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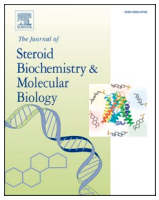

Sampling time for different matrices in stress assessment of farmed Atlantic salmon post-smolt

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ABSTRACT

The sustainability of commercial aquaculture production depends critically on prioritizing fish welfare management. Besides monitoring welfare parameters such as fish behaviour and water quality, fish stress level can also provide a reliable measure of the welfare status of farmed fish. Cortisol and 5 of its metabolites (5β-THF, cortisone, 5β-DHE, 5β-THE, β-cortolone) were previously identified by the authors as suitable stress biomarkers of farmed Atlantic salmon. Based on this knowledge, the present study aimed to investigate the time-related dynamics of these metabolites in plasma, skin mucus, bile and faeces over a 72 h- period. The objective was to determine the optimal sampling time for each matrix and to understand the clearance pathway of these metabolites following stress. An experiment was carried out using a total of 90 Atlantic salmon with an average weight of 438 (±132) g. The average sea temperature was 6.9 °C during the experimental period. A control group of 10 fish was first collected before the remaining 80 fish were submitted to a stress of netting and subsequent relocation into two separate cages. From each of these two stress groups, 10 fish were sampled at 1 h, 2 h, 4 h, 6 h and 12 h, 24 h, 48 h, 72 h after the stress event respectively. The concentrations of cortisol and its metabolites were measured at each of the sampling timepoint. The results demonstrated that plasma cortisol metabolites reached the highest concentration 4 h after stress and remained elevated despite the slight decrease for the remaining timepoints. The peak level was observed at 12 h post-stress in skin mucus and 24 h in bile and faeces. The findings suggest that these timepoints are the optimal for sampling Atlantic salmon post-smolt following stressful events in acute stress studies. Furthermore, the results reveal that analysing cortisol and its metabolites, both in free and conjugated forms, rather than free cortisol provides greater flexibility as their concentrations are less affected by sampling procedure. This study confirms the appropriateness of skin mucus and faeces as less-invasive sample matrices for fish stress evaluation and provides a basis for further developing low invasive tools for monitoring the welfare of farmed salmonid.

1. Introduction

The Atlantic salmon farming industry has grown rapidly since its first successful implementation in Norway in 1970 [1–3]. Over a period of just about five decades from 1970 to 2021, the global Atlantic salmon production has increased by almost 10 000-fold, from 294 to over 2.9 million tonnes live weight [1]. This corresponds to an average annual increase of 22 % [1]. However, the fast growth promoted by extensive salmon prices, successfully managed production, and major technological advances, brought about increasing challenges. These are either related to the marine environment or fish health and welfare and often influenced by production-related operations such as sea lice treatment [4–8]. The challenges cause considerable biomass losses, mainly in the form of fish mortality, which is among the significant problems in salmon aquaculture affecting the economy and reputation of the industry [5,8]. In Norway for example, 62.8 million farmed salmon (corresponding to 16.7 %) were lost to mortality in sea phase in 2023 [7]. A substantial amount of work remains to be done before the salmon farming industry can rightfully be called sustainable. Addressing the welfare issues will contribute to improving fish health which will result

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in lower mortality rate and enhanced growth and quality. Such advancements will exert a positive impact on the production cost and the reputation of the industry and will promote its further growth and competitiveness.

Reproducible and operative fish welfare evaluation techniques are thus important as tools for improving the production throughout the entire fish farming value-chain, from decision-making processes to demonstrating compliance to laws and regulations. Different welfare indicators, either environmental- or animal-based, measured on-site or laboratory-based, were explored and investigated over the years for this purpose. The FISHWELL handbook developed by Noble *et al.* [9] provides a comprehensive overview of the different fish welfare indicators available. It serves as a guideline for fish farmers and a basis for further development of fish welfare monitoring protocols [10].

Free plasma cortisol is one of the most common physiological response markers of stress and welfare in fish [11,12], although one of the limitations in using this hormone is that sampling of blood will severely disturb the fish and instantly increase cortisol level in plasma. Cortisol is known to be quickly metabolized upon release, both into other derivatives and through conjugation. Previous studies showed that these conjugated metabolites were in higher concentrations than the initial free cortisol in different matrices such as blood, bile and faeces [13–16], rendering them more suitable as fish stress biomarkers than free cortisol itself. Based on this knowledge, the present study was conducted to determine the optimal time for sampling four different matrices from fish, namely blood, skin mucus, bile and faeces, following episodes of an acute stress. The mutual correlations between the different compounds and the matrices were also investigated to assess the applicability of the matrices for stress measurement. Furthermore, the evacuation path of cortisol from fish was also addressed to better understand the metabolic clearance of this stress hormone.

2. Materials and methods

2.1. Stress experiment

This study was conducted on farmed Atlantic salmon post-smolt at the NTNU's farming site located in Aalesund municipality (Norway). The farming site, numbered 12265 in the Norwegian aquaculture register, is approved for research purposes by the Norwegian Directorate of Fisheries (approval number M-VS-0016). No further approval was required as the stress experiment was performed using netting and relocation of fish, stress factors normally encountered in a commercial aquaculture setting. The experiment and all samplings were conducted according to the Norwegian Animal Welfare Act.

Fish were transferred to sea in November 2021 and the experiment was carried out from the 22nd to the 25th of March 2022, i.e., 4 months after sea transfer. During the experiment, the Atlantic salmon post-smolt population exhibited an average weight of 438 (±132) g, ranging from 47 to 745 g, and an average fork length of 33 (±4) cm, ranging from 20 to 40 cm. Fish were reared in a large commercial net pen and fed daily with high-protein diet Rapid HP 500 50 A 500 (Cargill, Bergen). Sea temperature at 1.5 m depth was measured daily during the experimental period and averaged 6.9 °C, ranging from 6.8 to 7.0 °C.

2.1.1. Experimental design

On the 22nd of March 2022 at 08:25, a large net was immersed by a boat crane inside a full-scale production net pen (C0) of 39 m diameter and 27 m depth containing a population of about 195 000 Atlantic salmon. Fish were manually fed to attract them into the net. When approximately 100 fish were observed in the net, it was slightly lifted to avoid fish escaping and sampling was started by manually netting the fish, first into an anaesthetic bath of MS 222 (500 mg/mL) onboard of the boat (10 fish) and then to two smaller cages (80 fish). The remaining fish in the net were released back into C0. The first 10 fish were immediately killed by the anaesthetic overdose, aiming to minimize stress during sampling and served as a control (non-stressed) group. The additional 80 fish were divided into two batches of 40 fish each and transferred to the two small experimental cages designated as C1 and C2 respectively. Experimental cages C1 and C2 had approximately 3 m diameter and 2 m depth each and were maintained under the same condition within the large initial full-scale net pen C0. The process of capturing all 90 fish from C0 and either collecting them as control group or transferring them to C1 and C2 were performed as fast as possible (within 5 min) to ensure similar starting point of cortisol production in the stressed fish. The action of netting and relocating fish is assumed to be a stressful condition and is similar to what fish are subjected to during normal farming operations. Fish were divided into two groups to avoid crowding and reduce stress by repeated sampling. Fig. 1 shows a schematic overview of the experimental design.

Fish from cage C1 (Stress group 1) were sampled 1, 2, 4 and 6 h after transfer, corresponding to approximately 09:30, 10:30, 12:30 and 14:30 respectively on the 22nd of March 2022. Fish from cage C2 (Stress group 2) were sampled 12, 24, 48 and 72 h after transfer corresponding to approximately 20:30 (22.03.2022), 08:30 (23.03.2022), 08:30 (24.03.2022) and 09:40 (25.03.2022) respectively. At each sampling timepoint, 10 fish were carefully netted with a dip net from the small cage (C1 and later C2) and immediately euthanized by anaesthetic overdose as previously described. Care was taken to not disturb the remaining fish and minimize stress induced during each sampling. Fish in C1-the first small cage were sampled at tighter time intervals to identify the cortisol peak in blood and thus confirm that a stress response was initiated in the fish. The last sampling point of this group was set at 6 h post-stress as blood cortisol was expected to return to baseline level within 6–8 h post-stress, based on literature [17,18]. Fish in C2-the second experimental cage were kept undisturbed prior to the first sampling time of 12 h post-stress. Although the fish in the smaller cages were fed according to the feeding routine, the decreasing amount of gut content observed suggested that they refused eating (lack appetite) following the acute stress incident and during the three days of experiment.

2.1.2. Sample collection

Before collecting sample matrices, weight and fork length were recorded for each individual fish. The following matrices were then collected: blood, skin mucus, bile, mid-intestine (MI) and distal intestine (DI) contents, hereafter referred to as mid- and distal intestine faeces respectively. Blood from each individual was sampled from the caudal vein of the fish using Vacuette LH lithium heparin tubes (Greiner Bio-One, Austria), and centrifuged on site to collect plasma. Skin mucus was carefully scraped from the lateral surface of the fish with a cell scraper and collected into 15 mL centrifuge tubes. The mucus samples obtained from the 10 individuals at each timepoint were pooled due to too small sample amount from each fish. Previous study showed that sample pooling is feasible and provides results similar to that obtained with individual samples [13]. Bile and mid- and distal intestine faeces were sampled after dissecting the fish. Bile was extracted from the gallbladder with a 1 mL fine dosage syringe Injekt® with needle, while faeces were stripped separately from the respective segments of the intestine. Bile and faeces were stored in individual 2- or 4-mL test tubes, depending on the amount obtained. Samples were kept stored on ice immediately after sampling and frozen at -60 °C upon arrival to the lab and until further treatment and analysis.

2.2. Fish condition factor analysis

Fish condition factor K was calculated using the equation proposed by Barnham and Baxter [19] for salmonid fish, where W is fish weight in gram and L is fish fork length in cm:

$$K = \frac{100 \quad x \quad W}{L^3} \tag{1}$$

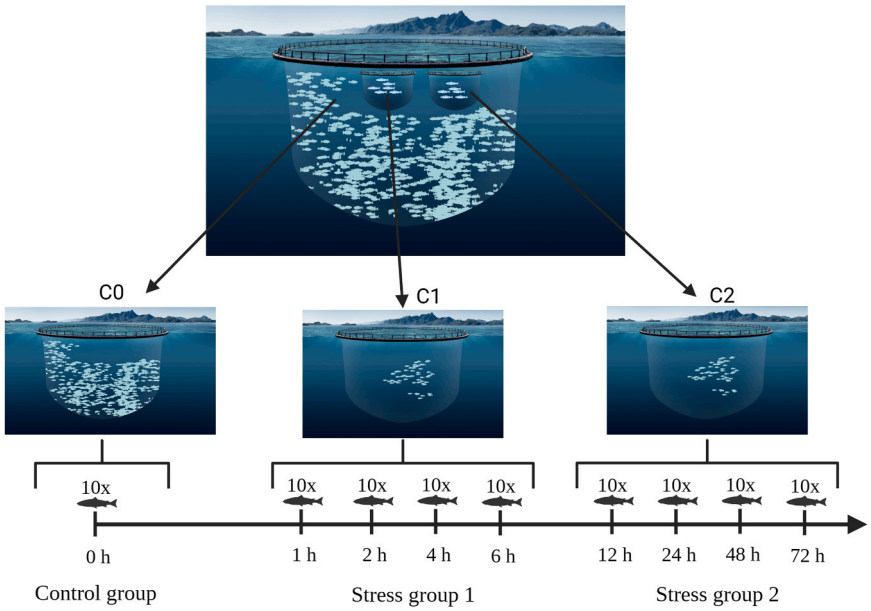

Fig. 1. Overview of the experimental design (created with Spillfree.no and BioRender.com).

2.3. Cortisol metabolites analysis

2.3.1. Chemicals and reagents

Table 1 shows the cortisol metabolites reference materials as well as the deuterated internal standards (IS) used in the present study. The reference materials were purchased from Steraloids (Newport, Rhode Island, USA) and the internal standards from Merck (Darmstadt, Germany). The enzyme β-glucuronidase (Type H-1 from *Helix pomatia*) was also obtained from Merck (Darmstadt, Germany). Methanol, formic acid and ammonium formate of LC-MS grades, sodium acetate trihydrate and glacial acetic acid of analytical grades, and *tert*-butyl methyl ether (TBME) of HPLC grade were supplied by VWR International (Oslo, Norway). Ultrapure water was produced in-house from an Omnia water system equipped with inline 0.2 μm-filter (Stakpure, Niederahr, Germany).

2.3.2. Sample preparation

The sample preparation method used in the present study was developed and validated in a previous study [13]. Briefly, about 100 mg raw faeces or 100 μL bile or plasma were firstly diluted with 1900 μL deionized water and centrifuged. The supernatant (100 μL for bile or 200 μL for plasma and faeces) was then subjected to hydrolysis with β-glucuronidase from *Helix pomatia* for 1 h at 55 °C and free cortisol metabolites extracted with 1 mL TBME. Liquid-liquid extraction of 200 μL skin mucus with 1 mL TBME was performed without hydrolysis. TBME extracts were dried at 40 °C under nitrogen gas flow and recovered in a solution of 15 % methanol for analysis with LC-MS/MS. Samples were prepared in duplicates and each replicate was analysed twice with the LC-MS/MS method.

2.3.3. Analytical method

The LC-MS/MS system used in the present study consisted of an Agilent 1200 series UPLC (Agilent, Santa Clara, California, USA) coupled to a high-resolution Compact Q-TOF MS with ESI source from Bruker (Billerica, Massachusetts, USA). An Acquity BEH C18 column (75 mm × 2.1 mm i.d., 1.7 μm) from Waters Associates, thermostated at 55 °C, was used for chromatographic separation. The latter was run with the following gradient, adapted from the work of Marcos *et al.* [20]: from 0 to 0.25 min: 15 % eluent B; 0.25–1.5 min: 15–40 % B; 1.5–9.5 min: 40–65 % B, 9.5–10 min: 65–90 % B, 10–11 min: 90 % B and 11.01–15 min: 15 % B for column conditioning. A solution of 0.01 % (v/v) formic acid and 1 mM ammonium formate was added to both eluents A (water) and B (methanol). The mobile phase was eluted at a flow rate of 0.3 mL/min and 5 μL sample was injected for analysis. Cortisol metabolites were detected in the positive ionization mode and scanned through multiple reaction monitoring (MRM) mode. The optimized parameters for the MRM method are presented in Table 2, and for the detection as follows: 500 V end plate offset, 4.5 kV capillary voltage, 2 bar nebulizer gas pressure, 9.0 L/min dry gas flow rate and 220 °C source temperature. Nebulizing and drying gas was nitrogen. Bruker's Compass QuantAnalysis software was used for quantitative analysis of the data.

Table 1
Nomenclature, abbreviation and formula of the analytes.

Trivial Name	Systematic name	Abbreviation	Formula
Reference materials			
Cortisol	11β,17,21-trihydroxypregn-4-ene-3,20-dione	F	$C_{21}H_{30}O_5$
Tetrahydrocortisol	3α,11β,17,21tetrahydroxy-5β-pregnan-20-one	5β-THF	$C_{21}H_{34}O_5$
Cortisone	17,21-dihydroxypregn-4-ene-3,11,20-trione	E	$C_{21}H_{28}O_5$
Dihydrocortisone	17,21-dihydroxy-5β-pregnane-3,11,20-trione	5β-DHE	$C_{21}H_{30}O_5$
Tetrahydrocortisone	3α, 17,21-trihydroxy-5β-pregnane-11,20-dione	5β-THE	$C_{21}H_{32}O_5$
β-Cortolone	3α,17,20β,21-tetrahydroxy-5β-pregnan-11-one	-	$C_{21}H_{34}O_5$
Deuterated internal standards (IS)			
Cortisol-9,11,12,12-d_4	11β,17,21-trihydroxypregn-4-ene-3,20-dione-D_4	F-d_4	$C_{21}D_4H_{26}O_5$
Tetrahydrocortisone-2,2,3,4,4-d_5	3α, 17,21-trihydroxy-5β-pregnane-11,20-dione -D_5	THE-d_5	$C_{21}D_5H_{27}O_5$

Table 2
Optimized MRM parameters for each reference material and IS.

Compound	MW g/mol	RT min	Precursor ion *m/z*	Species	CE eV	Product ions *m/z*		Ion ratio
Reference materials								
F	362	6.1	363	$[M+H]^+$	25	121	97	4.5
5β-THF	366	7.8	384	$[M+NH_4]^+$	15	313	301	2.2
E	360	5.7	361	$[M+H]^+$	30	163	121	6.1
5β-DHE	362	7.3	380	$[M+NH_4]^+$	20	315	327	1.3
5β-THE	364	8.2	382	$[M+NH_4]^+$	20	329	347	1.9
β-Cortolone	366	8.1	384	$[M+NH_4]^+$	20	331	271	4.9
Deuterated internal standards (IS)								
F-d_4	366	6.1	367	$[M+H]^+$	25	121		
5β-THE-d_5	370	8.2	387	$[M+NH_4]^+$	15	352		

2.3.4. Cortisol metabolites ratios

In humans, the activity of the enzyme converting cortisol to cortisone (11βHSD2) is usually inferred from the ratio between cortisol and cortisone [21]. Similarly, the ratios between 5β-THF and cortisol and 5β-THE and cortisone indicate the activity of the enzyme converting cortisol and cortisone to 5β-THF and 5β-THE respectively (5β-reductase) [21]. These ratios were also evaluated in the present study as they are assumed to reflect similar enzyme activities in fish. Furthermore, the ratios of the concentrations of the same metabolite in the different matrices were also calculated to assess the excretion pathway of the metabolite of interest in farmed Atlantic salmon following stress.

2.4. Statistical analysis

Results are presented as mean ± standard error (SEM). Model diagnostics were performed by first checking for the normality and the equal variances assumptions using Shapiro-Wilk and Levene's tests respectively. If the assumptions were met, as was the case of plasma cortisol and cortisone after excluding one extreme value from the control group, one-way analysis of variance (ANOVA) was performed to determine the differences between the means. Otherwise, as was the case for bile and faeces cortisol metabolites, Log_{10}-transformation of the data was used to stabilize the variance prior to ANOVA. When a significant difference was observed, Dunnett's test was performed to compare the stress groups to the control group. This approach was adopted as recommended by Yossa and Verdegem [22], considering fish stress status at each sampling timepoint as a qualitative independent variable without structure. Based on the suggestion of Rosner [23], non-parametric tests (Kruskal-Wallis) were also performed on the original, untransformed data and compared with the results from the parametric tests to strengthen the conclusions obtained.

For the correlation studies, Spearman rank-correlation (ρ) test was performed on the original data. The strength of association between the two variables studied was interpreted using the guidelines provided by Nolan and Heinzen [24] in which a correlation coefficient between 0.1 and 0.3 is considered small, between 0.3 and 0.5 medium and higher than 0.5 large.

All statistical analyses were carried out using IBM SPSS Statistics version 28.0.1.0 and graphs were plotted using OriginPro 2023b. Results were considered statistically significant when $p < 0.05$.

3. Results and discussion

3.1. Dynamics of cortisol metabolites concentrations following stress

In plasma and skin mucus, only cortisol and cortisone were identified whereas cortisol, 5β-THF, cortisone, 5β-DHE, 5β-THE and β-cortolone were detected in bile and faeces. Cortisol and cortisone were found in free form in skin mucus but mainly in conjugated form in plasma. Similarly, cortisol, 5β-THF, cortisone, 5β-DHE, 5β-THE and β-cortolone were also mainly found in conjugated form in bile and faeces (unpublished data).

3.1.1. Plasma

As shown in Fig. 2 and Table S1b, mean plasma cortisol for the control group (pre acute stressing) measured as baseline level was 72.68 ±4.96 ng/mL. Data obtained from the first stress group show that plasma cortisol concentration significantly increased to 172.44 ±4.64 ng/mL ($p < 0.001$, Table S2) 1 h post-stress and reached a maximal value of 177.26 ±11.78 ng/mL ($p < 0.001$, Table S2) after 4 h. Plasma cortisol concentration for the second stress group declined gradually towards the end of the experiment but remained significantly higher than that of the control group.

Mean baseline concentration of 8.40 ±1.06 ng/mL was measured for plasma cortisone in the control group (Fig. 2, Table S1b). It increased gradually in the first stress group and reached a peak value of 50.05 ±3.56 ng/mL ($p < 0.001$, Table S2) at 12 h in the second stress group. The concentration then declined slowly towards the end of the experiment but remained significantly higher than that of the control group.

Experiments conducted on Atlantic salmon exposed to acute stress showed that plasma cortisol reached a peak value within 20 min to 1 h post-stress which remained high up to 3 h, before declining to almost resting level already 6 h after the onset of stress [17,18]. The results obtained in the present study were more comparable to those found in fish subjected to continuous/chronic or repeated stress. Carbajal *et al.* [25] and Pottinger and Moran [26] found for instance that plasma cortisol concentrations in rainbow trout confined at high-density peaked at 45 min to 1 h post-stress and did not resume to baseline level even after 24 h and 9 days respectively. Although a one-time stress event was introduced at the beginning of the present experiment and care was taken to minimize disturbance during sampling, it cannot be excluded that the regular sampling of fish in the small cages resulted in a delayed return to baseline level. The high levels of metabolites observed 12 h post-stress (corresponding to 20:30 in the evening) can be attributed to the circadian rhythm (diurnal variation) of cortisol which naturally increases during the night and decreases during the day [27]. Another possible explanation may be the size of the small cages. Relocating fish to a smaller cage has been demonstrated to cause stress to fish even though biomass density does not increase. Nevertheless, the measurement of both free and conjugated compounds could also be a possible explanation. While free cortisol concentration may have returned to initial value at some point, cortisol metabolism and conjugation still continued and were reflected by the detection of a prolonged high concentration of conjugated cortisol in plasma. Similar observation was made by Pottinger *et al.* [16] in the bile of rainbow trout exposed to sampling stress. Even though both free and conjugated bile steroids increased to peak levels 24 h after the onset of stress, the former returned to normal levels within 48 h while the latter remained high through the end of the experiment (72 h post-stress). This suggests that fish stress could be more accurately evaluated in plasma by measuring total cortisol (conjugated + free) rather than solely free as the concentration of the former is less affected by sampling procedure and can be

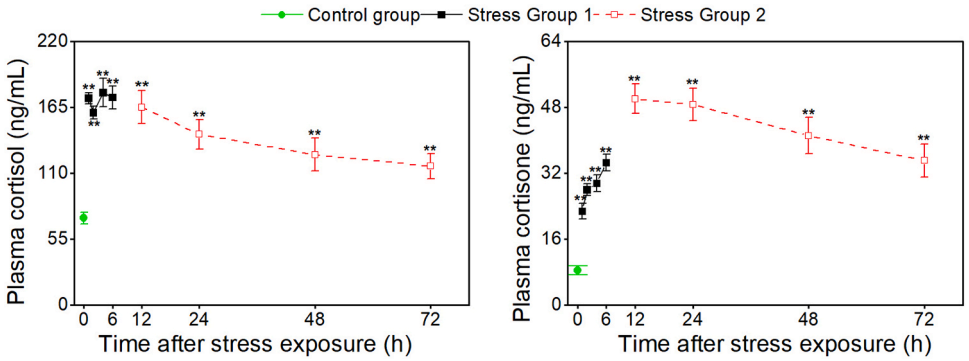

Fig. 2. Dynamics of cortisol and cortisone concentrations in Atlantic salmon plasma following acute handling stress. Each datapoint represents the Mean (±SEM) from 10 individuals except for the control group which was from 9 individuals. Fish from Stress group 1 were sampled at 1, 2, 4 and 6 h post-stress and Stress group 2 at 12, 24, 48 and 72 h.

detected over a longer period.

The plasma cortisone dynamics post-stress observed in the present study is similar to that found by Patiño *et al.* [28] in juvenile coho salmon and Pottinger and Moran [26] in rainbow trout. It is however worth noting that Pottinger and Moran [26] observed first a quick and sharp rise (peak) and then a drop of cortisone concentration from 10 to 20 min post-stress. This was not observed in the present study as no sampling was performed before 1 h post-stress. Assuming the early peak and decline have also occurred in the present experiment, the observed increase after 1 h through 12–24 h is a result of conjugated cortisone progressively released into the blood.

3.1.2. Skin mucus

In skin mucus, baseline levels of cortisol and cortisone were 3.88 and 0.30 ng/mL respectively (Fig. 3, Table S1b). Cortisol and cortisone concentrations increased gradually and reached highest values at 12 h (27.82 ng/mL) and 24 h (15.41 ng/mL) post-stress respectively before decreasing towards the end of the experiment. A comparison of means for control and stress groups was not performed since pooled samples were used for skin mucus.

The time lag in cortisol peaks between plasma and skin mucus observed in the present study is comparable to the results obtained on gilthead seabream *Sparus aurata* by Guardiola *et al.* [29]. It may correspond to the time needed for plasma cortisol to enter skin mucus through the secondary circulatory system as this connects the internal and external body surfaces of fish [30,31]. The time lag is an advantage in fish stress evaluation as cortisol peak in skin mucus is delayed and cortisol levels are not affected by sampling procedure. However, it may also be species-dependent since Franco Martínez *et al.* [32] observed similar timepoint (at 1 h) for cortisol peaks in plasma and skin mucus of rainbow trout.

3.1.3. Bile

Bile cortisol metabolites concentrations were increasing from the onset of stress until 24 h, at which peak values were reached (Fig. 4, Table S1b). From this timepoint and through the end of the experiment, the concentrations remained stable at the peak level. Based on the analysis of Log_{10}-transformed data, a significant difference from the baseline levels was already detected at 1 h post-stress for most of the compounds. However, the concentrations slightly decreased at 2 h before increasing and reaching statistically significant values again at 4 h for the first stress group. The concentrations were significantly higher than the baseline levels at all measured timepoints from 12 h, for all compounds except β-cortolone (Fig. 4, Table S2). The latter was first detected 6 h post-stress, the values prior to this timepoint were below the detection limit of the method (<LOD). As a result, a comparison of the stress groups to the control group was not feasible. However, a similar pattern as the other metabolites was also observed. The peak concentration was measured at 24 h post-stress and from this timepoint β-cortolone concentration remained high.

These results are in line with those found by Pottinger *et al.* [16] in the bile of stressed rainbow trout. The conjugated bile corticoids reached significantly elevated concentrations from 4 hours post-stress and continued to rise towards the end of the experiment. Trials on injection of radioactive cortisol in rainbow trout showed that 10 % and 25 % of the injected dose were detected in bile after 4 and 24 h respectively [15]. Likewise, Scott *et al.* [14] found 30 % of the injected dose in the bile of Atlantic cod after 22 h. This delay in cortisol metabolites accumulation in bile makes this matrix especially suitable for welfare evaluation in dead or slaughtered fish.

3.1.4. Faeces

Similar pattern was observed for cortisol metabolites concentrations

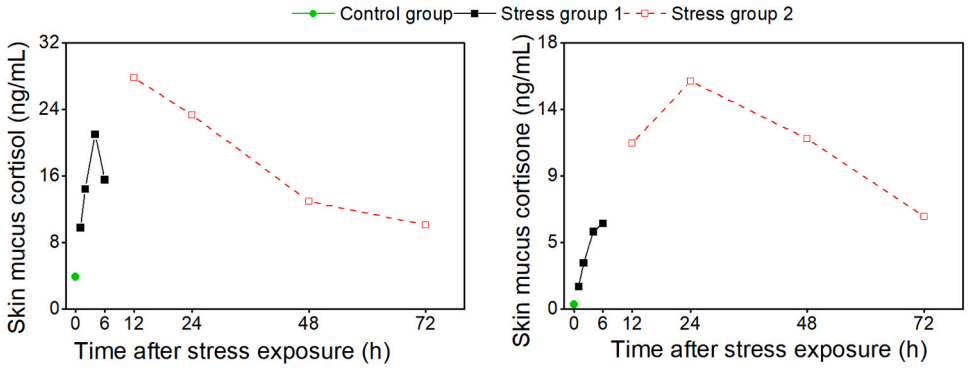

Fig. 3. Changes of skin mucus cortisol and cortisone concentrations over time following acute handling stress. Each datapoint was obtained from the measurement of pooled samples from 10 individuals. Fish from Stress group 1 were sampled at 1, 2, 4 and 6 h post-stress and Stress group 2 at 12, 24, 48 and 72 h.

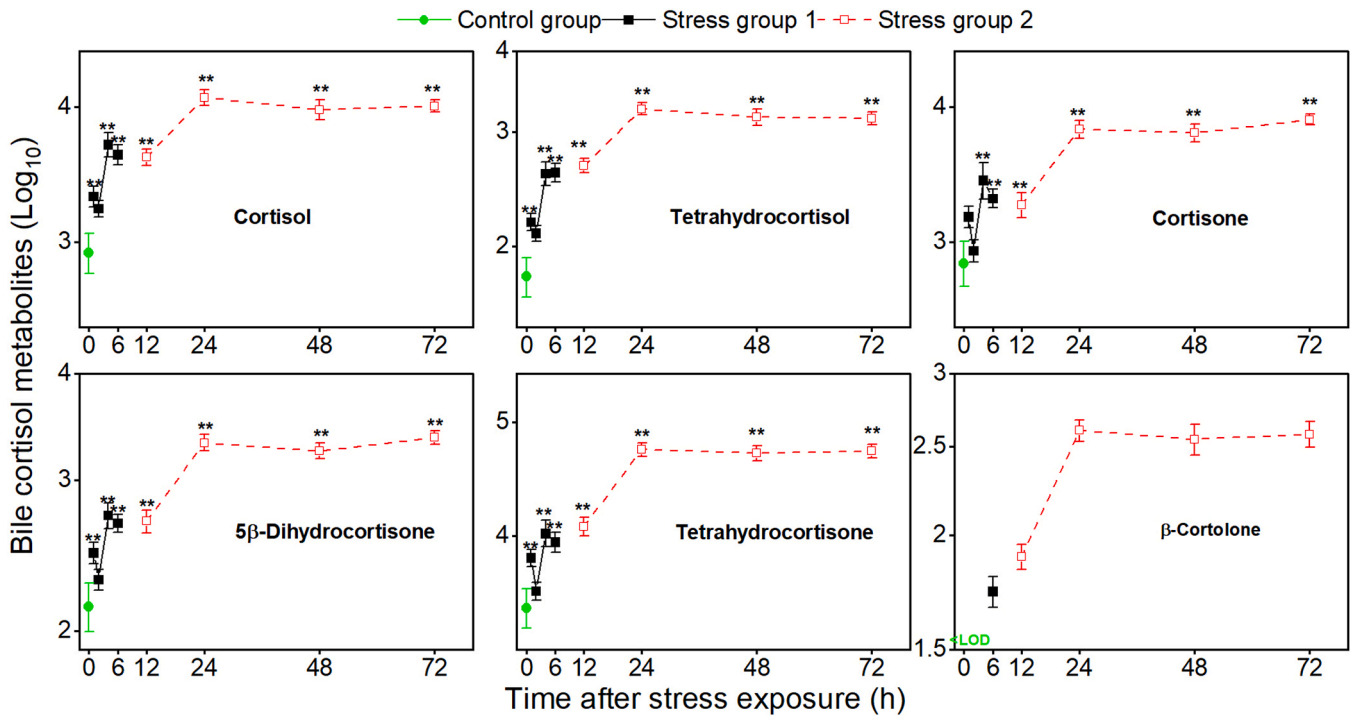

Fig. 4. Dynamics of cortisol metabolites concentrations in the bile of Atlantic salmon following acute handling stress. Each datapoint represents the Mean (±SEM) of Log_{10}-transformed values from 8 to 10 fish. Fish from Stress group 1 were sampled at 1, 2, 4 and 6 h post-stress and Stress group 2 at 12, 24, 48 and 72 h. β-Cortolone values in control group were below the limit of detection of the method (<LOD).

in both mid- and distal intestine faeces (Fig. 5, Table S1b). The compounds increased in concentration from the onset of stress to 6 and 24 h for the first and second stress groups respectively. After reaching the peak value at 24 h, the concentrations decreased until 48 h but slightly increased again towards the end of the experiment.

For cortisol and 5β-THF, a significant difference to the control group was already detected 1 to 6 h post-stress (Table S2). No statistically significant difference to the control group was observed for cortisone and 5β-DHE in the two stress groups. However, at 24 h the concentrations were still higher than the baseline levels. A significant difference to

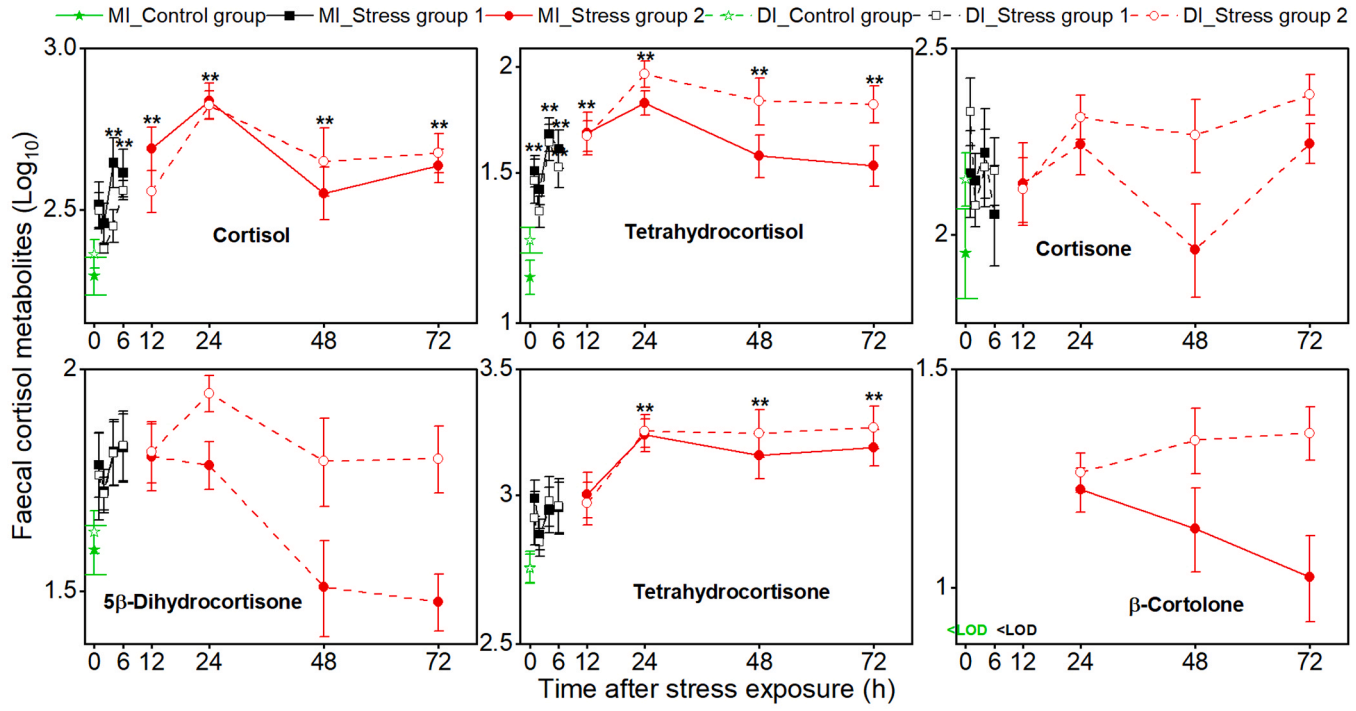

Fig. 5. Time-course of the changes of cortisol metabolites concentrations in Atlantic salmon faeces (MI: mid-intestine and DI: distal intestine) subjected to acute stress. Each datapoint represents the Mean (±SEM) of Log_{10}-transformed values from 10 fish. Fish from Stress group 1 were sampled at 1, 2, 4 and 6 h post-stress and Stress group 2 at 12, 24, 48 and 72 h. β-Cortolone values in Control group and Stress group 1 were below the limit of detection of the method (<LOD).

the control group was first observed from 24 h for 5β-THE. This was also the same timepoint β-cortolone was first detected in faeces.

High mean faecal cortisol value of about 200 ng/g was measured in the control group in the present study (Table S1a) while faecal cortisol values below 30 ng/g are usually reported in non-stressed Atlantic salmon [33,34], European sea bass [35,36] and rainbow trout [36]. This difference is assumed to result from the methodological approach used. Only free cortisol was most likely extracted and measured in these studies and not total (conjugated + free) as in the present study. Faecal cortisol concentration as high as 437 ±302 ng/g was also observed in Atlantic salmon sampled four days after sea transfer in which both free and conjugated cortisol were measured [37]. Furthermore, it must also be pointed out that, although we got a clear stress effect and measured high levels of cortisol metabolites, our enzyme hydrolysis system for plasma, bile and faeces was undoubtedly still not perfect and may have missed a lot of the metabolites. Truscott [15] and Scott *et al.* [14] showed that about 95% of radioactive cortisol metabolites in the bile are sulphates (as opposed to glucuronides) and both authors also showed that only about 20 % of the radioactive metabolites were susceptible to digestion by snail sulfatase. Scott *et al.* [14] showed that one of the steroids that was present in cod bile and yet was not susceptible to enzymatic digestion was 20β-cortolone that was sulphated via the 20β-hydroxyl group. This steroid could, however, as originally found with the 20β-sulphate of the fish maturation-inducing steroid, 17, 20β-dihydroxy-4-pregnen-3-one [38], be freed by acid solvolysis. This will need to be taken into account in future studies. Yet another observation that will need to be taken into account is that the effectiveness of acid solvolysis is highly influenced by the amount of bile in the reaction tubes [14]. With too much bile, the reaction will not take place. This was also observed with the enzymatic hydrolysis used in the present study where samples diluted 20 times prior to hydrolysis resulted in higher free metabolites yield than undiluted samples [13]. The observation of high cortisol metabolites concentrations at 48 and 72 h can be explained by the time the metabolites need to be completely cleared from fish. In the study of Meling *et al.* [37] for instance, cortisol level did not return to normal values before 7 days post-stress.

3.2. Comparison of cortisol metabolites concentrations

Correlation data for the two major metabolites in each sample matrix are presented in this section: cortisol and cortisone for skin mucus and plasma, and cortisol and 5β-THE for bile and faeces. Data for the other compounds are shown in Table S3 of the Supplementary material. Spearman rank-correlation coefficient (ρ) was computed using data from pooled samples for skin mucus and individual samples for plasma, bile and faeces.

3.2.1. Skin mucus vs. other matrices

Skin mucus cortisol and cortisone had higher and positive correlation with plasma cortisone than cortisol ($\rho \geq 0.72$, $p \leq 0.03$ for plasma cortisone vs. $\rho \leq 0.45$, $p \geq 0.22$ for plasma cortisol, Fig. 6). Bile cortisol metabolites had higher correlation with skin mucus cortisol (Fig. 7) and cortisone (Fig. 8), followed by mid- and distal intestine faecal cortisol metabolites respectively. Furthermore, skin mucus cortisone had higher and mainly significant correlation with bile and faecal cortisol metabolites compared to cortisol.

3.2.2. Plasma vs. bile and faeces

No to negligible correlations ($\rho \leq \pm 0.1$) were observed between plasma cortisol and bile cortisol metabolites. Low to moderate correlations ($\rho \leq \pm 0.5$) were obtained between plasma cortisol and faecal cortisol metabolites (Fig. 9). Plasma cortisone, on the other hand, had medium to high correlations ($\rho \geq 0.3$) with both bile and faecal cortisol metabolites (Fig. 10).

Although other studies found a high and positive correlation between cortisol in plasma and other matrices such as faeces [33–35], the

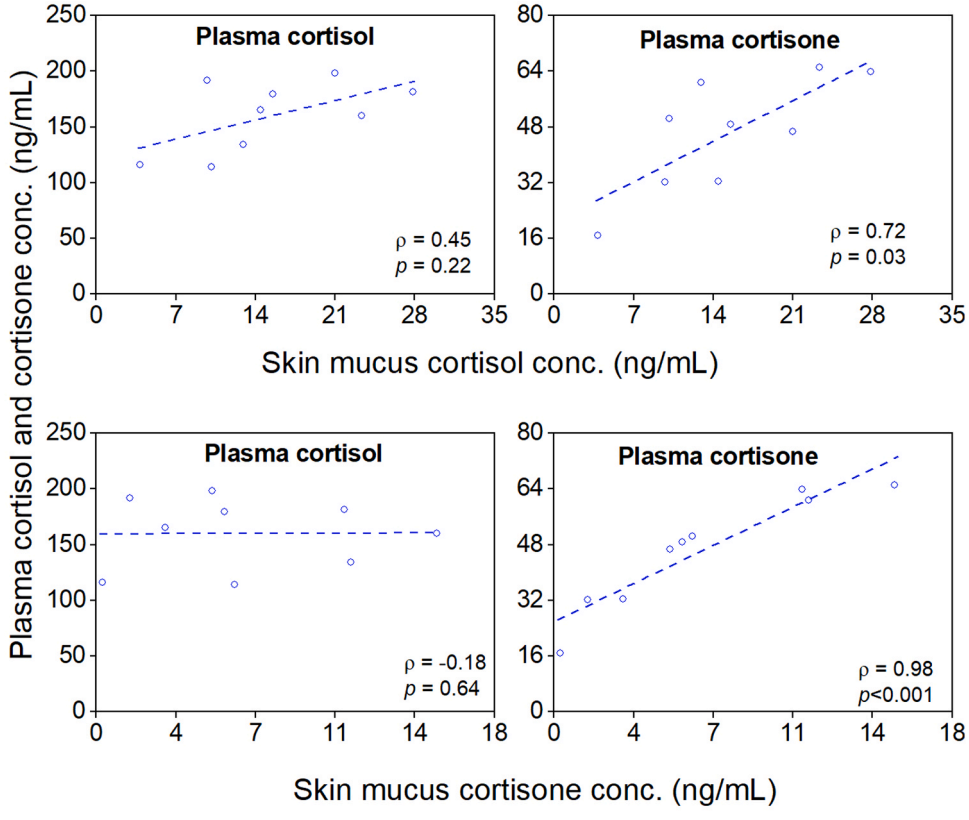

Fig. 6. Correlation between skin mucus cortisol and cortisone and plasma cortisol metabolites.

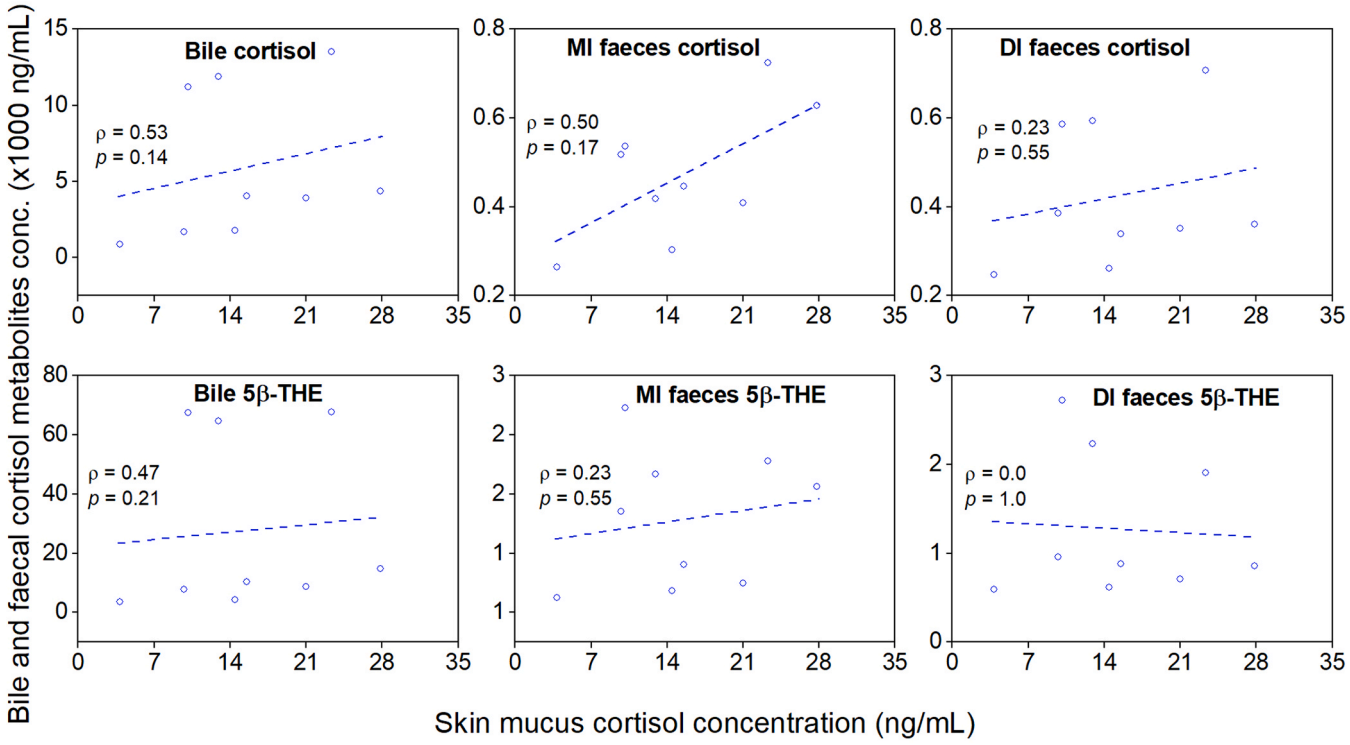

Fig. 7. Correlation between skin mucus cortisol and bile and faecal cortisol metabolites (MI: mid-intestine, DI: distal intestine, 5β-THE: Tetrahydrocortisone).

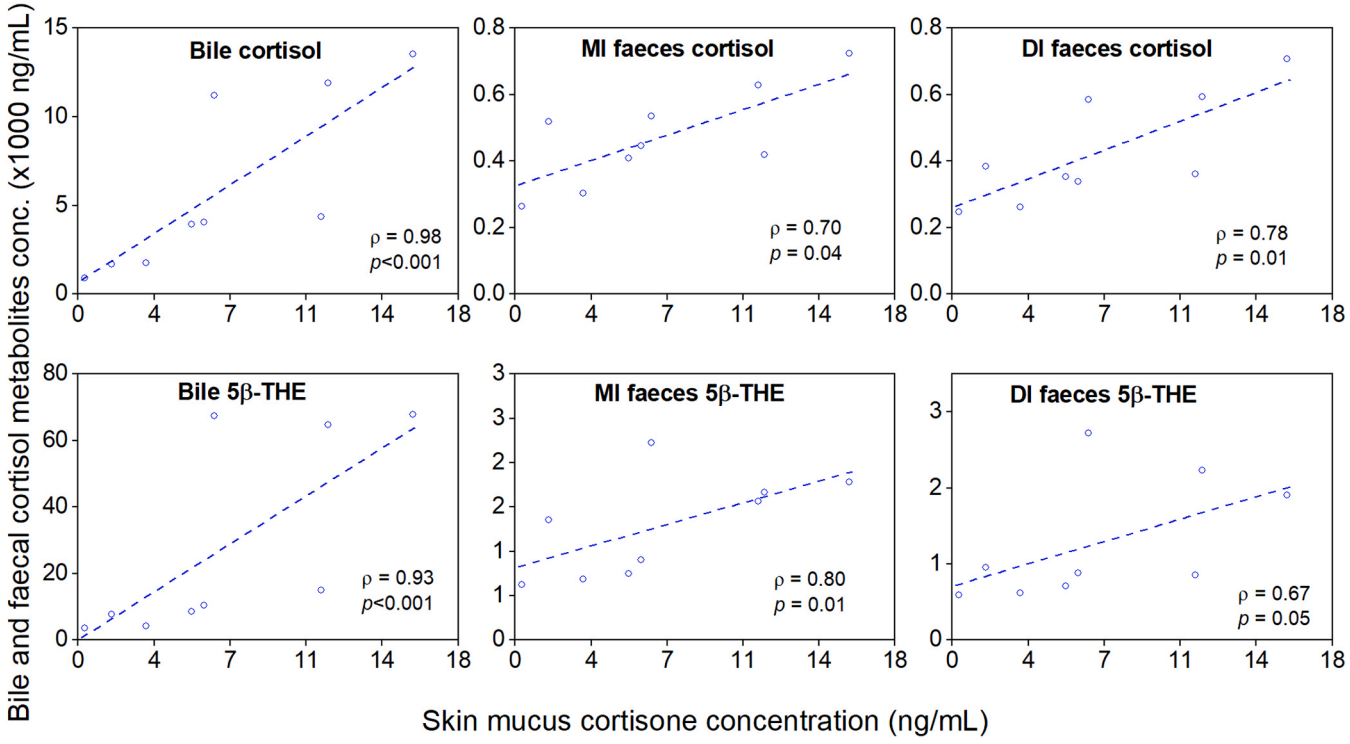

Fig. 8. Correlation between skin mucus cortisone and bile and faecal cortisol metabolites (MI: mid-intestine, DI: distal intestine, 5β-THE: Tetrahydrocortisone).

results obtained in the present study are more in agreement with the observations of Bertotto *et al.* [36]. No relationship between cortisol in plasma and gut content from European sea bass *Dicentrarchus labrax* subjected to 3 h transport stress (Pearson's r = 0.1, $p = 0.8$) was found by the authors. Whereas high correlations were observed between plasma and skin mucus cortisol of the same individuals (r = 0.63, $p < 0.05$). Nevertheless, the high correlation between plasma cortisone and cortisol metabolites in skin mucus, bile and faeces support the use of these matrices as alternative to blood.

3.2.3. *Bile vs. faeces*

As illustrated in Fig. 11 and Table S3b, the concentrations of the

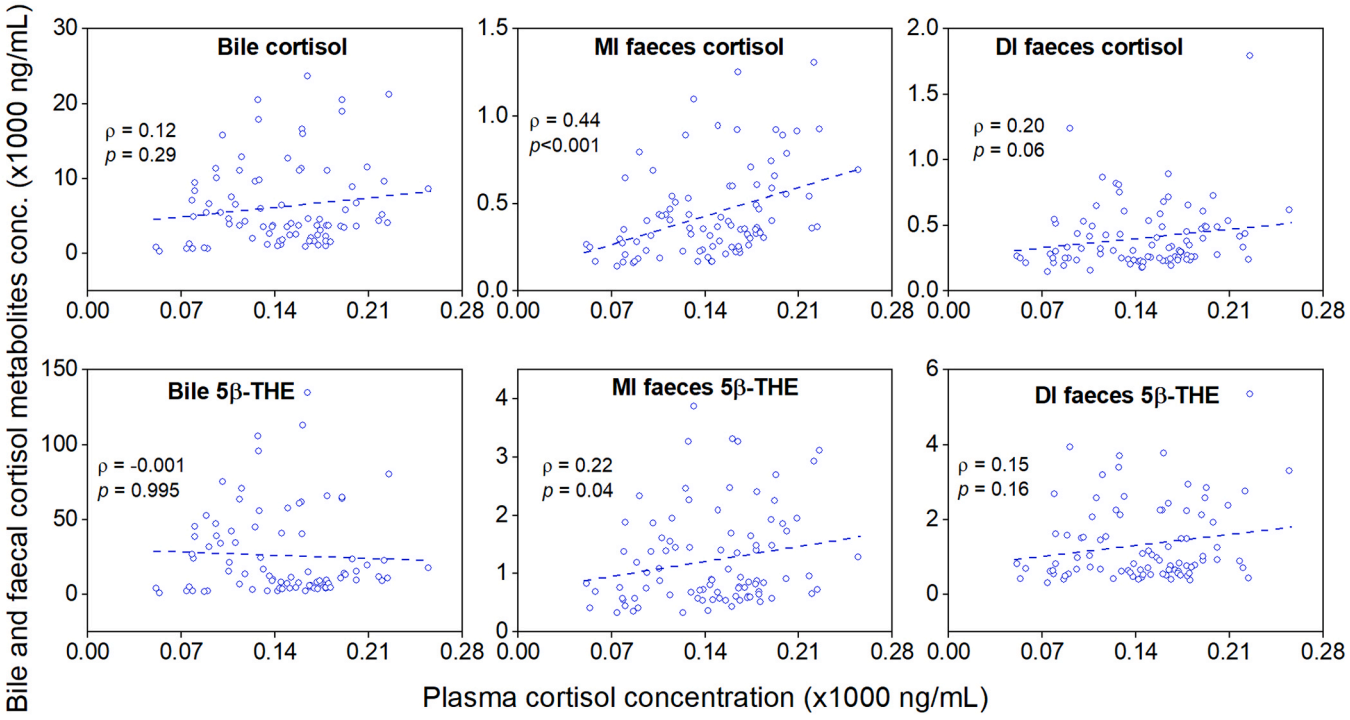

Fig. 9. Correlation between plasma cortisol and cortisol metabolites in bile and faeces (MI: mid-intestine, DI: distal intestine, 5β-THE: Tetrahydrocortisone).

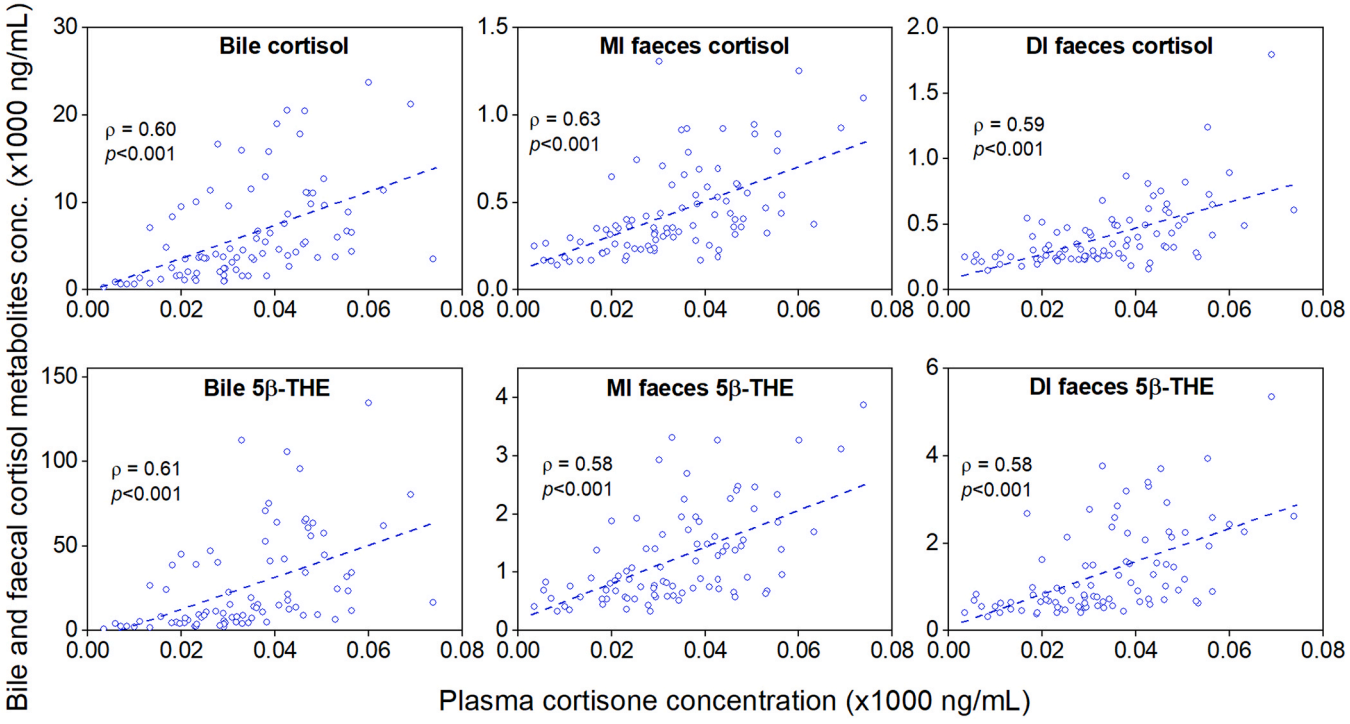

Fig. 10. Correlation between plasma cortisone and cortisol metabolites in bile and faeces (MI: mid-intestine, DI: distal intestine, 5β-THE: Tetrahydrocortisone).

same cortisol metabolite in the three sample matrices, bile, and mid- and distal intestine faeces, correlated well ($\rho \geq 0.5$). The exception was cortisone from mid-intestine faeces and faecal 5β-DHE which had low to moderate correlation with bile cortisone and 5β-DHE respectively ($\rho \leq 0.4$).

3.2.4. Cortisol vs. cortisol metabolites from the same sample matrix

As shown in Fig. 12, skin mucus cortisol had high but not significant correlation with skin mucus cortisone ($\rho = 0.63$, $p = 0.07$). Plasma cortisol displayed a moderate significant correlation with cortisone ($\rho = 0.35$, $p = 0.001$). Except for β-cortolone, bile and faecal cortisol correlated well with the other metabolites ($\rho \geq 0.7$, $p < 0.001$).

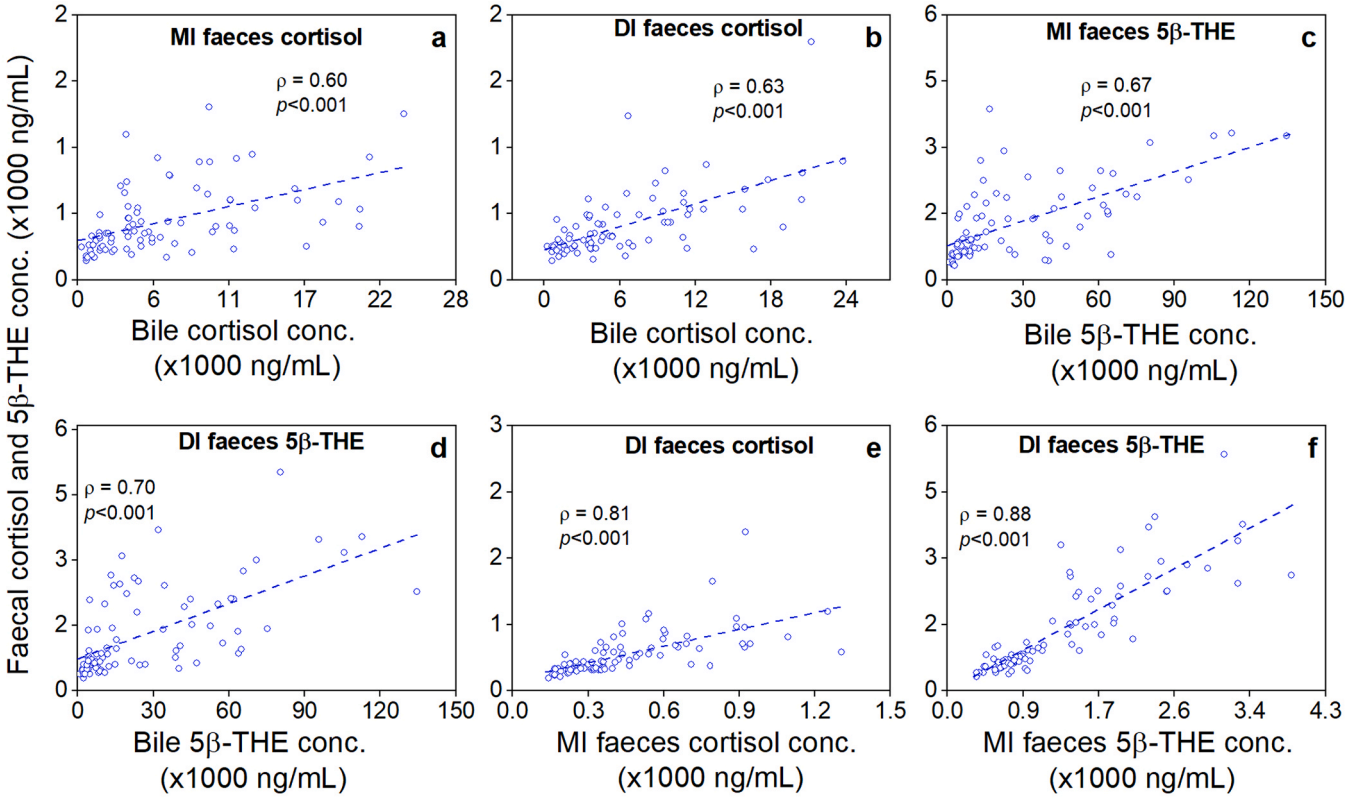

Fig. 11. Correlation between bile and faecal cortisol metabolites (a-d) and mid- and distal faecal cortisol metabolites (e-f). MI: mid-intestine, DI: distal intestine, 5β-THE: Tetrahydrocortisone.

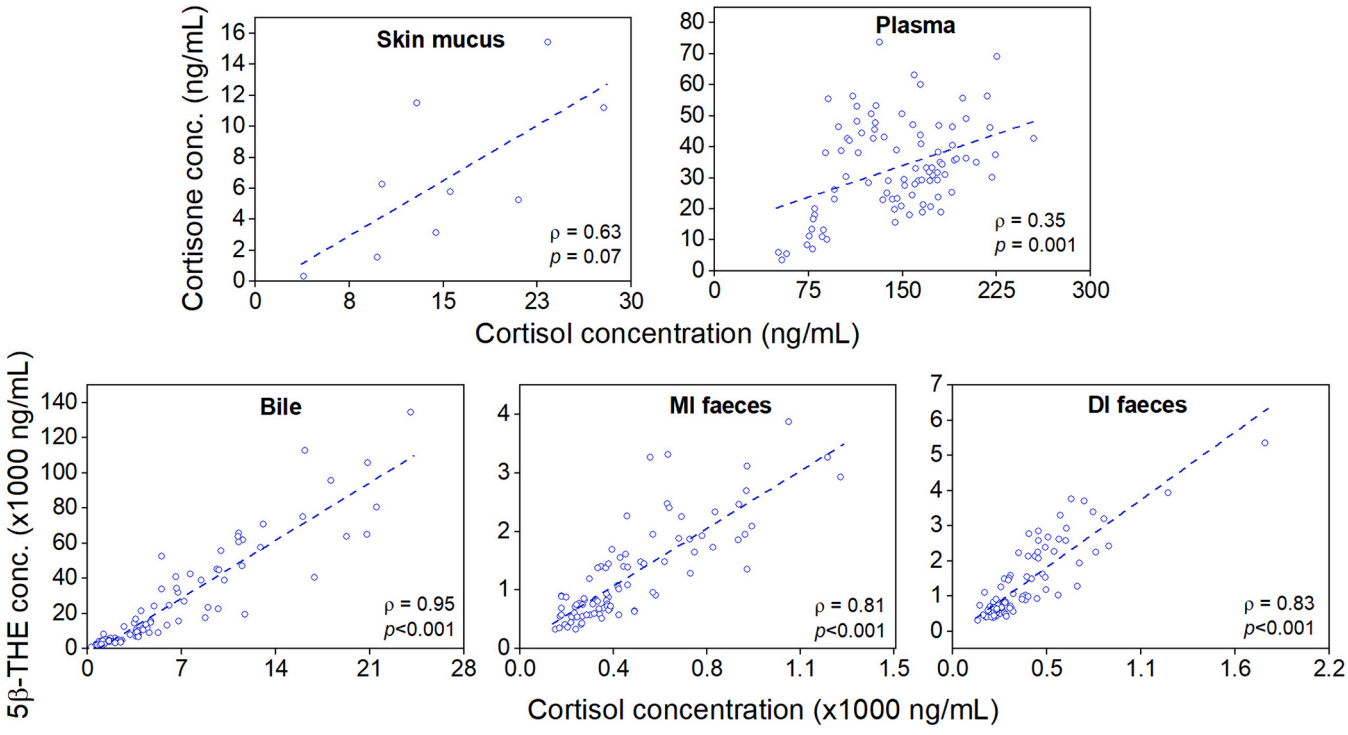

Fig. 12. Correlation between cortisol and cortisol metabolites from the same sample matrix (MI: mid-intestine, DI: distal intestine, 5β-THE: Tetrahydrocortisone).

3.2.5. Effect of fish condition factor on stress response

Low and negative correlations were obtained between fish condition factor K and plasma cortisol and cortisone (Fig. 13). Low to medium ($-0.2 \leq \rho \leq -0.4$) but significant negative correlations between cortisol metabolites and fish condition factor were observed in bile and faeces.

Similar to the observations made by Uren Webster *et al.* [33] on

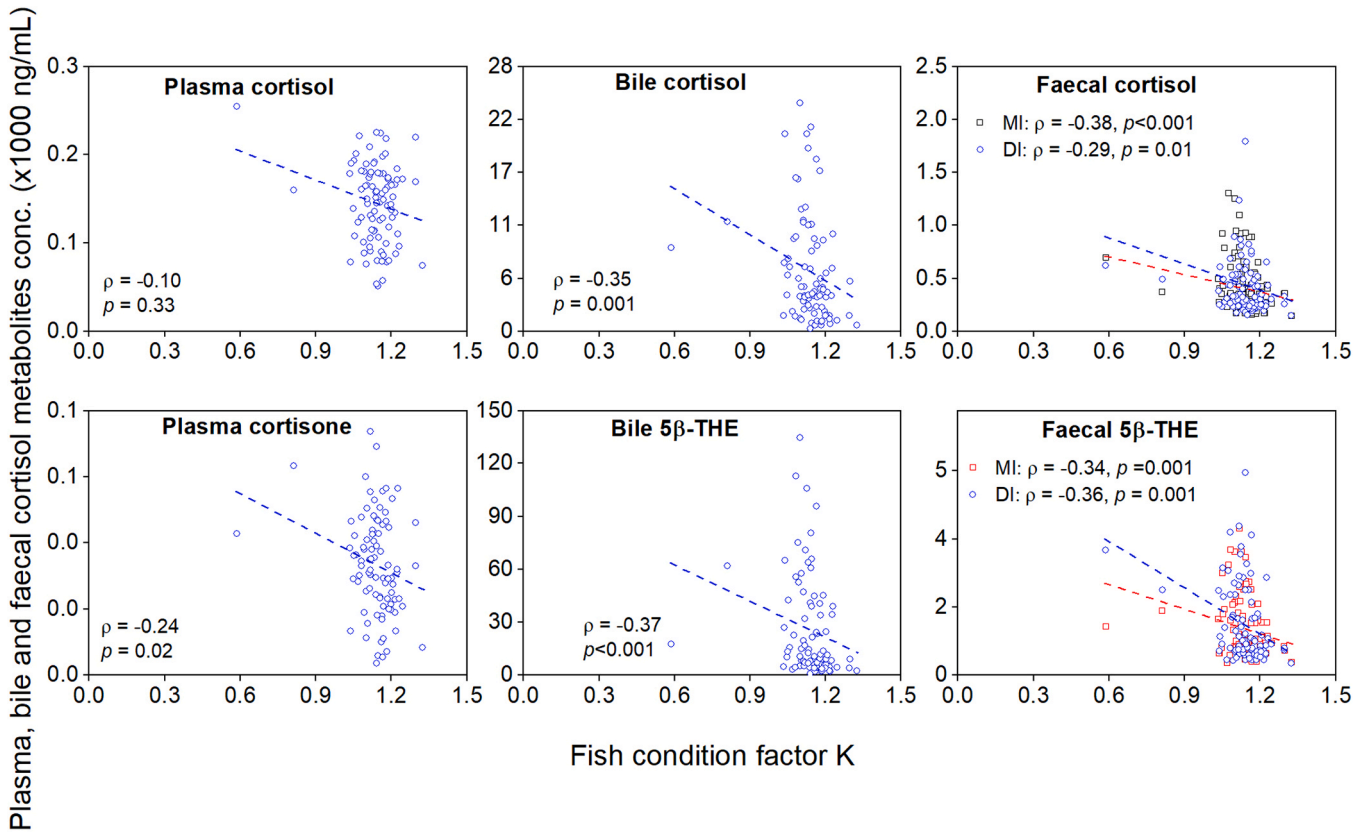

Fig. 13. Correlation between plasma, bile and faecal cortisol metabolites and fish condition factor K (MI: mid-intestine, DI: distal intestine, 5β-THE: Tetrahydrocortisone).

juvenile Atlantic salmon, fish body condition did not have any association with the concentrations of plasma cortisol. Fürtbauer and Heistermann [39] did not observe any linear relation between waterborne cortisol levels and fish weight as well. However, unlike the results obtained by Uren Webster *et al.* [33], a trend of decreasing faecal cortisol metabolites concentrations was observed with increasing fish condition factor in the present study. This may be either due to the differences in intestinal evacuation or metabolic clearance of cortisol metabolites in fasted fish depending on the fish body condition (fish in good condition or more robust may tackle stress better). In the present study, this parameter ranged from 0.59 to 1.32. Further studies are necessary to confirm if fish body condition can have an impact on fish's experience of stress through cortisol metabolism and clearance.

3.3. Cortisol clearance pathway in stressed Atlantic salmon

The clearance pathway of cortisol was evaluated using results obtained from the analysis of pooled samples to include skin mucus data (Table S4).

3.3.1. Clearance through metabolism

As shown in Fig. 14a and Table S4a, cortisol to cortisone ratios were similar for plasma and skin mucus: highest in control group, decreasing up to 24 h post-stress and stabilizing over the last 48 h. Also, cortisol to cortisone ratios were comparable for bile and faeces (Fig. 14a, Table S4a), increasing up to 6–12 h and slightly decreasing for the rest of the experiment. The ratios between 5β-THF and cortisol and 5β-THE and cortisone were similar for bile and faeces and had comparable pattern

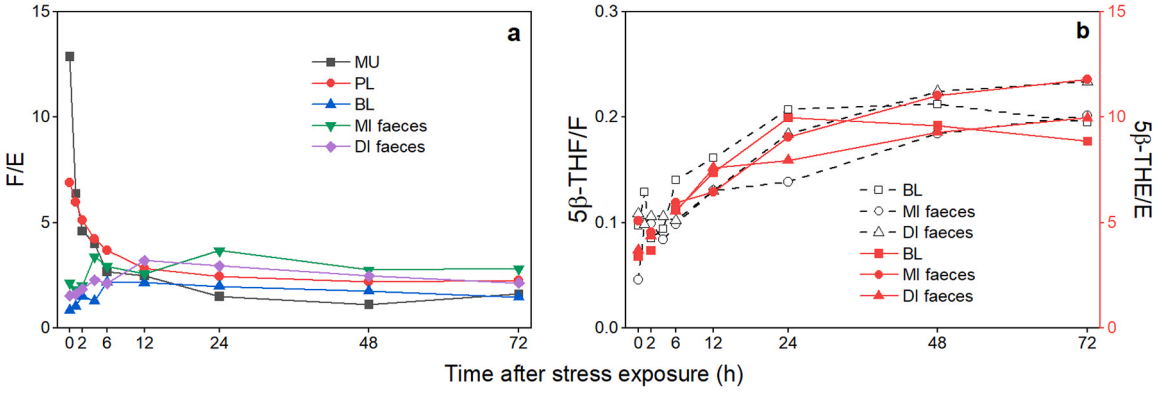

Fig. 14. **(a)** Cortisol to cortisone ratios (F/E) in plasma (PL), skin mucus (MU), bile (BL) and faeces (MI: mid-intestine and DI: distal intestine). **(b)** Tetrahydrocortisol to cortisol (5β-THF/F) and Tetrahydrocortisone to cortisone (5β-THE/E) ratios in bile and faeces.

although the former was lower in magnitude (Fig. 14b, Table S4a). The ratios were increasing from 0 to 24 h before flattening or slightly decreasing for the last 48 h.

The cortisol to cortisone and tetrahydrocortisol to cortisol ratios in non-stressed fish (sampled at 0 h) were similar to that found in free-ranging white-faced capuchins [40] indicating similarity to mammals at resting level (Table S4b).

The decrease in cortisol to cortisone ratios observed in plasma indicates the rate of conversion of cortisol to cortisone as a process of inactivation and clearance of this hormone. The results confirm that in fish, cortisol is mainly converted to cortisone by the enzyme 11βHSD1 and no activity of the enzyme 11βHSD2 which converts cortisone back to cortisol could be observed [26]. A high 5β-reductase activity can also be assumed based on the ratios of 5β-THF to cortisol and 5β-THE to cortisone observed [21]. It suggests that this is the main catabolic pathway of cortisol in Atlantic salmon, especially, the conversion of cortisone to 5β-THE.

3.3.2. *Clearance through evacuation*

The results in Table S4c and Fig. 15 show that skin mucus to plasma ratio for cortisol increased from 0.03 at 0 h to a maximum value of 0.15 at 12 h before dropping towards the end of the experiment. For cortisone, the ratio increased from 0.02 at 0 h to a peak value of 0.24 at 24 h. It then dropped for the rest of the experimental period. Bile to plasma ratios of cortisol and cortisone increased throughout the whole experiment, from 7.63 to 98.48 and 61.24 to 150.77 respectively. For all cortisol metabolites detected, a trend of decreasing faecal to bile ratios was observed from 0 to 24 h before the ratios stabilized during the two remaining days. For distal to mid-intestine faeces ratios, a moderate increase was observed over time.

These data support that cortisol and cortisone from blood slightly diffuse to skin mucus and are also evacuated through bile and faeces. The decrease of faecal to bile cortisol metabolites ratio over time indicates a higher accumulation rate of cortisol metabolites in bile compared to the clearance rate from bile to faeces. The metabolites are accumulating in bile but only a small fraction is evacuated through the intestine. This could be due to slow intestinal evacuation since fish did not eat after the onset of stress and through the end of the experiment as mentioned earlier. The profile could be different for fish eating regularly. Similar effect of stress on feeding behaviour was also observed by Pickering *et al.* [41] during an experiment involving handling and confinement of brown trout. Similarly, young coho salmon lost feeding behaviour up to 7 days after moderate crowding stress and did not resume feeding even after 16 days when highly crowded [42]. The moderate increase in ratio between distal and mid-intestine faecal cortisol metabolites suggests that the metabolites were slowly moving towards the distal intestine over time leading to higher proportion in that part of the intestine towards the end of the experiment.

The cortisol clearance pathway assumed to occur in Atlantic salmon upon onset of stress and the subsequent release of cortisol is summarized in Fig. 15. Cortisol is quickly converted to cortisone and a quite small fraction of these free compounds was then cleared through skin mucus while the majority reaches the liver, either through simple diffusion or carrier-mediated transport. Cortisol and (cortisone) are lipophilic hormones. To enhance their clearance, they are metabolized in the liver to other derivatives (5β-THF, 5β-DHE, 5β-THE and β-cortolone) and further

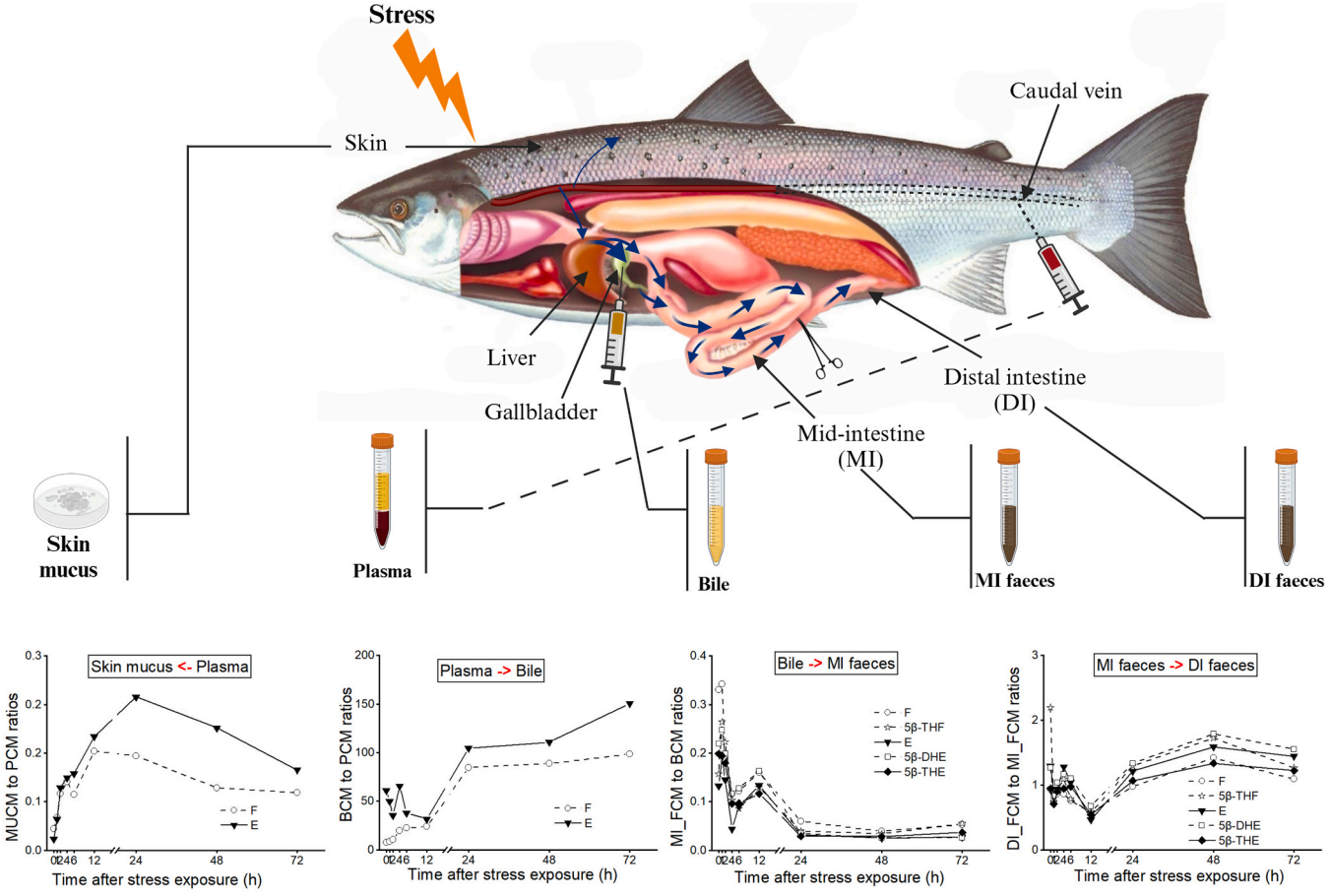

Fig. 15. Cortisol clearance pathway in stressed farmed Atlantic salmon. The size of the blue arrows illustrates the concentrations of cortisol metabolites (CM) in the tissue (F: cortisol, 5β-THF: Tetrahydrocortisol, 5β-DHE: 5β-Dihydrocortisol, 5β-THE: Tetrahydrocortisol). Salmon image courtesy of Robin Ade and figure created with BioRender.com.

conjugated with either glucuronide or sulphate group to render them more water-soluble. The derivatives are also mainly conjugated with either glucuronide or sulphate group. Some fraction of the parent compounds (cortisol and cortisone) in their conjugated form attains the blood stream while the remaining portion and the derivatives are evacuated into the bile and faeces.

4. Conclusion

To the best of our knowledge, the present study is the first to demonstrate the dynamics of total cortisol and cortisone in plasma and skin mucus, as well as cortisol and five of its metabolites (5β-THF, cortisone, 5β-DHE, 5β-THE and β-cortolone) in bile and faeces of farmed Atlantic salmon following a stress event. It reveals their suitability in reproducible stress evaluation in fish as an alternative to the traditional analysis of free blood cortisol. The results showed that skin mucus is a good alternative to blood and can reflect stress in a low invasive way although cortisol and cortisone were present in quite low concentration in this sample matrix. In starved, dead or slaughtered fish, the analysis of 5β-THE in bile proves to be an excellent tool for evaluating stress. Measurement of the same compound in faeces is also recommended for routine analysis as this matrix can be sampled in a low invasive way, during other operation like operational welfare evaluation or lice counting, and also provides results unaffected by sampling procedure. In the lack of faeces such as in starved fish and when skin mucus is unavailable or challenging to obtain (small fish), the analysis of conjugated plasma cortisol or cortisone could be more appropriate and less affected by sampling than measuring these compounds in free form in blood. This can be achieved by hydrolysing the sample prior to analysis or using appropriate standard materials for the LC-MS/MS measurement. Furthermore, owing to the hydrophilic properties of the cortisol metabolites and their higher concentrations in faeces compared to free cortisol, the analysis of these compounds opens new opportunities for non-invasive assessment of farmed fish welfare. This can for instance be achieved by utilizing faeces and sludge collected at the bottom of a net pen or a closed containment in commercial fish farming.

It was demonstrated that the concentrations of cortisol metabolites in plasma do not always correlate with those in other sample matrices due to the time-lag between their production in plasma and their clearance in the other tissues. Notably, skin mucus and plasma on one hand and bile and faeces on the other hand exhibited closer relationships with each other. As a result, a time window between 12 and 24 h after stress are suggested as suitable for sampling salmon post-smolt blood and skin mucus while 24 h post-stress is considered the most appropriate sampling timepoint for bile and faeces when using the analytical methods applied in the present study.

CRediT authorship contribution statement

Ernestine Fanjara: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Visualization, Writing – original draft preparation, Writing – review & editing. **Grete K. F. H. Aas**: Conceptualization, Supervision, Writing – review & editing. **Yanran Cao**: Conceptualization, Supervision, Writing – review & editing. **Vera Kristinova**: Conceptualization, Supervision, Writing – review & editing. **Asgeir Sæbø**: Conceptualization, Funding acquisition, Project administration. **Anne Stene**: Conceptualization, Supervision, Writing – review & editing.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. Ernestine Fanjara reports financial support was provided by Innolipid AS. Ernestine Fanjara reports a relationship with Innolipid AS that includes: employment. Vera Kristinova reports a relationship with Innolipid AS that includes: employment. Asgeir Sæbø reports a relationship with Innolipid AS that includes: board membership, employment, and equity or stocks. Asgeir Sæbø has patent Fish stress biomarker analysis pending to US Patent. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jsbmb.2024.106542.

References

[1] FAO. Fishery and Aquaculture Statistics. Global aquaculture production 1950-2021 (FishStatJ). 2023. Available from: https://www.fao.org/fishery/en/topic/166235.
[2] Norwegian Seafood Council. Celebrating 50 years of modern aquaculture. 2020.
[3] S. Tilseth, T. Hansen, D. Møller, Historical development of salmon culture, Aquaculture 98 (1991) 1–9, https://doi.org/10.1016/0044-8486(91)90367-G.
[4] D. Persson, A. Nødtvedt, A. Aunsmo, M. Stormoen, Analysing mortality patterns in salmon farming using daily cage registrations, J. Fish. Dis. 45 (2022) 335–347, https://doi.org/10.1111/jfd.13560.
[5] G.G. Singh, Z. Sajid, C. Mather, Quantitative analysis of mass mortality events in salmon aquaculture shows increasing scale of fish loss events around the world, Sci. Rep. 14 (2024) 3763, https://doi.org/10.1038/s41598-024-54033-9.
[6] Norwegian Directorate of Fisheries. Atlantic salmon, Rainbow trout and Trout - Grow out production. Official statistics. Updated 12.10.2023. 2023. Available from: https://www.fiskeridir.no/English/Aquaculture/Statistics/Atlantic-salmon-and-rainbow-trout/grow-out-production.
[7] I. Sommerset, J. Wiik-Nielsen, T. Moldal, V.H.S. Oliveira, J.C. Svendsen, A. Haukaas, E. Brun. Fiskehelserapporten 2023, Veterinærinstituttets rapportserie nr. 8a/2024, utgitt av Veterinærinstituttet. 2024.
[8] V.H.S. Oliveira, K.R. Dean, L. Qviller, C. Kirkeby, B. Bang Jensen, Factors associated with baseline mortality in Norwegian Atlantic salmon farming, Sci. Rep. 11 (2021) 14702, https://doi.org/10.1038/s41598-021-93874-6.
[9] C. Noble, K. Gismervik, M.H. Iversen, J. Kolarevic, J. Nilsson, L.H. Stien, J.F. Turnbull. Welfare Indicators for farmed Atlantic salmon: tools for assessing fish welfare. 2018.
[10] I. Sommerset, C.S. Walde, B.B. Jensen, J. Wiik-Nielsen, G. Bornø, V.H.S. de Oliveira, A. Haukaas, E. Brun. Norwegian Fish Health Report 2021. Norwegian Veterinary Institute Report. 2022.
[11] S.E. Wendelaar Bonga, The stress response in fish, Physiol. Rev. 77 (1997) 591–625, https://doi.org/10.1152/physrev.1997.77.3.591.
[12] T. Mommsen, M. Vijayan, T. Moon, Cortisol in teleost dynamics, mechanisms of action, and metabolic regulation, Rev. Fish. Biol. Fish. 9 (1999) 211–268, https://doi.org/10.1023/A:1008924418720.
[13] E. Fanjara, G.H. Aas, Y. Cao, V. Kristinova, A. Saeboe, A. Stene, Identification of cortisol metabolites with LC-MS/MS in plasma, skin mucus, bile and faeces for stress evaluation of farmed Atlantic salmon, J. Steroid Biochem. Mol. Biol. 234 (2023) 106401, https://doi.org/10.1016/j.jsbmb.2023.106401.
[14] A.P. Scott, T. Ellis, H. Tveiten, Identification of cortisol metabolites in the bile of Atlantic cod Gadus morhua L. Steroids 88 (2014) 26–35, https://doi.org/10.1016/j.steroids.2014.05.014.

[15] B. Truscott, Steroid metabolism in fish: Identification of steroid moieties of hydrolyzable conjugates of cortisol in the bile of trout Salmo gairdnerii, Gen. Comp. Endocrinol. 38 (1979) 196–206, https://doi.org/10.1016/0016-6480(79)90207-7.
[16] T.G. Pottinger, T.A. Moran, P.A. Cranwell, The biliary accumulation of corticosteroids in rainbow trout,Oncorhynchus mykiss, during acute and chronic stress, Fish. Physiol. Biochem. 10 (1992) 55–66, https://doi.org/10.1007/BF00004654.
[17] I.E. Einarsdóttir, K.J. Nilssen, Stress responses of Atlantic salmon (Salmo salar L.) elicited by water level reduction in rearing tanks, Fish. Physiol. Biochem. 15 (1996) 395–400, https://doi.org/10.1007/BF01875582.
[18] M.D. Fast, S. Hosoya, S.C. Johnson, L.O.B. Afonso, Cortisol response and immune-related effects of Atlantic salmon (Salmo salar Linnaeus) subjected to short- and long-term stress, Fish. Shellfish Immunol. 24 (2008) 194–204, https://doi.org/10.1016/j.fsi.2007.10.009.
[19] C.A. Barnham, A.F. Baxter. Condition factor, K, for Salmonid fish. Dept. of Primary Industries, Melbourne. 2003.
[20] J. Marcos, N. Renau, G. Casals, J. Segura, R. Ventura, O.J. Pozo, Investigation of endogenous corticosteroids profiles in human urine based on liquid chromatography tandem mass spectrometry, Anal. Chim. Acta 812 (2014) 92–104, https://doi.org/10.1016/j.aca.2013.12.030.
[21] M.J.J. Finken, R.C. Andrews, R. Andrew, B.R. Walker, Cortisol metabolism in healthy young adults: sexual dimorphism in activities of A-ring reductases, but not 11β-hydroxysteroid dehydrogenases1, J. Clin. Endocrinol. Metab. 84 (1999) 3316–3321, https://doi.org/10.1210/jcem.84.9.6009.
[22] R. Yossa, M. Verdegem, Misuse of multiple comparison tests and underuse of contrast procedures in aquaculture publications, Aquaculture 437 (2015) 344–350, https://doi.org/10.1016/j.aquaculture.2014.12.023.
[23] B. Rosner, Fundamentals of biostatistics, 8th edition ed, Cengage Learning, Boston, MA, 2016.
[24] S.A. Nolan, T.E. Heinzen, Statistics for the behavioral sciences, 2nd ed, Worth Publishers, New York, 2012.
[25] A. Carbajal, F.E. Reyes-Lopez, O. Tallo-Parra, M. Lopez-Beja, L. Tort, Comparative assessment of cortisol in plasma, skin mucus and scales as a measure of the hypothalamic-pituitary-interrenal axis activity in fish, Aquaculture 506 (2019) 410–416, https://doi.org/10.1016/j.aquaculture.2019.04.005.
[26] T.G. Pottinger, T.A. Moran, Differences in plasma cortisol and cortisone dynamics during stress in two strains of rainbow trout (Oncorhynchus mykiss), J. Fish. Biol. 43 (1993) 121–130, https://doi.org/10.1111/j.1095-8649.1993.tb00415.x.
[27] D.J. Nichols, M. Weisbart, Plasma cortisol concentrations in Atlantic salmon, Salmo salar: episodic variations, diurnal change, and short term response to adrenocorticotrophic hormone, Gen. Comp. Endocrinol. 56 (1984) 169–176, https://doi.org/10.1016/0016-6480(84)90027-3.
[28] R. Patiño, J.M. Redding, C.B. Schreck, Interrenal secretion of corticosteroids and plasma cortisol and cortisone concentrations after acute stress and during seawater acclimation in juvenile coho salmon (Oncorhynchus kisutch), Gen. Comp. Endocrinol. 68 (1987) 431–439, https://doi.org/10.1016/0016-6480(87)90082-7.
[29] F.A. Guardiola, A. Cuesta, M.Á. Esteban, Using skin mucus to evaluate stress in gilthead seabream (Sparus aurata L, Fish. Shellfish Immunol. 59 (2016) 323–330, https://doi.org/10.1016/j.fsi.2016.11.005.
[30] R.H. Easy, N.W. Ross, Changes in Atlantic salmon (Salmo salar) epidermal mucus protein composition profiles following infection with sea lice (Lepeophtheirus salmonis), Comp. Biochem. Physiol. Part D: Genom. Proteom. 4 (2009) 159–167, https://doi.org/10.1016/j.cbd.2009.02.001.
[31] Y. Tokushima, Y. Ito, M. Shimizu, N. Omoto, A. Hara, Immunochemical comparison between sera from the primary and secondary circulations in a Salmonid Fish, Sakhalin Taimen (Hucho perryi), Fish. Physiol. Biochem. 30 (2004) 179–188.
[32] L. Franco Martínez, I. Brandts Busom, F. Reyes-López, L. Tort, A. Tvarijonaviciute, M. Teles, Skin mucus as a relevant low-invasive biological matrix for the measurement of an acute stress response in rainbow trout (Oncorhynchus mykiss), Water 14 (2022) 1754, https://doi.org/10.3390/w14111754.
[33] T.M. Uren Webster, D. Rodriguez-Barreto, S. Consuegra, C. Garcia de Leaniz, Cortisol-related signatures of stress in the fish microbiome, Front. Microbiol. 11 (2020), https://doi.org/10.3389/fmicb.2020.01621.
[34] Y. Cao, A.-K. Tveten, A. Stene, Establishment of a non-invasive method for stress evaluation in farmed salmon based on direct fecal corticoid metabolites measurement, Fish. Shellfish Immunol. 66 (2017) 317–324, https://doi.org/10.1016/j.fsi.2017.04.012.
[35] C. Simontacchi, C. Poltronieri, C. Carraro, D. Bertotto, G. Xiccato, A. Trocino, G. Radaelli, Alternative stress indicators in sea bass Dicentrarchus labrax, L, J. Fish. Biol. 72 (2008) 747–752, https://doi.org/10.1111/j.1095-8649.2007.01717.x.
[36] D. Bertotto, C. Poltronieri, E. Negrato, D. Majolini, G. Radaelli, C. Simontacchi, Alternative matrices for cortisol measurement in fish, Aquac. Res. 41 (2010) 1261–1267, https://doi.org/10.1111/j.1365-2109.2009.02417.x.
[37] V.A. Meling, K. Berge, D.L. Knudsen, P.O. Rønning, C. Brede, Monitoring farmed fish welfare by measurement of cortisol as a stress marker in fish feces by liquid chromatography coupled with tandem mass spectrometry, Molecules 27 (2022), https://doi.org/10.3390/molecules27082481.
[38] A.P. Scott, A.V.M. Canario, 17α,20β-Dihydroxy-4-pregnen-3-one 20-sulphate: a major new metabolite of the teleost oocyte maturation-inducing steroid, Gen. Comp. Endocrinol. 85 (1992) 91–100, https://doi.org/10.1016/0016-6480(92)90176-K.
[39] I. Fürtbauer, M. Heistermann, Cortisol coregulation in fish, Sci. Rep. 6 (2016) 30334, https://doi.org/10.1038/srep30334.
[40] A. Weltring, F.S. Schaebs, S.E. Perry, T. Deschner, Simultaneous measurement of endogenous steroid hormones and their metabolites with LC–MS/MS in faeces of a New World primate species, Cebus capucinus, Physiol. Behav. 105 (2012) 510–521, https://doi.org/10.1016/j.physbeh.2011.09.004.
[41] A.D. Pickering, T.G. Pottinger, P. Christie, Recovery of the brown trout, Salmo trutta L., from acute handling stress: a time-course study, J. Fish. Biol. 20 (1982) 229–244, https://doi.org/10.1111/j.1095-8649.1982.tb03923.x.
[42] G.A. Wedemeyer, Physiological response of Juvenile Coho Salmon (Oncorhynchus kisutch) and Rainbow Trout (Salmo gairdneri) to handling and crowding stress in intensive fish culture, J. Fish. Res. Board Can. 33 (1976) 2699–2702, https://doi.org/10.1139/f76-320.