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Establishment of a non-invasive method for stress evaluation in farmed salmon based on direct fecal corticoid metabolites measurement

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Abstract:

Fish welfare is an important issue for growth of the aquaculture industry. Stress responses represent animal's natural reactions to challenging conditions and could be used as a welfare indicator. Cortisol level is relevant to fish welfare condition, and is a readily measured component of the primary stress response system. Generally, cortisol is measured by blood sampling. However, fish blood cortisol level could be instantly influenced by handling-stress at sampling. Fecal corticoid metabolites (FCM) are a mixture of several different metabolites with a wide range of polarities. Thus, feces could be promising alternative less handling-sensitive and non-invasive biological matrices for cortisol evaluation in Atlantic salmon. In this study we developed non-invasive method for determination of fecal corticoid metabolites in farmed Atlantic salmon (Salmo salar L.) using enzyme-linked immunosorbent assay (ELISA).

It was demonstrated that salmon FCM extracted from salmon feces is insoluble in non-polar solvents like diethyl ether, but well soluble in polar solvents like methanol. The proper extraction ratio could be one ml 100% methanol for 100 µL of the liquid part of salmon feces or 100 mg of the solid part. The FCM directly detected in unextracted liquid part of feces correlated well with the FCM extracted from both liquid and solid part of the corresponding samples, without significant difference. Thus, it is feasible to measure FCM directly in the liquid part of salmon feces without any extraction procedure.

Then, we applied this assay for FCM analysis in the group of salmon that experienced salmon pancreas disease (PD) and amoebic gill disease (AGD). We demonstrated 1) both plasma cortisol and FCM increased significantly during the outbreak of inflammatory disease ($P<0.01$). Plasma cortisol level was elevated from 28± 40 ng/ml to 164.4± 62.5 ng/ml, FCM from 14.4±13.2 ng/ml to 170.7± 89.7 ng/ml. 2) Growth and starvation has no significant impact on either cortisol or FCM level. 3) FCM correlated well with plasma cortisol level ($P<0.01$). Furthermore, there seems more individual variation in plasma cortisol levels than in FCM levels.

These results suggest FCM could be directly analyzed in liquid part of salmon feces without extraction. This directly detected FCM level could represent the total fecal FCM level and plasma cortisol level. This simple and non-invasive method makes FCM a proper indicator for salmon welfare.

1. Introduction

Fish welfare is an important issue for growth of the aquaculture industry, in terms of public perception, product acceptance, product quality, and marketing [1, 2]. Throughout the aquaculture industry, fish is subjected to many different sources of physiological stress, including high density, confinement, sex-separation, disturbance, and the threat of parasites. They have fundamental impact on fish performance, health and welfare [3]. Therefore, an objective and simple way to assess welfare, that would allow comparisons between different salmon farms, management procedures, and production strategies, offers great benefits.

Stress responses represent animal's natural reactions to challenging conditions and could be used as a welfare indicator. Thus, reproducible methods to confirm the presence of chronic stress in fish are of great importance for identifying stressful management procedures, as well as for evaluating and developing the official regulations and legislations to improve fish quality and survival. In the past decade, a number of indicators have been developed for direct and indirect assessment of physical or chemical stressors of salmon or other species of fish. However, many methods have not yet been used for official documentation of fish welfare in field salmon farms.

Cortisol is a readily measurable component of the primary stress response system [4]. Cortisol level is relevant to fish welfare condition, as it affects physiological and brain functions and modifies behaviour and susceptibility to diseases. Knowledge of cortisol production is therefore relevant to breeding and welfare in fish farming [5].

Generally, cortisol is measured by blood sampling. However, the blood cortisol level could be instantly influenced by handling-stress at sampling. Arguably, handling is more stressful, injurious, and problematic for fish than for higher vertebrates, especially since they are taken out of water [5]. It is crucial to assess corticosteroids in alternative less handling-sensitive and non-invasive biological matrices. These include faecal casts, which have been validated in parrotfish [3, 6]. Farmers can perform such sampling themselves, for instance during the weekly sea lice counting.

The aim of this study is to establish a non-invasive method for determination of fecal corticoid metabolites (FCM) in farmed Atlantic salmon (*Salmo salar* L.). This method based on non-invasive sampling is intended to reduce the impact of stressful and

invasive procedures such as capture and blood sampling. This method is validated in salmon that experienced inflammatory diseases. The FCM levels are correlated to the blood cortisol levels, as well as the fish health condition.

2. Materials and methods

2.1. Salmon sampling

Atlantic salmon used in this study were reared in a commercial marine fish farm in the northwestern coast of Norway. The salmon smolts were transferred to sea-water in August 2014 and slaughtered out in February 2016. Fish were stocked in cages for on-growning according to current industry standards for organic salmon production (max 10 kg/m^3). The fish were fed with standard feed developed for organic salmon production (Harmony Debio). The health status of the fish stock was monitored every month by the farms veterinarian. The diagnosis of infectious disease was based on the monthly clinical examination and participation in a Polymerase Chain Reaction (PCR) screening program. From March 2015 until February 2016, ten salmons were sampled every month from the same cage with a closing net. The fish were quickly removed from the net and immediately killed with a sharp blow to the head (handling < 5 minutes), according to farm site operational procedure. Procedure follow the Norwegian Animal Welfare Act (LOV-2009-06-19-97). The act (§ 13) and related regulation (FOR-2015-06-18-76) also cover the use of animals in research, education and medical testing. NTNU in Ålesund is approved by the Norwegian Food Safety Authority to do animal research according to the regulation. The sampling of fish as described here was in accordance with the acts and the approval.

A 4-ml blood sample was immediately drawn by caudal venipuncture using a 24-gauge needle, placed in a 4.5 ml Vacutainer tube containing lithium heparin as an anticoagulant (BD Vacutainer Systems, Plymouth, UK). Blood samples were transported ashore on ice. Plasma was separated by centrifugation (1500 rpm for 10 min) at 4℃, removed and aliquoted into 1.5 ml micr o centrifuge tubes. Samples were stored at −20℃ until subsequent analysis [7].

Fish feces samples were collected by manual stripping avoiding contamination of feces with water, mucous and urine. First, the moisture from the bellies of the fish was gently wiped with a paper towel. Then, using a moderate squeezing motion with

the thumb and forefinger, pressure was applied to the abdomen. Stripping started in line at the front of the pelvic fins and finished at the anus [8]. Feces samples were collected in a 15 mL plastic centrifuge tubes. To minimize the influence of sampling technique on fecal output between collection series, the same operator was assigned to each sample collection. Feces samples were kept on ice prior to centrifugation. After centrifugation (1500 rpm for 10 min), the liquid part was removed and aliquoted into 1.5 ml micro centrifuge tubes. Both the liquid part and the solid part of the feces were stored separately at −20℃ until subsequent an alysis.

2.2. Real-time RT-PCR Analysis

As a part of the farm site screening program for infectious disease, cardiac tissues and gill tissues were randomly collected from the same production units by the veterinarian in the fish farm. For identification of diseases, tissue samples were fixed for real-time quantitative reverse transcription PCR (RT-qPCR) analyses. The virus test for salmon pancreas disease (PD) in cardiac tissue, and amoebic gill disease (AGD) test in gill tissue, were performed by an accredited commercial firm, PatoGen Analyse AS (www.patogen.no). Real-time RT qPCR analyses were performed according to accredited procedures of PatoGen Analyse AS [9-11]. Table 1 shows the infection rate of AGD and PD in the studied salmon group. No evidence of disease was reported in the stock prior to the experiment. During August and September 2015, there was an outbreak of infectious diseases in the studied group, including salmon pancreas disease and amoebic gill disease (Table. 1). After November 2015, the disease symptoms were fading out. However, the PD virus was still detectable in 43-50% heart samples of the fish.

Date	PD	AGD
11.02.2015	0 %	0 %
12.05.2015	0 %	0 %
03.06.2015	0 %	0 %
02.07.2015	0 %	0 %
18.08.2015	52 %	0 %
15.09.2015	75 %	100 %
16.10.2015	38 %	100 %
17.11.2015	50 %	NA
09.12.2015	50 %	NA
12.01.2016	43 %	NA

Table 1. An overview of the incidence of infectious disease in studied cage detected by Real-time RT qPCR analysis. Salmon pancreas disease (PD) in cardiac tissue, and amoebic gill disease (AGD) were identified by PatoGen Analyse AS. The data from the fish farm indicated a high incidence of PD and AGD infection rate in September 2015 (NA: not available).

2.3. Blood cortisol extraction

The plasma cortisol was extracted according to the manufacturer's instructions for cortisol analyses by enzyme immunoassay (EIA)/ enzyme-linked immunosorbent assay (ELISA) kit (Neogen, Lexington, KY, USA), previously described by Afonso et al. [12]. Plasma 100 µL and diethyl ether (Sigma, St. Louis, MO, USA) 1 ml were mixed in a glass tube, vortexed for 30 seconds, and then the liquid phases were separated. The organic upper phase was transferred into a clean tube, and the solvent was evaporated with a stream of nitrogen. The residue was dissolved in 100 µL of diluted extraction buffer before plasma cortisol was measured.

2.4. Fecal corticoid metabolites extraction

The fecal corticoid metabolites was first extracted using diethyl ether follow the protocol for plasma cortisol extraction. Next, the FCM was extracted using methanol as previously described [13, 14]. The optimal ratio of feces to methanol as well as the concentration of methanol for the cortisol extraction was determined. Solid feces 100 mg and 300mg were well mixed with 1 ml aqueous methanol with different concentrations: 80%, 90%, and 100%. The mixture was vortexed for 2 hours at room temperature and then centrifuged 3000 rpm for 10 min. The extracted liquid part was transferred into a clean tube, and the solvent was evaporated with a stream of nitrogen. The residues were dissolved in 100 µL of diluted extraction buffer (from the kit).

In order to identify the relevant feces matrix for cortisol analysis, we extracted FCM from both solid part and liquid part of 14 salmon feces from the same study group. For FCM extraction, one ml 100% methanol was used for 100 µL of the liquid part or 100 mg of the solid part of feces.

2.5. Cortisol measurement

The cortisol levels were determined in duplicates using the cortisol ELISA kits. In this study, the samples for the cortisol test included extracted plasma and three different feces samples: unextracted liquid part of the feces (ULF), the extraction product of liquid part of feces (ELF) and the extraction product of solid part of feces (ESF). First, the prepared samples were 5 to 50 folds diluted. The standards provided by the kit were serially diluted in the EIA buffer (from the kit) and vortexed. Subsequently, 50 µL of diluted samples and the standards, as well as blank samples (buffer only) were added in duplicates to the assay tray, which was coated with anti-cortisol rabbit antibodies. Cross-reactivities of cortisol antibody were reported as: cortisol 100%, prednisolone 47.4% which would not be present in the samples [15], cortisone 15.8%, 11-Deoxycortisol 15%, prednisone 7.8%, corticosterone 4.8%, 6β-hydroxycortisol 1.4%, and 17-hydroxyprogesterone 1.4% respectively. The analytical sensitivity for the cortisol assay was at 0.04 ng/ml [15].

Secondly, 50 µL of the cortisol enzyme conjugate were added and the tray incubated at room temperature for 1 hour. After incubation the tray was washed with 3 x 300 µl/well wash buffer. Then 150 µl/well of substrate was added, and incubated for 30 min at room temperature. Finally 50 µl/well of 1 N hydrochloric acid (HCL) were added to stop enzyme reaction.

The absorbance at 450 nm was measured using a Synergy HTX Microplate Reader (BioTek Instruments). Endpoint readings were determined using Gen5 (Version 2.06). A standard curve was plotted using results of the cortisol standards, and the cortisol concentrations of the samples were estimated from this graph, as described by the kit's manufacturer.

2.6. Statistics

Statistical analysis was performed with IBM SPSS Statistics 22 (IBM Corp., Armonk, NY, USA). Data were presented as means ± standard deviation (SD) of the mean. Normality (W Shapiro-Wilk's test) and homogeneity (Levene's test) of variances were assessed prior to the use of statistical analysis. Significance was taken at $P < 0.05$. Due to non-normal distribution and non-homogenous variances of part of hormonal data and unequal sample sizes, a non-parametric test was applied. Monthly cortisol

levels were compared by Kruskal-Wallis's ANOVA rank test followed by a Mann–Whitney's U-test. Significance was considered at $P < 0.05$. Correlation between hormone levels in plasma and feces were assessed by Spearman's rank correlation analysis at a significance level of $P < 0.05$. [16].

3. Results

3.1. Salmon production data

The Atlantic salmon used in this investigation were reared in the open sea at a local fish farm. During the sampling period from March 2015 to February 2016, the weight and length of sampled salmons were from 884.7± 106.6 g and 40.9± 11 cm to 6131.8± 1264.8 g and 78.6± 5.7 cm. Sea temperature, the salmon growth rate and the mortality rate from sea transfer until slaughter, is shown in Fig. 1. Generally the sea temperature was positively correlated with the growth rate of farmed salmon. However, the growth rate in autumn 2015 was lower than that had been expected. This was probably due to the disease outbreaks, which was also reflected in the mortality rate in the same period.

3.2. FCM extracted by methanol

To extract FCM from the feces, we first attempted to use diethyl ether, which was suggested for plasma cortisol extracting– with no success. In a pre-experiment both methanol and ethanol had better capability to isolate corticoid metabolites from salmon feces samples (data not shown).

Next, we set out to determine the optimal ratio of feces to methanol as well as the concentration of methanol for the FCM extraction. It was shown that 100 mg feces in 1 ml methanol was the optimal ratio of feces to methanol, and the difference between 80% and 100% methanol was minor (Fig. 2) Considering that this method will be further applied for fluid part of feces, we decided to perform FCM extraction using 100% methanol.

3.3. Comparison of FCM in fecal fractions

The FCM detected in ELF and the ULF are shown in Fig. 3A. The FCM in ELF was lower than that directly detected in ULF of corresponding samples ($p<0.05$). The FCM in ELF correlated well with that directly detected in ULF ($p<0.001$, correlation coefficient 0.877).

The amount of FCM in ESF and that in the ULF was compared (Fig. 3B). The FCM in ESF were slightly lower than FCM directly detected in ULF from the corresponding samples. However, the difference was not statistically significant ($p>0.05$). The FCM in ESF correlated well with that directly detected in ULF ($p<0.001$, correlation coefficient 0.874)

We further compared the FCM in ELF and ESF of corresponding samples (Fig. 3C). More FCM was detected in ESF than in ELF ($p<0.05$), while the concentrations of the FCM extracted from both part correlated quite well ($p<0.01$, correlation coefficient 0.839).

3.4. Cortisol test in plasma

The results of the plasma cortisol analysis are shown in Fig. 4. The mean plasma cortisol concentration from March to July was 28± 40 ng/ml. Since August the plasma cortisol level was significantly elevated. In September it reached a peak value of 164.4± 62.5 ng/ml. This peak value was about 5.9-fold the level measured in the earlier months ($P<0.01$). Afterwards, the cortisol level declined to the normal level. From November 2015 to February 2016, the mean plasma cortisol level remained at 12.5± 21.5 ng/ml.

3.5. FCM test in feces

The results of the fecal corticoid metabolites analysis are shown in Fig. 5. The mean FCM concentration in the unextracted feces supernatant from March to July was 14.4±13.2 ng/ml. The FCM level was significantly elevated after August. In September, the peak value was 170.7± 89.7 ng/ml, which was about 11.9-fold the level of the fish at the rest of the time ($P<0.01$). Afterwards, the cortisol level declined to the normal level. From November 2015 to February 2016 the mean feces cortisol level was 10.9± 14.5 ng/ml.

3.6. Correlation between FCM and plasma cortisol level

We assessed the relationship between cortisol in plasma and feces. The analyses demonstrated that the plasma cortisol level correlated well with the level of cortisol detected in extracted liquid feces ($p<0.001$), as shown in Fig. 6. No significant difference was identified between the plasma cortisol and the fecal corticoid metabolites level ($p>0.05$). But it seemed that there was more individual variation in plasma cortisol levels than in FCM levels.

3.7. Impact of growth and starvation on cortisol level

In this study, we monitored the cortisol and health level of the salmon groups for one year. In August and September, both plasma cortisol and fecal corticoid metabolites were significantly elevated compared to the rest of the year. Except for the period of disease outbreak, cortisol and FCM level were relatively stable during the growing of these salmons. When we compared the plasma cortisol and FCM levels in the first and last two months of this experiment, no significant difference was evident ($p>0.0$), as shown in Fig. 4 and 5. This means no significant cortisol variation was identified during growing and starvation before slaughter.

In Sept 2015 when infectious diseases attacked the population, the salmons did not eat, and therefore they produced no feces. Instead, we achieved yellow casts from the digestive system. Similar fluids were collected in February 2016 during starvation prior to slaughter. In these seemingly similar fluids, significant different levels of cortisol was identified ($p<0.01$), as shown in Fig. 5. In plasma, a comparable result ($p<0.01$) (Fig. 4) was achieved. This may indicate lack of food uptake is not particularly stressful compared to the presence of an infectious disease.

4. Discussion

Cortisol is the principal glucocorticoid released by the activation of the hypothalamus-pituitary-interrenal axis (HPI axis), when an organism experiences stressful conditions [17]. Cortisol is involved in activation of a subsequent cascade of metabolic and physiological changes, which produces a burst of energy to prepare

the fish for an emergency situation [18]. The concentration of plasma cortisol represents species' responses to the environment. The response may be acute or chronic based on the duration, the magnitude, and severity of the stress stimuli. Cortisol has thus been widely used as a biomarker to assess an organism's response to stress [18].

In general, the main corticosteroids responding to stressors are cortisol in mammals, corticosterone in birds, and cortisol and cortisone in fish [19]. Most immunoassays with reasonable specificity for cortisol are adequate [20, 21]. Sink et al. detailed the methods of estimation of cortisol using ELISA assay in channel catfish, largemouth. Validation procedures were conducted for multiple commercial cortisol ELISA kits, and the results were compared with that of the Radioimmunoassay (RIA). They demonstrated that ELISA kits could be utilized for qualitative determination of cortisol in fish such as bass, red pacu, and golden shiners [21].

In salmonids the plasma cortisol was also used for stress assessment. Fast et al. showed for the first time that a salmonid subjected to short- and long-term stress exhibited the enhancement of cortisol level [22]. Sundell et al. observed an elevation in plasma cortisol level, when fish were transferred to sea water [23]. In Atlantic salmon parr, elevated plasma cortisol level was shown to lead to an impaired innate immune response, and higher infectious pancreatic necrosis virus (IPNV) prevalence in Atlantic salmon parr [24].

However, when sampling cortisol in fish, handling provokes an alarm reaction, upregulating the level of pituitary hormones, and thus influencing the precision of results. Anesthetics were used in other studies to reduce pain and awareness so as to reduce the sampling related stress. However, there were many inconsistent comments on anesthetics efficiency, as well as controversy regarding using of anesthetic due to animal management ethics [17].

Non-invasive methods were used to measure stress. The measurement of cortisol in water has the advantage that the sampling does not stress the fish. Free cortisol released into water by rainbow trout, common carp (Cyprinus carpio) and the rutilus (Rutilus rutilus) have been measured to evaluate stress status [25]. Cortisol secretion is in direct proportion to fish biomass and the flow rate through tanks. However, the measurement of cortisol in water in an open sea cage culture will be of limited

practical value since the flow rate through the cages are not known. In such cases cortisol in water will probably be too low to measure.

Another low invasive method to measure cortisol is to test it in feces. In general, the liver metabolizes circulating steroid hormones. Here steroids are inactivated by means of reduction and conjugation to glucuronides or sulfate to increase their water solubility, and then excreted as conjugates via the kidneys into the urine or via the bile into the gut. Consequently, specific steroid metabolites can be detected in the feces [19]. Unlike blood samples, fecal samples are less affected by episodic fluctuations or the pulsatile secretion of hormone. Thus, the FCM might represent the hormonal status of an animal more accurately than a single plasma sample. In the previous study stress hormones in mammals and birds were assessed by FCM tests [19]. Fecal corticoid studies on free-living parrotfish and common carp demonstrated that FCM in fecal casts correlated strongly to plasma cortisol level [6, 26, 27]. Our results demonstrates that it is possible to use FCM analysis as a non-invasive tool to assess stress levels in farmed salmon.

Determination of fecal corticoid metabolites could be very useful for assessment of fish welfare, as a complement to behavioral, physiological, and pathological studies. The first study of fecal corticoid metabolites in fish showed a relationship of the concentrations of plasma- cortisol, corticosterone, and testosterone to their fecal counterparts. Turner's study suggested that fecal measures reflected plasma measures under unstressed conditions [26]. This is in accordance with our results.

To measure free glucocorticoids and glucocorticoid metabolites in feces, high-performance liquid chromatography (HPLC) is an established method. This approach is very specific, but it is also technically demanding, time consuming, with relatively low throughput and limited sensitivity [20]. Lupica and Turner had validated an ELISA approach for FCM assessment using HPLC. The tests for parallelism and extraction efficiency showed that ELISA and HPLC assay generated almost identical values [27].

It was evidenced that ELISA is an efficient, sensitive and reliable method for FCM measurement in fecal extracts of mammals and birds. The common practice is to use commercially available immunoassay kits that have been designed to measure corticosteroids in blood samples, and to adapt the kit for other sampling media [20]. The cortisol ELISA Kit we used in this study had been used for plasma cortisol tests in fish species such as Atlantic cod (Gadus morhua) , common carp (Cyprinus carpio

L.), Nile tilapia, white sturgeon (Acipenser transmontanus), and rainbow trout (Oncorhynchus mykiss) [28-32]. Hormone concentrations could be very different between species. In wild migrating Pacific salmon (Oncorhynchus nerka) on spawning grounds, this kit was used for plasma cortisol tests to estimate the consequences of various stressful conditions on the physiology, behavior, and reproductive outcome [15, 33]. In Atlantic salmon, free and total plasma cortisol levels were quite often determined using commercial available ELISA kits [22]. In this study, we applied this ELISA assay for both plasma cortisol and FCM measurement in Atlantic salmon.

However, there are fundamental differences in the fecal media compared with plasma. Fecal corticoid metabolites are a mixture of several different metabolites with a wide range of polarities, and it was reported that in ruminants at least 21 FCM can be detected with great variation among species. Thus, no immunoassay will give complete accuracy because of the large numbers of metabolites [19, 20, 34] A relatively non-specific assay for FCM analysis was also suggested, using so-called group-specific antibodies that cross-react with a large number of structurally related corticoid compounds [35]. Currently, there are no standard assessment products covering all fecal FCM and valid for all species. To further validate the fish FCM method, future research might apply two or more EIA assays for corticosteroids and corticoid metabolites tests. HPLC could also be utilized for method validation and for identification of the substantive components of FCM in both the solid and liquid part of salmon feces.

For salmon FCM determination, the extraction of FCM from the fecal matrix prior to analysis was recommended. Before extraction, the feces sample was freeze dried and homogenized with a grinder, following a procedure which was technically demanding and time consuming [36]. However, FCM was also reported to be extracted directly from wet feces samples [14]. Palme reports the merits of collection of wet feces and further evaluated the protocols of storage, extraction, and immunoassay performance [37]. This provided an easier and simpler possibility for the salmon FCM assessment.

Since fecal corticoid metabolites are a mixture of several different metabolites, the selection of an appropriate extraction procedure was a serious issue for FCM assessment [19]. In the beginning, we failed to extract corticoid metabolites from the

salmon feces samples using diethyl ether. This could be due to conversion of cortisol to a more polar form in the liver. Flauger et al. compared extraction of horse FCM with methanol and diethyl ether. Three enzyme immunoassays that were already published were applied and HPLC was used for method validation. The results indicated that methanol suspension functions better than diethyl ether extraction [38]. Palme et al. has recently reviewed the differences between common extraction protocols used in combination with various immunoassays. Based on the results, they recommend to extract FCM by suspending the feces in a high percentage of primary alcohol, such as 80% aqueous methanol [13].

The collection of fecal casts from fish is difficult, particularly for free-living fish. For many small fish species, an insufficient volume of single casts could be a problem. However, these problems do not apply to farmed Atlantic salmon. Furthermore, the salmon feces sample is so moist that we were able to separate the liquid part by centrifuge, which made the direct test of FCM possible.

Our results showed that what we extracted from salmon feces, was insoluble in non-polar solvents like diethyl ether, but well soluble in polar solvents like methanol. We demonstrated that the FCM detected in unextracted liquid part of feces correlated well with the FCM extracted from both liquid and solid part of the corresponding samples without significant difference. This made it feasible to directly measure FCM in the liquid part of salmon feces without any extraction procedure.

Then, we applied this assay for FCM analysis in the group of salmon that experienced some completed infectious diseases. It was demonstrated that both plasma cortisol and FCM directly tested in the liquid part of salmon feces increased significantly during the outbreak of inflammatory disease, with the comparable peak values.

We noticed high standard deviation when summering the mean levels of plasma cortisol and FCM. This might because that mean cortisol level varied from month to month and between individual fish at each sampling. Unregistered stressful events happened in the cage, may affect the cortisol level, so also does the health condition of each fish. When we look into the data for each month shown in Fig. 4 and Fig. 5, the SD level was remarkably high in the period before and after the disease outbreak. This could be due to the different health condition of the fish. Namely, during that period, some fish were sick and had high cortisol level, while some were not.

Additionally, there seemed more individual variation in plasma cortisol levels than in FCM levels. It was demonstrated in Fig. 4 that plasma cortisol levels were especially high at the first month of the study, but the corresponding FCM levels were not. This variation might be induced by handling stress during the first sampling. Cortisol was instantly released into the blood in acute stressful situations, while cortisol in the corresponding feces sample was not affected by sampling. This indicated a skilled operator is required for the blood sampling to prevent handling related stress, while there is no such problem with FCM measurement. These results suggest the directly detected FCM could represent the total fecal FCM level and plasma cortisol level, and could be a proper indicator for salmon welfare.

Moreover, in this study, we compared the FCM in the outlooking similar fluide secretion from the digestive system collected during the disease outbreak period and pre-slaughtering starvation period. The significant difference indicated that several days-starvation might not be a strong stress factor for farmed salmon. No feeding and no feces do not directly increase plasma cortisol level, also not result in an enrichment of FCM in intestinal secretions. Nevertheless, the transmit time and volume of intestinal content were suggested to be important factors for incorporation of FCM into the sample, with respect to dilution or concentration effects [37]. Our future studies will also investigate the dynamic mechanism of FCM in the salmon digestive system, which is highly variable within and between species. Such studies could lead to a standard operating procedure for the collection, storage, and transport of fecal samples to the laboratory.

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Figure legend

Fig. 1. The variation of sea temperature, the salmon growth rate and the mortality rate from sea transfer until slaughter. Sea temperature is shown as a dashed line (℃), the salmon growth rate is shown as a solid li ne (%) and the mortality rate is shown as a dotted line (%)

Fig. 2. Extraction of salmon fecal corticoid metabolites using methanol. Two volumes of feces 100mg (shown as a solid line) and 300mg (shown as a dotted line) were well mixed with 1 ml methanol. Three concentrations of methanol were applied: 80%, 90%, and 100%. The final extraction products were dissolved in 100 µL of diluted extraction buffer (from the kit)

Fig. 3. Correlation between fecal corticoid metabolites (FCM) directly detected in unextracted liquid part of salmon feces (ULF) and FCM in methanol extraction product from liquid (ELF) and solid part of salmon feces (ESF). FCM concentrations directly detected in ULF were plotted versus FCM concentrations in ELF (A) and FCM in ESF (B). FCM concentrations detected in ELF were plotted versus FCM in ESF (C). The FCM directly detected in ULF correlated well with the FCM in ELF and ESF ($p<0.001$, correlation coefficient 0,874 (A) and 0.877 (B)); the latter two concentrations also correlated well with each other ($p<0.01$, $R=0.839$) (C).

Fig. 4. Box plots of serum cortisol concentration of salmons from March 2015 to February 2016. The median is the line within each box, the boxes indicate 25 – 75 percentiles, the whiskers 10 – 90 percentiles, and the non-filled circles the extreme values. Asterisk indicates a statistically significant difference between the relevant months and the other months ($P<0.01$)

Fig. 5. Box plots shows the concentration of fecal corticoid metabolites (FCM) directly detected in the unextracted liquid part of salmon feces (ULF) from March 2015 to February 2016. The median is the line within each box, the boxes indicate 25 – 75 percentiles, the whiskers 10 – 90 percentiles and the non-filled circles the extreme values. Asterisk indicates a statistically significant difference between the relevant months and the other months ($P<0.01$).

Fig. 6. Plot of plasma cortisol concentrations versus fecal corticoid metabolites (FCM) concentrations directly detected in unextracted liquid part of salmon feces (ULF).

Extracted plasma cortisol level correlated well with FCM level in ULF ($R=0.704$, $p<0.001$), without significant difference ($p>0.05$)

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NTNU

Figures for paper:

Establishment of a non-invasive method for stress evaluation in farmed salmon based on direct fecal corticoid metabolites measurement

Fig. 1

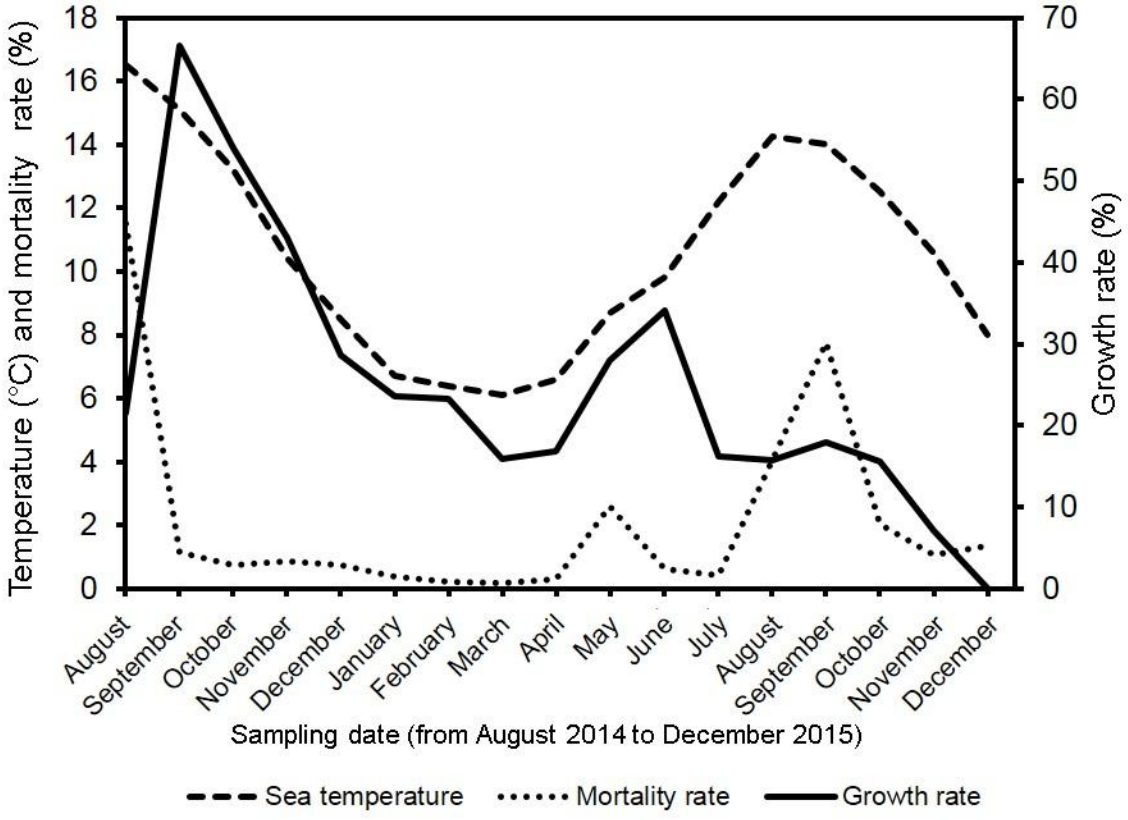

Temperature (°C) and mortality rate (%)
Growth rate (%)
0
2
4
6
8
10
12
14
16
18
0
10
20
30
40
50
60
70
August
September
October
November
December
January
February
March
April
May
June
July
August
September
October
November
December
Sampling date (from August 2014 to December 2015)
Sea temperature
Mortality rate
Growth rate

Fig. 2

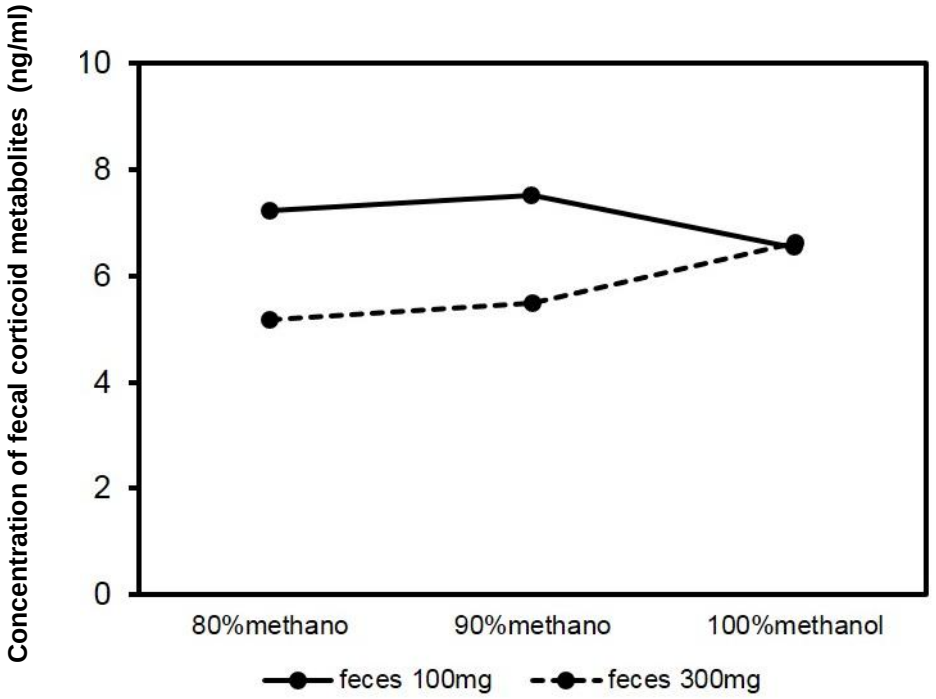

Concentration of fecal corticoid metabolites (ng/ml)
10
8
6
4
2
0
80%methano
90%methano
100%methanol
feces 100mg
feces 300mg

Fig. 3A

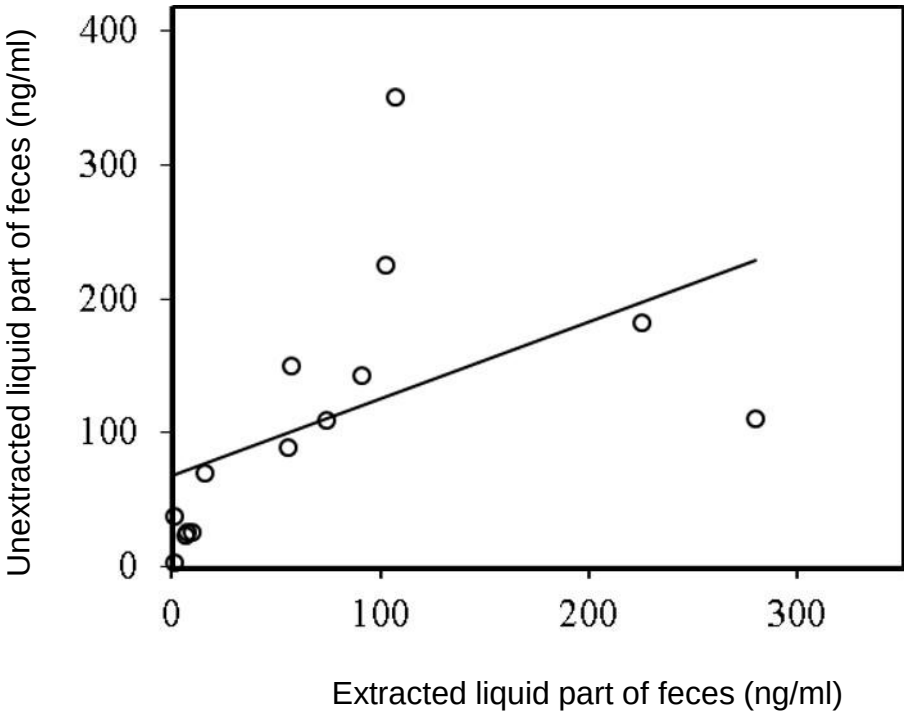

Unextracted liquid part of feces (ng/ml)
400
300
200
100
0
0
100
200
300
Extracted liquid part of feces (ng/ml)

Fig. 3B

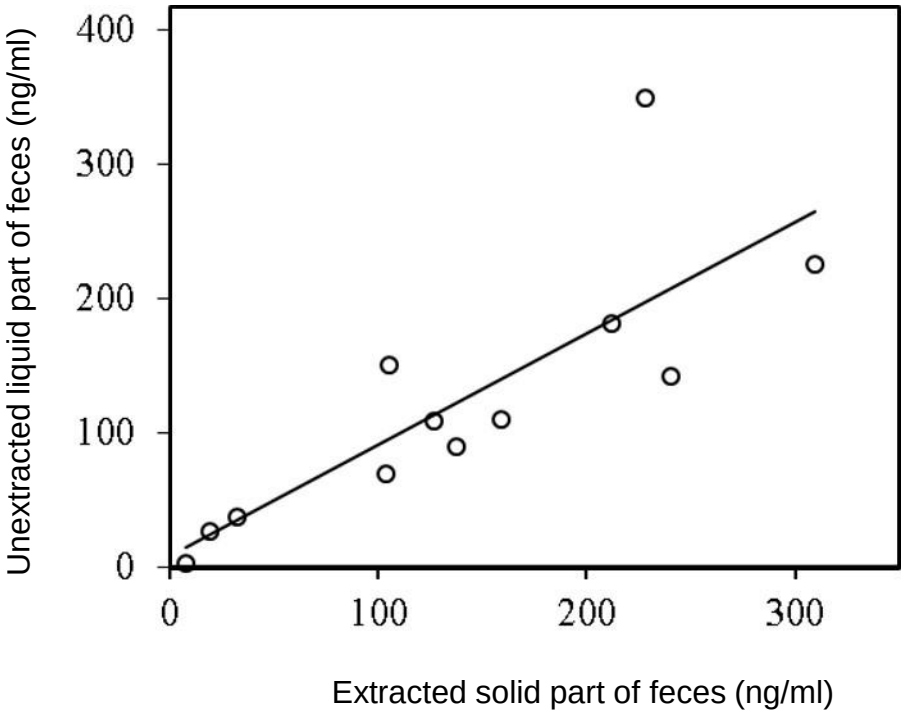

Unextracted liquid part of feces (ng/ml)
400
300
200
100
0
0
100
200
300
Extracted solid part of feces (ng/ml)

Fig. 3C

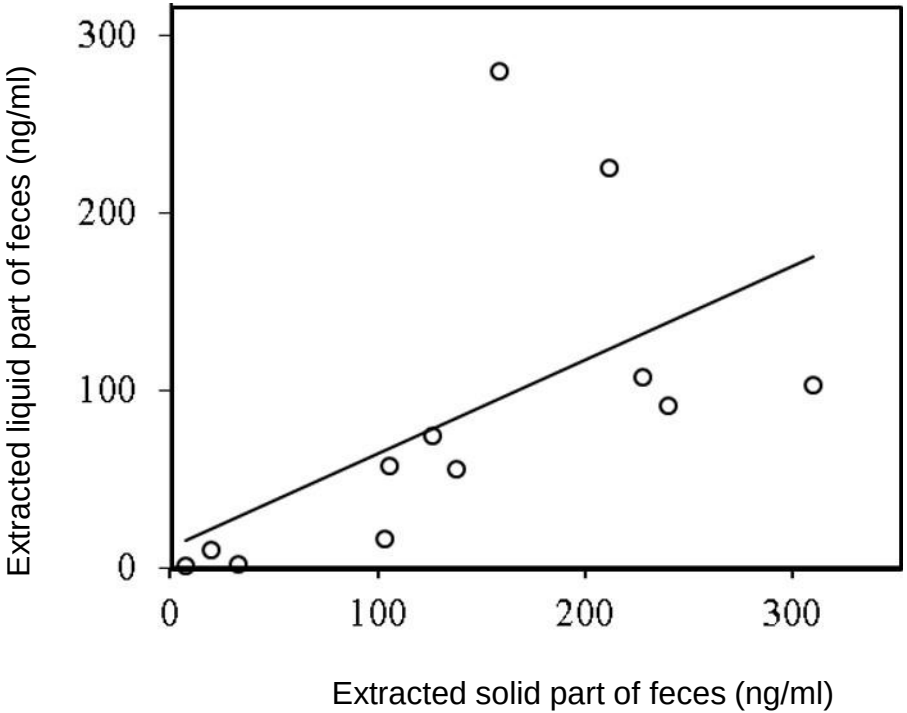

Extracted liquid part of feces (ng/ml)
300
200
100
0
0
100
200
300
Extracted solid part of feces (ng/ml)

Fig. 4

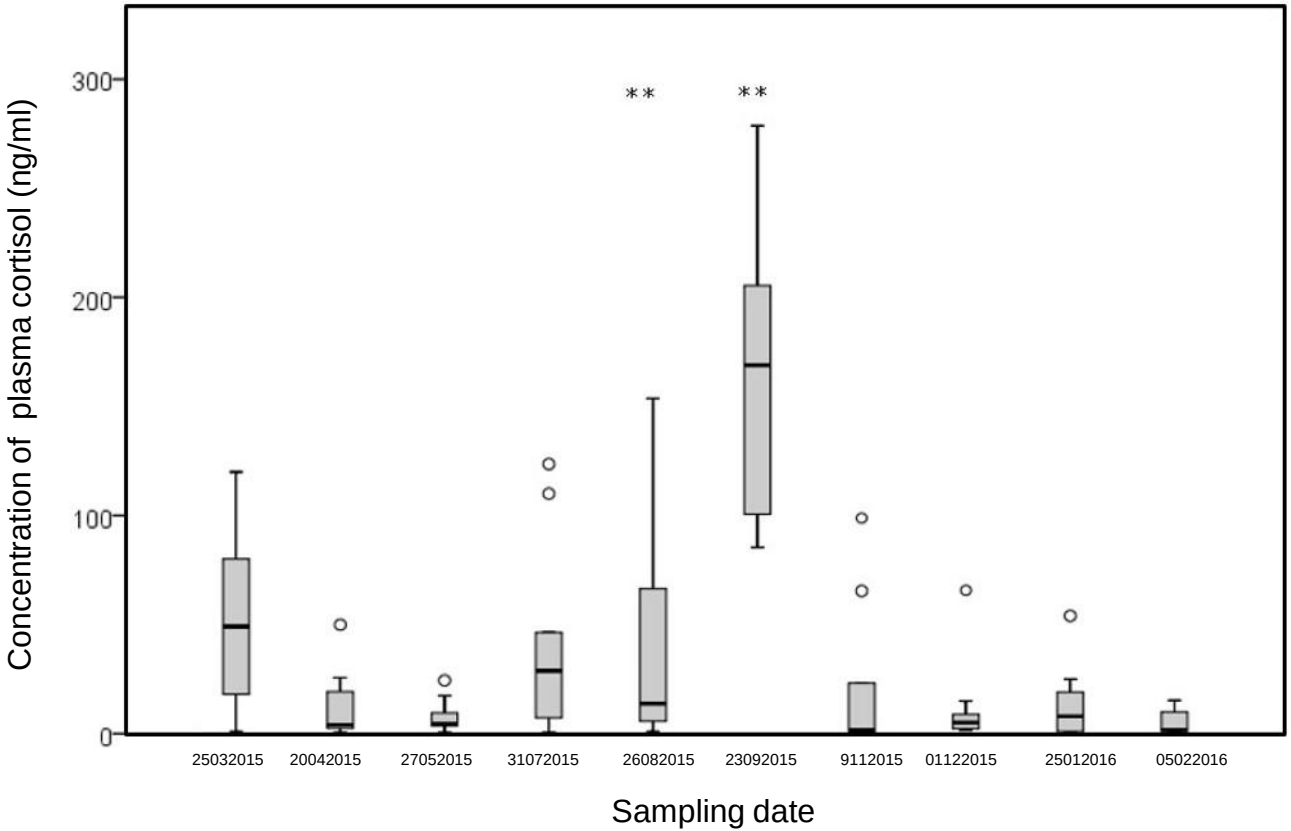

Concentration of plasma cortisol (ng/ml)
300
200
100
0
**
**
25032015
20042015
27052015
31072015
26082015
23092015
9112015
01122015
25012016
05022016
Sampling date

Fig. 5

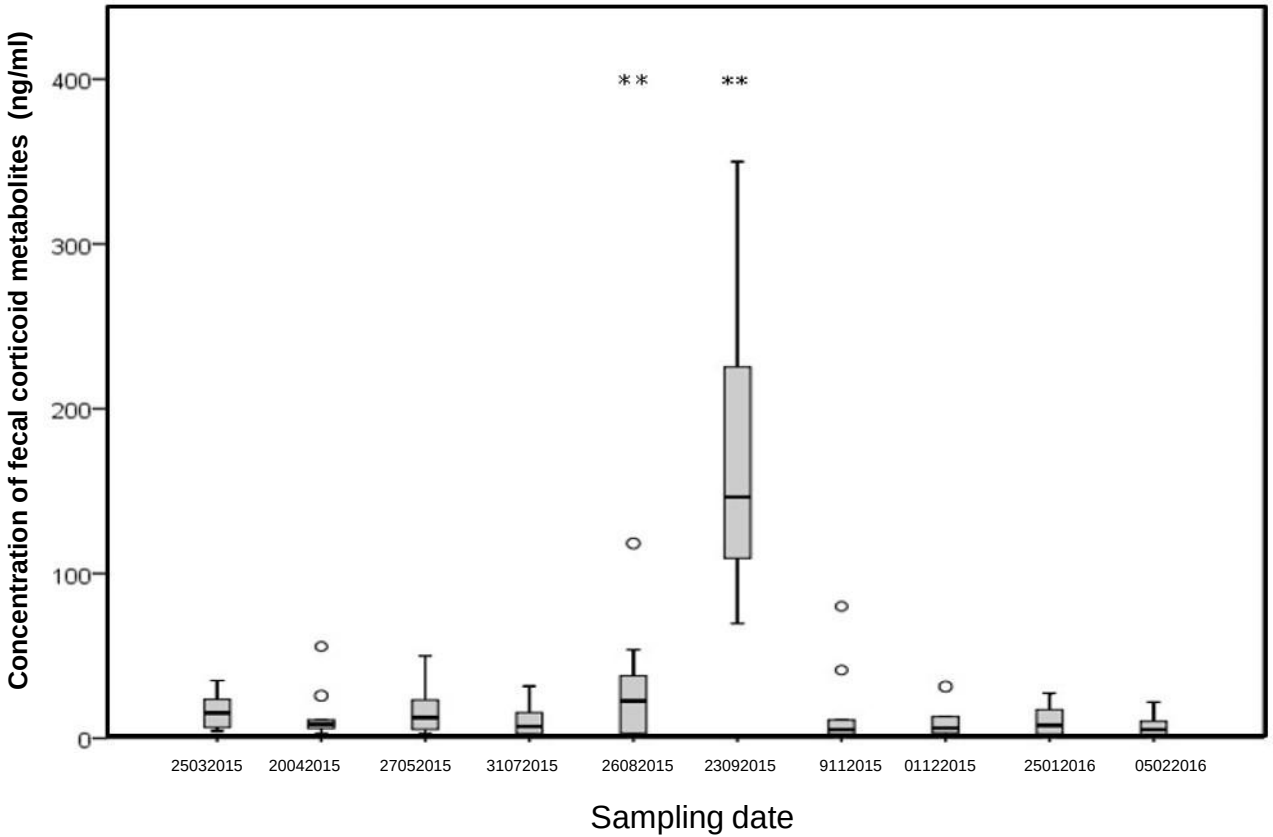
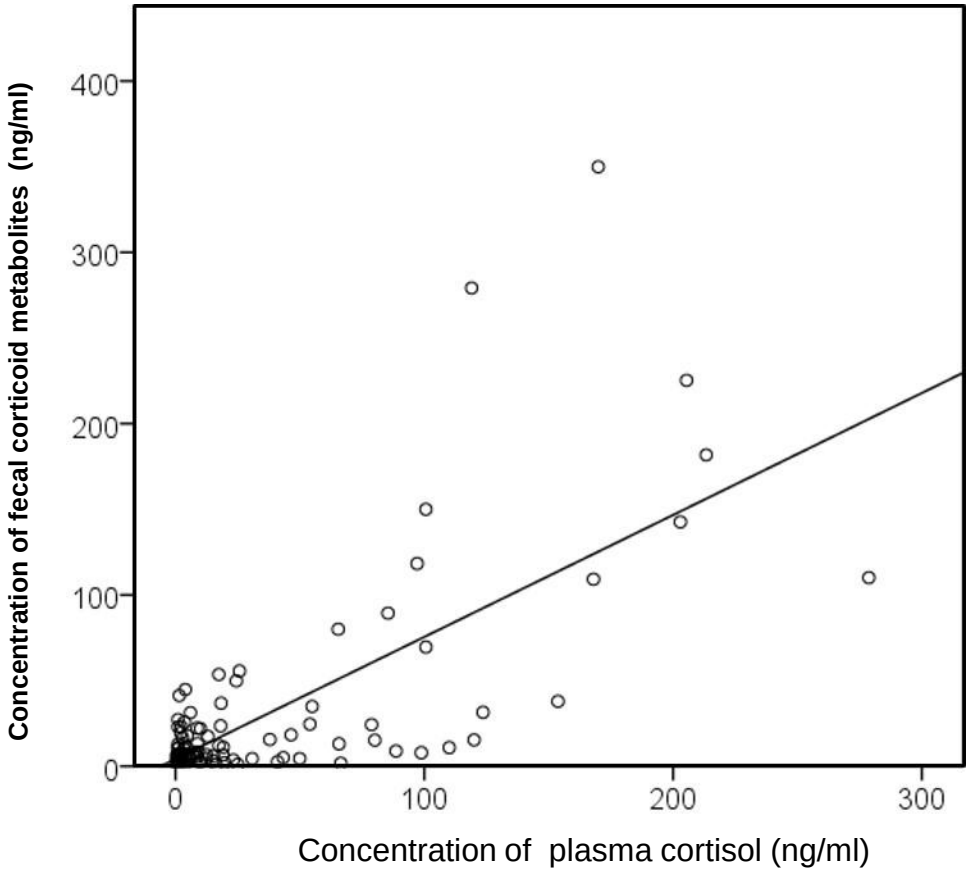

Concentration of fecal corticoid metabolites (ng/ml)
400
300
200
100
0
**
**
25032015
20042015
27052015
31072015
26082015
23092015
9112015
01122015
25012016
05022016
Sampling date

Fig. 6


Concentration of fecal corticoid metabolites (ng/ml)
400
300
200
100
0
0
100
200
300
Concentration of plasma cortisol (ng/ml)

PAPER REF. NO.: FSIM-D-16-01075

Title: “Direct fecal corticoid metabolites measurement - a non-invasive method for stress evaluation in farmed salmon

Highlights:

- Direct measurement of fecal corticoid metabolites (FCM) in the liquid part of salmon feces is proposed.
- Directly detected FCM level could represent the total fecal FCM and plasma cortisol level.
- The simple and non-invasive method makes FCM a proper indicator for salmon welfare.