Vitenskapskomiteen for mat og miljø
Norwegian Scientific Committee for Food and Environment

VKM Report 2019:02

The risk of transmission of infectious disease through trade of cryopreserved milt

Scientific opinion of the Panel on Animal Health and Welfare of the Norwegian Scientific Committee for Food and Environment

Report from the Norwegian Scientific Committee for Food and Environment (VKM) 2019:02
The risk of transmission of infectious disease through trade of cryopreserved milt

Scientific Opinion of the Panel on Animal Health and Welfare of the Norwegian Scientific Committee for Food and Environment
27.03.2019

ISBN: 978-82-8259-318-2
ISSN: 2535-4019
Norwegian Scientific Committee for Food and Environment (VKM)
Po 4404 Nydalen
N – 0403 Oslo
Norway

Phone: +47 21 62 28 00
Email: vkm@vkm.no

vkm.no
vkm.no/english

Cover photo: Colourbox

Suggested citation: VKM, Espen Rimstad, Dean Basic, Edgar Brun, Duncan Colquhoun, Niels Jørgen Olesen, Knut Egil Bøe, Tor Gjøen, Jacques Godfroid, Andrew M. Janczak, Knut Madslien, Rolf Erik Olsen, Øyvind Øverli, Angelika Agdestein (2019). The risk of transmission of infectious disease through trade of cryopreserved milt. Scientific Opinion of the Panel on Animal Health and Welfare of the Norwegian Scientific Committee for Food Safety. VKM report 2019:02, ISBN: 978-82-8259-318-2, ISSN: 2535-4019. Norwegian Scientific Committee for Food and Environment (VKM), Oslo, Norway.

The risk of transmission of infectious disease through trade of cryopreserved milt

Preparation of the opinion

The Norwegian Scientific Committee for Food and Environment (Vitenskapskomiteen for mat og miljø, VKM) appointed a project group to respond to the request from the Norwegian Food Safety Authority. The project group consisted of four persons, and a project leader from the VKM secretariat. Two external referees reviewed and commented the manuscript. The VKM Panel on Animal Health and Welfare evaluated and approved the final opinion drafted by the project group.

Authors of the opinion

Members of the project group that contributed to the drafting of the opinion (in alphabetical order after chair of the project group):

Espen Rimstad – Chair of the project group and member of the Panel on Animal Health and Welfare in VKM. Affiliation: 1) VKM; 2) Norwegian University of Life Sciences

Dean Basic – Member of the project group and project leader in the VKM secretariat. Affiliation: VKM

Edgar Brun – Member of the project group. Affiliation: Norwegian Veterinary Institute

Duncan Colquhoun – Member of the project group. Affiliation: Norwegian Veterinary Institute

Niels Jørgen Olesen – Member of the project group. Affiliation: Technical University of Denmark, DTU Aqua

Members of the Panel on Animal Health and Welfare that contributed to and approved the opinion:

In addition to Espen Rimstad, Dean Basic, Edgar Brun, Duncan Colquhoun and Niels Jørgen Olesen, these were (in alphabetical order before chair of the Panel):

Knut Egil Bøe – Member of the Panel on Animal Health and Welfare. Affiliation: 1) VKM; 2) Norwegian University of Life Sciences

Tor Gjøen – Member of the Panel on Animal Health and Welfare. Affiliation: 1) VKM; 2) University of Oslo

Jacques Godfroid – Member of the Panel on Animal Health and Welfare. Affiliation: 1) VKM; 2) UiT The Arctic University of Norway

Andrew M. Janczak – Member of the Panel on Animal Health and Welfare. Affiliation: 1) VKM; 2) Norwegian University of Life Sciences

Knut Madslien – Member of the Panel on Animal Health and Welfare. Affiliation: 1) VKM; 2) Norwegian Veterinary Institute

Rolf Erik Olsen – Member of the Panel on Animal Health and Welfare. Affiliation: 1) VKM; 2) NTNU – Norwegian University of Science and Technology

Øyvind Øverli – Member of the Panel on Animal Health and Welfare. Affiliation: 1) VKM; 2) Norwegian University of Life Sciences

Angelika Agdestein – Chair of the Panel on Animal Health and Welfare. Affiliation: 1) VKM; 2) Norwegian Veterinary Institute

Acknowledgment

VKM would like to thank Brit Hjeltnes and Paul Midtlyng for their valuable input on an early draft of the manuscript. VKM is grateful to the hearing experts Rudi Ripman Seim (Benchmark Genetics/SalmoBreed AS), Nina Santi and Torkjel Bruheim (AquaGen AS) and Per Helge Bergtun (Mowi Breeding AS) for sharing their views and experiences with the project group regarding cryopreservation of milt. VKM would like to thank Nina Santi for inviting the project group to Kyrksæterøra for demonstration of the procedures concerning milt extraction from broodfish and the subsequent cryopreservation process. VKM would also like to thank the external referees Maureen K. Purcell (U.S. Geological Survey, Western Fisheries Research Center, Seattle) and Lori L. Gustafson (The United States Department of Agriculture, Animal and Plant Health Inspection Service, Center for Epidemiology and Animal Health) for their valuable comments during critical review of the draft opinion. VKM emphasises that the two referees are not responsible for the content of the final opinion. In accordance with VKM's routines for approval of a risk assessment (VKM 2018), VKM received their comments before evaluation and approval by the Panel, and before the opinion was finalised for publication.

Competence of VKM experts

Persons working for VKM, either as appointed members of the Committee or as external experts, do this by virtue of their scientific expertise, not as representatives for their employers or third party interests. The Civil Services Act instructions on legal competence apply for all work prepared by VKM.

Table of Contents

Summary

Background

Control of reproduction is an important prerequisite for farming of fish. Trade of fertilized eggs has been important for the progression of farming of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Eggs from rainbow trout, in particular, have been extensively exported to many countries worldwide. Traits like growth, sexual maturation and disease resistance can be improved through selection and modern breeding programs. One example of the benefits of selective breeding in salmonids is the development of Infectious pancreas necrosis (IPN)-resistant Atlantic salmon and rainbow trout. Such efforts require significant resources. Sexual products are traded across borders. In salmonid fish farming this trade has traditionally consisted of fertilized eggs. Ensuring that fertilized eggs are free from infectious agents is, however, challenging. The potential for transmission through sexual products is dependent on control procedures, surveillance programs, clinical status in broodfish, numbers of infectious particles within the gonads, success/failure of disinfection process etc. Trade of cryopreserved milt represents an alternative and is widely used for terrestrial animals insemination. Knowledge on the extent to which infectious diseases may be transferred through cryopreserved milt is limited. An examination of the risks of disease transmission, as well as measures to address these risks, may initiate the development of international standards for the trade of cryopreserved milt. Such information could ease the adoption of advanced terrestrial animal breeding techniques in aquaculture species.

Terms of reference

In December 2017, the Norwegian Food Safety Authority requested the Norwegian Scientific Committee for Food and Environment (VKM) to provide an opinion on the risks of infectious disease transmission associated with the trade of cryopreserved milt from farmed Atlantic salmon and rainbow trout. VKM was also asked to investigate risk-reducing measures, as well as requirements for exporters to document freedom from infectious agents in cryopreserved milt.

Working group and evaluation of risk assessment

VKM established a working group consisting of one member from the Panel on Animal Health and Welfare and two external experts from the Norwegian Veterinary Institute and one from the Technical University of Denmark. Two external experts have reviewed and commented upon the manuscript. The Panel on Animal Health and Welfare has evaluated and approved the final report draft submitted by the project group.

Methodology

This report is a qualitative risk assessment. The risk of transmission of a particular pathogenic agent from salmon or rainbow trout milt to offspring is defined as the probability of the infectious hazard multiplied by the consequences of such an event occurring.

Published studies specifically addressing the role of fish semen in transmission of pathogens are very sparse. Most scientific data on the presence of infectious agents in sexual products from salmonids relates to eggs.

Empirical evidence suggest that surface disinfection of fertilized eggs is generally effective at breaking vertical transmission. This indicates that transport of sperm associated pathogens into the egg is not a significant route of transmission for most diseases. However, there are relatively few scientific reports that have studied such events in sufficient detail.

Different fish health related hazards associated with the trade of cryopreserved milt have been identified and characterized in the report. The working group used a qualitative approach with a 3-grade scale for assessing the risk of disease transmission via milt.

Hazard identification and characterization

Unlike internally fertilized animals, such as mammals for which infectious disease may be transmitted to the female through artificial insemination, sexual transmission of disease in salmonids applies only to the offspring. Milt may harbour infectious agents originating from the broodfish itself. Due to the open nature of the genital papilla during the later stages of sexual maturation, milt is likely to become contaminated with environmental agents. This may also occur during stripping or extraction of milt. The likelihood of transmission of any agent via milt will depend on the numbers of specific infectious particles within the broodfish gonads, the higher the number the greater the likelihood. The utilisation of specific pathogen free populations of broodfish would remove or minimize this possibility.

Salmonid eggs are routinely surface disinfected post-fertilization. Disinfection of milt, however, is not possible without compromising viability. The only known use of frozen milt is fertilization of eggs.

Infectious pancreatic necrosis virus and *Renibacterium salmoninarum* were identified as highly relevant followed by *Piscirickettsia salmonis*, *Flavobacterium psychrophilum* (rainbow trout) and *Infectious hematopoietic necrosis virus*. These infections may be transmitted via sexual products, potentially also frozen milt. It is crucial that the broodfish population is free of these agents.

Other salmonid pathogens were considered of lower relevance. Examples of agents that may contaminate or be present in milt are *Infectious salmon anaemia virus*, *Piscine orthoreovirus,* Piscine myocarditis virus*,* Salmonid alphavirus*, Viral haemorrhagic septicaemia virus, Yersinia ruckeri* and *Aeromonas salmonicida.*

The term ‘certified disease freedom’ means that the agent causing the disease is not present within a specific compartment/zone. Approved disease-free status for diseases described in present EU and Norwegian legislation (Infectious hematopoietic necrosis, Viral haemorrhagic septicaemia, Infectious salmon anaemia, Epizootic hematopoietic necrosis, Pancreas disease) is mandatory for all farms with brood-stock producing frozen milt.

For systemic infections and infections involving several organs it is possible that the agent may also be present in milt. For such infections, testing of tissue samples other than milt is applicable. Specific testing cannot certify freedom from infection.

Conclusions

Infectious agents present in milt originate either from the broodfish or via contamination during stripping/extraction of milt. However, the only known practical use of milt from atlantic salmon or rainbow trout is fertilization of eggs, and fertilized eggs can be surface disinfected. Some agents may be transmitted vertically, and although they may perhaps not be inside the egg, disinfection may nevertheless not be fully effective. Biosecurity measures and disinfection of fertilized eggs will reduce the probability of transmission through milt, provided that disinfection is properly performed and the agent is sensitive to the disinfection procedure.

Data gaps and uncertainties

Studies of milt as a vehicle for pathogen transmission are sparse. Studies of vertical transmission tend to give equivocal results. Experimental designs vary and are not always optimal. These factors cause uncertainty of the assessment.

Key words: Cryopreserved milt, Atlantic salmon, rainbow trout, infectious disease, compartment, *Infectious pancreatic necrosis virus, Renibacterium salmoninarum*, *Piscirickettsia salmonis*, *Flavobacterium psychrophilum*, *Infectious hematopoietic necrosis virus*, VKM, risk assessment, Norwegian Scientific Committee for Food and Environment, Norwegian Food Safety Authority

Sammendrag på norsk

Bakgrunn

Kontroll med reproduksjon er en viktig forutsetning for vellykket fiskeoppdrett. Befruktede egg har vært et viktig innsatsmiddel som har bidratt til avlsfremgangen i oppdrett av atlantisk laks (*Salmo salar*) og regnbueørret (*Oncorhynchus mykiss*). Spesielt, har befruktede egg fra regnbueørret blitt eksportert til mange land over hele verden. Egenskaper som vekst, kjønnsmodning og sykdomsresistens kan forbedres gjennom moderne avlsprogrammer. Et eksempel på fordelene med selektiv avl hos laksefisk er utviklingen av IPN-resistent atlantisk laks og regnbueørret. Slike forbedringer krever betydelige ressurser og fører til at det er behov for handel med befruktede egg over landegrensen. Det er en utfordring å sikre at befruktede egg er fri for smittestoffer. Potensialet for overføring av smitte gjennom kjønnsprodukter er avhengig av kontrollprosedyrer, overvåkingsprogrammer, klinisk status hos stamfisk, antall smittsomt agens i gonadene, eventuelle feil i desinfeksjonsprosedyrer etc. Handel med frossen fiskemelke representerer et alternativ og brukes mye hos landdyr. Kunnskap om i hvilken grad smittsomme sykdommer kan overføres gjennom frossen melke er begrenset. En oversikt over risikoen for overføring av sykdommer, samt tiltak for å håndtere disse, kan bidra til utvikling av internasjonale standarder for handel med frossen melke. Dette kan lette innføringen av tilsvarende avlsfremskritt som man har hatt hos landdyr til oppdrettsfisk.

Oppdrag

Mattilsynet ba i desember 2017 Vitenskapskomiteen for mat og miljø (VKM) om en vurdering av risiko for overføring av smittsomme sykdommer knyttet til handel med frossen melke fra atlantisk laks og regnbueørret. VKM ble også bedt om å undersøke risikoreduserende tiltak, samt krav til eksportører for å dokumentere frihet fra smittestoff i frossen melke.

Arbeidsgruppe og evaluering av risikovurdering

VKM nedsatte en arbeidsgruppe bestående av en medlem fra faggruppen for dyrehelse og dyrevelferd og to eksterne eksperter fra Veterinærinstituttet og en fra Danmarks tekniske universitet. To eksterne fagfeller har gått gjennom og kommentert rapporten. Faggruppen for dyrehelse og dyrevelferd evaluerte og godkjente den endelige rapporten.

Metodikk

Denne rapporten er en kvalitativ risikovurdering. Risikoen for overføring av et smittsomt agens i melke til avkom er definert som sannsynligheten for forekomst av agens multiplisert med konsekvensene av dette.

Publiserte studier som spesifikt omhandler melke som kilde til overføring av smittsomt agens er svært sparsom. De fleste vitenskapelige studier om infeksjonsstatus i kjønnsprodukter fra laks er utført på egg.

Overflatedesinfeksjon av befruktede egg er generelt ansett som en relativt effektiv metode for å bryte vertikal smitteoverføring. Dette indikerer at melke ikke er en viktig overføringsvei for de mange sykdommer. Det er imidlertid relativt få vitenskapelige rapporter som har studert dette i tilstrekkelig detalj.

Ulike fiskesykdommer forbundet med handel med frossen melke er identifisert og karakterisert i rapporten. Arbeidsgruppen brukte en kvalitativ tilnærming med en 3-graders skala for å vurdere risikoen for overføring av smittsomme agens gjennom melke.

Identifisering og karakterisering av fare

Hos pattedyr kan smittsomme sykdommer overføres til hunndyret gjennom kunstig befruktning. Hos laksefisk er det overføring til avkom som er aktuelt ved smitte gjennom kjønnsprodukter. Melke kan inneholde smittsomme agens som kommer fra stamfisken eller være kontaminert med agens fra miljøet. Siden kjønnsveien hos laksefisk er åpen hos kjønnsmoden fisk, kan melke bli kontaminert av agens i miljøet, og dette kan også skje under uttak av melke.

Sannsynligheten for overføring av et smittsomt agens gjennom frossen melke vil avhenge av mengden av agens i melken; jo mer agens, jo større sannsynlighet. Patogenfrie populasjoner av stamfisk ville fjerne / minimere denne sannsynligheten.

Overflatedesinfeksjon av lakseegg skal utføres etter befruktning. Desinfeksjon av melke er imidlertid ikke mulig i dag på grunn av at det medfører redusert levedyktighet av spermier. Den eneste kjente bruken av frossen melke er befruktning av egg.

De mest relevante smittsomme agens som kan overføres med frossen melke fra laks og regnbueørret er *Infeksiøs pankreas nekrose virus* og *Renibacterium salmoninarum* etterfulgt av *Piscirickettsia salmonis*, *Flavobacterium psychrophilum* (regnbueørret) og *Infeksiøs hematopoietisk nekrose virus*. Disse infeksjonene kan overføres med kjønnsprodukter, potensielt også frossen melke. Det er viktig at stamfisken er fri for disse smittestoffene.

Andre patogene agens ble ansett å være av lavere relevans. Eksempler på stoffer som kan forurense milt er: *Infeksiøs laksanemi virus, Piscint orthoreovirus,* Piscint myokarditt virus*,* Salmonid alfavirus*, Viralt hemorragisk septikemi virus, Yersinia ruckeri,* og *Aeromonas salmonicida*.

Sykdomsfrihet i et spesifikt område betyr at agens som forårsaker sykdommen ikke er tilstede i dette området / sonen. Stamfiskstasjoner som produserer frossen melke skal ha status som fri for sykdommer beskrevet i dagens lovgivning i EU og Norge (Infeksiøs hematopoietisk nekrose, viral hemorragisk septikemi, infeksiøs lakseanemi, epizootisk hematopoietisk nekrose, pankreas sykdom).

For systemiske infeksjoner og infeksjoner som involverer flere organer, er det mulig at agenset også kan være tilstede i melke. For slike infeksjoner kan testing av andre vevsprøver enn melke være aktuelt. Spesifikk testing av et agens kan ikke garantere frihet fra agenset.

Konklusjoner

Smittsomme agens i melke kommer enten fra stamfisken, eller fra kontaminering fra miljøet gjennom kjønnsåpning eller under uttak av melke. Den eneste kjente praktiske bruk av frossen melke fra laks eller regnbueørret er befruktning av egg, og befruktede egg kan overflatedesinfiseres. Noen smittsomme agens kan overføres vertikalt, de befinner seg kanskje ikke inne i egget, men desinfeksjonen er likevel ikke fullt ut effektiv. Biosikkerhetstiltak og desinfeksjon av befruktede egg vil redusere sannsynligheten for overføring gjennom melke, forutsatt at desinfeksjonen utføres riktig og agens er følsomt overfor desinfeksjonsmiddelet.

Kunnskapshull og usikkerheter

Det er få studier om betydningen av melke for overføring av smittsomme agens. Studier av vertikal overføring har en tendens til å gi ulike resultater. Eksperimentelle design for slike studier varierer og har ikke alltid vært optimal. Disse faktorene bidrar til usikkerhet i vurderingen.

Nøkkelord: Frossen melke, atlantisk laks, regnbueørret, smittsom sykdom, kompartment *infeksiøs pankreas nekrosevirus*, *Renibacterium salmoninarum*, *Piscirickettsia salmonis*, *Flavobacterium psychrophilum*, *infeksiøs hematopoietisk nekrosevirus*, VKM, risikovurdering, Vitenskapskomiteen for mat og miljø, Mattilsynet.

Abbreviations and glossary

Abbreviations

BKD = Bacterial Kidney Disease

CP = Cryopreservation

CMS = Cardiomyopathy syndrome

CWD = Coldwater disease

EHNV = Epizootic haematopoietic necrosis virus

ERM = Enteric redmouth disease

HIV = Human immunodeficiency virus

HPR = Highly polymorphic region

HSMI = Heart and skeletal muscle inflammation

IHNV = Infectious hematopoietic necrosis virus

IPNV = Infectious pancreatic necrosis virus

ISAV = Infectious salmon anaemia virus

OIE = World Organization for Animal Health

NFSA = Norwegian Food Safety Authority (In Norwegian: Mattilsynet)

NKEF = Natural killer cell enhancing factor

PMCV = Piscine myocarditis virus

PRV = Piscine orthoreovirus

RAS = Recirculating aquaculture system

RTFS = Rainbow trout fry syndrome

SRS = Salmonid rickettsial septicaemia

VHSV = Viral haemorrhagic septicaemia virus

VKM = Norwegian Scientific Committee for Food and Environment

Glossary

Compartment = one or more farms covered by a common biosecurity regime containing an aquatic animal population with a certified health status in respect to a specific disease. A compartment is a single epidemiological unit based on geographical location, distance from other farms, and a water source not influenced by the animal health status in the surroundings.

Epidemiological unit = a group of animals that share approximately the same risk of exposure to a pathogenic agent within a defined location.

Horizontal transmission = fish to fish transfer of a pathogen

Fry = recently hatched fish larvae

Infection = growth of pathogenic microorganisms in the body, whether or not body function is impaired

Infection pressure = concentration of infective pathogens in the environment of susceptible hosts

Milt = seminal fluid of male fish

Roe = unfertilized egg from female fish

Spermatozoa = motile sperm cells

Smolt/smoltification = juvenile salmon which has undergone physiological changes for a life in seawater/the process of physiological, morphological and behavioural changes that occur in juvenile salmonids to facilitate a transition from freshwater to seawater.

Stripping = Extraction of milt (including gonad removal) from brood fish.

Susceptible species = a species in which infection has been demonstrated by the occurrence of natural cases or by experimental exposure to the pathogenic agent that mimics natural transmission pathways

Vertical transmission = transmission of a pathogen from the parent fish to its progeny via its sexual products

Zone = a specific geographical area defined by natural or artificial barriers

Background as provided by the Norwegian Food Safety Authority

Improvement through breeding is an important prerequisite in all modern livestock production systems. Modern breeding is based on extensive breeding programs, the use of advanced statistical methods and modern biotechnology. The task requires both high levels of competence and financial resources. Consequently, the number of breeding companies in all types of livestock production, is limited. In order to transfer genetic progress to new and current markets, genetic breeding material has to cross borders. In aquaculture today, the traded commodities consist of smolt, fry and roe. In recent years, successful methods for cryopreservation of milt have been developed. This opens up the possibility of using frozen milt in the genetics trade.

All trade in living material entails a risk of transmission of infectious diseases. In cattle breeding, where frozen semen is the dominant traded product, there is continuous development of standards and protocols to eliminate this risk. In aquaculture, little work has been done in this area.

With the exception of Atlantic salmon and rainbow trout, breeding programs for fish are not widespread. When the Norwegian Food Safety Authority requested a risk assessment or an expert opinion, we thought it sensible to start up this work with Atlantic salmon and Rainbow trout. In connection with the literature search, that will be an important basis for this work, we therefore see that it may make sense to include all salmonids. Work done in connection with the cultivation of different species of salmonids could provide interesting information.

Terms of reference as provided by the Norwegian Food Safety Authority

The Norwegian Food Safety Authority asks VKM to assess the risk of transmission of infectious diseases in trade with frozen milt from Atlantic salmon and rainbow trout.

Specifically, we ask for answers to the following questions:

1. Relevant infectious diseases that are thought to be transmitted with frozen milt.
2. At what level will it be necessary to control diseases to ensure that frozen milt is not a source of infection for the relevant infectious diseases (national, regional, locations/sea farms, group and individual level)?
3. What requirements for screening; test material, sampling, method and biosafety are relevant to document freedom from relevant infectious agents in frozen milt?
4. Which measures would help ensure that frozen milt cannot transfer relevant infectious diseases? For example, adding antibiotics to control bacterial diseases in frozen milt.

Assessment

1 Introduction

1.1 General background

Control of reproduction is an important prerequisite for farming of aquaculture fish. Artificial insemination coupled with selective breeding is fundamental for improvement of domesticated stocks. Implementation of breeding programs targeting growth and other important traits has been central to the success of farming of Atlantic salmon (*Salmo salar)*.

Commercial scale artificial insemination of terrestrial animals (cattle) began in the 1930s. The discovery, in 1949, that glycerol protected the viability of sperm during cryopreservation made widespread application of artificial insemination possible. Assuming that advances made in terrestrial animal breeding techniques will be adopted for aquaculture species the future may allow separation of X- and Y-bearing sperm, targeted gene editing and the application of stem cell technology (Lonergan, 2018). Stem cell technology with *in vitro* gametogenesis can theoretically allow production of offspring from oocytes and *in vitro* derived sperm (Nagamatsu and Hayashi, 2017).

Trade of fertilized eggs has been important for the progression of Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) farming. Rainbow trout eggs, in particular, have been extensively exported to many countries worldwide. Although a large number of small producers remain, the global egg trade is dominated by large-scale brood-stock companies.

Some pathogens, such as *Renibacterium salmoninarum* and *Infectious pancreatic necrosis virus* (IPNV)*,* are well known to be transmitted via the sexual products of salmonids. The potential for transmission through sexual products is highly dependent on control procedures. Transmission via sexual products depends on factors such as clinical status, numbers of infectious particles within the brood fish or more specifically within the gonads, failure in the process of disinfection of fertilized eggs etc. In general, the higher a load of a particular infectious agent, the greater is the likelihood of transmission. Appropriate assessments of risk are based on knowledge of the characteristics of the pathogens and their infections. Fertilized eggs of salmonids can be disinfected; however, salmonid milt or non-fertilized eggs cannot currently be effectively disinfected without significant reduction in viability.

In mammals artificial insemination utilising thawed, cryopreserved sperm has been used for many decades. Semen may carry infectious agents originating from the male reproductive tract or from infected blood cells leaked into the semen. Risks of disease transmission following artificial insemination in mammals equals risk of transmission to the female animal, and it is thus different from that of salmonids where the risk of disease transmission is solely related to the offspring (Givens, 2018). Thus, sexually transmitted, venereal diseases in mammals caused by, for example, specialized agents such as *Tritrichomonas, Campylobacter, Brucella, Neisseria* have no counterparts in salmonid fish. Certain infections in mammals, e.g. human immunodeficiency virus, bovine herpesvirus 1, bovine viral diarrhoea virus and Schmallenberg virus, among others, may be transmitted through frozen semen to the female animal but may also cause foetal disease.

1.2 Development of genetic resources in salmonid aquaculture

1.2.1 The potential of fish milt for genetic resource management

An excellent example of the benefits of selective breeding in salmonids is the development of IPN-resistant salmon and rainbow trout (Houston et al., 2008). This has dramatically reduced the impact of IPN disease in Norwegian aquaculture. Trade in frozen fish milt could be an efficient way to exploit the benefits of selective breeding as demonstrated by the past and present use of frozen sperm in breeding of terrestrial production animals. The common trade product for salmonids is fertilized eggs (Tables 1.2.1-1 and 1.2.1-2). We are not aware that fish milt is presently traded to any significant extent.

Table 1.2.1-1. International trade in salmonid eggs. Export and import of fertilized salmonid eggs per year, Norway

	2013	**2014**	**2015**	**2016**	**2017**
Exported	30 419 698	54 625 618	60 041 838	52 650 262	48 485 698
Imported	10 887 000	17 276 700	35 032 200	29 850 900	33 812 400

Table 1.2.1-2. The total number of fertilized salmonid eggs incubated per year in Norway.

	2013	**2014**	**2015**	**2016**	**2017**
Fertilized eggs for incubation	636 038 000	719 009 000	560 668 000	450 208 000	460 905 000

1.2.2 Antibacterial characteristics of salmonid milt/seminal fluid

Seminal fluids are complex and may contain antibacterial proteins belonging to the complement system, lysozyme, antimicrobial peptides, phospholipases and iron sequestering proteins such as lactoferrin (Poiani, 2006). A recent study identified 345 individual proteins within the seminal plasma of Chinook salmon, including several members of the complement system (Gombar et al., 2017). Relatively little work is published regarding the antimicrobial properties of piscine seminal fluids but several studies have focused on characterisation of the proteome of fish spermatazoa (Ciereszko et al., 2017) with the majority of proteins identified being highly conserved between carp and rainbow trout (Ciereszko et al., 2017). Dominating proteins within both the seminal plasma and sperm include multitask proteins (including apolipoproteins, transferrin, and serum albumin), proteins related to the immune system (complements) and antioxidant proteins (thioredoxin, superoxide dismutase). An antimicrobial role for sperm-duct mucins in the marine fish *Zosterisessor ophiocephalus* has been postulated (Giacomello et al., 2008). It is apparent therefore that fish milt does contain factors that may limit or prevent survival of some microorganisms. The presence of a seminal microflora in mammals (Javurek et al., 2016) and the ability of seminal fluids to harbour pathogenic organisms does, however, illustrate that these factors do not eliminate microbial life from such fluids.

1.2.3 Seminal fluid as a source of infection

Recent studies suggest that the seminal fluid of mammals has its own microflora (Javurek et al., 2016) and that mammalian sperm may transmit an impressive array of microbial pathogens, both bacterial and viral (Poiani, 2006). Sperm associated pathogens may be transmitted either as a contaminant within the seminal fluid or bound to the surface of the sperm cell (Bielanski, 2007). Seminal fluid is recognised as responsible for a wide range of venereally transmitted infections in mammals including viral, fungal and bacterial diseases, but may also harbour possible probiotic species such as *Lactobacillus* spp. (Poiani, 2006). In humans, removal of seminal plasma and non-sperm cells utilising a technique known as 'sperm washing' has been used to prevent spread of HIV infection to the female or foetus. Such methods are successful because HIV does not attach to, or infect, the sperm cells (Nicopoullos et al., 2010). However, such a method may not be successful for all virus infections; for instance IPNV of salmonids, known to accumulate in ovarian fluid, adsorbs to the salmonid sperm cells (Mulcahy and Pascho, 1984), which may result in transport of the virus into the egg cell. If true, viruses inside the egg would not be susceptible to standard egg surface disinfection.

Several important fish pathogens have been isolated from milt. The fish pathogen *Piscirickettsia salmonis* was detected within the seminal fluid of experimentally inoculated rainbow trout brood-stock, as has vertical transmission of this pathogen to the offspring of such fish (Larenas et al., 2003). IPNV has been isolated from the seminal fluid of asymptomatic arctic char (Souter et al., 1984) and has been shown to adsorb to a high degree to the sperm surface membrane of rainbow trout (Mulcahy and Pascho, 1984). The rhabdoviruses *Viral haemorrhagic septicaemia virus* (VHSV) and *Infectious haematopoietic necrosis virus* (IHNV) have been isolated from the seminal fluid of adult Coho salmon and Sockeye salmon, respectively (Amos et al., 1998; Meyers et al., 1990). *Photobacterium damselae* subs. *piscicida* was identified in the seminal fluid of sea bream (Romalde et al., 1999).

1.3 Surveillance and epidemiological considerations

One prerequisite in the prevention of the spread of infectious diseases is an efficient surveillance system. This requires standardization of criteria for disease diagnostics and characterization. The diagnostic sensitivity of the surveillance system is important for determining whether a population can be declared positive or negative with regards to a specific infectious agent.

Important considerations for any surveillance programme are the laboratory tests used, assumed pathogen prevalence, and choice and number of tissues / animals sampled. Negative results have to be considered carefully, as while the presence of any particular agent can be demonstrated, its absence is less easily proven. Negative results may reflect the uneven distribution of the agent in the tissue sampled or could be due to sub-optimal sampling of tissue or animals, or that the prevalence of the agent is below the detection limit, given the number of animals sampled. In order to increase confidence in a negative result being indicative of an absence, the population should be followed over time and the sampling strategy carefully designed according to the characteristics of the agent being investigated.

The concept of freedom from listed diseases is based on the implementation and fulfilment of relevant international standards. The freedom from disease concept is based on the probability of freedom where the level of probability is dependent on historical data, the sensitivity of the surveillance system, and the probability of a new introduction to a population. It is, however, impossible to provide absolute certainty of the absence of a disease.

1.3.1 Compartments and zones

Norwegian salmon production is generally based on on-growing in semi-open cages in sea water. This is the traditional production system for both fish raised for consumption and for brood-stock. Focus on the production of disease-free populations has required the development of transparent documentation. Compartments and zones are two systems defined by the way animals are kept. Complying with one of these systems secures by definition a disease-free status.

A zone is a concept that covers a specific geographical area defined by natural or artificial barriers. In seawater such barriers may be defined by the distance relevant pathogens may be passively transported. Zones are used to structure containment of a disease outbreak and to (re-)establish freedom of disease status.

A compartment describes one or more farms covered by a common biosecurity regime containing an aquatic animal population with certified health status in respect to a specific disease. A compartment is a single epidemiological unit based on geographical location, distance from other farms, and a water source not influenced by the animal health status in the surrounding environments.

Required surveillance protocols are laid down by the World Organization for Animal Health (OIE) and in EU Council Directive 2006/88/EC on "animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals" with more detailed requirements described in Commission

Decision 2015-1554 "laying down rules for the application of Directive 2006/88/EC as regards requirements for surveillance and diagnostic methods". New EU requirements are under development and will be described in Annexes to the Delegated Act on Aquatic Animals under the new Animal Health Law that will be put into force from 2020. The requirements will most likely not differ significantly from the current ones.

The current categorization of zones and compartments provided in 2006/88/EC comprises 5 categories for each listed non-exotic disease with category 5 as 'infected', 4 'under eradication', 3 as 'undetermined' (not known to be present), 2 'under surveillance' and 1 as 'approved free of the disease in question'. Trade and movement of fish is only possible within categories 1, 3 and 5 and "downstream" to compartments with a higher category status. The categorization covers all fish and gametes from sites within a category. However, purchase of salmonid eggs and milt is only possible from farms with similar or higher health status.

Norway has established sea-water based ISA- free compartments (semi-open cage based farms) for brood-stock production approved by the Norwegian Food Safety Authority (NFSA). The system is further approved in accordance with EU -regulations.

For endemic diseases involving widely prevalent agents and/or agent precursors, it may be questionable whether traditional sea-based cage farms can strictly fulfil the requirements of a compartment. This would be easier to fulfil for land-based recirculating aquaculture system (RAS) farms.

1.3.2 Listing of aquatic animal diseases and susceptible species

OIE sets standards and lists diseases of aquatic animals that are important for the safe trade of aquatic animals and products thereof. National authorities can define additional diseases that are of particular importance within their country. The NFSA is responsible for surveillance of the health status of aquacultured animals in Norway. Fish diseases are listed to establish standards in the control of fish diseases. The OIE publishes health standards for international trade in animal and animal products. In the EU/EEA area, Lists 1 (exotic diseases) and 2 (non-exotic diseases) were originally established by the European Commission (Council Directive 91/67/EEC, Annex A) and are included in Part II of Annex IV of the Council Directive 2006/88/EU. The NFSA has a national List 3 that has been adopted by the Ministry of Trade, Industry and Fisheries under Annex I of Regulation 17 June 2008 on the placing on the market of aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals (FOR-2008-06-17-819, Norwegian Ministry of Trade, Industry and Fisheries). The fish species susceptible to infection with all notifiable diseases are listen by both the OIE and EU/EEA.

1.3.3 Vertical transmission of fish diseases

The spread of infectious diseases is a significant threat to the sustainability of aquaculture. The possibility of spread of pathogenic agents is an intrinsic risk during export and import of fertilized salmonid eggs. International agreements, legislation and certification are to a large extent based on the standards developed by OIE. The International Aquatic Animal Health Code (2008) has the following definition of vertical transmission: "*Vertical transmission means the transmission of a pathogen from a parent aquatic animal to its progeny via its sexual products".* The pathogen may be transmitted intra-ovum, adhered to the outside of the egg, be present in ovarian fluid, milt, blood, mucus or as a result of contamination from the environment.

Infectious organisms may be transmitted via milt. Infections of the male gonads or the migration/extravascular leakage of infected blood cells into the male reproductive tract can contaminate milt. The milt can also be contaminated during the manual milt collection process (Figure 1.3.3). This may result in the detection of an infectious pathogen or its DNA or RNA in milt. However, remnants of pathogen DNA or RNA and not an intact infectious agent may be detected, and a positive PCR result from milt does not necessarily imply that transmission to the offspring can occur. Likewise, isolation by virus culture does not necessarily imply that transmission can occur.

Figure 1.3.3. Milt collection from broodfish (Photo: AquaGen)

2 Literature search

Literature searches were performed in February-Mars 2019 in PubMed, Web of Knowledge and Scopus, and by searching databases at the Norwegian University of Life Sciences, the Norwegian Veterinary Institute and the Technical University of Denmark. The project group also provided information that was, if relevant, included in the assessment, based on their expertise on the topics.

The following search strings were performed (using the Advanced Search Builder), without any restrictions on date of publication:

PubMed:

- Searching for (fish [Title/Abstract] AND sperm [Title/Abstract] OR milt [Title/Abstract] AND virus [Title/Abstract]) resulted in 22 hits.
- Searching for (fish [Title/Abstract] AND sperm [Title/Abstract] OR milt [Title/Abstract] AND bacteria [Title/Abstract]) resulted in 18 hits.

ISI Web of knowledge:

- Searching for TOPIC=(milt AND disease AND transmission) resulted in 41 hits
- Searching for TOPIC=(aquaculture AND sperm OR milt AND bacteria) resulted in 448 hits
- Searching for TOPIC=(aquaculture AND sperm OR milt AND virus) resulted in 442 hits.

Scopus:

- Searching for (“milt” AND “disease” AND “transmission”) resulted in 102 hits.
- Searching for (“salmon” AND “milt” AND “virus” OR “bacteria”) resulted in 183 hits.
- Searching for (“trout” AND milt AND “virus” OR “bacteria”) resulted in 174 hits.

Relevance screening

The titles and abstracts of all hits were screened. Those that did not relate to the terms of references were omitted. The reference lists of remaining articles were screened to identify additional articles or reports that were not detected in the searches.

Other sources of information

Hearing experts from the breeding companies AquaGen, Benchmark Holding/SalmoBreed and Mowi Breeding presented their approach regarding the use of frozen milt in June and September 2018. Experts from these three companies presented their views and experiences, and answered questions from the working group.

3 Hazard identification and characterisation

The likelihood of transmission of any particular pathogenic agent via milt will depend on the number of infectious particles within the broodfish gonads, the more agent the greater the likelihood. The utilisation of specific pathogen-free populations of brood-fish would remove/minimize this possibility. However, milt may be contaminated with possible pathogenic agents from the environment during collection. The only known use of frozen salmonid milt is fertilization of eggs. Milt is not disinfected prior to this use, but fertilized salmonid eggs are surface disinfected. The recommendation from the NFSA for disinfection of salmonid eggs is bathing in a 100 mg/l iodine solution. The contact time should be 10 minutes and the pH of the solution must be between 6 and 8. It is recommended that the eggs be washed in fresh water before and after disinfection. The sensitivity of a pathogen to iodine is, therefore a prerequisite for effective disinfection. Surface disinfection may not completely inactivate pathogens that are able to enter or attach to the egg prior to disinfection. To compensate for the possible failure of routines in the field, iodine disinfection is usually performed at least twice; once just after fertilization and again prior to transport of eggs. In addition to iodine treatments, prophylactic anti-fungal treatment of eggs with formaldehyde (0.01%, 10-30 min) is routinely and repeatedly used during the eyed stage.

3.1 Viral agents

Infectious pancreatic necrosis virus

Infectious pancreatic necrosis virus (IPNV) belongs to the family *Birnaviridae*, genus *Aquabirnavirus*, which are small non-enveloped particles with two segments of dsRNA. Aquabirnaviruses have a very large host range that includes fish, crustaceans and bivalves in both freshwater and seawater. Infectious pancreatic necrosis is a highly contagious disease of young salmonid fish held under intensive rearing conditions (Wolf et al., 1960) and may occur in a variety of species of the genera *Salmo*, *Salvelinus* and *Oncorhynchus*. The disease is transmitted horizontally via water, but vertical transmission has been demonstrated for brook trout and rainbow trout (Ahne and Negele, 1985), and surface disinfection of fertilized eggs is not sufficient to block vertical transmission (Bullock et al., 1976). IPNV has strong affinity for spermatazoa (Mulcahy and Pascho, 1984), and may be present in ovarian fluid (Wolf et al., 1968) and in milt (Dorson et al., 1996). The spermatazoa may therefore act as a transport vehicle for virus to enter and infect the egg. The virus is not easily inactivated and is difficult to eradicate once introduced into a production facility.

Infectious pancreatic necrosis was one of the most common diseases in Norwegian aquaculture, and was present in both fresh water and marine phases of salmonid farming (Hjeltnes et al., 2016). The number of cases annually recorded has decreased substantially due to effective breeding programs. Infectious pancreatic necrosis is no longer on the Norwegian list of nationally notifiable diseases.

Infectious salmon anaemia virus

Infectious salmon anemia virus (ISAV) is an enveloped virus with a segmented ssRNA genome belonging to the genus *Isavirus* within the family *Orthomyxoviridae.* While the disease has only been detected in farmed Atlantic salmon in the field, ISAV has also been demonstrated to replicate in experimentally infected Atlantic salmon, brown trout, rainbow trout and Arctic char (Snow et al., 2001) as well as in several species of Pacific salmon and in some marine fish species (OIE, 2018).

ISAV can be genetically differentiated based on the highly polymorphic region (HPR) of the haemagglutinin-esterase (HE) protein (Rimstad et al., 2001). A deletion within the HPR region (HPRΔ), together with an insertion/site mutation in the fusion (F) protein is necessary for virulence (Markussen et al., 2008). Non-deleted ISAV (HPR0) has never been associated with disease. The current working hypothesis is that virulent HPRΔ ISAV is derived from HPR0. HPR0 virus is highly prevalent in farmed salmon populations. While waterborne (horizontal) transmission in fresh water has been demonstrated, vertical transmission is not considered to play a significant role in the transmission of ISA when disinfection of fertilized eggs is used (Lyngstad et al., 2011). The virus has, however, been recovered from non-disinfected ovarian fluid as well as from the interior of eggs originating from broodfish with significant viral loads in the ovarian fluid (Marshall et al., 2014).

Salmon pancreas disease virus - salmonid alphavirus

Salmon pancreas disease virus (SPDV, today commonly known as salmonid alphavirus (SAV), belongs to the genus *Alphavirus* of the family *Togaviridae*, and comprises enveloped particles with a ssRNA genome*.* In Norway, SAV is associated solely with disease during the seawater phase of commercial salmon and rainbow trout farming. The virus is also found in freshwater rainbow trout farms in continental Europe where the disease is called sleeping disease. The virus is sensitive to high and low pH, high temperature and common virucidal disinfectants (Graham et al., 2007a; Graham et al., 2007b). Salmonid alphavirus is currently classified into six subtypes, and most of these have specific geographic distributions in Europe (Graham et al., 2012). The virus transmits efficiently horizontally (McLoughlin et al., 1996). There is no evidence for vertical transmission of SAV in Atlantic salmon (Kongtorp et al., 2010). In the latter study, milt from uninfected brood fish was contaminated with SAV immediately prior to fertilization, and the offspring were followed until smoltification. All samples of progeny analyzed were negative for SAV infection; results from commercial hatcheries support these findings (Kongtorp et al., 2010).

Viral hemorrhagic septicemia virus

Viral hemorrhagic septicemia virus (VHSV) is an enveloped virus with a ssRNA genome belonging to the genus Novirhabdovirus, within the family Rhabdoviridae. There are currently four recognized major VHSV-genogroups and 9 subtypes with rather distinct geographical distributions and host ranges (OIE Aquatic Manual). VHSV primarily causes disease in farmed rainbow trout and is common in freshwater in Europe, but also infects a wide range of marine fish species (Skall et al., 2005). VHSV is thus present in marine fish populations in Norwegian coastal waters (Sandlund et al., 2014).

The virus is sensitive to high and low pH, high temperature and common virucidal disinfectants, such as UV, ozone, iodophors, and formalin (Ahne 1982; Jørgensen, 1974, Kurita, 2002; Øye and Rimstad, 2001). It is also rapidly inactivated by percolation through sand and gravel but resistant to high salt concentrations (Skall and Olesen, 2015).

In Denmark, it has been demonstrated that eradication of VHSV is possible utilising a combination of sanitization and fallowing (Jensen et al., 2015). Transmission of VHSV is horizontal via contaminated water/direct excretion of virus from infected fish. The virus may contaminate egg surfaces, but does not survive incubation. Jørgensen (1970) reported that viable VHSV could only be detected 3½ hrs after fertilization of naturally infected eggs and up to 10 days after fertilization of experimentally infected eggs. The adsorption of salmonid viruses to fish sperm was studied by Mulcahy and Pascho (1984). These authors were unable to demonstrate adsorption of VHSV to Chinook salmon sperm, whereas another rhabdovirus (IHNV) adsorbed readily and IPNV adsorbed but to a lesser degree than IHNV. To the best of our knowledge, there is no scientific report demonstrating the presence of VHSV in piscine seminal fluids, although it appears possible that contamination of the sperm may occur when stripping latently infected, mature rainbow trout males. VHSV has a limited survival time in the water, and is highly susceptible to ultraviolet (UV) light (Ahne, 1982). This may explain why there are no reports implicating egg-associated transmission of VHSV (Wolf, 1988).

Infectious hematopoietic necrosis virus

Infectious hematopoietic necrosis virus (IHNV) also belongs to the genus *Novirhabdovirus* within the family *Rhabdoviridae*. This virus comprises five geographically distinct genogroups U, M, L, J, and E (Kurath, 2012). Isolates from farmed rainbow trout in Europe appear to have evolved from the North American genogroup M (Enzmann et al., 2005). IHN is widespread in continental Europe and has never been detected in Norway, Denmark or Sweden, but was detected for the first time in Finland in 2017. The disease affects wild and farmed rainbow trout, many species of Pacific salmon and Atlantic salmon (OIE, 2018).

Disinfectants such as UV, chlorine, hypochlorite and iodophors are very efficient in inactivating rhabdoviruses. Nevertheless, disinfection with iodophore did not result in complete inactivation of IHNV on experimentally infected rainbow trout eggs (Goldes and Mead, 1995).

In the literature pertaining to IHN virus, ”vertical transmission” is often referred to as transmission of a virus from the parent fish to its offspring, regardless of whether the virus is located inside or outside the egg (Pilcher and Fryer, 1980a,b). This makes an assessment of the risk of true vertical or intra-ovum transmission of IHNV difficult to determine. The most frequent evidence cited for a "vertical transmission" event is the association of a shipment of salmonid eggs into a geographical area where IHNV has not been previously detected followed by subsequent detection of an IHN epizootic in those fish (Plumb, 1972; Holway and Smith, 1973; Sano et al., 1977; Niu and Zhao, 1988).

Further evidence cited for vertical transmission is that IHN has been reported in progeny from eggs disinfected with iodophor and incubated and raised in virus-free water (Wingfield and Chan, 1970; Ratliff, 1982; Mulcahy and Bauersfeld, 1983; Mulcahy and Pascho, 1985; Meyers et al., 1990; Roberts, 1993). However, if true vertical transmission does occur, it is most likely a very infrequent event. There are several reports of IHNV infected parents which did not produce IHNV-infected progeny when the eggs and fish were incubated and raised in virus-free water and/or disinfected with an iodophor solution (Amend, 1975; LaPatra, 1990; Engelking et al., 1991; LaPatra et al., 1991; Yamazaki and Motonishi, 1992; Traxler et al., 1997). Because of the number of tests that have been reported and the number of fish involved in each of those tests, this has raised significant doubts as to whether true vertical transmission of IHNV occurs. Mulcahy and Pascho (1985) also reported that it was difficult to demonstrate "vertical transmission" and they were only successful three times in isolating IHNV from live and dead eggs/fry of infected adult sockeye salmon. They found that only a small proportion of sockeye eggs and fry contained IHNV, that not all parents transmitted the virus to their progeny and that not every egg from infected females contained the virus. These observations could explain why studies to demonstrate vertical transmission have failed and why IHN epizootics appear intermittently in some groups of fish at only some aquaculture facilities. Alternatively, numerous other variables are unknown and/or uncontrollable that could initiate intermittent IHN epizootics in susceptible groups of fish.

Seminal fluids with high viral titres may represent a risk of transmission of infection equivalent to an ovarian fluid with high titres (Meyers et al., 1990). The virus strongly and quickly adsorbs to the surface membrane of steelhead trout and Chinook salmon sperm (Mulcahy and Pascho, 1984). Sperm-attached IHNV originating either from the male fish or from infected ovarian fluid could deliver the virus directly into the egg during fertilization (Mulcahy and Pascho, 1984; Meyers et al., 1990). However, the role of sperm in IHNV transmission remains unknown. Contamination of masu salmon or chum salmon milt with IHNV did not result in infection of eggs or fry (Yoshimizu et al., 1989) and there is no direct evidence that sperm can transmit IHNV into eggs.

Primary management strategies for minimizing the risk of vertical transmission include the use of a 100 mg/l iodophore solution for disinfection of the egg during water hardening, after fertilization, and also at the eyed-egg stage just prior to transport and hatching. In conclusion, there is some evidence that the virus may be truly vertically transmitted, but this appears to be a rare and infrequent event.

Piscine orthoreovirus

Piscine orthoreovirus (PRV) causes the disease heart and skeletal muscle inflammation (HSMI) which is common in seawater farmed Atlantic salmon in Norway (Wessel et al., 2017). The virus is also commonly found in salmon in the freshwater phase. It belongs to the genus *Orthoreovirus* within the family *Reoviridae* and is non-enveloped with a segmented dsRNA genome. It is divided into three genotypes, PRV-1-3, found most commonly in Atlantic salmon, coho salmon and rainbow trout, respectively. In Norwegian aquaculture, HSMI is a common diagnosis (Hjeltnes et al., 2016). PRV is ubiquitous in the marine phase of Atlantic salmon farming in Norway. While it may be detected, it is less common in the freshwater phase (Lovoll et al., 2012). Infection routes for PRV have not been thoroughly studied, but there are indications of the importance of the per oral infection route (Hauge et al., 2016). Erythrocytes are the major target cells, and contamination of sexual products with blood is common. The possibility of vertical transmission cannot, therefore, be excluded (Wiik-Nielsen et al., 2012).

Chilean and Canadian (British Columbia) Atlantic salmon PRV isolates are of a similar genotype to those found in the natural range for Atlantic salmon i.e. the North-Atlantic including Norway. This indicates transmission of this agent to Chile and BC, Canada from the North-Atlantic area. The effect of commonly used disinfectants on the infectivity of PRV is unknown, partly due to lack of an efficient *in vitro* cultivation system for the virus. However, avian and mammalian orthoreoviruses are remarkably stable and withstand temperatures up to 55 °C, pH values between 2 and 9, lipid solvents and detergents.

Piscine myocarditis virus

Piscine myocarditis virus (PMCV) is a non-enveloped dsRNA virus, tentatively placed in the family *Totiviridae*. Totiviruses cause latent infections in fungus and protozoans and transmit from cell to cell during cell division, sporogenesis and cell fusion. The virus is associated with cardiomyopathy syndrome (CMS) (Haugland et al., 2011; Lovoll et al., 2010), which is a disease mostly of larger Atlantic salmon farmed in seawater in Norway. Although the probability of vertical transmission of PMCV is unknown, it was not found to be an important risk factor for the development of CMS. The most important risk factors were identified as increasing time in the sea, infection pressure, cohort size and HSMI or CMS diagnoses in previous cohorts (Jensen et al., 2013). Little is known of the effect of disinfectants as the virus is not readily passaged in cell culture. PCR screening of Atlantic salmon for the presence of PMCV in Chile has so far, not identified any positive samples (Garseth et al., 2018), indicating a very low probability of vertical transmission of PMCV due to the lack of introduction to Chile despite large-scale import of eggs.

Epizootic hematopoietic necrosis virus

Epizootic haematopoietic necrosis virus (EHNV) belongs to the Genus *Ranavirus* within the Family *Iridoviridae* and has a large (150–180 nm), icosahedral virion with a double-stranded DNA genome of 150–170 kb. The virus replicates in both the nucleus and cytoplasm of infected cells with cytoplasmic assembly (Chinchar et al., 2005). EHNV is closely related to several other Ranaviruses that are of less concern and can only be identified by sequencing (Holopainen et al., 2009). EHNV is extremely resistant to drying and can survive for months in water (Langdon, 1989). EHNV is susceptible to 70% ethanol, 200 mg litre^{-1} sodium hypochlorite or heating to 60°C for 15 minutes (Langdon, 1989). There are no published data on its susceptibility to iodophores or other disinfectants.

No published or unpublished data are available providing evidence either for or against vertical transmission of EHN or the presence of the causative virus in the sexual products of susceptible host species. There are no field observations or other indirect evidence to indicate that vertical transmission of EHNV may occur. The risk of vertical transmission is therefore not possible to assess. The fact that EHN is categorized as a list A disease- and has only been observed in Australia does, however indicate that the risk of vertical transfer from infected brood fish in the rest of the world is negligible.

Salmon gill poxvirus

Salmon gill poxvirus belongs to the *Poxviridae*, a family of usually, but not always, enveloped viruses. They are very large and contain a single, linear, dsDNA genome (Gjessing et al., 2015; Nylund et al., 2008). Salmon gill poxvirus has not been cultured in cell culture. The effect of disinfectants on virus infectivity is unknown. *Salmon gill poxvirus* is one of several infective agents involved in multifactorial gill diseases in farmed salmon. The probability of vertical transmission of *Salmon gill poxvirus* is unknown, but as an apparently gill specific pathogen, vertical transmission seems unlikely.

Atlantic salmon calicivirus

The virus has properties typical of the family *Caliciviridae*, i.e. small, non-enveloped virus particles with a ssRNA genome. Although it can successfully be isolated in cell culture little is known of its susceptibility to disinfectants. The virus causes a systemic infection but is not associated with a specific disease (Mikalsen et al., 2014). The probability of vertical transmission of Atlantic salmon calicivirus is unknown.

Atlantic salmon paramyxovirus

Atlantic salmon paramyxovirus has the same properties as the family *Paramyxoviridae*, i.e. large, enveloped virus particles with a ssRNA genome. Although it can be successfully isolated in cell culture little is known of the effect of disinfectants (Kvellestad et al., 2003). *Atlantic salmon paramyxovirus* is one of several infective agents involved in multifactorial gill diseases in farmed salmon (Kvellestad et al., 2005). The probability of vertical transmission of Atlantic salmon paramyxovirus is unknown, but as it is assumed to be a gill specific pathogen, vertical transmission seems unlikely.

3.2 Bacterial agents

Renibacterium salmoninarum

Bacterial kidney disease (BKD) is caused by the facultative intracellular, short, gram-positive bacterium *R. salmoninarum*. BKD is a serious disease of wild and farmed salmonid fish throughout the world, with the possible exception of Australia and New Zealand and is a notifiable disease in Norway (List 3, national disease). The existence of an American genotype in Europe suggests that the bacterium has been spread by anthropogenic means from the American continent to Europe (Brynildsrud et al., 2014). While BKD normally manifests as a chronic eventually fatal infection, cases of acute high mortality can occur under farming conditions. While clinical BKD appears limited to salmonid fish (including whitefish Coregonidae) (Jonas et al., 2002; Loch and Faisal, 2010), *R. salmoninarum* has also been identified in moribund non-salmonid species (Kent et al., 1998). *Renibacterium salmoninarum* infections can partially resist to antibiotic treatment due to the bacterium's intracellular nature.

BKD, once a serious problem in Norwegian re-stocking hatcheries for Atlantic salmon, is now a rare finding due to the intensive screening of brood fish. Only occasional cases now occur in Norwegian farmed fish (both rainbow trout and Atlantic salmon), most commonly during the seawater phase of culture. In such cases, horizontal transmission from migrating wild salmon is suspected. The infection is transmitted both horizontally (Balfry et al., 1996) and vertically. Vertical transmission is via intra-ovum infection (Evelyn et al., 1986b) and iodine-based treatment of infected eggs is not effective (Evelyn et al. 1984). While erythromycin treatment of infected eggs is not successful, erythromycin injection of broodfish prior to spawning can reduce intra-ova infection (Evelyn et al., 1986a; Lee and Evelyn, 1994). Successful control of *R. salmoninarum* in Pacific Northwest Chinook salmon hatcheries is achieved by injecting spawning adults with an antibiotic and culling of highly infected females (Munson et al., 2010). *Renibacterium salmoninarum* has been demonstrated to agglutinate salmon sperm (Daly and Stevenson, 1989) but the possible role of milt in vertical transmission of the disease has not been studied. *Renibacterium salmoninarum* cells are known to bind to the tail, but not the head of rainbow trout spermatozoa, but the role if any of seminal fluid associated *R. salmoninarum* in vertical transmission has not been established (Daly and Stevenson, 1989).

Aeromonas salmonicida **subsp.** *salmonicida*

Furunculosis is the term normally associated with a serious disease in salmonid fish caused by the non-motile, gram-negative rod-shaped bacterium *Aeromonas salmonicida* subsp. *salmonicida*. Furunculosis is a serious threat to farmed salmonids throughout the world. While *A. salmonicida* subsp. *salmonicida* is most commonly associated with 'classical' furunculosis in salmonid fish, some other fish species e.g. turbot and lamprey may also be infected. While by far the majority of *A. salmonicida* infections in salmonids are associated with subsp. *salmonicida*, infections caused by other genotypes do occur. At the species level, *A. salmonicida* represents one of the most intensively studied fish pathogenic bacteria. Four other subspecies are described i.e. *achromogenes*, *masoucida*, *smithia* and *pectinolytica* (Martin-Carnahan and Joseph, 2005), but the current classification does not nearly describe the existing diversity within the species. The taxonomic situation is becoming clearer. However, Gulla and colleagues (2016) identified 14 (recently expanded to 21; Gulla and Colquhoun, unpublished) discrete, largely host-species specific genetic clusters within the species, with each cluster probably justifying description at the sub-species level. Infections with *A. salmonicida* subsp. *salmonicida* are notifiable (National list 3) in Norway. The disease is currently controlled in Norwegian aquaculture through vaccination, and outbreaks in salmonid fish in Norway are exceptionally rare.

Early experimental attempts to induce vertical transmission of furunculosis failed (Bullock and Stuckey, 1987) and intra-ova infections have not been identified (Cipriano et al., 2001). Kohara et al. (2012) found that experimental intra-ova *A. salmonicida* infection of rainbow trout or amago salmon eggs, in contrast to *F. psychrophilum*, steadily decreased and became undetectable. *Aeromonas salmonicida* may, however, be cultured from fertilised salmon eggs (Cipriano et al. 2001) and skin mucus of infected fish (Cipriano et al., 1992) so passive transmission via sexual fluids is apparently possible. Milt-associated transmission does not appear to have been studied.

Flavobacterium psychrophilum

Flavobacterium psychrophilum is a strictly aerobic, gram-negative, slender rod-shaped bacterium. *Flavobacterium psychrophilum* is the aetiological agent of coldwater disease (CWD) and rainbow trout fry syndrome (RTFS). As the name implies CWD normally occurs at low water temperatures, and may be topical or systemic in nature (Starliper, 2011). It affects mainly salmonid species but a range of non-salmonid species may also be affected. RTFS is a serious systemic infection affecting rainbow trout farmed worldwide. As its name implies the disease is most devastating in juvenile rainbow trout, but larger fish may also suffer severe disease. *Flavobacterium psychrophilum* appears ubiquitous in most temperate freshwater bodies and many genotypes with differing degrees of virulence and host specificity exist (Nilsen et al., 2014). The occurrence of identical genotypes in the same species of farmed fish over large geographic distances has been linked to international trade of brood fish and eggs (Nicolas et al., 2008). Many genetically diverse strains exist in the Nordic countries (Nilsen et al., 2014) and systemic disease occurs in both Atlantic salmon (Nilsen et al., 2011a) and rainbow trout (Nilsen et al., 2011b).

Systemic infection with *F. psychrophilum* in rainbow trout is a notifiable (national list 3) disease in Norway. Evidence exists that *F. psychrophilum* may be both horizontally and vertically transmitted via ova and ovarian fluids (Brown et al., 1997; Cipriano, 2005; Taylor, 2004). The bacterium has been associated with milt sampled from an Atlantic salmon showing signs of disease (Ekman et al., 1999) and culling of infected broodstock has been shown to reduce the prevalence in progeny (Long et al., 2014). While systemic disease caused by *F. psychrophilum* is a relatively uncommon disease in Norway, the possibility of further transmission via eggs or milt cannot be excluded. Iodophor disinfection of eggs appears to be effective (Kumagai and Nawata, 2010) as long as the bacterium is not present within the egg (Cipriano, 2005) although *F. psychrophilum* is reported to survive relatively high levels of iodophor (Brown et al., 1997).

Francisella spp.

Francisellosis in fish is caused by *Francisella noatunensis* an aerobic, gram-negative facultative intracellular cocco-bacilli (Colquhoun et al., 2014). Two subspecies have been described, *Francisella noatunensis* subsp. *noatunensis* and *F. noatunensis* subsp. *orientalis* which cause disease in temperate and warm water fish species respectively (Colquhoun and Duodu, 2011). Disease in salmonid fish has been restricted to a single genetic lineage of subsp. *noatunensis* which caused problems during the freshwater stage of Atlantic salmon farming in Chile (Birkbeck et al., 2007). This strain/genetic lineage has not been identified in Norway. The disease in Norway has been exclusively associated with farmed and wild cod. While vertical transmission has been considered likely (Karlsbakk et al., 2008) in the cod pathogen, the presence of subsp. *orientalis* has been confirmed in the ovaries/testes and milt of tilapia (Pradeep et al., 2017). There is little published literature regarding the effectiveness of disinfection against fish pathogenic *Francisella* species. Muller and colleagues (2011) found, however, that *Francisella* tolerated higher concentrations and exposure times of a commercially available disinfectant (superquats and glutaraldehyde) than *V. ordalii* or *V. anguillarum*.

Piscirickettsia salmonis

Piscirickettsia salmonis, a fastidious, Gram-negative bacterium causes the disease piscirickettsiosis or salmonid rickettsial septicaemia (SRS), a systemic infection of farmed salmonid (Bravo and Campos, 1989; Olsen et al., 1997; Rodger and Drinan, 1993) and marine fish (Arkush et al., 2005; McCarthy et al., 2005). Piscirickettsiosis is an extremely serious disease in Chilean salmon farming but is only an occasional finding in Norwegian aquaculture in recent years. Several genetic lineages exist (Brosnahan et al., 2018) and Norwegian strains appear much less virulent than the Chilean strains. Experimental vertical transmission of the bacterium (but not the disease) has been demonstrated via coelomic fluid and milt (Larenas et al., 2003b). It is therefore likely that *P. salmonis* can transmit via sexual products of brood-stock salmon. *Piscirickettsia salmonis* has, however, never been identified in the freshwater stage of salmon farming in Norway, suggesting that, while vertical transmission cannot be discounted completely, the probability of it occurring in Norway must be considered low. While the susceptibility of *P. salmonis* to several disinfectants has been tested (Muniesa et al., 2018) there does not appear to be any published literature regarding disinfection of eggs or milt.

Yersinia ruckeri

Yersinia ruckeri is a gram-negative enterobacteria which causes a systemic infection known as enteric redmouth disease (ERM) in salmonid fish. Internationally ERM is most significantly a disease of farmed rainbow trout, but in some countries, including Norway, Atlantic salmon are also affected. Norway is in an exceptional situation in which ERM is exclusively associated with disease in salmon but not in rainbow trout (Gulla et al., 2018). Until recently, little was known concerning the global *Y. ruckeri* population structure. We now know that several host-specific, disease-associated clonal complexes exist against a background of less virulent or putatively avirulent clones (Gulla et al., 2018).

Established serotyping systems previously used to classify this bacterium are now revealed to be of little use for epidemiological study or as a diagnostic markers, as both putatively avirulent and virulent clones have been identified within serotype O1, the serotype most commonly associated with disease outbreaks (Gulla et al., 2018; Verner-Jeffreys et al., 2011). A high degree of host-specificity has been associated with particular clonal complexes (Gulla et al., 2018) and the clonal complex associated with disease in rainbow trout worldwide has not yet been identified in Norway. *Yersinia ruckeri* has been identified in salmonid ovarian fluid (Glenn et al., 2015; Gulla et al., 2018) and it must be presumed to pose a risk of vertical transmission via contaminated roe. It does not appear that male sexual products have been examined for the presence of *Y. ruckeri* (Kumar et al., 2015). *Y. ruckeri* appears to be relatively susceptible to dilute concentrations of (amongst others) iodophor disinfectants (Verner-Jeffreys et al., 2009).

3.3 Diseases of fish listed by the OIE[1]

Disease/ Pathogen	Atlantic salmon susceptible[2]	Rainbow trout susceptible	Potential presence in salmonid milt	Relevant for further evaluation	Comments
Epizootic ulcerative syndrome */Aphanomyces invadans*	No	No	Negligible	No	Not infectious to Atlantic salmon
Epizootic haematopoietic necrosis virus	No	Yes	Yes	Yes	Exotic to Europe
Gyrodactylus salaris	Yes	Yes	No	No	Not relevant for milt
HPR-deleted or HPR0 *Infectious salmon anaemia virus*	Yes	Yes	Yes	Yes	HPR0 genotype most relevant for assessment of milt[3]
Infectious haematopoietic necrosis virus	Yes	Yes	Yes	Yes	Contaminated eggs suspected as the cause of transmission to Europe and Asia

[1] OIE - *Aquatic Animal Health Code.* Twenty-first Edition, 2018

[2] For species susceptibility see OIE, International Aquatic Animal Health Code

[3] Brood fish population with HPRΔ will not be used for breeding

Disease/ Pathogen	Atlantic salmon susceptible[2]	Rainbow trout susceptible	Potential presence in salmonid milt	Relevant for further evaluation	Comments
Koi herpesvirus	No	No	Negligible	No	Not infectious to Atlantic salmon
Red sea bream iridovirus	No	No	Negligible	No	Not considered infectious to Atlantic salmon
Salmonid alphavirus	Yes	Yes	Yes	Yes	
Spring viraemia of carp virus	No	No	Negligible	No	Not infectious to Atlantic salmon
Viral haemorrhagic septicaemia virus	Yes	Yes	Yes	Yes	

3.4 Norwegian national List 3 fish diseases[4]

Disease /Pathogen	Atlantic salmon susceptible	Rainbow trout susceptible	Potential presence in salmonid milt	Relevant for further evaluation
Bacterial kidney disease /*Renibacterium salmoninarum*	Yes	Yes	Yes	Yes
Viral encephalo- and retinopati (VER) Nodavirus	No	No	Negligible	No
Furunculosis/*Aeromonas salmonicida* **subsp.** *salmonicida*	Yes	Yes	Unknown/yes[5]	Yes
Systemic infection with *Flavobacterium psychrophilum* **in rainbow trout**	No[6]	Yes	Yes	Yes
Francisellosis/ *Francisella* **sp.**	Yes	No	Yes	Yes
Lepeophtheirus salmonis	Yes	Yes	No	No

[4] According to Norwegian Food Safety Authority. Infections also on the OIE list is not included.

[5] Disease controlled by vaccination, presence in milt unknown

[6] Not the sequence types associated with disease in rainbow trout

3.5 Other, non-listed diseases of potential interest[7]

Disease/Pathogen	Atlantic salmon susceptible	Rainbow trout susceptible	Potential presence in salmonid milt	Relevant for further evaluation
Infectious pancreatic necrosis **(IPN)**	Yes	Yes	Yes	Yes
Piscine orthoreovirus **(HSMI)**	Yes	Yes	Yes	Yes
Cardiopmyopathy syndrome/Piscine myocarditis virus	Yes	No cases known	Yes	Yes
Gill Pox Salmon gill poxvirus	Yes	Unknown	Unknown	Yes
Atlantic salmon calicivirus	Yes	Yes	Unknown	Yes
Atlantic salmon paramyxovirus	Yes	Yes	Unknown	Yes
Pisciricketsiosis	Yes	Yes	Yes	Yes
Enteric redmouth disease (ERM)/ *Yersinia ruckeri*	Yes	Yes	Yes	Yes
Classical vibrosis	Yes	Yes	Yes	Yes
Cold water vibrosis	Yes	Yes	Yes	Yes
White-spot *Ichthyophthirius multifiliis*	Yes	Yes	Unknown	No
Costiasis/ *Ichthyobodo necator*	Yes	Yes	Unknown	No
Hexamitiasis/ *Hexamita sp.*	Yes	Yes	Unknown	No
Amoebic gill disease (AGD)/ *Neoparamoeba perurans*	Yes	Yes	Unknown	No

[7] As assessed by the authors of the opinion

4 Exposure

Broodfish are regularly tested for various pathogens at different developmental stages. Testing is usually done by PCR and by autopsy. Today most broodfish are held for at least some time in open cages in the sea, i.e. in Norway, this is mostly in declared ISA-free zones. Some breeding companies have recently established fully land-based broodfish production systems and are also planning future land-based expansion.

Testing of brood fish for ISAV, SAV and BKD is mandatory. The breeding companies also regularly test for IPNV, PRV, PMCV, *Y. ruckeri*, *Paramoeba perurans*, *Branchiomonas cysticola* and Gill poxvirus. The latter two are normally only tested for if the broodfish are held in RAS facilities. Samples that are tested usually comprise organ tissues and/or sexual products. Experiences from testing sexual products indicate that ovarian fluid is better suited for PCR analysis than milt, and has, in general, a higher prevalence of pathogens compared to milt. Whether this reflects biological differences or assay sensitivity is unknown.

The prevalence of individual pathogens in broodfish will have annual variation. BKD is normally not found in brood-stock in Norway, but is occasionally found in large farmed salmon, with between zero to three cases per year, i.e., for both 2016 and 2017, there were single cases identified (Hjeltnes et al., 2018).

IPNV, ISAV-HPR0, PRV and PMCV are common infections in all salmon farming areas, and can, therefore, be expected to be present in broodfish. The prevalence of PMCV in broodfish identified by real-time PCR testing of heart and spleen was approximately 80 % (Wiik-Nielsen et al., 2012) and in another survey, heart samples from 128 of 132 brood fish were PMCV positive. Viral RNA was detected in 60% of milt samples and 69% of roe samples, although at levels close to the cut-off for the method (Garseth et al., 2018). The prevalence of PRV in broodfish identified by real-time PCR testing of heart and spleen was approximately 95-100% (Wiik-Nielsen et al., 2012). IPNV, PRV and PMCV cause persistent and systemic infections and broodfish that have been in contact with regular production fish will likely have a high prevalence, and viral presence in milt will always be a possibility. ISAV-HPR0 is predominantly an intermittent infection of gill tissues and less likely to be associated with milt.

Table 4-1. Description of terms used to describe the likelihood of an event occurring in Figure 4.1.

Likelihood	Description
High (H)	Expected to occur
Moderate (M)	Less than 50% probability of occurrence
Low (L)	Unlikely to occur

Different fish health related hazards associated with the trade of cryopreserved milt have been identified and characterized (Chapter 3). Figure 4.1 shows a scenario tree defining the chain of events needed to take place before an infection can be transmitted and established in a new population based on the trade of milt. The steps identified are awarded an assumed likelihood. These step- associated likelihoods are summarized as a conditional likelihood for the whole tree in Table 4.1.

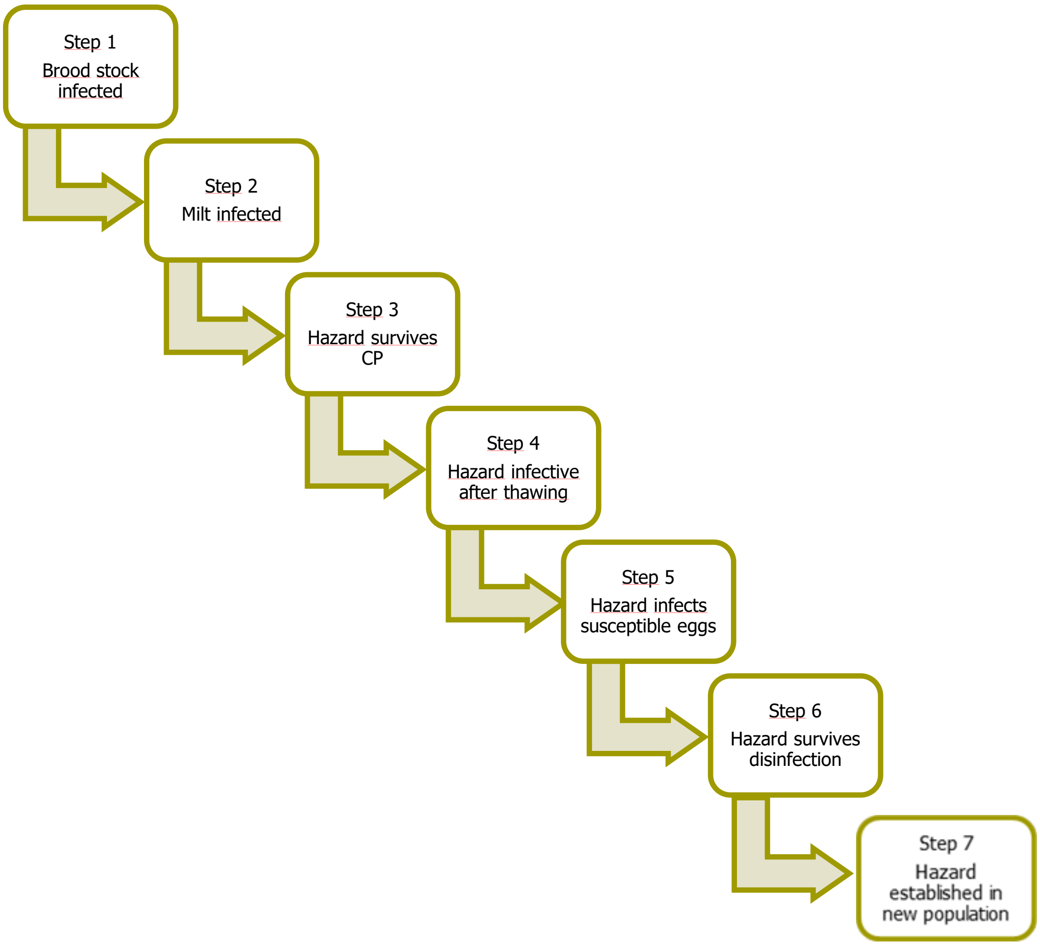

Figure 4-1. Scenario tree for the event of a hazard being introduced and established in a susceptible population via milt. CP refers to cryopreservation.

Description of each step

Step 1

IPN: Norwegian brood-stock companies are conducting selective breeding programs for salmon resistant to IPN. In addition, sanitation work has eradicated or reduced IPN from most hatcheries. During 2017, four salmon hatcheries, three rainbow trout hatcheries and 19 grow-out salmon farms reported outbreaks of IPN. No outbreaks were reported in brood stock (Hjeltnes et al., 2018). This is in line with a steady decrease in the numbers of IPN-outbreaks over the last ten years. All Norwegian brood-stock fish of both sexes are screened for IPNV prior to acceptance of roe batches into the production system.

BKD: The situation for the industry is favourable with no outbreaks reported in 2018 and single outbreaks (grow-out salmon) in both 2017 and 2016. No recent outbreaks are reported from brood fish stocks.

Flavobacterium psychrophilum: Present both in salmon and rainbow production, associated with skin ulcers as well as systemic infections, and both in hatcheries and brackish water facilities. Screening for *F. psychrophilum* in brood-stock is not performed to any significant degree, largely due to the low impact of this bacterium in Norwegian salmon farming, the ubiquitous nature of the bacterium and low specificity of PCR analyses towards virulent strains.

Step 2

There is little knowledge related to the presence of infectious agents in milt. However, we assume a potential risk that a hazard is present if a male brood stock-fish is infected (Tables 3.3 – 3.5). Both IPNV and *Renibacterium salmoninarum* are pathogens demonstrated to be associated with vertical transmission. Although never demonstrated conclusively, evidence also exists that *F. psychrophilum* may be vertically transmitted both via ova and ovarian fluids (Brown et al., 1997; Cipriano, 2005; Taylor, 2004) suggesting that this agent may be transmitted from parent to offspring via sexual products.

Step 3

It is assumed that all the described pathogens (Chapter 3) will survive cryopreservation (CP).

Step 4

All surviving pathogens will maintain their pre-freezing infectious capability after thawing. However, the freeze-thaw process will variably reduce the number of infectious particles, for instance freezing and thawing of IPNV cause a reduction of the virus titere (Mortensen et al., 1998). However, knowledge of reduced virus titer due to the freeze-thaw process is generic information and specific data for the various pathogens are not available.

Step 5

Seminal fluids carrying infectious pathogens may carry the pathogen to the egg, which may result in infections in fry after hatching.

Step 6

Pathogen survives disinfection of the egg post fertilization.

Step 7

Infected milt used to fertilize a batch of susceptible eggs can transmit the infection to a new batch of fish. If the agent is pathogenic such infections often cause disease in fry around start feeding. Examples of this are IPN and IHN, which typically occur in small fish.

Table 4-2. Summary of likelihoods for introducing and establishing a disease through cryopreserved (CP) milt.

Steps	Description	Likelihood, (conditional likelihood)			Uncertainties		
		IPNV	*R. salm.*	*F. psyc.*	IPNV	R.salm.	F.psyc.
1	Brood-stock infected	M	L	L	L	L	L
2	Milt infected	M	M (L)	M (L)			
3	Hazard survives CP	H (M)	H (L)	H (L)	L	L	L
4	Infectious when thawed	H (M)	H (L)	H (L)	L	L	L
5	Eggs infected	H (M)	H (L)	H (L)	L	L	L
6	Eggs disinfected	H (M)	H (L)	H (L)	L	L	L
7	Establishment	H (M)	H (L)	H (L)	L	L	L

*Assumed likelihood (steps 1-7) is given for each step/event occurring. The conditional likelihood (in parenthesis) for an event, is the likelihood of the event occurring given the likelihood of the previous event occurring. A moderate likelihood (M) for step 2 will therefore be reduced to low (L) when taken into account that step 1 (– which is necessary for 2 to occur) has only a low likelihood of occurring.

5 Risk characterisation

This report is a qualitative risk assessment. The risk of transmission of a particular pathogenic agent from salmon or rainbow trout milt to offspring in a non-endemic geographical area is defined as the probability of the infectious hazard (identified in the previous chapter), multiplied by the consequences of such an event occurring.

The probability of such an event occurring will be a function of the prevalence of the infection in the donor population, the probability of the agent surviving and retaining infectiousness through the process from stripping to fertilization, the frequency of export of milt and the length of time over which such trade will occur. Probabilities and consequences are discussed in previous chapters, and we have chosen a subjective compartmentalisation graphic to express these estimates (Figures 5-1 and 5-2). Each hazard represents either a low risk (green), a moderate risk (yellow) or a high risk (red).

The consequences are further assessed from a Norwegian perspective, i.e. all fish are vaccinated against *Aeromonas. salmonicida* subsp. *salmonicida*, *Moritella viscosa, Vibrio anguillarum, Aliivibrio salmonicida* and the fish are of IPN resistant genotypes. Transmission to a susceptible population without these measures may create more serious consequences than estimated in this assessment. Agents listed by OIE in the Aquatic Animal Health Code are automatically assessed as having serious consequences, due to potential direct disease related losses and implications for trade.

The probability of transmission assumes regular health monitoring, biosafety measures such as vaccination to reduce the potential for contaminated milt, good water quality, and that cautious handling procedures are used for donor brood fish.

The definitions used for categorizing probability are:

- Low probability of transmission occurring via milt.
- Moderate probability of transmission occurring via milt.
- High probability of transmission occurring via milt.

The definitions used for categorizing the consequence are:

- Limited consequence: Lack of or mild consequences for fish health, and the disease is easy to control. No changes in distribution of the infection.
- Moderate consequence: Moderate consequences for fish health, such as limited number of mortalities or morbidity with limited pathological changes, affecting a moderate number of fish. Limited changes in the epidemiological distribution of the infection.
- Serious consequence: Severe consequences for fish health, such as high mortality or high morbidity with significant pathological changes, affecting many fish. Significant changes in the epidemiological distribution of the infection.

	Likelihood of transmission via frozen milt, Atlantic salmon			
Consequences of transmission	**Severe**	**ISAV HPRΔ** **VHSV**	**IHNV**	
	Moderate	***A. salmonicida*** **subsp.** ***salmonicida*** ***Y. ruckeri***	**PRV** **PMCV** ***P. salmonis*** **ISAV HPR0**	***F. psychrophilum***
	Minor			**IPNV** ***R. salmoninarum***
		Low	**Moderate**	**High**

Figure 5-1. Risk characterisation of Atlantic salmon from a Norwegian perspective, i.e. fish are vaccinated against *Aeromonas salmonicida* subsp. *salmonicida*, *Moritella viscosa, Vibrio anguillarum, Aliivibrio salmonicida* and are of IPN resistant genotype. The assessment of the likelihood of transmission assumes that biosafety measures like vaccination, good water quality, and cautious handling procedures are used for the brood fish. Agents listed by OIE in the Aquatic Animal Health Code are automatically assessed as having serious consequences, due to potential disease losses and implications for trade.

Likelihood of transmission through frozen milt, rainbow trout				
Consequences of transmission	Severe	ISAV HPRΔ VHSV EHNV *F. psychrophilum*[8]	IHNV	IPNV[9] *R. salmoninarum*
	Moderate	PMCV ISAV HPR0	PRV *P. salmonis*	
	Minor	*A. salm.* subsp. *salmonicida.* *Y. ruckeri*		
		Low	Moderate	High

Figure 5-2. Risk characterisation of rainbow trout from a Norwegian perspective, i.e. fish are vaccinated against *A. salmonicida* subsp. *salmonicida*, *M. viscosa, V. anguillarum, Aliivibrio salmonicida* and are of IPN resistant genotype. The assessment of the likelihood of transmission assumes that biosafety measures like vaccination, good water quality, and cautious handling procedures are used for the brood fish. Agents listed by OIE in the Aquatic Animal Health Code are automatically assessed as having serious consequences, due to potential disease losses and implications for trade.

[8] In Norway the likelihood is low, but the consequences high as the prevalence in rainbow trout in Norway is extremely low. In the rest of Europe the likelihood is high and the consequences high, the bacterium is likely endemic over most of European countries and waterways.

[9] For IPN non-resistant genotypes

6 Uncertainties

Uncertainties may consist of components such as unsatisfactory testing methods, human error, lack of data and knowledge and biological variability. A classical approach to quantifying uncertainties is based on additively weighting single value estimations in e.g. the scenario tree given in Chapter 4. These estimations will, however, not reflect changes that may have occurred since the individual assessments were conducted. For the present assessment the uncertainties are addressed in the discussion and should be considered by the risk manager, the Norwegian Food Safety Authority.

Step 1[10]. For example IPN: The number of outbreaks have decreased significantly in Norway and thereby led to the assumption that the risk of a salmon being IPNV infected is very reduced. There are no brood-stock related IPN outbreaks. But this is based on the presence of disease and not the presence of the virus, which might be present in clinically healthy fish. As long as the prevalence of IPNV positive fish is unknown this might give high uncertainty. On the other hand, brood-stock testing would give a more reliable estimation of virus prevalence – with minor uncertainties connected to test sensitivities and sample handling.

Step 2. PCR of milt samples is regarded as problematic due to high concentrations of DNA in such material- thereby data from milt testing might be misleading due to lower performances of the tests used compared to e.g. kidney or heart tissues and must be taken into account in the assessment. Individual virus/bacteria have preferred compartments for persistent infection. In most cases, testing of kidney or spleen will indicate a higher prevalence than testing of milt.

Step 3. "All the pathogens will survive cryopreservation" is correct but it is known that IPNV titres are reduced between 1 and 3 log by lyophilization and that freeze/thawing inevitably will reduce viral (Mortensen et al., 1998) and bacterial titres. This will probably reduce the risk of pathogen transfer.

Step 4. "All the given pathogens will maintain their pre-freezing infectious capability when thawed" the uncertainty is that this might very well be untrue, as the infectious capability might very well be reduced due to freeze/thaw related reduction of the viable load.

Step 5. "Sperms carrying infectious pathogens when thawing will carry the pathogen to the egg, and where the infectious agent stays associated with the eggs may cause infection in fry after hatching. " this is an assumption that has never been scientifically demonstrated and is thereby coupled with a high level of uncertainty.

[10] The steps refer to Figure 4.1

Step 6. "Infected milt used to fertilize a batch of susceptible eggs, will transmit the infection to this new batch of fish". Again this has never been shown experimentally and is thereby coupled to a high level of uncertainty.

This risk analysis assesses the risk of milt transferring a disease in which the uncertainties in most cases will tend to decrease the risk. However, the uncertainties are many and would be problematic if, e.g. used for assessing the probability of milt not transferring a pathogen.

7 Risk-reducing measures

Prophylactic measures

While little is published regarding disinfection of fish milt, several methods of removal or inactivation of sperm/seminal fluid-associated pathogens in humans and farm animals have been described (reviewed by Bielanski 2007). These include washing (separation of the sperm fraction from the seminal fluid, applicable for both bacterial and viral pathogens), antibiotic treatment (bacteria), acidification (relevant for pH sensitive viruses), enzymatic treatment (viruses), use of photosensitive agents and dyes (viruses and bacteria), neutralising antibodies (virus) and ozonation (bacteria and viruses).

'Sperm washing' (Kim et al., 1999), which has been successfully used for removal of HIV from human seminal fluid and could conceivably be used for removal of pathogenic agents present in the seminal fluid of fish (Kim et al., 1999). This technique could be used for pathogens present in the seminal fluid but not directly bound to the spermatozoa, e.g. VHSV but not IHNV or IPNV which rapidly adsorb to the spermatozoa (Mulcahy and Pascho, 1984). Sperm washing using antibiotics has also been shown to effectively removal bacterial contamination, both gram-positive and gram-negative, from human seminal fluid/spermatozoa (Wong et al., 1986).

So-called extenders are commonly used in salmonid aquaculture due to the rapid loss in sperm motility when refrigerated in their natural state (Stoss, 1983). Extenders containing antibiotics have been found effective at killing *F. psychrophilum* present in the seminal fluid of rainbow trout (Oplinger and Wagner 2015) and extending the effective storage time of sperm from other species of fish by reducing bacterial growth (Brown and Mims, 1995; Christensen and Tiersch, 1996). While antibiotic treatment did not result in negative effects on rainbow trout sperm quality in the laboratory it did result in lower fertilisation rates when field tested (Oplinger and Wagner 2015). It would appear therefore that antibiotic treatment of sperm/seminal fluid may be effective in reducing the risk of transmission of *F. psychrophilum* via milt.

Prophylactic measures to prevent vertical transmission of bacterial diseases of fish include injection of brood-stock with the macrolide antibiotic erythromycin several weeks prior to spawning (Bullock and Leek, 1986) to prevent intra-ova transmission of *Renibacterium salmoninarum*. Disinfection of fertilised salmonid eggs with iodine-based solutions has been practised for many years and is proven effective for removal of non-intracellular and environmental bacteria (Cipriano et al. 2001). Glutaraldehyde was found to be better for disinfection of eggs from the marine fish species *Sparus aurata*, *Pagrus pagrus* and *Dentex dentex* (Can et al., 2010). Early work performed by Trust (1974) demonstrated that eggs, ovarian fluid and milt aseptically removed from healthy sexually mature fish are sterile (at least free from cultivable bacteria) prior to spawning. This work suggests that surgically removed roe/milt may reduce the risk of contamination of fertilised eggs with environmental opportunist pathogens compared to stripping-based collection of eggs/milt. However, given the volumes involved in aquaculture, while prophylactic treatment of eggs/milt may be effective in principle and screening of brood-stock for the presence of the pathogen in question using a suitable specific and sensitive molecular analysis, with subsequent removal of positive egg batches from the production system may be a more secure and biologically acceptable means of preventing vertical transmission.

As fertilized salmonid eggs can be disinfected, egg-associated pathogens can be efficiently inactivated provided good procedures are followed. Some pathogens, however, have the ability to bypass disinfection procedures possibly by penetration intra ovum, and thereby causing true vertical transmission. IPNV and *Renibacterium salmoninarum* are examples of this. *Piscirickettsia salmonis* and *Flavobacterium psychrophilum* may also be transmitted vertically, although perhaps not *intra ovum* but nevertheless disinfection may not be fully efficient (Larenas et al., 2003a; Madsen et al., 2005). The high affinity of IHNV for sperm cells and the introduction of this virus into new geographical areas through (illegal) distribution of eggs indicates that this virus may infrequently be vertically transmitted. However, this virus is sensitive to disinfection (Enzmann et al., 2010; Mulcahy and Pascho, 1984). To avoid the spread of agents where disinfection of fertilized eggs may not be sufficiently effective, the brood fish population should be maintained infection free. It is noteworthy that both IPNV and IHNV, both assessed as able to transmit vertically, classically cause disease in very young fish, around the start of feeding.

Agents that are not specialized for vertical transmission may contaminate sexual products during or after the stripping process. This could be agents assumed to be common in the environment, in mucus, in blood etc. Examples include ISAV genotype HPR0 (ISAV-HPR0), PRV and PMCV. There is no specific accumulation of such agents in the gonads, as compared to agents that are specialized for vertical transmission. During the last part of the sexual maturation of salmonids the environment may communicate with eggs/milt through an open uro-genital papilla. Agents in mucus and water may, therefore, contaminate the sexual products, however, as stated above: Trust (1974) found that eggs, ovarian fluid and milt aseptically removed from healthy sexually mature fish are sterile i.e. free from cultivable bacteria. Biosafety measures and disinfection of fertilized eggs will minimize the probability for transmission, but disinfection of fertilized eggs will not influence the probability of the presence of an agent in milt.

Barriers and biosafety measures such as RAS and limited use of seawater in land-based facilities, surface disinfection of fish when moved from sea cages to land tanks, routine formalin treatment of water, land facilities with separate recycling facilities for individual tanks, brood-stock screening and potential destruction of gametes from fish positive for listed agents, combined with washing of eggs before fertilization and disinfection of fertilized eggs will reduce the probability of vertical transmission. Vaccination, monitoring water quality, delousing, smolt quality, nutrition, handling procedures, light / temperature and prevention of fungal infections are examples of biosafety measures.

There are no effective disinfection procedures for fish seminal fluids described in the literature. This implies that infectious agents present in milt originating either from the brood fish or from contamination following stripping/extraction of gonads, may be present in frozen milt. However, the only practical use of salmonid milt is fertilization of salmonid eggs, which will be disinfected after fertilization.

Based on knowledge of the specific viral, bacterial and protozoal agents that may contaminate salmonid milt, practical control measures could be developed and implemented for the farm, geographical region or country. The absence of specific agents can be achieved through utilisation of specific pathogen-free breeding fish. Isolation and lack of indirect contact between brood fish and other populations of salmonids throughout the lifetime of the fish could be a method to achieve specific pathogen free brood-stock, but this is not always possible for every pathogen. It has, however, become common to separate brood fish production from ordinary food fish production, and to limit brood fish production to geographical zones declared as disease-free. Some breeding companies have established or are planning land-based production lines.

8 Conclusions (with answers to the terms of reference)

ToR 1. Relevant infectious diseases that are thought to be transmitted with frozen milt.

Infectious pancreatic necrosis virus (IPNV) and *Renibacterium salmoninarum* were identified as highly relevant, followed by *Piscirickettsia salmonis*, *Flavobacterium psychrophilum* (rainbow trout) and *Infectious hematopoietic necrosis virus* (IHNV). Other salmonid pathogens were of lower relevance.

IPNV and *R. salmoninarum* use vertical transmission as a natural route of infection. Both infections may also transmit horizontally.

Infrequent vertical transmission, not prevented by disinfection of fertilized eggs, may conceivably occur for *P. salmonis*, *F. psychrophilum* (rainbow trout) and IPNV. These infections may be transmitted by sexual products, including potentially, frozen milt. It is important that the broodfish population is free of these agents.

IHNV is listed by OIE, Aquatic Animal Health Code, and positive findings will cause mandatory eradication.

Bacterial kidney disease (BKD, caused by *R. salmoninarum*) and systemic infection with *F. psychrophilum* in rainbow trout are listed in the Norwegian national list 3 of the NFSA, and actions will be decided by NFSA.

Milt may become contaminated before or during milt extraction. Such agents include organisms normally associated with the environment, mucus or blood and exposure may occur via the open genital papilla during the later stages of sexual maturation or during stripping or milt extraction. Any agent present in mucus and water may potentially contaminate the milt. The list of all possible infectious agents, including pathogens and opportunists that potentially may be present in frozen milt is therefore large, and the list presented here will not be complete.

Examples of agents that may contaminate milt are:

Infectious salmon anaemia virus (ISAV), *piscine orthoreovirus* (PRV), piscine myocarditis virus (PMCV), salmon pancreas disease virus - Salmonid alphavirus (SAV), *Viral haemorrhagic septicaemia* virus (VHSV), *Yersinia ruckeri*, *Aeromonas salmonicida and Vibrio anguillarum*.

ISAV, SAV, VHSV cause infections that are listed by OIE in the Aquatic Animal Health Code. Furunculosis, (caused by *A. salmonicida* subsp. *salmonicida*), is listed on the Norwegian national list 3 of NFSA. If these infections are found in brood fish the population and site will be subjected to mandatory sanitation and any milt originating from these stocks will not be used. They are therefore disregarded in the following. However, ISAV genotype HPR0 (ISAV-HPR0) is listed by OIE, Aquatic Animal Health Code, and a common finding in Norwegian aquaculture including brood fish. The detection of ISAV genotype HPR0 (ISAV-HPR0) does not, however, trigger mandatory sanitation. Consequently, in the risk assessment in Fig. 5.1. ISAV HPR0 and ISAV HPRΔ are assessed differently.

Vaccination has practically eliminated the diseases furunculosis, classical vibriosis, and cold water vibriosis in Norway. However, all fertilized eggs in Norway are disinfected, and it is unknown whether the infectious agents of these diseases can at times be present in sexual products prior to disinfection. Their eventual presence in milt is therefore unknown. There have, however, been no known instances of vertical transmissions of these agents in Norway.

PCR detection of PRV and PMCV in eggs, milt and offspring has been reported (Wiik-Nielsen et al., 2012), but there are no reports of outbreak of disease linked to vertical transmission of these agents. CMS, which is caused by PMCV, has never been observed in the fresh water phase.

Biosecurity measures and disinfection of fertilized eggs will reduce the probability of transmission, provided that this is performed properly and the agent is sensitive to the disinfection procedure used.

ToR 2: At what level will it be necessary to control diseases to ensure that frozen milt is not a source of infection for the relevant infectious diseases (national, regional, locations/sea farms, group and individual level)?

When the term 'certified disease freedom' is used – it means that the agent causing the disease is not present within a compartment/zone.

Standardized procedures for certification of disease freedom regarding Category 1, 2, 3, 4 and 5 diseases of fish are described in present EU and Norwegian legislation (IHN, VHS, ISA, EHN, PD). Approved disease-free status for all these diseases is mandatory for all farms with brood-stock producing frozen milt.

Surveillance targeting the diseases VHS, IHN and PD demonstrate that Norwegian salmon farming is currently free of VHS and IHN and that PD is not present in the Northern part of the country. Norway has established ISA-free marine brood-stock production compartments approved by NFSA. The system is further approved in accordance with the EU-regulation for ISA HPR-deleted variants. However, for endemic, widely distributed disease causing agents it may be questionable whether traditional sea-based cage farms can strictly fulfil the requirements of a compartment.

For non-listed diseases/agents where the risk of vertical transmission is high, e.g. for IPN/V and BKD/*Renibacterium salmoninarum*, a program certifying freedom of the causative agent must be in place on brood-stock farms to ensure that frozen milt is not a source of infection. Such programs should be based either on individual testing of parental fish with exclusion of sexual products from positively testing parents, or as a disease free compartmentalisation of the breeding station and regular surveillance for these diseases/agents.

For the non-listed pathogens that are widespread in Norwegian salmonid farming (PRV-1, PRV-3, ISAV-HPR0, PMCV, *Aeromonas salmonicida*., *F. psychrophilium*., *Y. ruckeri*.) risk reduction strategies could vary from compartmental production of fish that spend their whole life in water free of the pathogens in question - combined with regular laboratory testing to specific brood stock testing with clear separation of batches and destruction of milt from parents testing positive for one or more of the causative pathogens; to accepting the risk and clearly stating that freedom for these pathogens cannot be certified in the frozen milt produced.

ToR 3. What requirements for screening; test material, sampling, method and bio-safety are relevant to document freedom from relevant infectious agents in frozen milt?

The freedom from disease concept relates to the probability of freedom where the level of probability is dependent on historical data, the sensitivity of the surveillance system, and the probability of a new introduction to a population. However, it is never possible to provide absolute certainty of the absence of any particular disease or infection.

The testing procedures for OIE listed diseases are available in the Manual of Diagnostic Tests for Aquatic Animals. EU regulations relating to listing of diseases and the basis for control and surveillance are provided in Council directive 2006/88/EC and the detailed procedures described in Commission decision 2015/1554/EC.

For systemic infections and infections involving several organs it is possible that the agent may also be present in milt. For such infections, testing of tissue samples other than milt is acceptable. The tissue material, organ, number and eventual fixation method chosen will depend upon the analysis method and properties of the agent e.g. currently dominating methods are PCR/RT-PCR and cultivation. All of the infectious agents listed in ToR 1 fall into the following categories; i.e. OIE-listed or causing systemic infection.

Testing of milt specifically for the presence of particular agents is a possibility. Due to the open nature of the genital papilla during the later stages of sexual maturation milt is likely to become contaminated with environmental agents. However, roe/milt aseptically removed from healthy sexually mature fish has been found to be sterile of cultivable bacteria (Trust, 1974). Detection of environmental opportunistically pathogenic agents can be performed but the sensitivity of molecular detection methods will often be impaired in milt due to the high levels of DNA present. This could possibly be overcome through assay optimization. Specific testing of milt can therefore reduce the risk of pathogens being present in frozen milt but cannot certify freedom.

ToR 4. Which measure would help ensure that frozen milt cannot transfer relevant infectious diseases? For example, adding antibiotics to control bacterial diseases in frozen milt

'Sperm washing' (Kim et al., 1999) of fish milt has not been described in the literature and is unlikely to reduce the burden of IPNV (Mulcahy and Pascho, 1984). Milt extenders containing antibiotics although effective in some cases for prolonging the effective storage time of sperm (Brown and Mims, 1995; Christensen and Tiersch, 1996) may have effects on sperm viability (Oplinger and Wagner 2015). As stated previously while erythromycin treatment of fertilised *Renibacterium salmoninarum* infected eggs was unsuccessful, erythromycin injection of brood fish prior to spawning did appear to prevent intra-ova infection (Evelyn et al., 1986; Lee and Evelyn, 1994). While antibiotic treatment will undoubtedly reduce the microbial flora of treated milt, there is, of course, a constant risk of development of resistance against any prophylactic antibiotic used. Reduced sensitivity to erythromycin has already been identified in strains of *R. salmoninarum* isolated from fish receiving multiple antibiotic treatments (Rhodes et al., 2008).

9 Data gaps

Studies of vertical transmission in general are difficult to perform and tend to give equivocal results. Published studies specifically addressing the role of fish semen in transmission of pathogens are very sparse. The experience of iodophor treatment of eggs, in general, is that it is effective at breaking vertical transmission. This indicates that sperm associated transport of the pathogen into the egg is not a major route of transmission. However, there are relatively few scientific reports that have studied this in sufficient detail.

Vertical transmission can be a rare event. Experimental designs vary and are not always optimal. The continuous debate regarding whether IHNV or ISAV are vertically transmitted via iodophor-treated eggs are examples of this. Laboratory experiments should reflect real-world conditions. The experience of real-world data indicate that that vertical transmission is a rare event.

Data are lacking on:

- Presence of almost all listed and non-listed fish pathogens in milt.
- Whether semen infected with pathogens infect eggs at fertilization.
- Whether fish semen have anti-bacterial properties.
- Persistence of viruses in milt.

10 References

Ahne, W. (1982) Vergleichende untersuchungen über die Stabilität von vier fischpathogenen viren (VHSV, PFR, SVCV, IPNV). Zentralblatt für Veterinärmedizinm, Serie B 29:457-476.

Ahne, W., & Negele, R. D. (1985). Studies on the transmission of infectious pancreatic necrosis virus via eyed roe and sexual products of salmonid fish. In A. Ellis (Ed.), *Fish and Shellfish Pathology* (pp. 262-270): Academic Press, London, UK.

Amend, D.F. (1975) Detection and transmission of infectious hematopoietic necrosis virus in rainbow trout. Journal of wildlife diseases 11:471-478.

Amos K., Thomas J., Hopper K. (1998) A case history of adaptive management strategies for viral hemorrhagic septicemia virus (VHSV) in Washington state. Journal of Aquatic Animal Health 10:152-159. DOI: 10.1577/1548-8667(1998)010<0152:achoam>2.0.co;2.

Arkush K.D., McBride A.M., Mendonca H.L., Okihiro M.S., Andree K.B., Marshall S., Henriquez V., Hedrick R.P. (2005) Genetic characterization and experimental pathogenesis of Piscirickettsia salmonis isolated from white seabass Atractoscion nobilis. Diseases of Aquatic Organisms 63:139-149. DOI: 10.3354/dao063139.

Balfry S.K., Albright L.J., Evelyn T.P.T. (1996) Horizontal transfer of Renibacterium salmoninarum among farmed salmonids via the fecal-oral route. Diseases of Aquatic Organisms 25:63-69. DOI: 10.3354/dao025063.

Biacchesi, S., Le Berre, M., Le Guillou, S., Benmansour, A., Bremont, M., Quillet, E., & Boudinot, P. (2007). Fish genotype significantly influences susceptibility of juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum), to waterborne infection with infectious salmon anaemia virus. *J Fish Dis, 30*(10), 631-636. doi:10.1111/j.1365-2761.2007.00854.x

Bielanski A. (2007) Disinfection procedures for controlling microorganisms in the semen and embryos of humans and farm animals. Theriogenology 68:1-22. DOI: 10.1016/j.theriogenology.2007.03.025.

Birkbeck T.H., Bordevik M., Froystad M.K., Baklien A. (2007) Identification of *Francisella* sp. from Atlantic salmon, *Salmo salar* L., in Chile. Journal of Fish Diseases 30:505-507. DOI: 10.1111/j.1365-2761.2007.00837.x.

Bravo S., Campos M. (1989) Coho salmon syndrome in Chile. Fish Health Section Newsletter, American Fisheries Society 17, 3.

Brosnahan C.L., Munday J.S., Ha H.J., Preece M., Jones J.B. (2018) New Zealand rickettsia-like organism (NZ-RLO) and *Tenacibaculum maritimum*: Distribution and phylogeny in farmed Chinook salmon (*Oncorhynchus tshawytscha*). J Fish Dis. DOI: 10.1111/jfd.12909.

Brown G.G., Mims S.D. (1995) Storage, transportation and fertility of undiluted and diluted paddlefish milt. Progressive Fish-Culturist 57:64-69. DOI: 10.1577/1548-8640(1995)057<0064:Stafou>2.3.Co;2.

Brown L.L., Cox W.T., Levine R.P. (1997) Evidence that the causal agent of bacterial cold-water disease *Flavobacterium psychrophilum* is transmitted within salmonid eggs. Diseases of Aquatic Organisms 29:213-218. DOI: 10.3354/dao029213.

Brynildsrud O., Feil E.J., Bohlin J., Castillo-Ramirez S., Colquhoun D., McCarthy U., Matejusova I.M., Rhodes L.D., Wiens G.D., Verner-Jeffreys D.W. (2014) Microevolution of *Renibacterium salmoninarum*: evidence for intercontinental dissemination associated with fish movements. Isme Journal 8:746-756. DOI: 10.1038/ismej.2013.186.

Bullock, G. L., Rucker, R. R., Amend, D., Wolf, K., & Stuckey, H. M. (1976). Infectious pancreatic necrosis – Transmission with iodine-treated and non-treated eggs of Brook trout (*Salvelinus fontinalis*). *Journal of the Fisheries Research Board of Canada, 33*(5), 1197-1198. doi:10.1139/f76-152

Bullock G.L., Leek S.L. (1986) Use of erythromycin in reducing vertical transmission of bacterial kidney disease. Vet Hum Toxicol 28 Suppl 1:18-20.

Bullock G.L., Stuckey H.M. (1987) Studies on vertical transmission of *Aeromonas salmonicida*. Progressive Fish-Culturist 49:302-303. DOI: 10.1577/1548-8640(1987)49<302:sovtoa>2.0.co;2.

Can E., Saka S., Firat K. (2010) Disinfection of Gilthead Sea Bream (*Sparus aurata*), Red Porgy (*Pagrus pagrus*), and Common Dentex (*Dentex dentex*) Eggs from Sparidae with Different Disinfectants. Kafkas Universitesi Veteriner Fakultesi Dergisi 16:299-306.

Chinchar V.G., Essbauer S., He J.G., Hyatt A.D., Miyazaki T., Seligy V., Williams T. (2005) Family *Iridoviridae*. In: Virus taxonomy: classification and nomenclature of viruses: Eighth report of the International Committee on the taxonomy of viruses. Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U. & Ball L.A., eds. Academic Press, San Diego, California, USA:145–162.

Christensen J.M., Tiersch T.R. (1996) Refrigerated storage of channel catfish sperm. Journal of the World Aquaculture Society 27:340-346. DOI: 10.1111/j.1749-7345.1996.tb00617.x.

Ciereszko A., Dietrich M.A., Nynca J. (2017) Fish semen proteomics - New opportunities in fish reproductive research. Aquaculture 472:81-92. DOI: 10.1016/j.aquaculture.2016.03.005.

Cipriano R.C. (2005) Intraovum infection caused by *Flavobacterium psychrophilum* among eggs from captive Atlantic salmon broodfish. Journal of Aquatic Animal Health 17:275-283. DOI: 10.1577/h05-003.1.

Cipriano R.C., Ford L.A., Teska J.D., Hale L.E. (1992) Detection of *Aeromonas salmonicida* the mucus of salmonid fishes. Journal of Aquatic Animal Health 4:114-118.

Cipriano R.C., Novak B.M., Flint D.E., Cutting D.C. (2001) Reappraisal of the federal fish health recommendation for disinfecting eggs of Atlantic salmon in iodophor. Journal of Aquatic Animal Health 13:320-327. DOI: 10.1577/1548-8667(2001)013<0320:rotffh>2.0.co;2.

Colquhoun D.J., Duodu S. (2011) *Francisella* infections in farmed and wild aquatic organisms. Veterinary Research 42. DOI: 10.1186/1297-9716-42-47.

Colquhoun D.J., Larsson P., Duodu S., Forsman M. (2014) The Family *Francisellaceae*. In The Prokaryotes pp. (287-314). Springer, Berlin, Heidelberg

Daly J.G., Stevenson R.M.W. (1989) Communications: Agglutination of Salmonid Spermatozoa by *Renibacterium salmoninarum* AU - Daly, James G. Journal of Aquatic Animal Health 1:163-164. DOI: 10.1577/1548-8667(1989)001<0163:CAOSSB>2.3.CO;2.

Dorson M., Torhy C., Billard R., Saudrais C., Maisse G., Haffray P., Hollebecq M.G. (1996) Infectious pancreatic necrosis of salmonids: Assessment of methods aiming at cutting off egg-associated transmission. Bulletin Francais De La Peche Et De La Pisciculture:1-14. DOI: 10.1051/kmae:1996001.

Ekman E., Börjeson H., Johansson N. (1999) *Flavobacterium psychrophilum* in Baltic salmon Salmo salar brood fish and their offspring. Diseases of Aquatic Organisms 37:159-163. DOI: 10.3354/dao037159.

Engelking, H.M.; Kaufman, J.,Groberg, W.J.Jr., LaPatra, S.E. (1991) Vertical transmission and covert infection studies of infectious hematopoietic necrosis virus. In: Fryer, J.L. (ed.) Proceedings: Second international symposium on viruses of lower vertebrates. July 29-31. 1991. Oregon State university, Corvallis, Oregon, pp.253-259.

Enzmann P.J., Kurath G., Fichtner D., Bergmann S.M. (2005) Infectious hematopoietic necrosis virus: monophyletic origin of European isolates from North American Genogroup M. Diseases of Aquatic Organisms 66:187-195. DOI: 10.3354/dao066187.

Enzmann P.J., Castric J., Bovo G., Thiery R., Fichtner D., Schutze H., Wahli T. (2010) Evolution of infectious hematopoietic necrosis virus (IHNV), a fish rhabdovirus, in Europe over 20 years: implications for control. Dis Aquat Organ 89:9-15. DOI: 10.3354/dao02182.

Evelyn T.P.T., Ketcheson J.E., Prosperi-Porta L. (1986a) Use of erythromycin as a means of preventing vertical transmission of *Renibacterium salmoninarum*. Diseases of Aquatic Organisms 2:7-11. DOI: 10.3354/dao002007.

Evelyn T.P.T., Prosperi-Porta L., Ketcheson J.E. (1986b) Experimental intra-ovum infection of salmonid eggs with *Renibacterium salmoninarum* and vertical transmission of the pathogen with such eggs despite their treatment with erythromycin. Diseases of Aquatic Organisms 1:197-202.

Falk, K., Namork, E., Rimstad, E., Mjaaland, S., & Dannevig, B. H. (1997). Characterization of infectious salmon anemia virus, an orthomyxo-like virus isolated from Atlantic salmon (*Salmo salar* L.). *J Virol, 71*(12), 9016-9023.

Garseth A.H., Fritsvold C., Svendsen J.C., Jensen B.B., Mikalsen A.B. (2018) Cardiomyopathy syndrome in Atlantic salmon *Salmo salar* L.: A review of the current state of knowledge. Journal of Fish Diseases 41:11-26. DOI: 10.1111/jfd.12735.

Giacomello E., Marri L., Marchini D., Mazzoldi C., Rasotto M.B. (2008) Sperm-duct gland secretion of the grass goby *Zosterisessor ophiocephalus* exhibits antimicrobial activity. Journal of Fish Biology 73:1823-1828. DOI: 10.1111/j.1095-8649.2008.02069.x.

Givens M.D. (2018) Review: Risks of disease transmission through semen in cattle. Animal 12:s165-s171. DOI: 10.1017/s1751731118000708.

Gjessing M.C., Yutin N., Tengs T., Senkevich T., Koonin E., Ronning H.P., Alarcon M., Ylving S., Lie K.I., Saure B., Tran L., Moss B., Dale O.B. (2015) Salmon Gill Poxvirus, the Deepest Representative of the *Chordopoxvirinae*. Journal of Virology 89:9348-9367. DOI: 10.1128/jvi.01174-15.

Glenn R.A., Taylor P.W., Pelton E.H., Gutenberger S.K., Ahrens M.A., Marchant L.M., Hanson K.C. (2015) Genetic Evidence of Vertical Transmission and Cycling of *Yersinia ruckeri* in Hatchery-Origin Fall Chinook Salmon *Oncorhynchus tshawytscha*. Journal of Fish and Wildlife Management 6:44-54. DOI: 10.3996/012014-jfwm-010.

Goldes S.A., Mead S.L. (1995) Efficacy of iodophor disinfection against egg surface-associated infectious hematopoietic necrosis virus Progressive Fish-Culturist 57:26-29. DOI: 10.1577/1548-8640(1995)057<0026:aeoida>2.3.co;2.

Gombar R., Pitcher T.E., Lewis J.A., Auld J., Vacratsis P.O. (2017) Proteomic characterization of seminal plasma from alternative reproductive tactics of

Chinook salmon (*Oncorhynchus tswatchysha*). Journal of Proteomics 157:1-9. DOI: 10.1016/jjprot.2017.01.019.

Graham D.A., Cherry K., Wilson C.J., Rowley H.M. (2007a) Susceptibility of salmonid alphavirus to a range of chemical disinfectants. J Fish Dis 30:269-77. DOI: 10.1111/j.1365-2761.2007.00810.x.

Graham D.A., Fringuelli E., Rowley H.M., Cockerill D., Cox D.I., Turnbull T., Rodger H., Morris D., Mc Loughlin M.F. (2012) Geographical distribution of salmonid alphavirus subtypes in marine farmed Atlantic salmon, *Salmo salar* L., in Scotland and Ireland. J Fish Dis 35:755-65. DOI: 10.1111/j.1365-2761.2012.01401.x.

Graham D.A., Staples C., Wilson C.J., Jewhurst H., Cherry K., Gordon A., Rowley H.M. (2007b) Biophysical properties of salmonid alphaviruses: influence of temperature and pH on virus survival. J Fish Dis 30:533-43. DOI: 10.1111/j.1365-2761.2007.00811.x.

Gulla S., Barnes A.C., Welch T.J., Romalde J.L., Ryder D., Ormsby M.J., Carson J., Lagesen K., Verner-Jeffreys D.W., Davies R.L., Colquhoun D.J. (2018) Multi-Locus Variable number of tandem repeat Analysis (MLVA) of *Yersinia ruckeri* confirms the existence of host-specificity, geographic endemism and anthropogenic dissemination of virulent clones. Appl Environ Microbiol. DOI: 10.1128/aem.00730-18.

Gulla S., Lund V., Kristoffersen A.B., Sorum H., Colquhoun D.J. (2016) vapA (A-layer) typing differentiates *Aeromonas salmonicida* subspecies and identifies a number of previously undescribed subtypes. Journal of Fish Diseases 39:329-342. DOI: 10.1111/jfd.12367.

Hauge H., Dahle M., Moldal T., Thoen E., Gjevre A.G., Weli S., Alarcon M., Grove S. (2016) Piscine orthoreovirus can infect and shed through the intestine in experimentally challenged Atlantic salmon (*Salmo salar* L.). Vet Res 47:57. DOI: 10.1186/s13567-016-0343-z.

Haugland, O., Mikalsen, A. B., Nilsen, P., Lindmo, K., Thu, B. J., Eliassen, T. M., Roos, N., Rode, M. & Evensen, O. (2011). Cardiomyopathy Syndrome of Atlantic Salmon (*Salmo salar* L.) Is Caused by a Double-Stranded RNA Virus of the *Totiviridae* Family. *J Virol, 85*(11), 5275-5286. doi:10.1128/jvi.02154-10

Hjeltnes B., Bornø G., Jansen M.D., Haukaas A., Walde C. (2016) The Fish Health Report 2015, Norwegian Veterinary Institute

Holopainen R., Ohlemeyer S., Schutze H., Bergmann S.M., Tapiovaara H. (2009) Ranavirus phylogeny and differentiation based on major capsid protein, DNA polymerase and neurofilament triplet H1-like protein genes. Diseases of Aquatic Organisms 85:81-91. DOI: 10.3354/dao02074.

Holway, J.E. & Smith, C.E. (1973) Infectious hematopoietic necrosis of rainbow trout in Montana: a case report. Journal of Wildlife Diseases 9:287-290.

Houston R.D., Gheyas A., Hamilton A., Guy D.R., Tinch A.E., Taggart J.B., McAndrew B.J., Haley C.S., Bishop S.C. (2008) Detection and confirmation of a major QTL affecting resistance to infectious pancreatic necrosis (IPN) in Atlantic salmon (*Salmo salar*). Dev Biol (Basel) 132:199-204. DOI: 10.1159/000317160.

Javurek A.B., Spollen W.G., Ali A.M.M., Johnson S.A., Lubahn D.B., Bivens N.J., Bromert K.H., Ellersieck M.R., Givan S.A., Rosenfeld C.S. (2016) Discovery of a Novel Seminal Fluid Microbiome and Influence of Estrogen Receptor Alpha Genetic Status. Scientific Reports 6. DOI: 10.1038/srep23027.

Jensen B.B., Brun E., Fineid B., Larssen R.B., Kristoffersen A.B. (2013) Risk factors for cardiomyopathy syndrome (CMS) in Norwegian salmon farming. Diseases of Aquatic Organisms 107:141-150. DOI: 10.3354/dao02678.

Jonas J.L., Schneeberger P.J., Clapp D.F., Wolgamood M., Wright G., Lasee B. (2002) Presence of the BKD-causing bacterium *Renibacterium salmoninarum* in lake whitefish and bloaters in the Laurentian Great Lakes. Archiv. fur Hydrobiologie 57:447-452.

Karlsbakk E., Isaksen T., Ottem K.F., Nylund A., Korsnes K., Nerland A.H., Patel S., Bergh Ø. (2008) The coast and aquaculture. Institute of marine research, Norway. Pathogens in the Coastal zone. (In Norwegian).

Kent M.L., Traxler G.S., Kieser D., Richard J., Dawe S.C., Shaw R.W., Prosperi-Porta G., Ketcheson J., Evelyn T.P.T. (1998) Survey of salmonid pathogens in ocean-caught fishes in British Columbia, Canada. Journal of Aquatic Animal Health 10:211-219. DOI: 10.1577/1548-8667(1998)010<0211:sospio>2.0.co;2.

Kim L.U., Johnson M.R., Barton S., Nelson M.R., Sontag G., Smith J.R., Gotch F.M., Gilmour J.W. (1999) Evaluation of sperm washing as a potential method of reducing HIV transmission in HIV-discordant couples wishing to have children. Aids 13:645-51.

Kohara M., Kasai H., Yoshimizu M. (2012) Intra-ovum Infection in Salmonid Eggs Artificially Contaminated with Fish Pathogenic Bacteria: *Flavobacterium psychrophilum, Renibacterium salmoninarum*, and *Aeromonas salmonicida*. Fish Pathology 47:49-55. DOI: 10.3147/jsfp.47.49.

Kongtorp, R. T., Stene, A., Andreassen, P. A., Aspehaug, V., Graham, D. A., Lyngstad, T. M., Olsen, A. B., Olsen, R.S., Sandberg, M., Santi, N., Wallace, C. & Breck, O. (2010). Lack of evidence for vertical transmission of SAV 3 using gametes of Atlantic salmon, *Salmo salar* L., exposed by natural and experimental routes. *J Fish Dis, 33*(11), 879-888. doi:10.1111/j.1365-2761.2010.01190.x

Kumagai A., Nawata A. (2010) Prevention of Flavobacterium psychrophilum Vertical Transmission by Iodophor Treatment of Unfertilized Eggs in Salmonids. Fish Pathology 45:164-168. DOI: 10.3147/jsfp.45.164.

Kumar G., Menanteau-Ledouble S., Saleh M., El-Matbouli M. (2015) *Yersinia ruckeri*, the causative agent of enteric redmouth disease in fish. Veterinary Research 46. DOI: 10.1186/s13567-015-0238-4.

Kurath, G., Garver, K. A., Troyer, R. M., Emmenegger, E. J., Einer-Jensen, K., & Anderson, E. D. (2003). Phylogeography of infectious haematopoietic necrosis virus in North America. *Journal of General Virology, 84*, 803-814. doi:10.1099/vir.0.18771-0

Kurath G. (2012) Fish novirhabdoviruses. In: Dietzgen RG, Kuzmin IV (eds) Rhabdoviruses: Molecular Taxonomy, Evolution, Genomics, Ecology, Host-Vector Interactions, Cytopathology and Control. Caister Academic Press, Norfolk, UK,:pp 89-116.

Kvellestad A., Dannevig B.H., Falk K. (2003) Isolation and partial characterization of a novel paramyxovirus from the gills of diseased seawater-reared Atlantic salmon (*Salmo salar* L.). Journal of General Virology 84:2179-2189. DOI: 10.1099/vir.0.18962-0.

Kvellestad A., Falk K., Nygaard S.M.R., Flesja K., Holm J.A. (2005) Atlantic salmon paramyxovirus (ASPV) infection contributes to proliferative gill inflammation (PGI) in seawater-reared *Salmo salar*. Diseases of Aquatic Organisms 67:47-54. DOI: 10.3354/dao067047.

Langdon J.S. (1989) Experimental transmission and pathogenicity of epizootic haematopoietic necrosis virus (EHNV) in redfin perch, *Perca fluviatilis* L., and 11 other teleosts. Journal of Fish Diseases 12:295-310. DOI: doi:10.1111/j.1365-2761.1989.tb00318.x.

LaPatra, S.E. (1990) Infectious hematopoietic necrosis virus (IHNV) transmission studies in Oregon. American Fisheries Society, Fish Health Section Newsletter 18(1):5-6.

LaPatra, S.E., Groberg, W.J., Rohovec, J.S., Ffryer, J.L. Delayed fertilization of steelhead (*Oncorhynchus mykiss*) ova to investigate vertical transmission of infectious hematopoietic necrosis virus (IHNV). In: Fryer, J.L. (ed.) Proceedings: Second international symposium on viruses of lower vertebrates, July 29-31, 1991. Oregon State University, Corvallis, Oregon, pp. 261-267.

Larenas J.J., Bartholomew J., Troncoso O., Fernandez S., Ledezma H., Sandoval N., Vera P., Contreras J., Smith P. (2003a) Experimental vertical transmission of

Piscirickettsia salmonis and in vitro study of attachment and mode of entrance into the fish ovum. Dis Aquat Organ 56:25-30. DOI: 10.3354/dao056025.

Larenas J.J., Bartholomew J., Troncoso O., Fernandez S., Ledezma H., Sandoval N., Vera P., Contreras J., Smith P. (2003b) Experimental vertical transmission of *Piscirickettsia salmonis* and in vitro study of attachment and mode of entrance into the fish ovum. Diseases of Aquatic Organisms 56:25-30. DOI: 10.3354/dao056025.

Lee E.G.H., Evelyn T.P.T. (1994) Prevention of vertical transmission of the bacterial kidney-disease agent *Renibacterium salmoninarum* by broodstock injection with erythromycin. Diseases of Aquatic Organisms 18:1-4.

Loch T.P., Faisal M. (2010) Infection of Lake Whitefish (*Coregonus clupeaformis*) with motile *Aeromonas* spp. in the Laurentian Great Lakes. Journal of Great Lakes Research 36:6-12. DOI: 10.1016/j.jglr.2009.07.001.

Lonergan P. (2018) Review: Historical and futuristic developments in bovine semen technology. Animal 12:s4-s18. DOI: 10.1017/s175173111800071x.

Long A., Call D.R., Cain K.D. (2014) Investigation of the link between broodstock infection, vertical transmission, and prevalence of *Flavobacterium psychrophilum* in eggs and progeny of Rainbow Trout and Coho Salmon. J Aquat Anim Health 26:66-77. DOI: 10.1080/08997659.2014.886632.

Lovoll M., Alarcon M., Bang Jensen B., Taksdal T., Kristoffersen A.B., Tengs T. (2012) Quantification of piscine reovirus (PRV) at different stages of Atlantic salmon *Salmo salar* production. Dis Aquat Organ 99:7-12. DOI: 10.3354/dao02451.

Lovoll M., Wiik-Nielsen J., Grove S., Wiik-Nielsen C.R., Kristoffersen A.B., Faller R., Poppe T., Jung J., Pedamallu C.S., Nederbragt A.J., Meyerson M., Rimstad E., Tengs T. (2010) A novel totivirus and piscine reovirus (PRV) in Atlantic salmon *(Salmo salar*) with cardiomyopathy syndrome (CMS). Virol J 7:309. DOI: 10.1186/1743-422x-7-309.

Lyngstad T.M., Hjortaas M.J., Kristoffersen A.B., Markussen T., Karlsen E.T., Jonassen C.M., Jansen P.A. (2011) Use of Molecular Epidemiology to Trace Transmission Pathways for Infectious Salmon Anaemia Virus (ISAV) in Norwegian Salmon Farming. Epidemics 3:1-11. DOI: 10.1016/j.epidem.2010.11.001.

Madsen L., Moller J.D., Dalsgaard I. (2005) *Flavobacterium psychrophilum* in rainbow trout, *Oncorhynchus mykiss* (Walbaum), hatcheries: studies on broodstock, eggs, fry and environment. J Fish Dis 28:39-47. DOI: 10.1111/j.1365-2761.2004.00598.x.

Markussen T., Jonassen C.M., Numanovic S., Braaen S., Hjortaas M., Nilsen H., Mjaaland S. (2008) Evolutionary mechanisms involved in the virulence of infectious salmon anaemia virus (ISAV), a piscine orthomyxovirus. Virology 374:515-527. DOI: 10.1016/j.virol.2008.01.019.

Marshall S.H., Ramirez R., Labra A., Carmona M., Munoz C. (2014) Bona fide evidence for natural vertical transmission of infectious salmon anemia virus in freshwater brood stocks of farmed Atlantic salmon (*Salmo salar*) in Southern Chile. J Virol 88:6012-8. DOI: 10.1128/jvi.03670-13.

Martin-Carnahan A., Joseph S.W. (2005) Aeromonadalesord. nov. In: Brenner, D.J., et al., (Eds) Bergey's Manual® of Systematic Bacteriology. Springer, Boston, MA. DOI: https://doi.org/10.1007/0-387-28022-7_12.

McCarthy U., Steiropoulos N.A., Thompson K.D., Adams A., Ellis A.E., Ferguson H.W. (2005) Confirmation of *Piscirickettsia salmonis* as a pathogen in European sea bass *Dicentrarchus labrax* and phylogenetic comparison with salmonid strains. Dis Aquat Organ 64:107-19. DOI: 10.3354/dao064107.

McLoughlin M.F., Nelson R.T., Rowley H.M., Cox D.I., Grant A.N. (1996) Experimental pancreas disease in Atlantic *salmon Salmo* salar post-smolts induced by salmon pancreas disease virus (SPDV). Diseases of Aquatic Organisms 26:117-124. DOI: 10.3354/dao026117.

Meyers T.R., Thomas J.B., Follett J.E., Saft R.R. (1990) Infectious Hematopoietic Necrosis Virus: Trends in Prevalence and the Risk Management Approach in Alaskan Sockeye Salmon Culture. Journal of Aquatic Animal Health 2:85-98. DOI: 10.1577/1548-8667(1990)002<0085:IHNVTI>2.3.CO;2.

Mikalsen, A. B., Nilsen, P., Froystad-Saugen, M., Lindmo, K., Eliassen, T. M., Rode, M., & Evensen, O. (2014). Characterization of a novel calicivirus causing systemic infection in atlantic salmon (*Salmo salar* L.): proposal for a new genus of caliciviridae. *PLoS One, 9*(9), e107132. doi:10.1371/journal.pone.0107132

Mortensen S.H., Nilsen R.K., Hjeltnes B. (1998) Stability of an infectious pancreatic necrosis virus (IPNV) isolate stored under different laboratory conditions. Dis Aquat Organ 33:67-71. DOI: 10.3354/dao033067.

Mulcahy, D. & Bauersfeld, K. (1983) Effect of loading density of sock-eye salmon, *Oncorhynchus nerka* (Walbaum), eggs in incubation boxes on mortality caused by infectious hematopoietic necrosis. Journal of fish diseases 6: 189-193.

Mulcahy D., Pascho R.J. (1984) Adsorption to fish sperm of vertically transmitted fish viruses. Science 225:333-5.

Mulcahy, D. & Pascho, R.J. (1985) Vertical transmission of infectious haematopoietic virus in sockeye salmon, *Oncorhynchus nerka* (Walbaum): isolation of virus from dead eggs and fry. Journal of fish diseases 8:393-396.

Muller M., Ilardi P., Avendano-Herrera R. (2011) Efficacy of a commercial disinfectant against *Vibrio ordalii*, *Vibrio anguillarum*, *Francisella* sp. and Infectious Pancreatic Necrosis Virus (IPNV) pathogens of Atlantic salmon (*Salmo salar*) farmed in Chile. Archivos de Medicina Veterinaria 43:73-78.

Muniesa A., Escobar-Dodero J., Silva N., Henriquez P., Bustos P., Perez A.M., Mardones F.O. (2018) Effectiveness of disinfectant treatments for inactivating *Piscirickettsia salmonis*. Prev Vet Med. DOI: 10.1016/j.prevetmed.2018.03.006.

Munson A.D., Elliott D.G., Johnson K. (2010) Management of bacterial kidney disease in Chinook Salmon hatcheries based on broodstock testing by enzyme-linked immunosorbent assay: A multiyear study. North American Journal of Fisheries Management 30:940-955. DOI: 10.1577/M09-044.1.

Nagamatsu, G., & Hayashi, K. (2017). Stem cells, in vitro gametogenesis and male fertility. *Reproduction, 154*(6), F79-f91. doi:10.1530/rep-17-0510

Nicolas P., Mondot S., Achaz G., Bouchenot C., Bernardet J.F., Duchaud E. (2008) Population structure of the fish-pathogenic bacterium *Flavobacterium psychrophilum*. Applied and Environmental Microbiology 74:3702-3709. DOI: 10.1128/aem.00244-08.

Nicopoullos J.D., Almeida P., Vourliotis M., Goulding R., Gilling-Smith C. (2010) A decade of sperm washing: clinical correlates of successful insemination outcome. Hum Reprod 25:1869-76. DOI: 10.1093/humrep/deq134.

Nilsen H., Johansen R., Colquhoun D.J., Kaada I., Bottolfsen K., Vagnes O., Olsen A.B. (2011a) *Flavobacterium psychrophilum* associated with septicaemia and necrotic myositis in Atlantic salmon *Salmo salar*: a case report. Diseases of Aquatic Organisms 97:37-46. DOI: 10.3354/dao02390.

Nilsen H., Olsen A.B., Vaagnes O., Hellberg H., Bottolfsen K., Skjelstad H., Colquhoun D.J. (2011b) Systemic *Flavobacterium psychrophilum* infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum), farmed in fresh and brackish water in Norway. Journal of Fish Diseases 34:403-408. DOI: 10.1111/j.1365-2761.2011.01249.x.

Nilsen H., Sundell K., Duchaud E., Nicolas P., Dalsgaard I., Madsen L., Aspan A., Jansson E., Colquhoun D.J., Wiklund T. (2014) Multilocus sequence typing identifies epidemic clones *of Flavobacterium psychrophilum* in Nordic countries. Appl Environ Microbiol 80:2728-36. DOI: 10.1128/aem.04233-13.

Niu, L. & Zhao, Z. (1988) The epidemiological IHN and IPN of rainbow trout in northeast China. Journal of fisheries of China 12: 351-352.

Nylund A., Watanabe K., Nylund S., Karlsen M., Saether P.A., Arnesen C.E., Karlsbakk E. (2008) Morphogenesis of salmonid gill poxvirus associated with proliferative gill disease in farmed Atlantic salmon (*Salmo salar*) in Norway. Archives of Virology 153:1299-1309. DOI: 10.1007/s00705-008-0117-7.

OIE. (2018) Section 2.3 Diseases of fish. Manual of Diagnostic Tests for Aquatic Animals.

Oplinger, R.W. & Wagner, E.J. (2015) Use of penicillin and streptomycin to reduce spread of bacterial coldwater disease I: antibiotics in sperm extenders. Journal of aquatic animal health 27(1):25-31 doi: 10.1080/08997659.2014.966211.

Olsen A.B., Melby H.P., Speilberg L., Evensen O., Hastein T. (1997) *Piscirickettsia salmonis* infection in Atlantic salmon *Salmo salar* in Norway - epidemiological, pathological and microbiological findings. Diseases of Aquatic Organisms 31:35-48. DOI: 10.3354/dao031035.

Plumb, J.A. (1972) A virus-caused epizootic if rainbow trout (*Salmo gairdneri*) in Minnesota. Transactions of the American Fisheries Society 101:121-123.

Pilcher, K.S. & Fryer, J.L. (1980a) The viral diseases of fish: a review through 1978. Part I: Diseases of proven viral etiology. CRC Critical Reviews in Microbiology 7:287-364.

Pilcher, K.S. Fryer, J.L. (1980b) The viral diseases of fish: a review through 1978. Part II Diseases in which a viral etiology is suspected but unproven. CRC Critical Reviews in Microbiology 8:1-25.

Poiani A. (2006) Complexity of seminal fluid: a review. Behavioral Ecology and Sociobiology 60:289-310. DOI: 10.1007/s00265-006-0178-0.

Pradeep P.J., Suebsing R., Sirithammajak S., Kampeera J., Turner W., Jeffs A., Kiatpathomchai W., Withyachumanarnkul B. (2017) Vertical transmission and concurrent infection of multiple bacterial pathogens in naturally infected red tilapia (*Oreochromis spp*.). Aquaculture Research 48:2706-2717. DOI: 10.1111/are.13102.

Ratcliff, D.E. (1982) Infectious hematopoietic necrosis: a comaprison of epizootic among fry hatched from saline-rinsed and unrinsed steelhead egg. Progressive fish culturist 44:217-220.

Rhodes L.D., Nguyen O.T., Deinhard R.K., White T.M., Harrell L.W., Roberts M.C. (2008) Characterization of *Renibacterium salmoninarum* with reduced susceptibility to macrolide antibiotics by a standardized antibiotic susceptibility test. Diseases of Aquatic Organisms 80:173-180. DOI: 10.3354/dao01959.

Rimstad, E., Mjaaland, S., Snow, M., Mikalsen, A. B., & Cunningham, C. O. (2001). Characterization of the infectious salmon anemia virus genomic segment that encodes the putative hemagglutinin. *J Virol, 75*(11), 5352-5356. doi:10.1128/jvi.75.11.5352-5356.2001

Roberts, S.D. (1993) IHN at Lyons Ferry Hatchery: a case study of vertical transmission. American Fisheries Society Fish Health Section Newsletter 21:13-14.

Rodger H.D., Drinan E.M. (1993) Observation of a Rickettsia-like organism in Atlantic salmon, *Salmo salar* L, in Ireland. Journal of Fish Diseases 16:361-369. DOI: 10.1111/j.1365-2761.1993.tb00869.x.

Romalde J.L., Magarinos B., Lores F., Osorio C.R., Toranzo A.E. (1999) Assessment of a magnetic bead-EIA based kit for rapid diagnosis of fish pasteurellosis. Journal of Microbiological Methods 38:147-154. DOI: 10.1016/s0167-7012(99)00088-3.

Sano, T., Nishimura, T. Okamoto, N. Yamazaki, T. Hanada, H. Watanabe, Y. (1977) Studies on viral disease of Japanese fish-VI. Infectious hematopoietic necrosis (IHN) of salmonids in the mainland of Japan.. Journal of the Tokyo University of fisheries 63:81-85.

Skall, H. F., Olesen, N. J., & Mellergaard, S. (2005). Viral haemorrhagic septicaemia virus in marine fish and its implications for fish farming - a review. *J Fish Dis, 28*(9), 509-529. doi:10.1111/j.1365-2761.2005.00654.x

Snow, M., Raynard, R. S., Inglis, J., & Bruno, D. W. (2001). Investigation into the potential for seawater rainbow trout (*Oncorhynchus mykiss*) to act as vectors of infectious salmon anaemia virus (ISAV). *Bulletin of the European Association of Fish Pathologists, 21*(6), 252-262.

Souter B.W., Dwilow A.G., Knight K., Yamamoto T. (1984) Infectious pancreatic necrosis virus: isolation from asymptomatic wild Arctic char (*Salvelinus alpinus* L.). J Wildl Dis 20:338-9.

Starliper C.E. (2011) Bacterial coldwater disease of fishes caused by *Flavobacterium psychrophilum*. Journal of Advanced Research 2:97-108. DOI: https://doi.org/10.1016/j.jare.2010.04.001.

Stoss J. (1983) Fish gamete preservation and spermatozoan physiology. in W. S. Hoar, D. J. Randall, and E. M. Donaldson, editors. Fish physiology volume IX, part B. Academic Press, New York.:pp. 305-350.

Taylor P.W. (2004) Detection of *Flavobacterium psychrophilum* in eggs and sexual fluids of Pacific Salmonids by a polymerase chain reaction assay: Implications for vertical transmission of bacterial coldwater disease. Journal of Aquatic Animal Health 16:104-108. DOI: 10.1577/h03-053.1.

Traxler, G.S., Roome, J.R. Lauda, K.A., LaPatra, S.E. (1997) The apperance of infectious hematopoietic necrosis virus (IHNV) and neutralizing antiboies in sockeye salmon (*Oncorhynchus nerka*) during their migration and maturation period. Disease of aquatic organisms 28:31-38.

Trust T.J. (1974) Sterility of salmonid roe and practicality of hatching gnotobiotic salmonid fish. Appl Microbiol 28:340-1.

Verner-Jeffreys D.W., Haig S.J., Welch T.J., Pond M.J., Stone D., Davies R.L., Gardiner R. (2011) Characterisation of a serotype O1 *Yersinia ruckeri* isolate from the Isle of Man: further evidence that O antigen serotype is not a reliable indicator of virulence. Bulletin of the European Association of Fish Pathologists 31:86-91.

Verner-Jeffreys D.W., Joiner C.L., Bagwell N.J., Reese R.A., Husby A., Dixon P.F. (2009) Development of bactericidal and virucidal testing standards for aquaculture disinfectants. Aquaculture 286:190-197. DOI: 10.1016/j.aquaculture.2008.10.001.

Wessel, O., Braaen, S., Alarcon, M., Haatveit, H., Roos, N., Markussen, T., Tengs, T., Dahle, M.K. & Rimstad, E. (2017). Infection with purified Piscine orthoreovirus demonstrates a causal relationship with heart and skeletal muscle inflammation in Atlantic salmon. *PLoS One, 12*(8), e0183781. doi:10.1371/journal.pone.0183781

Wiik-Nielsen, C. R., Ski, P. M., Aunsmo, A., & Lovoll, M. (2012). Prevalence of viral RNA from piscine reovirus and piscine myocarditis virus in Atlantic salmon, *Salmo salar* L., broodfish and progeny. *J Fish Dis, 35*(2), 169-171. doi:10.1111/j.1365-2761.2011.01328.x

Wingfield, W.H. & Chan, L.D. (1970) Studies on the Sacramento river chinook disease and its causative agent. In; Snieszko,S.F. (ed.) A symposium on diseases of fish and shellfish. Special Publication 5, American fisheries Society, Washington, DC, pp 307-318.

Wolf, K., Quimby, M. C., Carlson, C. P., & Bullock, G. L. (1968). Infectious pancreatic necrosis – Selection of virus-free stock from a population of carrier trout. *Journal of the Fisheries Research Board of Canada, 25*(2), 383-&. doi:10.1139/f68-030

Wolf, K., Snieszko, S. F., Dunbar, C. E., & Pyle, E. (1960). Virus nature of infectious pancreatic necrosis in trout. *Proceedings of the Society for Experimental Biology and Medicine, 104*(1), 105-108.

Wolf, K. (1988) Viral hemorrhagic septicaemia. Chapter 18. In: Wolf, K. Fish viruses and fish viral diseases. Cornell University Press, Itchaka, pp. 217-249.

Wong P.C., Balmaceda J.P., Blanco J.D., Gibbs R.S., Asch R.H. (1986) Sperm washing and swim-up technique using antibiotics removes microbes from human semen. Fertil Steril 45:97-100.

Yamazaki, T. & Motonishi, A. (1992) Control of infectious hematopoietic necrosis and infectious pancreatic necrosis in salmonid fish in Japan. In: Kimura, T. (ed.)

Proceedings of the Oji International symposium on salmonid diseases, Hokkaido University Press, Sapporo, Japan, pp. 103-110.

Yoshimizu, M., Sami, M., Kimura, T. (1989) Survivability of infectious hematopoietic necrosis virus in fertilized eggs of masu and chum salmon. Journal of aquatic animal health 1:13-20.