# Effects of the emerging marine aquafeed mycotoxins beauvericin (BEA) and enniatin B (ENNB) in Atlantic Salmon (Salmo salar)

– Implications on cellular pathways and functions, and tissue responses

Sofie Søderstrøm

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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UNIVERSITY OF BERGEN

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## Scientific environment

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While it is with mixed feelings, I am ready for this PhD journey to come to an end. There are a few people to whom I owe extra gratitude, and they are my supervisors who have guided, encouraged, and tirelessly supported me both in success and in setbacks. For all the hours they have spent reading and given feedback on manuscripts and especially this thesis. Liv Søfteland, my main supervisor, not only shared her knowledge about experimental design, but also accompanied me into the lab to show me the techniques you can't learn from books. She always lent a helping hand during large experiments (however, according to the PCA we should have synced up better and finished the experiment before lunch). Kai Kristoffer Lie, my co-supervisor and project leader, taught me that I should go easy on the red pills and that “less is more” when it comes to writing discussions. Nevertheless, we could discuss about connections between pathways and functions for hours, divert into Swedish crime series, to then try and remember what we were talking about in the first place. Anne-Katrine Lundebye, my co-supervisor, came back to the office at 22:00 just to read through my revised manuscript one last time so that I could meet the deadline at midnight. Her experience and feedback saved me from going down the wrong rabbit hole. I have been fortunate to have had these very people as my supervisors, they have always kept their doors open for questions, brainstorming, given good advice and shared their scientific knowledge.

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Finally, I would like to give a "Patience Award" to my office mate, Jojo Tibon. Not only did he show great patience and acceptance of having to sit in darkness in the office (I can't concentrate with bright industrial lights on), but he also took my side in this madness every time coworkers questioned why we sat in the dark.

## Abstract

Plant-based feed ingredients are a source of the mycotoxins enniatin B (ENNB) and beauvericin (BEA) to farmed Atlantic salmon (*Salmo salar*). This has raised concerns regarding fish health and feed safety, since these mycotoxins are currently not legislatively regulated in animal feed due to insufficient knowledge about their toxicity. In the first paper (**Paper I**), primary hepatocyte cells isolated from Atlantic salmon were used as an *in vitro* model to test the mycotoxins' cytotoxicity and to identify mechanism of toxicity with non-targeted transcriptomics and experimental assays. Both BEA and ENNB were highly cytotoxic to salmon hepatocytes and caused oxidative stress, increased energy consumption, disturbed iron homeostasis, and affected processes that triggered ferroptosis. In the second paper (**Paper II**), primary head kidney cells isolated from Atlantic salmon were used as an *in vitro* model to investigate whether BEA and ENNB could influence the innate immune response by modulating inflammatory signaling. In exposed head kidney cells, BEA and ENNB did not disrupt inflammatory signaling following bacterial and viral stimuli. However, exposure to high concentrations of BEA and ENNB in non-challenged cells induced the transcription of pro-inflammatory cytokines similar to the bacterial stimuli. The third paper (**Paper III**) comprises a short-term acute gavage feeding trial with Atlantic salmon smolt, describing the early transcriptional effects in the intestine and liver after dietary exposure to BEA and ENNB. Global transcriptomic analysis indicates that BEA interfered with heme biosynthesis in salmon liver tissue, which could further cause reduced hematocrit levels and anemic conditions in the liver. ENNB triggered an acute inflammatory response in the intestine of salmon that could potentially lead to compromised intestinal integrity if exposure continued. Concerning feed safety, although BEA can cause toxic effects, the risk of exposure through the feed is relatively low as it is not very prevalent. Similarly, ENNB can also cause adverse effects, and since it is prevalent and can occur at relatively high levels in marine aquafeeds, there is a possibility that Atlantic salmon may be exposed to harmful levels of ENNB.

# Abstrakt

Plantebaserte fôringredienser er en kilde til mykotoksinene enniatin B (ENNB) og beauvericin (BEA) for oppdrettslaks (*Salmo salar*). Dette har skapt bekymring for fiskehelse og fôrsikkerhet, siden disse mykotoksinene foreløpig ikke er lovregulert i dyrefôr på grunn av utilstrekkelig kunnskap om deres toksisitet. I den første artikkelen (**Paper I**) ble primære hepatocytter isolert fra laks og brukt som en *in vitro*-modell for å teste mykotoksinenes cytotoksisitet og virkemåte med transkriptomikk og eksperimentelle analyser. Både BEA og ENNB var svært cytotoksiske for lakse-hepatocyttene og forårsaket oksidativt stress, økt energiforbruk, forstyrret jernbalanse og påvirket prosesser som utløste ferroptotisk celle død. I den andre artikkelen (**Paper II**) ble primære hodenyreceller isolert fra atlantisk laks brukt som en *in vitro*-modell for å undersøke om BEA og ENNB kunne påvirke immunresponsen og inflammatorisk signalering. BEA og ENNB forstyrret ikke inflammatorisk signalering i eksponerte hodenyreceller etter bakteriell og viral stimuli. Imidlertid initierte den høyeste konsentrasjonen av BEA og ENNB alene en akutt inflammatorisk respons ved å stimulere transkripsjon av proinflammatoriske cytokiner. Den tredje artikkelen (**Paper III**) omfatter et akutt kort-tids sondefôringsforsøk med laksesmolt, som beskriver de tidlige transkripsjonelle effektene i tarmen og leveren etter BEA og ENNB eksponering via fôret. De globale transkriptomikk analysene viste at BEA forstyrret hemebiosyntesen i levervevet til laks, som videre kan forårsake redusert hematokrit og anemiske tilstander i leveren. ENNB utløste en akutt inflammatorisk respons i tarmen hos laks som kan mulig, ved forlenget eksponering, føre til redusert tarmintegritet. I forhold til fôrtrygghet, så kan eksponering for BEA føre til skadelige effekter, men risikoen er lav fordi nivåene er relativt lave i laksefôr. Eksponering for ENNB kan også gi toksiske effekter, og fordi den er relativt utbredt kan det være en risiko for at oppdrettslaksen blir utsatt for skadelige konsentrasjoner av ENNB.

# List of Publications

**Paper I:**

Søderstrøm, S., Lie, K. K., Lundebye, A. K., & Søfteland, L. (2022). Beauvericin (BEA) and enniatin B (ENNB)-induced impairment of mitochondria and lysosomes-Potential sources of intracellular reactive iron triggering ferroptosis in Atlantic salmon primary hepatocytes. Food and Chemical Toxicology, 161, 112819.
https://doi.org/10.1016/j.fct.2022.112819

**Paper II:**

Søderstrøm, S., Lie, K. K*., Berntssen, M. H. G., Lundebye, A. K., Holen, E., & Søfteland, L*. Stimulation of proinflammatory cytokines and heme biosynthesis by beauvericin (BEA) and enniatin B (ENNB) in Atlantic salmon (*Salmo salar*) primary head kidney cells. *Manuscript submitted to Fish and Shellfish Immunology*.

**Paper III:**

Søderstrøm, S., Søfteland, L., Sele, V., Lundebye, A. K., Berntssen, M. H., & Lie, K. K. (2023). Enniatin B and beauvericin affect intestinal cell function and hematological processes in Atlantic salmon (*Salmo salar*) after acute exposure. Food and Chemical Toxicology, 172, 113557.
https://doi.org/10.1016/j.fct.2022.113557

# Abbreviations

| | |
|---|---|
| 3Rs | Reduce, Refine, Replace |
| ACAT | Acyl-CoA cholesterol acyltransferase enzyme |
| ADP | Adenosine diphosphate |
| Ahr | Aryl hydrocarbon receptor |
| Alas1[b] | 5'-aminolevulinate synthase 1 |
| Alas2[b] | Mitochondrial 5-aminolevulinate synthase (erythrocyte-specific) |
| Ank1[b] | Ankyrin 1 |
| AOP * | Adverse outcome pathway |
| ATP | Adenosine triphosphate |
| BEA | Beauvericin |
| C(1-8) | Complement components 1-8 |
| $Ca^{2+}$ | Calcium ion |
| Cox-2 | Cyclooxygenase-2 |
| CYPs | Cytochromes P450 enzymes |
| DAMP | damage-associated molecular patterns |
| DMSO | Dimethyl sulfoxid |
| Dmtn[b] | Dematin actin binding protein |
| ENNs | Enniatins |
| ENNA | Enniatin A |
| ENNA1 | Enniatin A1 |
| ENNB | Enniatin B |
| ENNB1 | Enniatin B1 |
| Epb4.1[b] | Erythrocyte membrane protein band 4.1 |
| Epo[b] | Erythropoietin |
| Epor[b] | Erythropoietin receptor |
| $Fe^{2+}$ | Ferrous iron ion |
| $Fe^{3+}$ | Ferric iron ion |
| Fsp1[b] | Ferroptosis suppressor protein-1 |
| Gata1[b] | Gata binding protein 1 |
| Gcl[b] | Glutamate cysteine ligase |
| Gpx[b] | Glutathione peroxidase |
| GSH/GSSG | reduced/oxidized glutathione |
| Gsr[b] | Glutathione reductase |
| Gst[b] | Glutathione S-transferase |
| $H_2O_2$ | hydrogen peroxide |
| Hba | Hemoglobin subunit alpha |
| Hbb | Hemoglobin subunits zeta |
| Hbe | Hemoglobin subunit epsilon |
| Hbz | Hemoglobin subunit zeta |
| Hemgn[b] | Hemogen |
| Hmox1[b] | Heme oxygenase-1 |

| | |
|---|---|
| Il-1β | Interleukin 1 beta |
| Il-6β | Interleukin 6 beta |
| IMR | Institute of Marine Research |
| Itga (7-11) | Integrin alpha (7-11) |
| $K^+$ | Potassium ion |
| LDH | Lactate dehydrogenase |
| $Li^+$ | Lithium ion |
| LPO | Lipid peroxidation |
| LPS | Lipopolysaccharides |
| $Mg^{2+}$ | Magnesium ion |
| MIE[a] | Molecular initiating event |
| MMP | Mitochondrial membrane potential |
| $Mn^{2+}$ | manganese ion |
| MOA[a] | Mechanism of action<br>*"a complete and detailed understanding of every step in the sequence of events that leads to a toxic outcome" (ECETOC, 2007)* |
| MoA[a] | Mode of action<br>*"a common set of biochemical, physiological, or behavioral responses that characterize an adverse biological response, where some of the linkages between an initiating event and an adverse outcome are understood" (ECETOC, 2007)* |
| mRNA | Messenger ribonucleic acid |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| $Na^+$ | Sodium ion |
| NADPH/NADP+ | Nicotinamide adenine dinucleotide phosphate |
| NFSA | The Norwegian Food Safety Authority (Mattilsynet) |
| Ncoa4[b] | Nuclear receptor coactivator 4 |
| NR | Neutral red |
| PAMP | pathogen-associated molecular patterns |
| PIC | Polyinosinic:polycytidylic acid |
| PPP | Pentose phosphate pathway |
| Prkca[b] | Protein kinase C alpha |
| PRR | Pattern-recognition receptors |
| PUFA | Polyunsaturated fatty acid |
| Pxr[b] | Pregnane X receptor |
| Rhag[b] | Rhesus associated glycoprotein |
| ROS | Reactive oxygen species |
| Socs (1-6) | Suppressor of cytokine signaling (1-6) |
| Sptb[b] | Spectrin beta erythrocytic |
| Tfr1[b] | Transferrin receptor 1 |
| Tfr2[b] | Transferrin receptor 2 |
| TLRs | Toll-like receptors |
| Tnfα | Tumor necrosis factor alpha |
| | *Toxicity pathway |

[a]*Terms as defined and summarized in Willett et al. (2014) in Table 1.*
[b]*In this thesis, the nomenclature is in line with the ZFIN Zebrafish Nomenclature Conventions (https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Conventions), i.e. fish genes are written in lowercase italic, and fish proteins in non-italic and first letter uppercase.*

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# 1. General Introduction

## 1.1 Mycotoxins in feed and food

Mycotoxins produced by crop molds occur globally and contaminate food, animal feeds, and raw feed materials when the conditions are favorable, posing a challenge to both feed- and food safety (Hussein and Brasel, 2001). Field crops can become mold-infested pre-harvest, or molds can develop during storage of raw materials and commodities post-harvest (Bryden, 2012). However, the optimal conditions for mold growth do not necessarily correspond with increased production of mycotoxins. Enhanced production of mycotoxins often reflects a stress response in molds, for example triggered if the mold is unable to assimilate nutrients, or if exposed to a sublethal concentration of a biocide (Moss, 1991). A changing climate with rising global temperatures and increased rainfall causes fluctuating humidity vs drought cycles, (Rosenzweig et al., 2001), regionally shaping the composition of mold species, and more importantly their production of mycotoxins (Gruber-Dorninger et al., 2019). Climate changes may result in the loss of cultivation areas on a global scale, while "new" areas with suboptimal climatic conditions for crop growth may increase the risk of mold-susceptibility and thus mycotoxin contamination (Zingales et al., 2022). Mycotoxins of most concern are generally produced pre-harvest by *Fusarium* field molds, or by storage molds such as *Aspergillus* and *Penicillium* post-harvest (Sweeney and Dobson, 1998). Many species of *Fusarium* are plant pathogenic fungi of economic concern, since they can cause diseases (e.g., Fusarium head blight (FHB)) in crops leading to harvest yield losses and reduced grain quality (Dean et al., 2012, Timmusk et al., 2020). The severity of *Fusarium*-related diseases is increasing with the increase in climate-related weather fluctuations (Timmusk et al., 2020). Animal feeds and food contaminated with mycotoxins have in some instances been shown to cause acute mycotoxicosis or more diffuse chronic low-level effects in animals and humans (Hussein and Brasel, 2001, Richard, 2007). A few mycotoxins cause acute mycotoxicosis, which refers to a severe and sudden onset of toxic effects that occur after exposure to a high dose of mycotoxins over a short exposure period. The symptoms can include nausea, vomiting, diarrhea,

abdominal pain, fever, convulsions, and even death (Antonissen et al., 2014). More common is chronic low-level mycotoxin exposure over an extended period. Chronic exposure to mycotoxins can result in a range of health effects that may be subtle and difficult to detect, such as immune suppression, neurotoxicity, developmental delays, and in some cases cancer (D'mello et al., 1999, Antonissen et al., 2014, Kinoshita et al., 2018). In European terrestrial livestock feeds, the most prevalent mycotoxins are aflatoxins, fumonisins, ochratoxin A, deoxynivalenol, T-2 toxin, and zearalenone (Santos Pereira et al., 2019). For example, aflatoxin B1 and M1 are mycotoxins of major concern since they are known carcinogens to humans as well as to a wide range of animal species (Marchese et al., 2018). Aflatoxin was found to cause hepatic necrosis in poultry, referred to as "Turkey X disease" in 1960, which led to the first labelling of poultry feeds (Goldblatt, 2012). Due to the known hazards of these mycotoxins and to ensure feed and food safety, monitoring programs of European animal feeds routinely assess the prevalence of mycotoxins of concern. To control the risk associated with these mycotoxins (Streit et al., 2012, Cheli et al., 2014, Pinotti et al., 2016), maximum limits or guidance values have been assigned to feeds and feed commodities by the European Commission (EC, 2016).

## 1.2 Atlantic salmon farming and aquafeed – composition change over the past decades

Atlantic salmon (*Salmo salar*) aquaculture has become one of the largest seafood industries in the world, where Norway is the major producer of farmed Atlantic salmon accounting for more than 50 % of the global production (FAO, 2018). Initially in the early 1990s, fishmeal constituted 65.4 % and fish oil 24 % of the total aquafeed composition. Whereas in 2016, fishmeal was reduced to 14.5 % and fish oil to 10.4 % by instead incorporating 40.3 % plant-derived protein and 20.2 % plant-derived oil (Fig. 1) (Aas et al., 2019). Thus, the plant-based ingredients now constitute 60-65 % of the feed ingredients in Norwegian marine aquafeeds and thus reduce the "fish-in-fish-out" ratio (Aas et al., 2019, Mowi, 2021), i.e. the amount of wild fish used in feed for

every one kg farmed Atlantic salmon produced (Tacon and Metian, 2008, Jackson, 2009).

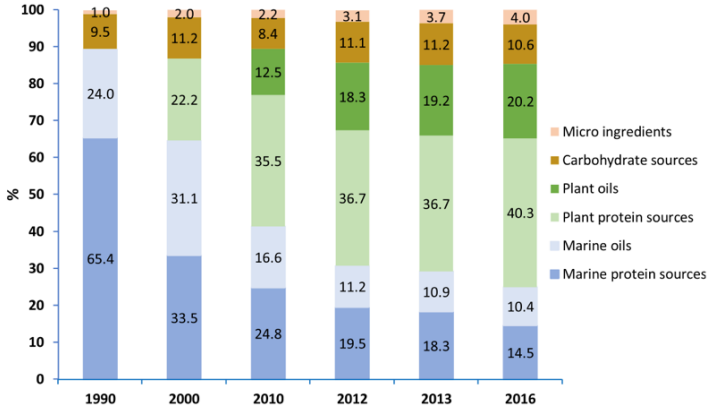



Fig. 1. Aquafeed composition changes (%) over time. (Aas et al., 2019).

This "blue-green-shift" (blue = marine-based feed ingredients, green = plant-based feed ingredients) (Sanden et al., 2016b) was partly driven by increased pressure on wild fish stocks and the increasing prices of marine feed ingredients, which did not support an upscaling of farmed fish production. At the same time, there was an increased demand from consumers for the development of more sustainable farmed salmon production (Ytrestøyl et al., 2015). A typical plant-based feed composition used for Atlantic salmon farming today is seen in Fig. 2.

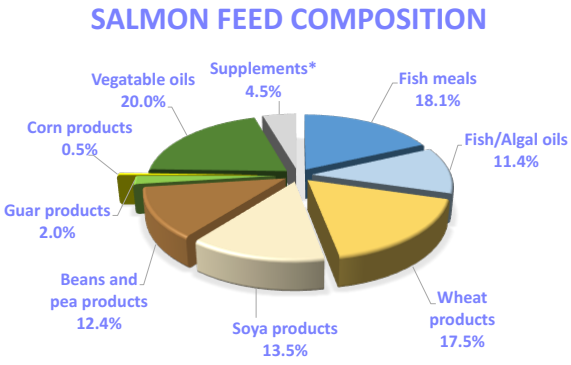



Fig. 2. Typical salmon feed composition today. Percentage of feed ingredient sources constituting Mowi-produced salmon feed (*vitamins, minerals, amino acids, yeast derivatives) (Mowi, 2021).

Atlantic salmon are fed plant-based feeds both in the freshwater- and seawater phases (E. Hevrøy, Mowi, personal communication, February 24, 2023) (Fig. 3). Smoltification and the freshwater/seawater transfer are vulnerable stages in salmon farming where increased mortality may occur.

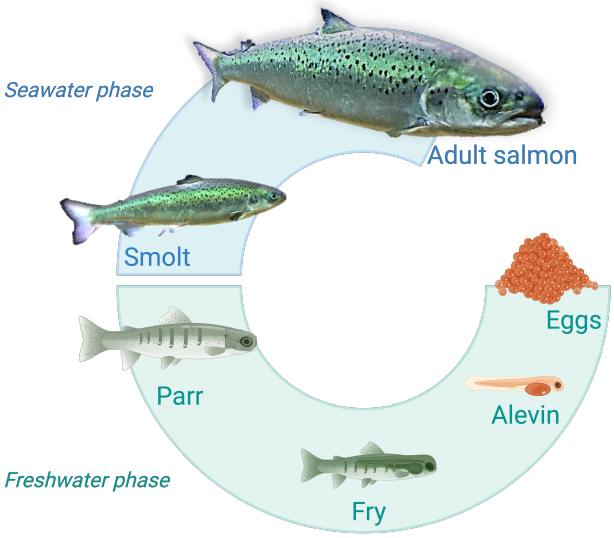



Fig. 3. The life and production cycle of farmed Atlantic salmon (*Salmo salar*) from the egg-stage in the freshwater phase to slaughter-ready fish in the seawater phase. Figure created in BioRender.com

Analysis of mortalities in Norwegian salmon farming indicates that the majority of the 50 million fish that die each year do so within the initial three months of being transferred to seawater (Oliveira et al., 2021). Premature death in the early phase of the seawater transfer is associated with poor smolt quality, whereas mortality occurring later during production is typically linked to illnesses and events related to handling (Persson et al., 2022). The reason for this increased mortality remains unknown, prompting the need for identifying factors that may affect the smolts' robustness and immune defense, which could impair their ability to withstand the stress the freshwater/seawater transfer generates. High mortality not only leads to significant economic losses but is also a key indicator of poor fish welfare (Ellis et al., 2012).

## 1.3 Emerging mycotoxins in aquafeeds

The feed compositional shift to mainly plant-based feed ingredients has introduced several novel contaminants (Berntssen et al., 2010), such as mycotoxins (Sele et al., 2019, Søderstrøm et al., 2022) previously not linked to farmed fish. Several studies have reported on the occurrence of mycotoxins in feed ingredients and marine aquafeeds (VKM et al., 2013, Nacher-Mestre et al., 2015, Bernhoft et al., 2017, Pietsch, 2020, Tolosa et al., 2021). These mycotoxins are introduced during the production of the marine aquafeeds through the use of mold- or mycotoxin-contaminated raw materials (Oliveira and Vasconcelos, 2020). Apart from aflatoxins and ochratoxin A, produced by the storage molds *Aspergillus* and *Penicillium*, most other mycotoxins associated with marine aquafeeds are produced by *Fusarium* field molds. *Fusarium* spp. produce mycotoxins such as beauvericin (BEA), deoxynivalenol, enniatins (ENNs), fumonisins, T-2 toxin, HT-2 toxin, moniliformin, nivalenol, and zearalenone (Jestoi et al., 2004, Uhlig et al., 2006, VKM et al., 2013). The Norwegian Food Safety Authority (NFSA) is responsible for implementing guidance values for safe levels of mycotoxins and fungi in the animal feed used in agriculture and aquaculture within Norway (NFSA, 2019). NFSA adheres to the regulations set forth by the European Commission for feeds and feed commodities (EC, 2016), but may also modify these guidelines to meet Norwegian safety requirements. Currently, only aflatoxin B1 has an established maximum allowable level (Lovdata, 2002). Available guidance values for mycotoxin levels permitted in marine aquafeeds and feed ingredients according to Norwegian legislation are summarized in Table 1.

Table 1. Norwegian guidance values on recommended limits for mycotoxin in salmon feed and feed ingredients (NFSA, 2019).

| Mycotoxin | Marine aquafeed/ Feed ingredient | Limit [µg/kg feed] |
|---|---|---|
| *Pre-harvest* | | |
| Deoxynivalenol | Feed | 2000 |
| | Cereals/ Corn | 8000/ 12,000 |
| Fumonisins | Feed | 10,000 |
| | Corn | 60,000 |
| T-2/HT-2 toxins | Feed | 250 |
| | Cereals/ Oat bran | 500/ 2000 |
| Zearalenone | Feed | 1000 |
| | Cereals/ Corn | 2000/ 3000 |
| *Post-harvest* | | |
| *Aflatoxin B1 | Feed | 10 |
| | Feed ingredients | 20 |
| Ochratoxin A | Feed | 1000 |
| | Cereals | 250 |

** Mycotoxin with established maximum limit (Lovdata, 2002).*

On behalf of the NFSA, the Institute of Marine Research (IMR) conducts a yearly monitoring of mycotoxin occurrence in commercial fish feed and feed materials (Sanden et al., 2016a, Sanden et al., 2017, Sele et al., 2018, Sele et al., 2019, Ørnsrud et al., 2020, Sele et al., 2021, Sele et al., 2022). The national monitoring program for fish feed screens for the following mycotoxins: aflatoxin B1, B2, G1, and G2, deoxynivalenol, fumonisin B1, fumonisin B2, T-2/HT-2 toxins, ochratoxin A, zearalenone, BEA, and enniatin A (ENNA), enniatin A1 (ENNA1), enniatin B (ENNB), and enniatin B1 (ENNB1).

In contrast to aflatoxin B1, deoxynivalenol, fumonisins, T-2/HT-2 toxins, ochratoxin A, and zearalenone, ENNs and BEA are currently not under any legislative regulation as no maximum limits or guidance values have been set due to insufficient knowledge about their toxicity *in vivo* (EFSA, 2014, NFSA, 2019). However, the highly lipophilic mycotoxins ENNB and BEA have been reported to be prevalent in European (Tolosa et al., 2014, Nacher-Mestre et al., 2020) (Table 2). These mycotoxins have also been found in Norwegian plant-based marine aquafeeds, where ENNB exhibited a higher prevalence, and the highest maximum concentration, in comparison to the other ENNs and the structurally similar BEA (Fig. 4) (Table 2) (Paper III).

Table 2. The prevalence (%) and mean, minimum and maximum concentrations (µg/kg feed) of BEA and ENNs in marine aquafeeds from Europe and Norway, with n = number of feed samples analyzed.

| Mycotoxin | Prevalence (%) | Mean [µg/kg] | Min - Max [µg/kg] | n | Reference |
|---|---|---|---|---|---|
| *Europe* | | | | | |
| BEA | 100 | 1.4 | 0.1 - 6.6 | 20 | (Tolosa et al., 2014) |
| ENNB | 100 | 0.89 | 0.1 - 3.2 | 20 | *sea bream/seabass feed* |
| BEA | 100 | 30 | <1.0 - 80.4 | 21 | (Nácher-Mestre et al., 2020) |
| ENNB | 100 | 19.9 | <1.0 - 32.8 | 21 | *n=17 salmon feed, n=4 seabream feed* |
| *Norway* | | | | | |
| BEA | 4 | 16 | 10 - 25 | 200 | |
| ENNA | 0.5 | <LOQ | 11 | 200 | (Paper III) |
| ENNA1 | 2 | 12 | 10 - 16 | 200 | *salmon feed* |
| ENNB | 80 | 37 | 10 - 250 | 200 | |
| ENNB1 | 27 | 19 | 10 - 54 | 200 | |

ENNB and BEA have never been reported to cause acute mycotoxicosis in animals or humans (VKM et al., 2013, EFSA, 2014). However, the occurrence of ENNB and BEA in feed and feed ingredients makes chronic low-dose exposure of particular concern (Bertero et al., 2018, EFSA, 2014). Especially with regards to potential health effects on farmed Atlantic salmon, since *in vivo* toxicity data for ENNB and BEA in salmon was completely lacking prior to Paper III and Berntssen et al. (2023). Elucidating ENNB's, and the structurally similar BEA's, mechanism(s) and modes of toxic action in Atlantic salmon were the main goals of this thesis.

## 1.4 The emerging mycotoxins BEA and ENNB - Current scientific understanding

ENNs and BEA are recognized for their cytotoxicity, phytotoxicity, antimicrobial- and insecticidal activity (Prosperini et al., 2017, Mallebrera et al., 2018). They have also shown a potential for cancer treatment (Heilos et al., 2017, Dornetshuber-Fleiss et al., 2015). The cytotoxicity of both ENNB and BEA has been well documented and described in a plethora of mammalian cell lines. These studies have been extensively reviewed by Prosperini et al. (2017) and Mallebrera et al. (2018). A limited number of studies have assessed the cytotoxicity of BEA or ENNB in fish using mitochondrial metabolism, membrane integrity, lysosomal function, and surface adhesion

measurements (Table 3). BEA exhibited high cytotoxicity in cell lines derived from desert topminnow (*Poeciliopsis lucida*) hepatocellular carcinoma (PLHC-1) and rainbow trout hepatoma (RTH-149) (García-Herranz et al., 2019) (Table 3). BEA was more cytotoxic than ENNB in rainbow trout gill cells (RTgill-W1) after 24 hours exposure (Bernal-Algaba et al., 2021) (Table 3). In primary hepatocytes from Atlantic salmon exposed for 48 hours, both mitochondria and lysosomes were affected to a similar extent by BEA, while ENNB had a more significant effect on mitochondrial metabolic activity compared to lysosomal function (Paper I).

Table 3. Cytotoxicity in different cell lines derived from fish elicited by enniatin B (ENNB) and beauvericin (BEA).

| *Fish cell line:* | | IC50 [µM] | | | |
|---|---|---|---|---|---|
| | | a**PLHC-1** *Desert topminnow* | a**RTH-149** *Rainbow trout* | b**RTgill-W1** *Rainbow trout* | c**Hepatocytes** *Atlantic salmon* |
| **ENNB** | Mitochondrial metabolism (AlamarBlue/MTT) | | | 112.19 | 4.2 - 5.66 |
| | Plasma membrane integrity (CFDA-AM) | | | >156.29 | |
| | Lysosomal function (NR) | | | 26.62 | 11.14 |
| | Surface adhesion/Morphology (xCELLigence) | | | | 3.23 |
| **BEA** | Mitochondrial metabolism (AlamarBlue/MTT) | 3.69 | 7.60 | 16.64 | 2.57 - 4.97 |
| | Plasma membrane integrity (CFDA-AM) | 5.97 | 17.36 | 16.75 | |
| | Lysosomal function (NR) | 4.63 | 14.42 | 3.84 | 2.58 |
| | Surface adhesion/Morphology (xCELLigence) | | | | 5.05 |

[a]*García-Herranz et al. (2019)*, [b]*Bernal-Algaba et al. (2021)*, [c]*Paper I – Primary salmon hepatocytes*

Ultimately, due to their cytotoxicity BEA and ENNB have been shown to initiate apoptosis and necrosis in mammalian cells (Prosperini et al., 2013a, Prosperini et al., 2013b, Jonsson et al., 2016).

### 1.4.1 Known mechanisms of toxicity

*Ionophoric properties*

ENNs and BEA are cyclic depsipeptide mycotoxins (Fig. 4) produced through secondary metabolism by several species of *Fusarium* molds. They have free electron pairs that can form weak interactions with ions giving them ionophoric properties (Jestoi, 2008), which is suggested to be BEA's and ENNB's underlying mechanism of toxicity (Mallebrera et al., 2018, Prosperini et al., 2017). Ionophores are lipid-soluble molecules produced by microorganisms such as fungi and bacteria. They can either be channel-forming or act as ion-selective carriers reversibly binding specific ions and

facilitating their transport across cellular lipid membranes, causing alteration of electrochemical gradients and osmotic balance (Bergen and Bates, 1984, Feher, 2017). The lipophilic characteristics of BEA and ENNB makes them prone to incorporation in the lipid bilayers of cellular- and organelle membranes (Kouri et al., 2003, Kamyar et al., 2004, Tonshin et al., 2010). BEA and ENNB can affect the transport of several essential cations ($K^+$, $Ca^{2+}$, $Na^+$, $Mg^{2+}$, $Li^+$) either by acting as ion carriers or as cation-selective channels (Ivanov et al., 1973, Prince et al., 1974, Hilgenfeld and Saenger, 1985, Kouri et al., 2003, Kamyar et al., 2004, Kouri et al., 2005). Through altering cellular ionic concentrations, BEA and ENNB disrupts pH homeostasis, a mode of toxic action which in turn disturbs normal cell- and organelle function (Kamyar et al., 2004, Kouri et al., 2005, Tonshin et al., 2010, Wu et al., 2018).

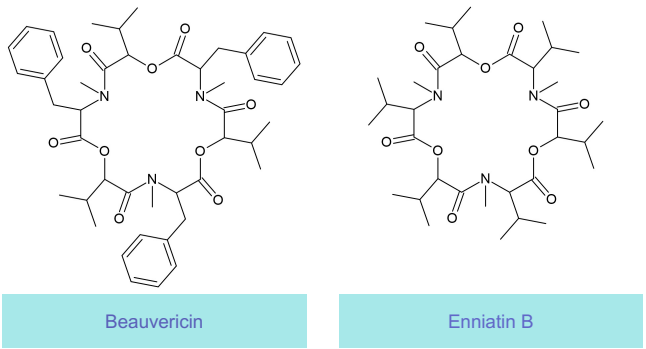



Fig. 4. The structures of beauvericin (BEA) and enniatin B (ENNB). Structure illustrations created by Carey Donald, 2022.

**Depolarization of membranes**

BEA (Kouri et al., 2003, Kouri et al., 2005) and ENNs (Kamyar et al., 2004) can, by influencing the transport of $Ca^{2+}$ and $K^+$ across membranes, disrupt physiological ion concentration gradients that are needed to uphold normal cellular and organelle function.

*Mitochondrial membranes*

Mitochondria have been revealed as the main targeted organelles *in vitro*, and the mitochondrial toxicity exerted by ENNB and BEA has been extensively researched in

mammalian cell lines (Tonshin et al., 2010, Prosperini et al., 2013a, Prosperini et al., 2013b, Alonso-Garrido et al., 2018, Escrivá et al., 2018, Escrivá et al., 2019, Alonso-Garrido et al., 2020a, Alonso-Garrido et al., 2021). Mitochondria are organelles reliant on a well-regulated cation influx/efflux and cycling in order to maintain proper function and signaling (Garlid and Paucek, 2003). A well-regulated cation balance is vital for the electron transport chain that drives oxidative phosphorylation of ADP to ATP during mitochondrial respiration (Stockwell et al., 2020). The mitochondrial membrane potential is the strongest electrochemical potential found within a cell, making these organelles especially susceptible to ionophore toxicity (Alonso-Garrido et al., 2018). Both BEA and ENNB depolarized mitochondrial transmembrane potential, uncoupled oxidative phosphorylation, and depleted ATP production in guinea pig (*Cavia porcellus*) myocytes and rat liver mitochondria (Kouri et al., 2005, Tonshin et al., 2010) (Table 4). In addition, BEA and ENNB were shown to cause depolarization of mitochondrial transmembrane potential in human colon carcinoma Caco-2 cells (Prosperini et al., 2013a, Prosperini et al., 2013b) (Table 4). ENNB cytotoxicity was also reported to cause mitochondrial permeabilization in Caco-2 cells (Ivanova et al., 2012) (Table 4). Similarly, transcriptomic and proteomic data indicated that ENNB and BEA modify pathways related to the electron transport chain and oxidative phosphorylation important for mitochondrial respiration in Jurkat and ECV304 cells (Escrivá et al., 2018, Alonso-Garrido et al., 2018, Manyes et al., 2018, Escrivá et al., 2019, Alonso-Garrido et al., 2020a, Alonso-Garrido et al., 2020b, Alonso-Garrido et al., 2021) (Table 4).

*Red blood cell membrane*

The electrochemical gradient across the red blood cell membrane is upheld by a low cation permeability to avoid osmotic dysregulation and shrinkage (eryptosis) or swelling (lysis) (Thomas and Egée, 1998). Both BEA and ENNA have been found to trigger eryptosis by disrupting the membrane of human red blood cells (Qadri et al., 2011, Jilani et al., 2011). In addition , BEA and ENNB to a lesser extent, were reported to exhibit weak hemolytic activity towards human red blood cells (Olleik et al., 2019).

*Bacterial membranes*

The mechanisms of ENNB and BEA antimicrobial activity were linked to their ability to interact with the lipid membranes of Gram-positive bacteria and fungi, though they had no effect on Gram-negative bacteria (Olleik et al., 2019). The authors found that the chemical structure of ENNs and BEA, in particular the presence and number of side chains, influenced their ability to interact with the microbial lipid membranes. Through their ionophoric properties, ENNs and BEA depolarized the membranes of Gram-positive bacteria (potentially acting as ionic channels), while causing low or no bacterial membrane permeabilization (Olleik et al., 2019). Furthermore, the bacterial membrane depolarization was not the sole mechanism of ENNs and BEA antimicrobial activity. While ENNB and BEA also inhibited protein synthesis, ENNA and ENNA1 inhibited RNA synthesis (Olleik et al., 2019).

*Other toxicological mechanisms*

Other reported mechanisms of by which BEA and ENNB exert their toxicity are 1) their ability to induce oxidative stress by production of reactive oxygen species (ROS), which in turn increases the level of lipid peroxidation (LPO). 2) The oxidative stress is counteracted by the activation of the antioxidant system, often reflected by a decrease in glutathione levels. 3) Ability to cause cell cycle alterations. 4) Their involvement in enzyme inhibition/activation. 5) Their immunomodulatory actions. These mechanisms of toxicity are summarized in Table 4.

Table 4. Described mechanisms of toxicity by BEA and ENNB *in vitro* other than, or downstream of their ionophore activity.

| Mycotoxin | Conc [μM] | Time | Cell model | Mechanism/Outcome | Reference |
|---|---|---|---|---|---|
| BEA | 1.0 - 30 μM | 2 - 3 days | Guinea pig myocytes | Mitochondrial membrane depolarization<br>Disrupted ionic homeostasis and pH<br>ATP depleation<br>Altered cellular metabolism | (Kouri et al., 2005) |
| BEA | 1.5 - 50 μM<br>(NR IC50 = 17.22 μM)<br>(MTT IC50 = 12.08 μM) | 2, 24, 48 h<br>24 h<br>24 h | Chinese hamster ovary cells (CHO-K1) | ROS and LPO generation<br>Lysosomal function impairment<br>Mitochondrial metabolic activity | (Ferrer et al., 2009) |
| BEA | 1.5 - 3.0 μM<br>(>12 μM)<br>(NR IC50 = 8.8, 3.4, 1.9 μM)<br>(MTT IC50 = 20.6, 12.8, 3.2 μM) | 24, 48, 72 h | Human colon carcinoma (Caco-2 cells) | Mitochondrial membrane depolarization<br>ROS and LPO generation<br>GSH decrease: GSSG increase<br>Cell cycle arrest<br>Apoptosis -> Necrosis<br>DNA damage<br>Lysosomal function impairment<br>Mitochondrial metabolic activity | (Prosperini et al., 2013a) |
| BEA | 0.1 - 5 μM | 24 h | Chinese hamster ovary cells (CHO-K1) | ROS and LPO generation<br>GST and GPx increased<br>GSH and GR decrease | (Mallebrera et al., 2015)<br>(Mallebrera et al., 2014) |
| BEA | 2.5 - 7.5 μM | 24 h | Bone marrow derived dendritic cells | Immunomodulation through the Toll-like receptor 4 (TLR4) pathway | (Yang et al., 2022) |
| BEA<br>ENNB | 0.1 - 150 μM<br>(IC50 BEA = 3.0/ 0.17 μM)<br>(IC50 ENNB = 113/ 0.81 μM) | 30 min<br>24 h | Rat liver microsomes/ J774 macrophages | Inhibited the acyl-CoA cholesterol acyltransferase enzyme (ACAT) | (Tomoda et al., 1992) |
| BEA<br>&<br>ENNmix | 1.0 - 10 μM<br>(IC50 = 1.41 - 3.18 μM) | 72 h | Human cancer cell lines ( KB-3-1, KBC-1, HL60, HL60/adr, HL60/vinc, GLC-4, GLC-4/adr, SW-1573, SW-1573/2R120, SW-1573/2R160, MDA-MB-231, MDA-MB-231/bcrp, A549, Caco-2) | short-term exposure:<br>ABCG2 weakly reduced cytotoxicity of BEA, but not by the ENNmix;<br>ABCC1 and Pgp (ABCB1) was not protective.<br>long-term exposure:<br>BEA>ENNmix inhibited ABCG2- and Pgp (ABCB1)-mediated efflux;<br>ABCG2 and Pgp (ABCB1) reduced cytotoxicity of ENNmix, and to a lesser extent BEA-induced cytotoxicity. | (Dornetshuber et al., 2009) |
| BEA<br>ENNB | 0.31 - 1.28 μM<br>0.39 - 4.69 μM | 35 min | Rat liver mitochondria | Mitochondrial membrane depolarization<br>Uncoupling of OXPHOS<br>Mitochondrial swelling, and decreased Ca2+ retention linked to K+ flux | (Tonshin et al., 2010) |
| BEA<br>ENNB | 0.1 - 15 μM<br>(IC50 BEA = 3 - 7.5, ENNB = 15 μM) | 24, 48, 72 h | Human T-lymphoblastoid cell line (Jurkat) | Mitochondrial metabolic activity impairment (BEA)<br>DNA damage (BEA)<br>Cell cycle arrest<br>Apoptosis -> Necrosis | (Manyes et al., 2018) |
| BEA<br>ENNB<br>1:1mix | 1.5 - 5 μM<br>1.5 - 5 μM<br>0.1 - 1.5 μM | 24 h<br>24 h<br>24 h | Human T-lymphoblastoid cells (Jurkat)<br>(Jurkat)<br>(Jurkat) | RNASeq/qPCR:<br>Mitochondrial metabolism/ damage<br>ETC complex gene transcription altered<br>Apoptosis (caspase depentent) (BEA)<br>Antioxidant-related genes downreg. | (Escrivá et al., 2018)<br>(Alonso-Garrido et al., 2018)<br>(Escrivá et al., 2019) |
| BEA:<br>ENNBmix | 0.01 - 0.5 μM | 24 h | (Jurkat) | Proteomic:<br>MT membrane proteins levels altered<br>ETC complexes levels altered<br>Ribosomes levels altered | (Alonso-Garrido et al., 2020a) |
| ENNmix<br>BEA | 0.1μM<br>0.1μM | 2 h<br>2 h | Blood brain barrier model (ECV304)<br>(ECV304) | qPCR:<br>ETC complexe gene transcripts | (Alonso-Garrido et al., 2020b)<br>(Alonso-Garrido et al., 2021) |
| ENNB | (AB IC50 = 2.6 μM)<br>(NR IC50 = 4.7μM)<br>0.05 - 100 μM<br>(10 μM) | 24 h | Murine macrophages (RAW 267.4) | Mitochondrial metabolic activity<br>Lysosomal damage releaseing DAMP<br>Inflammasome activation<br>Cell cycle arrest<br>Apoptosis | (Gammelsrud et al., 2012) |
| ENNB | 5 - 10 μM<br>1.0 - 25 μM<br>(NR IC50 = 10, 2.1μM) | 3, 24 h | Human colon carcinoma (Caco-2 cells) | Mitochondrial membrane permeabilization<br>Cell cycle arrest<br>Non-apoptotic, non-necrotic cell death<br>Lysosomal function impairment | (Ivanova et al., 2012) |
| ENNB | 1.5 - 3.0 μM<br>(NR IC50 = >15, 10.7, 1.4 μM)<br>(MTT IC50 = >15, >15, 11.7 μM) | 24, 48, 72 h | Human colon carcinoma (Caco-2 cells) | Mitochondrial membrane depolarization<br>ROS and LPO generation<br>Cell cycle arrest<br>Apoptosis -> Necrosis<br>Lysosomal function impairment<br>Mitochondrial metabolic activity | (Prosperini et al., 2013b) |
| ENNB | 10 - 20 μM | 1, 4 h | Rat primary hepatocytes | Altered energy metabolism<br>ETC complex gene transcription altered<br>Altered cell morphology<br>Necrotic cell death | (Jonsson et al., 2016) |

### 1.4.2 Toxicokinetics and toxicodynamics

The toxicity exerted by a xenobiotic, such as a mycotoxin, *in vivo* depends on both the toxicokinetics and toxicodynamics of the xenobiotic. Toxicokinetics is the rate of changes in concentration of a xenobiotic in an organism over time throughout the events of absorption, distribution, metabolism (biotransformation), and excretion/elimination which will determine the concentration of a xenobiotic reaching the target site (Ringot et al., 2006). When dietarily exposed to a xenobiotic, the gastrointestinal (GI) tract is the first barrier which is crossed, if not pre-systemically eliminated. When orally administered, the xenobiotic must first pass through the gastrointestinal tract, where it may be degraded by enzymes, pH changes, or gut microbiota. Then, the xenobiotic must cross the intestinal barrier before being absorbed and enter the bloodstream and becoming distributed to target organs. Thus, the oral bioavailability refers to the fraction of an orally administered xenobiotic that reaches the systemic circulation in an active form. However, a xenobiotic may not be bioavailable if it, for example, becomes bound to blood protein or is stored in tissue. Binding of xenobiotics to blood plasma proteins influences their distribution and metabolization, and hence also their toxicity as it determines the available fraction of xenobiotics that can cross cell membranes and reach biological targets (Smith et al., 2010). Biotransformation in fish, like in other vertebrates, protects against the bioaccumulation of xenobiotics. It is a process by which the xenobiotic is converted into a more water-soluble form that can be excreted from the body. This process occurs mainly in the liver, but also in other organs such as the intestine, and involves two main phases. During Phase I, the xenobiotic is chemically modified primarily by a group of enzymes called cytochrome P450 enzymes (CYPs). Phase I reactions include oxidation, reduction, and hydrolysis. These reactions introduce or expose a polar functional group, such as hydroxyl (-OH), carboxyl (-COOH), or amino (-NH2) group, to the molecule making it more water-soluble. In Phase II, the modified xenobiotic from Phase I is conjugated with an endogenous substrate, such as glucuronic acid, sulfate, glutathione, or amino acids, to form a more polar and water-soluble metabolite. This conjugation reaction is catalyzed by specific enzymes, such as UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), glutathione S-transferases

(GST, *also part of the antioxidant defense system against oxidative stress*), or amino acids-transferases. The resulting conjugate derivative is usually less toxic and can be excreted in urine or feces (Fig. 5). Alternatively, the xenobiotic metabolite from Phase I is acetylated or methylated to make it more excretable. Overall, the biotransformation process is crucial for maintaining the body's homeostasis and protecting it from toxic substances. However, the efficiency and speed of biotransformation can vary between individuals and species due to genetic, environmental, and lifestyle factors, which can affect the toxicity of a xenobiotic. Biotransformation can in some cases lead to *bioactivation* of a xenobiotic, where a more toxic metabolite is created (Yang et al., 2000, Wang-Buhler et al., 2005) (Fig. 5).

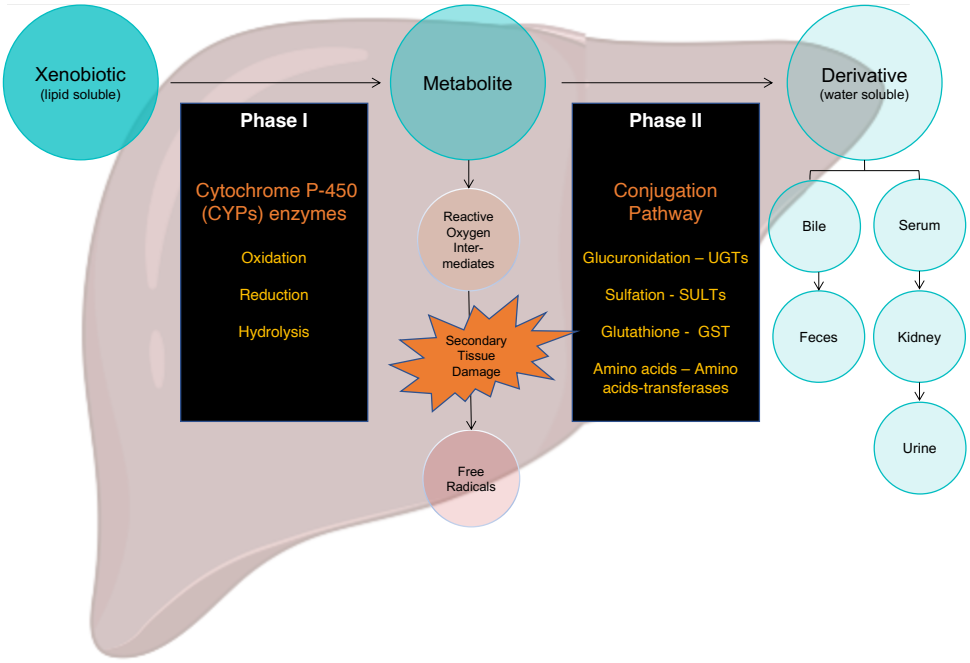



Fig 5. Phase I and Phase II in biotransformation. UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), glutathione S-transferases (GST). Illustration Sofie Søderstrøm.

The *in vivo* toxicokinetics of BEA and ENNB are not fully elucidated to date, and assessment of the toxicokinetics of BEA and ENNB in Atlantic salmon was not part of this thesis. However, based on *in vivo* data from ruminants, poultry, pigs, rabbits, guinea pigs, mice, and rats, the oral bioavailability of ENNB (including different ENN analogs) and BEA after dietary exposure appears to differ significantly between the different

mycotoxins, and is also highly influenced by animal species (Křížová et al., 2021). For example, the oral bioavailability of ENNB1 in pigs was found to be high (approx. 91 %) (Devreese et al., 2014). ENNA exhibits high binding to plasma proteins (99 %) in rat and human plasma (Bhateria et al., 2022). BEA, on the other hand, formed unstable complexes with human serum albumin proteins (Fliszár-Nyúl et al., 2022). In mice orally exposed to ENNB and BEA, Rodríguez-Carrasco et al. (2016) suggested that the mycotoxins diffused across the intestinal membranes, but were rapidly cleared from the bloodstream by hepatobiliary excretion. The authors further suggested that metabolism of ENNB occurred both in the intestine and in the liver explaining the low levels of ENNB in unmetabolized form found in liver [2.9 ug/kg] > adipose tissue [2.5 ug/kg] > serum [0.45 ug/kg] > colon [0.9 ug/kg] > muscle [0.12 ug/kg] ≈ kidney [0.1 ug/kg] ≈ brain [0.1 ug/kg]. Significantly higher levels of BEA were found in all sampled mice tissues, suggesting that BEA was not subjected to intestinal/hepatic metabolism to the same degree as ENNB. Instead, BEA appeared to be relatively stable in mice and was distributed to the liver [41.7 ug/kg] > adipose tissue [33.0 ug/kg] > colon [25.4ug/kg] > kidney [11.4 ug/kg] >> muscle [1.5 ug/kg] ≈ serum [1.3 ug/kg] ≈ brain [1.0 ug/kg] (Rodríguez-Carrasco et al., 2016) (Table 5). A recent study in Atlantic salmon exposed to dietary BEA and ENNB (Table 5) showed that, although low feed-to-organ transfer (<~0.01%), ENNB exhibited a higher transfer than BEA (kidney [ENNB: 24, BEA: 6 µg/kg] > liver [ENNB: 10, BEA: 4.7 µg/kg] > muscle [ENNB: 4.87, BEA: 0.118 µg/kg] > brain [ENNB: 2.82, BEA: nd µg/kg]) (Berntssen et al., 2023). Furthermore, Ivanova et al. (2017) reported that ENNB1 undergoes extensive metabolism (hydroxylation, carbonylation, carboxylation, and oxidative demethylation) both *in vitro* (liver microsomes) and *in vivo* in pigs. However, the formation of metabolites in pigs was higher when ENNB1 was administrated orally compared to intravenously, which suggested pre-systemic metabolism of ENNB1 prior to absorption from the gut. Similar to the *in vivo* experiments in chicken (Fraeyman et al., 2016), ENNs were rapidly metabolized through hydroxylation, carboxylation, and N-demethylation in liver microsomes of rat, dog, and human (Fæste et al., 2011, Ivanova et al., 2011, Ivanova et al., 2014). Phase I CYP enzymes (i.e., CYP3A4) have been reported to be important for metabolization and biotransformation of ENNB in human microsomes (Fæste et al.,

2011). The rapid metabolism of ENNs might suggest that phase I CYP biotransformation enzymes are the most relevant for the formation of metabolites and elimination of these particular mycotoxins (Ivanova et al., 2017). However, Cyp2k derived from rainbow trout (*Salmo trutta*) and zebrafish was responsible for the bioactivation of aflatoxin B1 making it carcinogenic (Yang et al., 2000, Wang-Buhler et al., 2005). ENNs have been reported to be transported through efflux pumps such as *P*-glycoprotein (*P*gp) (a.k.a multidrug resistance protein 1 (MDRP1)), and multidrug resistance protein 2 (MDRP2) across intestinal membranes *in vitro* (Ivanova et al., 2010). Thus, *P*gp activity can act protectively by lowering cellular accumulation of ENNB by pumping it back into the intestinal lumen (Pallarés et al., 2020). For example, cytotoxic effects induced by long-term exposure to $ENN_{mix}$, and to a lesser extent by BEA, were mitigated by breast cancer resistance protein (ABCG2) and *P*gp overexpression in human cancer cell lines (Dornetshuber et al., 2009). However, in a human gastrointestinal and colonic model, the ENNB-degradation was suggested to occur already in the digestive tract and gut, prior to absorption, and further hepatic metabolism (Pallarés et al., 2020). Based on rat trials, gut microbiota may also be important for degradation of mycotoxins such as ENNB and BEA (Manyes et al., 2014), and gut microbiota may thereby influence the bioavailability of ingested BEA and ENNB (Brown et al., 2021).

Toxicodynamics denotes the interactions between the xenobiotic, or its metabolites, and its biological targets with following downstream biochemical and physiobiological toxic effects (Ringot et al., 2006). Following EFSA's call for more *in vivo* toxicity data on BEA and ENNB (EFSA, 2014), a few *in vivo* toxicological studies with ENNB and BEA have been conducted. Of the most toxicologically relevant effects for this thesis, identified in these mammalian studies after acute exposure of mice exposed to BEA and ENNB, was immunosuppression. The articles summarized in Table 5 discuss the toxic effects of ENNB and BEA primarily in mammals and poultry including pigs, rats, mice, and broiler chickens. A limited number of studies have assessed the toxicity of BEA or ENNB *in vivo* in fish, and only two *in vivo* studies have been performed with Atlantic salmon (Paper III) (Berntssen et al., 2023). The mycotoxins were administered through different routes of exposure such as oral, injection, and water exposure. The effects of ENNB and BEA on animals varied from tissue bioaccumulation in broiler chickens (Fraeyman et al., 2016) and mice (Rodríguez-Carrasco et al., 2016), oxidative stress in Wistar rats (Cimbalo et al., 2021), and death in mice at high doses (McKee et al., 1997). Long-term exposure (42 days) of mice to these mycotoxins affected food consumption, body weight, caused immunosuppression and gender specific toxicity in several organs including reproductive systems (Maranghi et al., 2018). In zebrafish, ENNB and BEA affected embryonic mobility and cased death at high concentrations (Juan-García et al., 2021). Following long-term exposure (BEA 76 days, ENNB 69 days) of Atlantic salmon to these mycotoxins, BEA induced oxidative stress and reduced vitamin E levels and ENNB caused liver toxicity. In addition both mycotoxins affected growth, feed conversion and hematological parameters (Berntssen et al., 2023).

Table 5. *In vivo* toxicological studies of BEA and ENNB.

| Mycotoxin | Animal | Rout of exposure/ Dose | Effects | Reference |
|---|---|---|---|---|
| ENNB | Broiler chicken | *Acute toxicity, 10 h*<br>Oral<br>[200 µg/kg bw] | Tissue bioaccumulation | (Fraeyman et al., 2016) |
| ENNB | Pig | *Acute toxicity, 12 h*<br>Oral<br>[50 µg/kg bw] | Absorption in blood<br>Plasma (approx. 91 %) | (Devreese et al., 2013) |
| ENNB | Wistar rats | *Acute toxicity, 8 h*<br>Oral<br>[1,030 – 2,160 µg/kg bw] | No adverse effects | (Escrivá et al., 2015) |
| ENNB | Wistar rats | *Acute toxicity, 8 h*<br>Oral (liquid)<br>[296 - 593 µg/mL] | Oxidative stress in intestinal tissue, suggested to impair the intestinal barrier function. | (Cimbalo et al., 2021) |
| ENNB | Mice | *Acute toxicity, 6 days*<br>Injection (every 8 h)<br>[1,250 - 40,000 µg/kg bw] | Death in 2-5 days<br>[10,000 – 40,000 *µ*g/kg bw]<br>Reduced weight<br>No anti-HIV activity | (McKee et al., 1997) |
| BEA<br>ENNB | Mice | *Acute toxicity, 8 h*<br>Injection<br>3 consecutive days<br>2 consecutive days<br>[5,000 µg/kg bw] | No acute damage<br>Tissue bioaccumulation:<br>BEA in fat<br>ENNB in liver and colon | (Rodríguez-Carrasco et al., 2016) |
| BEA<br>ENNB | Mice | *Acute toxicity, 8 h*<br>Oral<br>[100 -10,000 µg/kg bw]<br>[180 -18,000 µg/kg bw] | Immunosuppression<br>(lymphoid organs) | (Maranghi et al., 2018)<br>*EFSA* |
| BEA<br>ENNB | Mice | *Sub-chronic toxicity, 42 days*<br>Oral<br>[100 -10,000 µg/kg bw]<br>[180 -18,000 µg/kg bw] | Increased food consumption<br>Decreased body weight<br>Reduced heart (BEA)<br>Increased white pulp in spleen (BEA)<br>*(Gender specific effects in tissues)*<br>BEA:<br>Both sexes - thyroid, kidneys, reproductive<br>Female - adrenals, duodenum;<br>Male - spleen.<br>ENNB:<br>Both sexes - spleen, brain, thyroid;<br>Female - thymus, kidneys, adrenals, reproductive<br>Male - duodenum. | (Maranghi et al., 2018)<br>*EFSA* |
| BEA<br>ENNB | Zebrafish<br>(larvae/embryo) | *Acute toxicity, 6 days*<br>Water exposure<br>[8 - 64 µM]<br>[3.15 - 25 µM] | Death with increasing conc.<br>Affected embryonic mobility | (Juan-García et al., 2021) |
| BEA<br>ENNB | Atlantic salmon | *Sub-chronic toxicity, 69 days ENNB/ 76 days BEA*<br>Oral<br>[300, 4800, 46000 µg/kg feed]<br>[300, 5200, 83000 µg/kg feed] | BEA:<br>Growth reduction (slimmer)<br>Increased FCR/ Decreased protein digestion<br>Oxidative stress response<br>Red blood cell shrinkage<br>Reduced vitamin E<br>ENNB:<br>Stunted growth (shorter)<br>Anemia<br>Liver toxicity (increased plasma ASAT)<br>Hematocrit (reduced)<br>Hemoglobin (increased)<br>Red blood cell count (reduced) | (Berntssen et al., 2023)<br>*AquaMyc* |

## 1.5 Experimental approach

*Toxicity testing – past and future*

Aquaculture faces an increasing number of undesirable substances, such as xenobiotics like mycotoxins and pesticides in the feed ingredients of aquafeeds for farmed Atlantic salmon, which need to be risk assessed. The risk assessment process traditionally follows the four steps of 1) hazard identification, 2) hazard characterization, 3) exposure assessment, and lastly 4) risk characterization. Thus, an initial characterization of novel xenobiotics' mode of toxic actions, and dose-response *in vivo* are important steps in the risk assessment process. However, the traditional use of whole-animal testing with high doses and qualitative endpoints not only requires many test animals, but such experiments are also time consuming and expensive. In 2007, the U:S National Academy of Sciences published *"Toxicity testing in the 21st century: a vision and a strategy"* (Council, 2007), emphasizing the need for integrating existing knowledge with advances in toxicogenomics using a systems biology approach. To facilitate a paradigm shift from low-throughput whole-animal testing, to an *in vitro* and *in silico*-mathematical model based integrative strategy, it is important to utilize quantitative endpoints measured in target cells and cell lines to generate more relevant and robust results with emphasis on perturbation of cellular pathways by xenobiotic (Council, 2007). This integrative *in silico* and *in vitro* high-throughput strategy would then allow toxicity testing of new xenobiotics to be upscaled and made more rapid and cost-efficient, whilst implementing the 3R principles (Krewski et al., 2010). The 3R principles involve reducing the number of animals used, refining procedures to minimize suffering, and replacing animals with non-animal alternatives (Russell and Burch, 1959).

Xenobiotics initially affect organisms at the molecular level and then progresses through biochemical, subcellular, cellular, tissue, organ, individual, and population levels (Van der Oost et al., 2003). The adverse outcome pathway (AOP) conceptual framework was created to clarify the usage of the terms mechanism of action (MOA) and mode of action (MoA) of a xenobiotic, and to provide a consistent structure and terminology for organizing toxicological data (Ankley et al., 2010). In an AOP, the

molecular initiating event (MIE) of the xenobiotic is linked to the adverse outcome (AO), creating a holistic view of the biological response pathway and highlighting gaps or uncertainties in existing knowledge (Ankley et al., 2010). A fully developed AOP can describe a xenobiotic's mechanism of action, while an AOP with knowledge gaps or uncertainties can be considered to denote its mode of action (Ankley et al., 2010). Furthermore, the integrative AOP conceptual framework allows incorporation of exposure (*internal dose, effective dose*) - and effect (*early biological effect, altered structure/function*) biomarkers, which can aid in risk assessments (Jeddi et al., 2021). Detecting sublethal effects at the molecular or biochemical level are useful early warning sign of potential adverse effects (Lee et al., 2015). Since the AOP connects the molecular initiating events to adverse outcomes in individuals, the AOP pipeline enhances the usefulness of short-term biomarker tests (Lee et al., 2015). High-throughput technologies such as transcriptomics, proteomics, and metabolomics have contributed to understanding a xenobiotics mechanisms and MoA (Song et al., 2019, Wissing et al., 2004, Aliferis and Jabaji, 2011). AOPs can aid in distinguishing between direct and secondary effects and guide the selection of appropriate tests and study endpoints throughout the project's progression (Coady et al., 2019).

### 1.5.2 Primary hepatocytes and head kidney cells isolated from Atlantic salmon as *in vitro* model systems

In line with the vision of *Toxicity testing in the 21st century* (Krewski et al., 2010), *in vitro* models and toxicogenomic were applied for the toxic characterization of the mycotoxins ENNB and BEA. Two primary cell models (hepatocytes and head kidney leukocytes) isolated from liver and head kidney tissues were used for the initial characterization of BEA and ENNB aiming to elucidate their mechanism of toxic actions. In favor of the 3R principles, the use of primary cell cultures instead of whole animals made it possible to reduce the number of fish needed for the research. *In vitro* assays using primary cell cultures are valuable approaches during hazard characterization of toxicants (Rehberger et al., 2018).

*Primary hepatocyte cells from Atlantic salmon*

The complex cell composition of the liver allows it to perform a wide range of functions including metabolizing and detoxifying xenobiotics, and immune defense. The liver of fish, like mammals, is composed of a variety of different cells. Hepatocytes are the most abundant cells in the liver, and they make up approx. 80 % of all the cells in the liver (Godoy et al., 2013). They are responsible for a variety of metabolic functions, including the production and secretion of bile, the detoxification of xenobiotics, and the regulation of carbohydrate, lipid, and protein metabolism. Other cell-types present are: biliary epithelial cells that line the bile ducts and are responsible for the secretion of bile into the digestive tract; stellate cells, which are involved in the storage and release of vitamin A and other lipid-soluble substances; endothelial cells that line the blood vessels in the liver and play a role in regulating blood flow and nutrient exchange; kupffer cells, which are specialized immune cells that help to remove foreign particles and microorganisms from the bloodstream as they pass through the liver; and pit cells which are also part of the immune system and are involved in the destruction of cancer cells and other abnormal cells (Ishibashi et al., 2009).

Primary hepatocytes a useful tool for screening of xenobiotic toxicity. An advantage of using primary cell cultures above cell lines, is that primary cells retain their natural cellular function to a greater extent than cell lines, in which certain characteristics tend to change or are lost during the process of immortalization (Pan et al., 2009). However, in contrast to cell lines, primary hepatocytes can only be cultured for a limited time-period, as they tend to undergo gradual dedifferentiation, and with time lose their native function, e.g., reduced levels of drug transporter proteins (Hu and Li, 2015). Atlantic salmon primary hepatocyte cell cultures have been successfully used in short-term exposure trials to assess hepatotoxicity while screening a range of xenobiotics that occur in marine aquafeeds (Olsvik and Søfteland, 2020, Olsvik et al., 2016, Olsvik et al., 2017, Søfteland et al., 2014)

*Primary head kidney cells from Atlantic salmon*

The immune system of Atlantic salmon is still not fully elucidated (del Mar Ortega-Villaizan et al., 2022), though similar to other vertebrates, it provides resistance against invading pathogens such as bacteria, viruses, fungi, and parasites through two main types of immune defenses i.e., innate immunity and adaptive immunity (Ashfaq et al., 2019). The innate immune system is the first line of defense and a rapid response present in all animals (Marshall et al., 2018). It includes physical barriers such as the fish's skin and mucus layer, as well as non-specific cellular- and humoral components that can recognize and destroy invading pathogens (Fig. 6). Examples of cellular components are monocytes (in blood), macrophages (in tissues), neutrophils, granulocytes, mast cells, natural killer-like cells, and pattern-recognition receptors (PRRs). Humoral components include e.g., compliment proteins, lysozyme, transferrin, lectins, chemokines, and cytokines (Firdaus-Nawi and Zamri-Saad, 2016, Abram et al., 2017) (Fig. X). The adaptive immune system is the second line of defense. It is specific to each individual and develops as a response to exposure to different pathogens over time when the innate immune system alone is insufficient (Natnan et al., 2021). This adaptive immunity involves specialized cellular components known as lymphocytes, such as B cells and T cells, that can recognize and remember specific pathogens and engage in a targeted response upon re-exposure (Marshall et al., 2018); (Ashfaq et al., 2019, Firdaus-Nawi and Zamri-Saad, 2016) (Fig. 6).

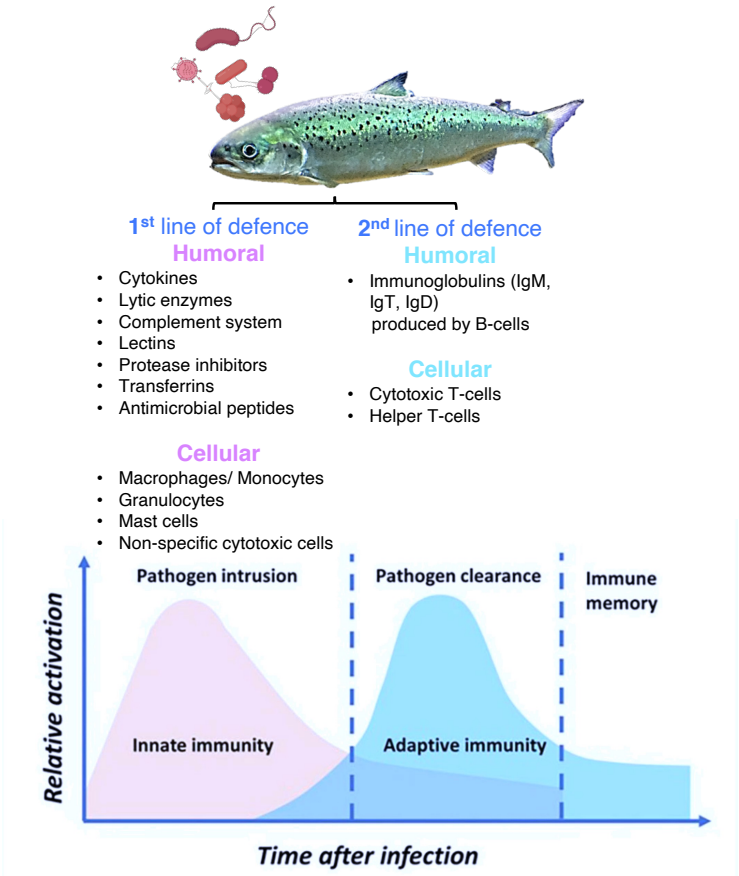



Fig. 6. Fish have a complex immune system that includes non-specific innate immunity (including physical barriers, cellular- and humoral components), and specific adaptive immunity (including cellular and humoral components). Figure adapted from Natnan et al. (2021).

The head kidney, as well as thymus, are the primary lymphoid organs and sites of hematopoiesis (blood cell formation) in fish (Cabillon and Lazado, 2019). Specifically, the head kidney is responsible for producing and regulating white blood cells (leukocytes) such as granulocytes, monocytes/macrophages, and lymphocytes for the immune defense (Reite and Evensen, 2006). The trunk kidney, spleen, liver, and mucosal-associated lymphoid tissues (MALT) serve as secondary lymphoid organs in fish (Natnan et al., 2021). In addition, the head kidney and spleen are also the primary

sites of erythropoiesis (red blood cell formation) in fish, having an equivalent role to mammalian bone marrow (Press and Evensen, 1999, Zapata and Amemiya, 2000, Chen et al., 2013). Fish primary head kidney leukocytes are therefore a valuable *in vitro* model for studying immune responses and diseases in fish. They have been successfully used as a model to assess immunotoxicity, immunesuppressive- and immune-stimulating modulating actions of the innate immune system by undesirable substances, feed components, or additives that occur in marine aquafeeds (Holen et al., 2011, Stenberg et al., 2019, Espe et al., 2020).

### 1.5.3 Short term in vivo dietary exposure using Atlantic salmon smolt

While *in vitro* models are more compliant with the 3R strategy and offers model systems with less variables, an advantage with *in vivo* dietary exposure trials is that they offer a realistic exposure route where cell-to-cell signaling and feedback-loops within and between tissues/organs are maintained. A short-term (12 h single dose exposure) gavage feeding trial was conducted on Atlantic salmon smolt to investigate early cellular and tissue responses in the intestine and liver following dietary exposure to BEA and ENNB. With this acute exposure study, we wanted to assess which early pathway perturbations (toxicity pathways) occur *in vivo*. A toxicity pathway denotes which pathway perturbations are sufficient to compromise a biological function, alternatively it indicates when the adaptive stress response is insufficient to restore homeostasis (Krewski et al., 2010). Previous evaluations of acute and sub-acute oral toxicity of ENNB and BEA have been assessed *in vivo* in mice which, depending on the endpoint, showed differences in tissue responses as well as gender differences in susceptibility (Maranghi et al., 2018).

The intestine, responsible for the absorption of nutrients and elimination of waste from the body, was chosen for evaluation since it constitutes the first protective barrier against feedborne xenobiotics. In addition, intestinal metabolization of ENNB has previously been reported in mice *in vivo* (Rodríguez-Carrasco et al., 2016), and in human gastrointestinal cells *in vitro* (Pallarés et al., 2020). Pre-systemic

degradation/metabolism of ENNB and BEA by gut microbiota could also influence their absorption from the gut (Gratz et al., 2013, Manyes et al., 2014, Brown et al., 2021). If the intestinal mucosa is compromised due to disease, inflammation, or exposure to harmful xenobiotics, it can lead to increased permeability or "leaky gut," allowing harmful xenobiotics (and bacteria) to enter the bloodstream and be transported to other target organs such as the liver, kidneys, and brain. For example, intestinal inflammatory response leading to release of pro-inflammatory cytokines by mycotoxins such as deoxynivalenol, can alter neurological-endocrine signaling between the intestine and the brain in humans (Maresca, 2013). Thus, potentially affecting physiological processes such as appetite, metabolism, and stress response.

The liver was evaluated since it plays a crucial role in the metabolism and detoxification of xenobiotics. The liver is responsible for the biotransformation of xenobiotics into more water-soluble and less toxic metabolites that can be excreted from the body. A process carried out by phase I (CYPs) and Phase II (GST, UGT, NAT, SULT) conjugation enzymes, which are primarily located in the liver. However, excessive exposure to certain xenobiotics can lead to liver damage and impaired liver function. Impairment of the liver can result in the accumulation of toxic metabolites in the body, leading to adverse health effects. In addition, some xenobiotics can interfere with the liver's ability to carry out its normal metabolic and detoxification functions, leading to further liver damage and dysfunction. Thus, the liver is an important target for toxicological evaluation of xenobiotics, and the assessment of liver function is an important part of toxicity testing. With the benefit of non-targeted transcriptomics, like RNA sequencing, it is possible to investigate non-lethal transcriptional endpoints, assess dose-responses, and identify toxicity pathways and pathway perturbations (Labib et al., 2017, Deng et al., 2018).

*AquaMyc: Tolerance of Atlantic salmon to novel feed mycotoxins - Implications on fish performance and nutrient interactions*

This PhD has been a part of the AquaMyc project funded by the Research Council of Norway, grant 281032 HAVBRUK2. The primary objective of AquaMyc was to

investigate Atlantic salmon's tolerance to the mycotoxin feed contaminants ENNB and BEA. This project has involved a combination of *in vitro* studies and *in vivo* feeding trials with Atlantic salmon. A systems biology approach was applied to gain fundamental knowledge on the mycotoxins' mechanisms and mode of toxic action, and their organismal (non-lethal) adverse effects (e.g., intestinal function, disturbed hematology, bone formation, feed utilization, lipid oxidative status, growth, health, immune-response etc.)

The Sub-objectives of AquaMyc were to:

1. Investigate the mycotoxins' toxicity *in vitro* in Atlantic salmon primary cells models by assessing concentration-dependent mechanisms of toxic actions, identifying toxicity pathways and pathway perturbations by using non-targeted omics with focus on non-lethal transcriptional endpoints.
2. Investigate immuno- and osteotoxicty of BEA and ENNB in *in vitro* Atlantic salmon primary head kidney and bone mineralization models.
3. Investigate how BEA and ENNB affect lipid metabolism, intestinal function, nutrient status, feed utilization, hematology, bone formation, growth, overall health, and immune-response *in vivo* in Atlantic salmon with acute and sub-chronic feeding trials.
4. Based on a sub-chronic feeding trial with Atlantic salmon, establish a benchmark dose limit (BMDL) for the investigated mycotoxins, as outlined by the European Food Safety Authority (EFSA) to assess the risk of ENNB and BEA causing adverse effects in farmed Atlantic salmon.
5. Provide an overall systems biology assessment of mycotoxin exposure based on omics data from *in vitro* and *in vivo* trials.

The project has been led by the Institute of Marine Research (IMR), with partners from industry (Cargill), international university sectors of fish models (CCMAR, Portugal), and bioinformatics and system biology (UCSD, USA) with an interest in implementing novel feed ingredients in sustainable marine aquafeeds. The work in this thesis has contributed to several of the objectives in AquaMyc. The main aim and sub-aims of this thesis are outlined in section 2.

# 2. Thesis Aims and Outline

The main aim of this thesis was to carry out initial characterization of BEA and ENNB toxicity using salmon specific *in vitro* models to elucidate their concentration-dependent mechanisms of toxic action (toxicity pathways) leading to toxic effects in Atlantic salmon. Further, with a short-term acute gavage feeding trial with Atlantic salmon smolt, identify toxicity pathways and pathway perturbations that are of potential biological relevance.

Sub-aim 1) Using salmon primary hepatocytes as an *in vitro* model to assess ENNB and BEA cytotoxic potential and mechanisms of toxicity, by assessing cell viability (xCELLigence system, MTT, and Neutral red), and assess morphological changes after 48 hours exposure. Thereafter, identifying perturbed pathways with non-targeted transcriptomics and validate the findings with enzymatic and labeling assays (ATP, $H_2O_2$, Iron, and Gpx/GSH).

Sub-aim 2) Using salmon primary head kidney cells as an *in vitro* model to assess if ENNB and BEA can modulate or disrupt immune cell signaling in cells challenged with LPS and PIC after 24 hours exposure, and to identify transcriptional markers of immune-related response and general toxicity using targeted RT-qPCR.

Sub-aim 3) Investigate *in vivo* toxic modes of action of BEA and ENNB at the acute phase (12 hours exposure) in Atlantic salmon smolt that potentially could lead to adverse effects in intestinal and liver tissue, by using non-targeted transcriptomics to identify toxicity pathways and pathway perturbations *in vivo*.

This thesis work is organized according to the AOP-framework (Fig. 7), to elucidate which pathway perturbations could potentially lead to adverse toxicological effects by assessing accumulation of inflicted damage, energy allocation, and compensatory mechanisms and adaptive responses leading to recovery of the inflicted stress.

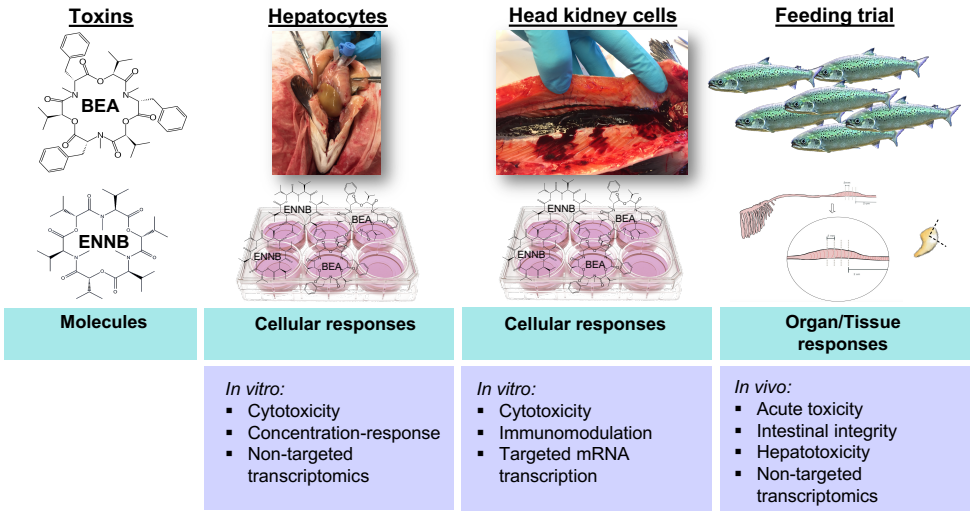



Fig. 7. Schematic overview of the outline of this thesis work (intestine illustration in the feeding trial column created by Åsne Omdal, 2022).

# 3. Methodology

As previously described, ENNB and BEA constitute two emerging mycotoxins found to be prevalent in aquaculture feed/feed commodities and due to limited toxicological data have not been risk assessed in fish. Primary cells isolated from the liver (tissue important for detoxification) and head kidney (involved in the immune system), were utilized to elucidate the mechanisms and mode of toxic action of BEA and ENNB in Atlantic salmon. In addition, an *in vivo* feeding trial examining initial effects of BEA and ENNB exposure in Atlantic salmon intestinal (first barrier) and liver tissue was performed. Non-targeted transcriptomic analysis, using RNA sequencing, and targeted mRNA transcription using RT-qPCR were used to identify possible toxic mechanism of ENNB and BEA in Atlantic salmon. An overview of experimental methodologies used in this thesis are summarized in Table 6 and further detailed in section 3.1.1 – 3.2.2.

Table 6. Overview of experimental methodologies used in Paper I-III.

| Paper # | I | II | III |
|---|---|---|---|
| ***In vitro/ In vivo*** | **Primary hepatocytes**<br><br>*Isolated from male juvenile Atlantic salmon weighing: initial) 216-326 g, n = 6 follow-up) 136-193 g, n = 4* | **Primary head kidney cells**<br><br>*Isolated from male Atlantic salmon weighing: 653-894 g, n = 6* | **Atlantic salmon**<br><br>*Freshwater-adapted pre-smolt weighing: 58-108 g (average 74 g) female:male ratio (14:17), n = 6* |
| **Conc./ Nominal conc.** | **Mycotoxin: 0.05, 0.5, 1.5, 2.5, and 5 µM**<br><br>*(48 h exposure, incubation at 10 °C)*<br><br>*(In the cell viability assays, i.e. xCELLigence, MTT, Neutral red, exposure at 10 µM was also included)* | **Mycotoxin: 0.05, 1.5, and 2.5 µM**<br>LPS: 100 µg/mL<br>PIC: 50 µg/mL<br><br>*(24 h exposure, incubation at 10 °C)* | **Mycotoxin: low = 50 µg/kg feed high = 500 µg/kg feed**<br><br>*(single-dose exposure, responses after 12 hours)* |
| **Cells/ tissues analyzed** | **Hepatocytes**<br><br>*(grows in monolayer)* | **Head kidney leukocytes**<br><br>*(grows in suspension)* | **Intestinal tissue**<br>**Liver tissue** |
| **Analyses** | **xCELLigence system**<br>**MTT**<br>**Neutral red (NR)**<br>**ATP levels**<br>**H2O2 levels**<br>**Iron (Fe2+, Fe3+) levels**<br>**Gpx enzyme activity**<br>**Non-targeted transcriptomics (RNA-Seq)**<br>*(KEGG pathway and module enrichment analyses)* | **LDH**<br>**Targeted mRNA transcription (RT-qPCR)** | **Non-targeted transcriptomics (RNA-Seq)**<br>*(IPA and DAVID pathway analyses)* |

## 3.1 Primary hepatocytes and head kidney cells isolated from Atlantic salmon as *in vitro* model systems

### 3.1.1 Primary hepatocytes

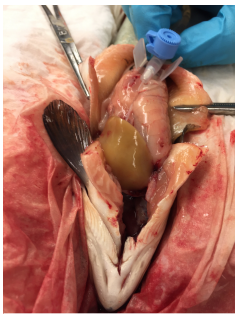


Fig. 8. Atlantic salmon liver perfusion

Atlantic salmon hepatocytes were isolated using the two-step liver perfusion method previously described by Søfteland et al. (2009) and is thoroughly explained in Paper III (Fig. 8). The use of all male subjects has long been common practice across different fields from human to animal *in vitro* model research (Beery and Zucker, 2011). The primary cell cultures used were collected from a clonal all-male Atlantic salmon strain, developed at the Institute of Marine Research (Fjelldal et al., 2020). Utilization of a clonal all-male Atlantic salmon strain, and their genetic uniformity, reduced the number of fish needed to achieve sufficient statistical power in the *in vitro* screening trail. However, gene expression is naturally variable among individuals of the same species. This variability can be influenced by factors such as age, sex, and genetics and can result in differences in toxicological responses between individuals or experimental conditions. Doing animal experiments is often a trade-off between variation (statistical power) and sex bias, since mixing male and female animals may result in excessive variation that could mask a toxicological response that is different in males and females. However, ultimately both sexes should be used for risk assessment, thus sex-dependent variation was accounted for in the final *in vivo* trial (section 3.2).

A single salmon liver gives a sufficient number of hepatocytes that can be seeded into the desired well-plate format for a large experimental setup. The amount of cellular material obtained makes this model well suited for carrying out targeted and non-targeted transcriptomics to identify affected cellular- and signaling pathways of the xenobiotic in question (Olsvik and Søfteland, 2020, Olsvik et al., 2016, Olsvik et al., 2017, Søfteland et al., 2014). With additional experimental assays (viability, enzymatic

activity, ROS etc.) it is possible to further elucidate mechanisms of toxicity at a hepatocellular biological organization level (Rehberger et al., 2018).

*Study design and analyses* – in vitro *hepatocytes*

## Study design

In the main experiment, the mycotoxins were exposed as single compounds at 5 different concentrations plus control. While in a complimentary follow-up experiment, 3 different concentrations were used plus control. In both experiments, 0.1 % dimethyl sulfoxide (DMSO) was used as the control. Six juvenile salmon weighing between 216-326 g were used in the main experiment, and four juvenile salmon weighing between 136-193 g were used in the follow-up experiment. The experimental design and exposure regimes are depicted in Table 7. Exposure concentrations were determined from a pilot trial prior to the current trail (data not shown). The follow-up experiment aimed to complement the main experiment and focused on 3 exposure concentrations that were selected to represent the entire concentration-response curve, as it was not possible to include all 5 original concentrations due to analytical constraints.

Table 7. Experimental design and exposure regimes for the main and follow-up Atlantic salmon primary hepatocyte *in vitro* trials.

| Trial | Fish # | Mycotoxin | Conc. 1 (μM) | Conc. 2 (μM) | Conc. 3 (μM) | Conc. 4 (μM) | Conc. 5 (μM) | Ctrl: DMSO % |
|---|---|---|---|---|---|---|---|---|
| Main trial | Fish 1 | BEA | 0.05 | 0.5 | 1.5 | 2.5 | 5 | 0.1 |
| | | ENNB | 0.05 | 0.5 | 1.5 | 2.5 | 5 | 0.1 |
| | Fish 2 | BEA | 0.05 | 0.5 | 1.5 | 2.5 | 5 | 0.1 |
| | | ENNB | 0.05 | 0.5 | 1.5 | 2.5 | 5 | 0.1 |
| | Fish 3 | BEA | 0.05 | 0.5 | 1.5 | 2.5 | 5 | 0.1 |
| | | ENNB | 0.05 | 0.5 | 1.5 | 2.5 | 5 | 0.1 |
| | Fish 4 | BEA | 0.05 | 0.5 | 1.5 | 2.5 | 5 | 0.1 |
| | | ENNB | 0.05 | 0.5 | 1.5 | 2.5 | 5 | 0.1 |
| | Fish 5 | BEA | 0.05 | 0.5 | 1.5 | 2.5 | 5 | 0.1 |
| | | ENNB | 0.05 | 0.5 | 1.5 | 2.5 | 5 | 0.1 |
| | Fish 6 | BEA | 0.05 | 0.5 | 1.5 | 2.5 | 5 | 0.1 |
| | | ENNB | 0.05 | 0.5 | 1.5 | 2.5 | 5 | 0.1 |
| Follow-up trial | Fish 7 | BEA | 0.05 | | | 2.5 | 5 | 0.1 |
| | | ENNB | 0.05 | | | 2.5 | 5 | 0.1 |
| | Fish 8 | BEA | 0.05 | | | 2.5 | 5 | 0.1 |
| | | ENNB | 0.05 | | | 2.5 | 5 | 0.1 |
| | Fish 9 | BEA | 0.05 | | | 2.5 | 5 | 0.1 |
| | | ENNB | 0.05 | | | 2.5 | 5 | 0.1 |
| | Fish 10 | BEA | 0.05 | | | 2.5 | 5 | 0.1 |
| | | ENNB | 0.05 | | | 2.5 | 5 | 0.1 |

**Analytical assays**

Real-Time Cell Analysis (RTCA) was carried out using the xCELLigence system to quantify biological factors, such as changes in cell morphology and the strength of the cells-substrate adhesion to the surface throughout the entire exposure duration (48 h). The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and Neutral red assays were used to assess the viability of the hepatocytes after exposure to BEA and ENNB at the end of the experiment, defined by a reduction in cell viability by more than 30 % (Iso, 2009). The MTT assay measures mitochondrial activity, which reflects the metabolic activity of cells. The Neutral Red assay is based on the ability of lysosomes in viable cells to take up and bind the dye Neutral Red. Both assays are easy to perform, relatively inexpensive, and provide reliable results. The ability of BEA and ENNB to affect energy production and the generation of reactive oxygen species was assessed by measuring ATP and hydrogen peroxide ($H_2O_2$) content in primary hepatocytes after 48 h exposure. The ATP and $H_2O_2$ detection assays were carried out as described in Paper I. However, assessing reactive oxygen species such as $H_2O_2$ can be challenging due to their highly reactive nature and short half-life. BEA and ENNB ability to affect cellular iron levels ($Fe^{2+}$ and $Fe^{3+}$) and Gpx activity were also assessed in the primary hepatocytes after 48 h exposure, using an iron assay kit and a glutathione peroxidase (Gpx) assay kit which are relatively simple, reliable, and accurate assays. In the initial experiment, the analytic assays used were xCELLigence, MTT, ATP, $H_2O_2$, in addition to RNA sequencing. In the follow-up experiment the analytic assays used were MTT, Neutral red, Iron, and Gpx (Table 6).

The hepatocytes from the main experiment intended for RNA sequencing were immediately lysed in a lysis buffer and stored at -80 °C until further processing for RNA extraction and purification. Total RNA samples were sent to the Norwegian Sequencing Centre (NSC), Oslo, Norway, for cDNA library preparation and sequencing using the NextSeq Illumina platform (Illumina, San Diego, California, USA). A total of 48 libraries were generated, 24 for BEA and 24 for ENNB, where (based on cell viability results) three experimental conditions below cytotoxic levels (0.05, 0.5, 2.5 µM) plus the control (0.1% DMSO) were selected for RNA sequence

analysis, n = 6. The average library size contained 10±2 million reads. The identification of differentially expressed genes (DEGs) and functional analyses are explained in section 3.2.2.

### 3.1.2 Primary head kidney leukocytes

Atlantic salmon primary head kidney cells (Fig. 9) were isolated using an established method (Holen et al., 2011, Holen et al., 2014), as described in Paper II, from clonal all-male Atlantic salmon (Fjelldal et al., 2020). These primary head kidney cell cultures, which mainly consist of leukocytes (Krøvel et al., 2011), represent a model for the innate immune system and provide insight into the immune system's function and response to pathogens and diseases (Cabillon and Lazado, 2019, Reite and Evensen, 2006). Here used to study the immunomodulation-potential of BEA and ENNB. Isolation of immune cells from the head kidney often results in low cell number, which becomes a limiting factor when designing the experimental set up and exposure regime. However, the amount of cellular material gained after exposure is sufficient for testing effects on mRNA transcription of a battery of immune related genes to identify affected immune signaling pathways. Thus, head kidney cell cultures are a useful tool when screening novel undesirable substances or toxicants for possible immunomodulatory activities, and to assess whether they can alter immune cell signaling and aggravate infections at a cellular immunity level (Holen et al., 2011, Stenberg et al., 2019, Espe et al., 2020).

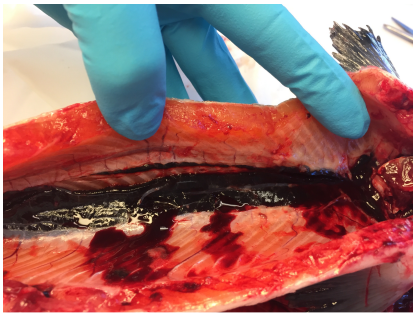


Fig. 9. Atlantic salmon head and trunk kidney

*Study design and analyses* – in vitro *head kidney leukocytes*

**Study design**

The fish used in this trial were from a clonal all-male Atlantic salmon line developed at the Institute of Marine Research (IMR), Matre, Norway (Fjelldal et al., 2020). The cells were isolated from 12 male salmon (n = 6 for BEA, and n = 6 for ENNB) weighing

between 653-894 g. The cells were cultivated in suspension and exposed for 24 hours to three different concentrations of either BEA or ENNB, either alone or in combination with either Lipopolysaccharides (LPS) or Polyinosinic:polycytidylic acid (PIC). The experimental design and exposure regimes used in the primary head kidney leukocyte trial are depicted in Table 8. The concentrations of BEA and ENNB in the present study were selected based on levels previously identified as non-cytotoxic for salmon primary hepatocytes.

Table 8. Experimental design and exposure regimes for the Atlantic salmon primary head kidney leukocytes *in vitro* trial.

| Mycotoxin | Fish # | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|---|
| BEA | Fish 1 | Ctrl (DMSO 0.1%) | LPS | LPS+BEA 0.05 | LPS+BEA 1.5 | PIC | PIC+BEA 0.05 | PIC+BEA 1.5 | BEA 2.5 |
| | Fish 2 | Ctrl (DMSO 0.1%) | LPS | LPS+BEA 0.05 | LPS+BEA 1.5 | PIC | PIC+BEA 0.05 | PIC+BEA 1.5 | BEA 2.5 |
| | Fish 3 | Ctrl (DMSO 0.1%) | LPS | LPS+BEA 0.05 | LPS+BEA 1.5 | PIC | PIC+BEA 0.05 | PIC+BEA 1.5 | BEA 2.5 |
| | Fish 4 | Ctrl (DMSO 0.1%) | LPS | LPS+BEA 0.05 | LPS+BEA 1.5 | PIC | PIC+BEA 0.05 | PIC+BEA 1.5 | BEA 2.5 |
| | Fish 5 | Ctrl (DMSO 0.1%) | LPS | LPS+BEA 0.05 | LPS+BEA 1.5 | PIC | PIC+BEA 0.05 | PIC+BEA 1.5 | BEA 2.5 |
| | Fish 6 | Ctrl (DMSO 0.1%) | LPS | LPS+BEA 0.05 | LPS+BEA 1.5 | PIC | PIC+BEA 0.05 | PIC+BEA 1.5 | BEA 2.5 |
| ENNB | Fish 7 | Ctrl (DMSO 0.1%) | LPS | LPS+ENNB 0.05 | LPS+ENNB 1.5 | PIC | PIC+ENNB 0.05 | PIC+ENNB 1.5 | ENNB 2.5 |
| | Fish 8 | Ctrl (DMSO 0.1%) | LPS | LPS+ENNB 0.05 | LPS+ENNB 1.5 | PIC | PIC+ENNB 0.05 | PIC+ENNB 1.5 | ENNB 2.5 |
| | Fish 9 | Ctrl (DMSO 0.1%) | LPS | LPS+ENNB 0.05 | LPS+ENNB 1.5 | PIC | PIC+ENNB 0.05 | PIC+ENNB 1.5 | ENNB 2.5 |
| | Fish 10 | Ctrl (DMSO 0.1%) | LPS | LPS+ENNB 0.05 | LPS+ENNB 1.5 | PIC | PIC+ENNB 0.05 | PIC+ENNB 1.5 | ENNB 2.5 |
| | Fish 11 | Ctrl (DMSO 0.1%) | LPS | LPS+ENNB 0.05 | LPS+ENNB 1.5 | PIC | PIC+ENNB 0.05 | PIC+ENNB 1.5 | ENNB 2.5 |
| | Fish 12 | Ctrl (DMSO 0.1%) | LPS | LPS+ENNB 0.05 | LPS+ENNB 1.5 | PIC | PIC+ENNB 0.05 | PIC+ENNB 1.5 | ENNB 2.5 |

**Analytical assays**

After 24 h exposure to BEA or ENNB, the viability of the head kidney cells was assessed by a Lactate dehydrogenase (LDH) Cytotoxicity Assay Kit (Cayman, Ann Arbor, Michigan, USA), and cells were harvested for RNA isolation and purification as described in Paper II. LDH is an enzyme that is present in most cells in the body, and it is released into the extracellular space following cell death and disruption of the cell membrane. Hence the amount of LDH released into the culture medium is commonly used as a marker of cell death. Although death and cell lysis are late endpoints compared to, for example, measuring mitochondrial metabolic activity (MTT), the advantage with the LDH assay is that the culture media is utilized for the analysis (Chan et al., 2013). In addition, since the primary head kidney cells are grown in suspension, MTT is not an option as it requires cells that attach to the plate bottom. Targeted mRNA transcription, using reverse transcription quantitative real-time PCR (RT-qPCR), was employed to investigate if BEA and ENNB exhibited immunomodulating

effects in the exposed primary head kidney. Transcriptional effects on immune receptors, suppressors, cytokines, inflammatory target genes, iron utilization genes, and gene markers for general toxicity was assessed with gene-specific primers (Table 9) (Paper II). RNA extraction, cDNA synthesis, and RT-qPCR was performed as described in Paper II.

Table 9. RT-qPCR primer sequences used for targeted mRNA transcription analyses.

| Target genes | Accession No./ Ref | Forward primer (5'-3') | Revers primer (5'-3') | Amplicon size (bp) |
|---|---|---|---|---|
| ***itgb2*** | 100306785 | CTGACGGGCAGAGGTGTTCT | TCCATGCAGCCCCTCTTTTC | 191 |
| ***il-1β*** | NM_001123582 | GTATCCCATCACCCCATCAC | GCAAGAAGTTGAGCAGGTCC | 127 |
| ***il-6b*** | 106570581 | TCACGAGGTAACCACCTTGC | CCTGGTGCTGTGAGAACGAT | 89 |
| ***tnfa*** | AY848945 | GGCGAGCGTACCACTCCTCT | CATTGTACCAGCCCTCGCC | 148 |
| ***cox2*** | AY848944 | GGAGGCCTACTCCAACCTAT | CGAACATGAGATTGGAACC | 179 |
| ***socs2*** | KF699316 | CACTGCCAACGAAGCCAAAGAGAT | CAAACTGCTTCAGCTTGGGCTTGA | 200 |
| ***tlr3*** | 106602560 | CCTTTGCTGCCCTACAGAGTC | GGCTTCAAGCAATCAGGCTC | 238 |
| ***isg15*** | AY926456 | GTGGCCACAACAAAGCAGAG | TGTGTGTGTCTGGCCCTTTT | 177 |
| ***ifna2*** | NM_001123570 | TACAATGCAGAGTTGGACGTG | GCTTTGTGATATCTCCTCCCATC | 149 |
| ***casp3*** | DQ008070 | ACAGCAAAGAGCTAGAGGTCCAACAC | AAAGCCAGGAGAGTTTGACGCAG | 92 |
| ***atp8*** | 808310 | CCTCCTAAAGTACTCGGCCA | CCTCCTAAAGTACTCGGCCA | 86 |
| ***gpx4*** | NM_001146603.1 | CGCGTGCTCTAGTTTTTGGT | GCAGACAAACCCCCTGTATTTC | 161 |
| ***FerH*** | NM_001123657.1 | CGTCAAGAAACCAGAGAAGGA | AGGTAGTGGGTCTCAATGAAGTC | 162 |
| ***FerM*** | NM_001123658.1 | ATCCGCCAGAACTATCACCA | CTGGCTTCTTGATGTCCTGG | 244 |
| ***alas1*** | XM_014168375 | CCCCATATCAGCTCACGCAT | CTCAGCTTTGGGACAGTCGT | 149 |
| ***alas2*** | XM_014146048.1 | TTTGCCCAATCAGCAACCCA | GGGGCCCACTGGGTTAGAAT | 135 |
| Ref. genes | | | | |
| ***arp*** | AY255630 | GAAAATCATCCAATTGCTGGATG | CTTCCCACGCAAGGACAGA | 106 |
| ***mhcII*** | XM_014133067 | TAAGATAGCCATCGGTGCGTC | GTACTACAGCACCCCAGAAGAC | 100 |

The geNorm VBA applet for Microsoft Excel version 3.4 was used to determine a normalization factor based on two reference genes, which was further used to calculate mean normalized expression (MNE) of the target genes (Vandesompele et al., 2002). Acidic ribosomal protein (*arp*) and H-2 class II histocompatibility antigen, I-E beta

chain (*mhcII*) exhibited a gene expression stability (M) of <0.4 and were therefore selected as reference genes for this trial. While *mhcII* is an immune-related gene (Haugland et al., 2005), in the current study it exhibited stable Ct levels among all exposure groups making it a suitable reference gene for this particular study. The expression of the target genes was depicted in boxplots as mean normalized expression data distribution (minimum, lower quartile, median, upper quartile, and maximum) based on n = 6 for each treatment group.

## 3.2 Short-term *in vivo* dietary exposure in Atlantic salmon smolt

A short-term (12 h single dose exposure) gavage feeding trial was conducted on Atlantic salmon smolt to investigate early cellular and tissue responses in the intestine and liver that could reduce its fitness following dietary exposure to BEA and ENNB. The exposure concentrations 50 and 500 µg/kg feed were selected for the dietary exposure trial with Atlantic salmon since they were in proximity to the mean and maximum measured levels of ENNB in commercial Norwegian marine aquafeed (37 and 250 µg/kg feed, Table 2) (Paper III). The exposure concentrations chosen for BEA were kept at the same levels as ENNB to make the toxicological effects more comparable in the *in vivo* trial (Paper III).

The prevalence, mean, minimum, and maximum concentration data of the mycotoxins surveyed in Norwegian marine aquafeed (and feed ingredients) presented in detail in paper III, was compiled from Sanden et al. (2016a), Sanden et al. (2017), Sele et al. (2018), Sele et al. (2019), Ørnsrud et al. (2020), Sele et al. (2021), Sele et al. (2022) and collected through the ongoing national monitoring program of fish feed and feed ingredients in Norway conducted for the NFSA. The NFSA collects samples of marine aquafeed and feed ingredients annually from authorized Norwegian feed production sites, with sampling randomized with regards to season and location. The samples are collected by inspectors from the NFSA and then transported to IMR for preparation, and subsequent reporting. The number of samples collected of plant-based protein meals (including all protein sources), plant-based oils, and marine aquafeeds between

2015-2021 are detailed in Paper III. The feed and feed ingredient samples were shipped to Eurofins WEJ Contaminants GmbH (Hamburg) for chemical analyses, where they use accredited methods (accreditation number D-PL-14602-01-00 and D-PL-14198-01-00). BEA and ENNs (A, A1, B, B1) were extracted with ACN/H20, SPE clean-up, and determined using LC-MS/MS. DON, FBs, HT-2, T-2, and ZEA were also determined using LC-MS/MS, while AFBs and OTA were analyzed by HPLC and fluorescence detection. The limits of quantification (LOQs, in µg/kg) were as follows: AFBs < 0.1/1.0, DON < 10 – 20, FFBs < 10 – 20, H2 < 10 – 20, OTA < 0.1, T2 < 10, ZEA < 10, ENNs < 10, BEA < 10. The prevalence (x%) of mycotoxins was calculated by the following equation:

$$\text{Prevalence}(\%) = \frac{Number\ of\ samples\ over\ LOQ}{Number\ of\ samples\ analysed} \cdot 100\%$$

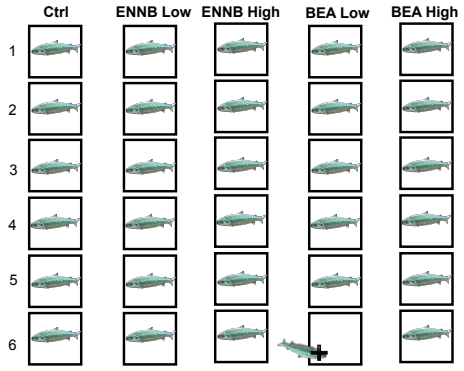
*Study design and RNA sequencing – in vivo*

The feeding trial was carried out at the IMR's facility at Matre (North of Bergen, Norway) between the 28th and 30th of January 2020. The pre-smolt fish were kept on-site, were adapted to freshwater, and weighed between 58-108 g. There were 30 tanks, each containing one fish, and the fish were kept in standard freshwater oxygen conditions at a temperature of 10 ± 0.2 °C with a 12:12 h light/dark cycle. The fish were gavage-fed, following the protocol from a previous study (Amlund and Berntssen, 2004). The mycotoxin-spiked (50 and 500 µg BEA or ENNB/kg feed) and control (0.075% DMSO) feed pastes were prepared the day before the trial as described in Paper III, using a commercial marine-based diet (produced by Cargill Aqua Nutrition) to assure background levels of mycotoxin-contamination was kept negligible. The experiment was designed as a single-dose exposure test, with the fish being tested for responses 12 hours after the oral administration. The five different exposure treatments (including control) were administrated, and 6 biological replicates (n) (5 in BEA-low) were used for each treatment test group (Table 10). The female:male ratio was 14:17. Detailed materials and methods are described in Paper III.

Table 10. Experimental design of exposure treatments with treatment groups, nominal concentration (µg/kg feed) and number of fish in each group.

| # | Treatment | Nominal conc. (µg/kg feed) | No. of fish (n) |
|---|---|---|---|
| 1 | Control | - | 6 |
| 2 | BEA-low | 50 | 5* |
| 3 | BEA-high | 500 | 6 |
| 4 | ENNB-low | 50 | 6 |
| 5 | ENNB-high | 500 | 6 |

*One fish died so n=5 for this group.




Precisely 12 hours later, samples of liver and intestinal tissue were collected, and flash frozen in liquid nitrogen and stored at -80 °C until further processing. RNA was extracted from intestinal and liver tissues, RNA purity, quantity and integrity assessment were carried out as described in paper III. Total RNA samples were sent to Novogene Europe, Cambridge, UK, for cDNA library preparation and sequencing using the Illumina NovaSeq 6000 platform (Illumina, San Diego, California, USA). In total, 58 libraries were created, 29 each for the liver and intestine, which included all five exposure groups. On average, each library contained 51±8 million reads.

### 3.2.2 Non-targeted transcriptomic analyses *in vitro* and *in vivo*

*Analytics - Differently expressed genes (DEGs)*

Targeted mRNA transcription (RT-qPCR) allows for the detection of low-abundance transcripts that might not be detectable by non-targeted methods, it is a cost-effective option for analyzing a small set of pre-defined target genes. While non-targeted transcriptomics (RNA-Seq) allows for analysis of all expressed genes in a sample, providing a comprehensive view of the transcriptome. Thus, non-targeted transcriptomics enables the discovery of novel genes or pathways that may not have been previously identified or considered, hence unbiased by prior knowledge. The large amount of data generated by omics technologies require specialized statistical

approaches to handle and extract meaningful insights from the data. Biostatistics provides the necessary tools and methods to analyze and interpret big biological data generated by high-throughput technologies. Following the revolution within sequencing technologies, a range of different analytical software has been developed to translate complex datasets into biological knowledge. These biostatistical tools help scientist to compile regulation of individual genes into effects on whole biological networks such as enzymatic and signaling pathways leading up to a biological outcome. In this thesis, transcriptomics was used to study the regulation of RNA transcripts at a global level (transcriptome) following exposure to BEA and ENNB. DNA hybridization-based microarrays are commonly applied for transcriptomic analysis prior to the introduction of high throughput sequencing technologies, often referred to as "next generation sequencing" technologies. The inherent bias of the DNA hybridization and limited dynamic range called for technical validation using alternative techniques such as RT-qPCR. RT-qPCR has been viewed as the gold standard for gene expression analysis. The reliability of the output from Illumina RNA-sequencing has greatly improved in recent times due to significant advancements and refinements made in next-generation sequencing technology (Coenye, 2021). Thus, Coenye (2021) concluded that "the data available suggest that RNA-seq methods and data analysis approaches are robust enough to not always require validation by qPCR and/or other approaches, although there are situations where this may be of added value". In this thesis conclusions were based on pathway analysis and not on a few specific DEGs. Thus RT-qPCR validation was excluded as it was not expected to have provided any additional knowledge. The transcriptomic data generated from the hepatocyte *in vitro* trial (Paper I) is a validation of the transcriptomic data generated through the *in vivo* feeding trial (Paper III). Next-generation Illumina RNA sequencing has become a widely used high-throughput method for studying gene expression levels and identifying differentially expressed genes (DEGs).

The process of generating DEGs from Illumina RNA sequencing data involved several steps:

1. Pre-processing of raw sequencing data: This involved trimming low-quality reads and removing adapter sequences. We used the TrimGalore 0.4.2 wrapper tool (https://github.com/FelixKrueger/TrimGalore) with the default parameters. Library quality was investigated using fastQC included in the TrimGalore wrapper.
2. Mapping reads to a reference genome: The high-quality reads were then aligned to a reference genome assembly. We used the Hisat2 short read aligner version 2.0.4 (Kim et al., 2015) and the Atlantic salmon NCBI gene annotation file (*Salmo_salar*, 24/01/2017 GCA_000233375.4_ICSASG_v2_genomic.gff) to map each individual library to the Atlantic salmon genome (RefSeq Assembly ICSASG_v2).
3. Quantification of gene expression levels: The mapped reads were assigned to individual genes and their expression levels were quantified using the tool FeatureCounts (Liao et al., 2014) of the Subread package (http://subread.sourceforge.net/), generating a count table that indicates the number of reads that map to each gene in each sample.
4. Normalization of expression data: The count data was normalized to account for differences in library size and sequencing depth across samples (prior to statistical analysis, pre-processed RNA sequencing data from the *in vitro* trial were log2 transformed) using the Bioconductor R package (version 3.4.4) DESeq2 (version 1.18.1) (Love et al., 2014).
5. Statistical analysis for identifying differentially expressed genes (DEGs): Once the data was normalized, the *in vitro* data was analyzed for DEGs in a pair-wise comparison of each contrast (Ctrl vs BEA 0.05, Ctrl vs BEA 0.5, Ctrl vs BEA 2.5 µM, and Ctrl vs ENNB 0.05, Ctrl vs ENNB 0.5, Ctrl vs ENNB 2.5 µM) ($q < 0.1$, fold change $> 1$) using Qlucore Omics Explorer 3.5 (Qlucore AB, Lund, Sweden) (the "eliminated factor" option was used to control for confounding factors in the transcriptomic analysis related to the isolation of primary hepatocytes (i.e., time of day, the person doing the isolation, differences in the

basal level of transcription) prior to statistical analysis.) (Qlucore, 2015). While for the *in vivo* data, DESeq2 was used for analysis for DEGs in a pair-wise comparison of each contrast (Ctrl vs BEA-low, Ctrl vs BEA-high, Ctrl vs ENNB-low, Ctrl vs ENNB-high) (Love et al., 2014). These method uses models that account for variation between replicates and estimates the statistical significance of differences in gene expression levels.

6. Annotation of DEGs: Lastly, the DEGs were annotated using databases such as Kyoto Encyclopedia of Genes and Genomes (KEGG; linked to the NCBI database) for the *in vitro* data, and BioMart (linked to the Ensembl database) for the *in vivo* data. The annotation of DEGs provides information about their biological function, including metabolic pathways, signaling pathways, processing pathways, and molecular interactions.

*Analytics – Functional annotation clustering analysis and pathway analysis*

To gain insights into the salmon DEGs underlying biological functions/processes, molecular functions, and involvement in pathways we used the KEGG for analyzing the *in vitro* data. For the *in vivo* trial we used Ingenuity Pathway Analysis (IPA) and the Database for Annotation, Visualization and Integrated Discovery (DAVID) for functional analysis. Furthermore, we carried out functional annotation analyzes of the DEG-lists we had generated from the trials.

KEGG (*in vitro*)

KEGG is a collection of databases providing functional annotations to individual genes/proteins/metabolites for a range of species including Atlantic salmon (Kanehisa and Goto, 2000, Kanehisa et al., 2007). The transcriptomic data generated from the hepatocytes *in vitro* study was used for KEGG pathways enrichment analyses as a hypothesis-generating tool, to identify affected metabolic pathways that could explain the mechanisms of BEA and ENNB causing cellular responses and toxicity in the exposed hepatocytes. Since a cell's biological function is controlled through a dynamic (spatio-temporal) and complex network of signaling within and between pathways through crosstalk and feedback loops (Kholodenko, 2006), a small change in gene

transcription could be important for the downstream effects (Zhan et al., 2017). With a good sample size (n = 6 per experimental condition) we did not wish to set a fold change cutoff criterion, at risk of losing valid data thereby failing to signify important pathways that contribute to producing the response (Zhan et al., 2017). Furthermore, independent follow-up enzymatic assays corroborated the hypothesis generated with non-targeted transcriptomics. Lists of DEGs (p.adjust < 0.1) from individual contrasts (control vs exposed) were analyzed for significantly enriched KEGG pathways and modules using ClusterProfiler v3.12.0 .

DAVID (*in vivo*)

DAVID provides a variety of web-based tools and resources for functional annotation and analysis, pathway analysis, and gene ontology analysis (Huang et al., 2009). It performs statistical analysis and generates graphical summaries of gene functions and pathways, searching a range of different databases such as KEGG, Gene Ontology, and Biocarta. Subsequent analyses were done separately for DEGs with positive and negative fold change (FC) values compared to the control from each exposure group. DAVID was asked to return functional annotation, pathway analysis, gene ontology analysis, and annotation clustering of functionally related DEGs together based on their annotations, making it easier to identify patterns in large gene sets. The cutoff criteria for the input data were set to p-adjusted < 0.2 (Benjamini and Hochberg correction) and gene count ≥ 2, and functional annotation clusters were considered significant when Benjamini was < 0.1.

IPA (*in vivo*)

QIAGEN's IPA tool provides a comprehensive database of curated biological pathways and networks (QIAGEN Inc., https://digitalinsights.qiagen.com/IPA) (Krämer et al., 2013). The IPA pathway analysis integrates gene expression data, data extracted from public databases and from full text scientific articles. Thus, IPA can link specific gene expression patterns to relevant findings in literature in a statistical framework, and identify the key biological pathways and networks involved in a particular disease or biological process. Both canonical pathway analysis and biological function analysis

predict affected biological events, while the upstream regulator analysis identifies the cascade of upstream transcriptional regulators that can explain the observed gene expression changes. We set the cutoff criteria for the DEG input data to p-adjusted < 0.2 (Benjamini and Hochberg correction). The results from IPA were then filtered using p-adjusted < 0.05 (Fisher exact test) and a Z-score > 2. IPA z-scoring is a statistical method used within the IPA software to evaluate the significance of biological functions and pathways. The IPA z-score is calculated by comparing the expression levels of the DEGs within a pathway or biological function, to the expected expression levels based on IPA's internal reference database of gene profiles. A positive IPA z-score indicates that the pathway or biological function is over-expressed, while a negative IPA z-score indicates that it is under-expressed.

*Why KEGG was used for analyzing in vitro DEGs, and IPA for analyzing in vivo DEGs*

The KEGG *Salmo salar* pathway analysis is good and specific and was therefore used for the large dataset from the *in vitro* hepatocytes study. However, the molecular toolbox for Atlantic salmon is in general not as extensive as it is for human/mice. Thus, since the number of DEGs from the *in vivo* trial was lower compared to the hepatocyte *in vitro* trail (total number of DEGs *in vivo* = 1319 vs *in vitro* = 3339), it became possible to manually annotate the DEGs and give the salmon genes their human ortholog name, which allowed us to use the IPA and DAVID databases which are more extensive, though only for human/mice genes. Both IPA and DAVID request human gene names as input, thus salmon gene names were converted to orthologs using the Biomart software and the Ensembl database. The Human – Salmon orthologs based on gene synteny and sequence similarity are mapped in the Ensembl database. Since not all salmon genes are automatically linked to a human ortholog in Ensembl, a manual annotation based on BLAST similarity was conducted on the remaining genes. This then allowed us to use the more comprehensive databases IPA in addition to DAVID.

*Considerations*

Gene orthologs refer to genes that have evolved from a common ancestral gene followed by a speciation event. Functionally, orthologs usually retain the same function, or a very similar function, across different species. However, they can differ in their expression patterns, regulatory mechanisms, or post-translational modifications. Therefore, the overall function of species orthologs may be similar, but not identical (Koonin, 2005, Gabaldón and Koonin, 2013). This is important to keep in mind when assigning an Atlantic salmon gene its human ortholog name and running analytics that are based on the human gene function. That is, while it is likely that the salmon gene has a similar function to its human ortholog, functional discrepancies might exist. Biostatistics are powerful tools that can help give insights into complex biological systems. In addition, biostatistics can be used for hypothesis generation and testing. However, it is important to be aware of their limitations and potential drawbacks when interpreting and applying the results of these analyses. One of the main drawbacks of biostatistics is that it is often reliant on assumptions about the data that may not be entirely accurate. For example, biostatistics cannot fully account for confounding factors that may affect the results of a study. In addition, statistical significance is not necessarily equivalent to biological relevance (Zhan et al., 2017). It is possible that different platforms and analytical methods can produce different results, which can make it difficult to compare data from different studies. However, if similar results were to be given with different platforms, it would instead corroborate the individual findings.

*Adverse outcome pathways development*

AOPs are conceptual frameworks aimed to organize relevant biological information in a logical and coherent manner. AOPs link MIEs following exposure to xenobiotics to AOs, through a sequence of key events (KEs) describing effects at cellular- tissue- or organ level (Fig. 11). The different components of an AOP have been well explained by Villeneuve et al. (2014) and can be summarizes as followed: The MIE is the upstream anchor of the AOP, which denotes the xenobiotics interaction with a biomolecule that causes a disturbance or alteration; A KE is a measurable biological

alteration that is necessary, but not always enough on its own, to advance from a biological disturbance to an adverse outcome; A key event relationship (KER) links how the intensity of a perturbation in an upstream key event affects the outcome of the subsequent key event downstream, supported by biological plausibility (existing knowledge), literature review, or experimental support; The AO is the downstream anchor of the AOP, denoting an adverse apical endpoint assessed at organ level or higher (Fig. 11).

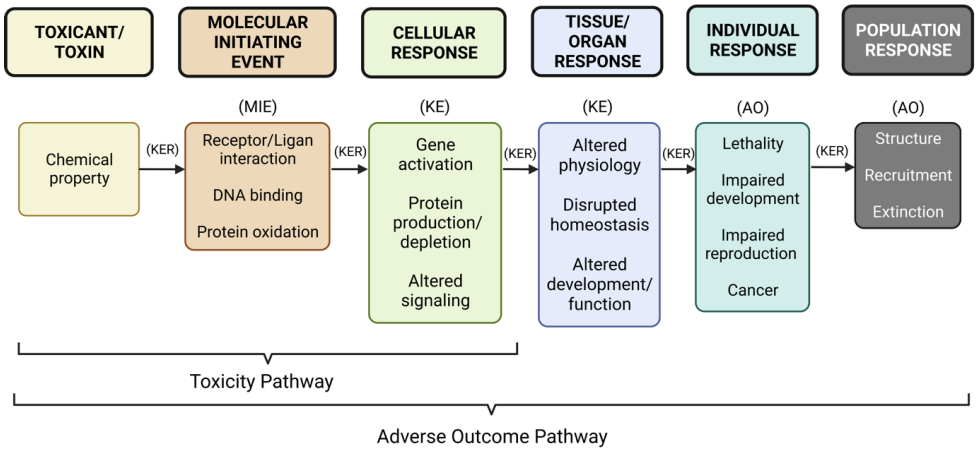



Fig. 11. Adverse outcome pathway (AOP) is a conceptual framework spanning multiple levels of biological organization. AOPs describe the current understanding of the connection between a specific molecular initiating event (MIE) and an adverse outcome (AO) at the level of an organism or population. Key event (KE) denoted by a measurable change in a biological parameter. Key event relationship (KER) are cause-and-effect linkages between upstream and downstream KEs. Adapted from Ankley et al. (2010) and (Willett et al., 2014), created in BioRender.com

There are three general approaches to developing an AOP described by Villeneuve et al. (2014): top-down, bottom-up, and middle-out and the choice of approach will depend on the initial knowledge. A top-down approach may be appropriate when there is a well-defined AO, and then to work backward to identify the key events and molecular initiating events that lead to that outcome. A bottom-up approach may be useful when the MIE is established, and then work upward to identify the key events and adverse outcomes that result from the molecular initiating event. A middle-out

approach can be valuable when there is some knowledge about the biological mechanisms. It starts with a limited set of KEs and then works both directions to fill in the gaps and eventually anchors the AOP to an upstream MIE and a downstream AO. If the molecular initiating event is not fully comprehended, transcriptomic data can provide insights into how exposure to a xenobiotic changes gene expression, giving indications of the cellular changes that may contribute to tissue or organismal alterations (Villeneuve et al., 2014). AOPs are dynamic documents that evolve with new knowledge. Putative AOPs are hypothetical pathways based on limited evidence, serving as a starting point for further research. Qualitative AOPs describe key events and their relationships, linked by biological plausibility and empirical evidence. Quantitative AOPs provide specific information on the magnitude and uncertainty of response, based on experimental data and computational models (Villeneuve et al., 2014). The data generated in Paper I, II, and III in this PhD, together with the work carried out in the AquaMyc project by Omdal (2022) and Berntssen et al. (2023), can be used to construct and populate a preliminary qualitative AOP. My approach was mainly centered around the middle-out approach, utilizing KEs obtained from Papers I, II, and III, in addition to the recent works by Omdal (2022) and Berntssen et al. (2023). To support the biological plausibility of BEA's and ENNB's MIE, I relied on literature reviews showing their ability to integrate into lipid membranes and function as ionophores.

# 4. Integrated Discussion

*"Unrevealing the mechanisms of action of mycotoxins should be viewed like a complicated puzzle for which no picture of the intact puzzle is available. When you find a few pieces that fit together, expand from what is certain, and eventually the picture will become clear. Unfortunately, if the first pieces that fit together are from the corners, then revealing the picture requires that more pieces must be fit together before the scenery becomes recognizable."*

*- Riley and Norred (1994)*

## 4.1 Toxicity exerted by BEA and ENNB in Atlantic salmon – mechanisms and toxicity pathways

### 4.1.1 Lipid membranes are biological targets for ionophores

BEA and ENNB can integrate into the lipid bilayers of cellular and organelle membranes (Kouri et al., 2003, Kamyar et al., 2004, Tonshin et al., 2010). While integrated into these membranes they act as ionophores (Ivanov et al., 1973, Hilgenfeld and Saenger, 1985, Kouri et al., 2003, Kouri et al., 2005, Kamyar et al., 2004). Consequently, the underlying MIE of BEA and ENNB toxicity is most likely their ability to transport ions across the membranes, thereby interfering with ion homeostasis (Mallebrera et al., 2018, Prosperini et al., 2017). For example, BEA caused a rapid influx of $Ca^{2+}$, and a sustained rise in intracellular $Ca^{2+}$ concentration in CCRF-CEM leukemia cells (Chen et al., 2006). The integrity of cell's and organelle's phospholipid membrane is essential for maintaining homeostasis. Several different repair pathways exist to repair injury or damage, caused by for example phospholipid peroxidation or altered fluidity (Ammendolia et al., 2021). *In vitro* and *in vivo* effects in Atlantic salmon following BEA and ENNB exposure are summarized in Table 11. Four main mechanisms of toxicity have been identified: 1) Impaired mitochondrial metabolic

activity and lysosomal function; 2) perturbation of iron homeostasis; 3) perturbation of heme biosynthesis; and 4) triggering of acute inflammation signaling. These mechanisms of toxicity will be further discussed in sections 4.1.2 – 4.1.5.

Table 11. Summary of effects following exposure to BEA and ENNB in Atlantic salmon *in vitro* and *in vivo*

| *in vitro* /*in vivo* | Early Cellular Response(s) | Adaptive Response(s) | ... | Cell/Tissue Injury |
|---|---|---|---|---|
| ***Primary hepatocytes*** | **Impaired mitochondrial metabolic activity and lysosomal function**<br>**Perturbed iron homeostasis**<br>(Paper I) | Antioxidant response<br>Cytoprotective response<br>Increased energy consumption<br>(Paper I) | | Ferroptosis<br>(Paper I) |
| ***Liver tissue*** | **Perturbed heme biosynthesis**<br>(Paper III) | **Acute inflammatory response**<br>(Paper III) | | Reduced hematocrit<br>Anemia<br>(Paper III) |
| ***Intestinal tissue*** | Cellular damage? | **Acute inflammatory response**<br>(Paper III) | | Compromised intestinal permeability? |
| ***Primary head kidney cells*** | Increased expression of pro-inflammatory cytokines (*tnfa, il-1β, il-6β, cox-2*)<br>Altered heme biosynthesis (*alas1*)<br>(Paper II) | | | |

### 4.1.2 Impaired mitochondrial metabolic activity and lysosomal function

The current work indicated that BEA and ENNB impaired both mitochondrial metabolic activity and lysosomal function *in vitro*, in a similar manner (Paper I). BEA- and ENNB-mediated mitochondrial toxicity has been reported previously in mammalian cell models (rat primary hepatocytes, Jurkat cells, ECV304 cells) (Jonsson et al., 2016, Escrivá et al., 2018, Alonso-Garrido et al., 2018, Alonso-Garrido et al., 2020a, Alonso-Garrido et al., 2020b, Alonso-Garrido et al., 2021) (Table 4), and fish cell models (PLHC-1, RTH-149) (García-Herranz et al., 2019) (Table 3). Mammalian *in vitro* studies have reported that BEA and ENNs decreased the mitochondrial membrane potential (MMP) and disrupted their ATP generating function in guinea pig myocytes and Caco-2 cells (Kouri et al., 2005, Ivanova et al., 2012, Prosperini et al., 2013a, Prosperini et al., 2013b) (Table 4). In addition to mitochondria, lysosomes have also been suggested as cytotoxic targets of BEA and ENNB-induced toxicity in CHO-K1 and Caco-2 cells (Ferrer et al., 2009, Ivanova et al., 2012, Prosperini et al., 2013a,

Prosperini et al., 2013b) (Table 4). Former studies found that BEA and ENNB already at transcriptional level and proteomic levels, affect genes related to mitochondrial respiration, by perturbating the electron transport chain and oxidative phosphorylation pathways in rat primary hepatocytes, Jurkat T cells, and ECV 304 cells (Jonsson et al., 2016, Escrivá et al., 2018, Alonso-Garrido et al., 2018, Alonso-Garrido et al., 2020a, Alonso-Garrido et al., 2020b, Alonso-Garrido et al., 2021) (Table 4). While these other studies have described ENNB to primarily down-regulate genes related to mitochondrial respiration, ENNB up-regulated the oxidative phosphorylation pathway in Atlantic salmon *in vitro* (Paper I). This discrepancy could be related to the investigated concentrations, which were lower in Paper I (0.05 - 2.5 µM) compared to those used in the mammalian studies by Jonsson et al. (2016) (rat primary hepatocytes, 1 - 20 µM), Escrivá et al. (2018) (Jurkat, 1.5 - 5 µM), and (Alonso-Garrido et al., 2018) (Jurkat, 1.5 - 5 µM) (Table 4). The ionophoric properties of ENNB and BEA at sub-cytotoxic concentrations could possibly have caused an alteration of the ion homeostasis that increased the rate of mitochondrial respiration, in particular following ENNB exposure. For example, an increase in intracellular [$Ca^{2+}$] can increase the rate of oxidative phosphorylation (Glancy et al., 2013). However, at cytotoxic concentrations, BEA and ENNB perturbated the production of ATP (Paper I) likely as a result of reduced cell viability, potentially causing a depolarization of the mitochondrial membrane potential (Tonshin et al., 2010), or permeabilization of the mitochondrial membrane (Ivanova et al., 2012).

### 4.1.3 Perturbed iron homeostasis

Iron homeostasis relies on a coordinated balance between iron storage, uptake, recycling, utilization, and export (Brown et al., 2020).

*Ferroptosis in hepatocyte - cell injury*

This thesis describes a novel mechanism of toxicity by the ionophoric BEA and ENNB as they affected cellular iron homeostasis, causing oxidative stress that exacerbated lipid peroxidation (LPO) and triggered a ferroptotic cell death in Atlantic salmon primary hepatocytes (Paper I). Earlier research has reported that exposure to BEA (1.5 µM, 24 h) and ENNB (3 µM, 48 h) lead to apoptotic cell death in Caco-2 cells (Prosperini et al., 2013b, Prosperini et al., 2013a). ENNB (10 µM, 4 h) has also been proposed to cause necrotic cell death (non-apoptotic) in rat primary hepatocytes (Jonsson et al., 2016). However, the data presented in this thesis indicated that the salmon primary hepatocytes became ferroptotic rather than apoptotic or necrotic. At cytotoxic concentrations (>2.5 µM, 48 h), both BEA and ENNB caused the hepatocytes to shrink and form protruding blister-like features on their cell membranes (Paper I), features consistent with ferroptosis (Magtanong et al., 2019, Dodson et al., 2019, Van der Meeren et al., 2020). This finding is in line with previous results that ENNB (>5 µM, 24 h) initiated a non-apoptotic cell death pathway with mitochondrial and lysosomal damage, which also lacked typical traits of necrosis in Caco-2 cells (Ivanova et al., 2012). Ferroptosis is described as an iron-dependent cell death pathway caused by toxic levels of lipid peroxidation in polyunsaturated fatty acid (PUFA)-rich phospholipid membranes, which will propagate if not repaired by the GSH/Gpx antioxidant defense system (Dixon et al., 2012, Xie et al., 2016). *In vitro*, both mycotoxins enriched the ferroptosis pathway, while also affecting the transcription of several genes important for iron cycling (transport, recycling, and storage) (Fig. 12).

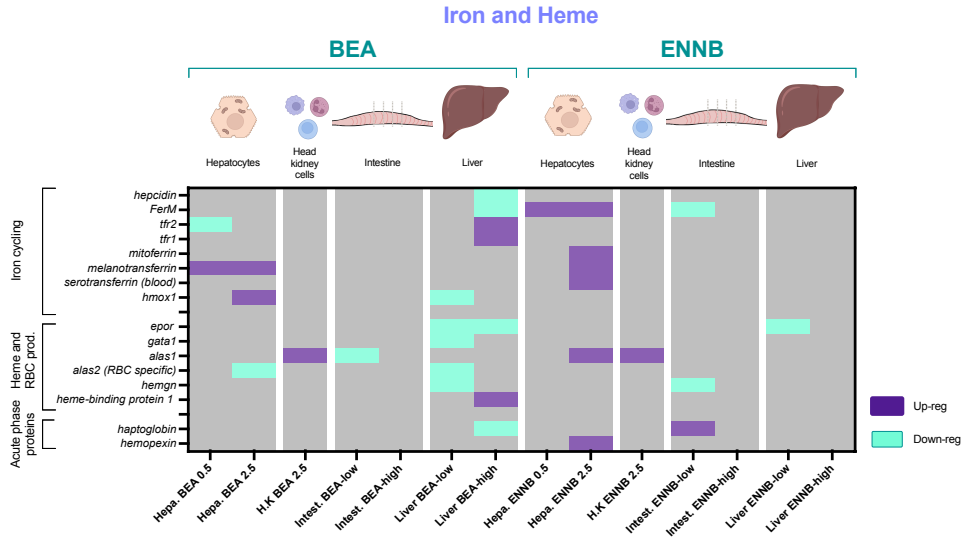



Fig. 12. Illustration showing BEA's and ENNB's effect on transcription of genes in the different *in vitro* primary cell model systems and *in vivo* related to iron cycling, heme biosynthesis and red blood cell production, and acute phase proteins. Genes from targeted mRNA transcription and differently expressed genes from RNA-Seq analysis are depicted in the same figure, to qualitatively illustrate which genes overlapped (and if up or downregulated) between the experiments conducted in Paper I, II, and III.

BEA significantly increased the intracellular iron ($Fe^{3+}$) levels displaying a perturbation of iron homeostasis (Paper I). While the result for ENNB was not significant, it showed a similar trend (Paper I). Unbound $Fe^{2+}$ iron in cells is toxic as it can react with $H_2O_2$ and, through a Fenton-like reaction, generate highly reactive hydroxyl free radicals ($^{\bullet}OH$) while the iron is oxidized to $Fe^{3+}$. These $^{\bullet}OH$ radicals can cause peroxidation of lipid membranes rich in PUFAs, initiating ferroptosis which is detrimental to the cells (Dixon and Stockwell, 2014, Jiang et al., 2021). Thus, the ability of BEA and ENNB to disrupt the iron homeostasis together with a suggested generation of LPO, sensitized the salmon hepatocytes to ferroptosis (Paper I) (Fig. 13). These mechanisms are likely a result of BEA's and ENNB's ionophoric properties, which is discussed in more detail in Paper I.

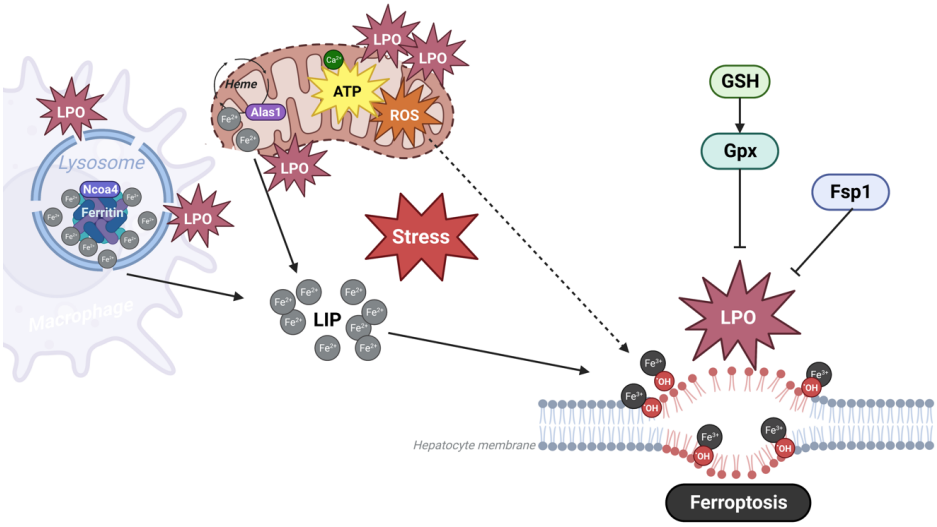



Fig. 13. Proposed model of how BEA and ENNB disrupts cellular iron homeostasis, causing oxidative stress that exacerbates lipid peroxidation (LPO), which triggers ferroptosis in Atlantic salmon primary hepatocytes. Hydroxyl radicals (•OH); Labile iron pool (LIP); Ferroptosis suppressor protein-1(Fsp1); Glutathione peroxidase (Gpx); 5'-aminolevulinate synthase 1 (Alas1); Nuclear receptor coactivator 4 (Ncoa4). Figure created in BioRender.com

Hepatocytes can store iron (Ganz and Nemeth, 2012), and can therefore be expected to be rich in iron. Other potential sources of stress-induced release of toxic intracellular iron levels have been suggested to occur from compromised iron-storing units, such as lysosomes or mitochondria (Fig. 13) (Terman and Kurz, 2013, Mena et al., 2015, Muñoz et al., 2016, Rizzollo et al., 2021). Both these organelles became impaired following BEA and ENNB exposure in the salmon hepatocytes study (section 4.1.2) (Paper I). Furthermore, mitochondria have been shown to be extremely sensitive to iron-induced lipid peroxidation, causing membrane permeabilization (Almeida et al., 2006) thereby diminishing their iron retention capacity which escalates the event. Whether or not BEA and ENNB toxicity is mediated by ROS generation is debated. For example Prosperini et al. (2013a) suggested BEA-mediated mitochondrial toxicity is caused by generation of ROS in Caco-2 cells. Ivanova et al. (2012) on the other hand, concluded that the observed ROS production was not the initiating factor to the loss of lysosomal function, but rather a downstream event in ENNB-mediated toxicity in Caco-2 cells. The authors instead hypothesized that ENNB's effect on mitochondrial

permeabilization and lysosomal destabilization were related to ENNB's ionophoric properties. However, whether the LPO of the salmon primary hepatocytes was directly caused by BEA's and ENNB's ionophoric action, or a downstream consequence of disturbed iron homeostasis needs further investigation.

*Antioxidant stress response counteracting ferroptosis*

Peroxidation of PUFA-rich phospholipid membranes may propagate and initiate ferroptosis if not sufficiently repaired by the glutathione peroxidase (Gpx) 4 antioxidant defense system (Dixon et al., 2012, Xie et al., 2016). In the salmon hepatocytes, the antioxidant defense system was sufficiently mitigating the ROS-generated damage at sub-cytotoxic concentrations of BEA and ENNB *in vitro*, keeping the $H_2O_2$ levels stable (Paper I). Activation of the antioxidant defense system was denoted by BEA and ENNB up-regulating several stress response genes related to glutathione metabolism and heat shock proteins (Fig. 12), that are known to protect against LPO and ferroptosis (Sun et al., 2015, Dixon et al., 2012). The concentration-dependent increase in total Gpx enzyme activity induced by both mycotoxins confirmed the transcriptomic analysis (Paper I). Furthermore, the ferroptosis suppressor protein 1 encoding gene (*fsp1*) that protects against phospholipid peroxidation equivalent to Gpx4 (Doll et al., 2019), was up-regulated by ENNB *in vitro* and by BEA *in vivo* (Fig. 12, 13) (Paper I, III), indicating LPO also occurred in the liver of Atlantic salmon. Both mycotoxins also appeared to up-regulate the pentose phosphate pathway (PPP) (Paper I), that supplies nicotinamide adenine dinucleotide phosphate (NADPH) to give reducing power to the antioxidant defense system during ROS scavenging (Chandel, 2021). However, redox cycling (GSH/GSSG, NADPH/$NADP^+$) is accompanied by an increased energy demand (Di Giulio and Hinton, 2008). This energy demand may be met by up-regulating functions related to oxidative phosphorylation, which is suggested by the results of Paper I for ENNB. In addition, both ENNB and BEA appeared to increase the biosynthesis of cofactors and metabolism of amino acids, as well as enriching the gluconeogenesis pathway. These results could imply the activation of the salmon *chemical defensome,* denoted as an integrative network that is comprised of receptors and transcription factors, biotransformation enzymes, transporters, antioxidants, and metal- and heat-

responsive genes (Eide et al., 2021) (Fig. 14). Support was given by the measured cellular ATP levels, which remained unchanged at sub-cytotoxic concentrations of the mycotoxins (Paper I). This indicated that the hepatocytes were able to sustain an increased energy consumption by the defensome, and other compensatory processes to restore homeostasis. At cytotoxic concentrations, however, both BEA and ENNB caused cellular ATP levels to plunge while compromising the hepatocytes viability, as shown through the reduction of mitochondrial metabolic activity (MTT) in Paper I.

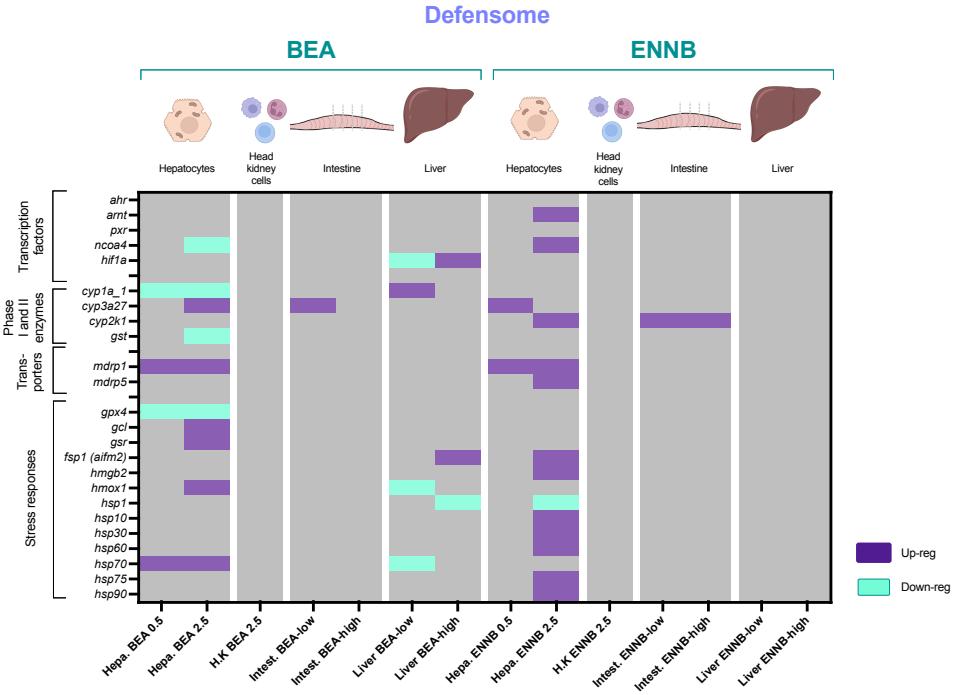



Fig. 14. Illustration showing BEA's and ENNB's effect on transcription of genes related to the Atlantic salmon defensome in the different *in vitro* primary cell model systems and *in vivo*. Genes from targeted mRNA transcription and differently expressed genes from RNA-Seq analysis are depicted in the same figure, to qualitatively illustrate which genes overlapped (and if up or downregulated) between the experiments conducted in Paper I, II, and III.

### 4.1.4 Perturbed heme biosynthesis

Transcriptomic data analysis following *in vivo* and *in vitro* exposure of BEA and ENNB suggested heme biosynthesis to be affected. Heme is a vital prosthetic group used by hemoglobin for oxygen carrying capacity, by cytochromes for electron transfer

capability, by CYPs for xenobiotic metabolism, and by glutathione peroxidases for ROS neutralization (Vijayan et al., 2018, Ryter, 2021). The initial and final steps of heme biosynthesis occur in mitochondria (Ryter and Tyrrell, 2000), where the rate-limiting step is controlled by Alas1, and by Alas 2 in red blood cells (Tanimura et al., 2016, Muckenthaler et al., 2017). *In vitro*, ENNB up-regulated *alas1* transcription in salmon hepatocytes and head kidney cells (Paper I, II) (Fig. 12). In addition, ENNB was also indicated to increase the production of porphyrin (precursor to heme) and *ncoa4* (nuclear receptor coactivator 4) involved in ferritin degradation in salmon hepatocytes (Paper I) (Fig. 14). Thus, ENNB appeared to increase accumulation and utilization of stored iron to synthesize heme *in vitro* at sub-cytotoxic concentration. While BEA up-regulated *alas1* transcription *in vitro* in salmon head kidney cells (Paper II), BEA decreased *alas1* expression in intestinal tissue (Paper III) (Fig. 12), and the erythrocyte-specific *alas2* in hepatocytes and liver tissue (Paper I, III) (Fig. 12). This suggested BEA primarily down-regulated heme biosynthesis in red blood cells both *in vivo* and *in vitro*. In addition, BEA appeared to increase heme degradation *in vitro* by upregulating *hmox1* expression in the hepatocytes (Fig. 12, 15). While the expression of *alas1* is dependent on the availability of both heme and iron, the expression of the erythrocyte specific *alas2* is only controlled by iron (Iolascon et al., 2009). Thus, as Alas2 becomes downregulated when intracellular iron levels are low (Poli et al., 2021), the data suggested that BEA exposure caused a reduced availability of iron that could be utilized for heme biosynthesis intended for hemoglobin *in vivo,* which would explain an inhibition of heme biosynthesis. BEA also down-regulated several genes important for regulating the development of red blood cells (*epor, gata1, alas2, hmox1, hemgn*) *in vivo* (Paper III). The suggested reduction in available cellular iron occurring at the low-dose of BEA in the liver, seemed to be counteracted at the high dose of BEA by the strong downregulation of hepcidin (Paper III). Hepcidin is a key regulator of iron homeostasis (Ganz, 2003). A down regulation of hepcidin would cause an increased release of intracellular iron stored in hepatocytes, macrophages, and other iron transporting units (Fig. 15). Furthermore, the up-regulated transcription of the iron transporter genes (*tfr1, tfr2*) (Fig. 12), could suggest an need to import more iron into the liver (Iolascon et al., 2009).

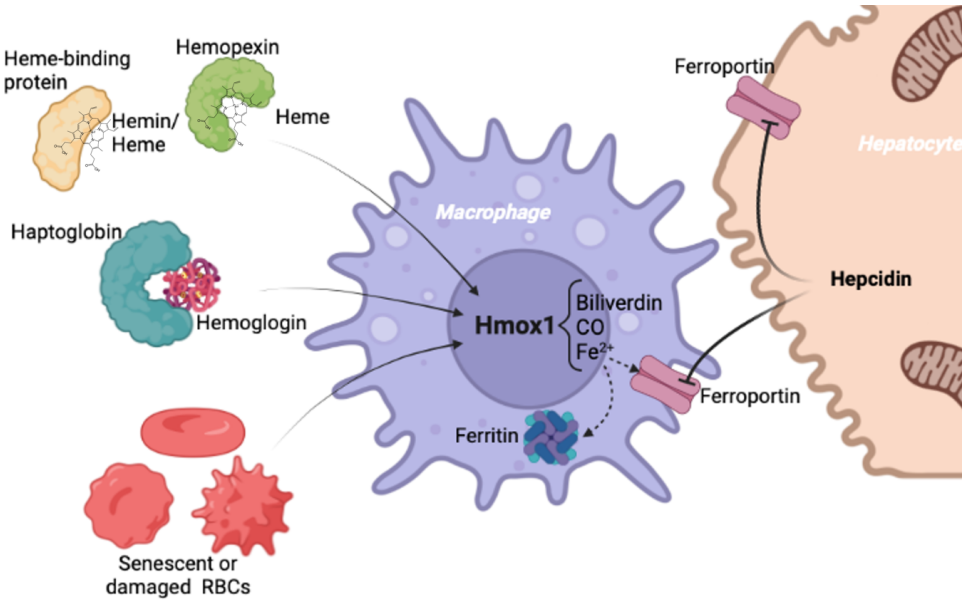



Fig. 15. Macrophages recycle iron through heme oxygenase-1 (Hmox1) activity from senescent or damaged red blood cells, or from haptoglobin-hemoglobin or hemopexin-heme complexes. Iron is then reutilized, stored, or exported through the iron transporter ferroportin. Hepcidin causes internalization and degradation of ferroportin, leading to iron sequestering in iron-recycling macrophages and iron-storing hepatocytes (Roth et al., 2019). Figure created in BioRender.com

The functional analyses indicated that BEA exposure led to reduced hematocrit levels and triggered (hemolytic) anemia in the liver tissue (Paper III), likely because of perturbed heme biosynthesis. Heme biosynthesis requires iron in its ferrous state ($Fe^{2+}$) (Heckl et al., 2021), suggesting a link between the disrupted iron homeostasis observed *in vitro* in Paper I and the suggested disruption of the heme biosynthesis *in vivo* in Paper III. The functional analysis also indicated that BEA exposure caused cell death of salmon blood cells and hemolytic anemia (Paper III), implying that BEA caused destruction of red blood cells *in vivo*. Fish red blood cells are different from mammalian red blood cells in that they have a nucleus, and contain various organelles such as mitochondria and lysosomes (Glomski et al., 1992). In addition, fish red blood cells contain multiple hemoglobin isoforms, some with higher oxygen affinity compared to hemoglobin in mammals and birds, allowing them to extract oxygen more efficiently from the water (Powers, 1980, Witeska, 2013). Despite these differences, several studies

have observed similar effects of BEA and ENNB on human erythrocytes. Olleik et al. (2019) showed BEA, and ENNB to a lesser extent, exhibited a weak hemolytic activity towards human erythrocytes. While, Jilani et al. (2011) found that ENNA triggered eryptosis to a larger extent than hemolysis, and Qadri et al. (2011) reported that BEA caused eryptosis by disrupting the red blood cell membrane. In contrast to hemolysis, eryptosis is programmed cell death of erythrocytes to remove old, defect, or damaged cells in a controlled manner without rupturing the membrane of the red blood cells (Lang et al., 2012b) (Fig. 18). In this thesis, both BEA and ENNB caused downregulation of several genes associated with the membrane of red blood cells in the liver of exposed salmon (Fig. 16) (Paper III). The genes *dmtn, sptb*, *ank1*, *epb4.1*, and *rhag* (Fig. 16) encode membrane-cytoskeleton-associated proteins that are all important for maintaining the shape of red blood cells and the properties of the membrane (Takakuwa, 2000, Nunomura and Takakuwa, 2006, Le Van Kim et al., 2006, Wang et al., 2022). Thus, the down-regulation of these gene indicates that both BEA and ENNB were able to alter the membrane and shape of salmon red blood cells. Further, the down-regulation of the transcription of the different hemoglobin-subunits (*hba, hbb, hbe, hbz*) primarily by BEA (Fig. 16), suggested an effect on the subunit assembly of hemoglobin.

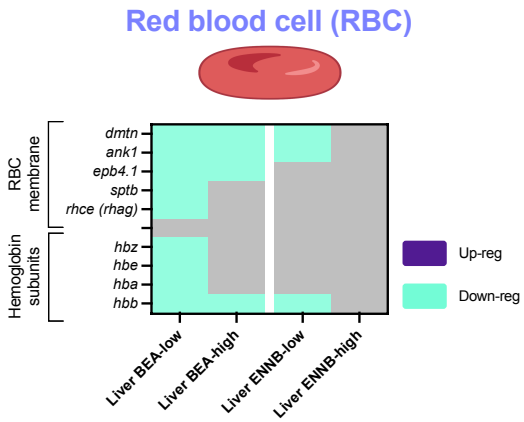



Fig. 16. Illustration showing BEA's and ENNB's effect on transcription of genes related to the membrane of red blood cells and hemoglobin subunits *in vivo*. The Illustration is based on the differently expressed gene data from Paper III.

The short-term feeding trial (Paper III) did not provide a definite answer as to whether BEA caused hemolysis or eryptosis, or if the indicated anemic condition in the liver was due to a disturbance in iron metabolism and heme biosynthesis. However, it is worth noting that red blood cells that are deficient in iron are more prone to oxidative stress and eryptosis (Kempe et al., 2006). In the sub-chronic feeding trial with Atlantic salmon conducted by Berntssen et al. (2023) exposure to BEA (300, 4800, and 46000 µg/kg feed) reduced the size of red blood cells, indicated by a significantly decreased mean corpuscular volume (MCV) consistent with eryptosis. This finding supports the indicated effects of BEA on the red blood cell membrane in the short-term feeding trial (Fig. 16) (Paper III). However, salmon sub-chronically exposed to BEA for 76 days did not exhibit an altered number of red blood cells, hemoglobin, or hematocrit (Berntssen et al., 2023). Interestingly, Berntssen et al. (2023) found that ENNB instead caused a significant reduction in hematocrit, number of red blood cells, and hemoglobin in the mid (5200 µg/kg feed) and the highest (83,000 µg/kg feed) dose. This was suggested to be due to a decrease in the formation of new red blood cells rather than hemolysis based on the mean corpuscular volume (MCV). However, the mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) increased with increasing dose of ENNB, indicated a higher hemoglobin concentration in the existing red blood cells. This was supposedly a compensatory mechanism for the reduced oxygen transport caused by the reduction in the red blood cells count (Berntssen et al., 2023).

### 4.1.5 Triggered acute inflammatory signaling

*In vitro*, BEA and ENNB affected immune related genes belonging to suppression of cytokine signaling and the compliment system in hepatocytes (Paper I). Both mycotoxins caused up-regulation of the pro-inflammatory cytokines *tnfa*, *il-1β*, *il-6β* and the *cox-2* enzyme to a lesser extent in head kidney cells (Paper II) (Fig. 17) but did not affect bacterial and viral induced immune signaling.

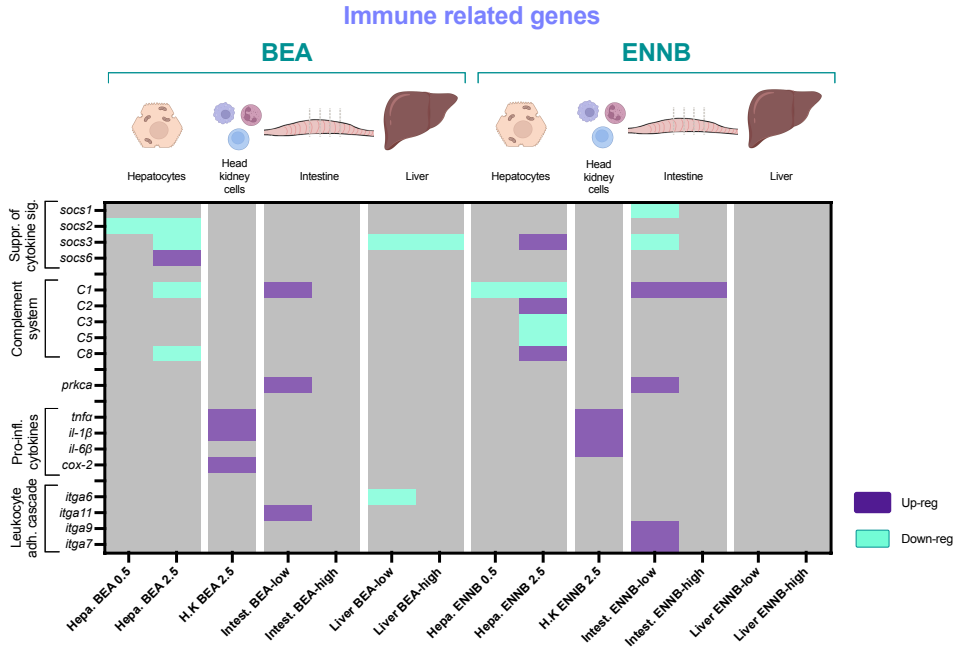



Fig. 17. Illustration showing BEA's and ENNB's effect on transcription of immune related gene in the different *in vitro* primary cell model systems and *in vivo*. The genes are categorized as suppressor of cytokine signaling, complement system, pro-inflammatory cytokines, and leukocyte adhesion cascade. Genes from targeted mRNA transcription and differently expressed genes from RNA-Seq analysis are depicted in the same figure, to qualitatively illustrate which genes overlapped (and if up or downregulated) between the experiments conducted in Paper I, II, and III.

*In vivo*, the functional analysis indicated that both mycotoxins triggered an acute inflammatory response, ENNB in the intestine and BEA in the liver (Paper III). The innate immune system of teleost fish includes PRRs which are activated by different immunostimulatory molecules that interact with the PRRs. Depending on the origin of the immunostimulatory molecules, they are divided into exogenous pathogen-associated molecular patterns (PAMPs) (i.e., bacterial, viral, fungal) or endogenous damage-associated molecular patterns (DAMPs) (i.e., cellular debris and inflammatory contents) (van de Veerdonk et al., 2008, Rebl et al., 2010). The third most up-regulated gene by ENNB-low in the intestine was haptoglobin (Paper III) (Fig. 12), an acute phase protein that binds free hemoglobin (Schaer et al., 2014) (Fig. 15), since free hemoglobin can be a source of ROS (Reddy et al., 2007). Tissue inflammation is often associated

with tissue damage, which tends to result in the release of ruptured red blood cells and thus release of hemoglobin (de la Rubia Ortí et al., 2021). Haptoglobin as an acute protein is therefore increased during inflammation, where IL-6 and Tnfα are known inducers (Wang et al., 2001, Chiellini et al., 2002). Interestingly, both ENNB and BEA had the ability to cause a strong induction of *tnfa* transcription *in vitro* in head kidney cells (Paper II). Haptoglobin has been reported to be the most sensitive marker of acute inflammation in Wistar Han rats (Giffen et al., 2003). This suggests that oral ENNB exposure can led to intestinal cell damage and subsequent release of DAMPs (Fig. 18). The implied inflammatory response triggered by BEA in the liver, could also indirectly become triggered by DAMPs released from damaged tissue or red blood cells (Fig. 18). Since, eryptosis remove old, defect, or damaged cells in a controlled manner without rupturing the membrane of red blood cells (Lang et al., 2012b) (Fig. 18), eryptosis would not be expected to trigger an inflammatory response to the same degree as hemolysis. However, excessive eryptosis, and engulfment and degradation of the eryptotic red blood cells by macrophages (Fig. 15), may lead to anemia if red blood cells are destroyed faster than they are replaced by erythropoiesis (Lang et al., 2012a). Furthermore, the suggested reduction in available iron for heme biosynthesis following BEA exposure (section 4.1.3 and 4.1.4), could also be related to an inflammatory response in the liver (Paper III). Inflammation is known to stimulate hepcidin synthesis, a protein that leads to the retention of iron in certain cells like macrophages and hepatocytes, as shown in Fig. 5. This mechanism is believed to serve as a "host defense" mechanism in the body during acute infections, limiting the availability of iron that pathogens require for growth at the acute phase (Ganz, 2003). The consequence of this process is to restrict iron availability for erythropoiesis, which can result in anemia (Pagani et al., 2019).

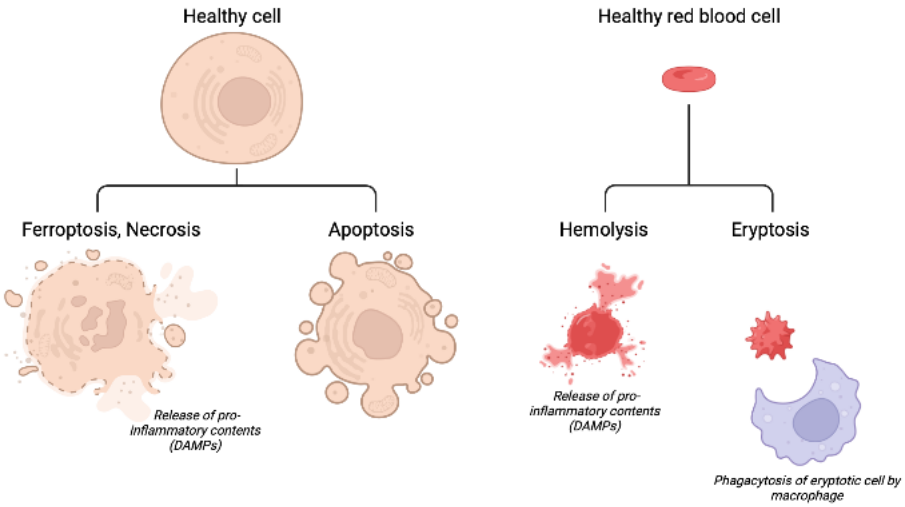



Fig. 18. Schematic of immunogenic and non-immunogenic cell deaths. Immunogenic cell deaths are characterized by membrane rupture and release of intracellular pro-inflammatory contents (damage associated molecular patterns (DAMPs)) (Jeney, 2018, Murao et al., 2021) that are recognized by the innate immune receptors (Tang et al., 2012) and causes a subsequent immune reaction. Figure created in BioRender.com

However, both BEA and ENNB induced the transcription of pro-inflammatory cytokines (similar to bacterial stimuli) in fully viable cells denoted by LDH measurements (Paper II), suggesting another inflammatory trigger than the release of DAMPs in the head kidney cells. Considering that these mycotoxins are known to target mitochondria in salmon primary hepatocytes (Paper I) and various mammalian cell lines (Tonshin et al., 2010, Prosperini et al., 2013a, Prosperini et al., 2013b, Alonso-Garrido et al., 2018, Escrivá et al., 2018, Escrivá et al., 2019, Alonso-Garrido et al., 2020a, Alonso-Garrido et al., 2021), it could be that BEA and ENNB cause damaged to mitochondria in the head kidney leukocytes without killing the cells. The LDH assay can only indicate cell lysis and not potential effects on mitochondria like the MTT assay. Mitochondria (mt) have namely been found to play a crucial role in regulating innate immune responses by releasing mtDAMPs (including mtDNA, ATP, cardiolipin, and formyl peptides). Since mitochondria are of bacterial origin, these mtDAMPs activate the same innate immune PRRs involved in detecting bacterial infections, thus triggering a similar inflammatory response (Rongvaux, 2018). Tonshin

et al. (2010) demonstrated that in rat liver mitochondria, BEA and ENNB caused a reduction in the cytoplasmic concentration of $K^+$, which they suggested could act as a danger signal to activate the NLRP3 inflammasome. Receptor-mediated activation of the NLRP3 inflammasome has been proposed to initiate an inflammatory response by ENNB in RAW 267.4 murine macrophages (Gammelsrud et al., 2012). The NLRP3 inflammasome is a protein complex that partakes in the body's immune response. Stimuli such as microbial infections, tissue damage, and xenobiotics can trigger the NLRP3 inflammasome to undertake a cysteine protease caspase-1-dependent production of pro-inflammatory cytokines (Sutterwala et al., 2014). It is possible that ENNB also can trigger the NLRP3 inflammasome in Atlantic salmon, however no caspase-1 transcription was observed *in vivo*.

Similar to ENNB and BEA, several other mycotoxins like aflatoxin B1, ochratoxin A, deoxynivalenol, T-2 toxin, fumonisin B1, and zearalenone can induce a pro-inflammatory effect (causing a significant increase of the pro-inflammatory cytokines e.g. TNFα, IL-1β, IL-6β, and COX-2) when immunologic stimulants are absent (review by Sun et al. (2022)). However, if the immune system is already activated by other stimulants e.g., a bacterial infection, mycotoxins it may instead act immunosuppressive by decreasing the cytokine production and down-regulating the inflammatory response. These findings are similar to the trends observed in exposed head kidney leukocytes where the mycotoxins did not intensify the impact (Paper II). Mechanisms for this two-way immunomodulation, both immunosuppressive- and immunostimulatory effects, have been suggested to be that mycotoxins may act immunosuppressive by being cytotoxic to immune cells inhibiting or killing them through apoptosis and autophagy (Brown et al., 2021, Sun et al., 2023). On the other hand, mycotoxins can impair the intestinal barrier function by killing intestinal epithelial cells and alerting tight junctions allowing inflammagens to enter the body causing an immunostimulant effect (Kraft et al., 2021, Sun et al., 2023). While future research is needed to fully clarify this, it could also be hypothesized that the acute inflammatory response is both time- and dose-dependent. For example, a higher dose could trigger a stronger initiation of the inflammatory response, that then would

level off as the response reaches the resolving phase and the damage is repaired faster than in a lower dose with a weaker inflammatory response. However, whether BEA and ENNB trigger the acute innate immune response by the same mechanisms, direct or indirect, and whether the mechanisms are cell/tissue dependent requires further research to elucidate.

### 4.1.6 Phase I metabolization enzymes

Detoxification includes a cytoprotective strategy against oxidative stress (Paraskeuas et al., 2021), where the cytosolic aryl hydrocarbon receptor (Ahr) and the nuclear pregnane X receptor (Pxr) pathways mediate the expression of phase I detoxification enzymes such as CYPs (De Anna et al., 2021). In Atlantic salmon, the Ahr pathway has been suggested to control the gene transcription of *cyp1a*, while Pxr controls the transcription of *cyp3a* and *cyp2k* (Søfteland et al., 2014, Yeh et al., 2021). In Atlantic salmon, BEA down-regulated the expression of *cyp1a* and up-regulated *cyp3a27*, while ENNB up-regulated *cyp3a27* (0.5 μM) and *cyp2k1* (2.5 μM) *in vitro* in exposed primary hepatocytes (Paper I) (Fig. 14). In agreement, the same CYPs were also affected *in vivo* by the respective mycotoxins, though BEA up-regulated *cyp1a* in the liver and *cyp3a27* in the intestine while ENNB up-regulated only *cyp2k1* in the intestine (Paper III) (Fig. 14). In contrast to salmon, the biotransformation enzymes CYP3A4, in addition to CYP1A2 and CYP2C19, were important for metabolizing ENNB in human liver microsomes (Fæste et al., 2011). The metabolism of ENNB has been reported to occur both in the intestine and liver in mice exposed to feedborne ENNB (Rodríguez-Carrasco et al., 2016). Due to differences in their evolutionary history, environmental pressures, and diet, isoforms and levels of CYP enzymes can differ between fish and humans. The salmonid Cyp1a1 (Cao et al., 2000) and Cyp3a27 (Lee et al., 1998) are orthologs to the human CYP1A1 and CYP3A4 respectively and have been shown to have similar substrate specificity (Buhler and Wang-Buhler, 1998, Celander et al., 1996). The Rainbow trout Cyp2k1 (Haasch et al., 1994, Buhler et al., 1994), has been suggested to have similarities to the mammalian CYP2 family (Buhler and Wang-Buhler, 1998). Salmonid Cyp2K-likes enzymes were found to be involved in metabolism of fatty acids, steroid hormones, and aflatoxin B1 (Bailey et al., 1996,

Buhler and Wang-Buhler, 1998, Thibaut et al., 2002). Furthermore, Rainbow trout Cyp2k1 was concluded to exhibit similar substrate specificity to aflatoxin B1 as human CYP3A4 (Yang et al., 2000). In fish, Cyp2k1 is primarily expressed in the digestive tract (Schlenk et al., 2008) and liver (Haasch et al., 1994, Buhler et al., 1994). Based on the transcriptomics of the CYP in Paper I and III, we hypothesized that Cyp2k1 is the main CYP responsible for metabolizing ENNB in the intestine and liver, and Cyp3a27 (and possibly Cyp1a_1) metabolizes BEA in the liver of salmon. However, assessments of CYP activity and of metabolites are needed to confirm whether these CYPs metabolize BEA and ENNB in Atlantic salmon.

### 4.1.7 Non-linear responses of *in vitro* and *in vivo* exposure to BEA and ENNB

In the *in vitro* study with primary Atlantic salmon hepatocytes, both BEA and ENNB produced a bell-shaped concentration-response according to the xCELLigence electric cell-substrate impedance assessment (associated with cellular changes e.g., cell adhesion, surface adhesion, cell migration, morphology) (Paper I). A similar bell-shaped response (though not significant) was observed in the MTT-based cell viability assay (mitochondrial metabolic activity) (Paper I). A dose-response relationship is not always linear, instead, the mycotoxins appeared to generate a bell-shaped stress response in the primary hepatocytes, causing them to change morphology and increasing their adhesion to the bottom of the plate (xCELLigence). Furthermore, the mycotoxins appeared to experience an increasing energetic cost following the bell-shaped trend indicated by the mitochondrial metabolic activity (MTT) (Paper I). Energy was likely required to support compensatory mechanisms and the antioxidant defense in the cells against the increased oxidative stress and disturbed iron homeostasis triggered by the mycotoxins (Paper I). However, at cytotoxic concentrations (>2.5 µM), the hepatocytes compensatory and defense mechanisms became overwhelmed and could no longer prevent the cells from becoming ferroptotic (Paper I).

Similarly, the feeding trial with Atlantic salmon showed that the low doses (50 µg/kg feed) of both BEA and ENNB led to a greater number of DEGs in the intestine than

the high dose (500 µg/kg feed) (Fig. 19. C, E) (Paper III). ENNB-low also affected more DEGs in the liver *in vivo* (Fig. 19. F) (Paper III). The reduction in detected DEGs by BEA-high and ENNB-high could indicate that the toxic reactions were overcoming the adaptive transcriptional stress responses in the intestine (Paper III). Sun et al. (2022) proposed that low-doses or short-term exposure to mycotoxins could induce an inflammatory response, while high-doses or long-term mycotoxin exposure could cause immunosuppression. Considering that the DEGs induced by ENNB in the salmon intestine were mainly related to inflammatory response (Paper III), this supports the findings reviewed Sun et al. (2022). Alternatively, the responses could be dose- and/or time-dependent (Sun et al., 2022, Sun et al., 2023). For example, in an *in vitro* study by Alonso-Garrido et al. (2018) with Jurkat cells exposed to ENNB (1.5, 3, and 5 µM), the largest number of DEGs was observed at the middle concentration. Thus, the authors proposed the transcriptomic damage caused by ENNB was dose-dependent.

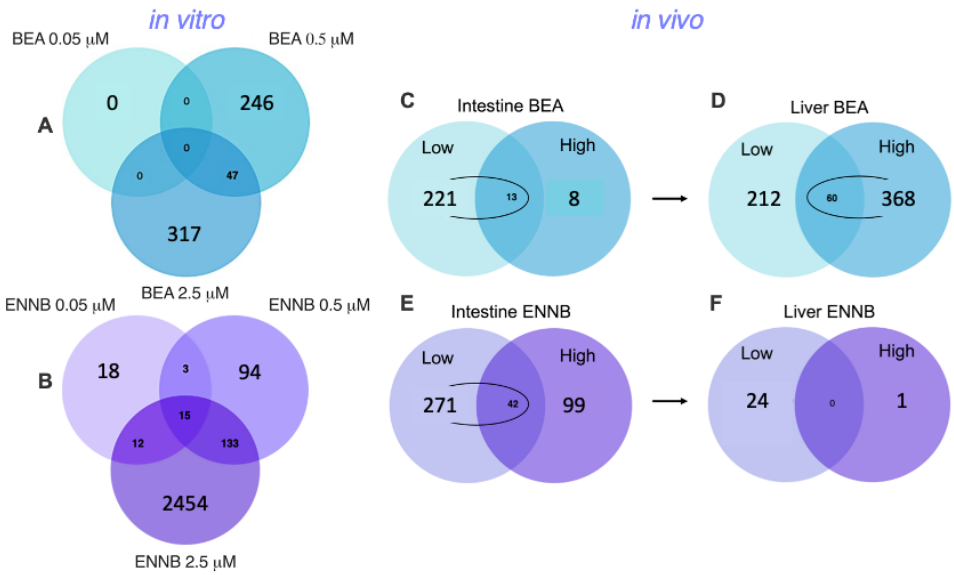



Fig. 19. Venn diagrams of number of DEGs from each exposure treatment *in vitro* (Paper I) and *in vivo* (Paper III). The total number of DEGs *in vitro* by A) BEA = 610, and B) ENNB = 2729, while *in vivo* C-D) BEA = 882, and E-F) ENNB = 437. Venn diagrams were created with EVenn (http://www.ehbio.com/test/venn/#/) (Chen et al., 2021) (Paper I and III).

Furthermore, while BEA-low and ENNB-low resulted in more DEGs in the intestine then their high dose (Fig. 19. C, E, F) (Paper III), the transcriptomic data from the primary hepatocyte *in vitro* study showed a concentration-dependent increase in number of DEGs for both mycotoxins (Fig. 19. A, B) (Paper I). This could indicate different toxicokinetics between the *in vivo* and *in vitro* system, which determines the concentration of the mycotoxin that reaches the intracellular space and ultimately its toxicity. However, in the short-term *in vivo* feeding trial in Atlantic salmon, BEA affected gene expression in the liver (totally 640 DEGs) to a larger degree than in the intestine (totally 242 DEGs). ENNB, on the other hand exhibited a larger effect on the gene expression in the intestine (totally 412 DEGs) and very little in the liver (totally 25 DEGs). This could reflect that ENNB and BEA exhibit different toxicokinetics *in vivo* in Atlantic salmon, that could either be denoted by a low or high oral absorption from the gut, a low or high intestinal/hepatic metabolization, and/or high elimination. Rapid liver metabolization has been reported for ENNB (or ENNB1) both *in vitro* in rat, dog, pig, chicken, and human liver microsomes (Fæste et al., 2011, Ivanova et al., 2011, Ivanova et al., 2017), and *in vivo* in mice, pigs, and rats (Rodríguez-Carrasco et al., 2016, Ivanova et al., 2017, Bhateria et al., 2022). Thus, ENNB could be proposed to undergo rapid metabolism also in the liver of Atlantic salmon, potentially explaining why ENNB had so little effect on gene transcription in the liver compared to the intestine (Fig. 19. E, F) (Paper III). However, similar to the findings in mice by Rodríguez-Carrasco et al. (2016), it is possible that metabolization of ENNB in Atlantic salmon occurred in both the intestine and liver. Consequently, it is plausible that excessive metabolization of ENNB in the intestine may have led to a reduction in the concentration of ENNB that reaches the liver. In mice BEA, did not undergo intestinal/hepatic metabolism to the same degree as ENNB, and was instead distributed to fat-rich tissue such as the liver, followed by adipose tissue and colon (Rodríguez-Carrasco et al., 2016). The findings by Rodríguez-Carrasco et al. (2016), could explain why BEA had a larger effect on gene transcription in liver compared to ENNB in Atlantic salmon *in vivo*. ENNB has also been suggested to be pre-systemically metabolized already in the digestive tract and gut, prior to absorption, in a human gastrointestinal and colonic model (Pallarés et al., 2020), and in pigs after oral administration of ENNB1 (Ivanova et al., 2017). Such a

pre-systemic degradation/metabolism of mycotoxins could be explained by the activity of some gut microbiota, which could influence the absorption of the mycotoxins in the gut (Gratz et al., 2013, Manyes et al., 2014, Brown et al., 2021). Interestingly, in the recent sub-chronic exposure study with Atlantic salmon, ENNB exhibited a higher transfer than BEA (kidney > liver > muscle > brain), although the organ transfer was generally low (<~0.01%) (Berntssen et al., 2023). The authors suggested that feedborne parental ENNB and BEA following sub-chronic exposure exhibited a low-feed-to-tissue transfer either due to a low oral absorption from the gut, high metabolization, and/or high excretion in Atlantic salmon *in vivo*. The degree of effect on gene transcription in intestinal and liver tissues observed in the short-term feeding trail with Atlantic salmon (Paper III), cannot be directly compared with the tissue transfer-measurements of parental ENNB and BEA carried out in the sub-chronic feeding trail with Atlantic salmon carried out by Berntssen et al. (2023). However, it is tempting to assume that the effect on gene transcription in the different salmon tissues observed in Paper III to some degree reflects the distribution of BEA and ENNB (or their metabolites).

But why does BEA have a larger effect on gene transcription than ENNB in the salmon liver following short-term *in vivo* exposure (Paper III), when ENNB exhibited a greater feed-to-organ transfer than BEA following the sub-chronic feeding trail with Atlantic salmon (Berntssen et al., 2023)? Since BEA and ENNB have been indicated to affect hematological parameters (Paper III), one explanation could be the blood protein albumin's affinity to form complexes with BEA and ENNB. ENNA exhibited high binding to blood proteins (99 %) in rat and human plasma (Bhateria et al., 2022). While BEA, on the other hand, formed unstable complexes with human serum albumin proteins (Fliszár-Nyúl et al., 2022). Plasma proteins such as albumin circulate in the blood and play a crucial role in transporting or storing various endogenous and exogenous substances, including xenobiotics, which can have both positive and negative effects on toxicity by influencing their distribution in the body and elimination (Smith et al., 2010, Tayyab and Feroz, 2021). Xenobiotics that bind tightly to albumin may become less toxic as a lower amount is free in the blood, while xenobiotics that bind weakly to albumin may exhibit an increased ability to interact with their targets

(Tayyab and Feroz, 2021). However, binding to serum albumin can also increase the half-life of xenobiotics in the body. Xenobiotics with a high albumin binding capacity (>90 %) can also displace endogenous or exogenous compounds already bound to albumin. This displacement thereby increases the unbound fraction of the displaced compound (endogenous or exogenous) and potentially increases its toxicity depending on the characteristics of the compound (Tayyab and Feroz, 2021). Thus, BEA's and ENNB's different effects on gene transcription in intestinal and liver tissue in Atlantic salmon (Paper III), might be due to different binding-capacity to plasma proteins such as albumin affecting their tissue distribution.

### 4.1.8 Potential implications of BEA and ENNB in dietary exposed Atlantic salmon

Prolonged exposure to BEA and ENNB could potentially cause negative effects in the salmon. The acute inflammatory response triggered in the intestine by ENNB (Paper III) is a natural response intended to mitigate the imposed damage inflicted by the mycotoxin thereby restoring the tissues homeostasis. But this mitigation comes with an energy cost, which will divert energy needed for growth and bone formation. The inflammatory response does indicate that ENNB causes tissue damage, potentially leading to compromised immunological and intestinal barrier integrity following prolonged exposure. While BEA did not appear to trigger any inflammatory response in the intestine, BEA exposure led to cell cycle arrest, inhibited cell proliferation, and made the intestinal cells sensitive to necrosis (Paper III), which is indicative of cellular stress. These types of cellular damage and onset of an inflammatory response in Atlantic salmon, could reduce its overall robustness and potentially have a negative impact on the survival rate during the freshwater/seawater transfer stage. In addition, preliminary results from a sub-chronic feeding trial with Atlantic salmon subjected to continuous dietary exposure of BEA and ENNB for 76 and 69 days, respectively, showed that BEA significantly increased the intestinal permeability, while no apparent histological signs of inflammation were observed (Omdal, 2022) (Unpublished MSc dissertation). In contrast, no significant effect was observed following ENNB exposure in the same study. In the same sub-chronic exposure feeding trial with Atlantic salmon, Berntssen

et al. (2023) reported that BEA exposure appeared to impair the intestinal function leading to a reduction in digestibility, decreased feed conversion, and reduced growth (in terms of fish weight). However, the salmon increased their feed intake, potentially as a compensatory mechanism for the reduced feed utilization (Berntssen et al., 2023). Similarly, Springler et al. (2016) reported that both ENNB and BEA exposure severely reduce intestinal barrier integrity in porcine epithelial cells. "Leaky gut" has been associated with reduced growth performance (Springler et al., 2016). Mycotoxins' potential triggering role in exacerbating epithelial dysfunction (a risk factor for inflammatory changes) prompts future research to address this issue, including the mycotoxins' effect on the gut microbiome as an important variable for intestinal homeostasis (Kraft et al., 2021).

The hematological effects in the liver observed 12 hours after a single-dose dietary exposure (Paper III), indicated that BEA can cause anemia with prolonged or chronic exposure. In the previous mentioned sub-chronic exposure feeding trial, BEA caused salmon red blood cells to shrink (possible eryptosis) though no reduction in the number of red blood cell was observed (Berntssen et al., 2023). Instead, ENNB was found to be more hemotoxic than BEA, causing anemia by reducing the number of red blood cells (not through hemolysis) and reduced hematocrit (Berntssen et al., 2023). Assessment of BEA's and ENNB's bioavailability have been carried out in rodents and pigs (Rodríguez-Carrasco et al., 2016), indicating large species differences. While Rodríguez-Carrasco et al. (2016) assumed a higher chronic toxicity of BEA compared to ENNB in mice, Berntssen et al. (2023) showed ENNB to exhibit higher sub-chronic toxicity than BEA in Atlantic salmon. The short-term exposure trail carried out in this thesis indicated that BEA and ENNB affect gene transcription differently (Paper III). Together, these findings demonstrate the intricate and dynamic nature of the responses following BEA and ENNB exposure, which are both time- and dose-dependent. This highlights the importance of gathering toxicity data at various biological levels (cellular, tissue/organ, organism) and exposure durations to explain the toxicodynamic, in addition to toxicokinetic from both *in vitro* and *in vivo* before a holistic view can be

depicted to gain a comprehensive understanding of the mechanisms and modes of action of these mycotoxins.

Tonshin et al. (2010) stated that ENNB's relatively weak toxicity in mammals is compensated for by its high prevalence in grain crops. In Paper III, we reported measured levels of ENNB with a mean of 37 µg/kg feed, and the maximum measured level was 250 µg/kg feed (Table 2), which falls within the range of doses used in the short-term gavage feeding trial (50 and 500 µg/kg feed). The data from the Norwegian feed surveillance program did not show a high prevalence of BEA which only present in 4 % of samples analyzed (Table 2). The mean measured level of BEA was 16 µg/kg feed, and the maximum level was 25 µg/kg feed (Table 2). Of note, Nácher-Mestre et al. (2020) reported the highest observed concentration of BEA in salmon feed to be 80 µg/kg (Table 2), which is comparable to our BEA-low (50 µg/kg feed). Thus, the low concentration used in our *in vivo* trial was still environmentally relevant, and the high dose (500 µg/kg feed) was added for early toxic effect assessment (still, a non-lethal dose). Furthermore, Pietsch (2020) theoretically estimated maximum mycotoxin levels in farmed fish based on different contamination scenarios of European feed ingredients with mean predicted concentrations of 41.5 µg/kg for BEA (predicted maximum 2692 µg/kg) and 107 µg/kg for total ENNs (predicted maximum 68472 µg/kg). Thus, the low doses used in the short-term feeding trial fall in close range of the predicted mean concentrations made by Pietsch (2020) and are well below the worst-case scenario.

Based on the data presented in Papers I, II, III, summarized in Table 12 showing the effect cascade in relation to the concentration/dose required to initiate the effect, the following toxicity assessment can be made for ENNB and BEA exposure to Atlantic salmon:

- The prevalence of BEA and ENNB in marine aquafeeds were 4 %, (maximum 25 µg/kg) and 80 %, (maximum 250 µg/kg), respectively (Paper III) (Table 2).
- The data indicate that exposure to BEA can cause toxic effects (Table 12) but is of low risk for Atlantic salmon due to the relatively low prevalence in marine aquafeeds.

- The results imply that exposure to ENNB can lead to toxic effects (Table 12), and due to ENNB's relatively high prevalence and concentration in marine aquafeeds, Atlantic salmon risk being exposed to adverse doses of ENNB.

Table 12. Summary of effects following exposure to BEA and ENNB in Atlantic salmon *in vitro* and *in vivo* and at which concentration/dose the effects were observed (Paper I, II, III).

| | *in vitro/ in vivo* | Mechanism/Outcome | Mycotoxin | Conc/Dose required | Exposure time |
|---|---|---|---|---|---|
| Paper I and II | in vitro | Altered cells-substrate adhesion/ Morphology | BEA | 1.5 µM | Paper I 48 h<br>Paper II 24 h |
| | | | ENNB | 0.05 µM | |
| | | Increased Gpx activity | BEA | 0.05 µM | |
| | | | ENNB | 2.5 µM | |
| | | Impaired lysosomal function | BEA | 2.5 µM | |
| | | | ENNB | 2.5 µM | |
| | | Pro-inflammatory cytokine induction | BEA | 2.5 µM | |
| | | | ENNB | 2.5 µM | |
| | | Perturbation of heme biosynthesis | BEA | 2.5 µM | |
| | | | ENNB | 2.5 µM | |
| | | Perturbation of iron homeostasis | BEA | 2.5 - 5.0 µM | |
| | | | ENNB | 2.5 - 5.0 µM | |
| | | Impaired mitochondrial metabolic activity | BEA | 2.5 - 5.0 µM | |
| | | | ENNB | 5.0 µM | |
| | | Ferroptosis | BEA | 2.5 - 5.0 µM | |
| | | | ENNB | 2.5 - 5.0 µM | |
| | | ATP depleation | BEA | 5.0 µM | |
| | | | ENNB | 5.0 µM | |
| Paper III | in vivo | Hematological effects (Liver) | BEA | 50 µg/kg feed | 12 h |
| | | Acute inflammation signaling (Intestine/ Liver) | BEA | 50 µg/kg feed | |
| | | | ENNB | 50 µg/kg feed | |

### 4.1.9 Toxicity pathways and AOP devlopment

The use of single gene biomarkers of exposure has long been the standard practice in toxicological studies (Gundert-Remy et al., 2005, Swenberg et al., 2008). However, implementing transcriptomics with pathway and functional enrichment analyses for identification of xenobiotics mechanisms of actions, gives a more complete picture of which biological functions initiating the toxicological response (Eide et al., 2021). In addition to transcriptomic data, other analyses, such as proteomics and metabolomics, can be incorporated into an AOP pipeline. In this thesis, all data generated from the global transcriptomic analyses, RT-qPCR and cell assays performed (Paper I-III), in addition to other results produced in the AquaMyc project, have been compiled according to an adverse outcome pathway (AOP) conceptual framework. A preliminary schematic overview of the toxicity pathways and adaptive stress responses triggered by BEA and ENNB in Atlantic salmon is presented in Fig. 20. The *in vitro* trial with hepatocytes showed that both BEA and ENNB exhibit a similar toxicity pathway, where a disturbed iron homeostasis (possibly augmented by impairment of iron-containing mitochondria and lysosomes) appeared to cause a redox imbalance with exacerbated lipid peroxidation leading to ferroptosis (Fig. 20) (Paper I). Following short-term exposure *in vivo*, the toxicity pathway of BEA appeared to be the perturbation of heme biosynthesis and affecting the shape of red blood cells indicated to cause reduced hematocrit and anemia (Paper III). This disruption is likely due to BEA ability to disrupt iron homeostasis (Paper I). The sub-chronic feeding trail supported BEA's ability to trigger eryptosis, where hematocrit was reduced after 32 days but became stabilized again after 76 days (Berntssen et al., 2023). This finding supported BEA's ability to cause regenerative anemia indicated in Paper III. Sub-chronic exposure to ENNB reduced hematocrit, hemoglobin, and red blood cells numbers, which is likely due to decreased formation of new red blood cells rather than hemolysis (Berntssen et al., 2023). ENNB's indicated ability to affect iron and heme metabolism *in vitro* (Paper I,II), could explain why ENNB hampered red blood cell formation *in vivo* and caused anemia following sub-chronic exposure (Berntssen et al., 2023). The ability of both ENNB and BEA to induce pro-inflammatory cytokine production *in vitro* (Paper II), supports ENNB's ability to trigger an acute inflammatory response in the salmon intestine *in vivo*

(Paper III). Following sub-chronic exposure, ENNB caused stunted growth that was not related to feed utilization capacity (Berntssen et al., 2023). The stunted growth is possibly a result of energy being diverted to mitigate an inflammatory response away from growth and bone formation. BEA increased the intestinal permeability *in vivo* (Omdal, 2022), which likely led to reduced digestibility, explaining the decreased feed conversion and reduced weight in exposed Atlantic salmon (Berntssen et al., 2023). Taken together, the elucidated toxicity pathways of BEA and ENNB show both mycotoxins have hazardous effects that could lead to adverse outcomes in farmed Atlantic salmon that can potentially lead to reduced survival/yield and performance.

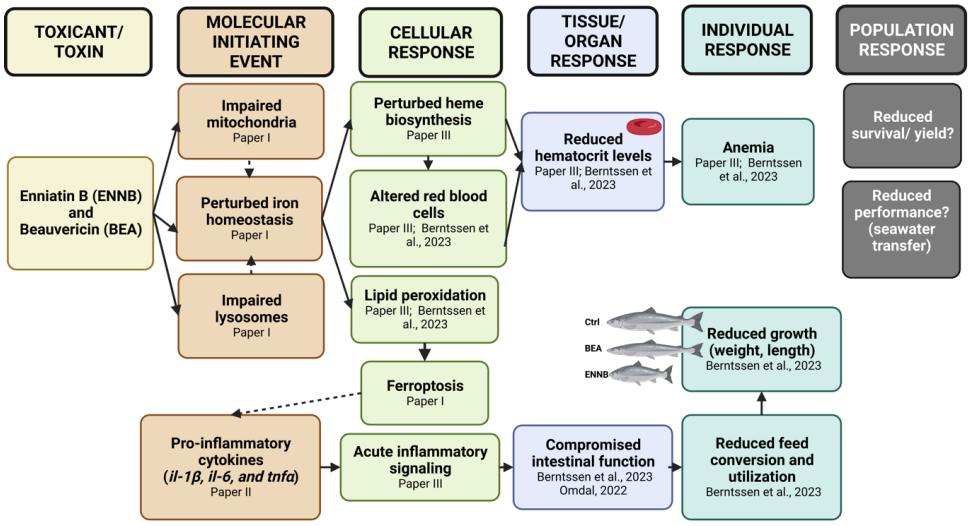



Fig. 20. Proposed schematic of a preliminary adverse outcome pathway network, depicting BEA's and ENNB's mechanisms and toxicity pathways based on the compiled data generated in the AquaMyc project presented in Paper I, II, III, in addition to Omdal (2022) and Berntssen et al. (2023). Figure created in BioRender.com

### 4.1.10 Reflections

When trying to elucidate the mechanism of toxic action of fungal mycotoxins in vertebrates, understanding their mechanism in fungal-fungal/fungal-plant interactions in nature will probably give valuable insights (Riley, 1998). Fungal mycotoxins have been proposed to be involved in fungal-bacterial competition, fungal-fungal/fungal-bacterial communication signals, fungal-fungal interaction, and fungal pathogenicity in plant and animal hosts (Venkatesh and Keller, 2019). The reason why fungi produce ionophoric mycotoxins such as BEA and ENNB is not fully understood (Moretti et al., 2002). Interestingly, in a recent study with bread wheat, ENNB was found to cause LPO and cell death, while also protecting against chlorophyll degradation in bread wheat leaf segments (Ederli et al., 2021). The authors hypothesized that the production of ENNB serves the *Fusarium* by prolonging the life of host plant leaves by halting chlorophyll from degradation. By delaying the yellowing process of the leaves, the production of pro-inflammatory cytokines by the host plant may also be postponed, allowing the *Fusarium* to access the necessary nutrients for the initial mold infection phase, as well as a means to outcompete other fungal competitors (Ederli et al., 2021). In bacteria, ionophores have been proposed as a means to "fight for iron" between competing bacteria (Raatschen et al., 2013). The authors showed that the ionophores calcimycin and ionomycin (which resemble BEA and ENNB) were able to deplete exposed bacterial cell of iron ($Fe^{2+}$) and manganese ($Mn^{2+}$) whilst causing an influx of calcium ($Ca^{2+}$), where the efflux/influx was suggested to be driven by the concentration gradients and the ionophores different binding affinities. Iron ion's ability to alternate between its ferric ($Fe^{3+}$) and ferrous ($Fe^{2+}$) state, gives catalytic function to enzymes important for (but not limited to) energy production, making iron an essential element most life-forms require for their growth (Verbon et al., 2017). Many microorganisms including fungi can produce specific iron($Fe^{3+}$)-scavenging compounds called siderophores, that can transport and store iron, allowing the microorganism to sequester iron and utilize it for their own growth, or withhold iron from competitor microorganism to suppress their growth (Verbon et al., 2017). Another interesting observation was also made by Pinsky et al. (2020), who found that human serum albumin facilitated heme-iron utilization by the human fungal pathogen, *Canadia*

*albicans*, which are able to extract and capture heme from hosts using a network of extracellular hemophores. Thus, similar to Raatschen et al. (2013) regarding bacterial ionophores, it is tempting to speculate that fungal ionophores such as BEA and ENNB could have a functional conformity to siderophores and hemophores, alternatively have a contributing function in fungal iron acquisition from competing microorganisms and hosts.

# 5. Conclusion

Four main mechanisms of toxicity by BEA and ENNB were identified in Atlantic salmon. 1) Impaired mitochondrial metabolic activity and lysosomal function - according to the present *in vitro* data, BEA and ENNB cause impairment of mitochondrial metabolic activity culminating in a drastic drop of ATP levels, as well as impeding lysosomal function (Paper I). 2) Perturbation of iron homeostasis - both mycotoxins disrupted the iron homeostasis *in vitro* and caused oxidative stress that initiated lipid peroxidation, which in turn triggered ferroptosis of salmon hepatocytes (Paper I). To counteract BEA's and ENNB's cytotoxicity, the hepatocytes increased their antioxidant defense (Paper I). 3) Perturbation of heme biosynthesis - BEA and ENNB affected heme biosynthesis, which was indicated by reduced transcription of the heme biosynthesis rate-limiting enzymes in all cells and tissues tested (Paper I, II, III). Further, BEA caused hematological effects in the liver (altered hematocrit and anemia) (Paper III), which were likely correlated to BEA's ability to disrupt iron homeostasis *in vitro*. 4) Acute inflammation signaling - both mycotoxins were indicated to trigger an acute inflammatory response, BEA in the liver and ENNB in the intestine (Paper III). Additionally, both BEA and ENNB exposure led to the up-regulation of genes encoding proinflammatory cytokines such as *tnfa* in exposed head kidney cells at non-cytotoxic concentrations (Paper II). In the case of prolonged exposure, the inflammatory response could predispose salmon to impaired intestinal barrier function. Regarding feed safety, exposure to BEA causes toxic effects but is of low risk due to its relatively low prevalence in marine aquafeeds. Similarly, exposure to ENNB causes adverse effects, and due to ENNB's relatively high occurrence and concentration in marine aquafeeds, Atlantic salmon could potentially be exposed to harmful levels of ENNB.

# 6. Future perspectives

The *in vitro* and *in vivo* trials comprised in this thesis showed that BEA and ENNB disrupted iron homeostasis, and caused oxidative stress that exacerbated lipid peroxidation, which triggered ferroptosis. It would therefore be interesting to carry out additional *in vitro* experiments with BEA and ENNB together with antioxidant and/or iron chelator supplementation to assess whether BEA's and ENNB's ability to disrupt iron homeostasis leading to LPO can be counteracted. Further, it would be useful to determine whether BEA and ENNB directly bind and transport iron across membranes, or if the release of cellular iron is secondary due to cell lysis. Using an iron probe and exposing different cell models (hepatocytes, leukocytes, enterocytes, erythrocytes) and tissues (intestine, liver), to test whether BEA and ENNB can change the flow or iron content (from intracellular to extracellular), while also performing cell viability and lipid peroxidation assessments, could help elucidate biological interactions occurring in this toxicity pathway. The use of transcriptomic analyses in this thesis has provided valuable information on affected biological functions, pathways, and processes following exposure to BEA and ENNB in Atlantic salmon. However, the biological relevance of changes at the transcriptional level can sometimes be difficult to assess, thus including other omics analyses, such as proteomics and metabolomics, which could elucidate which perturbations are biologically relevant, should be investigated.

BEA and ENNB were found to trigger acute inflammatory signaling in intestinal and liver tissue *in vivo*. However, it was difficult to determine whether this was a direct effect of the mycotoxins being recognized by the innate immune system, or if it was a secondary effect of cell and tissue damage. Due to the complexity of an *in vivo* setting, it would be interesting to carry out *in vitro* trials with co-cultures of primary hepatocytes, primary head kidney cells, and enterocytes. Primary cell co-cultures have been successfully used as a model before (Holen et al., 2019, Søfteland and Olsvik, 2022). Such a system would allow cell-to-cell communication signaling to be present, though reduce the number of the variables present *in vivo*. To then test effects on pro-

inflammatory signaling while performing parallel viability assessments, could help elucidate BEA's and ENNB's mechanism for triggering acute inflammation.

While the cytotoxicity of BEA and ENNB was similar *in vitro*, the mycotoxins behaved very differently *in vivo* in Atlantic salmon. The mycotoxins' toxicokinetics (uptake, metabolism, distribution, and elimination) will dictate their toxic effects *in vivo*, and the gut microbiome will influence the uptake of xenobiotics (Licht and Bahl, 2019). Since BEA and ENNB exhibit antimicrobial activity (Jestoi, 2008), it is plausible that the gut microbiota could be affected *in vivo*. Examining the effect of BEA and ENNB on the gut microbiome could add valuable information about their potential uptake. Further, the transcriptomic analyses based both on *in vitro* and *in vivo* work in this thesis showed that BEA exposure altered the expression of cyp1a and cyp3a27, while ENNB affected the expression of cyp2k1. It would therefore be interesting to test activity of these Cyp's. In addition, ligand activation assays could be performed using Atlantic salmon Pxr- and Ahr-constructs, to test whether BEA and ENNB can directly agonize these transcription factors and thus induce cyp3a27 and cyp2k1 transcription. Additionally, investigating the combined exposure of ENNB or BEA with other co-occurring mycotoxins, particularly deoxynivalenol, would provide valuable insights into their potential *in vivo* effects. This is because a study conducted by Ederli et al. (2021) revealed that ENNB and deoxynivalenol can exhibit both synergistic and antagonistic interactions.

# Glossary

**Alas1**: (ubiquitously expressed) rate-limiting enzyme in heme biosynthesis (Tanimura et al., 2016).

**Alas2**: (erythrocyte specific) rate-limiting enzyme in heme biosynthesis acting downstream of Gata1 (Tanimura et al., 2016).

**Aryl hydrocarbon receptor (Ahr)**: cytosolic ligand-activated transcription factor that mediates the expression of phase I detoxification enzymes such as CYPs (Hansson et al., 2004)

**Aryl hydrocarbon receptor nuclear translocator (Arnt)**: Ahr dimerization partner (Aranguren-Abadía et al., 2019).

**Erythropoietin (EPO):** facilitates the transcription of Gata1 (Zhao et al., 2006).

**Erythropoietin receptor (EpoR)**: binds and mediated Epo signaling essential for erythroid cell proliferation and differentiation (Broudy et al., 1991).

**Ferritin**: a $Fe^{2+}$ chaperone protein (Paul et al., 2017).

**Ferroptosis suppressor protein 1 encoding gene (*fsp1*)**: protect against phospholipid peroxidation equivalent to Gpx4 (Doll et al., 2019).

**Gata binding protein 1 (Gata1):** transcription factor that regulates the development of red blood cells (erythropoiesis) (Zhang and Hamza, 2019).

**Glutamate cysteine ligase (Gcl**), together with and glutathione synthetase (Gss) condense cysteine, glutamate, and glycine into glutathione through a two-step reaction (Miess et al., 2018).

**Glutathione peroxidase 4 (Gpx4)**: antioxidant enzyme important for repairing oxidized phospholipids (Pacitti et al., 2013)

**Glutathione reductase (Gsr)**: recirculates oxidized glutathione (GSSG) by reducing it back to GSH (Das and Roychoudhury, 2014).

**Haptoglobin**: an acute-phase responsive protein that binds free hemoglobin (Schaer et al., 2014).

**Heme-binding protein :** binds heme and heme-intermediates in cells to potentially remove these otherwise toxic compounds when free in the cytosol (Palmieri et al., 2015).

**Heme oxygenase-1 (Hmox1)**: enzyme carrying out the rate-limiting step of heme degradation and recycling (Ryter and Tyrrell, 2000).

**Hemogen**: regulates proliferation and development of hematopoietic cells in rodents (Yang et al., 2001).

**Hepcidin**: causes internalization and degradation of ferroportin, leading to iron sequestering in iron-recycling macrophages and iron-storing hepatocytes (Roth et al., 2019). Hemolytic anemia, iron deficiency, and erythropoiesis are known to suppress the expression of hepcidin (Nicolas et al., 2002, Nemeth, 2008, Hintze and McClung, 2011).

**Nuclear receptor coactivator 4 (NCOA4)**: mediates autophagy and degradation of ferritin (ferritinophagy) (Hou et al., 2016)

**Pregnane X receptor (Pxr)**: nuclear transcription factor that mediates the expression of phase I detoxification enzymes such as CYPs (Meucci and Arukwe, 2006).

**Transferrin receptor 1 (Tfr1)**: mediates uptake of transferrin carrying $Fe^{3+}$ through endocytosis (Mackenzie et al., 2008).

**Transferrin receptor 2 (Tfr2)**: a Trf1 homolog involved in maintaining iron homeostasis (Johnson and Enns, 2004).

# References


AAS, T. S., YTRESTØYL, T. & ÅSGÅRD, T. 2019. Utilization of feed resources in the production of Atlantic salmon (*Salmo salar*) in Norway: An update for 2016. *Aquaculture Reports,* 15**,** 100216.

ABRAM, Q. H., DIXON, B. & KATZENBACK, B. A. 2017. Impacts of low temperature on the teleost immune system. *Biology,* 6**,** 39.

ALIFERIS, K. A. & JABAJI, S. 2011. Metabolomics–A robust bioanalytical approach for the discovery of the modes-of-action of pesticides: A review. *Pesticide Biochemistry and physiology,* 100**,** 105-117.

ALMEIDA, A. M., BERTONCINI, C. R., BORECKÝ, J., SOUZA-PINTO, N. C. & VERCESI, A. E. 2006. Mitochondrial DNA damage associated with lipid peroxidation of the mitochondrial membrane induced by Fe2+-citrate. *Anais da Academia Brasileira de Ciências,* 78**,** 505-514.

ALONSO-GARRIDO, M., ESCRIVÁ, L., MANYES, L. & FONT, G. 2018. Enniatin B induces expression changes in the electron transport chain pathway related genes in lymphoblastic T-cell line. *Food and Chemical Toxicology,* 121**,** 437-443.

ALONSO-GARRIDO, M., FRANGIAMONE, M., FONT, G., CIMBALO, A. & MANYES, L. 2021. In vitro blood brain barrier exposure to mycotoxins and carotenoids pumpkin extract alters mitochondrial gene expression and oxidative stress. *Food and Chemical Toxicology,* 153**,** 112261.

ALONSO-GARRIDO, M., MANYES, L., PRALEA, I. & IUGA, C. 2020a. Mitochondrial proteomics profile points oxidative phosphorylation as main target for beauvericin and enniatin B mixture. *Food and Chemical Toxicology,* 141**,** 111432.

ALONSO-GARRIDO, M., TEDESCHI, P., MAIETTI, A., FONT, G., MARCHETTI, N. & MANYES, L. 2020b. Mitochondrial transcriptional study of the effect of aflatoxins, enniatins and carotenoids in vitro in a blood brain barrier model. *Food and Chemical Toxicology,* 137**,** 111077.

AMLUND, H. & BERNTSSEN, M. H. 2004. Arsenobetaine in Atlantic salmon (*Salmo salar L.*): influence of seawater adaptation. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* 138**,** 507-514.

AMMENDOLIA, D. A., BEMENT, W. M. & BRUMELL, J. H. 2021. Plasma membrane integrity: implications for health and disease. *BMC biology,* 19**,** 1-29.

ANKLEY, G. T., BENNETT, R. S., ERICKSON, R. J., HOFF, D. J., HORNUNG, M. W., JOHNSON, R. D., MOUNT, D. R., NICHOLS, J. W., RUSSOM, C. L. & SCHMIEDER, P. K. 2010. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environmental Toxicology and Chemistry: An International Journal,* 29**,** 730-741.

ANTONISSEN, G., MARTEL, A., PASMANS, F., DUCATELLE, R., VERBRUGGHE, E., VANDENBROUCKE, V., LI, S., HAESEBROUCK, F., VAN IMMERSEEL, F. & CROUBELS, S. 2014. The impact of *Fusarium* mycotoxins on human and animal host susceptibility to infectious diseases. *Toxins,* 6**,** 430-452.

ARANGUREN-ABADÍA, L., LILLE-LANGØY, R., MADSEN, A. K., KARCHNER, S. I., FRANKS, D. G., YADETIE, F., HAHN, M. E., GOKSØYR, A. & KARLSEN, O. A. 2019. Molecular and functional properties of the Atlantic cod (*Gadus morhua*) aryl hydrocarbon receptors Ahr1a and Ahr2a. *Environmental science & technology,* 54**,** 1033-1044.

ASHFAQ, H., SOLIMAN, H., SALEH, M. & EL-MATBOULI, M. 2019. CD4: a vital player in the teleost fish immune system. *Veterinary research,* 50**,** 1-11.

BAILEY, G. S., WILLIAMS, D. E. & HENDRICKS, J. D. 1996. Fish models for environmental carcinogenesis: the rainbow trout. *Environmental health perspectives,* 104**,** 5-21.

BEERY, A. K. & ZUCKER, I. 2011. Sex bias in neuroscience and biomedical research. *Neuroscience & Biobehavioral Reviews,* 35**,** 565-572.

BERGEN, W. G. & BATES, D. B. 1984. Ionophores: their effect on production efficiency and mode of action. *Journal of animal science,* 58**,** 1465-1483.

BERNAL-ALGABA, E., PULGARÍN-ALFARO, M. & FERNÁNDEZ-CRUZ, M. L. 2021. Cytotoxicity of mycotoxins frequently present in aquafeeds to the fish cell line RTGill-W1. *Toxins,* 13**,** 581.

BERNHOFT, A., HOGASEN, H. R., ROSENLUND, G., IVANOVA, L., BERNTSSEN, M. H. G., ALEXANDER, J., ERIKSEN, G. S. & FAESTE, C. K. 2017. Tissue distribution and elimination of deoxynivalenol and ochratoxin A in dietary-exposed Atlantic salmon (*Salmo salar*). *Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment,* 34**,** 1211-1224.

BERNTSSEN, M., FJELDAL, P., GAVAIA, P., LAIZÉ, V., HAMRE, K., DONALD, C., JAKOBSEN, J., OMDAL, Å., SØDERSTRØM, S. & LIE, K. 2023. Dietary beauvericin and enniatin B exposure cause different adverse health effects in farmed Atlantic salmon. *Food and Chemical Toxicology,* 174**,** 113648.

BERNTSSEN, M. H., JULSHAMN, K. & LUNDEBYE, A.-K. 2010. Chemical contaminants in aquafeeds and Atlantic salmon (*Salmo salar*) following the use of traditional-versus alternative feed ingredients. *Chemosphere,* 78**,** 637-646.

BERTERO, A., MORETTI, A., SPICER, L. J. & CALONI, F. 2018. Fusarium molds and mycotoxins: Potential species-specific effects. *Toxins,* 10**,** 244.

BHATERIA, M., KARSAULIYA, K., SONKER, A. K., YAHAVI, C. & SINGH, S. P. 2022. Cytochrome P450 isoforms contribution, plasma protein binding, toxicokinetics of enniatin A in rats and *in vivo* clearance prediction in humans. *Food and Chemical Toxicology,* 164**,** 112988.

BROUDY, V. C., LIN, N., BRICE, M., NAKAMOTO, B. & PAPAYANNOPOULOU, T. 1991. Erythropoietin receptor characteristics on primary human erythroid cells. *Blood,* 77**,** 2583-2590.

BROWN, R., PRIEST, E., NAGLIK, J. R. & RICHARDSON, J. P. 2021. Fungal toxins and host immune responses. *Frontiers in microbiology***,** 697.

BROWN, R. A., RICHARDSON, K. L., KABIR, T. D., TRINDER, D., GANSS, R. & LEEDMAN, P. J. 2020. Altered iron metabolism and impact in cancer biology, metastasis, and immunology. *Frontiers in oncology,* 10**,** 476.

BRYDEN, W. L. 2012. Mycotoxin contamination of the feed supply chain: Implications for animal productivity and feed security. *Animal Feed Science and Technology,* 173**,** 134-158.

BUHLER, D. R. & WANG-BUHLER, J.-L. 1998. Rainbow trout cytochrome P450s: purification, molecular aspects, metabolic activity, induction and role in environmental monitoring. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology,* 121**,** 107-137.

BUHLER, D. R., YANG, Y.-H., DREHER, T. W., MIRANDA, C. L. & WANG, J.-L. 1994. Cloning and sequencing of the major rainbow trout constitutive cytochrome P450 (CYP2K1): identification of a new cytochrome P450 gene subfamily and its expression in mature rainbow trout liver and trunk kidney. *Archives of biochemistry and biophysics,* 312**,** 45-51.

CABILLON, N. A. R. & LAZADO, C. C. 2019. Mucosal barrier functions of fish under changing environmental conditions. *Fishes,* 4**,** 2.

CAO, Z., HONG, J., PETERSON, R. E. & AIKEN, J. M. 2000. Characterization of CYP1A1 and CYP1A3 gene expression in rainbow trout (*Oncorhynchus mykiss*). *Aquatic toxicology,* 49**,** 101-109.

CELANDER, M., BUHLER, D. R., FÖRLIN, L., GOKSØYR, A., MIRANDA, C. L., WOODIN, B. R. & STEGEMAN, J. J. 1996. Immunochemical relationships of cytochrome P4503A-like proteins in teleost fish. *Fish Physiology and Biochemistry,* 15**,** 323-332.

CHAN, F. K.-M., MORIWAKI, K. & DE ROSA, M. J. 2013. Detection of necrosis by release of lactate dehydrogenase activity. *Immune Homeostasis: Methods and Protocols***,** 65-70.

CHANDEL, N. S. 2021. NADPH—the forgotten reducing equivalent. *Cold Spring Harbor Perspectives in Biology,* 13**,** a040550.

CHELI, F., BATTAGLIA, D., GALLO, R. & DELL'ORTO, V. 2014. EU legislation on cereal safety: An update with a focus on mycotoxins. *Food Control,* 37**,** 315-325.

CHEN, B.-F., TSAI, M.-C. & JOW, G.-M. 2006. Induction of calcium influx from extracellular fluid by beauvericin in human leukemia cells. *Biochemical and biophysical research communications,* 340**,** 134-139.

CHEN, T., ZHANG, H., LIU, Y., LIU, Y.-X. & HUANG, L. 2021. EVenn: Easy to create repeatable and editable Venn diagrams and Venn networks online. *Journal of genetics and genomics= Yi chuan xue bao,* 48**,** 863-866.

CHEN, Y.-H., FANG, S.-W. & JENG, S.-S. 2013. Zinc transferrin stimulates red blood cell formation in the head kidney of common carp (*Cyprinus carpio*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 166**,** 1-7.

CHIELLINI, C., BERTACCA, A., NOVELLI, S. E., GÖRGÜN, C. Z., CICCARONE, A., GIORDANO, A., XU, H., SOUKAS, A., COSTA, M. & GANDINI, D. 2002. Obesity modulates the expression of haptoglobin in the white adipose tissue via TNFα. *Journal of cellular physiology,* 190**,** 251-258.

CIMBALO, A., ALONSO-GARRIDO, M., FONT, G., FRANGIAMONE, M. & MANYES, L. 2021. Transcriptional changes after enniatins A, A1, B and B1 ingestion in rat stomach, liver, kidney and lower intestine. *Foods,* 10**,** 1630.

COADY, K., BROWNE, P., EMBRY, M., HILL, T., LEINALA, E., STEEGER, T., MAŚLANKIEWICZ, L. & HUTCHINSON, T. 2019. When are adverse outcome pathways and associated assays "fit for purpose" for regulatory decision-making and management of chemicals? *Integrated Environmental Assessment and Management,* 15**,** 633-647.

COENYE, T. 2021. Do results obtained with RNA-sequencing require independent verification? *Biofilm,* 3.

COUNCIL, N. R. 2007. *Toxicity testing in the 21st century: a vision and a strategy*, National Academies Press.

D'MELLO, J., PLACINTA, C. & MACDONALD, A. 1999. *Fusarium* mycotoxins: a review of global implications for animal health, welfare and productivity. *Animal feed science and technology,* 80**,** 183-205.

DAS, K. & ROYCHOUDHURY, A. 2014. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers in environmental science,* 2**,** 53.

DE ANNA, J. S., DARRAZ, L. A., PAINEFILÚ, J. C., CÁRCAMO, J. G., MOURA-ALVES, P., VENTURINO, A. & LUQUET, C. M. 2021. The insecticide chlorpyrifos modifies the expression of genes involved in the PXR and AhR pathways in the rainbow trout, *Oncorhynchus mykiss*. *Pesticide Biochemistry and Physiology,* 178**,** 104920.

DE LA RUBIA ORTÍ, J. E., PLATERO, J. L., BENLLOCH, M., FRANCO-MARTINEZ, L., TVARIJONAVICIUTE, A., ESCRIBÁ-ALEPUZ, J. & SANCHO-CASTILLO, S. 2021. Role of Haptoglobin as a Marker of Muscular Improvement in Patients with Multiple Sclerosis after Administration of Epigallocatechin Gallate and Increase of Beta-Hydroxybutyrate in the Blood: A Pilot Study. *Biomolecules,* 11**,** 617.

DEAN, R., VAN KAN, J. A., PRETORIUS, Z. A., HAMMOND-KOSACK, K. E., DI PIETRO, A., SPANU, P. D., RUDD, J. J., DICKMAN, M., KAHMANN, R. & ELLIS, J. 2012. The Top 10 fungal pathogens in molecular plant pathology. *Molecular plant pathology,* 13**,** 414-430.

DEL MAR ORTEGA-VILLAIZAN, M., COLL, J. & RIMSTAD, E. 2022. The role of red blood cells in the immune response of fish. *Frontiers in Immunology,* 13.

DENG, S., JIA, P.-P., ZHANG, J.-H., JUNAID, M., NIU, A., MA, Y.-B., FU, A. & PEI, D.-S. 2018. Transcriptomic response and perturbation of toxicity pathways in zebrafish larvae after exposure to graphene quantum dots (GQDs). *Journal of hazardous materials,* 357**,** 146-158.

DEVREESE, M., BROEKAERT, N., DE MIL, T., FRAEYMAN, S., DE BACKER, P. & CROUBELS, S. 2014. Pilot toxicokinetic study and absolute oral bioavailability of the *Fusarium* mycotoxin enniatin B1 in pigs. *Food and chemical toxicology,* 63**,** 161-165.

DI GIULIO, R. T. & HINTON, D. E. 2008. *The toxicology of fishes*, Crc Press.

DIXON, S. J., LEMBERG, K. M., LAMPRECHT, M. R., SKOUTA, R., ZAITSEV, E. M., GLEASON, C. E., PATEL, D. N., BAUER, A. J., CANTLEY, A. M. & YANG, W. S. 2012. Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell,* 149**,** 1060-1072.

DIXON, S. J. & STOCKWELL, B. R. 2014. The role of iron and reactive oxygen species in cell death. *Nature chemical biology,* 10**,** 9-17.

DODSON, M., CASTRO-PORTUGUEZ, R. & ZHANG, D. D. 2019. NRF2 plays a critical role in mitigating lipid peroxidation and ferroptosis. *Redox biology,* 23**,** 101107.

DOLL, S., FREITAS, F. P., SHAH, R., ALDROVANDI, M., DA SILVA, M. C., INGOLD, I., GROCIN, A. G., DA SILVA, T. N. X., PANZILIUS, E. & SCHEEL, C. 2019. FSP1 is a glutathione-independent ferroptosis suppressor. *Nature***,** 1-1.

DORNETSHUBER, R., HEFFETER, P., SULYOK, M., SCHUMACHER, R., CHIBA, P., KOPP, S., KOELLENSPERGER, G., MICKSCHE, M., LEMMENS-GRUBER, R. & BERGER, W. 2009. Interactions between ABC-transport proteins and the secondary Fusarium metabolites enniatin and beauvericin. *Molecular nutrition & food research,* 53**,** 904-920.

DORNETSHUBER-FLEISS, R., HEILOS, D., MOHR, T., RICHTER, L., SÜSSMUTH, R. D., ZLESAK, M., NOVICKY, A., HEFFETER, P., LEMMENS-GRUBER, R. & BERGER, W. 2015. The naturally born fusariotoxin enniatin B and sorafenib exert synergistic activity against cervical cancer *in vitro* and *in vivo*. *Biochemical Pharmacology,* 93**,** 318-331.

EC 2016. European Commission Recommendation (EU) 2016/1319 of 29 July 2016 amending Recommendation 2006/576/EC as regards deoxynivalenol, zearalenone and ochratoxin A in pet food. *Off. J. Eur. Union,* 208**,** 58-60.

ECETOC 2007. *Intelligent testing strategies in ecotoxicology: Mode of action approach for specifically acting chemicals*, European Centre for Ecotoxicology

Toxicology of Chemicals.

EDERLI, L., BECCARI, G., TINI, F., BERGAMINI, I., BELLEZZA, I., ROMANI, R. & COVARELLI, L. 2021. Enniatin B and Deoxynivalenol activity on Bread Wheat and on Fusarium species development. *Toxins,* 13**,** 728.

EFSA, P. O. C. I. T. F. C. 2014. Scientific Opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed. *EFSA Journal,* 12**,** 3802.

EIDE, M., ZHANG, X., KARLSEN, O. A., GOLDSTONE, J. V., STEGEMAN, J., JONASSEN, I. & GOKSØYR, A. 2021. The chemical defensome of five model teleost fish. *Scientific reports,* 11**,** 1-13.

ELLIS, T., BERRILL, I., LINES, J., TURNBULL, J. F. & KNOWLES, T. G. 2012. Mortality and fish welfare. *Fish physiology and biochemistry,* 38**,** 189-199.

ESCRIVÁ, L., ALONSO-GARRIDO, M., FONT, G. & MANYES, L. 2019. Transcriptional study after Beauvericin and Enniatin B combined exposure in Jurkat T cells. *Food and Chemical Toxicology,* 130**,** 122-129.

ESCRIVÁ, L., FONT, G. & MANYES, L. 2015. Quantitation of enniatins in biological samples of Wistar rats after oral administration by LC-MS/MS. *Toxicology Mechanisms and Methods,* 25**,** 552-558.

ESCRIVÁ, L., JENNEN, D., CAIMENT, F. & MANYES, L. 2018. Transcriptomic study of the toxic mechanism triggered by beauvericin in Jurkat cells. *Toxicology letters,* 284**,** 213-221.

ESPE, M., XIE, S., CHEN, S., AKSNES, A. & HOLEN, E. 2020. Surplus spermine does not protect salmon cells against lipopolysaccharide (LPS)-induced stress, but excess spermine is eliminated through spermidine/spermine N1-acetyltransferase (SSAT). *Aquaculture Nutrition,* 26**,** 946-953.

FÆSTE, C. K., IVANOVA, L. & UHLIG, S. 2011. In vitro metabolism of the mycotoxin enniatin B in different species and cytochrome p450 enzyme phenotyping by chemical inhibitors. *Drug metabolism and disposition,* 39**,** 1768-1776.

FAO, F. 2018. Agriculture Organization of the United Nations 2016. The state of world fisheries and aquaculture 2016. Contributing to food security and nutrition for all. Rome, 200 pp.

FEHER, J. J. 2017. *Quantitative human physiology: an introduction*, Academic press.

FERRER, E., JUAN-GARCÍA, A., FONT, G. & RUIZ, M. 2009. Reactive oxygen species induced by beauvericin, patulin and zearalenone in CHO-K1 cells. *Toxicology in Vitro,* 23**,** 1504-1509.

FIRDAUS-NAWI, M. & ZAMRI-SAAD, M. 2016. Major components of fish immunity: a review. *Pertanika Journal of Tropical Agricultural Science,* 39.

FJELLDAL, P. G., HANSEN, T. J., WARGELIUS, A., AYLLON, F., GLOVER, K. A., SCHULZ, R. W. & FRASER, T. W. 2020. Development of supermale and all-male Atlantic salmon to research the vgll3 allele-puberty link. *BMC genetics,* 21**,** 1-13.

FLISZÁR-NYÚL, E., FAISAL, Z., SKAPER, R., LEMLI, B., BAYARTSETSEG, B., HETÉNYI, C., GÖMBÖS, P., SZABÓ, A. & POÓR, M. 2022. Interaction of the Emerging Mycotoxins Beauvericin, Cyclopiazonic Acid, and Sterigmatocystin with Human Serum Albumin. *Biomolecules,* 12**,** 1106.

FRAEYMAN, S., DEVREESE, M., ANTONISSEN, G., DE BAERE, S., RYCHLIK, M. & CROUBELS, S. 2016. Comparative oral bioavailability, toxicokinetics, and biotransformation of enniatin B1 and enniatin B in broiler chickens. *Journal of agricultural and food chemistry,* 64**,** 7259-7264.

GABALDÓN, T. & KOONIN, E. V. 2013. Functional and evolutionary implications of gene orthology. *Nature Reviews Genetics,* 14**,** 360-366.

GAMMELSRUD, A., SOLHAUG, A., DENDELÉ, B., SANDBERG, W., IVANOVA, L., BØLLING, A. K., LAGADIC-GOSSMANN, D., REFSNES, M., BECHER, R. & ERIKSEN, G. 2012. Enniatin B-induced cell death and inflammatory responses in

RAW 267.4 murine macrophages. *Toxicology and applied pharmacology,* 261**,** 74-87.
GANZ, T. 2003. Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. *Blood,* 102**,** 783-788.
GANZ, T. & NEMETH, E. 2012. Hepcidin and iron homeostasis. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research,* 1823**,** 1434-1443.
GARCÍA-HERRANZ, V., VALDEHITA, A., NAVAS, J. & FERNÁNDEZ-CRUZ, M. 2019. Cytotoxicity against fish and mammalian cell lines and endocrine activity of the mycotoxins beauvericin, deoxynivalenol and ochratoxin-A. *Food and Chemical Toxicology,* 127**,** 288-297.
GARLID, K. D. & PAUCEK, P. 2003. Mitochondrial potassium transport: the K+ cycle. *Biochimica et Biophysica Acta (BBA)-Bioenergetics,* 1606**,** 23-41.
GIFFEN, P., TURTON, J., ANDREWS, C., BARRETT, P., CLARKE, C., FUNG, K.-W., MUNDAY, M., ROMAN, I., SMYTH, R. & WALSHE, K. 2003. Markers of experimental acute inflammation in the Wistar Han rat with particular reference to haptoglobin and C-reactive protein. *Archives of toxicology,* 77**,** 392-402.
GLANCY, B., WILLIS, W. T., CHESS, D. J. & BALABAN, R. S. 2013. Effect of calcium on the oxidative phosphorylation cascade in skeletal muscle mitochondria. *Biochemistry,* 52**,** 2793-2809.
GLOMSKI, C. A., TAMBURLIN, J. & CHAINANI, M. 1992. The phylogenetic odyssey of the erythrocyte. III. Fish, the lower vertebrate experience. *Histology and histopathology*.
GODOY, P., HEWITT, N. J., ALBRECHT, U., ANDERSEN, M. E., ANSARI, N., BHATTACHARYA, S., BODE, J. G., BOLLEYN, J., BORNER, C. & BOETTGER, J. 2013. Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. *Archives of toxicology,* 87**,** 1315-1530.
GOLDBLATT, L. 2012. *Aflatoxin: scientific background, control, and implications*, Elsevier.
GRATZ, S. W., DUNCAN, G. & RICHARDSON, A. J. 2013. The human fecal microbiota metabolizes deoxynivalenol and deoxynivalenol-3-glucoside and may be responsible for urinary deepoxy-deoxynivalenol. *Applied and Environmental Microbiology,* 79**,** 1821-1825.
GRUBER-DORNINGER, C., JENKINS, T. & SCHATZMAYR, G. 2019. Global mycotoxin occurrence in feed: A ten-year survey. *Toxins,* 11**,** 375.
GUNDERT-REMY, U., DAHL, S. G., BOOBIS, A., KREMERS, P., KOPP-SCHNEIDER, A., OBEREMM, A., RENWICK, A. & PELKONEN, O. 2005. Molecular approaches to the identification of biomarkers of exposure and effect—report of an expert meeting organized by COST Action B15. *Toxicology letters,* 156**,** 227-240.
HAASCH, M. L., GRAF, W. K., QUARDOKUS, E. M., MAYER, R. T. & LECH, J. J. 1994. Use of 7-alkoxyphenoxazones, 7-alkoxycoumarins and 7-alkoxyquinolines as fluorescent substrates for rainbow trout hepatic microsomes after treatment with various inducers. *Biochemical pharmacology,* 47**,** 893-903.
HANSSON, M. C., WITTZELL, H., PERSSON, K. & VON SCHANTZ, T. 2004. Unprecedented genomic diversity of AhR1 and AhR2 genes in Atlantic salmon (*Salmo salar L.*). *Aquatic toxicology,* 68**,** 219-232.
HAUGLAND, Ø., TORGERSEN, J., SYED, M. & EVENSEN, Ø. 2005. Expression profiles of inflammatory and immune-related genes in Atlantic salmon (*Salmo salar L.*) at early time post vaccination. *Vaccine,* 23**,** 5488-5499.

HECKL, C., EISEL, M., LANG, A., HOMANN, C., PAAL, M., VOGESER, M., RÜHM, A. & SROKA, R. 2021. Spectroscopic methods to quantify molecules of the heme-biosynthesis pathway: A review of laboratory work and point-of-care approaches. *Translational Biophotonics,* 3**,** e202000026.

HEILOS, D., RODRÍGUEZ-CARRASCO, Y., ENGLINGER, B., TIMELTHALER, G., VAN SCHOONHOVEN, S., SULYOK, M., BOECKER, S., SÜSSMUTH, R. D., HEFFETER, P. & LEMMENS-GRUBER, R. 2017. The natural fungal metabolite beauvericin exerts anticancer activity in vivo: a pre-clinical pilot study. *Toxins,* 9**,** 258.

HILGENFELD, R. & SAENGER, W. 1985. Structural chemistry of natural and synthetic ionophores and their complexes with cations. *Host Guest Complex Chemistry/Macrocycles.* Springer.

HINTZE, K. J. & MCCLUNG, J. P. 2011. Hepcidin: a critical regulator of iron metabolism during hypoxia. *Advances in Hematology,* 2011.

HOLEN, E., ARAUJO, P., XIE, S., SØFTELAND, L. & ESPE, M. 2019. Resveratrol inhibited LPS induced transcription of immune genes and secretion of eicosanoids in Atlantic salmon (*Salmo salar*), comparing mono-, co-and a novel triple cell culture model of head kidney leukocytes, liver cells and visceral adipocyte tissue. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* 224**,** 108560.

HOLEN, E., ESPE, M., ANDERSEN, S. M., TAYLOR, R., AKSNES, A., MENGESHA, Z. & ARAUJO, P. 2014. A co culture approach show that polyamine turnover is affected during inflammation in Atlantic salmon immune and liver cells and that arginine and LPS exerts opposite effects on p38MAPK signaling. *Fish & Shellfish Immunology,* 37**,** 286-298.

HOLEN, E., WINTERTHUN, S., DU, Z.-Y. & KRØVEL, A. 2011. Inhibition of p38 MAPK during cellular activation modulate gene expression of head kidney leukocytes isolated from Atlantic salmon (*Salmo salar*) fed soy bean oil or fish oil based diets. *Fish & shellfish immunology,* 30**,** 397-405.

HOU, W., XIE, Y., SONG, X., SUN, X., LOTZE, M. T., ZEH III, H. J., KANG, R. & TANG, D. 2016. Autophagy promotes ferroptosis by degradation of ferritin. *Autophagy,* 12**,** 1425-1428.

HU, C. & LI, L. 2015. In vitro culture of isolated primary hepatocytes and stem cell-derived hepatocyte-like cells for liver regeneration. *Protein & cell,* 6**,** 562-574.

HUANG, D. W., SHERMAN, B. T. & LEMPICKI, R. A. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols,* 4**,** 44-57.

HUSSEIN, H. S. & BRASEL, J. M. 2001. Toxicity, metabolism, and impact of mycotoxins on humans and animals. *Toxicology,* 167**,** 101-134.

IOLASCON, A., DE FALCO, L. & BEAUMONT, C. 2009. Molecular basis of inherited microcytic anemia due to defects in iron acquisition or heme synthesis. *haematologica,* 94**,** 395.

ISHIBASHI, H., NAKAMURA, M., KOMORI, A., MIGITA, K. & SHIMODA, S. Liver architecture, cell function, and disease. Seminars in immunopathology, 2009. Springer, 399-409.

ISO, I. 2009. 10993–5: 2009 Biological evaluation of medical devices—part 5: tests for in vitro cytotoxicity. *International Organization for Standardization, Geneva*.

IVANOV, V., EVSTRATOV, A., SUMSKAYA, L., MELNIK, E., CHUMBURIDZE, T., PORTNOVA, S., BALASHOVA, T. & OVCHINNIKOV, Y. A. 1973. Sandwich complexes as a functional form of the enniatin ionophores. *FEBS letters,* 36**,** 65-71.

IVANOVA, L., EGGE-JACOBSEN, W., SOLHAUG, A., THOEN, E. & FÆSTE, C. 2012. Lysosomes as a possible target of enniatin B-induced toxicity in Caco-2 cells. *Chemical research in toxicology,* 25**,** 1662-1674.

IVANOVA, L., FÆSTE, C. K. & UHLIG, S. 2011. In vitro phase I metabolism of the depsipeptide enniatin B. *Analytical and bioanalytical chemistry,* 400**,** 2889-2901.

IVANOVA, L., FÆSTE, C. K., VAN PAMEL, E., DAESELEIRE, E., CALLEBAUT, A. & UHLIG, S. 2014. Presence of enniatin B and its hepatic metabolites in plasma and liver samples from broilers and eggs from laying hens. *World Mycotoxin Journal,* 7**,** 167-175.

IVANOVA, L., UHLIG, S., DEVREESE, M., CROUBELS, S. & FÆSTE, C. K. 2017. Biotransformation of the mycotoxin enniatin B1 in pigs: A comparative in vitro and in vivo approach. *Food and Chemical Toxicology,* 105**,** 506-517.

IVANOVA, L., UHLIG, S., ERIKSEN, G. & JOHANNESSEN, L. 2010. Enniatin B1 is a substrate of intestinal P-glycoprotein, multidrug resistance-associated protein 2 and breast cancer resistance protein. *World Mycotoxin Journal,* 3**,** 271-281.

JACKSON, A. 2009. Fish in-Fish out. *Ratios explained,* 34.

JEDDI, M. Z., HOPF, N. B., VIEGAS, S., PRICE, A. B., PAINI, A., VAN THRIEL, C., BENFENATI, E., NDAW, S., BESSEMS, J. & BEHNISCH, P. A. 2021. Towards a systematic use of effect biomarkers in population and occupational biomonitoring. *Environment International,* 146**,** 106257.

JENEY, V. 2018. Pro-inflammatory actions of red blood cell-derived DAMPs. *Inflammasomes: Clinical and Therapeutic Implications.* Springer.

JESTOI, M. 2008. Emerging Fusarium-mycotoxins fusaproliferin, beauvericin, enniatins, and moniliformin—A review. *Critical reviews in food science and nutrition,* 48**,** 21-49.

JESTOI, M., ROKKA, M., YLI-MATTILA, T., PARIKKA, P., RIZZO, A. & PELTONEN, K. 2004. Presence and concentrations of the Fusarium-related mycotoxins beauvericin, enniatins and moniliformin in Finnish grain samples. *Food additives and contaminants,* 21**,** 794-802.

JIANG, X., STOCKWELL, B. R. & CONRAD, M. 2021. Ferroptosis: Mechanisms, biology and role in disease. *Nature Reviews Molecular Cell Biology***,** 1-17.

JILANI, K., QADRI, S. M., LANG, E., ZELENAK, C., ROTTE, A., BOBBALA, D. & LANG, F. 2011. Stimulation of erythrocyte phospholipid scrambling by enniatin A. *Molecular Nutrition & Food Research,* 55**,** S294-S302.

JOHNSON, M. B. & ENNS, C. A. 2004. Diferric transferrin regulates transferrin receptor 2 protein stability. *Blood,* 104**,** 4287-4293.

JONSSON, M., JESTOI, M., ANTHONI, M., WELLING, A., LOIVAMAA, I., HALLIKAINEN, V., KANKAINEN, M., LYSØE, E., KOIVISTO, P. & PELTONEN, K. 2016. Fusarium mycotoxin enniatin B: Cytotoxic effects and changes in gene expression profile. *Toxicology in Vitro,* 34**,** 309-320.

JUAN-GARCÍA, A., JUAN, C., BIND, M.-A. & ENGERT, F. 2021. Study of locomotion response and development in zebrafish (*Danio rerio*) embryos and larvae exposed to enniatin A, enniatin B, and beauvericin. *Science of The Total Environment,* 777**,** 146075.

KAMYAR, M., RAWNDUZI, P., STUDENIK, C. R., KOURI, K. & LEMMENS-GRUBER, R. 2004. Investigation of the electrophysiological properties of enniatins. *Archives of biochemistry and biophysics,* 429**,** 215-223.

KANEHISA, M., ARAKI, M., GOTO, S., HATTORI, M., HIRAKAWA, M., ITOH, M., KATAYAMA, T., KAWASHIMA, S., OKUDA, S. & TOKIMATSU, T. 2007.

KEGG for linking genomes to life and the environment. *Nucleic acids research,* 36**,** D480-D484.
KANEHISA, M. & GOTO, S. 2000. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic acids research,* 28**,** 27-30.
KEMPE, D. S., LANG, P. A., DURANTON, C., AKEL, A., LANG, K. S., HUBER, S. M., WIEDER, T. & LANG, F. 2006. Enhanced programmed cell death of iron-deficient erythrocytes. *The FASEB journal,* 20**,** 368-370.
KHOLODENKO, B. N. 2006. Cell-signalling dynamics in time and space. *Nature reviews Molecular cell biology,* 7**,** 165-176.
KIM, D., LANGMEAD, B. & SALZBERG, S. L. 2015. HISAT: a fast spliced aligner with low memory requirements. *Nature methods,* 12**,** 357-360.
KINOSHITA, A., KEESE, C., MEYER, U., STARKE, A., WRENZYCKI, C., DÄNICKE, S. & REHAGE, J. 2018. Chronic effects of fusarium mycotoxins in rations with or without increased concentrate proportion on the insulin sensitivity in lactating dairy cows. *Toxins,* 10**,** 188.
KOONIN, E. V. 2005. Orthologs, paralogs, and evolutionary genomics. *Annu. Rev. Genet.,* 39**,** 309-338.
KOURI, K., DUCHEN, M. R. & LEMMENS-GRUBER, R. 2005. Effects of beauvericin on the metabolic state and ionic homeostasis of ventricular myocytes of the guinea pig. *Chemical research in toxicology,* 18**,** 1661-1668.
KOURI, K., LEMMENS, M. & LEMMENS-GRUBER, R. 2003. Beauvericin-induced channels in ventricular myocytes and liposomes. *Biochimica et Biophysica Acta (BBA)-Biomembranes,* 1609**,** 203-210.
KRAFT, S., BUCHENAUER, L. & POLTE, T. 2021. Mold, Mycotoxins and a Dysregulated Immune System: A combination of concern? *International Journal of Molecular Sciences,* 22**,** 12269.
KRÄMER, A., GREEN, J., POLLARD, J., JR & TUGENDREICH, S. 2013. Causal analysis approaches in Ingenuity Pathway Analysis. *Bioinformatics,* 30**,** 523-530.
KREWSKI, D., ACOSTA JR, D., ANDERSEN, M., ANDERSON, H., BAILAR III, J. C., BOEKELHEIDE, K., BRENT, R., CHARNLEY, G., CHEUNG, V. G. & GREEN JR, S. 2010. Toxicity testing in the 21st century: a vision and a strategy. *Journal of Toxicology and Environmental Health, Part B,* 13**,** 51-138.
KŘÍŽOVÁ, L., DADÁKOVÁ, K., DVOŘÁČKOVÁ, M. & KAŠPAROVSKÝ, T. 2021. Feedborne mycotoxins beauvericin and enniatins and livestock animals. *Toxins,* 13**,** 32.
KRØVEL, A. V., WINTERTHUN, S., HOLEN, E. & OLSVIK, P. A. 2011. Development of a co-culture model for in vitro toxicological studies in Atlantic salmon. *Toxicology in Vitro,* 25**,** 1143-1152.
LABIB, S., WILLIAMS, A., KUO, B., YAUK, C. L., WHITE, P. A. & HALAPPANAVAR, S. 2017. A framework for the use of single-chemical transcriptomics data in predicting the hazards associated with complex mixtures of polycyclic aromatic hydrocarbons. *Archives of toxicology,* 91**,** 2599-2616.
LANG, E., QADRI, S. M. & LANG, F. 2012a. Killing me softly–suicidal erythrocyte death. *The international journal of biochemistry & cell biology,* 44**,** 1236-1243.
LANG, F., LANG, E. & FÖLLER, M. 2012b. Physiology and pathophysiology of eryptosis. *Transfusion Medicine and Hemotherapy,* 39**,** 308-314.
LE VAN KIM, C., COLIN, Y. & CARTRON, J.-P. 2006. Rh proteins: key structural and functional components of the red cell membrane. *Blood reviews,* 20**,** 93-110.

LEE, J. W., WON, E.-J., RAISUDDIN, S. & LEE, J.-S. 2015. Significance of adverse outcome pathways in biomarker-based environmental risk assessment in aquatic organisms. *Journal of Environmental Sciences,* 35**,** 115-127.

LEE, S.-J., WANG-BUHLER, J.-L., COK, I., YU, T.-S., YANG, Y.-H., MIRANDA, C. L., LECH, J. & BUHLER, D. R. 1998. Cloning, sequencing, and tissue expression of CYP3A27, a new member of the CYP3A subfamily from embryonic and adult rainbow trout livers. *Archives of biochemistry and biophysics,* 360**,** 53-61.

LIAO, Y., SMYTH, G. K. & SHI, W. 2014. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics,* 30**,** 923-930.

LOVDATA. 2002. *Forskrift om fôrvarer - Vedlegg 1. Grenseverdier for innhold av uønskede stoffer i fôrvarer* [Online]. Available: https://lovdata.no/dokument/SF/forskrift/2002-11-07-1290/KAPITTEL_9-1?q=mykotoksiner%20fôr%20og%20fôrvarer [14.04.2023].

LOVE, M. I., HUBER, W. & ANDERS, S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology,* 15**,** 1-21.

MACKENZIE, E. L., IWASAKI, K. & TSUJI, Y. 2008. Intracellular iron transport and storage: from molecular mechanisms to health implications. *Antioxidants & redox signaling,* 10**,** 997-1030.

MAGTANONG, L., KO, P.-J., TO, M., CAO, J. Y., FORCINA, G. C., TARANGELO, A., WARD, C. C., CHO, K., PATTI, G. J. & NOMURA, D. K. 2019. Exogenous monounsaturated fatty acids promote a ferroptosis-resistant cell state. *Cell chemical biology,* 26**,** 420-432. e9.

MALLEBRERA, B., PROSPERINI, A., FONT, G. & RUIZ, M. J. 2018. In vitro mechanisms of Beauvericin toxicity: A review. *Food and Chemical Toxicology,* 111**,** 537-545.

MANYES, L., ESCRIVÁ, L., RUIZ, M. & JUAN-GARCÍA, A. 2018. Beauvericin and enniatin B effects on a human lymphoblastoid Jurkat T-cell model. *Food and Chemical Toxicology,* 115**,** 127-135.

MANYES, L., ESCRIVÁ, L., SERRANO, A. B., RODRÍGUEZ-CARRASCO, Y., TOLOSA, J., MECA, G. & FONT, G. 2014. A preliminary study in Wistar rats with enniatin A contaminated feed. *Toxicology mechanisms and methods,* 24**,** 179-190.

MARANGHI, F., TASSINARI, R., NARCISO, L., TAIT, S., ROCCA, C. L., FELICE, G. D., BUTTERONI, C., CORINTI, S., BARLETTA, B. & CORDELLI, E. 2018. *In vivo* toxicity and genotoxicity of beauvericin and enniatins. Combined approach to study in vivo toxicity and genotoxicity of mycotoxins beauvericin (BEA) and enniatin B (ENNB). *EFSA Supporting Publications,* 15**,** 1406E.

MARCHESE, S., POLO, A., ARIANO, A., VELOTTO, S., COSTANTINI, S. & SEVERINO, L. 2018. Aflatoxin B1 and M1: Biological properties and their involvement in cancer development. *Toxins,* 10**,** 214.

MARESCA, M. 2013. From the gut to the brain: Journey and pathophysiological effects of the food-associated trichothecene mycotoxin deoxynivalenol. *Toxins,* 5**,** 784-820.

MARSHALL, J. S., WARRINGTON, R., WATSON, W. & KIM, H. L. 2018. An introduction to immunology and immunopathology. *Allergy, Asthma & Clinical Immunology,* 14**,** 1-10.

MCKEE, T. C., BOKESCH, H. R., MCCORMICK, J. L., RASHID, M. A., SPIELVOGEL, D., GUSTAFSON, K. R., ALAVANJA, M. M., CARDELLINA, J. H. & BOYD, M. R. 1997. Isolation and characterization of new anti-HIV and cytotoxic leads from plants, marine, and microbial organisms. *Journal of Natural Products,* 60**,** 431-438.

MENA, N. P., URRUTIA, P. J., LOURIDO, F., CARRASCO, C. M. & NÚÑEZ, M. T. 2015. Mitochondrial iron homeostasis and its dysfunctions in neurodegenerative disorders. *Mitochondrion,* 21**,** 92-105.

MEUCCI, V. & ARUKWE, A. 2006. The xenoestrogen 4-nonylphenol modulates hepatic gene expression of pregnane X receptor, aryl hydrocarbon receptor, CYP3A and CYP1A1 in juvenile Atlantic salmon (*Salmo salar*). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* 142**,** 142-150.

MIESS, H., DANKWORTH, B., GOUW, A. M., ROSENFELDT, M., SCHMITZ, W., JIANG, M., SAUNDERS, B., HOWELL, M., DOWNWARD, J. & FELSHER, D. W. 2018. The glutathione redox system is essential to prevent ferroptosis caused by impaired lipid metabolism in clear cell renal cell carcinoma. *Oncogene,* 37**,** 5435-5450.

MORETTI, A., BELISARIO, A., TAFURI, A., RITIENI, A., CORAZZA, L. & LOGRIECO, A. 2002. Production of beauvericin by different races of F*usarium oxysporum f. sp. melonis*, the *Fusarium* wilt agent of muskmelon. *Mycotoxins in Plant Disease.* Springer.

MOSS, M. 1991. The environmental factors controlling mycotoxin formation. *Mycotoxins and animal foods,* 23**,** 37-56.

MOWI, A. 2021. Integrated Annual Report. *Bergen, Norway: Mowi ASA*.

MUCKENTHALER, M. U., RIVELLA, S., HENTZE, M. W. & GALY, B. 2017. A red carpet for iron metabolism. *Cell,* 168**,** 344-361.

MUÑOZ, Y., CARRASCO, C. M., CAMPOS, J. D., AGUIRRE, P. & NÚÑEZ, M. T. 2016. Parkinson's disease: the mitochondria-iron link. *Parkinson's disease,* 2016.

MURAO, A., AZIZ, M., WANG, H., BRENNER, M. & WANG, P. 2021. Release mechanisms of major DAMPs. *Apoptosis,* 26**,** 152-162.

NACHER-MESTRE, J., BELTRAN, E., STRACHAN, F., DICK, J. R., PEREZ-SANCHEZ, J., BERNTSSEN, M. H. G. & TOCHER, D. R. 2020. No transfer of the non-regulated mycotoxins, beauvericin and enniatins, from feeds to farmed fish reared on plant-based diets. *Food Chemistry,* 323.

NACHER-MESTRE, J., SERRANO, R., BELTRAN, E., PEREZ-SANCHEZ, J., SILVA, J., KARALAZOS, V., HERNANDEZ, F. & BERNTSSEN, M. H. G. 2015. Occurrence and potential transfer of mycotoxins in gilthead sea bream and Atlantic salmon by use of novel alternative feed ingredients. *Chemosphere,* 128**,** 314-320.

NATNAN, M. E., LOW, C.-F., CHONG, C.-M., BUNAWAN, H. & BAHARUM, S. N. 2021. Integration of omics tools for understanding the fish immune response due to microbial challenge. *Frontiers in Marine Science,* 8**,** 668771.

NEMETH, E. 2008. Iron regulation and erythropoiesis. *Current opinion in hematology,* 15**,** 169-175.

NFSA 2019. Anbefalte Grenseverdier for sopp og Mykotoksiner in Fôrvarer. *Mattilsynet/ Norwegian Food Safety Authority*

NICOLAS, G., CHAUVET, C., VIATTE, L., DANAN, J. L., BIGARD, X., DEVAUX, I., BEAUMONT, C., KAHN, A. & VAULONT, S. 2002. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *The Journal of clinical investigation,* 110**,** 1037-1044.

NUNOMURA, W. & TAKAKUWA, Y. 2006. Regulation of protein 4.1 R interactions with membrane proteins by $Ca^{2+}$ and calmodulin. *Frontiers in Bioscience-Landmark,* 11**,** 1522-1539.

OLIVEIRA, M. & VASCONCELOS, V. 2020. Occurrence of mycotoxins in fish feed and its effects: A review. *Toxins,* 12**,** 160.

OLIVEIRA, V. H., DEAN, K. R., QVILLER, L., KIRKEBY, C. & BANG JENSEN, B. 2021. Factors associated with baseline mortality in Norwegian Atlantic salmon farming. *Scientific Reports,* 11**,** 1-14.

OLLEIK, H., NICOLETTI, C., LAFOND, M., COURVOISIER-DEZORD, E., XUE, P., HIJAZI, A., BAYDOUN, E., PERRIER, J. & MARESCA, M. 2019. Comparative structure–activity analysis of the antimicrobial activity, cytotoxicity, and mechanism of action of the fungal cyclohexadepsipeptides enniatins and beauvericin. *Toxins,* 11**,** 514.

OLSVIK, P. A., BERNTSSEN, M. H. & SØFTELAND, L. 2017. In vitro toxicity of pirimiphos-methyl in Atlantic salmon hepatocytes. *Toxicology in Vitro,* 39**,** 1-14.

OLSVIK, P. A. & SØFTELAND, L. 2020. Mixture toxicity of chlorpyrifos-methyl, pirimiphos-methyl, and nonylphenol in Atlantic salmon (*Salmo salar*) hepatocytes. *Toxicology reports,* 7**,** 547-558.

OLSVIK, P. A., WAAGBØ, R., HEVRØY, E. M., REMØ, S. C. & SØFTELAND, L. 2016. In vitro assessment of Hg toxicity in hepatocytes from heat-stressed Atlantic salmon. *Biological trace element research,* 174**,** 226-239.

OMDAL, Å. 2022. The effect of enniatin B and beauvercin–two novel feed contaminants in the intestinal barrier of Atlantic salmon *(Salmo salar).* The University of Bergen.

ØRNSRUD, R., SILVA, M. S., BERNTSSEN, M. H., LUNDEBYE, A.-K., STORESUND, J. E., LIE, K. K., WAAGBØ, R. & SELE, V. 2020. Program for overvåking av fiskefôr-Årsrapport for prøver innsamlet i 2019. *Rapport fra havforskningen*.

PACITTI, D., WANG, T., PAGE, M., MARTIN, S., SWEETMAN, J., FELDMANN, J. & SECOMBES, C. 2013. Characterization of cytosolic glutathione peroxidase and phospholipid-hydroperoxide glutathione peroxidase genes in rainbow trout (*Oncorhynchus mykiss*) and their modulation by in vitro selenium exposure. *Aquatic Toxicology,* 130**,** 97-111.

PAGANI, A., NAI, A., SILVESTRI, L. & CAMASCHELLA, C. 2019. Hepcidin and anemia: a tight relationship. *Frontiers in physiology,* 10**,** 1294.

PALLARÉS, N., RIGHETTI, L., GENEROTTI, S., CAVANNA, D., FERRER, E., DALL'ASTA, C. & SUMAN, M. 2020. Investigating the in vitro catabolic fate of Enniatin B in a human gastrointestinal and colonic model. *Food and Chemical Toxicology,* 137**,** 111166.

PALMIERI, O., MAZZOCCOLI, G., BOSSA, F., MAGLIETTA, R., PALUMBO, O., ANCONA, N., CORRITORE, G., LATIANO, T., MARTINO, G. & RUBINO, R. 2015. Systematic analysis of circadian genes using genome-wide cDNA microarrays in the inflammatory bowel disease transcriptome. *Chronobiology international,* 32**,** 903-916.

PAN, C., KUMAR, C., BOHL, S., KLINGMUELLER, U. & MANN, M. 2009. Comparative proteomic phenotyping of cell lines and primary cells to assess preservation of cell type-specific functions. *Molecular & Cellular Proteomics,* 8**,** 443-450.

PARASKEUAS, V., GRIELA, E., BOUZIOTIS, D., FEGEROS, K., ANTONISSEN, G. & MOUNTZOURIS, K. C. 2021. Effects of Deoxynivalenol and Fumonisins on Broiler Gut Cytoprotective Capacity. *Toxins,* 13**,** 729.

PAUL, B. T., MANZ, D. H., TORTI, F. M. & TORTI, S. V. 2017. Mitochondria and Iron: current questions. *Expert review of hematology,* 10**,** 65-79.

PERSSON, D., NØDTVEDT, A., AUNSMO, A. & STORMOEN, M. 2022. Analysing mortality patterns in salmon farming using daily cage registrations. *Journal of Fish Diseases,* 45**,** 335-347.

PIETSCH, C. 2020. Risk assessment for mycotoxin contamination in fish feeds in Europe. *Mycotoxin Research,* 36**,** 41-62.

PINOTTI, L., OTTOBONI, M., GIROMINI, C., DELL'ORTO, V. & CHELI, F. 2016. Mycotoxin contamination in the EU feed supply chain: A focus on cereal byproducts. *Toxins,* 8**,** 45.

PINSKY, M., ROY, U., MOSHE, S., WEISSMAN, Z. & KORNITZER, D. 2020. Human serum albumin facilitates heme-iron utilization by fungi. *MBio,* 11**,** e00607-20.

POLI, A., SCHMITT, C., MOULOUEL, B., MIRMIRAN, A., PUY, H., LEFÈBVRE, T. & GOUYA, L. 2021. Iron, Heme Synthesis and Erythropoietic Porphyrias: A Complex Interplay. *Metabolites,* 11**,** 798.

POWERS, D. A. 1980. Molecular ecology of teleost fish hemoglobins: strategies for adapting to changing environments. *American Zoologist***,** 139-162.

PRESS, C. M. & EVENSEN, Ø. 1999. The morphology of the immune system in teleost fishes. *Fish & shellfish immunology,* 9**,** 309-318.

PRINCE, R., CROFTS, A. & STEINRAUF, L. 1974. A comparison of beauvericin, enniatin and valinomycin as calcium transporting agents in liposomes and chromatophores. *Biochemical and biophysical research communications,* 59**,** 697-703.

PROSPERINI, A., BERRADA, H., RUIZ, M. J., CALONI, F., COCCINI, T., SPICER, L. J., PEREGO, M. C. & LAFRANCONI, A. 2017. A review of the mycotoxin enniatin B. *Frontiers in Public Health,* 5**,** 304.

PROSPERINI, A., JUAN-GARCÍA, A., FONT, G. & RUIZ, M. 2013a. Beauvericin-induced cytotoxicity via ROS production and mitochondrial damage in Caco-2 cells. *Toxicology letters,* 222**,** 204-211.

PROSPERINI, A., JUAN-GARCÍA, A., FONT, G. & RUIZ, M. 2013b. Reactive oxygen species involvement in apoptosis and mitochondrial damage in Caco-2 cells induced by enniatins A, A1, B and B1. *Toxicology letters,* 222**,** 36-44.

QADRI, S. M., KUCHERENKO, Y. & LANG, F. 2011. Beauvericin induced erythrocyte cell membrane scrambling. *Toxicology,* 283**,** 24-31.

QLUCORE. 2015. *Analyzing RNA-seq data with Qlucore Omics Explorer* [Online]. Available: https://qlucore.com/sites/default/files/2020-03/White%20paper%20rnaseqF.pdf [Accessed 15.04.2023 2023].

RAATSCHEN, N., WENZEL, M., OLE LEICHERT, L. I., DÜCHTING, P., KRÄMER, U. & ELISABETH BANDOW, J. 2013. Extracting iron and manganese from bacteria with ionophores—a mechanism against competitors characterized by increased potency in environments low in micronutrients. *Proteomics,* 13**,** 1358-1370.

REBL, A., GOLDAMMER, T. & SEYFERT, H.-M. 2010. Toll-like receptor signaling in bony fish. *Veterinary immunology and immunopathology,* 134**,** 139-150.

REDDY, C. S. S., SUBRAMANYAM, M., VANI, R. & DEVI, S. A. 2007. In vitro models of oxidative stress in rat erythrocytes: effect of antioxidant supplements. *Toxicology in vitro,* 21**,** 1355-1364.

REHBERGER, K., KROPF, C. & SEGNER, H. 2018. In vitro or not in vitro: a short journey through a long history. *Environmental Sciences Europe,* 30**,** 1-12.

REITE, O. B. & EVENSEN, Ø. 2006. Inflammatory cells of teleostean fish: a review focusing on mast cells/eosinophilic granule cells and rodlet cells. *Fish & shellfish immunology,* 20**,** 192-208.

RICHARD, J. L. 2007. Some major mycotoxins and their mycotoxicoses—An overview. *International journal of food microbiology,* 119**,** 3-10.

RILEY, R. & NORRED, W. 1994. 10 Mechanisms of Mycotoxicity. *Human and Animal Relationships,* 6**,** 193.

RILEY, R. T. 1998. Mechanistic interactions of mycotoxins: theoretical considerations. *Mycotoxins in Agriculture and Food Safety. Marcel Dekker, Inc, Basel, New York***,** 227-254.

RINGOT, D., CHANGO, A., SCHNEIDER, Y.-J. & LARONDELLE, Y. 2006. Toxicokinetics and toxicodynamics of ochratoxin A, an update. *Chemico-biological interactions,* 159**,** 18-46.

RIZZOLLO, F., MORE, S., VANGHELUWE, P. & AGOSTINIS, P. 2021. The lysosome as a master regulator of iron metabolism. *Trends in Biochemical Sciences,* 46**,** 960-975.

RODRÍGUEZ-CARRASCO, Y., HEILOS, D., RICHTER, L., SÜSSMUTH, R. D., HEFFETER, P., SULYOK, M., KENNER, L., BERGER, W. & DORNETSHUBER-FLEISS, R. 2016. Mouse tissue distribution and persistence of the food-born fusariotoxins Enniatin B and Beauvericin. *Toxicology letters,* 247**,** 35-44.

RONGVAUX, A. 2018. Innate immunity and tolerance toward mitochondria. *Mitochondrion,* 41**,** 14-20.

ROSENZWEIG, C., IGLESIUS, A., YANG, X.-B., EPSTEIN, P. R. & CHIVIAN, E. 2001. Climate change and extreme weather events-Implications for food production, plant diseases, and pests.

ROTH, M.-P., MEYNARD, D. & COPPIN, H. 2019. Regulators of hepcidin expression. *Vitamins and Hormones,* 110**,** 101-129.

RUSSELL, W. M. S. & BURCH, R. L. 1959. *The principles of humane experimental technique*, Methuen.

RYTER, S. W. 2021. Significance of heme and heme degradation in the pathogenesis of acute lung and inflammatory disorders. *International Journal of Molecular Sciences,* 22**,** 5509.

RYTER, S. W. & TYRRELL, R. M. 2000. The heme synthesis and degradation pathways: role in oxidant sensitivity: heme oxygenase has both pro-and antioxidant properties. *Free Radical Biology and Medicine,* 28**,** 289-309.

SANDEN, M., HEMRE, G., MÅGE, A., LUNESTAD, B., ESPE, M., LIE, K., LUNDEBYE, A., AMLUND, H., WAAGBØ, R. & ØRNSRUD, R. 2017. Program for overvåking av fiskefôr Årsrapport for prøver innsamlet i 2016. *Bergen: NIFES*.

SANDEN, M., HEMRE, G., MÅGE, A., LUNESTAD, B., ESPE, M., LUNDEBYE, A., AMLUND, H. & ØRNSRUD, R. 2016a. Program for overvåking av fiskefôr Årsrapport for prøver innsamlet i 2015. *Bergen: NIFES*.

SANDEN, M., LILAND, N. S., SÆLE, Ø., ROSENLUND, G., DU, S., TORSTENSEN, B. E., STUBHAUG, I., RUYTER, B. & SISSENER, N. H. 2016b. Minor lipid metabolic perturbations in the liver of Atlantic salmon (*Salmo salar L.*) caused by suboptimal dietary content of nutrients from fish oil. *Fish physiology and biochemistry,* 42**,** 1463-1480.

SANTOS PEREIRA, C., C. CUNHA, S. & FERNANDES, J. O. 2019. Prevalent mycotoxins in animal feed: Occurrence and analytical methods. *Toxins,* 11**,** 290.

SCHAER, D. J., VINCHI, F., INGOGLIA, G., TOLOSANO, E. & BUEHLER, P. W. 2014. Haptoglobin, hemopexin, and related defense pathways—basic science, clinical perspectives, and drug development. *Frontiers in physiology,* 5**,** 415.

SCHLENK, D., CELANDER, M., GALLAGHER, E., GEORGE, S., JAMES, M., KULLMAN, S., VAN DEN HURK, P. & WILLETT, K. 2008. Biotransformation in fishes In The Toxicology of Fishes (Di Giulio RT, Hinton DE, Eds.). CRC Press, Boca Raton.

SELE, V., BERNTSSEN, M. H., PRABHU, P., LUNDEBYE, A.-K., LIE, K. K., ESPE, M., STORESUND, J. E. & ØRNSRUD, R. 2021. Program for overvåking av fiskefôr-Årsapport for prøver innsamlet i 2020. *Rapport fra havforskningen*.

SELE, V., LUNDEBYE, A.-K., BERNTSSEN, M., STORESUND, J. E., LIE, K. K., PHILIP, A., NØSTBAKKEN, O. J., WAAGBØ, R. & ØRNSRUD, R. 2022. Program

for overvåking av fiskefôr-Årsrapport for prøver innsamlet i 2021. *Rapport fra havforskningen*.

SELE, V., SANDEN, M., BERNTSSEN, M., LUNESTAD, B., ESPE, M., LIE, K., AMLUND, H., LUNDEBYE, A., HEMRE, G., WAAGBØ, R. & ØRNSRUD, R. 2018. Program for overvåking av fiskefôr - Årsrapport for prøver innsamlet i 2017. *Rapport fra havforskningen*.

SELE, V., SANDEN, M., BERNTSSEN, M., STORESUND, J. E., LIE, K. K., ESPE, M., LUNDEBYE, A.-K., HEMRE, G. I., WAAGBØ, R. & ØRNSRUD, R. 2019. Program for overvåking av fiskefôr-Årsrapport for prøver innsamlet i 2018. *Rapport fra havforskningen*.

SMITH, D. A., DI, L. & KERNS, E. H. 2010. The effect of plasma protein binding on in vivo efficacy: misconceptions in drug discovery. *Nature reviews Drug discovery,* 9**,** 929-939.

SØDERSTRØM, S., SØFTELAND, L., SELE, V., LUNDEBYE, A.-K., BERNTSSEN, M. H. & LIE, K. K. 2022. Enniatin B and beauvericin affect intestinal cell function and hematological processes in Atlantic salmon (*Salmo salar)* after acute exposure. *Food and Chemical Toxicology***,** 113557.

SØFTELAND, L., EIDE, I. & OLSVIK, P. A. 2009. Factorial design applied for multiple endpoint toxicity evaluation in Atlantic salmon (*Salmo salar L.*) hepatocytes. *Toxicology In Vitro,* 23**,** 1455-1464.

SØFTELAND, L., KIRWAN, J. A., HORI, T. S., STØRSETH, T. R., SOMMER, U., BERNTSSEN, M. H., VIANT, M. R., RISE, M. L., WAAGBØ, R. & TORSTENSEN, B. E. 2014. Toxicological effect of single contaminants and contaminant mixtures associated with plant ingredients in novel salmon feeds. *Food and Chemical Toxicology,* 73**,** 157-174.

SØFTELAND, L. & OLSVIK, P. 2022. In vitro toxicity of glyphosate in Atlantic salmon evaluated with a 3D hepatocyte-kidney co-culture model. *Food and Chemical Toxicology,* 164**,** 113012.

SONG, Y., NAHRGANG, J. & TOLLEFSEN, K. E. 2019. Transcriptomic analysis reveals dose-dependent modes of action of benzo (a) pyrene in polar cod (*Boreogadus saida*). *Science of the Total Environment,* 653**,** 176-189.

SPRINGLER, A., VRUBEL, G.-J., MAYER, E., SCHATZMAYR, G. & NOVAK, B. 2016. Effect of Fusarium-derived metabolites on the barrier integrity of differentiated intestinal porcine epithelial cells (IPEC-J2). *Toxins,* 8**,** 345.

STENBERG, O. K., HOLEN, E., PIEMONTESE, L., LILAND, N. S., LOCK, E.-J., ESPE, M. & BELGHIT, I. 2019. Effect of dietary replacement of fish meal with insect meal on in vitro bacterial and viral induced gene response in Atlantic salmon (*Salmo salar*) head kidney leukocytes. *Fish & shellfish immunology,* 91**,** 223-232.

STOCKWELL, B. R., JIANG, X. & GU, W. 2020. Emerging Mechanisms and Disease Relevance of Ferroptosis. *Trends in Cell Biology*.

STREIT, E., SCHATZMAYR, G., TASSIS, P., TZIKA, E., MARIN, D., TARANU, I., TABUC, C., NICOLAU, A., APRODU, I. & PUEL, O. 2012. Current situation of mycotoxin contamination and co-occurrence in animal feed—Focus on Europe. *Toxins,* 4**,** 788-809.

SUN, X., OU, Z., XIE, M., KANG, R., FAN, Y., NIU, X., WANG, H., CAO, L. & TANG, D. 2015. HSPB1 as a novel regulator of ferroptotic cancer cell death. *Oncogene,* 34**,** 5617-5625.

SUN, Y., HUANG, K., LONG, M., YANG, S. & ZHANG, Y. 2022. An update on immunotoxicity and mechanisms of action of six environmental mycotoxins. *Food and Chemical Toxicology***,** 112895.

SUN, Y., SONG, Y., LONG, M. & YANG, S. 2023. Immunotoxicity of Three Environmental Mycotoxins and Their Risks of Increasing Pathogen Infections. *Toxins,* 15**,** 187.

SUTTERWALA, F. S., HAASKEN, S. & CASSEL, S. L. 2014. Mechanism of NLRP3 inflammasome activation. *Annals of the New York Academy of Sciences,* 1319**,** 82-95.

SWEENEY, M. J. & DOBSON, A. D. 1998. Mycotoxin production by Aspergillus, Fusarium and Penicillium species. *International journal of food microbiology,* 43**,** 141-158.

SWENBERG, J. A., FRYAR-TITA, E., JEONG, Y.-C., BOYSEN, G., STARR, T., WALKER, V. E. & ALBERTINI, R. J. 2008. Biomarkers in toxicology and risk assessment: informing critical dose–response relationships. *Chemical research in toxicology,* 21**,** 253-265.

TACON, A. G. & METIAN, M. 2008. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture,* 285**,** 146-158.

TAKAKUWA, Y. 2000. Protein 4.1, a multifunctional protein of the erythrocyte membrane skeleton: structure and functions in erythrocytes and nonerythroid cells. *International journal of hematology,* 72**,** 298-309.

TANG, D., KANG, R., COYNE, C. B., ZEH, H. J. & LOTZE, M. T. 2012. PAMP s and DAMP s: signal 0s that spur autophagy and immunity. *Immunological reviews,* 249**,** 158-175.

TANIMURA, N., MILLER, E., IGARASHI, K., YANG, D., BURSTYN, J. N., DEWEY, C. N. & BRESNICK, E. H. 2016. Mechanism governing heme synthesis reveals a GATA factor/heme circuit that controls differentiation. *EMBO reports,* 17**,** 249-265.

TAYYAB, S. & FEROZ, S. R. 2021. Serum albumin: Clinical significance of drug binding and development as drug delivery vehicle. *Advances in protein chemistry and structural biology,* 123**,** 193-218.

TERMAN, A. & KURZ, T. 2013. Lysosomal iron, iron chelation, and cell death. *Antioxidants & redox signaling,* 18**,** 888-898.

THIBAUT, R., DEBRAUWER, L., PERDU, E., GOKSØYR, A., CRAVEDI, J. P. & ARUKWE, A. 2002. Regio-specific hydroxylation of nonylphenol and the involvement of CYP2K-and CYP2M-like iso-enzymes in Atlantic salmon (*Salmo salar*). *Aquatic toxicology,* 56**,** 177-190.

THOMAS, S. & EGÉE, S. 1998. Fish red blood cells: characteristics and physiological role of the membrane ion transporters. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 119**,** 79-86.

TIMMUSK, S., NEVO, E., AYELE, F., NOE, S. & NIINEMETS, Ü. 2020. Fighting Fusarium pathogens in the era of climate change: a conceptual approach. *Pathogens,* 9**,** 419.

TOLOSA, J., FONT, G., MANES, J. & FERRER, E. 2014. Natural Occurrence of Emerging Fusarium Mycotoxins in Feed and Fish from Aquaculture. *Journal of Agricultural and Food Chemistry,* 62**,** 12462-12470.

TOLOSA, J., RODRIGUEZ-CARRASCO, Y., RUIZ, M. J. & VILA-DONAT, P. 2021. Multi-mycotoxin occurrence in feed, metabolism and carry-over to animal-derived food products: A review. *Food and Chemical Toxicology,* 158.

TONSHIN, A. A., TEPLOVA, V. V., ANDERSSON, M. A. & SALKINOJA-SALONEN, M. S. 2010. The Fusarium mycotoxins enniatins and beauvericin cause mitochondrial dysfunction by affecting the mitochondrial volume regulation, oxidative phosphorylation and ion homeostasis. *Toxicology,* 276**,** 49-57.

UHLIG, S., TORP, M. & HEIER, B. T. 2006. Beauvericin and enniatins A, A1, B and B1 in Norwegian grain: a survey. *Food Chemistry,* 94**,** 193-201.
VAN DE VEERDONK, F. L., KULLBERG, B. J., VAN DER MEER, J. W., GOW, N. A. & NETEA, M. G. 2008. Host–microbe interactions: innate pattern recognition of fungal pathogens. *Current opinion in microbiology,* 11**,** 305-312.
VAN DER MEEREN, L., VERDUIJN, J., KRYSKO, D. V. & SKIRTACH, A. G. 2020. AFM Analysis Enables Differentiation between Apoptosis, Necroptosis, and Ferroptosis in Murine Cancer Cells. *Iscience,* 23**,** 101816.
VAN DER OOST, R., BEYER, J. & VERMEULEN, N. P. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental toxicology and pharmacology,* 13**,** 57-149.
VANDESOMPELE, J., DE PRETER, K., PATTYN, F., POPPE, B., VAN ROY, N., DE PAEPE, A. & SPELEMAN, F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology,* 3**,** 1-12.
VENKATESH, N. & KELLER, N. P. 2019. Mycotoxins in conversation with bacteria and fungi. *Frontiers in microbiology,* 10**,** 403.
VERBON, E. H., TRAPET, P. L., STRINGLIS, I. A., KRUIJS, S., BAKKER, P. A. & PIETERSE, C. M. 2017. Iron and immunity. *Annual review of phytopathology,* 55**,** 355-375.
VIJAYAN, V., WAGENER, F. A. & IMMENSCHUH, S. 2018. The macrophage heme-heme oxygenase-1 system and its role in inflammation. *Biochemical pharmacology,* 153**,** 159-167.
VILLENEUVE, D. L., CRUMP, D., GARCIA-REYERO, N., HECKER, M., HUTCHINSON, T. H., LALONE, C. A., LANDESMANN, B., LETTIERI, T., MUNN, S. & NEPELSKA, M. 2014. Adverse outcome pathway (AOP) development I: strategies and principles. *Toxicological Sciences,* 142**,** 312-320.
VKM, BERNHOFT, A., ERIKSEN, G. S., SUNDHEIM, L., BERNTSSEN, M., BRANTSÆTER, A. L., BRODAL, G., FÆSTE, C. K., HOFGAARD, I. S., RAFOSS, T. & SIVERTSEN, T. 2013. Risk assessment of mycotoxins in cereal grain in Norway - Opinion of the Scientific Steering Committee of the Norwegian Scientific Committee for Food Safety (VKM). *VKM Report,* 21**,** 1-287.
WANG, M., CHEN, B., ZHANG, W., ZHANG, F., QIU, Y., LIN, Y. & YANG, S. 2022. Dematin inhibits glioblastoma malignancy through RhoA-mediated CDKs downregulation and cytoskeleton remodeling. *Experimental Cell Research,* 417**,** 113196.
WANG, Y., KINZIE, E., BERGER, F. G., LIM, S.-K. & BAUMANN, H. 2001. Haptoglobin, an inflammation-inducible plasma protein. *Redox report,* 6**,** 379-385.
WANG-BUHLER, J., LEE, S., CHUNG, W., STEVENS, J., TSENG, H., HSEU, T., HU, C., WESTERFIELD, M., YANG, Y. & MIRANDA, C. 2005. CYP2K6 from zebrafish (*Danio rerio*): cloning, mapping, developmental/tissue expression, and aflatoxin B1 activation by baculovirus expressed enzyme. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* 140**,** 207-219.
WILLETT, C., RAE, J. C., GOYAK, K. O., LANDESMANN, B., MINSAVAGE, G. & WESTMORELAND, C. 2014. Pathway-based toxicity: History, current approaches, and liver fibrosis and steatosis as prototypes. *ALTEX-Alternatives to animal experimentation,* 31**,** 407-421.
WISSING, J., GODL, K., BREHMER, D., BLENCKE, S., WEBER, M., HABENBERGER, P., STEIN-GERLACH, M., MISSIO, A., COTTEN, M. & MULLER, S. 2004.

Chemical proteomic analysis reveals alternative modes of action for pyrido [2, 3-d] pyrimidine kinase inhibitors. *Molecular & Cellular Proteomics,* 3**,** 1181-1193.
WITESKA, M. 2013. Erythrocytes in teleost fishes: a review. *Zoology and Ecology,* 23**,** 275-281.
WU, Q., PATOCKA, J., NEPOVIMOVA, E. & KUCA, K. 2018. A review on the synthesis and bioactivity aspects of beauvericin, a Fusarium mycotoxin. *Frontiers in Pharmacology,* 9**,** 1338.
XIE, Y., HOU, W., SONG, X., YU, Y., HUANG, J., SUN, X., KANG, R. & TANG, D. 2016. Ferroptosis: process and function. *Cell Death & Differentiation,* 23**,** 369-379.
YANG, L. V., NICHOLSON, R. H., KAPLAN, J., GALY, A. & LI, L. 2001. Hemogen is a novel nuclear factor specifically expressed in mouse hematopoietic development and its human homologue EDAG maps to chromosome 9q22, a region containing breakpoints of hematological neoplasms. *Mechanisms of development,* 104**,** 105-111.
YANG, Y.-H., MIRANDA, C. L., HENDERSON, M. C., WANG-BUHLER, J.-L. & BUHLER, D. R. 2000. Heterologous expression of Cyp2k1 and identification of the expressed protein (bv-Cyp2k1) as lauric acid (ω-1)-hydroxylase and aflatoxin b1exo-epoxidase. *Drug metabolism and disposition,* 28**,** 1279-1283.
YEH, A., MEADOR, J. P., LUNSMAN, T. D., MAYFIELD, D. B. & VERSLYCKE, T. A. 2021. Metabolic effects of pharmaceuticals in fish. *Pharmaceuticals in Marine and Coastal Environments***,** 457-499.
YTRESTØYL, T., AAS, T. S. & ÅSGÅRD, T. 2015. Utilisation of feed resources in production of Atlantic salmon (*Salmo salar*) in Norway. *Aquaculture,* 448**,** 365-374.
ZAPATA, A. & AMEMIYA, C. 2000. Phylogeny of lower vertebrates and their immunological structures. *Origin and evolution of the vertebrate immune system***,** 67-107.
ZHAN, X., LONG, Y., ZHAN, X. & MU, Y. 2017. Consideration of statistical vs. biological significances for omics data-based pathway network analysis. *Med One,* 2.
ZHANG, J. & HAMZA, I. 2019. Zebrafish as a model system to delineate the role of heme and iron metabolism during erythropoiesis. *Molecular genetics and metabolism,* 128**,** 204-212.
ZHAO, W., KITIDIS, C., FLEMING, M. D., LODISH, H. F. & GHAFFARI, S. 2006. Erythropoietin stimulates phosphorylation and activation of GATA-1 via the PI3-kinase/AKT signaling pathway. *Blood,* 107**,** 907-915.
ZINGALES, V., TARONCHER, M., MARTINO, P. A., RUIZ, M.-J. & CALONI, F. 2022. Climate Change and Effects on Molds and Mycotoxins. *Toxins,* 14**,** 445.

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# Beauvericin (BEA) and enniatin B (ENNB)-induced impairment of mitochondria and lysosomes - Potential sources of intracellular reactive iron triggering ferroptosis in Atlantic salmon primary hepatocytes

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ABSTRACT

Beauvericin (BEA) and enniatin B (ENNB) are emerging mycotoxins frequently detected in plant-based fish feed. With ionophoric properties, they have shown cytotoxic potential in mammalian models. Sensitivity in fish is still largely unknown. Primary hepatocytes isolated from Atlantic salmon (*Salmo salar*) were used as a model and exposed to BEA and ENNB (0.05–10 μM) for 48 h. Microscopy, evaluation of cell viability, total ATP, total $H_2O_2$, total iron content, total Gpx enzyme activity, and RNA sequencing were used to characterize the toxicodynamics of BEA and ENNB. Both mycotoxins became cytotoxic at ≥ 5 μM, causing condensation of the hepatocytes followed by formation of blister-like protrusions on the cell's membrane. RNA sequencing analysis at sub-cytotoxic levels indicated BEA and ENNB exposed hepatocytes to experience increased energy expenditure, elevated oxidative stress, and iron homeostasis disturbances sensitizing the hepatocytes to ferroptosis. The present study provides valuable knowledge disclosing the toxic action of these mycotoxins in Atlantic salmon primary hepatocytes.

## 1. Introduction

Plant-based feed ingredients are a source of novel contaminants to farmed fish. Traditional salmon feed ingredients, e.g., fishmeal and fish oil, have largely been replaced with vegetable feed ingredients making the feed more ecologically and economically sustainable (Ytrestøyl et al., 2015). Mycotoxins are secondary metabolites synthesized by crop molds, whereupon the use of mold-infested cereal grains and pulse crops for meal- and oil extraction can result in mycotoxins entering feed- and food production. Ingested mycotoxins have caused illness (mycotoxicosis) and even death in livestock and humans (Hussein and Brasel, 2001). The introduction of mycotoxins into aquafeeds from plant-based feed ingredients raises a concern regarding fish feed safety (Bernhoft et al., 2013b).

A study on mycotoxin levels in fish feeds in Kenya reported a high prevalence of several "emerging mycotoxins" (Jestoi, 2008), especially enniatin B (ENNB) but also beauvericin (BEA) (Mwihia et al., 2020). BEA and ENNs have been included in routine screening in the official annual fish feed surveillance program in Norway since 2016. Rapeseed oil has been identified as a major source of ENNB contamination in fish feed, whereas wheat- and corn gluten are sources of both BEA and ENNB (Sanden et al., 2017; Sele et al., 2018, 2019; Ørnsrud et al., 2020). ENNs and BEA have been found in the liver and fillets of commercially important fish species (e.g., sea bass (*Dicentrarchus labrax*), sea bream (*Sparus aurata*), Atlantic salmon (*Salmo salar*), and rainbow trout (*Oncorhynchus mykiss*)) (Tolosa et al., 2014, 2017). However, since existing toxicity data for BEA and ENNB are insufficient for establishing maximum contents, these mycotoxins are currently not legislatively regulated in feed or food (Lindblad et al., 2013; Bernhoft et al., 2013b; Vaclavikova et al., 2013). The occurrence of BEA and ENNB in salmon feed has highlighted the need for more knowledge regarding their potential chronic effects on fish health (Sanden et al., 2017; Sele et al., 2018, 2019; Ørnsrud et al., 2020; Bernhoft et al., 2013a). Toxicity studies on mycotoxins in fish species are limited, especially in salmonids. Atlantic salmon appear to be sensitive to dietary deoxynivalenol (DON) (Moldal et al., 2018), while Rainbow trout have been shown to be sensitive to dietary aflatoxin (Williams, 2012) and DON (Hooft et al., 2011). However, data on the dietary toxicity of BEA and ENNB in salmon and other fish are lacking. Advancing our understanding of the cellular toxicity of mycotoxins may improve the prediction of possible adverse effects in fish.

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**Abbreviations**

Enniatin B (ENNB)
Beauvericin (BEA)
Deoxynivalenol (DON)
Ochratoxin A (OTA)
Reactive oxygen species (ROS)
Ferric iron ($Fe^{3+}$)
Ferrous iron ($Fe^{2+}$)
Adenosine triphosphate (ATP)
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)
Ribonucleic acid (RNA)
RNA integrity number (Rin)
Hydrogen Peroxide ($H_2O_2$)
Differentially expressed genes (DEGs)
Tricarboxylic acid cycle (TCA cycle)
Peroxisome proliferator-activated receptors (PPARs)
Polyunsaturated fatty acid (PUFA)
Glutamate cysteine ligase (Gcl)
Glutathione synthetase (Gss)
Glutathione reductase (Gsr)
Reduced glutathione (GSH)
Oxidized glutathione (GSSG)
Glutathione peroxidase (Gpx)
Glutathione peroxidase 4 (Gpx4)
Polyunsaturated fatty acid (PUFA)

Few studies have assessed the cytotoxicity of BEA or ENNB in fish. However, BEA has been shown to exhibit high cytotoxicity in cell lines derived from rainbow trout hepatoma (RTH-149) ($EC_{50} = 5.96$ μg/mL), desert topminnow (*Poeciliopsis lucida*) hepatocellular carcinoma (PLHC-1) ($EC_{50} = 2.89$ μg/mL) (García-Herranz et al., 2019), and rainbow trout gill (RTgill-W1) ($EC_{50} = 3.01$ μg/mL) (Bernal-Algaba et al., 2021). Furthermore, García-Herranz et al. (2019) found that fish cell lines exhibited similar sensitivity to that of a rat (*Rattus norvegicus*) cell line (H4IIE) ($EC_{50} = 1.80$ μg/mL). Results from a mammalian cell model (Caco-2) have shown that both mycotoxins are cytotoxic at lower micro molar concentrations (BEA $IC_{50} = 12.80$ μM, ENNB $IC_{50} = 11.7$ μM) (Prosperini et al., 2013a, 2013b). These mycotoxins have also been shown to initiate mammalian cell death in an apoptotic or necrotic manner (Prosperini et al., 2013a, 2013b; Jonsson et al., 2016).

BEA and ENNB can act as ionophores, which has been suggested to be their underlying mechanism of toxicity (Mallebrera et al., 2018; Prosperini et al., 2017). Owing to their chemical structure, BEA and ENNB are lipophilic and prone to incorporation in the lipid bilayers of cellular- and organelle membranes (Kouri et al., 2003; Kamyar et al., 2004; Tonshin et al., 2010). When incorporated, these mycotoxins can disrupt the otherwise strictly regulated transport of ions. For example, BEA and ENNB have been shown to affect the transport of several essential cations (e.g., $K^+$, $Na^+$, and $Ca^{2+}$) either by acting as ion carriers or as cation-selective channels (Ivanov et al., 1973; Hilgenfeld and Saenger, 1985; Kouri et al., 2003, 2005; Kamyar et al., 2004). The proposed mode of toxic action of BEA and ENNB is the disruption of ion and pH homeostasis, which in turn disturbs normal cell- and organelle function (Kamyar et al., 2004; Kouri et al., 2005; Tonshin et al., 2010; Wu et al., 2018).

A variety of sub-lethal toxic effects have been reported from studies with different mammalian cell models. For example, BEA and ENNB have been demonstrated to depolarize mitochondrial transmembrane potential and to uncouple oxidative phosphorylation in rat liver mitochondria (Tonshin et al., 2010). BEA has been shown to cause mitochondria uncoupling and to deplete ATP in myocytes from guinea pig (*Cavia porcellus*) (Kouri et al., 2005). Either one or both mycotoxins have generated oxidative stress (i.e., the formation of reactive oxygen species (ROS) and/or lipid peroxidation) in exposed mammalian cell lines (Prosperini et al., 2013a, 2013b; Ivanova et al., 2012; Mallebrera et al., 2015; Klarić et al., 2007; Ferrer et al., 2009). Furthermore, BEA has been shown to inhibit the acyl-CoA cholesterol acyltransferase enzyme (ACAT) (Tomoda et al., 1992), and to affect the antioxidant system by decreasing glutathione levels in porcine-, rodent-, and human cell lines (Klarić et al., 2007; Mallebrera et al., 2014; Prosperini et al., 2013a). ABC transporter proteins such as ABCB1 and ABCG2 have, however, exhibited the ability to diminish the cytotoxic effects of BEA and ENNs in human cells (Dornetshuber et al., 2009).

Since Atlantic salmon is an important farmed fish species in Norway, the aim of this study was to generate salmon-specific toxicity data of these emerging mycotoxins. In compliance with the 3R principles (replace, reduce, refine), primary hepatocyte cells isolated from Atlantic salmon were used to assess the cytotoxicity of BEA and ENNB. With non-targeted transcriptomics, the aim was to discover the mycotoxins' mode of action by identifying affected pathways. The results of this study will be valuable for improving our understanding of how BEA and ENNB affect fish health and feed safety.

## 2. Materials and methods

### 2.1. Mycotoxins

The mycotoxins, beauvericin (BEA, cas 26048-05-5) and enniatin B (ENNB, cas 917-13-5) used in the exposure experiments were purchased from AdipoGen® (AdipoGen® Life Sciences, Nordic BioSite, Oslo, Norway). BEA and ENNB were dissolved individually in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Oslo, Norway) to constitute a 15 mM stock solution from which dilutions were made. In an initial experiment, the mycotoxins were exposed as single compounds at concentrations of 0.05, 0.5, 1.5, 2.5, and 5 μM, and in a follow-up experiment at 0.05, 2.5, and 5 μM. In both experiments, 0.1% DMSO was used as a control. In the cell viability assays (xCELLigence, MTT, Neutral red) exposure to 10 μM was also included. The mycotoxin stock solutions, as well as the control, were diluted in Leibovitz L-15 medium w/o phenol red (ThermoFisher, Oslo, Norway) supplemented with 9% FBS (Sigma-Aldrich, Oslo, Norway), 1% salmonid serum (#N82800F, Meridian Life Science, Inc. Memphis, USA), 1% Antibiotic Antimycotic Solution (10000 units penicillin, 10000 μg streptomycin, and 25 μg amphotericin per mL) (Sigma-Aldrich, Oslo, Norway), and from here on denoted as suppl. L-15.

### 2.2. Ethical statement

In these experiments, for the first time, a clonal all-male Atlantic salmon line was used that has been developed at the Institute of Marine Research (IMR), Bergen, Norway (Fjelldal et al., 2020). Using all-male salmon reduced the use of fish to a minimum (no females had to be discarded) thus favoring the 3R principles. All work was carried out in agreement with the current national *animal welfare act - the regulation on animal experimentation* approved by the Norwegian Animal Research Authority and overseen by the Norwegian Food Safety Authority (FOTS ID, 19351).

### 2.3. Isolation and exposure of primary cultures of Atlantic salmon hepatocytes

Atlantic salmon hepatocytes were isolated using the two-step liver

perfusion method described by Søfteland et al. (2009) under semi-sterile (hepatocyte cell isolation) and sterile (cell work) conditions. Prior to the start of the experiments, the fish were maintained in a fish-holding facility at IMR. Six juvenile fish weighing between 216 and 326 g were used in the initial experiment (analyses: xCELLigence, MTT, ATP, $H_2O_2$, and RNA sequencing), and four juveniles weighing between 136 and 193 g were used in the follow-up experiment (analyses: MTT, Neutral red, Iron, and Gpx). In brief, during the first step the liver was perfused through the hepatic vein with a perfusion buffer containing 17 mM EDTA (Sigma-Aldrich, Oslo, Norway) to clear the liver of blood, followed by the second perfusion step where the liver was digested by the addition of collagenase VIII (0.1 mg/mL) (Sigma-Aldrich, Oslo, Norway) to the perfusion buffer. The digested liver was transferred to a Petri dish with ice cold 1X PBS and gently pulled apart using tweezers, followed by pipetting the tissue suspension up and down using a 5-mL syringe. The homogenate was filtered through a 100 μm nylon mesh. The isolated hepatocytes were washed thrice with cold 1X PBS through centrifugation at 50 G for 5 min, and finally resuspended in suppl. L-15. Before seeding the hepatocytes, viability was confirmed to be $\geq$ 80% with trypan blue staining (BioRad) using a Bürker counting chamber (Tiefe 0.100 mm, 0.0025 $mm^2$) under a microscope (Axiovert 40 CFL, Carl Zeiss, Jena, Germany). Hepatocytes harvested from each of the biological replicate salmon (initial: n = 6, follow-up: n = 4) were seeded in precoated (2 μg/$cm^2$ laminin, Sigma-Aldrich, Oslo, Norway) well culture plates. Hepatocytes were incubated at 10 °C, without any additional $O_2$ or $CO_2$ throughout the duration of the experiments (MIR-554-PE Cooled Incubator, PHCbi, Etten-Leur, The Netherlands).

According to the methodology-optimization done by Søfteland et al. (2009), the hepatocytes were acclimatized for 48 h prior to exposure (given fresh suppl. L-15 after 24 h). On the third day, the hepatocytes were exposed to the mycotoxins for the following 48 h (given fresh exposure solution on the fourth day) (for exposure regimes see *2.1 Mycotoxins*). On the fifth and final day of the experiments, the predetermined endpoints were measured (initial: xCELLigence, MTT, ATP levels, $H_2O_2$ levels/follow-up: MTT, Neutral red, Iron levels, Gpx enzyme activity). All spectrophotometric measurements were done using a VICTOR X5 plate reader (PerkinElmer, Waltham, Massachusetts, USA). The hepatocytes from the initial experiment intended for RNA sequencing were immediately lysed in RLT Plus buffer (Qiagen, Crawley, UK) and stored at −80 °C until further processing for RNA extraction and purification.

### 2.4. Real-time viability assessment – xCELLigence

Real-Time Cell Analysis (RTCA) was carried out using the xCELLigence system (ACEA Biosciences, San Diego, CA, USA) according to manufactures' instructions to quantify biological factors, such as changes in cell morphology and the strength of the cells-substrate adhesion to the surface throughout the entire exposure duration (48 h). Primary hepatocytes (n = 6 per experimental condition) were seeded with a density of $2 \times 10^5$ cells per well in a 96-well electronic microtiter plate (E-plate) (ACEA Biosciences, San Diego, CA, USA), and cultured in 0.2 mL suppl. L-15. The hepatocytes were left to settle for 30 min before starting to monitor the response. As the cells adhere to the bottom of the E-plate with electrodes integrated into the bottom, the cell-electrode impedance was measured via the xCELLigence RTCA single plate (SP) station kept inside the incubator. After exposing the hepatocytes to BEA and ENNB, measurements were taken every 2 min during the first 12 h, then every 15 min of the remaining exposure time). Cell index (CI) values were then derived from the recorded cell-electrode impedance data, giving quantitative information about the cells' biological status and viability. The CI was normalized against the last point before starting the mycotoxin exposure (i.e., CI at a given time/CI at reference point (put to 1) using the RTCA Software v1.2.1.

### 2.5. Viability assessment - mitochondrial metabolic activity and lysosomal function

An MTT and a Neutral red-based *In Vitro* Toxicology assay kit (Sigma-Aldrich, St Louis, Missouri, USA) were used to assess the hepatocytes' mitochondrial metabolic activity and lysosomal function, respectively. Primary hepatocytes (initial experiment n = 6 and follow-up experiment n = 4 per experimental condition) were seeded with a density of $2 \times 10^5$ cells per well in a 96-well cell-culture plate (Nunc™, Roskilde, Denmark) and cultured in 0.2 mL suppl. L-15. The BEA and ENNB exposure solutions were removed after 48 h, and the cells were added either MTT (5 mg/mL) or solution neutral red (0.33%) (20 μL of MTT or neutral red solution was added to 200 μL fresh suppl. L-15) and placed back into the incubator for 4 h. Thereafter, the dye solutions were replaced with an assay solubilizing solution and stored at 4 °C in an airtight bag over the weekend. Spectrophotometric measurement of MTT was done at absorbance (abs) 570 nm, and neutral red at abs 540 nm (both corrected against background at 690 nm). The mycotoxins were regarded as cytotoxic when their concentration elicited a reduction in hepatocyte viability exceeding 30% relative to the control (ISO, 2009).

### 2.6. RNA extraction and sequencing

Hepatocytes for RNA sequencing were seeded with a density of $72 \times 10^5$ cells per well in 6-well cell-culture treated plates (Corning® Costar®, Sigma-Aldrich, Oslo, Norway) and cultured in 3 mL suppl. L-15. After 48 h exposure to BEA or ENNB, the cells were immediately lysed in RLT Plus buffer (Qiagen, Crawley, UK), flash frozen and stored at −80 °C. A RNeasy Plus Mini Kit (Qiagen, Crawley, UK) was used according to supplier's protocol to extract and purify total RNA from the cell lysate. Purified RNA was diluted in 30 μL RNase-free MilliQ $H_2O$ and stored at −80 °C until further analysis. RNA quantity and purity was measured using NanoDrop™ (One Microvolume UV–Vis Spectrophotometer, Thermo Fisher Scientific™, Waltham, MA, USA). The 260/280 and 260/230 nm ratios were 2.07 ± 0.03 and 2.38 ± 0.04, respectively, indicating pure samples of satisfactory quality (n = 6 per experimental condition, mean ± STD). RNA integrity was evaluated on an RNA 6000 Nano LabChips with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) according to manufacturer's protocol. The RNA integrity numbers (Rin) were 10.0 ± 0.0 (indicating non-degraded RNA) for all samples intended for RNA sequencing. RNA samples (2 μg) were sent to the Norwegian Sequencing Centre (NSC) (www.sequencing.uio.no) in Oslo, Norway, where the sequencing and library preparation were performed. DNA libraries were prepared using 90 ng total RNA input to the TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, California, USA) per manufacturer's protocol. For multiplexing, standard Illumina adaptors were used. The libraries were sequenced using the NextSeq Illumina platform (Illumina, San Diego, California, USA) according to the manufacturer's instructions, generating single end 75 bp read libraries with an average library size of 10 ± 2 million reads. A total of 48 libraries were generated, 24 for BEA and 24 for ENNB, where (based on cell viability results) three experimental conditions below cytotoxic levels (0.05, 0.5, 2.5 μM) plus control (0.1% DMSO) were selected for RNA sequence analysis, n = 6 per experimental condition. Raw reads were submitted to the gene expression omnibus https://www.ncbi. nlm.nih.gov/geo/( GEO accession: GSE193374).

### 2.7. RNA sequencing read mapping and differential gene expression

TrimGalore 0.4.2 wrapper tool (https://github.com/FelixKrueger/TrimGalore) was used for removing adaptors and quality trimming, applying the default parameters. Library quality was investigated using fastQC included in the TrimGalore wrapper.

Individual libraries were mapped to the Atlantic salmon genome (RefSeq Assembly ICSASG_v2) using the Hisat2 short read aligner

version 2.0.4 (Kim et al., 2015) and the Atlantic salmon NCBI gene annotation file (Salmon_salar, January 24, 2017 GCA_000233375.4_ICSASG_v2_genomic.gff), with a mapping efficiency rate of 87 ± 9%. Transcript levels for the individual libraries were estimated using FeatureCounts (Liao et al., 2014) of the Subread package (http://subread.sourceforge.net/). Read counts were normalized using Bioconductor R package (version 3.4.4) DESeq2 (version 1.18.1) (Love et al., 2014). Genes of which fewer than five samples had gene counts below or equal to 10 reads were excluded from further analysis prior to normalization. Differential gene expression was analyzed using Qlucore Omics Explorer 3.5 (Qlucore AB, Lund, Sweden). Prior to statistical analysis, pre-processed RNA sequencing data were log2 transformed.

### 2.8. Determination of cellular levels of ATP and $H_2O_2$

A Luminescent ATP Detection Assay Kit (ab113849, Abcam, Cambridge, UK), and a ROS-Glo™ $H_2O_2$ Assay (Promega, Madison, Wisconsin, USA) were used to measure total levels of ATP and hydrogen peroxide ($H_2O_2$), respectively. Hepatocytes (n = 6 per experimental condition) were seeded with a density of $2 \times 10^5$ cells per well in a 96-well cell-culture plate (Nunc™, Roskilde, Denmark) and cultured in 0.2 mL suppl. L-15. After 48 h exposure to BEA or ENNB, the hepatocytes were assayed according to supplier procedures (the non-lytic protocol for $H_2O_2$). ATP and $H_2O_2$ levels were measured through luminescence spectrophotometry in black opaque 96F-well OptiPlates (PerkinElmer, Waltham, Massachusetts, USA).

### 2.9. Determination of cellular iron and Gpx activity

An iron assay kit (ab83366) and a Glutathione peroxidase (Gpx) assay kit (ab102530) (Abcam, Cambridge, MA, USA) were used to assess the cellular ferric ($Fe^{3+}$) and ferrous ($Fe^{2+}$) iron levels and total Gpx activity, respectively. Hepatocytes (n = 4 per experimental condition) were seeded with a density of $26 \times 10^5$ cells per well in 12-well cell-culture plates (Corning® Costar®, Sigma-Aldrich, Oslo, Norway) and cultured in 2 mL suppl. L-15. After 48 h exposure to BEA or ENNB, the hepatocytes were washed with cold 1X PBS and harvested in either iron assay buffer, or cold Gpx assay buffer. The cells were then homogenized by pipetting (for the iron assay, cells were sonicated 3–4 short pulses at 30% amplitude, 0.5 cycle), and centrifuged at 13.000 G for 5 min at 4 °C. The supernatants were collected and assayed according to supplier procedures, using 100 μL-undiluted samples in the iron assay and 50 μL-undiluted samples in the Gpx assay. Spectrophotometric measurement of $Fe^{3+}$ and $Fe^{2+}$ was done at abs 570 nm, and Gpx activity at abs 340 nm after 5 min incubation at room temperature.

### 2.10. Statistics - transcriptomics and $IC_{50}$

Qlucore Omics Explorer 3.5 "eliminated factor" option was used to control for confounding factors in the transcriptomic analysis related to the isolation of primary hepatocytes (i.e., time of day, the person doing the isolation, differences in the basal level of transcription) prior to statistical analysis. The Two Group Comparison embedded in the Qlucore Omics Explorer 3.5 was used for significance analysis in the pairwise comparison of each contrast (Ctrl vs BEA 0.05, Ctrl vs BEA 0.5, Ctrl vs BEA 2.5 μM, Ctrl vs ENNB 0.05, Ctrl vs ENNB 0.5, Ctrl vs ENNB 2.5 μM) ($q < 0.1$, fold change >1) and to generate scatter plots of specific DEGs. NCBI gene ID's of differentially expressed genes (DEGs) combined with fold change was used for KEGG pathway analysis, and in addition KEGG module enrichment (enrichMKEGG) analysis returning the enriched module categories (functional units, e.g., pathway modules and structural complexes) of the gene set. Significantly enriched pathways and modules were investigated using ClusterProfiler v3.12.0 and differentially expressed genes of each contrast (p.adjust <0.1). PCA plots Qlucore Omics Explorer 3.5, and The Multi Group Comparison embedded in the Qlucore Omics Explorer 3.5 was used for generating principal component analysis (PCA) plots showing the different exposure groups of each mycotoxin relative control ($q < 0.1$). Venn diagrams were created with Venny 2.1.0 (Oliveros, 2007), and GraphPad Prism v.9 (GraphPad Software Inc., Palo Alto, CA, USA) was used to display dose-response relationships in the mycotoxin-exposed primary hepatocytes measured with the different assays. Results are showed as mean ± SD (n = 6 per experimental condition, initial: xCELLigence, MTT, ATP, $H_2O_2$, and RNA sequencing) (n = 4 per experimental condition, follow-up: MTT, Neutral red, Iron, and Gpx). A one-way ANOVA followed by Dunnet's post hoc test ($p < 0.05$) was used to calculate significant changes in the response relative to the control.

## 3. Results

### 3.1. Viability of salmon primary hepatocytes after BEA and ENNB exposure

In the initial experiment, both BEA and ENNB produced a bell-shaped dose-response according to the xCELLigence electric cell-substrate impedance assessment (associated to cellular changes e.g., cell adhesion, surface adhesion, cell migration, morphology) (Fig. 1, A). The dose-response curve of ENNB ($IC_{50}$ = 3.23 μM) appeared to peak at lower concentrations compared to BEA ($IC_{50}$ = 5.05 μM) (Fig. 1, A). The impedance was reduced by 29% and 34% at 10 μM of BEA and ENNB, respectively (Fig. 1, A), and 5 μM ENNB resulted in a 26% reduction of impedance (Fig. 1, A). Results obtained with the MTT-based cell viability assay (mitochondrial metabolic activity) also indicated effects at 5 and 10 μM of ENNB ($IC_{50}$ = 4.20 μM) as a reduction of metabolic activity in the cells by 50% and 75%, respectively (Fig. 1, B). BEA ($IC_{50}$ = 4.97 μM) decreased the cells metabolic activity by 50% at 10 μM (Fig. 1, B). In a follow-up experiment, using a new batch of fish and exposure solutions, the neutral red-based cell viability assay indicated that both mycotoxins impair lysosomal function at 2.5, 5, and 10 μM, BEA ($IC_{50}$ = 2.58) causing a reduction by 20, 55, and 53%, and ENNB ($IC_{50}$ = 11.14) by 11, 25, and 49%, respectively (Fig. 1, C). The MTT assay indicated that BEA ($IC_{50}$ = 2.57) exerted cytotoxicity at 2.5, 5, and 10 μM causing a reduction of metabolic activity by 25, 65, and 57%, respectively, and ENNB ($IC_{50}$ = 5.66 μM) at 5 and 10 μM caused a reduction of 27 and 60%, respectively (Fig. 1, D). Due to the high cytotoxicity at 10 μM of both BEA and ENNB, this concentration was excluded from any further testing. Since the degree of cytotoxicity at 5 μM started to become questionable, this concentration was excluded from RNA sequencing analysis. However, 5 μM was retained as the highest exposure concentration for dose-response purposes in the functional analyses (i.e., ATP, $H_2O_2$, Iron, and Gpx).

### 3.2. Morphological effects of BEA and ENNB in the salmon primary hepatocytes

In the initial experiment, visual examination of hepatocytes exposed to 5 μM ENNB showed changed morphology compared to the control cells (Fig. 2, A). The cells had contracted and exhibited a different growth pattern (though still attached to the culture plate) (Fig. 2, A). Some of the hepatocytes exposed to 5 μM ENNB also appeared to have protruding blister-like features on the cell membrane. Exposure to 5 μM BEA in the initial experiment did not cause any obvious visual changes to the cell's morphology (Fig. 2, A). When assessing the RNA integrity with Bioanalyzer to ensure good RNA quality of samples for RNA sequencing, the integrity of the RNA in cells exposed to 5 μM BEA showed no signs of degradation, all samples had an RNA integrity number (Rin) = 10 (Fig. 2, A, below cell picture). Cells exposed to 5 μM ENNB also exhibited high Rin number (Rin ≥9.5) (Fig. 2, A, below cell picture). However, collectively the results from xCELLigence, MTT, and visual examination of morphology indicated that ENNB (and to some degree also BEA) was cytotoxic to the hepatocytes at the relatively low micromolar concentration of 5 μM. In the follow-up experiment, the

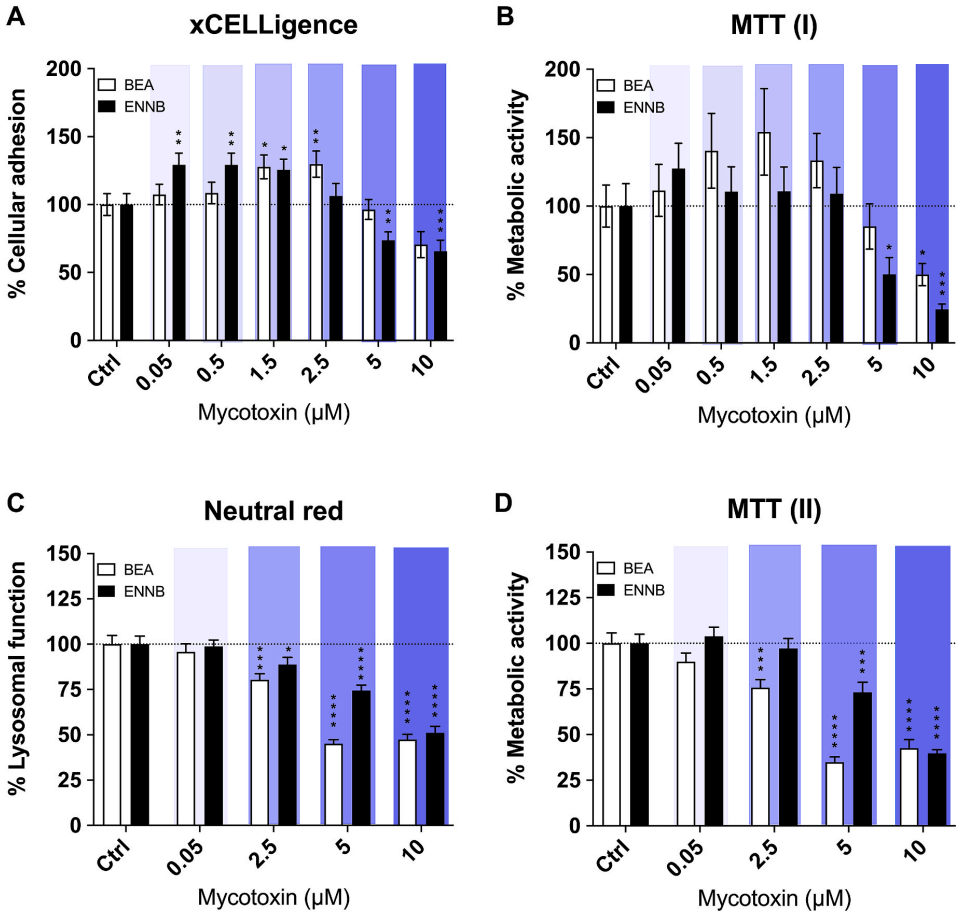


**Fig. 1.** Viability of primary hepatocytes exposed to BEA and ENNB. (A) xCELLigence, data shown as % cellular adhesion based on normalized cell index relative control. (B, D) MTT assay (I and II), data shown as % mitochondrial metabolic activity based on corrected abs (abs 570–690 nm) relative to control. (C) Neutral red assay, data shown as % lysosomal function based on corrected abs (abs 540–690 nm) relative to control. (A, B) n = 6. (C, D) n = 4 analyzed in duplicate. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

hepatocytes exposed to 5 μM of both BEA and ENNB exhibited obvious signs of morphological changes i.e., cell contraction and protrusion development (Fig. 2, B). The hepatocytes, which had been exposed to 2.5 μM of BEA and ENNB also exhibited altered morphology (though less obvious than in cells exposed to 5 μM), and these changes were more pronounced for BEA than ENNB (Fig. 2, B). The differences between the initial and follow-up experiment were reflected in both morphological changes and MTT results in exposed cells (Fig. 1, B). Thus, morphological changes became evident when the exposure concentration exceeded the $IC_{50}$ established in the MTT assays. In some cases, concentrations approaching $IC_{50}$ also produced an intermediate change in the cells.

### *3.3. Differential gene expression and affected pathways following BEA and ENNB exposure*

RNA sequencing technology revealed differentially expressed genes (DEGs) in Atlantic salmon hepatocytes exposed to 0.05, 0.5, and 2.5 μM of BEA and ENNB. The general trend was that the number of unique DEGs increased with increasing exposure concentration of both BEA and ENNB (Fig. 3A and B). Compared to the control, BEA 0.05 μM resulted in zero DEGs, BEA 0.5 μM in 293 DEGs (53% up-, and 47% down-regulated), and BEA 2.5 μM in 364 DEGs (49% up, 51% down) (Fig. 3, A, C). Whereas ENNB 0.05 μM resulted in 48 DEGs (46% up, 54% down), ENNB 0.5 μM in 245 DEGs (56% up, 44% down), and ENNB 2.5 μM in 2614 DEGs (63% up, 37% down) (Fig. 3B–D). Thus, in the initial experiment ENNB 2.5 μM appeared to have the greatest effect on gene transcription in the exposed Atlantic salmon primary hepatocytes also resulting in a larger proportion of up-regulated DEG than down-regulated. The PCA analysis showed the relationship between the different exposures concentration of BEA and ENNB to be best explained by principal component 1 (PC1), PC1 = 50% and PC1 = 55%, respectively (Fig. 3E–F).

To elucidate how the DEGs translate into functional cellular responses, a KEGG pathway enrichment analysis was run to identify enriched metabolic- and signaling pathways. No significantly enriched pathways were observed following 0.05 μM of BEA and ENNB exposure. The enrichment analysis indicated that 0.5 μM BEA enriched the ribosome pathway (sasa03010) (Fig. 4, A), causing down-regulation of all associated DEGs (Fig. 4, B). At 2.5 μM, BEA enriched 6 pathways (Fig. 4, A). The most significantly enriched pathway following 2.5 μM BEA

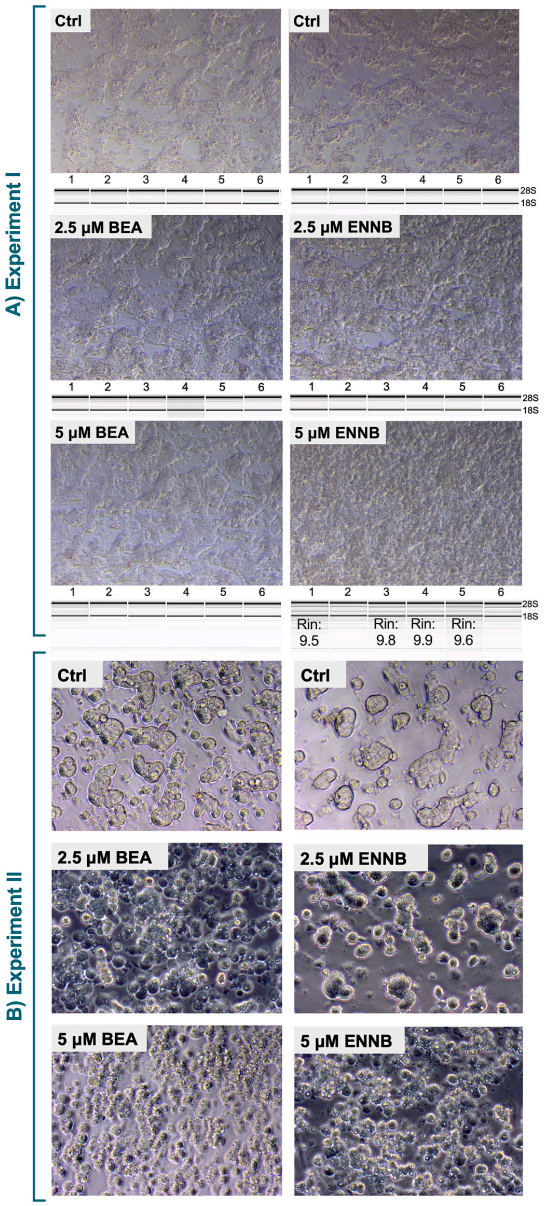


**Fig. 2.** Cell morphology. Primary hepatocytes imaged by light microscopy showing (A) control cells (Ctrl), and cells exposed to 2.5 and 5 µM of either BEA or ENNB (mag. 20x). RNA integrity in all 6 biological replicates is shown below cell pictures depicting 18 S and 28 S ribosomal subunit bands, RNA integrity number (Rin) = 10 if not otherwise indicated. (B) Control cells (Ctrl), and cells exposed to 2.5 and 5 µM of either BEA or ENNB (mag. 40x).

exposure was glutathione metabolism (sasa00480) (Fig. 4, A), and the transcript levels of the associated DEGs appeared to overall increase (Fig. 4, B). The number of increased and decreased DEGs attributed to ferroptosis (sasa04216) was the same, while the DEGs in the remaining pathways appeared to mainly decrease (Fig. 4, B). The top represented DEGs within glutathione metabolism with the highest increase in transcripts levels were the genes encoding glutamate cysteine ligase (Gcl) (gene: *gcl)*, and glutathione reductase (Gsr) (gene: *gsr*) (Fig. 4C and D). The DEG encoding glucose-6-phosphate1-dehydrogenase (*g6pd*) was up-regulated in the pentose phosphate pathway, while the gene encoding heme-oxygenase (*hmox*) was represented in the ferroptosis pathway (Fig. 4E and F) (for details on individual DEGs, see Supplemental Information BEA-Pathways).

Enrichment analysis of 0.5 µM ENNB indicated enhanced glycerolipid metabolism (sasa00561; Fig. 5, A), where the number of increased and decreased DEGs were equal (Fig. 5, B). At 2.5 µM, ENNB enriched 26 pathways (Fig. 5, A). The most significantly enriched pathway by 2.5 µM ENNB was the ribosome pathway (sasa03010), while the second most significant pathway was oxidative phosphorylation (sasa00190) (Fig. 5, A) (Supplemental Information Fig. S1.). All pathways affected by 2.5 µM ENNB appeared to primarily be up-regulated, with increased transcript levels of most DEGs (Fig. 5, B). The top represented DEGs within glycolysis/gluconeogenesis were the genes encoding phosphoenolpyruvate carboxykinase (Pepck) (gene: *pepck*) and glucose-6-phosphatase (G6Pase) (gene: *g6pase*) (Fig. 5C and D). The DEG encoding nuclear receptor coactivator 4 (*ncoa4*) was represented in the ferroptosis pathway (Fig. 5, E) (for details on individual DEGs, see Supplemental Information ENNB-Pathways). Affected pathways common to both mycotoxins were biosynthesis of cofactors (sasa01240), PPAR signaling pathway (sasa03320), ferroptosis (sasa04216), and pentose phosphate pathway (sasa00030) (Fig. 4, A, C). Of note, both BEA and ENNB enriched the ferroptosis pathway (sasa04216), i.e., cell death by iron-dependent lipid peroxidation (Fig. 4, A, C) and was the only enrich pathway related to regulated cell death. A detailed explanation of ferroptosis and how BEA and ENNB affected individual genes encoding key regulating proteins and rate limiting steps can be found in supplementary data (Supplemental Information Fig. S2. 1., S.2.2.).

In-depth analysis of functional units using enrichMKEGG indicated ENNB at 2.5 µM up-regulated three functional units related to central carbohydrate metabolism, four units related to ATP synthesis (representing complex III, IV, V), and one unit related to methane metabolism (Fig. 6).

### *3.4. Impact of BEA and ENNB on total ATP, total $H_2O_2$, cellular iron levels, and Gpx activity*

In the initial experiment, energy production in the Atlantic salmon primary hepatocytes was assessed after exposure to BEA and ENNB by measuring the total levels of cellular ATP, as other studies have reported effects on OXPHOS. No significant changes in ATP levels were detected at concentrations lower than 2.5 µM (apart from a weak trend of a bell-shaped response), though a decline exceeding 80% was observed at 5 µM for both BEA and ENNB (Fig. 7, A). The ability of BEA and ENNB to induce the generation of reactive oxygen species was assessed by measuring $H_2O_2$ content in exposed cells. No significant changes in $H_2O_2$ levels were detected at concentrations lower than 2.5 µM (apart from a weak dose-dependent reduction, and a weak increase in 2.5 µM ENNB), while a significant reduction (≥70%) was observed for both BEA and ENNB at 5 µM (Fig. 7, B). These results showed that 5 µM BEA and ENNB drastically reduced both the levels of ATP and $H_2O_2$ in exposed Atlantic salmon hepatocytes.

The RNA sequencing analysis done in the initial experiment indicated that BEA affected glutathione metabolism, ENNB affected amino acid syntheses of glutathione precursors (i.e., Glycine and Cysteine), and both mycotoxins enriched the ferroptosis pathway (iron-dependent regulated cell death) (Fig. A, C). Therefore, a follow-up experiment was carried out to further evaluate whether BEA and ENNB affected cellular iron levels as well as Gpx activity, since Gpx4 is important for the defense against ferroptosis. The iron assay, where both $Fe^{2+}$ and $Fe^{3+}$ are measured and together they constitute the total cellular iron content, showed BEA at 5 µM significantly increased the levels of $Fe^{3+}$ and hence

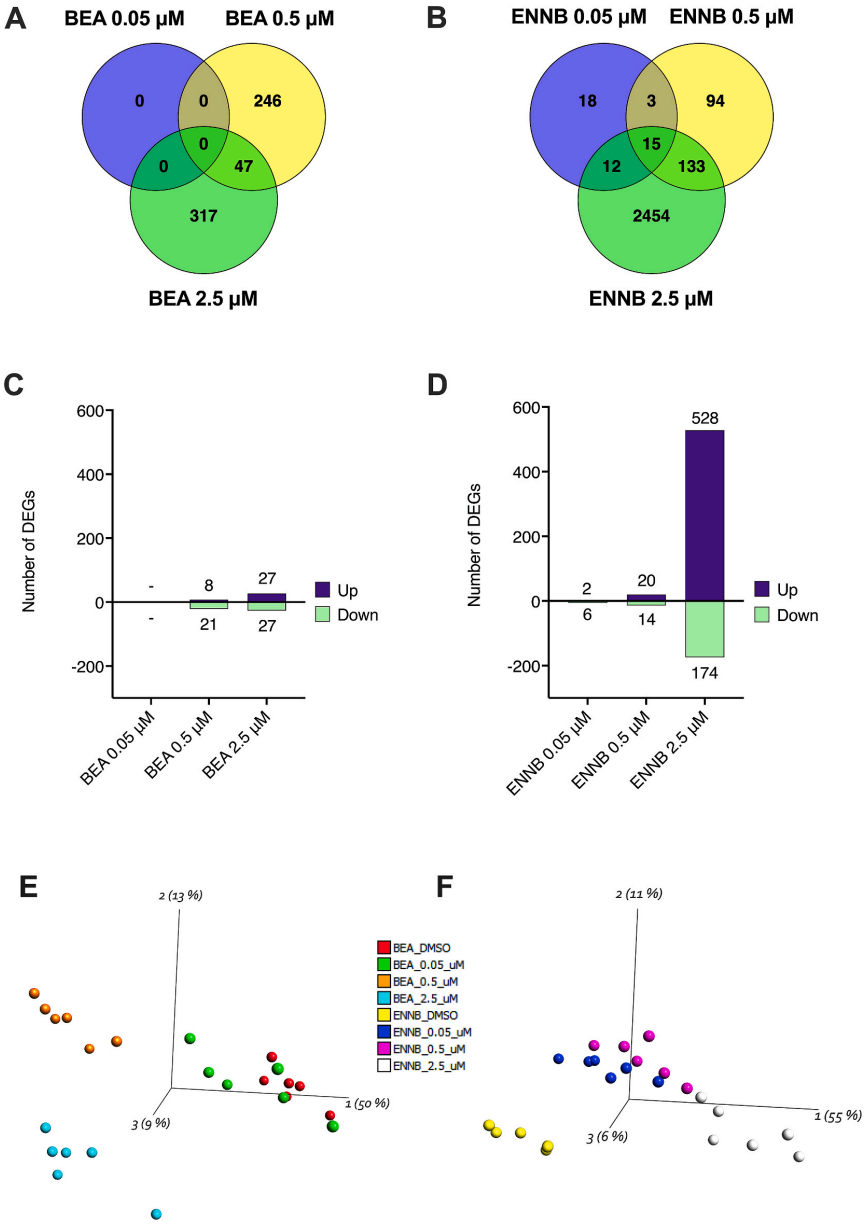


**Fig. 3.** Differentially expressed genes (DEGs). (A, B) Venn diagrams, (C, D) bar plots of the DEGs up- (purple) or down- (green) regulated, (E, F) PCA plots (legend indicating toxin and concentration) of exposure to three different concentrations of BEA and ENNB in relation to controls (q < 0.1, fold change >1.0). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

also the total level of iron in the cells (Fig. 7. C). The Gpx activity assay showed that all three concentrations of BEA (5, 2.5 and 0.05 µM), as well as 5 and 2.5 µM of ENNB significantly increased the activity of Gpx (Fig. 7. D).

## 4. Discussion

The prevalence of BEA and ENNB has increased in fish feed (Ytrestøyl et al., 2015), although the sensitivity of salmon to these emerging mycotoxins remains unknown. The toxicity exerted by BEA and ENNB in the present study was in general agreement with cytotoxic effects described in other studies, such as impairing lysosomal function and

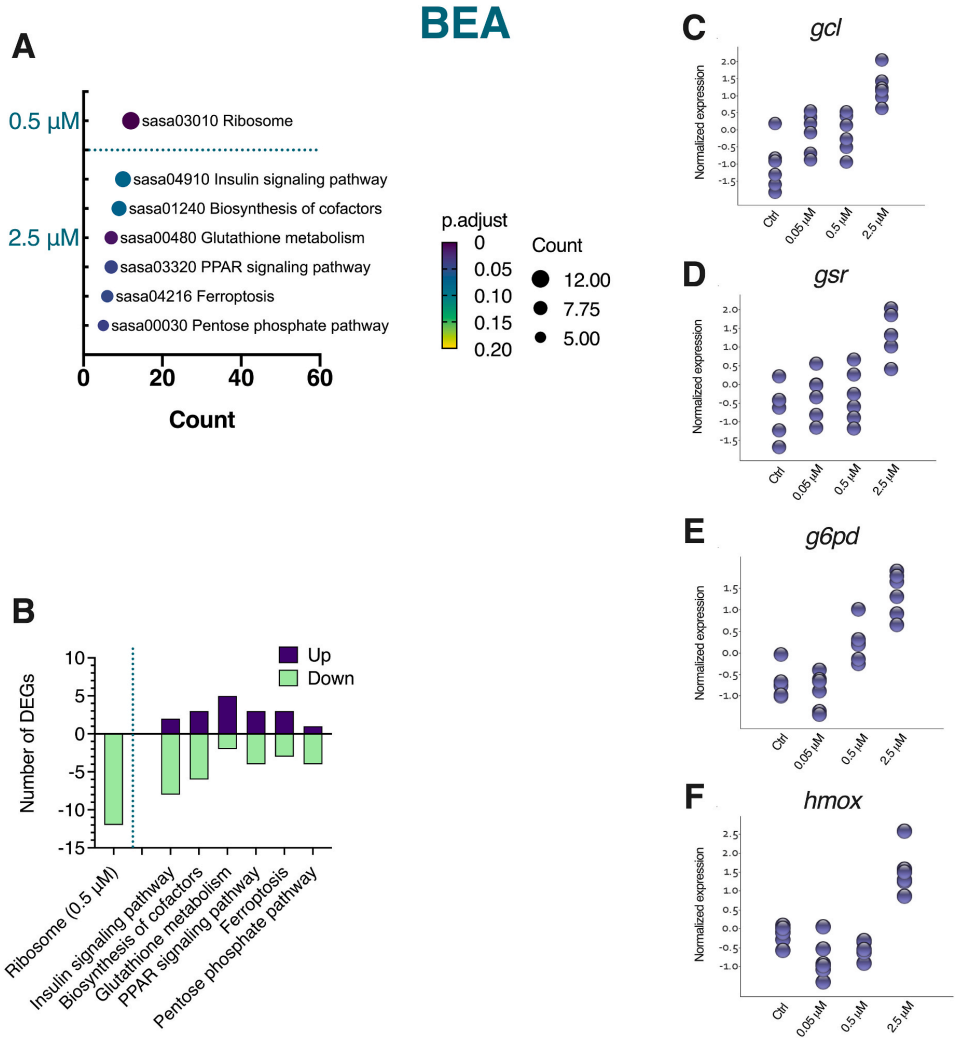


**Fig. 4.** KEGG pathway enrichment analysis of differentially expressed gene (DEG) counts. Enriched pathways in Atlantic salmon primary hepatocytes exposed to (A) 0.5 and 2.5 µM of BEA. X-axis and bubble size depict the count of attributed DEGs in each enriched pathway. Y-axis shows each enriched pathway where the fill colors from yellow to purple denotes the adjusted p-value (significant <0.1), the closer to purple the higher the significance. (B) Bar plot of the number of DEGs up- (purple) or down- (green) regulated, q < 0.1, fold change >1.0. (C-F) Scatter plots showing normalized expression of specific genes at the different exposure concentration including control. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

mitochondrial metabolic activity. RNA sequencing analysis at sub-cytotoxic levels further indicated BEA and ENNB-exposed salmon hepatocytes to experience an increased energy expenditure, oxidative stress, and disturbed iron homeostasis that sensitized the hepatocytes to ferroptotic cell death.

### 4.1. Cytotoxicity and morphology of salmon primary hepatocytes

The cell viability assessments (xCelligence, MTT and Neutral red) indicated that both mycotoxins were highly cytotoxic to the hepatocytes already at low µM concentration. In the initial experiment of the present study, ENNB exhibited higher cytotoxicity than BEA both in terms of effects on morphology and adhesion of the cells, as well as mitochondrial metabolic activity and impaired lysosomal function. However, in the follow-up experiment, BEA appeared more cytotoxic than ENNB. Thus, the dose-responses were fish and/or exposure batch-dependent although still within a narrow µM-range. We found that BEA affected both mitochondria and lysosomes to a similar degree, while ENNB had a greater impact on mitochondrial metabolic activity than lysosomal function. Similarly, Bernal-Algaba et al. (2021), found BEA to be more cytotoxic than ENNB when evaluating lysosomal function (Neutral red), mitochondrial metabolic activity (AlamarBlue) and plasma membrane integrity (CFDA-AM) in exposed rainbow trout gill cells (RTgill-W1). In contrast to the present study, they found lysosomal function to be more affected. Whereas García-Herranz et al. (2019) reported that BEA exerted higher toxicity to mitochondrial metabolic

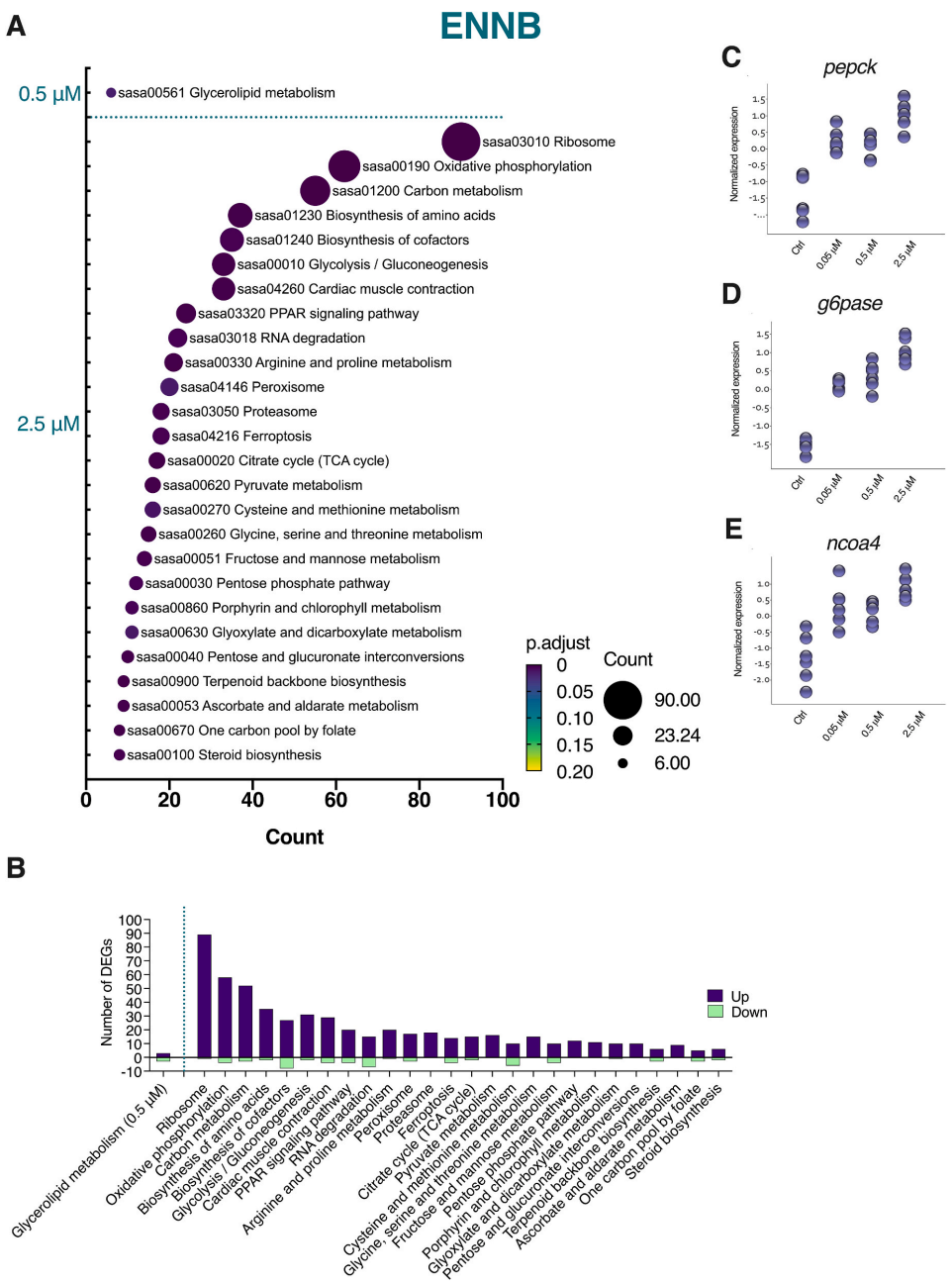


**Fig. 5.** KEGG pathway enrichment analysis of differentially expressed gene (DEG) counts. Enriched pathways in Atlantic salmon primary hepatocytes exposed to (A) 0.5 and 2.5 μM of ENNB. X-axis and bubble size depict the count of attributed DEGs in each enriched pathway. Y-axis shows each enriched pathway where the fill colors from yellow to purple denotes the adjusted p-value (significant <0.1), the closer to purple the higher the significance. (B) Bar plot of the number of DEGs up- (purple) or down- (green) regulated, q < 0.1, fold change >1. (C-F) Scatter plots showing normalized expression of specific genes at the different exposure concentration including control. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

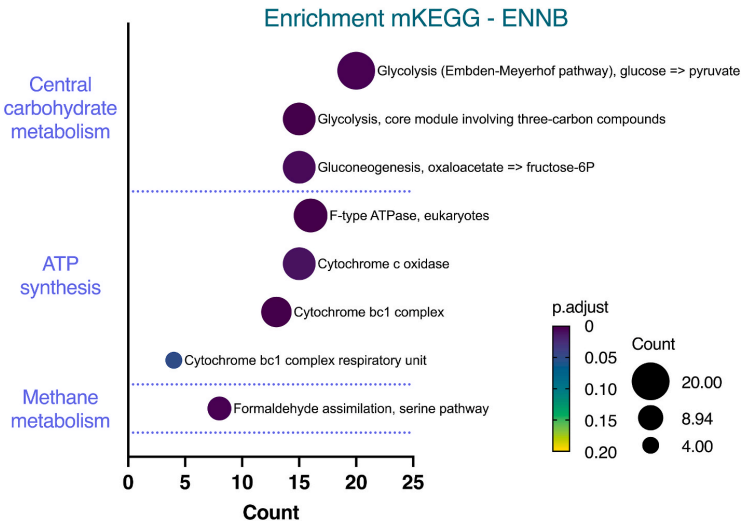


**Fig. 6.** EnrichMKEGG functional unit enrichment analysis of differentially expressed genes (DEGs) by 2.5 μM of ENNB. X-axis and bubble size depict the count of attributed DEGs in each functional unit. Y-axis shows significantly enriched functional (p-adjust < 0.1) units and the fill colors from yellow to purple denote statistical significance. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

activity than to lysosomal function, or plasma membrane integrity in the rainbow trout cell line RTH-149. In line with the present study which showed BEA and ENNB to be cytotoxic at low μM concentrations to the salmon hepatocytes, both García-Herranz et al. (2019) and Bernal-Algaba et al. (2021) reported high cytotoxicity of BEA (and ENNB) especially compared to the more well-studied mycotoxins ochratoxin A (OTA) and DON. This may be of potential concern since neither BEA nor ENNB are currently legislatively regulated in feed or food (Bernhoft et al., 2013b).

### 4.2. Increased energy expenditure with enhanced compensatory metabolic activity

RNA sequencing analysis showed that ENNB produced a more pronounced dose-response than BEA, both in terms of the numbers of unique DEGs and enriched pathways. Pathway enrichment analysis at the two lowest concentrations of BEA and ENNB sequenced (0.05 and 0.5 μM) gave few or no enriched pathways, whereas 2.5 μM of the mycotoxins enriched several pathways related to energy expenditure and homeostasis in the exposed hepatocytes. At 2.5 μM, ENNB significantly up-regulated genes related to the oxidative phosphorylation pathway. The electron transport chain drives oxidative phosphorylation of ADP to ATP during mitochondrial respiration (Stockwell et al., 2020), whereas the enrichMKEGG analysis especially emphasized ENNB's effect on complex II, IV, and V of the electron transport chain. Previous mammalian *in vitro* studies have also shown BEA and ENNB to cause transcriptional changes in genes related to oxidative phosphorylation (Jonsson et al., 2016; Alonso-Garrido et al., 2018; Escrivá et al., 2018). In contrast to the ENNB-induced up-regulation of oxidative phosphorylation in the present study, the previously mentioned studies indicated primarily down-regulation of genes related to the oxidative phosphorylation pathway. Further, ENNB appeared to cause a shift in the hepatocytes' metabolic state into glucose anabolism, consuming ATP at an increased rate. The measured cellular ATP levels indicated that the hepatocytes were able to sustain increased energy expenditure at sub-cytotoxic concentrations of the mycotoxins. However, 5 μM of both BEA and ENNB caused a sharp decline in cellular ATP levels, which coincided with reduced cell viability. BEA and ENNB's ionophoric properties could potentially cause a disruption of the ion homeostasis, affecting the rate of mitochondrial respiration (i.e., ATP production) (Hajnóczky et al., 1995), or cause a depolarization of the mitochondrial membrane potential (i.e., halting ATP production) (Tonshin et al., 2010).

The glycolysis/gluconeogenesis pathway was enriched by ENNB, although gluconeogenesis seemed to be favored since the genes encoding the two rate-limiting gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (Pepck) and glucose-6-phosphatase (G6Pase) (Klover and Mooney, 2004), were among the top up-regulated DEGs. This was further supported by the enrichMKEGG analysis of functional units enriched by the DEGs, which also indicated gluconeogenesis to be represented as one out of three functional units. In addition, several of the observed enriched metabolic pathways (i.e., pyruvate metabolism, TCA cycle, carbon metabolism, amino acid metabolism, PPAR signaling pathway, fructose and mannose metabolism) were implied to be up-stream events supplying precursors to gluconeogenesis (Rowsell et al., 1973; Sun et al., 2019; Klover and Mooney, 2004; Chinetti et al., 2000; Hannou et al., 2018). The production of such glucogenic precursors is ATP-dependent (Melkonian et al., 2021). In contrast to ENNB, BEA had limited effect on gluconeogenesis apart from one down-regulated DEG (fructose 1,6-bisphosphate) at 2.5 μM. Increased energy expenditure is often observed as an adaptive stress response (Manoli et al., 2007). For example, rats orally exposed to ochratoxin (OTA) exhibited disturbed energy metabolism at a transcriptional level in the liver (Qi et al., 2014).

### 4.3. Oxidative stress in exposed salmon primary hepatocytes

While oxidative phosphorylation is the main process providing the cells with ATP, it is also a significant intracellular source of reactive oxygen species (Brown et al., 2010). The transcriptional up-regulation of oxidative phosphorylation by ENNB in the present study may indicate an increased generation of reactive oxygen species. $H_2O_2$ levels in the hepatocytes were measured since this reactive oxygen species has the longest half-life and thus best chance of detecting (Zhao et al., 2018). However, neither BEA nor ENNB caused any significant changes in $H_2O_2$ levels at sub-cytotoxic levels. Only a drastic decrease in $H_2O_2$ was

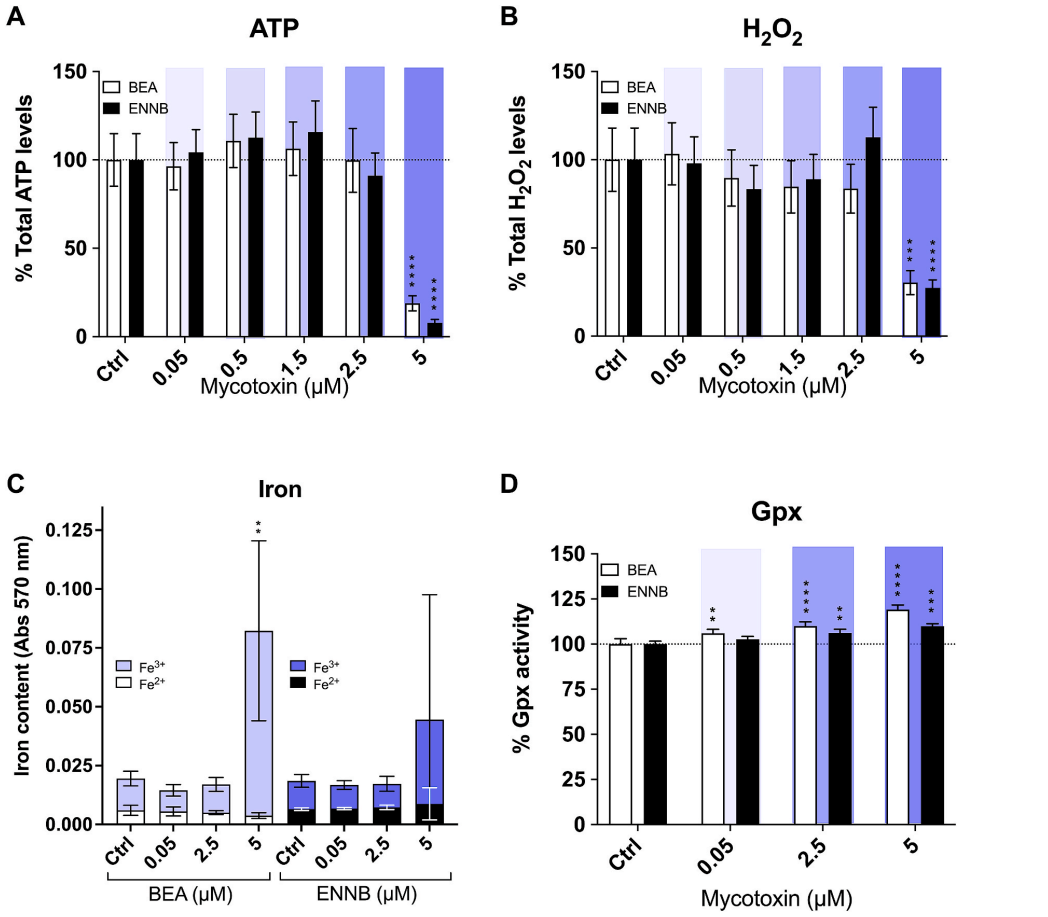


**Fig. 7.** Total levels of ATP, $H_2O_2$, Iron, and Gpx enzyme activity in salmon primary hepatocytes exposed to BEA and ENNB. (A) ATP assay, data shown as % total ATP levels based on average relative luminescence units (RLU) relative to control. (B) $H_2O_2$ assay, data shown as % total $H_2O_2$ levels based on average RLU relative to control. (C) Iron assay, data shown as cellular levels of ferrous ($Fe^{2+}$) and ferric ($Fe^{3+}$), stacked showing total iron content based on average corrected abs 570 relative to control. (D) Gpx assay, data shown as % Gpx enzyme activity based on average abs 340 nm after 5 min relative to control. (A, B) n = 6. (C, D) n = 4. **p < 0.01, ***p < 0.001, ****p < 0.0001.

observed at cytotoxic levels of both mycotoxins, which is probably correlated to the observed decline in ATP levels, and reduced cell viability at cytotoxic levels.

Inferred from the pathway enrichment analysis, BEA enriched both glutathione metabolism and biosynthesis of cofactors at sub-cytotoxic levels, where the top DEGs encoded glutathione reductase (Gsr), glutamate cysteine ligase (Gcl), and s-adenosylmethionine synthase isoform type-2 (Metk2; a synthase that activate a precursor needed to synthesize glutathione) (Lieber, 2002). Circulation of oxidized glutathione (GSSG) and reduced glutathione (GSH) through Gsr-activity, sustains the need for GSH, serving as a reducing agent for glutathione peroxidase (Gpx) during reactive oxygen species-scavenging (Lushchak, 2012; Das and Roychoudhury, 2014). Glutathione is synthesized from the precursor's cysteine, glutamate, and glycine, which are condensed into glutathione through a two-step reaction, where the first step is carried out by Gcl (Wu et al., 2004). The enrichment of peroxisomes, cysteine-, glycine-, and glyoxylate metabolism pathways by ENNB were likely up-stream events that supplied precursors needed for glutathione biosynthesis, as well as precursors for gluconeogenesis (Wu et al., 2004; Salido et al., 2012). The enrichment of the pentose phosphate pathway by both BEA and ENNB may also be related to the function of the antioxidant defense, as one of the primary products of the pentose phosphate pathway is nicotinamide adenine dinucleotide phosphate (NADPH), which is important for $H_2O_2$ savaging as NADPH gives reducing power to glutathione reductase (Gsr) (Chandel, 2021). The transcription of the rate-limiting enzyme, glucose-6-phosphate 1-dehydrogenase (G6pd), which directs glucose breakdown through the pentose phosphate pathway (Stanton, 2012; Tang, 2019), was up-regulated by BEA. Despite the ROS assay being unable to detect any significant increase in $H_2O_2$, the inferred elevation of the hepatocytes' redox state from the pathway enrichment analysis was supported by the dose-dependent increase in Gpx enzyme activity following BEA and ENNB exposure. BEA (0.1–5 μM) has previously been shown to significantly increase the Gpx activity in CHO-K1 cells (Mallebrera et al., 2014). An increased reactive oxygen species generation can distress cells and thereby trigger the onset of regulated cell death (Dixon and Stockwell, 2014).

### 4.4. Mitochondrial and lysosomal dysfunction – sensitizing the hepatocytes to ferroptosis?

Previous studies have described that BEA and ENNB induce apoptotic (Prosperini et al., 2013b) or necrotic cell death (Jonsson et al., 2016). Ivanova et al. (2012) on the other hand, reported that ENNB induced a cell death pathway in Caco-2 cells that did not exhibit any typical characteristics of classical apoptosis (PI/Hoechst assay) or necrosis (LDH assay). In the present study, hepatocytes exposed to cytotoxic levels of ENNB and BEA started to contract, exhibited a different growth pattern, and developed protruding blister-like features on the cell membrane consistent with the early onset of cell death. Xie et al. (2016) categorized typical morphological features of cell death into 1) necrosis - plasma membrane rupture; 2) apoptosis - rounded-up cell morphology with blebbing on plasma membrane; 3) ferroptosis - rounded-up cell morphology with intact plasma membrane free from "apoptotic" blebbing. Later studies on ferroptosis have described plasma membranes exhibiting "blisters", "ballooning" formations, or protrusions due to membrane integrity loss (Magtanong et al., 2019; Dodson et al., 2019, Van der meeren et al., 2020). These recent observations of ferroptosis are in line with the phenotype exhibited by the salmon hepatocytes exposed to cytotoxic levels of BEA and ENNB in the present study. In addition, the pathway enrichment analysis indicated that ENNB affected lipid membranes. ENNB enriched glycerophospholipid metabolism, and up-regulated the gene for the lipoprotein lipase (top DEG), which breaks down lipoproteins (e.g., triglycerides and cholesterol) into free fatty acids (Leaver et al., 2008), in the hepatocytes exposed to 0.5 μM. While at 2.5 μM, ENNB enriched terpenoid backbone synthesis producing terpenes, and steroid biosynthesis producing cholesterol via lysosomal acid lipase enzyme activity (top DEG). Both terpenes and cholesterol can be incorporated into phospholipid membranes to regulate their fluidity (Mendanha and Alonso, 2015; Nicholson et al., 2013).

The enrichment of genes related to the ferroptosis pathway in hepatocytes exposed to 2.5 μM BEA and ENNB added further support to this mode of cell death. Ferroptosis is described as an iron-dependent cell death pathway causing toxic levels of lipid peroxidation in PUFA-rich phospholipid membranes, which will propagate if not repaired by the GSH/Gpx antioxidant defense system (Dixon et al., 2012; Xie et al., 2016). Both mycotoxins affected transcript levels of key enzymes responsible for sensitizing cells to ferroptosis, such as enzymes important for polyunsaturated fatty acid phospholipid synthesis (e.g., *acsl4 and sat1*) (Yuan et al., 2016; Ou et al., 2016), and iron metabolism (e.g., *transferrin, steap3, dmt1, hmox, vdac2/3, ferritin, ncoa4*) (Paul et al., 2017; Zhang et al., 2012; Andrews, 1999; Choi and Alam, 1996; Colombini, 2004; Hou et al., 2016) (For a more detailed explanation of the ferroptosis pathway see Supplemental Information). Free $Fe^{2+}$ is toxic and can react with $H_2O_2$ and, through a Fenton-like reaction, generate highly reactive hydroxyl radicals prone to cause peroxidation of PUFA-rich lipid membranes, while the iron is oxidized to $Fe^{3+}$ (Dixon and Stockwell, 2014; Jiang et al., 2021). In the present study, cytotoxic levels of BEA caused a significant increase in $Fe^{3+}$ in the hepatocytes, thus indicating that a Fenton reaction had occurred. Lipid membranes which have suffered peroxidation form hydrophilic pores, can further impair the barrier function of membranes (Ju et al., 2021), potentially escalating the event. Ferritin is an iron chaperone protein which prevents $Fe^{2+}$ from reacting with hydroxyl free radicals (Paul et al., 2017), and ferritin transcription was increased by ENNB. Interestingly, ENNB also increased the transcription of the nuclear receptor coactivator 4 (*ncoa4*), which implied that iron was released from ferritin in the exposed hepatocytes. Overexpression of Ncoa4 has been shown to mediate ferritin-degradation, releasing unstable iron in fibroblasts and cancer cells which triggered the initiation of ferroptosis (Hou et al., 2016). Research suggests that stress-induced release of intracellular iron (to toxic levels) may occur from compromised iron-storing units such as lysosomes or mitochondrion (Terman and Kurz, 2013; Muñoz et al., 2016; Mena et al., 2015), and ferritin (Biasiotto et al., 2016). Thus, a plausible explanation is that the stress induced by BEA and ENNB caused an increased release of intracellular iron by damaging the lysosomes, the iron-storing ferritin, and mitochondria in the hepatocytes. Effects on lysosomes were supported by the results from the Neutral red assay showing that both mycotoxins significantly decreased lysosome function in a dose-dependent manner. Similarly, Ivanova et al. (2012) reported the loss of lysosomal integrity in ENNB-exposed Caco-2 cells. Almeida et al. (2006) demonstrated that isolated mitochondria were extremely sensitive to iron-induced lipid peroxidation, which caused permeabilization of their membranes. The observed reduction of the mitochondrial metabolic activity by both mycotoxins in the present study implied that mitochondrial iron homeostasis and retention might have been impaired. The presented data indicated that the salmon primary hepatocytes were becoming ferroptotic rather than apoptotic or necrotic. The mycotoxin aflatoxin B1 was recently shown to affect ferroptosis signaling in chicks (Zhao et al., 2021), and the mycotoxin DON caused iron imbalance in IPEC-1 cells (Lin et al., 2021). To our knowledge, this is the first time that BEA and ENNB have been associated with disruption of cellular iron homeostasis.

In this study we used the transcriptomic data for pathways enrichment analyses as a hypothesis-generating tool, to identify affected metabolic pathways that could explain the mechanisms of BEA and ENNB causing cellular responses and toxicity in the exposed hepatocytes. Since a cell's biological function is controlled through a dynamic (spatio-temporal) and complex network of signaling within and between pathways through crosstalk and feedback loops (Kholodenko, 2006), a small change in gene transcription could be important for the downstream effects (Zhan et al., 2017). With a good sample size (i.e., $n = 6$ per experimental condition) we did not wish to set a fold change cutoff criterion, at risk of losing valid data thereby failing to signify important pathways that contribute to producing the response (Zhan et al., 2017). As presented, the transcriptomics indicated that e.g., both the iron metabolism and the redox state of the exposed hepatocytes were significantly altered. Although statistical significance is not necessarily equivalent to biological relevance (Zhan et al., 2017), the independent follow-up experiment analyzing Gpx activity, lysosomal function, and cellular iron content in exposed primary hepatocytes, further corroborated our RNA-Seq generated hypothesis from the initial experiment. Whether the observed effects are secondary to the predicted primary mechanism of action of BEA and ENNB requires further studies to clarify. The level of toxicity exhibited by the exposed primary Atlantic salmon hepatocytes justifies a call for more *in vitro* and *in vivo* toxicity data in farmed Atlantic salmon to further elucidate the toxic mode of action of these prevalent mycotoxins.

## 5. Conclusion

Both BEA and ENNB were highly cytotoxic to Atlantic salmon primary hepatocytes, and impaired endpoints such as mitochondrial metabolic activity and lysosomal function in a dose-dependent manner. Cytotoxic levels of BEA and ENNB also caused shrinkage of the hepatocytes and the formation of protruding blister-like features on their cell membranes, consistent with the onset of cell death. At a transcriptional level, BEA and ENNB appeared to increase the hepatocytes' energy expenditure causing them to shift their metabolic state into an anabolic ATP-consuming state, and to trigger a general adaptive stress response in the hepatocytes by eliciting an increased redox balance to cope with the inflicted stress. This was supported by a dose-dependent increase in the GSH-dependent Gpx enzyme activity following exposure to both BEA and ENNB. Further, both BEA and ENNB enriched the ferroptosis pathway and measurement of intracellular iron, implying initiation of cell death through iron-dependent lipid peroxidation.

## CRediT authorship contribution statement

**Sofie Søderstrøm:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization. **Kai K. Lie:** Conceptualization, Methodology, Software, Formal analysis, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Anne-Katrine Lundebye:** Conceptualization, Formal analysis, Writing – review & editing, Supervision. **Liv Søfteland:** Conceptualization, Methodology, Formal analysis, Resources, Writing – review & editing, Supervision, Funding acquisition.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

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## References

Almeida, A.M., Bertoncini, C.R., Borecký, J., Souza-Pinto, N.C., Vercesi, A.E., 2006. Mitochondrial DNA damage associated with lipid peroxidation of the mitochondrial membrane induced by Fe2+-citrate. An Acad. Bras Ciências 78, 505–514.
Alonso-Garrido, M., Escrivá, L., Manyes, L., Font, G., 2018. Enniatin B induces expression changes in the electron transport chain pathway related genes in lymphoblastic T-cell line. Food Chem. Toxicol. 121, 437–443.
Andrews, N.C., 1999. The iron transporter DMT1. Int. J. Biochem. Cell Biol. 31, 991–994.
Bernal-Algaba, E., Pulgarín-Alfaro, M., Fernández-Cruz, M.L., 2021. Cytotoxicity of mycotoxins frequently present in aquafeeds to the fish cell line RTGill-W1. Toxins 13, 581.
Bernhoft, A., Eriksen, G., Sundheim, L., Berntssen, M., Brantsæter, A., Brodal, G., Fæste, C., Hofgaard, I., Rafoss, T., Sivertsen, T., 2013a. Risk Assessment of Mycotoxins in Cereal Grain in Norway. Opinion of the Scientific Steering Committee of the Norwegian Scientific Committee for Food Safety, Oslo, Norway.
Bernhoft, A., Eriksen, G.S., Sundheim, L., Berntssen, M., Brantsæter, A.L., Brodal, G., Fæste, C.K., Hofgaard, I.S., Rafoss, T., Sivertsen, T., 2013b. Risk Assessment of Mycotoxins in Cereal Grain in Norway. Opinion of the Scientific Steering Committee of the Norwegian Scientific Committee for Food Safety. VKM report.
Biasiotto, G., Di Lorenzo, D., Archetti, S., Zanella, I., 2016. Iron and neurodegeneration: is ferritinophagy the link? Mol. Neurobiol. 53, 5542–5574.
Brown, G.C., Murphy, M.P., Jastroch, M., Divakaruni, A.S., Mookerjee, S., Treberg, J.R., Brand, M.D., 2010. Mitochondrial proton and electron leaks. Essays Biochem. 47, 53–67.
Chandel, N.S., 2021. NADPH—the forgotten reducing equivalent. Cold Spring Harbor Perspect. Biol. 13, a040550.
Chinetti, G., Fruchart, J.-C., Staels, B., 2000. Peroxisome proliferator-activated receptors (PPARs): nuclear receptors at the crossroads between lipid metabolism and inflammation. Inflamm. Res. 49, 497–505.
Choi, A., Alam, J., 1996. Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. Am. J. Respir. Cell Mol. Biol. 15, 9–19.
Colombini, M., 2004. VDAC: the channel at the interface between mitochondria and the cytosol. Mol. Cell. Biochem. 256, 107–115.
Das, K., Roychoudhury, A., 2014. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Front. Environ. Sci. 2, 53.
Dixon, S.J., Lemberg, K.M., Lamprecht, M.R., Skouta, R., Zaitsev, E.M., Gleason, C.E., Patel, D.N., Bauer, A.J., Cantley, A.M., Yang, W.S., 2012. Ferroptosis: an iron-dependent form of nonapoptotic cell death. Cell 149, 1060–1072.
Dixon, S.J., Stockwell, B.R., 2014. The role of iron and reactive oxygen species in cell death. Nat. Chem. Biol. 10, 9–17.
Dodson, M., Castro-Portuguez, R., Zhang, D.D., 2019. NRF2 plays a critical role in mitigating lipid peroxidation and ferroptosis. Redox Biol. 23, 101107.
Dornetshuber, R., Heffeter, P., Sulyok, M., Schumacher, R., Chiba, P., Kopp, S., Koellensperger, G., Micksche, M., Lemmens-Gruber, R., Berger, W., 2009. Interactions between ABC-transport proteins and the secondary Fusarium metabolites enniatin and beauvericin. Mol. Nutr. Food Res. 53, 904–920.
Escrivá, L., Jennen, D., Caiment, F., Manyes, L., 2018. Transcriptomic study of the toxic mechanism triggered by beauvericin in Jurkat cells. Toxicol. Lett. 284, 213–221.
Ferrer, E., Juan-García, A., Font, G., Ruiz, M., 2009. Reactive oxygen species induced by beauvericin, patulin and zearalenone in CHO-K1 cells. Toxicol. Vitro 23, 1504–1509.
Fjelldal, P.G., Hansen, T.J., Wargelius, A., Ayllon, F., Glover, K.A., Schulz, R.W., Fraser, T.W., 2020. Development of supermale and all-male Atlantic salmon to research the vgll3 allele-puberty link. BMC Genet. 21, 1–13.
García-Herranz, V., Valdehita, A., Navas, J., Fernández-Cruz, M., 2019. Cytotoxicity against fish and mammalian cell lines and endocrine activity of the mycotoxins beauvericin, deoxynivalenol and ochratoxin-A. Food Chem. Toxicol. 127, 288–297.
Hajnóczky, G., Robb-Gaspers, L.D., Seitz, M.B., Thomas, A.P., 1995. Decoding of cytosolic calcium oscillations in the mitochondria. Cell 82, 415–424.
Hannou, S.A., Haslam, D.E., Mckeown, N.M., Herman, M.A., 2018. Fructose metabolism and metabolic disease. J. Clin. Invest. 128, 545–555.
Hilgenfeld, R., Saenger, W., 1985. Structural Chemistry of Natural and Synthetic Ionophores and Their Complexes with Cations. Host Guest Complex Chemistry/Macrocycles. Springer.
Hooft, J.M., Encarnação, P., Bureau, D.P., 2011. Rainbow trout (Oncorhynchus mykiss) is extremely sensitive to the feed-borne Fusarium mycotoxin deoxynivalenol (DON). Aquaculture 311, 224–232.
Hou, W., Xie, Y., Song, X., Sun, X., Lotze, M.T., Zeh III, H.J., Kang, R., Tang, D., 2016. Autophagy promotes ferroptosis by degradation of ferritin. Autophagy 12, 1425–1428.
Hussein, H.S., Brasel, J.M., 2001. Toxicity, metabolism, and impact of mycotoxins on humans and animals. Toxicology 167, 101–134.
Iso, I., 2009. 10993–5: 2009 Biological Evaluation of Medical Devices—Part 5: Tests for in Vitro Cytotoxicity. International Organization for Standardization, Geneva.
Ivanov, V., Evstratov, A., Sumskaya, L., Melnik, E., Chumburidze, T., Portnova, S., Balashova, T., Ovchinnikov, Y.A., 1973. Sandwich complexes as a functional form of the enniatin ionophores. FEBS Lett. 36, 65–71.
Ivanova, L., Egge-Jacobsen, W., Solhaug, A., Thoen, E., Fæste, C., 2012. Lysosomes as a possible target of enniatin B-induced toxicity in Caco-2 cells. Chem. Res. Toxicol. 25, 1662–1674.
Jestoi, M., 2008. Emerging Fusarium-mycotoxins fusaproliferin, beauvericin, enniatins, and moniliformin—a review. Crit. Rev. Food Sci. Nutr. 48, 21–49.
Jiang, X., Stockwell, B.R., Conrad, M., 2021. Ferroptosis: mechanisms, biology and role in disease. Nat. Rev. Mol. Cell Biol. 1–17.
Jonsson, M., Jestoi, M., Anthoni, M., Welling, A., Loivamaa, I., Hallikainen, V., Kankainen, M., Lysøe, E., Koivisto, P., Peltonen, K., 2016. Fusarium mycotoxin enniatin B: cytotoxic effects and changes in gene expression profile. Toxicol. Vitro 34, 309–320.
Ju, J., Song, Y.-N., Wang, K., 2021. Mechanism of ferroptosis: a potential target for cardiovascular diseases treatment. Aging Dis. 12, 261.
Kamyar, M., Rawnduzi, P., Studenik, C.R., Kouri, K., Lemmens-Gruber, R., 2004. Investigation of the electrophysiological properties of enniatins. Arch. Biochem. Biophys. 429, 215–223.
Kholodenko, B.N., 2006. Cell-signalling dynamics in time and space. Nat. Rev. Mol. Cell Biol. 7, 165–176.
Kim, D., Langmead, B., Salzberg, S.L., 2015. HISAT: a fast spliced aligner with low memory requirements. Nat. Methods 12, 357–360.
Klarić, M.S., Pepeljnjak, S., Domijan, A.M., Petrik, J., 2007. Lipid peroxidation and glutathione levels in porcine kidney PK15 cells after individual and combined treatment with fumonisin B1, beauvericin and ochratoxin A. Basic Clin. Pharmacol. Toxicol. 100, 157–164.
Klover, P.J., Mooney, R.A., 2004. Hepatocytes: critical for glucose homeostasis. Int. J. Biochem. Cell Biol. 36, 753–758.
Kouri, K., Duchen, M.R., Lemmens-Gruber, R., 2005. Effects of beauvericin on the metabolic state and ionic homeostasis of ventricular myocytes of the Guinea pig. Chem. Res. Toxicol. 18, 1661–1668.
Kouri, K., Lemmens, M., Lemmens-Gruber, R., 2003. Beauvericin-induced channels in ventricular myocytes and liposomes. Biochim. Biophys. Acta Biomembr. 1609, 203–210.
Leaver, M.J., Bautista, J.M., Björnsson, B.T., Jönsson, E., Krey, G., Tocher, D.R., Torstensen, B.E., 2008. Towards fish lipid nutrigenomics: current state and prospects for fin-fish aquaculture. Rev. Fish. Sci. 16, 73–94.
Liao, Y., Smyth, G.K., Shi, W., 2014. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30, 923–930.
Lieber, C.S., 2002. S-adenosyl-L-methionine: its role in the treatment of liver disorders. Am. J. Clin. Nutr. 76, 1183S–1187S.
Lin, J., Huang, F., Liang, T., Qin, Q., Xu, Q., Huang, X., Zhang, J., Xiao, K., Zhu, H., Zhao, J., 2021. EPA and DHA confer protection against DON-induced endoplasmic reticulum stress and iron imbalance in IPEC-1 cells. Br. J. Nutr. 1–29.
Lindblad, M., Gidlund, A., Sulyok, M., Börjesson, T., Krska, R., Olsen, M., Fredlund, E., 2013. Deoxynivalenol and other selected Fusarium toxins in Swedish wheat—occurrence and correlation to specific Fusarium species. Int. J. Food Microbiol. 167, 284–291.
Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 1–21.
Lushchak, V.I., 2012. Glutathione homeostasis and functions: potential targets for medical interventions. J. Amino Acids 2012, 736837.

Magtanong, L., Ko, P.-J., To, M., Cao, J.Y., Forcina, G.C., Tarangelo, A., Ward, C.C., Cho, K., Patti, G.J., Nomura, D.K., 2019. Exogenous monounsaturated fatty acids promote a ferroptosis-resistant cell state. Cell Chem. Biol. 26, 420–432 e9.
Mallebrera, B., Brandolini, V., Font, G., Ruiz, M., 2015. Cytoprotective effect of resveratrol diastereomers in CHO-K1 cells exposed to beauvericin. Food Chem. Toxicol. 80, 319–327.
Mallebrera, B., Font, G., Ruiz, M., 2014. Disturbance of antioxidant capacity produced by beauvericin in CHO-K1 cells. Toxicol. Lett. 226, 337–342.
Mallebrera, B., Prosperini, A., Font, G., Ruiz, M.J., 2018. In vitro mechanisms of Beauvericin toxicity: a review. Food Chem. Toxicol. 111, 537–545.
Manoli, I., Alesci, S., Blackman, M.R., Su, Y.A., Rennert, O.M., Chrousos, G.P., 2007. Mitochondria as key components of the stress response. Trends Endocrinol. Metabol. 18, 190–198.
Melkonian, E.A., Asuka, E., Schury, M.P., 2021. Physiology, Gluconeogenesis [Online]. StatPearls Publishing. Available: https://www.ncbi.nlm.nih.gov/books/NBK541119/. (Accessed 19 October 2021).
Mena, N.P., Urrutia, P.J., Lourido, F., Carrasco, C.M., Núñez, M.T., 2015. Mitochondrial iron homeostasis and its dysfunctions in neurodegenerative disorders. Mitochondrion 21, 92–105.
Mendanha, S.A., Alonso, A., 2015. Effects of terpenes on fluidity and lipid extraction in phospholipid membranes. Biophys. Chem. 198, 45–54.
Moldal, T., Bernhoft, A., Rosenlund, G., Kaldhusdal, M., Koppang, E.O., 2018. Dietary deoxynivalenol (DON) may impair the epithelial barrier and modulate the cytokine signaling in the intestine of Atlantic salmon (Salmo salar). Toxins 10, 376.
Muñoz, Y., Carrasco, C.M., Campos, J.D., Aguirre, P., Núñez, M.T., 2016. Parkinson's Disease: the Mitochondria-Iron Link. Parkinson's disease, 2016.
Mwihia, E.W., Lyche, J.L., Mbuthia, P.G., Ivanova, L., Uhlig, S., Gathumbi, J.K., Maina, J. G., Eshitera, E.E., Eriksen, G.S., 2020. Co-occurrence and levels of mycotoxins in fish feeds in Kenya. Toxins 12, 627.
Nicholson, C.K., Lambert, J.P., Molkentin, J.D., Sadoshima, J., Calvert, J.W., 2013. Thioredoxin 1 is essential for sodium sulfide–mediated cardioprotection in the setting of heart failure. Arterioscler. Thromb. Vasc. Biol. 33, 744–751.
Oliveros, J.C., 2007. VENNY. An interactive tool for comparing lists with Venn Diagrams. *http://bioinfogp.cnb.csic.es/tools/venny/index.html*.
Ørnsrud, R., Silva, M.S., Berntssen, M.H., Lundebye, A.-K., Storesund, J.E., Lie, K.K., Waagbø, R., Sele, V., 2020. Program for overvåking av fiskefôr-Årsrapport for prøver innsamlet i 2019. *Rapport fra havforskningen*.
Ou, Y., Wang, S.-J., Li, D., Chu, B., Gu, W., 2016. Activation of SAT1 engages polyamine metabolism with p53-mediated ferroptotic responses. Proc. Natl. Acad. Sci. Unit. States Am. 113, E6806–E6812.
Paul, B.T., Manz, D.H., Torti, F.M., Torti, S.V., 2017. Mitochondria and Iron: current questions. Expet Rev. Hematol. 10, 65–79.
Prosperini, A., Berrada, H., Ruiz, M.J., Caloni, F., Coccini, T., Spicer, L.J., Perego, M.C., Lafranconi, A., 2017. A review of the mycotoxin enniatin B. Front. Public Health 5, 304.
Prosperini, A., Juan-García, A., Font, G., Ruiz, M., 2013a. Beauvericin-induced cytotoxicity via ROS production and mitochondrial damage in Caco-2 cells. Toxicol. Lett. 222, 204–211.
Prosperini, A., Juan-García, A., Font, G., Ruiz, M., 2013b. Reactive oxygen species involvement in apoptosis and mitochondrial damage in Caco-2 cells induced by enniatins A, A1, B and B1. Toxicol. Lett. 222, 36–44.
Qi, X., Yang, X., Chen, S., He, X., Dweep, H., Guo, M., Cheng, W.-H., Xu, W., Luo, Y., Gretz, N., 2014. Ochratoxin A induced early hepatotoxicity: new mechanistic insights from microRNA, mRNA and proteomic profiling studies. Sci. Rep. 4, 1–14.
Rowsell, E.V., Al-Tai, A.H., Carnie, J.A., Rowsell, K.V., 1973. Increased liver l-serine–pyruvate aminotransferase activity under gluconeogenic conditions. Biochem. J. 134, 349–351.
Salido, E., Pey, A.L., Rodriguez, R., Lorenzo, V., 2012. Primary hyperoxalurias: disorders of glyoxylate detoxification. Biochim. Biophys. Acta (BBA) - Mol. Basis Dis. 1822, 1453–1464.
Sanden, M., Hemre, G., Måge, A., Lunestad, B., Espe, M., Lie, K., Lundebye, A., Amlund, H., Waagbø, R., Ørnsrud, R., 2017. Program for Overvåking Av Fiskefôr Årsrapport for Prøver Innsamlet I 2016. NIFES, Bergen.
Sele, V., Sanden, M., Berntssen, M., Lunestad, B., Espe, M., Lie, K., Amlund, H., Lundebye, A., Hemre, G., Waagbø, R., Ørnsrud, R., 2018. Program for overvåking av fiskefôr - Årsrapport for prøver innsamlet i 2017. *Rapport fra havforskningen*.
Sele, V., Sanden, M., Berntssen, M., Storesund, J.E., Lie, K.K., Espe, M., Lundebye, A.-K., Hemre, G.I., Waagbø, R., Ørnsrud, R., 2019. Program for overvåking av fiskefôr-Årsrapport for prøver innsamlet i 2018. *Rapport fra havforskningen*.
Søfteland, L., Eide, I., Olsvik, P.A., 2009. Factorial design applied for multiple endpoint toxicity evaluation in Atlantic salmon (Salmo salar L.) hepatocytes. Toxicol. Vitro 23, 1455–1464.
Stanton, R.C., 2012. Glucose-6-phosphate dehydrogenase, NADPH, and cell survival. IUBMB Life 64, 362–369.
Stockwell, B.R., Jiang, X., Gu, W., 2020. Emerging mechanisms and disease relevance of ferroptosis. Trends Cell Biol. 30, 478–490.
Sun, Y., Li, W., Shen, S., Yang, X., Lu, B., Zhang, X., Lu, P., Shen, Y., Ji, J., 2019. Loss of alanine-glyoxylate and serine-pyruvate aminotransferase expression accelerated the progression of hepatocellular carcinoma and predicted poor prognosis. J. Transl. Med. 17, 1–16.
Tang, B.L., 2019. Neuroprotection by glucose-6-phosphate dehydrogenase and the pentose phosphate pathway. J. Cell. Biochem. 120, 14285–14295.
Terman, A., Kurz, T., 2013. Lysosomal iron, iron chelation, and cell death. Antioxidants Redox Signal. 18, 888–898.
Tolosa, J., Font, G., Mañes, J., Ferrer, E., 2017. Mitigation of enniatins in edible fish tissues by thermal processes and identification of degradation products. Food Chem. Toxicol. 101, 67–74.
Tolosa, J., Font, G., Mañes, J., Ferrer, E., 2014. Natural occurrence of emerging Fusarium mycotoxins in feed and fish from aquaculture. J. Agric. Food Chem. 62, 12462–12470.
Tomoda, H., Huang, X.H., Cao, J., Nishida, H., Nagao, R., Okuda, S., Tanaka, H., Omura, S., Arai, H., Inoue, K., 1992. Inhibition of acyl-CoA: cholesterol acyltransferase activity by cyclodepsipeptide antibiotics. J. Antibiot. (Tokyo) 45, 1626–1632.
Tonshin, A.A., Teplova, V.V., Andersson, M.A., Salkinoja-Salonen, M.S., 2010. The Fusarium mycotoxins enniatins and beauvericin cause mitochondrial dysfunction by affecting the mitochondrial volume regulation, oxidative phosphorylation and ion homeostasis. Toxicology 276, 49–57.
Vaclavikova, M., Malachova, A., Veprikova, Z., Dzuman, Z., Zachariasova, M., Hajslova, J., 2013. 'Emerging'mycotoxins in cereals processing chains: changes of enniatins during beer and bread making. Food Chem. 136, 750–757.
Van der meeren, L., Verduijn, J., Krysko, D.V., Skirtach, A.G., 2020. AFM analysis enables differentiation between apoptosis, necroptosis, and ferroptosis in murine cancer cells. iScience 23, 101816.
Williams, D.E., 2012. The rainbow trout liver cancer model: response to environmental chemicals and studies on promotion and chemoprevention. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 155, 121–127.
Wu, G., Fang, Y.-Z., Yang, S., Lupton, J.R., Turner, N.D., 2004. Glutathione metabolism and its implications for health. J. Nutr. 134, 489–492.
Wu, Q., Patocka, J., Nepovimova, E., Kuca, K., 2018. A review on the synthesis and bioactivity aspects of beauvericin, a Fusarium mycotoxin. Front. Pharmacol. 9, 1338.
Xie, Y., Hou, W., Song, X., Yu, Y., Huang, J., Sun, X., Kang, R., Tang, D., 2016. Ferroptosis: process and function. Cell Death Differ. 23, 369–379.
Ytrestøyl, T., Aas, T.S., Åsgård, T., 2015. Utilisation of feed resources in production of Atlantic salmon (Salmo salar) in Norway. Aquaculture 448, 365–374.
Yuan, H., Li, X., Zhang, X., Kang, R., Tang, D., 2016. Identification of ACSL4 as a biomarker and contributor of ferroptosis. Biochem. Biophys. Res. Commun. 478, 1338–1343.
Zhan, X., Long, Y., Zhan, X., Mu, Y., 2017. Consideration of statistical vs. biological significances for omics data-based pathway network analysis. Med One 2.
Zhang, F., Tao, Y., Zhang, Z., Guo, X., An, P., Shen, Y., Wu, Q., Yu, Y., Wang, F., 2012. Metalloreductase Steap3 coordinates the regulation of iron homeostasis and inflammatory responses. Haematologica 97, 1826.
Zhao, L., Feng, Y., Xu, Z.-J., Zhang, N.-Y., Zhang, W.-P., Khalil, M.M., Sun, L.-H., 2021. Selenium mitigated aflatoxin B1-induced cardiotoxicity with potential regulation of 4 selenoproteins and ferroptosis signaling in chicks. Food Chem. Toxicol. 112320.
Zhao, M., Shi, J., Zhao, Z., Zhou, D., Dong, S., 2018. Enhancing chlorophenol biodegradation: using a co-substrate strategy to resist photo-H2O2 stress in a photocatalytic-biological reactor. Chem. Eng. J. 352, 255–261.

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# Enniatin B and beauvericin affect intestinal cell function and hematological processes in Atlantic salmon (*Salmo salar*) after acute exposure

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ABSTRACT

Unintentional use of mold-infested plant-based feed ingredients are sources of mycotoxins in fish feeds. The presence of the emerging mycotoxins ENNB and BEA in Norwegian commercial fish feeds and plant-based feed ingredients has raised concerns regarding the health effects on farmed Atlantic salmon (*Salmon salar*). Atlantic salmon pre-smolts were exposed to non-lethal doses of BEA and ENNB (ctrl, 50 and 500 μg/kg feed for 12 h), after which total RNA sequencing of the intestine and liver was carried out to evaluate gut health and identify possible hepatological changes after acute dietary exposure. ENNB and BEA did not trigger acute toxicity, however ENNB caused the onset of pathways linked to acute intestinal inflammation and BEA exposures caused the onset of hepatic hematological disruption. The prevalence and concentration of ENNB found in today's commercial feed could affect the fish health if consumed over a longer time-period.

## 1. Introduction

By the early 2010s, a substantial part of the fishmeal and fish oil used in traditional Norwegian salmon feed had been replaced with meal and oil of plant origin, to make the feed more ecologically and economically sustainable (Ytrestøyl et al., 2015). However, plant-based feed ingredients have introduced mycotoxins into marine farmed fish, through the unintentional use of mold-infested cereal grains and pulse crops that are used for production of the meal- and oil feed ingredients. Ingestion of mycotoxins by terrestrial livestock, as well as humans, have resulted in mycotoxicosis and even death (Hussein and Brasel, 2001). Some terrestrial livestock species (e.g., pig) are more sensitive to specific mycotoxins (e.g. deoxynivalenol (DON)) than other livestock species (e.g., ruminants) (Bernhoft et al., 2013a). The use of novel plant-based feed ingredients in marine aquafeeds has also raised concerns regarding the health effects on marine farmed fish species, such as Atlantic salmon (*Salmo salar*), which have not previously been exposed to the terrestrial plant associated mycotoxins (Bernhoft et al., 2013a). Atlantic salmon appears to better tolerate ochratoxin A (OTA) and zearalenone (ZEA), while being rather sensitive to DON exposure (Bernhoft et al., 2018; Moldal et al., 2018; Döll et al., 2010). Several reviews have reported the prevalence of mycotoxins in aquafeed ingredients, aquafeeds and/or transfer to tissues of European farmed fish species (Pietsch, 2020; Tolosa et al., 2021; Bernhoft et al., 2013b, 2017; Nacher-Mestre et al., 2015). As in terrestrial livestock feeds, DON and fumonisins (FBs) were the most prevalent mycotoxins in plant-based aquafeeds used for the two major marine farmed fish species in Europe (Atlantic salmon and gilthead seabream) (Nacher-Mestre et al., 2015).

More recently, the emerging mycotoxins beauvericin (BEA) and enniatin B (ENNB) have been reported in European marine aquafeeds, exhibiting a 100% prevalence in all tested feed samples (Nacher-Mestre et al., 2020; Tolosa et al., 2014). In an *in vitro* study, exposing Atlantic salmon hepatocyte to BEA and ENNB, reduced mitochondrial metabolic activity and altered cellular iron homeostasis were seen, implying that mitochondrial iron retention might have been impaired (Søderstrøm et al., 2022). However, the *in vitro* data on BEA and ENNB toxicity is still limited for fish, and to our knowledge the toxic mode of action and acute effect dose of BEA and ENNB *in vivo* have not been studied in farmed fish species, including Atlantic salmon. Currently, both BEA and ENNB remain non-regulated in the EU feed and food legislation (Lindblad et al., 2013; Bernhoft et al., 2013a; Vaclavikova et al., 2013), as opposed to more well-studied mycotoxins such as DON, FBs, and trichothecenes (e.g., T-2 HT-2) for which guidance values have been established for animal feed including fish feed (Cheli et al., 2014; Pinotti et al., 2016).

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Insufficient toxicity data for establishing tolerable maximum levels of BEA and ENNB (Chain, 2014), highlights the need for more knowledge regarding their potential adverse effects on fish health.

To identify relevant concentrations and prevalence, BEA and ENNB in addition to the mycotoxins aflatoxins (AFs), DON, FBs, T-2 and HT-2, OTA, and ZEA, were analyzed in commercial Norwegian salmon feeds collected from 2015 to 2021 through the National monitoring program for fish feed. Furthermore, a single-dose (one low and one high concentration) gavage feeding trial with Atlantic salmon was carried out to assess the potential acute toxicity of BEA and ENNB using transcriptomic screening. Since the gastrointestinal (GI) tract represents the first barrier after oral exposure, and the liver represents the major detoxification organ after intestinal absorptions, non-targeted intestinal and liver transcriptomics were used to identify dose-responses in the salmon defensome to dietary BEA and ENNB exposure.

## 2. Materials and methods

### 2.1. Fish feed samples

Data on mycotoxins in fish feed and feed ingredients were collected through the ongoing National monitoring program of fish feed in Norway, led by the Norwegian Food Safety Authority (NFSA). Commercial fish feeds and feed ingredients were collected annually from authorized Norwegian feed production sites, with sampling randomized with regards to season and location. The samples were collected by inspectors from the NFSA and transported to the Institute of Marine Research (IMR) for data analysis and reporting. In total, 78 plant-based meal and 51 plant-based oil intended for fish feed production, and 245 fish feeds were collected and analyzed for mycotoxins for the years 2015–2021. The plant-based meal ingredients composed of soy protein concentrates (SPC, n = 43), wheat gluten (n = 9), pea protein (n = 5), guar protein (n = 5), faba beans (n = 4), corn gluten (n = 4), sun flour (n = 4), soybean meal (n = 3), and unspecified plant protein meal (n = 1). The plant-based oils composed of rapeseed oil (n = 41), linseed oil (n = 1), camelina oil (n = 1), a blend of rapeseed and linseed oil (n = 1), whereas some of the oils were of unspecified plant origin (n = 7). The data were compiled from the IMR-generated reports Sanden et al. (2017), Sele et al. (2018), Sele et al. (2019), Ørnsrud et al. (2020), Sele et al. (2021).

#### 2.1.1. Analyzes of mycotoxins in feed and feed ingredients

The following mycotoxins were determined; aflatoxin B1, B2, G1, G2, (AFBs), deoxynivalenol (DON), fumonisins (FB1 and FB2), T-2 toxin (T-2), HT-2 toxin (HT-2), ochratoxin A (OTA), zearalenone (ZEA), beauvericin (BEA) and enniatin A, A1, B, B1) (ENNs). BEA and ENNs (A, A1, B, B1) were extracted with ACN/H20, SPE Clean-up, and determined using LC-MS/MS. DON, FBs, HT-2, T-2, and ZEA were also determined using LC-MS/MS, while AFBs and OTA were analyzed by HPLC and fluorescence detection. The limit of quantification (LOQs, in μg/kg) were AFBs <0.1/1.0, DON <10–20, FFBs <10–20, H2 < 10–20, OTA <0.1, T2 < 10, ZEA <10, ENNs <10, BEA <10. Feed and feed ingredient samples analyses were carried out by Eurofins WEJ Contaminants GmbH (Hamburg), using accredited methods (accreditation number D-PL-14602-01-00 and D-PL-14198-01-00). The prevalence (x%) of mycotoxins was calculated by the following equation:

$$\text{Prevalence}(\%) = \frac{\textit{Number of samples over LOQ}}{\textit{Number of samples analysed}} \bullet 100\%$$

The concentrations of ENNB and BEA chosen for the dietary exposure trial with Atlantic salmon were based on results from the monitoring program.

### 2.2. Fish and husbandry conditions

The feeding trial was carried out at the Institute of Marine Research's (IMR) facility at Matre (North of Bergen, Norway) between the 28th and 30th of January 2020. On-site farmed, freshwater-adapted pre-smolt weighing between 58 and 108 g (average 74 g) (female:male ratio (14:17)) were kept in flow-through tanks (32 tanks, 1 fish in each), in standard freshwater oxygen condition, 10 ± 0.2 °C, with a 12:12 h light/dark cycle regime. The fish was acclimatized for one month prior to the trial before they were placed into separate tanks and fed daily with standard commercial feed pellets through a disk feeder until 24 h prior to the start of the trial.

### 2.3. Mycotoxin dosing, feed paste preparation, and sampling

The gavage feeding trial was designed as a single-dose exposure experiment testing for non-lethal responses 12 h after the oral administration. The mycotoxins, beauvericin (BEA, cas 26048-05-5) and enniatin B (ENNB, cas 917-13-5), were purchased from AdipoGen® (AdipoGen® Life Sciences, Nordic BioSite, Oslo, Norway). Five different exposure treatments (including control) were administrated, and six biological replicates (n) were used for each treatment test group (Table 1).

The fish were gavage-fed based on the protocol from a previous study (Amlund and Berntssen, 2004). Mycotoxin-supplemented feed pastes where made the day before the trial. BEA and ENNB were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Oslo, Norway) to create two stock solutions of each mycotoxin (stock solution No. 1 = 1 mg/mL, and a stock solution No. 2 = 0.1 mg/mL). Stock solution No. 1 was diluted in the water fraction of the feed paste before being mixed with freshly grinded commercial fish feed in a 2:1 ration to constitute the "high exposure" [500 μg/kg feed], and in the same way stock solution No 2 was used to create the "low exposure" [50 μg/kg feed] feed pastes of BEA and ENNB. The control feed paste was prepared with 0.075% DMSO, equivalent to the concentrations used in the prepared BEA and ENNB feed pastes. The pastes were then pre-loaded into 1 mL syringes with cut off tips and smooth-polished edges and stored inside zip bags at 4 °C until the next day. Prior to administration, the fish were starved for one day to assure empty gastrointestinal tracts. The fish were sedated in 1 g base and 1 g Finquel MS-222 (Tricaine Methanesulfonate) per 10 L tank water before weight and length were recorded. The amount of feed paste administrated was corrected for the weight of the fish and corresponded to 0.85% of their body weight. The 1 mL syringe containing the treatment feed paste was inserted through the mouth and carefully pushed to the posterior end of the stomach, and then slowly retracted meanwhile gently pushing out the feed paste with the plunger. The fish were left to recover in a separate container with tank water, while assuring no regurgitation occurred, before being transferred back to their experimental tanks. One fish escaped from the tank and died during the trial. Exactly 12 h post gavage feeding, the fish were euthanized in 5 g Finquel MS-222 per 10 L tank water. Subsequently, liver- and intestinal tissue samples were dissected, collected, and flash frozen in liquid nitrogen, and stored at −80 °C until further processing for RNA extraction. This *in vivo* gavage feeding trial was approved and carried out in compliance with the current national *animal welfare act - the regulation on animal experimentation* approved by the Norwegian Animal Research Authority and overseen by the Norwegian Food Safety Authority (FOTS ID 21570).

**Table 1**
Experimental design of exposure treatments with treatment groups, nominal concentration (μg/kg feed) and number of fish in each group.

| # | Treatment | Nominal concentration (μg/kg feed) | No. of fish (n) |
|---|---|---|---|
| 1 | Control | – | 6 |
| 2 | BEA-low | 50 | 5[a] |
| 3 | BEA-high | 500 | 6 |
| 4 | ENNB-low | 50 | 6 |
| 5 | ENNB-high | 500 | 6 |

[a] One fish died so n = 5 for this group.

### 2.4. RNA extraction and sequencing

Total RNA was extracted from the distal intestine and liver tissues sampled using QIAzol® Lysis Reagent and the EZ1® RNA Tissue Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. Frozen liver tissue (50 mg), or intestinal tissue (100 mg), was homogenized in 750 μL QIAzol at 6000 rpm for 3 × 15 s in an Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Total RNA was collected and purified using an BioRobot EZ1 Nucleic Acid Purification System (Qiagen) running the program: Total RNA > universal tissue > including DNase. Purified RNA was eluted in 50 μL RNase-free MilliQ $H_2O$ and stored at −80 °C until downstream application. The RNA quantity and purity of all samples (mean ± STD) were measured spectrophotometrically (NanoDrop™ One Microvolume UV–Vis Spectrophotometer, Thermo Fisher Scientific™, Waltham, MA, USA). The A260/A280 and A260/A230 nm ratios for RNA extracted from intestine were 2.09 ± 0.01 and 2.26 ± 0.02, and from liver 2.10 ± 0.24 and 2.16 ± 0.02, respectively. RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) in combination with RNA 6000 Nano LabChips. The RNA integrity numbers (RIN) were 9.02 ± 0.50 for intestine and 9.08 ± 0.24 for liver (indicating non-degraded RNA) for all samples intended for RNA sequencing. Total RNA samples were sent to Novogene Europe, Cambridge, UK, for cDNA library preparation and sequencing using the Illumina NovaSeq 6000 platform for 150 bp paired end reads. Enriched mRNA was prepared using Oligo(dT beads). cDNA libraries were prepared following manufacturer's instructions per the Novogen pipeline (Novogene Europe, Cambridge, UK). A total of 58 libraries were generated, 29 for intestine and 29 for liver comprising all five exposure groups, n = 6 (5 in BEA-low) per experimental treatment. Each library contained an average of 51 ± 8 million reads. Raw reads were submitted to the gene expression omnibus https://www.ncbi.nlm.nih.gov/geo/(GSE213817).

### 2.5. Differential gene expression

TrimGalore 0.4.2 wrapper tool (https://github.com/FelixKrueger/TrimGalore) was used for removing adaptors and quality trimming, applying the default parameters. Library quality was investigated using fastQC included in the TrimGalore wrapper.

Individual libraries were mapped to the Atlantic salmon genome (RefSeq Assembly ICSASG_v2) using the Hisat2 short read aligner version 2.0.4 (Kim et al., 2015) and the Atlantic salmon NCBI gene annotation file (Salmon_salar, 24/01/2017 GCA_000233375.4_ICSASG_v2_genomic.gff). Transcript levels for the individual libraries were estimated using FeatureCounts (Liao et al., 2014) of the Subread package (http://subread.sourceforge.net/). Read counts were further normalized using Bioconductor R package (version 3.4.4) DESeq2 (version 1.18.1) (Love et al., 2014). Genes of which fewer than 5 samples had gene counts below or equal to 20 reads were excluded from further analysis prior to normalization. DESeq2 was further used for analysis of differentially expressed genes (DEGs) in a pair-wise comparison of each contrast (Ctrl vs BEA-low, Ctrl vs BEA-high, Ctrl vs ENNB-low, Ctrl vs ENNB-high). Venn diagrams were created with EVenn (http://www.ehbio.com/test/venn/#/) (Chen et al., 2021).

### 2.6. Functional annotation clustering analysis and pathway analysis

Functional annotation clusters enriched by the identified DEGs were generated using the Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis tool (https://david.ncifcrf.gov) (Huang et al., 2009). In short, the salmon NCBI gene ID's of DEG-names were given their human ortholog official gene symbol to generate a gene list. Subsequent analyses were done separately for DEGs with positive and negative fold change (FC) values compared to the control from each exposure group with the following settings: Functional Annotations (KW: disease (DIS), biological process (BP), molecular function (MF)), Gene Ontology (GOTERM: BP, MF). The cutoff criteria for the input data were set to p-adjusted <0.2 (Benjamini and Hochberg correction) and gene count ≥2, and functional annotation clusters were considered significant when Benjamini was <0.1. Pathway analyses were carried out using QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, (www.qiagen.com/ingenuity). The cutoff criteria for the input data were set to p-adjusted <0.2 (Benjamini and Hochberg correction), canonical pathway analysis, disease and biological function analysis and prediction of upstream regulator analysis were conducted. While both canonical pathway analysis and biological function analysis predict affected biological events, the upstream regulator analysis identifies the cascade of upstream transcriptional regulators that can explain the observed gene expression changes. IPA results were filtered using p-adjusted <0.05 (Fisher exact test) and a Z-score >2. The p-value filtering reflects the likelihood of a pathway/function being significantly enriched by the treatments; the Z-score designates the likelihood of a directional association in the dataset. Positive and negative Z-scores imply an increase or decrease in activity/activation of the enriched pathway, respectively.

## 3. Results

### 3.1. BEA and ENNB prevalence in Norwegian salmon feed and feed ingredients

Of the feed samples analyzed and above the LOQ, ENNB had the highest prevalence (80%) compared to the other mycotoxins analyzed (i.e., Afs, DON, FB1, FB2, T-2, HT-2, OTA, ZEA) while BEA occurred to a lesser extent (4%), with concentrations up to 250 μg/kg feed for ENNB and 25 μg/kg feed for BEA (Table 2). The highest concentrations were seen for FB1 and FB2, with approximately 30-50-fold higher concentrations than that of ENNB in fish feed (Table 2). In feed ingredients, plant-based protein meals showed a prevalence of 12% for BEA, and 15% of the samples contained ENNB above the LOQ (Table 3), with concentrations up to 2400 μg/kg for BEA, and 530 μg/kg meal for ENNB (Table 3). While the occurrence of ENNB prevailed over BEA, the concentration of BEA exceeded that of ENNB in plant ingredients, though the concentrations of FB1, FB2, and ZEA largely exceeded that of both BEA and ENNB (Table 3). Specifically, none of the plant-based protein meal of SPC contained BEA or ENNB above the LOQ. BEA were detected in guar meal (12–18 μg/kg, n = 3), wheat gluten/meal (11 μg/kg, n = 1)

**Table 2**
**Fish feed**. The prevalence (%) and concentrations (mean and standard deviation, μg/kg feed) and range (minimum to maximum concentrations, μg/kg) of mycotoxins in fish feed for the years 2015–2021, with the number of samples analyzed (N).

| Mycotoxin | Prevalence (%)[a)] | Mean ± SD (μg/kg) | Range (μg/kg) | N |
|---|---|---|---|---|
| Aflatoxin B1 | <1 | < LOQ | 0.1 | 245 |
| Aflatoxin B2 | n.d. | < LOQ | | 244 |
| Aflatoxin G1 | n.d. | < LOQ | | 244 |
| Aflatoxin G2 | n.d. | < LOQ | | 244 |
| Deoxynivalenol | 22 | 37 ± 18 | 20–99 | 245 |
| Fumonisin B1 | 8 | 205 ± 140 | 32–620 | 245 |
| Fumonisin B2 | 8 | 104 ± 72 | 30–280 | 245 |
| T-2 Toxin | <1 | < LOQ | 10 | 245 |
| HT-2 Toxin | <1 | < LOQ | 12 | 245 |
| Ochratoxin A | 2 | 1.6 ± 0.7 | 1.0–2.4 | 245 |
| Zearalenone | 11 | 74 ± 55 | 13–290 | 245 |
| Beauvericin | 4 | 16 ± 5 | 10–25 | 200 |
| Enniatin A | 0.5 | < LOQ | 11 | 200 |
| Enniatin A1 | 2 | 12 ± 27 | 10–16 | 200 |
| Enniatin B | 80 | 37 ± 35 | 10–250 | 200 |
| Enniatin B1 | 27 | 18 ± 9 | 10–54 | 200 |

n.d. = non-detected.
[a)] The number of samples above the LOQ of the total number of samples analyzed, in percentage.

**Table 3**
**Plant proteins**. The prevalence (%) and concentrations (mean and standard deviation (μg/kg) and the range: minimum to maximum concentrations, μg/kg) of mycotoxins in plant proteins intended for fish feed production. Data retrieved from the Norwegian monitoring program for fish feed (for the years 2015–2021), and the number of samples analyzed (N).

| Mycotoxin | Prevalence (%)[a)] | Mean ± SD (μg/kg) | Range (μg/kg) | N |
|---|---|---|---|---|
| Aflatoxin B1 | 10 | 4.7 ± 5.8 | 0.7–18 | 77 |
| Aflatoxin B2 | 5 | 0.9 ± 0.6 | 0.3–1.6 | 77 |
| Aflatoxin G1 | n.d. | < LOQ | | 77 |
| Aflatoxin G2 | n.d. | < LOQ | | 77 |
| Deoxynivalenol | 12 | 447 ± 406 | 35–1000 | 77 |
| Fumonisin B1 | 6 | 6825 ± 5165 | 25–12000 | 77 |
| Fumonisin B2 | 6 | 4084 ± 3350 | 21–8300 | 77 |
| T-2 Toxin | 1 | < LOQ | 30 | 77 |
| HT-2 Toxin | 3 | 10.1 ± 1.1 | 10–20 | 77 |
| Ochratoxin A | 19 | 2.0 ± 1.3 | 0.5–4 | 77 |
| Zearalenone | 10 | 1107 ± 1573 | 13–4470 | 77 |
| Beauvericin | 12 | 416 ± 814 | 11–2400 | 67 |
| Enniatin A | 1 | < LOQ | 90 | 67 |
| Enniatin A1 | 7 | 56 ± 49 | 15–140 | 67 |
| Enniatin B | 15 | 135 ± 186 | 11–530 | 67 |
| Enniatin B1 | 10 | 78 ± 63 | 16–190 | 67 |

[a)] The number of samples above the LOQ of the total number of samples analyzed (n.d. = non-detected).

and with the highest concentrations (220–2400 μg/kg, n = 4) in corn meal. ENNB was detected in samples of pea meal (11 μg/kg, n = 1), corn meal (11–130 μg/kg, n = 2), and more frequently in samples of wheat gluten/meal (13–530 μg/kg, n = 7) (Fig. 1). Of the plant-based oil samples analyzed, 10% of the samples contained BEA, and 88% of the samples contained ENNB above the LOQ (Table 4), with concentrations up to 24 μg/kg oil for BEA, and 450 μg/kg oil for ENNB.

### 3.2. Transcriptional effects of BEA and ENNB

Venn diagrams depicting the individual and overlapping differently expressed genes (DEGs) in the two different tissue types (Fig. 2A–D) resulting from the mycotoxin exposures. Low dose exposure to both BEA and ENNB resulted in a higher number of DEGs compared to the high doses in the intestine (Fig. 2, A, C). While in the liver tissue, the high dose of BEA resulted in 156 more DEGs than in its low dose (Fig. 2, B). BEA caused more transcriptional effects in the liver compared to the intestine (Fig. 2A and B) while ENNB primarily affected the transcription in the intestine, showing far less transcriptional effects in the liver (Fig. 2C and D).

The homeobox (Hox) genes were among the top upregulated DEGs in

**Table 4**
**Plant oil**. The prevalence (%) and concentrations (mean and standard deviation (μg/kg) and the range: minimum to maximum concentrations, μg/kg) of mycotoxins in plant oil intended for fish feed production, data retrieved from the Norwegian monitoring program for fish feed (for years 2015–2021), and the number of samples analyzed (N).

| Mycotoxin | Prevalence (%)[a)] | Mean ± SD (μg/kg) | Range (μg/kg) | N |
|---|---|---|---|---|
| Beauvericin | 10 | 16 ± 5 | 10–24 | 51 |
| Enniatin A | 10 | 20 ± 12 | 10–38 | 51 |
| Enniatin A1 | 29 | 22 ± 7 | 11–37 | 51 |
| Enniatin B | 88 | 114 ± 119 | 12–450 | 51 |
| Enniatin B1 | 65 | 38 ± 27 | 10–110 | 51 |

[a)] The number of samples above the LOQ of the total number of samples analyzed.

intestinal tissue exposed to BEA and ENNB (see Supplementary DEG list), of which *hoxa11ab* was represented in all exposure groups, and *hoxa9* and *hoxa7aa* in all but ENNB-low (Fig. 3, A). Several DEGs of cytochrome p450 (CYPs) phase I biotransformation enzymes exhibited increased expression following oral exposure to BEA and ENNB (Fig. 3, B, Supplementary DEG list). BEA-low increased the expression of the *cyp3a27* in the intestine and *cyp1a* in the liver, while ENNB increased the expression of the *cyp2k1* in the intestine (Fig. 3, B).

### 3.3. Common DEGs between annotations

The identified DEGs from each exposure group underwent a UniprotKB Keywords (KW) and Gene ontology (GO) functional enrichment annotation clustering analysis using the DAVID tool. The KW and GO clusters are comprised of the categories termed "disease" (DISs), "biological process" (BPs), and "molecular function" (MFs). In the intestine, the analysis revealed four annotation clusters in the low dose of BEA, and one annotation cluster in the high dose of BEA (Fig. 4). The low dose of ENNB resulted in three annotation clusters in the intestine, while the high dose did not result in any significant annotation clustering (Fig. 4). The top clusters in BEA-low contained downregulated DEGs connected to cell cycle and division, and upregulated DEGs related to developmental proteins (including many homeobox genes) and transcription in both BEA-low and BEA-high (Fig. 4). In the low dose of ENNB, the top cluster based on downregulated DEGs was associated with inflammatory response, followed by kinase activity (most significant) and apoptosis (Fig. 4). The cluster based on upregulated DEGs was related to molecular functions in the extracellular matrix (Fig. 4). ENNB-high did not significantly enrich any clusters.

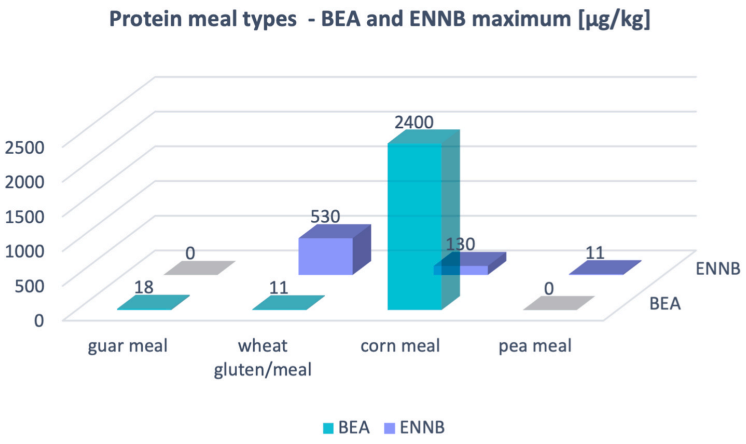


**Fig. 1. Plant protein meals**. Maximum concentrations (μg/kg) of BEA and ENNB measured in different protein meals: soy protein concentrate (SPC), guar meal, wheat gluten meal, corn meal, and pea meal. None of the SPC contained BEA or ENNB above the LOQ (n = 0 of 43); BEA was detected in guar (12–18 μg/kg, n = 3 of 5), wheat gluten (11 μg/kg, n = 1 of 9), corn (220–2400 μg/kg, n = 4 of 4); ENNB was detected in pea (11 μg/kg, n = 1 of 5), corn (11–130 μg/kg, n = 2 of 4), and wheat gluten (13–530 μg/kg, n = 7 of 9).

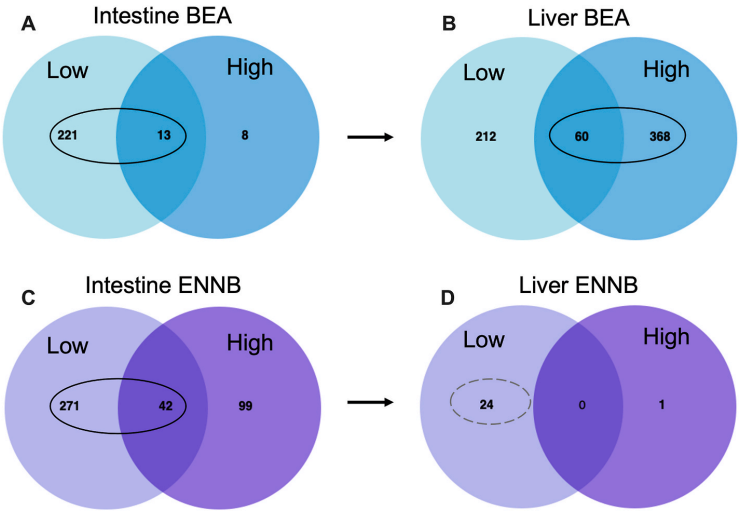


**Fig. 2.** Venn diagrams depicting the individual and overlapping differentially expressed genes (DEGs) identified in exposure groups of low and high doses of BEA in (A) intestine and (B) liver, as well as low and high doses of ENNB in (C) intestine and (D) liver. n = 6 (BEA-low n = 5), q < 0.2, fold change >1.0.

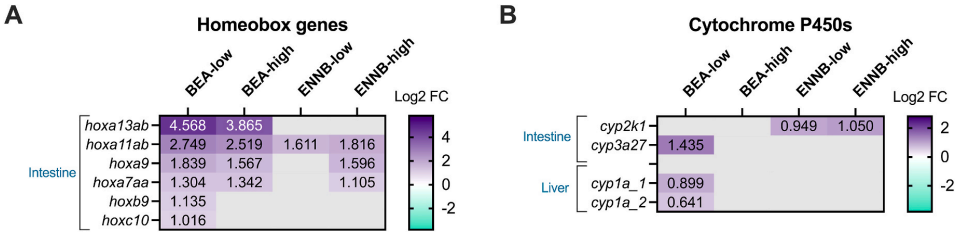


**Fig. 3.** Heatmaps of differentially expressed genes (DEGs) belonging to (A) homeobox genes, and (B) cytochrome P450 genes resulting from exposure to low and high doses of BEA and ENNB in intestine and liver (n = 6 (BEA-low n = 5), q < 0.2, fold change >1.0).

Only BEA resulted in a sufficient number of regulated DEGs for functional annotation clustering analysis in the liver. Low and high dose oral exposure to BEA resulted in seven annotated clusters (Fig. 5). For BEA-low, the most significant cluster based on DEGs with a negative fold change (FC) was related to hemolytic anemia, followed by actin-binding and detoxification (Fig. 5). In BEA-high, the most significant clusters were associated with the translation machinery (positive FC), and transcription factor activity (negative FC) (Fig. 5).

### 3.4. Pathway analysis

Ingenuity Pathway Analysis (IPA) of the Atlantic salmon intestine and liver transcriptome were carried out to further elucidate how the DEGs from the low- and high dose of BEA and ENNB translated into biological responses. The analysis of the intestine predicted ENNB-low to downregulate two canonical pathways (e.g., Th2 pathway) and BEA-low downregulated the cell cycle control of the chromosomal replication pathway, while the high doses did not show any effect in this category (Fig. 6, A). In the categories toxicity (Tox), disease and biological function in the intestine, ENNB-low was predicted to downregulate 34 and upregulate 6 pathways, ENNB-high to downregulate 8 and upregulate 2, BEA-low to downregulate 9 and upregulate 2, and BEA-high to downregulate 2 pathways (Fig. 6, B). Notably, both the low and high dose of ENNB upregulated organ inflammation, though the effect in the low dose appeared more pronounced since it also predicted upregulation of immune mediated inflammatory disease, while BEA-low mainly upregulated necrosis (Fig. 6, B). In the category of genes in the immune mediated inflammation/inflammation of organ pathways, ENNB-low exerted most transcriptional effect of the different exposure groups, where haptoglobin (*hp*) was the most upregulated DEG followed by integrin alpha 9 (*itga9*), protein kinase C alpha (*prkca*), while the most downregulated DEG was regenerating protein 1 alpha (*reg1a*) (Fig. 6, C). A similar effect on *prkca* and *reg1a* was observed following exposure to BEA-low (Fig. 6, C).

The liver ENNB-high group was excluded from the IPA since it only had 1 DEG. The analysis of gene expression in the salmon liver tissue predicted that both the low and the high dose exposure to BEA downregulated 4 canonical pathways, while ENNB-low showed no effect on such pathways (Fig. 6, A). In the category toxicity, disease and biological function in the liver, ENNB-low was predicted to downregulate 1 pathway, BEA-low to downregulate 16 and upregulate 5 pathways, while BEA-high downregulated 10 and upregulated 3 pathways (Fig. 7, B). Of note, while both the low and the high dose of BEA were predicted to downregulated hematocrit levels, both doses also upregulated anemia (Fig. 7, B). In addition, BEA-low was predicted to increase inflammatory response, cell death of immune cells, quantity of reticulocytes (immature red blood cells), and cell death of blood cells (Fig. 7, B). Processes related to proliferation of hematopoietic progenitor cells and erythroid precursor generation were among the pathways predicted to be downregulated by BEA-low (Fig. 7, B). In the category of upstream regulators

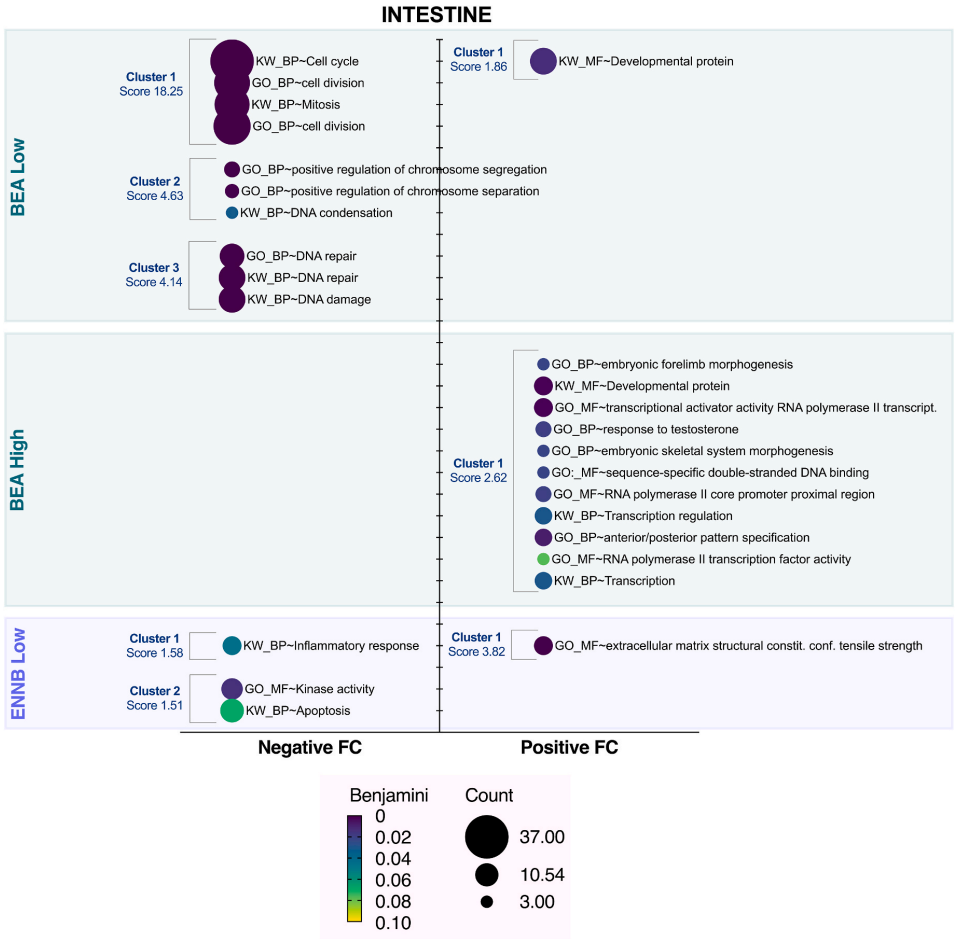


**Fig. 4.** Functional annotation clustering of differentially expressed gene (DEG) counts in the intestine. KW and GO clustering comprised of the categories disease (DIS), biological process (BP), and molecular function (MF) signified by up- and downregulated DEGs shown on the X-axis for each exposure group (A) BEA-low, (B) BEA-high, (C) ENNB-low, and (D) ENNB-high. The fill colors from yellow to purple denote the Benjamini (significant <0.1), the closer to purple the higher the significance. Bubble sizes depict the count of attributed DEGs in each cluster. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

in the liver, the GATA binding protein 1 (*gata1*) transcription factor gene was predicted to be downregulated by both the low and high dose of BEA and by ENNB-low, as were erythropoietin (*epo*) and interferon gamma (*ifng*) (Fig. 7, C). Both the low and the high dose of BEA upregulated the upstream regulators stearoyl-CoA desaturase (*scd*), lysosomal-associated membrane protein 2 (*lamp2*), dexamethasone, and glucocorticoid (in addition to the pharmaceuticals decitabine and budesonide) (Fig. 7, C). More specifically, BEA-low, BEA-high, and ENNB-low affected the genes involved in the hematocrit pathway that was indicated to be decreased (Fig. 7, B). Transcription of the CCAAT Enhancer Binding Protein Alpha (*cebpa*) and cytochrome P450 family 1 subfamily A member 1 (*cyp1a1*) was slightly increased, while and *gata1* and dematin actin binding protein (*dmtn*) were decreased in two and all three exposure groups, respectively (Fig. 7, D). The most downregulated gene was heme oxygenase 1 gene (*hmox1*) in the BEA-low exposure group (Fig. 7, D).

Since *gata1* was represented both on the upstream regulator category (Fig. 7, C), as well as in the hematocrit pathway (Fig. 7, D), the GATA binding protein 1 (GATA1) pathway was also signaled as significantly affected (Fig. 8, A). ENNB-low had the least effect and primarily downregulated predicted genes (Fig. 8, A). Notably, despite that ENNB-low only resulted in 24 DEGs in the liver (Fig. 2, D) five of these were represented in the GATA1 pathway. BEA-low had most effect and downregulated 15 and upregulated 5 genes, finally BEA-high affected 6 genes 3 were downregulated and 3 were upregulated (Fig. 8, A). Evident from the Ferris wheel plot (Fig. 8, B) showing how BEA-low affected genes downstream of GATA1, the mitochondrial 5-aminolevulinate synthase (*alas2*) was also predicted to be downregulated (Fig. 8, B).

## 4. Discussion

Evaluation of the prevalence and concentrations of mycotoxins in plant-based feed ingredients and Norwegian fish feeds identified ENNB as one of the most prevalent mycotoxins, and BEA to a lesser extent. To assess whether ENNB and BEA pose a concern to fish health, an acute

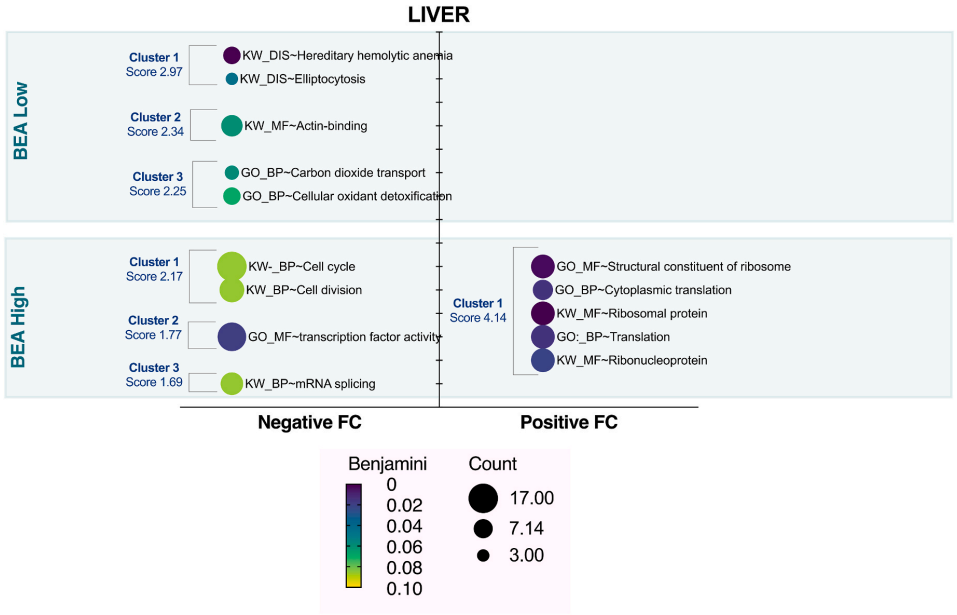


**Fig. 5.** Functional annotation clustering of differentially expressed gene (DEG) counts in the liver. Clustering comprised of CCs, BPs, and MFs signified by up- and downregulated DEGs shown on the X-axis for each exposure group (A) BEA-low, and (B) BEA-high. The fill colors from yellow to purple denote the Benjamini (significant <0.1), the closer to purple the higher the significance. Bubble sizes depict the count of attributed DEGs in each cluster. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

gavage feeding trail on Atlantic salmon was performed. To fully understand the complex xenobiotic defensome and stressor responses occurring in teleost (Eide et al., 2021), a non-targeted toxicological evaluation of the intestines and livers following short-term dietary exposure to BEA and ENNB were performed to map the initial transcriptomic response.

### 4.1. BEA and ENNB prevalence in Norwegian salmon feed (2015–2021)

Analysis of salmon feed and feed ingredients showed higher prevalence and concentrations of ENNB in comparison to other emerging mycotoxins such as other ENNs and BEA. Although the concentration of ENNB was lower than FBs and ZEA in some samples, the prevalence of ENNB in the surveyed salmon feeds was substantially higher than the more routinely monitored mycotoxins, i.e., $AFB_1$, FBs, HT-2, T-2, OTA, ZEA, and DON for which recommended or maximum levels in feed and commodities have been established by the European Commission (Cheli et al., 2014; Pinotti et al., 2016; EC, 2016). Analyses of plant-based feed ingredients indicated that rapeseed oil and wheat- and corn gluten were major sources of ENNB, and that corn gluten was the primary source of BEA. In a previous survey on Sea bream (*Sparus aurata*) and Seabass (*Dicentrarchus labrax*) aquafeeds from 2014, ENNB and BEA had a 100% prevalence with mean occurrence levels of 0.89 and 1.4 μg/kg (0.1–3.2 μg/kg and 0.1–6.6 μg/kg, min max, n = 20), respectively (Tolosa et al., 2014). In a more recent study on Atlantic salmon and Sea bream plant-based feeds all twenty surveyed feeds had detectable levels of BEA and ENNB, with the highest concentrations being 80.4 and 32.8 μg kg, respectively (Nacher-Mestre et al., 2020). In agreement with these surveys, ENNB showed a high prevalence in Norwegian fish feeds and feed ingredients surveyed in 2015–2020, and the concentrations of ENNB in fish feed were consistently higher than BEA (250 and 25 μg/kg, respectively). In plant-based feed ingredients, however, BEA was occasionally measured with extreme concentrations in corn meal.

### 4.2. Difference in BEA and ENNB intestinal-hepatic transcriptomic responses

In the current feeding trial with Atlantic salmon pre-smolt, the low environmentally relevant doses (50 μg/kg feed) of both BEA and ENNB caused a higher number of DEGs in the intestine than the non-lethal high doses (500 μg/kg feed). That fewer DEGs were observed in the high dose of BEA and ENNB could indicate that the toxic reactions are overcoming the adaptive transcriptional stress responses in the intestine. Similarly in an *in vitro* study, Jurkat cells exposed to three concentrations (1.5, 3, and 5 μM) of ENNB exhibited the highest number of DEGs in the mid-concentration (Alonso-Garrido et al., 2018). The authors suggested two hypotheses as to why the highest number of DEGs was observed in the mid-concentration, either due to time-dependent transcriptomic damage or that the toxicity of the highest concentration of ENNB was blocking it from entering the cells. However, while exposing the same cell line to BEA the number of DEGs increased with increasing concentration (Escrivá et al., 2018). The transcriptional effects following exposure to ENNB mainly occurred in the intestine while limited effects were observed in the liver. In comparison, BEA affected the liver transcriptome to a larger degree than the intestinal. Previous *in vivo* and *in vitro* studies on rodents indicated enterohepatic recycling with increased tissue levels of BEA, while the metabolism of ENNB occurred both in the intestine and liver in rodents (Rodríguez-Carrasco et al., 2016; Fæste et al., 2011; Ivanova et al., 2011). In the present *in vivo* study, ENNB increased the expression of *cyp2k1* in the intestine, while BEA increased the expression of *cyp3a27* in the intestine and *cyp1a* in the liver. This is in line with a previous salmon hepatocyte *in vitro* study, were ENNB affected the transcription of *cyp2k1*, while BEA affected *cyp3a27* and *cyp1a* transcription (Søderstrøm et al., 2022) (Supplementary DEG list). Phase I CYP enzymes have been reported to be important for biotransformation of ENNB in human microsomes (Fæste et al., 2011). In fish, cyp2k1 is primarily expressed in the digestive tract where it has been

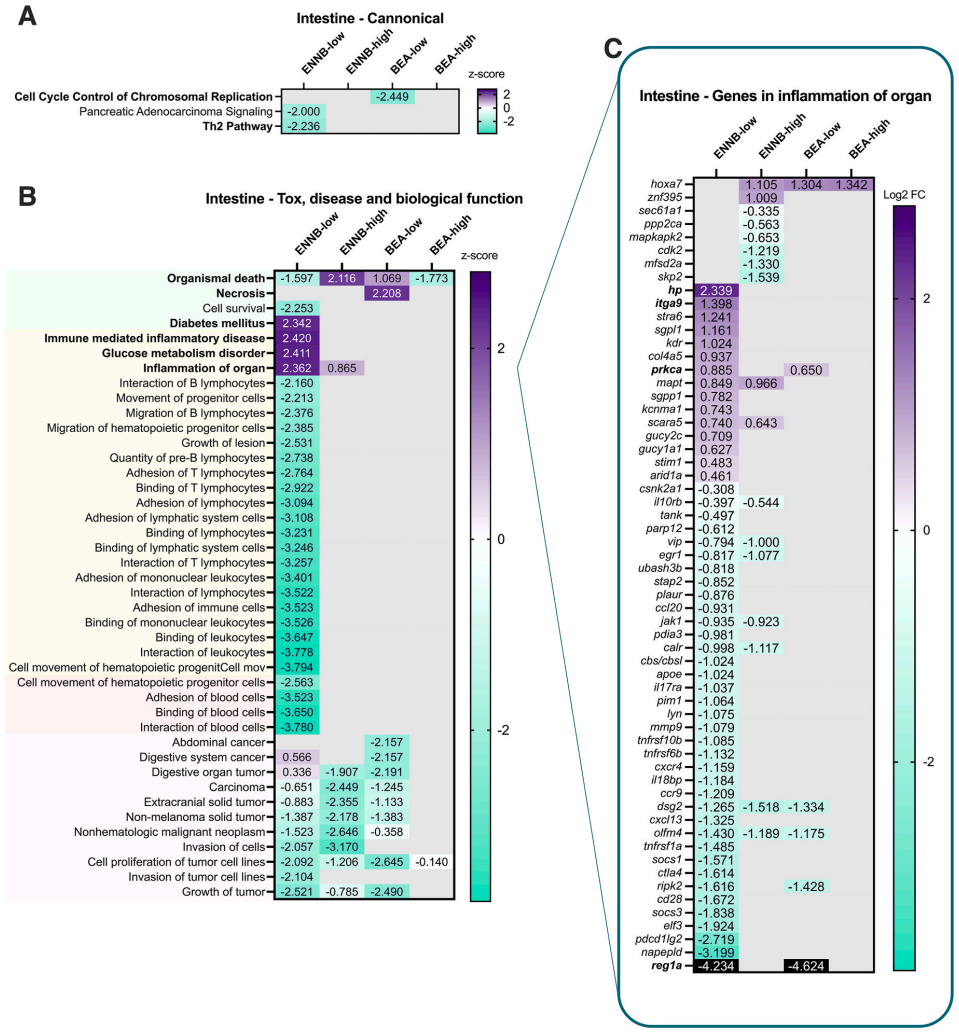


**Fig. 6.** IPA Pathway analysis of gene regulation patterns in the intestine in response to BEA and ENNB exposure. Heatmaps depicting (A) canonical pathways, (B) toxicity, disease and biological functions in intestine, and (C) genes in organ inflammation. Predicted upregulated pathways/genes indicated by dark purple, and downregulated genes indicated by turquoise. p-adjusted <0.2 and all pathways were subjected to z-score filtering >2, genes depicted as log2 fold change. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

shown to be involved in the metabolism (Schlenk et al., 2008) and bioactivation of aflatoxin B1 (AFB1) (Yang et al., 2000; Wang-Buhler et al., 2005). Thus, it could be hypothesized that *cyp2k1* prevents ENNB from reaching the liver which could explain the difference in transcriptomic response between BEA and ENNB in the present study. However, further toxicokinetic research is needed to establish the fate of ENNB and BEA in Atlantic salmon following dietary exposure.

### 4.3. Effects of dietary exposure to ENNB and BEA in the intestine

#### 4.3.1. Intestinal responses following ENNB exposure

In this study, transcriptional analysis of intestinal tissues from fish exposed to ENNB-low indicated an increase in inflammation-related processes (immune-mediated inflammation, glucose metabolism disorder, and organ inflammation). Haptoglobin (*hp*), an acute phase protein, was the most upregulated DEG in the immune mediated inflammation pathway in the intestine by ENNB. Tissue inflammation is often associated with tissue damage and the release of red blood cells that have ruptured (de la Rubia Ortí et al., 2021; Jeney, 2018). The main function of haptoglobin is to bind and remove free hemoglobin from damaged red blood cells to prevent heme iron ($Fe^{2+}$) causing oxidative damage such as lipid peroxidation (Alayash, 2011), or to reduce availability of free heme iron that could be utilized by invading pathogens (Díaz et al., 2021). Haptoglobin has previously been identified to be the most sensitive marker of acute inflammation in Wistar Han rats (Giffen et al., 2003). The observed upregulation of haptoglobin by ENNB-low suggested damage of intestinal cell and possibly red blood cells. Tissue injuries resulting in immunogenic cell death (e.g., necrosis, ferroptosis,

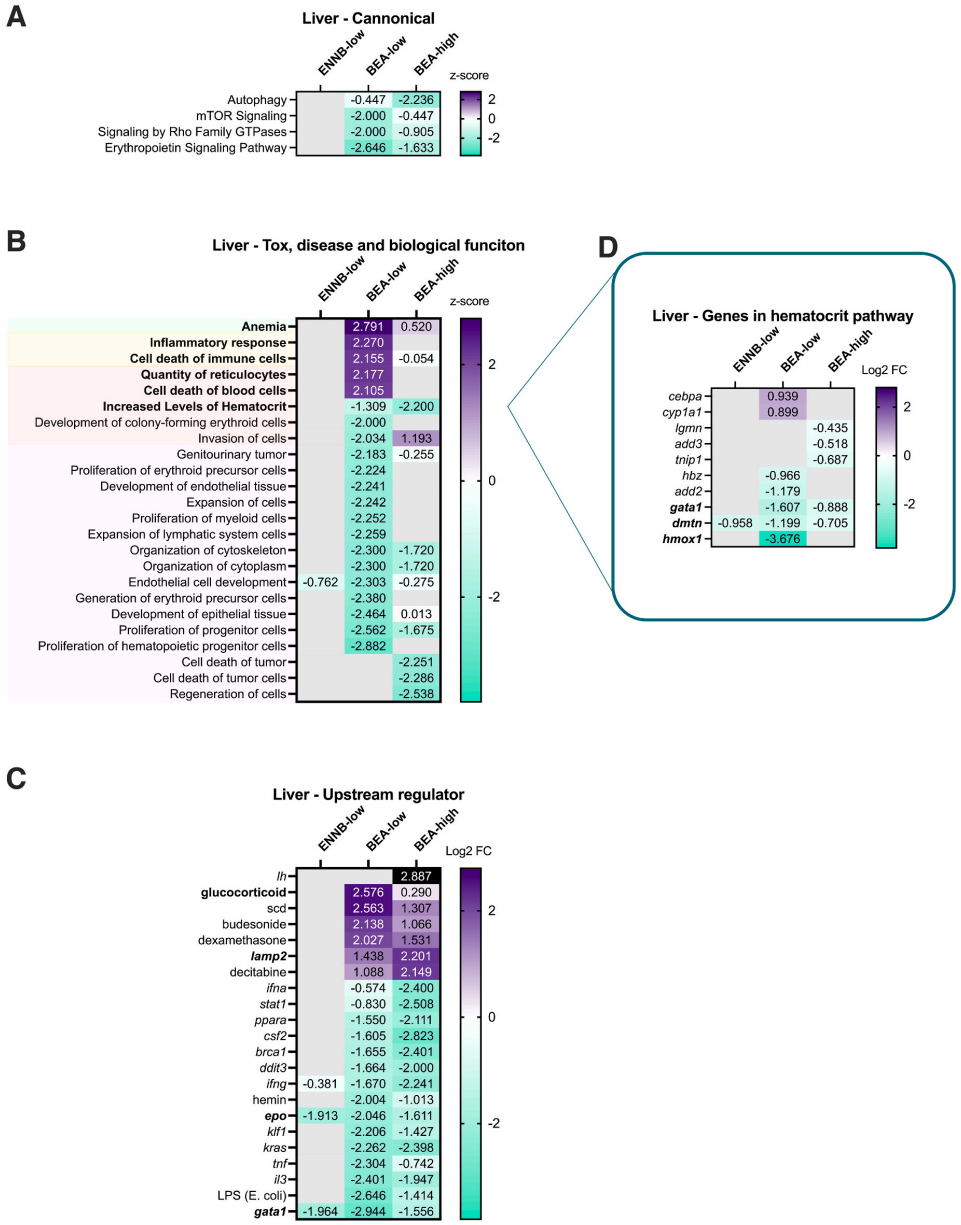


**Fig. 7.** IPA Pathway analysis of gene regulation patterns in the liver in response to BEA and ENNB exposure. Heatmaps depicting (A) canonical pathways, (B) toxicity, disease and biological functions, (C) upstream regulators, and (D) genes in hematocrit pathway in liver tissues. Predicted upregulated pathways/genes indicated by dark purple, and downregulated genes indicated by turquoise. p-adjusted <0.2 and all pathways were subjected to z-score filtering >2, genes depicted as log2 fold change. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

or excessive apoptosis), causes release of endogenous damage-associated molecular patterns (DAMPs) (i.e., cellular debris) that can activate the innate immune system and triggers acute inflammation to promote damage repair (Anders and Schaefer, 2014; Shi et al., 2021; Jaeschke, 2006). Meanwhile, pathogen-associated molecular pattern molecule (PAMP) (i.e., a foreign antigen), such as beta-glucan in the fungal cell wall, directly activates the receptors of the innate immune system (e.g., Toll-like receptors (TLRs)) (Medzhitov, 2008; Rauta et al., 2012). It might therefore also be possible that mycotoxins, such as ENNB and BEA, are recognized by TLRs of the innate immune system similar to fungal PAMP (Gruda et al., 2018; Perincherry et al., 2019), and directly trigger the receptors of the innate immune system

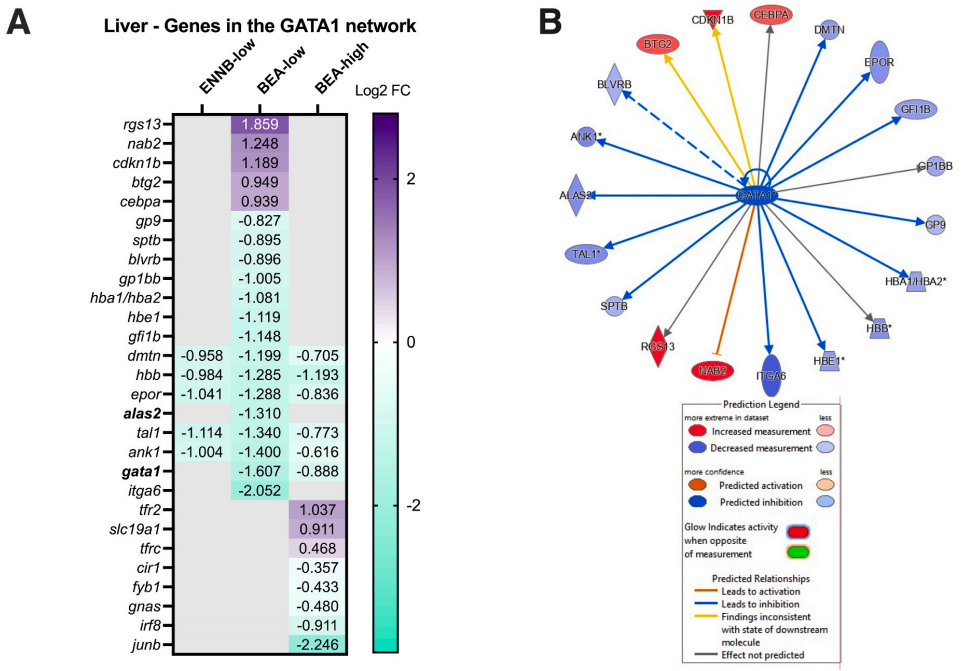


**Fig. 8.** IPA Pathway analysis of gene regulation patterns and Ferris wheel graph of GATA1 downstream target genes in response to ENNB-low, BEA-low, and BEA-high exposure. (A) Heatmap and (B) Ferris wheel showing predicted upregulated genes indicated by red, and downregulated genes indicated by blue. p-adjusted <0.2, and all pathways were subjected to z-score filtering >2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(Medzhitov, 2008; Rauta et al., 2012). Unlike mammalian red blood cells, in teleost these cells are nucleated and contain organelles such as mitochondria and lysosomes (Glomski et al., 1992), and teleost red blood cells partake in the immune response against infections since they can release cytokines, modulate leucocyte activity, and are able to phagocytose (Passantino et al., 2002, 2004, 2007). Recently, BEA was reported to exhibit immunostimulatory effects via the toll-like receptor 4 (TLR4) signaling pathway on murine dendritic cells (Yang et al., 2022). In a study with RAW 267.4 murine macrophages, ENNB was found to induce an inflammatory response that was proposed to occur through lysosomal damage releasing DAMPs that secondarily triggered the cellular inflammasome (Gammelsrud et al., 2012). ENNB was previously shown to impair mitochondria and lysosomes *in vitro* in a study with Atlantic salmon primary liver cells (Søderstrøm et al., 2022). However, in the current study, it remains to be elucidated whether ENNB secondarily triggers the cellular PRRs by causing DAMP release due to oxidative stress-induced cell damage, or if ENNB itself is recognized by PRRs and directly triggers the innate immune response.

ENNs ability to cause oxidative stress in intestinal tissue was previously suggested to impair the intestinal barrier function in Wistar rats (Cimbalo et al., 2021). Through their ionophoric activities, ENNs and BEA have been proposed to cause decreased tight junction resistance, and hence intestinal barrier impairment, by inducing the activity of protein kinase C alpha which is initiated by increased intracellular calcium ion levels (Tai et al., 1996; Bertero et al., 2018). Thus, the increase in *prkca* (protein kinase C alpha) expression in the intestine could indicate that ENNB has the potential to impair processes that affect the intestinal barrier if prolonged dietary exposure occurred. Homeobox genes (Hox genes) were among the most upregulated DEGs in the intestinal tissue responding to both mycotoxins. Hox genes have been shown to contribute to wound healing (Chuong, 2003), and adult cell renewal in mammals (Kachgal et al., 2012). Thus, the upregulation of Hox genes in the intestine may suggest that ENNB damaged the intestinal cells causing an upregulation of Hox genes to mitigate the damage.

While chronic inflammation could be harmful for tissues, acute inflammation is a rapid and short-term adaptive response characterized by initial recruitment of neutrophils to the injured site from the surrounding blood vessels, to resolve the damaging insult and reestablish homeostasis (Medzhitov, 2008; Germolec et al., 2018; Kolaczkowska and Kubes, 2013). Overall, the upregulation of responses related to inflammation indicate an early innate immune response to ENNB since immune functions related to the delayed adaptive immune system and the production of antibodies i.e., lymphocytes and the Th2 pathway, were downregulated by ENNB. The current study was a short-term trial (12 h) and activation of the adaptive immune response would have been expected to occur later (days, weeks) (Terrazas et al., 1998), if activated at all. Acute inflammation is a normal biological response that is rapidly triggered after an insult or injury, intended to quickly resolve and repair damaged cells and tissues to restore homeostasis (Medzhitov, 2008; Germolec et al., 2018; Kolaczkowska and Kubes, 2013). In this study, the upregulation of the function "inflammation of organ" (intestine) by the ENNB-low was stronger than by ENNB-high. While future research is needed to fully clarify this, it could be hypothesized that the acute inflammatory response is both time- and dose-dependent, where a higher dose would trigger a stronger initiation of the inflammatory response that would start to dab off as the response reaches the resolving phase and the damage is repaired. Thus, the transcriptomic data suggested that the cell damaging activity of ENNB most likely triggered the acute inflammatory response, which potentially could lead to immunological dysfunction of the intestinal barrier in Atlantic salmon if dietary

exposure would become prolonged.

#### 4.3.2. Intestinal responses following BEA exposure

Both the IPA (canonical) and the DAVID analyses indicated pathways involved in cell cycle arrest and DNA repair were affected in intestinal cells exposed to BEA-low. BEA-high on the other hand, appeared to impact the cells' transcriptional machinery, as seen from the absence of affected genetic pathways other than organismal death. The effects on intestinal cell cycle arrest and DNA repair at low BEA exposure, but absence of these biological process pathway responses at high BEA exposure, suggest that the intestinal cells temporarily stopped the cell cycle after BEA exposure to mitigate the inflicted stress and to allow cellular damage to be repaired (Pietenpol and Stewart, 2002). Similar disruption of cell cycle progression has previously been demonstrated in CHO–K1 cells (Mallebrera et al., 2016) and in SH-SY5Y neuronal cells (Agahi et al., 2021). The current transcriptomic analyses suggest that cell cycle control pathways were affected by BEA to prevent the development of necrosis in the salmon intestine. Like ENNB, BEA upregulated hox genes in the intestine which could mitigate the cell damage inflicted in the intestine.

### 4.4. Effects of dietary exposure to BEA in the liver

In the liver, representing the major detoxification organ, effects on gene transcription were primarily observed following BEA exposure. The functional analysis showed BEA to primarily cause hematological effects in the liver, such as decreased levels of hematocrit and anemia. Although kidney and spleen are the major erythropoietic organs, liver is the primary location for maintaining iron homeostasis and erythrocyte recycling involving Kupffer cells (Theurl et al., 2016). Gata1, one of the main upstream regulators in the production of red blood cell (erythropoiesis), was predicted downregulated in the present dataset. Improper expression of Gata1 has been reported to result in anemia (Gutiérrez et al., 2020). In addition, both doses of BEA downregulated the canonical erythropoietin (EPO) signaling pathway. EPO is a hormone-acing cytokine that can facilitates activation of Gata1 (Zhao et al., 2006), thereby linking the transcriptomics to dysregulation of maturation and production of red blood cell. Heme oxygenase 1 (*hmox1*), a rate-limiting enzyme in heme degradation and iron recycling (Bach, 2002), was the most downregulated gene (in terms of fold change) by BEA-low in the liver and related to the predicted reduction in hematocrit levels. Interestingly, mice lacking functional Hmox1 expression also developed anemia (Poss and Tonegawa, 1997). Alas2, the first rate limiting enzyme for heme biosynthesis acting downstream of Gata1 (Tanimura et al., 2016) was downregulated by BEA in the present study. Transcription of *alas2*, which is specific for blood cells (Muckenthaler et al., 2017), is normally inhibited when intracellular iron levels are low (Poli et al., 2021). This suggests exposure to BEA caused a reduction in bioavailable cellular iron in the salmon liver. BEA was shown to affect iron homeostasis in salmon primary hepatocytes *in vitro* (Søderstrøm et al., 2022), thus correlating to the transcriptional changes in the liver in the present *in vivo* trial, where BEA resulted in hematological effects (e.g., reduction of hematocrit levels and anemia). Although very few genes were affected by ENNB in the liver, like BEA, *dmtn*, *hbb*, *epor*, *tal1*, and *ank1* were downregulated, while *gata1* and *epo* were predicted as upstream regulators in ENNB-exposed livers. The *dmtn* gene was also denoted in the hematocrit pathway, and this gene encodes a membrane stabilizing protein important for the structure of erythrocytes (Wang et al., 2022). The function "*quantity of reticulocytes*" was predicted upregulated by IPA following exposure to BEA in the liver, and both IPA and DAVID suggested BEA to trigger regenerative hemolytic anemia, since increased levels of reticulocytes is characteristic of hemolytic anemia (Barcellini and Fattizzo, 2015; Grimes and Fry, 2015). While Olleik et al. (2019) reported BEA to exhibit a weak hemolytic activity towards human erythrocytes, Qadri et al. (2011) instead reported BEA to trigger eryptosis by scrambling the membranes of red blood cells. Ionomycin, a well-known calcium ($Ca^{2+}$) ionophore with high resemblance to the ionophoric BEA and ENNB, induced eryptosis and engulfment of injured and deformed human red blood cells by macrophages (Bigdelou and Farnoud, 2020). Eryptosis is a controlled removal of defective red blood cells to avoid hemolysis, though excessive eryptosis may however cause anemia (Lang et al., 2008; Lang and Lang, 2015).

Like the intestine, the liver transcriptome in BEA-exposed fish indicated promotion of cell death of immune cells and upregulation of inflammatory responses. Since oxidative stress can trigger cell death, it is interesting that the lysosomal-associated membrane protein 2 (*lamp2*) was predicted as an upstream regulator in the liver following BEA exposure. Lamp2 has been described to mediate removal of $Zn^{2+}$-induced reactive oxygen species in A549 cells (Qin et al., 2017), which suggests that BEA exposure caused oxidative stress in the salmon liver. This is in line with a previous *in vitro* study where BEA generated oxidative stress in primary salmon hepatocytes, impaired lysosomal and mitochondrial function, altered iron homeostasis, and increased Gpx activity in a concentration-dependent manner (Søderstrøm et al., 2022). Further, the inflammatory response triggered by BEA could be linked to the above-mentioned hematological effects and possible hemolytic activity. Previous studies have shown that increased production of pro-inflammatory cytokines (e.g., Tnfα, Il-1β, and Il-6) can lead to anemia by exerting a hampering effect on Epo production in mammals (Morceau et al., 2009). In the present study, Epo signaling was predicted by IPA to be downregulated in all exposure groups. Increased inflammatory signaling was also indicated by the predicted effect on the upstream regulators related to glucocorticoid, since glucocorticoids have been found to be anti-inflammatory and immune suppressive in various tissues e.g. mice intestine (Noti et al., 2010) and in airway epithelial cells and macrophages (Miyata et al., 2015). Since hemoglobin and red blood cell formation appeared affected by BEA in liver, it is noteworthy that the Hox genes were upregulated in the intestinal tissue by both mycotoxins. Hox genes in blood cells are key regulators during hematopoiesis (blood and plasma production), and dysregulation of their expression has been linked to various hematological abnormalities such as Hoxa9 (Sio et al., 2013; Morgan et al., 2005), that was upregulated by BEA-low and ENNB-high in intestinal tissue in the current study. Overall, the transcriptomics indicated BEA to primarily affect heme and blood related components negatively, in addition to hamper different transcriptional units and processes indicating oxidative stress, which was supported by earlier salmon hepatocyte *in vitro* studies showing an altered intercellular iron homeostasis and upregulated antioxidant defense (Søderstrøm et al., 2022). Whether the inflammatory response BEA triggered in the liver was due to oxidative stress-induced cell damage (including potential damage to red blood cells), or whether BEA itself directly triggers the innate immune response needs further research to elucidate.

### 4.5. Potential implications of exposure to BEA and ENNB for Atlantic salmon

Using earlier published data, Pietsch (2020) theoretically estimated maximum mycotoxin levels in farmed fish based on different contamination scenarios of European feed ingredients with mean predicted concentrations of 41.5 μg/kg for BEA (predicted maximum 2692 μg/kg) and 107 μg/kg for total ENNs (predicted maximum 68472 μg/kg). The low dose in the current study (50 μg/kg feed) was lower than the maximum measured concentration of ENNB in Norwegian commercial salmon feeds (250 μg/kg feed). Interestingly, the low dose in this study resulted in the onset of genes related to acute inflammation in intestinal tissue and possible impairment of intestinal barrier integrity. BEA-low (50 μg/kg feed) was comparable to the highest observed concentration (80 μg/kg) in salmon feed (Nácher-Mestre et al., 2020), and also indicated negative effects on intestinal barrier integrity and caused alterations in gene expression of the hematocrit and anemia pathways in the liver. Considering the measured concentrations of ENNB and BEA in

Norwegian salmon feed, and the concentrations of BEA and ENNs in the predicted scenarios by Pietsch (2020), the present study shows that the concentrations occasionally found in commercial fish feed could potentially constitute a problem for fish health if frequent re-exposure occurs. Thus, whether the toxic responses following repeated exposure to ENNB, and BEA can be mitigated and resolved needs further research, preferably by performing a long-term feeding trial using Atlantic salmon to test for chronic effects.

## 5. Conclusions

In accordance with other studies, ENNB was found to be one of the most prevalent mycotoxin in Norwegian fish feed, and the feed ingredients rapeseed oil and wheat gluten were major sources of ENNB, while BEA was primarily found in corn meal. Both BEA and ENNB elicited effects on transcription in the intestine, where the low doses caused the onset of adaptive transcriptional mechanisms, whereas the responses to high doses resulted in fewer DEGs indicating toxic reactions overcoming adaptive stress responses. BEA exerted greater transcriptional effects in the liver contra the intestine. The present acute dietary *in vivo* Atlantic salmon study showed that ENNB triggered an acute inflammatory response in the intestine, while BEA negatively affected heme biosynthesis and blood homeostasis in the liver.

## CRediT authorship contribution statement

**Sofie Søderstrøm:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing, Visualization. **Liv Søfteland:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition. **Veronika Sele:** Conceptualization, Formal analysis, Resources, Writing – review & editing, Visualization. **Anne-Katrine Lundebye:** Conceptualization, Writing – review & editing. **Marc HG. Berntssen:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition. **Kai K. Lie:** Conceptualization, Methodology, Software, Formal analysis, Investigation, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

## Declaration of competing interest

The authors 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

https://www.ncbi.nlm.nih.gov/geo/(GSE213817)

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2022.113557.

## References

Agahi, F., Juan, C., Font, G., Juan-García, A., 2021. Neurotoxicity of zearalenone's metabolites and beauvericin mycotoxins via apoptosis and cell cycle disruption. Toxicology 456, 152784.
Alayash, A.I., 2011. Haptoglobin: old protein with new functions. Clin. Chim. Acta 412, 493–498.
Alonso-Garrido, M., Escrivá, L., Manyes, L., Font, G., 2018. Enniatin B induces expression changes in the electron transport chain pathway related genes in lymphoblastic T-cell line. Food Chem. Toxicol. 121, 437–443.
Amlund, H., Berntssen, M.H., 2004. Arsenobetaine in Atlantic salmon (Salmo salar L.): influence of seawater adaptation. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 138, 507–514.
Anders, H.-J., Schaefer, L., 2014. Beyond tissue injury—damage-associated molecular patterns, toll-like receptors, and inflammasomes also drive regeneration and fibrosis. J. Am. Soc. Nephrol. 25, 1387–1400.
Bach, F.H., 2002. Heme oxygenase-1 as a protective gene. Wien Klin. Wochenschr. 114, 1–3.
Barcellini, W., Fattizzo, B., 2015. Clinical Applications of Hemolytic Markers in the Differential Diagnosis and Management of Hemolytic Anemia. Disease markers, 2015.
Bernhoft, A., Eriksen, G.S., Sundheim, L., Berntssen, M., Brantsæter, A.L., Brodal, G., Fæste, C.K., Hofgaard, I.S., Rafoss, T., Sivertsen, T., 2013a. Risk Assessment of Mycotoxins in Cereal Grain in Norway. Opinion of the Scientific Steering Committee of the Norwegian Scientific Committee for Food Safety *(VKM report*.
Bernhoft, A., Hogasen, H.R., Rosenlund, G., Ivanova, L., Berntssen, M.H.G., Alexander, J., Eriksen, G.S., Faeste, C.K., 2017. Tissue distribution and elimination of deoxynivalenol and ochratoxin A in dietary-exposed Atlantic salmon (Salmo salar). Food Addit. Contam. Part a-Chemistry Analysis Control Exposure & Risk Assessment 34, 1211–1224.
Bernhoft, A., Hogasen, H.R., Rosenlund, G., Moldal, T., Grove, S., Berntssen, M.H.G., Thoresen, S.I., Alexander, J., 2018. Effects of dietary deoxynivalenol or ochratoxin A on performance and selected health indices in Atlantic salmon (Salmo solar). Food Chem. Toxicol. 121, 374–386.
Bernhoft, A., Sundstøl Eriksen, G., Sundheim, L., Berntssen, M.H.G., Brantsæter, A.L., Brodal, G., Kruse Fæste, C., Skow Hofgaard, I., Rafoss, T., Sivertsen, T., Tronsmo, A. M., 2013b. Risk Assessment of Mycotoxins in Cereal Grain in Norway. Norwegian Scientific Committee for Food Safety (VKM), pp. 109–211. http://www.vkm.no/dav/eee04d10c4.pdf.
Bertero, A., Spicer, L., Caloni, F., 2018. Fusarium mycotoxins and in vitro species-specific approach with porcine intestinal and brain in vitro barriers: a review. Food Chem. Toxicol. 121, 666–675.
Bigdelou, P., Farnoud, A.M., 2020. Induction of eryptosis in red blood cells using a calcium ionophore. JoVE, e60659.
CHAIN, E. P. O. C. I. T. F, 2014. Scientific Opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed. EFSA J. 12, 3802.
Cheli, F., Battaglia, D., Gallo, R., Dell'Orto, V., 2014. EU legislation on cereal safety: an update with a focus on mycotoxins. Food Control 37, 315–325.
Chen, T., Zhang, H., Liu, Y., Liu, Y.-X., Huang, L., 2021. EVenn: easy to create repeatable and editable Venn diagrams and Venn networks online. Journal of genetics and genomics= Yi chuan xue bao 48, 863–866.
Chuong, C.-M., 2003. Homeobox genes, fetal wound healing, and skin regional specificity. J. Invest. Dermatol. 120, 9.
Cimbalo, A., Alonso-Garrido, M., Font, G., Frangiamone, M., Manyes, L., 2021. Transcriptional changes after enniatins A, A1, B and B1 ingestion in rat stomach, liver, kidney and lower intestine. Foods 10, 1630.
De La Rubia Ortí, J.E., Platero, J.L., Benlloch, M., Franco-Martinez, L., Tvarijonaviciute, A., Escribá-Alepuz, J., Sancho-Castillo, S., 2021. Role of haptoglobin as a marker of muscular improvement in patients with multiple sclerosis after administration of epigallocatechin gallate and increase of beta-hydroxybutyrate in the blood: a pilot study. Biomolecules 11, 617.
Díaz, R., Troncoso, J., Jakob, E., Skugor, S., 2021. Limiting access to iron decreases infection of Atlantic salmon SHK-1 cells with bacterium Piscirickettsia salmonis. BMC Vet. Res. 17, 1–11.
Döll, S., Baardsen, G., Möller, P., Koppe, W., Stubhaug, I., Dänicke, S., 2010. Effects of increasing concentrations of the mycotoxins deoxynivalenol, zearalenone or ochratoxin A in diets for Atlantic salmon (Salmo salar) on growth performance and health. In: 14th International Symposium on Fish Nutrition and Feeding. Qingdao, China.
EC, 2016. Commission Recommendation (EU) 2016/1319 of 29 July 2016 amending Recommendation 2006/576/EC as regards deoxynivalenol, zearalenone and ochratoxin A in pet food. Off. J. Eur. Union 208, 58–60.
Eide, M., Zhang, X., Karlsen, O.A., Goldstone, J.V., Stegeman, J., Jonassen, I., Goksøyr, A., 2021. The chemical defensome of five model teleost fish. Sci. Rep. 11, 1–13.
Escrivá, L., Jennen, D., Caiment, F., Manyes, L., 2018. Transcriptomic study of the toxic mechanism triggered by beauvericin in Jurkat cells. Toxicol. Lett. 284, 213–221.
Fæste, C.K., Ivanova, L., Uhlig, S., 2011. In vitro metabolism of the mycotoxin enniatin B in different species and cytochrome p450 enzyme phenotyping by chemical inhibitors. Drug Metab. Dispos. 39, 1768–1776.
Gammelsrud, A., Solhaug, A., Dendelé, B., Sandberg, W., Ivanova, L., Bølling, A.K., Lagadic-Gossmann, D., Refsnes, M., Becher, R., Eriksen, G., 2012. Enniatin B-induced cell death and inflammatory responses in RAW 267.4 murine macrophages. Toxicol. Appl. Pharmacol. 261, 74–87.

Germolec, D.R., Shipkowski, K.A., Frawley, R.P., Evans, E., 2018. Markers of inflammation. Immunotoxicity Testing 57–79.
Giffen, P., Turton, J., Andrews, C., Barrett, P., Clarke, C., Fung, K.-W., Munday, M., Roman, I., Smyth, R., Walshe, K., 2003. Markers of experimental acute inflammation in the Wistar Han rat with particular reference to haptoglobin and C-reactive protein. Arch. Toxicol. 77, 392–402.
Glomski, C.A., Tamburlin, J., Chainani, M., 1992. The phylogenetic odyssey of the erythrocyte. III. Fish, the lower vertebrate experience. Histol. Histopathol.
Grimes, C., Fry, M., 2015. Nonregenerative anemia: mechanisms of decreased or ineffective erythropoiesis. Veterinary Pathology 52, 298–311.
Gruda, M.C., Ruggeberg, K.-G., O'Sullivan, P., Guliashvili, T., Scheirer, A.R., Golobish, T. D., Capponi, V.J., Chan, P.P., 2018. Broad adsorption of sepsis-related PAMP and DAMP molecules, mycotoxins, and cytokines from whole blood using CytoSorb® sorbent porous polymer beads. PLoS One 13, e0191676.
Gutiérrez, L., Caballero, N., Fernández-Calleja, L., Karkoulia, E., Strouboulis, J., 2020. Regulation of GATA1 levels in erythropoiesis. IUBMB Life 72, 89–105.
Huang, D.W., Sherman, B.T., Lempicki, R.A., 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–57.
Hussein, H.S., Brasel, J.M., 2001. Toxicity, metabolism, and impact of mycotoxins on humans and animals. Toxicology 167, 101–134.
Ivanova, L., Fæste, C.K., Uhlig, S., 2011. In vitro phase I metabolism of the depsipeptide enniatin B. Anal. Bioanal. Chem. 400, 2889–2901.
Jaeschke, H., 2006. Mechanisms of Liver Injury. II. Mechanisms of neutrophil-induced liver cell injury during hepatic ischemia-reperfusion and other acute inflammatory conditions. Am. J. Physiol. Gastrointest. Liver Physiol. 290, G1083–G1088.
Jeney, V., 2018. Pro-inflammatory actions of red blood cell-derived DAMPs. Inflammasome: Clinical and Therapeutic Implications. Springer.
Kachgal, S., Mace, K.A., Boudreau, N.J., 2012. The dual roles of homeobox genes in vascularization and wound healing. Cell Adhes. Migrat. 6, 457–470.
Kim, D., Langmead, B., Salzberg, S.L., 2015. HISAT: a fast spliced aligner with low memory requirements. Nat. Methods 12, 357–360.
Kolaczkowska, E., Kubes, P., 2013. Neutrophil recruitment and function in health and inflammation. Nat. Rev. Immunol. 13, 159–175.
Lang, E., Lang, F., 2015. Mechanisms and pathophysiological significance of eryptosis, the suicidal erythrocyte death. In: Seminars in Cell & Developmental Biology. Elsevier, pp. 35–42.
Lang, F., Gulbins, E., Lerche, H., Huber, S.M., Kempe, D.S., Föller, M., 2008. Eryptosis, a window to systemic disease. Cell. Physiol. Biochem. 22, 373–380.
Liao, Y., Smyth, G.K., Shi, W., 2014. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30, 923–930.
Lindblad, M., Gidlund, A., Sulyok, M., Börjesson, T., Krska, R., Olsen, M., Fredlund, E., 2013. Deoxynivalenol and other selected Fusarium toxins in Swedish wheat—occurrence and correlation to specific Fusarium species. Int. J. Food Microbiol. 167, 284–291.
Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 1–21.
Mallebrera, B., Juan-Garcia, A., Font, G., Ruiz, M.-J., 2016. Mechanisms of beauvericin toxicity and antioxidant cellular defense. Toxicol. Lett. 246, 28–34.
Medzhitov, R., 2008. Origin and physiological roles of inflammation. Nature 454, 428–435.
Miyata, M., Lee, J.-Y., Susuki-Miyata, S., Wang, W.Y., Xu, H., Kai, H., Kobayashi, K.S., Flavell, R.A., Li, J.-D., 2015. Glucocorticoids suppress inflammation via the upregulation of negative regulator IRAK-M. Nat. Commun. 6, 1–12.
Moldal, T., Bernhoft, A., Rosenlund, G., Kaldhusdal, M., Koppang, E.O., 2018. Dietary deoxynivalenol (DON) may impair the epithelial barrier and modulate the cytokine signaling in the intestine of Atlantic salmon (Salmo salar). Toxins 10, 376.
Morceau, F., Dicato, M., Diederich, M., 2009. Pro-inflammatory Cytokine-Mediated Anemia: Regarding Molecular Mechanisms of Erythropoiesis. Mediators of inflammation, 2009.
Morgan, R., Begum, R., Theti, D., Chansa, M., Pettengell, R., Sohal, J., 2005. HOXA9 expression increases with age in human haemopoietic cells. Leuk. Res. 29, 1221–1222.
Muckenthaler, M.U., Rivella, S., Hentze, M.W., Galy, B., 2017. A red carpet for iron metabolism. Cell 168, 344–361.
Nácher-Mestre, J., Beltrán, E., Strachan, F., Dick, J.R., Pérez-Sánchez, J., Berntssen, M. H., Tocher, D.R., 2020. No transfer of the non-regulated mycotoxins, beauvericin and enniatins, from feeds to farmed fish reared on plant-based diets. Food Chem. 323, 126773.
Nacher-Mestre, J., Beltran, E., Strachan, F., Dick, J.R., Perez-Sanchez, J., Berntssen, M.H. G., Tocher, D.R., 2020. No transfer of the non-regulated mycotoxins, beauvericin and enniatins, from feeds to farmed fish reared on plant-based diets. Food Chem. 323.
Nacher-Mestre, J., Serrano, R., Beltran, E., Perez-Sanchez, J., Silva, J., Karalazos, V., Hernandez, F., Berntssen, M.H.G., 2015. Occurrence and potential transfer of mycotoxins in gilthead sea bream and Atlantic salmon by use of novel alternative feed ingredients. Chemosphere 128, 314–320.
Noti, M., Corazza, N., Mueller, C., Berger, B., Brunner, T., 2010. TNF suppresses acute intestinal inflammation by inducing local glucocorticoid synthesis. J. Exp. Med. 207, 1057–1066.
Olleik, H., Nicoletti, C., Lafond, M., Courvoisier-Dezord, E., Xue, P., Hijazi, A., Baydoun, E., Perrier, J., Maresca, M., 2019. Comparative structure–activity analysis of the antimicrobial activity, cytotoxicity, and mechanism of action of the fungal cyclohexadepsipeptides enniatins and beauvericin. Toxins 11, 514.
Ørnsrud, R., Silva, M.S., Berntssen, M.H., Lundebye, A.-K., Storesund, J.E., Lie, K.K., Waagbø, R., Sele, V., 2020. Program for overvåking av fiskefôr-Årsrapport for prøver innsamlet i 2019. *Rapport fra havforskningen*.
Passantino, L., Altamura, M., Cianciotta, A., Jirillo, F., Ribaud, M., Jirillo, E., Passantino, G., 2004. Maturation of fish erythrocytes coincides with changes in their morphology, enhanced ability to interact with Candida albicans and release of cytokine-like factors active upon autologous macrophages. Immunopharmacol. Immunotoxicol. 26, 573–585.
Passantino, L., Altamura, M., Cianciotta, A., Patruno, R., Tafaro, A., Jirillo, E., Passantino, G., 2002. Fish immunology. I. Binding and engulfment of Candida albicans by erythrocytes of rainbow trout (Salmo gairdneri Richardson). Immunopharmacol. Immunotoxicol. 24, 665–678.
Passantino, L., Massaro, M., Jirillo, F., Di Modugno, D., Ribaud, M., Di Modugno, G., Passantino, G., Jirillo, E., 2007. Antigenically activated avian erythrocytes release cytokine-like factors: a conserved phylogenetic function discovered in fish. Immunopharmacol. Immunotoxicol. 29, 141–152.
Perincherry, L., Lalak-Kańczugowska, J., Stępień, Ł., 2019. Fusarium-produced mycotoxins in plant-pathogen interactions. Toxins 11, 664.
Pietenpol, J., Stewart, Z., 2002. Cell cycle checkpoint signaling:: cell cycle arrest versus apoptosis. Toxicology 181, 475–481.
Pietsch, C., 2020. Risk assessment for mycotoxin contamination in fish feeds in Europe. Mycotoxin Res. 36, 41–62.
Pinotti, L., Ottoboni, M., Giromini, C., Dell'Orto, V., Cheli, F., 2016. Mycotoxin contamination in the EU feed supply chain: a focus on cereal byproducts. Toxins 8, 45.
Poli, A., Schmitt, C., Moulouel, B., Mirmiran, A., Puy, H., Lefèbvre, T., Gouya, L., 2021. Iron, heme synthesis and erythropoietic porphyrias: a complex interplay. Metabolites 11, 798.
Poss, K.D., Tonegawa, S., 1997. Heme oxygenase 1 is required for mammalian iron reutilization. Proc. Natl. Acad. Sci. USA 94, 10919–10924.
Qadri, S.M., Kucherenko, Y., Lang, F., 2011. Beauvericin induced erythrocyte cell membrane scrambling. Toxicology 283, 24–31.
Qin, X., Zhang, J., Wang, B., Xu, G., Zou, Z., 2017. LAMP-2 mediates oxidative stress-dependent cell death in Zn2+-treated lung epithelium cells. Biochem. Biophys. Res. Commun. 488, 177–181.
Rauta, P.R., Nayak, B., Das, S., 2012. Immune system and immune responses in fish and their role in comparative immunity study: a model for higher organisms. Immunol. Lett. 148, 23–33.
Rodríguez-Carrasco, Y., Heilos, D., Richter, L., Süssmuth, R.D., Heffeter, P., Sulyok, M., Kenner, L., Berger, W., Dornetshuber-Fleiss, R., 2016. Mouse tissue distribution and persistence of the food-born fusariotoxins Enniatin B and Beauvericin. Toxicol. Lett. 247, 35–44.
Sanden, M., Hemre, G., Måge, A., Lunestad, B., Espe, M., Lie, K., Lundebye, A., Amlund, H., Waagbø, R., Ørnsrud, R., 2017. Program for Overvåking Av Fiskefôr Årsrapport for Prøver Innsamlet I 2016. NIFES, Bergen.
Schlenk, D., Celander, M., Gallagher, E., George, S., James, M., Kullman, S., Van Den Hurk, P., Willett, K., 2008. In: Hinton, D.E. (Ed.), Biotransformation in Fishes in the Toxicology of Fishes (Di Giulio RT. CRC Press, Boca Raton ([Google Scholar]).
Sele, V., Berntssen, M.H., Prabhu, P., Lundebye, A.-K., Lie, K.K., Espe, M., Storesund, J.E., Ørnsrud, R., 2021. Program for overvåking av fiskefôr-Årsapport for prøver innsamlet i 2020. *Rapport fra havforskningen*.
Sele, V., Sanden, M., Berntssen, M., Lunestad, B., Espe, M., Lie, K., Amlund, H., Lundebye, A., Hemre, G., Waagbø, R., Ørnsrud, R., 2018. Program for overvåking av fiskefôr - Årsrapport for prøver innsamlet i 2017. *Rapport fra havforskningen*.
Sele, V., Sanden, M., Berntssen, M., Storesund, J.E., Lie, K.K., Espe, M., Lundebye, A.-K., Hemre, G.I., Waagbø, R., Ørnsrud, R., 2019. Program for overvåking av fiskefôr-Årsrapport for prøver innsamlet i 2018. *Rapport fra havforskningen*.
Shi, L., Liu, Y., Li, M., Luo, Z., 2021. Emerging roles of ferroptosis in the tumor immune landscape: from danger signals to anti-tumor immunity. FEBS J.
Sio, A., Chehal, M.K., Tsai, K., Fan, X., Roberts, M.E., Nelson, B.H., Grembecka, J., Cierpicki, T., Krebs, D.L., Harder, K.W., 2013. Dysregulated hematopoiesis caused by mammary cancer is associated with epigenetic changes and hox gene expression in hematopoietic cells. Cancer Res. 73, 5892–5904.
Søderstrøm, S., Lie, K.K., Lundebye, A.-K., Søfteland, L., 2022. Beauvericin (BEA) and enniatin B (ENNB)-induced impairment of mitochondria and lysosomes-Potential sources of intracellular reactive iron triggering ferroptosis in Atlantic salmon primary hepatocytes. Food Chem. Toxicol., 112819
Tai, Y.-H., Flick, J., Levine, S., Madara, J., Sharp, G., Donowitz, M., 1996. Regulation of tight junction resistance in T84 monolayers by elevation in intracellular Ca2+: a protein kinase C effect. J. Membr. Biol. 149, 71–79.
Tanimura, N., Miller, E., Igarashi, K., Yang, D., Burstyn, J.N., Dewey, C.N., Bresnick, E. H., 2016. Mechanism governing heme synthesis reveals a GATA factor/heme circuit that controls differentiation. EMBO Rep. 17, 249–265.
Terrazas, L.I., Bojalil, R., Govezensky, T., Larralde, C., 1998. Shift from an early protective Th1-type immune response to a late permissive Th2-type response in murine cysticercosis (Taenia crassiceps). J. Parasitol. 74–81.
Theurl, I., Hilgendorf, I., Nairz, M., Tymoszuk, P., Haschka, D., Asshoff, M., He, S., Gerhardt, L., Holderried, T.A., Seifert, M., 2016. On-demand erythrocyte disposal and iron recycling requires transient macrophages in the liver. Nat. Med. 22, 945–951.
Tolosa, J., Font, G., Manes, J., Ferrer, E., 2014. Natural occurrence of emerging Fusarium mycotoxins in feed and fish from aquaculture. J. Agric. Food Chem. 62, 12462–12470.
Tolosa, J., Rodriguez-Carrasco, Y., Ruiz, M.J., Vila-Donat, P., 2021. Multi-mycotoxin occurrence in feed, metabolism and carry-over to animal-derived food products: a review. Food Chem. Toxicol. 158.
Vaclavikova, M., Malachova, A., Veprikova, Z., Dzuman, Z., Zachariasova, M., Hajslova, J., 2013. 'Emerging'mycotoxins in cereals processing chains: changes of enniatins during beer and bread making. Food Chem. 136, 750–757.

Wang, M., Chen, B., Zhang, W., Zhang, F., Qiu, Y., Lin, Y., Yang, S., 2022. Dematin inhibits glioblastoma malignancy through RhoA-mediated CDKs downregulation and cytoskeleton remodeling. Exp. Cell Res. 417, 113196.
Wang-Buhler, J., Lee, S., Chung, W., Stevens, J., Tseng, H., Hseu, T., Hu, C., Westerfield, M., Yang, Y., Miranda, C., 2005. CYP2K6 from zebrafish (Danio rerio): cloning, mapping, developmental/tissue expression, and aflatoxin B1 activation by baculovirus expressed enzyme. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 140, 207–219.
Yang, X., Ali, S., Zhao, M., Richter, L., Schäfer, V., Diecks, J.S., Frank, M., Qi, J., Larsen, P.K., Skerra, J., 2022. The mycotoxin Beauvericin exhibits immunostimulatory effects on dendritic cells via activating the TLR4 signaling pathway. Front. Immunol. 1604.
Yang, Y.-H., Miranda, C.L., Henderson, M.C., Wang-Buhler, J.-L., Buhler, D.R., 2000. Heterologous expression of Cyp2k1 and identification of the expressed protein (bv-Cyp2k1) as lauric acid (ω-1)-hydroxylase and aflatoxin b1exo-epoxidase. Drug Metab. Dispos. 28, 1279–1283.
Ytrestøyl, T., Aas, T.S., Åsgård, T., 2015. Utilisation of feed resources in production of Atlantic salmon (Salmo salar) in Norway. Aquaculture 448, 365–374.
Zhao, W., Kitidis, C., Fleming, M.D., Lodish, H.F., Ghaffari, S., 2006. Erythropoietin stimulates phosphorylation and activation of GATA-1 via the PI3-kinase/AKT signaling pathway. Blood 107, 907–915.

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