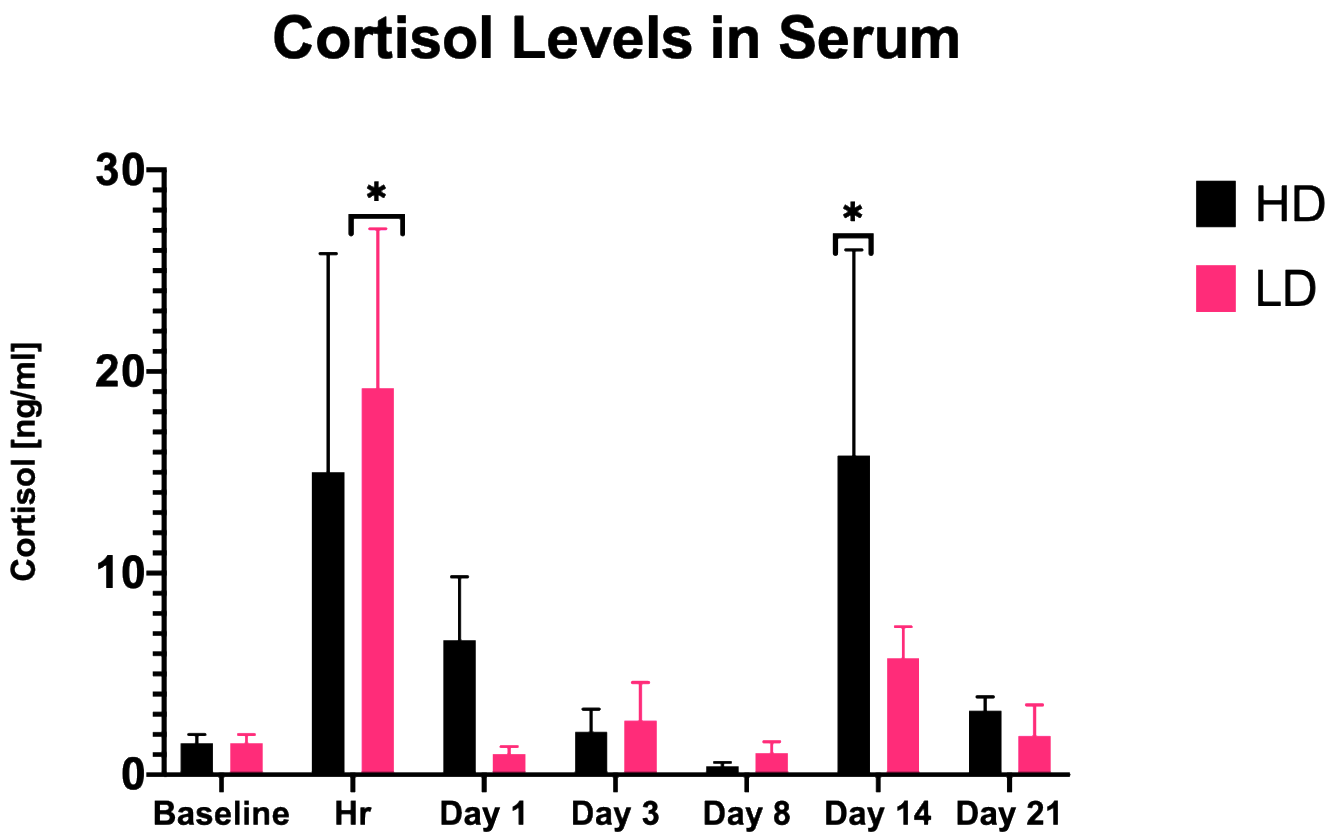


Figure 4. Water cortisol concentrations in ng/g/hr in HD versus LD conditions

Cortisol levels in water at high and low density conditions for a 21 day period in rainbow trout. Data shown as mean +/- SEM of pooled data from n3 tanks of 20 fish of each density condition per time point. Hr corresponds to a 1 hr timepoint measurement in HD tanks and 2 hr time measurement in LD tanks # indicate significance ($P<0.05$) between high and low density conditions at that time point (2-way ANOVA).

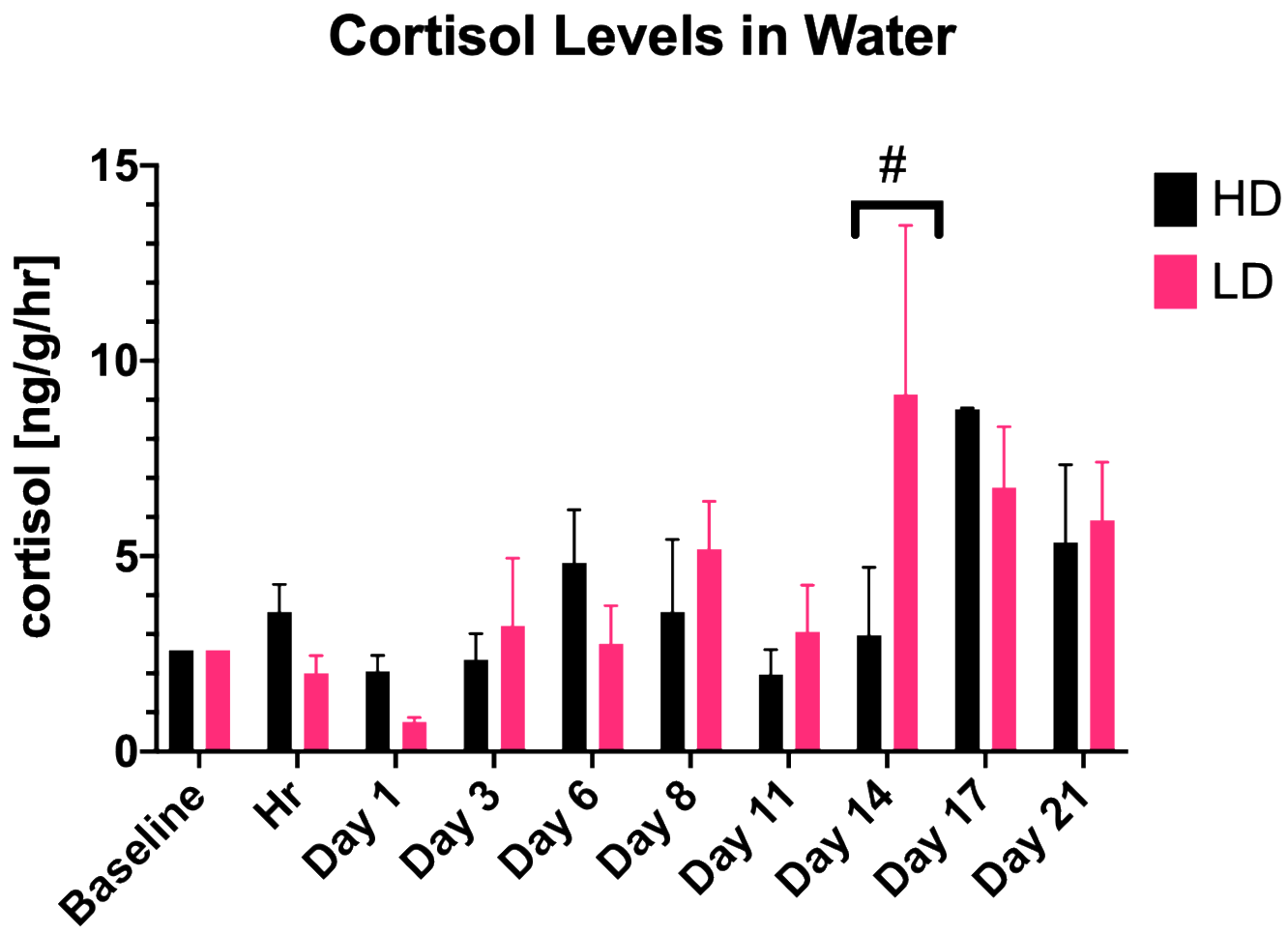


Figure 5. Comparison of Serum and Water Cortisol Concentration Patterns Over Time in HD and LD tanks A) pattern of cortisol release in HD and LD conditions in serum B) Pattern of cortisol release in HD and LD conditions in water

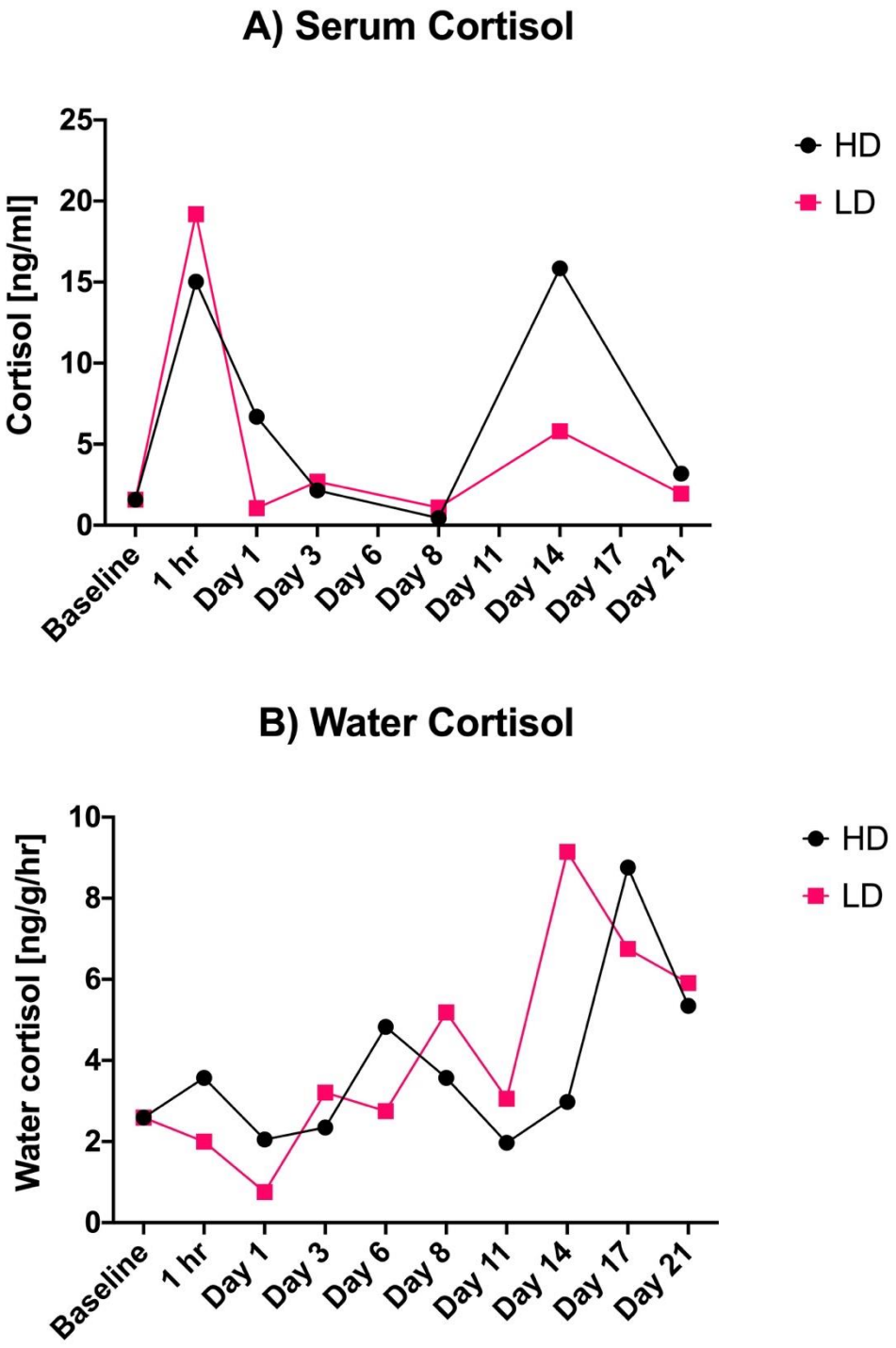
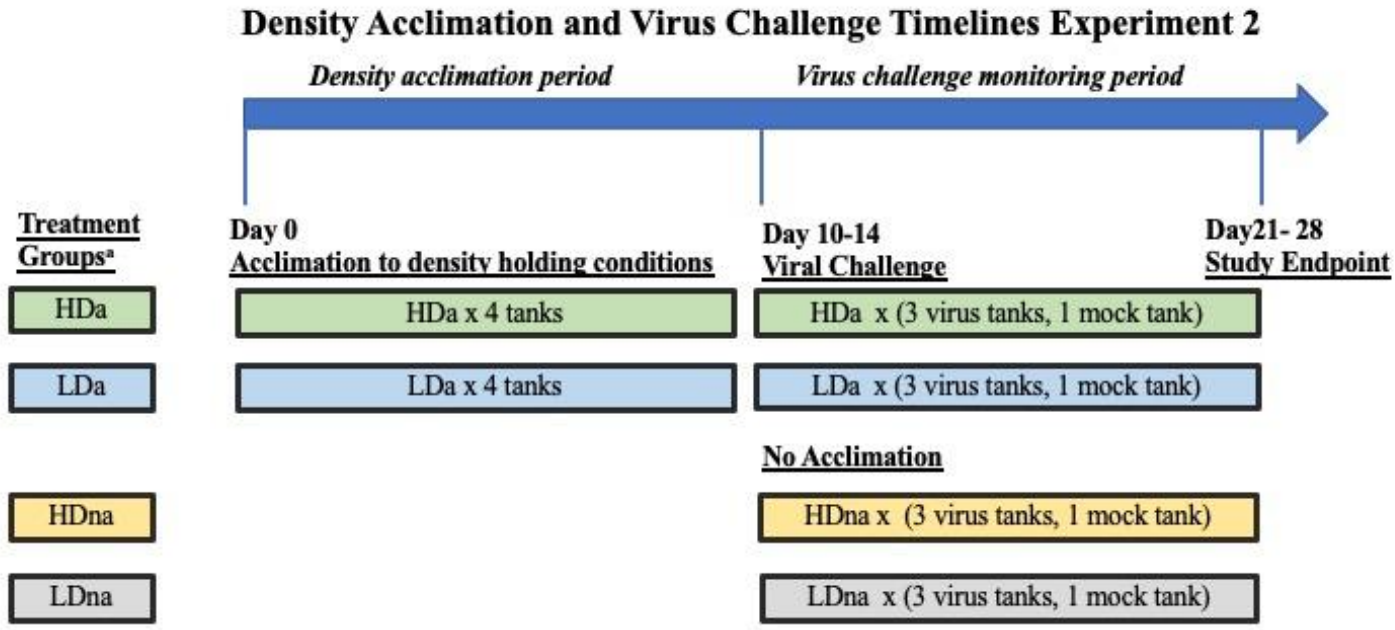


Figure 6. Virus Challenge Timelines Experiment 2. Fish were placed in tanks at specified densities and challenged with virus within an hour.



[a]HD, high density; LD, low density; a, acclimation; na = no acclimation

Figure 7. Cumulative Percent Mortality in Viral Challenge

Data shown as mean +/- Standard Deviation of pooled data from 3 replicate tanks per virus challenged treatment group (HDa, LDa, HDna, LDna) and pooled data for 4 mock challenged tanks. No significant differences were noted between virus challenge tanks (Wilcoxin).

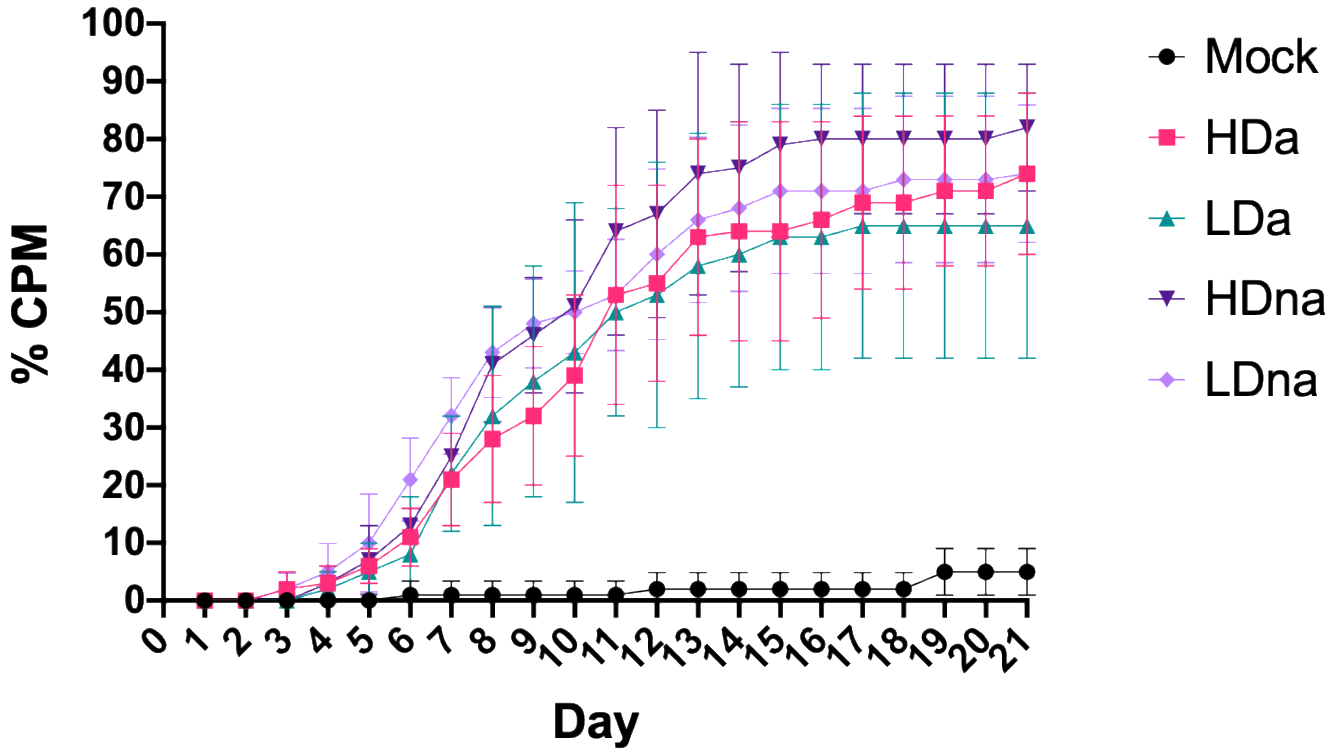
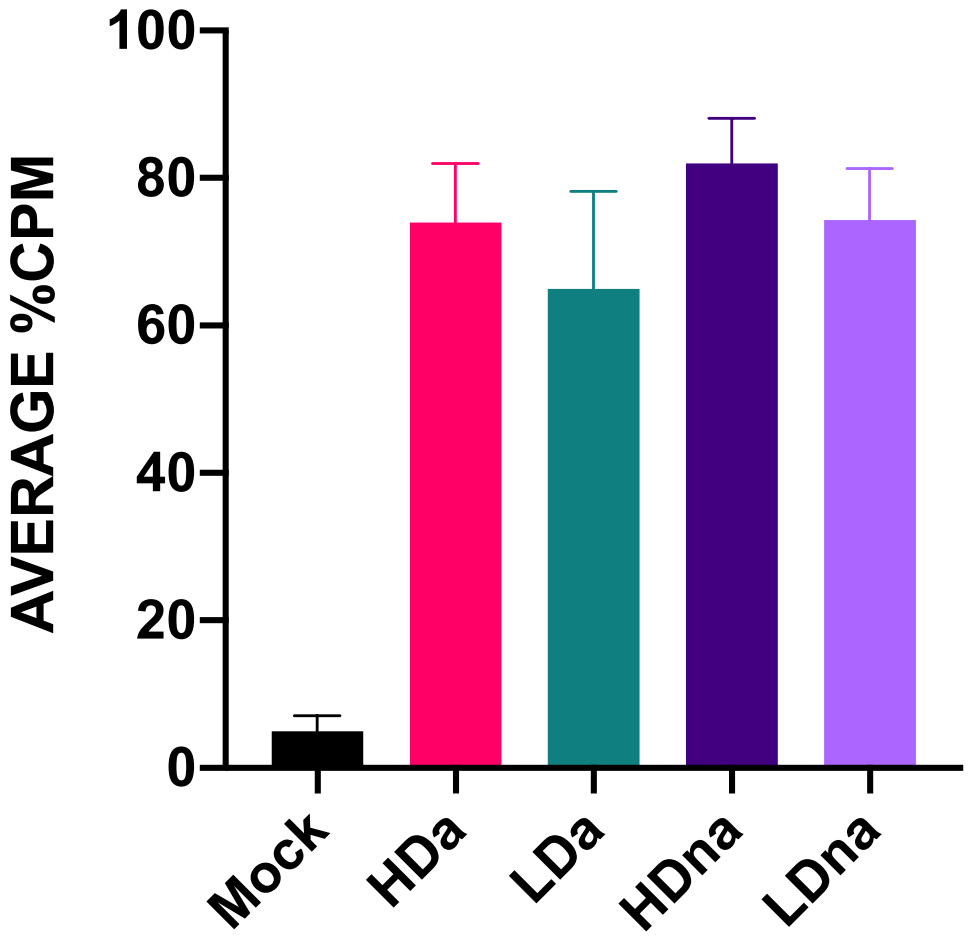


Figure 8: Final Cumulative Percent Mortality in Viral Challenge

Data shown as mean +/- SEM of pooled data from 3 replicate tanks per virus challenged tanks (HDa, LDa, HDna, LDna) and 4 mock challenged tanks. No significant differences were found between viral challenge tanks (Chi Square).



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