



A multi-biomarker study on Atlantic salmon (*Salmo salar* L.) affected by the emerging Red Skin Disease in the Baltic Sea

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Abstract

For half a decade, the Atlantic salmon in the Baltic Sea has been facing severe health issues. Clinical signs like haemorrhage, erosions and ulcerative/necrotic skin conditions in returning adults have been reported from different Swedish rivers. These primary disease signs precede a secondary, terminal fungal infection. As initial investigations of the disease did not provide conclusive answers regarding the pathogenesis, this study was initiated to gain insight into a possible link between this so-called Red Skin Disease and anthropogenic influences. Therefore, returning salmon were caught in rivers along the Swedish coast and different tissues were sampled. The focus was put on the measurements of a battery of biomarkers as well as biochemical and haematological parameters, which were analysed using multivariate statistics. The main findings were a severe osmotic haemodilution, an immune response and an alteration of the carbohydrate metabolism in diseased fish. Furthermore, oxidative stress does not seem to be a likely factor in the pathogenesis. Concluding, certain changes in physiological parameters were shown to be indicative for the disease patterns, while others were ruled out as significant factors. Thus, this study contributes to the understanding of the Red Skin Disease and may act as a hypothesis generator for future studies.

KEYWORDS

Atlantic salmon, Baltic Sea, biomarkers, ecotoxicology, Red Skin Disease

1 | INTRODUCTION

In the summer of 2014, reports on heavily diseased and dead Atlantic salmon (*Salmo salar*, LINNAEUS 1758) came from different Swedish rivers along the Baltic Sea coast. Descriptions of diseased fish included apathy, wounds and fungal infections (mycosis).

Due to reoccurring summer outbreaks, with severe outbreaks in the rivers Torneälven and Mörrumsån (2014 and 2015) and the

rivers Ume-/Vindelälven and Kalixälven (2015), and also reoccurring late autumn mass mortalities due to mycosis, the Swedish National Veterinary Institute (SVA) and the Finnish Food Safety Authority Ruokavirasto (formerly Evira) initiated an examination of the health issues of the salmon in the summer of 2016 (ICES, 2018; SVA, 2017). It was noted that outbreaks varied considerably between regions and time. Summer mortalities were noted in Sweden and Finland, whereas autumn mycoses were also reported from other Baltic

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countries, such as Poland and Latvia. Furthermore, the investigations showed that, besides Ulcerative Dermal Necrosis (UDN)-like lesions (Figure S1) and other skin erosions (Figure S2), fish also exhibited other clinical signs such as skin haemorrhage (Figure S3) and mechanical wounds (from hooks or seal attacks), fin damage and scale loss of varying degree. These lesions provide a gateway for secondary mycosis (Figure S4), which eventually will lead to the death of the returners. Also, it was noted that grilse (returners with just one sea winter) seem largely unaffected by the disease. The examination, including histopathology of lesions and analysis of common bacterial, fungal and viral specimen, did not provide conclusive answers as no specific pathogen was identified as a common cause of the disease and only about half of the fish with UDN-like lesions were affected by UDN based on the diagnostic criteria for that disease (ICES, 2018; SVA, 2017).

Because of the characteristic haemorrhages along the abdomen of the fish, the disease was later named Red Skin Disease (RSD). This name was agreed upon during a workshop in Norway in November 2019, where participants from Denmark, Finland, Ireland, Norway, Russia, Sweden and the UK came together (ICES, 2020). The persistence of the reported disease and the apparent spread of RSD to other countries (i.e. Denmark, Ireland, Norway and the UK) (ICES, 2020) gives rise to great concern, as a significant number of returners may not reach their spawning grounds and thus not contribute to the recruitment of the next generation. Especially, in rivers like Mörrumsån and Ume-/Vindelälven, where RSD outbreaks have been reported every year since 2014/2015, the wild population might be at high risk to lose their self-sustaining capability if no actions are taken.

The Baltic Sea is one of the most polluted seas in the world, and in addition eutrophication and accompanied toxic cyanobacterial blooms are considered to be of great environmental concern in the area. The pollution of marine systems is nowadays recognized as a possible direct and/or indirect driver of diseases in various aquatic species (Bojko et al., 2020). Thus, the exposure to the environmental pollutants in the Baltic Sea could make organisms such as the salmon more susceptible to diseases and possibly be a contributing factor to the outbreaks of the RSD. Monitoring the effects of exposure to hazardous substances such as polycyclic aromatic hydrocarbons (PAHs), metals, oestrogenic and neurotoxic chemicals are often performed using biological markers (biomarkers). In the Swedish national monitoring programs on eelpout (*Zoarces viviparus*) and European perch (*Perca fluviatilis*), a battery of well-established biological markers have been used since the late 1980s (Ronisz et al., 2005; Sandström et al., 2005). Biomarker analysis in fish or other aquatic organisms may act as an early warning signal for specific pollutants, serve as an indicator of habitat quality, support the evaluation of the health status of organisms and can also be used to understand the aetiology of diseases. Thus, studies with multi-biomarker approaches are vital parts of environmental monitoring programmes and help to obtain a holistic picture of fish health (Asker et al., 2015, 2016; Hylland et al., 2017; Kammann et al., 2014; Lang et al., 2017; Lehtonen et al., 2014; Sturve et al., 2005).

As no conclusive underlying cause of the disease was identified during the investigation in 2016, a broader strategy was applied to search for additional contributing factor to the outbreaks. A new investigation on returning salmon in the Baltic Sea was thus initiated in 2018, including salmon from the rivers Mörrumsån, Torneälven, Ume-/Vindelälven and Indalsälven on the Swedish east coast as well as salmon collected at Lagan, on the Swedish west coast. The aim of this study was to evaluate a possible link between RSD in Atlantic salmon and anthropogenic influences, thus increasing the knowledge of RSD pathogenesis. This was achieved by (a) determining a battery of biomarkers as well as biochemical parameters in different tissues and bodily fluids of diseased and non-diseased fish, (b) analysing histopathology of liver and kidney (as complementing internal organs that could indicate exposure to pollution and associated changes) in diseased and non-diseased fish and (c) statistical analysis of the generated data with regards to sampling sites and especially with regards to the correlation between the determined parameters and disease signs. This study adds new insight into the physiology of fish affected by the emerging RSD and may act as a hypothesis-generating investigation to set focus towards topics for future studies.

2 | MATERIALS AND METHODS

2.1 | Sampling sites

Adult salmon returning to their spawning grounds were sampled during May to October 2018 in four Swedish rivers discharging into the Baltic Sea (Mörrumsån, Torneälven, Ume-/Vindelsälven and Indalsälven) and one (Lagan) discharging into the Atlantic Ocean (Kattegat). Fish from Mörrumsån, Torneälven and Ume-/Vindelsälven were sampled during the usual peak of the migration season. For logistical reasons, fish from Indalsälven and Lagan were sampled later in their migration season, therefore, being already semi-mature or mature for spawning. Details about each sampling site including catching methods and “housing” are described in Table 1. A map of the sampling sites is enclosed in Figure S5.

2.2 | Sampling and necropsy procedure

Fish were anaesthetized by a sharp blow to the head. Euthanasia was carried out by exsanguination, where 10–20 ml blood was drawn from the caudal vein or the heart using heparinized syringes. The collected whole blood was used to create blood smears and to determine haematocrit (HAEMATOKRIT 200, Andreas Hettich GmbH & Co. KG), glucose and haemoglobin concentrations (HemoCue® Glucose 201+ System and HemoCue® Hb 201+ System, HemoCue AB, Ängelholm, Sweden). The remaining blood was centrifuged (5,000 g, 2 min), and the supernatant plasma, as well as the remaining red blood cells (RBCs), were aliquoted and stored on dry ice.

TABLE 1 Summary of important information concerning the sampling sites as well as the sampling conditions

Site and county	River system	Coordinates/distance from river mouth	Catchment and keeping methods	Date	No. of individuals		Additional information about the river systems (HELCOM, 2011)
					♀	♂	
Mörnum, Blekinge County	Mörrumsån	56°11'32"N, 14°45'00"E/~14 km from estuary	Rod angling and trapping in a fish ladder. Fish were sampled immediately.	22/05/2018, 04/06/2018 and 05/06/2018	4	–	Length: 186 km Drainage area: 3,369 km ² Discharging into: main basin of the Baltic Sea Accessibility: 31 km Mörrumsån is anthropogenically influenced by hydropower dams and holds a self-sustaining wild population of Atlantic salmon.
Hedenäset, Norrbotten County	Torneälven	66°11'28"N, 23°45'51"E/~55 km from estuary	Traditional drift gill-netting. Caught fish were shortly held in an aerated tank until sampling.	16/06/2018 and 17/06/2018	15	5	Length: 522 km Drainage area: 40,131 km ² Discharging into: Bothnian Bay Accessibility: fully accessible Torneälven holds a self-sustaining wild population of Atlantic salmon, has no major migration barrier, high water quality and anthropogenic influences are regarded as minor.
Umeå, Västerbotten County	Ume-/ Vindelälven	63°52'42"N, 20°00'49"E/~25 km from estuary	Taken from the fish ladder at Norrforss hydropower dam. Fish were held in aerated tanks until sampling.	02/07/2018 and 03/07/2018	22	8	Length: 467/453 km (Ume-/Vindelälven) Drainage area: 26,783/12,630 km ² (Ume-/Vindelälven) Discharging into: Bothnian Bay Accessibility: 47 km/fully accessible (Ume-/Vindelälven) Umeälven is highly influenced by hydropower generating dams, which partly or completely block the passage for migrating fish. Vindelälven, joining Ume älv about 32 km from the river mouth, is free of migration hindrances and holds wild self-sustaining salmon populations. At Norrforss hydropower dam (25 km upstream from the estuary) all migrating fish must pass the longest fish ladder in Sweden (300 m with 76 steps). Vindelälven salmon are restocked at Norrforss in addition to the wild population.

(Continues)

TABLE 1 (Continued)

Site and county	River system	Coordinates/distance from river mouth	Catchment and keeping methods	Date	No. of individuals		Additional information about the river systems (HELCOM, 2011)
					♀	♂	
Sundsvall, Västernorrland County	Indalsälven	62°31'14"N, 17°23'25"E/~10 km from estuary	Caught and held in fish traps	03/09/2018 and 04/09/2018	12	8	Length: 430 km Drainage area: 26,727 km ² Discharging into: Bothnian Sea Accessibility: 10 km Indalsälven holds no self-sustaining wild salmon population. At Bergeforsen hydropower dam restocking of salmon is performed to maintain the river population.
Laholm, Halland County	Lagan	56°31'04"N, 13°02'54"E/~9 km from estuary	Electrofishing. Fish were treated with formaldehyde and held in tanks until sampling.	22/10/2018	13	–	Length: 232 km Drainage area: 6,452 km ² Discharging into: Kattgat Accessibility: 9 km Lagan holds no self-sustained population of salmon. The waterway is blocked by a hydropower dam in the city of Laholm, where salmon is restocked to maintain the Lagan-strain salmon.

Necropsy included documentation of external and internal pathology and intestinal parasites. Findings were scored based on intensity (e.g. depth of erosions and skin area affected).

If possible, bile was retrieved for chemical analysis and stored on dry ice. The liver was removed and pieces were stored in liquid nitrogen for enzymatic measurements and on dry ice for thiamine measurements. Pieces of liver and mid-posterior kidney were stored at room temperature in 4% phosphate-buffered formaldehyde for histopathology. The brain was stored in liquid nitrogen for acetylcholinesterase (AChE) activity determination. Pieces of white dorsal muscle were stored in liquid nitrogen for AChE activity determination and on dry ice for biochemical analyses. All tissues or bodily fluids initially stored on dry ice were transferred to -80°C freezers upon arrival at the laboratories. All animals were handled in concordance with the ethical permit approved by the local animal committee in Gothenburg, Sweden and registered under the number 5.8.18-02260/2018 and the identification number 001380.

2.3 | Morphometric indices

Fork length (FL) in cm, whole weight (WW), somatic weight (SW), liver weight (LW) and gonadal weight (GW) (all weights in g) were recorded for each fish. Fulton's condition factor (CF), liver somatic index (LSI) and gonadal somatic index (GSI) were calculated as follows: $CF = 100 \cdot SW \cdot FL^{-3}$, $LSI = 100 \cdot LW \cdot SW^{-1}$, $GSI = 100 \cdot GW \cdot SW^{-1}$.

2.4 | Oxidative stress biomarkers

For the hepatic activity determination of catalase (CAT), glutathione reductase (GR), glutathione S-transferases (GST), and the content determination of reduced L-glutathione (GSH), its oxidized disulphide dimer (GSSG) and protein carbonyls, liver samples were prepared as described by Sturve et al. (2005). Protein concentrations were determined with Folin-Ciocalteu reagent, using the Lowry protein assay (Lowry et al., 1951). The activities of CAT, GR and GST were determined in S9 fractions using methods described in Asker et al. (2015, 2016) and Sturve et al. (2005) and references therein. Briefly, CAT activity was determined with the method by Aebi (1974), GR activity was analysed using a modified assay of Smith et al. (1988) and Cribb et al. (1989) and GST was assayed with a method described by Stephensen et al. (2002), which has been adapted from Habig et al. (1974). Content of GSH and GSSG were determined using modified methods by Vandeputte et al. (1994) and Baker et al. (1990) as described in Sturve et al. (2005). The content of protein carbonyls in liver homogenates was determined using a modified assay based on Levine et al. (1994) and Reznick and Packer (1994), as briefly described by Carney Almroth et al. (2010).

2.5 | Detoxification biomarkers

The hepatic activity of 7-ethoxyresorufin-O-deethylase (EROD) was measured fluorometrically as described by Förlin et al. (1994) in microsomal fractions. The concentration of two metabolites of PAHs, namely 1-hydroxy-pyrene (1-OH-pyrene) and 1-hydroxy-phenanthrene (1-OH-phenanthrene), were determined in bile fluids, using an HPLC system with fluorescence detection (LaChrom, Merck Hitachi) and a reversed-phase column (Multospher® 100–3 C18, 3 × 125 mm) as described in Kammann et al. (2014, 2017). Furthermore, bile pigments were determined by measurement of total absorbance at a wavelength of 380 nm, using a FLUOstar OPTIMA microplate reader (BMG Labtech).

2.6 | Neurotoxicity biomarker

For the determination of AChE activity, muscle and brain samples were homogenized in a Na-phosphate buffer and centrifuged at 10,000 g (20 min at 4°C). Aliquots of the supernatant were immediately stored at –80°C until further analysis. The AChE activity was determined in the S9 fraction of muscle and brain samples using a modified version of the method described in Ellman et al. (1961).

2.7 | Haematological parameters

Slides with dried blood smears were fixed in acetone for 5 min and stained with Giemsa. Blood cell counts included neutrophil leucocytes, monocyte/macrophage leucocytes, proerythrocytes and erythroblasts. Plasma ions (K⁺, Na⁺, Cl⁻ and Ca²⁺) and plasma pH were measured using an electrolyte analyser (Convergys® ISE comfort Electrolyte Analyzer, Convergent Technologies). The lytic activity of the complement system in plasma samples was determined using human red blood cells of type A RhD+ (obtained from Sahlgrenska Hospital) as target cells (Zhang et al., 2003) and a modified assay as described in Ortuño et al. (2002). In brief, plasma samples were 8 step serially diluted, mixed with red blood cell suspension and incubated (120 min) at room temperature. Subsequently, haemolysis was determined photospectrometrically (wavelength: 414 nm). The titre of the complement system equals the reciprocal of the median-effect concentration (i.e. the plasma concentration at 50% haemolysis).

2.8 | Histopathology

Fixed tissue pieces were embedded in paraffin, cut and put on microscope slides. Sections were stained with haematoxylin-eosin. Kidney histopathology included evaluation of nephrons and interstitium, with regards to degenerative, regenerative and inflammatory changes. Liver tissue was analysed with regards to vacuolation state of hepatocytes, degeneration and necrosis of hepatocytes,

inflammation, haemorrhage and parasitological infestation. Slides were evaluated at 40–1,000 X magnification.

2.9 | Biochemical analyses

The free (non-bound) and total (bound and free) form of the thyroid hormones triiodothyronine (T3) and thyroxine (T4) were determined in plasma with an electrochemiluminescence immunoassay, utilizing methodologies provided by the cobas® systems of Roche (F. Hoffmann-La Roche AG). In muscle, the total carotenoid concentration was measured by spectrophotometry according to Pettersson and Lignell (1999) with minor modifications. Muscular astaxanthin was analysed according to Schierle et al. (2014), with minor modifications, using HPLC techniques (Agilent 1100, normal phase column, UV detector). Vitamins A (retinol) and E (α -tocopherol) were analysed in muscle tissue by HPLC (Agilent 1100, reversed-phase column, UV detector), according to European Commission Regulation (EC) No 152/2009 Annex IV (European Commission, 2009) with minor modifications. In liver tissue, free thiamine (i.e. non-phosphorylated form) and the metabolites thiamine monophosphate and thiamine pyrophosphate were analysed according to Vuorinen et al. (2002), using the HPLC apparatus Waters Alliance 2690 coupled to a Waters 2475 fluorescence detector.

2.10 | Statistical analyses

All statistical analyses were conducted in R (R Core Team, 2018). To analyse possible relationships between biomarkers and disease patterns, redundancy analyses (RDAs) were conducted on six different sets of explanatory variables (Figure 1). The first set (a) comprised of morphometric data. The following five sets comprised of tissue- or bodily fluid-specific biomarkers which were: (b) whole blood-associated, (c) blood plasma-associated, (d) liver-associated, (e) muscle-associated and (f) histopathology-associated. An additional RDA was performed, using all significant explanatory variables from the abovementioned RDAs. All explanatory variable sets were log[x + 1]-transformed prior to RDA modelling. The response variables for each RDA were the scores of the following external disease signs: haemorrhages, UDN-like lesions, skin erosions and mycosis.

RDAs and subsequent permutation tests were performed using the R package {vegan} (Oksanen et al., 2019). The magnitude of multicollinearity among explanatory variables was examined by inspection of the variance inflation factors (VIFs).

3 | RESULTS

Eighty-seven fish were sampled: 4 females from Mörrumsån, 15 females and 5 males from Torneälven, 22 females and 8 males from Ume-/Vindelälven, 12 females and 8 males from Indalsälven and 13 females from Lagan (Table 1).

Since mainly female fish were caught and as it is known that certain biomarkers may be sex-biased, data analyses were focused on female fish. Furthermore, the results presented here are focused on the multivariate analyses. The site-wise presentation of the results and univariate analyses can be found in Tables S1, S2, S7–S12).

3.1 | Whole blood-associated parameters

All whole blood-associated parameters and site-specific differences are summarized in Table S8.

The RDA for whole blood (Figure 2) considered the parameters of haematocrit, haemoglobin, glucose, neutrophil leucocytes, monocyte/macrophage leucocytes, proerythrocytes and erythroblasts. Permutation test showed that the model was globally significant ($p \leq .001$) and explained 43.8% of total variance along the first two axes (RDA1: 40.8%, RDA2: 3.0%). After adjustment of constrained variance ($R^2 = .4452$; $R^2_{\text{adj}} = .3589$), the explained variances were 32.9% and 2.4% for the first two RDA axes, respectively. VIFs did not exceed a value of five. Permutation tests for explanatory variables showed that glucose ($p \leq .001$), monocyte/macrophage leucocytes ($p = .013$) and neutrophil leucocytes ($p = .039$) significantly correlated with the disease pattern (Table S3). Glucose levels, counts of monocyte/macrophage leucocytes and neutrophil leucocytes play an important role in the dispersion of data from Ume-/Vindelälven. Glucose levels and

monocyte/macrophage leucocytes showed a strong positive correlation with the prevalence of UDN-like lesions, haemorrhages, skin erosions and mycosis along the first axis. Glucose rather explained the prevalence of skin erosions, while monocyte/macrophage leucocytes accounted for mycosis (Figure 2). Along the second RDA axis, neutrophil leucocytes exhibited a negative correlation with UDN-like lesions.

3.2 | Blood plasma-associated parameters

A summary and site-wise comparison of all plasma-associated parameters are given in Table S9.

For the RDA of plasma-associated parameters (Figure 3), the following explanatory variables were considered: titre of the complement system, plasma pH, concentrations of K^+ , Na^+ , Cl^- and Ca^{2+} , free and total levels of thyroid hormones T3 and T4 as well as ratios between thyroid hormones. After inspection of multicollinearity by VIF analysis, it was decided to remove all measurements of total thyroid hormones, because they were strongly correlated with free thyroid hormones. Furthermore, the ratio of free thyroid hormones was removed because it correlated with free T3 and free T4. Therefore, the model (Figure 3, Table S4) included the titre of the complement system, plasma pH, concentrations of K^+ , Na^+ , Cl^- , Ca^{2+} , free T3 and free T4 as explanatory variables, with VIFs being below five. The model was highly significant ($p \leq .001$) on a global scale

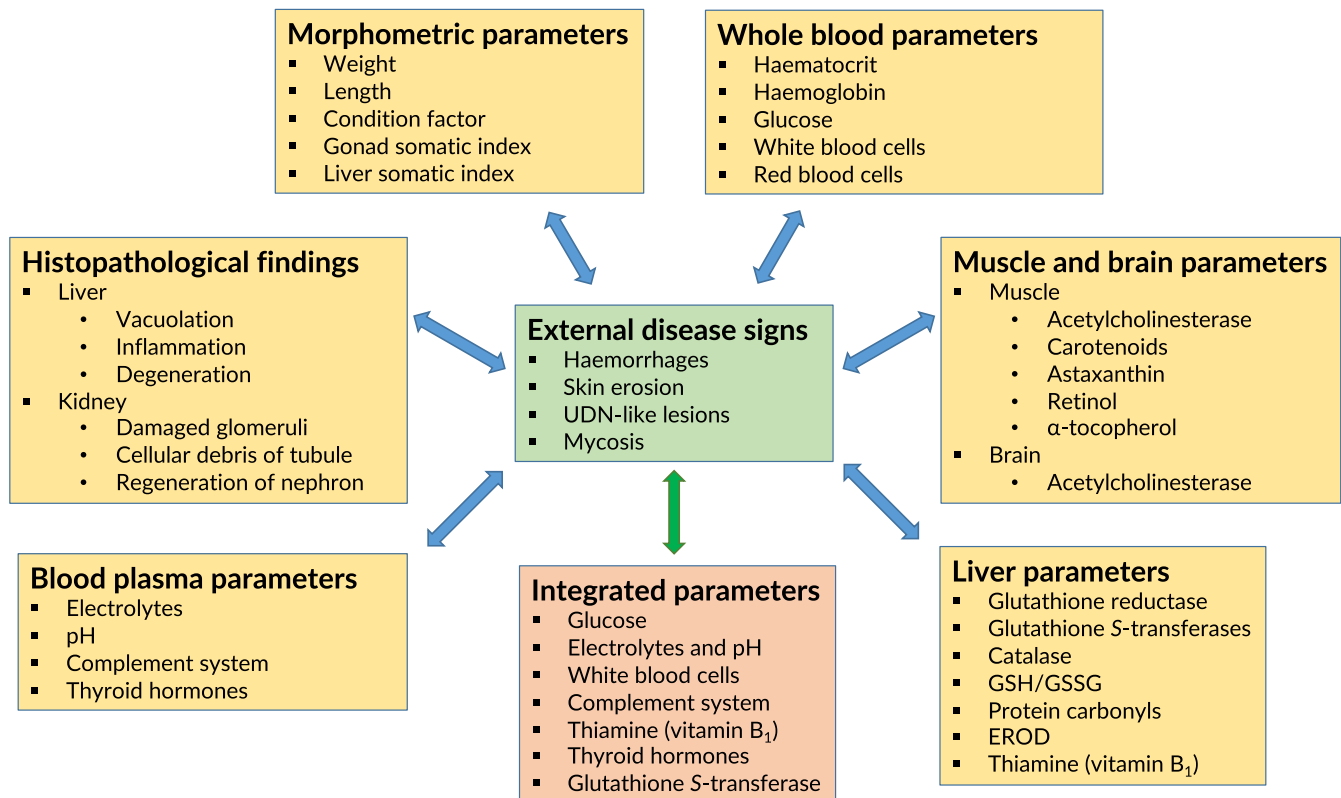


FIGURE 1 Overview over the seven parameter sets analysed in connection with gross external disease signs using redundancy analysis (RDA)

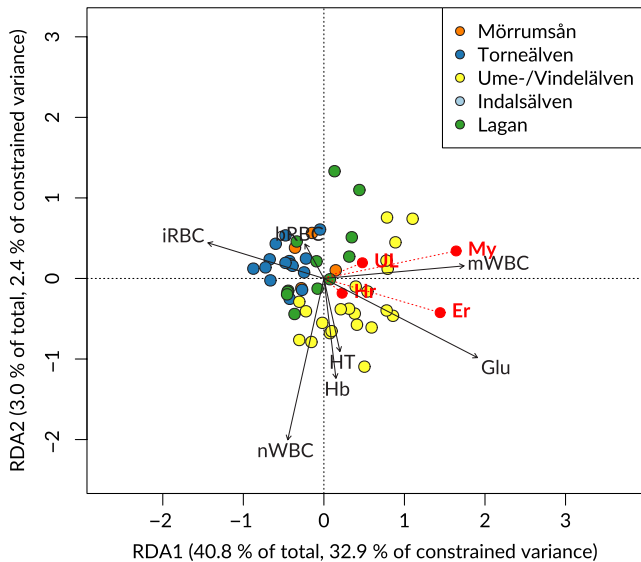


FIGURE 2 Redundancy analysis (RDA) of whole blood-associated biomarkers. Correlation triplot shows the ordination of clinical signs (red dots marked with Hr: haemorrhages, UL: UDN-like lesions, Er: other skin erosions and My: mycosis). Black arrows indicate the relation between whole blood-associated biomarkers—haematocrit (HT), haemoglobin (Hb), glucose (Glu), neutrophil leucocytes (nWBC), monocyte/macrophage leucocytes (mWBC), proerythrocytes (iRBC) and erythroblast (bRBC)—and disease pattern. Coloured dots show fitted site scores (linear combinations of explanatory variables) of individual samples. The cosine of the angle between the black arrows and the red dotted lines indicate an approximated correlation. Sample size per site: Mörrumsån ($n = 4$), Torneälven ($n = 15$), Ume-/Vindelälven ($n = 22$), Indalsälven ($n = 0$), Lagan ($n = 12$)

and explained 46.7% and 5.6% of total variance with the RDA axes 1 and 2, respectively. RDA1 accounted for 41.3% and RDA2 for 5.0% of constrained variance after adjustment ($R^2 = .5461$; $R^2_{adj} = .4824$). As described in Table S4, the explanatory variables of Na^+ , Cl^- , pH, the titre of the complement system, free T3 and free T4 were significantly related to the underlying disease pattern. Along RDA1, Na^+ , as well as Cl^- and free T3, correlated negatively with the manifestation of the disease pattern. Particularly, levels of Cl^- correlated negatively with the prevalence of skin erosions and as well as free T3 and Na^+ to mycosis (Figure 3). Levels of free T4 correlated positively with UDN-like lesions. A positive correlation was established between pH and the underlying disease pattern, while the correlations were apparent for mycosis, skin erosions and haemorrhages.

3.3 | Liver-associated parameters

In Tables S10 and S11, a summary of medians and site-specific differences for each liver-associated parameter is given.

The following parameters were used as explanatory variables in a liver-associated RDA: CAT, GR, GST, protein carbonyls, free thiamine and thiamine pyrophosphate. Due to the risk of severe data loss, PAH

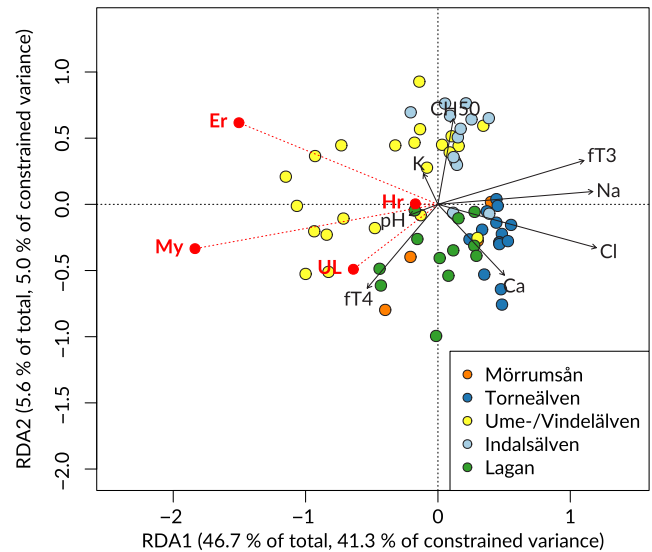


FIGURE 3 Redundancy analysis (RDA) of plasma-associated biomarkers. Correlation triplot shows the ordination of clinical signs (red dots marked with Hr: haemorrhages, UL: UDN-like lesions, Er: other skin erosions and My: mycosis). Black arrows indicate the relation between plasma-associated biomarkers—titre of the complement system (CH50), plasma pH and concentrations of potassium (K^+), sodium (Na^+), chloride (Cl^-) and calcium (Ca^{2+}), free levels of thyroid hormones T3 and T4 (ft3 and ft4)—and disease pattern. Coloured dots show fitted site scores (linear combinations of explanatory variables) of individual samples. The cosine of the angle between the black arrows and the red dotted lines indicate an approximated correlation. Sample size per site: Mörrumsån ($n = 4$), Torneälven ($n = 15$), Ume-/Vindelälven ($n = 22$), Indalsälven ($n = 13$), Lagan ($n = 12$)

metabolites were excluded from the model (only 40 female individuals had bile present at sampling). EROD was excluded as an explanatory variable, as the data showed a strong site-specific bias, which was also supported by the levels of PAH metabolites (Table S11). Furthermore, total thiamine content and thiamine monophosphate were excluded, as they correlated strongly with thiamine pyrophosphate and therefore yielded high VIFs, indicating multicollinearity. After exclusion of the abovementioned parameters, the VIFs were below five and permutation tests ascertained a highly significant global model ($p \leq .001$). The model explained 38.9% of total variance on the first two RDA axes (RDA1: 34.5% and RDA2: 4.4%) and after adjustment ($R^2 = .3996$; $R^2_{adj} = .2971$) it explained 25.7% (RDA1) and 3.3% (RDA2), adding up to 29.0% of constrained variance on its two first axes (Figure 4).

Permutation tests of explanatory variables showed that free thiamine ($p \leq .001$) and GST ($p = .037$) significantly explained the underlying data of disease (Table S5). Free thiamine levels correlated negatively with the prevalence of the clinical signs and explained predominately the occurrence of UDN-like lesions, skin erosions and mycosis. The activities of GST correlated positively with haemorrhages, but negatively with the occurrence of mycosis (Figure 4).

3.4 | Morphometric parameters

An RDA using the parameters CF, GSI and LSI as explanatory variables was performed but permutation tests did not show global significance ($p = .353$). Further details about the morphometric indices can be found in Table S7.

3.5 | Muscle- and histopathology-associated parameters

An RDA considering muscle-associated variables, namely muscular AChE, brain AChE (included even though not being muscle-associated), concentrations of carotenoids, astaxanthin, retinol and α -tocopherol was performed but did not prove to be globally significant ($p = .094$) according to permutation tests. A summary of all muscle-associated parameters and site-wise comparisons are given in Table S12.

Histopathological parameters of the liver and kidney were also analysed, using RDA. Explanatory variables for kidney tissue included counts of damaged glomeruli, debris in the tubular lumen, regenerated nephrons and a generalized kidney disease score, which

was calculated from presence and severity of the already mentioned histopathological findings and further abnormalities, such as macrophages, hyaline accumulation in renal tubuli or eosinophilia of renal tubuli cells. Liver tissue-associated histopathological parameters included vacuolation of hepatocytes, degeneration of hepatocytes, inflammation score (presence of macrophages and granulocytes) and a generalized liver disease score, which summarized the presence and degree of histopathological abnormalities already mentioned, as well as additional parameters like acute haemorrhages and parasitic infestation. Permutation tests of this histopathology-RDA did not show global significance ($p = .249$) and further analyses were therefore suspended.

3.6 | Integrated redundancy analysis

The previously significant parameters were used in an additional RDA. The parameters included were: glucose, neutrophil leucocytes, monocyte/macrophage leucocytes, the complement system titre, plasma pH and concentrations of Na^+ and Cl^- , free levels of thyroid hormones T3 and T4, GST and free thiamine. VIFs were below five and permutation tests indicated the global model as highly significant ($p \leq .001$). The model explained 55.1% of total variance on the first two RDA axes (RDA1: 50.1% and RDA2: 5.0%) and after adjustment ($R^2 = .5844$; $R^2_{\text{adj}} = .4671$) it explained 40.0% (RDA1) and 4.0% (RDA2), adding up to 44.0% of constrained variance on its two first axes (Figure 5).

Permutation tests of explanatory variables showed that glucose ($p \leq .001$), Na^+ ($p = .003$), monocyte/macrophage leucocytes ($p = .004$), neutrophil leucocytes ($p = .005$) and Cl^- ($p = .012$) significantly explained the underlying disease pattern (Table S6). Glucose levels and counts of monocyte/macrophage leucocytes were positively correlated with skin erosions and mycosis, respectively, while Na^+ and Cl^- were negatively correlated. Furthermore, neutrophil leucocytes were positively correlated with skin haemorrhage but negatively correlated to UDN-like lesions and mycosis (Figure 5).

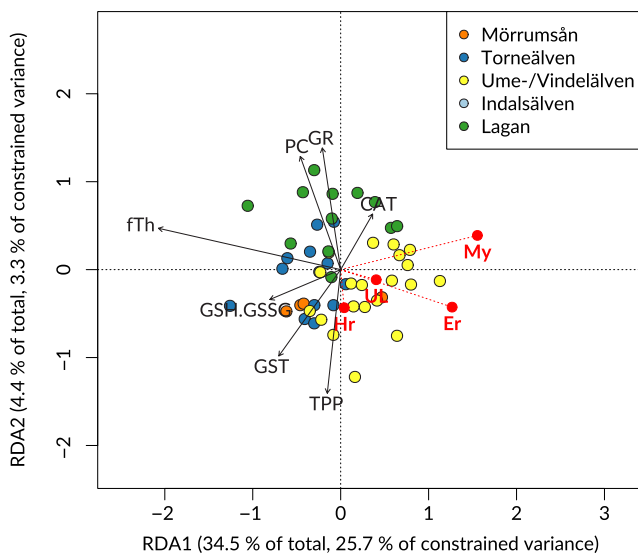


FIGURE 4 Redundancy analysis (RDA) of liver-associated biomarkers. Correlation triplot shows the ordination of clinical signs (red dots marked with Hr: haemorrhages, UL: UDN-like lesions, Er: other skin erosions and My: mycosis). Black arrows indicate the relation between whole blood-associated biomarkers—catalase (CAT), glutathione reductase (GR), glutathione *S*-transferases (GST), ratio between reduced and oxidized form of glutathione (GSH/GSSG), protein carbonyls (PC), free thiamine (fTh), thiamine monophosphate (TMP) and thiamine pyrophosphate (TPP)—and disease pattern. Coloured dots show fitted site scores (linear combinations of explanatory variables) of individual samples. The cosine of the angle between the black arrows and the red dotted lines indicate an approximated correlation. Sample size per site: Mörrumsån ($n = 4$), Torneälven ($n = 13$), Ume-/Vindelälven ($n = 20$), Indalsälven ($n = 0$), Lagan ($n = 12$)

4 | DISCUSSION

Due to the reoccurrence of severe RSD outbreaks among Atlantic salmon populations in the Baltic Sea and the apparent lack of an infectious cause (SVA, 2017), we initiated this study to gain a first insight into a possible anthropogenic influence as well as to increase the general understanding of the aetiology.

Therefore, we analysed a battery of different well-established biomarkers, histopathology and haematology as well as biochemical parameters in connection to the prevalence of the main clinical signs.

Examining our results, we found site-wise differences for several parameters measured, which in part may be due to temporal differences in sampling, as, for example displayed by the differences in gonadal development (GSI). However, here we present mainly the correlation analyses (RDA) in order to highlight parameters possibly involved in the pathogenesis of RSD, which will be discussed

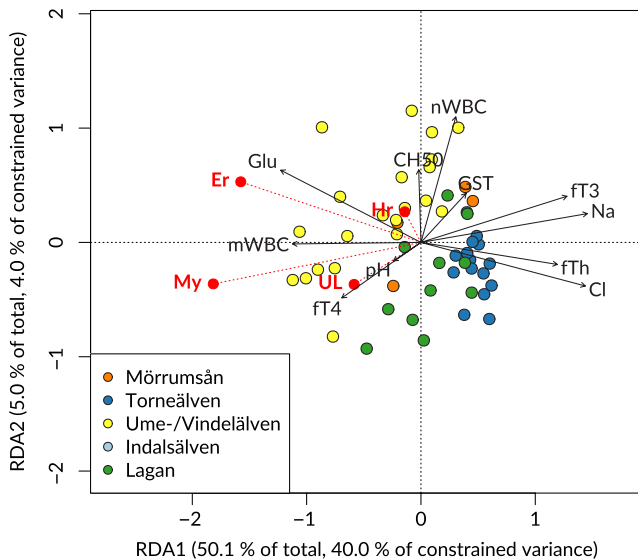


FIGURE 5 Redundancy analysis (RDA) of previously significant parameters. Correlation triplot shows the ordination of clinical signs (red dots marked with Hr: haemorrhages, UL: UDN-like lesions, Er: other skin erosions and My: mycosis). Black arrows indicate the relation between selected parameters—glucose (Glu), neutrophil leucocytes (nWBC), monocyte/macrophage leucocytes (mWBC), titre of the complement system (CH50), plasma pH and concentrations of sodium (Na^+) and chloride (Cl^-), free levels of thyroid hormones T3 and T4 (fT3 and fT4), glutathione S-transferases (GST) and free thiamine (fTh)—and disease pattern. Coloured dots show fitted site scores (linear combinations of explanatory variables) of individual samples. The cosine of the angle between the black arrows and the red dotted lines indicate an approximated correlation. Sample size per site: Mörrumsån ($n = 4$), Torneälven ($n = 15$), Ume-/Vindelälven ($n = 21$), Indalsälven ($n = 0$), Lagan ($n = 11$)

hereafter. Furthermore, we used a two-step approach in data analyses (integrated RDA) to find the most significant explanatory variables with regards to the disease prevalence.

Changes in haematological parameters may be due to a variety of alterations within the environment the fish inhabit (Barham et al., 1980; Bowers et al., 2000; Foda, 1973; Sandnes et al., 1988). In the present study, several haematological parameters were analysed. A first RDA revealed a highly significant correlation between glucose levels and the manifestation of the described disease. Glucose was also found to be a highly significant variable in the integrated RDA (Figure 5, Table S6). Changes in glucose levels have been studied extensively in fish for a variety of conditions (Polakof et al., 2012; Ytrestøy et al., 2001), and in most cases, hyperglycaemia was noted as a response to stress. In the present study, blood glucose was particularly high in diseased fish, compared with physiologically normal concentrations (Bowers et al., 2000).

Neutrophil counts were significant in both the RDA on whole blood-associated parameters (Figure 2, Table S3) and in the integrated RDA (Figure 5, Table S6). The RDAs indicated an initial neutrophilia and a possible end-stage neutropenia as a result of disease progression. Neutrophils are one of the first cells recruited to

a site of a pathogen insult and they play an important role in the anti-inflammatory response (Secombes & Wang, 2012), which will be exhibited by neutrophilia during early stages of a disease. As a pathogen load increases, acute neutropenia can suggest extravasation of neutrophils (Clauss et al., 2008) with a subsequent decrease of neutrophils in the blood. It has also been shown that neutrophils decrease drastically when an organism is unable to control, for example bacterial growth, and consequently is overwhelmed by the disease (Belon et al., 2014).

Furthermore, RDA showed that monocyte/macrophage counts were significantly correlated with the manifestation of the disease. Even though an increase of monocyte/macrophage leucocytes is not a marker for the identification of a specific pathogen, it indicates a response of the innate immune system. Macrophages play an important role in pathogen elimination but also in wound healing by promoting tissue repair and dampening inflammation (Secombes & Wang, 2012). The linear relationship between the severity of RSD and the immune response suggests that inflammation increases with the progression of clinical signs, which goes hand in hand with the development of mycosis in the terminal disease stage.

The complement system is an integral part of the innate immune system in fish. The RDA indicated a positive correlation between the activity of the complement system and the occurrence of skin erosions, while the other disease signs were negatively correlated. These results suggest that the skin erosions may trigger an immune response, but that when the disease progresses, the complement activity is down-regulated. This may show that potential pathogens responsible for skin erosions are initially detected by the complement system, while those responsible for the secondary mycosis do not trigger the activation of the complement system. However, the integrated RDA (Figure 5, Table S6) displayed the titre of the complement system as a less important explanatory variable.

Analysis of plasma electrolytes can reveal the effects of stress and impairment of the osmoregulatory system in fish (Waring et al., 1992; Wendelaar Bonga & Lock, 2008). In this study, RDA showed highly significant negative correlations between Na^+ and Cl^- and the disease manifestation, which was furthermore also shown in the integrated RDA (Figure 5, Table S6). These relationships indicate that diseased fish struggle to keep their osmoregulatory balance, which is manifested by a haemodilution in an environment with lower osmotic pressure (freshwater). Extensive skin erosions and especially fungal infection disturb the skin integrity so that its natural barrier function is severely diminished. Roberts (1993) described this effect for salmon affected by UDN—as soon as the basement membrane is eroded, lesions are infected by extended fungal growth and the fish will die of circulatory failure due to haemodilution.

In teleosts, thyroid hormones (T3 and T4) play major parts in a great array of vital processes involved in development, growth, behaviour, reproduction and osmoregulatory adaption. Throughout the life cycle of Atlantic salmon, large variations in T3 and T4 levels have been observed (Bowers et al., 2000; Brown et al., 2004; Persson et al., 1998; Stefansson et al., 2012; Youngson

& Webb, 1993). In the present study, greatly varying concentrations of thyroid hormones were found although the salmon were all at a similar stage of their life cycle. Considering the first RDA on blood plasma-associated variables, free T3 levels were found to be negatively correlated to the disease pattern, while free T4 levels rather exhibited a positive correlation. Interpretation of these patterns and correlations was difficult but the results are indicative of a disease-induced disruption in the thyroid hormone regulation. Whether this disruption is a general stress response (e.g. due to osmoregulatory stress) or is connected to a common cause of the pathogenesis remains unknown. However, the integrated RDA (Figure 5, Table S6) of all previously significant variables did not specify that the levels of free T3 and T4 are major indicators of the underlying disease.

Analyses of hepatic biomarkers were concentrated on oxidative stress and detoxification capacities. The site-wise comparisons of biomarkers directly involved in oxidative stress responses (i.e. GR, CAT, GSH/GSSG and PC) showed only small or no statistical differences and none of the abovementioned parameters appeared to correlate with the manifestation of RSD, indicating that oxidative stress has no major role in the pathogenesis. This is also supported by the fact that the correlation analysis with the non-enzymatic muscular antioxidants (i.e. total carotenoids, astaxanthin, retinol and α -tocopherol) did not show any significance. Only the hepatic GST activities were linked to the disease manifestation with a significant negative correlation in the liver-associated RDA. This significance was not found in the integrated RDA (Figure 5, Table S6), reducing the disease-indicating power of this explanatory variable.

As severe maternally transferred thiamine deficiencies were the major cause for the M74 syndrome—a reproductive disorder in yolk-sac fry of Atlantic salmon, which deeply affected salmon populations within the Baltic Sea—and widespread thiamine deficiencies in the Northern Hemisphere have been suggested (Balk et al., 2016; Bengtsson et al., 1999; Harder et al., 2018; Majaneva et al., 2020), the thiamine contents in liver samples were analysed. The free form of thiamine and the physiologically most important metabolite thiamine pyrophosphate were included in the RDA for liver-associated parameters and results suggested a negative correlation between the free form and the disease. The results from this study suggest that fish from Ume-/Vindelälven show signs of an altered thiamine metabolism, because of the proportional differences between the three forms in this population compared to the other sampled sites. Supportive of this hypothesis are the findings of highly influenced glucose levels, as thiamine is an important cofactor for enzymes involved in the carbohydrate metabolism (Harder et al., 2018; Manzetti et al., 2014). Nevertheless, it is the phosphorylated form of thiamine pyrophosphate that is the physiological important cofactor and levels of it did not differ, nor show a correlation to the disease manifestation. It appears that the diseased fish keep thiamine pyrophosphate levels constant, while free thiamine is depleted. This may reflect an overall lower level of free thiamine availability or indicate a higher demand for pyrophosphate metabolite (e.g. due to increased immune system activity) (Manzetti et al., 2014).

Supportive of the theory of lowered availability of free thiamine is that at physiological pH, free thiamine is existent in its cation form and it has been shown that uptake and distribution are connected to electrolyte balance (especially Na^+ and K^+) (Manzetti et al., 2014). The osmoregulation of diseased fish is strongly affected and thus, it might be that the cation form of free thiamine is susceptible to osmotic haemodilution as well. Furthermore, the integrated RDA (Figure 5, Table S6) did not show that free thiamine was a significant explanatory variable, diminishing its importance as an indicator for RSD.

4.1 | Conclusion

In conclusion, it was shown that the emerging RSD among Atlantic salmon is associated with a significant osmotic haemodilution, an interference with the carbohydrate, specifically the glucose metabolism and alteration of the immune system. Although the inflammatory response and haemodilution are definitely disease responses, it remains to be elucidated whether the other physiological alterations seen are part of the aetiology or a disease response.

Measurement of common oxidative stress biomarkers, as well as non-enzymatic antioxidants, suggested that a disturbance from oxidative stress does not play a significant role in the aetiology of RSD. Also, analyses of AChE activities in brain and muscle tissue did not show any connection with the disease prevalence. Thus, there were no indications that the disease may be caused by environmental pollutants that trigger neurotoxicity or oxidative stress.

The present study contributes to a better understanding of the new disease, as certain biomarkers were shown to be indicative of the disease and others were ruled out as possible explanatory factors. Thus, this first study helps setting focus for future investigations of the aetiology of RSD in Atlantic salmon (*Salmo salar*).

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CONFLICT OF INTERESTS

All authors declare that they do not have any conflict of interest.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article and its supporting materials.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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