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ORIGINAL ARTICLE

Leucine did not stimulate growth and accretion in either stressed or unstressed Atlantic salmon

Marit Espe[1] | Floriana Lai[2] | Ana S. Gomes[2] | Christian De Santis[3] | Mali B. Hartviksen[3] | Elisabeth Holen[1] | Ivar Rønnestad[2]

[1]Institute of Marine Research, Bergen, Norway

[2]Department of Biological Sciences, University of Bergen, Bergen, Norway

[3]Cargill Innovation Center, Dirdal, Norway

Correspondence
Marit Espe, Institute of Marine Research, Bergen, Norway.
Email: Marit.Espe@hi.no

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Abstract

The aim of the current trial was to test whether leucine affected growth and accretion including test any effects on leucine upon stress. Quadruplicate tanks each containing 50 Atlantic salmon (*Salmo salar*) (mean start body weight of 524 ± 28 g) were fed diets containing 27.3, 30.0, 35.0 and 41.0 g leucine/kg diet for 74 days. Two tanks per dietary group were exposed to a stressor (5 min chasing) three days a week to test whether enriched leucine diet aid coping with chronic stress, while two tanks per dietary groups were left unstressed. The stressed fish consumed less feed and grew less than the unstressed fish, irrespective of diet. Leucine inclusion did not affect protein accretion, but leucine retention declined with increasing dietary leucine in both stressed and unstressed fish. No difference between the stressed or unstressed fish was present. Leucine did not affect relative liver size, but unstressed fish had slightly higher relative liver size compared with stressed fish ($p = 0.05$). Free leucine in the muscle and liver was not affected by dietary leucine, but unstressed fish had higher concentration of valine and isoleucine in the muscle compared with the stressed fish. Muscle of fish fed elevated leucine had lower mRNA expression of *murf1* ($p = 0.037$) and higher expression of *ppara* ($p = 0.012$). Muscle of stressed fish had higher expression of the oxydative genes *mnsod* ($p = 0.049$) and *catalase* ($p = 0.037$) compared with the fish left unstressed, while in liver, there were no differences of expression of any of the genes tested. In conclusion, diets enriched in leucine had minor effects and neither protein accretion nor growth was affected in either stressed or unstressed fish.

KEYWORDS
Atlantic salmon, leucine, stress, growth, protein retention

1 | Introduction

The branched-chain amino acids (BCAA) comprise leucine, valine and isoleucine, which all are indispensable for Atlantic salmon (*Salmo salar*) (NRC, 2011). All BCAA share some common properties including increased insulin secretion (Nair & Short, 2005). In addition, leucine plays a critical role in promoting growth by increasing protein synthesis and inhibiting protein degradation depending on mTOR being phosphorylated or not. When phosphorylated, the mTOR stimulates amino acid-sensing p70 S6 kinase (S6K1), eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and eukaryotic translation initiation factor 4E (eIF4E) and thus promotes protein synthesis (Duan et al., 2015; Nie, He, Zhang, Zhang & Ma, 2018; Zou et al. 2018; Zhou et al., 2019). Increased leucine intake reduces diet-induced

obesity, hyperglycaemia and hypercholesterolemia in mice partly by increasing uncoupling protein 3 (UCP3) expression (Zhang et al., 2007). Leucine regulates mitochondrial functions and quality as it increases sirtuin and peroxisome proliferator-activated receptor-gamma coactivator1a (PGC1a) expression and, as such, reduces adiposity by increasing energy production (Kumai, Hatazawa, Uchitomi, Yoshimura & Miura, 2020; Brunetta et al., 2019; Yao et al., 2016). Long-term leucine supplementation reduces adiposity in ageing rats (Vianna et al., 2012) and in mice fed high-fat diets (Arakawa, Masaki, Nishimura, Seike & Yoshimatsu, 2011). Leucine regulates appetite, energy expenditure, lipid and glucose metabolism; thus, leucine management in the diet/feed has been used to attenuate obesity and metabolic disorders (Zhang et al., 2007). Furthermore, leucine increases intestinal oxidation status in grass carp (*Ctenopharyngodon idella*) (Deng et al., 2014) and reduces expression of pro-inflammatory cytokines in head kidneys (Giri, Sen, Jun, Sukumaran & Park, 2015). Muscle growth is highly correlated to the expression of insulin-like growth factor 1 (IGF-1), and any stress reduces IGF-1 expression (Hevrøy et al., 2013). Muscle degradation occurs by autophagy and leucine reduced protein degradation (Cleveland & Weber, 2010). The ubiquitin-proteasome and autophagy-lysosome systems are important pathways resulting in muscle protein degradation. The major markers for muscle catabolism are F-box only protein 32, also known as Atrogin or Mafbx, and members of the muscle RING-finger family (Murf). During muscle degradation, the ring finger protein 1 (Murf1) and atrogin increase (Bower, Neil, Taylor, & Johnston, 2009; Valente, Bower & Johnston, 2012). Thus, leucine may have several possible functions beyond protein deposition, but these effects are little studied in Atlantic salmon. This study aimed to test whether dietary leucine affects growth and adiposity in Atlantic salmon growing from about 500 g to 1.5 kg.

In our previous studies with Atlantic salmon, the diets used contained about 31–38 g leucine per kg feed (Espe et al., 2014, Espe et al., 2016a, 2016b; Espe et al., 2020). Therefore, in the current trial, we used diets ranging from 27 to 41 g leucine per kg diet, to address any functional properties of leucine management. In addition, unstressed and chronically stressed fish were fed the same diets to address the possible effect of leucine to attenuate the effect of chronic stress.

2 | MATERIAL AND METHODS

2.1 | Ethics statement

The experiments complied with the guidelines of the Norwegian Regulation on Animal Experimentation and European Community Directive 86/609/EEC. The trial was approved by The Norwegian Food Authority (FOTS no 20799).

2.2 | Feeding experiment

Four experimental diets of 7 mm pellet size were formulated and produced by Cargill Innovation Center (Norway), with 27.3 g and 35 g Leu/kg, and boosted with crystalline L-Leu to contain 30 g and 41 g Leu/kg. The composition of the diets and the dietary amino acid composition are given in Tables 1 and 2, respectively.

TABLE 1 Composition and analysed values (g/kg) of the four experimental diets used

Ingredients	Diet 1	Diet 2	Diet 3	Diet 4
Rapeseed oil	109	109	109	109
Fish oil	218.4	222.4	206.1	209.5
Fish meal	198	198	198	198
Corn gluten	-	-	150	150
Soy protein concentrate	53	59	14	18
Wheat gluten meal	85	78	20	20
Guar meal-Mahesh	110	100	50	40
Pea protein concentrate	101	102	127	123
Binders	102	102	103	103
Minerals and vitamins	18.5	18.5	18.4	18.6
L-Threonine	0.4	0.5	-	-
DL-Methionine	3.6	3.6	2.5	2.6
L-Histidine	0.9	1.0	1.0	1.2
L-Lysine (78%)	-	-	0.8	1.1
L-Leucine	-	5.8	-	5.8
Yttrium oxide	0.2	0.2	0.2	0.2
Analysed values:				
Dry matter	937	925	921	914
Protein	394	387	400	394
Lipid	342	353	332	337
Ash	52	49	50	46
Energy (MJ/kg)	25.4	25.5	24.9	25.4
Digestive Energy (MJ/kg)	22.5	22.5	22.5	22.5

Note: Diets were formulated with 27.3 g (diet 1) and 35 g (diet 3) Leu/kg, and boosted with equal amount of crystalline L-Leu (5.8 g/kg) to contain 30 g (diet 2) or 41 g (diet 4) Leu/kg, respectively.

2.3 | Fish and rearing facility

Atlantic salmon postsmolts with an initial weight of 524 ± 28 g were randomly distributed into 16 tanks (volume ca. 470 L; diameter 1.5 m (0.936 m^3); water flow 12–16 L/min), 50 fish per tank, at Cargill Innovation Center (Dirdal, Norway). Fish were maintained under controlled conditions with constant temperature (9 ± 0.01°C), salinity (28.39 ± 0.03‰), photoperiod (24:0, L:D) and oxygen levels (88 ± 0.42 %). Fish were acclimatized in the experimental tanks for four weeks and fed with a commercial pellet (Adapt Marine 80, Cargill) 4 times a day in periods of 70 min (19:00, 22:00, 01:00 and 06:00) by using automatic feeders (Hølland Technology, Sandnes, Norway). Following the acclimation period, fish were fed each of the four experimental diets, in quadruplicates, for 74 days. During the feeding period, feed was offered in slight excess (10%) to assure all fish in the tank had access to feed to satiation, and about 0.78–1.48% of the biomass was consumed daily. Uneaten feed was collected daily (Excess fish feed collector; Hølland Industries AS,

TABLE 2 Amino acid composition plus taurine (g/kg) of the experimental diets used

Amino acids	Diet 1	Diet 2	Diet 3	Diet 4
OH-pro	1.5	1.3	1.1	1.1
His	8.8	7.6	7.6	7.8
Ser	17.7	16.1	17.0	17.3
Arg	26.9	23.6	21.2	21.1
Gly	19.0	16.6	15.9	15.7
Asp	34.0	32.0	33.0	33.0
Glu	76.0	69.0	68.0	66.0
Thr	13.7	12.5	12.8	13.0
Ala	16.9	15.5	21.1	21.4
Pro	22.0	19.7	21.3	21.7
Lys	23.1	21.5	22.3	22.5
Tyr	12.7	10.8	12.6	12.9
Met	10.6	9.2	9.1	9.5
Val*	16.5	14.9	16.2	16.4
Ile*	15.1	13.60	14.6	14.8
Leu*	27.3	30.0	35.0	41.0
Phe	18.0	15.6	17.1	17.7
Taurine	1.1	0.9	0.9	0.9
BCAA*	58.9	58.5	65.8	72.2

*BCAA is sum of branched-chain amino acids.

Norway) to enable feed intake calculation. Four weeks after being fed with the experimental diets, half of the tanks (two replicates tanks per diet) were exposed to a stressor (chasing), while the rest of the tanks were left undisturbed, only subjected to routine practice of tank maintenance. The stressor consisted of manually stirring a hand net (spun clockwise and counter-clockwise) inside the tank for 5 min, which was performed for 3 days every week (Monday, Wednesday and Friday). Each day the chasing was performed between 10 and 11 am. A pilot study showed that this stress procedure increased plasma cortisol in postsmolt Atlantic salmon (Lai et al., 2021). No mortality occurred during the feeding trial.

2.4 | Sampling

Before sampling, fish were starved for 24 h. Fish were anaesthetized with a lethal dose of Tricaine (PHARMAQ Ltd., Hampshire, UK), and weight and length of the fish were recorded. The liver weight and viscera weight were recorded from five fish in each tank and used to calculate somatic indexes.

2.5 | Sampling for chemical analysis

At the beginning of the experiment, one pool of 10 whole fish was collected, while at the end of the experiment, a pool of 5 whole fish was sampled from each tank and used for chemical composition analyses. Liver and muscle tissues from 3 fish per tank were collected and transferred into RNAlater (Invitrogen, Carlsbad, CA, USA) and stored at −80°C until gene expression analyses. In addition, liver and muscle samples of the same 3 fish were flash-frozen in liquid nitrogen and used for chemical and biochemical analysis. Samples were pooled tank-wise before analysis.

2.6 | Chemical analysis

Energy, fat and protein content of the feeds and whole fish were determined as described by Espe, Lemme, Petri & El-Mowaffi (2006). Amino acid composition in the diets and whole fish was determined after hydrolysis for 22 h at 110°C in 6N HCl (chemically equivalent to 6 M HCl) containing 3.125 mM Norvalin (internal standard) and 3 mM DTT (to protect the sulphur amino acids against oxidation), and precolumn derivatization with AccQTag™ at 55°C as described by the producer Waters. Amino acids were separated on a UPLC-system (Waters Aquity UPLC BEH C18 column internal diameter of 1.7 μM at a flow rate of 0.7 ml/min using the gradient offered by the supplier). The concentration of each amino acid was calculated using external standards supplied by Sigma. Free amino acids and N-metabolites were determined in liver and muscle of pooled samples on Biochrom 20 plus amino acid bioanalyzer (Amersham Pharmacia Biotech, Sweden) using postcolumn derivatization with ninhydrin as described by (Espe et al., 2006).

2.7 | RNA extraction and gene expression analyses in muscle and liver

Total RNA was extracted from the liver and muscle ($n = 3$ individual fish per tank) using the BioRobot EZ1 and EZ1 RNA Universal Tissue Kit (Qiagen) according to the manufacture's description. The quality and quantity of RNA were analysed as described by (Espe et al., 2014). Reverse transcription followed by quantitative real-time PCR (RT-qPCR) was done as described by (Skjaerven, Olsvik, Finn, Holen & Hamre, 2011). The obtained cycle threshold (ct) values were normalized using two reference genes, *b-actin* and *ef1a*. The primer sequences used are described in Table 3. Normalization and calculation of relative gene expression were done using BioRad CFX Maestro version 1.1, Gene study tool. All the genes had an amplification efficiency between 90% and 110%.

2.8 | Calculations

Growth was calculated as specific growth rate (SGR):

$$\mathrm{SGR} = 100 * \left(\frac{\ln(\text{final mean body weight}) - \ln(\text{initial mean body weight})}{\text{days of feeding}} \right)$$

Feed utilization was calculated as feed conversion ratio (FCR):

TABLE 3 Primer pair sequences and GenBank accession numbers for 13 target genes and 2 reference genes used for RT-qPCR analyses

Genes	Primer pairs	Accession no	References
Target genes:			
IL-1β			
F	GTA TCC CAT CAC CCC ATC AC	NM_001123582	Holen et al., (2014)
R	GCA AGA AGT TGA GCA GGT CC		
IL-8			
F	GAG CGG TCA GGA GAT TTG TC	NM_001140710	Holen et al., (2014)
R	TTG GCC AGC ATC TTC TCA AT		
MnSOD			
F	GTT TCT CTC CAG CCT GCT CTA AG	XM_014145196.1	Remø et al., (2014)
R	CCG CTC TCC TTG TCG AAG C		
GPX-3			
F	CCT TCC AGT ACC TGG AGT TGA ATG C	XM_014213339.1	Remø et al., (2014)
R	CTC ATG ATT GTC TCC TGG CTC CTG T		
Catalase			
F	*GGG CAA CTG GGA CCT TAC TG*	*Ba935638*	Remø et al., (2014)
R	*GCA TGG CGT CCC TGA TAA A*		
Atrogin-1 (Mafbx)			
F	CGA GTG CTT CCA GGA GAA TCT G	NM_001185027.1	Valente et al., (2012)
R	GTC TGA AGG AGC TCC TTGA TGG		
Murf-1			
F	AGG CGG GAT CAG AGC TAA C	DN165465	Valente et al., (2012)
R	CGA CCA TTC CAA AGT CCA TC		
IGF-1			
F	*TGA CTT CGG CGG CAA CA*	M81904	Hevrøy et al., (2013)
R	GCC ATA GCC CGT TGG TTT ACT		
IGF-1r			
F	TGC ACA ACT CCA TCT TCA CC	EU861008	Hevrøy et al., (2013)
R	GGG GCT CTC CTT CTG TCC TA		
GHr			
F	TGG ACA CCC AGT GCT TGA TG	AF403539	Hevrøy et al., (2013)
R	TCC CTGAAGCCAATGGTGAT		
PPARa			
F	TCC TGG TGG CCT ACG GAT C	DQ294237	Holen et al., (2014)
R	CGT TGA ATT TCA TGG CGA ACT		
PGC1a			
F	GTC AAT ATG GCA ACG AGG CTT C	FJ710605	Castro et al., (2013)
R	TCG AAT GAA GGC AAT CCG TC		
Reference genes:			
Beta-actin			
F	CCA AAG CCA ACA GGG AGA A	BG933897	Stenberg et al., (2019)
R	AGG GAC AAC ACT GCC TGG AT		
ELF1a			
F	TGC CCC TCC AGG ATG TCT AC	AF321836	Holen et al., (2014)
R	CAC GGC CCA CAG GTA CTG		

TABLE 4 Growth performance, retention and total lipid in whole fish regression lines for the stressed and unstressed fish and comparison of differences in slopes or elevations fed the four diets having Leucine from 27.3 to 41 g/kg and the retention of Leucine

	Unstressed	R^2	*p*-Value unstressed	Stressed	R^2	*p*-Value stressed	*p*-Value elevation	*p*-Value slope
SGR	Y = 1.72 − 0.008×	0.29	0.13	Y = 1.42 − 0.003	0.16	0.17	**0.0004**	0.47
FCR	Y = 0.72 + 0.001×	0.07	0.51	Y = 0.73 + 0.002×	0.07	0.51	0.09	0.9
VFI	Y = 32009 − 173×	0.35	0.12	Y = 27494 − 99×	0.18	0.29	**0.008**	0.57
CF	Y = 1.34 + 0.001×	0.10	0.52	Y = 1.42 − 0.002×	0.07	0.45	0.12	0.33
HSI	Y = 1.76 − 0.004×	0.13	0.29	Y = 1.17 + 0.01×	0.18	0.38	0.05	0.17
VSI	Y = 10.71 − 0.003×	0.001	0.21	Y = 8.88 + 0.04×	0.25	0.93	0.33	0.38
PPV	Y = 60.01 − 0.19×	0.15	0.34	Y = 54.37 − 0.09×	0.02	0.74	0.16	0.76
PER	Y = 3.58 − 0.01×	0.08	0.49	Y = 3.48 − 0.01×	0.11	0.43	0.13	0.97
Leu retention	Y = 95.4 − 1.5×	0.92	**0.0001**	Y = 82.3 − 1.1×	0.77	**0.004**	0.59	0.27
Total lipid g/kg	Y = 212.7 − 0.39×	0.06	0.54	Y = 175.1 + 0.61×	0.23	0.23	0.32	0.21

Note: VFI is gram mean feed intake during the 74 days trial/tank.
Bold numbers are the statistical different.

$$\text{FCR} = \text{Consumed feed} * \left(\frac{\text{final biomass live fish} + \text{biomass dead fish}}{\text{body mass increase}}\right)$$

Calculation of nutrient deposition of protein productive value (PPV):

$$\text{PPV} = 100 * \left(\frac{\text{final protein content} - \text{initial protein content}}{\text{consumed protein}}\right)$$

Calculation of nutrient deposition of protein efficiency ratio (PER):

$$\text{PER} = \left(\frac{\text{final body weight} - \text{initial body weight}}{\text{protein consumed}}\right)$$

Calculation of nutrient deposition of retention of indispensable amino acids (AA):

$$\text{AA retention} = 100 * \left(\frac{\text{AA deposition}}{\text{AA consumed}}\right)$$

To address any changes in lipid deposition pattern, relative liver size as hepatosomatic index (HSI), condition factor (CF) and visceral index (VSI) were calculated

$$\text{HSI} = 100 * \left(\frac{\text{liver weight}}{\text{body weight}}\right)$$

$$\text{CF} = 100 * \left(\frac{\text{body weight}}{\text{fork length}^3}\right)$$

$$\text{VSI} = 100 * \left(\frac{\text{visceral weight}}{\text{body weight}}\right)$$

2.9 | Statistical methods

To address leucine effects, differences between tank mean values were calculated as regression lines for the unstressed and stressed diet groups, respectively. The stressed and unstressed regression lines were compared for differences in elevation and slope using GraphPad Prism (version 8.0) with the null hypothesis that the regression lines were similar. *p* values less than 0.05 were accepted as statistically different. Gene expression data were analysed by one-way ANOVA and Tukey's post hoc test on tank mean values using Statistica version 14.

3 | RESULTS

Feed intake was lower in stressed fish compared with unstressed fish, resulting in a better growth performance in the latter (Table 4). Leucine inclusion did not affect either feed intake or growth performance, irrespective of the application of stress. Furthermore, leucine did not have any effect on VSI or HSI, nor was protein accretion affected by either dietary leucine or stress. Generally, the retention of the indispensable amino acids was numerically higher in the unstressed fish as compared to the stressed fish, but only leucine retention was significantly affected by dietary treatments. When dietary leucine increased, both the unstressed and the stressed fish reduced the retention of leucine (Table 4, $p = 0.004$ and $p = 0.0001$, respectively), though there were no differences between the regression lines (i.e. slope or elevation) for the stressed or unstressed fish.

Table 5 lists free BCAA in muscle and liver. Leucine level in the liver was unaffected by treatment, so was muscle-free leucine. However, in the muscle, valine and isoleucine decreased as leucine in the diet increased. Also, the unstressed fish had a higher concentration of these two amino acids in the muscle compared with the stressed fish.

TABLE 5 Free isoleucine, leucine and valine in liver and muscle (μmol/100 g tissues) by the end of the feeding trial

	Unstressed	R^2	*p*-Value unstressed	Stressed	R^2	*p*-Value stressed	*p*-Value elevation	*p*-Value slope
Liver								
Ile	Y = 26.34 + 0.23×	0.09	0.45	Y = 44.6 − 0.32×	0.12	0.39	0.56	0.25
Leu	Y = 24.2 + 2.0×	0.43	0.68	Y = 100.4 − 0.4×	0.02	0.74	0.72	0.13
Val	Y = 45.5 + 0.8×	0.16	0.33	Y = 87.7 − 0.6×	0.06	0.55	0.44	0.26
Muscle								
Ile	Y = 29.6 − 0.4×	0.50	**0.05**	Y = 26.8 − 0.4×	0.70	**0.009**	**0.044**	0.92
Leu	Y = 32.6 + 0.02×	0.01	0.94	Y = 33.0 − 0.08×	0.03	0.69	0.08	0.75
Val	Y = 54.1 − 0.8×	0.51	**0.045**	Y = 43.7 − 0.6×	0.57	**0.031**	**0.033**	0.67

Bold numbers are the statistical different.

From the genes analysed in the muscle, only *murf1* and *ppara* expressions were affected by dietary leucine. Expression of *murf1* was higher in the muscle of fish fed with the lowest dietary leucine compared with fish fed higher leucine inclusion (Figure 1a, $p = 0.037$). *ppara* mRNA expression was lower in muscle of fish fed 30 g compared with fish fed the 41 g Leu/kg diet, while the other dietary groups were in between (Figure 1b, $p = 0.012$). Also, very few differences were observed in the muscle gene expression analyses between chronically stressed and unstressed fish, and only *mnsod* (Figure 1c, $p = 0.049$) and *catalase* expression (Figure 1d, $p = 0.037$) were affected, showing higher expression levels in the stressed fish compared with the unstressed fish. None of the other target genes (see Table 3) were significantly affected by treatments (data not shown). In the liver, there were no differences in cytokine or antioxidative gene expression between any of the treatments (data not shown). Thus, there was no effect of stress or dietary leucine treatment in the liver gene expression. The mean values of all treatments are given in supplementary Table 1.

4 | DISCUSSION

Leucine inclusion in the range of 27.3–41 g/kg did not affect feed intake, growth and protein accretion. However, retention of leucine reduced as dietary leucine increased in both stressed and unstressed fish. The decline in retention of individual amino acids upon increased dietary levels is in line with previous results testing diets with increased amino acid inclusions (Espe et al., 2020). The data showed that the fish that were chronical stressed by chasing consumed less feed and thus grew less when compared to the unstressed fish fed the same diets. The reduction in voluntary feed intake and lower growth following chronical stress is in line with our previous study on Atlantic salmon postsmolts exposed to a repeated stressor that resulted in a significantly lower growth (Lai et al., 2021). Additionally, Atlantic salmon stressed by elevated temperature also reduced their voluntary feed intake and consequently reduced their growth rates (Hevrøy et al., 2013). A reduced voluntary feed intake and growth was also observed in chronically stressed rainbow trout (*Oncorhynchus mykiss*) (Fontagne-Dicharry et al., 2020). Leucine reduced adiposity, but did not affect feed intake in mice (Zhang et al., 2007). In the current trial, leucine ranging from 27 to 41 g/kg did not affect voluntary feed intake, neither was the final total fat content affected in the whole fish (Table 4). Leucine also did not affect the crude protein growth in either stressed or unstressed fish. This is in contrast to results observed in hybrid grouper (*Epinephelus* sp.) where leucine improved growth and retention of protein (Zhou et al., 2019), but similar to results reported for chicken (*gallus gallus*) fed increasing levels of leucine (Zeitz et al., 2019). This supports the hypothesis that the diets used seem to contain sufficient leucine to support growth and protein retention in Atlantic salmon.

Leucine is reported to downregulate fatty acid synthesis leading to reduced lipid accumulation in the liver (Arakawa et al., 2011; Freudenberg, Petzke & Klaus, 2012; Freudenberg, Petzke & Klaus, 2013; Zhang et al., 2007). Mice fed a high-fat diet added surplus leucine got reduced amounts of white adipose tissue and triacylglycerol by increasing *ppara* expression (Chen et al., 2019). We hypothesize that the reason why we were unable to detect a clear effect of leucine on lipid metabolism was that the positive effects reported on leucine administration require high-fat levels, that is an animal with elevated lipid content. The diets used in the current study did not result in such a diet-induced adiposity in salmon as neither HSI nor lipid content was affected by leucine. Thus, any effects of leucine on lipid metabolism needs to be studied using a high-fat diet or fatty liver models. Deng et al. (2014) found that leucine had a positive effect on oxidation status, though we could not observe any effects of leucine on oxidation in the current trial. The reduced *murf1* expression indicates a reduced ubiquitination in fish fed the higher leucine diets. Muscle degradation occurs by autophagy and leucine is reported to attenuate this (Cleveland & Weber, 2010). The ubiquitin-proteasome and autophagy-lysosome systems are important pathways resulting in muscle protein degradation. The major markers for muscle catabolism are F-box only protein 32, also known as Atrogin or Mafbx, and members of Murf. During muscle degradation, *murf1* and *atrogin* expression increases (Bower et al., 2009; Valente et al., 2012). Here, we found no effect on *atrogin* mRNA levels, but *murf1* mRNA expression was reduced in the muscle of fish fed the higher leucine

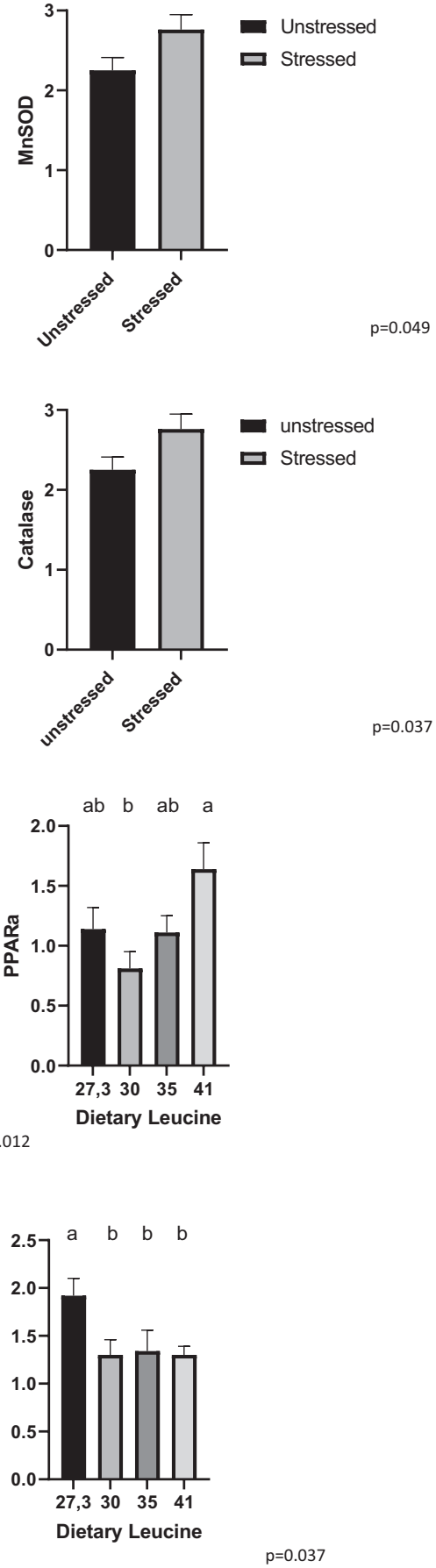

FIGURE 1 Normalized gene expression in muscle as affected by continuous stress and leucine inclusion in the diets. Values are tank means ± SE

inclusion indicating a lower ubiquitination in the muscle when dietary leucine is above 30 g/kg diet. However, these changes were not enough to affect the crude protein accretion or growth in the current trial. This suggests that leucine might have affected the degradation of globular proteins and not the structural proteins in the muscle, as also suggested by Vera et al. (2020) feeding salmon diets with increased nutrient package of minerals and vitamins reporting changes in *murf*, but not in crude protein accretion.

The higher gene expression of *catalase* and *mnsod* in stressed fish compared with unstressed fish suggests a higher oxidation in the stressed fish and thus a higher need for the antioxidative enzymes scavenging the H_2O_2 produced during oxidation. Leucine, however, had no impact on antioxidative-related gene mRNA expression. The increased *catalase* expression following chronic stress is in accordance with Fontagne-Dicharry et al. (2020) in rainbow trout juveniles during chronic stress. This is opposite to the effect observed after leucine supplementation of piglets where an increased expression of antioxidants genes in muscle and liver is observed (Chen et al., 2019), or as also reported in grass carp where leucine increased antioxidative enzymes levels (Deng et al., 2016). Opposite to the current trial, Giri et al. (2016) reported that leucine administration has anti-inflammatory effects in fish following lipopolysaccharide (LPS)-induced inflammatory signalling. However, we were unable to demonstrate any effect on pro-inflammatory cytokines in either muscle or liver in fish fed increasing dietary leucine.

In summary, we could not demonstrate a functional effect of leucine administration in Atlantic salmon. This was likely because the fish used in this study did not have a high-fat background, which is often reported to be a necessity to induce the functional effects of leucine. Another hypothesis that we cannot rule out is that the measured functional response following leucine administration occurs during the metabolic processing of a meal and any responses may have returned to lower or baseline values at the 24 h postprandial sampling in the current study. Thus, further studies should evaluate whether there is a positive effect on leucine administration on lipid accumulation and metabolism or oxidation in high-fat Atlantic salmon. Also, the temporal effects of leucine administration in postprandial phase following feeding should be further explored.

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AUTHOR CONTRIBUTIONS

ME, ASG, FL, CDS and IR conceived the study. FL, ASG, ME and MBH executed experiment and sampling. ME and EH performed the laboratory work and qPCR analysis. ME performed statistical analysis and related graphs. All authors contributed to the writing of the manuscript, read and approved the submitted version.

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