EAFP 19TH INTERNATIONAL CONFERENCE ON DISEASES OF FISH AND SHELLFISH SPECIAL EDITION WORKSHOP REPORT

Co-infections and multiple stressors in fish

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Introduction

Fish are typically exposed to multiple physical, chemical and biological stressors. The cumulative impact of co-infections between parasites, bacteria, viruses and (a)biotic environmental pressures may trigger complex interactions, eliciting different pathological and immunological outcomes than those classically assessed. New cross-disciplinary studies attempt to measure the impact of environmental stressors in modulating the host response to pathogens. Scientific advances are needed to reduce pressure on natural populations, improve fish stock management, and to design more efficient diagnostic tools or vaccination strategies. An EAFP-promoted workshop, held on 10th September 2019 in Porto, Portugal, was dedicated to sharing research experiences on the interaction between heterogenous pathogens and multiple stressors in fish. The workshop involved around 200 attendants, opened by a keynote talk (Fast), and followed by a further twelve oral presentations, including three in the format of flash poster presentations. Contributions illustrated cross-disciplinary approaches to study complex host-pathogen and stressors interactions.

Pathological synergies in co-infecting pathogens are impacted by exposure order, and host response to initial infection

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In wild and cultured fish populations, exposure and response to potential pathogens is

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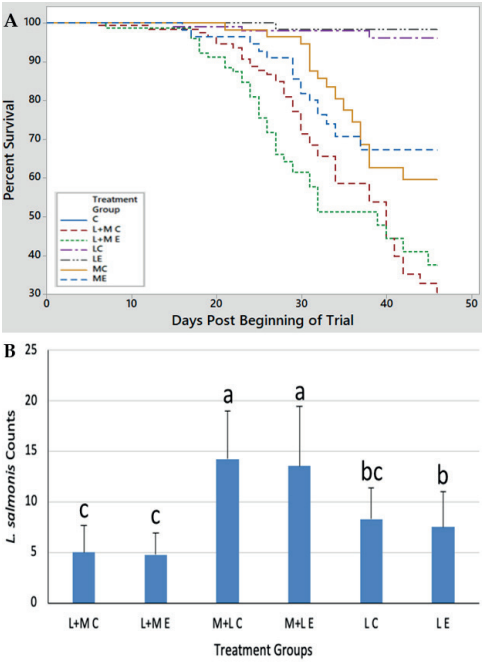

Figure 1. Differential survival (Kaplan-Meier plot) (A), and lice counts (B) following exposure to *Lepeophtheirus salmonis* (L) and/or *Moritella viscosa* (M), using a commercial (C) or experimental (E) diet. Letters on the bars denote significant differences in infection level (GLM; post-hoc Bonferroni, $p<0.05$).

a dynamic process. Until recently our understanding of host-pathogen interactions was limited to single exposures under individual pulsed conditions. In wild and cultured salmonids, the ectoparasitic copepod *Lepeophtheirus salmonis* (*L.s.*; aka "sea lice"), is highly prevalent and commonly causes acute and chronic immuno-physiological strain on its hosts in marine environments. Studies have shown that sea lice infection, can have significant impacts on immune responses of their host, such as decreased anti-viral responses (Barker et al., 2019), and anti-bacterial immunity (Figueroa et al., 2017). We have recently begun to examine the immunomodulatory and immuno-physiological effects sea lice infection has on the development of disease with other parasitic pathogens, viral and bacterial pathogens. Co-exposure of hosts to *Caligus elongatus* and *L.s.* copepodids, showed no impact on infection level or host impacts compared to single infection. However, in the case of bacterial and viral infections, prior infection with *L.s.* enhanced pathology and mortality associated with subsequent infecting organisms. This was not always linked to the intensity of sea lice infection. In the case of co-infection with the cold water ulcer-associated bacteria, *Moritella viscosa* (*M.v.*), our data suggest that the order of establishment impacted subsequent survival (Figure 1.A), and lice abundance (Figure 1.B). Fish exposed to co-infection with lice and *M.v.* showed enhanced inflammation and acute phase response signals in skin over time. However, fish with active lesions positive for bacterial growth showed the opposite, inhibited inflammatory signals in the skin, potentially linked to a higher mortality rate. Host immunomodulation through transcriptomic suppression is a hallmark of lice infections, likely enhancing colonisation of viral and bacterial pathogens. Iron regulation and transport are also commonly implicated in *L.s.* infection (Sutherland et al., 2014), and acutely (eg hepcidin) observed in lice and bacterial exposure in salmon (Martin et al., 2006). The order of infection with *L.s.* and *M.v.* impacted differential expression of iron homeostasis marker hepcidin in infected fish skin sites away from *L.s.* attachment. Functional feed properties did not have an overall influence on single and *M.v.* co-infection outcomes. However, multiple functional feeds significantly impacted survival following viral (eg infectious salmon anaemia) infection (eg CpG motifs) and lice counts (eg CpGs and %EPA:DHA inclusion).

In each case, feed gains in survival or reductions in lice number were all eliminated following addition of a co-infecting pathogen. These results demonstrate the need for a more complex approach to studying disease pathogenesis, to improve immune potentiating approaches in fish in the future.

The pathobiome in animal and plant health

D. Bass, C. Tyler, H-C. Wang, B. Koskella and G. Stentiford

A 'pathobiome' is the set of host-associated organisms (encompassing prokaryotes, eukaryotes, and viruses), linked to a reduced health status resulting from interactions between the members of that set of organisms and the host (Bass et al., 2019). These interactions are moderated by the environment within the host and immediately surrounding it. A pathobiome comprises a host with anything from two non-host lineages up to a complex symbiont community. Microbial diversity is now known to be much more diverse than previously thought in both environmental and host-associated habitats. There is a large and increasing literature reporting high levels of protistan, bacterial, and viral diversity revealed by eDNA/RNA-style analyses of host-associated (eg tissue, gut contents, skin epibionts) and environmental samples (e.g. water, sediment, soils) (Bass et al., 2015) (Figure 2). This diversity includes many lineages that are (or may become) host-associated and can form part of pathobiomes. These include cryptic, emerging, previously unrecognised, and opportunistic pathogens, including commensals switching to pathogenic modes (Overstreet and Lotz, 2016). Our appreciation of what constitutes

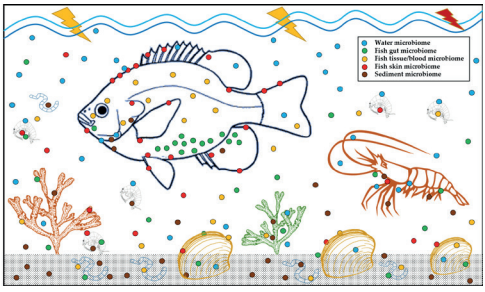

Figure 2. Microbial complexity in a fish-exemplar host-symbiont-environment system (Adapted from Bass et al., 2019). Green dots represent the fish gut microbiome (prokaryotes, eukaryotes, and viruses) and its exchange with other compartments of the habitat. Similarly for fish tissue/blood microbiome (yellow), skin (red), and the water and sediment microbiomes of the system (blue, brown).

a 'pathogen' has diversified and will continue to do so as we better understand the complexity of the pathogenic process. Pathobiotic mechanisms include antagonism or synergism among infecting agents, for example by one agent influencing the resistance of the host to another, or via signal sharing, metabolic interactions, etc. Genomic contributors to pathobiotic systems include co-existence of bacterial strains with complementary gene content, horizontal gene transfer via plasmids, transposons, genomic islands, and bacteriophages. However, pathobiotic mechanisms, particularly at the broader symbiont community level, are poorly understood. Diseases with indistinguishable clinical signs are often thought to be caused by the same primary pathogen, but may have different aetiologies, an example being white pox disease of the coral *Acropora* (Kemp et al., 2018). Even in cases of diseases for which a primary pathogen is identified, it is important to remember that that pathogen is operating within a complex microbial community, likely to modify its activities and their results in diverse ways. This aspect of pathogenesis and disease aetiology

is under-recognised but important for understanding different presentations of the same disease in different cases; a situation frequently complicating disease investigation. As symbiont community structures are often highly variable, functional traits such as gene expression patterns at different levels (eg core and non-core functions), and metabolic interactions provide complementary perspectives with different patterns of variability, from which to understand pathobiotic mechanisms. Understanding host symbiomes will be key to mitigating disease in global food production systems (Stentiford et al., 2017). Bodies such as the World Organisation for Animal Health define a list of emerging and notifiable diseases associated with infection by specific agents, against which diagnostic tests and management strategies can be designed. However, such legislation does not currently take in to account broader symbiont profiling of hosts, and the role that the symbiome may play in disease outbreaks.

Expecting the unexpected: an analysis of multiple stressors and their physiological consequences for rainbow trout

C. Bailey, E. Wernicke von Siebenthal, K. Rehberger, A. Ros, E.L. Herzog and H. Segner

The purpose of this study was to disentangle the molecular and organism level reactions of rainbow trout, *Oncorhynchus mykiss*, to the combined impact of two environmental stressors occurring in the natural habitat of salmonids. Fish were either exposed to: 1) a myxozoan parasite, *Tetracapsuloides bryosalmonae* (*T.b.*) causing proliferative kidney disease (PKD); 2) an estrogenic endocrine-disrupting compound ethinylestradiol (EE2); or 3) a combination of both (*T.b.*xEE2). To evaluate the chronic impact of these stressors exposures the fish response was investigated for 130 days post-exposure (dpe). In *T.b.* only exposed fish a greater parasite burden was observed at every time point, in contrast to fish exposed to *T.b.*xEE2. Moreover, in these fish at 90 dpe there was an increase in mRNA levels of immune genes in the anterior kidney that had shown to be reactive during PKD pathogenesis in our earlier studies (Bailey et al., 2017) (*blimp1*, *igm-sec*, *il-10* and *nkef*) and increased pathological alterations in primary and secondary lymphoid organs (anterior kidney, posterior kidney and spleen) relative to the EE2 exposed fish and *T.b.*xEE2 fish (Wernicke von Siebenthal et al., 2018). While in *T.b.*xEE2 fish there was lower parasite burden and lower expression of immune genes, but clear evidence of EE2 in the liver in terms of pathology and vitellogenesis production in contrast to the other treatments (Wernicke von Siebenthal et al., 2018). At 130 dpe, RNA-seq was applied to the posterior kidney of all experimental groups, this was a time when parasite intensity in the fish kidney started to decrease. A greater number of differentially expressed genes (DEGs) across major physiological and immunological processes was seen in the *T.b.*xEE2 group, not observed in or that could be deduced from either of the single stressor groups (Bailey et al., 2019). All groups exhibited a low intensity immune response, in contrast to what was reported for advanced PKD. This might suggest a trade-off where the host increases investment in recovery/resolution processes over immune responses at a later stage of disease. In the *T.b.*xEE2 group several T cell related genes (*tbet*, *cd8α*, *foxp3-1*, *foxp3-2*, *cd3e* and *mhc-II*) were downregulated (Figure 3.A) (Bailey et al., 2019). This could in-

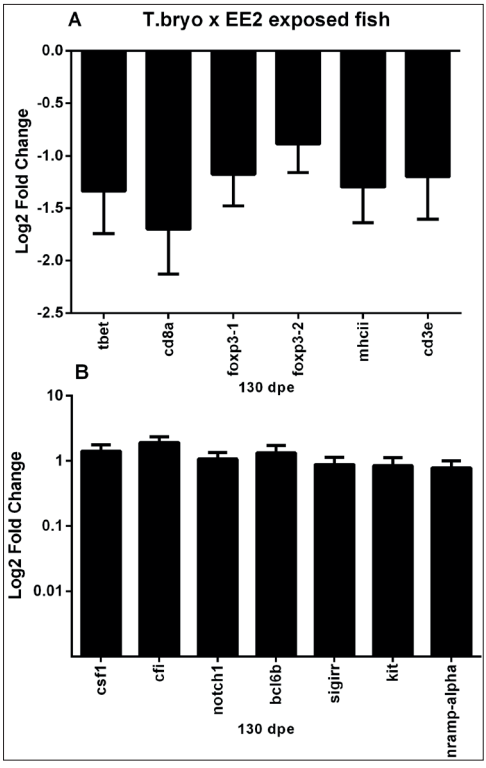

Figure 3. Impact of multiple stressor exposure (*T.b.*+EE2) on T cell-related genes at 130 dpe in the posterior kidney. T cell-associated genes shown to be significantly downregulated (A); immune genes shown to be significantly upregulated (B).

dicate that *T.b.*xEE2 might be having a greater immunomodulating role than in *T.b.* infected fish, leading to a reduction in PKD associated immune response and attenuating the disease impact (Bailey et al., 2019). Alternatively, as a greater number of immune genes were upregulated (*mcsf*, *cfi*, *notch1*, *bcl6b*, *sigirr*, *kit* and *nramp-α*) in these fish this may have translated into a lower parasite burden and reduced pathological alterations (Figure 3.B). In conclusion, the EE2 effects were paradoxical, that they exerted both immunosuppressive and immunoenhancing actions as reported in mammals. This is likely influenced by the pathogen (chronic/acute), the EE2 concentration, and physiological status of the host.

Saprolegniosis: who is out there?

M. Saraiva and P. van West

Saprolegnia is a eukaryotic pathogen of fish, endemic to all freshwater habitats around the world. Due to their filamentous hyphal structure oomycetes are often described as fungal-like microorganisms. *Saprolegnia parasitica* (*S.p.*) causes devastating infections to freshwater fish, leading to Saprolegniosis (Figure 4.A) (van West, 2006). *S.p.* tends to favour fish, whereas *S. diclina* prefers to infect fish eggs, although both species are found on all freshwater stages of both salmon and trout. *S.p.* was previously thought to be a secondary pathogen, but this is not the case since *Saprolegnia* is capable of actively suppressing the host immunity and expressing several effector proteins that can translocate into host cells, of which one is able to degrade fish RNA (Belmonte et al., 2014). However, it is likely that other microbes, such as true fungi, play a major role in the initiation of infection and possibly in increasing the severity of saprolegniosis. Fungal–bacterial interactions vary depending on species, strain and environments, and they can be endosymbiotic, synergistic or antagonistic. In general, polymicrobial infections are harder to treat due to increased resistance to antimicrobial therapy, as such, polymicrobial diseases can have increased mortality compared with their monomicrobial counterparts. These mixed communities possess an intercellular form of communication, often referred to as quorum

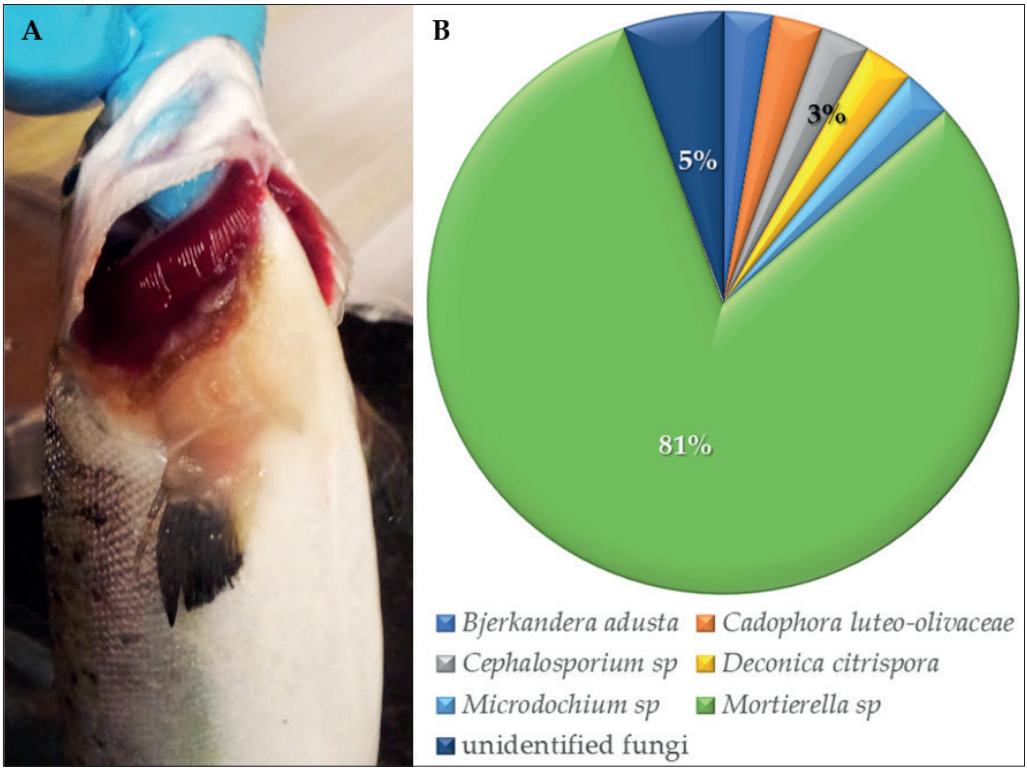

Figure 4. Atlantic salmon infected with *Saprolegnia parasitica* (A); fungal community found on fish in Scotland (B).

sensing (QS). QS is used to monitor the local environment to bring about a concerted change in behaviour beneficial to the community. QS molecules were characterised in several true fungal species (Barriuso et al., 2018) and are involved in growth regulation, stress resistance, morphogenesis, antibiotic production, motility, sporulation and biofilm formation, some produce quorum-sensing inhibitors that prevent communication, reducing their competitors' virulence (Rasmussen et al. 2005). More than 300 pure isolates were obtained from fish farms and hatcheries around Scotland. The second most abundant/represented genus was *Mortierella* (Figure 4.B), a soil fungus with species known to effectively transform a series of fish-toxic diterpenes and their chlorinated analogues into nontoxic metabolites (Kutney et al., 1985). It is speculative at this stage, but we believe this mechanism might aid *Saprolegnia* to infect fish. Metagenomic analysis is being undertaken to understand what species of bacteria and eukaryotes are present in different settings (before, during and after infection). Characterising the complex interactions that occur in these mixed-species communities will undoubtedly increase our understanding of host-pathogen relationships, the mechanisms that underly infection and help design/discovery of novel sustainable control strategies.

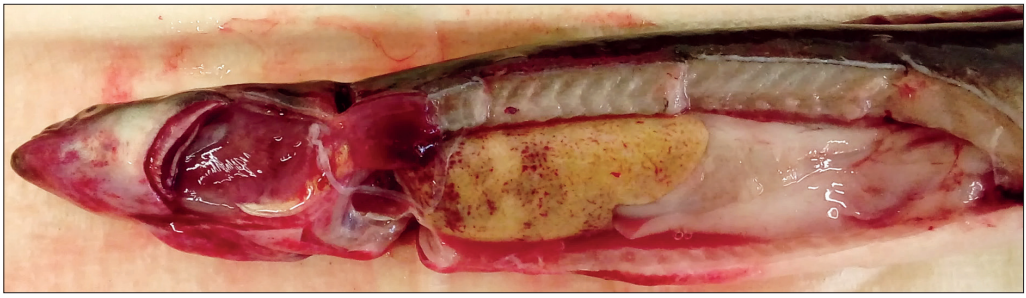

Figure 5. Farmed European eel from a Dutch eel farm with skin lesions, and extreme septicaemia with haemorrhages due to a severe triple infection with two viruses (EVEX, AngHV-1) and (*Vibrio vulnificus*/*navarrensis*).

Multi-causal eel diseases in the Netherlands

O. Haenen, M. Voorbergen-Laarman, E. van Gelderen, A. Davidse, I. Roozenburg, R. Vloet, S. van Beurden and M. Engelsma

Eel disease is seen as one of the factors in the decline of wild eel stocks (Haenen, 2019). Wild eels are farmed for consumption, and some are restocked into the wild against the eel population decline. This implies the transmission of pathogens at least from wild to farmed eel, and possibly back. In the past, global trade of stocking eels caused the introduction of eel parasites and pathogens from South East Asia into West Europe. Examples are the swim bladder nematode *Anguillicoloides crassus* (Van Banning and Haenen, 1990), gill trematodes *Pseudodactylogyrus anguillae* and *P. bini* (Buchmann et al., 1987), and *Herpesvirus anguillae* (AngHV-1) (Davidse et al., 1999). In our diagnostics, farmed eel suffered regularly from multi-causal infections, like *Pseudodactylogyrus* spp. with stress through poor water quality or handling as basis, with single or double viral infections by the AngHV-1, Eel Virus European (EVE), or Eel Virus European X (EVEX) (Haenen et al, 2002; Van Beurden et al., 2012), and single or double bacterial infections with e.g. *Pseudomonas anguilliseptica, Vibrio vulnificus* (Figure 5), *Edwardsiella tarda*, or *Aeromonas salmonicida*. The water temperature thereby is an important factor, with an optimum per virus, and a positive correlation for most bacteria and parasites in replication. It is recommended, to recruit healthy wild eels regionally, avoid global trade of live eels, avoid stress in eel farming, and check farmed eels for the absence of diseases before they are restocked regionally into the wild.

Flavobacterium branchiophilum co-infection can increase pathological changes during koi sleepy disease caused by carp edema virus infection in carp

M. Adamek, F. Teitge, V. Jung-Schroers, M. Heling, D. Gela, V. Piackova, M. Kocour and D. Steinhagen

Koi sleepy disease (KSD) affecting common carp is an often-fatal gill condition. Carp edema virus (CEV) is treated as the causative agent of KSD, however the disease often seems to have multifactorial causes (Way et al., 2017). Therefore, we hypothesised that CEV infection

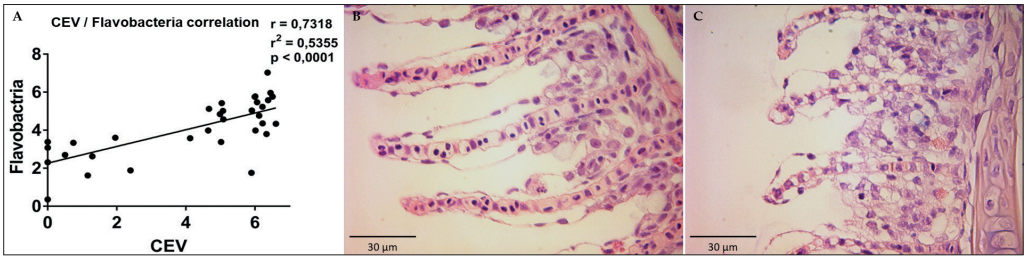

Figure 6. Correlation analysis between CEV and Flavobacteria loads in gills of common carp farmed in Germany and Hungary (A). Gill pathology in koi affected by CEV with flavobacterial co-infection, treated with 18 mg/l of florfenicol (B), or untreated (C); H&E.

may promote infections of gills with secondary pathogens, which subsequently increase the severity of pathology. We analysed the possibility of an interaction between infections with Flavobacteria and CEV during the development of clinical KSD. We examined gill samples of carp and koi from Germany and Hungary and performed infection experiments and antibiotic treatment of KSD affected fish. The amounts of Flavobacteria and CEV were evaluated by qPCR. The typing of Flavobacteria was performed by isolating the bacteria by culturing method using Cytophaga Agar Base and by molecular cloning and sequencing of the 16S amplicon. Pathogenesis was monitored by analysis of fish behaviour and by gill histology. The co-infection with CEV and Flavobacteria was diagnosed in samples collected from common carp aquaculture. There was a positive correlation ($r=0.7318$, $p<0.0001$) between CEV and Flavobacteria loads (Figure 6.A) in gills. Especially individuals with higher CEV loads (>10^5 virus copies/250 ng DNA) had statistically higher Flavobacteria loads in gills when compared to CEV-free fish. Infection experiments indicated a rapid transfer and progress of CEV and flavobacterial co-infections. Culturing and molecular methods identified *Flavobacterium branchiophilum* (*F.b.*) as the possible Flavobacteria species causing co-infections during KSD. Both CEV and Flavobacteria infections progressed faster in KSD-susceptible koi strain when compared to a KSD-resistant common carp strain (Amur wild carp), which could indicate possible co-dependency of the pathogens. Importantly, further experiments with an antibiotic treatment preventing *F.b.* infection confirmed that CEV is the main pathogen in KSD, as the presence of the bacterial co-infection was not required for KSD pathogenesis. However, CEV-*F.b.* co-infection could be responsible for increased levels of epithelial cells hyperplasia, epithelium lifting and proliferation of the intra-lamellar cellular mass, when compared to single CEV infection (Figures 6.B and 6.C). Therefore, *F.b.* co-infection could worsen pathological changes recorded during KSD outbreaks. Our results suggest that CEV gill infection may facilitate co-infections with other pathogens (Adamek et al., 2018).

Simultaneous and sequential co-infection patterns modulating rainbow trout response to BCWD and IHN

B. Gorgoglione, D.R. Jones, D.W. Leaman and A.R. Wargo

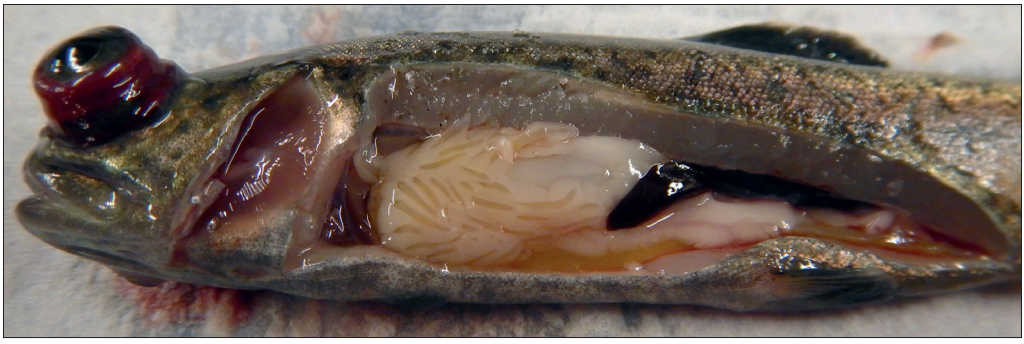

Figure 7. Necropsy of rainbow trout sequentially co-infected with *Flavobacterium psychrophilum* and IHNV, showing exacerbated exophthalmos and ascites.

Few studies have addressed co-infections with multiple pathogen species in salmonids. Those conducted have shown increased susceptibility and pathogenesis (Kotob et al., 2017; Nicholson et al., 2019), or enhanced or prolonged immune responses (Gorgoglione et al., 2019). *Flavobacterium psychrophilum* (*F.p.*), causing bacterial cold-water disease (BCWD), and *Salmonid novirhabdovirus* (IHNV), causing infectious hematopoietic necrosis (IHN), are major pathogens affecting rainbow trout in aquaculture, although little is known about their interaction. Our study investigated how *F.p.* and IHNV co-infections alter the host's ability to respond to these pathogens. Seventy fish per treatment underwent the following pathogen exposures: single IHNV; sequential *F.p.*+IHNV; single *F.p.*; simultaneous *F.p.*+IHNV; negative control. Trout were maintained in a flow-through system at 15°C in individual 0.8 l tanks. For single infections fish were respectively IP-injected with 50 µl *F.p.* suspension, with sterile TYES medium (*F.p.* control), bath-challenged with IHNV (C-genogroup), or with sterile medium (IHNV control). Fish allocated to the sequential co-infection group were infected with bacteria, and after 48 h with INHV, using the same dosage as single infections. Trout organs were sampled at 1,3,5,7,9 and 11 dpe. The simultaneous *F.p.*+IHNV co-infection induced the fastest and most exacerbated pathology, with mortality starting at 5 dpe and peaking between 6-7 dpe. These trout showed typical clinical signs of IHNV, including marked exophthalmos (Figure 7), anaemia, ascites often with diffuse haemorrhaging to abdominal organs, and a higher splenosomatic index at every time-point. The sequential infection group exhibited pathological signs, although milder, and with mortality starting at 8-9 dpe. A much milder gross pathology was observed in the single infection groups, with no mortality up to 11 dpe. Our results are in line with similar synergistic pathology and mortality results described upon *F.p.*+IHNV co-infections, from a different infection methodology (Ma et al., 2019). Sequential infections are likely to be more biologically relevant, thus more suitable to study pathobiological dynamics during co-infections, and better quantifiable immune responses, when compared to simultaneous co-infections where the combined pathogenetic effect of multiple pathogens exacerbates the pathology and drastically reduces host survival. Studies on heterogenous co-infections

Gill histopathology scoring vs gross morphology and transcriptome analysis in farmed Atlantic salmon

P. Noguera, E. Król, A. Douglas, R. Bickerdike, V. Valdenegro, K. Gajardo and S.A.M. Martin

are likely to lead to insights regarding disease management in the field, where co-infection is common.

As a multifunctional organ, gills are the primary site for respiration, osmoregulation, acid-base balance and metabolism of circulating hormones and xenobiotics. Gill diseases in farmed Atlantic salmon have been on the rise in recent years becoming a serious welfare concern and causing substantial economic losses. From the effects of well-characterised agents, eg *Neoparamoeba perurans* causing amoebic gill disease (AGD), to the combined response to AGD and other biological or non-biological factors, gill health represents a major challenge for salmon industry (Herrero et al., 2018). Aimed to support early actions on management strategies, disease control schemes implemented at farm level include periodical monitoring for scoring gross macroscopic changes associated with AGD and proliferative gill disease (PGD). Salmon gills from geographically diverse Scottish aquaculture sites were examined to explore the underlying molecular events associated with the progression of the above-mentioned conditions. After *in situ* macroscopic scoring of over 200 fish, with data recorded from both surfaces of all 8 arches. The gill arch with the highest PGD score was sampled for histopathology and RNA-seq analysis. The histological evaluation and scoring (Mitchell et al., 2012) was performed on digitalised images of routine H&E stained sections, while extracted RNA from a subset of 43 fish presenting low (=1) or medium score (=3) PGD scores were analysed by whole transcriptome analysis using RNA-seq. For each fish, 20 M reads were generated and mapped to the Atlantic salmon genome. The predominant histopathological features included hyperplastic and proliferative lesions, frequently associated with a degree of lamellar fusion, inflammatory reaction, presence of amoeba and relevant vascular lesions. Changes in gross morphology and histopathology were not consistent with each other but were rather linked to the sampling location. Results from RNA-seq analysis on same individuals showed a similar trend. The minimal common responses between different sites suggest spatio-temporal location represents an additional important factor influencing the gill response. Histopathology scores, and differences in gene expression, were driven by the site rather than by PGD scores, clustering together based on the sample origin. This suggests that macro and micro scores may inform on the overall progression of gill damage, but not on underlying pathology, providing support for a complex and multifactorial aetiology of the gill condition.

Multiple co-infections and environmental stressors as causes of chronic mortalities in juvenile sturgeons

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Sturgeon farming is an increasing activity with great interest in *Huso huso* species for caviar

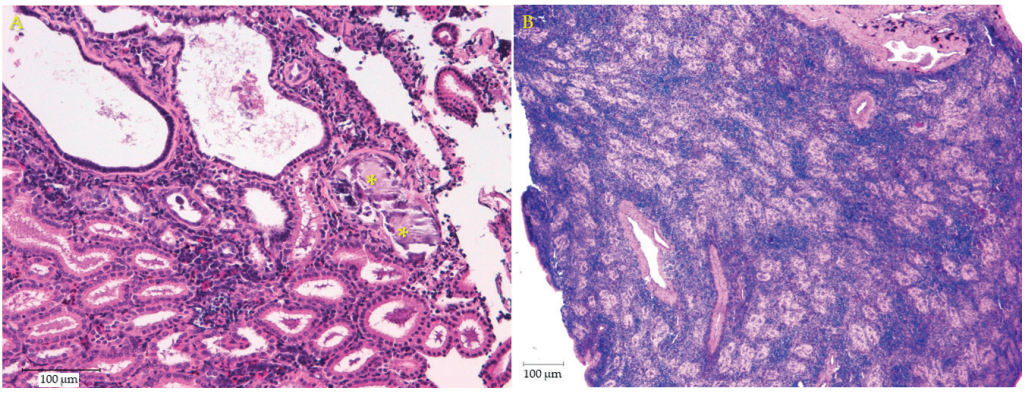

Figure 8. Nephrocalcinosis in kidney of sturgeon (A); spleen with vascular hyalinosis and depletion of the white and red pulp (B). H&E.

production and restocking. Currently, few data on *H. huso* health issues and on sturgeon pathology are available. We examined an episode of chronic mortality in juvenile *H. huso* in Italy, showing inverted and circular swimming, hyperactivity to stimuli alternated to prolonged resting on the bottom. Samples from the brain, kidney, spleen, gill, skin were collected for bacteriological and virologic analyses. Cytology of coelomatic effusions was performed and the main organs were sampled for histology. Body shape deformities, epaxial muscle softening, multifocal ulcerative dermatitis and a sero-haemorrhagic coelomatic effusion, which at cytological examination showed to be septic due to the presence of Gram-negative rods and pigmented-wall hyphae were observed. Bacteriology indicated septicaemia, due to *Aeromonas veronii*, *Shewanella* spp. and *Citrobacter freundii*. No viral growth was obtained in WSSK-1 cells, a known suitable cell line for sturgeon herpesviruses, nor investigated viruses (viz *Betanodavirus* and sturgeon nucleo-cytoplasmic large DNA viruses) were detected by PCR (Ciulli et al., 2016). Histology showed rarefaction and disorganisation of hematopoietic lymphoid tissue. Other findings, such as glomerular regeneration, sporadic intratubular mineral deposits in the renal parenchyma (Figure 8.A) and splenic vessel hyalinosis (Figure 8.B), were detected and interpreted as a response to negative environmental impacts, as previously reported in sturgeons (Romanov et al., 2006). The histology confirmed septicaemia, showing bacterial aggregates and pigmented-wall hyphae in several tissues. Current evidence suggests that pathological findings result from the sum of environmental stressors and multiple co-infections. Despite some of the bacteria found in this study being previously associated with mortality outbreaks in several fish species (Gholamhosseini et al., 2018; Volpe et al., 2019), other primary causes could be present, such as Herpesvirus or Adenovirus. In the absence of other primary biological agents, prolonged environmental stressors could be considered the cause of immune system impairment, facilitating the entry of opportunistic bacteria and mycetes. Indeed, the nephrocalcinosis and vessel hyalinosis suggested the presence of predisposing

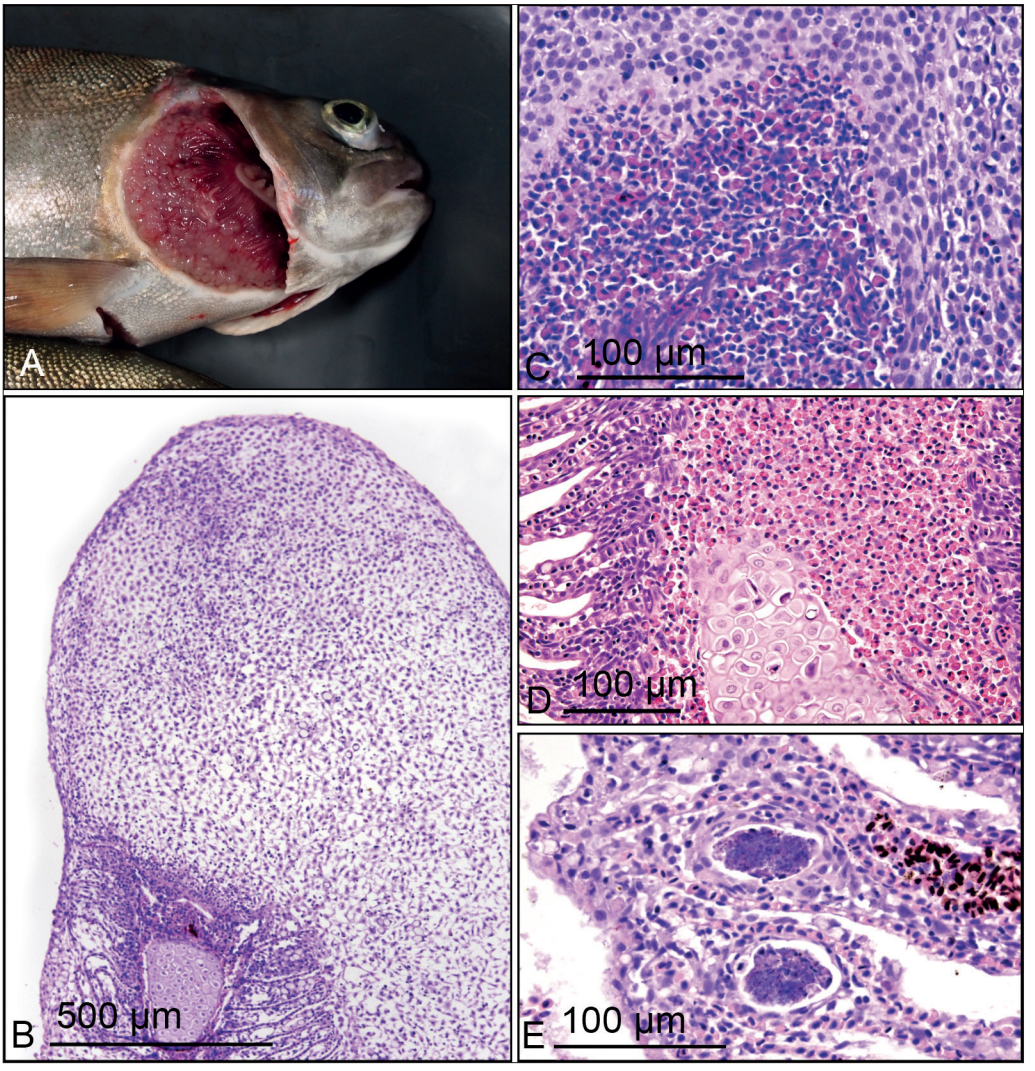

Figure 9. Macroscopic and histopathological findings in rainbow trout from RAS: marked gill swelling (A); hyperplasia of gill epithelium ("clubbing") (B); inflammatory changes in gill filaments with prevailing lymphocytes (C); eosinophilic granular cells predominating in inflammatory infiltrate along gill filaments axis (D); gill filament segments with lamellar fusion and channels with bacteria aggregates (E). H&E.

environmental stressors. Ongoing analyses, including electron microscopy and metagenomics, are further investigating the presence of viral pathogens that could have triggered the impairment of the *H. huso* immune system.

Component causes of severe gill damage in rainbow trout farmed under conditions of RAS

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In the fall of 2018, a rainbow trout farm using an indoor recirculating aquaculture system (RAS) reported disease issues, with mortality among stocks of marketing weight. To assess the health condition of the entire system, we combined detailed examinations with a series of tests of water quality and evaluation of water treatment effects. During five repeated samplings we performed necropsies, histopathological examination, and attempted the isolation of bacteria and amoebae. RAS water temperature ranged between 15.0 and 18.7°C. Clinical signs included frequent jumping of fish with grossly visible lesions, including marked gill swelling (Figure 9.A). The presence of *Ichthyophthirius multifiliis* (0–20 specimens in a microscopic field at 50X magnification) was only found in gills at the first sampling. Histopathological lesions were constantly including lamellar hypertrophy (like that described after exposure to high ammonia levels), lamellar fusion, and enormous hyperplasia of the epithelium in the distal part of filaments ("clubbing") (Figure 9.B). Chronic branchitis was found in a few instances (Figure 9.C), characterised by a dense accumulation of eosinophilic granular cells along the vascular axis of gill filaments (Figure 9.D). Bacterial clusters seen at the gill histological examination were identified upon the microbiological examination (Figure 9.E), revealing the multiple presences of *Pseudomonas koreensis*, *Aeromonas hydrophila*, *A. eucrenophila* and *A. bestiarum* (isolated using BA and TYES media). Amoebae isolation attempts were in contrast negative. Hydrochemical analysis revealed hypersaturation (oxygen up to 200%), corresponding to the presence of gas bubbles on fins at one sampling point. Therapeutic measures taken by the farmer included water ozonation, daily application of peracetic acid, or addition of salt and formaldehyde in a long-term bath. Based on retrieved results, the exact cause of gill damage could not be identified, thus it can be speculatively attributed to the conjunct action of bacteria and *I. multifiliis*, in combination with the long-term exposure to multiple chemicals used for treatment in combination with gas hypersaturation.

Pathogen interactions during experimental co-infection with *Piscirickettsia salmonis* and *Piscine Orthoreovirus* in *Salmo salar*

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Piscine orthoreovirus (PRV) infections are widespread in Chilean salmon farms, estimating that over 60% of freshwater Atlantic salmon is predominantly infected with PRV-1 (SUBPESCA, 2018). In this scenario, co-infections with other highly prevalent viruses or bacteria are likely to occur. Clinical signs could be misdiagnosed or be more severe due to different responses triggered during simultaneous infections with two or more pathogens. We evaluated the effect of the natural exposure to PRV-1 of a smolt population of Atlantic salmon on the outcome of a cohabitation challenge with *Piscirickettsia salmonis* (*P.s.*), the most relevant bacterial pathogen for the Chilean salmon industry (Rozas and Enríquez, 2014). From a population of PRV-1 positive salmon smolts (100 g), 84 shedder fish were intraperitoneally injected with $5.2x10^6$ CFU *P.s.* and placed together with 156 PRV-1 positive cohabitant fish to reach 35% pressure

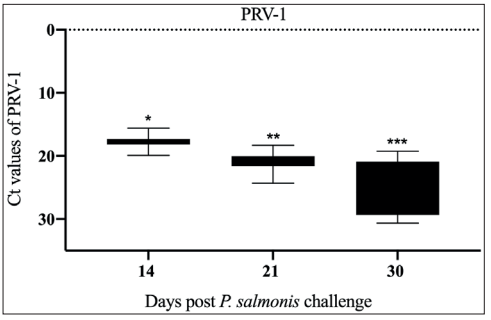

Figure 10. PRV-1 load during an experimental cohabitation challenge with *P. salmonis*. Significant differences between time-points are indicated (*p<0.0001).

of infection. Samples were taken at 14, 21, and 30 dpe to evaluate viral loads and immune gene expression in the head kidney by qPCR and RT-PCR. For histology, heart, head kidney, liver, spleen, and gill were sampled. Viral loads, and Type I Interferon transcripts, decreased significantly from 14 to 21 dpe and up to 30 dpe (Figure 10). This was accompanied by a rise in the percentage of *P.s.* positive co-habitants and IL-8 expression from 14 to 21 dpe, with a drop at 30 dpe. Our results indicate that the bacteria pressed against the viral infection, decreasing the viral load in the host. Histopathological examination showed only mild to moderate lesions compatible with initial SRS or HSMI in liver and heart, and only in samples at 21 dpe. Tissue examination at 14 and 30 dpe did not exhibit pathological evidence attributable to any disease. The previous natural exposure to PRV-1 did not increase the severity of SRS in a population of salmon smolts exposed to *P.s*. cohabitation challenge, however, the viral loads dropped as the proportion of *P.s*. positive fish increased. Co-infection with these two biological agents was achieved, but only mild to moderate clinical signs were observed, at 21 dpe.

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