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The optimum velocity for Atlantic salmon post-smolts in RAS is a compromise between muscle growth and fish welfare

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A B S T R A C T

It is increasingly common to rear Atlantic salmon smolts for longer periods in recirculating aquaculture systems (RAS) before transfer to sea cages. The conditions for this part of the life cycle are currently being optimized, especially the water velocity in the tanks since its impact has been correlated with.

growth. Earlier studies indicate that higher water velocities promote growth in salmon, nonetheless, the optimal velocity and its associated health and welfare consequences are yet to be established. In the present study, we determined the effects of different water velocities on growth, muscle development and welfare to estimate the optimum velocity for the rearing of post-smolts in RAS.

We divided 2400 salmon post-smolts (average start weight 80 g) into twelve tanks (200 fish per tank) and set the water velocities in four triplicate tanks to low (L) – 0.5 body length per second (BL/s); medium (M) – 1.0 BL/s; high (H) – 1.8 BL/s; and very high (VH) – 2.5 (BL/s). The velocity for the VH group was the highest tested for salmon post-smolts to date. The trial lasted for three months and organ samples were collected at three time points. Time-lapse cameras revealed a relatively even fish distribution in the tank in L and M groups. In contrast, fish in H and VH groups displayed strong schooling behavior at specific regions in the tanks. We observed a close to a linear relationship between water velocity and average growth rate, which resulted in a 5.7% higher average body weight in the VH group in comparison to the L group at termination. The condition factors of fish from the L group was lower than in the other three groups. Muscle cellularity analysis revealed smaller fibers in the L group, while bigger muscle fibers were identified in H and VH groups which contributed to somatic growth. Nonetheless, increased cases of inflammation were observed in H and VH groups. Muscle proteomics revealed decreased translation and carbohydrate activity in the L group. The overall external welfare status of the fish was favorable; however, increased incidence of skin damage (i.e., scale loss, hemorrhaging) and pelvic fin damage (i.e., splitting) in the H and VH groups was documented. Skin histology revealed relatively thinner epidermis at higher velocities. On the other hand, increased cases of aneurysm, lamellar clubbing and fusion were identified at higher velocities too.

In conclusion, the increased body weight of fish reared in high water velocities was likely mostly due to enhanced somatic growth of muscle fibers. These findings provide further evidence that elevated water velocities have positive effects on the growth rate of post-smolts even at the highest levels tested to date. On the other hand, the external welfare scores, histological analyses and the molecular data indicate that mucosal health was negatively affected by higher velocities. Thus, based on this study, the optimal water velocity for long term rearing of salmon post-smolts in RAS is most likely located slightly above 1 body length per second.

1. Introduction

The Atlantic salmon (*Salmo salar* L.) farming industry is expanding and progressively integrating recirculating aquaculture systems (RAS) into the production cycle. Juvenile salmon are produced in freshwater land-based facilities where they undergo the parr-smolt transformation which prepares them for migration to seawater, after which they are referred to as post-smolts. Salmon smolts are increasingly kept for longer periods in RAS in order to take advantage of the more controlled sea water environment and to produce larger fish prior to open sea cage transfer. The rising demand for prolonged land-based rearing has resulted in research initiatives (e.g. CtrlAQUA, https://ctrlaqua.no/) to optimize the conditions for post-smolt production. Some of the advantages of RAS-based production compared to flow through systems include minimal exposure to challenging conditions, better control of the rearing environment and less water consumption. The successful

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adoption of this technology during the parr-smolt transformation introduced some technological challenges. Although faster growth rates are often regarded as the primary indicator of rearing regime success, increased growth cannot come as an expense to fish welfare. Over and above ethical considerations around fish welfare, compromised early fish welfare may result in losses in production or a lower value product due to longer term associated effects on the resilience, disease resistance and health.

Salmonids are migratory fish, with an exceptional inherent capacity for sustained aerobic swimming. In wild Atlantic salmon this is particularly prominent in the early life stages where they are resident in fast-flowing streams and rivers before migrating downstream to the open seas. Thus, increasing the flow rate and the water current within the rearing tanks of juvenile Atlantic salmon as a long-term training regime, could simulate natural conditions. Improved growth due to exercise (i.e. increased water velocity) has been documented in different fish species, including salmonids (Castro et al., 2011; Davison, 1997; Good et al., 2016) and non-salmonids, (*Plecoglossus altivelis (*Nakagawa et al., 1991*)*; *Chalcarburnus chalcoides mento (*Hinterleitner et al., 1992*)*; *Morone saxatilis (*Young and Cech, 1993*)*; *Sparus aurata* (Ibarz et al., 2011); *Danio rerio (*Palstra et al., 2010*)*). These effects have been explained by improved feed efficiency, higher feed intake or a mix of both. A relationship between growth and disease resistance has been reported as well (see reviews by (Merrifield et al., 2010; Sweetman et al., 2010)). In addition, aerobic exercise of cultured fish has been linked to a myriad of benefits, including increased cardiac growth and performance, aerobic capacity of the muscle, oxygen carrying and extraction capacity, improved skeletal integrity (Anttila et al., 2008; Davie et al., 1986; Farrell et al., 1990; Gallaugher et al., 2001; Totland et al., 2011) and even plasticity of the brain (Mes et al., 2020). Several studies have investigated the effects of exercise on disease resistance in salmonids. A relatively lower mortality following seawater transfer was observed in exercised chum salmon (*Oncorhynchus keta*) compared with the group that was not subjected to exercise, implicating improved osmoregulatory capacity (Khovanskiy, and N.Y., Shakhmatova YI., 1993). In one of our previous studies, we found the survival rate of salmon pre-smolts subjected to moderate interval training was 13% higher during a challenge trial with infectious pancreatic necrosis virus in comparison to fish kept at low and constant water speed (Castro et al., 2011). Together these studies suggest moderate aerobic training of fish can induce similar beneficial effects as for humans, such as decreased risk of infections and chronic life-style diseases (Gleeson, 2007; Mathur and Pedersen, 2008).

Training intensities for fish are typically defined as water velocity relative to the body length of the fish. In previous training trials with salmon, including our own, medium velocities were usually defined as close to one body length (average of the population) per second of water velocity in the tank (BL/s). The lowest applicable water velocity is restricted by the self-cleaning capacity of the tank and assurance of a sufficient water exchange, which limits the build-up of bacteria, ammonia, CO_2 and particles. The hydraulic retention time must be controlled to stay within acceptable margins and the formation of "dead zones" (zones in the tank with low water exchange and thus, low oxygen levels) must be avoided. Long term tests with high-intensity training and with up to 2 BL/s have been reported (Good et al., 2016). However, in the case of salmon, the upper limits of training intensities were seldom addressed and in particular the effects on welfare remain unstudied.

Exercise regimes that increase the growth rate of salmon without compromising the welfare is a potential production strategy that is highly beneficial for the industry. It is likely, that an optimum swimming speed (U_{opt}), could be estimated, where the cost of transport is minimized in relation to energy consumption (Tucker, 1970) and healthy somatic development is most likely for most fish. Speeds higher than U_{opt} result in increased energy consumption for locomotion and may increase the stress level (Palstra et al., 2010). On the other hand, even lower speeds may have similar effects due to increased spontaneous activity and aggressive behavior. The upper limit of the swimming capacity is defined as critical swimming speed (U_{crit}). Both U_{opt} and U_{crit} are not trivial to determine since they may depend on various easily measurable parameters such as water temperature and quality. Further, it was shown that individual fish have a lower U_{crit} than groups (Remen et al., 2016). Thus, it is preferable for experiments with fish in high water velocities to be performed in environments, which reflect their natural habitat. Results from such experiments have the potential to contribute to a cost-benefit optimization of exercise regimes for increased growth and improved welfare and may help to define a best practice for the industry.

In this study, we aimed to identify the optimum water velocity for post-smolts in brackish water RAS. To achieve this, we used a range of water velocities from a minimum setting to ensure self-cleaning of the tanks, to the highest water velocity tested under experimental conditions (to our knowledge). A suite of tools was employed to examine the welfare, growth and the physiological responses of the fish to the different velocities.

2. Materials and methods

2.1. Compliance with ethical guidelines in animal experimentation

All fish handling procedures complied with the Guidelines of the European Union (2010/63/EU) and the trial was granted approval by the Norwegian Food Safety Authority (FOTS ID 13894).

2.2. Rearing of fish and experimental setup

The trial was performed at the Nofima Centre for Recirculation Aquaculture (NCRA) in Sunndalsøra, Norway. Fish eggs (Bolaks strain) were purchased from SalmoBreed and were hatched at Nofima in a freshwater flow-through system. When the fish reached an average body weight of 20 g they were exposed to a 12 h light and 12 h dark (12 L:12D) regime for eleven weeks, followed by a five-week period of 24 h light to induce smoltification (Handeland and Stefansson, 2001). During the last three weeks of the 24-h light regime, a subset of 10 fish was subjected to a seawater test at three time points to ensure seawater tolerance (Kolarevic et al., 2014). These fish were exposed to seawater (34 ppt) for 72 h, blood was drawn at the end of the trial and tested for plasma chloride levels. Chloride levels lower than 150 mmol/l were used as the critical threshold for seawater tolerance (Arnesen et al., 2003). Fish were fed with commercial diets in excess with monitoring of feed intake for optimal feeding and growth. At commencement of the training regime 3 mm *Skretting Nutra Olympic* pellets were fed (Skretting, Stavanger, Norway) and transitioned to 4 mm pellets after four weeks of training. Oxygen was monitored and kept >85% saturation, water temperature was 12 ± 0.3 °C (mean ± standard deviation). All fish were vaccinated with a 6-component (ALPHA JECT, Pharmaq, Norway) vaccine. At the beginning of the 24-h light regime, 2400 fish were injected with a passive integrated transponder tag (Jojo Automasjon AS, Sola, Norway).

Twelve octagonal tanks with a volume of $3.3m^3$, a diameter of 2 m and a water depth of ca. 1 m were prepared for the experiment and tested for the final maximum velocities. The water was recirculated between two recirculating aquaculture systems (RAS) with a salinity of 12 ppt and a constant temperature of 12 ± 0.5 °C, which was identified as optimum conditions in (Ytrestoyl et al., 2020). For specific technical details of the RAS, please refer to (Terjesen et al., 2013). The installed water inlets connected to vertical pipes with multiple outlets into the tanks were strong enough to reach the desired velocities for the L and M groups. For the H and VH velocity groups, additional pumps (Flygt DLM-50, Xylem Inc., New-York, USA) with attached horizontal pipes and outlets were installed on the bottom of the tanks and were activated when needed to maintain sufficient velocities. Time-lapse cameras (GoPro Hero 4) were installed over each tank to monitor fish

positioning/schooling behavior within the tanks. Pictures were taken every minute during the first week of the trial. Weight (average ± standard deviation 83.3 ± 9.8 g) and length (192.6 ± 8.0 mm) of all fish were recorded at transfer to the training tanks.

Groups of two hundred fish were randomly transferred to each of the twelve training tanks (density 5 kg/m^3) and the water velocity in all tanks was adjusted to 0.5 body length per second (BL/s). During the first week, the water velocities in the tanks were adjusted stepwise to the desired training velocities. At day four after the transfer, the velocity was adjusted to ca. 50% above the low starting velocity of the final values and at day seven, the desired velocities were achieved. The twelve tanks were divided into four training groups with three tanks per group (triplicates). The desired velocities in the four groups were: LOW (L): 0.5BL/s, MEDIUM (M) 1BL/s, HIGH (H) 1.8BL/s and VERY HIGH (VH) 2.5BL/s. Due to the length increase of the fish during the training period, the water velocity was adjusted weekly to the average body length in the respective tanks. The actual velocities in each tank were measured with a velocimeter (Nortek vector, Nortek, Norway) 45 cm away from the frontal tank wall and in three depths of 20, 40 and 60 cm. The average of velocities over depths was considered as the overall tank velocity. The measurement points were based on a detailed fluid-dynamics study, which was conducted previously in the same tanks and were found to be most representative to determine the average water velocities (unpublished). The weight and length measurements were conducted regularly (see Fig. 1), and the velocities were adjusted according to the measured or extrapolated average body lengths of the fish population in each tank. All tanks were inspected daily, and dead fish were removed from the tanks immediately and were registered.

The fish were subjected to the different velocities continuously for three months. Water parameters were regularly monitored and maintained at specific thresholds: Temperature at 12 ± 0.5 °C, salinity at 12 ppt, dissolved oxygen at >85% saturation, CO2 < 1450 vpm and pH at 7.5. Chemical analyses of ammonia and nitrogen oxide concentrations were performed at least twice per week. Water samples were taken from different tanks and no differences between tanks were found. The experimental hall was illuminated with white LED lights under a 24 L:0D photocycle. Samples were collected at three time points: (1) Nine days after start of the experiment. This time was chosen because the water velocity had stabilized at desired target values after a weeklong adjustment; (2) At the midpoint, after 6 weeks of training; (3) At the end of the experiment, after three months of training. The experiment was terminated three days after the last sampling and all remaining fish were scanned and measured for weight and length. During the three sampling time points, six fish were randomly removed from each tank and humanely euthanized with a lethal dose of Finquel (MSD Animal Health, Netherlands). These fish were scanned, measured for weight and length (fork length), whole-body pictures were taken in a custom-built photo box (manual settings, Canon 60D, 18-55 mm zoom lens, set to 28 mm), external welfare scores were evaluated according to the FISHWELL handbook (Noble et al., 2018), the heart weight was recorded, blood samples and tissue samples were taken. Tissue samples were collected below the dorsal fin – samples were preserved in RNAlater (ThermoFisher, USA) for gene expression analysis (i.e. skeletal muscle) and samples for histology (i.e., gills, skin and skeletal muscle) were preserved in 10% neutral buffered formalin (CellPath, UK).

2.3. Blood analysis

Blood was withdrawn from caudal artery using heparin-coated vacutainers (BD, United Kingdom) and the hemoglobin content was measured with a HemoCue™ analyser (HemoCue 201$^+$, Ängelholm, Sweden). Plasma was collected by centrifugation at 1000 xg for 10 mins at 4 °C, and thereafter kept at −80 °C until analysis. The plasma samples were used to profile key stress parameters, including cortisol (ELISA, Neogen, USA), glucose (Sigma-Aldrich, USA) and lactate (Sigma-Aldrich) using commercially available kits.

2.4. Gene expression

Total RNA was isolated from the skeletal muscle by Agencourt RNAdvanced Tissue Kit (Beckman Coulter, USA) using Biomek 4000 Automated Workstation (Beckman-Coulter, USA). The RNA concentration and quality were determined using a NanoDrop 8000 spectrophotometer (Thermo Scientific, USA). Complementary DNA (cDNA) was prepared from 1000 ng template RNA using Taqman RT Reagents Kit (Beckman-Coulter, USA).

The transcript levels of selected genes involved in muscle growth and metabolism were quantified using the PowerUp™ SYBR™ Green master chemistry (Applied Biosystems, CA USA) in a QuantStudio5 real-time quantitative PCR system (Applied Biosystems). The qPCR reaction mixture included 4 µll 1:10 dilution of cDNA, 5 µl SYBR™ Green Master, and 1 µl of the forward and reverse primer. All samples were run in duplicate, including minus reverse transcriptase and no template controls. The thermocycling protocol was as follows: a pre-incubation at 95 °C for 2 min, amplification with 40 cycles at 95 °C for 1 s, and 60 °C for 30 s, and a dissociation step series of 95 °C for 15 s, 60 °C for 1 min,

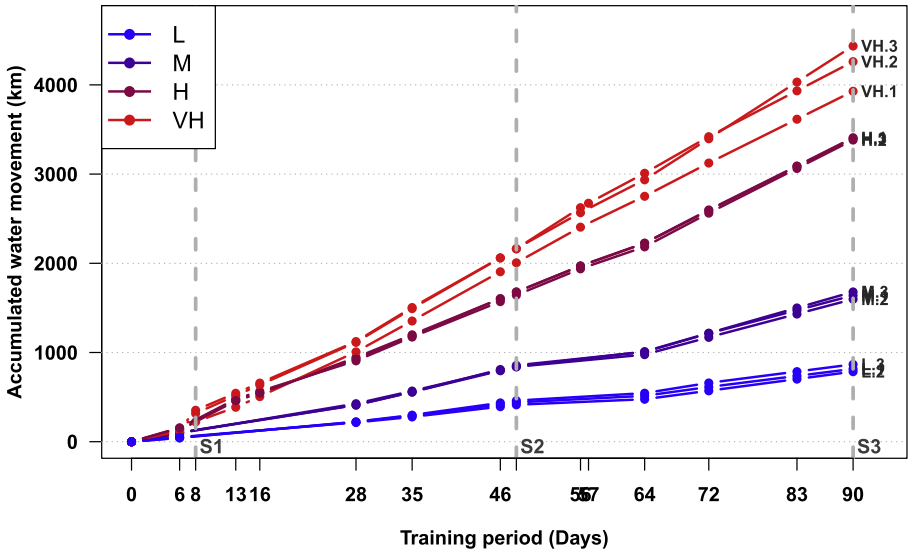

Fig. 1. Accumulated water movement during the training period. Water velocity measurements are represented by dots. The accumulated water movement was calculated based on these measurements. Tank replicate labels are shown at the end of the lines. The three sampling time points are indicated by dashed grey lines and the labels "S1" to "S3". The first sampling (S1) was performed two days after the desired water velocities in the training tanks were achieved. The second sampling (S2) took place on day 47 and the end point (S3) sampling on day 90.

and 95 °C for 15 s. The primers used in the study are given in Supplementary Table 1. A five-point standard curve of 3-fold dilution series was prepared from pooled cDNA to calculate the amplification efficiencies. Three reference genes namely *beta-actin* (*actb*), *eukaryotic translation elongation factor* (*eef1a1*) and *ribosomal protein S19* (*rps19*) were tested for their suitability for normalization. geNorm (Vandesompele et al., 2002) identified *actb* and *eef1a1* as the two most stable genes and their geometric average was used to normalize the expression data, as described earlier (Nagasawa et al., 2012).

2.5. Proteomics

Peptide digest from the muscle was prepared by a double digestion protocol (Pierce™ Mass Spec, ThermoFischer, USA), slightly modified for tissue samples. Briefly, 20 mg tissue was mixed with 4 volume of lysis buffer, vortexed vigorously and incubated at 95 °C for 5 mins. The lysate was pulse-sonicated and centrifuged at 16,000 ×*g* for 10 min at 4 °C before the protein content was determined by Qubit™ (ThermoFischer, UK) protein assay kit. The lysate was reduced for 45 mins in 10 mM dithiothreitol at 50 °C, alkylated for 20 mins in 50 mM iodoacetamide at room temperature and the proteins were acetone-precipitated overnight at 4 °C. Thereafter, the protein pellet was digested for 2 h in 1 μg Lys-C endoproteinase at 37 °C followed by an overnight trypsin treatment at 37 °C. Digestion was stopped by freezing the samples at −80 °C for 3 h. The proteolytic digest was concentrated in a vacuum centrifuge and stored at −80 °C until LC-MS analysis. Approximately 1 mg of tryptic peptides was loaded onto a trap column (Acclaim PepMap100, C18, 5 μm, 100 Å, 300 μm i.d. × 5 mm) and then backflushed onto a 50 cm × 75 mm analytical column (Acclaim PepMap RSLC C18, 2 mm, 100 Å, 75 mm i.d. × 50 cm, nanoViper). A 120-min gradient from 3 to 36% solution B (acetonitrile, supplemented with 0.1% [*v*/v] formic acid) was used for separation of the peptides, at a flow rate of 300 nl/min. The Q-Exactive mass spectrometer was set up in data-dependent acquisition method. Mass spectral data were analyzed using Mascot (Matrix Science, London, UK; version 2.6.1), set up to search the contaminants_20160129.fasta; S.salar_refseq_20181010 database with a fragment ion mass tolerance of 0.020 Da and a parent ion tolerance of 10.0 ppm. Carbamidomethyl of cysteine was specified as a fixed modification. Deamidated of asparagine and glutamine, oxidation of methionine and acetyl of the n-terminus were specified as variable modifications. MS/MS based peptide and protein identifications were validated in Scaffold (version Scaffold_4.8.9, Proteome Software Inc., Portland, OR). Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99,0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Unique peptide counts, UniProt accession numbers and protein names were extracted from the Scaffold file.

2.6. Histology and morphometry

Prior to processing, skin samples were decalcified in 10% Titriplex 3 (Sigma-Aldrich) for 3 days at room temperature with constant agitation. Tissue samples (i.e. decalcified skin, skeletal muscle, gills) were processed in a benchtop histoprocessor (Leica TP1020, Germany) following this protocol: 70%, 96%, and 3 × 100% ethanol, 3× xylene and 2× paraffin. Tissue blocks were cut into 5 μm sections and stained with haematoxylin-eosin (Thermo Scientific, USA). Photographs were taken in a Zeiss Axio Observer Z1 (Carl Zeiss, Germany) and all photoprocessing and measurements were carried out in AxioVision Rel. 4.8 (Carl Zeiss).

For skeletal muscle, the cross-sectional area (μm^2) of at least 900 muscle fibers per fish were measured. Muscle fiber density was determined by dividing the fiber number with the total cross-sectional area. For the gills, five filaments (2 upper half, 2 lower half and 1 middle) were randomly selected and evaluation was carried out in a region with 20 lamellae. A total of 100 lamellae were analyzed per fish. Eight key pathologies were scored including lamellar fusion, aneurysm, epithelial lifting, clubbing, hypertrophy, hyperplasia (Stiller et al., 2020), hyperemia and necrosis. A lamella that did not show any pathology was regarded as "healthy". Skin histomorphometry was conducted by measuring epidermal and dermal thickness, where 10 measurements were taken from 3 randomly selected fields per fish.

2.7. Welfare assessment

External welfare scoring is a systematic evaluation of morphological indicators according to a 0–3 ranking system, where 0 denotes no impairment/abnormality/deformity, *aka* healthy, while 3 indicates severe impairment/abnormality/deformity (Noble, 2018). The scoring system was specifically developed for controlled and field-based trials and the included damages were commonly occurring damages in these kind of production systems. The factors graded in this study were: Emaciation, eye damage (both sides), skin damages (both sides), operculum damage (both sides), snout damage, vertebral deformities, jaw deformities, dorsal fin damage, caudal fin damage, caudal fin damage, pectoral fin damage, pelvic fins damage. For fin damages, healed and active (characterized by splitting) damages are distinguished. Scores were assessed for twelve fish per group (four per replicate tank) at the first sampling time point (seven days after transfer to the training tanks) and for 18 fish (six per replicate tank) per group at the second (6 weeks of continuous training) and third (12 weeks of continuous training) sampling time point.

2.8. Data analysis

Statistical analysis and data presentation were performed in R (version 3.5.2, https://www.r-project.org/). ANOVA and *post-hoc* test, according to Tukey, were part of the *stats*-package (functions *aov()* and *TukeyHSD()*). Categorical count data were analyzed with chi-squared tests (*chisq.test()*, *stats* package) and chi-squared post-hoc tests (*chisq.post.hoc(), fifer* package). The cardio somatic index (CSI) was calculated by dividing the heart weight in milligrams (atrium and bulbus removed, remaining blood gently squeezed out onto tissue paper) by the body weight in grams. The thermal growth coefficient was calculated by the formula 1000 * (finalWeightˆ(1/3) - startWeightˆ(1/3)) * (1/(degreesC * days)).

Fish contour analysis: Prior to sampling, a picture was taken of each fish in a custom build box with internal LED lighting and on a green background. These pictures were used in an R script to calculate the contour of the fish. For each column of the pictures (with horizontally positioned fish), the first pixels from top and bottom were determined to calculate the outline positions. The outline was then rotated for identical horizontal positioning and scaled for length differences by scaling the outline by the same factor in X- and Y-directions. The mean and standard errors were then calculated for each position and for each of the four groups.

Picture analysis for fish positions in the tanks: The GoPro time laps cameras captured one picture per minute in a resolution of 2720 × 2040 pixels. To reduce the computation time, only every tenth picture was used in this analysis, which left 144 picture per tank per day. To process the pictures, randomly selected training pictures were read into R (package *jpeg*) and cut into 50 × 50 tiles. The resulting tiles were classified manually for a tile with a fish in it and without. These tiles were used to train an artificial neural network (package *nnet*) for classification of tiles with and without fish. Subsequently, all pictures were classified with the neural network and the results were further processed by

removing the area outside of the tanks (centering of tanks) and combining the three tanks for each training group. The resulting data were plotted for each day and group by the *smoothScatter()* function (package *graphics*) with the *spectral* colour palette (package *RcolorBrewer*).

For proteomics analysis, annotation and gene ontology (GO) terms were retrieved from the UniProt database (UniProt, 2019) for all identified proteins. In total, 1105 different proteins were found, and data was further processed in R. Low abundant results were removed by deleting all proteins with mean counts in the 24 samples smaller or equal than 1. The remaining 570 proteins were normalized by dividing the counts by the individual mean counts (lowest mean count was 3.7, highest was 6.8 and the mean was 5.8). To identify differences between groups, ANOVAs were calculated for each protein. A *p*-value cutoff of 0.05 was applied and resulted in 338 proteins. These proteins were prepared for cluster analysis by calculating group means, centered by dividing by row means and $\log_2$-transformed. The data were clustered by the function *hclust()* (*stats* package, for Euclidean distance and with complete linkage) and plotted with *heatmap.2()* (*gplots* package). Three sub-clusters were identified and mean values of these where plotted as bar plots with error bars, showing SEM. GO terms within the three clusters were counted for the GO aspects "biological process", sorted and the top 15 were added to the plot. A full list of GOs and proteomics results are provided in Supplement Table 2.

3. Results

3.1. Water velocity during the training period and sampling time points

Fig. 1 shows the accumulated water movement calculated based on the velocity measurements. The accumulated water movement was very similar within the L, M and H groups. After 90 days, the average water movement of group L was 823.2 km (standard deviation ±41.1), 1639.4 ± 41.0 km of group M and 3397.0 ± 12.3 km of group H. The average in group VH was 4209.0 ± 257.7 km, however, high variations were observed among replicate tanks. Replicate 1 of VH reached the lowest total distance with 3929.2 km.

3.2. Growth performance

The total number of fish with measurements at the beginning and the end of the experiment was 2252. During the experiment, eight fish died outside of samplings, the maximum of dead fish was recorded in two of the VH tanks with two dead fish each. The mean density increased to 27.87 kg/m^3 with a range from 27.2 to 28.7 kg/m^3 due to growth differences.

Body metrics of fish after three months of training are shown in Fig. 2. There is a visible and significant tendency that increasing water velocities resulted in increased weight gain. The lowest growth was found for the L group, which grew to an average weight of 463.1 ± 84.8 g. The relative weight increase in comparison to the L group was +2.3% for the M group, +3.4% for the H group and + 5.7% for the VH group, which reached an average weight of 489.5 ± 80.98 g. The thermal growth coefficient (TGC) followed a similar pattern as the body weight. The body length development showed a different pattern than the weight gain. The L and VH groups grew to a similar mean length (325 ± 16.52 mm and 325.4 ± 14.62 mm, respectively), while the M group was the shortest (322.6 ± 15.72 mm and the H group was in between the other groups (323.5 ± 14.99 mm. These differences in growth performance resulted in strong differences in condition factor. The L group had a significantly lower mean value (1.336 ± 0.11) than the other three groups (1.397 ± 0.11 and above), which were not significantly different to each other. Cardio somatic indices (CSI) were calculated for eighteen fish per group. However, no significant differences were found (data not shown).

Whole body pictures were taken from each sampled fish and a few additional ones to a total of 14 to 26 fish per group. These pictures were used to determine the outline of each fish and to calculate the average body contours for the four groups (Fig. 3). The largest differences in body heights were found at the widest part of the body with 1.1% for M, 3.7% for H and 4.3% for VH wider bodies relative to the L group. Differences were larger in front of the fish than in the back of the fish and the outlines of the M group were overall more similar to the L group, while H and VH were more similar to each other.

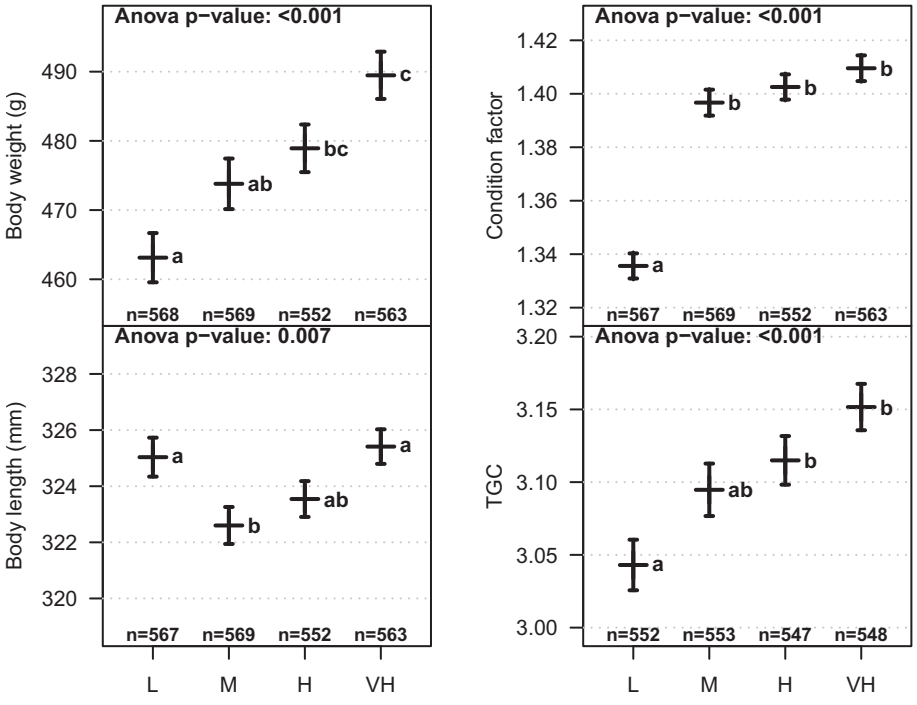

Fig. 2. Body metrics after three months of training (end of experiment). The plots show the means for the four training groups as black horizontal lines. The error bars show +/− the standard error of the mean. One-way ANOVA *p*-values for the respective variable are shown on top of each plot. Lower case letters indicate results of Tukey *post-hoc* tests. Groups which do not share a letter were significantly different to each other ($p < 0.05$). Numbers of samples within each group are indicated at the bottom of the plot. "TGC" = thermal growth coefficient.

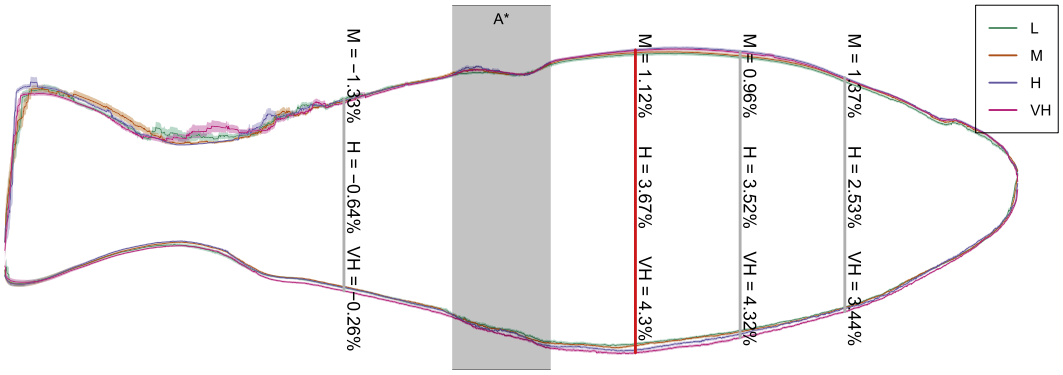

Fig. 3. Comparison of fish body contours after three months of training. Fish (n = 14 to 26 per group) sizes were determined from whole body pictures and normalized for differences in length. Body height differences were calculated for different positions (grey horizontal lines) and the widest position of the fish (red horizontal line). Relative differences in heights to the L group are indicated in the plot as percentage values. The grey area marked with "A*" had high interferences due to an uneven background. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Positioning of fish in the tanks and schooling behavior

The heat maps shown in Fig. 4 were generated from the time-lapse photos revealed the temporal progression of fish positions in the tank. During the first three days after transfer, the velocity was low (0.5 BL/s) in all tanks to allow the fish to acclimatize to the new environment. The tank profile was similar for all groups, where an even distribution with a visible low-density region at the center of the tank was observed. At day four, the velocity was increased to ca. the midpoint between the starting and the final velocity (L: 0.5 BL/s, M: 0.75 BL/s, H:1.1 BL/s, VH: 1.5 BL/s). The velocity did not change for the L group, as revealed by an unchanged pattern in the heatmaps. Similarly, the relatively low increase in velocity for the M group did not affect the distribution. In contrast, schooling behavior in the H and VH groups were visible and the fish formed a group at the corners of the tanks. At day seven, the velocities were adjusted to the final training intensities. The same trend as after the first increase was observed. The L and M groups did not change visibly in distribution; however, the H and VH groups increased the schooling behavior and the densities at the corners increased further. It was planned to monitor the fish distribution again before the midpoint and end point samplings, however, the turbidity of the water increased during the trial, which made further monitoring impossible.

3.4. Expression of selected genes in the skeletal muscle

The expression profile in skeletal muscle is shown in Fig. 5. Eight of the analyzed genes were involved in myogenesis (MG), for which three were markers for myogenic specification/commitment (*myod1b*, *myod1c* and *myf5*), one for proliferation (*pcna*), two for differentiation (*mrf4* and *myog*) and two for muscle-specific ubiquitin ligases (*murf1* and *mafbx*). Significant differences among the groups were only found for *myod1c*, *mrf4* and *myog*. The marker gene for MG specification (*myod1c)* was upregulated in the M group and was lowest in VH group. Similarly, the MG differentiation marker gene *mrf4* was slightly upregulated in M and L groups, compared with the two other groups that displayed almost similar expression pattern. However, the other MG differentiation gene (*myog*) was lowest expressed in the L group and expression increased with the training intensity. Six of the analyzed genes were involved in different metabolic pathways and three of these were significantly different among the training groups. *Phosphoglycerate kinase* (*pgk*) and *pyruvate kinase* (*pkm*), genes coding for key enzymes in glycolysis, were significantly upregulated in the VH group and in case of *pgk* to a lower extent in group H. *Aldolase B* (*aldob*) is coding for an enzyme involved in both glycolysis and glycogenesis and was downregulated in groups H and VH. The remaining three metabolic genes (*taldo*, *creb* and *fbp1*) were not significantly different among the training groups. *Lipoate-protein ligase* (*lpl*), one of the two lipid metabolism and adipocyte differentiation genes, was upregulated in the VH group, while the other marker, *adiponectin* (*adipoq*) was not differentially regulated. The analyzed insulin-like growth factor 1 (*igf1*) gene expression, which is a marker for anabolic effects, was significantly increased in the VH group and slightly higher expressed in the H group. Interleukin 1 beta (*il1b*) was analyzed as an inflammation marker and was significantly higher expressed in the VH group compared to the L group. The expression pattern of *il1b* followed a linear trend with increasing training intensity.

3.5. Muscle cellularity

Between 985 and 1330 muscle fibers were counted in each fish and the number of muscle fibers per µm of the white muscle cross-sections were estimated. Results of this analysis are shown in Fig. 6A. A significantly higher density of muscle fibers was found for the L group. The remaining groups M to VH were not significantly different to each other; however, a decreasing trend in density with increasing training intensity is observed. In addition to the density, areas of the muscle fibers were measured (Fig. 6B). The area of the fibers increased significantly with the training intensity. In comparison to the L group, the mean area of fibers increased by 9.9% for the M group, 20.1% for H and 28.1% for the VH group. Signs of inflammation were observed, thus the ratio of muscle fibers with inflammation of the total number of fibers was calculated for each fish. The mean ratios for the four groups are shown in Fig. 6C. Significant differences between the two lower intensity groups (L and M) to the two higher intensity groups (H and VH) were found. While ca. 1% of the counted fibers displayed signs of inflammation in the L and M groups, around 5.2% and 6.0% were accounted for in H and VH groups, respectively.

3.6. Skin histomorphometry

The epidermis was significantly thinner in the H and VH groups in comparison to the M group. Conversely, the thickness of dermis increased significantly with increasing water velocities (Fig. 7).

3.7. Gill quantitative pathology

The number of healthy lamellas, as well as six key branchial pathologies were evaluated, and the ratios of their occurrences relative to the total number of evaluated lamellas are shown in Fig. 8. The categorical counts were significantly different among the four groups (chi-squared test $p < 0.001$ and chi-squared post hoc test $p < 0.05$). There is an overall tendency that higher training intensities resulted in higher numbers of pathological alterations. While 90.6% of the evaluated lamellas of the L group were healthy, this ratio decreased to 80.6% in M, to 78.4% in H and finally to 73.6% in the VH group. Cases of hyperplasia

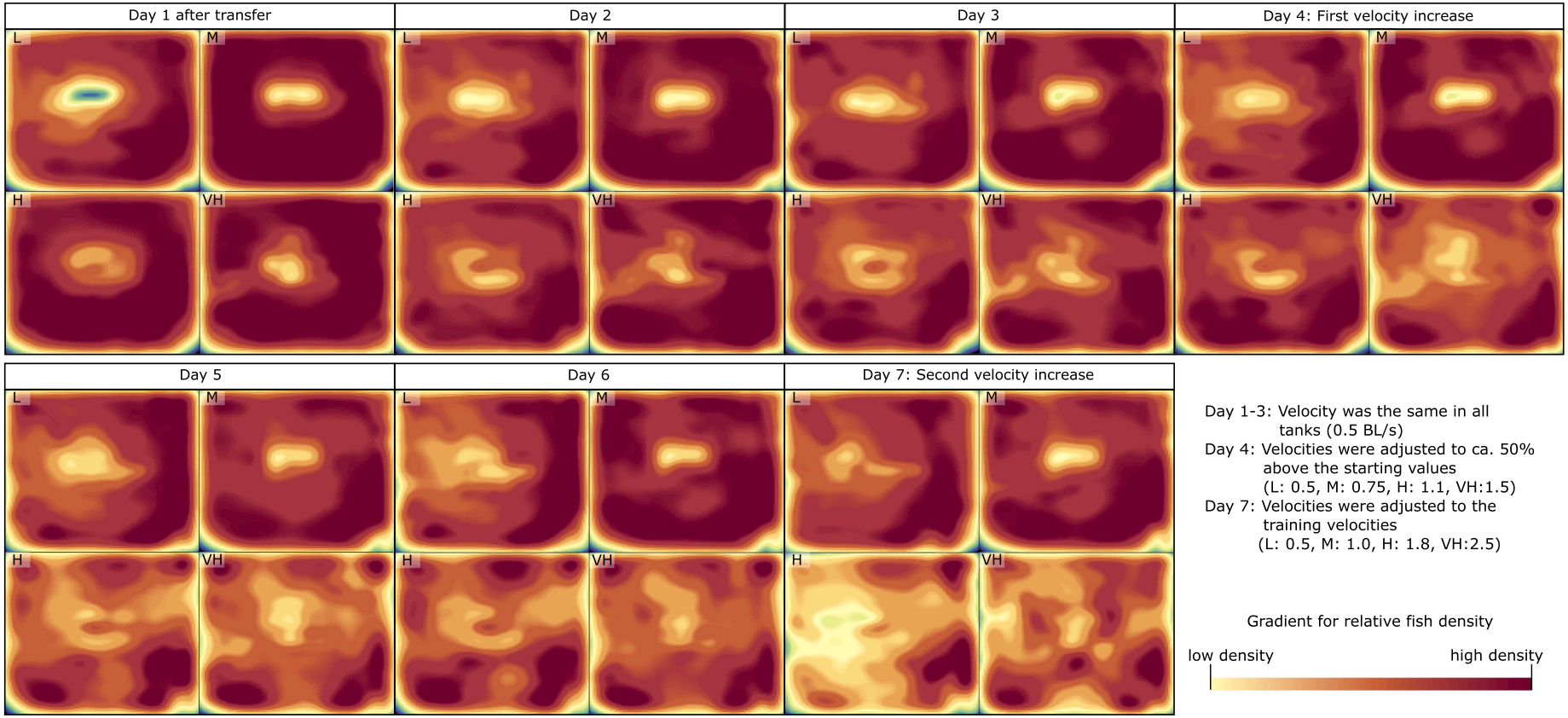

Fig. 4. Fish positions in the tanks during the first week after transfer into the training tanks. The velocities were adjusted at two time points, see explanation in figure for details (velocities are in BL/s). For each day, one heatmap was calculated per group. Each of these heatmaps represents the combined results for the triplicate tanks for one training group. The gradient from light yellow to dark red represents the detected relative fish density during the 24 h of each day. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

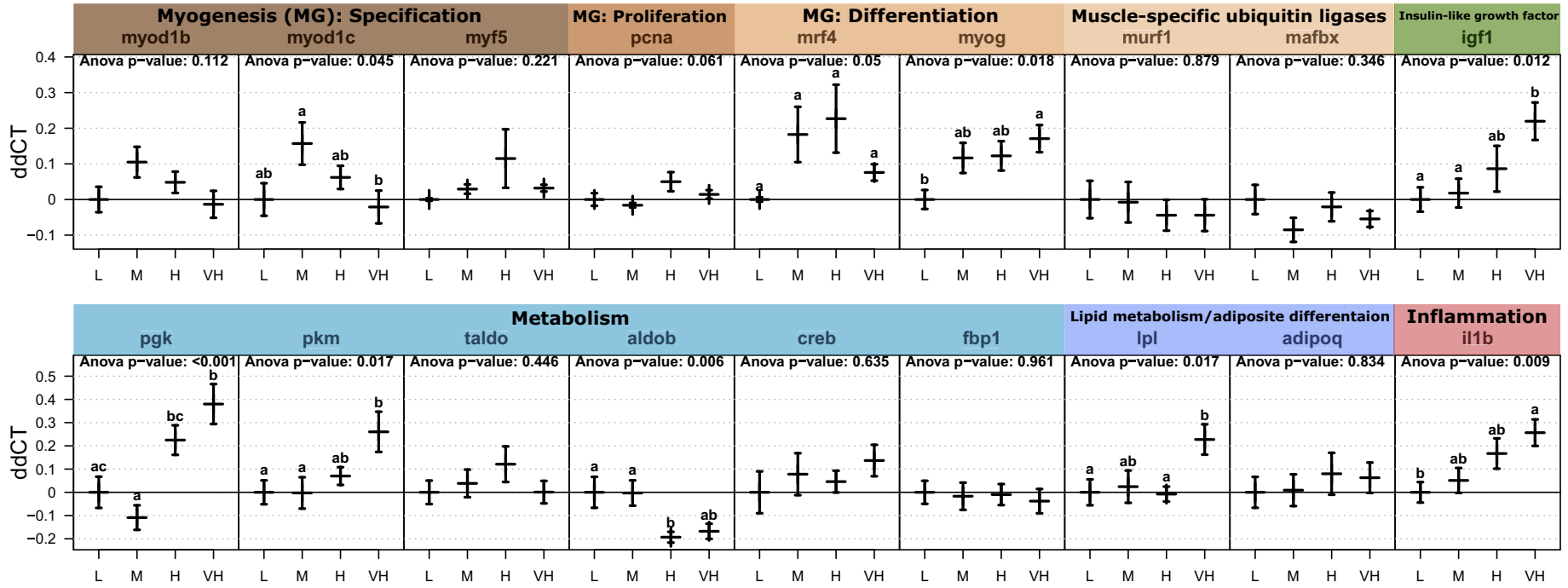

Fig. 5. Expression of selected genes, quantified by RT-qPCR. White muscle tissue from four fish from each replicate tank were analyzed to a total of twelve fish per group. Mean ddCT values are indicated by horizontal lines and error bars show +/− SEM. One-way ANOVA *p*-values for the respective genes are shown on top of each plot. In case of a significant ANOVA result ($p < 0.05$), lower case letters indicate results of a Tukey *post-hoc* test. Groups which do not share a letter were significantly different to each other ($p < 0.05$).

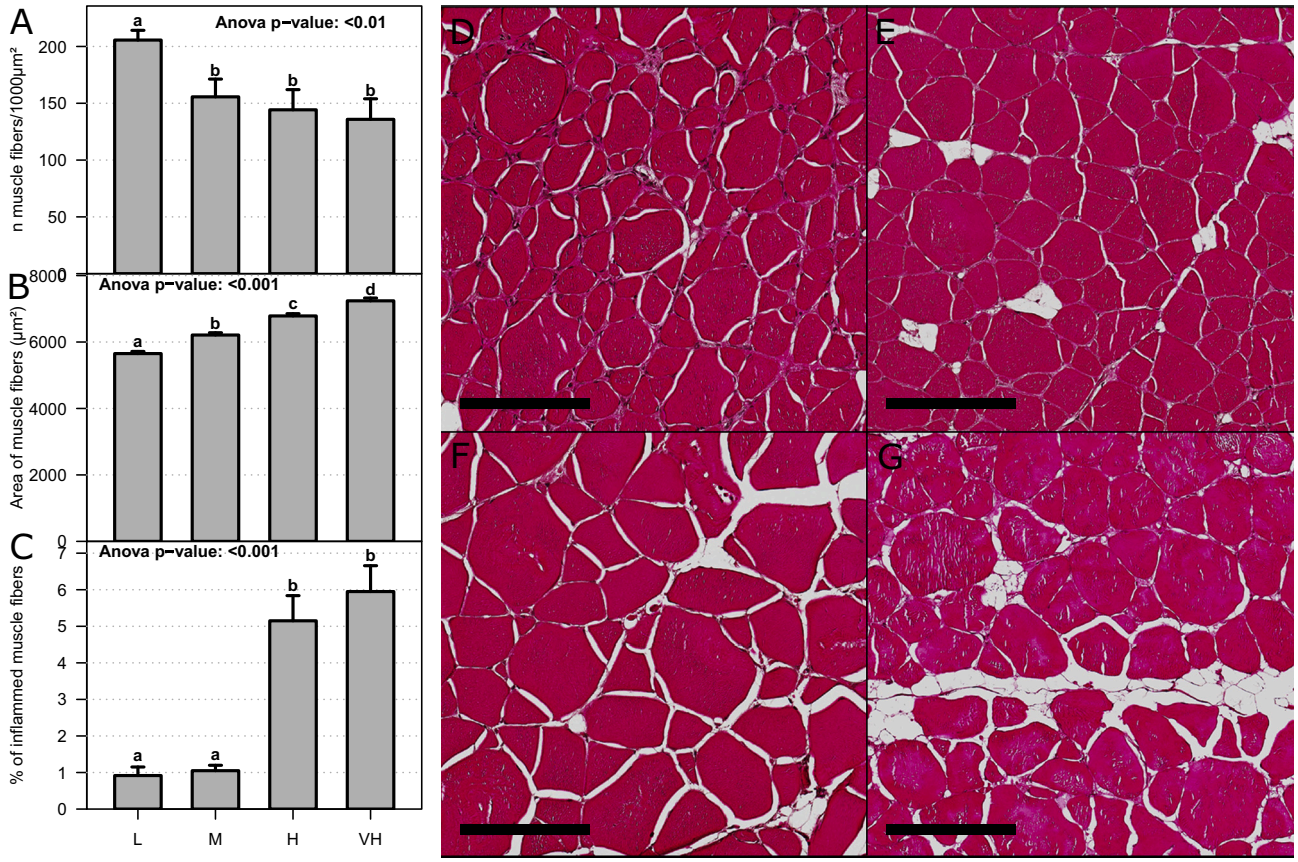

Fig. 6. Histological analysis of skeletal muscle fibers. A: Numbers of fibers per 1000µm^2 cross-sectional area of skeletal muscle tissue. Between 985 and 1330 fibers were counted per fish and six fish were analyzed per group (two per replicate tank). B: Mean values of the area of the cross sections of muscle fibers. C: Ratios (in percent) of inflamed muscle fibers. Error bars indicate the SEM. One-way ANOVA p-values are shown on top of each plot. Lower case letters indicate results of a Tukey *post-hoc* test. Groups which do not share a letter were significantly different to each other ($p < 0.05$). D-G: Histological example pictures of muscle fibers from group L to VH. The black bars indicate a distance of 200 µm.

were higher in the M and H groups (with 4.5 and 5.5%, respectively) than the L and VH groups (2.5 and 3.1%, respectively). On the other hand, hypertrophy was highest in the H group (5.6%). Increased cases of clubbing were found in H and VH groups (7.4 and 7.1%, respectively), compared with L and M (0.8% and 1.9%, respectively) groups. Epithelial lifting was observed in 4% of L, 9.5% of M and 5.8% of VH of the cases; however, this was only about 0.8% in the H group. Cases of aneurisms increased significantly in the VH group accounting to around 6.9% of the evaluated lamella, while such pathology was only around 2.3% in the H group and less than 0.3% in the other two groups. Lamellar fusion was only observed in the VH group and were accounted for to around 0.7% of the evaluated cases. Hyperemia and necrosis were not found in any of the examined gill lamellae.

3.8. External welfare scores

The overall external welfare of fish in the trial was favorable where the composite average score for majority of the indicators was below 2. Of the 192 fish from the three time points, very few fish were found with signs of emaciation (one fish), eye damages (none), operculum damages (10 fish with mild cases), snout damage (8 fish with mild cases), vertebral deformities (none) or jaw deformities (5 fish with mild cases). However, damages were prominent in skin and the fins. The assessment of the skin condition is shown in Fig. 9. After one week in the training tanks, no significant differences were found between the groups. At this time point, approximately half of the evaluated fish had minor cases of scale loss and almost 20% had severe cases (score ≥ 2); however, no hemorrhaging was found. In contrast, skin hemorrhages were documented in all groups for the following sampling time points. The severity of scale loss increased during the progression of the training and a tendency of more severe cases in all groups except L was apparent (although group-wise comparisons were only significant after 6 weeks of training). Hemorrhaging was detected more frequently on the right side of the fish (outwards side of the fish swimming against the current) after six weeks of training and a higher number of severe cases of scale loss were found after twelve weeks on the same side.

For fin health, dorsal, caudal, pectoral and pelvic fins were assessed (Fig. 10). In addition, it was distinguished if the damage was already healed or still active (i.e. fin splitting). Dorsal fin damages were prevalent at the first sampling time point, with ca. 40% of score 3; a slight recovery was observed mid-way, though damages increased again at the end of the trial. Nonetheless, no significant differences were found among the groups. Caudal fins were only mildly (score 1) affected, exhibited by approximately 75% of evaluated fish at the first time point to almost all fish at the end of the trial where almost all lesions were active. The most severe pectoral fin lesions (score > 2) were found after 7 days of training and decreased to mostly mild (score < 2) cases thereafter. However, at the first time point, lesions were significantly lower for the L group than the other three groups. Pelvic fins were in overall very good condition during the trial; however, mild lesions increased during the trial. At the end of the trial, significantly increasing numbers of fish with lesions were found in correlation with the training intensity.

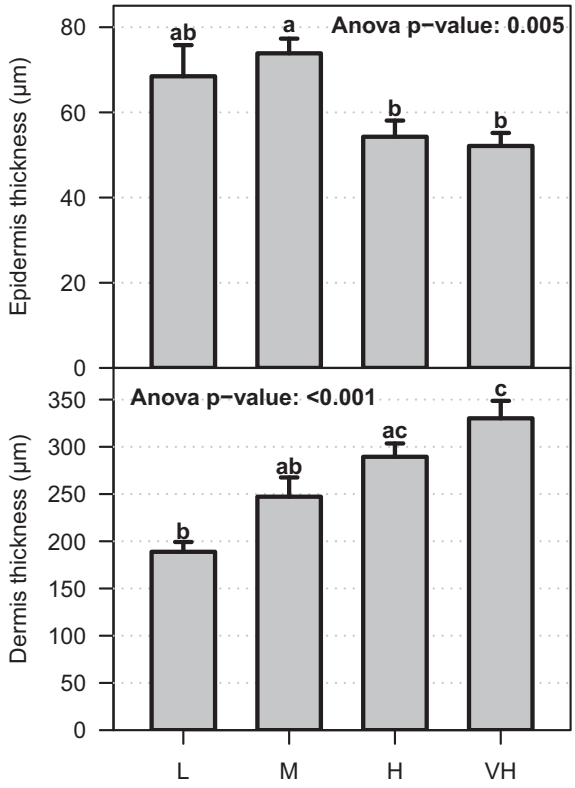

Fig. 7. Thickness of epidermis and dermis, measured for eight fish per group at the end of the trial. Bars show mean values and error bars indicate SEM. One-way ANOVA p-values are shown on top of each plot. Lower case letters indicate results of a Tukey *post-hoc* test. Groups which do not share a letter were significantly different to each other ($p < 0.05$).

3.9. Blood analysis

The plasma cortisol levels after the first week of training were significantly higher in VH fish than in L and M (Fig. 11). Glucose, lactate and hemoglobin were measured after twelve weeks of training (Table 1). All three parameters were not significantly different among the training groups.

3.10. Skeletal muscle proteome

The heatmaps for the proteins identified in the skeletal muscle identified three clusters (Fig. 12). The first two clusters showed a similar tendency – proteins were abundantly expressed in the L group and the expression gradually decreased with increasing training intensity. Though Cluster 2 ($n = 94$) was represented more than Cluster 1 ($n = 23$), the magnitude of change in the latter was higher than the former. Gene ontology terms (GO) for the biological process aspect, connected to the proteins were counted and are shown on the right side of the figure. In Cluster 1, only few GOs were counted more than once and a clear functional direction is not evident. Most GOs were related to regulation of transcription, protein folding and transport. In Cluster 2, highest GO counts were found for protein folding and cell redox homeostasis. This was followed by ATP synthesis, nucleotide excision repair and nucleo- and proteasome related proteins.

Cluster 3 was the largest of the major clusters with 221 proteins and exhibited a general profile of low expression in group L and elevated but similar expression in the other three groups. This cluster was dominated by proteins related to translation ($n = 45$) and protein folding ($n = 9$). This was followed by seven proteins of the tricarboxylic acid cycle, four of carbohydrate metabolic pathways and three proteins of the ATP synthesis. A full list of the proteins is provided in supplement Table 2.

4. Discussion

4.1. Technical challenges in continuous training in RAS

This trial successfully established an environment for controlled continuous training of salmon post-smolts in RAS. We managed to maintain the water velocity close to the desired levels expressed relative to body length per second. The differences between water velocities were small within the three replicate tanks for the groups L, M and H indicating that the training regimes were constantly established. One of the replicates for the VH group was lower than planned and this likely reduced the training effects in this group; however, this discrepancy did not have substantial effects on the experiment's outcome. This problem illustrates the technical challenges associated with the establishment of very high-water velocities (up to 75 cm per second in the end of this trial). It was necessary to install additional pumps in the VH tanks in order to increase the water current, since the current from the water inlet was not sufficient enough to achieve the desired velocity. On the other hand, we did not find differences in water quality between the tanks and we have tested the tanks in previous trials to ensure that even the lowest water velocities ensured sufficient water exchange and self-cleaning of the tanks. The main focus of this study lay on the physiological effects and challenges of different water velocities; thus, further studies are needed to fill in gaps, as hydrodynamic analyses and water quality assessments, especially for larger tanks than what was used in the present study. From a practical outlook, establishing higher velocities in industrial large-scale tanks entails a daunting challenge, requires high energy output and robust engineering design. From an academic perspective, very high-water velocities served as an upper treatment threshold from which to compare the more technically feasible water velocities.

The accumulated water movement plot distinguished a larger difference between the M and H groups than was expected based on the relative differences in their training regimes. The heatmaps for fish positioning and schooling behavior confirmed this by illustrating more evenly distributed fish in the groups L and M, while groups H and VH displayed more similar schooling behavior by forming denser groups at the corners of the tanks. The turbidity of the water increased rapidly during the trial and was most likely amplified by the additional pumps, which were installed in the H and VH tanks, causing shredding of excess feed and feces into difficult-to-filter fine particles. This prohibited further monitoring and inference into the schooling behavior after the first period. Thus, monitoring of schooling behavior of fish in higher densities, as they are used in commercial setups during smolt production (usually up to 50 kg/m^3), needs to be addressed in future trials.

4.2. Continuous training promotes higher body weight gain and skeletal muscle growth

Based on previous trials, where large inter-individual differences were observed, a number of statistical design steps were needed to ensure adequate statistical power to distinguish between treatments (Castro et al., 2011; Castro et al., 2013). Firstly, fish were size selected to have a similar starting weight (mean = 83.3 ± 9.9 g) and as expected, the variation increased during the trial (end point mean = 460.5 ± 108.0 g) Secondly, a high number of individually tagged fish per group (600 per treatment) was sufficient to detect statistically significant differences between groups. Body weight increased as a function of the water velocity and after twelve weeks of training the VH group was in average 5.7% heavier than the L group. This trend is congruent with similar findings in previous studies (Castro et al., 2011; Davison, 1997; Good et al., 2016). Interestingly, length followed a different trend, with the L group diverging from the other three groups, resulting in a relatively longer, thinner body shape and lower condition factor. Fish in the L groups were observed to swimming more freely as opposed to the other groups, where swimming position tended to be more static relative to

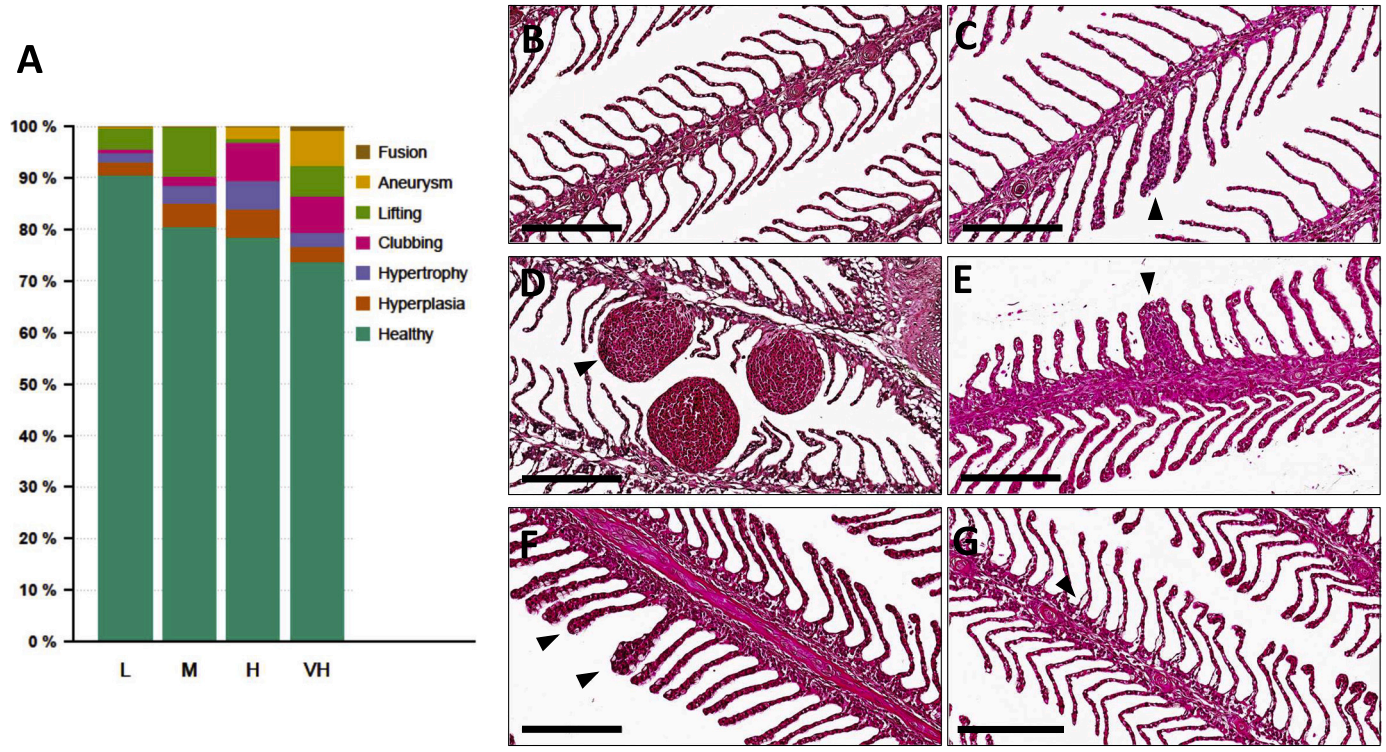

Fig. 8. A) Prevalence of histopathological alterations in the gills. 120 individual lamellas were scored per fish and 9 key pathologies were identified, including healthy, hyperplasia, hypertrophy, clubbing, lifting, aneurysm, fusion, hyperemia and necrosis. B) Healthy looking gills with well-defined branchial structures. Some of the pathologies (arrowhead) characterized: C) hyperplasia of the lamella; D) anuerism; E) fusion of 2 lamellae, with indication of hyplerplasia; F) different stages/degrees of distal lamellar clubbing; and G) epithelial lifting commonly found at the base of the lamella. Scale bar = 200 μm.

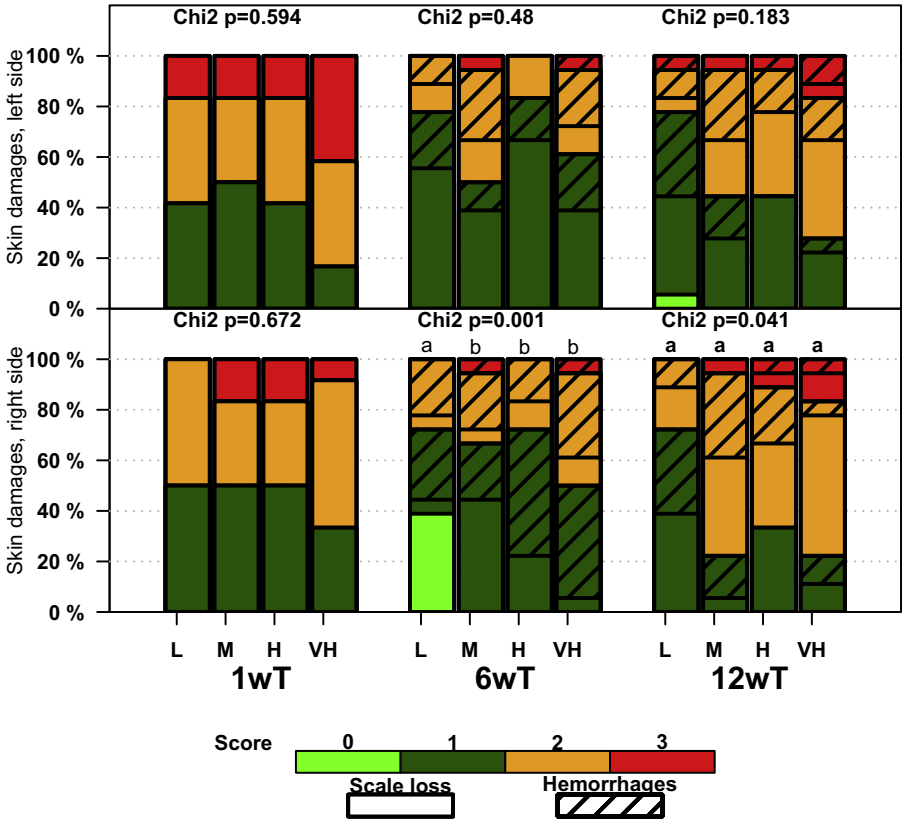

Fig. 9. Welfare scores for skin health. Results for the left side are shown in the top and for the right side in the bottom plot. Fish were swimming in a clockwise current; thus, the left side of the fish was pointing inwards of the tanks. The four score levels are indicated by colors green to red. The damages (score > 0) were distinguished between scale loss (no shading) and hemorrhages (shaded boxes). The assessment was conducted at each of the three sampling time points (after 1, 6, and 12 weeks of training (wT). Twelve fish per group (four per replicate tank) were examined at the first time point and eighteen (six per replicate tank) per group at the following time points. Chi-squared tests were conducted for each time point and in case of significant differences between groups, a chi-squared post hoc test was computed. Groups which do not share a lower-case letter were significantly different ($p < 0.05$). N per group and time point was 18. Scale loss score of 1 represents the loss of individual scales, score 2 a scale loss on less than 10% and score 3 a scale loss of more than 10% of the skin area. Hemorrhaging was assessed by area as the scale loss (from few local bleeding to large areas) and was always accompanied with scale loss. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

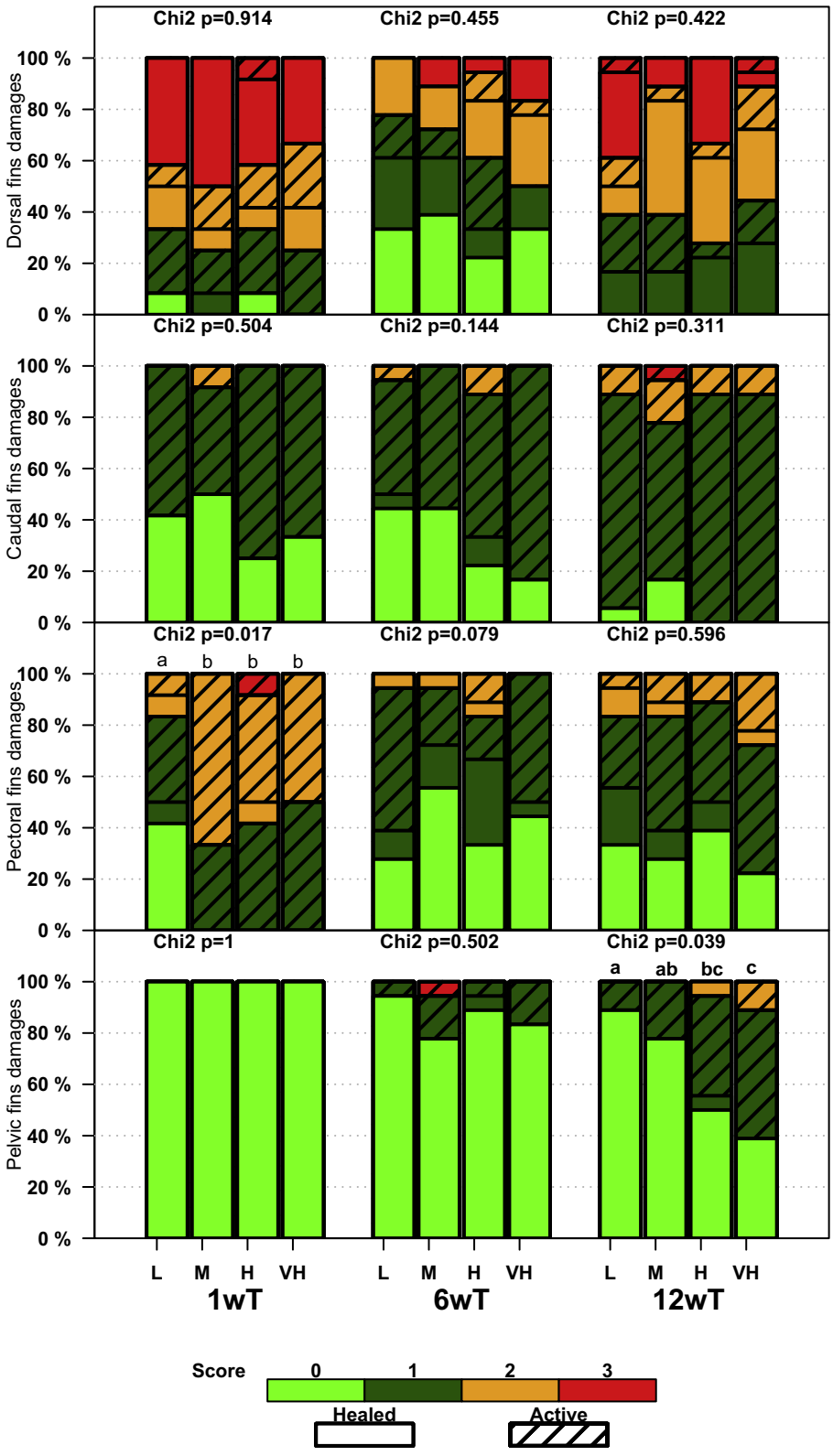

Fig. 10. Welfare scores for fin health. From top to bottom, results are shown for dorsal, caudal, pectoral and pelvic fins. The damages (score > 0) were distinguished between healed (no shading) and active damages (shaded boxes). For further explanations see caption of Fig. 9. Score 0 was given for healthy fins, score 1 for mostly intact fins, score 2 for ca. half of the fin remaining and score 3 in case very little of the fin was remaining.

the tank. A possible effector of the different growth development in the L group may be that the fish needed to invest more energy into swimming for ram-ventilation to sustain sufficient oxygen supplies and that the thinner body shape is an adaptation to this environment. Thus, velocities of 1 BL/s and higher may result in lower energy consumption for ventilation. However, these behaviors and effects were not sufficiently documented in this trial and further research is required to relate differences in body shape development to the swimming or ventilation

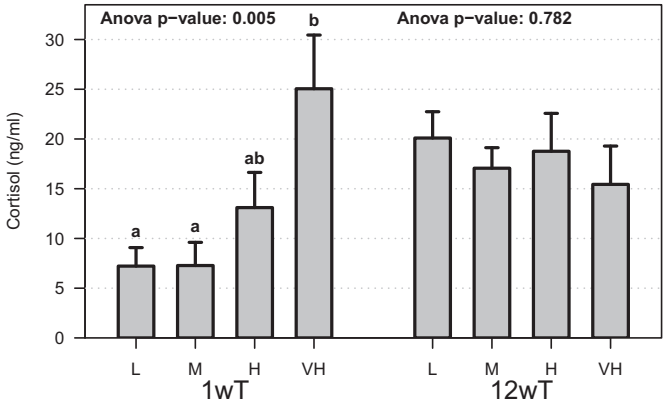

Fig. 11. Cortisol levels in blood, measured at 1wT and at 12wT. Bars show mean values and error bars indicate SEM. One-way ANOVA p-values are shown on top of each plot. Lower case letters indicate results of a Tukey *post-hoc* test. Groups which do not share a letter were significantly different to each other ($p < 0.05$). N = eight per group and time point.

Table 1
Glucose, lactate and hemoglobin levels in the blood after 12wT. Numbers are mean values for each training group with SEM (L: low, M: medium, H: high, VH: very high). No significant differences were found between groups (ANOVA). $N = 12$ fish per group for glucose and lactate and $N = 18$ for hemoglobin.

	L	M	H	VH
Glucose (ng/µl)	2.45 ± 0.58	2.4 ± 0.69	2.57 ± 0.67	2.51 ± 0.51
Lactate (ng/µl)	5.79 ± 2.16	6.55 ± 3.25	4.69 ± 1.94	6.37 ± 3.41
Hemoglobin (g/dl)	8.36 ± 0.14	8.44 ± 0.15	8.45 ± 0.15	8.62 ± 0.15

behavior.

The gene expression analyses revealed that three of the eight tested metabolic genes, involved in glycolysis and lipid metabolism, were upregulated in group VH. This may reflect the higher energy demand in this group which exceeded an unknown threshold not experienced in the lower velocity treatments. This is supported by the results of the proteomics analysis which provided a number of higher granularity molecular insights. Most prominently, there was a strong contrast between the L group and the higher water velocity groups in the abundance of proteins involved in translation, carbohydrate metabolism and ATP synthesis which demonstrate increased rates of translational activity and therefore protein synthesis. Conversely, the reduced translational activity and protein synthesis in the L group may further support our hypothesis of low water velocity impairing fish swimming behavior and resulting in an overall slimmer fish.

Muscle fiber analysis provided clear insight into the underlying phenotypic changes associated with the body weight increase in higher velocities. The number of muscle fibers per area decreased with increasing velocity and showed a strong positive association with condition factor. On the other hand, the mean area of individual muscle fibers increased with increasing water velocity and consequently increasing mean body weight across the groups. This was further complemented by contour analysis which revealed fish grew wider in the central and anterior regions as a result of increased training regimes. Hypertrophy in muscle fibers in fish especially following exercise regimes has been correlated with increased muscle mass and in turn, increased body, given that muscle occupies 50% of the fish body composition this will results in higher fillet yields (Palstra et al., 2014; Postlethwaite and McDonald, 1995). The optimal fiber size is not known for Atlantic salmon, however a study in arctic charr suggest a trade-off between diffusional constraints in the muscle and energy costs for maintaining ionic gradients. Thus, improved aerobic capacity due to continuous exercise may lead to improved gas exchange and metabolite provision to the fibers, which in turn allows for larger muscle fibers. The proteomics analysis showed a stark contrast between the L group and the training regime groups with the later showing increased protein translation and protein folding pathways. These phenotypic changes were also reflected in the gene expression analysis. Where the upregulation of the muscle differentiation marker *myog* and the anabolic marker, growth factor *igf1* followed an increasing trend with training regime, albeit with relatively weak differences. In addition, the inflammation marker *il1b* followed an increasing expression patter with training regime and complimented the histological observation of significantly increase in inflamed muscle fibers in the H and VH groups. The sharp increase of inflamed fibers between M and H groups could indicate that a threshold was passed at this point and may suggest potential longer-term negative effects of these training regimes. However, further research about long term effects of muscle fiber inflammations in post-smolts are needed to assess the severity of this finding.

4.3. Higher water velocities pose risks on mucosal health

Cortisol levels at the beginning of the trial indicated that the higher velocities induced an initial mild stress response. However, cortisol levels were similar in all four groups at the end of the trial, which indicated that the fish were coping successfully with the velocities over the training period. In addition, the overall mortality during the trial was low. On the other hand, histological analyses revealed possible negative effects of high water velocities. Skin morphometry revealed that increasing water velocities resulted in thinner epidermis and thickening of the dermal layer. Especially the epidermis, as the outermost layer of the fish skin is an important barrier against pathogens (reviewed by (Esteban, 2012)). Thus, thinning of this layer may for instance result in higher susceptibility against bacterial infections and in case of RAS-reared fish, this may result in more sensitive fish in regard to non-optimal water conditions.

The external welfare status of the experimental fish remained in an overall good state, though changes in the skin and fins provide some implications of the associated negative consequences of the training regimes. Scale loss was a common finding and was caused partly by netting out fish for sampling; however, higher occurrences of mild cases in all groups except L after twelve weeks of training indicated that moderate to high water velocities may promote scale loss. We documented skin hemorrhaging in all four groups after six and twelve weeks of training in ca. 30% to 50% of the fish. These occurrences were similar among the groups and the causative effects remain unknown. Another observation was that the tank wall facing sides of the fish exhibited increased cases of skin damages. The present study cannot provide further explanations for the cause of the observed skin lesions; however, it should be noted that the tested water velocities higher than L, all had similar effects on skin health and did not increase with higher velocities.

Fins with highest degrees of damages were the dorsal fins but similar to the hemorrhages, no differences among the groups were found and causal effects are unknown. Overall increasing tendencies of fin damages during the rearing of smolts have been reported in another study (Kolarevic et al., 2014) and are a frequent finding for smolts in aquaculture. An interesting pattern was found for pectoral fins after the first week of training, where the prevalence of damages was lower in the L group. The gradual increase of water velocities in the other groups possibly increased social interaction, thus resulted in increased damages. Unlike in the other groups, fish in the L group had relatively more available room in the tanks (due to calmer tank centers) and were swimming more freely. A similar profile was observed with pelvic fins, where increased lesions, mostly active, may be attributed to stress from the velocity and/or from social interaction. The schooling behavior resulted in denser fish groups at the corners of the tanks and thus the fish were experiencing higher densities with higher velocities. Fish may compete for the best spots within the schools and fin damages are perhaps a result of increased aggression. It has already been reported

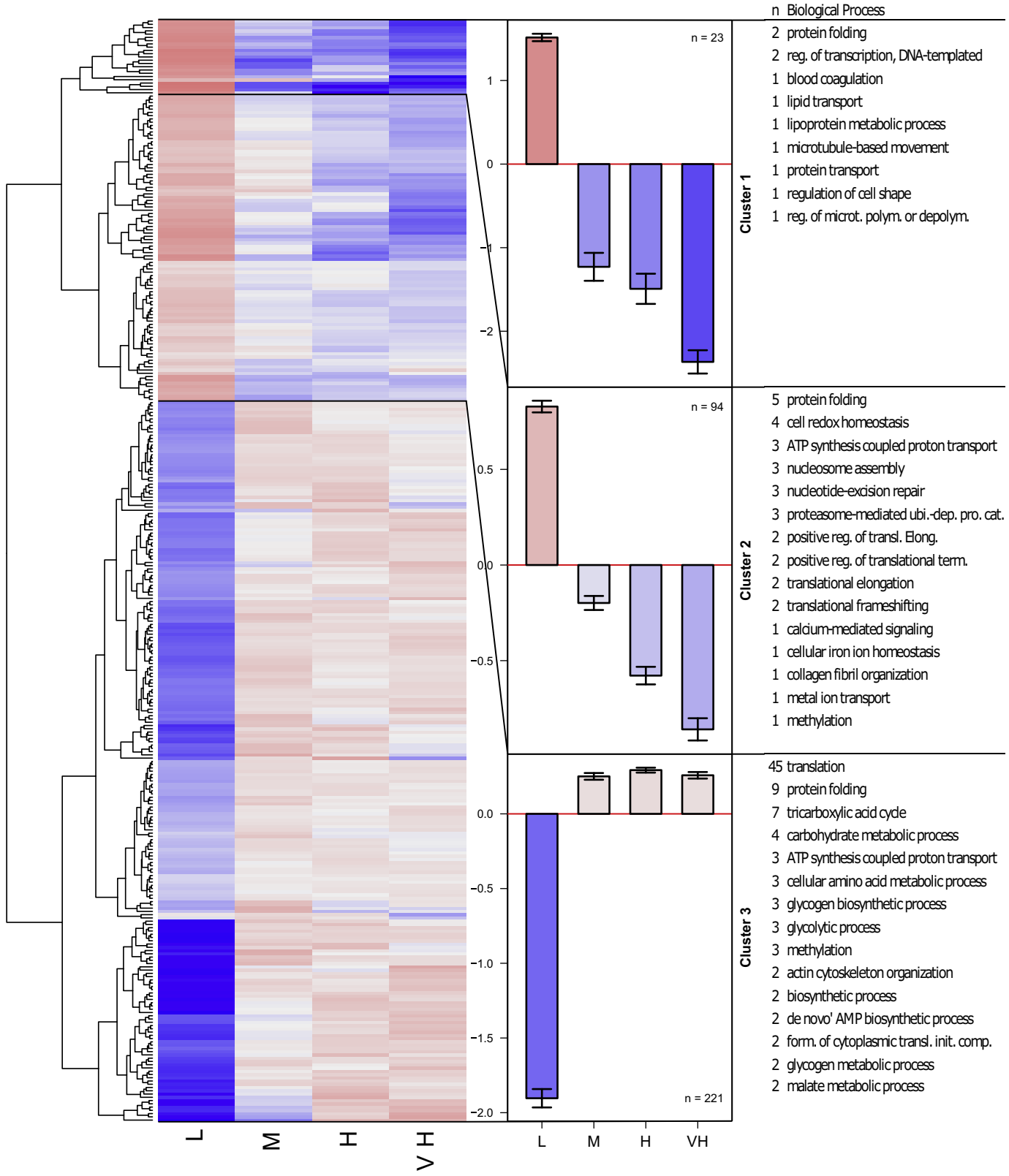

Fig. 12. Proteomics analysis of white muscle tissue after twelve weeks of continuous training. The heatmap on the left shows relative down- and upregulation of 338 proteins with colors from blue to red, which were differentially regulated in the four groups (ANOVA $p < 0.05$, $n = 6$ per group). The heatmap was divided into three sub-clusters and the means of the respective expression values with SEM-error bars are shown as bar plots in the middle (n: number of proteins in cluster). The table on the right shows the numbers of gene ontology (GO) terms (top 15), which were found for the proteins in the three clusters (biological process only). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that higher density may instigate aggression in salmon (Adams et al., 2007). However, further research is needed to shed light into the behavior of post-smolt schools within this context.

It is apparent that gill health was significantly affected by increasing water velocities, as shown by higher occurrences of branchial pathologies. Exercise carries a remarkable respiratory demand that is expected to influence ventilation volume, an increase in functional surface area, a reduction in diffusion distance and increased perfusion of blood (Fernandes et al., 2007). Cases of lifting, hypertrophy and hyperplasia were higher in M, H and VH groups compared with the L group, however, the tendencies did not reveal a compelling velocity-dependent relationship. Occurrences of such branchial pathologies are often related to the protective mechanisms in the gills - increase of the distance between the water and the blood, to act as a barrier against the external environment (Poleksic & Mitrovic-Tutundzic, 1994). One may assume that the earlier described rapid increase in turbidity and consequently, an increase of particles in the RAS water may be contributory to gill damages; however, if this would be a significant factor, gill damages would occur more evenly within the different velocity groups. Furthermore, it was shown that higher densities of particles did not have a significant effect on the gill health of rainbow trout (Becke et al., 2018). Aneurism and distal lamellar clubbing were the two pathologies that were observed to increase significantly at higher water velocities. Lamellar aneurism is associated with the rupture of pillar cells, that leads to increased blood flow and eventually the accumulation of blood in the secondary lamellae. This may be partly related to the respiratory pressure the fish is adapting to at higher velocities. It has been implicated in trout that susceptibility to aneurism is higher in RAS than in flow-through systems (Strzyzewska-Worotynska et al., 2017). It would be interesting to explore in the future whether the occurrence of such pathology is aggravated by combining RAS production and increased water velocity.

4.4. The optimum water velocity

This study has demonstrated that the effects of increased water velocities can be both beneficial and disadvantageous, hence, a production strategy that likely carries a compromise. The growth rate increased with the velocity, due to muscle growth. On the other hand, the number of inflamed muscle fibers, gill lesions and fin damages increased in velocities of 1.8 BL/s and higher. Fish in low velocities (0.5 BL/s) had the overall best gill, skin and fin condition but developed significantly lower condition factors. These findings illustrated that the growth rate of fish in aquaculture is closely linked with other parameters, e.g. a welfare assessments or other health parameters. Hence, such interaction provides a strong argument as to why optimization of rearing conditions must not only be defined by a single factor response, but most importantly, by the conjunction of multiple factors and parameters. Summarizing these results, a moderate velocity of 1 BL/s could be seen as the overall best performing training regime. However, the gap in training intensity between groups M and L was rather large and more research particularly on technical aspects of such a practice in RAS, is needed to narrow down the optimum water velocity for post-smolts; nevertheless, a healthy growth and minimal welfare implications can most likely be achieved with a velocity slightly above 1 BL/s.

4.5. Conclusion

Atlantic salmon post-smolts grow faster in higher water velocities and even in very high velocities up to 2.5 BL/s results in increased growth rate and muscle mass. However, multiple negative effects of velocities in the range from 1.8 BL/s and up have been identified, including higher numbers of inflamed muscle fibers, gill damages, thinner epidermis and fin damages. The protein synthesis and energy metabolism were decreased at a velocity of 0.5 BL/s and fish had lower condition factors. Thus, the optimum water velocity for post-smolts in RAS for a good welfare and a healthy growth is most likely slightly above 1 BL/s.

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Author contributions

G.T., C.C.T. and L.H.J. planned and coordinated the experiment. G. T., C.C.L. and N.A.C. collected the samples, conducted analyses and data visualizations. B.K.M.R. had the supervision over the training experiment at the research station. All authors contributed to the writing of the draft and review of the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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