A peer-reviewed version of this preprint was published in PeerJ on 27 April 2017.

Short-term starvation at low temperature prior to harvest does not impact the health and acute stress response of adult Atlantic salmon

Rune Waagbø [Corresp., 1] , Sven Martin Jørgensen [2] , Gerrit Timmerhaus [2] , Olav Breck [3] , Pål A Olsvik [4]

[1] National Institute of Nutrition and Seafood Research, Bergen, Norway

[2] Nofima, Ås, Norway

[3] Marine Harvest ASA, Bergen, Norway

[4] National Institute of Nutrition and seafood Research, Bergen, Norway

Corresponding Author: Rune Waagbø
Email address: rwa@nifes.no

Period of starvation is regarded as a sound practice in aquaculture prior to handling, transportation and harvest, to minimise impacts on welfare and ensure proper hygiene after harvest. However, documentation of welfare issues such as stress following starvation and handling in adult Atlantic salmon are lacking. This study aimed to examine gut emptying and potential stress during a two weeks starvation period, and whether this starvation period changes the tolerance for physical stress. The study confirmed slower emptying of the gut segments at low temperature. Plasma and bile cortisol, and selected clinical analyses were used to characterize potential stress, as well as the response to acute physical crowding stress during the starvation period. Neither the general stress level nor the ability to cope with handling stress was affected by a 14 days starvation period. Down-regulation of selected nutritional related gene markers in liver indicated classical starvation responses, with reduced metabolism and oxidative pressure, and sparing of nutrients. The response to acute handling stress was not affected by two weeks of starvation. There were minor effects of starvation on stress and health markers, as evaluated by plasma lysozyme activity and gene expression of selected inflammation marker proteins in heart and skin tissues.

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2 **health and acute stress response of adult Atlantic salmon**

3 Rune Waagbø[1], Sven Martin Jørgensen[2], Gerrit Timmerhaus[2], Olav Breck[3], Pål A. Olsvik[1]

4 [1] National Institute of Nutrition and Seafood Research (NIFES), Bergen, Norway

5 [2] Nofima, Ås, Norway

6 [3] Marine Harvest ASA, Bergen, Norway

7 Corresponding author:

8 Prof. Rune Waagbø, National Institute of Nutrition and Seafood Research (NIFES),

9 P.O. Box 2029 Nordnes, N-5817 Bergen, Norway

10 E-mail: rune.waagbo@nifes.no

11 Telephone: +47 48185036

Abstract

Period of starvation is regarded as a sound practice in aquaculture prior to handling, transportation and harvest, to minimise impacts on welfare and ensure proper hygiene after harvest. However, documentation of welfare issues such as stress following starvation and handling in adult Atlantic salmon are lacking. This study aimed to examine gut emptying and potential stress during a two weeks starvation period, and whether this starvation period changes the tolerance for physical stress. The study confirmed slower emptying of the gut segments at low temperature. Plasma and bile cortisol, and selected clinical analyses were used to characterize potential stress, as well as the response to acute physical crowding stress during the starvation period. Neither the general stress level nor the ability to cope with handling stress was affected by a 14 days starvation period. Down-regulation of selected nutritional related gene markers in liver indicated classical starvation responses, with reduced metabolism and oxidative pressure, and sparing of nutrients. The response to acute handling stress was not affected by two weeks of starvation. There were minor effects of starvation on stress and health markers, as evaluated by plasma lysozyme activity and gene expression of selected inflammation marker proteins in heart and skin tissues.

Introduction

Fish are exposed to periods of starvation or restricted feed intakes both in wild and for practical reasons in aquaculture. In these periods, the fish covers the energy requirements on the expense of body stores of nutrients (Lie & Huse, 1992). Along with the rapid expanding aquaculture production, increasing concerns on fish welfare and ethically acceptable production practices have called for scientific evaluation of biological and behavioural consequences of feeding and starvation practices (Lines & Spence, 2012).

Fasting and feed withdrawal periods prior to transportation and harvest of Atlantic salmon is practiced to obtain complete gut evacuation and a clean digestive tract, to ensure good water quality (e.g. minimize excretion of ammonia) and to reduce metabolic rate, physical activity, hierarchy and stress during transportation (Robb, 2008; VKM, 2008). According to the quality regulations in the Norwegian food legislation, fish should be starved to empty the gut prior to harvest to ensure proper hygiene in further processing. Temperature is the major factor influencing gut evacuation rate (Usher et al., 1991). They showed that other factors such as feed composition and physical quality may also influence the evacuation time, while fish size seems less important. Large cages in commercial scale aquaculture may need longer periods for harvesting and thus the fish population will be starved for longer periods for practical reasons.

Transportation of live salmon is a stressful event involving handling, crowding and exposure to varying water qualities (VKM, 2008). Starvation is practiced also of welfare concerns since there is a general understanding that starved fish is calmer and more tolerant to stress. However, the scientific rationale for this is not substantial and primarily related to reduced metabolic rate being indicative of higher stress tolerance (Petri, 2003). Fed fish may be less robust and more susceptible to stressors (e.g. handling) based on the notion that starvation save energy for digestion and metabolic processes, the fish has lower oxygen demands, less waste production and thereby conserving easily available energy for stress coping. There are few studies demonstrating additional beneficial effects on stress tolerance by starving fish for longer periods than three days (Einen et al., 1998). Mørkøre et al. (2008) concluded that a starvation period of

55   five weeks can apparently improve the resistant to acute stress prior to slaughtering of Atlantic

56   salmon. The pre-harvest starvation period is, however debated from a welfare perspective and

57   standard practices suggest between five and a maximum of 14 days with a priority of a cleared

58   gut (Robb, 2008).

59   Adaptation to starvation includes metabolic adjustments, such as reduced basal metabolic rate,

60   reduced activity in all organs related to exogenous nutrition and swimming activity (Petri, 2003).

61   The net result is a reduced spending of stored energy, mainly from intestinal and muscular lipid

62   stores (Lie & Huse, 1992; Waagbø et al., 1996). This adaptation can be observed as reduced daily

63   loss of body mass over time and this loss seems to be temperature dependent. The daily body

64   mass loss over a period of 28 days was higher for trout and carp reared at 20 °C than 10 °C

65   (Petri, 2003). Salem et al. (2007) reported that three weeks starvation of rainbow trout reduced

66   liver expression of genes involved in aerobic respiration, blood functions and immune responses,

67   associated with a decrease in tissue metabolism. Further, an overall reduction in protein

68   synthetic capacity was observed, and impairment of mitochondrial (aerobic) ATP production,

69   while maintaining liver glycolytic and gluconeogenic competence. In lipid metabolism, down-

70   regulated expression in pathways associated with hepatic lipid and fatty acid transport were

71   seen, while maintaining fatty acid oxidation mechanisms. Thus, fish may maintain tighter control

72   on the mechanisms of protein metabolism than metabolism of lipid or carbohydrate under short

73   term starvation.

74   In the present study, we aimed at examining the physiological response to starvation and if two

75   weeks of starvation affects the robustness to physical handling in adult Atlantic salmon farmed

76   under practical large scale farming conditions at low temperature (4-5 ℃).

77   **Materials and methods**

78   *Fish and sampling*

79 The present starvation study was conducted with adult Atlantic salmon (*Salmo salar* L.) at the
80 large scale R&D site Centre for Aquaculture Competence (CAC), located in Langavika in
81 Gardsundfjorden, Hjelmeland (Western Norway) with approval from the Norwegian authorities
82 (Directorate of Fisheries, approval # R-HM-20). The overall experiment and sampling were
83 controlled by veterinarian and conducted according to the Norwegian Animal Welfare Act. These
84 studies did not require special approval from the authorities. Technical details of the site were
85 previously described by Waagbø *et al.* (2013).

86 The study included examination of required days of starvation for a complete gut evacuation.
87 We examined selected clinical and gene expression markers from liver, muscle, heart and skin
88 tissues during the two weeks starvation time. The outcome was related to plasma cortisol as a
89 traditional primary response stress marker (Wedemeyer, 1996; Iwama, 2006). At each sampling
90 time, corresponding groups of fish were sampled either directly from the cages or following a 45
91 min period with practical relevant moderate confinement stress (crowding), to examine if
92 starvation affected the short time homeostasis to stress.

93 A population of adult Atlantic salmon [body weight 5608 ± 1205g (SD); length 73 ± 4 cm (SD) and
94 condition factor 1.43 ± 0.12 (SD); n=40 at start] had been reared in one of twelve 24 m x 24 m
95 steel cages (last period 30 m deep) from sea transfer until harvest. At the start of the production
96 in September 2012, the cage was stocked with 50 000 S0 smolts of approx. 80 g body weight.
97 Fish were fed a standard extruded diet (Skretting, Stavanger) of appropriate pellet sizes
98 according to the increasing fish size during the 18 months production. Details on feed and
99 biological performance are reported elsewhere (Sissener *et al.*, 2016). The water temperature at
100 the period of starvation averaged 4 °C (5 m depth; range 3.8-4.2 °C). The fish were sampled at
101 four time points after feeding was terminated; initially (March 6th 2014) and at days 3, 7 and 14
102 of starvation. At each of the sampling points, fish were either sampled immediately after careful
103 netting (Netted) or after a 45 min crowding stress in a narrowed catching net, similar to natural
104 handling during harvest operations (Stressed). Fifteen Netted fish were rapidly collected (3-5 fish
105 at a time) by careful netting and immediately killed by a blow to the head, aiming at minimizing

106 stress. Each fish was weighed and length measured. Blood was collected from the caudal vein

107 and selected tissues (liver, heart and skin) dissected and conserved. For Stressed fish, 15

108 fish were sampled after 45 min crowding stress, killed by a blow to the head, blood was

109 collected from the caudal vein and selected tissues (liver and heart) were sampled and

110 conserved as for Netted fish. Both groups were examined for gut content.

111 At each sampling, additional 10 fish was sampled for examining of gut content, making a total of

112 40 fish. At additional samplings at days 1, 2, 4, 5 and 6, forty fish was only weighed, length

113 measured and examined for gut content after removal of the intestine.

114 Blood was sampled on heparin vacutainers (BD Vacutainer, Boston US) and kept chilled until

115 centrifugation to collect plasma which was immediately frozen. Heart was sampled by dissecting

116 out a piece of the ventricle apex stored on RNAlater (Sigma-Aldrich, MO, US). Further, a piece of

117 the liver was stored on RNAlater. A piece of skin and muscle was sampled from the NQC region

118 (Norwegian Quality Cut - part of the salmon behind the dorsal fin, defined as reference fillet for

119 quality measures) from the same side and stored on RNAlater.

120 *Measurement of gut content*

121 The intestinal duct was carefully opened from oesophagus to hind-gut. The content of stomach,

122 pylorus, mid- and hind-gut was characterized separately, and the content was categorized as N

123 (normal), F (flocculants), M (mix/intermediate between normal and flocculants) or E (empty).

124 Flocculants were regarded as normal remnants of faeces following several days of starvation, not

125 negatively affecting slaughter hygiene (Figure 1). The gut evacuation time was evaluated as the

126 sampling day (0 to 7) where no gut content was observed, except remnants defined as

127 "flocculants".

128 *Clinical analyses in plasma and bile*

129   Plasma cortisol, glucose, lysozyme, protein and osmolarity were analysed from 15 Netted and 15

130   Stressed fish at day 0, 7 and 14 after starvation. Sampled bile from Netted fish only was analysed

131   for cortisol and osmolarity at day 0 and 14 (n=12-15). Plasma and bile were analysed for cortisol

132   using a commercially available radioimmunoassay (RIA) kit, GammaCoat^{TM} Cortisol ^{125}I radio

133   immune assay kit (DiaSorin CA1529E, Saluggia, Italy). Plasma glucose, lysozyme and protein

134   were analysed using a clinical bioanalyzer (Maxmat PL analyzer, Montpellier, France) according

135   to standardized procedures, reagents and controls. Osmolarity was analysed in plasma and bile

136   by measuring the freezing point with Fiske One Ten Osmometer (Fiske Associated, Norwood,

137   MA, US).

138   *Gene expression of metabolic markers in liver*

139   Table 1 shows selected metabolic gene markers (PCR primers and efficiencies) for starvation and

140   stress within energy metabolism, oxidative health and overall protein turnover in liver tissue.

141   Expression of these genes were analysed from 15 fish from day 0 and 14 post starvation, and

142   from Netted and Stressed groups. Total RNA from liver was extracted using the BioRobot EZ1 and

143   RNA Tissue Mini Kit (QiagenAB, Sollentuna, Sweden). Reverse transcription was performed using

144   Multiscribe reverse transcriptase (Applied Biosystems, Foster City, California US). Real-time PCR

145   was performed using SYBR Green Master Mix and the LightCycler 480 Real-Time PCR System

146   (Roche Applied Sciences, Penzberg, Germany). Mean normalized expression (MNE) of the target

147   genes was determined using a normalization factor based upon three un-regulated reference

148   genes (ß-actin, Elongation factor 1 alpha B and ubiquitin-60S ribosomal protein L40; Table 1), as

149   calculated by the *geNorm* software (Vandesompele et al., 2002).

150   *Gene expression of stress and health markers in heart and skin*

151   Table 1 shows the selected stress signalling, inflammation and muscle contraction gene markers

152   (PCR primers and efficiencies) analysed in heart and skin tissues. Heart samples were analysed,

153   with 15 fish per time point (0, 3, 7, 14 days starvation) for the Netted group, and 15 fish per time

154   point (0 and 14 starvation) for the Stressed group. Skin samples were analysed in 15 fish at start

155   and after 3, 7 and 14 days starvation from the Netted group only. Total RNA from heart and skin

156 tissues was extracted from frozen tissues and cDNA synthetized with SuperScript VILO kit

157 (Applied Biosystems) using standard operational procedures. The reference gene EF1A was used

158 for normalisation of gene expression in heart and skin tissues. Ct values were calculated by 2nd

159 derivative max method as part of the LightCycler software. Relative expression was calculated

160 according to the Pfaffl method (Pfaffl 2001) adjusted for PCR efficiency.

161 *Statistics*

162 The somatic data are given as mean (SD) while the other data are given as mean (SE or pooled

163 SE). The treatment groups were compared with two-way ANOVA (Starvation, Crowding stress

164 and their interaction term) and Tukey's post hoc test, and graphs prepared in Graphpad Prism.

165 Skin analyses in Netted fish only was analysed by one-way ANOVA. In case of unequal variances,

166 as determined by Bartlett's test, log-transformed data were used for the ANOVA (for liver

167 MnSOD, HSP70 and HMGCR; heart IERG2, JUNB, TNF1A and INOS, and skin MUC5 and MMP9

168 gene expressions). Individual correlation analysis between the parameters was done by a

169 Spearman rank order correlation test (significant at $p < 0.05$).

170 **Results**

171 *Somatic data and gut evacuation time*

172 Fish weight [5347 ± 940g (SD), n=360], length [72.0 ± 3.9 cm (SD), n=360] and condition factor

173 [1.42 ± 0.11 (SD), n=360] were similar for all sampled fish during the 14 day study, with only a

174 marginal increase in length from 72.8 ± 3.9 (SD) to 74.4 ± 3.2 (SD) cm (n=40 fish, p<0.047),

175 comparing the start and final samplings.

176 Regarding gut evacuation rates at low temperatures, Figure 1 shows % of fish (n=40 per day)

177 with gut content in four sections (stomach, pylorus, mid-gut and hind-gut) during 14 days. The

178 sections are gradually emptied with time, with 3, 5, 6 and 7 days in stomach, pylorus, mid-gut

179 and hind-gut, respectively. Overall, the results showed that faeces was still found in gut sections

180   from fish sampled on days 5 and 6 (mix/intermediate between normal and flocculants), implying

181   that 7 days of starvation was needed to completely empty the gut at low temperature (4-5 °C).

182   *Plasma clinical and bile analyses*

183   The plasma cortisol values (Table 2) were generally low and indicated a moderately transiently

184   increased concentrations with starvation (p<0.005) and increased levels after confinement stress

185   (p<0.001). Plasma cortisol increased temporarily from 62 to 97 ng/mL after one week starvation,

186   and returned to 69 ng/mL after 14 days (average levels of Netted and Stressed groups).

187   From Table 2, plasma protein increased significantly with starvation time (p<0.05), but was not

188   affected by confinement stress. Plasma osmolarity showed no changes with starvation, while an

189   approx. 5% rise in concentration was observed in Stressed versus Netted fish in all samplings

190   (p<0.001). For the entire sampled material, individual plasma cortisol was significantly positively

191   (p<0.05) related to plasma protein (Spearman rank order correlation r=0.39; n=45) and plasma

192   osmolarity (Spearman rank order correlation r=0.43; n=45). Plasma glucose declined moderately

193   but significantly with time of starvation (from 5.3 to 4.7 mM; p<0.02), and increased secondary

194   to stress (4.6 to 5.3 mM; p<0.001). Plasma lysozyme activity was neither affected by starvation

195   nor stress (Table 2). None of the clinical markers showed significant interaction terms between

196   starvation and stress.

197   *Bile cortisol and osmolarity*

198   Bile cortisol and osmolarity were analysed from the Netted samplings at start and after 14 days

199   starvation, meaning that starvation time was the only experimental variable. Despite that mean

200   cortisol values varied considerably at both time points, with 2210 ± 710 (SE) vs 350 ± 610 (SE)

201   ng/mL, individual bile cortisol concentration was significantly related to resting plasma cortisol

202   (Spearman rank order correlation 0.56; n=27). Bile cortisol was also related to bile osmolarity

203   (Spearman rank order correlation 0.76). Bile osmolarity declined significantly from 430 ± 21 (SE)

204   to 323 ± 18 (SE) mOsmol/L during starvation.

205 *Expression of metabolic gene markers in liver*

206 Gene expression markers of the liver were chosen to reflect changes in energy metabolism (lipid

207 and amino acid metabolism), oxidative status and overall protein turnover (Table 1), and how

208 the fish copes metabolically to acute stress at start and following 14 days of starvation.

209 Significant effects were observed after two weeks starvation on all genes (Figure 2), validating

210 the usefulness of the chosen genes in starvation with respect to oxidation (down-regulated

211 MnSOD; p=0.0118), cellular stress (up-regulated HSP70; p=0.0001), growth and energy

212 metabolism (down-regulated IGFBP1B; p=0.0006), nutritional stress (down-regulated GRP78;

213 p=0.0001), and lipid and steroid metabolism (down-regulated HMGCR; p=0.0001). In the present

214 study, ubiquitin (uba52) was included as reference gene, indicating that starvation or stress did

215 not have any major impact on protein degradation.

216 No significant effect was seen in the present gene markers in confined fish (Stressed) versus

217 gently Netted fish for any of the examined genes (p>0.05), including the HMGCR gene (Figure 2).

218 This latter gene was chosen to explore possible differences in cortisol synthesis from cholesterol.

219 *Expression of stress signalling and inflammation gene markers in heart*

220 Figure 3 presents expression of two early stress signalling markers (IERG2 and JUNB) in heart

221 tissue from Netted and Stressed fish over time after starvation. Both genes show similar

222 significant temporal changes in expression pattern during starvation (p=0.0066 and p=0.0006,

223 respectively). In the Netted group, both expression levels declined temporarily from 0 to 7 day of

224 starvation, and then returning to initial levels after 14 days starvation. In the Stressed group,

225 expression levels of both genes were generally significantly higher compared with the Netted

226 group, confirming their immediate response to crowding stress (both p<0.0001). Expression of

227 the cytokine TNF1a, a mediator of inflammation, cell survival and differentiation, showed higher

228 levels in Stressed versus Netted fish (p=0.0431; Figure 3). Within the groups there were no

229 changes in expression levels over the two weeks starvation. Expression levels of *inos*, a marker

230   of cardiovascular function and inflammation, was affected by stress but not starvation (Figure 3),

231   with reduced expression observed in Netted fish (p=0.029). Expression levels of *ryr1*, involved in

232   muscle contractility through regulation of myofiber contraction, showed neither changes with

233   starvation nor after confinement stress (Figure 3).

234   *Expression of mucosal and epithelial integrity gene markers in skin*

235   Skin tissue gene expression of mucin 5 (MUC5) and matrix metalloproteinase 9 (MMP9) were

236   assessed in Netted fish only, and showed minor non-significant declines from day 3 to 14 of

237   starvation (Figure 4), with lowest expression levels after 14 days of starvation.

238   **Discussion**

239   The objectives of this study were to examine welfare aspects, including the response to acute

240   stress, of two weeks starvation at low temperatures in adult Atlantic salmon prior to harvest.

241   According to the quality legislation, the fish should be starved so no feed remnants are found in

242   the stomach or intestines. The present study examined the gut evacuation time for fourteen

243   days starvation at low temperature, and underway the fish were examined for physiological,

244   metabolic and welfare issues, including the short-term response to confinement stress. The

245   frame of metabolic stress by starvation and acute confinement stress was measured by plasma

246   and bile cortisol concentrations, related plasma clinical markers, and the regulation of a number

247   of relevant genes in liver, heart and skin tissues.

248   *Gut evacuation time*

249   The present study demonstrates that current common practices with 3-4 days starvation prior to

250   harvest (Farmed fish quality, 2001; Einen et al., 1998) is not sufficient for complete removal of

251   feed from the stomach and gut under the low temperatures conditions in adult Atlantic salmon.

252   The results suggest that fish should be starved for at least 5-7 days at such low temperatures.

253   This is according to Usher et al. (1991), where calculated evacuation time was found two times

254 longer at 4 °C than at 13 °C. Earlier studies have also shown that the intestinal evacuation time

255 was inversely related to size of the previous feed intake in starved juvenile salmon (Talbot et al.,

256 1985). In adult commercially grown salmon like in the present study, the fish had an optimal

257 feed intake prior to starvation and the measured feed evacuation time is therefore

258 representative for present Atlantic salmon farming at cold temperature.

259 *Stress responses*

260 Plasma cortisol values were transiently and moderately increased at 7 days starvation, after

261 which it returned to initial levels after 2 weeks in non-stressed fish, and the relative rises after 45

262 min confinement stress did not seem to be influenced by starvation time. While plasma cortisol

263 is a classical primary response marker to physical stress (Wendelaar-Bonga, 1997), evidences of

264 changes in glucocorticoids in response to fasting in fish are contradictory. Plasma cortisol levels

265 in otherwise unstressed fish are variously reported to be unaffected, reduced or increased by

266 fasting. Starvation implies changes in circulating hormones and expression of their receptors in

267 target tissues, i.e. mainly affecting organs that take part in energy metabolism. Pottinger et al.

268 (2003) found no significant changes in plasma cortisol levels at any point during long-term

269 fasting of rainbow trout. Their results suggested that energy mobilisation during fasting may be

270 achieved without the endocrine involvement of growth hormone, cortisol or somatolactin.

271 Similarly, Rosten et al. (cited in VKM 2008) found that plasma cortisol recorded regularly during

272 15 hrs transportation was generally lower in salmon parr starved for 6 days than in fish starved

273 for 2 days prior to transportation. Mørkøre et al. (2008) indicated improved resistance to acute

274 stress in long-term starved fish (35 days), especially for *post mortem* quality aspects. The

275 mechanisms for this was, however not clear and the response to stress was not confirmed by

276 plasma cortisol analyses. In a marine species like gilthead seabream (*Sparus auratus*) exposed to

277 14 days starvation, however, several fold increase in plasma cortisol was observed (Polakov et

278 al., 2006), which was attributed increased carbohydrate (gluconeogenesis) and amino acid

279 (transamination) metabolism to support energy. Similarly, Costas et al. (2012) found elevated

280 cortisol and amino acids in plasma of Sengalese sole (*Solea senegalensis* Kaup, 1858) starved for

281 21 days and suggested a functional role of cortisol in energy mobilisation. There is obviously

282 both species, age and conditional differences in the endocrine regulation of metabolism during

283   starvation, where well-conditioned adult Atlantic salmon reared at low temperatures is less

284   dependent on cortisol induced energy mobilisation.

285   The present experiment included analyses of bile cortisol. It is assumed that bile, in line with

286   hair cortisol analysis in humans (Wikenius et al., 2016), might reflect longer-term and chronic

287   stress posed by for example starvation (Pottinger, 2008). The decline in bile cortisol and

288   osmolarity after 14 days of starvation reflected the severely reduced intestinal activity and

289   digestion after longer food deprivation. In line with plasma cortisol, the concentration of bile

290   cortisol decreased after 14 days starvation and there was a positive individual correlation

291   between the two in starved non-stressed fish. However, large variation in the bile cortisol

292   concentration reflected both inhomogeneous mucoid bile samples and the changes in

293   composition with time of starvation with reduced bile production and volumes of bile in the gall

294   bladder. The analysis of bile cortisol may therefore be useful to assess changes in cortisol over

295   time, like the endocrine elevation during parr-smolt transformation in salmonids (Shrimpton et

296   al., 1994) and in periods of chronic stress. Since the production and storage of bile varies and

297   declines under starvation, bile cortisol may be more successfully used as an indicator for

298   chronically stressed fish under normal feeding regimes.

299   Similarly to plasma and bile cortisol concentrations, the liver HMGCR gene expression,

300   representing cholesterol and cortisol synthesis was reduced with starvation. At start of the feed

301   deprivation, the short-term stress seemed to activate the HMGCR gene (not significant in the

302   applied two-way ANOVA model), while it was not responsive after 2 weeks of starvation.

303   Plasma osmolarity and protein were chosen as markers to examine secondary stress induced

304   physiological disturbances in the hydro mineral balance (increased osmolarity) and protein

305   mobilization during fasting, respectively. The moderate increase in plasma protein with time

306   seemed therefore to reflect a normal starvation metabolism, with mobilization of labile protein

307   reserves for energy purposes. Although lower than given reference values of plasma protein in

308   adult Atlantic salmon, the present rise was less than the seasonal variation (Sandnes et al.,

309   1988). The use of ubiquitin as reference gene supports the fact that starvation or stress did not

310   have any major impact on liver protein degradation. This is in contrast to 400 g gilthead

311   seabream, where moderate declines in plasma protein were observed in groups starved for 14

312   days, concomitantly to elevated liver transaminase activity, supporting breakdown of amino

313   acids for energy production (Polakof et al., 2006). Besides species differences, the discrepancy

314   may well be related to rearing temperature, as seen for plasma cortisol and amino acid

315   metabolism in Sengalese sole reared at cold and warm temperatures (Costas et al., 2012).

316   Plasma osmolarity was not affected by 14 days starvation, while it was increased after acute

317   stress in all samplings. This is in line with observations in gilthead seabream starved for the same

318   period (Polakof et al., 2006) and reflects the priority of homeostasis. Moderate decline in blood

319   glucose was seen with starvation, while hyperglycemia is a classical secondary response

320   confirming glucose mobilisation following stress and cortisol release (Pottinger, 2008). Together

321   with rise in plasma cortisol, the secondary moderately elevated osmolarity and hyperglycemia in

322   the confined fish, the clinical markers together demonstrated a classical mild response to stress

323   in the present experiment. In a recent study where 2 kg Atlantic salmon exposed to high

324   seawater temperature responded by reduced feed intake and anorexia, there was no impact

325   observed on clinical parameters or liver and white muscle fatty acid composition during a period

326   of 8 weeks (Hevrøy et al., 2010). The fish showed reduced metabolism, however without any

327   obvious physiological challenges. This is also in line with a previous study on adult salmon during

328   fasting (Waagbø & Hansen, 1997). Thus, the present study was suitable to further explore the

329   impact of pre-harvest starvation and responses to the physical acute stress on metabolic

330   adaptations and fish health and immunity at low temperature, as examined at gene expression

331   level in liver, heart and skin tissue.

332   *Metabolic responses*

333   When fish are starved, energy-saving strategies are elicited to maintain the supply of nutrients

334   to selected tissues, especially to the brain (Soengas et al., 1996). Liver is a central organ for

335   nutrient channelling during starvation, both through accumulated nutrients and as a metabolic

336   centre. This is observed by changes in metabolism, both by slowing down energy spenditure and

337 relative changes among the energy substrates at starvation. For example, enzymes involved in

338 lipid breakdown and protein degradation and turnover will generally be up-regulated during

339 starvation, and lipid anabolic enzymes will be down-regulated (Bauer et al., 2004; Costas et al.,

340 2011; Jagoe et al., 2002; Lange et al., 2003; Polakof et al., 2006; Salem et al., 2007; Suzuki et al.,.

341 2002). Starvation includes liver responses on transcriptional level for genes related to oxidative

342 stress, autophagy, energy metabolism, stress response, lipid and steroid metabolism and protein

343 degradation (Martin et al., 2010; Antonopoulou et al., 2013; Morales et al., 2004; Salem et al.,

344 2007). The liver expression markers analysed in the present fish material was selected to give

345 information on how the fish prioritised energy distribution and coped metabolically following 14

346 days of fasting and after exposed to stress. Significant effects were observed after two weeks

347 starvation on all genes (Fig. 2), validating the outcome with respect to redox defence (down

348 regulated MnSOD), cellular stress (upregulated HSP70), growth and energy metabolism (down

349 regulated IGFBP1B), nutritional stress (down regulated GRP78) and lipid and steroid metabolism

350 (down regulated HMGCR). All the nutritional related gene markers were down-regulated, and

351 indicated classical starvation responses like reduced metabolism, reduced oxidative pressure and

352 sparing of nutrients, including reduced igfbp1b expression, reflecting change in catabolism.

353 Circulating IGF-I has been regarded as an index of recent growth in fish under changing

354 nutritional conditions (Beckman 2011). According to Shimizu et al. (2005), the binding proteins

355 of IGF-I have important roles in regulating the metabolic actions of circulating IGF-I. In a later

356 paper, Shimizu et al. (2006) showed that the Chinook salmon circulating IGFBP-1, increased

357 during catabolic states such as fasting and stress. Results from a short-term starvation study (14

358 days) with Atlantic salmon also showed that the liver *igfbp1b* mRNA and protein levels of the

359 corresponding circulating IGFBP increased during 14 days of starvation (Hevrøy et al., 2010). The

360 discrepancy to the declined expression in present study may rely on both the initial nutritional

361 status of the fish and ambient temperature, where the present study was conducted with pre-

362 harvest salmon of good nutritionally condition at low temperature, as compared to 128 g

363 postsmolt salmon and 10°C in the study by Hevrøy et al. (2010). The present study could not

364 detect any weight reduction during the 14 days starvation. The maintenance of energy

365 homeostasis during food deprivation in fish is directly related to the capacity for mobilization of

366 energy reserves such as lipids and hepatic glycogen, at least during the initial stages of fasting,

367 and depends on subsequent activation of hepatic gluconeogenesis and reduction in the rate of

368   glucose utilization (Sheridan & Mommsen, 1991; Navarro & Gutiérrez, 1995). The high body fat

369   stores in the present pre-harvest salmon would ensure capacity to endure starvation, while

370   continuous use of labile protein reserves may impact immunity, as shown for selected functional

371   immunological systems in adult salmon under far longer starvation periods (Waagbø & Hansen,

372   1997; Waagbø, 2006). This may ultimately end in compromised immunity, increased

373   susceptibility to diseases and mortalities in less robust fish.

374   The moderately increased *hsp70* expression with 14 days fasting was observed, probably as a

375   protective measure corresponding to general gene down regulations. HSP70 has, however been

376   used with mixed success to reflect unfavourable farming conditions, like feed deprivation and

377   handling stress (Zarate & Bradley, 2003; Olsvik et al., 2011). Zarate & Bradley (2003) examined

378   how HSPs responded to hatchery stress in the Atlantic salmon and concluded that HSP70 is not a

379   sensitive indicator to aquacultural disturbances like feed deprivation, anesthesia, capture stress,

380   crowding stress, formalin, hyperoxia and hypoxia.

381   In accordance with Olsvik et al. (2008), most of the examined genes in liver were not significantly

382   regulated in confined fish versus gently netted fish, except for the trend of an upregulated

383   *hmgcr* gene after short-term confinement stress (45 min) at start of the starvation period (not

384   significant in the two-way ANOVA model). This gene was chosen to examine differences in

385   steroid and cortisol synthesis from cholesterol after stress. Although liver is not the major site

386   for cortisol synthesis, *hmgcr* gene expression was modestly upregulated in line with the

387   moderately elevated plasma cortisol. Gornati et al. (2005) showed that both the *hmgcr* and

388   *hsp70* genes were upregulated in the liver of fish reared at high rearing densities. HMGCR

389   therefore seems to be a useful early marker of the integrated stress response in Atlantic salmon,

390   reflecting changes in steroid and lipid metabolism with both starvation and shortly after

391   confinement stress.

392   The *igfbp1b* (both samplings) and *grp78* (start sampling) mRNA expressions indicated a trend

393   towards increased expression after stress (not significant), as found in earlier studies (Shimizu et

394   al., 2011). They also found that cortisol treatment induced both igfbp-1a gene expression and

395   igfbp-1a protein in the blood, confirming their role in catabolic conditions like stress. Circulating

396   IGFBP-1 is generally inhibitory to the IGF-1 action and the expression of *igfbp1b* is negatively

397   correlated to individual growth rates is salmonids (Kawaguchi et al., 2013). For the weak rise in

398   *igfbp1b* in the present study, one has to bear in mind that initiation of expression and the RNA

399   turnover may vary between the genes, and that the present short time between the stress and

400   sampling may have excluded genes as suitable stress markers in the present study. For example,

401   Olsvik et al. (2011) clearly suggested that *hsp70* mRNA was a good indicator recorded after two

402   days of handling stress in Atlantic salmon, while it was not affected in the present study,

403   recorded 45 min after stress. A study by Martin et al. (2010) in salmon parr reported that 28

404   days of starvation and bacterial infection (furunculosis) had profound effects on the liver

405   transcriptome, indicating that key components of the immune system were depressed during

406   starvation. However, following infection the starved fish attempt to compensate for this

407   immunosuppression by increasing expression of several key immune related genes to a greater

408   extent than seen in fish fed prior to infection. The principle of being prepared for coming feeding

409   event in periods of starvation have been seen in several fish species experiencing and

410   successfully surviving longer periods of starvation.

411   *Health and immunity*

412   Mounting an immune response requires energy and an increase in metabolic activity, and the

413   effectiveness of the response may be related to body energy reserves. Plasma lysozyme is a

414   simple and commonly used marker of innate immune competence in fish, as response to

415   vaccination, infection and immunosuppression (Waagbø, 2006). In the present study, plasma

416   lysozyme activity was related to protein concentration to prevent confounding effects of changes

417   in water balance in stressed fish. In line with other immunological markers, plasma lysozyme

418   values in our study did not indicate any impact of long time starvation and acute stress on

419   immunity. This confirms the stability of innate immunity during short term fasting and the lack

420   of priority of immunity at acute stress relative to physiologically regaining homeostasis.

421   The effect of starvation on cardiac stress and health markers was evaluated due to the

422   integrated role of the heart in maintaining physiological and metabolic homeostasis in salmon. A

423   previous study on mice showed a broad array of molecular events in response to starvation,

424   related to lipid and glucose energy metabolism, signalling, cell structure and the immune system

425   (Suzuki et al., 2002). The high on-growth of salmon during the last phase of the production cycle

426   may affect cardiac health (e.g. induced epicarditis, remnants of virus-induced myopathies)

427   possibly causing cardiorespiratory problems and risk of incidences following transport and other

428   stressful pre-harvest events. Thus, it was relevant to assess whether prolonged starvation would

429   affect myocardial function. The impact of starvation on cardiac stress was evaluated by two

430   genes, *ierg2* and *junb*, previously identified as immediate markers of the primary stress response

431   to diverse perturbations in salmon. The expression of *ierg2* showed a temporary decline, ending

432   with higher than initial levels after 14 days starvation. Both genes were significantly upregulated

433   in the Stressed versus Netted fish, confirming their response to confinement stress. The

434   significantly reduced expression levels of both markers in Netted fish at day 7, returning to initial

435   levels at day 14 suggests that fish exhibited a lower stress response compared to start and after

436   14 days. In response to crowding stress, there were no differences in expression levels and the

437   immediate stress response in fish between 0 and 14 days of starvation. Plasma cortisol levels

438   showed an opposite regulation of the heart stress markers, with rise in levels after 7 days

439   returning to initial levels after 14 days of starvation. The opposite results between stress markers

440   and cortisol is likely reflecting different kinetics of endocrine versus transcriptional regulation ,

441   or their involvement in different arms of the stress axis (immediate versus chronic stress). In

442   terms of interpretation, transient short-term stress is a healthy response to regain homeostasis

443   (allostasis), but long-term stress could indicate a negative impact on the fish health. Hence, the

444   reduced cortisol levels following 14 days starvation in addition to unchanged regulation of

445   immediate stress-marker suggest that starvation for two weeks has no impact on the stress

446   response, and may in fact be more adaptive and beneficial than one week of starvation at low

447   temperature.

448   Cardiac expression of the myokine TNF1a was induced in response to the crowding stress,

449   however levels of this gene, as well as *inos*, were unchanged with starvation time, suggesting

450   that prolonged starvation did not lead to elevated inflammatory levels in the heart. No changes

451   in the expression level of *inos* with time from start to day 14, further indicates that long

452   starvation is safe in this regard. One marker for myocardial function e.g. muscle contraction, the

453   ryanodine receptor (*ryr1*) was neither affected by prolonged starvation. This gene has also been

454   implicated in the regulation of ATP production in heart, thus steady state expression levels

455   suggested that prolonged starvation and short-term stress did not have any negative effects on

456   myocardial function.

457   The skin of fish is a dynamic tissue with cellular turnover known to be influenced by factors

458   including stress and environmental conditions (Iger et al., 1994). Maintenance of skin and

459   epidermal integrity during starvation and stress is crucial for a proper physical and chemical

460   barrier to challenging environmental conditions and salmon lice infestation (Jensen, 2015).

461   Unchanged expression of mucin 5 (*muc5*) and matrix metalloproteinase 9 (*mmp9*) genes in

462   starved fish suggests that prolonged starvation did not have any negative effects on skin integrity

463   in the present large scale experiment. A recent study on starvation of Atlantic salmon parr in

464   freshwater showed that food deprivation for 18 days caused a rapid decrease in the densities of

465   epidermal mucous cells, particularly in the lateral region of fish (Landeira-Dabarca et al., 2013).

466   These changes may reflect reduced tissue turnover and activity during prolonged starvation. This

467   is in line with previous observations in other fish species (Caruso et al., 2010 in the eel (*Anguilla*

468   *anguilla* L.) or Somejo et al., 2004 in the Nile tilapia (*Oreochromis niloticus* L.). The trend towards

469   reduced levels of skin *muc5* expression at days 7 and 14 could possibly imply a similar effect of

470   prolonged starvation. The same trend was observed for *mmp9*, a gene that is typically

471   upregulated in response to any perturbations of the skin tissue and extracellular environment.

472   *Conclusions*

473   The data from the reported study imply that at low temperature adult salmon with large

474   accumulated body reserves prior to fasting may well handle starvation periods for two weeks,

475   without any negative effects on general stress level, immunity or health, as well as on the ability

476   to cope with acute physical stress. A mild and temporary rise in plasma cortisol after 7 days

477   starvation was observed, while no change in primary and secondary responses to stress were

478   seen after two weeks starvation. Down-regulation of all the selected nutritional related gene

479   markers in liver indicated a classical response to starvation, like reduced metabolism, reduced

480   oxidative pressure, increased cell protection and sparing of nutrients, including reduced igfbp1b

481   expression reflecting catabolism. Starvation did not affect immunity, nor heart functions or skin

482   integrity. The stress markers in heart indicated a predictable response to acute handling stress.

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680 Table 1. *Tissue gene markers used in the experiment, their functional role and expected*

681 *response, as well as their detailed characteristics.*

Gene	Functional role	Expected response	Accession nu
Liver tissue	**Oxidative stress, cellular stress**		
Mn SOD	Manganese superoxide dismutase	Down regulated	DY718412
HSP70	Heat shock protein 70	Down regulated	C169R048
	Energy metabolism		
IGFBP1B	Insulin-like growth factor binding protein 1B	Up regulated	AY662657
	Stress response		
GRP78	78 kDa glucose-regulated protein precursor (GRP 78)	Up regulated	AM042306
	Lipid and steroid metabolism		
HMGCR	3-hydroxy-3-methyl-glutaryl-coenzymeA reductase gene	Up regulated	Contig1955_A n
ACTB	Beta-actin	Reference gene	BG933897
EF1AB	Elongation factor 1 alpha B	Reference gene	BG933853
UBA52	ubiquitin-60S ribosomal protein L40	Reference gene	GO050814
Heart and skin tissues	**Stress signaling**		
IERG2	Immediate early response gene 2		NM_00114012
JUNB	Jun B proto-oncogene		NM_0011399O
	Inflammation & muscle contraction		
TNF1A	Tumor necrosis factor 1 alpha		DQ787157.1
INOS	Inducible nitric oxide synthase		AF088999.1
RYR1	Ryanodine receptor isoform 1		DW541352
	Mucosal & epithelial integrity		
MUC5	Mucin-5B-like		XM_01418848
MMP9	Matrix metalloproteinase-9		NM_00114045
EF1A	Elongation factor 1 alpha	Reference gene	NM_00112362

682   *Table 2. Plasma clinical analyses of adult Atlantic salmon during after 7 and 14 days starvation*

683   *(Netted), including a short-time confinement stress (Stressed) at each time point.*

| Treatment	Day	Cortisol ng/mL	Glucose mM	Lysozyme U/g	Protein g/L	Osmolarity mOsm/L	n
Netted	0	48	5.21	0.32	32.1	332	15
Stressed	0	77	5.45	0.29	36.8	344	15
Netted	7	83	4.35	0.40	37.4	325	15
Stressed	7	112	5.44	0.36	37.9	342	15
Netted	14	49	4.39	0.32	37.7	328	15
Stressed	14	90	4.99	0.35	39.4	342	15
Pooled SEM		4	0.10	0.01	0.7	2	
Two-way ANOVA							
Starvation		$p < 0.005$	$p < 0.018$	ns	$p < 0.035$	ns	
Stress		$p < 0.001$	$p < 0.001$	ns	ns	$p < 0.001$	
Interaction term		ns	ns	ns	ns	ns	

684

Count of stomach amount

Count of pylorus amount
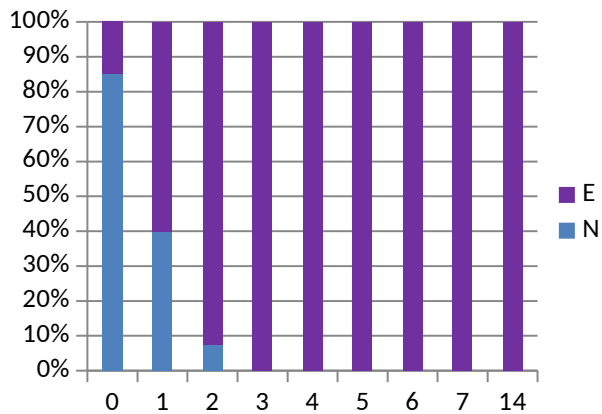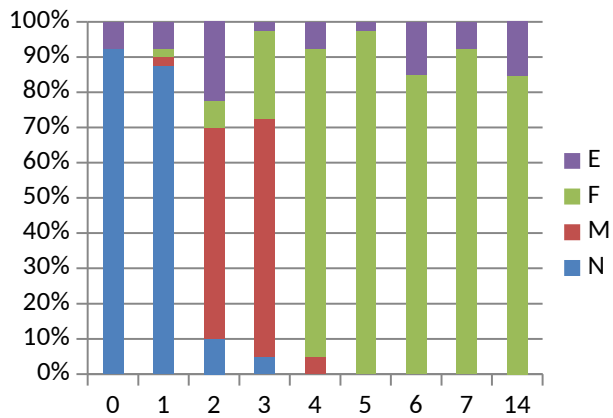
685

Count of mid-gut amount

Count of hind-gut amount
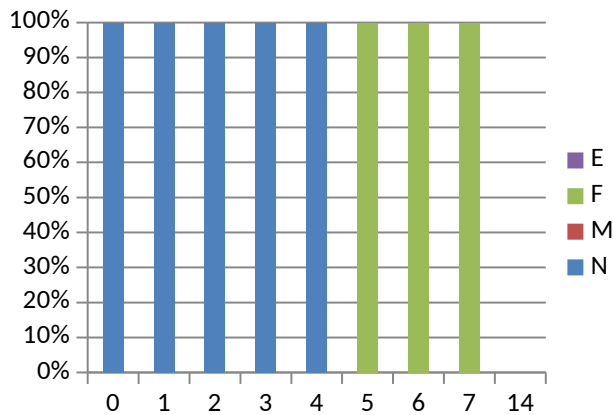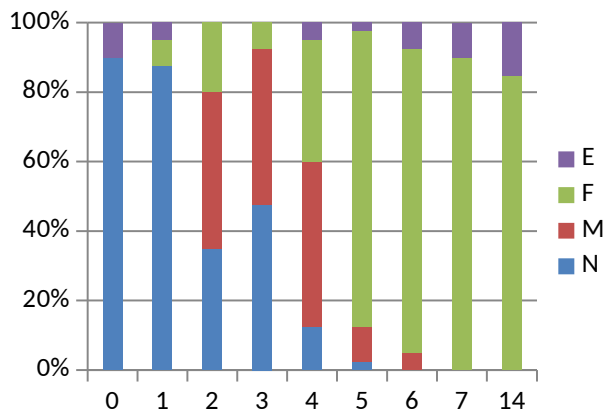
Figure 1. %-Distribution of in fish per sampling day (n=40 fish) with content in stomach, pylorus, mid- and hind-gut. The content was categorized as N (normal), F (flocculants), M (mix/intermediate between normal and flocculants) or E (empty). Flocculants was regarded as normal remnants of faeces following several days of starvation, not negatively affecting slaughter hygiene. The results in the graphs suggest that Atlantic salmon should be starved at least 5-7 days at low temperatures (4°C) to empty the intestine.
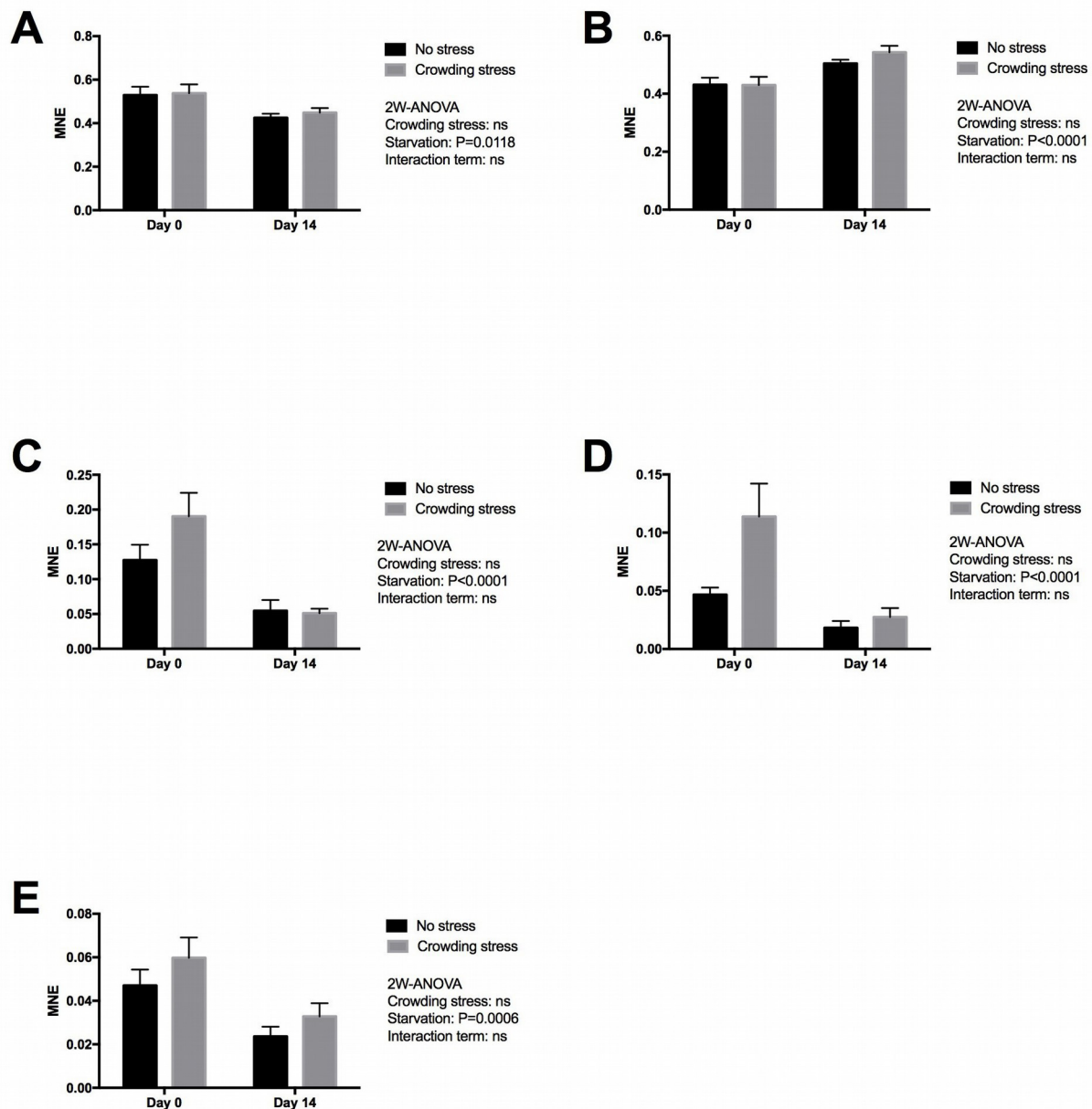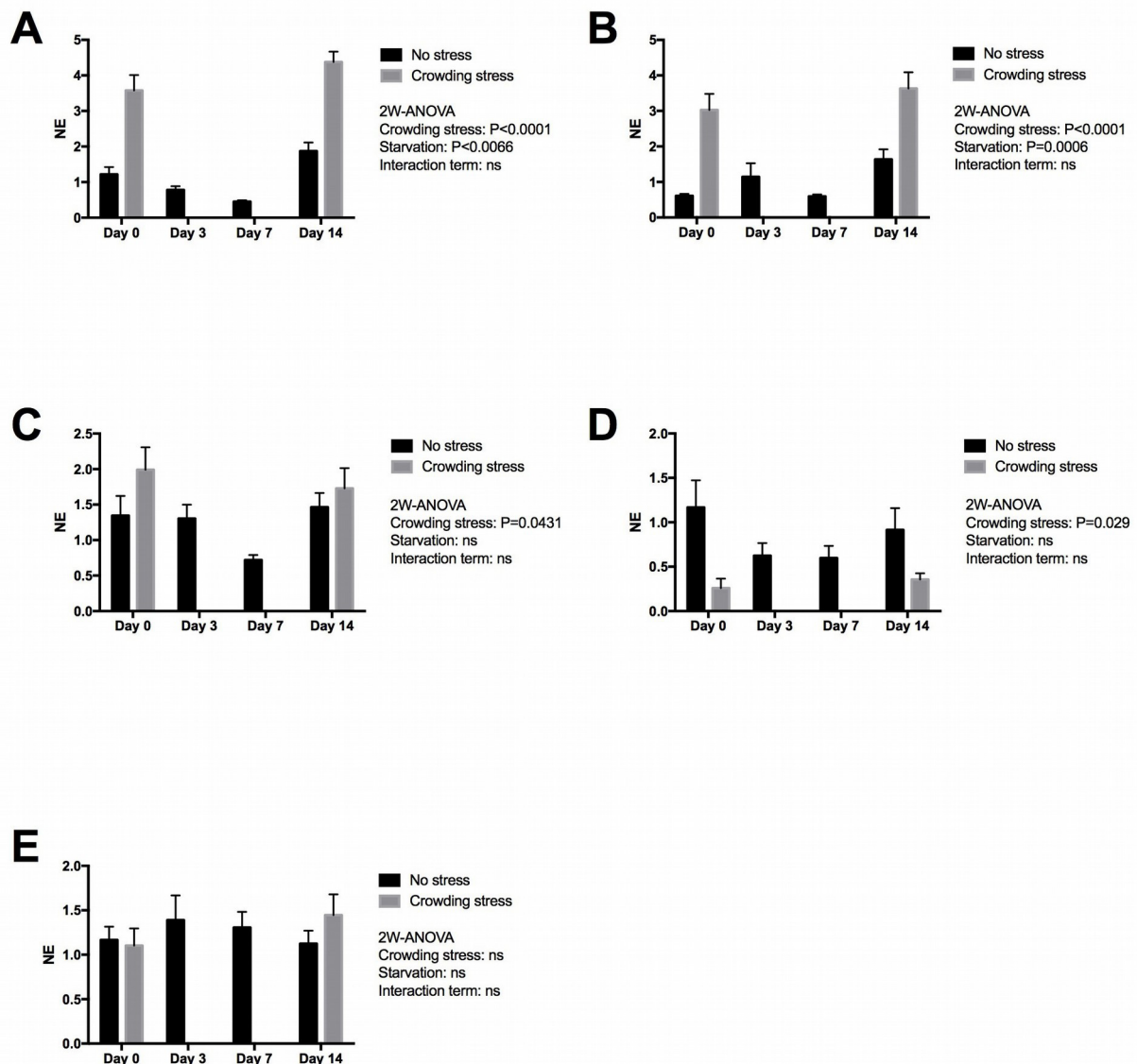
Figure 2. Gene expression of proteins in liver showed that all markers (MnSOD, HSP70, GRP78, HMGCR and IGFBP1 genes) responded to starvation, and none to confinement stress (the MnSOD, HSP70 and HMGCR data were log transformed). Two-way ANOVA was used to search for effects of crowding and starvation between Day 0 and Day 14. Significance levels of crowding stress, starvation and interaction terms are given. ns= not significant. MNE=mean normalized expression.

699 *Figure 3. Gene expression of proteins in heart tissue showed that the selected marker IERG2 and*
700 *JUNB responded differently to starvation, while the IERG2, JUNB, TNF1A (log transformed) and*
701 *INOS (log transformed) genes responded significantly to confinement stress. Two-way ANOVA*
702 *was used to search for effects of crowding and starvation between Day 0 and Day 14.*
703 *Significance levels of crowding stress, starvation and interaction terms are given. ns= not*
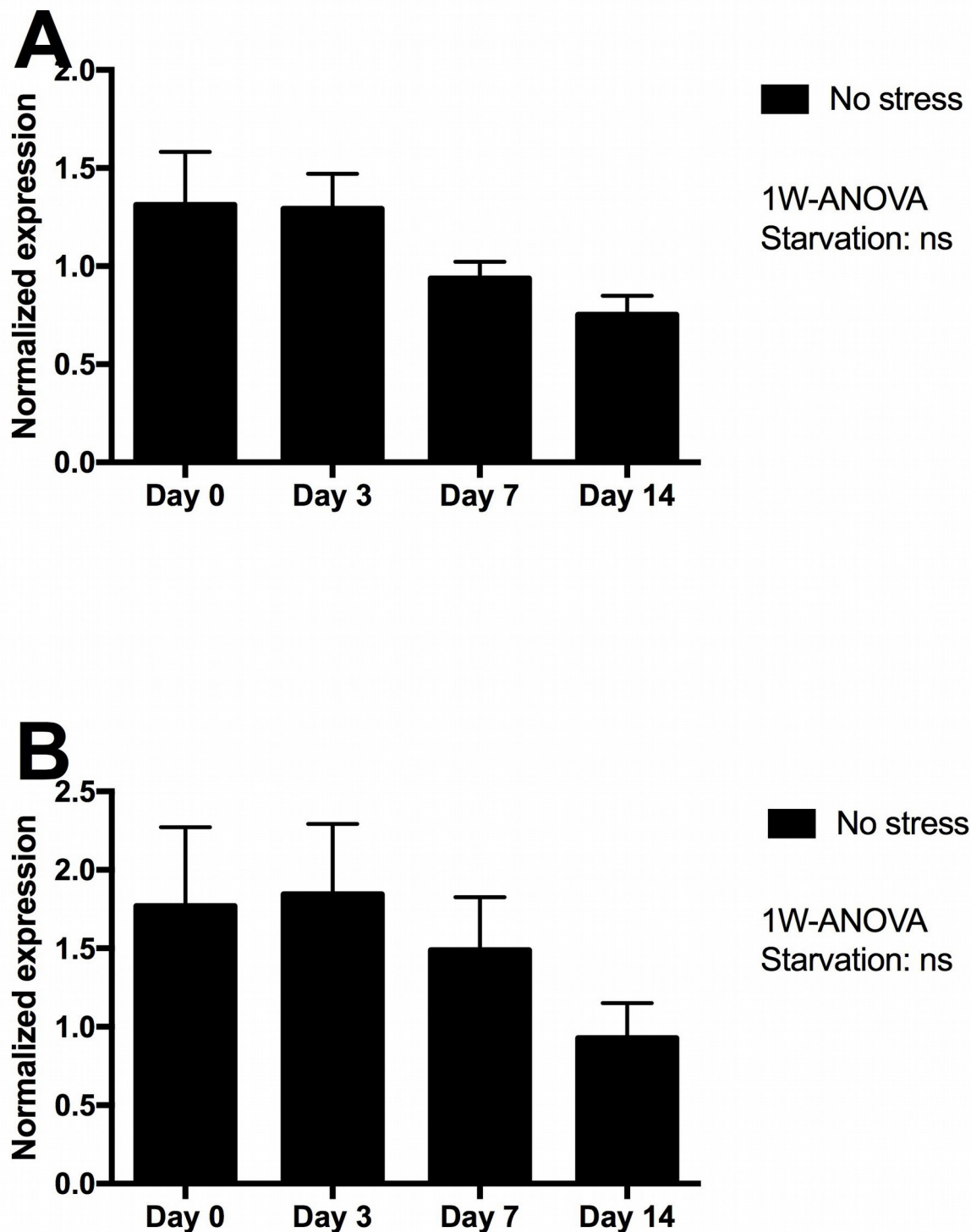704 *significant. NE= Normalized expression.*

Figure 4. *Gene expression of proteins in skin tissue from Netted fish (No stress) showed that neither MUC5 nor MMP9 genes (log transformed) responded significantly to starvation. ns=not significant.*