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Effects of Stocking Density on Stress Response and Susceptibility to Infectious Hematopoietic Necrosis Virus in Rainbow Trout

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The goals of this study were to examine the effect of stocking density on the stress response and disease susceptibility in juvenile rainbow trout (*Oncorhynchus mykiss*). Fish were sorted into one of 2 stocking densities (high density "HD", 20-40 kg/m^3) or (low density, "LD", 4-8 kg/m^3) and 3 stress indices (cortisol levels in serum and water, and neutrophil: lymphocyte (N:L) ratios from blood smears) were measured at multiple time points over 21 d. Serum cortisol was significantly increased at 1 h in LD samples and at 14 d in HD samples. Water cortisol concentrations were significantly higher in LD tanks as compared with HD tanks on day 14. N:L ratios were significantly higher in HD tanks on day 14 as compared with LD tanks and with baseline. The effect of stocking density on mortality after exposure to infectious hematopoietic necrosis virus (IHNV) was compared between fish held in HD or LD conditions, with or without prior acclimation to the different density conditions. No significant differences in survival were found between HD and LD treatments or between acclimated and nonacclimated treatments. Cumulative results indicate that 1) 1 to 4 gram rainbow trout did not generally demonstrate significant differences in stress indices at the density conditions tested over a 21-d period, 2) independent differences were found in 3 stress indices at day 14 after sorting into LD and HD holding conditions; and 3) LD and HD stocking densities did not have a significant effect on mortality due to IHNV.

Abbreviations: DO, dissolved oxygen; HD, high density; HDa, high density acclimated; HDna, high density no acclimation; IHNV, Infectious Hematopoietic Necrosis Virus; LD, low density; LDa, low density acclimated; LDna, low density no acclimation; N:L ratio, neutrophil: lymphocyte ratio; NH_3 un-ionized ammonia L/min, liters per minute

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Infectious hematopoietic necrosis virus (IHNV) is a common rhabdovirus of salmonid fish that has significant clinical and economic consequences. IHNV was first reported in fish hatcheries in Oregon and Washington state in the 1950s and is endemic among many wild and farmed salmonids in the Pacific Northwest.[6] The virus has been spread to Europe and Asia by the movement of infected fish and eggs.[6] Clinically, young fish are most susceptible to acute lethal disease associated with destruction and necrosis of the blood-forming tissues in the kidney and spleen.[20] In farmed fish, the disease can have a major economic impact, with reported cumulative mortality rates in young rainbow trout and salmon reaching 90% to 95%.[6] In this study, we examined the effect of rearing density on stress indices and on virus-induced mortality in juvenile rainbow trout (*Oncorhynchus mykiss*).

Developing recommendations for optimal ranges of housing density in captive fish populations is challenging, given the variety of species-specific preferences and tolerances.[12,18,37] Regardless, if densities are inappropriate for the species, fish may become stressed due to deterioration of water quality (physiologic requirements, "carrying capacity") and/or physical need for space (behavioral or interactive requirements, "density tolerance").[37] Previous experiments have shown that stress leads to increased 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.[35] In these cases, fish must acclimate, albeit at a reduced performance capacity, if they are to survive.[27,30]

The stress response can be measured at the level of the primary stress response (immediate neuroendocrine changes) and the subsequent effects (secondary and tertiary response).[31] Cortisol is the most frequently used indicator of stress in fish and represents a primary stress indice.[3] Its levels in blood rise rapidly with hypothalamic-pituitary-interrenal (HPI) axis activation, and it can be quantified in blood and tissues, or noninvasively through water samples.[7,11,29,32] A variety of secondary physiologic responses occur in the blood and tissues due to cortisol release. For example, cortisol induces alterations in circulating leukocytes across vertebrate taxa.[7] This generally consists of an increase in the number of neutrophils (N; neutrophilia) or a decrease in lymphocyte (L) numbers (lymphopenia) in peripheral blood samples (increased N:L ratio) that can be measured as a secondary stress indicator.[7,17,31]

When assessing stress responses, the duration of stressors is also important to consider because different effects will occur depending on whether the stressor is acute or chronic.[30,37] Acute stressors (minutes to a few hours) are those in which the duration of stress is shorter than the physiologic response. Examples of acute stressors in fish are handling and transport. Recovery from short-term acute stressors generally takes hours

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to a few days.[3,7,17] Chronic stressors (days to months) are those in which the stress is continuous. Examples include inappropriate densities for the fish species and water quality deterioration.[27] In contrast to well-developed models of acute stressors in fish (for example, 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.[30,31]

Relatively few studies have investigated the quantitative effect of housing density on stress and effects on IHNV-induced disease. A previously published paper demonstrated a density-dependent effect on virus transmission in juvenile rainbow trout when exposed to IHNV by cohabitation with a single infected donor fish. In that study, higher rearing densities (0.16 to 8 fish/L, 1 to 3 gram fish) resulted in higher probability of transmission.[26] However, the maximum density tested, 8 fish/L is considered very low for commercial aquaculture settings in this size of fish, which is often reared at high densities (>30 kg/m^3) due to the economic realities of large-scale production.[1,9,18,21,26]

The purpose of the current work was to examine 2 factors - stocking density and stress - that may influence IHN disease in fish farm settings in the host and size (juvenile rainbow trout 1 to 4 grams) in which mortality due to IHNV is most common.[6,9,20] To achieve this goal, 2 separate studies were performed. The first study assessed the kinetics of the chronic stress response profile to stocking density under 2 different density conditions over a 21-d period. Stocking densities were selected based on information from local trout production company staff and ranges reported in the literature.[1,12,21] Stress indices included cortisol concentrations in water and serum and differential WBC counts. The second study investigated the relationship between stocking density and IHNV-induced mortality by the use of a standardized disease exposure model.[15] Water quality parameters were measured and maintained at recommended safe levels for rainbow trout throughout the experiments to distinguish between the effects of density and potential of water quality deterioration.

Materials and Methods

Animals. Juvenile rainbow trout (1 to 4 grams) were procured from Troutlodge, Incorporated (Bonney Lake, WA, fish lots 1, 2, and 3) and Riverence, LLC (Rochester, WA, USA, fish lot 4). All fish housing and procedures occurred at the US Geological Survey (USGS) Western Fisheries Research Center (WFRC) in Seattle, WA, USA. Before the experiments, each lot of fish was housed in 278-L circular tanks with flow-through, membrane- or sand-filtered, UV irradiated fresh water at 15 °C. All tanks were aerated, unless otherwise described. Stock tank fish were fed commercial diet (#1 sinking pellets, Skretting, Tooele, UT) once a day at 1% of their body weight. Experimental fish were fed 1% body weight every other day. Fish were acclimated in stock tanks for a minimum of 12 d prior to use in experiments. The light cycle in which the fish were housed matched the ambient light cycle of the Pacific Northwest. All procedures complied with and were approved by the University of Washington Institutional Animal Care and Use Committee.

Fish husbandry. An initial study was conducted to assess experimental density conditions with the goal of validating methods of water quality measurements and documenting overall fish health and survival. For these experiments, fish (lot 1) were randomly sorted into 5 L circular tanks (25 cm diameter) of identical size supplied with flow-through water. Housing density was achieved by changing the volume of water in the tank. In LD (low density) treatments, 20 fish were held in 5 L of water (4 kg/m^3). This is the standard density used for virus infection studies at WFRC. In the HD (high density) treatment, 20 fish were held in 1 L of water (20 kg/m^3). This falls close to or within the range for commercial trout farm production (15 to 50 kg/m^3, with 25 to 50 kg/m^3 most common).[12,21]

The following water quality parameters were measured from the outflow of tanks: temperature and dissolved oxygen (DO) by total gas pressure meter (Pentair, Aquatic Eco-Systems, Point Four Tracker, Apopka, FL), alkalinity, general hardness, pH, nitrate, nitrite, total ammonia nitrogen by commercial test strips (Tetra, Blacksburg, VA), and unionized ammonia (NH_3) and carbon dioxide by Hach Aquaculture test kit (Fish Farmer's Water Quality Test Kit, Model FF-1A, Loveland, CO). Flow rates were measured from tank inflow in liters per min (L/min). Measurements were taken daily during initial experiments and after experimental time point collections in subsequent experiments (at a minimum once a week). All water quality parameters measured were within normal limits throughout experiments. No adjustments were needed in flow to maintain DO above 6 mg/L or NH_3 below 0.02 mg/L, and parameters of tanks showed little fluctuation each day. Average water quality parameters ± SEM for the HD tanks were: pH 7.0 ± 0, general hardness 51± 1.1 mg/L, alkalinity 32 ± 1.1 mg/L, total ammonia nitrogen 0 ± 0 mg/L, nitrate 0 ± 0 mg/L, nitrite 0 ± 0 mg/L, NH_3 0 ± 0 mg/L, carbon dioxide 8.5 ± 0.3 mg/L, DO 9.5 ± 0.04 mg/L, flow rate 0.32 ± 0.01 L/min and temperature 15° ± 0.1 °C. Average water quality parameters ± SEM for the LD tanks were: pH 7.0 ± 0, general hardness 51 ± 1 mg/L, alkalinity 33 ± 1 mg/L, total ammonia nitrogen 0 ± 0 mg/L, nitrate 0 ± 0 mg/L, nitrite 0 ± 0 mg/L, NH_3 0 ± 0 mg/L, carbon dioxide 8.0 ± 0.8 mg/L, DO 9.6 ± 0.05 mg/L, flow rate 0.30 ± 0.02 L/min and temperature 15 ± 0 °C.

Euthanasia method. For accurate cortisol measurement in fish serum as an indicator of fish stress, euthanasia must be rapid to avoid elevation due to the euthanasia process itself.[7,16,36] Based on published recommendations, our goal was to identify the dose of buffered MS-222 that resulted in rapid, consistent euthanasia within 1 min, as defined by cessation of all opercular motion within 1 min of immersion in buffered MS-222 and lack of response to tactile stimulation and deep pain (for example, tail pinch).[7,16,36] Testing was done in groups of 4 fish at successively higher concentrations of MS-222 (buffered to pH 7.0 to 7.5) starting at 240 mg/L and increasing until consistent euthanasia occurred for all fish within 1 min. A dose of 800 mg/L of MS-222 met the desired criteria within 1 min and was therefore selected as the dose for all subsequent experiments.

Experiment 1. Characterizing the stress response to HD and LD conditions for 21 d. Juvenile rainbow trout (lot 2) were acclimated to a stock tank for 12 d at 15 °C. The 278 L stock tank was supplied an average inflow of 3.23 ± 0.05 L/min, and contained 220 fish, with an average weight of 0.84 ± 0.06 grams per fish. The biomass was therefore 185 grams, stocking density 0.66 kg/m^3, and biomass loading rate 0.6 kg/L^{-1} min^{-1}. 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 700 mL water sample. A subset of fish was then randomly selected from the stock tank and allocated into triplicate tanks of HD or LD conditions, resulting in 20 fish per tank. After 1 h, a sample of 6 fish were euthanized (one fish per tank) for blood collection (serum cortisol and differential WBC count), and one 700 mL water sample was collected from the outflow of each HD and LD tank. On days 1, 3, 8, 14, and 21, the same samples were taken. At each time point, water samples were collected from all tanks before fish were taken. Additional water samples (without matched serum samples) were collected on

day 6, 11, and 17. All samples were collected between 1200 to 1800 to avoid diurnal effects.[13]

Blood collection and hematologic analysis. Within 5 min 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 2 drops of fresh whole blood were placed onto slides (1 drop per slide) and a blood smear prepared. The blood smears were then left to dry while the remainder of the blood from the fish was collected by centrifugal separation as described below for serum cortisol analysis. Blood smear slides were stained with Leishman–Giemsa on the same day of collection.[39] Differential WBC counts were performed using a battlement edge count method under 400× magnification.[23,34] Readouts consisted of counts of 200 WBCs per fish, unless the cell population was limited, in which case 100 WBCs were counted. Monocytes, lymphocytes, and neutrophils were quantified. N:L ratios were calculated as the ratio of the numbers of neutrophils (N) and lymphocytes (L) that were counted from each sample, with the resulting ratio value (N:L) recorded as a decimal.

In addition to experimental samples, control sample reference ranges were defined by conducting differential WBC counts in experimentally naive stock fish from 2 different lots of fish, (lots 2 and 3) which had been housed in the stock tanks for 21 d and 33 d, respectively, prior to euthanasia and blood smear collection. Average individual fish weights and stocking densities were similar, at an average of 1.27 ± 0.05 grams, 0.34 kg/m^3 for lot 3 fish and 2.13 ± 0.07 grams and 0.44 kg/m^3 for lot 2 fish.

Serum collection and cortisol measurement. Blood and serum collection were modified from a technique described previously.[2] Briefly, euthanized fish with severed caudal peduncles were placed in a fenestrated 5 mL tube (Falcon Round-Bottom Polypropylene Tubes, Corning, NY), which was then placed inside a 15 mL conical centrifuge tube (Falcon 15 mL Conical Centrifuge Tube, Corning, NY) for centrifugation at 400 x *g* for 5 min at room temperature (Sorvall Legend RT refrigerated centrifuge, Thermo Fisher Scientific, Waltham, MA). The resulting blood sample in the 15 mL centrifuge tube was transferred to a 0.6 mL microfuge tube and centrifuged at 13,000 x *g* for 15 min at 4 °C (Eppendorf model 5402 centrifuge, Eppendorf, Hamburg, Germany). Supernatant serum was collected and stored at −80 °C until analysis. Serum cortisol concentrations were measured using a cortisol ELISA kit (Salimetrics, State College, PA) following the manufacturer's instructions. Absorbances were measured at 450 nm in a Biotech model ELx808 automated plate reader (Winooski, VT). 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, Brighton, East Sussex, UK). Data were reported in units of µg cortisol per dL of fish serum. Final data were 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 less than 0.007 µg/dL (Salimetrics, State College, PA).

Water cortisol sampling and measurement. Tank water samples of 700 mL were collected from tank outflows into 1 gallon Ziploc freezer bags (Hefty, Reynolds Consumer Products, Lake Forest, IL) that were positioned in 1 L plastic beakers. Samples were frozen at −20 °C within 2 h of collection and stored until cortisol extraction. To assess background cortisol levels, water samples were collected from the inflow line connected to the experimental tanks (negative control). To assess the cortisol recovery rate, 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 of cortisol extraction.[8,10,11,14,24,40] In summary, samples were thawed overnight for up to 18 h at room temperature.[11] Once thawed, 500 mL of each sample was passed through a prefilter (Nalgene Rapid Flow Sterile Disposable Filter Units with PES membrane, 0.45 µm pore size, ThermoScientific, #295 to 454, Waltham, MA) into either a 500 mL polypropylene receiver unit, (Nalgene, Thermo Scientific, #2105-0016, Waltham, MA) or a 1000 mL pyrex glass bottle (Waltham, MA). Cortisol was extracted from water using a Sep Pak Vac C18 cartridge (Waters #WAT043395, Milford, MA) that was attached to a vacuum manifold (Millipore #WP6111560, Jaffrey, NH) via vinyl tubing (Tygon #57629, Akron, OH). Cartridges were conditioned prior to receiving the water sample by flushing with 5 mL of 100% methanol followed by 5 mL of distilled water at a rate of 5 to 10 mL/min. Water samples were loaded onto the cartridge at a rate of 2 to 10 mL/min. After loading, cartridges were removed from the manifold and stored at −20 °C until eluted. For elution, cartridges were thawed at room temperature for a minimum of 30 min, and then cortisol hormones were eluted from the cartridges with 5 mL of ethyl acetate (Sigma Aldrich, #319902, St Louis, MO) by positive displacement with a 10 mL luer lock syringe at a rate of 5 to 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) and then transferred into multiple microfuge tubes. Eluted samples of ethyl acetate were then immediately dried in a vacuum concentrator (SpeedVac, OligoPrep OP120 Savant, Holbrook, NY) for 6 h. The resulting dried steroid pellet was stored at −20 °C until resuspension.

Dried samples were resuspended in absolute ethanol (10% total volume, 30 µL) that was added directly onto the bottom of the microfuge tube, followed by rack shaking (Melrose Park, IL) at approximately 1100 rpm for 2 h. The resuspension volume in each tube was then brought up to 300 µL with 0.2% BSA and placed on a rack shaker at the same settings (1100 rpm) for another hour. Samples were stored at −20 °C for 1 to 2 d and then thawed and shaken again at approximately 1100 rpm for 2 h. Resuspended samples were stored at −20 °C for 1 wk or less before until the ELISA assay.

Cortisol concentrations in water were measured using a cortisol ELISA kit (Salimetrics, State College, PA) following the manufacturer's instructions. In each assay, samples were quantified in duplicate. 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. Raw data were analyzed as described above, and the sensitivity of the assay was again less than 0.007 ug/dL. Final water cortisol concentrations (in ng/g/hr) were calculated based on previously derived equations for flow through systems, 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 (grams).[10,11,32]

Experiment 2. Impacts of acclimation and holding densities on mortality due to virus exposure. Virus and host. For IHNV exposures, we used an M genogroup IHNV isolate, HG508, originally isolated from farmed rainbow trout in the Hagerman Valley of Idaho in 2014 (Fornshell and Kurath). The HG508 virus strain was propagated in Epithelioma papulosum cyprinid (EPC) cell

Table 1. Differential WBC counts (%) in experimentally naïve juvenile rainbow trout.

	Lot 3 (n = 9)			Lot 2 (n = 10)			
	Mean	**SEM**	**Range**	**Mean**	**SEM**	**Range**	**Reference intervals***
% Lymphocytes	91.8	1.4	85.5–97.5	94.2	0.7	90–97	89–98
% Neutrophils	5.5	1.2	0.5–10.5	4.1	0.5	2.5–8	1–9
% Monocytes	2.7	0.4	0.5–4.5	1.8	0.4	0.5–4	0–5
N:L ratio	0.06	0.01	0.01–0.12	0.04	0.01	0.03–0.09	

[a]Reference intervals for differential WBC count intervals measured in juvenile rainbow trout.[22,39]

line at 15 °C and quantified by plaque assay as previously described.[5] A dose of 4×10^2 pfu/mL was used to expose replicate groups of juvenile fish by use of a standard 1-h static immersion exposure.[15] This dose produced approximately 50% mortality in LD tanks based on dose-finding studies. Juvenile rainbow trout tested in the virus exposure experiment were from lot 4 and weighed an average of 1.3 ± 0.03 grams.

Virus exposure experiment. Exposure to IHNV was conducted on groups of fish that had been acclimated to HD and LD conditions and on nonacclimated fish that were placed into HD and LD conditions at the time of virus exposure. For density-acclimated fish groups, 160 fish were randomly sorted into HD or LD density conditions, with 20 fish per tank in 4 replicate tanks and were held for 14 d prior to virus exposure. These are referred to as acclimation groups HDa (high density acclimation) and LDa (low density acclimation) respectively. For nonacclimated groups additional subsets of 20 fish were sorted into HD or LD density conditions, with 4 replicate tanks for each condition. These fish were immediately exposed to virus; acclimated fish were exposed at the same time. The nonacclimated groups are referred to as HDna (high density no acclimation) and LDna (low density no acclimation) groups, respectively.

For the 4 replicate tanks in each of the 4 acclimation and density conditions tested, 3 tanks were exposed to virus, and 1 tank was mock exposed to medium (MEM-10) that had no virus. Fish were exposed to virus in the same volume of water and tanks (density conditions) in which they had been housed in throughout the experiment. At 1-h after virus exposure, water flow was turned back on, and fish were monitored daily until the study endpoint at 21 d after exposure. Clinical and behavioral signs and mortality in each tank were recorded daily. Fish that died were removed each day. Densities were not adjusted when dead fish were removed. At 21 d after exposure, all survivors were euthanized and counted to terminate the experiment.

Statistics. Data from the first experiment (stress response kinetics at high and low density) were analyzed in Prism (GraphPad Software, La Jolla, CA). Stress indices (serum cortisol, water cortisol, and N:L ratios) were analyzed using a 2-way ANOVA, with density and time as the 2 factors. A Tukey posthoc test was performed for pairwise comparisons. Data from the viral exposure experiment were assessed by survival analyses using R studio (Version 1.2.1335, Boston, MA). Differences in survival kinetics were assessed using Wilcoxon test, and the final cumulative mortality data were analyzed using χ^2 test. Results were considered significant if $P \leq 0.05$.

Results

Experiment 1. Define chronic stress response to HD and LD conditions. Leukocyte profiles measured as the ratio of neutrophils to lymphocytes (N:L ratio). To determine reference levels for experimentally naive rainbow trout, differential WBC counts were obtained from 2 different lots of fish used in this study (Table 1). Lymphocytes were the majority of leukocytes counted (94 ± 1% and 92 ± 1%) followed by neutrophils (4 ± 0% and 5 ± 1%) and monocytes (2 ± 0% and 3 ± 0%). Average N:L ratios were 0.04 ± 0.01 and 0.06 ± 0.01 in the 2 lots of fish. These results provided reference levels that were similar to differential WBC count ranges reported in the literature for juvenile rainbow trout.[22,37,39]

In experiment 1, the analysis showed a significant interaction between the effects of density and time on N:L ratios ($F_{10,34}$ = 11.20, $P < 0.0001$). On Day 14, the N:L ratios were significantly higher in HD fish as compared with baseline ($P < 0.0001$), and in HD fish as compared with LD fish ($P < 0.0001$), (Table 2, Figure 1).

Serum cortisol response. Compared with values at baseline, serum cortisol levels were significantly elevated in LD groups at 1 h ($P \leq 0.01$), and in HD groups on day 14 ($P \leq 0.05$, Figure 2 A). There were no significant differences between density groups at any time points.

Water cortisol levels. In tests of the water cortisol methodology our average recovery rate for spiked cortisol control samples of 10 ng/L was 79% (± 3.8%). Blank water samples from inflow water prior to exposure to fish were lower than test samples from tanks that contained stock fish.

In experiment 1, water cortisol values in LD groups were significantly elevated compared with HD groups at day 14 ($P < 0.05$, Figure 2 B). Neither density group showed significant differences over time as compared with baseline values.

Experiment 2. Impacts of acclimation and holding densities on mortality due to virus exposure. For the virus exposure experiment the 4 treatments groups included fish acclimated to either HD or LD conditions for 14 d prior to exposure (groups HDa and LDa), and fish placed into HD or LD conditions immediately before exposure (groups HDna and LDna). Mortality curves for each treatment group show the average cumulative percent mortality of triplicate tanks in the HDa, LDa, HDna, LDna groups, as well as low average mortality in 4 tanks of mock-exposed fish (Figure 3). The mortality curves for virus treated groups were typical for IHNV kinetics in terms of onset, duration, and mortality plateau.[15] In general, the first mortalities started to occur on day 3 to 5, with the bulk of mortality occurring between days 5 to 14 and starting to plateau at day 15. The average final cumulative percent mortalities for the 4 treatment groups were: HDa (74 ± 8%), LDa (65 ± 13%), HDna (82 ± 6%), and LDna (74 ± 7%) (Figure 3). Clinical signs noted during the observation period after exposure 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.[6]

Survival analysis found no significant differences between virus exposed groups ($P > 0.05$), although a tendency toward slightly higher mortality was found in non-acclimated groups as compared with acclimated groups ($P = 0.07$).

Table 2. Differential WBC counts (%) and relative N:L ratios in HD and LD conditions

		Differential WBC Counts			
Time	**Density**	**% Monocytes**	**% Lymphocytes**	**% Neutrophils**	**N:L Ratio ±/-SEM**
Baseline		4.3 ± 1.6	93 ± 1.6	2.7 ± 0.7	0.03 ± 0.01
Hr	HD	4.2 ± 1.4	90.7 ± 3.6	5.2 ± 2.2	0.06 ± 0.03
	LD	2.3 ± 1.3	94 ± 2	3.8 ± 0.8	0.04 ± 0.01
Day 1	HD	2.5 ± 0.8	90.3 ± 3.8	7.2 ± 3.2	0.08 ± 0.04
	LD	1 ± 0	91 ± 1.8	8 ± 1.8	0.09 ± 0.02
Day 3	HD	0.3 ± 0.2	96.2 ± 1.4	3.5 ± 1.3	0.04 ± 0.01
Day 8	HD	1.3 ± 0.9	96.7 ± 1.4	2 ± 0.8	0.02 ± 0.01
	LD	0.5 ± 0.3	96.5 ± 1.3	3 ± 1.3	0.03 ± 0.01
Day 14	HD	1.7 ± 0.7	61.5 ± 5.1	36.8 ± 5.3	0.62 ± 0.15
	LD	0.5 ± 0.3	93.3 ± 1.2	6.2 ± 1.4	0.07 ± 0.02
Day 21	HD	1 ± 0.6	85.2 ± 0.7	13.8 ± 1.3	0.16 ± 0.02
	LD	0.7 ± 0.2	87.3 ± 3.2	12 ± 3	0.14 ± 0.04

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).

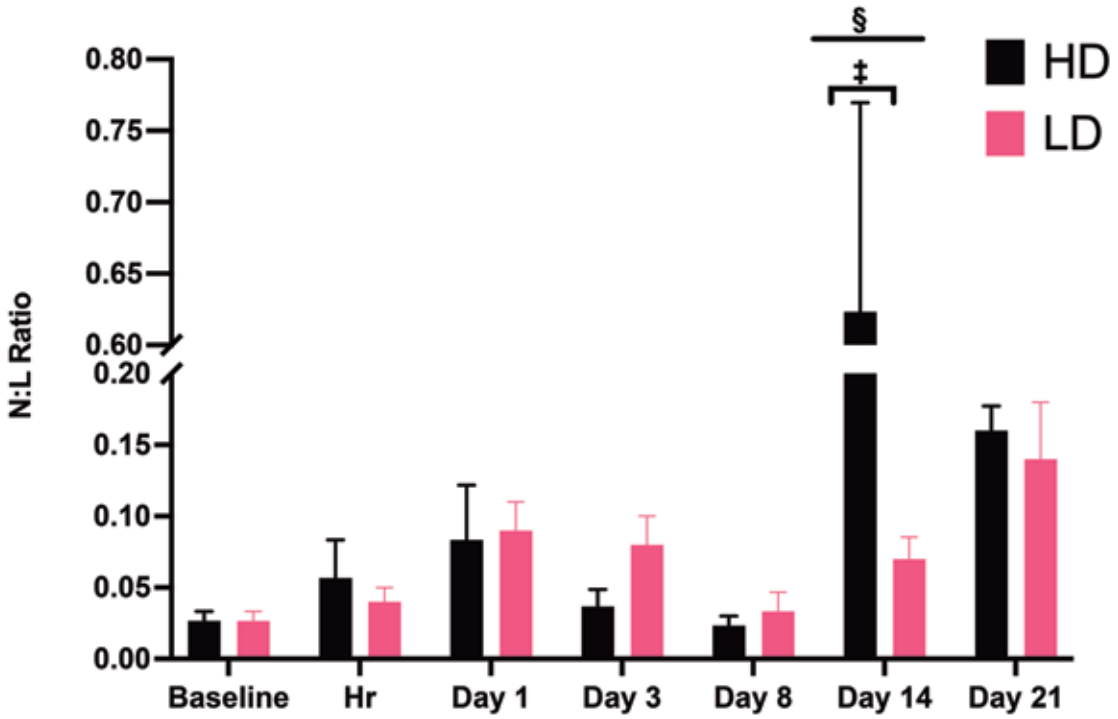

Figure 1. Relative N:L ratios from serum of rainbow trout held in HD and LD conditions and sampled at specified time points over a 21-d period. Data shown as mean ± SEM of pooled data from 3 fish/time point (1 from each of 3 replicate tanks of each density condition per time point). ‡, $P < 0.0001$ indicate significance between treatment and baseline; §, $P \leq 0.0001$; indicate significance between LD and HD at that time point.

Discussion

The results of this study indicated that juvenile rainbow trout did not demonstrate significant differences in stress indices at the density conditions tested (4 to 26 kg/m^3) over a 21-d period. In addition, density did not significantly affect IHNV mortality.

The stress response kinetics to density in the first experiment demonstrated 2 main findings. First, serum cortisol provided evidence of an acute stress response at the 1-h time point, which was significantly higher than baseline in LD fish serum, yet not significantly elevated for HD fish serum ($P = 0.07$). In addition, water cortisol and N:L ratio did not indicate an acute stress response. Our second main finding was that a secondary peak of 2 stress responses was detected in both HD and LD groups on day 14. Specifically, in HD groups, a significant peak was detected for serum cortisol levels and N:L ratios as compared with baseline and N:L ratios were increased as compared with LD groups. Water cortisol profiles for LD tanks on day 14 showed a peak that was significantly higher than HD tanks. Collectively, the baseline values, acute peak values, and secondary peak values of cortisol concentrations in water and serum were consistent with ranges reported for rainbow trout in the literature.[3,4,11,19,25,27,28,32] In general, after acute short term stress in salmonids, cortisol levels are usually elevated within minutes and water cortisol concentrations within several hours, each followed by a decay during the recovery from stress.[3,11] Subsequent rises or prolonged elevations in serum and water cortisol concentrations after initial acute stressors have been reported in various fish species in research, aquaculture, and farm settings usually under conditions associated with chronic

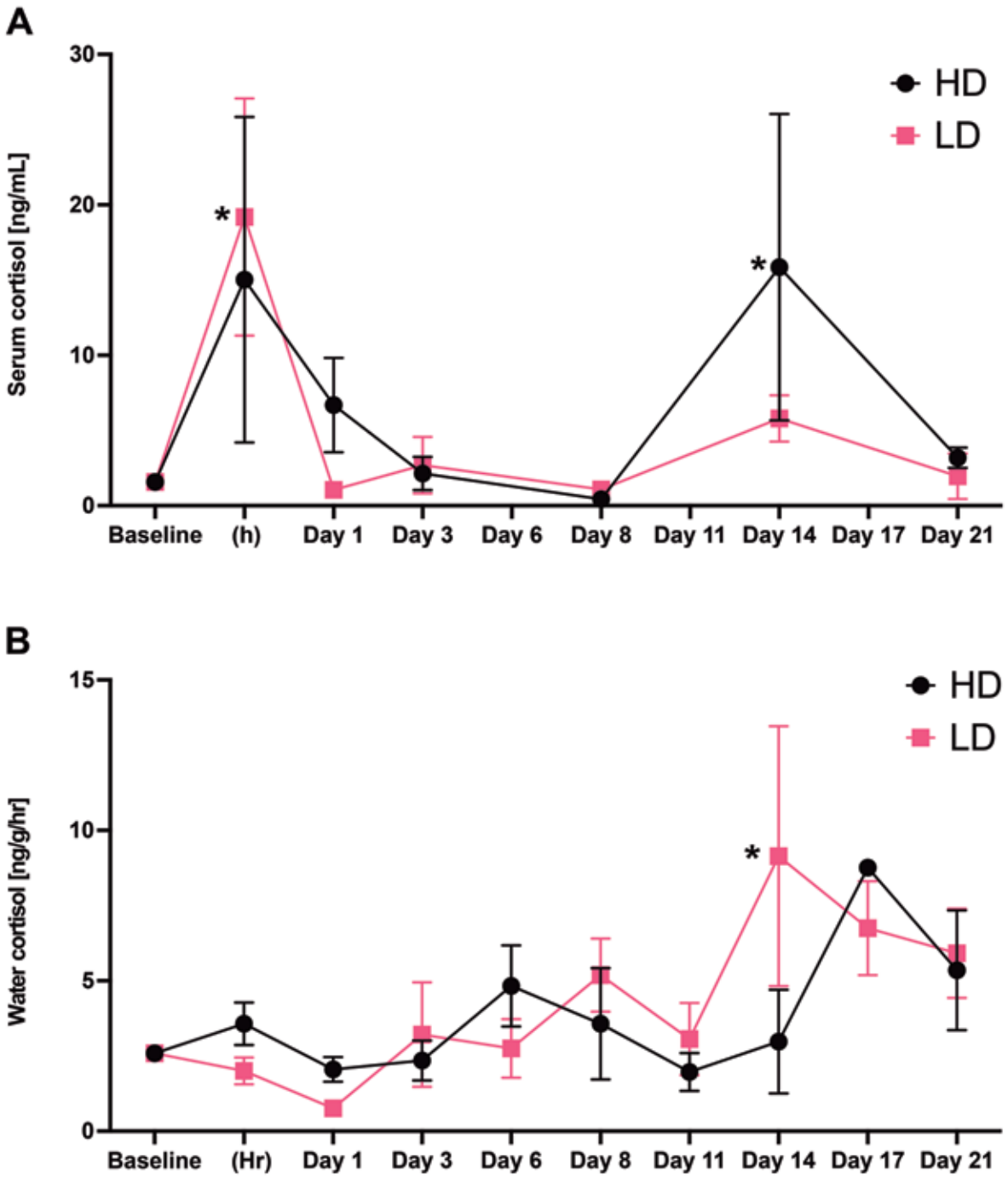

Figure 2. Comparison of serum and water cortisol concentration patterns over time in HD and LD conditions A) Serum cortisol levels [ng/mL] from rainbow trout held in HD and LD conditions and sampled at specified time points over a 21-d period. Data shown as mean ± SEM of pooled data from 3 fish/time point (1 from each replicate tank, 3 replicate tanks of each density condition per time point). * Indicate significant difference ($P < 0.05$) between treatment and baseline. B) Water cortisol levels [ng/g/hr] from tanks of rainbow trout held in HD and LD conditions and sampled at specified time points over a 21-d period. Data shown as mean ± SEM of pooled data from 3 tanks of 20 fish of each density condition per time point. * indicate significant differences ($P < 0.05$) between LD and HD at that time point.

or sequential stressors (confinement, crowding, acclimation post transport).[4,17,21,25] For our data, one potential explanation for the secondary stress response peak at day 14 after initiation of the experiment is a stress profile due to capture (netting) and confinement in the small experimental tanks over time, regardless of the different water volumes in the LD and HD conditions. Similar stress kinetics of blood cortisol have been detected in juvenile trout exposed to multiple stressors.[4] In that study, a similar rise in plasma cortisol was noted acutely after capture, followed by subsequent decreases that returned to baseline levels by day 8, and started to increase again at day 11 to 14.[4]

Experiment 1 also demonstrated that, with the exception of the acute stress response at 1 h, the kinetics of water cortisol concentrations in our experimental system approximated serum cortisol kinetics in both density conditions over time. Measurement of steroid hormone from water was based on previous studies that demonstrated periovulatory sex steroid pheromones released by female goldfish were detectable in water,

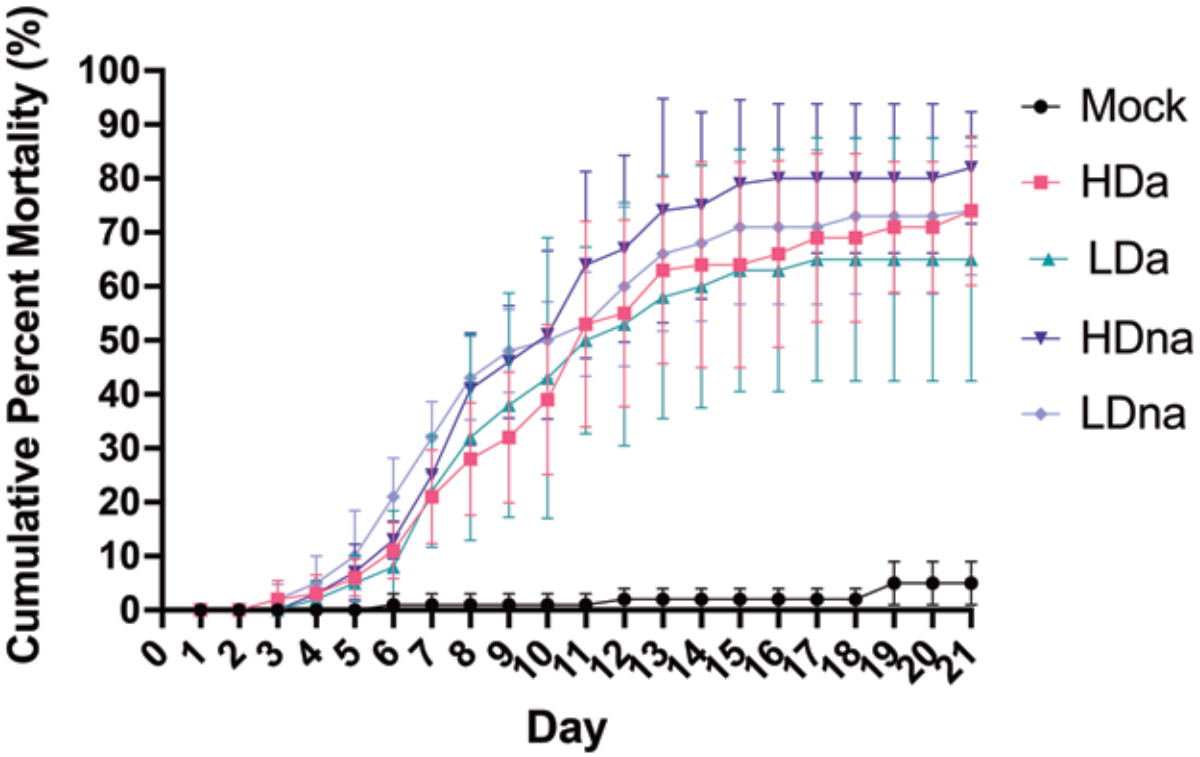

Figure 3. Cumulative percent mortality in viral exposure treatments that were either acclimated or nonacclimated to HD or LD conditions prior to virus exposure. Data shown as mean ± SEM of pooled data from 3 replicate tanks of 20 fish per virus exposed treatment group (HDa, LDa, HDna, LDna) and pooled data for 4 mock exposed tanks.

and that the pattern of release of the steroid approximated its secretion into the blood.[32] 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.[11,33] We validated the measurement in our experimental system and life stage (juvenile) of rainbow trout, demonstrating the pattern of changes of cortisol in water samples generally followed the changes in serum samples obtained over the 21-d period, but with some interesting differences. In our study, water cortisol was significantly different between HD and LD conditions on day 14, with LD tanks having higher water cortisol concentrations than did HD tanks. This difference was not evident in serum cortisol at day 14, where HD fish had significantly higher values than did LD fish. 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 in serum of 3 individual fish, at that time point. In addition to representing differences between individual and population responses, water cortisol measurements also represent a much wider interval of time, as steroid levels must accumulate to detectable levels in the water over time, and generally show a time lag to detection in tanks on flow-through systems.[11] The data for the HD groups suggest a time lag between peaks in serum and water cortisol in that HD groups had a significant peak in serum cortisol at day 14, followed by a near-significant elevation in water cortisol on day 17 ($P = 0.09$). For the LD groups, the observed peak in water cortisol on day 14 may have been preceded by a peak in serum cortisol that was not captured due to lack of sampling for serum cortisol between days 8 and 14. The idea of a time lag between serum and water cortisol responses could indicate that the peak of the acute stress response observed at 1 h for serum cortisol was followed by cortisol release into the water after the 1-h time point that we selected for in the first water measurements, peaking at some time point that was not sampled between 2 and 24 h.

Differential WBC counts performed on naive experimental fish in our study were consistent with ranges reported for this particular species in the literature.[22,39] Samples obtained from stock fish at baseline time 0 of our experiment were generally within these ranges, except for 1 fish with slightly high monocytes (7%), which was only mildly higher than our reference ranges or those in the literature, and most likely represented individual variation.[22,39] N:L ratios were significantly higher on day 14 in HD tanks, as compared with both baseline measurements and with LD tanks at the same time point. In general, fish can show increased N:L ratios or a lymphopenia in peripheral blood samples within hours after acute stressors, and generally return to baseline in 1 to 2 d.[3,7,17,28] Chronic stress-induced lymphopenia has been observed in both salmonids and zebrafish.[3,17] In some cases, changes in differential WBC parameters may be more reliable indicators of chronic stress than blood cortisol levels.[7,28] For example, previous studies in rainbow trout have shown that crowding produced a transient increase in plasma cortisol levels that returned to baseline levels after 10 d, suggesting acclimation, but lymphopenia was present for 21 d in adult rainbow trout and brown trout.[28] Although mechanisms other than stress can cause a prolonged lymphopenia, differential lymphocyte counts may be useful in conjunction with other stress indices, particularly over periods of chronic stress.[7,28] Interrenal production of cortisol may be inhibited by continuous stimulation of the hypothalamic-pituitary axis, resulting in lower cortisol levels.[4] In these cases, the fish may appear to have compensated, yet still be experiencing a disruption of homeostasis due to a stressor, manifested in other parts of the pathway.

To our knowledge, few studies to date have tested and measured simultaneous stress indices of serum, water, and N:L ratios in rainbow trout or other fish species.

The viral exposure experiment (experiment 2) did not demonstrate a significant impact of the housing densities tested on IHNV mortality. The kinetics of mortality curves (onset, duration, and plateau) and clinical signs were consistent with those reported for IHNV in rainbow trout at 15 °C.[15] The density ranges tested represent the lower end of stocking density used for this size of fish in commercial trout farm settings. Even the high-density condition used in this experiment may have been too low to reveal an effect of high-density stress. A previous study demonstrated a density-dependent effect on IHNV transmission by cohabitation.[26] Based on the size of fish and tank volume, the range of densities tested in their study was 0.03 to 24.8 kg/m^3, and IHNV transmission was demonstrated to occur at 0.45 to 24.8 kg/m^3.[26] The authors of that study noted that the highest density tested was much lower than densities reported to be used in commercial enterprises.[26] In our study, fish were exposed to IHNV in batch by immersion, and based on the weight of the fish, the high density conditions were roughly equivalent to the highest density tested in the study mentioned above, approximately 26 kg/m^3 at the time of exposure and prior to the reduction of biomass due to the onset of mortalities.[26] A density dependent effect may have been detected at even higher densities. The density conditions that we used were based on the shape, size, and volume of tanks and the size of fish. Considering fish size in relation to the volume of water in the tanks, then repeating the exposure at an even higher density in the same tanks would require housing different numbers of fish in the same volume of water to create desired HD and LD conditions, with increased water flow through the tanks used to ensure similar water quality conditions.

The results of the study collectively did not indicate significant differences in stress indices or on IHNV mortality at the 2 density conditions tested (4 or 26 kg/m^3). Overall, all 3 stress indices were similar for LD and HD groups. A rapid acute stress response was observed only for the serum cortisol measurements, but serum cortisol, N:L ratio, and water cortisol all appeared to show a peak in stress responses at 14 d after sorting the fish into either HD or LD experimental conditions. Therefore, the stress response profiles described here do not appear to be specific to the different density conditions as intended but rather may reflect the response of either group to being confined in small tanks, approximately 25 cm in diameter, regardless of actual water volume. If so, these data may define a stress response that is relevant to many experimental exposure systems used to investigate infectious diseases in fish. Whether this stress response affects the ability of experimental conditions to mimic disease as it progresses through fish populations in the much larger holding conditions found in aquaculture or wild fish environments remains to be investigated. Future studies could use stress indices to help characterize levels of stress at time points before, during and after exposure to infectious agents, and to examine the effects of even higher densities that might approach the upper end of densities found in captive fish settings (ex: commercial, research) relevant to the fish species under study.

The fish in the current studies originated from a domesticated stock. Compared with domesticated hatchery-reared fish, wild rainbow trout can exhibit more extreme stress responses when exposed to stressors encountered with common aquaculture management practices.[31,38] For example, stress responses measured by primary indices of plasma cortisol and secondary stress indices of glucose have been reported to be significantly higher in wild rainbow trout than hatchery-reared counterparts when exposed to common aquaculture management practices of confinement in a net and electroshock.[38] The domesticated fish used in our experiments are likely more resistant to these types of stressors, and thus the densities used here could still adversely affect wild populations. This concept may also extend to other fish species used in research, as they may show differences in stress-induced responses between generations of captive-bred as compared with wild populations.

Finally, for management implications, the collective data obtained from these studies indicate that the densities and water flows tested (4 to 26 kg/m^3, 1 to 4 grams, 0.3 L/min inflow) had no effect on water quality, stress, or other measures of fish welfare. Thus, these densities appear to be within an acceptable range for this fish species.

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