

Cortisol-induced signatures of stress in the fish microbiome

Tamsyn M. Uren Webster¹, Deiene Rodriguez-Barreto¹, Sofia Consuegra¹, Carlos Garcia de Leaniz¹

¹Centre for Sustainable Aquatic Research, College of Science, Swansea University, Swansea, SA2 8PP, UK

Abstract

Stress experienced in intensive aquaculture can compromise fish growth, condition and immunity. Microbiome disruption may contribute to these adverse health effects, but little is known about how stress affects fish microbial communities. Here, we specifically examined the effects of stress-induced cortisol production on the fish microbiome. We exposed juvenile Atlantic salmon to a mild confinement stressor for two weeks. We then measured cortisol in the plasma, skin-mucus and faeces, and characterised the skin and faecal microbiome. Faecal and skin cortisol concentrations increased in fish exposed to confinement stress, and were positively correlated with plasma cortisol. Elevated faecal cortisol was associated with pronounced changes in the diversity and structure of the faecal microbiome. In particular, we identified a marked decline in probiotic Lactobacillales (*Carnobacterium* sp.) and an increase in pro-inflammatory and pathogenic taxa within the classes Clostridia and Gammaproteobacteria. In contrast, skin-mucus cortisol concentrations were lower and not associated with any detectable changes in the skin microbiome. Our results demonstrate that cortisol disrupts the gut microbiome, which may, in turn, contribute to the adverse effects of stress on fish health. They also highlight the value of using non-invasive faecal samples to monitor stress, including simultaneous determination of cortisol and stress-responsive bacteria.

Keywords: Stress response, Microbiota, Glucocorticoid, *Salmo salar*, Lactic acid bacteria

33 **Introduction**

34 Aquaculture is the fastest growing food producing sector, and plays an increasingly
35 important role in global food security in the face of a growing human population, depletion of
36 capture fisheries and climate change (Teletchea & Fontaine 2014). However, the
37 intensification of aquaculture is often associated with an increase in stress, for example due
38 to crowding and handling, which can impact animal health and welfare, and threaten
39 aquaculture sustainability (Iwama et al. 2011). Stress response in fish includes activation of
40 the hypothalamus-pituitary-interrenal (HPI) axis, culminating in the release of glucocorticoids
41 from interrenal cells located in the head kidney (Barton 2002). As for mammals, cortisol is
42 the predominant glucocorticoid released as part of the primary stress response, and is
43 critical for mediating adaptive metabolic, physiological and behavioural adjustments
44 (Schreck & Tort 2016). However, prolonged elevation of cortisol is associated with adverse
45 health effects. In particular, stress-mediated impairment of immune function has been widely
46 described in cultured fish and has been associated with reduced disease resistance, which
47 is of critical importance for aquaculture sustainability (Ellison et al. 2018; Uren Webster et al.
48 2018b; Yada & Tort 2016).

49 Recent research has revealed the diverse influence of microbiota and their metabolites
50 on many aspects of host health and fitness, including digestion and nutrient uptake,
51 metabolism and immune development (Hooper et al. 2012; Rea et al. 2016). In mammals,
52 stress is well known to disrupt the diversity, structure and function of the microbiome which,
53 in turn, has been associated with long term health effects in the host, including metabolic
54 and immune impairment, and a range of diseases (Foster et al. 2017; Tetel et al. 2018). The
55 mechanisms by which stress impacts the microbiome are complex, and not fully understood.
56 Elevated plasma cortisol, resulting from social or psychological stress, has been associated
57 with alterations in the structure and/or diversity of the mammalian faecal or oral microbiome,
58 including the abundance of lactic acid bacteria and opportunistic pathogens (Galley et al.
59 2014; Jasarevic et al. 2015; Mudd et al. 2017). Direct administration of glucocorticoids has
60 also been shown to exert stimulatory and inhibitory effects on specific microbial taxa,
61 together with wider effects on host metabolism in some cases (Huang et al. 2015; Jentsch et
62 al. 2013; Petrosus et al. 2018; Wu et al. 2018). Additionally, while host stress response
63 influences the microbiome, the microbiome can also influence host stress response.
64 Microbiota and their metabolites are known to exert effects throughout the mammalian
65 hypothalamus-pituitary-adrenal (HPA) axis, influencing glucocorticoid synthesis, release and
66 signalling pathways (Burokas et al. 2017; de Weerth 2017; Simard et al. 2014; Vodicka et al.
67 2018).

68 Disruption of the microbiome is likely to represent an important mechanism by which
69 stress affects fish health, welfare and performance in aquaculture. There is some evidence

70 that environmental and social stressors disrupt microbial communities associated with the
71 fish gut and skin (Boutin et al. 2013; Sylvain et al. 2016; Uren Webster et al. 2019; Zha et al.
72 2018), however, a potential role of cortisol in mediating these effects is unknown. We
73 hypothesised that stress-induced cortisol production would directly disrupt the fish
74 microbiome. To test this, we examined the effects of cortisol on the skin and gut microbiome
75 of juvenile Atlantic salmon, following confinement, a mild aquaculture-relevant stressor.

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78 **Methods**

79

80 *Stress experiment*

81 Prior to the start of the experiment 0+ Atlantic salmon fry (mean mass 3.92 ± 0.11 g; fork
82 length 7.46 ± 0.07 cm), were housed in stock tanks (80 L) supplied with a constant flow of
83 aerated, de-chlorinated tap water in a recirculation system with a temperature of $15^{\circ}\text{C} \pm 0.5$
84 $^{\circ}\text{C}$ and photoperiod of 12L:12D. Water oxygen saturation was maintained above 90%, and
85 ammonia (<0.02 mg/L), nitrite (<0.01 mg/L), nitrate (<15 mg/L) and pH (7.5 ± 0.2) were
86 maintained within the optimal range for the species. Fish were fed with a commercial salmon
87 feed (Skretting Nutra Parr) at a rate of 3% body weight per day.

88 Experimental fish were assigned at random to the control and confinement-stress
89 treatment groups, with three replicate 20 L tanks per group, each containing 28 fish.
90 Confinement stress consisted of slowly lowering the water volume in each tank (via draining)
91 from 20 L to 5 L for one hour, and this was repeated every day at the same time (1100 hrs)
92 for two weeks. All other husbandry conditions were as before. At the end of the experiment,
93 fish were euthanised via an overdose of anaesthetic (Phenoxyethanol; 0.5 mg/L), followed
94 by destruction of the brain according to UK Home Office regulations. The fish were
95 measured (fork length), weighed (wet weight) and Fulton's condition factor was calculated as
96 a measure of body condition. Blood samples were collected from the caudal vein using
97 heparinised capillary tubes, centrifuged (5 min, $5000 \times g$) and the plasma supernatant
98 removed and stored at -80°C prior to cortisol analysis. For each fish, a sample of skin-
99 associated mucus for microbiome analysis was collected by swabbing the left side lateral
100 line five times using Epicentre Catch-All™ Sample Collection Swabs (Cambio, Cambridge,
101 UK). A sample of skin-associated mucus for cortisol analysis was collected by scraping
102 mucus from a 2 cm^2 region of skin on the left-hand side of the fish, above the lateral line
103 between the head and dorsal fin, using a scalpel blade. Faecal samples were collected from
104 each fish by gently pressing along the length of the abdomen and collecting expelled faeces,
105 which were then split evenly between samples for cortisol and microbiome analysis. All skin-
106 mucus and faecal samples were directly frozen at -80°C prior to analysis.

107

108 *Cortisol measurement*

109 Quantification of cortisol concentration in plasma, skin mucus and faecal samples was
110 performed using the DetectX Cortisol Enzyme Immunoassay Kit (Arbor Assays, Michigan,
111 USA), according to the manufacturer's recommendations. Briefly, plasma samples were first
112 pre-treated with dissociation reagent then diluted in assay buffer (1:50 final dilution) before
113 cortisol measurement. Faecal samples were suspended in 100 µl ethanol, vortexed for 30
114 minutes, centrifuged (5 min, 5000 x g), then the supernatant was then diluted in assay buffer
115 (1:20) before cortisol measurement. Skin-mucus samples were suspended in 100 µl 1M Tris-
116 HCl, vortexed for 30 minutes and centrifuged (5 min, 5000 x g), then the supernatant was
117 used directly in the assay without dilution.

118 Cortisol concentration was measured in the plasma, faeces and skin-mucus for a total of
119 60 individual fish (40 stressed fish and 20 controls; distributed evenly amongst replicate
120 tanks). Each of these 180 samples was analysed in duplicate, across five 96-well plates.
121 Cortisol concentration was calculated based on a standard curve run on each plate, and
122 adjusted for dilution factor and initial sample volume (plasma) or weight (skin-mucus/faeces).
123 Inter-assay variability, measured as the coefficient of variation (CV%) of four repeats across
124 the five plates, was 4.62% and the lower limit of detection was 76.4 pg/ml. We removed one
125 outlier of faecal cortisol (47.3 ng/g) from the stressed group (most likely resulting from an
126 error in sample preparation) using Tukey's 1.5*IQR method, as it was 3.4x higher than the
127 mean value (13.76 ng/g) and 1.6x higher than the next highest value in this group (29.1
128 ng/g).

129

130 *16S rRNA amplicon sequencing*

131 16S rRNA amplicon sequencing was performed using the faecal and skin mucus samples
132 for the same 60 individual fish for which cortisol quantification was performed (40 stressed
133 and 20 controls), as described previously (Uren Webster et al. 2018a). Briefly, DNA was
134 extracted from all samples using the MoBio PowerSoil® DNA Isolation Kit (Qiagen)
135 according to the manufacturer's instructions. Libraries were prepared amplifying the V4
136 hypervariable region of the bacterial 16S gene using the primers 341F and 785R, and
137 sequenced across two lanes of an Illumina MiSeq. Raw sequence reads were quality filtered
138 using Trimmomatic (Bolger et al. 2014), before analysis with mothur v1.39 (Kozich et al.
139 2013). Concatenated reads were aligned to the Silva seed reference database (version 128)
140 (Quast et al. 2013), chimeric reads were removed using UCHIME (Edgar et al. 2011), and
141 Bacteria and Archaea contigs were classified using the Silva reference taxonomy. Contigs
142 were clustered into operational taxonomic units (OTUs) using mothur, based on 97%
143 sequence similarity. Singleton OTUs were removed from the dataset then all faecal samples

144 were subsampled to an equal depth of 19,724 reads and all skin samples were subsampled
145 to 10,133 reads. Measures of alpha diversity (Chao1 richness and Shannon diversity) for
146 each sample were calculated in mothur.

147

148 *Statistical analysis*

149 All statistical analysis was performed in R v3.5.0. Firstly, we assessed whether non-
150 invasive measurements of faecal and skin cortisol were indicative of cortisol in blood plasma
151 by calculating the Pearson correlation coefficient. We then employed linear mixed effects
152 models (LMM) using the *lme4* package to examine the effects of confinement stress and fish
153 size on measured cortisol values in plasma, skin and faeces, using tank identity as a random
154 factor. We also used linear mixed effects models to examine the effects of confinement
155 stress, fish size and measured faeces/skin cortisol on faeces/skin microbial alpha diversity
156 (Chao1 richness and Shannon diversity), including tank as a random factor. We used fish
157 length as a covariate to control for size effects as it had a lower coefficient of variation (CV =
158 0.067) than fish mass (CV = 0.216). To achieve model simplification, we started with a
159 model with all main effects and selected the model with the lowest AIC value via backward
160 selection using the *step* and *drop1* functions and the *lmerTest* package (Kuznetsova et al.
161 2017). A minimal adequate model was then refitted via Restricted Maximum Likelihood, or
162 as a linear model when the random component (tank identity) did not improve model fit
163 compared to the fixed effects only model, as indicated by the Likelihood Ratio Test (LRT).
164 We used the *VCA* package to estimate the amount of variability in cortisol due to
165 confinement stress, tank effects, and differences among individual fish.

166 Analysis of microbial community structure (beta diversity) was performed within the
167 *Vegan* package in R (Oksanen et al. 2017), using the Bray-Curtis dissimilarity index. Non-
168 metric multidimensional scaling (NMDS) ordination of Bray-Curtis distances were visualised,
169 including measured cortisol concentration as an environmental vector. Multivariate statistical
170 analysis of microbial community separation in the faecal and skin samples was performed by
171 PERMANOVA using *Adonis* in the *Vegan* package with confinement stress and measured
172 faecal/skin cortisol as predictors. Statistical analysis of OTU abundance was performed
173 using DeSeq2 (Love et al. 2014). The effect of confinement stress and faecal/skin cortisol on
174 relative abundance of faecal/skin OTUs was tested using a multifactorial design. Within the
175 DeSeq model, low coverage OTUs were independently filtered to optimise power for
176 identification of differentially abundant OTUs at a threshold of $\alpha=0.05$. Outlier detection
177 and moderation of OTU level dispersion estimates were performed using default settings,
178 and OTUs were considered significantly differentially abundant at FDR <0.05.

179

180

181 **Results**

182 *Relation between plasma cortisol and non-invasive measures of cortisol in faeces and skin*

183 Cortisol concentrations ranged from 2.9 to 65.8 ng/ml in blood plasma, 3.6 to 29.1 ng/g in
184 faeces, and 0.14 to 9.45 ng/g in skin mucus across all samples. Significant positive
185 correlations were found between plasma and faecal cortisol (Pearson's $r_{56}=0.615$, $P <$
186 0.001), between plasma and skin cortisol ($r_{56}=0.289$, $P = 0.028$), and between faecal and
187 skin cortisol ($r_{57}=0.422$, $P < 0.001$; Figure 1a). Variance component analysis indicated that
188 82-85% of the variation in cortisol was due to variation between individuals, and 0-18% was
189 due to variation between tanks.

190

191 *Effects of confinement stress on cortisol*

192 There was a significant increase in cortisol in the faeces and skin of stressed fish
193 compared to unstressed controls (Welch two sample t-test; faeces, $t_{56,766} = 2.955$, $P = 0.004$;
194 skin, $t_{52,917} = 2.819$, $P = 0.007$), but not in blood plasma (LMM stress effect, $t_{4,785} = 0.603$, $P =$
195 0.574 ; Figure 1b), which showed a significant tank effect (LRT $\chi^2 = 6.01$, $P = 0.014$). There
196 was no association between cortisol and the length, weight or body condition of fish at the
197 end of the experiment ($P > 0.2$ in all cases).

198

199 *Effects of cortisol on microbial diversity*

200 Faecal cortisol was negatively correlated with faecal Chao1 microbial richness (Chao1
201 Cortisol estimate: -79.17 ± 32.33 , $t_{1,57} = -2.449$, $P = 0.017$), but positively correlated with
202 Shannon diversity (Shannon Cortisol estimate: 0.08 ± 0.024 , $t_{1,57} = 3.536$, $P < 0.001$; Figure
203 2). There was no effect of confinement stress, fish size or tank identity on faecal microbial
204 diversity beyond that accounted by an increase in cortisol ($P > 0.4$ in all cases). For the skin,
205 there was no significant effect of confinement stress, skin cortisol, or fish size on skin
206 microbial Chao1 richness or Shannon diversity ($P > 0.1$ in all cases), although there were
207 significant tank effects for skin Chao1 richness (LRT $\chi^2 = 15.53$, $P < 0.001$).

208 Microbial community structural diversity was performed based on the Bray-Curtis
209 dissimilarity metric, and visualised using NMDS analysis (Figure S1). As for alpha diversity,
210 there was a significant effect of faecal cortisol on faecal microbiome beta diversity (Cortisol:
211 $F_{1,56} = 9.525$, $P = 0.001$), but the confinement stress had no additional effect beyond that
212 caused by an increase in cortisol (Stress $F_{1,56} = 2.487$, $P = 0.064$). For the skin microbiome
213 there was no detectable effect of stress or skin cortisol concentration on beta diversity
214 (Stress: $F_{1,53} = 1.263$, $P=0.151$, Cortisol: $F_{1,53} = 0.960$, $P=0.475$).

215

216 *Effects of cortisol on microbial composition*

217 The effects of stress and cortisol concentration on OTU relative abundance was
218 investigated using DeSeq2. For the faecal microbiome, the abundance of 44 OTUs (27
219 increased, 17 decreased) were significantly associated with faecal cortisol concentration, but
220 only one OTU (*Vagococcus* sp.) was significantly elevated in the confinement stress group
221 independently of cortisol (Figure 3; Table S1). Strikingly, of the 17 OTUs which were
222 negatively associated with cortisol concentration, the vast majority (15) were classified as
223 belonging to the genus *Carnobacterium* sp. in the order Lactobacillales, including the most
224 abundant OTU overall. Of the OTUs that were positively associated with faecal cortisol
225 concentration, 10 (37%) were from the class Gammaproteobacteria and, notably, two highly
226 abundant OTUs from the family Clostridiaceae were also increased. In contrast, for the skin
227 microbiome, no OTUs were significantly associated with either confinement stress or
228 measured skin cortisol concentration.

229

230 Discussion

231 Our study indicates that the cortisol stress response modulates the intestinal, but not the
232 skin, microbiome of juvenile Atlantic salmon. Although the cortisol stress response to
233 confinement was variable amongst individuals, we identified a very distinctive relationship
234 between cortisol and the diversity and structure of the faeces microbiome, including a clear
235 inhibition of lactic acid-producing bacteria and the promotion of pro-inflammatory and
236 pathogenic taxa.

237 Exposure to a routine, aquaculture-relevant stressor (confinement) increased the cortisol
238 concentration in the skin and faeces of juvenile salmon compared to control fish, although
239 there was considerable variation among individuals. This variation is consistent with the
240 existence of low and high cortisol response fish (e.g. Pottinger & Carrick 1999; Samaras et
241 al. 2016). We also identified a positive association between plasma and faecal cortisol, and,
242 to a lesser extent, between plasma and skin-mucus cortisol. Plasma cortisol is typically used
243 to measure the stress response in fish, but blood sampling is invasive and may require
244 terminal sampling in the case of small fish (Sadoul & Geffroy 2019). Plasma cortisol
245 concentration is also known to be influenced by acute spikes in glucocorticoid production, for
246 example caused by handling stress prior to sampling, which may mask underlying stress
247 levels (Bertotto et al. 2010). Our results add to that of other recent studies suggesting that
248 faecal and skin sampling provide non-destructive alternatives to measuring plasma cortisol
249 in Atlantic salmon (Bertotto et al. 2010; Cao et al. 2017; De Mercado et al. 2018; Lupica &
250 Turner Jr 2009), which can also be linked directly to microbiome analysis.

251 Across all fish, we identified a strong association between cortisol in the faeces and both
252 alpha and beta measures of gut microbiome diversity. Faecal cortisol was negatively
253 associated with Chao1 richness, but positively associated with Shannon diversity,

254 suggesting that there were fewer, but more evenly distributed, bacterial taxa in the intestine
255 of stressed fish. This is likely to reflect an inhibitory effect of cortisol on dominant OTUs
256 normally present in non-stressed individuals. In particular, there was a striking decline in
257 *Carnobacterium* sp. with increasing levels of faecal cortisol, including the most abundant
258 OTU in non-stressed fish, together with +10 other OTUs assigned to this genus.
259 *Carnobacterium* (order Lactobacillales, class Bacilli, phylum Firmicutes) is a genus of
260 facultatively anaerobic, cold tolerant lactic acid bacteria comprising +12 species (Pikuta &
261 Hoover 2014). This genus, particularly *C. pisciola*, *C. divergens* and *C. inhibens*, is
262 commonly found in the intestinal communities of healthy fish, including Atlantic salmon
263 (Ringø et al. 2001). *Carnobacterium* sp. are also widely used as probiotics in aquaculture,
264 due to their beneficial effects on gut health and fish performance, and their ability to inhibit
265 the growth of several common fish pathogens (Ringø 2008; Ringø et al. 2001).

266 Individuals that displayed a high cortisol response to confinement stress had a distinct
267 faecal microbiome, that was very different from that of non-responsive fish, or from control
268 fish that had low baseline cortisol levels. Alongside a marked decline in *Carnobacterium* sp.,
269 this structural change was characterised by a notable increase in the relative abundance of
270 two Clostridiaceae OTUs. This family (class Clostridia, phylum Firmicutes) is commonly
271 found in the gut of healthy mammals and fish, but also includes a number of opportunistic
272 pathogens. An increased abundance of Clostridiaceae has been associated with microbial
273 dysbiosis, intestinal inflammation and gastrointestinal diseases (Lopetuso et al. 2013; Muñoz
274 Pedrego et al. 2018). Several OTUs within the class Gammaproteobacteria, including two
275 *Yersinia* sp., *Pseudomonas* sp., *Acinetobacter* sp. and *Aeromonas* sp, were also
276 particularly abundant in fish that had high levels of faecal cortisol. These genera include a
277 range of opportunistic fish pathogens (Austin & Austin 2007), and tend to increase following
278 exposure to different types of environmental stress in fish (Boutin et al. 2013; Uren Webster
279 et al. 2019), suggesting they may represent a common signature of stress exposure. In
280 mammals, experimental administration of cortisol results in a similar reduction in probiotic
281 lactic acid-producing bacteria and an increase in pro-inflammatory microbiota (Huang et al.
282 2015; Petrosus et al. 2018; Wu et al. 2018), suggesting that these bacteria taxa could
283 represent useful biomarkers of stress across vertebrates.

284 Our results demonstrate how an increase in cortisol can affect the diversity and structure
285 of the salmon gut microbiome, which may in turn contribute to the adverse effects of stress
286 on fish health. This is consistent with the results of previous studies that experimentally
287 administered glucocorticoids to rats, mice and pigs (Huang et al. 2015; Petrosus et al. 2018;
288 Wu et al. 2018). However, it is not clear exactly how cortisol may affect different taxa within
289 complex host-associated microbial communities. Potential inhibitory mechanisms could
290 include direct toxicity, metabolic impairment, disruption of ion-regulation, endocrine signalling

291 or nutrient depletion, similar to the effects of other chemical or physical stressors (Harms et
292 al. 2016; Weber et al. 2014). At the same time, taxa more tolerant of cortisol may flourish in
293 the absence of previous niche completion (Hibbing et al. 2010), and cortisol is also known to
294 specifically promote the growth of certain oral pathogens *in vitro* (Jentsch et al. 2013).
295 However, the overall relationship between stress response, cortisol and the microbiome is
296 complex. The microbiome may be influenced by other stress hormones, by interactions
297 amongst microbes or with the host immune system, while microbiota and/or their metabolites
298 are also well known to influence host stress response signalling (Burokas et al. 2017; de
299 Weerth 2017; Simard et al. 2014; Vodicka et al. 2018).

300 The impacts of cortisol on the microbiome are also likely to depend on the nature of the
301 microbial community, and the stress response. In contrast to the faecal microbiome, we
302 found no significant effects of confinement stress or skin cortisol on the diversity or structure
303 of the skin microbiome. It is possible that skin-associated communities, which are dominated
304 by Proteobacteria with much lower levels of Lactobacilliales, are less sensitive to cortisol
305 than faecal microbiota. On the other hand, cortisol concentrations in the skin mucus were
306 much lower than those measured in faecal samples, which may also help explain the lack of
307 observed effects of skin cortisol on the skin microbiome.

308 To conclude, our study shows, for the first time, that an increase in stress-related cortisol
309 alters the fish intestinal microbiome, notably by decreasing the abundance of
310 *Carnobacterium*, a Lactobacilliales commonly used as a probiotic in aquaculture, and
311 increasing pro-inflammatory and opportunistic bacterial pathogens. Given the fundamental
312 influence of microbiota and their metabolites on many aspects of host health, this suggests
313 that cortisol-mediated disruption of the intestinal microbiome is likely to contribute to the
314 adverse effects of stress on immune function and disease resistance. These results have
315 important implications for health and welfare of fish exposed to environmental stress, and
316 more broadly, to research on stress-related diseases, such as metabolic syndrome, obesity
317 and IBD, which have been associated with microbiome dysbiosis. Finally, our study
318 demonstrates that both cortisol measurements and microbiome analysis can be performed
319 simultaneously on faecal and skin samples collected non-invasively, which could represent a
320 valuable screening tool for evaluating stress in fish.

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331

332 **Author contributions**

333 TUW, DRB, CGL and SC designed the study; TUW and DRB performed the experiment;
334 TUW and CGL analysed the data; TUW drafted the manuscript. All authors contributed to
335 the final version of the manuscript.

336

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342

343 **Ethics**

344 All experimental procedures were approved by Swansea Animal Welfare and Ethical Review
345 Body (number IP-1415-2).

346

347 **Data accessibility**

348 All 16S rRNA sequence reads are available from the European Nucleotide Archive under
349 accession PRJEB32276. Full metadata, and measured cortisol concentrations are available
350 in the supporting information.

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353 **References**

354 Austin, B. & Austin, D. A. 2007 *Bacterial Fish Pathogens, Disease of Farmed and Wild Fish*.
355 Chichester: Springer-Praxis.

356 Barton, B. A. 2002 Stress in fishes: a diversity of responses with particular reference to
357 changes in circulating corticosteroids. *Integrative and comparative biology* **42**, 517-
358 525.

359 Bertotto, D., Poltronieri, C., Negrato, E., Majolini, D., Radaelli, G. & Simontacchi, C. 2010
360 Alternative matrices for cortisol measurement in fish. *Aquaculture Research* **41**,
361 1261-1267.

362 Bolger, A. M., Lohse, M. & Usadel, B. 2014 Trimmomatic: a flexible trimmer for Illumina
363 sequence data. *Bioinformatics* **30**, 2114-20.

- 364 Boutin, S., Bernatchez, L., Audet, C. & Derôme, N. 2013 Network analysis highlights
365 complex interactions between pathogen, host and commensal microbiota. *PLoS ONE*
366 **8**, e84772.
- 367 Burokas, A., Arboleya, S., Moloney, R. D., Peterson, V. L., Murphy, K., Clarke, G., Stanton,
368 C., Dinan, T. G. & Cryan, J. F. 2017 Targeting the Microbiota-Gut-Brain Axis:
369 Prebiotics Have Anxiolytic and Antidepressant-like Effects and Reverse the Impact of
370 Chronic Stress in Mice. *Biological Psychiatry* **82**, 472-487.
- 371 Cao, Y., Tveten, A.-K. & Stene, A. 2017 Establishment of a non-invasive method for stress
372 evaluation in farmed salmon based on direct fecal corticoid metabolites
373 measurement. *Fish & Shellfish Immunology* **66**, 317-324.
- 374 De Mercado, E., Larrán, A. M., Pinedo, J. & Tomás-Almenar, C. 2018 Skin mucous: A new
375 approach to assess stress in rainbow trout. *Aquaculture* **484**, 90-97.
- 376 de Weerth, C. 2017 Do bacteria shape our development? Crosstalk between intestinal
377 microbiota and HPA axis. *Neuroscience & Biobehavioral Reviews* **83**, 458-471.
- 378 Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C. & Knight, R. 2011 UCHIME improves
379 sensitivity and speed of chimera detection. *Bioinformatics* **27**, 2194-2200.
- 380 Ellison, A. R., Webster, T. M. U., Rey, O., de Leaniz, C. G., Consuegra, S., Orozco-
381 terWengel, P. & Cable, J. 2018 Transcriptomic response to parasite infection in Nile
382 tilapia (*Oreochromis niloticus*) depends on rearing density. *BMC genomics* **19**, 723.
- 383 Foster, J. A., Rinaman, L. & Cryan, J. F. 2017 Stress & the gut-brain axis: Regulation by the
384 microbiome. *Neurobiology of Stress* **7**, 124-136.
- 385 Galley, J. D., Nelson, M. C., Yu, Z., Dowd, S. E., Walter, J., Kumar, P. S., Lyte, M. & Bailey,
386 M. T. 2014 Exposure to a social stressor disrupts the community structure of the
387 colonic mucosa-associated microbiota. *BMC Microbiology* **14**, 189.
- 388 Harms, A., Maisonneuve, E. & Gerdes, K. 2016 Mechanisms of bacterial persistence during
389 stress and antibiotic exposure. *Science* **354**, aaf4268.
- 390 Hibbing, M. E., Fuqua, C., Parsek, M. R. & Peterson, S. B. 2010 Bacterial competition:
391 surviving and thriving in the microbial jungle. *Nature reviews. Microbiology* **8**, 15-25.
- 392 Hooper, L. V., Littman, D. R. & Macpherson, A. J. 2012 Interactions between the microbiota
393 and the immune system. *Science* **336**, 1268-1273.
- 394 Huang, E. Y., Inoue, T., Leone, V. A., Dalal, S., Touw, K., Wang, Y., Musch, M. W.,
395 Theriault, B., Higuchi, K., Donovan, S., Gilbert, J. & Chang, E. B. 2015 Using
396 corticosteroids to reshape the gut microbiome: implications for inflammatory bowel
397 diseases. *Inflamm Bowel Dis* **21**, 963-72.
- 398 Iwama, G. K., Pickering, A. & Sumpter, J. 2011 *Fish stress and health in aquaculture*:
399 Cambridge University Press.

- 400 Jasarevic, E., Howerton, C. L., Howard, C. D. & Bale, T. L. 2015 Alterations in the Vaginal
401 Microbiome by Maternal Stress Are Associated With Metabolic Reprogramming of
402 the Offspring Gut and Brain. *Endocrinology* **156**, 3265-76.
- 403 Jentsch, H. F., Marz, D. & Kruger, M. 2013 The effects of stress hormones on growth of
404 selected periodontitis related bacteria. *Anaerobe* **24**, 49-54.
- 405 Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K. & Schloss, P. D. 2013
406 Development of a dual-index sequencing strategy and curation pipeline for analyzing
407 amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ*
408 *Microbiol* **79**, 5112-20.
- 409 Kuznetsova, A., Brockhoff, P. B. & Christensen, R. H. B. 2017 lmerTest package: tests in
410 linear mixed effects models. *Journal of Statistical Software* **82**.
- 411 Lopetuso, L. R., Scaldaferri, F., Petito, V. & Gasbarrini, A. 2013 Commensal Clostridia:
412 leading players in the maintenance of gut homeostasis. *Gut pathogens* **5**, 23-23.
- 413 Love, M. I., Huber, W. & Anders, S. 2014 Moderated estimation of fold change and
414 dispersion for RNA-seq data with DESeq2. *Genome Biology* **15**, 550-555.
- 415 Lupica, S. J. & Turner Jr, J. W. 2009 Validation of enzyme-linked immunosorbent assay for
416 measurement of faecal cortisol in fish. *Aquaculture Research* **40**, 437-441.
- 417 Mudd, A. T., Berding, K., Wang, M., Donovan, S. M. & Dilger, R. N. 2017 Serum cortisol
418 mediates the relationship between fecal Ruminococcus and brain N-acetylaspartate
419 in the young pig. *Gut Microbes*, 1-12.
- 420 Muñiz Pedrogo, D. A., Chen, J., Hillmann, B., Jeraldo, P., Al-Ghalith, G., Taneja, V., Davis,
421 J. M., Knights, D., Nelson, H., Faubion, W. A., Raffals, L. & Kashyap, P. C. 2018 An
422 Increased Abundance of Clostridiaceae Characterizes Arthritis in Inflammatory Bowel
423 Disease and Rheumatoid Arthritis: A Cross-sectional Study. *Inflammatory bowel*
424 *diseases*.
- 425 Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.
426 R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E. &
427 Wagner, H. 2017 vegan: Community Ecology Package: [https://CRAN.R-](https://CRAN.R-project.org/package=vegan)
428 [project.org/package=vegan](https://CRAN.R-project.org/package=vegan).
- 429 Petrosus, E., Lay, D., Jr., Silva, E. B. & Eicher, S. D. 2018 Effects of orally administered
430 cortisol and norepinephrine on weanling piglet gut microbial populations and
431 Salmonella passage1. *Journal of Animal Science* **96**, 4543-4551.
- 432 Pikuta, E. V. & Hoover, R. B. 2014 The genus Carnobacterium. In *Lactic Acid Bacteria:*
433 *Biodiversity and Taxonomy* (ed. W. H. Holzapel & B. J. Wood): John Wiley & Sons,
434 Ltd.

- 435 Pottinger, T. G. & Carrick, T. R. 1999 A comparison of plasma glucose and plasma cortisol
436 as selection markers for high and low stress-responsiveness in female rainbow trout.
437 *Aquaculture* **175**, 351-363.
- 438 Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. &
439 Glöckner, F. O. 2013 The SILVA ribosomal RNA gene database project: improved
440 data processing and web-based tools. *Nucleic Acids Research* **41**, D590-D596.
- 441 Rea, K., Dinan, T. G. & Cryan, J. F. 2016 The microbiome: a key regulator of stress and
442 neuroinflammation. *Neurobiology of stress* **4**, 23-33.
- 443 Ringø, E. 2008 The ability of carnobacteria isolated from fish intestine to inhibit growth of fish
444 pathogenic bacteria: a screening study. *Aquaculture Research* **39**, 171-180.
- 445 Ringø, E., Wesmajervi, M. S., Bendiksen, H. R., Berg, A., Olsen, R. E., Johnsen, T.,
446 Mikkelsen, H., Seppola, M., Strøm, E. & Holzapfel, W. 2001 Identification and
447 Characterization of Carnobacteria Isolated from Fish Intestine. *Systematic and*
448 *Applied Microbiology* **24**, 183-191.
- 449 Sadoul, B. & Geffroy, B. 2019 Measuring cortisol, the major stress hormone in fishes.
450 *Journal of Fish Biology*.
- 451 Samaras, A., Dimitroglou, A., Sarropoulou, E., Papaharisis, L., Kottaras, L. & Pavlidis, M.
452 2016 Repeatability of cortisol stress response in the European sea bass
453 (*Dicentrarchus labrax*) and transcription differences between individuals with
454 divergent responses. *Scientific Reports* **6**, 34858.
- 455 Schreck, C. B. & Tort, L. 2016 1 - The Concept of Stress in Fish. In *Fish Physiology*, vol. 35
456 (ed. C. B. Schreck, L. Tort, A. P. Farrell & C. J. Brauner), pp. 1-34: Academic Press.
- 457 Simard, M., Hill, L. A., Underhill, C. M., Keller, B. O., Villanueva, I., Hancock, R. E. &
458 Hammond, G. L. 2014 *Pseudomonas aeruginosa* elastase disrupts the cortisol-
459 binding activity of corticosteroid-binding globulin. *Endocrinology* **155**, 2900-8.
- 460 Sylvain, F.-É., Cheaib, B., Llewellyn, M., Correia, T. G., Fagundes, D. B., Val, A. L. &
461 Derome, N. 2016 pH drop impacts differentially skin and gut microbiota of the
462 Amazonian fish tambaqui (*Colossoma macropomum*). *Scientific Reports* **6**, 32032.
- 463 Teletchea, F. & Fontaine, P. 2014 Levels of domestication in fish: implications for the
464 sustainable future of aquaculture. *Fish and fisheries* **15**, 181-195.
- 465 Tetel, M. J., de Vries, G. J., Melcangi, R. C., Panzica, G. & O'Mahony, S. M. 2018 Steroids,
466 stress and the gut microbiome-brain axis. *Journal of Neuroendocrinology* **30**, e12548.
- 467 Uren Webster, T., Consuegra, S. & Garcia de Leaniz, C. 2019 Early life stress causes
468 lasting impacts on the microbiome of Atlantic salmon. *BioRxiv*.
- 469 Uren Webster, T. M., Consuegra, S., Hitchings, M. & Garcia de Leaniz, C. 2018a Inter-
470 population variation in the Atlantic salmon microbiome reflects environmental and
471 genetic diversity. *Appl Environ Microbiol* **84**, e0061-18.

- 472 Uren Webster, T. M., Rodriguez-Barreto, D., Martin, S. A. M., Van Oosterhout, C., Orozco-
473 terWengel, P., Cable, J., Hamilton, A., Garcia De Leaniz, C. & Consuegra, S. 2018b
474 Contrasting effects of acute and chronic stress on the transcriptome, epigenome, and
475 immune response of Atlantic salmon. *Epigenetics* **13**, 1191-1207.
- 476 Vodicka, M., Ergang, P., Hrnčir, T., Mikulecka, A., Kvapilova, P., Vagnerova, K., Sestakova,
477 B., Fajstova, A., Hermanova, P., Hudcovic, T., Kozakova, H. & Pacha, J. 2018
478 Microbiota affects the expression of genes involved in HPA axis regulation and local
479 metabolism of glucocorticoids in chronic psychosocial stress. *Brain Behav Immun* **73**,
480 615-624.
- 481 Weber, M. F., Poxleitner, G., Hebisch, E., Frey, E. & Opitz, M. 2014 Chemical warfare and
482 survival strategies in bacterial range expansions. *Journal of The Royal Society*
483 *Interface* **11**, 20140172.
- 484 Wu, T., Yang, L., Jiang, J., Ni, Y., Zhu, J., Zheng, X., Wang, Q., Lu, X. & Fu, Z. 2018 Chronic
485 glucocorticoid treatment induced circadian clock disorder leads to lipid metabolism
486 and gut microbiota alterations in rats. *Life sciences* **192**, 173-182.
- 487 Yada, T. & Tort, L. 2016 Stress and disease resistance: immune system and
488 immunoendocrine interactions. In *Fish Physiology*, vol. 35, pp. 365-403: Elsevier.
- 489 Zha, Y., Eiler, A., Johansson, F. & Svanbäck, R. 2018 Effects of predation stress and food
490 ration on perch gut microbiota. *Microbiome* **6**, 28.

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492

493 **Figure Legends**

494

495 **Figure 1.** A) Relation between measured cortisol in the plasma, skin and faeces across
496 individual fish, and B) measured cortisol in the plasma, faeces and skin of Atlantic salmon
497 exposed to confinement stress compared to control fish.

498 **Figure 2.** Relationship between measured cortisol and microbial alpha diversity (Chao1
499 richness and Shannon diversity) in the faeces.

500 **Figure 3.** Genus-level composition of the faecal microbiome, and measured faecal cortisol
501 concentrations in individual fish. (C: control, S: confinement stress).

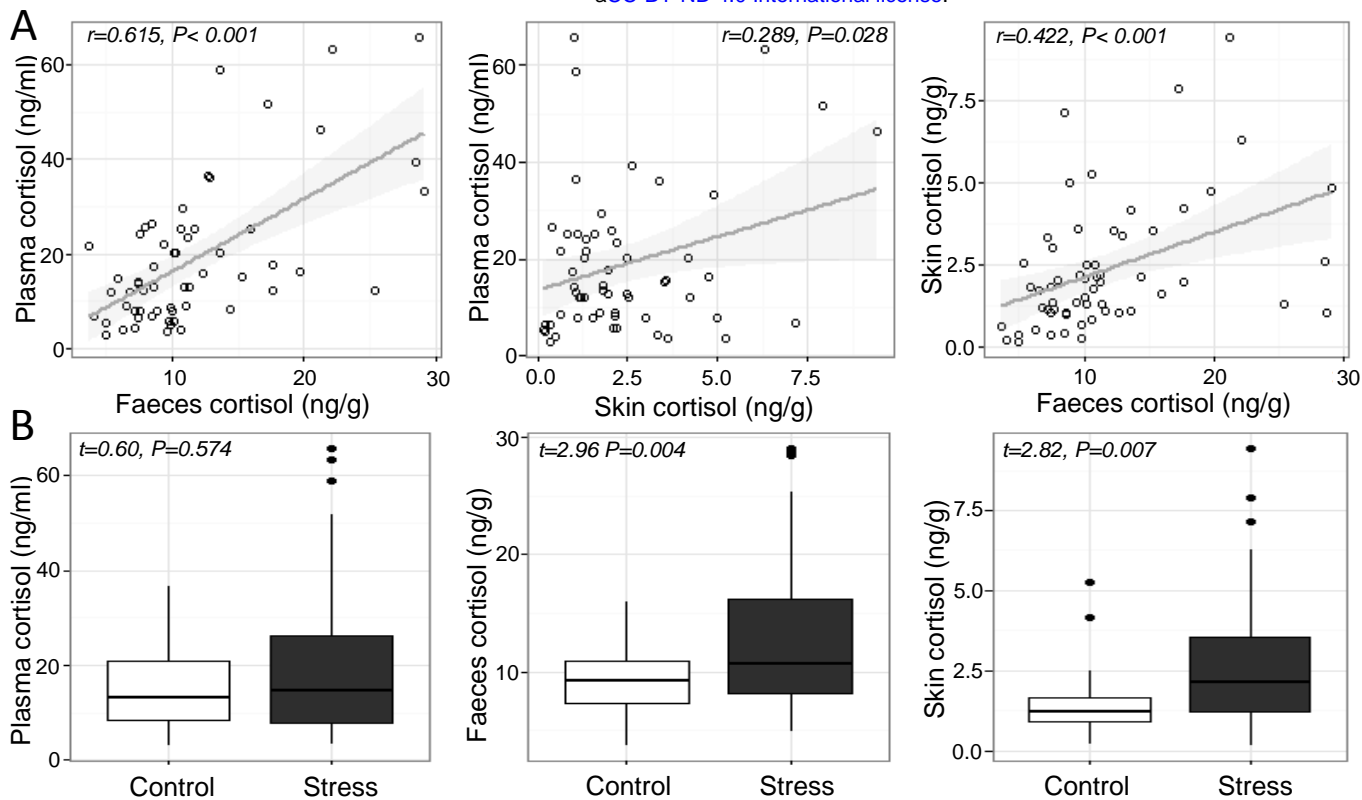


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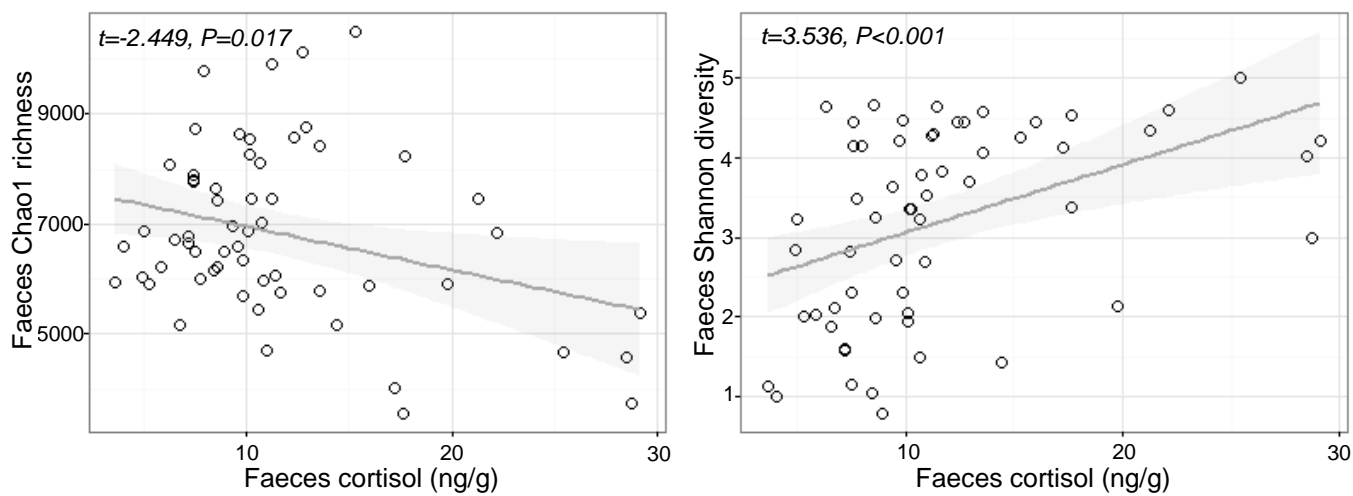


Figure 2. Relationship between measured cortisol and microbial alpha diversity (Chao1 richness and Shannon diversity) in the faeces.

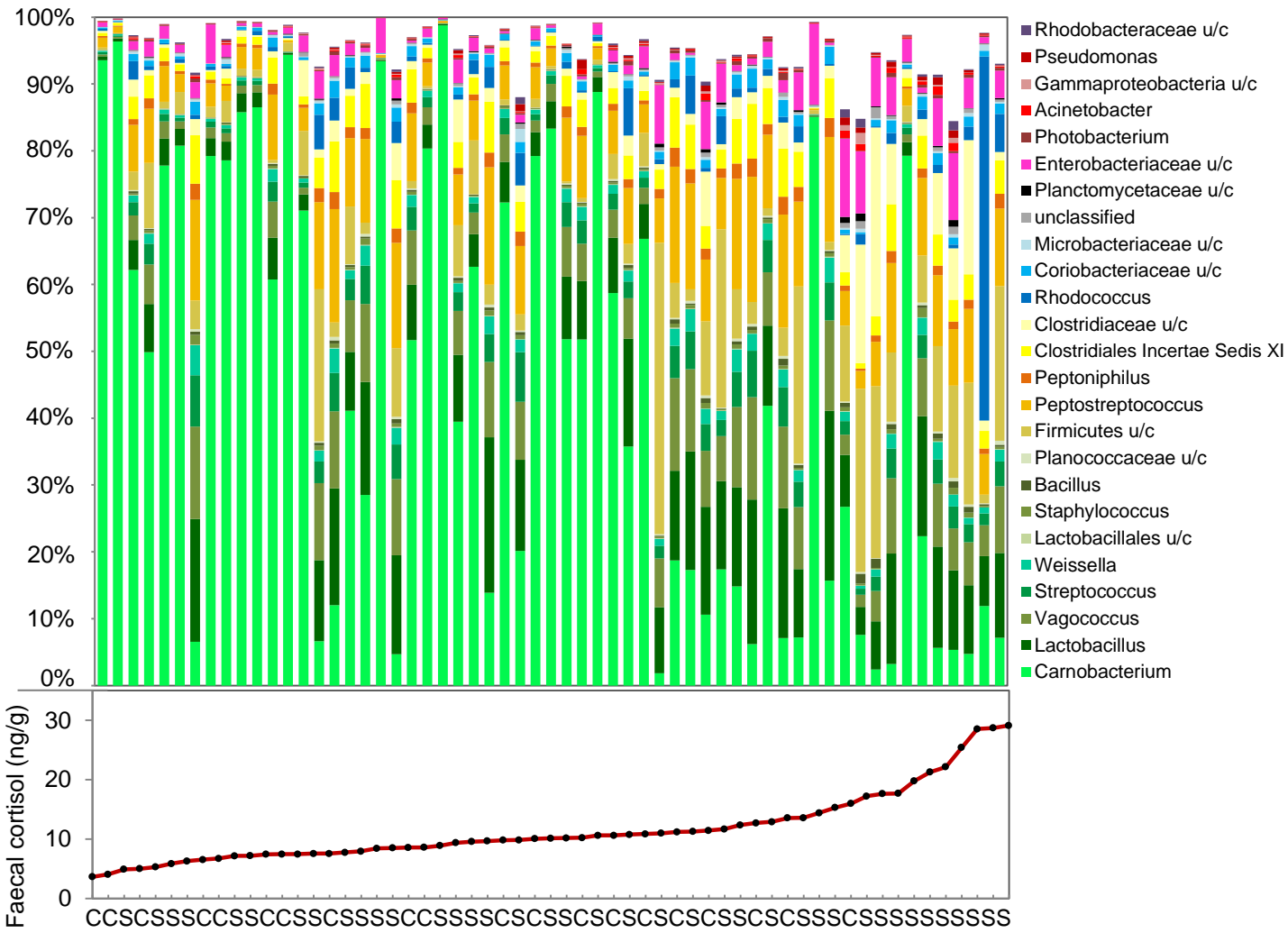


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